Assessment of Ionic Liquids for disinfection of healthcare surfaces

A thesis submitted for the degree

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By

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Summary

Surface disinfection for the prevention of healthcare associated infections (HCAI) is well recognised. Ionic liquids (ILs) possess antimicrobial activities that could make their inclusion into disinfectant products beneficial for the control of HCAI.

ILs were tested against microorganisms under conditions that affect antimicrobial activity concentration, contact time, organic soiling, and were compared to the commonly used cationic biocides benzalkonium chloride (BZC) and chlorhexidine gluconate (CHX). ILs had potent antimicrobial activity in the presence of organic soiling at a short contact time. At equivalent concentrations, BZC and CHX were not as effective at reducing viability of bacteria and the type of organism and organic soiling hindered the activity. The main factor that affected the antimicrobial activity of formulations was dilution. ILs were unable to inactivate *Bacillus subtilis* spores but were sporicidal when combined with hydrogen peroxide.

Cellular targets of ILs were investigated by potassium leakage from the cell and the uptake of DNA binding dyes. Significant release of potassium from the cell and uptake of dyes into the cell suggested membrane damage was caused by ILs. The ultrastructure of bacteria was assessed by scanning electron microscopy (SEM) and atomic force microscopy (AFM). Visually, cells lost structural integrity in a dose-dependent manner. Analysis by AFM shown development of valleys in the structure of *Staphylococcus epidermidis*. *B. subtilis* spore mutants lacking protective DNA proteins and spore coat were tested against ILs to assess any interaction of the formulations with intracellular biomolecules. There was no reduction in spore numbers indicating that intracellular components are not targets.

Finally, IL formulations were combined with wipe material to assess if antimicrobial activity would translate into a product. As a wet wipe the formulations reduced bacteria without transferring to subsequent surfaces and were more efficacious than commercial wipes. A spill wipe was also developed and was more effective than commercial spill wipes. Formulations were cytotoxic against skin cells in vitro.

Overall, ILs displayed greater antimicrobial activity against vegetative bacteria, than BZC and CHX. The proposed mechanism of action is through membrane damage of the cell and as wipe products the ILs were more efficacious than commercial products.

Abbreviations list

ATP	Adenosine Triphosphate
ATCC	American Type Culture Collection
AFM	Atomic Force Microscopy
CHX	Chlorhexidine Gluconate
BSA	Bovine Serum Albumin
BZC	Benzalkonium Chloride
CFU	Colony Forming Units
EVA	Ethylene-vinyl acetate
HCAI	Health Care-Associated Infection
ICD	Irritant contact dermatitis
ICU	Intensive Care Units
ILs	Ionic Liquids
MBC	Minimum Biocidal Concentration
MEA	Malt Extract Agar
MF1	1-dodecyl-3-methylimidazolium hydrogen carbonate
MF2	1-dodecyl-3-methylimidazolium bromide
MIC	Minimum Inhibitory Concentration
MOA	Mechanism of Action
MRSA	Methicillin Resistant *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NHS	National Health Service
PBS	Phosphate Buffer Saline
PPE	Personal Protective Equipment
QAC	Quaternary Ammonium Compounds
RCN	Royal College of Nursing
SASP	Small Acid-Soluble Proteins
SD	Standard Deviation
SDW	Sterile Distilled Water
SEM	Scanning Electron Microscopy
SICP	Standard Infection and Control Precautions
ss	Stainless steel
TAED	Tetraacetylethylenediamine
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
TSC	Tryptone Sodium Chloride
VRE	Vancomycin-Resistant Enterococci
WHO	World Health Organisation
WT	Wild Type

Table of Contents

List of Figures

List of Tables

1 General Introduction

1.1 Healthcare associated infections

Healthcare associated infections (HCAI), also known as nosocomial infections, occur in a patient during the process of care in a hospital or healthcare facility. According to the World Health Organisation (World Health Organisation), a HCAI can arise in a patient from direct contact with a healthcare environment, or from treatment such as an invasive surgical procedure (World Health Organisation, 2015). It is estimated that in Europe alone 4,131,000 patients every year are affected by HCAI, with 30% acquired from intensive care units (ICUs) and invasive intervention procedures such urinary catheters, central lines and ventilators (European Center for Disease Prevention and Control, 2010; World Health Organisation, 2011).

In the UK the cost of HCAI to the National Health Service (NHS) is estimated at £1 billion, with 56 million cases developing after the discharge of the patient (National Institute for Health and Clinical Excellence, 2011). In addition with the economical disadvantages of HCAI, they also burden the NHS due to increased morbidity and mortality rates and length of stay of patients (Plowman, 2000; Cosgrove *et al*., 2005; House of Commons Public Accounts Committee, 2009; Loveday et al., 2014a).

1.1.1 Micro-organisms that cause HCAI

1.1.1.1 Gram-positive bacteria

Staphylococcus aureus is a Gram-positive bacterium that can exist as part of the normal skin flora without causing harm. It can however cause infections ranging from minor skin conditions to life-threatening bacteraemia (Grice *et al.,* 2009; Public Health England, 2010). The ability of *S. aureus* to spread and lead to infection contributed to major concerns over infection control practices in UK hospitals during high media coverage of the 2009 outbreaks that lead to several deaths (Duerden, 2009; Chan *et al*., 2010; Chan *et al*., 2010; National Institue for Health and Care Excellence, 2011).

Enterococcus species are another example of Gram-positive bacteria that may reside in the human body asymptomatically but can result in blood stream infections, urinary tract infections and wound infections (Public Health England, 2008). The two major species (spp.) that cause disease in healthcare facilities are *Enterococcus faecalis* and *Enterococcus faecium*, that are usually resistant to glycopeptide antibiotics, vancomycin and teicoplanin (Public Health England, 2008)

Arguably, the most infectious Gram-positive bacterium is *Clostridium difficile*. An anaerobic spore-former, *C. difficile* may colonise the intestines of healthy adults and babies without causing infection. However, antibiotic use can increase numbers of *C. difficile* in the gut causing minor diarrhoea to more severe intestinal perforation. Since the late 20th century the increase in *C. difficile* outbreaks have been attributed to increased pathogenicity, transmission and higher severity of infections (Bartlett *et al*.,1977; Eyre *et al*., 2013). It is well-known that there is a greater acquisition rate of *C. difficile* upon admission to a healthcare facility, which increases during patient stay, although this varies between institutions (Loo *et al*., 2005; Jones *et al*., 2013).

1.1.1.2 Gram-negative bacteria

Escherichia coli is a Gram-negative facultative anaerobe found among intestinal flora. Whilst urinary tract infections and cystitis are more common conditions associated with *E. coli*, dissemination into the blood can also occur and lead to death (Public Health England, 2017). In the UK, rates of infections caused by *E. coli* rose 27 % from 32,309 to 41,060 cases annually, with 50 % of all bloodstream infections due to the bacteria (Public Health England, 2017).

Acinetobacter baumannii is an environmentally derived Gram-negative bacterium, typically isolated from healthcare facilities. It is an opportunistic pathogen that causes a broad range of infections including wound, urinary tract, meningitis, bacteraemia and ventilator associated pneumonia (Bergogneet *et al*., 1996; Dijkshoorn *et al*., 2007; Roca *et al*., 2012; McConnell *et al*., 2013). In a study analysing published outbreaks of *A. baumannii* and *Pseudomonas aeruginosa* findings concluded that *A. baumannii* was often found in intensive care units (ICU), transmission was by direct contact between staff/patients/surfaces and that mortality rates were higher upon infection with *A. baumannii* compared to *P. aeruginosa* (Wieland *et al*., 2018).

P. aeruginosa is also a major pathogen contributing to HCAI. A Gram-negative bacterium commonly found in soil and water. Infections caused by *P. aeruginosa* usually affects those with weakened immune systems such as neonates, cancer patients, burns patients and those with cystic fibrosis (Public Health England, 2018).

1.1.1.3 Fungi

Fungal infections are commonly associated with patients that are immunocompromised. The two most common microorganisms that are responsible for fungal infection in the UK belong to the *Candida* spp. and *Aspergillus* spp.

Candida are yeasts that grow in gastrointestinal tract of 40-60 % of the population without causing disease (Kerawala & Newlands, 2010; Erdogan & Rao, 2015). *C. auris* was first isolated in Japan in 2009 and has since been found in several other countries (Satoh *et al*., 2009; Magobo *et al*., 2014; Calvo *et al*., 2016; Schelenz *et al*., 2016; Lockhart *et al*., 2017; Schwartz & Hammond, 2017). In 2017, twenty NHS healthcare facilities reported outbreaks of the yeast that had been difficult to control, even with increased infection prevention measures resulting in 200 patients becoming colonised (Public Health England, 2017). The emerging pathogen *C. auris* presents a serious challenge to the healthcare system, but it is an emerging threat and only the second highest cause of candidaemia, as *Candida albicans* is still the largest contributor (Public Health England, 2018).

Aspergillus spp. are found ubiquitously throughout the environment and as with most fungal infections, patients most at risk are those with compromised immune systems. As the mould grows it produces airborne spores and so patients with lung conditions such as chronic obstructive pulmonary diseases (COPD) are also at risk of infection. Factors such as construction work near/in healthcare environments have been linked to higher incidences of aspergillosis (Arnow *et al*, 1978; Lentino *et al*, 1982; Flynn *et al*, 1993; Anderson *et al*, 1996; Singer *et al*, 1998; Oren *et al*, 2001; Raviv *et al*, 2007; Guinea *et al*, 2010; Etienne *et al*, 2011; Peláez *et al*, 2012)

1.1.1.4 Viruses

Viruses are also a major burden in healthcare environments where it is estimated that they contribute to 5 % of HCAI (Aitken, 2001). The most common cause of gastroenteritis is caused by norovirus, a highly infectious non-enveloped virus. The cost of annual outbreaks of norovirus are estimated around £107.6 million in England alone, and results in closed wards and Ill staff, putting increasing pressure on the NHS (Public Health England, 2014; Sandmann *et al*., 2018).

1.2 Infection control in the UK

The UK Department of Health provides Standard Infection and Control Precautions (SICPs) to instruct local authorities on how to implement cleaning practices within their own facilities. The guidelines are compiled from peer-reviewed research and healthcare professionals and is subject to a review every few years to incorporate advancements in technology and knowledge in the field (Loveday *et al*., 2014; Wigglesworth & Consultant, 2015).

The framework highlights five major aspects that need to be demonstrated within healthcare to reduce the number of HCAIs. These include hand hygiene, hospital environment cleanliness, the use of personal protective equipment (PPE), safe use and disposal of sharps and the principles of asepsis (Loveday *et al*., 2014).

The practice of hand hygiene is a well-established method for the control of HCAIs. Pittet *et al.* (2006), developed a five step model that is now accepted by WHO as five steps that occur during patient to patient transmission via healthcare workers hands: (I) patients are colonised with microorganisms and can contaminate surrounding areas; (II) healthcare workers touch contaminated patients and/or surfaces; (III) the organism can survive carriage on health workers hands; (IV) the washing of the healthcare worker is ineffective or non-existent; (V) the healthcare worker then touches patients or inanimate objects, transferring the contamination (Pittet *et al*., 2006; WHO, 2009).

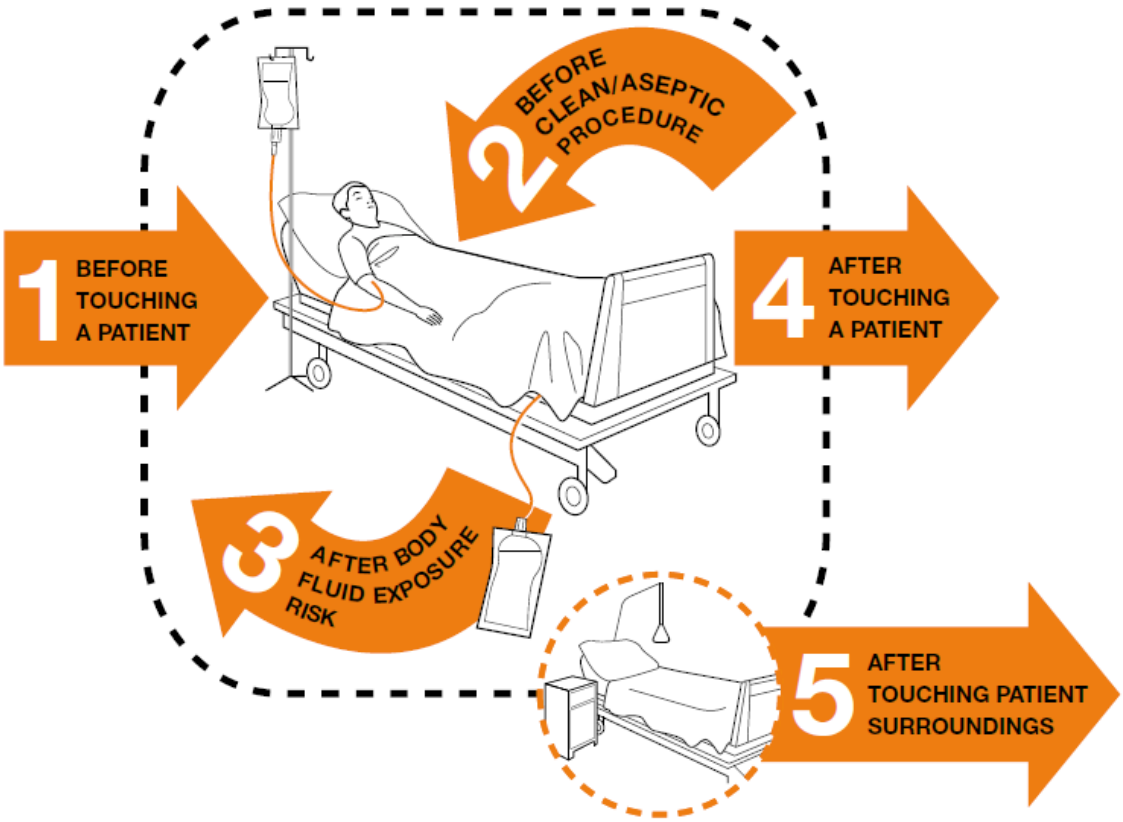
Substantial evidence has been collated to produce the model proposed by Pittet and co-workers, supporting the need for routine hand washing measures to reduce transmission of HCAIs. There are many cases in which implemented changes in hand washing practises have reduced the rate of HCAIs, for instance, two; independent studies have shown the ability to decrease cases of methicillin-resistant *Staphylococcus aureus* (MRSA) on neonatal units after introducing a new antiseptic hand wash containing triclosan (Webster *et al*., 1994; Zafar *et al*., 1995).

Whilst it is widely acknowledged that hand hygiene is a successful intervention for the control of hospital acquired infections and could be considered the most important step to control HCAI. However, it is now recognised that hand washing alone is not sufficient to prevent HCAI, as surfaces may be an indirect route of transmission (Sax *et al*., 2007; Dancer, 2009; Kundrapu *et al*., 2012) (Figure 1.1). It has been shown that cross contamination of surfaces to hands can attribute to 20-40 % of HCAI and that hand washing compliance is lower after touching a surface compared to touching a patient, further

emphasising the need for adequate surface disinfection (Weinstein, 1991; Randle *et al*., 2010).

Figure 1.1 Five moments of hand hygiene.

The events outlined indicated when a healthcare worker should be washing hands to prevent the spread of micro-organisms. Events 1-4 focuses on washing hands before and after patient contact and step 5 is a reminder that touching surfaces can spread HCAI. (Adapted from Sax *et al*., 2007)



1.3 Role of surfaces in the spread of HCAI

1.3.1 Does surface contamination lead to infection?

The presence of bacteria on a surface does not necessarily lead to infection of patients (Rhame, 1998). However, there are studies linking the presence of a pathogenic microbe on a surface to its subsequent transmission and infection of a patient. One such study conducted by Lawley and co-workers used mouse models to demonstrate that *C. difficile* infection was able to transmit in a dose-dependent manner in mice. From this study it was shown that disinfection of cages significantly lowered the rate of infection, providing a controllable example of how surface transmission of bacteria can lead to infection. Moreover, the research highlighted that disinfection is a valuable infection prevention procedure that can minimise transmission (Lawley *et al*., 2010).

Further emphasis has been placed on the role of environmental contamination in transmission of infection from studies highlighting the increased chances of acquiring a HCAI if patients are admitted to a room where the previous occupant was also colonised. This was observed with MRSA, *A. baumannii*, *C. difficile* and vancomycin-resistant *Enterococcus* (VRE) increasing the rate of colonisation by 73 % compared to rooms that were not previously contaminated (Martinez *et al.*, 2003; Hardy *et al.*, 2006; Hayden *et al.,* 2006; Wilks *et al*., 2006; Drees *et al*., 2008; Carling & Bartley, 2010; Shaughnessy *et al*., 2011)

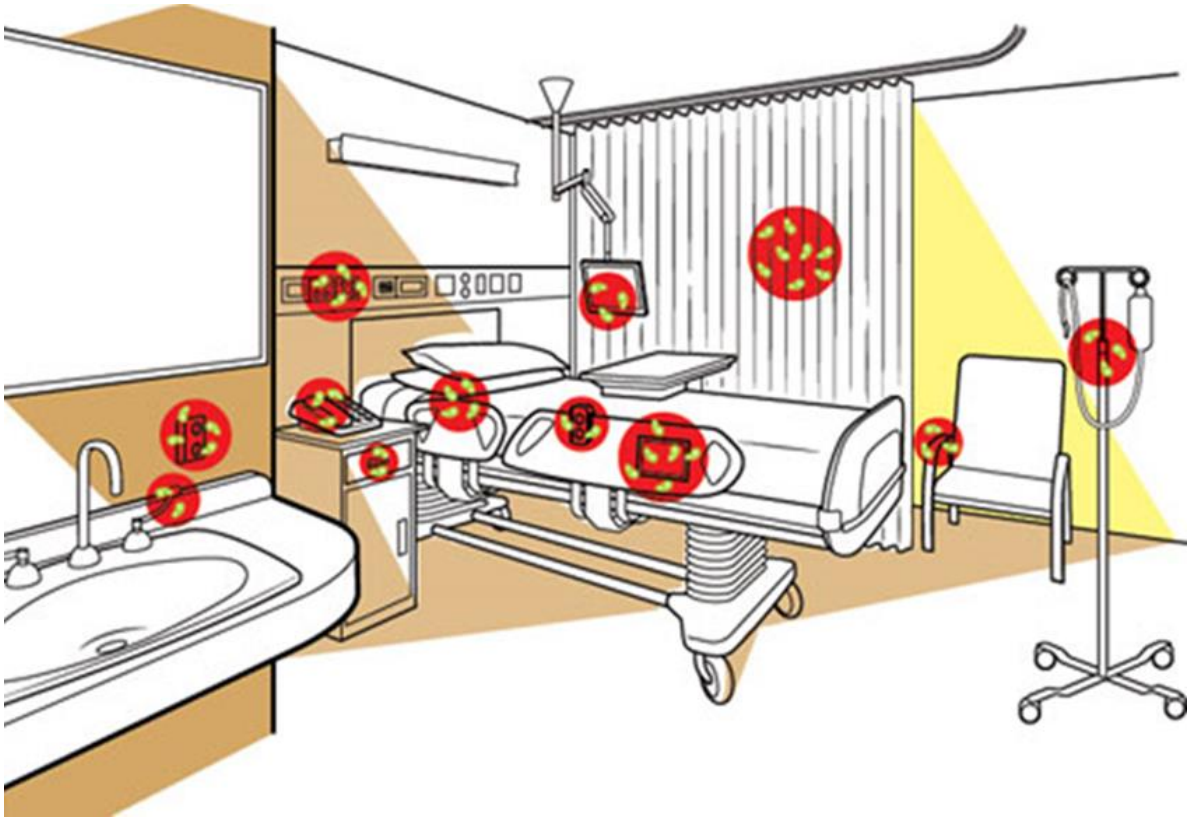
1.3.2 High touch surfaces

As patients are able to contaminate surfaces within the vicinity of their treatment, surfaces that are frequently accessed by healthcare workers and patients such as bedrails, bedpans and over-bed tables are termed ‘high-touch’ surfaces as they can often harbour high loads of bacteria (Figure 1.2) (Huslage *et al*., 2010). In fact, the amount times a surface is touched was shown to directly correlate with the amount of bioburden on surface, with a reported 12 colony forming units per cm^2 (CFU/cm^2) on bedrails. (Adams *et al*., 2017)

Surfaces found outside of hospitals, such as emergency transport vehicles can also harbour microbes that could potentially put patients and staff at risk. For example, MRSA was recovered from 47.6 % of surface swabs from 21 ambulances (Roline *et al*., 2007).

Figure 1.2 Example of high touch surfaces.

Areas in close proximity to patient are deemed high-touch surfaces as they often harbour microbes and require higher rates of disinfection. Examples of high-touch surfaces; bed rails, bed tray/table, IV stands, screens/televisions, chairs, phones, sockets, and sinks.



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Increased cleaning practises of high-touch surfaces is one intervention that can lower bioburden to an acceptable level. However, one problem faced when assessing cleanliness is there is no standard as to what constitutes a clean surface in a healthcare environment. Bacterial counts per cm^2 are the parameters usually applied when assessing surface bioburden, one standard suggests <1 CFU/cm^2 of an indicator organism such as MRSA, while others suggest a total aerobic colony count (ACC) should not be greater than 2.5-5 CFU/cm^2 (Dancer, 2004). It is not known, however, what level of surface contamination is needed to cause infection and can vary significantly depending on the bacteria or patient

susceptibility, as an example, infections caused by MRSA can be a result of several million or as little as 10 CFU (Dancer, 2008).

In whichever way cleaning is measured, the allocation of what a high touch surface is within a healthcare setting will allow for more focused cleaning with the possibility of increasing the frequency of cleaning of high touch surfaces.

Further problems that arise with the decontamination of surfaces are the different topographies found within a healthcare setting which may present uneven disinfection. For instance, disinfecting with hydrogen peroxide vapour against spores on a non-porous surface was shown to give a greater reduction than against spores on a porous surface (Rogers *et al*., 2007)

1.3.3 Microbes on surfaces

Microbes often associated with HCAI may possess key features that allow them to be transmitted to patients: (i) they can be shed from patients that are colonised or infected, (ii) they have the ability to survive for long periods of time on inanimate surfaces, and (iii) they are difficult to remove or disinfect on surfaces (Kramer *et al*., 2006; Otter *et al*., 2013). Table 1.1 shows key pathogens associated with HCAIs, and the length of time they can survive on surfaces.

Table 1.1 Length of time pathogens can remain of surfaces.

Commonly reported microbes that cause HCAI, their characteristics and ability to survive on surfaces.

Organism	Characteristics	Time of Survival on surface
MRSA	Gram positive cocci	7 days – 12 months (Wagenvoort *et al*., 2000)
C. difficile	Gram positive, anaerobe, spore forming, bacillus	Vegetative form; 6 hours on wet surfaces, 15 months on dry. Spores; highly resistant to drying, heat and disinfection (Kim *et al*., 1981; McFarland and Stamm, 1986)
Klebsiella pneumoniae	Gram negative, Facultative anaerobe, bacillus	2 days – 30 months (Neely, 2000; Scott and Bloomfield, 1990)
A. baumannii	Gram negative, aerobe, bacillus-cocci	3 days - 5 months (Getchell-White *et al*., 1989; Jawad *et al*., 1996; Wendt *et al*., 1997)
P. aeruginosa	Gram negative, aerobic, bacillus	6 hours – 6 months (Neely, 2000; Scott and Bloomfield, 1990)
VRE	Gram positive, facultative anaerobe, cocci	5 days – 4 months (Neely, 2000)
Norovirus	Single stranded RNA, non-enveloped	7 days on stainless steel surface (D'Souza *et al*., 2006)

1.4 Cleaning interventions in UK hospitals

An established infection prevention and control measure is routing cleaning of the healthcare environment (Carling & Bartley, 2010; Shaughnessy *et al*., 2011; Dancer, 2014; Otter *et al*., 2013, Sattar & Maillard, 2013). Routine cleaning within NHS hospitals, follows a detergent based regimen, with disinfection by biocides only occurring after spills of potentially contaminated body fluids, or after terminal cleaning if a room has been occupied by a patient with a known to be infected with *C. difficile* (terminal disinfection) (Griffith *et al*., 2000; British Standards Institute, 2014).

Whilst cleaning removes visible dirt and organic soiling (bodily fluids) it does not necessarily remove microbes and can spread them onto other surfaces (Ramm *et al*., 2015). Disinfection may use chemical liquids in the form of sprays/wipes or through automated disinfection technology (i.e. UV lighting), which aims to completely remove/kill microbes from surfaces.

Studies have linked the use of disinfectants as a valuable intervention to lower incidence rates of HCAI. For example, switching to sodium hypochlorite based-solutions to disinfect high-touch surfaces was shown to decrease VRE colonisation and bacteraemia in a haemodialysis unit, whilst in a separate study the rate of *C. difficile*-associated disease was significantly lower after use of hydrogen peroxide vapour (Boyce *et al*., 2008; Grabscha *et al*., 2012). Additionally, the use of antimicrobial wipes was shown to produce a significant decrease in surface bioburden and multidrug resistant organisms (MDRO) (Siani *et al*. 2018). Other studies have focused on implementing training and management programmes and monitoring of surface contamination which impacted rates of HCAI (Ray *et al*., 2017; Wong *et al*., 2018; Furlan *et al*., 2019).

Although disinfection can lower HCAI, issues remain with compliance among staff that carry out the cleaning as it is often considered repetitive, menial and for aesthetic purpose (Dancer, 1999). Moreover, the methods used for cleaning can also have a significant impact on compliance. More traditional methods such as cloth and bucket have shown significant downsides including improper dilution of disinfectant, choice of cloth used (microfiber or cotton) and re-use of cloths which could lead to spread of microbes (Wiemken *et al*., 2014; Siani *et al*., 2018). Compared to these traditional methods, ready-to-use cleaning/disinfecting wipes show a higher rate of compliance, a quicker turnaround time for cleaning and time related cost savings (Weimken *et al*., 2014).

Apart from manual cleaning of surfaces, other interventions to prevent and/or lower the bioburden on surfaces have been on the rise. Antimicrobial surfaces are a promising intervention to decrease numbers of microbes on surfaces. There are two main types of antimicrobial surfaces (i) anti-adhesive surfaces that prevent microbes sticking to the surface and (ii) those with antimicrobial coating that inhibit/kill bacteria.

An example of a surface where bacteria have difficulty sticking to a surface is the use of polyethylene glycol (PEG) coatings that repel the hydrophobic cell wall of bacteria, which was able to reduce bacteria by 3 Log_{10} (Chapman *et al*., 2001). However a major disadvantage with the surface was auto-oxidiation and loss of activity over time (Chapman *et al*., 2001). Surfaces containing copper have demonstrated effective antimicrobial activity, with one study showing complete reduction of MRSA over 45 minutes (Noyce *et al*., 2006).

Although promising, there are issues with antimicrobial surfaces and many have not shown effective activity against a range of pathogens, especially spores. Furthermore, surfaces can lose activity and are costly, with the benefits of investing in such surfaces compared to manual cleaning are not fully (Dancer, 2014).

Automated disinfection technologies such as UV light and hydrogen peroxide vapour have also demonstrated effective reduction of microbes on surfaces. In one study, *C. difficile*, MRSA and VRE were significantly reduced on surfaces after use of UV and hydrogen peroxide vapour lowered MRSA, spores *and Mycobacterium tuberculosis* in hospital rooms (Nerandzic *et al* 2010; Falagas *et al*., 2011; Ali *et al*., 2016).

As effective as automated disinfection technologies are, they have drawbacks that limit their use, such as: i) rooms cannot be occupied when machines are in use, ii) they require trained personnel for operation, iii) cost of equipment, and iv) the limited activity observed in soiled areas (Dancer, 2014). In light of the pitfalls that come with antimicrobial surface and automated disinfection machines, it has been suggested that they are used alongside manual disinfection procedures to prevent HCAI (Memarzadeh *et al*., 2010; Dancer, 2014).

1.5 Biocides in healthcare

1.5.1 Brief history of biocides

The definition of a biocide as defined by 528/2012 European legislature is one that contains one or more active ingredients that inactivate, destroy or control harmful pathogens, by

chemical or biological means, in the form that they are supplied to the user (European Union Biocides Regulation 528/2012). This includes antimicrobials that kill microorganisms, so are termed microbicidal (i.e. bactericidal, sporicidal, virucidal) or “statics” that inhibit their growth (bacteriostatic, fungistatic). The use of antimicrobials goes back thousands of years, seen in the expansion of the Persian empire when water was stored in silver and copper vessels to “preserve”, while vinegar and honey were recognised for their antiseptic properties in wound healing (Mcdonnell *et al*., 1999; Fraise *et al*., 2012). The beginning of the twentieth century lead to the introduction of quaternary ammonium compounds (QACs) and chlorine releasing agents, biguanides, phenols, peroxygens and aldehydes which are still widely used today (McDonnell *et al*., 1999). The increase in biocidal products after the 1950s, meant that many biocidal products on the market were unregulated and their misuse has now been linked to an increase in resistance to biocides, mainly for using sub-inhibitory levels, leading to exposure but not necessarily eliminating the microbes (Moken *et al*., 1997; Chuanchuen *et al*., 2001; Russell, 2002; Walsh *et al*., 2003).

Since the biocidal product regulations (BPR) were first rolled out in 1998, many biocides were taken off the market in the European Union. To prevent harm to people, animals or the environment, biocidal products need approval from the BPR before they can be used in the EU (European Union Biocides Regulation 528/2012).

1.5.2 Use of biocides in healthcare

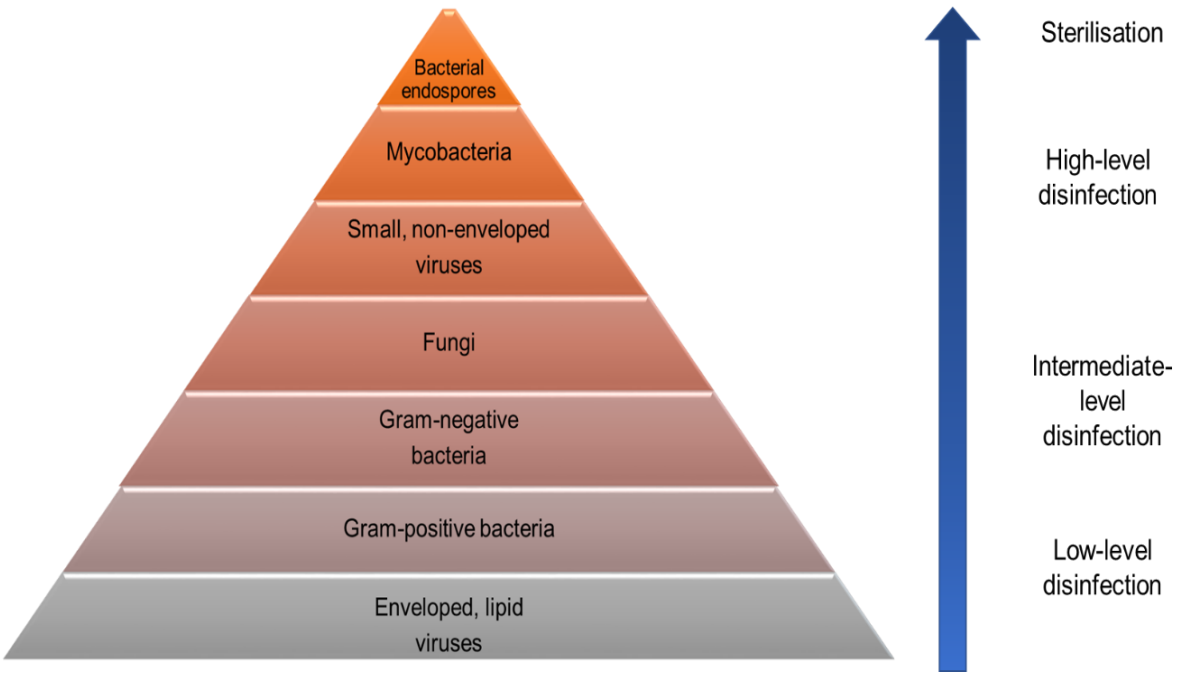
The use of biocides in healthcare can be diverse, from sanitising water, to preservation of formulations, disinfection of surfaces, in antiseptics for dermal application and to sterilise medical equipment.

It is not surprising that the varying physiology and cellular structure of microbes results in different susceptibility to biocides. Resistance to biocides can be naturally occurring (intrinsic) or it can result from mutations in DNA/acquisition of external DNA (acquired). Examples of mechanisms used by microbes as defence against antimicrobial agents include the outer layers of the cell that prevents uptake, enzymes that can degrade the compound and efflux pumps that can lower the intrinsic concentration of the compound (McNeil & Brennan, 1991; Heir *et al*., 1995; Kummerle *et al*., 1996; Ayres *et al*., 1998; Valkova *et al*., 2001; Denyer & Maillard, 2002; Lambert, 2002; Champlin *et al*., 2005; Davin-Regli *et al*., 2006; Piddock, 2006).

The level of disinfection needed for medical devices was first classed by Spaulding in 1957 and divided into high medium and low disinfection depending on the microbes (Spaulding, 1957) (Figure 1.3)

High-level disinfectants are implemented for use against bacterial endospores, bacteria and viruses that have innate mechanisms to protect them from disinfection. Examples of high-level disinfectants used in healthcare are glutaraldehyde, ortho-phthalaldehyde, oxidising agents such as peracetic acid and chlorine-based solutions (i.e. sodium hypochlorite). Intermediate-level disinfectants include phenolic compounds and low to intermediate include QACs, biguanides and phenolics.

Figure 1.3 Order of innate resistance of microbes to disinfection.
The resistance to disinfection among different microbes varies, with bacterial endospores requiring sterilisation before they are inactivated. Mycobacteria, non-enveloped viruses and fungi fall into the higher range of disinfection, Gram-negative and Gram-positive bacteria normally require intermediate to high levels of disinfection. Enveloped viruses are readily inactivated with lower levels of disinfection/cleaning although this is not always the case and high levels may be needed. Adapted from McDonnell and Burke, 2011.



1.5.3 Ionic liquids for disinfection

Ionic liquids (ILs) are defined as salts with melting points under 100°C with physiochemical properties including thermal stability, low vapour pressure and high polarity (Freemantle, 2009). ILs gained popularity in the chemical industry during the late 1990s as a 'greener' alternative to volatile organic solvents, more recently, their applications have expanded to the physical sciences for use as batteries and in life sciences as biocatalysts (Freemantle, 2009; Petkovic *et al*., 2011; Welton, 2018).

Typically, ILs are composed of a cationic head group with a small counter-anion, attached to an alkyl chain forming salts that can be solid or liquid at room temperature (Figure 1.4). There are many variants of ILs but only a few have been reported for their antimicrobial activity, the most common of which possess either an imidazolium, quaternary ammonium or pyrrolidinium head group. For example, several imidazolium and pyrrolidinonium salts were tested against *E. coli*, *S. aureus*, *B. subtilis* and *C. albicans* and antimicrobial activity was observed as the minimal concentration that could inhibit growth (MIC). Overall the activity of the ILs is dependent on alkyl chain length, with greater antimicrobial effect coinciding with increasing chain length, an observation that has been reported elsewhere. (Demberelnyamba *et al*., 2004; Docherty & Kulpa 2005).

Due to similar structural elements, ILs have comparable properties to surfactants which decrease surface tension of liquids, prevent beading and improve distribution of liquids across surfaces (Rutala & Weber, 2014). While not often cited as the active ingredient within a biocide, surfactants themselves can possess antibacterial qualities that can combine with the active ingredient in a synergistic manner to increase the antimicrobial properties of a biocide (Birnie *et al*., 2000). The antimicrobial activity of ILs has also been compared to QACs which possess surfactant qualities (Luczak *et al*., 2010).

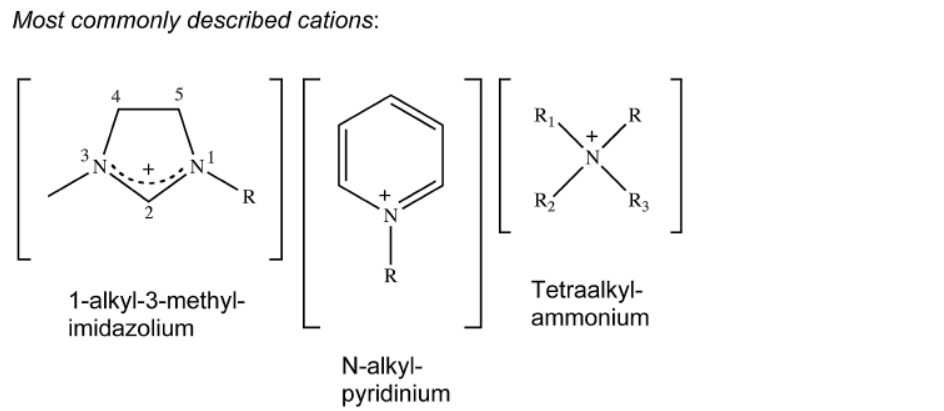
In formulation, surfactants often aggregate into self-assembling bodies of different shapes and forms depending on the concentration and molecular structure of the surfactant. To aid the formation of surfactant micelles a co-surfactant is often included in formulations allowing dispersion of the surfactants throughout the formulation, if co-surfactant is a short chain alcohol it also aids in increasing the fluidity of formulations (Paul & Moulik, 1997).

Whilst there is potential in ILs as antimicrobials, they are not currently applied as a disinfectant in healthcare or otherwise. Furthermore, current testing of ILs is by MIC

methods which does not fully challenge any disinfectant to conditions that may be encountered in a real-use basis (Anvari *et al*., 2016)

Figure 1.4 Examples of cationic head groups, anions and alkyl chains that commonly make ionic liquids.

Cationic head groups; imidazolium, pyridium and quaternary ammonium linked to an alkyl chain of varying length. The cations are combined with either organic or inorganic anions from least soluble such as hexafluorophosphate to halides.



1.6 Considerations for antimicrobial products

There are many factors that need to be considered when developing a disinfectant product for healthcare. First and foremost, the efficacy of the product needs to be appropriate for intended use. For example, if terminal disinfection requires a product that is sporicidal, the product needs to demonstrate activity against spores at the concentration and contact time it will be used. If using a pre-wetted wipe, can it be used on multiple surfaces or does efficacy decrease after use on one surface?

The efficacy is an important aspect of a product but there a many factor that need to be considered for a reliable product (Maillard & McDonnell, 2002). The main issues that are often raised are outlined below;

- Factors that influence efficacy – Type of microbe, organic soiling, contact time, concentration.
- Toxicity – does the product put staff at risk?
- Cost – would the product be cost effective vs. traditional methods such as 'cloth and bucket'?
- Ease of use – is the method more convenient than other methods?
- Organoleptic properties – disinfectants such as PAA create strong astringent odours that might deter their use over products that are perfumed or have no smell.
- Liquid disinfection products – stability of the disinfectant?
- Surface compatibility – is the formulation damaging to surfaces?
- Environmental issues – would chemicals accumulate in the environment and would they cause ecological damage?

1.7 Aims of thesis

The main objectives of this work was to measure the microbicidal efficacy of two imidazolium-based ionic liquids and explore potential use in products aiming to reduce occurrence of HCAI. The evaluation of the ionic liquids were compared to biocides/products that are used extensively in healthcare.

Specifically, this work aimed to evaluate the activity of ionic liquids against micro-organisms that are associated with HCAI, under conditions that challenge disinfectant activity, and to understand factors that would impinge efficacy. The hypothesis being that ILs will provide a broad spectrum activity against vegetative microorganisms.

As part of the understanding of the interactions between ILs and microorganisms, the mechanism in which the ionic liquids target cellular components of Gram-positive and Gram-negative cells were explored. Experiments focused on identifying damage related to cell membrane and outer structure in particular, hypothesising that ILs are membrane active agents.

Finally, as part of exploring the potential of product development, and in recognition that incorporating of ILs in a final product should be tested, the ionic liquids were combined with material for the development of wipe-based products, and tested using recognised international and European standard tests.

2 General Materials and Methods

2.1 Reagents

2.1.1 Media and diluents for microbiology

Deionised water was used for all diluents and media, which were sterilised at 121°C, 15 psi for 15 min where possible. Alternatively, heat-sensitive liquids were filter sterilised by passing through a cellulose acetate membrane, pore size 0.2 µm (Sartorius™ Minisart™, Fisher scientific, Loughborough, UK).

Tryptone Soya Broth (TSB) consisting of pancreatic digest of casein 17 g/ L, enzymatic digest of soya bean 3 g/ L, sodium chloride 5 g/ L, dipotassium hydrogen phosphate 2.5 g/ L, Glucose 2.5 g/ L, (Oxoid, dis, UK) and tryptone soya agar (TSA) (same composition as TSA plus 15g/ L agar; EO Labs, Cumbernauld, UK) were used for routine growth of microorganisms unless stated otherwise. Malt extract agar (MEA) made from 30 g/L malt extract, 5g/L mycological peptone and 5 g/L was used for the growth of yeast (Oxoid Basingstoke, UK).

BD Difco™ Columbia broth (Pancreatic digest of casein 10 g/L, yeast extract 5 g/L, proteose peptone No.3 5 g/L, tryptic digest of beef heart 3 g/L, L-cysteine HCl 0.1 g/L dextrose, 2.5 g/L sodium chloride 5 g/L, magnesium sulfate anhydrous 0.1 g/L, ferrous sulfate 0.02 g/L, sodium carbonate 0.6 g/L, tris-hydroxymethyl aminomethane 0.83 g/L, tris-hydroxymethyl aminomethane HCl 2.86 g/L; Fisher scientific, Loughborough, UK) was used to produce *B. subtilis* endospores.

Dey-Engley neutralising broth was used to stop the activity of biocides against bacteria; Casein enzymatic hydrolysate 5 g/L, yeast extract 2.5 g/L, dextrose 10 g/L, sodium thiosulfate 6.0 g/L sodium thioglycollate 1.0 g/L, sodium bisulfite 2.5 g/L, lecithin 7.0 g/L, polysorbate 80 5.0 g/L and bromocresol purple 0.02 g/L (Fisher scientific, Loughborough, UK)

Tryptone sodium chloride (TSC) was composed of 1 g/ L tryptone (Oxoid Basingstoke, UK) and 8.5 g/L sodium chloride (Fisher scientific, Loughborough, UK), and served as a general diluent for the suspension of the microorganisms used during this project.

2.1.2 Media for mammalian cell culture

Dulbecco's Modified Eagle Medium (DMEM) (4500 mg/ L Glucose, HEPES, no phenol red; Gibco, Fisher scientific, Loughborough, UK) was supplemented with 10% foetal bovine

serum (FBS),10 U/ mL penicillin and 10 µg/mL streptomycin (ATCC, LGC standards, Teddington, UK) for the routine growth of mammalian cells, unless started otherwise.

Phosphate Buffered Saline (PBS) (0.01 M phosphate, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4 at 25°C; Fisher scientific, Loughborough, UK) was used as a diluent for the suspension of microbes.

2.2 Microorganisms

2.2.1 List of bacterial and yeast strains

Table 2.1 Contains the list of strains used throughout this study

Table 2.1 Summary of bacterial strains used in this study

Strains were purchased from American Type Culture Collection (ATCC). *B. subtilis* were provided by P. Setlow.

Strain	Source	Traits
Pseudomonas aeruginosa **ATCC® 15442™**	ATCC LGC Standards, Teddington, UK.	Gram-negative Rod-shape Reference strain
Escherichia coli **ATCC® 10536™**	ATCC, LGC Standards Teddington, UK	Gram-negative Rods-shaped Reference strain
Staphylococcus **aureus ATCC® 6538™**	ATCC LGC Standards, Teddington, UK	Gram-positive Cocci Reference strain
Enterococcus hirae **ATCC® 10541™**	ATCC, LGC Standards, Teddington, UK	Gram-positive Cocci Reference strain
Staphylococcus epidermidis **ATCC® 14990™**	ATCC, LGC Standards, Teddington, UK.	Gram-positive Cocci Reference strain
Acinetobacter baumannii **ATCC® 19568™**	ATCC, LGC Standards, Teddington, UK.	Gram-negative Short rod-shape
Candida albicans **ATCC® 10231 ™**	ATCC, LGC Standards, Teddington, UK.	Yeast
Bacillus subtilis **PS533, Wild type and carrying plasmid pUB110 giving resistance to kanamycin (10µg/ml)**	Isogenic strain of 168 (Bacillus Genetic Stock Center, Columbus, OH, USA) provided by P. Setlow, UConn Health, Farmington, CT, USA.	Gram-positive Spore-forming
Bacillus subtilis **PS578 – lacking ~80% of the DNA protective alpha/beta-type small acid-soluble spore proteins (SASP), possess pUB110 and resistant to kanamycin (10 µg/ml)**	Isogenic strain of 168 (Bacillus Genetic Stock Center, Columbus, OH, USA) provided by P. Setlow UConn Health, Farmington, CT, USA.	Gram-positive Spore-forming
Bacillus subtilis **PS3394 – cotE and also carrying pUB110 and resistant to kanamycin (10µg/ml) and tetracycline (10µg/ml)**	Isogenic strain of 168 (Bacillus Genetic Stock Center, Columbus, OH, USA) provided by P. Setlow, UConn Health, Farmington, CT, USA.	Gram-positive Spore-forming

2.2.2 Storage

All microorganisms were revived from frozen -80 ºC glycerol stock cultures on to TSA plates and grown for 18-24 h at 37ºC ± 1ºC. To prepare future stocks, single colonies were aseptically transferred to Protect beads© (Fisher scientific, Loughborough, UK), in 20 % glycerol and stored at -80 ºC.

2.2.3 Preparation of bacteria and yeast suspensions

Frozen stock cultures were aseptically streaked on to TSA or MEA plates and grown at 37ºC ± 1ºC for 18-24 h or 30ºC ± 1°C for 36-48 h for bacteria and yeast, respectively. Once grown, single colonies were picked and streaked on a second plate and left to grow for a further 24 hours. From the second subculture single colonies were transferred to a sterile flask containing 10 mL TSC and 5 g of 3 mm glass beads (Sigma-Aldrich, Gillingham, UK) before vortexing for 3 min to obtain an even suspension.

2.2.4 Microbial enumeration

To determine the total number of viable cells in suspension, a spread plate method was performed in which 100 µL of the suspension was spread on to the surface of a TSA plate, and grown at 37ºC ± 1ºC for 18-24 h for bacteria and 30ºC ± 1°C for 36-48 h for *C. albicans*. After growth, plates with 30-300 colonies were used to calculate colony forming units per millilitre (CFU/mL).

2.2.5 Standardisation of CFU/mL

Each strain was prepared in suspension as detailed in (2.2.3) and was serially diluted 1:2 in TSC and vortexed for 30 s. The optical density of each dilution was measured at 600 nm with a spectrophotometer (Ultrospec 3100 pro; GE Healthcare, Buckinghamshire, UK). All dilutions underwent a second serial dilution (1:10) and the spread plate method was carried out (2.2.4). Finally, the CFU/mL for all dilutions was plotted against the OD_{600}, which was then used in future experiments to obtain the correct CFU/mL.

2.2.6 Preparation of bacterial endospores

Stains were defrosted cultures on TSA plates for 48 hours at 37ºC ± 1ºC. They were subcultured a second time and the purity of the culture was checked by Gram-stain. Single colonies were then picked inoculated into 99 mL Colombia broth (previously diluted 1:10 with deionised water) supplemented with 1 mL 10 mM $MnSO_4 \cdot H_2O$ (Sigma-Aldrich, Gillingham, UK) in a conical flask. After 72 hours incubation at 37ºC under constant agitation at 150 rpm, the culture was then centrifuged at 10,000 X g for 10 min at 4 ºC and the supernatant decanted. The pellet was resuspended in 20 mL sterile deionised water (SDW). To kill any remaining vegetative cells the suspensions were placed at 80ºC for 20 min, followed by two further centrifugation steps and the pellet adjusted to a final volume to obtain a ~1 x 10^8 spore per mL. The presence of mature spores and spore preparation purity (<20% vegetative cells per field view X 100 objective), was confirmed with phase microscopy. Spore suspensions were stored at 2ºC when not in use.

2.3 Mammalian cell culture

2.3.1 Revival of frozen cell culture stocks

HaCat cells were kindly provided by Prof. James Birchall (Cardiff University, UK). Cells were thawed from frozen at 37°C then aseptically transferred to 10 mL of pre-warmed DMEM, gently mixed, centrifuged at 200 x g for 8 min (MEGA STAR 600, VWR, Lutterworth, UK) and the supernatant discarded. The pellet was re-suspended in 8 mL of fresh DMEM before being transferred to a cell culture flask with a surface area of 25 cm^2 (T25) (Corning, Deeside, UK). T25 flasks were placed at 37°C ± 2°C with 5% CO_2 and 95% relative humidity for 24-36 h until cells covered 80% of the flask surface (80% confluence).

2.3.2 Viable cell counts

Once cells had reached 80% confluence (2.3.1) the DMEM was aseptically removed and 8 mL PBS was gently added to wash cells and then aspirated, this was repeated once more. To detach the cells from the flask, 1.5 mL pre-warmed 0.25% Trypsin (with 0.53 mM ethylenediaminetetraacetic acid (EDTA)) (Gibco™, ATCC, Teddington, UK) was added, and the flask was incubated at 37°C for 10-15 min. When cells had dissociated from the surface, they were placed in a 50 mL falcon tube containing 7 mL DMEM and aspirated/dispensed several times to avoid cell clumping. In a sterile Eppendorf tube, 100 µL of cells was mixed with 100 µL 0.4% Trypan blue (Gibco™, Fisher scientific, Loughborough, UK). The stained cells were loaded in to a haemocytometer and viewed with an inverted microscope at a low objective (x10) (Ceti Triton II, Medline, Oxon, UK). The total

amount of cells was counted and the ratio of stained cells (indicating non-viable cells) to unstained cells (live, metabolically active cells) was calculated.

2.3.3 Cryopreservation of cells

When HaCat cells had reached 80% confluency, they were washed and treated with trypsin as described previously (2.3.2). After trypsinization, cells were mixed with 7 mL DMEM and then centrifuged at 200 x g for 8 min. The cell pellet was then resuspended in DMEM containing 10% (v/v) filter sterilised Dimethyl sulfoxide ($DMSO_4$) (Fisher scientific, Loughborough, UK) and put in to sterile cryovials. To prevent ice crystal formation and subsequent cell rupture, cells were cooled at a rate of ~ 1°C/ min by placing cryovials in a Mr. Frosty™ Freezing Container (Fisher scientific, Loughborough, UK), which was placed at -20°C for 4 h, then -80°C for ~ 16 hours, then finally vials were put into the gas phase of a liquid nitrogen container for long-term storage.

2.4 Formulations

2.4.1 Hard water

Hard water was composed of magnesium chloride ($MgCL_2$) 0.12 g/L, calcium chloride ($CaCL_2$) 0.28 g/L and sodium bicarbonate $NaHCO_3$ 0.28 g/L, (Sigma-Aldrich, Gillingham, UK) made up to 1 L and sterilised with a membrane filter (2.1.1), and stored at 2 °C for 1 week.

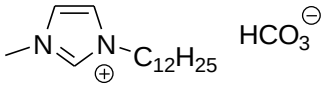
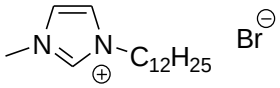
2.4.2 Biocides

Two imidazolium salts 1-dodecyl-3-methylimidazolium hydrogen carbonate (MF1) and 1-dodecyl-3-methylimidazolium bromide (MF2), were supplied by Ian Fallis, School of Chemistry, Cardiff University. They were made into a stock concentration by dissolving the salts in alcohol (butanol or ethanol) and water at a ratio of 1:1:7 The full composition is shown in Table 2.2.

All biocides including Ionic Liquids, benzalkonium chloride (BZC) (Sigma-Aldrich, Gillingham, UK).and chlorhexidine digluconate (CHX) (Sigma-Aldrich, Gillingham, UK) were diluted in hard water unless stated otherwise

Table 2.2 Composition of formulations MF1 and MF2

The formulations consisted of water, a butanol co-surfactant and Imidazolium salt with differing anions; 1-dodecyl-3-methylimidazolium hydrogen carbonate or 1-dodecyl-3-methylimidazolium bromide, which were termed MF1 and MF2, respectively.

Name	Surfactant/ Imidazolium	Co-surfactant	% as supplied
MF1		Butanol or Ethanol	1:1:7 Surfactant: co-surfactant : water
MF2		Butanol or Ethanol	1:1:7 Surfactant: co-surfactant : water

2.4.3 Hydrogen peroxide titration

MF1 and MF2 were used with and without Hydrogen peroxide (H_2O_2) (Fisher scientific, Loughborough, UK). Commercial H_2O_2 was titrated against potassium permanganate (KM_nO_4) (Fisher scientific, Loughborough, UK) to determine the percentage (w/v) of H_2O_2. Briefly, H_2O_2 was diluted to approximately 0.01% with deionised water and 5 M sulfuric acid (H_2SO_4) (Fisher scientific, Loughborough, UK) and transferred to an Erlenmeyer flask where H_2O_2 was titrated against 0.02 M KM_nO_4 until an end-point was reached (indicated by colourless H_2O_2 solution retaining a light purple colour) (British Pharmacopoeia, 2019).

$$\mathbf{[2\ KMnO_4 + 5\ H_2O_2 + 3\ H_2SO_4 = K_2SO_4 + 2\ MnSO_4 + 8\ H_2O + 5\ O_2]}$$

The amount (mL) of KM_nO_4 it took to degrade H_2O_2 was used to calculate % (w/v) of the commercial H_2O_2.

3 Ionic liquids: factors affecting antimicrobial efficacy

3.1 Introduction and aims

3.1.1 Parameters affecting efficacy of biocides

To ensure full and safe disinfection of surfaces there are several factors to take into consideration which can alter the efficacy of a biocide.

3.1.1.1 Concentration

Varying the concentration of a biocide can dramatically affect its activity on microbes (Russell and McDonnell, 2000). One way to evaluate the antimicrobial effectiveness of a chemical is by the concentration exponent (Ƞ) which relates the survival rates of the micro-organism to the dilution of biocide (Russell and Chopra, 1996). The antimicrobial activity of phenolics and alcohols is reduced upon dilution and have a higher Ƞ-value (6-10) compared to mercurials and aldehydes that have a lower Ƞ-value (approx. 1) and antimicrobial efficacy is not as affected by dilution. As concentration can have a significant impact on the activity of a biocide, it is important to take into consideration the effect of dilution. Commonly used disinfectants are often supplied as concentrated products that need dilution by the user, this can result in solutions that are too dilute. One study that investigated the over-dilution of QAC products reported that instead of the required 800 parts per million (ppm), approximately 50% of diluted solutions were at 200 – 400 ppm (Boyce *et al*., 2016).

Outbreaks within hospitals have been associated with biocides that have been over diluted and are no longer inactivating microbes but are instead a reservoir for pathogens (Tiwari *et al*., 2003; Tena *et al*., 2005).

3.1.1.2 Contact time

The period of time that a microbe and a biocide are in contact is another critical factor that determines the efficacy of the biocide. The treatment time needed to inactive microbes is dependent on the biocide, the concentration of the biocide and microorganism. For example, the contact time for microorganisms that have a high innate resistance to antimicrobials such as spores would require a longer contact time in comparison to vegetative bacteria on a surface (Wesgate *et al*., 2016)

The biocide of choice will also impact the contact time. For example, in a study that evaluated the impact of using sodium hypochlorite and a QAC against *S. aureus* and

P. aeruginosa, QAC had the greatest loss in activity whilst sodium hypochlorite remained effective (West *et al*., 2018).

It is an important consideration of biocidal products to show effective antimicrobial activity with a realistic contact time. In a busy healthcare environment, products that have long exposure times may not be adhered to.

3.1.1.3 Organic soiling

The presence of organic matter (soiling/interfering substance) on surfaces is inevitable in a healthcare setting and routine wiping of surfaces is needed to prevent build-up. Soiling can occur from dust, dirt and grime or can include bodily fluids such as blood, urine and faeces which are more likely to be encountered in healthcare. There are two possible mechanisms in which organic soiling can inhibit biocide activity. Firstly, the soil can act as a barrier between the target microbe and the biocide and second the organic soil can neutralise the active component (Guan *et al*., 2013).

The activity of QACs has been known to decrease in the presence of bovine serum albumin due to positively charged groups of QACs binding negatively charged proteins, thus quenching their activity. Other biocides also show altered activity in the presence of organic soiling (Jono *et al*., 1986). A peroxygen containing compound and a glutaraldehyde-based disinfectant showed significant differences in their ability to kill rotavirus at low organic loads (3% fetal bovine serum; FBS) compared to a high organic load (10% FBS; 20% yeast extract) (Chandler-Bostock and Mellits, 2015).

The effect of blood as a source of organic material has also been demonstrated to limit the efficacy against spores when using chlorine-based disinfectants. In the presence of 2 % blood, a disinfectant with 1200 parts per million (ppm) of chlorine was unable to reduce the number of spores compared to a 5 $\log_{10}$ reduction seen in the absence of blood (Coates, 1996). Therefore, when developing a biocidal product, it is important to consider if it will come into contact with organic matter and if so, will it retain activity.

3.1.1.4 Temperature and pH

Changes to temperature and pH can also influence the activity of a biocide. Increases in temperature have been linked a greater antimicrobial effect. This was observed when zinc oxide nanoparticles were tested against *S. aureus* and *E. coli* at 25°C, 37°C and 42°C and the greatest inhibition of bacteria was noted at the highest temperature. The authors

proposed that the greater activity of the nanoparticles was due to the excitement of electrons with increasing temperature which lead to production of reactive oxygen species (ROS), causing oxidative stress in the cells (Saliani *et al*., 2015). Other biocides such as sodium hypochlorite-based solutions and peracetic acid have also been shown to increase activity at temperatures between 30°C – 50°C (Stampi *et al*., 2001; Sirtes *et al*., 2005).

The storage of biocides at increased temperatures may have a negative impact on biocidal activity. The level of free available chlorine was shown to decline at 28°C – 30°C compared to storage at 4°C (Dash *et al*., 2017). Storage at low temperatures can also decrease activity, as seen with an antimicrobial lens solution against *Pseudomonas* (Leung *et al*., 2004). The studies that highlight changes in solutions with fluctuating temperature highlight the need for proper storage of biocides.

Altering the pH of biocidal formulations also impacts antimicrobial activity. An increase in pH can result in greater antimicrobial activity of glutaraldehyde and cationic biocides, others such as sodium hypochlorite are more active at neutral to acidic pH (Fraise *et al*., 2008; Frazer *et al*., 2013).

3.1.1.5 Formulation

Disinfectant formulations generally consist of an active compound with a range of additives/ excipients to help aid the active ingredients. Examples of excipients are metal chelators and surfactants. A common metal chelator is ethylenediaminetetraacetic acid (EDTA) which binds Mg^{2+} and Ca^{2+} that constitute the ions in hard water that can often impede the activity of biocides (Cousins & Clegg, 1956). Surfactants can lower the surface tension and can aid dispersion of other ingredients. Both EDTA and some surfactants also display their own antimicrobial activity by disrupting the outer layers of the cell (Farca *et al*., 1997; Ashoori *et al*., 1999; Gill & Holley, 2001).

3.1.1.6 Number and type of microorganisms

As mentioned in Chapter 1 (1.5.2), microbes possess mechanisms to survive stressful insults such as biocide exposure. Spores formed from Gram-positive bacteria are notoriously difficult to destroy. They possess several structural features such as a proteinaceous outer coat, low water content, highly compressed spore membrane and DNA-protective proteins (Leggett *et al*., 2012).

On surfaces bacteria can form biofilms, structured communities more resistant to chemical and physical stresses than planktonic bacteria. The mechanisms employed by biofilms include (i) reduced penetration of antimicrobials due to production of extracellular polymeric substances (proteins, extracellular DNA, polysaccharides, lipids) and increased cell density, (ii) reduced growth rate and bacterial metabolism (iii), development of a stress response and (iv) quorum sensing to communicate and upregulate the stress response mechanisms, and (v) gene mutations and gene transfer (Stewart & Olson, 1992; Lisle *et al*., 1998; Hassett *et al*., 1999; Hausner *et al*., 1999; Cochran *et al*., 2000; Maukonen et al., 2003; Coenye, 2010; Davison *et al*., 2010; Flemming & Wingender, 2010).

There are many tests that can be used to demonstrate biocidal product efficacy. The minimal inhibitory concentrations (MIC) and minimal bactericidal concentration (MBC) are high-throughput methods for screening of antimicrobials but do not consider factors that alter the activity of biocides (Patel *et al*., 2015).

Standardised efficacy tests use approved methods that specify contact times, soiling, temperature, and hard water to determine the activity of the biocide against microorganisms. They also enforce the use of standards of efficacy that relate to the end use of biocidal products. Furthermore, for the commercialisation of a biocidal product within the European Union (EU), the BPR requires that efficacy tests support the application for the antimicrobial product.

The efficacy tests are divided into phases with phase 2 tests used to make efficacy product claims on label.

Phase 1 tests are for initial evaluation of antimicrobial activity but, are not specific to product use and therefore can not be used solely for product claims. Phase 2 tests are categorised in two further steps; the first is a quantitative suspension test in which the in-use concentration of a product is in suspension with the microorganism with specified soiling conditions and suggested contact times; the second is a quantitative carrier test in which microbes are places on a surface and the in-use product is tested on the inoculated surface. Phase 3 tests are *in situ* tests which have not yet been developed.

3.1.2 Aims and objectives

In this chapter the main aim was to identify the key factors affecting the antimicrobial activity of ionic liquids MF1 and MF2.

This was assessed by adapting the following standards: BS EN 1276:2009 and BS EN 61324:2013 for bacteria and yeast, and BS EN 17126:2018 for bacterial endospores. These tests evaluate the activity of formulations in the presence of microorganisms and an interfering substance (organic soiling) for a specified contact time. Suspension tests were carried out against yeast, spores, Gram-negative and Gram-positive bacteria. Furthermore, two contact times and two organic soiling conditions have been selected to reflect the intended use.

The second aim was to compare MF1 and MF2 to two cationic biocides, benzalkonium chloride (BZC) and chlorohexidine digluconate (CHX) which are commonly used to disinfect healthcare facilities. This will provide more insight into the efficacy of ionic liquids in comparison to well-established biocides. This was performed by suspension tests alongside BZC and CHX at the same concentrations.

Finally, activity of MF1/MF2 in combination with hydrogen peroxide (H_2O_2) was assessed to determine if an increase in antimicrobial activity can be achieved when MF1/MF2 are at concentrations that are less efficacious.

3.2 Methods

3.2.1 Suspension tests

3.2.1.1 Microbial suspensions

Bacteria and yeast were prepared as mention in Chapter 2 Section 2.2.3 and *B. subtilis* spore preparation as described in Chapter 2 Section 2.2.6.

3.2.1.2 Preparation of interfering substances

Based on the BS EN 1276:2009 and BS EN 61324:2013 standards, the following organic soiling; bovine serum albumin (BSA) and BSA + erythrocytes were used for efficacy testing.

3.2.1.3 Bovine serum albumin

BSA was added to TSC at 30 g/L and filter sterilised (as described in Chapter 2, Section 2.1.1). The final concentration of BSA in the test mixture was equal to 3 g/L. BSA solution was stored at 2°C and used within one month.

3.2.1.4 Bovine serum albumin and sheep erythrocytes

Sheep erythrocytes were prepared from fresh defibrillated sheep blood (Fisher scientific, Loughborough, UK), by centrifugation 8 mL at x 800 g. The pellet was resuspended in TSC and washes were repeated three times until the supernatant was clear. Finally, the erythrocytes were reconstituted in 3 mL TSC and added to 97 mL BSA at 30 g/L. For suspension tests, BSA and sheep erythrocytes were diluted 10-fold, to give a final concentration of 3 g/L for both. The BSA/erythrocyte mix was stored at 2°C and used within one week.

3.2.1.5 Exposure of bacteria and yeast to formulations

The antimicrobial activity of MF1 and MF2 were evaluated in suspension against the strains listed in BS EN 1276:2009 and BS EN 13624:2013. Both standards were modified as followed for testing of bacteria and yeast: First, an aliquot of 100 µL bacteria/yeast prepared as described previously (chapter 2, section 2.2.3) and incubated with 100 µL of either 3 g/L BSA or 3 g/L BSA with 3 g/L sheep erythrocytes, at room temperature for 2 min.

Then formulations were diluted to 1.25 % (w/v), 0.125 % (w/v) and 0.0125 % (w/v), 800 µL of which was added to the bacteria:soiling mix to give a final concentration of 1 % (w/v), 0.1 % (w/v) and 0.01% (w/v), respectively. At a concentration of 0.01 % (w/v) MF1 and MF2 were also combined with 1 % (w/v) H_2O_2. Finally, after the addition of the biocides the suspension was vortexed briefly and incubated at room temperature for the allocated contact time of 5 or 0.5 min.

3.2.1.6 Exposure of spores to formulations

For spore testing, a modified BS EN 17126:2018 test was used as follows: 100 µL of spores (1 x 10^8 spores/mL) was mixed with 100 µL 3 g/L BSA and was combined with 800 µL 1% (w/v) MF1, 1% (w/v) MF2 alone and with the addition of 1 % (v/v) and 5% (v/v) H_2O_2 , and 1 % (v/v) and 5 % (v/v) H_2O_2 alone. Spores were left in contact with the formulations for 60 min before neutralisation.

3.2.1.7 Neutralisation of formulations

At the end of the contact time, formulations were neutralised by membrane filtration with cellulose nitrate membrane filters, pore size 0.2 µm (47 nm; Whatman, Fisher Scientific, Loughborough, UK). Briefly, 100 µL of test suspension was placed directly on to a wetted membrane filter and immediately rinsed with 150 mL TSC, followed by a final rinse with 50 mL SDW to prevent any salt crystal formation from the drying of TSC. Filters were aseptically transferred to TSA plates, ensuring there were no air bubbles between the filter and the agar and then incubated for 18-24 h at 37 ± 1ºC.

Both physical filtration and dilution of the biocide with TSC were considered to be sufficient to neutralise the biocides at the highest concentration used. The contact time was taken as the incubation time plus the time it took for 150 mL of TSC to filter through the membrane; contact times were established as 5 + 1 min and 0.5 + 1 min. For H_2O_2 tests catalase (500 U/ml) was added to the filter membrane after the addition of 150 mL TSC to ensure complete neutralisation of H_2O_2.

3.2.1.8 Exposure to formulations at low concentrations

When low concentrations of formulations were used, bacterial suspensions were too high to read single colonies on membrane filters (>300 CFU). In this case, membranes were not placed onto the surface of an agar plate, but instead transferred to 5 mL TSC containing

0.05% (v/v) Tween™ 80 (Fisher scientific, Loughborough, UK), 5 g glass beads (3 mm diameter) and vortexed for 1 min. The suspension was then serially diluted, and each dilution was plated out in duplicate.

3.2.1.9 Validation suspension

For all controls a dilute colony suspension (termed validation suspension) was used so that microorganisms were in a countable range on agar plates (30-300 CFU). To prepare the suspension, microorganisms were prepared as described in chapter 2 (sections 2.2.3 – 2.2.5) which gave a 1-2 x 10^8 CFU/mL concentration for bacteria and spores and 1-2 x 10^7 CFU/mL for yeast. The concentrated bacteria underwent a series of 1:10 serial dilutions in TSC and the dilution that fell in the range of 30-300 CFU/mL was used for suspension test controls.

3.2.1.10 Suspension test controls

To ensure any reductions in CFU were due to the presence of the formulations and not any experimental factors the following controls were carried out:

i) Filtration control. To rule out any effect that filtering could impact the tests, 100 µL validation suspension was aliquoted onto a membrane filter, followed by 150 mL TSC and 50 mL SDW. The membranes were placed onto sterile TSA plates before incubation

ii) Method validation control. 100 µL of 3 g/L BSA and 800 µL of SDW was added to 100 µL validation suspension. The mix was then vortexed and 100 µL was added to wetted membrane filters, before washing with 150 mL TSC followed by 50 mL SDW. Filters were then aseptically transferred to TSA plates.

iii) Neutralisation efficacy control. To confirm complete neutralisation of the formulations, 800 µL of the highest concentration of each of the biocides was added to 100 µL BSA and 100 µL TSC. This was then vortexed and 100 µL transferred membranes, which were washed with 150 mL TSC. Then 100 µL of the validation suspension was added to membranes and washed once more with 50 mL SDW. Membranes were placed on TSA plates.

All control plates were incubated at 37 ± 1ºC and the CFU counted after 18-24 hr. For each of the different contact times and biocide concentrations, the mean reduction of CFU/mL was calculated by subtracting recovered colonies from the original CFU/mL.

Following the BS EN 13624 suspension test for yeast the same procedure was followed for the above but *C. albicans* was plated on MEA agar for 48 hr at 30 ± 1°C.

3.2.1.11 H_2O_2 with MF1 and MF2

To determine if H_2O_2 could increase the efficacy of low concentrations of MF1/MF2, it was added to the formulations: 1 % (v/v) H_2O_2 was added to either 0.01 % (w/v) MF1 or MF2 for 1, 10, 30, 60, 120 and 240 min before neutralisation. The formulation was added to a 100 μL bacteria combined with 100 μL BSA and left for a contact time of 5 min. After the allocated contact time the formulations were neutralised as described in section 3.2.5. If the CFU count was too high (i.e. >300) after the test, membrane filters were transferred to 5 mL TSC and serially diluted as described in section 3.2.6. After neutralisation, 100 µL of each test was plated out onto TSA and incubated at 37 ± 1ºC and the CFU counted after 18-24 h.

3.2.2 Statistical analysis

For Log_{10} reduction of CFU/mL three biological replicates (n=3) were produced to obtain the average and the standard deviation (SD), unless stated otherwise. Statistical analysis was preformed using a one-way or two-way analysis of variance (ANOVA) followed by a post-hoc Turkey test. All statistical tests were performed with GraphPad Prism v.7 with a *p* value of < 0.001 (GraphPad Software, La Jolla California, US).

3.3 Results

3.3.1 Suspension tests to compare efficacy of MF1 and MF2

Suspension tests were used to determine the impact of different microorganisms, lowered concentration, lowered contact time and increased organic soiling on the biocidal activity of MF1 and MF2. For comparison two commonly used biocides benzalkonium chloride (BZC) and chlorhexidine digluconate (CHX) were used at the same concentrations as MF1 and MF2.

The results showed that MF1 and MF2 were efficacious against all the organisms tested at 1 % (w/v) with a ≥ 5 Log_{10} reduction (Figure.3.1-3.4). Moreover, shortening the contact time (5 to 0.5 min) and increasing the organic soil (3 g/L BSA to 3 g/L BSA + 3 g/L erythrocytes) did not decrease (p > 0.999; two-way ANOVA) the activity of MF1/MF2 at 1 % (w/v) or 0.1 % (w/v).

However, when diluted to 0.01 % (w/v) formulations had limited microbicidal activity. Only when tested against *E. coli* was MF2 at 0.01 % (v/v) able to achieve a ≥ 5 log_{10} reduction. All other microbes tested at 0.01% (w/v) did not reach a 5 Log_{10} reduction. Therefore at 0.01 % (w/v) MF1 and MF2 were not tested with the higher organic soil of 3 g/L BSA + 3 g/L erythrocytes.

In comparison to MF1 and MF2, the two cationic disinfectants BZC and CHX performed comparatively well at 1 % (w/v), 5 min contact time and with 3 g/L BSA. However, when lowered to 0.1 % (w/v) BZC did not reach a 5 log_{10} reduction against *P. aeruginosa* (Figure 3.1B) and CHX activity was lowered against *S. aureus* (Figure 3.1C) and *E. hirae* (Figure 3.1D. Lowering the contact time to 0.5 min and increasing the organic soil emphasised the loss activity of 1 % (w/v) BZC against *P. aeruginosa* in which only a 3.38 ± 0.17 Log_{10} reduction was observed (Figure 3.4B). For 1 % (w/v) CHX the lowered contact time and increase in soil resulted in the loss of activity (Log_{10} 0.74 ± 0.45) against *E. hirae* (Figure 3.4D).

Figure 3.1 Suspension test at 5 min contact time with 3 g/L BSA.

Figures A-F show Log_{10} reductions of *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C), *E. hirae* (D), *A. baumannii* (E) and *C. albicans* (F) after treatment with MF1, MF2, BZC and CHX at 1 % (w/v), 0.1 % (w/) and 0.01 % (w/v). Log_{10} reductions are the means of three biological repeats and two technical replicates (n=3). Dotted lines represent the required log_{10} reduction from standard protocols BS EN1267:2009 (5 Log_{10}) (A-E) and BS EN13624:2013 (4 $Log_{10;}$) (F). (*) represent a significant difference of $p < 0.001$.

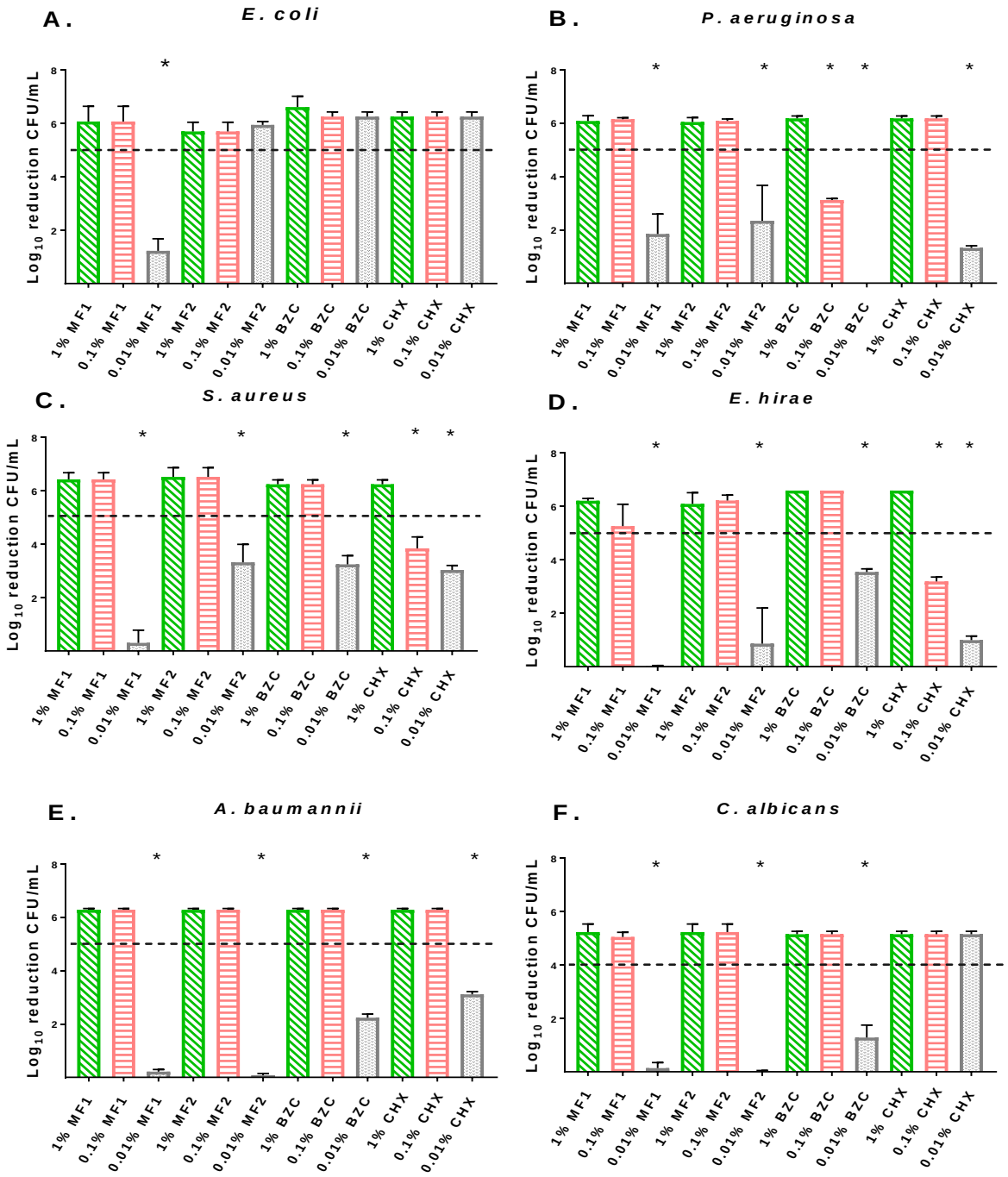


Figure 3.2 Suspension test at 0.5 min contact time with 3 g/L BSA.

Figures A-F show Log_{10} reductions of *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C), *E. hirae* (D), *A. baumannii* (E) and *C. albicans* (F) after treatment with MF1, MF2, BZC and CHX at 1 % (w/v), 0.1 % (w/v) and 0.01 % (w/v). Log_{10} reductions are the means of three biological repeats and two technical replicates (n=3). Dotted lines represent the required log reduction for the standard protocols BS EN 1267:2009 (5 Log_{10}) (A-E) and BS EN 13624:2 013 (4 $Log_{10;}$) (F). (*) represent a significant difference of $p < 0.001$.

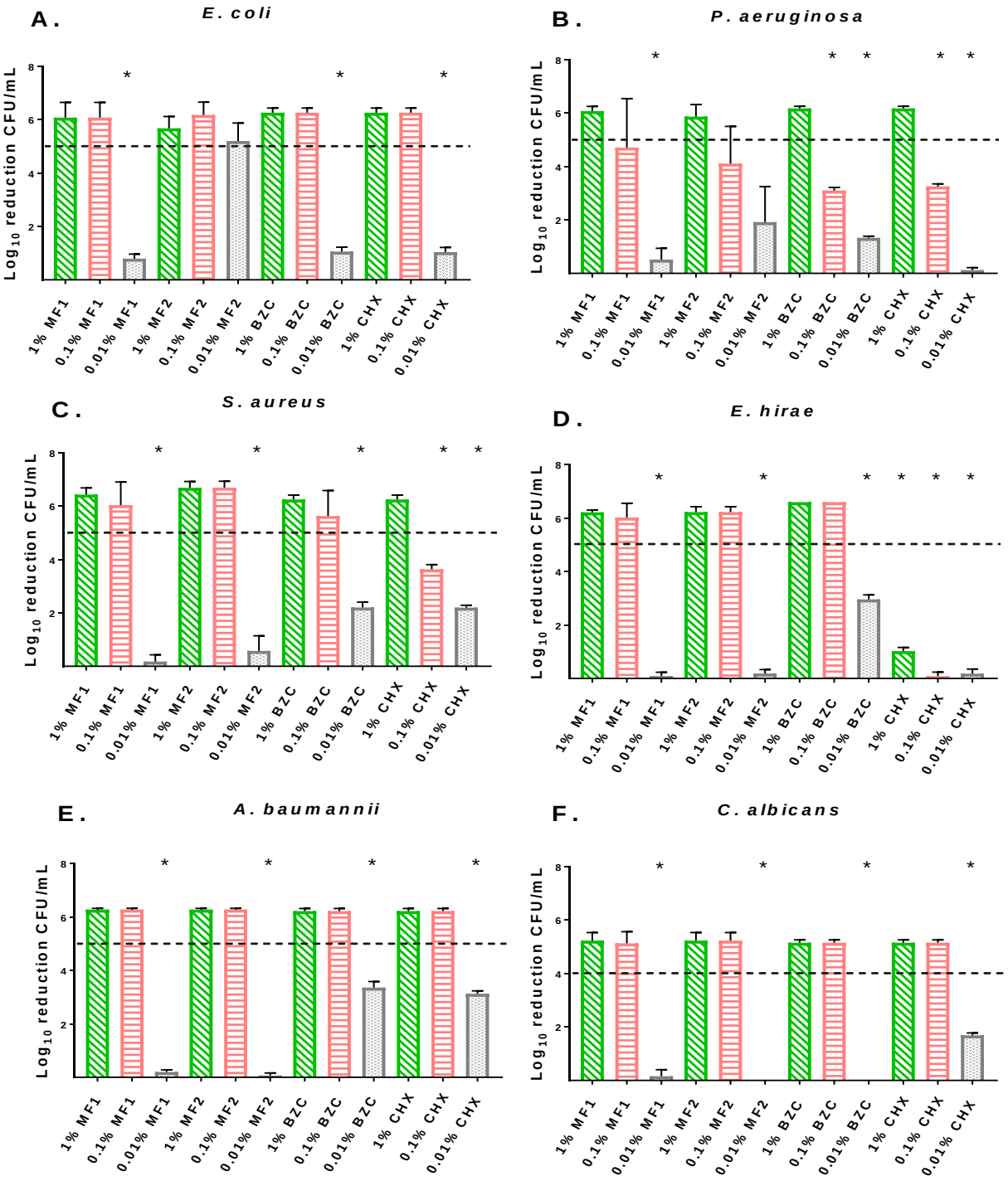


Figure 3.3 Suspension test at 5 min contact time with 3 g/L BSA and 3 g/L sheep erythrocytes.

A-F show Log_{10} reductions of *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C), *E. hirae* (D), *A. baumannii* (E) and *C. albicans* (F) after treatment with MF1, MF2, BZC and CHX at 1 % (w/v) and 0.1 % (w/v). Log_{10} reductions are the means of three biological repeats and two technical replicates (n=3). Dotted lines represent the required log reduction for the standard protocols BS EN 1267:2009 (5 Log_{10}) (A-E) and BS EN 13624:2013 (4 $Log_{10;}$) (F). (*) represent a significant difference of $p < 0.001$.

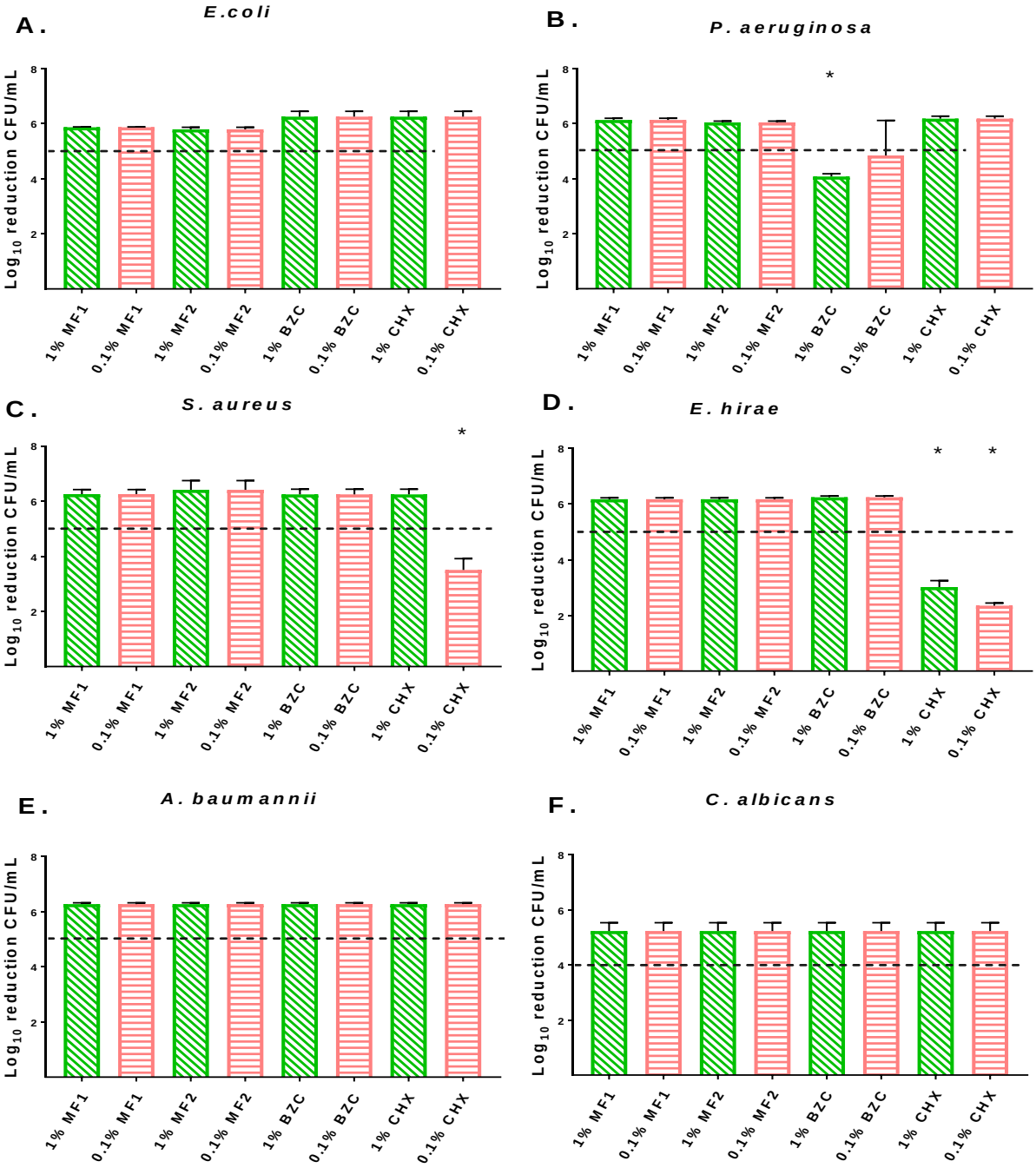
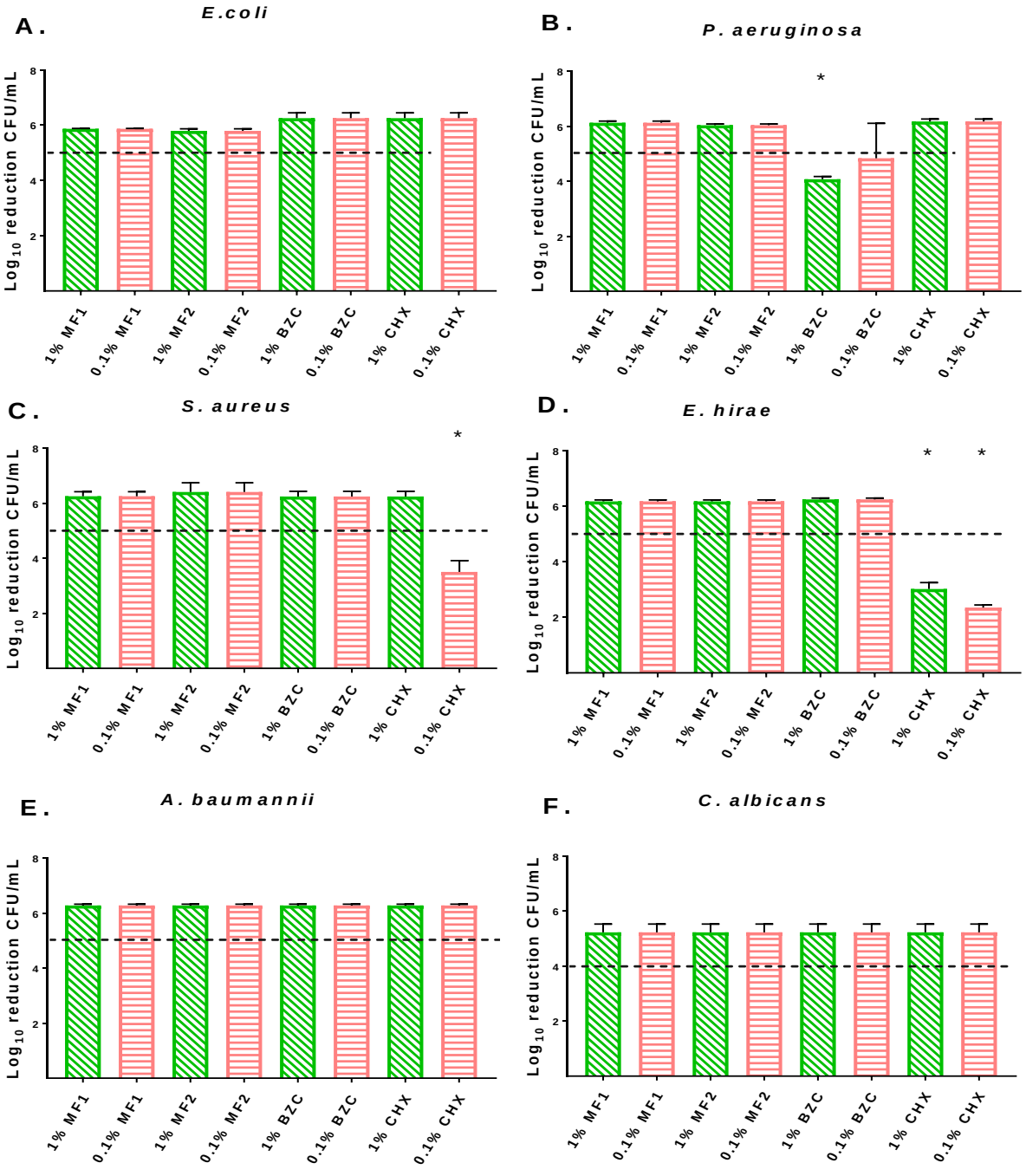


Figure 3.4 Suspension test at 0.5 min contact time with 3 g/L BSA and 3 g/L sheep erythrocytes.

A-F show Log_{10} reductions of *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C), *E. hirae* (D), *A. baumannii* (E) and *C. albicans* (F) after treatment with MF1, MF2, BZC and CHX at 1 % (w/v) and 0.1 % (w/v). Log_{10} reductions are the means of three biological repeats and two technical replicates (n=3). Dotted lines represent the required log reduction for the standard protocols BS EN 1267:2009 (5 Log_{10}) (A-E) and BS EN 13624:2013 (4 $Log_{10;}$) (F). (*) represent a significant difference of $p < 0.001$.



3.3.2 Effect of addition of H_2O_2 to the antimicrobial activity of MF1 and MF2

To determine if low concentrations (0.01 % w/v) of MF1 and MF2 could be enhanced in formulation with H_2O_2, suspension tests were performed. One factor that was addressed before suspension tests were carried out was at which time point after the addition of H_2O_2 to MF1/MF2 would be most active against *S. aureus* and *E. coli*.

After the addition of 1% (v/v) H_2O_2 to 0.01 % (w/v) MF1 or MF2, the formulations were left for 1, 10, 30, 60 and 120 min. H_2O_2, MF1 and MF2 were tested individually to observe any differences upon combing the formulations.

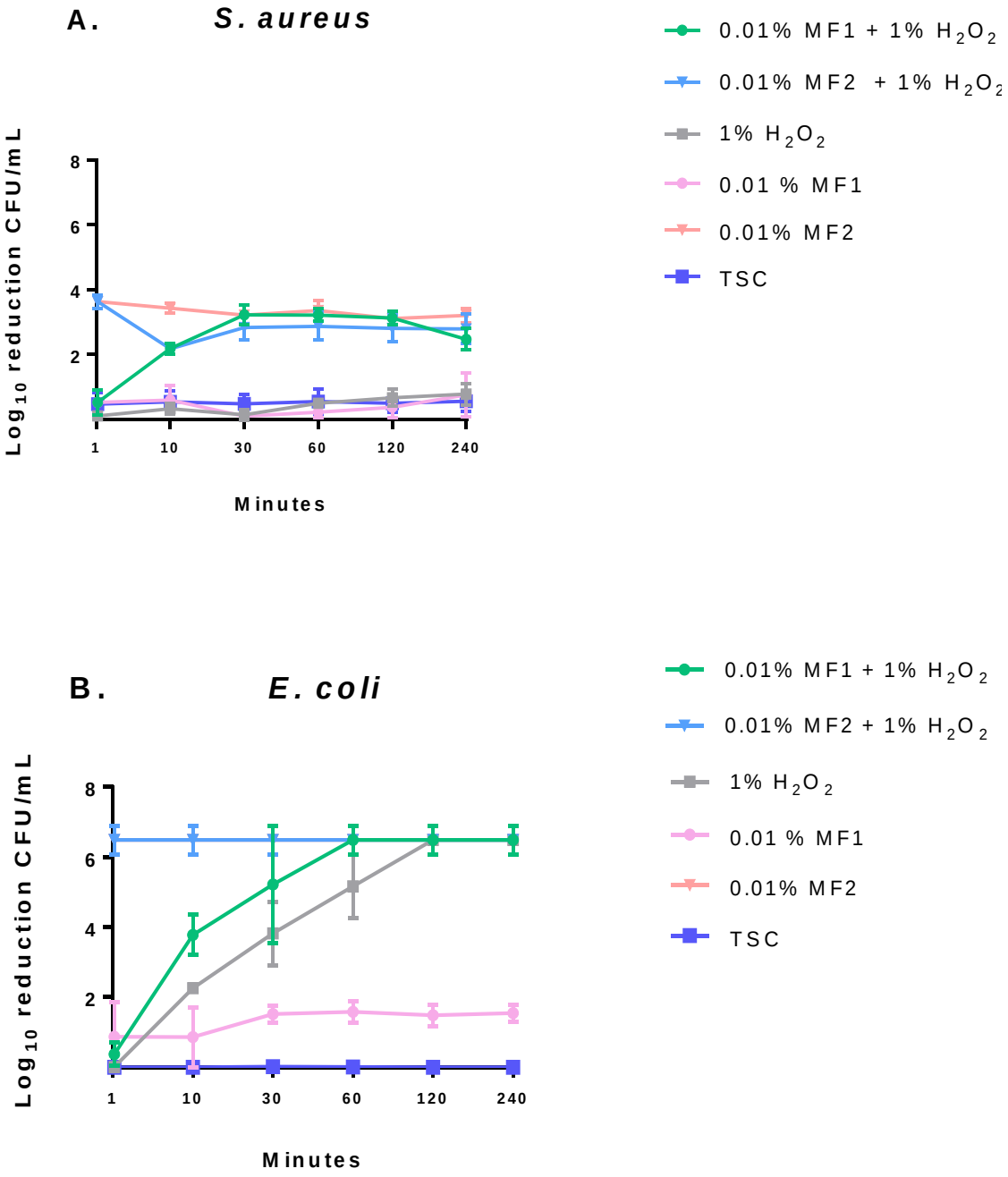
The addition of 1 % (v/v) H_2O_2 increased the Log_{10} reduction of *S. aureus* when added to 0.01 % MF1 from < 1 Log_{10} to 3.22 ± 0.29 after 30 min of addition. This was not significantly different (two-way ANOVA; $p > 0.50$) to other time points where H_2O_2 was added to MF1. The addition of H_2O_2 to 0.01 % (w/v) MF2 did not to increase the antimicrobial activity against *S. aureus* compared to 0.01 % (w/v) MF2 alone and the activity was not significantly different ($p > 0.999$; two-way ANOVA) at any of the time points. The activity of H_2O_2 alone gave <1 Log_{10} reduction.

E. coli was more susceptible to 1 % (w/v) H_2O_2 (Figure 3.5B) compared to *S. aureus.* The combination of H_2O_2 and MF1 increased in activity from < 1 Log_{10} at 1 min to 6.49 ± 0.41 at 60 min ($p < 0.001$; two-way ANOVA). There was no significant difference for between time points 10 – 240 min ($p > 0.001$; two-way ANOVA) and no notable difference observed with the combination of MF1 + H_2O_2 compared to H_2O_2 alone (two-way ANOVA; $p > 0.0001$). There was no significant difference between the time points upon addition of H_2O_2 to MF2 ($p > 0.001$; two-way ANOVA).

When tested against *E. coli*, solutions containing H_2O_2 had peak antimicrobial activity at 120 min (Figure 3.5B) and so all subsequent suspension tests were performed 120 min after the addition of H_2O_2.

Figure 3.5 Assessment of antimicrobial capabilities of solutions after the addition of H_2O_2 over a 4 hr time period.

A) *S. aureus* and B) *E. coli* averaged data of three biological repeats and two technical replicates (n=3) error bars indicate ± SD.



To determine if H_2O_2 could increase the activity of 0.01 % (w/v) MF1 and MF2, H_2O_2 was added to the two formulations and a reduction in CFU/mL was assessed after a contact time of 0.5 and 5 min (Figure 3.6).

When tested against *E. coli* the activity of 0.01 % MF2 was already efficacious at both contact times producing > 5 log_{10} reduction (Figure 3.6A). When 1 % (v/v) H_2O_2 was added to 0.01 % (w/v) MF1 the activity of MF1 increased three-fold at 5 min compared to the activity of 0.01 % (w/v) MF1 only. However, the increase was not significantly different (two-way ANOVA; $p > 0.0001$) to 1 % (v/v) H_2O_2 at 5 min. At 0.5 min the addition of H_2O_2 was not significantly different (two-way ANOVA; $p > 0.999$) to 0.01 % (w/v) MF1 or 1 % (v/v) H_2O_2.

For *P. aeruginosa*, the activity of 0.01 % (w/v) MF2 was not substantially increased with the addition of H_2O_2 and was lower than 0.01 % (w/v) MF2 alone, although not significantly (two-way ANOVA; $p > 0.999$). The addition to 0.01 % (w/v) MF1 did result in an increase from 1.75 ± 0.60 to 5.13 ± 1.40 at 5 min (two-way ANOVA; $p < 0.001$).

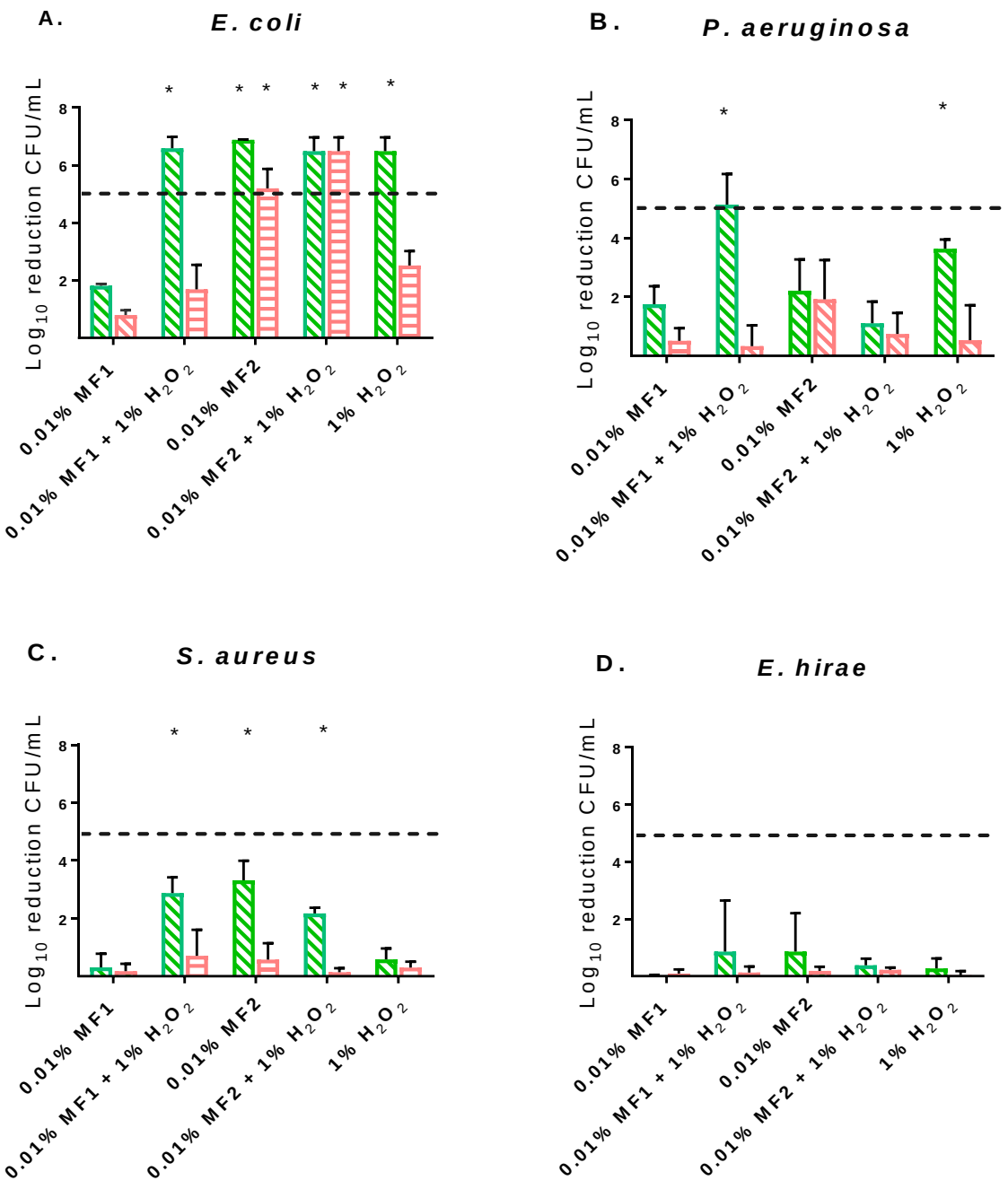
Against *S. aureus*, the addition of H_2O_2 did not recover the activity of MF1 and MF2 to the required 5 Log_{10} reduction. However, there was a significant increase in Log_{10} reduction of *S. aureus* (two-way ANOVA; $p < 0.001$) at 5 min when added to 0.01 % (w/v) MF1 (Figure 3.6C).

When tested against *E. hirae* (Figure 3.6D), the addition of H_2O_2 did not result in a significant increase (two-way ANOVA; $p > 0.001$) in the activity MF1 or MF2 at 5 and 0.5 min.

Figure 3.6 Log_{10} reductions of bacteria after treatment with 0.01 % (w/v) MF1 or MF2 with and without 1 % (v/v) H_2O_2.

Figure 3.6 Log_{10} reductions of bacteria after treatment with 0.01 % (w/v) MF1 or MF2 with and without 1 % (v/v) H_2O_2.

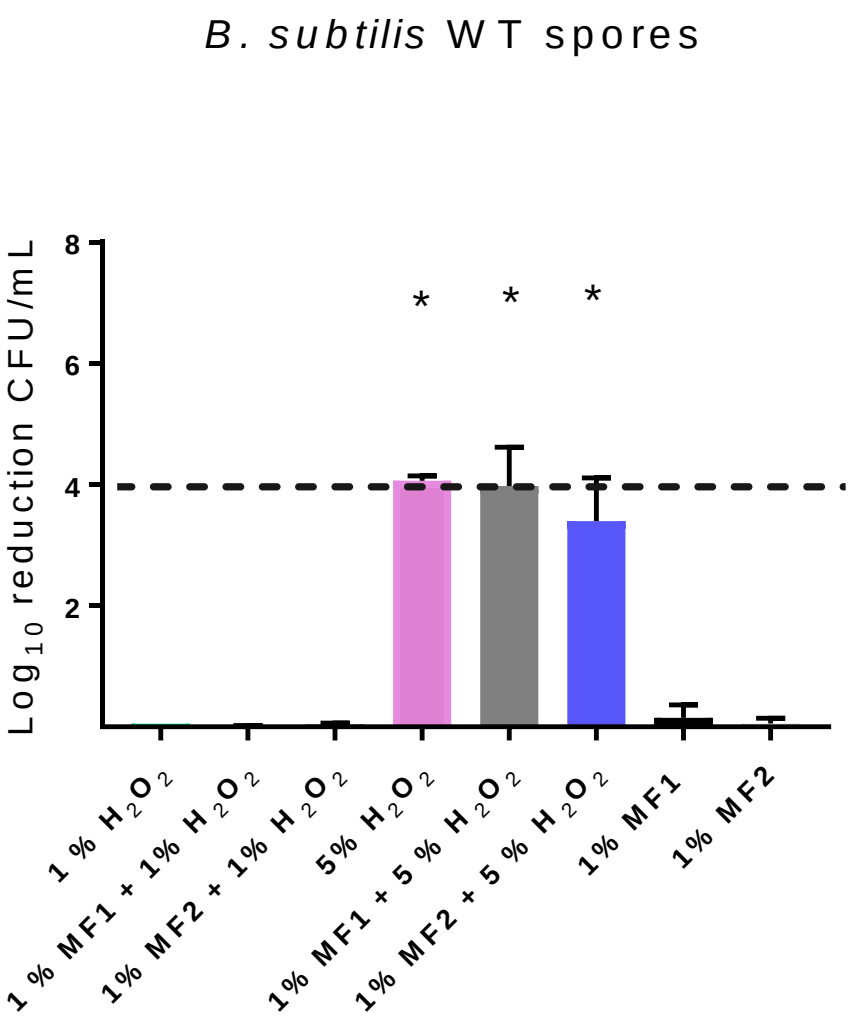
Log_{10} reductions are the means of three biological repeats and two technical replicates (n=3) *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C) and *E. hirae* (D). Green bars show 5 min contact time and red bars 0.5 min. Dotted lines represent the required log reduction for the standard protocols BS EN 1267:2009 (5 Log10). (*) represent a significant difference of $p < 0.001$.



3.3.3 Determination of the sporicidal activity of MF1 and MF2 following addition of H_2O_2

H_2O_2 was combined with MF1 and MF2 to determine if this would result in the reduction of *B. subtilis* spore viability. Shorter contact times were previously tested but no reduction in spores was observed so an increased contact time to 60 min was applied. 1 % (w/v) MF1 and MF2 alone did not possess any sporicidal activity (Figure 3.4.8). 1 % (v/v) H_2O_2 alone and in combination with MF1 and MF2 did not produce a significant reduction in spore viability ($p > 0.999$; one-way ANOVA). The concentration of H_2O_2 was increased to 5 % (v/v) which when tested individually gave a significant reduction of spore CFU/ mL ($p < 0.001$; one-way ANOVA). The addition of MF1 and MF2 did not produce significantly different reductions in spore viability compared to 5 % (v/v) H_2O_2 alone ($p > 0.999$; one-way ANOVA and $p = 0.5884$; one-way ANOVA, respectively) but gave significant reductions in spore titre compared to the surfactants alone ($p < 0.001$; one-way ANOVA) .

Figure 3.7 Efficacy of MF1 and MF2 with and without H_2O_2 against *B. subtilis* spores. Spores were tested at 1 % (w/v) MF1 and 1 % (w/v) MF2 with or without H_2O_2 at 1 % (v/v) or 5 % (v/v) for a contact time of 60 min. (*) indicates a significant increase in Log_{10} reductions ($p < 0.001$) (n=3).



3.4 Discussion

3.4.1 Factors affecting activity of formulations

The impact of two ionic liquids in formulation, concentration, contact time, organic soiling and microorganism were assessed on the microbicidal activity of two Ionic liquids MF1 and MF2 in comparison to two commonly used cationic biocides BZC and CHX.

3.4.2 Differences between MF1 and MF2

Both imidazolium salts showed potent antimicrobial activity against vegetative bacteria and yeast. There were no significant differences in efficacy following the suspension test between the formulations. This would suggest the antimicrobial activity is not due to the differing anions associated with the salts and is most likely a result of the cationic head group and the alkyl chain.

3.4.3 Concentration

The concentration of a disinfectant is an important factor for consideration for use in healthcare. If sold as concentrated product the user must be aware of any potential loss in antimicrobial potency that may occur as a result of dilution (Russell and Chopra, 1996). Most standard efficacy tests related to healthcare stipulate that a 4 or 5 Log_{10} reduction (depending on the test microorganism) must be achieved to make a product claims of efficacy (British Standards Institute, 2009; British Standards Institute, 2013).

Therefore, in this study the concentration of MF1 and MF2 were assessed by diluting the formulations 10-fold and testing against microorganisms for an allocated contact time. Overall the activity of MF1 and MF2 remained above the 5 Log_{10} reduction required for against Gram-negative and Gram-positive bacteria (BS EN 121276:2009) and the 4 Log_{10} reduction for yeast (BS EN61324:2013). In contrast, the dilution of BZC from 1 % (w/v) to 0.1 % (w/v) resulted in a loss in activity against *P. aeruginosa.* The tolerance of *P. aeruginosa* to BZC has previously been attributed to the inability of the compound to penetrate the outer-membrane and target the cytoplasmic membrane (Gilbert & Moore, 2005). Other argue that the decrease susceptibility of QACs is largely due to efflux pumps that can transport biocides out of the cell. (Braoudaki *et al*., 2004). It is possible that either of these reasons, or both are why BZC is not as effective against Gram-negatives.

Upon dilution CHX also lost activity to Gram-positives *S. aureus* and *E. Hirae*. It is unclear why this reduction in activity was observed against Gram-positives but reported reductions in susceptibly have been linked to the expression of efflux pumps (Horner *et al*., 2012).

Against spores, the highest concentration of MF1 and MF2 did not reduce the CFU/ mL. Possible explanations as why the ionic liquids could not reduce spore numbers are: firstly, spores have a physical barrier of a spore coat and second, the target of cationic biocides are lipid membranes (Maillard, 2002; Leggett *et al*., 2016a). Therefore, it unsurprising that at the highest concentration MF1 and MF2 were not sporicidal.

3.4.4 Contact times

The inactivation of microorganisms is dependent on the time that they are associated with biocides. It is acknowledged that shorter times may not always lead to suitable disinfection and so it is imperative that they are tested at contact times reflecting the *in-situ* use.

Here, two contact times (0.5 and 5 min) were chosen for the assessment of MF1 and MF2. There was no loss in antimicrobial activity for MF1 and MF2 when contact times were shortened to 0.5 min. Again, BZC and CHX lost antimicrobial activity at 0.5 min to *P. aeruginosa* and Gram-positives, respectively. This was especially true in the higher organic soiling conditions, suggesting BZC and CHX would need longer disinfection times in the presence of organic soiling. The recommended disinfectants should have a 1 minute 'wet' contact time against vegetative bacteria (Rutala & Weber, 2006). Product labels with higher contact times will most likely require re-application, as the drying time for water-based disinfectants is around 2 minutes (Rutala & Weber, 2013).

3.4.5 Soiling

The data presented indicate that the antimicrobial activities of MF1 and MF2 were not inhibited by the presence of organic soiling, However, for BZC and CHX there was a noticeable difference when erythrocytes were added to the BSA soiling mix. BZC was less effective against Gram-negatives and CHX against Gram-positives.

It is not surprising to see that soiling affected the activity of BZC against *Pseudomonas*, since reduced susceptibility to BZC has already been reported at lower organic loads of 0.3% BSA (Araujo *et al*., 2013). One suggestion for the lowered efficacy of QACs such as BZC in the presence of organic soiling is due to negatively charged protein ions binding to positively charged QACs, decreasing their availability, and subsequent ability to reduce

bacteria (Simoes *et al*., 2006). This was demonstrated by Jono and colleagues by increasing levels of BSA with BZC and analysing bound and unbound BZC by HPLC. They found a positive correlation to the amount of BSA with the levels of bound BZC which they then shown to decreased levels of antimicrobial activity (Jono *et al*., 1986). Although the mechanisms in which organic soiling may inhibit the activity of CHX have not been reported, it could be assumed that in a similar manner to BZC the positive charge may be sequestered by negatively charged proteins.

3.4.6 Combinations of ionic liquids with H_2O_2

There are multiple benefits to adding H_2O_2 to the formulations MF1 and MF2. Firstly, H_2O_2 breaks down into non-toxic products (water and oxygen) and so it is possible to use lower concentrations of other biocides that may not be as 'green'. Second, it can act synergistically with other agents to increase the activity of other biocides, this was seen by Leggett *et al* when H_2O_2 was used with peracetic acid (PAA) against highly resistant *B. subtilis* spores (Leggett *et al.,* 2016a). It was observed that PAA had increased antimicrobial activity as H_2O_2 damages the spore coat. The third reason H_2O_2 would be beneficial in formulation with ionic liquids is from a commercial viewpoint. Under the BPR, active substances are limited and need to be declared for product sales (European Union Biocides Regulation, 2012). Therefore, combining ionic liquids with H_2O_2, a recognised active substance would overcome this problem. Finally, producing formulations containing other excipients could circumvent the development of antimicrobial resistance compared to the active in aqueous solution only (Cowley *et al*., 2015).

In this chapter the combination of ionic liquids at low concentrations (0.01 % (w/v) was assessed in combination with H_2O_2. The low concentration of MF1 and MF2 was chosen on the basis that higher concentrations 1 % (w/v) - 0.1 % (w/v) were already active against bacteria and yeast.

When combined with the ionic formulations it is not known what affect H_2O_2 will have on the antimicrobial activity of MF1/ MF2 or *vice versa*. Therefore, after the addition of H_2O_2, the formulations were tested over a period of 4 hours and changes in activity were compared (Figure 3.5). For *E. coli*, the addition of H_2O_2 to MF1 did result in a rise of antimicrobial activity, but this was not significantly higher to H_2O_2 alone. After 30 minutes of adding H_2O_2 to MF1, there was increase in antimicrobial activity compared to individual effects of H_2O_2 or MF1. It is uncertain why when left for 30 minutes MF1 and H_2O_2 would generate greater activity, however, as the counter-ion of MF1 is sodium carbonate it could be that MF1 is

reacting with H_2O_2 to produce an antimicrobial effect, but without knowing what chemical species is responsible for the increase the reasons remain unclear.

The activity of the combined biocides appeared to be an additive effect which in contrast to a synergistic effect, is the overall sum of activity of the individual biocides (Lambert *et al*., 2003). Synergy on the other hand is when the activity of the biocides is greater than the sum of biocides alone. The additive effect was observed with *E. coli* when H_2O_2 was combined with MF2 at a 0.5 minute contact time (Figure 3.6A), the individual Log_{10} reductions of 1 % (v/v) H_2O_2 and 0.01 % (w/v) were 5.20 ± 0.67 and 2.51 ± 0.50, which when combined reached the upper limit of the test and no *E. coli* was recovered. This was also the case when MF1 and H_2O_2 were used against *P. aeruginosa* for a 5 min contact time (Figure 3.6B).

For the Gram-positive bacteria, the addition of H_2O_2 did not increase activity to the required 5 Log_{10} reduction required by the BS EN 1276:2009 standard test. The relative tolerance observed by *S. aureus* could be explained by the presence of intracellular catalases that can degrade H_2O_2 (McDonnell & Russel, 1999). Interestingly, others have shown that when tested against *S. aureus*, the activity of 0.5 % H_2O_2 was enhanced by combining with BZC and ethanol, thought to be due to BZC facilitating H_2O_2 entry into the cell (Ríos-Castillo *et a*l., 2017). The combination of an oxidising biocide with one that targets membranes could account for the increase in activity by 1.9 Log_{10} against *S. aureus* when MF1 and H_2O_2 were tested collectively.

The lowest antimicrobial activity observed when MF1 and MF2 were combined with H_2O_2 was against *E. hirae.* It is unclear why H_2O_2 shown negligible antimicrobial effect towards *E. hirae*, a catalase negative bacterium. Whilst others have demonstrated activity against *Enterococcus* when using H_2O_2, studies cannot be compared as different concentrations/ times and test methods are often used. Furthermore, most literature demonstrating efficacy of H_2O_2 against the strain *E. faecalis* as the test organism.

The formulations MF1 and MF2 were not sporicidal against *B. subtilis* spores. The addition of 5 % (v/v) H_2O_2 to 1 % (w/v) MF1 did lower the spore concentration to the required 4 Log_{10} reduction in 60 min as required by BS EN 17126:2018. However, as there was no significant difference between the combination of formulations and H_2O_2 individually, it can be argued that any sporicidal activity is due to H_2O_2 only. There are relatively few biocides that show sporicidal activity, those that do are usually oxidising or alkylating agents (Leggett *et al*., 2016b)

3.4.7 Use of standard tests for healthcare products

Standardised efficacy tests provide an insight into the microbicidal activity of a biocide and allow manufacturers to make a product claims on labels. One advantage of using standardised tests is that the multiple factors that influence activity are taken into account. A second benefit is that the use of these tests reduces variability of results between laboratories.

Phase 2 step 1 suspension tests used in this chapter, although invaluable, are carried out on vegetative bacteria or bacterial spores in suspension. However, in healthcare settings, realistically bacteria are dried on to a surface or are part of a biofilm. Currently, within the EU there are no standardised tests against biofilms. In the USA, two approved biofilm standard tests consist of i) a minimal biofilm eradication concentration (MBEC) (ASTM E2799, 2012) providing the lowest concentration needed to eradicate a biofilm, and ii) a method using a reactor that grows biofilms in batch phases upon coupons, which are then used as a carrier (ASTM E2562, 2012). However, both models grow biofilms in nutrient rich medium up until treatment that do not reflect the growth of biofilm in a healthcare surface. In fact, multiple groups are developing tests that reflect the wet and dry phases that a biofilm would experience of a surface in healthcare (Almatroudi *et al*., 2016; Ledwoch *et al*., 2019).

3.5 Conclusions

- MF1 and MF2 show bactericidal activity against *E. coli*, *P. aeruginosa*, *S. aureus*, *E. hirae*, *A. baumannii* and *C. albicans* producing a 5 Log_{10} (bacteria) and 4 Log_{10} (yeast) reduction following BS EN 1276:2009 and BS EN BS EN61324:2013
- MF1 and MF2 out-performed BZC and CHX at high organic soiling and with shorter contact times.
- The most limiting factor for MF1 and MF2 was dilution; bactericidal activity varied at 0.01 % (w/v).
- When combined with H_2O_2 the activity of 0.01 % (w/v) was not enhanced for all microbes tested.

- MF1 and MF2 were not sporicidal but when combined with 5 % (v/v) H_2O_2, were able to decrease spore viability to the required 4 Log_{10} using the BS EN 17126:2018 standard test.

4 Mechanism of Action of Ionic Liquids

4.1 Introduction and aims

An important consideration when developing a biocidal product is to understand the mechanisms in which the antimicrobial works against the target microbe. When cellular target(s) are identified the limitations of the active compound can be appreciated.

Additionally, understanding the MOA is key to assessing if biocide usage can lead to resistance to the biocide itself, other biocides with a similar MOA or to antibiotics. Commonly used biocides found in healthcare, veterinary care and in household products have been assessed in many studies as resistance has been associated with repeated exposure to low concentrations (Adair *et al*., 1971; Winder *et al*., 2000; Walsh *et al*., 2001; Romão *et al*., 2005; Martin *et al*., 2008) For example, exposure of BZC at concentrations below the MIC have resulted in increased tolerance to the biocide and to ampicillin, cefotaxime, and ceftazidime (Kampf, 2018).

4.1.1 Methods to assess MOA

The myriad of chemical groups that exhibit antimicrobial activity reflects the many mechanisms of action displayed by biocides. Table 4.1 shows some examples of biocides, their cellular target(s) and the MOA.

The cell structure of a microorganism presents the first barrier to an antimicrobial and is often a target itself (Denyer & Maillard, 2002; Maillard, 2002). The outermost layer of bacteria carries a net negative charge, formed from lipoteichoic acids and polysaccharides associated with Gram-positive cells and the lipopolysaccharides of Gram-negative bacteria (Figure 4.1). The cytoplasmic membrane also holds a negative charge, formed from a mix of phospholipids that develop a biolayer. Consequently, cationic antimicrobials tend to target negatively charged membranes, leading to destabilisation of the cell (Chawner & Gilbert, 1989). Once cationic biocides perturb the membrane, one of the first events to occur is efflux of potassium ions (K^+) out of the cell (Lambert & Hammond, 1973). Following this, other cellular constituents, usually those with higher molecular weight such as ATP and then DNA flow out of the cell indicating gross-membrane damage, which is followed by cell lysis.

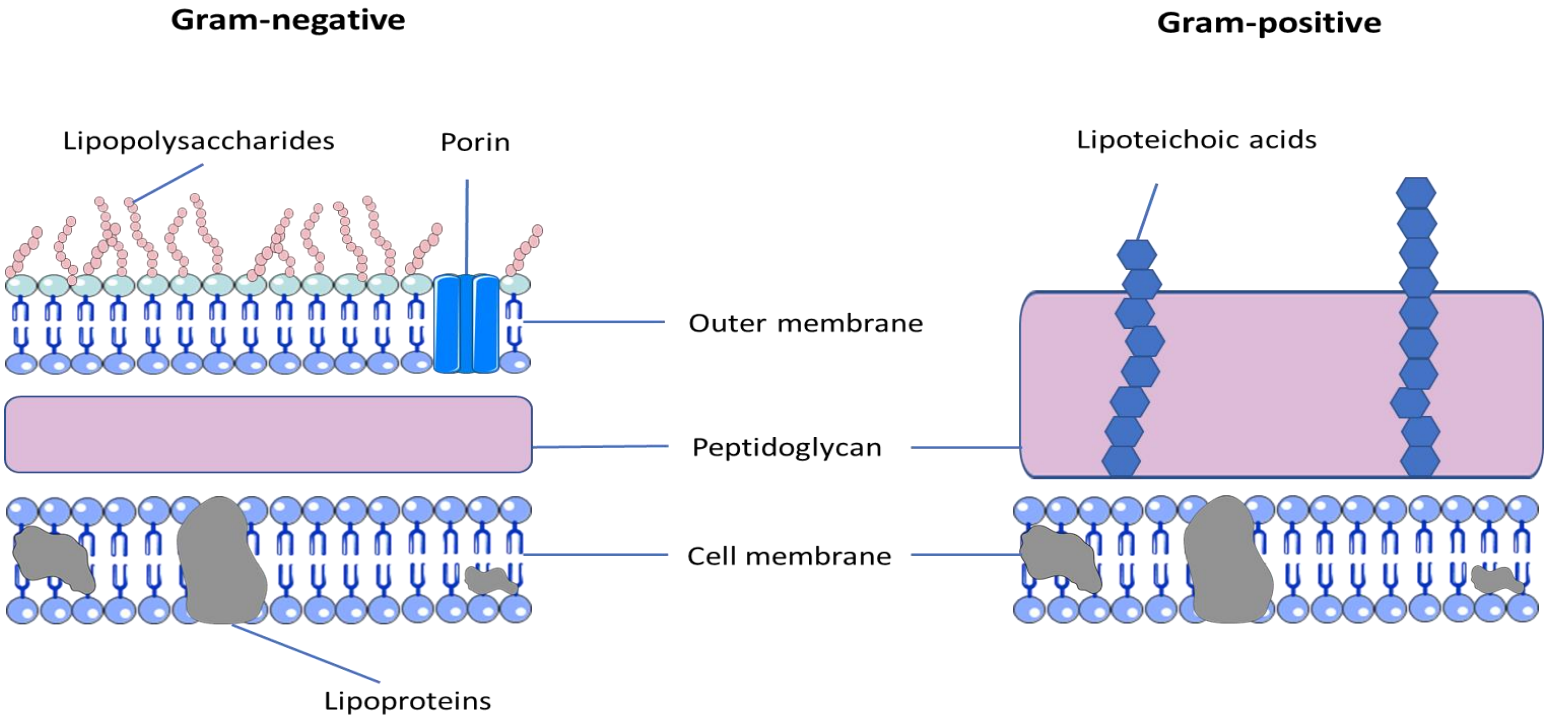
Table 4.1 Mechanism of action of common biocides.

Listed are the components of bacterial cells that are commonly a target for biocides and MOA that follows. (adapted from Maillard, 2002).

Cellular target	MOA	Biocides
Cell wall	Cross-linking proteins	Glutaraldehyde, *ortho*-phthalaldehyde, formaldehyde
Gram-negative outer membrane	Loss in membrane integrity	Phenolics, QACs, CHX
Cytoplasmic membrane	Loss of membrane integrity Membrane potential ATP synthesis	Alcohols, CHX, phenolics, anilides, Chlorine-releasing agents, acridines QACs, phenolics, acridines CHX, Copper (II) salts
Cytoplasmic components	Coagulation Interaction with Biomolecules (DNA, RNA)	Glutaraldehyde, CHX, QACs, phenolics, heavy metals Formaldehyde, glutaraldehyde, Acridine, H_2O_2, PAA
Thiol groups	Oxidation	Iodine, chlorine-releasing agents, heavy metals
Autocidal activity	Free radicals	H_2O_2, PAA

Figure 4.1 Cell wall structure of Gram-positive and Gram-negative bacteria.

Both Gram-positive and Gram-negatives have a cell membrane of phospholipids interspersed with lipoproteins. Gram-positive bacteria have a thick peptidoglycan layer which anchors anionic polymers such as teichoic acids. Gram-negatives have a thinner peptidoglycan layer and an outer membrane composed of phospholipids, lipopolysaccharides (LPS) and porins. Image adapted from Slavin *et al* 2017 drawn with Servier medical art.



As a result, a common method used to demonstrate the cell membrane is a target of biocide action is to detect and leakage of K^+ after treatment (Lambert & Hammond, 1973). Inductively coupled plasma – mass spectrometry (ICP-MS) is an analytical technique used for the detection of metals, by passing a sample through ICP, atoms become ionised and are detected in a mass spectrometer by their mass to charge ratio and so it is a useful tool for K^+ detection.

Another approach to assess membrane integrity, rather the detecting cytosolic components is to quantify the amount of nucleic dyes that can enter the cell. BacLight assay is a two-component system consisting of nucleic acid staining dyes SYTO 9 and Propidium iodide (PI). Whilst SYTO 9 can enter all cells, PI will only penetrate cells with damaged membranes (Warning, 1965; Arndt-Jovin & Jovin,1989). When used in conjugation, PI can displace SYTO 9 due to its higher binding affinity to DNA (Stocks, 2004). Consequently, as PI fluorescence intensifies, emissions of SYTO 9 decrease, so changes in fluorescence can be used as a marker for membrane integrity.

Often the in-use concentration of a biocide results in large amounts of damage to a bacterial cell that can be seen by microscopy techniques, a common and convenient method for investigating the MOA.

Scanning electron microscopy (SEM) is widely used as a visual aid for assessing any damage that might occur to the structure of the cell. Although there is no defined criteria to assess the damage to a cell, some authors have reported membrane blebbing (intracellular components leaking out of the cell), development of pores and cell disintegration (Codling *et al*., 2005; De Souza *et al*., 2010; Armas *et a*l., 2019)

Atomic force microscopy (AFM) is a powerful tool that can be utilised to investigate the morphological (size and shape) and topographical (surface) features of bacteria. Based on the mechanical scanning of surfaces, AFM can provide high resolution images and quantitative data regarding topographical features. It is a method commonly used in the field of material science but there has been a rise in its application in microbiology. Unlike SEM, it does not require fixation or coating of bacteria, providing a nanometre resolution.

Surface characterisation using AFM is achieved by a scanning probe (tip) that moves along the sample. The tip is attached to a cantileaver that bends as the tip moves over the surface. Directed at the cantileaver is a laser that is deflected to a photodiode, which is then used to record the changes in force between the sample and the tip.

Due to the cationic nature of MF1 and MF2 it is hypothesised that without the addition of H_2O_2, Ionic liquids will interact primarily with the cell membrane. As such, much of this chapter relates to the study of membrane damage.

MF1 and MF2 were not shown to be sporicidal in Chapter 3 without the addition of H_2O_2. The results showed that the sporicidal activity of the complete formulations resulted solely from H_2O_2. There are several mechanisms that spores have acquired to resist stress induced by biocides (Leggett *et al*., 2012). One such structure is the spore coat, a protein rich layer formed from soluble and insoluble proteins. One protein known to be important to the development and structure of the spore coat is CotE and spores lacking CotE have been shown to be more susceptible to oxidising agents (Leggett *et al*., 2012). SASPs are abundant proteins that bind spore DNA, once the spore germinates into a vegetative state the SASPs degrade and become a source of amino acid during outgrowth (Setlow, 1988; Setlow, *et al*., 1992). α/β type SASPs serve as the major proteins that protect DNA and, in their absence, oxidative biocides such as H_2O_2 can produce highly reactive radicals that are destructive to the DNA stand (Setlow 2006). One approach to understanding the lack of sporicidal activity of a formulation is the use of mutants (Legget *et al*., 2016). The use of spore mutants that are lacking an integral gene for the development spore coat (CotE) and another mutant without approximately 80 % of small acid-soluble proteins (SASPs) known to protect DNA can be used to assess if upon treatment spores become more susceptible.

4.1.2 Aims and objectives

The overall aim of this chapter was to understand the mechanism of action of the formulations MF1 and MF2. It was hypothesised that the ionic liquids because of their chemical structure, would affect bacterial membranes. With this mind MOA was investigated by using a number of different approaches compatible with indicating membrane damage including:

- Detection and quantification of K^+ a marker of membrane damage, using ICP-MS.
- Measure the fluorescence ratio of DNA binding dyes (PI and SYTO 9) after treatment to determine changes in membrane integrity.
- SEM to visualise absolute cell damage including release of cytoplasmic contents and collapsing of cell.
- AFM to quantify any fine structural damage to the cell such as surfaces roughness or development of pores.

The reason for the lack of sporicidal activity of the formulations without the addition of H_2O_2 was investigated using several spore mutants deficient in some of their structure

- Suspension tests of *B. subtilis* spore mutants CotE$^-$ and $\alpha^-\beta^-$ SASPs with MF1/MF2 with or without H_2O_2 to identify if any intracellular biomolecules are a possible target.

4.2 Methods

4.2.1 Potassium leakage

S. aureus ATCC 6538 and *E. coli* ATCC 10536 were grown overnight for 18 hours in 600 mL TSB at 37 °C ± 1 °C under agitation at 125 rpm. The bacterial cells were pelleted at 5500 x g for 10 min and the growth medium was removed. The cells were washed once more before resuspending in ultrapure water to final suspension of 5 x 10^{10} CFU/mL.

One mL of the 5 x 10^{10} CFU/mL bacteria was mixed with MF1 and MF2 to final concentrations of 1 % (w/v) and 0.1 % (w/v). CHX was used at 1 % (v/v) and ethanol at 70 % (v/v). All solutions were left in contact for 5 min and then passed through a 0.2 µm filter (Sartorius™ Minisart™, Fisher scientific, Loughborough, UK). All solutions were analysed for K^+ (mg/mL) by ICP-MS (Agilent 7900; Agilent Technologies LDA UK, Cheshire, UK) that had been calibrated using K^+ standards (Agilent Technologies LDA UK, Cheshire, UK). All tests were performed in triplicate (n=3) with three replicates for each. Formulations without bacteria were analysed and subtracted from the final readings. A p value of < 0.05 was considered significant (one-way ANOVA; Graphpad prism v.7)

4.2.2 BacLight™

4.2.2.1 Standard curve

S. aureus ATCC 6538 and *E. coli* ATCC 10536 were grown overnight in 30 mL TSB for 18 hours at 37 °C ± 1 °C under agitation at 125 rpm. The bacterial suspensions were pelleted by centrifugation at 5500 x g and resuspended in 0.85 % (w/v) NaCl solution. This wash step was repeated once more before resuspending the pellet in 2 mL 0.85 % (w/v) NaCl solution. 500 µL of concentrated bacteria (1x10^{10} CFU/mL) was added to 10 mL 70 % isopropanol (dead population) or 0.85 % (w/v) NaCl (live population) for 1 hr. Then bacterial cells were centrifuged at 10,000 x g for 10 min and the supernatant was removed. The cells were washed in 20 mL 0.85 % (w/v) NaCl solution and spun down at 10,000 x g once more.

S. aureus was adjusted to 0.60 OD_{670} and *E. coli* to 0.03 OD_{670}, and the live and dead populations were mixed to produce a series of live cells (10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % and 100 %). The bacterial cells were mixed with dye solution (SYTO 9 at 0.1 mM and PI at 0.6 mM) (LIVE/DEAD™ BacLight™, Invitrogen™, Fisher scientific, Loughborough, UK) and incubated in the dark at room temperature for 15 min before

fluorescent emissions were taken at 530 nm (green emission) and 630 nm (red emission) after excitation at 485 nm (Tecan infinite 200 Pro, Tecan UK Ltd, Reading, UK). Standard curves were produced (Figure 4.4A and Figure 4.5A) for live cells vs the ratio of green to red fluorescence (emission at 530nm /emission at 630 nm).

4.2.2.2 Treatments for Baclight™

S. aureus ATCC 6538 and *E. coli* ATCC 10536 were prepared as mentioned in section 4.2.2.1. In addition to 70 % (v/v) ethanol and 0.5 % (w/v) NaCl solution, bacteria were treated with 1 % (v/v) CHX, 1 % (w/v) MF1, 0.1 % (w/v) MF1, 1 % (w/v) MF2, 0.1 % (w/v) MF2 for a contact time of 5 minutes. Bacteria free controls were run in parallel and then subtracted from the treatment results. To determine the percentage of cells that had intact membranes the green emission was divided by red emission, which was then used in the line of best fit equation displayed on Figure 4.4A and Figure 4.5A. Tests were carried out in triplicate (n=3) and a p value of < 0.05 was considered significant (*) and $p < 0.001$ highly significant (**) (one-way ANOVA; Graphpad prism v.7)

4.2.3 Scanning electron microscopy

Following the suspension test procedure outlined in Chapter 3, *S. aureus* ATCC 6538 was treated with MF1 and MF2 at 1 % (w/v) and 0.1 % (w/v). Once the bacteria had been neutralised by membrane filtration, the bacterial cells were fixed by transferring the membrane (0.2 micron polycarbonate, to 2 % (v/v) glutaraldehyde in 0.1 M cacodylate buffer for 2 hours. The membrane was washed in 0.1 M cacodylate buffer before the cells were dehydrated with an ascending series of ethanol concentrations for 3 min each (10 % (v/v), 30 % (v/v), 50 % (v/v), 70 % (v/v), 90 % (v/v) and 100 % (v/v)). Hexamethyldisilazane (HMDS) was applied to the membrane to ensure critical point drying and left for 5 min. Finally, the membranes were splutter coated with 10 nm Au/Pd (80:20) (Quorum 150TES) and visualised with a field emission gun scanning electron microscope (FEG-SEM) operated at 5-25 kV (Tescan MAIA3; TESCAN-UK Ltd, Cambridge, UK).

4.2.4 Atomic force microscopy

4.2.4.1 Mica surfaces

Mica surfaces (Electron Microscopy Sciences, Ltd; Hatfield, UK) were prepared by cleaving the surface (removal of top layer) before the addition of 50 µL 0.01% poly-L-lysine solution (Sigma-Aldrich, Gillingham, UK. After 20 min lysine was removed and 50 µL of 20%

glutaraldehyde was applied to the mica for a further 20 min. The mica surface was then washed twice with sterile distilled water, covered, and stored at room temperature before use.

4.2.4.2 Preparation of bacteria

For AFM analysis, *S. epidermidis* was used in place of *S. aureus* due to restrictions on class II organisms in the AFM laboratory.

S. epidermidis ATCC 14990 was grown in 10 mL TSB for 18 hours at 37 °C, centrifuged at 5500 x g and washed twice in PBS. The cells were adjusted to 1 x 10^8 CFU/mL and 50 µL drops were placed onto chemically modified mica disks (4.2.4.1) then left to adhere to the surface for 30 min. Then 50 µL of 5 % (w/v), 1 % (w/v) and 0.1 % (w/v) MF1 and MF2 were applied for contact times of 5, 30, 60 and 120 min. The formulations were slowly removed, and the surfaces were washed twice with PBS to remove any remaining formulation.

4.2.4.3 Image acquirement and surface roughness measurements

Surface roughness measurements were performed in an AFM (Nanotec Electrónica, Madrid, Spain) by amplitude modulation mode (tapping mode). The microcantilever was silicon nitride (Olympus; 100 µm L x 20 µm W) with a tip radius of 20 nm, a spring constant of 0.39 N/m and a resonance frequency of 70kHz. For each treatment, 2 µm x 2 µm scans of six different bacteria were analysed and treatments were carried out in triplicate. Images were acquired using WSxM software. The root mean square roughness (*Rq*), the maximum profile peak height (*Rp*) and the maximum profile valley depth (*Rv*) were determined using Gwyddion software after second-order flattening (Antonio *et al*., 2012).

4.2.5 Spore mutants

Spores mutants were grown as described in Chapter 2 section 2.2.6. Suspension tests were performed as outlined in Chapter 3 section 3.2.1.

4.3 Results

4.3.1 Potassium leakage

Leakage of low molecular weight compounds from bacteria treated with an antimicrobial agent is considered the first indication of membrane damage (Lambert & Hammond, 1973). In this study, K^+ released from bacterial cells increased upon treatment of MF1 and MF2.

For *S. aureus* (Figure 4.2) and *E. coli* (Figure 4.3), all treatments released a significantly higher amount of K^+ from the cells than the untreated control (one-way ANOVA; $p < 0.05$). No differences were observed between treatments (one-way ANOVA; $p > 0.05$).

Figure 4.2 K^+ release form *S. aureus*.

Data show K^+ (mg/L) from cells after a 5 min treatment. Results are of three biological replicates n=3 and error bars represent SD. * indicates a $p < 0.05$ (one-way ANOVA).

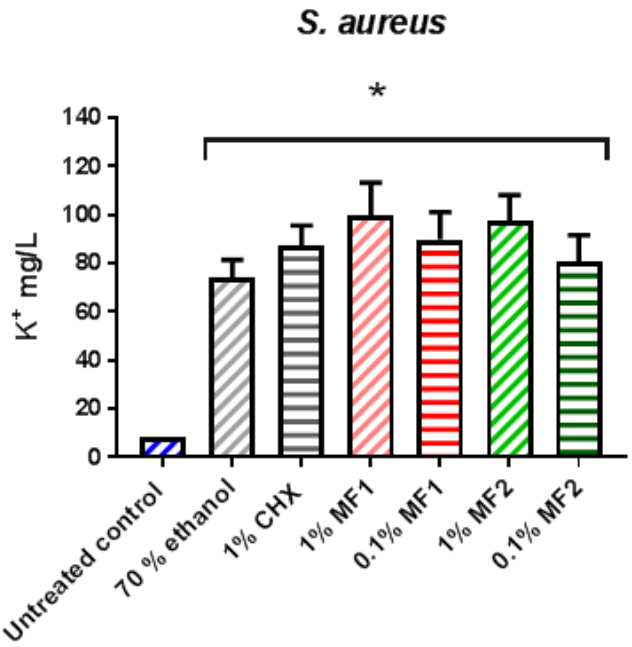
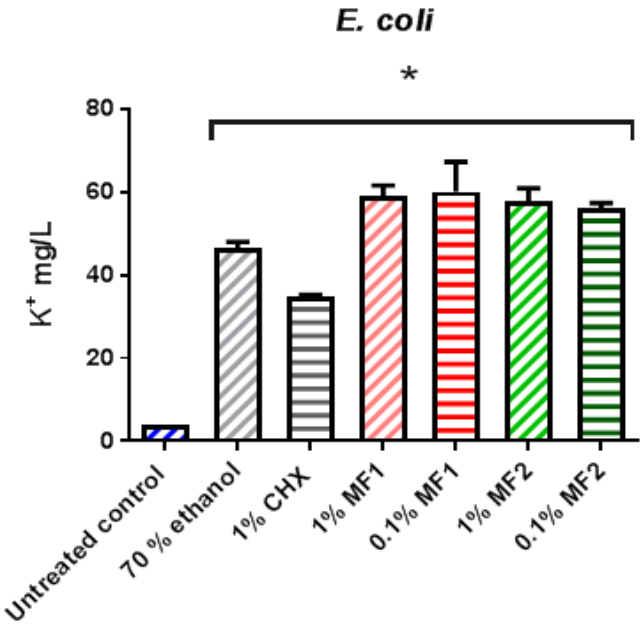


Figure 4.3 K^+ release from *E. coli.*

Data show K+ (mg/L) from cells after a 5 min treatment. Results are of three biological replicates n=3 and error bars represent SD. * indicates a $p < 0.05$ (one-way ANOVA).



4.3.2 BacLight™ assay

The fluorescent dyes SYTO 9 and PI were used in combination to determine if cell membranes of *S. aureus* and *E. coli* became damaged after treatment with MF1, MF2, 70 (v/v) % isopropanol and 1 % (v/v) CHX. For all the treatments tested, *S. aureus* (Figure 4.4B) had a significant (one-way ANOVA; $p < 0.001$) reduction in membrane integrity with ≥ 90 % of cells showing a lower ratio of green to red fluorescence. The results against *E. coli* followed a similar trend and all treatments lowered the % of cells with intact membranes (Figure 4.5B). Treatments with the highest impact on *E. coli* membranes were 1 % MF1 (w/v) and 1 % (w/v) MF2 with 14.87 ± 0.24 % and 16.30 ± 0.35 % intact bacterial cells remaining, respectively. The antimicrobial with the lowest effect on *E. coli* was 70 % (v/v) 65sopropanol, where only half of the cell population (53.20 ± 3.60 %) had an increase in the uptake of PI, but was still significantly lower than the untreated control (one-way ANOVA; $p < 0.05$).

Figure 4.4 Live dead staining with *S. aureus*.

(A.) Standard curve of Live/dead fluorescence *vs.* percentage of viable *S. aureus.* The equation for the slope of the curve and R^2 are displayed on the graph. (B.) The % of cells with intact membranes was determined using the slope equation in (A.) were x was the fluorescent measurement. All treatments were performed in triplicate (n=3) and error bars are of SD. ** indicates a $p < 0.001$ (one-way ANOVA).

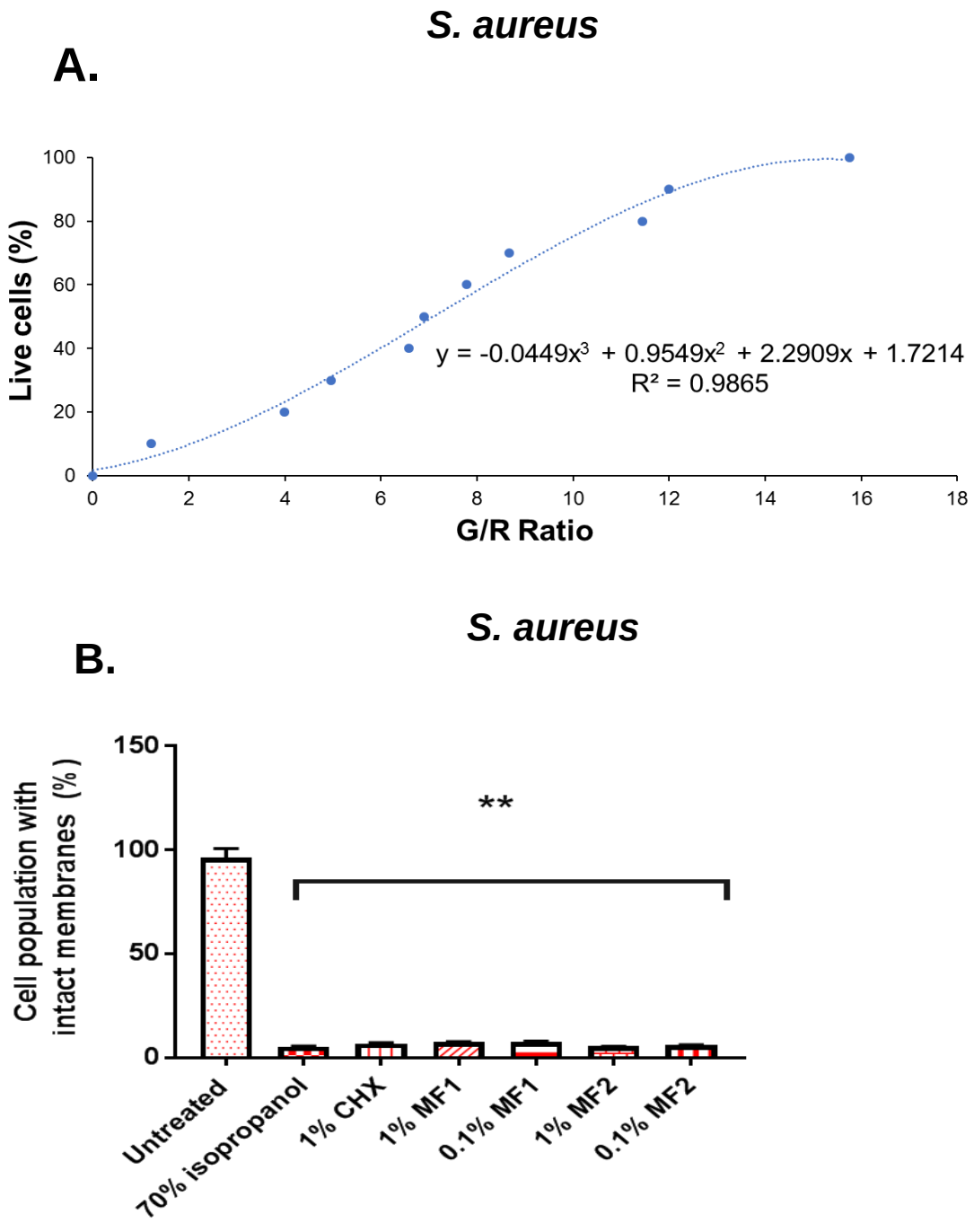
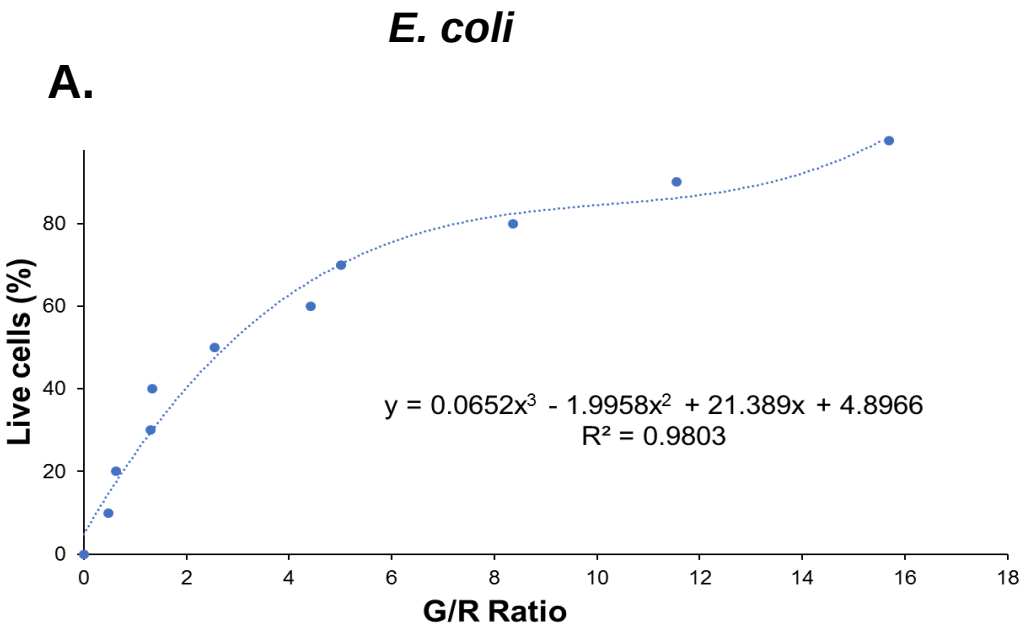
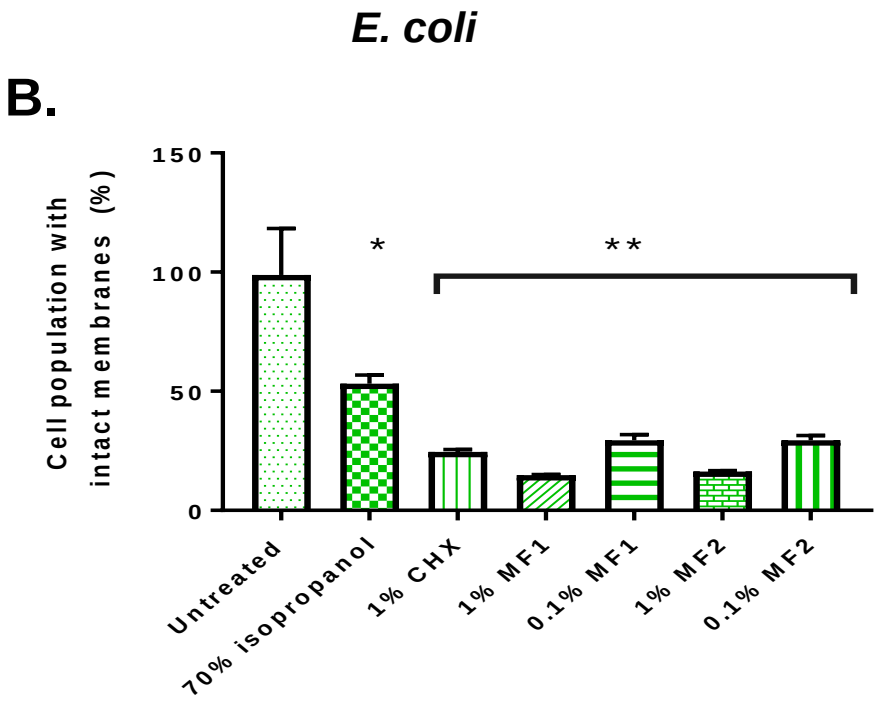


Figure 4.5 Live dead staining with *E. coli*.

(A.) Standard curve of Live/dead fluorescence *vs.* percentage of viable *E. coli.* The equation for the slope of the curve and R^2 are displayed on the graph. (B.) The % of cells with intact membranes was determined using the slope equation in (A.) were x was the fluorescent measurement. All treatments were performed in triplicate (n=3) and error bars are of SD. ** indicates $p < 0.001$ and * $p < 0.05$ (one-way ANOVA).





4.3.3 Scanning electron microscopy

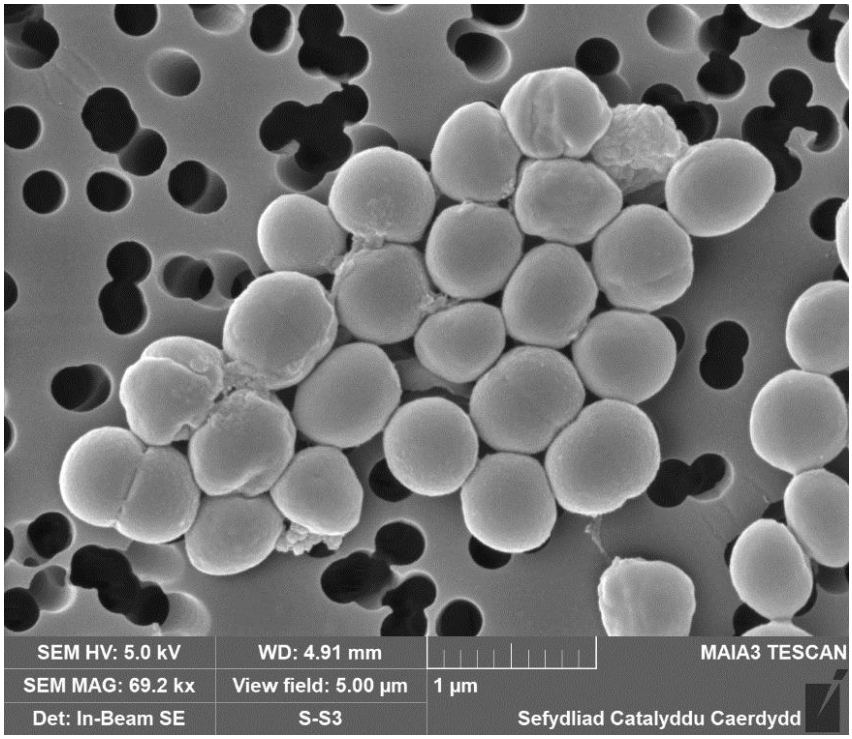
SEM images show increasing damage to cells and loss of spherical morphology with increasing concentration of both MF1 and MF2. Untreated cells (Figure 4.6) show a smooth cell surface, with no visible leaking of cytoplasm from the cell. The highest concentration of MF1 produced a loss of cell structure and cytoplasmic leaking (Figure 4.7A), notably, some cells still displayed a cocci form (Figure 4.7B) but edges of the cells appeared to be rougher. At a concentration 10-fold lower (0.1 % (w/v), cells were visible but had lost structural integrity (Figure 4.7C) and cellular contents were no longer kept within the cell (Figure 4.7D). At the lowest concentration of 0.01 % (w/v) cells remained spherical, clustered and no major changes to the cell occurred (Figure 4.7E & Figure 4.7F).

After treatment with 1 % (w/v) MF2 (Figure 4.8A & Figure 4.8B), remnants of the cells were clustered together, and there was no indication of intact cells. With 0.1 % (w/v) MF2, outlines of cell shape of *S. aureus* were observed but the cytoplasm was visible (Figure 4.8C) and cells appeared collapsed (Figure 4.8D). For 0.01 % (w/v) MF2 cells appear spherical and intact but the cell surface was rougher than untreated cells (Figure 4.8E & Figure 4.8F).

Figure 4.6 SEM of untreated *S. aureus.*

S. aureus at magnification 69.2 kx (A) and 60 kx.

A.



B.

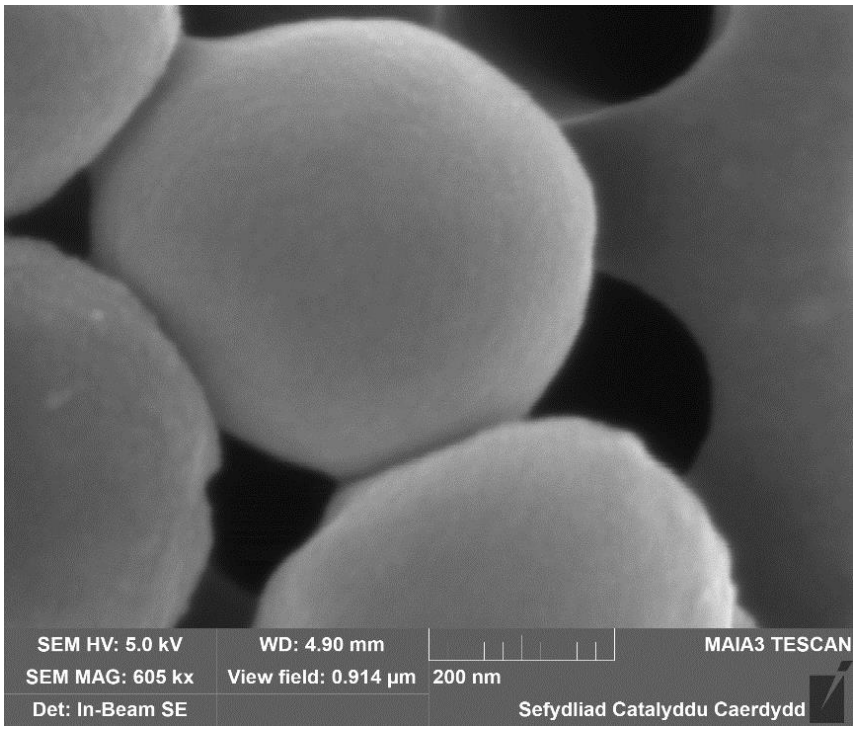
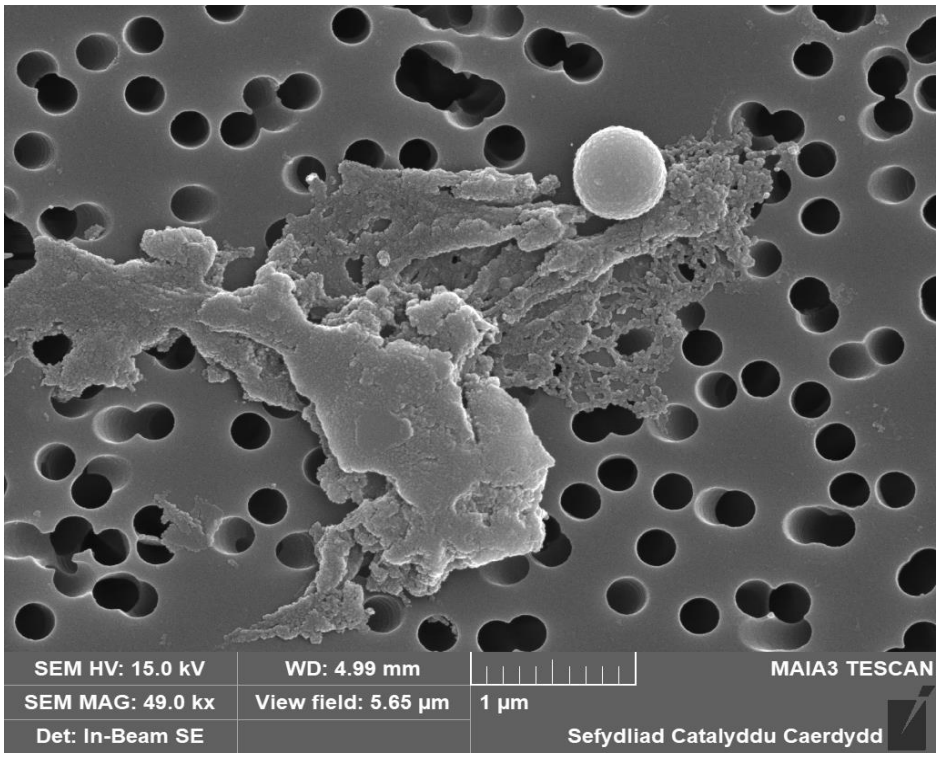


Figure 4.7 SEM of *S. aureus* after treatment with 1 % (w/v) MF1.

S. aureus at magnification 49.0 kx (A) and 185 kx.

A.



B.

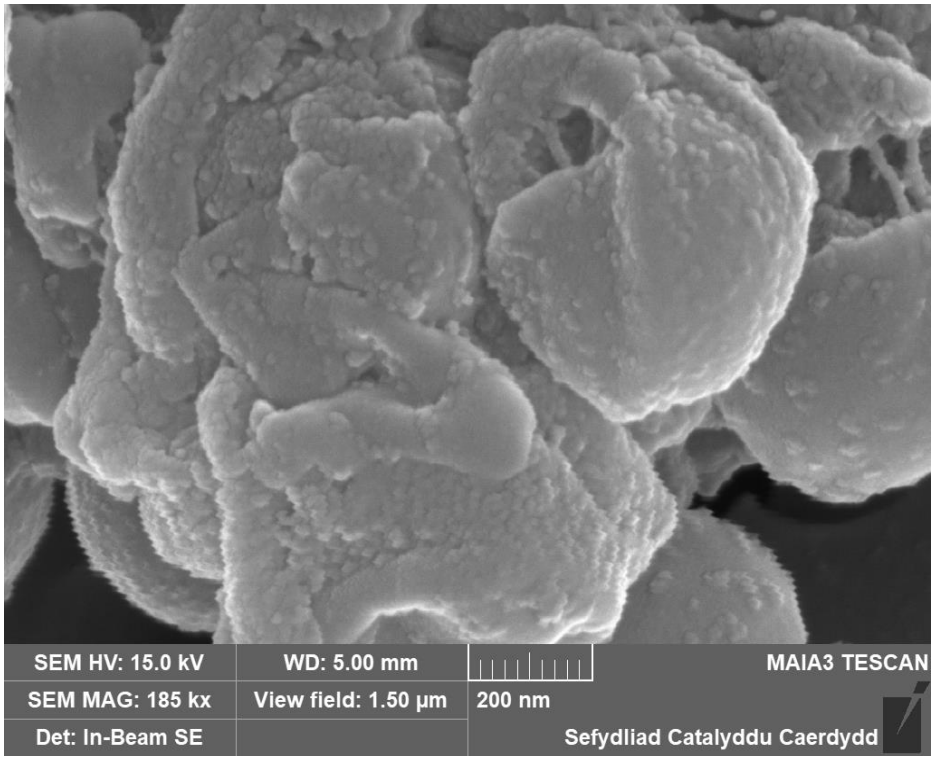
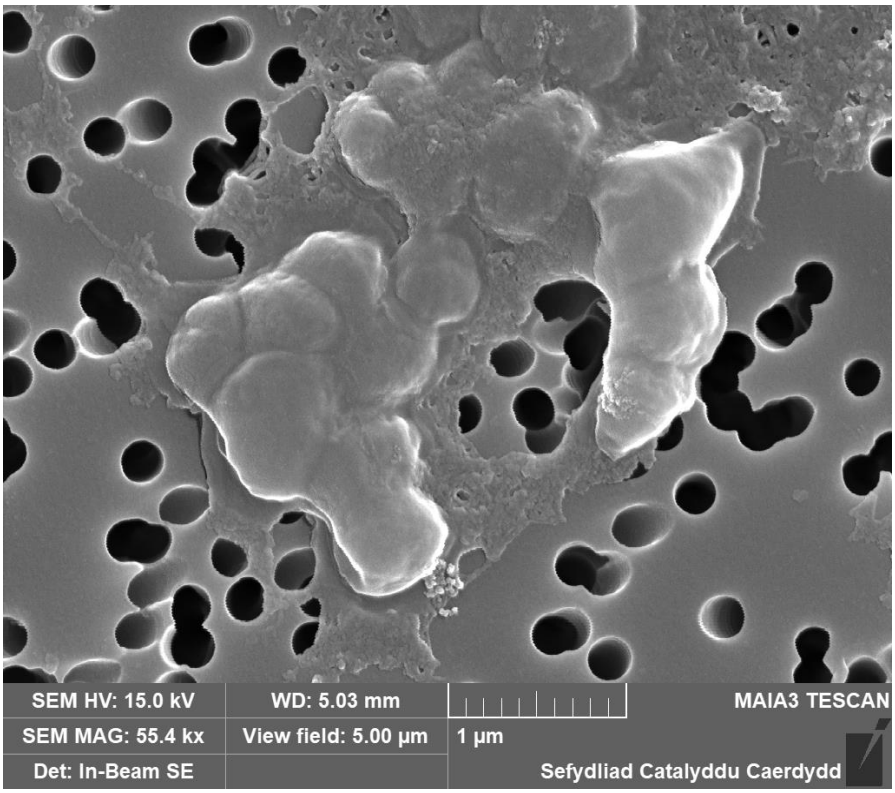


Figure 4.8 SEM of *S. aureus* after treatment with 0.1 % W/v) MF1.

S. aureus at magnification 55.4kx (A) and 92.3 kx (B).

A.



B.

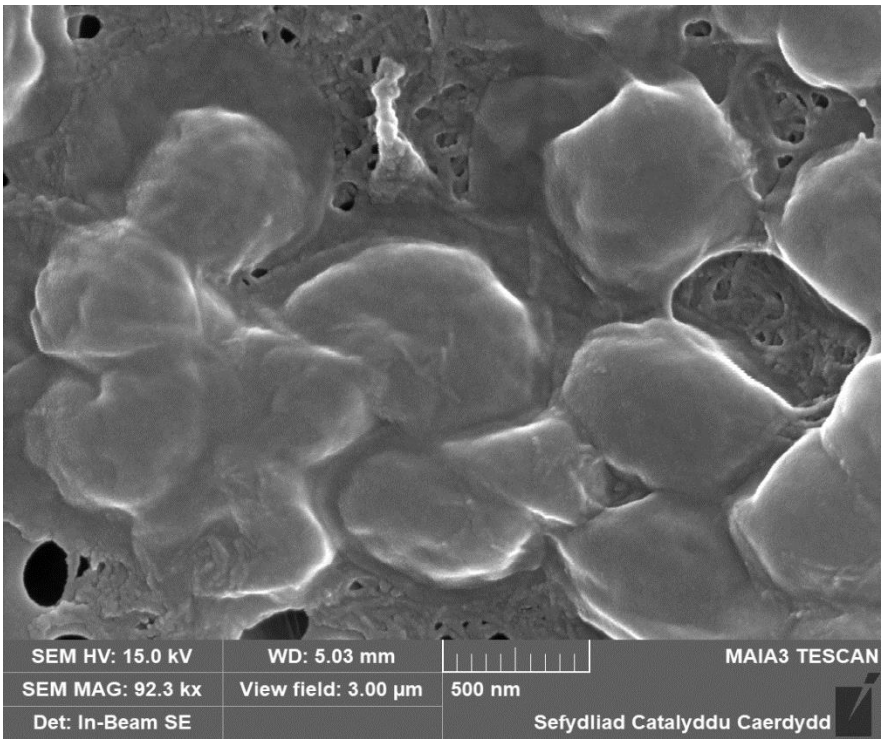
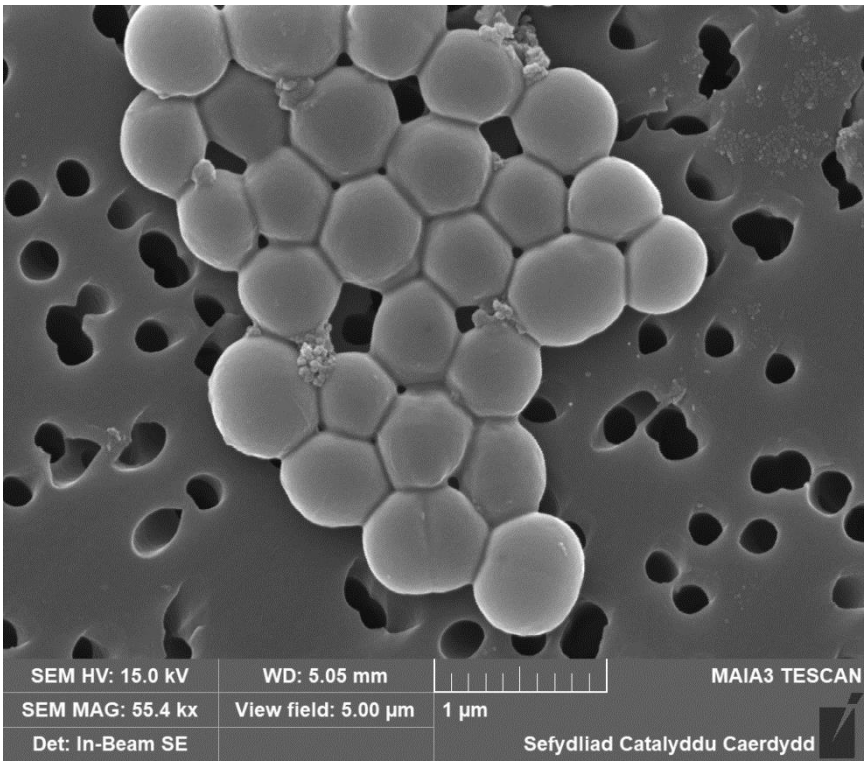


Figure 4.9 SEM of *S. aureus* after treatment with 0.01 % (w/v) MF1.

S. aureus at magnification 55.4 kx (A) and 185 kx (B).

A.



B.

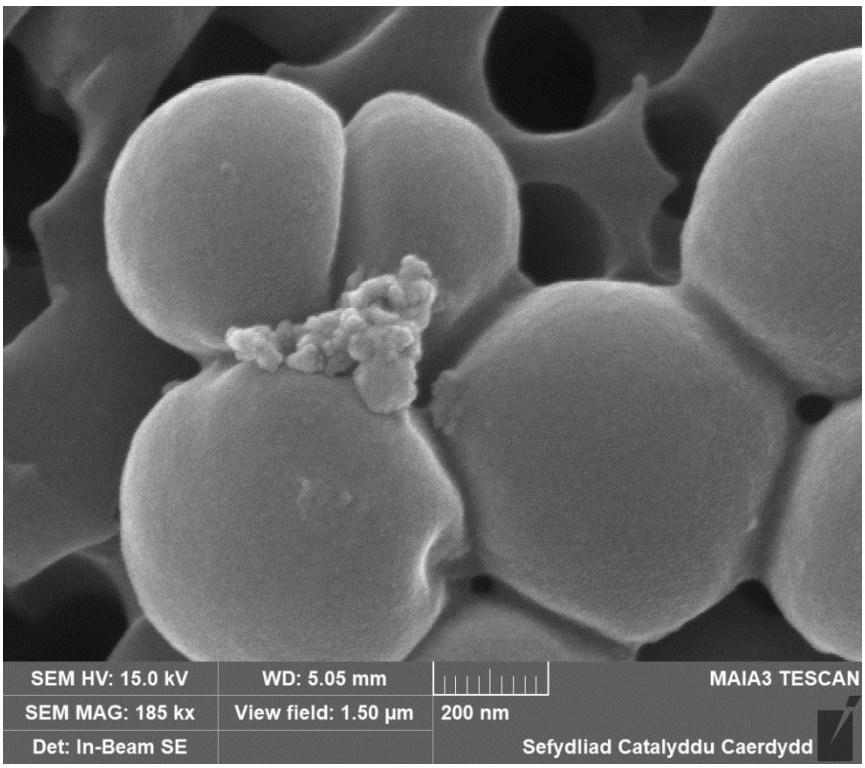
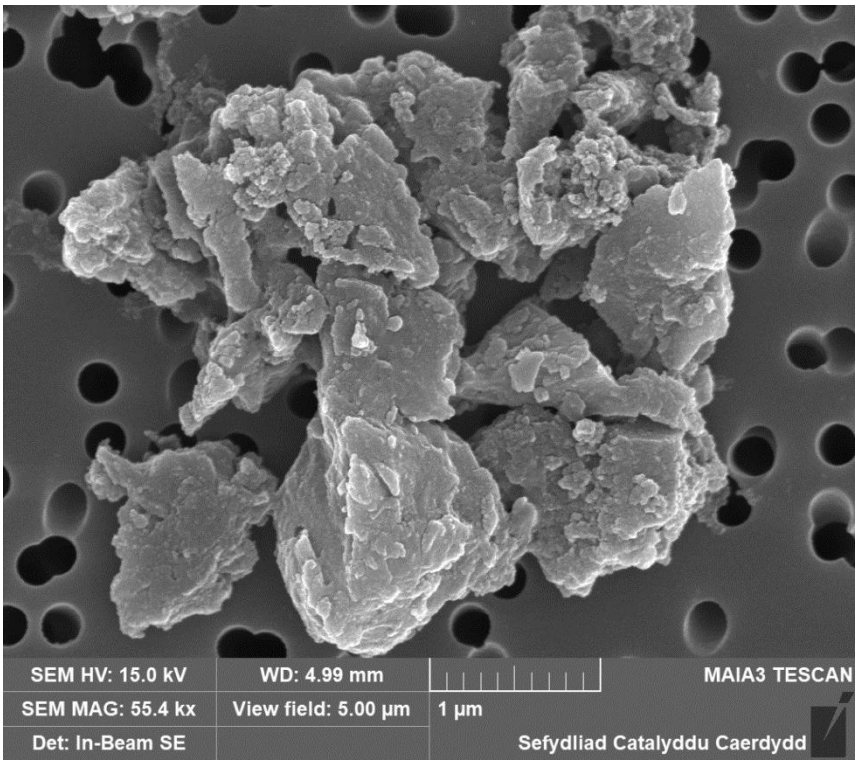


Figure 4.10 SEM of *S. aureus* treated with 1 % (w/v) MF2.

S. aureus at magnification 55.4 kx (A) and 92.3 (B).

A.



B.

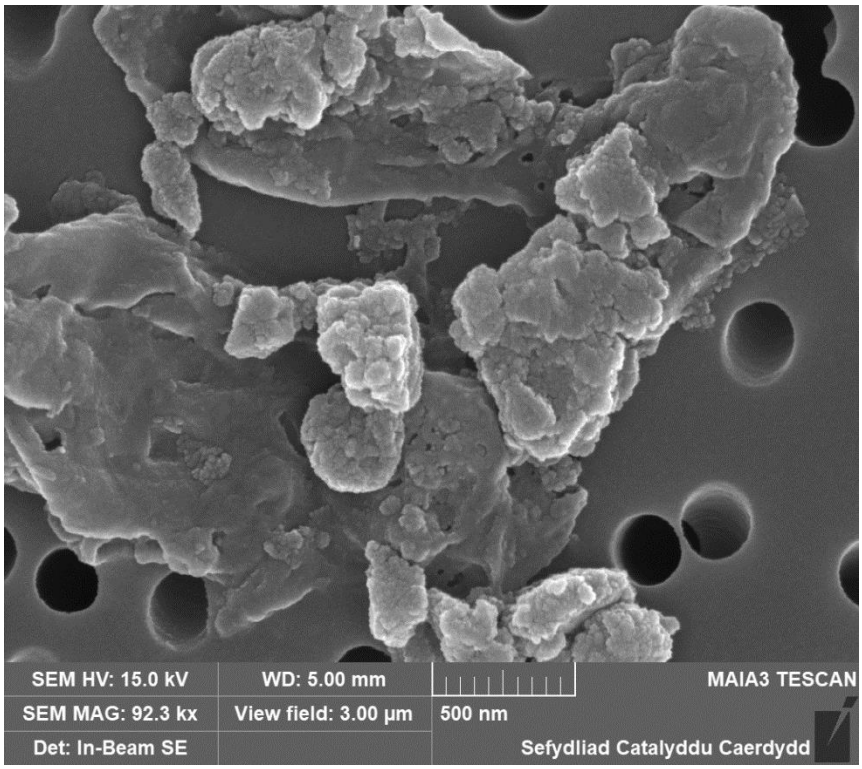
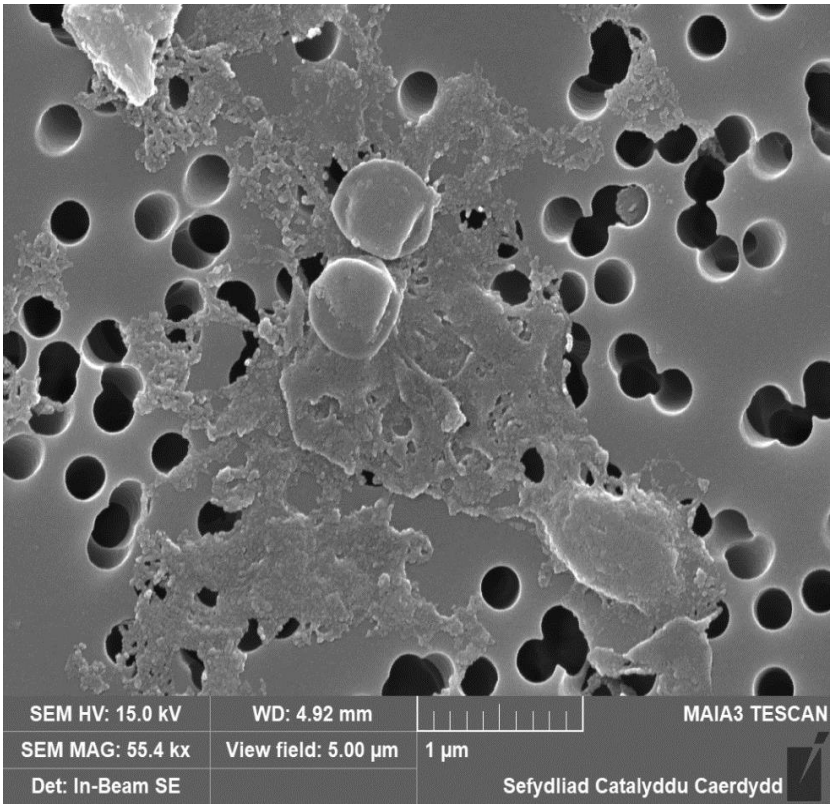


Figure 4.11 SEM of *S. aureus* treated with 0.1 % (w/v) MF2.

S. aureus at magnification 55.4 kx (A) and 92.3 kx (B).

A.



B.

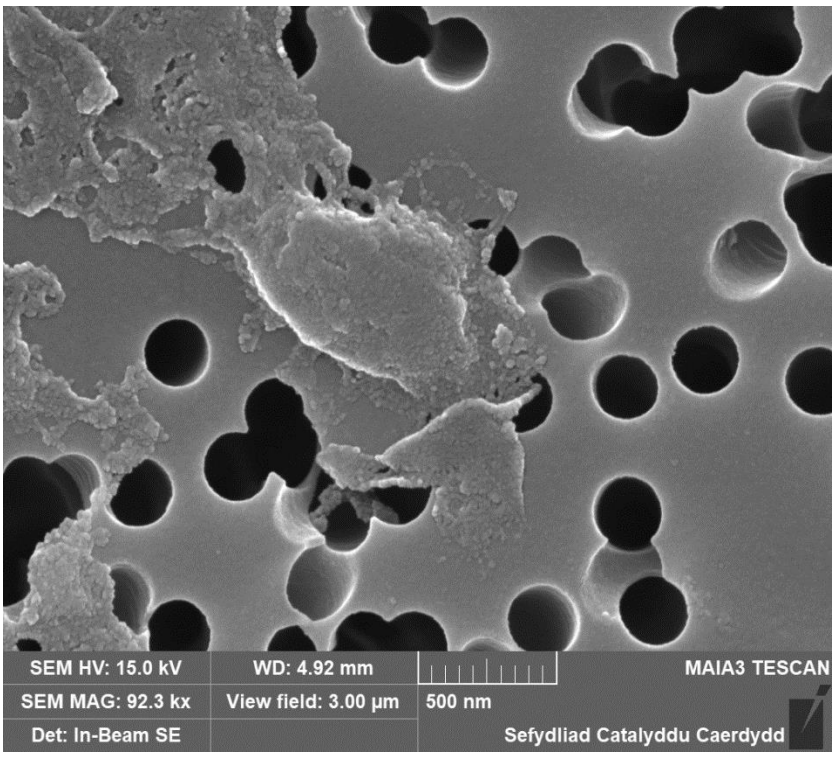
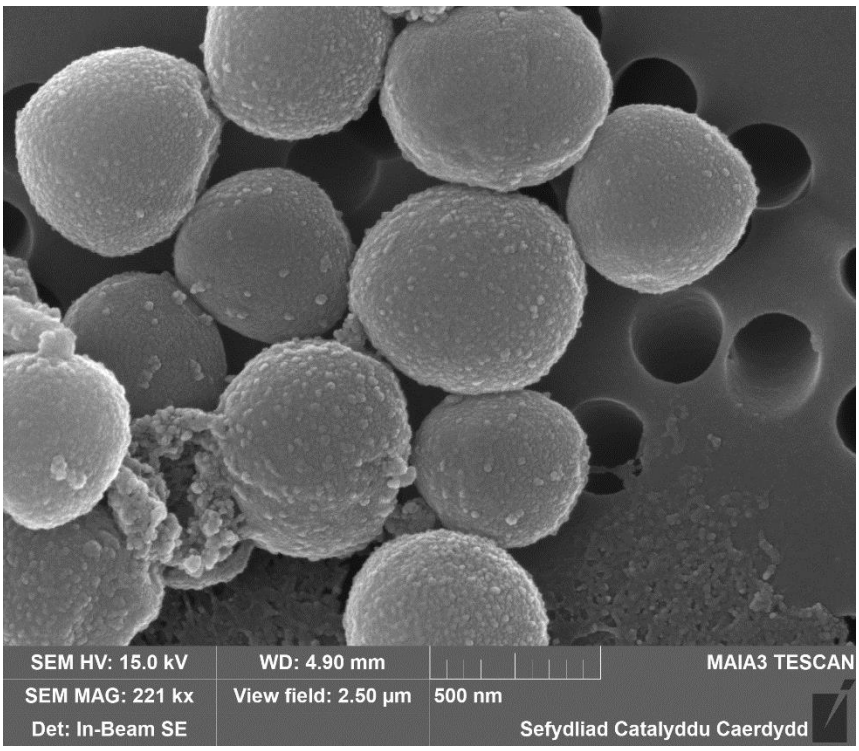


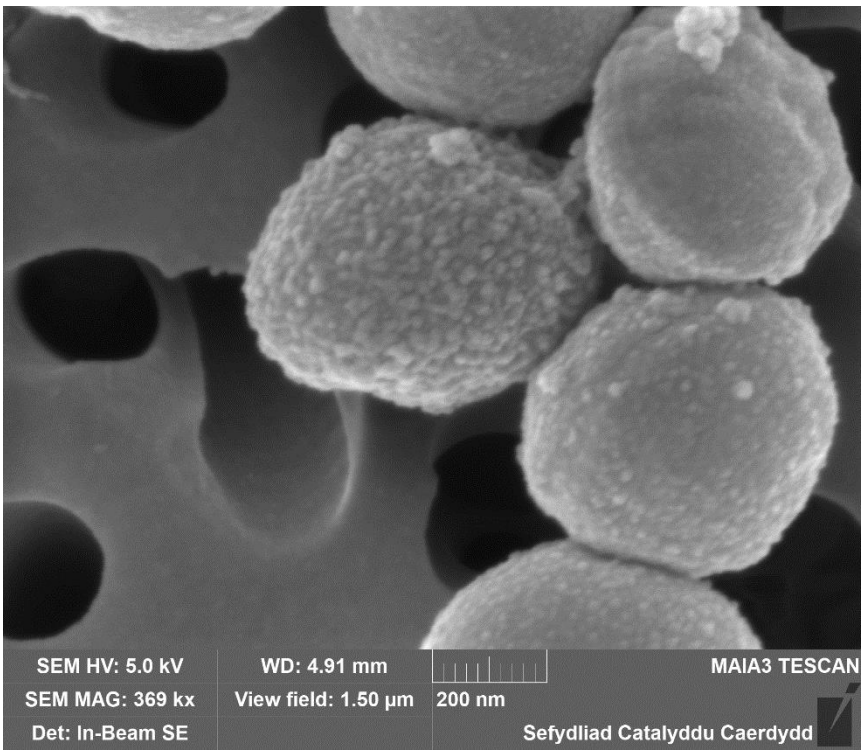
Figure 4.12 SEM of *S. aureus* treated with 0.01 % (w/v) MF2.

S. aureus at magnification 221 kx (A) and 369 kx (B).

A.



B.



4.3.4 Surface roughness

For each treatment the mean roughness squared (Rq), the maximum profile peak height (Rp) and the maximum profile valley depth (Rv) were calculated and the mean ± standard deviation are expressed as shown in table 4.2.

The results of the Rq and Rp analysis shown that in comparison to the untreated control all the conditions tested were not significantly different ($p > 0.05$; two-way ANOVA). However, there was an increase in the Rv at 5 % (w/v) MF2 at all the contact times tested.

When the surfaces were analysed for highest peaks in comparison to the untreated control there were no significant differences ($p > 0.05$; two-way ANOVA;). The treatments that recorded the highest peaks were 5 % (w/v) MF2 at contact times 120, 60 and 30 at 22.98 ± 12.55 nm, 18.96 ± 2.87 nm and 19.02 ± 17.75 nm, respectively.

There was an observed difference between maximum valley depths of untreated *S. epidermidis* (6.19 ± 3.04 nm) when compared to 5 % (w/v) MF2 at contact times 120 min (22.10 ± 0.91) 60 min (24.70 ± 1.28), 30 min (20.78 ± 3.05) and 5 min (18.97 ± 0.51) (Tab 4.2). Additionally, 70 % (v/v) ethanol treatment and 0.1 % (w/v) MF1 at 120 min had significantly deeper valleys in contrast to untreated bacteria ($p < 0.05$; two-way ANOVA).

Table 4.2 AFM results for treatments MF1 and MF2 against *S. epidermidis*.

The mean roughness squared (Rq), the maximum profile peak (Rp) and maximum profile valley depth (Rv) were measured in triplicate (n=3) ± SD. * indicates a significant difference (two-way ANOVA).

	120 min			**60 min**			**30 min**			**5 min**		
MF1 % (w/v)	Rq (nm)	Rp (nm)	Rv (nm)	Rq (nm)	Rp (nm)	Rv (nm)	Rq (nm)	Rp (nm)	Rv (nm)	Rq (nm)	Rp (nm)	Rv (nm)
5	2.70 ± 0.22	15.01 ± 6.81	12.37 ± 3.26	2.09 ± 0.95	7.31 ± 0.64	9.99 ± 3.59	2.69 ± 0.60	8.96 ± 1.22	15.16 ± 1.08	1.81 ± 0.19	6.02 ± 0.52	14.02 ± 1.00
1	3.20 ± 0.61	10.13 ± 2.61	11.61 ± 1.83	2.77 ± 1.62	8.29 ± 4.98	12.55 ± 9.62	1.28 ± 0.54	5.18 ± 2.80	4.82 ± 2.26	1.71 ± 0.77	5.96 ± 4.12	7.48 ± 3.47
0.1	4.21 ± 0.54	15.81 ± 0.54	***21.09* ± 4.82***	3.00 ± 1.17	12.17 ± 3.82	15.04 ± 1.24	3.10 ± 1.25	5.79 ± 1.41	11.10 ± 5.60	2.41 ± 1.22	7.79 ± 3.82	9.68 ± 4.69
MF2 % (w/v)	Rq (nm)	Rp (nm)	Rv (nm)	Rq (nm)	Rp (nm)	Rv (nm)	Rq (nm)	Rp (nm)	Rv (nm)	Rq (nm)	Rp (nm)	Rv (nm)
5	4.44 ± 1.13	22.98 ± 12.55	***22.10* ± 0.91***	5.21 ± 1.69	18.96 ± 2.87	***24.70* ± 1.28***	3.72 ± 1.34	19.02 ± 18.79	***20.78* ± 3.05***	4.31 ± 1.62	9.59 ± 4.17	***18.97* ± 0.51***
1	2.84 ± 1.92	9.39 ± 6.73	13.42 ± 10.41	2.63 ± 0.96	8.83 ± 2.69	13.16 ± 3.85	2.72 ± 1.84	8.71 ± 2.78	11.35 ± 5.70	2.10 ± 0.90	8.79 ± 3.49	8.75 ± 4.08
0.1	3.29 ± 0.90	10.95 ± 0.89	14.23 ± 3.83	2.55 ± 0.87	11.48 ± 0.61	12.10 ± 5.30	2.52 ± 0.30	7.15 ± 1.29	11.57 ± 0.73	3.13 ± 1.60	8.99 ± 4.38	11.30 ± 5.51

Table 4.3 AFM results for untreated *S. epidermidis* and 70 % (v/v) treatment.
The mean roughness squared (Rq), the maximum profile peak (Rp) and maximum profile valley depth (Rv) were measured in triplicate (n=3) ± SD. * indicates a significant difference (two-way ANOVA).

	Rq (nm)	**Rp (nm)**	**Rv (nm)**
Untreated	1.61 ± 0.68	6.22 ± 3.67	6.19 ± 3.04
70 % ethanol	4.65 ± 1.89	17.73 ± 14.09	**20.74* ± 8.98**

4.3.5 Spore mutants

B. subtilis spores have mechanisms that reduce their susceptibility to antimicrobial agents. These include but are not limited to; (i) a spore coat that prevents penetration, (ii) proteins that protect DNA from chemical interactions. Once these defences are removed, antimicrobials that damage DNA can access the macromolecular targets.

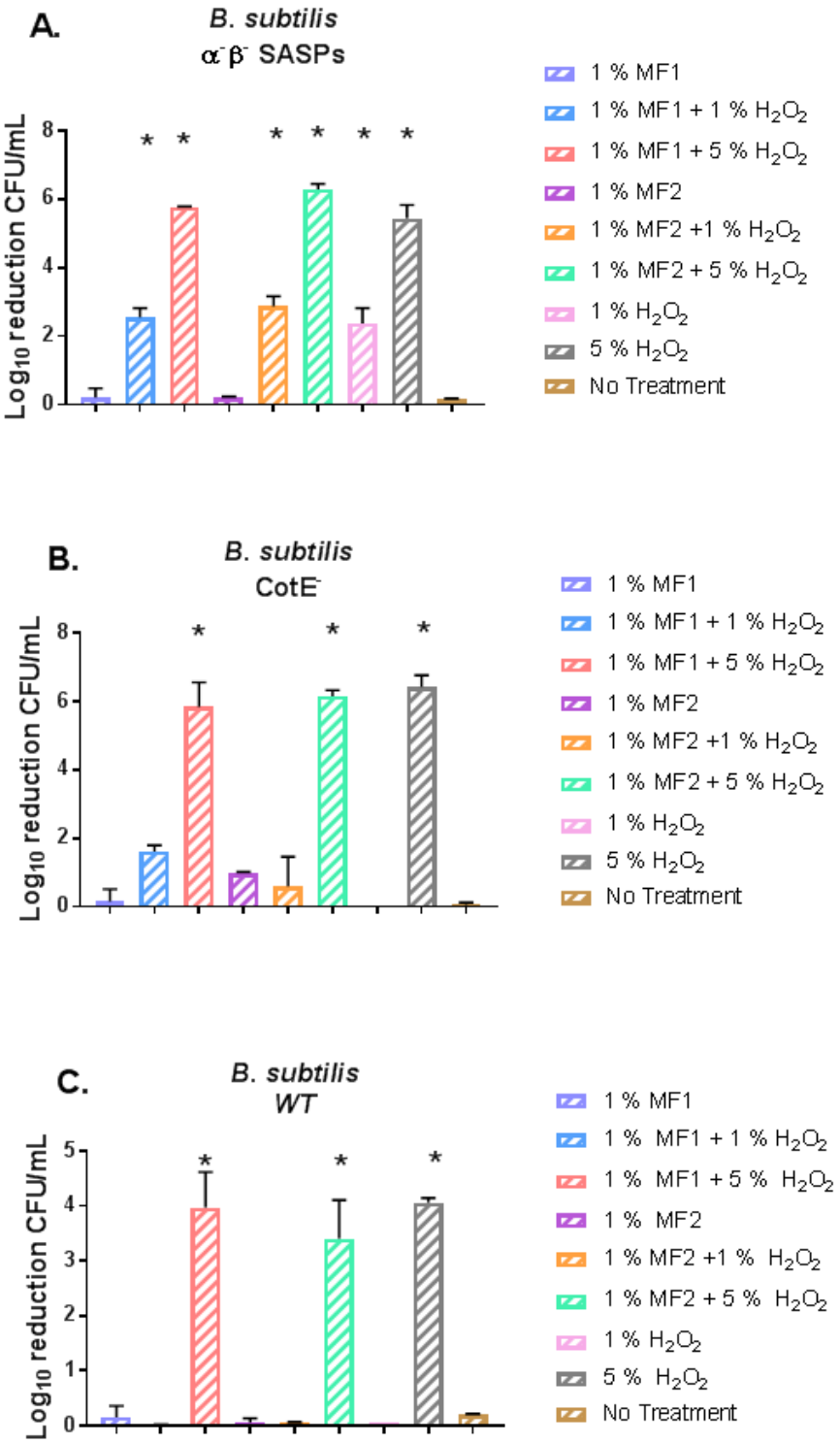
To understand what role, if any, MF1 and MF2 play in DNA damage to a cell, the two formulations were assessed against spore mutants lacking a either the spore coat ($CotE^-$) or protective DNA proteins ($\alpha^-\beta^-$ SASPs).

After a contact time of 60 minutes the activity of MF1 and MF2 did not lower the viability of spores lacking αβ SASPs (Figure 4.9A). When 1 % (v/v) H_2O_2 was added to the formulations, a significant increase ($p < 0.001$; one-way ANOVA) was observed in comparison to MF1/MF2 alone or the untreated control. There was no significant difference between the combined treatment of MF1/MF2 with 1 % (v/v) H_2O_2 and 1% (v/v) H_2O_2 alone ($p = 0.106$ and $p = 0.0078$; one-way ANOVA). Additionally, increasing the concentration of H_2O_2 to 5 % (v/v) resulted in no recovery of spores after treatment, However, there was not a significant difference when H_2O_2 was combined with MF1/MF2 to 5 % (v/v) H_2O_2 alone ($p > 0.9999$ and $p = 0.0328$; one-way ANOVA).

Similarly, when the spore coat (CotE$^-$) was defective (Figure 4.9B), MF1 and MF2 did not contribute to the reduction of spores. There was no significant increase when MF1 and MF2 were combined with H_2O_2 compared to H_2O_2 alone at 1 % (v/v) and 5 % (v/v) ($p > 0.9999$; one-way ANOVA.

Figure 4.7 Treatment of spore mutants with MF1 and MF2 with and without H_2O_2.

B. subtilis spores (A) lacking $\alpha^-\beta^-$ SASPs (B) lacking CotE and (C) Wild-type spores. n=3, error bars represent SD and * indicate $p < 0.001$.



4.4 Discussion

In order to fully understand the limitations of a biocide, the MOA must be investigated. As with efficacy there are many factors that influence MOA, such as concentration of a biocide or the type of microorganism. Often it is difficult to elucidate mechanisms based on in-use concentrations as biocides have multiple targets (Maillard, 2002). In this chapter, The MOA of two imidazolium-based salts in formulation (MF1 and MF2), were investigated at concentrations shown as highly efficacious against bacteria (1% (w/v) and 0.1 (w/v) (Chapter 3).

4.4.1 Membrane damage

The charge of an antimicrobial is an important factor for their potential efficacy. Antimicrobials that have a positive charge are commonly associated with negatively charged cell membranes (Gilbert & Moore, 2005).

The detection of K^+ is a common method for showing membrane disruption of bacteria after antimicrobial treatment (Lambert & Hammond, 1973; Maillard 2002; Denyer & Maillard, 2002). *S. aureus* and *E. coli* had a significant loss of K^+ after treatment with MF1 and MF2 and there was no difference between two concentrations (1 % (w/v) and 0.1 % (w/v)) that were previously shown to be bactericidal in Chapter 3. Differences in K^+ leakage often serve as an index for the severity of the membrane damage, but as no differences were observed between the concentrations tested it could be concluded that both cause irreversible damage and complete cell lysis (Kroll & Anagnostopoulos, 1981). Two other biocides that are known to effect membrane permeability, CHX and 70 % (v/v) ethanol were also tested and shown to cause significant K^+ loss from both *S. aureus* and *E. coli*, but there was no significant difference among the treatments.

Further evidence that MF1 and MF2 increases the permeability of the bacterial membranes was established by incubation of treated cells with DNA binding dyes PI (red: dead) and SYTO 9 (green: live). As only permeability is associated with the death of the cell, the ratio of dyes can be used to determine the number of cells with membrane damage (Warning, 1965; Arndt-Jovin & Jovin, 1989; Stocks, 2004). Against all treatments *S. aureus* membranes were susceptible and their G/R were significantly lower to the untreated control. Against *E. coli,* all treatment shown significant membrane damage although 70 % isopropanol left significantly lower membrane integrity than the untreated control, it appears

to not have caused as much damage as treatments with MF1, MF2 and CHX. This could be due to the outer membrane of *E. coli* which serves as an extra impermeability layer (Denyer & Maillard, 2002).

4.4.2 Cell ultrastructure

SEM allows for visual analysis to changes to the overall cell structure after antimicrobial treatment. After treatment with either MF1 or MF2, increasing cell damage was observed when the concentration of the formulations was increased. At 1 (w/v) MF1 cells were destroyed and the cell constitutes remained visible. However, this was not always the case as some cells kept their cocci shape and did not even appear to be badly damaged. This could be due to clustering of the cells so that the formulations have not fully penetrated through the cell mass. With 1 % (w/v) MF2, no intact cells were observed, and cell remnants appeared to coagulate. For both formulations at 0.1 % (w/v) outlines of cells are still shown but it is clear that cells have suffered great damage and the cytosolic components are leaking out of the cells leading to their collapse. At 0.01 % (w/v) for both formulations, cells still had a spherical structure, the surface *S. aureus* upon treatment of MF2 was noticeably rougher. This could be membrane blebbing in which the inner membrane coming out of the after cracks pores forming in the cell wall after treatment (Codling *et al*., 2005; De Souza *et al*., 2010).

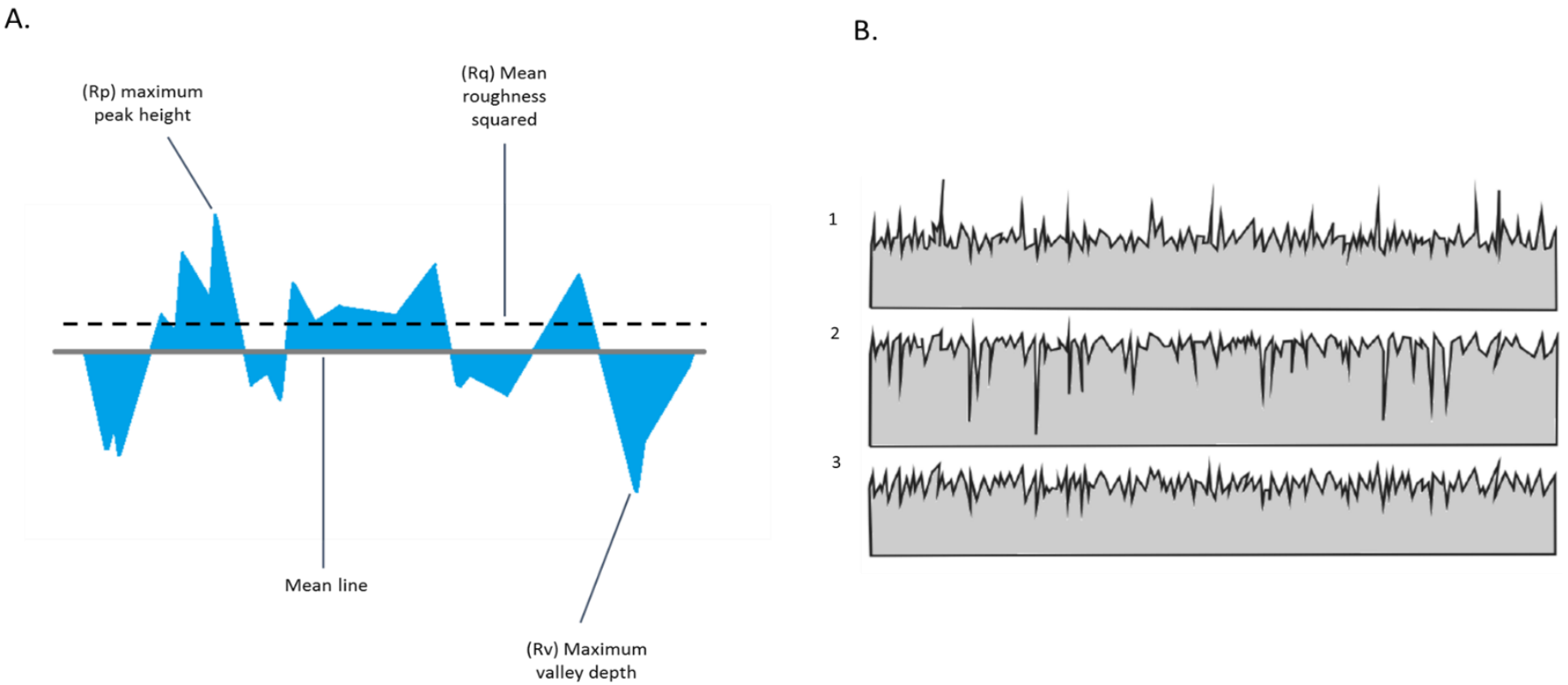
At high concentrations, biocides can have multiple targets and can cause gross cell destruction compared to antimicrobials which have a specific target, such as acridines that can target DNA polymerase, thus preventing the synthesis of polynucleotides (Kadohama & McCarter, 1971). The images acquired by SEM indicate that high concentrations of MF1 and MF2 are not target specific but lead to cell lysis.

Measuring the mean roughness squared (Rq) is a common method to determine the roughness profile of a surface (Deupree & Schoenfisch, 2009; López-Jiménez *et al*., 2015). From the mean line (Figure. 4.10A) it measures the mean roughness squared, i.e the average deviation from the mean line. In the literature those who have investigated changes of surface roughness of bacteria once treated with an antimicrobial, often report an increase in Rq. However, using only Rq as a measurement may be misleading as major peaks and valleys can average out and produce similar Rq, this is demonstrated in Figure 4.10B, where surface 1 has the highest peaks (Rp), surface 2 has the deepest valleys (Rv) yet all of them have the same Rq. For this chapter, the roughness parameters; Rq, Rp and Rv were

analysed to assess changes to the topography of *S. epidermidis* after treatment with MF1 and MF2.

Figure 4.8 Illustrations of different parameters of surface roughness.

(A.) demonstrated the how the three surface roughness measurements; Rq, Rp and Rv are measures from the mean line of the profile under study. (B.) shows the difference in (1) a surface with high peaks, (2) a surface with deep valleys and (3) a surface with no major peaks or valleys, all three surfaces would have the same Rq. (adapted from De Oliveira *et al*., 2012).



Treatment with 1 % (w/v) MF1 or MF2 did not show any apparent changes to the surface of *S. epidermidis*. Therefore, the concentration of MF1 and MF2 was increased to 5 % (w/v), in order to emphasis any changes. At 5 % (w/v) there was no significant differences in the mean roughness or the highest peaks. Interestingly, measurement of the deepest valleys (Rv) did yield significant results after treatment with 5 % (w/v) MF2 for all contact times. Significant valley depts were also recorded with 0.1 % (w/v) MF1 at 120 min and for 70 % (w/v) ethanol.

Overall, the surface topography and morphology *S. epidermidis* after treatment did not change drastically when measured with AFM. Gram-positive bacteria have a thick peptidoglycan wall, approximately 40-80 nm compared to 7-8 nm in Gram-negative bacteria, which could account for stability of the cell even after treatment (Vollmer & Holtje, 2004). The most likely explanation for the increased valley depths, particularly upon treatment with a high concentration of MF2, is the formation of pores in the membrane that results in collapsing of the cell.

The difference between MF1 and MF2 may be a consequence of the differing anions between the imidazolium salts. This was demonstrated by Luczak *et al*, through testing a range of ILs with different anions, in which they observed an increase in MICs with bulkier anions, which they attributed to steric hinderance, which would slow their transport to the cell membrane (Luckzak *et al*., 2010). Therefore, the smaller Br^- ion of MF2 be quicker at integrating into the membrane causing disruption compared to t HCO_3^- ion of MF1.

The disparities between the gross morphological changes seen with SEM and the less obvious surface changes recorded with AFM could be due to multiple factors. AFM was carried out with *S. epidermidis* due to restraints on containment of the AFM laboratory, and SEM was done with *S. aureus*. Although it is unclear why changes in activity would occur, they have been reported for other antimicrobial compounds (Nostro *et al*., 2007). Thus, the antimicrobial activity could be different between the species. Second as shown in Figure 4.7B, the treatment of 1 % (w/v) MF1 caused severe morphological damage to the cells, but some cells appear whole and unaffected. This possibility could be due to the formation of clusters of *Staphylococcus*, limiting the diffusion of the biocide throughout the bacteria, almost like a biofilm. This is likely with the AFM experiments as to allow cells to adhere to surfaces they were left to adhere for 30 min, so it is possible cells clumped together.

After the bacteria were adhered onto the surface for AFM examination, they were treated with either MF1 or MF2, followed by a wash step to remove any remaining biocide. This step could also have led to the loss of cells that were no longer whole and with severe damage which could be why huge of amounts of cell damage were not observed.

4.4.3 DNA damage

B. subtills spores are difficult to eradicate by disinfection due to several intrinsic resistance mechanisms including the spore coat and DNA protective proteins. Spore mutants lacking; CotE an essential protein for spore coat formation or αβ SASPs, proteins that protect DNA, were tested with the formulations MF1 and MF2.

With a defective spore coat the $CotE^-$ spores did not show a significant reduction in spores compared to the no treatment control. This suggests that the barrier effect of the spore coat did not limit the activity of ionic liquids at 1% (w/v), as no activity was observed compared to the WT. Addition of 1 % (v/v) H_2O_2 to MF1 and MF2 did not increase the antimicrobial activity. However, the reduction in viability of $CotE^-$ spores when treated with 5 % (v/v) H_2O_2 was significantly higher l compared to the untreated control, but there was no difference in the presence of MF1/MF2 to the individual effect of 5 % (v/v) H_2O_2. Treatment of 1 % (v/v) H_2O_2 against $CotE^-$ mutants has been shown by others to be insufficient at reducing spores in a 1 hour contact time and 5 hour treatment was required to fully eliminate spores suggesting the spore coat is not a limiting factor to the activity of H_2O_2 (Leggett *et al*, 2016a).

When treated with MF1 or MF2, spores that did not produce approximately 80 % of $\alpha^-\beta^-$ SASPs did not show any susceptibility. Interestingly, unlike WT or $CotE^-$ mutants, spores lacking $\alpha^-\beta^-$ SASPs had increased sensitivity when exposed to 1 % (v/v) H_2O_2, suggesting that the main MOA of H_2O_2 was DNA damage, a common mechanism reported for H_2O_2 against microbes (Imlay & Linn, 1988).

Both $CotE^-$ and $\alpha^-\beta^-$ SASPs were used to investigate if MF1 or MF2 could possibly target intracellular biomolecules located in the spore core. The spore coat acts as a first line of defence against biocides by limiting their entrance into the core. Once removed numerous oxidising biocides have shown increased activity towards spores (Riesenman & Nicholson, 2000; Young & Setlow, 2004). This was not the case for MF1 and MF2 and the relative inactivity of the formulations against spores, may not because the spore coat prevents access to DNA/ other biomolecules.

As SASPs play and important role in protecting DNA from chemical insults it can be argued that the target of MF1 and MF2 are not biomolecules such as proteins and DNA. This is not surprising as the activity of most cationic biocides is directed towards membranes (Gilbert & Moore, 2005). However, the resistance displayed by spore mutants after treatment with MF1 and MF2 may be because a double mutant (CotE$^-$ and $\alpha^-\beta^-$ SASPs) was not tested, and whilst one obstacle was removed, another could have prevented the formulations accessing bimolecular targets. However, this seems unlikely as H_2O_2 lowered spore numbers when single mutants were used.

B. subtilis spores have other mechanisms that help protect spores against chemical attack such as proteins involved in DNA repair (RecA). These were not studied in this work but mutants lacking RecA could be used to determine any DNA damage after treatment (Setlow, 2006).

4.5 Conclusions

Together the methods used in this chapter build a case for the disruption of membranes as the main MOA of MF1 and MF2, which subsequently leads to the breakdown of the cell structure and lysis.

- When treated with MF1 and MF2 *S. aureus* and *E. coli* lost a significant amount of K^+, this was independent of the two concentrations tested (1% (w/v) and 0.1 % (w/v)).
- After treatment *S. aureus* and *E. coli*, SYTO 9 was displaced with PI suggesting membrane integrity was lost.
- SEM imaging shown gross morphological damage at high concentrations and blebbing at concentrations shown to be infective against *S. aureus*.
- Analysis of the surface topography of *S. epidermidis* using AFM did not show obvious roughness to the surface but presented large surface indents in the cells at high concentrations of MF2.
- The viability of spore mutants was not reduced with treatment of MF1 and MF2 suggesting intracellular components are not the target of the MOA of MF1 and MF2.
- Thus, MF1 and MF2 appear to target cell membranes to induce activity.

5 Wipe Product Development

5.1 Introduction and aims

5.1.1 Wipe usage in healthcare

Examining the cleaning regimes in UK hospitals and epidemiological studies linking surface and infections are important to understand pitfalls in infection prevention and to improve interventions. One such intervention is the use of disposable wipes for the cleaning and disinfection of surfaces. In the UK, detergent wipes are used for routine cleaning of surfaces and when there is visible contamination or if there is known infection within a ward, disinfectant wipes are used (Loveday *et al*., 2014). One approach adapted by some healthcare settings is a two-step procedure of using a detergent wipe to remove the visible dirt and then to use a disinfectant wipe (Dancer, 2011).

The use of disposable disinfectant wipes has grown in healthcare settings, providing an easier alternative to spray and wipe methods (Boyce, 2016). Such “all-in-one” wipes prevent large amounts of residual liquid left on surfaces and any subsequent dripping, and their usage also reduces the risk of dilution errors that may occur from concentrated stock (Royal College of Nursing, 2011).

There are however, caveats to using disposable wipes which the Royal College of Nursing (RCN) has noted, warning that caution should be taken when deciding on which wipes to buy as the marketed efficacy of wipes may be misleading and there is no standard for selecting wipes (Royal College of Nursing, 2011). One of the main issues the RCN address, is some commercially available wipes, although effective under laboratory test conditions, may not be as effective for their intended in-field use. For example, if the efficacy of a wipe has been measured by at contact time of 60 minutes, efficacy may not be achievable as the wiping and surface drying may be under 30 s (Royal College of Nursing, 2011; Sattar and Maillard, 2013).

This was demonstrated by Siani *et al*. who assessed the ability of ten wipes that had claims of sporicidal activity against *C. difficile* spores. After a contact time of 10 s, the active ingredients of the wipes had low sporicidal activity with the highest reduction reaching just 1.96 Log_{10}. After a 5 min contact time, only one of the wipes tested had a significant reduction of *C. difficile* spores (Siani *et al*., 2011) . With label claims for the wipes reporting sporicidal action against *C. difficile*, it is alarming that high numbers of spores remained on surfaces after the recommended contact time.

Therefore, proper evaluation using standard methods that reflect the in-use application should be used to demonstrate wipe efficacy. In this thesis two protocols for assessing wipe efficacy were used, ASTM 2967-15 and the BS EN 16615:2018.

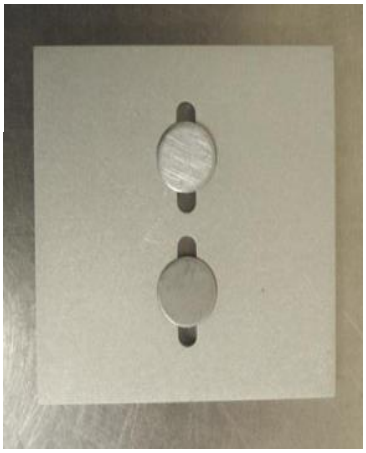
Importantly, both standards take into account the mechanical action (movement and applied pressure from wipe user) and not just the biocidal action of the formulation. Second, both methods consider the transfer of microbes from one surface to another surface, as wipes can be used on multiple surfaces or over large surface areas. Detergent wipes in particular have been shown to spread *C. difficile* on to numerous surfaces and can even break up aggregates increasing their dissemination (Siani *et al*., 2011; Ramm *et al*., 2015).

The ASTM 2967-15 controls the mechanical action through use of a machine the Wiperator® (Figure 5.1). Once the wipe is placed onto a plastic boss (Figure 5.1B) it attaches to a metal rod on the Wiperator®, once the inoculated disk (Figure 5.1C) is brought into contact with the wipe it performs an orbital rotation. The pressure and time of the wiping is controlled aiding repeatability.

Figure 5.1 The Wiperator® used for ASTM 2967-15.
(A) The Wiperator®, executes an orbital motion of 10-mm diameter at 1 orbit/sec (B) plastic boss test wipe is attached to and secured with O-rings (C) the test platform securing the stainless steel (ss) surface accommodates two 1 cm diameter disks.

A. 

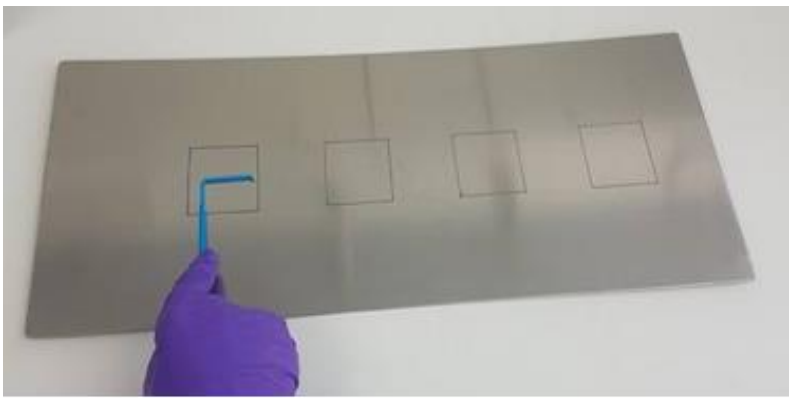
B. 

C. 

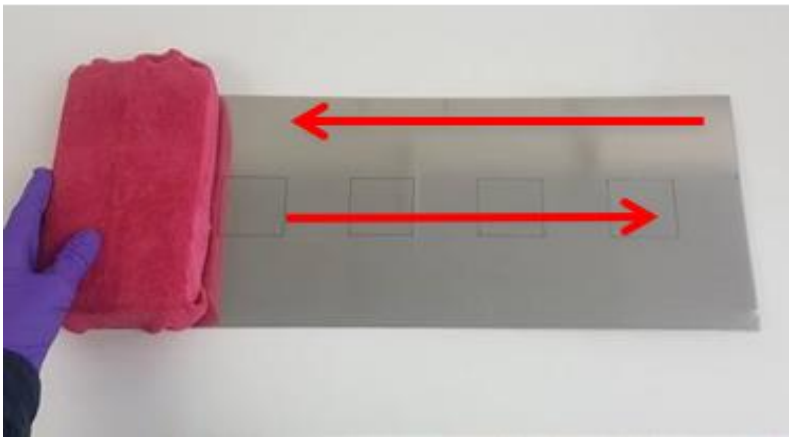
The BS EN 16615:2018 or the 4-field test is a surface with 4 marked squares, the first square is inoculated with bacteria and a wipe is moved across the surface from square 1 to 4 and back. Again, the wiping action is controlled with a unitary weight which the wipe is wrapped around.

Figure 5.2 Test procedure for the 4- Field BS EN16615:2018 wipe test.
Note the surface showed here is a ss surface. The BS EN16615:2018 makes use of a PVC surface. (A) inoculation of the square 1 (B) wipe direction.

A.



B.



There are notable differences between the standard tests used for wiping, which may impact the outcome of the results.

Table 5.1 Difference in variables between wipe efficacy standards ASTM 2967-15 and BS EN 16615:2018.

Notable differences between the two standard wipe tests that may impact efficacy.

	ASTM 2967-15 Wiperator	BS EN 16615:2018 4-field test
Surface	SS disks	PVC
Weight	150 g	2 kg
Contact time	Up to 45 s	1–5 min

5.1.2 Change in co-surfactants

Throughout this thesis the formulations were supplied as the imidazolium salt, butanol and water as outlined in Chapter 2 table 2.2. Formulations MF1 and MF2 were initially designed for the decontamination of sulphur mustards in a microemulsion system (Fallis *et al*., 2009). A microemulsion is a phase in which bipolar molecules such as surfactants, arrange into spherical aggregates/micelles (McClements, 2012). Often co-surfactants are used to lower water tension and disperse the surfactants (Paul and Moulik, 1997). In the study in which

these formulations were originally designed, and used to decontaminate sulphur mustards, butanol was chosen as co-solvent as it allowed for stability of micelle formation (Fallis *et al*., 2009). However, as keeping micelles of the ionic liquids was not an aim of this study and ethanol is a widely used and acceptable excipient, the efficacy of the formulations with butanol or ethanol were compared (Rowe *et al*., 2009).

5.1.3 Biocide cytotoxicity

In healthcare settings, disinfection procedures require use of PPE such as gloves to protect skin contact with chemicals, but contact can still occur through accidental exposure or failure to wear PPE. Irritant contact dermatitis (ICD) is a skin condition resulting from cytotoxic effects following single or repeated exposure to irritant stimuli (Ale & Maibach, 2006). Staff that come in to contact with biocides have an increased risk of developing ICD (Bauer, 2013; Malik & English, 2015; Pacheco, 2018). Therefore, assessment of the cytotoxicity of biocides is an important factor of product development, especially if products need to be used with proper PPE. One method for evaluating cytotoxicity is through *in vitro* testing using keratinocytes, which make up around 90 % of the skin epidermis (McGrath, *et al*., 2004). For the assessment of biocide cytotoxicity, a common protocol is colorimetric method with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Muller & Kramer, 2006; De Souza *et al*., 2007; Hirsch *et al*., 2009). Cells that are viable can metabolise the dye to purple formazan crystals, then the colour change can be analysed and correlated to the number of cells that were viable post-treatment

5.2 Aim and objectives

The aims of this chapter was to develop and assess wipe products containing MF1 and MF2. Not only would this provide insight into the efficacy of ionic liquids as products but could also improve existing products.

The first product was a wet wipe for general disinfection of surfaces and the second was a dry wipe with a primary purpose of absorbing bodily fluids/blood and disinfecting a surface simultaneously.

The wet wipes were prepared in a similar manner to wipes produced by GAMA Healthcare to compare activity to wipes already used in healthcare. The wet wipes were all made from the same material for direct comparison. The wipes were tested using the standards ASTM 2967-15 and BS EN 16615:2018.

The dry wipes were made from the same material as a spill wipe from GAMA Healthcare and was assessed using an adapted BS EN 16615:2018 in which sheep blood was used as a bodily fluid that this product would be aimed at clearing and disinfecting.

Finally, the cytotoxicity of the formulations MF1 and MF2 were assessed using an MTT assay with immortalised human keratinocytes (HaCat cells). The assay was also performed with biocides BZC and CHX at concentrations used in topical medicines/wipes/creams or which have been recognised as not possessing cytotoxic effects.

5.3 Methods

5.3.1 Wipe preparation

5.3.1.1 Wet wipes

Nonwoven wipes (90% polypropylene, 10% viscose; GAMA Healthcare Ltd, Halifax, UK), were cut to 4 cm^2 and formulations were added in a 2:1 ratio of mL per wipe weight (g). Control wipes were prepared by substituting the test formulations with TSC.

5.3.1.2 Dry wipes

Nonwoven wipe material (100% wood pulp; GAMA Healthcare Ltd, Halifax, UK) was cut to 20 cm (width) x 30 cm (Length). Between two sheets of material, 1g of powder MF2 was spread over the surface followed by 1.18 g adhesive ethylene-vinyl acetate (EVA) to facilitate bonding of the material. The impregnated wipes were passed through a heated flatbed laminator (Nova 45 PN; Reliant Machinery Ltd., Luton, UK) at 80°C at a speed of 12 seconds per meter. Control wipes were prepared with EVA only.

5.3.2 ASTM 2967-15 Wiperator

5.3.2.1 Inoculum preparation

Overnight cultures of *A. baumannii* ATCC 19568 and *S. aureus* ATCC 14990 were prepared by growing in 10 mL TSB for 18-22 hours at 37°C ± 1°C at 125 rpm. After growth, bacterial cells were spun down at 3000 x g for 20 min and resuspended in 5 mL TSC. Following resuspension, 4.5 mL of bacteria was added to 0.5 mL of 30 g/L of BSA or 30 g/L BSA + 30 g/L sheep erythrocytes (prepared as described chapter 3.2.2.1), vortexed for 30 seconds and 10 µL drops (final concentration on disks, 1-2 $x10^7$ CFU/mL) were placed onto stainless

steel disks (10 mm; Goodfellows Cambridge, Huntington, UK). Disks were left to dry for 60 minutes at room temperature.

5.3.2.2 Wiperator® test procedure

Following the ASTM 2967-15 standard, test wipe sections of 4 cm^2 were attached to a plastic boss and placed on the Wiperator® (Figure 5.1).

The inoculated disk was placed on a stainless steel carrier and brought into contact with the test wipe. In an orbiting motion the disk was wiped for a contact time of 30 s with 150 g weighted pressure. The same wipe was then used on a sterile stainless steel disk for 5 s to assess transfer of bacteria.

After wiping, disks were transferred to 1g glass beads in 1mL Dey-Engley neutralising broth. The 1 mL neutraliser broth was then plated on to two TSA plates and incubated at 37°C ± 1°C for 18-22 hours.

5.3.2.3 Validation suspension

A validation suspension was prepared as outlined in Chapter 3 section 3.2.7 for the use of all controls for the ASTM E2967-15 method.

5.3.2.4 ASTM E2967-15 test controls

(i) Neutraliser effectiveness: A sterile disk was wiped with a test wipe and placed in 1 mL neutraliser for 20 s, followed by 10 µL of validation suspension (30-300 CFU/mL). To determine if the neutraliser was effective in stopping any antimicrobial affect, the 1 mL suspension was plated out onto two TSA plates and incubated at 37°C ± 1°C for 18-22 hours. After growth, the neutraliser control was considered effective if the CFU/mL was 80%-120% in range of the validation suspension CFU/mL.

(ii) Drying control: As described in 5.2.1.2 bacteria were dried on to ss disks. After the bacteria were completely dried the disks were placed directly into 1g glass beads in 1mL Dey-Engley neutralising broth. After vortexing for 30 s, 0.1 mL of broth was serially diluted in TSC and 0.1 mL of each dilution was plated out using the spread plate method. After incubation at 37°C ± 1°C for 18-22 hours, the number of bacteria recovered were used as the starting inoculum in Log_{10} reduction calculations.

5.3.3 BS EN 16615: 2018 4-Field test

5.3.3.1 Bacteria preparation

S. aureus ATCC 14990 and *A. baumannii* ATCC 19568 suspensions were prepared as described in Chapter 2 section 2.2.3. to give a final suspension of 1-1.5 x 10^8 CFU/mL.

For wet wipes test: 9 mL of bacteria was mixed with 1 mL 30 g/L BSA, then 50 µL was placed on square 1 of the PVC surface, spread over the surface of the square using a L-shaped spreader and dried at room temperature (30-40 min).

For dry wipes: 9 mL of bacteria was mixed with 1 mL TSC and then 50 µL was placed on square 1 of the PVC surface, spread over the surface of the square using a L-shaped spreader and dried at room temperature (30-40 min). After the bacteria had dried, 50 µL of sheep's blood (Fisher, Loughborough, UK) was placed over the bacteria.

1.1.1.1 4-Field test procedure wet wipes

To ensure even distribution of weight throughout the wiping process, a granite block weighing 2.5 kg was covered with the test wipe. To begin, the test wipe was placed before square 1 on the PVC surface and moved horizontally from square 1 to square 4 in 1 second. The wipe was immediately turned after square 4 and returned back to square 1 in 1 second. The surface was left for a contact time of 5 min. The surface of square one was then rubbed using a cotton swab soaked in Dey-Engley neutraliser to recover any remaining bacteria. Once the full surface of square one had been rubbed, the swab was placed into 5 mL of Dey-Engley neutraliser. The recovery procedure was repeated with a second dry swab until the surface of square 1 was dry. The second swab was placed in the same 5 mL neutraliser as the first swab (two swabs per square).

5.3.4 MTT Assay

HaCat cells were defrosted and grown as outlined in chapter 2 section 2.3.1 and 2.3.2. Once cells had reached 80% confluence in a T25 flask the cells were washed twice with PBS and trypsinised for 10-15 minutes at 37°C ± 2°C. After cells had detached from flasks, 8 mLs of fresh medium with 10% FBS was added to the cells to inhibit trypsin. Cells were counted as outlined in Chapter 2 section 2.3.2 and adjusted to give a final cell count of 1.5 x 10^4 cells/well in 96-well plates.

Once cells were dispensed into 96-well plates, they were incubated at 37°C ± 2°C with 5% CO_2 and 95% relative humidity for 24 hours to reach 80% confluence. The DMEM was

aspirated and cells were washed twice to remove any remaining medium/FBS. Formulations were prepared in sterile water and 200 µl of each formulation was applied to the cells for 1 hour and incubated at 37 ± 2°C with 5% CO_2, 95% relative humidity. The formulations were removed and washed twice in PBS to remove any remaining formulations. In PBS, 200 µL of 0.5 mg/mL of MTT was applied to cells and incubated for 4 hours at 37 ± 2°C with 5% CO_2, 95% relative humidity. The MTT was then removed and 100 µL of dimethyl sulfoxide (DMSO) was applied to the wells and incubated at 37 ± 2°C for 30 minutes, the absorbance was determined at 550 nm (Tecan infinite 200 Pro, Tecan UK Ltd, Reading, UK). Background wells containing no cells were run in parallel and subtracted from final readings. Any viable cells remaining after treatment were calculated as a percentage of the untreated control.

5.4 Results

5.4.1 Wiperator

5.4.1.1 Wet wipes normal organic load

The removal and transfer of *S. aureus* and *A. baumannii* on ss disks was assessed after wiping with of pre-soaked wipes. The mechanical action was controlled using the Wiperator with a contact time of 30 s and transfer of 5 s.

At the 1 % (w/v) MF1 and MF2 with butanol as a co-surfactant, *S. aureus* and *A. baumannii* were completely removed from the inoculated disk and there was no transfer onto a second disk (Figure 5.3A & Figure 5.4A), this was statistically significant from the control wipe ($p < 0.001$; two-way ANOVA).

At the same concentration of MF1 and MF2, but with ethanol as the co-surfactant *S. aureus* was not entirely removed from the ss disk with MF1 at 4.75 ± 1.09 CFU/mL with a transfer of 0.24 ± 0.331 CFU/mL, for MF2 removal was 5.31 ± 0.95 CFU/mL and transfer 0.52 ± 0.48 CFU/mL (Figure 5.4B), however both were still significantly different to the control wipe ($p < 0.001$; two-way ANOVA). There was a complete removal of *A. baumannii* from stainless steel disks with 1 % (w/v) MF1 and MF2 wipes with no transfer to a second stainless steel disk. There was no significant difference observed between ethanol or butanol in the 1% (w/v) MF1/MF2 formulations against *S. aureus* and *A. baumannii* ($p > 0.001$; two-way ANOVA).

MF1 and MF2 at 0.1 % (w/v) (Figure 5.3 & Figure 5.4) were still efficacious and the Log_{10} removal was higher with transfer of colonies reduced for both *S. aureus* and *A. baumannii* compared to the control wipe ($p < 0.001$; two way ANOVA), with the exception of MF1 in butanol against *S. aureus* (Figure 5.3A) with a Log_{10} reduction of 3.22 ± 0.37 CFU/mL ($p = 0.111$; two-way ANOVA), however, transfer was still lower than the control wipe ($p < 0.001$; two-way ANOVA). Furthermore, against *S. aureus* there was significant difference between the reduction and transfer with 0.1 % (w/v) MF1 in butanol compared to ethanol ($p < 0.001$; two-way ANOVA) (Figure 5.3A & Figure 5.3B).

At the lowest concentration of 0.01 % (w/v) MF1 and MF2, wipes did not reduce the

CFU/mL of *S. aureus* or *A. baumannii* on stainless steel disks and no significant difference in transfer was seen in comparison to the control wipes ($p > 0.05$; two way ANOVA), with either co-surfactant.

Two commercially available wipes from GAMA Healthcare; a 'Universal' wet wipe and a detergent wet wipe were also assessed for their ability to remove and transfer *S. aureus* and *A. baumannii*. The Universal wipe removed *S. aureus* by Log_{10} 4.31 ± 0.26 CFU/mL and there was no transfer to a second disk. After a 30 s contact time with the detergent wipe, Log_{10} 3.14 ± 0.28 CFU/mL of *S. aureus* was removed from the inoculated disk and Log_{10} 3.68 ± 0.85 CFU/ mL was transferred to a second sterile disk.

Against *A. baumannii*, both commercial wipes resulted in complete removal of the bacteria and no transfer was observed. Compared to 1 % (w/v) MF1 and MF2 wipes, the Universal and detergent wipe were not significantly different ($p > 0.05$; two-way ANOVA) at removing and transferring the *A. baumannii.* At 0.1 % (w/v) only MF2 in butanol was comparable to the Universal and detergent wipes ($p > 0.999$; two-way ANOVA) and the of rest of the 0.1 % (w/v) MF1 and MF2 wipes were unable to completely remove *A. baumannii.* None of the MF1/MF2 wipes at 0.01 % (w/v) performed better than detergent or universal wipes against *A. baumannii* ($p < 0.05$; two-way ANOVA).

However, all the 1 % (w/v) MF1/MF2 wipes out-performed in the reduction in viability of *S. aureus* compared to the Universal and detergent wipes, however, statistically only the 1 % (w/v) MF2 in butanol wipe was significant ($p < 0.05$; two-way ANOVA). At lower concentrations only 0.1 % (w/v) MF2 in butanol was equivalent in the removal and transference of *S. aureus* to the Universal wipe, all other wipes were significantly lower ($p < 0.001$; two-way ANOVA).

Figure 5.3 Reduction and transfer of S. aureus using MF1 and MF2 wipes using the Wiperator®.

(A) Wipes were prepared with butanol co-surfactant or (B) ethanol co-surfactant. Results are of three biological repeats (n=3) with two technical repeats. A two-way ANOVA was carried out with Tukey's multiple comparison. * indicates significant difference ($p < 0.05$) to the control wipe. Squared boxes highlight a significant difference ($p < 0.05$) between the co-surfactants. Error bars represent SD.

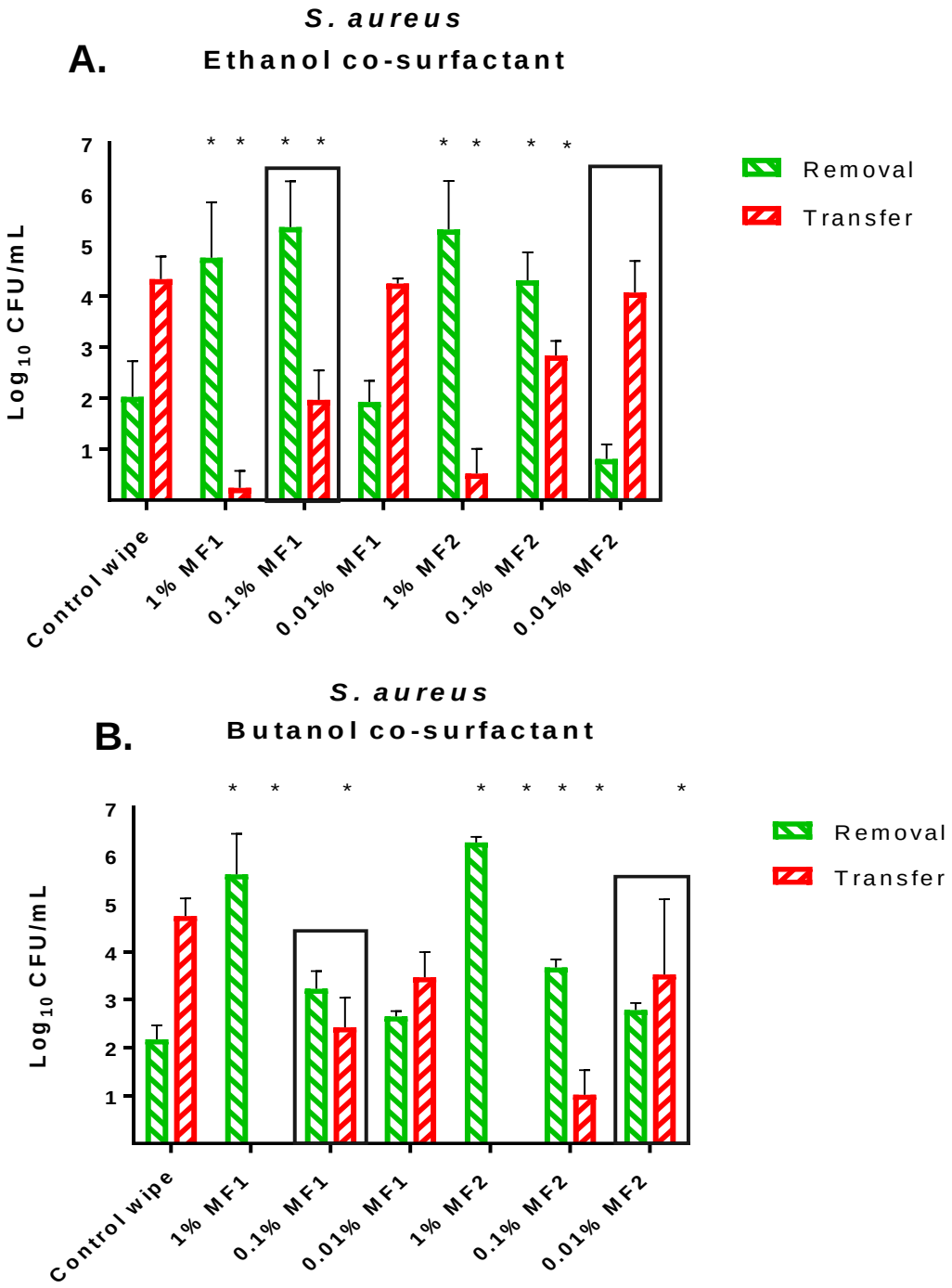


Figure 5.4 Reduction and transfer of *A. baumannii* using MF1 and MF2 wipes using the Wiperator®.

(A) Wipes were prepared with butanol co-surfactant or (B) ethanol co-surfactant. Results are of three biological repeats (n=3) with two technical repeats. A two-way ANOVA was carried out with Tukey's multiple comparison. * indicates significant difference ($p < 0.05$) to the control wipe. Squared boxes highlight a significant difference ($p < 0.05$) between the co-surfactants. Error bars represent SD.

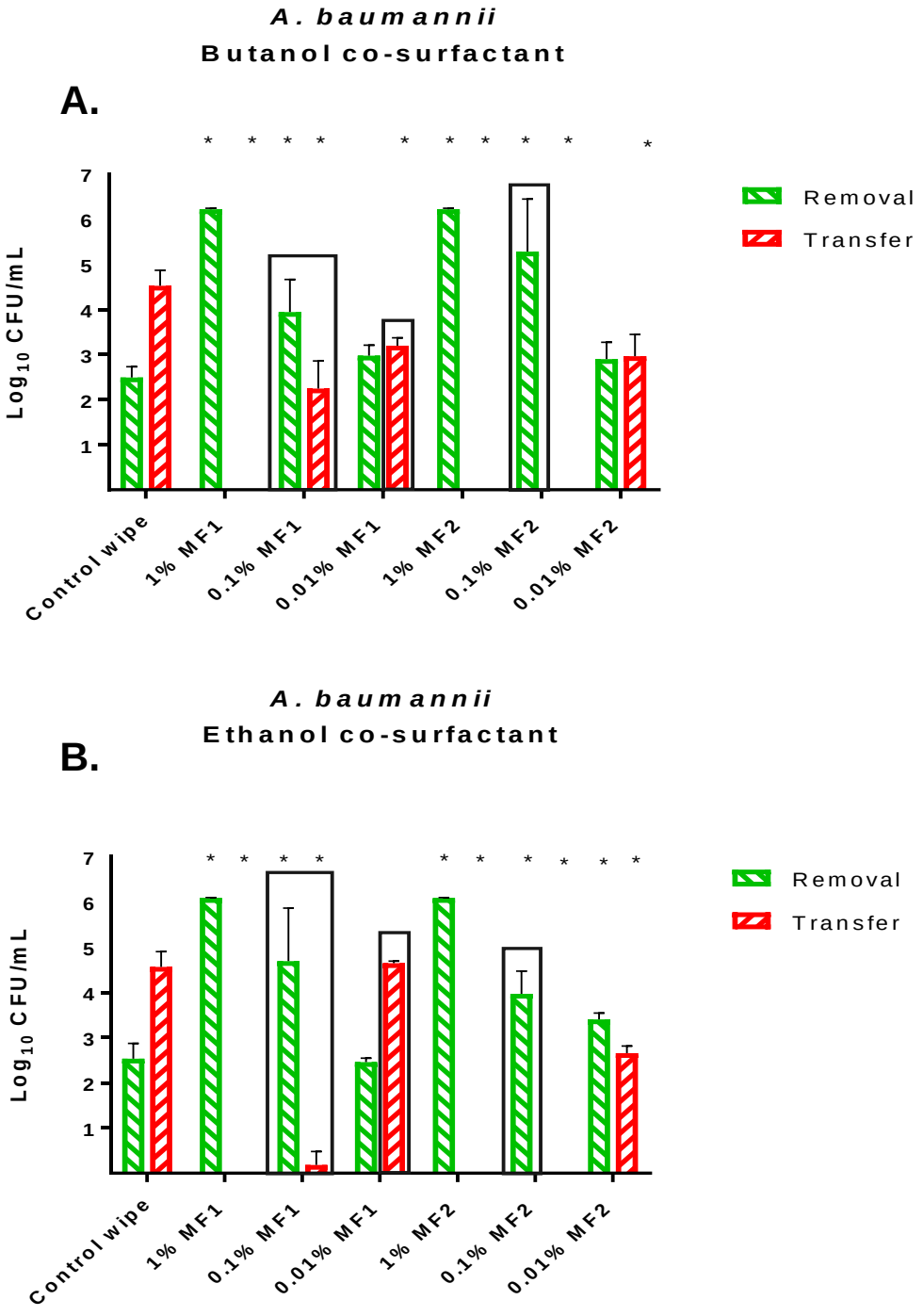
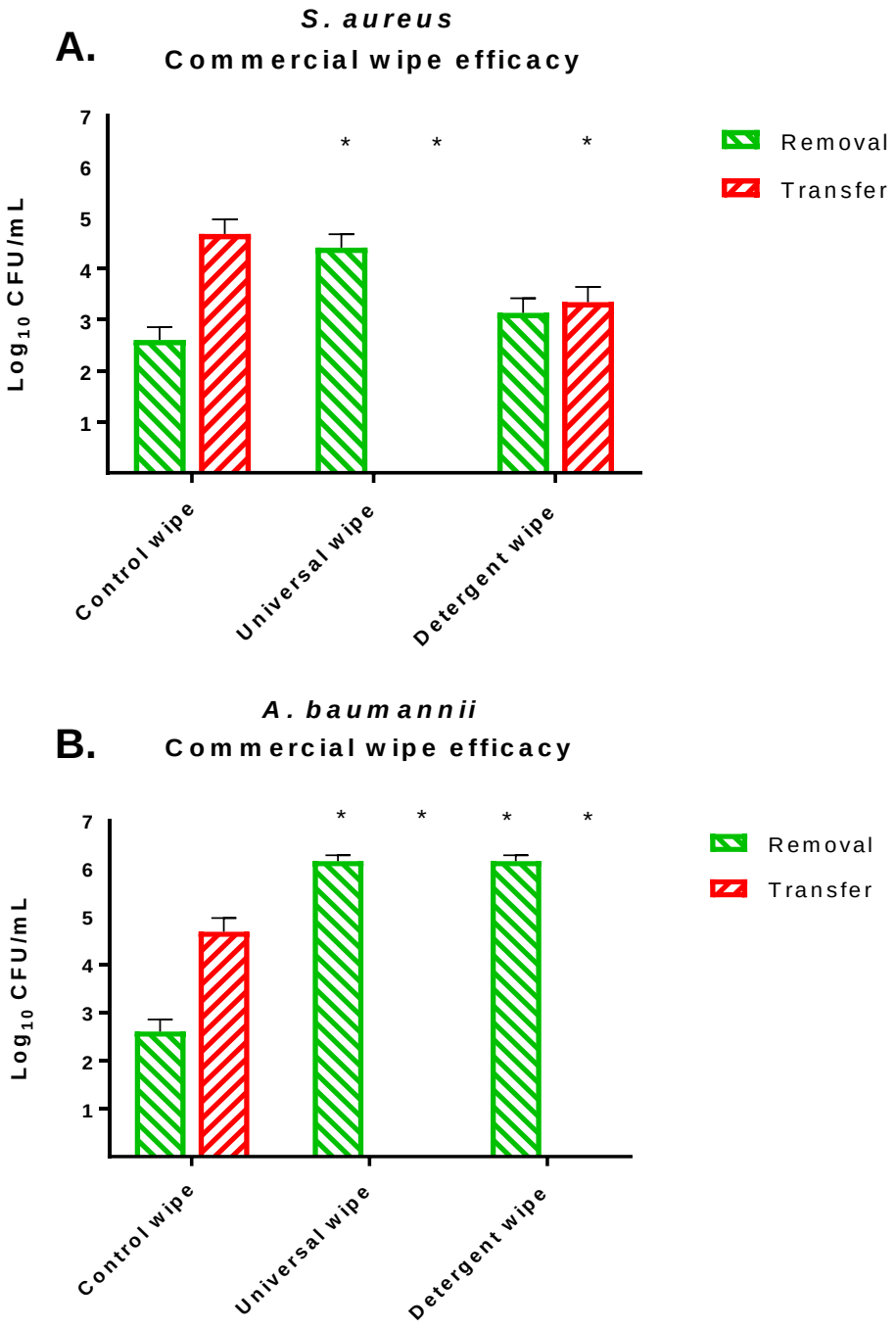


Figure 5.5 Reduction and transfer *S. aureus* and *A. baumannii* with commercial wipes using the Wiperator®.

(A) *S. aureus* tested with control wipe, Universal wipe and Detergent wipe (B) *A. baumannii* tested with control wipe, Universal wipe and Detergent wipe. Results are of three biological repeats (n=3) with two technical repeats. A two-way ANOVA was carried out with Tukey's multiple comparison. * indicates significant difference ($p < 0.05$) to the control wipe. Error bars represent SD.



5.4.1.2 Wet wipes higher organic load

As all 1 % (w/v) MF1/MF2 wipes had significant reductions and very low transference of Gram-positive and Gram-negative bacteria (Figure 5.3 & Figure 5.4), only this concentration was combined with wipe material and was tested with a higher organic load.

With 3 g/L BSA + 3 g/L sheep erythrocytes, all 1 % (w/v) MF1/MF2 wipes gave a full reduction against *A. baumannii* with no transfer, regardless of the imidazolium salt/ alcohol combination (Figure 5.7A). The commercial wipes were also effective, and no differences were observed between the commercial wipes and the MF1/MF2 wipes for *A. baumannii* ($p > 0.999$; two-way ANOVA).

For *S. aureus* with butanol as a co-surfactant the final reductions were Log_{10} 5.17 ± 0.26 CFU/mL and Log_{10} 5.64 ± 0.32 CFU/mL for MF1 and MF2, respectively. With ethanol as the co-surfactant the reductions were slightly lower at Log_{10} 4.59 ± 0.15 CFU/mL for MF1 and Log_{10} 4.85 ± 0.47 CFU/mL for MF2. However, reductions of *S. aureus* after use of the MF1/MF2 wipes were significantly higher than the control wipe ($p < 0.001$; two-way ANOVA).

Compared to the Universal wipe in which a Log_{10} 3.78 ± 0.70 reduction of *S. aureus* was reported, all the MF1/MF2 wipes gave a significantly higher reduction, apart from MF1 in ethanol which was not significantly different to the Universal wipe ($p = 0.127$; two-way ANOVA). The transfer of *S. aureus* form MF1/ MF2 wipes and the Universal wipe was negligible, and no difference was observed between any of the wipes ($p > 0.999$; two-way ANOVA). The reduction of *S. aureus* with the detergent wipe was significantly lower to the control wipe ($p < 0.05$; two-way ANOVA) but the reduction was lower than all other wipes tested ($p < 0.001$; two-way ANOVA). The transfer of *S. aureus* with the detergent wipe was no different to the control wipe ($p > 0.05$; two-way ANOVA) and was significantly higher than all the test wipes ($p < 0.001$; two-way ANOVA).

Figure 5.6 Reduction and transfer of *S. aureus* with higher organic soil using Wiperator® method.

(A) *S. aureus* tested with control, MF1 and MF2 wipes (B) *S. aureus* tested with commercial wipes; Universal and Detergent. Results are of three biological repeats (n=3) with two technical repeats. A two-way ANOVA was carried out with Tukey's multiple comparison. * indicates significant difference ($p < 0.05$) to the control wipe. Error bars represent SD.

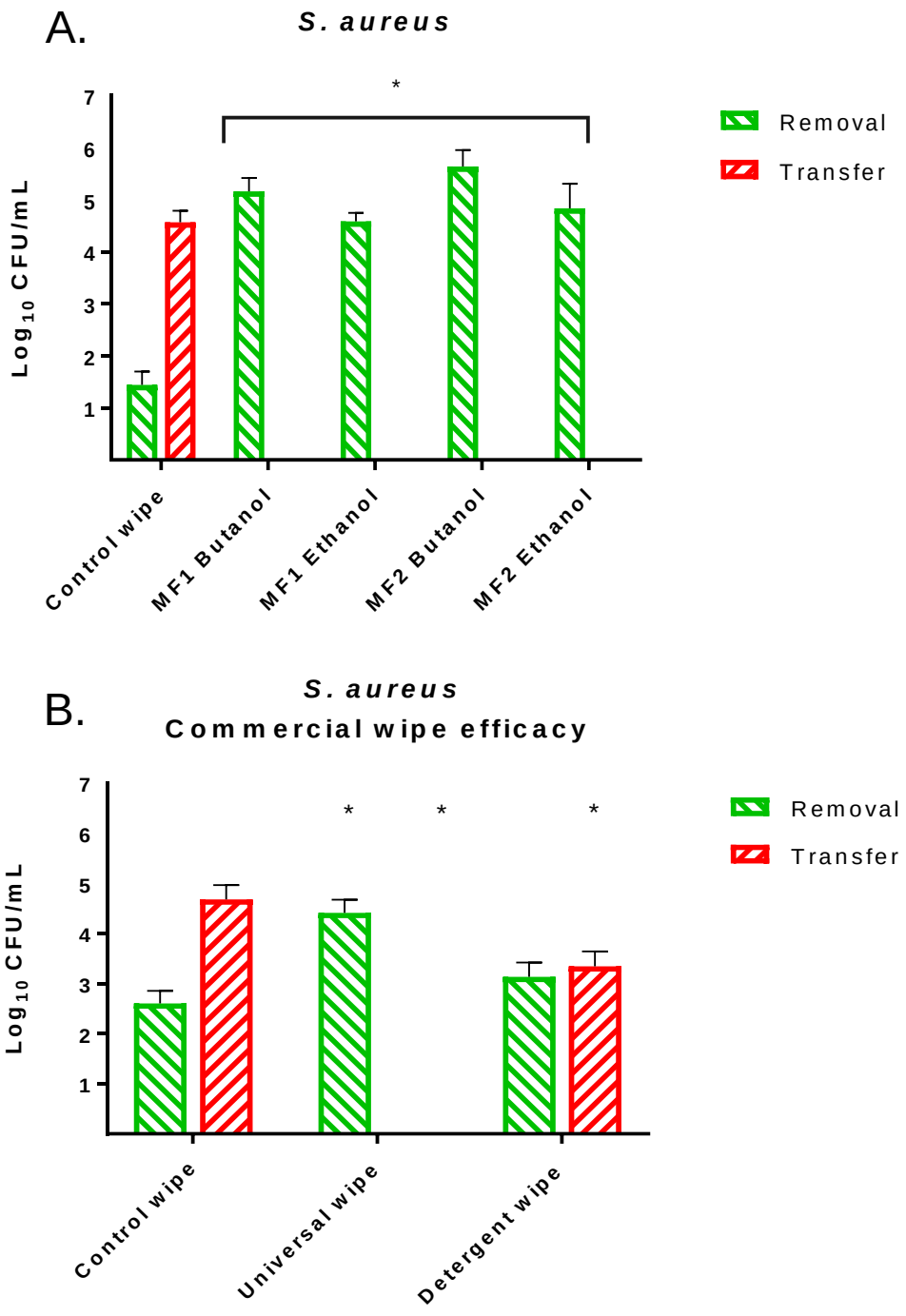
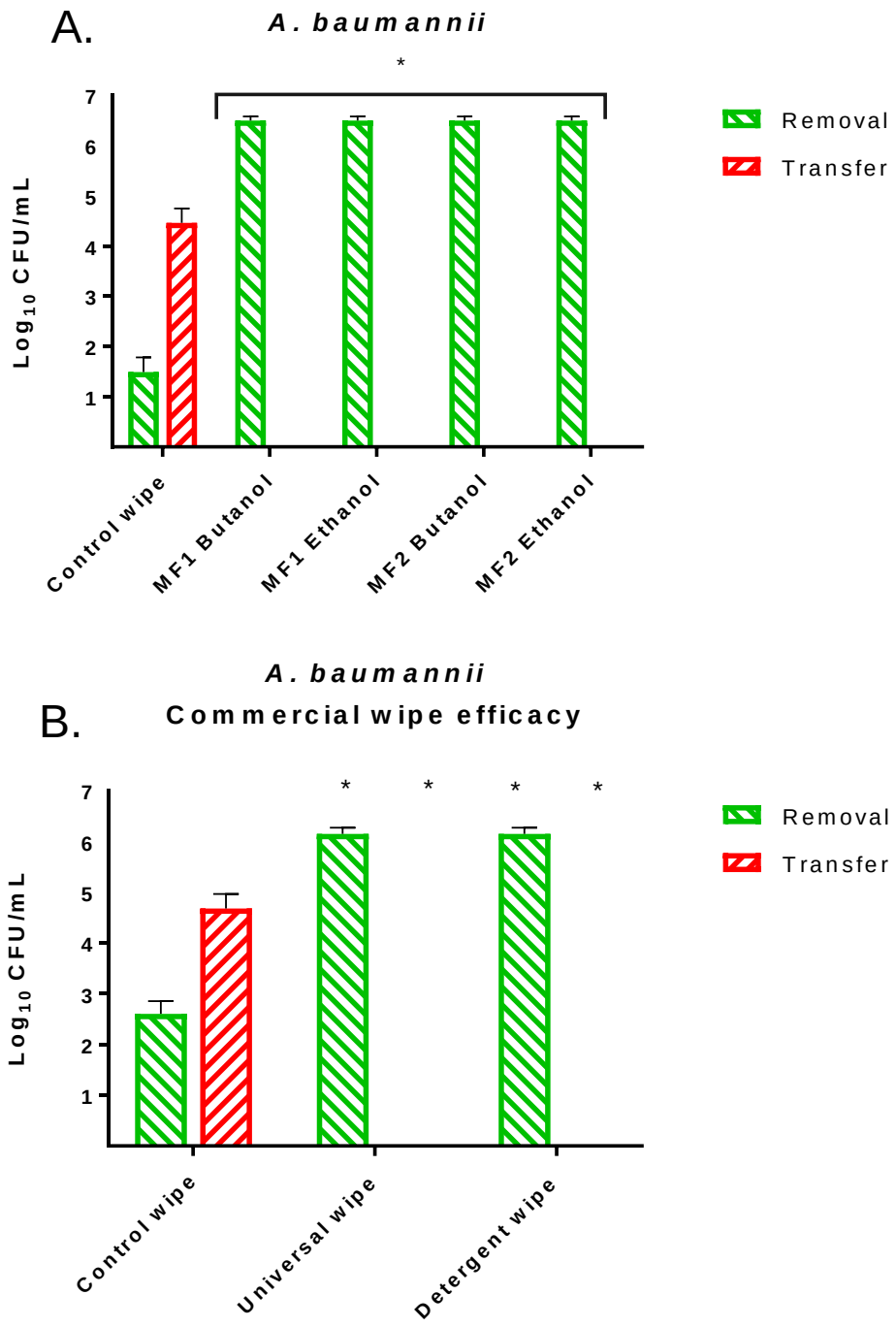


Figure 5.7 Reduction and transfer of *A. baumannii* with higher organic soiling using Wiperator® method.

(A) *A. baumannii tested* with control, MF1 and MF2 wipes (B) *A. baumannii* tested with control wipe, Universal wipe and Detergent wipe. Results are of three biological repeats (n=3) with two technical repeats. A two-way ANOVA was carried out with Tukey's multiple comparison. * indicates significant difference ($p < 0.05$) to the control wipe Error bars represent SD.



5.4.2 4-Field test

5.4.2.1 Wet wipes

Wipes were then tested following the BS EN 16615 4-field test.

Wet wipes soaked in 1% (w/v) MF1/MF2 with either butanol or ethanol co-surfactants completely removed *S. aureus* and *A. baumannii* from square 1 and had a significantly higher ($p < 0.001$; one-way ANOVA) (Figure 5.8 & Figure 5.9) Log_{10} reduction compared to the control wipes. Furthermore, there was no recovery from squares 2-4 for *S. aureus* or *A. baumannii*, when MF1/MF2 wipes were tested, in contrast the control wipe which recovered significantly more of both micro-organisms ($p < 0.001$; one-way ANOVA)

There was no difference between the Universal wipe and the MF1/MF2 wipes in reduction of *S. aureus* and *A. baumannii* on square 1 ($p > 0.999$; one-way ANOVA) or their recovery from squares 2-4 ($p > 0.999$; one-way ANOVA). The detergent wipe was not significantly different from the control wipe in reducing *S. aureus* from square 1 or recovery from squares 2-4 ($p > 0.999$; one-way ANOVA). Against *A. baumannii* the detergent wipe significantly lowered bioburden on square 1 ($p < 0.001$; one-way ANOVA) and reduced transfer to square compared to the control wipe ($p < 0.001$; one-way ANOVA), but recovery of *A. baumannii* from squares 3 ($p = 0.483$; one-way ANOVA) and 4 ($p = 0.728$; one-way ANOVA) were not significantly different from the control

Figure 5.8 Reduction and transfer of *S. aureus* after use of MF1, MF2 and commercial wet wipes using the 4-field test method.

(A) The reduction of *S. aureus* from sq 1 (B) Transfer of *S. aureus* to sq 2-4. Results are of three biological repeats (n=3) with two technical repeats. A one-way ANOVA used for (A) and a two-way ANOVA for (B) both were followed by Tukey's multiple comparison. * indicates significant difference ($p < 0.05$) to the control wipe. Error bars represent SD

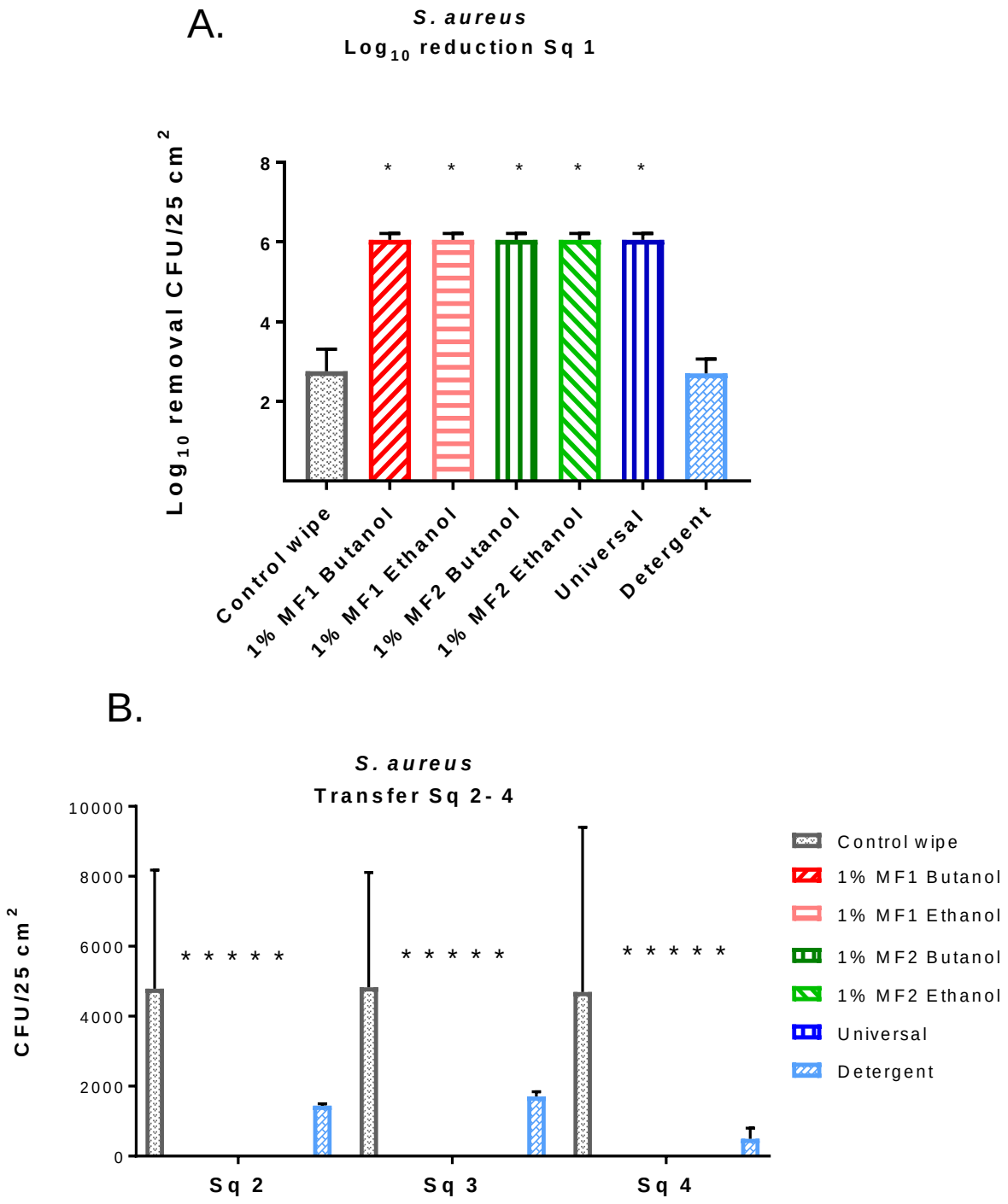
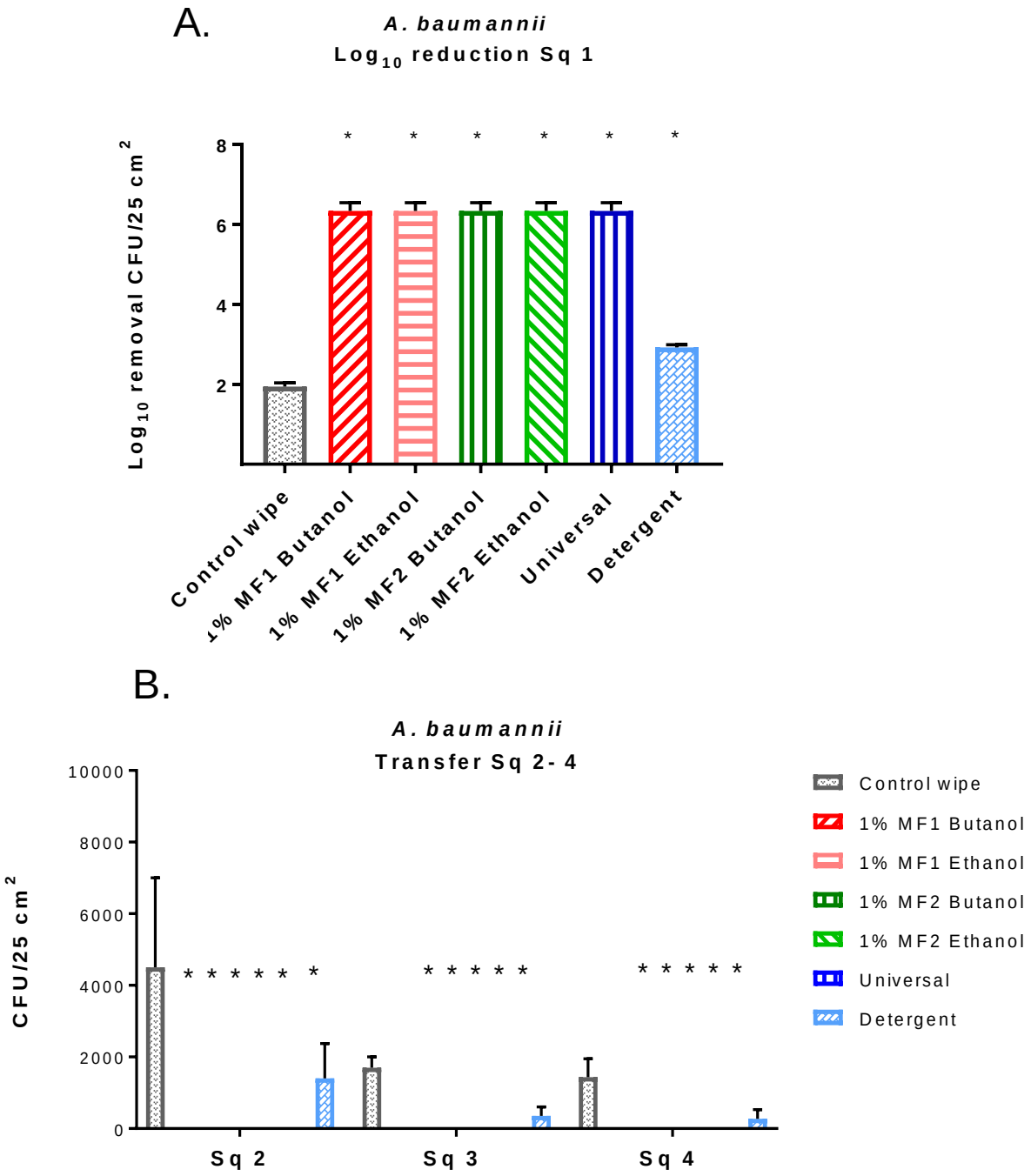


Figure 5.9 Reduction and transfer of *A. baumannii* after use of MF1, MF2 and commercial wet wipes using the 4-field test method.

(A) The reduction of *A. baumannii* from sq 1 (B) Transfer of *A. baumannii* to sq 2-4. Results are of three biological repeats (n=3) with two technical repeats. A one-way ANOVA used for (A) and a two-way ANOVA for (B) both were followed by Tukey's multiple comparison. * indicates significant difference ($p < 0.05$) to the control wipe. Error bars represent SD



5.4.2.2 Dry wipes vs. commercial spill wipes

Dry wipes were also tested using an adapted BS EN 16615 4-Field method.

Figures 5.10 and 5.11 show the reduction for all wipes fell below the required 5 Log_{10} to pass the 4-field test. However, the MF2 wipe did remove ≥ 4 Log_{10} of *S. aureus* and ≥ 3 Log_{10} of *A. baumannii*, which was significantly lower than the control wipe and the Gama spill wipe ($p < 0.05$; one-way ANOVA).

The transfer of *S. aureus* (Figure 5.10B) to squares 2 - 3 was also lower with the MF2 wipe than the control and the GAMA spill wipe ($p < 0.05$; two way ANOVA), but there was no difference for square 4 for any of the wipes ($p > 0.05$; two way ANOVA). Against *A. baumannii* the MF2 wipe resulted in lower transfer to square 2 and 3 compared to the control wipes but was only statistically lower than the GAMA spill wipe at square 2 ($p > 0.05$; two-way ANOVA) (Figure 5.11B). At square 4 there was no difference between the wipes ($p < 0.05$; two-way ANOVA).

Figure 5.10 Reduction and transfer of *S. aureus* after use of MF2 dry wipe, GAMA spill wipes and control wipe using the 4-field test method.

(A) The reduction of *S. aureus* from sq 1 (B) Transfer of *S. aureus* to sq 2-4. Results are of three biological repeats (n=3) with two technical repeats. A one-way ANOVA used for (A) and a two-way ANOVA for (B) both were followed by Tukey's multiple comparison. * indicates significant difference ($p < 0.05$) to the control wipe. Error bars represent SD

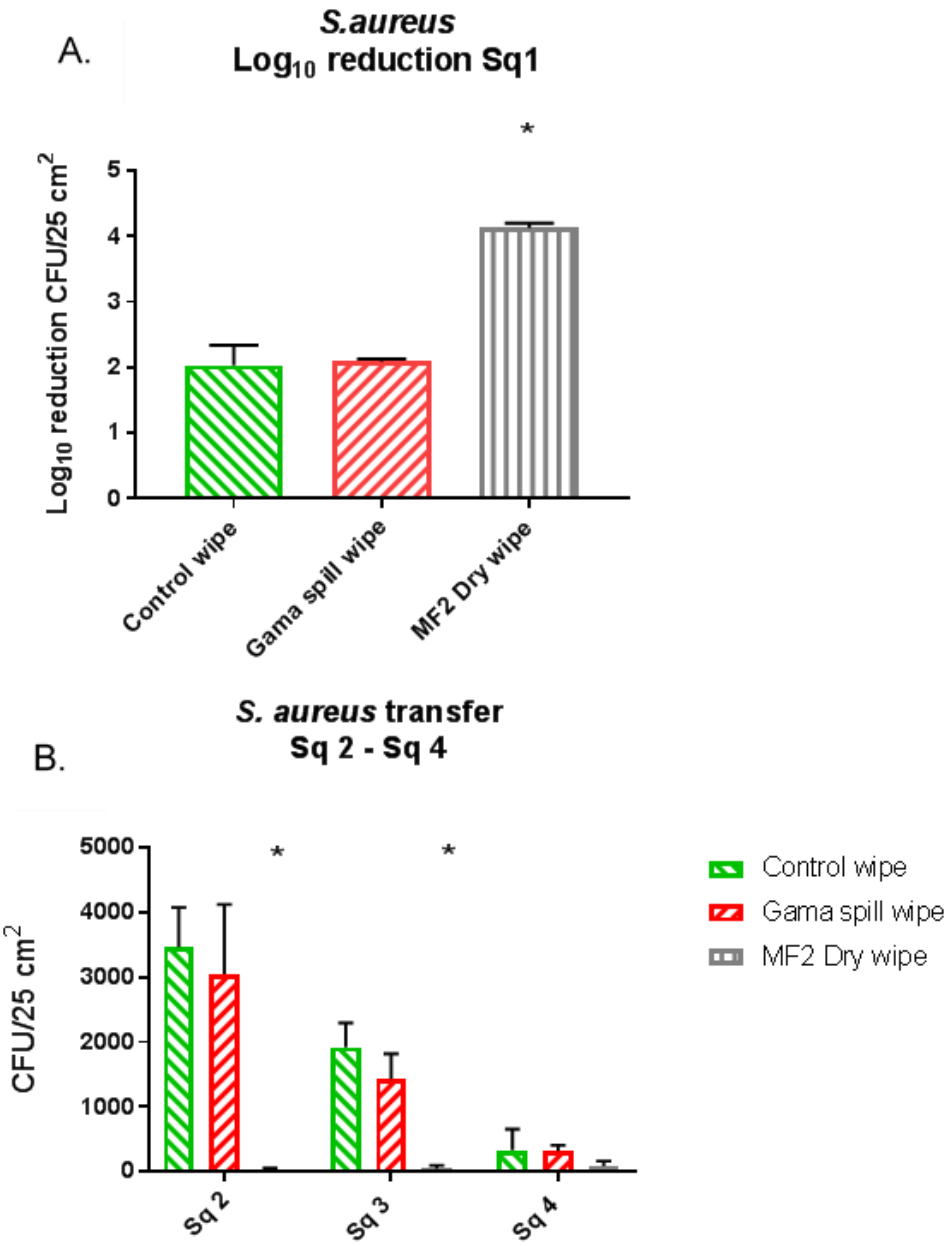
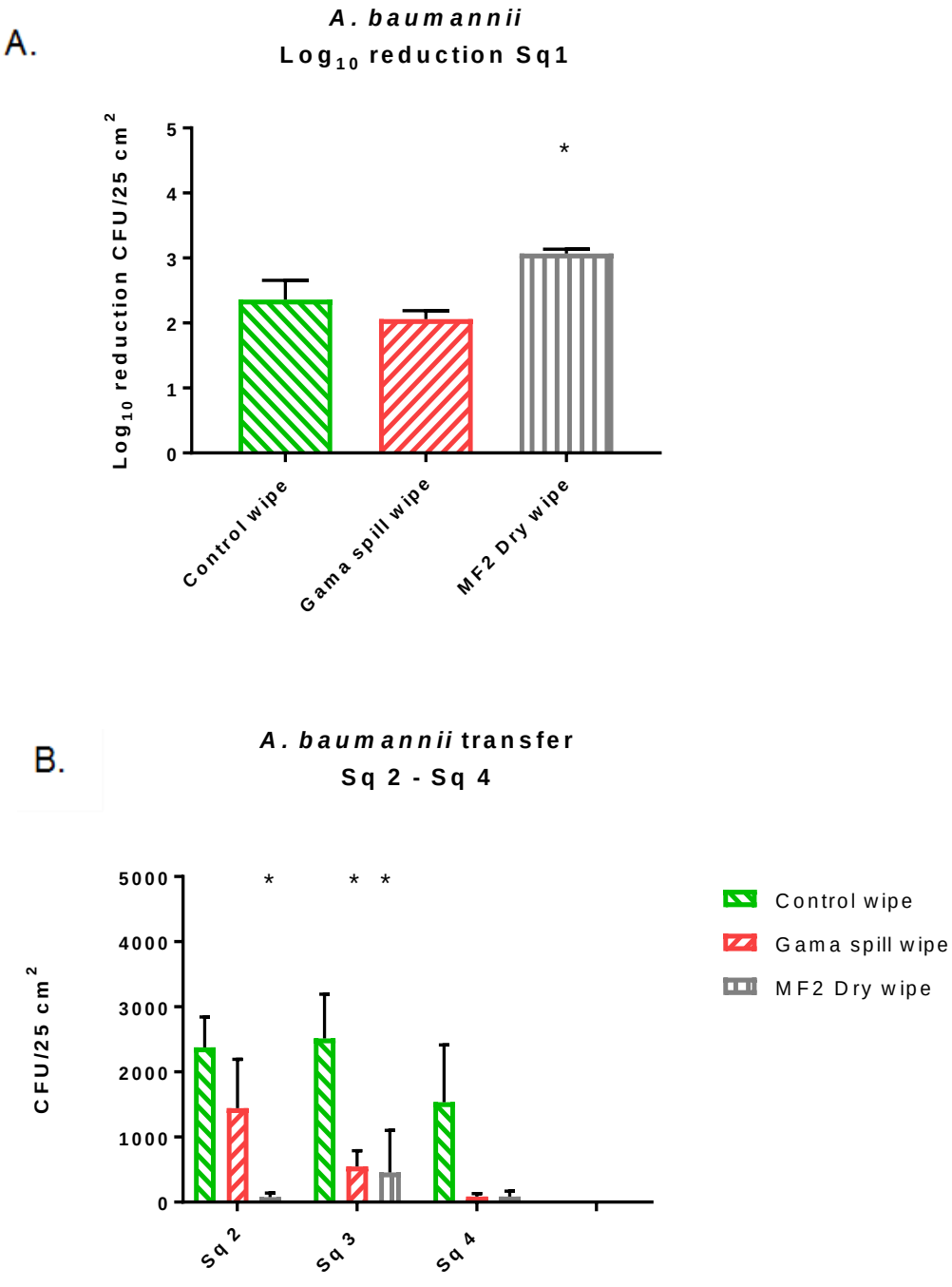


Figure 5.11 Reduction and transfer of *A. baumannii* after use of MF2 dry wipe, GAMA spill wipes and control wipe using the 4-field test method.

(A) The reduction of *A. baumannii* from sq 1 (B) Transfer of *A. baumannii* to sq 2-4. Results are of three biological repeats (n=3) with two technical repeats. A one-way ANOVA used for (A) and a two-way ANOVA for (B) both were followed by Tukey's multiple comparison. * indicates significant difference ($p < 0.05$) to the control wipe. Error bars represent SD.



5.4.3 MTT Assay

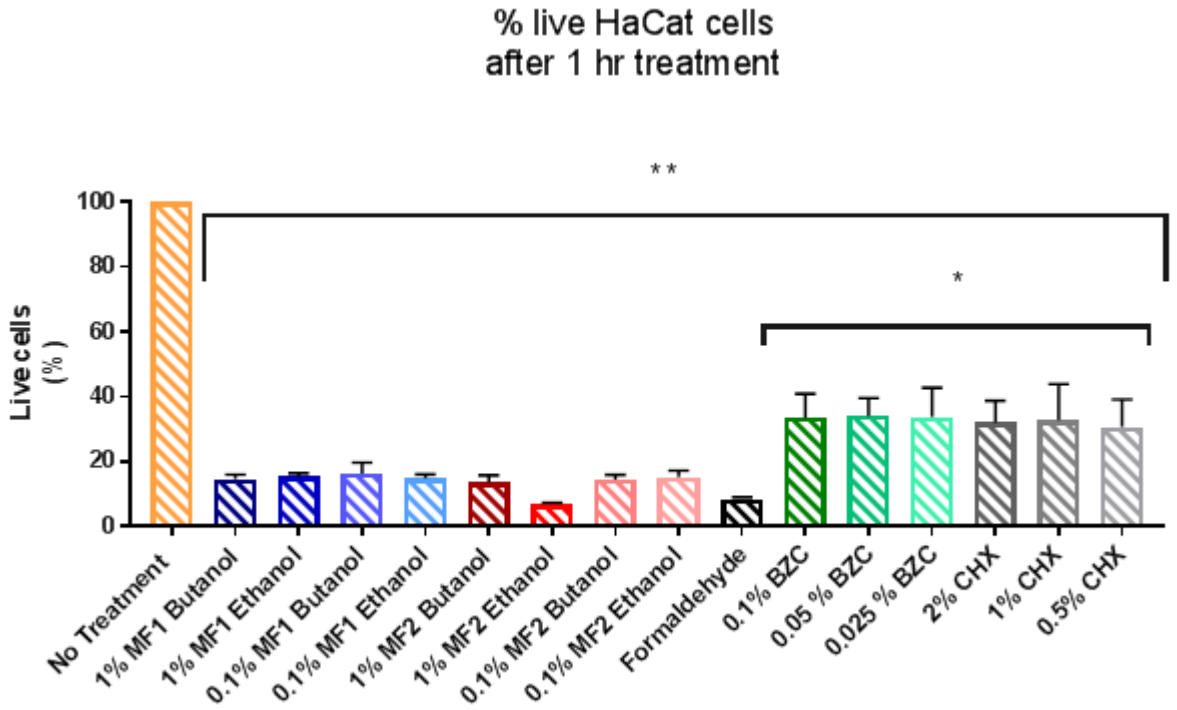
An MTT assay was performed to determine any cytotoxic effects of formulations against human keratinocytes (HaCat cells).

After a 1 hr treatment, Figure 5.12 shows that all solutions tested resulted in a significantly lower number of viable cells for the no treatment control ($p < 0.001$; two-way ANOVA). MF1 and MF2 solutions were not statistically significant ($p > 0.05$; two-way ANOVA) to the positive control (formaldehyde).

All BZC and CHX solutions had significantly more live cells remaining after treatment compared to any of the MF1, MF2 or formaldehyde solutions ($p < 0.05$; two-way ANOVA).

Figure 5.12 Cytotoxicity of biocides.

Cells were treated for 1 hr with MF1 (0.1 and 1 % (w/v)) with ethanol or butanol co-surfactant, MF2 (0.1 and 1 % (w/v)) with ethanol or butanol co-surfactant, formaldehyde (3 % v/v), BZC (0.025, 0.05 and 0.1 % (w/v) and CHX (0.5, 1 and 2 %). Tests were carried out in triplicate (n=3) and a one-way ANOVA was performed followed by Turkey's multiple comparisons. ** shows p< 0.001 compared to the no treatment control and * signifies *p* value of < 0.05 compared to the positive control. Error bars represent SD.



5.5 Discussion

The development of an antimicrobial product requires thorough evaluation to ensure antimicrobial efficacy translates from bench side to field use.

In this chapter the MF1 and MF2 were evaluated as wet wipes intended for disinfection of healthcare surfaces. They were compared to wipes widely used in the NHS. In addition, a second product a 'dry' wipe which incorporated 1-dodecyl-3-methylimidazolium bromide or 'MF2' between two sheets of wipe material was designed. The main aim of the dry wipe was to disinfect in the presence of large organic soiling such as blood. The efficacy of the 'MF2 dry wipe' was compared to a 'spill' wipes used in the NHS to decontaminate and absorb large spills.

First, the efficacy of the formulations as a wet wipe was tested to observe any differences in co-solvents (alcohol) that would affect the product. Also, the formulations were diluted and the efficacy in a wipe was compared to commercial wipes.

To assess efficacy the ASTM 2967-15 standard was performed to challenge wipes with orbital wiping motion weighted at 150 g. The advantage of using a device such as the Wiperator® for testing is that certain variables can be controlled, giving comparable results. One such factor that aids the repeatability of the test is the weight exerted by the machine when in use, the weight of 150 g was chosen after observing volunteers wiping on a single pan balance and noting that the weight applied varied between 100-300 g (Sattar *et al*., 2001).

As shown in Figure 5.3A and Figure 5.3B, at the highest concentrations tested (1 % w/v), MF1 and MF2 were highly efficacious against *S. aureus*, however the butanol formulations were more efficient at removing *S. aureus* from the first surface and did not lead to transfer on to a second surface which was seen when MF1/MF2 were mixed with ethanol. Against *A. baumannii* all 1 % (w/v) wipes removed the Gram-negative bacteria from the first disk and no transfer was observed (Figure 5.4A and Figure 5.4B), this was regardless of MF1/MF2 or the co-surfactant used.

At 0.1 % (w/v), both MF1 and MF2 were capable of removing *S. aureus* from stainless steel disks but transfer was observed for all conditions irrespective of MF1/MF2 or of co-surfactant used. At 0.1% (w/v) with *A. baumannii*, MF1 shown transfer of the bacteria with

both co-surfactants, but MF2 did not produce any transfer to a second surface and at this concentration MF2 performed better with butanol as a co-surfactant.

With 0.01 % (w/v) both MF1 and MF2 were ineffective at removal of *S. aureus* or *A. baumannii*, with either ethanol or butanol as the co-surfactant. This coincides with the suspension test results in Chapter 3, where very little activity was seen with the formulations at 0.01 % (w/v).

Two wipes supplied by GAMA healthcare were also assessed under the same conditions as the MF1/MF2 wipes. The 'Universal' wipe that contains surfactant, < 1 % cationic biocides, preservatives and EDTA, is designed as a 2-in-1 biocidal and detergent wipe for the cleaning and disinfecting of healthcare surfaces. The second wipe was detergent based containing: Capryl Glucoside 0.2-0.5 %, Lauryl Polyglucose 0.1-0.3 % and Butoxydiglycol 0.1-0.2 %, with an intended use of cleaning surfaces before disinfecting.

Both commercial wipes do not claim efficacy against any bacteria using the ASTM E2967-15 method, however the Universal wipe is commonly used for disinfecting surfaces and was chosen as a wipe for comparison of efficacy. The detergent wipe was not expected to lower the bioburden of bacteria and in fact detergents can spread bacteria from surface to surface, so it was chosen to demonstrate transfer compared to MF1 and MF2 wipes. For the ASTM E2967 – 15 standard, there is no minimum for the removal of bacteria from the stainless steel disks to 'pass', and therefore in order of highest removal of *S. aureus,* wipe efficacy was 1 % (w/v) MF2 butanol > 1 % (w/v) MF1 butanol > 1 % (w/v) MF2 ethanol > 1 % (w/v) MF1 ethanol > Universal > detergent.

As the activity at 0.1 % and 0.01 (w/v) MF1 and MF2 shows transfer of *S. aureus* and *A. baumannii*, they were not tested at a higher organic soiling of 3 g/L BSA + 3g/L sheep erythrocytes. Again, all wipes worked well against *A. baumannii*, with complete removal from the first surface and no transfer on to a second. The order of activity of the wipes against *S. aureus* with the higher organic soiling was that same as with just 3g/L BSA.

The wet wipes were also tested using the BS EN 16615 4-field test. Using the 4-field test, the required reduction for square 1 is a minimum of 5 Log_{10} and recovery of bacteria from each of the squares 2-4 should be ≤ 50 CFU/cm2. All wipes were used with a 2 second wipe motion and any remaining bacteria was recovered after 5 min. For *S. aureus* and *A. baumannii* all MF1/MF2 wipes and the Universal wipe gave a > 5 Log_{10} reduction with no

recovery of any of the bacteria on squares 2-4. In contrast, the detergent wipe, gave a < 3 Log_{10} removal and each square recovered over 50 CFU/cm^2.

Overall the 1 % MF1/MF2 wipes were effective with the wiperator and the 4-field standards. However, the wipes were shown to be more effective using the 4-field test, including the Universal wipe that had an increase of > 2 Log_{10} reduction of *S. aureus* compared to the Wiperator test. The difference in efficacy was also observed in another study that evaluated the antimicrobial wipes using the two wipe standards. The authors concluded that the differences between the weight used to wipe (2.5 kg for 4-field, 150g for wiperator) was a major cause for the difference in efficacy, mostly likely caused by an increased friction from the heavier weigh used (Wesgate *et al*., 2016). Other factors that were different between the two methods are also likely to impact the results, such as the longer contact time applied in the 4-field test. The surface materials that are used for each test will also impact the outcome of the results as the smoother the topography the easier it is to disinfect, which may explain why wipes performed better on the PVC surface (Sattar & Maillaird, 2013)

Both standard protocols for testing wipe efficacy are valuable in scrutinising a wipe product and the difference in methods (i.e different surfaces, different mechanical wiping) are more likely to reflect the variety of ways a wipe would be used in healthcare.

The second product, the dry wipe containing 1 g (w/w) powder MF2, was also assessed using the 4-field method. The purpose of the dry wipe was to absorb liquid and disinfectant the surface simultaneously. Currently, there are no standard methods to evaluate wipes with large amounts of soiling such as blood that would be encountered in healthcare. Therefore, an adapted method was used in which sheep blood was placed onto square 1 over dried bacteria. Alongside the MF2 wipe a ‘spill’ wipe from GAMA healthcare was also tested, the spill wipe contains powdered sodium percarbonate and tetraacetylethylenediamine (TAED), which when hydrated generates peracetic acid according to the manufacture.

When tested in the presence of blood the MF2 wipe produced a greater Log_{10} reduction against both *S. aureus* and *A. baumannii,* compared to the control wipe and the GAMA spill wipe. Moreover, there was less transfer to squares 2-3 with the MF2 wipe, although at square 4 there was little difference between wipes. Although the MF2 wipe did not show a 5 Log_{10} reduction at square 1, the method was adapted with higher organic soiling so a lower reduction is not surprising. Interestingly, the GAMA spill wipe did not show much activity compared to the control wipe, the wipe has not been tested using the 4-field test in

the presence of blood. The relatively low activity seen by the GAMA wipe could be explained by blood content, around 45 % is composed of red blood cells, thus lowering the amount of available water to activate the reaction needed to generate PAA (Beilin *et al.,* 1966). Secondly, as the PAA precursors need to react before generating PAA, the short contact time that the wipe is in touch with the surface (2 seconds) is unlikely to be long enough for the reaction to occur.

Cytotoxicity occurs as a result of toxic compounds leading to necrosis, apoptosis or a non-viable state of a cell and many biocides are known to possess cytotoxic effects. (Punjataewakupt *et al*., 2019). The cytotoxicity of ILs in formulations (MF1 and MF2) alongside BZC and CHX, two common biocides, were investigated using HaCat cells. After a 1 hr incubation, all biocides, at all concentrations tested resulted in a significant decrease in cells that could metabolise MTT to formazan. Although, BZC and CHX treatments were slightly less toxic, only 30-35 % of cells were viable. The highest concentration of CHX (2 % (v/v) is used in wipes designed for topical disinfection (Clinell skin disinfectant wipes), BZC is a common excipient in many products and is considered a skin irritants at concentrations greater than 0.1 % (Merianos, 2011; Committee for Human Medicinal Products, 2017; Bondurant *et al*., 2019).

The cytotoxicity of ILs have been noted elsewhere, with some researchers investigating the use ILs for topical drug use. In one study it was found that Imidazolium-based ILs were cytotoxic against HaCat cells and that cytotoxicity increased upon lengthening of the alkyl chain from ethyl (C2) to hexyl (C6) (Santos de Almeida *et al*., 2017). This was also highlighted by Yoo and colleagues, by molecular modelling studies in which an increase in hydrophobicity of ILs was related to the length of the alky chain, resulting in their insertion into the membrane, causing membrane disruption and cell death (Yoo *et al*., 2016).

5.6 Conclusions

Two wipe prototypes were developed, a wet wipe with MF1 and MF2 formulations and a dry wipe containing powdered MF2. Both were developed with the aim of showing that IL formulations can be used in combination with wipe material to develop products that have effective antimicrobial activity.

Different variations of formulations were combined with material and the following conclusions were found;

- The reduction and transfer of Gram-positive and Gram-negative bacteria was not greatly affected by the exchange of co-surfactant butanol for ethanol with 1% (w/v) MF1 or MF2.
- At lower concentrations, MF1 and MF2 wet wipes were more likely to transfer bacteria than at higher concentrations.
- The activity of the 1 % (w/v) MF1 and MF2 wet wipes was not hindered by increased organic load and was more effective at reducing bacteria than commercial wipes.
- For wet wipes, difference in efficacy was observed between the two standard methods ASTM 2967-15 and BS EN16615:2018.
- Using an adapted BS EN 4-field test, the dry MF2 wipe was unable to produce a ≥ 5 Log_{10} reduction, but out-performed the commercial wipe in the reduction and transfer of bacteria.
- MF1 and MF2 at 1 and 0.1 % (w/v) were cytotoxic to cells after 1 hour of treatment, this was biocides BZC and CHX

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6 General discussion

6.1 Introduction

6.1.1 Study overview

Infection and prevention and control measures are important interventions in limiting the rate of HCAI. One such measure is the disinfection of surfaces that can act as reservoirs for microorganisms and implementing disinfection practises can significantly lower the rate of HCAI (Cohen *et al*., 2010; Lawley *et al*., 2010; MacCannell *et al*., 2011; McDonald & Arduino, 2013; Muto *et al*., 2013; Watson *et al*., 2016). There are many issues to consider when selecting the right disinfection process for a healthcare setting, factors such as the type of microorganism, the time needed for a disinfectant to be in contact with microorganism, delivery of the formulation, aspects that can affect the disinfectant (organic soiling, pH, temperature etc.) (Sattar and Maillard 2013). Furthermore, ease of use will always need to be taken into account as inconvenient methods may result in noncompliance.

This thesis aimed to evaluate the use of ionic liquids (ILs) for the development of products that effectively disinfect healthcare surfaces. This was approached by assessing factors that hinder activity of disinfectants in healthcare. This was followed by understanding the mechanisms of action of ILs against Gram-negative and Gram-positive bacteria. Finally, cytotoxicity of the formulations was measured, and ILs were combined with material to produce a wet wipe and dry wipe and efficacy was compared to wipes that are commercially available.

6.2 Microbicidal activity

The formulations MF1 and MF2 containing the imidazolium-based salts; 1-dodecyl-3-methylimidazolium hydrogen carbonate and 1-dodecyl-3-methylimidazolium bromide, were assessed for their activity against yeast, Gram-negative and Gram-positive bacteria. The antimicrobial activity of ILs has been studied previously but is often examined by MIC testing, and factors that affect biocides in healthcare are not considered (Luczak *et al*., 2010). The effects of dilution, organic soiling, hard water, contact time and type of microorganism on the antimicrobial activity of MF1, MF2, BZC and CHX were assessed using a Phase 2 step 1 suspension test (British Standards Institute, 2002; British Standards Institute, 2013).

The main factor that impeded the activity of MF1 and MF2 was dilution. At 1 % (w/v) and 0.1 % (w/v) activity was observed against Gram-negative and Gram-positive bacteria and yeast that are associated with HCAI. However, activity at 0.01% (w/v) was insufficient to pass the BS EN 1276:2009 and BS EN BS EN61324:2013. Two commonly used biocides BZC and CHX were examined in parallel and comparisons between MF1/MF2 pass rates for the standard test are summarised in Table 6.1 and Table 6.2.

Table 6.1 Comparison of MF1, MF2, BZC and CHX at 5 min contact time.

P = pass of standard suspension test of ≥ 5 Log_{10} for bacteria and ≥ 4 Log_{10} for yeast. ***F*** = fails the standard test. White rows indicate organic soiling of 3 g/L BSA and red rows 3 g/L BSA + 3 g/L sheep erythrocytes.

Organism	MF1		MF2		BZC		CHX	
	1 %	0.1 %	1 %	0.1 %	1 %	0.1%	1 %	0.1 %
E. coli	P	P	P	P	P	P	P	P
	P	P	P	P	P	P	P	P
P. aeruginosa	P	P	P	P	P	***F***	P	P
	P	P	P	P	***F***	***F***	P	P
A. baumanniii	P	P	P	P	P	P	P	P
	P	P	P	P	P	P	P	P
S. aureus	P	P	P	P	P	P	P	***F***
	P	P	P	P	P	P	P	***F***
E. hirae	P	P	P	P	P	P	P	***F***
	P	P	P	P	P	P	***F***	***F***
C. albicans	P	P	P	P	P	P	P	P
	P	P	P	P	P	P	P	P

Table 6.2 Comparison of MF1, MF2, BZC and CHX at 0.5 min contact time.

P = pass of standard suspension test of ≥ 5 Log_{10} for bacteria and ≥ 4 Log_{10} for yeast. ***F*** = fails the standard test. White rows indicate organic soiling of 3 g/L BSA and red rows 3 g/L BSA + 3 g/L sheep erythrocytes.

Organism	MF1		MF2		BZC		CHX	
	1 %	0.1 %	1 %	0.1 %	1 %	0.1%	1 %	0.1 %
E. coli	P	P	P	P	P	P	P	P
	P	P	P	P	P	***F***	P	P
P. aeruginosa	P	***F***	P	***F***	P	***F***	P	P
	P	P	P	P	***F***	***F***	P	P
A. baumanniii	P	P	P	P	P	P	P	P
	P	P	P	P	P	P	P	P
S. aureus	P	P	P	P	P	P	P	***F***
	P	P	P	P	P	P	P	***F***
E. hirae	P	P	P	P	P	P	P	***F***
	P	P	P	P	P	P	***F***	***F***
C. albicans	P	P	P	P	P	P	P	P
	P	P	P	P	P	P	P	P

The antimicrobial activity of BZC and CHX were affected by a combination of increased organic soiling and the type of organism tested. As the net charge of BZC and CHX is positive it is likely that cationic biocides bind to the negatively charged proteins in BSA (Jono *et al*., 1986; Araújo *et al*., 2013). Therefore, it is possible that there is a decreased concentration of BZC and CHX that can enter the cell. Furthermore, loss in activity of BZC and CHX has been attributed to the expression of efflux pumps that actively relocate biocides outside of the cell (Braoudaki *et al*., 2004; Bjarnsholt *et al*., 2007; Horner *et al*., 2012). It could be that the lowered concentration of BZC and CHX as a result of binding to proteins, is reduced upon entering the cell, then the low concentrations may be pumped out of the cell. However, activity of MF1 and MF2 at 1 % (w/v) passed the standard tests against bacteria and yeast at both organic loads tested. The ability to remain active in the presence of organic soiling could be to the long alkyl chain (C12) as QACs with longer alkyl chains have retained activity compares to shorter chains (C8-C10) in the presence of BSA (Jono *et al*., 1986). Interestingly, at 0.5 min, 0.1 % MF1 and MF2 (Tab 6.2) performed better at a higher organic load than at 3 g/L BSA against *P. aeruginosa*. Whilst it is unknown why this occurred it could be that adding sheep erythrocytes to BSA prevented interaction of BSA with the formulations.

Another limitation of MF1 and MF2 was the lack of activity against *B. subtilis* spores. The unique structure of spores' present resistance mechanisms against many biocides, making them difficult to eliminate (Leggett *et al*., 2012). Even though, BZC and CHX were not tested against spores, others have reported that they are not sporicidal (Cook & Pierson, 1983; Russell *et al*., 1985; Russell, 2004).

Combining biocides has several benefits; (i) low activity could be enhanced through synergistic actions of multiple biocides or excipients (ii) biocides can be used at lower concentrations, so decreasing environmental impact (iii) biocides can circumvent the development of resistance (Cowley *et al*., 2015).

Here, MF1 and MF2 were combined with H_2O_2 to determine any synergistic activity against vegetative cells and *B. subtilis* spores. As cationic biocides/surfactants generally impact the cell membrane they allow easier access for biocides that have intracellular targets (Ríos-Castillo, González-Rivas & Rodríguez-Jerez, 2017). Overall, the combination of the two biocides in a formulation did not prove to enhance efficacy and activity appeared to be from the single biocides rather than collectivelly. This was possibly due to the concentration of MF1/MF2, at such low concentrations, the membrane of the cells most likely stays intact

and H_2O_2 does not reach cellular targets faster. The low concentration was chosen as at 0.1 % MF1/MF2 possess potent antimicrobial activity and benefits of adding another biocide would be over-shadowed. However, it could be that increasing the concentration between 0.1 % (w/v) and 0.01 % (w/v) would potentially enhance combined H_2O_2.

Testing efficacy of biocides against factors that may hinder their activity is an essential screening process. From suspensions tests it can be concluded that the role of the anion of the imidazolium salts did not play a major role in the activity (Lukzac *et al*., 2009). The contact time of 30 s is short enough for use in healthcare disinfection. Instead of cleaning surfaces before disinfection, formulations could be used as a '2-in-1' as formulations were still efficacious in the presence of organic soiling. The addition of an oxidising biocide could be included in formulations to increase the activity spectrum to other microbes such as spores.

6.3 Mechanisms of action

Assessment of the MOA is important aspect of biocide development as it aids optimisation and highlights any potential resistance mechanisms that may arise (Chapman, 2003). Multiple methods were used to assess if membrane damage was the leading cause of cell death. As cationic biocides such as BZC and CHX are known to destabilise membranes, it was hypothesised that MF1 and MF2 would be membrane active. After treatment with MF1 and MF2, there was leakage of K^+ from *S. aureus* and *E. coli.* As there was no difference between the concentrations tested indicating that both concentrations caused cell lysis and only with lower concentrations could a dose-dependent release of K^+ be possible.

Membrane damage was assessed further by use of a BacLight™ kit which consists of two fluorescent DNA-binding dyes PI and SYTO 9. As PI binds to DNA after entering through compromised membranes, increases in fluorescence intensity is an indicator that MF1 and MF2 target membranes. SYTO 9 on the other hand binds to DNA of cells with intact membranes but is displaced by PI which has a stronger binding affinity (Warning, 1965; Arndt-Jovin & Jovin,1989; Stocks, 2004). After treatment with MF1 and MF2 the ratio PI to SYTO 9 fluorescence was much greater indicating the majority of the cell population had membrane damage. For *S. aureus* there was no difference between the concentrations indicating that above 0.1 % the formulations caused the most membrane damage possible. Against *E. coli* the treatments also caused significant damage, a small difference was observed between 1 % (w/v) and 0.1 % (w/v) for MF1 and MF2, which could be due to the

outer membrane of Gram-negative bacteria reducing permeability of cationic biocides (McDonnell & Russel, 1999).

Using microscopy techniques, the aim was to visualise any damage to the cell using SEM and then quantify any changes in surface morphology using AFM. Unlike the assessment of K^+ leakage and BacLight™, with SEM there was difference observed between concentrations for both MF1 and MF2. At 1 % (w/v) cells were damaged and the spherical structure of *S. aureus* was lost. Interestingly, not all cells appeared to have lost their shape and it is unclear why some cells would be damaged and others not, one explanation could be that cells were clustered and the formulation did not fully penetrate through the mass of cells in the 5 minute contact time. Lowering the concentration of MF1 and MF2 correlated with a lessened impact on the cell structure. At 0.1 % (w/v) cell outlines were visible although it was clear that cells were damaged, at 0.01 % (w/v) cells retained their cocci structure.

Through SEM it appeared MF2 had a greater impression on the structure of Gram-positive cells as compared to MF1 at equivalent concentrations the structure of the cell appeared more damaged. This was especially true at 0.01 % (w/v) were cells treated with MF2 had a rougher surface topography. As the only difference between the formulations is the anion on the imidazolium salt, the increased roughness must be due to the bromide ion on MF2. The difference between MF1 and MF2 at 0.01 % (w/v) correlates with the suspension test performed in Chapter 3 (Figure 3.1C) in which MF2 caused a greater Log_{10} reduction against *S. aureus* than MF1.

The cells were then analysed after treatment with MF1 and MF2 using AFM. The surface topography did not appear rougher when 1 % (w/v) or 0.1 % (w/v) were used. So, the concentration was increased to 5 % (w/v) to emphasise any minute changes. After increasing the concentration there was a significant number of valleys found in the outer structure of *S. epidermidis* when treated with MF2. However, there was no difference between the contact times suggesting the valleys developed within 5 minutes and did not increase over time. It is not known how the indents in the structure developed after treatment, but the most likely explanation was that after the ILs interact with the cell membrane, the cell contents leak out leading to collapse of the cell from the inside, creating pits in the structure of *Staphylococcus*. The cells overall kept a resemblance of a cocci shape. Possible reasons *S. epidermidis* maintained its cell shape is due to the thick peptidoglycan layer of the cell wall and lack of outer membrane (Vollmer & Holtje, 2004). Rončević *et al* reported increased PI uptake by *Staphylococcus* upon treatment with the

antimicrobial peptide Melittin, but they did not report any significant structural disruption using AFM, emphasising that membrane damage may not always translate to structural damage for Gram-positives (Rončević *et al*., 2018).

Whilst K^+ leakage and BacLight™ studies do not differentiate between the antimicrobial activity of MF1 or MF2, SEM and AFM provide visual and quantitative evidence that MF2 causes greater structural damage. The difference in the anion has been noted as playing a subtle role in the antimicrobial activity of ILs, however, the small bromide ion of MF2 could eased the transport of the IL to the membrane increasing the rate at which it can interact with the membrane, however, bulkier anions such as CO_4^- may hindered transport of the IL to the membrane (Lukzac *et al*., 2010).

Cationic biocides often target the bacterial membrane, causing disruption and cell death. However, biocides often have multiple cellular targets. For instance, BZC is known to interact with the cell membrane at high concentrations but at lower concentrations can cause disruption of cytosolic proteins (Knauf *et al*., 2018). To demonstrate any potential intracellular targets, *B. subtillis* spores lacking mechanisms that protect biomolecules such as DNA were treated with ILs. *B. subtilis* spores lacking either protective DNA proteins ($\alpha^-\beta^-$ SASPs) or a fully functioning spore coat ($CotE^-$) were not susceptible to MF1 and MF2. On the other hand, H_2O_2 produces reactive oxygen species (ROS) that interacts with DNA, and so H_2O_2 had increased activity against spores, especially those deficient of $\alpha^-\beta^-$ SASPs (Imlay & Linn, 1988). Although, the interaction of MF1 and MF2 with DNA and other biomolecules can not be eliminated by this study, it does highlight that they do not interact as strongly with intracellular targets as H_2O_2, and so cytosolic components are probably not their main target.

6.4 Product development

To reduce the burden of HCAI, infection prevention and control measures need to be effective. Therefore, it is critical that products claiming efficacy against microbes at certain contact times and concentrations have been tested with their intended use in mind. Even though antimicrobial tests in suspension are a useful screening tool, they do not take into account the effect of; cells adhered to a surface, the combination of a biocide and mechanical action of wiping or how biocides perform when combined with material. For that reason, in Chapter 5, MF1 and MF2 were combined with material to develop products that would be efficacious against bacteria on surfaces.

Wet wipes were produced to first assess if the formulations retained antimicrobial activity in combination with material (non-woven fabric) (Sattar and Maillard 2013), and second they were compared to commercial wet wipes. The main findings from testing the wipes was that at 1 % (w/v) MF1 and MF2 were efficacious against *S. aureus* and *A. baumannii*, and transfer of bacteria was negligible. Changes in the co-surfactant were also assessed to a widely used excipient, ethanol (Rowe *et al*., 2009). At 1 % (w/v) MF1 and MF2, the change from butanol to ethanol did not make a significant difference to the antimicrobial activity of the product. When compared to two wipes; a 'Universal' wipe (for cleaning and disinfection) and a detergent wipe (intended for cleaning only) that are used in healthcare, the 1 % MF1 and MF2 wipes produced a greater reduction of *S. aureus* when tested using the Wiperator®, but there was no difference observed using the 4-field method. By using both protocols, this data agrees with other studies that have observed differences between the standard wipe tests (Wesgate *et al*., 2019). Although the 4-field aims to reflect a more realistic approach for how a wipe would be used, the unitary weight of 2 kg will contribute to effectiveness of the wipes and is most likely an over-estimate of the weight exerted by the user. So, even though the Universal wipe performed equally well using the 4-field method, the results for the Wiperator® would most likely represent the proper use of the wipe.

The second wipe product was developed with the intention of assessing MF2 as a product that would disinfect and absorb large amount of bodily fluids simultaneously. This wipe would provide a more convenient method of cleaning up hazardous fluids in lieu of spill kits that commonly used. However, there is no standard method for wipe testing in the presence of bodily fluids, so the 4-field test was adapted to included dried bacteria and liquid blood. Upon testing, the MF2 wipe was more effective at reducing the initial inoculum of *S. aureus* and *A. baumannii* and their subsequent transfer over the surface compared to the GAMA spill wipe that contains dry precursors for the development of PAA. The spill wipe produced by GAMA has not been tested in the presence of organic soiling. Although (when activated) the PAA wipe has demonstrated activity against various organisms, the wipe is inefficient at cleaning fluids such as blood (its intended use) as the precursors most likely do not have sufficient liquid and time to produce PAA. Therefore, the addition of the dry precursors in the current wipe does not add any advantage compared to a wipe without the chemicals.

The concentration at which MF1 and MF2 demonstrate potent antimicrobial activity (1 % (w/v)), proved to be cytotoxic to skin cells when tested *in vitro*. The net charge of mammalian cells is not as negative as prokaryotic cells; however, it is likely that ILs interact

with the membrane and cause lysis of the cells (Dougherty *et al*., 1987; Ingolfsson *et al*., 2014). As with most biocidal products, appropriate PPE would be recommended.

6.5 Limitations and future work

The microbes used throughout this thesis were chosen as they are common nosocomial pathogens and whist, they represent yeast, spores, Gram-positive and Gram-negative bacteria, there are many pathogens that contribute to HCAI (Haque, 2018). To develop a product to reduce HCAI, viruses, *Mycobacteria* spp. and moulds should also be tested.

The prevalence of biofilms on healthcare surfaces has become increasingly recognised over recent years. Biofilms on healthcare surfaces for long periods of time without constant nutrients and moisture (Hu *et al*., 2015; Ledwoch *et al*., 2018). Furthermore, biofilms have been recovered after multiple cleaning and disinfection practices highlighting that disinfection products should be tested against biofilms (Otter *et al*., 2011; Almatroudi *et al*., 2016).

Currently, standardised biofilm models used for the testing of antimicrobials employ nutrient rich media with constant moisture and a high density of cells, but biofilms on surfaces are much likely to desiccate, have a low supply of nutrients with a lower cell density (American Society for Testing and Materials E2562-12, 2012; American Society for Testing and Materials E2647-13, 2013; American Society for Testing and Materials E2799-12,2012; Otter *et al*., 2015; Ledwoch *et al*., 2018; Ledwoch *et al*., 2019) Thus, in standard biofilm models, susceptibility may be less than biofilms found in healthcare. Development of biofilms that are grown in dry and wet phases are now being recognised as *in vitro* models to assess hard surface disinfectants, and if grow on disks can be used with the Wiperator® to incorporate mechanical action of wiping (Ledwoch *et al*., 2019).

Although this thesis explored the MOA of MF1 and MF2, which is important to assess development of resistance, any increases in resistance were not investigated. For biocides that target cells walls and/or membranes, changes in composition of phospholipids, fatty acids and protein in these structures have been reported (Jones *et al*., 1989; Brözel & Cloete, 1994; Guérin-Méchin *et al*., 1999a; Guérin-Méchin *et al*., 1999b; Tattawasart *et al*., 2000; Winder *et al*., 2000; Boeris *et al*., 2007). As MF1 and MF2 target membranes, changes in the membrane composition leading to increased resistance after repeated exposure is a possibility. Protocols to examine the development of resistance usually involve serial passages in the biocide below the MIC, increases in the MIC after repeated

exposure are considered resistant. Issues with this method when assessing biocidal resistance is the concentration is not reflective of what is used to disinfect a surface.

One study that developed to a protocol to test biocide resistance, measure the MIC before and after a suspension test. Whilst this method highlighted that diluted 'worst-case' scenario use of biocides did select for increased MICs, it is unlikely that these dilutions would take place with MF1/MF2 in a wipe as it would need 100-fold dilution before it would be ineffective against bacteria (Wesgate *et al*., 2016).

To prevent the development of resistance, adherence to recommended concentrations and contact times would allow for proper disinfection and decreased risk of resistance developing. Moreover, the combination of MF1/MF2 with an oxidising biocide such as H_2O_2 could prevent resistance as biocides formulated with excipients can increase antimicrobial activity are less likely to lead to resistance than the biocides in an aqueous solution (Cowley *et al*., 2015).

In terms of wipe development, the wipe combined with MF1 andMF2 would not be sporicidal and to broaden activity and oxidising biocide could be included. For the dry wipe this would combine much of the contents of the current spill wipe containing sodium percarbonate and TAED to generate hydrogen peroxide *in situ.*

Following this initial assessment of MF1/MF2, the commercial use of the formulations will continue to be explored through application to BPR for use against vegetative bacteria.

6.6 Conclusions

Two imidazolium-based salts (MF1 and MF2) in formulation were assessed for their antimicrobial activity with factors that limit disinfectants in healthcare. Both MF1 and MF2 had potent activity against yeast, Gram-negative and Gram-positive bacteria, and were less susceptible to factors that impede activity compared to BZC and CHX. The proposed MOA is through cell membrane interaction which causes leakage of cellular components and collapsing of the cell, with MF2 demonstrating a greater impact on the cell structure. The formulations as wipe products proved to be more effective at reducing and preventing transfer of bacteria than products already used in healthcare. The formulations were cytotoxic to skin cells *in vitro*.

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