ANTIMICROBIAL EFFICACY OF XF-73 AND PHOTO-ACTIVATED XF-73 AGAINST CLINICALLY RELEVANT MICROORGANISMS IN PLANKTONIC AND BIOFILM MODES OF GROWTH

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Thesis abstract

Novel antimicrobials are urgently needed to combat the global increase in antimicrobial resistance. Exeporfinium chloride (XF-73) is a synthetic dicationic porphyrin antimicrobial, acting through membrane disruption; with a secondary mechanism of action, activated via blue light exposure. The aim of this study was to assess the antimicrobial efficacy of XF-73 and photo-activated XF-73 against a panel of clinically relevant Gram-positive and Gram-negative microorganisms in planktonic and biofilm modes of growth. The antimicrobial efficacy of XF-73 was assessed through minimum inhibitory and bactericidal concentration (MIC/MBC) assays. The biofilm Calgary device generated twenty-four hour biofilms to assess the anti-biofilm effect of XF-73 on biofilm viability through viable cell counts and through minimum biofilm inhibitory and eradication concentrations (MBIC/MBEC) assays. The antimicrobial activity of photo-activated XF-73 was also assessed following fifteen minutes blue light exposure at 420 nm (light dose = 13.8 J/cm^2). XF-73 expressed a greater bactericidal activity against Gram-positive planktonic bacteria in comparison to Gram-negative microorganisms as MBCs ranged from ≤ 0.125 – 4 μg/ mL and 128 – >256 μg/ mL respectively. Photo-activation of XF-73 enhanced the bactericidal properties against both Gram-positive and Gram-negative bacteria as MBCs decreased to ≤ 0.03 – 1 μg/ mL and 32 – 128 μg/ mL. Gram-positive biofilms were highly susceptible to XF-73 with MBEC's ranging from 1 – 2 μg/ mL. Photo-activation increased the bactericidal effect of XF-73 against Gram-positive biofilms with MBEC's ranging between ≤ 0.125 – 0.5 μg/ mL. XF-73 was unable to eradicate Gram-negative biofilms, but concentrations 128 – 512 μg/ mL significantly reduced biofilm viability (p= <0.0001). XF-73 is a potent antimicrobial against Gram-positive microorganisms in both planktonic and biofilm modes of growth. Photo-activation further enhances the bactericidal effect of XF-73 and potentially offers an adjunct form of treatment in comparison to current antimicrobial treatment strategies. Further research is warranted in this area.

Key words: Exeporfinium chloride, XF-73, antimicrobial, bactericidal, biofilm, photodynamic therapy, photosensitiser, anti-biofilm, minimum bactericidal concentration, minimum biofilm eradication concentration

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'A single chance is a galaxy of hope' – Star Wars: The clone wars, 2009

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List of Abbreviations

$ – United States dollar

% – percentage

£ – Pound sterling

€ - Euro

°C – degree Celsius

A. baumannii – *Acinetobacter baumannii*

ABC – ATP-binding cassette

ADL – argon dye lasers

agr – accessory gene regulator

AIP – autoinducing peptide

AMR – antimicrobial resistance

ATCC – American Type Culture Collection

ATP – adenosine triphosphate

C. acnes – *Cutibacterium acnes*

C. albicans – *Candida albicans*

C. striatum – *Corynebacterium striatum*

CAP – cationic antimicrobial peptide

CA-UTI – catheter associated urinary tract infection

CFSTRS – continuous flow stirred tank reactor system

CFU – colony forming units

CLSI – the Clinical Laboratory Standards Institute

cm – centimetre

CRBSI – catheter-related bloodstream infections

CVC – central venous catheter

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

E. coli – *Escherichia coli*

E. faecalis – *Enterococcus faecalis*

E. faecium – *Enterococcus faecium*

e.g. – example gratia/ for example

ESBL – extended spectrum beta-lactamases

ESCMID – the European Society of Clinical Microbiology and Infectious Diseases

EUCAST – the European Committee on Antimicrobial Susceptibility Testing

Ex-vivo – outside the living

FDA – Food and Drug Administration

GVL – gold vapour laser

HCAI – healthcare-associated infections

i.e. – id est/ that is

ICU – intensive care units

In-vitro – in glass

In-vivo – in a living organism

Isd system – iron-regulated surface determinant system

K. pneumoniae – *Klebsiella pneumoniae*

LED – light emitting diodes

LLoD – lower limit of detection

M – Molarity

MBC – minimum bactericidal concentration

MBEC – minimum biofilm eradication concentration

MBIC – minimum biofilm inhibitory concentration

MHA – Mueller-Hinton agar

MHB – Mueller-Hinton broth

MIC – minimum inhibitory concentration

mL – millilitre

mm – millimetre

MMSA – methicillin susceptible *S. aureus*

MRSA – methicillin resistant *S. aureus*

N. gonorrhoeae – *Neisseria gonorrhoeae*

NaOH – Sodium hydroxide

NHS – National Health Service

nm – nanometre

OD – optical density

P. aeruginosa – *Pseudomonas aeruginosa*

PBS – phosphate buffered saline

PDT – photodynamic therapy

PFRS – plug flow reactor system

PJI – periprosthetic joint infections

PS – photosensitiser

QS – Quorum sensing

QSI- Quorum sensing inhibition

RNA – ribonucleic acid

ROS – reactive oxygen species

RPMI media – Roswell Park Memorial Institute media

S. aureus – *Staphylococcus aureus*

S. epidermidis – *Staphylococcus epidermidis*

S. hominis – *Staphylococcus hominis*

S. lugdunensis – *Staphylococcus lugdunensis*

SSI – surgical site infections

TMTC – too many to count

UK – United Kingdom

USA – United States of America

UTI – urinary tract infection

VAP – ventilator-associated pneumonia

VRE – vancomycin resistant enterococci

W – Watt

WHO – World Health Organisation

XF – Exeporfinium chloride

XF-73 – Exeporfinium chloride 73

β-lactams – beta lactams

μg – microgram

μL – microliter

μM – micrometre

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1.0 Chapter 1: Introduction

1.1 Infectious diseases

The World Health Organisation (WHO) states that an infectious disease is capable of spreading directly or indirectly from person to person; these infections are caused by pathogenic microorganisms: bacteria, viruses, parasites and fungi (WHO, 2016). Infections are responsible for 15,454 doctor visits each year in the United States of America (USA) and 3,672 visits each year to the emergency department (Center for Health Statistics, 2016). Infectious diseases account for 7% of deaths, and 8% of hospital bed stays in the United Kingdom (UK) annually. These diseases also pose an economic problem for the country costing around £30 billion each year, including the costs of health care service and the labour market (Bunn, 2017). The most recent case of an emerging infectious disease was the COVID-19 virus in 2019 that quickly became a pandemic infecting over 153 million people and was reported to have caused 3.2 million death by May 2021, becoming one of the leading causes of death. The COVID-19 virus was reported to disproportionately impact more vulnerable populations such as older adults and areas that are economically disadvantaged. Similarly neglected tropical diseases affect over 1.6 billion people annually and account for infectious and non-infectious diseases commonly isolated in tropical or subtropical regions. The recent example of a neglected tropical diseases is the Ebola outbreak which caused over 11,000 deaths in Africa and was classified as an epidemic due to a 50 – 90% mortality rate in humans. Fortunately, two vaccines were designed to combat the virus and the Ebola vaccine is one of the first licenced human vaccine designed from a viral vector platform that was pioneering for the development of COVID-19 vaccines. Infectious diseases caused by bacterial infections make up some of the lead causes of global death, as lower respiratory infections and diarrhoeal diseases accounted for 2,590,000 and 1,450,000 deaths respectively (WHO, 2021; Vuitika *et al.*, 2022)

1.2 Bacterial biofilms

The term planktonic is a classification given to microorganisms that are free floating in the environment, alternatively microorganisms that grow together in an environment bound to a surface, either single species or multiple species of microorganisms are classified as biofilms. Biofilms play a key role in infectious diseases, as an estimated 75% of all bacterial infections are caused by bacterial species with the capability to form biofilms (Ahmed *et al.*, 2015).

1.2.1 The history of biofilms

Originally microorganisms were considered simple in comparison to higher organisms, with the belief they were free-floating, in a planktonic state in the environment. In 1933, Henrici showed that this was not the case, bacteria were able to grow on submerged surfaces rather than as free-floating cells in a liquid environment (O'Toole *et al.,* 2000). Claude ZoBell documented the 'bottle effect', he showed

bacteria introduced into a bottle disappear from the liquid phase, with a simultaneous rapid increase in the number of bacteria attached to the surface of the bottles. (Zobell, 1943).

It was not until nearly thirty years later that scientists began to understand that bacteria existed in biofilms. In the 1970's it was understood that sessile bacteria accounted for a large amount of the bacterial biomass in the environment (Costerton *et al.*, 1999). Costerton and his colleagues verified that microorganisms live in biofilms as a mode of life and that biofilms are not an uncommon occurrence, as previously believed (Coenye *et al.*, 2010). 'Biofilm' is a term given to describe complex communities of microorganisms attached to abiotic surfaces. The composition of these communities usually comprises of multiple species that interact within the environment and each other (Davey *et al.,* 2000).

Bacterial biofilms can consist of a single microbial species, but these biofilms are mainly present as infections or located on the surface of medical implants as mixed species biofilms are the most abundantly found in a wide variety of environments (O'Toole *et al.,* 2000). Bacterial biofilms are responsible for a magnitude of infections; the National Institutes of Health have reported that 65% of all microbial infections are associated with bacterial biofilm formation, additionally bacterial biofilms are responsible for 80% of chronic infections (Jamal *et al.*, 2018).

While antimicrobials are the current most effective form of treatment against microbial infections, bacterial biofilms are known to be naturally highly resistant to antibiotics, hard surface disinfectants and metal ions, in comparison to planktonic cells; due to microorganisms in a biofilm undergoing cell specialisation, leading to an increase of emergent functions in the biofilm community that differentiates microorganisms in biofilms from planktonic microorganisms (Harrison *et al.*, 2007).

1.2.2 Biofilm formation

Biofilm formation can be broken down to four main phases, starting with adhesion of the planktonic microorganisms to a surface of a substrate, followed by proliferation of the microorganisms, leading into biofilm maturation and ending with cellular detachment (Li *et al.*, 2018) (Figure 1.1).

Ia. Attachment to uncoated surface by physicochemical forces

1. Hydrophobic Interactions
2. Electrostatic Interactions
3. Van der Waals forces

Ib. Attachment to protein coated surface

1. Fibrinogen (Fg)
2. Vitronectin
3. Von Willebrand factor
4. Platelets/thrombin

II. Cell-cell proliferation To mature biofilm

1. Polysaccharide Intercellular Adhesin (PIA)
2. Accumulation Associated Protein (AAP)

III. Detachment

1. δ toxin
2. Degradation of Extracellular macromolecules

Figure 1.1: Diagram illustrating the process of biofilm formation of Staphylococcus epidermidis

Diagram included the key phases of formation: attachment to a substrate, biofilm formation and maturation, followed by detachment and finally dispersal of bacterial cells. Diagram includes factors involved throughout the process (Katsikogianni et al., 2004)

1.2.2.1 Bacterial adhesion to a substrate surface

Initial biofilm formation cannot occur unless a substrate surface is present in the environment. Bacteria have established ways to colonise a substrate, with type IV pili and flagella to allow controlled movement around an environment, and attachment on the surface of the substrate with the additional aid of adhesive membrane proteins and extracellular polysaccharides (Pasmore *et al.*, 2002). Furthermore, physical forces assist the movement of bacteria to a surface for colonisation. Gravitational settling classifies the movement of bacteria drifting down an environment and coming into contact of a surface through the forces of gravity alone. Brownian diffusion leads to the random movement of the bacteria which increases the possibility of locating a surface for attachment (Ammar *et al.*, 2015). Surfaces can facilitate cell movement via chemotaxis, by the secretion of solubilised attractants creating a concentration gradient, attracting bacterial cells. Alternatively hapotaxis occurs due to adhesive gradients located on the substrate itself triggering cell migration towards the substrate (Klominek *et al.*, 1993).

The adhesion and formation of a biofilm is effected by a range of variables, for instance hydrodynamic forces from the flow of water either at laminar or turbulent flow can alter the shape of a biofilm; therefore, the roughness and topography of the substrate surface is extremely influential for the attachment (Oh *et al.*, 2009). Previous studies have shown biofilms form weaker attachments to surfaces that are: smooth, hydrophilic, and neutrally charged were most easily removed with the addition of shear forces.

The longer the biofilm has to attach to a surface the stronger the bond between the biofilm and the substrate, increasing the difficulty for removal (Pasmore *et al*., 2002). The charge of a surface is one of the main factors that determines if the bacteria adhere to a surface or are repulsed away. Depending on the charge of both the bacteria and the surface, electrostatic interactions lead to repulsion from the substrate surface. Van der Waals interactions leads to an attraction to the surface and acid-base Lewis interactions depending on hydrophobic effects, e.g. the surface charge of the water/ environment the substrate and bacteria are present in, can cause either repulsion or attraction between the bacteria and the surface (Ammar *et al*., 2015). These interactions are classified as short-range interactions as they only take into effect when the bacterial cells come into close contact with the surface (< 5 nm), and only become relevant in bacterial adhesion following long range interactions.

The environment the bacteria and substrates are present in effects the adhesion and formation of biofilms. A conditioning film: a layer on the surface of the substrate made up of deposited organic matter present in the surrounding fluid, is created upon the substrate entering the environment. There are differences between conditioning films on the same substrate depending on the environment, as the surface will absorb the proteins present in specific environments. For example, if a biomaterial was inserted into a human, the surrounding fluid will vary depending on location with possibilities of tear fluid, tissue fluid, serum/ blood, urine, or saliva. Biomaterials are commonly inserted into an environment consisting of blood serum and from this environment the conditioning film will consist of the main proteins present in the environment: albumin, immunoglobulin, fibrinogen, and fibronectin. The physico-chemical properties of the substrate such as the hydrophobicity, chemical composition and charge leads to variation in the composition of the conditional film, affecting what microorganisms are able to attach and colonise the surface (Gottenbos *et al*., 2002).

1.2.2.2 Biofilm formation and maturation

Microorganisms capable of successful adhesion to the substrate surface are classified as primary colonisers, with suitable conditions the primary colonizers are able to propagate and form microcolonies: microscopic colony of bacterial cells capable of growing in suboptimal conditions. Once the primary colonisers cover the surface of the substrate, environmental conditions change within the biofilm allowing adhesion of other microorganisms, identified as secondary colonisers which leads to the development of a mixed species biofilm (Rickard *et al*., 2003). The density and thickness of a biofilm is dependent on the surface of the substrate, biofilm monolayers are located on soft substrates such as gingival tissue. In contrast, hard surfaces lead to biofilms that are several bacterial cell layers thick, if these biofilms are undisturbed they have the capability to reach thicknesses of several hundred micrometres (Kolenbrander, 2000).

Previous studies have showcased biofilms to be considered 'cities' of microorganisms, due to the high level of co-ordination and multicellular behaviour. Quorum sensing (QS) is a regulating mechanism

bacteria use to regulate their physiological processes and cooperative activities, facilitated by the production, detection, and response of small diffusible signalling molecules between bacterial cells. It has also been recorded that communication through QS is important in biofilm formation (Li *et al.*, 2012).

Originally the first recorded adhesion between genetically distinct suspended bacteria, termed coaggregation was recorded in human dental plaque. Adhesion was found to be highly specific between pairs of bacteria, mediated by complimentary systems where one strain of bacteria produces adhesion proteins, and the other strain produces saccharide receptors. Alternatively, co-adhesion is the adhesion between suspended cells and biofilm cells attached to a substrate. Currently there are two routes in which coaggregation interactions occur in the development of a biofilm; the first route occurs while in a suspension, the single cells recognise genetically distinct cells present in the developing biofilm and adhere to them, the second route involves coaggregation occurring while the cells are present in the suspension, followed by co-adhesion of the cell mass into the developing biofilm. Further studies have shown that coaggregation is no longer exclusively occurs in the dental environment, and has been recorded in the urogenital tract in humans, the gut of mammalian creatures and potable-water-supply systems (Kolenbrander, 2000; Rickard *et al.*, 2003).

Within a biofilm community the cells begin to produce and secrete a polymer matrix that will encase the microbial community, protecting the cells from the external environment in a three-dimensional structure. This polymer matrix consists of extracellular matrix and extracellular polymeric substances, on average the thickness of the extracellular polymer matrix ranges from 0.2 – 1.0 µM and the average size of a biofilm ranges from 10 – 30 nm (Jamal *et al.*, 2018; Nepper *et al.*, 2019). Regardless of biofilm size, the microorganisms present in a biofilm account for less than 10% of dry mass, with the biofilm matrix accounting for over 90% (Flemming *et al.*, 2010).

2.2.2.3 Biofilm disruption and detachment

A secondary maturation stage involves disruptive forces, used to aid in the formation of the biofilm structure and additional adhesive mechanisms. This process is mediated by enzymes capable of degrading biofilm polymers: proteases, nucleases, and surfactant-like molecules e.g. staphylococcal phenol-soluble modulins. These enzymes are then responsible for the detachment of a biofilm from a substrate and the dispersal of the biofilm (Otto, 2019).

Biofilm detachment occurs for a multitude of reasons; physical triggers such as fluid shear stress, loss of flow in environment and total bacterial biofilm mass. Biological causes for detachment include the depletion of metabolic substrate and accumulation of metabolic products. Detachment is facilitated by QS signals, matrix-degrading enzymes, and the activation of lytic bacteriophage. Hunt *et al.,* (2004) conducted a study on *Pseudomonas aeruginosa (P. aeruginosa)* biofilms showing that the biofilms detached following stoppage of a continuous flow of liquid medium and during a depletion of nutrients in the environment. Detachment was recorded in an environment where there was a continuous flow

but a loss of nutrients, indicating that starvation was a reason for detachment, not an accumulation of metabolic products (Hunt *et al.*, 2004).

Different bacterial species produce a range of saccharolytic enzymes: alginate ligate in *P. aeruginosa,* hyaluronidase in *Streptococcus equi* and *N*-acetyl-heparosan lyase is produced in *Escherichia coli (E. coli)*. These enzymes lyse the extracellular polymer matrix enabling biofilm detachment and the release of bacterial cells. During biofilm detachment bacterial cells also upregulate gene expression of proteins leading to flagella formation, allowing these released bacterial cells to travel greater distances in an environment before attaching to a new surface for biofilm formation (Jamal *et al.*, 2018).

Quorum sensing, while responsible for aiding gene expression required for biofilm growth and development, also has an involvement in biofilm disruption and detachment. Staphylococci contain an accessory gene regulator (*agr*) QS system (Figure 1.2); where upon the biofilm community reaching quorum, is responsible for upregulating the expression degradative exoenzymes and toxins, while downregulating surface adhesion proteins (Otto, 2013). The *agr* operon encodes four proteins: AgrA, AgrB, AgrC and AgrD. AgrD is made up of three domains: a 'pro-peptide' domain containing the precursor of the autoinducing peptide (AIP) QS signal, an N-terminal domain capable of localising the protein to the inner leaflet of the plasma membrane and a C-terminal recognition domain. The integral membrane endopeptidase, AgrB, recognises AgrD's C-terminal domain and cleaves it, the C-terminal residue is cyclized in the pro-peptide domain. AgrD, now modified, is transported outside the plasma membrane, following the cleavage of N-terminal domain the matured AIP signal is capable of leaving the cell. There is an accumulation of the AIP signal as the bacterial community continues to grow and cell density increases, once a threshold concentration of AIP has been reached the AIP binds to AgrC, a histidine kinase transmembrane receptor, at a specific target site; the AgrC transautophosphorylates and phosphorylates AgrA, an intracellular transcription factor. The phosphorylated AgrA binds to the P2 promoter and upregulates the *agr* operon, increasing the production of AIP QS signal, the phosphorylated AgrA also binds to a P3 promoter that upregulates a multitude of virulence factors: haemolysins and phenol-soluble modulins, these factors are associated with typical *Staphylococcus aureus (S. aureus)* infections (Vasquez *et al.*, 2019).
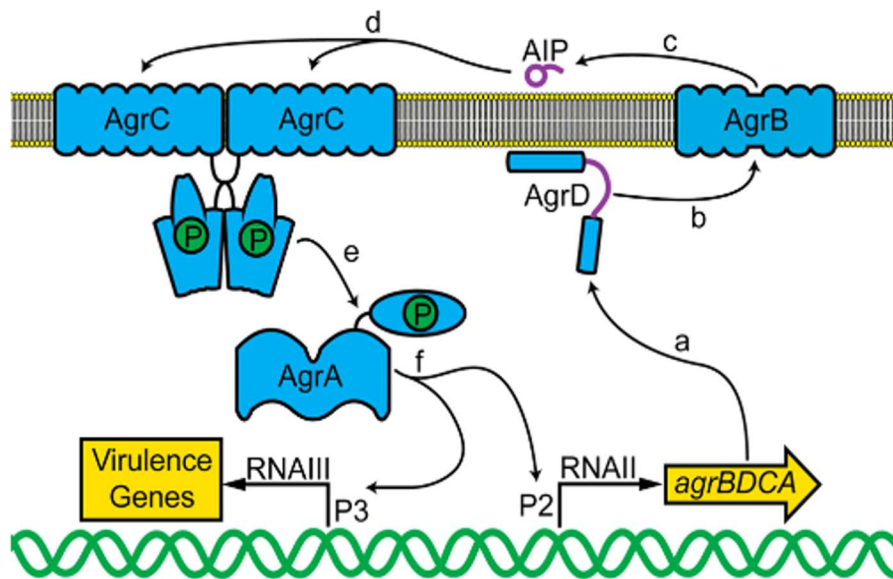
Figure 1.2: Diagram showcasing the agr QS system

Diagram labelled a – f to expand on the function of the agr QS system: a) Production of AgrA-D encoded by arg. b) AgrD's C-terminal domain is recognised by AgrB and is bound c) AgrB cleaves the C-terminal domain of AgrD and releases the AIP QS signal inside ArgD d) ArgC and the AIP signal bind together once there is an adequate accumulation of the AIP signal reaching the threshold concentration e) AgrC then transautophosphorylates ArgA and then phosphorylated it f) ArgA binds to the P2 promoter leading to the upregulation of the Agr operon, ArgA binding to the P3 promoter leads to the upregulation of virulence factors (Vasquez et al., 2019).

1.3 Antimicrobial resistance

Antimicrobial resistance (AMR) occurs when microorganisms survive a treatment of antibiotics that would usually inhibit growth or kill them. Surviving strains then thrive in the environment due to lack of competition of other organisms, leading to further growth and spread of resistance. 'Superbugs' is a title given to bacteria that have become difficult or even impossible to treat with current medicine (O'Neill, 2016). The WHO's priority list states a list of bacteria that need research and development for new antibiotics due to their resistance to current treatment, with *P. aeruginosa*, Enterobacteriaceae (including *E. coli*) and *Acinetobacter baumannii (A. baumannii)* ranked as in critical need of new antimicrobials. Followed by a high priority list consisting of *Enterococcus faecium (E. faecium)*, *S. aureus*, *Salmonella* spp., *Helicobacter pylori*, *Campylobacter* and *Neisseria gonorrhoeae (N. gonorrhoeae)* (WHO, 2017).

1.3.1 The problem with antimicrobial resistance

Antimicrobial resistance has been recognised as one of the greatest threats to human health globally, and are responsible for 25,000 deaths in Europe and an increase in healthcare costs by €1.5 billion each year (ECDC *et al.*, 2009). It has been estimated to cost the National Health Service (NHS) around £180 million per year to try and treat patients with resistant infections. Posing an even greater threat in the USA, accounting for 100,000 deaths and treatment costs for these infections ranging from $21- 31 billion (Oxford *et al.*, 2013; Williams, 2018)

Continuous use of current antibiotic treatments has triggered an emergence of multidrug resistant and extensively drug resistant bacterial species. The six nosocomial pathogens of concern that express multidrug resistance and virulence capabilities include a range of Gram-positive and Gram-negative microorganism: *E. faecium, S. aureus, Klebsiella pneumoniae (K. pneumoniae), A. baumannii, P. aeruginosa* and *Enterobacter* spp. and are termed ESKAPE microorganisms (Mulani *et al.*, 2019).

Resistance to antimicrobials has been recorded in a multitude of environments and is incredibly problematic in the healthcare environment. Specifically AMR biofilm infections pose an even greater threat to healthcare environments, with previous studies showing that minimum inhibition and bactericidal concentrations to treat biofilm bacterial cells are approximately 10 – 1000 times higher than the corresponding planktonic bacterial cells, due to increased resistance and protection the biofilm matrix offers (Hengzhuang *et al.*, 2011).

While the threat of AMR has been recognised, there has been no new class of antibiotics introduced as a treatment for over the past 30 years (Figure 1.3), highlighting the importance of drug discovery to tackle resistant infections. Infections caused by resistant strains of pathogens have been estimated to lead to 10 million deaths each year by 2050 (Williams, 2018). With recent studies showing that groundwater acts as a major global reservoir for AMR bacteria, where 76.9 % ± 22.7 of individual wells and springs tested in aggregated groundwater contained AMR bacteria and 57.2 % ± 36.8 of these isolates were resistant to up to 3 different antimicrobials. Emphasising the ease of how AMR bacteria can spread, as 2.2 billion people in developing countries use groundwater supplies as the primary supply of drinking water (Andrade *et al.*, 2020).

Antibiotic discovery and resistance timeline

Antibiotic class: PENICILLINS, MACROLIDES, CARBAPENEMS, TETRACYCLINES, FLUOROQUINOLONES

Date of resistance identified: 1940 1953 | 1985 1993

Date of discovery: 1928 1948 | 1985 | 30 years since a new class of antibiotics was last introduced

Year: 1920 1930 1940 1950 1960 1970 1980 1990 2000 2010 2020
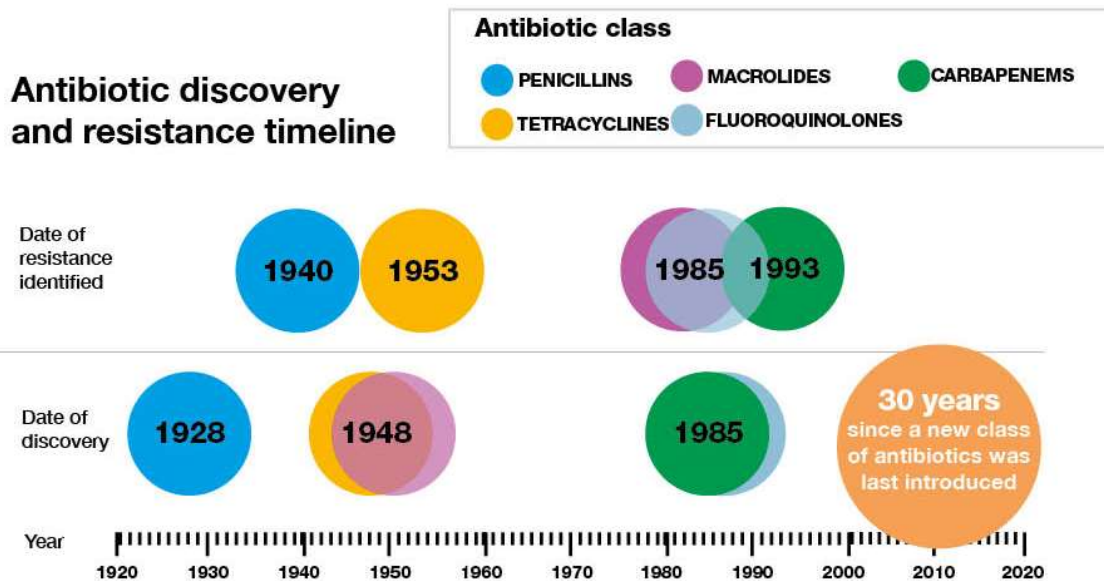
Figure 1.3: Timeline illustrating antimicrobial resistance over a hundred-year time period for all main antibiotic classes.

Timeline includes the date of discovery and introduction to an antibiotic class in comparison to the year of resistant strains were reported (Public Health England, 2017).

1.4 Healthcare associated infections

Healthcare-associated infections (HCAIs) are defined as infections that appear between forty-eight and seventy-two hours after admission to a hospital and up to 30 days after discharge, most commonly occurring during the first seven days. HCAIs cover a broad spectrum of infections, with urinary tract infections, surgical site infections and pneumonia accounting for the highest number of infections; in addition bacteraemia and pneumonia are recorded to have the highest death rates (Andersen, 2019). Bacteraemia, post-surgery infections, urinary infections, chest infections and skin infections are the five most common types of HCAIs and it is believed that 5 – 20% of these infections are preventable (Robinson, 2001).

These infections affect 5 – 20% of hospitalised patients, with roughly 7.5 million patients in Europe suffering from HCAIs, comprising of 4.2 million patients in long-term care facilities and 3.5 million patients in acute hospitals, with a 7.1% recorded prevalence rate of HCAIs. Each year HCAIs kill around 147,000 people in Europe alone (Andersen, 2019). The Coronavirus pandemic has brought the issue of the HCAIs to the forefront of concern in both the public and scientific community, highlighting how critical it is to prevent infections spreading in the hospital and healthcare environment (Abd, *et al.,* 2022).

It has previously been recorded that at any one time around 9% of patients have acquired an HCAIs during their hospital stay. These infections lead to extended stay in the hospitals with prolonged discomfort and the potential of a permanent disability. HCAIs put a large strain on the NHS, leading to around £1 billion each year spent to try and treat and deal with these infections, where 15% (roughly

£150 million) could be freed up if there was an improvement in good practice throughout hospitals (General *et al.*, 2004). A recorded 834, 000 cases of HCAI in NHS hospitals throughout the country in 2016/17 accounting for £2.7 billion and 28,500 patient deaths (Guest *et al.*, 2020).

Healthcare associated infections affect 1.7 million patients in the USA and lead to 99,000 – 103,000 deaths each year (Andersen, 2019). Treating HCAIs has shown to be costly for hospitals across the world with expenditures ranging from $28.4 – $33.8 billion in the USA (Scott, 2009) and €7 billion in Europe annually (Andersen, 2019). Around a third of HCAIs can be prevented simply by improved hygiene, detection and infection control (Kelly *et al.*, 2012; Andersen, 2019). Additionally, multidrug resistant bacteria are responsible for 15.5% of global HCAIs with ESKAPE microorganism as the most common cause for these infections. The Clinical and Laboratory Standards Institute (CLSI) has reported that multiple antibiotic treatments appropriate against ESKAPE infections in 2010 are no longer suitable, further highlighting the emergence of resistance (Mulani *et al.*, 2019).

1.4.1 Urinary tract infections

Urinary tract infections (UTIs) are one of the most common bacterial infections, with reports of 150 million people worldwide being affected by UTIs each year. Urinary tract infections occur when bacteria rise from the perianal region and enter the usually sterile urinary tract. Women are more commonly susceptible to UTIs in comparison to men at a ratio of 8:1, due to them having a shorter urethra. Chances of reoccurring UTI infections are likely to happen for at least one third of women between 25-29 years old, and over 50 (Kontiokari *et al.*, 2001; Al-Badr *et al.*, 2013).

Urinary tract infections are commonly caused by *E. coli,* as it is the most established bacteria located in the human faecal flora and is isolated in over 90% of patients suffering from UTIs, particularly in complicated infections. Additionally, uropathogenic *E. coli* are the cause of over 80% of all community-acquired infections. Around 5 – 10% of UTIs are caused by *Staphylococcus saprophyticus* in healthy women, although these infections are deemed uncomplicated (McLellan *et al.*, 2016; Gupta *et al.*, 2017).

1.4.1.1 Catheter associated urinary tract infections

Studies have shown that 70 – 80% of common HCAIs are originated from indwelling urethral catheters, termed catheter associated urinary tract infections (CA-UTIs). Urinary catheters are a prevalent site to infections due to urinary catheters being the most common indwelling device in hospitalised patients. In sixty-six European hospitals alone 17.% of patients have a catheter, out of 183 hospitals in the USA 23.6% of patients have catheters (Nicolle, 2014). CA-UTIs are responsible for 40% of HCAIs in the USA annually, with recorded mortality rates in long term care facilities at 5 – 10%, when long-term indwelling catheters are involved. CA-UTIs lead to an increased hospital stay between 2 – 4 days, resulting in estimated additional hospital costs to assess, treat and care for patients with UTIs at $676 per patient (Saint, 2000; Hooton *et al.*, 2010). Inert catheters are susceptible to microbial colonisation, due to these

catheters being inorganic material in the host, therefore biofilm formation is likely to occur. With free-flowing bacteria attaching to the catheter, followed by the recruitment of additional bacteria to build up a biofilm, catheters provide an excellent environment for biofilm infections. As catheters have a large surface area and the surrounding environment contains urine components: calcium ions, magnesium ions and Tamm-Horsfall proteins, these components are all amalgamated into the extracellular polymer matrix of the biofilm, aiding biofilm viability. CA-UTI can be prevented by proper use of catheters and their removal when they are no longer needed (Trautner, 2010; Nicolle, 2014). Studies have shown that infections can occur in the following forty-eight hours once the catheter has been inserted with the risk of infection increasing by 5% each day it remains (Boltz, 2012).

1.4.2 Central venous catheter infections

Central venous catheters (CVCs) have been in use since 1952, where they were originally used to resuscitate or cannulate the subclavian vein of wounded soldiers (Polderman *et al.*, 2002). Central venous catheters are now used in critical care for the delivery of medication directly into the bloodstream, monitoring blood flow and in haemodialysis (Eisen *et al.*, 2006). It is estimated in the UK that approximately 200, 000 CVCs are inserted into patients each year and out of an estimated 150 million intravascular devices used in the USA each year, 5 million are CVCs (Worthington *et al.*, 2005). The presence of bacteremia originating from the intravenous catheter leads to catheter-related bloodstream infections (CRBSI), which are the most common cause of nosocomial bacteremia. The microorganisms responsible for these infections are normally skin flora that colonise the catheter once it has been inserted. Colonisation at the tip of the catheter during insertion is the common source of infection for patients in intensive care units (ICU) (Gahlot *et al.*, 2014). The common pathogens responsible for CRBSI are *S. aureus* (accounting for 40%) followed by *P. aeruginosa* and *Candida* species (accounting for 16% of infections), with *E. coli,* coagulase negative staphylococci, *A. baumannii* and *K. pneumoniae* as less frequent causes of CRBSI (Parameswaran *et al.*, 2011).

As CVCs are used on critically ill patients, there is a greater risk for infection, higher than any other medical devices for infections. With CVC infections causing increased hospital stays from 7 – 14 days and mortality rates of 10 – 20%. Treating patients suffering with CRBSI results in additional hospital stay around 6.5 days leading to an additional cost of $28,690, with daily costs in the ICU at $1152 and $375 in a standard day ward (Veenstr *et al.*, 1999; Polderman *et al.*, 2002; Gahlot *et al.*, 2014).

1.4.2.1 Intravascular catheter and implant infections

Intravascular catheters are involved in many medical procedures e.g. bone marrow/ organ transplants, cancer therapies and trauma surgery. Around 150 million intravascular devices are implanted into patients each year in the USA (Raad, 1998), these devices are susceptible to pathogenesis in two forms: the planktonic microorganisms free floating and spreading over the surface of the catheter, or sessile microorganisms that attach to the surface of the catheter and form biofilms. Important implants

that are critical to keeping a patient alive: pacemakers, artificial/ prosthetic valves and implanted defibrillators are also at risk of bacterial infections; on top of the associated issue of an infection triggering localised destruction of the surrounding tissue around the implant this may lead to the inhibition of correct functioning of the implant which can be detrimental. There is a 20% chance of an infection occurring for pacemakers with a recorded increase for ventricular assist devices and intra-aortic balloon pumps (Raad, 1998; Gattringer *et al.*, 2010).

Skin bacteria adhering to the surface of a device is the predominant route of implant and catheter infections. These infections occur when bacteria located on the surface of the skin migrate to the external surface of the catheter at the insertion site on the skin and are capable of colonising the intravascular tip of the catheter, leading to bloodstream infections. *Staphylococcus epidermidis (S. epidermidis)* and *S. aureus* are the most commonly associated pathogens derived from 250, 000 bloodstream infections each year and bacteremia originating from catheter infections occurs in 19% of hospitalised patients (Raad, 1998; Gattringer *et al.*, 2010).

1.4.3 Ventilator infections

Patients dependent of mechanical ventilation (MV) are at risk of nosocomial pneumonia: ventilator-associated pneumonia (VAP) either occurs at early onset (within ninety-six hours of MV) or late onset (after ninety-six hours of MV). Ventilator-associated pneumonia affects 8 – 28 % of patients, with a 3- to 10- fold increase in infection rates for patients in intensive care units (Chastre *et al.*, 2002). Mortality rates of VAP after forty-eight hours of endotracheal intubation range from 24 – 50 %, with cases of mortality rates being as high as 76% if there is the addition of lung infections (Chastre *et al.*, 2002; Pneumatikos *et al.*, 2009). *A. baumannii,* is an opportunistic pathogen commonly linked to VAP in intensive care units, due to its ability to form biofilms on abiotic surfaces such as the endotracheal tubes. These biofilms formed in the endotracheal tubes play a role in the development of VAP and increase the pathogenesis of these infections (Khazaal *et al.*, 2020).

1.4.4 Wound infections

Due to the loss of barrier function once the skin has been damaged, the wounds created are either acute or chronic wounds and chances of infections are very high. Exposed damaged wound tissues are highly susceptible to the development of microbial biofilms. These biofilms are responsible for the ignition of infection in the wound and the prevention of wound healing (Percival *et al.*, 2012). In the UK the cost of treatment for skin infections ranges depending on the severity of the infection, pressure ulcers alone present a substantial burden on the NHS. The minimal treatment cost starts at around £1214 and costs increase substantially the more severe the ulcer; due to a range of factors such as a higher chance of complications to occur and increased hospital stay to allow the wound to heal, these infections can cost around £14,108 (Dealey *et al.*, 2013). Wound infections affect a great deal of the population particularly in the older population where diabetes and obesity are factors that increase the

frequency of these infections. In the UK alone up to 7% of the adult population suffer from chronic wounds, the costs of wound dressings and medication to treat these infections and the cost of staff in care facilities amounts to a £8.3 billion per year for the NHS. Of this total cost £5.6 billion was associated with the cost of wounds that were not healing, as only 45% of chronic wounds healed if an infection was involved or suspected to be involved with the wound (Guest *et al.*, 2020).

Previous studies have shown *P. aeruginosa* as one of the more commonly located microorganisms forming biofilms within burn wound sites, located on the tissue that surrounds blood cells and adipose cells. These *P. aeruginosa* biofilm infections cause both acute and chronic infections, and are the leading cause of mortality in burn wound patients (Schaber *et al.*, 2007). Biofilms are associated with other wound infections, with their presence recorded in diabetic wounds, pressure sores and venous stasis ulcers; *S. aureus* and *S. epidermidis* are two microorganisms commonly found in chronic wound infections, with slime layers from biofilms leading to a delayed wound healing response (Schierle *et al.*, 2009).

The main treatment strategy for acute and chronic wounds involves application of wound dressings, although depending on the severity of the wound, alternative treatments are available including: cell therapy, platelet therapy and instrumental based therapy consisting of laser therapy, hyperbaric oxygen therapy and negative pressure wound therapy. Out of all the treatments available, wound dressings are the most common form of treatment involving the application of different dressings depending on different factors of the wound i.e. size, location, depth, and type of wound. These dressings act as a physical barrier to prevent the transfer of microorganisms between the external environment and the wound leading to decrease wound healing time, current dressings release bioactive molecules to aid in maintaining the optimum environment for wound healing. Historically traditional wound dressings utilised a multitude of natural materials ranging from leaves, spider webs and even honey to protect the wound from the external environment and prevent bleeding. Currently there are passive wound dressing involving bandages, cotton pads and sterile gauze, the two classifications of passive wound dressings depend on the application, either applying dry dressings to the wound or wet dressings. The application of wet wound dressings leads to 3 – 5 times quicker wound healing in comparison to dry dressings; due to a moist wound bed being key for rapid wound healing and wet dressings maintains this environment. Dry dressings may adhere to the wound site making it painful to remove. The hydrophilic nature of biopolymer-based materials and the ability to absorb tissue exudates and prevent dehydration of the wound site while enabling the permeation of oxygen into the area. Modern dressings are the final classification of wound dressings and consist of a range of materials: film layers, foams, hydrocolloids, and hydrogels. These modern wound dressings also contain growth factors to stimulate wound healing and the first 'engineered skin' approved by the US Food and Drug Administration (FDA) incorporates Apligraf into the dressings; Apligraf (Organogenesis Inc.) is a living cellular skin substitute produced by keratinocytes and fibroblasts. Additionally alginate has been incorporated into multiple wound

dressings, leading to an increased hydrophilic nature increasing the rate of wound healing (Varaprasad *et al.*, 2020; Mirhaj *et al.*, 2022).

Bacterial species capable of forming biofilms are also reported to form on wound surfaces, these biofilm infections are reported to hinder the healing of wounds, 60% of chronic wounds and 6% acute wounds contained established biofilms, currently wound dressing containing antibiotic treatments are a promising form of treatment against biofilm related wound infections. Temperature is a limiting factor when it comes to wound heading and wound infections, the temperature of a wound can range between 33 – 41°C. When temperatures around a wound are below 33°C wound healing is hindered and in contrast temperatures above 37°C indicate inflammation localised around the wound, but a sudden increase in temperate signifies the occurrence of an infection. Therefore, alternative pioneering wound dressings containing temperature sensors can monitor the temperature, pH, moisture, and oxygen levels of wound, enabling real-time readings to flag when a potential infection may occur. As of 2019 the market for developing and utilising wound dressings is around $570.1 million (Darvishi *et al.*, 2022).

1.4.5 Joint infections

Arthroplasty is the term given to a replacement joint or surgical reconstruction of a joint. There is a high likelihood of bacterial infections in the form of biofilms due to the implant being an abiotic object. These infections around an implant can be classified as periprosthetic joint infections (PJIs), they are difficult to diagnose and treat, leading to a global issue (Li *et al.*, 2018). The colonisation of implants occurs as early as the time of implantation, with *Staphylococcus* species responsible for the majority of PIJs, in particular: *S. aureus* (22 – 23.6%) and *S. epidermidis* (19 – 37.5%) (Rohde *et al.*, 2007).

Cutibacterium acnes (*C. acnes,* formerly *Propionibacterium acnes*)*,* an anaerobic slow-growing Gram-positive microorganism was initially discounted as a pathogen located in surgical samples under the initial assumption of the presence being a sample contamination, due to *C. acnes* commonly located on the skin flora. Further studies have shown that C. *acnes* can act as an opportunistic pathogen, present in roughly 4 – 6% of all PJIs but its low virulence and low bacterial burden make these infections hard to detect. Due to the lack of symptoms, optimum treatment for PJIs that involve C. *acnes* infections has not yet been created. *C. acnes* has the virulent property of biofilm formation leading to the reduction of antibiotic susceptibility and acts a key agent in sever PJIs leading to polymicrobial biofilms with staphylococci (Söderquist *et al.*, 2010; Figa *et al.*, 2017).

1.5.6 Surgical site infections

Surgical site infections (SSIs) are common within the healthcare system and occur at the incision wound site or organs after surgery. The unintentional transfer of microorganisms from the hands of the surgical team into a wound site during an operation leads to SSI. While sterile gloves are worn, perforations can occur during surgery leading to a transfer of microorganisms. This form of infection makes up 20% of HCAIs, occurring in 2 – 5% of surgical patients annually, leading to an increase in hospital costs of

$20,000 per patient, for the treatment of the infection and for the increased hospital stay, which on average is an additional 10 days (Tanner *et al.*, 2016; Berriós-Torres *et al.*, 2017; Lawrence *et al.*, 2019).

The operating theatre itself, instruments and material used during the procedure as well as the patient's own endogenous flora can lead to SSIs; microorganisms commonly associate with SSIs include: *S. aureus, E. coli, Enterococcus* spp. and coagulase-negative staphylococci. Multiple measures and infection control strategies are in place to reduce the risk of SSIs. Where possible, skin carriage of potentially dangerous organisms and current known infections are treated before the operation. Pre-operative skin disinfection involves application of antiseptic solutions, e.g. chlorhexidine-based antiseptics: ChloraPrep to rapidly kill or remove skin microorganisms from the incision site and reduce the risk of contamination during surgery. All surgical staff ensure nails are kept short and wash hands up to the elbows for at least two to five minutes with an appropriate antiseptic (Owens *et al.*, 2008).

1.4.7 Dental biofilm infections

Restorative materials used in dental procedures are susceptible to biofilm formation as microorganisms can adhere to the hydrophobic and hydrophilic surfaces; these biofilms affect oral health, often leading to onset tooth decay. High levels of biofilm form on rough surfaces as it is easier for microbial attachment, with *Candida* species often found on acrylic dentures. *Streptococcus mutans*, present in the oral microbial community, is a highly acidogenic and aciduric microorganism, contributing to the pathogenesis of dental decay; with biofilms forming on the dental surface as a key virulence factor (Busscher *et al.*, 2010; Cocco *et al.*, 2020).

1.5 Antimicrobial resistance in hospitals and the community environments

Antimicrobial resistant bacteria are not exclusively a problem for healthcare institutions, with reports of AMR infections in community environments such as community associated *S. aureus* strains expressing resistance to methicillin. Community spread AMR bacteria pose increased difficulties regarding the control of infections, thus making them more difficult to treat, not only at long-term care environments but also common situations and places such as day care centres for children, sports facilities which can affect athletes and their teams as well as military bases (Tenover, 2006). In 2016 the reported death rates due to AMR infections was 700, 000 per year and if AMR continues, it has been estimated that by 2050 superbugs would be responsible for up to 10 – 50 million deaths and cost $100 trillion globally, making AMR the leading cause of death, overtaking cancer. The current concern with AMR is the current generation of novel antibiotic treatments is insufficient to counter the current rate of bacterial resistance, therefore it is imperative to generate novel antimicrobial treatment strategies to overcome resistant bacterial (Rappuoli *et al.*, 2017; Zhu *et al.*, 2022)

1.5.1 Methicillin resistant *Staphylococcus aureus*

S. aureus is a multidrug resistant pathogen, resistance to penicillin emerged two years after the introduction of penicillin as a viable antibiotic. To combat penicillin resistant *S. aureus*, methicillin, a semisynthetic antibiotic was introduced as a form of treatment in late 1950's. Methicillin resistant *S. aureus* (MRSA) outbreaks were first recorded in the UK in 1960. Following the outbreaks MRSA was mostly restricted to Europe, but after 1980, MRSA spread worldwide (Lakhundi *et al.*, 2018). *S. aureus* gained resistance through the acquisition and integration of the mobile genetic element staphylococcal cassette chromosome *mec* (SCCmec). This cassette is responsible for producing a methicillin-resistant transpeptidase (penicillin-binding protein 2a (PBP2a)) from the *mecA* gene. PBP2a also confers resistance to other β-lactam antibiotics. MRSA is responsible for many HCAIs: urinary tract infections, surgical site infections and central line-associated bloodstream infections. Although through prevention interventions there has been a decrease in MRSA from 2005-2008 but MRSA continues to be a major global healthcare problem (Kallen *et al.*, 2010).

Community infections due to MRSA were initially rare, although an increase of incidences are being reported. Five different SCCmec types (designated types I-V) have been identified in MRSA strains, community associated MRSA is mostly associated with type IV. Studies have shown that the type IV cassette carries the Panton-Valentine leucocidin (PVL) gene, leading to skin and soft tissue infections as well as severe pneumonia, which infects children and young adults, whereas MRSA usually infects older adults (Francis *et al.*, 2005).

Both methicillin resistant and susceptible strains of *S. aureus* (MSSA) are noted to be largely responsible for infections linked with biofilm development, having the potential to infect nearly all host sites present. Biofilm infections associated with MRSA strains are then capable of colonising different types of implantable medical devices, ranging from orthopaedic/ cardiac prostheses, tubes and catheters (Figueiredo *et al.*, 2017).

1.5.2 Vancomycin resistant Enterococcus

Enterococci are multidrug resistant Gram-positive bacteria. In 1988 there were reports of enterococci with vancomycin resistance, with 55 strains of vancomycin resistant enterococci (VRE) recorded from 22 patients in end-stage renal failure (Murray, 1990). Vancomycin resistance has been classified into five phenotypes: VanA, VanB, VanC, VanD and VanE. VanA and VanB are primarily found in *Enterococcus faecalis* (*E. faecalis*) and *E. faecium,* minimum inhibitory concentrations (MICs) range from 4 – 1024 µg/mL. *E. faecium* may also contain the phenotype VanD, while *E. faecalis* may also contain VanE, although these phenotypes can only resist lower levels of vancomycin, as MIC values ranged from 16 – 128µg/mL (Cetinkaya *et al.*, 2000).

Vancomycin resistant enterococci infections are one of the main causes of HCAI, in particular *E. faecium* which accounts for 77% of VRE. Infections predominantly occur in immunocompromised

patients e.g. those undergoing allogeneic hematopoietic cell transplantations (allo-HCT). Due to these patients receiving a variety of antibiotics to prevent any infections or to treat current infections during this procedure; the use of different antibiotics leads to a loss in the intestinal microbiota, where diverse species of bacteria are usually found in a healthy intestinal tract. VRE are able to inhabit and thrive in the decreased diversity in intestinal tract, dominating remaining bacteria in the microbiota, persisting for over four weeks after ampicillin treatment and a high density of VRE can persist for months in mouse models. These VRE infections in allo-HTC patients lead to increased risks for graft-versus-host diseases and bacteraemia (Dubin *et al.*, 2019).

1.5.3 Extended spectrum β-lactamase producing microorganisms

Beta lactams (β-lactams) are a classification of antibiotics named after the β-lactam ring. Discovered in the 1920's, they are the most commonly used antibiotics accounting for 65% of injectable antibiotic prescriptions in the United States. The mechanism of actions for β-lactam antibiotics is to inhibit bacterial cell wall synthesis by binding to penicillin-binding proteins that are involved in the terminal process of peptidoglycan cross-linking. Resistance to β-lactam antibiotics is due to the production of β-lactamases, which cleaves the amide bond in the β-lactam ring in the antibiotic rendering it harmless to the bacteria (Bonnet, 2004; Bush *et al.*, 2016). Biofilm production actively protects the bacterial cells from antibiotic treatments by preventing antimicrobials from passing through the extracellular polymer matrix; this biofilm capability paired with *P. aeruginosa* isolates that have a high expression of chromosomal β-lactamase enable proficient resistance to β-lactam antimicrobial treatments. The overproduction of β-lactamase leads to a recorded presence of β-lactamase in the extracellular polymer matrix, which in turn hydrolyses β-lactam treatment before the antibiotic is able to reach bacterial cells (Høiby *et al.*, 2010).

Cephalosporin C was naturally discovered during the 1950's, and was subsequently developed into the important group of semisynthetic cephalosporin antibiotics (Bush *et al.*, 2016). Third generation cephalosporins were introduced as antibiotics in the early 1980's to tackle the increasing problem of an increasing prevalence of β-lactamases in *E. coli* and *K. pneumoniae* as well as the spread of resistance to other pathogens, for example *N. gonorrhoeae*. Three years after the introduction of third generation cephalosporins, resistant strains started to occur, these strains contained β-lactamases, which had the capability of hydrolysing cephalosporins (Paterson *et al*, 2005). Extended spectrum beta-lactamases (ESBL) is the term given to enzymes capable of hydrolysing the extended spectrum of cephalosporins, these enzymes are produced by Gram-negative bacteria, particularly by *P. aeruginosa* and Enterobacteriaceae. Organisms producing ESBL are resistant to many cephalosporin antibiotics, for example ceftriaxone, cefotaxime, ceftazidime, and to oxyimino-monobactams (e.g. aztreonam). By 2010 the European Committee on Antimicrobial Susceptibility Testing (EUCAST) stated that ESBL-producing-Enterobacteriaceae (ESBL-E) were resistant to treatments of cephalosporins. Leading to the need of alternative treatment and the increased use of carbapenems (Fournier *et al.*, 2013).

Originally the Carbapenems: imipenem, panipenem, meropenem, ertapenem, biapenem and doripenemn were used to kill microorganisms that were resistant to penicillin and cephalosporin antibiotics, in addition to being a last-resort antibiotic to treat patients who had multidrug resistant infections (Pham *et al.*, 2020). From the increased use of carbapenems, bacteria are evolving to counter the treatment, leading to carbapenemase-producing Enterobacteriaceae (CPE) strains being reported worldwide. CPE are resistant to not only carbapenems but most of the other β-lactams and other classes of antibiotics leading to a limited line of treatment to combat these infections (Roujansky *et al.*, 2020).

1.6 Current strategies to treat biofilms

While antimicrobials are the current most effective form of treatment against microbial infections, bacterial biofilms are known to naturally be highly resistant to antibiotics, hard surface disinfectants and metal ions in comparison to planktonic cells; due to microorganisms in a biofilm undergoing cell specialisation, leading to an increase of emergent functions in the biofilm community that further differentiates biofilms from planktonic microorganisms (Harrison *et al.*, 2007). Previous studies have shown that antimicrobial concentrations required to treat biofilm bacterial cells are approximately 10 – 1000 times higher than the corresponding planktonic bacterial cells (Hengzhuang *et al.*, 2011).

1.6.1 Antimicrobial biofilm treatments

Antibiotics that are known for being highly penetrating are selected for long term type of treatment, usually in the form of a combination therapy with a range of mechanisms of action to combat biofilm infections. Therefore, β-lactams, aminoglycosides and polymyxin are not sufficient for treatment due to being weaker penetrators in tissue and cells in comparison to other antibiotics: macrolides, lincosamides, tetracyclines, rifamycins, quinolones, fusidic acid, nitroimidazole, sulfonamides and oxazolidinones. When a biofilm infection is caused by the insertion of a foreign body e.g. a CVC, removal of the object is most commonly required in order to eradicate the infection (Wu *et al.*, 2015).

1.6.1.1 Rifampicin based biofilm treatments

Rifampicin has alternative forms of treatment against biofilm infections i.e. using rifampicin in a combination therapy as a treatment against biofilm infections, but also the ability to topically apply and impregnate surfaces with rifampicin and additional antimicrobials to prevent infections occurring. Catheters impregnated with rifampicin-sparfoxacin were designed to slowly release the antimicrobials from the catheter surface, leading to a reduction of *S. epidermidis* adhesion to the treated catheters (Katsikogianni *et al.*, 2004). Topically applied rifampicin and minocycline have recorded success in preventing catheter related infections, additionally, rifampicin treatments are capable of preventing prosthetic vascular graft infections, through the pre-treatment of rifampicin on grafts with the combination of systemic vancomycin (Gattringer *et al.*, 2010).

Unfortunately, with the ever-growing issue of AMR, there is a recorded emergence of rifampicin resistant biofilm infections, leading to rifampicin no longer being an effective antimicrobial for biofilm treatment as a single agent. The glycopeptide daptomycin is a commonly used antibiotic to target a variety of staphylococcal infections including MRSA strains. When targeting staphylococcal biofilm infections daptomycin alone as a treatment showcases low levels of activity, though, when used in a combination treatment with rifampicin, daptomycin is capable of effectively preventing onset resistance to rifampicin (Gidari *et al.*, 2020).

Vancomycin and linezolid are also befalling a similar fate to rifampicin, with studies showing these antimicrobials missing an effective activity against staphylococci that are found embedded in biofilms. However, there are still antibiotics capable of inhibiting MRSA strains embedded into biofilms such as: daptomycin, minocycline and tigecycline with an increased efficacy in eradicating MRSA in biofilms with a combination of rifampicin (Raad *et al.*, 2007).

1.7 Alternative strategies for treatment of biofilm infections

With the ever growing need to tackle ARM infections, in particular biofilm infections, alternative treatment strategies are under development, these alternative strategies can be broken down into two categories: the creation of new treatments and reutilising treatments that were not initially designed to treat AMR infections. As antimicrobials alone are often inadequate to treat a biofilm infection, other strategies are required in an attempt to combat these resistant infections. Alternative treatments such as bacteriophage therapies and QS inhibition to target a biofilm infection are being researched as novel methods.

1.7.1 Quorum sensing inhibition

Quorum sensing is a regulating mechanism bacteria use to regulate their physiological processes and cooperative activities. It has also been recorded that communication through QS is important in biofilm formation (Li *et al.*, 2012).

P. aeruginosa is one of the most common Gram-negative microorganism associated with nosocomial infections and is frequently present in infections of immunocompromised patients. Nosocomial infections affect patients suffering with cystic fibrosis and these patients are susceptible to chronic lung infections caused by *P. aeruginosa*, as once biofilm formation occurs in the patients' lungs it is difficult to eradicate the infection (Johansen *et al.*, 2008). Studies have shown that QS inhibitors e.g. furanone compound 30 (C30) and 56 (C56), are known antagonists for QS in *P. aeruginosa.* Furanone is capable of inhibiting gene expression regulated by the QS systems in *P. aeruginosa*; leading to the disruption of biofilm formation. Quorum sensing inhibition (QSI) has the potential to be a new treatment for patients with cystic fibrosis to prevent further infections (Wu *et al.*, 2004)*.

Additional studies have further highlighted the effectiveness of QSI treatments. Due to the treatment strategy not affecting pathways that are essential for the viability of the bacteria, there is a lower likelihood for selective resistance to occur. Singh *et al.* tested the combination therapy of agents that disrupt biofilm formation and an antibiotic in a single formulation. The combination therapy consisted of ciprofloxacin and a known QSI for *P. aeruginosa*: 3-amino-7-chloro-2-ninylquinazolin-4(3H)-one via a delivery system of a bioresponsive polymer formulation, which was initially derived from a modified alginate nanoparticle, capable of forming a link between the QSI and encapsulate ciprofloxacin. These nanoparticles have the capability to effectively penetrate *P. aeruginosa* biofilm allowing ciprofloxacin to overcome the bacterial biofilm response to antimicrobials and effectively treat the infection. The combination treatment was recorded to be more efficient in eradicating *P. aeruginosa* biofilms in comparison to just antimicrobials alone, with the killing of bacterial cells recorded deep within the biofilm layer. Singh *et al.* has showcased the potential for QSI combination therapy with antimicrobials and the enhancement of this treatment through the addition of a nanoparticle delivery system to combat *P. aeruginosa* biofilm infections and the ability to prevent initial biofilm formation (Singh *et al.*, 2019).

1.7.2 Bacteriophage treatment

Bacteriophages are viruses capable of targeting specific strains of bacterial species, due to their level of selectivity and with no recorded instances of infecting human cells, making bacteriophage therapy a desirable form of treatment against bacterial human infections (Doub, 2020). For over 50 years scientists have utilised the ability of bacteriophages replicating and killing their host bacteria, as a therapy to treat antibiotic and multidrug resistant bacteria. With more recent studies showing bacteriophages are able to treat biofilm infections, including those present in implant, catheter and wound related infections (Wu *et al.*, 2015).

Phages have an advantage over standard antimicrobial treatments through their ability to counter defence mechanisms in bacteria, in particular the virulence response of biofilm formation; due to co-evolving with their hosts throughout the ages, therefore they are incredibly effective against antibiotic resistant biofilms (Burrowes *et al.*, 2014). An additional advantage of phages as a treatment strategy over standard antimicrobials is their innate ability to replicate in the presence of the host bacteria and limit replication when there is a lack of bacterial substrate present, they are able to penetrate biofilms and eliminate persister cells, through infecting persister cells and once these cells become metabolically active, the phages then lyse the cells.

Unfortunately, phages have the same shortcoming as antimicrobials as bacteria have the ability of evolving resistance to the phage infections. However, while bacterial evolution to resist phage infections is problematic, it is not a detrimental issue, this selective pressure can be utilised as an evolutionary-based strategy; where bacteria trade off their widespread antibiotic resistance to tackle phage

treatments. Phage therapy therefore offers a win-win treatment, with initial killing of target bacteria and the evolution of phage resistance leading to a sensitivity to antibiotics (Chan *et al.*, 2018; Doub, 2020).

1.7.3 Aromatic compounds inhibiting biofilm formation

The second messenger 3' – 5' cyclic dimeric guanosine monophosphate (c-dic-GMP) is a key nucleotide for biofilm formation, acting as a regulator for the transition of motile to non-motile lifestyles. While the majority of research on the role of c-dic-GMP has been conducted on Gram-negative microorganisms, further studies have shown the nucleotide plays a similar role in Gram-positive microorganisms although the mechanism of regulation may differ (Purcell *et al.*, 2016). Previous studies have shown that certain aromatic compounds i.e. acriflavine and proflavine interact with c-dic-GMP and form G-quadruplexes, inactivating the regulator, offering an alternative treatment to prevent biofilm formation (Nakayama *et al.*, 2011).

1.7.4 Novel antimicrobials

Scientific research is constantly underway to design novel antimicrobials to target a range of AMR infections. These novel antimicrobials are designed with the intention of working as a single form of treatment or in a combination therapy with commonly used current antimicrobials.

1.7.4.1 CBR-2092 anti-biofilm treatment

CBR-2092, a hybrid compound consisting of rifampicin and quinolone, has shown activity against wound infections caused by *S. aureus, S. epidermidis* and *Streptococcus pyogenes* in a mouse skin model, after three days of treatment. Biofilm infections in catheters, when treated for 14 days with CBR-2092 have shown a reduction in biofilm viability with no recorded resistance development. This lack of resistance shows the potential for this antimicrobial to be a highly effective anti-biofilm treatment (Lynch *et al.*, 2010).

1.7.4.2 Tedizolid anti-biofilm treatment with the addition of rifampicin combination therapy

Tedizolid (T-700), a novel oxazolidinone and the active drug of tedizolid phosphate was designed to combat linezolid resistance in Gram-positive biofilm infections. Tedizolids mechanism of action operates by inhibiting protein synthesis and has a recorded efficacy against Gram-positive biofilm infections including MRSA and MSSA. When used in combination with rifampicin, there was a significant increase in the efficacy of eradicating biofilms, with a considerable capability of breaking down the structure of pre-formed *S. aureus* biofilms. The presence of T-700 prevented the emergence of rifampicin resistance in MRSA and MSSA strains tested, with a similar effect recorded when rifampicin is used in a combination treatment with daptomycin against these biofilm infections (Gidari *et al.*, 2020).

1.7.4.3 Cationic antimicrobial peptides against biofilm infections

Cationic antimicrobial peptides (CAPs) are naturally forming peptides, located in a variety of organisms and account for a large portion of the innate immune system. CAPs also possess anti-biofilm activity, due to their ability to permeate the biofilm with mechanisms of action ranging from: the prevention of bacterial adhesion, modulating host response to the biofilm infection by increasing the activity of the host's immune cells and triggering the immune response, to interfering with gene expression relating to the bacteria's: motility, QS and matrix synthesis. These mechanisms of action are dependent on the specific CAPs, although their main mechanism of action is through the detachment of bacterial cells by increasing the permeability of the bacterial cell membrane. Naturally occurring CAPs are limited to functioning in their specific environment, therefore, synthetically created CAPs overcome this limitation and can be used as a targeted treatment.

6K-F17, a synthetic CAP, designed for non-specific physical disruption of membrane bacterial cells, but to also avoid targeting mammalian cells. The lack of specific membrane targeting allows a prolonged use of 6K-F17 for a treatment strategy, as bacteria are unlikely to quickly evolve resistance. 6K-F17 recorded efficacy against *P. aeruginosa* biofilms: PAO1 and multidrug resistant strains, all isolated from cystic fibrosis patients, and in comparison with tobramycin, the synthetic CAP was more effective at killing and greatly reducing biofilm viability of *P. aeruginosa* biofilms even in the presence of sputum. A combination approach led to a low dose of 6K-F17 aiding tobramycin and increasing its effectivity against eradicating biofilms (Beaudoin *et al*., 2018).

1.7.5 Novel wound dressing treatments against biofilm related wound infections

Currently, wound dressings and hydrogels are under development to contain antibiotic treatments to act as either prevention or treatment strategies against wound infections. Hydrogels are commonly used in wound dressings due to the ability to retain moisture and for the delivery of a drug into the target site. While gelatine hydrogels are structurally weak, crosslinks in hydrogels increase the tensile strength, glutaraldehyde is commonly used as the crosslink agent although the compound is known to be cytotoxic. Therefore, alternative crosslinking agents have been developed to reduce toxicity; genipin is a natural compound extracted from gardenia fruits and is capable of crosslinking with gelatine hydrogels with no toxicity. Alternate hydrogels are also being explored to enhance wound treatments. Currently hydrogels generated from chitin or chitosan's, polysaccharides derived from glucose, are recorded to be more compatible with fibroblasts to promote healing in comparison to current hydrogels made from alginate, gelatine or collagen, due to their recorded properties in regenerative medicine. Chitin and chitosan's activate macrophages and neutrophils through chemo-attraction and promote wound healing. Moreover these polysaccharides are non-toxic, biodegradable and have exhibited the capability of releasing cytokines or antibiotic treatments, highlighting the benefit of using these alternative hydrogels (Muzzarelli, 2009a, 2009b; Katas *et al*., 2021).

Recent studies have highlighted silver nanoparticles to have a promising antibacterial and anti-biofilm capability against a range of clinically relevant microorganisms, through *in-vitro* testing. Silver-nanoparticles were particularly effective against Gram-negative bacteria that are typically harder to treat. Hydrogels were generated through the incorporation of silver nanoparticles into gelatine hydrogels cross-linked with genipin, to increase tensile strength. Through agar well diffusion testing, the silver nanoparticles are released through the hydrogel and into agar inoculated with: *S. aureus, P. aeruginosa, E. coli* and *Bacillus subtilis* isolates and led to the inhibition of all bacterial growth (Katas *et al.*, 2021).

1.8 *In-vitro* development of bacterial infections

In order to assess the antibacterial properties of an antimicrobial against bacterial biofilms, *in-vitro* biofilm models are required to simulate a biofilm outside of its natural environment. A diversity in the composition of a biofilm containing the same bacterial species is reported depending on the origin of the biofilm, as physiochemical conditions lead to a variation in the metabolism and structural mechanisms to ensure survival; therefore, it is key to generate biofilm models that represent specific environments and are reproducible. Current biofilm models vary in complexity depending on the experimental needs, meaning no standardised biofilm model can be generated. These models range from simplistic biofilms formed on agar to continuous culture fermentation systems. *In-vitro* biofilm models are separated into two distinct categories depending on nutrient availability; closed systems biofilm model refers to a finite abundance of nutrients in the system whereas an open system has a continuous supply of nutrients into the environment (McBain, 2009).

1.8.1 Closed system biofilm models

Closed system biofilm models are often selected to study biofilm formation and early stages involved in the formation of biofilms in comparison to open systems due to their simplicity and reproducibility. No specialist equipment is required, allowing these experiments to be conducted in standard laboratories. Another benefit to closed system biofilm models is the variety of data that can be collected from testing ranging from microscopy to visualise bacterial cells to viability counts (Merritt *et al.*, 2005).

Historically, agar plates generated a simplistic biofilm model as bacterial growth on solid media had properties similar to bacteria found in biofilms, bacterial lawns were considered to be appropriate methods of generating bacterial cultures due to the high density of cells. Agar plate models have a limited nutrient availability so biofilm growth will be supported depending on the abundance of nutrients. This system simulates an environment similar to soft tissue infections. The addition of coupons i.e. filter paper placed onto the agar allows the formation of biofilms on abiotic surfaces that can be removed for further testing. The agar biofilm model was utilised to determine bacillus biofilm formation as the variation of bacterial morphology, in particular the formation of complex colonies, indicated biofilms (McBain, 2009).

1.8.1.1 Microtitre plate based system

One of the most commonly used closed system biofilm models throughout research utilises microtitre plates. Fletcher originally developed the microtitre plate based assay to investigate bacterial attachment to surfaces and the microtitre plate system also enables the study of sessile development in bacterial cultures (Fletcher, 1977). Alternatively, the addition of a coupon made of either a polymer or metal allows for biofilm formation to occur on a removable surface, these models use larger well microtitre plates (6 – 12 well plates) to enable larger coupons to be studied. The composition of these coupons can vary to ensure a relevant surface is available for specific biofilm formation. Due to the limitation of nutrients the environment will change throughout the study as nutrients will deplete during the formation and sustainment of biofilms, unless the medium is replaced. (Coenye *et al.*, 2010; Gabrilska *et al.*, 2015; Azeredo *et al.*, 2017).

1.8.1.2 The Calgary biofilm system

The traditional microtitre plate-based biofilm model was further developed in the form of the Calgary biofilm system and currently is one of the most popular models used in biofilm research (Ceri *et al.*, 1999). The key difference between the Calgary device and a standard microtitre plate are the cover lids; as the Calgary device has a cover lid with 96 pegs that become submerged into the media contained in the wells of the microtitre plate (Figure 1.4). The Calgary device utilises the formation of biofilms on the pegged lids, which in turn can be removed from the environment and transferred into new microtitre plates containing either fresh media for additional growth or antibacterial treatments. The pegged lid can also be used to quantify bacterial biomass once rinsing has occurred to remove any weakly attached bacterial cells. Furthermore, the Calgary device was designed and developed to overcome the limitation of standard microtitre assays, where the presence of sediment at the bottom of the microtitre wells becomes embedded in the biofilm matrix, leading to an inaccurate assessment of biofilm biomass remaining in microtitre wells (Azeredo *et al.*, 2017; Kragh *et al.*, 2019).
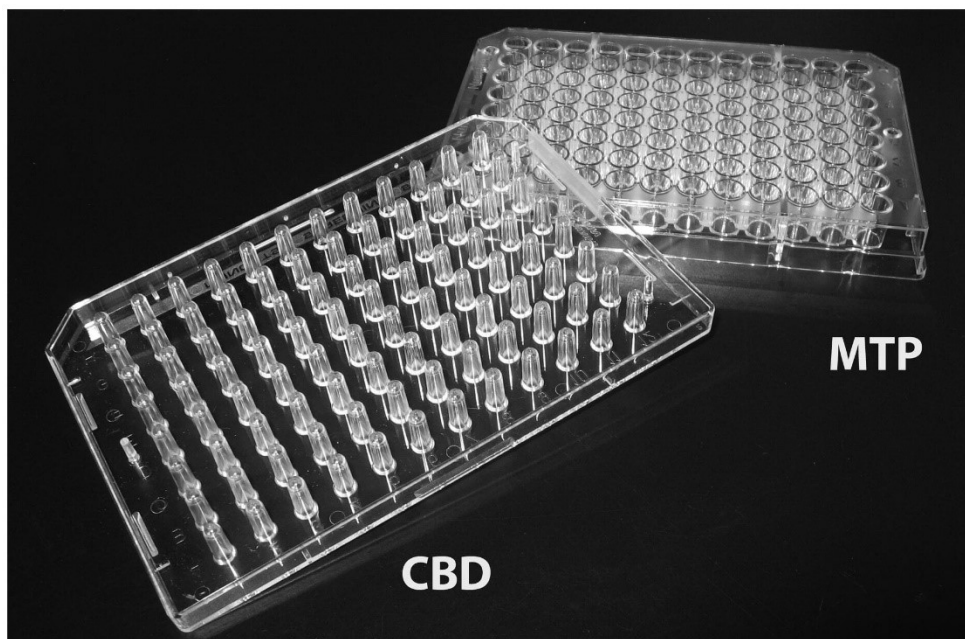
Figure 1.4 Photograph of the Calgary biofilm device

Cover lid containing 96 pegs (CBD) that fits into the wells of a standard 96 well microtitre plate (MTP). Image taken from: (Azeredo et al., 2017)

A limitation to both microtitre plate assays and the Calgary device is that the closed and static environment created within the microtitre plate promotes growth of planktonic cells due to the lack of selective pressures that would lead to presence of bacterial biofilms alone. Therefore the build-up of a planktonic population will compete with biofilms and use up available nutrients and other resources i.e. carbon and oxygen, reducing the total biomass of the established biofilms inside the wells of the microtitre plates and biofilms formed on the pegged lids (Azeredo *et al.*, 2017; Kragh *et al.*, 2019).

1.8.1.3 Biofilm ring test protocol

The biofilm ring test was designed to explore early stages of biofilm formation against a range of Gram-positive and Gram-negative bacteria (Chavant *et al.*, 2007). The protocol utilises paramagnetic microbeads as a surface for biofilm formation. A bacterial culture is mixed with the microbeads and then loaded into the wells of a microtitre plate before incubation. Following incubation, the plate is positioned over a specialised block containing individual magnets that are centred beneath each well in the microtitre plate. Low biofilm abundance on these magnetic microbeads allows magnetic attraction and the microbeads in the well cluster up under the magnet. The addition of contrast liquid allows for the visualisation of the microbeads and quantification occurs through specialised equipment. As the biofilm abundance increases on the surface of the microbeads, they become immobilised and are no longer magnetically attracted to the magnet, therefore no cluster of microbeads is observed. This protocol has been used to study the kinetics behind biofilm formation, the effect of co-administration of antibiotics on

biofilms and to compare the formation of biofilms between multiple bacterial species (Coenye *at al.*, 2010; Azeredo *et al.*, 2017).

1.8.2 Open system biofilm models

In contrast to closed system biofilm models, open systems are not as cost-efficient and require specialised equipment in order to run the models, additionally they are usually more complex than closed systems and are harder to use for high-throughput experiments. Consequently, standard laboratories are unable to use these models, although open system models have a range of advantages over closed system models as they better simulate a natural environment through the constant replenishment of nutrients and the addition of shear conditions. Certain bacterial species are unable to form biofilms without shear forces and flow displacement of the environment, therefore open system models are able to assess a wider variety of biofilms and the continuous supply of nutrients enables the sustainment of mature biofilms. Generally open system biofilm models are relevant to environmental biofilms, in particular those that form in aquatic environments; though this system also simulates an environment similar to the oral cavity and urinary tract (Gabrilska *et al.*, 2015).

Open system biofilm models fall into either category: continuous flow stirred tank reactor system (CFSTRS) or plug flow reactor system (PFRS). The CFSTRS operates without changes in concentrations over the time course, as the rate of growth medium added to the reactor is the same as the effluent removal rate, with the environmental conditions identical throughout the entire reactor. Planktonic cells are washed and removed from the reactor once the dilution rate is higher than the doubling time for the specific microorganism, ensuring that only sessile cells that have attached to surfaces remain in the reactor. In contrast the PFRS functions through the addition of growth media moving through the reactor as a plug in a singular motion in the direction of flow across the axial direction, leaving diffusion to occur along the radial direction, therefore there is a variation in the environmental conditions through the reactor (Coenye *et al.*, 2010).

1.8.2.1 Drip flow reactor

The drip flow reactor involves four individual test channels or chambers with vented lids where a coupon, usually a microscope slide, can be placed to offer a surface for biofilm formation. Alternatively, substrates could be ceramic or metal to simulate clinically relevant environments i.e. catheters tubing or the oral cavity to simulate biofilm formation in indwelling devices. Previous studies have also incorporated slices of pork or human tissue and sections of wound dressings to simulate a chronic wound infection (McBain, 2009). Growth medium or cell cultures enter each chamber through inlets in the lid releasing small volumes and the drip flow reactor system is set at an incline to generate a flow of the liquid, though in this model only low-shear fluid forces are used. This in turn promotes biofilm heterogeneity and a shift in dynamics as the biofilms mature due to the uneven distribution of media containing nutrients key for bacterial survival. In comparison to other open system wound models the

drip flow reactor has a limited number of samples, though is still considered a well-established model (McBain, 2009; Gabrilska *et al.*, 2015; Azeredo *et al.*, 2017).

1.8.2.2 Flow cell system

Flow cell system is a type of PFRS designed in the 1990's and is a specialised model for real time and non-destructive analysis of biofilms through confocal laser scanning microscopy. A standard flow cell system is made from polystyrene and the continuous flow of growth medium is mediated by a peristaltic pump, a microscope slide is the standard coupon for biofilm formation. Flow cell systems have been used to study a variety of bacterial biofilms including clinically relevant bacteria, in particular species found in the oral environment. The alteration of the rate of flow from laminar to turbulent allows for variation in the structure of biofilms to be observed (McBain, 2009; Coenye *et al.*, 2010)

1.8.2.3 The Robbins device

The Robbins device was originally designed by Robbins and McCoy to monitor the formation of biofilms depending on different fluid velocities simulated in drinking water facilities and recorded that high fluid velocity increased the induction time required for sessile cells to adhere and for biofilm formation to occur in comparison to low fluid velocities (McCoy *et al.*, 1981). The modified version of the Robbins device is at a much smaller size in comparison to the original, enabling the device to be utilised in standard laboratories. Biofilm formation occurs on plugs that are inserted into a line of ports along a tube either made of metal or plastic connected to a pump. The device is filled with an inoculum of planktonic cells and is then flipped over to aid in the adhesion of planktonic cells onto the discs located in the plugs, to further help biofilm formation on the plugs, either no flow or low flow of growth medium is run through the tube. Alternatively, biofilms are left to mature on the plug discs before the addition of liquid flow in the environment. Following biofilm formation, the device is turned back around and the pump is set to a continuous flow of the growth medium, though there is an uneven distribution of nutrients through the device, as a higher concentration of nutrients is present near the input and a build-up of waste is located around the output. The modified Robbins device model has been used to assess the formation of biofilms in varied environments i.e. rate of flow, nutrient availability; and to determine the effect of modifying materials on biofilm formation or retention under flow. The materials can be modified either through an antibacterial coating or altering the surface of the material. The modified Robbins device has also been selected as a biofilm model to assess antimicrobial lock therapy, where high concentrations of antimicrobials are added into tubing and locked in place to prevent flow throughout the tubing, to remove established biofilms (Coenye *et al.*, 2010; Gabrilska *et al.*, 2015; Azeredo *et al.*, 2017).

1.8.3 Quantification and visualisation of biofilm biomass

Through the use of available stains, the visualisation of bacterial biofilms can occur enabling the quantification of the total biomass or the assessment of biofilm components. Crystal violet is one of the

most commonly used stains due to its ability to stain negatively charged molecules, therefore crystal violet is retained in both bacterial cells and the extracellular polymer matrix that encases the biofilm. The density of a biofilm is determined through eluting the bacterial stain retained inside the bacterial biofilms and taking absorbance readings, the comparison of optical densities of biofilms treated with an antimicrobial vs untreated control biofilms enables the assessment of the effect of an antimicrobial on total biofilm density (Ebert *et al.*, 2021).

Alternative stains bind to different aspects of a bacterial biofilm, SYTO9 is a green fluorescent nucleic acid stain capable of binding to DNA, and the retention of this stain in biofilms highlights the presence of bacterial cells. Additionally, the reduction of certain dyes e.g. resazurin and fluorescein diacetate are used to distinguish live and dead cells based on cellular metabolic activity. The biofilm matrix is selectively stained with dimethyl methylene blue through the formation of complexes with sulphated polysaccharides (Gabrilska *et al.*, 2015).

1.8.4 Assessment of the anti-biofilm efficacy of antimicrobials

Minimum inhibitory and bactericidal concentration (MIC/ MBC) assays focus on determining the inhibitory and bactericidal effect of an antimicrobial against bacteria in planktonic mode of growth and are the most commonly used assays. In brief, MIC/ MBC assays utilise microtitre plates inoculated with bacterial cultures in growth media, followed by a doubling dilution of an antimicrobial. Treated plates are incubated overnight and the MIC is determined as the lowest concentration of the antimicrobial treatment able to inhibit bacterial growth. In contrast, the MBC is deemed the lowest concentration of the antimicrobial that has eradicated bacterial cells (or 99.9% reduction) as no visible growth is observed on agar following MIC testing (Lambert *et al.*, 2000; Andrews, 2001). Due to the reduced susceptibility to antimicrobial treatments against bacteria in biofilm mode of growth, MIC and MBC values of an antimicrobial are unsuitable for biofilm treatment. As biofilms are reported to require an antimicrobial treatment 10 – 1000 times greater than planktonic cells (Hengzhuang *et al.*, 2011). Therefore, specific assays are required to test the antibacterial efficacy of an antimicrobial against bacteria in biofilm mode of growth.

Both microtitre plate assay and the biofilm Calgary device can be used as a high throughput screening for antimicrobial susceptibility. The Calgary biofilm device was originally introduced to determine the antibiotic susceptibility of biofilms to antimicrobial treatments. The main commercial use of the Calgary device stated by Innovotech is the assessment of antimicrobial treatments against biofilms through minimum biofilm eradication concentration (MBEC) assay; where biofilms formed on the pegs of the microtitre pegged lid are transferred into solutions of antimicrobials at varying concentrations and the MBEC is determined either through transferring treated biofilms into fresh media or quantifying the number of viable cells following treatment by removing the pegs from the microtitre lid (Coenye *et al.*,

2010; Innovotech, 2012). Minimum biofilm inhibitory and eradication concentration (MBIC/ MBEC) assays are therefore the biofilm equivalent to MIC/ MBC assays.

Although unlike MIC/ MBC assays, there has been no standardised definition of an endpoint parameter published by official agencies i.e. CLSI or EUCAST to determine what classifies as the endpoint parameter for MBIC/ MBEC values. Therefore, the definition and interpretation of MBIC/ MBEC end point values vary between laboratories and current publications, making it difficult to compare results generated from different institutes. The definition of the MBIC varies between the lowest antimicrobial concentration causing no time-dependent increase in viable bacterial cells, or as the 'biofilm prevention concentration' and is the lowest concentration of an antimicrobial that significantly reduces the density of planktonic cells to prevent biofilm formation. Additionally, the MBEC value is either defined as the lowest antimicrobial concentration that eradicates 99.9% of bacteria located in the biofilm through viability testing; or the 'biofilm bactericidal concentration' as the lowest concentration of the antimicrobial treatment leading to no biofilm growth on agar, highlighting a similarly to MBC testing (Thieme *et al.*, 2019).

1.9 Photodynamic therapy as a strategy for treating problematic infections

The use of light as a form of treatment to improve health has occurred throughout the years and records of treatments are documented from around the world. While these therapies were not initially designed to treat bacterial infections, studies have shown the potential application of photodynamic therapy (PDT) to aid in the treatment of antibiotic resistant infections.

1.9.1 History of photodynamic therapy

The use of light for therapeutic treatment has been documented throughout the centuries, ancient Egyptians, Indians and Chinese civilisations have used light to treat a magnitude of diseases such as, skin cancer, psoriasis, rickets and vitiligo (Dolmans *et al.*, 2003). Ancient Greeks understood the benefits of sunlight, Herodotus, a famous Greek physician is regarded as the father of Heliotherapy: the therapeutic use of sunlight, and sunbathing was not only a pastime for ancient Greeks but also as a treatment for diseases, due to the belief that sun exposure was important for their health. France throughout the eighteenth and nineteenth century also used sunlight as a form of treatment for a variety of conditions: muscle weakness, paralysis, scurvy, rickets and even tuberculosis (Ackroyd *et al.*, 2007).

Further knowledge regarding PDT was discovered accidentally by a medical student Oscar Raab, who during 1897-1898 worked on investigating the toxicity of acridine. Initially he recorded that the toxicity of acridine varied each day; it was then he realised that due to it being winter there was a fluctuation of light intensity each day leading to the different results. Following this discovery, Raab was able to illustrate the effect of light on certain dyes for example eosin; low concentrations of acridine and the addition of eosin had no effect in the dark, but the same concentrations showed a rapid killing capability

of paramecia in the presence of light (Spikes, 1991). Professor Herman von Tappeiner who Raab worked under stated in a publication following Raab's findings that there was a future application of fluorescent substances in medicine (Ackroyd *et al.*, 2007).

The dawn of modern phototherapy began at the start of the 1900's, when Niels Finsen discovered the use of sunlight as well as a light source from a carbon arc lamp with a heat filter were able to treat the tubercular skin condition *lupus vulgaris.* He also learnt that red-light exposure was able to prevent the formation of smallpox and prevented the discharge of smallpox pustules. These pioneering discoveries led to Finsen being awarded a Nobel Prize in 1903. (Dolmans *et al.*, 2003; Hamblin, 2017)

Around the same time a French neurologist Jean Prime discovered the greatest negative effect that comes with PDT – photosensitivity. While attempting to treat epilepsy, Prime treated patients with an oral dose of eosin. Following treatment once patients were exposed to light blistering occurred as well as nail loss (Ross *et al.*, 2014). Thanks to this discovery von Tappeiner working with a dermatologist Jesionek was capable of treating skin tumours with a topical application of eosin and white light (Mitton *et al.*, 2008). Oxygen was soon identified as a key component for photosensitising reactions during 1904, through the work of von Tappeiner and Jodlbauer who later termed the phrase 'photodynamic action' years later in 1907 (Mitton *et al.*, 2008).

1.9.2 How does photodynamic therapy work?

In brief, PDT is a process involving a photosensitiser (PS) with the addition of a low intensity wavelength of visible light, which individually both are nontoxic. Treatment is completed in the presence of oxygen which combines with the PS and created a cytotoxic species (Ahmad *et al.*, 1998; Hamblin *et al.*, 2004).

When a PS is exposed to an appropriate wavelength of light, the PS is activated, leading to a series of photochemical reactions. Activation occurs by the light energy (photon of light) altering the PS via electron transfer, the PS then emits the energy in three different ways. Either through emission of heat, light or the conversion to an intermediate energy state, known as the triplet state and finally returns to its stable ground state (Figure 1.5). The two different reactions that can occur once the PS is in the triplet state is either a type 1 or type 2 reaction (Allison *et al.*, 2013).

Type 1 reactions occur when the excited state of the PS reacts with an organic substrate molecule, producing free radicals and radical ions. These free radicals then interact with endogenous molecular oxygen and produce reactive oxygen species (ROS): hydroxyl radicals, hydrogen peroxide and superoxide (Figure 1.5). These ROS cause irreversible biological damage by affecting the cell membrane integrity.

In contrast, during type 2 reactions the triplet state PS reacts directly with molecular oxygen, producing singlet oxygen, a highly reactive and electronically excited state of oxygen (Figure 1.5). PDT focuses

on type 2 reactions as the singlet oxygen reacts with lipids, proteins and nucleic acids in the cells, leading to cell death by functional and structural damage (Moore *et al.*, 2005; Takasaki *et al.*, 2009).
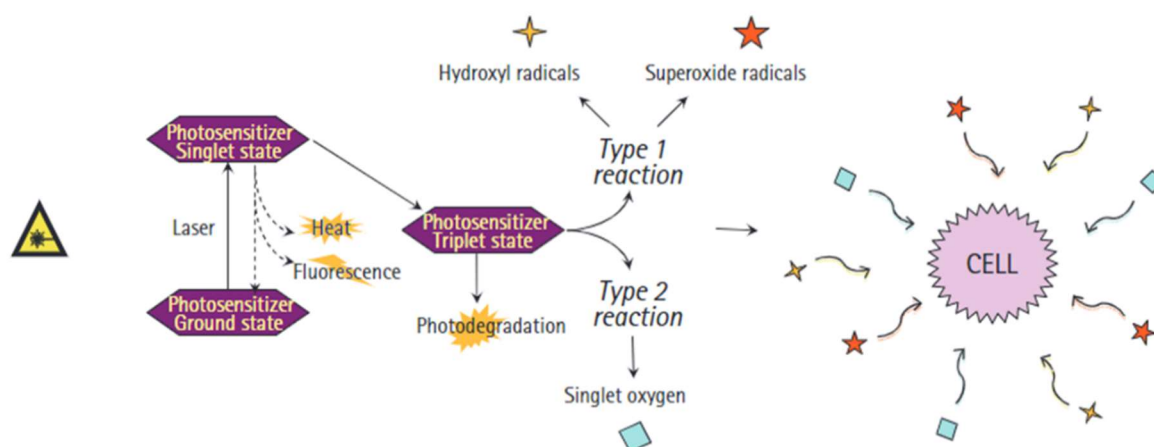
Figure 1.5: Diagram of the mechanism of action of PDT via type 1 and type 2 reactions

Diagram highlighting the two types of potential reactions that occur following the photoactivation of the photosensitiser in a singlet state from laser light to an excited triplet state. The diagram highlights the alternative routes the triplet state photosensitiser can go via a type 1 or a type 2 reactions or photo-degradation. A type 1 reaction consists of the photosensitiser reacting with organic substrates and produce free radicals: hydroxyl and superoxide radicals. Type 2 reaction occur when the photosensitiser reacts with molecular oxygen and produces singlet oxygen. These by-products of both reactions interact with cells and lead to cell death (Moore et al., 2005).

1.9.3 Photosensitisers

Haematoporphyrin, a precipitate isolated by Scherer from heated blood in 1841 was found to show fluorescent properties in 1867. It was not until 1911 that Hausmann recorded photosensitivity reactions following treatment of haematoporphyrin in mice, highlighting phototoxic properties in porphyrins, leading to the conclusion from his studies that PDT could target peripheral vasculature diseases. Following this discovery, in 1913 Meyer-Betz recorded prolonged pain and swelling in areas of his body exposed to light after injecting himself with 200mg of haematoporphyrin (Mitton *et al.*, 2008).

Haematoporphyrin is retained in tumour tissue and Dougherty showed this enabled selective targeted treatment of tumours. This was completed by exposing rat and mouse tumours to various types of light over 600 nm after injection of haematoporphyrin, the results showed that after treating over 300 tumours most mice have lived to over six months with no reappearance of a tumour after two months following treatment (Dougherty *et al.*, 1975).

Photofrin a derivative of haematoporphyrin, was the first PS to be approved worldwide for certain cancer treatments, although it has now been withdrawn from use in the European Union, replaced by multiple PS approved for basal cell carcinoma e.g. Levulan® (5-Aminolevulinic acid) and Metvix® (methyl 5-aminoleulinate).

Europe has only approved a handful of second and third generation PSs: Foscan® (temoporfin), Redaporfin® (LUZ111), Bremachlorin® and Photogem®. Foscan is such an effective PS that treatment times are measured in seconds and is the primary treatment for recurrent head and neck cancers; although the drug is so effective that after infusion a patient must avoid light for twenty-four hours and remain in a dark room to avoid severe burns. Different PSs are approved across the world, e.g. the dye Fotosens where therapy can occur the same day after PS infusion, due to how quickly it can be activated. Yet the illumination process can take up to 20 minutes per lesion and skin photosensitivity can last for up to several weeks (McFarland *et al.*, 2020, Allison *et al.*, 2013).

1.9.4 Light sources

The light source selected for PDT is dependent on the PS to be used, as the wavelength needs to match with the absorption spectra of the PS. Light at a higher wavelength has a greater penetrating depth into tissue up to 800 nm. Although many tumours and skin disorders are located on the surface, therefore, light penetration is not the limiting factor for treatment. In this case blue light, in particular at the Soret band – 410 nm is more effective at cell inactivation with a PS up to 2 mm down the surface of the skin in comparison to red light, which has a smaller extinction coefficient (Moan *et al.*, 1996).

Argon dye lasers (ADL) are considered as a standard source of light for PDT work, capable of delivering 1 – 7 W of continuous wave light energy, at 630 nm. Although early argon dye lasers were large and heavy, they provided the necessary power for early PDT studies involving treatment of malignant disease; producing 15 – 20 W of argon laser light, converted through the dye laser to produce 7 W of energy. Later models designed for the activation of Photofrin at 630 nm, were much smaller and were only capable of producing 2 W of usable power (Mang, 2004).

Alternative light sources were usually variations of the ADL system, i.e. the gold vapour laser (GVL). Though previous studies determined that GVL has no statistical difference in effectiveness of treating endobronchial tumours in comparison to ADL (McCaughan *et al.*, 1996).

Diode lasers are compact and versatile lasers capable of being used as either a continuous wave or pulsed. These lasers are often used due to the portability and ease of use. Once fitted with optical fibres they are capable of endoscopic PDT, as well as being used to treat skin lesions, the eyes and oral cavities. Although, diode lasers only offer a single output wavelength, limiting their versatility, but systems are being developed to allow interchangeable laser modules for different wavelengths (Brancaleon *et al.*, 2002).

Non-laser light sources may also be used for PDT. Through the use of light emitting diodes (LED's) light is produced by electroluminescence. The benefit of using LED light sources is that they are compact and lightweight, allowing ease of use and require a lot less energy in comparison to laser light

sources (Mang, 2004). With studies showing that non-laser light sources are capable and effective at treating Bowen's disease with only a few cases of scaring (Morton *et al.*, 1995).

1.9.5 Uses of photodynamic therapy in clinical practice

PDT has been proven to be an effective cancer therapy as both palliative and curative treatments; due to its capability of directly killing tumour cells. Additionally, tumour cells are also deprived of nutrients due to PDT causing vascular damage and blood flow stasis. Past studies have shown PDT can also lead to an anti-tumour immune response (Luo *et al.*, 2017). PDT is favourable for cancer treatments due to its intrinsic dual selectivity, from the PS being localised at the malignant tissue as well as the light treatment focused on the lesion/ treatment area. A benefit to PDT in comparison to radiation and chemotherapy is there is no cumulative toxicity in a patient with PDT (Castano *et al.,* 2005). With Canada being the first country to approve the use of PDT to treat bladder cancers in 1993, followed by FDA approval in USA in 1995 for esophageal cancer and lung cancer in 1998 (Ahn *et al.*, 2012).

The first recorded studies that highlighted the antibacterial capability of PDT against microorganisms occurred over 100 years ago, although limited research was followed through due to the success of antibiotics that took the focus of antibacterial research. Although due to the ever-growing issue of AMR, the search for alternative treatment strategies has rejuvenated antimicrobial PDT research. PDT as a form of treatment against bacterial infections is made possible as molecules can be up taken into Gram-positive microorganisms, therefore, PS are capable of entering the bacteria allowing photoactivation to occur. In contrast initial PDT was not effective to combat Gram-negative microorganisms, due to their structure and having a negatively charged surface, current PSs at the time were unable to enter the cell as they are anionic or neutral. Although, combination of a PS and a cationic agent i.e. polymyxin allows the disruption of the cell wall enabling the PS to enter. An alternative approach is cationic PSs that are capable of overcoming the natural defences of Gram-negative microorganisms, with previous studies showing that a PS: zinc pyridinium phthalocyanine were capable of killing Gram-positive microorganisms but also *E. coli* and *P. aeruginosa*. PDT requires no specific target site unlike antimicrobials, therefore, resistance to PDT is less likely to occur. Antimicrobial PDT could be a potential treatment for chronic ulcers, burn wound infections and oral infections, and has shown an anti-biofilm ability against bacterial infections (Minnock *et al.*, 1996; Jori *et al.*, 2004; Jia *et al.*, 2019).

Methylene blue and toluidine blue O are commonly used PSs used in antimicrobial PDT and are effective PS for inactivation of Gram-positive and Gram-negative microorganisms. Methylene blue is also effective at killing *Helicobacter pylori,* influenza virus and *Candida albicans (C. albicans)* (Rajesh *et al.*, 2011)*.*

1.10 Exeporfinium Chloride

Exeporfinium chloride (XF) is a novel antimicrobial series under development by Destiny Pharma Ltd (Brighton). Current research highlights the potential of these antimicrobials to be introduced as treatments against current AMR bacterial infections.

This thesis focuses on furthering current research on one compound in the XF series and assessing the efficacy of this compound against clinically relevant Gram-positive and Gram-negative infections in both planktonic and biofilm modes of growth.

1.10.1 Background and structure of XF-73

Exeporfinium chloride – 73 (XF-73) is the leading compound of the XF antimicrobial drug series produced by Destiny Pharma, named by the International Non-proprietary Name (INN) of Exeporfinium chloride by WHO. XF-73 is a dicationic porphyrin (Figure 1.6), unique in structure in comparison to known antibiotics (Ooi *et al.*, 2009b). The unique structure offers a novel mechanism of action, although not fully understood, it has been shown that XF-73 acts as a cell membrane disruptor but does not lyse the cells (Moir *et al.*, 2012). Rapid leakage of potassium and adenosine triphosphate (ATP) from cells exposed to XF-73, suggests that binding of XF-73 to the cell membrane causes damage to the lipid bilayer (Ooi *et al.*, 2009b).
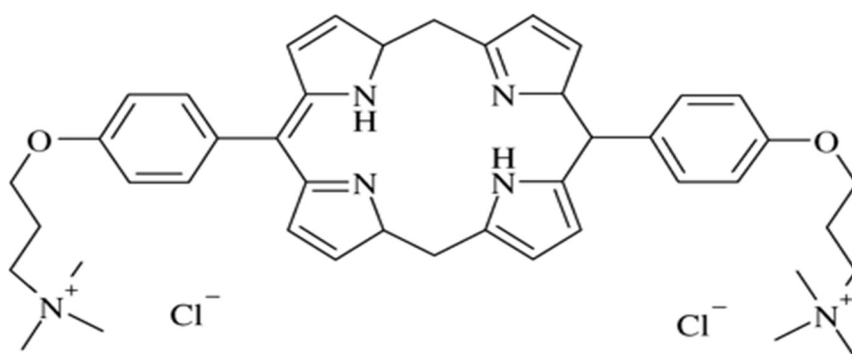
Figure 1.6: Chemical structure of XF-73

Diagram illustrating the chemical structure of XF-73 (Ooi, et al., 2009b).

XF-73 was initially designed as a treatment for the eradication of *S. aureus* carriage (Yendewa *et al.*, 2020). Currently XF-73 has undergone clinical evaluation in the USA as a nasal gel to assess its microbiological effect on commensal *S. aureus* nasal carriage in patients scheduled for surgical procedures deemed to be at high risk of post-operative *S. aureus* infection; this trial was completed in June 2022 (ClinicalTrials.gov, 2019). Destiny Pharma has also reported the approval given from the FDA for a phase 3 study of 'XF-73 nasal gel for the prevention of post-surgical staphylococcal infections' with feedback from the FDA stating the use of 'XF-73 Nasal gel' as a preventative to post-surgical staphylococcal infections following certain breast surgery operations, further expanding the potential applications of XF-73.

1.10.2 Antimicrobial activity of XF-73

As a novel antimicrobial XF-73 has shown to be a highly effective antibiotic against Gram-positive microorganisms. This has been highlighted by a study completed by Farrell *et al.,* (2010) testing the efficacy XF-73 against a panel of 65 Gram-positive microorganisms and 36 Gram-negative aerobic and anaerobic microorganisms, including strains with antimicrobial resistance to current antibiotics.

MICs were recorded for all Gram-positive microorganisms with a range between 0.25 – 4 µg /mL, followed by MBCs on all isolates to determine that XF-73 has bactericidal activity. Antibiotic resistant strains had recorded MIC values similar to antibiotic-susceptible strains. In contrast XF-73 is not as effective against Gram-negative microorganisms with MICs ranging from 1 µg/mL to > 64 µg/mL (Farrell *et al.*, 2010).

1.10.3 Low potential for resistance

Previous studies have highlighted that XF-73 exhibits a low potential for mutational resistance against four strains of *S. aureus*, including hospital and community acquired isolates. In this study the MICs were determined using the CLSI broth microdilution method against XF-73 and a panel of known antibiotics: vancomycin, fusidic acid, retapamulin, mupirocin and daptomycin. Multipassage resistance studies were completed against the panel of antibiotics by strains inoculated by a dilution series of twice the MIC followed by passaging the inoculum for 55 days. Resistance was determined by a fourfold increase of MIC. There was no recorded resistance against XF-73 for all *S. aureus* strains, whereas resistance to vancomycin occurred in the hospital associated MRSA and the common isolate of community associated MRSA strain after 32 and 20 passages respectively. The *S. aureus* strains were all reported to be resistant to each comparator antimicrobial; mupirocin, fusidic acid and daptomycin resistance occurred within seven passages. Resistance occurred against retapamulin within 3 – 16 passages (Farrell *et al.*, 2011).

1.10.4 Photodynamic effect on XF-73

An increased antimicrobial efficacy of XF-73 from exposure to blue light (380 – 480 nm) has been recorded in previous testing. This increased efficacy is concluded due XF-73 having the capability of being a porphyrin-based PS. Concentrations of XF-73 as low as 0.5 µM (0.383 µg/ mL) of XF-73 was capable of a 3-log reduction against a range of staphylococcal strains: MRSA, MSSA and methicillin-resistant *S. epidermidis*. XF-73 was confirmed to act as a PS via a type II reaction as it reacts with molecular oxygen only. Which was shown when sodium azide, a known quencher of singlet oxygen, was added and resulted in a decrease in the bactericidal capability of photo-activated XF-73 (Maisch *et al.*, 2005).

1.10.5 XF-73 against biofilm infections

Additional studies have shown the capability of the XF compounds killing slow growing and non-dividing *S. aureus*, highlighting the possibility of treatment for biofilms (Ooi *et al.*, 2009a). Initial studies into XF-73 as a biofilm treatment have shown its capability of killing *C. albicans* in planktonic and biofilm mode of life. Leading to the possibility of using XF-73 for treat infections of *C. albicans* including bloodstream infections (Gonzales *et al.*, 2013).

Further research has begun to investigate the effect of XF-73 against slow dividing microorganisms and microorganisms in biofilm mode of growth, XF-73 and XF-70 (another compound in the XF series) were both shown to be an effective treatment against *S. aureus* SH1000 biofilms, with the capability of eradicating the biofilms at the low concentration of 2 µg/ mL. Meanwhile all fifteen other antimicrobials tested in this study were unable to eradicate the *S. aureus* biofilm, these antimicrobials included: chlorhexidine, ciprofloxacin, vancomycin and tetracycline (Ooi *et al.*, 2009a).

1.11 Aims and objectives of this current research

The aim of this project was to investigate the antimicrobial efficacy of XF-73 against clinically relevant pathogens in both planktonic and biofilm forms of growth. With the potential of enhancing the effect of XF-73 with PDT. To achieve the aims the objectives were to:

- Conduct preliminary studies to assess the efficacy of XF-73.
  - Complete stability testing of XF-73 in aqueous solution.
  - Generate zones of inhibition of XF-73 against a panel of Gram-positive and Gram-negative microorganisms.
    - Compare zones of inhibition of XF-73 against an antibiotic panel of known antibiotics.
- Determine minimum inhibitory and bactericidal concentrations (MIC/ MBC) of XF-73 against clinically relevant pathogens.
  - Assess the enhancement of XF-73 using photodynamic therapy on MIC/ MBC values against clinically relevant pathogens.
  - Compare MIC/ MBC values of XF-73 against clinically relevant pathogens with a penal of known antibiotics.
- Assess the efficacy of XF-73 on microbial growth including time kill studies.
  - Assess the enhancement of XF-73 with photodynamic therapy on microbial growth via time kill studies.
- Determine the minimum biofilm inhibitory and eradication concentrations (MBIC/ MBEC) of XF-73 against clinically relevant biofilms at twenty-four and forty-eight hours of biofilm growth.
  - Record the enhancement of XF-73 against clinically relevant biofilms with the addition of photodynamic therapy.
    - Assess an alternative treatment strategy of XF-73 following photodynamic therapy against Gram-negative biofilms.
  - Compare efficacy of XF-73 against a panel of known antibiotics.
- Investigate potential mechanism of action of XF-73 through the delivery system of iron receptor channels of Gram-positive microorganisms.
  - Conduct competition assays of XF-73 and hemin in Mueller-Hinton broth.
  - Generate MIC/ MBC concentrations of XF-73 against Gram-positive microorganisms in an iron restricted media.
    - Conduct competition assay with XF-73 and hemin.

2.0 Chapter 2: Assessment of the antimicrobial efficacy of XF-73 through *in-vitro* testing against clinically relevant microorganisms in planktonic mode of growth

2.1 Introduction

The European Centre for Disease Prevention and Control (ECDC) released surveillance information regarding antimicrobial resistance in Europe from 2020 to 2022 (ECDC, 2022). Promisingly, around 86% of countries in the WHO European Region have currently reported to implement national action plans against AMR, a 36% increase in comparison to 2016; though the surveillance report stated that around 20% of the remaining countries do not have the capability of generating data for AMR surveillance. The AMR bacterial trends reported from 2020 data illustrated an overall decrease in multiple bacterial strains in comparison to the surveillance report in 2019. This current surveillance report recorded the prevalence of resistant microorganisms across forty countries in the European region. *E. coli* is one of the most common causes of a range of HCAIs including UTIs and community-acquired bloodstream infections; in this study 50% of the WHO European Regions reported a 25% or greater prevalence of *E. coli* isolates resistant to fluoroquinolone antibiotic treatments. A 50% or greater prevalence of these resistant *E. coli* strains was reported in Turkey, North Macedonia and the Russian Federation. *E. coli* isolates resistant to third generation cephalosporins was reported across the European countries and areas and 25% of these regions determined that 5 – 10% *E. coli* isolates expressed resistance. A recent emergence of carbapenem resistance in *E. coli* has been reported in the recent years, although 85% of European countries have reported a > 1% prevalence of these isolates, including the UK. In the UK 10 – 25% of *E. coli* isolates were reported as having resistance to either fluoroquinolone or third generation cephalosporins. Additionally, 5 – 10% of *P. aeruginosa* isolates documented in the UK have a reported resistance to carbapenems, whilst 15% and 10% of European countries reported carbapenem resistant *P. aeruginosa* in < 5% and ≥ 50% annual isolates respectively. This additional resistance in *P. aeruginosa* is a growing concern as *P. aeruginosa* already is naturally resistant to multiple antimicrobial treatments and this organism is a common cause of a range of HCAIs including UTIs, bloodstream infections and hospital-acquired pneumonia. Isolates of MRSA were reported across all European regions and these were the leading cause of surgical site infections following operations. MRSA isolates had an occurrence rate of 5 – 10% in the UK; less than 5% of *S. aureus* isolates were reported to have methicillin resistance in nine out of forty countries surveyed in this report, though ten countries reported a prevalence rate equal to or greater than 25%. The annual report recorded decreases in the total number of bacterial isolates and their prevalence in English hospitals in comparison to reports from 2017 – 2019. Although *E. coli* was still the most commonly reported bacterial infection with a recorded 21,063 isolates throughout the year, *S. aureus* had the second greatest prevalence with 6,975 reported isolates (ECDC, 2022).

Previous studies focusing on hospital and community acquired infections have shown that selective pressures act as a key driving force for microorganisms developing antibiotic resistance; therefore, an increase in antibiotic usage further increases the rate of resistance. Inappropriate use of antibiotics, such as healthcare professional prescribing antibiotics for viral infections or prescribing broad-spectrum antibiotics when an alternative or narrow-spectrum antibiotic is available, further aids the development of antimicrobial resistance. The ability to purchase antimicrobial medication over the counter at pharmacies also leads to the inappropriate usage of antimicrobials adding to increased AMR (Mcdermott *et al.*, 2008). Hospital-acquired infections impact the patient and are also linked to multidrug resistant infections at a community level; due to the acquisition of HCAIs from other patients in the facility or the members of staff. There are a range of risk factors related to the likelihood of a patient getting a HCAI, these include: age of patient, if the patient currently on immunosuppressants or has any underlying health issues, length of stay or admission into the intensive care unit, type of procedure related to admission (indwelling devices, ventilator support and invasive procedures increases the risk of HCAIs) and the frequency of visits to hospital or healthcare facilities. Therefore multiple hospitals have created surveillance systems to monitor infections and identify patients at risk for HCAIs and multidrug-resistant infections in order to minimise and prevent the occurrence of these infections (Monegro *et al.*, 2022).

Microbial identification is the first step when determining the appropriate treatment for an infection in healthcare environments. Sensitivity testing is conducted before a treatment is selected to determine which antimicrobials are appropriate to be used and out of these antimicrobials which would be the most effective. Microbiology departments in hospitals are responsible for aiding clinicians for the optimum antimicrobial agent for either treatment or prevention of a microbial infection. This is completed through microbial identification and antimicrobial susceptibility testing. The selection of a panel of antimicrobials is dependent on recent antimicrobial susceptibilities of local isolates, therefore varies between hospitals. A treatment strategy may be implemented before testing is completed, but once the results are collected, they are studied to determine if an alternative more appropriate treatment is possible or to explain a treatment failure. Susceptibility testing is beneficial for monitoring resistance to antimicrobials and offers national surveillance (NHS Oxford, 2020).

Disc diffusion assays are recorded as one of the oldest methodologies to test the susceptibility of microorganisms against an antimicrobial treatment and are commonly used in clinical laboratories to date. Through the aid of European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and EUCAST, a standardised antimicrobial disc diffusion protocol was designed to enable comparable results recorded from different laboratories. Un-supplemented Mueller-Hinton agar (MHA) is used for non-fastidious organisms and a supplemented MHA is required for fastidious organisms, enabling the majority of bacterial pathogens to be used cultured for testing. Antimicrobial discs are added to inoculated agar and a standard incubation time ranging from sixteen to twenty hours enables confluent

growth of the microorganism and the generation of zones of inhibition, should the antimicrobial effect bacterial growth (Matuschek *et al.*, 2014). EUCAST combined recorded breakpoints of a range of antimicrobial treatments against a range of microorganisms to conclude if a microorganism is susceptible, intermediate, or resistant to an antimicrobial and at what concentration. The recorded zones of inhibition are collected from worldwide sources and are regularly updated to ensure resistant patterns are monitored (EUCAST, 2021a). Quality assurance testing is also routinely conducted to ensure that high quality results are guaranteed during experimental testing. This is completed through the selection of control strains that are genetically stable and have susceptibility to specific antimicrobials. Quality assurance is completed by generating tables for acceptable diameter zones of inhibited growth against control microorganisms and comparing these results with published zones of inhibition (King *et al.*, 2001).

An alternative method to determine the susceptibility of a microorganism to an antimicrobial treatment is through minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. MIC values are recorded as the lowest concentration of an antimicrobial capable of inhibiting bacterial growth following overnight incubation with the antimicrobial. Initially MIC testing was conducted using tubes for each doubling dilution of antimicrobial, but a modernised version of the experiment utilises microtitre plates and the MIC value was determined as the lowest concentration of the antimicrobial with a clear solution, though the turbidity of the antimicrobial itself may affect the conclusion of a clear solution. This semi-quantitative experiment is considered as the gold standard to assess antimicrobial susceptibility against microorganisms. MIC testing is commonly used in diagnostic laboratories as definitive results are gained. MBC values are classified as the lowest concentration of an antimicrobial capable of eradicating microbial growth following incubation, assessed through sub-culturing samples on antimicrobial-free media. Disc diffusion assays are not always appropriate against specific microorganisms i.e. coagulase-negative staphylococci or specific antimicrobials i.e. teicoplanin, therefore an alternative experimental protocol is required and broth based MIC testing overcomes the issues of weak antimicrobial diffusion over agar (Lambert *et al.*, 2000; Andrews, 2001). EUCAST has also collated MIC breakpoints to determine the susceptibility or resistance of antimicrobial treatments against a magnitude of reference bacterial strains. Therefore *in-vitro* MIC testing in diagnostic laboratories influences the therapeutic strategy selected and the comparisons to reference MIC breakpoint values ensures an efficient antibacterial effect against the specific infection. (EUCAST, 2021b; Kowalska-Krochmal *et al.*, 2021)

Assessment of the antibacterial capability of XF-73 against a range of microorganisms was completed through MIC and MBC testing during previous studies. Gram-positive microorganisms have a greater susceptibility to XF-73 in comparison to Gram-negative bacterial cells; as Gram-positive MIC values ranged from 0.06 – 4 µg/ mL, in comparison to Gram-negative MIC values ranging between 1 – 1024

µg/ mL. These results were recorded against over two hundred bacterial isolates and multiple stains of species increasing the validity of these results (Farrell *et al.*, 2010; Board-Davies *et al.*, 2022).

Initial studies conducted by Ooi *et al.,* (2009) reported XF-73 inhibiting *S. aureus* growth at 1 µg/ mL and XF-73 maintained this inhibitor effect throughout two published studies (Ooi *et al.*, 2009a; Ooi, *et al.*, 2009b). Additionally Ooi *et al.,* (2009) recorded the killing kinetics of XF-73 against the *S. aureus* strain, a 4-log reduction in bacterial viability was observed following five minutes exposure to XF-73, increasing to a 5-log reduction after fifteen minutes exposure (Ooi *et al.*, 2009a). Similarly a > 5-log reduction in *S. aureus* viability was recorded following fifteen minutes of exposure to XF-73 (Farrell *et al.*, 2010).

2.2 Aim of the study

The aim of this study comprised two main stages. The first was to gather initial information on XF-73 before assessing its activity against Gram-positive and Gram-negative microorganisms in the planktonic mode of growth.

This was completed in the following stages:

- Creation of an aqueous stock solution of XF-73.
- Selection of a panel of clinically relevant Gram-positive and Gram-negative microorganisms to test the antimicrobial efficacy of XF-73.
- Assessment of the antimicrobial efficacy of XF-73 via disc diffusion assays against Gram-positive and Gram-negative microorganisms.
- Comparison of the antimicrobial efficacy of XF-73 with that of a panel of known antimicrobials via disc diffusion assays.

Once preliminary testing was conducted the main aim of the study was to assess the inhibitory activity of XF-73 against a panel of clinically relevant Gram-positive and Gram-negative microorganisms under planktonic growth conditions.

This was achieved in the following stages:

- Stability testing of XF-73 in aqueous solution.
- Measurement of the minimum inhibitory and bactericidal concentrations (MIC and MBC) of XF-73 against a panel of clinically relevant Gram-positive and Gram-negative microorganisms under planktonic growth conditions.
    - Comparison of the activity of XF-73 with that of a panel of known antibiotics.
- Assessment of the effect of XF-73 on microbial growth curves.

2.3 Materials

2.3.1 Microbial cultures

A panel of American Type Culture Collection (ATCC) microorganisms, consisting of both Gram-positive, Gram-negative bacteria, and a yeast strain, was selected for testing against the antimicrobial efficacy of XF-73 and a panel of known antimicrobials (Table 2.1). These microorganisms were selected due to their prevalence in a range of clinical infections and HCAIs and are commonly abundant microorganisms found these infections.

Table 2.1 Panel of clinically relevant microorganisms selected for treatment against XF-73 and known antimicrobials.

List of Gram-positive and Gram-negative microorganisms and a yeast strain, with corresponding American Type Culture Collection number when applicable

Microorganism	Gram Positive/Negative	American Type Culture Collection (ATCC)
Staphylococcus aureus	+ve	6538
Staphylococcus aureus	+ve	29213
Pseudomonas aeruginosa	-ve	15442
Pseudomonas aeruginosa	-ve	27853
Escherichia coli	-ve	35218
Escherichia coli	-ve	25922
Corynebacterium striatum	+ve	1293
Staphylococcus epidermidis	+ve	12228
Enterococcus faecalis	+ve	29212
Candida albicans	*Yeast*	76615
Escherichia coli DC0	-ve	* CGSC7138
Escherichia coli DC2	-ve	* CGSC7139

Note: DC0 is the parent E. coli K12 strain (also designated UB1005) from which the antibiotic hypersensitive mutant DC2 was derived as described (Clark, 1984).

2.3.2 Microbiological media and agar

Muller-Hinton broth (MHB) (Oxoid Ltd, Ireland) was used as the culture media for all microorganisms used in this study. Mueller-Hinton agar (MHA) (Oxoid Ltd) was used in this study for antimicrobial susceptibility testing and storage of all microorganisms.

2.3.3 XF-73 storage

The powder form of XF-73, provided by Destiny Pharma was stored in a 4°C fridge, encased in two opaque plastic containers to prevent exposure to light, in accordance with the guidelines provided by Destiny Pharma. Aqueous stock solutions of XF-73 were also stored at 4°C protected from exposure to light until needed.

2.3.4 Panel of comparator antimicrobials

A selection of five antimicrobials were selected for antimicrobial susceptibility testing on MHA (Table 2.2). The antibiotics were bought as commercial sensitivity discs (Oxoid Ltd) (6 mm) containing pre-set amounts of each antibiotic. Sensitivity discs were stored at 4°C until use.

Table 2.2 Panel of known antibiotics selected for comparator antimicrobial susceptibility testing

List of antibiotics used in the comparator antimicrobial including the concentration of each antibiotic present on sensitivity discs.

Antimicrobial	Disc Abbreviation	Amount (µg)
Ampicillin	AMP	10
Ciprofloxacin	CIP	5
Trimethoprim	W	5
Gentamicin	CN	10
Vancomycin	V	5

A different panel of antimicrobials comprising gentamicin, mupirocin, rifampicin and daptomycin was selected for study of activity against planktonic microorganisms (Table 2.1). These antimicrobials were purchased in powder form from Biosynth Carbosynth, UK, and were stored in accordance with the manufacturer's guidelines until required. Rifampicin and daptomycin were stored at -20°C, mupirocin at 4°C and gentamicin at room temperature. All liquid stocks of the antibiotics were stored at 4°C until required. Mupirocin and rifampicin were placed at room temperature to defrost before experiments were conducted.

2.3.5 Dimethyl sulfoxide (DMSO)

DMSO (Merck, Germany) was required to dissolve solid rifampicin and mupirocin to generate liquid stock solutions of the antimicrobials (2.3.4). DMSO was stored at room temperature in the absence of light until needed.

2.3.6 Specialist equipment

Optical density (OD) readings of microorganisms (Table 2.1) were taken using a spectrophotometer (Jenway Geneva Plus UV). A microtitre plate spectrophotometer (Biotech EL808) was used to record the OD readings throughout microbial growth experiments.

2.4 Methods

2.4.1 Preparation of media and agar

Media and agar were prepared in accordance with the manufacturer's instructions and sterilised before use by autoclaving.

2.4.1.1 Mueller-Hinton Broth

Following manufacturer's instructions 10.5 g of powdered MHB was dissolved in 500 mL of distilled water. Once the powder was fully dissolved by agitating the media was sterilised through autoclaving at 121°C for fifteen minutes, MHB was stored at room temperature before use.

2.4.1.2 Mueller-Hinton Agar

Nineteen grams of powdered MHA was dissolved in 500 mL of distilled water and agitated to fully dissolve the power. MHA was then sterilised by autoclaving the solution at 121°C for fifteen minutes. The molten MHA was then cooled in a water bath to 50°C before pouring into sterile petri dishes. Once the agar had cooled down and set the petri dishes were stored at 4°C until required.

2.4.2 XF-73 in aqueous stock solution

Aqueous stock solutions of XF-73 were made at a concentration of 1024 μg/ mL, where the solid XF-73 was vortexed in sterile distilled water to ensure complete solubility. The solution was then filtered using a 0.22 micron pore size cellulose acetate membrane filter (Minisart) to ensure sterility. XF-73 stocks were prepared in falcon tubes and wrapped in aluminium foil to exclude light before being stored in a plastic container at 4°C until needed in accordance with the guidelines provided by Destiny Pharma.

2.4.3 Stock solutions of antimicrobials

Solutions of antimicrobials were prepared in either sterile distilled water: gentamicin and daptomycin or dimethyl sulfoxide (DMSO): rifampicin and mupirocin, depending on the antimicrobial's solubility.

2.4.3.1 Gentamicin and Daptomycin

Aqueous stock solutions of gentamicin and daptomycin were made at the concentration of 1024 μg/ mL. Powdered gentamicin and daptomycin were vortexed in sterile distilled water. The solutions then underwent sterile filtration using a 0.22 micron pore size cellulose acetate membrane filter (Minisart) to ensure sterility and stored at 4°C until required.

2.4.3.2 Rifampicin and Mupirocin

Stock solutions of rifampicin and mupirocin were made at a concentration of 1024 μg/ mL in DMSO. Powdered rifampicin and mupirocin were vortexed in DMSO to create stock solutions and then stored at 4°C until needed.

2.4.4 Preparation of microorganisms for antimicrobial testing

The panel of microorganisms (Table 2.1) was selected from the Aston University Microbiology Research Group culture collection stored on Microbank beads (Pro-Lab Diagnostics) at –80°C and streak plates were used for samples of the microorganisms for experiments.

2.4.4.1 Glycerol stocks

Single colonies were selected from each microorganism (Table 2.1) and mixed into Microbank vials containing plastic beads and a glycerol-based cryopreservative medium (Pro-Lab Diagnostics), these vials were then stored in a freezer at -80°C until required.

2.4.4.2 Streak plates

Streak plates for each microorganism were prepared by taking a bead from each specific glycerol stock (2.4.4.1) and surface inoculating an MHA plate. Inoculated plates were then incubated overnight at 37°C to allow growth and then stored at 4°C until needed.

2.4.5 Antimicrobial susceptibility testing of XF-73 against the panel of clinically relevant Gram-positive and Gram-negative microorganisms

The antimicrobial efficacy of XF-73 (Batch 1) against the selected panel of microorganisms (Table 2.1) was initially tested through the disc diffusion assay method to identify the susceptibility of the microorganisms against XF-73.

MHA plates were surface inoculated with a suspension of each microorganism using a swab to produce semi-confluent lawns of growth after incubation. Blank sterile paper antibiotic sensitivity discs (6mm diameter, Whatman) were placed on the inoculated surface of each plate (6 per plate) and 20 μL of each concentration of XF-73: 1024 μg/mL; 102.4 μg/ mL, 10.24 μg/ mL and 1.024 μg/ mL, was applied to each disc (Figure 2.1). Three replicates were selected for each concentration of XF-73 and for the controls of: black discs and no discs on the agar.

Inoculated and treated plates were incubated at 37°C in aerobic conditions for 24 hours and the diameters of zones of inhibition of growth, including the diameter of the disc were measured in mm.

Key:
S = Stock: 1024 µg/ mL
1 = 102.4 µg/ mL
2 = 10.24 µg/ mL
3 = 1. 024 µg/ mL
B = Blank
C = Control: 0 µg/ mL
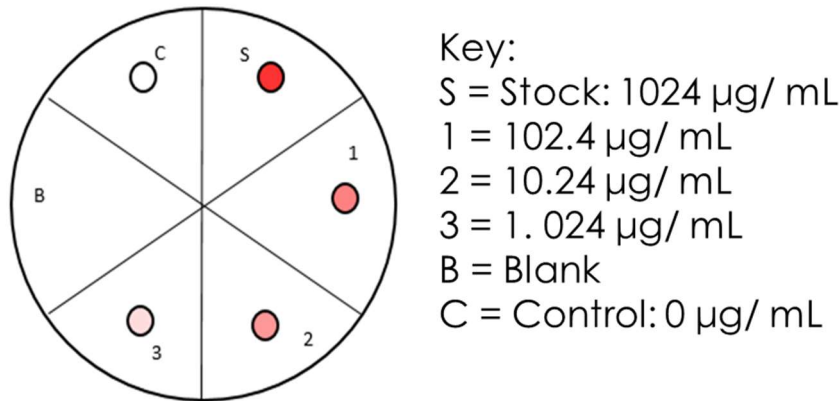
Figure 2.1 Layout of XF-73 disc diffusion assay plate

Diagram of layout of disc diffusion assay plates, with each concentration of XF-73 (µg/ mL) used.

2.4.6 Antimicrobial susceptibility testing of a panel of known antibiotics against panel of clinically relevant Gram-positive and Gram-negative microorganisms

The panel of known antibiotics (Table 2.2), each with a different mechanism of action was used for disc diffusion assay against the panel of microorganisms, to act as a comparison for XF-73. Disc diffusion assay was completed with the same method as the XF-73 disc diffusion assay (2.4.5), replacing XF-73 with the Oxoid sensitivity discs.

2.4.7 Minimum inhibition concentrations and minimum bactericidal concentrations of XF-73 and comparator antibiotics against Gram-positive and Gram-negative microorganisms

2.4.7.1 Minimum inhibition concentrations

MICs were determined using the broth microdilution method in line with CLSI guidelines (Patel *et al*., 2015). Round bottomed 96 well micro titre plates were prepared using 2-fold serial dilution of a stock concentration of 50 µL per antimicrobial in MHB (50 µL per well) to give a concentration range of 0.25 – 128 µg/ mL for Gram-positive microorganisms and 1 – 512 µg/ mL for Gram-negative microorganisms.

Each well was then inoculated with 50 µL of the test microorganism at 10^6 CFU (colony forming units)/ mL in MHB to give a final concentration of microorganism of 5 x10^5 CFU/mL in each well. Leading to further 2-fold dilution to give a final concentration range of 0.125 – 64 µg/ mL for Gram-positive microorganisms and 0.5 – 256 µg/ mL for Gram-negative microorganisms. Batches 1, 2 and 3 XF-73 were used in this study with three replicates per concentration and controls of: XF-73 alone, MHB and microorganism alone. The plates were incubated overnight at 37°C in aerobic conditions. Growth of each test organism was viewed as a visible cluster "button" of cells at the bottom of the well. MICs were recorded as the lowest concentration of antimicrobial that did not produce a visible "button" of cells.

2.4.7.2 Minimum bactericidal concentrations

MBCs were determined by streak plating 10 µL from each of the well of the microtitre plates, using a 10 µL inoculation loop onto MHA plates for each microorganism and incubated overnight at 37°C in aerobic conditions. Samples from each well were taken using a sterile loop and immersing into each test well, with agitation to ensure effective transfer to the spread plates. MBCs were recorded as the lowest concentration of antimicrobial that showed no growth on the streak plates.

2.4.7.3 DMSO control

To ensure that the concentration of DMSO alone was not affecting MIC or MBC results generated for rifampicin and mupirocin, an additional MIC and MBC was conducted using DMSO alone ranging from 0.78 – 50% (v/v) in MHB against the panel of microorganisms.

2.4.7.4 Daptomycin MIC/ MBC study with media enhancement of calcium chloride and magnesium chloride

An additional MIC/ MBC study was conducted with the daptomycin treatment, using an enhanced broth containing 50 mg/ L of calcium chloride and 50 mg/ L of magnesium chloride (Barry *et al.*, 2001) to compare the efficacy of daptomycin in standard and enhanced MHB.

2.4.7.5 Stability testing of XF-73 in aqueous solution

To determine the stability of XF-73 in aqueous solution, MIC and MBC testing of XF-73 against *S. aureus* ATCC 6538 was conducted every two weeks to monitor the MIC and MBC values.

2.4.8 Effect of XF-73 on the growth of *S. aureus* ATCC 6538

2.4.8.1 Varying inoculum density of *S. aureus* in lag phase of growth

A range of inoculum ODs of *S. aureus*: 0.01, 0.05, 0.1, 0.5 and 1 (at 570 nm, where OD0.01 = 10^6 CFU/ mL and OD1 = 10^9 CFU/mL) were selected to be treated with XF-73 at 2.5 µg/mL. OD readings were taken using the spectrometer (Novaspec 2, Pharmacia LKB), reading the OD of *S. aureus* in a 1 mL path length cuvette. Test wells of a flat bottom 96 well microtitre plate contained 10 µL of XF-73 and 90 µL of inoculum in MHB, with controls of 100 µL of *S. aureus* at the range of ODs, 100 µL of MHB and 100 µL of XF-73 2.5 µg/ mL. All test wells and controls were in triplicate. Plates were sealed with gas-permeable, transparent adhesive plastic film and placed in a microtitre plate spectrophotometer (Biotech EL808). OD (570nm) of each well was recorded over seventeen hours at twenty-minute intervals at 37°C in aerobic conditions, with the plate shaking between recording intervals.

Samples were streaked on MHA plates following the initial experiment to check for viability of organisms present and incubated overnight at 37°C in aerobic conditions.

2.4.8.2 Varying inoculum density of *S. aureus* in log phase of growth

Inocula prepared in MHB at ODs: 0.01 – 1 at 570 nm were determined using the spectrometer. The inocula were then incubated for two hours at 37°C in aerobic conditions to allow the cultures to reach

log phase growth. Two hours of growth following dilution to the required OD was determined to be an appropriate length of time to allow *S. aureus* to reach log phase growth. This was concluded following the growth curves observed through *S. aureus* control growths in the previous study. These log phase cultures were then used as inocula for the procedure as previously described (2.4.8.1).

2.4.8.3 Varying XF-73 concentrations against *S. aureus* in lag phase of growth

A range of XF-73 concentrations: 0.25 µg/ mL, 0.5 µg/ mL, 1 µg/ mL, 1.5 µg/ mL, 2 µg/ mL, and 2.5 µg/ mL in MHB were selected to treat *S. aureus* at OD 0.01 at 570 nm in MHB. ODs were determined using a spectrometer. Test wells of a flat bottom 96 well plate were inoculated with 90 µL *S. aureus* and treated with 10 µL of XF-73. With controls: 100 µL of *S. aureus*, 100 µL of MHB and 100 µL of the range of XF-73 concentrations. All test wells and controls were completed in triplicate. Plates were sealed with gas-permeable, transparent adhesive plastic film. ODs were recorded over seventeen hours with readings at 570 nm taken at twenty-minute intervals at 37°C in aerobic conditions using a microtitre plate spectrophotometer (Biotech EL808).

Samples were streaked on MHA plates after the initial experiment to check for viability of organisms present in test wells and incubated overnight at 37°C in aerobic conditions.

2.4.8.4 Varying XF-73 concentrations against *S. aureus* in log phase of growth

Inoculum at OD 0.01 at 570nm was incubated for two hours at 37°C in aerobic conditions to allow the culture to reach the logarithmic phase of growth, as determined through the previous studies (2.4.8.2). The log phase cultures were then used as inocula for the procedure as previously described (2.4.8.3).

2.4.9 Statistical analysis

To analyse data collected from multiple variables (2.4.8), end point OD values of each study were selected to run end point statistical analysis.

Initially, Column statistics was applied to the data test using the Shapiro-Wilk normality test, once normality of the data sets were confirmed a Brown-Forsythe test was also included to ensure there was no significant difference between the standard deviation of the data sets. Finally, to test for significance between the data sets an ordinary one-way ANOVA with the addition of Tukey's multiple comparisons *post-hoc* test. These tests were completed using the statistical software GraphPad Prism 7.

2.5 Results

2.5.1 Antimicrobial susceptibility disc diffusion assay of XF-73 and a panel of antimicrobials against Gram-positive and Gram-negative microorganisms

Zones of inhibition for XF-73 (2.4.5) and known antimicrobials (2.4.6) against Gram-positive microorganisms were grouped together and presented as a singular graph (Figure 2.2). Similarly, zones of inhibition of the panel of antimicrobials against Gram-negative microorganisms and *C. albicans* were grouped together to present results in a single composite graph (Figure 2.3).

2.5.1.1 Antimicrobial susceptibility disc diffusion assay of XF-73 and a panel of antimicrobials against Gram-positive microorganisms

Zones of inhibition were recorded for three concentrations of XF-73: 1024 μg/ mL, 102.4 μg/ mL and 10.24 μg/ mL for all Gram-positive microorganisms tested (Figure 2.2). *S. epidermidis* was the only microorganism with a recorded zone of inhibition at 1.024 μg/ mL. XF-73 at 102.4 μg/ mL was the concentration of XF-73 that produced the largest zone of inhibition for all test microorganisms, with both 1024 μg/ mL and 10.24 μg/ mL producing similar zones of inhibition to each other.

The comparator antimicrobials had a larger range of zones of inhibition in comparison to XF-73. The largest zone of inhibition recorded in this study was obtained for ampicillin against *S. aureus* ATCC 6538 at 40 mm, whereas for the same microorganism the discs containing 20 μ L of XF-73 at 102.4 μg/ mL only generated a mean zone of inhibition of 3 mm. All the comparators generated zones of inhibition against all Gram-positive microorganisms, with the exception of trimethoprim against *C. striatum*.

The zone sizes of the most effective antimicrobial varied between each microorganism: ciprofloxacin gave the greatest zones size for *C. striatum* and *S. aureus* ATCC 29213, trimethoprim gave the greatest zones size for *S. epidermidis*, ampicillin gave the greatest zones size for *S. aureus* ATCC 6538 and for *E. faecalis* ampicillin and trimethoprim gave the same maximum zones of inhibition at 22 mm.
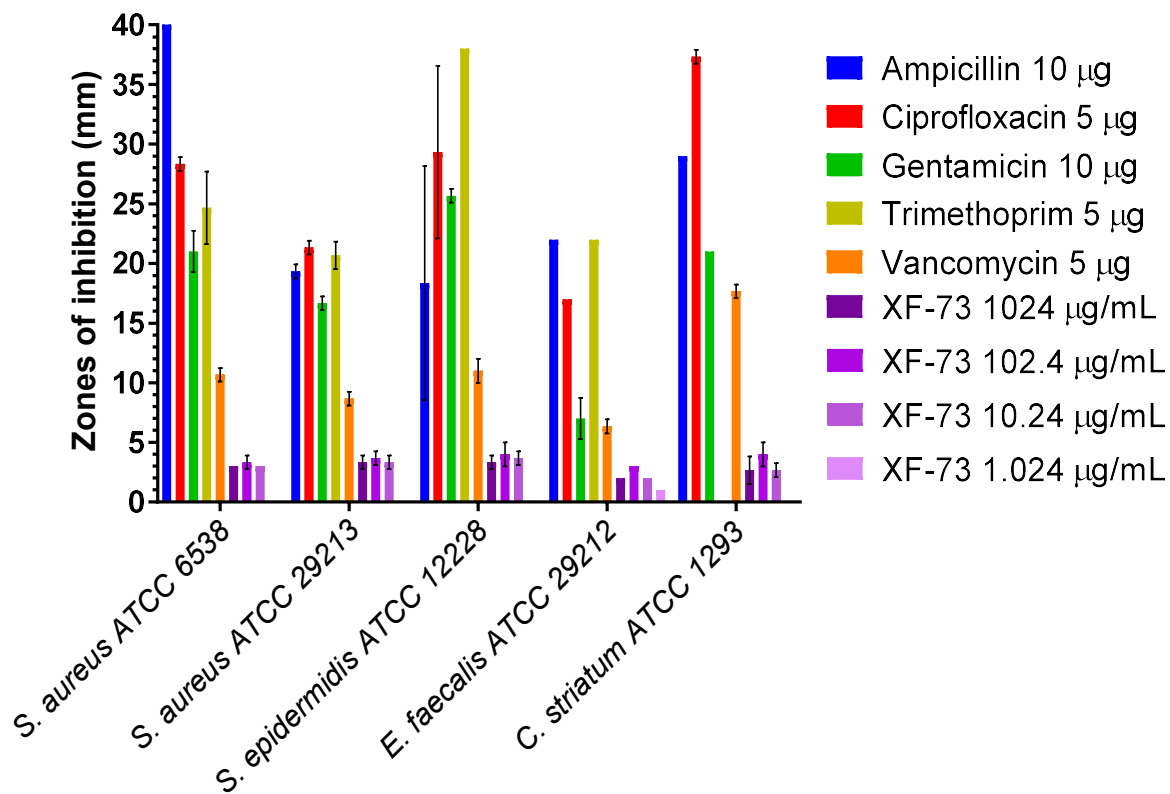
Figure 2.2 Antimicrobial susceptibility testing of Gram-positive microorganisms against a range of antimicrobials

Mean recorded zones of inhibition (mm) for a range of antimicrobials: XF-73, ampicillin, ciprofloxacin, gentamicin, trimethoprim, and vancomycin against a panel of Gram-positive microorganisms: S. aureus ATCC 6538, S. aureus ATCC 29213, S. epidermidis ATCC 12228, E. faecalis ATCC 29212 and C. striatum ATCC 1293. XF-73 was tested at a range of concentrations: 1024, 102.4, 10.24 and 1.024 (µg/ mL) with 20 µL of each concentration present on discs. Error bars generated as the standard deviation of the data sets from 3 replicates.

2.5.1.2 Antimicrobial susceptibility disc diffusion assay of XF-73 and a panel of antimicrobials against Gram-negative microorganisms

There was no recorded zone of inhibition for any concentration of XF-73 and vancomycin against the Gram-negative microorganisms tested (Figure 2.3). Ciprofloxacin and gentamicin were the only antimicrobials that were able to generate zones of inhibition for each Gram-negative microorganism. Additionally, ciprofloxacin produced the largest zones of inhibition against each Gram-negative microorganism, apart from *E. coli* DC2 where trimethoprim produced a 3 mm larger zone of inhibition. Trimethoprim generated a zone of inhibition against all *E. coli* strains, whereas no zones of inhibition were recorded against either *P. aeruginosa* strain. Similarly, ampicillin discs led to visible zones of inhibition against *E. coli* DC0, *E. coli* DC2 and *E. coli* ATCC 25922.

C. albicans was the only microorganism in this study with no recorded zone of inhibition against any antimicrobial.
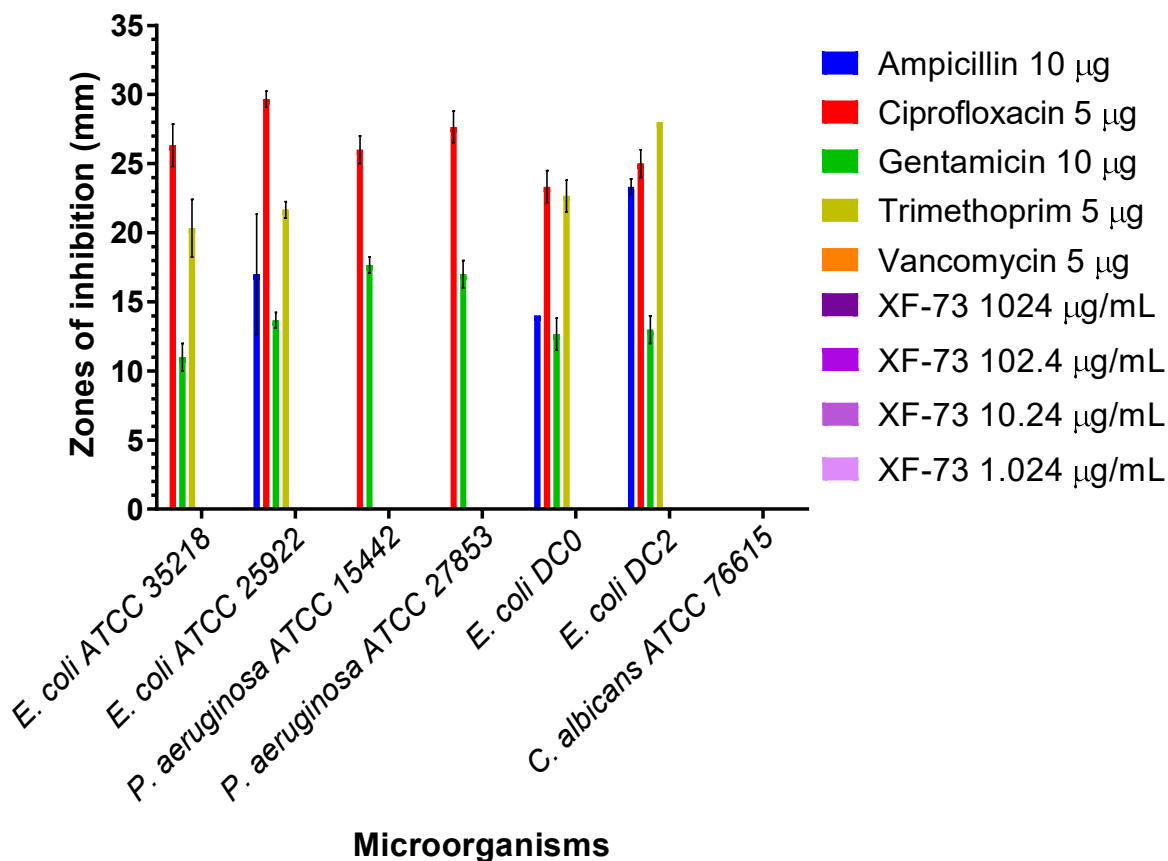
Figure 2.3 Antimicrobial susceptibility testing of Gram-negative microorganisms and a yeast strain against a range of antimicrobials

Mean recorded zones of inhibition (mm) for a range of antimicrobials: XF-73, ampicillin, ciprofloxacin, gentamicin, trimethoprim, and vancomycin against a panel of Gram-negative microorganisms: E. coli ATCC 35218, E. coli ATCC 25922, P. aeruginosa ATCC 15442, P. aeruginosa ATCC 27853, E. coli DC0 and E. coli DC2. The panel of microorganism also includes the yeast C. albicans ATCC 76615. XF-73 was tested at a range of concentrations: 1024, 102.4, 10.24 and 1.024 (µg/ mL) with 20 µL of each concentration present on discs. Error bars generated as the standard deviation of the data sets from 3 replicates.

2.5.2 Minimum inhibition and bactericidal concentrations of XF-73 against Gram-positive and Gram-negative bacteria and *C. albicans*

MIC and MBC values of different batches of XF-73 against Gram-positive and Gram-negative bacteria and *C. albicans* were recorded together and presented as a table (Table 2.3). *C. striatum* ATCC 1293 and *C. albicans* ATCC 76615 were only tested with batch 1 of XF-73. This table includes concentration ranges of MIC and MBC values of batch 1 and batch 2 XF-73 against: *S. aureus* ATCC 6538, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228 and *E. faecalis* as multiple MIC studies were conducted against these Gram-positive and the Gram-negative bacteria, although no change in MIC or MBC values was recorded against the Gram-negative bacteria. Batch 3 was only tested against these Gram-positive bacteria.

Every batch of XF-73 was capable of inhibiting and eradicating Gram-positive microorganisms.. Batch 1 contained the lowest recorded concentration range for MIC and MBC values out of all the batches, followed by batch 2 and then batch 3 (Table 2.3).

2.5.2.1 MIC and MBC values for batch 1 XF-73

The lowest concentration of XF-73 to inhibit and eradicate *C. striatum* ATCC 1293, *S. aureus* ATCC 29213 and *S. epidermidis* the MIC and MBC was determined as ≤ 0.125 µg/ mL Both *S. epidermidis* and *E. faecalis* had a 2-fold increase in the upper concentration range for the MBC values in comparison to the corresponding upper concentration range of XF-73 for MICs.

The same concentration range of XF-73 was shown to inhibit and eradicate both *S. aureus* strains; and XF-73 at 2 µg/ mL was the recorded upper concentration range to eradicate both *S. aureus* strains.

XF-73 also showed an antimicrobial effect against *C. albicans* ATCC 76615 and the same concentration of 4 µg/ mL XF-73 was observed for the MIC and MBC values.

No concentration of XF-73 used in this study was capable of inhibiting or eradicating any of the Gram-negative microorganisms tested; therefore, MIC and MBC values were determined as > 256 µg/ mL.

2.5.2.2 MIC and MBC values for batch 2 XF-73

All Gram-positive microorganisms selected for treatment against batch 2 XF-73 had the same respective lower concentration range to inhibit and eradicate the planktonic cells. Though an increase in the upper concentration range of XF-73 was observed for the MBCs collected against all Gram-positive microorganisms in comparison to corresponding MIC values. The same concentration range of XF-73 was recorded to inhibit both *S. aureus* strains at 0.5 – 1 µg/ mL. To eradicate *S. aureus* ATCC 6538 cells, a 4-fold increase in the upper inhibition concentration range was noted. Meanwhile, only a 2-fold increase in XF-73 concentration was required to eradicate *S. aureus* ATCC 29213 in comparison to the upper MIC concentration range.

Every concentration of XF-73 used in this study was capable of inhibiting and eradicating *S. epidermidis* and *E. faecalis*; therefore the lower concentration range for MICs and MBCs for both microorganisms were classified as ≤ 0.125 µg/ mL. A 4-fold increase in XF-73 was required to eradicate *S. epidermidis* at the upper concentration range. Similarly, a 2-fold increase was recorded between the upper concentration ranges from MIC to MBC values for *E. faecalis.*

Whilst no inhibition or eradication was recorded for any of the Gram-negative microorganisms treated with batch 1 XF-73; batch 2 XF-73 was capable of inhibiting all Gram-negative microorganisms at the same concentration of 128 µg/ mL.

E. coli ATCC 35218 was the only microorganism where no concentration of XF-73 was capable of eradicating the cells. In contrast, the same concentration of XF-73 was recorded to inhibit and eradicate *E. coli* ATCC 25992 planktonic cells. A 2-fold increase in the MIC XF-73 concentration was required to eradicate both *P. aeruginosa* strains.

2.5.2.3 MIC and MBC values for batch 3 XF-73

For each Gram-positive microorganism selected for XF-73 treatment, the same concentration was recorded to inhibit and eradicate each microorganism. The lowest MIC and MBC values of XF-73 were recorded at 0.5 µg/ mL against *S. epidermidis* and *E. faecalis.* XF-73 at 2 µg/ mL was determined to inhibit and eradicate *S. aureus* ATCC 6538, a 2-fold reduction in concentration was noted to have the same inhibitory and eradication effect against *S. aureus* ATCC 29213.

Table 2.3 MIC and MBC values (µg/ mL) of different batches of XF-73 against a panel of clinically relevant microorganisms

The panel of microorganisms varies depending on the batch of XF-73 used for the study, any microorganism not selected for a specific XF-73 treatment has 'N/A'.

Microorganism/ ATCC number	Batch 1: September 2018		Batch 2: March 2020		Batch 3: July 2021	
	MIC µg/ mL	MBC µg/ mL	MIC µg/ mL	MBC µg/ mL	MIC µg/ mL	MBC µg/ mL
S. aureus 6538	0.25 – 2	0.25 – 2	0.5 – 1	0.5 – 4	2	2
S. aureus 29213	≤ 0.125 – 2	≤ 0.125 – 2	0.5 – 1	0.5 – 2	1	1
S. epidermidis 12228	≤ 0.125 – 2	≤ 0.125 – 4	≤ 0.125	≤ 0.125 – 0.5	0.5	0.5
E. faecalis 29212	0.125 – 2	0.125 – 4	≤ 0.125 – 0.5	≤ 0.125 – 1	0.5	0.5
E. coli 35218	> 256	> 256	128	> 256	N/A	N/ A
E. coli 25922	> 256	> 256	128	128	N/A	N/ A
P. aeruginosa 15442	> 256	> 256	128	256	N/A	N/ A
P. aeruginosa 27853	> 256	> 256	128	256	N/A	N/ A
E. coli DC0	> 256	> 256	N/A	N/A	N/A	N/A
E. coli DC2	> 256	> 256	N/A	N/A	N/A	N/A
C. striatum 1293	0.125	0.125	N/A	N/A	N/A	N/ A
C. albicans 76615	4	4	N/A	N/A	N/A	N/ A

2.5.3 Minimum inhibition and bactericidal concentrations of comparator antibiotics against Gram-positive and Gram-negative microorganisms

A DMSO only MIC/ MBC study was conducted as rifampicin and mupirocin stocks were created in DMSO (Table 2.4). DMSO at 50% v/v was the recorded MIC concentration for all staphylococcal isolates in this study, but 50% v/v DMSO was unable to eradicate any Gram-positive microorganisms as growth was observed on MHA for all concentrations tested. A lower concentration of 25% v/v DMSO inhibited the growth of *E. faecalis.* DMSO at 25% inhibited growth of all Gram-negative microorganisms and 25% v/v also eradicated *P. aeruginosa* ATCC 27853; no concentration was capable of eradicating *E. coli* ATCC 35218, while 50% eradicated *E. coli* ATCC 25992 and *P. aeruginosa* ATCC 15442. Therefore, the maximum concentration of rifampicin and mupirocin that could be concluded as MIC and MBC values was 128 µg/ mL against Gram-negative microorganisms

Each antibiotic selected for this study was capable of inhibiting the Gram-positive and Gram-negative microorganisms, shown by an MIC value for each; with the exception of daptomycin, which was the only antibiotic unable to generate an MIC or MBC value against the Gram-negative microorganisms.

Whilst daptomycin produced MIC and MBC values against all four Gram-positive microorganisms in standard MHB, the addition of calcium chloride and magnesium chloride in media led to lower daptomycin concentrations inhibiting and eradicating the microorganisms.

Rifampicin had the lowest MICs recorded against all Gram-positive microorganisms in this study. For *S. aureus* ATCC 29213 and *S. epidermidis* the MIC and MBC concentrations were the same, and determined to be ≤ 0.125 µg/ mL as no growth was observed following XF-73 treatment. Despite this fact there was over a 256-fold increase in the MBC for *E. faecalis* as there was growth recorded throughout the concentration range. In comparison a 64-fold increase in concentration was determined as the MBC for *S. aureus* ATCC 6538.

Rifampicin was capable of inhibiting all Gram-negative bacteria at relatively low concentrations: 8 µg/ mL for both *P. aeruginosa* strains and 4 µg/ mL and 2 µg/ mL for *E. coli* ATCC 35218 and *E. coli* ATCC 25922 respectively. In contrast to inhibitory effects, no concentration of rifampicin used in this study was able to eradicate both *E. coli* strains; a 16-fold and 2-fold increase in MICs was required to eradicate *P. aeruginosa* ATCC 15422 and *P. aeruginosa* ATCC 27853 respectively.

The lowest MIC and MBC values recorded against all Gram-negative microorganisms were from gentamicin. *P. aeruginosa* ATCC 15442 and 27853 both had the same concentration at 1 µg/ mL for the MIC and MBC. *E. coli* ATCC 25992 also had 1 µg/ mL as the MIC but there was a 2-fold increase in concentration for the MBC. The same MIC and MBC was recorded for *E. coli* ATCC 35218 at 2 µg/ mL. Gentamicin was recorded to have equal concentrations (where MIC = MBC) for both strains of *S. aureus* at concentrations of 4 µg/ mL for ATCC 6538 and 2 µg/ mL for ATCC 29213. However, the MIC and the MBC were not the same for the other Gram-positive microorganisms, there was a recorded 2-fold increase in MBC compared with the MIC for both *S. epidermidis* and *E. faecalis*, though a higher concentration was required for *E. faecalis* at 16 µg/ mL while *S. epidermidis* only needed 0.25 µg/ mL.

Mupirocin was capable of inhibiting all Gram-positive microorganisms at low concentrations of ≤ 0.125 and 0.25 µg/ mL for the staphylococcal strains and 16 µg/ mL for *E. faecalis*, but no concentrations in the range selected were able to eradicate these microorganisms. Both *P. aeruginosa* strains were eradicated at 128 µg/ mL. In contrast, both *E. coli* strains were inhibited at 32 µg/ mL but no concentration used was able to eradicate them.

Table 2.4 MIC and MBC values (µg/ mL) of comparator antibiotics against a panel of Gram-positive and Gram-negative microorganisms

Microorganism/ ATCC number	DMSO % v/v MIC/ MBC	Rif µg/ mL MIC/ MBC	Gen µg/ mL MIC/ MBC	Mup µg/ mL MIC/ MBC	Dap µg/ mL MIC/ MBC	Dap + µg/ mL MIC/ MBC
S. aureus 6538	50/ > 50	≤ 0.125/ 8	4/ 4	≤ 0.125/ > 64	16/ 16	2/ 4
S. aureus 29213	50/ > 50	≤ 0.125/ ≤ 0.125	2/ 2	0.25/ > 64	16/ 16	4/ 4
S. epidermidis 12228	50/ > 50	≤ 0.125/ ≤ 0.125	≤ 0.125/ 0.25	≤ 0.125/ > 64	16 / 16	2/ 2
E. faecalis 29212	25/ > 50	0.25/ > 64	8/ 16	16/ > 64	> 64/ > 64	16/ 16
E. coli 35218	25/ > 50	4/ > 256	2/ 2	32/ > 256	> 256/ > 256	> 256/ > 256
E. coli 25992	25/ 50	2/ 256	1/ 2	32/ > 256	> 256/ > 256	> 256/ > 256
P. aeruginosa 15442	25/ 50	8/ 128	1/ 1	128/ 128	> 256/ > 256	> 256/ > 256
P. aeruginosa 27853	25/ 25	8/ 16	1/ 1	128/ 128	> 256/ > 256	> 256/ > 256

Antibiotic abbreviations: Rif: rifampicin, Gen: gentamicin, Mup: mupirocin, Dap: daptomycin.
Dap +: Daptomycin in enhanced MHB containing 50 mg/ L each of calcium chloride and magnesium chloride. DMSO control added to assess the effect of DMSO alone, due to Rifampicin and Mupirocin stocks containing DMSO.

2.5.4 Stability testing of XF-73 in aqueous solution against *S. aureus* ATCC 6538

As previously shown (Table 2.3), batch 1 XF-73 against *S. aureus* ATCC 6538 had an MIC and MBC value of 0.25 µg/ mL. Every two weeks and MIC test was conducted against *S. aureus* ATCC 6538 using batch 1 XF-73 to detect any shift in MIC/ MBC values. Results were collected and presented as a table (Table 2.5).

Throughout the study the concentration of the MIC and MBC was the same. For the first six weeks there was no shift in concentration. Although at the eighth week, 0.125 µg/ mL was recorded to eradicate *S. aureus* growth, though from week 10 to week 18 the MIC and MBC value remained at 0.25 µg/ mL. The first recorded increase in MIC and MBC above the expected value was on the eighteenth week, with a 2-fold increase in MIC and MBC. The final recording at twenty weeks showed a further increase in both the MIC and MBC value, with a minimum 4-fold increase. This was also the first time point where a different MIC and MBC was recorded between replicates.

Table 2.5 Stability testing of XF-73 in aqueous solution against S. aureus ATCC 6538 via MIC and MBC testing

Testing stability of batch 1 XF-73 against S. aureus ATCC 6538 in planktonic mode of growth

	XF-73 (µg/ mL)	
Week	MIC	MBC
0	0.25	0.25
2	0.25	0.25
4	0.25	0.25
6	0.25	0.25
8	0.125	0.125
10	0.25	0.25
12	0.25	0.25
14	0.25	0.25
16	0.25	0.25
18	0.5	0.5
20	1 – 4	1 – 4

Note: at week 20, a range of MIC/ MBC values were recorded between the triplicate tests

2.5.5 Varying inoculum density of *S. aureus* ATCC 6538

2.5.5.1 *S. aureus* in lag phase growth

A line graph was generated for each OD of *S. aureus* cultures in MHB treated with XF-73 over seventeen hours (Figure 2.4). Each experiment included a MHB + XF-73 control and throughout the timeframe there was no change in absorbance readings. While the OD was varied for each experiment *S. aureus* control followed a similar trend in absorbance readings. ODs: 0.01, 0.05, 0.1 and 0.5 (Figure A-D), showed a slow gradual increase in absorbance readings for the first two hours, absorbance readings then began to increase at a quicker rate before tapering off and remaining at similar readings after nine hours. Although a different trend was recorded for OD 1, within the first hour of the study a decrease in optical density was noted, followed by similar absorbance readings with a slight increase for the remainder of the timeframe (Figure 2.4 E).

Treatment of XF-73 against *S. aureus* at varying ODs led to different outcomes in growth rates in comparison to untreated *S. aureus* control growth. There was no increase in absorbance throughout the entirety of the seventeen hours for OD 0.01 and 0.05 (Figure 2.4 A, B); end point statistical analysis of both ODs showed a significant difference between *S. aureus* treated with 2.5 µg/ mL of XF-73 and the growth control at seventeen hours ($p=$ <0.0001).

OD 0.1 *S. aureus* treated with XF-73 followed a similar trend to OD 0.01 and 0.05 where there was no increase in absorbance readings for up to thirteen hours into the study, after this time point the absorbance began to slightly increase for the remaining four hours (Figure 2.4 C). After seventeen hours, treated *S. aureus's* absorbance had increased from 0.190 units at time zero to 0.208 units at 570 nm. In comparison at seventeen hours the growth control was at 0.459 units at 570 nm; end point

statistical analysis showed there was still a significant difference between the two absorbencies (p= 0.0007).

S. aureus OD 0.5 treated with XF-73 showed a slow gradual increase in absorbance readings throughout the entirety of the study, although each recorded absorbance was lower than the corresponding absorbance for the growth control (Figure 2.4 D). A significant difference was still recorded between treated *S. aureus* and the growth control (p= 0.0019).

Notwithstanding the other ODs treated with XF-73 in this study, OD 1 *S. aureus* treated with XF-73 had absorbance readings higher than the growth control, this difference between the other ODs was recorded since the first hour into the time course (Figure 2.4 E). In comparison to the growth control, which remained at a similar absorbance throughout the time course, the treated *S. aureus* exhibited absorbance readings similar to the growth control from other ODs. From time zero to seven hours treated *S. aureus* (OD 1) absorbance readings showed a steep increase, followed by no noteworthy increase or decrease in absorbance readings for the remainder of the study. End point statistical analysis showed a significant difference between treated *S. aureus* and the growth control (p= <0.0001).

Following the time course study, samples were then streaked out to check for viability, MHB+XF-73 showed no growth while the *S. aureus* growth control showed bacterial viability for all experiments. *S. aureus* at OD 0.01 treated with XF-73 was the only sample that showed no bacterial viability.

2.5.5.2 *S. aureus* in log phase growth

In these experiments, the inoculated cultures of *S. aureus* at each OD were pre-incubated for two hours, this allowed the organism to reach early log phase before the addition of XF-73 (Figure 2.5).

MHB + XF-73 controls for each OD in this study were recorded with no change in absorbance throughout the entirety of the time course. *S. aureus* growth controls for each OD grew throughout the study, as shown by an increase in absorbance readings throughout.

The absorbance readings for the growth control at OD 0.01 showed a gradual increase in absorbance readings up until seven hours into the study (Figure 2.5 A). There was then little change recorded in absorbance readings for the next three hours. At ten hours, an increase in absorbance began to occur again, although the last seven hours of the study showed a gradual increase in absorbance readings in comparison to the first seven hours. In contrast, *S. aureus* at an OD of 0.01 treated with XF-73 showed little change in absorbance readings over the seventeen hour study. When comparing the mean readings at the start (hour zero) and end of the study (hour seventeen) the absorbance readings are both 0.156 units at 570 nm. The difference between control growth and treated *S. aureus* was determined as significant through end point statistical analysis (p= <0.0001).

Both *S. aureus* control and the treated culture at OD 0.05 showed an increase of absorbance throughout the entirety of the study (Figure 2.5 B). Even though treated culture had lower absorbance readings at

each point in comparison to the control growth, there was no significant difference between the two samples at the end of the study ($p > 0.05$), with the growth control at 0.827 units and treated *S. aureus* at 0.786 units at 570 nm.

OD 0.1 had a recorded increase throughout the study for both the untreated and treated *S. aureus* at a steady rate (Figure 2.5 C). Although the rate of incline in absorbance readings for treated *S. aureus* was always less than the growth control. After seventeen hours there was a significant difference recorded between the growth control and treated *S. aureus* ($p = <0.0001$).

The same trend was recorded for OD 0.5 and OD 1 (Figure 2.5 D, E). The growth controls for both ODs had a gradual increase in absorbance throughout the study, treated cultures also showed an increase in absorbance but at a lower rate. The significance between treated and non-treated *S. aureus* for OD 0.5 ($p = <0.0001$) was greater than OD 1 ($p = 0.0006$).

Bacterial viability testing showed that only OD 0.01 *S. aureus* treated with XF-73 led to no viable cells, all other ODs showed growth on agar plates. Controls of *S. aureus* also showed growth and MHB + XF-73 had no bacterial cells present on agar plates.

A

B
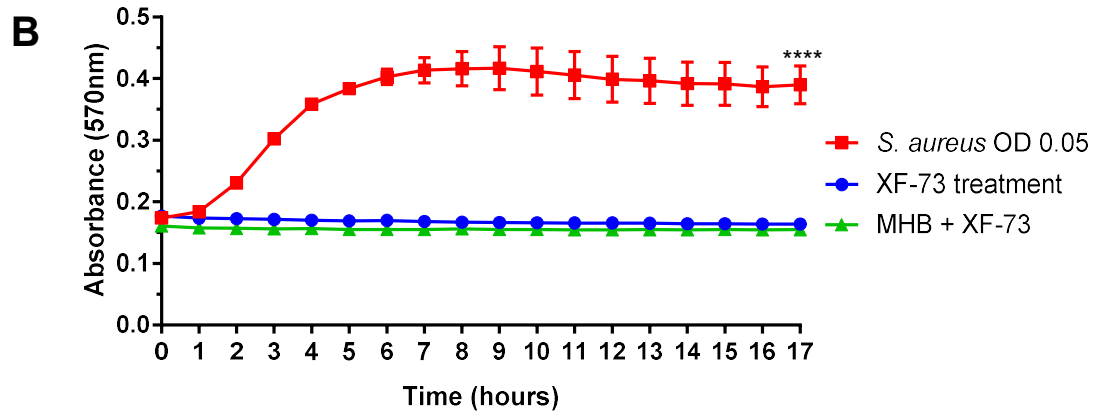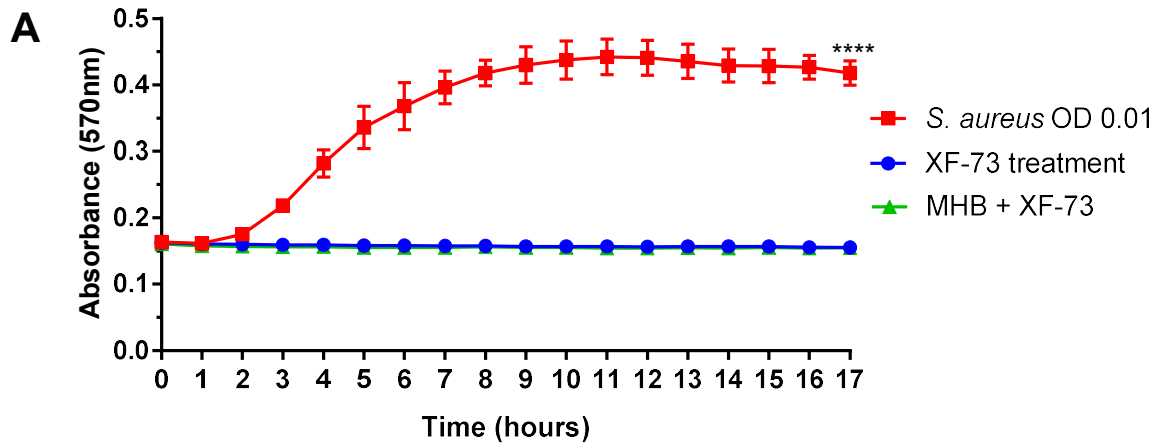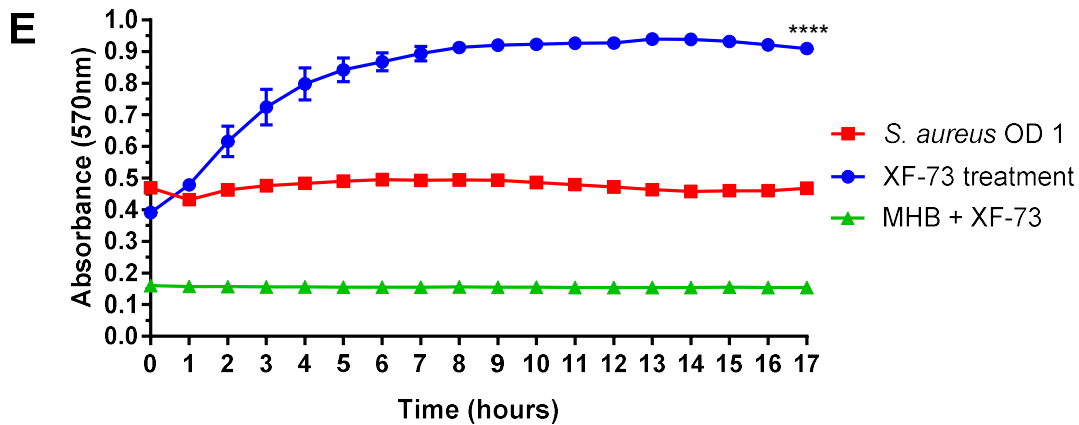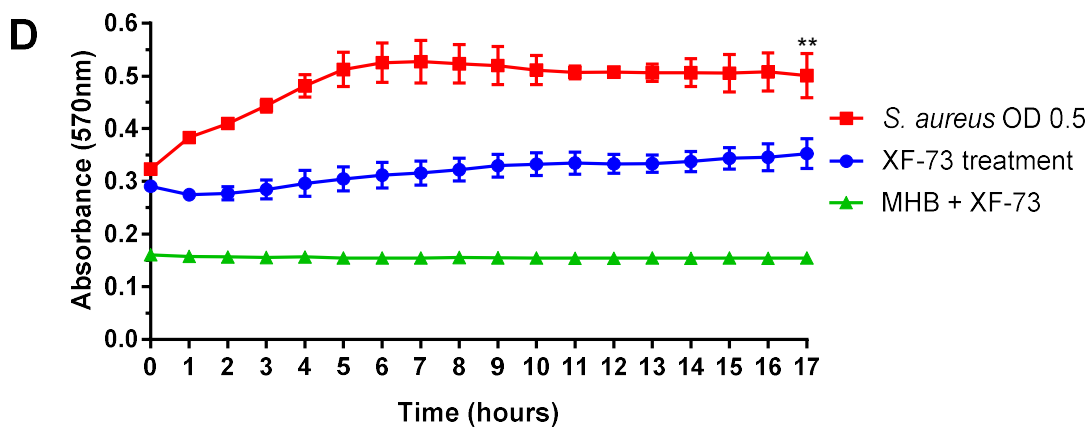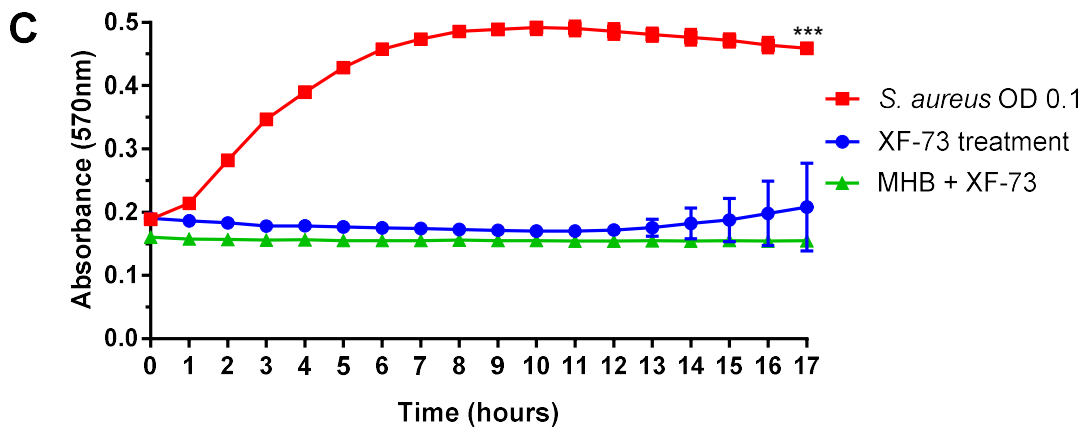
Figure 2.4 Absorbance of S. aureus ATCC 6538 cultures in MHB following inoculation with a range of ODs at lag phase growth treated with 10 x MIC (2.5 µg/ mL) of XF-73

Absorbance readings of S. aureus ATCC 6538 at a range of ODs: A) 0.01, B) 0.05, C) 0.1, D) 0.5 and E) 1 in lag phase growth treated with 2.5 µg/ mL of XF-73. Absorbance readings taken every hour for seventeen hours. Error bars generated from the standard deviation of the data sets from three replicates. End point statistical analysis on absorbance readings following treatment of XF-73 was completed via a one-way ANOVA with Tukey's multiple comparison test, where **: p= 0.0019, ***: p= 0.0007 and ****: p= <0.0001.

A
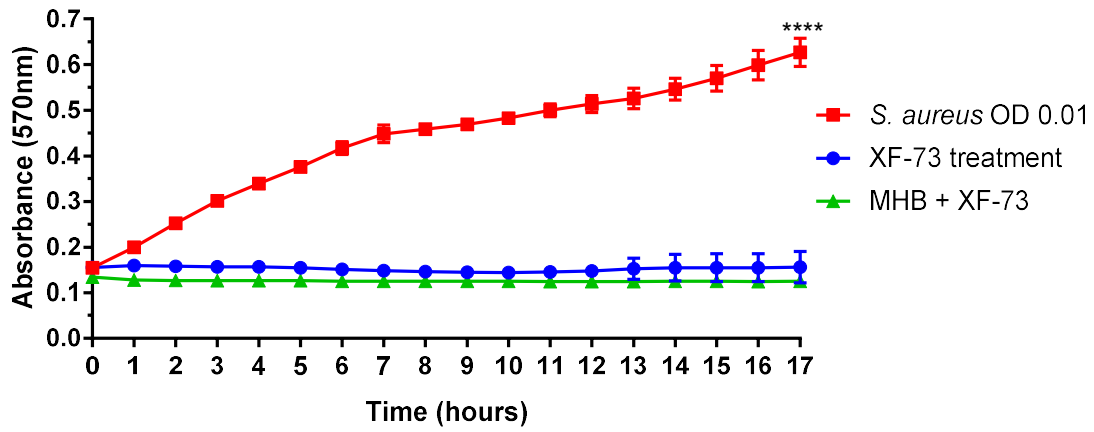
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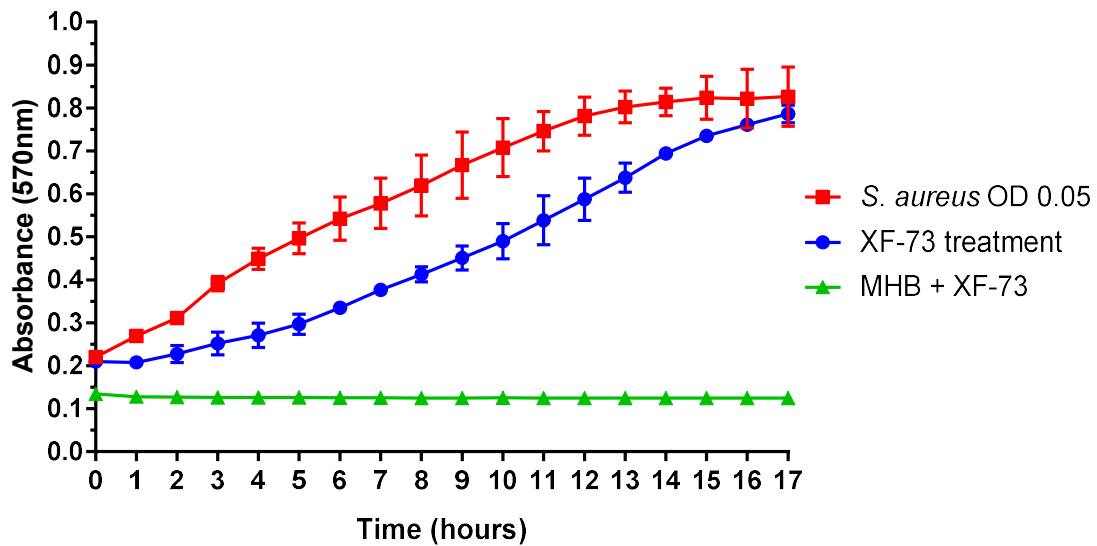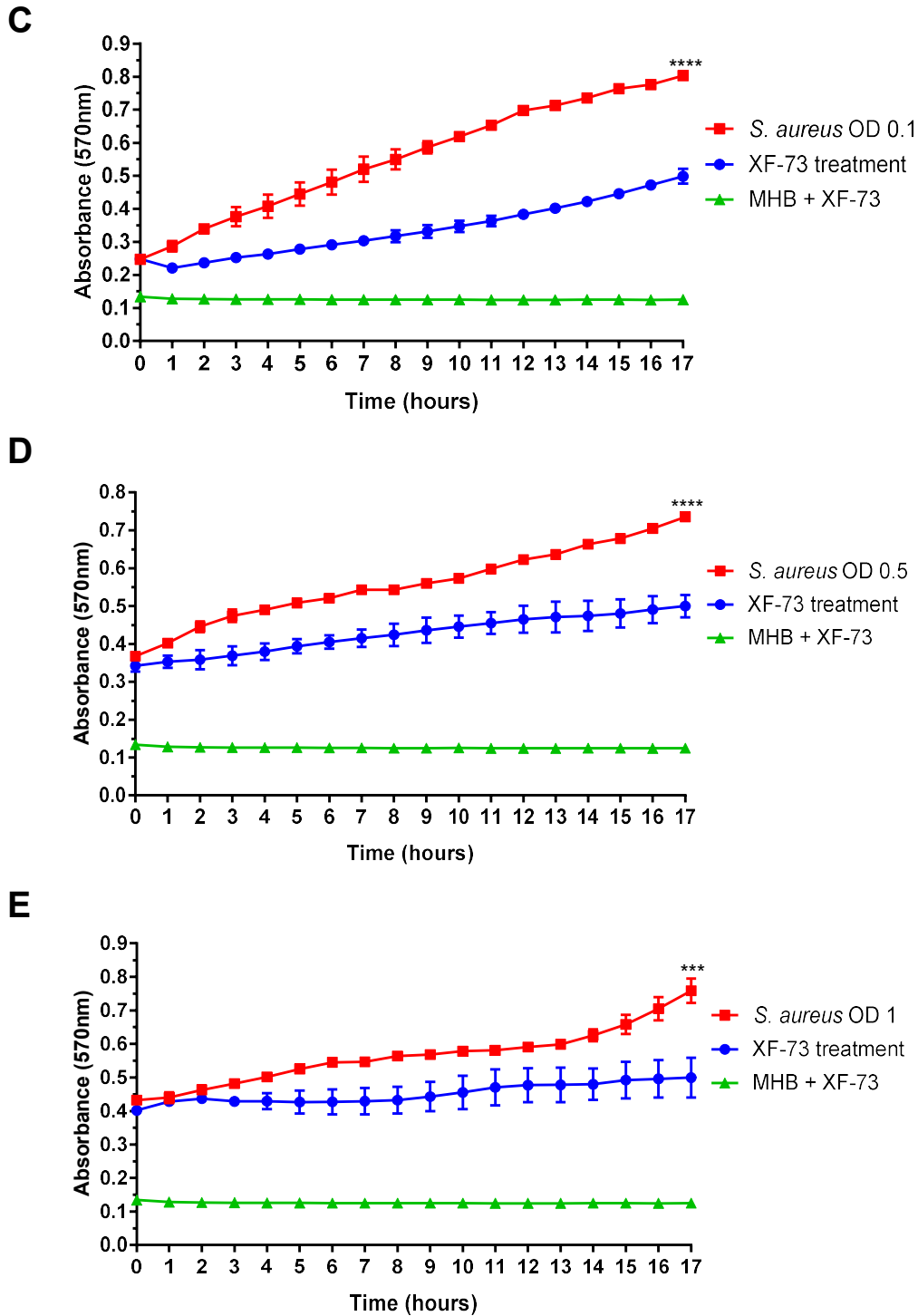
Figure 2.5 Absorbance of S. aureus ATCC 6538 cultures in MHB following inoculation with a range of ODs at log phase growth treated with 10 x MIC (2.5 µg/ mL) of XF-73

Absorbance readings (570 nm) of S. aureus ATCC 6538 at a range of ODs: A) 0.01, B) 0.05, C) 0.1, D) 0.5 and E) 1 in log phase growth treated with 2.5 µg/ mL of XF-73. Absorbance readings taken every hour for seventeen hours. Error bars generated from the standard deviation of the data sets from three replicates. End point statistical analysis on absorbance readings following treatment of XF-73 was completed via a one-way ANOVA with Tukey's multiple comparison test, where ***: p= 0.0006 and ****: p= <0.0001.

2.5.6 Varying XF-73 concentrations against *S. aureus* ATCC 6538

2.5.6.1 *S. aureus* in lag phase growth

The growth control of *S. aureus* (OD 0.01) increased slowly in absorbance readings for the first two hours of the study, followed by a further increase in absorbance readings for the next two hours (Figure 2.6). MHB alone showed no increase or decrease in absorbance readings throughout the entirety of the study.

S. aureus at an OD of 0.01 when treated with 0.25 μg/ mL showed similar absorbance readings to the growth control for the first seven hours of the study At eight hours, the absorbance of treated *S. aureus* began to increase and reach absorbance readings larger than the growth control for the remainder of the time course study. At seventeen hours 0.25 μg/ mL treated *S. aureus* had reached had a greater absorbance reading in contrast to the growth control, and statistical analysis determined the difference to be significant (p= <0.0001).

XF-73 at a concentration of 0.5 μg/ mL against *S. aureus* yielded no change in absorbance readings for the first eleven hours into the time course study, after this time point absorbance readings increased at a gradual rate. At the end of the study there was no significant difference found between the growth control and treated *S. aureus.*

Higher concentrations: 1 – 2.5 μg/ mL of XF-73 against *S. aureus* resulted in no change in absorbance readings throughout the entirety of the time course, leading to a significant difference between the growth control and XF-73 treatments (p= <0.0001).

Following the completion of the study, 2.5 μg/ mL was the only concentration of XF-73 that led to no growth of *S. aureus* on MHA, all other concentrations of XF-73 treatment and the *S. aureus* growth control showed growth on the agar plates. MHB alone showed no growth of microorganisms.

2.5.6.2 *S. aureus* in log phase growth

As previously described (2.5.5.1), incubating a culture of *S. aureus* OD 0.01 for two hours to allow the culture to reach log phase growth before conducting experiments with varying XF-73 concentrations. The *S. aureus* growth control showed an increase in absorbance readings throughout the entirety of the time study, increasing gradually for the first four hours (Figure 2.7). This increase occurred at a higher rate from the fifth to eighth hour of the study before absorbance readings began to even out, showing less change in absorbance readings for the remainder of the time. MHB alone showed no change in absorbance readings throughout the study. No significant changes in absorbance readings throughout the seventeen hours were recorded for concentrations of XF-73 at 1 – 2 μg/ mL, and end point statistical analysis indicated a significant difference between absorbance readings at these concentrations to the growth control (p= <0.0001).

Both 0.25 and 0.5 µg/ mL XF-73 treatments had recorded increases in absorbance throughout the study, with 0.25 µg/ mL having higher absorbance readings than 0.5 µg/ mL for the first seven hours of the study. Subsequently, XF-73 at 0.5 µg/ mL then generated increased absorbance readings for the remainder of the study. Only during the fifth hour did 0.25 µg/ mL of XF-73 have a similar absorbance reading to the growth control, all other time points both treatments had lower absorbance readings than the control. Statistical analysis showed the difference between both treatments and the control to be significant (p= <0.0001).

Streaked samples from each concentration of XF-73 used in this study gave confluent growth for concentrations: 0.25 – 1 µg/ mL. Growth was determined at concentrations 1.5 µg/ mL and 2 µg/ mL, although it was recorded that one of each triplicate from the samples showed no growth on MHA. XF-73 at 2.5 µg/ mL was the concentration where no growth was recorded on any triplicate on MHA. Growth was also noted for control *S. aureus* and MHB alone led to no growth on the agar.

S. aureus ATCC 6538 treated with a range of XF-73 concentrations at lag phase of growth
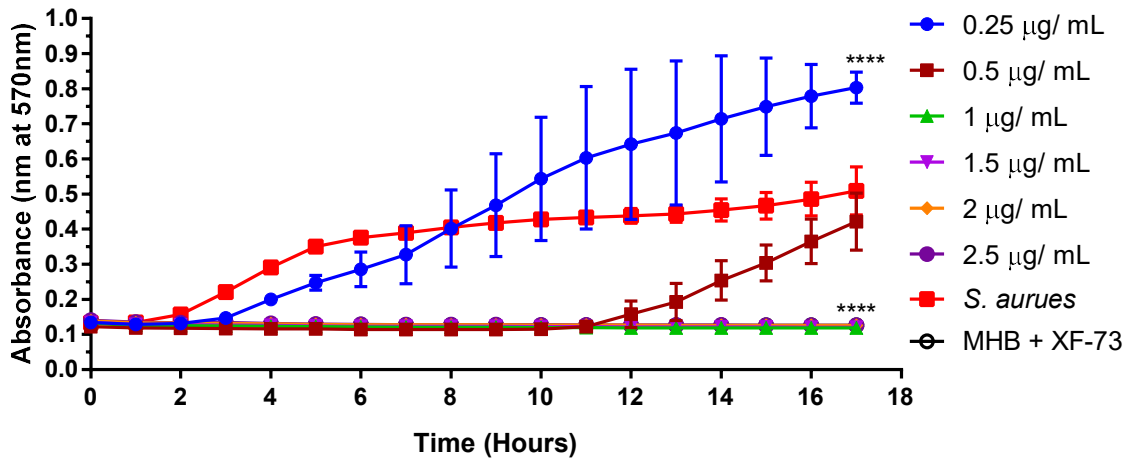
Figure 2.6 Absorbance of S. aureus ATCC 6538 culture in MHB at lag phase growth treated with XF-73 at a range of concentrations

*Absorbance readings (570 nm) of S. aureus ATCC 6538 at log phase growth treated with XF-73 at a concentration range: 0.25 µg/ mL, 0.5 µg/ mL, 1 µg/ mL, 1.5 µg/ mL, 2 µg/ mL, and 2.5 µg/ mL. Absorbance readings taken every hour for seventeen hours. Error bars generated from the standard deviation of the data sets from three replicates. End point statistical analysis on absorbance readings following treatment of XF-73 was completed via a one-way ANOVA with Tukey's multiple comparison test, where ****: p= <0.0001.*
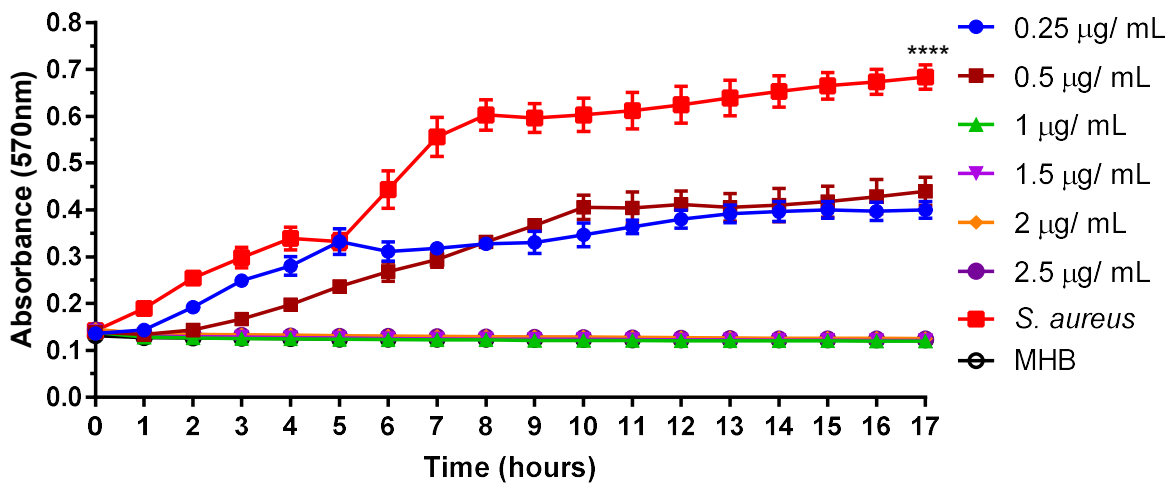
Figure 2.7 Absorbance of S. aureus ATCC 6538 culture in MHB at log phase growth treated with XF-73 at a range of concentrations

*Absorbance readings (570 nm) of S. aureus ATCC 6538 at lag phase growth treated with XF-73 at a concentration range: 0.25 µg/ mL, 0.5 µg/ mL, 1 µg/ mL, 1.5 µg/ mL, 2 µg/ mL, and 2.5 µg/ mL. Absorbance readings taken every hour for seventeen hours. Error bars generated from the standard deviation of the data sets from three replicates. End point statistical analysis on absorbance readings following treatment of XF-73 was completed via a one-way ANOVA with Tukey's multiple comparison test, where ****: p= <0.0001.*

2.6 Discussion

The aims of this part of the study were successfully met through the production of an aqueous stock solution of XF-73, with additional stability testing to determine how long XF-73 remains active and in solution. The antibacterial effect of XF-73 was recorded through antibiotic susceptibility testing through disc diffusion assays and MIC/ MBC testing against Gram-positive and Gram-negative microorganisms compared to a panel of established antibiotics.

The panel of Gram-positive and Gram-negative microorganisms was selected for this project due to their clinical relevance as pathogenic microorganisms. Each microorganism, excluding *E. coli* DCO and *E. coli* DC2, are ATCC strains and these microorganisms are commonly used for susceptibility testing and to quantify the effect of antimicrobials through media testing, *E. coli* ATCC 35218 is one of the quality control microbes selected for MHB (Sigmaalrdich, 1989; ATCC, 2022).

The ATCC strains of both Gram-positive and Gram-negative microorganisms are also clinically relevant as they were initially collected from infection sites or responsible for internal infections. *S. aureus* ATCC 6538 and *S. aureus* ATCC 29213 were both isolated from lesions and wound infections. Similarly, *E. faecalis* ATCC 29212 was isolated from urine samples. Out of the Gram-negative microorganisms selected *P. aeruginosa* ATCC 27853 was isolated from a blood culture. In contrast, *P. aeruginosa* ATCC 15442 originated from a water bottle in an animal room and *E. coli* ATCC 35218 was found in a canine (ATCC, 2022). Therefore, these are appropriate microorganisms selected for antimicrobial testing as multiple strains are relevant to human infections and each species of microorganism selected are known to cause clinical infections. Of these strains selected, both *E. coli* strains: ATCC 35218, 25922, *P. aeruginosa* ATCC 15442 and *E. faecalis* ATCC 29212 have all been used for the evaluation and quality control testing of culturing microorganisms in MHA (Sigmaalrdich, 1989). *C. albicans* ATCC 76615 was also isolated from a clinical infection (ATCC, 2022). Including this strain into the study offered insight into the effect of XF-73 against a clinically relevant yeast.

To determine the antimicrobial efficacy of XF-73 against these microorganisms, a panel of common antibiotics with different modes of action were selected for additional testing against the same panel of microorganisms to act as comparators. For the preliminary studies in this part of the study, which focused on the antimicrobial susceptibility of XF-73 against a panel of Gram-positive and Gram-negative microorganisms through disc diffusion assays, commonly used antimicrobials for disc diffusion assays were selected.

Further testing to explore the efficacy of XF-73 against planktonic Gram-positive and Gram-negative microorganisms through MIC and MBC assays; the panel of known antimicrobials was selected for this study due to their relevance as comparators to XF-73 for antibacterial efficacy.

Mupirocin was selected to be a comparator as it is currently used as a form of nasal decontamination; a potential route for XF-73 application. Mupirocin has shown potent activity against Gram-positive microorganisms, especially against *S. aureus* and has shown to be effective against MRSA present in neonatal intensive care units, with mupirocin used for nasal decolonisation of MRSA and other *S. aureus* strains (Henkel *et al.*, 1999; Pierce *et al.*, 2016). Mupirocin has also been shown to be an effective treatment for primary and secondary superficial skin infections due to *S. aureus* leading to 90% eradication in the isolates and 80% improvement for infected patients. The mechanism of action for mupirocin is the inhibition of protein synthesis by binding to the bacterial isoleucyl-tRNA synthetase (Khoshnood *et al.*, 2019). The extensive use of mupirocin has led to resistance found in *S. aureus* and coagulase-negative staphylococci, in cases of both high-level resistance and low-level resistance (Critchley *et al.*, 2005).

Daptomycin is used to treat skin and soft tissue infections, and was selected as a comparator against XF-73 as this is an additional area for future clinical application of XF-73. Bactericidal activity of daptomycin has been reported *in-vitro* against clinically important Gram-positive microorganisms and to treat resistant organisms such as: MRSA and vancomycin resistant enterococci (Silverman *et al.*, 2001). Daptomycin has the capability to be bactericidal through the disruption of the bacterial plasma membrane causing a calcium-dependent dissipation of the membrane potential documented in *S. aureus* (Alborn *et al.*, 1991). As XF-73 also imparts efficacy through membrane disruption, daptomycin acts as a direct comparator for assessing efficacy as they act upon similar target sites in bacteria. Daptomycin is effective at treating skin and soft tissue infections (Tally *et al.*, 1999). Although resistance to daptomycin has started to increase over the past years in coagulase-negative staphylococci with *Staphylococcus capitis* showing the highest levels of resistance (Jiang *et al.*, 2019).

Rifampicin blocks bacterial RNA synthesis by the specific binding site on the RNA polymerase which leads to the inhibition of RNA chain initiation and chain elongation (Hartmann *et al.*, 1985). Since its discovery in 1965, rifampicin is classified as one of the most important drugs for treatment of tuberculosis including chronic drug resistant pulmonary tuberculosis. Additionally, this ansamycin derivative has activity against Gram-positive microorganisms including multidrug resistant *S. aureus*, but is less effective against Gram-negative microorganisms. Microorganisms have shown resistance to rifampicin through multiple defence mechanisms such as mutating or duplicating the target site of rifampicin or altering the permeability of the cell, preventing rifampicin from entering (Tupin *et al.*, 2010; Grobbelaar *et al.*, 2019). Rifampicin was selected to be a comparator against XF-73 due to its effectiveness against Gram-positive microorganisms.

Gentamicin is a commonly used aminoglycoside antibiotic, often used in hospitals, with potent efficacy against aerobic microorganisms and is commonly used to treat serious infections caused by Gram-negative bacterium. Gentamicin has a range of applications through topical or systemic treatments

against the Enterobacteriaceae. Furthermore gentamicin demonstrates activity against MRSA and a range of coagulase-negative staphylococci (Chaves *et al.*, 2022). Gentamicin acts through the inhibition of protein synthesis as well as inducing an increase in misreading messenger RNA through binding on the 30s ribosomal subunits (Tangy *et al.*, 1985). Resistance to gentamicin is recorded in *Enterococcu*s species, in particular *E. faecalis* through production of four aminoglycoside-modifying enzymes that inactivate gentamicin. This resistance to gentamicin reduces previously effective antimicrobial cocktail treatments involving gentamicin and β-lactams or glycopeptides which are disruptive to the bacterial cell well (Aslangul *et al.*, 2006). Gentamicin was selected to be a comparator antimicrobial in this study due to its wide application range as an antimicrobial treatment and its efficacy against both Gram-positive and Gram-negative microorganisms.

Initial testing for the efficacy of XF-73 via disc diffusion assays, highlighted XF-73 as the least effective antimicrobial treatment in comparison to the comparators against Gram-positive microorganisms, due to all concentrations of XF-73 used having the smallest recorded zones of inhibition (Figure 2.2). A dose dependent response was recorded for the concentrations of XF-73 against each Gram-positive microorganism. This was determined as 20 µL of 102.4 µg/ mL solution of XF-73 applied to the discs (equivalent to 2 µg XF-73) yielded the largest zone of inhibition, signifying that concentrations of XF-73 greater than 102.4 µg/ mL did not further increase the antimicrobial efficacy as determined by inhibition zone size. XF-73 showed no antibacterial effect against Gram-negative microorganisms. No zones of inhibition for any antimicrobial treatment were recorded against *C. albicans* (Figure 2.3). From these data sets XF-73 shows little antimicrobial effect against Gram-positive or Gram-negative microorganisms and current antimicrobials treatments such as ciprofloxacin are more effective in comparison.

In this study, XF-73 showed a bactericidal effect against a range of Gram-positive and Gram-negative microorganisms, with a potent effect highlighted during MIC/ MBC testing against the Gram-positive samples (Table 2.3). This antibacterial effect of XF-73 was not recorded during preliminary testing via disc diffusion assays, signifying that this form of antibacterial susceptibility testing is not an appropriate form of testing to measure the antibacterial properties of XF-73. Disc diffusion assays are known to have limitations. Zones of inhibition are measured as the diameter around the antibiotic discs and these circles are often irregular. Therefore, the diameter may be uneven around the disc, making it difficult to accurately measure the zone and the zone is dependent to where an individual decides to measure the diameter. Zones of inhibition are known to be imprecise and are not comparable to MIC values. Additionally these tests are unable to differentiate if the antimicrobial treatment is causing a bacteriostatic or bactericidal effect against the microorganism. Previous studies have highlighted that a lack of a zone of inhibition does not necessarily equate to no antibacterial effect, as some antimicrobials, in particular low polarity compounds, will diffuse slowly across agar   (Liu *et al.*, 2016; Bubonja-Šonje *et al.*, 2020). XF-73 has been shown to diffuse poorly through agar; Destiny Pharma has also confirmed

that XF-73 is unable to efficiently diffuse across agar, supporting the recorded results in this preliminary study. Diffusion assay discs containing XF-73 have not been developed by Destiny Pharma to monitor activity against clinical isolates. Some small variations in MIC and MBC values were recorded between the different batches of XF-73 used throughout the study (Table 2.3), although each batch of XF-73 was always more effective at eradicating Gram-positive microorganisms in comparison to the selected Gram-negative isolates. Similarly, as multiple MIC and MBC assays were conducted using batches 1 and 2 of XF-73, a further variation was observed. Fluctuations in bacterial behaviour are to be expected as the research incorporates living microorganisms, therefore it is highly likely that small differences would be recorded during different MIC testing. Batch 2 of XF-73 exhibited a bactericidal effect against Gram-negative microorganisms, but at a much higher concentration (128 µg/ mL) in comparison to MIC values for Gram-positive bacteria. Generally, Gram-negative microorganisms are known to be more resistant to antimicrobial treatments in comparison to Gram-positive microorganisms, due mainly to the different permeability properties of the cell envelopes.

A main difference between Gram positive and negative bacteria is the presence of an outer membrane that only Gram-negative bacteria possess. This outer membrane surrounds the bacterial cell wall made up of peptidoglycan; in contrast, Gram-positive bacteria have a much thicker cell wall to overcome the absence of an outer membrane. Anionic polymers are located throughout the peptidoglycan to support and maintain the structure and protects the cell membrane. These external structures are classified as the bacterial cell envelope and protect the cytoplasm, additionally the cell envelope has evolved to allow the passage of nutrients into the cells and exporting metabolically generated waste from the cell (Silhav *et al.*, 2010).

The outer membrane of Gram-negative bacteria aids in the resistance to antimicrobial treatment. The presence of the outer membrane alone leads to vancomycin resistance in Gram-negative microorganisms as it is unable to pass through and reach its target site, due to its large chemical structure. This resistance was illustrated during the disc diffusion assays, as vancomycin failed to generate a zone of inhibition against any Gram-negative microorganism (Figure 2.3). Additionally vancomycin is classified as having a large chemical structure, and is composed of a tricyclic ring system made up of a seven-membered peptide chain, with glucose and vancosamine attached to the tricyclic ring (Biondi *et al.*, 2016). Diffusion for large compounds across agar is limited, leading to small zones of inhibition recorded for larger compounds, this was highlighted as vancomycin had small zones of inhibition against Gram-positive microorganisms (Figure 2.2).

The outer membrane of Gram-negative microorganisms are made up of three zones: a hydrophilic outer lipopolysaccharide layer, a hydrophilic oligosaccharide core and a hydrophobic inner lipid layer. This composition limits the membrane permeability to hydrophobic molecules; therefore, antimicrobial treatments that target components within the cytoplasm may be unable to enter the cell to cause an

antibacterial effect. Resistance to antimicrobial treatments for Gram-negative microorganisms also occurs through mutations of the drug target site and the expression of porin efflux pumps to prevent antimicrobial treatments entering the cell. Gram-negative microorganisms are also known to have additional resistant mechanisms including altering the permeability of the outer membrane, to prevent antimicrobials from entering the cell, the expression of enzymes capable of inactivating antibiotics also increases resistance. Horizontal gene transfer allows resistance genes to be transferred between species or between the same species, this is conducted through transduction, transformation and conjugation, these resistant genes are passed between bacterial cells in a variety of vectors including: plasmids, transposons or integrons. Horizontal gene transfer of plasmids containing β-lactamases led to an increased resistance pattern occurring between Gram-negative microorganisms and β-lactam treatments (Exner *et al.*, 2017; Gupta *et al.*, 2019; Breijyeh *et al.*, 2020).

The membrane disruptive effect of XF-73 is less effective against Gram-negative microorganisms in comparison to Gram-positive planktonic cells; this may be due to the presence of the outer membrane restricting XF-73 from entering the cells. These results correlate with current findings that Gram-negative microorganisms are naturally more resistant to antimicrobial treatments (Gupta *et al.*, 2019).

The mutant *E. coli* DC0 and *E. coli* DC2 were also selected for this study, *E. coli* DC0 is the parent strain and the mutant *E. coli* DC2 has a hyper-permeable cell wall and outer membrane. Though this mutant showed no change in lipid composition, nor any changes observed in the lipopolysaccharide sugar composition, or in the cross-linking of peptidoglycan. Previous studies have illustrated that the mutant *E. coli* DC2 is more susceptible to a range of antimicrobial treatments, with different mechanisms of action in comparison to *E. coli* DC0; for example penicillin had an MIC at 200 µg/ mL against *E. coli* DC0 but a 400-fold decrease in concentration was recorded against *E. coli* DC2 at 0.5 µg/ mL (Clark, 1984). Due to this increase in antimicrobial susceptibility against *E. coli* DC2, in particular the antimicrobials that operate via membrane disruption, the mutant and parent strains were added into this study to determine if an increase in efficacy of XF-73 would be observed, as previous work conducted on XF-73 has highlighted its antibacterial activity through membrane disruption (Ooi *et al.*, 2009b). Though no decrease in concentrations of XF-73 were recorded against both strains. Therefore, the permeability of Gram-negative cell envelops does not appear to influence the effectiveness of XF-73 against these microorganisms.

The findings from this study are supported by previous research which also conducted MIC and MBC testing against a range of clinically relevant microorganisms. Throughout previous and current research XF-73 has shown a potent anti-staphylococcal effect, with a recorded MIC value of 1 µg/ mL against *S. aureus* SH1000*,* comparator testing in this study also included daptomycin and rifampicin with MIC values at 1 µg/ mL and 0.008 µg/ mL respectively (Ooi *et al.*, 2009b). Board-Davies *et al.,* (2022) conducted MIC testing against fifty five Gram-positive isolates and reported MIC values of XF-73

against these microorganisms ranging from 0.06 – 2 µg/ mL (Board-Davies *et al.*, 2022). XF-73 MIC values recorded in previous studies are within the MIC ranges collected between the different batches of XF-73 in chapter two (Table 2.3), additionally rifampicin and daptomycin had similar MIC values reported in previous work in comparison to values recorded in chapter two (Table 2.3), highlighting that XF-73 retains its potent inhibitory effect against multiple strains of *S. aureus*, similar MIC values for daptomycin and rifampicin also support the validity of the current study conducted (Table 2.6).

Farrell *et al.,* (2010) conducted MIC testing against a panel of 101 Gram-positive and Gram-negative microorganisms. XF-73 had an MIC range of 0.5 – 1 µg/ mL against a range of *S. aureus* strains including methicillin-resistant, mupirocin-resistant, and vancomycin-intermediate-resistant strains. This concentration range correlates to the concentration ranges of XF-73 collected throughout this current study. Recorded MIC values of XF-73 against *E. faecalis* ATCC 29212 and *S. epidermidis* ATCC 12228 were similar to those recorded against different multiple strains of *E. faecalis* and *S. epidermidis* in Farrell's study, signifying that the efficacy of XF-73 against these microorganisms is retained between different strains, making XF-73 a potentially effective treatment against infections involving these Gram-positive microorganisms. Farrell *et al.,* (2010) recorded MICs against multiple strains of *E. faecalis* ranging from 0.5 – 2 µg/ mL, from the five strains tested antimicrobial resistant strains of *E. faecalis* were included: vancomycin-resistant and gentamicin-resistant. Similarly, MIC values ranged from 0.25 – 1 µg/ mL (Farrell *et al.*, 2010).

Previous research also supports the data collected in this study showing XF-73 to be less effective against Gram-negative bacteria in comparison to Gram-positives microorganisms. Farrell *et al.,* (2010) conducted MIC testing at a lower concentration range where 64 µg/ mL was the highest concentration and each *P. aeruginosa* strain selected in the study had a concluded MIC value greater than 64 µg/ mL, similarly *E. coli* strains had MICs ranging from 32 – >64 µg/ mL (Farrell *et al.*, 2010). Recent studies incorporated a greater concentration range of XF-73 for MIC testing against a range of fifty nine Gram-negative isolates, including multiple strains of *E. coli* and *P. aeruginosa,* and reported that Gram-negative bacterial cells were less susceptible to XF-73 treatment in comparison to Gram-positive microorganisms as MIC values ranged from 128 – > 1024 µg/ mL. (Board-Davies *et al.*, 2022). The concentration ranges of XF-73 capable of inhibiting Gram-negative bacterial growth (Table 2.3) fit into the concentration ranges collected from previous research; signifying XF-73 has a uniform response against Gram-negative microorganisms.

Similarly, XF-73 showed a bactericidal effect against *C. albicans* ATCC 76615 at 4 µg/ mL, indicating an effective antimicrobial effect against the yeast. Previous study assessing the efficacy of XF-73 against *C. albicans* ATCC-MYA-273 planktonic cells showed that XF-73 at 1 µM (0.766 µg/mL) was unable to decrease the number of viable cells (Gonzales *et al.*, 2013). Signifying that low concentrations of XF-73 is ineffective against different strains of *C. albicans*.

In comparison to mupirocin, XF-73 showed a greater efficacy against Gram-positive microorganisms as mupirocin was only able to inhibit bacterial growth (Table 2.6). Whereas XF-73 showed potent bactericidal effect where the same concentration of XF-73 was recorded to inhibit and eradicate the Gram-positive microorganisms. Although Mupirocin was shown to be more effective at inhibiting both *E. coli* strains.

Rifampicin and XF-73 showed similar antibacterial properties against the panel of Gram-positive microorganisms, with the same MIC and MBC values recorded against *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 (Table 2.6). Whilst rifampicin at a lower concertation in comparison to XF-73 was capable of inhibiting *S. aureus* ATCC 6538 planktonic cells, XF-73 had a lower MIC value against *E. faecalis* ATCC 29212. Rifampicin demonstrated a bacteriostatic effect against *S. aureus* ATCC 6538 and *E. faecalis* ATCC 29212, whereas XF-73 had a recorded bactericidal effect against all Gram-positive microorganisms. Highlighting that XF-73 is a more efficient antibacterial treatment against the panel of Gram-positive microorganisms.

When the antimicrobial efficacy of XF-73 was compared to daptomycin, XF-73 had a greater antibacterial efficacy against Gram-positive microorganisms, shown by the lower MIC and MBC values generated by XF-73 (Table 2.6). No antibacterial properties were recorded for daptomycin against the panel of Gram-negative microorganisms whereas batch 2 XF-73 could eradicate them.

Gentamicin had the lowest recorded bactericidal effect against Gram-negative microorganisms in comparison to the panel of antimicrobials used in this study. This was expected as previous studies had highlighted the efficacy of gentamicin as more potent against Gram-negative microorganisms in comparison to Gram-positive microorganisms. As antimicrobial resistance has been recorded in *E. faecalis*, the results collected from this study supports this statement as *E. faecalis* ATCC 29212 required the greatest concentration of gentamicin to eradicate the planktonic cells at 16 μg/ mL out of the entire panel of microorganisms (Table 2.6). XF-73 was identified to be a more effective at eradicating all Gram-positive microorganisms, as lower MBC values were recorded against the panel of Gram-positive microorganisms in comparison to corresponding gentamicin MBC values.

Table 2.6 MIC and MBC values (µg/ mL) of a range of antimicrobials against a panel of clinically relevant Gram-positive and Gram-negative microorganisms

Values are a combination of MIC and MBC values collected from Table 2.3 and Table 2.4, XF-73 values are lowest MIC and MBC values collected from each batch of XF-73 used.

Microorganism/ ATCC	XF-73 µg/ mL MIC	MBC	Gentamicin µg/ mL MIC	MBC	Mupirocin µg/ mL MIC	MBC	Rifampicin µg/ mL MIC	MBC	Daptomycin µg/ mL MIC	MBC
S. aureus 6538	0.25	0.25	4	4	≤ 0.125	> 64	≤ 0.125	8	2	4
S. aureus 29213	≤ 0.125	≤ 0.125	2	2	0.25	> 64	≤ 0.125	≤ 0.125	4	4
S. epidermidis 12228	≤ 0.125	≤ 0.125	≤ 0.125	0.25	≤ 0.125	> 64	≤ 0.125	≤ 0.125	2	2
E. faecalis 29213	0.125	0.125	8	16	16	> 64	0.25	> 64	16	16
E. coli 35218	128	> 256	2	2	32	> 256	4	> 256	> 256	> 256
E. coli 25992	128	128	1	2	32	> 256	2	256	> 256	> 256
P. aeruginosa 15442	128	256	1	1	128	128	8	128	> 256	> 256
P. aeruginosa 27853	128	256	1	1	128	128	8	16	> 256	> 256

Note: table contains the lowest recorded MIC and MBC values across all batches of XF-73 used throughout the study (Table 2. 3). Daptomycin MIC and MBC values are used from supplemented media containing calcium chloride and magnesium chloride (Table 2.4).

This part of the study has given an insight into the potential efficacy of XF-73 particularly against Gram-positive microorganisms. The benefit of including technical and experimental replicates during MIC and MBC testing of XF-73 against the panel of Gram-positive and Gram-negative microorganisms, sans *C. striatum* ATCC 1293, *E. coli* DC0 and *E. coli* DC2, increases the validity of the recorded results, signifying that the recorded MIC and MBC values are an accurate representation of the efficacy of XF-73 against these microorganisms. Concentration ranges recorded for MIC and MBC values against the Gram-positive microorganisms is expected due to biological variation when testing involves the use of bacterial species.

Future research would involve repeating MIC and MBC testing for the panel of antimicrobials against Gram-positive and Gram-negative microorganisms. As these experimental replicates may lead to potential inhibition and eradication ranges, similar to those recorded for XF-73, supporting the idea of biological variation leads to variation in XF-73 concentrations capable of inhibiting or eradicating Gram-positive microorganisms. As XF-73 showed a bactericidal effect against Gram-negative bacteria but required high concentrations of XF-73 to be effective, future testing involving combination treatments with gentamicin could aid in decreasing the concentration of XF-73 required to eradicate these bacterial cells.

Stability testing of XF-73 in aqueous solution highlighted that XF-73 remains in solution and retains its antimicrobial efficacy for up to 16 – 18 weeks, as the MIC/ MBC values against *S. aureus* ATCC 6538 remained the same for 16 weeks (Table 2.5). There was only a 2-fold increase in MIC and MBC values on the 18th week. Therefore, 18 weeks is possibly the maximum time XF-73 remains active in aqueous solution as at 20 weeks a range of MIC/ MBC values were recorded between replicates and a minimum 4-fold increase in concentration was now observed. One of the benefits of XF-73 remaining stable in solution for long periods of time is the reduced need to prepare fresh aqueous stock solutions of XF-73. Also, potential antimicrobial XF-73 treatments would have an expected shelf life of around 4 months.

Recording absorbance readings of *S. aureus* ATCC 6538 following XF-73 treatment offers an opportunity to observe the immediate inhibitory effect XF-73 has against the microorganism. This intermediate effect of XF-73 is not recorded through standard susceptibility testing or MIC/ MBC assays, as these studies only show the end point effect of antimicrobials.

Varying the optical density of the microorganism, simulated different levels of an infection, as the greater the optical density the more established the infection. Throughout the seventeen-hour absorbance readings for untreated *S, aureus* ATCC 6538 planktonic cells OD: 0.01 – 0.5, a standard bacterial growth curve was observed (Figure 2.4 A – D). A bacterial growth curve represents the different phases of growth for a bacterium, following introduction into a new environment. Lag phase is the first phase for bacterial growth curves, where the microorganism is adjusting to the new environment, during this time bacterial replication is not usually observed though the cells themselves may grow. *S. aureus* remained in lag phase for the first two hours of the study at OD 0.01, as the optical density increased: OD 0.05, 0.1, *S. aureus* remained in lag phase for only one hour.

Log or exponential phase follows lag phase and at this timeframe the bacterial cells regularly divide and expand in this new environment, using up the nutrients and materials present while cell division occurs. As only a finite amount of nutrients are present in the environment, especially during biological testing. It could be suggested that *S. aureus* OD 0.5 begins in log phase growth as within the first hour of the time course study, cell division is occurring as the absorbance readings are increasing. A bacterial culture reaches a maximum growth rate and the number of bacterial cells remains the same during stationary phase, where the number of dividing cells is equal to number of cells that are dying due to a lack of nutrients and exhaustion. *S. aureus* at OD 1 has a constant absorbance reading recorded throughout the study, therefore is determined to be at stationary phase throughout the study. This may be due to the large volume of bacterial cells entering the environment and this volume of cells already reaches the maximum potential growth rate in the environment due to nutrient availability.

The final phase of bacterial growth in an environment is death phase as there is a greater abundance of dead cells in comparison to living cells and there is no longer enough nutrients in the environment to support cellular growth and division (Wang *et al.*, 2015). Although absorbance readings will not be able

to indicate death phase as the absorbance of *S. aureus* will not decrease following cellular death. Therefore, the stationary phase recorded for *S. aureus* throughout these studies may also include death phase but absorbance readings alone will not be able to differentiate between them. Plating up bacterial cultures following the time study allows further testing to determine if XF-73 inhibited or eradicated the bacterial cells. Overcoming the issue of identifying the antibacterial effect of XF-73 from absorbance readings alone.

The absorbance readings of OD 0.01 and 0.05 did not increase throughout the study (Figure 2.4 A, B), signifying that cell replication is not occurring and from these results XF-73 is either inhibiting cellular growth or has eradicated *S. aureus* cells. Following plating up the samples onto MHA XF-73 only exhibited bactericidal efficacy against OD 0.01 of *S. aureus*, shown by no viable cells present on MHA following the time studies. XF-73 displayed bacteriostatic properties against *S. aureus* at ODs 0.05 – 0.5 (Figure 2.4 B – D).

Inhibition of cell replication was observed at OD 0.1 of *S. aureus* where no change in absorbance readings occurred for the first thirteen hours of the study (Figure 2.4 C). At the fourteenth hour replication occurs as the absorbance readings begin to increase, though XF-73 decreased *S. aureus* growth and treated cells did not reach absorbance readings recorded for the growth control. As the OD of *S. aureus* increased, the inhibitory effect of XF-73 decreased and at OD 0.5, *S. aureus* treated with XF-73 showed a steady increase in absorbance readings signifying cell growth (Figure 2.4 D). But at a reduced rate as each time point readings of treated *S. aureus* had a lower absorbance reading in comparison to corresponding growth controls.

Unlike the growth control at OD 1, XF-73 treatment at 2.5 µg/ mL led to an increase in absorbance readings indicating log phase growth throughout the entirety of the time course study (Figure 2.4 E). A possible explanation for these results is immediate exposure to XF-73 treatment decreased the starting inoculum of *S. aureus* highlighted from the lower absorbance reading observed at time 0. As XF-73 at 2.5 µg/ mL no longer inhibits the bacterial growth, *S. aureus* begins to replicate, utilising the available nutrients before reaching stationary phase growth around the eight hour.

S. aureus at each OD was recorded to reached log phase growth after incubation in MHB for two hours; therefore, two hours was the selected incubation time for each OD of *S. aureus* to enable log phase growth before XF-73 treatment was conducted. Log phase growth was confirmed following two hours of incubation for each OD, as absorbance readings increased throughout the time study, indicating log phase growth (Figure 2.5). Allowing *S. aureus* to reach log phase growth before XF-73 treatment simulates an a more realistic effect of XF-73 against an established infection, as infections are more likely to be rapidly replicating before a treatment strategy occurs as the bacteria is established in the environment.

Whilst the same bactericidal effect was recorded in this study; only OD 0.01 was eradicated following XF-73 treatment as no viable cells were present on MHA after the time course study, a different inhibitory effect was noted between the optical densities. As the density of *S. aureus* increased, the inhibitory effect of XF-73 decreased. *S. aureus* OD 0.01 was the only density where XF-73 treatment led to no increase in absorbance readings throughout the seventeen hours (Figure 2.5 A). All other optical densities of *S. aureus* had a reduced growth rate with the addition of XF-73 in comparison to the growth control, but the cells continued to grow throughout the time frame (Figure 2.5 B – E). *S. aureus* OD 0.05 was the density determined to be least effected by XF-73 treatment as at the end of the study treated and untreated planktonic cells had similar absorbance readings (Figure 2.5 B).

These results have highlighted that once *S. aureus* has established itself in an environment and begins to regularly replicate, XF-73 at 2.5 µg/ mL becomes less effective at inhibiting bacterial growth for densities of *S. aureus* greater than OD 0.01.

Varying the concentration of XF-73 against OD 0.01 *S. aureus* ATCC 6538 in lag and log phase growth highlighted the distinction in antimicrobial effect of XF-73 at different concentrations (Figure 2.6, 2.7). As expected, following the previous study varying optical densities, 2.5 µg/ mL of XF-73 inhibited bacterial growth for the entire seventeen-hour study, illustrated by no increase in absorbance readings for lag (Figure 2.6) and log (Figure 2.7) phase growth, whilst no viable cells grew on MHA following the studies it was determined that XF-73 at 2.5 µg/ mL retained its bactericidal effect against OD 0.01 *S. aureus* in lag and log phase growth (2.5.6).

Concentrations of 2 – 1 µg/ mL of XF-73 also inhibited the growth of *S. aureus* in both lag and log phase growth (Figure 2.6, 2.7), as no change in absorbance readings were recorded, viable cells present on MHA following the time course confirmed that these concentrations of XF-73 only had an inhibitory effect (2.5.6).

Lower concentrations of XF-73 showed a variation on inhibitory effects depending on the growth phase of *S. aureus*. XF-73 at 0.5 µg/ mL inhibited *S. aureus* in lag phase growth for eleven hours before bacterial growth and cell division occurred for the remaining six hours, end point absorbance readings highlighted treated *S. aureus* having a similar absorbance to the growth control (Figure 2.6). *S. aureus* in log phase growth decreased the inhibitory effect of 0.5 µg/ mL of XF-73 as the antimicrobial treatment was only able to inhibit growth for two hours before cell replication occurred. Although after ten hours a plateau in absorbance readings occurred, indicating a potential reduction in growth rates (Figure 2.7). Unlike all other concentrations of XF-73 used in the study, XF-73 at 0.25 µg/ mL led to growth rates that surpassed corresponding absorbance readings the growth control in lag phase growth after eight hours (Figure 2.6). Though inhibition did occur for three hours following XF-73 treatment. XF-73 has the potential to eradicate *S. aureus* cells during the first three hours, as absorbance readings are unable to differentiate between cell death or inhibition, there are more nutrients available in the environment for

the remaining planktonic cells, therefore cells are no longer competing for nutrients to aid growth. XF-73 treatment at 0.25 μg/ mL against *S. aureus* in log phase growth yielded a different result in comparison to the lag phase growth study. Treated *S. aureus* cells followed a similar replication trend to untreated cells for the initial five hours, but for the remainder of the study the absorbance readings plateaued and yielded similar results to *S. aureus* cells treated with 0.5 μg/ mL, this may present a potential bacteriostatic effect of XF-73 that occurred once bacterial cells reached stationary phase growth and the remaining cells were more susceptible to the antimicrobial treatment.

As absorbance readings following XF-73 treatment were only conducted on a single strain of *S. aureus,* it is difficult to draw conclusions from, though it has highlighted an immediate inhibitory effect that is not documented from MIC / MBC testing alone. Additional testing focusing on the inhibitory effect of XF-73 against the remaining panel of Gram-positive microorganisms could support these current findings on the immediate inhibitory effect of XF-73. Conducting the same studies using the panel of known antimicrobials would also aid in determining if this inhibitory effect is observed for all antimicrobials or is unique to XF-73. XF-73 has shown a bactericidal effect against some Gram-negative microorganisms from MIC and MBC testing. Assessing the antibacterial effect of the antimicrobial through time course studies would help in expanding the current understanding of XF-73 against Gram-negative microorganisms. High concentrations of XF-73 are capable of eradicating Gram-negative planktonic cells, potential retreatment strategies with XF-73 may reduce the concentration of XF-73 to have the same effect.

2.7 Conclusion

This initial part of the study has highlighted the antibacterial properties of XF-73 against a range of clinically relevant Gram-positive, Gram-negative microorganisms and *C. albicans* in planktonic mode of growth via MIC and MBC assays. Whilst disc diffusion assay offered some insight into the antimicrobial efficacy of XF-73 and antibiotic comparators, the technique offered little scientific value overall. The MIC and MBC values generated from this study are similar to values recorded in previous research which has focused on the effect of XF-73 against a range of clinically relevant bacteria. Therefore, research outside of this study supports the findings that XF-73 retains its efficacy against a range of microorganisms and keeps its inhibitory effect against a variety of strains of the same microorganism.

XF-73 is classified as a bactericidal antimicrobial treatment against Gram-positive and Gram-negative planktonic microorganisms, as the same concentration of XF-73 was recorded as the MIC and MBC value for the panel of microorganisms. The antibacterial efficacy of XF-73 is deemed to be greater against Gram-positive microorganisms, as ≤ 0.125 µg/ mL of XF-73 eradicated *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228 and *E. faecalis* ATCC 29212 planktonic cells, in comparison greater concentrations of XF-73 was required to eradicate Gram-negative bacterial cells (Table 2.3).

While XF-73 is less effective against Gram-negative planktonic cells, potential combination therapies with antimicrobials known to be effective against these infections may aid in the antimicrobial effect of XF-73. Concentration ranges for MIC and MBC values were recorded for XF-73 against the Gram-positive microorganisms, but the lower concentration ranges for MBC values indicated XF-73 to be a greater antimicrobial treatment at eradicating a panel of four Gram-positive microorganisms in comparison to a panel of four known antimicrobials. Indicating XF-73 has the potential to be a potent antibacterial treatment against clinically relevant staphylococcal infections.

This study has demonstrated the immediate inhibitory and killing effect that XF-73 has against *S. aureus* ATCC 6538 planktonic cells, with time course studies exploring the effect of XF-73 on the growth rate of the microorganisms. These results provide a greater insight in what happens during the exposure of XF-73 to bacterial cells, an area that MIC/ MBC assays alone does not show. Further testing involving time course studies of the efficacy of XF-73 against a range of Gram-positive and Gram-negative microbes would advance our current understanding on the efficacy of XF-73.

3.0 Chapter 3: Assessing the antimicrobial efficacy of XF-73 through *in-vitro* testing against clinically relevant microorganisms in biofilm mode of growth

3.1 Introduction

Each year in England alone 1,000,000 cases of HCAIs are reported, these cases are not limited to hospitals alone but are also present in care homes and homes of the patients themselves. These infections have a prevalence rate of 6.4% (Percival *et al.*, 2015). Biofilm formation poses a significant risk in HCAIs due to their ability to form on abiotic surfaces. Medical devices inserted into a patient are at a greater risk of biofilm infections, these sites include: respiratory devices, central venous catheters, urinary catheters and mechanical heart valves. Biofilm infections also have the capability to form on biotic surfaces and have been responsible for wound infections including non-healing wound infections, in particular those caused by *P. aeruginosa*. The most commonly isolated microorganisms from HCAI biofilm infections are Gram-positive bacteria: *S. aureus*, *S. epidermidis, E. faecalis* and viridans streptococci and Gram-negative bacteria: *E. coli, P. aeruginosa, K. pneumoniae* and *Proteus mirabilis*. The origin of these microorganisms can vary from the environment to the skin microbiota of either the patient or the healthcare worker i.e. *S. aureus* and *S. epidermidis.* These infections not only lead to an increased financial burden for healthcare systems, due to prolonged treatment and hospitalisation but lead to an increased mortality and morbidly for a patient suffering with these infections (Donlan, 2001; Percival *et al.*, 2015).

An increased difficulty is observed when trying to treat biofilm infections in comparison to planktonic infections, due to the high antibacterial tolerance biofilms exhibit due to their extracellular polymer matrix, composed of extra-cellular polymeric substances encasing them in a protective slime layer; therefore, microorganisms in biofilm mode of growth are well protected from immune responses and antimicrobial treatments (Percival *et al.*, 2015). Mottola *et al.* showcased that concentrations required to treat *S. aureus* biofilm infections, commonly isolated in diabetic foot ulcers, are up to 10 – 1000 times greater in comparison to corresponding planktonic *S. aureus* cells (Mottola *et al.*, 2016).

Initial biofilms formed on indwelling medical devices such as CVC or wound sites may be comprised of a single microorganism species; though, the longer these biofilms remain untreated, it becomes unavoidable that the growth and development of the biofilm leads to multiple microorganisms becoming encased in the biofilm generating a polymicrobial biofilm. These multispecies biofilms are then considerably harder to treat as treatment needs to eradicate different species of microorganisms and a single antimicrobial may no longer be suitable to treat the infection.

Alternative treatments have been developed to tackle these infections, such as antimicrobial lock treatment to salvage tunnelled CVCs with catheter colonization and catheter-related bloodstream

infection. High concentrations of antimicrobials are locked within the catheter containing the biofilm and maintained within the catheter lumen for a sufficient time frame to eradicate the biofilms. Although this type of exposure treatment for biofilm infections poses the additional risk associated with all antimicrobial treatments: microorganisms gaining a resistance to the antimicrobial, leading to a need for combination antimicrobial therapies and the addition of agents that are capable of eradicating biofilms that are not classified as antimicrobials (Donlan, 2001, 2008). Zanwar *et al.*, (2019) tested the benefit of antimicrobial lock treatments, as removal of a CVC colonised by *S. aureus* and *C. albicans* is standard procedure to combat the infection. Whilst this removes the infection from the patient, re-insertion of a new catheter leads to added costs and poses the risk of iatrogenic issues such as: bleeding, nerve, and soft tissue injuries. Zanwar recorded that catheters colonised with *S. aureus, C. albicans* and Gram-negative bacilli could be salvaged following antimicrobial lock treatment and systemic antibiotics, 86% of catheters were salvaged due to this treatment strategy in comparison to only a 55% salvage of CVCs from systemic antibiotics alone (Zanwar *et al.*, 2019).

Other forms of treatment to combat biofilm infections in catheters is to try and prevent initial adherence of microorganisms to prevent biofilm formation. Previous research has determined the antibacterial ability of silver and has shown that silver damages bacterial cell membranes, cell walls and has the capability to inhibit bacterial genome replication. Due to the high reactivity and small nanometric sizes, silver nanoparticles at a low concentration have been used to coat the surfaces of biomedical instruments. Recent studies have investigated applying silver nanoparticles as a surface coating for catheters to try and prevent biofilm formation. Zare *et al.*, (2017) pre-coated catheters with an organic polymer parylene (PPX-N) that is biocompatible with silver nanoparticles to improve adhesion of silver nanoparticles to the surface of the catheter. PPX-N also protects the silver coating on catheter surface, as the polymer creates a hydrophobic layer, preventing diffusion of silver ions from the higher concentration located on the catheters to silver present in synthetic urine. The combination of silver coating and an protective polymer layer inhibits the growth of *E. coli* and *Staphylococcus cohnii* and prevents bacterial adhesion to the catheter (Zare *et al.*, 2017).

Whilst XF-73 has demonstrated a bactericidal efficacy against planktonic microorganisms, previous studies have also reported XF-73 to have activity against microorganisms in biofilm mode of growth. Ooi *et al.*, (2009) documented that XF-73 was capable to effectively eradicate *S. aureus* in both planktonic and biofilm modes of growth as only a 1-fold increase from XF-73 MIC value was recorded to eradicate biofilms with an MBEC value of 2 µg/ mL. In contrast, commonly used antimicrobial treatments i.e. mupirocin, tetracycline, chlorohexidine, rifampicin and vancomycin were unable to maintain their antimicrobial efficacy recorded against planktonic cells when used as a treatment against biofilms as concentrations of 265 µg/ mL were unable to eradicate *S. aureus* biofilms (Ooi, *et al.*, 2009a).

3.2 Aims of the study

The aim of this study was to further assess the antimicrobial activity of XF-73 against clinically relevant microorganisms in biofilm mode of growth. This study was completed using the same panel of clinically relevant Gram-positive and Gram-negative microorganisms used in the previous planktonic study (Table 2.1).

The study was carried out in the following stages:

- Determine the difference in biofilm load after twenty-four, forty-eight and seventy-two hours of growth.
- Assess the antimicrobial efficacy of XF-73 against clinically relevant microorganisms in biofilm mode of growth.
    - Determine minimum biofilm inhibitory and eradication concentrations of XF-73 against a panel of clinically relevant microorganisms at twenty-four and forty-eight hour biofilms.
    - Determine the antimicrobial efficacy of XF-73 on the viability of Gram-positive and Gram-negative biofilms following twenty-four exposure to XF-73 treatment.
    - Compare the efficacy of XF-73 to a panel of known antibiotics commonly used against clinically relevant biofilms.

3.3 Materials

3.3.1 Microbial cultures

A reduced panel of ATCC microorganisms (Table 2.1) were selected for biofilm mode of growth sensitivity testing against XF-73 (Table 3.1). A further reduction of microorganisms (Table 3.2) was selected for susceptibility testing against the panel of known comparator antimicrobials. The antimicrobial efficacy of XF-73 was investigated against Gram-positive forty-eight hour biofilms. Gentamicin was tested against forty-eight hour biofilms of the microorganisms previously used for comparator antimicrobial testing (Table 3.2).

Streak plates were prepared on MHA plates for each test microorganism, using a bead from the glycerol stock (2.4.4.1). Stored at -80°C. Streak plates were subsequently stored at 4°C. The microorganisms were selected for this study due to them all being commonly found in HCAIs involving biofilm formation in indwelling devices and wounds.

Table 3.1 Panel of Gram-positive and Gram-negative microorganisms selected for biofilm studies against XF-73

Reduced panel of Gram-positive and Gram-negative microorganisms are the same microorganisms used in previous planktonic studies against XF-73 (Table 2.1).

Microorganism	Gram Positive/ Negative	ATCC Number
Staphylococcus aureus	+ve	6538
Staphylococcus aureus	+ve	29213
Staphylococcus epidermidis	+ve	12228
Enterococcus faecalis	+ve	29212
Escherichia coli	-ve	35218
Escherichia coli	-ve	25922
Pseudomonas aeruginosa	-ve	15442
Pseudomonas aeruginosa	-ve	27853

Table 3.2 Reduced panel of Gram-positive and Gram-negative microorganisms selected for biofilm studies against comparator antimicrobials

Panel of microorganisms to be grown as biofilms for treatment against: gentamicin, mupirocin, rifampicin and daptomycin.

Microorganism	Gram Positive/ Negative	ATCC Number
Staphylococcus aureus	+ve	29213
Enterococcus faecalis	+ve	29212
Escherichia coli	-ve	35218
Pseudomonas aeruginosa	-ve	27853

3.3.2 Microbiological media and agar

MHB and MHA were used for this study. MHB was used to grow the cultures and to conduct MBIC testing and MHA for MBEC testing. Both the media and agar were made up according to the manufacturer's instructions following the same protocol used from the previous study (2.4.1.1, 2.4.1.2).

3.3.3 Comparator antibiotics

Batch 1 and 2 XF-73 in aqueous solution was used for this study and its antimicrobial efficacy was compared to a panel of known comparator antibiotics used for assessing antimicrobial activity against microorganisms in planktonic mode of growth (2.3.4): rifampicin, gentamicin, mupirocin and daptomycin. The powdered antibiotics were stored in accordance with the manufacturer's guidelines until required. Following preparation, rifampicin and daptomycin were stored at -20°C, mupirocin at 4°C and gentamicin at room temperature.

Stocks of gentamicin and daptomycin were made in aqueous solution. Dimethyl sulfoxide (DMSO) (100%) was used to create stocks of mupirocin and rifampicin. All liquid stocks of antimicrobials were stored at 4°C until required. Mupirocin and rifampicin were removed from 4°C storage and left at room temperature to liquefy the DMSO solutions before each experiment.

3.3.4 Crystal violet

Crystal violet (Merck) was used to stain biofilms. Crystal violet was made following manufacturer's instruction, where solid crystal violet was dissolved in distilled water to give a final concentration of 0.06% w/v.

3.3.5 Phosphate buffered saline

Phosphate buffered saline (PBS) (Oxoid Ltd) was used to remove planktonic/ not fully adhered microorganism from the biofilms. PBS was prepared in accordance with the manufacturer's instructions of diluting 1 PBS tablet in 200 mL of distilled water. Once the tablet was fully dissolved, the PBS was

sterilised through autoclaving at 121°C for fifteen minutes. PBS solution was stored at room temperature until required.

3.3.6 Dimethyl sulfoxide (DMSO)

DMSO was required to dissolve solid rifampicin and mupirocin to generate liquid stock solutions of the antimicrobials (3.3.3).

3.3.7 Specialist equipment

Optical density (OD) readings of microbial growth were taken using a spectrophotometer (Jenway Geneva Plus UV).

3.4 Methods

3.4.1 Biofilm formation

S. aureus ATCC 6538 and *E. coli* ATCC 35218 were selected for initial testing to determine the biofilm viability at different time points: twenty-four, forty-eight and seventy-two hours of growth.

3.4.1.1 Twenty-four hour biofilms

An overnight culture of each microorganism, grown in MHB was diluted to give a concentration of 10^5 CFU/ mL in MHB, and 150 µL of these diluted suspensions of each microorganism was added to test wells of microtitre plates for a total of six replicates. In line with the Calgary method of biofilm development, microtitre plate lids containing 96 plastic pegs (Nunc-Immuno™ TSP plates) were inserted into the wells of each microtitre plate. The plates were then incubated overnight at 37°C and shaken at 180 rpm in aerobic conditions.

3.4.1.2 Forty-eight and seventy-two-hour biofilm growth

Following biofilm formation after twenty-four hours of incubation (3.4.1.1), biofilms formed on the pegged lid were removed from the microtitre plate and immersed five times in 200 µl of PBS, before being transferred to an additional PBS microtitre plate and immersed a further five times.

Forty-eight hour biofilms were created by taking twenty-four biofilm hour peg lids and transferring them into microtitre plates containing 150 µL of MHB. These plates were then further incubated overnight at 37°C and shaken at 180 rpm in aerobic conditions.

Removal of the peg lid after forty-eight hours of incubation was followed by the washing of the biofilm growing on each peg using the previously described method of immersion in PBS, before transferal into new microtitre plates containing 150 µL of MHB, for a further incubation overnight at 37°C and shaken 180 rpm in aerobic conditions to generate seventy-two-hour biofilms.

3.4.1.3 Biofilm viability testing

Once the biofilms had reached the desired ages: twenty-for, forty-eight and seventy-two hours of growth. The microtitre lid containing the biofilm pegs was immersed into a microtitre plate containing 200 µL of PBS per well, and sonicated by floatation on the surface of an ultrasonic water bath for 30 minutes following Innovotech guidelines (Innovotech, 2012), to release and disperse residual biofilm organisms. The numbers of viable organisms released by sonication were determined by serial dilution, with dilutions plated on MHA using the Miles Misra method (Miles *et al.,* 1938). In brief, 20 µL of sonicated biofilm in PBS was transferred into 180 µL of PBS and serially diluted to 10^{-7}; 20 µL of each dilution was then plated onto MHA and incubated overnight.

Biofilm viability was determined by recording the number of colonies present at the lowest serial dilution giving countable colonies on the Miles Misra spread plates for each biofilm treatment. Biofilm viability results were then calculated as the number of viable bacteria per mL (CFU/ mL) released from the pegs

by sonication into the 200 μL of PBS. These measures of biofilm viability were used in all figures and statistical analyses throughout.

3.4.2 Antimicrobial efficacy assays against twenty-four hour Gram-positive and Gram-negative biofilms

Twenty-four hour biofilms (3.4.1.1) grown on the pegs of three microtitre plate lids were immersed in microtitre plates containing PBS following the method previously described (3.4.1.2), to allow for the removal of any weakly attached planktonic microorganism from the biofilms.

Washed microtitre lids were then transferred into a 'challenge plate' consisting of 200 μL of each antimicrobial in MHB at a range of concentrations depending upon the microorganism and the selected antimicrobial.

XF-73 concentration ranges varied depending on if the microorganism was Gram-positive or Gram-negative. Gram-positive microorganisms had an XF-73 concentration range of 0.125 μg/ mL – 64 μg/ mL. An increased concentration range was used against all Gram-negative microorganisms: 16 μg/ mL – 512 μg/ mL. As previous work (2.5.2) showed that XF-73 was effective against Gram-positive planktonic microorganisms but less effective against Gram-negative microorganisms. Batch 2 XF-73 was used for this study. The range of concentrations for the panel of known antimicrobials for each microorganism are as follows (Table 3.3). Each antimicrobial treatment and controls: microorganism only, antimicrobial only and MHB had three replicates.

Table 3.3 Concentration ranges (μg/ mL) for the panel of known antimicrobials selected for testing against twenty-four hour Gram-positive and Gram-negative biofilms

Antimicrobial	Concentration range per microorganism (μg/ mL)			
	S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*
Gentamicin	1 – 64	2 – 128	1 – 64	0.5 – 32
Mupirocin	8 – 512	8 – 512	8 – 512	8 – 512
Rifampicin	8 – 512	8 – 512	2 – 128	4 – 256
Daptomycin	8 – 512	8 – 512	8 – 512	8 – 512

The challenge plates, now containing biofilms formed on the microtitre pegged lids, were then incubated overnight at 37°C and shaken 180 rpm in aerobic conditions. Three microtitre challenge plates were generated per antimicrobial treatment.

3.4.3 Efficacy of antimicrobials against microorganisms in biofilm mode of growth

Peg lids containing treated biofilms were removed from the challenge plates and washed in PBS before being transferred into one of the following assay plates to assess the antimicrobial efficacy of the antimicrobials against formed biofilms.

3.4.3.1 Minimum biofilm inhibitory concentration and minimum biofilm eradication concentration assays

A microtitre lid was transferred into a microtitre plate containing fresh MHB at 200 µl and incubated overnight at 37°C at 180 rpm in aerobic conditions to determine the MBIC. Following MBIC determination, wells of the microtitre plate containing no growth were sub-cultured onto MHA plates and incubated overnight at 37°C to check for growth to determine the MBEC.

3.4.3.2 Biofilm viability

Another microtitre lid containing biofilm pegs was selected for biofilm viability testing, which was completed following the method previously stated (3.4.1.3).

3.4.3.3 Crystal violet staining

The final triplicate microtitre lid was stained with 200 µL of crystal violet (0.06% w/v) in each well for five minutes. Followed by removal of excess stain through PBS washes (3.4.1.2). Images were then taken of the stained biofilm pegs and control pegs for qualitative analysis.

3.4.4 Antimicrobial treatments against forty-eight hour Gram-positive biofilms

A further study was conducted, assessing the efficacy of XF-73 against forty-eight hour biofilms focusing only the Gram-positive microorganisms (Table 3.1). To compare the efficacy of XF-73 against forty-eight hour biofilms, gentamicin was selected as a comparator antimicrobial and was used as treatment against the same panel of microorganisms used in previous comparator testing (Table 3.2).

Each microorganism was left to develop to a forty-eight hour biofilm (3.4.1.2) before the addition of antimicrobial treatments (3.4.2). Antimicrobial concentration ranges varied between antimicrobials and microorganism; for *S. aureus* ATCC 6538 XF-73 treatment ranged from 8 µg/ mL – 512 µg/ mL, for the remaining Gram-positive microorganisms XF-73 had a lower concentration range: 0.5 µg/ mL – 32 µg/ mL. Gentamicin was diluted to a concentration a range of 8 µg/ mL – 512 µg/ mL. Each antimicrobial treatment and controls: microorganism only, antimicrobial only and MHB had three replicates.

3.4.4.1 Analysis of antimicrobial treatments against forty-eight hour biofilms

Analysis of the efficacy of XF-73 and gentamicin against forty-eight hour biofilms was undertaken via MBIC/ MBEC testing (3.4.3.1) and crystal violet staining of the treated biofilms (3.4.3.3).

3.4.5 Statistical analysis of biofilm viability

To analyse data collected from biofilm viability (3.4.1.3, 3.4.3.2), all recorded viable cell counts were log_{10} transformed before statistical analysis was conducted. Shapiro-Wilk normality testing was initially used to ensure the log transformed data sets were normally distributed. A one-way ANOVA, with

Tukey's multiple comparison *post-hoc* test then conducted to determine any significant differences in biofilm viability between treated and untreated data sets.

3.5 Results

3.5.1 Viability of *S. aureus* ATCC 6538 and *E. coli* ATCC 35218 biofilms after twenty-four, forty-eight and seventy-two hours of growth

Recorded viability of *S. aureus* ATCC 29213 and *E. coli* ATCC 35218 biofilms at a range of maturation ages: twenty-four, forty-eight and seventy-two hours are presented as graphs for each microorganism (Figure 3.1).

Viable cell counts of *S. aureus* biofilms increased over time (Figure 3.1 A) from 1.4 x10^8 CFU/ mL at twenty-four hours to 3.5 x10^8 CFU/ mL at forty-eight hours and 2.9 x10^9 CFU/ mL at seventy-two hours. A significant statistical difference was concluded between the biofilm viabilities after each time point. A greater significance was recorded between seventy-two hour biofilms and twenty-four and seventy-two and forty-eight hour biofilms determined for both (p= <0.0001).

The viability of *E. coli* biofilms also increased with the age of the biofilm (Figure 3.1 B). Twenty-four and forty-eight hour biofilms had a lower recorded cell concentration in comparison to corresponding viabilities of *S. aureus* biofilms. After seventy-two hours the viability of *E. coli* biofilms were higher than *S. aureus* with a 2.6 x10^9 CFU/ mL difference between the two microorganisms.

Analysing the difference in viabilities between *E. coli* biofilms at different ages showed a statistical difference determined between them all. A greater significance was recorded between twenty-four and seventy-two hours, and between forty-eight and seventy-two hour (p= <0.0001). Significance was still noted between twenty-four and forty-eight hour biofilm viabilities, though at a lower value (p= 0.0152).
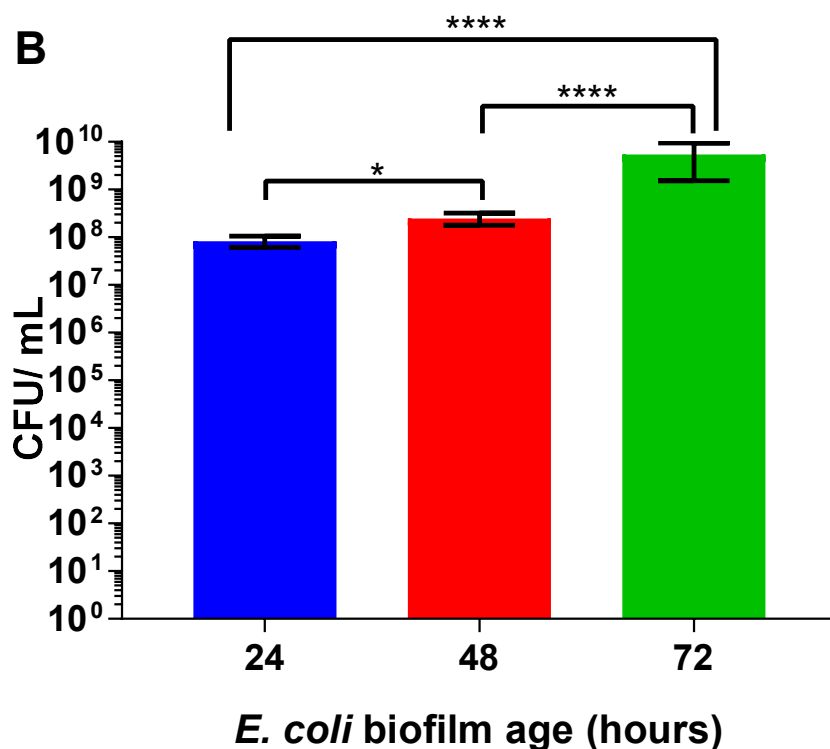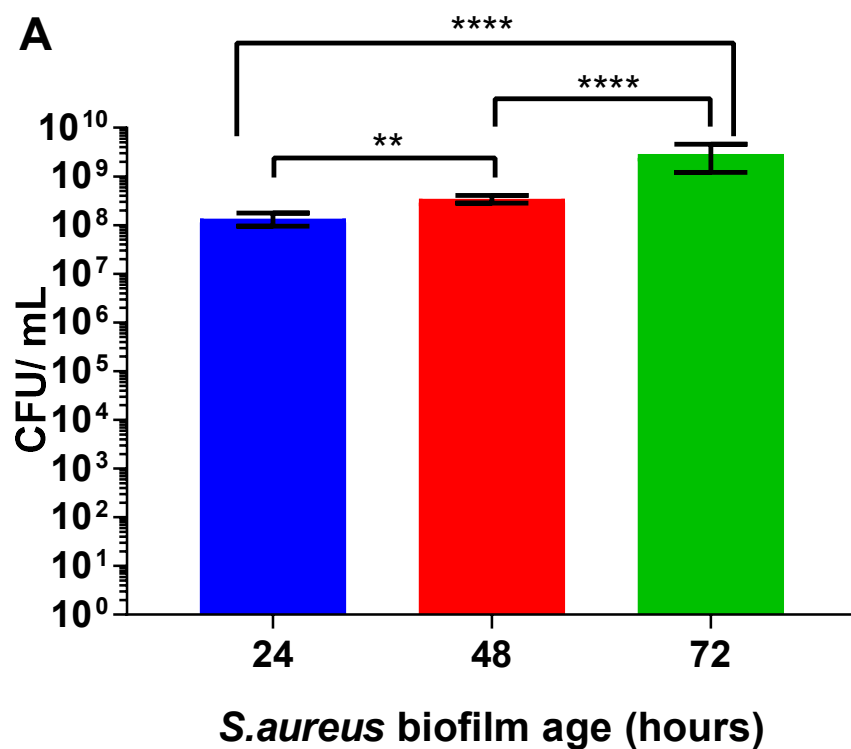
Figure 3.1 S. aureus ATCC 6538 and E. coli ATCC 35218 biofilm viabilities at a range of biofilm maturities

*Viability of (A) S. aureus and (B) E. coli biofilms at a range of ages: 24, 48 and 72 hours of growth at CFU/ mL. Error bars generated using the standard deviation of the data sets from six replicates. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between each time point per microorganism. For S. aureus biofilms ****: p= <0.0001 and **: p= 0.0026. Statistical analysis of E. coli biofilms showed the differences between biofilm ages where ****: p= <0.0001 and *: p= 0.0152.*

3.5.2 Minimum inhibition and eradication concentrations of XF-73 and comparator antibiotics against twenty-four hour Gram-positive and Gram-negative biofilms

3.5.2.1 MBIC and MBEC values of XF-73 against Gram-positive and Gram-negative biofilms

MBIC and MBEC values for XF-73 against Gram-positive and Gram-negative microorganisms are presented as a table (Table 3.4). Gram-positive biofilms were more susceptible to XF-73 in comparison to Gram-negative biofilms, shown by the low concentrations of XF-73 able to inhibit and eradicate these biofilms.

For each Gram-positive microorganism tested in this study, the same concentration of XF-73 was recorded for inhibition and eradication of the biofilms, where MBIC = MBEC. All staphylococcal biofilms had MBIC and MBEC values of 1 µg/ mL. A 2-fold increase in XF-73 concentration was required to eradicate *E. faecalis* biofilms (MBEC 2 µg/ mL).

In contrast to the Gram-positive results, no concentration of XF-73 was capable of inhibiting or eradicating any of the Gram-negative biofilms tested in this study, as MBIC and MBEC values were > 512 µg/ mL for each microorganism.

Table 3.4 Recorded MBIC and MBEC values (µg/ mL) of XF-73 against twenty-four hour Gram-positive and Gram-negative biofilms

Microorganism/ ATCC	MBIC µg/ mL	MBEC µg/ mL
S. aureus 6538	1	1
S. aureus 29213	1	1
S. epidermidis 12228	1	1
E. faecalis 29212	2	2
E. coli 35218	> 512	> 512
E. coli 25922	> 512	> 512
P. aeruginosa 15442	> 512	> 512
P. aeruginosa 27853	> 512	> 512

3.5.2.2 MBIC and MBEC values of a panel of antimicrobials against Gram-positive and Gram-negative biofilms

MBIC and MBEC values for the panel of antimicrobials against the panel of Gram-positive and Gram-negative biofilms were recorded and presented as a (Table 3.5). Included in this table are the corresponding XF-73 MBIC and MBEC values and a DMSO only control, due to mupirocin and rifampicin stocks being made in DMSO.

No concentration of DMSO was recorded to inhibit or eradicate *E. faecalis* or *E. coli* biofilms, in contrast 50% (v/v) DMSO both inhibited and eradicated *S. aureus* and *P. aeruginosa* biofilms. Therefore, 512 µg/ mL of mupirocin and rifampicin cannot be classified as either MBIC's or MBEC's against *S. aureus* or *P. aeruginosa* biofilms due to the DMSO concentration in the antimicrobials affecting biofilm growth.

For each of the comparator antibiotics used against *S. aureus* ATCC 29213 biofilms, the same concentration was recorded for the MBIC and MBEC. Gentamicin had the lowest recorded values against *S. aureus* ATCC 29213 at 8 µg/ mL for both inhibition (MBIC) and eradication (MBEC). Rifampicin generated the second lowest MBIC and MBEC values at a 2-fold increase in comparison to gentamicin, daptomycin also eradicated *S. aureus* biofilms at a 4-fold increase to gentamicin at 32 µg/ mL. No concentration of mupirocin was capable of inhibiting or eradicating biofilms of *S. aureus*. While gentamicin had the lowest recorded MBIC and MBEC values out of all the antimicrobials used in the comparator study, though the concentration was still an 8-fold increase in comparison to XF-73.

No concentration of mupirocin, rifampicin or daptomycin used in this study was capable of inhibiting or eradicating *E. faecalis* ATCC 29212 biofilms, therefore the MBIC and MBEC values are referred to >512 µg/ mL. Gentamicin was the only comparator antibiotic that inhibited biofilm growth at 64 µg/ mL, a 32-fold increase in comparison to XF-73. Another difference between the two antimicrobials is that whilst MBIC = MBEC for XF-73, a concentration higher than the MBIC was required to eradicate the biofilms for gentamicin, with a 64-fold increase compared to XF-73.

Out of the panel of antimicrobials used against *E. coli* ATCC 35218 biofilms, gentamicin was the only antimicrobial that inhibited and eradicate the biofilms. Whilst gentamicin inhibited growth at a relatively low concentration at 8 µg/ mL, a higher concentration was required to eradicate the biofilm at 64 µg/ mL, an 8-fold increase in concentration in comparison to the MBIC. Despite the increase in concentration to eradicate *E. coli* biofilms, gentamicin was able to generate an MBIC and MBEC concentration whilst XF-73 was unable to do so.

Similarly, to the results recorded for *E. coli* biofilms, gentamicin was the only antimicrobial, including XF-73 that generated an MBIC against *P. aeruginosa* ATCC 27853 biofilms. Though 16 µg/ mL gentamicin only inhibited biofilm growth, the MBEC was determined to be greater than the upper concentration of gentamicin used in this study. Mupirocin and rifampicin both had MBIC and MBEC

values greater than 256 μg/ mL, although concentrations above 256 μg/ mL were unsuitable for use in this study as the DMSO concentration present at these concentrations had an antimicrobial effect against *P. aeruginosa* biofilms.

Table 3.5 Collected MBIC and MBEC values (μg/ mL) of: XF-73, gentamicin, mupirocin, rifampicin and daptomycin against twenty-four hour Gram-positive and Gram-negative biofilms.

Results includes a DMSO only treatment to ensure that DMSO concentration in mupirocin and rifampicin stocks does not pose and antimicrobial effect.

Organism & ATCC number	DMSO MBIC/ MBEC % (v/v)	Gentamicin MBIC/ MBEC μg/ mL	Mupirocin MBIC/ MBEC μg/ mL	Rifampicin MBIC/ MBEC μg/ mL	Daptomycin MBIC/ MBEC μg/ mL	XF-73 MBIC/ MBEC μg/ mL
S. aureus 29213	50/ 50	8/ 8	> 256/ > 256	16/ 16	32/ 32	1/1
E. faecalis 29212	> 50/ > 50	64/ 128	> 512/ > 512	512/ > 512	> 512/ > 512	2/2
E. coli 35218	> 50/ > 50	8/ 64	> 512/ > 512	> 128 / > 128	> 512/ > 512	> 512/ > 512
P. aeruginosa 27853	50/ 50	16/ > 32	> 256/ > 256	> 256/ > 256	> 512/ > 512	> 512/ > 512

3.5.3 Biofilm viability of Gram-positive and Gram-negative microorganisms treated with a range of antimicrobials

Biofilm viability was determined by counting any single colony forming units (CFUs) present on MHB for the three sample replicates after treatment with XF-73 or a range of comparator antimicrobials. CFUs were multiplied by the dilution factor selected for CFU counts. From the 200 μL of PBS solution containing the sonicated biofilm from each peg, 20 μL was either plated directly onto MHA or following appropriate serial dilution to 10^{-7} then plated onto MHA. The CFU count was multiplied by the serial dilution factor and then by 50 to generate CFUs per mL (CFU/ mL):

Calculation formula: CFU/ mL = viable cell count x dilution factor x 50

Any concentration with no colonies present during viable cell counts on MHA was concluded as having a biofilm viability below the lower limit of detection. As 20 μL of 200 μL PBS containing sonicated biofilm was plated onto MHA, the lower limit of detection was 10 CFU/ mL.

3.5.3.1 XF-73 treatment against Gram-positive biofilms after twenty-four hours of growth

3.5.3.1.1 *S. aureus* ATCC 6538 biofilms

Treatment of XF-73 at all concentrations used in this study was shown to exert a significant decrease in biofilm viability of *S. aureus* ATCC 6538 in comparison to the growth control, statistical analysis generated a p value of for each antimicrobial treatment (p= <0.0001). As the concentration of XF-73

increased, the biofilm viability decreased (Figure 3.2). Concentrations of 1 – 64 μg/ mL showed no viable colonies during the viable cell count and therefore are classified as having a biofilm viability below the lower limit of detection.

A 2-log reduction in biofilm viability was recorded at the lowest concentration of XF-73 used in this study. XF-73 at 0.5 μg/ mL had the lowest recorded biofilm viability out of concentrations that had cell counts above the lower limit of detecting, leading to a 4-log reduction in comparison to the growth control. There was a significant statistical difference between XF-73 concentrations that generated a biofilm viability value above the lower limit of detection (0.125 – 0.5 μg/ mL); each concentration had the same significant difference calculated for one another ($p = <0.0001$) except between 0.125 μg/ mL and 0.25 μg/ mL ($p = 0.0426$).

3.5.3.1.2 *S. aureus* ATCC 29213 biofilms

The lowest concentration of XF-73, 0.125 μg/ mL had a similar biofilm viability value in comparison to the growth control at 6.83×10^7 CFU/ mL and 6.5×10^7 CFU/ mL respectively (Figure 3.3). No statistical difference was determined between 0.125 μg/ mL and the growth control ($p > 0.05$). The remaining concentrations all showed a decrease in biofilm viability, following a trend of the higher the concentration of XF-73 the lower the viability. A significant difference ($p = <0.0001$) was recorded for all other concentrations of XF-73 against *S. aureus* ATCC 29213 biofilms.

Concentrations of 1 – 64 μg/ mL showed no viable colonies during the viable cell count and is therefore classified as having a biofilm viability under the lower limit of detection.

XF-73 treatment at 0.5 μg/ mL showed the greatest decrease in biofilm viability against *S. aureus* biofilms, out of XF-73 concentrations with biofilm viabilities above the lower limit of detection, at 1.43×10^4 CFU/ mL, a 3-log reduction in comparison to the control. A significant difference was also recorded between 0.25 μg/ mL and 0.5 μg/ mL ($p = <0.0001$).

3.5.3.1.3 *S. epidermidis* ATCC 12228 biofilms

Each concentration of XF-73 used in this study decreased the viability of *S. epidermidis* biofilms, the higher the concentration of XF-73 the lower the biofilm viability (Figure 3.4). This decrease was determined to be significantly different ($p = <0.0001$). Concentrations 1 – 64 μg/ mL showed no viable colonies during the viable cell count and therefore is classified as having a biofilm viability under the lower limit of detection.

Both 0.125 μg/ mL and 0.25 μg/ mL treatments of XF-73 led to a 3-log reduction in biofilm viability in comparison to the growth control, when comparing the viability generated by both concentrations: 1.08×10^6 CFU/ mL for 0.125 μg/ mL and 9×10^5 CFU/ mL for 0.25 μg/ mL, there was found to be significant difference between them ($p = <0.0001$).

XF-73 at 0.5 µg/ mL led to the second lowest recorded biofilm viability in comparison to *S. epidermidis* alone at 1.65 x10^4 CFU/ mL and 1.05 x10^9 CFU/ mL respectively. A significant difference (p= <0.0001) was found between the viability of biofilms treated with 0.5 µg/ mL and 0.25 µg/ mL and 0.125 µg/ mL XF-73 treatments.

3.5.3.1.4 *E. faecalis* ATCC 29212 biofilms

All concentrations of XF-73 used to treat *E. faecalis* biofilms in this study yielded a difference in biofilm viability in comparison to the growth control of *E. faecalis* biofilms, which had a recorded biofilm viability at 6.33 x10^6 CFU/ mL (Figure 3.5). For concentrations ranging from 0.25 – 64 µg/ mL the statistical difference was recorded (p= <0.0001). While 0.125 µg/ mL had the minor change in viability at 9.5 x10^5 CFU/ mL, the difference was still determined as significant (p= 0.0259).

Biofilm viability of *E. faecalis* decreased as the concentration of XF-73 treatment increased, with the exception of 0.5 µg/ mL, a higher recorded viability than 0.25 µg/ mL, with values at 8.5 x10^4 CFU/ mL and 1.38 x10^4 CFU/ mL respectively. XF-73 at 1 µg/ mL led to a 4-log reduction in viability against *E. faecalis* biofilms.

Concentrations 2 – 64 µg/ mL showed no viable colonies during the viable cell count and therefore is classified as having a biofilm viability under the lower limit of detection.
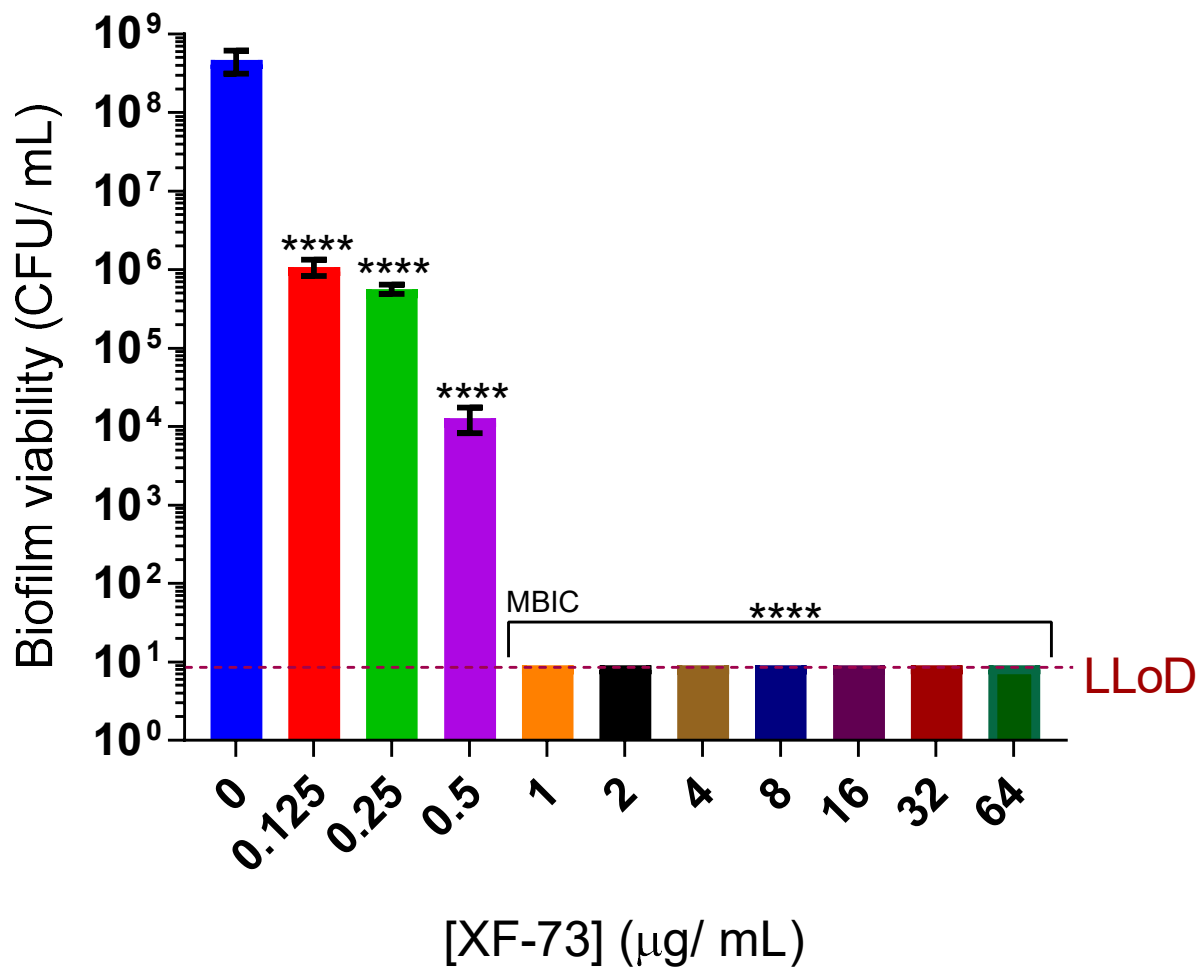
Figure 3.2 Twenty-four hour S. aureus ATCC 6538 biofilms treated with XF-73 at a range of concentrations

*Recorded viabilities of S. aureus ATCC 6538 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 0.125 – 64 µg/ mL. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated S. aureus biofilm viability, where ****: p= <0.0001. MBIC value (Table 3.4) also highlighted.*

Figure 3.3 Twenty-four hour *S. aureus* ATCC 29213 biofilms treated with XF-73 at a range of concentrations

*Recorded viabilities of S. aureus ATCC 29213 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 0.125 – 64 μg/ mL. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated S. aureus biofilm viability, where ****: p= <0.0001. MBIC value (Table 3.4) also highlighted.*

Figure 3.4 Twenty-four hour S. epidermidis ATCC 12228 biofilms treated with XF-73 at a range of concentrations

*Recorded viabilities of S. epidermidis ATCC 12228 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 0.125 – 64 µg/ mL. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated S. epidermidis biofilm viability, where ****: p= <0.0001. MBIC value (Table 3.4) also highlighted.*

Figure 3.5 Twenty-four hour *E. faecalis* ATCC 29212 biofilms treated with XF-73 at a range of concentrations

Recorded viabilities of E. faecalis ATCC 29212 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 0.125 – 64 µg/ mL. Error bars generated using the standard deviation of the three replates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated E. faecalis biofilm viability, where **: p= 0.0259 and ****: p= <0.0001. MBIC value (Table 3.4) also highlighted.

3.5.3.2 XF-73 treatment against Gram-negative biofilms after twenty-four hours of growth

3.5.3.2.1 *E. coli* ATCC 35218 biofilms

Each concentration of XF-73 used against *E. coli* biofilms led to a decrease in biofilm viability at a minimum 6-log reduction (Figure 3.6); this decrease in biofilm viability was determined to be significantly different to untreated *E. coli* biofilms (p= <0.0001). Concentrations 256 µg/ mL and 512 µg/ mL showed no viable colonies during the viable cell count and therefore is classified as having a biofilm viability under the lower limit of detection.

The same viability of *E. coli* biofilms, 1.23 x10^4 CFU/ mL, was recorded following treatment of XF-73 at 32 µg/ mL and 64 µg/ mL. Additionally, a low biofilm load was also recorded at 128 µg/ mL, statistical analysis showed there was no significant difference (p > 0.05) between 32 µg/ mL and 64 µg/ mL.

3.5.3.2.2 *E. coli* ATCC 25922 biofilms

A minimum 4-log reduction in viability of *E. coli* biofilms was recorded when treated with XF-73 at any concentration used in this study (Figure 3.7). Concentrations 128 µg/ mL - 512 µg/ mL showed no viable colonies during the viable cell count and therefore is classified as having a biofilm viability under the lower limit of detection.

A significant difference (p= <0.0001) was determined for each concentration of XF-73 against untreated *E. coli* biofilms. A similar CFU/ mL was noted between concentrations ranging from 16 – 64 µg/ mL of XF-73 and statistical analysis showed there was no significant difference (p > 0.05) between these concentrations.

3.5.3.2.3 *P. aeruginosa* ATCC 15442 biofilms

All concentrations of XF-73 led to a significant reduction (p= <0.0001) in *P. aeruginosa* viability (Figure 3.8). However, when increasing the concentration of XF-73 there was no trend of a decrease in biofilm viability; this was highlighted as 128 µg/ mL had the highest recorded biofilm viability out of the other concentrations of XF-73, with a 3-log reduction: 4.33 x10^7 CFU/ mL in viability comparison to the control growth: 1.83 x10^{10} CFU/ mL.

The lowest recorded viability of *P. aeruginosa* biofilms was with the addition of 512 µg/ mL of XF-73, causing a 7-log reduction and a viability count of 483 CFU/ mL. No significant difference was determined between the viability of *P. aeruginosa* biofilms when treated with 16 µg/ mL, 32 µg/ mL, 64 µg/ mL and 256 µg/ mL of XF-73 (p > 0.05).

3.5.3.2.4 *P. aeruginosa* ATCC 27853

An increase in biofilm viability was determined with the addition of XF-73 at concentrations 16 µg/ mL and 32 µg/ mL, with recorded viabilities: 1.115 x10^{10} CFU/ mL and 1.07 x10^{10} CFU/ mL respectively and were found to be higher than that of the growth control of untreated *P. aeruginosa* ATCC 27853 biofilms:

1.67 x10^9 CFU/ mL (Figure 3.9). The difference between viabilities of these concentrations and control growth was deemed statistically significant, (p= 0.0017 for 16 μg/ mL and p= 0.0025 for 32 μg/ mL). Furthermore, no significant difference was found between viabilities of these two concentrations of XF-73 (p > 0.05).

Concentrations of XF-73 ranging from 64 – 512 μg/ mL against *P. aeruginosa* biofilms showed a lower biofilm viability in comparison to viability of the growth control and were shown to been statistically different (p = <0.0001). XF-73 at 512 μg/ mL showed the greatest decrease in biofilm viability with a 5-log reduction in comparison to the viability of the growth control.
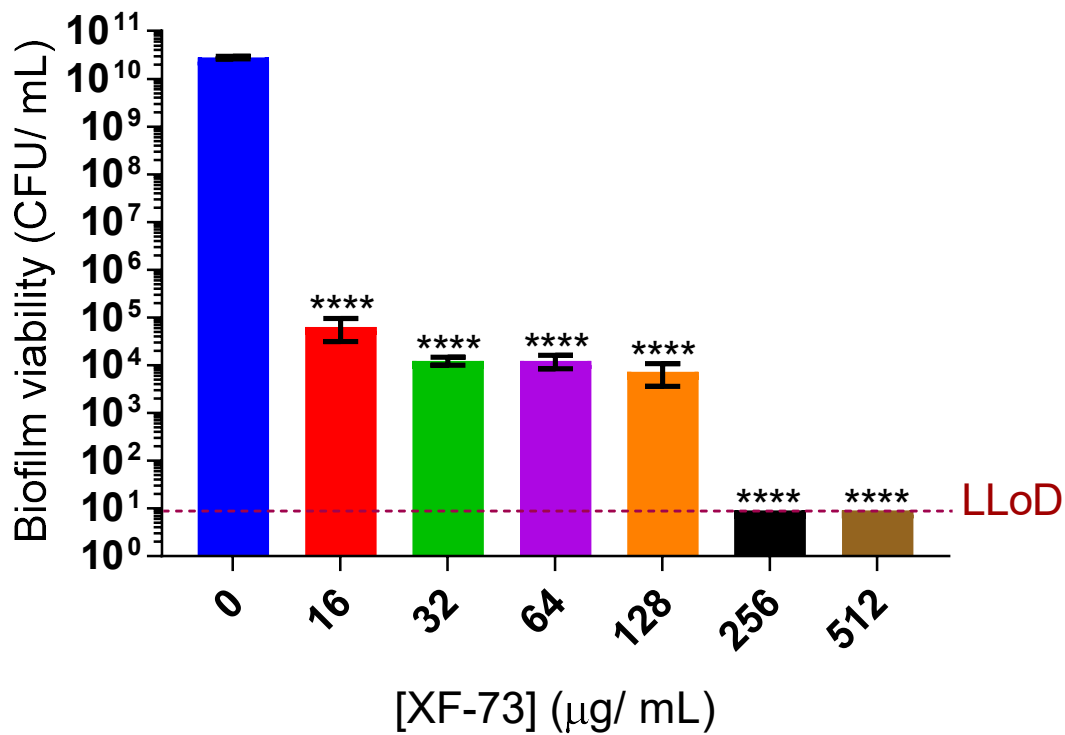
Figure 3.6 Twenty-four hour E. coli ATCC 35218 biofilms treated with XF-73 at a range of concentrations

*Recorded viabilities of E. coli ATCC 35218 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 16 – 512 µg/ mL. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated E. coli biofilm viability, where ****: p= <0.0001.*
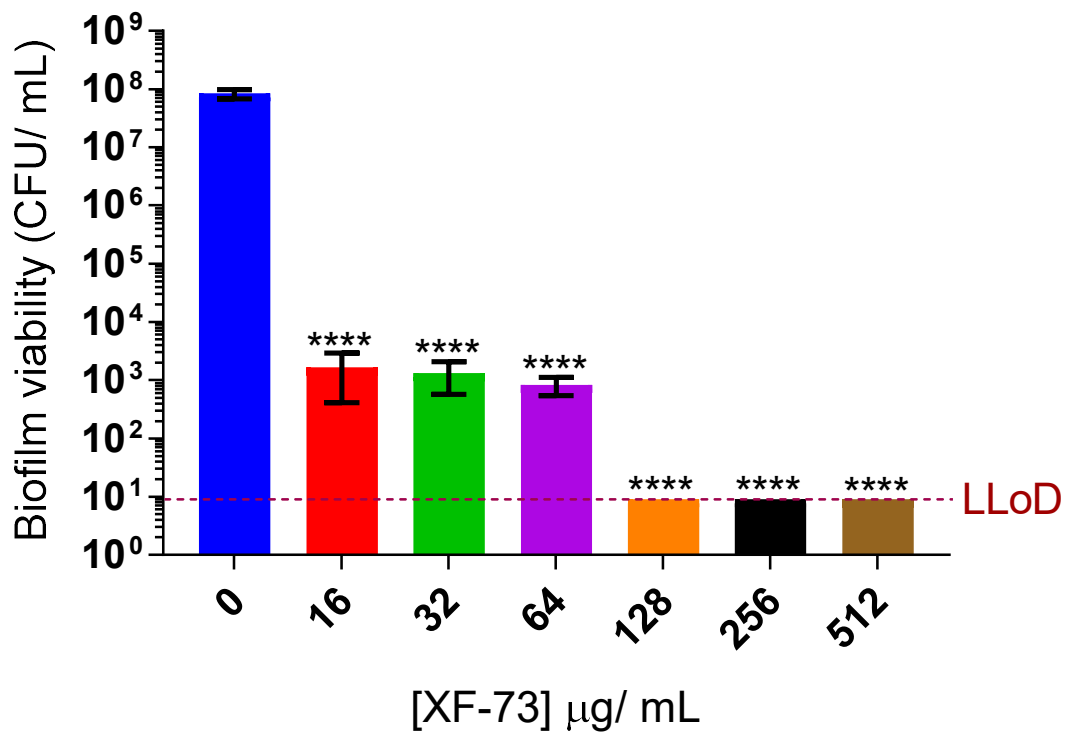
Figure 3.7 Twenty-four hour E. coli ATCC 25922 biofilms treated with XF-73 at a range of concentrations

*Recorded viabilities of E. coli ATCC 25922 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 16 – 512 µg/ mL. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated E. coli biofilm viability, where ****: p= <0.0001.*
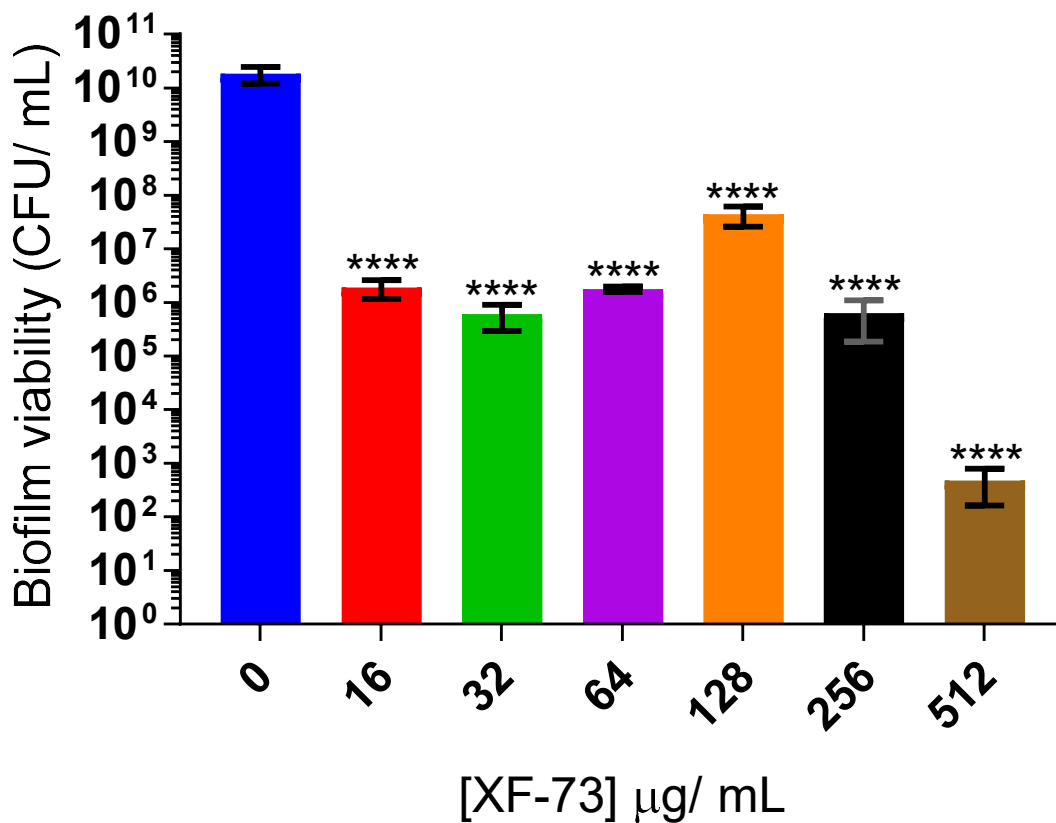
Figure 3.8 Twenty-four hour P. aeruginosa ATCC 15442 biofilms treated with XF-73 at a range of concentrations

*Recorded viabilities of P. aeruginosa ATCC 15442 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 16 – 512 µg/ mL. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated P. aeruginosa biofilm viability, where ****: p= <0.0001.*
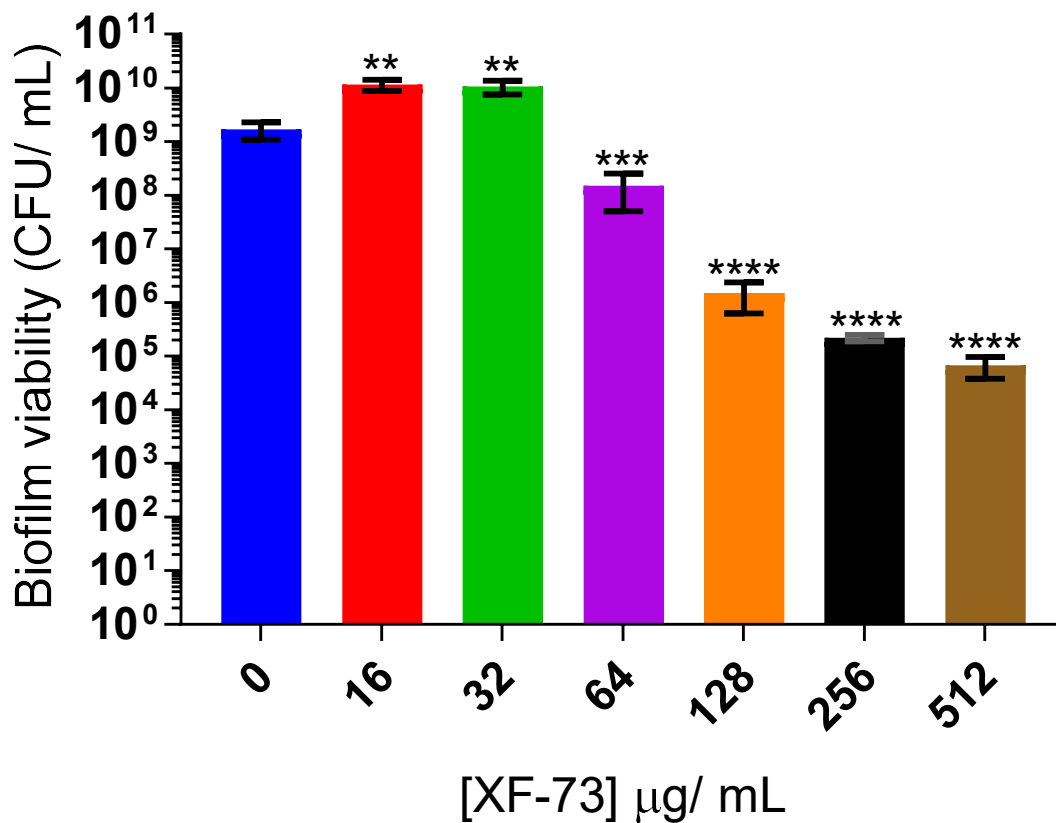
Figure 3.9 Twenty-four hour P. aeruginosa ATCC 27853 biofilms treated with XF-73 at a range of concentrations

Recorded viabilities of P. aeruginosa ATCC 27853 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 16 – 512 µg/ mL. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated P. aeruginosa biofilm viability, where **: p= 0.0017 (16 µg/ mL) and 0.0025 (32 µg/ mL), ***: p= 0.0001 and ****: p= <0.0001.

3.5.3.3 Comparator antimicrobials against Gram-positive biofilms after twenty-four hours of growth

3.5.3.3.1 *S. aureus* ATCC 29213 biofilms

The addition of gentamicin to *S. aureus* biofilms led to a decrease in biofilm viability. Concentrations of 2 – 16 µg/ mL showed no viable colonies during the viable cell count and therefore are classified as having a biofilm viability under the lower limit of detection (Figure 3.10 A). The lowest concentration of gentamicin used in this study led to a 3-log reduction in biofilm viability. The difference between biofilm viabilities recorded after treatment of each concentration of gentamicin was shown to be significant in comparison to the growth control (p= <0.0001).

Each concentration of mupirocin used against *S. aureus* biofilms had a recorded biofilm viability lower than the growth control (Figure 3.10 B); statistical analysis of these viabilities showed the same significant difference at each concentration of mupirocin against the control viability (p= <0.0001). Mupirocin at 512 µg/ mL showed no viable colonies present during the viable cell count and is therefore classified as having a biofilm viability under the lower limit of detection. Concentrations 8 – 64 µg/ mL, showcased that as the concentration of mupirocin increased the biofilm viability decreased. Viability of *S. aureus* biofilms treated with 64 µg/ mL of mupirocin had an 8-log reduction in comparison to the growth control, with viability readings at: 8.33 x10^2 CFU/ mL and 2.35 x10^{10} CFU/ mL respectively. While only 64 µg/ mL showed an 8-log reduction in viability, no significant difference between biofilm viability values was found between concentrations: 64 µg/ mL vs 128 µg/ mL, 64 µg/ mL, and 256 µg/ mL; furthermore, no significant difference was determined between viabilities of 128 µg/ mL vs 256 µg/ mL (p > 0.05). Additionally, no significant differences in biofilm viabilities were found between concentrations: 8 µg/ mL vs 16 µg/ mL, 16 µg/ mL vs 32 µg/ mL, 128 µg/ mL, 256 µg/ mL and 32 µg/ mL vs 128 µg/ mL, 256 µg/ mL (p > 0.05).

When rifampicin was added to *S. aureus* biofilms, a significant difference (p= <0.0001) in viability was recorded at each concentration in comparison to the growth control (Figure 3.10 C). At concentrations 32 µg/ mL, 64 µg/ mL and 512 µg/ mL, no viable colonies were present during viable cell counts and therefore are classified as having a biofilm viability under the lower limit of detection. A greater viability of *S. aureus* biofilms was recorded at higher concentrations of rifampicin: 128 µg/ mL and 256 µg/ mL in comparison to the lower concentrations. Rifampicin at 16 µg/ mL showed the lowest recorded biofilm viability at 53 CFU/ mL.

Although, a decrease in biofilm viability was recorded at each concentration of daptomycin used against *S. aureus* biofilms, there was no significant difference (p > 0.05) between the growth control and 8 µg/ mL treatment (Figure 3.10 D). The decrease in viability with the addition of the higher concentrations of daptomycin: 16 – 512 µg/ mL was shown to be significant through statistical analysis (p= <0.0001). Concentrations 32 – 512 µg/ mL showed no viable colonies during the viable cell count and therefore

is classified as having a biofilm viability under the lower limit of detection. Daptomycin at 16 µg/ mL showed an 8-log reduction in biofilm viability, *S. aureus* alone had a biofilm viability of 5.33 x10^9, meanwhile 16 µg/ mL a mean viability of 89 CFU/ mL.

3.5.3.3.2 *E. faecalis* ATCC 29212 biofilms

A decrease in biofilm viability was recorded as the concentration of gentamicin increased throughout this study (Figure 3.11 A). A significant difference (p= <0.0001) was recorded for each treatment of gentamicin against in *E. faecalis* biofilms in comparison to the growth control of *E. faecalis* alone. Concentrations 16 – 128 µg/ mL are classified as having a biofilm viability below the lower limit of detection, due to no viable colonies visible during the viable cell counts. Gentamicin at 8 µg/ mL had the lowest recorded biofilm mean viability at 89 CFU/ mL and a 7-log reduction in comparison to the growth control. Treatment of gentamicin at 2 µg/ mL led to a 2-log reduction and a 5-log reduction at 4 µg/ mL.

Higher concentrations of mupirocin: 32 – 512 µg/ mL used in this study, led to a lower biofilm viability in *E. faecalis* biofilms in comparison to the growth control (Figure 3.11 B); the difference between viabilities was determined to be significant through statistical analysis for all mupirocin concentrations (p= <0.0001). In contrast the lowest concentrations of mupirocin: 8 µg/ mL and 16 µg/ mL showed biofilm viabilities greater than the growth control, with large lower error bars as one replicate for each concentration had a biofilm viability 2.8 x10^{10} and 1.8 x10^{10} CFU/ mL greater than the highest recorded biofilm viability of the three replicates recorded for the growth control. Although these differences were deemed to be not significant (p > 0.05) from the growth control. Mupirocin at 512 µg/ mL has a recorded biofilm viability under the lower limit of detection as no viable colonies were observed during the viable cell count. Mupirocin at 256 µg/ mL led to the lowest recorded biofilm viability at 817 CFU/ mL, an 8-log reduction in comparison to the growth control: 7.67 x10^9 CFU/ mL. Whilst mupirocin at 256 µg/ mL had the lowest recorded biofilm viability, there was no significant difference between *E. faecalis* biofilms treated with 128 µg/ mL of mupirocin (p > 0.05).

All concentrations of rifampicin used against *E. faecalis* biofilms yielded a lower viability in comparison to the control growth (Figure 3.11 C); despite this fact, no significant difference was determined between viability of 128 µg/ mL and 256 µg/ mL rifampicin treatments and untreated *E. faecalis* biofilms (p > 0.05). Rifampicin treatment at 512 µg/ mL had the lowest recorded biofilm viability at 5.6 x10^1 CFU/ mL, a 5-log reduction in comparison to the growth control. Statistical analysis of biofilm viability of rifampicin treatments: 8 – 64 µg/ mL and 512 µg/ mL against the growth control showed a significant difference, where 32 µg/ mL and 512 µg/ mL had the same difference (p= <0.0001). Whilst 8 µg/ mL, 16 µg/ mL and 64 µg/ mL all had a recorded 2-log reduction in viability, each concentration had a different statistical difference (p= 0.001, p= 0.002 and p= 0.0024).

Daptomycin, the final antimicrobial treatment against *E. faecalis* biofilms, showed a decrease in viability at all concentrations used; concentrations 64 – 512 µg/ mL are classified as having a biofilm viability below the lower limit of detection, due to no viable colonies visible during the viable cell counts (Figure 3.11 D). All concentrations were shown through statistical analysis to significantly reduce the viability of *E. faecalis* compared with the growth control (p= <0.0001). Daptomycin at 32 µg/ mL had the lowest recorded biofilm mean viability at 86 CFU/ mL, an 8-fold reduction in comparison to the growth control at 4.83 x10^9 CFU/ mL.

C

D
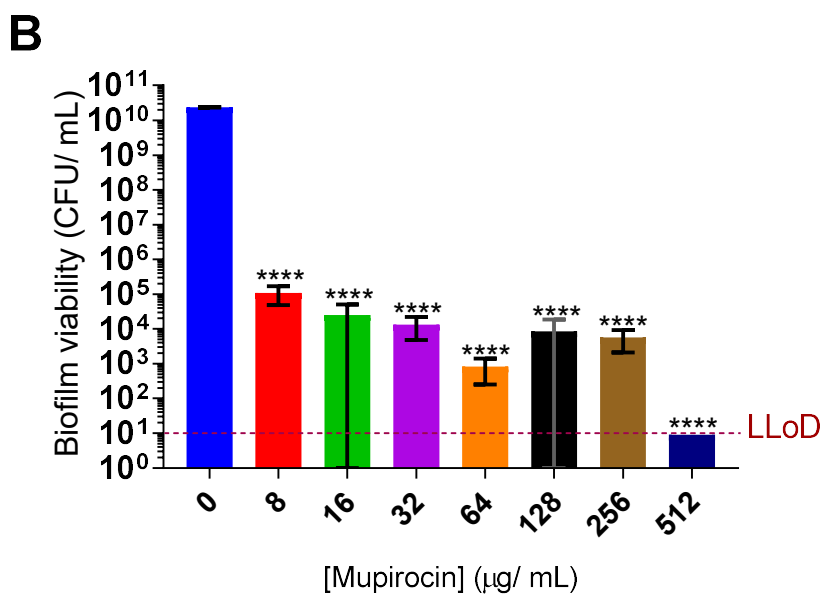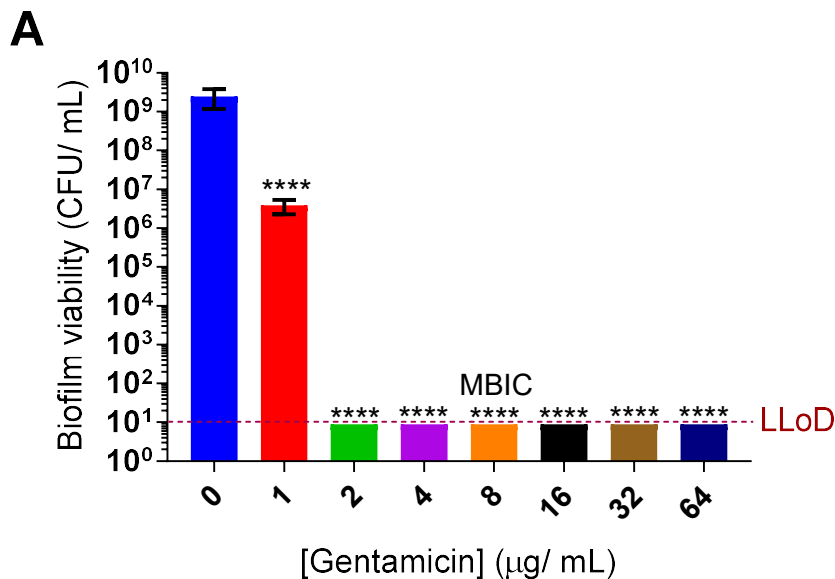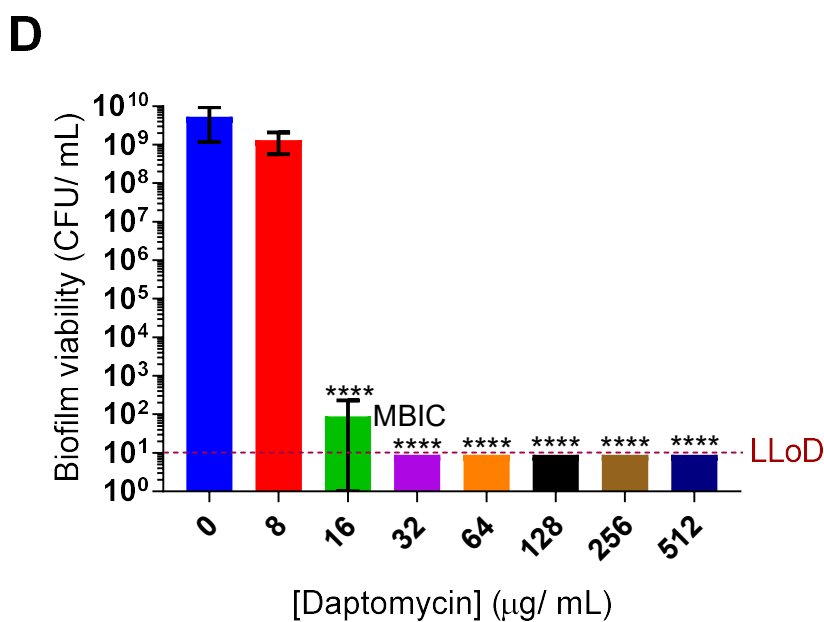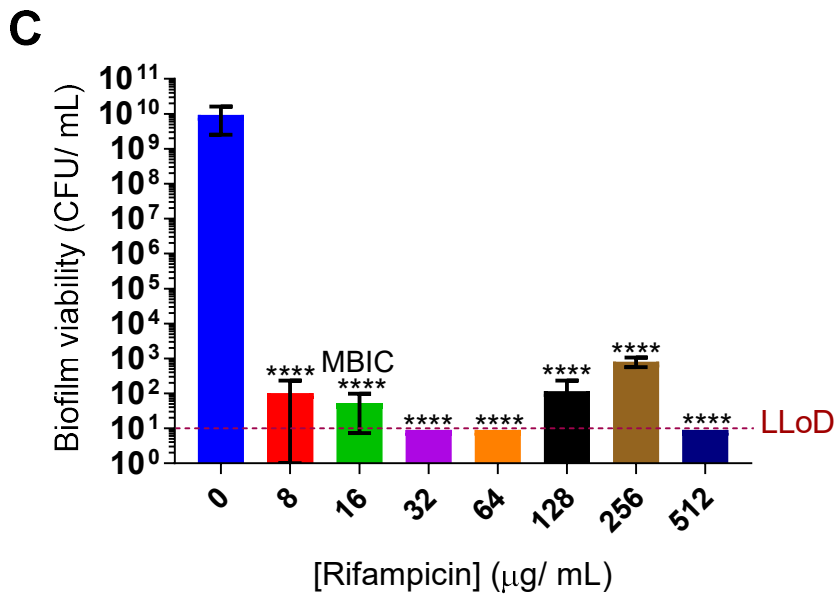
Figure 3.10 Twenty-four hour S. aureus ATCC 29213 biofilms treated with a panel of antimicrobials

*Recorded viabilities of S. aureus ATCC 29213 biofilms (CFU/ mL), after twenty-four hours of growth treated with (A) gentamicin, (B) mupirocin, (C) rifampicin and (D) daptomycin at a concentration range of 1 – 512 μg/ mL. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of antimicrobial compared against untreated S. aureus biofilm viability, where ****: p= <0.0001. MBIC values also highlighted for each antimicrobial (Table 3.5).*

A

B

C

Biofilm viability (CFU/ mL) vs [Rifampicin] (µg/ mL)

D

Biofilm viability (CFU/ mL) vs [Daptomycin] (µg/ mL)

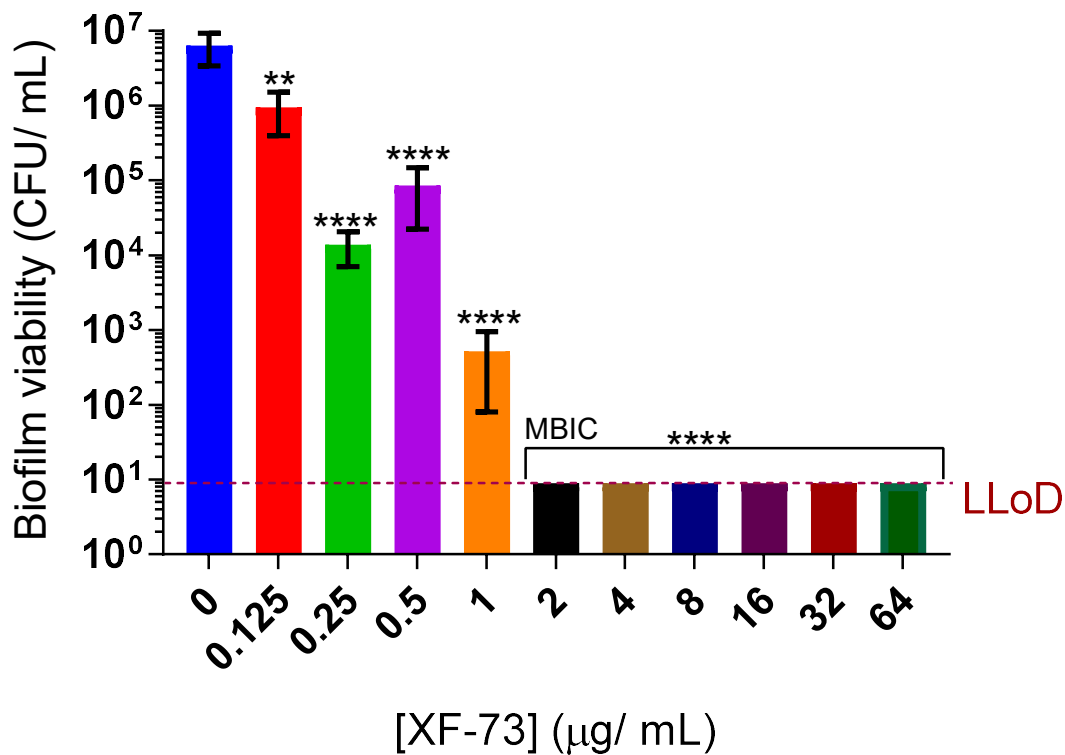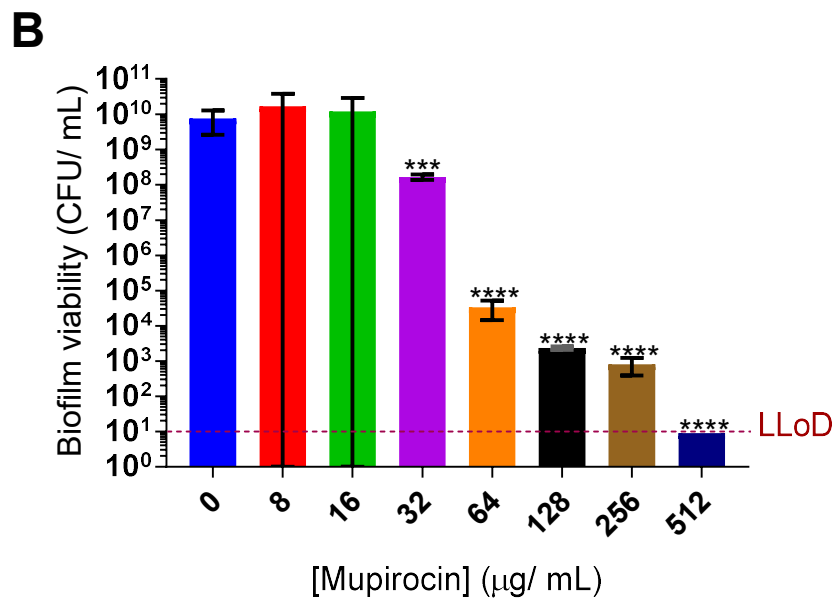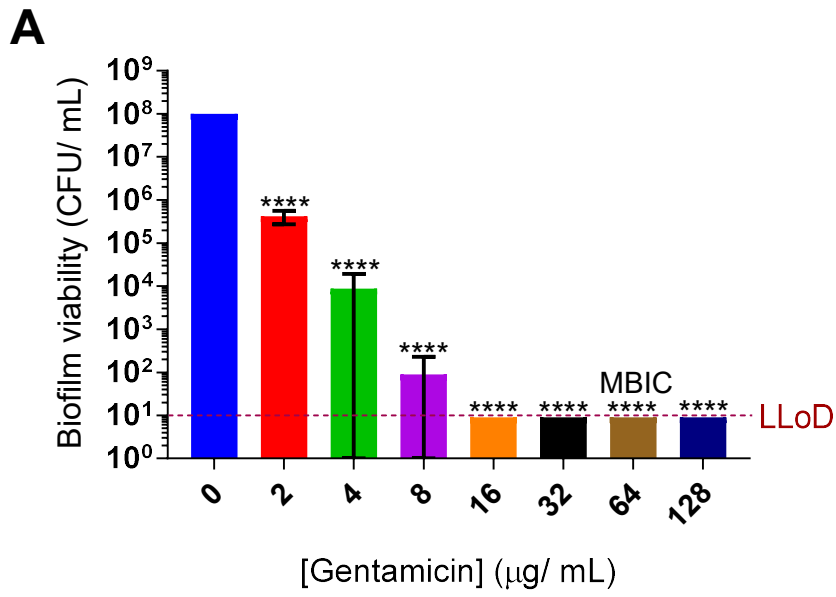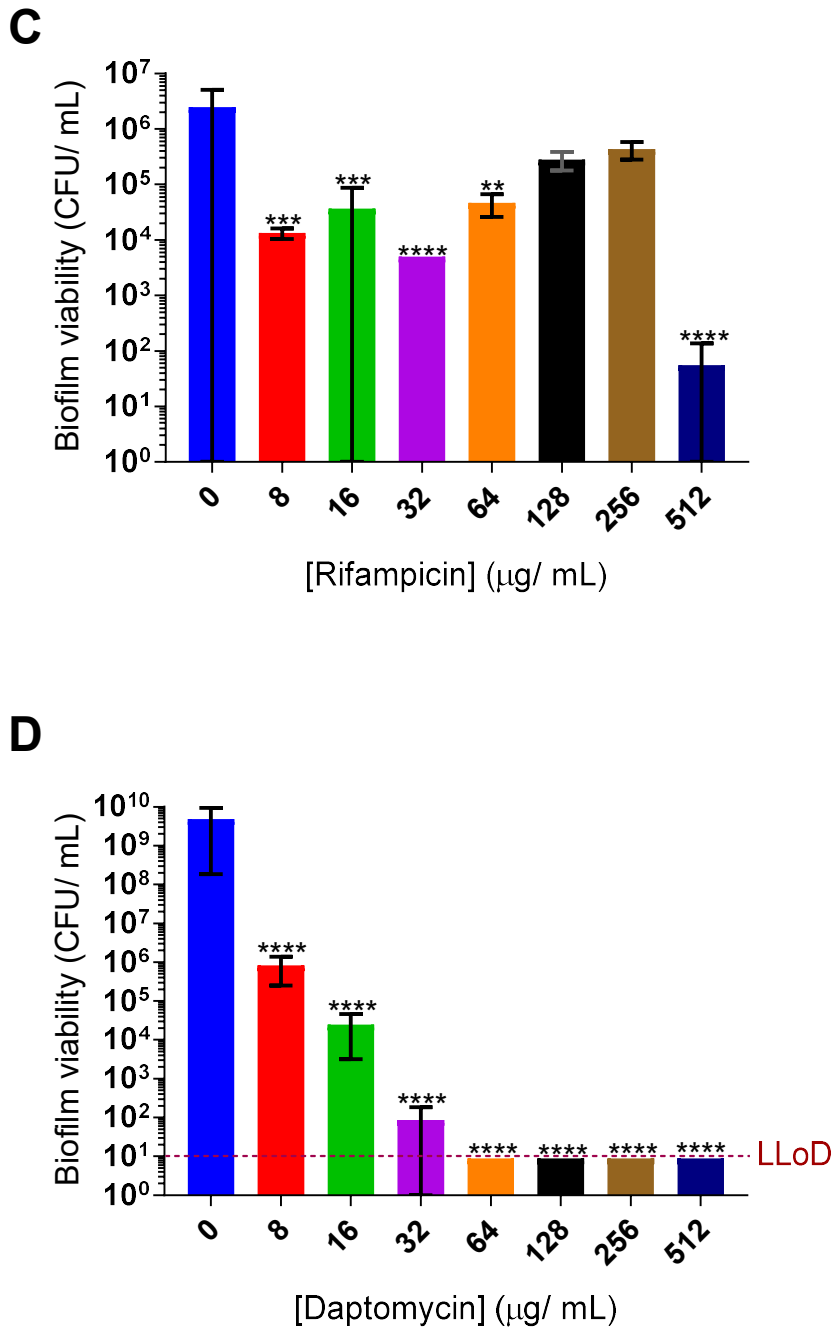*Figure 3.11 Twenty-four hour E. faecalis ATCC 29212 biofilms treated with a panel of antimicrobials*

Recorded viabilities of E. faecalis biofilms (CFU/ mL), after twenty-four hours of growth treated with (A) gentamicin, (B) mupirocin, (C) rifampicin and (D) daptomycin at a concentration range of 2 – 512 µg/ mL. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of antimicrobial compared against untreated E. faecalis biofilm viability, where **: p= 0.0024 (64 µg/ mL of rifampicin), ***: p= 0.0001, 0.0002 (8 µg/ mL and 16 µg/ mL of rifampicin) and 0.0009 (64 µg/ mL of mupirocin) and ****: p= <0.0001. MBIC value also highlighted for gentamicin (Table 3.5).

3.5.3.4 Comparator antimicrobials against Gram-negative biofilms after twenty-four hours of growth

3.5.3.4.1 *E. coli* ATCC 35218 biofilms

Gentamicin when used against *E. coli* biofilms showed a lower biofilm viability in comparison to untreated *E. coli* with the exception of 8 µg/ mL, whose viability value: 2.83×10^9 CFU/ mL was very similar to the growth control: 3.0×10^9 CFU/ mL (Figure 3.12 A). Although lower viabilities were recorded, statistical analysis showed no significant difference between the growth control and gentamicin concentrations 1 – 8 µg/ mL ($p > 0.05$). The lowest biofilm viability recorded was 1.3×10^3 CFU/ mL from gentamicin at 32 µg/ mL, resulting in a 6-log reduction in comparison to the control growth; and was determined to be statistically significant different ($p = <0.0001$). Concentrations 16 µg/ mL and 64 µg/ mL also showed a significant difference in viability compared to the growth control ($p = 0.0011$ and 0.0003). No significant difference between gentamicin treatments: 1 µg/ mL, 2 µg/ mL, 4 µg/ mL and 16 µg/ mL ($p > 0.05$); additionally, no significance was found between higher gentamicin concentrations: 16 µg/ mL, 32 µg/ mL and 64 µg/ mL ($p > 0.05$).

Every concentration of mupirocin used as a treatment against *E. coli* biofilms had a lower biofilm viability in comparison with the growth control (Figure 3.12 B), the difference between viabilities at all concentrations to the control viability was deemed significant through statistical analysis ($p = <0.0001$). XF-73 at 512 µg/ mL had a biofilm viability determined under the lower limit of detection. Concentrations 8 µg/ mL and 16 µg/ mL had a 2-log reduction in viability in comparison to the growth control, with no statistical difference between the two viabilities, while concentrations 32 – 256 µg/ mL had a 3-log reduction, similarly there was no statistical difference between viabilities recorded at these concentrations ($p > 0.05$).

Reduced viabilities of *E. coli* biofilms were recorded with the addition of rifampicin at each concentration used in the study, although there was no trend following the increase in rifampicin concentrations to biofilm viabilities (Figure 3.12 C). The lowest biofilm viability: 1.33×10^7 CFU/ mL, was recorded at 32 µg/ mL of rifampicin and was a 3-log reduction in comparison to the growth control: 2.73×10^{10} CFU/ mL. Biofilm viability of *E. coli* treated with 4 µg/ mL of rifampicin had a similar viability to the growth control: 1.45×10^{10} CFU/ mL. Regardless of the decreased viabilities in treated *E. coli* biofilms, no significant difference was found at any rifampicin concentration against the growth control, similarly there was no significant difference found between viabilities of each concentration of rifampicin ($p > 0.05$).

The addition of daptomycin at each concentration to *E. coli* biofilms showed similar viability readings in comparison to untreated *E. coli* biofilms (Figure 3.12 D). Statistical analysis determined that there was no significant difference between daptomycin treatments to the growth control and no statistical difference amongst any of the concentrations of daptomycin ($p > 0.05$).

3.5.3.4.2 *P. aeruginosa* ATCC 27853

Concentrations marked with 'x' signifies a biofilm viability reading that had a 'Too Many to Count' (TMTC) viable colonies during the viable cell count (Figure 3.13); therefore, a viability reading was calculated using the mean previously collected biofilm viability readings of untreated *P. aeruginosa* throughout the biofilm studies. The calculated value: 2.63 $\times 10^{11}$ CFU/ mL was determined to be the minimum potential biofilm viability and was inputted into the biofilm viability graphs to visualise the effect of the antimicrobials on viability against *P. aeruginosa* biofilms and conduct statistical analysis.

The lowest concentrations of gentamicin: 0.5 µg/ mL and 1 µg/ mL, used against *P. aeruginosa* biofilms showed no decrease in viability, in comparison to the growth control (Figure 3.13 A). Although the growth control, *P. aeruginosa* biofilms treated with 0.5 µg/ mL and 1 µg/ mL of gentamicin had a viability classified as TMTC. In contrast, the remaining concentrations 2 – 32 µg/ mL all had recorded biofilm viabilities; 32 µg/ mL was recorded with the lowest biofilm viability at 3.33 $\times 10^3$ CFU/ mL, which can be concluded as a minimum 6-log reduction compared to the growth control. Concentrations 2 µg/ mL, 4 µg/ mL and 16 µg/ mL had similar biofilm viabilities and statistical analysis concluded no significant difference between any of those concentrations ($p > 0.05$). Further statistical analysis concluded concentrations 2 – 32 µg/ mL had a significant difference in viability in comparison the viability of untreated biofilms for all concentrations ($p = <0.0001$).

Viability of *P. aeruginosa* biofilms was classified as TMTC for the growth control and mupirocin at concentrations 8 – 64 µg/ mL, thus no statistical difference ($p > 0.05$) was determined between these concentrations of mupirocin against the growth control (Figure 3.13 B). Even though mupirocin at 128 µg/ mL had a recorded biofilm viability and was therefore lower than viability of untreated *P. aeruginosa* biofilms, no statistical difference was concluded ($p > 0.05$). The final concentrations of mupirocin were both deemed to have a statistically significant difference between the growth control ($p = <0.0001$); whilst 256 µg/ mL had a minimum 6-log reduction and 512 µg/ mL a minimum 8-log reduction in comparison to the growth control, there was no significant or statistical difference between the viability of these two concentrations ($p > 0.05$).

Rifampicin as an antimicrobial treatment against *P. aeruginosa* biofilms had a recorded increase in viability at the lowest concentrations used: 4 µg/ mL and 8 µg/ mL, where viability for these two concentrations was TMTC (Figure 3.13 C). The remaining concentrations 16 – 256 µg/ mL all had lower *P. aeruginosa* biofilm viabilities recorded in comparison to the growth control. The viabilities recorded from these rifampicin treatments had a significant statistical difference between the growth control that varied between concentrations; rifampicin at 32 – 256 µg/ mL had a greater statistical difference ($p = <0.0001$) in comparison to 16 µg/ mL ($p = 0.425$). No significant difference for biofilm viabilities was determined between concentrations 64 µg/ mL and 128 µg/ mL ($p > 0.05$). The lowest recorded viability of *P. aeruginosa* biofilms was with the addition of rifampicin at 256 µg/ mL, leading to a 7-log decrease

in viability in comparison to the growth control at 7.67 x10^4 CFU/ mL and 1.84 x10^{11} CFU/ mL respectively.

All concentrations of daptomycin used in this study against *P. aeruginosa* biofilms had a viability classification as TMTC (Figure 3.13 D). The viabilities of each concentration were greater than the viability of the growth control, this difference was deemed significant through statistical analysis (p= <0.0001).

C

D
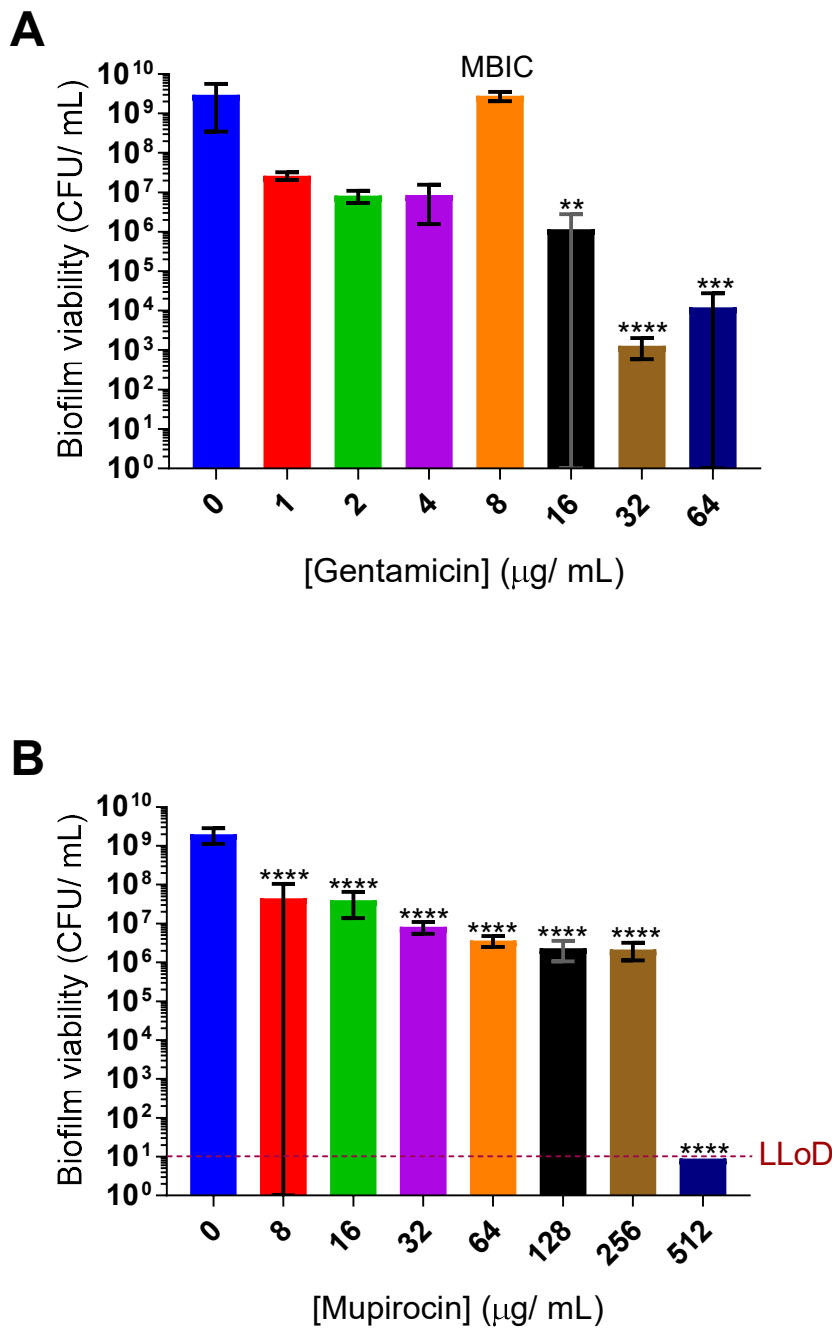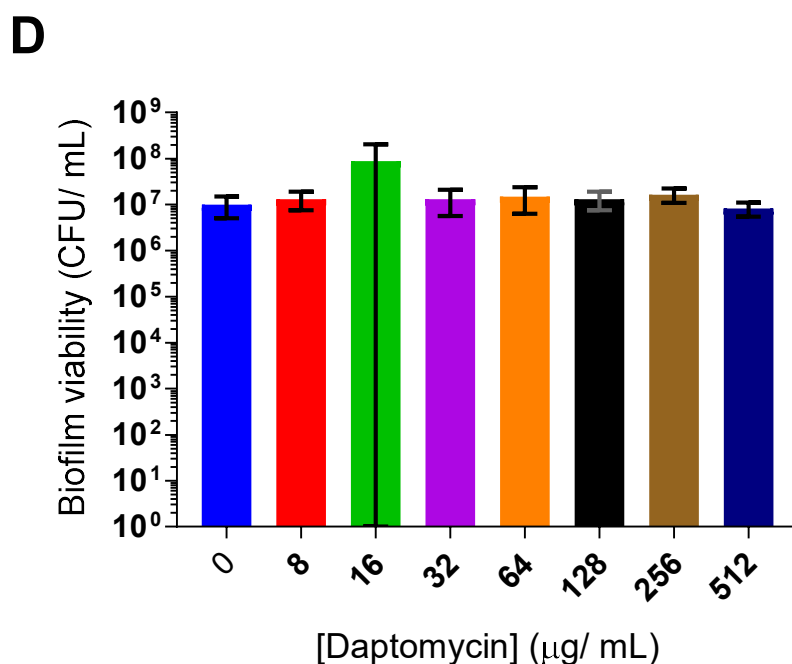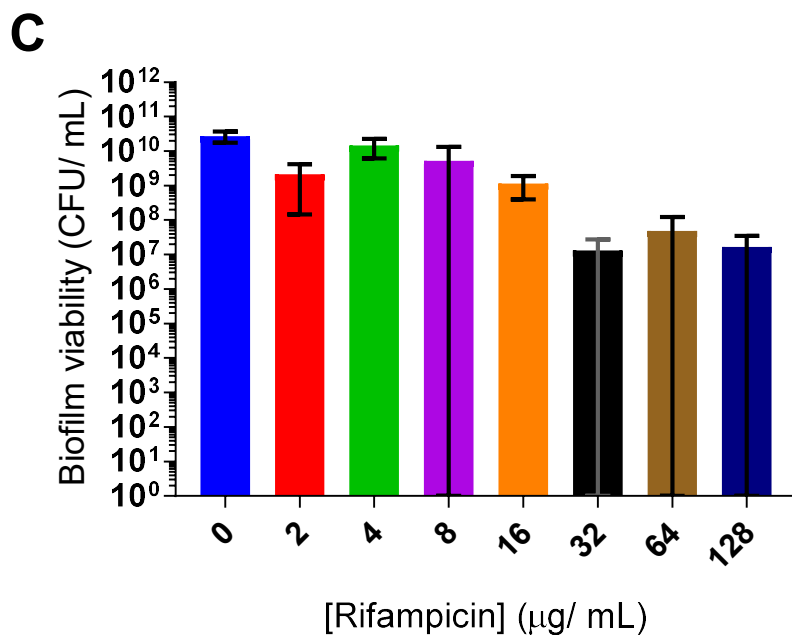
Figure 3.12 Twenty-four hour E. coli ATCC 35218 biofilms treated with a panel of antimicrobials

Recorded viabilities of E. coli biofilms (CFU/ mL), after twenty-four hours of growth treated with (A) gentamicin, (B) mupirocin, (C) rifampicin and (D) daptomycin at a concentration range of 2 – 512 µg/ mL. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of antimicrobial compared against untreated E. coli biofilm viability, where **: p= 0.0011 (16 µg/ mL of gentamicin), ***: p= 0.0003 (64 µg/ mL of gentamicin) and ****: p= <0.0001. MBIC value also highlighted for gentamicin (Table 3.5).

A

B

C

D
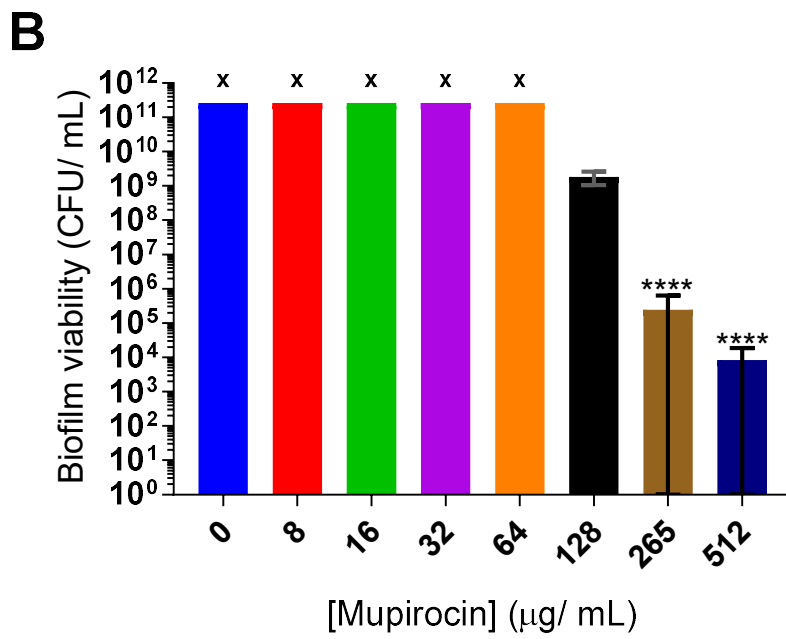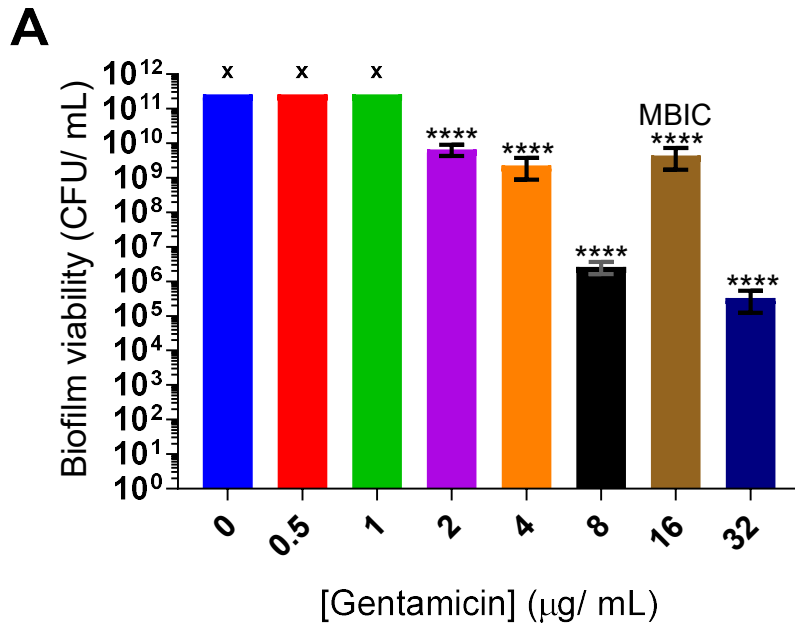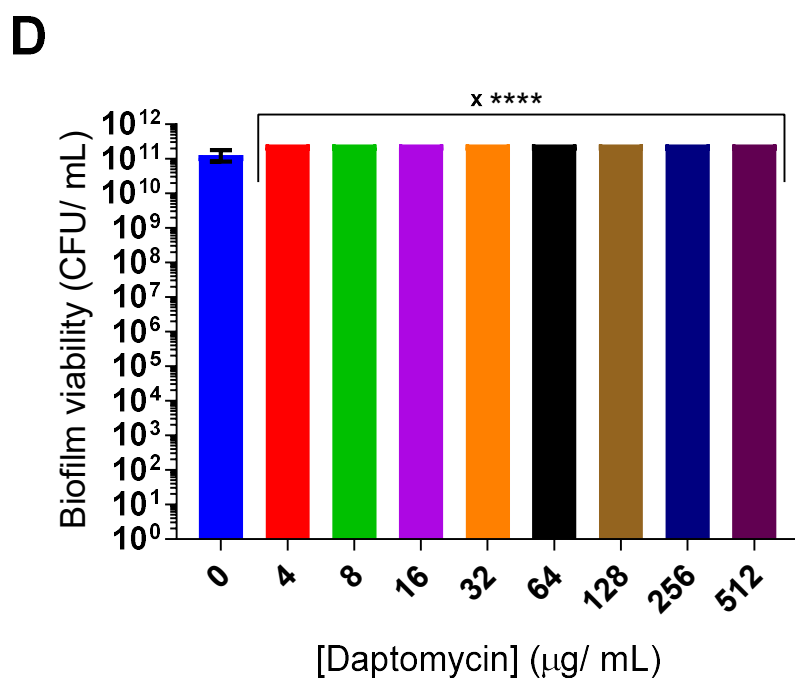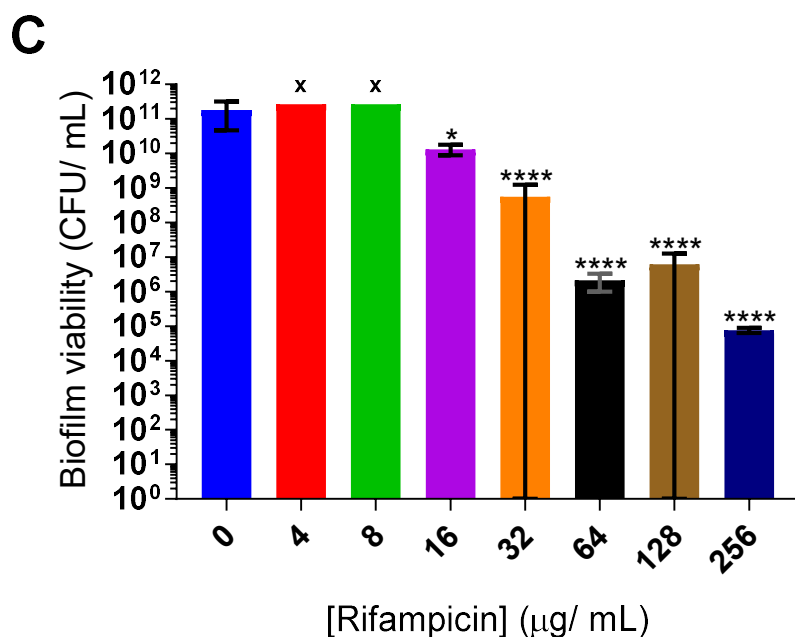
Figure 3.13 Twenty-four hour *P. aeruginosa* ATCC 27853 biofilms treated with a panel of antimicrobials

Recorded viabilities of P. aeruginosa biofilms (CFU/ mL), after twenty-four hours of growth treated with (A) gentamicin, (B) mupirocin, (C) rifampicin and (D) daptomycin at a concentration range of 2 – 512 µg/ mL. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. An 'x' above biofilm viability signifies there was 'too many to count' colonies during viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of antimicrobial compared against untreated P. aeruginosa biofilm viability, where *: p= 0.425 (32 µg/ mL of rifampicin) and ****: p= <0.0001. MBIC values also highlighted for gentamicin (Table 3.5).

3.5.4 Biofilm density of twenty-four hour Gram-positive and Gram-negative microorganisms visualised through crystal violet staining following a panel of antimicrobial treatments

Photographs of microtitre pegged lids containing Gram-positive and Gram-negative biofilms stained with crystal violet, following a range of antimicrobials treatments were collected to visualise biofilm density. Antimicrobial treatments consisted of: XF-73 (Figure 3.14, 3.15), gentamicin, mupirocin, rifampicin and daptomycin (Figure 3.16, 3.17, 3.18, 3.19)

3.5.4.1 Biofilm density of Gram-positive and Gram-negative microorganisms visualised through crystal violet staining following XF-73 treatment

3.5.4.1.1 Gram-positive biofilms

Untreated *S. aureus* ATCC 6538 biofilms had the greatest density of crystal violet on the sample pegs, as shown by the darker colouration in comparison to all biofilms treated with XF-73 (Figure 3.14 A). Each peg containing *S. aureus* biofilms were stained with crystal violet, whereas control pegs of XF-73 and MHB had no purple colouration. The greater the concentration of XF-73 used against *S. aureus* biofilms; the lower density of crystal violet staining was present on the pegs. Treatments 0.125 – 0.5 µg/ mL showed the greatest level of crystal violet staining out of the range of XF-73 treatments. Whilst concentrations of XF-73 at 16 µg/ mL – 64 µg/ mL had a visible crystal violet stain on the pegs, the colour was a darker purple in comparison to all other stained pegs.

There was no apparent presence of crystal violet staining of *S. aureus* ATCC 29213 biofilms (Figure 3.14 B); no stain was observed on untreated *S. aureus* biofilms and only a faint band was visualised around the pegs of XF-73 treatments ranging 8 – 64 µg/ mL. No crystal violet was recorded at concentrations 0.125 – 4 µg/ mL or XF-73 and MHB controls.

Only a faint presence of crystal violet staining was observed on *S. epidermidis* biofilm pegs, including untreated *S. epidermidis* biofilms where only a slight purple colouration was present further up the peg (Figure 3.14 C). At one replicate of each XF-73 concentration ranging: 8 – 64 µg/ mL, a darker, more abundant stain was present, covering only a portion of the peg as a ring. A faded thin band of crystal violet located further up the sample pegs was present at concentrations 0.125 – 0.5 µg/ mL. No stain was present on XF-73 and MHB controls pegs.

A lower abundance of stained *E. faecalis* biofilms was observed following XF-73 treatments ranging from 1 – 64 µg/ mL in comparison to untreated biofilms, as shown by only a thin band of purple around the treated pegs (Figure 3.14 D). In contrast concentrations 0.125 µg/ mL and 0.25 µg/ mL showed a greater density of crystal violet staining with a darker purple band three quarters of the way up the pegs

in comparison to untreated biofilms; though, untreated *E. faecalis* biofilms had a greater area of pegs stained in crystal violet. No crystal violet was found on XF-73 and MHB pegs.
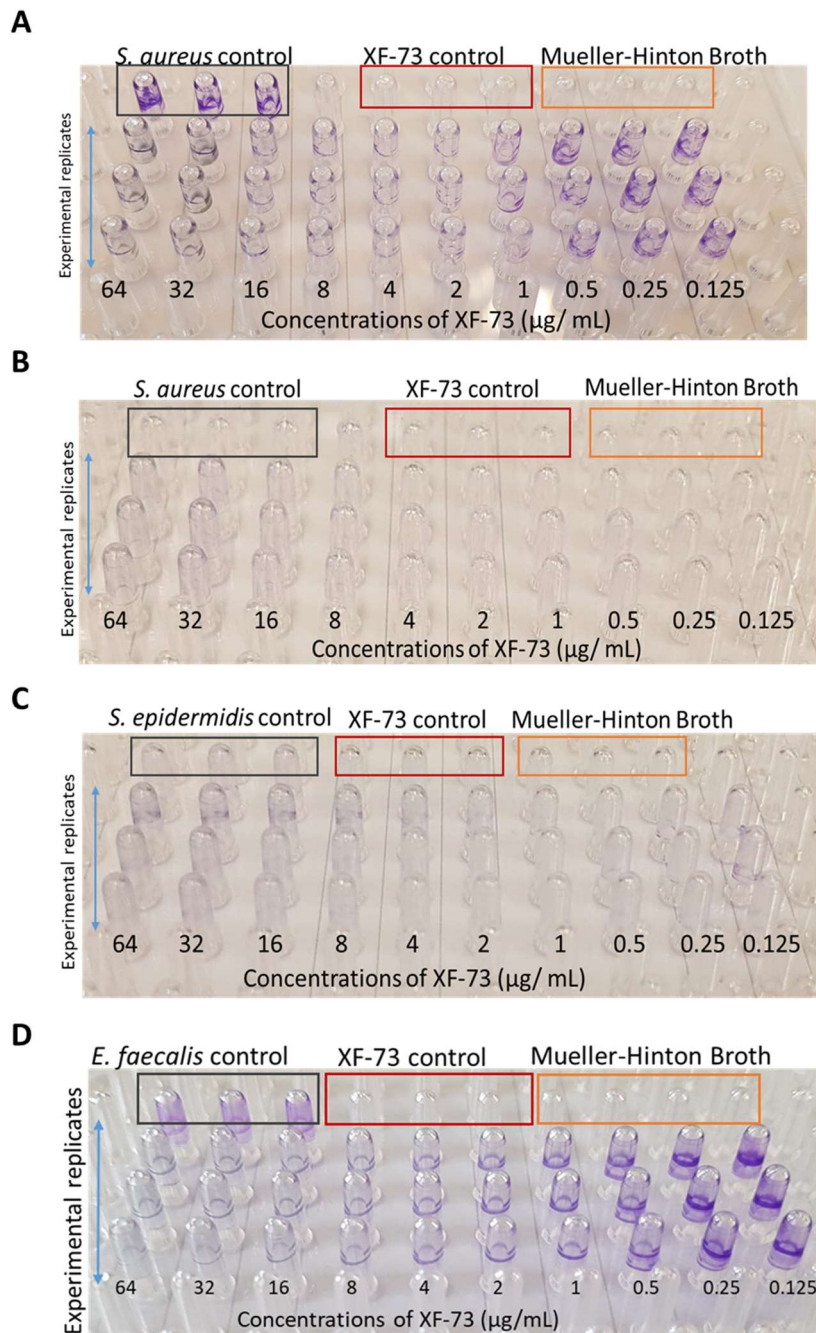
Figure 3.14 Density of twenty-four hour Gram-positive biofilms following XF-73 treatment

Density of Gram-positive biofilms: (A) S. aureus ATCC 6538, (B) S. aureus ATCC 29213, (C) S. epidermidis ATCC 12228 and (D) E. faecalis ATCC 29212 via crystal violet staining following XF-73 treatments at a concentration range of 0.125 µg/ mL – 64 µg/ mL. Controls of untreated biofilm, XF-73 alone and MHB alone are highlighted on each image.

3.5.4.1.2 Gram-negative biofilms

For each Gram-negative microorganism selected for this biofilm study, a higher density of crystal violet staining was present on untreated biofilm pegs (Figure 3.15) in comparison to untreated Gram-positive biofilms (Figure 3.14).

The highest density of crystal violet staining on *E. coli* ATCC 35218 biofilms was observed on the untreated biofilm pegs, with a dark purple colouration covering the entirety of the pegs except for the rounded ends of the pegs (Figure 3.15 A). A slight decrease in the stain density was recorded at concentrations 128 – 512 µg/ mL, meanwhile the lowest density of crystal violet was observed at the lower concentrations of XF-73 treatments: 16 – 64 µg/ mL. No stain was recorded on control pegs of XF-73 and MHB.

All XF-73 treatments against *E. coli* ATCC 25922 showed a decrease in crystal violet staining on the microtitre pegs (Figure 3.15 B), the greater the concentration of XF-73, the less residual crystal violet remained on the pegs. Compared to untreated *E. coli* biofilms, all XF-73 treated biofilms had a lower density of crystal violet staining, located mainly around the centre of the pegs and with no stain present on the ends of the treated biofilm pegs. The crystal violet stain present on biofilms treated with 512 µg/ mL XF-73 had the darkest colouration of crystal violet appearing dark blue in contrast to the standard purple colouration viewed on all other *E. coli* pegs. No crystal violet stain was observed on microtitre pegs containing no *E. coli* biofilms.

Thin bands of dark pigmented crystal violet were present at concentrations 256 µg/ mL and 512 µg/ mL of XF-73 treatments against *P. aeruginosa* ATCC 15442 biofilms (Figure 3.15 C). A greater density of crystal violet was recorded at XF-73 treatments ranging from 16 – 128 µg/ mL in comparison to untreated biofilms, though there was no stain visible on the ends of the pegs. No crystal violet was observed on pegs for XF-73 and MHB controls.

No crystal violet was present along the ends of the microtitre pegs containing *P aeruginosa* ATCC 27853 biofilms (Figure 3.15 D). Following XF-73 treatment at 16 – 64 µg/ mL, a similar abundance of crystal violet stain was observed along the triplicate pegs in comparison to untreated biofilms. After treatment of XF-73 at 128 µg/ mL XF-73, a higher density of crystal violet was present on the microtitre pegs compared to untreated *P. aeruginosa* biofilms. The lowest density of crystal violet staining remaining on the microtitre pegs was visualised on treated biofilms at concentrations of 256 – 512 µg/ mL of XF-73, where the crystal violet staining was darker when compared to the other stained pegs. Meanwhile control pegs of XF-73 and MHB alone showed no residual crystal violet.
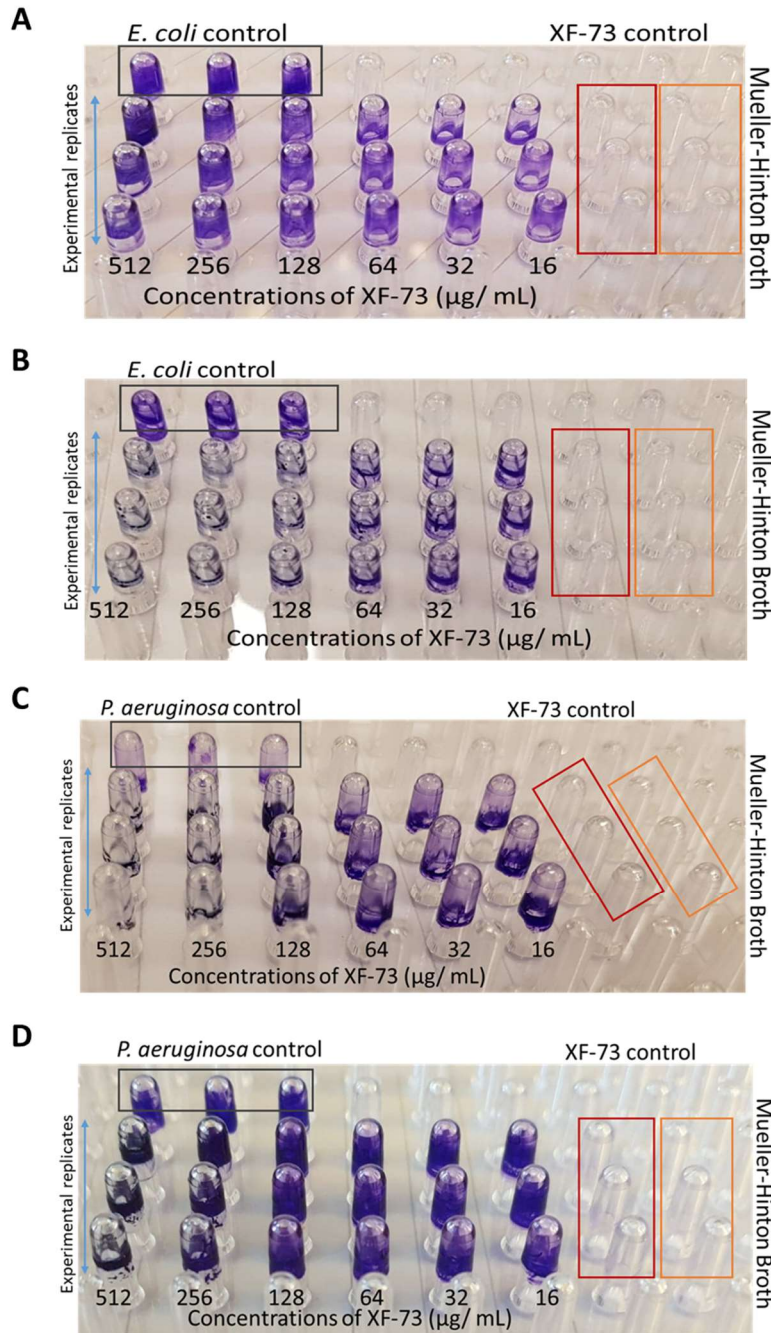
Figure 3.15 Density of twenty-four hour Gram-negative biofilms following XF-73 treatment

Density of Gram-negative biofilms: (A) E. coli ATCC 35218, (B) E. coli ATCC 25922, (C) P. aeruginosa ATCC 15442 and (D) P. aeruginosa ATCC 27853 via crystal violet staining following XF-73 treatments at a concentration range of 16 µg/ mL – 512 µg/ mL. Controls of untreated biofilm, XF-73 alone and MHB alone are highlighted on each image.

3.5.4.2 Biofilm density of Gram-positive and Gram-negative microorganisms visualised through crystal violet staining following antimicrobial treatment

3.5.4.2.1 *S. aureus* ATCC 29213

No crystal violet staining was present on any microtitre pegs containing *S. aureus* ATCC 29213 biofilms treated with a range of antimicrobials nor on any control pegs which were exposed to each antimicrobial alone or MHB alone (Figure 3.16 A, B, C). A faint purple colouration was only recorded on the microtitre peg lid representing daptomycin treatment, visible on all three replicates of untreated *S. aureus* biofilms (Figure 3.16 D).

3.5.4.2.2 *E. faecalis* ATCC 29212

Control wells containing each antimicrobial alone and MHB alone had not retained any crystal violet stain on any of the replicate pegs on each microtitre lid, per antimicrobial treatment (Figure 3.17). Similarly, no crystal violet stain was present on *E. faecalis* biofilms treated with daptomycin at every concentration (Figure 3.17 D); only untreated *E. faecalis* biofilms showed a faint amount of stain on each replicate peg.

Whilst there was only a slight presence of crystal violet as a band around the middle of untreated *E. faecalis* biofilm pegs, more distinct bands were noted at 2 µg/ mL of gentamicin treatment (Figure 3.17 A). As the concentration of gentamicin increased the presence and density of crystal violet staining decreased.

A similar amount of crystal violet was retained on untreated *E. faecalis* biofilms and biofilms treated with 8 – 32 µg/ mL of mupirocin (Figure 3.17 B). From 64 µg/ mL of mupirocin onwards, the greater the concentration the less crystal violet was present on the pegs, at 512 µg/ mL crystal violet was barely present on any of the replicate pegs. Out of all antimicrobial treatments, mupirocin showed the greatest retention of crystal violet staining.

A similar density of crystal violet staining was present on all *E. faecalis* biofilms treated with rifampicin (Figure 3.17 C). In comparison, untreated *E. faecalis* had the greatest density of crystal violet staining, present faintly along a greater surface area of the pegs.
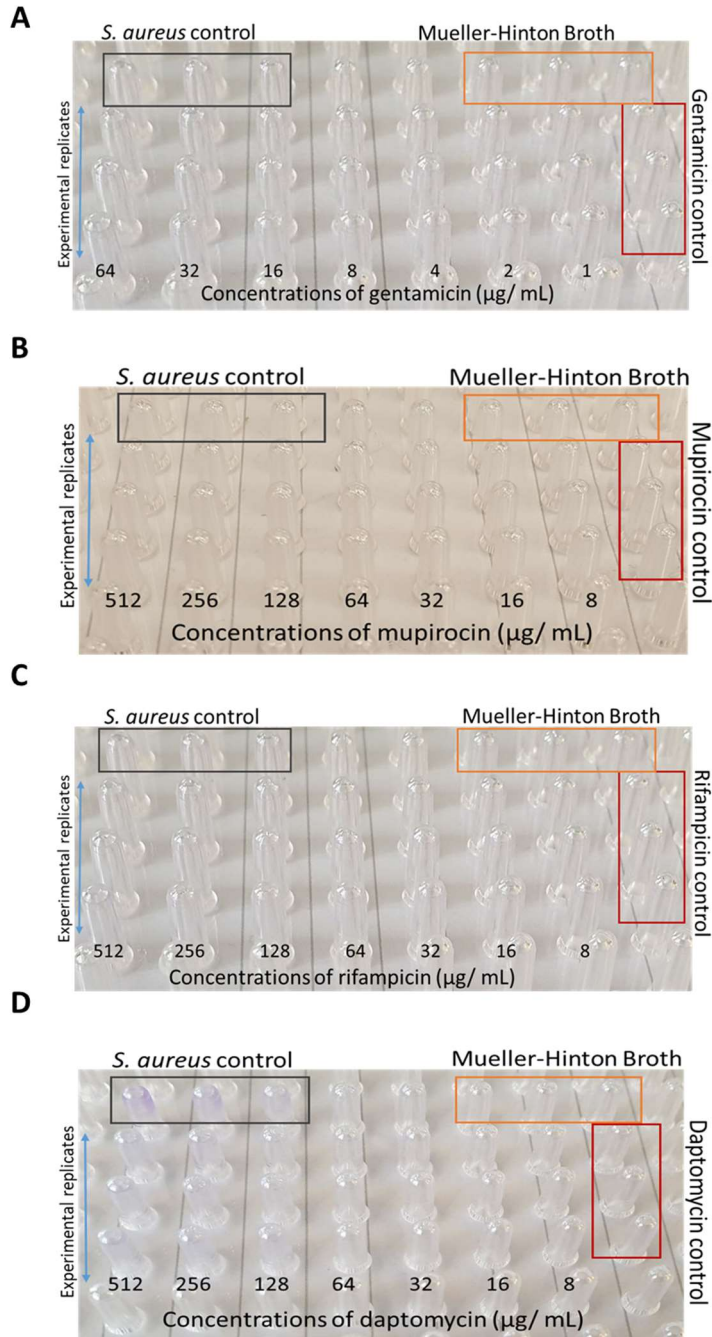
Figure 3.16 Density of twenty-four hour S. aureus ATCC 29213 biofilms following treatment with a panel of antimicrobials

Density of S. aureus ATCC 29213 biofilms via crystal violet staining following treatment against a panel of antimicrobials: (A) gentamicin, (B) mupirocin, (C) rifampicin and (D) daptomycin at a concentration range of 1 – 512 μg/ mL. Controls of untreated S. aureus, antimicrobial alone and MHB alone are highlighted on each image.
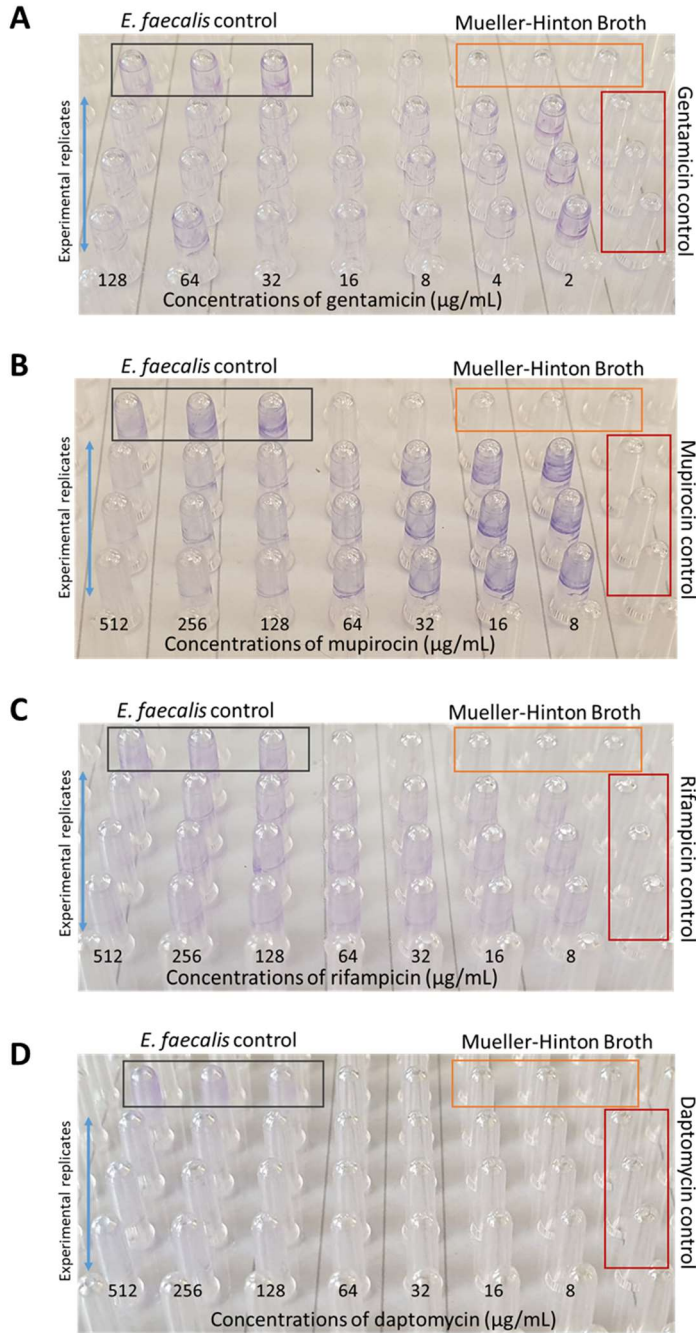
Figure 3.17 Density of twenty-four hour E. faecalis ATCC 29213 biofilms following treatment with a panel of antimicrobials

Density of E. faecalis ATCC 29213 biofilms via crystal violet staining following treatment against a panel of antimicrobials: (A) gentamicin, (B) mupirocin, (C) rifampicin and (D) daptomycin at a concentration range of 2 – 512 µg/ mL. Controls of untreated E. faecalis, antimicrobial alone and MHB alone are highlighted on each image.

3.5.4.2.3 *E. coli* ATCC 35218

Crystal violet staining was present on all microtitre pegs bearing *E. coli* ATCC 35218 biofilms and corresponding pegs with biofilms subjected to each antimicrobial treatment (Figure 3.18). The only pegs containing no stain were control pegs of antimicrobial alone and MHB alone.

Dense crystal violet staining was present on untreated *E. coli* pegs, though the tips of the pegs contained no stain. The addition of gentamicin led to a dramatic decrease in the presence of crystal violet staining (Figure 3.18 A), with only a thin band of crystal violet present at each concentration range. Concentrations 2 – 64 μg/ mL had a similar density of crystal violet staining on each peg.

Similar to the recorded presence of crystal violet on *E. coli* biofilms treated with gentamicin; mupirocin and rifampicin treatments led to a notable decrease in crystal violet staining, in comparison to their corresponding untreated control pegs (Figure 3.18 B, C). The lowest concentration of mupirocin and rifampicin yielded the greatest retention of stain.

Alternatively, untreated *E. coli* biofilms and biofilms treated with daptomycin at a range of concentrations showed no difference in density of crystal violet staining (Figure 3.18 D). Daptomycin treated biofilms had the greatest density of crystal violet staining out of all other antimicrobial treatments against *E. coli* (Figure 18).

3.5.4.2.4 *P. aeruginosa* ATCC 27853

The lowest concentrations of gentamicin, rifampicin and daptomycin treatments used against *P. aeruginosa* ATCC 27853 biofilms had the greatest density of crystal violet retained on the pegs, and their corresponding untreated *P. aeruginosa* replicates (Figure 3.19 A, C, D). As the concentration of each antimicrobial increased the density of crystal violet decreased. Concentrations 2 – 32 μg/ mL of gentamicin (Figure 19 A), 32 μg/ mL – 256 μg/ mL of rifampicin (Figure 3.19 C) showed lower densities of crystal violet staining in comparison to their corresponding untreated biofilm pegs.

The density of crystal violet staining showed no correlation with the concentrations of mupirocin used against *P. aeruginosa* biofilms (Figure 3.19 B). A greater density of crystal violet staining was observed on one replicate at each concentration of mupirocin ranging from 64 – 512 μg/ mL. Concentrations 32 μg/ mL and 64 μg/ mL showed the greatest density of staining against treated and untreated *P. aeruginosa* biofilms.
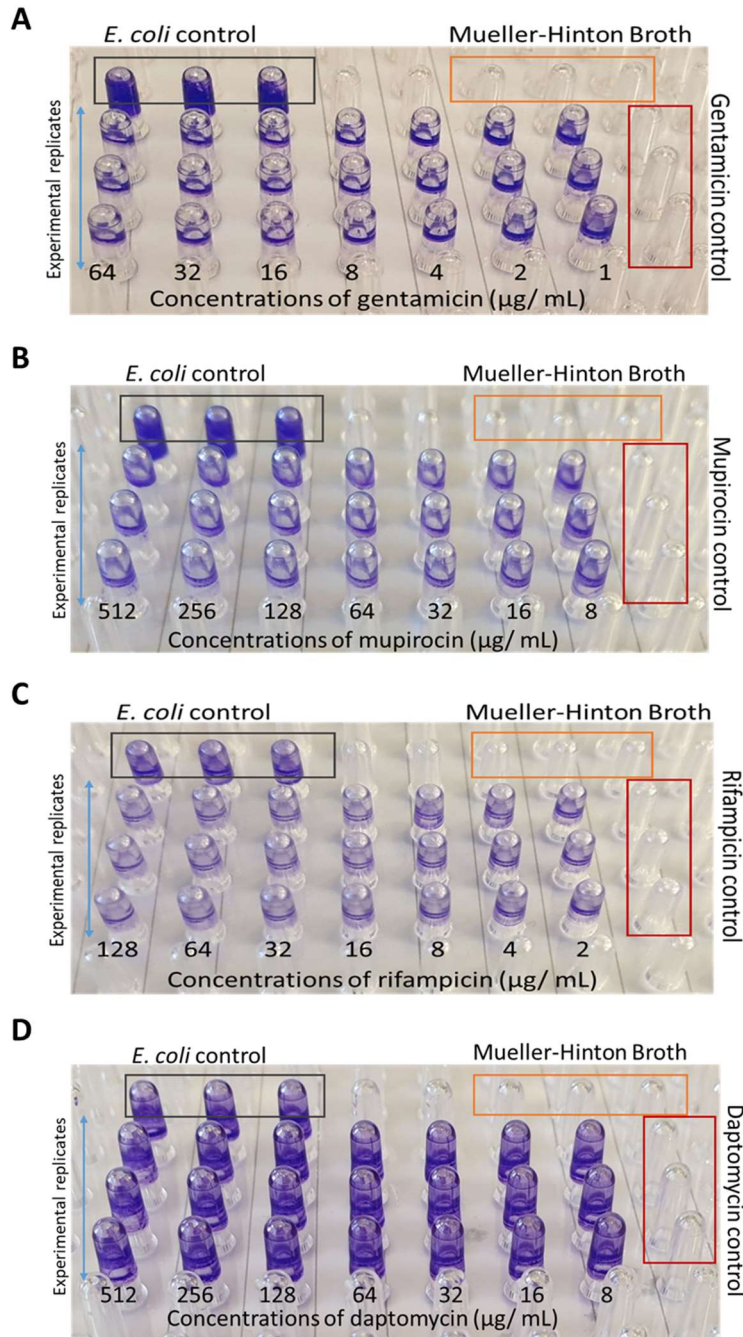
Figure 3.18 Density of twenty-four hour E. coli ATCC 35218 biofilms following treatment with a range of antimicrobials

Density of E. coli ATCC 35218 biofilms via crystal violet staining following treatment against a panel of antimicrobials: (A) gentamicin, (B) mupirocin, (C) rifampicin and (D) daptomycin at a concentration range of 1 – 512 μg/ mL. Controls of untreated E. coli, antimicrobial alone and MHB alone are highlighted on each image.
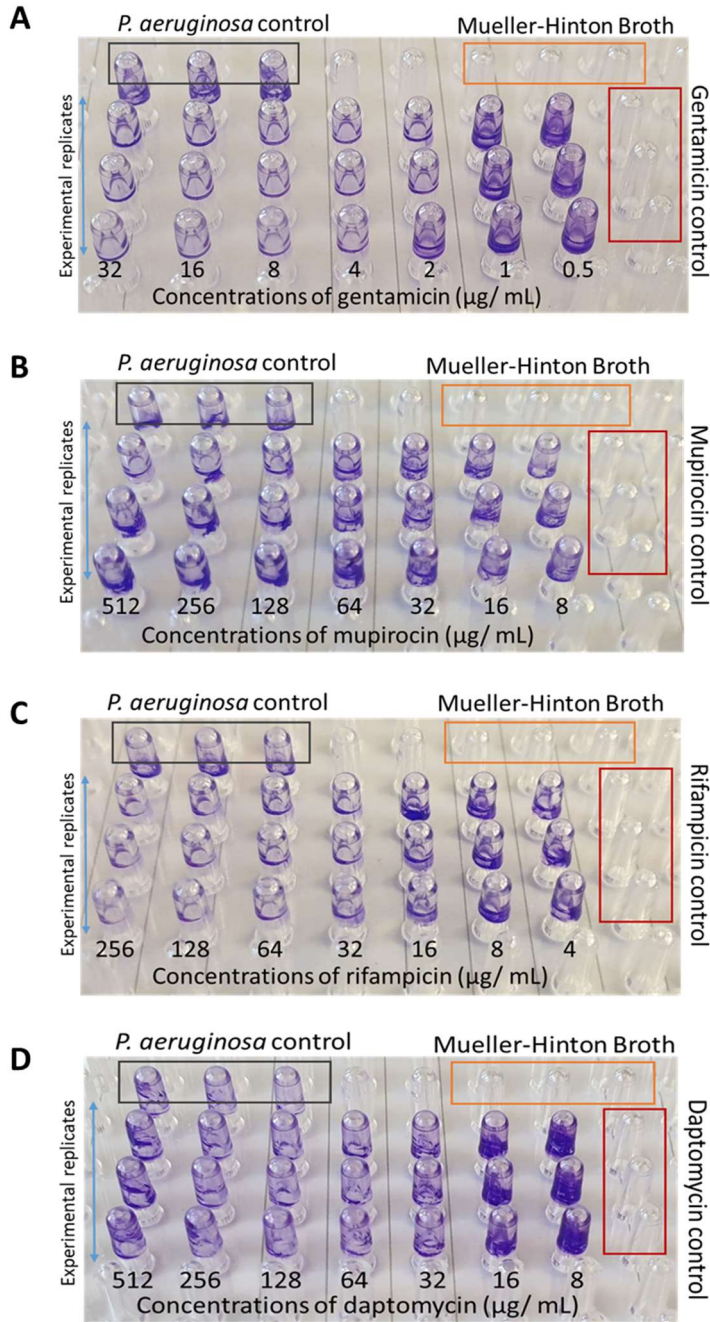
Figure 3.19 Density of twenty-four hour *P. aeruginosa* ATCC 27853 biofilms following treatment with a range of antimicrobials

Density of *P. aeruginosa* ATCC 27853 biofilms via crystal violet staining following treatment against a panel of antimicrobials: (A) gentamicin, (B) mupirocin, (C) rifampicin and (D) daptomycin at a concentration range of 0.5 – 512 µg/ mL. Controls of untreated *P. aeruginosa*, antimicrobial alone and MHB alone are highlighted on each image.

3.5.5 Gram-positive and Gram-negative biofilms after forty-eight hours of growth treated with XF-73 and gentamicin

3.5.5.1 Minimum inhibition and eradication concentrations of XF-73 and gentamicin against forty-eight hour Gram-positive and Gram-negative biofilms

MBIC and MBEC values for XF-73 against forty-eight hour Gram-positive biofilms are presented as a table (Table 3.6). Gentamicin MBIC and MBEC values against the panel of Gram-positive and Gram-negative biofilms after forty-eight hour growth are presented in a separate table (Table 3.7), corresponding XF-73 MBIC/ MBEC values were included for Gram-positive biofilms.

XF-73 was capable of inhibiting and eradicating biofilms of all Gam-positive microorganisms tested in this study (Table 3.6). Inhibition and eradication concentrations were recorded as the same for each microorganism. Low concentrations of XF-73 were capable of eradicating *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 at 1 µg/ mL, a slight increase in concentration was required to eradicate *E. faecalis* biofilms at 4 µg/ mL. In contrast, a greater concentration was necessary to eradicate *S. aureus* ATCC 6538 biofilms, at a 256-fold increase in concentration in comparison to the other staphylococcal biofilms.

In comparison to XF-73, gentamicin was also capable of eradicating *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 biofilms, although at a higher concentration (Table 3.7). A 4-fold increase in concentration was required to eradicate *S. aureus* ATCC 29213 biofilms and a 64-fold increase to eradicate *E. faecalis* biofilms.

P. aeruginosa ATCC 27853 in biofilm mode of growth was the only microorganism gentamicin was unable to eradicate, with an MBEC value of > 512 µg/ mL. Eradication of *E. coli* ATCC 35218 biofilms was recorded at 128 µg/ mL of gentamicin.

There are no corresponding XF-73 MBIC/ MBEC values against *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 35218 forty-eight hour biofilms due to inability of XF-73 to inhibit nor eradicate any Gram-negative biofilms at twenty-four hours of growth (Table 3.7).

Table 3.6 Collected MBIC and MBEC values (μg/ mL) of XF-73 against forty-eight hour Gram-positive biofilms.

Microorganism/ ATCC	XF-73 (μg/ mL)	
	MBIC	MBEC
S. aureus 6538	256	256
S. aureus 29213	1	1
S. epidermidis 12228	1	1
E. faecalis 29212	4	4

Table 3.7 Collected MBIC and MBEC values (μg/ mL) of XF-73 and gentamicin against forty-eight hour Gram-positive and Gram-negative biofilms.

Organism/ ATCC number	Gentamicin (μg/ mL)		XF-73 (μg/ mL)	
	MBIC	MBEC	MBIC	MBEC
S. aureus 29213	4	4	1	1
E. faecalis 29212	256	256	4	4
E. coli 35218	128	128	N/A	N/A
P. aeruginosa 27853	16	> 512	N/ A	N/A

Note: As E. coli and P. aeruginosa MBIC and MBEC values were > 512 μg/ mL for XF-73 at 24 hours, XF-73 testing at 48 hours was not applicable for these microorganisms

3.5.5.2 Biofilm density of forty-eight hour Gram-positive and Gram-negative microorganisms visualised through crystal violet staining following antimicrobial treatment

For each concentration of XF-73 used against forty-eight hour *S. aureus* ATCC 6538 biofilms, a decrease in crystal violet staining density was observed on these pegs in comparison to untreated *S. aureus* biofilms (Figure 3.20). A relatively similar level of crystal violet staining was observed between XF-73 ranging from 16 – 128 μg/ mL. Although, at higher concentrations: 256 μg/ mL and 512 μg/ mL of XF-73, crystal violet-stained pegs had a darker appearance when compared to the retained stain on other *S. aureus* pegs. Controls of XF-73 and MHB alone had no crystal violet stain present on any pegs.

There was an absence of crystal violet staining on all pegs involved in XF-73 and gentamicin treatments against *S. aureus* ATCC 29213 biofilms, including untreated *S. aureus* (Figure 3.21 A, B). Similarly, MHB, XF-73 and gentamicin controls showed no crystal violet staining.

Untreated *S. epidermidis* biofilms showed a retention of crystal violet, located as a purple band further up the microtitre pegs (Figure 3.22). With the addition of XF-73, a dramatic decrease in crystal violet

stain density was observed in comparison to untreated *S. epidermidis,* where only a faint purple band was present around the centre of the pegs. The greater the concentration of XF-73, the more crystal violet remained on the pegs. At 2 μg/ mL, no crystal violet was present on the pegs, along with control pegs of XF-73 and MHB.

A greater density of crystal violet staining was recorded on forty-eight hour *E. faecalis* biofilms treated with gentamicin, in comparison to XF-73, even though a greater concentration range of gentamicin was used in comparison to XF-73 (Figure 3.23 A, B). Crystal violet stained the middle of the untreated *E. faecalis* pegs in the gentamicin study, the addition of gentamicin reduced the retention of crystal violet on the treated *E. faecalis* pegs, where only a purple band was visible towards the top of the pegged microtitre lid (Figure 3.23 B). Between each gentamicin concentration the density of crystal violet retained on these treated pegs was comparable. In contrast, the greater the concentration of XF-73 used against *E. faecalis* biofilms, the less crystal violet remained on the treated pegs (Figure 3.23 A). Even at the lowest concentration of XF-73 at 0.5 μg/ mL, the amount of crystal violet retained on the replicate pegs was less than that on untreated *E. faecalis* pegs. The only pegs showing no crystal violet staining were MHB and antimicrobial alone on each treatment plate.

Crystal violet covered the entirety of untreated *E. coli* ATCC 35218 biofilms after forty-eight hours of growth, with a dark purple colouration recorded (Figure 3.24). Crystal violet was absent from pegs treated with gentamicin and MHB alone. Meanwhile the addition of gentamicin against these *E. coli* biofilms led to a marked decrease in the density of crystal violet staining when compared to untreated crystal violet presence. All biofilm pegs treated with gentamicin had a similar band of crystal violet stained material, located around the top of the pegs.

A relatively low density of crystal violet staining was observed near the top of replicate pegs of forty-eight hour old *P. aeruginosa* ATCC 27853 biofilms (Figure 3.25). Alternatively, with the addition of gentamicin at 512 μg/ mL a greater staining density was observed in comparison to the stain present on untreated biofilm pegs. The density of crystal violet staining decreased as the concentration of gentamicin treatments also decreased. The overall quantity of crystal violet on these pegs was less than the crystal violet stain on the untreated *P. aeruginosa* biofilms. Gentamicin at 128 μg/ mL led to the least amount of crystal violet retained on the microtitre pegs. Controls of gentamicin and MHB alone had no crystal violet present on the pegs.
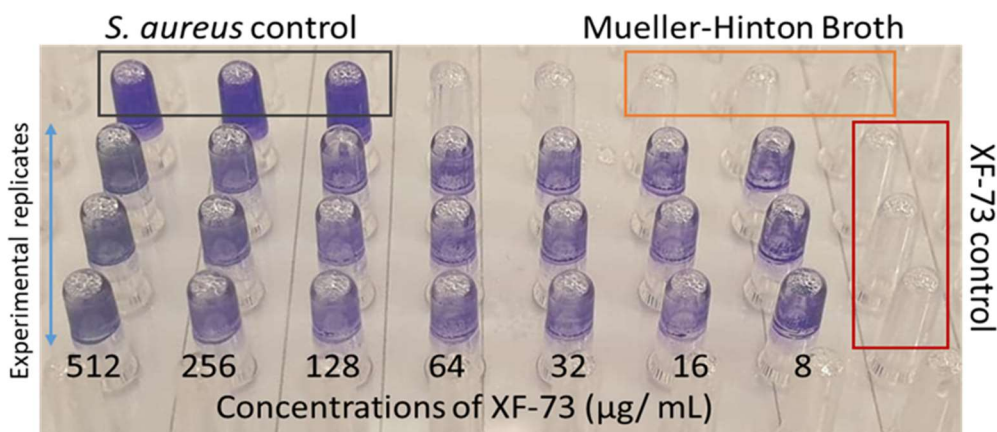
Figure 3.20 Density of forty-eight hour S. aureus ATCC 6538 biofilms following XF-73 treatment

Density of S. aureus ATCC 6538 forty-eight hour biofilms treated with XF-73 at a concentration range of: 8 – 512 µg/ mL. Controls of S. aureus alone, XF-73 alone and MHB alone are highlighted on the microtitre peg lid.
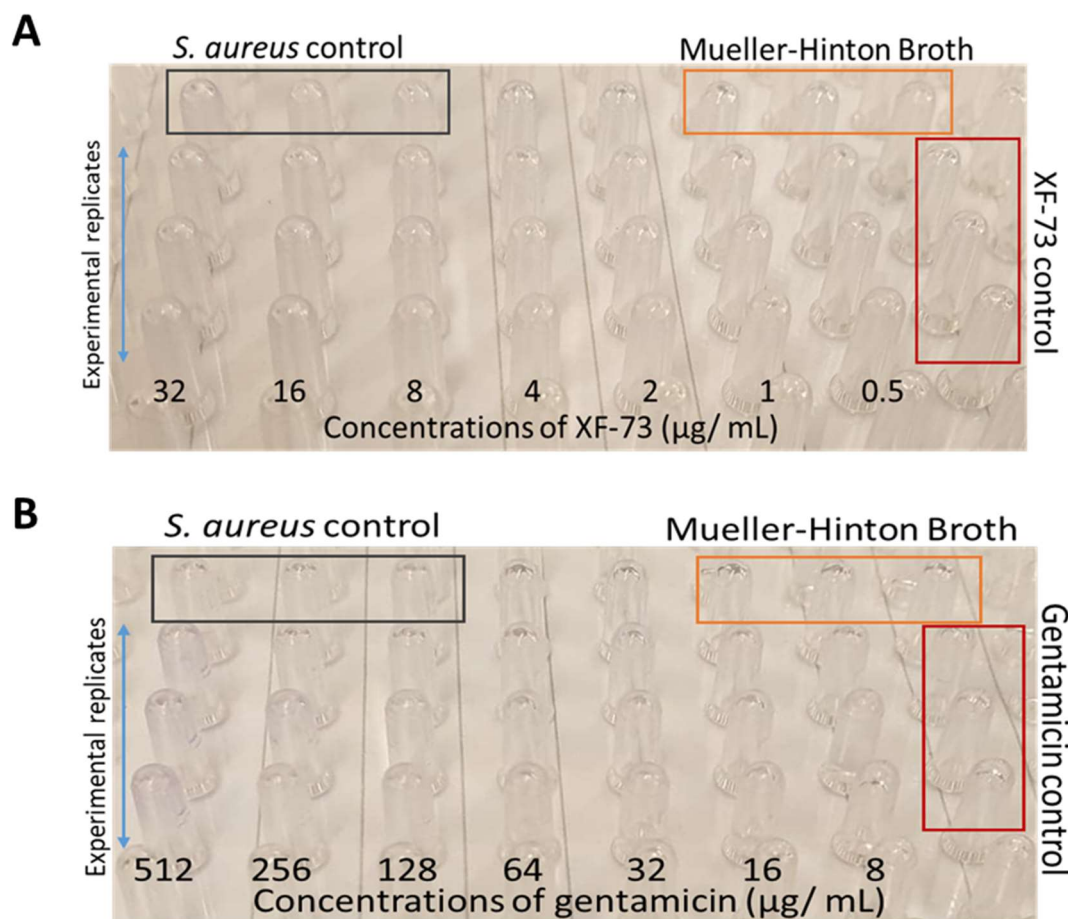
Figure 3.21 Density of forty-eight hour S. aureus ATCC 29213 biofilms following XF-73 and gentamicin treatments

Density of S. aureus ATCC 29213 forty-eight hour biofilms treated with (A) XF-73 at a concentration range of: 0.5 – 32 µg/ mL and (B) gentamicin at a concentration range of: 8 – 512 µg/ mL. Controls of S. aureus alone, MHB alone, XF-73 and gentamicin alone are highlighted on the microtitre peg lid.
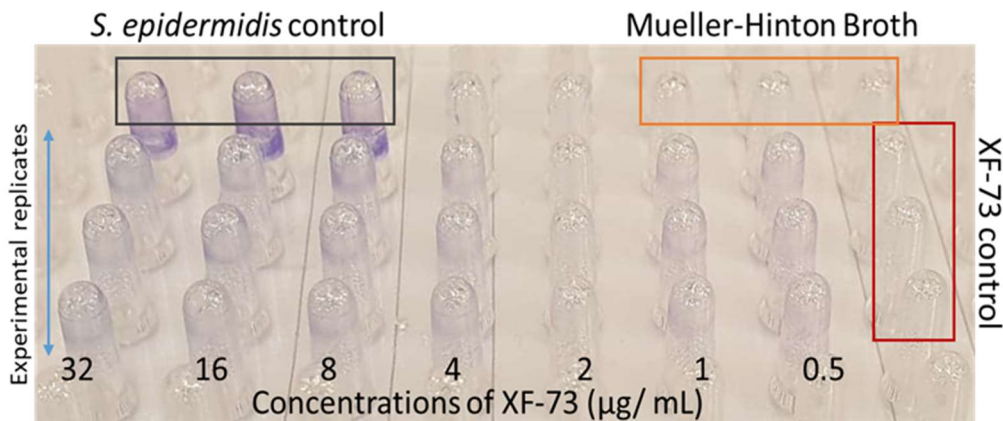
Figure 3.22 Density of forty-eight hour S. epidermidis ATCC 12228 biofilms following XF-73 treatment

Density of S. epidermidis ATCC 12228 forty-eight hour biofilms treated with XF-73 at a concentration range of: 0.5 – 32 µg/ mL. Controls of S. epidermidis alone, XF-73 alone and MHB alone are highlighted on the microtitre peg lid.
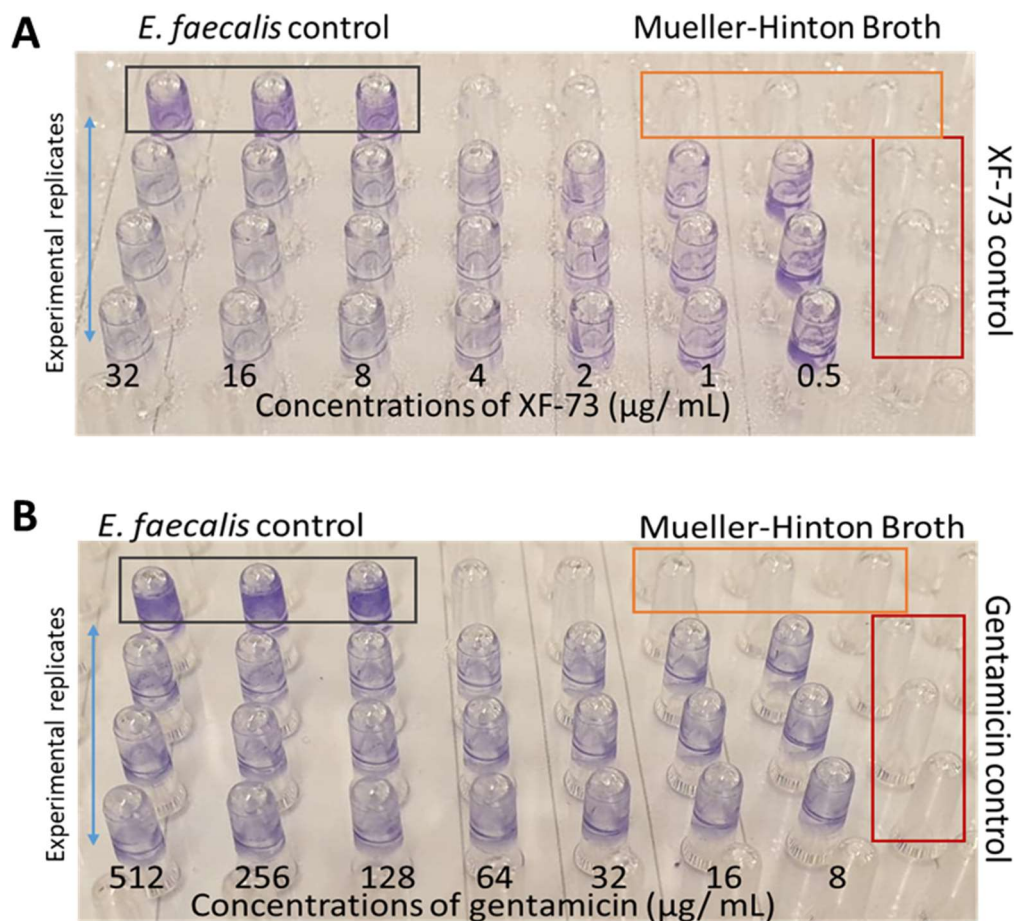
Figure 3.23 Density of forty-eight hour E. faecalis ATCC 29212 biofilms following XF-73 and gentamicin treatments

Density of E. faecalis ATCC 29212 forty-eight hour biofilms treated with (A) XF-73 at a concentration range of: 0.5 – 32 µg/ mL and (B) gentamicin at a concentration range of: 8 – 512 µg/ mL. Controls of E. faecalis alone, MHB alone, XF-73 and gentamicin alone are highlighted on the microtitre peg lid.
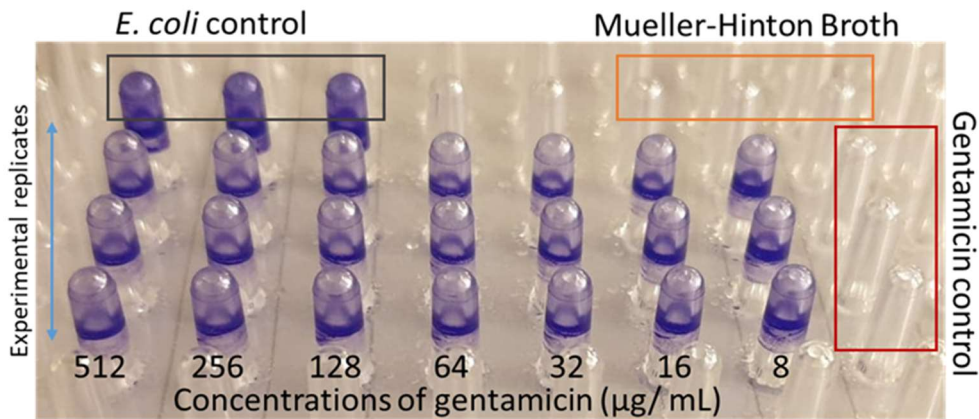
Figure 3.24 Density of forty-eight hour E. coli ATCC 35218 biofilms following gentamicin treatment

Density of E. coli ATCC 35218 forty-eight hour biofilms treated with gentamicin at a concentration range of: 8 – 512 µg/ mL. Controls of E. coli alone, gentamicin alone and MHB alone are highlighted on the microtitre peg lid.
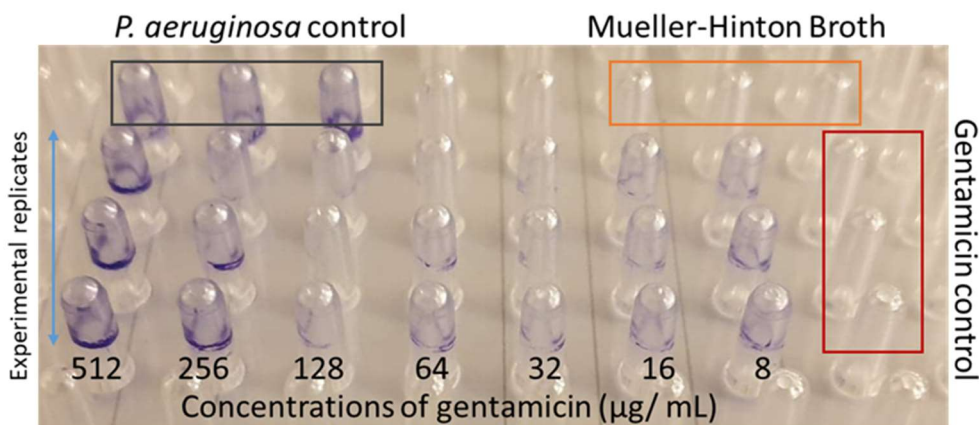
Figure 3.25 Density of forty-eight hour P. aeruginosa ATCC 27853 biofilms following gentamicin treatment

Density of P. aeruginosa ATCC 27853 forty-eight hour biofilms treated with gentamicin at a concentration range of: 8 µg/ mL – 512 µg/ mL. Controls of P. aeruginosa alone, gentamicin alone and MHB alone are highlighted on the microtitre peg lid.

3.6 Discussion

The aim of this study was achieved through the completion of each objective previously stated (3.2). A difference in biofilm load was determined between *S. aureus* and *E. coli* biofilms as an increase in viable cell numbers was recorded over the increased incubation time. There was a difference between microorganisms at each time-point, indicating Gram-positive and Gram-negative biofilms grow at different rates. The efficacy of XF-73 against a range of Gram-positive and Gram-negative biofilms was determined through MBIC/ MBEC assays and additional information about the antibacterial effect of XF-73 was collected through crystal violet staining and calculating the biofilm viability following XF-73 treatment. By including a panel of known antimicrobials into the study, the results gained from these antimicrobials act as a direct comparator to the antibacterial effect of XF-73. As a difference in biofilm load was recorded between twenty-four and forty-eight hours of incubation, further testing was conducted to assess XF-73 against older biofilms at forty-eight hours of growth via MBIC/ MBEC assays and crystal violet staining.

Initial biofilm testing highlighted that the longer a biofilm is incubated in a nutritional environment, the greater the viability. This was observed for both Gram-positive and Gram-negative microorganisms and statistical analysis concluded that the differences between each biofilm age were significant (Figure 3.1). These results illustrate the growth and maturation rates of Gram-positive and Gram-negative bacteria in biofilm mode of growth, the longer a biofilm is left undisturbed in an environment, and the bacterial cells grow and divide, increasing the biomass of the biofilm. Previous studies have also highlighted an increase in viability for Gram-positive and Gram-negative biofilms over different time periods. An increase in CFUs was recorded for *S. aureus* and *P. aeruginosa* biofilms after seventy-two hours of growth in comparison to twenty-four hours of growth, where a 1-log increase in CFUs was recorded between twenty-four and seventy-two hour *S. aureus* and *P. aeruginosa* biofilms (Chen *et al.*, 2020). Similarly, a 1-log increase in CFUs was also recorded between twenty-four and seventy-two hour *S. aureus* biofilms signifying that *S. aureus* biofilms grow and mature at a similar rate, a 2-log increase in viable cells was recorded for *E. coli* biofilms (Table 3.8), indicating that Gram-negative biofilms mature and grow at different rates.

Table 3.8 Viability of S. aureus and E. coli biofilms at a range of maturation ages

Biofilm viability (CFU/ mL) of S. aureus and E. coli biofilms after twenty-four, forty-eight and seventy-two hours of growth. Including 95% confidence interval of data sets.

Time Hours	*S. aureus* CFU/ mL (95% CI)	*E. coli* CFU/ mL (95% CI)
24	1.4×10^8 ($9.4 \times 10^7 - 1.8 \times 10^8$)	8.3×10^7 ($6.1 \times 10^7 - 1.1 \times 10^8$)
48	3.5×10^8 ($2.8 \times 10^8 - 4.2 \times 10^8$)	2.5×10^8 ($1.8 \times 10^8 - 3.2 \times 10^8$)
72	2.9×10^9 ($1.1 \times 10^9 - 4.7 \times 10^9$)	5.5×10^9 ($1.3 \times 10^9 - 9.7 \times 10^9$)

MBIC/ MBEC assays via the Calgary device was selected for this study, as this method was used in previous studies assessing the anti-biofilm effect of XF-73 (Ooi *et al.*, 2009a), therefore results collected from those studies and this current study can be directly compared, increasing the validity of the results. Additionally microtitre assays involving MBIC/ MBEC analysis is one of the most commonly used methods to determine the anti-biofilm properties of an antimicrobial (Kragh *et al.*, 2019). Throughout this study, XF-73 displayed a potent antibacterial effect against twenty-four hour Gram-positive biofilms; as MBIC = MBEC, the antibacterial effect was concluded to be bactericidal (Table 3.4). The same concentration of XF-73 was capable of eradicating all staphylococcal biofilms at 1 µg/ mL highlighting XF-73 exhibits a uniform effect against this species of biofilms. Similarly, only 2 µg/ mL of XF-73 was recorded to eradicate *E. faecalis* biofilms. Out of all antimicrobials used against the Gram-positive microorganisms *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212, XF-73 was the most effective. Mupirocin had no effect against either microorganism. Daptomycin was also ineffective against *E. faecalis* and rifampicin was only able to inhibit *E. faecalis* biofilm growth at the highest concentration used in this study (Table 3.5). The addition of known antimicrobials in this study aided in emphasising the efficient anti-biofilm effect of XF-73, as XF-73 was capable at eradicating Gram-positive biofilms at a lower concentration in comparison to the known antimicrobials. This illustrates the potential for XF-73 to be used as a treatment against clinically relevant biofilm infections involving Gram-positive microorganisms, in particular staphylococcal microorganisms.

These results are supported by previous research conducted on XF-73. Ooi *et al.*, (2009) also recorded an effective anti-biofilm effect against *S. aureus* biofilms, where 1 µg/ mL inhibited and 2 µg/ mL eradicated *S. aureus* SH1000 biofilms using the Calgary biofilm device. The study also added a wide range of comparator antimicrobials, to act as comparisons against the efficacy of XF-73, these antimicrobials included: gentamicin, mupirocin, rifampicin and daptomycin; therefore, these results collected in Ooi's study can be compared to the results collected from this current study. An inhibitory effect was recorded for each comparator, gentamicin had the same MBIC value as XF-73 at 1 µg/ mL whereas a 2-fold increase in concentration was required for daptomycin to inhibit biofilm growth. In contrast rifampicin and mupirocin had MBIC values less than XF-73 at 0.02 µg/ mL and 0.25 respectively µg/ mL. The comparator antimicrobials showed a greater inhibitory effect against *S. aureus* SH1000 in comparison to *S. aureus* ATCC 29213 (Table 3.5), signifying that variation in the anti-biofilm

effect of the antimicrobials vary between strains of *S. aureus.* In addition to varied inhibitory effects against *S. aureus* biofilms, the comparator antimicrobials were unable to eradicate *S. aureus* SH1000 biofilms with an MBEC value concluded as greater 256 µg/ mL (Ooi *et al.*, 2009a). Mupirocin also had a MBEC value greater than 256 µg/ mL against *S. aureus* ATCC 29213 biofilms but gentamicin, rifampicin and daptomycin all MBIC = MBEC values (Table 3.5). Though XF-73 was shown to retain its anti-staphylococcal effect against a range of staphylococcal strains from both studies, highlighting the efficient anti-biofilm effect of XF-73 showing the potential for XF-73 becoming a potent anti-biofilm treatment, with a superior bactericidal effect in comparison to current antimicrobials.

The results recorded in this study are also supported by additional recent research, focusing on the anti-biofilm effect of XF-73 against a range of staphylococcal biofilms. XF-73 once again had a recorded bactericidal effect against three strains of *S. aureus* including an MRSA strain and a *Staphylococcus hominis* (*S. hominis*) strain with MBEC values ranging from 2 – 8 µg/ mL (Board-Davies *et al.*, 2022). These concentrations are all greater than the MBEC values recorded for XF-73 against the staphylococcal strains used in this study (Table 3.4) with a maximum 8-fold increase in XF-73 concentration, signifying that relatively low concentrations (1 – 8 µg/ mL) of XF-73 is capable of eradicating a range of staphylococcal biofilms.

S. epidermidis ATCC 12228 has been identified as a strain of *S. epidermidis* that does not form a biofilm in an environment from the ATCC identification information (ATCC, 2022). Previous studies involving this strain of *S. epidermidis* support this statement and state that ATCC 12228 is a non-infection, non-biofilm forming strain (Zhang *et al.*, 2003). Results collected from this study clearly indicate a biofilm-forming capability for this strain of *S. epidermidis,* as biofilms were visualised during crystal violet staining, where a purple pigment was present on pegs, indicating bacterial adhesion and biofilm formation (Figure 3.14 C, 3.22). Biofilm viability was also recorded during viable cell counts (Figure 3.4). Microtitre pegged lids were immersed in PBS before any testing involving biofilm quantification occurred, therefore, planktonic cells were removed from the environment and only cells adhered to the pegs were tested. Similarly other studies have also confirmed that *S. epidermidis* ATCC 12228 can form a biofilm on abiotic surfaces and biofilm growth was determined on a range of surfaces including silicone and acrylic surfaces; though the biomass of *S. epidermidis* ATCC 12228 was less than the biomass of *S. epidermidis* ATCC 35984, a strain of *S. epidermidis* that is known to biofilm (Okajima *et al.*, 2006). Therefore *S. epidermidis* ATCC 12228 has a biofilm-forming capability although it is less efficient growing in a biofilm mode of growth in comparison to other *S. epidermidis* strains known to biofilm. Additional research further supported this as *S. epidermidis* ATCC 12228 was able to biofilm in certain conditions, for example in a platelet rich plasma environment, but at a lower density in comparison to *S. epidermidis* ATCC 35984 biofilms (Greco *et al.*, 2007).

Although XF-73 was shown to have no effect at inhibiting or eradicating any of the Gram-negative biofilms used in this study, similarly mupirocin, rifampicin and daptomycin were also unable to effect the Gram-negative biofilms (Table 3.4, 3.5). There is a lack in previous research focusing on the anti-biofilm effect of XF-73 against Gram-negative biofilms, though Board-Davies *et al.,* (2022) included a *P. aeruginosa*, *E. coli* and *A. baumannii* strains in their study and XF-73 showed no anti-biofilm capabilities against the biofilms with an MBEC value concluded as greater than 1024 µg/ mL (Board-Davies *et al.*, 2022). This result further highlights that XF-73 has limited antimicrobial action against Gram-negative biofilms, in comparison to Gram-positive biofilms and would not be suitable as a treatment strategy against clinical infections involving these microorganisms.

The capability for microorganisms to form a biofilm as a virulence mechanism offers protection to the bacterial cells from the external environment and to resist antibacterial treatments. Previous studies have highlighted the relationship with Gram-negative bacteria forming biofilms and antimicrobial resistance, e.g. *E. coli* biofilms having resistance against gentamicin and ceftazidime and *P. aeruginosa* having resistance to ciprofloxacin (Cepas *et al.*, 2019). The extracellular polymer matrix secreted by the bacterial cells in a biofilm is responsible for the increased level of antimicrobial resistance, as this slime layer offers an additional layer of protection, preventing antimicrobial treatments from entering the bacterial community (Steven *et al.*, 2015). Due to the structural differences between Gram-positive and Gram-negative planktonic cells, where Gram-negative cells contain an outer membrane which decreases the susceptibility of these microorganisms to many antibiotic treatments as they are unable to cross the outer membrane. Additional resistance mechanisms include efflux pumps to further prevent antibiotics entering the bacterial cells (Exner *et al.*, 2017). The resistance factors found in Gram-negative bacterial cells in planktonic mode of growth are also retained in biofilms, therefore, aiding in the increased resistance to antibiotic treatments in comparison to Gram-positive biofilms.

The results collected in this study correspond with previous research, highlighting that Gram-negative biofilms are less susceptible to antibacterial treatments, as XF-73 and the panel of known antimicrobials were less effective against Gram-negative biofilms in comparison to the Gram-positive biofilms tested.

As gentamicin was the only comparator antimicrobial capable of inhibiting the reduced panel of twenty-four hour biofilms and was recorded to eradicate both Gram-positive biofilms and *E. coli* ATCC 35218 (Table 3.5); gentamicin was therefore selected to be the direct comparator to XF-73 against forty-eight hour biofilms. Mupirocin, rifampicin and daptomycin were not used as comparators against forty-eight hour biofilms, as they showed weak anti-biofilm effects against the panel of twenty-four hour biofilms. Mupirocin lacked any anti-biofilm effect against any of the twenty-four biofilms, similarly rifampicin was unable to inhibit or eradicate either of the Gram-negative biofilms and could only inhibit *E. faecalis* ATCC 29212 biofilms. Daptomycin was only able to eradicate *S. aureus* ATCC 29212 biofilms. Initial testing concluded a significant difference in the viability of Gram-positive and Gram-negative biofilms

at different time points (Figure 3.1); therefore, as mupirocin, rifampicin and daptomycin showed a weak antibacterial effect against twenty-four hour biofilms, it was expected that these antimicrobials would be incapable of causing an anti-biofilm effect against older biofilms.

Though comparing the anti-biofilm effect of gentamicin against twenty-four and forty-eight hour *S. aureus* ATCC 29213 biofilms, gentamicin proved more effective against the older biofilms as a 2-fold decrease MBIC and MBEC values were recorded (Table 3.9). This highlights that the other comparator antimicrobials may have had a potential increased effect against forty-eight hour Gram-positive biofilms and future testing involving mupirocin, rifampicin and daptomycin against forty-eight hour would be required to prove this theory. This increased efficacy was not recorded between gentamicin treatments against forty-eight hour *E. faecalis* biofilms, where a 4-fold and 2-fold increase in gentamicin concentration was required to inhibit and eradicate the bacterial growth respectively.

XF-73 retained its bactericidal effect against all Gram-positive forty-eight hour biofilms; additional XF-73 retained the same killing effect against *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 forty-eight hour biofilms, as the same MBEC value of XF-73 was recorded for twenty-four and forty-eight hour biofilms (Table 3.9). Similarly, only a 2-fold increase in XF-73 concentration was recorded to eradicate forty-eight hour *E. faecalis* biofilms in comparison to eradicating twenty-four hour biofilms. In contrast, this efficacy of XF-73 was not retained against *S. aureus* ATCC 6538 biofilms and was the only Gram-positive microorganism where this trend was observed where a much greater concentration of the antimicrobial (256-fold increase) was required to eradicate the older biofilms (Table 3.9).

Previous research focusing on the antibacterial properties of XF-73 against bacterial biofilms have only ever focused on twenty-four hour biofilms; therefore these novel results collected offer an initial insight on the efficacy of XF-73 against older biofilms. These results also simulate a more realistic clinical biofilm infection as an established biofilm infection will have a greater abundance of bacterial cells.

Table 3.9 MBIC and MBEC values (µg/ mL) of XF-73 and gentamicin against twenty-four and forty-eight hour Gram-positive biofilms

| Microorganism/ ATCC | XF-73 (µg/ mL) | | | | Gentamicin (µg/ mL) | | | |
| | 24-hour biofilms | | 48-hour biofilms | | 24-hour biofilms | | 48-hour biofilms | |
	MBIC	MBEC	MBIC	MBEC	MBIC	MBEC	MBIC	MBEC
S. aureus 6538	1	1	256	256	N/A	N/A	N/A	N/A
S. aureus 29213	1	1	1	1	8	8	4	4
S. epidermidis 12228	1	1	1	1	N/A	N/A	N/A	N/A
E. faecalis 29212	2	2	4	4	64	128	256	256

Note: No gentamicin MBIC/ MBEC values recorded for S. aureus ATCC 6538 and S. epidermidis ATCC 12228 as these microorganisms were not selected for comparator testing.

Through serial dilutions and viable cell counts, the anti-biofilm effect of XF-73 and the panel of known antimicrobials was assessed through the changes in biofilm viability following treatment. Therefore, an in-depth look into the effect of the antimicrobial at specific concentrations can be assessed, an area that is not explored through MBIC and MBEC studies, which only focus on the outcome of inhibition or eradication of a biofilm. Although biofilm viability testing through the calculation of CFUs present in the biofilms are not without limitations, as only culturable cells are tested, therefore biofilm viability does not illustrate the full composition of bacterial biofilm cells as dormant or non-culturable cells are not included in the testing (Azeredo *et al.*, 2017).

Interestingly, the lowest concentrations of XF-73 leading to a biofilm viability reading below the lower limit of detection against each Gram-positive twenty-four hour biofilms (Table 3.10) were the same concentration as the corresponding MBEC values (Table 3.5). Therefore, the concentration of XF-73 that led to a biofilm viability below the lower limit of detection against Gram-positive biofilms can be concluded to have no viable cells. This trend was also recorded for daptomycin against *S. aureus* ATCC 29213 biofilms. Although concentrations 64 – 512 µg/ mL of daptomycin had viability readings below the lower limit of detection (Figure 3.11 D), the MBEC was determined to be > 512 µg/ mL (Table 3.5), the viabilities are therefore confirmed to have ≤ 10 cells.

Gentamicin had concentrations below the recorded MBIC/ MBEC value: 8 µg/ mL (Table 3.5), leading to biofilm viability readings below the lower limit of detection from 2 – 64 µg/ mL against twenty-four hour *S. aureus* ATCC 29213 biofilms (Figure 3.10 A). This indicated that concentrations of 2 µg/ mL and 4 µg/ mL of gentamicin had ≤ 10 viable cells present in the samples and concentrations 8 – 64 µg/ mL had no viable cells present. Similarly, gentamicin concentrations ranging from 16 – 128 µg/ mL had biofilm viability readings below the lower limit of detection for twenty-four hour *E. faecalis* ATCC 29212 biofilms (Figure 3.11 A), as the MBEC of gentamicin against *E. faecalis* biofilms was 128 µg/ mL (Table 3.5), concentrations 16 – 64 µg/ mL had ≤ 10 viable cells remaining in the sample and no viable cells were present in the biofilm sample following 128 µg/ mL.

Mupirocin at 512 µg/ mL led to a biofilm viability below the lower limit of detection against both Gram-positive biofilms; although the concentration of DMSO present in the mupirocin solution at 512 µg/ mL was determined to have an bactericidal effect against *S. aureus* ATCC 29213 biofilms and therefore the antibacterial effect is due to DMSO not mupirocin (Table 3.10). Whilst 512 µg/ mL of mupirocin against *E. faecalis* biofilms led to a viability reading below the limit of detection, the MBEC was concluded to be greater than 512 µg/ mL (Table 3.5), confirming that ≤ 10 cells were present in the biofilm samples.

Twenty-four hour *E. faecalis* biofilms had viable cells present following each concentration of rifampicin treatment (Figure 3.11 C). These results are supported by MBIC/ MBEC testing as the MBEC of rifampicin against *E. faecalis* biofilms was > 512 µg/ mL. In contrast, a fluctuation in rifampicin

concentrations led to *S. aureus* ATCC 29213 biofilm viability readings below the lower limit of detection at 32 μg/ mL, 64 μg/ mL, and 512 μg/ mL (Figure 3.10 C). Rifampicin at 512 μg/ mL had a concentration of DMSO present in the solution that was determined to have a bactericidal effect against *S. aureus* ATCC 29213 biofilms and therefore the antibacterial effect is due to DMSO not rifampicin (Table 3.5). However, through MBIC and MBEC analysis, 16 μg/ mL of rifampicin was shown to eradicate *S. aureus* biofilms (Table 3.5). Therefore, the bactericidal effect of rifampicin against *S. aureus* biofilms varies between experimental replicates.

XF-73 was the only antimicrobial capable of generating a biofilm viability below the lower limit of detection against *E. coli* ATCC 35218, similarly XF-73 also had a recorded biofilm viability below the lower limit of detection against *E. coli* ATCC 25922 at 256 μg/ mL and 128 μg/ mL respectively (Figure 3.6, 3.7). While no MBEC value recorded against either *E. coli* biofilms (Table 3.4), each concentration of XF-73 that led to a biofilm viability below the lower limit of detection therefore had ≤ 10 cells present in the biofilm samples. Indicating that whilst XF-73 was unable to eradicate *E. coli* biofilms, XF-73 as an anti-biofilm treatment still led to a 10-log and 7-log reduction in viable bacterial cells in *E. coli* ATCC 35218 and *E. coli* ATCC 25922 biofilms respectively.

Neither XF-73 nor the panel of antimicrobials were able to generate a biofilm viability below the lower limit of detection against *P. aeruginosa* ATCC 27853 biofilms (Table 3.10). XF-73 was capable of a 5-log reduction in biofilm viability, similarly a 7-log reduction was recorded for XF-73 against *P. aeruginosa* ATCC 15442 biofilms. These results indicate that a single dose treatment of XF-73 and the comparator antimicrobials were unable to fully eradicate *P. aeruginosa* biofilms. Thus, future testing involving *P. aeruginosa* biofilms retreated with antimicrobials could increase the anti-biofilm efficacy and potentially lead to eradication of biofilms. Whilst XF-73 was unable to fully inhibit or eradicate *E. coli* or *P. aeruginosa* biofilms, XF-73 was able to dramatically decrease the number of viable cells remaining in the Gram-negative biofilms in particular *E. coli* biofilms, therefore further testing involving retreatment may lead to an efficient killing effect of XF-73 against these biofilms.

Table 3.10 Lowest recorded concentration of XF-73 and comparator antimicrobials (µg/ mL) leading to a biofilm viability reading below the lower limit of detection against a panel of twenty-four hour Gram-positive and Gram-negative biofilms

Microorganism/ ATCC number	Lowest LLoD concentration (µg/ mL)				
	XF-73	Gentamicin	Mupirocin	Rifampicin	Daptomycin
S. aureus 6538	1	N/A	N/A	N/A	N/A
S. aureus 29213	1	2	512	32	32
S. epidermidis 12228	1	N/A	N/A	N/A	N/A
E. faecalis 29212	2	16	512	N/A	64
E. coli 35218	256	-	-	-	-
E. coli 25922	128	N/A	N/A	N/A	N/A
P. aeruginosa 15442	-	N/A	N/A	N/A	N/A
P. aeruginosa 27853	-	-	-	-	-

Note: - indicates that no concertation of XF-73 or gentamicin led to a biofilm viability reading below the lower limit of detection. N/A inputted to signify the antimicrobial treatment was not used against the microorganism. Red value highlights concentration of mupirocin where the concentration of DMSO present has a bactericidal effect against the microorganisms.

Another reason why rifampicin was selected as an antimicrobial comparator against XF-73 is due to its anti-biofilm effect. Rifampicin has the capability of penetrating *S. epidermidis* biofilms, this was recorded through diffusion bioassays and transmission electron microscopy, although cell death was not recorded, rifampicin was only able to act on *S. epidermidis* cells located along the edges of the biofilm (Zheng *et al*., 2002). These findings are supported by the results collected throughout this study (Table 3.5, 3.10), indicating that rifampicin alone is not an effective anti-biofilm treatment. Although, combination therapies involving rifampicin overcomes the inability of rifampicin eradicating bacterial cells in a biofilm; previous studies have shown rifampicin and Fosfomycin as a combination therapy leads to a viable treatment against MRSA biofilms with a recorded cure rate of 83% (Mihailescu *et al*., 2014). Previous studies have highlighted that combination therapies such as minocycline-rifampicin and chlorohexidine coated catheters have a greater efficacy in preventing biofilm colonisation of Gram-negative bacteria involved in central line blood steam infections i.e. *P. aeruginosa, E. coli* and *K. pneumoniae* in comparison to treatments of chlorhexidine or minocycline-rifampicin alone (Jamal *et al*., 2014). These results indicate that future testing involving combination therapies of rifampicin, XF-73 and gentamicin may increase the anti-biofilm properties of these antimicrobials.

Biofilm abundance was visualised by staining the microtitre pegged lids with crystal violet. The standard protocol for crystal violet staining biofilms involves eluting the stain and taking absorbance readings to compare the optical density of treated biofilms to untreated biofilms (Ebert *et al*., 2021). Although this method was inapplicable for this study, due to the red pigment present in XF-73 at interacting with the purple pigment of crystal violet. Therefore, absorbance readings are skewed for biofilms treated with higher concentrations of XF-73. This was visualised as a dark purple/ black pigment was present on bacterial biofilms treated with 256 – 512 µg/ mL of XF-73 (Figure 3.15, 3.20).

Through staining control pegs that were immersed in MHB alone or a mix of MHB and the antimicrobial treatment it was shown that the crystal violet stain did not stain the microtitre pegs, nor did the stain interact with XF-73 or MHB, therefore the presence of stained material on the microtitre pegs directly correlated with the density of biofilms present.

Both *S. aureus* ATCC 6538 and *E. faecalis* ATCC 29212 showed a reduction in crystal violet retention at concentrations above the MBEC (Table 3.4), biofilm viability testing also determined no viable cells present at these concentrations, > 1 µg/ mL and > 2 µg/ mL for *S. aureus* ATCC 6538 and *E. faecalis* ATCC 29212 respectively (Figure 3.2, 3.4). Therefore, the retained stain present on these treated pegs must represent dead bacterial cells still attached to the pegs. Signifying that XF-73 treatment did not always lead to the dispersal of biofilms following eradication. These results highlight a limitation to crystal violet staining, as there was no variation between live or dead cells following staining, as crystal violet does not differentiate from live or dead cells present on microtitre pegs. Crystal violet stains negatively charged molecules located on the bacterial cells and the extracellular polymer substances surrounding the biofilm (Ebert *et al*., 2021). Alternatively, biofilm eradication may not lead to the full removal of the extracellular polymer matrix, therefore, whilst cell death has occurred within the biofilm at concentrations of XF-73 above the MBEC and no living cells remain, crystal violet stains the extracellular polymer matrix that is still attached to the pegs, leading to a false positive result of biofilm presence following XF-73 treatment (Latka *et al*., 2020).

Whilst no concentration of XF-73 used against Gram-negative biofilms led to total inhibition or eradication, biofilm viability testing indicated a decrease in the total number of viable cells remaining in the biofilms following treatment (Figure 3.6 – 3.9). This reduced viability was recorded during crystal violet staining as less stain was retained on test pegs following XF-73 treatment at concentrations 128 – 512 µg/ mL (Figure 3.15). The reduced crystal violet retention observed on the treated pegs in comparison to the control pegs of untreated *E. coli* and *P. aeruginosa* growth illustrates that the bacterial biofilms had been compromised by XF-73, leading to bacterial shedding and a reduced biofilm size.

A greater density of crystal violet was retained on the growth control pegs of forty-eight hour Gram-positive or Gram-negative biofilms (Figure 3.20 – 3.25) in comparison to the corresponding twenty-four hour biofilm growth (Figure 3.14, 3.15, 3.17 A, 3.18 A, 3.19 A). These results visualise and support the initial research that an increased incubation time leads to a greater density of bacterial cells present in both Gram-positive and Gram-negative biofilms (Figure 3.1).

A lack of crystal violet stain was observed against twenty-four and forty-eight hour *S. aureus* ATCC 29213 biofilms, following antimicrobial treatment of each microorganism used in this study, and a lack of stain was also recorded on control growth of *S. aureus* alone (Figure 3.14 B, 3.16, 3.21). These results collected from crystal violet staining alone would indicate that *S. aureus* ATCC 29213 is unable to form biofilms due to the lack of stain present on the bacterial sample pegs, though this is proven to

be incorrect as untreated *S. aureus* ATCC 29213 had a biofilm viability of 6.5 x10^7 CFU/ mL (Figure 3.3). Additionally, a weak presence of crystal violet was recorded on *S. epidermidis* ATCC 12228 biofilms (Figure 3.14 C), though a greater density of crystal violet was observed on forty-eight hour *S. epidermidis* control biofilms. These results correspond with previous research signifying that this strain of *S. epidermidis* is a less effective at forming a biofilm (Okajima *et al.*, 2006), but given a greater incubation period, *S. epidermidis* ATCC 12228 can establish a more prominent biofilm, capable of being visualised following crystal violet staining. Though through the addition of biofilm viability testing, control growth of twenty-four hour *S. epidermidis* biofilms had a recorded viability greater than the viability of the other Gram-positive biofilm growth controls at 1.05 x10^9 CFU/ mL (Figure 3.2 – 3.5). Therefore *S. epidermis* ATCC 12228 exhibited a potent ability to effectively form biofilms that lead to efficient bacterial growth, highlighting that the ability of *S. epidermidis* ATCC 12228 to form biofilms varies between studies.

The visualisation of crystal violet staining is due to the binding between the positively charged crystal violet and negatively charged components located in the biofilm including: nucleic acids, proteins and polysaccharides. As the composition of biofilms vary between strain, it is possible that the composition of *S. aureus* ATCC 29212 biofilms differs from the composition of *S. aureus* ATCC 6538 biofilms, leading to a weak retention of crystal violet in *S. aureus* ATCC 29213 biofilms. Alternatively, if a biofilm increases production of polysaccharides as a defence mechanism against XF-73 treatment, a greater retention of crystal violet would be recorded, this would indicate that *S. epidermidis* ATCC 12228 and *S. aureus* ATCC 29213 do not have such mechanisms as no increase in crystal violet stain was recorded following any antimicrobial treatment (Latka *et al.*, 2020).

The benefit of running MBIC/ MBEC assays, biofilm viability analysis and crystal violet staining to visualise biofilm density in conjunction with one another, is that each experiment adds additional information the other experiments lack. Offering a more complete picture into the anti-biofilm effect XF-73 has against these microorganisms. If MBIC/ MBEC testing alone was conducted for XF-73 against Gram-negative biofilms, XF-73 would appear to have no effect against the twenty-four hour biofilms at the concentration range used throughout the study; though biofilm viability readings highlight that XF-73 is capable of greatly reducing the total volume of viable cells present in these biofilms. Similarly, crystal violet staining allowed the visualisation of the antibacterial effect of XF-73 against the range of Gram-positive and Gram-negative biofilms, supplementing the results gathered from MBIC/ MBEC testing and assessing the biofilm viability following XF-73 treatment.

The results collected from this study highlight the use XF-73 as an anti-biofilm treatment against clinically relevant Gram-positive biofilm infections. Roughly 75% of all bacterial infections are biofilm related, staphylococcal species in particular *S. aureus* are commonly located and responsible for a range of healthcare associated infections, locating on foreign objects: prosthetic joints, prosthetic heart

valves and central venous catheters (Ahmed *et al.*, 2015). XF-73 has a particularly strong anti-staphylococcal effect, meaning XF-73 has the capability of becoming an efficient antimicrobial treatment to target these staphylococcal biofilm infections.

A reduced panel of microorganisms was selected for this study, due to biofilm experiments taking a longer time compete compared to standard planktonic testing, therefore a reduced panel of microorganisms was required. Three technical replicates were included in each experiment focusing on the antibacterial effect of XF-73 and comparator antimicrobials against the panel of microorganisms to increase validity of results, although time restraints prevented the occurrence of experimental replicates.

Future testing involving experimental replicates would aid increasing the validity of the results and confirm the strong anti-bacterial effect of XF-73 against twenty-four and forty-eight hours Gram-positive biofilms. Increasing the panel of microorganisms used in this study would also expand on determining if XF-73 remains effective against a range of Gram-positive biofilms. Additionally, as a significant difference in biofilm viability was recorded between seventy-two, forty-eight and twenty-four hour biofilms (Figure 3.1), further studies assessing the antibacterial capability of XF-73 against older biofilms i.e. seventy-two hour Gram-positive and Gram-negative biofilms would aid in simulating the anti-biofilm properties of XF-73 against established biofilms that would have similar bacterial densities to actual clinical infections. Therefore these results would offer a realistic recorded effect of XF-73.

3.7 Conclusion

XF-73 exhibited an anti-biofilm capability against a range of eight Gram-positive and Gram-negative biofilms used in this study, highlighted through MBIC/ MBEC assays, and measuring the biofilm viability of each microorganism following treatment. Additional testing involving XF-73 was determined to be more efficient at eradicating Gram-positive biofilms, in particular staphylococcal strains and had MBEC values recorded at lower concentrations in comparison to a panel of current antimicrobials. Whilst XF-73 was unable to generate MBIC or MBEC values against Gram-negative biofilms, biofilm viability testing highlighted that XF-73 was still capable of dramatically reducing the total number of viable cells present in the biofilms. Crystal violet staining also illustrated this effect, showing a reduced abundance of *E. coli* and *P. aeruginosa* biofilms following XF-73 treatments at high concentrations.

Older biofilms are known to have a greater density of bacterial cells present, as preliminary studies confirmed a significant difference in biofilm abundance for Gram-positive and Gram-negative biofilms over twenty-four, forty-eight and seventy-two hours of growth. XF-73 retained its anti-biofilm properties against forty-eight hour Gram-positive biofilms and was more effective in eradicating the biofilms in comparison to gentamicin. Although forty-eight hour *S. aureus* ATCC 6538 biofilms required a high concentration of XF-73 to eradicate bacterial growth, signifying a variation in XF-73 treatments against different strains of *S. aureus* biofilms.

The results collected throughout this study indicate the potential application for XF-73 as an anti-biofilm treatment against clinically relevant staphylococcal biofilms, at different maturation ages. Further testing involving combination treatments of XF-73 and current anti-biofilm antimicrobials may increase the efficacy of XF-73 against Gram-negative biofilms.

4.0 Chapter 4: Activation of XF-73's secondary mechanism of action via blue light exposure and *in-vitro* assessment of its antimicrobial efficacy against clinically relevant microorganisms in planktonic and biofilm modes of growth

4.1 Introduction

In the race to combat and overcome antibiotic and multidrug resistant bacterial infections, treatment strategies used before the introduction of antibiotics are being revitalised. Photodynamic antibacterial therapy is a promising approach for dealing with resistant bacterial infections, including those associated with biofilms. Bacteria are less likely to develop resistance to PDT because no specific target interaction is required between a PS and bacteria. The methodology of PDT involves the activation of a PS through excitation via a specific light source, generation of ROS, which in turn cause oxidative damage to all surrounding biomolecules depending on location of ROS. Current clinical use of antibacterial PDT is focused on localised refractory diseases, such as oral infections. The increased usage of PDT against these treatments will aid in the decrease in AMR development; and by targeting localised infections with PDT this treatment strategy will help reduce the risk of the infection spreading and becoming systemic (Wainwright *et al.*, 2017; Jia *et al.*, 2019).

Localising ROS around the cell membrane of a microorganism damages the cell's permeability barrier, leading to an ionic imbalance which interrupts homeostasis. Therefore, designing a delivery strategy for PSs to receptors found on the bacterial cell wall is a desirable target for PDT. One potential location is extracellular haemin receptors that are commonly found on many bacterial pathogens, including *S. aureus* (Tripathi *et al.*, 2022). Targeting hemin receptors on antimicrobials for antimicrobial PDT was utilised through the use of gallium protoporphyrin IX, a PS and a hemin analogue, encapsulated in haemoglobin and mounted on silver nanoparticles. Following administration of the nanoparticles to MRSA and *S. aureus,* eradication of microorganisms was recorded following ten seconds of irradiation from LED light (Morales-De-Echegaray *et al.*, 2020).

PDT against biofilm infections is able to overcome current issues faced by antimicrobial treatments, such as the inability to effectively penetrate extracellular matrix. ROS produced through the excitation of PSs have the capability to damage the extracellular matrix through oxidative destruction of excreted products responsible for the formation of the extracellular polymer matrix (Jia *et al.*, 2019). Clinical trials are currently underway with a range of PSs against bacterial infections e.g. phenothiazinium compounds: methylene blue, toluidine blue O and PPA-904 (3,7-bis(di-n-butylamino)phenothiazin-5-ium bromide), which all have a broad spectrum of antibacterial activity, including MRSA skin and surgical site infections, diabetic ulcers and wounds including burn wounds; ALA-PPIX (5-aminolevulinic acid induced protoporphyrin IX) has a narrow spectrum of activity targeting different forms of acne,

chronic skin ulcers and *P. aeruginosa* infections (Wainwright *et al.*, 2017). Studies have also demonstrated the efficacy of PDT against biofilm infections in the oral cavity where methylene blue, toluidine blue O, curcumin and photogem were shown to be highly effective PSs against cariogenic biofilms, responsible for the loss of enamel and tooth cavities (Italo *et al.*, 2021).

Maisch *et al.,* (2007) has reported the efficient antibacterial activity of photo-activated XF-73 against methicillin-resistant and methicillin-sensitive *S. aureus* strains, highlighting XF-73 as a possible treatment for superficial skin infections. The study was conducted using an *ex-vivo* porcine skin model, and a reduction in viable bacteria was recorded with the addition of XF-73 followed by light activation, with a 3-log reduction in viability recorded at 10 µM (7.66 µg/ mL) of XF-73. A further reduction was recorded when XF-73 was pre-incubated with MRSA before photo-activated XF-73 treatment (Maisch *et al.*, 2007). Additional studies have further shown photo-activated XF-73's capability of killing a range of staphylococcal strains, where only five minutes of light exposure led to a recorded 3-log to 5-log reduction CFU/ mL in viable bacterial cells. Photo-activated XF-73 was also shown to have little direct effect on skin cells, further supporting its capability as a treatment against skin infections (Maisch *et al.*, 2005). XF-73 also has the capability to be an effective PS against *C. albicans* in both planktonic and biofilm modes of growth. Incubation with 0.5 µM (0.383 µg/ mL) of XF-73 with fifteen minutes exposure to blue light led to over a 6-log decrease in planktonic *C. albicans* planktonic cells and after one hour exposure to blue light, XF-73 at 1 µM (0.766 µg/ mL) led to a 5-log reduction in biofilm viability. In this study XF-73 was concluded to be more effective against *C. albicans* both as planktonic cells and in biofilms in comparison to TMPyP, a known PS capable of eradicating planktonic *C. albicans* (Gonzales *et al.*, 2013)*.

4.2 Aims of the study

The aim of this study was to assess the antibacterial efficacy of the secondary mechanism action of XF-73, activated through blue light (420 nm) exposure, against the same panel of clinically relevant Gram-positive and Gram-negative microorganisms in planktonic and biofilm mode of growth.

This study was completed through following these objectives:

- Assess the direct phototoxic effect of blue light on Gram-positive and Gram-negative microorganisms.
- Determine the optimum exposure time of blue light on XF-73 to enhance its antimicrobial effect against Gram-positive and Gram-negative microorganisms following fifteen and thirty minute's exposure.
- Assess the efficacy of PDT on enhancing the antimicrobial activity of XF-73 via MIC/ MBC assays.
- Assess the antimicrobial efficacy of blue light activated XF-73 against Gram-positive and Gram-negative biofilms after twenty-four hour biofilm growth.
- Determine the efficacy of blue light activated XF-73 against Gram-positive microorganisms after forty-eight hours of growth.

4.3 Materials

4.3.1 Microbial cultures

The same panel of ATCC microorganisms selected for biofilm studies in natural light (Table 3.1) was selected for this study to assess the enhancement of XF-73 through blue light activation. A reduced panel of microorganisms was adopted for forty-eight hour biofilm testing, focusing only on the Gram-positive microorganisms.

4.3.2 Microbial media and agar

MHB and MHA were used for this study. MHB was used to grow overnight microbial cultures and for conducting MIC and MBIC studies. MHA was used to determine the MBC for planktonic testing and MBEC's for biofilm studies. Both MHB and MHA was prepared following the previous methods: 2.4.1.1, 2.4.1.2.

4.3.3 XF-73 in aqueous stock solution

XF-73 in aqueous solution was the only antimicrobial used for this study. Due to the panel of known antibiotics previously used: gentamicin, mupirocin, rifampicin and daptomycin lacking the capability to be enhanced by blue light. Aqueous stocks of XF-73 were prepared following the previously described method: 2.4.2.

4.3.4 Crystal violet

Crystal violet (Merck) was used to stain biofilms. Crystal violet was made following manufacturer's instruction, where solid crystal violet was dissolved in distilled water to give a final concentration of 0.06% (w/v).

4.3.5 Phosphate buffered saline

Phosphate buffered saline (PBS) (Oxoid Ltd) was prepared in accordance with the manufacturer's instructions of diluting 1 PBS tablet in 200 mL of distilled water. Sterilised PBS solution was stored at room temperature until required.

4.3.6 Specialist equipment

Optical density (OD) readings of microbial growth were taken using a spectrophotometer (Jenway Geneva Plus UV).

A microtitre plate spectrophotometer (Biotech EL808) was used to record the OD readings throughout microbial growth experiments.

A Waldmann Therapy System Series light box (Herbert Waldmann GmbH & Co) fitted with two blue (V 36W) light tubes emitting blue light: 380 – 480 nm (with a maximum of 420 nm) was used during photo-activation experiments.

Light intensity at the surface of the light box was measured at intervals using a Waldmann Variocontrol light meter to ensure that reproducible illumination was achieved throughout the project. Light intensity readings taken from around the surface of the light box were used to calculate the average light dose. The light meter determined that the average light dose from the specific light box used for this study was 13.7 J/cm^2. This value was within the light dose range (12.1 – 14.0 J/cm^2) used in previous studies (Gonzales *et al.*, 2013; Board-Davies *et al.*, 2022).

4.4 Methods

4.4.1 Preliminary blue light study assessing the phototoxic effect of blue light alone against *S. aureus* and *E. coli*

Initial testing was conducted to determine the antimicrobial efficacy of blue light (420 nm) on Gram-positive and Gram-negative microorganisms. *S. aureus* ATCC 6538 and *E. coli* ATCC 35218 were the test microorganisms selected for this study.

Overnight cultures of the test organisms were diluted to a final OD of 0.01 at 570 nm in MHB (*S. aureus*: 10^6 CFU/ mL, *E. coli*: 10^7 CFU/mL), followed by incubation for two hours at 37°C in aerobic conditions to reach log phase growth. A 100 µL volume of each test organism was added to the wells of a flat bottom 96 well microtitre plate. For each microorganism, two flat bottom microtitre plates were used each with sixteen replicates: one for a control which was left to grow in natural light, and one placed on the Waldmann Therapy blue light box (4.3.6) with OD readings (at 570 nm) taken every five minutes using a microtitre plate spectrophotometer.

Following exposure, streak plates on MHA were completed to check for cell viability and purity of each organism and incubated overnight at 37°C in aerobic conditions.

4.4.2 Photo-activated XF-73 against *S. aureus* and *E. coli* in log phase of growth

4.4.2.1 *S. aureus* ATCC 6538 inoculum

An overnight culture of *S. aureus* was diluted to give a final OD of 0.01 in MHB (10^6 CFU/ mL). The inoculum was then incubated at 37°C in aerobic conditions for two hours to reach log phase growth. A 90 µL volume of inoculum was treated with 10 µL of XF-73 (Batch 1) at 2.5 µg/ mL to give a final concentration of 0.25 µg/ mL, in flat bottom 96 well microtitre plates with twelve replicates. A duplicate plate set up as a control was left to grow in natural light. The test plate was exposed to blue light for thirty minutes with ODs taken every five minutes at 570nm using a microtitre plate spectrophotometer.

Samples were streaked on MHA plates after exposure to check for viability of *S. aureus* present in the well and was incubated overnight at 37°C in aerobic conditions.

4.4.2.2 *E. coli* ATCC 35218 inoculum

An overnight *E. coli* culture diluted to a final concentration of OD 0.01 in MHB (10^7 CFU/ mL), was incubated for two hours at 37°C in aerobic conditions to reach log phase growth. A 90 µL volume of inoculum was treated with 10 µL of XF-73 at 1024 µg/ mL to give a final concentration of 102.4 µg/ mL, in a flat bottom 96 well microtitre plates with twelve replicates. A duplicate plate was used as a control plate left to grow in natural light, while the test plate was exposed to blue light for one hour with ODs taken every five minutes at 570nm using a microtitre plate spectrophotometer.

Following exposure test wells from the 96 microtitre plates were streaked on MHA plates to check for viability and purity of organisms present in the well and incubated overnight at 37°C in aerobic conditions.

4.4.3 Varying blue light exposure of XF-73 against *S. aureus* in log phase growth

4.4.3.1 Thirty minutes blue light exposure

An overnight culture of *S. aureus* ATCC 6538 was diluted in MHB to give an inoculum at an OD of 0.01 at 570 nm. The inoculum was then incubated for two hours to reach log phase growth. Ninety microliters of inoculum were treated with 10 µL of XF-73 at 2.5 µg/ mL to give a final concentration of 0.25 µg/ mL, in flat bottom 96 well microtitre plates. These plates were then exposed to blue light for thirty minutes, and ODs taken every ten minutes at 570 nm. Following exposure, microtitre plates were incubated at 37°C in aerobic conditions and ODs taken every 30 minutes for four and a half hours using a microtitre plate spectrophotometer (Biotech EL808). A control plate was set up with the same volumes of inoculum and XF-73 and was left to grow in natural light for thirty minutes before incubation, with readings taken at the same intervals. Each plate contained twelve replicates of XF-73 treatment and *S. aureus* alone.

Samples were streaked on MHA plates after the initial experiment, to check for cell viability and incubated overnight at 37°C in aerobic conditions. The microtitre plates were incubated overnight and after a total of twenty-four hours, samples were then streaked on MHA once more to compare cell viability to the MHA plates streaked after the five-hour initial study.

4.4.3.2 Fifteen minutes blue light exposure

The experiment was conducted as previously stated (4.4.3.1), with the alteration of decreasing blue light exposure to XF-73 from thirty minutes to fifteen minutes.

4.4.4 Minimum inhibitory and bactericidal concentrations of photo-activated XF-73 against Gram-positive and Gram-negative microorganisms

MIC experiments were completed following the same method previously stated (2.4.7.1). A lower concentration range of XF-73 was selected for batch 1 XF-73 against Gram-positive microorganisms at: 0.03 – 16 µg/ mL; whereas batch 2 XF-73 used the original concentration range: 0.125 – 64 µg/ mL.

Before incubation, the microtitre plates were exposed to blue light for fifteen minutes at room temperature and then incubated overnight at 37°C at 180 rpm in aerobic conditions. MBCs were conducted following the same method as previously described (2.4.7.2).

4.4.5 Activity of photo-activated XF-73 against twenty-four hour Gram-positive and Gram-negative biofilms

Twenty-four hour biofilms (3.4.1.1) were selected for initial analysis of blue light activated XF-73 (Batch 2) against Gram-positive and Gram-negative microorganisms. Where treatment was conducted following the previously mentioned method (3.4.2).

XF-73 concentrations varied depending on the test microorganism. Gram-positive microorganisms had an XF-73 concentration range of 0.125 µg/ mL – 64 µg/ mL. An increased concentration range was used against all Gram-negative microorganisms at 16 µg/ mL – 512 µg/ mL.

4.4.5.1 Gram-positive biofilms

Following the introduction of the microtitre lids containing twenty-four hour biofilms into challenge plates consisting of XF-73 (3.4.2), the microtitre plates were then placed on the light box (4.3.6) and exposed to blue light for 15 minutes at room temperature. Plates were incubated overnight at 37°C at 180 rpm in aerobic conditions.

4.4.5.2 Gram-negative biofilms

Following twenty-four hour biofilm formation, microtitre biofilm lids were placed into XF-73 challenge plates (3.4.2). The microtitre plates were then exposed to blue light for 15 minutes at room temperature and then incubated overnight at 37°C at 180 rpm in aerobic conditions.

4.4.5.2.1 Gram-negative biofilms with photo-activated XF-73 and XF-73 retreatment

An additional treatment after blue light exposure (4.4.5.2) was undertaken. The microtitre peg lids were transferred into a new challenge plate containing the same concentrations of XF-73 that had not been exposed to blue light. These plates were then incubated overnight at 37°C at 180 rpm in aerobic conditions.

4.4.5.3 Biofilm analysis following blue light activated XF-73 treatment

Peg lids containing treated biofilms were removed from the challenge plates and washed in PBS following the previously mentioned method (3.4.1.2), before being transferred into one of the following tests to analyse the biofilm. MBIC/ MBEC assays (3.4.3.1), biofilm viability testing (3.4.3.2) and crystal violet staining (3.4.3.3) were all conducted following the same method previously stated using XF-73 treatments in natural light.

4.4.6 Photo-activated XF-73 against forty-eight hour Gram-positive and Gram-negative biofilms

A further study was conducted, that assessed the efficacy of blue light activated XF-73 against forty-eight hour Gram-positive biofilms. Each microorganism was left to grow to a forty-eight hour biofilm

(3.4.1.2) before blue light activated XF-73 treatment (4.4.5.1). A higher concentration range of XF-73 was selected for this study: *S. aureus* ATCC 6538 XF-73 treatments ranged from 8 µg/ mL - 512 µg/ mL, for the remaining Gram-positive microorganisms XF-73 had a lower concentration range: 0.5 µg/ mL – 32 µg/ mL.

4.4.6.1 Analysis of forty-eight hour biofilms treated with blue light activated XF-73

To analyse the effect of blue light activated XF-73 against forty-eight hour biofilms, MBIC/ MBEC assays and crystal violet staining were selected to use in this study (3.4.3.1, 3.4.3.3).

4.4.7 Statistical analysis

Statistical analysis was completed using GraphPad Prism (Version 7). Normality testing was conducted via a Shapiro-Wilk normality test on end point absorbance readings taken throughout the study (4.4.1, 4.42 & 4.4.3), followed by an unpaired t-test with Welch's correction to test for significance between absorbance readings of *S. aureus* and *E. coli* in natural and blue light (4.4.1) and the effect of XF-73 against *S. aureus* and *E. coli* at log phase growth in the presence and absence of blue light (4.4.2). A one-way ANOVA with Tukey's multiple comparison *post-hoc* test was used to determine the significance of varying blue light exposure of XF-73 against *S. aureus* in log phase growth (4.4.3).

To analyse data collected from biofilm viability (4.4.6), all viable cell counts were log_{10} transformed before statistical analysis was conducted. Shapiro-Wilk normality testing was initially used to ensure the log transformed data sets were normally distributed. A one-way ANOVA, with Tukey's multiple comparison *post-hoc* test was then conducted to determine any significant differences in biofilm viability between treated and untreated data sets.

4.5 Results

4.5.1 Phototoxic effect of blue light alone against *S. aureus* and *E. coli*

Absorbance readings of *S. aureus* ATCC 6538 (Figure 4.1) and *E. coli* ATCC 35218 (Figure 4.2) in log phase growth in the presence and absence of blue light are presented as line graphs to visualise the effect of blue light alone on the microorganisms.

4.5.1.1 *S. aureus* exposed to blue light

Throughout the first twenty-five minutes of the study *S. aureus* in natural and blue light exhibited similar absorbance readings, at thirty minutes there was only a 0.001 absorbance unit difference between the two treatments (Figure 4.1). For the remaining thirty minutes *S. aureus* treated with blue light showed only a slight increase in absorbance readings while *S. aureus* in natural light continued to increase in a gradual incline. After one hour in the presence and absence of blue light a significant difference was determined between the two *S. aureus* cultures ($p = <0.0001$).

Samples plated on MHA showed confluent growth for *S. aureus* in the presence and absence of blue light.

4.5.1.2 *E. coli* exposed to blue light

E. coli in the presence and absence of blue light showed similar absorbance readings throughout the entirety of the time study (Figure 4.2). The difference in recorded absorbances at the end of the hour study between both treatments was 0.005 nm and statistical analysis of the data sets determined no significant difference ($p > 0.05$).

After the time course study confluent growth was then observed on MHA for *E. coli* in natural and blue light.
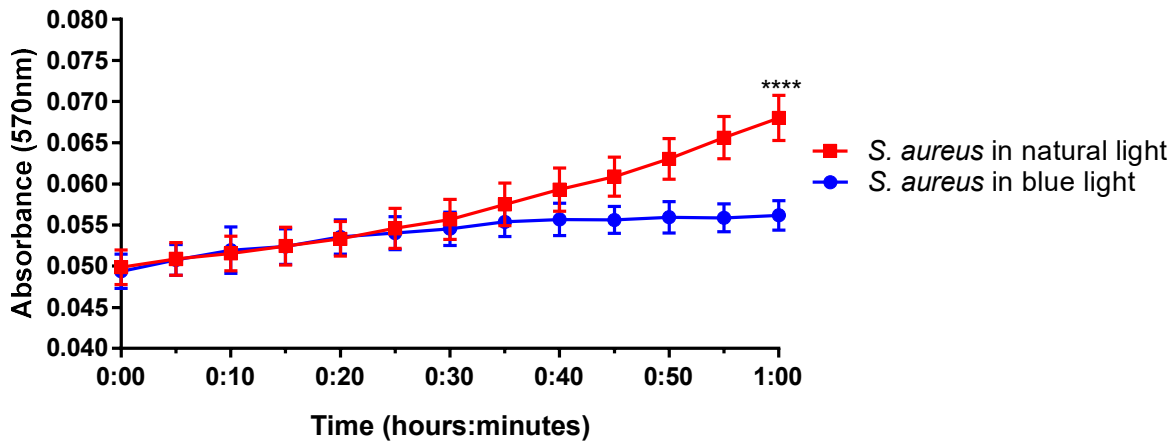
Figure 4.1 Growth of S. aureus ATCC 6538 in log phase growth in the presence and absence of blue light

Absorbance readings (570 nm) of S. aureus ATCC 6538 in log phase growth in the presence and absence of blue light for one hour. Absorbance readings taken every five minutes. Error bars generated from the standard deviation of the sixteen replicates. End point statistical analysis was completed via an unpaired t test with Welch's correction between 1 hour absorbance readings of S. aureus in natural and blue light, where **** p= <0.0001.
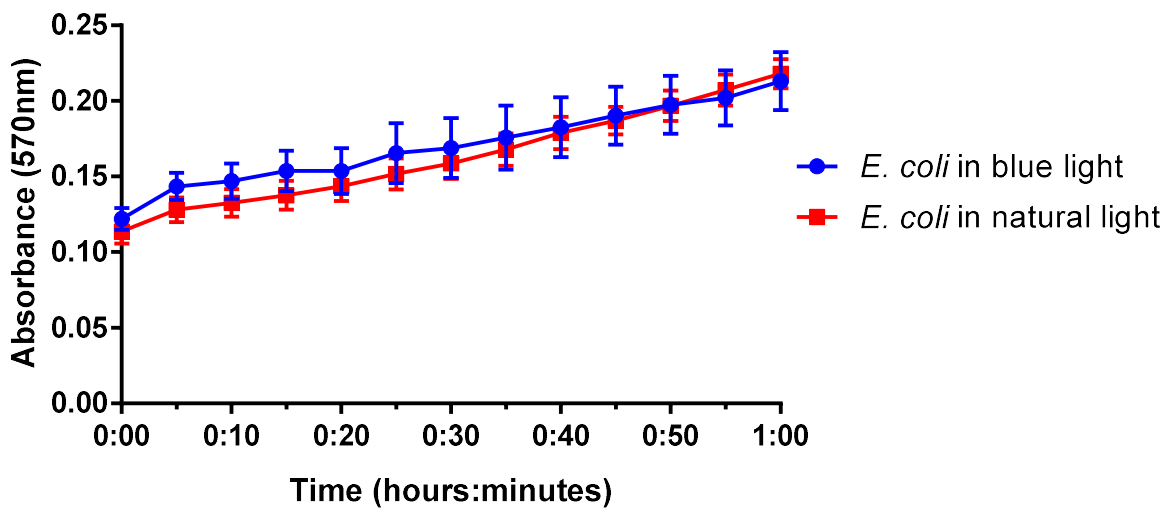
Figure 4.2 Growth of E. coli ATCC 35218 in log phase growth in the presence and absence of blue light

Absorbance readings (570 nm) of E. coli ATCC 35218 in log phase growth in the presence and absence of blue light for one hour. Absorbance readings taken every five minutes. Error bars generated from the standard deviation of the sixteen replicates.

4.5.2 Photo-activated XF-73 against *S. aureus* and *E. coli* in log phase of growth

Line charts were generated from absorbance readings of *S. aureus* ATCC 6538 (Figure 4.3) and *E. coli* (Figure 4.4) treated with XF-73 in the presence and absence of blue light to visualise the difference in absorbance readings between these two treatments.

4.5.2.1 *S. aureus* ATCC 6538 in log phase growth

Over the recorded time periods in this study, *S. aureus,* when treated with XF-73 in both the presence and absence of blue light had lower absorbance readings compared to corresponding growth controls of untreated *S. aureus* (Figure 4.3).

The growth control of *S. aureus* in natural light showed a distinct increase in absorbance readings after the first five minutes of the study and continued for the remaining time frame (Figure 4.3 C). In contrast blue light exposed *S. aureus* only showed a slight increase in absorbance readings throughout the thirty minutes, this trend was also recorded for both XF-73 treatments in natural and blue light (Figure 4.3 B).

Both treatments of XF-73 showed a significant difference in comparison to their corresponding growth controls (Figure 4.3 A). A greater significance was recorded in the natural light environment (p= < 0.001), in comparison to the blue light environment (p= 0.0159). A statistically significant difference between both XF-73 treatments was also recorded (p= 0.0442).

Although a significant difference was recorded for both XF-73 treatments in the two different environments, only XF-73 in blue light led to no viable growth on MHA, the controls in both environments and XF-73 treatment in natural light all showed growth on MHA.

4.5.2.2 *E. coli* ATCC 35218 in log phase growth

E. coli showed increased absorbance readings throughout the study regardless of the environment or XF-73 treatment, but there was a decrease in absorbance readings after fifty minutes for *E. coli* alone exposed to blue light (Figure 4.4).

Forty minutes into the time study was the first recorded instance where *E. coli* in blue light treated with XF-73 had a greater absorbance reading than the corresponding readings for the growth control in blue light (Figure 4.4 B). For the remaining twenty minutes treated *E. coli* continued to have greater absorbance readings. End point statistical analysis showed the difference between treated and control *E. coli* in blue light and XF-73 treatment was not significant (p > 0.05).

Throughout the hour study, *E. coli* treated with XF-73 in natural light had lower absorbance readings than *E. coli* alone (Figure 4.4 C). At the end of the time course a significant difference was recorded between the two samples (p= 0.0026).

E. coli treated with XF-73 in blue light had similar absorbance readings to the growth control in natural light; similarly, the growth control in blue light and XF-73 treatment in natural light also have comparable absorbance readings (Figure 4.4 A).

At the end of the time course study, confluent growth was recorded on MHA for *E. coli* treated with XF-73 in the presence and absence of blue light. Controls of *E. coli* alone in natural and blue light also showed growth.

A
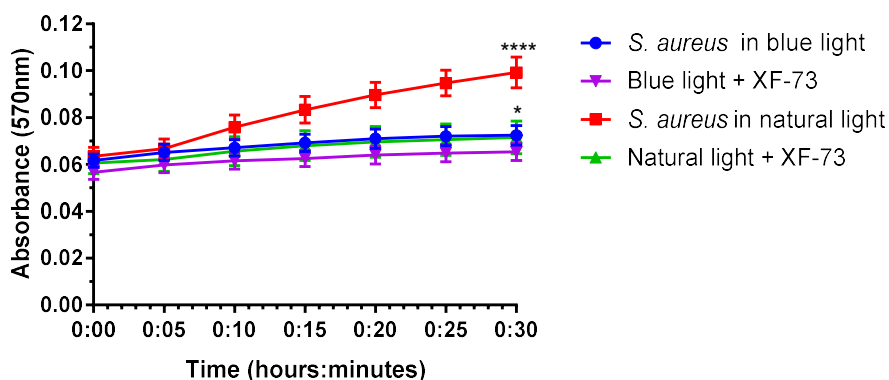
B
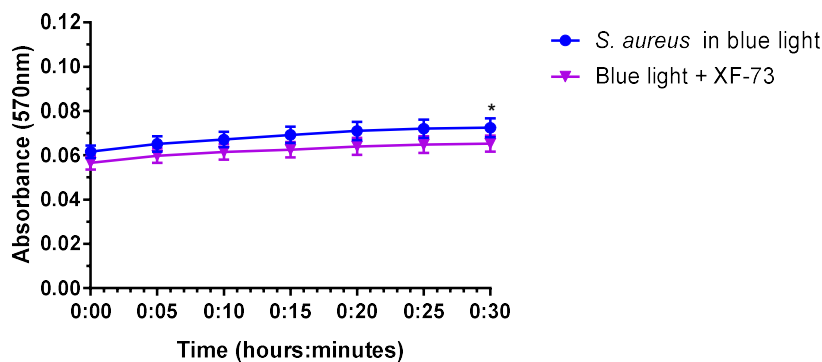
C
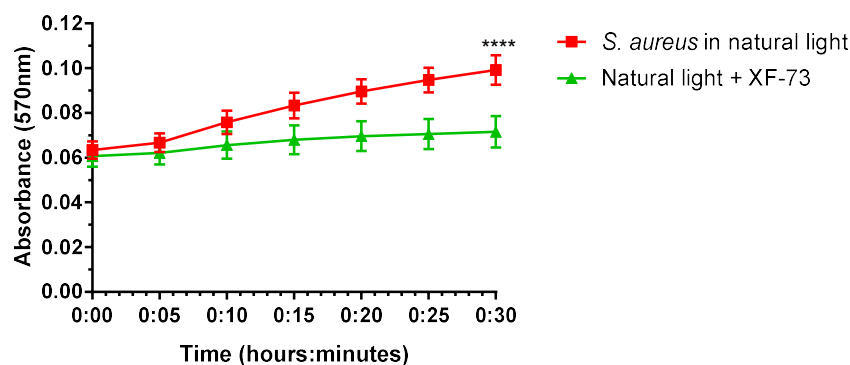
Figure 4.3 S. aureus ATCC 6538 in log phase growth, treated with XF-73 (0.25 µg/ mL) in the presence and absence of blue light

*Absorbance readings (570 nm) of S. aureus ATCC 6538 in log phase growth, treated with XF-73 (0.25 µg/ mL) in the presence (B) and absence (C) of blue light for thirty minutes. With a line graph combining both absorbance readings (A). Error bars generated from the standard deviation of the twelve replicates. End point statistical analysis on absorbance readings following treatment of XF-73 in the presence and absence of blue light was completed via a one-way ANOVA with Tukey's multiple comparison test, where *: p= 0.0159 and ****: p= <0.0001.*
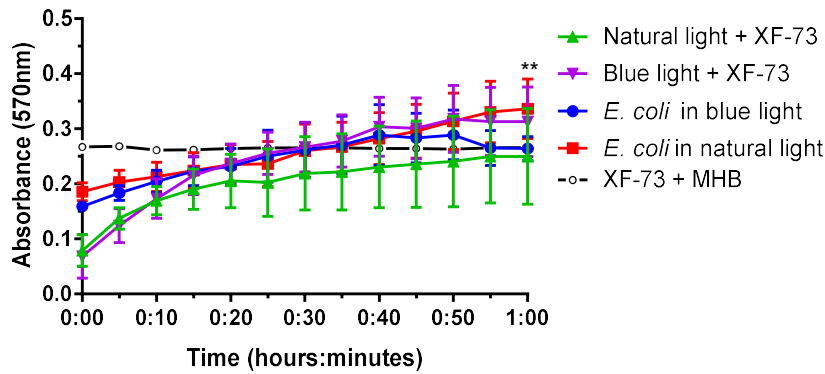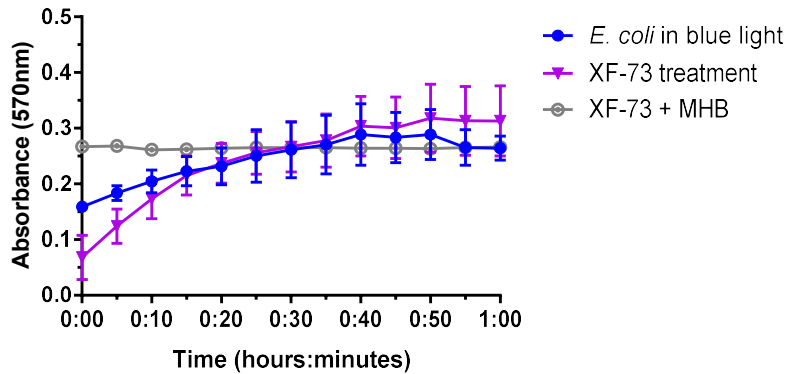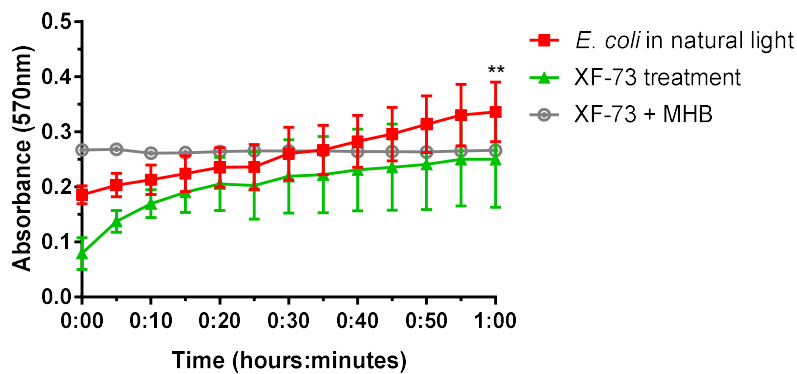
Figure 4.4 E. coli ATCC 35218 in log phase growth treated with XF-73 (102.4 μg/ mL) in the presence and absence of blue light

Absorbance readings (570 nm) of E. coli ATCC 35218 in log phase growth, treated with XF-73 (102.4 μg/ mL) in the presence (B) and absence (C) of blue light taken over one hour. With a collection of both absorbance readings (A). Error bars generated from the standard deviation of the twelve replicates. End point statistical analysis on absorbance readings following treatment of XF-73 in the presence and absence of blue light was completed via a one-way ANOVA with Tukey's multiple comparison test, where **: p= 0.0026.

4.5.3 Photo-activated XF-73 at different exposure times against *S. aureus* in log phase growth

Both thirty minutes (Figure 4.5) and fifteen minutes (Figure 4.6) exposure of blue light activated XF-73 against *S. aureus* ATCC 6538 in log phase growth are presented as line graphs.

4.5.3.1 Thirty minutes blue light exposure

In natural light, the growth control increased throughout the entirety of the time course, with a steeper recorded increase in absorbance readings from the first hour to the fifth (Figure 4.5). On the other hand, the absorbance readings for *S. aureus* treated with XF-73 stayed constant throughout the five hours. The difference between treated and untreated *S. aureus* in natural light was found to be statistically significant (p= <0.0001). There was no noteworthy increase in absorbance readings between the growth control and XF-73 treatment in blue light and were deemed not significantly different (p > 0.05); the difference between absorbance readings at the fifth hour was determined to be not significant through end point statistical analysis. A statistically significant difference was determined between absorbance readings for both XF-73 treatments in the presence and absence of blue light (p= 0.0275).

Samples streaked onto MHA after the initial five-hour time course study yielded confluent growth for *S. aureus* in natural light and less growth visible on XF-73 treated *S. aureus* in natural light. Only a few single colonies of *S. aureus* were observed after blue light exposure and no growth was recorded for the corresponding XF-73 treatment in blue light.

Twenty-four hour samples streaked on MHA showed confluent growth for *S. aureus* with and without XF-73 treatment in natural light. No visible growth was recorded for untreated and treated *S. aureus* following blue light exposure.

4.5.3.2 Fifteen minutes blue light exposure

Both *S. aureus* absorbance readings in natural and blue light increased throughout the five hours, the readings for the growth control in blue light had lower readings in comparison to natural light readings at each time point (Figure 4.6); this difference between untreated *S. aureus* absorbance readings was determined to be significant (p= <0.0001).

Both XF-73 treatments showed no increase in absorbance readings and remained constant throughout the study. A significant statistical difference was noted between XF-73 treatments in the presence and absence of blue light and their corresponding untreated *S. aureus* absorbance readings (p= <0.0001).

Initial plated samples on MHA following the time course study yielded confluent growth for *S. aureus* in the presence and absence of blue light. Less growth was observed for the XF-73 treatment in natural light and only a few individual colonies were present on the MHA plates showing XF-73 treatment in blue light. In contrast, plated samples following twenty-four hours showcased growth for all test conditions of *S. aureus*.
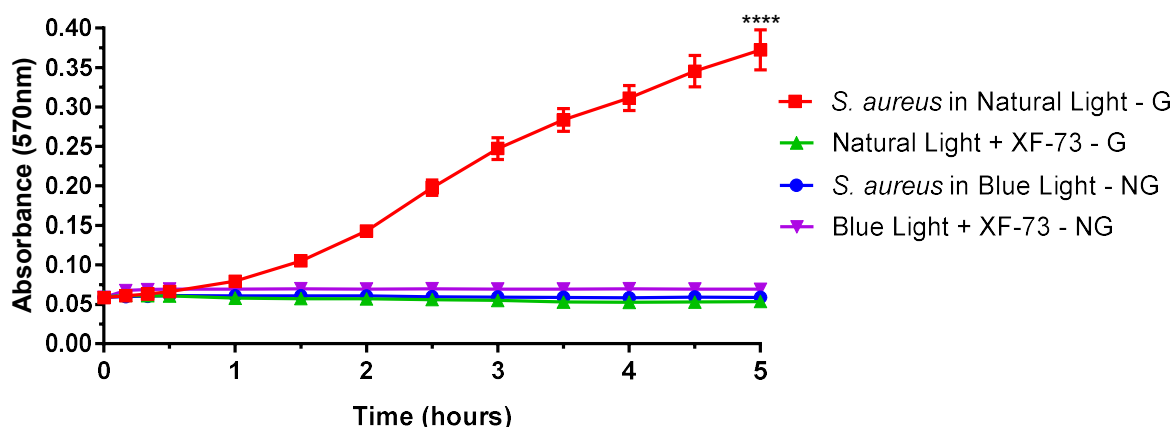
Figure 4.5 S. aureus ATCC 6538 in log phase growth treated with XF-73 (0.25 µg/ mL) in the presence and absence of blue light for thirty minutes

*Absorbance readings (570 nm) of S. aureus ATCC 6538 in log phase growth, treated with XF-73 (0.25 µg/ mL) in natural light and thirty minutes of blue light exposure. Error bars generated from standard deviation of the twelve replicates. Statistical analysis on absorbance readings following treatment of XF-73 in the presence and absence of blue light was completed via a one-way ANOVA with Tukey's multiple comparison test, where ****: p= <0.0001. 'G' indicates growth and 'NG' indicates no growth for samples streaked on MHA following twenty-four hours after the time course study.*
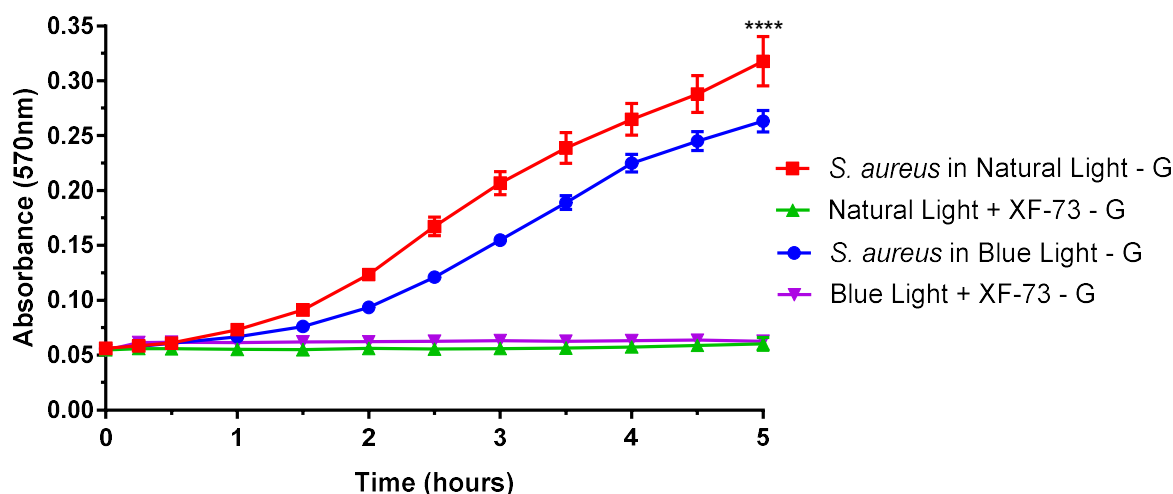
Figure 4.6 S. aureus ATCC 6538 in log phase growth treated with XF-73 (0.25 µg/ mL) in the presence and absence of blue light for fifteen minutes

*Absorbance readings (570 nm) of S. aureus ATCC 6538 in log phase growth, treated with XF-73 (0.25 µg/ mL) in natural light and fifteen minutes of blue light exposure. Error bars generated from standard deviation of the twelve replicates. Statistical analysis on absorbance readings following treatment of XF-73 in the presence and absence of blue light was completed via a one-way ANOVA with Tukey's multiple comparison test, where ****: p= <0.0001. 'G' indicates growth for samples streaked on MHA following twenty-four hours after the time course study.*

4.5.4 Minimum inhibitory and bactericidal concentrations of photo-activated XF-73 against Gram-positive and Gram-negative microorganisms

Throughout this study, two batches of XF-73 were used: batch 1 and batch 2. *C. striatum* ATCC 1293 and *C. albicans* ATCC 76615 were only tested against batch 1 XF-73. Gram-positive microorganisms: *S. aureus* ATCC 6538, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228 and *E. faecalis* ATCC 29213 treated with batch 1 XF-73 have concentration ranges due to multiple MIC and MBC tests (Table 4.1).

C. albicans was the only microorganism where an increase in XF-73 concentration was required to inhibit and eradicate the planktonic cells with the addition of blue light activation, with recorded 2-fold increase in XF-73 concentration in comparison to natural light MIC and MBC values (Table 4.1).

4.5.4.1 Batch 1 XF-73 MIC and MBCs following blue light activation

Lower concentrations of XF-73 were capable of inhibiting and eradicating all Gram-positive microorganisms following blue light activation in comparison to XF-73 in natural light (Table 4.1).

A 4-fold to 8-fold reduction was observed in XF-73 concentrations following blue light activation to inhibit *S. aureus* ATCC 6538 planktonic cells. A 4-fold reduction of blue light activated XF-73 was also noted to eradicate the cells; but 1μg/ mL of XF-73 was also recorded to eradicate *S. aureus* in the presence and absence of blue light.

The lowest concentration of XF-73 following blue light activation used in the study was capable of inhibiting and eradicating *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228 and *E. faecalis* ATCC 29212 planktonic cells at ≤ 0. 003 μg/ mL. Activated XF-73 had a minimum 64-fold decrease in comparison to XF-73 alone in natural light to inhibit and eradicate *S. aureus* ATCC 29213 cells.

The same concentration range of blue light XF-73 was able to inhibit and eradicate *S. epidermidis* and had the lowest concentration range of blue light activated XF-73 in comparison to all other microorganisms, at ≤ 0.003 – 0.06 μg/ mL and had a minimum 4-fold decrease in concentration compared to XF-73 in natural light. Similarly, the same concentration range of activated XF-73 was recorded as the MIC and MBC against *E. faecalis* and had a minimum 4-fold decrease in concentration was determined in comparison to corresponding natural light XF-73 treatments.

A large reduction in XF-73 concentrations following blue light enhancement was found to inhibit and eradicate *C. striatum,* with an 8-fold reduction in comparison to XF-73 in natural light. Similar to natural light XF-73 against *C. striatum*, the same concentration of activated XF-73 was capable of inhibiting and eradicating the microorganism.

Similar to XF-73 in natural light, blue light activated XF-73 was unable to inhibit nor eradicate Gram-negative microorganisms (Table 4.1). Therefore, microorganisms had an MIC and MBC value determined as greater than the maximum concentration of XF-73 tested at > 265 µg/ mL.

4.5.4.2 Batch 2 XF-73 MIC and MBCs following blue light activation

The lowest concentration of blue light enhanced XF-73 was found to inhibit and eradicate all Gram-positive microorganisms and were therefore classified as having an MIC and MBC concentration ≤ 0.125 µg/mL. A reduced concentration of XF-73 was found to eradicate all Gram-positive and Gram-negative microorganisms following blue light activation (Table 4.2).

A minimum 8-fold reduction in XF-73 following blue light exposure was found to inhibit both strains of *S. aureus* in comparison to XF-73 in natural light. Meanwhile, a minimum 32-fold and 16-fold decrease in XF-73 concentration was capable of eradicating *S. aureus* ATCC 6538 and *S. aureus* ATCC 29213 respectively.

XF-73 in the presence and absence of blue light had the same recorded MIC value against *S. epidermidis* ATCC 12228 planktonic cells. Though a minimum 4-fold decrease in XF-73 concentration following blue light activation was able to eradicate *S. epidermidis* in comparison to XF-73 in natural light.

Similar to both *S. aureus* strains used in this study, a decrease in XF-73 concentration was sufficient following blue light activation to inhibit *E. faecalis* ATCC 29213 and a further decrease in concentration was found to eradicate the planktonic cells. As a minimum 4-fold and 8-fold reduction calculated between MIC and MBC values in comparison to XF-73 in natural light.

Out of all Gram-negative microorganisms tested against blue light activated XF-73, *E. coli* ATCC 35218 was the only strain where MIC = MBC and had a 4-fold and minimum 8-fold reduction in concentration of blue light activated XF-73 needed to inhibit and eradicate the planktonic cells respectively, in comparison to XF-73 in natural light. The same concentration of blue light activated XF-73 was found to eradicate both strains of *E. coli* cells tested in this study. The lowest recorded concentration of blue light activated XF-73 to inhibit Gram-negative microorganisms was found against *E. coli* ATCC 25922 at 16 µg/ mL, an 8-fold decrease in concentration in comparison to the corresponding MIC value of XF-73 in natural light.

A 4-fold decrease in XF-73 concentration was found following blue light activation to inhibit the growth of both *P. aeruginosa* strains tested. A lower concentration of enhanced XF-73 was found to eradicate *P. aeruginosa* ATCC 27853 cells at 64 µg/ mL whereas 128 µg/ mL was the minimum concentration of blue light activated XF-73 needed to eradicate *P. aeruginosa* ATCC 15442.

Table 4.1 MIC and MBC values (µg/ mL) of batch 1 XF-73 in the presence and absence of blue light against a panel of Gram-positive and Gram-negative microorganisms

Microorganism/ ATCC number	XF-73 Batch 1: September 2018 Natural Light		Blue Light	
	MIC µg/ mL	MBC µg/ mL	MIC µg/ mL	MBC µg/ mL
S. aureus 6538	1 – 2	1 – 2	0.25	0.25 – 1
S. aureus 29213	2	2	≤ 0.03 – 0.25	≤ 0.03 – 0.5
S. epidermidis 12228	1 – 2	1 – 4	≤ 0.03 – 0.06	≤ 0.03 – 0.06
E. faecalis 29212	0.125 – 2	0.125 – 4	≤ 0.03 – 1	≤ 0.03 – 1
C. striatum 1293	2	2	0.125	0.125
C. albicans 76615	4	4	8	8
E. coli 35218	> 256	> 256	> 256	> 256
E. coli 25922	> 256	> 256	> 256	> 256
P. aeruginosa 15442	> 256	> 256	> 256	> 256
P. aeruginosa 27853	> 256	> 256	> 256	> 256

Table 4.2 MIC and MBC values (µg/ mL) of batch 2 XF-73 in the presence and absence of blue light against a panel of Gram-positive and Gram-negative microorganisms

Microorganism/ ATCC number	Batch 2: March 2020 Natural Light		Blue Light	
	MIC µg/ mL	MBC µg/ mL	MIC µg/ mL	MBC µg/ mL
S. aureus 6538	1	4	≤ 0.125	≤ 0.125
S. aureus 29213	1	2	≤ 0.125	≤ 0.125
S. epidermidis 12228	≤ 0.125	0.5	≤ 0.125	≤ 0.125
E. faecalis 29212	0.5	1	≤ 0.125	≤ 0.125
E. coli 35218	128	> 256	32	32
E. coli 25922	128	128	16	32
P. aeruginosa 15442	128	256	32	128
P. aeruginosa 27853	128	256	32	64

4.5.5 Activity of photo-activated XF-73 against Gram-positive and Gram-negative twenty-four hour biofilms

As previously mentioned (3.5.3), biofilm viability was calculated through counting CFUs present on MHB following blue light activated XF-73 treatment and multiplied by the dilution factor and 50 to determine the CFU/ mL (4.5.5.2, 4.5.5.3). As the original PBS suspension was 200 mL, any concentration with no viable colonies were determined to have a biofilm viability below the lower limit of detection with a biofilm viability as ≤ 10 CFU/ mL.

4.5.5.1 MBIC and MBEC values of blue light activated XF-73 against Gram-positive and Gram-negative biofilms

With the exception of *S. aureus* ATCC 6538, XF-73 activated by blue light was capable of inhibiting and eradicating Gram-positive biofilms at a lower concentration in comparison to XF-73 in natural light (Table 4.3).

A greater concentration of XF-73 was required when blue light was involved to inhibit *S. aureus* ATCC 6538 biofilms and was determined to be greater than the maximum concentration of XF-73 used in this study, indicating a greater than 8-fold increase in comparison to natural light XF-73 treatment.

The same concentration of XF-73 was determined for the MBIC and MBEC of each microorganism. A 4-fold reduction in XF-73 concentration was found to eradicate *E. faecalis* biofilms following blue light treatment. Meanwhile an 8-fold reduction in MBIC and MBEC was noted against *S. aureus* ATCC 29213 in comparison to natural light XF-73 treatment. At each concentration of blue light activated XF-73, no recorded growth of *S. epidermidis* biofilms was observed throughout the study. Therefore, MBIC/ MBEC values of blue light activated XF-73 were recorded as less than or equal to 0.125 µg/ mL.

Similar to the equivalent natural light results, blue light activated XF-73 was not able to inhibit or eradicate either *P. aeruginosa* biofilms or *E. coli* ATCC 53218 biofilms in the study. The final treatment involving activated XF-73 via blue light followed by an additional retreatment with XF-73 was also unable to have an effect against these biofilms.

Both blue light activated XF-73 and blue light activated XF-3 followed by an additional XF-73 treatment were able to eradicate *E. coli* ATCC 25922 biofilms, at 512 µg/ mL for both treatments, whereas in natural light XF-73 was unable to inhibit nor eradicate these biofilms.

Table 4.3 MBIC and MBEC values (μg/ mL) of XF-73 in the presence and absence of blue light against Gram-positive and Gram-negative twenty-four hour biofilms

Table includes additional XF-73 treatment against Gram-negative twenty-four hour biofilms consisting of blue light activated XF-73 treatment followed by an additional XF-73 treatment in natural light

Microorganism/ ATCC	Natural Light		Blue Light		Blue Light + Retreatment	
	MBIC (μg/ mL)	MBEC (μg/ mL)	MBIC (μg/ mL)	MBEC (μg/ mL)	MBIC (μg/ mL)	MBEC (μg/ mL)
***S. aureus* 6538**	1	1	> 8	> 8	N/A	N/A
***S. aureus* 29213**	1	1	0.125	0.125	N/A	N/A
***S. epidermidis* 12228**	1	1	≤ 0.125	≤ 0.125	N/A	N/A
***E. faecalis* 29212**	2	2	0.5	0.5	N/A	N/A
***E. coli* 35218**	> 512	> 512	> 512	> 512	> 512	> 512
***E. coli* 25922**	> 512	> 512	512	512	512	512
***P. aeruginosa* 15442**	> 512	> 512	> 512	> 512	512	> 512
***P. aeruginosa* 27853**	> 512	> 512	> 512	> 512	> 512	> 512

Note: The final treatment strategy consisting of blue light with XF-73 and an additional XF-73 retreatment was not used against Gram-positive biofilms

4.5.5.2 Viability of Gram-positive twenty-four hour biofilms treated with blue light activated XF-73

Biofilm viability following blue light activated XF-73 treatment against Gram-positive twenty-four hour biofilms were collected and recorded as bar charts, with the addition of corresponding biofilm viabilities of XF-73 treatment in natural light (Figure 4.7 – 4.10).

4.5.5.2.1 *S. aureus* ATCC 6538 biofilms

Concentrations 0.125 – 1 μg/ mL of blue light activated XF-73 led to similar viabilities of *S. aureus* ATCC 6538 biofilms in comparison to untreated biofilms, statistical analysis determined no significant difference ($p > 0.05$) between the viable counts over this concentration range (Figure 4.7).

An increase in XF-73 concentration above 1 μg/ mL showed a decrease in biofilm viability, with a 4-log and 7-log reduction in comparison to untreated *S. aureus* biofilms at concentrations 2 μg/ mL and 4 μg/ mL respectively. At 8 μg/ mL, no viable cells were recorded during the viable cell count and was therefore determined as having a viability below the lower limit of detection. The differences in biofilm viability at concentrations ranging from 2 – 8 μg/ mL in comparison with the growth control were statistically significant ($p = <0.0001$).

4.5.5.2.2 *S. aureus* ATCC 29213 biofilms

The addition of blue light activated XF-73 against *S. aureus* ATCC 29213 biofilms, at any concentration had a recorded biofilm viability below the lower limit of detection (Figure 4.8). The difference between XF-73 treatments and the growth control was statistically significant (p= <0.0001). XF-73 treatment in natural light only had viability readings under the lower limit of detection at concentrations 1 – 8 μg/ mL.

4.5.5.2.3 *S. epidermidis* ATCC 12228 biofilms

In natural light, XF-73 treatments at 1 – 8 μg/ mL had recorded viabilities of *S. epidermidis* biofilms below the lower limit of detection; meanwhile, treatment with every concentration of blue light activated XF-73: 0.125 – 8 μg/ mL resulted in biofilm viabilities below the lower limit of detection (Figure 4.9). Statistical analysis determined significance (p= <0.0001) for each biofilm viability generated from blue light activated XF-73 treatment compared to the growth control.

4.5.5.2.4 *E. faecalis* ATCC 29212 biofilms

Each concentration of blue light activated XF-73 treatment led to a decrease in *E. faecalis* biofilm viability (Figure 4.10), as the concentration of XF-73 increased the viability of *E. faecalis* biofilms decreased. A significant statistical difference in biofilm viabilities following blue light activated XF-73 treatment at every concentration in comparison to the growth control was determined (p= <0.0001).

Blue light activated XF-73 at 0.125 μg/ mL and 0.25 μg/ mL caused a 2-log decrease in biofilm viability in comparison to the growth control at 6.67×10^4 CFU/ mL and 1.93×10^4 CFU/ mL respectively. Although there was a 4.73×10^4 CFU/ mL difference in biofilm viability between these two concentrations of XF-73, there was no significant difference (p > 0.05) between them. Concentrations 0.5 – 8 μg/ mL resulted in biofilm viabilities under the lower limit of detection, whereas only concentrations of 2 – 8 μg/ mL of XF-73 in natural light resulted in biofilm viabilities above the lower limit of detection.
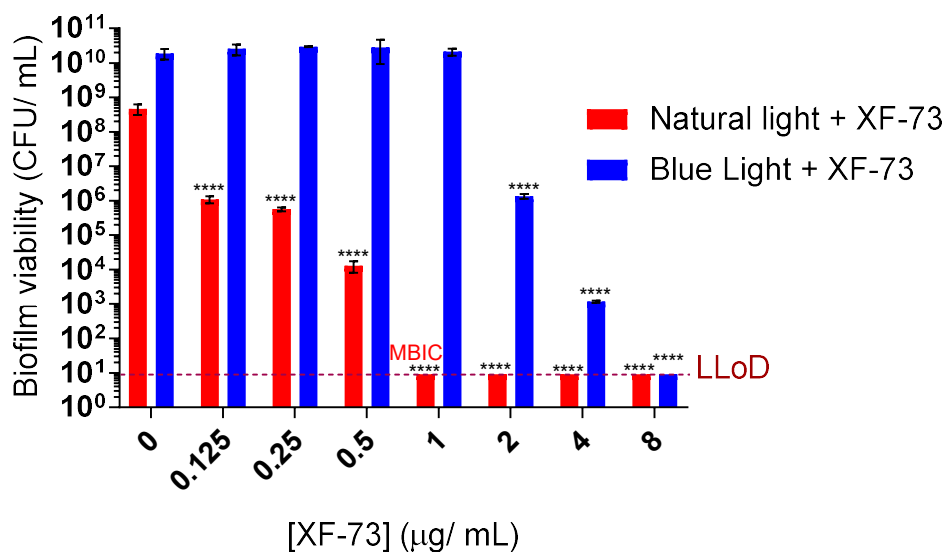
Figure 4.7 S. aureus ATCC 6538 twenty-four hour biofilms treated with XF-73 in the presence and absence of blue light

*Recorded viabilities of S. aureus ATCC 6538 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 0.125 – 8 µg/ mL in the presence and absence of blue light. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated S. aureus biofilm viability, where ****: p= <0.0001. Natural light MBIC value also highlighted, blue light MBIC >8 µg/ mL (Table 4.3).*
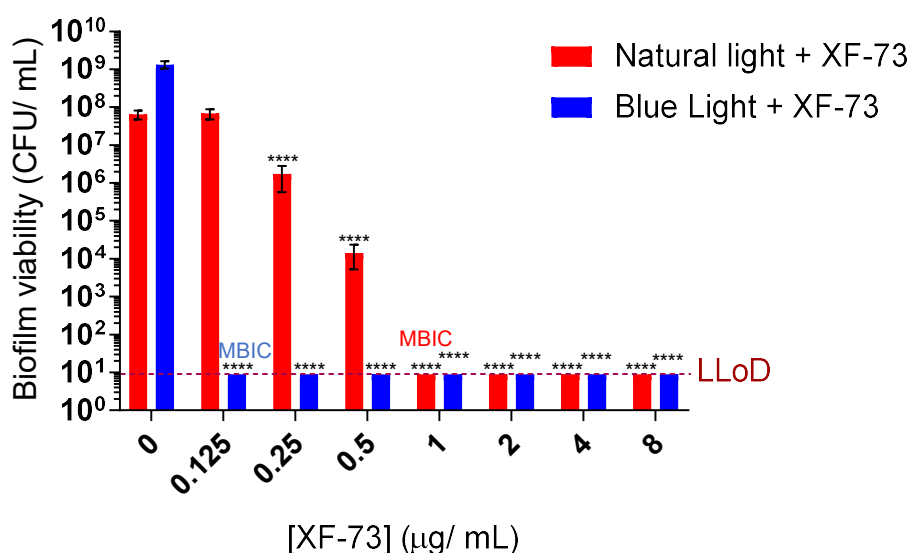
Figure 4.8 S. aureus ATCC 29213 twenty-four hour biofilms treated with XF-73 in the presence and absence of blue light

*Recorded viabilities of S. aureus ATCC 29213 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 0.125 – 8 µg/ mL in the presence and absence of blue light. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated S. aureus biofilm viability, where ****: p= <0.0001. Natural light MBIC and blue light MBIC (Table 4.3) values also highlighted.*

Figure 4.9 S. epidermidis ATCC 12228 twenty-four hour biofilms treated with XF-73 in the presence and absence of blue light

*Recorded viabilities of S. epidermidis ATCC 12228 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 0.125 – 8 µg/ mL in the presence and absence of blue light. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated S. epidermidis biofilm viability, where ****: p= <0.0001. Natural light MBIC value also highlighted, blue light MBIC ≤ 0.125 (Table 4.3).*
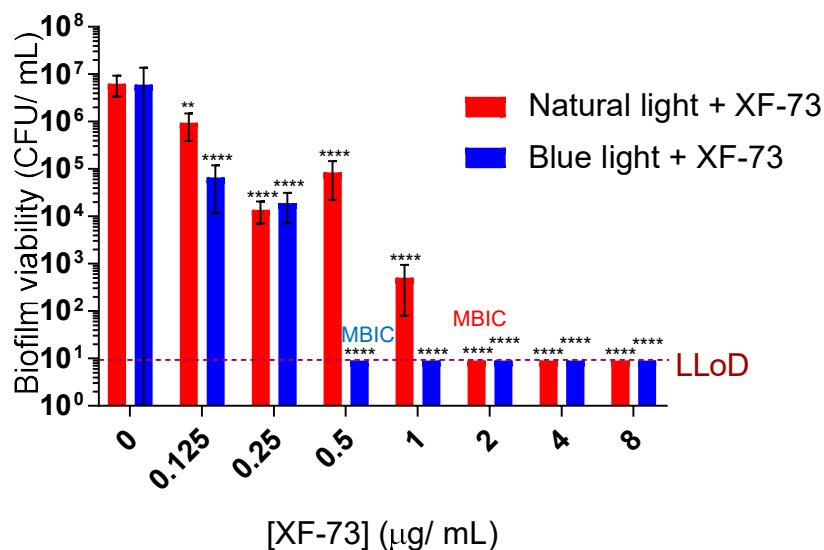
Figure 4.10 E. faecalis ATCC 29212 twenty-four hour biofilms treated with XF-73 in the presence and absence of blue light

*Recorded viabilities of E. faecalis ATCC 29212 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 0.125 – 8 µg/ mL in the presence and absence of blue light. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated E. faecalis biofilm viability, where **: p= 0.007 and ****: p= <0.0001. Natural light MBIC and blue light MBIC (Table 4.3) values also highlighted.*

4.5.5.3 Viability of Gram-negative twenty-four hour biofilms treated with blue light activated XF-73

Biofilm viability following blue light activated XF-73 treatment against Gram-negative twenty-four hour biofilms and the additional treatment of blue light activated XF-73 followed by an additional retreatment of XF-73 were collected and recorded as bar charts, corresponding biofilm viabilities of XF-73 treatment in natural light were also included (Figure 4.11 – 4.14).

4.5.5.3.1 *E. coli* ATCC 35218 biofilms

XF-73 exposed to blue light resulted in a decrease in viability of *E. coli* ATCC 35218 biofilms at each concentration used (Figure 4.11). Similar biofilm viabilities were recorded at concentrations 16 µg/ mL and 32 µg/ mL between blue light enhanced and natural light XF-73. A lower viability was noted for blue light activated XF-73 at concentrations 64 µg/ mL and 128 µg/ mL compared to XF-73 in natural light. Concentrations 128 – 512 µg/ mL of blue light activated XF-73 resulted in biofilm viabilities below the lower limit of detection. An 8-log reduction was observed at biofilm 64 µg/ mL of blue light activated XF-73 in comparison to the growth control.

In contrast, each concentration of blue light enhanced XF-73 followed by a retreatment of XF-73 in natural light, led to biofilm viabilities under the lower limit of detection. A statistically significant difference between biofilm viabilities was recorded for both blue light treatments and their corresponding growth controls (p= <0.0001).

4.5.5.3.2 *E. coli* ATCC 25922 biofilms

Similar to blue light activated XF-73 against *S. aureus* ATCC 6538 biofilms (Figure 4.7); a greater viability of *E. coli* ATCC 25922 biofilms was noted at each concentration of blue light activated XF-73, in comparison to viabilities recorded for natural light XF-73 treatment (Figure 4.12). There was no correlation between an increase in activated XF-73 concentration to *E. coli* biofilm viability, as concentrations 32 µg/ mL and 256 µg/ mL had the highest recorded viability in comparison to the other concentrations; although these concentrations of activated XF-73 still led to a 5-log and 6-log reduction respectively in comparison to the growth control. An 8-fold decrease in viability was recorded at 512 µg/ mL of activated XF-73. A statistically significant difference was determined at each concentration of XF-73 enhanced by blue light, although there was a different p value for concentrations 16 – 256 µg/ mL of XF-73 (p= 0.0011 – 0.0066). Blue light activated XF-73 at 512 µg/ mL had the lowest p value (p= 0.0009). No statistical difference (p > 0.05) was calculated between biofilm viabilities at each concentration of blue light activated XF-73.

A distinct difference was observed between biofilm viabilities generated by blue light activated XF-73 treatment and blue light activated XF-73 with an additional XF-73 retreatment. Each concentration of XF-73 used in the treatment strategy involving XF-73 retreatment had biofilm viabilities below the lower

limit of detection, a significant statistical difference was determined between these concentrations and untreated *E. coli* biofilms at each concentration (p= <0.0001).

4.5.5.3.3 *P. aeruginosa* ATCC 15442 biofilms

Treatments of blue light activated XF-73 and a further XF-73 retreatment decreased *P. aeruginosa* ATCC 15442 biofilm viability at each concentration used, in comparison to corresponding untreated biofilms (Figure 4.13). Both blue light treatments resulted in biofilm viabilities greater than the corresponding viabilities recorded from XF-73 in natural light treatment at concentrations ranging from 16 – 64 µg/ mL.

Blue light treatment followed by retreatment of XF-73 had the highest recorded biofilm viabilities out of all other XF-73 treatments at concentrations: 32 µg/ mL, 64 µg/ mL, and 512 µg/ mL. XF-73 activated by blue light at 512 µg/ mL was the only treatment resulting in a biofilm viability below the lower limit of detection.

Similar biofilm viabilities were recorded between untreated *P. aeruginosa* and blue light enhanced XF-73 at 16 µg/ mL, though a 3.82×10^{10} CFU/ mL difference was recorded between them; this difference was determined to be significant by statistical analysis (p= 0.0302). Each concentration of blue light enhanced XF-73 greater than 16 µg/ mL had a statistically significant difference between the growth control (p= <0.0001).

A log reduction in viability was recorded between untreated biofilms and biofilms treated with blue light enhanced XF-73 and retreatment of XF-73 at 16 µg/ mL. As the concentration of retreatment XF-73 increased following 16 µg/ mL, the viability of *P. aeruginosa* biofilms decreased, with a maximum 7-log reduction observed at 512 µg/ mL of retreated XF-73. Biofilm viabilities recorded at each concentration was shown to be statistically significant when compared to the viability of the growth control (p= <0.0001).

4.5.5.3.4 *P. aeruginosa* ATCC 27853 biofilms

The viability of *P. aeruginosa* ATCC 27853 biofilms following blue light activated XF-73 treatment at 16 µg/ mL and 32 µg/ mL, was found to be greater than the growth control (Figure 4.14). At 16 µg/ mL a 1-log increase was demonstrated between untreated *P. aeruginosa* and was determined to be statistically significant (p= <0.0001). No statistically significant difference (p > 0.05) was calculated between untreated biofilm viability and viability readings after 32 µg/ mL of blue light activated XF-73. Concentrations ranging from 64 – 256 µg/ mL of blue light activated XF-73 led to a decrease in biofilm viability in accordance with the increase in XF-73 concentrations. Although at 512 µg/ mL of blue light activated XF-73, viability was greater than the recorded viability at 256 µg/ mL at 1.83×10^5 CFU/ mL and 7.67×10^5 CFU/ mL respectively. Statistical analysis indicated a significant difference between each

antimicrobial treatment of blue light enhanced XF-73 and untreated *P. aeruginosa* biofilms (p= <0.0001) for each concentration.

As the concentration of XF-73 involved in the blue light activation followed by XF-73 retreatment increased, biofilm viability decreased. Each recorded biofilm viability following this treatment strategy was less than the growth control. These reduced viabilities were determined to be statistically significant, and a greater significance was recorded at concentrations 32 – 512 μg/ mL (p= <0.0001) in comparison to 16 μg/ mL (p= 0.0088). XF-73 retreatment at 512 μg/ mL was responsible for the lowest viability of *P. aeruginosa* biofilms with a 5-log decrease in comparison to untreated viability.
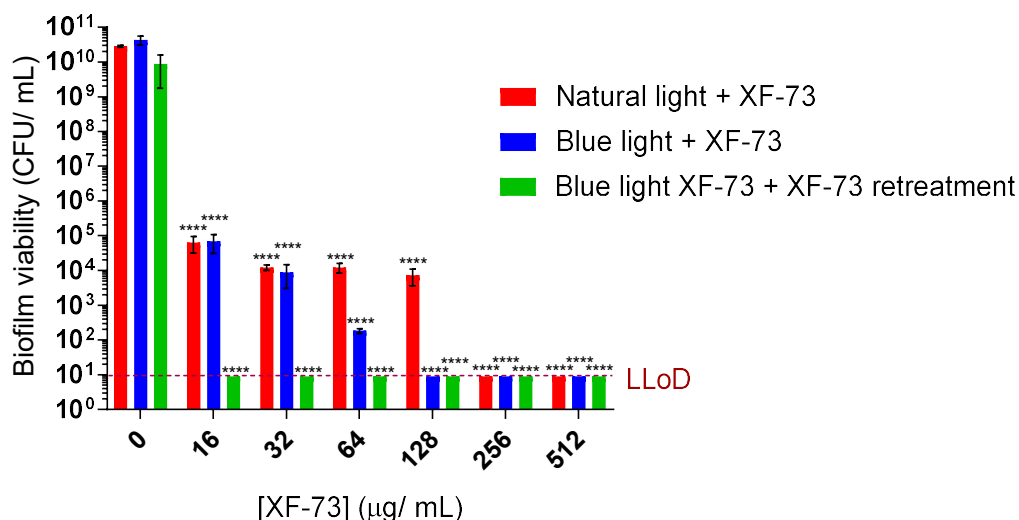
Figure 4.11 E. coli ATCC 35218 twenty-four hour biofilms treated with XF-73 in the presence and absence of blue light

Recorded viabilities of E. coli ATCC 35218 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 16 – 512 μg/ mL in the presence and absence of blue light; with an additional treatment strategy of blue light enhanced XF-73 followed by XF-73 retreatment. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated E. coli biofilm viability, where ****: p= <0.0001.

Figure 4.12 E. coli ATCC 25922 twenty-four hour biofilms treated with XF-73 in the presence and absence of blue light

Recorded viabilities of E. coli ATCC 25922 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 16 – 512 μg/ mL in the presence and absence of blue light; with an additional treatment strategy of blue light enhanced XF-73 followed by XF-73 retreatment. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated E. coli biofilm viability, where **: p= 0.0011 – 0.0066, ***: p= 0.0009 and ****: p= <0.0001. Blue light MBIC and blue light + retreatment MBIC (Table 4.3) values also highlighted.
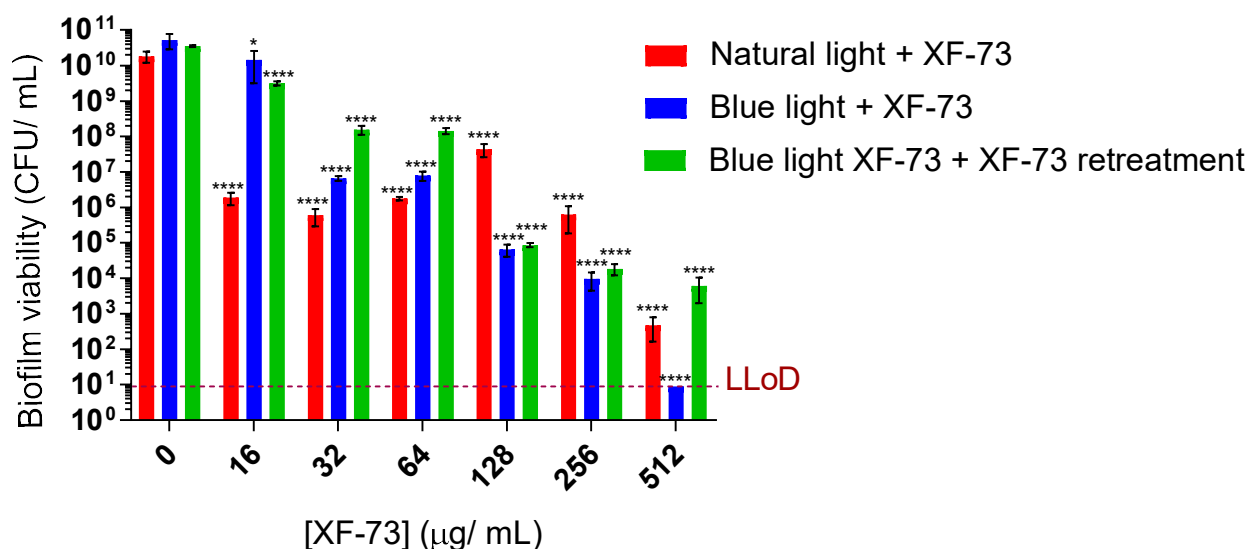
Figure 4.13 P. aeruginosa ATCC 15442 twenty-four hour biofilms treated with XF-73 in the presence and absence of blue light

Recorded viabilities of P. aeruginosa ATCC 15442 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 16 – 512 µg/ mL in the presence and absence of blue light; with an additional treatment strategy of blue light enhanced XF-73 followed by XF-73 retreatment. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated P. aeruginosa biofilm viability, where *: p= 0.0302 and ****: p= <0.0001.
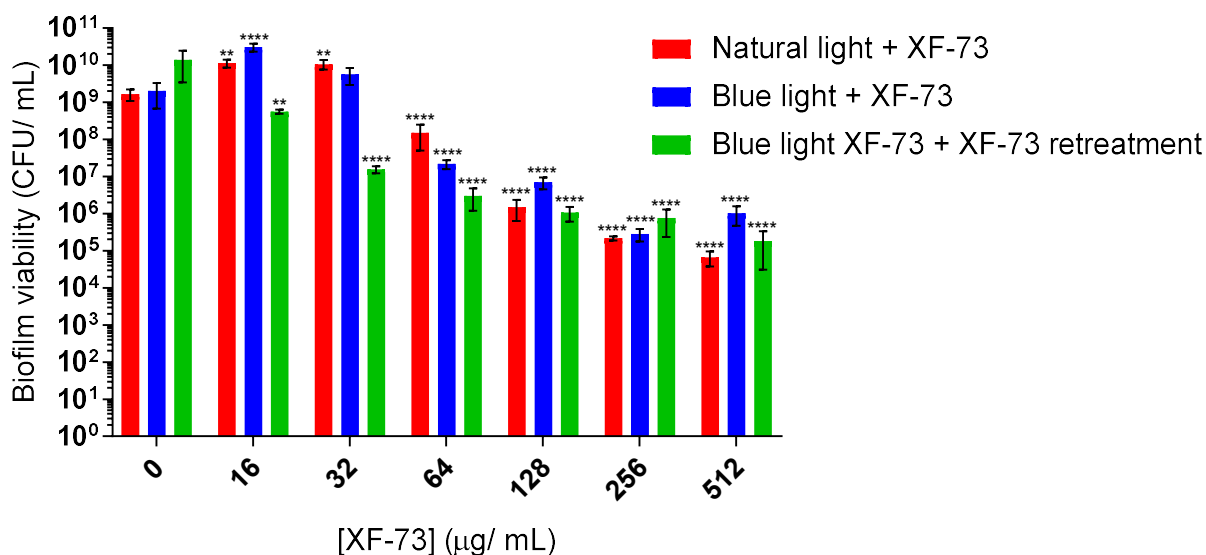
Figure 4.14 P. aeruginosa ATCC 27853 twenty-four hour biofilms treated with XF-73 in the presence and absence of blue light

Recorded viabilities of P. aeruginosa ATCC 27853 biofilms (CFU/ mL), after twenty-four hours of growth treated with XF-73 at a concentration range of 16 µg/ mL – 512 µg/ mL in the presence and absence of blue light; with an additional treatment strategy of blue light enhanced XF-73 followed by XF-73 retreatment. Error bars generated using the standard deviation of the three replicates. Dotted line labelled LLoD signifies the 'lower limit of detection' for viable cell counts. Statistical analysis was completed via a one-way ANOVA and a Tukey's multiple comparison test between viability of each concentration of XF-73 compared against untreated P. aeruginosa biofilm viability, where **: p= 0.0017, 0.0025 (natural light XF-73) and 0.0088 (XF-73 retreatment) and ****: p= <0.0001.

4.5.5.4 Crystal violet staining of Gram-positive and Gram-negative biofilms treated with blue light activated XF-73

Photographs are given of microtitre pegged lids containing Gram-positive (Figure 4.15) and Gram-negative biofilms (Figure 4.16, 4.17), following blue light activated XF-73 treatments and subsequently stained with crystal violet to visualise biofilm density.

4.5.5.4.1 Gram-positive biofilms

Blue light activated XF-73 decreased the density of crystal violet retained on *S. aureus* ATCC 6538 biofilms, due to only a band of dark purple present around the top of each pegs containing treatments ranging from 0.125 – 4 µg/ mL (Figure 4.15 A). In contrast untreated *S. aureus* biofilms had crystal violet covering the entirety of the microtitre pegs, bar the ends. A lighter purple colouration was observed at 8 µg/ mL of blue light activated XF-73 and had the lowest density of crystal violet out of all XF-73 treatments. Controls of MHB and XF-73 alone had no crystal violet present on the sample pegs.

A low density of crystal violet was retained on *S. aureus* ATCC 29213 biofilms (Figure 4.15 B), this was visualised by a faint band of light purple pigment present on the untreated *S. aureus* biofilm pegs. No crystal violet was present on control pegs of MHB and blue light activated XF-73, similarly no stain was present on *S. aureus* biofilms treated with blue light activated XF-73 at concentrations 0.125 – 0.5 µg/ mL. *S. aureus* biofilms treated with higher concentrations of XF-73 (1 – 8 µg/ mL) were shown to have a faint presence of crystal violet, but only on a maximum of one or two replicates each concentration. A lower density of crystal violet was observed on treated *S. aureus* biofilms in comparison to untreated biofilms.

S. epidermidis biofilms had a weak density of crystal violet retained on only one of the replicate pegs (Figure 4.15 C), the stain present on this peg was located in small patches on the peg. In contrast, with the addition of blue light activated XF-73 against these biofilms, a greater density was recorded at 4 µg/ mL and 8 µg/ mL of XF-73. No crystal violet was retained at XF-73 treatments ranging from 0.125 – 2 µg/ mL, MHB and XF-73 alone pegs.

No crystal violet was retained on *E. faecalis* biofilms and control pegs of MHB and XF-73 alone (Figure 4.15 D). Despite no stain present on untreated *E. faecalis* biofilms, crystal violet was present on all *E. faecalis* biofilms pegs that were treated with blue light activated XF-73. The density of crystal violet increased as the concentration of XF-73 treatments increased.

4.5.5.4.2 Gram-negative biofilms

Blue light activated XF-73 and the treatment strategy involving blue light activated XF-73 followed by another XF-73 treatment against *E. coli* ATCC 35218 biofilms showed to have similar densities of crystal violet retained on microtitre pegs (Figure 4.16 A, B). Not only was the stain coverage comparable

between the two treatment strategies, but there was also no difference between crystal violet density between the different concentrations of XF-73 used.

The density of crystal violet on untreated *E. coli* ATCC 35218 biofilms was also comparable to the corresponding blue light activated XF-73 treatment (Figure 4.16 A); meanwhile, untreated *E. coli* biofilms had a greater retention of the stain in comparison to XF-73 retreatment sample pegs (Figure 4.16 B). MHB and XF-73 alone were the only pegs on both treatment plates that lacked crystal violet.

E. coli ATCC 25922 biofilms had a greater retention of crystal violet in comparison to both treatments of XF-73: blue light activated XF-73 and blue light activated XF-73 with an additional XF-73 retreatment (Figure 4.16 C, D); out of the two untreated sets of *E. coli* biofilms, the greater density was recorded on the microtitre pegs for blue light activated XF-73 plate (Figure 4.16 C).

Whilst crystal violet covered the entirety of untreated *E. coli* ATCC 25922 pegs, a lower density of the stain was present, primarily as a band located further up the pegs containing *E. coli* treated with blue light activated XF-73 (Figure 4.16 C). Concentrations of XF-73 ranging from 16 – 256 µg/ mL had comparable densities of crystal violet, whereas at 512 µg/ mL a lower density was recorded. No trend in crystal violet density was recorded between concentrations of photo-activated XF-73 (Figure 4.16 C). Concentrations 64 – 256 µg/ mL of retreated XF-73 had the greatest retention of crystal violet, while 512 µg/ mL of retreated XF-73 had the lowest density of the stain, where the pigment was a darker blue (Figure 4.16 D). MHB had no retention of crystal violet on either plates nor did XF-73 alone on the photo-activated XF-73 treatment plate (Figure 4.16 C), a faint presence of crystal violet was recorded on the XF-73 controls on the XF-73 retreatment plate (Figure 4.16 D).

A decrease in crystal violet density was recorded between replicate pegs of *P. aeruginosa* ATCC 15442 biofilms following photo-activated XF-73 treatment (Figure 4.17 A). In comparison a lower crystal violet density was recorded between all replicate pegs of *P. aeruginosa* biofilms on the XF-73 (Figure 4.17 B). No stain was viewed on any control pegs of XF-73 or MHB alone on either treatment plates.

An increase in crystal violet retention was recorded on microtitre pegs containing *P. aeruginosa* ATCC 15442 biofilms treated with 16 – 64 µg/ mL of retreated XF-73 in comparison to untreated biofilms (Figure 4.17 B). XF-73 at 256 µg/ mL had the lowest density of crystal violet and a darker blue/ black pigment was observed at this concentration and at 512 µg/ mL, two replicates of 128 µg/ mL treated biofilms also had the same darker pigment. Neither MHB nor XF-73 alone retained crystal violet on the microtitre pegs.

Untreated *P. aeruginosa* ATCC 27853 biofilms had a lower density of crystal violet retained on replicate pegs in comparison to biofilms treated with 16 – 256 µg/ mL of photo-activated XF-73 (Figure 4.16 C). As the concentration of XF-73 increased from 16 – 128 µg/ mL a greater retention of crystal violet was observed. A change in pigment colouration from purple to black was noted at concentrations 256 µg/

mL and 512 µg/ mL of XF-73, a similar density of the stain was present between these two concentrations.

As the concentration of XF-73 on the retreatment plate decreased from 128 µg/ mL against *P. aeruginosa* ATCC 27853 biofilms, the presence of crystal violet also decreased, where 16 µg/ mL and 32 µg/ mL had a lower density in comparison to untreated biofilms (Figure 4.17 D). A darker purple pigment was recorded at concentrations 256 µg/ mL and 512 µg/ mL of XF-73 and the volume of crystal violet was greater than the crystal violet retained on untreated biofilms, the greatest density was recorded at 128 µg/ mL. Out of both XF-73 treatments used against *P. aeruginosa* ATCC 27853 biofilms, biofilms treated with photo-activated XF-73 followed by an additional XF-73 treatment yielded the greatest abundance of crystal violet. No crystal violet was present on control pegs of XF-73 and MHB alone on both treatment plates (Figure 4.17 C, D).
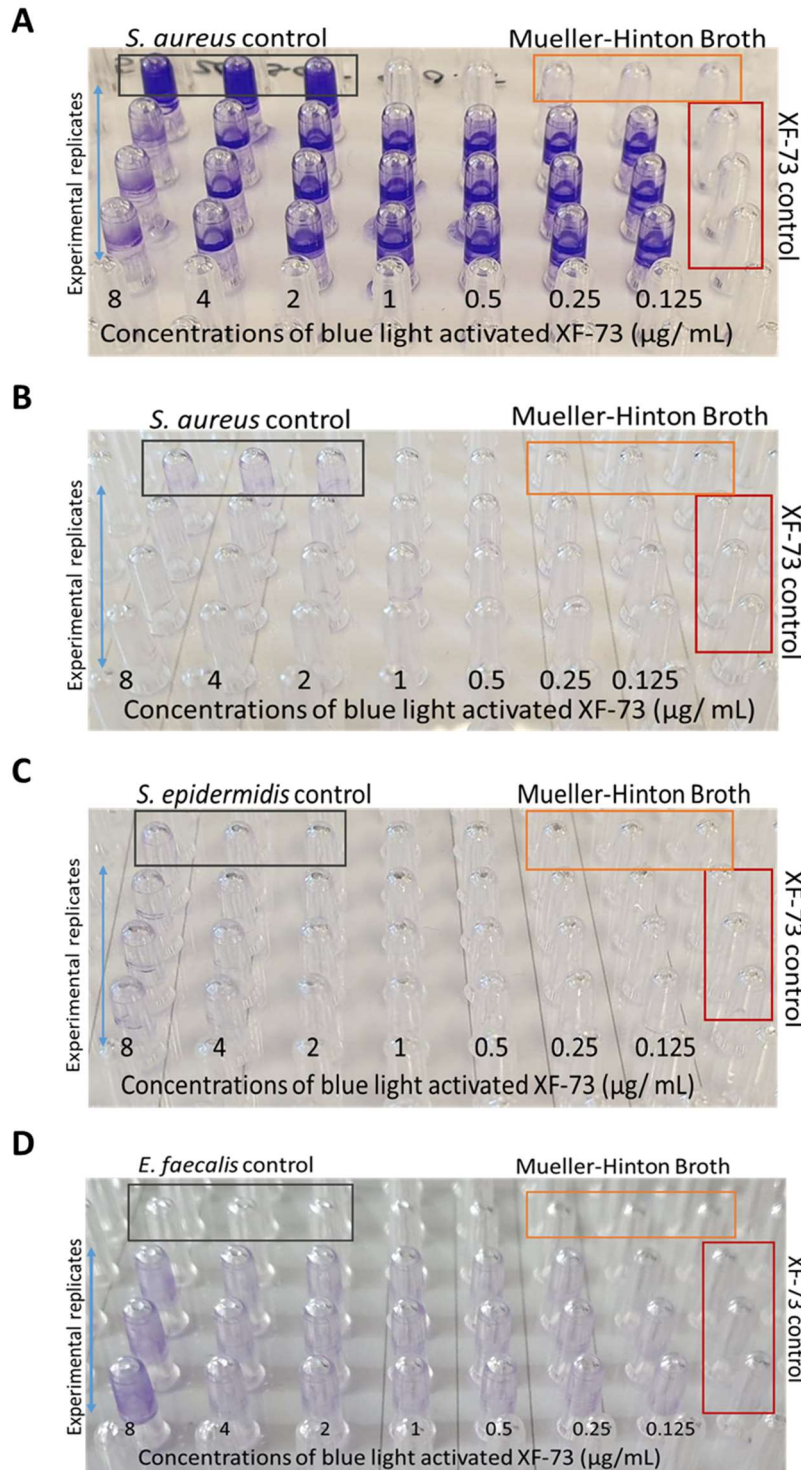
Figure 4.15 Density of Gram-positive twenty-four hour biofilms following blue light enhanced XF-73 treatment

Density of Gram-positive biofilms: (A) S. aureus ATCC 6538, (B) S. aureus ATCC 29213, (C) S. epidermidis ATCC 12228 and (D) E. faecalis ATCC 29212 via crystal violet staining following blue light activated XF-73 treatments at a concentration range of 0.125 – 8 µg/ mL. Controls of untreated biofilm, XF-73 alone and MHB alone are highlighted on each image.
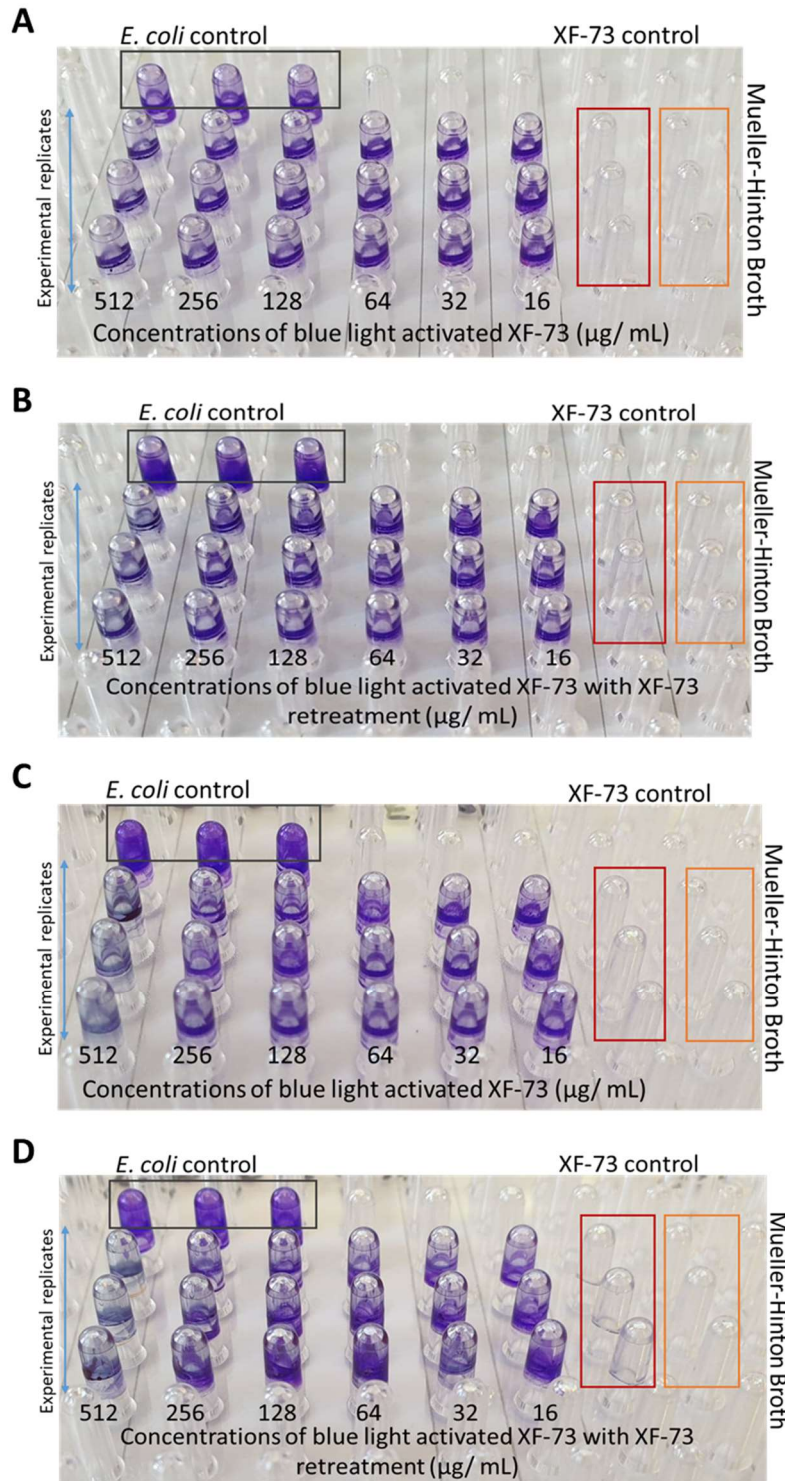
Figure 4.16 Density of E. coli twenty-four hour biofilms following blue light enhanced XF-73 treatment

Density of E. coli biofilms: E. coli ATCC 35218 (A & B) and E. coli ATCC 25922 (C & D) via crystal violet staining following blue light activated XF-73 treatments (A & C) and blue light activated XF-73 followed by XF-73 retreatment (B & D) at a concentration range of 16 – 512 µg/ mL. Controls of untreated biofilm, XF-73 alone and MHB alone are highlighted on each image.
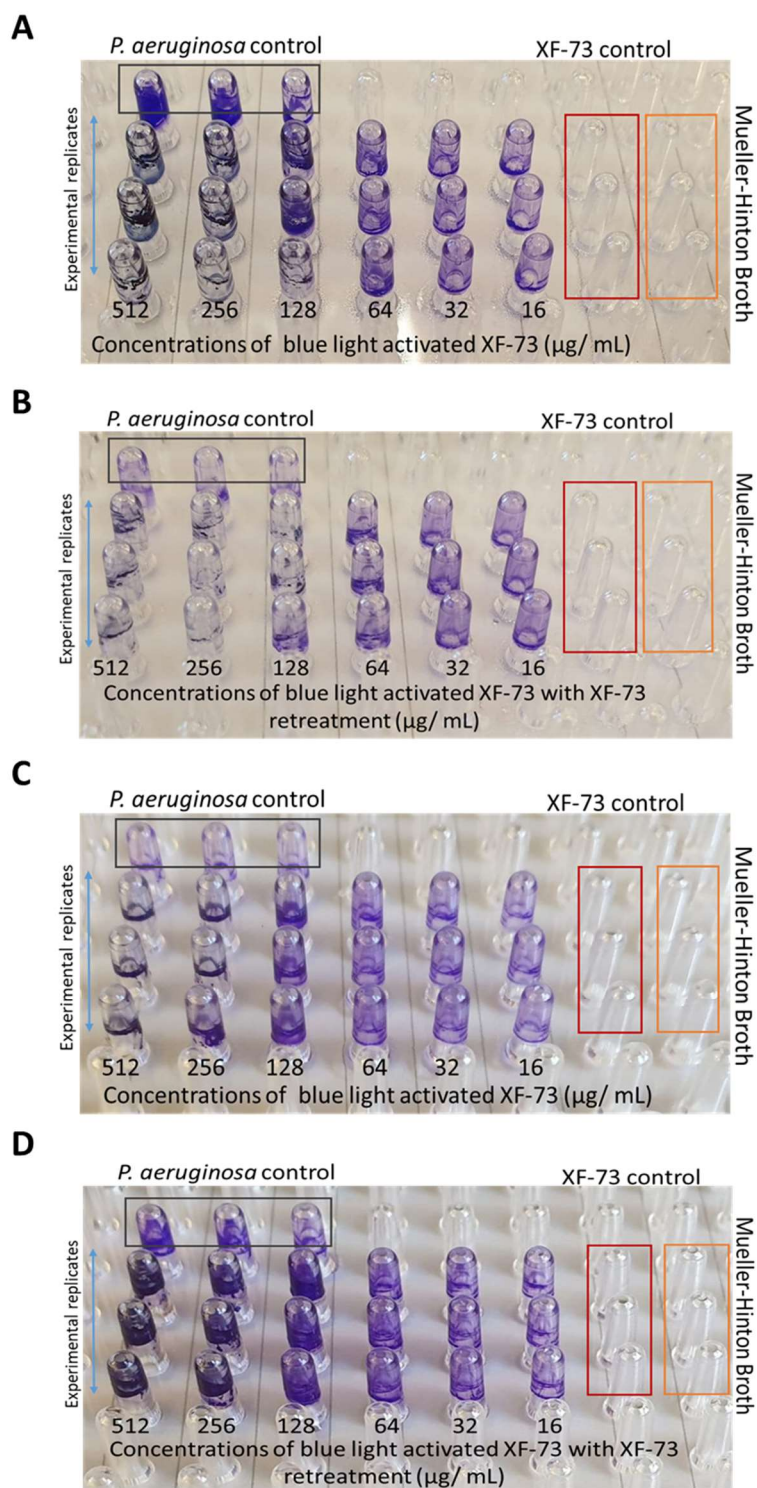
Figure 4.17 Density of P. aeruginosa twenty-four hour biofilms following blue light activated XF-73 treatment

Density of P. aeruginosa biofilms: P. aeruginosa ATCC 15442 (A & B) and P. aeruginosa ATCC 27853 (C & D) via crystal violet staining following blue light activated XF-73 treatments (A & C) and blue light activated XF-73 followed by XF-73 retreatment (B & D) at a concentration range of 16 – 512 µg/ mL. Controls of untreated biofilm, XF-73 alone and MHB alone are highlighted on each image.

4.5.6 Photo-activated XF-73 against forty-eight hour Gram-positive biofilms

4.5.6.1 MBIC and MBEC values of photo-activated XF-73 against forty-eight hour Gram-positive biofilms

Similar MBIC and MBEC values were recorded for each forty-eight hour Gram-positive biofilm treated with blue light activated XF-73 (Table 4.4). There was a 2-fold recorded increase in MBIC and MBEC concentrations for *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 in comparison to natural light XF-73 treatments. Despite the activation of XF-73 via blue light exposure, the MBIC and MBEC values for *S. aureus* ATCC 6538 and *E. faecalis* ATCC 29212 biofilms remained the same as the respective values determined for XF-73 in natural light.

Table 4.4 MBIC and MBEC values (µg/ mL) of blue light enhanced XF-73 against forty-eight hour Gram-positive biofilms

Table includes corresponding MIC and MBEC values (µg/ mL) of XF-73 in natural light

Microorganism/ ATCC	Natural Light (µg/ mL)		Blue Light (µg/ mL)	
	MBIC	MBEC	MBIC	MBEC
S. aureus 6538	256	256	256	256
S. aureus 29213	1	1	2	2
S. epidermidis 12228	1	1	2	2
E. faecalis 29212	4	4	4	4

4.5.6.2 Crystal violet staining of forty-eight hour Gram-positive biofilms treated with photo-activated XF-73

Microtitre pegs containing *S. aureus* ATCC 6538 biofilms after forty-eight hours of growth, had a crystal violet presence around the entirety of the peg with a strong purple pigment; the ends of the pegs were the only area lacking crystal violet (Figure 4.18 A). The addition of blue light activated XF-73 decreased the density of crystal violet, leaving only a faint purple colouration at concentrations ranging from 16 – 128 µg/ mL, density of crystal violet at these concentrations are all comparable; although 8 µg/ mL of XF-73 had a greater retention of crystal violet. Similarly, XF-73 at the highest concentrations used in this study: 256 µg/ mL and 512 µg/ mL also had a greater retention of crystal violet, compared to the other concentrations of XF-73, although lower than untreated *S. aureus* biofilms, a visible difference in crystal violet colouration was observed at these concentrations, where the pigment was a darker purple at higher XF-73 concentrations. MHB and XF-73 alone had no retained crystal violet.

No crystal violet was retained on any pegs containing *S. aureus* ATCC 29213 forty-eight hour biofilms in the presence and absence of blue light activated XF-73 (Figure 4.18 B). Additionally, no crystal violet was present on control pegs of MHB and XF-73 alone.

A faint trace amount of crystal violet was only recorded on *S. epidermidis* forty-eight hour biofilms (Figure 4.18 C), the abundance of crystal violet varied between each replicate, where one peg contained a greater amount of crystal violet in comparison to the other two pegs. The addition of any concentration of blue light activated XF-73 led to no retention of crystal violet. In addition, XF-73 and MHB alone also lacked crystal violet on these control pegs.

Each concentration of blue light activated XF-73 used against forty-eight hour *E. faecalis* biofilms led to a decrease of crystal violet retention in comparison to untreated biofilms, leaving only a thin purple circle around the tops of the microtitre pegs (Figure 4.18 D). A similar abundance of crystal violet was recorded between each XF-73 treatment. Controls of MHB and XF-73 alone lacked any crystal violet stain on each replicate peg.
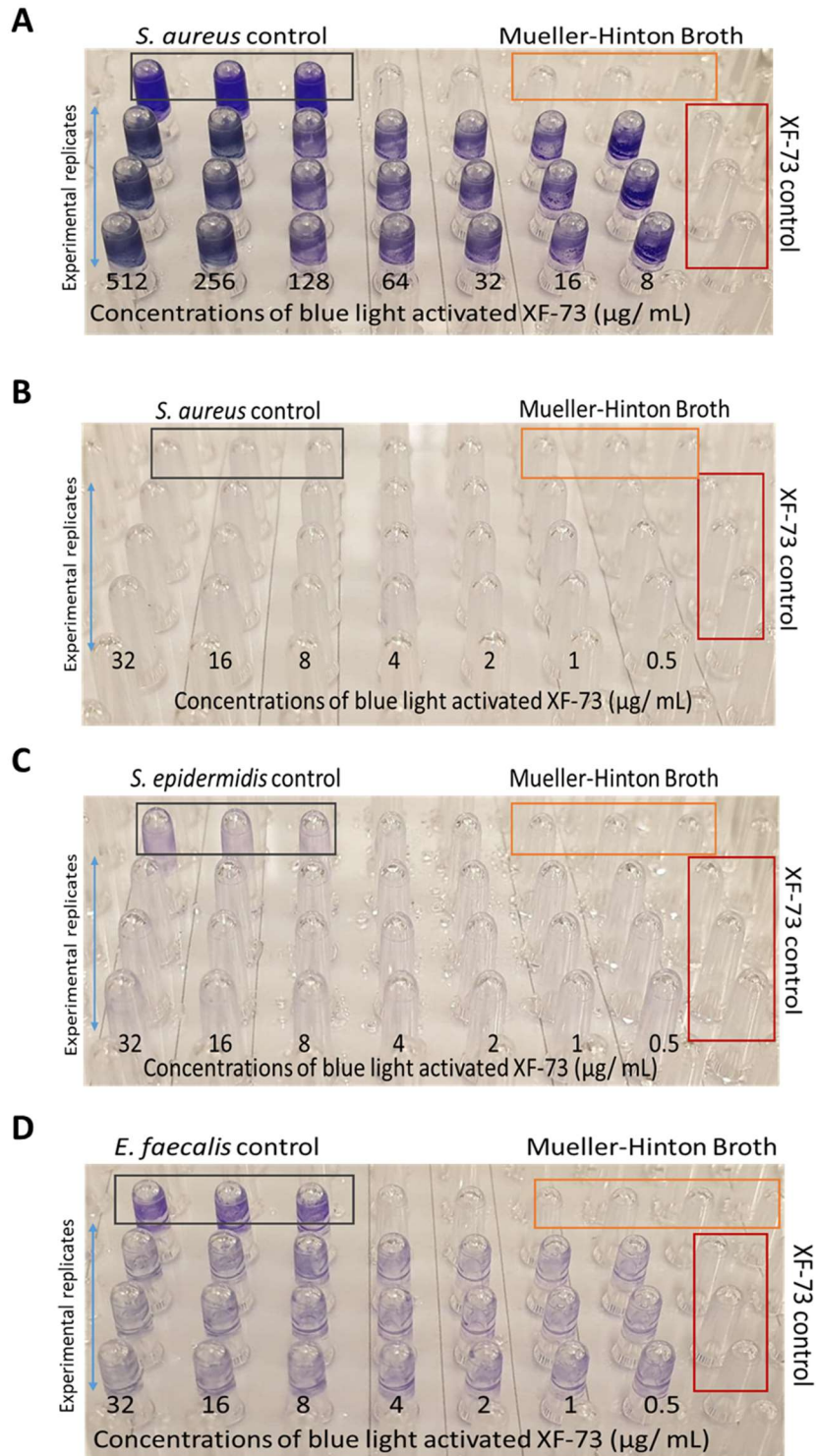
Figure 4.18 Density of Gram-positive forty-eight hour biofilms following blue light enhanced XF-73 treatment

Density of forty-eight hour Gram-positive biofilms: (A) S. aureus ATCC 6538, (B) S. aureus ATCC 29213, (C) S. epidermidis ATCC 12228 and (D) E. faecalis ATCC 29212 via crystal violet staining following blue light activated XF-73 treatments at a concentration range of 0.5 – 512 µg/ mL. Controls of untreated biofilm, XF-73 alone and MHB alone are highlighted on each image.

4.6 Discussion

The antimicrobial properties of XF-73's secondary mechanism of action through blue light enhancement was successfully assessed against a range of Gram-positive and Gram-negative microorganisms in planktonic and biofilm modes of growth. MIC/ MBC testing demonstrated the enhanced bactericidal properties of XF-73 through blue light activation against planktonic microorganisms, whilst MBIC/ MBEC testing recorded an enhanced anti-biofilm capability of XF-73 following blue light exposure against a range of clinically relevant twenty-four Gram-positive biofilms. Though the enhanced anti-biofilm capability of XF-73 in comparison to XF-73 in natural light was not retained against forty-eight hour Gram-positive biofilms.

Preliminary testing demonstrated that blue light alone had no direct inhibitory effect against planktonic *E. coli* ATCC 35218 growth (Figure 4.2). In contrast, thirty minutes' exposure to blue light inhibited growth of *S. aureus* ATCC 6538 (Figure 4.1). All further experiments involving blue light exposure were limited to a maximum of thirty minutes. Previous studies have also focused on the antibacterial properties of blue light alone. Gupta *et al.,* (2015) tested the antibacterial properties of blue light (at 405 nm) against a range of Gram-positive and Gram-negative planktonic cells; the results of which demonstrated Gram-positive microorganisms to be more susceptible to inactivation in comparison to Gram-negative bacterial cells. *S. aureus* strains were inactivated following twenty minutes of blue light exposure whereas *E. coli* was inactivated following three hundred minutes of exposure. The study also included strains of *S. epidermidis, E. faecalis* and *P. aeruginosa*; *S. epidermidis* was inactivated after the shorter exposure time of sixteen minutes, whereas *E. faecalis* required one hundred and twenty minutes of blue light exposure. *P. aeruginosa* was more susceptible to blue light in comparison to *E. coli* and was inactivated after ninety minutes (Gupta *et al.*, 2015). These results support the findings collected in the initial part of this study, where *S. aureus* showed a greater susceptibility to blue light inhibition in comparison to *E. coli*. As this preliminary study was limited to a single Gram-positive and a single Gram-negative microorganism, further testing would include the full panel of microorganisms (Table 3.1). Additionally, a longer period of blue light exposure would be required to enable an inhibitory effect of blue light against Gram-negative microorganisms, as previous studies have illustrated a long exposure time is required to affect Gram-negative microorganisms.

Further testing involving the combination treatment of XF-73 and blue light against *S. aureus* and *E. coli* planktonic cells in log phase growth confirmed that thirty minutes exposure to blue light alone inhibited *S. aureus* growth, therefore the combined effect of XF-73 and blue light exposure against bacterial growth could not be determined. This was highlighted by the different absorbance readings of *S. aureus* growth controls in the presence and absence of blue light (Figure 4.3). This was supported as the natural light study recorded XF-73 inhibiting *S. aureus* growth after five minutes as the absorbance readings plateaued and end point statistical analysis indicated a significant difference in

growth (p= <0.0001), meanwhile the difference between the growth control and treated *S. aureus* in blue light was less significantly different (p= 0.0159). These results influenced further testing involving blue light enhanced XF-73 against bacterial growth, as a decreased exposure time was included; similarly, future experiments were conducted over a longer time frame in order to conclude if the inhibitory effect of XF-73 was increased following blue light enhancement (Figure 4.5, 4.6).

Following the five-hour time course studies, thirty minutes of blue light exposure led to complete inhibition of *S. aureus* growth, as both the growth control and XF-73 treatment in blue light showed no change in absorbance readings throughout the study. Meanwhile the growth control in natural light exhibited bacterial growth throughout, indicated by the increase in absorbance readings, whereas XF-73 treatment showed no change in absorbance readings (Figure 4.5). Therefore, blue light enhanced XF-73 treatment cannot be concluded as the only factor leading to bacterial inhibition. By reducing the exposure time of blue light to fifteen minutes, the growth control was no longer inhibited by blue light alone, as a similar growth curve was recorded between the two *S. aureus* growth controls in natural light and fifteen minutes blue light exposure (Figure 4.6). Both XF-73 treatments, in the presence and absence of blue light led to the inhibition of *S. aureus* growth as no increase in absorbance readings was recorded for either treatment. The limitation of this study was the inability to differentiate the antibacterial effect of the two different mechanisms of action XF-73 exhibits. The results gained from this study concluded that fifteen minutes of blue light exposure was the appropriate exposure time where blue light alone had no antibacterial effect against bacterial growth. Absorbance readings can only determine inhibition of bacterial growth, and as XF-73 in natural and blue light led to immediate inhibition of *S. aureus* growth, an assessment between the efficacies of both treatments cannot be concluded from these tests alone. Therefore, future testing employed a longer time frame to determine how long XF-73 retained its inhibitory effect in natural and blue light and to record any potential difference between the two.

In accordance with the results previously collected (Figure 4.2), *E. coli* showed no susceptibility to blue light exposure or blue light enhanced XF-73 treatment (Figure 4.4). *E. coli* had greater absorbance readings after thirty minutes exposure to blue light and XF-73 treatment in comparison to the growth control in blue light alone, though there was no significant difference between growth rates. In contrast a significant difference was recorded between the growth control and XF-73 treatment of *E. coli* in natural light (p= 0.0026). These results indicate that there was a decrease in the efficacy of XF-73 following blue light exposure against *E. coli* planktonic cells and that the secondary mechanism of action of XF-73 activated via blue light was less effective than the standard membrane disruption mechanism of action in natural light, where previous studies have recorded potassium and ATP leakage from the bacterial cells (Ooi *et al.*, 2009b).

Through MIC and MBC assays, both batches of XF-73 used in this study showed an enhanced bactericidal effect against Gram-positive microorganisms following blue light exposure, as blue light enhanced XF-73 was capable of eradicating Gram-positive planktonic cells at a lower concentration in comparison to XF-73 treatments in natural light (Table 4.1, 4.2). Blue light enhanced XF-73 was determined to have a greater efficacy against Gram-positive microorganisms in comparison to Gram-negative microorganisms, as only batch 2 of XF-73 also exhibited an enhanced bactericidal effect against all Gram-negative microorganisms tested (Table 4.2). Batch 1 XF-73 was also shown to be less effective against Gram-negative microorganisms in natural light in comparison to batch 2 XF-73, as batch 1 XF-73 failed to generate MIC/ MBC values, therefore no change in efficacy was recorded for batch 1 XF-73 against the Gram-negative microorganisms (Table 4.3). These results indicate that the antibacterial efficacy of different batches of XF-73 fluctuates against Gram-negative microorganisms, and without experimental replicates and different batches of XF-73, this aspect would have been missed.

Limited research has previously been conducted on the antibacterial properties of photo-activated XF-73 against a range of microorganisms, indicating this is a novel area of research focusing on enhancing the efficacy of XF-73 against a range of microorganisms. Furthermore, the research also highlights a potential application for this form of treatment. Board-Davies *et al.,* (2022) also reported an enhanced inhibitory effect of XF-73 following fifteen minutes blue light exposure against a range of staphylococcal strains and Gram-negative microorganisms, through MIC and MBC assays. Similar concentration ranges were recorded in comparison to results collected in this current study as photo-activated XF-73 was noted to inhibit multiple strains of *S. aureus* growth at concentrations ranging from 0.03 – 0.05 μg/ mL (Board-Davies *et al.*, 2022). Therefore, the anti-staphylococcal efficacy of photo-activated XF-73 is retained between different staphylococcal strains, further highlighting the potential for XF-73 to be a potent antibacterial treatment against staphylococcal infections, where blue light exposure further aids in the antibacterial capability of XF-73. Board-Davies *et al.,* (2022) also reported an enhanced antibacterial effect of XF-73 following blue light activation against *P. aeruginosa* and *E. coli* planktonic cells at 64 μg/ mL and 256 μg/ mL respectively. Similar concentrations of XF-73 were shown to inhibit *P. aeruginosa* between this study and previous research, whereas the results collected from this study showed *E. coli* to have a greater susceptibility in to photo-activated XF-73 as concentrations 16 μg/ mL and 32 μg/ mL led to bacterial inhibition (Table 4.2). Therefore, the antibacterial properties of photo-activated XF-73 is not maintained between different species of *E. coli.*

Gram-negative bacteria are encased in an outer membrane, which protects the cell from antibiotic treatments by acting as a penetration barrier. The outer component of this membrane comprises lipopolysaccharides and is responsible for the prevention of hydrophobic solutes entering the cell through passive diffusion (Zhang *et al.*, 2013). Initial research which focused upon the antibacterial effect of light treatments against microorganisms has illustrated that Gram-negative bacteria are less

susceptible to toxicity via the production of singlet oxygen in comparison to Gram-positive bacteria, due to the structural differences between the two types of microorganisms. Due to the size of standard PSs, whose molecular masses range between 1500 – 1800 Da (Dalton), the cell envelope of Gram-negative microorganisms prevents large molecules from entering the cell. Therefore, PSs with a small molecular mass have a greater likelihood of entering into Gram-negative bacterial cells which may subsequently impact upon the overall efficacy of PDT. The lipopolysaccharide outer layer of the outer membrane acts as a barrier, preventing singlet oxygen accessing target sites inside the cells. Additionally, the outer membrane offers target sites for singlet oxygen that are not detrimental to bacterial survival, as the outer membrane is comprised of unsaturated fatty acids and proteins, compounds known to react with singlet oxygen (Dahl *et al.*, 1989; Meerovich *et al.*, 2020).

E. coli, P. aeruginosa and other proteobacteria produce glutathione, a tripeptide that has a key role by protecting the microorganism from oxidative stress; additionally glutathione also maintains the oxidation states of protein thiols to prevent a build-up of ROS and offers the cells further protection from osmotic stress, low pH environments and from the presence of chlorine compounds (Masip *et al.*, 2006). Reduced and oxidized forms of glutathione exist in cells and the ratio of these two states determines the redox status of the cells, aiding in cell survival during oxidative stress (Pizzorno, 2014). Previous research has determined that many Gram-positive microorganisms are unable to synthesise glutathione (Vergauwen *et al.*, 2013).

Whilst photo-activated XF-73 showed an enhanced killing activity against Gram-negative microorganisms in comparison to XF-73 in natural light, Gram-negative bacteria are less susceptible to ROS due to additional mechanisms of resistance and in turn are less susceptible to activated XF-73 in comparison to Gram-positive bacterial cells.

Blue light exposure has a promising clinical application against dermatology-related infections, in particular surface tissue infections. Prior research has shown that blue light kills *S. aureus*, a microorganism commonly found on the surface of the skin. Additionally, blue light is absorbed in multiple mammalian cell types which have the capability of producing ROS, therefore can act as natural PSs for PDT (Gwynne *et al.*, 2018). The incorporation of XF-73 in blue light treatments against dermal *S. aureus* infections has the potential of enhancing the efficacy of the treatment, offering a potential alternative therapeutic use of XF-73.

Whilst an enhanced effect was recorded for photo-activated XF-73 against the panel of Gram-positive and Gram-negative planktonic cells, blue light exposure, reduced the efficacy of XF-73 against *C. albicans* as a 2-fold increase in XF-73 concentration was required to eradicate the growth of the yeast in comparison to XF-73 in natural light (Table 4.1). Previous studies focusing on the antibacterial properties of photo-activated XF-73 against planktonic *C. albicans* showed an enhanced killing effect in comparison to the efficacy of XF-73 in natural light. A 6-log reduction in *C. albicans* viable cells was

recorded following XF-73 treatment at 0.5 µM (0.383 µg/ mL) and fifteen minutes blue light exposure (Gonzales *et al.*, 2013). Therefore, comparing the data collected from this study and results recorded from previous studies highlights a variation between the antimicrobial efficacy of photo-activated XF-73 against different strains of *C. albicans*.

As photo-activated XF-73 exhibited an enhanced bactericidal effect against a range of microorganisms in planktonic mode of growth, subsequent experiments in this study focused on assessing the antimicrobial efficacy of photo-activated XF-73 against the same panel of microorganisms in biofilm mode of growth. An enhanced anti-biofilm effect was recorded with photo-activated XF-73, following fifteen minutes exposure to blue light against a range of biofilms: *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *S. epidermidis* ATCC 12228 and *E. coli* ATCC 25922 (Table 4.3). These results indicate that XF-73 exposed to blue light leads to an enhanced anti-biofilm effect against a range of Gram-positive biofilms. However, the efficacy of XF-73 against *S. aureus* ATCC 6538 biofilms decreased following blue light exposure as no concentration under 8 µg/ mL was able to eradicate biofilm growth, whereas 1 µg/ mL of XF-73 eradicated the bacterial biofilms in natural light. Previous studies have also reported a decreased efficacy of XF-73 against Gram-positive biofilms following blue light exposure. Out of three *S. aureus* strains tested, a 2-fold increase in the MBEC values were recorded against two strains, whilst the MBEC remained the same for the final strain, additionally an increased concentration of XF-73 was required to eradicate *S. hominins* biofilms following blue light exposure (Board-Davies *et al.*, 2022). These results indicate that the anti-biofilm capability of photo-activated XF-73 fluctuates against staphylococcal biofilms as an enhanced and reduced activity of XF-73 was recorded between different staphylococcal strains.

A key virulence factor for *S. aureus* is the production of staphyloxanthin, and over 90% of isolates produce this carotenoid pigment. Staphyloxanthin is located in the bacterial membrane and is key to the environmental fitness of the microorganism, especially in harsh environments; by increasing resistance to dehydration and fatty acids in the skin, such as linoleic acid (Zhang *et al.*, 2018; Sun *et al.*, 2020). Historic research has also highlighted that carotenoid pigments have the capability to quench singlet oxygen, therefore protecting bacterial cells from the toxicity of PDT using either endogenous or exogenous PSs (Dahl *et al.*, 1989). As staphyloxanthin possesses an antioxidant capability, *S. aureus* strains containing the pigment are protected from the host immune response of neutrophils producing ROS, further increasing the virulence factor of *S. aureus* (Sun *et al.*, 2020). The presence of staphyloxanthin is visualised due to the pigment being located in the membrane and the golden-yellow pigment influences the colour of the bacterial cell colonies. *S. aureus* ATCC 6538 has been previously selected for testing the staphyloxanthin inhibition due to the strong golden colouration of the cells. Askoura *et al.*, (2022) reported that when staphyloxanthin synthesis was inhibited by terbinafine, an allylamine used as an antifungal agent, *S. aureus* became more susceptible to oxidative and acidic stress. Additionally membrane fluidity increased following alteration of the abundance of

staphyloxanthin in the membrane, indicating that the pigment is also responsible for regulating the membrane fluidity (Askoura *et al.*, 2022). Whilst *S. aureus* ATCC 29213 has a lighter colour pigment in comparison to *S. aureus* ATCC 6538, staphyloxanthin is still present in the membrane of *S. aureus* ATCC 29213 cells (Suzzi *et al.*, 2020). It might be concluded that the decreased efficacy of photo-activated XF-73 against *S. aureus* ATCC 6538 biofilms and the *S. aureus* strains used in the study conducted Board-Davies *et al.,* (2022) in comparison to natural light treatment of XF-73 is due to these strains having a greater abundance of staphyloxanthin present in the bacterial membrane, protecting the organisms from the secondary mechanism of action of XF-73. As *S. aureus* ATCC 29213 is less pigmented than *S. aureus* ATCC 6538, this strain may have less staphyloxanthin present leading to no resistance to XF-73 treatment in the presence of blue light.

Whilst a promising anti-biofilm effect was recorded for photo-activated XF-73 against twenty-four hour Gram-positive biofilms, blue light activation was shown not to improve the anti-biofilm capability of XF-73 against forty-eight hour Gram-positive biofilms. In contrast, this treatment strategy was determined to be less effective in comparison to XF-73 in natural light against *S. aureus* ATCC 29213 and *S. epidermidis* biofilms and had no effect upon the killing capability of XF-73 against *S. aureus* ATCC 6538 and *E. faecalis* ATCC 29212 biofilms (Table 4. 4). These initial results indicate that photo-activated XF-73 does not retain its anti-biofilm efficacy against mature Gram-positive biofilms in comparison to twenty-four hour Gram-positive biofilms. However, as 8 µg/ mL of photo-activated XF-73 was the maximum concentration used against twenty-four hour biofilms no MBIC/ MBEC values were recorded against *S. aureus* ATCC 6538, suggesting that MBIC/MBEC values for twenty-four and forty-eight hour biofilm growth cannot be compared (Table 4.5). This is the first reported study assessing the antibacterial properties of photo-activated XF-73 against forty-eight hour biofilms, and no previous work is available for comparison. As four Gram-positive microorganisms were incorporated in this study, a larger sample size would be required in further work to fully determine the anti-biofilm effect of photo-activated XF-73 against mature biofilms.

Previous studies have also reported that the age of *Streptococcus mutans* biofilms influenced the efficacy of PDT against biofilm viability; where seven-day and ten-day biofilms showed a decreased susceptibility to PDT involving toluidine blue O and light-emitting diode exposure, or exposure to helium/ neon laser light. Supporting the results of a reduced susceptibility of PDT involving XF-73 against older biofilms (Table 4.5). Confocal laser scanning microscopy has also shown that PDT treatment led to cell death at the outermost layers of the biofilms, leaving bacterial cells located in the centre of the biofilms untouched by treatment. These results indicate that PDT treatments are unable to fully penetrate into the biofilm, leading to cell survival allowing the biofilm to re-establish (Zanin *et al.*, 2005).

Table 4.5 MBIC and MBEC values (µg/ mL) of photo-activated XF-73 against twenty-four and forty-eight hour Gram-positive biofilms

Microorganism/ ATCC	Twenty-four hour biofilms XF-73 (µg/ mL)		Forty-eight hour biofilms XF-73 (µg/ mL)	
	MBIC	MBEC	MBIC	MBEC
S. aureus 6538	> 8	> 8	256	256
S. aureus 29213	0.125	0.125	2	2
S. epidermidis 12228	≤ 0.125	≤ 0.125	2	2
E. faecalis 29212	0.5	0.5	4	4

Interestingly, *E. coli* ATCC 25922 was the only Gram-negative biofilm with a recorded MBEC following photo-activated XF-73 treatment (Table 4.3), indicating Gram-negative bacterial biofilms are less susceptible to ROS generated by photo-activating XF-73 in comparison to Gram-positive biofilms. Similarly, previous studies support the data reported in this current study. In the study by Board-Davies *et al*., (2022) the antibacterial property of XF-73 following fifteen minutes blue light exposure against twenty-four hour *P. aeruginosa, E. coli* and *A. baumannii* biofilms; only *A. baumannii* biofilms showed susceptibility to photo-activated XF-73 as the MBEC concentration decreased from 1024 µg/ mL (XF-73 in natural light) to 128 µg/ mL (Board-Davies *et al*., 2022). As Gram-negative biofilms remained unaffected by photo-activated XF-73 treatment, an additional treatment strategy was used against these biofilms. This involved the Gram-negative biofilms treated with XF-73 and photo-activated by blue light for fifteen minutes. Following this treatment, the microtitre peg lids containing the biofilms were transferred into a fresh challenge plate containing the range of XF-73 solutions which had not been exposed to blue light before incubation.

This additional treatment strategy was added to the study to try and further explore the resistance Gram-negative biofilms exhibit against PDT and was determined to be successful against *E. coli* biofilms. Both blue light and the blue light retreatment strategy generated an MBEC value of 512 µg/ mL against *E. coli* ATCC 25922 twenty-four hour biofilms. However, through MBIC/ MBEC studies alone, no increase in the anti-biofilm effect of XF-73 was recorded against the remaining Gram-negative biofilms following the additional treatment strategy (Table 4.3).

Previous research has shown that Gram-negative microorganisms are less susceptible to ROS in comparison to Gram-positive microorganisms in planktonic mode of growth (Dahl *et al*., 1989); Gram-negative microorganisms in biofilm mode of growth also have a greater resistance to ROS in comparison to Gram-positive biofilms. This was highlighted in previous research, where PDT treatment was recorded to generate a greater reduction of biofilm viability against *E. faecalis* biofilms in comparison to *P. aeruginosa* biofilms (Garcez *et al*., 2013). Although both Gram-positive and Gram-

negative microorganisms are less susceptible to PDT in biofilm mode of growth in comparison to planktonic cells. This was illustrated in the current study as greater concentrations of photo-activated XF-73 was required to eradicate microorganisms in biofilm mode of growth in comparison to planktonic cells (Table 4.2, 4.3). Previous research also supports this statement as planktonic cells were determined to be more susceptible to PDT in comparison to the same microorganisms in biofilm mode of growth (Fontana *et al.*, 2009; Garcez *et al.*, 2013).

The inclusion of biofilm viability testing illustrated an anti-biofilm capability of photo-activated XF-73 against Gram-negative biofilm that was previously missed from MBIC/ MBEC assays alone. Additionally, the enhanced effect of photo-activated XF-73 against Gram-positive biofilms was further observed.

The decreased efficacy of XF-73 following blue light exposure against twenty-four hour *S. aureus* ATCC 6538 biofilms was illustrated during biofilm viability testing as concentrations 0.125 – 1 μg/ mL of photo-activated XF-73 had no effect on bacterial growth; as no statistically significant difference ($p > 0.05$) was recorded between these concentrations and untreated *S. aureus* growth (Figure 4.7). Originally, XF-73 at 1 – 8 μg/ mL led to a biofilm viability reading below the lower limit of detection, but only 8 μg/ mL of photo-activated XF-73 yielded a viability reading below the lower limit of detection (Table 4.6). MBEC testing concluded that no concentration of XF-73 used in this study eradicated *S. aureus* ATCC 6538 biofilms (Table 4.3), therefore ≤ 10 cells were determined to be present in biofilm samples following 8 μg/ mL of photo-activated XF-73 treatment. For the remaining Gram-positive biofilms, the lowest concentration of blue light activated XF-73 leading to a biofilm viability below the lower limit of detection (Table 4.6) was the same concentration to corresponding MBEC values for each microorganism (Table 4.3). Therefore, no viable cells were present at concentrations of XF-73 with classified viabilities below the lower limit of detection.

Photo-activated XF-73 at each concentration used in this study was capable of reducing the viability of *E. coli* biofilms in comparison to corresponding control growth (Figure 4.11, 4.12), signifying that whilst blue light activated XF-73 was unable to fully eradicate *E. coli* ATCC 35218 (Table 4.3), concentrations 128 – 512 μg/ mL of photo-activated XF-73 and concentrations 16 – 512 μg/ mL of retreated XF-73 reduced the biofilm viability below the lower limit of detection (Table 4.6).

Photo-activated XF-73, and retreatment XF-73 at 512 μg/ mL eradicated *E. coli* ATCC 25922 biofilms (Table 4.3). Therefore, the corresponding biofilm viability reading, below the lower limit of detection at 512 μg/ mL of XF-73 retreatment is concluded to have no viable cells present in the sample and 16 – 256 μg/ mL is determined to have ≤ 10 viable cells remaining in the bacterial samples (Table 4.6). The retreatment strategy of XF-73 was determined to be most effective XF-73 treatment and had the lowest recorded biofilm viability readings. In contrast, photo-activated XF-73 led to biofilm viability readings greater than natural light XF-73 treatment and XF-73 retreatment and had no concentration capable of

reducing the biofilm viability below the lower limit of detection (Table 4.6). *E. coli* ATCC 25922 had a biofilm viability of 203 CFU/ mL following treatment of 512 µg/ mL photo-activated XF-73. In contrast MBIC/ MBEC studies concluded that 512 µg/ mL eradicated biofilm growth (Table 4.3), therefore the bactericidal effect of photo-activated XF-73 was found to fluctuate between experimental replicates.

Photo-activated XF-73 and the retreatment strategy of XF-73 were unable to inhibit or eradicate either *P. aeruginosa* biofilms (Table 4.3). These results were supported by biofilm viability testing as no concentration of XF-73 used in this study led to a biofilm viability reading below the lower limit of detection against *P. aeruginosa* ATCC 27853 biofilms.

Table 4.6 Lowest concentration of XF-73 (µg/ mL) in the presence and absence of light against a range of twenty-four hour Gram-positive and Gram-negative biofilms leading to a biofilm viability below the lower limit of detection

Microorganism/ ATCC number	Lowest concentration of XF-73 (µg/ mL) leading to a LLoD		
	Natural Light	Blue Light	Blue Light + retreatment
S. aureus 6538	1	-	N/A
S. aureus 29213	1	0.125	N/A
S. epidermidis 12228	1	0.125	N/A
E. faecalis 29212	2	0.5	N/A
E. coli 35218	256	128	16
E. coli 25922	128	-	16
P. aeruginosa 15442	-	512	-
P. aeruginosa 27853	-	-	-

Note: - indicates that no XF-73 concentration was capable of generating a biofilm viability below the lower limit of detection. N/A indicates where the study was not conducted

As previously stated (3.6), eluting crystal violet following staining of treated biofilms was not possible due to the red pigment of XF-73 interfering with the colour of crystal violet, leading to inaccurate absorbance readings for biofilms treated with high concentrations of XF-73. Therefore, crystal violet stains of biofilms following photo-activated XF-73 treatment was purely to visualise the effect of the antimicrobial on biofilm density retained on the microtitre pegs.

While no concentration of photo-activated XF-73 was able to eradicate twenty-four hour *S. aureus* ATCC 6538 biofilms, crystal violet staining illustrated each concentration of photo-activated XF-73 led to a decrease in biofilm density as less stain was retained on the treated pegs, in comparison to the growth control (Figure 4.15 A).

A weak retention of crystal violet was recorded on microtitre pegs containing twenty-four hour *S. epidermidis* ATCC 12228 biofilms. As previously discussed (3.7), these microorganisms have previously shown a weak capability to retain crystal violet stain, highlighted by the growth controls (Figure 4.15 C). Since 0.125 µg/ mL of photo-activated XF-73 eradicated bacterial growth (Table 4.3), the lack of pigment present during crystal violet staining signifies that biofilm eradication has occurred

and the biofilms are no longer adhered onto the microtitre pegs. *S. epidermidis* ATCC 12228 treated with photo-activated XF-73 at 4 µg/ mL and 8 µg/ mL showed a faint presence of crystal violet, whilst no viable cells remained in the biofilm following XF-73 treatment, portions of the extracellular matrix may have remained on the microtitre pegs leading to the retention of crystal violet stain (Latka *et al.*, 2020).

Similarly, the retention of crystal violet on twenty-four hour *E. faecalis* ATCC 29212 biofilms following 0.5 – 8 µg/ mL of photo-activated XF-73 may also be due to extracellular polymer matrix remaining on the microtitre pegs as the MBEC was concluded to be 0.5 µg/ mL (Table 4.3), therefore no viable cells are present in these samples. Alternatively, as biofilm eradication was confirmed through MBIC/ MBEC testing and biofilm viability testing (Figure 4.10), whilst no viable cells remain on the microtitre pegs, biofilm dispersal may not have occurred, and dead bacterial cells may still remain on the pegs along with the biofilm matrix. As crystal violet does not differentiate between live or dead cells, the stain present following antibacterial treatment may signify the presence of dead biofilms still adhered to the pegs. However, no crystal violet as present on microtitre pegs of untreated *E. faecalis* biofilms, indicating that crystal violet staining could not be used to determine effect of treatments upon biofilm cell presence for this organism.

S. aureus ATCC 29213 biofilms were also shown to be unable to retain crystal violet as well as *S. aureus* ATCC 6538, as little crystal violet stain was retained on untreated biofilm control pegs (Figure 4.15 A, B). All concentrations of photo-activated XF-73 used in this study eradicated bacterial growth of *S. aureus* ATCC 29213 biofilms (Table 4.3); therefore, as only a few replicate pegs per XF-73 treatment retained the stain, this indicates that biofilm dispersal occurs but not to the same extent for all *S. aureus* biofilms.

The MBEC of photo-activated XF-73 against forty-eight hour *S. aureus* ATCC 6538 biofilms was recorded to be 256 µg/ mL (Table 4.4). Therefore, as a high density of crystal violet was retained on biofilms treated with 256 µg/ mL and 512 µg/ mL of photo-activated XF-73 these results indicate that while the bacterial cells have been killed at these concentrations, biofilm dispersal had not occurred, hence the density similar to that of the growth control was observed (Figure 4.18 A). Concentrations of XF-73 below 256 µg/ mL had a recorded decrease in crystal violet density in comparison to the growth control, signifying that whilst these concentrations were unable to inhibit or eradicate bacterial growth, the density of the biofilm was reduced. In contrast, no crystal violet was retained on microtitre pegs containing forty-eight hour *S. aureus* ATCC 29213 biofilms (Figure 4.18 B), further signifying a difference between strains in the composition of extracellular polymer matrix.

A greater density of crystal violet was noted on the control pegs of forty-eight hour *S. epidermidis* ATCC 1228 biofilms, highlighting that a greater density of bacterial cells are present in older biofilms (Figure 4.18 C). The lack of crystal violet present on the bacterial pegs following blue light and XF-73 treatment

indicates an absence of biofilms. However, MBIC/MBEC testing contradicts this observation, as the MBEC concludes that biofilm eradication occurs following XF-73 treatment at 2 µg/ mL (Table 4.4). Therefore, the failure to retain crystal violet in *S. epidermidis* biofilms leads to false positives of biofilm eradication following low concentrations of photo-activated XF-73.

Untreated *E. faecalis* ATCC 29212 biofilms retained crystal violet following forty-eight hours of growth (Figure 4.18 C), once again highlighting that mature biofilms have a greater abundance of negatively charged molecules present in the biofilm, enabling an increased binding of crystal violet to the biofilms (Latka *et al.*, 2020). As every concentration of photo-activated XF-73 used in this study led to a decreased density of crystal violet present on the sample pegs in comparison to the growth control, the presence of photo-activated XF-73 at each concentration greatly decreased the density of *E. faecalis* biofilms remaining on the microtitre pegs. Photo-activated XF-73 at concentrations of 4 µg/ mL led to the eradication of forty-eight hour *E. faecalis* biofilms (Table 4.4); therefore the presence of crystal violet on *E. faecalis* biofilms treated with XF-73 at concentrations 4 – 32 µg/ mL is determined to be the remnants of eradicated biofilms that had not yet dispersed from the sample pegs.

A greater density of crystal violet was retained on untreated microtitre pegs containing twenty four-hour *E. coli* and *P. aeruginosa* biofilms (Figure 4.16) in comparison to untreated *S. aureus* ATCC 29213, *S. epidermidis* and *E. faecalis* biofilms (Figure 4.15 B, C, D). Biofilm viability testing supported this observation as, on average, the Gram-negative untreated biofilms had a larger biofilm viability in comparison to Gram-negatives (Figure 4.7 – 4.14). Therefore, Gram-negative biofilms have a larger bacterial abundance and possibly contain a greater presence of anionic molecules in the extracellular polymer matrix in comparison to Gram-positive biofilms, leading to a greater retention of crystal violet.

A similar density of crystal violet was recorded for untreated *E. coli* ATCC 35218 biofilms and biofilms treated with photo-activated XF-73, illustrating that XF-73 was unable to reduce the density of the biofilms (Figure 4.16 A). These results are supported by MBIC/ MBEC testing as no concentration of blue light activated XF-73 used in this study inhibited or eradicated the biofilms (Table 4.3). However, photo-activated XF-73 followed by XF-73 retreatment against *E. coli* ATCC 35218 biofilms had less crystal violet retained in comparison to untreated biofilm (Figure 4.16 B), indicating that a reduction in biofilm density has occurred, biofilm viability testing confirmed that all concentrations of XF-73 used in this treatment greatly decreases the number of replicating cells (Table 4.6).

MBIC/ MBEC testing revealed that both photo-activated XF-73 and retreatment of XF-73 eradicated *E. coli* ATCC 25922 biofilms at 512 µg/ mL (Table 4.4). This is supported through crystal violet staining of the biofilms following XF-73 treatments, as 512 µg/ mL of XF-73 for both treatments had the least crystal violet retained on the microtitre pegs, illustrating non-viable biofilm cells still attached to the pegs (Figure 4.16 C, D). Concentrations below the MBEC for both treatment strategies had a decreased density of crystal violet in comparison to the growth controls, highlighting that whilst these concentrations of XF-

73 were unable to fully eradicate *E. coli* biofilms, a reduction in the density of viable cells occurred, meaning that some biofilm shedding had occurred.

No concentration of XF-73 used in this study was effective in inhibiting or eradicating twenty-four hour *P. aeruginosa* biofilms (Table 4.3). Biofilm viability testing indicated that both treatment strategies of photo-activated XF-73 and photo-activated XF-73 followed by an additional XF-73 treatment led to a concentration dependent decrease in the number of viable cells remaining in both *P. aeruginosa* ATCC 15442 and *P. aeruginosa* ATCC 27853 biofilms (Figure 4.13, 4.14). A decrease in the density of crystal violet was retained on *P. aeruginosa* ATCC 15442 biofilms following both treatment strategies, illustrating the dose dependent response observed during biofilm viability testing leading to the shedding of parts of the biofilm that was affected by the antimicrobial treatment (Figure 4.17 A, B). The same trend was also recorded for photo-activated XF-73 against *P. aeruginosa* ATCC 27853 biofilms (Figure 4.17 C); in contrast a greater density of crystal violet was retained on *P. aeruginosa* ATCC 27853 biofilms treated with higher concentrations of photo-activated XF-73 followed by a retreatment of XF-73 at 128 – 512 μg/ mL (Figure 4.17 D). Biofilm viability testing demonstrated a greater number of viable *P. aeruginosa* cells following XF-73 treatment at 16 – 32 μg/ mL (Figure 4.14). Therefore, the increased retention of crystal violet at higher XF-73 concentrations indicate bacterial cells that have been killed following treatment, but dispersal of these cells has not occurred. Meanwhile dispersal was illustrated at the lower XF-73 concentration shown by a decreased density of retained stain. This indicates that high concentrations of XF-73 led to cell death within the biofilm but not dispersal, which prevents the spreading of bacterial cells.

Each experiment conducted for the anti-biofilm properties of photo-activated XF-73 against Gram-positive and Gram-negative biofilms offered a different insight into different aspects of XF-73 against biofilms. A standard measurement for the anti-biofilm capability of an antimicrobial *in-vitro* is MBIC/ MBEC testing, therefore the results collected in this study can be compared to current anti-biofilm treatments. Crystal violet staining enables the visualisation of biofilms following blue light and XF-73 treatment, showing a direct effect XF-73 had on the density of the biofilms remaining on the microtitre pegs, and calculating the biofilm viability of a microorganism following treatment allows a direct assessment of the efficacy of photo-activated XF-73 against the number of culturable cells present in a biofilm. Without biofilm viability testing, the potent killing effect of photo-activated XF-73 and a retreatment of XF-73 as a treatment strategy would not have been noted through MBIC/ MBEC assays alone. These results inspire future work as there is a potential to increase the anti-biofilm capability of XF-73 against Gram-negative biofilms.

Variation was recorded between experimental replicates of different microbial biofilms during biofilm viability testing and MBIC/ MBEC assays. To overcome this issue, further testing to include experimental replicates for each experiment would increase the validity of the results and determine

the anti-biofilm effect of photo-activated XF-73 accurately. Additionally, XF-73 was the only antimicrobial used in this study, therefore the efficacy of photo-activated XF-73 against a range of microorganisms could only be compared against XF-73 in natural light. Future testing involving additional PSs would enable a comparison between current PSs against photo-activated XF-73. Hypericin is a potential PS that could be selected for this study, it is a naturally occurring PS and had an excitation maximum at 593 nm (Liu *et al.*, 2015). Previous studies have highlighted hypericin having photo-toxicity against both Gram-positive and Gram-negative microorganisms such as *E. coli*, MSSA and MRSA. Additionally, an anti-biofilm capability was reported for hypericin against *S. aureus* biofilms, although similar to XF-73, *S. aureus* biofilms were less susceptible to hypericin in comparison to planktonic cells (Liu *et al.*, 2015).

A benefit of PDT in comparison to antimicrobial treatments against biofilm infections is the ability of ROS interacting with the polysaccharides present on the extracellular matrix of the biofilm, therefore biofilms have a greater susceptibility to PDT in comparison to antimicrobials alone (Plotino *et al.*, 2019). XF-73 has an advantage over standard antimicrobials due to its ability to act as a PS in the presence of blue light, expanding the potential applications of XF-73 as an antimicrobial treatment.

4.7 Conclusion

XF-73 has a secondary mechanism of action against bacterial cells that is activated through the exposure of blue light. Exploring the antibacterial effect of this secondary mechanism of action has highlighted that photo-activated XF-73 has a greater bactericidal effect against Gram-positive and Gram-negative microorganisms in planktonic mode of growth than the primary mechanism of action of XF-73 in natural light. Therefore photo-activated XF-73 is potentially a more desirable treatment strategy (if applied clinically) than XF-73 alone against bacterial infections located on the skin surface, and photo-activated XF-73 may increase the efficacy of the antimicrobial as a staphylococcal nasal decolonisation agent.

Similarly, blue light exposure enhanced the anti-biofilm capability of XF-73 against three Gram-positive biofilms and an *E. coli* strain, as a reduced concentration of XF-73 was recorded to eradicate these biofilms in comparison to XF-73 in natural light. Although no change in the anti-biofilm capability of XF-73 was observed against the remaining three Gram-negative biofilms and a reduced efficacy was recorded against *S. aureus* ATCC 6538 biofilms. Mature, forty-eight hour biofilms were also included in the study and photo-activated XF-73 concentrations greater than XF-73 in natural light were required to eradicate mature *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 biofilms. The combination treatment involving photo-activated XF-73 and *E. coli* biofilms exposed to blue light followed by an additional retreatment of XF-73 remaining in natural light led to a greater reduction in biofilm viability in comparison to photo-activated and XF-73 in natural light alone. Therefore, future experiments focusing on XF-73 and photo-activated XF-73 in retreatment therapies may increase the killing effect against *E. coli* biofilms and potentially a range of Gram-negative biofilms.

These results indicate that blue light enhancement of XF-73 leads to an increased antibacterial capability against some microorganisms in biofilm mode growth and would be a beneficial treatment strategy against bacterial biofilm infections susceptible to PDT. But the primary mechanism of action for XF-73 in natural light as a membrane disruptor offers a greater antibacterial effect against a wider range of microorganisms.

5.0 Chapter 5: Exploring the antimicrobial efficacy of XF-73 against clinically relevant Gram-positive microorganisms in planktonic mode of growth under iron restriction and the potential effects of competition with haemin

5.1 Introduction

Iron is a key component for the biochemical processes for nearly all life forms, aiding in catalysing reactions, shuttle elections and forming a range of co-ordinated complexes. Iron as a metallic cofactor is irreversibly intertwined with biochemistry due to its high abundance and availability throughout the early evolution of life. Critical biological processes for both prokaryotes and eukaryotes rely on proteins containing iron e.g. DNA synthesis, regulation of gene expression, and transportation of oxygen. Multiple inorganic compounds of iron are found in the human body, these compounds can be classified as either: haemoproteins, iron-sulphur cluster proteins and iron-containing proteins lacking haem or sulphur. Humans ingest on average 20 – 25 mg of iron a day, inorganic iron accounts for the majority of this iron at 85 – 90 % with the remaining iron as haem-bound iron. Whilst a great volume of iron is ingested each day, only roughly 1% is absorbed into the body, due to highly regulated iron homeostasis. In humans and all other mammals, iron homeostasis is attained through the regulation of iron absorption in the intestines and colon (Sousa Gerós *et al.*, 2020).

In order for bacteria to survive acquisition of iron from its external environment is critical. Haemin and ferric chloride haem, the oxidized form of haem, is the primary iron source for bacteria in mammals. Although, an exceptionally low abundance of free haemin is found in blood, extracellular fluid, and mucosal surfaces due to strict haemin regulation. Transferrin and lactoferrin, iron-binding proteins found in mammals are responsible for this strict regulation of iron to prevent oxidative damage. A further reduction in free iron is also a standard non-specific immune response to prevent bacterial infections from attaining an iron source (Brown *et al.*, 2002; Maltais *et al.*, 2016).

To counter the immune response and general low levels of iron in a host, bacteria have evolved to overcome the issue. Certain microorganisms tried to remove the need of iron for survival. These bacteria for example *Borrelia burgdorferi,* focus on using alternative cations as cofactors to produce metalloproteins, and genes that encode for proteins that are iron-dependent are eliminated from the genome. On the other hand, the majority of bacteria instead chose a less drastic approach and instead chose to use high-affinity iron-uptake mechanisms to obtain iron directly from host proteins, these proteins are dependent on the host environment. In the blood microorganisms interact with haemoglobin, haptoglobulin and haemopexin; meanwhile iron-binding glycoproteins transferrin are the primary focus in the extracellular fluid and lactoferrin in mucosal surfaces and secretions. Studies into the mechanisms of the uptake of iron have shown Gram-positive microorganisms to contain less

mechanisms in comparison to Gram-negative microorganisms and iron transportation from the external environment to the cytosol is mainly facilitated by membrane-bound ABC transporters. Although *S. aureus* is the exception to this trend and has multiple transporters and uptake systems (Brown *et al.*, 2002).

S. aureus has two optimised mechanism specific iron uptake systems to obtain haem from haemoglobin in a host: obtaining iron from transferrin through secretion of siderophores capable of binding to iron in the environment, then up-taking these now iron-bound molecules, and an iron-regulated surface determinant (Isd) system that reversibly binds haem directly to *S. aureus* (Bowden *et al.*, 2018).

The Isd system is broken down into nine proteins, expression of the Isd system is regulated by the Fur regulator and is repressed in the presence of iron (Figure 5.1). Four proteins: IsdA, IsdB, IsdC and IsdH are surface bound via covalent bonds to peptidoglycan. IsdB and IsdH are the only two proteins in this system responsible for the direct interaction and binding of haem. IsdB binds to haem from haemoglobin and haemoglobin-haptoglobin complexes, whereas IsdH can interact with haem from haemoglobin, haptoglobin and haemoglobin-haptoglobin complexes. IsdA is partially buried in the cell wall, while IsdC is completed buried within the cell wall. The four Isd proteins located in the cell wall all have three shared features: N-terminal secretion signal, C-terminal sortase signal for cell wall adhesion and a varying abundance of copies of a Near transporter (NEAT) domain. IsdA and IsdC contain a single NEAT domain while IsdB contains two NEAT domains, and three NEAT domains are located in IsdH. The N-terminal and central NEAT domains of IsdH are able to bind to haemoglobin, haptoglobin and haemoglobin—haptoglobin complexes, meanwhile the N-terminal of the NEAT domain of IsdB is unable to bind to haptoglobin alone. The single NEAT domain in IsdA and IsdC and the C-terminal NEAT domain are the only domains capable of binding to haem.

Once haem has been removed from haemoglobin by IsdB and IsdH it is then transferred by a unidirectional relay from IsdA and IsdC to IsdE, a lipoprotein associated an ATP-binding cassette (ABC) made up of IsdF, an ABC transporter and IsdD, a membrane protein whose function remains unknown. The haem is then transported through the cell wall and into the cell by IsdF. Finally the haem inside the cell is degraded by the IsdG and IsdI, cytoplasmic haem-degrading enzymes to release the iron atoms into the cell to be used for cellular processes. (Grigg *et al.*, 2010; Fonner *et al.*, 2014; Bowden *et al.*, 2018).
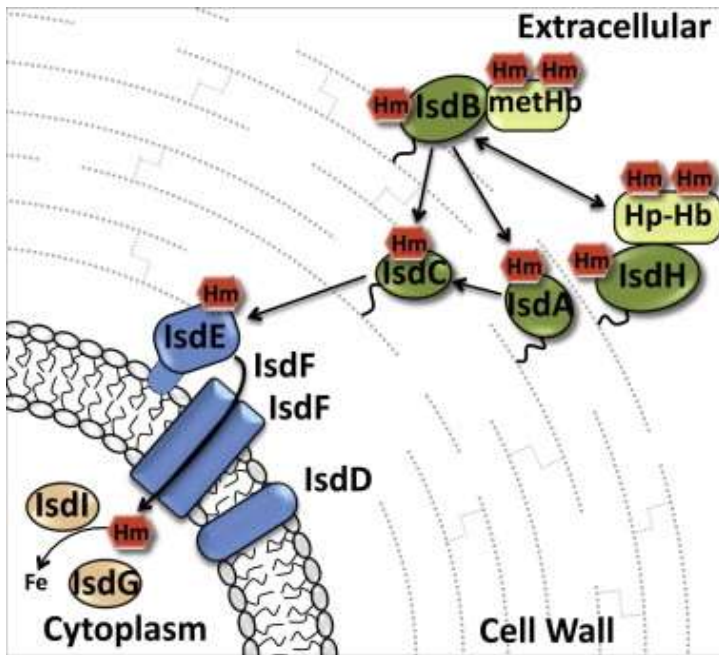
Figure 5.1 Acquisition of iron from the environment into S. aureus bacterial cells via the Isd system

Diagram illustrating the interactions between S. aureus and environmental iron through the Isd system. IsdB and IsdH are surface bound receptors that bind: haem, methemoglobin (haemoglobin carrying oxygen) or haptoglobin-haemoglobin complexes to the Isd system. IsdA and IsdC are membrane bound proteins interlinked with IsdB and IsdH and aid in the transport of haem to IsdE; an ATP binding cassette that transports haem into the cell through an ABC transporter IsdF. IsdD is a membrane bound protein present in the Isd system but its function is unknown. Finally IsdI and IsdG release iron into the cell as these enzymes degrade haem (Grigg et al., 2010).

The FDA and WHO recommend the use of MHB for MIC testing, due to the enriched media having the capability to grow the most commonly isolated aerobic and facultative anaerobic bacteria located in clinical environments and food material. MHB contains sufficient iron for growth of bacteria without the need for expression of specific high-affinity iron uptake systems. Media containing very low levels of iron molecules that bind iron tightly are required to grow bacteria under iron-restricted conditions, expressing their high affinity uptake systems (Sigmaalrdich, 1989).

Roswell Park Memorial Institute (RPMI) media is primarily used as a culturing media for a multitude of mammalian cells and unlike other culturing media, RPMI media contains high concentrations of vitamins and glutathione, a tripeptide that has many roles, some of these key roles are: the maintenance and function of mitochondrial DNA and shielding macromolecules from ROS and nitrogen species (Pizzorno, 2014; Fisher Scientific, 2022). Previous studies have shown an alternative use of RPMI media, where *S. aureus* has been left to grow in the media to simulate an iron restricted environment. Zapotoczna *et al.,* (2012) utilised RPMI media to determine that in an iron restricted environment, the IsdB and IsdC proteins found in *Staphylococcus lugdunensis* (*S. lugdunensis*) have the same function as the corresponding IsdB and IsdC found in the iron uptake system found in *S. aureus*. Zapotoczna also discovered that IsdB is specialised to interact with human haemoglobin as bacterial growth was

only recorded in RPMI with the presence of human haemoglobin and no growth was found with the addition of mouse haemoglobin (Zapotoczna *et al.*, 2012).

Ythier et al., (2012) discovered that IsdA IsdB and IsdC protein expression is upregulated in iron-restricted conditions, through growth of *S. aureus* in RPMI media and this upregulation is greater in comparison to growth in an iron rich environment. Similarly, IsdH is not present when the microorganism is grown in an iron-rich media. *S. aureus* grew at a much lower rate in RPMI media in comparison to the iron rich media (Ythier *et al.*, 2012).

The chemical structure of XF-73 illustrates that the compound contains a porphyrin ring (Figure 1.6), as haem and haemin are porphyrin ring complexes with iron (Figure 5.2); XF-73 may potentially interact with the iron-uptake mechanisms present on the surface of the bacterial cells leading to the update of XF-73 into the microorganisms. Therefore, the presence of haemin in an environment may act as a direct competitor for cellular interactions with XF-73 and could affect the antibacterial capabilities of XF-73, alternatively haemin may directly interact with XF-73 reducing the availability of XF-73 to act against bacterial cells.
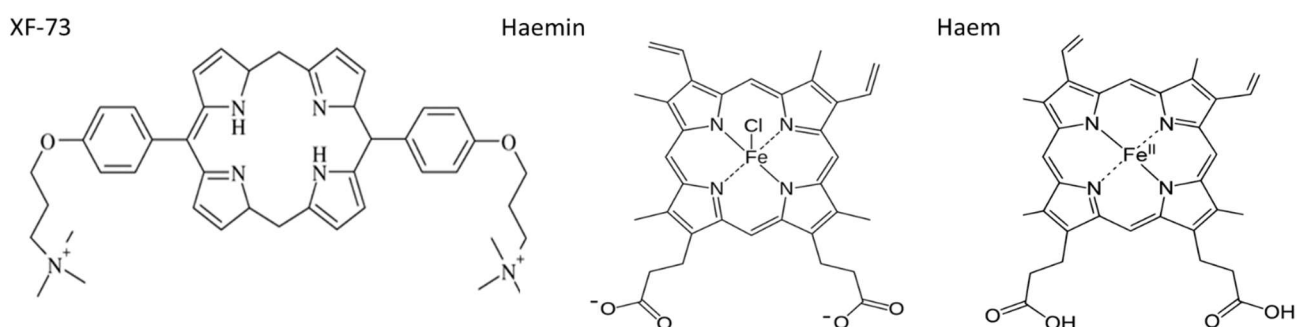
Figure 5.2 Chemical structures of XF-73, haemin and haem

Diagram illustrating the chemical structures of XF-73: (Ooi, *et al.*, 2009b)*, haemin and haem: (Wikimedia Commons).*

5.2 Aims of the study

The aim of this study was to assess the potential effect of the presence of haemin on the antimicrobial efficacy of XF-73 against Gram-positive microorganisms. Additionally, further studies involved exploring the antimicrobial efficacy of XF-73 against planktonic Gram-positive microorganisms in an iron restricted environment to represent that within a human infection.

The study was carried out in the following stages:

- Assess the antimicrobial properties of haemin alone against Gram-positive microorganisms.
    - In enriched media.
    - In iron restricted media.
- Determine the antagonistic effect (through competition) of haemin on the efficacy XF-73 against Gram-positive microorganisms determined through MIC and MBC assays.
    - In enriched media.
    - In iron restricted media.

5.3 Materials

5.3.1 Microbial cultures

A reduced panel of Gram-positive microorganisms (Table 5.1) was selected for this study to assess the antimicrobial efficacy of XF-73 in the presence of haemin. These microorganisms were selected as they showed the greatest susceptibility to XF-73 treatment in planktonic and biofilm mode of growth as shown from previous work (Table 2.3, 3.4).

Streak plates were prepared on MHA for each microorganism (Table 5.1) using a bead from glycerol stocks previously described (2.4.4.1).

Table 5.1: Panel of Gram-positive microorganisms with corresponding ATCC numbers.

Microorganism	ATCC Number
Staphylococcus aureus	6538
Staphylococcus aureus	29213
Staphylococcus epidermidis	12228
Enterococcus faecalis	29212

5.3.2 Microbiological media and agar

MHB and MHA were selected for initial studies to assess the effect of haemin on the antimicrobial efficacy of XF-73 through competition assays. Both MHB and MHA were prepared following the previous methods: (2.4.1.1, and 2.4.1.2).

Roswell Park Memorial Institute (RPMI) 1640 medium (ThermoFisher Scientific, UK) was also selected for this study to simulate the nutritional environment of an infection, this medium was modified with L-glutamine and phenol red but did not contain HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). RPMI was stored at 4°C until needed.

RPMI was supplemented with fetal bovine serum (FBS) (ThermoFisher Scientific) at 10% per final volume of RPMI to ensure an iron restricted media, for haemin and XF-73 competition assays against Gram-positive microorganisms. Both supplemented RPMI and FBS were stored at 4°C until required.

5.3.3 Antimicrobial agents

XF-73 in aqueous solution was selected for this study and was prepared following the method previously described (2.4.2). Stock XF-73 was stored at 4°C until required.

Bovine haemin (>90% (Merck)), in powdered form was stored at 4°C until required to make stock solutions for competition assays against XF-73. Haemin stocks were prepared either in DMSO to dissolve the haemin directly or in water containing sufficient sodium hydroxide to convert the carboxylic acid residues to their water-soluble sodium salts.

DMSO was stored at room temperature in the absence of light until required and sodium hydroxide (NaOH) was stored at room temperature in a glass bottle until needed.

5.3.4 Specialist equipment

Optical density (OD) readings of microbial growth were taken using a spectrophotometer (Jenway Geneva Plus UV).

5.4 Methods

5.4.1 Preparation of haemin stocks

5.4.1.1 Haemin in DMSO

Stock solutions of haemin in DMSO were made at a final concentration of 1000 µg/ mL, solid haemin was vortexed in DMSO at room temperature to ensure complete solubility. Haemin stocks were transferred to 1.5 mL Eppendorf tubes and stored in a black plastic container to protect the solutions from light and stored at 4°C until needed.

5.4.1.2 Haemin in aqueous solution

Aqueous haemin stock solutions were made at a final concentration of 1000 µg/ mL, where solid haemin was suspended in sterile distilled water. Sufficient NaOH (0.02 mL of 1M solution for 2 mg of haemin) was added to the aqueous solution to neutralise the two carboxylic acid groups in haemin to soluble sodium salt. The solution was then vortexed to ensure complete solubility. The solution then underwent sterile filtration using a 0.22 micron pore size cellulose acetate membrane filter (Minisart) to ensure sterility of the solution. Haemin stocks were then stored in 1.5 mL Eppendorf tubes protected from light at and stored at 4°C until needed.

5.4.2 Assessing the antimicrobial properties of haemin against Gram-positive microorganisms in an enriched environment

5.4.2.1 Antimicrobial properties of haemin against *S. aureus* planktonic cells

Initial testing to observe the potential antimicrobial effect of haemin in both DMSO and aqueous solutions was conducted against planktonic *S. aureus* ATCC 29213 bacterial cells. Testing was completed through MIC and MBC assays (2.4.7) using both DMSO and aqueous stock solutions of haemin at a final concentration range of 7.8 – 250 µg/ mL. With controls of microorganism, haemin and broth alone. This study was also conducted to determine which stock solution of haemin was suitable for use in further studies.

5.4.2.2 Antimicrobial efficacy of haemin against Gram-positive microorganisms

Following initial testing of both haemin stocks against *S. aureus* ATCC 29213 (5.4.2.1), the antimicrobial properties of haemin in aqueous solution was further assessed against the remaining Gram-positive microorganisms (Table 5.1). MIC and MBC assays (2.4.7) were conducted using haemin in aqueous solution at a concentration range of 5 – 160 µg/ mL.

5.4.3 Assessing the antimicrobial properties of haemin against Gram-positive microorganisms in an iron restricted media

MIC and MBC assays (2.4.7) were conducted using haemin in aqueous solution against the Gram-positive microorganisms in RPMI to simulate an iron restricted environment at a concentration range of 5 – 160 µg/ mL. MBC assays were conducted on MHA. Overnight cultures of *S. epidermidis* in RPMI showed no growth and were incubated for seventy-two hours to try and observe growth. Following twenty-four hours incubation, no growth was observed for MIC plates containing *E. faecalis*, at forty-eight hours there was still no growth, although MIC/ MBC values were obtained following seventy-two hours of incubation.

5.4.4 Minimum inhibition and bactericidal concentrations of XF-73 against Gram-positive microorganisms in the presence and absence of haemin in an enriched environment

To determine the effect of haemin on XF-73 treatments against Gram-positive microorganisms, a modified MIC/ MBC study (2.4.7) was conducted with the addition of haemin. Initial experimental setup remained the same, where XF-73 (Batch 3) was serially diluted in MHB to give an initial concentration range of 1 – 512 µg/ mL for all Gram-positive microorganisms.

The wells of microtitre plates were then inoculated with 50 µL of each test microorganism at a concentration of 10^6 CFU/ mL in MHB and haemin, to give a final concentration of 5 x10^5 CFU/ mL. Each bacterial culture contained a specific concentration of haemin per study, leading to a final concentration of haemin ranging from 2.5 – 40 µg/ mL.

Following the addition of bacterial cultures, the final concentration range of XF-73 ranged from 0.5 – 256 µg/ mL. Control MIC/ MBC testing of XF-73 alone had a final concentration range of 0.125 – 64 µg/ mL. Following this modification, MIC/ MBC methodology remained the same as previously stated (2.4.7). MBC testing for both enriched and iron restricted mediums were conducted using MHA.

5.4.5 Minimum inhibition and bactericidal concentrations of XF-73 against Gram-positive microorganisms in the presence and absence of haemin in an iron restricted media

The same modified MIC/ MBC testing was used to assess the efficacy of XF-73 against Gram-positive microorganisms in the presence and absence of haemin (5.4.4). However, MHB was replaced in this study with RPMI media containing 10% FBS. As *S. epidermidis* ATCC 12228 was unable to grow in RPMI, the microorganism was not included in this study. Final concentration ranges for XF-73 ranged from 1 – 64 µg/ mL for *S. aureus* ATCC29213 followed by a decreased concentration range: 0.03 – 2 µg/ mL *S. aureus* ATCC 6538 and *E. faecalis* ATCC 29213. Following the data collected from 5.4.3, *E. faecalis* MIC plates were incubated for seventy-two hours to generate MIC values.

5.5 Results

5.5.1 Antimicrobial efficacy of haemin against Gram-positive microorganisms in an enriched environment

5.5.1.1 Antimicrobial properties of haemin in DMSO and in aqueous solution against *S. aureus* ATCC 29213

Haemin in DMSO and in water were both capable of inhibiting *S. aureus* ATCC 29213 growth (Table 5.2), though a 2-fold increase in concentration was required to inhibit growth for haemin in water in comparison to the MIC recorded for haemin in DMSO.

The same concentration of haemin in DMSO was found to inhibit and eradicate *S. aureus* planktonic cells. In contrast no concentration of haemin in aqueous solution was capable of eradicating *S. aureus* as the MBC was concluded as > 250 μg/ mL.

Table 5.2: MIC and MBC values (μg/ mL) of haemin in DMSO and in water against S. aureus ATCC 29213 planktonic cells

Microorganism/ ATCC	Haemin in water (μg/ mL)		Haemin in DMSO (μg/ mL)	
	MIC	MBC	MIC	MBC
S. aureus 29213	125	> 250	62.5	62.5

5.5.1.2 Antimicrobial properties of haemin in aqueous solution against Gram-positive microorganisms

The addition of haemin at a range of concentrations had different affects against the planktonic Gram-positive microorganisms used in this study (Table 5.3). No concentration of haemin used against *E. faecalis* inhibited or eradicated growth. Conversely, *S. epidermidis* required the lowest concentration of haemin in order to inhibit growth (Table 5.3).

Whist haemin was capable of inhibiting the growth of *S. aureus* ATCC 29213, *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 1228, a concentration greater than the MIC was required to eradicate these microorganisms; a 2-fold increase to the MIC were required to eradicate *S. aureus* ATCC 6538 and *S. epidermidis*. No concentration tested was able to eradicate *S. aureus* ATCC 29213 (MBC >250 μg/ mL).

From these recorded results, concentrations of haemin greater than the MIC for each microorganism would not be used in further studies.

Table 5.3 MIC and MBC values (µg/ mL) of haemin in aqueous solution against a panel of Gram-positive microorganisms in MHB.

Microorganism/ ATCC	Haemin (µg/ mL) MIC	MBC
S. aureus 29213	125	> 250
S. aureus 6538	80	160
S. epidermidis 12228	40	80
E. faecalis 29212	> 160	> 160

5.5.2 Antimicrobial efficacy of XF-73 against Gram-positive microorganisms in the presence and absence of haemin in MHB

The addition of haemin at concentrations greater than 2.5 µg/ mL led to an increase in MIC and MBC values for XF-73 against all Gram-positive microorganisms tested (Table 5.4). For each microorganism and concentration of haemin, the concentration of XF-73 for the MIC and MBC was the same; this trend was also recorded for XF-73 alone.

5.5.2.1 *S. aureus* ATCC 6538

S. aureus ATCC 6538 was the only microorganism tested where the addition of haemin at 2.5 µg/ mL did not lead to an increase in XF-73 MIC and MBC values in comparison to XF-73 alone. As the concentration of haemin increased following 2.5 µg/ mL the XF-73 MIC and MBC values increased.

A 2-fold increase in MIC/ MBC values was recorded at 5 µg/ mL of haemin and a further 2-fold increase was observed between the concentrations 5 – 10 µg/ mL, and 10 – 20 µg/ mL of haemin. The highest concentration of haemin used in this study (40 µg/ mL) led to a 32-fold increase in XF-73 MIC/ MBC values compared to XF-73 alone.

5.5.2.2 *S. aureus* ATCC 29213

As the haemin concentration increased, the MIC/ MBC values of XF-73 against *S. aureus* ATCC 29213 increased, with a minimum 4-fold increase in concentration recorded at the lowest concentration of haemin used in the study. Haemin at 5 µg/ mL and 10 µg/ mL had the same effect on shifting the MIC/ MBC of XF-73 from 1 – 8 µg/ mL.

Out of all Gram-positive microorganisms tested, the greatest increase in MIC and MBC values due to the addition of haemin at 20 µg/ mL of was recorded against *S. aureus* ATCC 29213 planktonic cells at 32 µg/ mL. Similarly, to the results obtained against *S. aureus* ATCC 6538, 40 µg/ mL of haemin led to an increase in XF-73 MIC/ MBC values to 64 µg/ mL, although against *S. aureus* ATCC 29213 there was a 64-fold increase in XF-73 concentration.

5.5.2.3 *S. epidermidis* ATCC 12228

Haemin at 40 μg/ mL was not used for the study of assessing the effect of haemin on XF-73 against *S. epidermidis* ATCC cells, due to 40 μg/ mL of haemin having the capability of inhibiting *S. epidermidis* growth itself (Table 5.3). Though all concentrations of haemin used to antagonise the efficacy of XF-73 against *S. epidermidis* planktonic cells led to an increase in XF-73 MIC/ MBC.

Haemin at 2.5 μg/ mL and 5 μg/ mL both led to a 4-fold increase in XF-73 MIC and MBC values. Additionally, haemin at 10 μg/ mL resulted in a 16-fold increase in XF-73's concentration required to inhibit and eradicate *S. epidermidis* cells. The greatest increase in XF-73 MIC and MBC values was recorded with the addition of haemin at 20 μg/ mL, responsible for a 32-fold increase.

5.5.2.4 *E. faecalis* ATCC 29213

Each concentration of haemin used in the study led to a shift in XF-73 MIC/ MBCs against *E. faecalis* ATCC 29212; as the concentration of haemin increased the corresponding XF-73 MIC and MBC values. No concentrations of haemin shared the same MIC/ MBC value of XF-73 against *E. faecalis*. At 40 μg/ mL of haemin a 64-fold increase in XF-73 MIC and MBC was recorded when compared to XF-73 alone. This 64-fold increase in MIC and MBC values was also recorded for XF-73 against *S. aureus* ATCC 29213 and *S. aureus* ATCC 6538 with 40 μg/ mL of haemin.

Table 5.4 MIC and MBC values (μg/ mL) of XF-73 against Gram-positive microorganisms in the presence and absence of haemin in MHB.

Haemin in aqueous solution, ranged from 2.5 – 40 μg/ mL, though 20 μg/ mL was the maximum haemin concentration for S. epidermidis ATCC 12228.

Haemin (μg/ mL)	*S. aureus* ATCC 6538		*S. aureus* ATCC 29213		*S. epidermidis* ATCC 12228		*E. faecalis* ATCC 29212	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
40	64	64	64	64	N/A	N/A	32	32
20	16	16	32	32	16	16	16	16
10	8	8	8	8	8	8	4	4
5	4	4	8	8	2	2	2	2
2.5	2	2	4	4	2	2	1	1
0	2	2	1	1	0.5	0.5	0.5	0.5

5.5.3 The antimicrobial effect of haemin against Gram-positive microorganisms in an iron restricted environment

Overnight cultures of *S. epidermidis* ATCC 12228 showed no growth in RPMI and were then incubated for up to seventy-two hours incubation at 37°C. No viable growth was observed at twenty-four, forty-eight and seventy-two hours of incubation, therefore *S. epidermidis* was not included for any studies involving RPMI. *E. faecalis* ATCC 29212 showed no MIC/ MBC values of haemin after twenty-four and forty-eight hours of incubation, though at seventy-two hours MIC/ MBC values were recorded. *S. aureus* ATCC 29213 and *S. aureus* ATCC 6538 only required twenty-four hours of incubation.

Haemin in aqueous solution was capable of inhibiting the growth of all remaining Gram-positive microorganisms (Table 5.5). A lower concentration was required to inhibit *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 planktonic cells at 80 μg/ mL, in comparison to *S. aureus* ATCC 6538, which had a 2-fold increase in concentration at160 μg/ mL.

In contrast to MIC values, no concentration of haemin used in this study was capable of eradicating *S. aureus* ATCC 29213 and *E. faecalis* cells; meanwhile 160 μg/ mL of haemin was able to eradicate *S. aureus* ATCC 6538 and was the only microorganism where the same concentration was recorded for the MIC and MBC.

Table 5.5 MIC and MBC values (μg/ mL) of haemin in aqueous solution against Gram-positive microorganisms in RPMI media.

Haemin MIC and MBC values for E. faecalis ATCC 29212 were generated following seventy-two hours incubation, both S. aureus strains required twenty-four hours of incubation.

Microorganism/ ATCC	Haemin (μg/ mL)	
	MIC	MBC
S. aureus 29213	80	> 160
S. aureus 6538	160	160
E. faecalis 29212	80	> 160

5.5.4 Antimicrobial efficacy of XF-73 against Gram-positive microorganisms in the presence and absence of haemin in an iron restricted environment

S. epidermidis ATCC 12228 was unable to grow in RPMI media from up to seventy-two hours incubation at 37°C. No viable cells were present, and this microorganism was therefore unable to be included in the study. *E. faecalis* ATCC 29212 MIC/ MBC values of XF-73 were recorded following seventy-two hours of incubation at 37°C, whereas *S. aureus* ATCC 29213 and *S. aureus* ATCC 6538 only required twenty-four hours of incubation.

XF-73 alone was capable of inhibiting and eradicating all Gram-positive microorganisms used in this study in iron restricted media (Table 5.6); *S. aureus* ATCC 29213 was the only microorganism tested that had the same XF-73 concentrations recorded for the MIC and MBC.

5.5.4.1 *S. aureus* ATCC 29213

The addition of haemin at each concentration altered the MIC and MBC of XF-73 against *S. aureus* ATCC 29213 planktonic cells; although the effect haemin had on XF-73 MIC and MBC values varied at each concentration.

Haemin at 5 – 40 µg/ mL had the potential to increase XF-73's MIC and MBC values by a 2-fold increase, but these concentrations of haemin were also recorded with MIC/ MBC values below the MIC and MBC of XF-73 alone.

Haemin at 2.5 µg/ mL had the capability of decreasing XF-73 MIC and MBC values below the recorded concentration ranges of XF-73 alone in RPMI media. Though the upper range of MIC and MBC values for XF-73 in the presence of 2.5 µg/ mL of haemin was the same concentration of XF-73 alone.

5.5.4.2 *S. aureus* ATCC 6538

XF-73 alone was capable of inhibiting *S. aureus* ATCC 6538 cells at a concentration range of 0.063 – 0.25 µg/ mL, meanwhile a slightly higher concentration range of XF-73 was able to eradicate the planktonic cells at 0.125 – 0.25 µg/ mL. The addition of 2.5 µg/ mL of haemin led to no change to the concentration range of XF-73's MIC and MBC values. Haemin at 5 µg/ mL also had the same concentration range for XF-73 MICs in the absence of haemin; but only 0.25 µg/ mL of XF-73 was able to eradicate *S. aureus* bacterial cells.

The addition of 10 µg/ mL of haemin did not impact upon the lower concentration range of XF-73 for inhibition and eradication of planktonic *S. aureus*; a 2-fold increase in the upper concentration range of XF-73 was recorded for the MIC and MBC values in comparison to XF-73 alone.

The highest concentrations of haemin used in this study: 20 µg/ mL and 40 µg/ mL had the same recorded MIC and MBC concentration ranges of XF-73 and had the lowest concentration of XF-73 recorded as the lower concentration range for MIC and MBCs in comparison to XF-73 alone at < 0.03 µg/ mL and 0.063 µg/ mL respectively. Although at these concentrations of haemin, a 2-fold increase at the upper concentration range of XF-73 MIC and MBC values were observed when compared to XF-73 alone.

5.5.4.3 *E. faecalis* ATCC 29212

A lower concentration range of XF-73 was capable of inhibiting *E. faecalis* growth at 0.125 – 1 µg/ mL. To eradicate the planktonic cells a 4-fold increase at the lower concentration range of XF-73 was required as the MBCs ranged from 0.5 – 1 µg/ mL. No change in XF-73 MIC and MBC values was recorded with the addition of haemin at 2.5 µg/ mL and 10 µg/ mL. Additionally, no change in XF-73 MBCs were recorded with the addition of 5 µg/ mL of haemin, but a decrease in the lower concentration range of XF-73 was capable of inhibiting *E. faecalis* growth at 0.063 µg/ mL.

Haemin at 40 µg/ mL caused a shift in XF-73 MICs and MBCs with an upper concentration range greater than the corresponding MIC and MBCs of XF-73 alone. Although in this environment the recorded lower concentration range of XF-73 to eradicate *E. faecalis* was less than the lower concentration range of XF-73 alone; and the lower limit XF-73 inhibition has the potential to also be less XF-73 alone with a

value of < 0.5 µg/ mL. The same concentration range was recorded for MIC and MBC values of XF-73 against the planktonic cells with 40 µg/ mL of haemin.

Table 5.6 MIC and MBC values (µg/ mL) of XF-73 in the presence and absence of haemin at a range of concentrations in RPMI media

Haemin MIC and MBC values for E. faecalis ATCC 29212 were generated following seventy-two hours incubation, both S. aureus strains required twenty-four hours of incubation.

Haemin (µg/ mL)	*S. aureus* ATCC 29213 MIC	MBC	*S. aureus* ATCC 6538 MIC	MBC	*E. faecalis* ATCC 29212 MIC	MBC
40	< 1 – 2	< 1 – 2	< 0.03 – 0.5	0.063 – 0.5	< 0.5 – < 2	< 0.5 – < 2
20	< 1 – 2	< 1 – 2	< 0.03 – 0.5	0.063 – 0.5	0.5 - 1	1
10	< 1 – 2	< 1 – 2	0.063 – 0.5	0.125 – 0.5	0.125 – 1	0.5 – 1
5	< 1 – 2	< 1 – 2	0.063 – 0.25	0.25	0.063 – 1	0.5 – 1
2.5	< 1 – 1	< 1 – 1	0.063 – 0.25	0.125 – 0.25	0.125 – 1	0.5 – 1
0	1	1	0.063 – 0.25	0.125 – 0.25	0.125 – 1	0.5 – 1

5.6 Discussion

The aim of this study was to determine the antibacterial properties of haemin against Gram-positive microorganisms and assess the potential effect haemin had on XF-73; the efficacy of XF-73 was assessed against Gram-positive microorganisms in an iron restricted media, with the potential effect of haemin in the environment were successfully met through the experiments conducted throughout. The antibacterial property of haemin alone (in both an enriched medium through MHB and an iron restricted medium through the use of RPMI) was demonstrated against Gram-positive microorganisms selected for this study. An antagonistic effect of haemin was also demonstrated against XF-73, resulting in an increase in MIC and MBC values against the Gram-positive microorganisms, when haemin at a range of concentrations was present in the enriched environment of MHB. In contrast, in an iron restricted environment, haemin no longer contributed an antagonistic effect against the efficacy of XF-73 against these Gram-positive microorganisms. This posed the question as to why the antagonistic effect of haemin against XF-73 was lost in an iron restricted media.

Whilst iron is key to the survival of microorganisms and noted to be a critical cofactor for multiple biochemical processes, this study has highlighted an inhibitory effect of high concentrations of haemin against *S. aureus* ATCC 6538, *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228. Haemin at a concertation double to the corresponding MIC values was then recorded to have a bactericidal capability against *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228 in MHB. In contrast, *E. faecalis* ATCC 29212 was the only microorganism that remained unaffected against exposure to haemin. This was illustrated as no concentration of haemin used in the study and was determined to inhibit or eradicate bacterial growth (Table 5.3).

The data generated in this study is supported by over seventy years of previous research, highlighting toxicity of haem/ haemin against microorganisms, although sensitivity to haem/ haemin toxicity varies depending on microorganisms; Gram-positive microorganisms in particular are more susceptible to this toxicity. Nitzan *et al.,* (1994) determined that concentrations of haemin as low as 10 – 20 µg/ mL was able to inactivate a range of anaerobic Gram-positive microorganisms (Nitzan *et al.*, 1994). The mechanism leading to haem/ haemin toxicity has not yet been determined, though it is speculated that a potential mechanism is due to damaging the bacterial DNA (Anzaldi *et al.*, 2010). This theory is supported as Aft *et al.* expanded on the role of haemin modulating gene expression during cellular differentiation. Haem/haemin in the presence of oxygen and 2-mercaptoethanol (a reducing agent) has the capability to cause strand scissions in DNA (Aft *et al.*, 1983).

Later studies have determined that haem/ haemin toxicity against microorganisms, in particular Gram-positive microorganisms, is due to the multiple effects on the microorganism. Iron is freed either by haem oxygenases or through reactions with ROS. Iron has the ability to cycle between excitation states: ferrous (Fe^{2+}) and ferric (Fe^{3+}) via the Haber-Weiss/ Fenton reaction; where the reversible reaction of

ferric iron and singlet oxygen generates oxygen and ferrous iron, the ferrous iron then reacts with hydrogen peroxide and generates ferric iron and hydroxide ions. These processes generate more ROS in the environment which in turn damage the DNA, lipids and proteins of the microorganisms. For *S. aureus*, haem/ haemin toxicity leads to oxidative stress and the membrane proteins are oxidised leading to the formation of superoxide's causing further oxidative damage to the bacterial cells (Choby *et al.*, 2016).

E. faecalis was noted not to be inhibited or eradicated by the maximum dosage of haemin used in this study (Table 5.3). Therefore, it is concluded that these concentrations of haemin have no antibacterial efficacy against the microorganism in an iron enriched media. Previous studies have highlighted that *E. faecalis* is not dependent on haem for growth and is unable to synthesise haem, a common trait associated with intestinal bacteria. Whilst haem is known to cause toxicity, *E. faecalis* has evolved several mechanisms to control haem homeostasis. Research into the mechanisms controlling haem homeostasis in *E. faecalis* has shown a unique haem transport regulator: faecalis haem transport regulator (FhtR). When haemin is present in the environment FhtR has the capability of maintaining haem homeostasis via regulating the expression of a haem-dependent catalase A (KatA), a mono-functional catalase, to break down haem in the environment. *E. faecalis* is one of only a few species of lactic acid bacteria that has the capability to utilise catalase to combat hydrogen peroxide stress, though haem has to be taken from the environment in order to generate an active KatA. The FhtR system also regulates a haem efflux pump through haem-regulated transport proteins and an ABC transporter to ensure no build-up of iron occurs inside the bacteria leading to iron toxicity (Baureder *et al.*, 2012; Saillant *et al.*, 2021).

When haemin was added in MHB it demonstrated an antagonistic effect on the efficacy of XF-73 against Gram-positive microorganisms, shown by the increase in XF-73 concentrations required to inhibit and eradicate each microorganism used in the study (Table 5.4). The highest concentration of haemin used for each microorganism: 40 μg/ mL for *S. aureus* ATCC 6538, *S. aureus* ATCC 29213 and *E. faecalis* and 20 μg/ mL for *S. epidermidis* were responsible for the greatest increase in XF-73 MIC and MBC values; at a 32-fold increase for *S. aureus* ATCC 6538 and *S. epidermidis* and a 64-fold increase for *S. aureus* ATCC 29213 and *E. faecalis.* A concentration dependent increase was observed for each Gram-positive microorganism, where increasing haemin concentration led to an increase in MIC and MBC values of XF-73 (Table 5.4).

When the solid chloride salt of XF-73 is dissolved in water, it dissociates to the dicationic porphyrin ions and chloride ions. The solid dicarboxylic acid form haemin used in this study is highly insoluble in water.

It was rendered water soluble by conversion to the disodium salt by addition of sufficient sodium hydroxide to neutralise the two carboxylic acid residues on the haemin ring system (Figure 5.3).
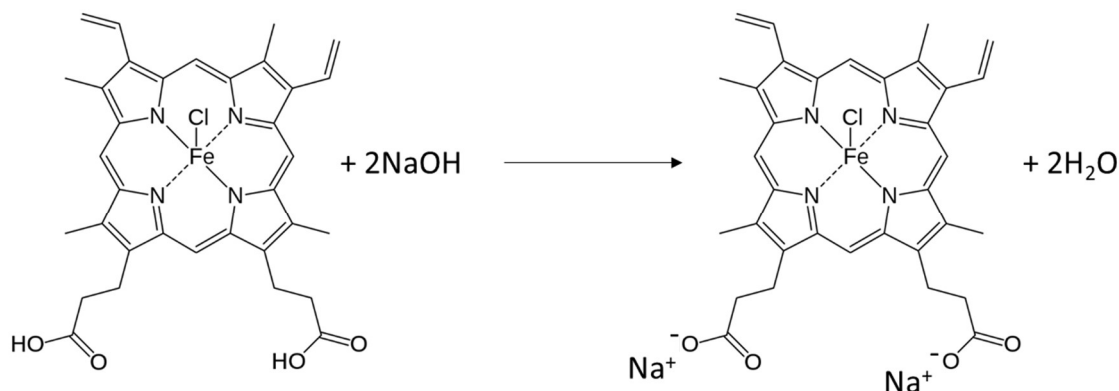
Figure 5.3 Neutralisation of haemin via the addition of sodium hydroxide

Diagram illustrating the neutralisation reaction of haemin with sodium hydroxide, generating water and dianionic haemin with sodium association. Chemical structure of haemin: (Wikimedia Commons).

One possible explanation for the antagonistic effect of haemin on XF-73 efficacy may be due to ionic associations between the positively charged XF-73 cations and negatively charged haemin anions, leading to an XF-73-haemin ion-pair complex, through electrostatic forces of attraction. No evidence of precipitation was observed in any microtitre test wells following XF-73 treatment against Gram-positive microorganisms in the presence of haemin at each concentration of haemin used throughout. Therefore, if haemin-XF-73 complexes are formed they do not form an insoluble precipitate but may remain in solution in the form of an ion pair. If XF-73-haemin complexes are formed in solution via ion pair bonding, additional interactions may aid in maintaining this complex once the two compounds are brought together as ion pairs are temporary and the formation and dissociation of iron pairs happens continuously in the environment (Britannica, 2019). For example, close contact would enable further stabilisation of the complexes by pi-pi interactions between the planar porphyrin ring systems of XF-73 and haemin XF-73. In the absence of an electric charge, pi-pi interactions form assemblies between porphyrins involving dispersion forces and form a stacking structure between them. Previous studies have determined pi-pi interactions as a form of stacking porphyrins and oppositely charged porphyrins come together through ion pairs (Yamasumi *et al.*, 2021). Recent studies have also reported the formation of porphyrin dimers through ion pairs between a range of anionic and cationic porphyrins (Natali *et al.*, 2016). Figure 5.4 highlights the potential zones where ion pairs and pi-pi interactions would form between XF-73 and haemin.
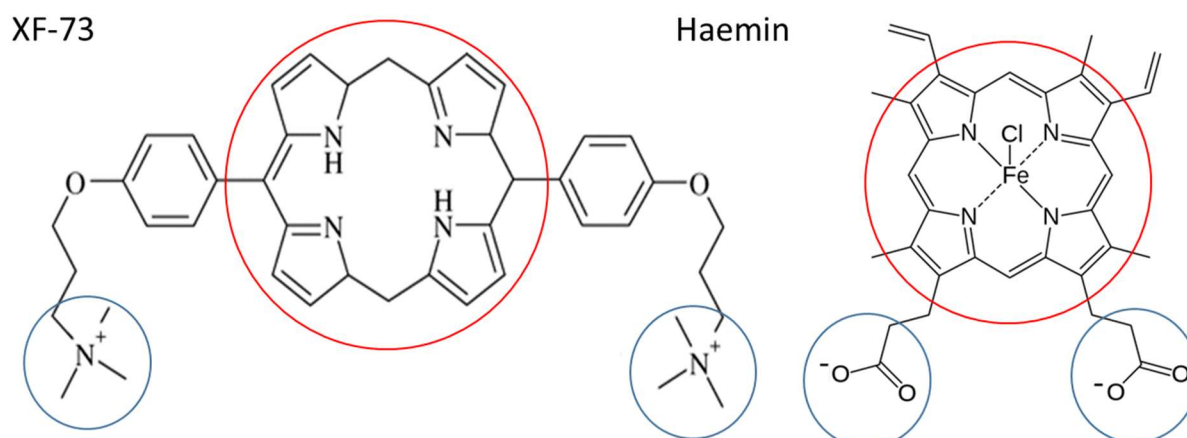
Figure 5.4 Chemical structures of dicationic XF-73 and dianionic haemin

Chemical structures of XF-73 and haemin, blue regions on each compound shows the cationic regions on XF-73 or the anionic regions present in haemin and are the potential regions for ion pairs. The red regions of both compounds highlight the net neutral porphyrin rings and the potential area for pi-pi interactions. Chemical structure of haemin: (Wikimedia Commons), chemical structure of XF-73: (Ooi, et al., 2009b)

S. epidermidis ATCC 12228 could not be used for testing in the iron restricted RPMI media because no growth was present after incubation for seventy-two hours. Massonet *et al.,* (2006) also reported a decrease in the growth rate of planktonic *S. epidermidis* cells in an iron limited environment in comparison to an iron rich environment (Massonet *et al.*, 2006). This inability to grow in iron restricted media after seventy-two hours of incubation highlights that the iron uptake system for *S. epidermidis* is not as effective as the uptake systems found in *S. aureus* when these microorganisms are left to grow in an iron restricted environment. While the Isd system is located in *S. aureus*, only a few other staphylococcal species such as *S. lugdunensis*, *S. caprae* and *S. capitis* also encode the Isd locus (Horswill *et al.*, 2020). *S. epidermidis* does not encode an entire Isd system, but previous research has shown that *S. epidermidis* encodes for homologues of IsdG and IsdI, the enzymes capable of degrading haem in the Isd system (Skaar *et al.*, 2003). *S. epidermidis* also encodes a two component regulatory system, HssSHssR which maintains intracellular haem homeostasis. In response to haem, haemin, haemoglobin or blood this regulator system activates the HrtAB efflux pump which removes the potentially toxic haem or haemoglobin from the cells (Gill *et al.*, 2005).

In human infections, previous research has shown that both *S. aureus* and *S. epidermidis* induce lipoproteins located in the cell wall and cytoplasmic membrane in response to a lack of free iron present in the environment. One of these proteins present on the cell wall is a receptor for human transferrin in *S. epidermidis;* the lipoprotein bound to the cytoplasmic membrane has been determined to be a part of a coupled iron-regulated operon. This operon is made up of three genes *sit*A, *sit*B and *sit*C, the products of these genes make up an ABC transporter; *sitA* encodes an ATP binding protein whilst *sitB* encodes for a membrane protein, finally *sitC* encodes for a cytoplasmic membrane anchored lipoprotein. This *sitABC* operon has also been suggested to mediate the uptake of iron collected from

siderophores that chelate free iron in an environment as well as releasing iron from transferrin with SitC as the potential ferric siderophores receptor (Hill *et al.*, 1998; Massonet *et al.*, 2006).

A reduced growth rate was also recorded for *E. faecalis* ATCC 29212 planktonic cells in RPMI media. Whilst overnight cultures of *E. faecalis* were created, any experiment set up to test the efficacy of haemin alone or XF-73 in the presence and absence of haemin in RPMI media required seventy-two hours of incubation before viable cells were recorded. This reduced growth rate of *E. faecalis* may be due to the fact that *E. faecalis* is unable to utilise haem to aid in bacterial growth and as haemin was the only iron source supplemented into the media for the studies; *E. faecalis* therefore struggled to grow in this restricted environment in comparison to both *S. aureus* strains that were able to make use of haemin. A study conducted by López *et al.*, (2012) supported these findings, where concentrations of excess iron in an environment lead to a doubling time in the growth of *E. faecalis,* where growth was inhibited with the presence of 40 nM of ferric nitrilotriacetate (López *et al.*, 2012)*.*

A difference in the antibacterial properties of haemin against Gram-positive microorganisms was recorded in the iron restricted RPMI environment in comparison to haemin in MHB. A decrease in inhibitory concentrations was recorded for haemin in RPMI against *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212; whereas a 2-fold increase in haemin was required to inhibit *S. aureus* ATCC 6538. Similarly, to the effect of haemin in MHB, *S. aureus* ATCC 6538 was the only microorganism recorded to be eradicated by haemin in RPMI media (Table 5.7). A potential explanation to this change in antibacterial efficacy of haemin in an iron restricted media is that the Gram-positive microorganisms were more susceptible to haem toxicity as an uptake of iron from haem was now critical for the survival of the microorganisms. Alternatively, the levels of induction and expression of the Isd systems in the two *S. aureus* strains in RPMI might not have been the same, resulting in the recorded variation to sensitivity to haemin.

Table 5.7 MIC and MBC values (µg/ mL) of haemin against a panel of Gram-positive microorganisms in an enriched or iron restricted environment

Microorganism/ ATCC	Haemin in MHB (µg/ mL)		Haemin in RPMI (µg/ mL)	
	MIC	MBC	MIC	MBC
S. aureus 29213	125	> 250	80	> 160
S. aureus 6538	80	160	160	160
E. faecalis 29212	> 160	> 160	80	> 160

An antagonistic effect was demonstrated between haemin and XF-73, leading to a decrease in the antimicrobial efficacy of XF-73 against Gram-positive microorganisms in an enriched media. Once the experiment was repeated in RPMI media, to simulate an iron restricted environment, the antagonistic effect of haemin was lost against XF-73. No marked increases in XF-73 concentrations were required

to inhibit or eradicate the Gram-positive microorganisms in the presence of haemin at each concentration (Table 5.6), compared with the concentrations observed in an enriched media (Table 5.4).

Gram-positive microorganisms are known to upregulate iron acquisition mechanisms, i.e. the Isd system found in *S. aureus*, when the microorganisms are in an environment where iron is a limiting factor for the growth and survival of the bacteria (Ythier *et al*., 2012). This upregulation of iron acquisition system leads to an increase of iron receptors on the surface of *S. aureus* cells, these receptor sites have a greater affinity to interact with haem in comparison to XF-73, reducing the abundance of haemin in the environment. This decrease of free haemin therefore reduces the likelihood for XF-73-haemin complex forming, allowing XF-73 to maintain its antibacterial effect against the Gram-positive microorganisms.

Additionally, decreased XF-73 concentrations were able to inhibit *S. aureus* ATCC 6538 and *E. faecalis* ATCC 29212 in RPMI media in comparison to corresponding MIC and MBC values recorded for XF-73 in MHB (Table 5.8). A minimum 8-fold increase in XF-73 concentration was recorded to inhibit and eradicate *S. aureus* ATCC 6538 planktonic cells. The concentration ranges for MIC and MBC values of XF-73 against *S. aureus* ATCC 6538 in RPMI were all lower than corresponding XF-73 MIC and MBC values in MHB. In contrast, no changes in XF-73 MIC and MBC values were recorded against *S. aureus* ATCC 29213 in RPMI in comparison to MHB.

Whilst a concentration of XF-73 below the MIC value of XF-73 against *E. faecalis* in MHB was demonstrated to inhibit bacterial growth in RPMI media; through repeating the study the upper concentration range of XF-73 for MIC and MBC values in RPMI media were greater, with a 2-fold increase in concentration in comparison to recorded MIC and MBC values in MHB. Highlighting that the efficacy of XF-73 varies in RPMI media against Gram-positive microorganisms.

Table 5.8 MIC and MBC values (µg/ mL) of XF-73 against Gram-positive microorganisms in an enriched and iron restricted environment

Microorganism ATCC number	XF-73 in MHB (µg/ mL)		XF-73 in RPMI (µg/ mL)	
	MIC	MBC	MIC	MBC
S. aureus 6538	2	2	0.063 – 0.25	0.125 – 0.25
S. aureus 29213	1	1	1	1
E. faecalis 29212	0.5	0.5	0.125 – 1	0.5 – 1

By including two stains of *S. aureus* in this study, a comparison can be conducted between the MIC and MBC values of XF-73 recorded against both strains to determine if a similar antibacterial efficacy of XF-73 is present against both. This study has highlighted that whilst the efficacy of XF-73 increases against *S. aureus* ATCC 6538 in an iron restricted medium in comparison to control growth in MHB, no changes in MIC or MBC values were recorded against *S. aureus* ATCC 29213. Therefore, the efficacy of XF-73 varies between strains of *S. aureus.*

Only Gam-positive microorganisms were selected for this study; the reasoning behind this choice was due to the lack of antimicrobial efficacy of XF-73 recorded against Gram-negative microorganisms, shown through the results collected throughout the studies conducted in this project (Table 2.3, 3.4). Therefore, Gram-negative microorganisms may have a potential susceptibility to XF-73 in an iron restricted media due to a different growth rates for the microorganisms in RPMI, as the environment is no longer optimal for bacterial growth. Further work in this area of research would focus on assessing the growth of Gram-negative microorganisms in an iron restricted media, in particular against the panel previously selected (Table 3.1) and the potential efficacy of XF-73 in this restricted environment.

The findings generated from this study highlight the potential of using XF-73 as a potential antimicrobial treatment in wound infections. As the efficacy of XF-73 against Gram-positive microorganism recorded in an enriched media was also retained in an iron restricted environment; akin to an environment present in a wound infection due to iron being limited in the body to combat infections. Though the presence of haemin, even at low concentrations, began to antagonise XF-73; iron is key for bacterial survival and in an iron restricted environment, bacterial iron uptake systems are upregulated, and receptors have a greater affinity for haemin in comparison to XF-73. Thus, XF-73 is free to exert antimicrobial activity against these microorganisms in an iron limited environment, as found in a wound infection site.

From the data generated in this study, it is clear that further experiments would be critical to expand upon the current findings. Historically it was believed that porphyrin structures involved in both plant and bacterial photosynthesis synthesise porphyrin dimers through covalent bonding. Metalloporphyrins e.g. haem b and chlorophyll a, chemical reactivity's and catalytic properties were dependent on interactions between proteins or structures in the environment through energy and electron transfer between the interactions; additionally the formation of these porphyrin dimers or higher complex structures was believed to be through covalent bonding, and experimental models focused mainly on covalent bonds. Though during the 1980's it was speculated that porphyrin dimers or 'accordion-type aggregates' multi-structures porphyrins can spontaneously form through associations between cationic and anionic porphyrins. To test this theory, experiments were conducted to measure the absorbance spectra of an anionic and cationic porphyrins separately, followed by absorbance readings conducted on a mixture of the two porphyrins. The absorbance spectra of the mixture was not a combination of the two spectra readings of the individual porphyrins, as energy peaks different to either porphyrin were recorded. Indicating that anionic and cationic porphyrins interact with one another and form complexes, even at low concentrations (Shimidzu *et al.*, 1981; Ojadi *et al.*, 1985).

Current spectroscopic technique utilises UV or natural light to explore the consistency and structures of biological molecules. Through designing a spectroscopic study as a future experiment to measure the absorption spectra of XF-73 and haemin separately and compare the readings against a mixture of the two compounds, could aid in determining any potential ion-pair associates or pi-pi interactions

between XF-73 and haemin (Hofmann *et al.*, 2010). These studies would then either support or prove otherwise the current theory of XF-73-haemin complexes via ion pairs and pi-pi interactions.

Alternative studies to assess the potential XF-73-haemin complex may include conductivity testing. Previous research in the field has shown that the formation of ion pairs between cations and anions leads to a decrease in the conductivity of a liquid solution, due to a decrease in free ions present in the solution (Dukhin *et al.*, 2018). Through varying the concentration of haemin in a solution of XF-73, if a decrease in conductivity is recorded then this supports the theory of XF-73-haemin complexes forming via ion pair binding.

Furthermore, the addition of comparator testing with known antimicrobials (2.3.4) against Gram-positive microorganisms in iron limited media would be beneficial to allow a comparison of these results to the recorded antimicrobial efficacy of XF-73. If any potential decrease in efficacy of these comparators were demonstrated against Gram-positive microorganisms in RPMI, this would further support XF-73 becoming a viable antimicrobial treatment for wound infections.

5.7 Conclusion

Whilst a decrease in the efficacy of XF-73 was demonstrated against a panel of four Gram-positive microorganisms following the addition of haemin in MHB, XF-73 was still able to eradicate these planktonic microorganisms at all concentrations of haemin added. A dose dependent response between haemin and the efficacy of XF-73 as an antimicrobial treatment against Gram-positive microorganisms signifies an antagonistic effect, though no conclusions can be drawn from this research to determine what the effect is. Further studies are required to determine the type of interaction haemin has with XF-73 as current speculation theorises a XF-73-haemin complex forming in the media before treatment against the Gram-positive microorganisms can occur.

XF-73 successfully retained its antimicrobial efficacy against Gram-positive microorganisms in planktonic mode of growth in an iron restricted media, and the presence of haemin no longer demonstrated an antagonistic effect against XF-73 in eradicating the bacterial cells. This further highlights the potential to apply XF-73 as an efficient antimicrobial treatment against wound infections, where the presence of haemin and free iron from bloody will not alter the efficacy of XF-73.

6.0 Chapter 6: General discussion and future studies

The WHO has stated in the past few decades that the spread and emergence of AMR is of global concern. Pathogens have the ability to express or gain resistance to multiple drug therapies leading to a prevalence of multidrug resistant species, increasing mortality and morbidity rates. AMR infections also pose a substantial burden on the economy through the costs of prolonged hospital stays and increased medical bills for different treatments to overcome the infections. Whilst AMR is a global issue, the greater affliction is found in low-income and middle-income countries as the inability to overcome AMR infections threatens sustainable development of these areas. Bacterial species are resistant to antimicrobial treatments, one such mechanism of resistance is through the upregulation of efflux pumps to remove the antimicrobial from the bacterial cells, altering the properties and expression of the drug target site or through production of enzymes to inactivate the antimicrobials (Priyamvada *et al.*, 2022; Waddington *et al.*, 2022).

Currently, there is a concerning lack of new antibiotics in the pipeline with novel mechanisms of action to combat resistant pathogens and, if the spread of AMR continues without suitable treatment strategies, as of 2050 the leading cause of human death shall be due to AMR infections accounting for 10 – 50 million deaths annually (Rappuoli *et al.*, 2017; Zhu *et al.*, 2022). It is now imperative to develop new treatment strategies, either through the generation of novel antimicrobials or alternative treatments that do not require antibiotic drugs to prevent these projections from becoming a reality.

The overall aim of this research project was to further assess the antimicrobial efficacy of the novel compound XF-73, and photo-activated XF-73, against a range of clinically relevant microorganisms in planktonic and biofilm mode of growth, expanding on the results collected from previous studies. Both of XF-73's mechanisms of action were assessed in this study, the primary mechanism of action occurs in natural light, whereas the secondary mechanism of action can only be triggered through photo-activation using blue light. XF-73 in natural light kills bacterial cells through the disruption of their bacterial membranes leading to rapid cell death (Ooi *et al.*, 2009b; Moir *et al.*, 2012). During blue light exposure, XF-73 acts as a PS and, through generation of ROS by a type 2 reaction, leads to bacterial cell death (Maisch *et al.*, 2005). When activated through blue light exposure, the excited state of XF-73 directly interacts with molecular oxygen, leading to the production of singlet oxygen, which in turn interacts with bacterial cells leading to cell death through targeted damage to the structural and functional components of the cells i.e. proteins and nucleic acids (Moore *et al.*, 2005; Takasaki *et al.*, 2009). Throughout this study XF-73 exhibited an antibacterial effect against both Gram-positive and Gram-negative microorganisms in planktonic and biofilm mode of growth, though was more effective against Gram-positive microorganisms (Table 2.3, 3.4). Furthermore, through the activation of XF-73's secondary mechanism of action, an enhanced killing capability of XF-73 was recorded against these microorganisms in both planktonic and biofilm mode of growth (Table 4.1, 4.2 and 4.3).

In chapter two, preliminary testing via antimicrobial susceptibility testing confirmed that XF-73 had an antibacterial effect against Gram-positive microorganisms, though no effect was recorded against Gram-negative microorganisms as no zones of inhibition were recorded for XF-73 against these bacterial cultures in agar diffusion assays (Figure 2.2, 2.3). These initial results indicated that XF-73 was less effective at inhibiting bacterial growth in comparison to the panel of antimicrobials selected for this study, as gentamicin and ciprofloxacin were able to inhibit bacterial growth against all Gram-positive and Gram-negative microorganisms selected for this study. Previous studies conducted on XF-73 have not assessed the antibacterial property of XF-73 through agar diffusion assays as Destiny Pharma have stated that XF-73 does not diffuse efficiently through agar media. Further experiments conducted in chapter two progressed onto broth-based studies i.e. MIC/ MBC testing (2.5.2, 2.5.3) and time-kill studies (2.5.5, 2.5.6) and it was during these experiments that the antimicrobial capability of XF-73 was truly expressed.

The overarching conclusion from the results collected in chapter two is that XF-73 has a potent bactericidal activity against Gram-positive planktonic cells. XF-73 was able to inhibit bacterial growth and eradicate bacterial cells at concentrations ranging from ≤ 0.125 – 4 µg/ mL (Table 2.3). Additionally, different batches of XF-73 received from Destiny Pharma retained their enhanced killing effect against the Gram-positive microorganisms, in particular the staphylococcal strains (Table 2.3). These results are concurrent with previous research, where MIC testing was conducted with XF-73 and a range of Gram-positive and Gram-negative microorganisms and generated MIC and MBC values were similar to the values recorded in this current study (Ooi *et al.*, 2009a; Farrell *et al.*, 2010; Board-Davies *et al.*, 2022)

Through the comparison of batch 1 and batch 2 of XF-73, a bactericidal capability of batch 2 XF-73 was recorded against *E. coli* and *P. aeruginosa* strains. The variation of MIC and MBC values between different batches of XF-73 indicate that the antibacterial properties of the compound are dependent on XF-73 synthesis. Each batch of XF-73 received from Destiny Pharma throughout the project was prepared differently. There was a clear difference in appearance between the crystalline compounds of XF-73 per batch. XF-73 in batch 2 and 3 had much finer granules in comparison to batch 1, which had larger granules that clustered together. The synthesis and preparation for each batch of XF-73 remains confidential to Destiny Pharma, therefore further comparison and analysis of the different preparation methods could not be conducted.

Gram-negative microorganisms were determined to be less susceptible to XF-73 treatment, as a greater concentration of XF-73 was required to eradicate bacterial growth in comparison to lower concentrations eradicating Gram-positive bacterial cells (Table 2.3). Our current understanding into the specific mechanism of action of XF-73 is limited, but potassium and ATP leakage from Gram-positive bacterial cells was reported following XF-73 treatment (Ooi *et al.*, 2009b). Since previous studies have

primarily focussed upon the antibacterial properties of XF-73 against Gram-positive microorganisms, little research focuses on the lack of susceptibility of XF-73 against Gram-negative microorganisms.

The presence of an outer membrane in Gram-negative microorganisms is thought to be responsible for the reduced susceptibility. As XF-73 acts via membrane disruption, the presence of an outer membrane may be responsible for decreasing susceptibility for Gram-negative microorganisms against XF-73, as XF-73 may be unable to effectively enter the cell to cause an antibacterial effect. To test this theory, mutant stains of *E. coli* (*E. coli* DC0/ DC2) were introduced into the panel of microorganisms, *E. coli* DC2 was classified as having a hyper-permeable cell envelope in comparison to standard *E. coli* strains (Clark, 1984). Interestingly, this study showed no enhanced antibacterial effect of XF-73 against this mutant strain (Table 2.3); as neither inhibition nor eradication of the bacterial cells was recorded during MIC/ MBC assays. Consequently, these results indicate that the permeability of the cell envelope: outer membrane and cell wall, does not influence the antibacterial capability of XF-73.

Once the antibacterial capability of XF-73 was established against a range of microorganisms in planktonic mode of growth, the study then progressed to test XF-73 against the same microorganisms growing in established biofilms in chapter three. The ability to form biofilm is classified as a virulence factor for microorganisms and biofilm formation is noted as an adaptation strategy to survive hostile environments. Due to advances is modern day medicine, there is an increased use of implanted medical devices and indwelling medical equipment such as: urinary catheters, intravenous catheters, dialysis catheters, prosthetic heart valves, breast implants, dentures and even contact lenses. In turn this increases the presence of abiotic surfaces, leading to a greater prevalence biofilm formation. An estimated 80% of human microbial infections are classified as biofilm infections and in the USA alone 50% of HCAIs are due to infected indwelling devices. Another issue with biofilm infections is during the end of a biofilm life cycle planktonic bacterial cells are released from the biofilm, further spreading the infection. A distinct difference between planktonic and biofilm infections are the molecular mechanisms for survival, planktonic bacterial cells tend to operate through pathogenic infections and kill host cells to further growth; in contrast bacteria growing in biofilms favour defensive mechanisms to survive for extended periods of time in their niche environment and reduce susceptibility to antibacterial treatments or the host immune response. Therefore, chronic biofilm infections are difficult to treat with antibiotics alone, as antimicrobial treatments may overcome the acute planktonic infections caused by the dispersal of planktonic cells from the biofilm, but the biofilm itself remains undisturbed from these treatments (Phillips *et al.*, 2012; Paredes *et al.*, 2014; Wu *et al.*, 2015).

Whilst the formation of a biofilm protects bacterial cells in a hostile environment, the extracellular polymer matrix encasing the biofilm community also aids in resisting antimicrobial treatments. Indeed, approximately 10 – 1000 times the concentration of an antimicrobial to treat planktonic cells is required to combat biofilm infections. At these high concentrations, many antimicrobial treatments are no longer

feasible due to toxicity levels and potential side effects (Hengzhuang *et al.*, 2011; Wu *et al.*, 2015). Therefore, it is critical to assess the anti-biofilm properties of XF-73 against infections in biofilm mode of growth as novel antimicrobial treatments need to eliminate both planktonic and biofilm infections.

Through the comparison of MIC and MBC values determined in chapter two (Table 2.3) to MBIC and MBEC values collected in chapter three (Table 3.4), an assessment of the antibacterial properties of XF-73 demonstrates how effective XF-73 is against planktonic cells and established biofilms (Table 6.1). Additionally, a comparison between the bactericidal effect of XF-73 and the panel of known antimicrobials can be undertaken against *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212, in both planktonic and biofilm mode of growth (Table 6.2). These two data sets yield a promising capability of XF-73, as it maintained a potent bactericidal effect against Gram-positive microorganisms in both planktonic and biofilm modes of growth.

Concentrations of XF-73 ranging from 1 – 2 μg/ mL eradicated all four Gram-positive biofilms used in this study. A 4-fold and 16-fold increase in XF-73 concentration was required to eradicate *S. aureus* ATCC 6538 and *E. faecalis* ATCC 29213 biofilms respectively, in comparison to corresponding bactericidal concentrations against planktonic cells. Similarly, a minimum 8-fold increase in concentration to eradicate *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 biofilms in contrast to planktonic cells (Table 6.1).

Gentamicin retained its bactericidal capability against both *S. aureus* ATCC 29213 and *E. faecalis* biofilms with a 4-fold and 8-fold increase in concentration respectively in comparison to eradication of planktonic cells. Although when comparing XF-73 to gentamicin, an 8-fold and 64-fold increase in concentrations was required for gentamicin to eradicate these Gram-positive biofilms. Mupirocin was shown to eradicate neither planktonic nor biofilm cells of *S. aureus* ATCC 29213 and *E. faecalis* whilst rifampicin and daptomycin lost their bactericidal capability against *E. faecalis* biofilms. Moreover, a minimum 128-fold increase in rifampicin concentration was required to eradicate *S. aureus* ATCC 29213 biofilms in comparison to planktonic cells. Whilst only a 2-fold increase in daptomycin concentration was required for *S. aureus* ATCC 29213 biofilms compared to planktonic cells, higher concentrations of daptomycin led to eradication in comparison to XF-73 (Table 6.2).

Table 6.1 Inhibition and eradication concentrations (µg/ mL) of XF-73 against Gram-positive microorganisms in planktonic and biofilm modes of growth

Antimicrobial Microorganism	XF-73 (µg/ mL) MIC	MBC	MBIC	MBEC
S. aureus ATCC 6538	0.25	0.25	1	1
S. aureus ATCC 29213	≤ 0.125	≤ 0.125	1	1
S. epidermidis ATCC 12228	≤ 0.125	≤ 0.125	1	1
E. faecalis ATCC 29212	0.125	0.125	2	2

Table 6.2 Eradication concentrations (µg/ mL) of XF-73 and a range of antimicrobials against S. aureus and E. faecalis planktonic cells and established biofilms

Antimicrobial (µg/ mL) Microorganism	XF-73 MBC	MBEC	Gentamicin MBC	MBEC	Mupirocin MBC	MBEC	Rifampicin MBC	MBEC	Daptomycin MBC	MBEC
S. aureus ATCC 29213	≤ 0.125	1	2	8	> 64	> 256	≤ 0.125	16	16	32
E. faecalis ATCC 29212	0.125	2	16	128	> 64	> 512	> 64	> 512	16	> 512

Note: Lowest recorded MBEC values of XF-73 (µg/ mL) selected from all batches of XF-73 used throughout MIC/ MBC testing

Whilst a reduced panel of microorganisms was selected for the comparator study against bacterial biofilms (Table 6.2) the efficacy of XF-73 was tested against four Gram-positive microorganisms in planktonic and biofilm mode of growth (Table 6.1). The results gained suggest that XF-73 could be a proficient antimicrobial treatment, as no reduction in antibacterial efficacy was noted for XF-73 between eradicating planktonic cells or established biofilms. Furthermore, XF-73 at concentrations lower than all comparator antimicrobials was recorded to eradicate Gram-positive biofilms (Table 6.2), illustrating that, unlike currently used antimicrobials, XF-73 does not lose its effective antibacterial properties against bacterial biofilms.

Previous research conducted by Ooi *et al.,* (2009) supports these findings (Table 6.2), as fifteen commercial antimicrobials including: daptomycin, gentamicin, mupirocin and rifampicin were used as comparators against XF-73 against a strain of *S. aureus.* Whilst all comparator antimicrobials were effective in inhibiting growth of planktonic and biofilm cells, each antimicrobial was unable to eradicate *S. aureus* biofilms and MBEC's were determined as > 256 µg/ mL. In contrast XF-73 inhibited planktonic cells at 1 µg/ mL and eradicated the bacterial cells in a biofilm mode of growth at 2 µg/ mL (Ooi *et al.*, 2009a). A recent study also presented similar results, Board-Davies *et al.*, (2022) reported XF-73 inhibiting a range of *S. aureus* strains planktonic cells at concentrations 1 – 2 µg/ mL, a slight increase in XF-73 concentration was recorded to eradicate biofilms of the same *S. aureus* strains at 2 – 8 µg/

mL. The inhibitory effect of four antimicrobials were also included with XF-73 against a MRSA strain, mupirocin inhibited *S. aureus* at 0.125 µg/ mL (Board-Davies *et al.*, 2022), this result is similar to the results gained in chapter two study where the MIC values for mupirocin for *S. aureus* strains ranged from ≤ 0.125 – 0.5 µg/ mL (Table 2.3).

Chapter three focused on the anti-biofilm capability of XF-73 against single species biofilms after twenty-four hours of growth but also included an assessment of XF-73 against forty-eight hour Gram-positive biofilms, through MBIC/ MBEC (Table 3.6) testing and crystal violet staining (Figure 3.20 – 3.23). XF-73 maintained its bactericidal effect at low concentrations against *S. aureus* ATCC 29213, *S. epidermidis* and *E. faecalis* forty-eight hour biofilms and eradicated *S. aureus* ATCC 6538 forty-eight hour biofilms at a concentration of 256 µg/ mL (Table 3.9). The results gained during this study offered an insight into the anti-biofilm capability of XF-73 against mature biofilms; a novel area of research that previous studies have not explored.

XF-73 was unable to eradicate any established biofilm consisting of Gram-negative microorganisms (Table 3.4), and no anti-biofilm activity of XF-73 against Gram-negative biofilms has been recorded in previous studies (Board-Davies *et al.*, 2022). Though through biofilm viability testing and statistical analysis, it was shown that each concentration of XF-73 significantly reduced the viability of *E. coli* ATCC 35218, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 15442 biofilms at concentrations 64 – 512 µg/ mL of XF-73 and 128 – 512 µg/ mL of XF-73 also significantly reduced *P. aeruginosa* ATCC 27853 biofilms (p= <0.0001) (Figure 3.6 – 3.9). These novel data sets show an anti-biofilm capability of XF-73 that was previously unknown due to only limited research conducted in the area.

Although chapter three increased our current understanding on the anti-biofilm properties of XF-73, only single species biofilms were tested. Whilst single species biofilms occur naturally, mixed biofilms are also a common natural occurrence; mixed biofilms are often formed when multiple bacterial species are within close proximity of one another. Mixed biofilms, also known as polymicrobial biofilms have an increased virulence ability and a decreased susceptibility to antimicrobial treatments in comparison to single species biofilms. The multiple species located in a biofilm promote the development of larger structures, enabling the organisation of different microbial communities through structural patterns now present in the biofilm, leading to a larger biofilm superstructure (Røder *et al.*, 2016; Costa *et al.*, 2022).

Metagenomics of bacteria capable of forming biofilms have shown that these bacterial cells contain mechanisms to encourage diversity, enabling polymicrobial biofilms and the potential addition of fungal species into the biofilm communities. The benefits for microorganisms existing in polymicrobial biofilms include an increased level of antimicrobial resistance, as passive resistance increases in the biofilm if one species present possess resistance to an antimicrobial treatment as the sharing of these genes can occur between the bacteria; quorum sensing between the multiple species enables metabolic cooperation, preventing competition between the bacterial species and multiple species present in the

same location leads to a larger gene pool allowing DNA sharing to occur. Additional research has also highlighted that polymicrobial biofilms are better adapted to survive in nutrient limited environment in comparison to single species biofilms, due to an increased efficiency in degrading organic compounds (Wolcott *et al.*, 2013; Røder *et al.*, 2016). An improved ability of horizontal gene transfer is also recorded between different bacterial species in polymicrobial biofilms. Recent studies have recorded that this feat, where the carbapenemase resistance gene bla_{NDM-1} located in a plasmid in *E. coli* strains was transferred into *P. aeruginosa* and *A. baumannii* via conjugation; this transferal of resistance has not been recorded between these bacterial species as planktonic cells. MRSA strains have also gained vancomycin and tetracycline resistance *in-vivo* from *E. faecium* bacterial cells present in a polymicrobial through the transference of a *vanA*-containing plasmid and the resistant gene *tetU* respectively (Orazi *et al.*, 2020).

Polymicrobial biofilms are prevalent across a range of human diseases, with reported instances in oral infections, respiratory infections, UTIs, wound sites and diabetic foot ulcers. *P. aeruginosa* is one of the most reported bacterial species routinely found in polymicrobial biofilm infections, in particular respiratory infections relating to patients suffering with cystic fibrosis, chronic obstructive pulmonary disease or asthma. These polymicrobial biofilms include *P. aeruginosa* and *S. aureus,* although yeast strains such as *C. albicans* have also been reported with *P. aeruginosa* in mixed biofilms found in cystic fibrosis patients. Similarly, polymicrobial biofilms containing *P. aeruginosa* and *S. aureus* or *E. faecalis* are reported in wound infections such as burn wounds (Kulshrestha *et al.*, 2022). Due to the frequency of polymicrobial biofilms in clinical infections and HCAIs it is critical to test the efficacy of an antimicrobial against these mixed bacterial communities. Hence further testing to assess the efficacy of XF-73 against polymicrobial biofilms is warranted.

While polymicrobial biofilms form naturally in a multitude of environments, it is often challenging to generate polymicrobial biofilms *in-vitro,* as the correct media and growth conditions i.e. temperature and motion must be selected to support the growth of multiple species at a normalised rate to prevent one species out-competing the other as naturally they co-exist (Gabrilska *et al*, 2015). Research focusing on the optimum media composition for polymicrobial biofilm formation has shown promising results. The Lubbock chronic wound biofilm model was recorded as the first *in-vitro* model capable of simulating polymicrobial biofilms similar to those found in chronic wounds *in-vivo* (Sun *et al.*, 2008). The model enables cooperative growth between *S. aureus, E. faecalis* and *P. aeruginosa* through the creation of a novel media consisting of Bolton broth supplemented with 50% (v/v) plasma and 5% (v/v) freeze thaw haemolysed horse red blood cells. Equal amounts of each microorganism were inoculated in glass test tubes, a pipette tip remains in the test tube to operate as the surface for biofilm formation. This model enables the visualisation of biofilms via scanning electron microscopy and is capable of generating MBEC values (Sun *et al.*, 2008; Di Giulio *et al.*, 2020). Previous studies have also utilised the Lubbock chronic wound biofilm model to generate forty-eight hour *in-vitro* polymicrobial biofilms and

transfer these established biofilms *in-vivo* to generate chronic wounds in a mouse model (Dalton *et al.*, 2011).

Static or closed system models are frequently used due to their technical simplicity and are the least expensive form of model and rely on plastic polymers to act as the surfaces for biofilm adhesion and formation. Therefore, commonly used microtitre assays are Calgary biofilm devices for single species biofilm formation can be used to generate polymicrobial biofilms (Gabrilska *et al.*, 2015). Future studies involving XF-73 against polymicrobial biofilms containing both Gram-positive and Gram-negative species could be attained via the Calgary device and the growth media used in the Lubbock chronic wound biofilm model.

The results from chapter two and chapter three have determined the potent bactericidal effect of XF-73 against Gram-positive microorganisms in planktonic and biofilm mode of growth. Previous research has also assessed the antibacterial capability of XF-73's secondary mechanism of action. Board-Davies *et al.,* (2022) reported an enhanced bactericidal effect of photo-activated XF-73 against Gram-positive planktonic cultures, although greater concentrations of photo-activated XF-73 were noted to eradicate *S. aureus* biofilms in comparison to the primary mechanism of action of XF-73 in natural light (Board-Davies *et al.*, 2022). *Ex-vivo* testing involving a porcine skin model conducted by Maisch *et al.* 2005, 2007 illustrated that photo-activated XF-73 at 7.66 μg/ mL led to a 3-log reduction in viability of MRSA bacterial cells and a 3-log to 5-log reduction bacterial viability against multiple *S. aureus* strains (Maisch *et al.*, 2005, 2007). Chapter four then progressed into exploring the secondary mechanism of action of XF-73 against Gram-positive and Gram-negative microorganisms in both planktonic and biofilm modes of growth, to further expand on the antibacterial properties photo-activated XF-73.

Compared to XF-73 in natural light, photo-activated XF-73 retained its killing capability as MIC = MBC and MBIC = MBEC against each Gram-positive microorganism (Table 6.3), indicating that XF-73 in natural light and photo-activated XF-73 are both bactericidal antimicrobial treatments. Photo-activated XF-73 exhibited a greater bactericidal capability in comparison to XF-73 in natural light against all Gram-positive planktonic cells as MIC/ MBC values decreased from 0.125 – 2 μg/ mL to ≤ 0.03 – 0.25 μg/ mL following fifteen minutes blue light exposure (Table 6.3). The same enhanced effect was also recorded from the recent study conducted by Board-Davies *et al.,* (2022) where the MIC concentration of XF-73 decreased from 1 – 2 μg/ mL to 0.03 – 0.5 μg/ mL following blue light exposure against three *S. aureus* strains and *S. hominis* (Board-Davies *et al.*, 2022).

Variations in the anti-biofilm capability of photo-activated XF-73 was noted between the results gained in chapter four to previous research. Board-Davies *et al.,* (2022) reported that photo-activated XF-73 had a decreased efficacy against Gram-positive microorganisms where MBEC values increased from 2 – 8 μg/ mL to 4 – 16 μg/ mL following photo-activation via blue light (Board-Davies *et al.*, 2022); with the exception of *S. aureus* ATCC 6538, the remaining Gram-positive biofilms had an increased

susceptibility to XF-73 and blue light exposure as MBEC values decreased from 1 – 2 µg/ mL to ≤ 0.125 – 0.5 µg/ mL (Table 6.3).

Table 6.3 Inhibition and eradication concentrations (µg/ mL) of XF-73 in natural light and photo-activation via blue light exposure against Gram-positive microorganisms in planktonic and biofilm modes of growth

Antimicrobial (µg/ mL) Microorganism	XF-73 Natural light				XF-73 Blue light			
	MIC	MBC	MBIC	MBEC	MIC	MBC	MBIC	MBEC
S. aureus ATCC 6538	1	1	1	1	0.25	0.25	> 8	> 8
S. aureus ATCC 29213	2	2	1	1	≤ 0.03	≤ 0.03	0.125	0.125
S. epidermidis ATCC 12228	1	1	1	1	≤ 0.03	≤ 0.03	≤ 0.125	≤ 0.125
E. faecalis ATCC 29212	0.125	0.125	2	2	≤ 0.03	≤ 0.03	0.5	0.5

Note: lowest recorded MIC and MBC values selected from batch 1 of XF-73 (Table 4.1)

It is speculated that the decreased activity of photo-activated XF-73 against different the strains of *S. aureus* biofilms, including *S. aureus* ATCC 6538 (Table 6.3), may be due to the expression of staphyloxanthin; a carotenoid pigment with a recorded capability of quenching singlet oxygen during PDT and other ROS generated from neutrophils as an immune response (Dahl *et al.*, 1989; Sun *et al.*, 2020). Interestingly, the reduced susceptibility of *S. aureus* strains against photo-activated XF-73 was only recorded against established biofilms. This was reported in chapter four (Table 4.1, 4.2, 4.3) and the study undertaken by Board-Davies *et al.,* (2022) as MIC testing recorded an increased susceptibility to photo-activated XF-73. Therefore, staphyloxanthin alone is unable to fully reduce *S. aureus* susceptibility to ROS but does so once the *S. aureus* strains have formed biofilms. Staphyloxanthin is located in the cytoplasmic membrane of bacterial cells (Zhang *et al.*, 2018); however, if it is also expressed in the extracellular polymer matrix of the biofilm, the pigment may quench singlet oxygen produced via type 2 reactions through the photo-activation of XF-73 before it can interact with the bacterial cells, decreasing the efficacy. However, as photo-activated XF-73 was still reported to eradicate each *S. aureus* biofilm, this staphyloxanthin-mediated defence mechanism was unable to fully prevent the photo-toxic effect of XF-73 following blue light exposure.

There was no enhanced anti-biofilm effect of photo-activated XF-73 against forty-eight hour Gram-positive biofilms in comparison to XF-73 in natural light (Table 4.4). As mature biofilms have a greater density, a greater abundance of staphyloxanthin may also be present in the biofilms leading to a reduced susceptibility.

The results obtained in chapter four also illustrated an enhanced killing capability of XF-73 against Gram-negative microorganisms, as MIC and MBC values of XF-73 decreased following blue light activation in comparison to XF-73 in natural light. MBC values decreased from 128 – > 256 µg/ mL to 32 – 128 µg/ mL following photo-activation (Table 4.2). The enhanced antibacterial capability of XF-73

was not maintained against all Gram-negative biofilms as only *E. coli* ATCC 25922 biofilms were eradicated following 512 µg/ mL of XF-73 (Table 4.3). The additional treatment strategy against Gram-negative biofilms, consisting of photo-activated XF-73 treatment for fifteen minutes followed by XF-73 treatment overnight in natural light also failed to eradicate the remaining Gram-negative biofilms. Board-Davies *et al.,* (2022) also reported no enhanced anti-biofilm capability of photo-activated XF-73 against *E. coli* and *P. aeruginosa* biofilms (Board-Davies *et al.*, 2022).

Biofilm viability testing determined that whilst no concentrations of photo-activated or retreated XF-73 led to the total eradication of *E. coli* ATCC 35218, *P. aeruginosa* ATCC 15442 and *P. aeruginosa* ATCC 27853 biofilms, both treatments led to a reduction in the total viability of remaining bacterial cells. Photo-activated XF-73 and retreated XF-73 had a greater reduction in *E. coli* ATCC 35218 biofilm viability at concentrations 32 – 512 µg/ mL in comparison to XF-73 in natural light (Figure 4.12). *P. aeruginosa* biofilms were concluded to be less susceptible to PDT treatment involving XF-73 as neither treatment led to an enhanced reduction in biofilm viability in comparison to XF-73 in natural light, though 512 µg/ mL of photo-activated XF-73 led to a viability reading of *P. aeruginosa* ATCC 15442 below the lower limit of detection (Figure 4.13, 4.14).

The research undertaken in chapter four lacked any additional antimicrobials to compare against the photo-activated antimicrobial efficacy of XF-73. This was due to the fact that the comparator antimicrobials used in chapter two and chapter three were not classified as PSs, therefore light activation would not increase their bactericidal properties. Board-Davies *et al*., (2022) illustrated that of the four antimicrobials selected, retapamulin exhibited an enhanced inhibitory effect against MRSA with a 1-fold reduction in MIC values following blue light exposure and polymyxin B had a 4-fold reduced in MIC values following photo-activation against *P. aeruginosa*. Mupirocin and ertapenem remained unaffected by blue light (Board-Davies *et al.*, 2022). Therefore, further testing including PSs would be appropriate comparators against photo-activated XF-73. Potential PSs include hypericin as this natural anthraquinone PS has shown a bactericidal effect against Gram-positive and Gram-negative planktonic cells and an anti-biofilm capability against *S. aureus* biofilms after yellow light exposure at 593 nm (Liu *et al.*, 2015). An additional PS includes 5-aminolevulinic acid as after LED light activation at 630 nm, it showed a promising bactericidal effect against *P. aeruginosa* bacterial cells in planktonic and biofilm mode of growth. 5-aminolevulinic acid following LED exposure decreases the expression of mRNA relating to QS-related genes and reduces virulence factor secretion (Tan *et al.*, 2018). Clinical testing involving photo-activated 5-aminolevulinic acid and red light treatments against patients suffering from *P. aeruginosa* chronic skin ulcers reported that PDT involving 5-aminolevulinic acid led to a greater reduction in the ulcer surface area in comparison to red light alone (Lei *et al.*, 2015).

The experiments conducted in chapters two, three and four focused only on the antibacterial properties of XF-73 alone against a range of clinically relevant microorganisms. As XF-73 was less effective

against Gram-negative microorganisms in comparison to Gram-positive microorganisms in both planktonic and biofilm modes of growth (Table 2.3, 3.4), combination therapies involving antimicrobials with a known antibacterial effect against Gram-negative microorganisms may aid in the increasing the efficacy of XF-73. Rifampicin is a potential antimicrobial for combination therapy with XF-73 against Gram-negative planktonic cells due to the results gained from chapter two indicating low concentrations of rifampicin inhibited bacterial growth (Table 2.4). Gentamicin showed a greater antibacterial effect against Gram-negative microorganisms in planktonic and biofilm mode of growth and had the lowest MBC and MBEC values out of the range of antimicrobials (Table 2.4, 3.5); therefore, combining XF-73 and gentamicin may lead to an increased antibacterial effect of XF-73 against planktonic cells and established biofilms of Gram-negative microorganisms. Board-Davies *et al.,* (2022) showed synergistic effect between XF-73 and polymyxin B against *P. aeruginosa* planktonic cells, additionally once XF-73 was photo-activated through blue light exposure an enhanced inhibitory effect was recorded with polymyxin B. A synergistic effect was also recorded between XF-73 and ertapenem against MRSA planktonic cells, illustrating that the already potent antibacterial effect of XF-73 can be further enhanced through combination therapy (Board-Davies *et al.*, 2022).

The results gained throughout chapters two, three and four were all conducted using MHB and MHA. MHB was selected for these studies as the enriched media is the recommended media for MIC testing due to its ability to facilitate growth for a range of aerobic and facultative anaerobic bacteria and is commonly used in research studies (Sigmaalrdich, 1989). Additionally previous studies assessing the antibacterial properties of XF-73 via MIC testing were conducted using MHB, therefore direct comparisons are possible between this study and previous research if the same media is used (Ooi *et al.*, 2009a; Ooi *et al.,* 2009b Farrell *et al.*, 2010; Board-Davies *et al.*, 2022). Though it was noted that due to the chemical structures of haemin and haem (Figure 5.2), interactions with haemin at wound sites or in the blood may occur with XF-73, or due to the similarities of XF-73 and haemin as porphyrin structures, XF-73 may interact with the iron uptake systems on the bacterial cell walls leading to the antibacterial effect. Therefore, chapter five aimed to assess any potential interactions between XF-73 and haemin and to try and further enhance our understanding of XF-73's mechanism of action. Additional testing in chapter five included an alternative medium for MIC testing to simulate a restricted environment that would occur at an infection site. RPMI was the selected restricted media, as previous bacterial studies have used the media to simulate an iron restricted environment. Staphylococcal strains i.e. *S. aureus* and *S. lugdunensis* have been shown to grow in RPMI media, though at a reduced rate in comparison to an iron enriched media (Ythier *et al.*, 2012; Zapotoczna *et al.*, 2012). Through the use of an iron restricted medium, the novel results gained in this chapter offer an insight into how effective XF-73 may be against bacterial infections in a wound site or potential blood stream infection.

Initial testing conducted in chapter five indicated that in MHB the presence of haemin decreased the antimicrobial capability of XF-73; a concentration dependent increase in XF-73 MIC and MBC values

was observed following an increase of haemin concentration. This trend was recorded for each Gram-positive microorganism selected for this study (Table 5.4). These results indicate that haemin is either a direct competitor to XF-73 for interaction between bacterial cells, or haemin interacts directly with XF-73, decreasing the antimicrobial capabilities of XF-73. Due to the different charges of XF-73 and haemin it is unlikely that both compounds have the same interaction pathway between bacterial cells. XF-73 is a dicationic porphyrin ion in aqueous solution whereas haemin is a dianionic porphyrin ion. Bacterial iron uptake systems have the capability to interact directly with environmental haem, for example *S. aureus* species have surface bound receptors IsdB and IsdH to facilitate haem binding (Grigg *et al.*, 2010). Therefore, it is highly unlikely that positively charged XF-73 ions have the capability to interact with haem receptor sites located on the bacterial cell wall.

Interestingly, once MIC and MBC testing was repeated in the iron-restricted RPMI media, haemin no longer negatively affected the antibacterial capability of XF-73 against both *S. aureus* strains and *E. faecalis* (Table 5.6). It is critical for bacteria to acquire iron from external sources to maintain biochemical processes inside the cells, but due to strict iron regulation maintained in humans to prevent oxidative damage, there is a limited abundance of free iron located in the blood and extracellular fluids. Additionally, an innate immune response to bacterial infections is the further regulation and reduction of free iron. To survive in these hostile environments, Gram-positive bacteria utilise iron-uptake systems to aid in the acquisition of the remaining free iron (Brown *et al.*, 2002; Maltais *et al.*, 2016). Therefore, the studies conducted in RPMI media, stimulate *S. aureus* and *E. faecalis* to upregulate their iron-uptake mechanisms, increasing the bacterial affinity to haemin; previous research supports this theory as *S. aureus* upregulated the Isd system in RPMI media (Ythier *et al.*, 2012). These surface bound receptors of the Isd system have a higher affinity for haemin in comparison to XF-73, preventing the occurrence of XF-73-haemin complexes, leading to XF-73 maintaining its bactericidal capability against Gram-positive bacterial cells as similar MIC and MBC results were noted between XF-73 in MHB and RPMI media (Table 5. 8).

Whilst the results generated from the research presented in chapter five are unable to increase our understanding into the mechanism of action of XF-73; the decreased antibacterial efficacy of XF-73 against Gram-positive planktonic cells in MHB following the addition of haemin indicates a potential interaction between the two compounds. Whilst no conclusion can be drawn for the type of interaction occurring between haemin and XF-73, no solid precipitate was observed in test wells containing haemin and XF-73, indicating that any XF-73-haemin complex that might be formed remains soluble in solution. Current speculations regarding the XF-73-haemin complex involved the combination through ion pairings between from the electrostatic attraction between the dicationic XF-73 ions and the dianionic haemin ions. Additional pi-pi interactions may occur between the porphyrin rings in XF-73 and haemin, further stabilising the complex (Natali *et al.*, 2016; Yamasumi *et al.*, 2021). The results gained from MIC MBC studies in RPMI media (5.5.3) support the current theory of ion pairs between the different charged

compounds and pi-pi interactions between the porphyrin rings of XF-73 and haemin as these are classified as weaker electrostatic forces of attraction in comparison to covalent bonds or active binding. Therefore, the loss of the antagonistic effect of haemin against XF-73 in RPMI illustrates that only weak interactions keep the XF-73-haemin complex together.

Natural pi-pi interactions have also been recorded between biofilms and antimicrobial treatments, where aromatic surfaces of both the antimicrobial and the extracellular polymeric substances that are located in the extracellular polymer matrix that encases the biofilm community. Additionally, negatively charged polymeric substances are able to bind to positively charged antimicrobials or react with them through redox-processes; all of these protection mechanisms prevent the antimicrobial treatments to diffuse through the biofilm matrix (Cieplik *et al.*, 2014). It is possible that these processes occur in Gram-negative biofilms preventing the bactericidal effect of XF-73, hence the reduced susceptibility in comparison to Gram-positive biofilms; the decreased antibacterial capability of XF-73 following haemin introduction into the environment supports the theory that ion pairs or pi-pi interactions happen between haemin and XF-73 and could potentially also occur between XF-73 and the extracellular polymeric substances making up the biofilm matrix of Gram-negative microorganisms.

These results have laid a foundation to further explore the interactions between XF-73 and haemin, where spectroscopic testing involving the potential change in absorption spectra of XF-73 following the addition of haemin in the solution indicating the formation of XF-73-haemin complexes. Solutions containing electrolytes are known to facilitate the occurrence of ion pairs (Britannica, 2019). Chloride and sodium ions are present in solutions of MHB and RPMI media due to the disassociation of chloride ions from XF-73 and sodium ions from haemin; further supporting the likelihood of XF-73-haemin complexes forming via ion pairs.

Both Gram-positive and Gram-negative bacteria contain endogenous PSs, many of these PSs are determined to be intermedia species involved in haem biosynthesis i.e. protoporphyrin, uroporphyrin and coproporphyrin, and are potentially localised in the cytoplasmic matrix of the bacterial cells. These intermediate species are also known to be photo-activated by blue light ranging from 400 – 450 nm (Hadi *et al.*, 2020). As previously stated (4.1) clinical trials are currently underway exploring the photo-toxic capabilities of protoporphyrin against bacterial infections including surgical site infections, diabetic ulcers and wound infections; the combination of protoporphyrin mounted on silver nanoparticles has also increased eradication times of *S. aureus* from as little as ten seconds of LED light exposure (Morales-De-Echegaray *et al.*, 2020; Italo *et al.*, 2021). Therefore, from current knowledge and the data generated in this thesis, the photo-toxic effect of XF-73 following blue light exposure may be aided by the naturally occurring PSs located in the bacterial cells. Additionally, in iron restricted media a greater abundance of these endogenous PSs may occur due to the increased expression of iron-acquisition systems leading to a further enhanced bactericidal efficacy of XF-73 following blue light exposure. To

test this hypothesis further MIC/ MBC assays involving photo-activated XF-73 against the panel of Gram-positive and Gram-negative microorganisms should be carried out in RPMI media. The research conducted in this thesis has further confirmed the potent bactericidal capability of XF-73 against clinically relevant Gram-positive microorganisms. As different bacterial isolates were selected for this thesis in comparison to previous research, the recorded MIC and MBC values attained in this study add to the list of historic MIC and MBC values. The addition of three consecutive methods to assess the anti-biofilm effect of XF-73 against bacterial biofilms offered a greater insight into the immediate effect of XF-73 against bacterial biofilms, that previous studies had not previously explored; without biofilm viability testing the killing capability of XF-73 would have been missed against Gram-negative biofilms through MBIC MBEC assays alone; and viability testing further highlighted a promising potential for XF-73 as an anti-biofilm treatment against Gram-positive biofilms.

Blue light exposure triggered the activation of XF-73's secondary mechanism of action offering the alternative treatment strategy involving PDT. Due to the dual mechanisms of action XF-73 has an advantage over standard antimicrobial treatments, as bacterial species are less likely to develop resistance to PDT due to no specific target site and treatments can be localised (Wainwright *et al.*, 2017; Jia *et al.*, 2019). Blue light alone has a recorded antibacterial property against dermal infections in particular *S. aureus,* one of the most commonly isolated bacteria related to skin infections. Additionally blue light is absorbed into a range of mammalian cell types, and the addition of a PS further enhances the antibacterial effect (Gwynne *et al.*, 2018).

Initial testing conducted on the photo-dynamic effect on XF-73 as an antibacterial treatment conducted by Maisch *et al.* 2005 also assessed the effect of photo-activated XF-73 against human dermal keratinocytes and fibroblast cells, to ensure that potential of XF-73 as a PS for PDT against skin infections. XF-73 at 0.039 µg/ mL following blue light exposure was recorded to have no toxicity against the dermal cell lines, but led to a 3-log reduction in bacterial viability of all staphylococcal strains used in the study (Maisch *et al.*, 2005). Further testing led by Maisch *et al.* 2007 focused on *ex-vivo* involved porcine skin models and recorded that XF-73 remained localised in the outermost layer of the epidermis and had a dose dependent response to the inhibition and eradication of MRSA bacterial cells (Maisch *et al.*, 2007). Therefore, due to the results collected in this study and previous experiments, XF-73 has the capability to be a potent PS for PDT against wound or surface skin infections involving Gram-positive microorganisms, in particular *S. aureus* strains in planktonic or biofilm mode of growth; with a reduced chance of resistance occurring in comparison to XF-73 treatments in natural light. The results gained from chapter five supplement the potent capability for XF-73 to be used against surface infections as the presence of free iron will not affect the antibacterial properties of XF-73.

Skin is the largest organ in the human body, providing protection for the internal organs from the external environment as a physical and chemical barrier. Although the skin is classified as the most

frequently injured human organ and bacterial infections leads to acute or chronic wound infections, if these bacterial infections have the capability to form biofilms, 60% of chronic wound infections are related to bacterial biofilms, the healing of these wounds is dramatically reduced (Darvishi *et al.*, 2022). As previously stated (1.4.4), wet wound dressings are the most common treatment strategy to aid in the healing of wounds and the prevention of bacterial infections by acting as a physical barrier; modern wound dressings incorporate factors to aid in wound healing and skin regrowth while incorporating antibacterial agents to combat wound infections. These wound dressings containing antimicrobial agents include a range of treatments to combat localised wound infections that require high concentrations of antimicrobial treatments. A wide variety of antimicrobial treatments have the capability to be incorporated into the wound dressings including cephalosporins, quinolones, tetracycline's and aminoglycosides; additionally essential oils that exhibit an antibacterial capability are also selected for wound dressings (Varaprasad *et al.*, 2020; Katas *et al.*, 2021; Darvishi *et al.*, 2022). Further development involving the incorporation of novel antimicrobial treatments including silver nanoparticles capable of inhibiting planktonic and biofilm cultures of a range of Gram-positive and Gram-negative microorganisms (Katas *et al.*, 2021)

XF-73 already has an application as a nasal gel to decrease *S. aureus* presence in the nose before surgeries to prevent post-operative *S. aureus* infections (ClinicalTrials.gov, 2019), and is currently in the design stage for a phase 3 trial to prevent post-surgical *S. aureus* infections following breast surgeries. The results from this thesis propose that additional clinical applications of XF-73 could include the prevention or treatment of wound infections caused by *S. aureus* or *S. epidermidis* i.e. diabetic wounds, pressure sores and venous stasis ulcers (Schierle *et al.*, 2009). As it has already been proven that XF-73 is active in a gel formulation, further testing involving the application of XF-73 in hydrogels or wound dressings may expand the clinical uses for XF-73; with photo-activation offering a further enhancement in the bactericidal capability against Gram-positive microorganisms in planktonic and biofilm modes of growth and combating the ever-growing issue of AMR due to the reduced likelihood of resistance occurring against PDT.

7.0 Chapter 7: References

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8.0 Chapter 8: Appendices

8.1 Conferences and presentations

Bioscience Research Day, 2022, Birmingham, 10-minute presentation with 5-minutes of questions: Antibacterial efficacy of XF-73 via two mechanisms of action against Gram-positive microorganisms in biofilm mode of growth. Isabella Romeo-Melody, Daniel Hynes, William Rhys-Williams, Bill Love, Peter Lambert, Tony Worthington

European Congress of Clinical Microbiology and Infectious Diseases annual 32nd Conference, 2022, Lisbon, poster with presentation session, online presentation submitted for hybrid conference (4656/L0321): Antibacterial efficacy of XF-73 via two mechanisms of action against Gram-positive microorganisms in biofilm mode of growth. Isabella Romeo-Melody, Daniel Hynes, William Rhys-Williams, Bill Love, Peter Lambert, Tony Worthington

ABX Antibiotic Discovery Accelerator Network Meeting, 2021, Birmingham, 10-minute presentation with 5-minutes of questions: Antimicrobial efficacy of XF-73 against Gram-positive microorganisms. Isabella Romeo-Melody, Daniel Hynes, William Rhys-Williams, Bill Love, Peter Lambert, Tony Worthington

Microbiological Society Roadshow, 2021, Birmingham, 5-minute presentation with 3-minutes of questions: Antimicrobial efficacy of XF-73 against *E. faecalis* biofilms. Isabella Romeo-Melody, Daniel Hynes, William Rhys-Williams, Bill Love, Peter Lambert, Tony Worthington

Medical Research Foundation National PhD Training Programme in AMR Research Annual Conference, 2021, online, 3-minute presentation: Antimicrobial efficacy of XF-73 and antibiotic comparators against Enterococcus faecalis in biofilm mode of growth. Isabella Romeo-Melody, Daniel Hynes, William Rhys-Williams, Bill Love, Peter Lambert, Tony Worthington

European Congress of Clinical Microbiology and Infectious Diseases annual 31st Conference, 2021, online, poster with audio description (1435): Antimicrobial efficacy of XF-73 and antibiotic comparators against clinically relevant Gram-positive microorganisms in biofilm mode of growth. Isabella Romeo-Melody, Daniel Hynes, William Rhys-Williams, Bill Love, Peter Lambert, Tony Worthington

European Congress of Clinical Microbiology and Infectious Diseases annual 30st Conference, 2020, online, abstract (3606): Antimicrobial efficacy of XF-73 against Gram-positive microorganisms. Isabella Romeo-Melody, Daniel Hynes, William Rhys-Williams, Bill Love, Peter Lambert, Tony Worthington

Medical Research Foundation National PhD Training Programme in AMR Research Annual Conference, 2020, online, 3-minute presentation: Enhancement of XF-73 against *Staphylococcus aureus* with photodynamic therapy. Isabella Romeo-Melody, Daniel Hynes, William Rhys-Williams, Bill Love, Peter Lambert, Tony Worthington

Medical Research Foundation National PhD Training Programme in AMR Research Annual Conference, 2019, Bristol, poster with presentation: Antimicrobial efficacy of XF-73. Isabella Romeo-Melody, Daniel Hynes, William Rhys-Williams, Bill Love, Peter Lambert, Tony Worthington

8.2 Manuscripts in preparation

Working titles for publications:

- The antimicrobial efficacy of XF-73 and mupirocin against a panel of clinically relevant microorganisms. Is XF-73 a novel anti-staphylococcal agent in nasal decontamination strategies?
- The antimicrobial efficacy of photo-activated XF-73 against a panel of clinically relevant microorganisms in planktonic and biofilm modes of growth.
- The antimicrobial efficacy of XF-73 against clinically relevant Gram-positive microorganisms in the planktonic mode of growth under iron restriction and the potential effects of competition with haemin.

8.3 Copies of conference presentations

8.3.1 Medical Research Foundation annual conferences

Aston University Birmingham

Antimicrobial Efficacy of XF-73

Isabella Romeo-Melody

Dr Tony Worthington and Professor Peter Lambert

Destiny Pharma

Introduction: Antimicrobial Resistance

Antimicrobial resistance (AMR) is regarded as one of the greatest threats to human health globally. Infections caused by AMR microorganisms has led to 25,000 deaths and an increase in healthcare costs by €1.5 billion each year in Europe[1]. In addition there has been a lack in the development of novel antibiotics over the last 30 years (Fig 1). This highlights the need for the discovery and development of effective novel antimicrobials.
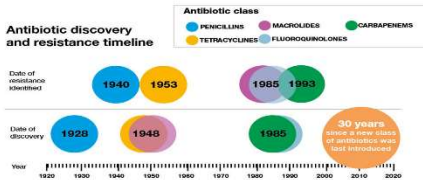

Figure 1 Timeline illustrating antimicrobial resistance over a 100 year time period for all main antibiotic classes. Showing the date of discovery and introduction to an antibiotic class in comparison to the year of resistant strains were reported. Taken from: Public Health England Guidance, health matters: antimicrobial resistance (December 10th 2013)

Exeporfinium Chloride – XF-73

XF-73 is the leading compound of the XF-73 antimicrobial drug series produced by Destiny Pharma; as a dicationic porphyrin (Fig 2), this unique structure is different to known antibiotic classes[2], offering a novel mechanism of action as a membrane disruptor[3]. XF-73 offers a low probability of antimicrobial resistance occurring, as shown by a previous study for AMR of *Staphylococcus aureus*[4].
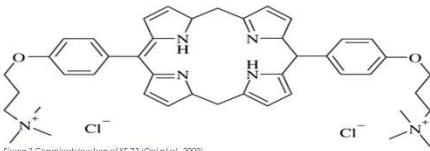

Figure 2 Chemical structure of XF-73 (Ooi et al., 2009)

Aims and Objectives

The aim of the current research, was to investigate the antimicrobial efficacy of XF-73 against clinically and industrially relevant pathogens. To achieve this aim the objectives were to:

- Determine the efficacy of XF-73 through disc diffusion assays.
- Determine minimum inhibitory and bactericidal concentrations (MIC/MBC) of XF-73 against clinically and industrially relevant pathogens.

Methods and Results: Disc Diffusion Assay

Mueller-Hinton agar plates were surface inoculated with a 10^6 CFU/mL suspension of each organism, to produce lawns on incubation. Blank sterile paper antibiotic sensitivity discs (6mm diameter), were placed on the inoculated surface of each plate (6 per plate) and 20 µL of each concentration of XF-73 was applied to the discs (Fig 3). XF-73 concentrations used were; 1024 µg/ mL, 102.4 µg/ mL, 10.24 µg/ mL and 1.024 µg/ mL. Plates were incubated overnight at 37°C in aerobic conditions, and diameters of zones of inhibition of growth were measured in mm (including the diameter of the paper disc).
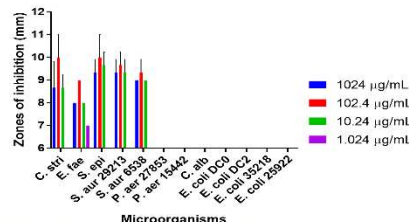

Figure 3 Mean values of recorded zones of inhibition for XF-73 from disc diffusion assays, against a panel of Gram-positive and Gram-negative organisms and a fungus, with error bars indicating the standard deviation of the data from 3 replicates. Diameter of blank disc 6mm.

Minimum Inhibition Concentrations (MIC's) and Minimum Bactericidal Concentrations (MBC's)

MIC's were determined using the broth microdilution method, following the Clinical and Laboratory Standards Institute (CLSI) guidelines. MIC's were prepared by 2-fold dilution of stock concentration of XF-73, with final concentrations ranging from: 256 µg/ mL – 0.5 µg/ mL. Each well was inoculated with test microorganism at 10^6 CFU/ mL, in Mueller-Hinton broth and incubated overnight at 37°C in aerobic conditions (Table 1). MBC's were determined by subculture of MIC assay onto Mueller-Hinton agar plates, incubated overnight at 37°C in aerobic conditions.

Table 1 Recorded MIC and MBC values against a panel of Gram-positive and Gram-negative microorganisms, including American Type Culture Collection strain numbers.

Microorganisms	American Type Culture Collection (ATCC)	MIC/ MBC µg/ mL
Pseudomonas aeruginosa	15442	>256/ >256
Escherichia coli	35218	>256/ >256
Staphylococcus aureus	6538	0.25/ 0.25
Staphylococcus aureus	29213	0.125/ 0.125
Staphylococcus epidermidis	12228	0.125/ 0.125
Enterococcus faecalis	29212	0.25/ 0.25
Corynebacterium striatum	1293	0.125/ 0.125

Discussion: Disc Diffusion Assay's

XF-73 produced small zones of inhibition: 7 mm to 11 mm (Fig 3), suggesting that XF-73 may not be effective in inhibiting growth against a range of microorganisms, when using this method. However, it is possible that XF-73 does not easily diffuse through agar, causing the relatively small zones of inhibition observed; this might explain why previous work using XF-73 has involved testing in Mueller-Hinton broth and conducting tests focusing on minimum inhibition concentrations.

MIC's and MBC's

XF-73 is highly effective against Gram-positive microorganisms (*S. aureus, S. epidermidis, E. faecalis* and *C. striatum*), shown by the low concentrations required to inhibit growth (Table 1). XF-73 is classified as an bactericidal antimicrobial, as the MIC's are equal to the MBC's. Though, XF-73 is not an effective antimicrobial against Gram-negative microorganisms (*E. coli* and *P. aeruginosa*), due to the absence of zones of inhibition during disc diffusion assays and no recorded MIC's from the high concentrations of XF-73 (256 µg/ mL) used. The lack of effectiveness of XF-73 against Gram-negative microorganisms may be due to their structure which increases the difficulty for antibiotic treatment. Gram-negative microorganisms possess an outer membrane, which prevents large or hydrophobic molecules entering and cytoplasmic efflux pumps, which are capable of pumping out antibiotics that were able to enter the cell[5]. XF-73 therefore, may be unable to pass through the outer membrane. Alternatively XF-73 has entered the cell, thought the efflux pumps have removed XF-73, before an effect can occur.

Future work

Following the aim of this study, future research objectives are to:

- Assess the efficacy of XF-73 on microbial growth, including time kill studies.
- Assess the efficacy of photodynamic therapy, on enhancing the antimicrobial activity of XF-73 against planktonic microorganisms.
- Assess the efficacy of XF-73 against single organism and polymicrobial biofilms in the presence and absence of photodynamic therapy.
- Determine if the composition of a biofilm alters the efficacy of XF-73, in regards to polymicrobial biofilms containing both Gram-positive and Gram-negative microorganisms.

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Aston University Birmingham

Antimicrobial efficacy of XF-73 and antibiotic comparators against *Enterococcus faecalis* in biofilm mode of growth.

Destiny Pharma

Introduction

- Infections involving **bacterial biofilms** are harder to treat due to the biofilm matrix:
  - Offering increased **resistance** and **protection** from antimicrobials.

- Therefore there is a need for novel antimicrobials capable of penetrating the biofilm matrix.

Aim of the study:

- Investigate the **antimicrobial efficacy of XF-73** against *E. faecalis* ATCC 29212 in a **biofilm mode of growth**.

Exeporfinium Chloride: XF-73

- XF-73 is an antimicrobial with a novel rapid mechanism of action as a membrane disruptor.
  - Leading to potassium and ATP leakage without lysing the cells.
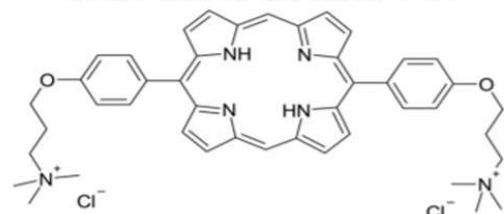
Figure 1: chemical structure of XF-73.

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Minimum biofilm inhibition concentrations and minimum biofilm eradication concentrations

- Biofilm formation occurred via the Calgary Biofilm Device using a starting inoculum of 10^5 CFU/mL of *E. faecalis* ATCC 29212.

- Biofilms were treated with: XF-73, gentamicin, mupirocin and rifampicin at concentrations ranging from **0.125 – 512 μg/mL** for 24 hours.

- Treated biofilms were transferred into MHB for an additional 24 hours to determine the **MBIC**, recorded as the **lowest** concentration of antimicrobial capable of inhibiting biofilm growth. Concentrations above the MBIC were cultured on Mueller-Hinton agar and the **MBEC** was determined as the lowest concentration that showed no growth.

Table 1: recorded MBIC and MBEC concentrations (μg/ mL) of a range of antimicrobials against E. faecalis biofilms.

Antimicrobial	MBIC (μg/ mL)	MBEC (μg/ mL)
XF-73	2	2
Gentamicin	64	128
Mupirocin	>512	>512
Rifampicin	512	>512

- XF-73 was the **most effective** antimicrobial at **eradicating** *E. faecalis* biofilms (Table 1).
- Gentamicin was the only comparator recorded with an MBIC and MBEC.
- Mupirocin **did not inhibit or eradicate** *E. faecalis* biofilms.
- Rifampicin **only inhibited** the biofilms at the highest concentration tested.

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Crystal violet imaging of *E. faecalis* treated with XF-73 and comparator antibiotics.

- **Decreased** biofilm load of *E. faecalis* biofilms recorded at concentrations **below MBIC/ MBEC** of **XF-73** (Table 1) at 1 and 0.5 μg/mL (Figure 2 A).
- Once *E. faecalis* biofilms were treated with an antimicrobial there was a **concentration dependent decrease** in biofilm load in comparison to control growth (Figure 2 A-D).

- **XF-73 was the most effective antimicrobial tested against *E. faecalis* biofilms**

Figure 2: images of microtitre peg lids containing E. faecalis biofilm treated with A) XF-73, B) Gentamicin, C) Mupirocin and D) Rifampicin stained with crystal violet (0.06% w/v) to visualise biofilm load. Including E. faecalis growth control, MHB control and a drug control for each test plate. **Note:** **Lower** concentration range of **XF-73** was used for crystal violet testing in comparison to other antimicrobials.
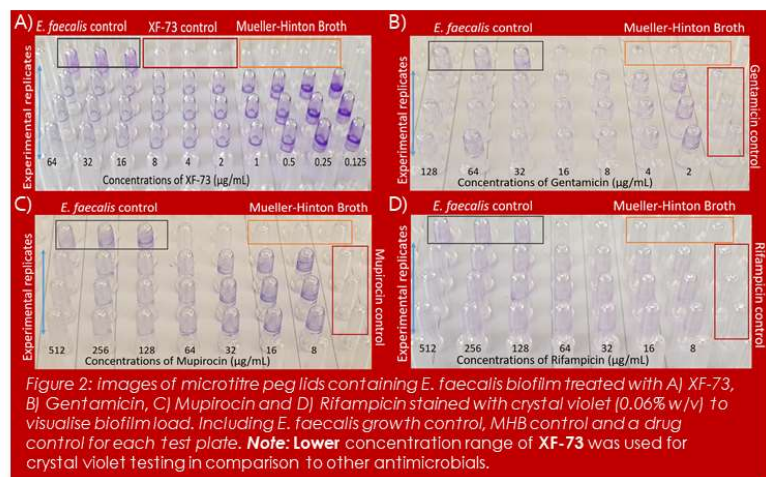
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Wound infections

- Healthcare-associated infections affect **6%** of hospitalised patients. Costing the NHS roughly **£2.7** billion each year.
  - Surgical site infections, wound infections, urinary tract infections and central line-associated blood-stream infections.

- Wound infections cost the NHS on average **£7300** per wound each year, with wounds having **68%** chance of being clinically infected.

- Soft tissue infections, in particular **chronic** and **burn wounds** can be caused by *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

- *S. aureus* most commonly isolated bacteria located in wounds.

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Exeporfinium Chloride: XF-73

- Synthetic dicationic porphyrin derivative – has a **novel mechanism of action** compared to existing antibiotics, acting as a membrane disrupter, **without** lysing the cells.

- XF-73 has the capability to be **photoactivated** via blue light exposure.
  - Second mechanism of action: singlet oxygen mediated bacterial kill.
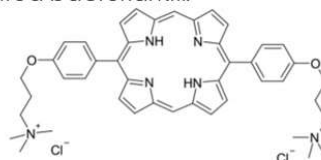
- Highly effective against **Gram-positive bacteria** including Methicillin-resistant *Staphylococcus aureus* (MRSA) in the absence of light activation (MIC 2μg/ mL).

- In phase II development as a topical agent for the prevention of post-surgical infections.

Determination of 'no effect' blue light dose level on *S. aureus*

- Wells of a 96 well microtitre plate were inoculated with *S. aureus* (ATCC 6538) at log phase growth. With starting OD 0.01 at 570 nm.

- Control plate exposed to natural light with test plate exposed to blue light (380-480 nm).

- Absorbance readings taken every 5 minutes at 570 nm.

- Blue light began to inhibit growth after **30 minutes**.

- **No effect after 15 minutes illumination.**

Growth of *S. arueus* ATCC 6538 in the presence and absence of blue light
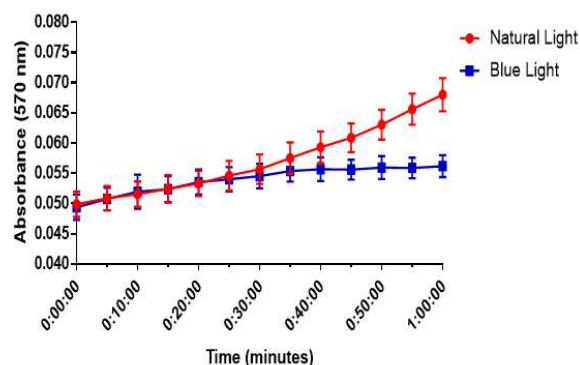
Isabella Romeo-Melody, Aston University, Destiny Pharma

Enhanced photodynamic killing of *S. aureus* with XF-73

- MIC's determined following the broth microdilution method in Mueller-Hinton Broth (CLSI guidelines).

- XF-73 range: 64 – 0.125 µg/ mL.

- Control plate exposed to natural light while test plate exposed to blue light (light dose 13.7 J/cm^2) for **15 minutes**. Following incubation for 18 hours at 37°C in aerobic conditions.

Organism	Natural light MIC	Blue Light MIC
S. aureus	2 µg/ mL	0.25 µg/ mL

- Enhanced activity of XF-73 following exposure to blue light.

- **8 - fold** reduction of MIC.

- Indicated photoactivation of XF-73 by blue light and generation of reactive oxygen species (ROS).

Isabella Romeo-Melody, Aston University, Destiny Pharma

8.3.2 European Congress of Clinical Microbiology and Infectious Diseases annual conferences

Abstracts 2020

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Abstract 3606

Antimicrobial activity of XF-73 against clinically relevant Gram-positive bacteria

Isabella Romeo-Melody*[1], Daniel Hynes[2], William Rhys-Williams[2], Bill Love[2], Peter Lambert[1], Tony Worthington[1]

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Background: Antimicrobial resistance is a global health issue and the need for new, effective antimicrobials is crucial. The synthetic dicationic porphyrin, Exeporfinium chloride (XF-73), is a proprietary, clinical stage drug with a novel bacterial membraneacting mechanism of action. In addition, XF-73 is an antimicrobial agent with photosensitising potential, able to generate singlet oxygen and free radicals which may further enhance its antimicrobial activity following exposure to blue light. The aim of the current study was to determine the antimicrobial efficacy of XF-73 against a range of clinically relevant Gram-positive bacteria in the presence and absence of blue light.

Materials/methods: Minimum inhibition concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined by micro-broth dilution in Mueller-Hinton Broth in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines. The panel of Gram-positive bacteria consisted of: *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* ATCC 29213, *Corynebacterium striatum* ATCC 1293, *Staphylococcus epidermidis* ATCC 12228 and *Enterococcus faecalis* ATCC 29212. Enhancement of XF-73 activity following exposure to blue light was investigated by placing the inoculated microtiter plate on the surface of a Waldmann Therapy System light box incorporating 36W Blue bulbs (emission peak 420 nm) for 15 minutes prior to incubation (light dose 13.7 J/cm^2).

Results: XF-73 demonstrated significant intrinsic antimicrobial activity against the panel of test bacteria with MICs ranging from 0.25 – 0.125 μg/mL. MBCs recorded for all bacteria equated to the MIC values thus demonstrating bactericidal activity. Following exposure of XF-73 to blue light, the MICs and MBCs were significantly reduced with an approximate 8-fold reduction (0.25 – 0.03 μg/mL) for *E. faecalis*.

Conclusions: XF-73 has potent bactericidal activity against Gram-positive bacteria, which is further enhanced by 15 minute exposure to blue light. Further work is currently in progress to determine MIC/MBC values for Gram-negative bacteria, and the potential effect of XF-73 against single cell and polymicrobial bacterial biofilms with and without photoactivation.

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Antimicrobial efficacy of XF-73 and antibiotic comparators against clinically relevant Gram-positive microorganisms in biofilm mode of growth

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INTRODUCTION

Bacterial infections involving biofilms are harder to treat due to the increased resistance and protection a biofilm matrix offers[1], leading to the need of novel antimicrobials capable of penetrating the biofilm matrix. **Exeporfinium chloride** (XF-73) is currently under clinical development for the prevention of post-surgical staphylococcal infections; with a **novel mechanism of action** as a membrane disruptor, leading to potassium and ATP leakage[2] without the lysing of the cells[3]

AIM

The aim of this study was to investigate the **antimicrobial efficacy of XF-73** against clinically relevant **Gram-positive microorganisms** in a **biofilm mode of growth**. To achieve this aim the objectives were to:
- Determine the **minimum biofilm inhibition concentration** (MBIC) and **minimum biofilm eradication concentration** (MBEC) of XF-73 against Gram-positive biofilms.
- Determine the MBIC/ MBEC concentrations of known antibiotics for comparison against XF-73.

METHOD

Staphylococcus aureus ATCC 29213 and ***Enterococcus faecalis*** ATCC 29212 were selected for treatment against **XF-73, gentamicin, rifampicin** and **mupirocin** in biofilm mode of growth.

Biofilm formation and treatment: Biofilm formation occurred on polystyrene microtitre plate pegs in Mueller-Hinton broth (MHB) using a starting inoculum of 10^5 CFU/mL of each microorganism via the Calgary Biofilm Device[4]. Following biofilm formation, the biofilm pegs were placed into microtitre plates containing each antimicrobial at concentrations ranging from **0.125 – 512 μg/mL** for 24 hours.

MBIC/ MBEC: Treated biofilms were transferred into MHB for an additional 24 hours to determine the **MBIC**, recorded as the **lowest** concentration of antimicrobial capable of inhibiting biofilm growth. Concentrations above the MBIC were cultured on Mueller-Hinton agar and the **MBEC** was determined as the lowest concentration that showed no growth.

Crystal Violet staining: Treated biofilm pegs were stained with crystal violet (0.06% w/v) for 5 minutes with excess stain removed through washes of phosphate-buffered saline solution (PBS) to visualise biofilm formation.

RESULTS

Table 1: Table of recorded minimum inhibition concentrations (MIC) and minimum bactericidal concentrations (MBC) (μg/mL) concentrations of gentamicin, mupirocin, rifampicin and XF-73 against planktonic S. aureus and E. faecalis. Table includes DMSO control due to mupirocin and rifampicin solutions made up in DMSO.

Organism/ ATCC	DMSO (%)	Gentamicin	Mupirocin	Rifampicin	XF-73
S. aureus 29213	50/ >50	2/ 2	0.25/ >64	≤0.125/ ≤0.125	≤0.125/ ≤0.125
E. faecalis 29212	25/ >50	8/ 16	16/ >64	0.25/ 64	0.25/ 0.25

Table 2: Table of recorded MBIC and MBEC (μg/mL) concentrations of gentamicin, mupirocin, rifampicin and XF-73 against S. aureus and E. faecalis biofilms. Table includes a DMSO control due to mupirocin and rifampicin solutions made up in DMSO.

Organism/ ATCC	DMSO (%)	Gentamicin	Mupirocin	Rifampicin	XF-73
S. aureus 29213	50/ 50	8/ 8	>256/ >256	16/ 16	1/ 1
E. faecalis 29212	>50/ >50	64/ 128	>512/ >512	512/ 512	2/ 2

Figure 1: Image of microtitre peg lids containing E. faecalis biofilm treated with A) XF-73, B) Gentamicin, C) Mupirocin and D) Rifampicin stained with crystal violet (0.06% w/v) to visualise biofilm load. Including E. faecalis growth control, MHB control and a drug control for each test plate.

- Gentamicin, mupirocin and rifampicin were selected for comparator testing due to their MIC/ MBC values being similar to XF-73 against planktonic Gram-positive microorganisms (Table 1).
- XF-73 was capable of **eradicating** all Gram-positive biofilms tested as MBIC = MBEC and had the **lowest** recorded MBIC/ MBEC for both S. aureus and E. faecalis in comparison to the other antibiotics tested (Table 2).
- Gentamicin was the only comparator antibiotic recorded with a MBIC and MBEC against both biofilms, whereas mupirocin was unable to inhibit or eradicate either biofilm. Rifampicin was effective at eradicating S. aureus biofilms, though the highest concentration tested was only capable of **inhibiting** E. faecalis biofilms (Table 2).
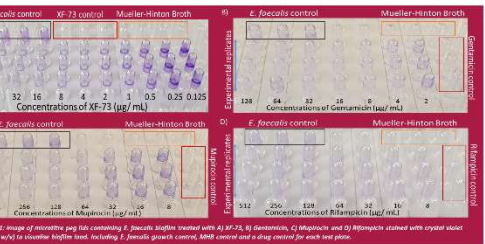- Crystal violet stains both the matrix surrounding the biofilm as well as the bacteria present[5], therefore, the lighter purple staining on the pegs indicates a lower residual biofilm load after antibiotic exposure.
- **Lower** concentration range of XF-73 was used for crystal violet testing in comparison to other antimicrobials, to record the change in biofilm viability after XF-73 treatment (Figure 1 A).
- **Decreased** biofilm load of E. faecalis biofilm recorded concentrations **below MBIC/ MBEC** of XF-73 at 1 and 0.5 μg/mL.
- Once E. faecalis biofilms were treated with an antimicrobial there was a **concentration dependent decrease** in biofilm load in comparison to control growth (Figure 1 A-D).

CONCLUSIONS

XF-73 was the **most effective** antimicrobial at eradicating Gram-positive biofilms, shown by the low concentrations of XF-73 required to **eradicate** the biofilms in comparison to the known antibiotics. XF-73 only required **1 μg/mL** to treat **S. aureus** biofilms whereas the known antimicrobials ranged from **8 - >256 μg/mL**; highlighting the potential for XF-73 to be used as a treatment for against infections involving Gram-positive microorganisms (Table 1 & 2, Figure 1).

Further work is ongoing to record the effect of XF-73 against Gram-negative biofilms and the potential to **increase** efficacy of XF-73 against Gram-positive biofilms with the addition of **photodynamic therapy.**

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Antibacterial efficacy of XF-73 via two mechanisms of action against Gram-positive microorganisms in biofilm mode of growth

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Introduction:

Staphylococcus aureus is the most **commonly** isolated bacteria located in **wound infections**[1]; **Enterococci** are one of the main causes of healthcare associated infections predominantly in **immunocompromised patients**[2]. Infections involving bacterial biofilms are becoming increasingly more difficult to treat due to the **biofilm matrix**, which offers **increased resistance** and **protection** from **antimicrobials**[3].

Exeporfinium chloride (XF-73) (Figure 1), has a **novel mechanism of action** as a membrane disruptor, leading to **potassium** and **ATP leakage**[4] **without the lysing of the cells**[5]. and has the potential for **photo-activation by blue light** to enhance its antibacterial activity. XF-73 currently under clinical development for the prevention of post-surgical staphylococcal infections.

Project Aim:

Investigate the **antimicrobial efficacy of XF-73** under **natural and blue light** against *Staphylococcus aureus* and *Enterococcus faecalis* in a **biofilm mode of growth**. Compare activity with that of **gentamicin, mupirocin, daptomycin and rifampicin.**
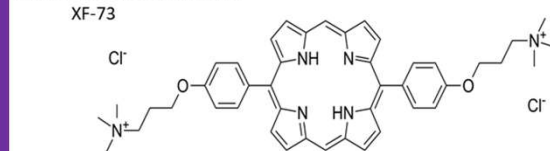
Figure 1: chemical structure of XF-73

Methods:

Biofilm formation: *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 biofilms were grown were on polystyrene microtitre plate pegs in Mueller-Hinton broth (MHB) using a starting inoculum of 10^5 CFU/mL , via the Calgary Biofilm Device[6].

Biofilm treatment: Formed biofilms were transferred into microtitre treatment plates containing each antimicrobial: **XF-73**, **gentamicin**, **rifampicin**, **mupirocin** and **daptomycin**; at concentrations ranging from **0.125 – 512 µg/mL** for 24 hours. **Blue light activation of XF-73** was achieved by placing the pegs lids in microtitre plates on the surface of a Waldmann Therapy System, blue light (420nm) for **15 minutes.**

MBIC/ MBEC: Treated biofilms were transferred into MHB for an additional 24 hours to determine the **MBIC**, recorded as the **lowest** concentration of antimicrobial capable of inhibiting biofilm growth. Concentrations above the MBIC were cultured on Mueller-Hinton agar and the **MBEC** was determined as the lowest concentration that showed no growth.

Crystal Violet staining: Treated biofilm pegs were stained with crystal violet (0.06% w/v) for 5 minutes with excess stain removed through washes of phosphate-buffered saline solution (PBS) to visualise biofilm formation.

Results:

Table 1: Table of recorded MBIC and MBEC (µg/mL) concentrations of XF-73, blue light enhance XF-73, gentamicin, mupirocin, rifampicin and daptomycin against S. aureus and E. faecalis biofilms.

Antimicrobial	S. aureus MBIC/ MBEC µg/ mL	E. Faecalis MBIC/ MBEC µg/ mL
XF-73	1/ 1	2/ 2
XF-73 + blue light	0.125/ 0.125	0.5/ 0.5
Gentamicin	8/ 8	64/ 128
Mupirocin	>256/ >256	>512/ >512
Rifampicin	16/ 16	512/ >512
Daptomycin	32/ 32	>512/ >512

Crystal Violet staining:

Crystal violet stains both **bacterial cells** and **biofilm matrix**, the stain present on the pegs indicates biofilm load.

Lower concentration range required for **both XF-73 treatments** for crystal violet staining, to record decrease in viability.

Concentration dependent decrease in biofilm load after all antimicrobial treatments except blue light enhanced XF-73 treatment. May be caused by eradication of biofilm, but no release from the pegs at higher concentrations.

MBIC/ MBEC results:

XF-73 was the **most effective** antimicrobial in eradicating the biofilms.

Blue light treatment further enhanced XF-73 activity, leading to an **8-fold** and **4-fold** decrease in MBIC and MBEC for *S. aureus* and *E. faecalis*.

Gentamicin was the **only comparator** capable of **eradicating both biofilms.**

Mupirocin neither **inhibited nor eradicated** either biofilm.

Rifampicin **eradicated** *S. aureus* biofilms but was only able to **inhibit** *E. faecalis* biofilms.
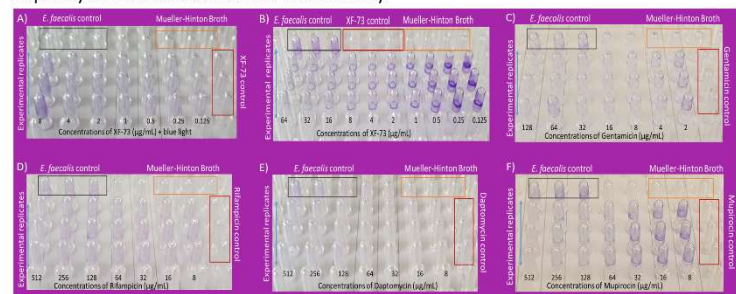
Daptomycin **eradicated** *S. aureus* biofilms only.

Figure 2: images of microtitre peg lids containing E. faecalis biofilm treated with A) blue light enhance XF-73, B) XF-73, C) gentamicin, D) rifampicin, E) daptomycin and F) mupirocin and stained with crystal violet (0.06% w/v) to visualise biofilm load. Including E. faecalis growth control, MHB control and a drug control for each test plate.

Conclusion:

XF-73 was the **most effective** antimicrobial at **eradicating Gram-positive biofilms**, shown by the low concentration of **1 µg/mL** to treat *S. aureus* biofilms whereas the known antimicrobials ranged from **8 - >256 µg/mL**; XF-73 showed a greater efficacy in eradicating *E. faecalis* biofilms requiring only **2 µg/mL** in comparison to the known antimicrobials that ranged from **128 - >512 µg/mL** (Table 1 & Figure 2).

The **addition of blue light** leading to the activation of XF-73's **secondary mechanism of action,** further **enhanced the killing activity** against both microorganisms; highlighting the potential of XF-73 to be used as a future antimicrobial treatment in natural light and additional blue light treatment. Further work is ongoing to **assess the efficacy of XF-73** in **natural** and **blue light** against a **range of Gram-positive microorganisms.**

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Antibacterial efficacy of XF-73 via two mechanisms of action against Gram-positive microorganisms in biofilm mode of growth

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23–26 April 2022

- Healthcare-associated infections (HCAI's) affect **6% of hospitalised patients**, with at least **50% of HCAI's being preventable.**

- **Vancomycin resistant Enterococci** are one of the main causes of healthcare associated infections predominantly in **immunocompromised** patients.

- *Staphylococcus aureus* is the most **commonly** isolated bacteria located in **wound infections.**

- Infections involving bacterial biofilms are becoming increasingly more difficult to treat due to the **biofilm matrix**, which offers **increased resistance** and **protection** from **antimicrobials.**

- **XF-73** is the leading compound of the XF series, currently in clinical trials in the UK and USA.

- Synthetic dicationic porphyrin derivative, with a **novel membrane disruption mechanism of action.** With potential to **be photo-activated** by **blue light** to enhance antibacterial activity.

Chemical structure of XF-73
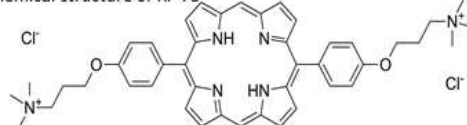
- **AIM:** investigate the **antimicrobial efficacy of XF-73** via **two mechanisms of action: natural and blue light** against clinically relevant **Gram-positive microorganisms** in a **biofilm mode of growth** with comparison to the efficacy of **known antibiotics.**

1

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Experimental methods

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- **1)** Biofilm formation occurred via the **Calgary Biofilm Device** using a starting inoculum of 10^5 CFU/ mL of *E. faecalis* ATCC 29212 or *S. aureus* ATCC 29213 in Mueller-Hinton broth for 24 hours in microtitre plates.

- **2)** Formed biofilms were treated with: **XF-73, gentamicin, mupirocin, daptomycin** and **rifampicin** at concentrations ranging from: **0.125 – 512 µg/ mL** for 24 hours. Blue light activation of XF-73 was achieved by placing microtitre plates on the surface of a Waldmann Therapy System, blue light (420nm) for **15 minutes.**

- **3a)** Minimum biofilm inhibition concentration (MBIC): **lowest** recorded concentration of antimicrobial capable of **inhibiting biofilm growth.**

- **3b)** Minimum biofilm eradication concentration (MBEC): **lowest** concentration of antimicrobial recorded with **no bacterial growth** on Mueller-Hinton agar plates.

- **4)** Treated biofilm pegs were stained with **crystal violet** (0.06% w/v) for **5 minutes** with excess stain removed through washes of phosphate-buffered saline solution (PBS) to **visualise biofilm formation.**

2

MBIC/ MBEC results and conclusions

- **XF-73** was the **most effective** antimicrobial in eradicating the biofilms
- **Blue light treatment further enhanced XF-73**, leading to an **8-fold** and **4-fold** decrease in concentration for *S. aureus* and *E. faecalis*
- Gentamicin was the **only comparator** capable of **eradicating both biofilms.**
- Mupirocin neither **inhibited nor eradicated** either biofilm.
- Rifampicin **eradicated** *S. aureus* biofilms but was only able to **inhibit** *E. faecalis* biofilms.
- Daptomycin **eradicated** *S. aureus* biofilms only.

Antimicrobial	*S. aureus* MBIC/ MBEC µg/ mL	*E. Faecalis* MBIC/ MBEC µg/ mL
XF-73	1/ 1	2/ 2
XF-73 + blue light	0.125/ 0.125	0.5/ 0.5
Gentamicin	8/ 8	64/ 128
Mupirocin	>256/ >256	>512/ >512
Rifampicin	16/ 16	512/ >512
Daptomycin	32/ 32	>512/ >512

3

Crystal Violet staining of *E. faecalis* biofilms treated with antimicrobials

Crystal violet stains **bacterial cells** and **biofilm matrix**, therefore, the light stain present on the pegs indicates a lower biofilm load following treatment.

Lower concentration range required for **both XF-73 treatments** for crystal violet staining, to record decrease in viability after treatment.

Concentration dependent decrease in biofilm load after all antimicrobial treatments bar blue light enhanced XF-73 treatment. May be caused by eradication of biofilm, but no shedding off the pegs at higher concentrations.