Project Title:

Antibacterial activity of Polyunsaturated Fatty Acids related to Chronic Wound Infections

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BY

MOHAMED ESHLAK

@00407504

SUPERVISOR: DR CHLOE JAMES

CO-SUPERVISOR: DR JOE LATIMER

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List of Abbreviations

Antimicrobial peptides (AMPs)

Antimicrobial resistance (AMR)

Antimicrobial sensitivity test (AST)

Artemis Comparison Tool (ACT)

Auto inducer (AI)

Autoinducing peptide (AIP)

Extra polysaccharide (EPS)

Bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP)

Basic fibroblast growth factor (BFGF)

Biofilm-associated protein (BAP)

Brain Heart infusion (BHI)

Clumping factor, A and B (ClfA and ClfB)

Coagulase negative staphylococci (CoNS)

Colony forming units (CFU)

Congo Red Agar assay (CRA)

Converts adenosine monophosphate (AMP)

Crystal Violet (CV)

Delta-5-desaturase (D5D)

Docosahexaenoic acid (DHA

Eicosapentaenoic acid (EPA)

Epidermal growth factor (EGF)

European Committee on Antimicrobial Susceptibility Testing (EUCAST)

Exopolysaccharides (EPS)

Extracellular matrix (ECM)

Extracellular polymeric matrix (EPM)

Fibrinogen binding protein A, B (Efb A, B)

Fibroblast growth factor (FGF)

Horizontal Gene Transfer (HGT)

Human microvascular endothelial cell line (HMEC-1)

Insulin-like growth factor-1 (Igf-1)

Interleukin 1 beta (IL-1β)

Keratinocyte growth factors (KGF)

Keratinocyte-derived anti-fibrogenic factor (KDAF)

Luria Bertani broth (LB)

Multidrug organisms (MDRO)

Methicillin resistant *S. aureus* (MRSA)

Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs)

Minimal inhibitory concentration (MIC)

Minimum bactericidal concentrations (MBC)

Minimum biofilm eradication concentration (MBEC)

Minimum biofilm inhibitory concentration (MBIC)

Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)

Muller Hinton (MH)

Next-Generation Sequencing (NGS)

Panton-Valentine leucocidin (PVL)

Phosphate-buffered saline (PBS)

Platelets derived growth factor (PDGF)

Polyhexamethyl biguanide (PHMB)

Polymorph nuclear neutrophils (PMNs)

Poly-Nsuccinyl-β-1, 6-glucosamine (PNAG)

Polysaccharide adhesin (PS/A)

polysaccharide intercellular adhesin (PIA)

Polyunsaturated Fatty Acids (PUFA)

Pyrogenic toxin super-antigens (PTSAgs)

Quorum Sensing (QS)

Reactive oxygen species (ROS)

Real Time quantitive Polymerase chain reaction (RT-qPCR)

Single nucleotide polymorphisms (SNPs)

Standard Deviation (SD)

Toxic shock syndrome toxin-1 (TSST-1)

Transforming growth α and β (TGF-α -β)

Tryptic soy broth (TSB)

Tumour Necrosis Factor α (TNF-α)

Vancomycin-resistant enterococcus (VRE)

Vascular endothelial growth factor (VEGF)

Abstract

Wound infections present major health problems worldwide. It has been revealed that bacterial biofilm communities frequently colonise chronic wounds.Biofilms with Multidrug-resistant organisms (MDRO) such as Methicillin Resistant*Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*are increasingly implicated in chronic wound infections. The limited therapeutic options are further compromised by the fact that wound-infecting bacteria often co-exist within a polymicrobial biofilm community, which enhances bacterial tolerance or resistance to antimicrobials.The polyunsaturated fatty acid (PUFA) compounds including Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) offer both immunomodulatory and antimicrobial properties that could enhance wound healing.

This study evaluated the activity and efficiencyof these compounds against wound pathogens and compared them to common antibiotics Linezolid (LZD) and vancomycin (VAN).Standard antibiotic sensitivity assays were used to determine the ability of PUFAs to inhibit growth and biofilm formation. Most activity was observed against *S. aureus*strains. The bacteriostatic activity (minimum inhibitory concentration - MIC) of DHA and EPA for *S. aureus*SA3 and *P. aeruginosa* PA01 was 65 and 117 $\mu g ml^{-1}$respectively. Meanwhile, the bacteriocidal activity required higher concentrations (MBC) of 260 and 468$\mu g ml^{-1}$respectively. The antibiotic LZD was most active against Gram positive bacteria (MIC range from 1.9 $\mu g\ ml^{-1}$to 3.9 $\mu g\ ml^{-1}$). However, vancomycin was the most potent biofilm disrupter, with MBECs ranging from 5-12.5 $\mu g\ ml^{-1}$. These concentrations were 35-fold lower than the MBECs recorded for linezolid (125 -500 $\mu g\ ml^{-1}$). Considerably higher concentrations of PUFA compounds were required to disrupt biofilms. The MBECs of DHA ranged between 750 and 3125 $\mu g\ ml^{-1}$. EPA was found to be more disruptive than DHA (MBECs ranging

between 500 and 2250 µg ml^{-1}), while the MBECs of combination treatment with both PUFAs (DHA+EPA) ranged from 750 - 1500 µg ml^{-1}.

The antimicrobial activity of different concentrations of PUFAs was further assessedby monitoring inhibition of growth of a clinical wound isolate of *S. aureus*(SA3)for 24 h. PUFAs were shown to differentially affect expression of global gene regulators using RT-qPCR.DHA and EPA were found to upregulate agrA and *icaA* butdownregulate *sarA*at the same concentrations and exposure times.

Two different infection models were used to assess the activity of PUFAs *in vivo*(*Galleria mellonella*) and on *ex-vivo* skin compared to common antibiotics (LZD and VAN). PUFAs reduced virulence of SA3 in *G.mellonella,* protecting the larvae from death. Furthermore, prolonged exposure to sub-inhibitory concentrations affected growth efficiencyof SA3*in vivo.*Whole genome sequencing of parent vs PUFA and antibiotics-adaptedstrains of *S. aureus*SA3 revealed some common mutations in *sarA* and *clfB*genes, that may contribute to altered virulence and susceptibility to treatment. Preliminary studies with the *ex-vivo* skin model suggested that this may be a useful system for testing the efficacy of antimicrobial compounds for wound-treatment. Differences in SA3 survival and growth were measurable in the presence of PUFAs or absence of PUFAs.

In conclusion, PUFAs (DHA and EPA) showed weak antimicrobial activity compared to common antibiotics against common wound-associated bacteria. However, this study shows evidence that PUFAs have additional desirable properties, including disruption of global gene regulation, reduction of virulence and low potential to promote development of resistance.

Chapter One

Introduction

Chapter 1 Introduction

1.1 Pathology of chronic wounds

1.1.1 Definition and epidemiology of chronic wounds

Skin is the body's largest organ and is the first line of the innate immune system. The surface of the skin is colonised by complex microbiota that form a dynamic barrier to compete with potential pathogens (Rosenthal *et al*., 2011). Usually, the healthy skin microbiota, including potential opportunistic pathogens, do not cause infection or stimulate the immune system, unless there is disorder of the skin structure or the immune system becomes compromised. When the skin barrier is breached, any of the microorganisms' present can cause a wound or skin infection (Stevens *et al*., 2014).

A wound is a type of injury (trauma) that cause an acute disturbance of tissues by an external force in which skin is torn, cut, or punctured (open wound), or where strong force trauma causes a contusion (a closed wound). Wounds are usually classified according to the etiological agent and anatomical site, whether it is acute or chronic or according to presentation of symptoms or appearance of tissue type in the wound location (Seidel *et al*., 2013). An acute wound is by definition expected to progress through the stages of normal healing, resulting in closure of the wound (Guo & Dipietro, 2010). However, without proper dressing and wound care management the acute wound can develop complications, poor healing and infection. Any acute wound can progress to a chronic wound if it does not heal within the expected period, because of poor blood supply, oxygen, nutrients or hygiene (Anderson & Hamm, 2014). Acute wounds should be promptly treated to avoid infection, inflammation or progression to a chronic, un-healing wound (Seidel *et al*., 2013). The most common definition that has been used for chronic wounds, is that of any wound that has not

healed within 3 months of the initial trauma. Some ulcers have been observed to persist for years (Iqbal *et al*., 2017).

There are three main types of chronic wound: venous leg ulcers, diabetic ulcers, and pressure ulcers. These ulcers are usually a gradual disturbance of tissues caused by triggers such as diabetes or high cholesterol. Slow blood flow in veins (ischemia) leads to secondary physiological changes due to lack of nutrients and oxygen supply to the tissue. This can lead to tissue damage and impaired healing (Avishai *et al.*, 2017). Classical signs of chronic wounds include the development of necrotic tissue, foul odour and a cumulative breakdown of the tissue structure (Kane *et al.*, 2014). A deterioration and discoloration of granulation tissue and increased fragmentation in the site of infection are also often described (Leaper*et al*., 2015).

Many risk factors are associated with the development of chronic wounds, such as diabetes, arterial diseases, microbial infection, and old age (Frykberg & Banks, 2015). The increasing elderly population, growing incidence of diabetes and obesity and cardiovascular diseases are significant contributions to the increasing incidence of chronic wounds (Avishai *et al*., 2017). For instance, leg ulceration affects 15–18 per 1000 adults in developed countries (Agale, 2013). The estimated annual cost of wound management (Dressing, nursing, practicing, and patient visit) to the NHS in the UK is between £4.5 and £5.1 billion (Guest *et al*., 2015).

1.1.2 Stages of wound healing

Normal wound healing is a dynamic process consisting of four continuous, overlapping, and programmed phases: (i) haemostasis; (ii) inflammation; (iii) cell differentiation and proliferation to the wound site; (iv) maturation and remodelling (Gosain & DiPietro, 2004; Mathieu *et al*., 2006). Briefly, coagulation at the site of injury leads to formation of platelet thrombus, followed by haemostasis, with vascular constriction and formation of fibrin clot. Bleeding is controlled, and the inflammatory phase is promoted by infiltration of polymorphonuclear cells (PMNs), macrophages and lymphocytes (Beldon, 2010; Campos *et al*., 2008). A major function of PMNs is as a first defence barrier against invasion of microorganisms, although they produce proteases and reactive oxygen species (ROS) that cause additional parallel damage (Dunnill *et al*., 2017). Macrophages are also important mediators of wound healing by secreting pro-inflammatory cytokines (Figure 1), such as Tumour Necrosis Factor α (TNF-α) and interleukin 1 beta (IL-1β), that stimulate the production and release of matrix metalloprotease (MMP) enzymes and enhance the production of growth factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF). The second function of phagocytic cells is the engulfment of the microorganisms and apoptotic cells. Phagocytic cells undergo a transition to a reparative state that stimulates keratinocytes, fibroblasts, and angiogenesis to regulate tissue regeneration (Meszaros *et al*., 2000; Mosser & Edwards, 2008).

The proliferative phase of healing is characterised by synthesising collagen as well as glycosaminoglycan and proteoglycan, which are major components of the extracellular matrix (ECM) (Teller & White, 2009). This phase, involves modulation of immune response and T-lymphocytes migrate to the wound site and reach their peak activation during the late-proliferative/ early-remodelling phase (Ghatak *et al*., 2015). The activity of T-lymphocytes

within an inflammatory exudate is reflected in the relative proportions of T-lymphocyte subsets such as CD4+ T helper cells and CD8+ T suppressor cytotoxic cells. These T lymphocytes modulate the wound cytokine environment and regulate keratinocyte, fibroblast and endothelial cell function (Dunnill *et al*., 2017; Toben & Baune, 2015). Three distinctive cell types; fibroblasts, endothelial cells, and keratinocytes are supported by new blood vessel growth via vascular endothelial cell formation (angiogenesis). These mature cells are activated by transformed keratinocytes and produce fibroblast growth factor 7 (FGF-7), keratinocyte growth factors (KGF), and insulin-like growth factor-1 (Igf-1), to support keratinocyte proliferation and cell survival. The fibroblast cells are under the control of cytokines and growth factors that are released from platelets and macrophages. Keratinocytes synthesize other growth factors transforming growth (TGF-α -β), and keratinocyte-derived anti-fibrogenic factor (KDAF), while endothelial cells synthesize vascular endothelial growth factor (VEGF), basic fibroblast growth factor (BFGF). The interaction between these growth factors and re-epithelialization of the epidermis over the extracellular matrix contributes to the maintenance of normal skin and wound healing.

The final stage of wound healing is the remodelling phase. The critical feature of this phase is ECM rebuilding the architecture of normal tissue (Figure 1.2). This process reduces cell migration and allows cell differentiation (Diegelmann & Evans, 2004). Several enzymes are produced during the remodelling phase such as hyaluronidase, plasminogen activators, collagenase, and elastase. Hyaluronidase plays a role in epidermises-epithelialization and synthesis of extracellular matrix (Fronza *et al*., 2014). Plasmin, formed from plasminogen, degrades fibrin and urokinase, produced by fibroblasts, endothelial cells, keratinocytes, and leukocytes, activates collagenase and elastase (Clark *et al*., 2007). Collagenase is secreted by macrophages, fibroblasts, epithelial cells, and leukocytes and can break down the collagen

triple helix. Collagenase activity has been detected at the tissue recovery site for many months (Campos *et al.*, 2008; Gosain & DiPietro, 2004).

The interruption and failure of wound healing leads to the development of chronic wounds (Figure 1.3). The major factors that prevent wound healing are ischemia, bacterial colonisation, inflammation and oxidative damage (Guo & Dipietro, 2010). Ischemia is caused by narrowing of the blood vessels in tissues and is common in old age or individuals with diabetes. This causes tissue to become inflamed and release interleukins, chemokines and complement factors that attract PMNs (Iqbal *et al*., 2017; Turner *et al*., 2014). Further complications can be caused by microbial colonisation of the wound attracting a greater number of PMNs (Sorg *et al*., 2017). The PMNs release inflammatory mediators such as degrading proteases, collagenase, and reactive oxygen species (ROS) (Falanga, 2000). ROS and enzymes such as peroxidase are part of the immune response important for killing invading pathogens. However, they have also been shown to prevent host cell proliferation and wound closure (McCarty & Percival, 2013). ROS are also the main cause oxidative stress in the skin. Excessive production of ROS leads to sustained stimulation of pro-inflammatory cytokines and induction of matrix metalloproteases. This can activate proteolysis, degrading extracellular matrix proteins and decreasing the integrity of connective tissue. Other effects include interference with growth factors and epithelial cell proliferation, impaired dermal fibroblast and keratinocyte function and continuing influx of PMNs (Dunnill *et al*., 2017). During the ischemic period, depletion of oxygen and nutrients results in poor blood circulation, causing further damage and wound chronicity (Schultz *et al*., 2003).

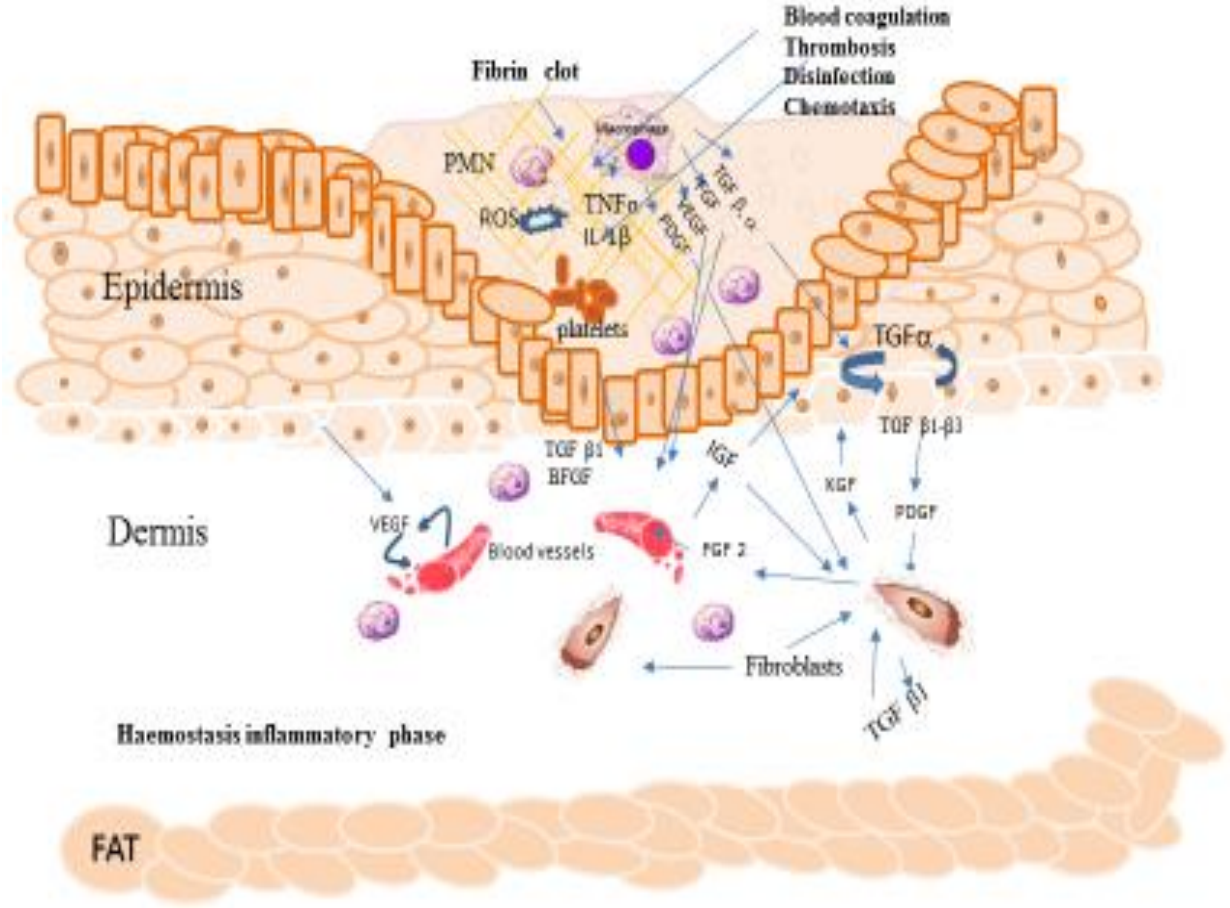


Figure1.1 Initiation of wound healing: Blood coagulation by thrombosis, inflammatory response by release the PMNs mediators degrading proteases. Collagenase, and excessive production reactive oxygen species (ROS)leads to sustained stimulation of pro-inflammatory cytokines and induction of matrix metalloproteases, activate proteolysis, secretion of growth factors such as TGF-b and PDGF and epithelial cell proliferation. Neutrophils and Macrophages clean, ingest cellular debris and the invading bacteria, Anti-inflammatory macrophages are cleared by migration to lymphatic vessels, impaired dermal fibroblast and keratinocyte function and continuing influx of PMNs (Singer & Clark, 1999).

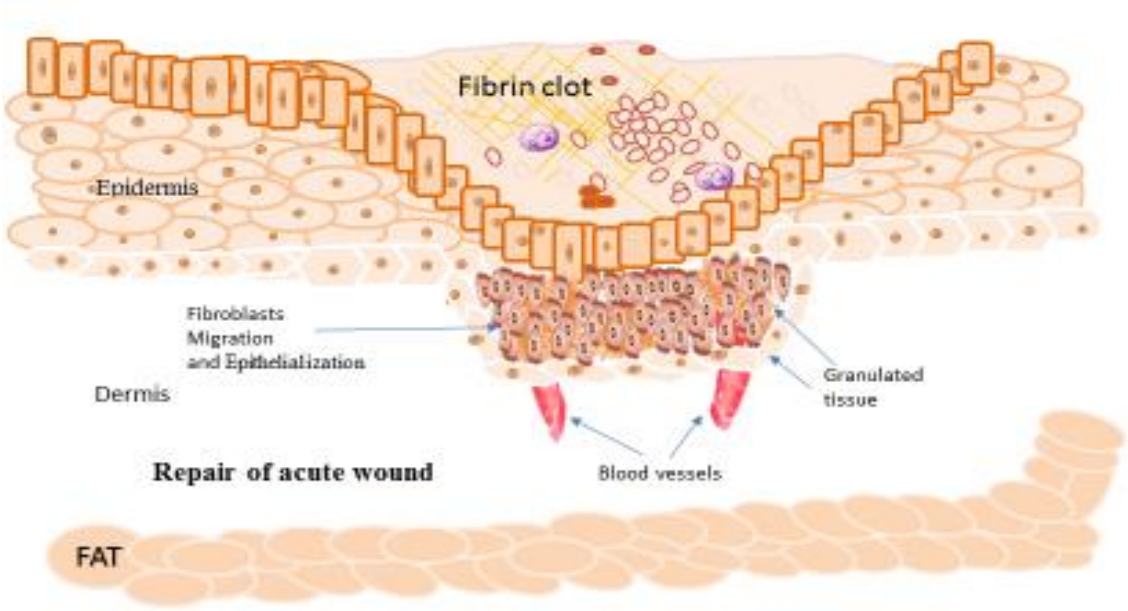


Figure1.2 Rebuilding normal tissue: maintenance of fibrin clot, restoration of tissue architecture by extracellular matrix, rebuilding of new blood vessels, fibroblast and keratinocyte migration to start re-epithelialisation and proliferation in wound healing.

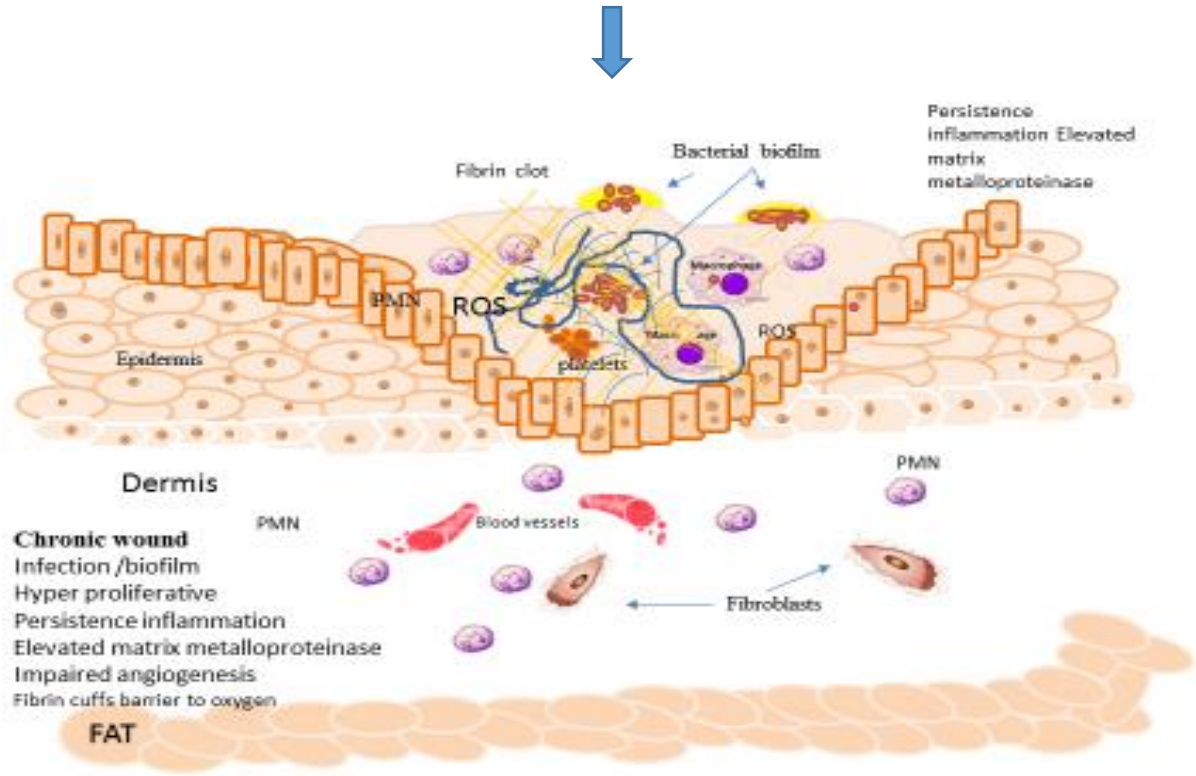


Figure1.3 Chronic wound: characterised by persistent inflammation. Macrophage response triggers cellular signalling by excessive secretion of ROS, cytokines, growth factors, and Matrix metalloproteinases (MMPs). Bacterial colonisation and biofilm formation, and or ischemia occur. All these factors contribute to a delay in healing.

1.1.3 Microbial burden of wounds

All chronic wounds are usually colonised by microorganisms from the normal skin microbiota (McCarty & Percival, 2013) or from environmental sources such as air, water, sand, bullets, medical instruments or staff (Gardner & Frantz, 2008). There is a distinct difference between wound contamination and wound colonisation. The term contamination refers to microbial attachment but no proliferation. In contrast, colonisation requires attachment of microorganisms at the wound surface followed by proliferation, without clinical signs of host injury (Leaper *et al*., 2015; Sibbald, 2011). The wound environment provides favourable conditions of warmth, moisture and nutrition for easy colonisation of opportunistic pathogens. Therefore non-healing wounds are mostly colonised by polymicrobial communities (Bjarnsholt *et al*.,2011; Leaper *et al*., 2015; Phillips *et al*., 2016). Some microorganisms have been shown to excrete substances, such as bacteriocins, within the wound that will reduce or inhibit the growth of others. For example, *Escherichia coli* secrets colicins that will inhibit growth of closely related strains (Johnson *et al*., 2013).

Following colonisation of a wound, heavy proliferation of pathogens and production of virulence factors such as adhesins and surface-associated endotoxins (of Gram negative bacteria) or exotoxins can overcome the immune response, causing significant damage to host tissue and resulting in infection (Galán, 2005; Wilson *et al*., 2002). According to Hamed *et al*, (2015) and Agren, (2016) the microbial burden of $> 10^5$ CFU g^{-1} of tissue is considered to indicate infection (Agren, 2016; Hamed *et al*., 2015). Several classical signs of wound infection have been identified (Table1.1). An infected wound will usually have the appearance of abnormal granulation tissue with changes in colour due to inflammatory state and increased exudate from abscesses around the infected site (McCarty & Percival, 2013). Most practitioners depend on clinical symptoms to confirm wound infections, especially increasing pain and wound tissue damage (Iqbal *et al*., 2017).

Table1.1 Development and characterisation of infection in chronic wound (Leaper et *al*., 2015).

Abnormal granulation tissue (excessive 'vascular' hyper granulation)
Bleeding from friable granulation tissue at the wound surface
Epithelial bridging and pocketing in granulation tissue
Wound breakdown and enlargement
Changes in the wound bed colour from red to green/ yellow or black
Increasing inflammatory signs and abscess formation
Increasing pain
Increasing odour
Increased exudate and maceration of surrounding skin
Delayed healing (beyond expectation)

1.1.4 Bacteria commonly isolated from chronic wounds

Chronic wounds are usually co-infected with multiple bacterial species (Table 1.2) and several studies have demonstrated their complex ecology (Wolcott *et al*., 2013; Zhao *et al*., 2013). Traditional microbial culture techniques have found a mean number of bacterial species per wound ranging from 1.6-4.4 (Howell-Jones *et al*., 2005). *Staphylococcus aureus* and coagulase negative *Staphylococci* (CoNS) have been the predominant organisms isolated from both prospective, purpose-collected samples, and retrospective analysis of clinical investigations (Zhao *et al*., 2013). However, molecular techniques such as 16srRNA profiling have revealed a more diverse polymicrobial nature of chronic wounds with approximately four times more diversity of species in wounds than isolated by culture (Lavigne *et al*., 2015). The dynamics of polymicrobial communities colonising chronic wounds changes over time.

S. aureus, CoNS, *Streptococcus spp*, and *Corynebacterium spp*, are primary colonisers before the accumulation of facultative anaerobic Gram-negative bacilli, such as *Escherichia coli*, *Enterobacter cloacae, Klebsiella spp,* and *Proteus spp. P. aeruginosa* and anaerobes such as *Bacteroides fragilis and Propionibacterium acnes* have been identified later, usually days to weeks later (Bessa *et al*., 2013; Martin *et al*., 2010).

A major problem with wound species profiles reported so far is the incomplete isolation of anaerobes, which is both difficult and time consuming by traditional culture method (Charles *et al*., 2015). For example, anaerobic bacteria were not thought to be present in 73% of non-infected wound ulcers (Bowler & Davies, 1999) and 82% of infected leg ulcers. However, more thorough studies that focused on the isolation of anaerobic bacteria, have yielded a high frequency (Zhao *et al*., 2017). 16S rRNA profiling and metagenomic approaches are increasingly useful for exploring such complex bacterial communities including anaerobic bacteria (Salipante *et al*., 2013; Sprockett *et al*., 2015). *S. aureus* and *P. aeruginosa* are frequently found together in chronic wound infections (Trivedi *et al*., 2014). For example, a retrospective study was carried out on 217 infected wounds that were found to have polymicrobial infections. *S. aureus* and *P. aeruginosa* were found together in 59 (27·1%) of the samples (Bessa *et al*., 2015a). Another study showed that the predominant organisms in chronic wounds from both surface swabs and tissue biopsies were *Staphylococcus spp*. and *P. aeruginosa* present in 71.2% of patients (Davies *et al*., 2007). Gjødsbøl *et al*., (2006) found *S. aureus* in 93.5% and *P. aeruginosa* in 52.2% of chronic venous leg ulcers, making these species most common and significant for the nature of bacterial communities in wound (Gjødsbøl *et al*., 2006).

Table1.2 The most common pathogens detected in wounds (Bessa *et al*., 2015; Yali *et al*., 2014).

Group	**Organisms**
Gram-positive bacteria	***S. aureus*** ***S. aureus* (methicillin resistant)** *Coagulase Negative S. aureus (CoNS)* *Streptococcus pyogenes* ***Enterococcus faecalis*** *Corynebacterium spp*
Gram-negative bacteria	***Pseudomonas aeruginosa*** *Acinetobacter baumannii* ***Escherichia coli*** *Klebsiella species* *Proteus species* *Enterobacter spp*
Anaerobic bacteria	*Clostridium species* *Bacteroides species* *Propionibacterium acnes*
Fungi	*Candida spp*

1.2 Defining characteristics of *Staphylococcus aureus*

Staphylococci are Gram positive bacteria usually arranged as cluster of coccoid cells. These facultative anaerobes are nonmotile, and non-spore forming. They are members of the family *Micrococcaceae* consist of more than 47 species and subspecies (Becker *et al*., 2014). These opportunistic bacteria commonly colonise human skin and mucosal membranes as commensal normal flora (Schmidt *et al*., 2014). The staphylococci produce catalase enzyme, a feature differentiating them from *Streptococcus spp*. and they are oxidase-negative and require complex nutrients, e.g., many amino acids and vitamins B, for growth. *S. aureus* is known to be tolerant of high concentrations of sodium chloride (Choi *et al.*, 2014). Another feature of the genus *Staphylococcus* is the cell wall peptidoglycan structure that contains multiple glycine residues in the cross bridge, which causes susceptibility to lysostaphin (Bastos *et al*., 2010). *S. aureus* can be distinguished from CoNS using a coagulase test. *S. aureus* produces coagulase

which interacts with prothrombin in the blood causing plasma to coagulate by converting fibrinogen into fibrin.

Antibiotic resistance is widespread amongst *S. aureus*. The incidence of methicillin resistant *S. aureus* (MRSA) associated wound infection has been well published (Almeida *et al*., 2014; Bowling *et al*., 2009). Reports revealed that 25%-50% of all hospital *S. aureus* infections in Europe were resistant to methicillin (Johnson, 2011). MRSA strains express a modified penicillin-binding protein encoded by the *mec A* gene which has lower binding affinity forβ-lactam antibiotics (Taitt *et al.*, 2015). Over the past three decades MRSA strains have become a major public health concern commonly causing health care (HA-MRSA) and community acquired (CA-MRSA) infections (Montgomery *et al*., 2010). The CA-MRSA are distinguished genetically by their ability to produce toxins such as Panton-Valentine leucocidin (PVL) which kills the leucocytes, contributing to recurrent infections such as boils and abscess (David & Daum, 2010). The *mec A* gene is a major determinant of methicillin resistance in both *S. aureus* and CoNS (Shrestha *et al*., 2002). This gene has been used for rapid identification of methicillin resistant *S. aureus* (MRSA) (Appelbaum, 2007). Vancomycin resistant *S. aureus* (VRSA) are also developing as an increasing burden on public health (Akhi *et al*., 2017; Taitt *et al*., 2015). The *S. aureus* genome is dynamic and includes a number of mobile genetic elements, such as transposons, pathogenicity islands, insertion sequences, and a staphylococcal cassette chromosome (Alibayov *et al*., 2014; Lindsay, 2010). These elements of horizontal gene transfer are prevalent amongst staphylococci and many carry antibiotic resistance genes and virulence factors. For example, study analysing the genotypic pattern for *S. aureus* isolated from wound infection found the typical staphylococcal cassette chromosome *mec IV* elements in 88% of isolates. Panton-Valentine leukocidin virulence genes were identified in 88% of isolates (Murray *et al*., 2010). In another study, clinical isolates were investigated for resistance to methicillin encoded by

the *mecA* gene carried on a mobile staphylococcal cassette chromosomal mec element (SCCmec). These genes were present in up to 49% of non-device associated infection and all biofilm-positive isolates except four harboured *ica* ADBC genes (Mesrati *et al*., 2018). The *ica*ADBC operon is responsible for the synthesis of PIA, the main component of staphylococcal biofilm (Cramton *et al*., 1999).

1.2.1 *Staphylococcus aureus* infection

S. aureus is the most common bacterium isolated from both acute and chronic wounds (Bessa *et al*., 2015; Gjødsbøl *et al*., 2006), but is also capable of causing a wide range of other infections (Das *et al.*, 2016; Tong*et al*., 2015). This opportunistic pathogen frequently colonises the human skin and is present in the nasal cavity of 25-30% of healthy people without causing harm to the host or any symptoms (Bhattacharya *et al*., 2015). However, if there is a defect in the human skin from a wound, surgery or indwelling device such as intravenous catheters; or if there is a suppression of the immune system, then colonising *S. aureus* can cause an infection (Foster *et al*., 2014). Transmission of *S. aureus* can occur through close contact from both infected individuals and carriers to other healthy subjects through poor hand hygiene or through sharing contaminated objects, such as towels or razors. *S. aureus* can cause localized skin infections, such as folliculitis and impetigo (Dhawan *et al*., 2015). It can also cause abscesses and spread into blood stream (bacteraemia or sepsis) (Fowler *et al.*, 2006), pneumonia (Gillet *et al*., 2002), bones (osteomyelitis) (Elasri *et al*., 2002), and damage heart valves (endocarditis) (Fernández Guerrero *et al.*, 2009). The ability of *S. aureus* to form a biofilm matrix enables this organism to resist the host immune response protecting it from opsonisation or phagocytosis and antimicrobial agents (Snowden, 2016).

1.2.1.1 Pathogenesis of *S. aureus*

S. aureus coordinates several mechanisms to colonise and survive *in vivo.* A wide range of cell surface-associated adhesins, secreted cytotoxins and enzymes have been described, as well as methods of escape from the host immune system (Das *et al*., 2016). The fact that *S. aureus* has the ability to cause such arrange of different infections is partly due to the expression of different cell wall-associated proteins and extra-cellular virulence factors which enable the bacteria to attach to and colonise host tissues (Pietrocola *et al*., 2017). The ability of bacteria to adhere to the host surface is a crucial first step to initiate infection and is essential for biofilm formation. *S. aureus* possess a group of microbial adhesins referred to Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMMs) that help the bacterium to bind antibodies and extracellular proteins such as fibrinogen, fibronectin and collagen (Foster *et al*., 2014). The MSCRAMMs can coat the bacteria surface thereby evading detection by immune defences. A biofilm-associated protein (BAP) has also been found to be involved in *S. aureus* adhesion and biofilm formation (Lister & Horswill, 2014). Collagen Binding Protein (CAP), is also essential for adherence of *S. aureus* to collagenous tissues and cartilage (Hauck & Ohlsen, 2006). It has been shown that anti-collagen antibodies block the bacterial attachment to those tissues (Speziale *et al*., 2018). Plasma-sensitive proteins (Pls), on the *S. aureus* surface are processed by plasmin, and contribute to binding to both fibrinogen and fibronectin (Crosby *et al*., 2016). Surface protein A binds to von-Willebrand factor, a blood glycoprotein that contributes to haemostasis at sites of endothelial damage, contributing to endovascular diseases by *S. aureus* (Hartleib *et al*., 2000). *S. aureus* infections associated with implanted biomedical devices rely on the ability of this pathogen to form biofilms on the surface of the biomaterial (Lister & Horswill, 2014; McAdow *et al*., 2012).

S. aureus produces an array of exotoxins, such as haemolysin, leukocidin, kinases, coagulase and hyaluronidase, that damage host tissues, destroy leukocytes or stimulate symptoms of disease such as Toxic shock syndrome (TSS) (de Haas *et al*., 2004; Rooijakkers *et al*., 2005). These enzymes act locally to damage host cells providing nutrients for growth. Additional sets of exotoxins produced by *S. aureus* which include the pyrogenic toxin super-antigens (PTSAgs) toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins are able to stimulate proliferation of T-lymphocytes (Janik & Lee, 2015). Staphylococcal enterotoxin has been shown to cause symptomatic food poisoning disease even in the absence of viable bacteria (Dinges *et al*., 2000). However, Staphylococcal toxic shock syndrome (TSS) is only sufficient to cause symptomatic disease, if the toxin is secreted and concentrated at colonised sites (Kulhankova *et al*., 2014). Staphylococcal Scalded Skin Toxin SSS causes a skin disease mainly affecting children and immunocompromised patients (Brewer *et al*., 2008).

A number of *S. aureus* virulence factors are important for immune evasion, preventing recognition by antibodies and complement, and inhibiting phagocytic activation (Bien *et al* 2011; DeLeo *et al*., 2009). *S. aureus* produce capsular polysaccharides, protein A, coagulase and extracellular fibrinogen binding proteins (Efb A, B) all of which play a role in the inhibition of phagocytic engulfment. Coagulase interacts with fibrinogen to form fibrinogen clots through activation of prothrombin. Efb proteins stimulate fibrinogen to the bacterial surface and promotes clot formation that protects the bacterium from phagocytic clearance. These surface-associated proteins also enhance attachment to extra-cellular matrix component (McAdow *et al*., 2012). Clumping factor, A and B (ClfA and ClfB) mediate clumping and adherence of bacterial cells to fibrinogen in the presence of fibronectin. Clumping factors are thought to play a significant role in wound infections and it has been shown that a*clfA* mutant was less virulent than the wild type isogenic strain (Foster *et al*.,

2014). Moreover, *S. aureus* possesses a cell wall anchored enzyme known as adenosine synthase A (AdsA enzyme), that converts adenosine monophosphate (AMP) to adenosine, which has immune suppression properties (Thammavongsa *et al*., 2011). Protein A, encoded by the *spa* gene is cell wall-associated and binds to the Fc domain of immunoglobin G (IgG) in the "wrong orientation" on the surface of *S. aureus* cells which is thought to disrupt opsonisation and phagocytosis (Switalski *et al*., 1993).

1.2.2 *Enterococcus faecalis*

E. faecalis is a Gram-positive cocci, that are common inhabitants of the human intestinal. They are opportunistic pathogens that infected urinary tract infections, blood-stream, surgical sites, and wounds (Yali *et al*., 2014). Enterococcal infections are polymicrobial in nature, often biofilm-associated, and resistant to antibiotics. Several virulence factors have associated enterococcal infection such as gelatinase production, *Enterococcus* surface protein (Esp), aggregation substance (AS) and biofilm formation (Comerlato *et al*., 2013). The gelatinase is a metalloprotease, encoded by *gelE,* that is capable of hydrolysing gelatine, collagen, haemoglobin and other peptides (Lindenstrau *et al.*, 2011). The Enterococcus surface protein (Esp) protein, encoded by the *esp* gene, contributed to the colonisation and persistence of *E. faecalis* strains in several infections including wound (Rahimi *et al*., 2018). The protein Esp may mediate the interaction with primary surfaces and participate in biofilm formation. The aggregation substance (AS) is plasmid encoded by *prgB* gene that play role in cellular aggregation required for conjugative plasmid transfer and facilitates the adherence to contributes to biofilm formation (Wardal *et al*., 2013). In addition, *E. faecalis* encodes 2 sortase enzymes, SrtC and SrtA, that associated with surface cell wall structured and pilli (Mandlik *et al*., 2007). These components play major role in initial stage

of biofilm formation. The expression of the SrtA substrate aggregation substance along with biofilm formation, can evade and protect *E. faecalis* from macrophages and neutrophils. The antimicrobial Resistance of *E. faecalis* such as vancomycin-resistant *E. faecalis* (VREfs) are the most clinically significant in the genus. These resistance genotypes are commonly disseminated on the mobile elements Tn1546 like transposon *vanA* and Tn1549 conjugative transposon *vanB*, which are generally encoded on plasmids and chromosome, respectively (Zhong *et al*., 2017).

1.2.3 *Pseudomonas aeruginosa*

An opportunistic human pathogen that causes life-threatening infections in patients with cystic fibrosis (CF), endocarditis, wounds or artificial implants. In addition, *P. aeruginosa* is one of the most common pathogens in chronic wounds due to its ability to form resistant biofilms (Hotterbeekx *et al.*, 2017). It has been estimated that about ten percent of all hospital-acquired infections are caused by *P. aeruginosa*, and for immune-compromised patients the mortality rate ranges from 20 to 70 % (Parsons *et al*., 2007). *P.* aeruginosa contain a battery of virulence factors that help facilitate colonization of the burn wound and translocation through the vasculature and into the bloodstream; *P. aeruginosa* produce both extracellular and cell-associated virulence factors products (Kipnis *et al.*, 2006). The secreted virulence factors maintain the chronic wound in a persistent inflammatory phase. Exotoxins are secreted which bind to specific sites on the ribosomes in host cells, resulting in inhibited protein synthesis and cell death (Leid *et al.*, 2005). Several enzymes are produced by *P. aeruginosa.* For instance, proteases and phospholipases that disrupt host cell structures, and adhesins that allow for binding to epithelial cell receptors (Gellatly & Hancock, 2013). Bjarnsholt *et al* (2008) suggest that the virulence factor rhamnolipid plays an important role

in chronic *P. aeruginosa* infections. Rhamnolipids destroy neutrophils, resulting in leakage of several different substances with harmful effects on both tissue cells and bacteria (Bjarnsholt *et al*., 2008). On the outer surface of the bacterial cell are lipopolysaccharides deposited, which contribute to attachment to host cells but also stimulate inflammatory responses (Kipnis *et al* 2006). Other cell surface structures such as flagella also allow for adherence to endothelial cells. Another important virulence factor for *P. aeruginosa* is the toxic blue pigment phenazine pyocyanin, which is a redox-active toxin that causes cellular destruction, increased expression of IL-8 and disruption of calcium homeostasis (Winstanley & Fothergill, 2009).

The ability of *P. aeruginosa* to form biofilms is critical for its survival in wound infection and enhances its resistance to antimicrobial treatment and host defense mechanisms (Meluleni *et al.*, 1995). For instance, *P. aeruginosa* PA 01 produce at least three exopolysaccharides alginate, Psl, and Pel polysaccharides, that play major role in biofilm formation and resistance to antimicrobial agents (Periasamy *et al*., 2015). One third of *P. aeruginosa* clinical isolates are resistant to three or more antibiotics, including third-generation cephalosporins and imipenem, which have been the gold standard antibiotics for *P. aeruginosa* infection (Hirsch & Tam, 2010).

1.2.4 *Escherichia coli*

Gram-negative rods ,facultative anaerobic, normal flora of the lower intestine and are part of the normal microbiota of the gut. Most *Es. coli* strains are non-pathogenic, and can assists in digestion by producing vitamins B12 and K2 (Blount, 2015). There are more than 700 different serotypes of *Es. coli*, distinguished by different surface proteins and polysaccharides (Kumar *et al*., 2015). Many serotypes can cause variety of infection such as

urinary tract infection, bacteraemia , neonatal meningitis, soft tissue infection and wound infection. However, various virulence factors associated structures or produced by *Es. coli* such as Pfimbriae, type I fimbriae, capsule, siderophores, haemolysin, proteases and cytotoxic necrotizing factors (Kumar *et al*., 2015). These bacterial toxin production in wound infection, causing collagen degradation, stress, and malnutrition, preventing the normal healing of wounds. In addition, pathogenic *Es. coli* produce biofilm on acute wounds to turn into chronic infection. The biofilm structure is significant factor that contributes to antibiotic resistance. Estimates spectrum beta lactamase (ESBL) are encoded on plasmids on *Es. coli* and other member of *Enterobactericeae* are typical example of antibiotic resistance in wound infection (Palzkill, 2018).

1.3 Bacterial Biofilms and Implications in chronic Wound Infections

The microbial populations of infected wounds are typically biofilm associated and have shown significant resistance to antimicrobial agents (James *et al*., 2008; Zhao *et al*., 2013).

1.3.1 Biofilm formation

A biofilm is a complex and well-coordinated structure of adherent microorganisms surrounded by an extracellular polymeric matrix (EPM). The EPM is mainly composed of secreted polysaccharides, proteins and extracellular DNA (Bhattacharya *et al*., 2015). The phenotypic and biochemical properties of bacteria within biofilm matrices differ considerably from planktonic cells. They are more resistant to immune responses and antimicrobial agents and are, therefore, strongly associated with persistent infections (Sadekuzzaman *et al*., 2015).

The process of bacterial biofilm formation is complex and requires coordinated microbial activities, to pass through several stages Figure 1.4). The initial stage involves the reversible attachment of cells to a surface. Stronger adhesion then takes place via specific bacterial surface structures such as pili or lipo-teichoic acid and polysaccharides**.** Multiplication of bacterial cells occurs at the site of adhesion (Cramton *et al*., 1999). This increased cell density enables cells of immature biofilms to respond to quorum sensing signals with global changes in gene expression (Atshan *et al*., 2013). During these early stages, attached bacteria modify the expression of key genes involved in adhesion and motility. In particular, the production of flagella and pili and secretion of exopolysaccharides (EPS) are affected, resulting in more firm "irreversible" attachment and development of biofilm microcolonies. Maturation of biofilm architecture occurs over several days, with the formation of columns and channels through which nutrients and toxic metabolites can flow. This structure enables maintenance of polymicrobial communities within the matrix; as the biofilm develops, different micro-niches are established with varying oxygen and pH gradients (Koo & Yamada, 2016). Eventually, the mature biofilm will breakdown, followed by dispersion of single cells from the matrix (Kostakioti *et al*., 2013). Each stage of biofilm formation has distinguishing characteristic features depending on the species involved and each requires a complex network of regulatory systems to co-ordinate expression of specific adhesins, secretion, metabolism and signalling (Fleming & Rumbaugh, 2017).

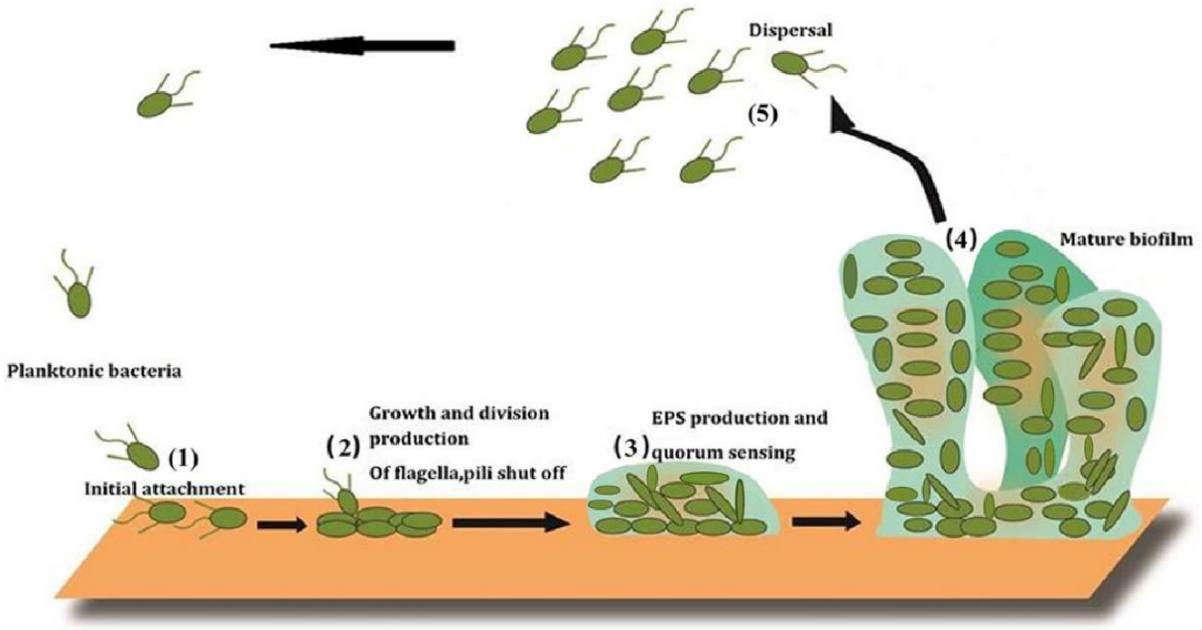


Figure1.4 **Development of biofilm formation** (Sadekuzzaman *et al*., 2015). The biofilm formation passes via several stage 1) attachments and adhesion to surface. 2) Bacterial multiplication and irreversible mobility 3) EPS and quorum sensing production4) Biofilm growth and mature 5) planktonic cell dispersal.

The key components of a biofilm matrix are exopolysaccharides. For example, the production and secretion of alginate exopolysaccharide, encoded by *alg* genes, is thought to be one the main survival strategies of *P. aeruginosa* in hostile environments. Alginate provides the bacteria with a selective advantage for colonisation, through increased resistance to opsonisation and phagocytic engulfment and binds many cationic antibiotics such as aminoglycosides (Fleming & Rumbaugh, 2017; Høiby *et al*., 2010a; Nivens *et al*., 2001). Other studies have shown an important role for capsular polysaccharide adhesin (PS/A) and intercellular polysaccharide adhesin (PIA) or poly-N succinyl-β-1, 6-glucosamine (PNAG), in biofilm formation by *S. aureus*, whose synthesis is encoded by the *ica ABCD* operon (Anjuman *et al*., 2014; Nourbakhsh & Namvar, 2016). *S. aureus* can develop at least two types of biofilm: intra cellular adhesion (*ica)*-dependent (promoted by the *ica* operon) and

ica-independent biofilm matrixes (Archer *et al*., 2011). The *ica* dependent biofilm formation plays an important role in persistent infections (Ghasemian *et al*., 2015)

The other main characterisation in biofilm formation is Quorum-sensing signals. For example, *S. aureus* have chromosomal locus responsible for QS is accessory gene regulator (*agr*), RNA III, and *luxS* (Gupta *et al*., 2015; Le & Otto, 2013; Wang & Muir, 2016). The *agr* locus encodes a signalling circuit that both produces and senses the autoinducer, peptide (AIP). The *agr* system has been shown to contribute to virulence, biofilm formation and wound infection (Brackman & Coenye, 2015; Rutherford & Bassler, 2012). Agr-controlled pore-forming toxins, which includes γ-hemolysin,, Leuco toxin LukDE, LukAB and Panton Valentine Leukocidin (PVL) (Alonzo *et al*., 2014; Cheung *etal*., 2011; Le & Otto, 2013). The *agr* locus functionality and phenol soluble modulin PSM production play a major role in disrupting interactions of biofilm matrix molecules, such as the polysaccharide intercellular adhesin (PIA), with the bacterial cell surface (Dastgheyb *et al*., 2015), and decrease the expression of several surface adhesions, including protein A and the fibronectin-binding protein (Otto, 2013). The global transcriptional regulator RNAIII acts to increase secretion of virulence factors genes such as phenol-soluble-modulin (PSM), which play role in immune system evasion especially in CA-MRSA (Fechter *et al*., 2014). In addition, the autoinducer (AI-2)which is synthesized by the LuxS enzyme in a metabolic pathway in *S. aureus*, the LuxS/AI-2 system play role in bacterial behaviours and infection including biofilm formation and virulence factors (Zhao *et al*., 2010). The composition of biofilms matrix often varies considerably between different bacteria strains and between different sites of infection by the same strain (Cue *et al*., 2012).

1.3.2 *Staphylococcus aureus* regulation genes

There is considerable variation between strains of *S. aureus*, which differ in the array of survival strategies and virulence factors that they express. For instance, the invasive *S. aureus* strain 6850 invades host cells and expresses a wide range of virulence factors that are absent in other strains that persist *in vivo* without causing inflammatory affects (Strobel *et al*., 2016). Over 100 transcriptional factors (TFs) and a wide range of sigma factors have been identified within the *S. aureus* pan genome and these have been classified into 36 regulatory families. However, less than 50% of the identified sigma factors have been experimentally characterised (Ibarra *et al*., 2013). Diverse *S. aureus* virulence and survival strategies are controlled by multiple inter-linking regulatory systems (Bronesky *et al*., 2016). *S. aureus* uses these regulatory systems to modulate the expression to adapt to changing environmental conditions such as sensing nutrient concentration, the antimicrobial agents, and the regulation of virulence factor expression including hemolysins, leukocidins, super antigens, surface proteins, and proteases (Liu *et al*., 2016). Two-component systems (TCSs) of *S. aureus* consists of transcriptional adaptation system membrane-bound histidine kinase (HK) and a cytosolic response regulator and often circuit regulate and affect each other's expression(Haag & Bagnoli, 2015). For example, *S. aureus* has encoded the BraSR/BraDE determinant that are two-component signal transduction systems and regulate bacitracin resistance. BraDE is involved in bacitracin sensing and signalling through BraSR, whereas the BraSR activate transcription of two operons encoding ABC transporters in presence of low concentration bacitracin, the two ABC transporters play significant role in Bacitracin antibiotic resistance (Hiron *et al*., 2011). In addition, *S. aureus* encoded VraSR two-component sensory regulatory system. Inactivation of vraS by cell wall inhibitor vancomycin, overexpressed several genes such as (two-component response regulator *vraR*, Penicillin binding protein 2*pbp2*, Peptide methionine sulfoxide reductase *msrA*), at least 1.5-fold genes

expression increase in different *S. aureus* strains after treatment with vancomycin, which also causes reduction in the levels of resistance to beta-lactam antibiotics and vancomycin (Gardete *et al*., 2006).

S. aureus co-ordinates global gene expression in response to density via a quorum sensing (QS) system named accessory gene regulator(agr) (Figure 1.3). The agr system is composed of two divergent transcriptional units, RNAII and RNAIII, which are driven by two P2 and P3 promoters, respectively. The RNAII locus contains four genes, *agrB, agrD, agrC and agrA.* Activation of the P2 promoter drives the expression of the components of the QS system (agrBDCA). The *agrD* and *agr B* combined in this system to control gene expression in a cell density-dependent fashion through the production an autoinducing peptide (AIP), which in turn acts as the ligand binding to the *agrC* receptor (Rutherford & Bassler, 2012). The *agrC* and *agrA* genes encode a two-component signal transduction system involving a histidine kinase sensor *agrC*, a transmembrane protein that is phosphorylated upon the binding of AIP, leading to dimerization and binding to the intergenic region between P2 and P3 promoters upregulating their expression (Wang & Muir, 2016). Expression from the P3 promoter drives the synthesis of RNAIII, the molecule RNAIII is the major effector of agr system, affecting many cell-wall-associated and extra-cellular proteins. RNAIII plays major role in controlling the production of the *hla* locus, which is one of the α-exotoxins produced by *S. aureus* during infection. Furthermore, RNAIII also has a role in mediating the switch from expression of surface proteins such as protein A (SpA) to enzymes and toxins such as alpha haemolysin and proteases (den Reijer *et al*., 2016).

The staphylococcal accessory regulator (*sarA*), family protein encodes at least 10 transcriptional regulators that initiate intricate molecular cascades (Gordon *et al*., 2013). Transcriptional gene patterns have demonstrated that *sarA* regulates (either directly or

indirectly) over 120 genes including up-regulation of the expression of extracellular proteins such as TSST-1, staphylococcal enterotoxin B, fibronectin binding protein and α- and β-hemolysins (Dunman *et al*., 2001). Sar A is a DNA-binding protein that positively regulates biofilm promotion factors, such as the biofilm-associated protein (bap) and the *ica* locus. The intracellular adhesion (*ica*) locus consists of the four genes ica ABCD, these genes encode proteins that mediate the synthesis of the polysaccharide intercellular adhesin PIA (Cramton *et al*., 1999). Furthermore, the accessory regulator *sarA* negatively regulates proteases and nucleases that can breakdown the biofilm matrix (Lister & Horswill, 2014). Expression of the *sarA*gene is increased under biofilm conditions compared with planktonic growth and *sarA* mutants are unable to form biofilms (Lister & Horswill, 2014). The *sarA* biofilm-negative phenotype is thought to be due to the over-expression of secreted proteases and nuclease as inhibiting these enzymes using chemical inhibitors restores *sarA* biofilm-forming capacity (Rowe *et al*., 2016).

The stress response sigma factor B (*sigB*) is also key for the control of virulence gene expression. Sigma *B*(*sigB*) is an alternate sigma factor that is necessary for stringent responses to conditions including high temperature, and oxidative or alkaline stress (Ahmed *et al*., 2016). In addition, it has known roles in virulence, intracellular survival, and persistence within the host. Loss of function mutations in *sigB* up-regulate the *agr* system and result in the inability of the strain to form a biofilm (Lauderdale *et al*., 2009). While the mechanisms behind these phenotypes are not completely understood, it is known that *sig B* is a negative regulator of secreted virulence factors, which is largely due to the repressive effect exerted on the *agr* system (Mootz *et al*., 2013; Pané-Farré *et al*., 2006).

At high bacterial cell density (Figure 1.3), AIP accumulates in the extracellular environment and triggers the agr QS circuit AIP binds to the *agrC* receptor, a membrane-bound histidine kinase activates the *agrC* kinase, resulting in

phosphorylation of the *agrA* response regulator and activation of the P2 and P3 promoters. The transcription of *sarA* activation or repression regulator ultimately affects RNAIII expression which is the effector molecule of *S. aureus* virulence. RNAIII expression can be grouped into four strategies: competitive inhibitors of *agrC* (the agr histidine kinase receptor), inhibition of *agrA* P2/P3 interactions, RNAIII transcription inhibitors, and transcriptional regulator. The RNAIII transcript yields a regulatory RNA molecule that acts as the primary effector of the agr system by up-regulating extracellular virulence factors and down-regulating cell surface proteins.

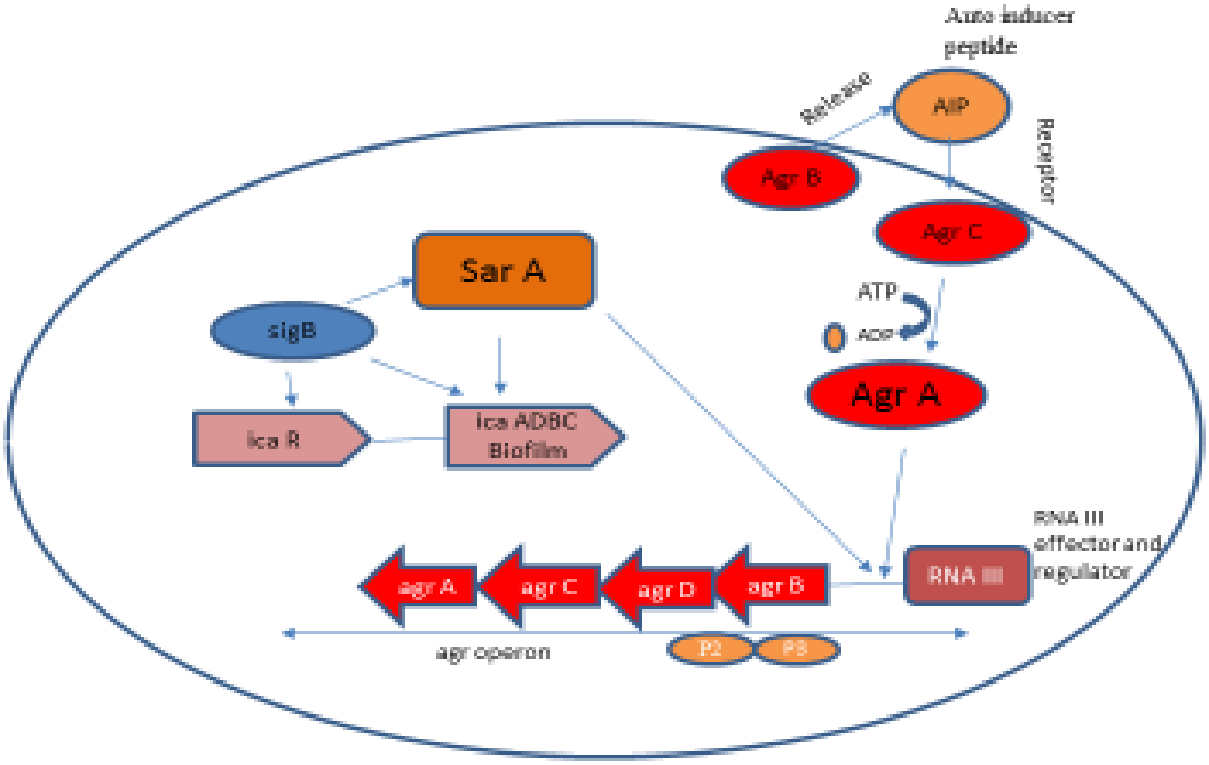


Figure1.5 Schematic of the agr system and the SarA proteins family in *S. aureus*. AIP binding activates the *agrC* kinase, resulting in phosphorylation of the *agrA* response regulator and activation of the P2 and P3 promoters. *sarA* contributes to the activation of *agr* expression and ultimately affects RNAIII expression. The *sigB* system is linked to the complex *S. aureus* regulatory network, as it increases *sarA* expression, but decreases RNA III production.

1.4 Management and treatment of wounds

The variety of causes of chronic wounds and complexity of the healing process are major hurdles to effective treatments, which must be tailored to the individual. Medical history and physical examination of wounds are essential for every patient. Appropriate treatments of chronic wounds are determined according to location, severity and pathology (Anderson & Hamm, 2014). For example, a variety of strategies are used to relieve pressure on wounds and sores. Pressure ulcers are treated with off-loading devices and ambulation, whereas venous ulcers are relieved with compression therapy and diabetic ulcers with off-loading and management of ischemia (Mrdjenovich, 2010). Since most chronic wounds are complicated by infection, some form of antimicrobial therapy is required for effective healing. Elimination of localised necrotic tissue is achieved using surgical, enzymatic, mechanical, biological or autolytic debridement. These processes minimize the bioburden of the wounds by decreasing the presence of bacteria, reducing the hypoxic part of the wound and diminishing the local inflammatory reaction (Avishai *et al*., 2017; Leaper *et al*., 2015). Surgical debridement is the fastest method to remove the necrotic tissue from the wound. It is particularly important in life- or limb-threatening infections with necrotic eschar or gangrene (Leaper *et al*., 2015). Mechanical debridement methods include wet-to-dry dressings, which are performed by leaving wet gauze in direct contact with wound surfaces and removing when dry, together with any adhering slough. This is usually followed by pressure irrigation, which uses a syringe to force saline through cannulas to remove necrotic tissues that are loose and superficial (Fonder *et al*., 2008). Enzymatic debridement eliminates necrotic tissue using endogenous enzymes, derived from plants or micro-organisms (e.g. collagenase, varidase, papain, and bromelain) (Strohal *et al*., 2013). Occlusive (air and water-tight) and semi-occlusive dressings maintain a moist wound environment, which speeds up the processes of epithelialisation and collagen synthesis (Kirketerp *et al*., 2011). However, occlusive dressings

can also promote the growth of anaerobic bacteria (Nayeri, 2016). In contrast, where moisture retention is apparent in the site of wound bed, highly exudative wounds require moisture removal. This has been achieved using absorbent dressings. Selection of a wound dressing should, therefore, consider quantities of exudate, level of necrotic tissue and bacterial burden. Dressing choice can thus change as the wound progresses or deteriorates (Benbow, 2016).

1.4.1 Antimicrobial treatments and resistance profiles of chronic wound infections

It has been reported that more than quarter of patients suffering with chronic wounds are receiving antibiotics at any one time. Furthermore, 60% will have received systemic antibiotics during the previous 6-month period (Landis, 2008). Management of acne relies on the application of benzoyl peroxide (BPO), salicylic acid and certain antibiotics such as tetracyclines, levofloxacin and doxycycline MR (Sardana *et al*., 2015). *S. aureus* skin and wound infections are typically treated with fusidic acid, mupirocin, neomycin or retapamulin (Williamson *et al*., 2017). Alternatively, polyhexamethyl biguanide (PHMB),used in cosmetics and cleaning solutions, has been formulated as foam and shown to significantly reduce bacterial load of chronic wounds when impregnated into dressings (Sibbald *et al*., 2011). Wound dressings have become available that contain antiseptic agents such as silver. Nano-crystalline silver has been shown to kill a wide range of wound-infecting microorganisms including MRSA and vancomycin-resistant enterococcus (VRE) (Boonkaew *et al*., 2014). However, some of these agents are associated with undesirable side effects such as skin irritation and, of wider concern, is the increasing incidence of antibiotic resistance (Castanheira *et al*., 2010; Fritz *et al*., 2013).

Bacterial resistance to antibiotics can occur by de-activation of the antibiotic activity (by modification or destruction); alteration of the target site to prevent binding; or

modification of surface permeability to prevent an effective concentration of antibiotic reaching the target site. All of these strategies can be exemplified in different resistance mechanisms against β-lactam antibiotics, including the production β-lactamase enzymes, alternative cell-wall trans-peptidases known as penicillin-binding proteins, or over-expression of efflux pumps to reduce the concentration of antibiotics at the target site (Høiby *et al*., 2010; Molin *et al*., 2003). The presence of an antibiotic within a chronic wound bed, exerts selective pressure on the microbial colonisers and promotes the emergence of resistant microbial populations. MRSA is considered one of most common multidrug resistant pathogens in chronic wounds. A longitudinal study of diabetic foot ulcer infections demonstrated the rapid evolution of MRSA, whose prevalence almost doubled over a three year period (Howell-Jones *et al*., 2005). One hospital in-patient study found as many as half of all *S. aureus* isolates from infected leg ulcers to be MRSA and more than one-third of *P. aeruginosa* isolates to be resistant to ciprofloxacin (Bowling *et al*., 2009).

More recently VRSA have presented new challenges. The first clinical VRSA was isolated from diabetic foot ulcers in 2002 (Appelbaum, 2007). These strains reportedly acquired the *van A* gene from vancomycin resistant enterococci (VRE). Vancomycin is currently the most frequently used antibiotic in the treatment of severe MRSA infections. Therefore, there is an urgent need for alternative treatments for such infections. Both forms of methicillin sensitive *S. aureus* (MSSA) and MRSA, are common opportunistic pathogens, responsible for most of acute and chronic wound infections, resulting in significant increases in health-care costs and morbidity (Berenholtz *et al*., 2004; Bode *et al*., 2010).

P. aeruginosa has also been shown to adapt to sub-inhibitory antibiotic treatment of wounds. For instance, exposure of *P. aeruginosa* biofilms to azithromycin induced the expression of the *MexCD-OprJ* efflux pump (Gillis *et al*., 2005). Another study reported that *P. aeruginosa* can modify the targets of antibiotic action, for example by methylation of

16srRNA to prevent aminoglycoside binding, or modification of DNA topoisomerase to prevent binding of quinolones (Gorityala *et al*., 2016). This species has also been reported to possess multidrug efflux pumps, such as AdeABC and AdeDE systems that confer resistance against a number of antibiotics (Chua *et al*., 2015; Kobayashi *et al*., 2015).

Increasing resistance to macrolides, such as erythromycin, is of major concern. This antibiotic has a relatively narrow spectrum of activity against Gram-positive bacteria, mainly staphylococci and streptococci (Deng *et al*., 2015). Resistance has developed by ribosomal modification, mediated by 23S rRNA methylases, encoded by erythromycin ribosomal methylase (*erm*) genes. Erythromycin can also be expelled via active (ATP-dependent) efflux systems (Gatermann *et al*.,2007).

The incidence of wound treatment failures due to MDR infections is of major concern worldwide and there is increasing demand for novel treatment strategies.

1.4.2 Effects of Polyunsaturated Fatty Acids (PUFA) on wound healing.

Unsaturated fatty acids have the potential to decrease inflammation and it has been suggested that they may be effective in treatment of wound infection (Alexander & Supp, 2014; McDaniel *et al*., 2008). Polyunsaturated fatty acids (PUFAs), which cannot be synthesized *de novo* by mammals, consist mainly of two families, n-6 (omega-6, found in soybean oil) and n-3 (omega-3, found in fish oil) (Arnold & Barbul, 2006). Fish oil has been widely reported to have health benefits for brain function, support cardiovascular conditions and inflammatory disease (Assiri & Hassanien, 2013; Calder, 2013; Domingo, 2016). Omega-3 poly-unsaturated fatty acids include Eicosapentaenoic acid (EPA; C20:5 double bond) and Docosahexaenoic acid (DHA; C22:6 double bond) and their chemical structure are shown (Figure 1.6). Furthermore, PUFAs have been shown to exhibit antimicrobial activity

against several Gram-positive and some Gram negative bacterial species (Chitra Som & Radhakrishnan, 2011; Desbois & Lawlor, 2013; Kim *et al*., 2018; Mil-Homens *et al*., 2012; Sun *et al*.,2017). Classical resistance mechanisms against these compounds have not, so far, been identified (Desbois *et al*., 2013; Sun *et al*., 2016). PUFAs are suggested to have multiple effects, such as alteration cell membrane hydrophobicity, disruption cell surface charge, and membrane integrity, which lead to electron leakage resulting in bacterial cell death (Chanda *et al*., 2018; Desbois & Smith, 2010; Sun *et al*., 2016).

DHA, C_{22}:6n-3

EPA, C_{20}:5n-3

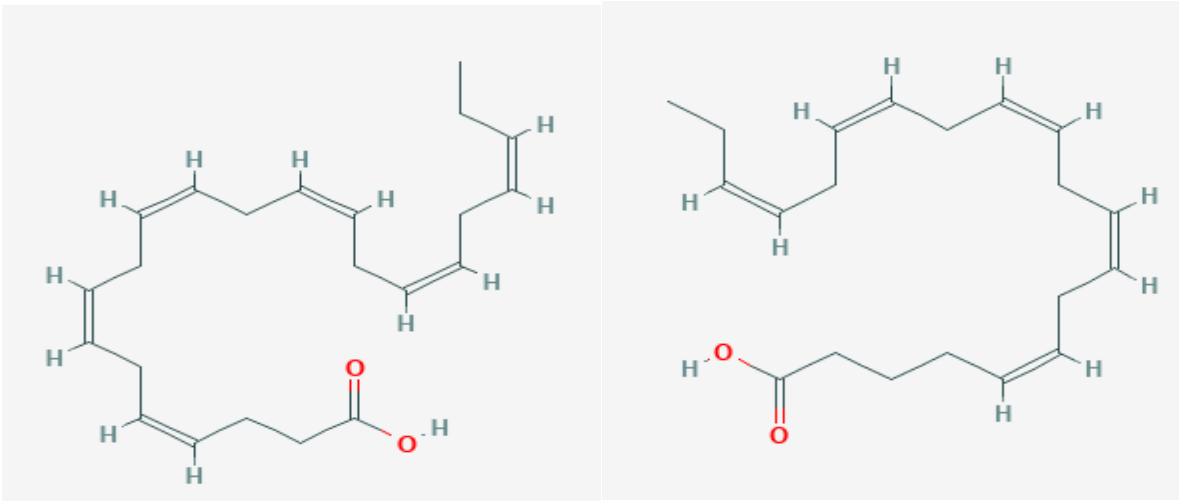


Figure1.6 Chemical structure of Docosahexaenoic acid (DHA, $C_{22}H_{32}O_2$) and Eicosapentaenoic acid (EPA, $C_{2O}H_{30}O_2$) (Assiri & Hassanien, 2013).

DHA and EPA are expected to trigger anti-inflammatory protective effects by acting as antagonists to arachidonic acid metabolism (Das, 2018). The arachidonic acid are prostaglandins such as prostacyclin (PGI2), leukotrienes (LTs) and thromboxane's (TXs). They are produced by immune cells that inhibit the production of inflammatory eicosanoids, adhesion molecules, and cytokines (Calder, 2013). Thus, by preventing the breakdown of arachidonic acid, PUFAs could prolong their anti-inflammatory effects and have been

suggested widley to enhance healing of wounds (Oh *et al*., 2015). *In vivo* experiments provide evidence for the value of PUFAs as wound-healing treatments. For example, the DHA-derived pro-resolving mediatorResolvin D1 (RvD1) has been shown to reduce the volume of necrotic cells, macrophages and microorganisms in chronically inflamed tissues of theexperimetaldibetic mouse model (Tang *et al*., 2013). Topical treatment with DHA has also been shown to significantly enhance healing in a wounded-rat model (Arantes *et al*., 2016). This PUFA has been shown to reduce production of pro-inflammatory cytokines such as IL-1b whilst causing increases in the expression of IL-6 and transforming growth factor b (TGF-b). These cytokines play important roles in the recruitment of inflammatory cells to the lesion site and activation of keratinocytes to promote wound healing (Arantes *et al*., 2016). Zhuang *et al*., (2013) used cell culture models to investigate the role of EPA and DHA on vascular endothelial wound repair *in vitro*. Anti-proliferative and anti-migratory effects of both DHA and EPA treatments were observed, evidenced by decreased proliferation of human microvascular endothelial cell lines (HMEC-1) and reduced expression of vascular endothelial growth factor (VEGF)was confirmed (Zhuang *et al*., 2013). Clinical studies of the effects of PUFAs on the healing of blisters have reported a significant increase in re-epithelialisation and collagen synthesis compared to the control group (McDaniel *et al*., 2011). There is published evidence that PUFAs have antibacterial activity against *S. aureus* and *Propionibacterium acnes* (Desbois & Lawlor, 2013). They have also been reported to have intrinsic antibacterial properties, shown by findings of activity against oral pathogens(Choi *et al*., 2013). Furthermore, PUFA compounds have shown no cytotoxic effect on human tissue cells (Yang *et al*., 2013). DHA or EPA did not reduce cell viability when at concentrations between 100 and 200 μM (Sun *et al*., 2016; Yang *et al*., 2013). A similar study reported 95% viability of C2 C12 myoblast cells after exposure to 50 μM and 100 μM

of EPA and DHA respectively (Peng *et al.*, 2012). All these studies indicated that EPA and DHA did not show any cytotoxicity or reduce cell viability at 100 μM concentrations.

Taken together, there is clearly sufficient evidence to support further research to explore the therapeutic potential of PUFAs. Thus, the aims of this present study were to evaluate the properties of two PUFA compounds (DHA, and EPA) against biofilm formation and resistance development by bacterial species commonly involved in chronic wound infection. The antimicrobial effects of PUFAs were investigated using *in vitro; in vivo* and *ex-vivo* experiments.

1.5 Models for testing antimicrobial efficacy

1.5.1 *In vitro* Antimicrobial sensitivity tests and biofilm assays

Antimicrobial sensitivity tests (AST), to determine the minimum inhibitory concertation (MIC), play a significant role in selection of an appropriate antimicrobial agent, particularly in an environment where the prevalence of resistant strains is high (Wellington *et al*., 2013). *In vitro* susceptibility data from wound isolates cannot always predict therapeutic success, as *in vitro* and *in vivo* conditions are variable (Dowd *et al*., 2008; Høiby *et al*., 2010b). Laboratory investigation does not reflect the reduced metabolic activity that is characteristic of bacterial cells within an extracellular biofilm matrix (Høiby *et al*., 2010). Often the MIC required to neutralize a microbial biofilm population is well above the concertation that is clinically and safely achievable (Fricks-Lima *et al*., 2011; Wu *et al*., 2015). Some antibiotics, such as linezolid, rifampicin, and daptomycin can penetrate biofilms (Barber *et al*., 2014; Seaton *et al*., 2013). However, limited antimicrobial penetration, poor nutrient acquisition, slow growth, adaptive stress responses, and formation of phenotypic variants are hypothesized to mediate resistance to a wide range of antibiotics and biocides (Song *et al*.,*et al*., 2016; Zhao *et al*., 2013). In certain conditions, biofilm bacteria have a 10-1000fold higher intracellular survival rate than planktonic bacteria (Fastenberg *et al*., 2016; Rasmussen & Givskov, 2006; Song *et al*., 2016). In addition, the physiological conditions of biofilms promotes an increase in the rate of horizontal gene transmission among microorganisms (Periasamy *et al*., 2012). These physiological conditions have been shown to enhance β-lactam resistance in *S. aureus* and *P. aeruginosa* biofilms by promoting over-production of β-lactamases that are concentrated in the biofilm matrix. These enzymes will hydrolyse the β-lactam antibiotics before they reach the bacterial cells (Hodille *et al*., 2017; Moradali *et al*., 2017). Tests to assess the efficacy of antimicrobials on biofilm shave been implemented over the last decade. Microtiter plate-based assays, the Calgary biofilm device,

substratum suspending reactors and the flow cell system are some of the most used *in vitro* biofilm models for susceptibility studies. The application of pharmacodynamic parameters, including minimal biofilm inhibitory concentration, minimal MBIC, biofilm-eradication concentration MBEC, have been shown in recent years to quantify antimicrobial activity in biofilms. Theses parameters have shown several advantageous for assessing the effect of quantitative and qualitative differences for the effects of most antibiotics when acting on planktonic cells or biofilm matrix (Macià, Rojo-Molinero, & Oliver, 2014).

1.5.2 Skin/wound models

Normal wound healing is a series of dynamic and complex biological processes that cooperate to reorganise the injury site. Wound healing processes are subdivided into three distinct phases: an inflammatory phase, a proliferative phase and a remodelling phase (Harper *et al*., 2014). Experimental wound models aim to recapitulate each phase to understand the mechanistic features of wound repair and to evaluate the efficacy of wound-healing treatments (Nunan *et al*., 2014). Different models (animal, cell -culture, 3D cell culture and *ex-vivo* skin models) have been developed, using a range of parameters that can vary depending on the research questions being asked (Antoni *et al*., 2015; Gottrup *et al*., 2000; Pastar *et al*., 2014).

Animal skin models have largely focused on mice, rats and guinea pigs (Delfan *et al*., 2014; Fukunaga *et al*., 2017), attempting to cover different phases of wound healing. The advantage of animal models is the host's vasculature, immune systems, and the external environment, can be considered to most-closely resemble human cases (Dorsett-Martin, 2004). Moreover, these models are well-characterised, and the availability of mutant clonal replicates allows for controlled experiments. However, the cost of experimental animal

facilities is high. More crucially, the contradiction between human skin and rat skin are clearly evident. For example, wounded rats heal by contraction of a subcutaneous muscle layer (panniculus carnosus), these layers do not exist in human skin and the re-epithelialization process contributes to a different healing mechanism (Li *et al*., 2015). Furthermore, the tissue of mice, rats and other animals possess the enzyme L-gluconolactone oxidase (not present in human skin) that converts L-gluconogamma lactone to vitamin C (Blais *et al.*, 2017), and plays- a major role in collagen synthesis (Takahashi *et al*., 2014). The variation in anatomy and physiology and the fundamental genetic differences between human and rat skin should be considered.

In vitro cell culture experiments, using either transformed skin cell lines or primary skin cells are cheaper and more easily reproduced in a well-controlled environment that contains suitable medium with essential nutrients such as (amino acids, vitamins, carbohydrates growth factors, and minerals), (Coulomb & Dubertret, 2002). Carefully controlled CO_2 and O_2 levels can create the correct physio-chemical environment. Most cells are adherent on surfaces by using artificial substrate or others can grow free floating in culture medium (Mehling&Matthias, 2014). These experimental conditions allow for high-throughput testing of wound-healing treatments, using scratch test assays. However, they provide limited information about skin structure or the role of immune-modulators.

More sophisticated 3D cell culture wound healing models have been developed, using fibroblast cells cultured in 3D matrices, generally containing fibrin or type I collagen. Collagen production is decreased whereas collagenase is increased by fibroblasts integrated into relaxed type I collagen gels (Eckes *et al*., 1995). The gel composition can be modified by incorporating different types of collagen, fibronectin or glycosaminoglycans to imitate the early granulation tissue deposited during wound repair (Xu & Clark, 1996). Another example of a novel 3D wound healing model was presented by Chen *et al*., (2014). This model

introduces multiple cell types such as fibroblasts, keratinocytes, monocytes, and mesenchymal stem cells. Cell migration and proliferation can be screened by adding different growth factors during the 3D fibrin construction. This model has been used in to study the effect of different drugs on cell migration during wound healing process (Chen *et al*., 2014).

There are several limitations to the use of *in-vitro* and animal models to investigate wound healing. High cost, poor reproducibility or poor resemblance of actual human skin are common pitfalls. Therefore, researchers are keen to find alternative models able to screen the effects of different compounds and conditions on the wound healing process, in a system that more closely resembles natural human wounds. *Ex-vivo* skin models have been developed, using human skin left over from routine elective surgery such as cosmetics (Groeber *et al*., 2011). These skin explants are cut with a full thickness punch biopsy and contain both dermis tissue (fibroblast and collagen) and epidermis tissue (keratinocytes). The punch biopsies are then placed in tissue support systems and growth media. This approach has an advantage over other model systems, as it enables the study of re-epithelialization that occurs in epidermal and dermal layers. The *ex-vivo* skin can be maintained under controlled experimental conditions, however, variation in host factors of the donor can affect reproducibility. Cell proliferation and death during reepithelization can be measured by immunofluorescence markers (Mendoza-Garcia *et al*., 2015).

Thus, a further aim of this project is to investigate the effects of PUFA compounds on the growth of *S. aureus* on wounded and non-wounded *ex vivo* skin.

1.5.3 Invertebrate and non-vertebrate models of infection

The study of infectious diseases and toxicity using invertebrate models has given insights into host pathogen interaction for several bacteria. In addition, several alternative invertebrate models have been developed based on their experimental advantages, low cost, ease of maintenance, high throughput capabilities and the various aspects of the innate immune response are conservative between invertebrates and mammals, makes these invertebrates an effective method for identifying microbial virulence factors and testing potential antimicrobial agents.

Among the alternative models to study microbial infection are amoebas (Trevijano-Contador & Zaragoza, 2014). For example, the *acanthamoeba* model which are considered a typical model for studying microbial phagocytosis. Macrophages and *acanthamoeba* have lysosomal enzymes responsible for the digestion and nutrient acquisition (Marciano-Cabral & Cabral, 2003), which gives advantageous to evaluate the microbial interaction and virulence factors. In addition, comparison the gene expression between the *A. canthamoeba* model and mammalian cells, might provide the knowledge on how pathogenicity enhanced and on how pathogens evolved and adapted to distinct hosts.

Drosophila melanogaster has been used to investigate whether these insects are potential vectors of human and plant pathogens. Genomic analysis of *Drosophila melanogaster* revealed that similarity to the mammalian innate immune system (Marcu *et al*., 2011). Furthermore, *D. melanogaster* have been used to explore pathogen interactions and host factors that promote or inhibit infection by pathogens like *S. aureus* (Tabuchi *et al*., 2010), and *P. aeruginosa* (Kim *et al*., 2008).

The nematode *Caenorhabditis elegans* is multicellular organism and has been the subject of intense study for decades. The genome sequence for *C. elegans* is available and

many functional genomic approaches have been developed to study the innate immunity of humans or other animal hosts. *C. elegans* has an innate immune response, involving the activation of specific signalling pathways and leading to the production and release of defence molecules such as antimicrobial peptides, caenopores, caenacins, and lysozymes (Dierking *et al*., 2016). This nematode worm has been applied to the study of host-pathogen interactions, immune response and used for antimicrobial discovery and development. For example, community-associated MRSA strains have been shown to be more pathogenic than hospital-associated MRSA in *C. elegans* and support the use of this model for studying the virulence of *S. aureus* strains (Wu *et al*., 2010). This model has also been useful in testing the efficacy of novel antibiotics. For example, cytolex, a magainin AMP derived from frogs, has been identified as a topical antibiotic for treating diabetic foot infection. Its efficacy has been tested using the *C. elegans* model, with promising result for AMP derivatives. The nematode could be a rich source of potential leads for the future development of such drugs (Ewbank & Zugasti, 2011).

Galleria mellonella is high-throughput model that mimics the key features of microbial pathogenesis, interactions, and studying the microbial virulence. It can also be used to screen novel antimicrobial drug candidates. *G. mellonella* has two major parts of innate immune system, the cellular and the humoral immune response that play significant role to eliminate infecting microbes. The cellular response includes phagocytosis, encapsulation and clotting. The humoral response is coordinated by soluble complement-like proteins, melanin, and anti-microbial peptides. Several studied have been established that use the invertebrate *G. mellonella* model for virulence determinant, host response and antimicrobial efficacy, with a range of pathogens such as *S. aureus* (Desbois & Coote, 2011; Silva *et al*., 2017), *P. aeruginosa* (Koch *et al*., 2014), and *Candida albicans* (Mowlds *et al*., 2008). The

invertebrate *G. mellonella* are ethically more acceptable and practically more-challenging than the use of vertebrates meaning larger group sizes can be used to enhance reproducibility.

This study focused on exploring the interaction between *S. aureus* and PUFA compounds using *ex-vivo* skin and *G. mellonella* as infection models.

1.6 Aims and Objectives

This study focused mainly on a clinical wound isolate of *S. aureus,* because it is the pathogen most commonly associated with wound infection. The experimental approach had three major aspects, the first aim was to evaluate the activity of polyunsaturated fatty acids (PUFA) (DHA, EPA and both DHA+EPA) *in-vitro* as potential antimicrobial and anti-biofilm agents. Furthermore, to assess the effect of the inhibitory levels of PUFAs on global gene regulation involved in virulence and biofilm formation. The second aim of this study was to assess the effect of PUFAs *in vivo* on *S. aureus* on the growth and infection using *G. mellonella* model and *ex-vivo* skin models. Finally, this study observed the phenotypic and mutational variance that occurred after environmental stress caused by PUFA compounds compared to standard antibiotics (LZD and VAN).

Specific objectives

1. Assess the antibacterial and anti-biofilm activity of PUFAs on bacterial species that commonly cause wound infection using MIC, MBC, MBC and MBEC assays and *in-vitro* biofilm assays.
2. Estimate the effect of the inhibitory concertation's of PUFAs on global regulatory genes of *S. aureus* using RT-q PCR.
3. Assess the efficiency of PUFA compounds and standard antibiotics (LZD and VAN) on the virulence of *S. aureus* using *G. mellonella* and *ex-vivo* skin models.
4. Assess variation in antibiotic sensitivity and virulence of *S. aureus* SA3 following prolonged exposure to PUFAs and clinically relevant antibiotics.
5. Evaluate single nucleotide polymorphisms (SNPs) associated with *S. aureus* adaption to PUFAs, LZD and VAN using the whole genome sequencing.

Chapter Two

Materials and Methods

Chapter 2 Materials and Methods

2.1 Bacterial culture

All bacterial strains and species used for this study are listed (Table 2.1). These species were chosen as they have been commonly associated with wound infections (Cooper *et al.*, 2014). Each strain/isolate was identified and confirmed using standard microbiological culture techniques such as Gram stain, Coagulase test, Catalase test, and Analytical Profile Index (API 20 E). The range of solid media used in this study included Columbia agar (Lab M, UK) for inoculating organisms from -80°C, for propagation of organisms for total viable count and for growing organisms for stock cultures. The liquid media include Luria-Bertani (LB) broth (Oxoid, UK), for liquid cultures of reference organisms. *S. aureus* isolates were sub-cultured on mannitol-salt agar plates (Oxoid, UK). Muller Hinton broth (MHB, Oxoid, UK) was used for antimicrobial susceptibility tests by broth dilution to determine the minimum inhibitory concentrations. All confirmed isolates were stored in LB with 20% glycerol and frozen at -80°Cuntil required for further investigation.

Table 2.1 Bacterial species/strains and their origin

Strains /isolates	Strain	Origin and source	Source	References
S. aureus	*SA2	Forearm wound	SRFTΨ	This study
	*SA3	Ulcer swab	SRFTΨ	This study
	*SA4	Ulcer swab	SRFTΨ	This study
	#6538	ATCC reference strain Major	Salford University stock	(Nicolaou *et al.*, 2007)
	Ψ252	MRSA strains found in British Hospitals	Salford University stock	(Holden *et al.*, 2004)

E. faecalis	#13280	NCIMB reference strain	Salford University stock	(Thomson *et al*.,2011)
P. aeruginosa	#PA01	Wound isolate	IGH, University of Liverpool	(De Soyza *et al*.,2013)
	#PA14	Human burn isolate	IGH, University of Liverpool	(De Soyza *et al*.,2013)
Es. coli	#10536	ATCC reference strain	Salford University stock	(Mogna *et al*., 2012)

*Clinical wound isolate; #Standard reference strain; ᴪSalford Royal Foundation Trust Hospital; IGH – Institute of infection and Global Health.

2.2 Antimicrobial susceptibility testing (AST)

2.2.1 Disk diffusion assays

The disk-diffusion (Kirby Bauer) method is the traditional method used in clinical setting when testing bacteria for antimicrobial susceptibility patterns. This method has been standardised as detailed in the standard susceptibility testing guidelines of European Committee on Antimicrobial Susceptibility Testing EUCAST (EUCAST, 2018). Overnight bacterial cultures were prepared on Luria agar and suspended in phosphate-buffered saline (PBS Thermo Fisher Scientific, UK) then adjusted to a 0.5 McFarland turbidity standard and swabbed evenly onto Mueller Hinton agar in accordance with EUCAST guidelines. Appropriate antibiotic discs (Oxoid, UK), were then placed on the agar surface (Table 2.2). The antibiotics used were chosen as important representatives of ten different antibiotic classes. After incubation at 37 ºC, for 18 h. The break points were determined by measuring the diameter of each zone of inhibition, as a function of the amount of drug in the disk and susceptibility of the microorganism. Each bacterial strain or isolate was classified as

susceptible or resistant by comparing the diameters of inhibition zones with the breakpoints according to EUCAST recommendations (EUCAST, 2018).

Table 2. 2 Antibiotics and break points (EUCAST Clinical Breakpoint Tables v. 6.0)

Classes	**Antibiotics (Oxoid)**	**Disk content (µg)**	**Inhibition Zone diameter (mm)**	
			Target	**Range**
β-lactam	Tazobactam (TAZ) Cefoxitin (FOX) *	85 30	Vary 17-28	NA 14-20
Glyopepetide	Vancomycin (VAN)	5	13	10-16
Aminoglycoside	Gentamicin (CN) Tobramycin (TOB)	10 10	22 22	19-26 18-26
Lincosamide	Lincomycin (MY))	2	26	22-29
Macrolide	Erythromycin (E)	15	26	23-29
Fluoroquinolone	Ciprofloxacin (CIP)	5	24	21-27
Tetracycline	Tetracycline (TE)	30	27	23-31
Oxazolidinone	Linezolid (LZD)	10	24	21-27

*Cefoxitin (FOX)30 µg used for identification of MRSA in accordance with EUCAST recommendations.

Notes: - Break points size are used for most *Staphylococcus aureus* references strains (EUCAT, 2018), therefore the size of inhibition is varying that dependent on the strains type.

2.2.2 Detection of PUFA antimicrobial activity by disk diffusion assay

Polyunsaturated fatty acid such as DHA and EPA have been recognised as antimicrobial inhibitors for several pathogens (Desbois and Lawlor, 2013; Sun *et al*., 2016). This study sought to confirm these reports and further characterise such activity against several clinical isolates of *S. aureus*. Briefly, overnight cultures of *S. aureus* isolates SA2, SA3, SA4, SA 6538 and MRSA 252 were prepared on Luria agar and 2 -3 colonies of each isolate are inoculated into 5ml of Phosphate buffer saline (PBS, Thermo Fisher Scientific, UK). These bacterial suspensions were adjusted to a turbidity of 0.5 McFarland standard. Bacterial suspensions were swabbed onto Mueller Hinton agar plates using a sterile cotton swab within 15 min of preparation. DHA and EPA (Cambridge Bioscience) were solubilised in Ethanol and diluted with sterile H_2O to 100µg ml^{-1}. Diluted compounds (25 µL) were absorbed onto 6mm sterile filter paper discs (Whatman) and allowed to dry for 1h. Control discs were prepared using diluted ethanol. Disks were placed onto inoculated MH agar, incubated and interpreted as described for AST (section 2.2).

2.3 Detection of Minimum Inhibitory Concentration (MIC) and Minimum bactericidal concentration (MBC)

The antimicrobial effect of PUFAs (DHA and EPA, Cayman, Cambridge Bioscience) and relevant antibiotics was tested by determining the minimal inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC). All bacterial cultures were grown for 18h at 37°C under aerobic conditions on Columbia base agar (Oxoid, UK), then inoculated in to 5 ml Mueller Hinton (Oxoid, UK) broth for further incubation at 37 °C with shaking 18-20h. The OD_{600} nm of bacterial cultures were measured and adjusted to OD_{600} 0.05 in MH Broth. The MIC assay was carried out according to standardised broth dilution

methods (Andrews, 2001). Briefly, DHA and EPA (100 mg) were solubilised in ethanol (100%) to prepare stock concentrations of 50 mg ml^{-1}. Two-fold serial dilutions (100 µL) were then prepared in MH broth in a sterilized 96-well compound plate (Sigma-Aldrich, Costar, USA) as detailed in Table 2.3. The Gentamycin antibiotic (20 µg ml^{-1}) was 2- fold serial diluted in ds H_2 O as positive control. Each dilution was then diluted further (1:20) by introducing to a dosing plate, each plate containing 190 µL of MH broth. A final MIC plate was prepared using 100 µL from each well of dosing plate plus 100 µL diluted bacterial suspension (Table 2.3). Broth only wells were used as negative controls. MIC plates were incubated for 18-20 h at 37ºC. The MIC values were determined as the minimum concentration of PUFA or antibiotic that prevented any bacterial growth (measured as turbidity above background levels) and recorded using a plate reader (FLUOSTAR omega, BMG, UK) at wavelength 600 nm.

Table 2.3 Preparation template for dilution of PUFA compounds for MIC assays

Compound plate50mg ml^{-1}stock	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**
Dosing plate (1:20)	1:40	1:80	1:160	1:320	1: 640	1:1280	1:2560
MIC plate (100uL of dosing plate+100uL bacterial suspension)	1250µg ml^{-1}	625µg ml^{-1}	312µg ml^{-1}	156µg ml^{-1}	78µg ml^{-1}	39 µg ml^{-1}	19.5µg ml^{-1}

The Minimum Bactericidal concertation (MBC) values were determined by spreading50 µL of bacterial culture from wells containing the determined MIC, and wells on either side, to MH agar plates. The solution was spread using a sterile loop and each plate was inverted and incubated for 24h at 37°C. In order to determine the MBC, the colonies were counted and the lowest concentration plates that contained no growth were designated the MBC**.**

2.4 Detection of antimicrobial tolerance.

The measurement of MBC/MIC ratios to detect the ability of bacteria for prolonged survive underexposure of bactericidal without acquired phenotypic resistance (Biedenbach *et al*., 2007; Jones, 2006).

2.5 Biofilm assays

2.5.1 Congo red agar assay

The Congo Red Agar assay (CRA) allows for detection of certain exopolysaccharides as an indicator of biofilm forming capability by variation in the colour of bacterial colonies grown on the specialised solid medium (Kaiser *et al*., 2013). Congo Red agar was prepared using sterile Brain Heart Infusion (BHI) broth (Oxoid, UK) supplemented with sucrose (5% w/v); and Congo red stain indicator (0.08% w/v; Oxoid, UK; Sharvari & Chitra, 2012). Bacterial isolates were streak inoculated on to Congo Red Agar plates and incubated at 37ºC under aerobic conditions for (24 and 48 h). The biofilm producer strains formed black colonies with a dry crystalline consistency indicating a good capacity for biofilm production, while the non-biofilm producer strains formed red colonies. The experiment was performed in triplicate and repeated three times.

2.5.2 Crystal Violet Assay

The crystal violet microtiter plate assay is a simple method for biofilm detection. This semi-quantitative test, was performed according to the method (O'Toole, 2011). Bacterial strains were grown aerobically (16-18h) on Columbia base agar (Oxoid, UK). Selected colonies were inoculated in 5 ml of Brain Heart Infusion (BHI) broth and incubated at 37ºC for further

24 h. The cultures were then adjusted to OD_{600} 0.05 by diluting in Tryptic soy broth (TSB) supplemented with 1% glucose for enhancing the biofilm formation in staphylococci (Reiter *et al*., 2013). The bacterial culture was adjusted to OD_{600} 0.05 and subsequently 200 µL were filled in sterile 96 well flat-bottom polystyrene plates (Sigma-Aldrich, Costar, USA). Negative control wells, containing broth only were included. Two microtiter plates were incubated aerobically at 37°C for 24 h and 48 h. A reading of optical density (OD_{600}) was taken as an estimation of the total growth in each well. Planktonic cultures were removed, and the wells were washed with 200 µL of PBS (pH 7.2) x3. This removed any non-biofilm-associated bacteria. Biofilms adhered to the bottom and side of the wells were stained by adding crystal violet (0.1%; SIRCHIE, USA) to each well and incubating at room temperature for 15 min. Excess stain was removed and biofilms were washed again with PBS as previously. Plates were dried completely at incubator for 2 h before 100 µL of 100% ethanol was added (Cargill, UK). All plates were incubated at room temperature for 15min with gentle shaking. Total biofilm density was quantified by reading OD at wavelength (OD_{600}) using a plate reader (FLUOSTAR omega, BMG, UK). Each experiment was performed in triplicate and repeated 3times. The biofilm index score based on the optical density and crystal violet stain were recorded. The average OD values were calculated for all tested strains and negative controls. Final OD values of a test strain was expressed as average OD value of the strain reduced by OD cut-off value (ODc) of negative control. Then the interpretation for biofilm production was carried out according to the criteria of Stepanovic *et al*.(Stepanovic *et al*., 2007). ODc was defined as three standard deviations (SDs) above the mean OD of negative control.

2.5.3 Detection of Minimum Inhibitory Concentration (MIC) of PUFA on the biofilm formation

The effect of PUFAs on biofilm formation was assessed by setting up 96-well plates in the same way as the MIC assay (section 2.3), then measuring biofilm formation in each well after 24-48 h using the crystal violet assay (Stepanovic *et al*., 2007)**.**

2.5.4 Assessment of PUFA compound efficacy on biofilm disruption by using (MBIC and MBEC assays)

The minimum biofilm inhibitory concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC) methods are easy to perform and applicable to high throughput screening for assessing anti-biofilm activity using the Calgary Biofilm Device (MBEC™ Biofilm Technologies Ltd. Calgary, AB, Canada). The Calgary biofilm system was designed to allow growth of biofilms, for longer periods in 96 wells values in clinical situations; determination of Antibiotic Susceptibilities of Bacterial Biofilms and assessing potential efficacy of treatments for chronic biofilm-associated infections (Ceri *et al*., 1999). The Calgary system is a two-piece disposable plastic 96-well plate designed for the growth of biofilms on pegs that are attached to the lid and bathed in liquid contained within each well. This system was used to evaluate the ability of PUFA compounds to disrupt biofilms of nine bacterial strains / clinical isolates, as previously described (Girard *et al*., 2010; Reiter *et al*., 2013) with slight modifications. Briefly, single colonies of test bacteria were inoculated into 5 ml of sterile tryptone soy broth (TSB) with1% glucose and incubated at 37°C in a shaking aerobic incubator (100 rpm) for 18-20 h. Bacterial culture densities were normalised by diluting to McFarland standard 0.5 using fresh growth medium. Next, 100 µL of bacterial inoculum was added to each well of the Calgary biofilm plate and incubated at 37°C and 30 rpm for 48 h to allow biofilm formation on the pegs. Biofilms were exposed to doubling

dilutions of PUFAs (150 µL) in sterile broth across a 96-well microtiter plate by transferring the Calgary plate lid into the freshly prepared challenge plate and incubated for 24 h at 37°C and 100 rpm. After incubation, the lid was transferred to a 96-well recovery plate containing 200 µL of sterile broth and incubated for 18 h at 37°C. Different controls of broth only and bacterial suspension (containing the equivalent concentration of ethanol used to initially solubilise each PUFA), were prepared as negative controls. The MBECs were determined as the lowest concentration for which bacterial growth did not occur. Growth was viewed as turbidity compared to an un-inoculated well (negative control) and was detected using a microtiter plate reader (FLUOSTAR omega, UK). The cut-off was defined as three standard deviations above the mean ODc. Each isolate was classified as follows: weak biofilm producer OD = 2 × ODc, moderate biofilmproducer 2 × ODc < OD = 4 × ODc, or strong biofilm producer OD > 4 × ODc.

2.6 *Galleria mellonella* as a model to assess virulence of *S. aureus* and antimicrobial activity of PUFAs *in vivo.*

2.6.1 *G. mellonella* challenge experiments to compare virulence of *S. aureus* strains

G. mellonella larvae were purchased (next-day delivery) from Live Foods Direct (Sheffield, United Kingdom), stored in the dark at 4°C and used within 14 d of delivery. The virulence of 5 different strains (or adapted strains) of *S. aureus* were compared using the *Galleria mellonella* (wax moth larvae) infection model, carried out according to Latimer *et al* (Latimer *et al*., 2012). Briefly, all bacterial cultures were grown in on Luria agar plates before overnight culture in LB broth at 37°C with 200 rpm shaking. Three times in PBS washing and diluting appropriately to a desired OD_{600} of 0.1 (5×10^5 - 8×10^5 $CFUml^{-1}$). Colony counts were confirmed by preparing a dilution series and spreading 100 µL on Mannitol Salt Agar (MSA) (Lab M, UK) for each suspension then incubating at 37°C for 30 min. All experiments used groups of 16 larvae per treatment. Each larva from each group was injected with 5µL prepared inoculum into the hemocele of the last left proleg larvae by using Hamilton syringe (Sigma-Aldrich Ltd, UK) with needle. The syringe was cleaned between tested strains with sequential washes of PBS, 70 % ethanol and sterile water. Two negative control groups were always prepared: one group that underwent no manipulation to control for background larval mortality and other group was injected with PBS only (uninfected control) to control any effect of physical trauma. Larvae were incubated in sterile Petri dishes in the dark at 37°C for up to 7 days and inspected every 24h so that the number of dead larvae could be recorded and removed, and percentage survival could be calculated for each group. The experiment was terminated once two larvae from either control group had died. Larvae were considered dead if they turned black in colour due to melanisation as a result of bacterial multiplication and infection. Each experiment was repeated 3x. To assess the bacterial burden of infection, each dead larva was crushed and homogenised in sterile PBS.

The homogenate was serially diluted, and the colony counts were performed by spreading 100 µL on MSA. Viable colony forming units per mL were determined by calculating the mean colony count x dilution factor x 10 for each treatment.

2.6.2 *G. mellonella* challenge experiments in the presence of PUFAs

Further challenge experiments were carried out using the clinical *S. aureus* wound isolate, SA3. Assays were set up as described in section 2.4.1, but bacterial cultures were inoculated in the presence or absence of different concentrations (MIC or 2x MIC) of PUFAs (DHA or EPA) or antibiotics (LZD or VAN).

2.7 *Ex-vivo* Human Skin Model

2.7.1 Development, and testing of a bacterial burden on wound infection based on *ex vivo* human skin model

Fresh human skin specimens were obtained from (a licenced research tissue bank housed at the University of Bradford). Skin samples (5 x 5 cm) were taken from abdominoplasty patients at Bradford Royal Infirmary, processed at Ethical Tissue and dispatched by courier to Salford. Samples were typically processed in our laboratory within 24 h of surgery and were transported on cooling packs (dry ice box) in a sealed container of saline solution. All Details of the facility and Ethics are supplemented (Supplement 12 chapter3 in appendices) and more details : https://www.bradford.ac.uk/business/ethical-tissue/about-us/.

The experiments were performed using antiseptic technique in a containment level 2 biological safety cabinet. Briefly, the surface of the *ex vivo* skin (free of injuries or redness) was cleaned with double distilled water. All excess subcutaneous adipose tissue and fats were

removed using a pair of blunt stainless-steel scissors and coated stainless steel dissection forceps (Fisher Scientific UK Ltd) to yield full-thickness skin. Skin samples were washed twice in Williams E medium (Fisher Scientific UK Ltd), in a sterile plastic Petri dish and then bathed in the medium for 10 min to refresh the skin. The skin biopsies were prepared using a 5.0 mm Biopsy Punch (Selles Medical Ltd, UK) and transferred in to fresh Williams E medium. For the wounded-skin model, superficial wounds were introduced using a 2.0mm Biopsy Punch (Selles Medical Ltd, UK), taking care to only cut through the surface epidermis. Unwounded and wounded skin biopsies were mounted into 6-insert Trans-wells (24mm) containing TC Treated PC Membrane (pore size 0.4µm) (Fisher Scientific UK Ltd). Biopsy skin samples were pulled through a pin-prick hole in the centre of each trans-well membrane as shown (Figure 2.1). Biopsies were bathed, from the basal side in wells containing 1 ml serum-free Williams E culture medium (Thermo-Fisher Scientific™, Basingstoke, UK) supplemented with 10 $\mu L ml^{-1}$ insulin, 10 $ng ml^{-1}$ hydrocortisone and 2mmol L-glutamine (Sigma-Aldrich Company Ltd). A series of experiments were designed and set up in sterile 6 well tissue culture plates and trans-well inserts (Fisher Scientific UK Ltd) as shown (Table 2.4). Overnight bacterial cultures of clinical *S. aureus* isolate SA3 were prepared by incubating for 20 h in tryptic soy broth at 37˚C with shaking (180 rpm), then washed 3x in sterile PBS to desired OD_{600} nm of 0.1 (3.5×10^7 to 8×10^7 $CFU ml^{-1}$), the colony counts of each inoculum was confirmed on MSA agar. A total volume of 5 µL of each inoculum (containing ≅ 3.5×10^4 $CFU mm^2$) was deposited on to the surface of each wounded or non-wounded biopsy sample. For treatments in the presence of PUFA, DHA or EPA was solubilised in ethanol and diluted in MH broth to a final concentration of 130 $\mu g\ ml^{-1}$ (2x MIC). PUFA preparations (100 µL at 2 MIC $\mu g\ ml^{-1}$) were mixed with 100µl suspensions of diluted SA3 in sterile microfuge tubes. Mixtures (5 µL) were pipetted on to the surface of wounded and non-wounded biopsy samples. An alternative experiment was set up, in which

bacteria were inoculated on to the skin surface and allowed to absorb, 15 min before the addition of PUFAs (Table 2.4). In this case, SA3 suspensions (5 µL) were laid on top of wounded and non-wounded biopsy samples then after 15 min, 2x MIC of DHA compound (5µl) was added. Media was added to the skin surface as negative control. Each test setting was performed in triplicate (3 wells) with a total of at least three repeat plates. All plates were incubated overnight in a CO_2 incubator (5% CO_2) (LEEC, UK) at 37˚C.

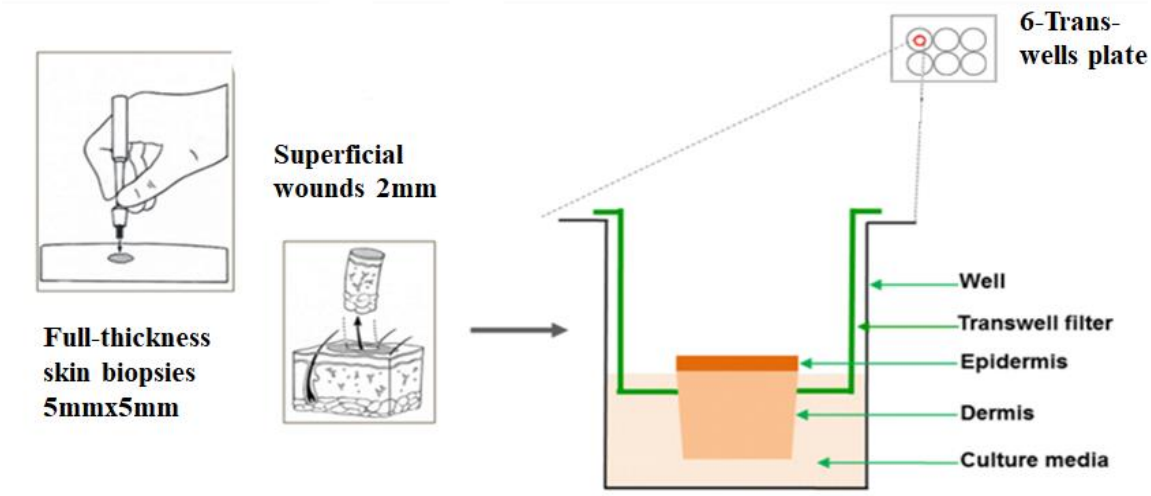


Figure 2.1 **Schematic of *ex-vivo* skin model set up. Modified from ("Skin - Predictive Drug Discovery Services").** Biopsy samples taking from Human skin ex-vivo model for 1stExperiment wounded and Nonwounded biopsy, 2nd Experiment were treated with SA3 + DHA (2 MIC,130µgml^{-1}), Biopsy samples with bacteria only and with DHA 2 MIC only were included as controls. Each experiment was done 3times in triplicate.

Each time skin was delivered, 4 assay plates were prepared, 5mm skin biopsies were inserted into the centre of each trans-well and bacterial culture (SA3), PUFA (DHA) or media (5µL) was inoculated on to the surface.

Table 2.4 Conditions used for each *ex-vivo* skin model assay

Assay Plate	**Row 1: Wounded (3 replica wells)**	**Row 2 Non-wounded (3 replica wells)**
1	SA3 ≅1 x10^7 CFUmm^2	SA3 ≅1 x10^7 CFUmm^2
2	Media only control	Media only control
3	SA3 ≅1 x10^7CFUmm^2+ DHA (2 MIC130 µg ml^{-1})	SA3 ≅1x10^7CFUmm^2 + DHA (2 MIC130 µg ml^{-1})
4	SA3 + delayed* DHA (2 MIC µg ml^{-1})	SA3 + delayed* DHA (2 MIC µg ml^{-1})

Determination of bacterial burden and the activity of PUFAs on the *ex-vivo* human skin model was performed by using a sterile cotton swab to collect bacteria from the surface of each skin biopsy. Bacteria were recovered from the swab by agitation in sterile PBS (1 ml), which was serially diluted and then 100 µL of each dilution was spread plated on MSA for overnight culture at 37˚C. Colony forming units on each skin biopsy (5 mm^2) were calculated.

2.8 Effect of the inhibitory concentrations of DHA and EPA on growth of *S. aureus* clinical wound isolate SA3

This study aimed to investigate potential mechanisms by which PUFAs affect the growth or virulence of *S. aureus.* Growth and expression of key virulence regulator genes were observed in the presence of different concentrations of DHA and EPA. Briefly, fresh cultures (50 ml) of clinical wound isolate, SA3 were prepared in MH broth from overnight cultures to a starting density of OD_{600} 0.1 corresponding to 10^6 $CFUml^{-1}$ (as confirmed by serial dilution and spread plating on MSA). Cultures were exposed to two different concentrations (50µg ml^{-1} or 100 µg ml^{-1}) of DHA, EPA or both together, in triplicate and incubated at 37ºC. Samples (4 x 1.5 ml) were taken from each culture vessel at time points 0, 6, 12 and 24 h. Optical density measurements and broth dilution CFU ml^{-1} assays were performed at each time point. Negative controls used MH, supplemented with diluted Ethanol in place of PUFAs. Bacterial cells were harvested from each 1.5 ml sample by centrifugation (13,000 rpm, 2min) using bench top microfuge, bacterial pellets were stored at -20ºC for total RNA extraction.

2.9 Adaption Experiments

Since PUFAs could be applied to wound dressings to enhance the treatment of chronic infections, this study investigated whether *S. aureus* was able to adapt to prolonged exposure of PUFAs or clinically relevant antibiotics. Experiments were set up to monitor resistance, tolerance and virulence of the clinical wound isolate, SA3 after sequential sub-culture in below-MICs of DHA, EPA, or both DHA+EPA, vancomycin or linezolid. Briefly, single colonies of SA3 were inoculated in to MH broth for culture at 37ºC for 24h. Bacterial suspensions were standardised by dilution to OD_{600} 0.05 using fresh growth medium. MIC assays were prepared

in 96-well microtiter plates (96-well) (according to section 2.3), using a 2-fold dilution series of each test agent (2x above and 2x below the MIC – see table 2.5). 'Bacteria only' and 'MH broth only' conditions were included in triplicate as negative controls. Growth was observed after 20 h by measuring absorption using a microplate reader (FLUOSTAR omega, BMG, UK) and compared to negative control. Each day, after recording MIC, wells containing bacterial growth at the highest possible concentration of antimicrobials, were sub-cultured (2 μl) in to a fresh MIC assay plate, prepared as before. Concentrations were adjusted each time, according to any change in MIC, to ensure that an appropriate range was used (2x above and 2x below MIC). A loopful of culture from the same well was streaked onto MSA to check for purity. The remaining cultures, from each assay plate, were stored in 20% glycerol at - 80°C.

Table 2.5 Concentrations of test agents (PUFA or antibiotics) used for adaptation experiments

Test agent	Concentration range (μg ml^{-1})					
DHA	312	156	78	**65**	52	39
EPA	312	156	**78**	65	52	39
DHA+EPA	312	156	**78**	65	52	39
Linezolid	16	8	**4**	2	1	0.5
Vancomycin	0.25	0.12	0.06	**0.03**	0.015	0.0075

*Concentrations were adjusted each time to maintain 2 below and 2 above the MIC (MIC highlighted in grey)

2.10 Detection and quantification of *S. aureus* enzymes

2.10.1 Coagulase Assay

Coagulase production was measured following the method of (Aldridge *et al.*, 1984). Briefly, bacterial suspensions were prepared in MH broth (1 ml, OD_{600} 0.4) and added to 3 ml rabbit plasma supplemented with EDTA (Sigma-Aldrich., UK). The suspensions were then incubated at 37 °C and monitored for signs of coagulation for up to 3 h. Extent of coagulation was judged using a 4-timepoints scale.

2.10.2 Deoxyribonuclease Assay

Deoxyribonuclease (DNase) production was measured following the method of (Choi & Szoka, 2000). Briefly, fresh bacterial cultures were inoculated onto DNase test plates containing Soya peptone, Deoxyribonucleic acid (DNA), Sodium chloride and agar (Thermo Fisher Scientific, UK) and incubated for 18-20 h at 37 °C. Once cultures had grown, the surface of the plate was flooded with 1N HCl (Sigma-Aldrich, UK), poring the excess into a designated waste pot. Polymerised DNA precipitated in the presence of HCl, making the medium opaque. If the bacteria growing on the agar produced DNase enzymes in sufficient quantity to hydrolyse the DNA, then clear zones were observed around the bacterial growth.

2.10.3 Haemolysin Assay

Haemolysin production was measured following the method of (Cheung *et al*., 2012). Briefly, fresh bacterial cultures were grown to early exponential phase (OD_{600} 0.3) in MHB. Positive and negative controls were prepared using sd H_2O and sterile MHB respectively. Whole defibrinated horse blood (TCS) (50 µL) was added to each culture (0.95 ml) to a final

concentration of 5%. Mixtures were incubated at 37°C for up to 3 h. After incubation, un-lysed red blood cells were removed from suspension by centrifugation (13,000 rpm) using a table top Microfuge for 4 min at room temperature. The supernatant was transferred to a clean cuvette and absorbance was measured at OD_{540} using a spectrophotometer (WPA Biowave, fisher-scientific, UK).

2.11 DNA Extraction

Bacterial cultures were grown in Luria Bertani (LB) broth for 18-24h at 37°C and DNA was extracted from 1ml using the Isolate II Genomic DNA Kit (Bioline, UK) according to the manufacturer's instructions. An extra pre-treatment step was conducted for efficient lysis of Gram-positive cells. Briefly pellets of harvested bacteria were washed twice with 0.15 M NaCl (Fisher Scientific, UK), 10 mM EDTA (pH 8.0) (Fisher Scientific, UK) and re-suspended in lysozyme (Sigma Aldrich, UK) solution (10 mM Tris-HCl (pH 8.0), 0.1 m M EDTA, 10 mg ml^{-1} lysozyme and 1mg ml^{-1} lysostaphin (Sigma Aldrich, UK), and incubated for one hour at 37°C. Further breakdown of proteins was achieved by adding proteinase K (20 ug ml^{-1}) (Bioline, UK) and 200 µL GL buffer, samples were mixed and incubated at 56 °C for 30 min. DNA was precipitated by adding 210 µL 100% ethanol and vortexed to homogenise the solution. DNA was purified by transferring in to a mini spin column and centrifuged for 1 min at 11,000 x g. Columns were placed in new 2 ml collection tubes, 500 µL GW1 buffer were added and centrifuged for 1 min at 11,000 x g, finally 500 µl GW2 buffer were add and centrifuged for 3 min at 11,000 x g. Pure DNA was eluted from the column with sterile, distilled H_2O. DNA quality was verified by agarose gel electrophoresis and quantified using a Nanodrop spectrophotometer.

2.12 DNA and RNA Quality Control

In this study, both the purity and concentration of DNA and RNA samples was measured by the 'Nano Drop 2000' spectrophotometer (Thermo-Scientific, UK). This was done by Dispensing 1 µl of dsH_2O (Elution buffer) a blank onto the lower optical pedestal of the spectrophotometer, then 1 µl of extracted nucleic acid samples was measured. Sample purity ratios were calculated at 260/230 nm and 260/280 nm for the purity.

2.13 Polymerase Chain reaction (PCR)

Standard PCR technique was used to confirm the identification of *S. aureus* and detection of antimicrobial resistance genes in particular erythromycin resistance (*erm A, erm B* and *erm C*). PCR reactions were prepared using 1x of My Taq Red Mix (Bioline, UK), 10 pmole of each primer; 1 µL template DNA and sdH_2O to final volume 25 µL. Cycling conditions and primer sequences are in detailed (Table 2.6). Positive controls (University confirmed stock strain) and negative controls (sdH_2O) were used as appropriate. After thermocycling had completed 5 µL was removed and subjected to agarose gel electrophoresis to determine quantity, quality, purity, and appropriate size of amplicons.

2.14 Agarose gel Electrophoresis

Extracted DNA and PCR amplicons were analysed by gel electrophoresis. Agarose gels (1.5% (w/v)) (Sigma Aldrich, UK) were prepared using 1x Tris Borate buffer (TBE, Thermo Scientific) supplemented with 1.5 % gel red (Biotium, USA). Sizes were estimated by comparison with 1kb Hyper ladder (Bioline, UK). Samples were separated by size at 70v for

1 h in an electrophoresis tank (Life Technologies, USA; model 250) filled with 1x TBE. DNA was visualized using UV transilluminator (SYNGENE G: BOX-XT14).

Table 2.6 Primers and cycling conditions

Primers	Sequences (5′-3′)	Cycling condition	Size bp	Ref
16SrRNA	F**5′**CGGTCCAGACTCCTACGGGAGGC AGCA'**3′** R**5′**GCGTGGACTACCAGGGTATCTAA TCC**3′**	96°C 3 min 96°C 10 sec 60°C 10 sec 30 cycle 72°C 50 sec 72°C 5 min	191	(Schlegelova *et al*., 2008)
ermA	F- **5′**GCGGTAAACCCCTCTGAG**3′** R- **5′**GCCTGTCGGAATTGG**3′**	96°C 3 min 96°C 10 sec 57°C 10 sec 30cycle 72°C 50 sec 72°C 5 min	434	(Schlegelova *et al*., 2008)
ermB	F- **5′**CATTTAACGACGAAACTGGC**3′** R- **5′**GGAACATCTGTGGTATGGCG**3′**	96°C 3 min 96°C 10 sec 57°C 10 sec 30 cycle 72°C 50 sec 72°C 5 min	425	(Schlegelova *et al*., 2008)
ermC	F- **5′**ATCTTTGAAATCGGCTCAGG**3′** R- **5′**CAAACCCGTATTCCACGATT**3′**	96°C 3 min 96°C 10 sec 57°C 10 sec 30 cycle 72°C 50 sec 72°C 5 min	295	(Schlegelova *et al*., 2008)

2.15 RNA extraction and purification

RNA was extracted from frozen bacterial pellets using the RNeasy Mini Kit (Qiagen, Germany) in combination with the RNase-free DNase kit (Qiagen, Germany) according to the manufacturer's instructions. *S. aureus* cell pellets were re-suspended in lysozyme solution [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 10 $mgml^{-1}$ lysozyme, 1 $mgml^{-1}$ lysostaphin and Proteinase K (Bioline, UK) and incubated for 30 min at 37°C with shaking. Lysis buffer (350 µL) was prepared with β - mercaptoethanol (10 μl^{-1}, Sigma- Aldrich) to reduce the disulfide bound and then vortexed for 1 min. 700 µL of ethanol (70%) was added to the lysate. A binding step was carried out by adding 700 µL of the lysate to a Mini Spin column and RNA purified according to the manufacturer's instructions (Qiagen, Germany). For DNase digestion, 80 µL of DNase (10 µL of DNase stock solution to 70 µL buffer RDD was added to the column and incubate for 15min). As DNA digested, the RDD buffer provides efficient bound to the RNA in the column. The washing step was proceeded by adding 350 µL of washing Buffer RW1 that removes biomolecules such as carbohydrates, proteins, and fatty acids and then added 500 µL Buffer RPE to spin column respectively, then centrifuged for 15 secs at 8000 xg. Finally, the spin column was placed in new 2ml collection tube with 30-50 µL RNase free water for elution RNA. Eluted RNA was further treated with Turbo DNase (Thermo-Fisher, UK), 1 U/ 1 µg of total RNA at 37°C for 20-30 min according to the manufacturer's instructions to digest single and double stranded DNA. DNase I was inactivated by addition of 2 µL DNase inactivation reagent and incubation for 5 min at room temperature. Finally, the mixture was centrifuged for 2 min at 10,000 xg, and the pure RNA without genomic DNA contamination was aliquoted to a fresh tube. Removal of genomic DNA was confirmed by PCR with *erm C* primers (Table 2.6). The SA3 was previously confirmed positive for this gene. Integrity of total RNA was evaluated by gel electrophoresis at 70 V for 1 h using a 2% (w/v) agarose gel and stained with Gel Red (Biotium, UK), RNA

concentrations were measured by Nano Drop 2000 spectrophotometer (Thermo Scientific, UK) and three independent measurements of the same sample were performed. All RNA isolations resulted in acceptable levels of purity (A260/A280 ≥1.95), ratio of sample absorbance at 260 and 280 nm of ~2.0, samples were stored at - 80°C for further use.

2.16 Realtime PCR

2.16.1 Complement DNA (cDNA) synthesis

Complement (c)DNA was synthesized with integrated removal of genomic DNA contamination by using QuantiTect Reverse Transcription (Qiagen, Germany). One microgram of each purified RNA sample was incubated in gDNA Wipe-out Buffer at 42°C for 2 minutes to effectively remove any traces of contaminating genomic DNA. Then the reverse-transcription master mix (containing 1 µL of Quantiscript Reverse Transcriptase and 4 µL of Quant script RT Buffer) was prepared on ice, and then the Reverse-transcription reaction components added to purified g DNA. Wipe-out Buffer and incubated at 42°C for 15 min. The cDNA sample was incubated for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase. The whole cDNA volume was diluted 10-fold for subsequent quantitative real-time RT-PCR reactions. The quality of the cDNA was assessed by using standard PCR with specific primers (*erm C* gene). The SA3 was previously confirmed positive for this gene. The cDNA samples were stored at –20°C.

2.16.2 Design of Primers for RT-PCR gene expression studies

The assembled sequence output from whole genome sequencing of clinical *S. aureus* isolates (see section 2.17) were used for the design of primers. The Artemis Comparison Tool (ACT)

(Sullivan *et al*., 2011), an online software tool for visualizing and comparing multiple DNA sequences was used to identify regions of similarity, insertions and rearrangements across the whole genome of three *S. aureus* wound isolates (SA2, SA3 and SA4). Primers (Table 2.7) were designed to investigate the expression of several genes involved in global regulation, adhesion and biofilm formation by Quantitative real time PCR for all 3 isolates. The following parameters were used for primer design: length (between 18 and 25 residues), 40 to 50% GC content, and melting temperature (ranging from 57 to 63°C), amplicon less than 200 bp. Primer sequences were chosen using primer 3 software (Wang & Seed, 2003) and further verified for absence of dimerization or hairpin formation using the web-enabled Auto Dimer interface.

Table 2.7 Nucleotide sequences and anticipated amplicon sizes for the *S. aureus* SA3 gene-specific oligonucleotide primers used for RT-q PCR in this study (Atshan *et al*., 2013).

Gene	**Function**	**primers sequences (5′-3′)**	**Size**	**Ref**
16S rRNA	*Internal standard gene*	F-5′-CGGTCCAGACTCCTACGGGAGGCAGCA 3′ R 5′-GCGTGGACTACCAGGGTATCTAATCC 3′	191 bp	(Sugiyama *et al*., 2009)
agr A	*Accessory gene regulator*	F/ 5′-TTCCCTCGCAACTGATAAT R/ 5′-ACCAACTGGGTCATGCTTAC	149 bp	This study
sar A	*Synthesis of fibronectin and fibrinogen binding proteins*	F/5′-ACGTAATGAGCATGATGAAAGAAC3′ R/5′-TGTTTGCTTCAGTGATTCGTTT3′	110 bp	This study
ica A	*Intracellular adhesion*	F/5′-GTAAAGCCAACGCACTCAATC3′ R/5′-ACCTGTAACTGCACCAAGTT3′	149 bp	This study
pro C	*Pyrroline-5-carboxylate reductase*	F/5′-GGCAGGTATTCCGATTGA 3′ R/5′-CCAGTAACAGAGTGTCCAAC 3′	106 bp	This study
pyk A	*Pyruvate kinase*	F/5′-ATCGCGGCGTTATTTCATTCG 3′ R/ 5′-ATCCGGCAACGTACTTACTC 3′	99bp	This study
*f*abD	*Malonyl CoA-acyl carrier protein transacylase*	F/5′-TGGCTGCAGTATTGGGATTAG 3′ R/5′- GGGCAATTAATGTTTGCTGGT 3′	100 bp	This study

2.16.3 Real-time- quantitative PCR

cDNA samples generated from SA3cultures exposed to different concentrations of DHA and EPA were used as templates in RT-PCR assays to identify any changes in the regulation of gene expression. As described elsewhere (Paniagua-Contreras *et al.*, 2012), RT-q PCR was performed using SYBER green super-mix (SensiFAST™ SYBR, Bioline) and various genes (*16SrRNA, pro C, fab D*, and *pyk A*) were explored for use as internal controls (Theis *et al*., 2007). Reactions were set up in triplicate with 1µL cDNA, primers (250µM) and 1x SYBR green supermix. The RT-PCR cycling conditions included initial denaturation at 95^{o} C for 5min followed by 40 cycles of denaturation at 95^{o}C for 15 secs, annealing at 60^{o}C for 15 sec and extension at 72^{o}C for 30 sec. The delta-delta ct method was used to assess changes ion gene expression (Rao *et al*., 2013). Briefly, differences in gene expression between "treated" and "untreated" cultures were compared to differences in control gene (*proC*) expression at each time point.

2.17 Whole Genome sequencing

Whole genome sequencing of 3 clinical wound isolates of *S. aureus* and the well-characterised MRSA 252 strain was performed as part of a demonstration by representatives from Illumina during onsite installation of a MiSeq (illumina, USA). The genome libraries were prepared using a Nextera XT library preparation kit according to the manufacturer's instructions. Samples were multiplexed on a single MiSeq flowcell using Nextera XT barcodes and normalised using the bead-based normalisation kit. Sequencing was performed using a TruSeq v2 500 cycle kit (2x250bp reads). Raw data was processed and assembled by Dr Ian Goodhead. Reads were trimmed for adapter sequences and low quality using trimmomatic (v 0.35) and assembled using SPADES v3.8.2. The resulting assemblies were

annotated using PROKKA v 1.11. These assembled genome sequences were compared using the Artemis Comparison Tool (ACT) (Sanger) and used to design primers for real time (RT) PCR.

The genomes of parent and adapted strains of *S. aureus* clinical isolate SA3 exposed to DHA (SA3-DHA); EPA (SA3-EPA); both PUFAs (SA3-DHA+EPA); linezolid (SA3-LZD) and vancomycin (SA3-VAN) were sequenced by Microbes NG. Bacterial cultures were sent to the service on beads according to their instructions. Microbes NG completed DNA extraction, sequencing, trimming and assembly of each genome. Further bioinformatics analysis was performed by Dr Ian Goodhead. The online webtool: Pathogen Watch (formerly WGSA; https://pathogen.watch)software was used to cluster the annotated genomes of each isolate with other *S. aureus* genome sequences from other submitted sequences and to generate a phylogenetic tree to demonstrate relatedness, and to predict antibiotic resistance profiles. Pathogen Watch uses PAARSNP, bespoke software that uses BLAST against a custom database (combined from public AMR databases such as CARD, ResFinder and NCBI) to highlight the presence or absence of genes and SNPs implicated in antimicrobial resistance and infers their combined effect (https://cgps.gitbook.io/pathogenwatch/technical-descriptions/antimicrobial-resistance-prediction/paarsnp). Single-nucleotide polymorphisms (SNPs) in evolved strains compared to parent SA3, were detected using “Snippy” and “Free-bayes bioinformatics analysis software tools. This program identified synonymous (silent) and nonsynonymous (missense or nonsense) SNPs. Synonymous mutations do not change the encoded amino acid but a change in general the DNA sequence, while nonsynonymous SNPs do change the amino acid sequence, more likely resulting of differences in the encoding protein function. The nonsynonymous SNPs consist of two types of mutation, missense and nonsense mutation.

Chapter Three

Antimicrobial and Anti-Biofilm Activity of PUFAs and Antibiotics against Wound Pathogens

Chapter 3 Antimicrobial and Anti-Biofilm Activity of PUFAs and Antibiotics against Wound Pathogens

3.1 Detection of antimicrobial activity

3.1.1 Introduction

Treatment of wound infections has become more challenging due to widespread antimicrobial resistance (AMR), which can develop and spread by mutation selection and horizontal gene transfer (HGT) in protected polymicrobial biofilms within the wound (Bjarnsholt, 2013; Kibret & Abera, 2011; M. Wu *et al*., 2018). Multidrug resistant strains of species such as *S. aureus, E. faecalis, P. aeruginosa, and Es. coli spp.* are commonly found in skin and wound infections (Bessa *et al*., 2015; Kane *et al*., 2014; Phillips *et al.*, 2016). Thus, investment has promoted the development of alternative wound-treatment strategies. Dressings have been developed that are impregnated with metal nanoparticles such as silver (Raghavan *etal*., 2016), gold (Cabuzu *et al*., 2015)or zinc (Yang *et al*., 2017) or with natural antimicrobials such as honey or Manuka honey (Kwakman *et al*., 2010), and essential oils (Seow *et al*., 2014). These antibacterial agents have showed impressive properties that accelerate wound healing processes (Negut *et al*., 2018). However, a more detailed investigation is required to justify the use of alternative antimicrobial agents in wound treatment.

As most infected wounds are complicated by biofilms, it is important to consider the efficacy of any wound treatment in this context. Bacterial cells within a biofilm matrix are less susceptible to antibiotic treatments due to slowed diffusion through the matrix and

slower growth, promoting persistence. The cells are protected within a biofilm matrix that contains mainly bacterial extra polysaccharide(EPS), extracellular protein and DNA as well as excreted host cellular products such as muco-polysaccharides, fibrin, collagen and water (up to 95–99%) (Kostenko *et al*., 2007).This physiological composition plays a significant role in preventing the binding or transport of antimicrobial agents to the cells by either sorption with the bactericides, or electrostatic and hydrophobic interactions, or size exclusion, or degradation of antimicrobial agent (Stewart, 2015). For example, a comparison study of clinical *S. aureus* wound isolates revealed that 87%of biofilm-positive isolates were MDR organisms, and all the biofilm non-producers were non-MDR isolates ($P<0.05$) (Neopane *et al*., 2018).

Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) are two major compounds of omega-3 polyunsaturated fatty acids (n-3 PUFAs) that have been shown to have diverse beneficial effects on human health, including wound healing (Turk *et al*., 2013a). The beneficial effects are believed to be related to the anti-inflammatory and antimicrobial action of PUFAs (Park *et al.,* 2015; Choi *et al*., 2015). The antimicrobial activity of polyunsaturated fatty acids (PUFAs) has been demonstrated against several pathogens, including *S. aureus*, *S. mutans*, *B. cereus*, *P. gingivalis, P. aeruginosa* and *Es.coli* (Chitra Som & Radhakrishnan, 2011; Kim *et al*., 2018; Le & Desbois, 2017; Sun *et al*., 2016).

Several studies are attracting attention to PUFA compound as potential antimicrobial agents, due to their safety, high efficiency, wide spectrum of activity and the lack of resistance mechanism (Chanda *et al*., 2018; Desbois *et al*., 2013). Other studies have shown differences in effectiveness and the potency of DHA and EPA as antibacterial and anti-biofilm agents against several Gram positive and Gram-negative bacteria (Arantes *et al*., 2016; Ilievska *et al*., 2016; Sun *et al*., 2017). The high genetic variations between different

strains would lead to different results (Correia *et al*., 2012; Andrew P. Desbois & Smith, 2010)

This results chapter details an extensive study of the antibacterial activity of DHA and EPA on common wound pathogens. The minimum inhibitory and bactericidal concentrations (MIC and MBC) were determined for these compounds and compared with commonly used antibiotics against a range of clinical and reference strains of bacteria. Biofilm assays provided information about the ability of these strains to form biofilms, and of PUFAs to disrupt their biofilm formation. Whole genome comparison of clinical wound isolates identified a candidate strain on which to base further investigations.

3.1.2 Antibiotic sensitivity tests (AST)

Phenotypic antimicrobial susceptibility testing (AST) measures the susceptibility of microorganisms to different concentrations of antimicrobial agents and enables a drug resistance profile to be determined. Antibiotic susceptibility assays were performed to determine the sensitivities of wound pathogens to various antibiotics that are commonly administered for Gram positive and Gram-negative bacteria in wound infections. Standardised EUCAST protocols were used to expose each strain to set of recommended antibiotic concentrations impregnated into filter paper disks. The method enabled a simple screen of susceptibilities to multiple antibiotics on agar plates. Resistance profiles were determined by comparing zones of inhibition to breakpoint tables for each antibiotic according to EUCAST V8 2018 guidelines (Table 3.1).

Gram-positive bacteria and Gram-negative bacteria did not show the same resistance profiles. Reference strain MSSA 6538 was susceptible to all tested antibiotics while *S. aureus* 252 (MRSA) was resistant to five out of ten antibiotics including imipenem, remaining

susceptible to gentamycin, vancomycin and tetracycline. The *E. faecalis* reference strain 13280 showed resistance to vancomycin, erythromycin and tetracycline. Reference strains of Gram-negative bacteria (*P. aeruginosa* PA01, PA014 and *Es. coli* 10536) were susceptible to the all antibiotics tested. Several clinical wound isolates of *S. aureus* (SA2, SA3 and SA4) were provided by Salford Royal Foundation Trust. SA2 showed resistance to representatives from two different classes of antibiotics lincomycin and erythromycin, whereas the clinical isolate SA3, showed resistance to three different classes of antibiotics (lincomycin, erythromycin, cefoxitin). The cefoxitin is indicative of MRSA.

Conversely, clinical isolate SA4 was susceptible to all classes of antibiotics tested except for erythromycin, lincomycin and cefoxitin. This demonstrated that there is considerable diversity of resistance profiles of *S. aureus* that infect wounds. The most effective antibiotic treatments for this group of isolates and reference strains would be the aminoglycosides (gentamycin and tobramycin), and ciprofloxacin. Finally, these assays suggest that linezolid would be the most effective antibiotic for treating *S. aureus* infection.

Table 3.1 Antimicrobial sensitivity test (AST) against wound pathogens

Antibiotics		**Aminoglycoside**		**β-lactam**	**Glycopeptides**	**Lincos-amides**	**Macrol-ides**	**Oxazolidine**	**Quino-lone**	**Tetra-cycline**	**Carbapenem**
	species	**CN 10 µg**	**TOB 10µg**	**FOX 30µg**	**VAN 5 µg**	**MY 2 µg**	**E 15 µg**	**LZD 10 µg**	**CIP 5 µg**	**TE 30 µg**	**IMP 10 µg**
S. aureus	***SA 2***	S (23)	S (19)	S (22)	S (12)	**R (12)**	**R (13)**	S (26)	S (25)	S (25)	S (26)
	SA 3	S (25)	S (23)	**R (10)**	S (25)	S (14)	**R (15)**	S (24)	S (23)	**R (7)**	S (22)
	SA4	S (19)	S (21)	**R (13)**	S (24)	**R (12)**	**R (14)**	S (25)	S (24)	S (25)	S (25)
	6538	S (25)	S (25)	S (17)	S (17)	S (23)	S (24)	S (27)	S (26)	S (25)	S (26)
	MRSA 252	S (25)	**R (8)**	**R (10)**	S (11)	**R (11)**	**R (12)**	S (22)	**R (16)**	S (25)	**R (8)**
E. faecalis 13280		S (14)	S (10)	S (20)	S (17)	S (19)	**R (16)**	S (22)	S (20)	**R (8)**	S (25)
P. aeruginosa	***PAO1***	S (20)	S (24)	NA	NA	NA	NA	NA	S (27)	S (24)	S (24)
	PA 14	S (23)	S (19)	NA	NA	NA	NA	NA	S (32)	S (25)	S (21)
Es. coli 10536		S (23)	S (20)	NA	NA	NA	NA	NA	S (38)	S (23)	S (23)

KEY: S= Sensitive R= Resistance NA = Not applicable for these strains according to EUCAST 2018

3.1.3 Minimum Inhibitory Concentration (MIC) of PUFA compounds compared to Linezolid and Vancomycin against wound pathogens

Using a broth microdilution method to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents. The PUFA compounds (DHA and EPA) were found to have varied inhibitory effects on the growth of Gram-positive and Gram-negative bacteria (Table 3.2) compared to the gold standard antibiotics linezolid and vancomycin that are commonly used for treating wounds infected with MRSA.

Table 3.2 **Minimum inhibitory concentration (MIC) of PUFAs and antimicrobial agents against common wound pathogens**

Species	Strain	DHA	EPA	DHA +EPA	LZD	VAN
	MIC (µg ml^{-1})					
S. aureus	*SA2	78	117	78	3.9	0.18
	*SA3	65	78	65	3.9	0.093
	*SA4	65	156	156	1.9	0.062
	#6538	65	78	78	1.9	0.12
	ψ252	78	156	117	3.9	0.25
E. faecalis	#13280	156	312	156	3.9	0.062
P. aeruginosa	#PA01	117	234	156	94	0.62
	#PA14	156	234	234	125	1.25
Es. coli	#10536	156	312	156	62.5	0.74

The values in the table represent the minimum inhibitory concentration (MIC), a measure of the effectiveness of PUFAs compounds and the antibiotic concentrations in µg ml^{-1} against wound pathogens. *Clinical wound isolate; # Standard reference strain; ᵠMRSA

The MIC values of DHA against clinical wound isolates SA2, SA3, SA4, and reference strains MSSA6538 and MRSA 252 were very similar 65 - 78 µg ml^{-1}. DHA

displayed different activity against *E. faecalis* 13280, PA14 and *E coli*10536 (156 µg ml^{-1}) and PA01 (117 µg ml^{-1}). The PUFA compound, EPA showed slightly different levels of inhibition, with the strongest activity against SA3 and MSSA 6538 (MIC 78 µg ml^{-1}) and weakest activity against *E. faecalis* 13280 and *E coli*10536 (MIC 312 µg ml^{-1}). Interestingly, the combination of DHA+EPA together did not result in any synergistic effect. The MIC of the combined compounds was comparable with DHA alone and lower than that of EPA alone against clinical isolates SA2 and SA3 and reference strains of *E. faecalis* and *Es. coli*. Conversely, the activity of the combination was more comparable with EPA alone against SA4, MSSA6538 and PA14. Finally, the MIC of the combination was in between the MICs for DHA and EPA when tested against PA01 and MRSA reference strains.

In general, linezolid was observed to be more potent than the PUFA compounds and showed higher inhibitory activity against Gram-positive bacteria than Gram-negatives. This antibiotic was most active against MRSA252 and the clinical isolate SA4, with MICs of 1.9 µg ml^{-1}. Linezolid also effectively inhibited the Gram-positive bacteria SA2, SA3, MSSA6538 and *E. faecalis* at a MIC of 3.9 µg ml^{-1}. However, this antibiotic linezolid showed much less potency against Gram-negative bacteria as expected. The MIC for *Es. coli* was 62.5 µg ml^{-1} and even less active against *P. aeruginosa* strains PA01 and PA14 with MICs of 94, 125 µg ml^{-1} respectively. These MICs are in line with expectations since linezolid has been reported to be effective against these Gram-positive bacteria (MIC 0.5 – 4 µg ml^{-1}) and that aerobic Gram-negative bacteria are largely resistant (Livermore, 2003).

This study showed vancomycin to be the most highly potent inhibitor of the Gram-positive bacterial strains tested, with the highest activity against clinical *S. aureus* isolate SA4 and *E. faecalis* reference strain 13280 (0.062 µg ml^{-1}). The lowest activity was observed against MRSA (MIC 0.25 µg ml^{-1}). Vancomycin was less active against Gram-negative bacteria (*P. aeruginosa* strains PA01 and PA14, and *Es. coli* strain 10536) with MICs of 0.62,

1.25 and 0.74 µg ml^{-1} respectively. However, these MICs were lower than reported elsewhere (4 – 128 µg ml^{-1}; Hamoud *et al*., 2014).

3.1.4 Minimum bactericidal concentration (MBC) of PUFA compounds compared to Linezolid and Vancomycin against common wound pathogens

Further analysis of the antimicrobial effect of PUFAs was determined using bactericidal assays. The minimum bacteriocidal concentration (MBC) for each PUFA compound was compared to that of classic antibiotics linezolid and vancomycin. Liquid culture from each well of the micro-dilution plate was spread onto MH agar plates and colony forming units (CFU ml $^{-1}$) were recorded (Table 3.3).

Table 3.3 **Minimum bactericidal concentration (MBC) of PUFAs and antibiotics against common wound pathogens**

		MBC (µg ml^{-1})				
Species	Strain	DHA	EPA	DHA +EPA	LZD	VAN
Staphylococcus aureus	*SA2	312	468	312	15.6	0.72
	*SA3	260	312	260	31.2	0.37
	*SA4	520	520	625	31.2	0.48
	#6538	260	312	312	31.2	0.48
	ψ252	520	625	520	15.6	1
E. faecalis	#13280	625	936	936	31.2	0.48
P. aeruginosa	#PA01	468	625	625	376	1.86
	#PA14	520	625	520	375	2.5
Es.coli	#10536	625	936	625	250	2.96

* Clinical wound isolate; #Standard reference strain; ΨMRSA

Of the PUFAs, the MBC of the DHA compound exhibited the highest bactericidal effect, with most killing activity against *S. aureus.* The highest activity was observed against the clinical wound isolate SA3 and the MSSA reference strain 6538 (MBC 260 µg ml^{-1}). The lowest killing activity was observed against *Es. coli* and *E. faecalis* reference strains (MBC 625 µg ml^{-1}). Differences were observed in the bactericidal activity of EPA compared to DHA. Slightly higher MBCs of EPA were recorded for all bacteria tested, except for clinical isolate SA4, which was equally susceptible to the bactericidal effects of EPA and DHA (MBC 520 µg ml^{-1}). The highest activity was still observed against SA3 and 6538 (312 µg ml^{-1}) and the lowest against *E. faecalis* 13280 and *Es. coli* 10536 (MBC 936 µg ml^{-1}). As observed during MIC assays, the combination of PUFAs (DHA+EPA) had no synergistic effect on killing. The lowest MBC of the mixed compound was 260 µg ml^{-1}, comparable with DHA killing of SA3. The highest concentration of this mixture of PUFAs was required to kill the reference strain of *E. faecalis* 13280 (936 µg ml^{-1}), comparable with EPA alone.

Linezolid was clearly more effective at killing Gram-positive bacteria than Gram-negative bacteria. This antibiotic showed most killing activity against clinical *S. aureus* strains, SA2 and MRSA (MBC 15.2 µg ml^{-1}), while still effectively killing SA3, MSSA 6538, MRSA 252 and *E. faecalis* 13280 (MBCs 31.2 µg ml^{-1}). Considerably higher concentrations of linezolid were required to kill *P. aeruginosa* strains (PA01 and PA14 – MBC 375 µg ml^{-1}) and *Es. coli* strain 10536 (MBC 250 µg ml^{-1}). The bactericidal activity of vancomycin was the strongest, with an MBC of 0.37 – 1.0 µg ml^{-1} against all *S. aureus* and *E. faecalis* strains. Higher MBCs were observed for Gram-negative bacteria (between 1.86 and 2.96 µg ml^{-1}).

3.1.5 Tolerance of common wound pathogens to PUFAs and antibiotics

Tolerance and persistence refer to the capacity of bacteria for prolonged survival under exposure to bacteriocidal agents without acquiring phenotypic resistance. Treatment failures can often result from tolerance of the infecting pathogen to antibiotics (Gefen *et al*., 2017; Gonzalez *et al*., 2013). One method for quantifying tolerance is by calculating the ratio MBC/ MIC.Table 3.4 reveals that *P. aeruginosa* strain PA14 has the lowest tolerance capacity (in the range of 2 – 3.3) and *S. aureus* strain SA4 had an increased tolerance to all compounds overall (in the range of 3 – 16). In general, the bacterial strains tested showed least tolerance to the PUFA compounds (MBC/MIC ratios of 2.2 - 8). Moreover, the most predominant tolerance value was 4 with most pathogens. Surprising, the SA4 strain and MSSA 6538 strain had the highest tolerance to Linezolid (16.4) compared to other strains, while SA4 and *E. faecalis* 1328 showed high tolerance to vancomycin (7.7).

Table 3.4 Wound pathogens tolerance to antimicrobial compounds.

Species	Strain	DHA	EPA	DHA +EPA	LZD	VAN
S. aureus	*SA2	4	4	4	4	4
	*SA3	4	4	4	4	3.9
	*SA4	8	3.3	4	16.4	7.7
	#6538	4	4	4	16.4	4
	ψ252	6.6	4	4.4	4	4
E. faecalis	#13280	4	3	6	4	7.7
P. aeruginosa	#PAO1	3	2.6	4	4	3
	#PA14	3.3	2.6	2.2	3	2
Es. coli	#10536	4	3	4	4	4

*Clinical wound isolate; #Reference strain; ψMRSA; LAZ: Linezolid; VAN: Vancomycin

3.2 Quantification of biofilm formation by wound pathogens

3.2.1 Biofilm detection on Congo Red Agar plates (CRA)

The growth of common wound pathogen representatives was observed on Congo red agar (CRA) plates. Black colonies with a dry crystalline consistency reported exopolysaccharide production in the presence of Congo red indicator, suggesting capacity to form biofilms (Sharvari & Chitra, 2012). The clinical *S. aureus* isolates SA2 and SA3, and the characterised MRSA 252 strain, produced dark black colonies within 24h, indicating strong production of exopolysaccharides which is the staphylococcal polysaccharide intercellular adhesin (PIA) and capacity to form biofilms (Otto, 2008). Conversely MSSA reference strain 6538, clinical *S. aureus* SA4 and *Es. coli* 10536 all produced colonies that appeared brown to black after 24 h, being classified as weak exopolysaccharide production (Sharvari & Chitra, 2012). *P. aeruginosa* strains PA01 and PA14 showed dark black colonies within 24 h and were classified as being strong biofilm producers.

3.2.2 Crystal violet assay to compare densities of biofilms formed by wound pathogens

The Congo red assay only indicates the production of certain exopolysaccharides. Biofilm formation can be screened using a basic crystal violet assay to measure the density of biofilms produced on the solid surfaces of microtiter plates. This study grew each representative wound pathogen in Luria Bertani broth (LB) and measured biofilm density after 24h and 48 h. After removal of planktonic cells and washing, crystal violet stained the biofilm complex that was adherent to the wells of the microtiter plate. Following solubilisation with ethanol, the optical density of biofilms was measured (OD_{600}).

Biofilm density readings indicated that all representative wound pathogens were strong biofilm producers. *S. aureus* SA2, SA3, SA4 and MSSA 6538 showed a similar pattern of

biofilm growth after 24 h, with the mean OD_{600} of 1.1. These densities all increased after 48h incubation to 2.0, significantly denser compared to 24 h biofilms (P = 0.00037). MRSA strain 252 produced the thickest biofilm compared to other *S. aureus* strains (mean OD_{600} 1.9 after 24h, increasing to 2.4 after 48h (P= 0.000135). *P. aeruginosa* strains, PA01 and PA14, were indicated as the strongest biofilm producers, with OD_{600} 2.2 within 24 h, increasing to 3.3 (P=0.00008). *E. faecalis* 13280 and *Es. coli* 10536 produced the weakest biofilm growth measurements of 0.35 and 0.8 respectively, increasing to 0.75 and 1.5 respectively after 48 h with (P =0.003473).

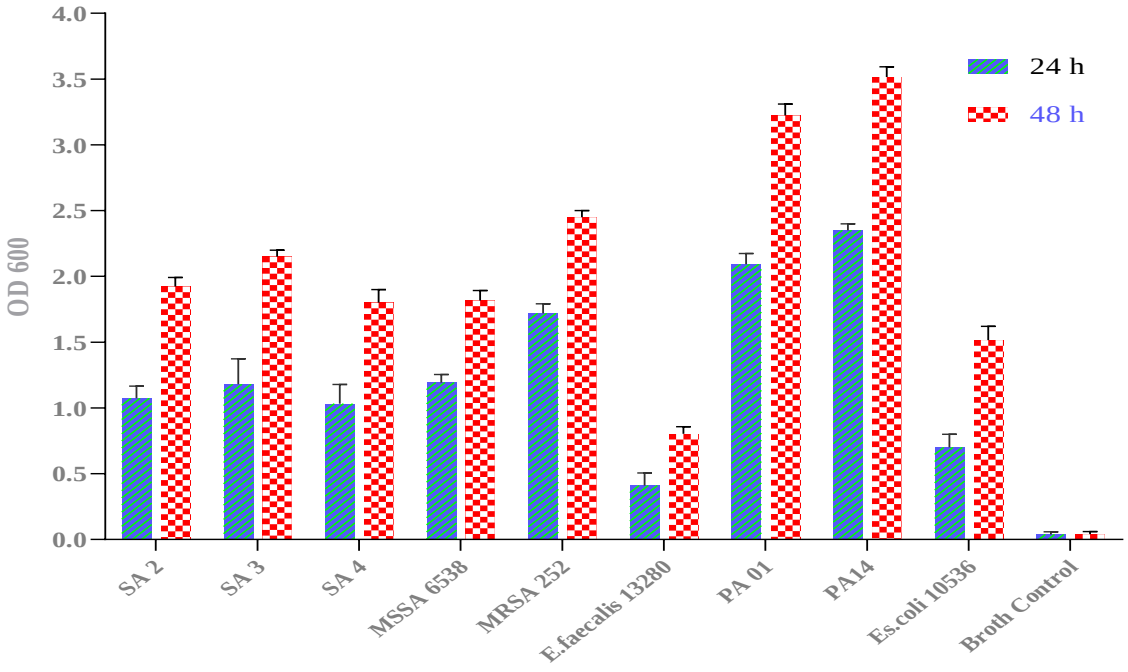


Figure 3.1 **Quantification of biofilm density formed by representative wound pathogens** Detection of biofilm-formation against wound pathogens within 24h and 48h. This assay was performed using the CV method, the error bars represent standard deviation. Un-inoculated, sterile LB was used as a negative control. The values are plotted as mean ± SD obtained from three representative experiment performed in triplicate.

3.2.3 The efficacy of antimicrobial agents on biofilm disruption

The Calgary biofilm device was designed to allow growth of biofilms for longer periods than the basic microtiter plate assays. A 96 well plate with pegs built into the lid allowed the biofilm to develop on the pegs attached to the lid, which can be transferred to a 2nd plate containing fresh media. The device is also useful for directly comparing the ability of multiple test agents to inhibit or eradicate biofilm formation. The Minimum biofilm inhibitory concentration (MBIC) is the lowest concentration that inhibits and prevents initial biofilm formation, defined as the last well in which there is no visible biofilm growth after incubation. The minimum biofilm eradication concentration (MBEC) is defined as the minimum concentration of antimicrobial agent required to disrupt the already formed biofilm (Reiter *et al*, 2013). MBIC and MBEC assays were used to quantify the ability of PUFAs and antibiotics (linezolid and vancomycin) to disrupt biofilms formed by representative wound pathogens. Sterile MH broth alone served as a negative control. OD values of these wells were subtracted from the OD values of the test wells (Figure 3.2).

Antibiotics were clearly the most effective biofilm inhibitors, with much lower MBIC values than those for the respective PUFAs. Vancomycin inhibited Gram-positive bacterial biofilms with MBICs between 1.25 and 2.5 µg ml^{-1}. Linezolid was more effective at inhibiting biofilms formed by Gram-positive bacteria (MBIC ranging from 20.5 to 83.5 µg ml^{-1}) than Gram-negative bacteria (MBIC ranging from (300 to 416 µg ml^{-1}). PUFAs were also more effective at inhibiting biofilms formed by Gram-positive bacteria than Gram negative bacteria. DHA treatment (MBIC 195- 468µg ml^{-1}) was more effective than EPA (MBIC 260- 520 µg ml^{-1}) and co-treatment with both PUFAs (MBIC 260 - 520 µg ml^{-1}). Although less effective, DHA was also better than EPA at inhibiting the biofilms formed by *Es. coli* 10536 (MBICs 625 and 520 µg ml^{-1} respectively) and *P. aeruginosa* strains (MBICs 937 - 1250 µg ml^{-1} respectively) (Figure 3.2A)

MBEC assays were used to assess the disruption of biofilms that were grown on pegs in 96 well micro-titre plates. As expected, a higher concentration of each agent was required to disrupt pre-formed biofilms than to inhibit their growth. Vancomycin was the most potent biofilm disrupter, with MBECs ranging from 5-12.5 µg ml^{-1}. These concentrations were 35-fold lower than the MBECs recorded for linezolid (125 -500 µg ml^{-1}). Considerably higher concentrations of PUFA compounds were required to disrupt biofilms. The MBECs of DHA ranged between 750 and 3125 µg ml^{-1}. EPA was found to be more disruptive than DHA (MBECs ranging between 500 and 2250 µg ml^{-1}), while the MBECs of combination treatment with both PUFAs (DHA+EPA) ranged from 750 - 1500 µg ml^{-1} (Figure 3.2B). Although the antimicrobial properties of PUFAs have already been reported, the data presented in this chapter demonstrate antimicrobial activity against growth and biofilm formation of clinical wound isolates of *S. aureus* with different antibiotic sensitivity profiles. Although the antibiotics (vancomycin and linezolid), were more potent inhibitors, the PUFA compounds have additional immunomodulatory effects for the promotion of wound healing (Allam-Ndoul *et al.*, 2017; Magee *et al.*, 2008; Turk *et al.*, 2013). In addition, bacteria generally showed reduced tolerance to PUFAs compared to antibiotics.

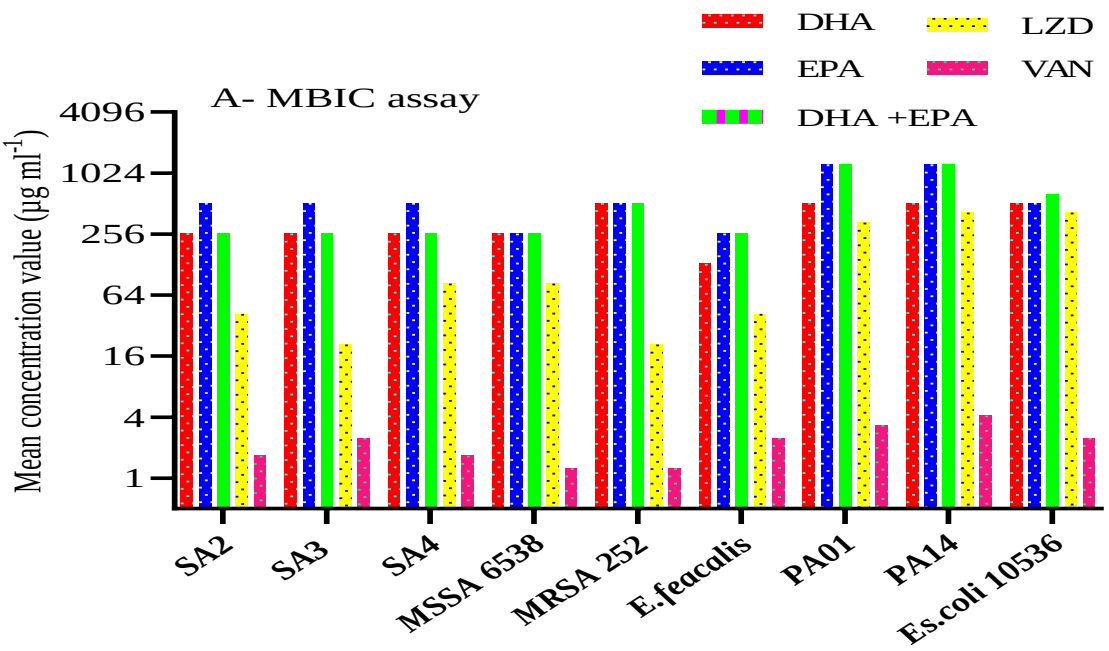


Figure 3.2**: A- Minimum biofilm inhibitory concentration (MBIC) of PUFAs compound and antibiotics against wound pathogens**. The static biofilm assay was performed with the Calgary biofilm device., bar graph shown the difference between PUFAs compounds and Antibiotics groups. Statistical analysis was performed using the unpaired t test using graph pad V8. Significant differences with all mean concentrations of MBICs antibiotics vs All mean MBIC PUFA s = <0.0001, significant differences MBICs antibiotics Gram +ve vs MBICs antibiotics Gram -ve = 0.012.

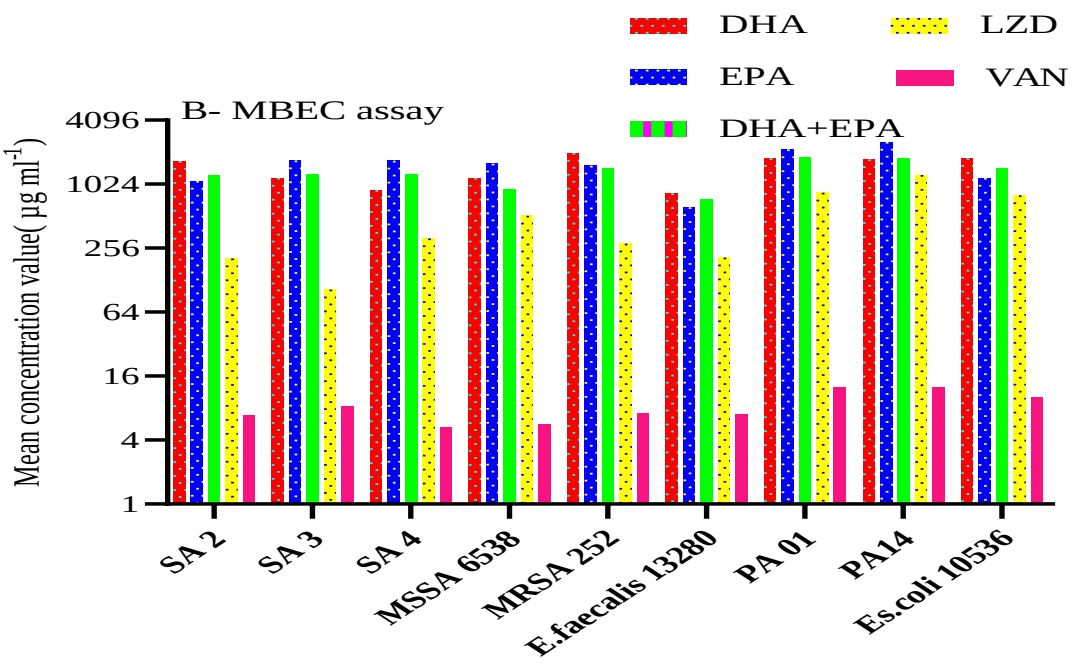


Figure 3. 3**: B- Minimum biofilm eradication concentration (MBEC) of PUFAs compound and antibiotics against wound pathogens**. Significant difference to All MBICS VS All MBECS P value = <0.0001, significant difference to all MBECs antibiotics vs all MBEC PUFA= <0.0001, No significant MBECs antibiotics Gram +ve vs MBECs antibiotics Gram -ve = 0.053, significant MBEC PUFA Gram +ve VS MBEC PUFA Gram-ve = 0.0074.

3.2.4 Selection of isolates for further investigation

The whole genome of each clinical *S. aureus* isolate (SA2, SA3 and SA4) was sequenced to determine their relatedness and their similarities to other reported *S. aureus* strains. The genetic basis of their antibiotic resistance phenotypes was also investigated. Isolates SA3 and SA2 clustered most closely with the sequence types (ST) 398 and 36 that included the MRSA strain 252. Isolate SA4 clustered more closely with a separate clade, showing most similarity to ST 72 (Figure 3.3). Genes associated with resistance to β lacatams antibiotics such as *mecA gene*, which encodes for an alternative penicillin-binding protein, PBP 2a were identified in SA2 and SA3 (Wielders *et al*., 2002). The genes encoding for the ribosomal methylase have been designated *ermA, ermB, and ermC* that are involved in macrolides, lincosamide, and streptogramin B (MLS_B) (Ghanbari *et al*., 2016) and were also detected in SA2 and SA3.No obvious antibiotic resistance genes were identified in the SA4 genome (Table 3.5). These genetic profiles do not fully match the phenotypic profiles observed using the Kirby Bauer disk diffusion assay. This differences may be of multiple resistance alleles occur on the same genome or plasmid (Allen *et al*., 2017). Since SA3 most resembled an MRSA profile, this isolate was chosen for further analysis of PUFA activity.

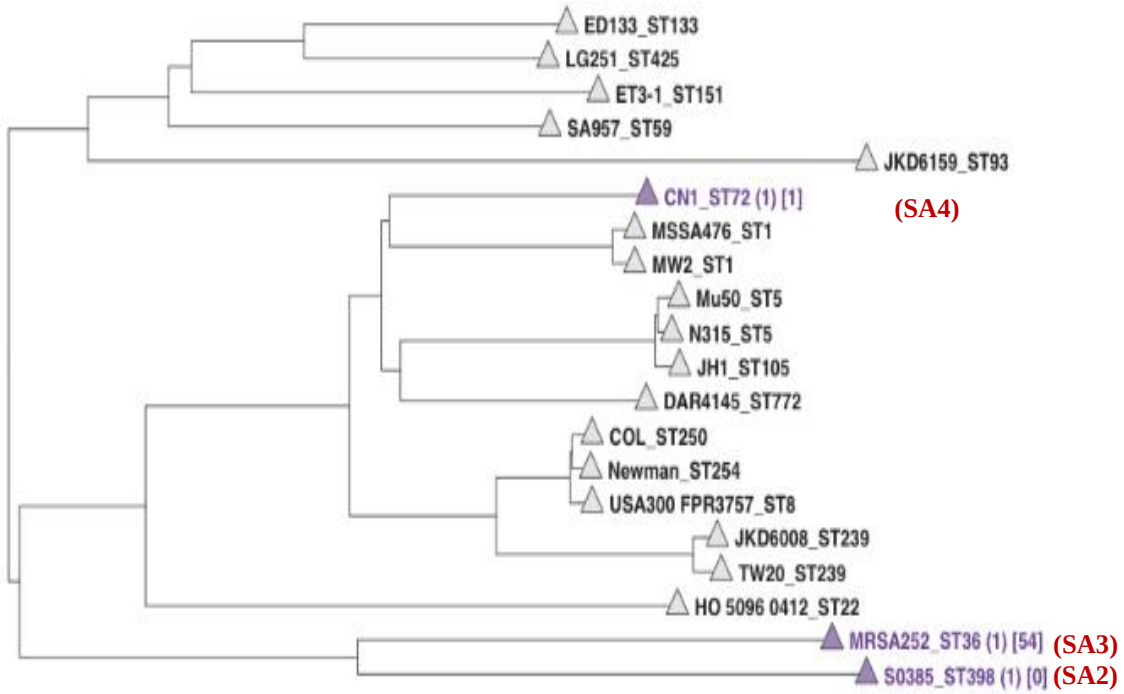


Figure 3.4 **Relatedness of Clinical *S. aureus* wound isolates.** Comparison of phylogenetic tree of the staphylococcal species and clinical *S. aureus* isolates generated under the illumina Next generation sequencing

Table 3.5 **Presence of antibiotic resistance genes in clinical *S. aureus* genomes (from pathogen watch online webtool** https://pathogen.watch)

Strains	Ampicillin	Tobramycin	Kanamycin	Methicillin	Penicillin	Vancomycin	Ticarcillin	Clindamycin	Erythromycin	Mupirocin	Linezolid	Trimethoprim	Daptomycin	Rifampicin	Ciprofloxacin
SA2	-	-	-	-	R	-	-	R	R	-	-	-	-	-	-
SA3	-	-	-	-	R	-	-	R	R	-	-	-	-	-	-
SA4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MRSA 252	R	-	-	R	R	-	-	R	R	-	-	-	-	-	-

- The antimicrobial susceptibility from whole-genome sequencing within a single species, three clinical isolates of *S. aureus* compared to the reference strain MRSA 252 strain. - = sensitive, and R =Resistance

3.3 Discussion

The major findings presented in this chapter suggest that PUFAs have bacteriostatic, bactericidal and anti-biofilm activity against wound pathogens, particularly *S. aureus*. A diversity of resistance profiles was recorded for different *S. aureus* strains. As expected, the MSSA strain 6538 was susceptible to all antibiotics used in the study and the MRSA strain 252 strain showed resistance to cefoxitin, erythromycin, lincomycin, ciprofloxacin and imipenem. Clinical isolates of *S. aureus* were resistant to lincomycin and erythromycin.

This study compared the antimicrobial activity of PUFAs with the gold standard antibiotics currently recommended for treating *S. aureus* infected wounds. The data presented in this chapter shows that linezolid and vancomycin were much more active than PUFAs against a range of common wound pathogens. The MICs presented in this chapter were similar to the findings of others. For example, Vanegas *et al*., (2017) found an isolate of MRSA to be sensitive to linezolid (ranged from 0.38 to 4.0μgml^{-1}) (Vanegas *et al*., 2017). Similar findings were reported by Mottola *et al*., (2016) who found no resistance to linezolid when testing bacterial isolates from diabetic foot patients (MICs ranged from 1-2 μg ml^{-1}). Vancomycin was also highly effective against these strains (MICs 0.062 - 0.25μg ml^{-1}) (Mottola *et al*., 2016) . These results are in range with another study (Gonzalez *et al*., 2013), and within the desired range (0.5–2 μg ml^{-1}) as recommended by the EUCAST 2018 V8 for vancomycin MICs guidance (EUCAST, 2018). MICs of vancomycin against 30 clinical isolates of *S. aureus* were all observed to be ≥1.5μg ml^{-1} (Fabri *et al*., 2018). Despite evidence that vancomycin and linezolid are still largely effective, there have been reports of resistance developing. The determination of MIC by using Epsilometer test (E test) study found that 3.1% of MSSA isolates and 14.8 % of MRSA isolates to be resistant to vancomycin (Azhar *et al*., 2017). These resistance profiles confirm the urgent need for alternative therapeutic strategies for wound treatment.

The antimicrobial effects of DHA and EPA observed during this investigation are consistent with previously published studies. In general, there are consistent reports that PUFAs have bacteriostatic activity; that they are more effective against Gram positive bacteria than Gram negative bacteria; that DHA is more potent than EPA and that there is no synergistic effect, when bacterial cultures are exposed to the two PUFAs together. There are however, differences in reported MICs. For example, Le and Desbois (2017) found that EPA inhibited the growth of *S. aureus* with MICs of 64 µg ml^{-1} (Le & Desbois, 2017). Others have reported that PUFAs are slightly less effective against MRSA, with MIC 128 µg ml^{-1} (Desbois & Lawlor, 2013). Furthermore, Shin *et al.,* (2007) reported much higher MIC of EPA against *S. aureus* strains ranging from 750 to 1350 µg ml^{-1}. This same study reported higher MICs against *P. aeruginosa* (MIC 500 µg ml^{-1}) compared to this study (MIC 156 µg ml^{-1}), but they used strain *KCTC2004,* whereas this study used strains PA14 and PA01 (Shin *et al*., 2007). These observed differences in MICs can be explained by different strains been used or genetic variation of the strains.

The antibacterial activity of DHA and EPA against other bacterial species have been demonstrated in several studies. For example, DHA has been shown to inhibit the growth for *B. cenocepacia* K56-2 with comparable MICs ranging from 100 - 175 µg ml^{-1} (Mil-Homens *et al*., 2012) and EPA is effective against *Bacillus cereus* (MIC128 µg ml^{-1}) (Le & Desbois, 2017a) and *Bacillus subtilis* (MIC 500 µgml^{-1}) (Shin *et al.*,2007). There is also reported activity against non-aerobic species. PUFAs have been shown to inhibit growth of the oral pathogen *Porphyromonas gingivalis* KCTC 381 (EPA MIC 9.7µg ml^{-1}) and *P. gingivalis* KCTC 5352 (EPA MIC 78 µg ml^{-1}) and DHA MIC 9.7, 312 µg ml^{-1}respectively , and *Streptococcus mutans* KCTC 3065 (DHA MIC 625µgml^{-1}) and *S. mutans* KCTC 3289 (DHA MIC1250 µgml^{-1})and EPA MIC 625,9.7 µgml^{-1}) respectively (Choi *et al*., 2013). The same

authors also reported antifungal activity against *Candida albicans* KCTC 17484 (DHA MIC 1250 µgml^{-1} and EPA MIC 625µgml^{-1}) (Choi *et al*., 2013).

This chapter observed the bacteriocidal activity of DHA and EPA and revealed a broad spectrum of activity against various Gram-positive bacteria and Gram-negative bacteria. Whilst this study shows MBC of 260 - 625 µgml^{-1} against *S. aureus* strains, PUFAs were less effective at killing other species (*E. faecalis* MBC, 625 -936 µgml^{-1}). These results suggest that PUFAs are less bactericidal than other studies, that report MBC of 128 µgml^{-1} for *S. aureus* (Le & Desbois, 2017). Other have shown that both DHA and EPA were capable of totally killing of *S. aureus* UA159, with the same MBC value of 100 mM (Sun *et al*., 2017). Again, this current study suggests that, as with bacteriostatic activity, the killing activity of PUFAs was much weaker against Gram-negative species (MBC range 468- 936 µgml^{-1}) compared to Gram-positive species.

The MIC and MBC activity of PUFAs compound showed slightly differences in bacteriostatic and bacteriocidal effect, This may be of unsaturation and chain length between the two compound (DHA and EPA) positively correlated with higher cytotoxic potencies of the tested compounds (Kinsella & Black, 1993). However, the concentrations of inhibition and killing vary depending on the bacterial strains and species, these results confirmed that PUFA have bacteriostatic and bactericidal activity against Gram positive and negative bacteria.

Bacterial biofilms add challenges to the management of wound infection (Bjarnsholt, 2013; Rhoads *et al*., 2008). Biofilm formation is a significant factor in the development of chronic infection and allows for immune evasion as well as resistance to antimicrobial agents (Bjarnsholt, 2013). The tissues surrounding a biofilm-infected wound remain in a persistent

inflammatory state (Zhao *et al*., 2016). The biofilm matrix has been widely reported to confer decreased susceptibility to antibiotics (100 –1000 fold) (Beyth *et al*., 2015). It has been suggested that decreased susceptibility results from slower metabolic activity and growth rates, slower diffusion through extracellular polymeric substances and other specific resistance mechanisms. Thus, when assessing the efficacy of potential wound treatments, it is not sufficient to rely on the results of standard MIC and MBC assays that only involve planktonic culture. It is crucially important to assess the activity of wound treatments on biofilms.

This study used semi-quantitative techniques to compare the biofilm forming capacity of a range of bacterial strains representative of common wound pathogens. The Congo red agar plate method was used to assess the production of exopolysaccharides. Some colony growth on the medium was observed to be typical dry crystalline and black in colour. Others produced black colonies but appeared smooth and non-crystalline when the test was repeated in triplicate. This method was used as an indirect indication of biofilm formatting capacity, but the results suggested it to be un-reliable. The micro-titre plate assay is a more reliable method for comparing the differences in the biofilm density. The results for this assay depend on the medium composition (Haney., 2018), growth phase, timescales and other environmental parameters such as temperature and pH (Speranza, 2011).

This study observed that *Pseudomonas* species were the strongest biofilm producers, in particular, PA14 produced the most dense biofilms, and this corresponds with published data that investigate the metabolic activity of the biofilms formed by *P. aeruginosa* and MRSA following exposure to different antibiotics (Haney *et al*., 2018). The weakest biofilm producer was *E. faecalis*13280. All of the *S. aureus* strains showed similar biofilm forming capacities. MRSA252 was the strongest biofilm producer, followed by clinical isolates SA3 and SA2, with strains 6538 and SA4 producing the least dense biofilms. Differences in

biofilm density compared to other studies may be due to strain differences. However, similar trends can be observed. For example, Sigh *et al*., (2012) investigated the effect of meropenem and vancomycin on biofilm production by *P. aeruginosa* ATCC 700888andmucoid *S. aureus* ATCC 29213. The biofilm density was significantly higher at all time points sampled for the *P. aeruginosa* strain compared to the *S. aureus*, and antibiotics were more effective in disruption of *S aureus* biofilms than *P. aeruginosa* biofilms (Singh *et al*., 2012). Meanwhile, Furukawa *et al*.,(2010) showed that *S. aureus* A 7510 formed thick biofilms and was more resistant to strong acidic /alkaline cleaning agents than *Es. coli* K-12 MG 1655 (Furukawa *et al.*, 2010).

The antibiofilm activity of PUFA compounds and antibiotics were measured using a microtiter plate Calgary device assay with pegs to determine MBIC and MBEC. PUFA compounds were found to be more effective at inhibiting biofilm formation by Gram-positive bacteria than Gram-negative species. The MBIC is a measure of early inhibition of biofilm formation rather than disruption of mature biofilms which required higher concentrations (MBEC). The same trend was observed, in both assays, with more efficient disruption of biofilms formed by Gram-positive bacteria. As expected, the observed MBICs of PUFAs were considerably higher than the MICs, and more closely resembled the MBCs recorded for each strain. These results are consistent with published data (Kim *et al*., 2018). However, there is considerable variation in the effective concentrations reported. This can be explained by an inconsistency in techniques used to assess the extent of biofilm inhibition. Kim *et al* (2018) used confocal laser scanning microscopy to quantify the effect of DHA and EPA on biofilm formation. They showed that concentrations as low as 20 µg ml $^{-1}$ (>10-fold lower than this study) markedly inhibited biofilm formation by *S. aureus* 6538 (Kim *et al*., 2018). Confocal microscopy is a more precise and sensitive method, and conditions differed considerably to the parameters used for the Calgary plate assays. The most effective

treatment against wound infections is not only to prevent early biofilms but also to inhibit biofilms that have already formed. Interestingly, in our study, DHA and EPA not only inhibited biofilm formation but also disrupted pre-formed biofilms. The MBEC assays show that PUFAs, in the range of 750- 3125 µg ml^{-1}, disrupted biofilms formed by all wound pathogens used in the study. However, the microtiter plate assay has limitations, and does not fully reflect a true biofilm. The nature of a closed system, as well as inconsistencies in crystal violet binding and in washing can contribute to misleading results. A further investigation of this activity with continuous flow and confocal laser microscopy would give a more accurate indication of antibiofilm activity. Sun *et al* (2016) used confocal laser microscopy to evaluate the activity of PUFAs on *P. gingivalis* biofilms. They revealed that the viability and thickness of cells were reduced in the presence of PUFAs, and characterized obvious cellular distortion when mature biofilms were exposed to 100 mM of DHA or EPA (Sun *et al*., 2016).

The present study shows that the antibiotics, linezolid and vancomycin have stronger anti-biofilm activity than PUFA compounds and that vancomycin was by far the most potent biofilm inhibitor in all cases. The activity against Gram-positive bacteria generally agree with other studies. However, this study has showed the disruption of biofilm with vancomycin in Gram positive bacteria (MBIC 1-2.5µg ml^{-1}, and MBEC 5-8µg ml^{-1}), while a higher concentration of linezolid was required to disrupt biofilms with (MBIC 20-85µg ml^{-1}, and MBEC 62-500µg ml^{-1}). Others have reported a wide range of MBICs for linezolid (1-1024 µg ml^{-1}) and vancomycin (1 -16 µg ml^{-1}) depending on several clinical isolates of *S. aureus* from diabetic foot ulcers. The same study reported similar MBECs (4 - >1024 µg ml^{-1} for linezolid and 8 ->1024 µg ml^{-1} for vancomycin (Mottola *et al*., 2016). Another study on clinical methicillin-resistant isolates of *S. epidermidis* showed the MBIC for linezolid and vancomycin were 2-128µg $ml^{-1}$and 4-256 µg ml^{-1}respectively, while the MBECs of linezolid, ranging from 4-256 µg $ml^{-1}$and 32 -256µg ml^{-1} for vancomycin (Reiter *et al*., 2013).

Vancomycin and linezolid clearly have better antibacterial and antibiofilm activity against common wound pathogens. However, DHA and EPA exhibited some activity and may be of additional use in wound treatments since they may impose less selective pressure and have other wound healing properties. Several studies have revealed that both DHA and EPA play significant role for resolving inflammation by reducing the levels of proinflammatory cytokines (IL-1, IL-2, IL- 6, IL-8, IL-1β, TNFa, TNF-2, and MCP1) (Cheng *et al*., 2015; Lu, *et al* ., 2010; Saini & Keum, 2018). Chang*et al.,* (2015) demonstrated that DHA enhanced the survival of transgenic zebrafish when challenged with *Vibrio vulnificus.* Anti-inflammatory effects of DHA were observed, resulting in decreased mortality and reduced hepatocyte damage (Cheng *et al*., 2015).

In conclusion, PUFAs could be considered as key alternatives to antimicrobial treatment for multidrug resistant organisms that have developed resistance to vancomycin and linezolid. Although a higher concentration is required. PUFAs at these concentrations can be considered non-toxic (Sun *et al*., 2017), and have additional anti-inflammatory effects to promote wound healing. However, it is important to consider the efficacy of these compounds further *in vivo* and the likelihood of resistance development following long term exposure to sub-inhibitory concentrations.

Chapter Four

Effect of PUFA compounds and antibiotics on the virulence of *S. aureus* in infection models

Chapter 4 Effect of PUFA compounds and antibiotics on the virulence of *S. aureus*

4.1 Introduction

It is well known that antibiotics select for antibiotic resistant bacteria. The speed of bacterial replication and high rate of mutation allows rapid adaptation to stressful conditions. Any mutation that allows a bacterium to avoid growth inhibition or killing, confers a selective advantage, even if it affects important phenotypes. For example, the acquisition of phage-resistance to MRSA and VRSA strains can alter several characteristics including reduced growth rate, and reduced expression of virulence factors such as terminal N - acetylglucosamine from cell wall teichoic (Capparelli *et al*., 2010). In the search for novel antimicrobials, agents have developed that have low killing activity, but inhibit virulence instead. For example, isothiocyanate iberin compound extracted from the crude horseradish plant exhibited remarkable activity against *P. aeruginosa* by interfering with quorum sensing (Jakobsen *et al*., 2012). Other quorum sensing inhibitors such as colostrum hexa saccharide, derived from mare colostrum showed significant inhibition of virulence factors secreted by *S. aureus* such as protease, haemolysis, and lipase activities, when applied at a rate of 5 mg ml^{-1} (Srivastava *et al*., 2015). This strategy puts less selective pressure on the target bacteria, reducing the chance of resistance development.

The data presented in chapter 3 show that PUFAs have some antimicrobial activity, particularly against *S. aureus* strains, but that they are much less potent than recommended antibiotics. The *in vitro* assays presented in chapter 3 quantify antimicrobial activity in laboratory conditions that do not represent the situation *in vivo.* This chapter investigated

whether the same PUFAs have any effect on the virulence of *S. aureus in vivo.* Two different infection model systems were used, an invertebrate and an *ex-vivo* skin model.

Advanced next generation sequencing technology has revealed the whole genome sequences of wide-ranging pathogens, and their genes can provide clues about how they cause disease. Many novel virulence genes have been suggested and decoding their functions will increase our understanding of disease mechanisms and may ultimately represent novel targets for the development of novel therapeutic agents. Researchers studying the pathogenicity of *Staphylococci* face the challenge that many mammalian models, such as mice or pigs, are relatively resistant to staphylococcal infection (Polakowska *et al*., 2012). In addition, the amount of inoculum and the route delivery of bacteria cells significantly differ from that which occurs naturally, raising questions about the relevance of results.

A variety of experimental animal models have been developed to help further understand the process of infection and the key determinants of pathogenicity. Widely used examples include rodents, such as the murine and rat models; the nematode worm, *Caenorhabditis elegans*; and the common fruit fly, *Drosophila melanogaster.* A range of larger mammalian models have been proposed, that better reflect human infection. For example, rabbit and guinea pigs have been used for skin infection models. These experimental models need special equipment and facilities to ensure the welfare of animals in the laboratory. As such, these experiments are expensive and require highly trained researchers and management and lengthy ethical approval procedures.

4.1.1 *Galleria mellonella* as a model to study virulence and antimicrobial activity *in vivo*

Invertebrate models offer a simpler alternative to study bacterial virulence and antimicrobial activity. *Galleria mellonella* are larvae of the greater wax moth and have been developed as a simple model of infection, used widely in the past decade. This infection model is an alternative approach to studying microbial virulence factors, host immune responses to infection, antimicrobial activity and toxicity of drugs (Tsai *et al*., 2016). Unlike immune responses in vertebrates, the adaptive immune response in insects contains a limited type of B cells and T cells (Ribeiro *et al*., 2017). The immune response of *G. mellonella* has two major components; humoral and cellular. The cellular immune responses are mediated by phagocytic cells, named haemocytes. These cells are found within the haemolymph, which functions analogously to mammalian blood cells. Besides phagocytosis, these cells play a role in encapsulation and clotting. The humoral response is coordinated by soluble effector molecules that immobilize or kill invading pathogens. These effectors include complement-like proteins, melanin, and anti-microbial peptides. The antimicrobial peptides (AMPs) are host defence peptides, found among all classes of life in *G. mellonella* and play a major role in innate immunity showing a broad-spectrum of bacteriocidal activity (Tsai *et al*., 2016).

The major advantages of *G. mellonella* model over other invertebrate models are that it is considered the least complex model, easy to use and maintain and cheap to obtain in large numbers. These factors make it possible to carry out high throughput studies. The larvae are of undefined age or weight compared to mouse model, and do not need feeding. They can also survive at 37^{o}C. Limited specialist equipment is needed to carry out *G. mellonella* experiments and there is no need of application for ethical approval. Thus, many have reported the usefulness of the *G. mellonella* model for evaluation of the efficacy of antimicrobial agents *in vivo*.

Several studies have demonstrated the susceptibility of *G. mellonella* larvae to infection. For example, 90% of larvae died 24h post infection with10^7 CFU *S. pyogenes* M3 (Olsen *et al*., 2011). The efficacy of antimicrobial compounds can be tested *in vivo* to determine whether they can protect a host from infectious disease. These experiments can be done by injecting *G. mellonella* with both an infectious agent and an antimicrobial agent to demonstrate their abilities to control infections caused by a range of pathogens. A positive correlation has been found between the results of studies in humans and in *G. mellonella* (Kavanagh & Sheehan, 2018; Lange *et al*., 2018). For instance, microbial virulence in the *G. mellonella* infection model, larvae survival is typically assessed within 5-7 days post inoculation (Yang *et al*., 2017). When larvae are accurately injected with defined doses of bacteria and antimicrobial concentrations, the survival rate at different time points is endpoint can be calculated. Bacterial inoculum can be normalised and monitored by measuring CFU. ml^{-1}. Several reports indicate that the *G. mellonella* model system has largely been one of convenience, to compare the virulence of different microbial strains or pathogenic species, by observing the relative time it takes for them to kill the larvae. Death is easily recognised, as the larvae stop moving and turn black (melanisation). Complete melanisation is typically distinctive with deposition of black spots on the cream-coloured larvae. Complete black larvae correlate with death. The time it takes for a larvae to die, post-injection, has been shown to correlate with the pathogenic potential of an infectious agent (Bokhari *et al*., 2017; Gundogdu, 2015). The health status of the larvae can be defined by assigning scores according to different observations: larvae mobility, cocoon formation, accumulation of melanin (melanisation) on the surface of larvae and survival rate (Loh, *et al*., 2013).

Investigators have carried out a variety of measurements, including survival rate and importance of key virulence factors. For example, when 2.5 x 10^6 CFU ml^{-1} of *S. aureus* Newman strain were inoculated into the larvae, the survival rate was~10% at 120 h post infection (Quiblier *et al*., 2013). Others have shown that MICs of cefotaxime, ciprofloxacin, and imipenem significantly prolonged the live of *G. mellonella* infection with *E. cloacae* GN1059 (inoculum 5×10^5 $CFUml^{-1}$; Yang *et al*., 2017). In assessing the efficacy of anti-staphylococcal agents against *S. aureus* infection, the survival rate of larvae was enhanced by treating with MICs of daptomycin or vancomycin (Desbois & Coote, 2011).

Furthermore, host responses to pathogen challenge can be investigated in *G. mellonella.* For example, Sheehan and Kavanagh (2018) measured the expression of anti-microbial proteins in response to *Candida albicans* infection (Sheehan & Kavanagh, 2018), and Wanda *et al.,* (2013) monitored the production of lactate dehydrogenase as a marker of cell damage during infection of *G. mellonella* with *K. pneumoniae* (Wand *et al*., 2013)

G. mellonella has been used to study host-pathogen interactions for a range of organisms, including studying the virulence factors of *Streptococcus suies* (Velikova, K & Wells, 2016) and *Shigella spp.* Mortality rates were found to be dependent on the *Shigella* strain, the infectious dose, and the presence of the virulence plasmid (Barnoy *et al*., 2017). The model has been used to assess the activity of Myricetin *in vivo,* which was found to inhibit multiple virulence factors in *S. aureus* and protect *G. mellonella* larvae from death (Silva *et al*., 2017).

There are few publications reporting the antimicrobial properties of PUFAs *in vivo.* Mil-Homens *et. al.,* (2012) have studied the antimicrobial effects of DHA on *Burkholderia cenocepacia* using the *G. mellonella* model. A single dose of DHA (50 Mm and100 mM)) was injected 6 h post infection and the PUFA compound was found to significantly inhibit

the growth of *B. cenocepacia* and reduced the number of viable cells (Mil-Homens *et al*., 2012). Taken together, previous infection experiments with *G. mellonella* suggest it to be a valid model for assessing the action of PUFAs *in vivo.* However, the model does not accurately reflect the conditions found in human wound infections.

4.2.1 An *ex vivo* human skin model for assessing antimicrobial efficacy for wound treatment

Patients with non-healing wounds are at a high risk of infection by colonisation of the wound with multi-drug resistant, biofilm forming bacteria. Chronic non-healing wounds are often inflamed due to an imbalance of the immune system (Metcalf *et al*., 2016; Percival *et al*., 2015). It is thus important to assess the efficacy of wound treatments using infection models that better reflect these conditions. Several skin models have been developed to help better understand the pathogenicity of wound infections and the processes that delay wound healing. *Ex vivo* human skin models use skin biopsies from healthy volunteers and variations have been used to investigate the molecular interactions and cellular responses that occur during treatment of wound infection. A reliable *ex vivo* skin model must include the distinctive features of human skin such as full-thickness and multiple layers to reflect relevant tissue structures. Fresh skin is also required to enable investigation of relevant immune responses to control environmental changes. The model must be easy to generate wounds and wound infection and efficiently support the growth of clinically relevant bacterial species. *Ex-vivo* skin models that can maintain relevant structure, infection and host responses are of considerable value for investigating the efficacy of antimicrobial wound treatment (Schaudinn *et al*., 2017).

A further aim of this study was to demonstrate relevant wound infection with the clinical SA3 isolate and to evaluate the antimicrobial activity of PUFA compounds using an established *ex vivo* human skin model. These activities could then be compared to findings from *in vitro* and *in vivo* (*G. mellonella*) assays.

S. aureus expresses an array of virulence factors that contribute to the pathogenesis of infection. Simple infection and treatment experiments with wildtype strains cannot identify whether PUFAs affect a particular virulence trait (e.g. surface adhesion, immune escape toxin production or tissue damage). However, they can determine whether the PUFAs inhibit growth or kill infectious agents *in vivo*, or whether they affect virulence in general. The antimicrobial and antibiofilm properties of PUFA compounds and antibiotics have been evaluated *in vitro* in the previous chapter 3. Thus, the aims of this chapter were:

1) Compare the virulence of different strains of *S. aureus* (SA3, 252 and 6538) *in vivo* using the *G. mellonella* model.

2) Evaluate the ability of PUFAs to protect *G. mellonella* from fatal infection.

3) Evaluate the usefulness of an *ex vivo* skin model to assess the antimicrobial properties of PUFAs under more relevant conditions to chronic wounds.

4.2 Comparing the virulence of different *S. aureus* strains in the *G. mellonella* infection model

The *G. mellonella* infection model has been used by others to assess the virulence of *S. aureus* and the ability of antimicrobial agents to protect the larvae from lethal infection (Latimer *et al*., 2012). The first experiment presented in this chapter was designed to determine how quickly the study strains of *S. aureus* killed the *G. mellonella* larvae. Three *S. aureus* strains were selected for this study: 1) SA3clinical isolate (resistant to erythromycin and lincomycin and susceptible to PUFA (MIC DHA 65µgml^{-1}, EPA 78 µgml^{-1}); 2) MSSA-6538 (a standard reference strain, originally collected from a human lesion and considered susceptible to all antibiotics used in this study); 3) MRSA-252 (considered to be a major cause of community-acquired and hospital-acquired infections; resistant to the most antibiotics used in this study).

Washed suspensions of each *S. aureus* strain were adjusted to OD 0.1 (5 x10^5 – 8 x10^{5}CFU ml^{-1}) then injected in to the *G. mellonella* larvae (n = 16 each strain) that were incubated in the dark at 37 ^{o}C for up to 7 days. Non-injected and mock-PBS-injected larvae (n=16 each) were included as controls. The number of surviving larvae under each condition were counted every 24 h (supplementary Table S1; appendix 1). Death of larvae was distinguished by melanisation of the larvae and lack of movement. All three *S. aureus* strains were observed to be virulent in the *G. mellonella* model, but to different levels. The percentage survival of larvae challenged with SA3, MSSA6538, and MRSA252 at day seven was 12.5%, 50%, and 6.5% respectively (Figure 4.1). The SA3 infected group were the most affected, with 6 larvae being killed rapidly within the first 48h, then 2 larvae died at each day until day 7. The 6538-infected group was least affected. One larva was observed to die each day of incubation, indicating this strain to be less virulent. The 252-infected group showed a similar trend to the SA3-infected group, with 2/16 larvae surviving the full 7 days. These

findingsdemonstrate that *G. mellonella* were more sensitive to infection by the *S. aureus* SA3 and MRSA 252 than by MSSA 6538. No death rate was recorded with un-inoculated larvae and PBS inoculated controls over the seven days. These data indicate that the *G. mellonella* model was a valid system for evaluating the antimicrobial activity of PUFAs *in vivo*.

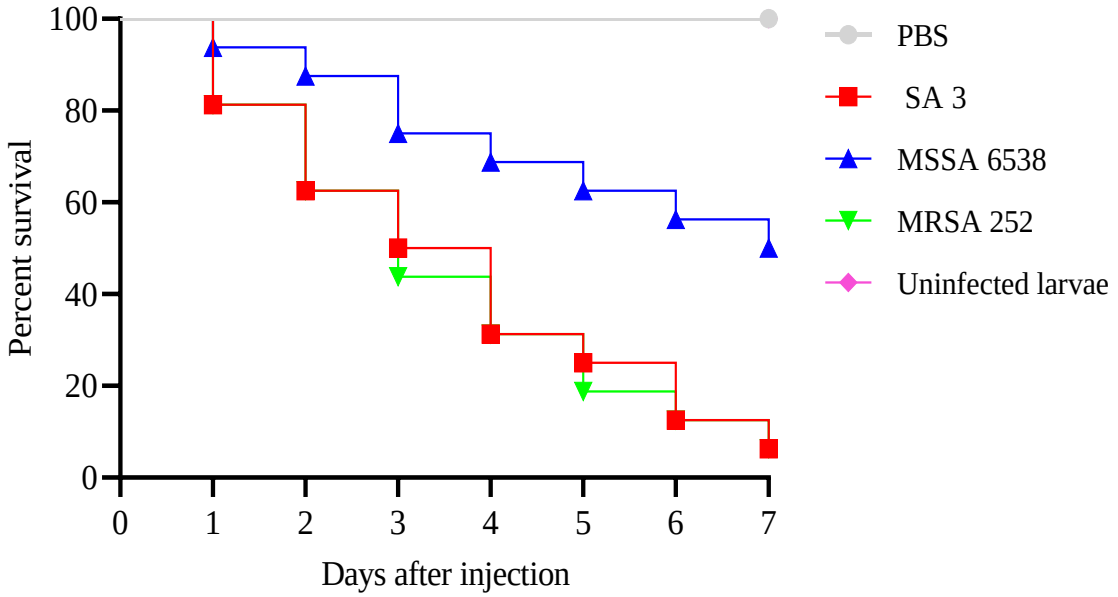


Figure 4.1 **Survival of *G. mellonella* following challenge with different strains of *S. aureus***. Larvae (n=16) were injected with MSSA6538, MRSA 252 or SA3 and survival was monitored for 7d. Negative controls included uninfected larvae (n=16) and injection with PBS (n=16). The figure shows example data chosen from 3 experimental replicates. Survival analysis was done using log-rank Mantel-Cox test in GraphPad prismV8. Statistically significant differences in virulence depending on the strain (significant difference SA3 vs MSSA P = 0.0051; significant difference MRSA vs MSSA P =0.0038; and not significant difference withSA3 vs MRSA P<0.837).

4.2.1 *S. aureus* growth recovery (CFU) from *G. mellonella* post infection

The survival/growth of bacteria in the infected larvae was assessed by quantifying CFU ml^{-1} recovered from each dead larva that was homogenized in PBS, diluted and spread onto MSA. Uninfected and mock-infected larvae were included as negative controls. Colony counts recovered from dead larvae were compared to the initial inoculum (5 $x10^5$ - 8x 10^5CFU. ml^{-1}). The bacteria recovered from dead larvae infected with SA3 was between 6.8 x 10^8 - 8.2x10^9 CFU $ml^{-1}$and for MSSA 6538was 8.3 x 10^8 - 5.8x10^9 CFU $ml^{-1}$and for MRSA 252 was 3.7 x 10^8 - 7.4 x10^9 CFU ml^{-1} (Figure 4.2). These data suggest that *S. aureus* was able to survive and multiply within *G. mellonella* and that each strain had a similar fitness *in vivo* despite differences in virulence. Although *S. aureus* SA3 growth were confirmed on MSA, Gram stain, and coagulase test, a few colonies of *S. epidermidis* and *Es. coli* were recovered from the dead larvae. However, these numbers were insignificant compared to the number of *S. aureus* recovered in each case. There was no a statically significant difference between infection groups of staphylococci *P-value*= 0.123.

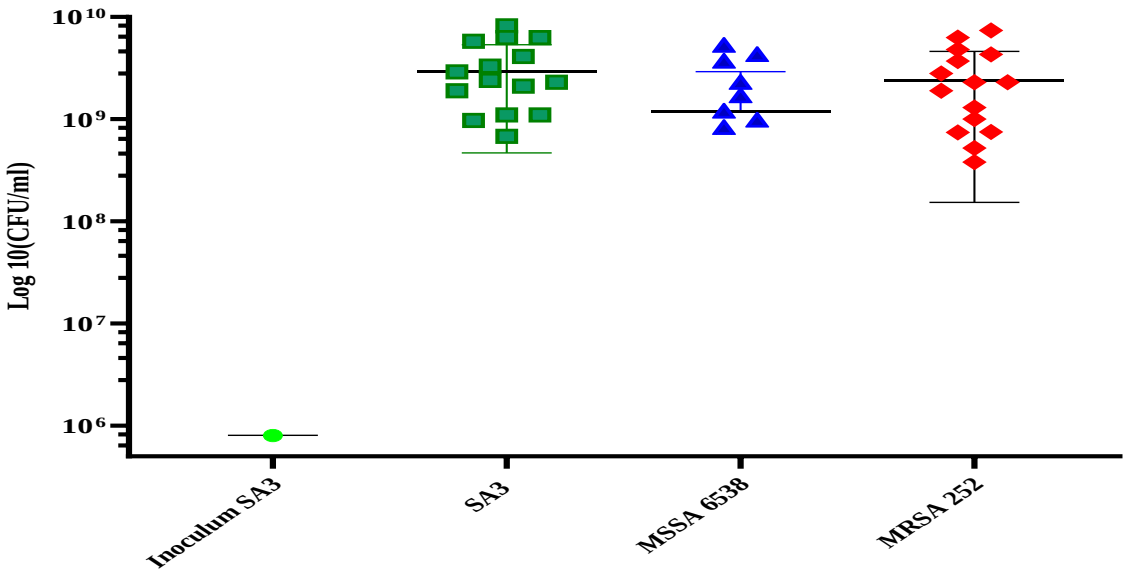


Figure 4.2 **Recovery of viable bacteria from *G. mellonella* post infection**. Each dot represents the CFU recovered from each larva on death. Mean CFU and standard

deviation values are shown from three independent experiments. Even there are no statistically significant ($P < 0.123$) and even though bacterial numbers are the same – there is still a difference in larvae survival thus suggesting differences in virulence.

4.2.2 The efficacy of PUFA compounds in the *G. mellonella* model

In vitro assays suggested that PUFA compounds have both antibacterial and anti-biofilm activity against different wound pathogens (chapter 3). Survival assays confirmed that *G. mellonella* larvae are a valid model for assessing the efficacy and toxicity of antimicrobial agents *in vivo*. These survival assays were therefore repeated, with additional challenge groups that were treated with different concentrations of DHA and EPA. Following initial inoculation with SA3 suspensions, larvae in the PUFA-treated groups were injected with either MIC or 2 MIC of DHA or EPA or both together. Control groups included SA3 challenge only; PUFA-treated in the absence of challenge; un-infected and mock-infected (15 groups in total; n = 16 per group as detailed in supplementary Table S2 in appendix 2). The survival of *G. mellonella* in each group was compared over a period of 7 days. A significant decrease in mortality was observed in the challenge groups that were treated with PUFAs. Percentage survival of groups treated with MICs of DHA, EPA and both PUFAs together were 68%, 56% and 62% respectively, compared to 6.25% survival in the SA3 untreated group after 7 days. Survival rates were observed to increase further when PUFAs were applied at 2 MICs 87%,75% and 75% respectively (Figures 4.3 and 4.4).

Each larva was challenged with SA3 infection and treated with different concentrations of DHA (MIC 65µg ml^{-1} and 2 MIC 130 µg/ml) simultaneously. The experiment was repeated three times. Full details of the experiment can be found (supplementary table S2, appendix 1). Of the *G. mellonella* infected with SA3 and treated with DHA (65µg ml^{-1}); only one larvae

had died on the 1st day, compared to 3 deaths in the untreated group. Eleven out of sixteen (68%) larvae remained alive on day 7 in the presence of DHA. The second group of *G. mellonella* larvae infected with SA3 and treated with 2x MIC DHA (130µg ml^{-1}), were completely protected from *S. aureus* killing for the 1st 4 days of the experiment, then two larvae died each day from day 5 to day 7. All the larvae in the un-infected control groups (with and without DHA) survived for the entire 7 days (Figure 4.3).

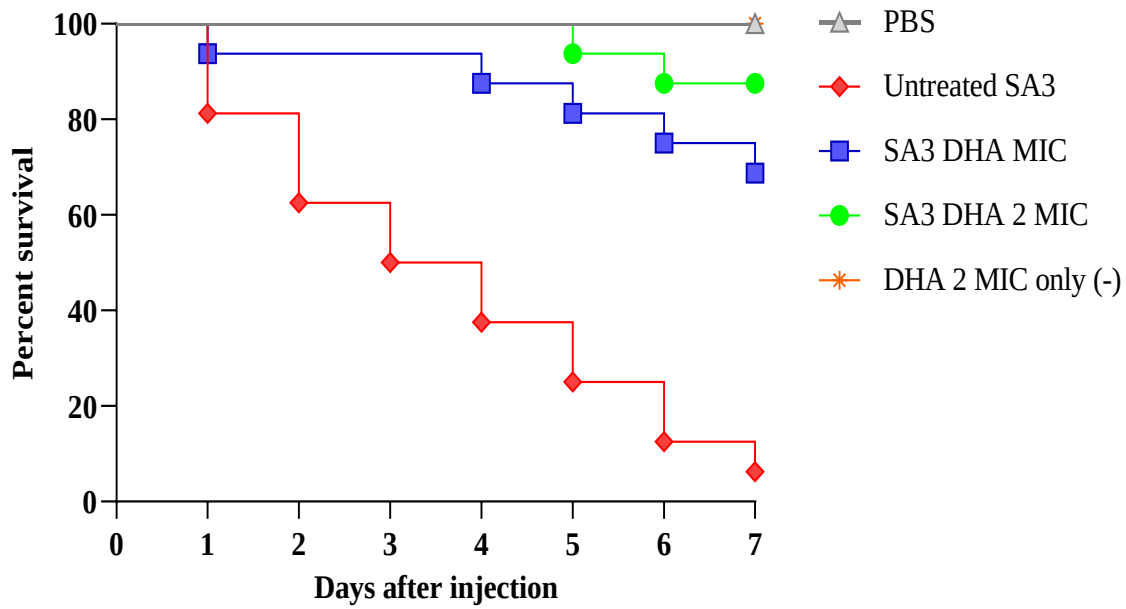


Figure 4.3 **Effect of DHA treatment on the survival of *G. mellonella* larvae.** Survival analysis was done using log-rank Mantel-Cox test in GraphPad prismV8. Statistically significant differences in virulence depending on the strain (Untreated SA3 vs SA3 DHA MIC significant P = <0.0001; Untreated SA3 vs SA3 DHA2MIC significant P = < 0.0001, and SA3 DHA MIC vs SA3 DHA 2MIC not significant P value = 0.1952).

Treatment with EPA had similar, but slightly weaker protective effects compared to DHA. Each larva was challenged with SA3 infection and treated with different concentrations of EPA (MIC 78µg $ml^{-1}$and 2 MIC 156µg ml^{-1}) simultaneously. The

experiment was repeated three times and full details can be found (supplementary table S2, appendix 1). Groups of *G. mellonella* showed survival rates of 56% and 75% seven days post challenge and treatment with MIC (78µg ml^{-1}) and 2 MIC (156 µg ml^{-1}) respectively (Figure 4.4). Of the *G. mellonella* group infected with SA3 and treated with EPA at MIC two larvae died on the first day, then one more died on each of days 2, 4, 5, 6 and 7 (56 % survival on day 7). The second group, infected with SA3 and treated with 2 MIC of EPA (156 µg ml^{-1}), were completely protected by the PUFA from SA3 killing for the 1st 3 days. Only one larvae died on day 4, followed by one each day until day 7 (75% survival).

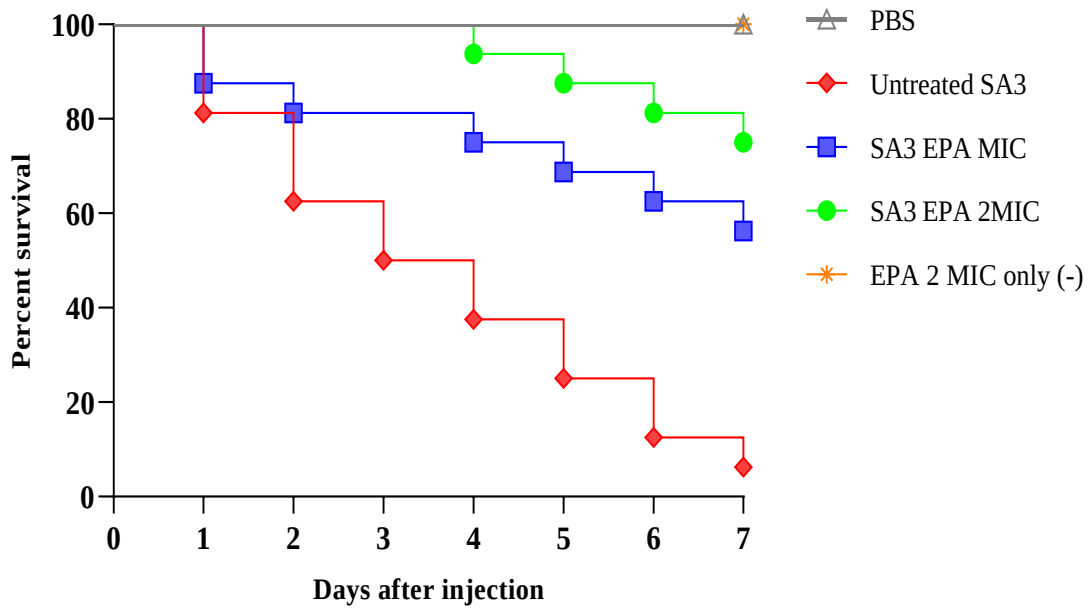


Figure 4.4 **Effect of EPA treatment on the survival of *G. mellonella* larvae.** Survival analysis of treatment group of EPA MIC and 2 MIC was done using log-rank Mantel-Cox test in GraphPad prismV8. Statistically significant differences in virulence depending on the strain (Untreated SA3 vs SA3 EPA MIC significant P =<0.0022; Untreated SA3 vs SA3 EPA 2 MIC significant P = < 0.0001, and SA3 DHA MIC vs SA3 DHA 2MIC not significant P value = 0.21).

The percentage survival of *G. mellonella* was monitored over 7 days following infection with SA3 and treatment with two concentrations of both PUFAs together (MIC 78µg ml^{-1} and 2 MIC 156 µgml^{-1}). Each larva was challenged with SA3 infection and treated with different concentrations of DHA+ EPA (MIC 78µg $ml^{-1}$and 2 MIC 130µg ml^{-1}) simultaneously. Full details can be found (supplementary table S2, appendix 1). On day 7, survival was recorded as 62% and 75% for each of these groups respectively (Figure 4.5). When treated with MIC most larvae were protected from SA3 by DHA+EPA. One larvae were killed by the infection on each of days 1, 2, 4, 5, 6 and 7. When treated with 2 MIC, the larvae were completely protected for 3 days before the first killing was observed. Then one larvae died each day from day 4 to day 7, with a final percentage of survival of 75%.

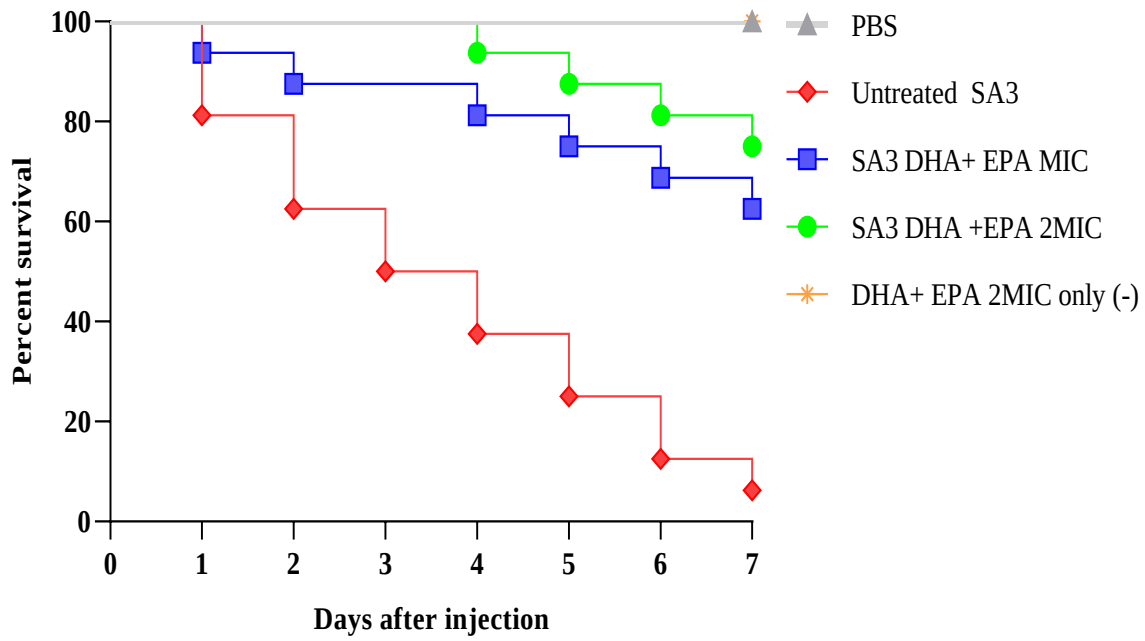


Figure 4.5 **Effect of DHA+EPA treatment on the survival of *G. mellonella* larvae.**. Survival analysis was done using log-rank Mantel-Cox test in GraphPad prismV8. Statistically significant differences in virulence depending on the strain (Untreated SA3 vs SA3 DHA+ EPA MIC significant P =<0.0004; Untreated SA3 vs SA3DHA+ EPA 2 MIC significant P = < 0.0001, and SA3 DHA+EPA MIC vs SA3 DHA+EPA 2MIC not significant P value = 0.39).

Finally, both linezolid and vancomycin protected the *G. mellonella* larvae from SA3 killing considerably. Each larva was challenged with SA3 infection and treated with concentrations of LZD MIC 3.9µg $ml^{-1}$and VAN MIC 0.093µg ml^{-1}simultaneously. Full details can be found (supplementary table S3, appendix 1). Only 1 larvae died on day 6 after being co-treated with MIC of linezolid. Similarly, only 2 larvae died after co-treatment with SA3 and MIC of vancomycin, 1 on day 5 and a second on day7. By comparison, in the absence of antibiotics, 93% (15/16) larvae died from SA3 infection within 7 days.

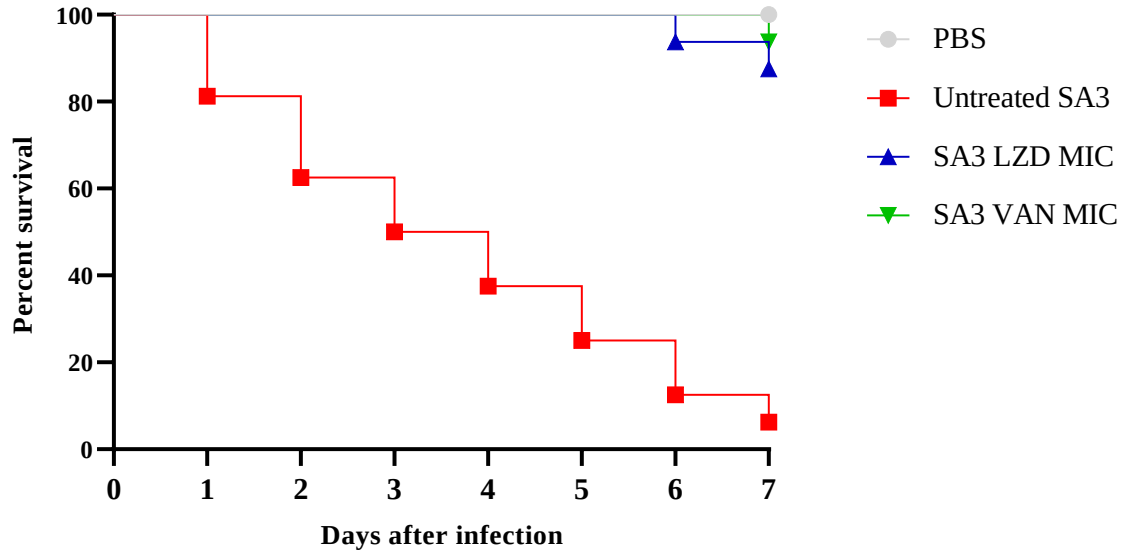


Figure 4.6 **Effect of LZD or VAN treatment on the survival of *G. mellonella* larvae.** Survival analysis was done using log-rank Mantel-Cox test in GraphPad prismV8. Statistically significant differences in virulence depending on the strain (Untreated SA3 vs SA3 LZD MIC significant P =<0.0001; Untreated SA3 vs SA3VAN MIC significant P = < 0.0001, and SA3 LZD MIC vs SA3 VAN MIC not significant P value = 0.53).

Taken together, these results demonstrate that, although *S. aureus* SA3 had significant virulence activity in *G. mellonella*, the PUFA compounds clearly protected the larvae from

lethal infection compared to the untreated groups, and this effect appears to be dose-dependent, with higher concentrations of PUFAs delaying death for much longer. These experiments could not determine whether PUFAs were acting by preventing growth or inhibiting virulence of SA3. This could be better assessed by quantifying bacterial numbers following challenge and treatment.

4.2.3 Bacterial growth (CFU) after infected *G. mellonella* with different concentrations MICs of PUFAs compounds

Following the death of each larvae during the challenge-treatment experiment, *G. mellonella* were homogenized and serially diluted in PBS for bacterial counts, which were compared to the initial inoculum of SA3 ($5x10^5$ CFU.). The *G. mellonella* were not sterilised before inoculation, hence a small number of non-SA3 colonies were observed on recovery plates. However, this did not affect the recovery of high numbers of SA3, that were confirmed by plating on Baird-parker agar plates, coagulase test, catalase test and Gram stain. The recovered CFU from all treated *G. mellonella* groups was considerably lower that the numbers recovered from the untreated group in each case (Figure 4.7). Whilst 6.8×10^8 - 8.2×10^9 CFU. were recovered from each infected, untreated larva, roughly 10-fold less were recovered from larvae treated with DHA (9.7×10^7 - 5.6×10^8CFU. $larvae^{-1}$). Similar numbers were recovered from larvae treated with EPA (1.1×10^8 - 6.1×10^8 CFU. $larvae^{-1}$), and both PUFAs together (1.4×10^8 - 7.3×10^8 CFU. $larvae^{-1}$). There was no significant difference in recovery between the 3 treated groups. The SA3 numbers recovered from *G. mellonella* treated with double concentrations (2 MIC) of each PUFA were slightly reduced, but not to a level of statistical significance (3.9×10^7 - 3.4×10^8 CFU. $larvae^{-1}$).

These data indicate that PUFAs can either hinder growth or kill a proportion of SA3 *in vivo*, but that much higher concentrations would be needed to eradicate the infection. Growth was not hindered completely, as SA3 was recovered from all treatments in much higher numbers than the initial inoculum. Interestingly, when the PUFA dose was doubled, the *G. mellonella* survival rate increased, but there was no significant decrease in SA3 numbers recovered from dead larvae. This might suggest that the higher dose may have more effect on virulence, rather than a cumulative bacteriostatic or bacteriocidal effect. However, this might just be a reflection of genetic variation and susceptibility of individual larvae. Quantification of bacteria from the surviving larvae would have provided more insight into this. Uninfected larvae, that were treated with PUFAs remained 100% viable, confirming the non-toxic nature of the PUFAs at these concentrations.

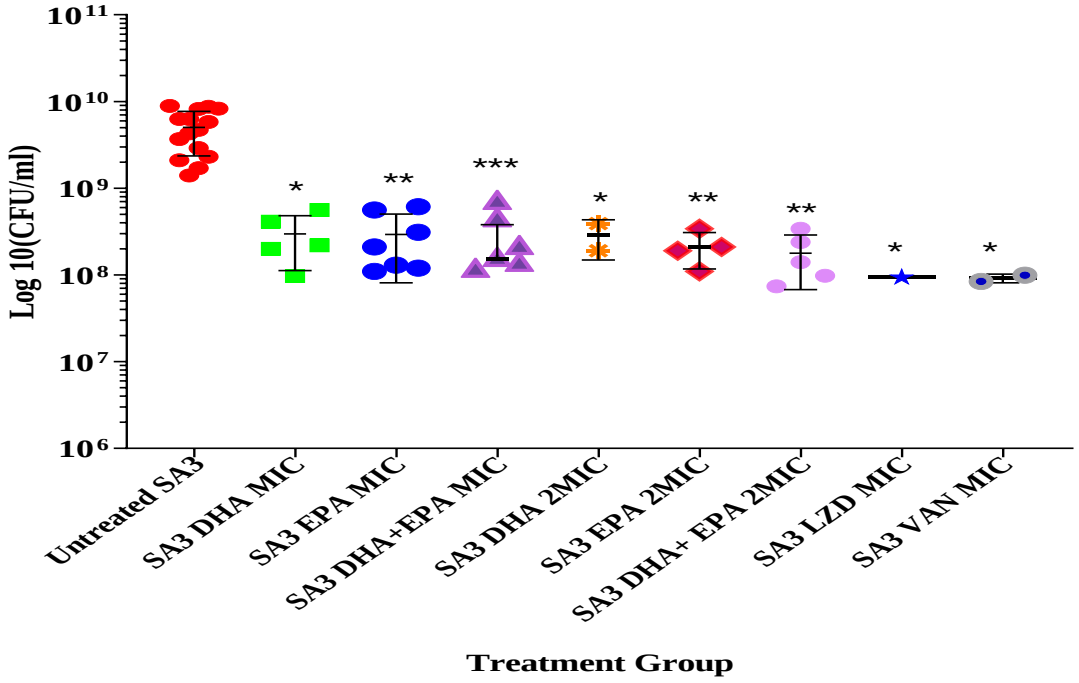


Figure 4.7 **Effect of different PUFA compounds on bacterial growth (CFU) of SA3 *in vivo*.** *G. mellonella* were inoculated with various concentrations of PUFA compounds MIC and 2 MIC) and LZD, VAN antibiotics. Each dot represents the number of viable bacterial cells recovered from dead larvae. All these experiments represent the means ±

SEM of three independent determinations. All medial the medians vary and statically significant compared to Untreated SA3.

- *SA3 DHA MIC vs Untreated SA 3 P value = 0.0314
- **SA3 EPA MIC vs Untreated SA 3 P value =0. 0092
- ***SA3 DHA+EPA MIC vs Untreated SA 3 P value = 0.002
- * SA3 DHA 2 MIC vs Untreated SA 3 P value = 0.0276
- **SA3 EPA 2 MIC vs Untreated SA 3 P value = 0.026
- ***SA3 DHA+EPA 2 MIC vs Untreated SA 3 P value = 0.024
- *SA3 LZD MIC vs Untreated SA 3 0.020-

*SA3 VAN MIC vs Untreated SA 3 = 0.022

This chapter demonstrates the activity of PUFAs in more realistic conditions, but does not provide any information about how the PUFAs inhibit SA3 growth, or how they might interfere with virulence. *G mellonella* infection model is an alternative approach to studying microbial virulence factors, host immune responses to infection, antimicrobial activity and toxicity of drugs. There are several limitations of the *G. mellonella* model; and there is need for more realistic models to assess host responses through the development of standardised larvae for research, the whole genome sequencing projects and the development of pathology scores to achieve more reliable model.

4.3 Detectionof the growth of SA3 on the *ex vivo* human skin model

An *ex-vivo* human skin model was used to evaluate PUFAs as viable interventions for wound infection (Latimer *et al*., 2012). The skin model consists of punch biopsies, taken

from consenting donors undergoing elective surgery. The skin structure is maintained in culture well inserts, with a small amount of the bottom layer pulled through a central hole that feeds into the tissue culture media below. This set up allowed for multiple replicates (n=6) per plate. The skin can be "wounded", using a smaller biopsy punch to remove a small amount of the top layer of skin. Experiments were performed, in which bacteria, PUFAs and controls were applied in different combinations. The initial inoculum of SA3 $\cong 1x10^7 CFUmm^2$. Triplicate swabs samples were taken at time zero to evaluate the number of bacterial cell that located on biopsy samples. The growth and response of *S. aureus* SA3 on non-wounded and wounded skin were assessed 24 h post inoculation. The first experiment showed that SA3 could multiply on both the wounded and non-wounded skin surface to similar levels (Figure 4.8). The mean CFU. mm^{-1} of SA3 was 2.6 $x10^8$ CFU. mm^{-1} on wounded skin and 3.4 $x10^8$ CFU. mm^{-1} on non-wounded skin after 24 h recovery. These viable bacterial cell counts were significantly more than the "uninfected" wound inoculated at t= 0 with of 8 $x10^4$ CFU mm^{-1} (P =0.0160). Slightly more SA3 growth was observed on infected non-wounded biopsies compared to the "uninfected" (inoculated) nonwounded biopsies, (P =0.0028). No viable bacterial cells were recovered from negative controls biopsies (biopsy skin only).

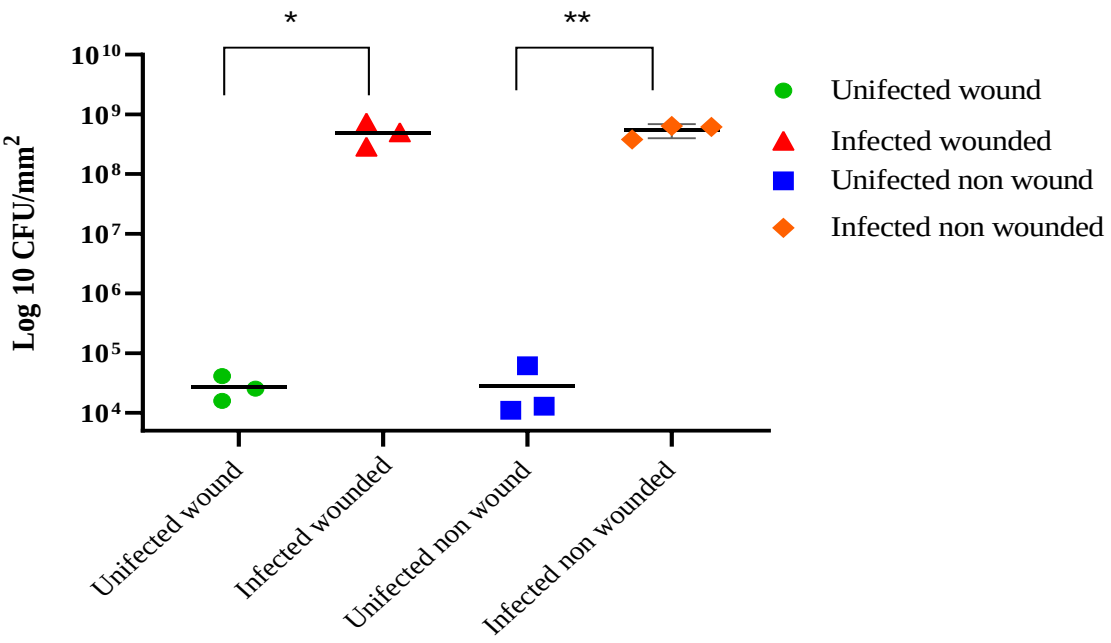


Figure 4. 8 **CFU viable SA 3 from wounded and non-wounded skin biopsies**. The dots are represented as the mean ± SD of three donors. The term "un-infected" refers to skin biopsies at t = 0 that had just been inoculated with SA3. The term "infected" refers to skin biopsies 24 h post inoculation with SA3. CFU growth of SA3 analysis was done using un-paired t test in Graph Pad prismV8. The CFU growth of SA3 revealed statistically significant differences between t = 0 h vs t = 24 h P value= 0.016, **significant uninfected non-wounded vs and infected non-wounded sample P value= 0.0028, No significant wounded sample and Non-wounded sample P value= 0.97.

The second experiment using the ex vivo human skin model was carried out to explore the wound model's potential as a test system for local antimicrobial DHA treatment. The wounds and non-wounded samples were treated with DHA 130 µg ml $^{-1}$ to evaluate the antimicrobial activity of DHA in a skin or wound environment. Briefly, the SA3 inoculum of SA3 ≅ 1x10^{7} CFU mm^{2} was loaded on to the surface of wounded and non-wounded skin and allowed to adsorb. Triplicate swabs samples were taken at time zero to evaluate the number of bacterial cells that located on biopsy samples. The topical DHA (2 MIC130 µg ml $^{-1}$) was added 15 min after inoculation. After 24 h bacterial cells were recovered from the surface of

each biopsy using a sterile swab. A decreased viable cell density was recovered from treated samples compared to non-treated samples (Figure 4.9), regardless of whether or not the skin was wounded. The mean number of SA3 cells recovered from infected wounded samples treated with DHA was 5.3 x 10^4 mm^{-1} compared to 2.6 x10^8 CFU mm^{-1} from untreated samples (P = 0.017) from the same experiment. Similarly, although significant mean number of SA3 cells (P = 0.0028), recovered from infected non-wounded samples treated with DHA, was slightly lower (1.3 x10^4 CFU mm^{-1}), this number was still much less than recovered from the non-treated equivalents. The results show no significant differences between infected wound and infected non-wounded both were treated with DHA (P =0.658). No bacteria count could be recovered from negative controls (biopsy skin only).

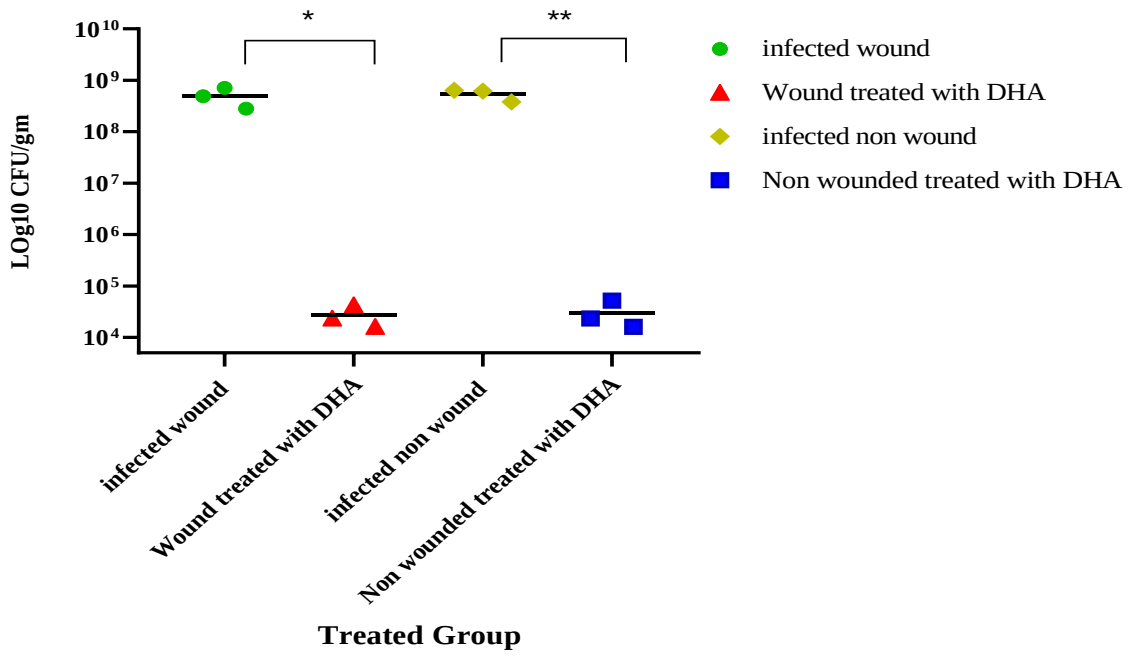


Figure 4.9 **Bacteria quantification and effects of topical DHA 130 µg ml $^{-1}$treatment on wounded and non-wounded skin sample**. The dots represent the mean ± SD of three experiments, each performed with skin biopsies from different donors. Recovery growth of SA3 analysis was done using un-paired t test in Graph Pad prismV8. The growth of SA3 revealed statistically *significant differences between the infected wound vs treated wound

with DHA 130 µg ml $^{-1}$ P value=0.017, ** significant infected non-wounded vs non-wounded treated with DHA 130 µg ml $^{-1}$ sample P value= 0.0028.

These data suggest that PUFAs are likely to have antimicrobial effects during wound treatment. As no growth of bacterial cells at negative control (biopsy skin only) suggest a thorough skin disinfection of each patient before surgery. However, the whole Williams E medium were preserved at -80 ^{o}C for future investigation such as the host immune response towards bacterial infection. Moreover, experiments are required to compare the action of PUFAs to the action of antibiotics in this model.

4.4 Discussion

This study agrees with others, that *G. mellonella* is a useful alternative infection model for studying microbial virulence and the efficacy of antimicrobial activity *in vivo*. The virulence of *Staphylococci* in *G. mellonella* has been demonstrated by Silva *et al* (2017). They revealed that Myricetin, as alternative compound for inhibiting virulence factors of *S. aureus* (α- haemolysin activity, coagulase and staphylo xanthin production)without interfering with growth, and protecting *G. mellonella* infection (Silva *et al*., 2017). Although the antimicrobial activity of PUFA compounds has been studied *in vitro* against potential wound pathogens in chapter 3, this invertebrate model provided more realistic conditions for testing activity and for assessing the effect of PUFAs on virulence of SA3 during proliferation of the bacterial cells inside the larvae. These experiments demonstrated that *G. mellonella* was sensitive to infection by *S. aureus* SA3 and MRSA 252, and slightly less sensitive to MSSA 6538. An inoculum of 5 x 10^5 CFU. enabled comparison of virulence over a 7-days period. By contrast, Ignasiak *et al*, observed very rapid killing 60–80% of *G. mellonella* within 48h post infection with *S. aureus* ATCC 29213 (Ignasiak & Maxwell, 2017). Others have compared a wider range of different strains and stratified them into high (90–100% mortality within 1-2 days), intermediate (50-90% mortality between 3-5 days) virulence phenotypes (Mannala *et al*., 2018). These reports demonstrate that the virulence and killing of *G. mellonella* by *S. aureus* are dependent on the strain. Differences in rates of killing by *S. aureus* indicate variation in the production of virulence factors during different stages of host colonization and infection (Kobayashi *et al*., 2015).

The clinical wound isolate, SA3 was chosen as a relevant target to assess the pathogenic consequences of antimicrobial activity of PUFA compounds in *G. mellonella*. This strain was previously confirmed as a multidrug resistant organism, with a profile resembling MRSA determined by observing resistance to cefoxtime (chapter 3). This study

suggests that DHA was more effective at preventing SA3 from killing *G. mellonella* than EPA. The recovery of SA3 was significantly higher from EPA-treated larvae than from DHA-treated larvae. This might be due to activity of EPA that promoted more reduction of cellular inflammation than DHA. EPA is an inhibitor of the enzyme delta-5-desaturase (D5D) that produces arachidonic acid, which is precursor to pro-inflammatory eicosanoids, and could result in synergistic improvement in immune response (Yashiro *et al*., 2016).

The killing activity of SA3 was significantly reduced when larvae were treated with higher concentrations of PUFAs compound. These findings indicate that PUFA compounds have encouraging inhibitory effects on the growth of the clinical SA3 isolate *in vivo*. Relatively high densities of viable SA3 were recovered from treated larvae, despite longer survival of the larvae. This suggests that the action of PUFAs affected virulence, not just growth inhibition. These findings agree with other study by Kim *el al.*, (2018) investigated the effects of DHA and EPA on *S. aureus* virulence in the *C. elegans* model. The survival rate of *C. elegans* was increased more than seven and nine fold when worms were exposed to *S. aureus* in the presence of DHA or EPA at 20 µg ml^{-1} respectively (Kim *et al*., 2018)

No toxic effects were observed when non-infected larvae were exposed to PUFAs, DHA, EPA or both DHA+EPA at 2MICs concentrations up to 156 μgm^{-1}. These data agree with others who also report no toxic effects of PUFAs (up to 200 µM) on gingival epithelial cells (Sun *et al*., 2016).

The experiments performed using the *ex vivo* human skin model presented encouraging results to further investigate the antimicrobial efficacy of PUFA compounds using this skin model. The clinical SA3 isolate grew well on wounded and non-wounded skin. The significant increase in bacterial density after 24 h incubation may be explained by the presence of nutrients in the skin wound biopsy, furthermore, our data from of infected ex

vivo wounds and infected non-wound treated with DHA 130 resulted in a reduction from ~$10^8$to ~10^4 bacteria per wound, these results are consent with other study using the same model with other antibiotics. For example, Schaudinn *et al*., (2017) had demonstrated that topical treatment with ciprofloxacin (375 μg ml^{-1}) for *P. aeruginosa* PA01 had reduced the bacterial count from 10^9 to 10^4 bacteria per wound, the same study revealed that interleukinIL-1α expression was significantly affected and distinguished between infected and uninfected wounds, indicating a response of skin cells to the infection of the PA01 strain. Furthermore, scanning electron microscopy confirmed the high number of bacteria that were interwoven in collagen bundles and apparently attached to the collagen fibres (Schaudinn *et al*., 2017).

The significant reduction of bacterial cells at both infected wound and infected non-wounded biopsies provide encouraging evidence for further investigation, such as comparison with other topical antibiotics for wound healing. Moreover, further investigation for testing different immune responses to infections and treatment. This is possible because the skin is maintained in a viable state by bathing the under-side in tissue-culture medium (Shah *et al*., 2012). Cytokines such as (IL-6, IL-8, IL-1α and IL-1β) are expressed and increase in inflammatory state and infections. The cytokines IL-6 and IL-8, are released by skin cells (fibroblasts and macrophages) and normally up-regulated during an infection (Arango Duque & Descoteaux, 2014). In addition, IL-1α is related to tissue damage and bacterial infections. IL-1β is known to be secreted by several skin cell types such as macrophages and dendritic cells, when bacterial components bind to the Toll-like receptor (Chamberlain *et al*., 2013). Measurement of these cytokines in skin biopsies exposed to SA3 and PUFAs, would provide insight into the multi-faceted roles of PUFAs in chronic wound healing.

Chapter Five

Effects of the inhibitory concentrations of PUFAs on regulatory genes of *S. aureus* SA3

Chapter 5 Effects of the inhibitory concentrations of PUFAs on regulatory genes of *S. aureus* SA3

5.1 Introduction

S. aureus is an opportunistic pathogen that causes a wide range of acute and chronic infections including wound infections (Nair*et al*., 2014). These pathogenic bacteria have been shown to form extracellular biofilm matrix in the wound bed, and employ a wide range of virulence factors, such as adhesins, enzymes, toxins, and immune evasion proteins (Phillips *et al*., 2016; Powers & Wardenburg, 2014). There has been a significant increase in reports of antibiotic-resistant *S. aureus* such as MRSA and VRSA. With the increasing challenges of AMR, there is an urgent need for novel treatment strategies and, ideally, antimicrobial agents that impose less selective pressure but may inhibit virulence and biofilm formation. Previous chapters demonstrate antimicrobial and antibiofilm activity of PUFAs *in vitro* and in infection models. Results from *G. mellonella* challenge with SA3 show reduced killing of larvae, but little reduction on growth of the invading pathogens. These data suggest that PUFAs may have an inhibitory effect on the virulence of *S. aureus*. Others have reported that PUFAs may affect the expression of genes involved in adhesion, biofilm formation and virulence gene regulation (Choi *et al*., 2013). This chapter explores the effect of different concentrations of PUFAs on the expression of key target genes involved in the global regulation of biofilm formation and virulence.

The formation of a mature biofilms is a complex process that can be subdivided into distinct phases of attachment, accumulation, maturation, and dispersal. The early stages of Staphylococcal biofilms (attachment and accumulation) rely on different types of adhesins. *S. aureus* adhesion occurs via a group of surface-exposed proteins collectively referred to as

MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) and the polysaccharide intercellular adhesin (PIA) operon, whose biosynthesis is encoded by *icaA, icaD, icaB,* and *ica C* genes. *S. aureus* controls expression of these genes using different regulatory systems. The accessory gene regulator (Agr BDCA) is a quorum-sensing system (Foster *et al*, 2014)that regulates genes via an autoinducing peptide (AIP) in response to cell density. The response regulator (*agrA*) is activated on detection of AIP and acts as a global regulator of a wide range of virulence genes such as hemolysins, staphylococcal proteases, and lipases (Mannala *et al*., 2018). The Agr system is part of a more complex regulatory system and is linked to the staphylococcal accessory regulator nucleic acid-binding protein, SarA. The *sar* locus consists of transcriptional units (*sarB, sarC* and *sarA*). The SarA subunit is constitutively produced throughout *S. aureus* growth phases (Morrison, 2012) and plays a significant role in the control of virulence and biofilm formation (Arya *et al*., 2015; Dunman *et al*., 2001). For example, the study by Balamurugan *et al*., (2017) showed that the compound 2-[(Methylamino)methyl] phenol has significant inhibition the representative virulence genes *RNAIII, fnbA*, and *hla* genes, these genes are under control of global regulator *sarA* gene. Furthermore >70% of clinical *S. aureus* biofilm inhibition was observed when treated with 1.25 μM of this compound (Balamurugan *et al*., 2017).

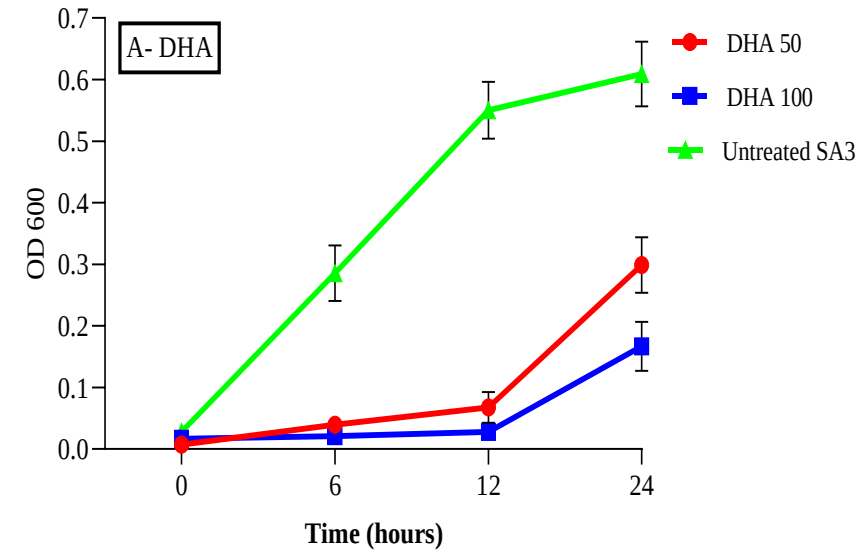
This chapter aimed to gain a deeper understanding of the mechanism of antibacterial action of PUFAs and assess whether PUFAs interfere with the activity of global virulence gene regulators. The main objective was to use RT-PCR to measure the differential expression of *sarA* and *agrA* by SA3 in the presence and absence of PUFAs.

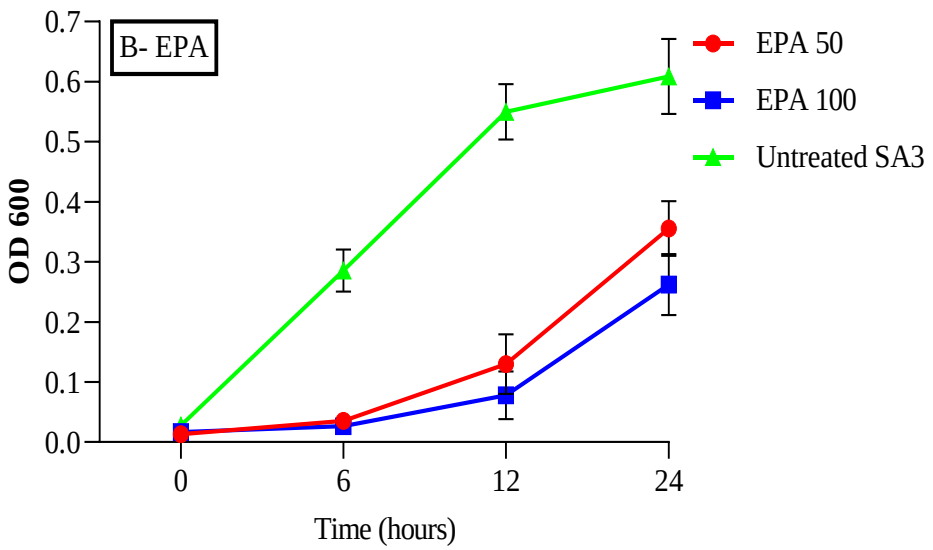
5.1.1 Effect of DHA and EPA on SA3 growth curves

The effect of different concentrations of PUFA compounds on the growth of SA3 was measured in liquid culture. Briefly, a fresh suspension (20ml) of SA3 was prepared in MH broth from overnight cultures to the density of $1.5x10^4$ CFUml^{-1} as confirmed by serial dilution and spread plating on Columbia agar. Cultures were set up in triplicate (Table S4 in appendix) and exposed to two different concentrations (50µgml^{-1} and 100 µg ml^{-1}) of DHA EPA or both together in triplicate for 24 h at 37 ºC. Cultures were sampled for RNA extraction at several time points (t = 0, 6, 12 and 24 h). Optical density measurements (OD_{600}) and broth dilution CFU ml^{-1} assays were performed at each time point. Negative controls were not treated with PUFAs, instead, they were exposed to dilute ethanol (1:1000), used to solubilise PUFAs. Aliquots (1.5 ml) of bacterial suspension were collected at each time point and bacterial cells were harvested by centrifugation (15.000rpm, 2min) using a benchtop microfuge. Bacterial pellets were kept at -20 ºC for total RNA extraction.

DHA and EPA delayed the growth of SA 3 during the first 12 h. Cultures treated with 50 µgml^{-1} and 100 µgml^{-1} DHA grew to OD 0.067 and 0.028 respectively compared to the untreated SA3 which grew to OD 0.55 (Figure 5.1-A). EPA showed slightly less inhibitory effect after 12 h, with treated cultures reaching OD_{600} 0.13 and 0.077 when treated with 50 µg ml^{-1} and 100 µg ml^{-1} EPA respectively (Figure5.1-B). When SA3 was treated with a mixture of both compounds (DHA+ EPA) growth inhibition was similar to that observed with EPA alone (Figure 5.1-C). After 24 h exposure to PUFAs, SA3 growth showed signs of recovery. The cultures treated with 50 µgml^{-1} and 100 µgml^{-1} DHA grew to OD_{600} 0.29 and 0.15 respectively, whereas those treated with EPA grew to OD_{600} 0.35 and 0.25 respectively. Although growth was recovering, the optical density of all PUFA-treated cultures was still

considerably lower than the untreated cultures after 24 h (OD_{600} 0.6). This experiment was repeated three times. Therefore, antimicrobial activity of PUFA against *S aureus* waspresent and considerable.





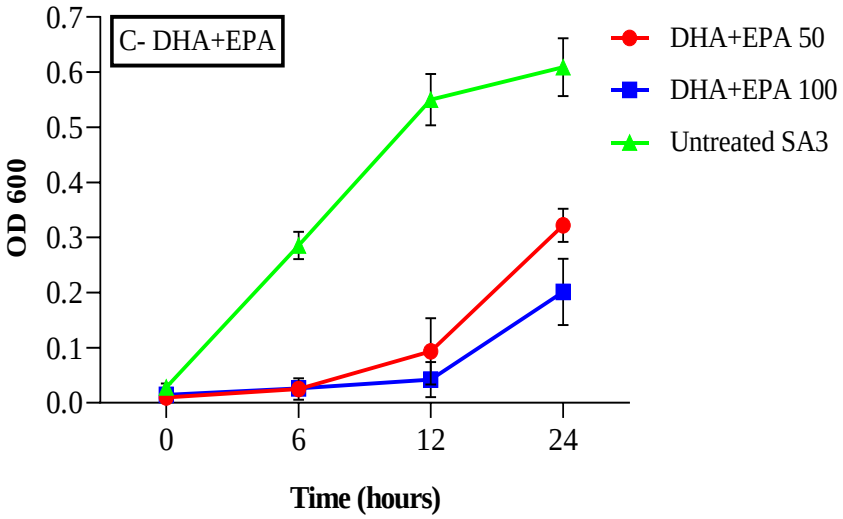
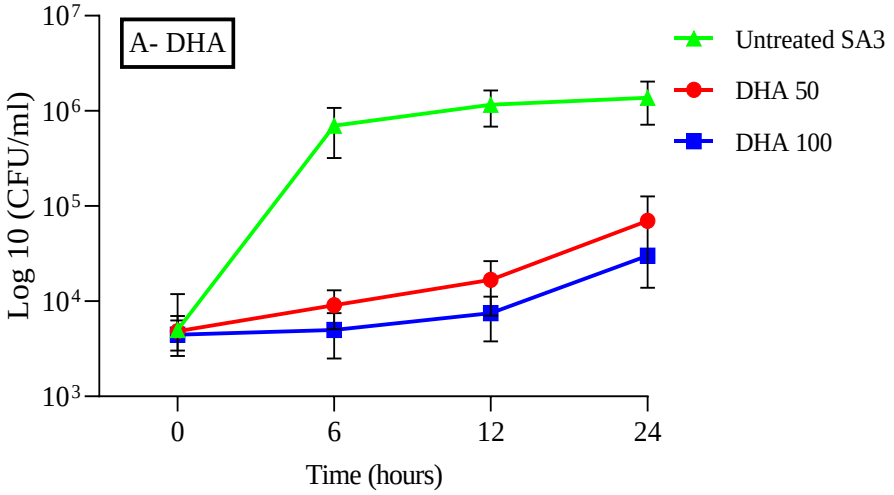
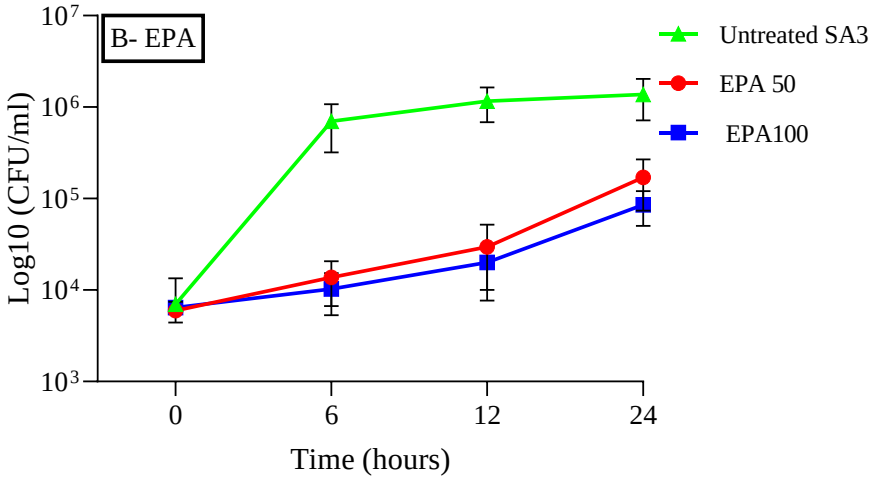


Figure 5.1 **The mean OD_{600} measurements of SA3 in presence of different concentrations of PUFAs compounds. A)** DHA with two concentrations 50 and 100 µg ml^{-1}. **B)** EPA with two concentrations 50 and 100 µg ml^{-1}. **C)** Both mixture of DHA+EPA with two concentrations 50 and 100 µg ml^{-1}. The data represent the average of triplicate independent essays, Bars indicate standard deviations.

Growth of SA3 exposed to 100 µgml^{-1} PUFAs was surprising, given that previous experiments determined the MIC of DHA and EPA to be below this concentration (65 and 78 µgml^{-1} respectively). This may be explained by different culture volumes. To further establish the exact effects of DHA and EPA on the planktonic growth of SA3, viable bacterial cells were quantified at each time point. The number of viable SA3 cells did not increase from the initial inoculum for the first 6 h exposure to DHA. Colony counts from cultures exposed to 50 and 100 µg ml^{-1} were observed to be 9x 10^{4} and 5x10^{3} CFUml^{-1} respectively, compared to untreated SA3 that grew to 69 x 10^{5} CFU ml^{-1} within 6 h. A slow, steady increase in growth was observed between 6 and 24 h with PUFA exposed cultures reaching between 16 x10 ^{4} and 7 x 10^{3} CFU ml^{-1} compared to untreated cultures that reached 13 x 10^{6} CFU ml^{-1} after 12 h and remained at this number after 24 h (Figure 5.2, A). A similar effect was observed in EPA treated cultures (Figure 5.2, B and C) with viable cells remaining at 13x 10^{3} and 1x 10^{3} CFU ml^{-1} for the first 6 h treatment with 50 and 100 µg ml^{-1} respectively. Then slow growth was observed, reaching 17 x 10^{4} and 85 x 10^{3} CFU ml^{-1} after 24 h. The growth of SA3 was inhibited in a similar way by combined treatment with DHA and EPA and there was no additive effect of the two (Figure 5.2 C). RNA was extracted from culture samples at 6 and 12 h for gene expression studies.





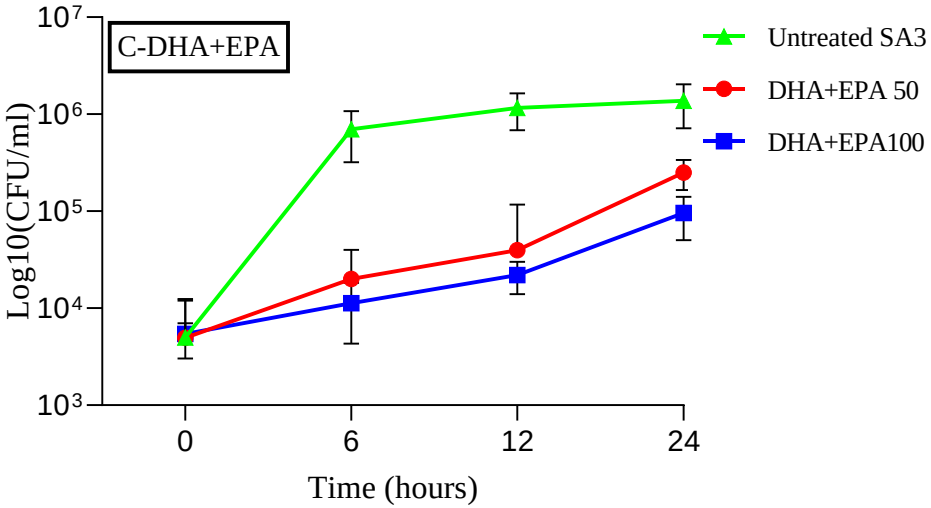


Figure 5.2 **Inhibition of SA3 growth over 24 h by different concentrations of PUFA compounds. A)** CFU ml^{-1} SA3 exposed to DHA at 50 and 100 µg ml^{-1}. **B)** CFU ml^{-1} SA3 exposed to EPA at 50 and 100 µg ml^{-1}. **C)** CFU ml^{-1} SA3 exposed to a 50:50 mixture of DHA and EPA at 50 and 100 µg ml^{-1}. Viable cell counts from all treated cultures were compared to untreated cultures. Bars indicate standard deviations. The data represent the average of the triplicate independent essay.

5.1.2 The efficacy of complementary DNA (c DNA) synthesis

To determine the effect of PUFAs on the expression of global gene regulators, RNA was extracted from untreated and PUFA treated cultures at 6 and 12 h. Complimentary DNA (cDNA) was synthesised from each sample and quality was determined by analysis on a Nanodrop 2000 and by checking for the presence of contaminating DNA. Since SA3 was found to be resistant to erythromycin, and the genome sequence confirmed the presence of the *erm*C gene (chapter 3), *ermC* primers were used to assess the quality of the cDNA samples by PCR. The primers amplified a 285 bp product from initial RNA samples, suggesting DNA contamination. Therefore, a second DNAse treatment was performed and the treated RNA samples no longer contained contaminating DNA. All cDNA was synthesised from DNAse treated RNA and the *ermC* product was amplified to confirm quality of the cDNA (Figure5.3).

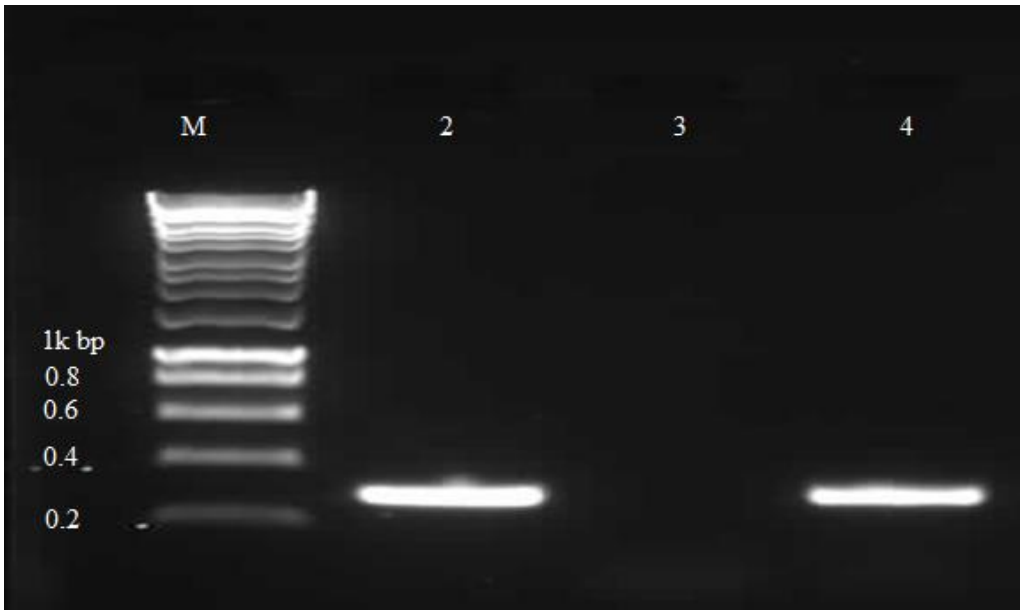


Figure 5.3 **Validation of the quality of RNA and cDNA**. 1% agarose gel stained with Gel Red. PCR amplification of *ermC* (285 bp) confirms presence of SA3 DNA. Lane 1, Molecular weight marker (Hyperladder I – Bioline); Lane 2, *ermC* PCR product from example DNA contaminated RNA sample; Lane 3, no PCR product indicates pure RNA; Lane 4, *ermC* PCR product from example cDNA synthesised from pure RNA.

5.4 Optimisation of primers for RT-PCR

The optimisation of primers is important for improving the quality of RT-qPCR performance. Artemis Comparison Tool (ACT) software was used to view the SA3 whole genome and design primers for target genes. Since previous chapters indicated that the PUFAs affected both biofilm formation and virulence, primers were designed to quantify expression of global regulators of virulence and biofilm formation (*agrA, sarA and ica A*). Different house-keeping genes were also assessed as internal control genes (*16S rRNA, fabD, pyk* and *proC*). Standard PCR was performed validate each primer set and optimise cycling conditions. The cDNA template was used for each prime set, which all amplified the expected product size for each gene (Figure 5.4).

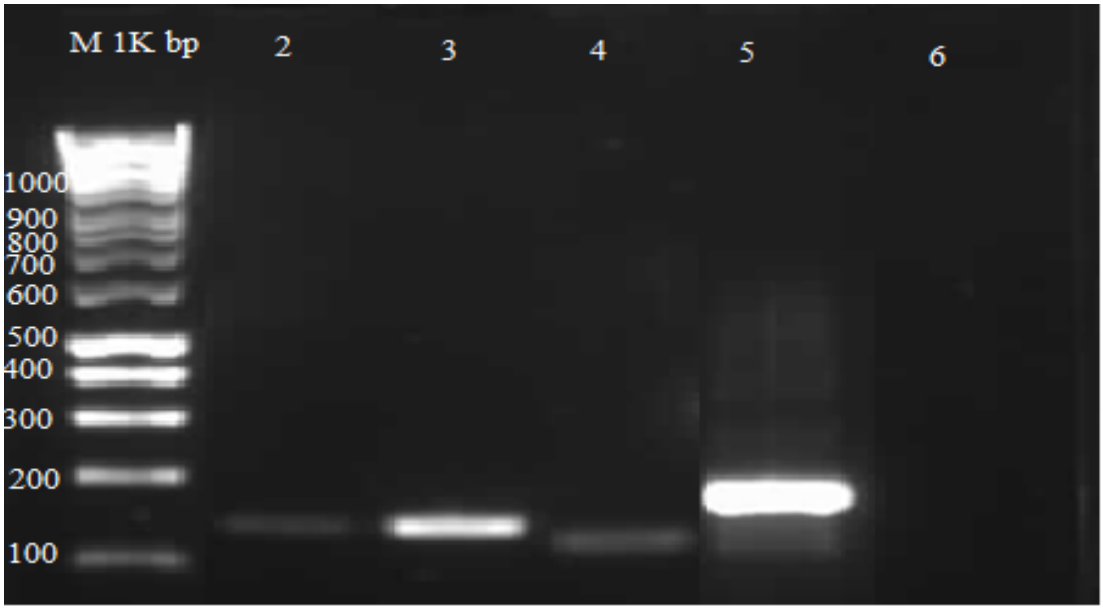


Figure 5.4 **Confirmation of amplicon sizes for the RT-qPCR target genes.** 1.5% agarose gel loaded with PCR products and run at 70V for 1 h. Lane 1, MW marker 1kbase pair (Hyperladder I – Biolone); Lanes 1*ica A*149bp, Lanes 2-*agrA*149bp, Lanes 3-*sarA*110bp, Lanes4-*16s RNA*191bp and Lanes 5-negative control H_2O.

5.5Identification of suitable internal controls for gene regulation in SA3

Three staphylococcal house-keeping genes (*fabD, proC* and *pyk*) were assessed for their suitability as internal control genes according to published work (Theis, *et al*., 2007). These house keeping genes are typically used because they are believed to be stable inthe presence of a range of concentrations of four structurally different antimicrobial compounds (Theis *et al*., 2007). The PCR produced of *fabD* and *pyk*, did not give reliable quantification as shown in figure 5.5. The *proC* gene (106 bp amplicon) was chosen in this study as an optimum internal control. Each single band of the correct size was purified by gel extraction to produce standard concentration controls (Figure 5.5).

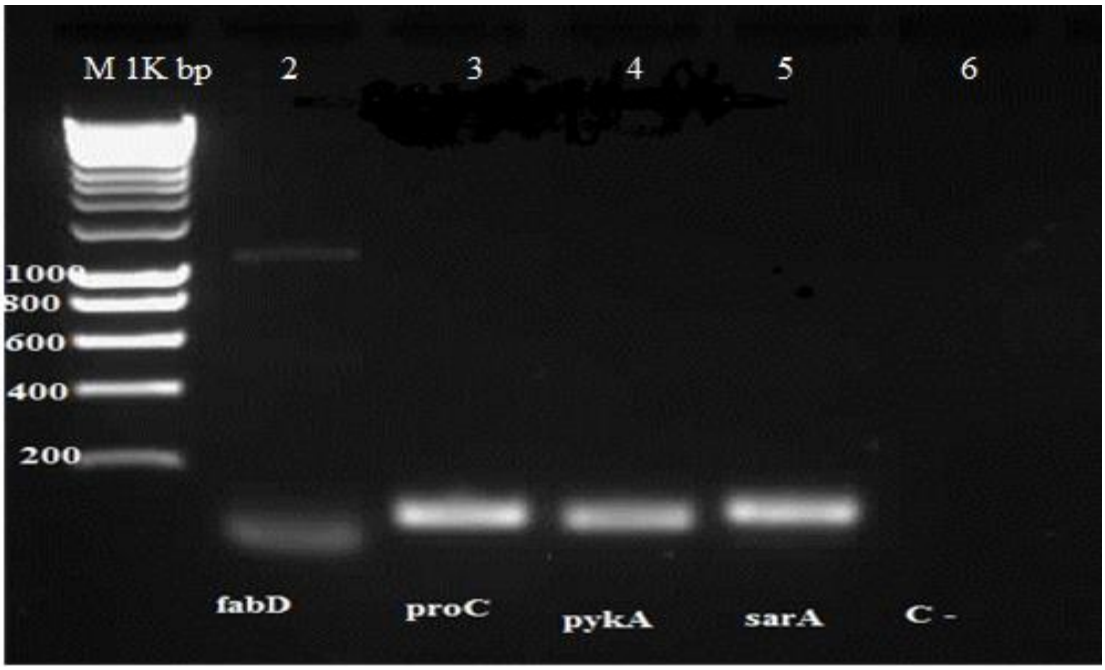


Figure 5.5 **Primer validation for *S. aureus* housekeeping genes in SA3**. 1.5% agarose gel analysis of housekeeping genes amplicons from standard PCR. Lane 1 MW marker (Hyperladder I – Bioline); Lanes 2-5 gene amplicons [2, *fabD.* 3, *proC.*4, *pyK*]. Lane 5, *sarA* as a positive control. Lane 6, negative control H_2O template.

5.1.3 Effect of PUFAs on the expression of virulence genes and regulators regulation

S. aureus coordinates the expression of a wide range of virulence genes by a complex network including multiple global regulator genes such as *agr A, sarA*. Disruption of these regulatory networks is an attractive strategy for novel therapeutics. This chapter focusses on the effect of PUFAs on the associated regulatory genes of virulence of clinical *S. aureus* isolate SA3. The Quantitative real-time PCR (qRT-PCR) of relative expression levels of three virulence-relatedeach gene *agrA,icaA* and *sarA* were determined using the 2−ΔΔCT method (Livak & Schmittgen, 2001). Gene specific primers were used, and *pro C* was used as the housekeeping control (Supplementary Table S1) to normalize the expressions of genes of interest. The q RT-PCR technique employed was an adaptation of a previously described method (Montgomery *et al*., 2010). RT-qPCR was performed using a SYBR Green supermix system (details in Chapter 2).

Treatment of SA3 with DHA or EPA caused changes in expression of global regulator genes compared to no treatment. Relative transcriptional profiles were determined for 6 and 12 h cultures by qRT-PCR with respect to *proC* expression. Within 6h, treatment of cultures with 50 µg ml^{-1} DHA up-regulated the expression of *agr A* and *ica A* genes by 4.7 and 5.5-fold, but dramatically down-regulated *sarA* 10-fold. This effect was more pronounced when cultures were treated with double the DHA concentration (100 µg ml^{-1}). The expression of *agrA* and *icaA* increased 7.4 and 9-fold respectively, while *sarA* expression decreased 11.1-fold compared to untreated cultures (Figure 5.5-A). After 12 h treatment with 50 µg ml^{-1} DHA, at the same trend of differential expression was observed, but to different levels (Figure 5.5-B). The *agrA* gene was up regulated more (7.6 fold), but the *icaA* gene was only upregulated 4.6-fold, while *sarA* was down-regulated much more (16.6-fold) compared to untreated cultures. Moreover, when cultures were treated with double concentrations of DHA

(100 µg ml^{-1}) the effect was more pronounced, with upregulation of *agrA* and *ica A* 33 and 10-fold respectively, and downregulation of *sarA* 20-fold.

EPA had a weaker effect on gene expression, but still induced the same general effect. After the first 6 h of 50 µg ml^{-1}EPA treatment, the expression of *agr A* and *ica A* was up-regulated by 3.5 and 2.8-fold, while *sarA* was down-regulated 5.5-fold (Figure 5.6-A). This effect was more evenly amplified when higher concentrations of EPA (100 µg ml^{-1}) were used. The *agr A* and *ica A* genes were upregulated 6.6 and 5-fold respectively, while *sarA* was down-regulated 9-fold. After 12 h 50µg ml^{-1} EPA differential gene expression was further amplified, with *agrA* and *icaA* genes up-regulated 10 and 8.3-fold and *sarA* down-regulated 9-fold (Figure 5.6-B). The higher concentrations (100 µg ml^{-1}) caused much higher changes in expression of *agrA* (up 20-fold) and *sarA* (down 25-fold). However, the *icaA* gene expression was only increase 8.3-fold (the same effect as 50 µg ml^{-1}) compared to untreated cultures.

Thus, the present study indicates that PUFA compounds, DHA and EPA have the capacity to attenuate *S. aureus* virulence, as evidenced by considerably altering the expression of global gene regulators and adhesins.

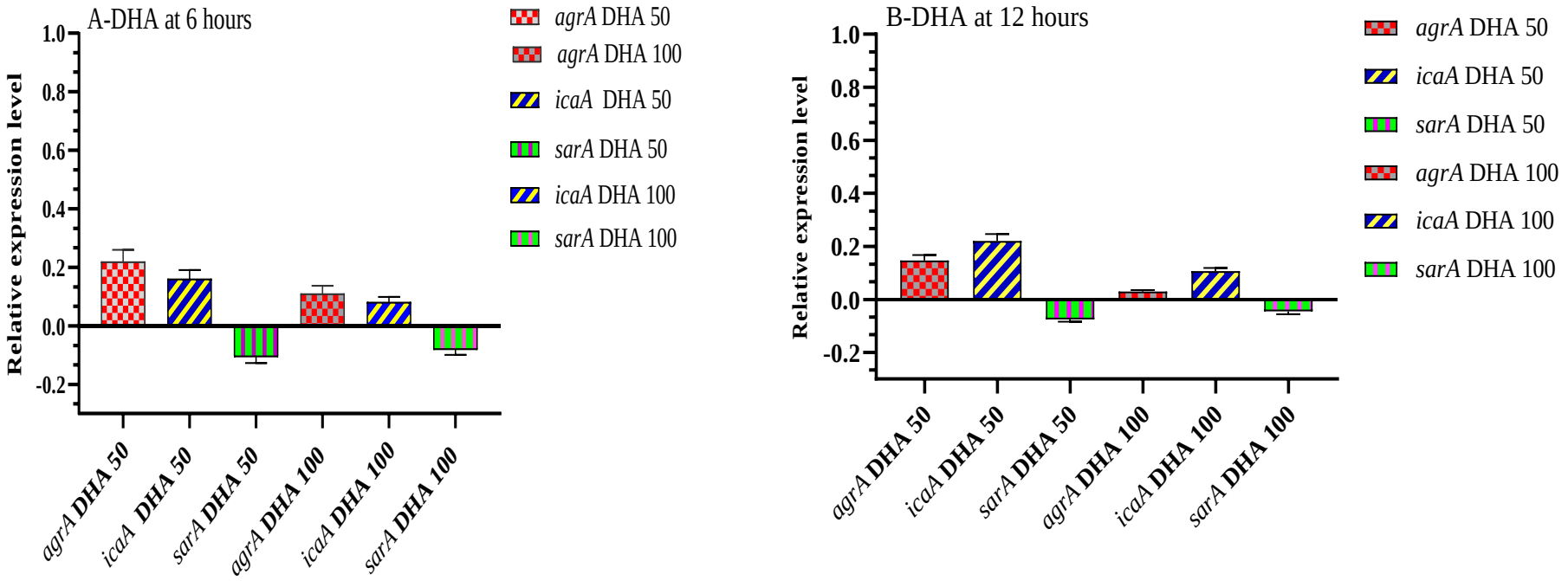


Figure 5.6 **RT-PCR analysis to quantify expression of three virulence-associated genes (*agrA*, *icaR*, and *sarA*) in SA3 cultures exposed to DHA(A)** Treatment at the time six hours and **(B)** Treatment at the time of twelve hours. Data were expressed as the mean ± standard deviation (n = 3). Relative transcriptional profiles were determined with respect to the internal control gene *proC* expression. Statistical analyses were performed by GraphPad Prism v8.00, and an unpaired Student's t-test with Welch's correction was considered statistically significant $P < 0.05$. The p value table for both DHA at 6 and 12h are in Table S6 in appendixes.

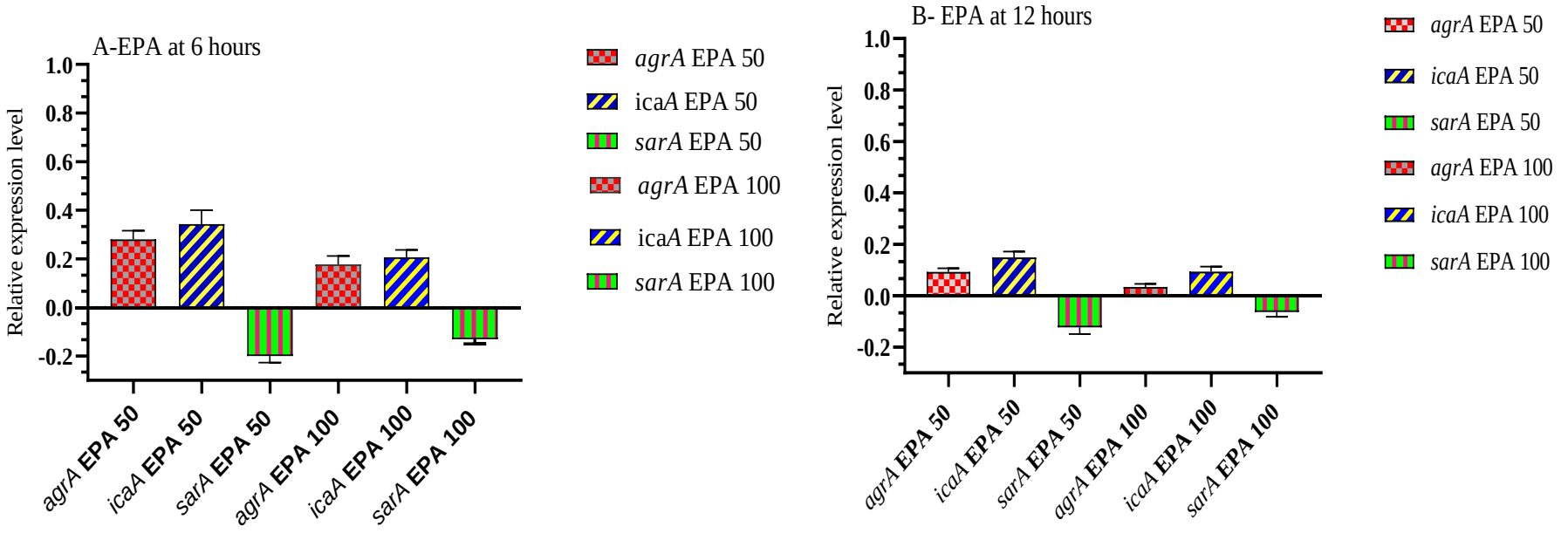


Figure 5.7 **RT-PCR analysis to quantify expression of three virulence-associated genes (*agrA*, *icaR*, and *sarA*) in SA3 cultures exposed to EPA(A)** Treatment at the time six hours and **(B)** Treatment at the time of twelve hours. Data were expressed as the mean ± standard deviation (n = 3). Relative transcriptional profiles were determined with respect to the internal control gene *proC* expression. Statistical analyses were performed by GraphPad Prism v8.00, and an unpaired Student's t-test with Welch's correction was considered statistically significant P< 0.05. The p value table for both EPA at 6 and 12 h are in Table S 7 in appendixes.

5.2 Discussion

S. aureus pathogenicity involves coordinated regulation of a wide range of genes that encode virulence factors, including adhesins, toxins and biofilm-associated proteins. Global gene regulators such as the accessory gene regulator, *agrA* and staphylococcal accessory gene regulator, *sarA* control the expression of most exotoxins produced by *S. aureus* during different stages of infection (Lee *et al*., 2014). In addition, *S. aureus* has been shown contain the intracellular adhesion operon *ica* which is responsible for slime layer production in biofilm formation (Cramton *et al*., 1999). Furthermore, the *ica A* gene encoded the enzyme N-acetyl glucosamyl transferase which is involved in synthesised of N Acetylglucosamine oligomers from UDP N acetylglucosamine, with the regulation of i*ca ADBC* orthologous genes are playing significant role for synthesising the Polysaccharide Intercellular Adhesion (PIA) (Anjuman *et al*., 2014; Nourbakhsh & Namvar, 2016), which are highly contributed for cell to cell interaction during biofilm formation. However, its widely published that the global regulator, *sarA*, has a direct effect on the on P2 and P3 promoters, to which *agrA* binds, and on *agrC-agrA* interaction (Cheung & Manna, 2005; Reyes *et al*., 2011). Furthermore, *SarA* has been demonstrated to enhance biofilm formation directly by increasing PIA production (Valle *et al*., 2003). For example, mutation of *sarA* results in increased expression of proteinases and nucleases, which both enzymes have a negative impact on biofilm formation (Beenken *et al*., 2010). The transcriptional downregulation of *agr* expression has been attributed to the sequestration of the quorum-signalling molecule auto-inducing peptide (AIP) (James *et al*., 2013). A study by Otto, 2011 showed that expression of *agr* leads to increase expression of several toxins such as, serine protease, DNase, enterotoxin B, and toxic shock syndrome toxin-1, and the *agr* locus decreases the expression of colonization factors including *ica* gene (Otto, 2001). These studies suggested

that both global regulators genes *agr A* and *sarA* and the *ica A* gene play significant role in biofilm formation and *S. aureus* infections.

The major aim of this study was to investigate the effect of PUFA compounds on global gene regulators *agrA* and *sarA* and further investigate the effect of PUFA compounds on *icaA* gene in biofilm formation. Disruption of virulence and biofilm gene regulation would be an attractive action for new wound treatments. Disruption of virulence, and growth rate, would exert less selective pressure and reduce the likelihood of resistance development.

Notably, DHA and EPA significantly affected the expressions of all three genes (*agrA, icaA* and *sarA*). Thus, the present study indicates that DHA and EPA could attenuate *S. aureus* virulence. This is supported by evidence presented in chapter 3 that showed reduced biofilm formation in the presence of PUFAs and in chapter 4 that indicated reduced virulence in *G. mellonella*. Whilst *agrA* and *icaA* genes were up-regulated, *sarA* was down-regulated by PUFAs. This study has shown the effect of DHA was more pronounced, with upregulation of *agrA* and *ica A* 33 and 10-fold respectively, and downregulation of *sarA* 20-fold. Similarly, the higher concentrations of EPA caused much higher changes in expression of *agrA* (up 20-fold) and *sarA* (down 25-fold). These results disagree with Kim *et al*., (2018), who found that neither DHA, or EPA, significantly affected the expression of, *agrA, sarA, icaA*, and other related genes such as *sigB*, or *spa*. However, same study showed that DHA, and EPA attenuate *S. aureus* virulence, and reduction in biofilm formation (Kim *et al.*, 2018). This may be because Kim *et al.*, (2008) used much lower concentrations of PUFAs (20 μgml^{-1}) compared to this study (50 and 100 μgml^{-1}). Sun *et al*., (2016) showed that exposing *P. gingivalis* to 100 Mm DHA significantly decreased expression of several virulence genes, including *hagA* and *hagB* genes by 3.8-, 7.3-fold, respectively, as well as proteases genes *rgpB* and *kgp* (5.5- and 4.2-fold, respectively). Similarly, treatment with 100 mM EPA also

markedly downregulated the expression of*hagA, hagB, rgpB* and *kgp*by 5.5-, 10.53.4, and 9.4- fold, respectively (Sun *et al*., 2016).

The differential up and down regulation of *agrA*, and s*arA* loci during infection is widely reported. For example, the exposure to sub MICs of oxacillin resulted in higher levels of expression of*agr A*in *S. aureus*CC15 and CC45 strains while the same gene was down-regulated in the CC30 strain (Viedma *et al*., 2018). It is conceivable that downregulation of *agr* activity, as part of bacterial colonisation, as *agr* dysfunction has been associated with persistent bacteraemia in MRSA, the innate immune response could in fact contribute to the formation of staphylococcal persisters leading to chronic infections (Chong *et al*., 2013).

This effect on global regulators has been observed in response to other treatments, but usually, both *agrA* and *sarA* are down-regulated. For example, the novel drug candidate baicalein (a flavonoid, originally isolated from the *Scutellaria baicalensis*) has been investigated, for treatment *S. aureus* infections. At 32 μg ml^{-1} and 64μg ml^{-1}this compound downregulated the quorum-sensing system regulators *agrA*, RNAIII, and *sarA*, and the gene *ica* (Chen *et al*., 2016). Choi *et al*., (2015) showed that the ethanolic extract of *Artemisia princeps* significantly down-regulated *agrA*, and *sarA*, expression by MRSA at the concentrations higher than 1mg ml^{-1} (Choi *et al*., 2015).

The variation of activity of PUFAs on gene regulation could be dependent on the study strain or the concentration of PUFA used. This study just looks at two global genes and the *ica* gene at 6h and 12 h, and expression changes have only been assessed at the transcript level (not protein level changes). So, the actual overall effect requires clarification. Changes in *agrA* and *sarA* transcription does not fully explain the reduced virulence *in vivo.* It is important to consider the wide network of global regulator genes and other virulence regulators involved in the pathogenicity of *S. aureus.* For example *sarU, sarX, codY, mgrA,*

and *sigB* (Junecko *et al*., 2012; Lauderdale *et al*., 2009) that have a direct or indirect effect on the *agr, sarA* and *icaA,* that all play role for *S. aureus* infection. Although, it is widely reported and accepted that PUFA compounds could be used to treat *S. aureus* infections. The effects of the inhibitory concentrations of PUFAs on *S. aureus* infection need more assessment by looking to other genes regulators and to look for the activity of EPA and both mixture of these compound at different time point.

Furthermore, it is important to consider the effect of sustained exposure to PUFAs on *S. aureus* isolates and to evaluate the effect on susceptibility and cross-resistance profiles as well as any effect on virulence.

Chapter Six

Effects of sustained exposure to PUFAs and clinically relevant antibiotics on *S. aureus* resistance profiles and virulence.

Chapter 6 Effects of sustained exposure to PUFAs and clinically relevant antibiotics on *S. aureus* resistance profiles and virulence.

6.1 Introduction

Bacterial antibiotic resistance has become one of the greatest threats to public health throughout the world. According to the World Health Organisation, antibiotic resistant infections could claim at least 50,000 lives across the US and Europe each year, and that by 2050 antibiotic resistance could account for 10 million deaths per year (O'Neill, 2014). Treatment of infections, such as in chronic wound infection, is further complicated due to their polymicrobial nature, biofilm formation and the prevalence of horizontal gene transfer (HGT). As a result, many wound-associated bacteria become multi-drug resistant (MDR). These bacteria have developed a diversity of mechanisms to overcome and survive environmental stresses posed by antibiotics and spread their resistance genes. Adaptation to antibiotics can be partly evidenced by comparing the genomes of resistant and sensitive strains. For instance, many strains of *S. aureus* have acquired resistance via external genetic transfer of the *mecA* gene that encodes an alternative penicillin-binding protein (PBP), conferring methicillin resistance (MRSA). Other resistance mechanisms can occur by mutations. For example, modifications of antibiotic target sites such as domain V of the 23S rRNA subunit, and/or the ribosomal proteins L3 and L4 *rplC and rplD*, interfering with linezolid binding, hence encoding resistance (Munita & Arias, 2016).

Development of resistance by mutation can be a stepwise and transient process in response to prolonged exposure to sub-inhibitory concentrations of antibiotics. Stress responses such as reduced porin channel or increased efflux pump expression, can limit the

concentration of antibiotics at the target site and allow for the further development of resistance mechanisms (Pagès *et al*., 2008). The ability of bacteria to survive under treatment without developing resistance is called tolerance, which is a transient, non-heritable phenotype. A slow or non-growing tolerance phenotype can enable the bacteria to adapt and survive most bacteriocidal agents, which require active growth for action. Many reports have shown that interrupted antibiotic treatment leads to tolerance which could accelerate the evolution of antibiotic resistance (Fridman *et al*., 2014; Haaber *et al*., 2015; Novak *et al*., 1999). For example, Habber *el. al.,* (2015) revealed that when *S. aureus* strain USA300 was exposed to sub-inhibitory concentrations of colistin, a 50% increase in the vancomycin resistance was observed (MIC increase from 1.1 to 1.6 g ml^{-1}). Colistin was found to induce a reversible tolerance to vancomycin action by reducing cell wall thickness, altered cell wall surface charge, and altered gene expression (Haaber *et al*., 2015). Others have shown that staphylococcal biofilm formation and density increased following pre-exposure to sub-inhibitory concentrations of antibiotics, enhancing antibiotic tolerance (Felden & Cattoir, 2018; Kaplan *et al*., 2012; Levin-Reisman *et al*., 2017). Adaptive responses have also been reported during treatment of *S. epidermidis* biofilms with sub-inhibitory concentrations of vancomycin. A two-fold increase in abundance of eDNA was observed, which has a strong binding affinity for vancomycin, preventing its access to the cells encased in the biofilm matrix (Hall & Mah, 2017).

The global burden of multi-drug resistant bacterial infections has reached a critical point. Although effective new anti-bacterial therapies are dripping through the pipeline, resistant clinical isolates are usually reported shortly after their introduction. So far, this study has demonstrated the antibacterial activity of PUFAs and quantified their effective MICs *in vitro* and under more realistic conditions using an *in vivo* invertebrate model and *ex-vivo* skin model. PUFAs did not show any toxicity in the *G. mellonella* model. Furthermore, other

studies have shown that PUFAs can be used in humans from up to 200 µM. For instance, PUFAs have been shown to enhance cancer cell sensitivity to chemotherapy and radiation, and DHA and EPA concentration of 25–100 µM did not inhibit the growth of fibroblasts cells (Zajdel *et al*., 2015). Moreover, DHA and EPA have been shown to exhibit no cytotoxicity on human oral tissue cells when used at concentrations of 100 - 200 mM (Sun *et al*., 2016). However, little is known about the potential for bacteria to adapt to PUFAs and develop resistance and what effects this might have on virulence *in vivo*.

Clinical surveillance and experimental evolution approaches have demonstrated the rate at which bacteria can adapt to stresses such as antibiotics. For example, Latimer *et. al*., (2012) demonstrated that during exposure of *S. aureus* 6538 to concentration gradients of triclosan, the phenotypic growth showed Small-Colony Variant (SCV) and significant reduction in biofilm formation capacity, and reduction in virulence factors such as coagulase, DNase, haemolysin, and attenuated virulence in the *G. mellonella* model of infection (Latimer *et al*., 2012). Others have reported reduced coagulase production when *S. aureus* was exposed to sub-inhibitory concentrations of roxithromycin, while cefalexin induced biofilm formation at a wide range of sub-inhibitory concentrations (Haddadin *et al*., 2010). Another recent study used the Hollow Fiber Infection model and showed that exposure of USA300 to lower vancomycin concentrations (0.5 - 4 g every 12h regimen) resulted in reduced expression of the global gene regulator *agr* genes. These genes have been shown to coordinate *S. aureus* virulence and reduced expression enhanced survival rate of infected *G. mellonella* larvae. In addition, as the exposure concentration of vancomycin increased the MICs of daptomycin and telavancin also increased, suggesting cross-resistance. Conversely, the bactericidal effects of Vancomycin (4 g every 12h regimen) suppressed vancomycin resistance and did not significantly alter the survival rate of *G. mellonella* model (Lenhard *et al*., 2016).

This study adopted an experimental evolution approach to investigate the ability of *S. aureus* SA3 to adapt to continuous exposure to PUFAs compared to antibiotics and to assess the effects on virulence using the *G. mellonella* model.

6.2 Altered resistance profiles following prolonged exposure to sub-inhibitory concentrations of PUFAs and antibiotics.

Following extended exposure of SA3 to sub-inhibitory concentrations of PUFA compounds (DHA and EPA) and antibiotics (vancomycin and linezolid), changes in resistance profiles were assessed. Notable differences in resistance profiles of evolved strains were observed compared to the parent SA3 strain (Table 6.1). The DHA and EPA-evolved strains (SA3-DHA, and SA3-DHA), tested after 14 passages in sub-inhibitory DHA or EPA, exhibited no reduced susceptibility to EPA, but surprisingly, a slight increase in susceptibility to DHA (MIC change from 65 to 39 µg ml^{-1}) and linezolid (MIC change from 3.9 to 1.9 µg ml^{-1}). Furthermore, both evolved strains were considerably more sensitive to vancomycin. SA3-DHA was 23 x more sensitive (MIC change from 0.093 to 0.0039) and SA3-EPA was 13x more sensitive (MIC change from 0.093 to 0.007).

Interestingly, the linezolid adapted strain (SA3-LZD) became more sensitive to inhibition by DHA (MIC reduced from 65 – 19.5 µg ml^{-1}), but not to EPA after 5 passages in sub-inhibitory concentrations of linezolid. Conversely, SA3-LZD was found to have developed increased resistance to linezolid (MIC increased 8-fold from 3.9 – 31.2 µg ml^{-1}). Moreover, there was slight increased resistance to vancomycin (MIC increased from 0.093 to 0.12 µg ml^{-1}). A similar phenomenon was observed for the vancomycin-evolved strain (SA3-VAN) after 8 passages in sun-inhibitory concentrations of vancomycin. No significant change in sensitivity to PUFAs was observed, but a considerable increase in cross-resistance to both

linezolid (4 fold) and vancomycin (2.7 fold) was evident. Surprisingly, SA3-VAN showed a bigger increase in resistance to linezolid (MIC increased from 3.9 – 15.6 µg ml^{-1}) than to vancomycin (MIC increased from 0.093 – 0.25 µg ml^{-1}).

Table 6.1 Sensitivity of SA3 to bacteriostatic effects of PUFAs and antibiotics following sustained exposure to sub-inhibitory concentrations.

Strain	DHA	EPA	DHA +EPA	LZD	VAN
Parent SA3	65	78	65	3.9	0.093
Adapted SA3-DHA (passage 14)	39	78	65	1.9	0.0039
Adapted SA3-EPA (passage 14)	39	78	65	1.9	0.007
Adapted SA3-DHA+EPA (passage 14)	39	65	65	1.9	0.015
Adapted SA3-LZD (passage 5)	19.5	78	65	31.2	0.12
Adapted SA-VAN (passage 8)	39	65	65	15.6	0.25

Colours indicate extent of decreased (blue) or increased (green) resistance to bactericidal effect of each agent. Deeper colours indicate more pronounced change in MIC. Parent phenotypes are shaded in grey and white cells indicate no change from parent phenotype.

The DHA and EPA-evolved strains (SA3-DHA, and SA3-DHA) exhibited 2-fold increased susceptibility to killing by both PUFAs (MBC change from 520 to 260 µg ml^{-1} DHA and 312 µg ml^{-1}). SA3-DHA also showed increased sensitivity to killing by linezolid, but SA3-EPA did not. Both evolved strains were considerably more sensitive to killing by vancomycin. SA3-DHA was 23 x more sensitive (MIC change from 1 to 0.062).

Again, the linezolid adapted SA3-LZD was observed to have become more sensitive to killing by DHA (MBC reduced from 520 – 78 µg ml^{-1}), but not to EPA. This contrasted with a 4-fold increase in resistance of SA3-LZD to killing by both linezolid and vancomycin (MBC increased from 31.2 – 125 µg ml^{-1} linezolid and from 1 to 4.1 µg ml^{-1} vancomycin). Interestingly, the vancomycin-evolved strain (SA3-VAN) was somewhat sensitized to killing by DHA (MBC decrease from 512 to 156 µg ml^{-1}), but not EPA. A 2-fold increase in cross-resistance to linezolid killing activity was observed. Surprisingly, no change was observed in sensitivity of SA3-VAN to killing by vancomycin.

Table 6.2 Sensitivity of SA3 to bactericidal effects of PUFAs and antibiotics following sustained exposure to sub-inhibitory concentrations.

Strain	DHA	EPA	DHA +EPA	LZD	VAN
Parent SA3	520	520	625	31.2	1
Adapted SA3-DHA (passage 14)	260	312	260	15.6	0.062
Adapted SA3-EPA (passage 14)	312	312	312	31.2	0.062
Adapted SA3 DHA+EPA(passage 14)	260	260	260	31.2	0.062
Adapted SA3-LZD (passage 5)	78	312	260	125	4.1
Adapted SA-VAN (passage 8)	156	260	312	62.5	1

Colours indicate extent of decreased (blue) or increased (green) resistance to bacteriocidal effect of each agent. Deeper colours indicate more pronounced change in MBC. Parent phenotypes are shaded in grey and white cells indicate no change from parent phenotype.

6.3 Changes in tolerance levels of SA3 evolved with prolonged exposure to sub-inhibitory concentrations of PUFAs or antibiotics.

According to the ratio MBC/MIC, SA3 that had been exposed to prolonged sub-inhibitory levels of PUFAs or antibiotics showed stable or slightly reduced tolerance to DHA (ratios 8 - 6.6). However, a slightly more marked reduction in tolerance to EPA was observed, with rations reducing from 6.6 to 4 (Table 6.3). Conversely SA3 tolerance to antibiotics was increased following pre-exposure to PUFAs (ratios ranging from 8 to 16.4) but decreased following pre-exposure to antibiotics (ratios ranging from 4 – 0.5). A reduced tolerance value suggests that the evolved strain had become more able to grow but less able to resist killing by the antimicrobial agent.

Table 6.3 Antimicrobial tolerance of SA3 to PUFAs and antibiotics

Strain	DHA	EPA	DHA +EPA	LZD	VAN
Parent SA3	8	6.6	8	8	10.7
Adapted SA3-DHA (passage 14)	6.6	4	4	8.2	15.9
Adapted SA3-EPA (passage 14)	8	4	4.8	16.4	8.8
Adapted SA3-DHA+EPA (passage 14)	6.6	4	4	16.4	4.1
Adapted SA3-LZD (passage 5)	4	4	4	4	0.5
Adapted SA-VAN (passage 8)	4	4	4.8	4	4

Tolerance = ratio of MBC/MIC *higher value = increased tolerance. Colours indicate extent of decreased (blue) or increased (green) tolerance to each agent. Deeper colours indicate more pronounced change in tolerance. Parent phenotypes are shaded in grey and white cells indicate no change from parent phenotype.

In summary, SA3 did not show any notable capacity to develop resistance or tolerance to PUFAs, which in turn were able to sensitize SA3 to antibiotics, particularly linezolid. By contrast, SA3 showed rapid development of resistance to linezolid and vancomycin, but generally no change in sensitivity to PUFAs. However, long-term exposure to linezolid resulted in slightly increased susceptibility to DHA only.

6.3.1 Detection of Cross-resistance:

Given the reduced sensitivity of SA3 following sustained exposure to antibiotics. This study used Kirby Bauer disk diffusion assays to investigate whether any cross-resistance had also developed to other antibiotics (Table 6.4). The antibiotic adapted strains (SA3-LZD and SA3-VAN) showed little change in susceptibility to most other tested antibiotics compared to the parent. However, a marked difference in sensitivities of the vancomycin adapted strain was observed for lincomycin, and erythromycin but not tetracycline. PUFA adapted strains showed no notable change in sensitivity to gentamycin, tobramycin, cefoxitin, vancomycin, oxazolidine or ciprofloxacin (despite small fluctuations in size of zones of inhibition). However, a marked difference in sensitivities was observed for lincomycin, erythromycin and tetracycline. For example, according to EUCAST guidelines, the SA3 parent was considered sensitive to lincomycin, with a zone of inhibition of 21mm diameter. This sensitivity did not change following prolonged exposure to linezolid (SA-LZD). However, SA3-DHA, SA3-EPA and SA3-VAN showed reduced sensitivity and were considered resistant, with zones of inhibition measuring 13, 14- and 12-mm diameter respectively. The same phenomenon was observed with respect to sensitivities to erythromycin. Conversely, all adapted strains became considerably more sensitive to tetracycline compared to the parent SA3 (zones of inhibition increasing from 7 to 21-27 mm diameter).

Table 6.4 **Antibiotics sensitivity test of SA3 after prolonged exposure to sub-inhibitory concentrations of PUFAs or antibiotics**

Antibiotics Disks (µg)		**Aminoglycoside**		**β-lactam**	**Glycopeptides**	**Lincos-amides**	**Macrol-ides**	**Oxazolidine**	**Quinolone**	**Tetracycline**
Adaption of *S. aureus* (SA3)	Strains	**CN 10**	**TOB 10**	**FOX 30**	**VAN 5**	**MY 2**	**E 15**	**LZD 10**	**CIP 5**	**TE 30**
	Parent SA3	S (25)	S (23)	**R (10)**	S (25)	S (21)	S (19)	S (24)	S (23)	**R (7)**
	Adapted DHA SA 3	S (20)	S (24)	**R (15)**	S (15)	**R (13)**	**R (12)**	S (24)	S (25)	S (25)
	Adapted EPA SA3	S (22)	S (25)	**R (11)**	S (14)	**R (14)**	**R (13)**	S (21)	S (22)	S (26)
	Adapted EPA+DHA SA3	S (23)	S (24)	**R (13)**	S (14)	**R (16)**	**R (15)**	S (24)	S (28)	S (27)
	Adapted Linezolid 32µgml^{-1} SA3	S (19)	S (21)	S (25)	**R (10)**	S (24)	S (22)	**R (11)**	S (27)	S (22)
	Adapted Vancomycin 0.25µgml^{-1} SA3	S (20)	S (24)	S (26)	**R (9)**	**R (12)**	**R (10)**	**R (14)**	S (26)	S (21)

6.5 Quantification of biofilm formation by adapted SA3 strains

RT-PCR data from chapter 5 of this study suggest that exposure to PUFAs can affect the expression of global regulators and genes associated with biofilm formation. Since prolonged exposure to PUFAs has affected resistance phenotypes, it was hypothesised that the PUFAs may also drive changes in biofilm forming capacity. Standard crystal violet assays in micro-titre plates were used to compare the biofilm densities of parent and adapted SA3 within 24 h and 48 h. Biofilm density readings OD_{600} indicated that all adapted strains of SA3 had 3-fold reduced capacity to form biofilms compared to parent SA3 (Figure 6.1). The mean OD_{600} of 24 h biofilms produced by adapted strains ranged from 0.14 to 0.31 (Table of P-valuedata are supplemented in appendices). In all cases, adapted strains showed a decreased capacity to form biofilms. Biofilms formed by the vancomycin adapted strain were denser than those formed by the other adapted strains, but still 2-fold reduction compared to the parent SA3.

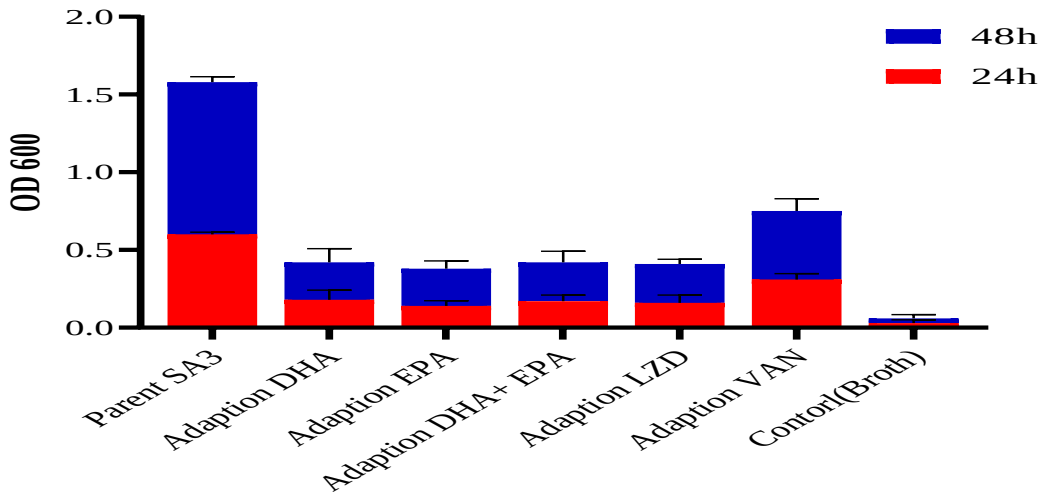


Figure 6.1 **Quantification of biofilm formation by adapted SA3 compared to parent.** The crystal violet microtiter plate assay was used to quantify biofilm density after 24h and 48 h. Un-inoculated, sterile LB was used as a negative control. The values are plotted as mean ± SD obtained from three representative experiments performed in triplicate. By using unpaired

t test data showed statically significant with all strains compared to Parent SA3, the statically table are in appendices (table S 7 chapter 6).

6.4 Effect of prolonged exposure to PUFAs and antibiotics on susceptibility of SA3 biofilms to inhibition and disruption

Minimum biofilm inhibitory concentration (MBIC) and the minimum biofilm eradication concentration (MBEC) assays were used to quantify the ability of PUFAs and antibiotics to disrupt biofilm formation by adapted strains that were pre-exposed to sequential sub-inhibitory concentrations of each agent (Figure 6.2). In all cases, biofilm formation by adapted strains was much more readily inhibited and disrupted compared to the parent SA3 (data for parent shown in chapter 3). Antibiotics were clearly the most effective biofilm inhibitors and disrupters. Vancomycin inhibited biofilm formation by the parent SA3 at MBIC 2 μg ml^{-1} but adapted strain biofilms were inhibited with much lower MBICs between 0.019 and 0.0048 μg ml^{-1}. Linezolid was less efficient than vancomycin at inhibiting biofilms formed by parent (MBIC 32 μg ml^{-1}) and adapted strains (MBIC ranging from 0.97 to 3.9 μg ml-1). PUFAs were also more effective at inhibiting biofilms formed by adapted strains compared to the parent. The mean MBIC of DHA was between 6.1- 24.4 μg ml^{-1} for adapted strains compared to 256 μg $ml^{-1}$for the parent SA3. The same was observed for EPA inhibition of biofilm formation by adapted strains, although it was less effective than DHA. The mean MBIC of EPA was 48.8- 97.7 μg ml^{-1} for adapted strains, compared to 384 μg ml^{-1} for the parent SA3.

MBEC assays were used to measure the eradication of biofilms formed around pegs after recovery in 96 wells plates. As shown in chapter 3, the concentrations required to disrupt biofilms (MBEC) were much higher than those required to inhibit biofilm formation (MBIC). The MBEC of vancomycin for adapted strains was between 0.125 and 0.5 μg ml^{-1};

considerably higher than the MBIC (0.08 - 0.125 µg ml^{-1}), but at least 16 x lower than the MBEC for parent SA3 (8 µg ml^{-1}). Again, linezolid was a less potent biofilm disrupter, with a MBECs of between 3.9 and 15.6 µg ml^{-1} compared to MBICs ranging from 0.97 to 3.9 µg ml^{-1} for adapted strains. A much higher MBEC of 128 µg ml^{-1} was required to disrupt parent SA3 biofilms. As expected, higher MBECs of PUFA compounds were needed to disrupt biofilms compared to antibiotics. MBECs of DHA and EPA ranged between 24.4 and 97.7 µg ml^{-1} to disrupt biofilms formed by evolved strains.

It should be noted that the evolved SA3 variants showed reduced ability to form biofilms that were much less dense than the parent SA3 biofilms after 24 and 48 h. This may explain why lower concentrations of PUFAs and antibiotics were needed to inhibit their growth or disrupt them.

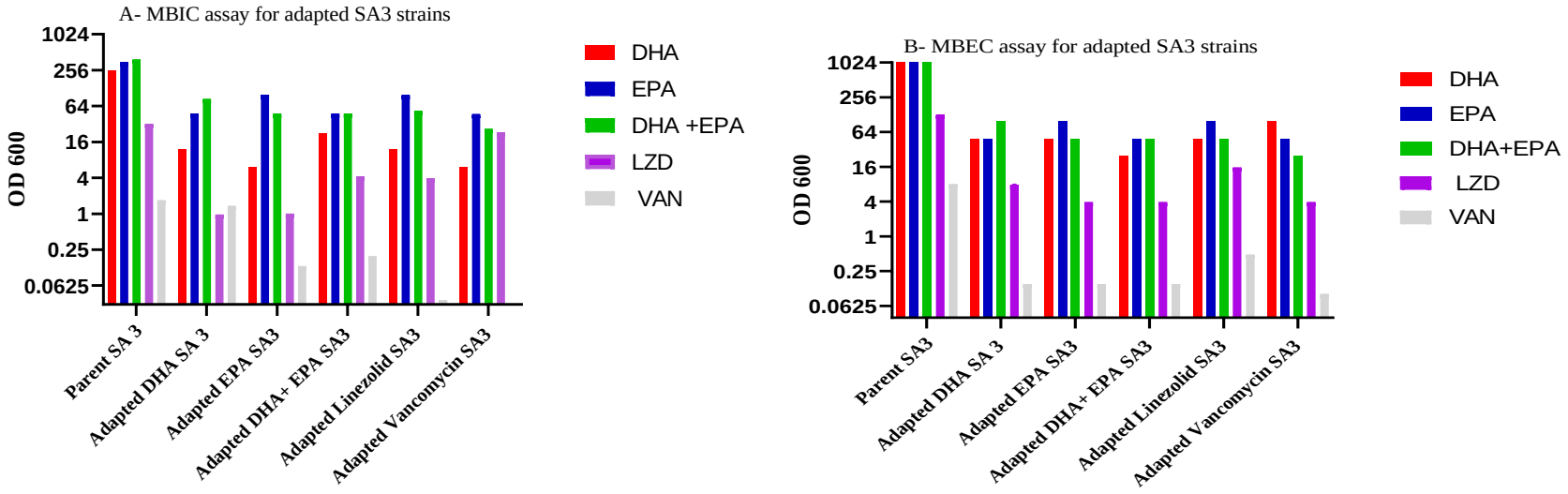


Figure 6.2 **Susceptibility of evolved SA3 biofilms to PUFA and antibiotic compounds. A)** Minimum biofilm inhibitory concentration (MBIC). **B)** Minimum biofilm eradication concentration (MBEC). Statistically significant reductions were found in biofilm production according to the t-test (GraphPad v8). The multiple comparison data using unpaired test showed that No difference between all MBICS VS all MBECS P value= 0.3568, significant VAN MBICs vs All other MBICs =0.0467, significant VAN MBECs vs All other MBICs= 0.0476, significant LZD MBICs vs all PUFA MBICs= 0.0091, significant LZD MBECs vs all PUFA MBECs= 0.0320, significant DHA MBICs vs EPA MBICs= 0.0020, No significant DHA MBECs vs EPA MBECs= 0.4113.The statically table are in appendices (table S 8 chapter 6)

6.5 Effect of prolonged exposure to PUFAs and antibiotics on virulence of SA3 in the *G. mellonella in vivo* model of infection.

Since biofilm formation and expression of global gene regulators are affected by PUFA compounds, this study investigated whether prolonged exposure to sub-inhibitory concentrations of PUFAs would also affect *S. aureus* virulence. This would be a desired effect for an ideal therapeutic. Virulence was determined by observing survival of *G. mellonella* infected with parent and PUFA-evolved variants (SA3-adaptes DHA and SA3-adapted EPA and SA3-adapted DHA+EPA), compared to the virulence of antibiotic-evolved variants (SA3-adaptedLZD and SA3-adaapted VAN) and infection controls (PBS and mock-infected) (Table S 6 chapter6 in appendices). *G. mellonella* larvae were incubated in dark at 37^{o}C for 7 days.

The survival rate of each group is shown (Figure 6.3). The parent SA3 control group remained the most virulent strain, killing 6 larvae in the first 48 h then approximarely two larvae each day after that with 93% of all larvae killed within 7 days (6% survival). The DHA-evolved variant (SA3-DHA) had reduced virulence compared to the parent. All larvae in this group (16/16) survived for the first 5 days, and on day 7, 12/16 larvae (75%) had survived. The EPA-evolved variant was more virulent than SA3-DHA, with a survival rate of 63%. Two larvae died after 72 h then approximately one larvae died each day until day 7. Similarly, both compound DHA+ EPA had evolved variant more a bit virulent with a survival rate of 50%. Two larvae died after 72 h then approximately two larvae died each day until day 7. The infection experiments were repeated 3 times and there was slight variation in number of larvae that died each day, but all experiments followed the same trend (Table S 6 chapter6 in appendices).

The virulence of PUFA-evolved variants was compared to that of SA3 that had been exposed to prolonged sub-inhibitory linezolid (SA3-LZD) or vancomycin (SA3-VAN). In both cases, the survival rate if the infected *G. mellonella* larvae was 81% and 88% respectively. One

larva died 48 h after being infected with SA3-LZD, then a second died on day 6 and a 3rd on day 7 (81% survival). The SA3-Van infection took longer to show any effect, killing 1 larva on day 5 then a second on day 7 (88% survival). No larvae in the negative control groups were observed to die during the 7-day experiment.

These results demonstrate that, although *S. aureus* SA3 showed significant virulence in the *G. mellonella* model, sub-inhibitory concentrations of PUFA compounds and antibiotics (LZD and VAN) had driven a reduction in virulence. It is possible that the reduced virulence is due to serial passage in media, and not specifically driven by the antimicrobial agents. However, the different levels of virulence demonstrated by each evolved variant, and consistently across three repeat experiments, suggests that the may have been an agent-specific effect.

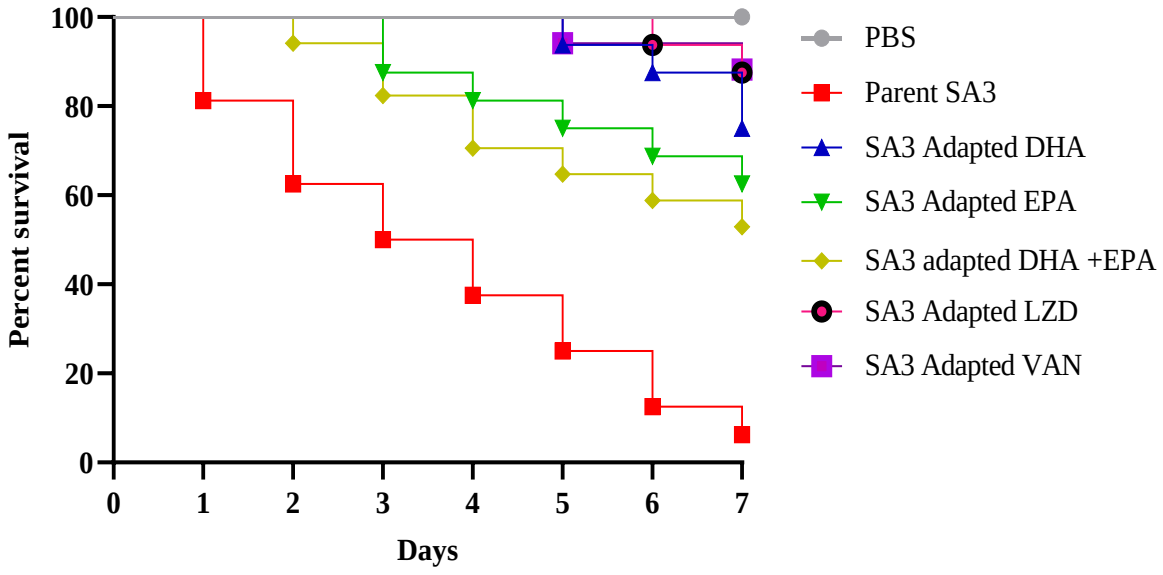


Figure 6.3 **Survival of *G. mellonella* following challenge with PUFA and Antibiotics-evolved variants of *S. aureus SA3***. Groups of larvae (n = 16 each group) were injected with either SA3 or a variant that had been passaged (x 14) in sub-inhibitory PUFAS (SA3-DHA, SA3-EPA and both SA3 DHA+EPA) or antibiotics (SA3-LZD – 5 passages and SA3-VAN – 8 passages). Experimental controls included the PBS control group (n=16), and uninfected larvae (n=16). The figure shows example data chosen from 3 experimental replicates. Survival analysis was done using log-rank Mantel-Cox test in Graph Pad prismV8. Parent SA3 vs SA3 adapted DHA significant P = <0.0001; Parent SA3 vs SA3 adapted EPA significant P =0.0002; Parent SA3 vs SA3 adapted DHA+ EPA

significant P =0.0017; Parent SA3 vs SA3 adapted LZD significant P = < 0.0001, and Parent SA3 vs SA3 adapted VAN significant P = < 0.0001.

6.6 Quantification of viable bacterial cells recovered from *G. mellonella* lethally infected with evolved and parent SA3.

Bacteria were recovered from each infected *G. mellonella* at time of death and numbers were compared to the initial inoculum (5 $x10^5$ - 8x 10^5 CFU ml^{-1}). The results are summarised (Figure 6.4). The parent *S. aureus* strain SA3 was the most virulent and "fit" compared to the evolved strains. SA3 was recovered at densities between 4.6 x 10^8 - 1.1$x10^{10}$ CFU ml^{-1}, a 100-fold increase in viable cell numbers compared to the initial inoculum. SA3-DHA killed just four of the larvae during the experiment. Between 2.8 x 10^7 and 6.8 $x10^8$ CFU ml^{-1} of this variant were recovered, while between 2.1 x 10^8 and 1.2 $x10^9$ CFU ml^{-1} SA3-EPA were recovered (10 fold less than SA3 parent). In addition, SA3 DHA+EPA 8/16 larvae killed about 50 %, between 3.2 x 10^8 and 1.0 $x10^9$ CFU ml^{-1}. Similarly, significant growth of the antibiotics-evolved variants was observed *in vivo* with 6.5 x 10^8 - 3.1$x10^9$ CFU ml^{-1} SA3-LZD and 9.9 x 10^8 - 3.7$x10^9$ CFU $ml^{-1.}$ Recovered from the SA-VAN infected group.

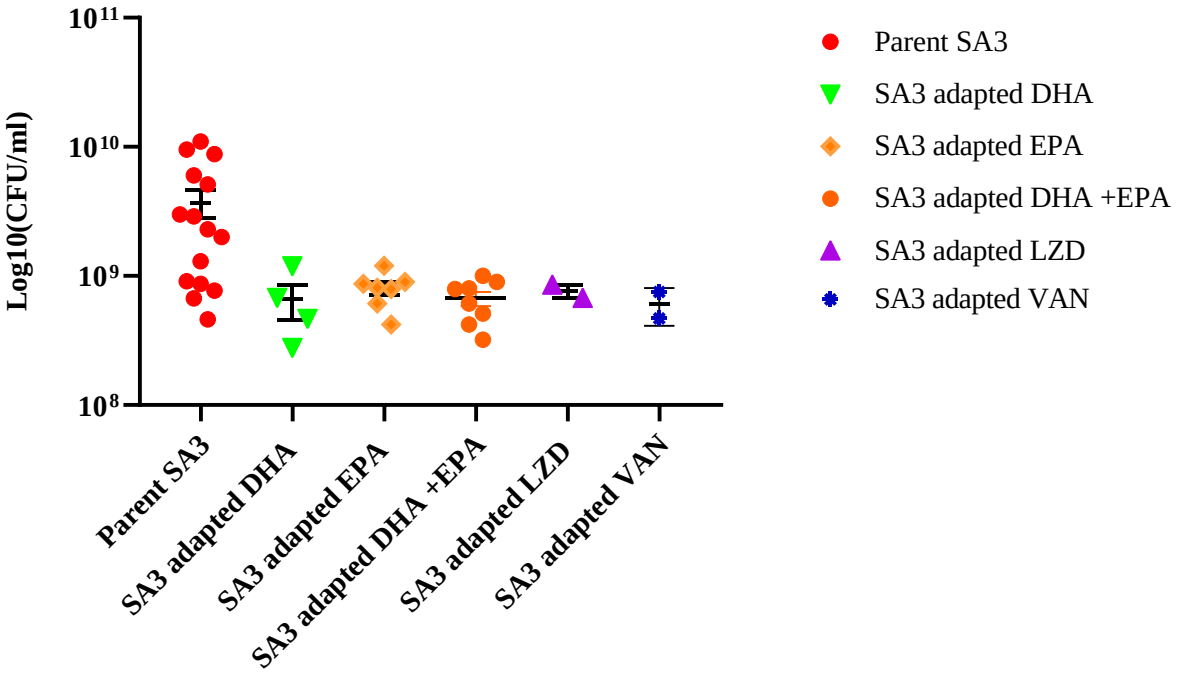


Figure 6.4 **Recovery of viable bacteria from *G. mellonella* post infection with parent SA3 and variants evolved in the presence of PUFAs or antibiotics (LZD or VAN)**. Larvae (n=16) were injected with parent or evolved variants. Negative controls included injection with PBS (n=16), and uninfected larvae (n=16). Each dot represents viable bacteria recovered from each dead larva. Initial inoculum of SA3, and the recovery from adapted DHA, adapted EPA, adapted DHA+EPA, adapted LZD, and adapted VAN. Mean CFU and standard deviation values are shown from three independent experiments. There are no statistically significant ($P < 0.135$) and even though bacterial numbers are the same – there is still a difference in larvae survival thus suggesting differences in virulence.

6.7 Effect of prolonged exposure to PUFAs and antibiotics on coagulase activity of SA3

Coagulase activity of SA3 parent and evolved variants was examined periodically over 24 h (1, 2, 4, and 24 h) at 37°C incubation. Formation of a complete clot that stayed in place when the assay tube was tilted or inverted was considered positive for coagulase activity. Clot formation was observed in all strains within 24 h (Table 6.5). The parent SA3 had the strongest coagulase activity, causing full clotting within 1 h. By comparison, all evolved variants had weaker coagulase activity, only causing partial clotting within the first hour, and then a gradual increase in clotting, which was only complete after 24h.

Table 6.5 Coagulase activity of *S. aureus*SA3 and PUFA or antibiotics-evolved variants

Time (h)	**Coagulase activity of strains**					
	Parent SA3	**Adapted DHA**	**Adapted EPA**	**Adapted DHA+EPA**	**Adapted LZD**	**Adapted VAN**
1	++++	+	++	++	++	+
2	++++	++	++	++	++	++
4	++++	++	+++	+++	+++	++
24	++++	+++	++++	++++	++++	+++

>75% coagulase +ve, coagulase Negative: No clot (plasma remains wholly liquid). Data represents the mean of triplicate experiments with H_2O as a negative control

6.8 Effect of prolonged exposure to PUFAs and antibiotics on haemolytic activity of SA3

The ability of SA3 to lyse erythrocytes was investigated compared to evolved variants. The haemolysin assay measured the release of haemoglobin by lysed horse blood erythrocytes in the supernatant. The parent SA3 showed the strongest haemolysin activity, and SA3-DHA had the weakest haemolysin activity (Figure 6.3). T-test analysis identified a significant difference in reduction of haemolytic activity compared to the Parent SA3.

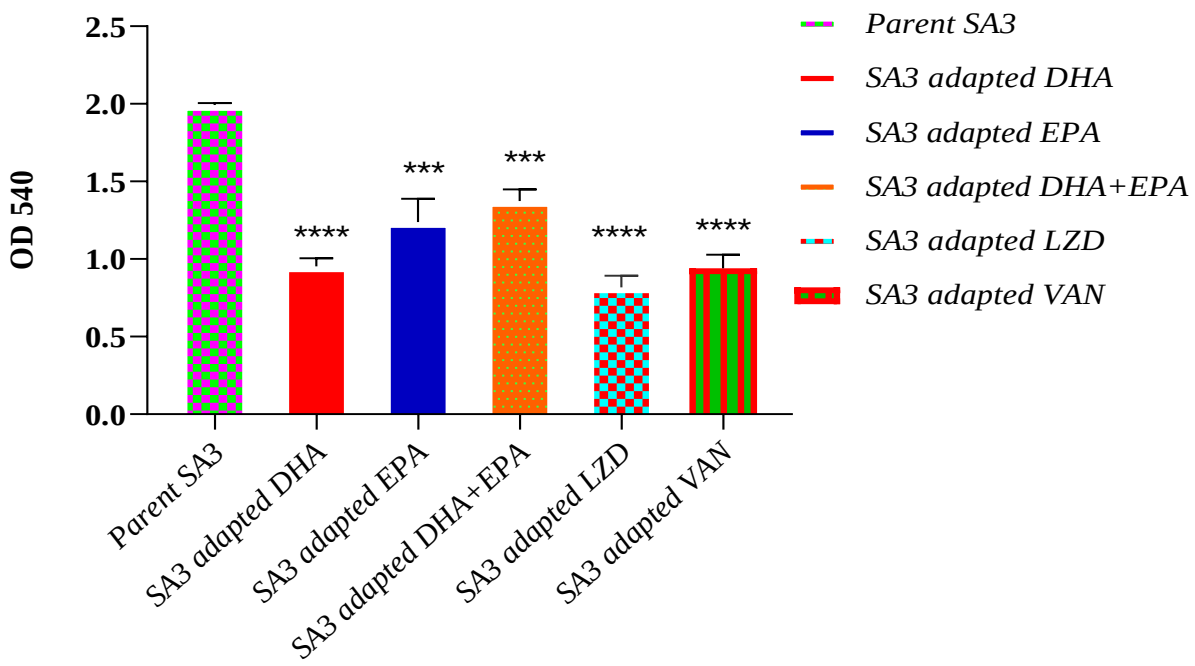


Figure 6.5 **Haemolytic test of *S. aureus* SA3 and evolved variants.** Cultures were incubated with horse blood and haemolytic activity was measured after 1 hour of incubation (n = 3). Data are expressed as mean value by using GraphPad prismV8 un paired t test showed that all adaption strain compared Parent SA3 are statically difference and the P value = < 0.002

6.9 Effect of prolonged exposure to PUFAs and antibiotics on DNase activity of SA3

The activity of DNase was detected by culturing SA3 and evolved strains on DNase detection agar, which changes colour on addition of HCl in the presence of the degraded DNA. This assay indicated that the parent SA3 had much more DNase activity than evolved variants (mean zone diameter, 22 ± 0.50 cm). The PUFA-evolved variants had almost a 50% reduced activity (mean zone diameter 12 ± 0.50 cm), and the antibiotics-evolved variants had lost even more DNase activity. SA3-VAN produced a mean zone diameter, 11 ± 0.55 cm and SA3-LZD produced a mean zone diameter, 8 ± 0.53 cm. Data are presented in Figure 6.4.

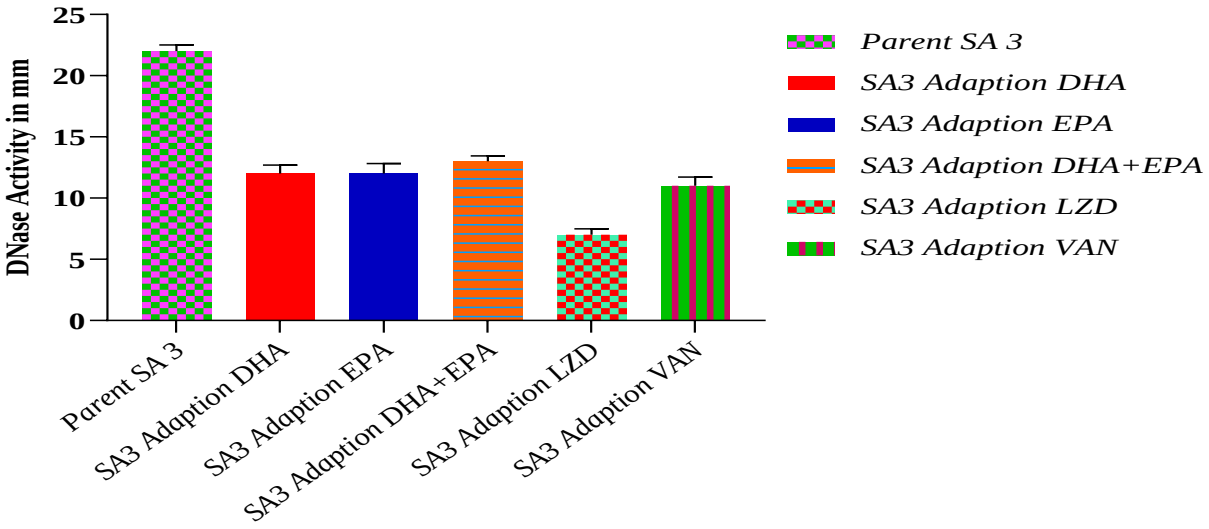


Figure 6.6 **DNase activity in mm of *S. aureus* SA3 and evolved strains:** DNase activity measured by comparing clearing zone diameters (mm) after 24 h incubation. The mean and standard divisions are shown from triplicate experiments.

Reduced coagulase, haemolysin and DNase activity of evolved variants could, in part, explain their reduced virulence *in vivo*.

6.10 Whole genome sequencing to investigate the genetic basis for altered resistance and virulence phenotypes after exposure to prolonged sub-inhibitory concentrations of antimicrobials

This study has identified differences in resistance and virulence profiles of the *S. aureus* clinical wound isolate (SA3), when it was evolved in sub-inhibitory concentrations of PUFA compounds and antibiotics (linezolid or vancomycin). It is likely that these changes have resulted from 1) genetic mutations in key genes that have been selected for by the presence of stressful conditions. 2) a biochemical effect (incorporation of PUFAs into bacterial membranes, affecting function) or 3) stress responses that lead to differential expression of key resistance and virulence determinants.

To investigate the likelihood of genetic mutations as drivers of observed phenotypic changes, the whole genomes of parent SA3, and each evolved strain, were sequenced by Microbes NG University of Birmingham. Raw sequence reads were assembled, annotated and compared by Dr Ian Goodhead (University of Salford). Single-nucleotide polymorphisms (SNPs) in evolved strains compared to parent SA3, were detected using "Snippy" and "Free-bayes bioinformatics analysis software tools. This program identified synonymous (silent) and nonsynonymous (missense or nonsense) SNPs. Synonymous mutations do not change the encoded amino acid but a change in general the DNA sequence, while nonsynonymous SNPs do change the amino acid sequence, more likely resulting of differences in the encoding protein function. The nonsynonymous SNPs consist of two types of mutation, missense and nonsense mutation.

This study focussed on missense and nonsense mutations, since they are more likely to confer a functional effect (Table 6.6).

Table 6.6 **Non-synonymous SNPs in evolved genomes compared to parent SA3**

nt	SA3	DHA	EPA	PUFA	LZD	VAN	*GENE*	PRODUCT
119633	C	C	C	T	C	C	*odh*	Opine dehydrogenase
406314	G	G	G	G	G	A	*isaA*	putative transglycosylase IsaA precursor
467840	C	C	C	C	A	T	*clfB*	Clumping factor B precursor
467843	A	A	A	A	G	T	*clfB*	Clumping factor B precursor
467948	C	C	C	C	C	G	*clfB*	Clumping factor B precursor
467984	C	C	C	C	G	C	*clfB*	Clumping factor B precursor
467987	A	A	A	A	G	A	*clfB*	Clumping factor B precursor
468531	C	C	C	C	C	T	*clfB*	Clumping factor B precursor
504148	C	C	C	C	A	C	*SecY*	preprotein translocase subunit
589448	C	C	C	T	C	C	*walR*	Transcriptional regulatory protein WalR
14541	C	C	C	C	C	G	*yabJ*	Enamine/imine deaminase
110532	C	C	T	C	C	T	*oppC*	Oligopeptide transport system permease
68847	C	T	C	C	C	C	*treR_1*	Trehalose operon transcriptional repressor
68898	C	C	T	C	C	T	*treR_1*	Trehalose operon transcriptional repressor
84890	G	G	G	A	G	G	*ywqD_1*	Tyrosine-protein kinase YwqD
87768	A	A	A	A	A	G	*pglF*	UDP-N-acetyl-α-D-glucosamine C6 dehydratase
87205	C	**G**	C	C	C	C	***pglF***	UDP-N-acetyl-alpha-D-glucosamine C6 dehydratase
124672	G	G	T	G	G	T	*rocD2_1*	Ornithine aminotransferase 2
256455	G	G	G	G	T	G	*yezG_3*	putative antitoxin YezG
365873	G	G	G	G	T	G	*speC_2*	Exotoxin type C precursor
146542	C	C	C	C	C	G	*cymR*	HTH-type transcriptional regulator CymR
151383	G	G	A	G	G	A	*hisS*	Histidine--tRNA ligase
308293	T	T	T	T	T	A	*ribD*	Riboflavin biosynthesis protein RibD
7614	G	G	G	G	A	G	*sarA*	Transcriptional regulator SarA
39605	G	G	A	G	G	A	*feuC_1*	Iron-uptake system permease protein FeuC
51999	G	G	G	G	G	T	*bceB_1*	Bacitracin export permease protein BceB
83534	G	G	G	G	G	A	*chp*	Chemotaxis inhibitory protein precursor
56649	C	C	A	C	C	C	*sdrD*	Ser-asp repeat-containing protein D precursor
56861	G	G	A	G	G	G	*sdrD*	Serine-aspartate repeat-containing protein C precursor
347877	T	T	T	T	**A**	T	***rsmB***	rRNA small subunit methyltransferase B
		2	**7**	**3**	**9**	**15**		**Total number of non-synonymous SNPs**

Shaded cells indicate SNP compared to parent. Red letters indicate non-sense mutations. Full details of amino acid changes are shown in a more detailed table in appendix Table S9

A total of 32 missense and 2 nonsense SNP mutations were detected in the genomes of the evolved strains compared to the parent SA3 (reference genome). The antibiotics-evolved variants had accumulated more SNPS than the PUFA-evolved variants, with most mutations detected in SA3-LZD (15 SNPs) followed by SA3-VAN (9 SNPS); SA3-EPA (7 SNPs); SA3-DHA+EPA (3 SNPs). SA3-DHA carried just 2 SNPs and the other PUFA-evolved variants possessed different SNPS. The absence of any strong pattern in variant genomes of the PUFA-evolved strains, suggests that the PUFAs did not specifically select for many mutations, which are more likely to be random. However, SA3-DHA and SA3-EPA did both harbour a SNP in the same *treR_1* gene, which encodes a trehalose operon transcriptional repressor. The SA3-DHA mutation occurred at position 68847 with a C-T substitution converting the glycine to glutamic acid in the protein sequence. The SA3-EPA variant SNP (C-T, histidine – arginine) occurred 51 nt up-stream on the same gene (nt 68898). This mutation was also detected in the SA3-LZD variant genome, suggesting parallel evolution. *S. aureus* are able to produce acid aerobically from trehalose, mutation in the transcriptional regulation of this process may affect metabolic flexibility of the pathogen in certain environments (Ibarra *et al*., 2013a).

Other notable mutations found in the genomes of PUFA-evolved strains include a nonsense mutation in *pglF* (UDP-N-acetyl-α-D-glucosamine C6 dehydratase) of SA3-DHA (also mutated in SA3-VAN); the UDP-GlcNAc C6 dehydratase is involved in biosynthesis and production of capsular polysaccharides in *S. aureus* (Li *et al*., 2014). A missense SNP in *hisS* which encodes for Histidine-tRNA ligase which play significant role in protein biosynthesis and was found in both SA3-EPA and SA3-VAN genome; and a *feuC_1* (iron uptake) which is siderophores ABC-transporter and permease enzymes for acquisition of iron during infection, the siderophores produced by pathogenic bacteria including *S. aureus* are critical for their survival and often represent virulence factors (Hammer & Skaar, 2011). The *feuC* gene mutation was found in both SA3-EPA and SA3-LZD. Two different SNPs of *sdrD* were also found in SA3-EPA, the *sdrD* gene

encodea a surface component belonging to a subfamily of the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) and is involved in adhesion to human squamous cells (Askarian *et al*., 2016). Other missense mutations in *oppC, rocD2_1, odh, walR, and ywqD_1* were only found in single variants. It would be interesting to sequence more isolates from these experiments and determine whether these SNPs were conserved in the populations and whether they would be maintained in the absence of PUFAs. Of the missense mutations detected in the antibiotics-evolved strains, the *clfB* gene was a SNP hotspot, with 4 mutations in SA3-LZD and 4 mutations in SA3-VAN, none of which were shared by any of the variants. This gene encodes a clumping factor B precursor which is a staphylococcal surface adhesin for the attachment to the anterior nares during colonisation (Sakr *et al*., 2018). Furthermore, *ClfB* has high affinity to bind to cytokeratin 10 and plasma fibrinogen (Walsh *et al*., 2004), which is the dominant component of the interior of squamous cells and binds loricrin which is the most abundant protein of the cornified envelope of squames (Mulcahy *et al*., 2012).

Exposure to linezolid produced the most SNPs, none of which were shared with SA3-VAN. Of note, there was a mutation in the *speC_2* gene, which encodes for Exotoxin Type C which is likely to be involved in virulence. There was also a mutation in the *sarA* gene, which encodes a global transcriptional regulator. Evidence in chapter 5 suggests that both PUFAs and antibiotics also affect expression of *sarA.* A study by Zielinska *et al* (2012) demonstrate that *sarA* mutants of *S aureus* USA300 increased production of extracellular proteases and simultaneously promote the production of virulence factors, extracellular toxins such as alpha toxin and phenol-soluble modulins and reduced biofilm formation (Zielinska *et al*., 2012). This gene is well known as a keystone of *S. aureus* virulence control, therefore, long-term exposure to PUFAs may disrupt this system and reduce virulence. However, more replicate populations would need to be screened to determine whether this SNP was driven by linezolid exposure, or just a random mutation.

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6.11 Discussion

The prevalence of antimicrobial resistant strains could seriously limit the ability to treat bacterial diseases such as wound infections. In addition, with presence of polymicrobial infection and the escalating levels of resistance to linezolid and vancomycin, PUFAs have been reported as attractive potential for alternative treatments with no effect on antimicrobial resistance (Desbois and Smith, 2010). PUFA compounds such as DHA and EPA have exhibited a comparatively broad range of antimicrobial activity and anti-biofilm efficacy against different wound pathogens including SA3 (as shown in chapter3). This final chapter focused on the ability of SA3 to adapt to sub-inhibitory concentrations of PUFAs and antibiotics (LZD, VAN) and the affect that this would have on antibiotic susceptibility and virulence. This was tested using a range of *in vitro* sensitivity and activity assays and the *in vivo G. mellonella* infection model. Finally, the genetic basis of the observed phenotypic changes was investigated by comparing the whole genome sequence of evolved variants with the parent SA3.

Resistance profiles of evolved strains were observed compared to the parent SA3 strain following extended exposure to PUFAs and antibiotics. However, the PUFA evolved strains exhibited increased sensitivity to Lincomycin and Vancomycin. Interestingly, the linezolid adapted strain became more sensitive to inhibition by DHA. Conversely, a marked increase in resistance to linezolid and vancomycin was observed for the SA3 that was evolved in the presence of antibiotics. In addition, from published studies suspected lack of acquired bacterial resistance mechanisms to PUFA has been encountered (Desbois, 2010). Although, this study has shown cross resistance when adapted PUFA compound are exposed to antibiotics (LZD or VAN), our serial passage that employ sub-inhibitory PUFA concentrations against adapted SA3 strains did not lead to the emergence or reduced the susceptibility of these compounds. Further study is required to determine the likelihood of resistance development with other clinical stains and species.

These observations, of altered susceptibilities after sustained exposure to PUFAs or antibiotics, are consistent with the suggestion that it is more difficult to select for resistance against PUFAs compounds that exert their antibacterial action by acting on multiple cellular targets and the cell membrane. Future investigations will use further adapted bacterial species and strains.

Biofilm density by each adapted strain was significantly reduced compared to the parent (~3 fold). Biofilms formed by the vancomycin adapted strain were denser than those formed by the other adapted strains, but still showed a 2-fold reduction compared to the parent SA3.MBIC and MBEC data suggest that all adapted strains formed weaker biofilms that were much more susceptible to disruption.

The PUFA and antibiotics adapted strains clearly had reduced virulence in *G. mellonella* compared to the parent SA3. The adapted strains also showed reduced fitness, evidenced by a significantly reduced number of viable cells recovered from dead *G. mellonella* larvae. Furthermore, a all adapted strains were impaired in production of key virulence traits. SA3-DHA and SA3-VAN showed reduced coagulase activity, whilst SA3-DHA, SA3--LZD and SA3- VAN showed reduced haemolytic activity and all adapted strains showed reduced DNase activity compared to Parent SA3 strain. The present study revealed that sustained exposure to PUFA compounds may have important implications for virulence. However, further investigation is required to confirm whether these reductions in enzyme production were driven by the compounds, or simply by sub-culture in broth in the absence of a host.

Chapter seven

General Discussion and Conclusion

Chapter 7 General Discussion and Conclusion

There is a major need for novel antimicrobials and anti-inflammatory agents to treat complicated wound infections. This study further explores the potential of DHA and EPA; two major compounds of n-3 PUFAs that have been shown to have diverse beneficial effects on human health, including wound healing (Turk *et al*., 2013a). The beneficial effects are believed to be related to the anti-inflammatory and antimicrobial action of PUFAs (Choi *et al*., 2013; Dyall, 2015; Park *et al.*, 2016). However, not much is known about their mechanisms of action on clinical bacterial isolates from wounds. Furthermore, there is little known about the long-term effects of PUFA treatment on bacterial populations at wound sites.

This thesis has worked on the hypothesis that PUFAs areas key alternative antimicrobial and antibiofilm treatments for bacterial wound infection, but also have an effect on the virulence of *S. aureus in vivo.* Furthermore, it was important to test PUFA activity under more relevant conditions to wound infection and with a clinically relevant wound isolate (SA3). This was achieved using an established *ex vivo* human skin model. This study also aimed to investigate the mechanisms of PUFA antimicrobial activity and postulated that PUFAs interfere with global virulence gene regulators. Finally, considering the fact that bacteria can rapidly develop resistance, this study investigated the effect of long-term exposure to PUFAs vs antibiotics on resistance and virulence. It was hypothesised that identification of SNPs in adapted vs parent strain would provide clues to explain such phenotypic changes.

Chapter 3 is an extensive study of antimicrobial and antibiofilm properties of PUFAs on common wound pathogens. This research found that DHA and EPA have a broad spectrum of bacteriostatic and bacteriocidal activity on wound pathogens. DHA and EPA activity inhibited pre-formed biofilms and also disrupted biofilm formation. However, this activity could be further explored using more sophisticated biofilm models such as in flow cells that support more mature

biofilm formation. Although PUFAs compounds were less potent inhibitors compared to standard antibiotics (vancomycin and linezolid), some clinical *S. aureus* have been reported to have developed resistance. Wound bacteria showed reduced tolerance to PUFAs compared to antibiotics. In additions, although a higher concentration of PUFAs is required to inhibit wound pathogens, they can be considered non-toxic, and have additional immunomodulatory effects to promote wound healing.

Chapter 4 involved in the study of *S. aureus* virulence and the antimicrobial properties of PUFAs in 2 different models of infection. The clinical *S. aureus*SA3 isolate was virulent in the *G. mellonella* model to a similar level as MRSA strain 252, killing 87.5% of the larvae within 7 days of infection. Furthermore, the PUFAs were found to either hinder growth or kill a proportion of SA3 *in vivo*, but much higher concentrations of PUFAs would be needed to eradicate the infection. This might suggest that PUFAs may have more effect on virulence, rather than a cumulative bacteriostatic or bacteriocidal effect. Uninfected larvae, that were treated with PUFAs remained 100% viable, confirming the non-toxic nature of the PUFAs at these concentrations.

The second infection model aimed to better reflect more realistic wound conditions using a novel *ex vivo* human skin model to assess the activity of DHA. This study validated the use of this model to assess the efficacy of novel antimicrobials. The data showed significant reduction of bacterial cells on wounded and non-wounded skin biopsies. These data should be compared to other topical antibiotics. This model also provides the possibility of exploring host-pathogen interactions, by measuring cytokine responses to bacteria with and without treatment. This would be a valuable line of research in the future.

Chapter 5 explored the effects of different concentrations of PUFAs on the expression of key target genes involved in global regulation, biofilm formation and virulence. It was hypothesised that PUFAs may act as quorum sensing inhibitors, as a novel strategy to treat *S aureus* infection.

Target genes *agr A*, and *sarA* were chosen because they are the most common regulatory genes of *S. aureus* used to modulate behaviour in response to changing environmental conditions, including antimicrobial agents. Two different concentrations of PUFAs (50 and 100 $\mu g ml^{-1}$) were chosen because they represent sub and ultra- MICs. Each concentration delayed growth during the first 12 h, but cultures recovered after 24 h. Differential expression of global regulators was observed at all concentrations and times. This suggests that PUFAs can disrupt quorum sensing. The adhesin gene (*icaA*) was also affected. Together, these findings may explain the reduced killing activity of SA3 *in vivo* in response to PUFAs. However, further work is needed to confirm the downstream effects of gene regulator disruption. A transcriptomics, proteomics or gene knockout approach couldshed light on the detailed mechanism of action.

Disruption of bacterial regulatory networks is an attractive strategy for novel therapeutics. Reduction of virulence, and growth rate, would exert less selective pressure than antimicrobial killing activity and reduce the likelihood of resistance development. Chapter 6 explored the likelihood of resistance development to PUFAs by exposing SA3 to prolonged PUFA treatment compared to prolonged treatment with vancomycin and linezolid. The main finding from this research confirmed that PUFAs do not easily promote resistance development. In fact, PUFA-adapted strains showed slightly increased susceptibility to PUFAs and antibiotics. Conversely, Antibiotics-adapted strains rapidly developed resistance to vancomycin and linezolid. All adapted strains showed reduced virulence, biofilm formation and enzyme production compared to the parent SA3. The final stage of this thesis was to investigate the genetic basis of these phenotypic changes. Whole genome sequences were compared to identify SNPs in adapted strains. Most non-synonymous SNPs were found in the linezolid adapted strain, with multiple SNPs located in the clumping factor B gene, which may explain reduced virulence. However, these data do not provide enough evidence to suggest that PUFAs and antibiotics have driven or selected for these mutations. It is possible that they would have occurred randomly anyway through sequential subculture. This

experiment would need to be repeated with more replicates and sequencing more members of each population. Only when multiple versions of the same SNP are identified, would this suggest that PUFAs have driven the adaptation. Furthermore, this analysis only considers genetic mutation and does not identify any effect that antimicrobial agents may have on gene expression or biochemical surface structure. Further transcriptomic, proteomic and microscopic investigation would be needed to explore this. It would also be interesting to analyse the bacteria that were recovered from treated and untreated *G. mellonella* and skin to determine adaptation to PUFAs in the host environment.

In conclusion this research suggests that PUFAs are an attractive alternative treatment for chronic wounds infected with *S. aureus*, that are unlikely to develop resistance. The PUFAs appear to interfere with global regulation and virulence, with the added benefit of non-toxicity and immunomodulatory properties.

Chapter seven

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Chapter 8 References:

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Appendices

Appendices

Table S1 chapter 4 the virulence of Staphylococci strains in the *G.mellonella* model

A number of larvae survive:								
Strains / Days	0	1	2	3	4	5	6	7
PBS	16	16	16	16	16	16	16	15
SA3	*16*	*13*	*10*	*8*	*5*	*4*	*2*	*2*
#MSSA 6538	*16*	*15*	*14*	*12*	*11*	*10*	*9*	*8*
#*MRSA 252*	16	13	10	7	5	3	2	1
Uninfected Larvae	16	16	16	16	16	16	16	16

Table S2 chapter 4 *Galleria mellonella*infected with different MICs of PUFAs compound

Larvae / Days	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
PBS	16	16	16	16	16	16	16	16
SA3	16	13	10	8	6	4	2	2
SA3 DHA MIC	16	15	15	15	14	13	12	11
SA3 EPA MIC	16	14	13	12	12	10	10	9
SA3 DHA+ EPA MIC	16	15	14	13	13	12	11	10
SA3DHA 2 MIC	16	16	16	16	16	15	14	14
SA3EPA2 MIC	16	16	16	16	15	14	13	12
SA3 DHA+EPA 2MIC	16	16	16	16	15	14	13	12
MIC DHA only	16	16	16	16	16	16	16	16
MIC EPA only	16	16	16	16	16	16	16	16
MIC DHA+EPA only	16	16	16	16	16	16	16	16
2 MIC DHA only	16	16	16	16	16	16	16	16
2 MIC EPA only	16	16	16	16	16	16	16	16
2 MIC DHA+EPA only	16	16	16	16	16	16	16	16
Uninfected Larvae	16	16	16	16	16	16	16	16

Table S3 chapter 4 Infected *G. mellonella* model with MICs of LZD, VAN

Number of larvae survive								
Strains / Days	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
PBS	16	16	16	16	16	16	16	15
SA3	16	13	10	8	6	4	2	2
SA3 LZD MIC	16	16	16	16	16	16	15	14
SA3 VAN MIC	16	16	16	16	16	16	15	15
Uninfected Larvae	16	16	16	16	16	16	16	16

Table S4 Chapter 5 Sets of the-inhibitory DHA and EPA in two concentrations (50 µg ml^{-1}, 100 µgml^{-1}) and Ethanol.

S. N	Samples sets	Conc in µg ml^{-1}	Time/hour	O.D at 600
1-	DHA	50 µg ml^{-1}	0h	0.01
2-	EPA	50 µg ml^{-1}	0h	0.013
3-	DHA+EPA	50 µg ml^{-1}	0h	0.04
4-	ETOH	50 µg ml	0h	0.027
5-	DHA	100 µg ml^{-1}	0h	0.01
6-	EPA	100 µg ml^{-1}	0h	0.016
7-	DHA+EPA	100 µg ml^{-1}	0h	0.01
8-	ETOH	100 µg ml^{-1}	0h	0.028
9-	DHA	50 µg ml^{-1}	6h	0.031
10-	EPA	50 µg ml^{-1}	6h	0.032
11-	DHA+EPA	50 µg ml^{-1}	6h	0.028
12-	ETOH	50 µg ml	6h	0.3
13-	DHA	100 µg ml^{-1}	6h	0.021
14-	EPA	100 µg ml^{-1}	6h	0.026
15-	DHA+EPA	100 µg ml^{-1}	6h	0.026
16-	ETOH	100 µg ml^{-1}	6h	0.28
17-	DHA	50 µg ml^{-1}	12h	0.067
18-	EPA	50 µg ml^{-1}	12h	0.13
19	DHA+EPA	50 µg ml^{-1}	12h	0.097
20	ETOH	50 µg ml	12h	0.62
21-	DHA	100 µg ml^{-1}	12h	0.026
22-	EPA	100 µg ml^{-1}	12h	0.073
23-	DHA+EPA	100 µg ml^{-1}	12h	0.046
24	ETOH	100 µg ml^{-1}	12h	0.55
25-	DHA	50 µg ml^{-1}	24h	0.29
26-	EPA	50 µg ml^{-1}	24h	0.35
27-	DHA+EPA	50 µg ml^{-1}	24h	0.39
28-	ETOH	50 µg ml	24h	0.72
29-	DHA	100 µg ml^{-1}	24h	0.16
30-	EPA	100 µg ml^{-1}	24h	0.26
31	DHA+EPA	100 µg ml^{-1}	24h	0.21
32-	ETOH	100 µg ml^{-1}	24h	0.6

Table S 5-chapter 5 Template for calculating 2 DD CT,

This table with CT value results from Rtq PCR was calculated in original sheet in Excel

	Experimental Well 1	Experimental Well 2	Experimental Well 3	Control Well 1	Control Well 2	Control Well 3	Average Experimental Ct Value	Average Experimental Ct Value
	Raw Ct Value	**Raw Ct Value**	**Raw Ct Value**	**Raw Ct Value**	**Raw Ct Value**	**Raw Ct Value**	**TE**	**HE**
Housekeeping Gene	21.00	20.50	20.60	20.00	20.50	20.30	-	20.70
Gene being Tested	24.00	23.00	20.50	26.00	24.20	26.40	22.50	-

Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
TC	**HC**	**ΔCTE**	**ΔCTC**	**ΔΔCt**	**2^-ΔΔCt**
-	20.27	1.80	5.27	-3.47	11.05530304
25.53	-				

Table S6 chapter 5 p value of RTq PCR for the inhibitory DHA against regulation of several genes at 6 and 12 hours

DHA at 6 hours	*P value*	**Significantly different (P < 0.05)?**
agr A DHA 50 µg ml^{-1}	0.0014	Yes
sarA DHA 50 µg ml^{-1}	0.007	Yes
icaA DHA 50 µg ml^{-1}	0.001	Yes
agr A DHA 100 µg ml^{-1}	0.004	Yes
sarA DHA 100 µg ml^{-1}	0.0003	Yes
ica A DHA 100 µg ml^{-1}	0.0001	Yes
DHA at 12 hours		
agr A DHA 50 µg ml^{-1}	0.0009	Yes
sarA DHA 50 µg ml^{-1}	0.0019	Yes
icaA DHA 50 µg ml^{-1}	0.0002	Yes
agr A DHA 100 µg ml^{-1}	0.0003	Yes
sarA DHA 100 µg ml^{-1}	0.0005	Yes
ica A DHA 100 µg ml^{-1}	0.0002	Yes

Table S7 chapter 5 p value of RTq PCR for the inhibitory DHA against regulation of several genes at 6 and 12 hours

DHA at 6 hours	*P value*	**Significantly different (P < 0.05)?**
agr A EPA 50 µg ml^{-1}	0.0026	Yes
sarA EPA 50 µg ml^{-1}	0.0080	Yes
icaA EPA 50 µg ml^{-1}	< 0.0001	Yes
agr A EPA 100 µg ml^{-1}	0.0009	Yes
sarA EPA 100 µg ml^{-1}	0.0011	Yes
ica A EPA 100 µg ml^{-1}	< 0.0001	Yes
DHA at 12 hours		
agr A EPA 50 µg ml^{-1}	0.0009	Yes
sarA EPA 50 µg ml^{-1}	0.0015	Yes
icaA EPA 50 µg ml^{-1}	0.0003	Yes
agr A EPA 100 µg ml^{-1}	0.0006	Yes
sarA EPA 100 µg ml^{-1}	0.0010	Yes
ica A EPA 100 µg ml^{-1}	0.0004	Yes

Table S 8 chapter6 *G. mellonella* virulence assay with adaptions strain

A number of larvae survive:								
Strains / Days	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
PBS	16	16	16	16	16	16	16	16
Parent SA3	16	13	10	8	6	4	2	1
SA3 adapted DHA	16	16	16	16	16	15	14	12
SA3 adapted EPA	16	16	16	14	13	12	11	10
SA3 adapted DHA + EPA	16	16	16	14	12	10	9	8
SA3 LZD adapted	16	16	16	16	16	16	15	14
SA3 VAN adapted	16	16	16	16	16	15	15	14
Uninfected Larvae	16	16	16	16	16	16	16	16

Table S 9 chapter 6 multiple comparisons of basic t test between different procedures and compounds

Procedure	*P value*	**Significantly different (P < 0.05)?**
All MBICS VS All MBECS	0.3568	No
VAN MBICs vs All other MBICs	0.0467	Yes
VAN MBECs vs All other MBICs	0.0476	Yes
LZD MBICs vs all PUFA MBICs	0.0091	Yes
LZD MBECs vs all PUFA MBECs	0.0320	Yes
DHA MBICs vs EPA MBICs	0.0020	Yes
DHA MBECs vs EPA MBECs	0.4113	No

Table S 10 chapter 6 the average of un paired t test

24 h		**48 h**	
Strains	***P value***	***Strains***	***P value***
Adapted DHA	0.0289	***Adapted DHA***	0.0162
Adapted EPA	0.0328	***Adapted EPA***	0.0169
Adapted DHA+EPA	0.0021	***Adapted DHA+EPA***	0.0165
Adapted LZD	0.0037	***Adapted LZD***	0.0173
Adapted VAN	0.0044	***Adapted VAN***	0.0292

Table S11: Amino acid changes of SNPs of adapted strains compared to parent SA3

POS	Reference	SA3_DHA	SA3_EPA	SA3_EPADHA	SA3_LZD	SA3_VAN	GENE	EFFECT	Amino Acid Change
59463	G	G	G	**A**	G	G	**rpoA**	**synonymous**	**p.Asp111Asp**
119633	C	C	C	**T**	C	C	**odh**	**missense**	**p.Val201Met**
406314	G	G	G	G	G	**A**	isaA	missense_	p.Thr169Ile
467819	C	C	C	C	**T**	C	**clfB**	**synonymous**	**p.Ser884Ser**
467840	C	C	C	C	**A**	**T**	clfB	missense	p.SerGlu876ArgGlu
467843	A	A	A	A	**G**	**T**	**clfB**	**missense**	**p.SerGlu876ArgGlu**
467852	G	G	G	G	G	**A**	clfB	synonymous	p.SerAsp872SerAsp
467855	C	C	C	C	**T**	**T**	**clfB**	**synonymous_**	**p.SerAsp872SerAsp**
467873	C	C	C	C	**T**	**A**	clfB	synonymous_	p.Ser866Ser
467927	C	C	C	C	**T**	**T**	**clfB**	**synonymous_**	**p.Ser848Ser**
467948	C	C	C	C	C	**G**	clfB	missense_	p.Glu841Asp
467963	C	C	**T**	C	C	C	**clfB**	**synonymous_**	**p.Ser836Ser**
467984	C	C	C	C	**G**	C	clfB	missense_t	p.SerGlu828SerAsp
467987	A	A	A	A	**G**	A	**clfB**	**missense_**	**p.SerGlu828SerAsp**
468017	G	G	**T**	G	**T**	G	clfB	synonymous_	p.Ser818Ser
468531	C	C	C	C	C	**T**	**clfB**	**missense_**	**p.Gly647Asp**
504148	C	C	C	C	**A**	C		missense_	p.Met174Ile
589448	C	C	C	**T**	C	C	**walR**	**missense_**	**p.Arg119Cys**
21474	A	A	A	**T**	A	A	**scrB**	**synonymous_**	**p.Leu196Leu**
14541	C	C	C	C	C	**G**	yabJ	missense_	p.Pro15Ala
38892	T	T	T	T	**C**	T		**non_coding_transcript_t**	
793	G	G	G	G	**A**	G		non_coding_transcript_	
2103	C	C	**T**	C	**T**	C		**non_coding_transcript_**	
12526	C	C	C	C	C	**T**	clfA	synonymous_	p.Asp806Asp
12547	C	C	C	C	C	**A**	**clfA**	**synonymous_**	**p.Ser813Ser**
12565	G	G	G	G	G	**A**	clfA	synonymous_	p.Ser819Ser
110532	C	C	**T**	C	C	**T**	**oppC**	**missense_**	**p.Ala177Val**

347877	T	T	T	T	**A**	T	rsmB	stop_gained	p.Cys264*
409267	G	G	G	G	G	**C**	**infB**	**synonymous_**	**p.Thr283Thr**
413094	G	G	G	G	G	**A**	ribF	synonymous_	p.Gly240Gly
1313	**C**	**C**	T	T	T	T		**non_coding_transcript_**	
419	T	**C**	**C**	T	T	T			
68847	C	**T**	C	C	C	C	**treR_1**	**missense_**	**p.Gly65Glu**
68898	C	C	**T**	C	C	**T**	treR_1	missense_variant	p.Arg48His
84890	G	G	G	**A**	G	G	**ywqD_1**	**missense_**	**p.Asp79Asn**
87205	C	**G**	C	C	C	C	pglF	stop_gained	p.Tyr359*
87768	A	A	A	A	A	**G**	**pglF**	**missense_**	**p.Glu547Gly**
124672	G	G	**T**	G	G	**T**	rocD2_1	missense_	p.Ala153Glu
256455	G	G	G	G	**T**	G	**yezG_3**	**missense_**	**p.Asp59Tyr**
365873	G	G	G	G	**T**	G	speC_2	missense_	p.Asp188Tyr
146542	C	C	C	C	C	**G**	**cymR**	**missense_**	**p.Gly47Ala**
151383	G	G	**A**	G	G	**A**	hisS	missense_	p.Arg366Cys
308293	T	T	T	T	T	**A**	**ribD**	**missense_**	**p.Glu62Asp**
7614	G	G	G	G	**A**	G	sarA	missense_	p.Pro65Leu
39605	G	G	**A**	G	G	**A**	**feuC_1**	**missense_variant**	**p.Ala261Thr**
51999	G	G	G	G	G	**T**	bceB_1	missense_	p.Ser505Ile
83534	G	G	G	G	G	**A**	chp	missense_	p.Glu95Lys
51258	C	C	C	**A**	C	C	**ywlC**	**synonymous_**	**p.Ser103Ser**
52009	G	G	G	G	G	**A**	**sdrC**	**synonymous_variant**	**p.Ser757Ser**
52156	C	C	C	C	C	**T**	sdrC	synonymous_	p.Asp806Asp
52495	G	G	G	G	**A**	**A**	**sdrC**	**synonymous_**	**p.Ser919Ser**
56574	C	C	**T**	C	C	C	sdrD	synonymous_	p.Asp1128Asp
56586	C	C	**T**	C	C	C	**sdrD**	**synonymous_**	**p.Asp1132Asp**
56607	G	G	**A**	G	**A**	**A**	sdrD	synonymous_	p.Ser1139Ser
56616	T	T	**C**	T	T	T	**sdrD**	**synonymous_**	**p.Asp1142Asp**
56631	T	T	**C**	T	T	**C**	sdrD	synonymous_	p.Ser1147Ser
56643	G	G	**A**	G	G	**A**	**sdrD**	**synonymous_**	**p.Ser1151Ser**

56649	C	C	**A**	C	C	C	sdrD	missense_	p.Ser1153Arg
56700	T	T	T	T	T	**C**	**sdrD**	**synonymous_**	**p.Asp1170Asp**
56718	T	T	T	T	T	**C**	sdrD	synonymous_	p.Asp1176Asp
56784	C	C	**T**	C	C	**T**	**sdrD**	**synonymous_**	**p.Asp1198Asp**
56802	C	C	**T**	C	C	**T**	sdrD	synonymous_	p.AspSerAsp1204AspSerAsp
56805	T	T	**C**	T	T	**C**	**sdrD**	**synonymous_**	**p.AspSerAsp1204AspSerAsp**
56808	T	T	**C**	T	T	**C**	sdrD	synonymous_	p.AspSerAsp1204AspSerAsp
56811	G	G	**A**	G	**A**	G	**sdrD**	**synonymous_variant**	**p.Ser1207Ser**
56832	C	C	**T**	C	C	**T**	sdrD	synonymous_	p.Asp1214Asp
56859	T	T	**C**	T	T	**C**	**sdrD**	**synonymous_**	**p.Ser1223Ser**
56861	G	G	**A**	G	G	G	sdrD	missense_variant	p.SerGly1223SerAsp
56868	T	T	**C**	T	T	**C**	**sdrD**	**synonymous_**	**p.Asp1226Asp**
56886	C	C	**T**	C	C	**T**	sdrD	synonymous_	p.Asp1232Asp
56892	C	C	**T**	C	C	**T**	**sdrD**	**synonymous_**	**p.Asp1234Asp**
56904	T	T	T	T	T	**C**	sdrD	synonymous_	p.Asp1238Asp
60592	C	C	C	C	C	**T**	**sdrE**	**synonymous_**	**p.Asp989Asp**
96316	G	G	G	G	**C**	G	yhdN_1	synonymous_	p.Thr55Thr

Supplement 12 chapter3

Title: An *ex-vivo* skin model to characterise the interplay between bacteria, host and antimicrobials

Aims:

1) Profile the dynamic interactions between bacteria and antimicrobials in the context of human skin

2) Determine the antibacterial efficacy of antimicrobials (including bioactive lipids) in a wound environment

3) Detect host responses to bacteria and antimicrobials (including bioactive lipids) – consequences for wound healing

Research Outline:

The bacteria that live on our skin are an important part of our microbiota as they can inhibit invasion of pathogens and signal appropriate immune responses, but can also cause chronic disease. However, we do not yet know the potentially damaging effects of long-term sub-lethal exposure to antibiotics on this microbiota. As the human microbiota is influenced strongly by interactions with the host, it is essential that this research is done in the context of live human tissue.

Furthermore, chronic wounds caused by commensal and pathogenic bacteria represent a significant burden to patients and current approaches to wound treatment are limited and far from ideal. At least 60 % of chronic wounds are infected with microbial biofilms[1] that show increased resistance to antibiotics and play a crucial role in preventing wound healing [2, 3]. This evidence, along with the increasing reports of multi-drug resistant bacterial wound infections[4] reflect the need for new treatment regimens for wound care that address both host and microbial contributors.

To research these themes, we will use *ex-vivo* skin as a realistic intact/wounded skin model (JL *in prep*). The model utilises full thickness 'plugs' of human skin tissue taken from a larger sheet. Skin biopsies (5 mm^2) will be maintained in the correct orientation by incorporating them into trans-well cell culture inserts, with the basal dermal surface continually bathed in culture medium, whilst the upper apical epidermis will remain exposed and 'dry'. A smaller wound will be created using a biopsy punch and can be of variable thickness. This model has been successfully developed using samples from Ethical Tissues with Catherine O'Neill at the University of Manchester.

Objective 1: Long-term effects of exposure of the human skin microbiome to antimicrobials

Bacteria are able to adapt very quickly to changes in their environment, but these adaptations may come with other costs[5]. However, we do not yet know the effects of long-term sub-lethal exposure to antibiotics on the microbiota we rely upon to defend us against disease.

Elucidating mechanisms of adaptive resistance in these species may help prevent further complications in these infections. This project aims to characterise the adaptation to antibiotic exposure skin bacteria in the context of viable human skin.

Objective 2a: Anti-bacterial Activityof bioactive lipids from marine-sourced polyunsaturated fatty acids (PUFA)

The aim of this project is to assess the antibacterial and immunomodulatory activity of PUFAs in the context of an infected wound. An attractive aspect of potential PUFA therapies is their safety and lack of significant side effects. They have been found to reduce systemic levels of IL-1β, IL-6 and TNF-α[6] but their effects in an infected wound setting have yet to be investigated.

We will simulate wound infection; initially using single clinical isolates of *Staphylococcus aureus* obtained from infected wounds, and treat with PUFAs. Bacterial viability will be monitored by swabbing the skin surface to recover bacterial cells after 0 – 96 h. Later work will involve the incorporation of pre-formed biofilms within acollagen gel matrix with serum protein, mimicking the wound bed of chronic wounds[7]. We will establish such biofilms using key bacterial wound isolates and monitor bacterial viability in the presence or absence of PUFAs.

Objective 2b: Immunomodulatory Activity ofPUFAs.

PUFAs also have potent anti-inflammatory and antibacterial properties. They have been shown to reduce pro-inflammatory cytokines *in vitro*[8,9] and to regulate fibrogenic activities of TGF-β1 that can contribute significantly to wound repair processes (unpublished data). PUFAs have previously been incorporated into 'solid emulsion gel' dressings, with demonstrable stimulation of wound repair in an acute model[10].

Skin biopsies will be exposed to PUFAs, frozen and stored for immunological analysis. Key responses of the skin to the presence of bacteria or PUFAs alone and in combination will be assessed. Histological staining of formalin-fixed paraffin embedded sections will be performed using monoclonal antibodies. Re-epithelialization will be evaluated by image analysis measurements taken from the edge of the wound origin to the leading edge of re-epithelialization, according to keratinocyte staining. Cell culture supernatants will be recovered for analysis of markers such as matrix metalloproteinases.

References:

1. James GA et al (2008) Biofilms in chronic wounds. Wound Repair Regen. 16:37-44.
2. Gurjala AN *et al* (2011) Development of a novel, highly quantitative in vivo model for the study of biofilm-impaired cutaneous wound healing. Wound Repair Regen. 19(3):400-10.
3. Bjarnsholt T *et al* (2007), Silver against *Pseudomonas aeruginosa* biofilms. APMIS, 115: 921–928.
4. Godebo G et al (2013)Multidrug-resistant bacterial isolates in infected wounds at Jimma University Specialized Hospital, Ethiopia. *Ann Clin Microbiol Antimicrob* 12: 17.
5. Latimer J et al (2012). Attenuated virulence and biofilm formation in Staphylococcus aureus following sublethal exposure to triclosan. AAC. 56(6):3092-100
6. McDaniel JC, Belury M, Ahijevych K, et al. Omega-3 fatty acids effect on wound healing. Wound Rep. Regen. (2008) 16:337-345.
7. Werthén M, Henriksson L, Jensen PØ, Sternberg C, Givskov M, Bjarnsholt T. An in vitro model of bacterial infections in wounds and other soft tissues. APMIS. 2010 Feb; 118(2):156-64.
8. Magee P, Pearson S, Allen J. (2008) The omega-3 fatty acid, eicosapentaenoic acid (EPA) prevents the damaging effects of tumour necrosis factor (TNF)-alpha during murine skeletal muscle cell differentiation. *Lipids in Health and Disease* 7:24
9. Magee P, et al. (2012). PPAR as a molecular target of EPA anti-inflammatiry activity during TNF-impaired skeletal muscle cell differentiation. *J. Nut. Biochem.*23 (11):1440-8.
10. Shingel KI et al. (2008) Solid emulsion gel as a vehicle for delivery of polyunsaturated fatty acids: implications for tissue repair, dermal angiogenesis and wound healing. J. Tissue Eng. Regen. Med. 2:383-393.

ETHICAL TISSUE

MATERIAL TRANSFER AGREEMENT

Definitions:

"Materials" means, respectively the human tissue supplied as outlined below including without limitation, tissue, tissue derivatives, cell lines, blood, blood derivatives, bio-fluids and isolated cells.

"Data" refers to any and all clinical or research information about the individual from whom the Materials were obtained, or about the Materials themselves.

"Services" shall refer to any and all human tissue research related services as set forth in more detail in the applicable Part 2 Application.

"Institution" shall refer to the academic or commercial entity that falls under the supervision of the Principle Investigator listed in the full Part 2 Application.

"Recipient" shall refer to the named Principle Investigator listed in the full Part 2 Application

"Third Party Recipient(s)" shall refer to an academic or commercial entity or institution other than ETHICAL TISSUE and the named Principle Investigator/ Institution in the Part 2 Application

"Principle Investigator" shall refer to the representative of an Institution in a position of authority to ensure suitable practices are followed to maintain compliance with applicable laws and this MTA. Will be expected to take responsibility for integrity and use of relevant material within the institution

"Authorised Representative" shall take legal responsibility for all activities and agreements within the Institution, having the authority to execute this agreement on behalf of the Institution

TERMS OF THE MATERIAL TRANSFER AGREEMENT

The Recipient agrees that the Material and Data provided by ETHICAL TISSUE will be used only for the purposes specified in the Part 2 Application. The Recipient agrees not to attempt to obtain information identifying the individuals providing the Material and Data to ETHICAL TISSUE. The Recipient agrees that all payments made hereunder are for the Services provided by ETHICAL TISSUE and are not for the Material or Data themselves. The Recipient also agrees that they shall not transfer Material and Data (or any portion thereof) supplied by ETHICAL TISSUE, to Third Party Recipients without the prior permission of ETHICAL TISSUE. Similarly, Material must not be transferred

to Third Party Recipients without an agreement in place that the Third Party Recipient complies with ETHICAL TISSUE Material Transfer Agreement and all applicable laws and regulations relating to the use, storage, handling and disposal of the Material and Data including but not limited to those set down by the Human Tissue Authority or other foreign regulatory bodies.

The Recipient understands that while ETHICAL TISSUE, attempts to avoid providing Material that is contaminated with highly infectious agents such as hepatitis and HIV, all Material should be handled as if potentially infectious. The individuals who have supplied Material to ETHICAL TISSUE, have not agreed to have clinical tests performed on this Material (e.g. for the presence of infective agents such as hepatitis). If these tests are carried out, the Recipient agrees that no results of these tests are reported back to ETHICAL TISSUE. The Recipient acknowledges that the Institution where the Material and Data will be used follows Human Tissue Authority or appropriate local regulations if outside England, Wales and Northern Ireland, for handling human Material and Data and will instruct their staff to abide by those rules. The Recipient further agrees to assume all responsibility for informing and training personnel in the dangers and procedures for safe handling and disposal of human Material.

Material and Data are provided as a service to the research community without warranty of merchantability or fitness for a particular purpose or any other warranty, expressed or implied. ETHICAL TISSUE accepts no responsibility for any injury (including death), damages or loss that may arise either directly or indirectly from their use, except to the extent caused by the negligence or wilful misconduct of ETHICAL TISSUE.

Specific questions about your application should be directed to Ethical Tissue, University of Bradford, West Yorkshire, BD7 IDP, United Kingdom.
Tel: +44 (0)1274 235897, Fax: +44 (0)1274 233234, Email: enquiries@ethicaltissue.org

The Recipient agrees that the Material and Data provided by ETHICAL TISSUE: Will not be used in any activity that contravenes the Human Tissue Act 2004 or other legislative body if outside the UK; Will not be used for human application such as transplant use; Will not be used in research that falls under the Human Fertilisation and Embryo Authority (HFEA) or other foreign national equivalent bodies and involve activities such as reproductive cloning and cell nuclear replacement as described by HFEA; Will not be tested for known inherited diseases; Will onlyl be used for biomedical research and not for the testing of cosmetics.

Although desirable, it is not essential for the Recipient to acknowledge the contributions of ETHICAL TISSUE, in publications (including posters and presentations at conferences and symposia). It is also desirable, but not essential, for the Recipient to report back to ETHICAL TISSUE any significant research findings and publications resulting from the use of the Material and Data. ETHICAL TISSUE acknowledges that in some cases confidentiality issues may prevent this from happening. The Recipient agrees to return or destroy any unused Material and Data required by applicable legislation. ETHICAL TISSUE however accepts that, it may not be possible to remove Data from analyses that has already been carried out.

The Institution acting for the Recipient agrees to assume all risks and responsibility in connection with the receipt, handling, storage and use of Material from ETHICAL TISSUE. It further agrees to indemnify and hold harmless ETHICAL TISSUE from any claims, costs, damages or expenses resulting from the use of the Material and Data provided by ETHICAL TISSUE. The indemnity in this clause will not apply where such claims, demands, actions, costs, expenses, losses and damages arise from the negligence or wilful misconduct of ETHICAL TISSUE.

The undersigned warrant that they have authority to execute this agreement on behalf of the Recipient Institution. (NB Ifthe Authorised Representative and Principle Investigator are one, both sections should be signed off by this person).

BY MY SIGNATURE 1 AGREE TO THE TERMS SET FORTH IN THE ABOVE AGREEMENT

Printed Name of Authorised Representative : Prof. Sheila Pankhurst Date 03 5

Signature of Authorised Representative SJPankhurst

cipal Investigator Dr Joe LaTimer

l Investigator

UNIVERSITY OF SALFORD

Name of Institution UNIVERSITY OF SALFORD

Printed Name of Principal Investigator Date 04 os- 2017

Signature of Principal Investigator

Name of Institution

UPON RECEIPT OF THESE SIGNED UNDERSTANDINGS AND THE INFORMATION REQUESTED IN THE FULL PART 2 APPLICATION, ETHICAL TISSUE WILL CONSIDER THIS REQUEST.

Specific questions about your application should be directed to Ethical Tissue, University of Bradford, West Yorkshire, BD7 IDP, United Kingdom.
Tel: +44 (0)1274 235897, Fax: +44 (0)1274 233234, Email: enquiries@ethicaltissue.org

The Licensed Research Tissue Bank (Ethical Tissue)

PART 2 APPLICATION FOR ACCESS TO HUMAN MATERIAL, DATA AND/OR SERVICES

This application should be completed when Material, Data and/or Services are being requested from ETHICAL TISSUEand utilized by:

- The laboratory and/or personnel at the Institution that falls under the supervision of the Principal Investigator listed in this application.

- The third party recipient for whom the Principle Investigator from the Institution listed in this application is acting. **In the case where human Materials and Data are to be transferred, Section 5of this Part 2 Application must be completed.** As stated on ETHICAL TISSUE Material Transfer Agreement, Material (or any portion thereof) must not be transferred to third party recipients without prior permission of ETHICAL TISSUE. Similarly, Material must not be transferred to third party recipients without an agreement in place that the third party recipient complies with ETHICAL TISSUE Material Transfer Agreement and all applicable laws and regulations relating to the use, storage, handling and disposal of the Material including but not limited to those set down by the Human Tissue Authority or other foreign regulatory bodies.

ETHICAL TISSUEis authorized by the Leeds flagged REC to release samples to researchers. Researchers receiving samples from ETHICAL TISSUE are NOT required to have approval from NRES for the use of these samples, as samples will be provided anonymously with only the minimum data set. This application is also for the Services of ETHICAL TISSUE to obtain Ethical Approval on behalf of the Recipient's Institution for the collection, use and storage of Material from the Institutions personnel and associated data. In both cases, researchers must be able to satisfy the Independent Scientific Advisory Committee (ISAC) and the Tissue Bank Management Team (TBMT) of ETHICAL TISSUE that the project they submit is both ethically and scientifically valid. IF researchers are already in possession of NRES approval for their projects, a copy of the NRES letter should be supplied with the application. Researchers are advised that it is their responsibility to ensure that they comply with the Human Tissue Act or other appropriate laws that cover the use of human material in research. An HTA licence is NOT needed to store tissue sourced from ETHICAL TISSUEfor an approved project that is subject to a signed Material Transfer Agreement. Where a Service Agreement is requested, the Institution is responsible for obtaining an HTA licence which will allow the collection, storage and use of human tissue for research purposes.

The information requested in this form is necessary in order to document correctly your request for material and other services and to ensure that ETHICAL TISSUEoperates within the guidelines of the Human Tissue Authority. When submitting a written request for supply of material:

- Please e-mail the completed Part 2 Application form to ETHICAL TISSUE (enquiries@ethicaltissue.org) and send hard copies of both signed off documents (Part 2 Application and Material Transfer Agreement. The latter is required only when tissue is requested) by post to:

  ETHICAL TISSUE,

The ICT Bioincubator
Tumbling Hill Street
University of Bradford
West Yorkshire
BD7 1DP

- Patient identity is confidential. Material and Data will be coded with codes supplied by Ethical Tissue with a minimum data set.
- Details of the Service provided or processing fees for Material and Data will be sent to you following receipt of your full application.
- Any transfer of samples from ETHICAL TISSUE to researchers will be by Courier. Researchers arerequired to cover the cost of transport of their samples and supply appropriate customs declarations if appropriate.

For additional information please contact ETHICAL TISSUE on +44 (0)1274 235897

For Ethical Tissue Office Use Only

Date of receipt of application		
Date reviewed by **TBMT**		
Please tick	**Approved**	**Rejected**
Date submitted to **ISAC**		
Please tick	**Approved**	**Rejected**
Project Registration Code		

PLEASE COMPLETE THE FOLLOWING SECTIONS

SECTION 1 – INVESTIGATOR DATA

(Please note that the Principle Investigator named below must have the authority to sign the relevant section of ETHICAL TISSUE Material Transfer Agreement and will be required to accept the terms set forth in the agreement).

Principle Investigator	Dr Joe Latimer		
	Title	*First Name*	*Surname*

Qualifications	BSc(Hons), MSc, PhD
Address Post code	Environment and Life Sciences, University of Salford, Crescent, Salford, M5 4WT

Phone Number	0161 2950283
Fax Number	
E-mail	j.latimer2@salford.ac.uk

Contact person*(if different from above)*	
Name	
Contact Number	
E-mail	

Shipping Address*(if different from above)*

Post code

Invoice Information

Is a purchase order required for shipment of specimens or Services provided by Ethical Tissue? *(Please tick box below)*	
Yes ✓	**No**
Purchase order number *Please supply when the project has been approved*	

Currently invoices are included with the tissue shipment to the shipping address listed above. If you would like the original invoice to be provided by post to another location (e.g. your finance department), please enter that address below. A copy of the invoice will also be included with your shipment. Where Services are provided, a Contract will be drawn up by the University of

Bradford Legal department, which includes a Schedule of payment for Services provided by Ethical Tissue.

Person to whom invoice should be addressed ***(if different from above)***

Name:	
Invoice address *(If different from above)* Post code	

SECTION 2 - FUNDING INFORMATION

Please indicate the likely source(s) of funding for the proposed project and the period of support applied for.

Funding Source(s) and period of support:

__The Government of Libya – PhD studentship awarded to Mohamed Eshlak – 12 months remaining

__Studentship from the Doctoral Training Alliance – 3 years_____

SECTION 3 – RESEARCH OUTLINE

Please provide a brief outline of the proposed research that is intended to be carried out involving the Material, data and/or Services requested from ETHICAL TISSUE (*use separate pages*). This should take the form of:

- **Title**
- **Aims**
- **Research Outline**
- **References**

Sufficient information (approx 600 words or 2 pages of A4) should be provided to enable the Independent Scientific Advisory Committee to determine whether the research outputs justify the use of material and data. If the project has already been reviewed as part of a grant application, you may supply a copy of the scientific part of that grant application.

Where Services are being requested, this will form the basis of the contract drawn up by the legal department of the University of Bradford.

Please include a CV for the principle investigator with your application.

SECTION 4 - SPECIMENS REQUESTED

(Please refer to ETHICAL TISSUE website (www.ethicaltissue.org) or contact ETHICAL TISSUE directly for an overview of the type of tissue or tissue product available).

Please specify exactly what you require e.g. 20 samples of RNA extracted from superficial TCC bladder tumours – G1pTa (male). Please also include the time frame involved.

Material Information Required: *(Anatomic site of tissue, pathological diagnosis, patient age, sex will be provided for all samples.)***Additional patient information may be available, but you must request it in this application and justify its necessity in the research project. It may be possible to provide some samples with details of treatment and outcome – although this may not be possible for all samples.**

- Sections of whole skin isolated during surgery from otherwise healthy volunteers, to be delivered as soon as possible after excision. Approximately 2 samples per month for four years.

SECTION 5 – UTILISATION OF MATERIAL AND DATA

*Please delete **will /willnot** as appropriate in the statement below.*

The Principle Investigator must sign and date this statement.

- Material and/or Data **will not** be transferred to third party recipient(s)

Signature of Principle Investigator ____ _

Date ___26/04/2017_____

If material and/or data are to be transferred to a third party recipient, please include a justification for this action here.

_____

SECTION 6 – ETHICAL TISSUE

Please state how you found out about sourcing human Material and Data from ETHICAL TISSUE

I have used ETHICAL TISSUE as a source of human material for a very similar project in a previous role