Functional role of polysaccharide intercellular adhesin during Staphylococcus epidermidis biofilm interaction with the innate immune system.

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Functional role of Polysaccharide Intercellular Adhesin during *Staphylococcus epidermidis* biofilm interaction with the innate immune system

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Summary

Synthesis of polysaccharide intercellular adhesin (PIA), accumulation associated protein (Aap) and extracellular matrix binding protein (Embp) are major mechanisms used by *Staphylococcus epidermidis* to evade immunity through biofilm formation. These evasion strategies are particularly suited for colonisation of medical devices such as heart valves, joint prostheses and central venous catheters resulting in significant patient morbidity. Two biological activities of PIA, Aap and Embp contribute to their role as an evasion molecules. Firstly their 'barrier' function limiting penetration of immune cells and antibiotics. Secondly, their 'immunomodulatory' properties which influence cytokine responses. At present little is known about these functional activities in physiological media and biological fluids.

This thesis uses a cell biology approach to study the environment necessary for PIA production. Specifically *in vitro* modelling of biofilm formation, PIA production and *S. epidermidis* leukocyte co-culture experiments have been used to assess conditions that are conducive for PIA production.

This thesis has identified that:

- Specific cell culture media cause unique profiles of biofilm accumulation with differential production of PIA, Aap and Embp.
- Fetal bovine serum and pooled human serum support *S. epidermidis* growth but differentially affect biofilm formation by PIA, Aap and Embp.
- Large scale production of PIA (~20mg) can be achieved by culturing in Iscove's Modified Dulbecco's Media which has allowed streamlining of current isolation procedures.
- PIA induction of cytokines, including IL-8 and TNF is dependent on being tethered to the bacterial membrane.
- Macrophages can penetrate into a *S. epidermidis* produced PIA 'barrier'.
- Immunosuppression of whole blood with dexamethasone unmasks the pathogenic advantage of PIA in *S. epidermidis* expressing PIA compared to negative controls.
- Whole blood killing of *S. epidermidis* is dependent on CD11b/CD18.
- PIA induces whole blood killing dysfunction which is likely related to C5a production.
- PIA can be produced in a whole blood environment.
- Inocula of ~10^3 colony forming unit of *S. epidermidis* are required to form biofilms in whole blood.

This study suggests the importance of studying clinically important biofilm production mechanisms under conditions that closely resemble those in human disease.

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Abbreviation

Aae	Autolysin
AtlE	Autolysin
Aap	Accumulation associated protein
APP	Acute phase proteins
AMPs	Antimicrobial peptides
agr	quorum-sensing accessory gene regulator
BSIs	Blood stream infections
Bhp	Bap homologous protein
BSA	Bovine Serum Albumin
BL	Blood
CoNS	Coagulase-negative staphylococci
CoPS	Coagulase-positive staphylococci
CBA	Columbia blood agar
CLSM	Confocal scanning laser microscopy
CFU	colony forming unit
CSF	colony-stimulating factors
CKs	Chemokines
Cyto.D	Cytochalasin D
CFU	Colony forming unit
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DAI	Device associated infection
Dex	Dexamethasone
Dsp.B	Dispersin B
EbpS	Elastin binding protein S

EPS	Extracellular polymeric substance
Embp	Extracellular matrix binding protein
ELISA	Enzyme-linked immunosorbent assay
Fbe	Fibrinogen-binding protein
FIVAR	Found in Various Architectures domains
FBS	Fetal bovine serum
GehD	Extracellular lipase glycerol ester hydrolase
GlcNAc	N-acetylglucosamine
GFs	Growth factors
G-CSF	Granulocytes colony stimulating factor
HHW	Hussain-Hastings-White modified medium
HAI	Health care associated infection
ICU	Intensive care unit
ICC	Immune cytochemistry
IMDM	Iscove's Modified Dulbecco's Media
*Ica*R	intercellular adhesin regulator
IL-	Interleukin
iNOS	Inducible nitric oxide synthase
IFNs	Interferons
IFN-γ	interferon-γ
LPS	Lipopolysaccharide
LAL	*Limulus* Amebocyte Lysate
MDR	Multi-drug resistant
MRSA	Methicillin resistance *Staphylococus aureus*
MSSA	Methicillin sensitive *Staphylococus aureus*
MW	Molecular weight
MHC	major histocompatibility complex

MAC	membrane attack complex
MBL	Mannose binding lectin
NK	natural killer cells
NETs	Neutrophil extracellular traps
NC	Negative control
PIA	polysaccharide intercellular adhesion
PMN	Polymorphonuclear leukocytes
PGN	peptidoglycan
PSMs	Phenol soluble modulins
PI	Propidium iodide
PBS	Phosphate buffered saline
PRRs	Pattern recognition receptors
PAMPs	Pathogens –associated molecules pattern
PS	Pooled human serum
PMA	phorbol myristate acetate
ROS	Reactive oxygen species
RPMI+	Roswell Park Memorial Institute media with GlutaMAX
RPMI-	Roswell Park Memorial Institute media without L- glutamine
S. epidermidis	*Staphylococcus epidermidis*
S. aureus	*Staphylococcus aureus*
SSI	Surgical site infection
Ssp1 and Ssp2	Surface proteins 1 and 2
SarA	Staphylococcal accessory regulator A
SigB	alternative sigma factor Sigma B
SEM	standard error of the mean
TcaR	teicoplanin –associated locus regulator
TCA	tricarboxylic acid

TNF	tumor necrosis factor alpha
TLRs	Toll-like receptors
TSB	Tryptic Soy Broth
TMB	Tetramethylbenzidine / Microwell Peroxidase Substrate
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
WBK	Whole blood killing
WGA	Wheat Germ Agglutinin conjugates
WHO	World health organization

Chapter 1

Introduction

1.1 Infectious Diseases

1.1.1 Introduction

The World Health Organization (WHO) defines infectious diseases as the diseases caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi. These diseases can be spread, directly or indirectly, from one person to another (WHO, 2012). Infectious diseases are the leading causes of death in low income countries, and in 2011 infectious diseases killed 7.7 million people all over the world (WHO, 2011).

1.1.2 Healthcare associated infections (HAI)

HAI are defined as infections acquired in hospital or other healthcare facilities by a patient admitted for reasons other than that infection, and includes infections acquired in the hospital, but appearing after discharge and occupational infections among staff of health facilities (Benenson, 1995; Ducel G., 2002).

The term ' Healthcare associated infections ' (HAI) describe infections related to medical care; and can be caused by a wide variety of bacteria, fungi and viruses. These infections can be very severe and sometime fatal (WHO, 2012). HAI play a great role in increasing the cost of the healthcare, through increasing the length of stay in the hospital and the use of antibiotics (Rosenthal VD, 2005; Higuera and Rangel-Frausto, 2007; Rosenthal, 2008).

HAIs are a significant global problem as they continue to increase even in highly developed countries. For example the cost of HAIs in USA increased from $4.5 billion in 1992 (Scott, 1992) to $28.4-33.4 billion in 2007 (Rosenthal, 2008). There are over 1.4 million people worldwide suffering from an HAI at any one time (Tikhomirov, 1987). The rates of HAIs in developing countries are 3 to 5 times higher than those reported globally (Rosenthal, 2008).

1.1.2.1 Predisposing factors for healthcare associated infections

Many factors contribute to the increasing burden of HAIs across the world. A key factor is in the injudicious use of antimicrobial agents in hospitals, which has contributed to the emergence of multi-drug resistant (MDR) microorganisms. For example, 60-70% of *Staphylococcus aureus* strains isolated among inpatients in many countries are resistant to methicillin and often several other first-line antibiotics (Levy and Marshall, 2004; Aboelela, 2007). In addition the lack of compliance with hygiene guide lines by healthcare personnel, especially hand hygiene facilitates the transmission of pathogens to and between patients, thereby promoting spread of HAIs (Allegranzi and Pittet, 2009). Furthermore, patients in hospitals can be more vulnerable to infection because their immune systems are often in a weakened state either due to their illnesses or certain treatment regimens. Thus immuno-suppression in organ transplant patients and chemotherapy treatment for cancer patients, the elderly and newborn are all considered at risk because of reduced immune function (Abramczyk, Carvalho et al., 2003; Pittet, 2008). Finally, the increased use of implanted medical devices and invasive medical procedures including attachment of urinary catheters, endotracheal intubation and ventilation of patients in the intensive care unit (ICU), intravascular catheter, prosthetic heart valve, surgical drains and tracheostomy, all bypass the body's natural lines of defense against pathogens and provide an easy route for infection (Collignon, 1994; Richards, Edwards et al., 1999). Furthermore, the medical devices provide a surface for bacterial attachment, aggregation and secretion of extracellular matrix to form biofilm (Schierholz and Beuth, 2001). Recent studies show that at least half of all cases of HAIs are associated with medical devices (Guggenbichler, Assadian et al., 2011).

1.1.2.2 Pathogenesis of healthcare associated infections

The most common pathogens causing HAIs are methicillin sensitive and resistant *Staphylococcus aureus* (MSSA and MRSA), *Escherichia coli,*, Enterococci, Pseudomonads spp, and coagulase-negative Staphylococci such as *S. epidermidis* (Jarvis and Martone, 1992). *Candida* species account for (72.1%) of fungal nosocomial infection especially in immunocompromised patients who are at high risk for

candidemia (Jarvis, 1995). Also, *Aspergillus fumigatus* is a common nosocomial fungal infection in the immunocompromised especially in patients with leukaemia, undergoing bone marrow transplant, and organ transplant recipients on long term immunosuppressive therapy, where inhalation of fungal spores can lead to aspergillosis (French, 2005; Nicolle, Benet et al., 2011). The most common viral nosocomial infections are caused by Respiratory Syncytial Virus (especially in children), influenza and para influenza virus, and norovirus (Welliver and McLaughlin, 1984; Sukhrie, Teunis et al., 2012). Cryptosporidiosis, caused by the protozoa *Cryptosporidium parvum* is a gastrointestinal pathogen associated with nosocomial transmission and is responsible for multiple outbreaks in child care centers and amongst immunocompromised patients in the hospital environment (Navarrete, Stetler et al., 1991; Ravn, Lundgren et al., 1991; Tangermann, Gordon et al., 1991). Frequently encountered healthcare associated infections and their causative pathogens are summarized in Table 1.1.

Table 1.1: Examples of healthcare associated infections and their causes

Body site	Infection type	Common causative microorganisms	References
Surgical Site	Surgical site infection	*S. aureus*, Coagulase-negative Staphylococci (CoNS), Enterococcus spp.	(Mangram, Horan et al., 1999)
Implanted Medical Device	Prosthetic joint infection, Cardiac pace maker	Coagulase-negative staphylococci(CoNS), *S. aureus*	(Trampuz and Zimmerli, 2005)
Gastro- intestinal tract	Gastroenteritis, Nosocomial Diarrhea.	*Clostridium difficile*, Rotavirus	(Barbut and Petit, 2001; Ogilvie, Khoury et al., 2012)
Urinary tract	Urinary tract infection, Urinary catheter infection	*S. epidermidis*, *S. aureus*, *S. saprophyticus*	(Orrett and Shurland, 1998)
Central nervous system	Ventriculo- artrial and Ventriculo-peritoneal shunt infection, Meningitis	CoNS especially *S. epidermidis* and *S. aureus*	(Pople, Bayston et al., 1992)
Respiratory System	Ventilator associated Pneumonia, Tuberculosis, Nosocomial Pneumonia	*Pseudomonas aeruginosa* , *Klebsiella pneumoniae* , methicillin-resistant *S. aureus* (MRSA) *Mycobacterium tuberculosis*	(Werarak, Kiratisin et al., 2010; Hamid, Malik et al., 2012)
Circulatory System (blood)	Bacteremia and intravascular catheter associated infection, Endocarditis, Phlebitis, vascular graft infection	Coagulase-negative Staphylococci (CoNS), Enterococci, *S. aureus.*	(National Nosocomial Infections Surveillance, 1999)
Skin and soft tissue	Infective cellulitis	*S. aureus*, Coagulase -negative staphylococci, *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *Streptococcus pyogenes*	(Carratala, Roson et al., 2003)

1.1.2.3 Mode of transmission of healthcare associated infections

There are five main routes of transmission of pathogenic microorganisms that cause HAIs:

1) Contact transmission is the most common method and can be direct and indirect. Direct-contact transmission happens when the infectious agent is transmitted from an infected or colonized person to a susceptible host. This can occur between medical staff and patients during patient care activities, or between two patients (Rosen, 1997). Indirect-contact transmission occurs by contact with contaminated objects such as instruments, needles, dressings or contaminated gloves (Zachary, Bayne et al., 2001; Siegel, Rhinehart et al., 2007).

2) Droplet transmission occurs when droplets carrying infectious agents are generated from infected individuals during coughing, sneezing, talking or when performing certain procedures such as bronchoscopy or endotracheal intubation and then travel to mucosal surfaces of the recipient (Papineni and Rosenthal, 1997; Fowler, Guest et al., 2004).

3) Airborne transmission of infectious microorganisms occurs with droplets or dust particles of less than 5μm, which are generated again by coughing, sneezing, talking or procedures such as bronchoscopy. Microorganisms transmitted this way can remain in the air for several hours and can be spread widely within a room or over longer distances and then inhaled by susceptible individuals who have not had direct contact with the infectious individual. Microorganisms frequently transmitted by this route include *Mycobacterium tuberculosis*, spores of Aspergillus *spp*, and the measles virus (Bloch, Orenstein et al., 1985; Beck-Sague, Dooley et al., 1992).

4) Vehicle transmission includes microorganisms transmitted to the host by contaminated items such as food, water, devices, and equipment.

5) Vector borne transmission occurs when vectors such as mosquitoes, flies and rats transmit microorganisms (Siegel, Rhinehart et al., 2007).

1.1.3 Medical device-related infections

Infections related to implanted medical devices are frequent HAIs. During recent years, and as a result of increased use of interventional instrumentation and invasive procedures (e.g. intravascular catheters, indwelling urinary catheters, ventriculo-peritoneal shunt, prosthetic joint and prosthetic cardiac valves) there has been an increase in the incidence of medical device associated infections. To highlight this, the numbers of total hip and knee replacement procedures in England and Wales have increased from around 130,000 in 2005–2006 to about 179,000 in 2010–2011(National Joint Registry 8th Annual Report, 2011). In the USA alone, each year there are 2 million cases of nosocomial infection with half of them being associated with indwelling devices (Darouiche, 2004). Medical device-related infection is considered to be a leading cause of morbidity and mortality in hospitalized patients, with infections usually leading to device failure and the need for replacement (Bjornson, 1993).

1.1.3.1 Pathogenesis of medical device-related infection

The presence of foreign material within patients is known to increase their vulnerability to infection. Even when small numbers of microorganisms colonize implanted plastic devices, they can form thick biofilms within 24 hours (Chambless, Hunt et al., 2006). The presence of a foreign body has been found to decrease by >100,000-fold the minimal infecting dose of *S. aureus* and can lead to permanent abscess (Zimmerli, Waldvogel et al., 1982).

There are many factors that play a role in the pathogenesis of medical device infections including those related to the host, the device, and the causative microorganisms. It is the sum of these factors which influence pathogenesis.

Important host factors include any underlying disease, nutritional status, obesity, immune status and age. A wide range of medical devices have been implicated in device related infections. These include prosthetic joints often used in patients with rheumatoid arthritis (Chu, Crosslin et al., 2005), intravenous catheters (Raad, Hanna et al., 2007), urinary catheters (Ostrowska, Strzelczyk et al., 2013), prosthetic heart valves (Krikunov, Kharchenko et al., 2013), endotracheal tubes (Machado, Tarquinio et al., 2012) and oral

prostheses (Sakka, Baroudi et al., 2012). Even when such devices are placed in a patient, additional variables can determine likelihood of infection. For example patients with rheumatoid arthritis have a higher risk of prosthetic joint infection than those with osteoarthritis (Sia IG, 2005). Factors influencing infection risk after placement of a catheter included the condition under which the catheter was placed (e.g., elective or urgent), catheter materials; silicone catheters more susceptible to infection than catheters made from other materials (O'Grady, Alexander et al., 2002).

The fundamental factor associated with the microbe is its ability to adhere and accumulate on biomaterials to form a multilayered structure called a biofilm (discussed in section 1.1.3.2) which is central to the pathogenesis of prosthetic device infection (Mack, Rohde et al., 2006). *S. epidermidis* and *S. aureus* are the most common microorganisms causing implanted device associated infection. Pooled data from 1992 - 1999 show that coagulase-negative staphylococci (CoNS) followed by *S. aureus*, are the most frequently isolated causes of hospital-acquired bloodstream infections (BSI)s, accounting for 37% and 12.6% respectively (National Nosocomial Infections Surveillance, 1999). Prosthetic joint infection occurs at a frequency of 1-2% after joint replacement, the major causative organisms being CoNS and *S. aureus*, accounting for 30-43% and 12-23% respectively (Trampuz and Zimmerli, 2005), urinary tract infection (UTI) in patients with indwelling catheters also have a high incidence of CoNS such as *S. epidermidis* and *S. haemolyticus* isolates accounting for 45.9% and 34% respectively (Nicolle, Hoban et al., 1983; Orrett and Shurland, 1998; Kumari, Rai et al., 2001). The ability of CoNS and to a lesser extent *S. aureus* to form biofilms on a biomaterial surface is a major reason behind their high incidence in medical device related infections (Laverty, Gorman et al., 2013)

1.1.3.2 Biofilms

Biofilms (Figure 1.1) are communities of microorganisms that are attached to a surface or connected to each other, embedded in an extracellular matrix. The extracellular polymeric substance (EPS) matrix accounts for ~90% of the biofilm biomass. Extracellular matrix composed of exopolysaccharides, proteins and extracellular DNA that was secreted by the microorganisms themselves (Vilain, Pretorius et al., 2009; Flemming and Wingender, 2010). Extracellular DNA is vital for early attachment of *S. epidermidis* to both plastic and glass surfaces (Qin, Ou et al., 2007), and for *P. aeruginosa* biofilms formation (Whitchurch, Tolker-Nielsen et al., 2002).

There are three essential components needed for biofilm formation; a surface, microorganisms and a polymeric matrix (Dunne, 2002). In general the basic steps of biofilm formation are: 1. The movement of planktonic (free floating cells) to a surface 2. Adhesion and attachment of planktonic cells to a surface 3. Growth and accumulation of cells in multiple layers 4. Secretion of extracellular matrix 5. Maturation and 6. Detachment and dispersal of planktonic cells (Figure 1.1).

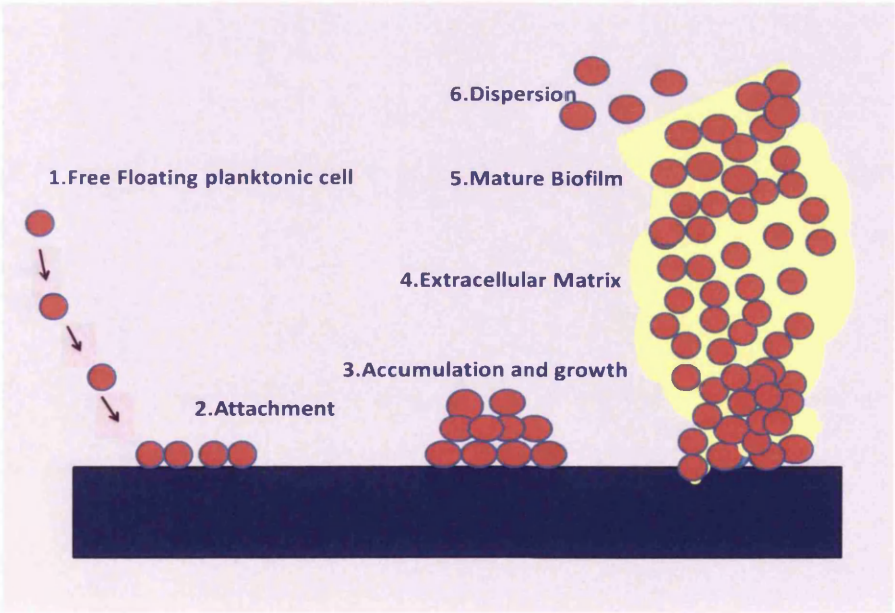


Figure 1.1 Schematic representation of the steps in biofilm formation.

In case of biofilms associated with chronic infections, the bacteria in the biofilm are often protected from the killing effect of the immune system either by impaired neutrophils and macrophages phagocytosis, limited penetration of leukocytes into the biofilm, or by prevent bacterial opsonization through prevention of immunoglobulins and complement deposition on bacterial surface (Jesaitis, Franklin et al., 2003; Leid, Willson et al., 2005; Cerca, Jefferson et al., 2006; Kristian, Birkenstock et al., 2008; Fey, 2010; Kostakioti, Hadjifrangiskou et al., 2013). In addition, bacteria in biofilm produce catalase enzyme that decay hydrogen peroxide produced by phagocytic cells (Estela and Alejandro, 2012). Bacteria in biofilm also resist the killing effect of administrated antibiotics either by produce a small number of dormant cells called persisters that survive lethal concentrations of antibiotics without any specific resistance mechanisms (Lewis, 2005; Lewis, Spoering et al., 2006; Lewis, 2008), or biofilm matrix itself delay antibiotic penetration through acting as a physical barrier (Duguid, Evans et al., 1992; Souli and Giamarellou 1998, Aparna and Yadav, 2008). Antimicrobial concentrations needed to inactivate biofilm associated microorganisms are much higher compared to planktonic cells (Donlan, 2001). Furthermore, bacteria inside biofilm produce enzyme can degrade or modify the activity of antibiotics via different mechanisms like hydrolysis (β-lactams), phosphorylation, glycosylation and transfer of thiol groups (Wright, 2005; Gallant, Daniels et al., 2005). Multidrug efflux pumps are another mechanism of antibiotic resistance (Leid, 2009). Efflux pumps are transport proteins that are able to expel toxic compounds such as antibiotics from the cell into the outside (Gallant, Daniels et al., 2005).

Shifting from planktonic microorganisms to cells that are part of surface-attached community occurs in a sequence of different events, including initial cell-to-surface or cell-to-cell attachment, micro-colony formation, biofilm maturation, and biofilm dispersal (Costerton, Montanaro et al., 2005). Quorum sensing is an important signaling process triggering biofilm formation by which bacterial cells communicate with each other. Quorum sensing uses signaling molecules called auto inducers to activate specific sites on DNA that induce protein formation involved numerous processes including biofilm formation (Hentzer, Eberl et al., 2003; Daniels, Vanderleyden et al., 2004).

The increased use of implanted medical devices has lead to an associated increase in infections associated with biofilm formation (Tenke, Riedl et al., 2004; Fey, 2010).
In 1988, Gristina described foreign body implant infections as a "race for the surface"; a competition between host tissue cells and bacteria to occupy the biomaterials surface, if the host wins it greatly reduces the possibility of bacterial colonization and as a result decreases the chance of infection (Gristina, Naylor et al., 1988). When an implanted device surface is covered by a film of the host serum including protein like albumin, collagen, fibrin or cells, it also presents conditions conducive for bacteria to attach (Gristina, 1994).
The first step in biofilm formation is attachment, which is largely dependent on the properties of the biomaterial surface and the bacterial cell surface. Hydrophobic materials like Teflon, plastics, and silicone are more liable to develop biofilm than hydrophilic materials like metals (Donlan, 2001).

A study on oral biofilm formation shows that surface roughness of dental/oral implants like crowns, implant abutments, and denture bases play an important role in bioflm formation by stabilizing the initial bacterial adhesion to the surface, which is usually weak and reversible (Teughels, Van Assche et al., 2006). Regarding the bacterial cell surface properties, the presence of flagella, pili, or fimbriae, may affect the rate of microbial attachment to the surfaces (Donlan, 2001), the presence of thin pili in Acinetobacter spp play important roles in their ability to attach to biotic and abiotic surface and form biofilms (Gohl, Friedrich et al., 2006).

After bacteria attach to a surface they begin to divide, proliferate and accumulate to form micro-colonies, then secrete an extracellular matrix and become a mature biofilm (McCann, Gilmore et al., 2008). There are different inter cellular adhesions that support biofilm accumulation, including the extracellular polysaccharide polysachariede intercellular adhesin (PIA)(Mack, Nedelmann et al., 1994) and the proteinous dependent biofilm accumulations; accumulation associated proteins (Aap)(Rohde, Burdelski et al., 2005) and extracellular matrix binding proteins (Embp)(Christner, Franke et al., 2010).
From mature biofilm matrix, bacteria begin to disperse and may attach to other sites to form new biofilm. The loss of bacterial cells from biofilm occurs passively due to shear

stresses forces or as a result of alteration in nutrient availability, oxygen deprivation or accumulation of toxins inside the biofilm (Costerton, Stewart et al., 1999; Sauer, Cullen et al., 2004; Rowe, Withers et al., 2010; Kostakioti, Hadjifrangiskou et al., 2013).

1.2 Staphylococci and human disease

1.2.1 Microbiology

Staphylococcus species are Gram-positive bacteria that come from the family Staphylococcaceae. Under the microscope these bacteria arrange in grape-like clusters; they are facultative anaerobes and when grown on solid media, form white colonies as in the case of *S. epidermidis* or golden colonies in the case of *S. aureus*. Staphylococci are catalase positive and oxidase negative bacteria. Staphylococci may be coagulase positive (CoPS) like *S. aureus* and *S. intermedius* or coagulase negative (CoNS) like *S. epidermidis, S. saprophyticus,* and *S. haemolyticus*. Although the *Staphylococcus* genus includes more than 40 species, only *Staphylococcus aureus* and *Staphylococcus epidermidis* are considered important human pathogens (Kloos and Schleifer, 1975; Kloos, 1980; Kloos and Bannerman, 1994; Harris, Foster et al., 2002). In the annual report of the National Healthcare Safety Network between 2006-2007 which involved 463 hospitals, the CoNS and *S. aureus* were top of the 10 most common microorganisms causing healthcare associated infections (accounting for 84% of any HAIs) (Hidron, Edwards et al., 2008). In the USA, MRSA USA300 genotype emerged as a significant cause of nosocomial pneumonia in the intensive care unit (Pasquale, Jabrocki et al., 2013).

1.2.2 Disease cause by Staphylococci

Diseases caused by Staphylococci vary in severity according to the species. The CoNS like *S. epidermidis* and *S. haemolyticus* which are part of the normal flora of the human skin, used to be considered commensal organisms; however during recent years there has been an increase in the incidence of HAIs and medical device infection attributed to these two strains; recent studies revealed that CoNS are the third major cause of HAIs and a common cause of bloodstream infections (BSI) (Rahman, Hosaain et al., 2012).

Both *S. epidermidis* and *S. haemolyticus* resist the immune system by their ability to produce biofilm and overcome the effect of antibiotics through methicillin resistant mechanisms (de Allori, Jure et al., 2006). Moreover, 15-40% of prosthetic valve endocarditis are due to CoNS (Wang, Athan et al., 2007). In contrast *S. aureus* is a human pathogen which causes different types of infections that vary in severity from mild skin infection to severe surgical site infections, pneumonia and osteomyelitis. *S. aureus* can colonize the nasal passages in healthy individuals, and play important roles in HAIs. A recent study showed that pretreatment of *S. aureus* nasal carriers with mupirocin ointment and chlorhexidine can reduce incidence of surgical site infection (SSI) to 4% compared to 11% in untreated *S. aureus* carriers, and 3% in non-carriers (Tai, Borchard et al., 2013).

1.3 *Staphylococcus epidermidis*

S. epidermidis is a Gram positive coccus whose cells arranged in grape-like clusters. This species is non-motile, and forms white colonies, approximately 1–2 millimeters in diameter after overnight incubation. They are facultative anaerobes, catalase positive, oxidase negative, and have the ability to produce urease and are non-hemolytic on blood agar.

S. epidermidis is part of the normal microflora that colonizes the human skin and mucosal surface and maintains a healthy component of skin microflora by out competing more harmful bacteria (Lina, Boutite et al., 2003). Studies show that the interaction of *S. epidermidis* peptides, the phenol soluble modulins (PSMs) gamma and delta, with human skin antimicrobial peptides (AMPs) enhances killing of some skin pathogens like *S. aureus* (Cogen, Yamasaki et al., 2010a; Cogen, Yamasaki et al., 2010b).

In the immunocompetent, host *S. epidermidis* acts as a commensal but it can cause severe infection in immunocompromised patients (von Eiff, Proctor et al., 2001). Currently CoNS (mostly *S. epidermidis*) are considered to be a major cause of HAIs and a common cause of infections on indwelling medical devices (CDC., 2004). The increase in the rate of HAIs attributed to *S. epidermidis* during the recent years is due to increases in the use of invasive medical techniques and implanted medical devices.

Colonization of the skin by *S. epidermidis* provides the opportunity for contamination of the medical device during implantation and can result in numerous infections, like intravenous catheter infection, prosthetic joint infection, surgical site infection, central nervous system shunt infection, ventilator-associated pneumonia, and catheter-associated urinary tract infection (Longauerova, 2006; Piette and Verschraegen, 2009). CoNS were the most common pathogens isolated from BSI in pediatric and intensive care units in the United States (Richards, Edwards et al., 1999; Richards, Edwards et al., 1999).

Multiple virulence factors play a role in the ability of CoNS to cause HAIs. These factors include biofilm formation, antibiotic resistance (as 55-75% of CoNS HAIs isolates are methicillin resistant), expression of surface proteins with adhesive properties which enable *S. epidermidis* to adhere to abiotic surfaces and to host matrix proteins that coat implants after implantation, and production of phenol soluble modulins (PSMs). Studies show a correlation between biofilm formation properties and antibiotic resistance compared to biofilm-negative isolates (Francois, Vaudaux et al., 1998; Otto, 2004; Klingenberg, Ronnestad et al., 2007; Granslo, Gammelsrud et al., 2008).

Treatment of *S. epidermidis* infections is difficult and thus, measures directed towards prevention are particularly important. Improvement in hygiene standards applied during implantation of peripheral intravenous cannula can reduce the possibility of infection which lead to a reduction in patient morbidity rates and the duration of the treatment (Morris and Heong Tay, 2008; Aziz, 2009).

1.4 *Staphylococcus epidermidis* virulence

1.4.1 Biofilm formation in Staphylococci

Biofilm formation is a major virulence mechanism involved in *S. epidermidis* infection particularly in those associated with an indwelling medical device (Fey and Olson, 2010). Biofilm formation is the major cause of implant failure in orthopaedic joint replacements, which often demand orthopaedic revision surgery as bacteria inside the biofilm remain protected from the immune system and antibiotic killing (Neut, van Horn et al., 2003). Often it is difficult to identify the causative agent of these infections using

routine methods of swabbing of the infected area, taking small tissue samples or pre-prosthetic tissue for culture and sensitivity always helpful (Costerton, 2005; Donlan, 2005). Recent studies by Trampuz et al (Trampuz, Piper et al., 2007) show that sonication of the removed hip or knee joint prostheses can help in diagnosis of causative agents by separating and dislodging bacteria embedded in biofilm formed on these prostheses. A case study by Palmer et al (Palmer, Costerton et al., 2011) demonstrated the presence of methicillin-resistant *Staphylococcus epidermidis* (MRSA) biofilm in a 3 year non healing tibia bone fracture where other standard cultures were negative. Increased use of more advanced techniques like the Ibis T5000 Universal Biosensor; pathogens diagnoses based on broad-range PCR and high performance mass spectrometry (Ecker, Drader et al., 2006) and fluorescence *in situ* hybridization (FISH) can detect the presence of pathogens in previously negative samples.

Biofilm structure comprises a multilayered community of bacteria encased in a self-produced polymeric matrix, which is thought to protect it from the effect of antibiotics and the immune response (Mack, Rohde et al., 2006).

After the free floating *S. epidermidis* reversibly attach to a surface, the second stage of biofilm formation starts as the cells attach irreversibly to the surface via adhesions. There are 10 adhesins found in *S. epidermidis* which have been reported to bind to a number of host cell extracellular matrix proteins summarized in (Table 1.2). Cell wall teichoic acid also bind to fibronectin (Gross, Cramton et al., 2001). These extracellular substance (DNA, surface proteins, lipids, and lipopolysaccharides) facilitates binding to materials coated with proteins such as fibrinogen, fibronectin, vitronectin, and collagen (Von Eiff, 2002; Renner and Weibel, 2011).

Table 1.2 Adhesins found in *S. epidermidis*

Adhesive	Attachment	References
Autolysin (Aae)	Binds fibrinogen, vitronectin, and fibronectin	(Heilmann, Thumm et al., 2003)
Autolysin (AtlE)	Binds vitronectin	(Heilmann, Hussain et al., 1997)
Fibrinogen-binding protein (Fbe) Also identified as SdrG (serine-aspartate repeat protein G)	Binds fibrinogen	(Davis, Gurusiddappa et al., 2001)
SdrF surface proteins belong to the serine-aspartate repeat (Sdr) family	Binds Collagen	(Arrecubieta, Lee et al., 2007; Arrecubieta, Toba et al., 2009)
Surface proteins 1 and 2 (Sspl and Ssp2)	Binds to polystyrene surface	(Veenstra, Cremers et al., 1996)
Extracellular lipase glycerol ester hydrolase (GehD)	Binds collagen	(Bowden, Visai et al., 2002)
Extracellular matrix binding protein (Embp)	Bind fibronectin	(Christner, Franke et al., 2010)
Elastin binding protein S (EbpS)	Binds elastin	(Downer, Roche et al., 2002)
Bap homologous protein (Bhp)	Binds polystyrenes	(Tormo, Knecht et al., 2005; Lasa and Penades, 2006)

In the third stage of biofilm formation bacteria start to proliferate and form microcolonies, and in the fourth stage bacteria secret an extracellular matrix. There are three different mechanisms mediating biofilm accumulation. They are: production of polysaccharide intercellular adhesin (PIA), accumulation associated protein (Aap) protein, and extracellular matrix binding protein (Embp) (Mack, Haeder et al., 1996; Rohde, Burdelski et al., 2005; Rohde, Burandt et al., 2007; Christner, Franke et al., 2010). In addition extracellular teichoic acids are also considered as integral parts of the extracellular matrix and mature biofilm.

In the fifth stage of biofilm formation the bacterial cells begin to disperse from the mature biofilm under the influence of peptides called phenol-soluble modulins and become free floating bacteria leading to dissemination of the infection and repeated cycles of biofilm formation (Sadovskaya, Vinogradov et al., 2005; Yao, Sturdevant et al., 2005; Wang, Khan et al., 2011).

Studies shows that biofilm production is the most important virulence factor attributed to invasiveness of *S. epidermidis* infection (Gad, El-Feky et al., 2009; Mekni, Bouchami et al., 2012), followed by antibiotic resistance. Indeed the majority of *S. epidermidis* strains causing catheter infection and catheter-related bacteremia are biofilm-producing and methicillin-resistant bacteria (Abbassi, Bouchami et al., 2008; Kitao, Ishimaru et al., 2010). Methicillin resistance, mediated by the *mecA* gene, is present in 80% of device associated infections caused by *S. epidemidis* (Kozitskaya, Cho et al., 2004). However, there are several biofilm properties that influence antibiotic resistance such as limiting antibiotic penetration (Mah and O'Toole, 2001) and reduction of metabolic activity of bacteria (e.g. cell division, protein synthesis, and DNA replication) due to decreases in the amount of oxygen and nutrient diffusing within biofilm. As antimicrobial often act on actively dividing cells, a low metabolic activity by biofilm cells leads to greater tolerance of biofilm cells to the agent. Also, the presence of persister cells in biofilm mean that once treatment is over the biofilm cells can regrow and cause a recurrence of infection (Lewis, 2008; Fauvart, De Groote et al., 2011).

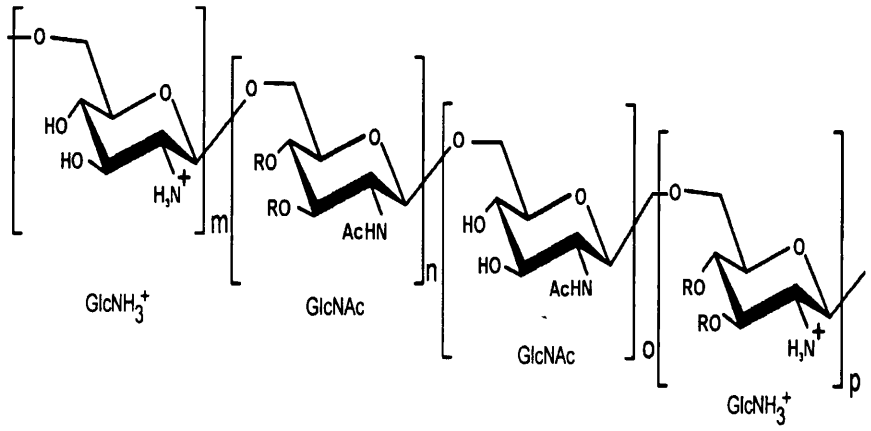
1.4.1.1 Mechanisms of biofilm accumulation

1.4.1.1.1 Polysaccharide intercellular adhesin (PIA)

PIA is the main functional element for intercellular adhesion in *S. epidermidis* biofilms (Mack, Nedelmann et al., 1994; Mack, Davies et al., 2009). PIA is also detected in other bacterial species, e.g. *S. aureus*, *Escherichia coli*, *Yersinia pestis*, Bordetella spp, *Aggregatibacter actinomycetemcomitans* (Mack, Haeder et al., 1996; Cho, Naber et al., 2002; Cafiso, Bertuccio et al., 2004; Rohde, Burandt et al., 2007; Rohde, Frankenberger et al., 2010) suggesting that it is a more general mechanism of biofilm formation.

PIA is a homoglycan of β-1, 6-linked *N* –acetylglucosamine of at least 130 residues, PIA both positively charged due to free 2-amino groups (no *N* -acetylation) and negatively charged due to *O* -succinoyl ester residues. These charge properties are essential for intercellular adhesion (Figure 1.2).

A



B

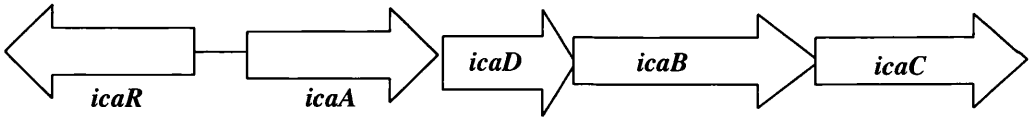


Figure 1.2 PIA structure and ica operon

A. Schematic representation of PIA structure. B. Schematic representation of *ica* operon.

PIA is encoded for by the *icaADBC* locus which produces a membrane bound enzyme complex (Heilmann, Schweitzer et al., 1996; Gerke, Kraft et al., 1998). PIA existing as two polysaccharide species, major polysaccharide I (>80%) and minor polysaccharide II (<20%) which is moderately anionic (Mack, Fischer et al., 1996; Mack, Davies et al., 2009). Polysaccharide I is a linear homoglycan of β-1, 6-linked 2-amino-2- deoxy-D-glucopyransoyl residues, about 80-85% are N-acetylated and the rest non N-acetylated. Polysaccharide II, structurally related to polysaccharide I but contain less N-acetylated D- glucosaminyl residues, in addition to ester- linked succinate and phosphate (Mack, Nedelmann et al., 1994; Mack, Fischer et al., 1996).

Thus *icaADBC* is a major virulence factor that is found in most *S. epidermidis* strains associated with severe nosocomial and medical device associated infection. Importantly this operon is uncommon in *S. epidermidis* isolated from the community (Arciola, Campoccia et al., 2006; O'Gara, 2007). The operon comprises four open reading frames; *Ica*A, *Ica*C and *Ica*D are all membrane protein, while *Ica*B is a secreted protein. Synthesis of functionally active PIA molecules requisite all the four *ica*ADBC genes (Gerke, Kraft et al., 1998; Gotz, 2002). *Ica*A is a glycosyltransferase that directs the syntheses of β-1,6-linked N-acetylglucosamine (GlcNAc) oligosaccharides using Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), IcaD is required for full activity of IcaA. IcaC is responsible for elongation and externalization of the growing polysaccharide across the cytoplasmic membrane (Gerke, Kraft et al., 1998). IcaB, is deacetylase responsible for de-N-asetylation of PIA, which is crucial for PIA activity, biofilm formation and immune evasion of *S. epidermidis* (Vuong, Kocianova et al., 2004). Deletion of IcaB gene from *S. epidermidis* prevented deacetylation and produced PIA poorly attached to the cell surface (Vuong, Kocianova et al., 2004; Cerca, Jefferson et al., 2007). IcaB deacetylated around 3-8% of the amino groups (Maira-Litran, Kropec et al., 2002), Figure 1.3 Summarize the PIA biosynthesis mechanism.

The expression of *ica* locus regulated by both positive and negative regulators. The intercellular adhesin regulator (*Ica*R) is a negative regulator act as a transcriptional repressor of *ica* operon in *S. epidermdis*. *Ica*R is located upstream of the *icaADBC* operon (Conlon, Humphreys et al., 2002). Ethanol represses icaR expression, lead to induction of *ica* operon (Conlon, Humphreys et al., 2002).

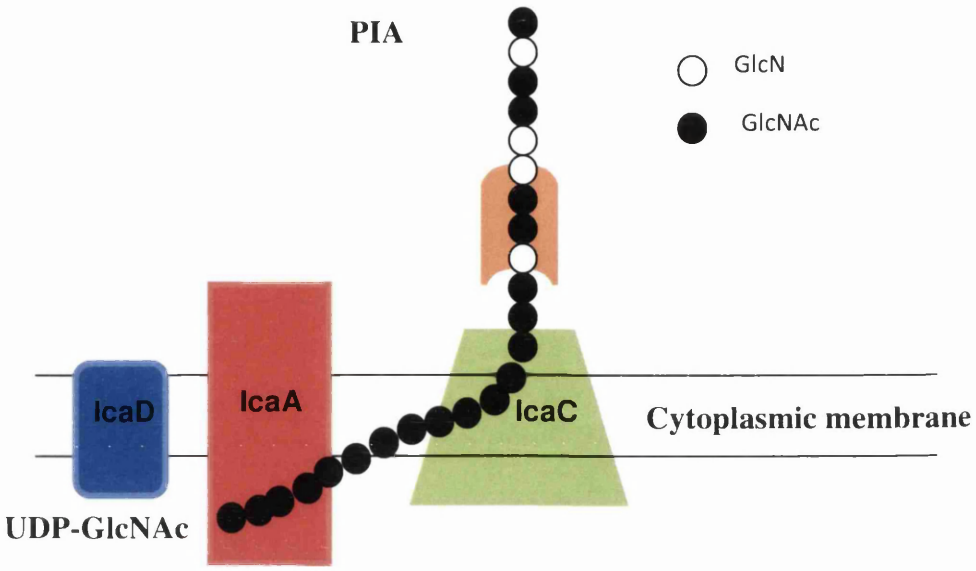


Figure1.3 Scheme of the PIA biosynthesis mechanism in *S. epidermidis* as proposed by Gotz(Gotz 2002). IcaA by the help of IcaD using UDP-GlcNAc to synthesis β-1, 6-linked GlcNAc oligomers, which is elongated and translocate across the cytoplasmic membrane by IcaC. IcaB is responsible for removing some (approximately 15%) of acetyle groups from GlcNAc residues.

The teicoplanin –associated locus regulator (TcaR) also acts as a transcriptional repressor of *ica* operon (Jefferson, Pier et al., 2004). The Staphylococcal accessory regulator A (SarA) and the general stress alternative sigma factor Sigma B (SigB) are a positive transcriptional regulator of *ica* operon (Handke, Slater et al., 2007; Pintens, Massonet et al., 2008).

The positive regulation of SigB on *ica* operon in *S. epidermidis* is due to its negative regulation on icaR (Handke, Slater et al., 2007; Jager, Jonas et al., 2009). In addition to the above regulators, there are several environmental and nutritional conditions were proving to regulate PIA biosynthesis, e.g. anaerobic growth conditions lead to increased polysaccharide expression in *S. epidermidis* (Cramton, Ulrich et al., 2001), Subinhibitory concentrations of different antibiotic (e.g. tetracycline, streptogramin and quinupristin-dalfopristin) were found to enhance *ica* expression in *S. epidermidis* 9- to 11-fold (Rachid, Ohlsen et al., 2000). Studies suggested a relationship between

*ica*ADBC expression and metabolism; the presence of glucose in growth media affected *ica*ADBC expression and PIA production (Dobinsky, Kiel et al., 2003). Also, PIA synthesis enhanced during tricarboxylic acid (TCA) cycle stress (Vuong, Kidder et al., 2005).

1.4.1.1.2 Accumulation associated protein (Aap)

Accumulation associated protein (Aap) is a cell surface associated protein mediating intercellular adhesion and biofilm accumulation in a PIA-independent way (Sun, Accavitti et al., 2005). Aap is synthesized as a pre-protein and is anchored via an LPXTG motif and comprises domain A, which itself contains a lectin-like domain, and a repetitive domain B, composed of a variable number of 128 amino acid repeats. Domine B repeats contain G5 domains which may bind *N*-acetylglucosamine (Bateman, Holden et al., 2005). G5 domines mediate intercellular accumulation through Zn (2+)-mediated homodimerization (Gruszka, Wojdyla et al., 2012). *S. epidermidis* biofilm formation repressed by Zn (2+) chelation (Conrady, Brescia et al., 2008). Proteolytic processing of Aap leads to removal of the *N* -terminal domain A, exposing domain B, which gives Aap intercellular adhesive properties causing biofilm accumulation. The proteolytic activation may be mediated either by staphylococcal exoproteases or by the host, as part of the early immune response against *S. epidermidis* (Rohde, Burdelski et al., 2005). Protein-dependent biofilms mediated by Aap were found in *S. epidermidis* strains isolated from total hip arthroplasty (Rohde, Burandt et al., 2007)

1.4.1.1.3 Extracellular matrix binding protein (Embp)

The Extracellular matrix binding protein Embp is a cell surface associated protein that mediates attachment to host extracellular matrix and biofilm accumulation. The intercellular adhesive properties first revealed in a clinical isolates of *S. epidermidis* 1585 from blood culture of catheter infection which was icaADBC negative and Aap negative did not form biofilm in *vitro*, with subsequent culturing a biofilm positive *S. epidermidis* 1585V variant obtained. Embp is a giant 1MDa fibronectin-binding protein, harboring 59 so-called Found in Various Architectures (FIVAR) domains and 38 protein G-related albumin-binding (GA) domains. The FIVAR domains of Embp mediate

binding of *S. epidermidis* to fibronectin, the first step of biofilm formation on conditioned surfaces (Williams, Henderson et al., 2002; Christner, Franke et al., 2010). Under standard *in vitro* conditions, Embp is not expressed; however Embp expression induced in biofilm negative wild type *S. epidermidis* 1585 in the presence of goat serum, leading to biofilm formation (Christner, Franke et al., 2010). The Embp mediated biofilm protects *S. epidermidis* from phagocytosis by macrophages (Schommer, Christner et al., 2011).

1.4.2 Phenol soluble modulins

S. epidermidis produces a number of exoenzymes including lipases, proteases, hemolysin, nuclease (DNAse) and Thermonuclease (TNAse). In addition, *S. epidermidis* synthesizes toxins like phenol soluble modulins (PSMs) and enterotoxin C (SEC). These enterotoxins have superantigens properties and can stimulate the overproduction of cytokines such as interferon-γ (IFN-γ), interleukin-1 (IL-1), interleukin-2 (IL-2), and tumor necrosis factor alpha (TNFα). The release of these enzymes and toxins contribute to the persistence of *S. epidermidis* infection and also results in injury to host tissue (Otto, 2004; Cunha Mde, Rugolo et al., 2006). Phenol soluble modulins are peptide toxins with pro- inflammatory effects on the host. The production of PSMs is controlled by the quorum-sensing accessory gene regulator (*agr*) (Vuong, Durr et al., 2004) and *agr*-dependent PSMs production could disrupt cell-cell contacts in the biofilm and encourage cell detachment. There are three PSMs peptides encoded in *S. epidermidis*, PSM-α, PMS-β and PSM-δ. These peptides facilitate biofilm dispersion and dissemination of biofilm-associated infection, especially PSM-β which is a key effecter of *S. epidermidis* biofilm maturation and detachment (Wang, Khan et al., 2011). PSM-δ has strong cytolytic activity, lysing neutrophils and erythrocytes when present at high concentrations (Cheung, Rigby et al., 2010).

1.5 Immune system

The immune system is responsible for protecting the host against microorganisms that invade the body such as bacteria, viruses, fungi and protozoa. The host immune defense can be divided into two major categories: the innate (natural) immune system which recognizes conserved residues in microorganisms and the adaptive immune system which recognizes unique structural motifs (Beck and Habicht, 1996) and is beyond the scope of this thesis.

1.6 Innate immunity

Innate immunity is the first line of defense against infections. Innate immune mechanisms are rapidly activated by microbes before adaptive immune responses, and are nonspecific. The innate immune system uses cellular immunity and humoral immunity to complete its function. Thus, cellular innate immunity involves pathogen recognition through cell surface pattern recognition receptors (such as toll-like receptors) and pathogen removal through phagocytosis and killing by phagocytic cells (such as neutrophils and macrophages). Humoral innate immunity involves intercellular signaling and amplification of the inflammatory response by cytokines and complement cascades. Innate immunity plays an important role in limiting infection in its early stages (Abbas, Lichtman et al., 2010).

1.6.1. Barriers as part of the innate immune system

Anatomical barriers covering the outer body surface (e.g. skin), and mucus membranes covering the inner body surface (respiratory system, urinary system, gastrointestinal system) are also part of the innate immune system. These membranes act as mechanical barriers preventing pathogens from penetrating and entering the body. Any damage to the integrity of these barriers caused by factors such as burns, wounds or surgical procedures, give pathogens an opportunity to enter the body, multiply and cause infection. In addition skin and mucous membranes contain chemical barriers. Epithelial cells produce antibacterial peptides (e.g. defensins), sebum secreted by skin sebaceous glands can inhibit bacterial and fungal growth (fatty acid and lactic acid), gastric acid

kills microorganism and lysozyme in tears and nasal secretion have antibacterial properties, secretary immunoglobulin A found in tears, saliva and on the nasal and bronchial surface plays an important role in pathogen opsonization. Finally, the normal microbial flora of the skin and mucus membrane also acts as a microbiological barrier to prevent the growth of pathogenic bacteria. Disturbance to the normal microflora caused by antibiotic usage can lead to decreased normal flora and enhanced growth of pathogenic bacteria (Helbert, 2006; Parham, 2009).

1.6.2 Cellular immunity

1.6.2.1 Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes (PMN), are considered a major defence mechanism against bacterial invasion. Neutrophils which represent two-thirds of peripheral blood leukocytes, have a major function of killing invading microbes. Neutrophils have a short life span, 8-12 hours in circulation and 1-4 days in tissues, with everyday turnover of human neutrophils approximately $0.8–1.6 \times 10^9$ cells per kg of body weight. Thus to maintain cellular homeostasis under physiological conditions equivalent numbers of neutrophils are cleared (Akgul, Moulding et al., 2001; Xu, Loison et al., 2009). Severe bacterial infections occur if neutrophils are few in number (neutropenia) or are deficient in function, as in chronic granulomatous disease (CGD) (Lakshman and Finn, 2001).

Neutrophils have multi-lobed nuclei and cytoplasmic granules called lysosomes and contain a variety of degradative enzymes which are important in the bactericidal action of these cells (Yamashiro, Kamohara et al., 2001; Levinson, 2006). After migrating to the site of infection, neutrophils encounter invading microbes and engulf pathogens into a phagosome. In phagosomes, there are two major antimicrobial effects. The first effect involves fusion of phagosomes with neutrophil granules. Azurophilic granules contain lysozyme, defensins, proteases, whilst specific granules contain lactoferin, lysozyme and several membrane proteins including NADPH oxidase. All these are essential enzymes for neutrophil function and assembled in phagosomes following fusion with azurophilic and specific granules (Hampton, Kettle et al., 1998). The second antimicrobial effect

involves pre-synthesized subunits of the NADPH oxidase assembling at the phagosomal membrane and transfering electrons to oxygen to form superoxide anions. These anions dismutate spontaneously or catalytically to oxygen and hydrogen peroxide and collectively are called reactive oxygen species (ROS). Discharging antimicrobial peptides, enzymes and ROS, are collectively responsible for microbial digestion and killing in neutrophils (Parham, 2009).

Activated neutrophils undergo programmed cell death or apoptosis and this prevents the release of enzymes and ROS in to surrounding tissue (Savill, Wyllie et al., 1989; Haslett and Savill, 2001). The dead neutrophils are then phagocytes by macrophages which prevent local tissue damage that would otherwise occur through exposure to the retuned enzymes and toxin from the neutrophils (Akgul, Moulding et al., 2001; Maianski, Maianski et al., 2004).

Recently, another mechanism of killing has been identified in neutrophils which is the generation and release of neutrophil extracellular traps (NETs) from gaps or breaks in the cell membrane. NETs are extracellular fibrous structures composed of granule protein and chromatin that have the ability to bind and kill invading pathogens and prevent them from spreading (Brinkmann, Reichard et al., 2004; Brinkmann, Laube et al., 2010). This antimicrobial process is also aided by the generation of ROS by NADPH oxidase (Fuchs, Abed et al., 2007).

The vast array of toxic products produced by neutophils have two contrasting effects. Firstly a powerful microbicidal activity essential for an effective host defence and secondly a toxic effect resulting in excessive inflammation and tissue damage as observed in rheumatoid arthritis and inflammatory bowel disease and acute lung injury (Cook, Pisetsky et al., 2004; Himmel, Hardenberg et al., 2008).

1.6.2.2 Macrophages

Macrophages are found in all body tissues, and in considerable numbers in the liver, spleen and lung, where they are responsible for activating acquired (adaptive) immune processes. Their anatomical location in tissues means they are the first cells to deal with invading pathogens and they also send chemical messages in the form of chemokines CXCL8 (IL-8) to recruit other cells of the immune system from the blood, e.g.

neutrophils and monocytes (monocytes differentiated into macrophages when enter the tissue) into the infected area. Macrophages express different surface markers such as CD11b, CD18, CD68 and FC receptors (Murray and Wynn, 2011). Both CD11b/CD18 (also called CR3) function as adhesion molecules and complement receptors in the binding of C3b, and thus stimulate recognition and phagocytosis of the invading pathogens (Ehlers, 2000).

Macrophages have several key function and these are: 1) The phagocytosis of opsonized organisms, cellular debris and apoptotic cells 2) The killing of phagocytosed organisms by reactive nitrogen species (RNS) 3) Antigen presentation on class II major histocompatibility complex (MHC) molecules, and 4) Secretion of cytokines e.g. IL-1, IL-6, IL-12, TNF –α that promote immune responses (Levinson, 2006; Menendez-Benito and Neefjes, 2007).

Macrophages are activated either by cytokines (e.g. gamma interferon (IFN-γ) produced by innate immune cells such as natural killer cells (NK), or Th1 T cells), or by microbial products such as lipopolysaccharide (LPS), peptidoglycan (PGN) and mannans collectively called pathogen-associated molecular patterns(PAMPs). PAMPs stimulate macrophage activation through toll-like receptors (TLRs) (Schnare, Holt et al., 2000; Young, 2006). Bacterial pathogens prompt inducible nitric oxide synthase (iNOS) enzyme which induce release of antimicrobial molecules RNS derived from nitric oxide (NO), RNS act with ROS as potent antimicrobial to damage pathogens (Lyons, Orloff et al., 1992; Aktan, 2004; Fang, 2004).

On recognition of a pathogen in host tissues, macrophages utilise cytokines in several ways in order to generate an effective response. Secreted chemokines such as IL-8 serve to attract neutrophils to the site of infection, whilst the release of cytokines such as TNF, prostaglandin and platelet activating factor act on endothelium and change blood vessels permeability to allow neutrophils and monocytes to leave the blood and enter the tissues. Macrophages do not just kill invading pathogens, but also eliminate the waste and debris of dead or damaged tissues. Macrophages also ingest apoptotic neutrophils thereby acting as scavengers that continue to survive after phagocytosis (Dale, Boxer et al., 2008; Silva, 2010).

1.6.2.3 Phagocytosis

Phagocytosis is the process of ingestion and killing of microbes by phagocytic cells, the main phagocytes are neutrophils and macrophages along with dendritic cells. Phagocytes use different methods to recognize pathogens. These include the presence of pattern recognition receptors like toll-like receptors, complement receptors, or Fc receptors to recognize pathogens–associated molecular patterns (PAMPs) present on microbes. These recognition mechanisms are important to facilitate the attachment of the pathogen to the phagocyte (Aderem and Underhill, 1999; Underhill and Goodridge, 2012). Phagocytosis is an actin- dependent mechanism that involves polymerization of monomeric G- actin to polymeric F-actin (Swanson and Baer, 1995; Greenberg and Grinstein, 2002). Phagocytes extend membrane protrusions called pseudopodia around ingested particles to engulf them into the phagosome (Dale, Boxer et al., 2008). Phagosomes fuse with endosomes and lysosomes to form phagolysosomes (Stuart and Ezekowitz, 2005). Pathogens inside the phagolysosomes are killed by the effect of respiratory burst, which involves production of hypochlorous acid, hydrogen peroxide and nitric acid. Additional antimicrobial effect occure due to antimicrobial peptides e.g. defensins that are secreted into the phagosome (Underhill and Ozinsky, 2002; Flannagan, Jaumouille et al., 2011; Sarantis and Grinstein, 2012). Furthermore, NADPH-oxidase kill the internalized pathogens by release of reactive oxygen intermediates and activation of proteases (Reeves, Lu et al., 2002).

An important function of phagocytes is to act as the link between the innate and acquired immune systems. This link occur due to the phagocyte presenting antigen via class II MHCs to activate CD4 T cells (Stuart and Ezekowitz, 2005; Kagan and Iwasaki, 2012).

1.6.2.4 Pattern recognition receptors

To detect pathogens, cells of the innate immune system have receptors called pattern recognition receptors (PRRs) which detect conserved structural motifs on pathogenic organisms called pathogens–associated molecular patterns (PAMPs) (Reynolds and Dong, 2013). Important PRRs are Toll-like receptors (TLRs), (Hallman, Ramet et al., 2001; Nishiya and DeFranco, 2004), mannose receptors and, collectins such as C1q and mannose binding lectin (MBL). Both neutrophils and macrophages express TLRs on

their cell surfaces, endosomes and in their cytoplasm (Underhill and Ozinsky, 2002; Parker, Whyte et al., 2005; Takahashi, Ishida et al., 2010). TLR activation leads to up-regulation of phagocytosis, maturation of leukocytes and cytokine release (Heine and Ulmer, 2005). Young children with deficiencies in the complement recognition molecule mannose-binding lectin are more susceptible to *S. aureus* infections (Park, Kurokawa et al., 2010).

There are 11 TLRs each recognizing different PAMPs. For example TLR-4 binds lipopolysaccharide (LPS), TLR-2 binds peptidoglycan and lipopeptides, and TLR-3 binds viral RNA. The interaction of TLRs with their specific PAMP induces activation of signal transduction pathways including activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway, leading to secretion of pro-inflammatory cytokines such as tumour necrosis factor (TNFα), IL-1β, IL-6 and IL-12. Furthermore, activation of mitogen-activated proteins (MAP) kinase family pathways, including extracellular signal-regulated protein kinases ERK, c-Jun amino-terminal kinases (JNK) and p38 MAP kinases, are also fundamental for complete host defence including cell growth and differentiation, control of inflammation, apoptosis, macrophage activation and the expression of IL-12. Molecules released following TLR activation, signal to other cells of the immune system to increase production of neutrophils in bone marrow and recruit neutrophils to site of the infection making TLRs essential elements of both innate and adaptive immunity (Pearson, Robinson et al., 2001; Zhang and Dong, 2005; Carpenter and O'Neill, 2007).

1.6.3 Humoral immunity

1.6.3.1 Complement system

The complement system is composed of more than 30 serum and cell membrane proteins forming an enzymatic cascade (Walport, 2001). Hepatocytes produce the majority of serum complement proteins, as inactive zymogens. Once activated, zymogens are cleaved and become proteases activating downstream zymogens in the cascade. The complement system plays an important role in host defence against bacterial and viral infection (Wood, 2011). Activation of the complement cascade is initiated by three

mechanisms; the classical pathway, the lectin-binding pathway and the alternative pathway. The three pathways join in a final common pathway through cleavage of the central component C3. C3 cleavage subsequently activates C5, C6, C7, C8 and C9 initiating the aggregation of C5b, C6, C7, C8 and C9 into the membrane attack complex (MAC) that bind to the pathogen cell wall inducing cytolysis (Cole and Morgan, 2003).

The classical complement pathway is activated by antigen-antibody complexes. C1q (pattern recognition receptor; PRR) interacts with the Fc portion of immunoglobulin (IgG, IgM) to activate C1s protease leading to cleavage of C4 into C4a and C4b, and cleavage of C2 into C2a and C2b. The C4bC2a complex is the formed which promotes C3 cleavage into C3a and C3b. C3b activates the common final pathway allowing MAC assembly (Pettigrew, Teuber et al., 2009; Carroll and Georgiou, 2013). The Lectin–binding pathway is similar to the classical pathway and is activated when the PRR mannose–binding lectin (MBL) binds to monosaccharides such as mannose, N-acetylglucosamine, N-acetylmannosamine on microbial surfaces. This serves to activate MBL-associated serine protease MASP-2 leading to cleavage and activation of C4 and C2 which finally cleave C3 into C3a and C3b which activate the common final pathway (Sorensen, Thiel et al., 2005). The alternative pathway is activated by microbial surfaces and cell surface components (e.g. LPS and teichoic acid) which lead to cleavage of C3 into C3a and C3b. In addition to forming MAC, the C3a fragment increases vascular permeability stimulating phagocyte transmigration to the site of infection and C3b acts as an opsonin to coat pathogens and to enhance phagocytosis (Pettigrew, Teuber et al., 2009).

Activation of the complement system produces a plethora of cleavage fragments with important functions in host defence; 1) C3b is an opsonin that binds to pathogens and interacts with phagocytic receptors (CR1, CR2, CR3, and CR4) on the surface of phagocytes; 2) C3a and C5a induce phagocyte chemotaxis and increase vascular permeability to enhance cell migration; 3) The MAC complex induces direct killing of pathogens by inducing pores in the cell membrane; 4) C3b and C4b bind immune complexes to aid clearance from the circulation in the liver, by recognition of C1

receptors on the erythrocytes; 5) C3d coating of antigens activates B lymphocytes which promote antibody production (Actor, 2012).

In contrast, excess releases of complement cleavage fragment have harmful effects on cellular function within the innate immune system, especially in the critically ill patient. For example in patient with sepsis, C5a has inhibitory effects on neutrophil phagocytic function, migration and release of ROS in response to pathogens (Conway Morris, Kefala et al., 2009; Morris, Brittan et al., 2011).

1.6.3.2 Cytokines

Cytokines are protein messenger molecules used for intercellular communication, between cells of the immune system. The main families of cytokines are Interleukins (ILs), Colony Stimulating Factors (CSFs), Interferons (IFNs), Tumour Necrosis Factors (TNFs), Chemokines (CKs) and Growth Factors (GFs). Cytokines control cell growth, proliferation, function and leukocyte migration (Helbert, 2006).

Interleukins (ILs) are a large group of proteins with immune modulatory function that control cell growth, differentiation and migration during the immune response. Interleukins have both pro-inflammatory and anti-inflammatory actions. Based on structural features, ILs can be divided into 4 main groups; IL1-like cytokines, the class I helical cytokines (IL4-like, γ-chain and IL6/12-like), the class II helical cytokines (IL10-like and IL28-like) and the IL17-like cytokines (Commins, Borish et al., 2010).

The colony-stimulating factors (CSFs) are a group of cytokines that play important roles in the regulation of hematopoiesis of blood cells through their effect on hematopoietic stem cells. This group consists of the macrophage-CSF (M-CSF), granulocyte-CSF (G-CSF), granulocyte/macrophage-CSF (GM-CSF), and multi-CSF (IL-3) (Barreda, Hanington et al., 2004). M-CSF controls the proliferation, differentiation, and survival of macrophages, monocytes and their precursors cells and increases their phagocytic and chemotactic activity (Stanley, Berg et al., 1997). G-CSF promotes neutrophil proliferation and maturation, whereas, GM-CSF has much broader effects on multiple cells including macrophages, eosinophils and neutrophils. G-CSF and GM-CSF are produced by macrophages, monocytes, fibroblasts, stromal cells and endothelial cells.

The production of both G-CSF and GM-CSF is triggered by bacterial LPS, but GM-CSF production can also be triggered by antigens which induce its production and release from T lymphocytes as part of the specific immune response (Barreda, Hanington et al., 2004).

The tumour necrosis factor (TNFs) cytokine family includes numerous cytokines. TNFs include TNF-α (cachectin) which is primarily produced by macrophages, T lymphocytes and natural killer cells. TNF-α plays a major role in the inflammatory response and induce the acute phase reaction with IL-1 and IL-6, causing fever and cachexia. The antitumor effects of TNF-α are used in treatment of sarcoma and melanoma patients. TNF-β (Lymphtoxin-alpha and beta) is secreted by lymphocytes with cytotoxic effects on tumors. Fas ligand (FasL or CD95L) is a cytokine that induces cell apoptosis (Vilcek and Lee, 1991; Suda, Takahashi et al., 1993; van Horssen, Ten Hagen et al., 2006).

Chemokines (CKs) are a family of more than 50 small proteins secreted by a variety of cell types. CKs have the ability to chemoattract cells of the immune system to the site of infection (Ding, Xiong et al., 2001). IL-8 is a major chemokine produced mainly by macrophages and epithelial cells during the inflammatory response. IL-8 attracts neutrophils, lymphocytes and basophils to the site of infection (Actor, 2012).

Growth factors (GFs) are a large family of proteins able to stimulate cell proliferation and growth, regulate tissue morphogenesis, angiogenesis, cell differentiation, maintenance of tissue homeostasis and wound healing. The most important GF is Transforming growth factor beta (TGF-β) which is produced by activated Th1 cells (T-helper), macrophages and NK cells. TGF-β has an anti-inflammatory effect and inhibits the inflammatory response in macrophages, attenuates lymphocyte proliferation, and promotes wound healing (King, 2013; Peplow and Chatterjee, 2013; Seaman and Gold, 2013).

Interferons (IFNs) are cytokines with antiviral and antitumor activity. Type I interferons include IFN-α, IFN-β and IFN- ω and these have antiviral activity by preventing viruses from replicating within host cells (Fensterl and Sen, 2009). Type II IFNs include IFN-γ which is secreted primarily by cytotoxic $CD8^+$ T-cells to enhance the presentation of

antigen to T-helper ($CD4^+$) cells (Helbert, 2006) and increases the bacteriocidal capacity of macrophages by inducing RNS through inducible nitric oxide synthase (Lyons, Orloff et al., 1992)

1.7 Immune evasion by Staphylococci

The role of the immune system is to eradicate microorganisms and return the body to a state of homeostasis. Successful pathogens have however evolved numerous strategies to evade eradication and thus allow their successful survival in the host niche.

Strategies of evasion include *S. aureus* changing its cell membrane charge by incorporating positively charged lipoteichoic and teichoic acid in the cell wall which increases electrical dissonance with the cationic AMP (Peschel, Otto et al., 1999). Secretions of proteolytic enzymes which inactivate AMP, and down regulate bacterial surface binding proteins prevent AMP effects (Sieprawska-Lupa, Mydel et al., 2004). Furthermore, *S. aureus* secretes proteins that inhibit complement activation and neutrophil chemotaxis and lyse neutrophils (Foster, 2005). Also, *S. aureus* expresses a number of exotoxins with superantigen properties that alter the humoral immune response, resulting in immunosuppression (Foster, 2005; Iwatsuki, Yamasaki et al., 2006).

In addition, some bacteria prevent recruitment of phagocytes to the site of infection by interfering with the chemoattractants IL-8 and complement component C5a. This is accomplished in many strains of *S. aureus* by production of the chemotaxis inhibitory protein (CHIPS), which binds to the leukocyte receptors for C5a, preventing attachment of specific chemo attractants (Postma, Poppelier et al., 2004; Edwards, Taylor et al., 2005).

Other strategies by which *S. aureus* evade the innate immune system are by secreting a number of other proteins. The extracellular complement-binding protein (Ecb) impairs complement activation by binding to the C3d domain of C3. The effect of this is to inhibit C3 convertases (alternative pathway) and C5 convertases (all complement pathways) and block C5a formation. The extracellular fibrinogen-binding protein (Efb)

also blocks both complement activation and neutrophil adhesion to fibrinogen (Hammel, Sfyroera et al., 2007; Jongerius, Kohl et al., 2007).

1.8 *Staphylococcus epidermidis* interaction with innate immune system

It is well established that a biofilm growth phase protects bacteria within the polymeric matrix from the phagocytic effect of polymorphonuclear neutrophils (Rodgers, Phillips et al., 1994; Vuong, Voyich et al., 2004). To date, knowledge on the effect of phagocytes on *S. epidermidis* biofilms, is largely confined to PIA interactions. These studies have identified two contrasting roles for PIA-one of 'immune priming' and the other of 'immune evasion'. This situation is often complicated by the fact that priming and evasion can often be measured simultaneously in the same model. Thus, Stevens and co-workers (Stevens, Sadovskaya et al., 2009) showed that purified PIA could stimulate IL-8 production through toll-like receptor- 2 (TLR-2). Granslo *et al* (Granslo, Klingenberg et al., 2013) also found that the PIA biofilm forming strain of *S. epidermidis* (1457) induced higher IL-8 and IL-6 secretion, but less complement activation in neonatal cord blood compared to adult blood. Kristian *et al* (Kristian, Birkenstock et al., 2008) demonstrated induction of complement component 3, but decreased deposition of C3b and IgG on the bacterial surface. Similar findings were confirmed by Fredheim *et al* who showed that PIA induced complement activation together with a simultaneous decrease in the activation of granulocytes (Fredheim, Granslo et al., 2011). Furthermore, a study by Heinzelmann *et al* (Heinzelmann, Herzig et al., 1997) showed that planktonic, slime-producing *S. epidermidis* RP62A induced increased phagocytosis and production of reactive oxygen intermediates (ROI) during oxidative burst by PMNs compared with its non-slime-producing phenotypic variant RP62A-NA. The importance of the specific mechanism of biofilm accumulation was confirmed by Schommer *et al* (Schommer, Christner et al., 2011) who demonstrated that PIA, Aap and Embp dependent *S. epidermidis* biofilms can escape phagocytic uptake by macrophages and reduce NF-κB activation and IL-1β production. Clinical studies also support the case that immune evasion by biofilm positive strains could be due to an attenuated immune response. In an *ex vivo* study using whole cord vein blood from term and preterm infants, Hartel *et al* found significant decreases in IL-6 production from

whole blood culture infected with a biofilm positive *S. epidermidis* compared to those infected with biofilm negative strains (Hartel, Osthues et al., 2008). In addition, lower C- reactive protein (CRP) levels have been detected in serum samples of neonates infected with biofilm positive *S. epidermidis* sepsis (Klingenberg, Aarag et al., 2005).

Animal models also suggest a survival advantage of *S. epidermidis* biofilm positive strains. Begun *et al* (Begun, Gaiani et al., 2007) showed that PIA-producing *S. epidermidis* has a significant survival advantage over *ica*-deficient *S. epidermidis* within the intestinal tract of the nematode *Caenorhabditis elegans*. Similarly, in models of mouse biomaterial associated infection and rat intravascular catheter related infection PIA positive *S. epidermidis* caused a more severe infection than its isogenic mutant (Rupp, Ulphani et al., 1999a; Rupp, Ulphani et al., 1999b; Rupp, Fey et al., 2001).

To date, few clinical studies have assessed the function of leukocytes isolated from patients suffering from biofilm positive *S. epidermidis* directly. However studies in patients with post-traumatic osteomyelitis, where a high percentage of isolates are Staphylococci, show that neutrophils migrate to the infection site but are unable to control the infection and instead cause tissue damage due to excessive release of superoxides (Wagner, Deppisch et al., 2003; Wagner, Kaksa et al., 2004; Wagner, Hansch et al., 2006). In an *in vitro* catheter infection, Leid *et al* showed that leukocytes penetrated *S. aureus* biofilm, but were unable to engulf and kill bacteria (Leid, Shirtliff et al., 2002). Interestingly, Guenther *et al* found that the neutophils response to *S. aureus* biofilm differed to that of *S. epidermidis* biofilm. Time-lapse video demonstrated that neurophils could pass through *S. aureus* biofilm and phagocytose bacteria while they became paralysed when in contact with *S. epidermidis* biofilm. Furthermore, *S. aureus* biofilm induced apoptotic killing in 80% of neutrophils in comparison to *S. epidermidis* for which this figure was 50%. The effect seen with *S. epidermidis* was due to phagocytosis of biofilm cells and not due to associated toxic effects (Guenther, Stroh et al., 2009). Finally, implanted medical devices in osteomyelitis associated with biofilm formation lead to T lymphocytes activation and infiltration to the sites of infection with excessive synthesis of IFN-γ which activate phagocytic cells (Wagner, Heck et al., 2006; Kotsougiani, Pioch et al., 2010).

1. 9 Aims of the study

Both *in vitro* and *in vivo* studies suggest a role for *S. epidermidis* derived PIA in immune priming and evasion. To date, the physiological/pathological conditions that determine which of those responses predominates and whether PIA is produced at all is unknown. Furthermore, very little data is available on the physiological cues responsible for PIA production.

This thesis uses a cell biology approach;

i) To compare PIA production and biofilm formation in *S. epidermidis* cultured in cell culture media and human serum versus traditional broth culture.

ii) To investigate the inflammatory response produced by purified PIA and PIA dependent biofilm using *in vitro* and *ex vivo* modelling in both a macrophage cell line and whole blood.

Specifically the thesis has been organized as follows:

- In chapter 3 the nutrients necessary for PIA production will be identified.
- In chapter 4 PIA will be purified in cell culture media supporting biofilm formation.
- In chapter 5 the effect of soluble, cell-associated and biofilm- associated PIA on THP-1 cells will be studied thereby identifying the importance of tethering for cytokine production.
- In chapter 6 the effect of soluble, cell-associated and biofilm-associated PIA on whole blood will be analysed and along with the importance of humoral immunity, C5a and immunosuppression.

Chapter 2

Materials and Methods

2.1 Chemicals and reagents

2.1.1 Chemicals

Table 2.1: Chemicals used in this study

Chemicals	Sources
Triton X-100	Sigma Aldrich
Trypan Blue	Fisher Scientific
Tween 20	Sigma Ultra
Trisbase	Fisher Scientific
Sodium nitrite ($NaNO_2$)	Sigma Aldrich
Iron chloride hexahydrate ($FeCl_3.H_2O$)	Sigma Aldrich
Picric acid	VWR/PROLABO
Acetic acid glacial	Fisher Scientific
37% Formaldehyde	Fisher Scientific
Sodium chloride (NaCl)	Fisher Scientific
Disodium hydrogen (Na_2HPO_4)	Fisher Scientific
Potassium dihydrogenphosphate (KH_2PO_4)	Fisher Scientific
Ammonium sulfamate	Sigma Aldrich
Saturated Phenol	VWR international LTD
Crystal violet ACS reagent	Sigma Aldrich
Glycerol	Sigma Aldrich
Agar bacteriological	Oxoid
Sodium azide (NaN_3)	Fisher Scientific
Sure Blue TMB 1-component Microwell Peroxidase	KPL/Insight Biotechnologies
Bradford Reagent	Sigma Aldrich
3-Methyl-2-benzothiazolonehydrazone hydrochloride (MBTH)	Sigma Aldrich
Hydrochloric acid (HCl)	Sigma Aldrich

Bovine serum albumin fraction v	Fisher Scientific
Fixing solution (Methyl alcohol)	Merck Millipore
Color reagent red (eosin)	Merck Millipore
Color reagent blue (azur)	Merck Millipore

2.1.2Enzymes

Table 2.2: Enzymes used in this study

Enzymes	Sources
Dispersin B	Gift from Kaplan JB (Kaplan 2009)
Trypsin	Invitrogen

2.1.3. Fluorescent stains

Table 2.3: Reagents used for fluorescent staining experiments

Fluorescent stain	Source
SYTO9	Invitrogen
Rhodamine-conjugated Wheat Germ Agglutinin	Invitrogen
Propidium iodide	Invitrogen
AlexaFluor 488	Invitrogen
Cell mask cell membrane stain	Invitrogen

2.1.4 Blocking Antibodies

Table 2.4 Blocking antibodies used in this study

Blocking Antibodies	Sources
Antihuman CD11b	Biolegend
Antihuman CD18	Biolegend
Antihuman CD16	Biolegend
Mouse IgG, K Isotype	Biolegend

2.1.5 Miscellaneous materials

Table 2.5: Miscellaneous materials used in this study

Materials	Sources
Nunclon cell culture plates (6, 24, and 96) wells	Thermo Scientific
Microplate, 96 well, half-area, flat bottom for ELISA	Greiner bio-one
Disposable sterile cell lifters	Fisher Scientific
Cuvettes	Fisher Scientific
Vectashield mounting medium for fluorescence	Vector
Single use syringe filter 0.45µm	Sartorius stedim
DPX Mountant for histology	Sigma Aldrich
Shandon cytoclip	Thermo Scientific
Shandon single cytofunnel	Thermo Scientific
Shandon Filter card	Thermo Scientific
Shandon* Single Cytofunnel* with white filter cards	Thermo Scientific
FACS Lysis solution for lysing whole blood	BD Biosciences
Centrifugal filter units Millipore (3K, 10K, and30K)	Amicon Ultra
Combitips plus 1.0 ml	Eppendorf
ELISA Kits	R and D system
RayBio Human Cytokine Antibody Array	Ray Biotech

NUNC NUNCLON cell culture dishes 100mm	Invitrogen
L-Glutamine	Invitrogen
Penicillin/Streptomycin	Invitrogen
Cytochalasin D	Sigma Aldrich
Anti-rabbit IgG, HRP-linked Antibody	Sigma Aldrich
The Pierce LAL Chromogenic Endotoxin Quantitation kit	Thermo Scientific

2.2 Instruments

Table 2.6: Instruments used in this study

Instruments	Sources
Light Microscope	Zeiss Axio vision
Epi-fluorescent Microscope	Zeiss Axio vision
Confocal Laser Scanning Microscope	Carl Zeiss
Fluo Star Plate Reader	Omega Fluo Star
Centrifuge	Eppendorf Centrifuge 5810R
Digital dry bath	Jencons-PLS
CO_2 Air Jacketed incubator supply	NUAIRE
Shandon Cytospin Cytocentrifuge	Thermo Scientific
Cell culture Hood	ScanLAF cell culture hood Mars
Sonicator	Branson digital Sonifer
Vacuum system	Vacusafe Comfort
Axiovert 40C Inverted Microscope	Carl Zeiss
Avanti J-20XP Centrifuge	Beckman Coulter
Rotator	Stuart
Gel Doc Chemiluminescence imaging system	BIO-RAD

2.3 Staphylococci used in this study

2.3.1 *Staphylococcus epidermiduis* strains used in this study

Table 2.7: *S. epidermidis* strains used in this study were:

Strain	Source	Biofilm accumulation	Reference
1457	Central venous catheter infection	PIA dependent	Mack *et al.* (1992)
1457-M10	A PIA negative isogenic mutant of strain 1457 that was produced by insertion of transposon Tn*917* at nucleotide 931 in the *icaA* gene of the *icaADBC* gene locus	Biofilm negative	Mack *et al.* (1999)
8400	Central venous catheter infection, biofilm positive, and PIA positive	PIA dependent	Mack *et al.* (1992)
9142	Central venous catheter infection, biofilm positive, and PIA positive	PIA dependent	Mack *et al.* (1992)
5179	CSF-Shunt Infection, biofilm negative, and PIA negative strain; *icaA*:IS*257*	Biofilm negative	Mack et al. (1992)
5179-R1	Biofilm positive revertant of the respective parent strain	Aap dependent	Mack *et al.* (1992)
1585	Isolated from port-catheter infection; biofilm negative, Aap negative, *icaADBC* negative and PIA negative	Biofilm negative	Rohde *et al.* (2005)
1585-RA	Biofilm positive revertant of the respective parent strain	Embp dependent	Rohde *et al.* (2005)

2.3.2 *Staphylococcus aureus* strain used in this study

S. aureus Cowan I strain used in this study which obtained from human, from septic arthritis fluid, was obtained from HPA cultures.

2.4 Cell sources

- Human monocytic cell; THP-1 cell line, derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia (Tsuchiya, Yamabe et al. 1980; Tsuchiya, Kobayashi et al. 1982).
- Whole blood from healthy volunteers.

2.5 Media

2.5.1 Tryptose Soy Media (TSB) based broth (+/- Dextrose)

Thirty grams of TSB powder (+/- Dextrose) was added to 1000 ml of distilled water (dH_2O) mixed, and autoclaved in a pressure vessel autoclave at 121°C for 20 minutes and then stored at room temperature.

2.5.2 Cell culture media

Five different cell culture media were used in this study all obtained from Invitrogen: Iscove's Modified Dulbecco's Media (IMDM), Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), Roswell Park Memorial Institute media with GlutaMAX (RPMI+), and Roswell Park Memorial Institute media without glutamine (RPMI-). Table 2.8 summaries media specification.

Table 2.8 Cell culture media formulations:

Components	IMDM 21056-023	DMEM 41965- 039	DMEM/F12 31331	RPMI+1640 72400- 021	RPMI-1640 31870- 025
Amino acids					
Glycine	+	+	+	+	+
L-Alanine	+		+		
L-Alanyl –L-Glutamine			+	+	
L-Arginine hydrochloride	+	+	+		
L-Arginine				+	+
L-Asparagine-H2O			+	+	
L-Asparagine	+			+	+
L-Aspartic acid	+		+	+	+
L-Cysteine hydrochloride			+		
L-Cystine 2HCL	+	+	+		
L-Cystine				+	+
L-Glutamic acid	+		+	+	+
L-Glutamine	+	+			
L-Histidine hydrochloride H2o	+	+	+		
L-Histidine				+	+
L-Hydroxyproline				+	+
L-Isoleucine	+	+	+	+	+
L-Leucine	+	+	+	+	+
L-Lysine hydrochloride	+	+	+	+	+
L-Methionine	+	+	+	+	+

L-Phenylalanine	+	+	+	+	+
L-Proline	+		+	+	+
L-Serine	+	+	+	+	+
L-Threonine	+	+	+	+	+
L-Tryptophan	+	+	+	+	+
L-Tyrosine disodium salt	+		+		
L-Tyrosine		+		+	+
L-Valine	+	+	+	+	+
Vitamins					
Biotin	+		+	+	+
Choline chloride	+	+	+	+	+
D-Calcium pantothenate	+	+	+	+	+
Folic acid	+	+	+	+	+
Niacinamide	+	+	+	+	+
Para-Aminobenzoic Acid				+	+
Pyridoxal hydrochloride	+	+	+	+	+
Riboflavin	+	+	+	+	+
Thiamine hydrochloride	+	+	+	+	+
Vitamin B12	+		+	+	+
i-Inositol	+	+	+	+	+
Inorganic Salts					
Calcium chloride	+	+	+		
Calcium nitrate				+	+
Cupric sulfate			+		

Ferric Nitrate		+	+		
Ferric sulfate			+		
Magnesium Chloride			+		
Magnesium Sulfate	+	+	+	+	+
Potassium Chloride	+	+	+	+	+
Potassium Nitrate	+				
Sodium Bicarbonate	+	+	+	+	+
Sodium Chloride	+	+	+	+	+
Sodium Phosphate monobasic	+	+	+		
Sodium Phosphate dibasic			+	+	+
Sodium Selenite	+				
Zinc sulfate			+		
Other components					
D-Glucose	+	+	+	+	+
Glutathione				+	+
HEPES	+			+	
Sodium Pyruvate	+				
Phenol Red		+	+	+	+
Hypoxanthine Na			+		
Linoleic Acid			+		
Lipoic Acid			+		
Putrescine 2HCL			+		

2.6 Agar

2.6.1 TSB agar plates

Thirty grams of TSB powder and 100g agar (1%) was added to 1000 ml of dH_2O, mixed together, and then autoclaved at 121°C for 20 minutes. The agar was cooled to around 50°C, then poured into sterile plates and placed stored inverted at 4°C.

2.6.2 Columbia blood agar with horse blood

Ready-made blood agar plates were used in this study (Oxoid).

2.7 Buffers and Stains

2.7.1 Phosphate buffered saline (PBS)

Eight grams of sodium chloride (NaCl), 0.2g of potassium chloride (KCl), 1.44g sodium phosphate (Na_2HPO_4) and 0.24g potassium phosphate (KH_2PO_4) were mixed with 1L dH_2O before autoclaving and storage at room temperature.

2.7.2 1M Tris-HCl, PH=7.5

Prepared by adding 2.36g of Trisbase, 12.7 g Tris-HCl to 89ml dH_2O, then autoclaved and stored at room temperature.

2.7.3 Sodium Phosphate buffer pH=7.5

Two solutions were prepared separately and then combined:

Stock solution A; 2M monobasic sodium phosphate / sodium dihydrogen $NaH_2PO_4.2H_2O$, 78.005g was added to 250 ml of dH_2O

Stock solution B;2M dibasic sodium phosphate /disodium hydrogen $Na_2HPO_4.12H_2O$, 179.07g was added to 250 ml dH_2O

Then 16ml of solution A was mixed with 84 ml of solution B and corrected to pH =7.5, then diluted to 200ml to give a 1M phosphate buffer.

2.7.4 ELISA wash buffer

Prepared by adding 500µl Tween 20 and 8gNaCl to 500ml PBS.

2.7.5 RBC Lysis Buffer pH=7.5

Prepared by dissolving 4.15g Ammonium Chloride in 50ml 0.1M Tris HCL. The solution was then adjusted to 500ml with dH_2O.

2.7. 6 Reagent diluent

Prepared by dissolving 0.1% BSA and 0.05% Tween 20 in Tris-buffer saline, pH 7.2-7.4.

2.7.7 Triton 1%

One ml of Triton X-100 was added to 100ml of PBS and then filtered sterilised.

2.7.8 Phenol

Five milliliters of phenol was dissolved in 95ml dH_2O to generate a 5% phenol solution.

2.7.9 Bouin´s fixative

In a fume hood, 7.5ml of picric acid, 2.5ml of 40% paraformaldehyde, and 0.5ml of concentrated glacial acetic acid were mixed together and the fixative used immediately.

2.7.10 Crystal violet

Prepared by adding 0.1g of crystal violet to 100ml dH_2O. The stored bottle was covered with foil to avoid light exposure.

2.8 Microbiological Methods

2.8.1 Biofilm assay

A single bacterial colony was picked from a blood plate and suspended in 5 ml of TSB broth and then incubated at 37°C for 18 hours with shaking at 200rpm. Subsequently pre-culture was diluted (1:100) with fresh TSB, and 200µl aliquoted into each well of NUNC 96-well plate. The plate was incubated for 18 hours at 37°C without shaking. After incubation the media was carefully removed and the wells washed (X3) with 200µl PBS. In a fume hood, 150µl of Bouin´s fixative was added to each well and incubated for 15 minutes. The Bouin´s fixative was removed and the wells washed once with PBS and left to air dry. Adherent biofilms were then stained with 150µl of crystal violet for 5 minutes, and washed (X5) under running tap water and left it to air dry. The

optical density of biofilms was measured at 570 nm using an Omega Fluo Star plate reader.

2.8.2 Determination of bacterial growth

A single bacterial colony was picked from a blood plate and suspended in 5 ml of TSB broth and then incubated for 18 hours. The pre-culture was diluted (1:100) with fresh TSB or cell culture media (IMDM, DEMEM/F12, and RPMI-) and 200µl aliquoted into a 96-well plate and incubated for 18 hours at 37°C. The optical density of each well was then determined using an Omega stare plate reader at 600nm.

2.8.3 Determination of viable counts (colony forming units)

Experimental samples (whether in TSB, cell culture media, blood or serum) were diluted 50:50 in 0.2% triton and pipetted gently for 10 seconds. Serial dilutions ranging from 10^1-10^6 (depending on specific experiment) were generated. A 50µl volume of the final 2 dilutions were spread using a sterile disposable spreader on a TSB agar plate in duplicate. Plates were incubated overnight at 37°C and the viable count then determined by enumerating visible colonies.

2.8.4 Preparation of crude overnight culture supernatants

Overnight cultures of *S. epidemidis* 1457 and its isogeic mutant *S. epidermidis* 1457-M10 in IMDM were sonicated twice at 70% power for 10 seconds, centrifuged at 4000rpm for 30 minutes. The supernatant was then decanted and filtered with 0.45µm syringe filter.

2.8.5 Bradford protein assay

The Bradford assay (Bradford 1976; Redinbaugh and Campbell 1985) was done using bovine serum albumin (BSA) as a standard protein. One hundred microlitres of Bradford Reagent was added to each well of a 96 well plate pre-coated with different concentration of pooled human serum. The preparation was incubated for 5 minutes and absorbance read on a plate reader at OD= 595 nm.

2.9 Polysaccharide intercellular adhesin (PIA): Purification and conformation assays

2.9.1 Purification of PIA from *S. epidermidis* 1457

Five milliliters of IMDM was inoculated with one colony of *S. epidermidis* 1457 and incubated overnight (18 hours) at 37°C with shaking at 200rpm (Pre-culture). On the next day 20ml volume of sterile IMDM was then added to each of 24 sterile NUNC cell culture plates. To each plate 200µl of the pre-culture was then added. Plates were incubated for 48 hours without shaking at 37°C. Biofilms were removed from the plates using a sterile cell scraper and decanted into centrifuge tubes. Bacteria were sedimented by centrifugation at 6000rpm (JA 8.1000 rotor) for 30min in two 1L bottles, the medium was discarded by inverting the bottles (<30 min to prevent drying out). The cell pellets were re-suspended in a combined volume of 20 ml of 50mM Sodium Phosphate Buffer (pH=7.5) in a 50 ml centrifuge tube and sonicated (X4) on ice with consecutive bursts at intervals of 30s at 70% power (Branson Digital Sonifier). Bacteria were sedimented at 4000 rpm for 15 min and the supernatant transferred (containing PIA) to a 50ml tube. Supernatant was aliquoted to 2 ml microcentrifuge tubes and any remaining bacteria / debris were sedimented by centrifugation at 12000rpm for 5 min in a minifuge, prior to filter sterilization (0.22µm).

The PIA supernatant was concentrated 5 fold by using centrifugal filter units millipore (Amicon Ultra) by centrifugation at 3000 rpm in 10 min intervals. It was found that 5ml of PIA preparation took 10 minutes to run through the amicon. Then two Q-Sepharose anionic exchange columns were prepared by adding filter disks to two PD-10 columns and a packed bed of 7 cm was generated.

The columns were equilibrated with at least 20ml of 50mM Sodium Phosphate buffer (pH=7.5). A 2 ml PIA preparation was gently applied and allowed to flow into each column. Fifteen consecutive 2ml fractions were then taken by washing successive 2ml fractions of 50mM sodium phosphate buffer through the column. PIA fragment 1 does not bind to this column so will elute in fractions following the void volume (V_0). Experiments demonstrated PIA was in fractions 3-7 giving 5 fractions per column in a volume of 20ml.

2.9.2 PIA confirmatory assay

PIA confirmatory assays were done to test all the primary fractions before testing pooled fractions with higher titers for PIA. Assays included: PIA ELISA, Co-agglutination assay, Hexosamine assay and Hexose assay.

2.9.2.1 PIA ELISA

2.9.2.1.1 Optimization of PIA ELISA

PIA ELISA confirmatory assay performed on the pooled fractions of two preparation (P1 and P2) of PIA obtained from growing of *S. epidermidis* 1457 (PIA/biofilm+) in TSB and one preparation of *S. epidemidis* 1457-M10 (PIA/biofilm-) which used as negative control (NC). However the ELISA readings were high even in the negative control extract and so an attempt to optimize PIA ELISA was performed by changing the block (Bovine serum albumin (BSA) is the usual block) and the concentration of the primary antibody (anti-PIA antibody usual dilution 1:800 in PBS) and the dilution of the secondary antibodies (anti-rabbit IgG HRP antibody usual dilution 1:2000 in PBS).

Optimization of the block

One hundred microlitres per well of 4% BSA in PBS was the usual block for PIA ELISA. However different types of block were investigated including dried Milk and Fetal bovine serum (FBS) in addition to BSA. BSA, milk and FBS were used at 4%. Pooled fractions of PIA were obtained from growing *S. epidermidis* 1457 (PIA/biofilm+) in TSB diluted 1:500, the negative control extract (NC) obtained from *S. epidermidis* 1457-M10 (PIA/biofilm-) was used with the same dilution. The best block was still 4% BSA in compared to milk and FBS as shown in Table 2.9, BSA shows the highest reading in fractions 3-7 in compare to milk and FBS when read by the microplate reader.

Table 2.9 The PIA ELISA blocked with BSA, milk and FBS.

BSA

Fractions	1457 (P1)/nm	1457 (P2)/nm	1457-M10/nm
1-2	0.452	0.368	0.355
3-7	1.276	0.974	0.369
8-10	0.442	0.37	0.395
11-15	0.379	0.394	0.452

Milk

Fractions	1457 (P1)/nm	1457 (P2)/nm	1457-M10/nm
1-2	0.363	0.334	0.291
3-7	0.823	0.584	0.296
8-10	0.331	0.305	0.335
11-15	0.319	0.317	0.371

FBS

Fractions	1457 (P1)/nm	1457 (P2)/nm	1457-M10/nm
1-2	0.314	0.37	0.328
3-7	1.241	0.821	0.36
8-10	0.406	0.343	0.422
11-15	0.373	0.379	0.445

Optimization of PIA ELISA antibody conditions

ELISA optimization was further investigated by adjusting detergent and secondary antibody concentration. The ELISA plate was prepared as before with samples diluted 1:500, blocked with 4% BSA and treated with three different detergent concentrations; PIA without Tween, PIA with 0.05% Tween and PIA with 2% Tween. Furthermore, secondary antibody dilutions of anti-rabbit antibody were investigated from 1/2000 to 1/5000. The 0.05% and 2% Tween had a mild inhibitory effect on the readings of the PIA extract and NC extract by the plate reader. Increasing the dilution of secondary anti body (anti rabbit) to 1/5000 decreased the reading of the extract and thus the background signal (that can be seen in the second column) as in Table 2.10.

Table 2.10 Optimization of detergent and secondary antibody concentration

1457/P1 fraction	PIA without Tween/nm	PIA 0.05% Tween/nm	PIA 2% Tween/nm
1-2	0.225	0.178	0.172
3-7	1.677	1.586	1.454
8-10	0.399	0.332	0.275
11-15	0.286	0.192	0.184

1457/P2 fractions	PIA without Tween/nm	PIA 0.05% Tween/nm	PIA 2% Tween/nm
1-2	0.168	0.186	0.195
3-7	1.12	1.248	1.231
8-10	0.151	0.182	0.178
11-15	0.181	0.183	0.187

1457-M10 fractions	PIA without Tween/nm	PIA 0.05% Tween/nm	PIA 2% Tween/nm
1-2	0.222	0.198	0.171
3-7	0.19	0.214	0.119
8-10	0.249	0.226	0.218
11-15	0.215	0.192	0.192

Optimization of primary anti-PIA antibody

In order to define the optimal concentrations of anti-PIA antibody for use in the ELISA, various dilutions of anti-PIA antibody were added to PIA ELISA plate whilst maintaining all other condition constant; i.e. blocking with 4% BSA, use of anti-rabbit antibody diluted 1/5000 and the test samples diluted 1:500.

The 1/8000 dilution of anti-PIA antibody proved the optimal dilution; as the reading were high in the 3-7 fractions (3rd column) which should contain PIA extract obtained from *S. epidermidis* 1457 whilst the readings were low in all negative control extracts (NC) as in Table 2.11.

Table 2.11 Optimization of anti PIA antibody concentration

Dilution of primary antibody	Fractions 1-2 /PIA nm	Fractions 3-7/ PIA nm	Fractions 8-10/PIA nm	Fractions 1-2/ NC nm	Fractions 3-7/ NC nm	Fractions 8-10/ NC nm
1/1000	1.882	2.857	2.473	2.317	2.113	2.383
1/2000	1.29	3.255	1.279	1.172	2.277	1.348
1/4000	0.596	2.483	0.639	0.577	0.762	0.665
1/8000	0.304	1.308	0.295	0.287	0.281	0.277
1/16000	0.15	0.677	0.155	0.132	0.141	0.14
1/32000	0.083	0.404	0.083	0.084	0.087	0.084
1/64000	0.05	0.199	0.052	0.05	0.053	0.054
1/128000	0.048	0.143	0.048	0.049	0.05	0.044

2.9.2.1.2 Optimized PIA ELISA

Fifty microlitres of sample (PIA extract and NC extract) was added to a 96-well flat bottom half area plate and incubated over night at 4° C. This was then washed 3 times with 120 µl of ELISA wash buffer. Then 100 µl of BSA (4%) in PBS was added and incubated for 1h. The preparation was washed 3 times with 120 µl buffer before 50 µl of primary rabbit anti-PIA antibody (diluted 1/8000 in PBS) was added and incubated for 1h. Plates were washed 3 times with 120 µl buffer, before 50 µl of secondary antibody (anti-rabbit HRP diluted 1/5000 in PBS) was added and incubated for 1h. Plates were washed 3 times with 120 µl buffer, before 50µl of TMB substrate was added. The plate was incubated for up to 30 minutes in the dark and then 50µl of stop solution (1M H_2SO_4) was added before the plate was read at OD_{450}nm by the Omega plate reader.

2.9.2.2 Detection of PIA by coagglutination in bacterial extracts

Preparation of *S. aureus* Cowan I for coagglutination

A bacterial colony of *S. aureus* Cowan I was inoculated into 5ml TSB and allowed to grow overnight. A 5 ml volume of this pre-culture was then inoculated into 800ml of TSB and incubated for 20h at 37°C with shaking. Bacterial cells were sedimented by centrifugation at 6000rpm for 30min in 1L bottles (JA 8.1000 rotor) and the pellets washed twice in PBS containing 0.05% NaN_3. The wet weight of the cell pellet (approximately 5.5g) as suspended to a density of 10% (w/v) in the same buffer.

Formaldehyde was added to a final concentration of 1.5% and cells were gently mixed on a magnetic stirrer for 90 minutes at room temperature. Cells were sedimented, by centrifugation at 4000rpm and suspended in the same volume of buffer as described above, before being transferred to a glass beaker. The cell suspension was heated in a water bath at 80°C for 5 min before being cooled rapidly in an ice bath. Cells were washed twice in PBS containing 0.05% (w/v) NaN_3, and weighed. A 3 gram pellet was suspended in the same buffer at a concentration of 10% (w/v). Suspensions were stored in 1 ml volumes at -80° until use.

Preparation of coagglutination reagent

On milliliter of *S. aureus* Cowan I suspension was centrifuged at 12000 rpm for 5 minutes and the pellet washed twice in PBS containing 0.05%(w/v) NaN_3 and re-suspended in 900 µl of the same buffer. Rabbit anti-PIA antiserum (100µl) (Mack, Siemssen et al., 1992) was added and incubated at room temperature for 15 min with occasional mixing. Then the sensitized *S. aureus* Cowan I cells were sedimented in a microcentrifuge (13000 rpm for 1 min) and washed three times with PBS containing 0.05% (w/v) NaN_3. Finally, the sensitized cells were re-suspended in 10 ml of PBS containing 0.05% (w/v) NaN_3 and stored at 4°C for a maximum of 2 weeks.

Detection of PIA by coagglutination in bacterial extracts

Five microliters of purified PIA fractions were mixed with 15 µl of coagglutination reagent on microscope slides. One standard microscope slide allowed three different samples to be tested simultaneously. Then the slides were inverted back and forth for 3 minutes and agglutination evaluated in bright light against a dark background. Controls included bacterial extracts from PIA negative strains (e.g. *S. epidermidis* 1457-M10) and PBS. These were used to evaluate the cutoff point for positive coagulation. Quantitation of antigen in bacterial extracts was performed by analysis of serial 2–fold dilutions of the respective extracts in PBS and the antigen titers were defined as the highest dilution displaying a positive coagulation test.

2.9.2.3 Hexosamine assay (Smith and Gilkerson 1979)

N-acetylglucosamine used as a positive control in concentration of 1000µg/ml, then a 1:2 serial dilution done to create a standard curve. Two hundred microliters of standard samples and purified PIA were decanted to small glass bottles with lids before adding 200 µl of 0.5M HCl. Bottles were vortexed and heated to 110°C for 2 h. Samples were then cooled to room temperature in a water bath. Within a fume hood, 400 µl of 2.5% $NaNO_2$ was added, vortexed and allowed to stand at room temperature for 15 minutes. Following this, 200 µl of 12.5% ammonium sulfamate was added and the bottles vortexed, and allowed to stand at room temperature for 5 minutes, to allow the liberation of excess $NaNO_2$. Then 200µl of 0.25% 3-Methyl-2-benzothiazolonehydrazone hydrochloride (MBTH) was added and the bottles, vortexed before incubating at 37°C for 30 minutes. Finally 200 µl of 0.5% $FeCl_3$ was added and incubated at 37°C for an additional 5 minutes, before allowing the samples to cool to room temperature. The optical density of the solutions was determined at 650 nm in an Omega plate reader.

2.9.2.4 Hexose assay

Two hundred microliters of samples, glucose as standard (containing up to 100µg carbohydrates) or control solutions were mixed with 200µl of 5% phenol. Then, 1 ml of concentrated sulphuric acid was rapidly added directly to the solution surface without touching the sides of the tube. Solutions were left undisturbed for 10 minutes before shaking vigorously and further incubated for 30 minutes before the determination of absorbance at 490nm.

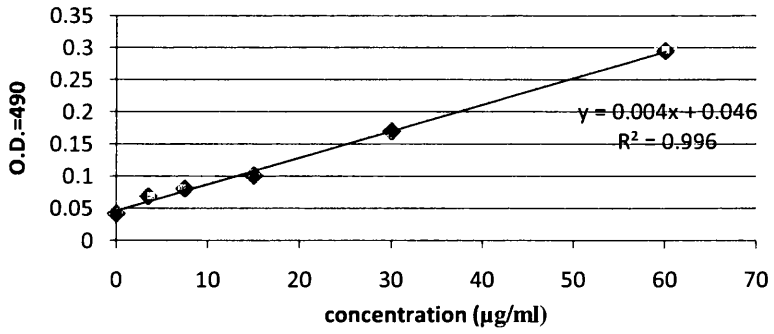


Figure 2.1 Standard curve of Hexose assay.

2.9.2.5 Lipopolysaccharide (LPS) detection assay

The Pierce LAL Chromogenic Endotoxin Quantitation kit was used to measure the amount of lipopolysaccharide (LPS) in the purified PIA samples.

The kit contains the following reagents: *Escherichia coli* endotoxin standard, lyophilized, reconstituted in 1ml of endotoxin-free water provided with the kit to working concentrations of 15-40 EU/ml (one endotoxin unit/mL (EU/mL) equals approximately 0.1ng endotoxin/mL of solution). *Limulus* Amebocyte Lysate (LAL), lyophilized, 2 vials, reconstituted immediately before use with 1.4mL/vial of endotoxin-free water. Chromogenic substrate contains ~7mg of lyophilized substrate, 1 vial, reconstituted with 6.5ml/vial of endotoxin-free water to yield a final concentration of ~2mM. The standard stock solutions prepared from the endotoxin standard were with final endotoxin concentration (1.0, 0.5, 0.25, 0.1) EU/ml.

All reagents equilibrated to room temperature before use, and the microplate equilibrated in a heating block for 10 minutes at 37°C. A 50µL of each standard or sample was added in replicate into the microplate well, the plate covered with the lid and incubated for 5 minutes at 37°C. Then 50µL of LAL was added to each well, plate covered with the lid and gently shake the plate on a plate shaker for 10 seconds. The plate incubated at 37°C for 10 minutes. Then 100µL of substrate solution added to each well. The plate covered with lid and gently shakes on a plate mixer for 10 seconds. The plate incubated at 37°C for 6 minutes. Then 50µL of stop reagent (25% acetic acid) added. The plate gently shakes on a plate mixer for 10 seconds. Then absorbance measured at 405-410nm on a microplate reader.

2.10 Immunological methods

2.10.1 DuoSet Enzyme-linked immunosorbant assays (ELISA) development system

Six different human ELISAs were used in this study obtained from R and D system; these were specific for Human CXCL8/IL-8, Human IL-1β/IL-1F2, Human TNF-α, Human IL-10, Human complement component C5a, Human CCL17/TARC. Reagents for these ELISAS included:

Capture Antibodies: Mouse anti-human IL-8, IL-1β, and TNF-α (720µg/ml), IL-10, Thymus and activation-regulated chemokine (TARC) and C5a (360µg/ml). These were reconstituted in 1ml PBS and diluted to working concentrations of 4µg/ml (in PBS) for IL-8, IL-1β, and TNF-α, 2µg/ml (in PBS) for IL-10 and TARC and 1µg/ml (in PBS) for C5a.

Detection Antibodies: the following biotinylated detection antibodies were used in the ELISA: goat anti-human IL-8 (3.6µg/ml), goat anti-human IL-1β (36µg/ml), goat anti-human TNF-α (45µg/ml), goat anti-human IL-10 (27µg/ml), goat anti-human TARC (18µg/ml) and mouse anti-human C5a (360µg/ml). Secondary antibodies were all reconstituted in reagent diluents provided by the manufacture.

Standards: The following recombinant human standards used in the ELISAS: recombinant human IL-8 (110ng/ml), recombinant human IL-1β (75ng/ml), recombinant human TNF-α (320ng/ml), recombinant human IL-10 (210ng/ml), recombinant human TARC (110ng/ml), and recombinant human C5a (130ng/ml) when reconstituted with 0.5ml reagent diluent. Standard curves had seven points using 2-fold serial dilutions in reagent diluent with a high standard of 2000pg/ml for IL-8 and IL-10, 250pg/ml for IL-1β, and 1000pg/ml for TNF-α and 2000pg/ml for C5a.

Streptavidin-HRP was diluted 1:200 to the working concentration specified on the vial using reagent diluent.

General method- Fifty-µl of capture antibody previously diluted to the working concentration in PBS was added to wells of a 96-well microtiter plate (ELISA half plate). The plate was sealed with parafilm and incubated overnight at room temperature. Each well was then washed 3 times with 200µl wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4) and remaining wash buffer was then removed by inverting the plate and blotting it against clean paper towels. To each well, 150µl of block buffer (1% BSA, in PBS with 0.05% NaN_3) was added and the plate incubated at room temperature for a minimum of 1 hour. The plate was then washed as before. A 50µl volume of samples and standards were then added to appropriate wells, the plate was then covered with parafilm and incubated for 2 hours at room temperature. The plate was washed as before prior to adding 50µl of the detection antibody to each well. The plate was again covered with parafilm and incubated for 2 hours. The plate was washed prior to adding 50µl of the working dilution of Streptavidin-HRP to each well. The plate was covered and incubated for 20 minutes in the dark, washed and 50µl of Substrate Solution (1-component TMB) added to each well and incubated for 20 minutes at room temperature in the dark. Finally, 25µl of stop solution (HCl 44ml water + 4ml 37% HCl) was added to each well and the optical density at 450nm of each well determined using a microplate reader.

2.10.2 RayBio Human inflammation Antibody Array 3

The RayBiotech system which is able to detect the expression of 42 cytokines in samples simultaneously was also used to detect specific cytokines in this study. The cytokines antibody array 3 map showed in figure 2.2 . Methodology for this system is described below.

Blocking and Incubation

Sample from THP-1 cells in RPMI- media were diluted (1:5) with 1X Blocking Buffer. Serum samples were diluted 1:10 in 1X Blocking Buffer. All membranes were placed in an eight-well tray (one/well), which was handled using sterile forceps. Membranes were inspected for the (-) mark to ensure correct antibodies orientation prior to being completely immersed with 2ml 1X Blocking Buffer. The preparation was then incubated at room temperatures for 30 minutes. After removing Blocking Buffer, membranes were

incubated with 1ml of samples at room temperature for 1-2 hours. Samples were decanted from each container and washed (X3) with 2ml of 1X Wash Buffer I for 5 minutes with shaking. This was followed by 2 washes with 2 ml of 1X Wash Buffer II, each time for 5 minutes with shaking. The working solution for primary antibody was prepared by adding 100µl of 1X blocking buffer to the Biotin-Conjugated Anti-Cytokines tubes. The preparation was mixed and transferred to a tube containing 2ml of 1X blocking buffer. One milliliter of diluted biotin—conjugated antibodies was added to each membrane and incubated overnight at 4°C. The membranes were then washed 3 times with wash buffer I and twice with wash buffer II. A 2ml volume of 1,000 fold diluted HRP-conjugated streptavidin (prepared by adding 2µl of HRP-conjugated streptavidin concentrate to 1998µl 1X Blocking Buffer) was added to each membrane, and incubated at room temperature for 2 hours. Membranes were washed 3 times with wash buffer I and twice with wash buffer II as before.

Detection

Membranes were drained of excess wash buffer and placed on a clean plastic sheet. Detection Buffer (250µl of 1X Detection Buffer C and 250µl of 1X Detection Buffer D for one membrane) was added to the membrane which was then incubated at room temperature for 2 minute. Any excess detection reagent was drained off by holding the membrane vertically with a forceps and touching the edge against a tissue. The membrane was then placed protein side up, on a piece of plastic sheet and covered with another plastic sheet.

Signals from the membrane were detected using a chemiluminescence imaging system (Gel Doc with Quantity One software).

Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α	I-309	IL-1α	IL-1β
Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α	I-309	IL-1α	IL-1β
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12 p40p70	IL-13	IL-15	IFN-γ
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12 p40p70	IL-13	IL-15	IFN-γ
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1
TNF-α	TNF-β	EGF	IGF-I	Angiogenin	Oncostatin M	Thrombopoietin	VEGF	PDGF BB	Leptin	Neg	Pos
TNF-α	TNF-β	EGF	IGF-I	Angiogenin	Oncostatin M	Thrombopoietin	VEGF	PDGF BB	Leptin	Neg	Pos

Figure2.2 RayBio Human cytokine antibody array 3 map.

2.11 Cell culture

2.11.1 Culture of THP-1 cells

The human Acute Monocytic Leukemia Cell Line, THP-1, was used in this study. Cells were cultured in RPMI- supplemented with 10% FBS, 1% penicillin/streptomycin (P/S) and 1% L-Glutamine, and incubated in a 5% CO_2 incubator at 37˚C.

2.11.2 Thawing stocks of frozen THP-1 cells

Frozen stocks were thawed rapidly at 37ºC and transferred to a 50ml centrifuge tube containing 30 ml of warm RPMI- media with 1% L-glutamine, 10% FBS and 1% P/S. The suspension was mixed gently and centrifuged at 350g for 5 minutes. The supernatant was decanted and the washing step repeated again to remove cell debris. Cell pellet was re-suspended in 15 ml fresh medium and the cells incubated in a 5% CO_2 incubator at 37˚C in a NUNC cell culture dish.

2.11.3 Sub-culturing of THP-1 cell

THP-1 cells were sub-cultured every 3-4 days by gently lifting cells using a sterile cell scraper. Cells were then transferred to a 50ml centrifuge tube and centrifugated at 350g for 5 minutes and the supernatant decanted. Cells were resuspended gently by flicking the tube before 5ml of fresh media was added to the cell suspension. Cell viability was examined by using a Naubeuer Haemocytometer to calculate the number of the viable cell. To determine cell viability, 10µl of cell suspension was mixed with 10µl of Trypan Blue and added to the haemocytometer before visualising under a light microscope. Viable cell concentrations were estimated by multiplying cell counts by 20,000 and the dead cell percentage was calculated by counting clear and blue cells. Cells were seeded at a density 2-3x10^5/ml viable cell in each dish with 15 ml of cell culture media and then incubated in 5% CO_2 incubator at 37˚C.

2.11.4 THP-1 cell killing assay

An overnight culture of *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 was re-suspended to an OD595 = 0.1. A 1:1000 dilution (~1 x 10^4/ml) of these suspensions was made into 3% FBS /IMDM. To 1ml volume of these bacterial suspensions 2x10^6 THP-1 monocyte macrophages was added and incubated for 2 hours. Control cultures at time zero and those without macrophages were included. At defined time points, interaction mixes were diluted 1:1 with 0.2% triton and mixed by pipetting to digest macrophages. Dilutions were plated onto TSB agar and incubated overnight at 37°C. Colonies were counted and the number used to estimate remaining viable bacteria.

2.11.5 Stimulation of THP-1 cells with bacterial products

2.11.5.1 Cytokine expression in response to challenge with *S. epidermidis* culture supernatant

Various dilutions of crude supernatant (1:5000, 1:500, 1:10 and 1:2.5) µl/ml were added to 2x10^5 THP-1 cells/500µl of RPMI- containing 10% FBS and 1% L-glutamine and P/S. Controls included a time zero sample, a sample containing PBS in place of supernatant and finally a sample containing phorbol myristate acetate (PMA) instead of supernatant. Supernatants were collected at 6 and 24 hours and ELISA performed as described in section 2.10.1.

2.11.5.2 Cytokine expression in response to PIA challenge

Increasing concentrations of PIA extract (0.5, 1.5, 12, 30, 60µg/ml) were added to 2x10^5 THP-1 cells in 500µl RPMI- containing 1% L-glutamine and 1% P/S and different FBS concentration (10%, 3%, 1%). Controls were a time zero sample, a sample with sodium phosphate buffer or PMA instead of PIA extract. The plates were incubated at 37°C in 5% CO_2 for 6 and 24 hours, before the media was aspirated and centrifuged at 2000rpm for 5 minutes. Supernatants were decanted and stored at -20°C until use. Samples were analyzed by ELISA as described in section 2.10.1.

2.11.5.3 Cytokine expression in response to *S. epidemidis* 1457 biofilm

Biofilms were prepared in a 24 cell culture plate using a modification to the method previously outlined in section 2.8.1. After incubation TSB media was removed and the biofilm washed three times with PBS. IMDM containing $5x10^5$ THP-1 cells, 10% FBS and 1% LG was then added to each well prior to addition of increasing concentrations of Dispersin B (Disp.B) (0.001, 0.01, 0.1, 1µg/ml). Plates were incubated at 37°C and 5% CO_2 for 6 hours, before the media was aspirated and centrifuged at 2000rpm for 5 minutes. The supernatant was decanted and kept frozen at -20°C prior to further use. Samples were analyzed for cytokine production by ELISA as described in section 2.10.1.

2.11.5.4 Cytokine expression in response to planktonic *S. epidermidis* bacteria.

Overnight culture of *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 were prepared in TSB. After incubation, bacterial suspensions with OD_{600}nm=1 were prepared in IMDM. A 10µl volume of this suspension was then added to 500µl of IMDM containing 10%. FBS and 1% LG and THP-1 at $5x10^5$/well in a 24 well cell culture plate. PMA (100nM) was used as a positive control. Cultures were incubated at 37°C with 5% CO_2 for 6 hours. The media was aspirated and centrifuged at 2000rpm for 5 minutes before the supernatant was decanted and frozen at -20°C until use. Samples were analyzed for cytokine production by ELISA as described in section 2.10.1.

2.12 Whole blood methods

2.12.1 Whole blood isolation

Whole blood was collected on the day of the experiment from healthy volunters using the vacutte blood collection system with sodium heparin (5ml-9ml). Volunteers gave their consent and the work is assessed by the local Human Tissue Act committee at College of Medicine, Swansea University.

2.12.2 Whole blood killing

One millilter of blood was added to a 1.5ml microcentrifuge tube. A 10µl volume of *S. epidermidis* 1457 or *S. epidermidis* 1457-M10 was added directly to blood. The bacterial dose used was 1:10,000 dilution of OD_{600}nm=1 of an overnight broth in TSB or IMDM. Blood with bacteria was incubated with rotation at 10rpm at 37˚C for different time points (1,2,3,6,and 24 hours) according to the experiment and viable counts assessed according to section 2.8.3. Remining whole blood was centrifuged at 12,000rpm for 5 min and the platelet poor plasma / serum removed and stored at -20°C prior to further analysis.

2.12.3 Pretreatment of whole blood with immunsuppresive agents

2.12.3.1 Pretreatment with dexamethasone

One millilitre of whole blood was placed into a 1.5ml microcentrifuge tube before adding different dilutions of Dexamethasone (1mg/ml) in 100% ethanol (1:4, 1:2500, 1:25000) and incubated overnight with rotation at 10rpm at 37°C. On the next day bacteria were added to blood as described in section 2.12.2. Whole blood was incubated with rotation (10rpm) at 37˚C for 6 hours and then and then viable counts assessed. Platelet poor plasma/serum was isolated as described in section 2.12.2.

2.12.3.2 Pretreatment with cytochalasin D

One millilitre of whole blood was placed in 1.5ml microcentrifuge tubes, and incubated with increasing concentration of Cytochalasin D (0.2µg/ml, 1µg/ml, 3µg/ml) for 15 minutes. Bacteria were added to blood as described in section 2.12.2. Whole blood was incubated with bacteria on rotation (10rpm) at 37˚C for different time points (3, 6,and 24

hours) according to our experiment and viable counts were then assessed. Platelet poor plasma/serum was isolated as described in section 2.12.2.

2.12.4 Whole blood killing with PIA Extract

One millilitre of whole blood was added to a 1.5ml microcentrifuge tube and incubated with purified PIA extract and *S. epidermidis* 1457-M10 extract at increasing concentration (10, 30, and 60µg/ml) for 3 hours before adding *S. epidermidis* 1457-M10, as described in section 2.12.2. Suspensions were incubated with rotation (10rpm) at 37˚C for 3 hours and viable counts assessed. Platelet poor plasma/serum was isolated as described in section 2.12.2.

2.12.5 Whole blood killing with antibody receptor blocking antibodies

One millilitre of whole blood was added to a 1.5ml microcentrifuge tube, and incubated the with anti-human CD11b, CD18 and CD16 and their Isotype (all at 2.5µg/ml) for 30 minutes. Then *S. epidermidis* 1457 and its isogenic mutant *S. epidermidis* 1457-M10, was added as described in section 2.12.2. Suspensions were incubated with rotation (10rpm) at 37˚C for 6 hours and viable counts assessed. Platelet poor plasma/serum was isolated as described in section 2.12.2.

2.13 Cytospin preparations

Cytospin preparations were used for phagocytosis and immunocytochemistry assays.

2.13.1 Sample preparation: Two hundred microliters of whole blood was added to 8ml polystyrene tubes and 3ml of RBC lysis buffer added. Samples were incubated for a maximum of 30 minutes at 37˚C. Tubes were centrifuged at 700g for 5 minutes, the supernatant discarded and 1ml of lysis buffer added. Suspensions were mixed and centrifuged as before. The supernatant was discarded and 100µl of PBS added to the tube.

2.13.2 Chamber preparation: A microscope slide was put into the cytospin clip and a filter card was positioned between the sample container and the slide before securing the stack in place. Then 70-120µl of sample was aliquoted to an upright cytochamber

prior to inserting it into the cytospin machine. Samples were centrifuged at 300rpm for 3 minutes prior to removing the filter paper and allowing slides to air dry.

2.14 Hemocolor staining procedure for Microscopy

Slides prepared by cytospin were stained to assess phagocytosis. All the staining was done in coplin jars by placing air dried slides in fixative (5 seconds), then blotting excess fixative on clean tissue paper. Slides were placed in red color reagent (3 seconds), before blotting removed of excess stain. Slides were then placed in blue colour reagent (6 seconds) prior to blotting removal of excess stain. Finally slides were placed in buffer solution (twice over10 seconds) and left to air dry.

2.15 Immunocytochemistry

Cytospin preperations were blocked with 200µl 1% BSA for 1 hour, then washed X3 with PBS. A 200µl volume of anti-rabbit PIA antiserum (diluted 1:50) was applied, the slides covered and after 30 minutes the slides washed X3 with PBS. Slides were then stained with fluorescein–conjugated anti-rabbit IgG (Alexa flour 488, diluted 1:100) and propidium iodide (PI) (diluted 1:1000) for 30 minutes. Slides were washed X3 wih PBS then 25µl of vectashield hardset was added. Preparation was overland with a coverslip and left in the fridge to harden.

2.16 Microscopy

2.16.1 Epifluorescent microscopy

Epifluorescent microscopy was used to examine the stained immunocytochemistry slides prepared with anti-PIA antibody, anti-Embp antibody and propidium iodide (PI) to detect the *S. epidermidis* biofilm formation in different physiological media and at different time points.

2.16.2 Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy was used for characterisation of *S. epidermidis* biofilm structure, charactarisation of *S. epidermidis* biofilm viability and migration of macrophages into preformed biofilm.

Four and 8 well chamber slides were used to stain both *S. epidermidis* 1457 and 5179-R1 strains. Then up to 500µl of a 1:100 dilution of an overnight suspension culture of each strain was aliquoted into each well of a chamber slides and incubated at 37°C overnight.

2.17 Fluorescent staining

Fluorescent assays were done to differentiate between bacteria and matrix in biofilms and to assess bacterial viability.

2.17.1 Fluorescent staining of biofilms

Bacterial biofilms were generated as described in section 2.8.1 and the cells were labeled by incubating with Syto 9 (20µM) for 60 minutes. The matrix was stained by incubating with rhodamine-conjugated wheat germ agglutinin (which binds to N-acetylglucosamine residues in PIA) (20µg/ml) for 60 minutes. Excess stain was removed by three gentle washes in PBS. Signal was detected using a plate reader or CSLM at excitation/ emission wavelengths of 485/498nm for Syto 9 and 555/580nm for rhodamine.

2.17.2 Live / dead cell staining assay

Biofilms were produced as described in section 2.8.1 and syto 9 staining was similar to the above with the exception of the last 5 minutes where propidium iodide (300ng/ml) was added. Biofilms were washed gently and then visualised using CSLM at excitation / emission wavelengths of 485/498nm for Syto 9 and 535/617nm for propidium iodide.

2.18 Software programs used in this study:

Table 2.12 Software programs used in this study

Program	Version	Manufacturer
Image J Image processing program	V. 1.45	National Institutes of Health
Axio vision for Fluorescent Microscope	V. 4.8	Carl Zeiss
LSM image browser for Laser Scanning Microscope	V. 4.2	Carl Zeiss
Quantity One for Gel Doc	V4.62	Bio-Rad
Microsoft Office word and power point	2007	Microsoft
Microsoft office Excel	2007	Microsoft
IBM SPSS Statistics	16.0 for Windows	IBM

2.19 Data evaluation and statistical analysis

All results were expressed as Mean ± standard error of the mean (SEM). To compare groups an ANOVA and T- test was used with $p<0.05$ being considered significant.

Chapter 3

Biofilm formation by *S. epidermidis* cultured in different physiological media

3.1 Introduction

Biofilm formation is the major virulence factor of *S. epidermidis* and plays an important role in protecting the bacterial cell from the killing effect of antimicrobial agents and the immune system (Fey and Olson, 2010). To achieve this, staphylococci limit antibiotic diffusion into the biofilm and restrict leukocyte penetration which attenuates the host immune response (Mack, Rohde et al., 2006; Kokare, Chakraborty et al., 2009). There are three main mechanisms of biofilm formation by *S. epidermidis*. These include production of polysaccharide intercellular adhesin (PIA) through the *icaADBC* locus (Mack, Haeder et al., 1996), production of accumulation associated protein (Aap) which is activated by proteolytic cleavage (Rohde, Burdelski et al., 2005) and production of extracellular matrix binding protein (Embp) which is an fibronectin-binding protein found on cell surface (Christner, Franke et al., 2010).

Recent studies have suggested that the host plays a major role in promoting biofilm phenotype in *S. epidermidis*. For instance, PIA expression and biofilm formation are subjected to phase variation induced by the insertion sequence element IS*256* which reversibly inactivates the *ica* operon (Ziebuhr, Krimmer et al., 1999; Conlon, Humphreys et al., 2004). Aap is activated by a host/bacterial protease enzyme (Rohde, Burdelski et al., 2005). Embp biofilms are induced by serum in biofilm negative *S. epidermidis* 1585 (Christner, Franke et al., 2010), which suggests that specific physiological conditions may promote the biofilm phenotype.

Studies investigating the nutritional requirements for *S. epidermidis* biofilm formation have confirmed the essential need for glucose which is routinely added to TSB (TSB/G+) when generating *S. epidermidis* biofilms *in vitro* (Mack, Siemssen et al., 1992; Dobinsky, Kiel et al., 2003). Defining a physiological medium that supports *S. epidermidis* growth and biofilm formation is essential for PIA production with few contaminations, and allows study host-PIA interaction (as addressed in chapter 5 and 6). Study investigated host-pathogens interaction, demonstrated an apoptotic changes in macrophages cultivated with Staphylococci in TSB medium (Thurlow, Hanke et al., 2011). The minimal nutritional requirement needed to generate a *S. epidermidis* biofilm has been identified in a chemically defined medium called Hussain-Hastings-White

modified medium (HHWm) in which basic components were glucose, 18 amino acids, two purines and six vitamins (Hussain, Hastings et al., 1991).

Less is known about the effect of physiological media on biofilm formation. The effect of cell culture media on *S. epidermidis* biofilm formation was investigated in this chapter. Cell culture media can be more reliable media compare to TSB, as it may reflect a more realistic picture of the environment in which *S. epidermidis* grows biofilm. Biofilm assays were performed in five different cell culture media, these were RPMI with glutamine (RPMI+), RPMI without glutamine (RPMI-), DMEM, DMEM/F12 and IMDM.

RPMI is a basic media that supports the growth of mammalian cells, it contains the reducing agent glutathione and high concentrations of the vitamins, inositol and choline, along with the vitamins biotin, vitamin B_{12} and para-Aminobenzoic acid (PABA) (Moore and Woods, 1976; Invitrogen RPMI, 2013). Two RPMI media were used so that the effect of glutamine (+/- Glutamine) on biofilm formation could be studied.

DMEM is a basal medium for mammalian cell culture which contains a high concentration of amino acids and vitamins. Also used was DMEM/F12 which contains a 1:1 mixture of DMEM and Ham's F-12 Nutrient Mixture (contains ingredients not found in other basal media; zinc, putrescine, hypoxanthine, and thymidine) (Dulbecco and Freeman, 1959; Rutzky and Pumper, 1974; Invitrogen DMEM, 2013). IMDM is a highly enriched media modified from DMEM media, lacking iron, and replaced by potassium nitrate and sodium selenite (Iscove and Melchers, 1978; Invitrogen IMDM, 2013).

In this study, eight strains of *S. epidermidis* were used to investigate biofilm formation under different physiological media, these were PIA positive *S. epidermidis* 1457 from central venous catheter infection and its isogenic mutant *S. epidermidis* 1457-M10 (non biofilm forming) that was produced by insertion of transposon Tn*917* at nucleotide 931 in the *icaA*gene of the *icaADBC*gene locus, in addition to two other PIA forming biofilm *S. epidermidis* 9142 and *S. epidermidis* 8400 which isolated from central venous catheter infection (Mack, Siemssen et al., 1992). *Staphylococcus epidermidis* 1457 was originally used to determine PIA structure through analysis of biofilm obtained by 1457 grown in TSB (Mack, Davies et al., 2009). Biofilm negative *S. epidermidis* 5179 (non Aap forming) isolate from CSF-shunt infection and biofilm positive *S. epidermidis* 5179-R1

(Aap forming biofilm) subpopulation of *S. epidermidis* 5179 also been used (Mack, Siemssen et al., 1992). In addition to wild type *S. epidermidis* 1585 which is a biofilm negative strain isolated from blood of a patient with an infected catheter, and an Embp positive biofilm forming *S. epidermidis* 1585-RA generated by culture through multiple passages (Christner, Franke et al. 2010).

In this chapter:

- The ability of specific cell culture media to support biofilm formation was studied.
- Nutritional differences between each biofilm mechanism were identified.
- The effect of human and bovine serum on biofilm formation was investigated.

3.2 Methods

To assess bacterial growth, an overnight (18h) pre-culture of *S. epidermidis* in TSB was diluted 1:100 and grown in microcentrifuge tubes for 18h at 37°C, with different concentrations of FBS and pooled human serum (PS) (0.1%, 0.3%, 1%, 3%, and 10%) in TSB, RPMI+, RPMI-, DMEM, DMEM/F12 and IMDM, the subsequent growth assessed by reading absorbance at OD_{600}nm. Each experiment was undertaken three times.

To assess serum effects on biofilm formation in different media, biofilm assays were undertaken as described before (section 2.8.1) in 96 well microtitre plates with different serum concentration (0.1%, 0.3%, 1%, 3%, and 10%) and without serum, by adding 200µl per well of a 1:100 dilution of pre-culture to TSB/G+, and different cell culture media including IMDM, DMEM, DMEM/F12, RPMI- and RPMI+. Each experiment was performed three times

The inhibitory effect of serum on biofilm formation further investigated by fractionating serum samples (FBS and PS) using amicon centrifugation to generate samples with a defined molecular weight ranges. Then the effect of these fractions on biofilm formation assess by performing biofilm assay (for each defined molecular weight ranges) as described in section 2.8.1 in 96 well microtitre plates with different serum concentration (0.1%, 0.3%, 1%, 3%, and 10%) and without serum. All experiments were performed independently 3 times.

Experiments were undertaken to assess the effect that pre-coating the wells of the microtiter plate with serum had on subsequent biofilm formation. In these studies wells were pre-coated overnight with 200µl of 10% FBS or PS at 37°C. After this period the coating solution was removed and the wells inoculated with *S. epidermidis* as previously described (section 2.8.1). After about 18 hours incubation, biofilms were washed X3 with PBS and fixed with Bouin's fixative, left to air dry, stained with crystal violet and the biofilm determined at OD_{570}nm, the experiment was performed three times.

Immunocytochemistry (ICC) performed to identify Embp expression in Embp biofilm positive *S. epidermidis* 1585-RA and biofilm negative *S. epidermidis* 1585 cultured in cell cultured media. Microscope slide was prepared as in section (2.13.2) using 100µl of sample from *S. epidermidis* 1585-RA and *S. epidermidis* 1585 incubated for 18h in cell culture media. Cytospin preperations were blocked with 200µl 1% BSA for 1 hour, then washed X3 with PBS. A 200µl volume of anti- Embp specific abbsorbed antiserum (diluted 1:50) was applied, the slides covered and after 30 minutes the slides washed X3 with PBS. Slides were then stained with fluorescein–conjugated anti-rabbit IgG (Alexa flour 488, diluted 1:100) for 30 minutes. Slides were washed X3 wih PBS then 25µl of vectashield hardset was added. Preparation was overland with a coverslip and left in the fridge to harden. Slides examined using epifluorecent microscopy.

Results

3.3 Screening of cell culture media supporting PIA dependent biofilm formation

In order to investigate the effect of cell culture media on *S. epidermidis* biofilm formation, biofilm assays were performed in five different cell culture media with three strains of *S. epidermidis* that produce PIA dependent biofilms including *S. epidermidis* 1457, 8400 and 9142. The cell culture media chosen were RPMI+, RPMI-, DMEM, DMEM/F12 and IMDM.

All PIA producing *S. epidermidis* generated large quantities of biofilm in TSB/G+ as determined by absorbance measurements (Figure 3.1). For cell culture media, it was found that *S. epidermidis* biofilm formation was specific to certain media. DMEM/F12 and IMDM were the best cell culture media in supporting biofilm formation by *S. epidermidis* 1457, ($P<0.05$) in comparison to other media. There was an advantage of growing *S. epidermidis* 1457 in RPMI without glutamine (RPMI-); as removing glutamine enhanced biofilm formation in comparison to RPMI with GlutaMax (RPMI+). Cell culture media did not support high level of biofilm formation by both *S. epidermidis* 8400 and *S. epidermidis* 9142 (Figure 3.1).

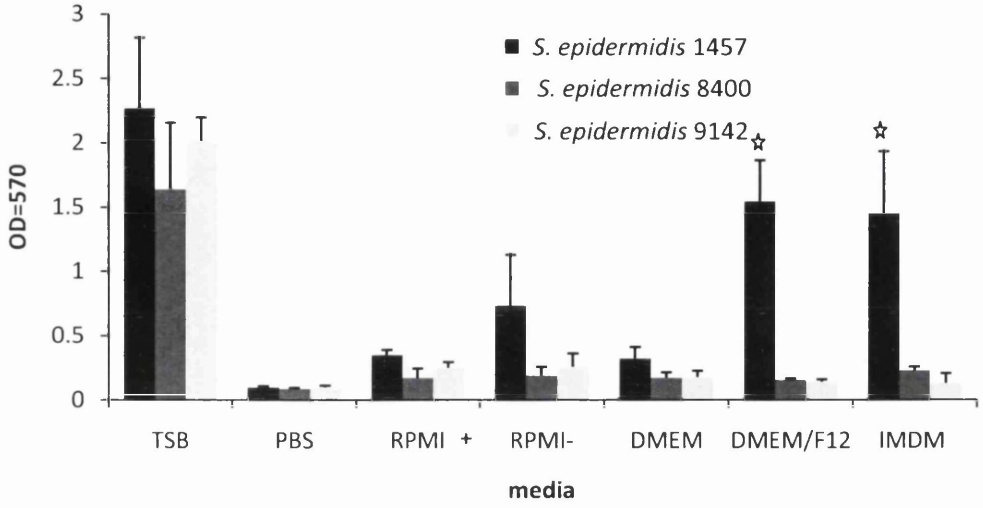


Figure 3.1 Screening of cell culture media supporting PIA dependent biofilm formation. The three PIA forming biofilm strains of *S. epidermidis* used were 1457, 8400 and 9142. These strains were cultured for 18h in five different cell culture media, namely RPMI+, RPMI-, DMEM, DMEM/F12 and IMDM. The columns represent the mean of three independent experiments. Stars indicate significant difference within groups *P<0.05.

3.4 Screening of cell culture media supporting Aap and Embp dependent biofilm formation

In order to investigate the effect of the five cell culture media previously mentioned on Aap and Embp dependent biofilms, biofilm assays were performed using *S. epidermidis* 5179-R1 (Aap dependent) and *S. epidermidis* 1585-RA (Embp dependent).

In TSB/G+ *S. epidermidis* 5179-R1 formed greater quantities of biofilm in comparison to *S. epidermidis* 1585-RA. RPMI with GlutaMax (RPMI+) was the best cell culture media to support biofilm formation by *S. epidermidis* 5179-R1. RPMI without L-glutamine (RPMI-) and IMDM media also supported biofilm formation by *S. epidermidis* 5179-R1, but to a lesser extent. *S. epidermidis* 1585-RA formed weaker biofilms, RPMI+, IMDM and DMEM/F12 media were also able to support biofilm formation (Figure 3.2).

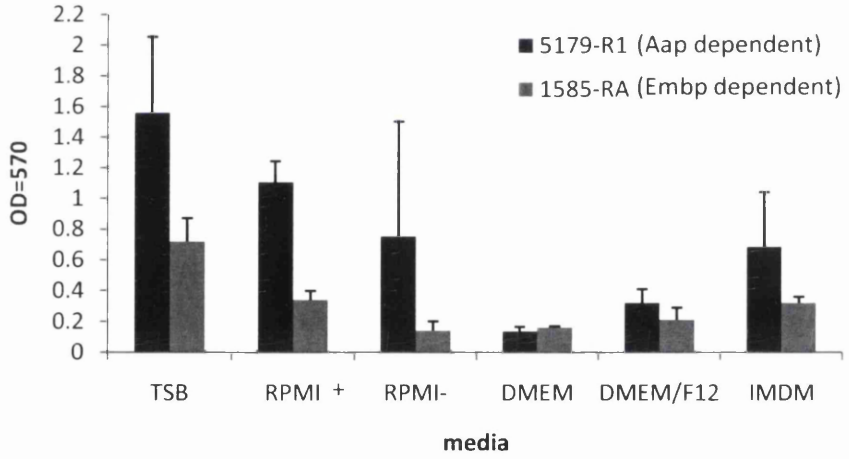


Figure 3.2 Screening of cell culture media supporting Aap and Embp dependent biofilm formation. *S. epidermidis* 5179-R1 (Aap dependent) and *S. epidermidis* 1585-RA (Embp dependent) were cultured for 18h in five different cell culture media namely, RPMI+, RPMI-, DMEM, DMEM/F12 and IMDM. The results represent mean of three independent experiments.

3.5 Screening of cell culture media inducing Embp biofilm formation in biofilm negative *S. epidermidis* 1585

To explore the ability of cell culture media to induce Embp biofilm formation in biofilm negative *S. epidermidis* 1585, biofilm assays were performed with TSB and also the five different cell culture media (RPMI+, RPMI-, DMEM, DMEM/F12 and IMDM).

RPMI+ induced biofilm formation by *S. epidermidis* 1585 ($P<0.05$) with an optical density reading of 0.56 followed by DMEM and IMDM with readings of 0.41 and 0.31 respectively (Figure 3.3). Importantly TSB did not induce biofilm formation by this strain.

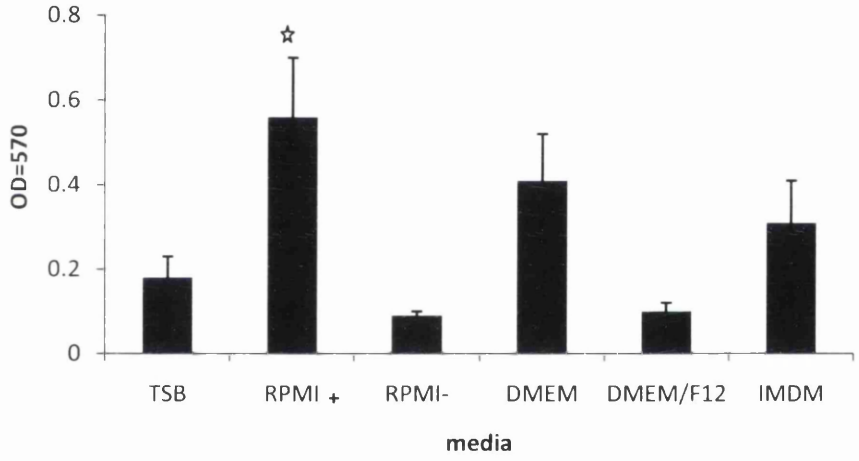


Figure 3.3 Screening of cell culture media inducing Embp biofilm formation in biofilm negative *S. epidermidis* strain 1585. The incubation was for 18 hours. The results represent mean of three independent experiments. Star indicate significant difference within groups *$P<0.05$.

To further investigate the Embp expression and confirm the Embp biofilm expression by biofilm negative *S. epidermidis* 1585 grown in cell culture media, an Immunocytochemistry (ICC) undertaken for both Embp biofilm positive *S. epidermidis* 1585-RA and Embp biofilm negative *S. epidermidis* 1585, using Embp specific absorbed antiserum for Embp detection.

ICC demonstrated Embp expression in *S. epidermidis* 1585 cultured in DMEM and RPMI+ (Figure 3.4 C and D) compared to Embp positive *S. epidermidis* 1585-RA (Figure 3.4 A and B).

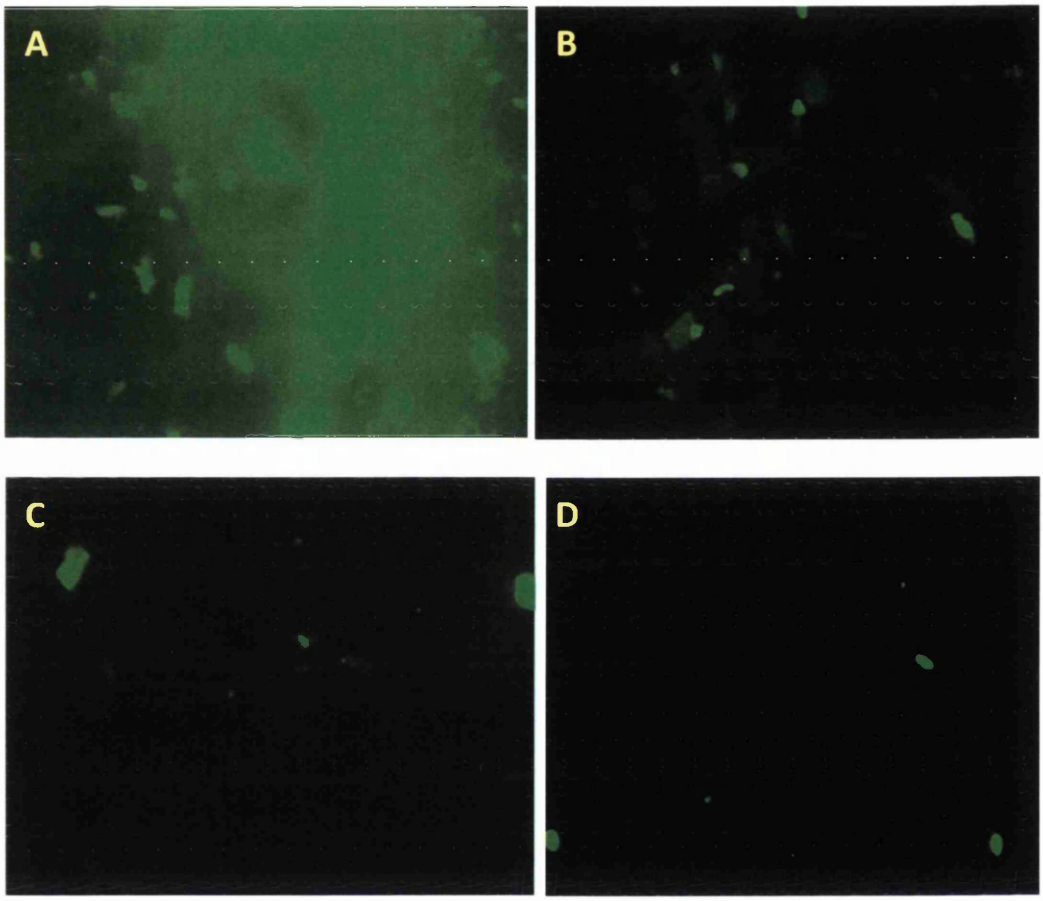


Figure 3.4 Immunofluorescence detection of Embp expression in *S. epidermidis* 1585-RA and *S. epidermidis* 1585 using Embp –specific absorbed antiserum. Immunocytochemistry (ICC) demonstrated Embp expression in Embp biofilm negative *S. epidermidis* 1585 grown in cell culture media compared to Embp positive *S. epidermidis* 1585-RA. **A.** *S. epidermidis* 1585-RA in TSB, **B.** *S. epidermidis* 1585-RA in RPMI+, **C.** *S. epidermidis* 1585 in DMEM, **D.** *S. epidermidis* 1585 in RPMI+.

Christner et al (Christner, Franke et al., 2010) showed that goat serum could induce biofilm formation in biofilm negative *S. epidermidis* 1585. Biofilm assays were carried out using three different goat serum concentrations (50%, 60%, 70%) in TSB/G+, and compared to biofilm formation in IMDM, DEME and RPMI+ which were the media provoke biofilm formation in this negative strain.

Growth of *S. epidermidis* 1585 under different concentrations of goat serum (50%, 60%, and 70%) was shown not to induce biofilm formation after both 24h and 48h compared to the culture in IMDM, DMEM and RPMI+ which significantly induced biofilm formation ($P<0.05$) (Figure 3.5).

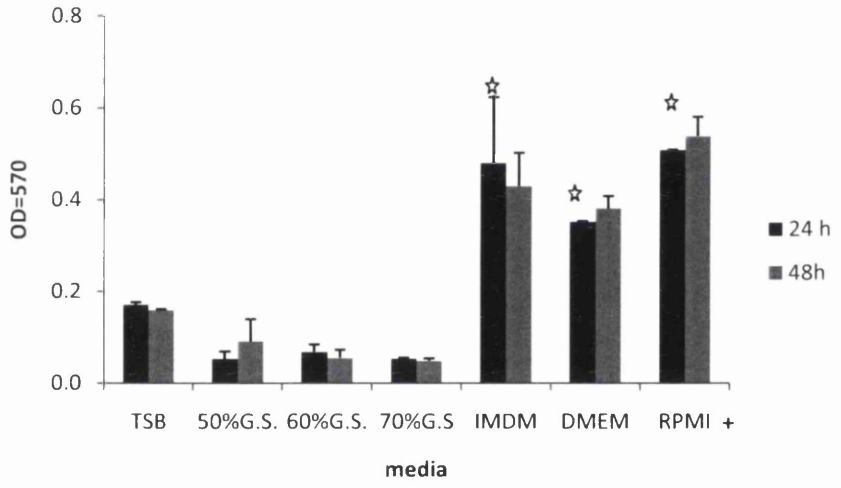


Figure 3.5 Investigating cell culture media ability to induce biofilm formation in *S. epidermidis* 1585 in comparison to goat serum in 24 and 48 hours. Results represent the data from three independent experiments. Stars indicate significant difference between cell culture media and goat serum *$P<0.05$.

3.6 Effect of mixing cell culture media on biofilm formation

To investigate a universal culture media supportive of biofilm formation by all *S. epidermidis* strains (with different accumulation mechanisms) a mixed media containing components that supported good biofilm formation by each strain was prepared. This mix contained DMEM/F12 which was the best at supporting biofilm formation by *S. epidermidis* 1457, and RPMI+ was also included as it was the best media to support biofilm formation of *S. epidermidis* 5179-R1.

Combining DMEM/F12 and RPMI+ cell culture media in a 1:1 ratio lead to reduced biofilm formation by both *S. epidermidis* 1457 and *S. epidermidis* 5179-R1 strains in compared to DMEM/F12 and RPMI+ alone. Furthermore control samples diluted in PBS gave similar response to the original media (Figure 3.6).

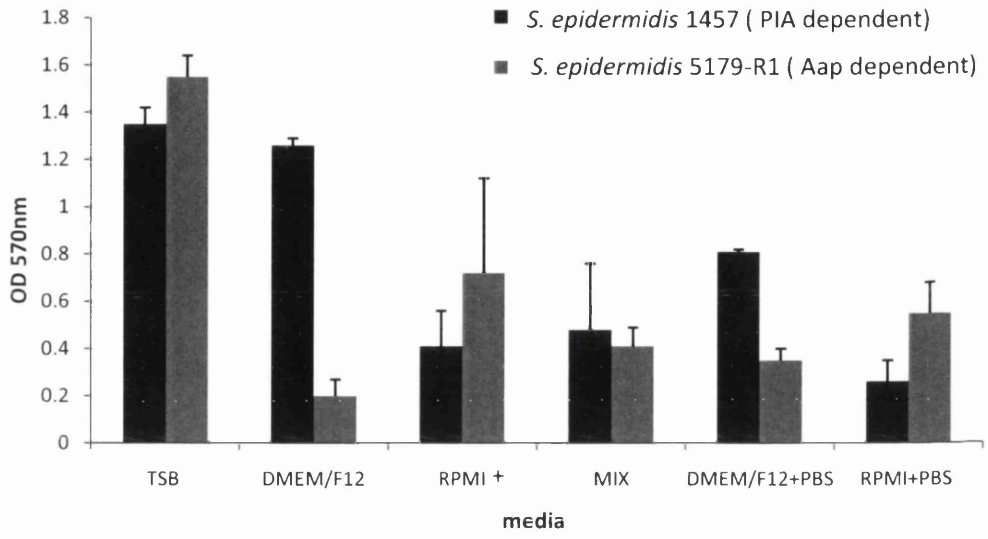


Figure 3.6 Effect of mixing cell culture media DMEM/F12 and RPMI+ on biofilm formation. The figure shows the relative biofilm formation of both *S. epidermidis* 1457 (PIA dependent) and *S. epidermidis* 5179-R1 (Aap dependent) in separate culture media as well as a mixed preparation. The 1:1 mix of DMEM/F12 and RPMI+ was found to generate lower level of biofilm by each of these strains compared with the medium previously identified as giving best biofilm formation for strains. Results represent the data from three independent experiments.

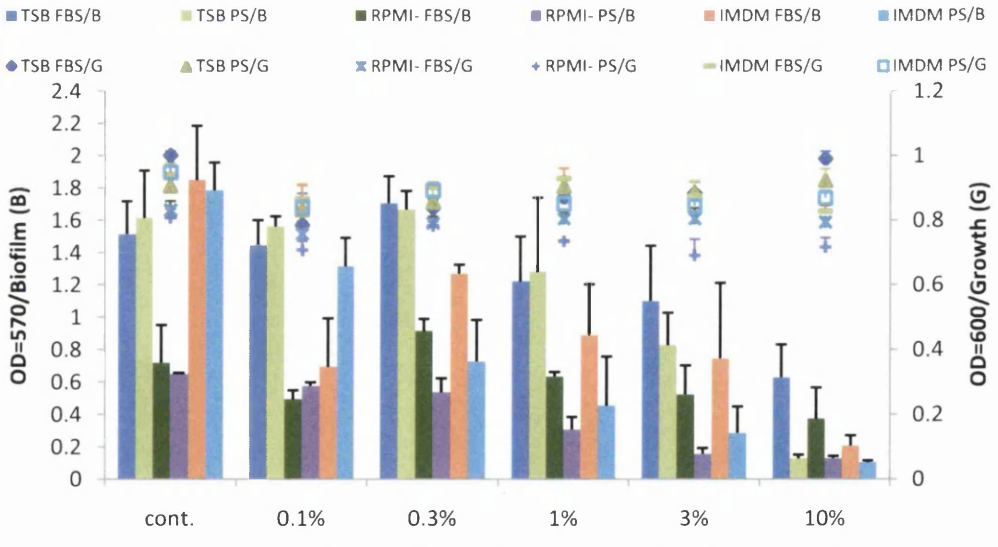
3.7 The effect of serum on growth and biofilm formation of *S. epidermidis* 1457 and *S. epidermidis* 5179-R1

To study the effect of FBS and human serum on the growth and biofilm formation of *S. epidermidis* 1457 and *S. epidermidis* 5179-R1, pooled serum (PS) was generated from 7 different human sera and added to TSB, IMDM and RPMI- media and incubated for 18 hours. Serum concentrations used were (0.1%, 0.3%, 1%, 3%, and 10%).

Adding FBS and PS had no effect on the growth of *S. epidermidis* 1457 and *S. epidermidis* 5179-R1 in any of the three media (Figure 3.7 A and B).

Regarding effects of FBS on biofilm formation, *S. epidermidis* 1457 grown in TSB, IMDM or RPMI-, showed a dose dependent decrease with maximum effect at 10% serum. For PS the dose dependent inhibitory effect was more powerful, resulting in complete inhibition of biofilm formation at 10% serum in TSB, RPMI- and IMDM (Figure 3.7A).

In *S. epidermidis* 5179-R1, both FBS and PS had a powerful inhibitory effect on the biofilm formation especially in cell culture media. In TSB 1-10% serum inhibited biofilm formation, while adding 0.1% FBS or PS to RPMI- and IMDM completely prevented biofilm formation (Figure 3.7 B).



A

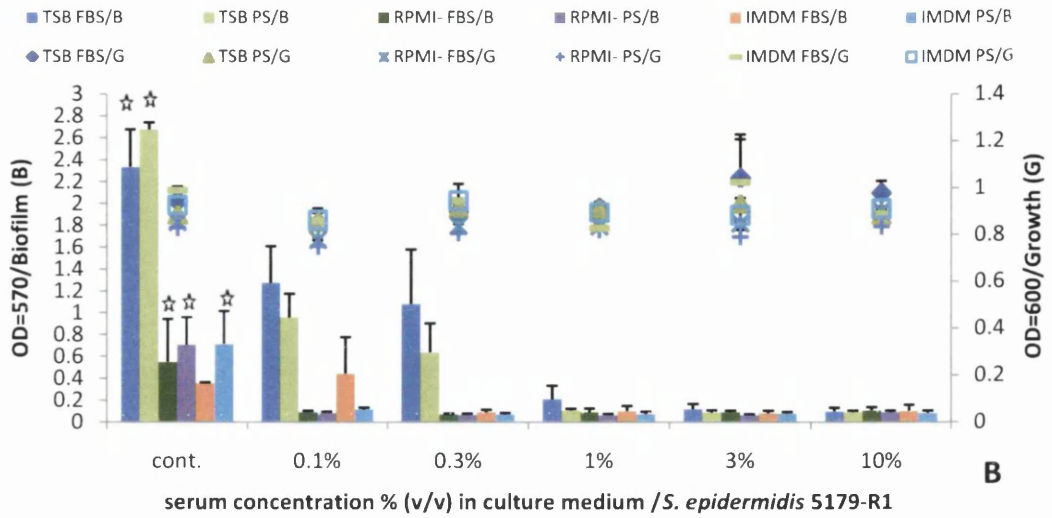
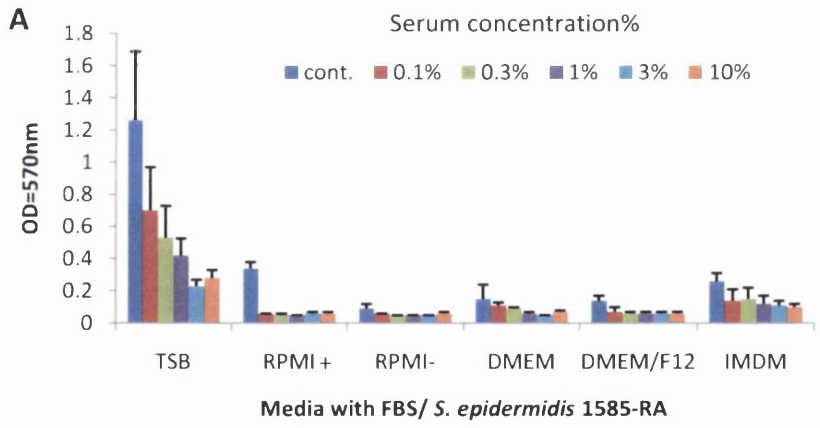


Figure 3.7 Effect of FBS and PS on the growth and biofilm formation of *S. epidermidis*. A. *S. epidermidis* 1457 (PIA dependent) B. *S. epidermidis* 5179-R1 (Aap dependent). The markers on the top of graph represent the effect of different concentration of both FBS and PS (0.1%, 0.3%, 1%, 3%, and 10%) on the growth (G) of *S. epidermidis* 1457 and *S. epidermidis* 5179-R1 in three different media namely, TSB, RPMI- and IMDM. The columns represent the effect of different concentration of both FBS and PS (0.1%, 0.3%, 1%, 3%, and 10%) on the biofilm formation (B) of *S. epidermidis* 1457 and *S. epidermidis* 5179-R1 in three different media namely, TSB, RPMI- and IMDM. Results represent the data from 3 independent experiments. Stars indicate significant difference between control and different serum concentrations * $P<0.05$. FBS/B; biofilm formation with presence of fetal bovine serum in media, PS/B; biofilm formation with presence of pooled human serum in media, FBS/G; growth with presence of fetal bovine serum in media, PS/G; growth with presence of pooled human serum in media.

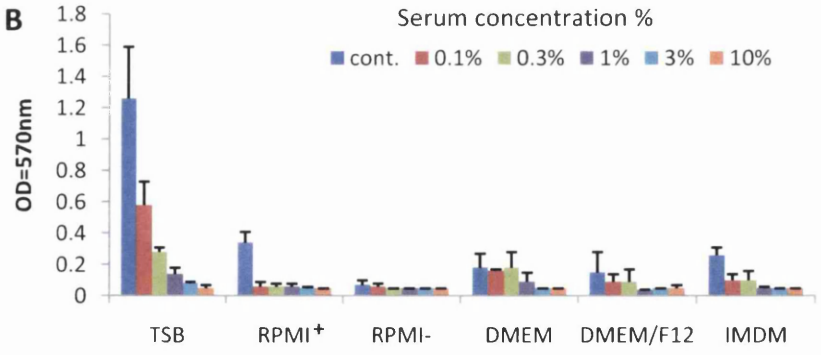
3.8 The effect of serum on biofilm formation by *S. epidermidis* 1585-RA and 1585

The effect of FBS and PS on biofilm formation by Embp biofilm positive *S. epidermidis* 1585-RA, and biofilm negative *S. epidermidis* 1585 was also assessed. Increasing concentrations of serum were added to TSB and the cell culture media; RPMI+, RPMI-, DMEM, DMEM/F12 and IMDM, prior to biofilm assays.

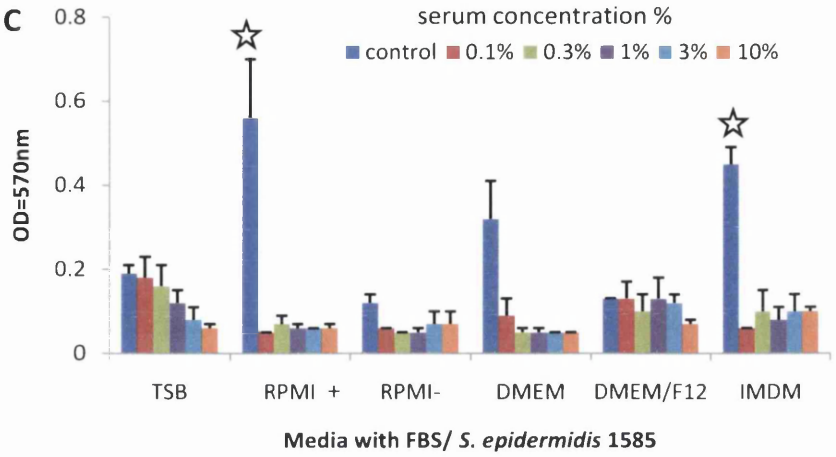
For *S. epidermidis* 1585-RA, adding FBS and PS serum reduced biofilm formation in TSB, RPMI+ and IMDM (Figure 3.8 A and B). For biofilm negative *S. epidermidis* 1585 adding serum did not induce biofilm formation in TSB. However serum did reduce the biofilm induced by RPMI+, DMEM and IMDM and this was statistically significant $P<0.05$ (Figure 3.8 C and D).



Media with FBS/ *S. epidermidis* 1585-RA



Media with PS/ *S. epidermidis* 1585-RA



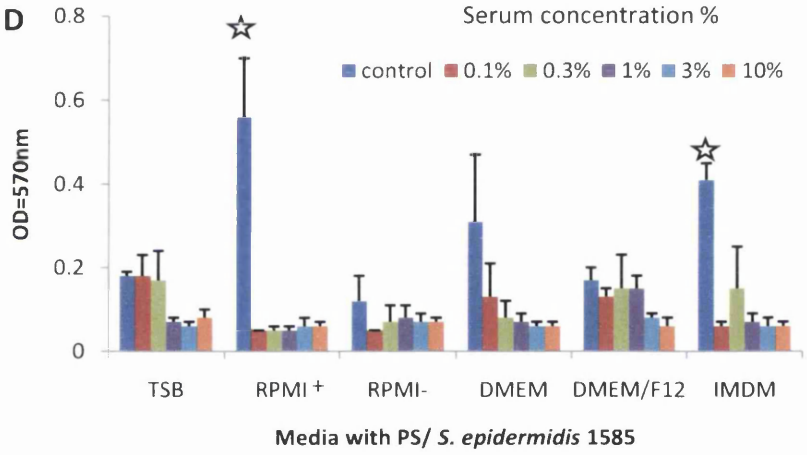


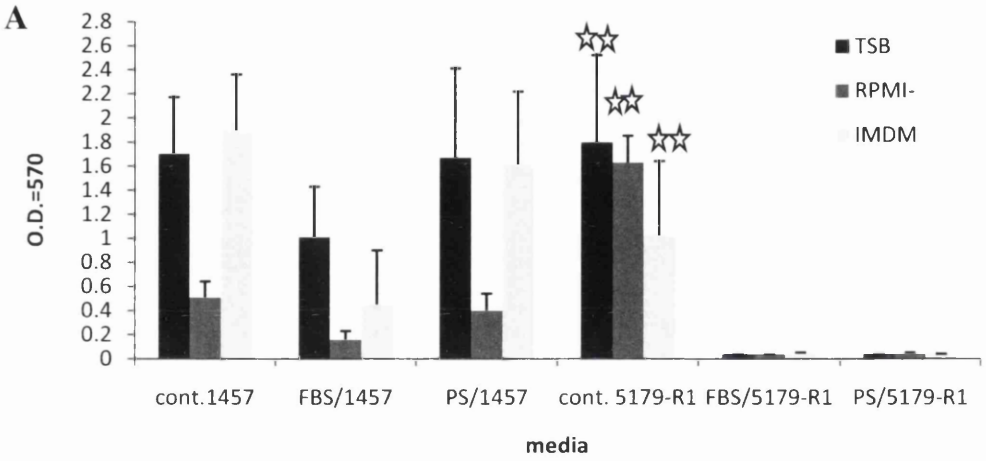
Figure 3.8 Serum effect on biofilm formation by *S. epidermidis* 1585-RA and *S. epidermidis* 1585. A. *S. epidermidis* 1585-RA biofilm formation with FBS, **B.** *S. epidermidis* 1585-RA biofilm formation with PS, **C.** *S. epidermidis* 1585 biofilm formation with FBS, **D.** *S. epidermidis* 1585 biofilm formation with PS. The columns represent the effect of different concentration of both FBS and PS (0.1%, 0.3%, 1%, 3%, and 10%) on the biofilm formation of *S. epidermidis* 1585-RA (Embp dependent) and *S. epidermidis* 1585 (biofilm negative) in different media namely, TSB, RPMI+, RPMI-, DMEM, DMEM/F12 and IMDM. Results represent the data from 3 independent experiments. Stars indicate significant difference between control and different serum

concentrations * $P<0.05$. Media with FBS; biofilm formation with presence of fetal bovine serum in culture media, media with PS; biofilm formation with presence of pooled human serum in culture media, control is biofilm formation in serum free medium.

3.9 The effect of coating microtiter plate wells with serum on biofilm formation by *S. epidermidis* 1457, 5179-R1 and 1585-RA

The effect of serum coating the microtiter plate wells on subsequent biofilm formation by *S. epidermidis* 1457, 5179-R1 and 1585-RA, was performed in 96 well plates whose wells were coated overnight with 10% PS and FBS. The culture media used for biofilm formation were TSB, RPMI+, RPMI- and IMDM. After incubation the biofilm was assessed by staining with crystal violet and the measuring OD_{570}.

For *S. epidermidis* 1457, pre-coating wells with FBS resulted in slight reduction in biofilm formation in all media tested. Coating with PS had no effect on biofilm formation in all the three media. For *S. epidermidis* 5179-R1, pre-coating wells with FBS or PS completely inhibited biofilm formation in all the three media and this was statistically significant, $P< 0.01$ (Figure 3.9 A). Pre-coating wells with FBS significantly reduced biofilm formation by *S. epidermidis* 1585-RA in TSB, and prevented biofilm formation in IMDM and RPMI+ media. Pre-coating wells with PS inhibited biofilm formation completely in all media (Figure 3.9 B).



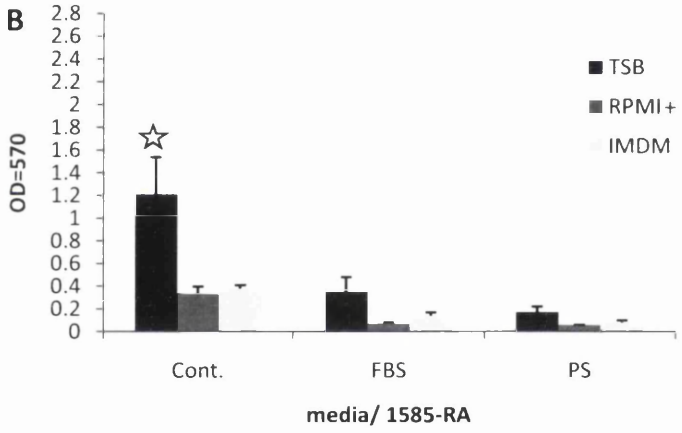


Figure 3.9 Effects of pre-coating microtitre plate wells with serum on *S. epidermidis* biofilm formation. **A.** *S. epidermidis* 1457 and *S. epidermidis* 5179-R1 cultured in TSB, RPMI- and IMDM. **B.** *S. epidermidis* 1585-RA cultured in TSB, RPMI+ and IMDM. The figure shows the effect of pre-coating microtiter plate wells with pooled human serum (PS) and fetal bovine serum (FBS) on biofilm formation of *S. epidermidis* 1457 (PIA dependent), *S. epidermdis* 5179-R1 (Aap dependent) and *S. epidermidis* 1585-RA (Embp dependent). Results represent the data from 3 independent experiments. Cont.; control which represent biofilm formation in non serum pre-coated microititre plate wells. Stars indicate significant difference between different serum concentrations **P<0.01, *P<0.05.

3.10 The effect of molecular weight (MW) size fractions of FBS and PS on biofilm formation by *S. epidermidis* strains

The effect of different fractionated serum samples on biofilm formation was studied to determine whether the molecular weight of serum used was an important factor in affecting biofilm formation.

Both FBS and PS were fractionated into four groups: fractions above 30000 kDa, fractions between 30000-10000 kDa, fractions between 10000-3000kDa and fractions below 3000kDa. Biofilm assays were performed with different fractions of serum to characterise the size (according to their MW) of the biofilm inhibitory factors.

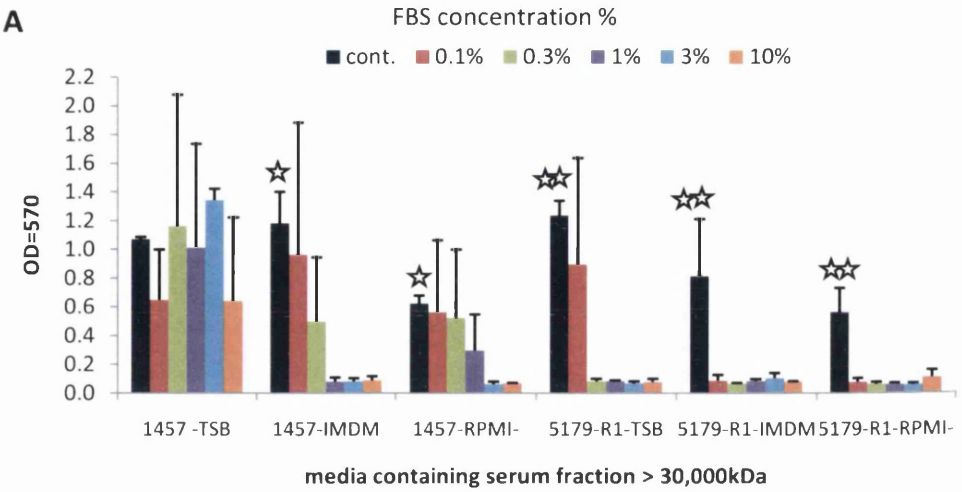
3.10.1 The effect of molecular weight (MW) size fractions of FBS on *S. epidermidis* biofilm formation

Biofilm assays for *S. epidermidis* 1457 and *S. epidermidis* 5179-R1 were performed with different fractions of FBS. For fractions with MW above 30000kDa, the effect on *S. epidermidis* 1457 biofilm was limited when added to TSB, while for IMDM and RPMI- there was a trend toward a dose dependent decrease in biofilm formation until 3-10% serum.

Complete inhibition of biofilm formation was observed for *S. epidermidis* 5179-R1 with serum preventing biofilm formation in all media even with lowest concentrations of serum except for 0.1% TSB (Figure 3.10 A). It appears that the major inhibitory effect of serum lay in fractions with MW above 30000kDa and was consistent with earlier findings with FBS and PS on biofilm formation (section 3.5).

For serum fractions with MW between 30000-10000kDa, 10000-3000 kDa and below 3000 kDa, there were mild or no effects on *S. epidermidis* 1457 biofilm formation in all three media. For *S. epidermidis* 5179-R1 there was no or limited effects when added to TSB and IMDM. In RPMI-, the serum actually induced biofilm formation and this effect increased with increasing the serum concentration 0.3-10% ($P<0.05$) (Figure 3.10 B,C and D).

A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cont.
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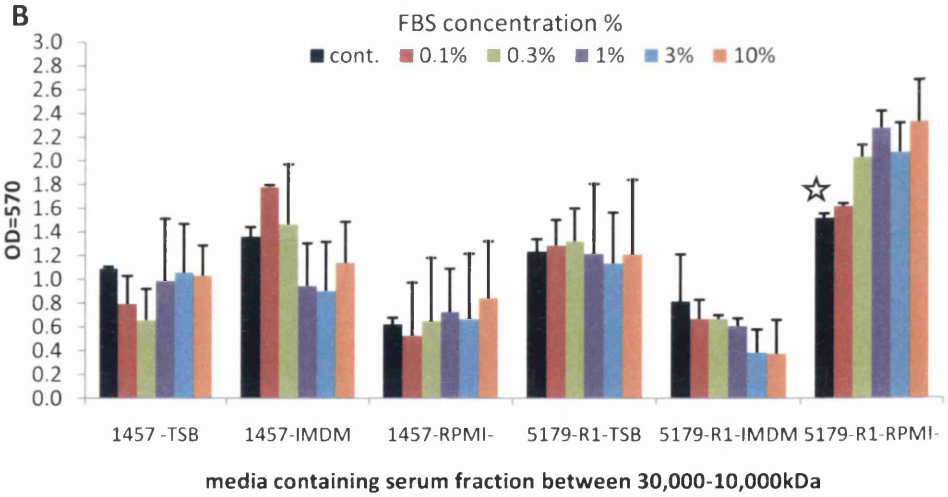
0.3%
1%
3%
10%
OD=570
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1457 -TSB
1457-IMDM
1457-RPMI-
5179-R1-TSB
5179-R1-IMDM
5179-R1-RPMI-
media containing serum fraction > 30,000kDa

B


FBS concentration %
cont.
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0.3%
1%
3%
10%
OD=570
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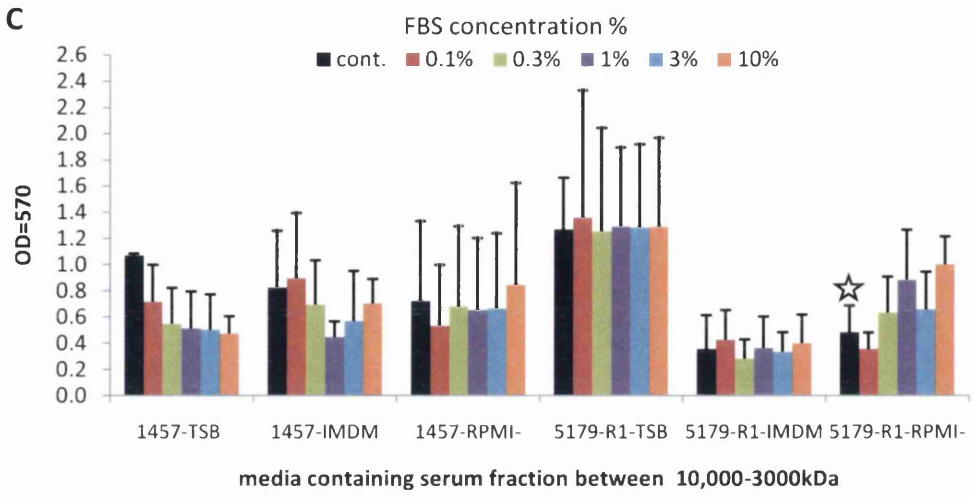
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1457 -TSB
1457-IMDM
1457-RPMI-
5179-R1-TSB
5179-R1-IMDM
5179-R1-RPMI-
media containing serum fraction between 30,000-10,000kDa



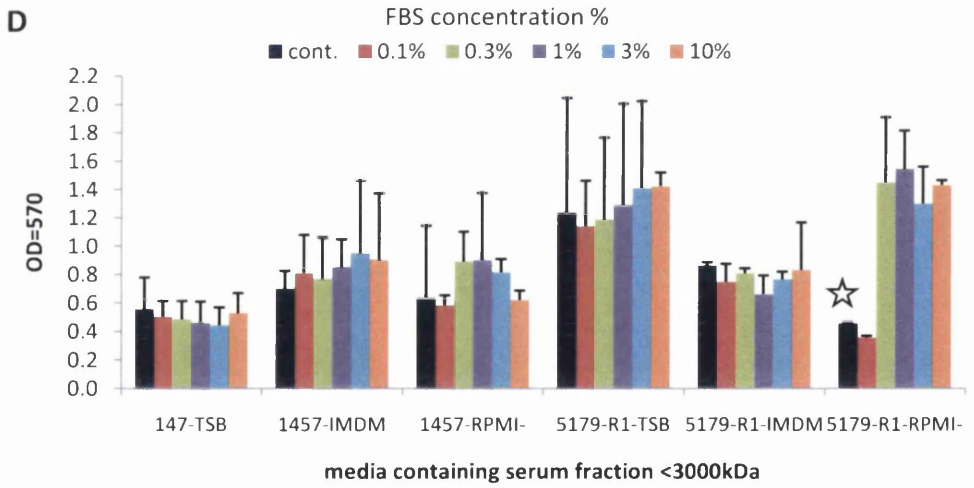
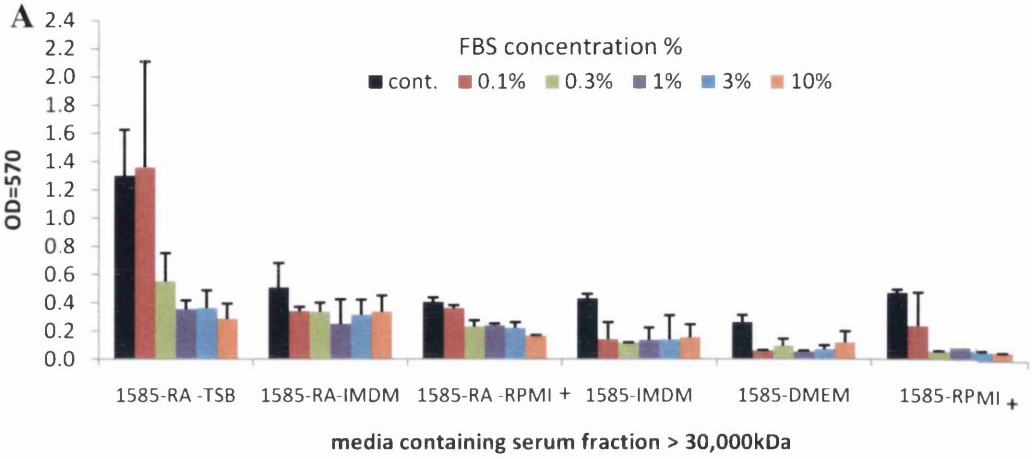


Figure 3.10 The effect of different molecular weight fractions of FBS on biofilm formation by *S. epidermidis* 1457 and *S. epidermidis* 5179-R1. A. FBS fractions with MW > 30000kDa. **B.** FBS fractions with MW between 30000-10000kDa. **C.** FBS fractions with MW between 10000-3000kDa. **D.** FBS fractions with MW < 3000kDa. Three different media TSB, IMDM and RPMI- used to cultured *S. epidermidis* 1457 (PIA dependent) and *S. epidermidis* 5179-R1 (Aap dependent) and the effect of different molecular weight fractions of FBS on biofilm formation assessed. The fraction

> 30,000 kDa had powerful inhibitory effect compared to other frctions. Results represent the data from 3 independent experiments. Stars indicate significant difference between control and different serum concentrations **P<0.01, *P<0.05. Cont. is control medium used to cultured *S. epidermidis* without serum, FBS id fetal bovine serum.

For *S. epidermidis* 1585-RA and *S. epidermidis* 1585, biofilm assays were performed with different fractions of FBS in TSB, IMDM and RPMI+ (1585-RA) and IMDM, DMEM and RPMI+ (1585) (Figure 3.11 A-D). For fractions with MW above 30000kDa, inhibitory effects on biofilm formation in TSB occurred with serum concentration >0.3%. For *S. epidermidis* 1585-RA, whilst in IMDM and RPMI- the serum effect was limited (Figure 3.11 A). For *S. epidermidis* 1585 all concentrations of FBS prevented biofilm formation in all media (Figure 3.11 A). For all other FBS fractions, FBS had no effect on biofilm formation for both *S. epidermidis* 1585-RA and *S. epidermidis* 1585 (Figure 3.11 B-D).


A
FBS concentration %
cont.
0.1%
0.3%
1%
3%
10%
OD=570
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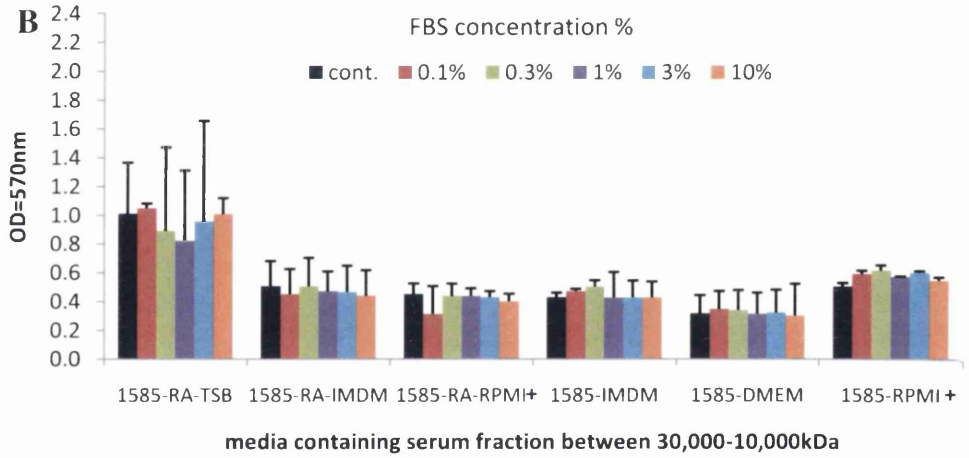
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1585-RA -TSB
1585-RA-IMDM
1585-RA -RPMI +
1585-IMDM
1585-DMEM
1585-RPMI +
media containing serum fraction > 30,000kDa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FBS concentration %
cont.
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1%
3%
10%
OD=570nm
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2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1585-RA-TSB
1585-RA-IMDM
1585-RA-RPMI+
1585-IMDM
1585-DMEM
1585-RPMI +
media containing serum fraction between 30,000-10,000kDa

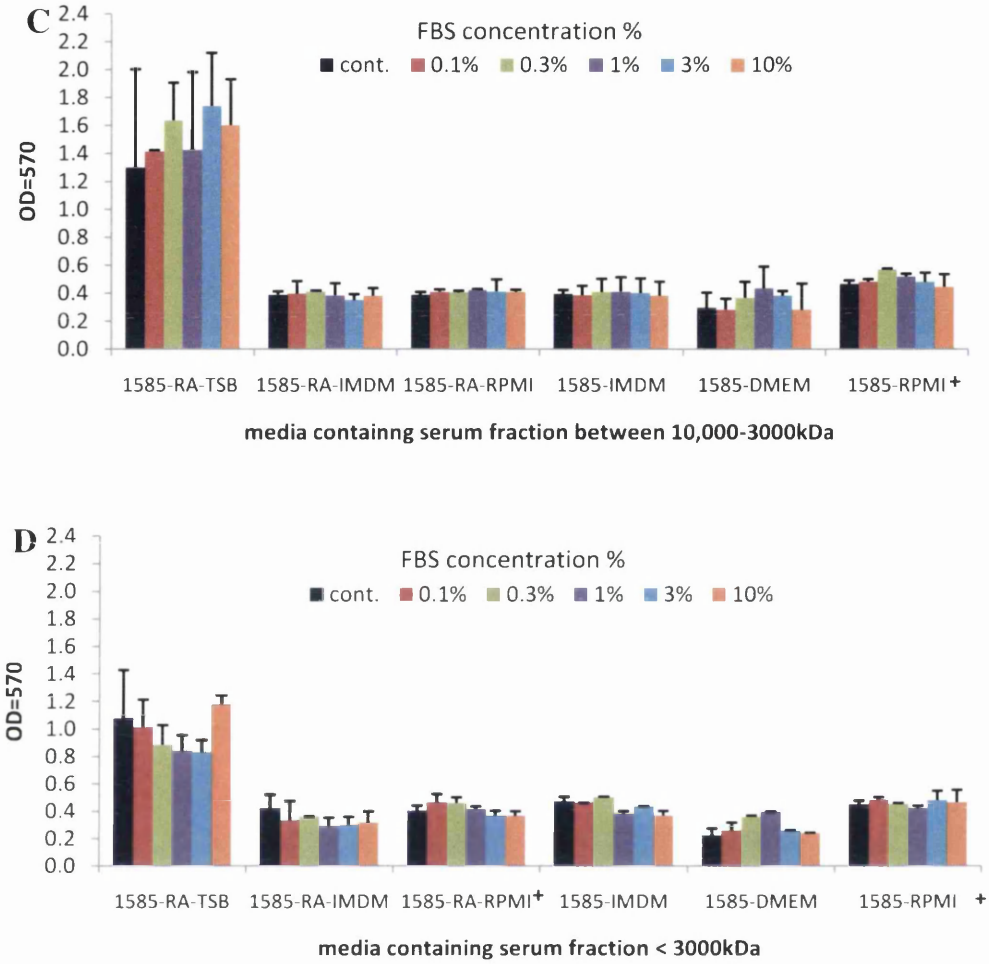


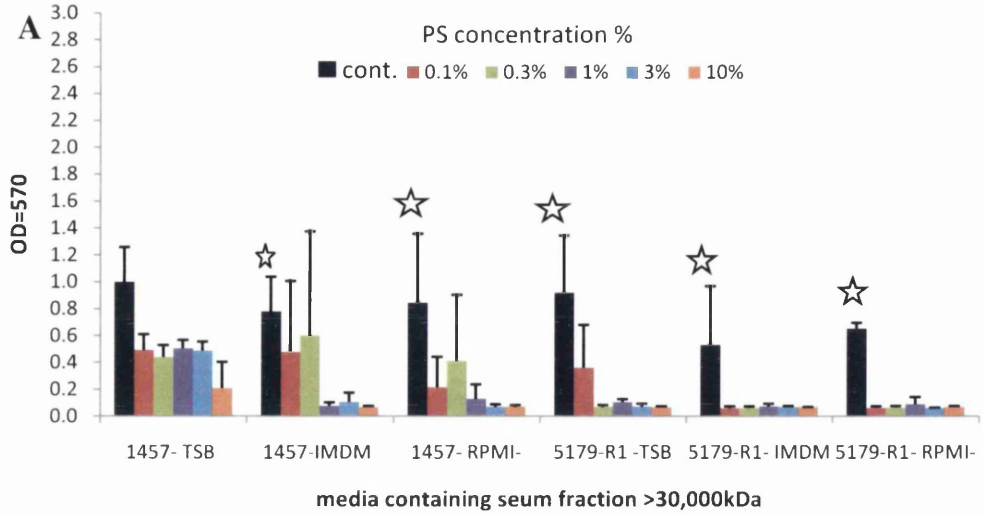
Figure 3.11 The effect of different molecular weight fractions of FBS on biofilm formation in 1585-RA and 1585 *S. epidermidis* strains. A. FBS fractions with MW above 30000kDa. **B.** FBS fractions with MW between 30000-10000kDa. **C.** FBS fractions with MW between 10000-3000kDa. **D.** FBS fractions with MW below 3000kDa. Three different media TSB, IMDM and RPMI- used to cultured *S. epidermidis* 1585-RA (Embp dependent) and *S. epidermidis* 1585 (biofilm negative). The fraction > 30,000 kDa had a powerful inhibitory effect compared to other fractions. Results represent the data from 3 independent experiments. Cont. is control medium used to cultured *S. epidermidis* without serum, FBS is fetal bovine serum.

3.10.2 The effect of different molecular weight (MW) fractions of PS on *S. epidermidis* biofilm formation

Biofilm assays of *S. epidermidis* 1457 and *S. epidermidis* 5179-R1 incubated with MW fractions of PS above 30000kDa in TSB led to greater than 50% biofilm reduction for *S. epidermidis* 1457. In IMDM and RPMI- concentrations of PS >1% prevented biofilm formation ($P<0.05$). For *S. epidermidis* 5179-R1 adding PS prevented biofilm formation in all three media at PS $\geq$0.1% and these effects were statistically significant $P<0.05$ (Figure 3.12 A).

PS fractions with MW between 30000-10000kDa, 10000-3000kDa and below 3000kDa had no significant effect on 1457 biofilm formation in TSB, IMDM and RPMI- (Figure 3.12 B,C,D). For *S. epidermidis* 5179-R1, adding PS fractions with MW between 30000-10000kDa had no effect on biofilm formation in TSB and IMDM, but decreased biofilm formation in RPMI- when PS >0.3% (Figure 3.12B). For the PS fractions with MW between 10000-3000kDa, no effect on 5179-R1 biofilm formation was evident in TSB. Increased biofilm formation occurred in IMDM and no biofilm formation was evident in RPMI- ($P<0.05$) (Figure 3.12 C). Finally, adding PS fractions with MW below 3000kDa had no effect on biofilm formation of *S. epidermidis* 5179-R1 in TSB and IMDM, but still prevented biofilm formation in RPMI- ($P<0.05$) (Figure 3.12 D).

PS had a significantly higher inhibitory effect on biofilm formation compared with FBS. The inhibitory effect of PS on biofilm formation in *S. epidermidis* 1457 was due to serum components present in fractions above 30000kDa, whilst in 5179-R1 the effect was limited in TSB and IMDM. In contrast, in RPMI-, PS inhibited biofilm formation unlike FBS which induced biofilm.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OD=570
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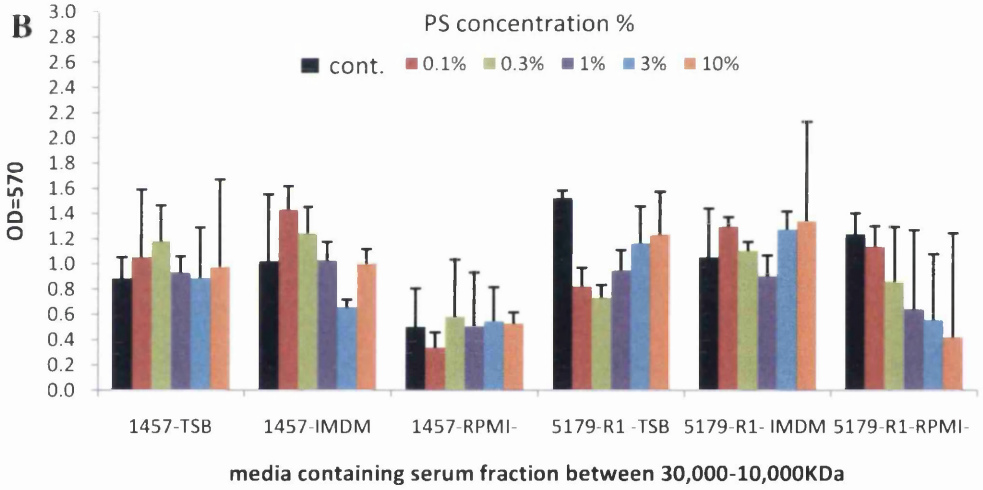
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1457- TSB
1457-IMDM
1457- RPMI-
5179-R1 -TSB
5179-R1- IMDM
5179-R1- RPMI-
media containing seum fraction >30,000kDa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PS concentration %
cont. 0.1% 0.3% 1% 3% 10%
OD=570
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2.4
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2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1457-TSB
1457-IMDM
1457-RPMI-
5179-R1 -TSB
5179-R1- IMDM
5179-R1-RPMI-
media containing serum fraction between 30,000-10,000KDa

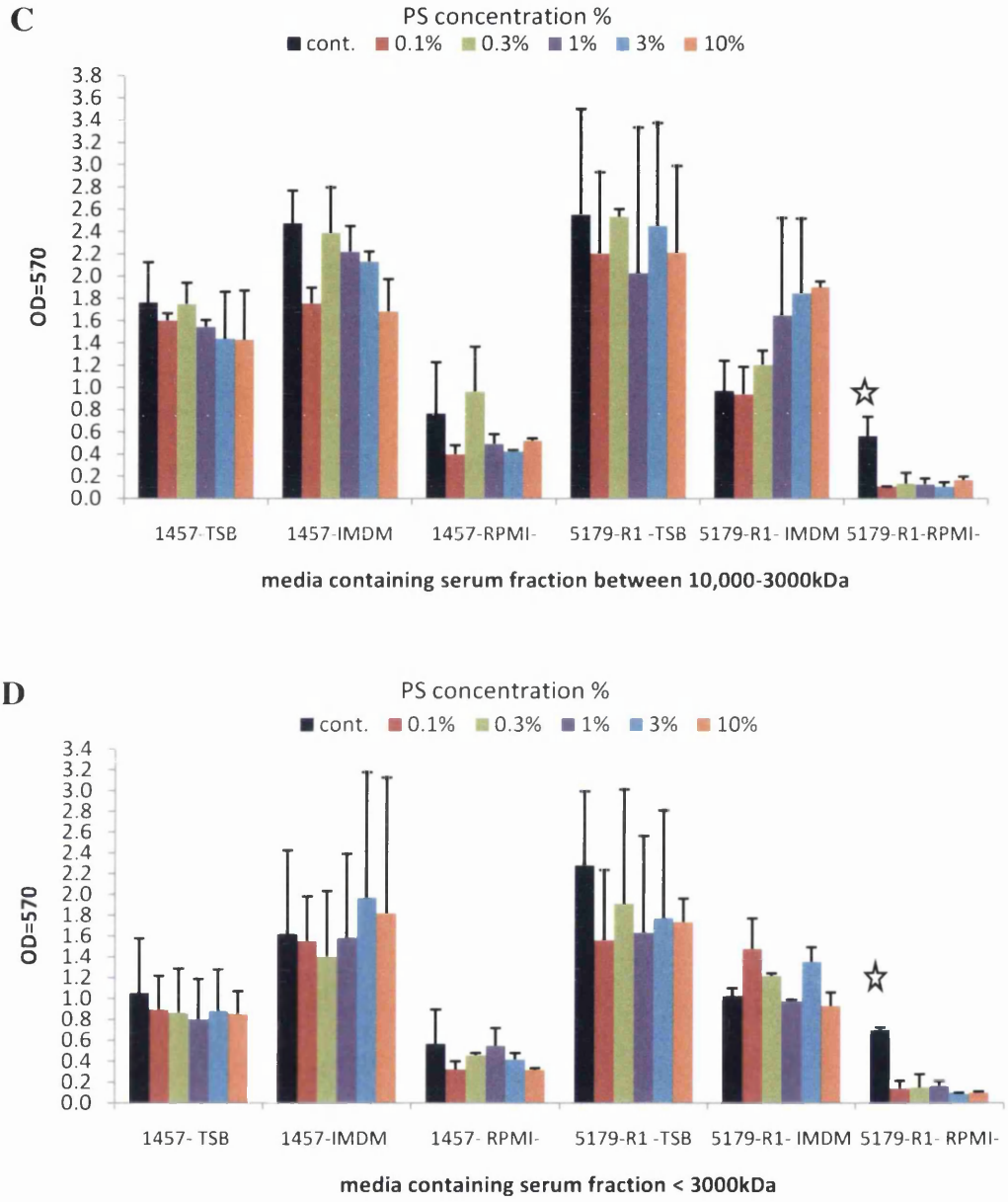


Figure 3.12 The effect of different molecular weight fractions of PS on biofilm formation in *S. epidermidis* 1457 and *S. epidermidis* 5179-R1. A. PS fractions with MW above 30000kDa. **B.** PS fractions with MW between 30000-10000kDa. **C.** PS fractions with MW between 10000-3000kDa. **D.** PS fractions with MW below 3000kDa. Three different media TSB, IMDM and RPMI- used to cultured *S. epidermidis* 1457 (PIA dependent) and *S. epidermidis* 5179-R1 (Aap dependent). The fraction > 30,000

kDa had a powerful inhibitory effect compared to other fractions. Results represent the data from 3 independent experiments. Stars indicate significant difference between control and different serum concentrations *P<0.05. Cont. is control medium used to culture *S. epidermidis* without serum, PS is pooled human serum.

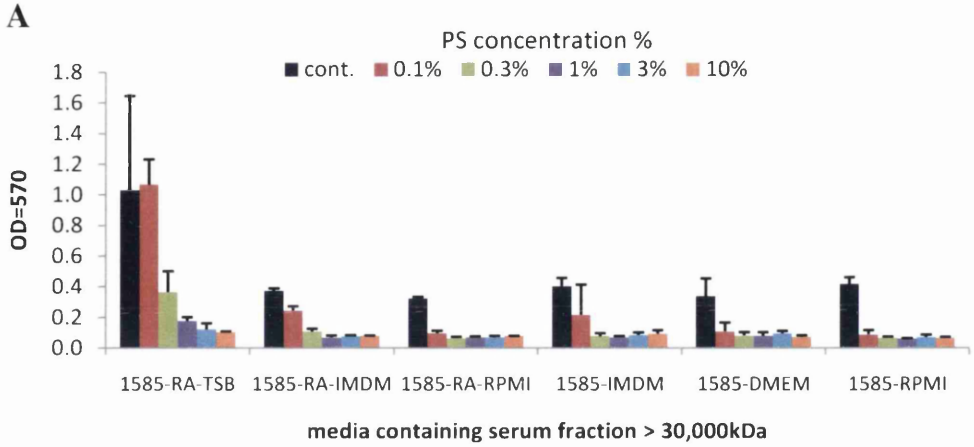
Biofilm assays were undertaken with *S. epidermidis*1585-RA and *S. epidermidis* 1585 using culture media to which different MW fractions of PS had been added. Using PS > 30,000kDa and at concentrations > 0.3% in TSB, biofilm inhibition was evident with *S. epidermidis* 1585-RA. For this strain, the addition of this fraction of PS completely inhibited biofilm formation in the other test media. For *S. epidermidis* 1585 adding PS prevented biofilm formation in all the three media (Figure 3.13A).

Adding PS fractions between 30000-10000kDa had no effect on biofilm formation of 1585-RA in the three media; whilst for *S. epidermidis* 1585, PS prevented biofilm formation when added to DMEM, with no effect on biofilm formation in IMDM and RPMI+ (Figure 3.13B).

PS fractions between 10000-3000kDa and < 3000kDa, in TSB, inhibited *S. epidermidis* 1585-RA biofilm at 10%, but had no effect on biofilm formation in IMDM and RPMI+. In the case of *S. epidermidis* 1585, PS had no effect on biofilm formation in IMDM and RPMI+, but prevented biofilm formation in DMEM (Figure 3.13 C and D).

In summary, PS had more potent inhibitory effect on biofilm formation compared with FBS on *S. epidermidis* 1585-RA and *S. epidermidis* 1585. The inhibitory effect of PS on biofilm formation by *S. epidermidis* 1585-RA and *S. epidermidis* 1585 was due to serum components present in fractions above 30000kDa. For other fractions the inhibitory effect on *S. epidermidis* 1585 was observed in DMEM only.

A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3%
10%
OD=570
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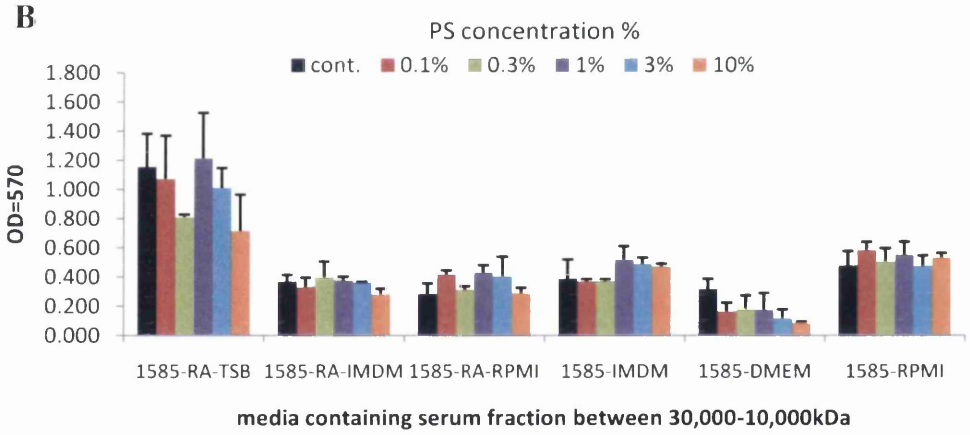
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1585-RA-TSB
1585-RA-IMDM
1585-RA-RPMI
1585-IMDM
1585-DMEM
1585-RPMI
media containing serum fraction > 30,000kDa

B


PS concentration %
cont.
0.1%
0.3%
1%
3%
10%
OD=570
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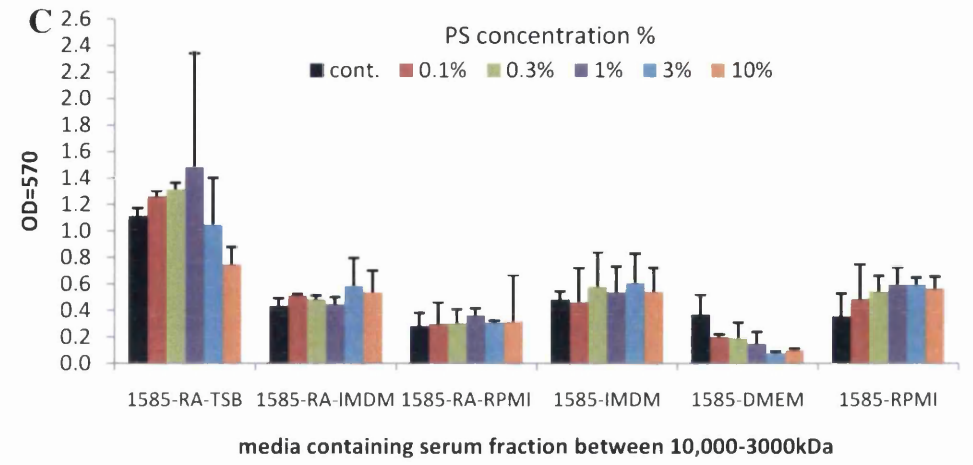
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
1585-RA-TSB
1585-RA-IMDM
1585-RA-RPMI
1585-IMDM
1585-DMEM
1585-RPMI
media containing serum fraction between 30,000-10,000kDa



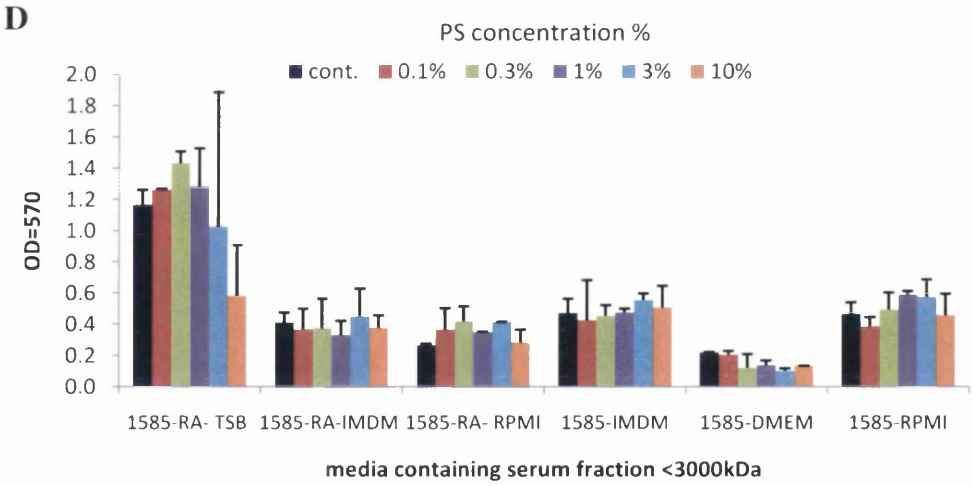


Figure 3.13 The effect of different molecular weight fractions of PS on biofilm formation in *S. epidermidis* 1585-RA and *S. epidermidis* 1585. A. PS fractions with MW above 30000kDa. **B.** PS fractions with MW between 30000-10000kDa. **C.** PS fractions with MW between 10000-3000kDa. **D.** PS fractions with MW below 3000kDa. Four different media TSB, IMDM, DMEM and RPMI+ used to culture *S. epidermidis* 1585-RA (Embp dependent) and *S. epidermidis* 1585 (biofilm negative). The fraction > 30,000 kDa had a powerful inhibitory effect on biofilm formation compared with other fractions. Results represent the data from 3 independent experiments. Cont. is control medium used to culture *S. epidermidis* without serum, PS is pooled human serum.

3.11 Discussion

The purpose of the present study was to determine the significance of the growth conditions on the growth and biofilm formation of different *S. epidermidis* strains with different mechanisms of biofilm accumulation. The ability of specific cell culture media to support biofilm formation was investigated, and a difference in the nutritional requirement needed for biofilm formation between different *S. epidermidis* strains with different mechanism of biofilm accumulation was identified. In addition, serum effect (human and bovine) on biofilm formation was studied.

This study demonstrated that cell culture media can support the growth and biofilm formation of *S. epidermidis* strains exhibiting different mechanisms of biofilm formation (PIA, Aap and Embp). This effect could be due to the effect of specific nutrients in these media that regulate PIA, Aap and Embp expression with the resultant formations of biofilm. Dobinsky *et al* (Dobinsky, Kiel et al., 2003) showed that glucose was essential for PIA synthesis and biofilm formation and in this study glucose was a component in all cell culture media studied. In addition, all cell culture media used contained the inorganic salts magnesium (Mg) and calcium (Ca), which are important for biofilm formation according to Ozerdem *et al* who demonstrated a significant increase in biofilm formation by adding Mg and Ca to TSB (Ozerdem Akpolat, Elci et al., 2003).

There was a difference in biofilm formation between RPMI + and RPMI- suggesting that glutamine has differential effects on biofilm growth by *S. epidermidis*. Thus glutamine induces biofilm development in strains like *S. epidermidis* 1585-RA and *S. epidermidis* 1585 and appears non-essential for biofilm development in *S. epidermidis* 5179-R1 and is inhibitory for *S. epidermidis* 1457. This is interesting as to the author's knowledge this is the first report of a specific nutrient having differential effects on specific biofilm mechanisms. L-glutamine is an important precursor for peptide and protein synthesis, amino sugar synthesis, purine and pyrimidine for nucleic acid and nucleotide synthesis, (Matthews and Anderson, 2002; Forchhammer, 2007). The metabolic effect of glutamine on PIA synthesis was partly demonstrated by Vuong *et al* as factors able to repress tricarboxylic acid (TCA) cycle activity induced PIA production (Vuong, Kidder et al., 2005). Furthermore, activation of TCA by inactivation of the glutamine permease gene (*glnP*) (which prevents glutamine transport from the media to the cell) led to

decreased PIA synthesis and biofilm accumulation (Zhu, Xiong et al., 2009). The importance of glutamine to cell survival and proliferation *in vitro* was described by Eagle *et al* (Eagle, Oyama et al., 1956) who developed the first chemically defined synthetic medium that contained glucose, 19 essential and nonessential amino acids and a high concentration of glutamine.

In addition, RPMI media contains glutathione in a reduced form which is an essential element in DNA synthesis, protein synthesis, and enzyme activation, and also acts as an antioxidant (Moore and Woods, 1976; Invitrogen RPMI, 2013). Indeed adding glutathione to cell culture media enhances cell growth and proliferation (Ozawa, Nagai et al., 2006).

IMDM was the media that best supported the growth and biofilm formation of *S. epidemidis* strains with different mechanisms of biofilm formation including *S. epidermidis* 1457, *S. epidermidis* 5179-R1, and *S. epidermidis* 1585-RA. Furthermore this medium was also able to induce biofilm formation in the biofilm negative *S. epidermidis* 1585. IMDM is a highly enriched medium modified from DMEM. The positive effect of IMDM on biofilm formation may be due to presence of sodium pyruvate which is known to act as a source of energy and prevent hydrogen peroxide accumulating in the medium. IMDM also contains selenium which is an essential trace element for cell growth and development and functions as an antioxidant. Furthermore, IMDM lacks iron supplementation which could induce biofilm formation (Johnson, Cockayne et al., 2005; Johnson, Cockayne et al., 2008; Invitrogen IMDM, 2013). Iron plays an important role in all aspects of cell growth, proliferation and differentiation in bacteria (Cornelis and Andrews, 2010). Interestingly, innate immunity resists bacterial infection by promoting an iron deprived state in blood and tissues leading to inhibition of pathogen growth (Symeonidis and Marangos, 2012). The growth and biofilm formation of *S. epidermidis* under iron deprived condition could be a survival mechanism, which explains the ability of *S. epidermidis* to withstand unsuitable environmental conditions and cause persistent and chronic infections.

This research was also able to identify culture conditions that promoted biofilm production in the biofilm negative strain *S. epidermidis* 1585. Thus biofilm production and Embp expression was demonstrated in IMDM, RPMI+ and DMEM, which may be

due to the lack of iron. This finding correlates with those of Deighton *et al* who found that stress conditions (like iron depletion) can induce biofilm formation in biofilm negative strains (a situation which is present in IMDM) or provoke biofilm formation under low iron conditions (Deighton and Borland, 1993; Baldassarri, Bertuccini et al., 2001; Vuong, Kidder et al., 2005). Furthermore, these conditions could induce phenotypic variation which convert normal skin flora to pathogenic microorganisms upon entering the host and the low iron environment.

Biofilm induction in *S. epidermidis* 1585 was examined using cell culture media and serum. Previous work by Christner *et al* (Christner, Franke et al., 2010) showed that biofilm could be induced in this particular strain by goat serum although this could not be reproduced in this present study using three different serum concentration (50%, 60%, and 70%). The possible reason for this could be due to the different sources of goat serum (Difco and Invitrogen). However, cell culture media including RPMI+, IMDM and DMEM induced biofilm formation by *S. epidermidis* 1585, which could be due to glutamine, as all the three media contain glutamine. The observed biofilm formation in selected media could indicate the important of certain amino acids for recognised mechanisms of *S. epidermidis* biofilm formation.

Amino acid catabolism is important for staphylococcal biofilm formation (Resch, Rosenstein et al., 2005; Resch, Leicht et al., 2006). Bacteria have established mechanisms for the selective transport of amino acids into the cytosol. This allows acquisition of the necessary amino acids from the local environment (Beenken, Dunman et al., 2004; Horsburgh, Wiltshire et al., 2004). The metabolic requirement of *S. aureus* growing as a biofilm differs from that of planktonic growth. In a biofilm *S. aureus* selectively utilises certain amino acids compared to planktonic cells (Zhu, Weiss et al., 2007). The ability of staphylococci to use amino acids present in the host environment during infection is an important factor contributing to growth and pathogenesis (Horsburgh, Wiltshire et al., 2004). These finding suggest that different nutritional requirement are needed for different *S. epidermidis* strains to induce biofilm formation *in vitro*. The cell culture media used and resulting biofilm formation summarized in Table 3.1.

Table 3.1 Biofilm formation by *S. epidermidis* strains cultured in different cell culture media.

	Culture medium and resulting biofilm formation					
Biofilm dependent mechanism/strain	**TSB**	**RPMI+**	**RPMI-**	**DMEM**	**DEMEM/F12**	**IMDM**
PIA/ *S. epidermidis* 1457	+++*	+	++ *	+	+++	+++*
PIA/ *S. epidermidis* 8400	+++	+	+	+	+	+
PIA/ *S. epidermidis* 9142	+++	+	+	+	+	+
Embp/ *S. epidermidis* 1585-RA	++ *	+ *	- *	- *	- *	+ *
Aap/ *S. epidermidis* 5179-R1	+++*	++	+ *	-	-	+ *
S. epidermidis 1585	-	++	-	+	-	+

Addition of whole fetal bovine serum * or whole human serum reduced biofilm development; +++, sunstantial biofilm formation, ++, moderate biofilm formation, +, limited biofilm formation, -, no biofilm formation.

S. epidermidis is part of the normal skin flora, however when entering the human host it develops a pathogenic phenotype. This study hypothesizes that there are certain factors in host blood or tissues which modify this transformation. To explore this theory, serum was added to TSB and cell culture media and examined the effect of this on growth and biofilm formation.

FBS and PS (0.1-10%) had no effect on *S. epidermidis* growth, although inhibition of biofilm formation was evident. Interestingly, the test strains had different sensitivities to serum, with PIA dependent biofilm accumulation being less sensitive to that of Aap and Embp dependent biofilm development.

Patel *et al* demonstrated that the presence of adsorbed serum proteins suppresses the initial adhesion of *S. epidermidis* (Patel, Ebert et al., 2007) and Liu *et al* demonstrated an inhibitory effect of FBS coated surface on bacterial adhesion (Liu, Strauss et al., 2008) which supports findings from this study. In contrast, the presence of 20% serum increased bacterial adhesion and aggregation on both anionic and cationic charged surfaces (MacKintosh, Patel et al., 2006).

The presence of PS in the media appeared to have a more potent inhibitory effect on biofilm formation than FBS, especially for Aap biofilm dependent strains. These finding correlate with those of Abraham and Jefferson (Abraham and Jefferson, 2010) who demonstrated that 50% FBS prevented *S. aureus* biofilm formation, whilst only 10% human serum was needed. Others have suggested the importance of certain human serum proteins such as apo-transferrin, which had an inhibitory effect on bacterial adhesion and biofilm formation in *S. aureus* and *S. epidermidis* (Ardehali, Shi et al., 2002; Ardehali, Shi et al., 2003).

To study the influence of adhesion on biofilm formation, this research examined *S. epidermidis* biofilm development on polystyrene surface pre-coated with serum. Coating had no effect on *S. epidermidis* 1457 biofilm formation, whilst it prevented biofilm formation by the protein dependent biofilm of *S. epidermidis* 5179-R1 and *S. epidermidis* 1585-RA and the biofilm negative *S. epidermidis* 1585 (induced). This may indicate a variation in surface requirement to achieve primary adhesion between carbohydrate mediated biofilm and protein mediated biofilm formation. The influence of a conditioning film has generated variable result regarding biofilm development. For instance, Pihl *et al* (Pihl, Arvidsson et al., 2013) showed that *S. epidermidis* adherence to 10% serum-coated peritoneal catheters was four times greater than to uncoated ones. Furthermore, growing *Candida* spp on surfaces coated with serum showed that biofilm formation increased with increasing serum concentration (Nikawa, Nishimura et al., 2000). Others have suggested that adsorption with 20% serum inhibits the initial stages of adhesion, but within 24 hours incubation, a mature biofilm developed that did not differ from biofilm formed on non-adsorbed surfaces (Patel, Ebert et al., 2007). It was therefore assumed that in the case of Aap and Embp dependent biofilm, the coating serum completely prohibited primary attachment as within 24h a measurable biofilm

was still not evident. This is similar to studies with *Pseudomonas aeruginosa* biofilm formation where pre-coating with adult bovine serum and bovine serum albumin has been shown to be inhibitory (Hammond, Dertien et al., 2010).

To further investigate the effect of serum on biofilm formation, the FBS and PS was fractionated according to MW using Amicon Ultra centrifugal filter units. It was found that the serum inhibitory effect observed in whole serum was present in the serum fractions with MW above 30000kDa. PS fractions had greater inhibitory effects compared to equivalent FBS fractions. This effect may be attributed to albumins, transferrin, immunoglobulins and lipoproteins (Tirumalai, Chan et al., 2003) which are the major serum proteins with MW above 30000kDa. It seems that the factors in serum fractions above 30000kDa are highly efficient at inhibiting biofilm formation as all dilutions (0.1-10%) of this fraction inhibited biofilm formation. This may be due to blocking effect of the proteins in these fractions that interfere with adhesive capability of different *S. epidermidis* adhesins.

There are many surface proteins that can initiate primary attachment of *S. epidermidis* to surfaces and promote adhesion including *Staphylococcus* surface proteins SSP-I and SSP-II (Veenstra, Cremers et al., 1996), extracellular matrix binding proteins like Autolysin AtlE, fibronectin binding protein (Embp) (McCann, Gilmore et al., 2008), fibrinogen binding (Fbe) (Nilsson, Frykberg et al., 1998), collagen binding protein (GehD) (Bowden, Visai et al., 2002), and the cell surface associated proteins, Sdrf, SdrG and SdrH (McCrea, Hartford et al., 2000).

This present study also demonstrated biofilm induction under certain conditions. In *S. epidermidis* 5179-R1, FBS fractions below 30000kDa in RPMI- stimulated biofilm formation (the opposite effect was found in the same fractions of PS). This might be due to the 'lack of glutamine' which is unmasked when the inhibitory effect of high MW fractions is removed. Our FBS data contrasts, whilst the PS data concurs with findings of Abraham and Jefferson (Abraham and Jefferson, 2010), where an inhibitory effect on *S. aureus* biofilm formation was identified with serum fractions below 3000kDa. The effects of serum on biofilm formation suggested that there are host factors responsible for both inducing and inhibiting biofilm formation confirming the importance of the host in the final biofilm response Figure 3.14.

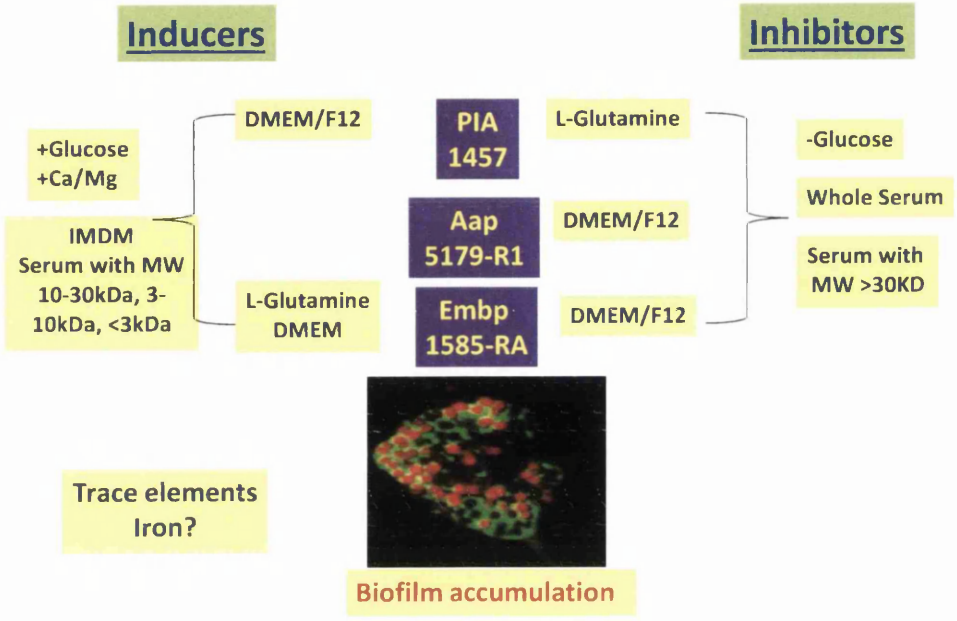


Figure 3.14 Factors affecting *S. epidermidis* biofilm formation.

Chapter 4

Purification of PIA produced by *S. epidermidis* biofilms cultured on different substrates and growth media

4.1 Introduction

PIA purification was first described by Mack *et al* in 1994 (Mack, Nedelmann et al., 1994) when *S. epidermidis* was grown in TSB, the supernatants recovered, dialyzed, and chromatography used to separate PIA fractions. However difficulties exist in using PIA purified by this method for study of host pathogens interactions. These difficulties include the fact that i) TSB is not sufficient to support human cells in culture, as TSB induced apoptosis in immune cells (Thurlow, Hanke et al., 2011; Hanke and Kielian, 2012); ii) TSB could be contaminated with lipopolysaccharide (LPS) which could interfere with interpretation of results, as macrophages can be activated by LPS concentrations as low as 15pg/ml (Wakelin, Sabroe et al., 2006); iii) Purified PIA could be contaminated with TSB media components; iv) PIA is lost during the dialyses process.

Defining a physiological medium that supports both bacterial and human cells and allows for PIA production with few contamination so that their interaction may be studied was partly addressed in chapter 3. Thus, identifying cell culture media that supports the growth and biofilm formation of different strains of *S. epidermidis*, in Chapter 3 will inform the larger scale production of purified PIA in this chapter (Figure 4.5) and will be used to allow study of host pathogens interactions (Chapter 5 and 6). The importance of medium and atmosphere for Staphylococcus biofilm formation was addressed in previous studies. Hussain *et al.* found that biofilm formation of *S. epidermidis* cultured in a chemically defined medium called Hussain-Hastings-White modified medium (HHW) with 5% Co_2 unaffected compared to *S. epidermidis* cultured under same condition in TSB were biofilm formation prohibited (Hussain, Wilcox et al. 1992). Similar effect was confirmed by Stepanovic *et al.* who found that incubation in Co_2 rich environment significantly reduce biofilm formation by both *S. aureus* and *S. epidermidis* (Stepanovic, Djukic et al., 2003).

In this chapter:

- PIA was purified on a large scale (milligrams) for cell culture studies.
- Purification methods from planktonic bacteria and from adherent biofilms were compared.

4.2 Methods

This present study modified a previously described methodology, referred from here on in as the (Mack Method). This method was modified by using IMDM instead of TSB/G+ to culture *S. epidermidis* 1457. Initially, *S. epidermidis* 1457 cultured into 6 glass beakers, a negative control culture of the biofilm negative *S. epidermidis* 1457-10 was similarly prepared. The biofilms obtained from culturing *S. epidermidis* 1457 in IMDM in glass beakers was weak and small in amount (Figure 4.1) and the amount of PIA was low in compare to Mack method as will show in 4.3. Thus, cell culture plates used as opposed to glass beakers. This was undertaken in order to try and increase PIA yield. The PIA purification using IMDM in cell culture plates is described in the materials and methods (section 2.9.1). In brief, on day 1, 5ml of IMDM was inoculated with *S. epidermidis* 1457 and incubated overnight at 37°C (Pre-culture). Twenty ml of IMDM was added to NUNC cell culture plates followed by 200µl of the pre-culture. This preparation was incubated for 48 hours without shaking at 37°C. After 72 hours the biofilms were scraped from the plates using a sterile cell scraper and the purified extracts were applied directly to Q-Sepharose columns without the need for a dialysis step (preventing potential PIA loss in this process). Then, 15 consecutive 2ml fractions were collected. Confirmatory assays for PIA included ELISA, Hexosamine assay, Coagglutination assay and Hexose assay and were done on all the 15 fractions and the pooled fractions (tubes 1-2, 3-7, 8-10 and 11-15) pooled together as described in section 2.9.2 (Mack, Nedelmann et al., 1994; Mack, Bartscht et al., 2001).

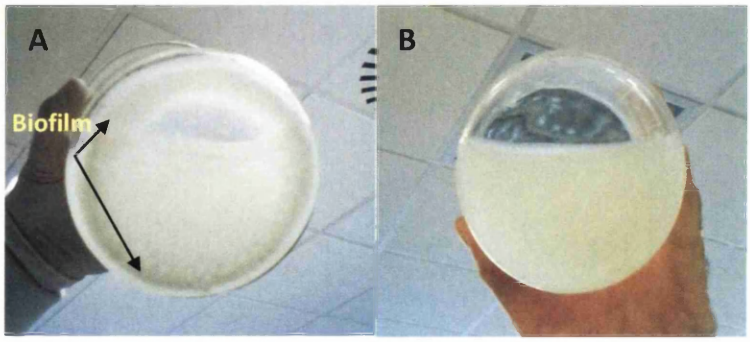


Figure 4.1 PIA purification from biofilm cultured in IMDM in a glass beakers.
A. Biofilm formation of 1457 *S. epidermidis* cultured in IMDM **B.** Biofilm negative *S. epidermidis* 1457-M10 cultured in IMDM.

Results

4.3 Comparison of PIA preparation by the Mack and IMDM methods

To compare PIA production from the Mack and IMDM methods, several confirmatory assays were performed on pooled extracts from six glass beakers cultures. Two isolates was cultured either in TSB (Mack method) or IMDM and there were *S. epidermidis* 1457 (PIA dependent) and a negative control NC of *S. epidermidis* 1457-M10. Fractions obtained from chromatography (section 2.9.1) were pooled as following: fractions 1-2, fractions 3-7, fractions 8-10 and fractions 11-15 together.

A total Hexosamine assay was undertaken as described previously in the materials and methods (section 2.9.2.3) to estimate the amount of PIA produced. The amount of PIA in purified extracts obtained by Mack method was more than that obtained by growing *S. epidermidis* 1457 in IMDM in glass beakers (Table 4.1). Purity of the pooled fractions was restricted to one pooled fraction in extracts isolated from IMDM, whereas, hexosamine sugars could be detected in other pooled fractions isolated from TSB. In addition, biofilms produced by IMDM were noticeably weaker, detaching easily from the surface of the beaker as in Figure 4.1 A.

An optimized PIA ELISA (section 2.9.2.1.2) was used to detect the antigenic presence of the PIA molecule. Pooled PIA fractions and the NC extract from the IMDM method were assayed by ELISA (1:20, 1:100 and 1:500 dilutions) together with the pooled fractions of PIA (tubes 3-7), and pooled fractions of NC (tubes 3-7) prepared by the Mack method (1:500 dilution) (Table 4.2). The optimized PIA ELISA confirmed the presence of PIA in extracts isolated by both methods and alluded to higher levels generated by the Mack method (Table 4.2).

Hexose sugar was quantified in the purified PIA extract by a Hexose assay (section 2.9.2.4) with a robust standard curve. This assay confirmed the absence of hexose in all samples tested.

Table 4.1 Hexosamine assay of PIA fractions obtained by Mack and IMDM method.

Pooled fractions	1457-TSB P1	1457- TSB P2	NC- TSB	1457-IMDM	NC-IMDM
1-2	1 µg/ml	21 µg/ml	0µg/ml	0 µg/ml	0µg/ml
3-7*	224 µg/ml	156 µg/ml	0µg/ml	131 µg/ml	0µg/ml
8-10	21 µg/ml	42 µg/ml	0µg/ml	0 µg/ml	0µg/ml
11-15	22 µg/ml	24 µg/ml	0µg/ml	0 µg/ml	0µg/ml

P1, preparation one; P2, preparation two; NC, negative control. * The pooled fractions should contains PIA.

Table 4.2 PIA ELISA of the pooled fractions of PIA prepared by Mack and IMDM method

	PIA extract/nm			NC extract/nm		
	1:20	**1:100**	**1:500**	**1:20**	**1:100**	**1:500**
IMDM 1-2	0.144	0.108	0.119	0.32	0.112	0.114
IMDM 3-7	0.803	0.438	0.202	0.209	0.204	0.246
IMDM 8-10	0.22	0.139	0.121	0.243	0.166	0.133
IMDM 11-15	0.209	0.174	0.152	0.251	0.171	0.131
TSB 3-7			0.768			0.118

The numbers represent the absorbance value at OD=450nm.

4.4 Up-scaling of PIA preparation using adherent biofilms produced in IMDM using cell culture plates

IMDM cell culture media supported *S. epidermidis* 1457 biofilm formation, however it was reasoned that glass was not the optimum surface for biofilm formation. Indeed biofilm assays are routinely done in NUNC 96 well cell culture plates. An approach was used involving the inoculation of a NUNC 90mm cell culture plate with an *S. epidermidis* 1457 pre-culture grown in IMDM. Robust biofilms could be seen at 24 and 48 hours following inoculation.

To quantify the amount of PIA purified from *S. epidermidis* 1457 grown in IMDM using 6 and 24 cell culture plates, hexosamine assays were carried out as described in materials and methods (section 2.9.2.3) for all the purified fractions 1-15 (Figure 4.2). There was a large increase in the amount of PIA obtained from 24 cell culture plates in comparison to the amount of PIA obtained from 6 cell culture plates. Critically for both preparations, PIA as determined by hexosamine was highest in fractions 3-7. In both preparations fraction 3 resulted in peak PIA output of 1187 µg/ml (from 24 plates) and 200µg/ml (from 6 plates) of PIA respectively (Figure 4.2).

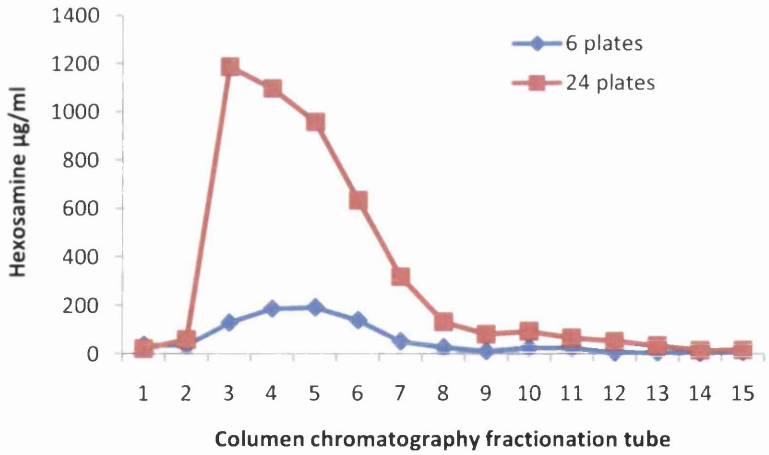


Figure 4.2 Hexosamine assays of PIA fractions obtained by the IMDM method using 6 and 24 cell culture plates. Assay carried out on all 15 fractions for both 6 and 24 plates preparations.

After confirming the amount of PIA in all purified fractions obtained from growing *S. epidermidis* 1457 in 6 and 24 cell culture plates with IMDM, the PIA fractions were pooled together (1-2, 3-7, and 8-15) and the Hexosamine assay repeated. In the pooled fraction of tubes 3-7 which contained the majority of Hexosamine, the PIA increased from 103µg/ml in 6 plates to 603µg/ml in preparation 1(P1) of 24 plates and 1065µg/ml in preparation 2 (P2) of 24 plates .

To determine antigenic presence of PIA the optimized ELISA was used as previously described. The optimized PIA ELISA confirmed the presence of PIA in extracts isolated by both methods, PIA levels were higher in fractions obtained from 24 cell culture plates in comparison with PIA obtained from 6 cell culture plates, and higher levels were detected in pooled fractions 3-7 as shown in table 4.3

Table 4.3 PIA ELISA of pooled fractions obtained by IMDM method

Pooled fractions	6 plates/nm	24 plates/nm
1-2	0.12	0.38
3-7	2.57	2.36
8-10	1.83	1.63
11-15	1.36	0.37

The numbers represent the absorbance value at OD=450nm.

To further quantify the presence of functional PIA, a coagglutination assay (section 2.9.2.2) was performed on pooled fractions (3-7) obtained from 6 and 24 plates preparations. Furthermore, extracts from biofilm negative *S. epidermidis* 1457-M10 and PBS were used as negative controls. PIA fractions from the 6 plate preparation had minimum active concentrations at 1/2 dilutions, whilst fractions from 24 plate preparations had minimum active concentrations at 1/502. Similar fractions from *S. epidermidis* 1457-M10 (NC) showed no activity in this assay (Table 4.4).

Table 4.4 Coagglutination assay for PIA detection

	Neat	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/502	1/1024
6 PIA	+	+	-	-	-	-	-	-	-	-	-
24 PIA	+	+	+	+	+	+	+	+	+	+	-
24 PIA	+	+	+	+	+	+	+	+	+	+	-
NC	-	-	-	-	-	-	-	-	-	-	-
PBS	-	-	-	-	-	-	-	-	-	-	-

PIA, polysaccharide intercellular adhesin; NC, negative control; PBS, phosphate buffered saline; neat, used samples without any dilution; +, coagglutination detected; -, absence of coagglutination.

4.5 Lipopolysaccharide (LPS) detection assay

The LAL Chromogenic Endotoxin Quantitation Kit (section 2.9.3) was used to measure the amount of LPS in our purified PIA samples. The assay produced a robust standard curve (Figure 4.3) and reading were negative in all the purified samples obtained by IMDM methods, involving both samples obtained from biofilm forming *S. epidermidis* 1457 and samples obtained from biofilm negative *S. epidermidis* 1457-M10 which was used as negative controls.

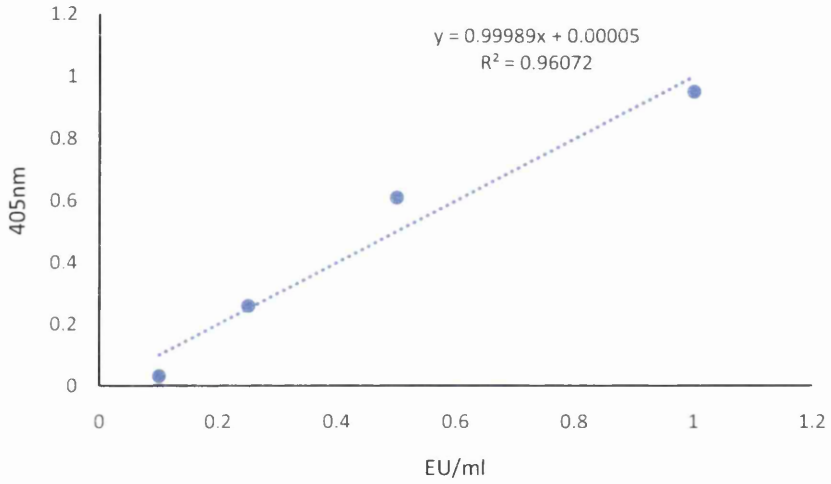


Figure 4.3 Standard curve of LPS detection assay.

4.6 Confirmation of improved IMDM method

To compare material input and PIA output between the methods modified within this chapter an assessment of the volume of starting culture, the surface, including its area and the constituent material, and the final yield of PIA within the purified sample was done (Table 4.5). In moving from 6 beakers to 6 tissue culture plates, the total volume of starting culture was decreased 12-fold, despite the surface area and bacterial yield remaining equal. Furthermore a 4-fold increase in the number of tissue culture plates resulted in a 6-10-fold increase in yield, but with 3-fold less starting culture from the original Mack method (Table 4.5).

Table 4.5 Comparison of modified PIA preparation methods

Method	Surface Material	Volume (ml)	Surface area cm^2	PIA (µg)	PIA /18ml
Mack / P1	6 Beaker-Glass	6 x 300 = 1800	6 x 55 = 330	224	4 mg
Mack/P2	6 Beaker-Glass	6 x 300 = 1800	6 x 55 = 330	156	2.8mg
IMDM	6 Beaker-Glass	6 x 300 = 1800	6 x 55 = 330	131	2.35mg
IMDM	6 NUNC-TC dish	6 x 25 = 150	6 x 55 = 330	103	1.85 mg
IMDM/P1	24 NUNC-TC dish	24 x 25 = 600	24 x 55 = 1320	603	10 mg
IMDM/P2	24 NUNC-TC dish	24 x 25 = 600	24 x 55 = 1320	1065	19 mg

P1, preparation one; P2, preparation two.

4.7 Discussion

Production of polysaccharide intercellular adhesin (PIA) is a major mechanism of *S. epidermidis* biofilm formation (Mack, Riedewald et al., 1999; Rohde, Frankenberger et al., 2010; Spiliopoulou, Krevvata et al., 2012), and plays an important role in bacterial primary adhesion to a surface and subsequent intercellular adhesion within the biofilm (Vuong, Voyich et al., 2004). PIA is considered a major virulence factor of *S. epidermidis* (Rupp, Ulphani et al., 1999) that helps *S. epidermidis* evade the immune system (Vuong, Voyich et al., 2004).

The ability to grow *S. epidermidis* biofilm and isolate purify PIA without any cross contamination from the culture medium is essential in order to study PIA composition and determine its biological effect on various aspects of the immune system. TSB has previously been the medium of choice to culture *S. epidermidis* biofilms (Christensen, Simpson et al., 1982), with the resultant biofilm undergoing a complex purification process to get a highly purified PIA without contamination from medium components. Studies carried out by Tojo *et al* and Ludwicka *et al* (Ludwicka, Uhlenbruck et al., 1984; Tojo, Yamashita et al., 1988) used a complex medium to grow biofilms and a complicated procedure for PIA purification in which contamination could not be excluded. Others developed a chemically defined medium that induced biofilm formation, in which different combinations of amino acids, vitamins and glucose produced biofilm by *S. epidermidis* (Hussain, Hastings et al. 1991).

To study PIA properties and its subsequent effects on the host, it was necessary to obtain an efficient method to purify PIA. Previously, Mack *et al* developed a purification process (Mack, Nedelmann et al., 1994) in which they used a TSB/G+ medium to culture *S. epidermidis* 1457 biofilm and used chromatography for the purification process. This method was modified by Gerke et al who extracted PIA attached to bacterial cell surfaces by boiling in 0.5M EDTA buffer (ethylene-diamine-tetraacetic acid) for 5 min at 100°C (Gerke, Kraft et al., 1998). Further modifications from Vuong and Otto (Vuong and Otto, 2008) involved boiling and dialysis of PIA extract, followed by enzyme digestion steps including treatment with DNase, RNase, Lysostaphin, Lysozyme and proteinase K before chromatography.

The disadvantage of these methods have been; i) the use of TSB as the growth medium may lead to PIA contamination; ii) the potential loss of PIA in the dialysis step. Due to these limitations the need for a highly purified PIA without contamination to asses host / PIA interaction, this study modified the protocol developed by Mack by replacing TSB/G+ with IMDM which supported biofilm formation to a similar extent to TSB (Chapter 3, Figure 3.1).

S. epidermidis 1457 grown in glass beakers using IMDM produced detectable PIA but the amount was much less compared to Mack *et al*, although purity appeared concentrated in discrete fractions. The reduced yield under these circumstances was not surprising considering the major roles of growth media (as shown in Chapter 3) and (Deighton and Balkau 1990) and surface substrate play in biofilm formation (Bos, van der Mei et al. 1999; Patel, Ebert et al. 2003; MacKintosh, Patel et al. 2006).

Staphylococcus epidermidis is a major cause of device related infection, in which biofilm formation is the main virulence factor (O'Gara and Humphreys, 2001). Bacterial adherence and biofilm formation of *S. epidermidis* can be affected by the type of the biomaterials and their chemical and physical surface properties (Patel, Ebert et al., 2007). Bacterial adherence and biofilm formation to intraocular lenses are increased when the lenses are made from polymethyl methacrylate as bacteria have greater avidity for the irregular surface of these lens type (Pinna, Zanetti et al., 2000; Kodjikian, Roques et al., 2006).

Surface properties play an important role in biofilm formation and subsequent development of catheter associated urinary tract infections. The use of antiseptic- coated silver oxide urinary catheters reduces bacterial colonization and biofilm formation (Schumm and Lam, 2008) compared with antiseptic-coated silver alloy catheters and standard polytetrafluoroethylene coated catheters (Pickard, Lam et al., 2012).

In this study it was hypothesised that the presence of a suitable surface would enhance primary bacterial attachment, which is essential for subsequent biofilm formation in IMDM (Chapter 3). Thus inoculating *S. epidermidis* 1457 into cell culture plates overcame this inhibitory effect (in beakers) and supported primary attachment, which

consequently led to formation of a larger quantities of biofilm. Furthermore, increasing the surface area 4X (6 to 24 plates) increased the amount of PIA obtained by 6-10 folds (100µg/ml from 6 plates to 600-1000µg/ml in 24 plates). These simple findings demonstrate that increasing the adherent surface area available to bacteria is a key point in obtaining significantly increased biofilm biomass, which ultimately leads to dramatic increases in the amount of PIA purified (Figure 4.4).

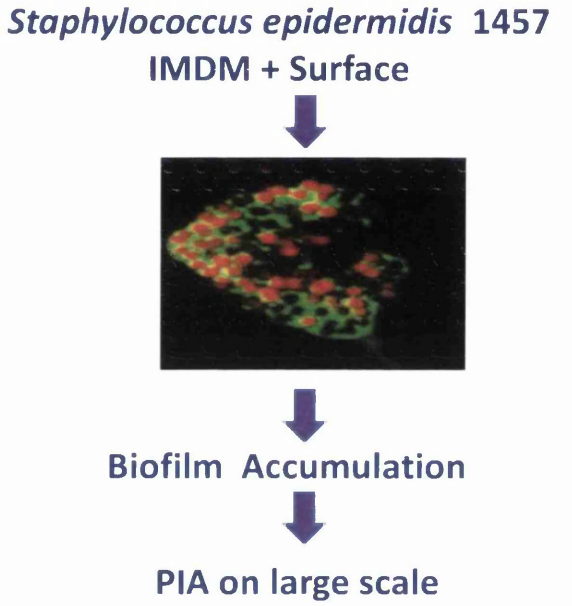


Figure 4.4 Biofilm formation by *S. epidermidis* 1457 in IMDM using cell culture plates.

Finally, bacterial lipopolysaccharides (LPS) are a component of the Gram-negative bacteria cell wall and as such are considered major virulence factors (Fujihara, Muroi et al., 2003). LPS activates macrophages through Toll –like receptor 4 (TLR4), which produces a variety of inflammatory cytokines (Heumann and Roger, 2002). The ubiquitous nature of LPS results in cell culture contamination and increased background levels during *in vitro* experiments (Wakelin, Sabroe et al., 2006). Removing LPS can prove difficult and use of TLR-4 deficient cells is not always practical, thus in these studies the confirmatory results that our PIA preparations contain <15pg/ml LPS is

particularly encouraging. PIA produced by this method will be used in the host pathogen interaction experiments described in Chapter 5 and 6.

Chapter 5

The effect of soluble, cell- associated and biofilm- associated PIA on THP-1 cells: importance of tethering for cytokine production

5.1Introduction

PIA is major virulence factor of *S. epidermidis* and essential for its pathogenesis (Nedelmann, Sabottke et al., 1998; Rupp, Ulphani et al., 1999). PIA is a linear β1,6- N-acetylglucosamine containing positive charges due to deacetylated amino groups and negative charges due to O-succinoyl ester residues (Heilmann, Schweitzer et al., 1996; Mack, Fischer et al., 1996), PIA is produced by *ica* locus, which is composed of the operon encoding icaADBC-membrane bound enzyme complex (Vuong, Kocianova et al., 2004; Cerca, Jefferson et al., 2007).

PIA has dramatic effects on the innate immune system when tested during *in vitro* assays. Indeed, PIA has been shown to protect planktonic wild-type *S. epidermidis* from the killing effects of neurophils and antimicrobial peptides to a greater extent than its PIA negative mutant (Vuong, Kocianova et al., 2004; Vuong, Voyich et al., 2004). Kristian *et al* (Kristian, Birkenstock et al., 2008) demonstrated that PIA dependent biofilms protected *S. epidermidis* from killing by neutrophils through increasing C3 complement secretion and prevention of C3b and IgG deposition on the bacterial cell surface. PIA expressing biofilm positive *S. epidermidis* were protected from phagocytosis by macrophages compared to their isogenic deficient mutants through diminished activation of the inflammatory response by a mechanism involving reduced NF-κB activation and IL-1β production (Schommer, Christner et al., 2011). Leid *et al* (Leid, Shirtliff et al., 2002) demonstrated that leukocytes could penctrate PIA biofilm, but were unable to engulf bacteria inside biofilm compared to their planktonic counterparts. This finding suggests that there are mechanisms other than biofilm matrix that serve to discourage phagocytosis. In contrast, Wagner *et al* found that Polymorphonuclear leukocytes (PMN) were unable to penetrate biofilm and whilst 'paralysed' retained the ability to produce antimicrobial superoxides (Wagner, Kaksa et al., 2004).

The studies in this chapter investigate the effect of PIA on the immune system have used the purified PIA molecule (generated in chapter 4), PIA positive *S. epidermidis* and their negative controls in both planktonic and mature biofilm models. Little is known about the effect of purified PIA, cell associated PIA and biofilm associated PIA on isolated cells *in vitro*. For that PIA effect on cytokine release (IL-8, IL-10 and TNF-α) was

studied. IL-8 and TNF-α are pro-inflammatory cytokines release by PMN cells rapidly after exposure to infectious agent (Baggiolini and Clark-Lewis, 1992), IL-8 is a powerful chemotactic, attract neutrophils to the site of infection (Desbaillets, Diserens et al., 1997), whilst IL-10 is anti-inflammatory cytokines inhibit IL-8 and TNF-α release from macrophages (Cassatella, Meda et al., 1993). Furthermore, the traditional microbiology approache of using biofilm positive wild type *S. epidermidis* with an isogenic biofilm negative mutant often results in two dramatically different phenotypes that become difficult to compare due to their different forms. Recent studies demonstrated that PIA dependent biofilms can be disrupted by the enzyme dispersinB (DspB) which is a beta-1,6-N-Acetylglucosaminidase, originally identified in *Actinobacillus actinomycetemcomitans* (Kaplan, Ragunath et al., 2004; Ramasubbu, Thomas et al., 2005; Chaignon, Sadovskaya et al., 2007). DispB has the ability to digest PIA, removing the biofilm matrix and exposing biofilm bacteria to the host immune system and antibiotics (Venketaraman, Lin et al., 2008; Arciola, 2009; Kaplan, 2009).
In this chapter the studies undertaken were to:

- Investigate the influence of different forms of PIA on the immune response
- Model biofilm structure and leukocyte sedimentation
- Use Dsp.B to examine biofilm associated PIA effects

5.2 Methods

THP-1 cells were added to planktonically grown *S. epidermidis* 1457, preformed *S. epidermidis* 1457 biofilm or purified PIA to assess THP-1 effects on bacterial viability (section 2.11.4) and cytokines profiles (section 2.11.5) through using ELISA.

For killing assays, bacterial suspensions of *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 were incubated with THP-1 monocytes for 2 hours. Control cultures at zero time and without macrophages were also included. Bacterial viability was assessed through enumerating colony forming unit as described in section 2.8.3. For cytokine production assays, samples from THP-1 cells incubated with planktonic *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 for 6 hours, were tested for expression of IL-8, TNF-α and IL-10 using an ELISA kit as described in section 2.10.1.

For crude lysate effect, different concentration of crude supernatant prepared as in section 2.8.4 (1, 10, 50, 200) µl/ml added to $2X10^5$ THP-1 cells / 500µl of RPMI- made with 10%FBS and 1% L-glutamine and P/S. Control culture at zero time with PBS and PMA were included. Supernatant were collected at two time points, 6 and 24 hours, and an IL-8 ELISA was done for the samples as mentioned previously.

With regards to the effect of purified PIA on THP-1 cells, 60µg/ml of PIA and an equivalent volume of PIA- extract (as a negative control; NC) was incubated with THP-1 cells for 6 and 24 hours. IL-8 was then quantified by ELISA. THP-1 cells with and without Phorbol myristate acetate (PMA) was used as the controls (to assess cytokines production in PMA treated THP-1 cells).

For characterization of *S. epidermidis* 1457 biofilm structure, *S. epidermidis* 1457 was stained with SYTO9 (green) and the bacterial PIA matrix stained with tetramethylrhodamine conjugate of Wheat Germ Agglutinin (WGA, orange) (as in section 2.17.1). A 4 and 8 chamber slide was used to incubate *S. epidermidis* for 24 hours and 48 hours. The biofilm was then stained with SYTO9 and WGA fluorescent stain for 60 minutes. CLSM was then used to image the biofilm.

To examine the ability of THP-1 cells to penetrate *S. epidermidis* 1457 biofilm, THP-1 cells were added to preformed biofilm and migration assessed at three different time intervals (0-1, 1-2, 2-3 hours). *S. epidermidis* 1457 expressing green fluorescent protein (GFP) (Franke, Dobinsky et al. 2007) was also used along with THP-1 cells stained with

Cell Mask. CLSM was again used to investigate the penetration of leukocytes into the biofilm.

Results

5.3 The effect of cell associated PIA on THP-1 cells.

To investigate the effect of cell-associated PIA on THP-1 cells, planktonic *S. epidermidis* 1457 (biofilm/PIApositive) and 1457-M10 (biofilm/PIA negative) were both used to challenge THP-1 cells and functional killing of bacteria, phagocytosis and cytokine production then analysed.

Leukocyte killing assays showed that THP-1 monocytes had no significant effect on the growth or killing of either *S. epidermidis* 1457 or 1457-M10 after two hours, with both strains growing over the course of the assay (Figure 5.1). Phagocytosis assays confirmed there was no difference in the number of THP-1 cells containing *S. epidermidis* 1457 (1.25± 0.3%) bacteria/cell+/-SD and *S. epidermidis* 1457-M10 (0.5± 0%) bacteria/cell+/-SD of three independent experiments.

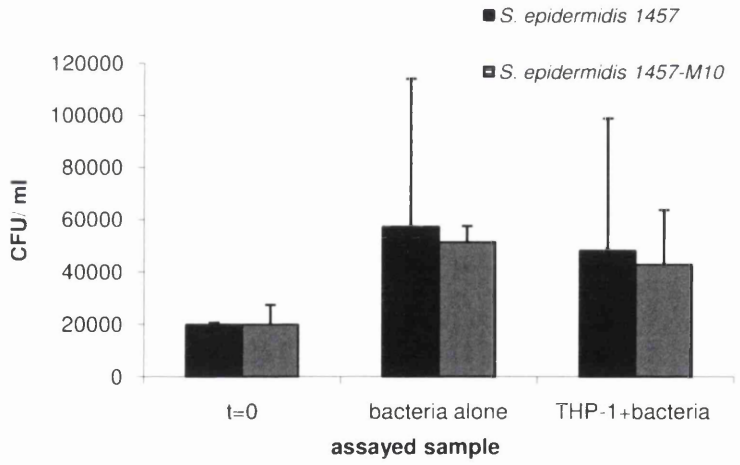


Figure 5.1. Effect of THP-1 leukocytes on bacterial viability. t=0 represents control culture at the start of the experiment, bacteria alone represents control culture after two hours without macrophages, THP-1+ bacteria is the combination of THP-1 cells and bacteria after two hours. Results represent data from 3 independent experiments.

Cytokine ELISA assays showed that planktonic *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 induced IL-8 release by THP-1 cells after 6 hours. Specifically, IL-8 release in response to *S. epidermidis* 1457 was nearly double the amount induced by *S. epidermidis* 1457-M10 bacteria and was statistically significant $P<0.05$ (Figure 5.2). Furthermore, adding planktonically grown *S. epidermidis* 1457 to THP-1 cells promoted TNF-α release compared with *S. epidermidis* 1457-M10 and this was statistically significant $P< 0.05$ (Figure 5.3).

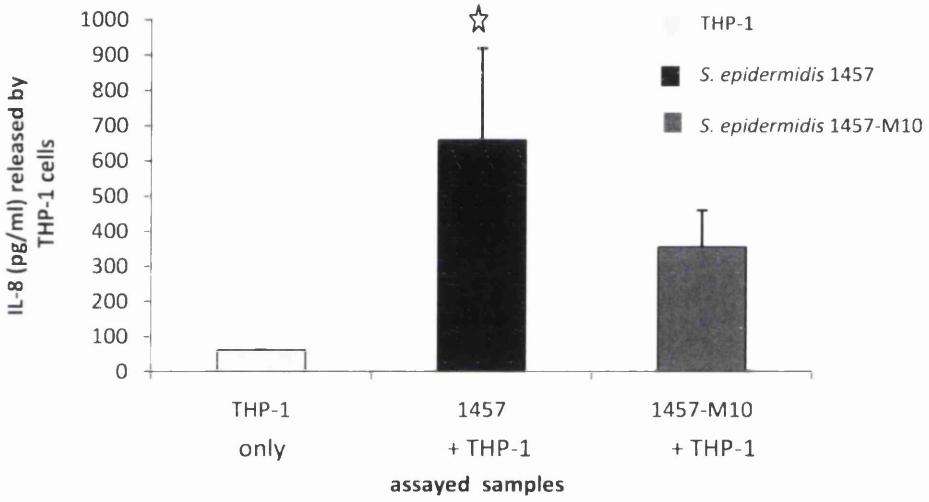


Figure. 5.2 Effect of cell associated PIA on IL-8 release from THP-1 cells. Comparison between *S. epidermidis* 1457 (PIA/biofilm) positive and *S. epidermidis* 1457-M10 (PIA/biofilm) negative. Results represent data from 3 independent experiments. Star indicates significant difference $P< 0.05$.

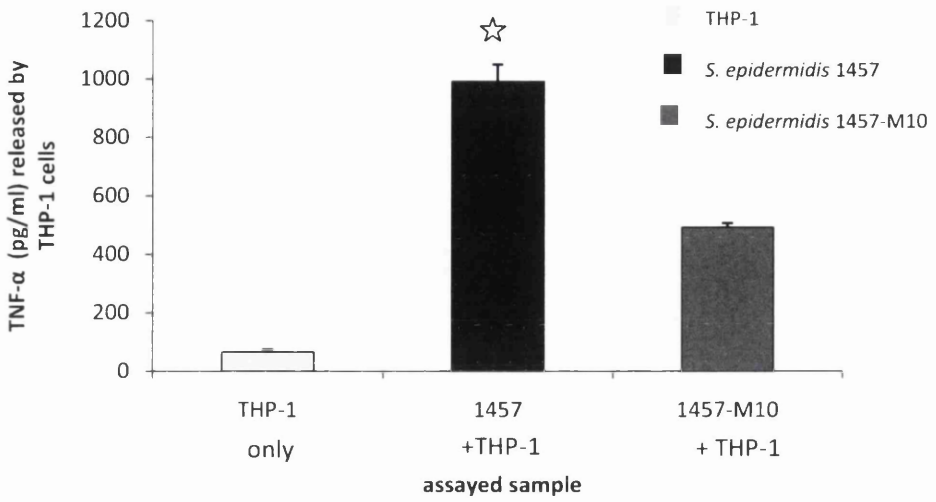


Figure. 5.3 Effect of cell associated PIA on TNF-α release from THP-1 cells. Comparison between *S. epidermidis* 1457 (PIA/biofilm positive) and *S. epidermidis* 1457-M10 (PIA/biofilm) negative. Results represent data from 3 independent experiments. Star indicates significant difference $P< 0.05$.

In contrast, IL-10 release from THP-1 cells in response to *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 showed no difference between each other or control THP-1 cells (Figure 5.4).

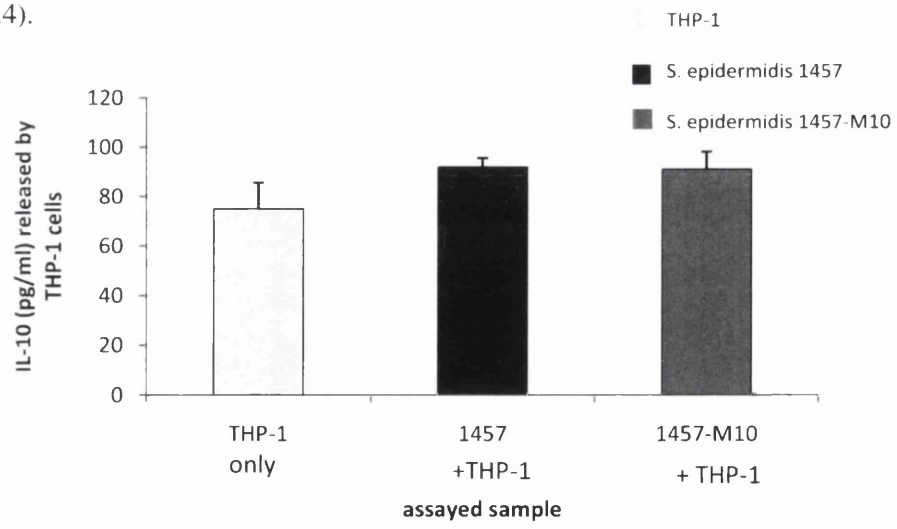


Figure. 5.4 Effect of cell associated PIA on IL-10 release from THP-1 cells.
Comparison between 1457 (PIA/biofilm) positive and 1457-M10 (PIA/biofilm) negative. Results represent data from 3 independent experiments.

5.4 The effect of soluble PIA on THP-1 cells

To investigate the effect of soluble PIA on THP-1 cells, crude lysate prepared from *S. epidermidis* 1457 (biofilm/PIA positive) and *S. epidermidis* 1457-M10 (biofilm/PIA negative), and purified PIA obtained by the IMDM method (Chapter 4) were used to examine their effects on cytokine production by THP-1 cells after 6 hours.

Cytokine assays showed that crude extracts induced IL-8 release from THP-1 cells, in a dose dependent manner (1:500, 1:50, 1:10, and 1:2.5 dilutions). IL-8 released in response to *S. epidermidis* 1457 crude lysate was higher than that induced by *S. epidermidis* 1457-M10 (Figure 5.5A). IL-8 production was 350pg/ml and 236pg/ml for *S. epidermidis* 1457 and *S. epidermidis* 1457-M10, respectively. Increasing the incubation time to 24 hours, increased IL-8 levels further, and at the highest dose of crude lysate IL-8 a peak level was achieved of 640pg/ml and 510pg/ml for *S. epidermidis* 1457 and *S. epidermidis* 1457-M10, respectively (Figure 5.5 B).

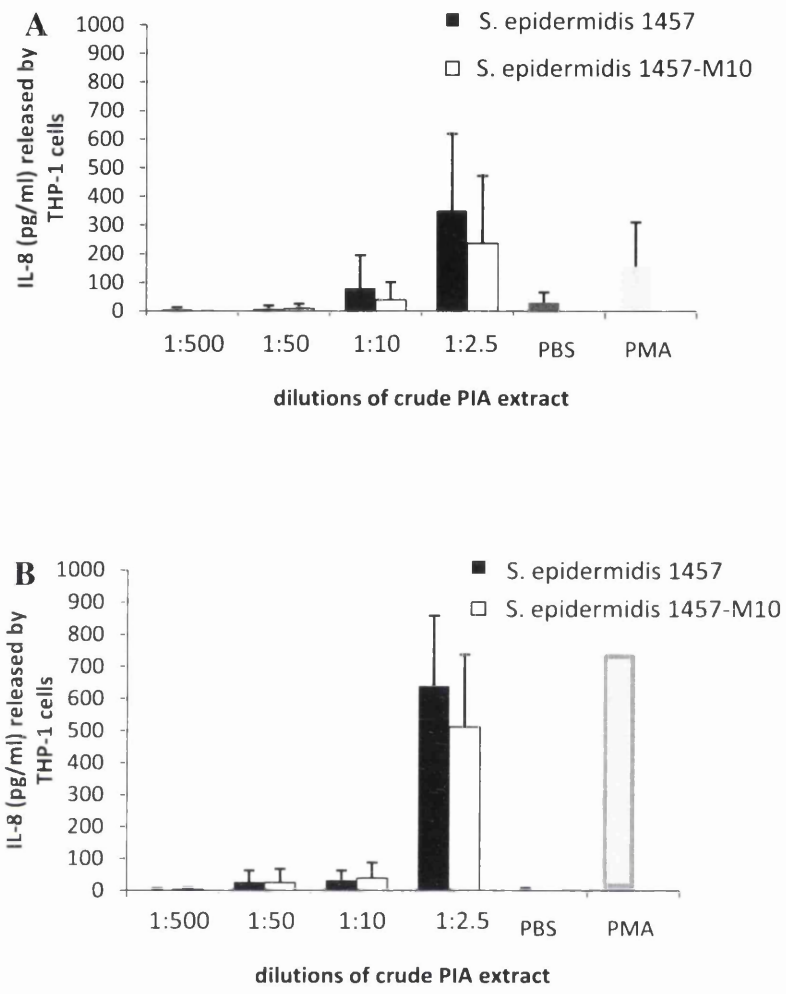


Figure 5.5 IL-8 cytokine release by THP-1 cells in response to dilution of *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 crude lysate. A. IL-8 release in 6 hours, **B.** IL-8 release in 24 hours. Results represent data from 3 independent experiments. PBS is phosphate buffered saline, used as negative control. PMA is phorbol myristate acetate, used as positive control.

To improve the specificity of these experiments the purified PIA (60µg/ml) produced in Chapter 4 was used. This PA had previously been shown to be biologically active by the co-agglutination assay (Figure 4.4).

Adding purified PIA to THP-1 cells did not induce IL-8 secretion after both 6 and 24 hours incubation, despite significant IL-8 release following stimulation with PMA and LPS (Figure 5.6).

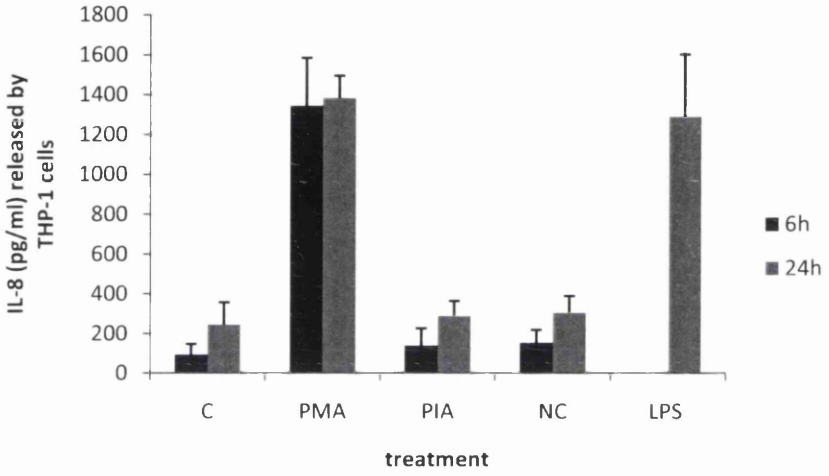


Figure 5.6 IL-8 cytokines release in THP-1 cell line in response to purified PIA extract. IL-8 ELISA preformed at 6 and 24 hours to estimate IL-8 release in response to different stimuli. The results shown are representative of three independent experiments. C, THP-1 cells only. PMA, phorbol myristate acetate, used as positive control. PIA, polysaccharide intercellular adhesin (purified) from *S. epidermidis* 1457. NC, extract from (PIA/biofilm) negative *S. epidermidis* 1457-M10. LPS, lipopolysaccharide, used as positive control.

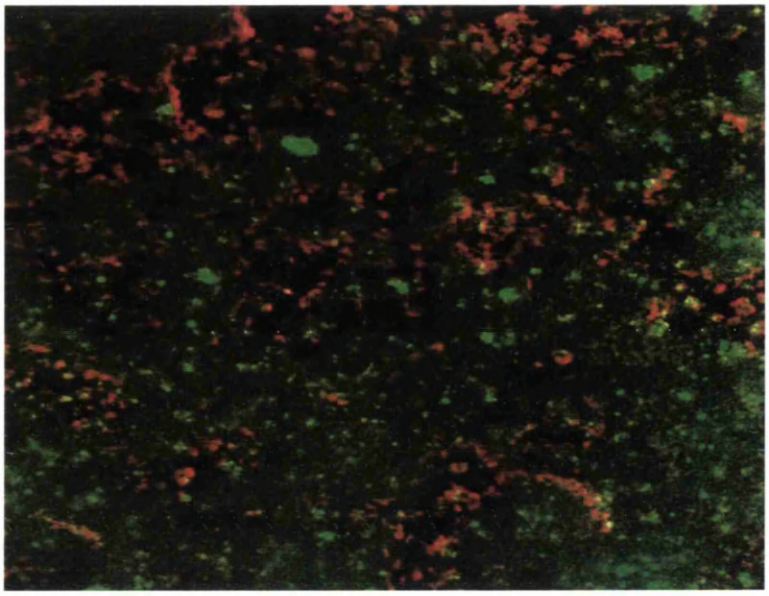
5.5 Structure of *S. epidermidis* biofilm associated PIA as determined by confocal laser scanning microscopy

To investigate the effect of biofilm associated PIA on THP-1 monocytes, the temporal changes taking place when moncytes were added to *S. epidermidis* biofilms had to be determined. These experiments served to inform later experiments on cytokine production.

Confocal scanning laser microscopy (CLSM) was used to define biofilm structure and follow the time course of monocytes penetration.

Confocal microscopy images revealed good staining of bacteria using SYTO9 (Green), whilst WGA (Orange) stained the PIA matrix surrounding and covering the bacteria (Figure 5.7A). *Staphylococcus epidermidis* 1457 formed robust biofilms (Figure 5.7 B-C) where bacterial microcolonies were embedded in extracellular PIA. Black areas devoid of stain were also observed. *Staphylococcus epidermidis* 1457 was present in high densities close/bound to the substratum and were embedded in the extracellular polymeric substance (EPS) of biofilm. Thus, moving from the bottom to the top of the biofilm, the numbers of bacteria gradually decrease with increasing amounts of EPS becoming evident (Figure 5.7B). This pattern was enhanced after 48 hours due to further production of EPS/PIA with a resulting increase in the thickness of biofilm (Figure 5.7C).

A



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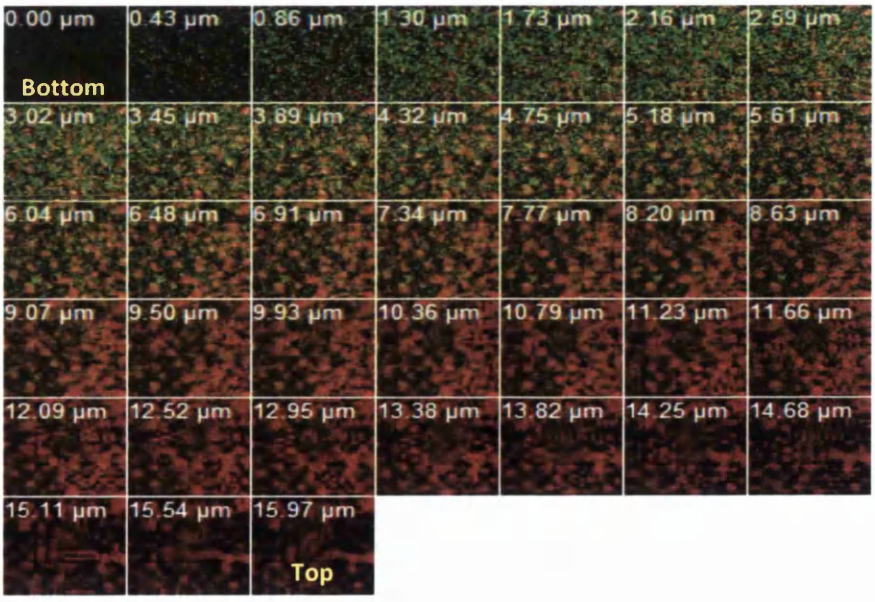
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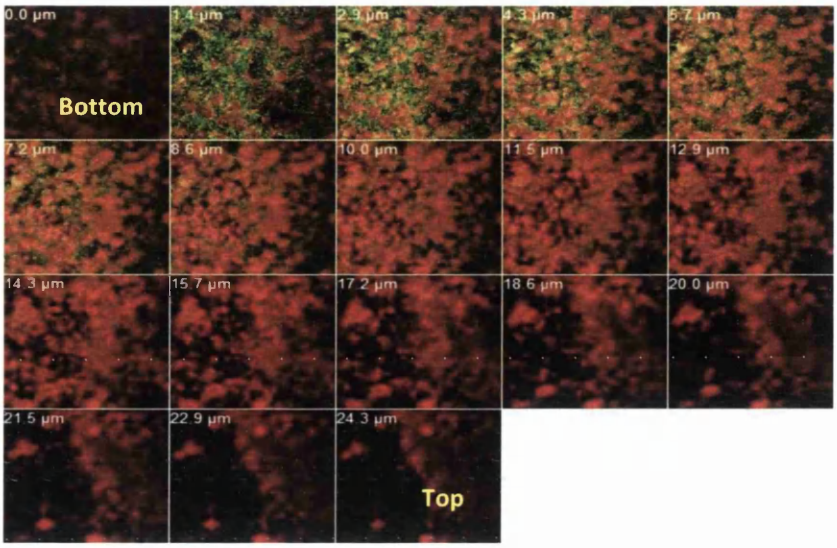


Figure 5.7 Characterisation of *Staphylococcus epidermidis* 1457 biofilm structure. Biofilm growth was viewed by CLSM, the green colour represents the *S. epidermidis* 1457 bacteria, and the orange colour represents the PIA matrix. **A.** Bacteria embedded in PIA matrix; **B.** Biofilm formation in 24 hours; **C.** Biofilm formation in 48 hours. The depth of the biofilm is indicated in the panels of 5.7b and 5.7c.

For characterisation of *S. epidermidis* 1457 biofilm viability, the live/dead ratio of cells in the biofilm was assessed by Syto 9 and Propidium Iodide (PI) fluorescent stain. Using CLSM, live / dead staining revealed that under normal conditions (nutrient, temperature and suitable environmental condition), over 90% of the cells in the normal biofilm were viable (Figure 5.8).

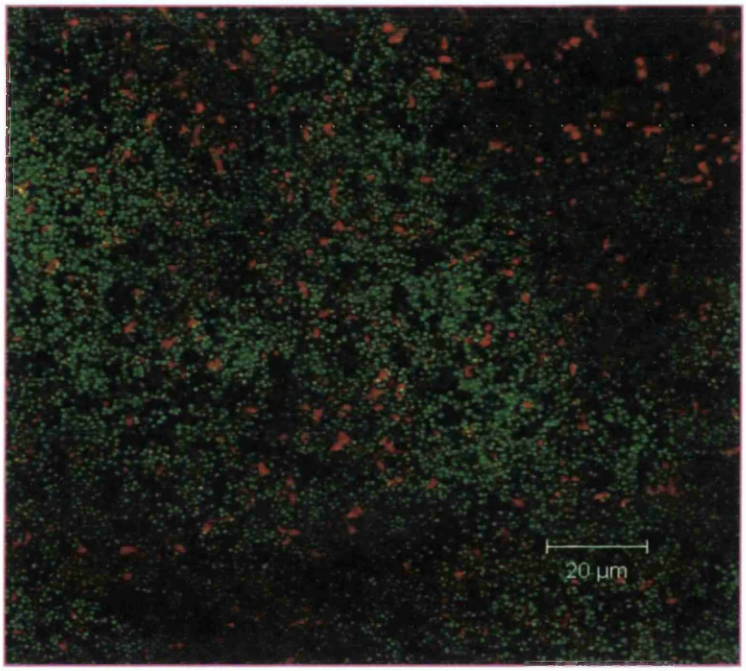


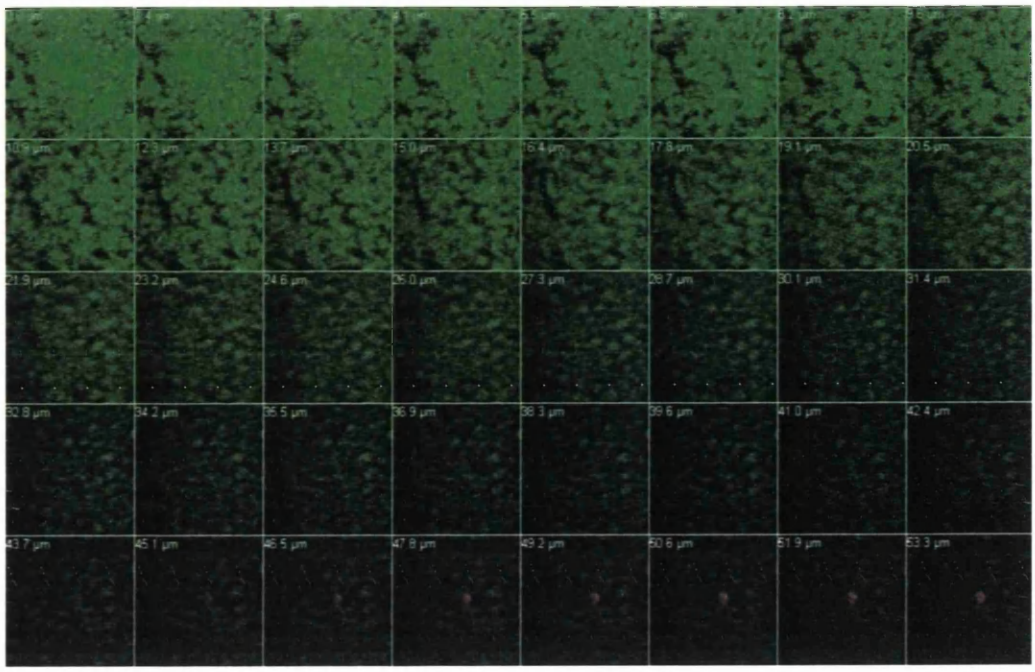
Figure 5.8 Characterisation of *Staphylococcus epidermidis* 1457 biofilm viability. Biofilm growth as viewed by CLSM. Green fluorescence represents viable cells, and red fluorescence represents non viable cells.

5.6 The effect of biofilm associated PIA on penetration of THP-1 cells

In order to evaluate biofilm – monocyte interaction, and to examine the ability of THP-1 cells to penetrate *S. epidermidis* 1457 biofilm, THP-1 cells were added to preformed biofilm and their migration assessed at three different time intervals (0-1, 1-2, 2-3 hours). *Staphylococcus epidermidis* 1457 expressing GFP (Franke, Dobinsky et al., 2007) was used and the THP-1 cells were stained with Cell Mask. CLSM was used to investigate the penetration of the leukocytes into the biofilm.

Serial sections of biofilm were obtained at 1.4µm intervals between 0-53.3µm from the substrate to the surface of the biofilm. THP-1 cells stained with Cell Mask were pseudocoloured purple, and the GFP expressing strain of *S. epidermidis* 1457 (Franke, Dobinsky et al. 2007) was depicted as green staining bacteria embedded in the PIA matrix. The biofilm matrix was seen as an unstained gap between the bacteria and monocytes (Figure 5.9 A-C). Biofilm penetration by the leukocytes was assessed in serial sections at different time points and the velocity of leukocyte migration measured as µm/h. Confocal images revealed that THP-1 cells had the ability to penetrate the biofilm. During the first hour of incubation THP-1 cells migrated about 6.8µm (53.3 to 46.5µm), in the second hour migration was 19.2 µm (46.5 to 27.3µm), then in the third hour the THP-1 cells moved 5.5µm (27.3 to 21.8µm).

A



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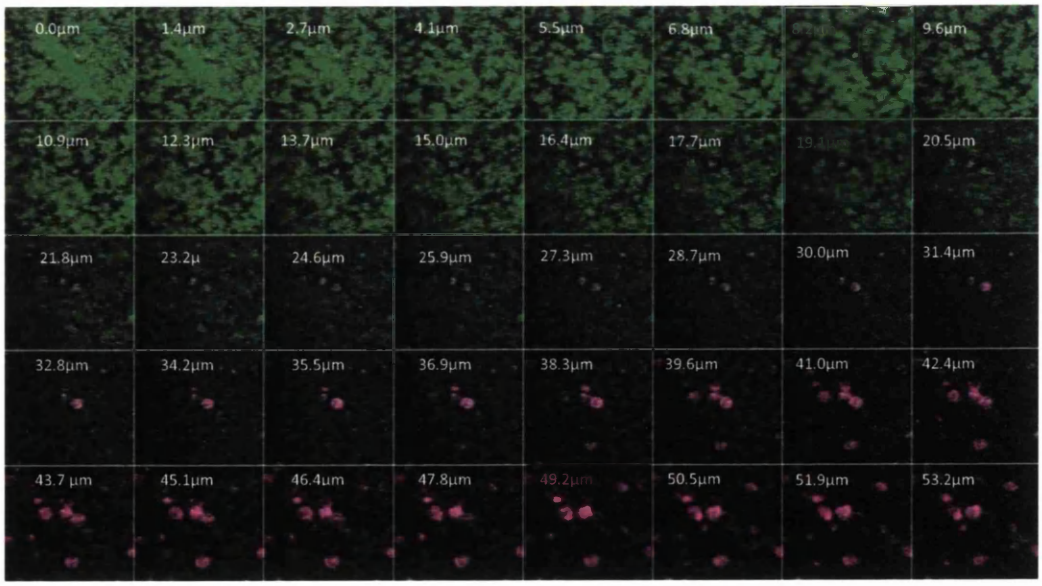
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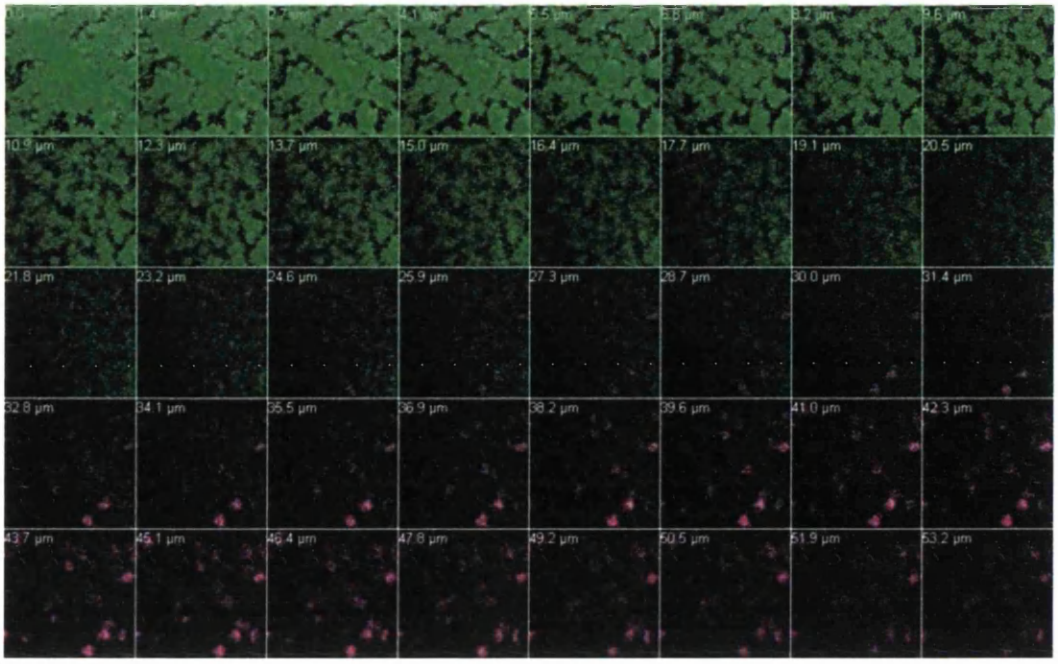


Figure 5.9 The effect of biofilm associated PIA on penetration of THP-1 cells.
A. 0-1 hours, **B.** 1-2 hours, C. 2-3hours.

5.7 The effect of biofilm associated PIA on cytokine production by THP-1 cells

Knowing that THP-1 cells can penetrate into *S. epidermidis* biofilm following 3 hours, the effect of biofilm associated PIA on the THP-1 cell cytokine profile following 3 hours was determined. Mature *S. epidermidis* 1457 biofilms were incubated with THP-1 cells for 6 hours in the presence of increasing doses of Dispersin B (DspB, 0.001, 0.01, 0.1, 1 μg/ml). THP-1 cells with 1μg/ml Dsp.B alone were used as controls and IL-8, TNF-α and IL-10 quantified by ELISA as previously described.

Biofilm associated PIA enhanced IL-8 release from THP-1 cells. Subsequent digestion of biofilm matrix with DspB diminished IL-8 release in a dose dependent manner. The IL-8 release in response to biofilm declined by more than the 57% when the biofilm was digested with1μg/ml DspB (decreasing from 714 to 312pg/ml respectively, $P<0.05$) (Figure 5.10).

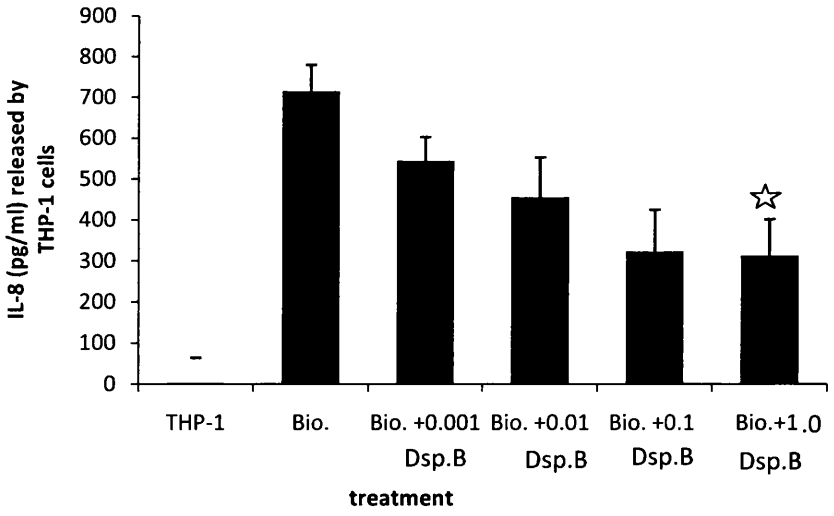


Figure 5.10 IL-8 cytokine release in THP-1 cells in response to *S.epidermidis* 1457 biofilm treated with Dsp.B. The results shown are representative of 3 independent experiments. Star indicates significant difference $P<0.05$. Bio, biofilm. Dsp.B, Dispersin B concentratin provided in μg/ml.

Furthermore, digestion of biofilm by Dsp.B decreased TNF-α release by THP-1 cells compared with biofilm, although results were not significant (Figure 5.11).

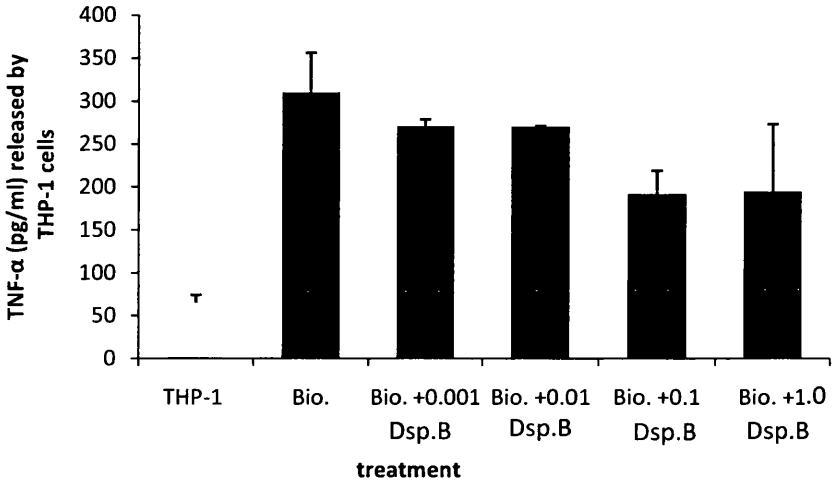


Figure 5.11 TNF-α cytokine release by THP-1 cells in response to *S.epidermidis* 1457 biofilm treated with Dsp.B. The results shown are representative of three independent experiments. Bio, biofilm. Dsp.B, Dispersin B concentratin provided in μg/ml.

Adding THP-1 cells to mature biofilm had no effect on IL-10 release in comparison to control THP-1 cells. In contrast complete digestion of biofilm with Dsp.B increased IL-10 release, P< 0.05 (Figure 5.12).

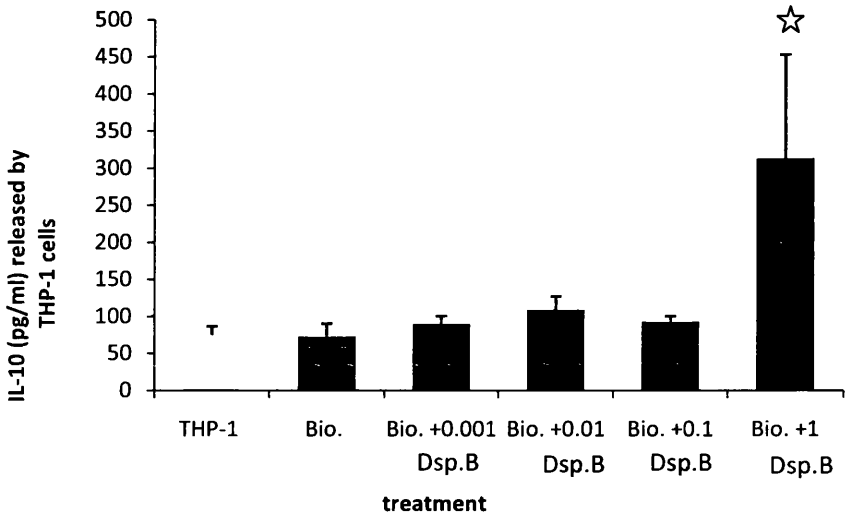


Figure 5.12 IL-10 cytokines release by THP-1 cells in response to *S.epidermidis* 1457 biofilm treated with Dsp.B. The results shown are representative of 3 independent experiments. Star indicates significant difference P<0.05. Bio, biofilm. Dsp.B, Dispersin B concentratin provided in µg/ml.

5. 8 Discussion

To date, it is still unclear how *S. epidermidis* eludes killing by the human innate immune system. The role of cytokines and cell types other than IL-8 and neutrophils in *S. epidermidis* infection is not well studied. Monocytes are an important cell type which *S. epidemidis* may interact with to induce an inflammatory response.

In this chapter it was shown that cytokine responses by monocytic THP-1 cells were dependent on the form of PIA (cell associated, soluble or biofilm associated). These results showed that biofilm bacteria have phenotypically distinct characteristics from their planktonic forms, as when purified PIA was added to THP-1 cells there was no IL-8 release, but after addition of *S. epidermidis* 1457 in suspension, there was an enhanced IL-8 release compared with PIA negative *S. epidermidis* 1457-M10. Furthermore, digestion of *S. epidermidis* 1457 biofilms with Dsp.B led to reduced IL-8 release. These results suggest that PIA may need to be bound to the bacterial surface to simulate cytokines release by macrophages. That is when it is bound to the bacterial surface it is held in the correct conformation to stimulate a receptor on the surface of THP-1 cells. Interestingly, for complete biofilm forming activity PIA needs to be deacetylated, through the function of the *IcaB* gene, the product of which is a surface protein that coordinate polysaccharide de-N-acetylation (Vuong, Kocianova et al., 2004; Cerca, Jefferson et al., 2007; Itoh, Rice et al., 2008). To date, it is unknown whether *icaB* is essential for inducing cytokine release.

The difference in the inflammatory response between *S. epidermidis* 1457 biofilm associated PIA and purified PIA could be because the biofilm matrix contains other antigens. These other antigen include extracellular DNA (eDNA) (Qin, Ou et al., 2007) and protein in addition to the carbohydrates poly-N-acetylglucosamine polysaccharides and extracellular teichoic acids (Mehlin, Headley et al., 1999; Sadovskaya, Vinogradov et al., 2004; Jones, Perris et al., 2005; Arciola, 2009; Jabbouri and Sadovskaya, 2010). Therefore, digestion of PIA by Dsp.B (Kaplan, 2009) could have caused disruption of biofilm structure and exposed bacteria and other components of the biofilm matrix and cell-walls leading contribute to a differential cytokine release as planktonic cells produced IL-8 and IL-10 but biofilm cells only produced IL-8. These findings correlate with those of Secor *et al* (Secor, James et al., 2011) who found a difference in

inflammatory cytokine production between *S. aureus* biofilm and their planktonic equivalent. Similar findings were also demonstrated by Schommer *et al* and Spiliopoulou *et al* (Schommer, Christner et al., 2011; Spiliopoulou, Krevvata et al., 2012). In contrast to the finding of Stevens *et al* (Stevens, Sadovskaya et al., 2009), our purified PIA did not induce cytokine release in THP-1 which could be due to difference in the purification method by which the present study obtained PIA. In addition, this present study used THP-1 cells whilst Steves *et al* employed astrocytes, which could be more sensitive to external stimuli (Sofroniew and Vinters, 2009).

The simultaneous up regulation of pro-inflammatory cytokines IL-8 and TNF-α and down regulation of the anti-inflammatory cytokine IL-10 by biofilm associated PIA is likely to increase the inflammatory response at the site of infection resulting in further damage to host tissue, as IL-8 induces infiltration of more neutrophils to the site of infection. However, macrophages use cytokines to deal with infection by different methods. Secreted chemokines like IL-8 will attract neutrophils to the site of infection, release cytokines and soluble mediators such as TNF, prostaglandin and platelet activating factor. These mediators then act on the endothelium to modify blood vessel permeability and allow more neutrophils and monocytes to leave blood and enter the tissues (Wood, 2011). Certainly, there is a suggestion that monocytic cells are an important contributor to the tissue damage caused by device associated infections. Similar findings have also been demonstrated in animal models of *S. aureus* infection where there was a strong inflammatory response induced at site of infection (Prabhakara, Harro et al. 2011). Figure 5.13 summarizes the inflammatory response toward different form of PIA.

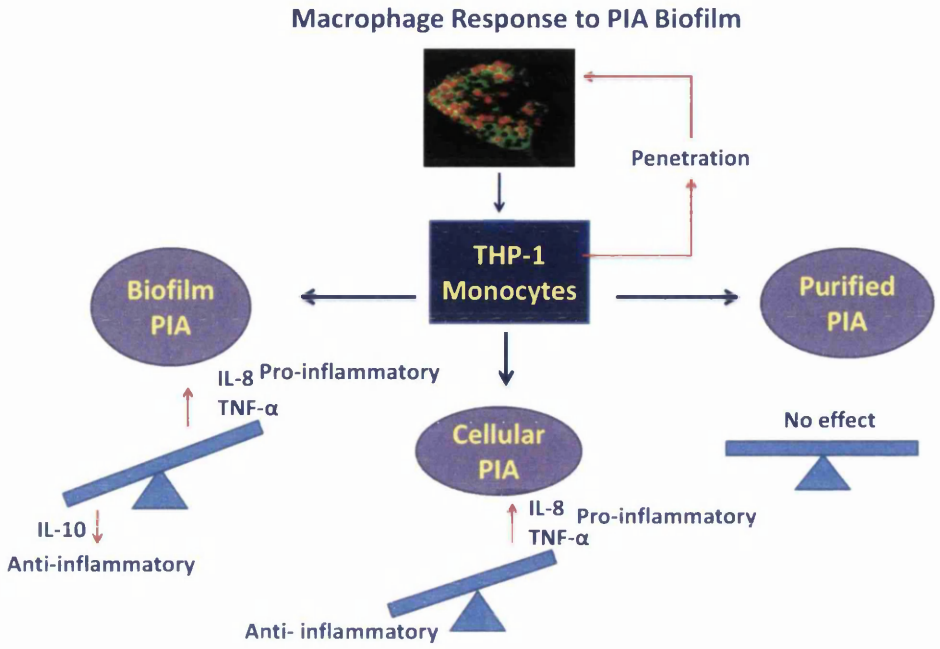


Figure 5.13 Inflammatory response of THP-1 cells toward different form of PIA.

CSLM images revealed the unique structure of biofilm plays an important role in bacterial protection, with the bacteria near the substratum being surrounded by heterogeneously distributed extracellular polysaccharide which may protect against host immune cell penetration and antibiotic diffusion. The increasing biofim thickness with time suggested the continuing synthesis of the biofilm and may explain the difficulty of eradication of chronic implanted infection associated with biofilm formation, as increased biofilm thickness may further reduce the penetration for antibiotic and immune cells (Patel, 2005; Anderson and O'Toole, 2008). Kirby *et al* found that biofilm may act as a diffusion barrier protecting bacteria from the killing effect of antibiotics like Colistin, Streptomycin, and Gentamicin as planktonic bacteria were killed to a greater extent than those embedded within biofilms (Kirby, Garner et al., 2012). Therefore the organization of bacterial cells within biofilms could in part explain the resistance of this organism to antimicrobial agent. Studies by Schommer *et al* (Schommer, Christner et al., 2011) demonstrated the importance of macroscopically and microscopically distinctive structure of *S. epidermidis* 1457 biofilm, as

immunofluorescence microscopy images showed PIA as tether-like fibers pervading the biofilm in a string-like manner.

This present study also demonstrated that THP-1 cells had the ability to penetrate the biofilm over 1-3 hours. Interestingly, the penetration rate slowed with increased time and cells did not reach the bacteria bound to the substratum. Leid *et al* (Leid, Shirtliff et al., 2002) also demonstrated that leukocytes penetrate the biofilm, but they were not able to engulf bacteria inside the biofilm. This brake or paralysis at a certain point following contact with the biofilm remains a critical area for research as the phenotype of these penetrated leukocytes is unknown.

In conclusion, the structural characteristics of *S. epidermidis* biofilms together with the tethering of PIA to the bacterial cell surface (Ganesan, Stewart et al., 2013) have significant impact on the inflammatory response in THP-1 cells. In Chapter 6 these findings are extended to an *ex vivo* model of whole blood infection.

Chapter 6

The effect of soluble, cell-associated and biofilm-associated PIA on whole blood: importance of humoral immunity and C5a

6.1 Introduction

Biofilm formation is a leading cause of health care associated infection involving implanted medical devices such as urinary catheters (Niveditha, Pramodhini et al., 2012), central venous catheters, heart valves, coronary stents, neurosurgical ventricular shunts, and fracture-fixation devices (Costerton, Montanaro et al., 2005). In addition, there are many chronic diseases associated with biofilms, e.g. native valve endocarditis, periodontitis, chronic rhinosinusitis, and otitis media (Hou, Sun et al., 2012).

In previous studies investigating killing and phagocytosis of biofilm positive bacteria using cells such as neutrophils and monocytes, biofilm forming *S. aureus* were more resistant to neutrophils compared to planktonic counter parts (Barrio, Vangroenweghe et al., 2000). However, isolated leukocytes do seem to adhere and penetrate into *S. aureus* biofilms (Leid, Shirtliff et al. 2002).

In vivo, biofilm formation has been associated with persistent CoNS bacteremia in neonates in intensive-care units (Dimitriou, Fouzas et al., 2011). Furthermore, PIA forming Staphylococcal biofilms appear to be a major virulence factor in a biomaterial-associated infection model in rate and mice (Rupp, Ulphani et al., 1999; Rupp, Ulphani et al., 1999). The killing effect of *S. epidermidis* on *Caenorhabditis elegans* (nematode) is also diminished by infecting with PIA negative *S. epidermidis* strains (Begun, Gaiani et al., 2007). Gunether *et al.* found that *S. epidermidis* biofilm were more resistance to phagocytosis by polymorphonuclear cells (PMN) than *S. aureus* biofilm (Guenther, Stroh et al. 2009). In a mouse model of catheter-associated biofilm infection, *S. aureus* biofilms evad innate immunity, through inhibited macrophages phagocytosis and reduced proinflammatory immune responses (IL-1β and TNF-α) (Thurlow, Hanke et al. 2011).

Less is known about the functional effects of PIA dependent biofilms on human systems *in vivo* during infection. In this chapter an *ex vivo* whole blood model has been developed to display function and dysfunction. Using this model, exploration of the effect of functional modulation on whole blood killing and cytokine release, and whether killing and inflammatory responses to PIA/biofilm positive *S. epidemidis* differed to PIA/biofilm negative strain was undertake. The phagocytic receptor pathways by which the immune cells cleared *S. epidermidis* were examined. The different forms of PIA

including purified PIA, cell associated PIA and biofilm associated PIA were all used in these experiments.

Specifically, this chapter investigated the:

- Development of an *ex vivo* model of whole blood infection in health and under immunosuppressive conditions
- Comparison of planktonic and biofilm associated PIA effects on whole blood
- PIA expression in whole blood
- Formation of biofilm in normal or immunosuppressed whole blood

6.2 Methods

For whole blood killing assays, bacterial suspensions of *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 were incubated with rotation at 37°C in whole blood or TSB for 30 min, 1h, 2h and 6h. Control cultures at zero time and those without blood were included. Bacterial viability was quantified through viable counts (section 2.12.2).

To study the effects of purified PIA extracts, 1ml of whole blood was added to a 1.5ml microcentirfuge tube and incubated with purified PIA extract and *S. epidermidis* 1457-M10 extract. Extracts at different concentrations (10, 30, and 60μg/ml) were incubated for 3 hours before adding *S. epidermidis* 1457-M10, as described in section 2.12.4. Suspensions were incubated with rotation (10rpm) at 37°C for 6 hours and then viable counts assessed. Platelet poor plasma/serum was isolated as described in section 2.12.2.

To investigate potential phagocytic receptors pathway blood was pretreated with either 5μg/ml anti CD11b antibody, 20μg/ml anti CD18, or anti CD16. Mouse IgG Isotype was used as a control. Incubation was for 30 minutes, before adding *S. epidermidis* 1457 and *S. epidermidis* 1457-M10. The blood was then incubated with the bacteria for 6 hours with rotation at 37°C. Bacterial viability was then assessed by counting CFU.

For cytokines production assays, samples from whole blood (pretreated with Dex., CytoD and PIA) were incubated with planktonic *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 for 6 hours and then tested for IL-8, TARC, IL-1β,TNF-α , IL-10 and C5a using ELISA as described in section 2.10.1. The RayBioHuman cytokine assay was used to determine cytokine expression profile in samples of whole blood preincubated with

planktonic *S. epidermidis* 1457 and *S. epidermidis* 1457-M10, purified PIA and controls as described in section 2.10.2.

For Immunocytochemistry ICC, cytospin preperations were prepared as described in section 2.13. Preparations were blocked with 200µl 1% BSA for 1 hour, then washed X3 with PBS. Then, 200µl of anti-rabbit PIA antiserum (diluted 1:50) was applied. The slides were covered and after 30 minutes washed X3 with PBS. Slides were then stained with fluorescein–conjugated anti-rabbit IgG (Alexa flour 488, diluted 1:100) and propidium iodide (diluted 1:1000), covered and incubated for 30 minutes. Slides were washed X3 wih PBS and 25µl of vectashield hardset was added. A coverslip was gentaly placed on the preparation which was left in the fridge to harden. Slides were examined using an epifluorecent microscope as mentioned in section 2.16.1.

Results

6.3 The effect of cell associated PIA on whole blood

To investigate the effect of cell associated PIA on whole blood, planktonic *S. epidermidis* 1457 (biofilm/PIA positive) and *S. epidermidis* 1457-M10 (biofilm/PIA negative) were compared in a variety of assays including; whole blood killing of bacteria, cytokine production and C5a production in blood.

Preliminary dose finding experiments used high (5.3 x10^{10} bacteria/ml), medium (2.4 x 10^{8} bacteria/ml) and low (5.1 x 10^{7} bacteria/ml) doses of *S. epidermidis* 1457-M10 incubated for 30, 60 and 120 minutes with whole blood, before assesment of bacterial viability. Whole blood killing of *S. epidermidis* 1457-M10 was dose dependent with the lowest dose (5.1 x 10^{7}) used for the remaining whole blood killing experiments as it resulted in the greatest killing response (Figure 6.1).

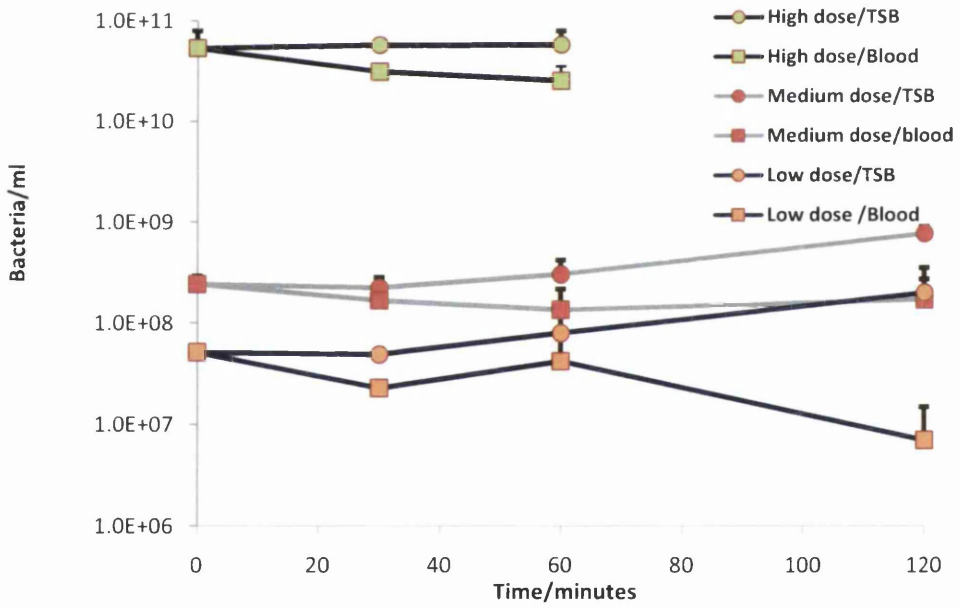


Figure 6.1 Dose responses of *S. epidermidis* 1457-M10 to whole blood killing. Three different doses of *S. epidermidis* 1457-M10 were used, and bacteria were incubated in either TSB or whole blood over 120 minutes. Results represent data from 3 independent experiments.

To study the effect of cell associated PIA on whole blood killing assays bacterial suspensions of *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 were incubated with rotation at 37°C in whole blood or TSB for 30, 60 and 120 minutes. Control cultures at zero time and without blood were also included and bacterial viability quantified through viable counts. No significant differences in whole blood killing (Figure 6.2, t=0 compared to t=120) was detected between *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 at early time points post infection (Figure 6.2). At t=120 there was a trend towards slower growth by *S. epidermidis* 1457 compared to its isogenic control (Figure 6.2 orange vs yellow circles).

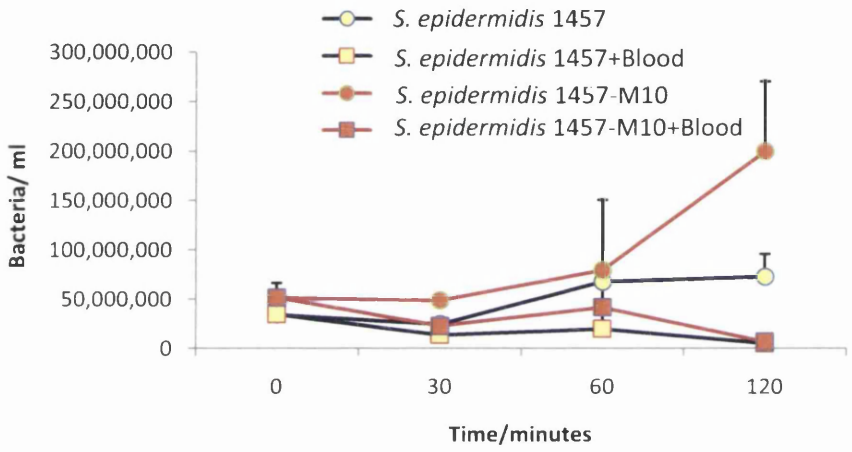


Figure 6.2 Whole blood killing of *S. epidermidis* 1457 and *S. epidermidis* 1457-M10. Killing of both 1457 and 1457-M10 in blood compared to the normal growth in TSB at different time intervals (0, 30, 60, and 120 minutes). Results represent data from 3 independent experiments.

Further characterization of whole blood killing of *S. epidermidis* after 6 hours demonstrated a 50-fold reduction in bacterial counts, but no differences between *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 (Figure 6.3, Bar 1 and 2 vs Bar 5 and 6). Antibody blocking experiments showed that clearance of both *S. epidermidis* 1457 (biofilm/PIA positive) and *S. epidermidis* 1457-M10 (biofilm/PIA negative) were CD11b/CD18 dependent (complement receptor 3), but independent of CD16 (Fc receptor) (Figure 6.3).

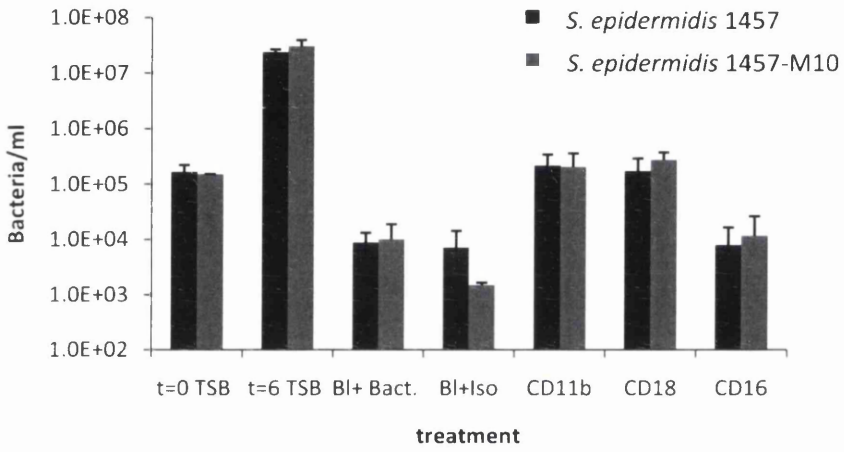
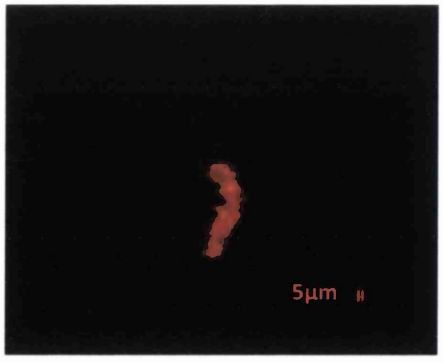


Figure 6.3 Phagocytic receptors pathway. A comparison in killing rate between *S. epidermidis* 1457 (PIA/biofilm +) and *S. epidermidis* 1457-M10 (PIA/biofilm-) in blood pretreated with CD11b, CD18 and CD16 blocking antibodies. Bl+Bact., blood with bacteria only. Bl+Iso, blood with isotype. CD11b, blood blocked with antihuman CD11b blocking antibody. CD18, blood blocked with antihuman CD18 blocking antibody. CD16, blood blocked with antihuman CD16 blocking antibody. Results represent data from 3 independent experiments.

The consistently observed similarities between *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 prompted sudies to examine PIA expression in *S. epidermidis* 1457 in whole blood. Immunocytochemistry (ICC) assays were performed to detect PIA expression by *S. epidermidis* 1457 in whole blood and cell culture media. In these studies *S. epidermidis* 1457 was grown in TSB/G- (without glucose) overnight (which inhibite PIA expression) and subcultured into different media including TSB/G-, TSB/G+ (with glucose), IMDM, blood, blood+cytochalasin D (cyto.D). Samples were then taken after 3h, 5h, and 24h (Figure 6.4). Samples were then deposted on to microscope slides and stained as previously described for PIA using anti-PIA rabbit antibodies. Propidium iodide (PI) was used as a counterstain for bacteria and blood cells. *S. epidermidis* 1457 grown in TSB/G- showed no PIA expression both prior to and following subculture. In contrast, subculture into TSB/G+ and IMDM resulted in *S. epidermidis* 1457 producing PIA as early as 3h to 5h with significantly robust staining by 24 hours.

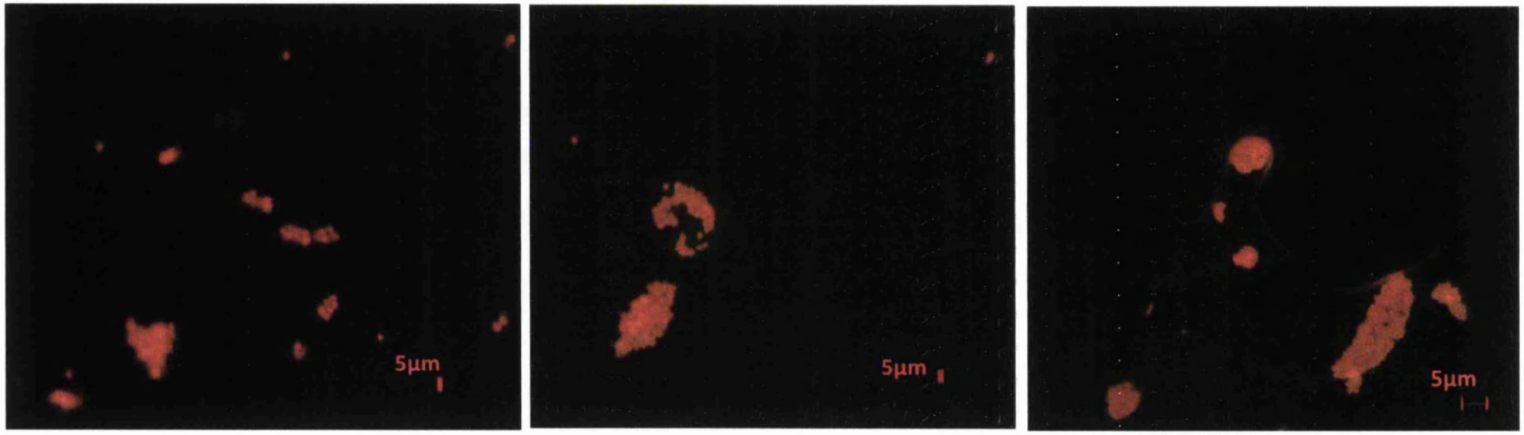
In whole blood, no bacteria could be detected; suggesting clearance by blood leukocytes. Adding cytochalasin D to whole blood prevented killing and *S. epidermidis* 1457 was then able to grow in this environment. Furthermore, PIA could be detected at t=5h to t=24h (Figure 6.4). Indeed PIA was observed surrounding bacteria, but also covering leukocytes. These studies suggested that PIA could be produced in whole blood (when treated with cytochalasin B) over the course of the earlier killing assays, and immune suppression was an advantage for bacterial growth and PIA expression.

T=0h

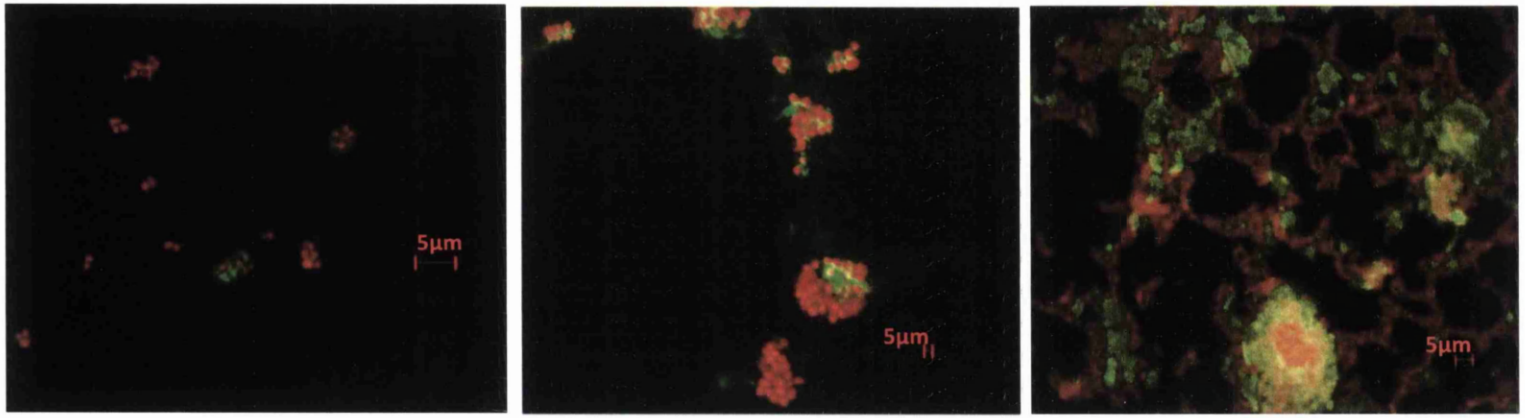


T=3h T=5h T=24h

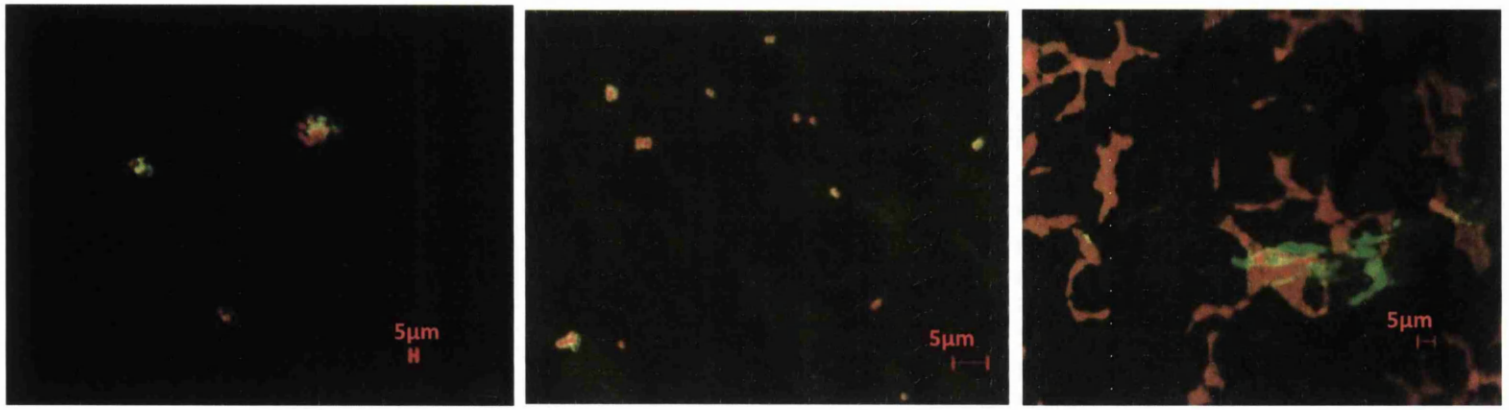
A. TSB/Glucose -



B.TSB/Glucose +



C. IMDM



D. Blood

E. Blood+Cyto.D

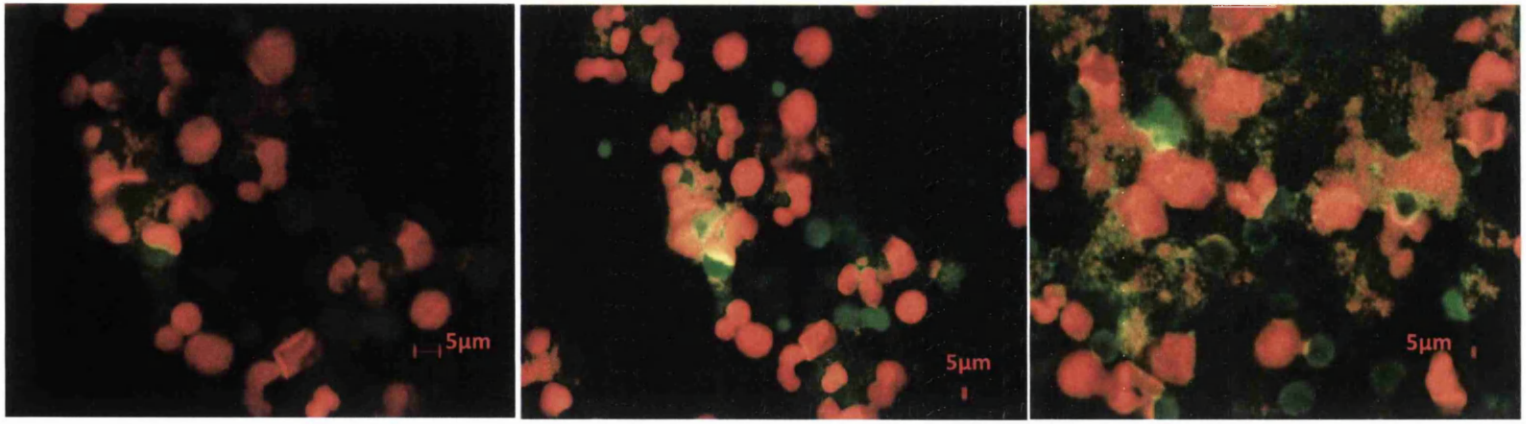


Figure 6.4 Comparison of PIA expression of *S. epidermidis* 1457 cultured in different media (t=0 was the same for all the media). A. TSB/Glucose-, **B.**TSB/Gloucose+, **C.** IMDM, **D.** Blood, **E.** Blood +Cyto.D. PIA was detected using antiPIA antibody coupled to Alexa 488 (green). *Staphylococcus epidermidis* bacteria and neutrophils stained with propidium iodide (red).

In addition, cytokine release was measured in whole blood in response to *S. epidermidis* 1457 (PIA/Biofilim positive) and *S. epidermidis* 1457-M10 (PIA/biofilm negative) using a RayBioHuman cytokine array. Following 3 hours, cytokines could be detected in untreated whole blood. These cytokines included GRO-α, IL-7, IL-10, TARC, TGF-β1, TNF-α, Oncostatin M, Thrombopoietin, VEGF (Figure 6.5A). Infection of whole blood with *S. epidermidis* 1457 induced the release of IL-2, IL-6, SDF-1, EGF and MIG, in addition to cytokines detected in control blood (Figure 6.5 B). Whole blood infected with *S. epidermidis* 1457-M10 had increased levels of IL-1α and IL-1β in addition to detectable levels of all the cytokines release in response to *S. epidermidis* 1457 (Figure 6.5 C).

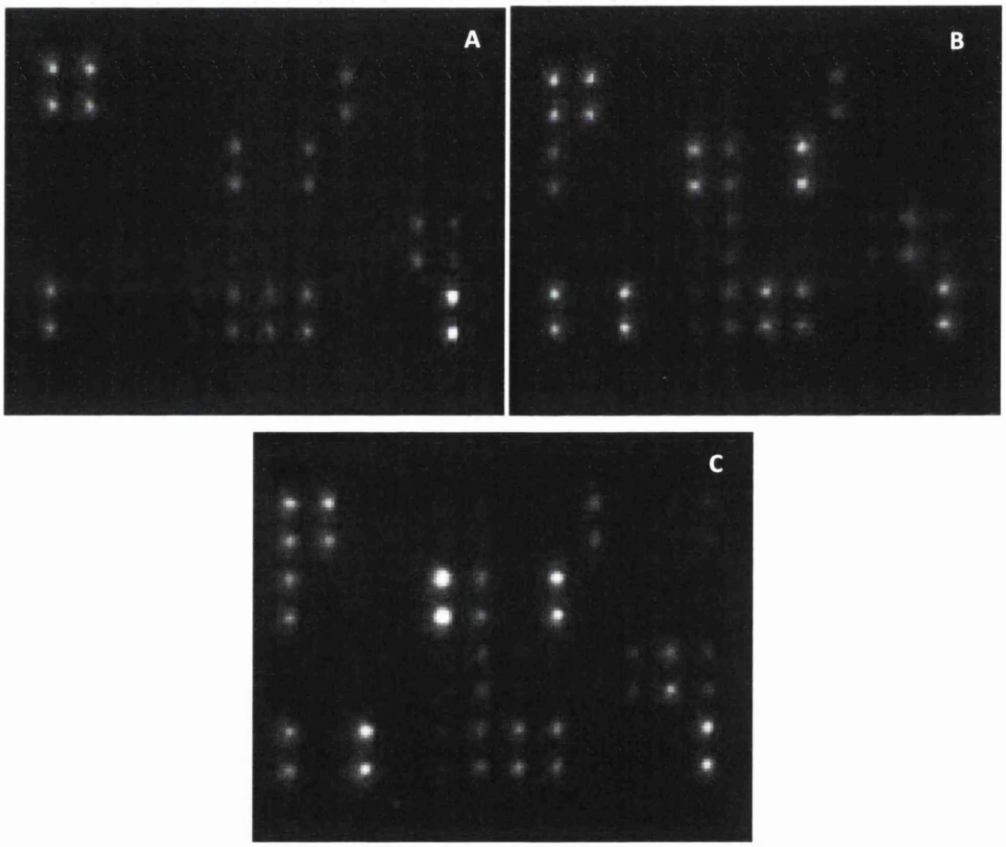


Figure 6.5 RayBioHuman cytokine array of blood incubated with planktonic *S. epidermidis*. A. Control blood, **B.** Blood with *S. epidermidis* 1457, **C.** Blood with *S. epidermidis* 1457-M10

Table 6.1 Cytokines release in blood in response to planktonic *S.epidermidis* bacteria

Cytokines	Control blood	*S. epidermidis* 1457	*S. epidermidis* 1457-M10
Positive control	+++	+++	+++
IL-10	++	+++	+++
TARC	++	++	++
IL-7	++	++	++
IL-6		+++	+++
IL-1α			+
IL-1β			+
IL-2		+++	+++
EGF		+++	+++
TGF-β1	+	++	++
MIG		++	++
SDF-1		++	++
TNF-α	++	+++	+++
Angiogenin	+	+	+
Oncostatin M	++	++	++
Thrombopoietin	++	+++	+++
VEGF	++	+++	+++
GRO-α	++	++	++

Semi quantitative analyses of signals detected are: +, weak, ++; moderate, +++, strong signals.

6.4 The effect of cell associated PIA on immunosuppressed whole blood

Cell associated PIA had very little effect on the killing response of whole blood from healthy volunteers. It was hypothesized that cell associated PIA may have more of an effect on whole blood killing using injured/dysfunctional phagocytes. A model of immunosuppressed whole blood was therefor developed by pre-treatment protocols including use of Cytochalasin D (cyto.D) and Dexamethasone.

Whole blood pre-incubated with two different concentrations of Cyto.D (1μg/ml and 15μg/ml) was infected with *S. epidermidis* 1457 and *S. epidermidis* 1457-M10. The preparation was then incubated at 37°C for 6 hours with rotation. After incubation bacterial viable counts were determined. Whole blood killing at 6 h confirmed a 10-50 fold decrease in bacterial numbers (Figure 6.6 vs Figure 6.3). Addition of Cyto.D resulted in a dose dependent inhibition of killing with complete suppression at the highest dose. No significant difference was observed between the killing of *S. epidermidis* 1457 and *S. epiddermidi* 1457-M10 (Figure 6.6).

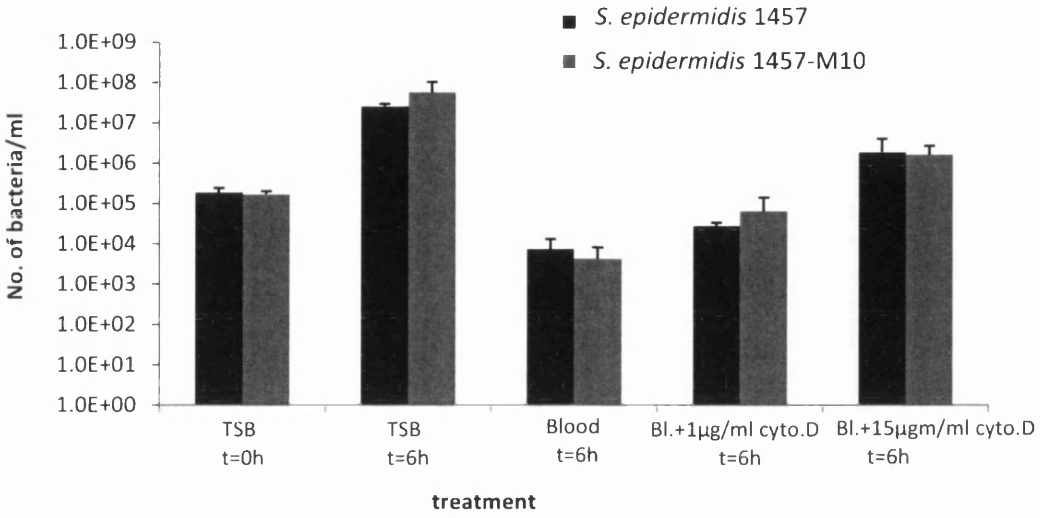


Figure 6.6 Whole blood killing of *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 in immuno suppressed blood. Whole blood was pre-incubated with two different concentrations of Cyto.D (1µg/ml and 15µg/ml) or control media (Blood) and infected with *S.epidermidis* 1457 and *S. epidermidis* 1457-M10 and incubated at 37°C for 6 hours with rotation. Bacterial viable counts were determined after 6 hours. Viable counts were determined at t=0 in TSB and compared with counts 6h later in TSB, whole blood, and whole blood treated with Cyto.D (Bl + 1µg/ml and Bl +15µg/ml). Results represent data from four independent experiments.

Serum collected from the same experiment was used to evaluate the inflammatory response toward *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 by ELISA. Cyokines and immune factors analysed included Interleukin 8 (IL-8), Thymus and activation-regulated chemokine (TARC), Interleukin-1 beta (IL-1β), Tumor necrosis factor alfa (TNF-α), Interleukin 10 (IL-10) and complement component 5 (C5a).

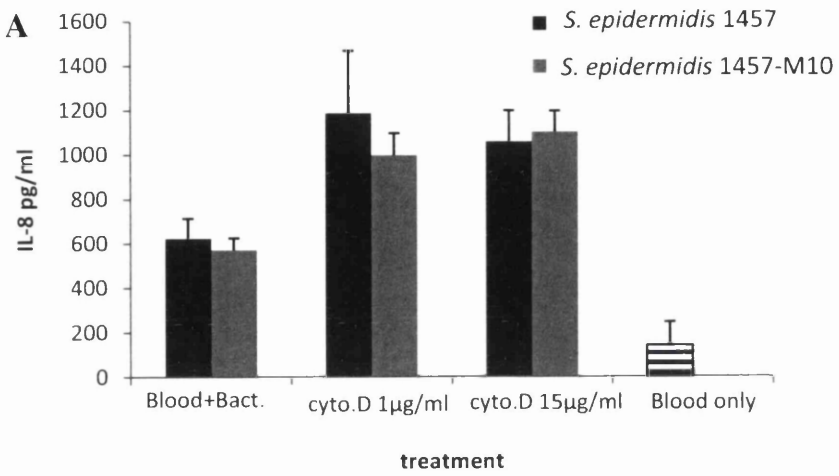
IL-8 levels were increased in blood infected with *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 compared to control blood without bacteria. There was however no difference in IL-8 level between whole blood infected with either of the two strains. Pretreatment with Cyto.D increased IL-8 levels, but no difference was detected following challenged with *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 at the two Cyto.D concentrations used (Figure 6.7A).

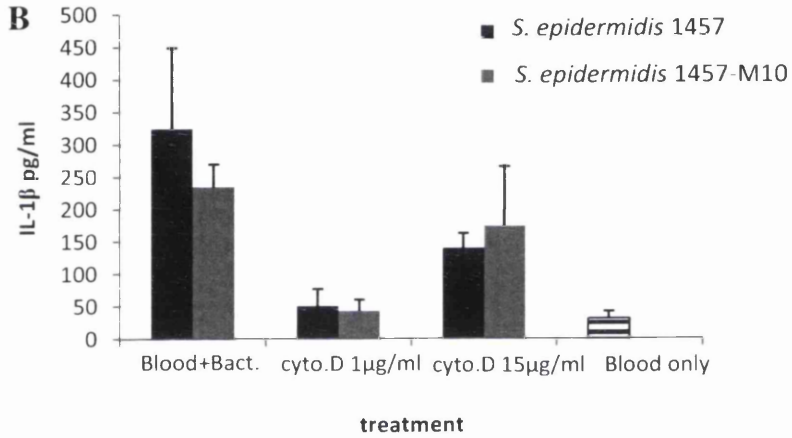
IL-1β also increased in blood infected with *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 6 hours following infection, compared to untreated control blood. There was a trend towards increased IL-1β levels in blood containg *S. epidermidis* 1457 compared with *S. epidermidis* 1457-M10. Pretreatment with 1μg/ml Cyto.D decreased IL-1β levels but there was no difference between the two strains. Pretreatment with 15μg/ml Cyto.D resulted in higher levels of IL-1β being generated than the 1μg/ml cyto.D treatment (Figure 6.7B).

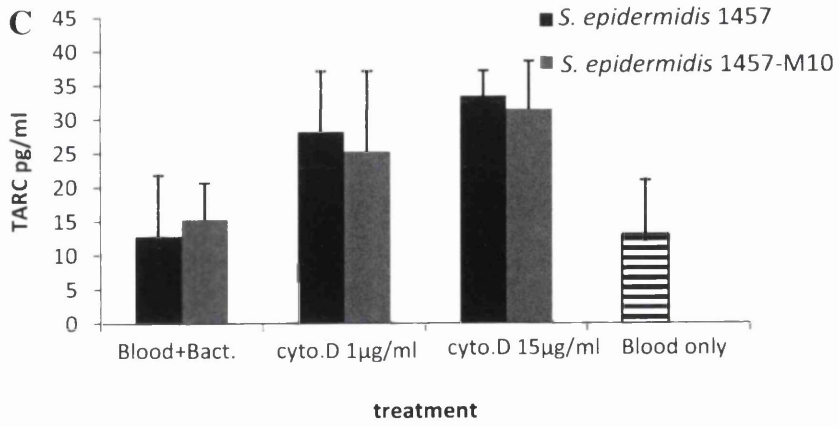
In blood infected with *S. epidermidis* 1457 and *S. epidermidis* 1457-M10, TARC levels were similar to untreated blood, whilst pretreatment of blood with Cyto.D increased TARC levels with no difference in response between *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 (Figure 6.7 C).

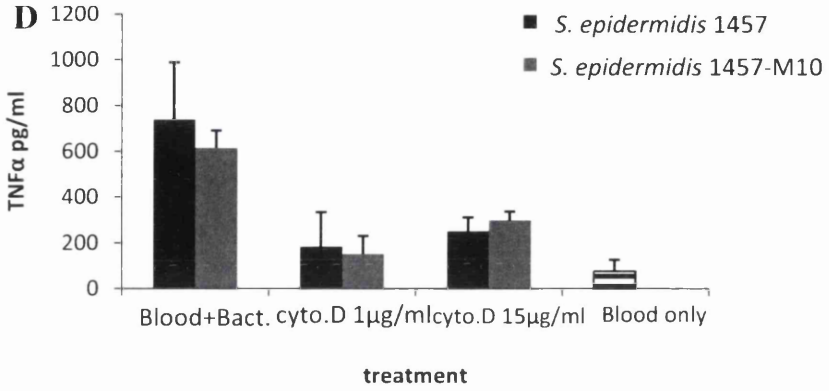
TNF-α levels increased in blood infected with *S. epidermidis* 1457 and *S. epidermidis* 1457-M10, whilst pretreatment with Cyto.D decreased TNF-α release. No differences were detected between strains (Figure 6.7 D).

Finally, there were no differences in IL-10 levels in blood infected with *S. epidermidis* 1457 and *S. epidermidis*1457-M10 compared to untreated controls or blood pretreated with Cyto.D (Figure 6.7 E).


A
1600
1400
1200
1000
800
600
400
200
0
IL-8 pg/ml
S. epidermidis 1457
S. epidermidis 1457-M10
Blood+Bact.
cyto.D 1µg/ml
cyto.D 15µg/ml
Blood only
treatment


B
500
450
400
350
300
250
200
150
100
50
0
IL-1β pg/ml
S. epidermidis 1457
S. epidermidis 1457-M10
Blood+Bact.
cyto.D 1µg/ml
cyto.D 15µg/ml
Blood only
treatment


C
45
40
35
30
25
20
15
10
5
0
TARC pg/ml
S. epidermidis 1457
S. epidermidis 1457-M10
Blood+Bact.
cyto.D 1µg/ml
cyto.D 15µg/ml
Blood only
treatment



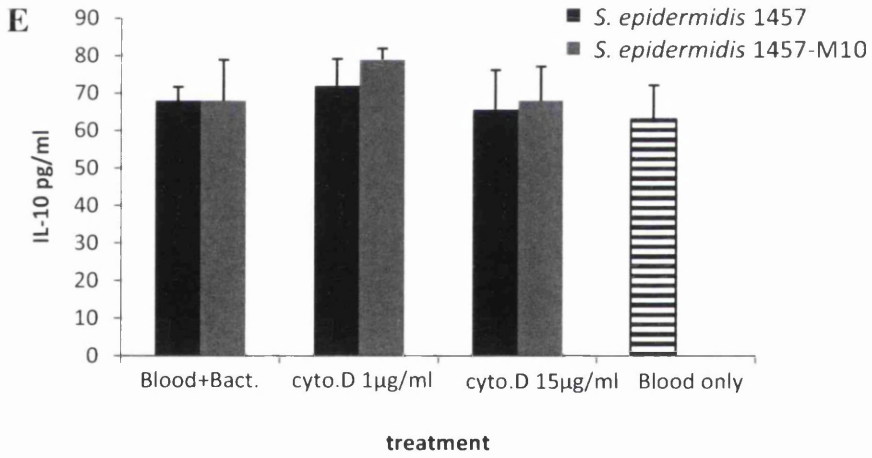


Figure 6.7 Cytokine expression in blood pretreated with Cyto.D.

Serum collected from whole blood pretreated with (1, 15 μg/ml) Cyto.D and infected with *S. epidermidis*1457 and *S. epidermidis* 1457-M10 was subjected to ELISA determination. Panels represent **A.** IL-8, **B.** IL-1β, **C.** TARC, D. TNFα, E. IL-10. Results represent data from four independent experiments.

C5a complement levels in whole blood infected with *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 increased slightly (but not significantly) compared to untreated blood. Pretreatment of blood with Cyto.D increased C5a level in a dose dependent manner (Figure 6.8).

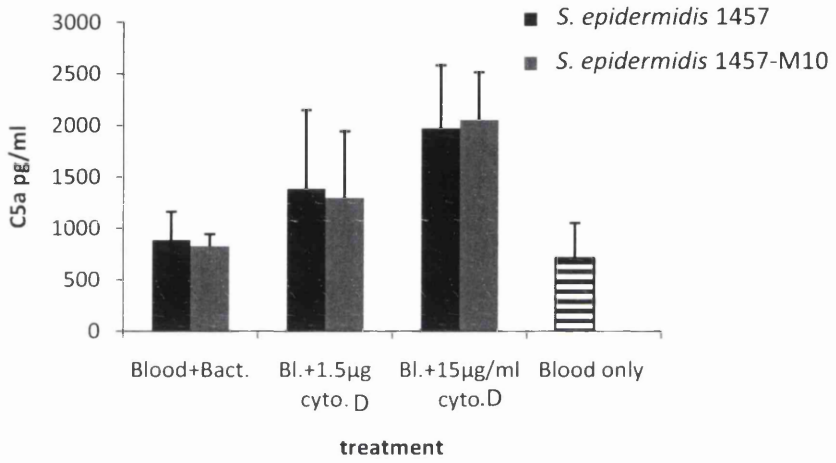


Figure 6.8 C5a complement expression in blood pretreated with Cyto.D. C5a levels in serum collected from whole blood pretreated with (1, 15 µg/ml) Cyto.D and infected with *S. epidermidis*1457 and *S. epidermidis* 1457-M10. Results represent data from four independent experiments.

Whole blood was pre-incubated overnight with three different doses of dexamethasone (0.1, 1 and 10μM). *Staphylococcus epidermidis* 1457 and *S. epidermidis* 1457-M10 were then added to the blood and incubated at 37°C with rotation for 6 hours. Bacteria were plated at t=0, and t=6 from TSB culture to assess bacterial viable counts and serum was collected for cytokine analysis using ELISA.

Healthy whole blood killed ~80% of *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 with no difference between the isogenic strains (Figure 6.9). Addition of dexamethasone (0.1-10μM) had very little effect on *S. epidermidis* 1457-M10 killing, but resulted in a decrease in the killing of *S. epidermidis* 1457 which was statistically significant ($P < 0.05$) (Black vs grey bars Figure 6.9).

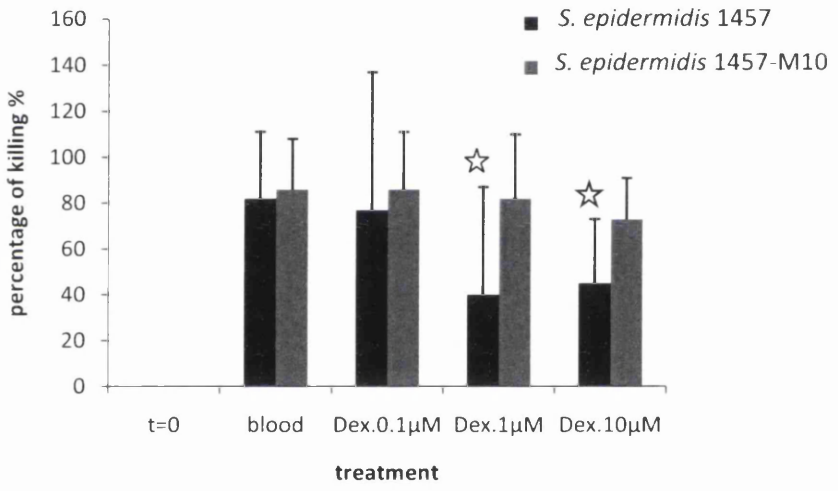
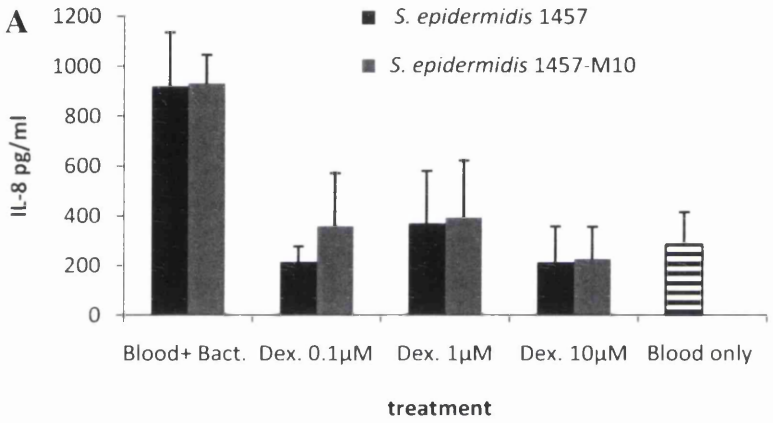
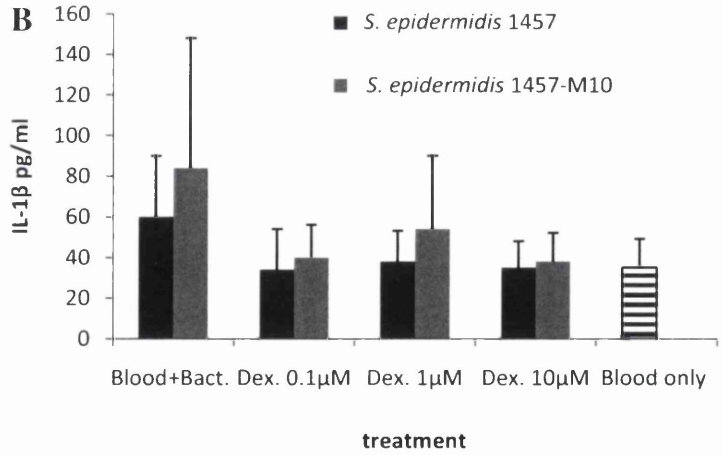


Figure 6.9 Whole blood killing with dexamethason pre-treatment. The graph shows the percentage killing of *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 with different dexamethasone concentrations (0.1-10μM). Whole blood was pre-incubated with three different concentrations of Dex. (0.1, 1, and 10μM) and infected with *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 and incubated at 37°C for 6 hours with rotation, before bacterial viable counts were determined after 6 hours. Original percentage of killing of bacteria (t=0) was considered zero (blood). Stars indicate significant difference between *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 $P<0.05$. Results represent data from four independent experiments.

To investigate the effect of dexamethasone on cytokine release in whole blood, sera also collected and assayed by ELISA for IL-8, TARC, IL-1β, TNFα, IL-10 and complement C5a, as described before in materials and methods (section 2.10.1).

Dexamethasone had a noticeable inhibitory effect on the release of the pro-inflammatory cytokines such as IL-8 (Figure 6.10A), IL-1β (Figure 6.10B), and TNFα (Figure 6.10D), compared to whole blood infected with *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 alone, but no statistical difference observed between the two strains. There was no difference in TARC or IL-10 (Figure 6.10 C and E) levels in blood pre-treated with dexamethasone compared to blood infected without pretreatment.





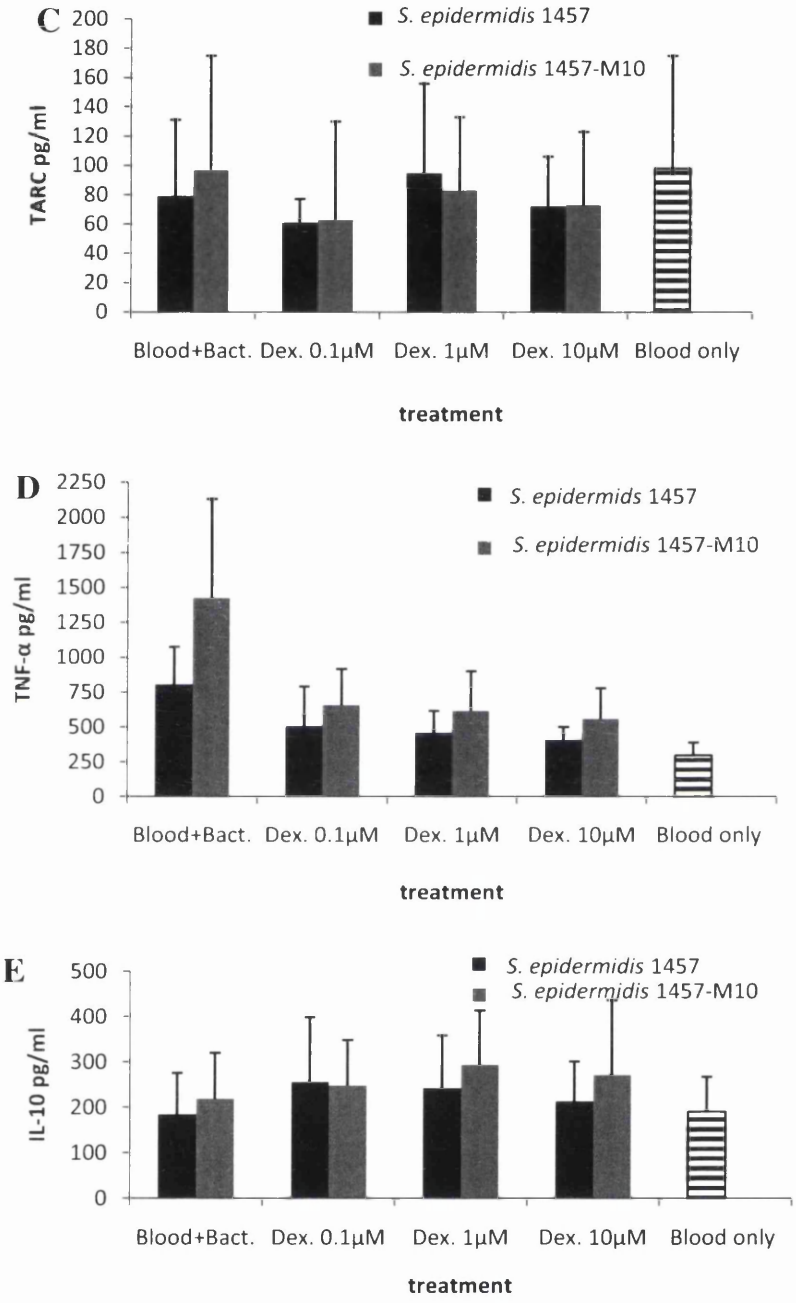


Figure 6.10 Cytokines expression in blood pretreated with dexamethasone. Cytokine analysis by ELISA of serum collected from blood pre-treated with different concentrations of dexamethason, blood only and blood infected with bacteria alone. **A.** IL-8, **B.** IL1-β, **C.** TARC, D. TNFα, E. IL-10. Result represent data from four independent experiments.

Complement component C5a was also measured in sera from infected whole blood pretreated with 0.1-10μM dexamethasone (Figure 6.11). Here, infection of whole blood did not induce C5a, although the basal levels were relatively high. Pretreatment with 0.1μM dexamethasone inhibited C5a release, but higher doses had no effect compared to infected controls (Figure 6.11).

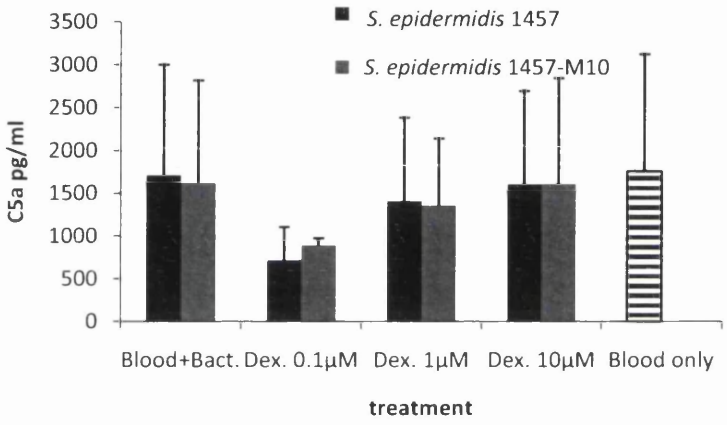


Figure 6.11 C5a Complement expression in blood pretreated with dexamethasone
Complement C5a ELISA of serum collected from blood pre-treated with different concentration of dexamethasone (0.1-10μM). Results represent data from four independent experiments.

6.5 The effect of soluble PIA on whole blood

To explore the effect of soluble PIA on killing effects of whole blood, increasing concentrations (10,30,60μg/ml) of purified PIA extract (as obtained by IMDM method in Chapter 4) were pre-incubated with whole blood for 3 hours, then co-cultured with (PIA/biofilm) negative *S. epidermidis* 1457-M10 for 6 hours. The extract produced from *S. epidermidis*1457-M10 was used as negative control (NC). The effects of PIA and NC extract were compared in a variety of assays including; functional killing of bacteria, cytokine and C5a ELISA and RayBio Human cytokines arrays.

Pre-incubation of whole blood with purified PIA extract significantly ($P<0.05$) attenuated killing of *S. epidermidis*1457-M10 in comparison to whole blood pre-incubated with NC extract. The killing was inversely related to PIA concentration; as PIA concentration increased the killing rate decreased (Figure 6.12).

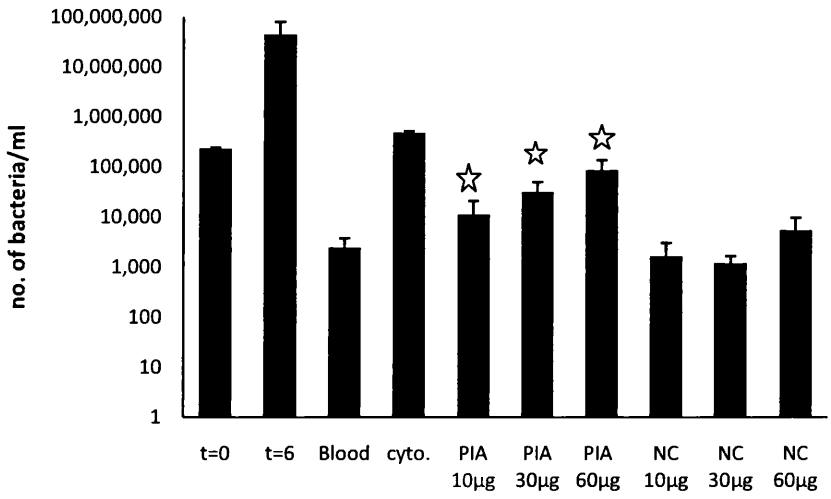
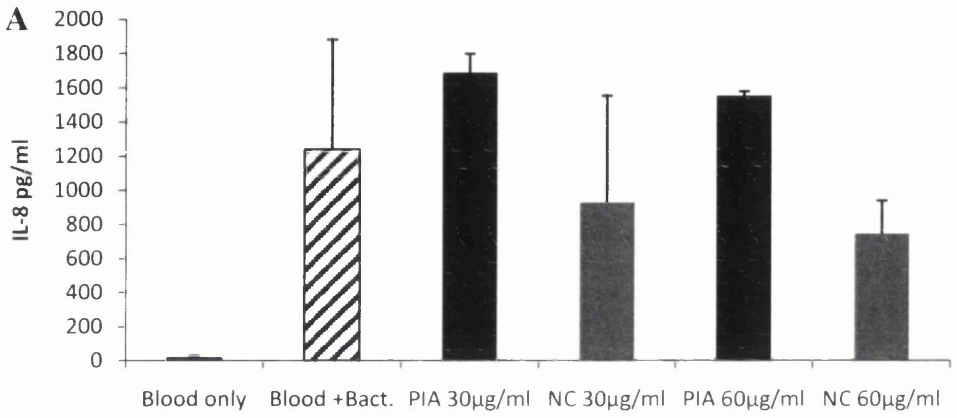
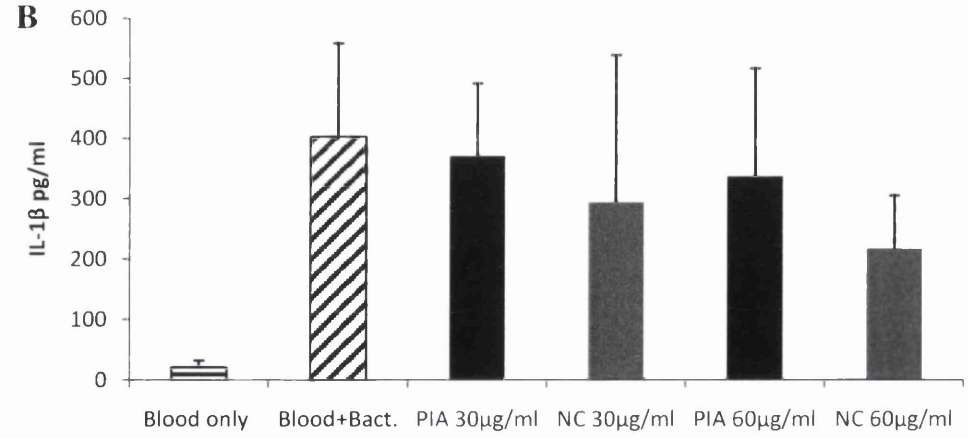


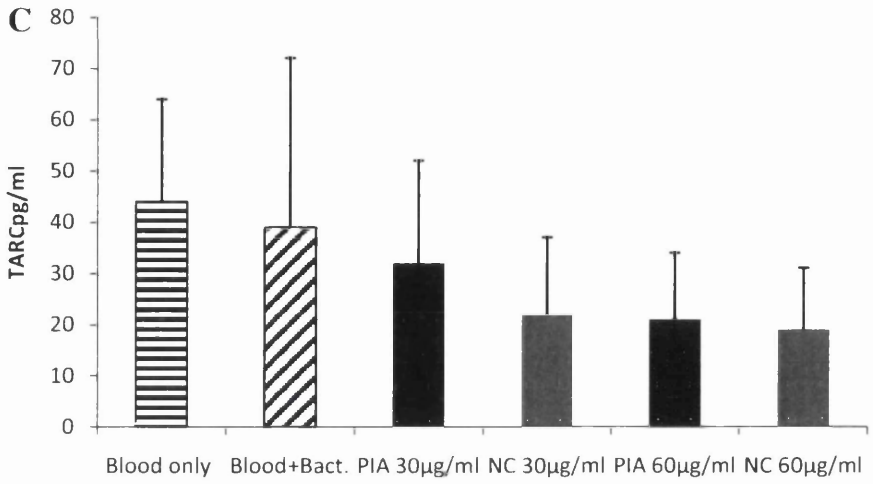
Figure 6.12 The effect of soluble PIA on whole blood killing of *S. epidermidis* 1457-M10.

Whole blood was pre-treated with three different concentrations of PIA (10µg/ml, 30µg/ml and 60µg/ml) or equal volumes of negative control (NC) and infected with *S. epidermidis* 1457-M10 and incubated at 37°C for 6 hours with rotation, before bacterial viable counts were determined after 6 hours. Viable counts was established in TSB at t=0, t=6, whole blood alone (Blood), or blood treated with cyto.D (cyto.), PIA or NC. Stars indicate significant difference $P<0.5$. Results represent data from four independent experiments.

Cytokine (IL-8, TARC, IL-1β, TNF-α and IL-10) and complement C5a levels in sera were determined by ELISA (Figure 6.13 A-D and 6.15). IL-8 release increased in PIA pre-treated blood compared to NC pretreated blood and blood infected with bacteria but effect was statistically not significant (Figure 6.13A). Pretreatment with PIA had no effect on IL-1β, TARC, TNFα and IL-10 cytokine release (Figure 6.13 B-E).



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60
50
40
30
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TARCpg/ml
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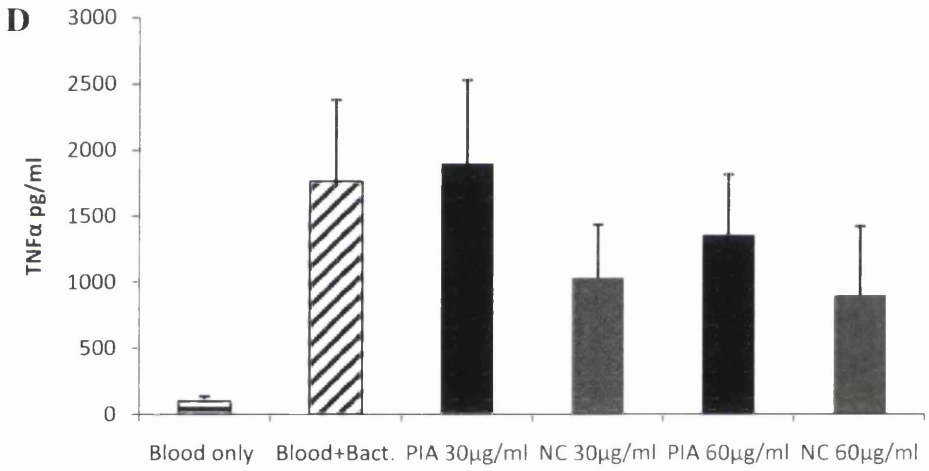
PIA 30μg/ml
NC 30μg/ml
PIA 60μg/ml
NC 60μg/ml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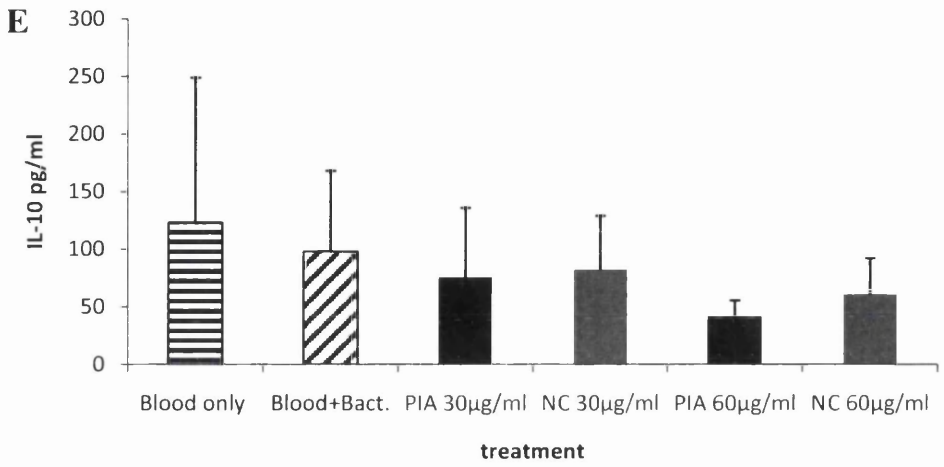

Figure 6.13 Cytokine expression in blood pretreated with PIA.

Cytokine ELISA of serum collected from blood pre-treated with different concentration of PIA, blood only and blood infected with bacteria alone used as control. **A.** IL-8 ELISA, **B.** IL-1β ELISA, **C.** TARC ELISA, **D.** TNF-α ELISA, **E.** IL-10 ELISA. Blood+Bact., blood infected with *S. epidermidis* 1457-M10. PIA, Polysaccharide intercellular adhesin purified extract obtained by IMDM method from PIA/biofilm positive *S. epidermidis* 1457. NC, negative control extract obtained from PIA/biofilm negative *S. epidermidis* 1457-M10. Results represent data from four independent experiments.

In contrast, pretreatment with PIA increased C5a release in compared to infected blood alone and this was statistically significant (P<0.01) (Figure 6.14).

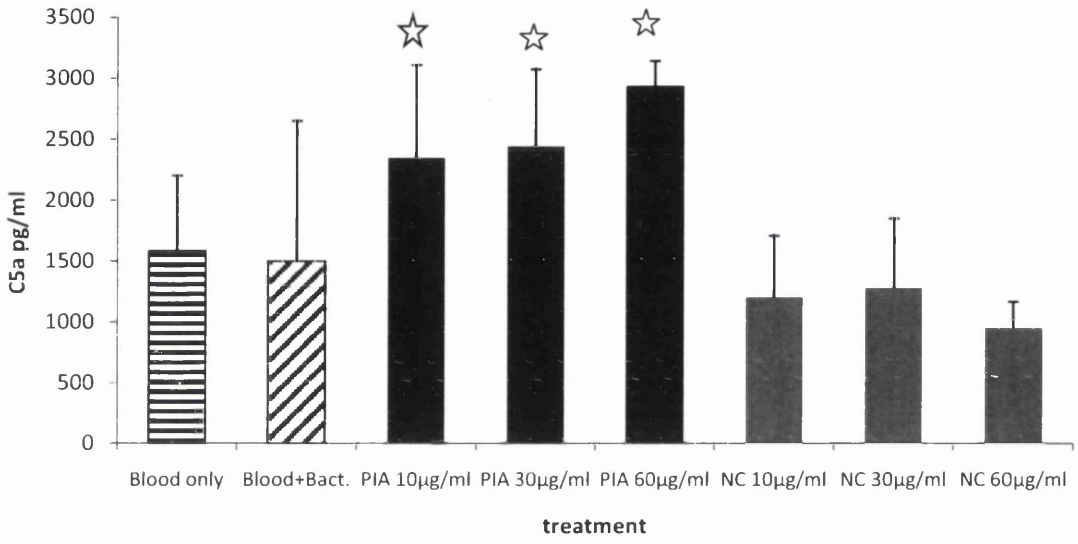

Figure 6.14 C5a Complement expression in blood pretreated with PIA extract. The C5a levels in response to pretreatment with different concentration of PIA (10, 30 and 60) µg/ml and equal volume of NC extract, blood and blood infected with bacteria alone were used as control. Stars indicate significant difference P<0.01. Blood+Bact., blood infected with *S. epidermidis* 1457-M10. PIA, Polysaccharide intercellular adhesin purified extract obtained by IMDM method from PIA/biofilm positive *S. epidermidis* 1457. NC, negative control extract obtained from PIA/biofilm negative *S. epidermidis* 1457-M10. Results represent data from four independent experiments.

To investigate the cytokine profiles induced in whole blood in response to purified PIA extract, serum from whole blood incubated with 60µg/ml of purified PIA extract and NC extract were analysed using the RayBio cytokine array (Figure 6.15 A-C). Cytokines detected in whole blood in response to purified PIA extract were similar to those found in untreated blood and whole blood treated with NC. Semi quantative differences are summerised in Table 6.1. Specifically, increases in PIA or NC induced cytokines compared to the control were observed for TNF, Oncostatin M, Thrombopoietin and VEGF. Differences between PIA and NC treated blood were observed for IL-10 and MIG. No differences were observed for TARC, IL-7, TGFβ, Angiogenin and GROα. The following cytokines were undetectable in whole blood; ENA-78, GCSF, GM-CSF, GRO, I-309, IL-1α, IL-1β,IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-12p40p70, IL-13, IL-15, IFN-γ, MCP-1, MCP-2, MCP-3, MCSF, MDC, MIP-1δ, RANTES, SCF, SDF-1, TNF-β, EGF, IGF-I, PDGF-BB, and Leptin.

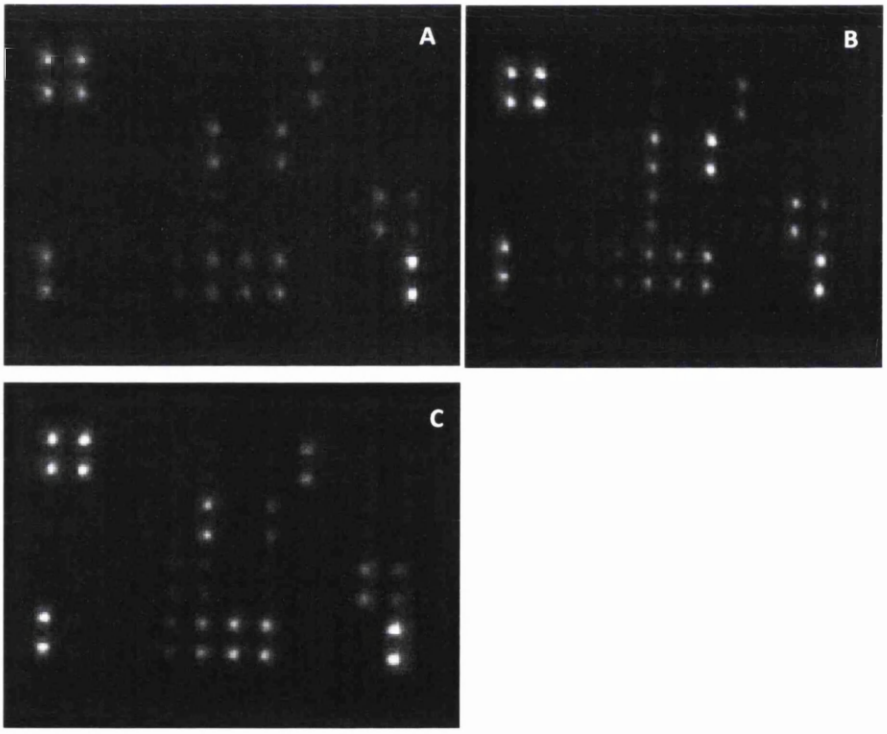

Figure 6.15 RayBio cytokines array of blood in response to PIA extract.

A. Control blood **B.** Blood and purified PIA extract **C.** Blood and NC extract

Table 6.2 Cytokines release in blood in response to PIA extract

	Control Blood	PIA extract	NC extract
Pos. control	+++	+++	+++
IL-10	++	+++	+
TARC	++	++	++
IL-7	++	++	++
TGF-β1	+	+	+
TNF-α	++	+++	+++
Angiogenin	+	+	+
Oncostatin M	++	+++	+++
Thrombopoietin	++	+++	+++
VEGF	++	+++	+++
GRO-α	++	++	++
MIG		++	+

Semi quantitative analyses of signals detected are: +, weak, ++; moderate, +++, strong signals.

6.6 The effect of biofilm associated PIA on whole blood

It was shown previously in this chapter (Section 6.1) that *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 can grow in immune suppressed blood and furthermore, immunocytochemistry (Figure 6.4) demonstrated that PIA can be secreted in immunosuppresed blood (pretreated with cyto.D). To test whether mature biofilms could form in both normal and/or immunosuppressed whole blood, biofilm and growth assays at four different dilutions (1:100 ($2.4x10^5$), 1:1000($2.4x10^4$), 1:10000($2.4x10^3$), 1:100000 ($2.4x10^2$)) of overnight culture (18 hours) were investigated.

Growth results showed that healthy whole blood eradicated all bacteria at lower inocula (1:1000, 1:10000, and 1:10000 dilution), but not at the highest inocula (1:100 dilution), (Figure 6.16A). In contrast, *S. epidermidis* 1457 grown in immunosuppressed (Cyto. D treated) whole blood resulted in to noticeable increases in bacterial growth at all dilutions of starting inocula (Figure 6.16 B).

Regarding biofilm formation, untreated normal whole blood completely inhibited biofilm formation (Figure 6.17A), however under immunosuppressive conditions the lowest dilution of starting inocula progressed to form a defined biofilm (0.256 at OD=570), as biofilm positive is defined at OD=2nm and above (Figure 6.17 B).

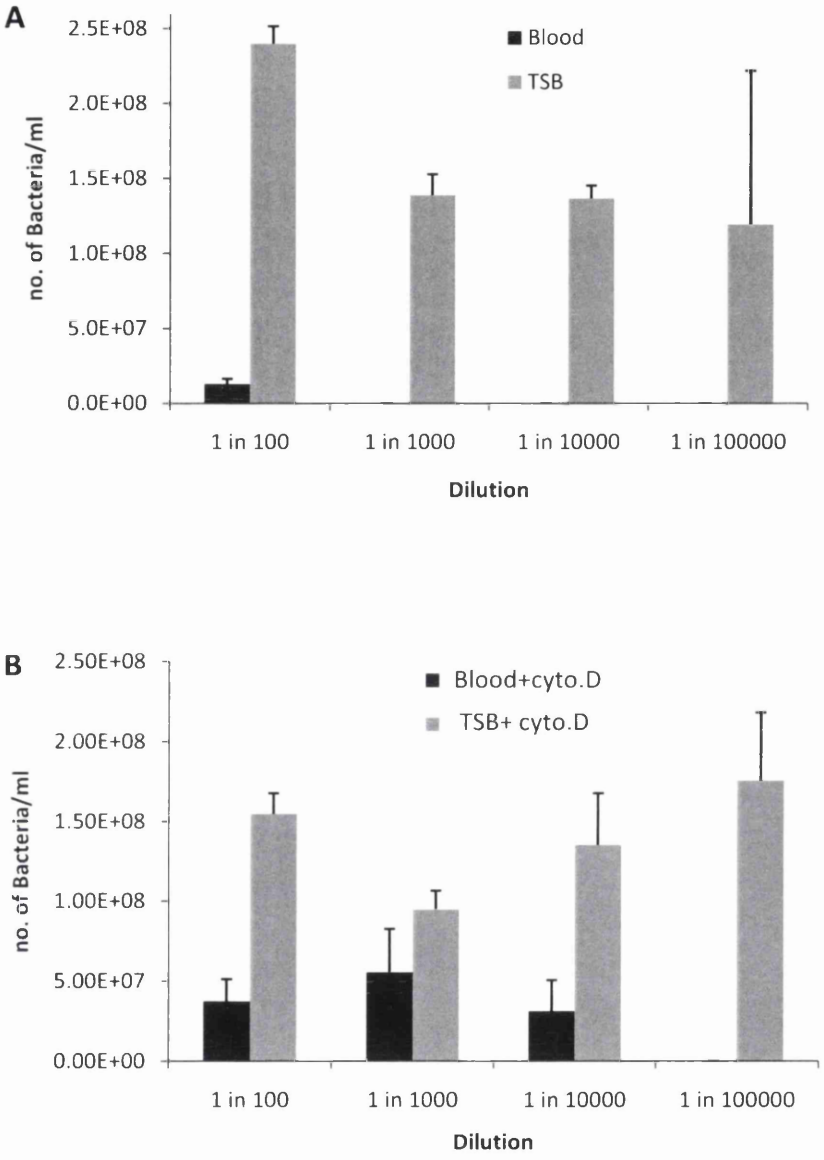

Figure 6.16 Growth of *S.epidermidis* 1457 in blood and TSB in presence and absence of cyto.D. A. Growth in normal whole blood **B.** Growth in whole blood with cyto.D (immunosuppressed). Results represent data from three independent experiments.

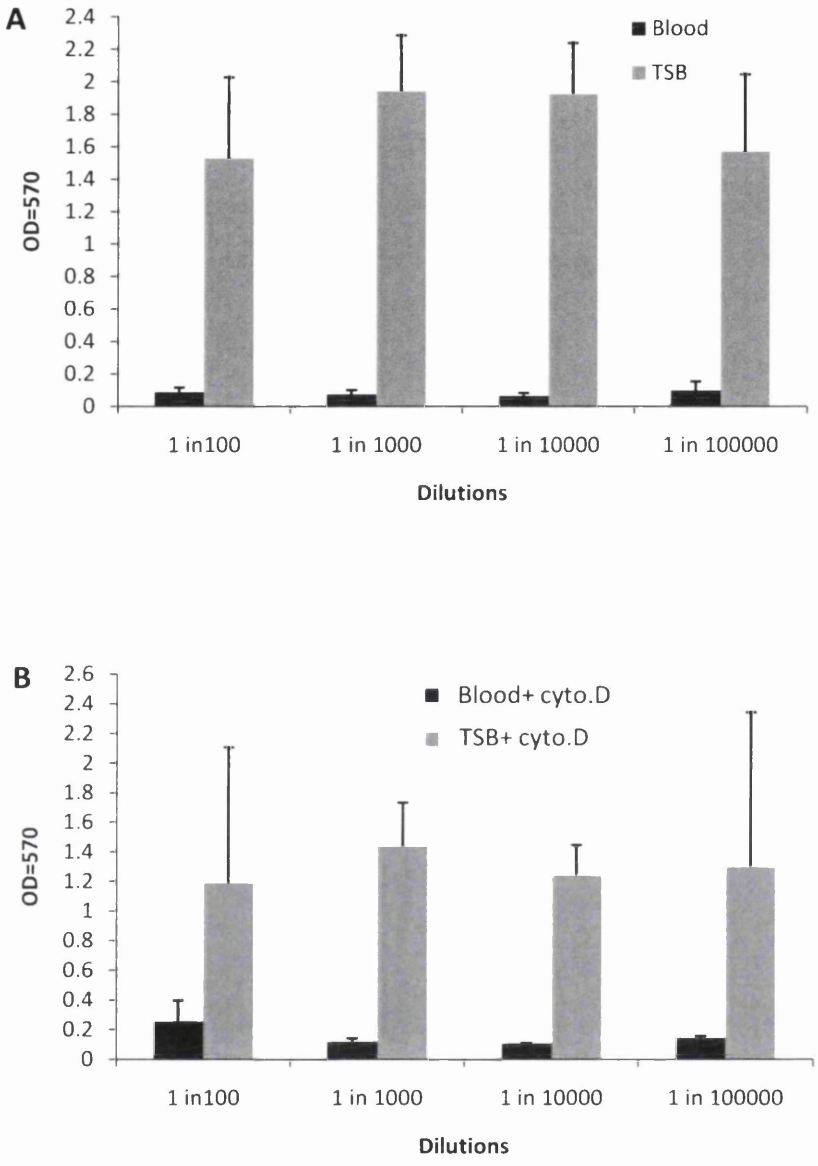

Figure 6.17 Biofilm formation of *S. epidermidis* 1457 in blood and TSB in presence and absence of cyto.D.

A. Biofilm formation in normal whole blood **B.** Biofilm formation in whole blood with Cyto.D (immunosuppressed). Results represent data from three independent experiments.

Finally, mature biofilms were used to investigate the effect of biofilm associated PIA on whole blood killing and cytokine production.

Previously in this chapter, whole blood had an inhibitory effect on bacterial growth and biofilm formation. Here whole blood killing (WBK) was undertaken on preformed *S. epidermidis* 1457 biofilm. Blood was added to biofilm for 6 hours at 37˚C, after which the biofilm was digested with Dis.B and bacterial viability assessed through viable counts. Results showed that there was no difference in the number of viable bacteria from blood incubated with preformed biofilm for 6 hours compared with biofilm incubated with TSB (Figure 6.18).

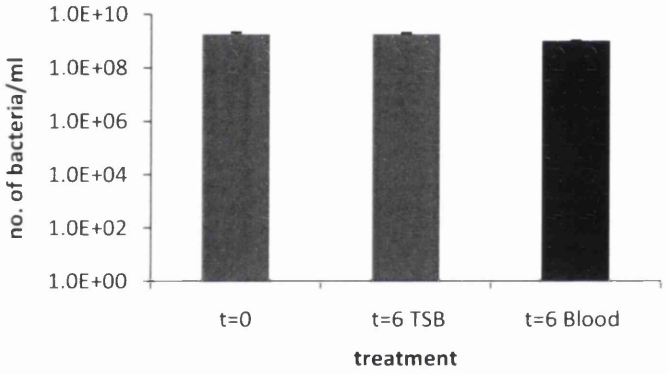

Figure 6.18 Whole blood killing on preformed biofilm. T=6 blood, represented viable bacterial count after 6 hours incubation with blood compared to original number of bacteria (t=0) and counts following 6 hours in TSB (t=6 TSB). Results represent data fron three independent experiments.

Finally, to examine the effect of biofilm associated PIA on cytokine profiles produced by whole blood, preformed *S. epidermidis* 1457 biofilm was incubated with 1ml of blood at 37°C with shaking for 3 hours, and the serum analysed using the RayBioHuman cytokine array.

The RayBioHuman cytokine array showed that *S. epidermidis* 1457 biofilms had an inhibitory effect on the release of specific cytokines and growth factors (TGF-β1, Angiogenin, Oncostatin M, Thrombopoietin, VEGF, TARC, TNF-α and GRO-α) compared to control blood. In addition, biofilms induced the release of other cytokines and growth factors (SDF-1, EGF and GRO-α) (Figure 6.19). Furthermore, biofilm had an inhibitory effect on cytokine release (IL-2, IL-7, MIG, and EGF) in response to *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 (section 6.5).

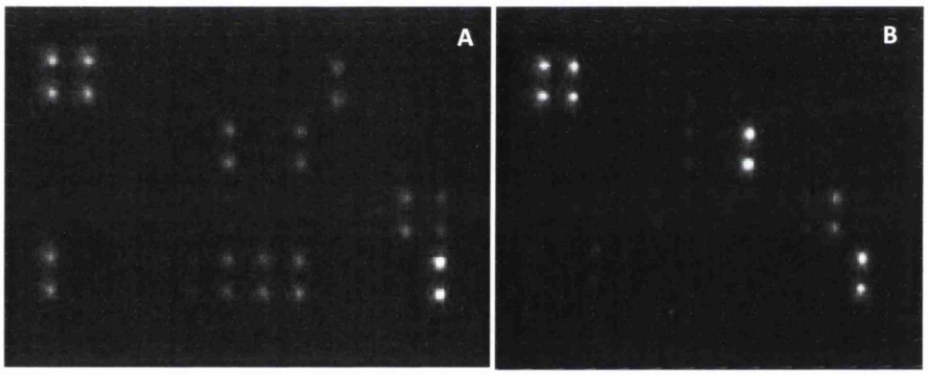

Figure 6.19 RayBioHuman cytokines array detected from blood in response to *S. epidermidis* biofilm. A. RayBio array of blood only **B.** Raybio array of *S. epidermidis* 1457 biofilm

6.7 Discussion

Studies on the effect of PIA and/or biofilm positive Staphylococci on whole blood are limited, as most previous studies have been done on isolated cells such as neutrophils and macrophages (Shiau and Wu, 1998; Leid, Shirtliff et al., 2002; Vuong, Voyich et al., 2004). Whole blood experiments are an alternative method to reflect physiological conditions *in vivo* regarding cellular function and cytokine production as the normal constituents of the media are present and, in addition immune cells, are present in normal percentages (Hermann, von Aulock et al., 2003).

The whole blood experiments presented interesting data regarding whole blood killing (WBK) and inflammatory response. Immunocompetent blood killing of planktonic *S. epidermidis* was found to increase with incubation period (from 2-6 hours). However, there were no statistical differences between biofilm positive and negative strains. There are only a few studies on the killing effect of whole blood on biofilm forming bacteria. Anwar *et al* found that *Pseudomonas aeruginosa* in biofilms were more resistant to whole blood killing than their planktonic equivalents (Anwar, Strap et al., 1992). Blood platelets have also been shown to provide an antimicrobial effect (reduction in viable counts 50-87%) on planktonically grown *S. aureus* and an ability to reduce metabolic activity in biofilm cells by 7-38% (Rozalski, Micota et al., 2013).

To this author's knowledge, this study is the first demonstration of an *ex vivo* experimental model showing that immunosuppression is an advantage for the growth of PIA/biofilm positive *S. epidermidis*. Despite the low amounts of cell associated PIA produced, sufficient advantage is gained following treatment with the glucocorticoid drug dexamethasone. Glucocorticoids are immunosuppressive and anti-inflammatory agents that interfere with leukocytes function (Boumpas, Chrousos et al., 1993; Franchimont, Galon et al., 2000; Barnes, 2010). This property facilitated their use in whole blood experiments to investigate WBK under immunosuppressive conditions. Similar effects can be observed in the immature neonatal innate immune system (Levy, 2007) where low cytokine production and complement response increase susceptibility to biofilm positive *S. epidermidis* sepsis (Sonntag, Brandenburg et al., 1998; Hartel, Osthues et al., 2008). In this regard, *S. epidermidis* is the most common causative agent

of neonatal catheter-related blood stream infection and sepsis (Marchant, Boyce et al., 2013). Interestingly, the difference in killing rate between *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 could not be demonstrated with cyto.D treated blood, where both strains had an equal advantage. This was likely due to very different mechanism of action between glucocorticoids and chytochalasins. Cyto.D interferes with phagocytosis, which is actin-polymerization dependent, and sensitive to actin-polymerisation inhibitors (Elliott and Winn 1986). Cytochalasin D is a fungal metabolite with actin – polymerization inhibition ability (Weihing, 1978) that decreases oxidative burst of blood neutophils (Smits, Burvenich et al., 1997) and interferes with killing, regardless of PIA status. Dexamethasone inhibits the production of inflammatory cytokines from the immune cells, the proliferation of stimulated T and B cells, and leukocyte motility (Kurihara, Ojima et al., 1984; Zhang and Mosser, 2008).
Interestingly, differences in the inflammatory response were also noticed between dexamethasone and Cyto.D treatment of whole blood. Whilst immunosuppresed blood with dexamethasone had anti inflammatory properties seen as a decrease in IL-8, IL-1β and TNF-α, Cyto.D actually increased production of IL-8, TARC and complement C5a.

The experiments on soluble PIA pretreated whole blood support its role as an inhibitor of whole blood killing or an inducer of leukocyte dysfunction. Pre-incubation of whole blood with purified PIA extract (obtained by the IMDM method) inhibited killing of *S. epidermidis* 1457-M10 in a dose dependent manner. These results are consistent with previous studies showing that cell associated and biofilm associated PIA inhibit the killing effects of the immune system including action of antimicrobial peptides and phagocytosis (Vuong, Voyich et al., 2004; Conway Morris, Kefala et al., 2009; Morris, Brittan et al., 2011). Soluble PIA induced IL-8 and complement fragment C5a release, whilst decreasing IL-10 when compared to untreated blood. These findings are consistent with a study showing that PIA from *S. epidermidis* 1457, whether cell associated or biofilm associated, induced stronger complement activation when compared to *S. epidermidis* 1457-M10 (Fredheim, Granslo et al. 2011). Interestingly, neutrophils have recently been shown to be activated by *S. epidermidis* biofilm matrix components (Meyle, Brenner-Weiss et al., 2012).

This present study is the first *ex vivo* model of WBK (both immune competent and immune suppressed) for investigating of *S. epidermidis* growth and biofilm formation.
The data presented in this chapter indicated that whole blood collected from healthy volunteers had the ability to clear *S. epidermids* bacteria and also prevented biofilm formation. This effect appeared dependent on the number of bacteria in the initial inoculum as blood completely cleared 240 bacteria/ml, with $2.4X10^3$ and $2.4X10^4$ CFU /ml persisting with restrained growth. The highest bacterial inoculum ($2.4X10^5$ CFU/ml) continued to grow to ($1.2X10^7$CFU/ml) which was significantly higher than for other bacterial inocula used. In immunosuppressed blood, bacteria grew even when used at the lowest bacterial concentration ($2.4X10^2$ CFU/ml) and also had biofilm formation ability. This findings correlate with these of Tunney *et al* (Tunney, Dunne et al., 2007) who demonstrated the importance of initial inculum size on antibiotic sensitivity and biofilm formation, as low initial inocula (~$1x10^3$CFU/ml) of *S. epidermidis* were killed by antibiotics, whilst high initial inocula resisted antibiotic killing and adhered to surfaces forming biofilms.
Neonateal sepsis with or without indwelling catheters is mainly caused by *S. epidermidis* and associated with the immature neonatal immune system (Stoll, Gordon et al., 1996; Stoll, Gordon et al., 1996; von Eiff, Jansen et al., 2005). In these cases quantitative neonatal blood cultures often reveal CoNS at concentrations of around 10^3 CFU/mL (Buttery, 2002). Bacteremia does not generally lead to sepsis as the bacteria tend to be are present in small numbers which are rapidly removed by a competent immune system. However, if bacteria are present in large enough numbers, in people with weakened immune systems, bacteremia can lead to sepsis.

In this present study whole blood was unable to clear preformed *S. epidermidis* biofilm and this has been noticed by others. Shiau and Wu (Shiau and Wu, 1998) found that "extracellular slime" produced by *S. epidermidis* interfered with macrophage phagocytic activity. Guenther *et al* demonstrated that *S. epidermidis* biofilm was more resistant to phagocytosis by neutrophils compared with *S. aureus* biofilm (Guenther, Stroh et al., 2009). Other researchers have found the same protective effect of biofilm (Steinberg, Poran et al., 1999; Kristian, Birkenstock et al., 2008) or biofilm modulation of the

immune response through attenuating leukocyte penetration into biofilm (Costerton, Stewart et al., 1999; Hoiby, Krogh Johansen et al., 2001).

To the author's knowledge this current study is the first demonstration of PIA expression in whole blood. ICC images revealed *S. epidermidis* 1457 expression of PIA under immnosuppressed conditions, whilst immune competent blood cleared the bacteria. It was also demonstrated that PIA expression by *S. epidermidis* 1457 in IMDM cell culture media occurred confirming the results of Chapter 3.

Phagocytosis of both *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 was dependent on complement receptors CD11b/CD18. The complex of CD11b/CD18 is present on neutrophils and mediate cellular clearance of pathogens (Hickstein, Ozols et al., 1987). Patients with leukocyte adhesion deficiency type I (LAD I) have severe or total deficiency of the CD11/CD18 on the cell surface, which leads to serious and fatal infections (Lekstrom-Himes and Gallin, 2000; Bonilla and Geha, 2003). Interestingly CD11b/CD18 expression on human neutrophils has been shown to be upregulated by staphylococcal supernatant (Veldkamp, Van Kessel et al., 1997; Meyle, Brenner-Weiss et al., 2012) but this was not measured in our study, and could be an area for future work.

The inhibitory effect of PIA/biofilm on WBK and inflammatory responses supports the growing evidence of a pathogenic role of this molecule. In this research the mechanism behind this effect was studied. The research demonstrated that purified PIA increased blood C5a level in a dose dependant manner, which may cause dysfunction of phagocytic cells (Figure 6.20). Interestingly, it was previously been shown that neutrophil dysfunction is medicated by C5a through inhibition of phagocytosis through preventing actin polymerization, in critically ill patients (Conway Morris, Kefala et al., 2009; Morris, Brittan et al., 2011). This also correlates with work in this chapter where Cyto.D increased C5a levels, suggesting an extra activity for Cyo.D in addition to its role as an actin –polymerization inhibitor (Weihing, 1978).

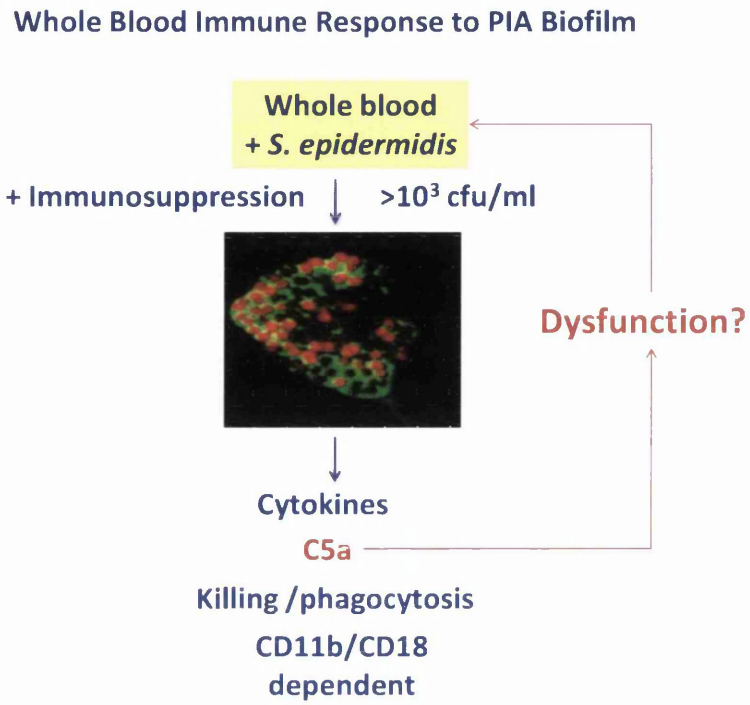

Figure 6.20 Whole blood immune response toward PIA/ biofilm. Immunosuppresion and size of initial bacterial inoculum > 10^3 CFU/ml are an advantage for growth and biofilm formation of *S. epidermidis* 1457 in blood, lead to increase in IL-8 cytokine and C5a complement release. C5a complement may have a role in phagocytosis dysfunction. Phagocytosis of *S. epidermidis* is CD11b and CD18 complement receptors dependent.

Chapter 7

Discussion

Bacterial biofilms are acknowledged as causes of persistent infection. In the case of health care associated infections (HAIs) related to implantation of a medical device theses are often accompanied with a destructive inflammatory response (Broekhuizen, de Boer et al., 2007). *Staphylococcus epidermidis* causes about 50% of infections associated with catheters, artificial joints and heart valves (Khalil, Williams et al., 2007). According to the National Healthcare Safety Network (NHSN) the most common cause of (HAIs) are CoNS (15%), followed by *S. aureus* (14%) (Jernigan and Kallen, 2008). In the UK, the cost of implant-associated infections is approximately £7-11 million per year (Harris and Richards, 2006). Given the importance of such infections, this research thesis investigated biofilm molecules associated with *S. epidermidis*, including PIA, Aap and Embp, under conditions that more accurately reflect those of the host environment.

In chapter 3, biofilm formation by *S. epidermidis* was investigated under physiological media. It has been long-established that glucose is an essential nutritional requirement for *S. epidermidis* biofilm formation (Mack, Siemssen et al., 1992; Dobinsky, Kiel et al., 2003). Few studies have been undertaken to identify minimal nutritional requirement needed to generate a *S. epidermidis* biofilm. Hussain *et al* identified a chemically defined medium called Hussain-Hastings-White modified medium (HHWm) that supported biofilm formation (Hussain, Hastings et al., 1991). Chapter 3 describes the study of specific cell culture media to support biofilm formation of *S. epidermidis* strains with different mechanisms of biofilm accumulation. The study showed that cell culture media support growth and biofilm formation to different degree depending on the mechanisms of biofilm accumulation. This effect is likely due to the specific nutrients in these media that regulate PIA, Aap and Embp expression and resultant biofilm formation. For instance, it was shown that the amino acid glutamine, induces biofilm in *S. epidermidis* 1585-RA and *S. epidermidis* 1585 whilst was non-essential for biofilm formation by *S. epidermidis* 5179-R1 and was inhibitory for *S. epidermidis* 1457 biofilm. Recently, other purified nutrients have proved to be important with D-glucosamine addition to TSB shown to inhibit *S. epidermidis* biofilm formation (Aiassa, Barnes et al., 2012). Indeed, a similar effect was demonstrated by Enche *et al*, with adherence of *Candida albicans* to human epithelial oesophageal cell line partially inhibited in the presence of D-glucosamine, but not with D-glucose, L-fucose or D-galactose (Enache,

Eskandari et al., 1996). In addition, the presence of Mg and Ca are important for biofilm formation (Ozerdem Akpolat, Elci et al., 2003). A study by Cerca *et al* showed the importance of environmental conditions, such as high levels of Ca and Mg in cell culture medium for *S. epideremidis* evasion of the host immune response, as they supported dormancy within the biofilm which was more resistant to antibiotic and immune effects (Cerca, Andrade et al., 2011).

IMDM was able to support the growth and biofilm formation of *S. epidemidis* strains with different mechanisms of biofilm formation including *S. epidermidis* 1457, *S. epidermidis* 5179-R1 and *S. epidermidis* 1585-RA. Furthermore, culture conditions that promoted biofilm production in the biofilm negative strain *S. epidermidis* 1585 were also identified. Accordingly, biofilm production was demonstrated in IMDM, RPMI+ and DMEM, which may be due to the lack of iron. Some studies have previously suggested the role of iron deprivation on biofilm formation (Johnson, Cockayne et al., 2005; Johnson, Cockayne et al., 2008).

To investigate the host factors responsible for the pathogenic transformation of *S. epidermidis* from skin commensal to pathogenic microorganism, the effect of human and bovine serum on biofilm formation was assessed. Both FBS and PS had no effect on *S. epidermidis* growth, however biofilm formation by strains had differing sensitivities to serum. Accumulation of PIA dependent biofilm was less sensitive than Aap and Embp dependent biofilms. In addition, PS was more inhibitory than FBS, which correlated with findings of Abraham and Jefferson (Abraham and Jefferson, 2010). The inhibitory activity of FBS and PS was found to be due to high molecular weight components of serum. Previously, a number of surface factors have been shown to regulate biofilm formation including surface chemistry (Patel, Ebert et al., 2003; MacKintosh, Patel et al., 2006; Abraham and Jefferson, 2010), surface roughness (Harris and Richards, 2006) and bacterial surface hydrophobicity (Sudagidan, Erdem et al., 2010). In this present study, pre-coating the polystyrene surface of the wells of microtiter plates with serum was found to prevent biofilm formation by the protein -dependent biofilm former, *S. epidermidis* 5179-R1, *S. epidermidis* 1585-RA and the biofilm negative (induced) *S. epidermidis* 1585.

The findings from Chapter 3 informed experiments undertaken in chapter 4 to purify PIA on a larger scale (milligrams). Characterisation of a purification method that can

purify PIA on a large scale (milligrams) for use in cell culture studies, without any contamination with LPS, was essential for accuracy of subsequent host pathogens interaction work. This is because macrophages can be activated by LPS concentrations as low as 15pg/ml (Wakelin, Sabroe et al., 2006). Therefore IMDM cell culture medium was used that supported *S. epidermidis* 1457 biofilm formation. The amount of PIA purified by the IMDM method in glass beakers was lower than the PIA obtained by the Mack Method (Mack, Nedelmann et al., 1994). However, using cell culture plates and increasing the surface area (from 6-24 plates) to which the bacteria adhered, the amount of PIA was greatly enhanced in comparison to the Mack method. Increasing the surface area 4X (6 to 24 plates) increased the amount of PIA obtained by 6-10X (100µg/ml from 6 plates to 600-1000µg/ml in 24 plates). It seems that using cell culture plates also provided different substrata to glass beakers which increased biofilm accumulation. Adherence and biofilm formation by *S. epidermidis* can be affected by biomaterials and their chemical and physical surface properties (Patel, Ebert et al., 2003; Patel, Ebert et al., 2007). Bacterial adherence and biofilm formation to intraocular lenses are increased when they are made from polymethyl methacrylate, silicone as bacteria have greater avidity for the irregular surface (Kodjikian, Roques et al., 2006; Baillif, Ecochard et al., 2009).

In chapter 5 the effect of PIA on THP-1 macrophages were investigated. Biofilm formation mediated by PIA is a major virulence factor of *S. epidermidis* and important for its pathogenesis (Nedelmann, Sabottke et al., 1998; Li, Xu et al., 2005; Fredheim, Granslo et al., 2011). PIA is synthesized by enzymes encoded in the *icaADBC* operon (Heilmann, Schweitzer et al., 1996). The majority of commensal *S. epidermidis* lack the *icaADBC* operon (Kozitskaya, Olson et al., 2005).

Remarkably, in chapter 5 it was shown that different forms of PIA had varying influences on the immune response elicited by monocytic THP-1 cells. Cytokine responses were dependent on cell associated or biofilm associated PIA, as when purified PIA was added to THP-1 cells there was no IL-8 release. After *S. epidermidis* 1457 was added in suspension there was enhanced IL-8 release than that generated by *S. epidermidis* 1457-M10. Furthermore, digestion of *S. epidermidis* 1457 biofilms with Dsp.B reduced IL-8 release. These results confirm that PIA may need to be bound to the bacterial surface to cause its effects. These results highlight the importance of PIA tethering; that is when PIA is bound to the bacterial surface it

is held in the correct conformation to stimulate a receptor on the THP-1 cells. Interestingly, for complete biofilm forming activity, PIA needs to be deacetylated, through the function of the *IcaB* gene, the product of which is a surface protein in charge of polysaccharide de-N-acetylation (Vuong, Kocianova et al., 2004; Cerca, Jefferson et al., 2007; Itoh, Rice et al., 2008). Deacetylation has been shown to be essential for *S. epidermidis* biofilm formation, immune evasion and virulence in animal models of implant infection (Vuong, Kocianova et al., 2004). Another explanation for the effects of PIA biofilm is mediator synergy, in which PIA requires another stimulus to be present; PIA plus other component of biofilm such as extracellular DNA, teichoic acids and peptidoglycans to show its biological effect. Thus, when PIA is added alone there is no response, but when PIA is bound to *S. epidermidis* there is a biological effect (Mehlin, Headley et al., 1999; Sadovskaya, Vinogradov et al., 2004; Jones, Perris et al., 2005; Qin, Ou et al., 2007). Interestingly, other researchers have added combinations of purified PIA, LPS, peptidoglycans and pro-inflammatory cytokines without cytokines induction suggesting that mediator synergy may not be so important (Wilkinson, Yokoyama and Armstrong unpublished observations).

CSLM revealed that the unique structure of biofilm may play an important role in bacterial evasion of leukocytes. *Staphylococcus epidermidis* were seen to locate near the substratum and were surrounded by heterogeneously distributed extracellular polysaccharide which could protect the bacteria against host immune cell penetration and antibiotic diffusion. Schommer *et al* (Schommer, Christner et al., 2011) demonstrated that biofilm embedded bacteria were killed to a lesser degree by macrophages than planktonic equivalents. Kirby *et al* found that the biofilm matrix may act as a diffusion barrier protecting bacteria from the killing effect of antibiotics like Colistin, Streptomycin, and Gentamicin, as planktonic bacteria were killed to a greater extent than those embedded within biofilms (Kirby, Garner et al., 2012). Furthermore, *S. epidermidis* biofilm restricted THP-1 cell migration into biofilms *in vivo*; similar findings were also demonstrated by Thurlow *et al*, as *S. aureus* biofilm limited macrophages penetration (Thurlow, Hanke et al., 2011).

In chapter 6 biofilm interactions with the host was examined in an *ex vivo* model of infection to demonstrate the immune response toward different forms of *S. epidermidis* PIA (cell associated, biofilm associated and purifies). Pre-treatment of

blood with purified PIA extract significantly reduced *S. epidermidis* killing in dose dependant manner which supports the role of PIA as an inducer of leukocyte dysfunction. Studies have demonstrated the role of PIA in protecting *S. epidermidis* from phagocytosis and reducing opsonization with C3b and IgG binding on the bacterial surface (Vuong, Voyich et al., 2004; Cerca, Jefferson et al., 2006; Kristian, Birkenstock et al., 2008). However, the exact mechanism of phagocytosis inhibition is not clear. *Staphylococcus epidermidis* PIA was found to induce C5a, which may lead to phagocytosis inactivation thus possibly explaining the observed decrease in WBK in blood pre-treated with PIA. Previous studies have suggested that C5a induces paralysis of phagocytic cells (Ward, 2004; Ward, 2008; Conway Morris, Kefala et al., 2009). Fredhem *et al* showed that whilst purified PIA induced complement activation it also decreased activation of granulocytes (Fredheim, Granslo et al., 2011). Satorious *et al* showed that C5a levels induced by *S. epidermidis* PIA biofilm could be lower than needed for leukocytes activation (Satorius, Szafranski et al., 2013). In addition, bacteria inside the biofilm appear more resistant to killing by whole blood. Indeed Thurlow *et al* confirmed the protective mechanisms of biofilm against host innate immune response (Thurlow, Hanke et al., 2011). Killing bacteria in a biofilm has been reported to require antibiotic concentrations 10 to 1,000 times that needed to kill free-living bacteria (Drinka, 2009).

This study is the first demonstration of an *ex vivo* experimental model showing that immunosuppression is an advantage for the growth of PIA/biofilm positive *S. epidermidis*. Here, dexamethasone pre-treated blood significantly decreased killing of *S. epidermidis* 1457 PIA/biofilm positive bacteria in comparison to *S. epidermidis* 1457-M10 PIA /biofilm negative bacteria. This study is also the first *ex vivo* model of the use of WBK (both immune competent and immune suppressed) for investigating of *S. epidermidis* growth and biofilm formation. The importance of blood immune status and size of bacterial initial inocula in the outcome of bacterial infection has been demonstrated. Immune suppression was also shown to be advantageous for bacterial growth and biofilm formation. Inocula of ~10^3 cfu of *S. epidermidis* were required to form biofilms in whole blood. This finding correlates with those of Tunney *et al* who demonstrated the importance of initial inoculm size on antibiotic sensitivity and biofilm formation, as low initial inocula (~$1x10^3$CFU/ml) of *S.*

epidermidis were killed by antibiotic, whilst high initial inoculum resisted antibiotic killing and generated biofilm (Tunney, Dunne et al., 2007).

This study is also the first to demonstrate PIA expression in whole blood, as ICC images revealed the ability of *S. epidermidis* 1457 to express PIA under immnosuppressed conditions. Immunocompetent blood did however have the ability to clear bacteria. Moreover, phagocytosis of both *S. epidermidis* 1457 and *S. epidermidis* 1457-M10 was dependent on complement receptors CD11b/CD18. The expression of CD11b/CD18 on human neutrophils has been shown to be upregulated by staphylococcal supernatant (Veldkamp, Van Kessel et al., 1997; Meyle, Brenner-Weiss et al., 2012).

This thesis has therefore investigated and identified host factors that stimulate and regulate biofilm formation in *S. epidermidis*. The study has brought attention to the importance of differential nutrient requirements needed for each biofilm accumulation mechanism. Studies using macrophages *in vitro* have identified the importance of PIA forms for final biological response. 3D modelling stressed the importance of structural characteristic of PIA biofilm which may have a barrier function that protects bacteria inside biofilm from the killing effect of immune system and antibiotic. Studies in *ex vivo* whole blood have suggested that PIA can induce C5a which is a critical mediator of leukocyte dysfunction and has allowed a biological advantage for biofilm under immunosuppressive conditions to be demonstrated (Figure 7.1).

Recognizing the nature of the immune response towards biofilms, as well as the optimal conditions needed for PIA expression and biofilm formation in blood, will be vital for the development of treatments for *S. epidermidis* biofilm infection on implanted medical devices.

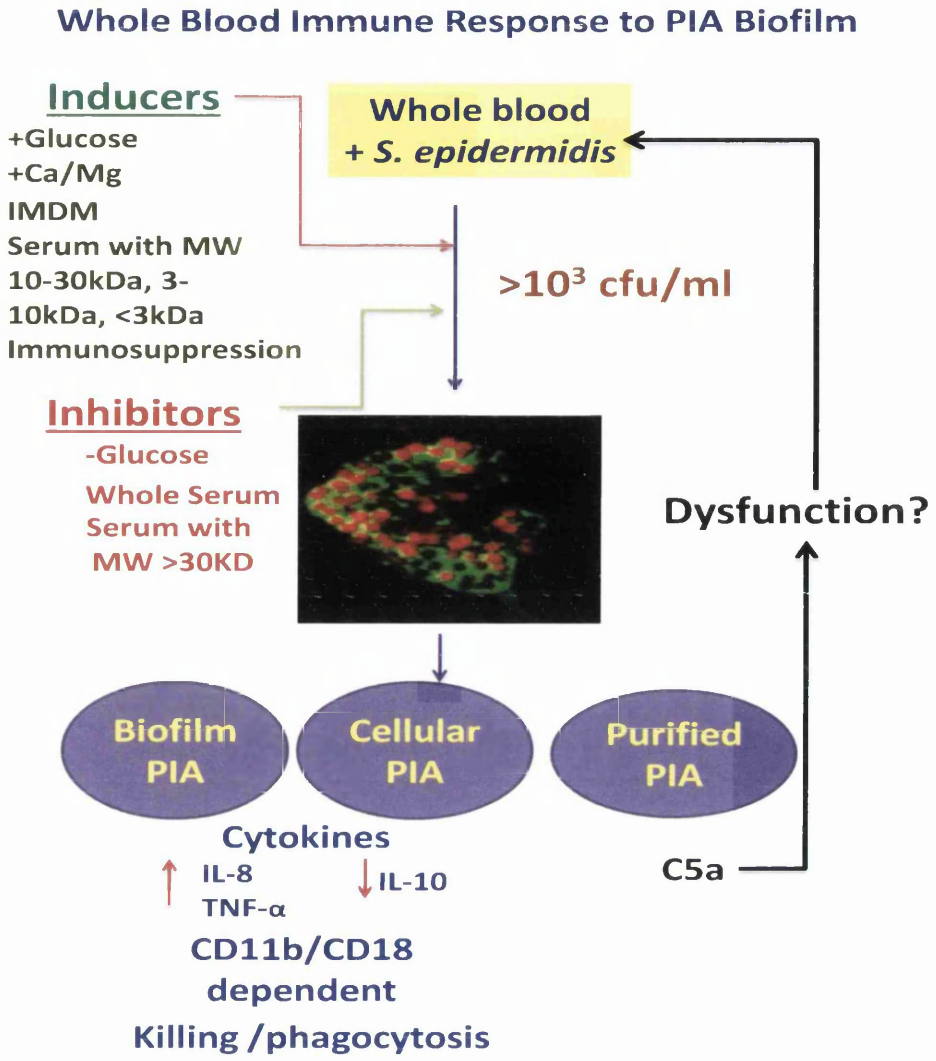

Figure 7.1 Schematic representations of results from this thesis. This thesis identified the importance of initial bacterial inoculums size (> 10^3 CFU/ml) on *S. epidermidis* biofilm formation. In addition, it recognized factors that induce or inhibit biofilm formation. A difference in the inflammatory response detected with different form of PIA (soluble PIA, biofilm associated PIA or cell associated PIA). Also, it was found that PIA induces C5a complement which may lead to dysfunction of immune cells and inhibit phagocytosis.

Future perspective

This study has identified a number of issues that may form the basis for future experiments. There is a need to:

1. Further investigate the nature of high molecular weight serum components that inhibits biofilm formation.
2. Further investigate the inhibitory effect of serum on biofilm formation by determination of the key interactions between the serum component and the bacterial cell surface through antibody blocking and Western blot analysis of cell extracts.
3. Investigate whole blood killing in both immune competent and immune compromised whole blood towards different forms of biofilm accumulation mechanisms (Aap and Embp).
4. Investigate serum responses in patients with medical device related infections.
5. Study the cytokines profile of whole blood in response to different forms of biofilm accumulation mechanism; comparison between PIA, Aap and Embp in different form (cells associated and biofilm associated).
6. Undertaken antibody blocking experiments to confirm that C5a blocking antibodies can inhibit PIA induced leukocyte dysfunction.

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