Investigating Physiological and Genetic Characteristics of Community Acquired Infections and Potential Antimicrobial Interventions

Submitted for the Degree of Doctor of Philosophy
At the University of Northampton

2013

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Acknowledgement

I would like to express my gratitude to many people who have been influential in my progress throughout the PhD and directly or indirectly contributed to the work presented in this thesis. Firstly, I would like to thank my supervisors Professor Carol Phillips for her guidance, continued support throughout the PhD process and Dr Stuart Allen for his meticulous contributions and advice. I would like to thank the Centre of Health and Well Being Research (CHWR) and Mrs Lynn Marriot at the University of Northampton for the research studentship and the financial support towards the PhD.

The essential oils used in this study were provided by Belmay Plc. and I would like to thank Lynne Wright and Glenn Moran for providing the oils and for performing the GC-MS analysis. My appreciation also goes to Professor Mark Fielder and Dr Simon Gould from Kingston University who donated the community acquired isolates used in this study.

I would also like to thank the staff at the Graduate School: David, Twiggy, Simone and Dr Miggie Pickton for support and fascinating workshops, Dr Kostantinos Gzaktionis and Dr Monna Arvinen-Barrow for advice and support throughout the PhD and the laboratory staff; Val, Linda and Tanya and for support with orders, laboratory consumables and equipment training.

I am particularly indebted to Professor Val Edwards-Jones from Manchester Metropolitan University for her mentorship throughout my studies and to Society for Applied Microbiology for giving me the platform to disseminate my research and or many conference grants and development opportunities.

My friends and family have been immense throughout my programme. To my parents, and to Arnold, Amara, Grace, Victor, Devlina, Daisy, Chisom and my colleagues on the SfAM Postgraduate and Early Career Scientist committee, I am incredibly thankful. Finally, I give thanks to God for health, wisdom and strength to accomplish all that I have to this point and for being with me on this amazing journey.

I dedicate this thesis to all these people and without a doubt these people contributed to ensuring I had a worthwhile experience.

Abstract

Staphylococcus aureus and *Enterococcus* sp. infections occur in hospital and, increasingly, in community settings, with the potential of having different susceptibility to antimicrobial agents. The purpose of this study was to investigate the effect of antimicrobial agents against community acquired S.aureus and investigate antibiotic characteristics, biofilm formation and gene expression following exposure to an antimicrobial agent.

The susceptibility of *S. aureus* isolates and a vancomycin resistant *Enterococcus faecium* isolate to antibiotics, essential oils and disinfectants were investigated under planktonic conditions using standardised antimicrobial susceptibility tests and the Quantitative suspension and surface tests (EN 1276 and EN 13697) for the disinfectants. Biofilm formation, inhibition and eradication was investigated using the crystal violet (CV) assay while the viability of treated biofilms were investigated using the 2, 3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) reduction assay and CFU/ml assay. Multiplex PCR was used to determine the presence of PVL, icaA and IcaD genes as well as SCC*mec* typing while RT-PCR used to investigate changes in gene expression in five target genes following treatment of PVL CA MSSA and CA MRSA MW2 biofilms with grapefruit EO.

The *S. aureus* isolates all formed biofilms and had similar molecular characteristics however one isolate (CA MRSA SR) was multidrug resistant and PVL negative. The VRE isolate was negative for biofilm formation. In suspension, household bleach and NaDCC caused >5 log reduction in viable counts and on stainless steel surfaces, there was ≤3.5 log reduction. Against biofilms, Household bleach at 5000ppm caused 100% biofilm eradication within 10 minutes while NaDCC eradicated <50% of the biofilm within one hour at 10,000ppm.The eco-friendly product did not demonstrate any antimicrobial activity against planktonic cells or biofilms. Antimicrobial activity of six essential oils (EO) (lime, lemon, lemongrass, geranium, grapefruit, bergamot), and two components (limonene and citral) was investigated for the *S. aureus* isolates. Following exposure to lemongrass EO extensive disruption to *S. aureus* biofilms was shown under scanning electron microscopy. The most notable changes in gene expression following exposure to grapefruit EO were the *icaD*, *luxS* and *sodA* genes when the PVL CA MSSA biofilms was compared to the prototype community acquired strain, CA MRSA MW2.

The *S. aureus* isolates were susceptible to the essential oils with the exception of limonene and lemon EO. Lemongrass EO inhibited biofilm formation, metabolic activity and viability. No anti-biofilm activity was observed for Grapefruit EO against *S. aureus* except for one isolate (PVL positive CA MSSA), where an increase in metabolic activity was observed following treatment. Lemongrass EO was effective as an antibacterial and antibiofilm agent and could be a potential alternative to chemical based antimicrobial agents in both healthcare and non-healthcare environments.

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List of Abbreviations

agr	accessory gene regulator	**IRT**	Inhibitor removal technology
NaDCC	Sodium dichloroisocyanurate	**MBC**	Minimum bactericidal concentration
ANOVA	One way analysis of variance	**MBEC**	Minimum biofilm eradication concentration
ATCC	American Type Culture Collection	**MBIC**	Minimum biofilm inhibitory concentration
BHI	Brain heart infusion	**MIC**	Minimum inhibitory concentration
BSA	Bovine serum albumin	**MLST**	Multi locus sequence typing
BSAC	British Society for Antimicrobial Chemotherapy	**MRSA**	Methicillin resistant *Staphylococcus aureus*
CA	Community acquired	**MSSA**	Methicillin sensitive *Staphylococcus aureus*
CAI	Community acquired infection	**NCBI**	National Center for Biotechnology Information
CCR	Chromosomal cassette recombinase	**NCTC**	National Collection of Type Cultures
CDAD	*Clostridium difficile* associated disease	**OD**	Optical density
CDC	Centers for Disease Control and Prevention	**PBS**	Phosphate buffered saline
cfu/ml	colony forming units per millilitre	**PCR**	Polymerase chain reaction
CRA	Congo red agar	**PFGE**	Pulsed-field gel electrophoresis
Ct	Cycle threshold	**PIA**	Polysaccharide intercellular adhesin
CV	Crystal violet	**ppm**	Parts per million
DEFRA	Department for environment, food and rural affairs	**PVL**	Panton valentin leukocidin
DNA	Deoxyribonucleic acid	**QS**	Quorum sensing
EFCP	Environmentally friendly cleaning product	**RNA**	Ribonucleic acid
EO	Essential oil	**ROS**	Reactive oxygen species
EPA	Environmental Protection Agency	***SarA***	Staphylococcal accessory regulator
FAC	Free available chlorine	**SCCmec**	Staphylococcal chromosomal cassette mec
GCMS	Gas chromatography–mass spectrometry	**SEM**	Scanning electron microscopy
HA	Hospital acquired	**SOD**	Superoxide dismutase
HAI	Hospital acquired infection	**spa**	Staphylococcal protein A
HB	Household bleach	**USD**	US dollars
HCAI	Healthcare associated infection	**VRE**	Vancoymycin resistant enterococci
HPA	Health Protection Agency	**XTT**	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide
ICU	Intensive care unit	**ZOI**	Zone of inhibition

CHAPTER ONE

Introduction

Since the 1950s the most reported infections in healthcare have been hospital acquired infections (HAIs), also known as nosocomial infections (Burke, 2003). The effect of HAIs has been felt worldwide and in Europe, about 1.75 million people are affected annually (Guggenbichler *et al.* 2011). In the UK, the cost of these infections has been calculated at over £1 billion annually (Graves, 2004), which highlights the impact of these infections on the economy.

HAIs remain a problem due to factors such as the development of multidrug resistant organisms and consequent difficulties, whilst the emergence of the infections with community origins has altered the landscape of the infection burden in healthcare. These infections have been termed community acquired infections (CAIs). The first known reports of CAIs were in the early 1980s and numbers have been on an increase since (Saravolatz *et al.* 1982; Conly and Johnston, 2003; Moran *et al.* 2006; File, 2007).

Some of the organisms linked to infections in the community include those of bacterial origin such as *Staphylococcus aureus*, *Clostridium difficile*, *Escherichia coli*, *Enterococcus sp.*, and various other Gram-negative bacteria; those of viral origin, such as respiratory viruses and those of fungal origin such as *Candida* sp. (Lina *et al.* 1996; Warnock, 2007; Yumuk *et al.* 2008; Allard *et al.* 2011; Marcus *et al.* 2012). These CAIs are important as they lack the risk factors usually associated with the HAIs such as prolonged hospital stay and previous antibiotic therapy (Witte, 2009).

Some of the key problems encountered in healthcare settings with bacteria of either healthcare or community origin include antibiotic resistance, ease of transmission within hospitals and closed communities and the formation of biofilms. Since the emergence of

methicillin resistant strains of *S. aureus* (MRSA) in the 1960s (Jevons *et al.* 1963), antibiotic resistance has been an important concern for healthcare professionals. Thus, the emergence of community acquired (CA) *S. aureus* infections especially CA-MRSA, poses a significant challenge for the prevention and control of MRSA (File, 2007).

Alongside MRSA, another pathogen recognised for diverse antibiotic resistance characteristics is *Enterococcus* sp. Both MRSA and antibiotic resistant *Enterococcus* sp. are versatile organisms that possess transmissible genetic elements, the physiological mechanisms to form biofilms and to persist in the environment (Simjee and Gill, 1997; Wenzel and Edmond, 1998; Donlan, 2002; Hiramatsu, 2009).

To combat infections in healthcare the UK National Institute for Health and Clinical Excellence (NICE) published a public health guidance document (NICE, 2011) on prevention and control of healthcare associated infections (HCAIs) and highlighted key areas that form the basis of infection control policies which included hand hygiene, antimicrobial stewardship and environmental cleanliness. Disinfectants and antiseptics are key examples of conventional products used in healthcare and non-healthcare settings and are regarded as important in combating infections caused by bacteria (McDonnell and Russell, 1999; Dancer, 2009). However, there have been reports of resistance to some conventional disinfectants (Suller and Russell, 2000) and problems of toxicity reported in some disinfectant products (Rutala and Weber, 1997).

In recent years, the interest in environmental friendliness and sustainability has risen (DEFRA, 2011; Yates, 2009). Research into the antimicrobial potential of plant based products such as those of essential oils (EOs) has increased (Hammer *et al.* 1999; Edwards-Jones, 2004; Fisher and Phillips, 2006). These products are now visible in various applications such as cosmetics and antibacterial products (Bakkali *et al.* 2008; Barker and Altman 2010).

Recently, the effects of these EOs on biofilms have been demonstrated (Kwiesincki *et al.* 2009; Nuryastuti *et al.* 2009; Laird *et al.* 2012a). Biofilms are a complex community of cells attached to each other or to a surface (Davey and O'Toole, 2000) and studies have shown that they are highly resistant to the killing effects of antimicrobial agents (Spoering and Lewis, 2001) and, when compared to planktonic cells, require higher concentrations of antimicrobial agents (Yarwood *et al.* 2004).

Although antimicrobial agents have been useful alongside other infection control practices in managing HAIs in recent years, the emergence of CAIs with strains differing in susceptibility patterns and molecular features creates a need for further investigations. Firstly, there is a need to understand the differences between the isolates of CAI and HAI origin and subsequently identify tools to prevent, eradicate and/or manage infections especially those of CA origin.

CHAPTER TWO

Literature Review

2.1 Community acquired infections (CAIs)

CAIs have been defined by the Centre for Disease Control and prevention (CDC) as 'infections acquired by persons without recent hospitalization or invasive medical procedure'. Infections of community acquired origin have been identified worldwide in Europe, North-America, Asia, and Africa (Naimi *et al.* 2001; Dufour *et al.* 2002; Holmes *et al.* 2005; Miyashita *et al.* 2005; Reddy *et al.* 2010). A large number of cases reported for infections in community settings are caused by *S. aureus* with more mention of MRSA than other organisms. For many years, MRSA infections were limited to hospitals (Panhotra *et al.* 2005; Otto, 2009) and persons predisposed to infections in hospital environments such as elderly individuals, immune-compromised persons and patients undergoing surgery. However, MRSA have also been isolated from skin and soft tissue infections and from bacteremias among persons in the community without any health care contact (Chambers, 2001).

Apart from MRSA, other infections which have been identified as community acquired include: community acquired methicillin sensitive *S. aureus* infections (CA-MSSA) (Sattler *et al.* 2002), community acquired *C. difficile* infection (CA-CDI) (Dial *et al.* 2005), community acquired pneumonia (CAP) (Francis *et al.* 2005; Mandell, 2010) and community acquired urinary tract infections (CA-UTIs) (Prais *et al.* 2003).

2.1.1 Community acquired *S. aureus* infections

Among the early reports of CA *S. aureus* infections included one outbreak of CA-MRSA between 1980 and 1981 among injection drug users in community settings in Detroit, USA (Saravolatz *et al.* 1982) and another between 1989 and 1991, where CA-MRSA infections were identified in persons without contact with healthcare in Western Australia

(Udo *et al.* 1993). Prior to 1999, there were few reports worldwide of *S. aureus* infections of CA origin however these reports according to Conly and Johnston (2003) increased exponentially afterwards.

CA-MRSA infections have been reported in diverse groups such as healthy children, sports teams, prison inmates, and among other groups with often close physical contact and low hygiene (Gould, 2006). The types of infections associated with CA Staphylococcal infections include; skin and soft tissue, pneumonia, upper respiratory, urinary tract, conjunctivitis (Naimi *et al.* 2001). There have also been reports of fatalities caused by CA-MRSA and CA-MSSA (Naimi *et al.* 2001; Hota *et al.* 2007).

Said-Salim *et al.* (2005) suggested dividing infections caused by CA-MRSA into two groups i.e. infections from persons with predisposing risk factors, and from those without predisposing factors. Using genotyping methods, the authors found the group without predisposing risk factors to be mono-beta-lactam or beta-lactam (β-lactam), erythromycin resistant, with the ability to infect healthy patients. Within this group, they described MW2 as the prototype strain. In the second group, the MRSA strains are commonly resistant to clindamycin and fluoroquinolones and have a background referred to as archaic or historic. The common feature of both groups is that they both harbour a Staphylococcal cassette chromosome mec (SCC*mec*) type IV and carry the Panton Valentin Leukocidin (PVL) gene (Said-Salim *et al.* 2005; Zetola *et al.* 2005).

With regard to CA-MRSA origin, two hypotheses currently exist. The first suggests that the CA-MRSA strains originated from HA-MRSA and, due to selective antibiotic pressure, lost antibiotic genes. The second hypothesis suggests that the origin of CA-MRSA was a result of SCC*mec* transfer into MSSA and other staphylococci (Kwon *et al.* 2005; Mongkolrattanothai *et al.* 2011). Diep and Otto (2008) described five CA-MRSA lineages worldwide. These lineages are the ST1-IV (USA400); ST8-IV (USA300); ST30-IV

(Pacific/Oceania); ST59-IV and V (USA1000, Taiwan) and ST80-IV (European) while Wulf and Voss (2008) described a worldwide emerged CC398 lineage isolated from livestock suggesting that some livestock are a potential source of CA-MRSA.

Two *S. aureus* strains have been described in the majority of CA-MRSA infections. They are the USA 300 and USA 400 and, in comparison to the known hospital acquired methicillin resistant *S. aureus* (HA-MRSA) strains, these strains have shown enhanced virulence due to an increased ability to evade killing by human neutrophils (Palazzolo-Ballance *et al.* 2008). These two strains are clones which are widely disseminated contain SCC*mec* type IVa producing PVL (Davis *et al.* 2007), cause leucocyte destruction, and tissue necrosis (Loughrey *et al.* 2007). Within the USA 400 cluster, one strain known to be associated with fatal infections is MW2 (Kaplan *et al.* 2005). This strain has been fully sequenced (Baba *et al.* 2002) and shown to carry unique CA-MRSA specific virulence factors; staphylococcal enterotoxins H and C (*seh* and *sec*) and PVL (Said-Salim *et al.* 2005). *Sec* and *seh* are among a group of staphylococcal enterotoxins (SEs) which are extracellular proteins produced by some strains of *S. aureus* and are known causative agents of staphylococcal food poisoning, skin disorders, toxic shock syndrome (TSS) and auto-immune disorders (Jett *et al.* 2001). Wan *et al.* (2011) suggested a significant role of the enterotoxins in staphylococcal food poisoning following molecular analysis which showed most of the SE carrying MRSA strains belong to SCCmec types IV and V.

2.1.1.1 Panton-Valentin Leukocidin (PVL)

PVL was first reported in 1932 and has been described as the main virulence factor in some community-acquired MRSA strains (Gillet *et al.* 2002; Elston, 2007). Although the PVL genes are present in almost all CA-MRSA strains, they are largely absent in HA-MRSA strains (Gordon and Lowy, 2008; Wallin *et al.* 2008). In the USA, the PVL genes are in 40-95% of the CA-MRSA isolates but significantly lower in the UK (Hidron *et al.*

2009). Denis *et al.* (2005) relates the acquisition of the *PVL* gene to horizontal transfer into resident *S. aureus*.

At present, the epidemiology of PVL-positive MSSA is not well known although MSSA can cause infections similar to CA-MRSA (Mongkolrattanothai and Daum, 2005). PVL-MSSA strains have been associated with skin infection outbreaks in Swiss school children, a German village and French soldiers in Cote d'Ivoire (Tinelli *et al.* 2009). The authors also identified a PVL-positive outbreak MSSA strain, characterized by staphylococcal protein a (spa) type t005 and ST22, related to one of the major MRSA clones (EMRSA-15) also known as ST22-IV. This strain is SCC*mec* type IV and is now emerging as a successful clone in several areas of the world.

There are conflicting opinions on the true role of PVL in CA-MRSA pathogenesis (Hidron *et al.* 2009). Multiple animal infection studies using isogenic deletions of the *PVL*-encoding genes in CA-MRSA strains revealed that PVL has no or only a transient role in CA-MRSA pathogenesis however it may still have an important role in explaining the pathogenic potential when CA-MRSA is compared with other MRSA strains (Otto, 2009).

2.1.1.2 SCC*mec* types in CA-MRSA

SCC*mec* is a member of the SCC family. SCC are mobile genetic elements that drive gene exchange among staphylococcal species (Ito *et al.* 2004). SCC*mec* is an important feature in MRSA epidemiological studies as it is a large mobile genetic element which carries the gene responsible for methicillin resistance i.e. *mecA*, in *S. aureus* (Kwon *et al.* 2005). SCC*mec* is known to carry other resistance genes such as those for resistance to aminoglycosides, macrolides, tetracyclines, and heavy metals such as cadmium and mercury (Smyth *et al.* 2011) as well as genes for specific recombinases (*ccr*) necessary for its excision and integration (Ito *et al.* 2004; Deurenberg *et al.* 2007). Two specific genes *ccrA* and *ccrB* are carried on SCC*mec* which allow for movement and for specific

integration and excision of SCC*mec* to and from the *S. aureus* chromosome (Katayama *et al.* 2000; Hiramatsu *et al.* 2001).

According to The International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC), there are eleven different SCC*mec* types (I to XI) noted to date. The CA-MRSA strains based on SCC*mec* typing have been categorized to be within SCC*mec* types IV, V and VI (Kwon *et al.* 2005; Milhierico *et al.* 2007a).

The SCC*mec* type IV element is smaller in size (21 to 24 kb in comparison to the other SCC*mec* types (34 to 67 kb) found in the HA strains (Ito *et al.* 2001; Ma *et al.* 2002) but similar to the SCC*mec* type I element, referred to as the archaic SCC*mec* type (Fey *et al.* 2003). In the SCC*mec* type IV, there are also subtypes. Eight subtypes of the SCC*mec* type IV have been described and designated; IVa – IVj (IWG-SCC, 2009). The differences in the subtypes have been ascribed to polymorphisms and variations in the "joining-regions" or "J region" previously described as "junkyard regions" within the same *ccr* gene complex and *mec* gene complex combination (IWG-SCC, 2009; Smyth *et al.* 2011). Owing to its small size, there is the possibility that more genetic backgrounds of MRSA will be discovered with this SCC*mec* type (Fey *et al.* 2003) due to the mobility of the SCC*mec* element (Baba *et al.* 2002; Noto and Archer, 2006).

2.1.1.3 Methicillin resistance and the *mecA* gene

SCC*mec* carries the *mecA* gene, which encodes a low affinity penicillin binding protein PBP2A (Wielders *et al.* 2002), a key factor in beta-lactam resistance in MRSA due to its low affinity for β-lactam antibiotics (Lim *et al.* 2002). Development of methicillin resistance in *S. aureus* is associated with acquisition of the *mecA* gene (Lowy, 2003) although the origin of the *mecA* gene is unclear (Deplano *et al.* 2000). Tsubakishita *et al.* (2010) found the original chromosomal locus which they believed served as a template for the *mec*

gene complex of SCC*mec* from *Staphylococcus fleurettii*, a commensal bacterium in animals. Other reports have suggested that another microorganism *Staphylococcus sciuri* is a natural reservoir for the *mecA* gene (Wu *et al.* 1996; Kloos *et al.* 1997) adding to the suspicion that *mecA* is transmissible among staphylococcal species (Katayama *et al.* 2000). There is documented evidence of *mecA* in other *Staphylococcus* sp. For example *mecA* has been identified in *Staphylococcus lugdunensis*, a coagulase negative, biofilm forming pathogen isolated in Northern Europe (Starlander *et al.* 2011).

2.1.2 Community acquired *C. difficile* infection (CA-CDI)

C. difficile is a Gram-positive bacterium first described in the 1930s and initially referred to as *Bacillus difficilis*. It was identified in 1978 as the main cause of pseudomembranous colitis following its isolation from faeces of patients undergoing clindamycin treatment (Voth and Ballard, 2005).

The first report of CA-CDI was in 1984 and the reports of the infection increased afterwards although, to date, little is known about the strains responsible (Limbago *et al.* 2009). Although *C. difficile* is widely regarded as a nosocomial pathogen, the emergence of a hypervirulent strain, North American pulsed-field type 1 (NAP-1/B1/027) was linked to the increase in the rates and severity of *C. difficile*-associated disease (CDAD), (McDonald *et al.* 2005, Warny *et al.* 2005). The NAP-1/B1/027 isolates have been characterised and shown to be positive for a binary toxin CDT, and an 18-bp deletion in *tcdC* (McDonald *et al.* 2005). *tcdC* is one of three accessory genes located in a pathogenicity locus which are involved in regulation of the toxin genes *tcdA and tcdB* in *C. difficile* (Dupuy *et al.* 2008).

There is a consensus that CDI is increasing worldwide (Khanna *et al.* 2012; Leffler and Lamont, 2012). Khanna *et al.* (2012) reported a rise in CA-CDI infections between 1991 and 2005 in a population-based study which showed a rise from < 2.8 cases per 100,000 persons between 1991 and 1993 to about 15 cases for CA per 100,000 persons between 2003 and 2005. There have been other reports of CA-CDI. Kutty *et al.* (2010) found 20% of all CDI cases to be CA which was similar to 22-28% found in studies conducted in Sweden (Noren *et al.* 2004) and in Manitoba, Canada, the incidence of CA-CDI infection was 23.4 cases per 100,000 (Lambert *et al.* 2009).

Although the structural and virulence characteristics of *C. difficile* are known, a common feature in strains associated with CA-CDI pathogenesis is the production of a binary

toxin. However the role of this toxin in CA-CDI pathogenesis remains unclear (Terhes *et al.* 2004). Recently, ribotyping of strains isolated from CA cases showed that in Sherbrooke, Canada, *C. difficile* ribotype 027 accounted for a higher proportion of HA cases than CA cases (67% vs. 37%) contrary to the reports from cases in Calgary, (16.6%, CA vs. 7.4%, HA) (Bignardi and Settle, 2008).

2.1.3 Community acquired pneumonia (CAP)

It is estimated that about 5-6 million cases of CAP are diagnosed annually in the United States causing over 1 million hospitalizations and 10 million physician visits as well as an approximate annual cost of $8.4 billion for treatment (Colice *et al.* 2004). According to Andrews *et al.* (2003), CAP mortality rates are low at levels less than 1-5% in the outpatient setting, although in persons requiring hospital care, the mortality rate is an average of 12%, but is higher in some groups such as those with bacteremia and nursing home residents.

Two thirds of CAP is caused by *Streptococcus pneumoniae*, however other microorganisms have been associated with cases of CAP including and not limited to:- *Haemophilus influenzae, Mycoplasma pneumoniae, Legionella* spp., *Pseudomonas aeruginosa, S. aureus*, respiratory viruses such as respiratory synctical virus and coronaviruses (File, 2003). Two types of CAP have been described; the typical pneumonia caused by *S. pneumoniae* and the atypical pneumonia caused by the influenza virus, mycoplasma, chlamydia, *Legionella sp.*, adenovirus, or other unidentified microorganisms with the age of the infected patient the difference between CAP types (Lutfiyya *et al.* 2006). Normally, cases of pneumoniae are diagnosed in older persons (≥ 60 years) however, in the atypical cases of CAP, the infections have been observed in younger persons i.e. < 60 years (Ruiz *et al.* 1999; Lutfiyya *et al.* 2006). Apart from age as a factor in CAP pathogenesis, other risk factors identified as independent in a population-based study by Almirall *et al.* (2008) included:- being underweight, cigarette smoking,

high alcohol consumption in men, contact with children and pets and other environmental factors. Cigarette smoking and alcohol abuse had previously been identified as risk factors in another study (Ruiz *et al.* 1999). Cigarette-smoke linked CAP was associated with *Legionella* sp. and *Chlamydophila pneumoniae* while alcohol related CAP was associated with *S. pneumoniae* (Ruiz *et al.* 1999).

The association of different organisms in cases of CAP highlights the difficulty that is encountered in managing the associated infections. Some of the obvious problems include choice of antimicrobial treatments, prescription of appropriate antibiotics as well as antibiotic resistance. Guest and Morris (1997) reported that 45% of all prescriptions for CAP are broad-spectrum antibiotics, 23% are cephalosporins, 22% are macrolides, 3% are aminoglycosides and 3% are nitroimidazoles. To treat CAP, the pathogens should be eradicated which will in turn resolve the clinical features of the disease, minimize hospitalization and prevent reinfection (Lutfiyya *et al.* 2006).

2.2 Hospital acquired infections (HAIs)

HAIs were defined by Emori and Gaynes, (1993) as infections with no evidence of presence or incubation at the time of admission to hospital. The definition of nosocomial infections has been extended to include infections in any health care setting and also including infections acquired by staff or visitors to the healthcare or hospital setting (Ducel *et al.* 2002).

The requirements for transmission of HAIs include a source of infecting microorganism, a susceptible host and a transmission mechanism (Collins, 2008). In the pre-antibiotic era, the main pathogens responsible for nosocomial infections were Gram positive cocci – *S. aureus* and *Streptococcus* species mainly *S. pneumoniae* and S. *pyogenes* (Swartz, 1994). Both MSSA and MRSA are by far the most significant hospital acquired pathogens and have been implicated in surgical site infections (SSI), hospital-acquired pneumonia, septic arthritis, osteomyelitis, discitis, prosthesis-associated infection, and intra-abdominal infections (Gould, 2006). Of the nosocomial infections, UTIs have been reported as the most common (Mylotte *et al.* 2000; Ducel *et al.* 2002). Other important HAIs include skin and soft tissue infections (Gaynes *et al.* 2001), respiratory tract infections (Jarvis and Martone, 1992), bacteraemia (Correa and Pittet, 2000) and gastrointestinal infections (Archibald *et al.* 2004).

According to Guggenbichler *et al.* (2011) HAIs affect 1.75 million people in Europe annually and the authors suggest that despite a reduction in hospital admissions and length of stay the rates of HAIs is still increasing in Europe and in developing countries. The numbers of such infections are on a decline in the UK as the recent report from the Health Protection Health protection Agency (HPA) suggests (HPA, 2012). According to the report, infections caused by MRSA or *C. difficile* fell by 30% and 26% respectively between the second quarter of 2011 and the second quarter of 2012 (HPA, 2012).

2.2.1 Risk factors for HAIs

The documented risk factors associated with the HAIs include: intrinsic factors such as immunosuppression and age, extrinsic factors such as use of high risk medical device and surgical operations (Emori and Gaynes, 1993). Hospital personnel are among those implicated as possible sources of these nosocomial infections (Cespedes *et al.* 2002). Gastric acid suppression following use of proton pump inhibitors has been implicated as a risk factor associated with *C. difficile* (Dial et al. 2004). This is also the case with antimicrobial drug therapy (Kutty *et al.* 2010) with antibiotics such as clindamycin, cephalosporin and ampicillin known to induce CDAD (Louie and Meddings, 2004). Older and severely ill hospitalized patients or residents of long-term care facilities are all at risk of infection caused by *C. difficile* (Kutty *et al.* 2010).

Similar factors have been described in infections of MRSA origin in nosocomial settings. These factors include recent hospitalization, antibiotic misuse or overuse, older age, intensive care unit (ICU) stay, use of intravenous devices, mechanical ventilators and more severe clinical conditions (Wang *et al.* 2008; Hidron *et al.* 2009; Vidal *et al.* 2009). Presence of a skin and soft tissue infection and HIV-seropositive status has also been identified as risk factors of nosocomial MRSA colonization (Hidron *et al.* 2009).

Nosocomial infections caused by the Gram negative bacteria *P. aeruginosa* and *Acinectobacter baumannii* have also shown similar risk factors to that of MRSA and *C. difficile*. However in the case of *A. baumannii*, unique factors such as large burn wound areas, mechanical ventilation, multiple surgical procedures, parenteral nutrition, decreased fluid, electrolyte and serum protein levels have been reported to be of potential significance in acquisition of the infection (Wisplinghoff *et al.* 1999).

2.3 Differences between HAIs and CAIs

Several characteristics have been identified that are used in the differentiation of bacterial strains associated with both HA and CA infections. For example, the CA-MRSA strains fall into the SCC*mec* groups IV-VI while the HA-MRSA strains belong to the SCC*mec* groups I-III (Ito *et al.* 2004; Oliveira *et al.* 2006). Other factors have been identified that differentiate the CA from HA strains. These differences include virulence mechanisms and antibiotic susceptibility patterns. The differences in clinical and epidemiological features between the HA-MRSA and CA-MRSA are highlighted in Table 2.1.

Table 2.1. A summary of the differences between CA and HA MRSA infections. (Source: Health Protection Agency, 2008).

	HA-MRSA	CA-MRSA
Typical patients	Elderly, debilitated and/or critically or chronically ill	Young healthy people, students, athletes, military service personnel
Infection site	Wounds/invasive devices often cause bacteraemia	Often spontaneous, skin, cellulitis, abscess
Transmission	Within healthcare settings; little spread among household contacts	Community-acquired; many spread in close community settings e.g. Families, sports teams, via pets (not in UK)
Diagnosis is typically made	In an in-patient setting	In an out-patient setting
Medical history	History of MRSA colonization/infection, recent surgery, admission to hospital or nursing home, antibiotics, renal dialysis, permanent indwelling catheter, skin ulcers, diabetes	No significant history
Virulence factors	Community spread limited, PVL genes absent	Community spread readily, PVL genes present, predisposition to necrotising skin and soft tissue infections
Antibiotic susceptibility	Choice of antibiotic agents limited	Currently more susceptible antibiotics

2.3.1 Molecular identification methods for HA and CA isolates

Molecular methods have been used in numerous applications including characterising and identifying the evolutionary and epidemiological origins of microorganims. Some of the methods currently used in microbiological applications and in identification of infections caused by CA and HA associated *S. aureus* infections include: multi-locus sequence typing (MLST), staphylococcal protein A typing (*spa* typing), mass spectrophotometry, Pulsed-field gel electrophoresis (PFGE), plasmid analysis, microarray analysis, Matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF), restriction endonuclease analysis (REA), polymerase chain reaction (PCR) based methods which include accessory *gene* regulator (*agr*) grouping and SCC*mec* typing and ribotyping (Mongkolrattanothai and Daum, 2005; Faria *et al.* 2008; Tenover *et al.* 2011).

In differentiation and characterisation of *C. difficile* infection from hospital and community settings, PCR ribotyping has been used (Noren *et al.* 2004). In Europe and North America, PFGE and PCR ribotyping are the methods of choice for fast and convenient detection of *C. difficile* infection, whilst modified PCR ribotyping methods have been used for direct detection of the bacteria in stool samples (Janezic *et al.* 2011). PCR based methods are very versatile and have proved successful in determination of molecular characteristics of community acquired pathogens (Morozumi *et al.* 2006).

For MRSA investigations, the three main strain typing methods are PFGE, MLST and *spa* typing (Tenover and Pearson, 2004). Multiplex PCR was developed by Chamberlain *et al.* (1998) and has since been shown to be successful in differentiating MRSA strains. Numerous studies have used this approach for SCC*mec* typing (McClure *et al.* 2006; Faria *et al.* 2008). This method has also been used to differentiate other microorganisms (Rodas *et al.* 2009; Vankerckhoven *et al.* 2004).

2.4 Transmission of CA-MRSA and HA-MRSA into 'non traditional settings'

Although differences have been identified between CA and HA-MRSA infections, the continuous evolution of MRSA infections poses challenges to healthcare professionals. CA-MRSA strains have now been identified in nosocomial infections while the HA-MRSA strains have been identified as the causative agents of MRSA infections in community settings. This development according to several authors has made it difficult to differentiate between CA-MRSA and HA-MRSA strains (File, 2003; David and Daum, 2010).

A CA-MRSA strain (USA 300) was identified as the cause of nosocomial infections and responsible for 28% of healthcare associated blood stream infections (BSI) and 20% of nosocomial MRSA BSI (Seybold *et al.* 2006). Other studies that have demonstrated the movement of CA-MRSA into nosocomial settings include that of Saiman *et al.* (2003) who identified a community strain, MRSA USA 400, as the causative agent of skin and soft tissue infections in eight postpartum women. Eckhart *et al.* (2003) also reported a case of CA-MRSA transmission in a neonatal ICU.

On the other hand, Adedeji *et al.* (2007) identified HA-MRSA strains circulating in community settings in Birmingham, UK following molecular typing of isolates while Rollason *et al.* (2008) also identified strains genetically related to HA-MRSA strains circulating in persons without hospital contact within the West Midlands community. The observation of HA strains circulating in the community is not limited to the UK as transmission of HA-MRSA in community settings was observed in Taiwan between 1999 and 2002 (Chi *et al.* 2007).

According to Eckhart *et al.* (2003) the prevalence of MRSA in the community is likely to influence the number of nosocomial infections and Popovic *et al.* (2008) suggested the likelihood that CA-MRSA strains may be replacing the traditional HA-MRSA strains. This

raises other problems as the spread of strains from community settings into the hospital environment could have an effect on infection prevention and control measures already in practice (File, 2003). Benoit *et al.* (2008) highlighted the possibility of exchange of genetic material between both CA-MRSA and HA-MRSA. If this happens, there could be the potential for CA-MRSA strains circulating in nosocomial settings to acquire antibiotic resistance genes and increase the severity of disease in older persons within healthcare (Leclercq, 2009).

2.5 Economic burden of CA and HA MRSA

The economic impact of CA-MRSA infections remains unclear as the data on this subject if limited. Lee *et al.* (2012) recently developed a simulated model to calculate the costs of CA-MRSA infections. In their study, with the exception of data from infection control interventions, hospital transmission and associated costs, the authors found that, in the US, the annual burden of CA-MRSA is between $478 million and $2.2 billion on third party payers and between $1.4 and $13.8 billion on society (Lee *et al.* 2012). The third party payers act as intermediaries between the healthcare providers and the users of healthcare services and the third party payers include: insurance companies, health service providers and the government (Chen and Feldman, 2000).

The factors associated with the infection costs caused by CA-MRSA are similar to that from other studies on HA-MRSA and traditional MRSA infections. These factors include screening costs, hospitalization, treatment, management of patient care and more recently litigation costs (Stone, 2009; Gould *et al.* 2010; Lee *et al.* 2012). Although published data on CA-MRSA infections are lacking, economic cost calculations of the burden of HA-MRSA infections have been available since 2002. In the UK, the cost of healthcare associated infections was calculated at over £1 billion annually (Plowman *et al.* 2001; Graves, 2004). In the US, the CDC estimated as many as two million infections a year in hospital led to about 90,000 deaths with associated costs at over $5 billion per

year (Guggenbichler *et al.* 2011; Shannon, 2011). Furthermore, direct health care costs linked to MRSA infections in Canada amounted to an average of $82 million USD in 2004 and was projected to increase to $129 million by 2010 (Goetghebeur *et al.* 2007). These figures all highlight the economic impact these infections have on overall national budgets.

Combining the cost of CA-MRSA infections from the simulated model (Lee *et al.* 2012) and the established costs associated with nosocomial infections suggests that both infections represent a huge burden to the overall costs of healthcare spending and national budgets. Thus, it is imperative that measures to prevent the transmission of both CA and HA MRSA infections and to manage cases of both infections are continuously developed and implemented.

2.6 Managing the problems of HAIs and CAIs

The UK House of Commons committee offered two suggestions for managing HAIs (Mayor, 2009). Firstly, that the NHS should monitor the extent and costs of the HAIs and secondly an indiscriminate approach is required for prevention of the HAIs involving everyone not just healthcare workers. Plowman *et al.* (2001) also suggested using surveillance detection methods and improved infection control strategies as a way to prevent HAIs.

Following the introduction of legislation on prevention of healthcare associated infections in the UK since 2003/2004, the rates of MRSA bloodstream infections (BSI) and *C. difficile* infections declined by 57% and 41% respectively. Treatment costs were also reduced as a result by £45 to £59 million for MRSA BSIs and between £97 and £204 million for *C. difficile* according to the UK National Audit Office (2009). Current guidelines available for managing infections caused by antibiotic resistant pathogens are focused on the HAIs. However, Barton *et al.* (2006) developed guidelines for managing CAIs in

Canada and suggested incorporating surveillance for CA-MRSA infections into the national surveillance programs, and reiterated the importance of good hygiene practices including hand washing in the prevention of CA-MRSA infections. Other factors have been addressed that could have an impact in reducing infections caused by CA-MRSA including early identification and proper treatment which will reduce transmission and infection incidences and, in turn, save the associated cost of infections (Lee *et al.* 2012).

2.6.1 Antimicrobial agents

Antimicrobial agents have for many years been used in treatment of infections of bacterial, parasitic, viral and fungal origin. In the early twentieth century, the discovery by Alexander Fleming that bacterial growth was prevented by a fungus, *Penicillium notatum*, heralded the development of antibiotics and in turn, a fall in mortality and morbidity (Singh and Barrett, 2006). Different classes of antibiotics have been developed over the years to treat bacterial infections. The main targets of activity have included inhibition of functions such as: cell wall synthesis, protein synthesis, RNA synthesis, DNA synthesis and metabolism (Singh and Barrett, 2006; Gwynn *et al.* 2010). Observation of antimicrobial resistance in different microorganisms, very notably in cases of MRSA (Jevons, 1963) led to decreased efficiency and reduced usage of certain antimicrobial agents (Coates and Hu, 2007). This has also led to the search for new methods, targets and products with antimicrobial potential such as bacteriophages, plant and animal peptides and in other cases combinations of antimicrobial treatments (Andreu and Rivas, 1998; Hancock and Sahl, 2006; Coates and Hu, 2007; Kollef, 2008). Strategies to prevent antimicrobial infections in both healthcare and non-healthcare settings have also seen the use of environmental decontaminants and recently the re-introduction of natural products as antimicrobial agents. Examples of environmental decontaminants and natural antimicrobials with known antimicrobial activity include: chlorine releasing agents (bleach, sodium dichloroisocyanurate), phenolics, silver, honey and EOs (Hammer *et al.* 1999; McDonnell and Russell, 1999; Cooper *et al.* 2002; Chopra, 2007; Edwards-Jones, 2009).

2.6.2 Cleaning and disinfection

According to Sattar, (2011) cleaning, disinfection and sterilisation are key tools in preventing spread of infection. The benefits of cleaning in the hospital, in the household and in industry have been investigated extensively (Salvat and Colin, 1995; Cooper *et al.* 2007; Dancer *et al.* 2009; Marshall *et al.* 2012). It has been shown that cleaning as a standalone activity is not sufficient for environmental decontamination (Cooper *et al.* 2007; Dancer, 2008). The current indicators for assessing cleaning effectiveness are by visual inspections which are unreliable and impractical as this approach does not consider the invisibility of bacteria to the naked eye (Cooper *et al.* 2007; Dancer, 2008). It has been demonstrated that detergent-based cleaning reduces the risk of cross contamination (Scott *et al.* 1984; Barker *et al.* 2003). However in both studies, greater reduction of contamination and risk of infection was only achieved following the use of a disinfectant product. McMullen *et al.* (2007) reported a fall in the rates of *C. difficile* infection in two hospital ICUs following enhanced environmental cleaning using a hypochlorite solution. The effect of cleaning against antibiotic resistant pathogens has also been reported, as a fall in rates of VRE was observed after routine enhanced cleaning measures (Hayden *et al.* 2007). Goodman *et al.* (2008) also reported a fall in rates of vancomycin resistant enterococci (VRE) and MRSA following increased application of disinfectants, staff education on environmental cleaning practices and monitoring.

The effect of disinfectants against bacteria in the healthcare setting has been studied extensively. Using disinfectants correctly in healthcare and non-healthcare settings could significantly reduce the number of infections which in turn will reduce the need for antibiotic therapy (Weber and Rutala, 2006). Some of the disinfectants known to exhibit antimicrobial activity against hospital acquired pathogens include; chlorine releasing agents (sodium hypochlorite, chlorine dioxide, sodium dichloroisocyanurate), hydrogen

peroxide and glutaraldehyde (Coates, 1996; Rutala and Weber, 1997; Fawley *et al.* 2007).

Several factors have been identified as important in the activity of disinfecting agents. These factors include prior cleaning of the object or surface, contact time between the disinfectant and contaminated object or surface, temperature, organic and inorganic load, physical nature of the object or surface, biofilm formation and pH of the disinfection process (Maillard, 2005; Rutala *et al.* 2008). The spectrum of activity of disinfectants is broader than that of antibiotics (McDonnell and Russell, 1999; Rutala *et al.* 2008). According to Maillard (2002), the mechanism of action of a biocidal product is dependent on the bacterial structure against which it exerts its main activity. The author also described three levels of interaction between the biocidal products and bacteria. These areas of biocidal interaction in the bacteria include: the outer cellular components such as the cell wall and outer membrane, the cytoplasmic membrane with possible effects such as inhibition of enzyme activity and adenosine triphosphate (ATP) synthesis and thirdly, the cytoplasmic constituents which includes nucleic acids and ribosomes.

2.6.3 Environmentally friendly cleaning agents

In recent years there has been a rise in the development of household and commercial cleaning products considered 'environmentally friendly' and/or 'green'. The Department for Environment Food and Rural Affairs (DEFRA) defined the term "environmentally friendly" as a vague and non-specific environmental claim which suggests a product is friendly and/or harmless to the environment (DEFRA, 2003). According to a report by Air Quality Sciences (2011) there was a major increase in sales of green cleaning products in the USA between 2003 and 2008 from $17.7 million to $64.5 million USD and the report also projected that by 2013, sales of these products would rise to about $623 million USD. Based on the report, the factors driving the growth of these products include a need to create effective cleaning products for a variety of cleaning applications, to

minimise health risks from indoor contaminants such as volatile organic compounds and to protect the outdoor environment.

In the UK, a report from the Consumer Focus Group investigating consumers understanding of 'green' claims in advertising showed that there was a 54% rise in consumer purchase of environmentally preferable products between 2007 and 2009 (Yates, 2009). In the UK, there is a target to deliver products and services which aims to cut carbon emissions by 80% by the year 2050 (DEFRA, 2011). To ensure that goods and services developed with environmental claims are implemented correctly, the International Organisation for Standardisation (ISO) developed an international standard (ISO 14021:1999) on self-declared environmental claims with the aim of regulating and motivating the use of good quality claims (Palerm, 2000). The objective of this standard is to harmonize the use of self-declared environmental claims and the key elements of this standard include: use of symbols, evaluation and claim verification requirements and specific requirements for selected claims

Although the suggestion is that 'green' cleaning products pose less health concerns, there is currently no assurance of risk reduction (Markkannen *et al.* 2009). This is because of a lack of data on associated hazards, clarity on the application and disposal of the products, evidence of antimicrobial activity and certification issues. Currently in the US, the Environmental Protection Agency (EPA) does not permit products to be marketed as green if they do not kill microorganisms, however the EPA are exploring changing policies to incorporate green claims for disinfectants and sanitizers (Markkanen *et al.* 2009). Green cleaning products are marketed as containing natural materials. They are designed to be drop-in substitutes or potential replacements for the "highly toxic" conventional cleaning agents or disinfectants. However factors such as the effect on pathogen control, impact on applicators, sensitive occupants and environment also need be considered (Light, 2009).

Published data on the effects of 'green' cleaning products are limited, however data from laboratory experiments investigating the effects of alternative products are available. Olsen *et al.* (1994) compared the effects of vinegar, baking soda, ammonia and lemon juice with conventional hard surface disinfectants and found the alternative products to be less effective in both microbial reduction and soil removal. In another study, Rutala *et al.* (2000) found little or no effect of vinegar and baking soda against either Gram-positive and Gram-negative, bacteria which was also observed by Bauer *et al.* (1995) using an EPA Non-Food Contact Sanitizer Test.

Sattar (2010) in a review article suggested caution in the widespread use of green products without properly addressing any potential risks they might pose. The author also highlighted principles that should govern 'green' products which are used for disinfection purposes. These principles infer that the 'green' products and chemicals should be effective with no toxicity, safe, degradable and possess minimal potential for accidents and environmental release.

2.7 Natural products as antimicrobial agents

Natural products have aided the discovery of new leads in the search and development of drugs for treatment of human diseases (Newman and Cragg, 2007). Some of the natural products with known antimicrobial potential have been identified and harnessed from various sources including: bacteria, fungi, animals, insects and plant sources. Naturally occurring products known to possess antimicrobial activity with potential healthcare applications include honey, chitosan, silver (Cooper *et al.* 1999; Chopra, 2007; Raafat and Sahl, 2009). The potential of natural antimicrobial products is not only applicable in the health sector as some of these natural compounds have been used in other areas such as food preservation, household cleaning and detergent products, cosmetics and beauty products (Roller, 2003; Tiwari *et al.* 2009; Ponce *et al.* 2011).

2.7.1 EOs

EOs are defined as "complex mixtures of volatile compounds produced by living organisms and isolated by physical means only (pressing and distillation) from a whole plant or plant of known taxonomical origin" (Franz and Novak, 2009). The use of essential oils for medicinal purposes is dated back to 3000BC (Haber, 2010) and in the 16th century, EOs and separation methods for essences from aromatic waters were established while the 21st century has seen a rise in popularity of these compounds (Vidal, 2010).

To obtain essential oils from the plants, different extraction methods have been used. Commercially, steam or hydrodistillation is the most commonly used while other methods such as cold pressing and dry distillation have been described (Kubeczka, 2009). Over the years numerous studies have shown essential oils to be antibacterial, antitoxigenic, antimycotic, antiparasitic, antiviral and possessing insecticidal properties (Burt, 2004).

2.7.1.1 Essential oils as antimicrobial agents

In recent years the antimicrobial properties of essential oils have been investigated *in vitro* against Gram-positive bacteria, Gram-negative bacteria and fungi (Hammer *et al.* 1998; Hammer *et al.* 1999; Dorman and Deans, 2000; Prabuseenivasan *et al.* 2006). Some of the essential oils studied for antimicrobial potential include: tea tree oil, thyme, geranium, manuka, lemongrass, lavender and citrus-based oils (Lis-Balchin *et al.* 1998; Williams *et al.* 1998; Lis-Balchin and Roth, 2000; Takarada *et al.* 2004; Fisher and Phillips, 2009). In a study by Hammer *et al.* (1999), the minimum inhibitory concentration (MICs) of 52 plant oils against ten organisms were investigated and of those, lemongrass, oregano and bay inhibited all the organisms which included Gram-positive bacteria, Gram-negative bacteria and fungi at the concentration ≤ 2% (v/v) while nine other essential oils (rosewood, coriander, palmarosa, tea tree, niaouli, peppermint, spearmint, sage and majoram) inhibited all the strains at a concentration ≤ 2% (v/v) with the exception of *P. aeruginosa*.

The antimicrobial effects of essential oils have also been observed when different essential oils were combined with each other. Fisher and Phillips (2009), blended two EOs (orange and bergamot) at a ratio 1:1 and 2% (v/v) concentration with the EO combination demonstrating antimicrobial activity by growth inhibition and cell death in *E.faecium* and *E.faecalis*. Patrone *et al.* (2009) also observed synergistic properties when essential oils with surfactants were combined with cosmetic preservatives suggesting potential for enhancement of biocidal activity and improvement of cosmetic preparations. In other studies, Hendry *et al.* (2009) and Karpanen *et al.* (2008), both assessed the combination of eucalyptus oil and 1, 8-cineole with chlorhexidine digluconate (CHG) against planktonic and biofilm cells and observed synergistic activity both in suspension and against biofilms. The antibiofilm effects of essential oils against biofilms of bacteria and fungi have been demonstrated previously (Kwiesincki *et al.* 2009; Budzynska *et al.* 2011; Laird *et al.* 2012a).

2.8 Surface attached bacteria and biofilms

Bacteria attached to a surface experience different conditions in comparison to planktonic cells. These conditions include higher osmolarity, differences in nutrient availability (limited in embedded bacteria), higher cell density, different growth rates and genetic responses (Fux *et al.* 2003).

2.8.1 Surface attached bacteria

Investigations on the effect of antimicrobial compounds on bacteria adhered to surfaces have been performed for many years. Contact with inert surfaces and attachment to these surfaces has been described as a contributing factor in infection outbreaks in food processing environments (Mafu *et al.* 1991). Surface adhered bacteria have been investigated in numerous studies and Wider *et al.* (1991) reported the potential to successfully treat established device related infections by using drugs or treatment that kill adherent and non-adherent bacteria. Surface adhered bacteria have been isolated from various surfaces. For example, *Salmonella enteritidis* Phage type 4 (PT4) was isolated from domestic toilets and bathrooms (Barker and Bloomfield, 2000) while in a different study, *Listeria monocytogenes* was found to be adherent to materials in food processing environments (Beresford *et al.* 2001). In laboratory investigations, other surfaces such as ceramics, stainless steel and polyvinyl chloride (PVC) plastic have been used to determine the effect of antimicrobial agents (Norwood and Gilmour, 1999; Katsikogianni and Missirlis, 2004; Laird *et al.* 2012a). Although non-sporing bacteria such as *Enterococcus* sp. and *S. aureus* have been isolated from inert surfaces, spore-forming bacteria such as *Bacillus cereus*, *Bacillus subtilis* and *C. difficile* have also been isolated (Waligora *et al.* 1999; Faille *et al.* 2002).

It is still unknown what the key factor is in initial bacterial attachment to a surface. However in a review article; Palmer *et al.* (2007) discusses a number of factors which included surface conditioning, mass transport, surface charge, hydrophobicity, surface

roughness and surface micro-topography. Busscher *et al.* (2012) highlighted that the forces of adhesion tend to strengthen during the first minutes of contact leading to a switch from reversible to irreversible adhesion. The authors also proposed three adhesion force regimes once bacteria come in contact with a surface; the planktonic regime, the interaction regime and the lethal regime. The first phase of adherence of bacteria to surfaces is however said to be as a result of Van der Waals forces (Skvarla, 1993). Bacterial attachment to surfaces is important as it is the first stage in development of biofilms following irreversible attachment (Hinsa *et al.* 2003).

2.8.2 Biofilms

Biofilms have been defined as communities of microorganisms attached to a surface (O'Toole *et al.* 2000). Donlan and Costerton (2002) have also provided a definition of biofilms as; *"microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription"*.

Formation of biofilms has been observed in both bacteria and fungi and is an important factor in survival and virulence (Vuong *et al.* 2004). Examples of organisms studied extensively for the ability to form biofilms include *P. aeruginosa* (O'Toole and Kolter, 1998), *E. coli* (Pratt and Kolter, 1998), *C. albicans* (Ramage *et al.* 2002), *S. aureus* and *Staphylococcus epidermidis* (Otto, 2008) among others. According to Prigent-Combaret *et al.* (1999), biofilm formation occurs as a response to certain environmental signals such as nutrient availability, cell to cell signalling, oxygen concentration, osmolarity and genetic mechanisms.

Biofilm formation has also been associated with treatment failures in healthcare and increased antibiotic resistance (Fux *et al.* 2003). The antimicrobial susceptibilities

demonstrated by bacteria in biofilms differ from planktonically growing bacterial cells as the cells in biofilms show reduced susceptibility to the effect of antimicrobial agents (Ceri *et al.* 1999).

The key stages of biofilm development have been demonstrated in Figure 2.1. These stages are: attachment (this could be either to other bacteria or a surface) an irreversible binding or attachment phase usually on surfaces, maturation of the biofilm, maintenance of the biofilm and dissolution or cell dispersal from the biofilms (O'Toole *et al.* 2000; Aparna and Yadav, 2008). Thomas *et al.* (2006) described three spatial regions within the biofilm. These three levels or spatial regions were the glycocalyx layer also referred to as the slime layer which serves a protective barrier, a resistance transfer layer and thirdly the innermost part of the biofilm which constitutes cells that are metabolically inactive. The latter is perhaps the layer harbouring persister cells.

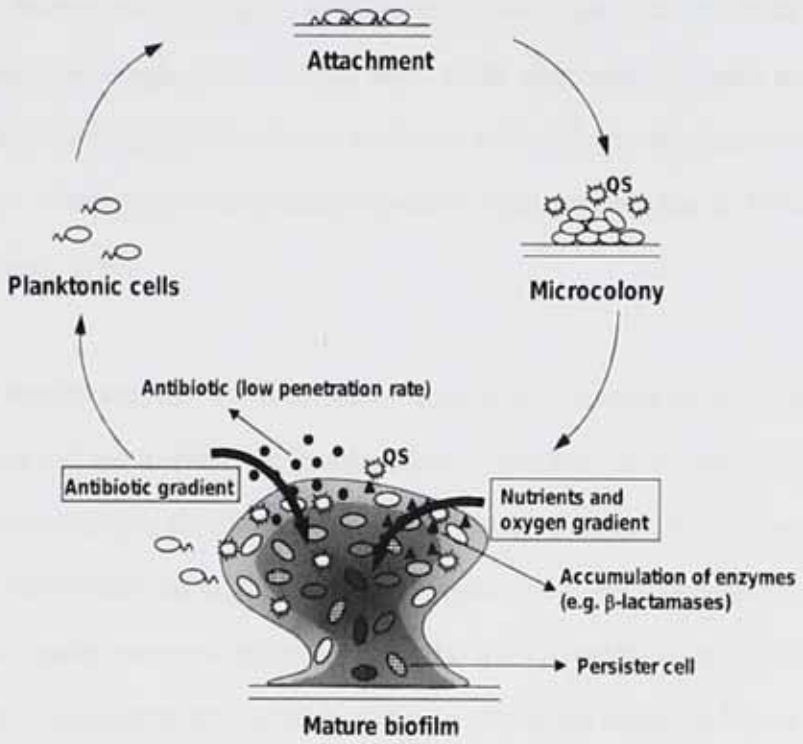


Figure 2.1. Stages in the development of a biofilm. (Schematic image from Fernández *et al.* 2011). The process of biofilm formation begins with the attachment of planktonic cells to a surface or to each other and a subsequent period of irreversible attachment, maturation and finally dispersal of the biofilm. (O'Toole *et al.* 2000).

2.8.2.1 Biofilm formation in *S. aureus*

S. aureus is known to form biofilms (Gotz, 2002) and, in other staphylococci, biofilm formation is a common feature, for example, in *S. epidermidis* (Knobloch *et al.* 2001), *S. saprophyticus* (Cernohorská and Votava, 2010), *Staphylococcus haemolyticus* (Fredheim *et al.* 2009) and in *S. sciuri* (Stepanovic *et al.* 2003). Biofilm formation in the staphylococci begins when the organism attaches to host tissue or a surface (Heilmann and Gotz, 2010; Periasamy *et al.* 2012). In *S. aureus*, initial adherence to abiotic surfaces is associated with the charge of its techoic acid, while attachment to both abiotic and biotic surfaces such as implanted material is linked to host-factor binding proteins, the microbial surface components recognizing adhesive matrix molecules or MSCRAMMS (Heilmann and Gotz, 2010).

Following initial attachment, formation of biofilm occurs in *S. aureus* by accumulation of bacteria mediated by extracellular polysaccharides (Fluckiger *et al.* 2005). The best known mechanism mediating attachment and accumulation of bacteria in *S. aureus* is the polysaccharide intercellular adhesin (PIA) or polymeric N-acetyl-glucosamine (PNAG) produced by *ica* operon-encoded enzymes (O'Gara, 2007; Heilmann and Gotz, 2010). The model of PIA biosynthesis and function of the *ica* genes are shown in Figure 2.2. PIA plays a role in biofilm formation in both *S. epidermidis* and *S. aureus* and is the main molecule responsible for intracellular adhesion supposedly working like 'glue' sticking cells together by electrostatic interaction (Otto, 2008). Fluckiger *et al.* (2005) demonstrated that, during infections associated with medical devices, PIA production is induced and the authors concluded that, for colonization and survival of *S. aureus* on implants, the genes on the *ica* locus and biofilm formation are important factors.

The *ica* locus is made up of five genes: a regulatory gene *icaR* and four other genes on the locus *icaADBC*. Several laboratory-based assessments have investigated the role of the *ica* genes in biofilm development in both *S. epidermidis* and *S. aureus* (Arciola *et al.*

2001; Gad *et al.* 2009). The *icaA* and *icaD* genes work together to synthesise sugar oligomers *in vitro* with UDP-*N*-acetylglucosamine as substrate (Crampton *et al.* 1999). The *icaD* acts as a chaperone directing the folding and membrane insertion of *icaA* and also as a link between *icaA* and *icaD* although the true function of *icaC* is unclear (Gotz *et al.* 2006). The *icaB* is regarded as important as it has a crucial function in biofilm formation and virulence and is the only extracellular protein of the *ica* system (Vuong *et al.* 2004).

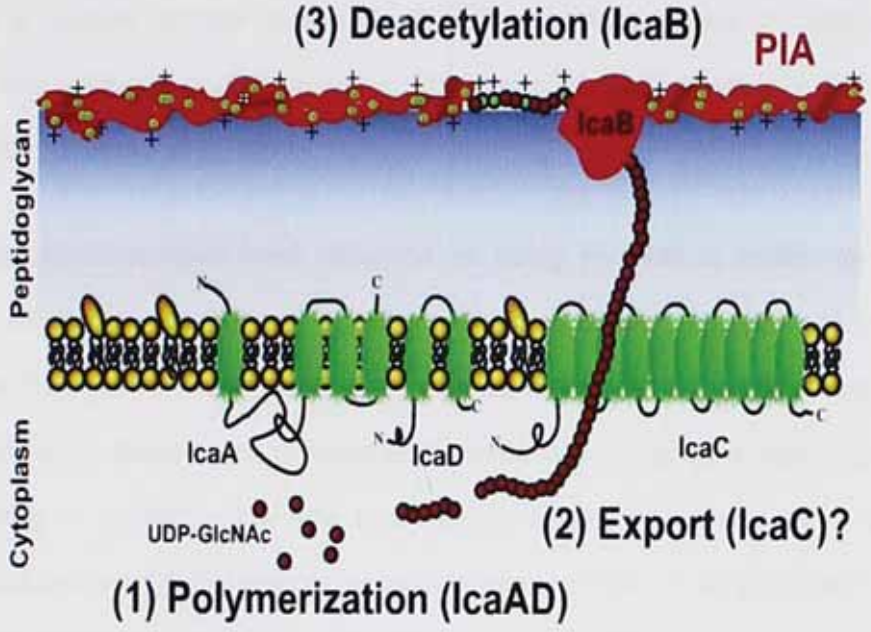


Figure 2.2. The model of PIA biosynthesis showing the genes of the ica locus and their functions. Source: Vuong *et al.* 2004)

The role of the *ica* locus in biofilm development is under debate as biofilm phenotypes have been observed which are PIA independent (Qin *et al.* 2007; Rohde *et al.* 2007). In cases of PIA-independent biofilm formation, adhesive proteins are the 'most likely substitute for PIA' (Otto, 2008). Examples of biofilm associated proteins in *S. aureus* include *S. aureus* surface protein G, *S. aureus* surface protein C, biofilm associated protein and catabolite control protein A (Corrigan *et al.* 2007; Seidl *et al.* 2008; Schroeder *et al.* 2009; Gruszka *et al.* 2012).

Other mechanisms have been observed as being involved in biofilm formation in *S. aureus*. Of these mechanisms, quorum sensing (QS) and regulation of certain genes have been studied in recent years. In *S. aureus*, cell to cell signalling is mediated by the genes of the agr locus which consists of five genes including; *agrA, agrC, agrD, agrB* and *hld* (Vuong *et al.* 2000; Sakoulas *et al.* 2002). However, recently, other studies have suggested another QS system governed by a gene *luxS* (Xu *et al.* 2006) although the role of this gene in QS has been debated (Doherty *et al.* 2006). Unlike previous observations in *P. aeruginosa* biofilms, in *S. aureus*, the *agr* mediated QS system impedes attachment and low *agr* is important for biofilm development (Boles and Horswill, 2008). It has been suggested that perhaps the QS system in *S. aureus* (*agr*) is involved in detachment of the biofilms (Yarwood *et al.* 2004).

Expression of the factors described as associated with biofilm formation is due to the activity of regulatory genes. To identify these genes, molecular methods have been used such as microarrays, western blotting analysis and gene expression studies. For regulation of the *ica* and the *agr* dependent genes, a gene staphylococcal accessory regulator (*SarA*) is responsible. Beenken *et al.* (2003) showed that mutation in the *SarA* gene led to a reduced ability for *S. aureus* to form biofilms while *agr* mutation is associated with increased biofilm formation suggesting opposing activity. Beenken *et al.* (2010) also demonstrated that *SarA* has an epistatic relationship to *agr* and regardless of the presence of mutation in the *agr*, the biofilm formation is dependent on *SarA*. Other

factors that have been identified in *S. aureus* biofilm development include proteins such as the stress induced sigma factor *SigB* and *rbf*. The role of *SigB* is still unclear (Rachid *et al.* 2000), however Lim *et al.* (2004) suggested that *rbf* is associated with regulation of multicellular aggregation in response to glucose and salt in *S. aureus* biofilms. Cue *et al.* (2009) further demonstrated that *rbf* promotes biofilm formation in *S.aureus* by controlling an unknown factor which represses *icaR* which is a negative regulator of the *icaADBC* locus.

2.8.2.2 Biofilm formation in Enterococci

The *enterococci* are opportunistic Gram-positive bacteria linked to a range of infections in humans and in animal populations. The most common of the *Enterococcus* species are *E. faecalis* and *E. faecium* which cause over 95 % of enterococcal infections (Mohammed and Huang, 2007). Both *E. faecalis* and *E. faecium* are clinically relevant organisms and have been associated with infections from indwelling medical devices, venous and urinary catheters (Garsin and Willems, 2010). It is apparent that more is known about *E. faecalis* than *E. faecium* strains with regard to their pathogenic factors and biofilm determinants (Di Rosa *et al.* 2006).

Biofilm formation is regarded as an important virulence factor in pathogenesis of enterococcal infections (Kouldhi *et al.* 2011) and is also associated with increased antibiotic resistance (O'Toole *et al.* 2000). Di Rosa *et al.* (2006) found higher numbers of biofilm formers in *E. faecalis* (85.2%) compared with *E. faecium* isolates (31.1%) suggesting that, regardless of the source, biofilm formation is common in *E. faecalis*. In another study, Ramadhan and Hegedus (2005) investigated biofilm formation in VRE and VSE isolates from a single hospital and found no biofilm formation in seven VRE isolates although nine out of twenty eight VSE isolates were positive for biofilm formation. In a review article by Mohamed and Huang (2007), the authors concluded that, *E. faecalis*

produces more biofilm than *E. faecium* and that biofilm formation may be an important pathogenicity factor in infections caused by *Enterococcus* sp.

Factors that have been associated with biofilm formation in *Enterococcus* sp. include nutritional factors such as nutrient availability, changes in osmotic strength and other environmental cues (Mohamed and Huang, 2007). Also important in the process of biofilm formation are involvement of surface proteins, QS systems and other gene products (Tendolkar *et al.* 2004; Mohamed and Huang, 2007).

The presence of an enterococcal surface protein (esp) was suggested as an important factor in biofilm formation in *Enterococcus* sp. (Toledo-Arana *et al.* 2001; Tendolkar *et al.* 2004). This was thrown into question when *esp*-independent biofilm formation was observed in *E. faecalis* (Kristich *et al.* 2004). Ramadhan and Hegedius (2005) concluded that this gene is neither necessary for biofilm formation nor sufficient for biofilm production in enterococci. However Di Rosa *et al.* (2006) observed that clinical isolates of *E. faecium*, positive for biofilm formation also possessed the *esp* gene and further concluded that although neither *esp* nor gelatinase are responsible for biofilm formation in *E. faecium* strains, both factors act in synergy in the establishment of infection.

A two-component QS system designated *fsr* has been demonstrated as a factor in biofilm formation in *E. faecalis*. Pillai *et al.* (2004) demonstrated that losing the function of *fsr* led to a reduced ability of *E. faecalis* to form biofilms. *Fsr* is a homologue of the *agr* locus, a QS cluster in *S. aureus* with known involvement in virulence and biofilm formation (Qin *et al.* 2000). The *fsr* locus is composed of three genes *frsA*, *fsrB* and *fsrC* and the resulting proteins FsrABC are important for producing two secreted proteases, gelatinase and serine proteinase (Hancock and Perego, 2004), with the former associated with the early stages of biofilm formation in *E. faecalis* (Kristich *et al.* 2004; Wang *et al.* 2011).

Other genes that have been associated with biofilm formation include; *epa* (enterococcal polysaccharide antigen), *bee* (biofilm enhancer in enterococcus), *bop* (biofilms on plastic surface), *eta* (Enterococcal two-component system regulator) as described by Mohamed and Huang (2007).

2.8.2.3 Biofilms in diverse environments

In healthcare settings biofilms have been observed in medical devices such as contact lenses (McLaughlin-Borlace *et al.* 1998), venous and urinary catheters (Sandoe *et al.* 2003), intrauterine devices (Auler *et al.* 2010), and dental applications (Subramani *et al.* 2009). In humans, a known environment of biofilm development is in dental plaques (Ten Cate, 2006) and in chronic wounds (James *et al.* 2008). Outside the healthcare environment, biofilms have been observed in food processing environments (Wong, 1998) and in swimming pools, hot tubs and piping systems (Lutz and Lee, 2011).

2.8.2.4 Significance of biofilms

Biofilms are important in both healthcare and non-healthcare settings. Biofilms demonstrate certain characteristics which are of clinical relevance such as resistance to antimicrobial agents, a harbour for pathogenic organisms with potential for resistance plasmid exchange, and a persistent source of infection (Donlan, 2001). An important aspect of biofilm development is the ability of bacteria in the biofilms to resist the action of antimicrobial compounds and protection against some antimicrobial compounds (Yarwood *et al.* 2004), by reducing antimicrobial susceptibility and increasing tolerance up to 1,000-fold (Thomas *et al.* 2006).

According to Anwar *et al.* (1992), formation of biofilms generates a sheltered encapsulated community of cells and this makes the cells less vulnerable to different stresses and the action of phagocytes. Stewart and Costerton (2001) offered three

hypotheses for biofilm resistance mechanisms; firstly, slow or incomplete penetration of the antibiotic into the biofilm, secondly, altered conditions in the microenvironment of the biofilms such as changes in depleted oxygen concentrations and altered osmotic environment, and thirdly, cell differentiation in a biofilm similar to spore formation. In a review article by Mah and O'Toole (2001), it was reported that lack of inhibition of diffusion of an antimicrobial into a biofilm does not always explain resistance. The authors also suggested that other factors such as slow growth rate of the bacteria in biofilms, heterogeneity of cells within the biofilms, general stress responses, involvement of genes in the biofilm phenotype and QS could be involved.

According to Hoiby *et al.* (2011), bacteria remain non-pathogenic and only colonise their hosts until QS is activated. The QS system was described by the authors as one which allows bacteria to sense the concentration of other bacteria within a limited microenvironment which in turn leads to gene activation that produces proteins, enzymes or toxins. To date, QS systems have been identified in many pathogens including *S. aureus* (Yarwood *et al.* 2004), *Vibrio chlorerae* (Hammer and Bassler, 2003), *Streptococcus* sp. (Cvitkovitch *et al.* 2003), *P. aeruginosa* (Hentzer *et al.* 2002).

QS in Gram-positive and Gram-negative bacteria uses different signalling molecules. Those in Gram-negative bacteria use the N-acyl homoserine lactones (AHL) which in high concentrations bind to activate a transcriptional regulator while in Gram-positive bacteria, posttranslational peptide signal molecules are the signalling molecules (Kievit and Iglewski, 2000). In *S. aureus* strains, QS is encoded by the accessory gene regulator (*agr*) locus which produces a molecule called autoinducing peptide (AIP) (Boles and Horswill, 2008). While QS has been suggested to be involved in biofilm development, Boles and Horswill (2008) reported the importance of *agr* repression in biofilm development in *S. aureus*.

2.9 Conclusion

The emergence of CAIs has altered the way the health community views and responds to certain pathogenic infections. At present the investigative tools which use phenotypic, genetic and epidemiological methods have been helpful in providing information on characteristics of the causative organisms of CAIs and the spectrum of disease. Also, good personal hygiene, early detection of CA-MRSA and decolonisation are measures that could control, limit or prevent infection spread (Witte, 2009).

Although disinfectants have been important in infection prevention to control environmental contamination and nosocomial infections, the emergence of new strains and the continuously growing knowledge of bacterial biofilms shows that there is a need for development of new products to support the already existing tools used in infection prevention. Also as the demand for products of natural origin continues to increase and the quest for greener and safer alternatives for the environment remains, there is a need to increase research activity on naturally occurring plant based materials such as essential oils which have shown promising results in many antimicrobial studies.

2.10 Aims

The aims of this study were to evaluate the differences in physiological and genetic characteristics between *S. aureus* isolates of CA and non-CA origin and a vancomycin resistant *E. faecium* strain and to determine the sensitivity of the isolates to antimicrobial agents.

This would be achieved by -

- Identifying the phenotypic characteristics of *S. aureus* strains including antibiotic susceptibility, slime formation and biofilm formation.
- To determine the SCC*mec* type of the *S. aureus* isolates and the presence of certain genes including: *mecA*, *ccrB2*, *icaA*, *icaD* and *pvl*.
- To investigate the effect of disinfectants and an environmentally friendly cleaning product against one VRE strain and six *S. aureus* strains, including a HA-methicillin sensitive and two CA *S. aureus* strains.
- To determine the antibacterial and antibiofilm effect of range of EOs against *S. aureus* strains
- To identify the differences in gene expression between two *S. aureus* strains following exposure to EOs.

CHAPTER THREE

General materials and methods

3.1 General Methods

3.1.1 Microorganisms

The test organisms used in this study included: vancomycin resistant *E. faecium* NCTC 12202 (Health protection Agency, London, UK), hospital MSSA isolate, methicillin sensitive *S. aureus* NCTC 13297, PVL positive CA MSSA, PVL positive CA MRSA, CA MRSA MW2 and CA MRSA SR donated by Prof. Mark Fielder from the Kingston University culture collection.

3.1.2 Preparation and maintenance of cultures

All culture media were prepared as recommended by the manufacturers. The list of microbiological media and chemicals used are shown in Appendix I. Stock cultures were stored on beads (Microbank PL 160/M, Pro-lab diagnostics, Cheshire, UK) at -80°C. Working cultures were maintained on BHI agar, sub-culturing weekly for a maximum of three weeks to maintain viability and colony characteristics. The isolates were subcultured on brain heart infusion (BHI) agar (70138-500G; Fluka Analytical, Sigma-Aldrich, UK) at 37°C and recultured on fresh BHI agar plates weekly to maintain viability and phenotypic characteristics.

3.1.3 Preparation of inoculum

Single colonies were picked from a BHI agar plate into BHI broth (CM225, Oxoid Ltd, Basingstoke, UK) and incubated overnight at 37°C. Enumeration of viable bacterial colonies was on BHI agar and the colony forming units (cfu) for each isolate determined using spread plating or the Miles and Misra method (1938). In each case, 20 µl of serial 10-fold dilutions of the overnight cultures of each organism was spotted onto BHI agar,

left to dry and incubated overnight at 37°C after which the numbers of colonies were enumerated.

3.1.4 Formation and quantification of biofilms in *S. aureus* and VRE

The method used was adapted from Stepanovic *et al.* (2007). 100 µl of overnight culture was diluted 1:100 in BHI broth supplemented with 1% (w/v) glucose. 200 µl was pipetted into the wells of a sterile 96-well plate. Negative controls were wells containing the BHI broth without organism. The contents of the wells were decanted, discarded and each well washed three times with 300 µl of sterile PBS (pH: 7.3 ± 0.3). Prior to fixation the plates were drained in an inverted position. The plates were then air dried for one hour and stained with 0.1% crystal violet (w/v) for 30 minutes at room temperature (Wijman *et al.* 2007), aspirated, washed three times with PBS (200 µl per well) before drying. The crystal violet was then solubilised using 10% (v/v) glacial acetic acid and the resultant OD measured at 595nm using a Microplate reader (Bio-Rad 680XR, Hertfordshire, UK).

3.1.5 Statistical Analysis

Statistical analysis was conducted using SPSS v17.0. Significance levels were set at P=0.05. Assumptions of normality of the distributions and homogeneity of variances were checked. For parametric data, where assumptions of normality and variance were met, t-tests and one way analysis of variance (ANOVA) was performed while in cases were assumptions of normality and variance were not met, the Mann Whitney and Kruskall-Wallis tests were used for non-parametric data.

CHAPTER FOUR

Identification of phenotypic and genetic characteristics of the bacterial isolates.

4.0 Introduction

S. aureus remains a very important human pathogen and infections caused by the methicillin resistant strains continue to be a problem within healthcare. Since the detection of methicillin resistance in the 1960s (Jevons, 1963), there has been a rise in the rates of methicillin resistance and resistance to antibiotics in general (Brown *et al.* 2005) and also other antimicrobial agents such as triclosan and quartenary ammonium compounds (McDonnell and Russell, 1999). There has also been the emergence of HA-MRSA and, even more recently, CA-MRSA infections with phenotypic and genetically distinct characteristics from HA-MRSA (Nathwani *et al.* 2008). An important characteristic used in differentiating HA from the CA MRSA is in SCC*mec* type. Of the eleven SCC*mec* types, I to III are associated with traditional hospital infections while the types IV to VI are characteristic of CA-MRSA (Chen *et al.* 2009; Boyle-Vavra and Daum, 2007). The differences observed between CA strains and HA strains also include antibiotic susceptibility where currently CA-MRSA are known to be more susceptible to non β-lactam antibiotics (Elston *et al.* 2009; Fey *et al.* 2003) than HA-MRSA.

Identifying antibiotic susceptibility patterns in microorganisms is important in antibiotic treatment, epidemiological investigations and development of infection control policies (Marias *et al.* 2009; Brown *et al.* 2005). Alongside the development of resistance traits, another important feature of *S. aureus* is the ability to form biofilms. Biofilm formation in bacteria has been described as a means of survival (Donlan and Costerton, 2002) and a virulence characteristic (Vuong and Otto, 2002). Certain genetic characteristics in *S. aureus* are related to virulence, resistance and survival. One of the genes associated with virulence, is PVL linked with virulence in CA-MRSA (Boyle-Varva and Daum, 2007). Another gene associated with biofilm formation and virulence in *S. aureus* is the

intracellular adhesion gene (*ica*), which is involved in mediation of cell to cell adhesion and initiation of biofilm formation (Gardner *et al.* 2011).

The objective of this study was to investigate the phenotypic characteristics of the *S. aureus* strains including antibiotic susceptibility and biofilm formation and subsequently investigate the genetic characteristics of the *S. aureus* strains. Multiplex PCR was used to determine genetic characteristics of the *S. aureus* isolates in this study. Since it was first developed by Chamberlain *et al.* (1988), multiplex PCR has also been applied in other investigations to determine genetic characteristics of MRSA (Oliveira and Lencastre, 2002; Mason *et al.* 2001).

4.1 Materials and Methods

4.1.1 Antibiotic Screening Tests

4.1.1.1 Bacterial strains

Six bacterial strains were investigated for antibiotic susceptibility (section 3.1.1). The control strain was *S. aureus* NCTC 12981 based on British Society for Antimicrobial Chemotherapy (BSAC) Guidelines; version 10.2 (Andrews and Howe, 2011).

4.1.1.2 Inoculum preparation

Following BSAC guidelines three to four colonies of each organism were taken from Iso-Sensitest Agar (ISA) (Oxoid Basingstoke, UK) using a sterile loop, added to 1ml sterile PBS and vortexed for one minute. Optical densities (OD) were measured using a CE 1011 spectrophotometer (Cecil Instruments Ltd, Cambridge, UK) at 600nm for each strain and standardised to between OD 0.8 and OD 1.2 to attain semi-confluent growth. The cultures were then spread onto agar plates using sterile cotton swabs.

4.1.1.3 Selection of antibiotics

The antibiotics selected for this study including the concentrations and breakpoints are shown in Table 4.1.

4.1.1.4 Disc diffusion

Susceptibility of the bacteria to antibiotics was assessed using the disc diffusion assay according to BSAC guidelines on Iso-Sensitest agar (ISA) plates. The antibiotic discs (Table 4.1) were deposited on the ISA agar within 30 minutes of spreading the cultures and the plates incubated overnight at 37°C. After incubation, diameters of the zones of inhibition were measured using vernier callipers to the nearest millimetre at the inner edges of the zones observed. Interpretation of sensitivity or resistance was confirmed using the recommended breakpoints.

Table 4.1. Antibiotics Tested.

Antibiotic group	Antibiotics	Concentration of antibiotic disk(μg)	Zone interpretation (mm)		
			R ≤	I	S ≥
Quinolones	Ciprofloxacin	1	13	-	14
Aminoglycosides	Gentamicin	10	19	-	20
β-lactam	*cefoxitin	10	21	-	22
Miscellaneous antibiotics	Rifampicin	2	23	24-29	30
Macrolides, lincosamines and streptogramins	Erythromycin	5	16	17-19	20
Tetracyclines	Tetracycline	10	19	-	20

*Cefoxitin was selected for measurement of methicillin resistance (following BSAC recommendations).

4.1.2 Phenotypic determination of slime and biofilm formation

4.1.2.1 Mannitol fermentation using mannitol salt agar (MSA)

MSA was prepared according to manufacturer's instructions. Single colonies of *S. aureus* strains were streaked onto the MSA and incubated overnight at 37°C for 24 hours. Observation of yellow colonies indicated positive mannitol fermentation, hence confirming the isolates as *S. aureus* while pink colonies were negative for mannitol fermentation hence non-*S. aureus* isolates. All tests were performed in triplicate and on three separate occasions. The negative control strains were *S. epidermidis* NCTC 13360 and *S. epidermidis* NCTC 11047.

4.1.2.2 Determination of slime formation using Congo red agar (CRA)

This was performed according to the methods of Mathur *et al.* (2006) and Mariana *et al.* (2009). Briefly, the *S. aureus* strains and the VRE NCTC 12202 isolate were streaked onto media containing BHI (52g/L, Sigma), sucrose (36g/L), congo red dye (0.8g/L) and incubated at 37°C for 24 hours . Black colonies indicated the strain was positive for slime production while pink colonies indicated weak slime production. All tests were performed in triplicate and on three separate occasions. The control strain for this experiment was the *S. epidermidis* NCTC 13360, known to be negative for slime formation.

4.1.2.3 Time dependent determination of biofilm formation

Overnight cultures of the *S. aureus* isolates and the VRE NCTC 12202 strain were diluted 1:100 to give approximately 1 x 10^8 Cfu/ml in BHI supplemented with glucose and added to wells of a 96 well plate (Nunclon™ surface, Denmark). To determine the time required for biofilm formation, 200 µl of diluted overnight cultures were incubated on 96-well plates for 24 hours, 48 hours or 72 hours. At each time point the wells were rinsed and the crystal violet staining method was applied (Section 3.2) and the absorbances were measured at 595nm using the Bio-Rad 680XR Microplate reader.

4.1.2.4 Biofilm growth with adjustment of growth media

200 µl of overnight cultures of each *S. aureus* strain at approximately 1 x 10^8 cfu/ml were added to the wells of 96-well plates and incubated at 37°C for 24 hours. Following overnight incubation, 100 µl was gently removed from the wells and replaced with 100 µl sterile broth. The 96-well plates were incubated for a further 24 or 48 hours dependent on the organism after which, the plates were rinsed, stained with crystal violet (Section 3.2) and the absorbances measured at 595nm using the Bio-Rad 680XR Microplate reader.

4.1.3 Detection of *mec*A, PVL, SCC*mec* and IcaA and IcaD in *S. aureus* strains.

4.1.3.1 Selection of bacterial strains

Six bacterial strains were investigated for antibiotic susceptibility (Section 3.1.1). The control strains were *S. epidermidis* NCTC 11047 which is negative for both PVL and the *mec*A gene, and *S.epidermidis* NCTC 13360 which is negative for both *Ica*A and *Ica*D genes.

4.1.3.2 Primers

The primers used in this study were purchased from MWG Biotech, UK (Table 4.2).

Table 4.2. Selected genes and primer sequences

	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Control strain
Staph 756-F	AACTCTGTTATTAGGGAAGAACA	756	McClure *et al.* 2006
Staph 750-R	CCACCTTCCTCCGGTTTGTCACC		
luk-PV-F	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	McClure *et al.* 2006
luk-PV-R	GCATCAAGTGTATTGGATAGCAAAAGC		
*mec*A - F	GTAGAAATGACTGAACGTCCGATAA	310	McClure *et al.* 2006
*mec*A - R	CCAATTCCACATTGTTTCGGTCTAA		
*lca*A- F	CCTAACTAACGAAAGGTAG	1315	Dhanawade *et al.* 2010
*lca*A - R	AAGATATAGCGATAAGTGC		
*lca*D- F	AAACGTAAGAGAGGTGG	381	Dhanawade *et al.* 2010
*lca*D - R	GGCAATATGATCAAGATA		
ccrB2 F	GAAGGTTATAGTATCGACGGACAAATC	66	Valvatne *et al.* 2009
ccrB2 R	ACCACGGTCAGCGTATATATCTTTAA		
Type IVa F	ATAAGAGATCGAACAGAAGC	278	Milheirico *et al.* 2007
Type IVa R	TGAAGAAATCATGCCTATCG		

Primer specificity for each gene was checked using single PCR analysis. The internal control reference gene was the 16S rRNA gene.

4.1.3.3 DNA extraction

DNA was extracted from cultures of each of the strains using the GenElute bacterial Genomic DNA kit (Sigma-Aldrich, UK) and performed according to the manufacturer's instructions (Appendix I).

4.1.3.4 Quantification of Deoxyribonucleic acid (DNA)

Following isolation of DNA using the GenElute kit, the yield and concentration of the extracted DNA was measured using the GeneQuant-Pro Quartz spectrophotometer (Amersham Bioscience, UK) measuring absorbances at 260, 280 and 320 nm. Absorbance of 1.0 at 260 nm for DNA corresponds to approximately 50 mg/ml of double-stranded DNA and the value of the $A_{260}–A_{320}/A_{280}–A_{320}$ ratio should be 1.6–1.9. To quantify the DNA, 6 µl DNA sample was added to 114 µl of the elution solution in a cuvette and the absorbances measured. 20 ng of extracted DNA per sample was subsequently subjected to amplification by multiplex PCR.

4.1.3.5 Multiplex PCR protocol

Single PCR amplification reactions were used to test for primer adequacy before performing the multiplex PCR assay. The total volume used for the multiplex PCR amplification was 25 µl and the PCR reaction was performed according to the manufacturer's instructions. 1 µl of genomic DNA was added to 24 µl of the QIAGEN Multiplex PCR Master mix which also included 0.2 µM of the forward and reverse primers. The PCR was performed using the *MyCycler*™ Thermal Cycler (Bio-Rad, UK) using the following parameters: an initial PCR activation step for 5 minutes at 95 ^{0}C to activate the HotStar Taq *Plus* DNA polymerase, followed by 35 cycles at 95 ^{0}C for 30 seconds (denaturation), 57 ^{0}C for 90 seconds (annealing), 72 ^{0}C for 30 seconds (extension) and a final extension step at 68 ^{0}C for 10 minutes.

4.1.3.6 *MecA* and *PVL* gene

The method for simultaneous detection of the *mec*A and PVL genes by multiplex PCR approach was adapted from and McClure *et al.* (2006). A single target PCR assay was applied with each primer pair to check for amplification and specificity of each gene target. The expected sizes of each fragment and the genes are shown in Table 4.2. The internal control was the 16S rRNA gene. The primers were the Staph756F and Staph750R and the control strain was *S.epidermidis* (NCTC11047) which is negative for both the *PVL* and *mec*A genes.

4.1.3.7 *IcaA* and *IcaD* Genes

Simultaneous detection of *IcaA* and *IcaD* was performed. The primers used were chosen from studies by Duran *et al.* (2010) and Crampton *et al.* (1999). The control strain was *S. epidermidis* NCTC 13360 which is negative for both *Ica*A and *IcaD* genes and the 16S rRNA gene was the internal control.

4.1.3.8 SCC*mec* type IV Screening

Determination of the SCC*mec* type for the *S. aureus* strains was also performed using multiplex PCR. The CA MRSA (MW2) strain was used as a positive control as it is positive for the SCC*mec* type IV (Maree *et al.* 2007) and the 16S rRNA was used as an internal control. The PVL CA MSSA strain was used as a negative control as it is negative for *mecA* encoded by SCC*mec*.

4.1.3.9 Gel Electrophoresis

For resolution of amplification products, a 2% (w/v) agarose gel (Duran *et al.* 2010; Arciola *et al.* 2001) in 1 x Tris-acetate-EDTA (TAE) buffer was used at 100V and this was visualised using the Gel Doc 1000 (Bio-Rad) following addition of ethidium bromide (Sigma-Aldrich, UK). An aliquot of 0.8 µl of a 100 bp DNA ladder (Promega, UK) was used to determine the size of the amplified products.

4.2 Results

4.2.1 Antibiotic Screening Results

In the strains under investigation methicillin sensitivity and/or resistance was confirmed using cefoxitin following the BSAC guidelines. There was no difference in the antibiotic sensitivity profiles between the hospital, type and community acquired assigned MSSA strains as all three strains were sensitive to all six antibiotic groups with no antibiotic resistance patterns determined (Table 4.1).

Methicillin resistance was confirmed in the three CA-MRSA strains as these strains were resistant to cefoxitin. The CA-MRSA (MW2) and the PVL CA MRSA strains showed a similar antibiotic profile with sensitivity to the other antibiotic groups. However the CA-MRSA (SR) strain showed a different susceptibility pattern in that it was resistant to four antibiotics: ciprofloxacin (quinolones), Gentamicin (aminoglycosides), cefoxitin (β-lactam) and the erythromycin (macrolides, lincosamines and streptogramins). Antibiotic sensitivity was observed with rifampicin (miscellaneous antibiotics) and tetracycline (tetracyclines). The control strain (*S. aureus* NCTC 12981) was sensitive to all the antibiotics selected (Table 4.3).

Table 4.3. Antibiotic susceptibility profiles of *S. aureus* strains.

	Interpretation of zone diameters (mm)			Hospital MSSA	PVL CA MSSA	MSSA NCTC 13297	MRSA MW2	PVL CA MRSA	CA MRSA (SR)	Control
	R ≤	I	S ≥							
Ciprofloxacin	13	-	14	29.30 (S)	23.50 (S)	21.41 (S)	20.87 (S)	16.63 (S)	0.00 (R)	14.42 (S)
Gentamicin	19	-	20	25.21 (S)	23.30 (S)	22.46 (S)	22.80 (S)	24.04 (S)	7.27 (R)	28.71 (S)
Cefoxitin	21	-	22	28.96 (S)	28.39 (S)	26.88 (S)	15.63 (R)	17.48 (R)	19.48 (R)	29.98 (S)
Rifampicin	23	24-29	30	31.51 (S)	30.74 (S)	30.03 (S)	31.25 (S)	29.88 (S)*	29.58 (S)*	31.98 (S)
Erythromycin	16	17-19	20	26.28 (S)	25.96 (S)	24.33 (S)	24.04 (S)	24.37 (S)	0.00 (R)	24.25 (S)
Tetracycline	19	-	20	30.34 (S)	29.43 (S)	28.89 (S)	29.63 (S)	28.24 (S)	28.47 (S)	30.20 (S)

Key – (S) sensitive, (I) intermediate, (R) resistant; Control strain - *S. aureus* NCTC 12981

4.2.2 Phenotypic identification by MSA and Congo red agar (CRA)

All the *S. aureus* strains tested were positive for mannitol salt fermentation while the *S. epidermidis* strains (NCTC 11047 and NCTC 13360) were both negative demonstrated by the colonies on the plates remaining pink (Table 4.4).

Table 4.4. Determination of mannitol fermentation by *S. aureus* strains

Bacterial isolates	Mannitol fermentation (MSA agar)
Hospital MSSA isolate	positive
PVL CA MSSA	positive
MSSA NCTC 13297	positive
CA MRSA (MW2)	positive
PVL CA MRSA	positive
CA MRSA (SR)	positive
S. epidermidis NCTC 11047	negative
S. epidermidis NCTC 13360	negative

For slime production by the Congo red method, the six *S. aureus* strains were positive as the enumerated colonies were black however the *S. epidermidis* strains and VRE NCTC 12202 were negative producing pink colonies.

Table 4.5. Slime production on Congo red agar. Classification of slime formation was adopted from Mariana *et al.* (2009) with the slime producers forming black to slightly black colonies while the non slime producing strains produce strong red colonies.

Bacterial isolates	Phenotype	Slime formation (CRA Agar)
Hospital MSSA	Black	positive
MSSA NCTC 13297	Strong black	positive
PVL CA MSSA	Strong black	positive
CA MRSA MW2	Strong black	positive
PVL CA MRSA	Strong black	positive
CA MRSA (SR)	Strong black	positive
S. aureus NCTC 12981	Strong black	positive
S. epidermidis NCTC 13360	Red	negative
VRE NCTC 12202	Red	negative

4.2.3 Biofilm formation in *S. aureus* strains

All the *S. aureus* strains were positive for biofilm formation after incubation for 48 hours using the 96-well plate method. The PVL CA MSSA strain formed a higher amount of biofilm with an OD of 1.4 compared to the other strains ranging between OD 0.54 and1.18 (Figure 4.1). When biofilm formation was compared between the strains, ANOVA showed there was a significant difference $F(5) = 21.199$, $p < 0.001$. Post hoc analysis further showed that biofilm formation in the PVL MSSA strain was significantly higher than in Hosp MSSA, MSSA NCTC 13297, CA MRSA MW2 and the PVL CA MRSA isolates $p \leq 0.007$, while there was no significant difference between biofilm formation in PVL CA MSSA and CA MRSA SR isolates, $p = 0.229$.

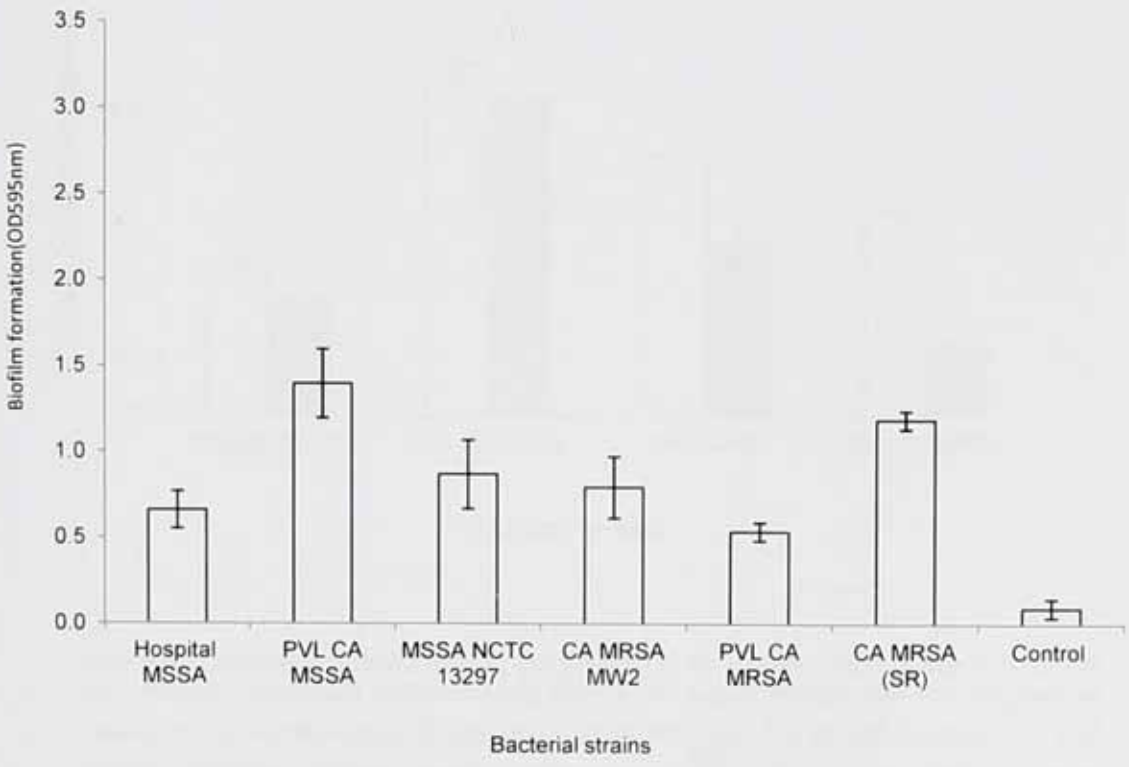


Figure 4.1. Biofilm formation (mean ± SE), after 24 hours in microtitre wells in six *S. aureus* strains and *S. epidermidis* NCTC 13360 (control contained BHI broth without any organism). The experiment was performed in quadruplicate wells and at three separate occasions.

4.2.3.1 Effect of nutrient media change

Biofilm formation was higher in the wells with the changed media compared to the wells without (Figure 4.2). Comparison of the results following media change showed differences between biofilms with and without changing the media in all four strains. Assumptions of normality were met and independent *t*-tests showed that there were no significant differences between changing the media at the 24 hour time point for the Hosp MSSA and PVL CA MSSA isolates $p \geq 0.05$. However, t-test analysis showed significant differences in biofilm formation after the media was changed for CA MRSA MW2 $t\,(14) = 0.010$, $p = 0.010$ and PVL CA MRSA $t\,(14) = 11.014$, $p < 0.001$.

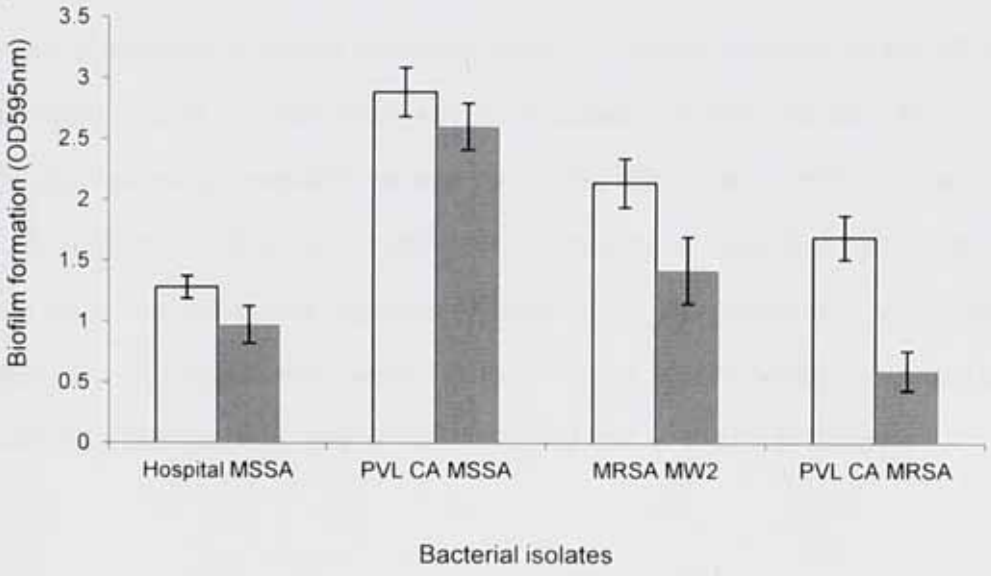


Figure 4.2. Preliminary data showing comparison of biofilm formation (mean ± SE), with and without replenished nutrient media (BHI broth supplemented with 1% glucose) as measured by the Microplate reader (Bio-Rad 680XR) with OD at 595nm. Key: ☐ (OD results following nutrient media replenishment), ☐ (OD results without media replenishment). The experiment was performed in quadruplicate wells and at three separate occasions.

4.2.3.2 Time dependent measurement of biofilm formation

Figure 4.3 demonstrates the growth of PVL CA MSSA, MSSA NCTC 13297 and *E. faecium* NCTC 12202 over a 72 hour period following initial incubation in a 96-well plate. The *E. faecium* NCTC 12202 strain did not form a biofilm as measured by CV staining with absorbance measurements below OD=0.2 at all time points. Between 24 and 48 hour time points, biofilm formation in the MSSA NCTC 13297 increased from OD 1.74 to OD 1.97 but reduced at 72 hours with an OD 1.0. Comparison of biofilm formation over the three day period showed significant difference $F(2) = 45.049$, $p < 0.001$. Post hoc analysis within the group showed that at 72 hours, biofilm formation was significantly reduced $p < 0.001$.

There was a reduction in biofilm formation in PVL CA MSSA between 24 and 48 hours from OD 2.05 to 1.84 however there was an increase in biofilm formation at 72 hours. ANOVA was carried out between the time points, the difference in biofilm formation was significant $F(2) = 68.725$, $p < 0.001$ and post hoc analysis showed that biofilm formation between each time point was significantly different for this isolate $p \leq 0.002$. Biofilm formation was also significantly greater in the PVL CA MSSA isolate compared to the MSSA NCTC 13297 as confirmed by t-test $t(22) = 3.356$, $p < 0.001$ at 72 hours.

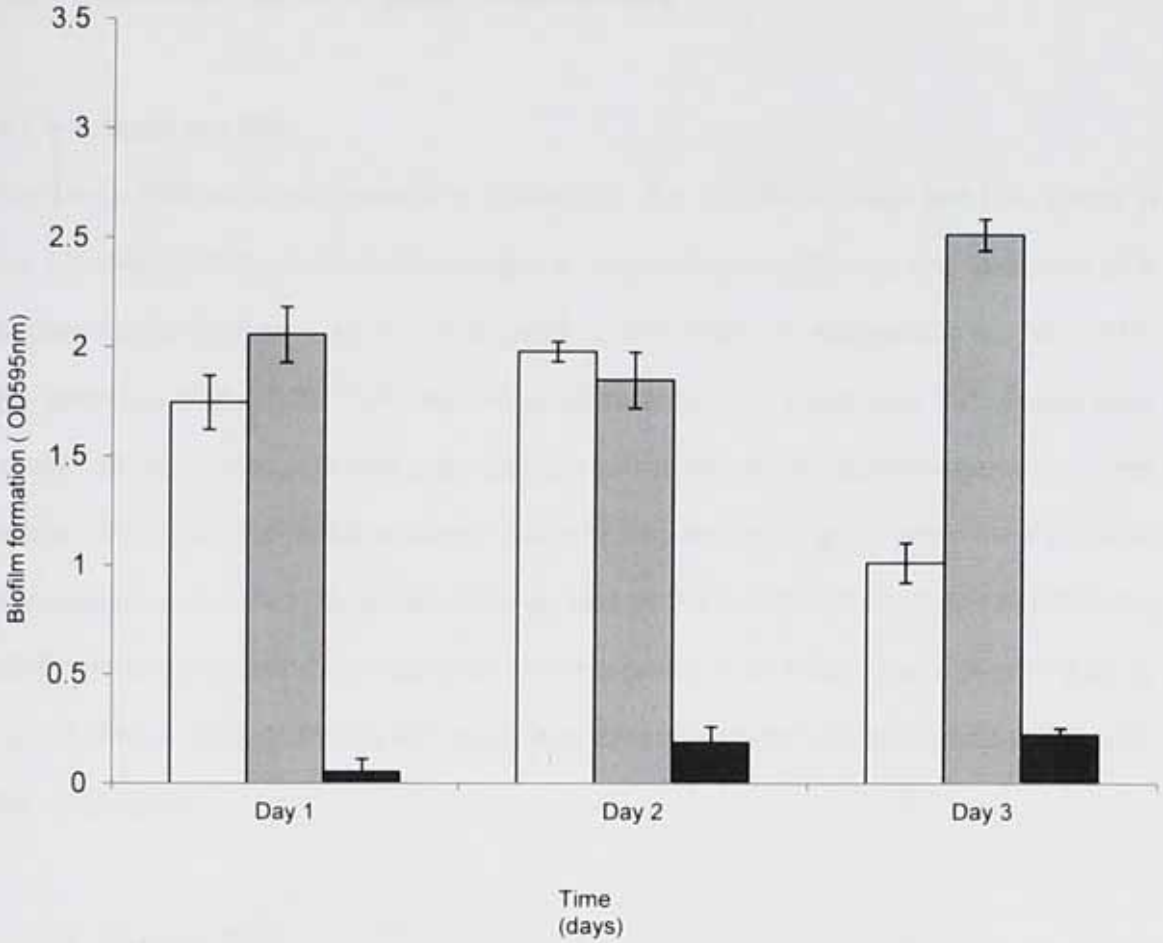


Figure 4.3. Preliminary data showing time dependent measurement of biofilm formation ($OD595_{nm}$) in ☐ MSSA NCTC 13297, ▒ PVL CA MSSA and ■ *E. faecium* NCTC 12202 over a three day incubation at 37 °C in 96-well plates as measured by the Microplate reader (Bio-Rad 680XR) with OD at 595nm (mean ± SE). The experiment was performed in quadruplicate wells and at three separate occasions.

4.2.4 Molecular analysis of genetic characteristics

4.2.4.1 *MecA* and PVL

The single PCR protocol showed the presence of the 16S rRNA, *mec*A and PVL genes in the CA MRSA MW2 strain which is known to possess all three genes and was used as a positive control (Figure 4.4). In the negative control strain (*S. epidermidis* NCTC 11047) the presence of the 16S rRNA was demonstrated but both *mec*A and PVL genes were absent. All six *S. aureus* strains were positive for the 16S rRNA reference gene: the three MSSA strains and the negative control strain lacked the *mecA* gene, while the PVL gene was present in the PVL CA MSSA strain as well as the CA MRSA MW2 and the PVL CA MRSA strains confirming the presence of these genes in all three strains (Figure 4.5). In the CA MRSA (SR) strain, the PVL gene was absent although this strain was positive for the *mecA* gene.

4.2.4.2 *IcaA* and *IcaD*

The presence of both *IcaA* and *IcaD* genes was confirmed in the CA MRSA MW2 strain using the single PCR (Figure 4.6). Figure 4.7 shows the presence of both *IcaA* and *IcaD* genes in all six *S. aureus* strains. In the *S. epidermidis* NCTC 13360 (the negative control strain), both genes were absent (Figure 4.6 and 4.7).

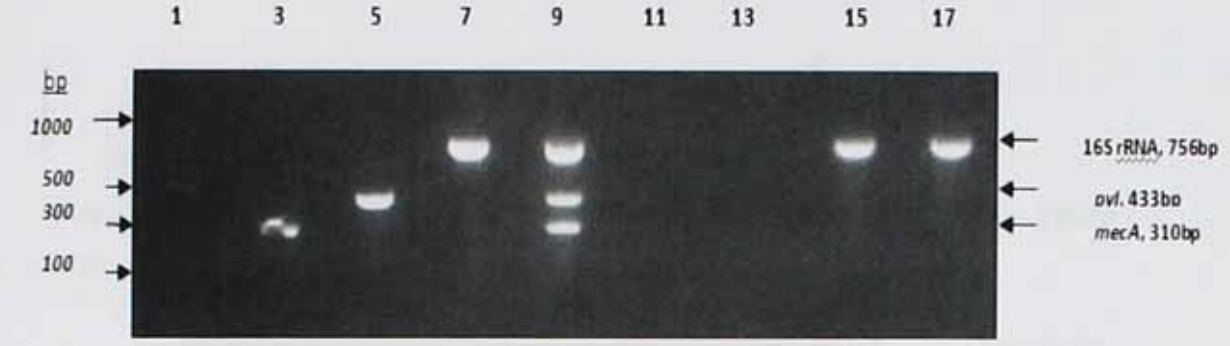


Figure 4.4. PCR amplification of 16S rRNA, PVL and *mecA* genes in CA-MRSA (MW2) and *S. epidermidis* NCTC 11047. Lane 1: DNA molecular size marker (100 bp ladder); Lane 3: *mecA* (MW2); Lane 5: PVL (MW2); Lane 7: 16S RNA (MW2); Lane 9: 16S RNA, PVL & *mec*A (MW2); Lane 11: *mec*A (*S.epidermidis* NCTC 11047); Lane 13: PVL (*S.epidermidis* NCTC 11047); Lane 15: 16S RNA, PVL, *mec*A (*S.epidermidis* NCTC 11047); Lane 17: 16S RNA, PVL, *mec*A (*S.epidermidis* NCTC 11047).

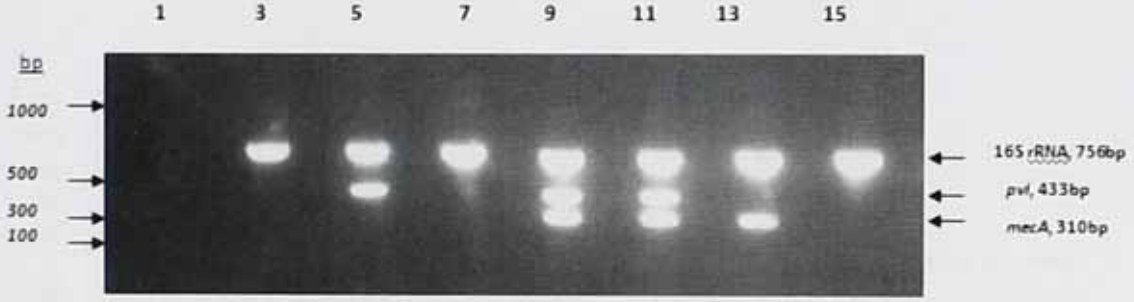


Figure 4.5. Multiplex PCR amplification of 16S, PVL and *mecA* genes in six *S. aureus* strains and *S. epidermidis* NCTC 11047. Lane 1: DNA molecular size marker (100 bp ladder); Lane 3: 16S RNA (Hospital MSSA); Lane 5: 16S RNA, PVL, *mec*A (PVL CA MSSA); Lane 7: 16S RNA, PVL, *mec*A (MSSA NCTC 13297); Lane 9: 16S RNA, PVL, *mec*A (CA MRSA MW2); Lane 11: 16S RNA, PVL, *mec*A (PVL CA MRSA)Lane 13: 16S RNA, PVL, *mec*A (CA MRSA (SR); Lane 15: 16S RNA, PVL, *mec*A (*S. epidermidis* NCTC 11047).

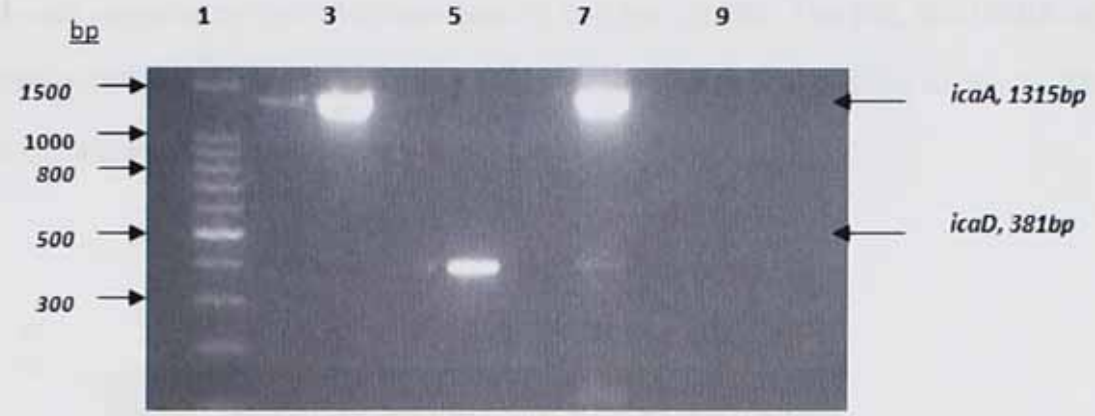


Figure 4.6. PCR amplification of *icaA* and *icaD* genes in the CA MRSA (MW2) strains and controls strain *S. epidermidis* NCTC 13360. Lane 1: DNA molecular size marker (100 bp ladder); Lane 3: IcaA (MW2); Lane 5: *IcaD* (MW2); Lane 7: *IcaA* and *IcaD* (MW2); Lanes 9-13 negative for *IcaA*, *IcaD* and both *IcaA* and *IcaD* in the control strain.

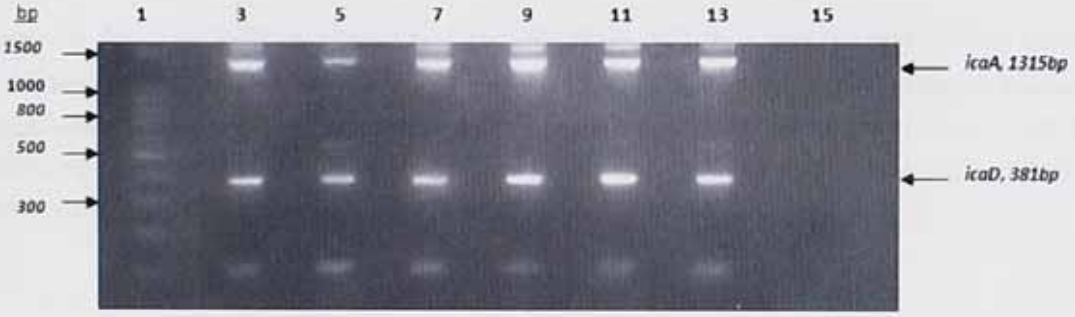


Figure 4.7. Multiplex PCR amplification of *icaA* and *icaD* genes in six *S. aureus* strains and *S. epidermidis* NCTC 13360. Lane 1: DNA molecular size marker (100 bp ladder); Lanes 3 to 13 were positive for *IcaA* and *IcaD* for the following organisms in the order (L3 – Hospital MSSA, L5 – PVL CA MSSA, L7 – MSSA NCTC 13297, L9 – CA MRSA MW2, L11 – PVL CA MRSA, L13 – CA MRSA (SR); Lane 15 was negative for both IcaA and IcaD (*S. epidermidis* NCTC 13360).

4.2.4.3 Detection of Type IVa SCC*mec* and CCRB2

Lanes 3, 5 and 7 in Figure 4.8 show that both the SCC*mec* type IVa and *CCRB2* genes were present in the CA MRSA MW2 strain while lane 9 showed that the PVL CA MSSA strain was negative for both SCC*mec* type IV and the *CCRB2*. The PVL CA MRSA and CA MRSA (SR) strains in Lanes 11 and 13 respectively were both positive for the *CCRB2* gene but lacked the SCC*mec* type IVa.

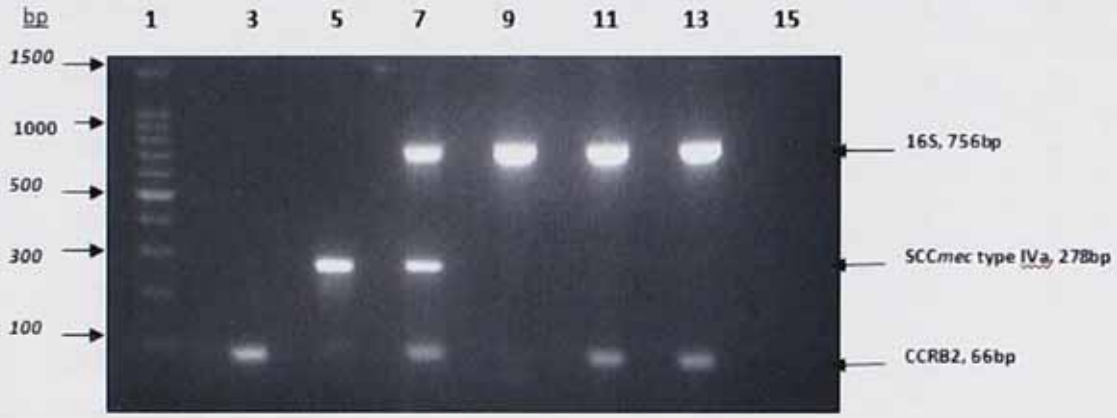


Figure 4.8. Multiplex PCR amplification of the 16S rRNA, SCC*mec* type IVa and CCRB2 genes in the CA MRSA MW2 and the experimental control (PVL CA MSSA). Lane 1: DNA molecular size marker (100 bp ladder); Lanes 3: CCRB2 (CA MRSA MW2); Lane 5: Type IVa (CA MRSA MW2); Lane 7: 16S RNA, CCRB2 (?bp) and SCC*mec* (CA MRSA MW2); Lane 9: 16S RNA (PVL CA MSSA); Lane 11: 16S RNA and CCRB2 (PVL CA MRSA) and Lane 13: 16S RNA and CCRB2 [CA MRSA (SR).

Summary

Table 4.6 shows a summary of the physiological and genetic characteristics identified in the *S. aureus* strains used in this study.

Table 4.6. Summary table of the phenotypic and genetic characteristics identified in six *S. aureus* test strains.

	Hospital MSSA	PVL CA MSSA	MSSA NCTC 13297	CA MRSA (MW2)	PVL CA MRSA	CA MRSA (SR)
Antibiotic susceptibility						
Ciprofloxacin	S	S	S	S	S	R
Gentamicin	S	S	S	S	S	R
cefoxitin	S	S	S	R	R	R
Rifampicin	S	S	S	S	S	S
Erythromycin	S	S	S	S	S	R
Tetracycline	S	S	S	S	S	S
Mannitol fermentation (MSA)	+	+	+	+	+	+
Slime formation (CRA)	+	+	+	+	+	+
Biofilm formation (CV method)	+	+	+	+	+	+
Investigated genes						
mecA	-	-	-	+	+	+
PVL	-	+	-	+	+	-
IcaA	+	+	+	+	+	+
IcaD	+	+	+	+	+	+
SCC*mec* type IVa	ND	-	ND	+	-	-
CCRB2	ND	-	ND	+	+	+

Key – (S) – sensitive; (R) – resistant; (+) – positive; (-) – negative.

4.3 Discussion

Determination of methicillin susceptibility in the *S. aureus* strains was confirmed using cefoxitin and all the strains were confirmed as either sensitive or resistant to methicillin using this antibiotic based on BSAC guidelines. The hospital MSSA strain, the PVL CA MSSA and MSSA NCTC 13297 strains were all sensitive to all the antibiotic groups with no difference between the Hospital MSSA and the PVL CA MSSA strain in antibiotic profile. With the MRSA strains, cefoxitin resistance was observed which confirmed methicillin resistance in these strains. Both PVL CA MRSA and CA MRSA MW2 strains demonstrated similar antibiotic susceptibility patterns as both strains were susceptible to five of six antibiotic groups. The antibiotic susceptibility profiles of both CA-MRSA strains have been demonstrated previously and these strains are sensitive to the effects of the antibiotic groups tested in this study (Vandenesch et al. 2003; Millar et al. 2008).

The antibiotic susceptibility profile of the CA MRSA SR isolate was different from the other two strains and fits the profile of the HA-MRSA strains according to the susceptibility patterns described by Millar *et al.* (2008) as HA-MRSA are not susceptible to fluoroquinolones, aminoglycosides, erythromycin and clindamycin. However the CA-MRSA strains are generally sensitive to both tetracycline and rifampicin (Vandenesch *et al.* 2003; Delorme *et al.* 2009) which was observed in the CA MRSA SR strain. The CA-MRSA (SR) strain was multidrug resistant (resistant to four of six antibiotics classes). Multidrug resistance is defined as resistance to three or more representatives of the following classes of antibiotics: quinolones, extended-spectrum cephalosporins, β-lactams, aminoglycosides and carbapenems (Hujer *et al.* 2006).

Slime production was determined in the six *S. aureus* strains although absent in the two *S. epidermidis* strains, NCTC 11047 and NCTC 13360. The *S. aureus* strains were all positive for biofilm formation using the crystal violet staining method and in previous studies by Jain and Argawal, (2009) and Grinholc *et al.* (2007), there was a 91% and

96% correlation between the Congo red agar (qualitative) and CV staining (quantitative) methods. Kouidhi *et al.* (2011) also showed a correlation between slime production and biofilm formation using the CV staining method following investigation of biofilm formation in *Enterococcus* sp. However according to Croes *et al.* (2009) CRA screening should not be used as an alternative method to other methods of biofilm detection. In this study, although a small sample size, the *S.aureus* strains that were positive for slime production were also found to be positive for biofilm formation.

It has been suggested that differences exist in the mechanisms and regulation of biofilm formation in MRSA and MSSA (O'Neill *et al.* 2007) and that biofilm development in MRSA is primarily glucose-induced while in MSSA NaCl is more likely to induce biofilm development. However according to Fitzpatrick *et al.* (2006) in media supplemented with glucose, both MSSA and MRSA are likely to form biofilms. In this study, both MSSA and MRSA formed biofilms in the presence of glucose as observed in previous studies (Stepanovic *et al.* 2007; Croes *et al.* 2009). Biofilm formation was determined to be highest in the PVL CA MSSA strain compared with the other MSSA and MRSA strains. When the two strains with the most biofilms were compared i.e. PVL CA MSSA and CA MRSA (SR), the difference in the absorbance measurement (1.395 for PVL CA MSSA and 1.183 for CA MRSA SR) between the strains was not significant p=0.229.

In a review article by O'Toole (2000), it was reported that changes in environmental cues including nutritional conditions, temperature, osmolarity, pH, iron and oxygen, can influence biofilm formation. After changing the nutrient media in the microtitre plates after 24 hour biofilm formation, a higher amount of biofilm was formed. However as the biofilms formed withought nutrient media change were sufficient to perform experiments, subsequent biofilm formation experiments were performed without changing the growth media over 48 hour incubation.

Using multiplex PCR, methicillin susceptibility was confirmed in the strains tested. The cefoxitin resistant strains were mecA positive thus methicillin resistant while the cefoxitin sensitive strains were mecA negative hence methicillin sensitive. In the CA-MRSA strains the PVL genes are common (File, 2007) and (Zhao *et al.* 2012) demonstrated a high prevalence of the PVL (41.5%) in CA-MSSA infections. In this study, only six *S. aureus* strains were analysed and of these strains, three of the four community strains were PVL positive while one the CA-MRSA (SR) was PVL negative.

According to Vourli *et al.* (2009) introduction of a PVL-positive CA-MRSA strain (ST80-MRSA Type IV) into hospitals in Greece was linked to an increased severity of disease mainly due to the presence of the PVL gene, and other studies have shown that PVL does indeed contribute to disease severity (Etienne, 2005; Lipinska *et al.* 2011). Etienne *et al.* (2005) suggested that clinical laboratories routinely screen for the PVL toxin in *S. aureus* isolates due to the increasing prevalence of PVL positive CA-MRSA. Absence of the *PVL* gene in the CA-MRSA (SR) strain does not automatically suggest a lack of pathogenic potential as PVL negative CA-MRSA have been identified in other studies and are known to be pathogenic. For example, PVL negative CA-MRSA strains have been disseminated in Japan (Hisata *et al.* 2005; Kikuta *et al.* 2011) and Kikuta *et al.* (2011) found that their PVL negative CA-MRSA strains also caused impetigo which had previously been associated with the SCC*mec* type IVa (Takizawa *et al.* 2005) and SCC*mec* type V CA-MRSA (Yamamoto *et al.* 2010).

In the HA-MSSA strain in this study, *mec*A and *PVL genes* were not found and where this strain had previously shown sensitivity to cefoxitin hence methicillin sensitivity, the absence of the PVL was unsurprising as Dufour *et al.* (2002) previously found that MRSA strains isolated from hospital acquired infections were negative for the PVL gene.

SCC*mec* types are defined based on the ccrAB allotype and *mec*A gene locus in staphylococci (Ito *et al.* 2001). The *ccrB2* gene had been used previously as a internal positive control in the SCC*mec* IV multiplex (Milheirico *et al.* 2007b). Oliveira *et al.* (2008) proposed that sequencing an internal fragment of the ccrB is a promising tool for typing SCC*mec* as there is a correlation between ccrB allelic clusters and SCC*mec* types. Following multiplex PCR analysis it was determined that all three CA-MRSA strains were positive for *ccrB2* although the CA-MRSA MW2 strain was the only strain positive for the SCC*mec* type IVa which was expected. It is also worth noting that the *mecA* negative PCL CA-MSSA strain used in this study as a negative control strain in multiplex PCR for SCC*mec* did not possess the *ccrB2* gene. In a previous study (Sabet *et al.* 2012) the methicillin sensitive strains lacked the *ccr* genes hence SCC*mec* was deemed absent in these strains. The SCC*mec* IV observed in CA-MRSA is important and there is also the suggestion that the SCC*mec* IV subtypes play a role in spread of beta-lactam resistance between species and populations and could provide a selective advantage to staphylococci (Smyth *et al.* 2011).

Another important characteristic of *S. aureus* is the presence of intracellular adhesion genes which has been associated with biofilm formation (Crampton *et al.* 1999). In this study, both the *ica*A and *icaD* genes were identified in the six *S. aureus* strains tested. Crampton *et al.* (1999) suggested that the *ica* locus is present in nearly all *S. aureus* strains. In the study by Arciola *et al.* (2001), the authors reported that the *ica* locus was present in a majority of clinical slime producing staphylococcal isolates and suggested that the *icaA* and *icaD* genes play a role in the pathogenesis of catheter associated infections.

All six *S. aureus* strains in this study were shown to be positive for biofilm formation and were also positive for slime formation following enumeration on Congo red agar. Several studies have shown the association between the *ica* locus and biofilm formation in

staphylococci (Rohde *et al.* 2001; Cerca *et al.* 2005; Oliveira and Cunha, 2010) and the data presented here suggest a link between presence of the *icaA* and *icaD* genes with the formation of slime and biofilms in the *S. aureus* strains tested. Arciola *et al.* (2001) suggested a strong link between the phenotypic characteristic of slime production and the genotypic detection of the *ica* locus. Molecular analysis of the *ica* genes by Resch *et al.* (2005) also showed that the *ica* genes are up-regulated in a *S. aureus* biofilm and are necessary for adhesion and the initiation of biofilm formation. This further highlights the importance of the *ica* locus and determination of the genes present in the *ica* locus could help in routine diagnostic identification of particularly virulent *S. aureus* strains (Arciola *et al.* 2001). The presence of the *icaA* and *icaD* genes in all six investigated *S. aureus* strains in this study, coupled with the knowledge that these genes are present in majority of *S. aureus* isolates (Arciola et al. 2001) suggest a potential role of the *ica* locus as genetic markers in the identification of *S. aureus*.

Understanding the antibiotic susceptibility profiles, phenotypic and molecular characteristics of the community acquired isolates would provide support to the development of effective therapeutic options. However as shown in this study, the genetic changes that have occurred in *S. aureus* since the emergence of methicillin resistance continues to pose a problem for healthcare. Further research is required to identify the origin of the CA MRSA (SR) strain as this organism showed a multidrug resistant profile which is not a common characteristic of CA-MRSA strains.

CHAPTER FIVE

Antibacterial and antibiofilm activity of two disinfectants and an ecofriendly cleaner on *Staphylococcus aureus* and vancomycin resistant *Enterococcus faecium*

5.0 Introduction

In the past 20 years, there has been an increase in the number of antibacterial products and disinfectants sold commercially. For example, in 1994, only a dozen products containing antibacterial agents were marketed for use in the home. However by 2001, more than 700 were found to be available in the US for the same purpose (Levy, 2001). This observed rise in the number of antibacterial and disinfectant products has been attributed to media attention and subsequently an increase in products labelled as antibacterial has followed (Larson *et al.* 2004).

Disinfectants have been used extensively for topical and hard surface applications in healthcare and non-healthcare settings (Kagan *et al.* 2002; McDonnell and Russell, 1999) and remain an important tool in reducing microbial infections (Dancer, 2009). However, there are concerns of toxicity from conventional disinfectants such as chlorine releasing agents (Rutala and Weber, 1997). Cleaning products labelled and marketed as 'environmentally' friendly, with potentially less harmful health and environmental effects, have been developed in recent years (Markkanen *et al.* 2009). This is possibly due to the movement towards 'green consumerism' and the resurgence and development of supposedly less harmful natural animal and plant based products (Tuley de Silva, 1996). From *in vitro* studies, certain factors have been identified which cause variations in the efficacy of disinfectant products. These factors include those relating to the disinfectant or biocidal product such as concentration, contact time, organic load, formulation, temperature, pH and those which relate to the

organism such as the type of the organism, the amount or numbers of organisms present and/or presence of biofilms Maillard (2005).

The effect of disinfectants and cleaning agents has been studied extensively against hospital acquired infections (Rutala and Weber, 1997) however data on the effect of these products against bacteria of community origin is limited. There is also a lack of information made available to consumers on eco friendly or 'green' products despite an increase in the number of marketed products regarded as environmentally friendly, making product comparison difficult (Yates, 2009).

The objective of this study was to investigate the antibacterial activity and biofilm eradication ability of two chlorine based disinfectants and a commercially available eco friendly cleaning product (EFCP) against a vancomycin resistant *E. faecium* strain and four *S. aureus* strains.

5.1 Materials and Methods

5.1.1 Micro-organisms

Four *S. aureus* strains, MSSA NCTC 13297, HA-MSSA isolate, a PVL CA-MSSA and the PVL CA-MRSA and VRE strain *E. faecium* NCTC 12202 (Section 3.1.1) were used in this study. The media used and culture maintenance procedures are as described in Section 3.1.2.

5.1.2 Disinfectants

Two chlorine releasing disinfectants were selected for susceptibility testing; household bleach with sodium hypochlorite as the active ingredient and effervescent chlorine tablets containing sodium dichloroisocyanurate (NaDCC) as the active ingredient. An EFCP containing alcohol, sodium citrate, lactic acid, limonene and other ingredients in its composition was also tested (the amounts of each component was not specified by the manufacturer). The 'use' dilution for these products was 1.2% (v/v) or 500 ppm free available chlorine (FAC) for the household bleach, 1000 ppm FAC for the NaDCC and 1.2% (v/v) for the EFCP. The household bleach and the ecofriendly cleaner were purchased from a retail store while the NaDCC based disinfectant was purchased directly from the suppliers (Hydrachem, UK). The disinfectants were stored in the dark and in dry conditions and were prepared at the concentrations required on the day of the experiments. The disinfectant products were used within the shelf life as instructed by the manufacturer.

5.1.3 Determination of disinfectant activity against planktonic cells

5.1.3.1 Neutralizer efficacy

A neutralizer assay was performed based on previous methods (Rutala *et al.* 2000; Johnston *et al.* 2002) as neutralizer efficacy is critical in determining disinfectant activity (Sutton, 2002). Briefly, 1 ml of the disinfectant at the recommended use concentration was added to 9 ml of

neutralizer 1.1% sodium thiosulphate for the household bleach, and a universal quenching agent consisting of 1.0% (w/v) sodium thiosulphate, 0.07% (w/v) lecithin, 0.5% (v/v) Tween 20 and 0.1% (w/v) peptone for the NaDCC and 0.1 ml of an overnight culture at a final concentration of 10^4 cfu/ml was added to the mix and left for 60 minutes. Aliquots (0.1 ml) were spread on duplicate BHI agar plates and incubated at 37 °C for 24 hours before enumeration. Log recovery was calculated from each test sample. The experimental Controls contained neutraliser but no disinfectant/cleaner solution.

5.1.3.2 Quantitative suspension test (QST)

This was performed according to EN 1276:1997 with adaptations in the contact times and enumeration media. The experiments were performed under clean and dirty (in the presence of 0.3% w/v BSA) conditions at 1 minute, 5 minutes and 10 minutes. 0.1 ml of an overnight culture at approximately 10^7 cfu/ml was added to 9.9ml of disinfectant at the recommended use-dilution and to the control (sterile distilled water) at the contact times. After each contact time, 1 ml of the challenged sample was added to 9 ml of appropriate neutralizer, from which 200 µl was spread onto BHI agar and incubated at 37°C for 24 hours before enumeration. For dirty conditions, the same experimental method was used however, 1 ml of 0.3% (w/v) BSA was mixed with 1 ml of an overnight culture at ratio 1:1 and incubated at room temperature for 5 minutes before mixing with 9 ml of the disinfectant solution (test sample) or into 9 ml of sterile distilled water (control).

5.1.3.3 Quantitative surface test

This was performed in accordance with EN 13697 - Quantitative Surface test under both clean and dirty conditions with adaptations in the contact times and enumeration media. 0.1 ml of an overnight culture at approximately 10^7 cfu/ml was inoculated onto 2 cm stainless steel discs (Goodfellows Cambridge Ltd, UK) and left to dry in a sterile laminar flow cabinet

for 30 minutes. Once the discs were dried, 0.1 ml of the disinfectant or sterile distilled water (control) was gently deposited on the surface of the discs. The discs were then transferred into 50ml polypropylene centrifuge tubes (Scientific Laboratory Supplies, UK) containing the neutralizer and 3.5 – 4g of sterile glass beads (3-7mm; Scientific Laboratory Supplies, UK) at the contact times; 1 minute, 5 minutes and 10 minutes and immediately vortexed for two minutes (Autovortex SA6; Stuart Scientific, UK). Recovery was calculated using the Miles and Misra (1938) method after incubation on BHI at 37°C for 24 hours. For dirty conditions, 1 ml of 0.3% (w/v) BSA was mixed with 1ml of an overnight culture at ratio 1:1 for 5 minutes to attain an approximate concentration of 10^7 cfu/ml after which 0.1 ml of the culture was deposited on the discs and similar procedure to that of the clean conditions was performed.

5.1.4 Determination of disinfectant activity against biofilms

5.1.4.1 Bacterial strains

Two *S. aureus* strains were selected for biofilm experiments. The two *S. aureus* strains investigated for were PVL CA MSSA and MSSA NCTC 13297 as both strains had been demonstrated previously as efficient biofilm formers and demonstrated similar amounts of biofilm formation following 48 hour incubation (Figure 4.3).

5.1.4.2 Biofilm Formation

The method used was adapted from Stepanovic *et al.* (2007). 100 µl of overnight culture was diluted 1:100 in BHI broth supplemented with 1% (w/v) glucose. 200 µl of this was pipetted into the wells of a sterile 96-well plate and incubated at 37°C for 48 hours. Negative controls were wells containing BHI broth without the organism. The contents of the wells were decanted and discarded and each well washed three times with 300 µl of sterile PBS (pH: 7.3 ± 0.3) using a micropipette. Prior to fixation the plates were drained in an inverted position. The plates were then air dried for one hour, stained with 0.1% (w/v) CV diluted in sterile

distilled water for 30 minutes at room temperature (Wijman *et al.* 2007), aspirated and washed three times with PBS (200 µl per well) before drying. The CV was then solubilised using 10% (v/v) glacial acetic acid and OD measured at 595nm using a Microplate reader (Bio-Rad 680XR, Hertfordshire, UK).

5.1.4.3 Biofilm Eradication

After formation of biofilms in microtitre wells for 48 hours, the contents of the wells were discarded and 200 µl of either household bleach, NaDCC or the cleaning product added into the wells for the following contact times; 10 minutes, 30 minutes and 1 hour, after which the disinfectants were neutralized with sodium thiosulphate and universal quenching agent for one minute, and the wells rinsed with PBS three times. CV staining for biofilm quantification was performed and absorbances measured at 595nm using the Microplate reader (Bio-Rad 680XR, Hertfordshire, UK).

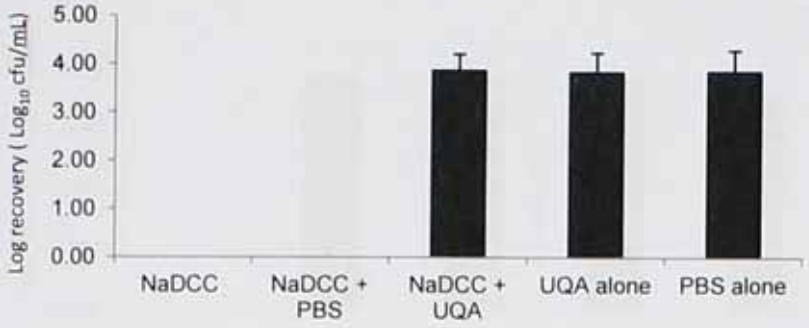
5.1.5 Statistical Analysis

Statistical analysis was conducted as described in Section 3.3.

5.2 Results

Sodium thiosulphate at 1.1% (v/v) was effective at neutralizing the household bleach and the universal quenching agent was effective at neutralizing NaDCC and the EFCP (Figures 5.1 and 5.2). In the suspension test household bleach reduced the viable counts of all the strains tested by ≥5 log within one minute at both 500 ppm (FAC) and 50 ppm (FAC) even in the presence of 0.3% (w/v) BSA (Table 5.1). Although the recommended use concentration for household bleach is 500 ppm (FAC), general use of this product by consumers in household environments is not regulated hence the rationale for testing its effect at a lower concentration.

NaDCC-based disinfectants are marketed under different brand names but generally used in industrial and healthcare environments with a recommended use concentration of 1,000 ppm. When tested at 1,000 ppm a ≥5 log reduction occurred within one minute for all strains tested both in the absence and presence of 0.3% (w/v) BSA (Table 5.1). Following exposure to the EFCP for 10 minutes, no significant reduction was determined for any of the strains in clean conditions (Table 5.1) and there was no significant difference between the strains after 10 minutes $F(4) = 2.34$, $p = 0.07$ determined by ANOVA. As a result, the effect of organic contamination was not determined and the product was not tested for activity against surface contamination.



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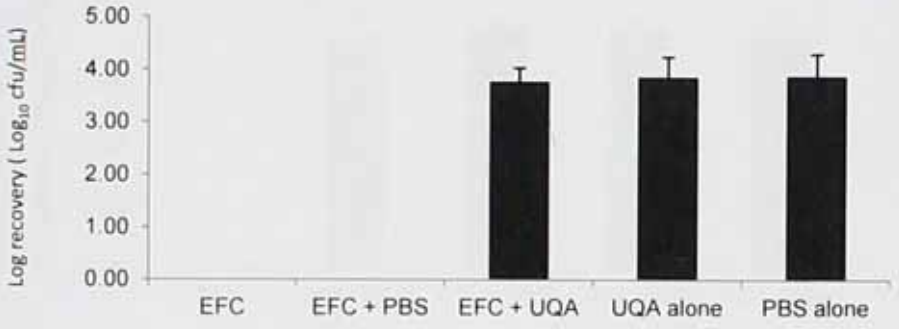

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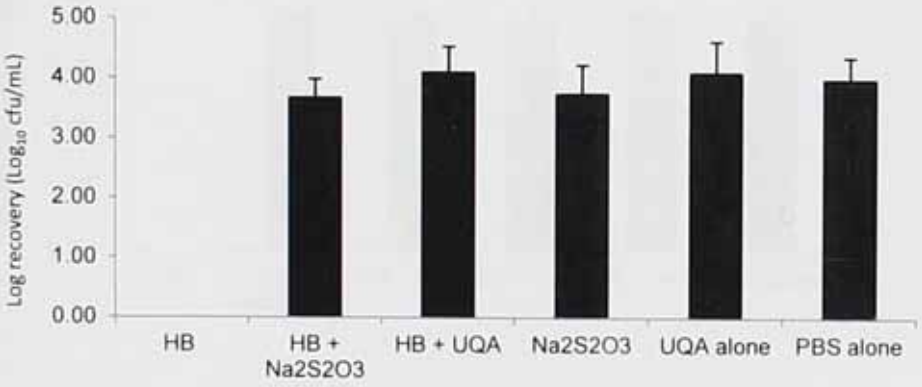
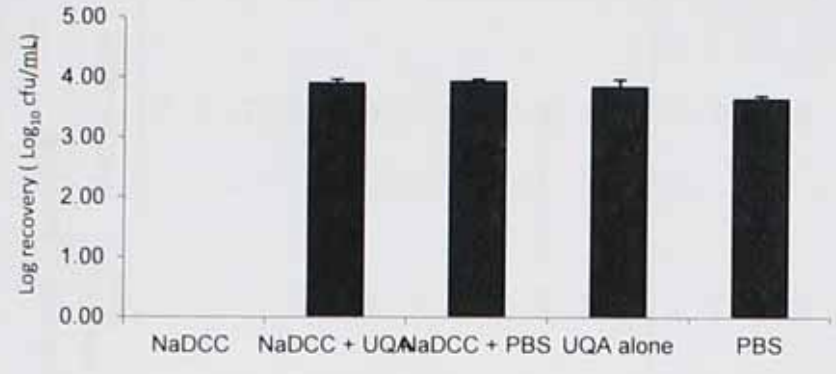


(c)

Figure 5.1 Log $_{(10)}$ recovery (mean ±SE; n=3) of MSSA NCTC 13697 after quenching of (a) NaDCC at 1000 ppm FAC (b) EFCP at 1.2% (v/v) (c) HB at 500 ppm FAC. The contact time was 60 minutes. N=3

Key: HB – household bleach; EFC – Environmentally friendly cleaning product; $Na_2S_2O_3$ – Sodium thiosulphate; UQA – universal quenching agent; PBS – Phosphate buffered saline.



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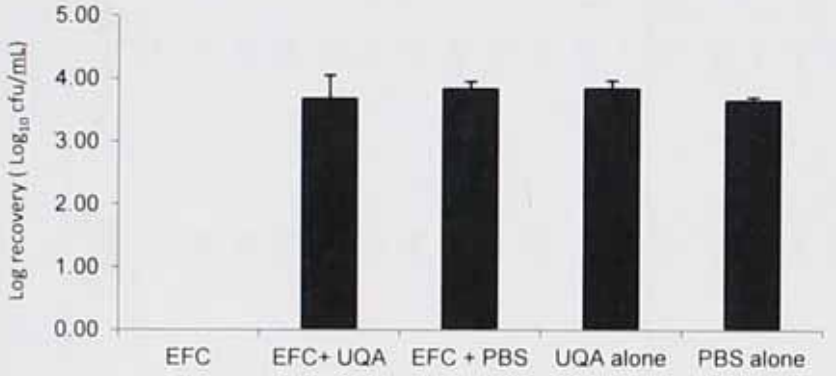

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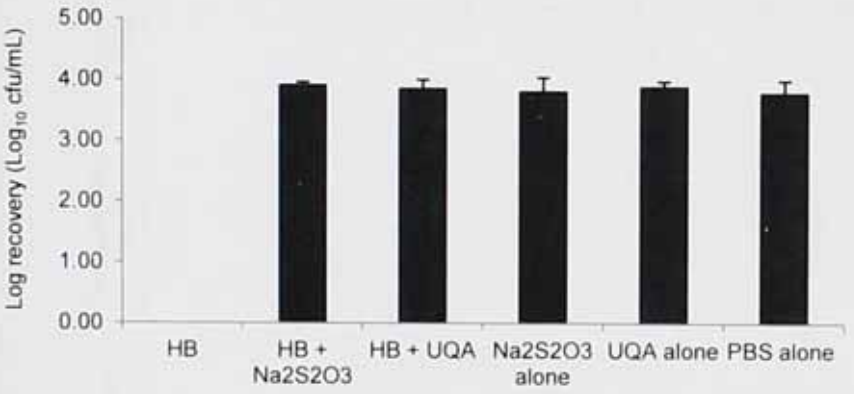


(c)

Figure 5.2 Log $_{(10)}$ recovery (mean ±SE; n=3) of *E. faecium* NCTC 12202 after quenching of (a) NaDCC at 1000 ppm FAC (b) EFCP at 1.2% (v/v) (c) HB at 500 ppm FAC. The contact time was 60 minutes. N=3

Key: HB – household bleach; EFCP – Environmentally friendly cleaning agent; $Na_2S_2O_3$ – Sodium thiosulphate; UQA – universal quenching agent; PBS – Phosphate buffered saline.

Table 5.1. Log $_{(10)}$ reduction in viable counts (mean ±SE; n=3) on exposure to household bleach, NaDCC and eco-friendly cleaning product using EN 1276 protocol.

				Log reduction (log_{10} cfu/ml)				
Product	**Conditions**	**Concentration (ppm)**	**Contact time (minutes)**	**Hospital MSSA**	**PVL CA MSSA**	**MSSA NCTC 13297**	**PVL CA MRSA**	***E. faecium* NCTC 12202**
Bleach	*Clean*	*500ppm*	1	>5	>5	>5	>5	>5
			5	>5	>5	>5	>5	>5
			10	>5	>5	>5	>5	>5
		50ppm	1	>5	>5	>5	>5	>5
			5	>5	>5	>5	>5	>5
			10	>5	>5	>5	>5	>5
	Dirty	*500ppm*	1	>5	>5	>5	>5	>5
			5	>5	>5	>5	>5	>5
			10	>5	>5	>5	>5	>5
		50ppm	1	>5	>5	>5	>5	>5
			5	>5	>5	>5	>5	>5
			10	>5	>5	>5	>5	>5
NaDCC	*Clean*	*1,000ppm*	1	>5	>5	>5	>5	>5
			5	>5	>5	>5	>5	>5
			10	>5	>5	>5	>5	>5
	Dirty	*1,000ppm*	1	>5	>5	>5	>5	>5
			5	>5	>5	>5	>5	>5
			10	>5	>5	>5	>5	>5
Ecofriendly cleaning product	*Clean*	*500ppm*	1	0.11 (-0.07)	0 (0.06)	0.11 (0)	0.1 (0.16)	-0.2 (0.1)
			5	0.02(-0.02)	0.01 (-0.05)	0.14 (0)	0.4 (0.04)	0.02 (-0.11)
			10	0.1 (-0.03)	-0.01 (-0.10)	0.11(0.02)	0.68 (0.05)	-0.21 (-0.1)

Dirty conditions = in presence of 0.3% (w/v) BSA.

In the surface tests, treatment with household bleach caused a log reduction ranging between 0.11-1.03, 1.06-1.80 and 1.57-2.51 at 1, 5 and 10 minutes respectively in clean conditions (Table 5.2). In the presence of 0.3% (w/v) BSA, the corresponding log reductions in viable counts were 0.30-1.03, 0.71-2.10 and 1.64-3.57. The greatest $\log_{(10)}$ reduction in viable count was observed with the PVL CA MRSA isolate at 2.35 (±0.55) in clean conditions and in dirty conditions, the greatest $\log_{(10)}$ reduction in viable counts at 10 minutes was observed with the Hosp MSSA isolate at 3.57 (±0.40). The $\log_{(10)}$ reduction in viable counts following exposure to NaDCC under clean conditions were between 0.86-1.02, 1.09-2.99 and 2.15–3.48 at 1 minute, 5 and 10 minutes respectively for all five strains tested with the greatest reduction observed for *E. faecium* NCTC 12202 at 3.48 (±0.39). In dirty conditions, the $\log_{(10)}$ reductions ranged from 0.91–1.51, 1.40–2.16 and 2.29–3.07 at 1 minute, 5 and 10 minutes respectively (Table 5.2).

The differences in $\log_{(10)}$ reductions between clean and dirty conditions were also compared using *t*-test where assumptions of normality were met and at 5 minutes, following exposure to household bleach at 500 ppm, the $\log_{(10)}$ reduction was significantly greater in dirty conditions than in clean conditions t (16) = 3.391, p = 0.002 for the Hosp MSSA isolate and in the *E. faecium* NCTC 12202 isolate, the $\log_{(10)}$ reduction was significantly greater in clean conditions than in dirty conditions (U = 15.5, p = 0.024) while there were no significant differences between both conditions in the other strains $p > 0.05$.

When the same isolates were exposed to 1000 ppm NaDCC at the 5 minute contact time, the $\log_{(10)}$ reduction in *E. faecium* NCTC 12202 was significantly lower in dirty conditions than in clean conditions (U = 3.00, p = 0.01) however there were no significant differences between both conditions in the other strains $p > 0.05$. At the 10 minute contact time, when the effect of clean and dirty conditions were compared between all the isolates tested, with the exception

of *E. faecium* NCTC 12202, there were no significant differences between the isolates. The $\log_{(10)}$ reduction was significantly greater in clean conditions $t(14) = 26.692$, $p = 0.02$ following exposure of *E. faecium* NCTC 12202 to household bleach and $U = 15.00$, $p = 0.024$, after exposure to NaDCC.

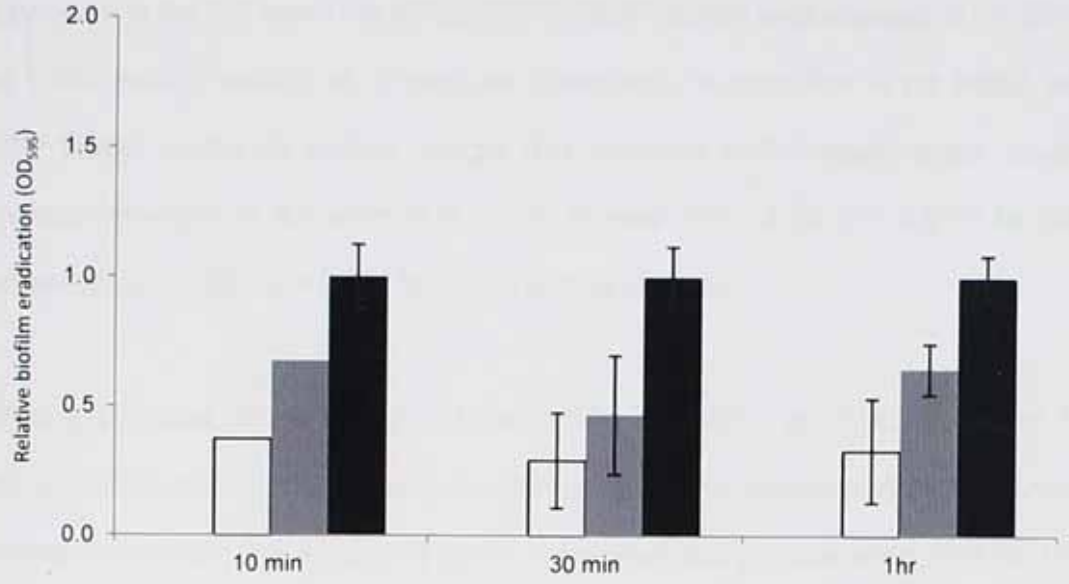
Table 5.2. Log $_{(10)}$ reduction in viable counts (mean ±SE; n=3) on exposure to household bleach and NaDCC using EN13697. These figures have been corrected for any log reduction due to drying alone.

				Log reduction (log_{10} cfu/ml)				
Product	**Conditions**	**Concentration (ppm)**	**Contact time (minutes)**	**Hospital MSSA**	**PVL CA MSSA**	**MSSA NCTC 13297**	**PVL CA MRSA**	***E. faecium* NCTC 12202**
Bleach	*Clean*	*500ppm*	1	0.49 (0.03)	0.11 (0.10)	0.93 (0.14)	0.96 (0.09)	1.03 (0.01)
			5	1.07 (0.14)*	1.06 (0.19)	1.42 (0.14)	1.49 (0.62)	1.80 (0.66)*
			10	1.98 (0.69)	1.74 (0.19)	1.57 (0.08)*	2.51 (0.45)	2.35 (0.55)*
	Dirty	*500ppm*	1	1.03 (0.09)	0.33 (0.20)	0.54 (0.05)	0.30 (0.70)	0.99 (0.03)
			5	2.10 (0.31)*	0.71 (0.10)	1.26 (0.00)	0.98 (0.31)	1.47 (0.02)*
			10	3.57 (0.40)	1.83 (0.03)	2.10 (0.02)*	1.64 (0.66)	2.08 (0.04)*
NaDCC	*Clean*	*1,000ppm*	1	1.02 (0.08)	0.86 (0.09)	1.65 (0.23)	1.20 (0.09)	1.80 (0.64)
			5	1.70 (0.05)	1.35 (0.23)	2.11 (0.12)	1.09 (0.86)	2.99 (0.48)*
			10	2.25 (0.09)	2.61 (0.03)	3.05 (0.42)	2.15 (0.63)	3.48 (0.39)*
	Dirty	*1,000 ppm*	1	1.44 (0.01)	0.95 (0.18)	1.51 (0.15)	1.00 (0.71)	0.91 (0.00)
			5	1.56 (0.05)	1.63 (0.16)	2.16 (0.22)	2.14 (0.21)	1.40 (0.05)*
			10	3.07 (0.05)	2.52 (0.18)	2.32 (0.37)	2.33 (0.79)	2.29 (0.08)*

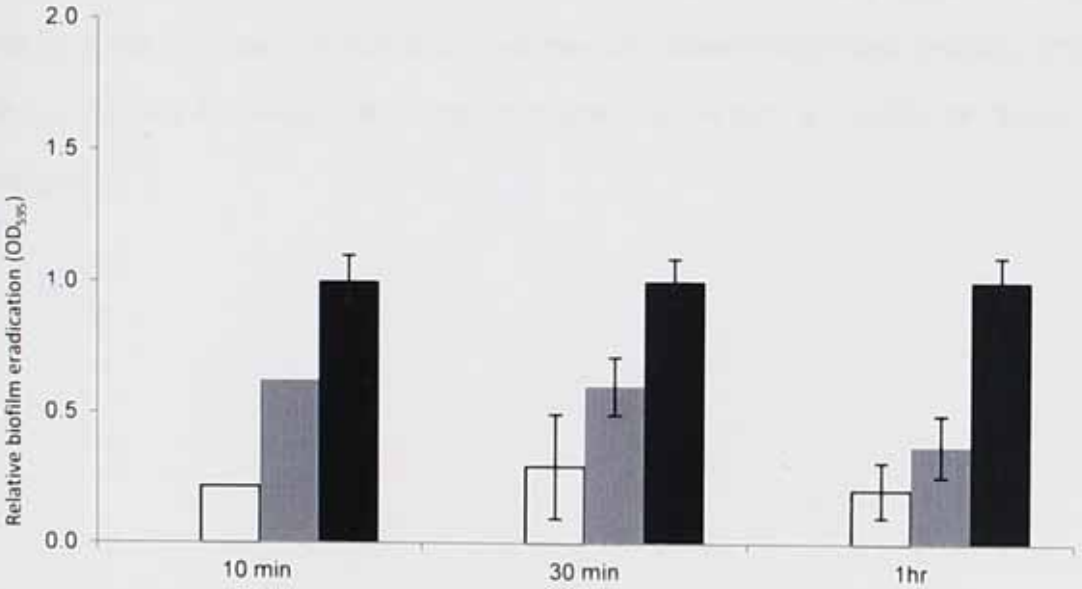
Dirty conditions = in presence of 0.3% (w/v) BSA

* Significantly different between clean and dirty conditions

Biofilm formation was observed in the two *S. aureus* strains (MSSA NCTC 13297 and PVL CA MSSA) but the *E. faecium* strain (NCTC 12202) did not form any biofilms (Figure 4.3). Exposure to household bleach at 5,000 ppm caused a total eradication of both PVL CA MSSA and MSSA NCTC 13297 biofilms within 10 minutes. Relative biofilm eradication at 500 ppm was 62% for MSSA NCTC 13297 and 78% for PVL CA MSSA within 10 minutes (Figure 5.3). When the biofilms were exposed to household bleach at 500 ppm, there was no significant difference in biofilm eradication at the 10 minutes, 30 minutes and one hour as determined by ANOVA, $F(2) = 0.450$, $p = 0.643$ for MSSA NCTC 13297 where assumptions of normality were met and by the Kruskall-Wallis test where assumptions of normality were not met, $H(2) = 2.668$, $p = 0.263$ for PVL CA MSSA. At 50 ppm, household bleach caused a 33 - 53% reduction in relative biofilm formation for MSSA NCTC 13297 at the three contact times while for PVL CA MSSA biofilms, relative biofilm formation was reduced by 38% at 10 minutes, 40% at 30 minutes and 63% at one hour (Figure 5.3).



(a)

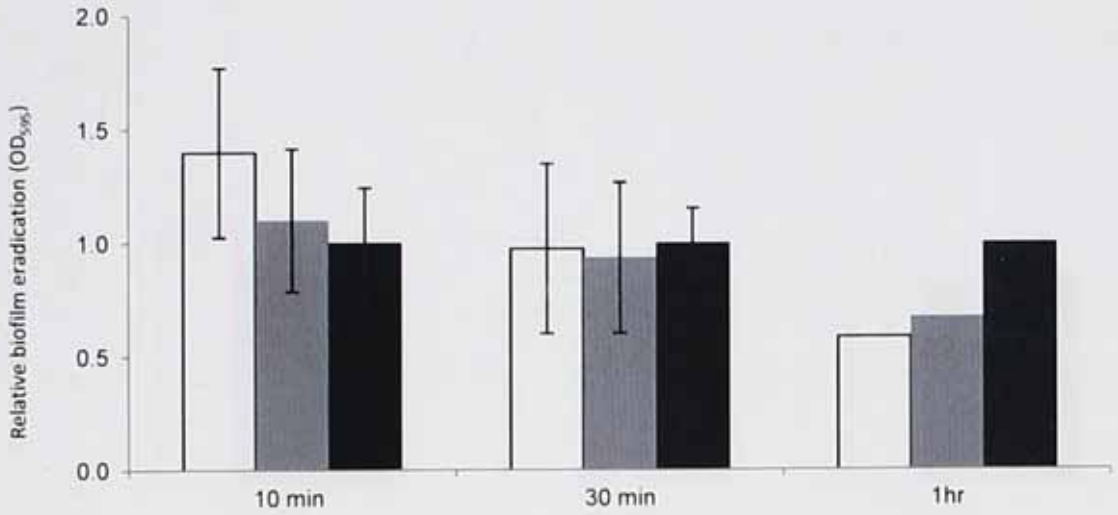


(b)

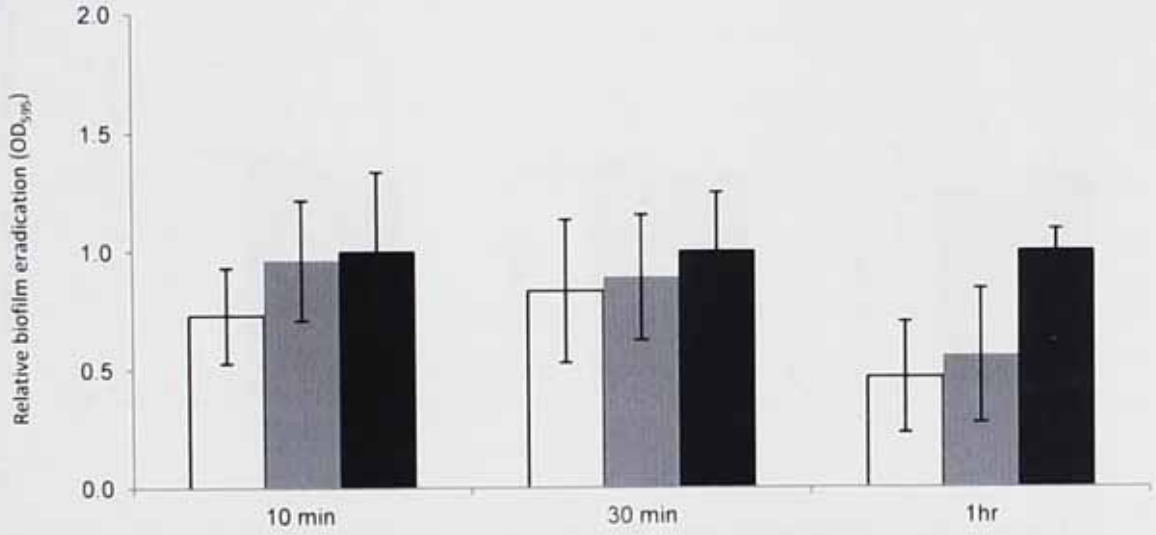
Figure 5.3. Relative biofilm eradication (mean ±SE; n=3) of (a) MSSA NCTC 13297 and (b) PVL (+) CA MSSA biofilms after 48 hour incubation and exposure to household bleach for 10 minutes, 30 minutes and 1 hour. (□ 500 ppm, ▒ 50 ppm and ■ 0 ppm)

Exposure of established biofilms to NaDCC at 10,000 ppm and 1,000 ppm resulted in a reduction in relative biofilm formation at one hour only for MSSA NCTC 13297. There was an increase in the OD when the MSSA NCTC 13297 biofilm was exposed to 10,000 ppm and 1,000 ppm of NaDCC at 10 minutes. There was no reduction in the MSSA NCTC 13297 biofilm at the 30 minute contact time however both concentrations caused a significant reduction in the amount of biofilm formed ($U = 12.00$, $p = 0.011$) for 10,000 ppm and $t(16) = 3.550$, $p = 0.027$ for 1,000 ppm at one hour.

Following exposure of the PVL CA MSSA biofilm to NaDCC at 10,000 ppm and 1,000 ppm within 30 minutes there was a approximately a 27% reduction in biofilm formation however at one hour, the amount of biofilm eradicated was approximately 54% for 10,000 ppm and 44% for 1,000 ppm (Figure 5.4). The reduction in biofilm formation was significant when the PVL CA MSSA biofilm was exposed to both concentrations of NaDCC at the one hour contact time confirmed by independent *t*-test analysis; $t(16) = 6.651$, $p < 0.001$ for NaDCC at 10,000 ppm and $t(15) = 8.881$, $p = 0.002$ for NaDCC at 1,000 ppm.



(a)



(b)

Figure 5.4. Relative biofilm eradication (mean ±SE; n=3) of (a) MSSA NCTC 13297 and (b) PVL (+) CA MSSA biofilms after 48 hour incubation and exposure to NaDCC for 10 minutes, 30 minutes and 1 hour. (□ 10,000 ppm, ▒ 1000 ppm and ■ 0 ppm)

Exposure of the biofilms to the EFCP at 100% and 1.2% (v/v) did not cause any significant reduction in relative biofilm formation at 10 minutes, 30 minutes or one hour. At 10 minutes there was an increase in relative biofilm formation of 12% and 21% for PVL CA MSSA and MSSA NCTC 13297 respectively (Figure 5.5).

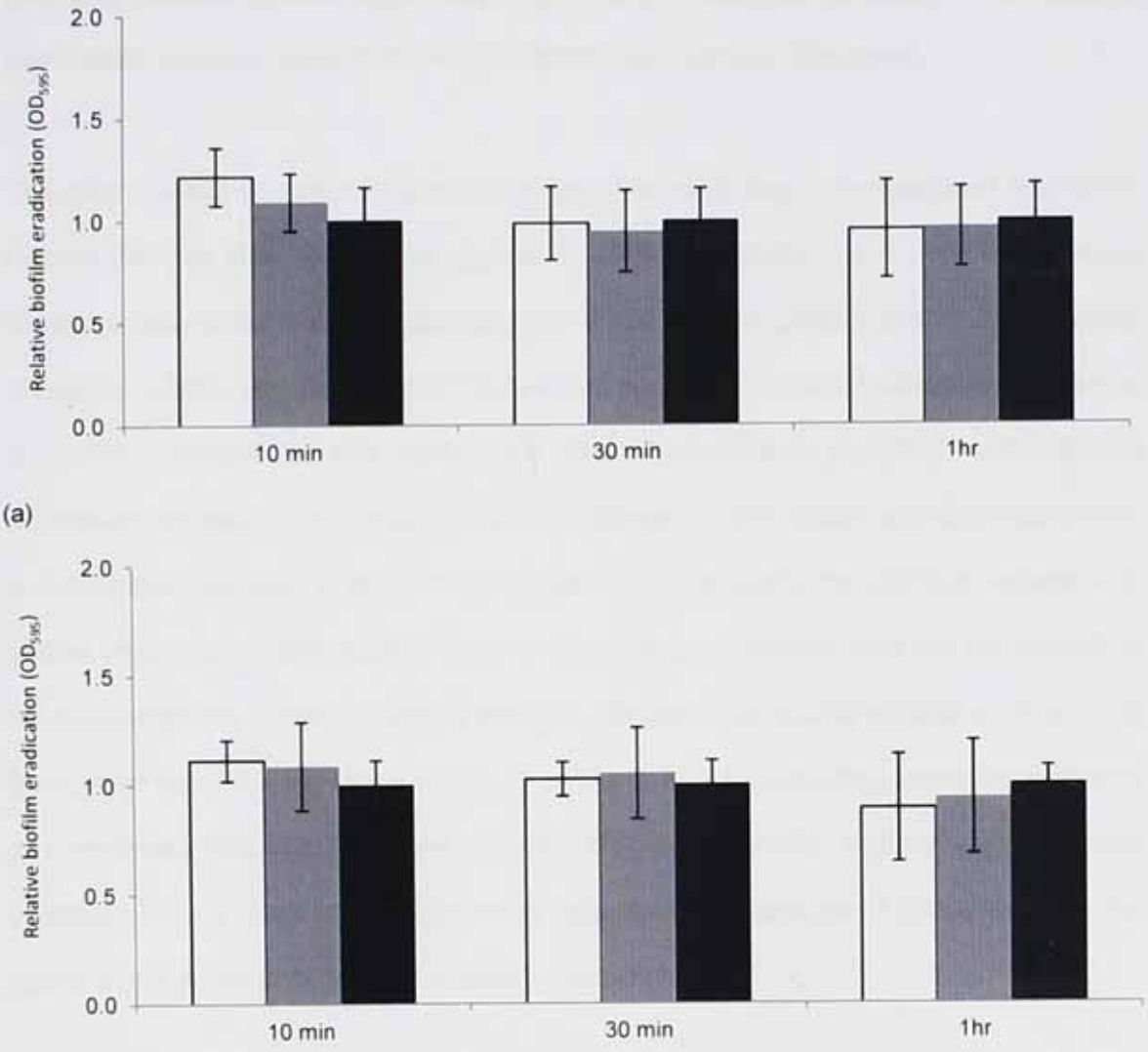


Figure 5.5. Relative biofilm eradication (mean ±SE; n=3) of (a) MSSA NCTC 13297 and (b) PVL CA MSSA biofilms after 48 hour incubation and exposure to the EFCP for 10 minutes, 30 minutes and 1 hour. (□ 10 min, ▒ 30 min and ■ 1hr)

5.3 Discussion

The antibacterial and sporicidal effect of sodium hypochlorite in household bleach and NaDCC has previously been demonstrated (Block, 2004; Heling *et al.* 2001; Rutala *et al.* 2000), however the continued emergence of community acquired infections and newly emerging bacterial strains emphasizes the need to investigate the effect of conventional disinfectant products and eco-friendly products against these pathogens.

The effectiveness of sodium hypochlorite has previously been demonstrated against *E. faecalis* (Gomes *et al.* 2001), and against *S. aureus* (Bloomfield and Uso, 1985), which corresponds with the results presented here. It has been suggested that, in the presence of organic matter, compounds such as sodium hypochlorite is less effective (Pappen *et al.* 2010). However in this study, both household bleach and the NaDCC-based disinfectant caused >5 log reduction within 1 minute in both 'clean' and 'dirty' conditions in suspension. Marques *et al.* (2007) did observe a reduction in the cfu/ml of biofilms of *S. aureus* after treating with NaDCC however the authors concluded that this disinfectant at the concentrations tested (100mg/L) was not effective at removing surface adhered cells from glass and stainless steel surfaces. In this study, no reduction in biofilm formation was observed following treatment with NaDCC using the CV method and this could perhaps be as a result of the differences between the strains used in this study or the nature of the surfaces in which the biofilms were formed.

The effect of disinfectants in suspension does not necessarily reflect their ability to eliminate microorganisms dried on surfaces (Rutala *et al.* 2000). Surface disinfection is an important hygiene control measure which can control dissemination of pathogens (Exner *et al.* 2004). For a disinfectant to be regarded as effective on surfaces, a 4 log reduction of a test organism must be achieved under defined conditions (Holah, 1996; Moretro *et al.* 2009). Both chlorine based disinfectants tested in this study in clean and dirty conditions caused less than 4 log reduction against all five organisms on stainless

steel surfaces with the greatest log reduction (3.48) in viable counts observed with the *E. faecium* strain at 10 minutes following NaDCC exposure.

Comparisons have been made in previous studies between sodium hypochlorite and associated products and NaDCC, with the latter being recommended as an alternative to sodium hypochlorite for disinfection (Clasen and Edmondson, 2006; Mazzola *et al.* 2003). This is because of its slower decomposition, and factors such as release of hypochlorous acid, stable chlorine levels, low toxicity and low corrosivity (Mazzola *et al.* 2003). In this study, both household bleach and the NaDCC-based disinfectant showed similar activity in suspension but on surface-attached bacteria, the NaDCC-based disinfectant was more effective. However this effect was reversed when the disinfectants were compared for anti-biofilm activity, with household bleach at the different concentrations (including the use dilution) removing biofilms while the NaDCC-based product was less effective. There have been a few previous reports on the anti-biofilm effect of NaDCC (Morgenthau *et al.* 2012; Marques *et al.* 2007).

The antibiofilm activity of the disinfectants was not investigated against the VRE isolate in this study as this isolate was negative for biofilm formation (Figure 4.3). In a previous study by Tote *et al.*(2010), exposure of *S. aureus* biofilms to sodium hypochlorite for 60 minutes demonstrated an increase in antibiofilm activity (0 to 55%). In this study, higher concentrations of household bleach resulted in greater antibiofilm activity; 5,000 ppm completely eradicating established biofilms while at the use dilution (500 ppm), only 63% of the biofilm was eradicated. The use of sodium hypochlorite at a 2% (v/v) concentration has been recommended for biofilm removal (Lee *et al.* 2009).

Marketing of eco-friendly products is on the basis that they contain natural materials and are designed as potential replacements or drop-in substitutes for disinfectants (Light, 2009) and have been used in the hospital environment (Markkanen *et al.* 2009) although

they are also likely to be used in community settings. The product tested in this study showed no anti-biofilm activity against the community isolates. Biofilms of the community *S. aureus* strain were more susceptible to both bleach and NaDCC than MSSA NCTC 13297 suggesting that household bleach would be most effective at reducing bacterial load in a non healthcare setting. There is therefore a need to provide information for consumers to enable proper selection of antibacterial products and their appropriate use in order to protect the population from infectious diseases (Bloomfield, 2007; Larson *et al.* 2004).

The results in this study demonstrate that disinfectants are effective in reducing bacterial contamination in different conditions and diverse *S. aureus* strains. The lack of antimicrobial activity in the eco-friendly cleaning product highlights the need for further research on the antimicrobial potential of these products occasionally marketed as 'alternatives' to conventional disinfectants and cleaning products.

CHAPTER SIX

Antimicrobial activity and antibiofilm activity of essential oils against *S. aureus* strains.

6.0 Introduction

Interest in natural antimicrobials has grown in recent years and the most important and well researched of these are plant products which have many medicinal and antimicrobial properties (Bourne *et al.* 1999; Cowan, 1999). EOs extracted from plants have been shown to possess antimicrobial activity in *in vitro* assays against a range of bacteria including known antibiotic resistant strains (Fisher and Phillips 2006; Warnke *et al.* 2009). EOs have been used as topical antimicrobials (Barker and Altman 2010; Dai *et al.* 2010), as dental and oral treatments (Palombo 2011; Jeon *et al.* 2011), and for burns and wound healing (Edwards-Jones *et al.* 2004; Thakur *et al.* 2011). Recently the use of EOs in vapour phase has also been shown to be antibacterial and antifungal, as reviewed by Laird and Phillips (2012).

In recent years, reports of studies on the anti-biofilm activity of EOs have been increasing. For example, cinnamon EO against *Candida* sp. (Pires *et al.* 2011) a citrus EO in vapour phase against *E. faecium* (Laird *et al.* 2012) and lemongrass EO against biofilm formation in *L. monocytogenes* (De Oliveira *et al.* 2010). The resistance of biofilm-associated organisms is estimated at 50 to 500 times more than planktonic cells (Jabra-Rizk *et al.* 2006). The effect of different EOs on biofilms has been investigated with the effects identified ranging from inhibition, reduction, attenuation and eradication of biofilms in both Gram positive bacteria, Gram negative bacteria and fungi (Kavanaugh and Ribbeck, 2012; Jia *et al.* 2011; Nuryastuti *et al.* 2009; Argawal *et al.* 2008; Nostro *et al.* 2007). In other *in vitro* studies, there was increased activity in biofilms exposed to EOs when compared to the effect of the essential oils against cells in suspension (Kavanaugh and Ribbeck, 2012; Karpanen *et al* 2008; Al-Shuneigat *et al.* 2005).

The objective of this study was to determine the anti-staphyloccocal activity of a range of EOs against six strains which included those of community acquired origin using *in vitro* screening assays and to further investigate the anti-biofilm effect of the EOs found to be the most effective after initial screening.

6.1 Materials and Methods

6.1.1 Essential Oils and Components

The EOs used in this study were lemongrass (*Cymbopogon flexuosus*), grapefruit (*Citrus paradisi*), lime (*Citrus aurantifolia*), bergamot (*Citrus bergamia*) and lemon (*Citrus limon*) obtained from Belmay Plc., Northampton, UK. Two known EO components limonene (Sigma-Aldrich, Dorset, UK) and citral (95%, natural; SAFC Supply Solutions, St Louis, USA) were also investigated.

6.1.2 Microorganisms

The Six *S. aureus* strains listed in section 3.1.1 were investigated for susceptibility to the essential oils. The media used and culture maintenance procedures are as described in Section 3.1.2.

6.1.3 Disc Diffusion

The screening method was adapted from Prabuseenivasan *et al.* (2006) and the British Society for Antimicrobial Chemotherapy (BSAC) guidelines Version 10 (Andrews and Howe 2011). Briefly, 100 µl of each of the EOs was deposited onto sterile 2cm diameter filter paper discs placed on the surface of BHI plates previously spread with 10^7 cfu/ml. The plates were left to dry for 15 minutes in a sterile environment, inverted and incubated at 37°C for 24 hours. The diameters of zones of inhibition (ZOI) were measured using Vernier callipers. The controls were bacterial cultures without EO exposure.

6.1.4 Minimum Inhibitory and Minimum Bactericidal Concentrations

The method used was adapted from Hammer *et al.* (1998). An aliquot (20 µl) of a 10^8 cfu/ml overnight culture was added to wells of a sterile 96-well microtitre plate. Each EO was diluted in BHI broth containing 0.5% (v/v) Tween 20 and added to wells to give final EO concentrations of 0.03%, 0.06%, 0.12%, 0.5%, 1%, 2% and 4% (v/v). The positive

control wells contained BHI broth and cells without EOs while the negative control wells contained BHI only. Optical density was measured at 595nm using a microplate reader (Bio-Rad 680XR, Hertfordshire, UK) and again after incubation for 24 hours at 37°C. The MIC was determined as the lowest EO concentration at which the OD at 24 hours of the inoculum remained the same or reduced compared with the initial reading.

For MBC determination, 10 µl was taken from each well after incubation and spot inoculated (Hammer *et al.* 1998) onto BHI agar and incubated for 24 hours at 37°C. The concentration at which no growth was observed on subculture was determined as the MBC.

6.1.5 Minimum Biofilm Inhibitory Concentration (MBIC)

Inhibition of biofilm formation was assessed using a method adapted from Nostro *et al.* (2007). An aliquot (100 µl) from an overnight culture diluted in BHI broth supplemented with 1% (w/v) glucose to 10^8 cfu/ml was dispensed into each test well of a 96 well plate. 100µl of the EOs at concentrations 0.06% to 4% (v/v) for lemongrass EO and 1% to 4% (v/v) for grapefruit EO were added into the wells. The negative control was BHI broth only while the positive control contained cell cultures alone with no added EO. Following 24 hours incubation at 37 °C, the contents of the wells were decanted and each well gently rinsed twice with 300 µl of sterile phosphate buffered saline (PBS) (pH: 7.3 ± 0.3). The plates were air dried for 30 minutes, stained with 0.1% (w/v) crystal violet for 30 minutes at room temperature (Wijman *et al.* 2007), washed three times with PBS (200 µl per well) and dried. The crystal violet was then solubilized using 10% (v/v) glacial acetic acid and the OD measured at 595nm using a Microplate reader (Bio-Rad 680XR, Hertfordshire, UK). The MBIC was determined as the EO concentration at which the OD ≤ negative control (Pettit *et al.* 2005; Sandoe *et al.* 2006). Each experiment was performed in quadruplicate and performed on four separate occasions.

6.1.6 Minimum Biofilm Eradication Concentration (MBEC)

The method used was similar to that described by Kwiecinski *et al.* (2009). After biofilm formation for 48 hours, the medium was discarded and the wells gently rinsed twice with PBS. 200 µl of the EOs (lemongrass or grapefruit) were serially diluted and added into the wells ranging from 0.06% to 4% (v/v) for lemongrass EO and 1% to 4% (v/v) for grapefruit EO. The plates were then incubated for 24 hours at 37°C after which the wells were washed with PBS and stained using the CV staining method as described previously. The positive control was biofilm without EO. The concentration at which already established biofilms were removed from the bottom of the treated wells was determined as the minimum biofilm eradication concentration or MBEC (Muli and Struthers 1998; Ceri *et al.* 1999). Each experiment was performed in quadruplicate and performed on four separate occasions.

6.1.7 Biofilm metabolism assay - XTT reduction

This method is based on reduction of tetrazolium salt XTT [2, 3-bis (2-methyloxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilalide] and was performed to determine the metabolic activity of the biofilm formed using methods described by Cerca *et al.* (2005) and Laird *et al.* (2012). Stock solutions of XTT in PBS (cfu/ml) and menadione (1 mM) were prepared. At the start of each experiment, a fresh solution of XTT/menadione was prepared at a ratio of (12.5/1 v/v). Biofilms were formed for 48 hours in the wells of 96 well plates and 200 µl of the XTT/menadione mix was added into each test and control well, incubated in the dark at 37 °C for 24 hours and the OD measured at 450nm.

6.1.8 Biofilm viability assay (cfu/ml)

Biofilm viability was measured using a method adapted from Pettit *et al.* (2005). Following 24 hours exposure of biofilms to lemongrass or grapefruit EO, a sterile scraper was used to dislodge each biofilm into the micro-titre wells, 100 µl of the well contents removed and spread onto BHI agar. Plates were incubated for 24 hours at 37 °C before enumeration.

6.1.9 Gas Chromatography Mass Spectrometry (GC-MS)

GC/MS analysis of the EOs was performed at Belmay Plc. This analysis was using the Perkin-Elmer (Turbo Mass™) instrument with column 1 stationary phase Rtx 1 column (60m x 0.25 mm I.D.; film thickness: 0.25 µm; Restek). The oven temperature program was: initial temperature of 50 °C; increasing by 3 °C/min to 265 °C; and held for 13 minutes. Helium was used as the carrier gas with a 1 µl injector volume, an injector temperature of 285 °C and a split ratio 30:1. The MS was performed with an EI+ source and operated in scan mode, from 35 to 350 m/z at a detector temperature of 300 °C. The compounds were identified by comparing retention times and mass spectra with those of standards or their retention indices (RI) with published data and their mass spectra with the National Institute of Standards and Technology (NIST) library.

6.1.10 Scanning Electron Microscopy (SEM)

PVL CA MSSA was selected for SEM observations due to its higher biofilm OD values compared with the other strains (Figure 4.2). 2 cm diameter sterile stainless steel discs (Goodfellows Cambridge Ltd, UK) were immersed in 6 well plates (NunclonTM surface, Denmark) containing 5 ml of BHI broth supplemented with 1% (w/v) glucose for 48 hours. 100 µl of a 10^8 cfu/ml overnight culture was then added and the plates incubated for 48 hours in a shaking incubator. After incubation the discs were removed and gently rinsed with sterile PBS to remove loosely attached cells and re-suspended in 0.125%, 0.5% and 1% (v/v) lemongrass or 4% (v/v) grapefruit EO. After exposure to the EOs, the discs were washed three times with PBS and fixed with 2.5% (v/v) glutaraldehyde in PBS solution for 2 hours at 4 °C, washed twice with PBS and dehydrated for 10 minutes using a graded ethanol series; 30, 50, 70, 90, 100% (v/v). The samples were then dried prior to coating with gold and observed using a Hitachi S-3000 Scanning Electron Microscope (Hitachi High-Technologies Europe, Maidenhead, UK).

6.1.11 Statistical Analysis

Statistical analysis was conducted as described in Section 3.3.

6.2 Results

Screening - No ZOIs were observed with either lemon EO or limonene while lemongrass EO and citral, the major component in lemongrass EO both demonstrated total zone inhibition with ZOI of >8.60 cm (Table 6.1). Grapefruit, lime and bergamot EOs produced ZOIs ranging from 2.85 cm to 4.63 cm (Table 6.1). Consequentially lemongrass and grapefruit EOs were selected for determination of MICs and MBCs and for anti-biofilm activity as these were the most effective at >8.60 cm and 3.48 cm respectively (Table 6.2). Although ZOI produced by the latter was only marginally more effective than lime EO (ZOI =3.47 cm) it was selected for further studies since it has been shown to have anti-bacterial activity and potential in other antimicrobial applications (Williams *et al.* 2007; Uysal *et al.* 2011).

Table 6.1. Zones of inhibition (cm) ±SE measured after exposure to EOs and components against *S. aureus* strains. (n=3)

	Lemongrass	Grapefruit	Lime	Bergamot	Citral*	Geranium	Lemon	Limonene*
Hospital MSSA	8.6 (±0)	3.96 (±0.17)	4.17 (±0.18)	3.36 (±0.07)	8.6 (±0)	3.06 (±0.12)	0 (±0)	0 (±0)
PVL CA-MSSA	8.6 (±0)	3.23 (±0.06)	2.90 (±0.17)	2.89 (±0.07)	8.6 (±0)	2.81 (±0.05)	0 (±0)	0 (±0)
MSSA NCTC 13297	8.6 (±0)	3.35 (±0.07)	3.27 (±0.05)	3.12 (±0.11)	8.6 (±0)	3.03 (±0.13)	0 (±0)	0 (±0)
CA-MRSA (MW2)	8.6 (±0)	3.43 (±0.04)	3.57 (±0.12)	2.85 (±0.09)	8.6 (±0)	3.05 (±0.12)	0 (±0)	0 (±0)
PVL CA-MRSA	8.6 (±0)	3.42 (±0.11)	3.58 (±0.08)	2.85 (±0.09)	8.6 (±0)	2.98 (±0.14)	0 (±0)	0 (±0)
CA-MRSA (SR)	8.6 (±0)	2.85 (±0.11)	3.13 (±0.09)	3.24 (±0.06)	8.6 (±0)	4.17 (±0.10)	0 (±0)	0 (±0)

The MIC for lemongrass EO at 0.06% (v/v) was lower than that of geranium 0.13% (v/v) and grapefruit EO [range 0.5-2% (v/v)] for all strains tested. The MBC was also lower than that of lemongrass EO and the other EOs. The MBC for lemongrass EO was at the same concentration for all the strains tested (0.125% v/v) while geranium EO had MICs at 0.13% for all the strains and MBCs ranging between 0.25% (v/v) for the MSSA NCTC 13297 and 4% (v/v) for the two PVL positive strains. Of the EOs and components tested the MIC was highest with grapefruit EO ranging between 0.5% (v/v) for MSSA NCTC 13297 and 2% (v/v) for the CA MRSA (SR) while the MBC was 2% (v/v) for the hospital MSSA isolate and MSSA NCTC 13297 and 4% (v/v) for the three community strains; PVL CA MSSA, CA-MRSA (MW2) and PVL CA MRSA (Table 6.2). The MBC for the CA MRSA (SR) strain was >4% (v/v).

Lemongrass EO prevented biofilm formation at 0.06% (v/v) for the hospital MSSA strain and 0.125 % (v/v) for the other strains tested (Table 6.3) which, for four of the five strains was the same concentration as the MBC. However lemongrass EO did not remove already formed biofilms (MBEC) at any of the concentrations tested i.e. 0.06% to 4% (v/v). Grapefruit EO did not either prevent biofilm formation or remove already formed biofilms at 1% to 4% (v/v).

Table 6.2. Minimum inhibitory concentration and minimum bactericidal concentration, (% v/v) for lemongrass EO, citral, grapefruit EO and geranium EO against *S. aureus* strains (N=4).

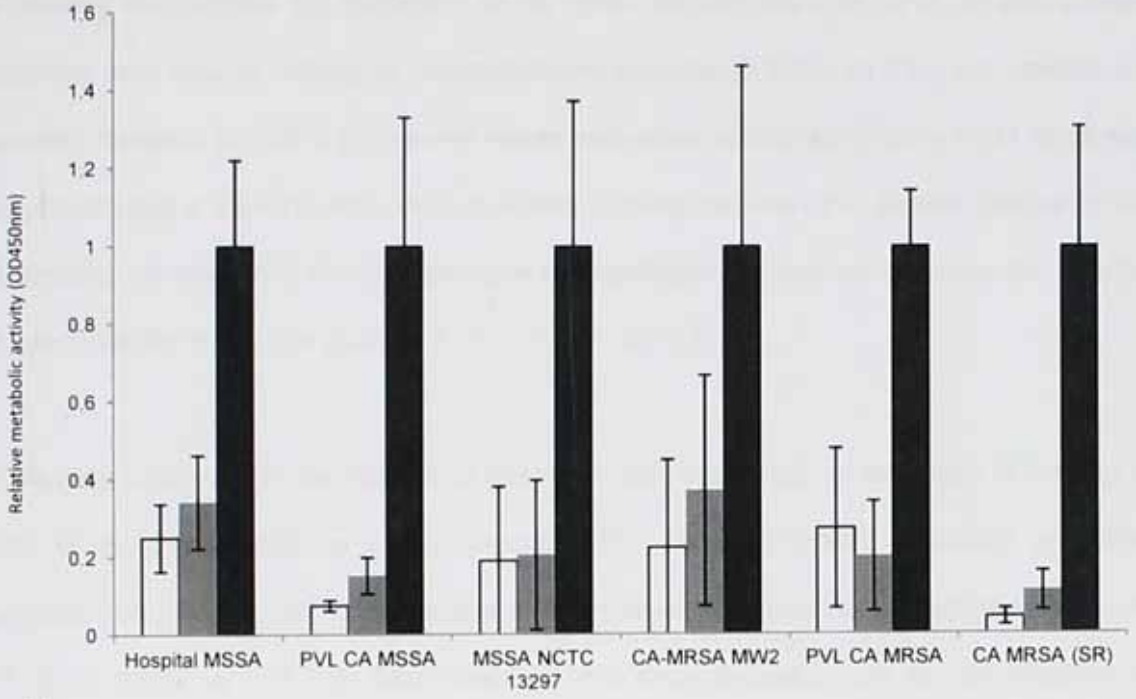
Bacterial strains	Lemongrass		Citral		Grapefruit		Geranium	
	MIC	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***
MSSA 110	0.06%	0.13%	0.02%	0.06%	1%	2%	0.13%	2%
PVL CA-MSSA	0.06%	0.13%	0.03%	0.06%	1%	4%	0.13%	4%
MSSA NCTC 13297	0.06%	0.13%	0.03%	0.06%	0.5%	2%	0.13%	0.25%
MRSA MW2	0.06%	0.13%	0.03%	0.06%	1%	4%	0.13%	1%
PVL CA-MRSA	0.06%	0.13%	0.03%	0.06%	1%	4%	0.13%	4%
CA MRSA (SR)	0.06%	0.13%	0.03%	0.06%	2%	> 4%	0.13%	2%

Table 6.3. Minimum biofilm inhibitory concentration and minimum biofilm eradication concentration (% v/v) for lemongrass EO, citral, grapefruit EO and geranium EO against *S. aureus* strains (N=4)

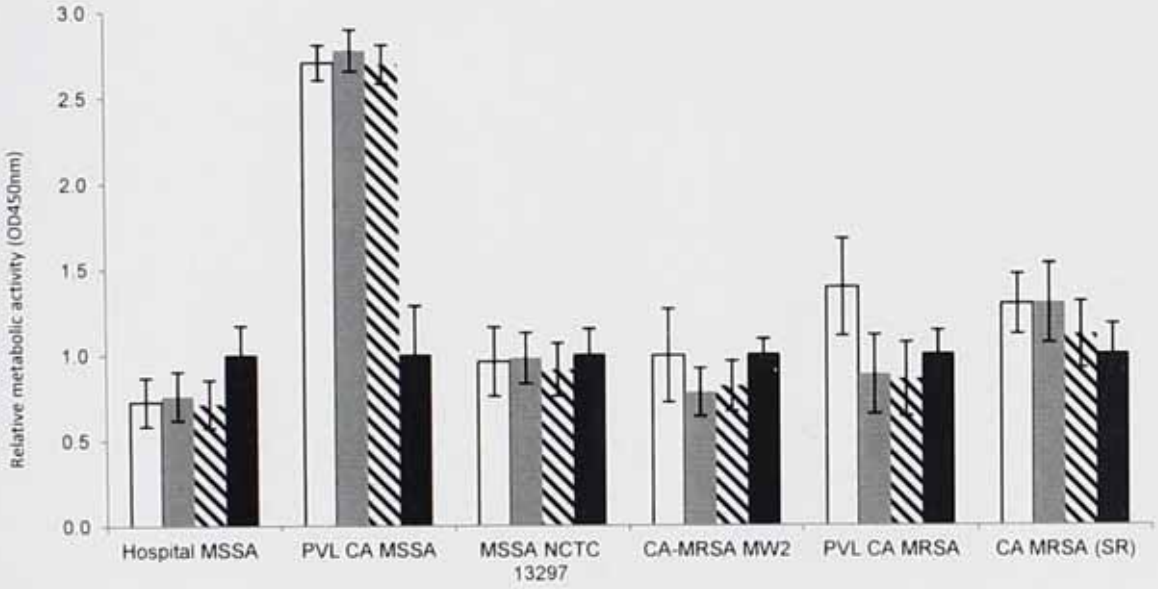
Bacterial strains	Lemongrass EO		Grapefruit EO	
	MBIC	***MBEC***	***MBIC***	***MBEC***
Hospital MSSA	0.06%	>4%	>4%	>4%
PVL CA-MSSA	0.13%	>4%	>4%	>4%
MRSA NCTC 13297	0.13%	>4%	>4%	>4%
CA MRSA (MW2)	0.13%	>4%	>4%	>4%
PVL CA-MRSA	0.13%	>4%	>4%	>4%
CA -MRSA (SR)	0.13%	>4%	>4%	>4%

Inhibition of metabolic activity occurred in the presence of lemongrass EO after 24 hours for all six *S. aureus* strains at 0.125% and 0.06% (v/v) with no significant difference in the reduction brought about by these two concentrations (Figure 6.1a). At 0.25% (v/v), no metabolic activity was observed (results not shown). Grapefruit EO did not reduce the metabolic activity as measured by the XTT assay after 24 hours incubation. When the effect of grapefruit EO was compared for four strains (not including PVL CA MSSA) there was no significant difference in metabolic activity between the strains as determined by the ANOVA for 1% $F(4) = 1.753$, $p = 0.152$, 2% $F(4) = 2.248$, $p = 0.076$ EO however at 4% there was a significant difference $F(4) = 2.793$, $p = 0.035$ with post hoc analysis showing metabolic activity in PVL CA MRSA biofilm as significantly different from Hosp MSSA $p = 0.001$ following treatment with 4% grapefruit EO.

There was a significant difference between the metabolic activity of PVL CA MSSA and the other strains at all the concentrations of grapefruit EO tested at 4%, $F(5) = 2.574$, $p = 0.035$; at 2%, $F(5) = 3.010$, $p = 0.017$; at 1%, $F(5) = 3.013$, $p = 0.016$ with PVL CA MSSA having a metabolic activity approximately 2.7 times that of the control compared to the other four strains (Figure 6.1b).



(a)

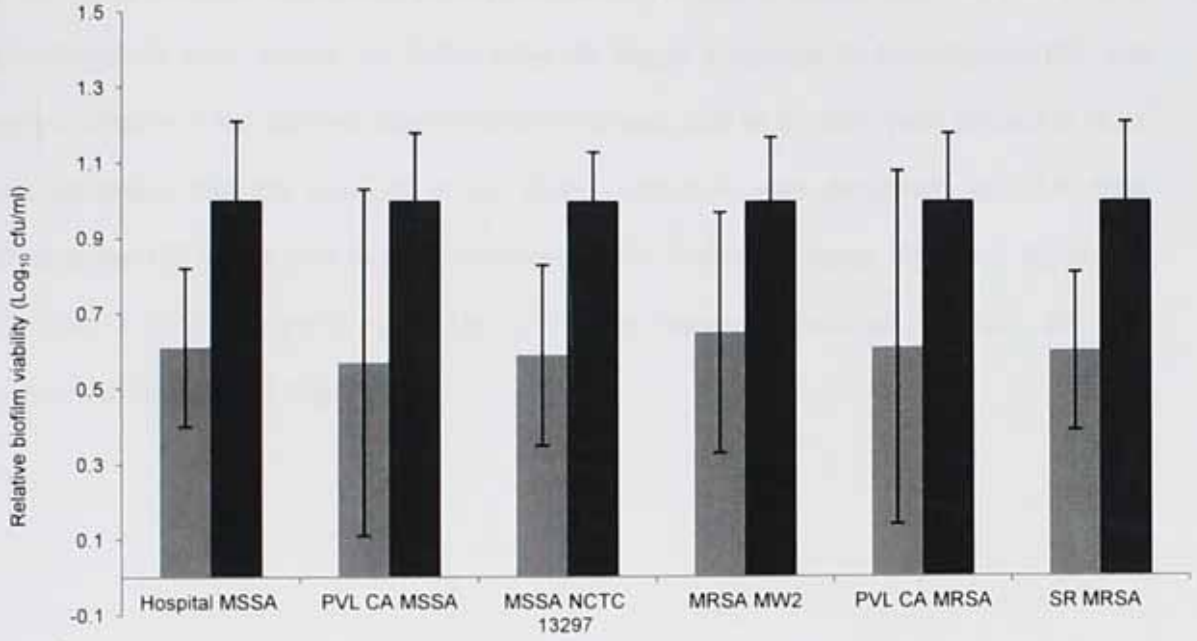


(b)

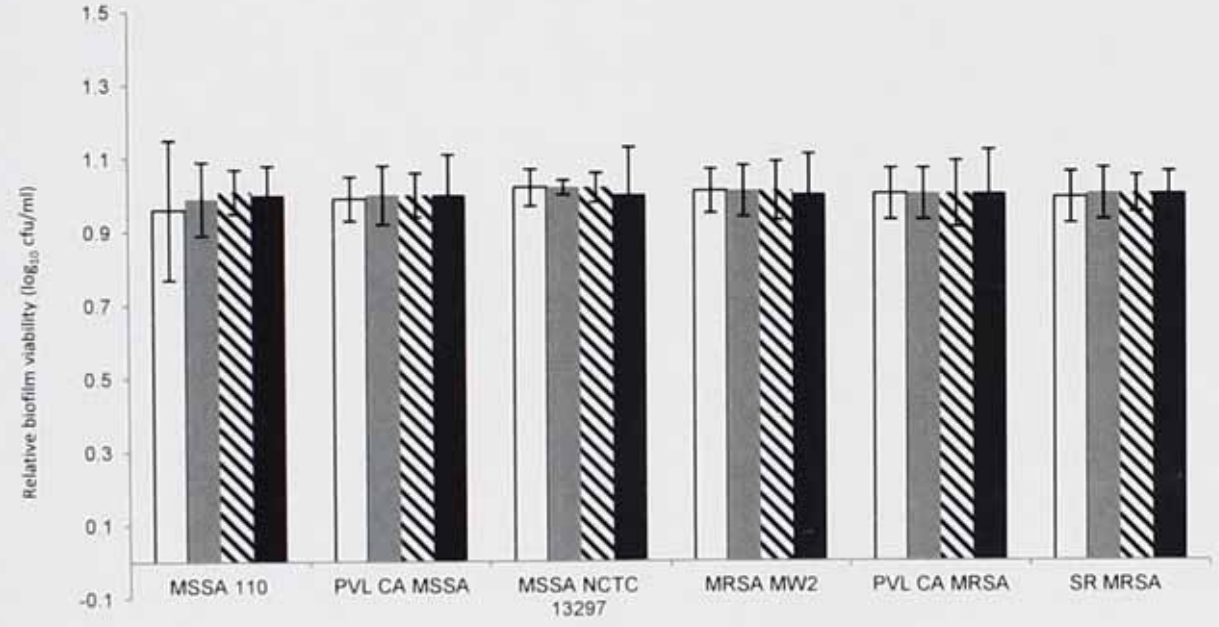
Figure 6.1. Changes in metabolic activity following 24 hours exposure of biofilms of *S. aureus* strains to a) lemongrass EO (☐ 0.125%, ▒ 0.06% and ■ 0%) and b) grapefruit EO (☐ 4%, ▒ 2%, ▨ 1% and ■ 0%) as determined by the XTT assay (control = biofilms not exposed to EO; N=4 for each treatment and for each strain).

Following lemongrass EO treatment for 24 hours, biofilms from all six S. aureus isolates showed total loss of viability at concentrations between 0.125% to 4% (v/v) (results not shown) however at 0.06% (v/v) some viable cells were recovered (Figure 6.2a) there was approximately a 35-43% reduction in biofilm viability between the strains relative to the untreated controls. A 0.06%, there were no significant differences between the relative biofilm viability for the six isolates $F(5) = 0.637$, $p = 0.673$.

Following exposure of the biofilms to grapefruit EO, there was no reduction in viability at any of the concentrations tested (Figure 6.2b). The only visible reduction in biofilm viability was the Hospital MSSA isolate at 4% however this was not statistically significant $H(5) = 6.977$, $p = 0.222$ determined by the Kruskall-Wallis test as assumptions of normality were not met.



(a)



(b)

Figure 6.2. Effects of a) lemongrass EO (0.06% and 0%) and b) grapefruit EO (4%, 2%, 1% and 0%) on the relative biofilm viability of *S. aureus* strains following 24 hour exposure as determined by the cfu ml^{-1} assay (control = biofilms not exposed to EO; N=4 for each treatment and for each strain).

Following biofilm quantification by the CV staining method the PVL CA MSSA strain consistently showed increased biofilm formation compared to the other strains tested, and therefore was chosen for SEM. After 24 hours exposure to lemongrass EO, the control (Figure 6.3a) showed intact biofilm structure, and at 0.125% (v/v) (Figure 6.3b) it was observed that the integrity of the biofilm structure was disrupted. At 0.5% (v/v) lemongrass EO, there was evident damage on the biofilm structure (Figure 6.3c) and at 1% (v/v) of lemongrass EO treatment, no biofilms were observed on the discs although biofilm debris remained (Figure 6.3d).

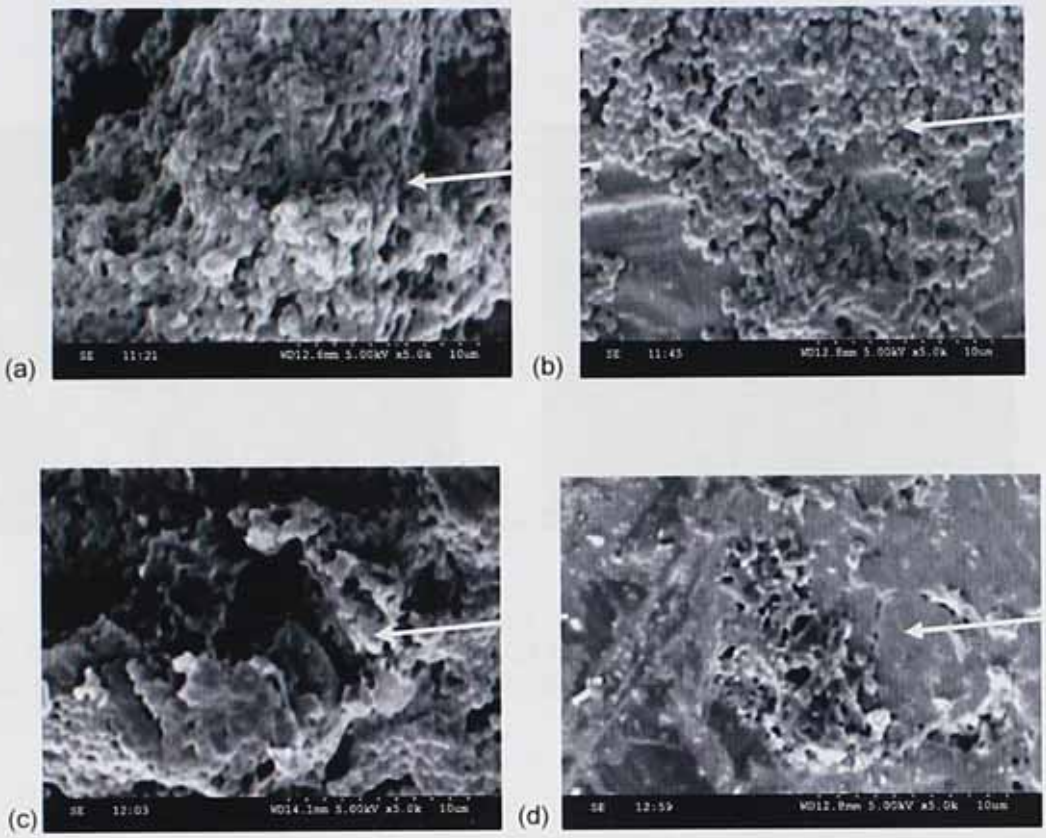
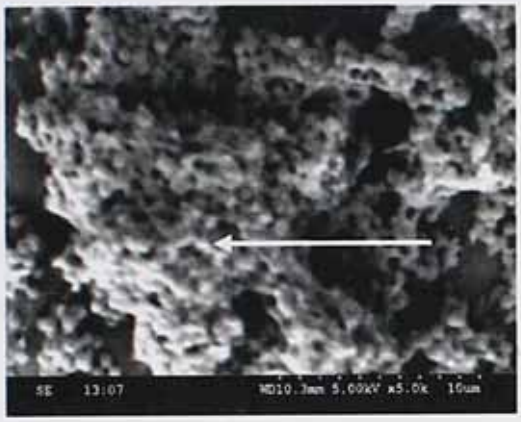


Figure 6.3. Scanning electron micrographs of a PVL CA MSSA biofilm following treatment with lemongrass EO at a) 0% (control) b) 0.125% c) 0.5% and d) 1% (v/v) after 24 hour exposure (Magnification x5000, Scale 10µm). Arrows indicate biofilm formation (a, b), biofilm disruption (c), and biofilm debris (d).

When PVL CA MSSA was treated with 4% (v/v) grapefruit EO, no effect on biofilm formation and integrity was observed (Figure 6.4b) in comparison to the control (Figure 6.4a).

(a) 

(b) 

Figure 6.4. Scanning electron micrographs of a PVL CA MSSA biofilm following treatment with grapefruit EO at a) 0% (control) b) 4% (v/v) after 24 hour exposure (Magnification x5000, Scale 10µm). Arrows indicate biofilm formation although b suggests lack of penetration of the grapefruit EO into the biofilm.

6.3 Discussion

Lemongrass EO at concentrations of between 0.03% and 0.06% (v/v) was effective at inhibiting the growth of all six *S. aureus* strains and at 0.125% (v/v) the effect of lemongrass EO was bactericidal. The results presented here are consistent with those of a previous study (Barbosa *et al.* 2009) in which it was demonstrated that lemongrass EO inhibited the growth of Gram positive bacteria, including *S. aureus* at a concentration of 0.05% (v/v). In this present study the MIC for grapefruit EO was higher than that for lemongrass EO for all the strains i.e. between 0.5% and 2% (v/v), while bactericidal activity was observed between 2% and 4% (v/v) EO. To date, there are very few studies that have investigated the antimicrobial activity of grapefruit EO however it has been shown to possess both antifungal and anti-bacterial activity (Viuda-Martos *et al.* 2008; Uysal *et al.* 2011).

When the effects of the components were compared to the overall effect of the EO, contrasting results were observed. Firstly, citral, the major component in lemongrass EO, showed total zone inhibition at screening with MICs ranging from 0.02 - 0.03% (v/v), and approximately one fold lower than that of lemongrass EO. Therefore citral may be considered as being responsible for the majority of the antibacterial activity observed in lemongrass EO. This activity by citral has been previously reported (Hayes and Markovic 2002; Da Silva *et al.* 2008; Aiemsaard *et al.* 2011). Limonene is the major component in the grapefruit EO used, at approximately 94% (v/v), (Appendix IV: GC-MS results), but it did not show any antimicrobial effect as demonstrated by the screening results (Table 6.1) which has also been observed in previous studies by Fisher and Phillips (2006) and Inouye *et al.* (2001). In comparison to this inactivity by limonene, grapefruit EO produces inhibition zones for the *S. aureus* strains of between 2.85 and 3.96 cm which suggests that other components of the grapefruit EO are involved in the antibacterial activity observed. Previous reported that myrcene show no antibacterial activity *per se* but its presence enhanced activity of other EO components suggesting that the presence of

other components in small amounts enhances the EO antimicrobial activity (Onawunmi *et al.* 1984). Although the individual components of EOs are important, they act in a synergistic manner so that the EO exhibits a greater antibacterial activity than the sum of that brought about by its components (Gill *et al.* 2002).

The results of this study demonstrate that lemongrass EO possesses anti-biofilm activity at low concentrations of between 0.06% and 0.125% (v/v) which has been reported previously (Aiemsaard *et al.* 2011). Since biofilm formation is a survival mechanism but also contributes to virulence and persistence (Vuong *et al.* 2004; Soto *et al.* 2006) it has been suggested that preventing biofilm attachment is a way of dealing with the problem of biofilms in the food industry (Sinde and Carballo 2000). Therefore considering the results presented here there may be a possible potential for lemongrass EO use in food processing environments. The effect on the organoleptic properties of the foodstuff at the anti-biofilm concentrations would need to be determined, although lemongrass *per se* is GRAS (Generally Recognised as Safe) and is used a food ingredient world-wide (USFDA, 2012).

This is the first time the anti-biofilm activity of grapefruit EO has been reported. The results described here demonstrate that, although grapefruit EO is bactericidal at 2% to 4% (v/v) against the different *S. aureus* strains tested, it has limited or no activity against biofilm formation (Table 6.3). This suggests that biofilm formation could offer protection against EOs or at least against grapefruit EO. In a previous study, it was reported that some biofilms could be more susceptible to EOs than their planktonic counterparts (Kavanaugh and Ribbeck, 2012). The authors suggested that this action could be as a result of differential gene expression within the biofilm or an increase in local concentration of the essential oils in the extracellular matrix of the biofilm. Previous studies have shown however that when grapefruit EO was combined with other EOs against MRSA (not in biofilms), there was synergistic activity and improved antimicrobial

potential (Edwards-Jones *et al.* 2004) hence, combining grapefruit EO with other EOs or antimicrobial compounds might also enhance its activity against biofilms. The synergistic action of EOs against surface adhered cells has previously been demonstrated by the results of a study by De Oliveira *et al.* (2010) who reported a 100% log reduction of a mature *L. monocytogenes* biofilm after 60 minute contact time with a combination of *Cymbopogon citratus (D.C) Staph* and *Cymbopogon nardus (L)* EOs.

Both lemongrass and grapefruit EOs were unable to eradicate already established biofilms (Table 6.3). The inability of antimicrobial compounds to remove biofilm deposits has been observed previously (Lin *et al.* 2011). As biofilms develop, they undergo irreversible attachment leading up to maturation (Mittelman, 1998) and at this point, removal of biofilms is said to be difficult and would require mechanical force or chemical disruption (De Oliveira *et al.* 2010). In addition to this, Pitts *et al.* (2003) after investigating reductions in *Ps. aeruginosa* and *S. epidermidis* biofilms using chemical agents such as hydrogen peroxide and 1M NaCl, suggest that such reductions are microorganism and antimicrobial agent specific highlighting the difficulty with regard to biofilm removal. For example, 1M NaCl significantly reduces *P. aeruginosa* biofilms but not those of *S. epidermidis* while hydrogen peroxide has the reverse effect (Pitts *et al.* 2003). To control biofilms, and to avoid the associated problems with biofilm formation such as antibiotic resistance, persistence and treatment difficulties, preventing the formation of biofilms is an important measure (Kelly *et al.* 2012).

The difference in anti-biofilm activity of various interventions i.e. the ability of treatments to prevent formation and eradicate pre-formed biofilms or reduce metabolic activity has been previously reported. For example, Babu *et al.* (2012), using a high molecular weight component extracted from cranberry reported they were unable to detach *Streptococcus gordonii* from the preformed biofilm mass however they inhibited biofilm metabolic activity. On treatment with a citrus EO in vapour form MSSA and MRSA biofilms were

reduced both during and after formation, while *Enterococcus* sp. biofilms were significantly reduced ($p \leq 0.05$) only after formation (Kelly *et al.* 2012; Laird *et al.* 2012) Complete suppression of metabolic activity indicates effective killing (Flemming *et al.* 2009) although even cells that have lost metabolic activity are able to contribute to the total biofilm biomass (Pitts *et al.* 2003). In this study the lemongrass EO treated biofilms showed loss of metabolic activity as well as total loss of viability at low concentrations of EO of between 0.125% and 0.25% (v/v) dependent on strain.

Biofilms tolerate higher amounts of antibiotic between 10 to 1000 fold when compared to planktonic cells (Yarwood *et al.* 2004; Resch *et al.* 2006; Kelly *et al.* 2012). However in this study, lemongrass at twice the MIC and same concentration as the MBC prevented biofilm formation highlighting antimicrobial activity as well as its potential as an anti-biofilm agent. Exposure of the biofilms to grapefruit EO showed no reduction in metabolic activity in five of the six *S. aureus* strains and an increased metabolic activity in the PVL positive MSSA strain (Figure 6.1b). The reason for such increase in metabolic activity is unclear. However, a study by Kwiecinski *et al.* (2009) using the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay reported an increase in metabolism in *S. aureus* biofilms when treated with tea tree oil (TTO) at concentrations lower than the MBEC which, it was suggested, could be a result of a stress response. Whether this is the case for the increase in metabolic activity in PVL positive MSSA strain when treated with grapefruit EO is unclear and will be investigated further.

Lemongrass EO at 0.125% (v/v), the MBC and MBIC concentrations, disrupted the biofilms of PVL CA MSSA (Figure 6.3b) and at 0.5% (v/v) of lemongrass EO, damage to the biofilm was increased further (Figure 6.3c) while at 1% (v/v) of the EO treatment, total destruction of the biofilm was observed (Figure 6.3d). Kwiecinski *et al.* (2009) has suggested that TTO treatment of *S. aureus* biofilms causes damage to the extracellular

matrix and in this present study, damage to the biofilm structure was observed on treatment with 1% (v/v) lemongrass EO suggesting a similar mode of action. With the grapefruit EO, SEM images did not show any disruption of the biofilm structure (Figure 6.4b) which further confirms the lack of anti-biofilm activity and indicates the importance of biofilm formation as a protective mechanism against EOs.

This study is one of few that have investigated the anti-biofilm properties of EOs especially the effect of lemongrass EOs against *S. aureus* biofilms. Where previously (Bearden *et al.* 2008), investigated commercial formulations containing EOs against community acquired MRSA, this is first study that has demonstrated the anti-biofilm activity of lemongrass EO against biofilms of community acquired MSSA and MRSA including PVL positive strains. The results of this study also demonstrate that EOs exhibit antimicrobial potential and could offer a promising development in the search for new and novel antimicrobial agents.

CHAPTER SEVEN

Investigating the effect of grapefruit EO on metabolic activity in two *S. aureus* strains by RT-PCR.

7.0 Introduction

In chapter 6, it was demonstrated that exposure of the PVL CA MSSA biofilms to grapefruit EO yielded different responses as measured by the XTT reduction assay compared with five other *S. aureus* strains. PVL CA MSSA showed more than twice the metabolic activity in the biofilms treated with 1-4% (v/v) grapefruit EO than in the untreated biofilms while there was little or no difference in metabolic activity in the other strains tested (Figure 6.1b).

According to Secor *et al.* (2011), there are links between metabolic processes in *S. aureus* and its pathogenicity and these metabolic states are different in planktonic cells compared with biofilms. Following extensive studies on biofilm characteristics in *S. aureus* and the advancement of molecular identification analytical tools, numerous factors have been identified which are involved in the process of biofilm formation. Some of the factors identified include genetic mechanisms such as the genes of the *ica* operon known to be involved in intercellular adhesion, regulatory genes such as accessory gene regulator *agr* and staphylococcal accessory regulator *sarA* and the QS system. In *S. aureus*, previous studies have also suggested the role of a gene *luxS* in bacterial metabolism and as a QS regulator (Lebeer *et al.* 2007).

In this study, five genes were investigated for the change in metabolic activity observed in the PVL CA MSSA biofilm (Figure 6.1b). The genes investigated were *icaA*, *icaD*, *cap8C*, *luxS* and *sodA*. These genes have been implicated in previous studies in biofilm formation and

involvement in metabolic processes in (Doherty *et al.* 2007; Fuchs *et al.* 2007; Resch *et al.* 2005; Beenken *et al.* 2004).

The objective of this study was to investigate the differences in expression for five target genes in CA MRSA MW2 and PVL CA MSSA biofilms following exposure to grapefruit EO.

7.1 Materials and Methods

7.1.1 Bacterial strains and culture conditions

CA MRSA MW2 and PVL CA MSSA were used in this study. Maintenance of cultures was performed as previously described in Section 3.1.2.

7.1.1.1 Exponential phase cultures

Sterile polypropylene tubes containing 10 ml BHI broth were inoculated with single colonies of each microorganism and incubated at 37°C in a shaking incubator, SI500 (Fisher Scientific, Loughborough, UK) for six hours.

7.1.1.2 Biofilm formation

Overnight cultures at approximately 1 x 10^8 cfu/ml were dispensed into wells of a 24 well plate and incubated under shaking conditions for 48 hours in BHI broth supplemented with 1% (w/v) glucose. Following incubation, planktonic cells were gently discarded and the grapefruit EO was added at 2% (v/v) and incubated for another 24 hours under shaking conditions at 37°C. After incubation, the contents of the wells were discarded, the bottom of the wells scrapped and the biofilms collected.

7.1.2 Isolation and quantification of RNA

7.1.2.1 Nucleic acid stabilization

This was performed using the RNA Protect Bacteria Mini Prep kit (Qiagen, Manchester, UK). Briefly, 0.5 ml bacterial culture (exponential phase or biofilms) was added to 1 ml of RNAprotect Bacteria Reagent, vortexed for 5 seconds and centrifuged at 5,000*g* for 10 minutes. The supernatant was discarded and the pellet collected and stored at -20°C until needed.

7.1.2.2 Initial lysis step

Before RNA extraction, the pellet was removed from storage at -20°C, centrifuged at 10,000*g* for 1 minute to remove any residual liquid and resuspended in 10 µl (v/v) of lysostaphin (1 mg/ml; Sigma-Aldrich, UK) for 5 minutes at 37°C. This was then followed by the addition of 40 µl Tris EDTA (TE) buffer (30mM Tris-Cl, 1 mM EDTA, pH 8.0), 40 µl lysozyme (40 mg/ml; Sigma-Aldrich, UK) and 20 µl (v/v) of Proteinase K (Qiagen, UK) and incubated further for 30 minutes, vortexing every 5 minutes.

(a) RNA extraction using the Qiagen RNeasy kit - planktonic cells and biofilms

This was performed according to manufacturer instructions with minor adjustments (Appendix I: materials and methods). On-column DNA digestion using RNase-free DNase (Qiagen) was also performed and the total RNA was eluted using 100 µl RNase-free water.

(b) RNA extraction using the PowerBiofilm™ RNA Isolation Kit – biofilms only

Following biofilm formation, initial lysis was performed prior to extraction of the RNA using the PowerBiofilm™ RNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, California, USA) according to manufacturer's instructions. This protocol included the utilization of a patented Inhibitor Removal Technology® (IRT) and on-column RNase-Free DNase I digestion (Appendix I: materials and methods).

7.1.3 RNA quantification

RNA quantification and checks for purity were performed using the Nanodrop 2000 (Thermo fisher Scientific, Loughborough, UK). Briefly 2 µl of RNase free water was used as controls and subsequently 2 µl of the eluate was measured for concentration, purity and yield.

7.1.4 Primers

The primers used were those previously used in published reports. In the absence of any such data, they were designed online using the NCBI database (http://www.ncbi.nlm.nih.gov/). The primers were purchased from MWG Biotech, UK and details of the genes and primer sequences are shown in Table 7.1.

Table 7.1. Sequences of primers used for RT-PCR amplification assay

Gene		Sequence (5–3')	References
GyrB	F	AGTAACGGATAACGGACGTGGTA	Bore *et al.* (2007); Beenken *et al.* (2004)
	R	CCAACACCATGTAAACCACCAGAT	
16S	F	CCAGCAGCCGCGGTAAT	Bore *et al.* (2007)
	R	CGCGCTTTACGCCCAATA	
Cap8C	F	CCAGAGCGGAATAAAGCAATAAG	Korem *et al.* (2010)
	R	TGACGCCGTTGTCACTTGAC	
SodA	F	TATTTGGATCAGGTTGGACTTGGT	Korem *et al.* (2010)
	R	TGGTTTGGCGTTGTCACAAT	
LuxS	F	GGGTTGCCAAACTGGTTTCT	*
	R	GCCCAGCCACATTGTACTTC	
IcaD	F	GGGTGGATCCTTAGTGTTACAATTTT	Korem *et al.* (2010)
	R	TGACTTTTTGGTAATTCAAGGTTGTC	
IcaA	F	TATGAACCGCTTGCCATGTG	Korem *et al.* (2010)
	R	TCACGCGTTGCTTCCAAAG	

*Primers designed using the NCBI BLAST software.

7.1.5 Quantitative reverse transcriptase PCR (QRT-PCR)

The RNA was reverse transcribed using the Power cells to CT OneStep RT-PCR kit (Life Technologies, UK) was used to perform the RT-PCR according to manufacturer's instructions. the reaction mixture contained: 10µl of 2x PowerSYBR® Green RT-PCR Mix containing (SYBR® Green I dye, AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure), dNTPs, ROX™ passive reference and Optimized buffer components); 0.16 µl per reaction of RT Enzyme Mix containing ArrayScript™ UP Reverse Transcriptase and RNase inhibitor; 1 µl/reaction of each primer;100 ng/reaction of the template RNA and RNase free water for a final volume of 10 µl.

The conditions selected for the RT-PCR amplification protocol is shown in table 7.2.

Table 7.2. Amplification conditions for RT-PCR reaction

Stage	Step	Temperature	Time
Holding	Reverse transcription	48°C	30 minutes
Holding	Activation of AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure)	95°C	10 minutes
Cycling (40 cycles)	Denaturation	95°C	15 seconds
	Annealing /Extension	60°C	1 minute

7.1.6 Analysis of RT-PCR Data

The RT-PCR experiment was performed using the MJ MiniOpticon real-time PCR detection system (Bio-Rad, Hertfordshire, UK). For both planktonic cells and biofilms, analysis of data was performed using the delta-delta Ct method without efficiency correction (Pfaffl, 2006). For the biofilms, following exposure to the EOs, the RT-PCR was only performed once.

The formula for this method is $R = 2^{-[\Delta CP\ sample - \Delta CP\ control]}$.

7.2 Results

7.2.1 *RNA Extraction*

Using the Qiagen RNA extraction kit, exponentially growing planktonic *S. aureus* cells (CA MRSA MW2 and PVL CA MSSA) yielded RNA concentrations of 286.6 ng/µl and 329.45 respectively. The measurements for RNA purity based on A260/280 and A260/230 respectively gave values at 2.07 for the MW2 strain and 2.08 for PVL CA MSSA indicating high purity levels from the extracted RNA samples. The A260/230 values were 2.21 and 1.97 for CA MRSA MW2 and PVL CA MSSA respectively (Table 7.3). Since the results were acceptable, no other kits were used for extraction of RNA from planktonic cells.

When RNA was extracted from biofilms using the Qiagen kit, lower RNA concentration (A260/280 and A260/230) values than that of planktonic cells were determined (Table 7.3). RNA concentrations from the treated and untreated biofilms ranged between 28.85 and 42.25 ng/µl, the A260/280 values ranged between 1.72 and 1.99 and the A260/230 values were between 0.65 and 0.92. When the PowerBiofilm™ RNA Isolation Kit was used for RNA extraction from biofilms, RNA concentrations ranged between 95 and 248.5 ng/µl for the two strains tested (Table 7.3). The A260/230 was between 1.9 and 2.15 while the A260/230 values ranged between 1.17 and 1.78.

Table 7.3. RNA yields and purity from exponential and biofilm cells of CA MRSA MW2 and PVL CA MSSA.

Bacterial strains	Qiagen kit			Power Bio		
	RNA Concentration ng/µl	$A_{260/280}$	$A_{260/230}$	RNA Concentration ng/µl	$A_{260/280}$	$A_{260/230}$
CA MRSA (MW2) Exponential cells	329.45	2.07	2.21	ND*	ND*	ND*
PVL CA MSSA Exponential cells	286.6	2.08	1.94	ND*	ND	ND*
CA MRSA (MW2) Untreated biofilms	28.85	1.88	0.65	149.7	2.14	1.78
CA MRSA (MW2) Treated biofilms	39.7	1.72	0.67	118.6	2.01	1.2
PVL CA MSSA Untreated biofilms	42.25	1.99	0.77	95.0	2.15	1.72
PVL CA MSSA Treated biofilms	33.15	1.94	0.92	248.5	1.87	1.17

*RNA extraction was not performed with this kit for the biofilm cells as the Qiagen kit had provided RNA of good yield and purity.

7.2.2 *RT-PCR Results*

Two housekeeping genes (*16S* and *gyrB*) were checked for suitability in this experiment against both strains (Figure 7.1). There was a similarity in the C_t values for the *16S* gene between (28.15 and 28.91) for the two strains tested (Figure 7.1a) while the variation between the C_t values for the *gyrB* gene was larger, between 23.06 and 29.06 for both strains (Figure 7.1b). As a result, the *16S* gene was selected as the reference gene for the RT-PCR reaction because there was less variation in the Ct values between both strains.

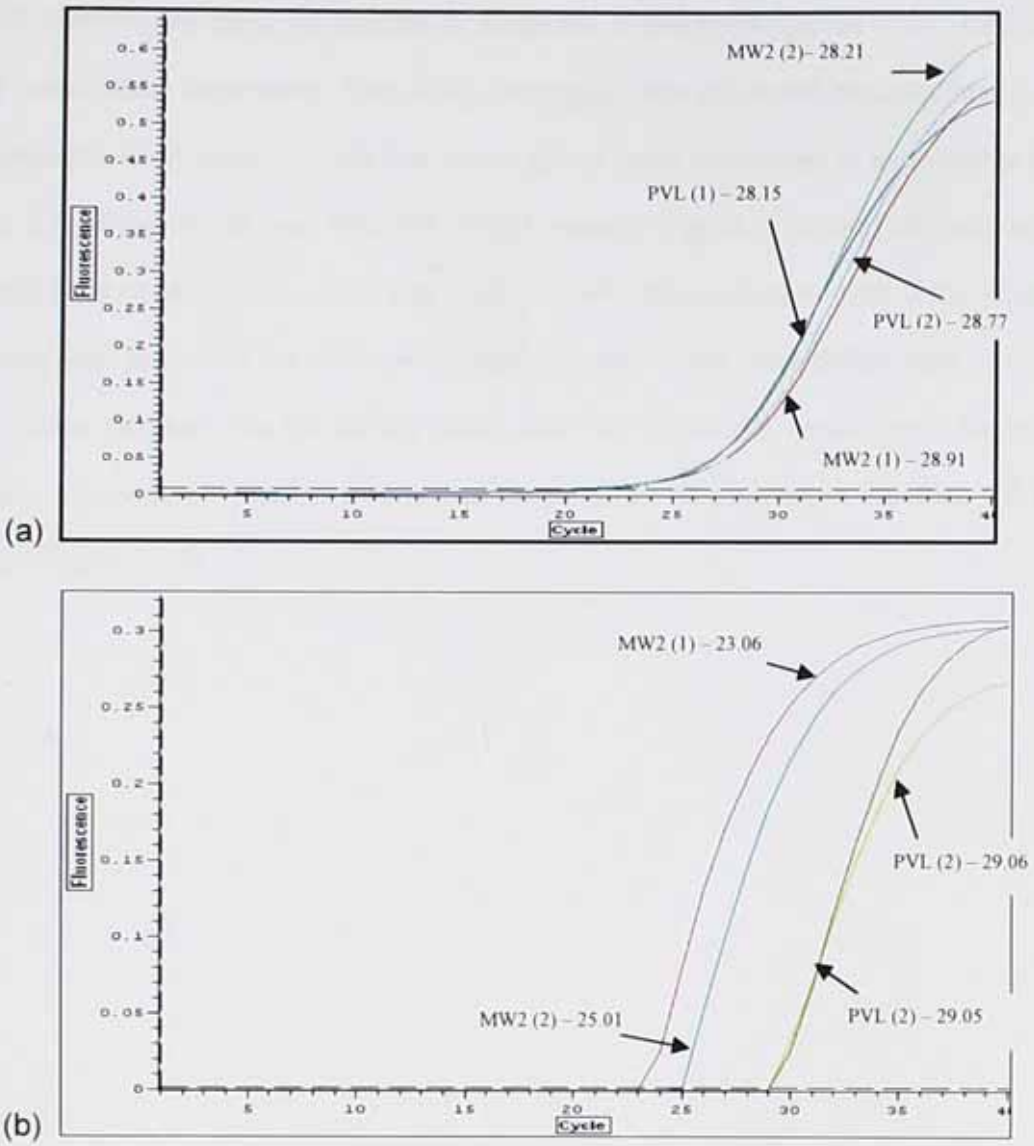
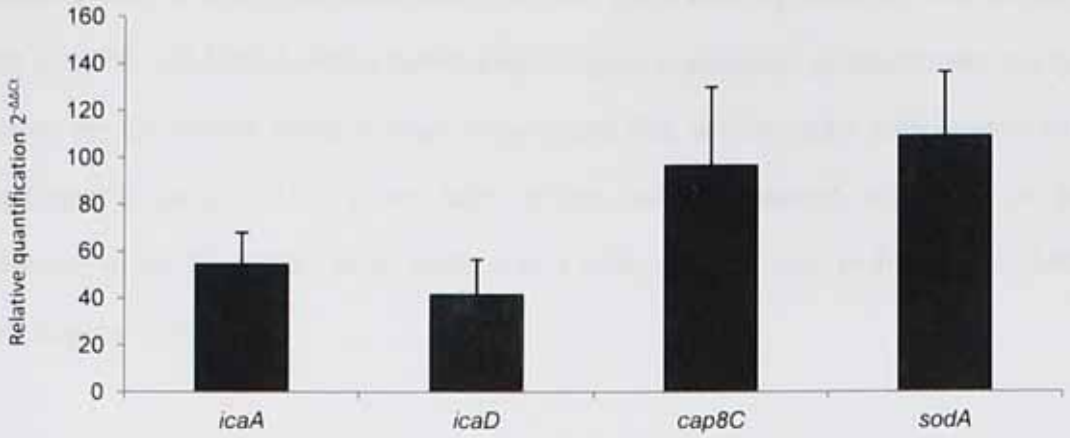
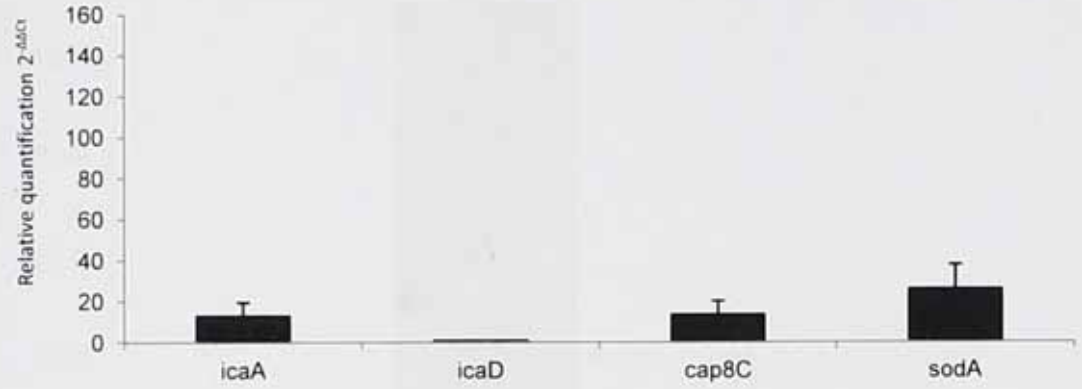


Figure 7.1. Amplification curves for the (a) *16S* and (b) *gyrB* genes in CA MRSA MW2 and PVL CA MSSA strains. The C_t values are shown for each strain.

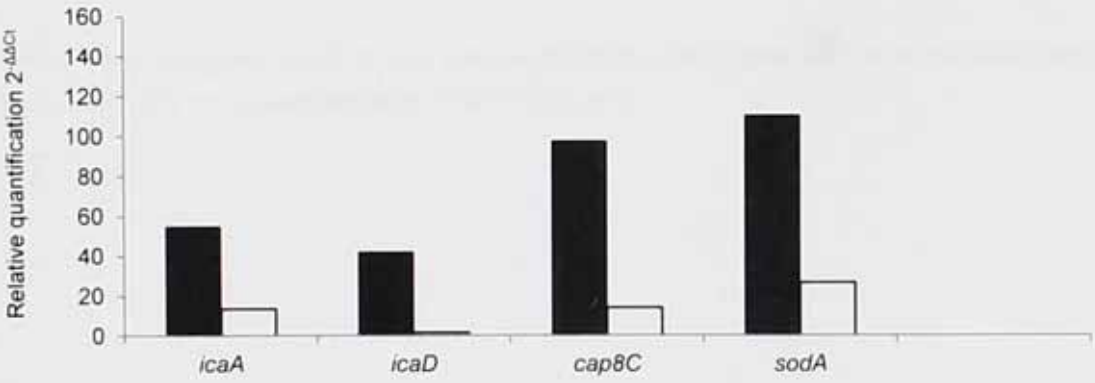
Prior to investigating the effect of grapefruit EO on the expression of the selected genes in the biofilms, the gene expression or presence of the target genes (*icaA*, *icaD*, *cap8C* and *sodA*).were determined from RNA extracted from untreated exponentially growing planktonic cells (Figure 7.2). All four target genes were expressed in exponential cells in both CA MRSA MW2 and PVL CA MSSA strains (Figure 7.2a and 2b) although the lowest level of gene expression was observed with the *icaD* gene with a fold change of 1.5 between icaD and the *16S* housekeeping gene. There were differences in the gene expression between the CA MRSA MW2 and PVL CA MSSA strains and, for all target genes, the gene expression was higher in the MW2 strain than in the PVL CA MSSA strain (Figure 7.2c).



(a)



(b)



(c)

Figure 7.2. Relative quantification of four target genes relative to the 16S housekeeping gene in planktonic (a) CA MRSA MW2 (b) PVL CA MSSA and (c) comparison between the relative quantities of ■ MW2 and □ PVL CA MSSA (n=3).

In exponentially growing planktonic cells, the *luxS* gene was expressed in both CA MRSA MW2 and PVL CA MSSA with a fold change in gene expression approximately fourty six times in the CA MRSA MW2 and ten times in the PVL MSSA strain both relative to the housekeeping gene (16S). When both strains were compared, the level of gene expression in the CA MRSA MW2 strain was 4.7 times higher than in the PVL CA MSSA strain (Figure 7.3)

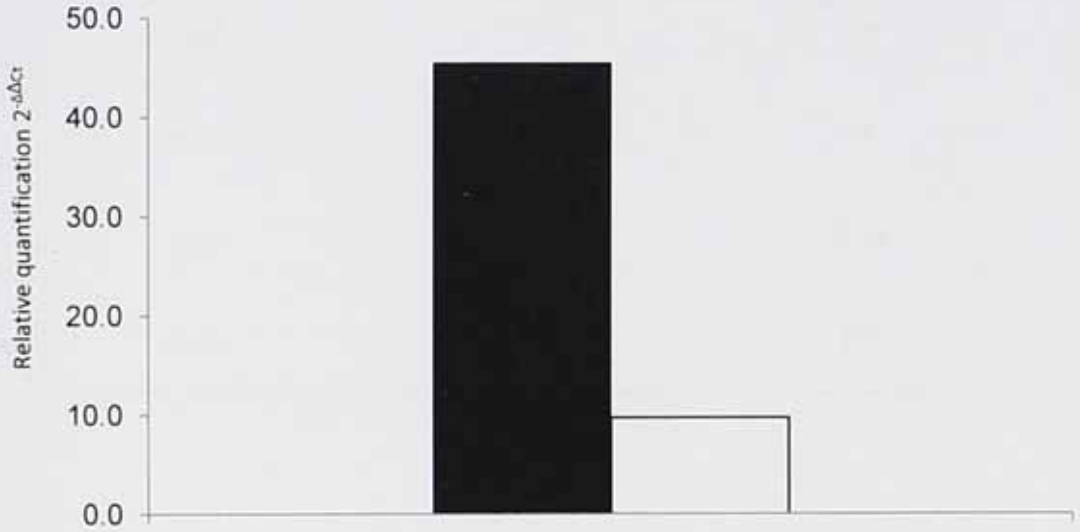


Figure 7.3. Relative quantification of *luxS* gene expression in planktonic ■ CA MRSA MW2 and □ PVL CA MSSA. Housekeeping gene (16S rRNA) (n=1).

Table 7.4 shows a summary of the relative quantities of the five target genes comparative to the housekeeping gene in the exponentially growing planktonic cells.

Table 7.4. Summary table of the quantities of the five target genes comparative to the housekeeping gene in CA MRSA MW2 and PVL CA MSSA.

Genes	CA MRSA (MW2)	PVL CA MSSA
icaA	54.77	13.5
icaD	42	1.5
cap8C	97.53	14
sodA	110.07	26.33
luxS	45.6	9.7

Although in the exponential cells, the *icaA* gene expression was higher in the CA MRSA MW2 strain than in the PVL CA MSSA strain, in biofilms following exposure to the EO, there was no difference in relative quantification between both strains (Figure 7.4). The *icaD* gene was up-regulated in the biofilms of MW2 with a fold change of 3.2. However, in the PVL MSSA strain, this gene was down-regulated by more than a 100 fold.

The *cap8C* gene was up-regulated in both strains with a fold change of 4.8 and 3.1 for the MW2 and PVL CA MSSA strains respectively. With the *cap8C* the gene expression was higher in the CA MRSA MW2 biofilms than the PVL MSSA biofilms following treatment with grapefruit EO by 1.5 times.

The *luxS* gene was up-regulated in the biofilms of both strains however the relative quantities for this gene was 12.4 for the MW2 strain and 3.1 for the PVL CA MSSA strains with the gene expression in the MW2 strain four times that of the PVL CA MSSA strain following treatment with grapefruit EO. With the sodA gene, following treatment of the MW2 biofilm with grapefruit EO, this gene was down regulated by 1.1 fold relative to the housekeeping gene while in the PVL CA MSSA biofilm, this gene was up-regulated by 3.4 fold (Figure 7.4).

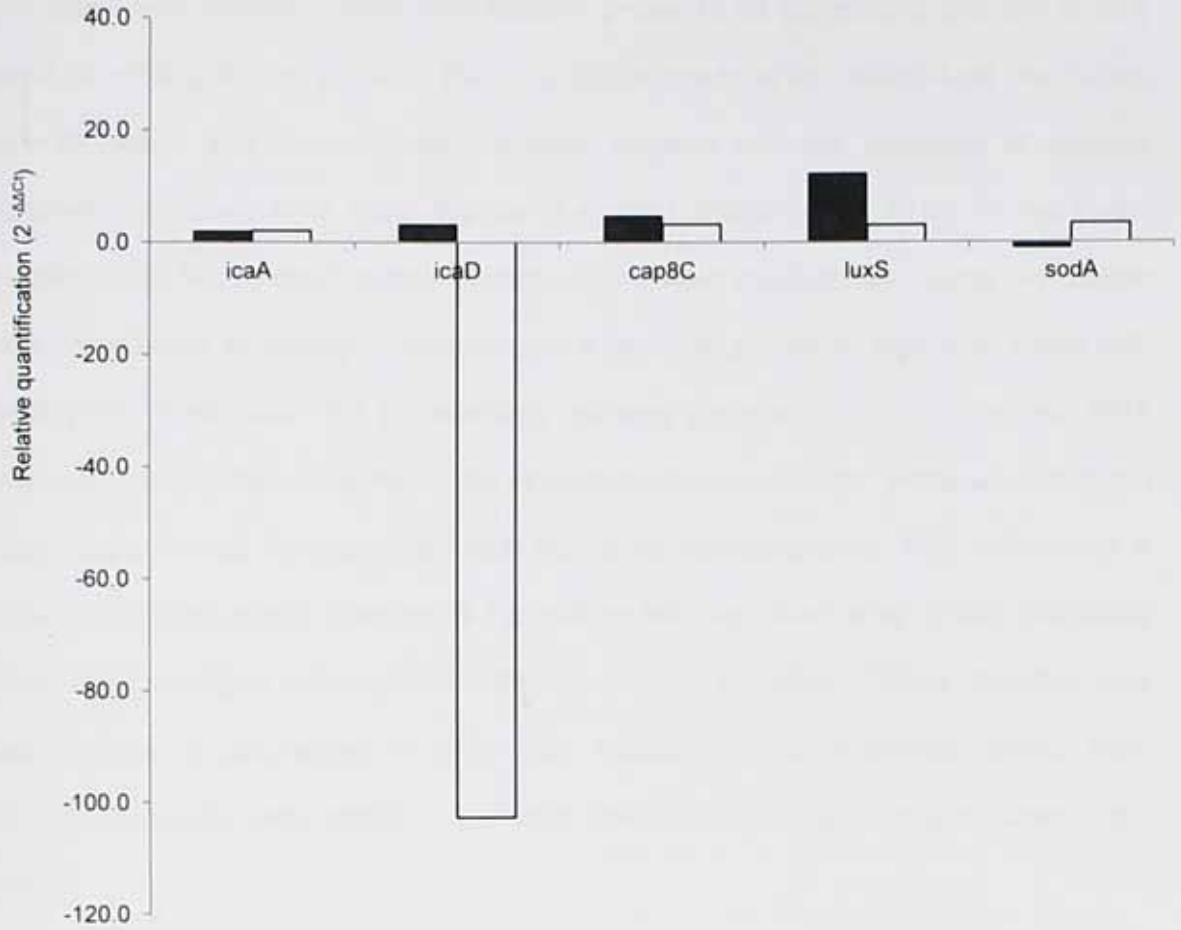


Figure 7.4. Changes oberserved in expression of five target genes between treated [2% (v/v) Grapefruit EO] and untreated biofilm cells of ■ CA MRSA MW2 and □ PVL CA MSSA as determined by fold change measurements using the delta-delta Ct method. (N=1)

7.3 Discussion

The process of extracting RNA from biofilms proved to be challenging and this is as a result of varying factors including the extracellular matrix which affects both the nucleic acid extraction and the purification process coupled with the presence of residual genomic DNA (Cury *et al.* 2008; Franca *et al.* 2011; Asthan *et al.* 2012). In this study, problems with RNA extraction from biofilm cultures were encountered. Using the Qiagen RNA isolation kit according to manufacturer's protocols produced high RNA yields with good purity levels from the exponentially growing planktonic cells. In contrast, RNA extraction from biofilms required many extraction attempts and the yields were at much lower levels; almost 10 times lower than that of the planktonic cells. This could be as a result of the extracellular matrices in the biofilms although, Cury *et al.* (2008) previously found that the Qiagen RNeasy RNA extraction kit using on-column DNase digestion was less effective in comparison to other RNA isolation kits in recovering purified RNA samples especially with regard to genomic DNA contamination of the extracted RNA sample.

The PowerBiofilm™ RNA isolation kit used in this study was more effective at extracting RNA from the *S. aureus* biofilms however this protocol was supported with prior stabilization of the cells and an initial lysis step which involved the use of lysozyme and lysostaphin. This kit has not been used extensively in studies involving RNA extraction from bacteria however it has been used in some recent studies (Callahan, 2010; Zahreddine *et al.* 2012). Callahan (2010) however identified that the PowerBiofilm™ RNA and DNA extraction kits produce optimal yields and inhibitor free extracts and related that to the patented inhibitor removal technology (IRT) utilised by the both kits.

In this study, five genes were investigated for possibility of involvement in the observed increase in the metabolic activity of the PVL CA MSSA biofilms following exposure to

three different concentrations of grapefruit EO (Figure 6.1b). Using RT-PCR, the selected genes (*icaA*, *icaD*, *cap8C*, *luxS* and *sodA*) were all expressed in the exponential cultures without treatment with the grapefruit EO albeit at varying levels. The relative quantities of all five genes were higher in the exponential cells than in biofilm cells. Lenz *et al.* (2008) also found mRNA levels of two genes to be higher in exponentially growing planktonic cells than in an equivalent volume of biofilms while Beaudoin *et al.* (2012) observed increased gene expression in biofilms than in planktonic cells, however according to Lenz *et al.* (2008), mRNA amounts are at the highest at the top zone of biofilms, with RNA abundances not uniformly distributed throughout the biofilms which could lead to varying and generally reduced amounts of gene expression in comparison to the planktonic cells.

The *16S* rRNA was selected as the housekeeping gene and according to Lenz *et al.* (2008), the amounts of this gene is fairly consistent throughout the biofilm and is used as an internal control for differentially regulated genes in biofilms. Following treatment of the two *S.aureus* biofilms with grapefruit EO, the preliminary results showed differences in gene expression between the two strains tested. For four of the five target genes, gene expression was observed to be higher in the CA MRSA MW2 strain than in the PVL CA MSSA strain following treatment with the EO even though the same RNA templates were used and at the same concentrations. As the results from a single experiment and as the PCR efficiency was not tested for the housekeeper or target, it is difficult to determine the significance and conclusivity of the data.

From the observed data, looking at the five target genes under investigation, the variability in gene expression was greater with the *luxS*, *icaD* and *sodA* genes between the two strains tested. The *luxS* gene was upregulated in both CA MRSA MW2 and PVL CA MSSA biofilms although the gene expression was higher in the CA MRSA MW2 strain by four fold. The expression of this gene was also higher in the exponentially growing planktonic cells by approximately five fold in the CA MRSA MW2 strain which may

suggest an increased expression of this gene in one strain than the other. The *luxS* gene is a QS gene which is involved with the production of an auto inducer molecule, AI-2 (Winzer *et al.* 2002; Xu *et al.* 2006). When these molecules reach concentrations above their threshold, the QS system is activated leading to the control and regulation of target genes (Xu *et al.* 2006).

There are contrasting opinions on the exact role of the *luxS* gene. In relation to metabolic activity, Xu *et al.* (2006) did not find any significant effect of *luxS* on basic cellular metabolic processes *in vitro* in *S. epidermidis* however in other studies (McNab *et al.* 2003; Doherty *et al.* 2006; Lebeer *et al.* 2007) the *luxS* gene was associated with sulphur, amino acid and carbohydrate metabolism in different organisms including *S. aureus*. The absence of the *luxS* gene has also been shown to affect formation of biofilms (Merritt *et al.* 2003) and in a study by McNab *et al.* (2003) a luxS mutant *Streptococcus gordonii* strain was unable to form normal biofilms. It is still possible however, that the production and degradation of the AI-2 molecule which is produced by *luxS* could affect the metabolic state of the cells as suggested by Winzer *et al.* (2002). Surette and Bassler (1998) previously demonstrated a strong induction of AI-2 when glucose was present in growth media. With both strains expressing the *luxS* gene, it is difficult to determine if the *luxS* gene was of any impact in this investigation. It might perhaps be worth assessing the effect of the *luxS* gene in both strains without glucose supplementation to ascertain whether the presence of glucose in the growth media influences the metabolic activity in the PVL CA MSSA strain.

Following treatment of the *S. aureus* biofilms with the grapefruit EO, the preliminary results showed two interesting observations. Firstly, there was an upregulation of the *icaD* in the CA MRSA MW2 biofilm while the opposite was observed in the PVL CA MSSA biofilm even though the *icaA* gene was expressed at similar levels in the biofilms of both strains. In a previous study, Vandecasteele *et al.* (2003) observed similar levels of

expression of *icaA* and *icaC* and suggested that this similarity should be expected from two genes transcribed in the same operon. The exact function of the *icaD* is unclear (Vuong *et al.* 2004) although for the *icaD* gene to function, interaction with *icaA* is required prior to membrane integration (Gerke *et al.* 1998) therefore suggesting a chaperone-like action of the *icaD* gene, directing the membrane insertion of *icaA*. Cafiso *et al.* (2004) also investigated the transcriptional activity of the *ica* genes in biofilm positive and negative isolates and observed that although the *icaD* was always expressed, biofilm formation only occurred when the *icaA* was also expressed. According to Oliveira and Cunha (2010), gene expression of the *ica* locus is variable and is induced by variations in culture conditions, such as increase in sugar or other stress inducing substances. Chang *et al.* (2006) previously observed that the *ica ADBC* locus was repressed due to oxidative stress following exposure to hydrogen peroxide.

The second observation, was the downregulation of the *sodA* gene in the CA MRSA MW2 biofilm with the opposite response in the PVL CA MSSA biofilm this. In the *S. aureus* strains, the genes which encode the superoxide dismutases (SODS) are the *sodA* and *sodM* genes. Ballal and Manna (2009) demonstrated that expression of the *sodA* gene is dependent on oxygen availability. However in biofilms, bacterial growth is under microaerophillic or anaerobic conditions (Resch *et al.* 2005). Whilst in another study by Jakubovics *et al.* (2002), the SOD band was barely visible in extracts from cells grown anaerobically and this band was also absent in *sodA* mutant cell extracts in *S. gordonii*. Bizzini *et al.* (2009) reported no change in viability of a *sodA* mutant *E. faecalis* strain following exposure to bacteriostatic agent. No change in viability was observed in the biofilms of both CA MRSA MW2 and PVL CA MSSA after grapefruit EO exposure (Figure 6.2b) and in Chapter 6, the effect of grapefruit EO was determined to be bacteriostatic against both *S. aureus* strains in planktonic conditions. Roslev and King (1993) observed an increase in XTT reduction in the presence of methanol and suggested this action to be caused by a general increase in electron transport system activity. This observation by

Roslev and King and the data from another study by Martinez and Casadevall (2006) who observed greater metabolic activity in biofilms than in planktonic cells following exposure to nitric oxide suggests the problem of oxidative stress. Oxidative stresses in bacteria can be caused by reactive oxygen species (Cabiscol *et al.* 2010). Endogenous production of ROS in biofilms causes variability in the biofilm cells as well as acting as a signal which mediates cell death of subpopulation of the cells leading to metabolic differentiation in other parts of the biofilm (Cap *et al.* 2012). Hence, there is a possibility that the changes observed in metabolic activity in the PVLCA MSSA strain may have been mainly affected by the production of ROS which mediated the upregulation of the *sodA*.

Recent studies have shown that oxidative stresses are produced within biofilms and are capable of affecting the growth of the biofilm and subsequently leading to production of ROS and decrease in the extracellular matrix (Arce Miranda *et al.* 2011). The increase in *sodA* expression and the decrease in *icaD* in the PVL CA MSSA suggests that oxidative stress following exposure to the EO was perhaps responsible for the increase in XTT reduction observed in PVL CA MSSA however as the result was from a single experiment, this is not conclusive and requires further investigation. The differences in gene expression observed between the CA MRSA MW2 and PVL CA MSSA biofilms does suggest that gene expression is strain dependent which adds to the current ideas surrounding the interactions of the genes involved in biofilm formation and the multivariable activity of the genes in biofilm conditions.

CHAPTER EIGHT

General Discussion

8.1 Physiological differences between the *S. aureus* strains

Results from antibiotic susceptibility testing of the six *S. aureus* strains (Chapter 4) showed that the strains mostly confirmed the sources from which the organisms were collected. Four strains PVL CA MSSA, MRSA MW2, PVL CA MRSA and MRSA SR were provided as community acquired isolates while the other two strains were a hospital strain (Hospital MSSA) and a type MRSA strain (MRSA NCTC 13297). The CA strains are known to be more susceptible to antibiotics than the HA isolates (Rice, 2006; Boyle-Vavra and Daum, 2007). In this study, the CA isolates were found to be sensitive to the different antibiotic groups with the exception of the β-lactam antibiotics as was expected (Vandenesch *et al.* 2003; Wallin et al. 2008) however, one of the strains provided was multi drug resistant. Multidrug resistance has been reported predominantly in the HA rather than CA MRSA strains (Wallin *et al.* 2008; Chambers and DeLeo, 2009) and according to Chambers and DeLeo (2009), this is a problem as it limits options for treatment.

According to Vandenesch *et al.* (2003) although CA-MRSA isolates are more susceptible to non β-lactam antibiotics, European CA MRSA isolates appear more resistant to several antibiotics than US and Oceanian isolates. Also, a recent study by Wang *et al.* (2012) identified multidrug resistant clones of CA-MRSA from Chinese children. The origin of the multidrug resistant CA MRSA (SR) strain used in this study needs to be investigated further as the antibiotic profiles and genetic characteristics suggest a different background from the other CA MRSA isolates.

The ability of *S. aureus* to colonise different surfaces including those of medical devices and to form biofilms is an important phenotypic characteristic which enhances its pathogenicity. The investigated *S. aureus* strains were all positive for biofilm formation regardless of the origins of the strains (Chapter 4) when the 96-well plate method was used. There was a correlation between biofilm formation using this method and the CRA method for slime formation. Although there was a correlation between the two methods, some authors have suggested the CRA method should not be used as a standalone method in determining biofilm formation as clinical *S. aureus* strains have been observed which, although positive for CRA method, were negative for biofilm formation and vice versa (Fitzpatrick *et al.* 2006; Croes *et al.* 2009; Nasr *et al.* 2012). It is currently known that biofilm formation is a means of survival (Donlan, 2002) and the ability of the CA *S. aureus* strains to form biofilms could be a factor in survival and dissemination within community settings.

8.2 Molecular differences between the CA and non-CA isolates

The *PVL* gene was present in three of the four CA strains confirmed by multiplex PCR, where the only exception was the CA MRSA (SR) strain, which was negative for this gene. The *PVL* gene is rarely found in HA strains and frequent in the CA isolates (David and Daum, 2010) however PVL negative CA *S. aureus* strains have been identified previously (Takizawa *et al.* 2005; David and Daum, 2010). There is still debate on the main role of PVL in pathogenesis, which has been reviewed by David and Daum (2010).

Biofilm formation had been demonstrated in all six *S. aureus* strains and was negative in the *E. faecium* isolate in this study (Chapter 4). In early stages of biofilm development genes of the *ica* operon are usually expressed leading to production of PIA (Moretro *et al.* 2003). The investigation in Chapter 4 using multiplex PCR demonstrated the presence of both *icaA* and *icaD* genes in all six *S. aureus* strains. As these strains were also biofilm positive and CRA positive, the results observed suggested that the *ica* operon is present

in a high proportion and supports other studies which have reported that the majority of clinical *S. aureus* strains have both the *icaA* and *icaD* genes (Crampton *et al.* 1999; Arciola *et al.* 2001).

Although various methods are available for typing MRSA including PFGE, MLST and *spa* typing, SCC*mec* typing of *S. aureus* was used in this investigation as variations in SCC*mec* types have been used in differentiation of MRSA (Mehndiratta and Bhalla, 2012). Results from the SCC*mec* typing (Chapter 4; Section 4.2.2.4) suggested that the community strains were all type IV based on the presence of the *CCRB2* gene. There were differences between the strains with regard to the SCC*mec* type IV status as only the MW2 was SCC*mec* type IVa and this raises the possibility that the other strains are of different subtypes of the SCC*mec* type IV. To clarify the exact origins of the other CA isolates, identifying what subtypes the other strains belong to would be necessary as the reports suggest that the presence of these different subtypes of the SCC*mec* IV contributes to the ease of transmission of the SCC*mec* type IV CA MRSA (David and Daum, 2010).

8.3 Effects of the disinfectants against *S. aureus* and *E. faecium*

McDonnell and Russell (1999) reviewed the activity and action of disinfectants and antiseptics and described the chlorine and iodine–based compounds as the most significant microbicidal halogens used in the clinic. However the authors suggested that the actual mechanism of action is not fully known. In this study (Chapter 5) the antibacterial activity of household bleach containing sodium hypochlorite and a disinfectant with NaDCC as the main compound were effective as antibacterial agents when the bacteria were in suspension. Both compounds release hypochlorous acid in aqueous solution (Bloomfield, 1996). Hypochlorous acids primarily affect the oxidation of sulfhydryl groups of essential enzymes and antioxidants and also have an effect on DNA synthesis (Fukuzaki, 2006).

Against surface-attached and biofilm cells of *S. aureus* and VRE, both disinfectants were less effective than in suspension, confirming the reports from previous studies (Rutala *et al.* 2000). The factors considered to cause a reduced effect on surfaces include the contact times and the concentrations used. These two factors have been mentioned as the two key variables when determining disinfectant efficacy (Fukuzaki, 2006). Although these two disinfectant products at the recommended concentrations were effective in suspension within one minute, against the same organisms on surfaces, the reduction by these disinfectants was more than two logs. However, it was observed that as the concentrations increased the log reduction increased, hence an extended contact time might be required for the cleaning and disinfection of surface-adhered cells.

The results obtained following exposure of the mature *S. aureus* biofilms to the disinfectants suggest that extending the contact time and increasing the concentration of the disinfectant (to safe and non-toxic levels) could be an important factor in reducing bacterial colonisation. Increasing the concentration of household bleach to 5000 ppm caused a 100% effect within 10 minutes against the biofilms while extending contact time to one hour caused a reduction in biofilm formation by both household bleach and NaDCC at the concentrations tested.

As Kim *et al.* (2007) demonstrated in their study, levels the disinfectant efficacy ranged from planktonic cells, to surface-attached cells and then biofilms. This highlights that although both disinfectants are chlorine releasing agents and have been used for cleaning and disinfection purposes, the effect of these products against biofilms requires further investigation and the concentrations required for biofilm killing and removal needs to be established.

The EFCP showed no antibacterial activity and did not possess any antibiofilm effect against the *S. aureus* strains and VRE. The product contains approximately 5% of non-ionic surfactants which are generally used in detergents and cleaning products. In other

studies, cleaning using surfactants in combination with other disinfectants and/or components was effective in reducing bacterial contamination (Barker *et al.* 2004; Lankford *et al.* 2006) however the assumption is that non-ionic surfactants are inactive (Glover *et al.* 2006).

An observation made on purchase of this EFCP was the lack of distinction between the placement of conventional antibacterial disinfectants and EFCPs at the time of purchase as well as the absence of clearly defined labels which inform the consumers on the lack of antibacterial properties of these products. In the US, standards based certifications such as Green Seal and Ecologo with standard labels are in place to help the end users in identification of green cleaning or EFCPs (Green Seal, 2012). Alongside the current guidelines produced for companies involved in developing EFCPs (DEFRA, 2011), there is a need for adherence to the international standards (ISO 14021:1999) and better information for consumers to restore confidence in making choices (Yates, 2009).

8.4 Effects of the essential oils against *S. aureus* isolates

Essential oils at low concentrations have been demonstrated in a number of studies to possess antimicrobial activity (Hammer *et al.* 1999; Fisher and Phillips, 2006). At 0.06% lemongrass oil (*Cymbopogon flexuosus*) was able to inhibit the growth of six *S. aureus* strains of diverse origins (Chapter 6). The type strain, hospital acquired and community acquired strains were all susceptible to the action of this EO. This EO was the most effective following screening by the disk diffusion assay in comparison to the other EOs; lime, geranium, grapefruit, lemon and bergamot. With the exception of lemon, the other EOs demonstrated antimicrobial activity with ZOIs observed after 24 hour incubation (Table 6.1) demonstrating that essential oils from different sources possess antimicrobial activity albeit at different levels.

The effect of essential oils including lemongrass has been investigated previously however this study is the first to our knowledge that has demonstrated the effect of this essential oil on community acquired *S. aureus* MSSA and MRSA PVL and non-PVL producing isolates. The effect of lemongrass oil was also observed against biofilms and at 0.13% (v/v), biofilm inhibition was achieved (Chapter 6).

Evaluation of the GCMS data provided by Belmay Plc. (Appendix II) showed that the lemongrass EO was composed of approximately 80% citral. Lemongrass EO and citral and its derivatives have been investigated and the effects on bacteria and other microorganisms suggest a broad spectrum of activity (Saddiq and Khayyat, 2010; Tyagi and Malik, 2010). The antimicrobial activity demonstrated by the lemongrass EO included biofilm inhibition, inhibition of metabolic activity and reduction of viability of the cells within the biofilm. The ability of this essential oil to prevent biofilm formation and inhibit metabolic activity in biofilms of clinical *S. aureus* coupled with lack of data demonstrating resistance of these organisms to the effect of EO exposure suggest that this essential oil, if used correctly could prevent infection and reduce bacterial contamination in healthcare and non-healthcare settings.

8.5 Effect of grapefruit EO on *S.aureus* gene expression

In bacteria, genes are expressed differently when the organisms are in either planktonic or biofilm conditions. The observation in this study was that gene expression of the target genes in planktonic cells was much higher than that observed in the untreated biofilm cells. In the planktonic cells it was also observed that the gene expression of the target genes in the untreated planktonic MRSA strain was higher than in the untreated MSSA strain. The differences between the MSSA and MRSA strains have been investigated in previous studies (Edwards-Jones *et al.* 2000; Hallin *et al.* 2007; Rozgonyi *et al.* 2007).

According to Rozgonyi *et al.* (2007), there is a need for comparison of congenic MRSA and MSSA sub-populations to understand what differences exist between these groups however, the authors mentioned that the MRSA strains consist of heterogenous populations of cells which consist of methicillin-sensitive, borderline resistant and methicillin resistant sub-populations. The authors further discuss differences between the MSSA and MRSA (Rozgonyi *et al.* 2007) however they and others (Hallin *et al.* 2007) call for further genetic studies to understand the differences and interaction between MRSA and MSSA as knowledge of this would help in tackling the associated infections. The results presented in Chapter 7 suggest that there is a difference in the expression of certain genes in MRSA and MSSA however more studies would be needed with a larger number of strains for efficient comparison before any conclusions can be drawn from these observations.

The changes in gene expression observed following grapefruit EO exposure could be attributed to different factors. In terms of the expressed genes, it is possible that the responses observed in the *icaD* and the *sodA* genes were responsible for the changes in metabolic activity in the PVL CA MSSA strain. The increase in *sodA* and decrease in *icaD* gene expression in PVL CA MSSA may be as a result of oxidative stress however, this needs to be investigated further as the experiment was only performed once.

In recent studies, changes in gene expression following exposure of *S. aureus* and *Saccharomyces cerevisiae* to essential oils and components have been demonstrated using DNA microarrays (Bi *et al.* 2010; Cuaron *et al.* 2012; Muthaiyan *et al.* 2012). Due to time and resource constrains, this was not performed in this current study. Data on the changes in gene expression in biofilms exposed to EO's is currently unavailable and armed with the knowledge that biofilm development in *S. aureus* is multifactorial (Belion *et al.* 2004; Belion and Gigho, 2005; Lin *et al.* 2012) performing a global gene expression analysis using microarray technology with the same strains and treatment would provide

more information about the metabolic changes in the biofilms observed following EO exposure.

8.6 Conclusion

Biofilm formation is an important factor in survival and virulence of *S. aureus* and in all the *S. aureus* isolates tested in this study were of a biofilm positive phenotype however the VRE strain (*E.faecium* NCTC 12202) was biofilm negative. The CA *S. aureus* isolates demonstrated the characteristic antibiotic susceptibility profiles as the isolates were generally sensitive to the different antibiotic groups however one of the isolates (CA MRSA SR) was multi drug resistant as this strain was resistant to four of six antibiotics (ciprofloxacin, gentamicin, cefoxitin and erythromycin),

Although this CA MRSA isolate was multidrug resistant, this isolate and the other *S. aureus* isolates were sensitive to the effect of five EOs (bergamot, lime, lemongrass, grapefruit, geranium) and citral. The effect of lemongrass EO also included prevention of biofilm formation and inhibition of metabolic activity and viability of the cells in the biofilm of all six *S. aureus* isolates.

In this study, the effect of disinfectants was also demonstrated against both *S. aureus* isolates and the VRE strain. Both household bleach and NaDCC were effective against the isolates tested in suspension with >5 $\log_{(10)}$ reduction although on surface-attached cells and biofilms they were less efficient. However, the EFCP was ineffective against both *S. aureus* and VRE isolates in suspension or against biofilms.

As this study demonstrates, disinfectants when used correctly are still effective in reducing bacterial contamination even against CA *S. aureus* isolates and the effect of lemongrass EO demonstrated in this study suggests that EOs could provide another

option for antimicrobial interventions in clinical, domestic, food and industrial environments.

8.7 Future work

Following all the investigations described in this thesis, a number of aspects have been identified that could result in further research. Firstly, the multidrug resistant MRSA (SR) isolate needs to be characterised further due to the different antibiotic profiles from the traditional CA-MRSA strains. Also this isolate possessed the *ccrB2* gene suggesting this isolate belonged to the SCC*mec* type IV however, as there are different subtypes of the SCC*mec* IV further typing of this strain would be provide some important clues about the origin of this isolate.

Secondly, as there was no antimicrobial activity observed with the ecofriendly cleaning product used in this study, investigating antimicrobial activity in other products advertised as 'green' or ecofriendly from other manufacturers should be performed. Also, a survey to assess the awareness and attitudes of consumers to ecofriendly products and their understanding of the exact function and performances of these products in comparison to conventional sanitizers and disinfectants, would inform consumers and retailers.

As the results from Chapter 7 on the effect of grapefruit EO on the biofilms of the *S. aureus* isolates were preliminary, there is a need to perform further RT-PCR to provide clearer and conclusive results on the changes in gene expression observed. This should also be supported with an initial determination of the amplification efficiencies of the housekeeper and target genes. At present, no published data on the global changes in gene expression in biofilms of *S. aureus* is available. With this in mind, it would be important to determine the changes in gene expression using microarray technology to identify the genes involved in cellular metabolism and oxidative stresses in the PVL CA MSSA biofilm following exposure to grapefruit EO.

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APPENDICES

Appendix I - Materials and Methods

Table I. List of culture media, chemicals and reagents.

Media and Reagents	Codes	Source
Brain Heart Infusion	-	-
Agar	70138-500G	Fluka Analytical, Sigma-Aldrich, UK
Broth	CM1135	Oxoid Ltd, Basingstoke, UK
Mannitol Salt Agar	CM0085	Oxoid Ltd, Basingstoke, UK
Congo Red dye		
Tween 20 (Polyoxyethylene 20-Sorbitan Monolaurate)	BP337-500	Fisher Scientific, Loughborough, UK
Peptone from casein	1.07213.1000	Merck Darmstadt, Germany
L-alpha-Phosphatidylcholine (lecithin)	61755-25G	Sigma-Aldrich, UK
Albumin, from bovine serum (BSA)	A7906-10G	Sigma-Aldrich,Dorset, UK
XTT Sodium salt	X4626	Sigma-Aldrich, Dorset, UK
Menadione	M5625	Sigma-Aldrich, Dorset, UK
Methylated spirit	M/4450/17	Fisher Scientific, Loughborough, UK
Crystal Violet	C.I.42555	SLS, Nottingham, UK
Acetic Acid Glacial	A/0360/PB08	Fisher Scientific, Loughborough, UK
Glutaraldehyde		
D-Glucose anhydrous	G/0500/53	Fisher Scientific, Loughborough, UK
Sodium thiosulphate		

DNA Extraction - GenElute™ Bacterial Genomic DNA Kit

Protocol specific for Gram-Positive Bacterial Preparation

Prepare Lysozyme Solution
Prepare a 2.115 × 106 unit/mL Lysozyme Solution using the included Gram-Positive Lysis Buffer as the diluent. 200 µL of Lysozyme Solution is needed for each prep. Make extra to account for pipetting error.

1. Harvest Cells

Pellet 1.5 mL of bacterial broth culture at 12,000–16,000 × g for 2 minutes, discard media.

2. Digest Cell Wall

Resuspend pellet in 200 µL Lysozyme Solution and incubate at 37 °C for 30 minutes.

3. Lyse Cells

Add 20 µL Proteinase K and 200 µL Lysis Solution C to cell suspension, vortex or pipette to mix. Incubate at 55 °C for 10 minutes.

4. Prepare Column

Add 500 µL of Column Preparation Solution to each binding column. Spin at ≥12,000 × g for 1 minute. Discard flow-through.

5. Bind DNA to Column

Add 200 µL ethanol to the lysed cells, vortex or invert to mix. Transfer EtOH mixture to binding column. Spin at ≥ 6500 × g for 1 minute.

6. Wash Column

Transfer column to new collection tube. Add 500 µL Wash Solution 1 to column. Spin at ≥ 6500 × g for 1 minute. Transfer column to new collection tube. Add 500 µL Wash Solution Concentrate to column. Spin at ≥12,000 × g for 3 minutes to dry column.

7. Elute DNA

Transfer column to new collection tube. Add 200 µL of Elution Solution. Spin at ≥ 6500 × g for 1 minute.

Full details of extraction protocol can be found at http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/na2110bul.Par.0001.File.tmp/na2110bul.pdf

Stepwise procedures from manufacturer.

Protocol 4: Enzymatic Lysis and Proteinase K Digestion of Bacteria

Important points before starting

- Before growing and harvesting bacteria, read "Optimal culture conditions" (page 13).
- If preparing RNA for the first time, read Appendix A (page 38).
- Perform all steps of the procedure at room temperature (15–25°C) without interruption.
- If RNeasy Kits will not be used for RNA purification, follow the procedure up to step 7 only.
- If RNeasy Kits will be used for RNA purification, read "Determining the correct amount of starting material" and "Quantifying bacterial cells" (pages 13–14). Depending on the amount of starting material, choose whether to use the RNeasy Mini Kit or RNeasy Midi Kit.
- Bacterial lysis in this protocol consists of treatment with 15 mg/ml lysozyme for 10 min, which is optimal for *B. subtilis*. Since the conditions for enzymatic lysis are affected by bacterial species, cell number, and culture medium, it may be necessary to adjust enzyme concentration and/or to adjust enzyme incubation time. For some bacteria, other enzymes may be more effective (e.g., we recommend lysostaphin for disrupting the cell wall of *Staphylococcus aureus*).

Things to do before starting

- If using RNeasy Kits for RNA purification, add 10 µl β-mercaptoethanol per 1 ml Buffer RLT, and mix. Buffer RLT is stable for 1 month after addition of β-mercaptoethanol.

Procedure

1. **Prepare TE buffer (30 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 15 mg/ml lysozyme.**
2. **Calculate the required volume of bacterial culture (1 volume).**
   See "Determining the correct amount of starting material", page 13.
3. **Pipet 2 volumes of RNAprotect Bacteria Reagent into a tube (not supplied).**
   For example, if the volume of bacterial culture is 500 µl, add 1000 µl RNAprotect Bacteria Reagent.
   The volume of the tube must be 4-times that of the bacterial culture (e.g., if the volume of bacterial culture is 500 µl, use a 2 ml tube).
   Optional: Entire bacterial cultures can be stabilized by adding 2 volumes of RNAprotect Bacteria Reagent to the culture.
4. **Add 1 volume of bacterial culture to the tube. Mix immediately by vortexing for 5 s. Incubate for 5 min at room temperature (15–25°C).**
5. **Centrifuge for 10 min at 5000 x *g*.**
   A pellet may not be visible after centrifugation. This is due to an interaction between the cells and the stabilization reagent that causes a change in the optical density of the cells. The procedure is not affected.
   Note: For tubes larger than 50 ml, the centrifugal force or centrifugation time may be increased. However, using excessive centrifugal force may make resuspension of the pellet difficult.
6. **Decant the supernatant. Remove residual supernatant by gently dabbing the inverted tube once onto a paper towel.**
   After dabbing the tube onto a paper towel, do not remove the remaining supernatant by pipetting, as this may lead to loss of the pellet. If the tube is larger than 15 ml, we recommend removing residual supernatant by leaving the tube inverted on a paper towel for 10 s.
   Note: The remaining supernatant should not exceed approximately 80 µl per 100 µl TE buffer containing lysoyzme used in step 7.
   Optional: Pellets can be stored at –20°C for up to 2 weeks or at –70°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature (15–25°C) and proceed with step 7 of the procedure.
7. **Add 10–20 µl QIAGEN Proteinase K to the appropriate volume of TE buffer containing lysozyme (see Table 6), and add the mixture to the pellet. Carefully resuspend the pellet by pipetting up and down several times.**
   The amount of QIAGEN Proteinase K required depends on the bacterial species. If using the RNeasy Midi Kit for RNA purification, use 20 µl QIAGEN Proteinase K.

Table 6. Reagent Volumes for Enzymatic Lysis and Proteinase K Digestion of Bacteria

Number of bacteria*	RNeasy spin column	TE buffer containing lysozyme (step 7)	Buffer RLT (step 9)	Ethanol (96–100%) (step 10)	Ethanol (80%) (step 10)
$<1 \times 10^8$	Mini	100 µl	350 µl	250 µl	–
$1 \times 10^8 - 2.5 \times 10^8$	Mini	200 µl	700 µl	500 µl	–
$<2.5 \times 10^8 - <1.5 \times 10^9$	Midi	200 µl	2000 µl	–	1750 µl
$7.5 \times 10^8 - 1.5 \times 10^9$	Midi	200 µl	4000 µl	–	3500 µl

* The cell numbers are optimized for *B. subtilis*, and may need to be optimized for other bacteria. See "Determining the correct amount of starting material", page 13.

8. **Mix by vortexing for 10 s. Incubate at room temperature (15–25°C) for 10 min. During incubation, incubate on a shaker-incubator, or vortex for 10 s at least every 2 min.**

   Note: Since the RNA is stabilized, the incubation time can be extended without causing any adverse effects, and may increase the RNA yield.

9. **Add the appropriate volume of Buffer RLT (see Table 6) and vortex vigorously. If particulate material is visible, pellet it by centrifugation, and use only the supernatant in step 10.**

   For tubes of up to 2 ml, centrifuge for 2 min at maximum speed in a microcentrifuge. For tubes larger than 2 ml, centrifuge for 5 min at 3000–5000 × *g*.

   Note: Ensure that β-mercaptoethanol is added to Buffer RLT before use (see "Important points before starting", page 25).

10. **Add the appropriate volume of ethanol (96–100% for the RNeasy Mini procedure, or 80% for the RNeasy Midi procedure) (see Table 6). Mix by pipetting (RNeasy Mini procedure) or by shaking vigorously (RNeasy Midi procedure). Do not centrifuge.**

    After adding ethanol, a precipitate may form. This will not affect the RNeasy procedure.

11. **If using the RNeasy Mini Kit, proceed to Protocol 7. If using the RNeasy Midi Kit,**

Appendix B: Optional On-Column DNase Digestion Using the RNase-Free DNase Set

The QIAGEN RNase-Free DNase Set provides efficient on-column digestion of DNA during RNA purification using the RNeasy Mini or Midi Kit. The DNase is efficiently removed in subsequent wash steps.

Note: Standard DNase buffers are not compatible with on-column DNase digestion. Using other buffers may affect the binding of the RNA to the RNeasy spin column membrane, reducing the yield and integrity of the RNA.

Preparation of bacterial lysates and binding of RNA to the RNeasy spin column membrane are performed according to the protocols in this handbook. After washing with a reduced volume of Buffer RW1, RNA is treated with DNase I while bound to the spin column membrane. DNase I is removed by a second wash with Buffer RW1. Washing with Buffer RPE and elution are then performed according to the protocols in this handbook.

Important points before starting

- Generally, DNase digestion is not required since RNeasy silica-membrane technology enables efficient removal of most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., RT-PCR analysis with a low-abundant target). DNA can also be removed by a DNase digestion following RNA purification.
- **Do not vortex the reconstituted DNase I.** DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the vial.

Things to do before starting

- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. **Do not vortex.**
- For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots.

Procedure

Prepare bacterial lysates as described in Protocols 1–6. Then perform Protocol 7 (■) or Protocol 8 (▲). Instead of following step 2 of Protocol 7 or 8 (i.e., the wash with Buffer RW1), follow steps B1–B4 below.

B1. **Add ■ 350 µl or ▲ 2 ml Buffer RW1 to the RNeasy spin column, and centrifuge for ■ 15 s at ≥8000 x *g* or ▲ 5 min at 3000–5000 x *g* to wash the spin column membrane. Discard the flow-through.* Reuse the collection tube in step B4.**

B2. **Add ■ 10 µl or ▲ 20 µl DNase I stock solution (see above) to ■ 70 µl or ▲ 140 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.**

Buffer RDD is supplied with the RNase-Free DNase Set.

Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

B3. **Add the DNase I incubation mix (■ 80 µl or ▲ 160 µl) directly to the RNeasy spin column membrane, and incubate at room temperature (20–30°C) for 15 min.**

Note: Be sure to add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

B4. **Add ■ 350 µl or ▲ 2 ml Buffer RW1 to the RNeasy spin column, wait for 5 min, and then centrifuge for ■ 15 s at ≥8000 x *g* or ▲ 5 min at 3000–5000 x *g*. Discard the flow-through and collection tube.* Continue with step 3 of ■ Protocol 7 or ▲ Protocol 8 (i.e., the first wash with Buffer RPE).**

RNA Extraction - PowerBiofilm™ RNA Isolation Kit

Important Notes Before Starting

Solution BFR1 must be warmed at 55°C for 5-10 minutes to dissolve precipitates prior to each use. Solution BFR1 should be used while still warm. Shake to mix Solution BFR6 before use.

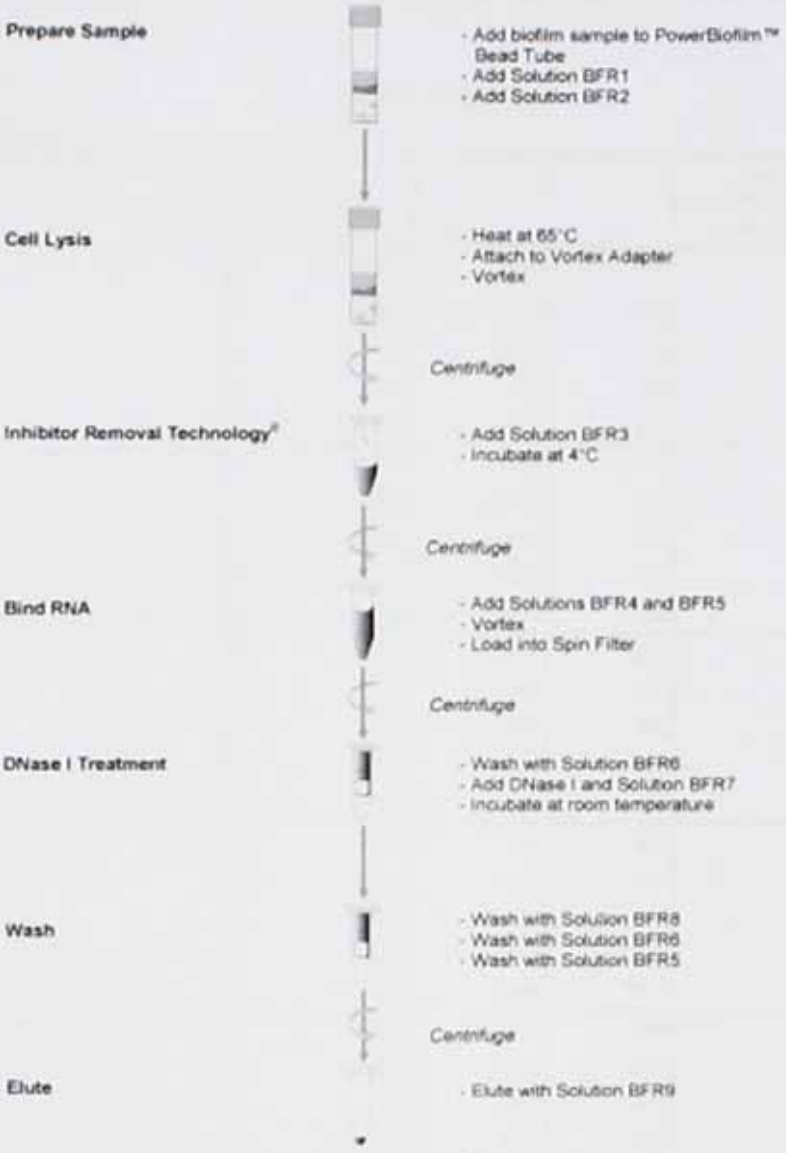
Prepare Solution BFR1 by adding β- mercaptoethanol (βME)
Add 5 µl of β- mercaptoethanol (βME) for every 345 µl of the Solution BFR1 for all samples to be processed. For each prep, 350 µl of the Solution BFR1/ β- mercaptoethanol (βME) will be needed. Note: Prepare Solution BFR1 in smaller aliquots with fresh βME according to the number of samples you need to process that day instead of adding βME to the whole bottle. Use a fume hood when opening βME to avoid exposure to the chemical.

DNase I Preparation and Storage
To prepare DNase I stock solution add RNase-Free water (Solution BFR9) to the lyophilized DNase I according to the table below and mix gently. Aliquot the enzyme in 50 µl portions and store at -20°C for long term storage. Note: The enzyme can be freeze/thawed up to three times without loss of activity.
To prepare the DNase I Solution, thaw the volume of enzyme needed according to the number of samples. Per prep, combine 5 µl of DNase I enzyme with 45 µl of Solution BFR7.

PowerBiofilm™ RNA extraction – Procedure

MO BIO Laboratories, Inc.

PowerBiofilm™ RNA Isolation Kit



Appendix II (Chapter 4 - Statistics)

Biofilm formation in *S. aureus* strains

ANOVA

Strains

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.165	5	.633	21.199	.000
Within Groups	.896	30	.030		
Total	4.061	35			

Multiple Comparisons

Dependent Variable Strains

	(I) VAR00007	(J) VAR00007	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Games-Howell	1	2	-.7343*	.1198	.004	-1.190	-.278
		3	-.2077	.1396	.679	-.698	.282
		4	-.1318	.1189	.863	-.588	.324
		5	.1252	.1134	.863	-.334	.584
		6	-.5222*	.1303	.028	-.991	-.054
	2	1	.7343*	.1198	.004	.278	1.190
		3	.5267*	.0993	.007	.162	.891
		4	.6025*	.0671	.000	.369	.836
		5	.8595*	.0568	.000	.653	1.066
		6	.2122	.0858	.229	-.094	.518
	3	1	.2077	.1396	.679	-.282	.698
		2	-.5267*	.0993	.007	-.891	-.162
		4	.0758	.0982	.965	-.287	.439
		5	.3328	.0915	.070	-.028	.694
		6	-.3145	.1118	.138	-.706	.077
	4	1	.1318	.1189	.863	-.324	.588
		2	-.6025*	.0671	.000	-.836	-.369
		3	-.0758	.0982	.965	-.439	.287
		5	.2570*	.0548	.012	.060	.454
		6	-.3903*	.0845	.012	-.694	-.087
	5	1	-.1252	.1134	.863	-.584	.334
		2	-.8595*	.0568	.000	-1.066	-.653
		3	-.3328	.0915	.070	-.694	.028
		4	-.2570*	.0548	.012	-.454	-.060
		6	-.6473*	.0766	.001	-.942	-.353
	6	1	.5222*	.1303	.028	.054	.991
		2	-.2122	.0858	.229	-.518	.094
		3	.3145	.1118	.138	-.077	.706
		4	.3903*	.0845	.012	.087	.694
		5	.6473*	.0766	.001	.353	.942

*. The mean difference is significant at the 0.05 level.

Effect of nutrient media change

CA MRSA MW2

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MRSA MW2	Equal variances assumed	.001	.978	2.954	14	.010	.71488	.24200	.19583	1.23392
	Equal variances not assumed			2.954	13.866	.011	.71488	.24200	.19536	1.23439

PVL CA MRSA

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
PVL MRSA	Equal variances assumed	11.014	.005	8.762	14	.000	1.09013	.12442	.82328	1.35697
	Equal variances not assumed			8.762	10.004	.000	1.09013	.12442	.81292	1.36733

Time dependent measurement of biofilm formation

MSSA NCTC 13297

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.130	2	2.065	45.049	.000
Within Groups	1.513	33	.046		
Total	5.643	35			

Multiple Comparisons

Games-Howell

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
24 hours	48 hours	-.16942	.07413	.093	-.3645	.0257
	72 hours	.61867*	.10453	.000	.3559	.8814
48 hours	24 hours	.16942	.07413	.093	-.0257	.3645
	72 hours	.78808*	.08062	.000	.5751	1.0010
72 hours	24 hours	-.61867*	.10453	.000	-.8814	-.3559
	48 hours	-.78808*	.08062	.000	-1.0010	-.5751

*. The mean difference is significant at the 0.05 level.

PVL CA MSSA

ANOVA

PVL CA MSSA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.941	2	.971	68.725	.000
Within Groups	.466	33	.014		
Total	2.407	35			

Multiple Comparisons

PVL CA MSSA
Games-Howell

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
24 HR	48 HR	.15158*	.03901	.002	.0535	.2496
	72 HR	-.39900*	.05197	.000	-.5318	-.2662
48 HR	24 HR	-.15158*	.03901	.002	-.2496	-.0535
	72 HR	-.55058*	.05328	.000	-.6861	-.4151
72 HR	24 HR	.39900*	.05197	.000	.2662	.5318
	48 HR	.55058*	.05328	.000	.4151	.6861

*. The mean difference is significant at the 0.05 level.

PVL CA MSSA vs MSSA NCTC 13297

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MICRORGANISMS	Equal variances assumed	3.356	.081	-15.396	22	.000	-1.37483	.08930	-1.56002	-1.18965
	Equal variances not assumed			-15.396	17.654	.000	-1.37483	.08930	-1.56270	-1.18697

Appendix III – (Chapter 5 - Statistics)

Surface test data output

Hospital MSSA isolate (clean vs. dirty – 5 minutes) with 500 ppm household bleach

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Hosp MSSA 5 min HB	Equal variances assumed	3.391	.084	-3.713	16	.002	-1.73000	.46595	-2.71778	-.74222
	Equal variances not assumed			-3.713	14.463	.002	-1.73000	.46595	-2.72638	-.73362

E. faecium NCTC 12202 isolate (clean vs. dirty – 5 minutes) treated with 500 ppm household bleach

Test Statistics[b]

	E.faecium NCTC 12202 5 min HB
Mann-Whitney U	15.500
Wilcoxon W	60.500
Z	-2.209
Asymp. Sig. (2-tailed)	.027
Exact Sig. [2*(1-tailed Sig.)]	.024[a]

a. Not corrected for ties.

b. Grouping Variable: VAR00002

E. faecium NCTC 12202 isolate (clean vs. dirty – 5 minutes) treated with 1000 ppm NaDCC

Test Statistics[b]

	E.faecium NCTC 12202 5 min HB
Mann-Whitney U	3.000
Wilcoxon W	39.000
Z	-3.046
Asymp. Sig. (2-tailed)	002
Exact Sig. [2*(1-tailed Sig.)]	.001[a]

a. Not corrected for ties.

b. Grouping Variable: VAR00002

E. faecium NCTC 12202 isolate (clean vs. dirty – 10 minutes) treated with 500 ppm Household bleach

Test Statistics[b]

	VRE NCTc 12202 10 min HB
Mann-Whitney U	15.000
Wilcoxon W	60.000
Z	-2.253
Asymp. Sig. (2-tailed)	.024
Exact Sig. [2*(1-tailed Sig.)]	.024[a]

a. Not corrected for ties.

b. Grouping Variable: VAR00002

E. faecium NCTC 12202 isolate (clean vs. dirty – 5 minutes) treated with 1000 ppm NaDCC

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VRE 10 min NaDCC	Equal variances assumed	26.692	.000	4.466	14	.001	2.35750	.52782	1.22544	3.48956
	Equal variances not assumed			4.466	7.579	.002	2.35750	.52782	1.12846	3.58654

Biofilms

MSSA NCTC 13297 biofilm vs 500 ppm household bleach

ANOVA

Biofilms v HB)

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		.444	2	.222	.450	.643
	Linear Term	Contrast	.007	1	.007	.015	.903
		Deviation	.437	1	.437	.885	.356
Within Groups			11.846	24	.494		
Total			12.291	26			

PVL CA MSSA biofilm vs 500 ppm household bleach

Test Statistics[a,b]

	PVL CA MSSA
Chi-Square	2.668
df	2
Asymp. Sig.	.263

a. Kruskal Wallis Test

b. Grouping Variable: ContactTime

MSSA NCTC 13297 biofilm vs 10,000 ppm NaDCC

Test Statistics[b]

	CONCENTRATIONS
Mann-Whitney U	12.000
Wilcoxon W	57.000
Z	-2.517
Asymp. Sig. (2-tailed)	012
Exact Sig. [2*(1-tailed Sig.)]	.011[a]

a. Not corrected for ties.

b. Grouping Variable: VAR00002

MSSA NCTC 13297 biofilm vs 1,000 ppm NaDCC

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
CONCENTRATIONS	Equal variances assumed	3.550	.078	-2.475	16	.025	-.71167	.28758	-1.32131	-.10202
	Equal variances not assumed			-2.475	13.548	.027	-.71167	.28758	-1.33040	-.09293

PVL CA MSSA biofilm vs 10,000 ppm NaDCC

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
CONCENTRATIONS	Equal variances assumed	6.651	.020	-5.019	16	.000	-1.26189	.25142	-1.79488	-.72890
	Equal variances not assumed			-5.019	10.295	.000	-1.26189	.25142	-1.81992	-.70386

PVL CA MSSA biofilm vs 1,000 ppm NaDCC

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
concentrations	Equal variances assumed	8.881	.009	-3.707	15	.002	-1.04812	.28277	-1.65084	-.44541
	Equal variances not assumed			-3.525	8.404	.007	-1.04812	.29733	-1.72806	-.36819

Appendix IV (GC-MS analysis and Chapter 6 statistics)

Belmay Fragrances Ltd Analytical Report.

Table I. GCMS analysis of Lemongrass essential oil.

Component	Typical Value
alpha Pinene	0.20
methyl heptenone	2.00
myrcene	1.00
limonene	0.40
linalool	1.50
citronellal	1.00
neral	33.00
geranial	47.00
geranyl acetate	1.50
beta carypohyllene	4.00

Table II. GCMS analysis of Geranium essential oil (African).

Component	Typical Value
rose oxide	1.50
laevo menthone	1.50
isomenthone	6.00
citronellol	30.00
geraniol	15.00
citronellyl formate	8.00
geranyl formate	3.00
beta bourbonene	1.00
guaia-6,9-diene	0.10
10-epi gamma eudesmol	4.00

Table III. GCMS analysis of Grapefruit oil Expressed

Component	Typical Value
alpha pinene	0.50
beta pinene	0.40
myrcene	2.32
limonene	93.50
decanal	0.26
nootkatone	0.10

Table IV. GCMS analysis of Lime essential oil (Expressed Mexico).

Component	Typical Value
alpha pinene	2.30
beta pinene	22.70
myrcene	1.30
alpha terpinene	0.25
para cymene	0.25
limonene	47.30
gamma terpinene	7.50
linalool	0.15
neral	2.00
geranial	2.90
beta caryophyllene	1.00
trans alpha bergamotene	1.10
beta bisabolene	2.70

Table V. GCMS analysis of Bergamot essential oil Reggio (bergaptene free)

Component	Typical Value
alpha thujene	0.30
alpha pinene	1.30
sabinene	1.10
beta pinene	7.20
myrcene	1.00
alpha terpinene	0.15
para cymene	0.50
limonene	38.50
gamma terpinene	6.00
terpinolene	0.30
linalool	11.40
neral	0.20
linalyl acetate	27.90
geranial	0.40

Data output for 1% Grapefruit EO (All strains excluding PVL MSSA)

ANOVA

1% No PVL CA MSSA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.687	4	.422	1.753	**.152**
Within Groups	13.231	55	.241		
Total	14.918	59			

Data output for 2% Grapefruit EO (All strains excluding PVL MSSA)

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.271	4	.568	2.248	**.076**
Within Groups	13.886	55	.252		
Total	16.157	59			

Data output for 4% Grapefruit EO (All strains excluding PVL CA MSSA)

ANOVA

4% No PVL MSSA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.409	4	1.602	2.793	.035
Within Groups	31.553	55	.574		
Total	37.961	59			

Post Hoc Tests

Multiple Comparisons

4% No PVL MSSA. Games-Howell

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Hosp MSSA	MSSA NCTC 13297	-.40400	.34307	.764	-1.4893	.6813
	CA MRSA MW2	-.29267	.28316	.836	-1.1803	.5949
	PVL CA MRSA	-.97833*	.19903	.001	-1.5870	-.3696
	CA MRSA SR	-.61067*	.15991	.009	-1.0908	-.1306
MSSA NCTC 13297	Hosp MSSA	.40400	.34307	.764	-.6813	1.4893
	CA MRSA MW2	.11133	.42645	.999	-1.1585	1.3812
	PVL CA MRSA	-.57433	.37589	.560	-1.7191	.5704
	CA MRSA SR	-.20667	.35672	.976	-1.3137	.9003
CA MRSA MW2	Hosp MSSA	.29267	.28316	.836	-.5949	1.1803
	MSSA NCTC 13297	-.11133	.42645	.999	-1.3812	1.1585
	PVL CA MRSA	-.68567	.32214	.249	-1.6540	.2827
	CA MRSA SR	-.31800	.29955	.823	-1.2354	.5994
PVL CA MRSA	Hosp MSSA	.97833*	.19903	.001	.3696	1.5870
	MSSA NCTC 13297	.57433	.37589	.560	-.5704	1.7191
	CA MRSA MW2	.68567	.32214	.249	-.2827	1.6540
	CA MRSA SR	.36767	.22173	.480	-.2948	1.0301
CA MRSA SR	Hosp MSSA	.61067*	.15991	.009	.1306	1.0908
	MSSA NCTC 13297	.20667	.35672	.976	-.9003	1.3137
	CA MRSA MW2	.31800	.29955	.823	-.5994	1.2354
	PVL CA MRSA	-.36767	.22173	.480	-1.0301	.2948

*. The mean difference is significant at the 0.05 level.

Data output for all strains (including PVL CA MSSA) following exposure to 1% Grapefruit EO

ANOVA

1% All strains (inc PVL MSSA)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.585	5	.717	3.013	.016
Within Groups	15.709	66	.238		
Total	19.294	71			

Multiple Comparisons

1% All strains (inc PVL MSSA) Games-Howell

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Hosp MSSA	PVL CA MSSA	-.68558*	.14933	.004	-1.1708	-.2004
	MSSA NCTC 13297	-.31125	.23080	.755	-1.0808	.4583
	CA MRSA MW2	-.12017	.13668	.946	-.5610	.3207
	PVL CA MRSA	-.35008	.11466	.064	-.7142	.0140
	CA MRSA SR	-.46783	.16040	.093	-.9918	.0562
PVL CA MSSA	Hosp MSSA	.68558*	.14933	.004	.2004	1.1708
	MSSA NCTC 13297	.37433	.26174	.709	-.4562	1.2048
	CA MRSA MW2	.56542	.18418	.055	-.0089	1.1397
	PVL CA MRSA	.33550	.16848	.381	-.1943	.8653
	CA MRSA SR	.21775	.20241	.886	-.4132	.8487
MSSA NCTC 13297	Hosp MSSA	.31125	.23080	.755	-.4583	1.0808
	PVL CA MSSA	-.37433	.26174	.709	-1.2048	.4562
	CA MRSA MW2	.19108	.25474	.972	-.6230	1.0052
	PVL CA MRSA	-.03883	.24363	1.000	-.8297	.7520
	CA MRSA SR	-.15658	.26822	.991	-1.0032	.6900
CA MRSA MW2	Hosp MSSA	.12017	.13668	.946	-.3207	.5610
	PVL CA MSSA	-.56542	.18418	.055	-1.1397	.0089
	MSSA NCTC 13297	-.19108	.25474	.972	-1.0052	.6230
	PVL CA MRSA	-.22992	.15738	.691	-.7224	.2626
	CA MRSA SR	-.34767	.19327	.487	-.9517	.2563
PVL CA MRSA	Hosp MSSA	.35008	.11466	.064	-.0140	.7142
	PVL CA MSSA	-.33550	.16848	.381	-.8653	.1943
	MSSA NCTC 13297	.03883	.24363	1.000	-.7520	.8297
	CA MRSA MW2	.22992	.15738	.691	-.2626	.7224
	CA MRSA SR	-.11775	.17838	.984	-.6813	.4458
CA MRSA SR	Hosp MSSA	.46783	.16040	.093	-.0562	.9918
	PVL CA MSSA	-.21775	.20241	.886	-.8487	.4132
	MSSA NCTC 13297	.15658	.26822	.991	-.6900	1.0032
	CA MRSA MW2	.34767	.19327	.487	-.2563	.9517
	PVL CA MRSA	.11775	.17838	.984	-.4458	.6813

*. The mean difference is significant at the 0.05 level.

Data output for all strains (including PVL CA MSSA) following exposure to 2% Grapefruit EO

ANOVA

2% GRA Inc PVL CA MSSA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.685	5	.737	3.010	.017
Within Groups	16.162	66	.245		
Total	19.847	71			

Multiple Comparisons

2% GRA Inc PVL CA MSSA
Games-Howell

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Hosp MSSA	PVL CA MSSA	-.56583*	.17311	.037	-1.1062	-.0255
	MSSA NCTC 13297	-.22883	.22523	.906	-.9463	.4886
	CA MRSA MW2	.03625	.17330	1.000	-.5047	.5772
	PVL CA MRSA	-.25617	.18532	.737	-.8370	.3246
	CA MRSA SR	-.49992	.16814	.067	-1.0242	.0243
PVL CA MSSA	Hosp MSSA	.56583*	.17311	.037	.0255	1.1062
	MSSA NCTC 13297	.33700	.23503	.707	-.4045	1.0785
	CA MRSA MW2	.60208*	.18586	.038	.0231	1.1811
	PVL CA MRSA	.30967	.19712	.625	-.3051	.9244
	CA MRSA SR	.06592	.18107	.999	-.4983	.6301
MSSA NCTC 13297	Hosp MSSA	.22883	.22523	.906	-.4886	.9463
	PVL CA MSSA	-.33700	.23503	.707	-1.0785	.4045
	CA MRSA MW2	.26508	.23517	.864	-.4768	1.0070
	PVL CA MRSA	-.02733	.24417	1.000	-.7931	.7385
	CA MRSA SR	-.27108	.23140	.845	-1.0034	.4613
CA MRSA MW2	Hosp MSSA	-.03625	.17330	1.000	-.5772	.5047
	PVL CA MSSA	-.60208*	.18586	.038	-1.1811	-.0231
	MSSA NCTC 13297	-.26508	.23517	.864	-1.0070	.4768
	PVL CA MRSA	-.29242	.19729	.678	-.9077	.3228
	CA MRSA SR	-.53617	.18125	.069	-1.1009	.0286
PVL CA MRSA	Hosp MSSA	.25617	.18532	.737	-.3246	.8370
	PVL CA MSSA	-.30967	.19712	.625	-.9244	.3051
	MSSA NCTC 13297	.02733	.24417	1.000	-.7385	.7931
	CA MRSA MW2	.29242	.19729	.678	-.3228	.9077
	CA MRSA SR	-.24375	.19278	.800	-.8457	.3582
CA MRSA SR	Hosp MSSA	.49992	.16814	.067	-.0243	1.0242
	PVL CA MSSA	-.06592	.18107	.999	-.6301	.4983
	MSSA NCTC 13297	.27108	.23140	.845	-.4613	1.0034
	CA MRSA MW2	.53617	.18125	.069	-.0286	1.1009
	PVL CA MRSA	.24375	.19278	.800	-.3582	.8457

* The mean difference is significant at the 0.05 level.

Data output for all strains (including PVL CA MSSA) following exposure to 4% Grapefruit EO

ANOVA

4% GRA incl PVL CA MSSA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.661	5	1.332	2.574	.035
Within Groups	34.162	66	.518		
Total	40.824	71			

Multiple Comparisons

4% GRA incl PVL CA MSSA
Games-Howell

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig	95% Confidence Interval	
					Lower Bound	Upper Bound
Hosp MSSA	PVL CA MSSA	-.61608*	.16668	.017	-1.1438	-.0884
	MSSA NCTC 13297	-.40400	.34307	.839	-1.5473	.7393
	CA MRSA MW2	-.29267	.28316	.898	-1.2272	.6419
	PVL CA MRSA	-.97833*	.19903	.002	-1.6185	-.3382
	CA MRSA SR	-.61067*	.15991	.012	-1.1151	-.1063
PVL CA MSSA	Hosp MSSA	.61608*	.16668	.017	.0884	1.1438
	MSSA NCTC 13297	.21208	.35981	.990	-.9586	1.3828
	CA MRSA MW2	.32342	.30322	.888	-.6492	1.2960
	PVL CA MRSA	-.36225	.22666	.609	-1.0717	.3472
	CA MRSA SR	.00542	.19321	1.000	-.5967	.6075
MSSA NCTC 13297	Hosp MSSA	.40400	.34307	.839	-.7393	1.5473
	PVL CA MSSA	-.21208	.35981	.990	-1.3828	.9586
	CA MRSA MW2	.11133	.42645	1.000	-1.2222	1.4449
	PVL CA MRSA	-.57433	.37589	.652	-1.7779	.6293
	CA MRSA SR	-.20667	.35672	.991	-1.3717	.9584
CA MRSA MW2	Hosp MSSA	.29267	.28316	.898	-.6419	1.2272
	PVL CA MSSA	-.32342	.30322	.888	-1.2960	.6492
	MSSA NCTC 13297	-.11133	.42645	1.000	-1.4449	1.2222
	PVL CA MRSA	-.68567	.32214	.315	-1.7031	.3317
	CA MRSA SR	-.31800	.29955	.889	-1.2828	.6468
PVL CA MRSA	Hosp MSSA	.97833*	.19903	.002	.3382	1.6185
	PVL CA MSSA	.36225	.22666	.609	-.3472	1.0717
	MSSA NCTC 13297	.57433	.37589	.652	-.6293	1.7779
	CA MRSA MW2	.68567	.32214	.315	-.3317	1.7031
	CA MRSA SR	.36767	.22173	.572	-.3281	1.0635
CA MRSA SR	Hosp MSSA	.61067*	.15991	.012	.1063	1.1151
	PVL CA MSSA	-.00542	.19321	1.000	-.6075	.5967
	MSSA NCTC 13297	.20667	.35672	.991	-.9584	1.3717
	CA MRSA MW2	.31800	.29955	.889	-.6468	1.2828
	PVL CA MRSA	-.36767	.22173	.572	-1.0635	.3281

* The mean difference is significant at the 0.05 level.

Lemongrass EO Treated cells (Cell viability data output)

Comparison between the recovered cells from all six *S. aureus* strains

ANOVA

Biofilm viability

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.037	5	.007	.637	.673
Within Groups	.480	41	.012		
Total	.517	46			

Multiple Comparisons

Biofilm viability
Games-Howell

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Hosp MSSA	No treatment control	.05386	.05603	.920	-.1425	.2502
	3.00	.01400	.03255	.998	-.0909	.1189
	4.00	-.04432	.03575	.810	-.1639	.0752
	5.00	.00011	.05112	1.000	-.1908	.1910
	6.00	.00161	.05787	1.000	-.2019	.2052
No treatment control	Hosp MSSA	-.05386	.05603	.920	-.2502	.1425
	3.00	-.03986	.05660	.977	-.2369	.1571
	4.00	-.09818	.05850	.571	-.2989	.1025
	5.00	-.05375	.06898	.966	-.2856	.1781
	6.00	-.05225	.07411	.978	-.2954	.1909
3.00	Hosp MSSA	-.01400	.03255	.998	-.1189	.0909
	No treatment control	.03986	.05660	.977	-.1571	.2369
	4.00	-.05832	.03663	.617	-.1801	.0635
	5.00	-.01389	.05174	1.000	-.2050	.1772
	6.00	-.01239	.05841	1.000	-.2166	.1918
4.00	Hosp MSSA	.04432	.03575	.810	-.0752	.1639
	No treatment control	.09818	.05850	.571	-.1025	.2989
	3.00	.05832	.03663	.617	-.0635	.1801
	5.00	.04443	.05382	.955	-.1497	.2386
	6.00	.04593	.06026	.968	-.1616	.2534
5.00	Hosp MSSA	-.00011	.05112	1.000	-.1910	.1908
	No treatment control	.05375	.06898	.966	-.1781	.2856
	3.00	.01389	.05174	1.000	-.1772	.2050
	4.00	-.04443	.05382	.955	-.2386	.1497
	6.00	.00150	.07047	1.000	-.2352	.2382
6.00	Hosp MSSA	-.00161	.05787	1.000	-.2052	.2019
	No treatment control	.05225	.07411	.978	-.1909	.2954
	3.00	.01239	.05841	1.000	-.1918	.2166
	4.00	-.04593	.06026	.968	-.2534	.1616
	5.00	-.00150	.07047	1.000	-.2382	.2352

Grapefruit EO Treated cells (Cell viability data output)

Comparison between the recovered cells from all six *S. aureus* strains

Test Statistics[a,b]

	GRA 4% ALl strains CFU/ML
Chi-Square	6.977
df	5
Asymp. Sig.	.222

a. Kruskal Wallis Test

b. Grouping Variable: Isolates

Appendix V (List of publications and presentations)

SCIENTIFIC PUBLICATIONS

Adukwu EC, Allen SCH, Phillips CA. (2012). The effect of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils on *Staphylococcus aureus* biofilms. *Journal of Applied Microbiology*. 113(5), 1217-27.

CONFERENCE PRESENTATIONS & SEMINARS

Adukwu EC, Allen SCH, Phillips CA. Antimicrobial activity of Lemongrass essential oil (*Cymbopogon flexuosus*) against *Staphylococcus aureus* biofilms. 112th General Meeting, ASM, San Francisco, California, USA. June 2012

Adukwu EC. Research Dissemination: Challenges and Opportunities. CHWR seminar, The University of Northampton, Northampton, UK. Oct 2011

Adukwu EC, Allen SCH, Phillips CA. Antimicrobial activity of disinfectants against different strains of *Staphylococcus aureus*" SfAM Summer conference, Dublin, Ireland. July 2011

Adukwu EC, Allen SCH, Phillips CA. Investigating the potential of commercially available environmental decontaminants against strains of *Staphylococcus aureus* and antibiotic resistant enterococci" 111th General Meeting, ASM, New Orleans, Louisiana, USA. May 2011

Adukwu EC, Allen SCH, Phillips CA. Community acquired infections and its implications in the elderly'. East Midlands Universities Association annual research conference, Nottingham, UK. October 14th 2010

Adukwu EC, Allen, S.C.H, Phillips, C.A. Antimicrobial effect of sodium hypochlorite, sodium diisochlorocyanurate and a commercial available cleaning product on VRE and *Clostridium difficile*". SfAM Summer conference, Brighton, UK. July 2010

Adukwu EC, Allen SCH, Phillips CA. Community acquired infections (CAIs) – reducing the burden of infections. Annual poster competition, The University of Northampton, UK. May 19th 2010

Adukwu EC. Community acquired infections (CAIs). The School of Health Learning and Teaching Conference. The University of Northampton, UK. Dec 2009

Adukwu EC, Allen SCH, Phillips CA. Community acquired infections (CAIs). CHWR annual conference, Northampton, UK. Sep 2009

INVITED SEMINARS AND PRESENTATIONS

Adukwu EC. Antimicrobial activity of disinfectants and natural products against *Staphylococcus aureus* in culture and biofilms. Research Group Seminar, OARDC, Ohio State University, Wooster, Ohio, USA. August 1st 2012.

Adukwu EC. Antimicrobial activity of Lemongrass essential oil (*Cymbopogon flexuosus*) against staphylococcus aureus biofilms. Research Group Seminar, Haverford College, Philadelphia, USA. June 22nd 2012.