The role of growth conditions on the interactions of *Pseudomonas* extracellular secretions with keratinocytes

Sophie Joanne Glossop BSc (Hons)

A thesis submitted to the University of Huddersfield in partial fulfilment of the requirements for the degree of Doctor of Philosophy

University of Huddersfield

January 2020

Copyright Statement

Acknowledgements

Firstly I would like to express my sincerest gratitude to my supervisor, Dr Andrew Collett for all his knowledge, support and encouragement that I have received throughout this research project and for his continued guidance and advice which has helped me develop as a researcher. I would also like to thank my co-supervisor Professor Paul Humphreys for providing the *Pseudomonas* strains and laboratory materials and for contributing a rich wealth of knowledge relating to all things microbial.

I would like to thank all my laboratory colleagues, with special mention to both Dr Simon Rout for his ideas and sharing of knowledge with various microbial aspects of this study and Dr Ana Vitlic for her help with flow cytometry and for her company on the many long trips to Sheffield. In addition I would like to thank the Biology technicians for all their help during the laboratory period.

Thank you to all my friends from home and in Huddersfield - particularly to all residents of ‘Girl Street’, with special mention to my very good friend Miss Zoe Hollis who has always been there as a source of support when it was most needed.

Finally, thank you to my amazing family and boyfriend for their continued encouragement and support, both physically and financially, throughout these (at times) stressful few years.

This thesis is dedicated to my parents, Laureen and Kevin.

Thank you for everything you have done for me to get me where I am today

I hope I have made you proud.

Abstract

The skin is the largest organ in the body and acts as a barrier to protect from the external environment as well as having an important immunogenic function. Damage to the skin such as wounding results in the loss of protection to the host and can permit the invasion of opportunistic pathogens, which can cause disruption to the healing process resulting in a chronic wound. *Pseudomonas aeruginosa* is an opportunistic pathogen and a coloniser of chronic wounds and is becoming increasingly recognised as major cause of hospital acquired infections due to its ability to secrete a variety of virulence and pathogenic factors such as toxins, proteases, vesicles and in addition biofilm formation. One of the main controls of preventing infections in hospitals is the use of biocides such as ethanol, which are frequently used in routine hospital cleaning.

Two strains of *Pseudomonas* (*aeruginosa,* hospital strain (PS3) isolated from a chronic wound dressing and (*fluorescens,* a laboratory reference strain (PF) that had no known virulence factors were grown in different media, supplemented with ethanol or glucose and in an additional media, simulated wound fluid for 24 or 80 hours. The effects of culture conditions on the response of the bacteria and their secretions was investigated directly and also by studying their effects on the keratinocyte cell line (HaCaT).

PS3 80 hour cultures showed an increased production of all the virulence factors tested compared to 24 hours cultures. In addition there was some differences between culture conditions with PS3 grown in ethanol producing a greater amount of hemolysin and pyocyanin, however, live bacteria from these cultures had little effect on keratinocytes unlike the corresponding extracellular secretions. The secretions from cultures grown with ethanol for 80 hours produced increased toxicity resulting in greater keratinocyte death and longer healing times in a scratch assay model of wound healing, in addition there was a high pro-inflammatory response from the keratinocytes compared to those exposed to live bacteria grown in the same conditions and also when compared to secretions from glucose grown PS3. Generally the secretion of CXCL8 was higher from cells exposed to PS3 secretions when grown in ethanol, however there was greater expression of MAMP receptors in keratinocytes exposed secretions SWF and glucose grown PS3. PF cultures grown in the same conditions produced no measurable virulence factors and the secretions had no toxic effects on the keratinocytes, however faster healing in the scratch assay occurred for some conditions.

The increase in virulence factors seen from ethanol grown PS3 in addition to the high toxicity in keratinocytes from 80 hour cultures indicates that prolonged exposure to trace amounts of ethanol within the bacterial microenvironment can influence the production of immunogenic and increase virulence factor production from PS3. Considering PS3 is a clinical isolate and there is increasing wide spread use of ethanol based products within clinical environments these studies highlight how the improper use of disinfection products may be enhancing bacterial pathogenicity and virulence within clinical isolates and enhancing microbial fitness potentially leading to an emergence of bactericidal resistant bacteria.

Table of Contents

Table of figures

Table of tables

Abbreviations

AHLs	Acyl homoserine lactones
AIs	Autoinducers
AIPs	Autoinducing peptides
ALI	Air liquid interface
ATCC	American type culture collection
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
CD	Cluster of differentiation
CF	Cystic Fibrosis
CLR	C-type lectin receptor
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CXCL	Chemokine ligand
DAMP	Damage associated molecular patterns
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	double stranded Ribonucleic acid
EC	Extracellular
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EPS	Extracellular polymeric substance
Exo	Exotoxin
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FMO	Fluorescence minus one

H & E	Hemotoxylin and Eosin
HBSS	Hanks balanced salt solution
HCAIs	Health care acquired infections
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
ICF	Instrument cleaning fluid
IFN	Interferon
IL	interleukin
IRAK	Interleukin receptor associated kinases
IMS	Industrial methylated spirit
KSFM	Keratinocyte serum free media
LB	Luria Bertani
LPS	Lipopolysaccharide
MAMP	Microbial associated molecular pattern
min	Minutes
MM EtOH	Mineral Media Ethanol
MM Glu	Mineral media glucose
MOI	Multiplicity of infection
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRD	Maximum recovery diluent
mRNA	messenger Ribonucleic acid
MyD88	Myeloid differentiation primary response 88
NaCl	Sodium chloride
NF-κB	Nuclear factor kappa B
NHS	National health service
NLR	NOD like receptor
NICE	National institute for health and care excellence
NOD	Nucleotide-binding oligomerization domain
OCT	Optimal cutting temperature compound

OD	Optical density
OMVs	Outer membrane vesicles
PBS	Phosphate buffered saline
PF	*Pseudomonas fluorescens*
PFA	Paraformaldehyde
PGN	Peptidoglycan
PRRs	Pattern recognition receptors
PS3	*Pseudomonas aeruginosa* hospital strain
QS	Quorum sensing
RLR	Retinoic acid-inducible gene1 receptors
RNA	Ribonucleic acid
rpm	revolutions per minute
RT-PCR	Real time – polymerase chain reaction
rRNA	ribosomal Ribonucleic acid
SEM	Standard error of the mean
ssRNA	single stranded Ribonucleic acid
SWF	Simulated wound fluid
TBS	Tris buffered saline
TGF	Transforming growth factor
TIR	Toll IL-1 receptor
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRAF	Tumour necrosis factor receptor–associated factor
TSA	Tryptone soy agar
UV	Ultraviolet
WHO	World health organisation

Chapter 1
Introduction

1.1 The skin

The skin is the largest and potentially the most complex organ in the human body, it acts primarily as a physical barrier between the host and the external environment to provide protection to the host (Miller, 2008) . As part of the barrier function, the skin constantly receives signals from the external environment which are detected through receptors which aid in the regulation of body temperature and prevention of water loss (Abdallah et al., 2017). In addition to its barrier and protective function, the skin is made up of a variety of cell types that provide a vast cellular network with cellular functions involved in structure, immunity and circulation (Menon, 2002). The skin is made up of 3 layers; epidermis, dermis and hypodermis (figure 1.1) (Kanitakis, 2002) each of which have a slightly different function and different cell types to reflect this.

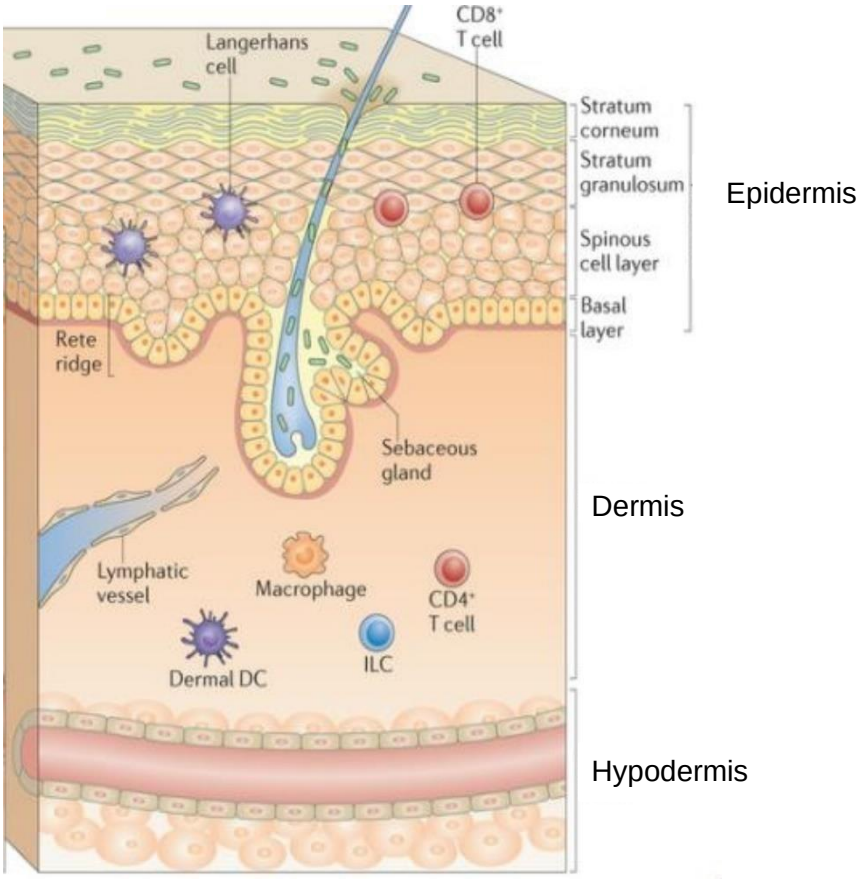

Figure 1.1 The structure of the skin. Diagram of the skin showing the location of the three layers; epidermis, dermis and hypodermis where the epidermis forms the most outer layer and the associated cells types found there. Adapted from Pasparakis et al. (2014)

The hypodermis is the inner layer of the skin and is mainly composed of fibroblasts and adipose tissue but also contains many immune cells, and acts to attach the dermis and epidermis to the under lying tissue such as bones and muscles. The next layer the dermis is made primarily of collagen fibres which provide flexibility and mechanical strength to the skin as well as containing blood vessels, nerve endings and sweat glands which are involved in the control of body temperature, in addition it also contains immune cells such as macrophages and mast cells (Nguyen and Soulika, 2019). It also acts to support the function of the epidermis and facilitates the delivery of nutrients and removal of waste products to contribute to the health of the epidermis. The epidermis forms the outer layer of the skin and is primarily composed of three main cell types; keratinocytes, which contribute to immune function, melanocytes which are responsible for pigmentation and Langerhans cells which are a type of dendritic cell involved in antigen presentation. The epidermis is made up of five different layers, each of which contribute to providing a strong protective function and continued renewal of the skin.

Stratum corneum uppermost top layer.
Stratum lucidum mainly located on the palms and soles
Stratum granulosum
Stratum spinosum
Stratum basale deepest layer

As the epidermis forms the outer layer of the skin and is in constant contact with the external environment it can be susceptible to damage (Dabboue et al., 2015) which can permit the invasion of potential harmful pathogens and thus specific healing responses are required. As part of its protective function, the skin has an

important immunologic role and can provide a rapid first line of defence against invading microbes (Hirobe, 2014).

1.1.1 Keratinocytes

Keratinocytes are the most abundant cell types in the epidermis, accounting for around 90% of the cells found there (Nestle et al., 2009). In the stratum basale, keratinocytes exist in an undifferentiated state before they undergo cell division to begin the maturation process which involves change of shape and the production and expression of different keratin filaments, (Matsui and Amagai, 2015) which occurs throughout the different layers of the epidermis until full maturation has been reached in the stratum corneum, where they become terminally differentiated and undergo desquamation (skin cell shedding) (Baroni et al., 2012). As keratinocytes are one of the main cell types found in the epidermis it is important that they can support the epidermis structure as well as its immune function. They are also essential in maintaining the skins barrier function, particularly in the stratum corneum where they can form tight junctions between other keratinocytes to promote the protection of the host to invading microorganisms such as bacteria, viruses and fungi as well as protecting against biological and chemical damage such as by ultra-violet (UV) radiation. More recently, keratinocytes have been found to contribute to both innate and adaptive immune functions within the skin (Nestle et al., 2009) and have been identified as being pivotal in initiating innate immune and inflammatory responses in the skin.

1.1.2 Keratinocytes in culture

The use of keratinocyte cell lines to study the normal physiology of dermal keratinocytes is well established (Boelsma et al., 1999). One main cell line used to study keratinocytes in culture is HaCaT, which is a spontaneously immortalized human keratinocyte cell line often used to investigate epidermal keratinocytes (Deyrieux and Wilson, 2007, Smits et al., 2017). An adaptation methodology was recently used to transform the culture conditions of HaCaT cells to serum free low calcium medium which involved the gradual replacement of serum supplemented media with keratinocyte serum free media (KSFM) over a period of 6 passages which resulted in an adapted (less differentiated) cell line termed HaCaTa, which more closely represent primary cells (Al Tameemi et al., 2014). HaCaT cells are often used in culture in order to investigate host-pathogen interactions relating to the skin, an area of research which is important in understanding pathogenic and host defence mechanisms (Pan et al., 2014).

1.2 The skin as an immune organ

The theory that the skin acts as an immune organ was first proposed by Streilein (1983) who suggested that the skin had specialised functions involved in immune surveillance. The skin is now widely recognised as being an active immune organ with the ability to provide resistance to microorganisms through both innate and adaptive immune responses (Guttman-Yassky et al., 2019). Immune responses are defined as the way a host defends itself against foreign material. The aim of the immune response is to destroy invading organisms that would otherwise harm or kill the host (Hoebe et al., 2004).

Immune responses within the skin are dependent on surveillance and a network of communication between both non-immune and immune cells each of which can contribute to immune and inflammatory responses (Salmon et al., 1994). In addition the skin is the location for a wide variety of soluble immune mediators such as chemokines and cytokines which aid in the orchestration and regulation of immune responses and inflammation (Dinarello, 2000) which are important in the innate immune response. Innate immune responses are the first to be triggered, and provide a rapid first line of defence to invading pathogens and can be initiated by both immune and non-immune cells throughout the dermis and epidermis (Coates et al., 2018). Due to their abundance within the epidermis and having the greatest exposure to the external environment keratinocytes have receptors that aid in the recognition of foreign microbes and thus have been considered true innate immune cells within the skin (Bernard et al., 2012).

1.2.1 Pattern recognition receptors

Pattern recognition receptors (PRRs) are defined as non-clonal germ line encoded receptors that are important in the initiation of innate immune responses (Suresh and Mosser, 2013). First discovered by Janeway (1989) PRRs are involved in the recognition of microbial patterns present on microorganisms known as microbial associated molecular patterns (MAMPs). Upon MAMP stimulation of PRRs, cell pathways are initiated leading to the activation of a cell-signalling cascades resulting in cytokine production, recruitment of phagocytes and stimulation of the adaptive immune response (Palm and Medzhitov, 2009, Kumagai and Akira, 2010, Moretti and Blander, 2014) leading to the eventual clearance of the invading microbe (Mogensen, 2009).

PRRs have also been found to be stimulated by danger associated molecular patterns (DAMPs) which unlike microbial ligands, are released or expressed by host cells in response to stress or injury (Land, 2015). Several families of PRRs have been identified, including toll like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) and retinoic acid-inducible gene1 receptors (RLRs) (Jang et al., 2015) (Amarante-Mendes et al., 2018) which are found in a variety of cell types involved in innate immunity and can be expressed in both intracellular compartments and as transmembrane receptors. Of all the PRR families, the toll like receptors have been the most extensively studied especially their role in the innate immune response.

1.2.2 Toll like receptors

TLRs are expressed on a variety of different cell types that are involved with initiating early components of the innate immune response within the skin (Hari et al., 2010) including keratinocytes (Valins et al., 2010). Much research has shown that TLRs 1-6 and 9 are expressed on keratinocytes (Baker et al., 2003, Lebre et al., 2007) and in addition to these findings Kollisch et al. (2005) demonstrated by RT-PCR that TLR10 mRNA is expressed in keratinocytes supported by Lebre et al. (2007).

TLRs are type 1 trans-membrane receptors that consist of an N terminal ecto domain that is involved in the recognition of MAMPs that form a unique horse-shoe structure (Botos et al., 2011) a single transmembrane helix and a C-terminal cytoplasmic signalling domain, also known as Toll IL-1 receptor (TIR) domain which is involved in activating intracellular signalling pathways (Jin and Lee,

2008; Botos et al, 2011). TLR synthesis occurs in the endoplasmic reticulum and they are then passed to the golgi where they can be localised to the cell surface or remain in the endosomes or lysosomes (Lee and Barton, 2014, Kawasaki and Kawai, 2014).

TLRs are all involved in the recognition of specific MAMPs presented by microorganisms (Tanimura and Miyake, 2014) (table 1.1). Microbes can express many different MAMPs depending on the species of bacteria, and these are generally conserved within species (Newman et al., 2013). Whilst conserved, MAMPS can differ between strains with some strains having more virulent and pathogenic properties which can also be influenced by other factors including the environment and the length of time the bacteria grow (Mogensen, 2009).

TLRs 1, 2, 4, 5 and 6 are all cell surface transmembrane receptors (Barton and Kagan, 2009) and as such they are responsible for the recognition of MAMPs presented extracellularly. TLR 2 can form heterodimers with TLR 1 and 6 mostly recognise Gram positive MAMPs including lipopeptides, peptidoglycan (PGN) and lipoteichoic acid (Oliveira-Nascimento et al., 2012) TLR 4 and its co-receptor CD-14 is involved in the recognition of lipopolysaccharide (LPS) from Gram negative bacteria and TLR 5 is involved in the recognition of bacterial flagellin (Borish and Steinke, 2003). TLRs 3, 7, 8 and 9 are all found in intracellular compartments (Barton and Kagan, 2009) and are responsible for the recognition of MAMPs presented intracellularly (Kawasaki and Kawai, 2014) and are mainly involved in the recognition of single and double stranded RNA, which are associated with viral infections (Kawai and Akira, 2011). TLR 9 can recognise hypo-methylated CpG DNA which can be found in both viral and bacterial DNA

(Pohar et al., 2017). A summary of the TLR's, cellular location and their ligand is shown in table 1.1.

TLR	MAMP	Special Features	Location
1	Bacterial tri-acyl lipopeptides	Heterodimers with TLR2	Cell membrane
2	Peptidoglycan (PGN), lipopeptides, and lipoteichoic acid, some LPS. Mainly gram positive MAMPs	Heterodimers with TLR1/6	Cell membrane
3	Viral dsRNA		Intracellular compartments
4	Gram-negative bacterial endotoxin (LPS)		Cell membrane
5	Flagellin		Cell membrane
6	Bacterial di-acyl lipopeptides	Heterodimers with TLR2	Cell membrane
7	Viral ssRNA		Membranes - intracellular compartments
8	Viral ssRNA		Membranes - intracellular compartments
9	Hypomethylated CpG motifs of bacterial and viral DNA		Membranes - intracellular compartments
10	Unknown	Not much known	Not identified

Table 1.1 Toll like receptors and their ligands. Specific Toll like receptors and their corresponding microbial ligand and location in different human cell types (Miller and Modlin, 2007b) (Takeda and Akira, 2005).

Once a TLR has been activated by a specific MAMP, a number of cell signalling cascades are initiated which leads to the activation of innate and adaptive immune responses often via the Myleoid Differentiation factor 88 (MyD88) dependent pathway, which is common to most TLRs (Takeda and Akira, 2005).

When activated TLRs will interact with MyD88, this is done via the Toll/IL-1 receptor domain present on the TLR (Miller and Modlin, 2007a). MyD88, once activated can recruit other signalling molecules including IRAK-1, and IRAK-4 and TNF receptor associated factor (TRAF6), which leads to the activation of nuclear factor-κ-B (NF-κB), which acts as a transcription factor for immunomodulatory genes involved in the immune response including the stimulation of cytokines, chemokines, antimicrobial peptides (AMPs) and co-stimulatory molecules such as CD40, a crucial modulator of the adaptive immune response (Chandel et al., 2014).

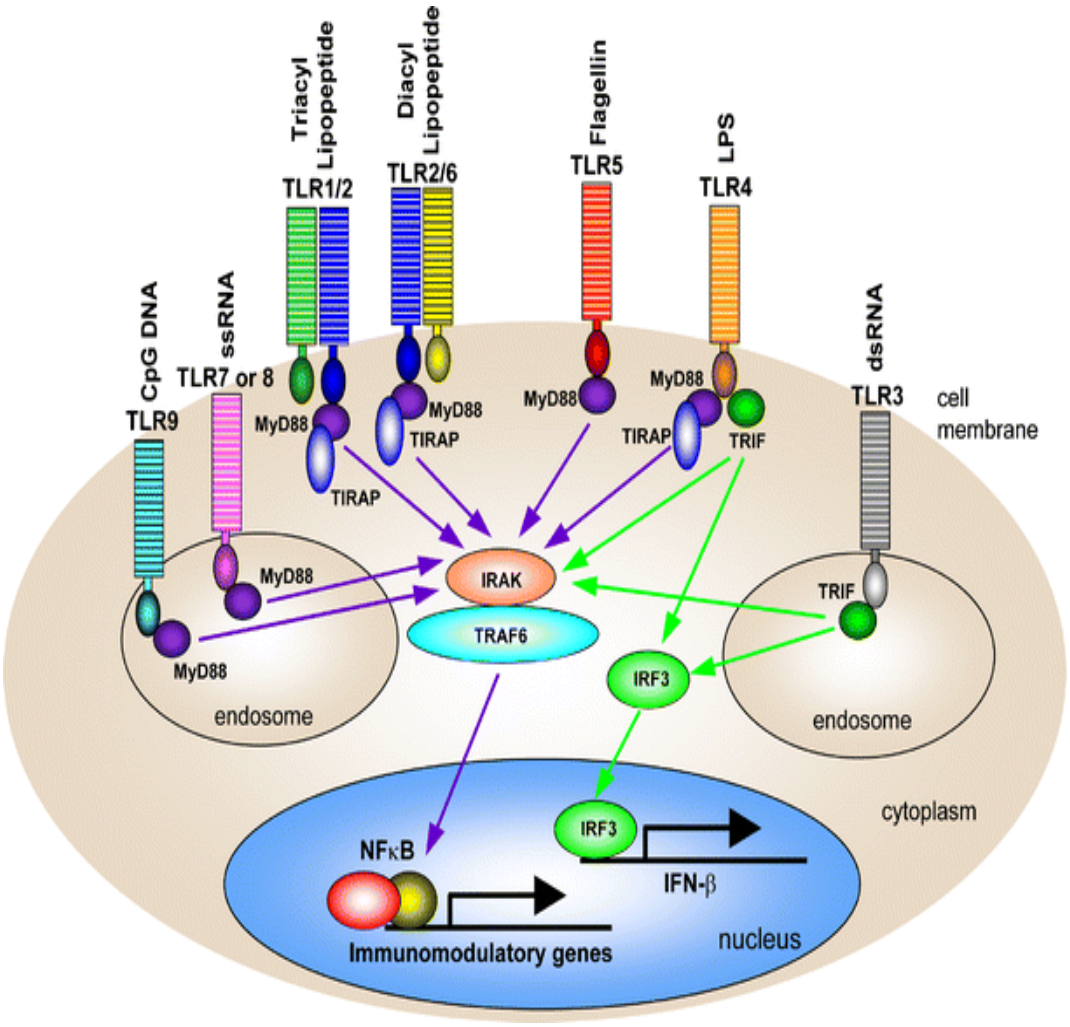

Figure 1.2 MyD88 Toll like receptor signalling pathways. Schematic diagram showing location of TLR's, the MAMPs that activate them and the subsequent signalling pathways. As shown on the diagram, each of the signalling pathways leads to the activation of NF- κB, which in turn promotes transcription of immunomodulatory genes. Adopted from (Miller and Modlin, 2007b).

Whilst recognition of MAMPs from invading microbes leads to the initiation of immune responses via TLR signalling, microorganisms have evolved ways to modulate host responses including, blocking of TLR signalling molecules such as NF- κB, evasion of phagocytosis and the modification of MAMPs to weaken the activation of TLRs (Arpaia and Barton, 2013, McGuire and Arthur, 2015). All of these factors aid in the invasion process of microorganisms and allow evasion from the immune system.

1.2.3 Pro-inflammatory cytokines

Cytokines are small chemical mediators that contribute to communication between cells (Zhang and An, 2007). Cytokines can be released by a variety of cell types in response to invading microorganisms and are key in the regulation of host immune responses to infection, inflammation and trauma (Dinarello, 2000). The activation of TLRs by their MAMPs initiates a cell signalling cascade that results in cytokine release from the host cell as described in section 1.2.2. Cytokines can signal to the host immune system to attract other immune cells such as macrophages to the site of infection and mediate interactions between immune cells to link the innate and adaptive immune responses (Sokol and Luster, 2015).

Cytokines can be categorised as either promoting or attenuating inflammation (pro/anti-inflammatory). Pro-inflammatory cytokines include interleukin (IL) 1 (CXCL1) and 8 (CXCL8) and Interferon gamma (IFN-γ), and anti-inflammatory cytokines include IL-10 and IFN-α (Cavaillon, 2001). One of the most important pro-inflammatory cytokines produced by a variety of cells in the skin including keratinocytes is IL-8 (CXCL8), where it can recruit neutrophils and macrophages

in response to inflammatory stimuli as well as promoting keratinocyte migration through binding to CXC receptors present on these cells (Sauder, 1990, Brat et al., 2005).

CXLC8 has an important role in the skin immune system such as aiding in tissue repair following damage (Gillitzer and Goebeler, 2001) however the over production of CXCL8 has been linked to pathogenesis of wounds and inflammatory skin disorders such as psoriasis (Kemény et al., 1994). It is important that the secretion of both pro and anti-inflammatory cytokines from host cells is balanced to ensure clearance of the microorganism without incurring damage to the host (Cicchese et al., 2018).

1.3 Pseudomonas

The *Pseudomonas* genus is the largest genus of Gram negative bacteria, with over 140 species currently identified (Gomila et al., 2015) with species classification based on 16S rRNA gene sequence, cellular fatty acid analysis and various biochemical tests (Ozen and Ussery, 2012), common species of *Pseudomonas* include *aeruginosa, fluorescens* and *syringae*, *Pseudomonas* is regarded an important genus of Gram negative bacteria consisting of species with both medical and biotechnological applications (Ozen and Ussery, 2012). In addition, it has a vastly diverse genome with many species having the ability to thrive in a wide variety of environments including water and soil (Winsor et al., 2016) and it can cause a number of plant and animal diseases. The highly diverse *Pseudomonas* genus is reflected in the genomic analysis with most genes being species specific or shared by a subset of the species (figure 1.4). This gives flexibility to the *Pseudomonas* genus and allows adaptation to specific

environments (Gross and Loper, 2009) some overlap seen between species (figure 1.3).

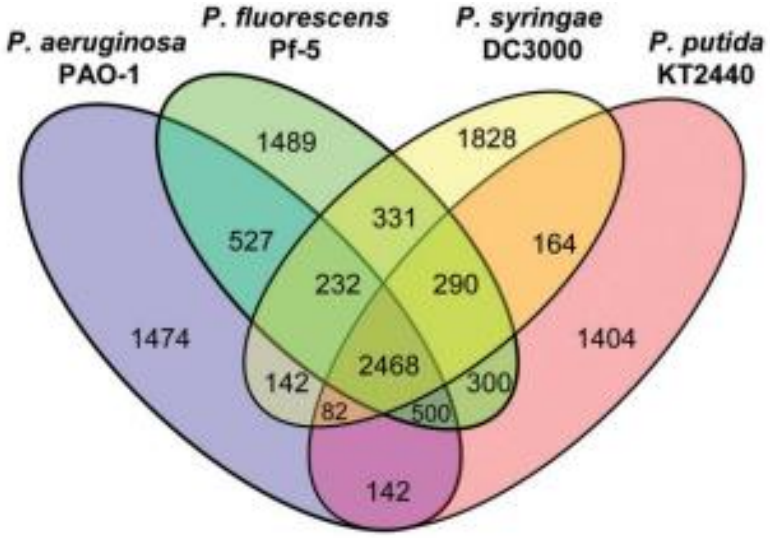

Figure 1.3 Genomic analysis of *Pseudomonas* strains Venn diagram illustrating the genomic analysis of 4 different *Pseudomonas* strains from 4 different species, adopted from Gross and Loper (2009).

1.3.1 *Pseudomonas fluorescens*

Pseudomonas fluorescens (PF) are obligate anaerobes that thrive in a variety of mineral salts as well as in the presence of a wide range of carbon sources (Ganeshan and Arthikala, 2005) and have an optimum growth temperature of 25-30°C (Donnarumma et al., 2010). They are commonly found in soils and water and are often present on the roots and leaves of plants (Silby et al., 2009, Trippe et al., 2013). They are widely regarded as a plant commensal and provide a useful biotechnological tool in controlling plant pathogens due to their ability to produce secondary metabolites that can act as antimicrobials with (Paulsen et al., 2005, Haas and Defago, 2005).

PF is also associated with humans, for example it can colonise the circulatory system of patients who have undergone blood transfusions, it can also be found in patient samples from the mouth and lungs however it is not thought to cause any specific diseases in these organs (Scales et al., 2014).

1.3.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa was first isolated from a bandage from a cutaneous wound in 1882 by Carle Gessard (Lister et al., 2009) and was identified as a pathogen in 1890 by Charrin (Bodey et al., 1983). *Pseudomonas aeruginosa* is now widely recognised as an opportunistic pathogen capable of causing plant and animal diseases. It is a rod - shaped bacteria approximately 1-5µm long and 0.5-1.0µm wide (Bhawsaw and Singh, 1989) which produces a grape like fruity aroma and has a blue-green colouration caused by the production of two main pigments pyocyanin and pyoverdine (Wu et al, 2011). It has an extremely broad metabolic diversity, which allows it to thrive in a wild variety of environments including soil, water, plants and vegetables and hospitals and which permits it to succeed as an opportunistic pathogen (LaBauve and Wargo, 2012).

Pseudomonas aeruginosa is the most important infective agent in the lungs of individuals with cystic fibrosis (CF) as well as other pulmonary diseases such as pneumonia and chronic obstructive pulmonary disorders (COPD) (Hurley et al., 2012). CF is caused by mutations in an epithelial chloride channel which causes a reduced NaCl and water secretion in the lungs resulting in thickened mucus accumulating on the surfaces of the lungs, leading to decreased bacterial clearance resulting in the persistent colonisation and infection (Al-Wrafy et al., 2017). The continued presence of *Pseudomonas aeruginosa* within the lungs leads to chronic infection and promotes the decline of lung function leading to poor clinical outcomes which results in increased mortality and morbidity (Smith et al., 2017).

Pseudomonas aeruginosa has also been linked to a broad spectrum of other diseases in humans including urinary tract infections and burn infections (Fazeli et al., 2012) where it can cause both acute and chronic infections. *Pseudomonas aeruginosa* rarely infects healthy individuals with those most at risk including patients who are immuno-compromised, undergoing chemotherapy or have a physical break in the skin barrier either from a wound or following surgery (Kerr and Snelling, 2009).

The majority of *Pseudomonas aeruginosa* infections occur in health care or hospital settings and is becoming increasingly recognised as a leading pathogen involved in both acute and chronic hospital acquired infections (El Zowalaty et al., 2015). Once infections are established, traditional treatment options such as antibiotics are limited due to increased emergence of antibiotic resistant strains within the *aeruginosa* species (Bassetti et al., 2018). The selective pressure amongst clinical strains of Gram negative bacteria provides resistance to antibiotic killing mechanisms, in addition antibiotics can affect characteristics that promote infection, particularly in hospital environments (Beceiro et al., 2013). For example, within the *aeruginosa* species, antibiotic resistance mutations have be found to alter characteristics associated with bacterial fitness and virulence (Geisinger and Isberg, 2017) which produces great versatility within the *aeruginosa* species making it a high risk pathogen for public health (Rodulfo et al., 2019).

1.4 Pathogenicity and virulence

The term pathogenicity refers to the ability of a pathogen to cause host damage, with virulence often referring to the degree of the damage caused, which is normally relative to the pathogenicity (Casadevall and Pirofski, 1999) and both terms are often used interchangeably to describe ways in which microorganisms can successfully invade a host cell and cause disease. Virulence is controlled by specialised cell appendages termed virulence factors, which can be cell attached and/or secreted and can interact with specific features of host cells including receptors and signalling pathways to cause disruption to defence mechanisms (Doxey et al., 2019). Virulence factors are under tight genetic control of the pathogen and their expression can change dependent on the pathogens ability to sense multiple environmental clues including changes in pH, temperature or metal ion availability (Thomas and Wigneshweraraj, 2014).

1.4.1 *Pseudomonas aeruginosa* and associated virulence factors

Pseudomonas aeruginosa possess an arsenal of different virulence factors that are cell-attached and/or secreted, which ultimately work together to allow the pathogen to invade host cells and cause disease. Each virulence factor can act in a different way and has its own specific role in promoting initial infection, bacterial survival, evasion of host immune responses and dissemination into the host (Ballok and O'Toole, 2013). Examples of virulence factors in *Pseudomonas aeruginosa* include flagella, hemolysins, proteases lipopolysaccharide (LPS) production, exotoxin (Exo) secretion and biofilm production (figure 1.4).

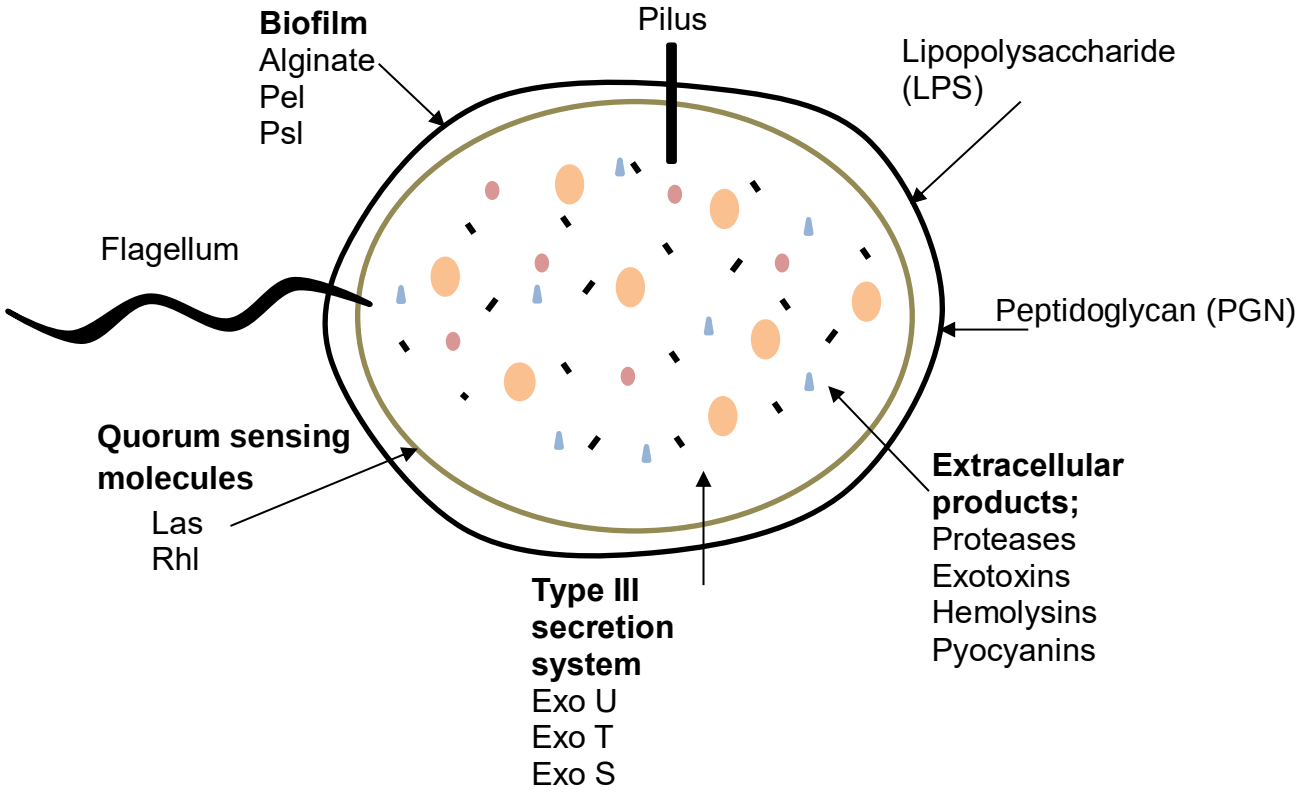

Figure 1.4 Virulence factors of *Pseudomonas aeruginosa*. Schematic diagram showing the cell attached and secreted virulence factors associated with *Pseudomonas aeruginosa.* Created by author based on Sadikot et al. (2005).

Flagella and pili are cell-attached appendages that have been identified as having roles in the motility and adhesion of *Pseudomonas aeruginosa* (Tran et al., 2011). Flagella are long whip like appendages which protrude through the bacterial cell membrane and are primarily composed of flagellin. The flagella rotate rapidly in a corkscrew like motion to act as a motor to aid in motility and swarming, a term that refers to the movement of bacteria over semi-solid surfaces (Kearns, 2010). By contrast, pili are small hair like projections, composed of pilin which are involved in promoting adhesion to host environments as well as twitching motility by the reversible assembly and disassembly to pull the bacteria along a surface (Persat et al., 2015). For *Pseudomonas aeruginosa,* motility and adhesion is of particular importance to allow movement through viscous environments such as the thick lung mucus of cystic fibrosis patients and sloughed skin cells often found in wound beds that it often colonises.

Once initial attachment has occurred, *Pseudomonas aeruginosa* can secrete a variety of virulence factors to promote acute infection. Secretion of molecules from Gram negative bacteria is an important physiological characteristic which allows them to interact with their wider environment without the need to utilise energy (Kulp and Kuehn, 2010). One of the main secreted virulence factors expressed upon attachment to host cells, is the activation of the type three secretion system, which allows exotoxins to be directly injected into the host cell cytoplasm causing direct cell damage (Galle et al., 2012). Other secreted virulence factors involved in promoting acute infections include proteases and lipases which are involved in the degradation of host cell proteins and lipids, particularly those within host cell membranes (Gellatly and Hancock, 2013). In addition to these, *Pseudomonas aeruginosa* can also exploit its pigments as

secreted virulence factors, with pyocyanin having the ability to interfere with electron transfer pathways in host cells (Rada and Leto, 2013). Whilst *Pseudomonas aeruginosa* has a multitude of virulence factors, not all are needed or expressed at the same time. Some virulence factors are only expressed within certain environments for example pyoverdines, which are involved in iron scavenging, are only secreted in iron limiting conditions (Voulhoux et al., 2006).

In addition to the cell attached and secreted virulence factors, *Pseudomonas aeruginosa* also has another mechanism that can deliver virulence factors into host cells, through the formation and secretion of outer membrane vesicles.

1.5 Outer membrane vesicles

Outer membrane vesicles (OMVs) are small (50-250nm) spherical, membrane bound structures that are secreted from the outer membrane of Gram negative bacteria (Beveridge, 1999). Production of OMVs by Gram negative bacteria is part of the normal growth process and has been found in a variety of environments including planktonic cultures, fresh and salt water sources as well as infected human tissue (Schwechheimer and Kuehn, 2015, Ellis et al., 2010). OMVs have been found to have a wide variety of biological functions dependent on the species of bacteria, with one of the main common functions being to facilitate interactions between the pathogen and its environment and to promote bacterial survival (Kuehn and Kesty, 2005).

As OMVs are shed from the outer membrane of Gram negative bacteria and are derived from the cell envelope they contain many different fragments and proteins that make up cell envelope of the parent cell (Jan, 2017). The outer membrane is often rich in immunogenic and virulence factors including LPS, and PGN which is often found in the periplasmic space (Anand and Chaudhuri, 2016). The secretion of OMVs by pathogenic Gram negative can aid in the delivery of these virulence factors and thus promote infection within the host cells.

1.5.1 Outer membrane vesicles of *Pseudomonas aeruginosa*

Considering *Pseudomonas aeruginosa* is well-established as a prevalent pathogen and coloniser of human tissue, it is unsurprising that the secretion of OMVs has been identified as essential for its pathogenesis (Choi et al., 2011). Due to the small size of OMVs, the virulence factors and other toxins associated with them can penetrate much further into host tissue than the bacterium itself and thus allows the delivery of virulent material to sites distant from the original site of colonisation (Macdonald and Kuehn, 2013). The delivery of virulence factors via vesicles occurs through fusion with the host cell membrane where they can then cause direct cell cytotoxicity or distribute to the specific locations and target a range of host cell processes (Bomberger et al., 2009).

OMVs derived from *Pseudomonas aeruginosa* could potentially be relevant in the pathogenesis of diseases, and have been detected in infected human tissue (Metruccio et al., 2016). The activation of TLRs is primarily through the recognition of immunogenic factors, and OMVs from *Pseudomonas aeruginosa* have been found to possess LPS, flagellin and CpG DNA all of which are capable of binding to host cell TLRs triggering cytokine release and the generation of an

immune response (Ellis et al., 2010). In addition, OMVs can act as decoy agents which can divert host immune responses away from the original site of colonisation (Cooke et al., 2019, O'Donoghue and Krachler, 2016).

Production of OMVs from *Pseudomonas aeruginosa* can be influenced by a variety of factors and their secretion is thought to be an environmental -dependent process (Orench-Rivera and Kuehn, 2016). Factors present in the bacterial micro-environment that can influence OMV secretion include bacterial population, temperature, pH and nutrient availability and potentially cause bacterial stress which can often lead to increased vesicle production (Klimentova and Stulik, 2015). In addition, *Pseudomonas aeruginosa* can alter the expression of outer membrane MAMPs and associated virulence factors (Schwechheimer and Kuehn, 2015) depending on environmental cues present within the bacterial micro-environment, via quorum sensing. As OMVs are derived directly from the parent cell it is likely that the OMVs will reflect this altered expression which in turn will promote virulence and bacterial survival within the host.

1.6 Quorum sensing

The ability of bacteria to alter gene expression based on environmental conditions is a highly controlled and regulated process that involves quorum sensing (QS) (Venturi, 2006). Quorum sensing is a cell to cell communication mechanism that allows bacteria to share information with other bacteria within a population via the release of extracellular (EC) signalling molecules (Rutherford and Bassler, 2012). These signalling molecules are known as autoinducers (AIs) and are produced and released by bacteria into the immediate environment (Abisado et al., 2018) . As the bacterial population increases, AIs in the local environment accumulate

which allows bacteria to detect and respond to the increased numbers. The increased concentration of AIs within the population is detected by receptors present in the cytoplasm or in the cell membrane which can then activate specific genes allowing the whole population to respond in a co-ordinated manner by the collective altering of gene expression (Diggle et al., 2007). Both Gram positive and Gram negative bacteria are capable of using quorum sensing however the signalling mechanisms are different, Gram positive bacteria use small post-translationally modified peptides named auto-inducing peptides (AIPs) as AIs whereas Gram negative bacteria use acyl-homoserine lactones (AHLs) (Miller and Bassler, 2001).

Quorum sensing can be utilised by bacteria to regulate gene expression mediating a range of biological functions including motility, antibiotic resistance and virulence factor production, all of which become more efficient when undertaken in a whole population (Papenfort and Bassler, 2016). The role of QS is critical for the control and orchestration of virulence factor production and as such has become an increasing area of research (Antunes et al., 2010).

There are 4 main intracellular communication signals that contribute to the quorum sensing network of *Pseudomonas; las, rhl, PQS and IQS* (Lee and Zhang, 2015). However much of the research has focused on the two main systems termed las and rhl both of which are AHLs, and have been identified as being responsible for the collective control of *Pseudomonas* virulence factors (Pearson et al., 1997). Genes coding for virulence factors that are regulated by QS systems include those responsible for the production of elastase, proteases, rhamolipids, exotoxins and pyocyanins (Antunes et al., 2010). In addition to this, AHLs produced by *Pseudomonas* have also been shown to directly interact with

mammalian host cells and their signalling pathways in both *in-vivo* and *in-vitro* models (Holm and Vikstrom, 2014). The ability of *Pseudomonas* to utilise QS mechanisms to collectively alter gene expression involved in virulence factor production provides *Pseudomonas* with the adaptability that facilitates its pathogenesis and its ability to cause disease.

In addition to the control of genes that directly code for virulence factors, another important function controlled by the environment and QS is the ability to produce generalised phenotypic changes, with one of the most important change being the ability of *Pseudomonas* to form biofilms (Sauer and Camper, 2001).

1.7 Biofilms

Biofilms are an organised community of surface associated microorganisms encased in an extracellular matrix consisting of bacterium derived secretions collectively termed extracellular polymeric substances (EPS) (Flemming et al., 2007). In nature bacteria often exist in the form of biofilms rather than in planktonic (freely-suspended) cultures and these can be composed of a mix of bacterial species, however due to the difficulty of replicating mixed-species biofilms *in vitro* much of the research has focused on single population biofilms (Elias and Banin, 2012). Biofilms can form on a wide variety of surfaces including human tissue, water pipes and medical devices (Donlan, 2002). The ability of microorganisms to form biofilms is an essential survival strategy as it can protect the bacterium from environmental stresses such as extreme pH, metal toxicity and host immune responses (Chen et al., 2018). In addition to this, biofilms can enhance resistance to killing mechanisms such as antimicrobials or traditional

disinfectants (Ghafoor et al., 2011, Lineback et al., 2018) and thus can lead to persistent colonisation.

The transition of planktonic culture to biofilm occurs through changes within the bacterial environment detected by QS mechanisms which then leads to collective alteration of the expression of genes mediating a variety of cellular functions including surface molecules, nutrient acquisition and virulence factors which allows bacteria to temporarily act as a multicellular organisms to promote survival in harsh unfavourable conditions (Kostakioti et al., 2013). Biofilm formation is dependent on the attachment of bacteria to surfaces, which once irreversibly attached allows further growth and the formation of micro-colonies which can then begin to form organised structures and become encased in EPS until eventual dispersal and break-up of the biofilm which allows released bacteria of increased virulence to colonise other environmental niches (Chang, 2017) (figure 1.5). The EPS surrounding the biofilm can account for up to 85% of the total biofilm mass and plays a central role in the establishment and development of the biofilm architecture and acts as a structural scaffold as well as providing protection from harsh environments (Wei and Ma, 2013).

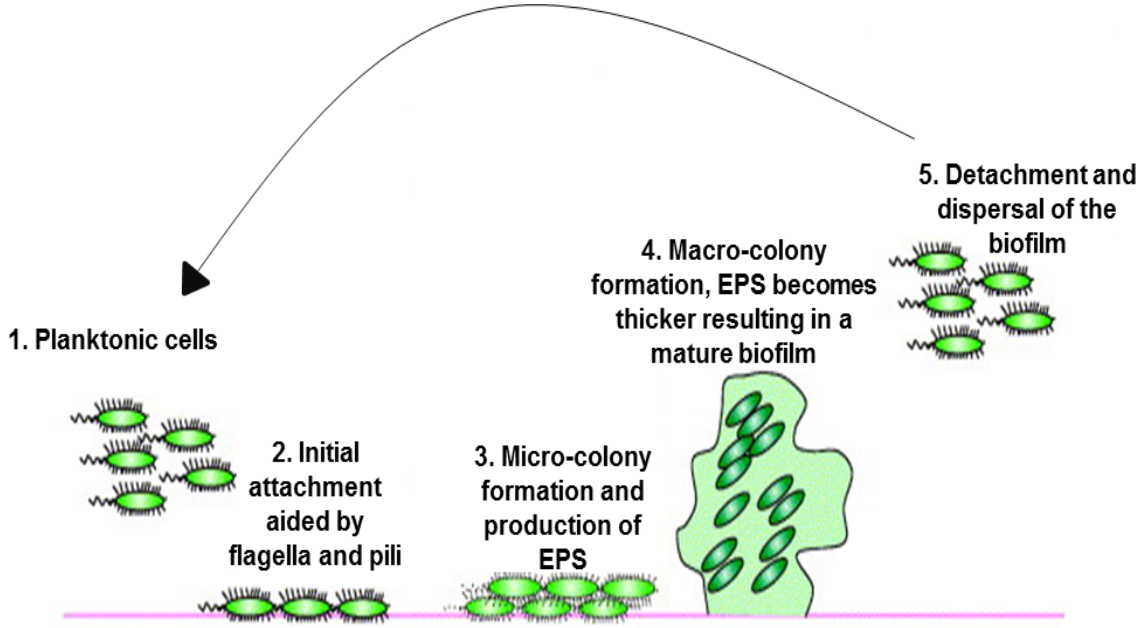

Figure 1.5 Formation and life cycle of biofilms in *Pseudomonas aeruginosa.* Schematic diagram showing each of the different stages of biofilm formation from initial attachment to maturing of the biofilm and then detachment and dispersal of the biofilm where cells become free growing (planktonic) again. Adapted from Lau et al. (2005)

Pseudomonas aeruginosa is widely recognised as a biofilm producer and depending on the strain and culture conditions phenotypic differences between different biofilms occur which is reflected in the composition of the EPS. In *Pseudomonas aeruginosa* biofilms, key EPS components include extracellular DNA, lipids, proteins, exopolysaccharides, as well as flagella and pili which have been shown to support biofilm attachment and structure (Chang, 2017, Rasamiravaka et al., 2015).

Pseudomonas can produce three main exopolysaccharides that are important in determining biofilm structure Pel, Psl and alginate (Alg) (Ghafoor et al., 2011). Pel polysaccharide has been identified as important in pellicle formation, a specialised biofilm which occurs only at air-liquid interphases such as in wound beds (Limoli et al., 2015, Ryder et al., 2007). Psl polysaccharide has been identified as being important in the maintenance of cell-cell interactions within biofilms and cell-surface interactions on mucosal surfaces and airway epithelial cells. Alginate polysaccharide has been associated with mucoid strains of *Pseudomonas aeruginosa*, such as those that reside in the lungs of CF patients and has been found to contribute to biofilm stability, protection and water retention (Rasamiravaka et al., 2015).

Due to the increased expression of virulence factors, the protection of the bacteria from the host and increased antibiotic resistance within biofilms, *Pseudomonas aeruginosa* biofilms become almost impossible to eradicate with traditional methods such as the use of antibiotics or other bactericidal products (Koo et al., 2017). Many *Pseudomonas aeruginosa* infections are often associated with biofilm formation which causes persistent colonisation leading to chronic infection (Rasamiravaka et al., 2015). The main biofilm associated infections from

Pseudomonas aeruginosa are in the lungs of patients with CF and in the wound bed of chronic wounds. In CF patients *Pseudomonas aeruginosa* has been shown to form small biofilm like micro-colonies and visual analysis of chronic wounds has also shown small biofilm like colonies, which were not found in acute infections (Mulcahy et al., 2014) however currently there is no definitive method for the detection of biofilms within clinical settings (Percival et al., 2015).

In addition, *Pseudomonas aeruginosa* biofilms have been found on a variety of medical devices and surfaces within clinical environments including water systems, venous catheters and urinary catheters (Walker and Moore, 2015, Percival et al., 2015).

1.8 Wounds and wound healing

A wound is defined as a disruption to the normal skin structure, function and/or architecture (Enoch and Leaper, 2005). One of the main functions of the skin is to act as a physical barrier between the host and the external environment and any breaks in the skin through physical or chemical damage, results in a loss of protection to the host. The ability of the skin to repair itself following the formation of a wound is essential to allow the restoration of the skins function (Ca et al., 2019).

Wound healing is a complex, orchestrated series of biological processes which consists of three main phases, inflammation, tissue formation and tissue remodelling (Rittie, 2016) (figure 1.6). The inflammatory stage is initiated in the early stages of wound repair and involves the formation of blood clots and the stimulation and recruitment of inflammatory cells such as monocytes and neutrophils, through the activation of DAMPs, to the site of infection to begin the removal of any foreign cells and infective agents (Landen et al., 2016). The tissue formation phase consists of the restoration of different components of the skin that have been damaged and involves formation of a new extracellular matrix (ECM) and the proliferation and migration of cells from the edge of the lesion resulting in re-epithelisation (Guo and Dipietro, 2010). The final phase, tissue remodelling, is responsible for the formation of a new strong and structured epithelium and the formation of scar tissue (Enoch and Price, 2004). The tissue remodelling phase can take up to 2 years depending on the severity of the wound, which is why closed wounds can deteriorate quickly if there is inadequate after care.

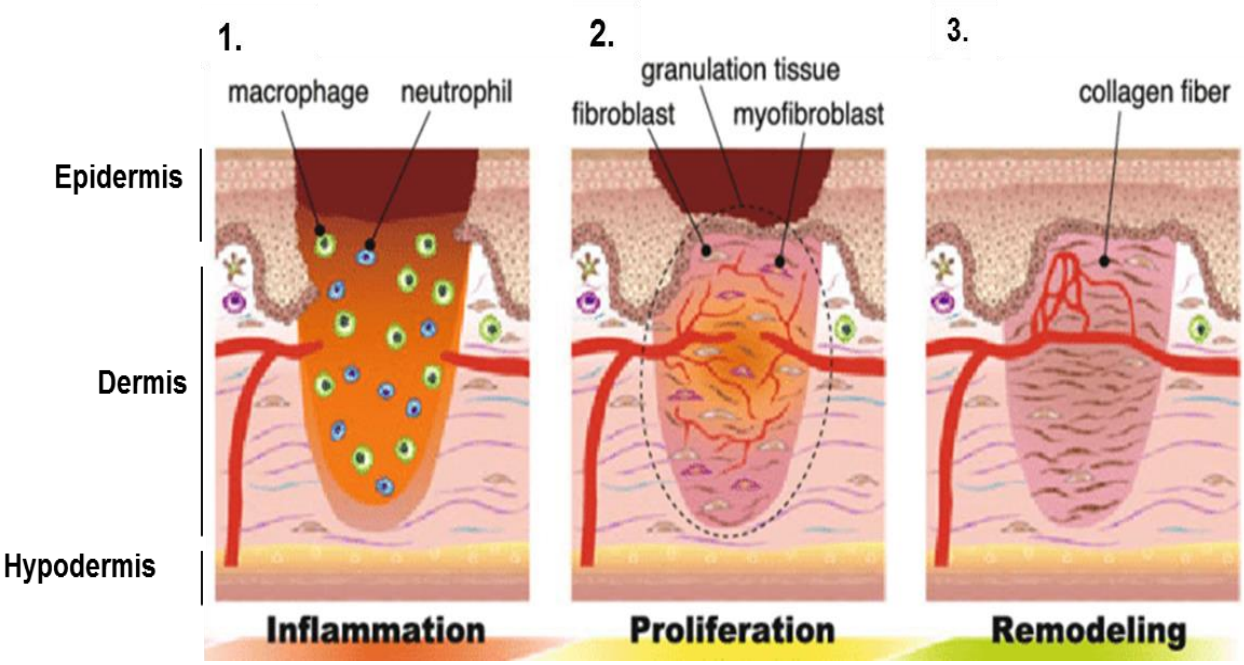

Figure 1.6 Stages of wound healing. Schematic diagram of the three main stages of wound healing that occurs within the skin and the cell types that carry out each stage to result in effective healing. Adapted from Kawasumi et al. (2012).

1.8.1 Models of wound healing

There are a wide variety of experimental methodologies which can be employed to provide insight into the effects of a range of chemical and biological compounds on wound healing including the use of in-vivo, in-vitro and ex-vivo models (Ud-Din and Bayat, 2017). In-vivo models are currently the most clinically relevant model of wound healing and often encompass both human and animal models (Sami et al., 2019). The use of animal models in the investigation of wound healing has been well documented, with animal models often used as an attempt to replicate normal wound healing in humans, with both murine and porcine models being commonly used to investigate incisional wound healing as well as burn models (Ashrafi et al., 2018). Due to limitations in differences in skin architecture and mechanisms of wound healing as associated costs and ethical issues surrounding the use of animals in research (Wong et al., 2011) (Barré-Sinoussi and Montagutelli, 2015) other models are often employed, such as in-vitro models which can provide valuable information with regards to wound healing.

One of the most common in-vitro models is the scratch assay which involves the creation of an artificial gap within a confluent single monolayer of cells which is then monitored for closure, generally over a few days until the gap has fully closed (Pastar et al., 2018). Other in-vitro models involve the migration of cells across a membrane where a chemoattractant is placed on one side of the membrane, which is then stained to determine the number of migrating cells known as the Boyden chamber assay (Guy et al., 2017), however this method is limited as it can only provide information regarding cell migration rather than wound closure. In-vitro models can provide an easy and cost-effective way to investigate both

proliferation and migration, both of which are essential for successful wound healing, however due to their reductive nature, in-vitro models may not be reflective of full wound healing within the skin. Ex-vivo models are often favoured over in-vitro models as they can provide a more relevant model of the skin architecture and often use cells and tissue obtained from donors commonly from surgical procedures (Corzo-León et al., 2019). Similar to the scratch assay, these models are often artificially wounded through incisions or burning to create a cell free area which can then allow the effects of re-epithelisation to be studied (Rakita et al., 2020). Ex-vivo wound healing models are advantageous in that they allow the effects of biological compounds to be investigated in a model that more closely resembles the skin (Ud-Din and Bayat, 2017).

1.8.2 Pathology of wounds and chronic wounds

The initiation and timings of each of the three main stages of wound healing is essential in maintaining the order of the healing process and any alterations to this or the cellular components involved can cause slow or impaired healing (Enoch and Leaper, 2005) that results in a chronic wound, which is defined as a wound that has not progressed orderly through the normal healing process (Frykberg and Banks, 2015). A variety of factors can cause delayed wound healing including ageing and pre-existing health conditions such as diabetes, where wounds of the lower leg are common due to nerve damage found in the lower extremities (Pendsey, 2010). Delayed wound healing often results in the wound held in a constant inflammatory state which can cause further tissue damage due to the constant release of pro-inflammatory cytokines and can allow the formation of an infection from invading microorganisms (Negut et al., 2018).

A wound infection can be local or invasive and is defined as the presence of multiplying microorganisms which can initiate a host immune response and causes delayed wound healing (Enoch and Price, 2004). Colonisation of the wound bed with microorganisms alone does not necessarily mean the wound bed is infected (Hanft and Smith, 2005). In acute wounds microbial colonisation can occur due to the prevalence of normal commensal bacteria on the skin however this is often not detrimental to the wound and orderly healing can progress (Bowler et al., 2001). If wounds become infected with pathogenic bacteria, or are exposed to a high bacterial load (over 10^5 bacteria per gram of wound tissue) or if there is biofilm formation, this leads to an invasive infection and a chronic wound can develop (Enoch and Price, 2004).

The main colonisers of wounds including burns and other soft tissue lesions including diabetic ulcers is *Pseudomonas aeruginosa,* where it is responsible for both acute and chronic infections (Schaber et al., 2007). Acute infections with *Pseudomonas aeruginosa* often spread rapidly and can lead to sepsis resulting in poor clinical outcomes and high rates of mortality (Turner et al., 2014). In chronic infections, *Pseudomonas aeruginosa* can readily form biofilms within the wound bed leading to long term impaired healing leaving very limited treatment options and thus may result in amputation (Järbrink et al., 2017). The chronic wound bed provides a warm, and moist environment and the presence of necrotic debris within the wound provide an ideal surface for bacterial attachment (Zhao et al., 2013). In addition, the inflammatory state of chronic wounds can be enhanced further by the presence of a biofilm. In the case of *Pseudomonas aeruginosa*, this can be by the activation of TLRs through MAMP recognition such as LPS as well as QS molecules which can act on host cells to induce expression

of pro-inflammatory cytokines, creating a hyper inflammatory environment which enhances nutrient acquisition to the biofilm (Wolcott et al., 2008).

1.8.3 Management of chronic wounds

The management of chronic wounds is becomingly increasingly critical due to their increased prevalence linked to an increasing ageing population, and the morbidity associated with them (Han and Ceilley, 2017, Järbrink et al., 2017). In the UK, data from the NHS showed that in 2012 2.2 million patients were diagnosed with a wound that required health care treatment, with annual cost for the direct management and associated morbidities estimated to be around £5.3 billion with patient care costs associated with non-healing wounds being 135% higher compared to healing wounds (Guest et al., 2017). In addition to this, it is estimated that the global wound care product market is set to exceed $15 billion by 2022 (Sen, 2019).

As those most at risk of developing a chronic wound include the elderly and those with other health conditions such as diabetes, who are more likely to frequent hospital environments, and given the fact that *Pseudomonas aeruginosa* is an opportunistic pathogen that is frequently found in hospital environments, it is likely that infection with *Pseudomonas aeruginosa* could occur within hospital environments.

1.9 Infections in health care settings

Healthcare associated infections (HCAIs) can be caused by a wide range of infective agents and are defined as infections that develop as a result of receiving health care either directly in a hospital or health care facility. To be classified as

a HCAI the infection must appear in the first 48 hours following admission to hospital or appear within the first month of receiving health care and not be present at the time of patient admission (Haque et al., 2018). Some of the most common HCAIs that can occur within hospitals include surgical site infections, blood stream infections and soft tissue infections such as wounds (Feleke et al., 2018). Healthcare associated infections are a major cause of morbidity and mortality worldwide and are becoming increasingly recognised as a global burden, however the extent of this is unknown due to the difficulty gathering reliable data particularly from developing countries due to lack of surveillance systems and issues surrounding the complexity of diagnosing infections (WHO, 2011). According to the World Health Organisation (WHO) it is estimated that patients in developing countries are more likely to suffer from HCAIs, with infections associated with medical devices up to 13 times higher, and the risk of HCAIs in new-borns up to 20 times higher than in developed countries. In the UK alone it is estimated that 300,000 people acquire an HCAI every year costing the NHS approximately £1 billion, with £56 million estimated to be spent after patients are discharged (NICE, 2012). In the USA, around 1.7 million patients acquire HCAIs annually with a mortality rate of about 1 in 17 patients (Haque et al., 2018).

Pseudomonas aeruginosa is the most common cause of HCAIs in health care settings (Kerr and Snelling, 2009, Pachori et al., 2019). Within clinical environments it has been found on a variety of surfaces including sinks and medical devices such as respirators and on medical practitioners' hands (Wilson and Dowling, 1998). Currently it is estimated that *Pseudomonas aeruginosa* accounts for around 7% of all HCAIs in the USA (Harris et al., 2016) and is the second most frequent cause of both acute and chronic infection in patients with

skin burns (Andonova and Urumova, 2013). Due to *Pseudomonas aeruginosa* strains increasing resistance to antibiotics and disinfectants, particularly those residing in hospitals there is an urgent need for adequate control measures to be adapted to prevent the opportunistic infections caused by these potentially pathogenic bacteria.

1.9.1 Infection control and prevention methods

According to the WHO, infection control and prevention is defined as a practical solution designed to prevent harm to patients and health workers from infectious agents. In hospitals, infection control and prevention methods are widely recognised as an important aspect in the maintenance of a safe hospital environment and in patient care and safety (Peters et al., 2018).

The hospital environment is known to harbour many species of pathogenic bacteria with them being found to be prevalent in high contact areas such as telephones, keyboards, nurse call buttons, doorknobs and medical charts, where they can survive for long periods of time (Russotto et al., 2015, Saka et al., 2017). The contamination of these high-contact areas results in them acting as a reservoir of pathogenic bacteria and as they are high contact areas, they can easily contaminate hands of medical staff who can then transfer these pathogens to patients during care (Saka et al., 2017, Boyce, 2007) and it has been estimated that up to 40% of HCAIs are caused by cross infection via the hands of medical staff (Weber et al., 2010).

One of the main infection control and prevention methods employed by hospitals and other clinical environments is routine environmental cleaning to maintain hygiene. During the 1990s in UK hospitals, cost saving reductions to cleaning

staff and services lead to reduced cleaning and a decrease in hospital hygiene (Dancer, 1999). The reduced level of cleaning, lead to a rapid increase of antibiotic resistant strains of *Staphylococcus aureus* (MRSA) the emergence of which generated interest surrounding pathogens in hospitals and their transmission, and as such there was an increased focus on hospital cleaning (Dancer, 2014) with cleaning now widely recognised as an effective way to manage the spread of infections, an important principle in controlling infections within hospital and clinical settings (Leas et al., 2015). The management of routine cleaning is critical in hospital environments, with importance placed on cleaning guidance, methods and the reporting of cleaning outcomes (NHS, 2009). Whilst these policies are in place to facilitate the improvement of hospitals cleaning, its global application is limited due to inadequate facilities, such as lack of clean water or untrained staff often found in hospitals in developing countries (Dancer, 2014). In addition to this, staff members tasked with hospital cleaning often receive very little recognition, leading to decreased job satisfaction and performance (Cross et al., 2019).

Cleaning involves the removal of solid debris such as dirt and grease however the act of cleaning itself does not eliminate microorganisms and must be followed by the use of disinfectants to ensure microorganisms are removed from the environment (Rutala and Weber, 2004). Disinfectants are commonly used in hospitals as part of routine hospital cleaning in addition to the sterilisation of medical equipment and skin decontamination prior to surgery (Huet et al., 2008). Both cleaning and disinfectants are required for decontamination in hospitals, in which environmental contamination is reduced to a level not considered harmful for health (Otter and Gellatly, 2018). Disinfectant products often contain biocides

which have a broad range of antimicrobial activity including disruption of bacterial cell membranes and inhibition of metabolic pathways resulting in cell lysis (Denyer and Stewart, 1998) examples of biocides include alcohols, iodine and chlorine (McDonnell and Russell, 1999). In addition to their use as part of environmental cleaning, biocides are often found in hand washes and hand rubs designed for use in hospitals with alcohol based hand rubs now being widely used in hospitals (Peters et al., 2018) with their use in place of traditional hand washing with soap and water recommended by the WHO before giving patient care, due to time efficiency and ease of use (Saito et al., 2017).

1.9.2 Effects of biocides on virulence and pathogenicity

The increase of HCAIs has led to the wide spread use of biocides in hospitals, however as their mechanism of action is similar to that of traditional antibiotics there is a possibility that microorganisms may develop resistance mechanisms to biocidal products (Capita et al., 2019, Ghanem and Haddadin, 2018). Some clinical isolates of *Staphylococcus aureus* have been found to have an increased expression of efflux pumps that are associated with antibiotic resistance following exposure to some biocidal compounds (Huet et al., 2008). In addition the exposure of clinical isolates of *Escherichia coli (E.coli)* to some biocides resulted in increased biofilm formation and increased pathogenicity in a wax worm model (Henly et al., 2019). The pathogenicity to wax worms of a clinical isolate of *Pseudomonas aeruginosa* has also been shown to increase when it is grown with ethanol as the main carbon source (Akbar, 2016).

The increased number of biocidal resistant bacteria is thought to be a result of long term exposure to sub lethal concentrations of biocides (Maillard, 2005,

Forbes et al., 2014).The increased use of biocidal products paired with inefficient cleaning methods in hospital environments and the presence of opportunistic bacteria may result in favourable environmental conditions to select for of highly pathogenic and resistant bacteria.

1.10 Aims

Pseudomonas is becoming increasingly recognised as a leading cause of HCAIs and the role of the bacterial microenvironment within clinical settings may cause alterations of immunogenic and virulence factors which can aid in its ability to colonise human hosts, with particular importance placed on the use of biocidal products. This project aimed to investigate the role of media supplemented with different carbon sources (ethanol or glucose) as well as an additional media (simulated wound fluid) for 2 culture times (24 and 80 hours) on two *Pseudomonas* species, a clinical isolate of *aeruginosa* isolated from a discarded bandage from a chronic wound (PS3) and a laboratory reference strain *Pseudomonas fluorescens* (PF). The specific project aims are;

- To investigate the role of growth condition and time in culture on the effects of specific virulence factors from both live bacteria and corresponding extracellular secretions.
- To investigate the toxicity of extracellular secretions on keratinocytes in culture from both live bacteria and corresponding extracellular secretions grown in each of the different conditions,
- To quantify the secretion of the pro-inflammatory cytokine from keratinocytes exposed to extracellular secretions from bacteria grown in each of the different conditions.
- To investigate the role of extracellular secretions from each of the different growth conditions on a keratinocyte model of wound healing.

These aims will help to achieve further understanding the effects of how the bacterial microenvironment within clinical settings can influence mechanisms that can contribute to the pathogenicity and virulence of *Pseudomonas* how these can affect keratinocyte cells in culture to potentially improve the clinical outcomes of chronic wounds and other bacterial skin diseases.

Chapter 2
Materials and Methods

HaCaT cells were kindly donated by Dr. Nikolaos Georgopoulos, Department of Biological and Geographical sciences, University of Huddersfield, UK. *Pseudomonas* strains and transcriptomic data for PS3 were kindly provided by Professor Paul Humphreys, Department of Biological and Geographical sciences, University of Huddersfield, UK. *Staphylococcus epidermidis* strain was purchased from ATCC. Materials used in ex-vivo skin models were kindly donated by Dr Joanna Shepherd, School of Dentistry, University of Sheffield UK.

- *A full list of reagents and suppliers can be found in appendix 1*

2.1 Cell culture

HaCaT cells (passage 43-53) were grown in culture and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with L-glutamine (1%) and FBS (10%) Cells were routinely grown in 75cm^3 culture flasks with vented caps to allow gaseous exchange during incubation (37° C and 5% CO_2). The incubator contained sterile deionised water supplemented with aquaguard to keep contamination at a minimum. Cells were grown to approximately 80% confluence (figure 2.1) before being passaged to ensure their successful growth.

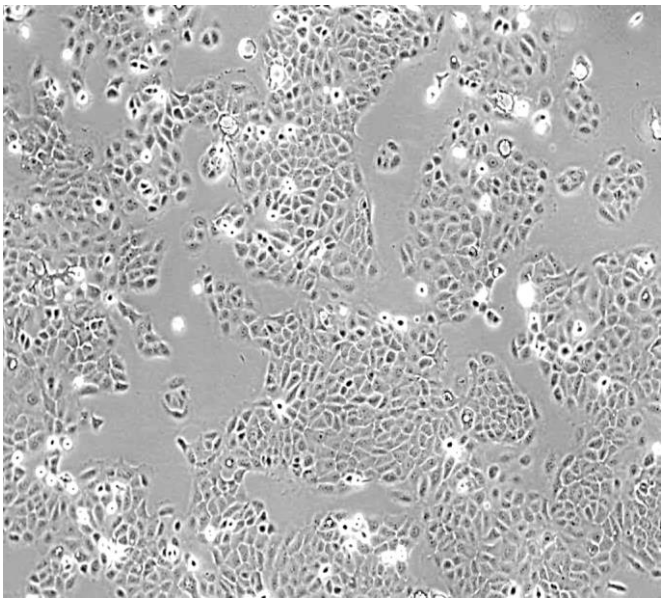

Figure 2.1 HaCaT cell monolayer. HaCaT cells in culture in 75 cm^3 tissue culture flasks at 80% confluence in preparation for passage. Monolayers were imaged and photographed by phase contrast microscopy using an EVOS XL core inverted microscope (Peqlab) at x10 magnification.

2.1.1 Sub culture of HaCaT cells

Media was removed from the confluent flask using a sterile aspirator tip and the cells were washed with (0.1%) ethylenediaminetriacetic acid (EDTA) in phosphate buffered saline (PBS) and incubated (37°C, 5% CO2) for 10 minutes to allow the EDTA to break cell-cell interactions. EDTA was removed using a sterile aspirator tip and 0.8 ml of 0.25% trypsin-EDTA was added to the flask. Flasks were then tilted from side to side to ensure an even contact of trypsin on the cells. Cells were left to incubate (37°C, 5% CO2) for 2 minutes to allow the trypsin to break contacts between the cells and the bottom of the flask. Flasks were then tapped lightly and checked by microscopy to ensure all cells had lifted. After all the cells became detached complete media was added to the flask, a wash was performed and all cells were collected from the confluent flask. Continuation 75cm^3 flasks were seeded at a split ration of 1:3 and media added to a total volume of 12ml.

2.1.2 Preparation of Sub-banks

Sub-banks of HaCaT cells (passage 40 and 43) were prepared in order to maintain cell stocks throughout the project. CryoPure tubes were labelled with the cell line, date, passage number and split ratio, split ratio was 1:3 to ensure approximately 2 million cells per vial. Freezing media was prepared (80% complete cell culture media, 10% FBS and 10% DMSO). Cells were lifted from the flask and collected in media as described in section 2.1.1, cells were then transferred to a 50ml centrifuge tube and were centrifuged at 1200rpm/5min to pellet the cells. Media was removed from the tube via aspiration and the pellet was re-suspended in freezing media with 1ml added to each CryoPure tube. Tubes were then placed in a "MrFrosty" cryogenic freezing container containing isopropanol and placed at -80˚C overnight to ensure a slow and consistent reduction in cell temperature. CryoPure tubes were then removed from the cryogenic container and transferred into a cell dewar containing liquid nitrogen for long term storage.

2.1.3 Seeding Cells

Once a flask of cells had reached approximately 80% confluency, determined by microscopic observation, the cells could be used to seed various size well plates in order to perform experiments as shown in table 2.1

Plate used	Seeding Density/well
6 well plate	3.0×10^5
24 well plate	1.1×10^5
96 well plate	7.5×10^3

Table 2.1 Seeding densities. Initial cell numbers per well used to seed different multi-well plates for each of the different experiments in this study.

One confluent $75cm^3$ culture flask gave approximately 8×10^6 cells which were lifted using EDTA and trypsin-EDTA as described in section 2.1.1, media was added to the flask and the cells were mixed, subsequently media was collected and placed in a 25ml tube. Of this cell suspension 10µl was taken and placed on a haemocytometer so cells could be counted. Each of the 4 corner squares were counted, this was done twice for both grids on the haemocytometer. Once counted, cell numbers were averaged and used to determine volume of cell suspension needed to achieve the correct seeding density.

2.2 Bacterial Culture

Bacterial strains used in these experiments were *Pseudomonas aeruginosa* clinical isolate PS3, *Pseudomonas fluorescens* (PF) and *Staphylococcus epidermidis* (ATCC 12228). Bacterial strains were maintained on agar plates throughout experiments with stock plates kept at 4˚C. *Pseudomonas* stock plates were freshly prepared from -80˚C Microbank stock vials every 4 weeks. *Staphylococcus epidermidis* stock plates were freshly prepared from ATCC cultiloops.

2.2.1 Preparation of Microbanks

Microbanks of bacterial strains were prepared in order to keep stocks of bacterial strains throughout the project. Microbank tubes were labelled with the date, and the name of organism including the strain. Bacteria were streaked out and grown on agar plates overnight before preparing bacterial stocks, to ensure colonies would be at the same growth stage. Sterile loops were used scrape bacteria from the agar plate until the loop was covered with bacteria, loops were then used to inoculate the cryopreservative fluid and swivelled around the porous beads. Tubes were then mixed several times to ensure beads were thoroughly coated with the microorganism. The cryopreservative liquid was then pipetted off to leave beads as liquid free as possible and the lid was placed tightly back on the tube (figure 2.2) tubes were then placed in -80˚C freezer for long term storage.

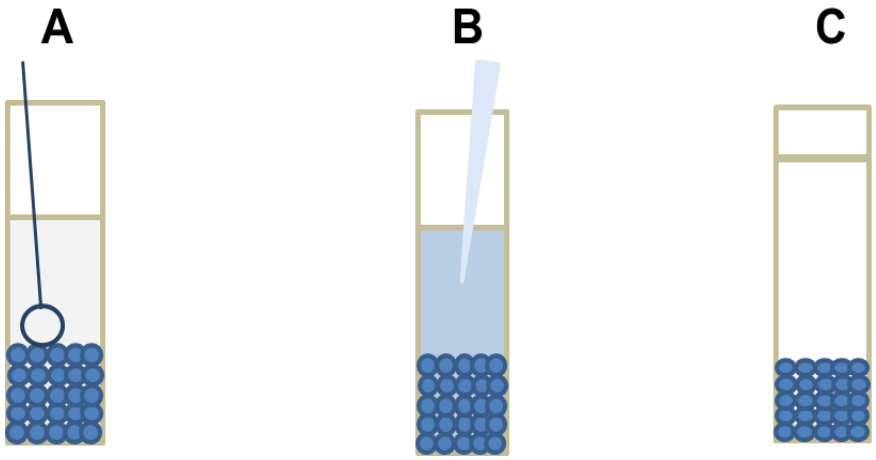

Figure 2.2 Microbank preparation. Schematic showing the Microbank tube preparation for long term storage of microorganisms used in these experimental procedures. A, shows the mixing of the bacterial coated loop in the cryoperservative fluid and beads, B, shows removal of the liquid by pipetting and C shows the final Microbank tube ready for long term storage at -80˚C.

2.2.2 *Pseudomonas* Growth

Pseudomonas strains were sub-cultured onto fresh tryptone soy agar (TSA) plates from -4˚C stock plates and grown overnight at 37˚C (PS3) or 30˚C (PF) before being used for suspension culture growth. For experimental procedures three liquid medias were used, 2 liquid mineral medias supplemented with either glucose or ethanol and simulated wound fluid (SWF) (1:1 FBS and maximum recovery diluent (MRD). The recipe for the mineral media is shown below in table 2.2.

Component	Mineral Media Glucose (MMG) g/l	Mineral Media Ethanol (MME) g/l or ml/l
Dipotassium Phosphate	4.5	4.5
Ammonium Sulphate	1	1
Magnesium Sulphate Heptahydrate	1	1
Sodium Chloride	0.1	0.1
Calcium Chloride	0.1	0.1
Ferric Chloride	0.02	0.02
Casein	1	1
Glucose	10	-
Ethanol	-	13.4 (1.34%)
pH	7.4	7.4

Table 2.2. Preparation of Mineral Media. Each component of mineral media shown in the left column with the corresponding amounts used per litre.

For suspension growth PF and PS3 were prepared to an OD of 0.27 at 620nm in MRD, which represented approximately 10^7 bacteria based on previous growth curves. For each growth media, (MMG, MME or SWF) 45ml of each was placed in sterile conical flasks, to this 5ml of *Pseudomonas* either PS3 or PF suspension was added before being placed in an incubator at 30˚C shaking at 100rpm. Bacterial cultures were left for 24 or 80 hours, these time points were chosen as previous work suggests the pathogenicity is increased in the hospital strain after 80 hours of growth (Akbar, 2016).

2.2.3 *Staphylococcus epidermidis* growth

Staphylococcus epidermidis was sub-cultured onto Luria-Bertani (LB) agar from sterile culti-loops coated with *Staphylococcus epidermidis*. Agar plates were warmed to room temperature before use, loops were placed on the surface of the plate and were held for approximately 15 seconds to allow absorption of moisture before being streaked out and incubated overnight at 37°C. Following overnight incubation, 1 or 2 colonies were picked from the plate and used to set up suspension cultures in LB broth for 24 hours.

2.2.4 Preparation of *Pseudomonas* cell-free supernatant

Pseudomonas strains were grown as described in section 2.2.2 and cultures were centrifuged in 50ml centrifuge tubes at 4000 rpm for 12 minutes, conditioned supernatant containing the extracellular (EC) products was transferred into a fresh 50ml centrifuge tube and the pellet was discarded into 2% trigene. The supernatant pH was adjusted to 7.4 before being sterile filtered through 0.45µm and 0.22µm pore filters to ensure any remaining bacteria was removed. Supernatants were diluted in complete DMEM in 1:2, 1:5 and 1:10 dilutions.

2.2.5 Cell free supernatant exposure assays

It was investigated whether bacterial growth conditions would have an effect on the secretion of the pro-inflammatory cytokine CXCL8 and have an effect on viability of the cells in culture.

Viability assays performed prior to full experiments, showed 7500 cells per well was the optimal seeding density to use for a 3 day experiment (figure 2.3).

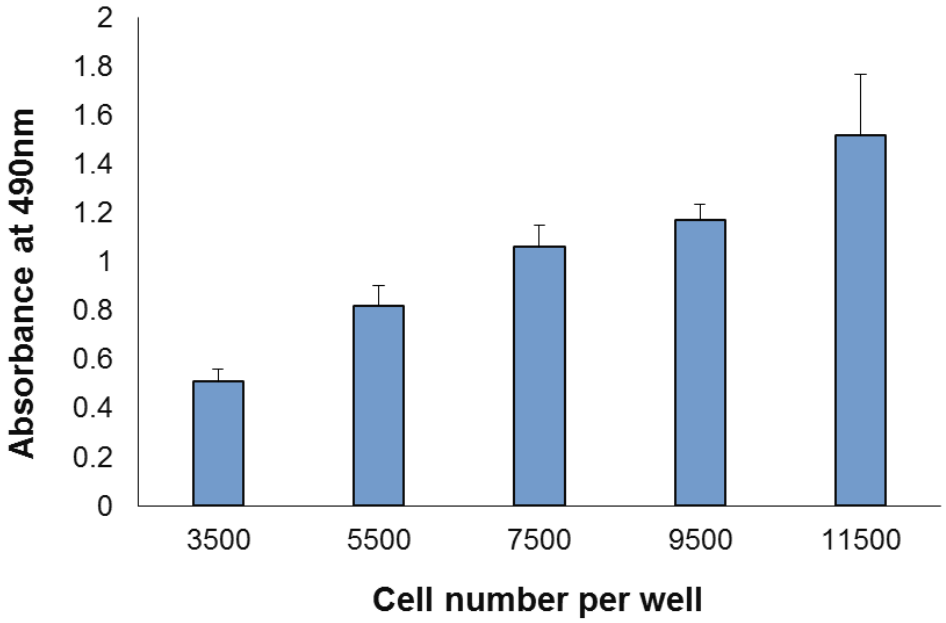

Figure 2.3 Absorbance of different cell densities at 490nm. Absorbance of 5 different cell densities at 490nm 3 days after initial seeding in 96 well plates, where cell number per well represents the cell number when plates were initially seeded into 96 well plates. Data are mean + standard deviation of 6 replicates where n=1.

HaCaT cells were seeded in 96 well plates at a density of 7.5 x 10^3 using a multichannel pipette and grown for 48 hours to around 80% confluence before being challenged with bacterial conditioned media. Conditioned media containing EC products was prepared as described in section 2.2.4, in addition, positive controls (10% DMSO) and negative controls of growth medium only were also prepared. Media was removed from the well and wells were treated with 200µl of corresponding dilution with each dilution replicated 6 times. Cells were then incubated for 4 hours at 37°C, after which the solutions were removed by aspiration and the cells washed with DMEM. Complete media was replaced and cells were incubated for a further 24 hours at 37°C and 5% CO_2 to allow time for CXCL8 secretion into the surrounding media. Supernatants from individual wells for each condition were pooled and placed into labelled 1.5ml microtubes. These were then placed at -80˚C for storage before being analysed for CXCL8 secretion and quantification by ELISA. A schematic diagram of the methodology is shown in figure 2.4.

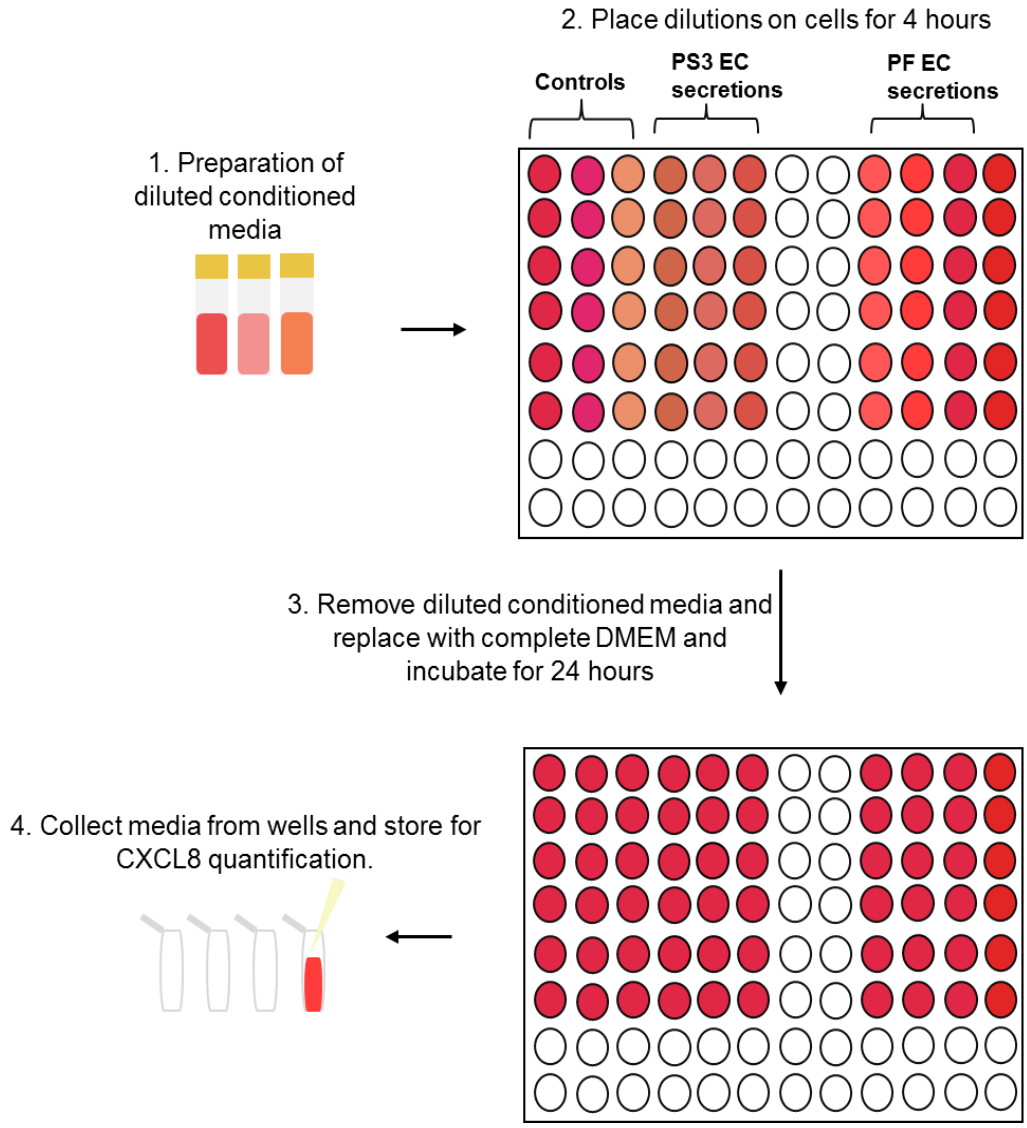

Figure 2.4: Flow through of cell free supernatant exposure assays. Schematic diagram showing the flow through of the methodology as described in section 2.2.5. Cells were seeded into 96 well plates and challenged with diluted bacterial conditioned media from each of the different *Pseudomonas* culture conditions. Media was collected 24 hours after initial exposure and stored for CXCL8 analysis.

2.3. MTS Assay

Once supernatants were collected MTS assays were performed to ascertain if any death had occurred in the cells. MTS assays were performed using CellTiter 96® Aqueous One Solution Cell Proliferation Assay following the manufacturer's guidelines. The MTS tetrazolium compound is reduced by cells into a coloured formazan product that is soluble in cell culture medium which can then be measured by absorbance at 490nm.

Fresh media was placed back onto the cells with each well receiving 200µl, followed by 20µl of CellTiter, this was performed in darkness due to light sensitivity of the reagent, cells were incubated for 4 hours at 37°C, 5% CO2 to allow the reaction to take place and the formazan colour to develop, after which the absorbance of each of the wells was read at 490nm using a FLUOstar OPTIMA plate reader. Experiments were performed in triplicate, in the three different growth conditions (MME, MMG SWF) for the 2 different strains of bacteria (PS3, PF).

2.4 CXCL8 quantification

To quantify secretion of the pro-inflammatory cytokine CXCL8, enzyme linked immune-sorbent assay (ELISA) were performed using the R&D CXLCL8 kit, with the addition of the supplementary ancillary kit (R&D). Instructions were followed as per the manufacturer's protocols. Each of the antibodies, and reagents were prepared according to the manufacture's guidelines (table 2.3).

Reagent/Antibody	
Capture Antibody	reconstituted in 1ml of PBS
Detection Antibody	reconstituted in 1ml of assay reagent diluent
IL-8 Standard	reconstituted in 0.5ml deionised water
Reagent Diluent	Diluted 1:10 in deionised water for blocking buffer. Diluted 1:00 in TBS-Tween for assay reagent diluent.
Streptavidin HRP	Diluted 1:40 in assay reagent diluent

Table 2.3. Reagents and antibodies used in the human CXCL8 ELISA.

Capture antibody was diluted in plate coating buffer to a concentration of 1µg/ml and used to coat ELISA 96 well plates which were then sealed using disposable plate sealers and left overnight at room temperature. Wells were then rinsed with washing buffer with each well receiving 400µl this was repeated a total of 3 times with thorough blotting of the plate after each wash to ensure there was no remaining wash buffer in the wells. Blocking of the plate was done using blocking buffer with each well receiving 300µl and plates were sealed and left for 1 hour

at room temperature. Standard concentrations of CXCL8 were serially diluted in reagent diluent to give final concentrations of 2000, 1000, 500, 250, 125, 62.5 and 31.3 pg/ml in addition samples were defrosted and thawed in preparation for ELISA analysis. Following blocking, plates were rinsed 3 times with washing buffer and blotted, 100µl of each standard was added, in duplicate, to the appropriate wells, 100µl of each sample was also added in duplicate to the corresponding wells, the plate was then sealed and left for 2 hours at room temperature. The plates were then washed again 3 times and blotted before 100µl of the detection antibody (diluted in reagent diluent to 1µg/ml) was added to each well, plates were then sealed and left for a further 2 hours at room temperature. Following this, plates were washed three times and blotted before 100µl Strep-HRP diluted 1 in 40 in reagent diluent was added to each well the plates were then sealed and kept in darkness for 20 minutes. Subsequently, plates were washed for a final time and blotted before the addition of solution A and B (1:1 ratio), with each well receiving 100µl. Upon addition a blue colour became evident and plates were sealed and left for 20 minutes in darkness to allow the colour to develop. Once colour had developed, 50µl of stop solution was added to each of the wells causing a change in colour from blue to yellow. The plate was then read using a FLUOstar OPTIMA plate reader. CXCL8 values were quantified according to the standards of the plate, standard curves were constructed using the plate reader OPTIMA software (appendix 2).

2.5 Bacterial Translocation Assays

To investigate whether the role of bacterial growth conditions had an effect on the ability of *Pseudomonas* to attach and translocate into keratinocytes in culture, HaCaT cells were seeded on 24 well plates at a density of $1.1x\ 10^5$ and left for 24 hours to allow a monolayer of early confluence to be established. *Pseudomonas* strains were grown in the different growth conditions for both 24 and 80 hours as described in section 2.2.2 after which the cultures were centrifuged at 4,000rpm for 12 minutes to pellet the bacteria. The supernatant was removed by aspiration and bacterial pellets were re-suspended in Hanks Balanced Salt Solution (HBSS) supplemented with 10mM sodium bicarbonate, 10mM glucose and 10mM Tris-base. HBSS was chosen due to its ability to minimise bacterial growth 1000 fold compared to DMEM (Clark et al., 2003). For some experiments this method was adapted such that the bacterial conditioned media supernatant was retained from the centrifuged cultures and used to pre-treat the cells before incubation with live bacteria.

To quantify the number of bacteria added to the cells, ten-fold serial dilutions were prepared from the bacterial suspension and plated using the spread plate technique and plates were left to incubate overnight at 30°C, with serial dilutions kept at 4°C to prevent bacterial growth. Following incubation plates were counted and multiplicity of infection (MOI)'s were calculated using the following equation.

$$\text{MOI} = \frac{\text{Colony forming units per ml (cfu/ml)}}{\text{Number of keratinocytes in culture}}$$

Once MOI's were determined, tubes containing serial dilutions of the bacteria suspension were warmed to room temperature before 1ml of each dilution was added to each well with a confluent layer of cells. Cells were exposed to the bacterial suspensions at MOIs of 100, 10 and 1 for 2, 4 or 6 hours. After this the bacteria from each well was removed and serial dilutions were prepared and plated. The cells were then washed with HBSS either with or without the antibiotic (polymixin B sulphate (1µg/ml)), and lifted from the wells using EDTA and trypsin. Presence of antibiotics allowed remaining external bacteria to be killed leaving internalised bacteria only. Once lifted cells were burst using sterile ultrapure water and pipetted up and down vigorously to ensure lysing had occurred, 100µl of this was taken and added to a TSA plate. A schematic diagram of the method used is shown in figure 2.5. Experiments were performed in triplicate, bacteria from wells was serially diluted once each time and plated out in duplicate using the spread plate technique.

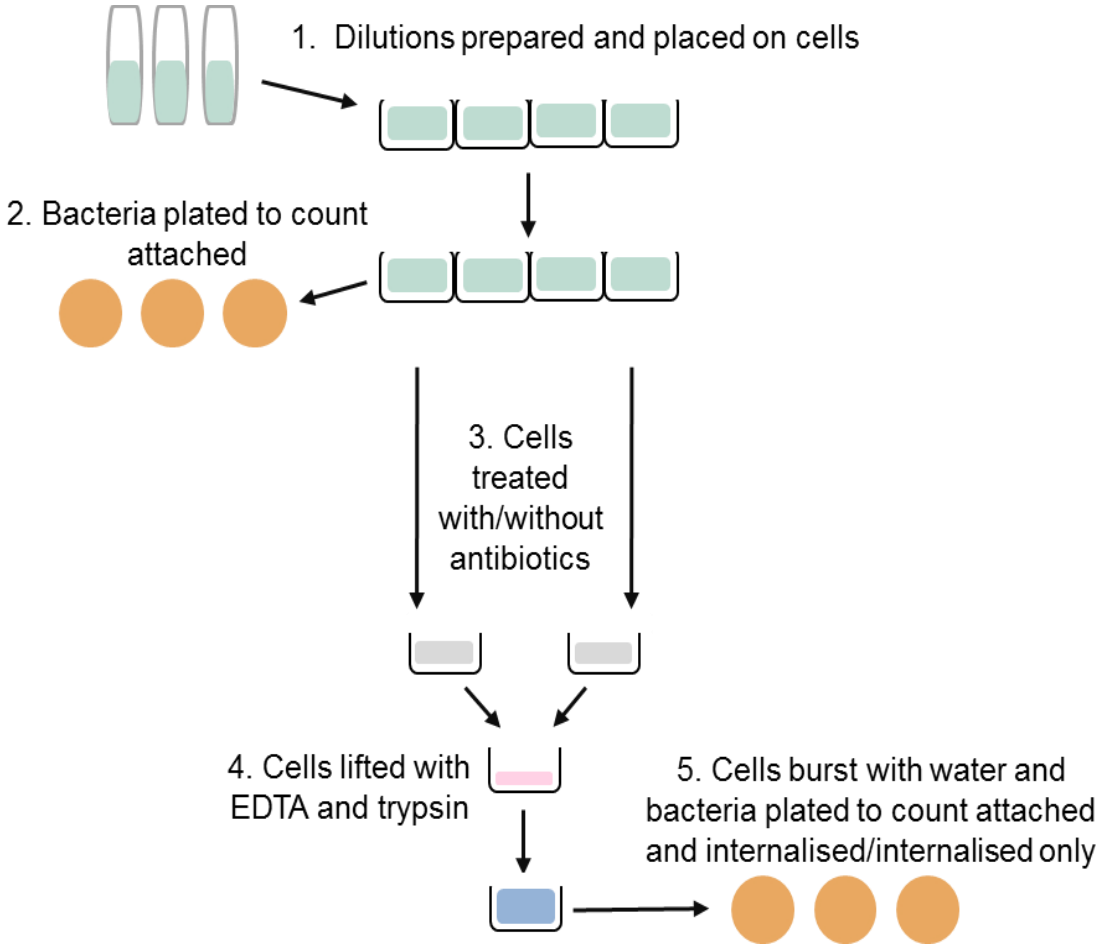

Figure 2.5 Flow through of bacterial translocation assay. Schematic diagram showing the flow through of the methodology as described in section 2.5. Cells were seeded into 24 well plates and challenged with live PS3 either with or without a pre-treatment of bacterial conditioned media.

2.6 Scratch Assay

Scratch assays were performed to investigate further the role of growth conditions on the closure of a scratch made in a layer of cells in culture. In order to develop a suitable methodology, various biological objects were used in initial scratch assays to determine which would give a consistent neat scratch and would allow suitable time frame in which to measure closure. Scratches were photographed every 24 hours and measured using ImageJ software. Cells scratched with a P200 tip were almost 100% healed after 24 hours (figure 2.6), with the P1000 tip and aspirator tip showing similar rates of healing throughout all time points, with all scratches healed after 3 days (figure 2.6).

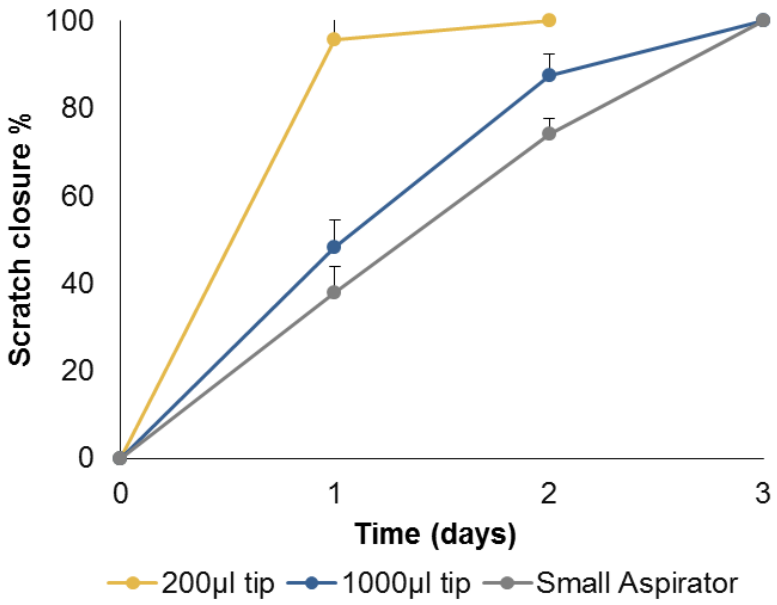

Figure 2.6 Determination of methodology for scratch assays. Scratch closure over time from HaCaT monolayer scratched with three different pointed objects. Data are expressed as mean + standard deviation of 3 replicates where n=1.

Based on the data presented in figure 2.6 the aspirator tip was chosen to perform the scratch assay experiments as this gave the most consistent, clean and smooth scratch.

HaCaT cells were seeded into 6 well plates at a density of 3 x 10^5 as described in section 2.1.3 and left for approximately 48 hours until early confluency. The media was then removed from the wells by aspiration and cell monolayers were 'scratched' using an aspirator tip attached to a vacuum pump, fresh media was then placed back onto the cells. A line across one part of the scratch was marked on the underside of the wells with a fine liner pen and cells were photographed using an EVOS XL core inverted microscope at x4 magnification, representing time 0. Marking of the wells ensured the same area of scratch was imaged at each time point to avoid inconsistencies within the data.

Media was then removed from the wells and cells were then exposed to dilutions of cell free media as prepared in section 2.3 for either 4 or 6 hours. Once exposure times were complete, DMEM was placed back on the cells and cells were incubated at 37°C, 5% CO2 for 5 days with cells imaged every 24 hours. The area of scratches was measured using ImageJ software.

2.7 Expression of TLR's

Flow cytometry was used to investigate the role of growth conditions on the activation of TLR's on HaCaT cells. Flow cytometry is a laser-based technique involving the use of fluorescently labelled antibodies and dyes for the that can provide information about protein expression in individual cells within a population (McKinnon, 2018).

2.7.1 Preparation for flow cytometry

HaCaT cells were seeded in 24 wells at a density of 1.1 x 10^5 as described in section 2.1.3 and left for 24 hours to allow cells to form a monolayer of around 80% confluence. Cells were exposed to cell free supernatant from each of the different growth conditions (1:2 dilution) for 4 hours before supernatant was removed and replaced with complete media. In addition to experimental conditions, controls were also included. Cells were then lifted using 0.5ml EDTA only, trypsin was omitted as this stage due to potential damage to cell receptors. Once cells had been lifted they were placed into corresponding labelled 1.5ml tubes and mixed thoroughly. 500µl of ice cold FACS buffer was added to each tube which were then centrifuged at 400g for 5 minutes.

Antibodies were then prepared, and used are shown in table 2.4. Antibodies for control tubes were prepared first, controls included 3 fluorescence minus one (FMO) tubes, 3 compensation tubes, 1 test and 1 unstained tube. A summary of control tubes and corresponding antibodies can be found in table 2.5.

Antibody
CD282 (TLR2)
CD284 (TLR4)
CD14

Table 2.4 Antibodies used for flow cytometry experiments.

Conditions	Antibodies used
Control - FMO (CD14)	CD282, CD284
Control - FMO (TLR2)	CD14, CD284
Control - FMO (TLR4)	CD14, CD282
Control - Compensation TLR 2	CD282
Control - Compensation TLR 4	CD284
Control - Compensation CD14	CD14
Test Condition	CD282, CD284, CD14
Unstained	None
All experimental conditions	CD282, CD284, CD14

Table 2.5 Control conditions and antibodies used.

For staining with antibodies 2µl of required antibody was used and tubes received a total volume of 50µl (antibody and FACS buffer). Tubes were then left to incubate for 30 minutes on ice in darkness, cells were then washed in FACS buffer and re-suspended in 300µl PFA (1%) and kept at 4°C overnight. Once samples had been stained and fixed flow cytometry could be performed.

2.7.2 Flow Cytometry

Prior to running experimental samples on the flow cytometer (Guava easyCyte), cleaning was performed using deionised water and instrument cleaning fluid (ICF). Threshold changes to forward and side scatter were set based on controls (test) to ensure correct gating of the cell population. Once gating was performed and cell populations were gaged the samples were vortexed before ran on the flow cytometer, quick cleaning of the machine was performed between every 3 samples. Once all samples had been analysed a deep clean of the machine was performed using deionised water and ICF.

2.8 Virulence Quantification

Pseudomonas possess a variety of virulence factors which contribute to both virulence and pathogenicity. Quantification of virulence factors was used to investigate the role of growth conditions on their expression.

2.8.1 Biofilm Formation.

Pseudomonas strains, PS3 and PF were prepared to an OD of 0.27nm at 620nm in MRD as described in section 2.2. Sterile boiling tubes were prepared containing 18ml of fresh growth media MME, MMG or SWF, these were then inoculated with 2ml of bacteria either PS3 or PF. Tubes were then left to incubate at 30°C for up to 80 hours with photos taken at 24 hours and 80 hours. Experiments were performed in triplicate, in the three different growth conditions (MME, MMG and SWF) for the 2 different strains of bacteria (PS3 and PF).

2.8.2 Pyocyanin Quantification

Pyocyanin production was quantified using methodology adapted from Schaible et al. (2017). *Pseudomonas* cell free supernatant was prepared as described in section 2.2.4, and 5ml was taken and placed in a 25ml tube and the pyocyanin extracted with 3ml of chloroform. Subsequently this was then back extracted with 1ml of 0.2M Hydrochloric acid (HCl), the aqueous phase of the second extraction was then measured in duplicate via spectrometry at 520nm. These experiments were performed in triplicate, in the three different growth conditions (MME, MMG and SWF) for the 2 different strains of bacteria (PS3 and PF).

2.8.3 Hemolysin and Protease Quantification

Hemolysin and protease production was quantified using blood and milk agar plates. For hemolysin quantification, blood agar plates were prepared using TSA agar containing 5% horse blood, for protease quantification milk agar plates were prepared using TSA containing 10% milk. *Pseudomonas* was grown as described in section 2.2.2, once growth times were complete, cultures were centrifuged and cell free supernatant was prepared as described in section 2.2.4, of this 10μl was added to the plates in a single drop form, with each plate receiving 5 drops of 10μl. The remaining bacterial pellet was re-suspended in MRD and serial dilutions were prepared to 10^{-6}, of this 10μl was added to separate plates in a single drop form, representing approximately 10^{3} bacteria/10μl. Plates were marked to indicate where the drops were placed, and were then then incubated at 30°C for up to 5 days hours with plates checked for any clear zones every 24 hours. Clear zones were measured using ImageJ software and expressed as a percentage area of the total plate. Experiments were performed in triplicate, in the three different growth conditions (MME, MMG and SWF) for the 2 different strains of *Pseudomonas* (PS3 and PF).

2.9 Ex-vivo skin models

This work was performed as part of a collaboration with Dr Joey Shepherd at the University of Sheffield. Skin sections were obtained from Euroskin bank via cadaveric donations and models were constructed using methodologies adapted from (MacNeil et al., 2011) and (Shepherd et al, unpublished). Models consisted of dermal scaffolds populated with cultured dermal fibroblasts and keratinocytes to give a more realistic model of human skin.

2.9.1 Fibroblast culture

Dermal fibroblasts (p8-12) were grown in DMEM supplemented with FBS (10%) and fibroblast growth supplement containing basic fibroblast growth factor (1ng/µl) and insulin (1ng/µl) and were maintained in 75cm^3 tissue culture vented flasks. Cells were grown to around 80% confluence before being passaged as described in section 2.1.1. Slight modification was needed due to the nature of the cell line, with EDTA exposure time reduced to 1 minute and trypsin exposure reduced to 1 minute also. Cells were passaged in a 1:4 ratio to ensure successful growth.

2.9.2 HaCaT culture

HaCaT cells (p32-38) were grown and maintained as described in section 2.1 with the addition of TGF-alpha (2ng/ml) supplement in the media. Cells were passaged as described in section 2.1.1 in a 1:3 ratio to ensure successful growth.

2.9.3 Construction of 3D ex-vivo skin models

Skin sections were placed in 100ml sterile PBS and incubated at 37°C for 5 days before being placed in 100ml 1M NaCl for 24 hours in order to begin the detachment process of epidermis from the dermis. Once detachment was visible at the edges, the epidermis was removed by gentle scraping using a scalpel and subsequently discarded. The dermis was then washed with complete DMEM containing antibiotics and placed in 100ml complete DMEM and stored at 4°C.

Once the dermis had been prepared, this was then used as a scaffold for the ex vivo wound healing model. The dermis was cut using a scalpel into circular shapes approximately 2cm x 2cm, sterile forceps were used to handle the dermis to keep contamination to a minimum. Circular scaffolds were placed in 6 well inserts to check sizing before being placed in DMEM media to keep moist until all dermal scaffolds were prepared and 6 dermal scaffolds were set up per experiment. Once dermal scaffolds were prepared, HaCaT cells and cultured dermal fibroblasts were lifted from confluent 75cm^3 flasks and counted via a haemocytometer as described in section 2.1.3. Scaffolds were individually placed in 6 well 'bucket' inserts which were then placed inside wells of a 6 well plate. Sterile metal 'donut' shaped rings were placed centrally on top of the dermal scaffolds, and were pressed down firmly. Once rings were in place 1.5ml of complete DMEM supplemented with TGF alpha (2ng/ml) was placed between the edge of the insert and the well to allow the underside of the dermis to be in contact with the media. Cells were seeded at a 1:3 ratio (2 x 10^5 fibroblasts: 6 x 10^5 HaCaT) between the outer edge of the ring and the insert. The inner ring of the donut was treated with 200μl dispase (2mg/ml) for 4 hours to allow enzymatic

break down of the dermis so that 'healing' could occur within the model (figure 2.7). Following 4 hour dispase treatment, the area was washed with EDTA to inactivate the dispase, and was replaced with 100µl complete DMEM. Models were left to incubate at 37°C/5% CO_2 for 48 hours, before all media was removed from the surface, leaving media only in the bottom of the well forming an air liquid interface (ALI). Models were then incubated for 14 days, with media changes occurring every 2-3 days with freshly prepared TGF alpha.

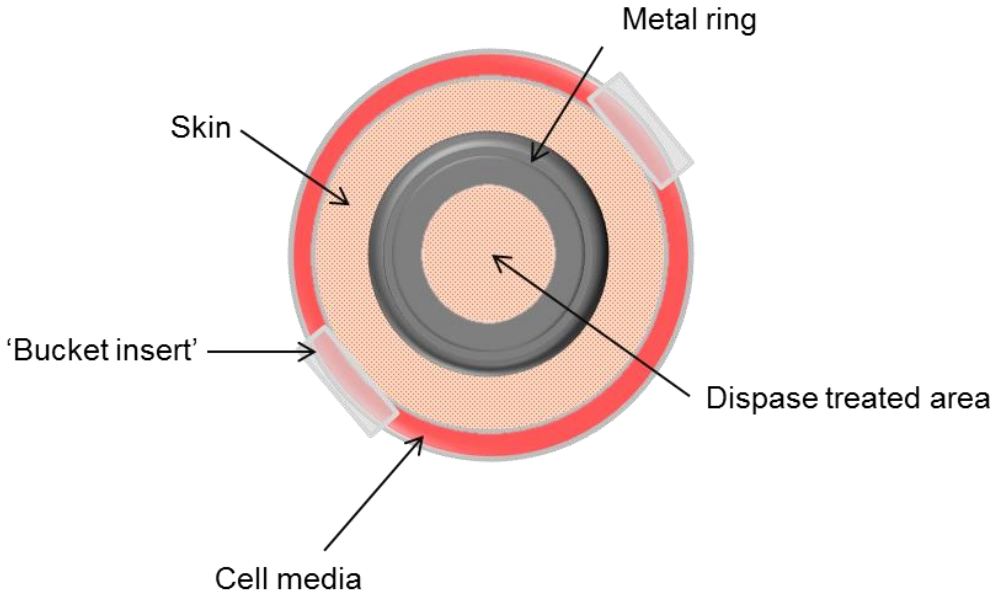

Figure 2.7 Construction of ex-vivo skin models. Schematic diagram showing the construction of the ex-vivo skin models as described in section 2.9.3. The dispase treated area creates an artificial wound which, when the metal ring is removed, cells seeded on the outside can begin to 'heal'.

2.9.4 Exposure to cell free supernatant and CXCL8 secretion.

Cell free supernatant from PS3 grown in mineral media supplemented with ethanol or glucose for 24 and 80 hours was prepared as described in section 2.2.4 and diluted 1:2 in complete DMEM. In addition to these controls were set up containing non-conditioned growth media diluted 1:2 in DMEM. Once prepared, metal rings were removed and cell free supernatants were placed on top of the skin, with each well receiving 0.5ml of the corresponding dilution. Skin models were incubated for 4 hours, after which supernatants were removed using a pipette, and the skin returned to ALI. Skin models were incubated for 24 hours before the media was collected from underneath the skin models and stored for IL-8 quantification as described in section 2.4. Fresh complete DMEM supplemented with TGF alpha was then placed underneath the skin to maintain ALI, and models were incubated for a further 14 days with media changed every 2-3 days.

2.9.5 Tissue Sectioning

Following 14-day incubation, skin models were prepared for sectioning and staining. Foil was moulded around 6 well inserts to form the circular shape similar to the skin and a drop of Neg-50 frozen section medium was place in the centre. The skin was lifted out of the insert using forceps and placed in the centre of the foil, the skin was then further covered with Neg-50 frozen section medium before being placed in liquid nitrogen for around 2 minutes until fully frozen, once frozen samples were stored at -80ºC. Tissue was sectioned using a cryostat (Leica CM1900), skin models were cut in half and 7mm sections were placed on glass slides, these were then checked under a microscope (Leica CTR6500) to ensure

good sections had been taken and slides were stored at -80°C until ready for staining.

2.9.6 Staining of tissue

Prior to hemotoxylin and eosin (H&E) staining, slides were retrieved from -80ºC storage and allowed to air dry at room temperature for 10 minutes before samples were fixed on the slide in 4% paraformaldehyde (PFA) for a further 10 minutes. Once fixed, slides were treated as shown in 2.6 before being mounted using pertex, slides were then left to dry overnight before being imaged.

Reagent/Treatment	Time
Running tap water	1 min
Hematoxylin (Mayers)	7 mins exactly
Running tap water	2 mins exactly
0.2% acid/water	20 secs exactly
Running tap water	1 min
37mM ammonia (or blueing reagent)	2 mins
Running tap water	1 min
70% IMS	1 min
Eosin	2 mins exactly
70% IMS	20 secs exactly
100% IMS	15 secs exactly
50:50 Xylene/100% IMS	30 secs exactly
Xylene	1 min
Xylene	1min

Table 2.6 Hemotoxylin and Eosin staining procedure.

2.10 Statistics

Results are expressed as mean +/- standard error of the mean (SEM) for the specified number of technical repeats, given by (n). Where standard error is not possible standard deviation of the mean is given, as indicated. Statistical analysis was performed using GraphPad (Prism8), with statistical significance determined using unpaired Student's T-test or ANOVA analysis with Tukey's post hoc test (used for multiple comparisons within one data set). Statistical differences are indicated by * $p<0.05$, ** $p<0.01$, ***$p<0.001$ or **** $p<0.0001$.

Chapter 3
Characterisation of *Pseudomonas* virulence and toxicity

3.1 Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that can cause both acute and chronic infections within a clinical environment (Sadikot et al., 2005). *Pseudomonas aeruginosa* can cause serious infections in critically ill and immune compromised hospital patients, often leading to high morbidity and mortality (Bassetti et al., 2018).

Pseudomonas aeruginosa infections are often mediated by the presence of virulence factors, *Pseudomonas aeruginosa* possess a wide variety of virulence factors that aid in its ability to colonise and invade human tissue. (Vasil, 1986). Colonisation can often lead to biofilm development, which can form on human tissue such as wound beds and on a variety of medical devices such as catheters which then become difficult to remove with traditional methods such as cleaning with anti-bacterial agents and the use of antibiotics (Chen et al., 2018). Quantification of virulence factors can be done by investigating genomic data or traditional laboratory techniques, however this gives little information regarding effects of these factors on the host.

In-vitro models can provide a robust and cost effective method to investigate the possible in-vivo effects of a wide variety of factors and can reduce the need for animal experiments (Bocheńska et al., 2017). The spontaneously immortalised HaCaT cell line is often used as a keratinocyte model to study bacterial-host interactions due to its resemblance to primary cultured keratinocytes (Edwards et al., 2011). As keratinocytes account for around 90% of the epidermis and often form the skin's first line of defence against toxic substances, the HaCaT cell line

makes an appropriate model to investigate effects of skin sensitivity to cytotoxic agents (Hoh and Maier, 1993).

3.2 Quantification of *Pseudomonas* virulence factors.

Pseudomonas possess a wide variety of virulence factors that mediate its pathogenicity and toxicity. Virulence factors are of particular importance for hospital strains of *Pseudomonas,* where they can aid in its ability to cause nosocomial infections potentially leading to serious complications in hospital patients.

3.2.1 Biofilm formation occurs in *Pseudomonas aeruginosa* but not *fluorescens*

Visual analysis of boiling tubes showed PS3 could form well established biofilms, within 24 hours (figure 3.1). PS3 grown in MM EtOH and SWF produced thicker and more structured biofilms compared to MM Glu tubes (figure 3.1). In addition there was a distinct green pigmentation directly below the biofilm in SWF grown PS3 (figure 3.1). Biofilms were not present in tubes inoculated with PF in any of the conditions (figure 3.1), with the cultures appearing similar to controls.

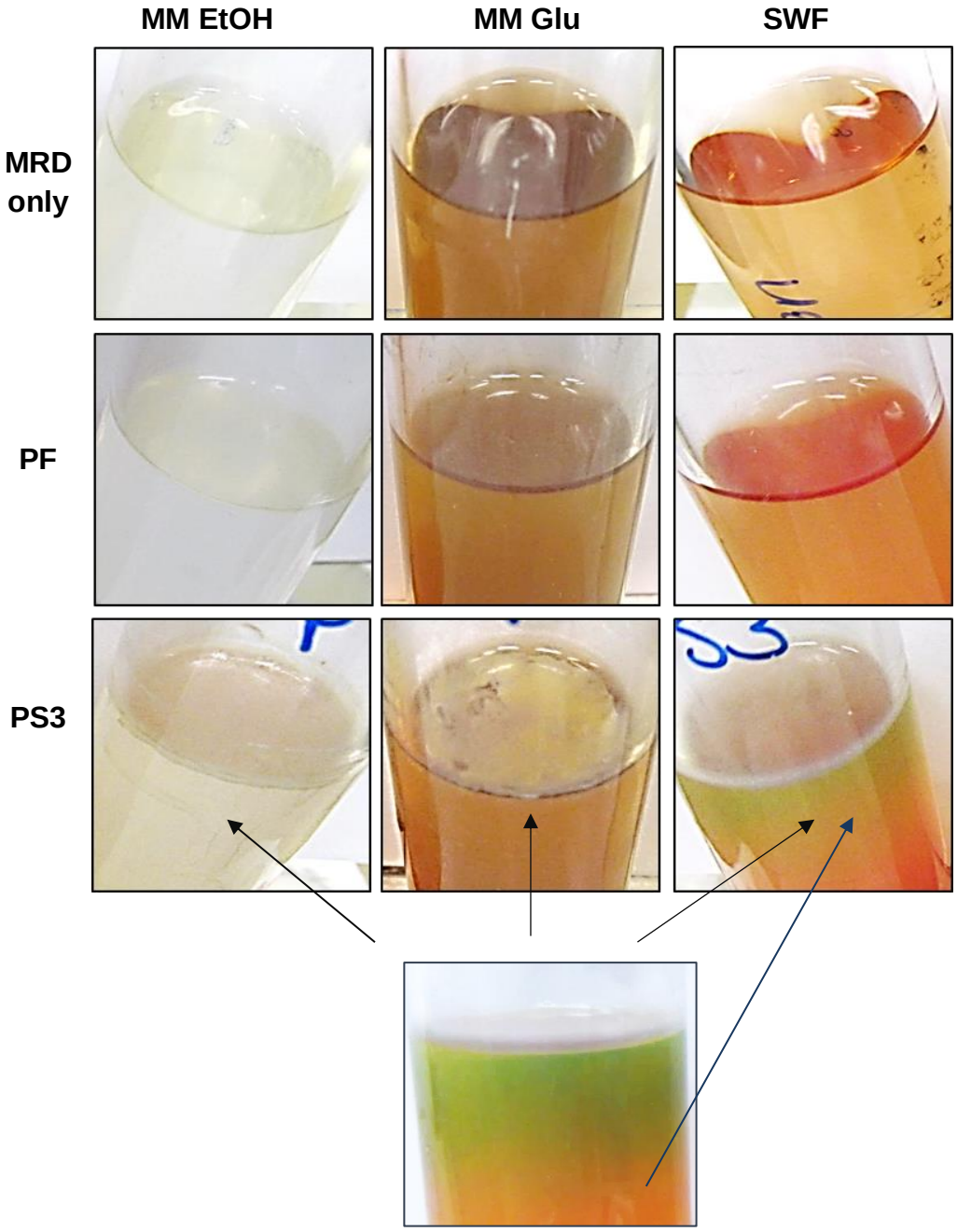

Figure 3.1 Biofilm formation from *Pseudomonas* grown for 24 hours. Boiling tubes containing growth media and inoculated with MRD only (control), PF or PS3 and incubated for 24 hours. Tubes contained 18ml of growth media and were inoculated with 2ml of MRD only, PF or PS3 and incubated for 24 hours before being photographed. Images shown are representative of 3 independent experiments.

The cultures were incubated for a total of 80 hours, after which biofilms had not formed in any tubes inoculated with PF (figure 3.2) For PS3 the appearance was similar to 24 hour incubation for MM EtOH and SWF, with a thick, well established biofilm and green pigmentation visible in SWF cultures was more vivid compared with 24 hour biofilms (figure 3.2). Tubes containing MM Glu inoculated with PS3 showed disintegration of the biofilm, with holes being clearly visible and detachment occurring from the glass surface coupled with a thinning of the biofilm (figure 3.2). Biofilm that had become disintegrated was floating as clumps in the tube (figure 3.3).

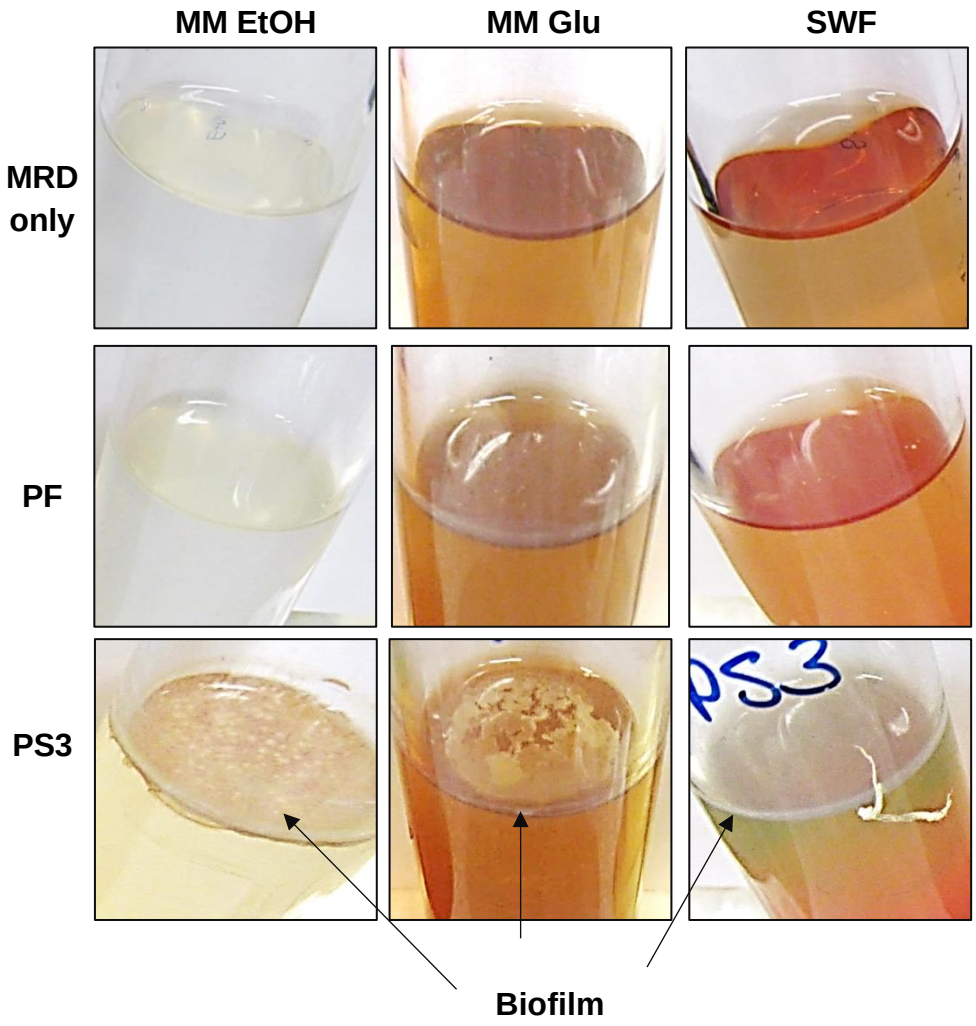

Figure 3.2 Biofilm formation from *Pseudomonas* grown for 80 hours. Boiling tubes containing growth media and inoculated with MRD only (control), PF or PS3 and incubated for 80 hours. Tubes contained 18ml of growth media and were inoculated with 2ml of MRD only, PF or PS3 and incubated for 80 hours before being photographed. Images shown are representative of 3 independent experiments.

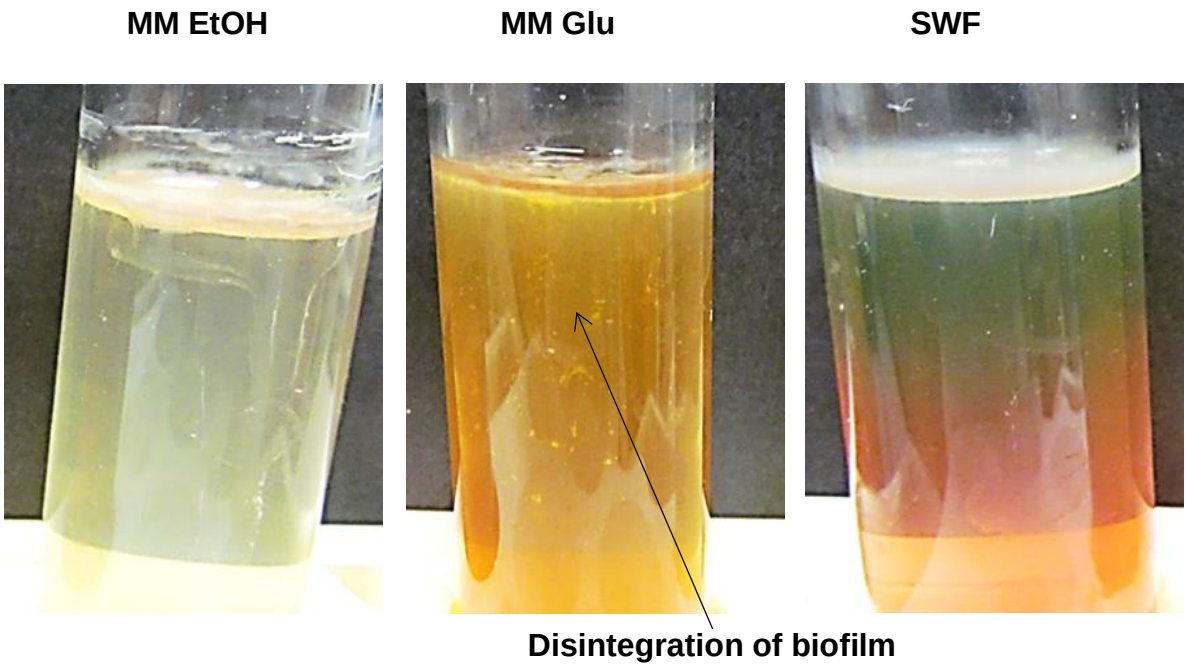

Figure 3.3 Biofilm formation from PS3 only grown for 80 hours. Boiling tubes containing growth media and inoculated with PS3 after 80 hour incubation period. Images show biofilms imaged from the side of the boiling tubes, with differences in biofilm thickness being more clearly visible. Disintegration of the biofilm is also clearly visible in the tube containing MM Glu.

3.2.1.1 Expression of biofilm genes in PS3

Currently 3 genes have been associated with *Pseudomonas biofilms;* Psl, Pel and Alg. Analysis of transcriptomes indicated an increased expression of nearly all Psl associated genes in MM Glu 24 hour cultures (figure 3.4). In 80 hour MM EtOH cultures there was an increased expression in all Psl genes compared to 24 hours further establishing the biofilm components at 80 hours (figure 3.5). There was a decrease in some Psl genes in MM Glu in 80 hour cultures compared to 24 hours, contributing to the break-up of the biofilm. There were minimal differences in the expression of genes involved in Pel and Alg expression within 80 hours cultures as well as minimal differences in the fold change of expression for Pel and Alg for both MM EtOH and MM Glu between 24 and 80 hours (appendix 3).

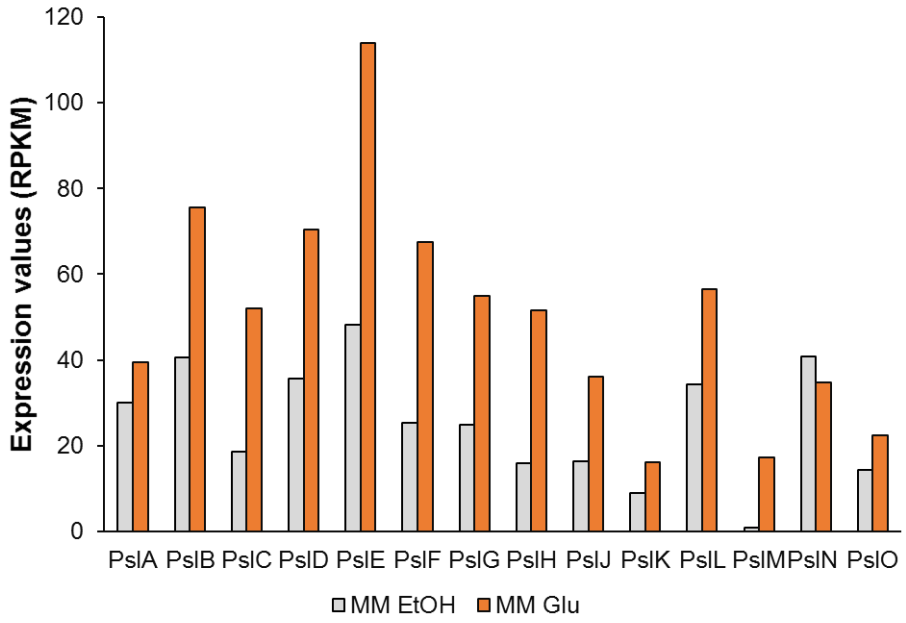

Figure 3.4 Expression values of Psl genes from PS3. Transcriptome analysis showing the total expression values, expressed as reads per kilo base per million mapped reads, of genes involved in Psl production (Psl A-O) from PS3 cultures grown in MM EtOH and MM Glu for 24 hours.

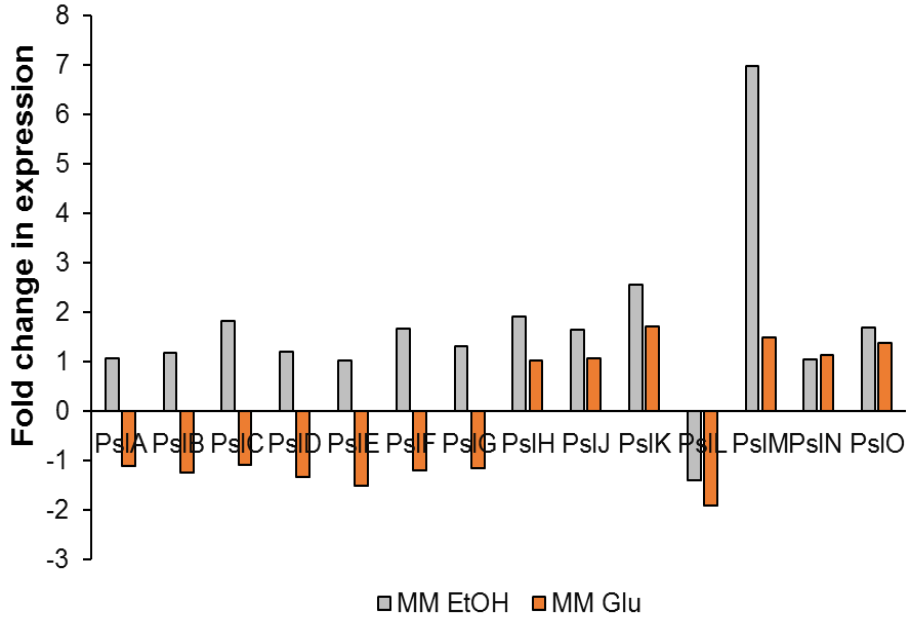

Figure 3.5 Fold change in the expression values of Psl genes of PS3. Transcriptome analysis showing the fold change of the expression of genes involved in Psl (Psl A-O) production from PS3 cultures grown in MM EtOH and MM Glu between 24 and 80 hour culture times.

3.2.2 Pyocyanin production from PS3 cultures is increased following 80 hour culture time.

The effect of growth conditions on the production of pyocyanin from PS3 was investigated. For PS3 grown for 24 hours the highest concentration of pyocyanin was produced by MM EtOH with an absorbance of 0.16 at 520nm. The corresponding value for SWF was 0.1, and for MM Glu it was 0.02 (figure 3.6), but there was no significant differences found between these conditions.

When PS3 was grown for 80 hours there was greater absorbance in all conditions with MM EtOH producing the highest absorbance of 0.4, with MM Glu 0.35 and SWF 0.15 (figure 3.6). Both ethanol and glucose conditioned media were significantly greater than SWF cultures ($p \leq 0.0001$) (figure 3.6).

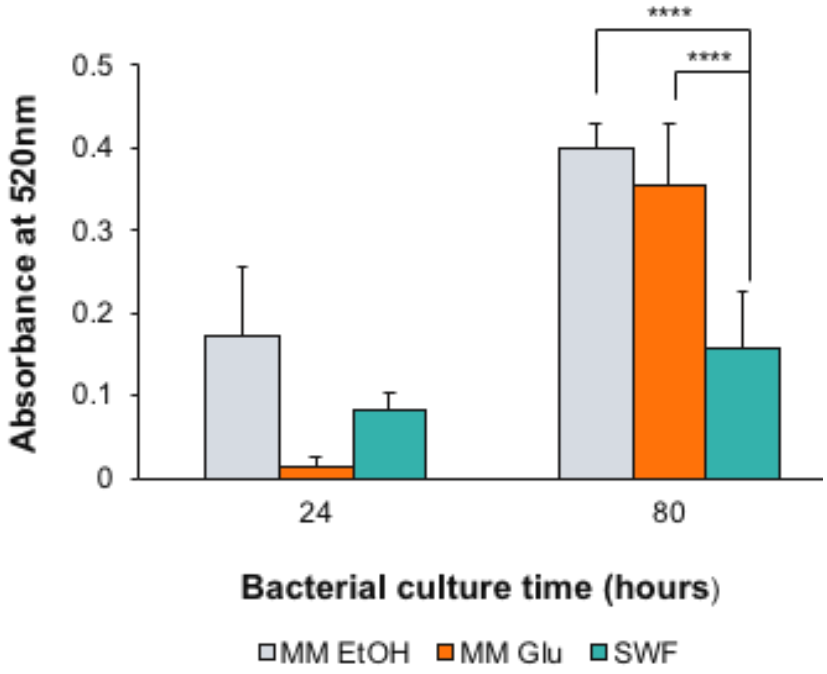

Figure 3.6 Pyocyanin production from cell free conditioned PS3 media grown for 24 and 80 hours. Absorbance of pyocyanin at 520nm from PS3 cell free conditioned media from each of the growth conditions for 24 and 80 hour cultures is shown. Cell free media was measured via spectrometry at 520nm. Data is expressed as mean of 3 biological repeats + SEM. Two way ANOVA with Tukey's post hoc test showed significance as indicated on the graph (****p ≤0.0001).

3.2.3 Quantification of protease production from PS3 cultures

It was investigated whether protease production could be detected in the supernatants from PS3 cultures and whether growth condition had an effect on its production. However no protease activity could be detected throughout the incubation period for any of the culture conditions.

To provide a more sensitive test of protease activity, live PS3 from suspension cultures were centrifuged, diluted and transferred to plates that contained milk agar and incubated for either 24 or 48 hours at 30°C. For PS3 from cultures grown for 24 hours in all conditions there was a 15-20% clear zone area on the plate after a 24 hour incubation (figure 3.7). After 48 hours the clear zone had increased to approximately 40% of the total plate area (figure 3.7).

Plates inoculated with PS3 that had been grown as a suspension culture for 80 in any of the conditions showed no protease production after a 24 hour incubation of the plate, however after 48 hours the clear zone had increased to approximately 55-60% of the total plate area which was observed on plates inoculated with cultures grown in all conditions (figure 3.7).

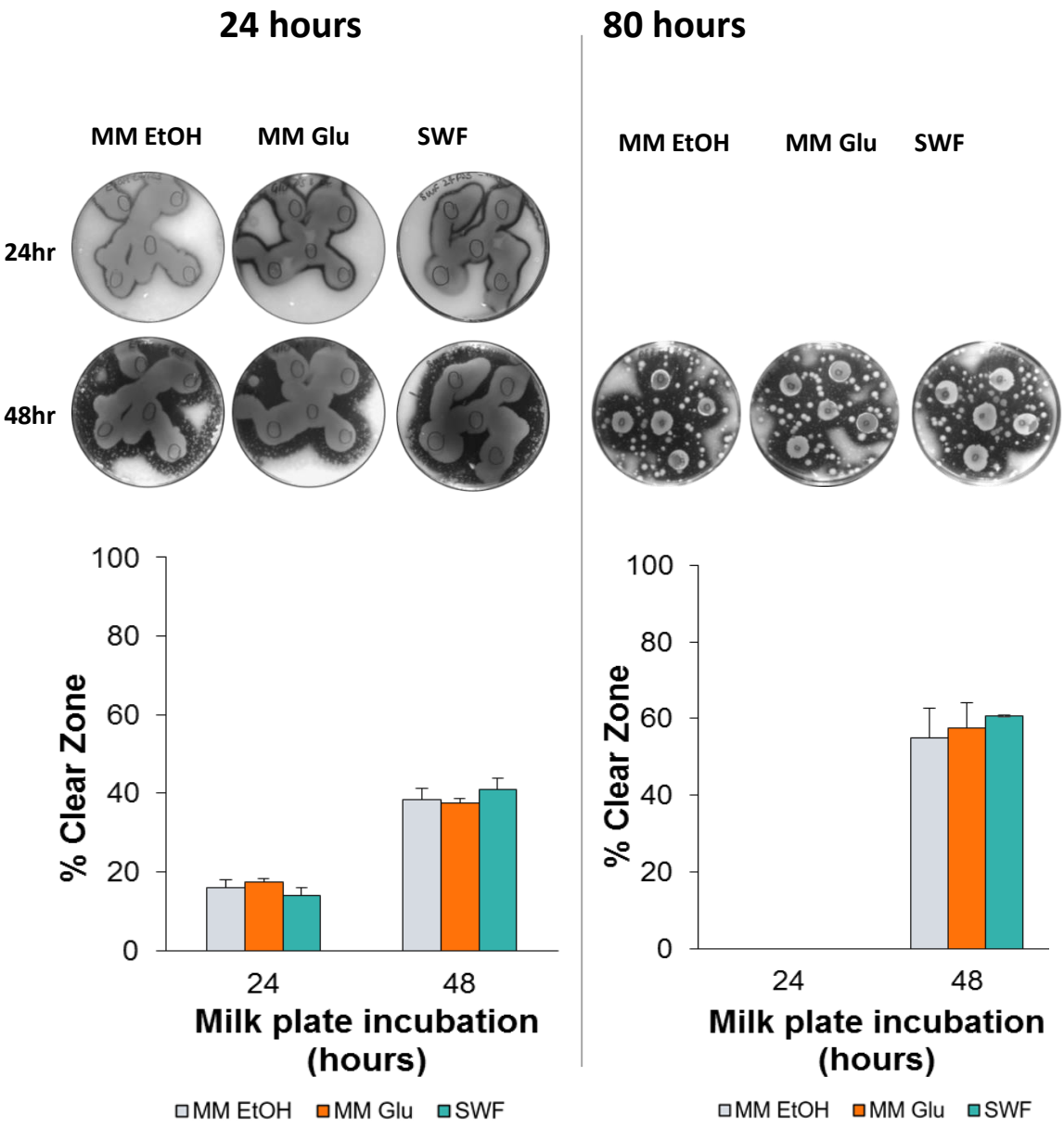

Figure 3.7 Plate images and corresponding protease production from live PS3 grown for 24 and 80 hours. Area of clear zones from milk agar plates inoculated with live PS3 from each of the different growth conditions from 24 and 80 hour cultures as indicated is shown. Milk agar plates were inoculated with 5 drops of approximately 1 x 10^3 live PS3 and incubated for 48 hours. Plates were imaged every 24 hours and clear areas measured using ImageJ software and expressed as a percentage of the total plate area**.** Data is expressed as mean of 3 biological repeats + SEM. Two way ANOVA showed no significant difference between any of the conditions.

3.2.4 Quantification of hemolysin production from PS3 cultures

It was investigated whether hemolysin production could be detected from PS3 and whether growth conditions have an effect on its production. No hemolysin production was detected in cell free conditioned media from PS3 cultures grown in any of the growth conditions throughout the incubation period.

Hemolysin was not detected in plates inoculated with live PS3 and incubated at 30°C for 24 hours for either the 24 or 80 hour cultures after a 48 hour plate incubation there was also no activity from the 24 hour cultures (figure 3.8). After incubating the plates for 120 hours (5 days), hemolysin production was detected from plates inoculated with PS3 grown for 24 hours with MM EtOH producing an 8% clear zone, MM Glu 12% and SWF showing 5% clear zones (figure 3.8), these values were not significant from each other.

Plates inoculated with live PS3 from 80 hour cultures produced hemolysin after a 48 hour plate incubation (figure 3.8), and this increased after 120 hours (5 days) plates incubation with PS3 grown in MM EtOH having the greatest hemolysin production (45% clear zone), This was significantly greater than PS3 grown in MM Glu (26% clear zone) ($p \leq 0.01$) and PS3 grown in SWF (28% clear zone) ($p \leq 0.05$) (figure 3.8).

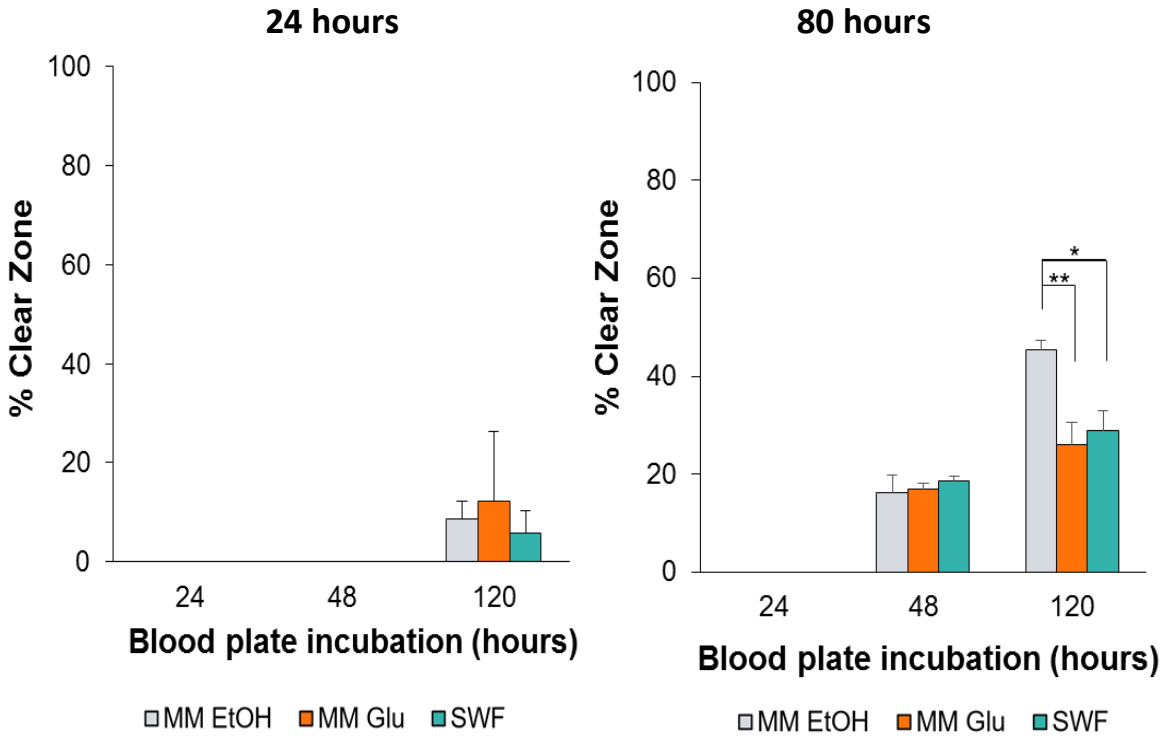

Figure 3.8 Hemolysin production from live PS3 grown for 24 and 80 hours. Area of clear zones measured from blood agar plates inoculated with live Ps3 from each of the different growth conditions from 24 hour cultures 80 hour cultures as indicated. Blood agar plates were inoculated with 5 drops of approximately 1 x 10^3 live PS3 and incubated for up for 120 hours (5 days). Plates were imaged every 24 hours and clear areas measured using ImageJ software and expressed as a percentage of the total plate area. Data is expressed as mean of 3 biological repeats + SEM. Two way ANOVA with Tukey's post hoc test showed significance as indicated on the graph (*p ≤0.05, **p≤0.01).

3.2.5 *Pseudomonas fluorescens* did not express virulence factors

PF was used as an additional reference strain as a comparison to the clinical isolate PS3. No positive results were observed for hemolysion or pyocyanin quantification from any PF cultures.

Minimal protease activity was observed on plates inoculated with PF grown for 24 hours, with 1-2% clear zones after 48 hour incubation of the plate, no protease activity was observed from plates inoculated with PF grown for 80 hours (data not shown).

3.3 Quantification of toxicity of non-conditioned growth media on cultured keratinocytes.

Initial experiments investigated whether exposure of HaCaT cells to different dilutions of the media used to grow the bacteria produced any acute toxic effects

Keratinocytes were exposed to diluted non-conditioned growth media for 4 hours, cells were visually examined 24 hours after initial exposure and viability of the cells was determined by an MTS assay. No change in the appearance of the cells exposed to any of the bacterial growth media was observed. In addition the MTS assay data indicated no toxic effects of any of the growth media, with a small, but not significant, increase in biomass compared to controls being observed (figure 3.9).

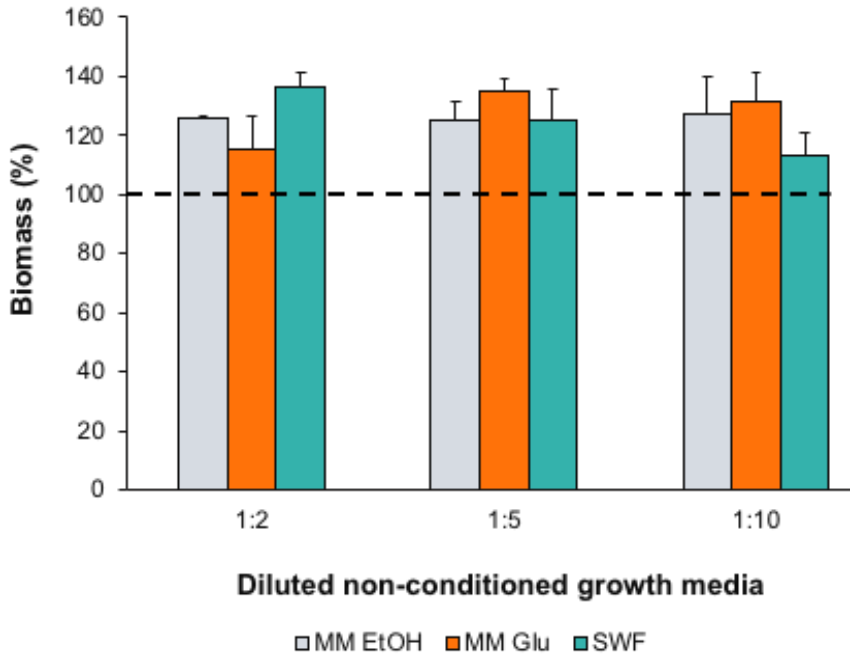

Figure 3.9 HaCaT viability following exposure to non-conditioned bacterial growth media. Percentage biomass change relative to control (complete DMEM) for each condition is shown, controls are indicated by the dashed line (100%). MTS data was measured 24 hours after an initial 4 hour exposure to non-conditioned growth media diluted 1:2 in complete DMEM. Data is mean + SEM of 3 independent experiments, (n=6 for each experiment). A two-way ANOVA showed no significance differences between growth conditions.

3.4 Investigation of *Pseudomonas* extracellular secretion toxicity on cultured human keratinocytes.

Once it had been established that non-conditioned growth media alone had no acute toxic effects were observed, keratinocytes were exposed to cell free conditioned media from both *Pseudomonas* strains (PS3 and PF) grown in the three different conditions (MM EtOH, MM Glu and SWF) to investigate if growth conditions affected the toxicity of extra cellular secretions in either of these strains. Cells were checked visually 24 hours after initial exposure and visual observations were then confirmed via MTS assay.

3.4.1 *Pseudomonas* conditioned media from 24 hour bacterial cultures does not cause toxicity in cultured human keratinocytes

Visual analysis of the cells exposed to conditioned media from PS3 grown for 24 hours in MM EtOH or MM Glu showed no apparent adverse effects on the morphology of the cells in culture, with cells exposed to the highest concentration (1:2 in complete DMEM) looking similar in size, shape and cell density to controls (growth media diluted 1:2 in complete DMEM) (figure 3.10). Conditioned media from the laboratory strain PF grown for 24 hours also did not have any visual effect on the cells in culture (figure 3.10). The lack of toxicity was confirmed by MTS data where PS3 and PF grown in MM EtOH, MM Glu or SWF showed no decrease in viability, with a small increase in biomass above control in almost all conditions and dilutions (figure 3.10). PS3 grown in MM Glu and diluted 1:2 in complete DMEM produced a small but not significant decrease in biomass compared to control. No statistically significant differences were observed between conditions (figure 3.10).

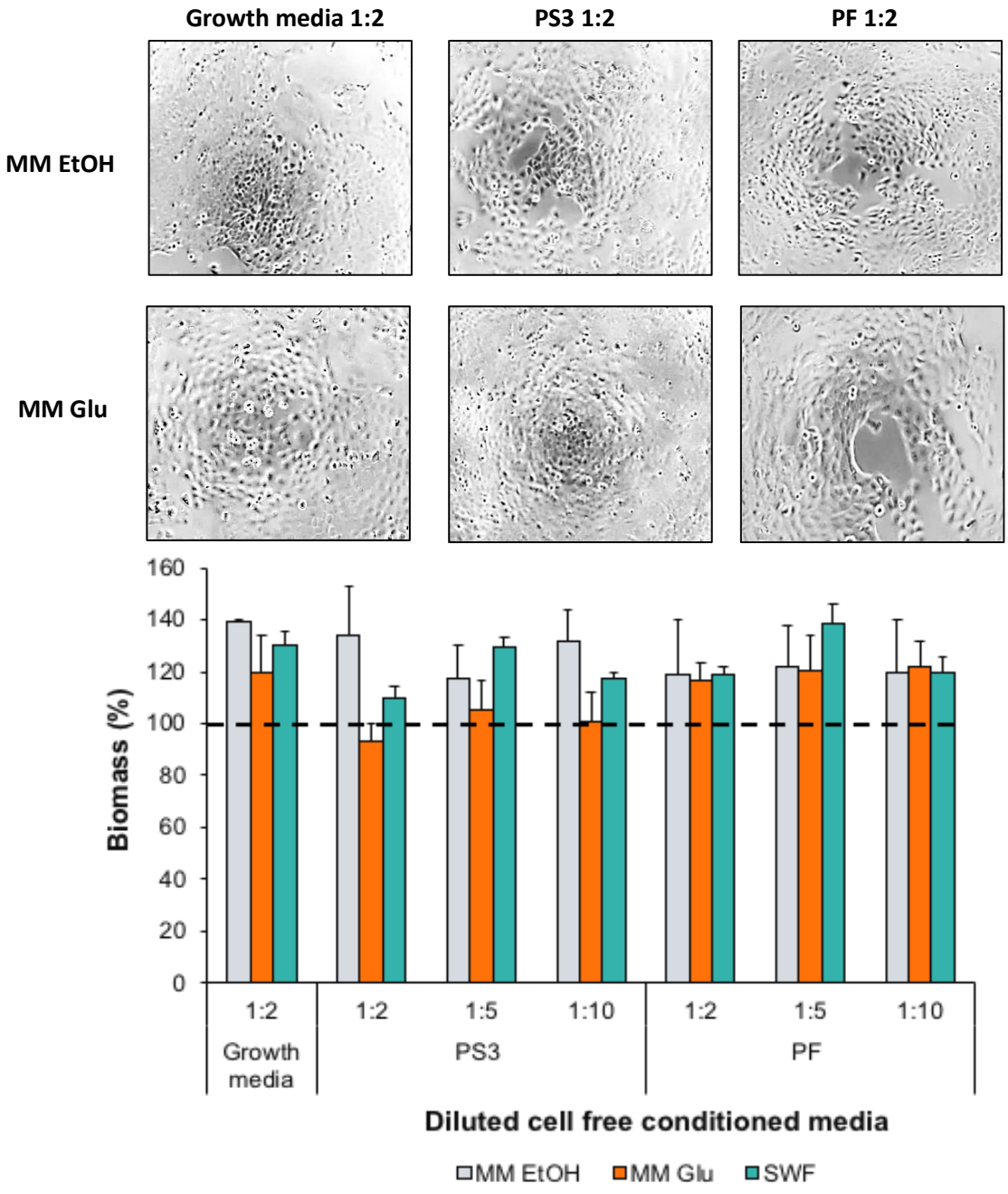

Figure 3.10 HaCaT images and corresponding viability data following exposure to 24 hour *Pseudomonas* cell free conditioned media. HaCaT images and percentage biomass change relative to control (complete DMEM) for each condition is shown, DMEM only treated cells are indicated by the dashed line (100%). MTS data was measured 24 hours after an initial 4 hour exposure to cell free conditioned media grown for 24 hours and diluted in complete DMEM as indicated. Data is expressed as an average of three independent experiments + SEM (n=6 for each experiment). A two-way ANOVA showed no significant differences between conditions.

3.4.2 *Pseudomonas* conditioned media from 80 hour bacterial cultures from ethanol supplemented cultures shows toxic effects in cultured keratinocytes

Visual analysis of HaCaT cells exposed to cell free conditioned media from PS3 grown in MM EtOH for 80 hours showed a visible stress response, with less cells visible and those that are being more rounded in appearance when exposed to conditioned media diluted 1:2 in complete DMEM compared to control (growth media diluted in DMEM) (figure 3.11). This was not observed in PS3 grown in MM Glu for 80 hours, where no visible stress response occurred (figure 3.11) with cells appearing similar to control (growth media diluted in complete DMEM). With PF conditioned media no visual stress was observed (figure 3.11) for either growth condition at a 1:2 dilution.

The visual observations were confirmed by MTS data where cell free conditioned media from PS3 grown in ethanol for 80 hours produced a significant decrease in viability compared to MM Glu. This appeared to be concentration dependent (figure 3.11) resulting in 0% biomass at the 1:2 dilution, 90% biomass at the 1:5 dilution and around 100% biomass at the 1:10 dilution (all diluted in complete DMEM). Cell free conditioned media from PF grown for 80 hours had a biomass similar to controls from both MM EtOH and MM Glu (figure 3.11).

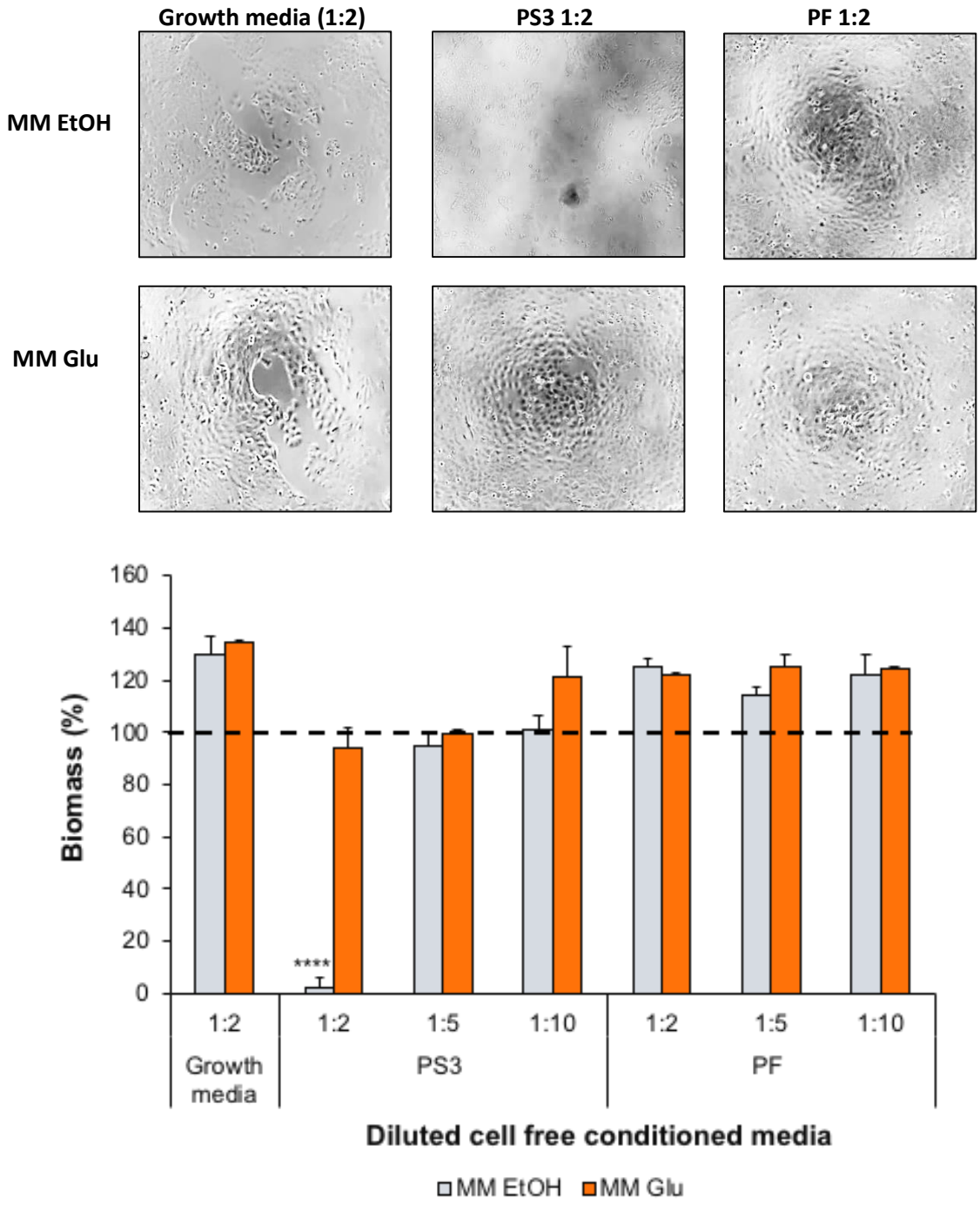

Figure 3.11 HaCaT images and corresponding viability data following exposure to 80 hour *Pseudomonas* cell free conditioned media. HaCaT images and percentage biomass change relative to control (complete DMEM) for *Pseudomonas* cell free conditioned media for MM EtOH and MM Glu is shown, complete DMEM treated cells are indicated by the dashed line (100%). MTS data was measured 24 hours after an initial 4 hour exposure to cell free bacterial conditioned media that had been grown for 80 hours and diluted in complete DMEM. Data is expressed as average of three independent experiments (+) SEM (n=6 for each experiment). A two-way ANOVA with Bonferroni post hoc showed significance (**** $p<0.001$) between MM EtOH and MM Glu at the 1:2 dilution as indicated on the graph.

3.3.3 PS3 grown in simulated wound fluid for 80 hours shows varied toxicity on cultured keratinocytes.

PS3 grown in SWF gave varied results on HaCaT viability with no or minimal toxicity seen at the at a 1:2 dilution for 2 biological repeats however the third biological repeat showed a reduction in biomass to 19% compared to control (figure 3.12). These results lead to large variation particularly at the highest concentration (1:2) of PS3 cell free media (figure 3.12).

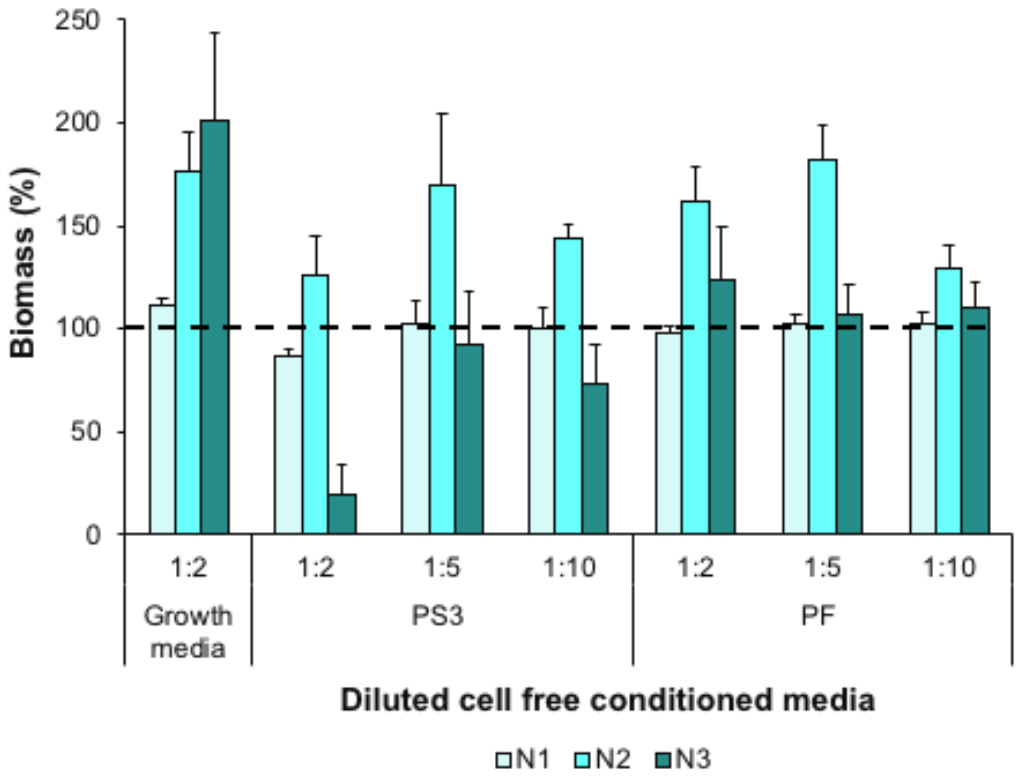

Figure 3.12 Keratinocyte viability for each biological replica following exposure to *Pseudomonas* grown in simulated wound fluid for 80 hours. Percentage biomass change relative to controls (complete DMEM) for 80 hour *Pseudomonas* cell free conditioned SWF, complete DMEM treated cells are indicated by the dashed line (100%). MTS data was measured 24 hours after initial 4 hour exposure to cell free conditioned SWF grown for 80 hours and diluted in complete DMEM as indicated on the graph. MTS data expressed is the mean of 6 technical repeats + standard deviation for each experiment.

3.4.4 Comparisons of time in culture within ethanol or glucose grown PS3 on keratinocyte viability.

In order to directly compare the effects of cell free conditioned media from 24 and 80 hour cultures, MTS data from the two time points for PS3 grown in MM EtOH or MM Glu were plotted together. This clearly shows the significant loss of viable cells ($p \leq 0.0001$) produced by the 1:2 dilution occurs only in response to secretions obtained from the 80 hour cultures grown in MM supplemented with EtOH with no significant effect being produced by 24 hour cultures or with glucose supplementation for either culture time (figure 3.13).

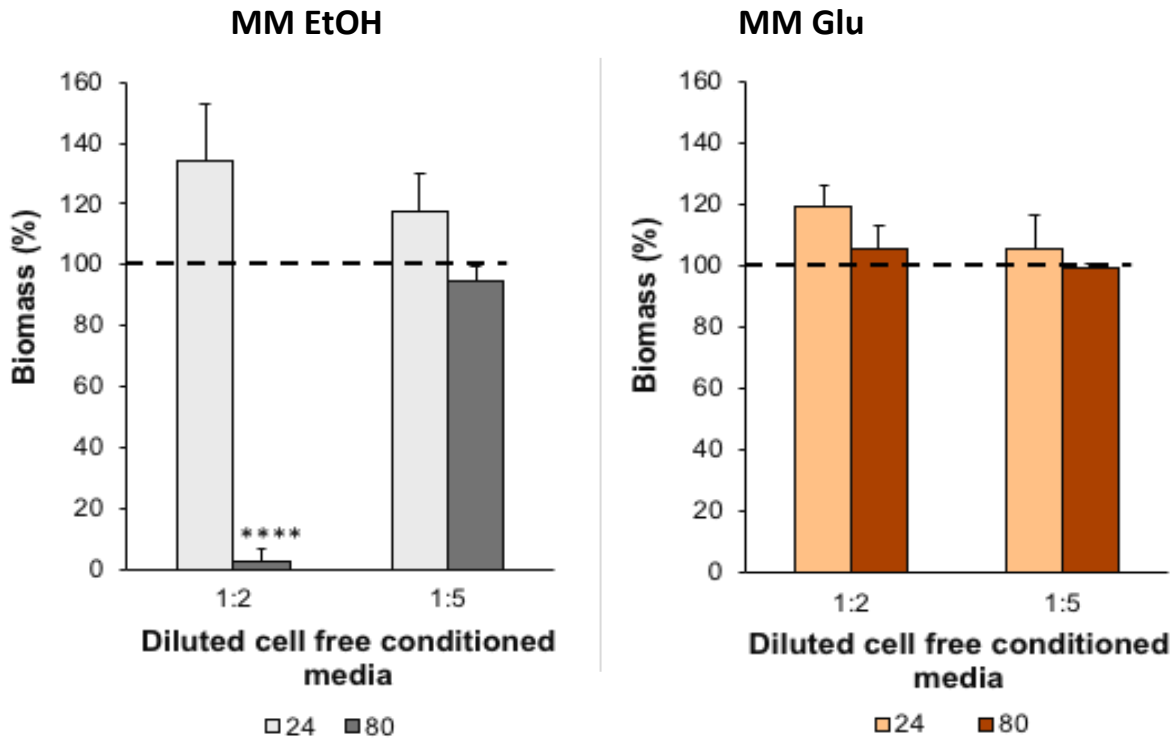

Figure 3.13 MTS data for keratinocyte viability following exposure to PS3 grown in MM EtOH or MM Glu for 24 and 80 hours. Percentage biomass change relative to controls (complete DMEM) for *Pseudomonas* cell free conditioned media with either EtOH or glucose as indicated DMEM treated cells are indicated by the dashed line (100%). MTS data was measured 24 hours after a 4 hour exposure to cell free conditioned media grown for 24 and 80 hours and diluted in complete DMEM. MTS data is expressed as the mean + SEM for 3 biological replicates (n=6 for each experiment). T test performed showed significance between time points as indicated on the graph (**** $p \leq 0.0001$).

3.4.5 Determination of concentration dependent toxicity on cultured keratinocytes following exposure to PS3 conditioned cell free media

To determine the concentration dependency of PS3 cell free conditioned media on cultured keratinocytes, the cells were exposed to additional dilutions (1:3 and 1:4 in complete DMEM) from 80 hour cultures of PS3 grown in MM EtOH or MM Glu. Cultured keratinocytes were exposed for 4 hours, with visual checks and viability determined 24 hours after the 4 hour exposure. MTS data showed a concentration dependent relationship on keratinocyte toxicity for PS3 grown in MM EtOH with percentage biomass relative to control increasing from 3% to 90% with concentration (figure 3.14). This concentration dependency was not observed with PS3 grown in MM Glu, with the percentage biomass relative to control around 90-100% for all dilutions (figure 3.14). There was a significant difference between MM EtOH and MM Glu at the 1:2 dilution ($p \leq 0.01$) and at the 1:5 dilution ($p \leq 0.05$). Due to the variation in the response of keratinocytes to 80 hour cultures of PS3 grown in SWF, the concentration dependency was not investigated.

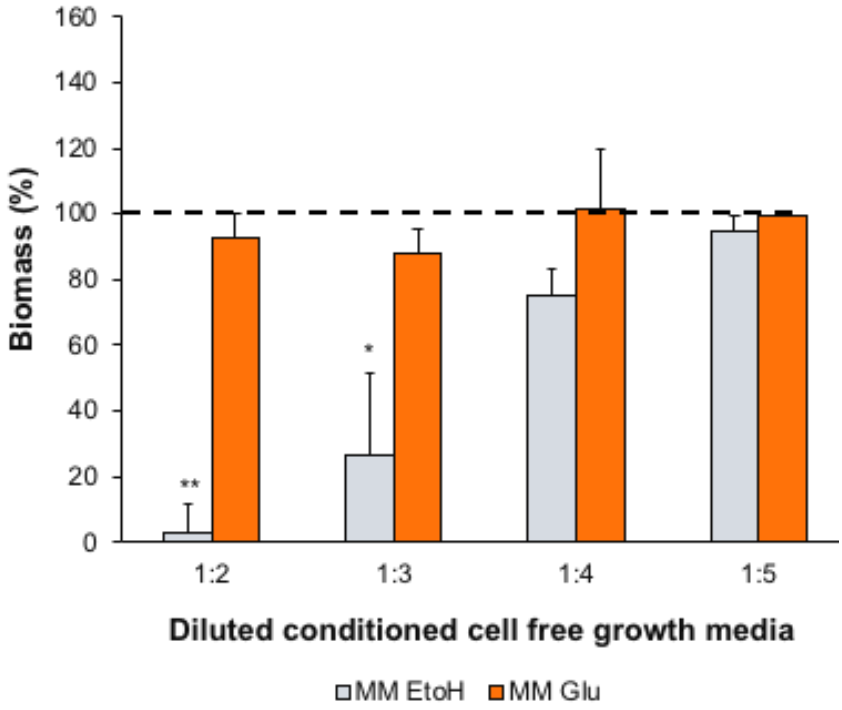

Figure 3.14 MTS data for keratinocyte viability following exposure to PS3 grown in MM EtOH or MM Glu for 80 hours. Percentage biomass change relative to control (complete DMEM) for *Pseudomonas* cell free conditioned media in MM EtOH or MM Glu is shown, complete DMEM treated cells are indicated by the dashed line (100%). MTS data was measured 24 hours after a 4 hour exposure to cell free conditioned media diluted in complete DMEM. MTS data is expressed as the mean +/- SEM for 3 biological repeats (n=6) A two-way ANOVA with Bonferroni post-hoc showed significance between MM EtOH and MM Glu at the 1:2 and 1:5 dilution (** $p \leq 0.01$, * $p \leq 0.05$).

3.5 Investigation of toxicity mediated by direct exposure to *Pseudomonas* on cultured human keratinocyte viability.

Once the effects of *Pseudomonas* conditioned cell free media on keratinocyte viability had been established, cells were exposed directly to live PS3 grown in the growth conditions to investigate the toxicity of the bacterial cells directly. Keratinocytes were exposed to approximately 1×10^5 and 1×10^3 bacteria per well for 2 hours and cells were checked visually after 8 hours, visual observations were then confirmed by MTS assay.

3.5.1 Keratinocyte viability following exposure to live PS3 grown for 24 hours

Observations of the cultured keratinocytes showed no visual appearance of stress in response to direct exposure to PS3 at the highest concentration of bacterial number of 1×10^5 per well from any of the growth conditions with all cells looking similar to the control (HBSS only) (figure 3.15). There was also no effect of the direct exposure of PS3 grown in any condition of the viability of the keratinocytes as determined by an MTS assay (figure 3.15). Keratinocytes exposed to higher bacterial number of 1×10^5, all showed 100% biomass compared to control, in comparison, cells exposed to the lower bacterial number of 1×10^3 produced an increase in biomass conditions of around 20% above control (figure 3.15).

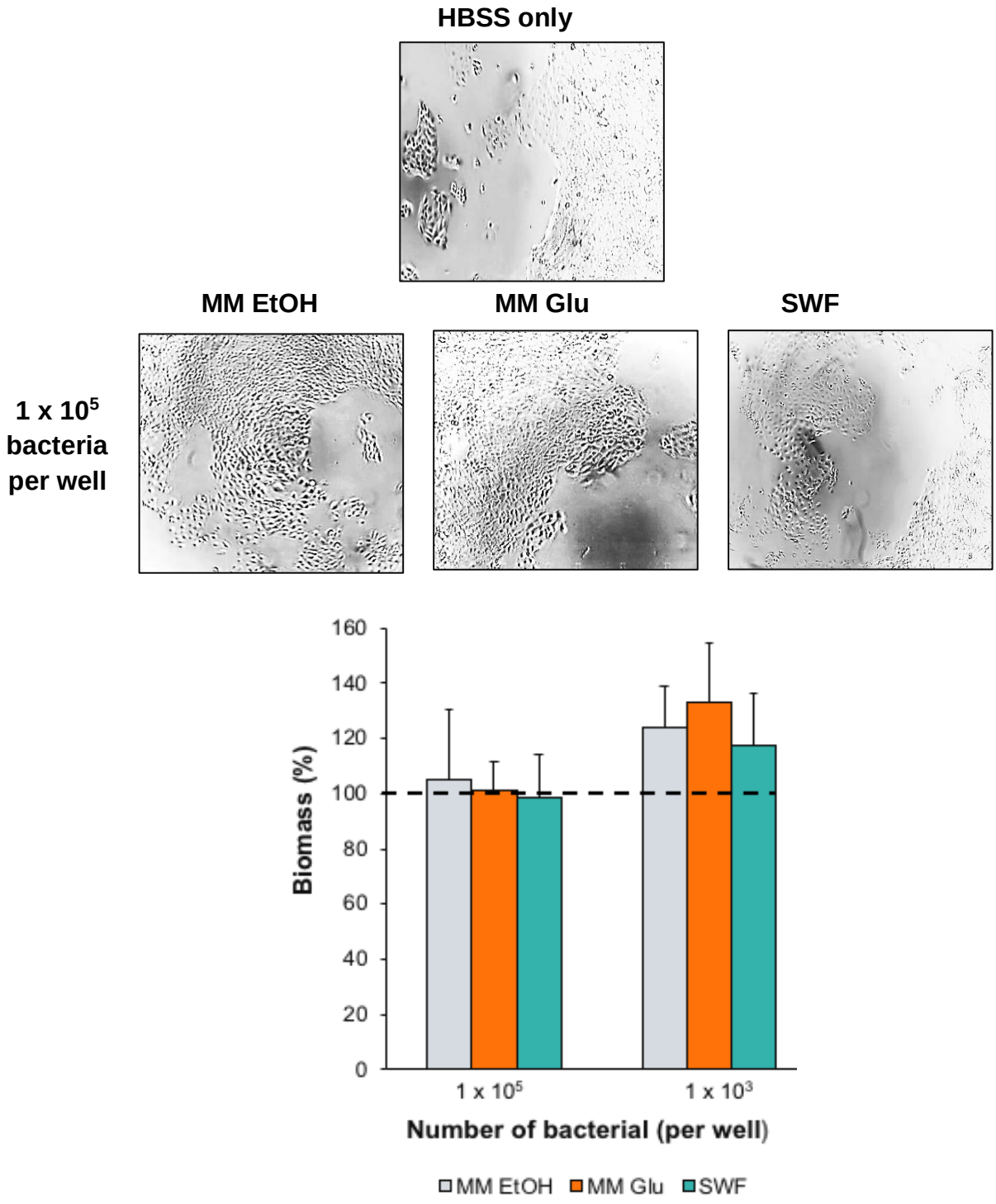

Figure 3.15 HaCaT images and corresponding viability data following exposure to live PS3 grown for 24 hours. Keratinocyte images and percentage biomass change relative to control (HBSS only) after exposure to live PS3 grown in each of the conditions is shown, controls are indicated by the dashed line (100%). MTS assay was performed 8 hours after a 2 hour exposure to live PS3, data is mean (+) standard deviation of 6 technical replicates. HaCaT images show control cells (HBSS only) and treated cells 8 hours after 2 hour exposure to 1 x 10^5 bacteria from each of the growth conditions.

3.4.2 Keratinocyte viability following direct exposure to live PS3 grown for 80 hours

PS3 grown in MM EtOH for 80 hours, showed no significant effect on the viability of keratinocytes when exposed to both concentrations of bacteria with the biomass of keratinocytes being about 90% of the control (HBSS only) (figure 3.16).

PS3 grown in MM Glu produced a small decrease in biomass to about 80% of the control when exposed to 1 X 10^5 bacteria and about 90% of the control when exposed to 1 x 10^3 bacteria (figure 3.16). PS3 grown in SWF, produced a slight decrease in biomass to about 90% of the control upon exposure to both bacterial numbers (figure 3.16).

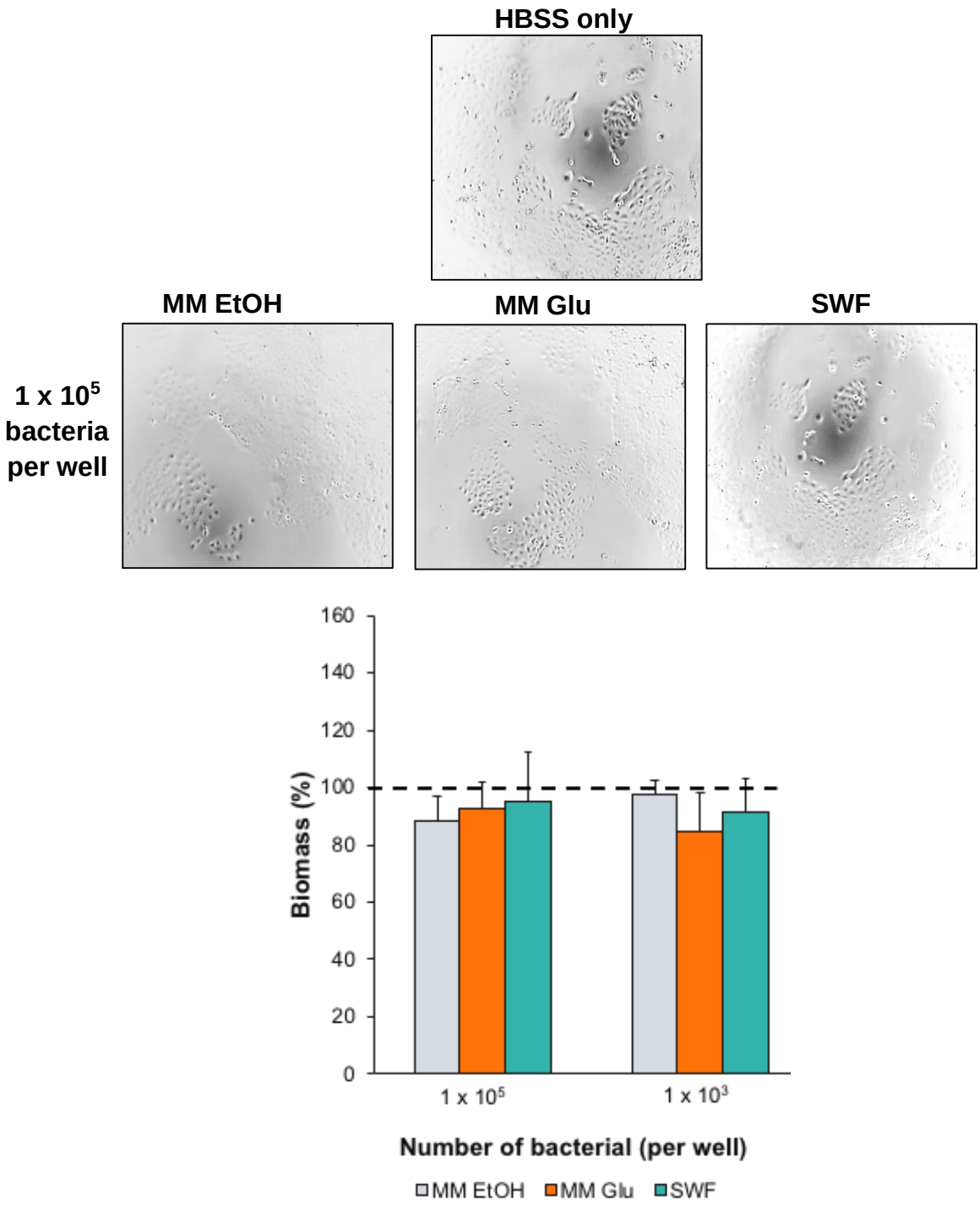

Figure 3.16 HaCaT images and corresponding viability data following exposure to live PS3 grown for 80 hours. Keratinocyte images and percentage biomass change relative to control (HBSS only) for exposure to live PS3 grown in each of the conditions is shown, controls are indicated by the dashed line (100%). MTS data was measured 8 hours after initial 2 hour exposure to PS3. Data is expressed as an average of one experiment + standard deviation of 6 technical replicates. Keratinocyte images show control cells (HBSS only) and keratinocytes 8 hours after initial 2 hour exposure to 1 x 10^5 bacteria from each of the growth conditions.

3.5.3 Reduction of exposure time still shows toxicity of PS3 cell free conditioned media

To confirm that the lack of toxicity observed on direct exposure of PS3 to the keratinocytes was not due to shortened exposure times of the cells to the live bacteria, additional experiments using 80 hour cell free conditioned media in which cells were exposed for the shorter time period of 2 hours were carried out. A reduction in biomass was seen 8 hours after a 2 hour exposure to PS3 cell free conditioned media with SWF showing the biggest decrease in biomass at a 1:2 dilution to 6% compared to MM EtOH (58% biomass) ($p \leq 0.0001$) and MM Glu (87% biomass) ($p \leq 0.0001$) at a 1:2 dilution (figure 3.17). In addition the decrease in biomass in response to PS3 grown in MM EtOH was significantly lower than that for MM Glu at a 1:2 dilution ($p \leq 0.0001$). A reduction in biomass also occurred at a 1:5 dilution, with biomass in response to SWF conditioned media being 60% and MM EtOH having 66% biomass, both of which were significantly lower than MM Glu (100%) ($p \leq 0.0001$). No significant differences were observed between conditions at a 1:10 dilution.

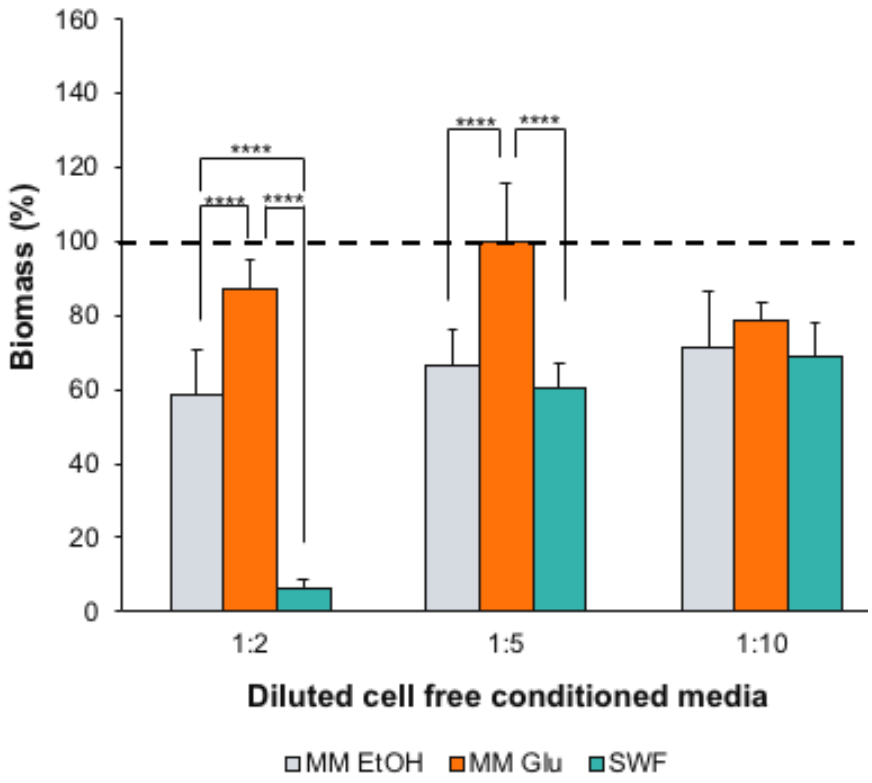

Figure 3.17 Keratinocyte viability data following shortened 2 hour exposure time to PS3 cell free conditioned media Percentage biomass change relative to control (HBSS only) for exposure PS3 cell free conditioned media from each of the different growth conditions, controls are indicated by the dashed line (100%). MTS data was measured 8 hours after a 2 hour exposure. Data is expressed as an average of one experiment + standard deviation of 6 technical replicates. A two way ANOVA with Tukey's post hoc showed significance between groups as indicated on the graph (**** $p \leq 0.0001$).

3.6 Bacterial attachment and internalisation into cultured keratinocytes from PS3 grown for 24 hours

Bacterial attachment and/or internalisation into keratinocytes could represent factors in the pathology of chronic wounds. It was therefore investigated whether culture conditions had an effect on the attachment and internalisation of PS3 to keratinocytes. In addition, having shown virulence factors can be secreted by the bacteria and that at a high concentration these caused cellular stress, the effect of cell free conditioned media on *Pseudomonas* attachment or internalisation into keratinocytes was also investigated. Keratinocytes were exposed directly to PS3 for 2 hours, with bacterial pellets re-suspended with HBSS only. In additional experiments, to investigate the role of secreted factors on this process cell free media was diluted 1:5 in HBSS and used as a pre-treatment before keratinocytes were exposed to live PS3.

Further experiments were performed with an increased exposure time of keratinocytes to the bacteria of 4 hours but this approach was discontinued due to high levels of variance seen in MOI after the 4 hour incubation time (data not shown). Due to the lack of virulence seen from PF attachment and internalisation was not investigated.

3.6.1 Attachment and internalisation of live PS3 only from 24 and 80 hour cultures.

Initial experiments investigated attachment and internalisation from PS3 grown for 24 and 80 hours in each of the different culture condition resuspended in HBSS only, keratinocytes were exposed for 2 hours at an MOI of 100, 10 or 1.

PS3 grown for 24 hours in MM Glu showed the greatest level of attachment and internalisation with approximately 2-3 bacteria per keratinocyte across each MOI (figure 3.18), this was significantly greater than SWF grown bacteria at an MOI of 1 or 10 ($p \leq 0.01$), and significantly greater than MM EtOH and at MOI of 100 ($p \leq 0.05$) (figure 3.18). MOI had minimal effects on the degree of bacterial attachment and or internalisation for MM Glu and MM EtOH, with a slight reduction in attachment and internalisation occurring at MOI 100 for MM EtOH (figure 3.18). For SWF grown PS3, bacterial attachment and internalisation increased proportionally with MOI (figure 3.18).

In contrast to 24 hour data PS3 grown in MM EtOH, showed the greatest amount of attachment and internalisation with approximately 2 bacteria per keratinocyte across each MOI (figure 3.18), this was significantly greater to PS3 grown in MMG and SWF which showed minimal attachment and translocation at an MOI of 1 and 10 ($p \leq 0.0001$). Significant differences were also seen at MOI 100 between MM EtOH and Glu ($p \leq 0.001$) and MM EtOH and SWF ($p \leq 0.01$) (figure 3.18). Similar to the data for 24 hour cultures, there was little effect of MOI on the number of bacteria attached or internalised, particularly for PS3 grown in MM EtOH, a small increase was seen at MOI 100 for MM Glu and SWF (figure 3.18).

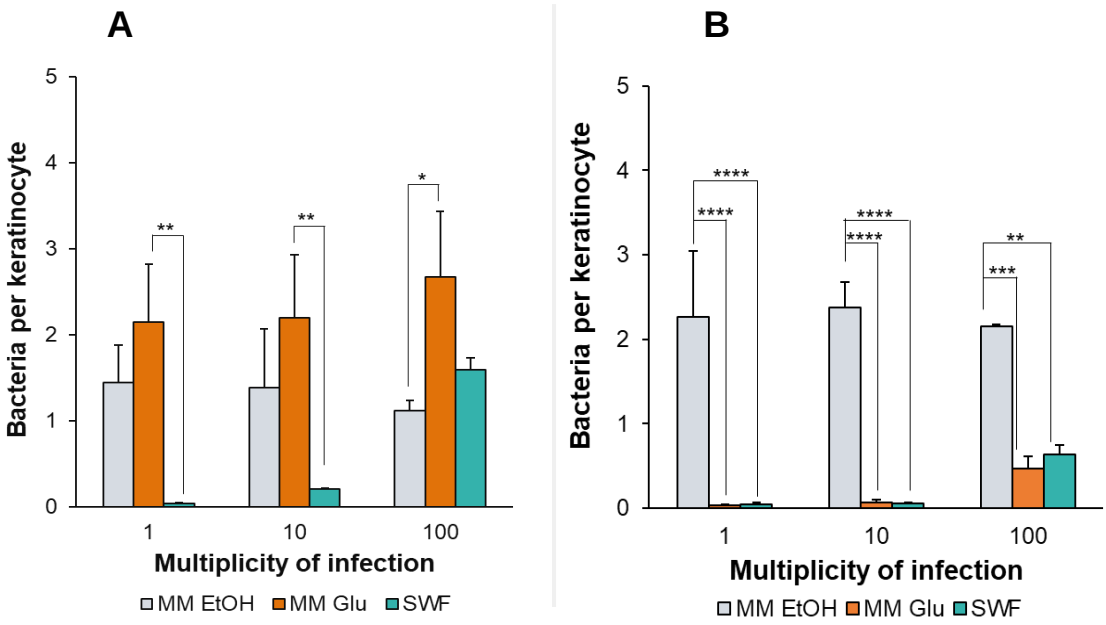

Figure 3.18 Attachment and internalisation of PS3 grown for 24 and 80 hours into cultured keratinocytes. Bacterial attachment and translocation of PS3 grown in each of the different growth conditions to cultured keratinocytes at an MOI of 1, 10 and 100, where A represents 24 hour cultures and B represents 80 hour cultures. Keratinocytes were washed thoroughly after 2 hour exposure time and burst to get viable bacterial cell counts. Bacterial numbers were calculated from plate counts and are expressed as bacteria per cultured keratinocyte. Data is expressed as mean + SEM for independent experiments, with the plate counts duplicated for each repeat. A two way ANOVA with Tukey's post hoc showed significant differences as indicated on the graph (* p≤.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001).

3.6.2 Internalisation from of live PS3 only from 24 and 80 hour cultures.

Once the amount of attachment and internalisation of PS3 resuspended in HBSS only grown for 24 and 80 hours, had been determined, further experiments were carried out to determine the amount of internalisation of PS3 grown for 24 hours, into culture keratinocytes. These data quantifying the numbers of bacteria that had internalised into the keratinocytes were obtained from cells treated with the same bacterial suspension at the same time as the attachment and internalisation data previously reported (figure 3.18).

For 24 hour conditions, whilst MM EtOH and MM Glu showed greater overall attachment and internalisation (figure 3.18) bacteria grown in SWF showed internalisation in greater numbers compared to MM EtOH and MM Glu (figure 3.19), with MOI also having an effect. SWF grown bacteria showed the greatest level of internalisation for each MOI tested, and this was significant compared to MM EtOH at MOI 10 and 100 ($p \leq 0.01$) and to MM Glu at MOI 10 ($p \leq 0.001$) and MOI 100 ($p \leq 0.0001$) (figure 3.19).

For 80 hours growth conditions PS3 grown in MM EtOH showed the greatest amount of internalisation across each MOI (figure 3.19). As these data are quantifying the internalised bacteria that were obtained from cells on the same plate and time as the attached and internalisation data (figure 3.18), it indicates that PS3 grown on MM EtOH has greater attachment to keratinocytes, whereas PS3 grown in MM Glu and SWF internalise more, particularly at an MOI of 1 (figure 3.19). MOI had little effect on bacterial internalisation, particularly for PS3 grown in MM EtOH and MM Glu, for SWF however again there is a positive correlation between bacteria internalised and MOI (figure 3.19).

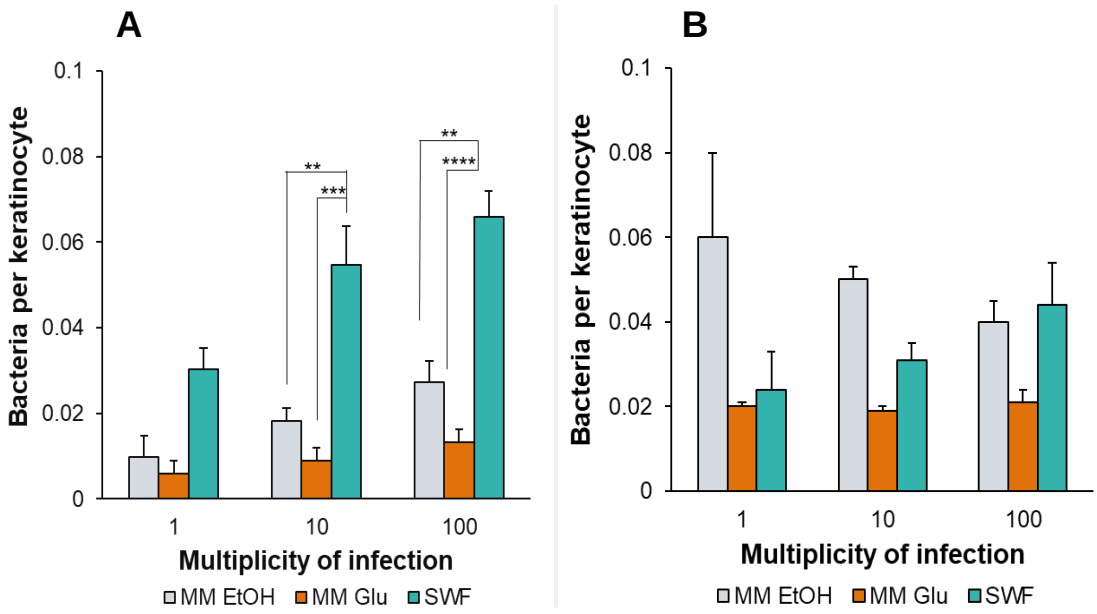

Figure 3.19 Internalisation only of PS3 grown for 24 and 80 hours into cultured keratinocytes. Bacterial internalisation of PS3 grown in each of the different growth conditions to cultured keratinocytes at an MOI of 1, 10 and 100 is shown, where A represents 24 hour cultures and B represents 80 hour cultures. Keratinocytes were incubated with antibiotics to remove external bacteria before being burst to internalised bacteria only. Bacterial numbers were calculated from plate counts and are expressed as bacteria per cultured keratinocytes. Data is expressed as mean + SEM for 3 independent experiments, with plate counts duplicated for each repeat. A two way ANOVA with Tukey's post hoc showed significant differences as indicated on the graph (** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

3.6.3 Attachment and internalisation of live PS3 24 and 80 hour cultures with the addition of cell free media

Once the amount of attachment and internalisation and internalisation only had been established for PS3 only grown in each of the conditions for 24 and 80 hours, further experiments were performed to investigate the role of the cell free conditioned media. Cell free media was diluted 1:5 in HBSS used as a pre-treatment before keratinocytes were exposed to live PS3.

The addition of diluted cell free conditioned media generally had little effect on attachment and internalisation, with PS3 grown in MM Glu showing the greatest level of attachment across each MOI similar to when cells were exposed to live PS3 only (figure 3.18). This was significantly greater than PS3 grown in SWF at each MOI ($p \leq 0.05$) and PS3 grown in MM EtOH at an MOI of 1 ($p \leq 0.05$) and an MOI of 10 and 100 ($p \leq 0.01$) (figure 3.20).

Similar to 24 hours, at 80 hours the addition of diluted cell free media as a pre-treatment generally had little effect on attachment and internalisation of PS3 grown in MM EtOH, with the amount of attachment and internalisation being similar to keratinocytes that received no pre-treatment (figure 3.18) (figure 3.20), this was statistically significant to both MM Glu and SWF at MOI 1 and 10 at ($p \leq 0.001$, $p \leq 0.0001$). There was an increase in attachment and internalisation for PS3 grown in SWF which was related to MOI, with significant differences seen between MM Glu and SWF ($p \leq 0.01$) which was only observed at MOI 100 (figure 3.20).

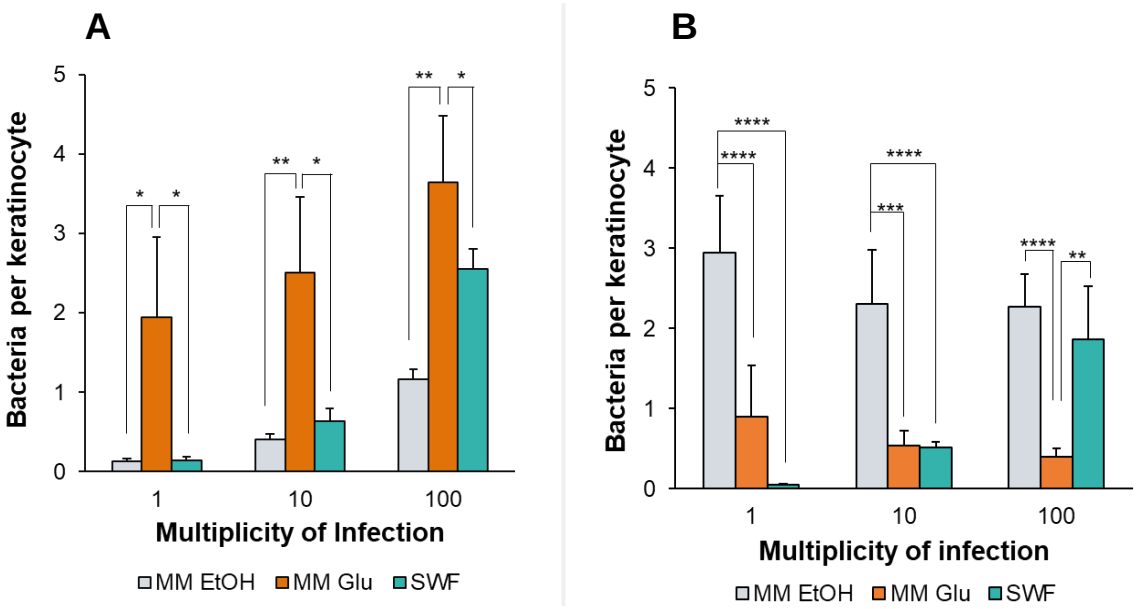

Figure 3.20 Attachment and internalisation of PS3 grown for 24 and 80 hours to cultured keratinocytes pre-treated with cell free conditioned media. Bacterial attachment and internalisation of PS3 grown in each of the different growth conditions to cultured keratinocytes at an MOI of 1, 10 or 100 is shown, where A represents 24 hour cultures and B represents 80 hour cultures. Keratinocytes were washed thoroughly after 2 hour exposure time and burst to get viable bacterial cell counts. Bacterial numbers were calculated from plate counts and are expressed as bacteria per cultured keratinocytes. Data is expressed as mean + SEM for 3 independent experiments with the plate counts duplicated for each repeat. A two way ANOVA with Tukey's post hoc showed significant differences as indicated on the graph (** p≤0.01 *** p ≤0.001, ****p ≤0.0001).

3.6.4 Internalisation of PS3 24 and 80 hour cultures with the addition of cell free media

Once the attachment and internalisation had been determined for PS3 with the addition of cell free media as a pre-treatment, the role of the addition of cell free media was assessed on internalisation only. These data quantifying the numbers of bacteria that had internalised into the keratinocytes were obtained from cells treated with the same bacterial suspension at the same time as the attachment and internalisation data previously reported (figure 3.20).

For 24 hour cultures, PS3 grown in SWF showed the greatest level of internalisation at MOI 10 and 100 compared to MM EtOH ($p \leq 0.01$) and MM Glu ($p \leq 0.001$, $p \leq 0.0001$) (figure 3.21), which was similar to when keratinocytes were exposed to live PS3 only (figure 3.19). At an MOI of 1 PS3 grown in MM EtOH showed the greatest level of internalisation (figure 3.21) however this was not significant to the other conditions.

For the 80 hour conditions, PS3 grown in MM EtOH had the greatest level of internalisation across each MOI with statistical significances seen at MOI 1 between MM Glu and SWF ($p \leq 0.0001$) (figure 3.21). MOI did not have an effect on the amount of internalisation of PS3 grown in MM EtOH or MM Glu, but again it did for PS3 grown in SWF (figure 3.21).

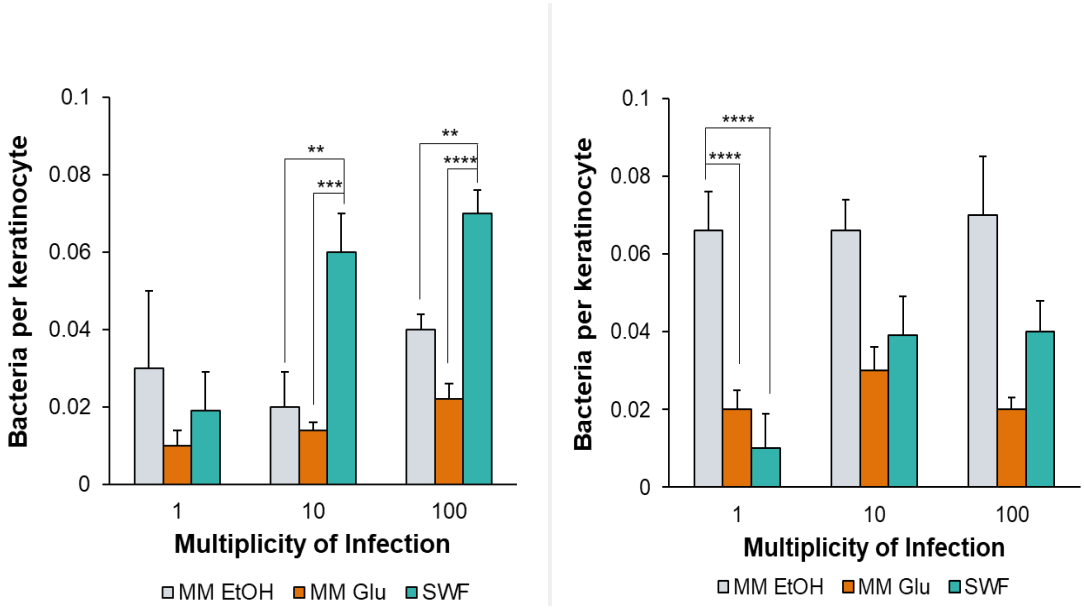

Figure 3.21 Internalisation only of PS3 grown for 24 and 80 hours into cultured keratinocytes pre-treated with cell free conditioned media. Bacterial internalisation of PS3 grown in each of the different growth conditions to cultured keratinocytes at an MOI of 1, 10 and 100 is shown, where A represents 24 hour cultures and B represents 80 hour cultures. Keratinocytes were incubated with antibiotics to remove external bacteria before being burst to internalised bacteria only. Bacterial numbers were calculated from plate counts and are expressed as bacteria per cultured keratinocytes. Data is expressed as mean + SEM for 3 independent experiments, with plate counts duplicated for each repeat. A two way ANOVA with Tukey's post hoc showed significant differences as indicated on the graph (** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

3.6.5. Expression of virulence factors associated with PS3 attachment

Whilst there was a low level of attachment, there was some differences between conditions and time points, suggesting potential differences in expression of genes involved in attachment. Transcriptome analysis showed increased expression of two genes involved in attachment, FleQ involved in expression of flagella (Hickman and Harwood, 2008) and PilA, involved in pili assembly (Persat et al., 2015) from 24 hour cultures of MM Glu, however after 80 hours, expression of both of these was higher from MM EtOH cultures (figure 3.22).

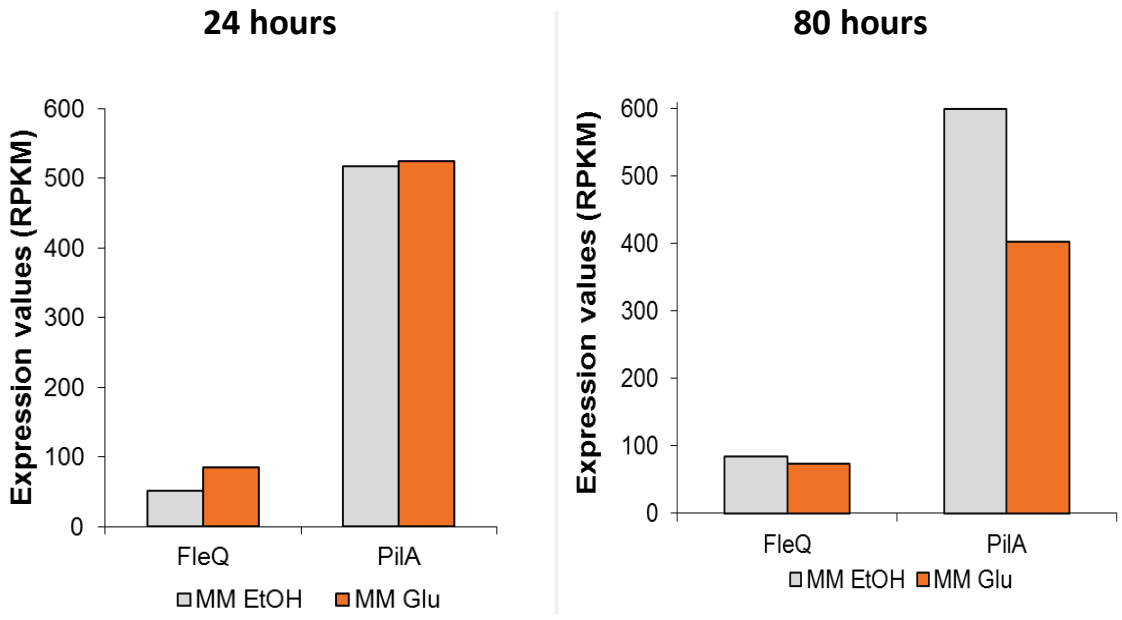

Figure 3.22 Expression of FleQ and Pil A genes from PS3 grown for 24 and 80 hours. Expression values expressed as reads per kilo base per million mapped reads of FleQ and Pil A genes from PS3 grown in MM EtOH and MM Glu for 24 and 80 hours.

3.6.6 The skin commensal bacteria *Staphylococcus epidermidis* can internalise into keratinocytes more than PS3

To determine whether the low levels of internalisation that occurred with the PS3 was specific to this strain, the amount of internalisation of a common non pathological skin commensal *Staphylococcus epidermidis* was ascertained.

*Staphylococcu*s epidermidis at an MOI of 100 could internalise into cultured keratinocytes in higher numbers than PS3, with internalisation of 1-15 bacteria per 10 keratinocyte (figure 3.23). By comparison the highest amount of internalisation for PS3 was from PS3 grown in SWF for 80 hours at an MOI of 100 and this was an average 0.8 bacteria per 10 keratinocytes (figure 3.21).

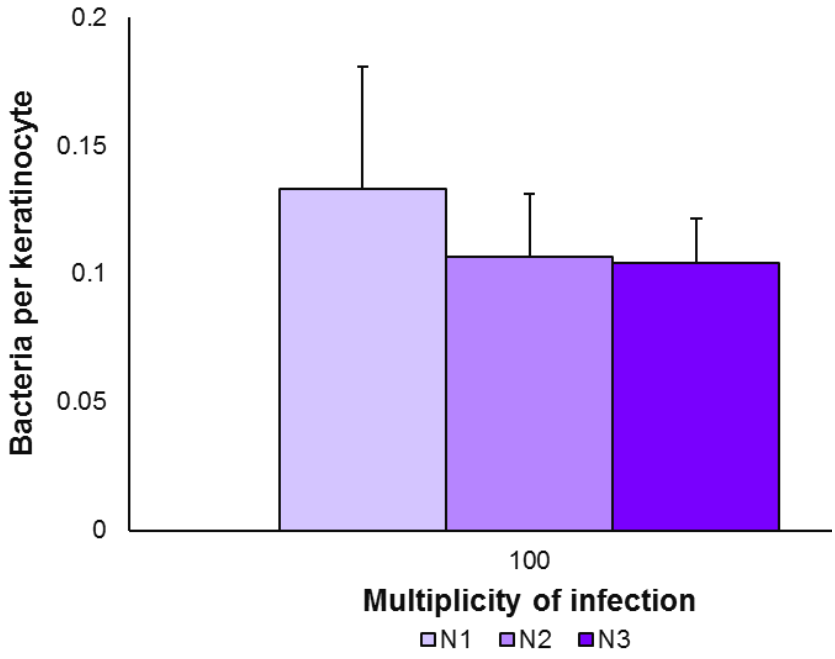

Figure 3.23 Internalisation of *Staphylococcus* epidermidis grown for 24 hours in to cultured keratinocytes. Bacterial internalisation of *Staphylococcus epidermis* grown in TSB for 24 hours at an MOI of 100 is shown. Bacterial numbers were calculated from plate counts and are expressed as bacteria per cultured keratinocyte. Data is expressed as the mean + standard deviation for 3 independent experiments

3.7 Summary and key findings

Results in this chapter showed that PS3 cell free conditioned media caused toxicity of cultured human keratinocytes and that growth condition of the bacteria can influence toxicity. A total loss of viable cultured keratinocytes occurred at high concentrations of cell free conditioned media from PS3 grown in MM EtOH for 80 hours, but this was not observed for PS3 grown in MM Glu or for PF grown in the same conditions. This was also not observed for PS3 grown for 24 hours indicating time in culture has an effect on the toxicity of secreted factors from PS3.

Analysis of some virulence factors showed that PS3 could express virulence factors, all of which were increased for 80 hour culture times compared to 24 hours, showing potential for culture time to have an effect of virulence of *Pseudomonas*. At the level of individual virulence factors there was no consistent significant difference PS3 grown in MM EtOH compared to other growth conditions. In addition, no protease or hemolysin activity could be detected in cell free conditioned media from PS3 grown in any of the conditions. No virulence factors were detected from PF in any of the assays used.

PS3 showed a general low level of attachment and internalisation into cultured keratinocytes. For 24 hour cultures, PS3 grown in MM Glu showed the greatest level of attachment, by comparison at 80 hours, PS3 grown in MM EtOH showed greater attachment which was highly significant compared to other conditions. PS3 grown in MM EtOH for 80 hours also showed a greater amount of internalisation into keratinocytes however there was no consistent significance difference compared to the other conditions.

Chapter 4
Generation of immune responses from *Pseudomonas*

4.1 Introduction

Whilst the skin acts as a physical barrier, damage to the skin can potentially lead to microbial invasion of the damaged and surrounding tissue, which is commonly found in hospital patients (Ki and Rotstein, 2008). HaCaT cells make a useful model to investigate immunological and inflammatory responses of the skin (Colombo et al., 2017) similar to dermal keratinocytes, they are known to express PRR's, known as MAMP receptors which make them a suitable model to investigate host - pathogen interactions relating to the skin (Pivarcsi et al., 2003).

Innate immune responses initiated within keratinocytes occur via MAMP receptors which upon recognition of bacterial MAMPs become activated. *Pseudomonas* possess a variety of MAMPs which can be cell attached or secreted and are specific in activating certain MAMP receptors (Lavoie et al., 2011). Upon activation, a cell signalling cascade is generated resulting in pro-inflammatory cytokine release, the pro-inflammatory cytokine CXCL8 is central to the innate immune response due its ability to target and recruit neutrophils to the site of infection through chemotaxis (Jundi and Greene, 2015). Following neutrophil activation other professional immune cells such as macrophages are recruited to the site of infection leading to inflammation which is an important and tightly regulated process which aids in the ability to clear invading microbes and promote healing (Piktel et al., 2019). In the case of persistent microbial invasion which often occurs in chronic wounds, clearance of invading microbes is not always achieved and this can lead to the overproduction of inflammatory mediators which can in turn be of benefit to invading microbes (Lin and Zhang, 2017). In addition, dysregulation of inflammatory responses can cause further damage to host tissue (Ruffin and Brochiero, 2019). Whilst a lot of research has

focused on cell attached MAMPs, little work has been carried out to investigate the interactions between host cells and secreted bacterial products (Kuipers et al., 2018).

4.2 CXCL8 secretion from keratinocytes direct exposure to PS3 vs cell free conditioned media.

In order to investigate the secretion of CXCL8 from keratinocytes in response to direct contact with PS3 compared to PS3 cell free conditioned media, keratinocytes were exposed to 1 x 10^5 bacteria or 1:2 dilution (in complete cell culture media) of the corresponding bacterial cell free growth media for 2 hours. This was then replace with complete culture media (supplemented with antibiotics (1μg/ml) for cells exposed to live bacteria). Supernatants from the cells were collected 8 hours after initial exposure and CXCL8 was quantified by ELISA.

4.2.1 CXCL8 stimulation from live PS3 and EC secretions from 24 hour cultures

Direct exposure of keratinocytes to direct PS3 from 24 hour cultures grown in all 3 conditioned stimulated CXCL8 secretion. PS3 grown in MM Glu or SWF produced the largest stimulation of CXCL8 secretion of 1250-1300 (pg/ml) (figure 4.1), which was approximately 2 fold higher than Ps3 grown in MM EtOH ($p \leq 0.05$).

The corresponding cell free conditioned media derived from the same cultures differed, with MM EtOH and MM Glu producing a concentration of 1100-1250 (pg/ml) CXCL8. These were both significantly greater than SWF cell free

conditioned media (p ≤0.001, p≤0.01) for which there was a lower CXCL8 secretion compared to exposure to live PS3 (figure 4.1).

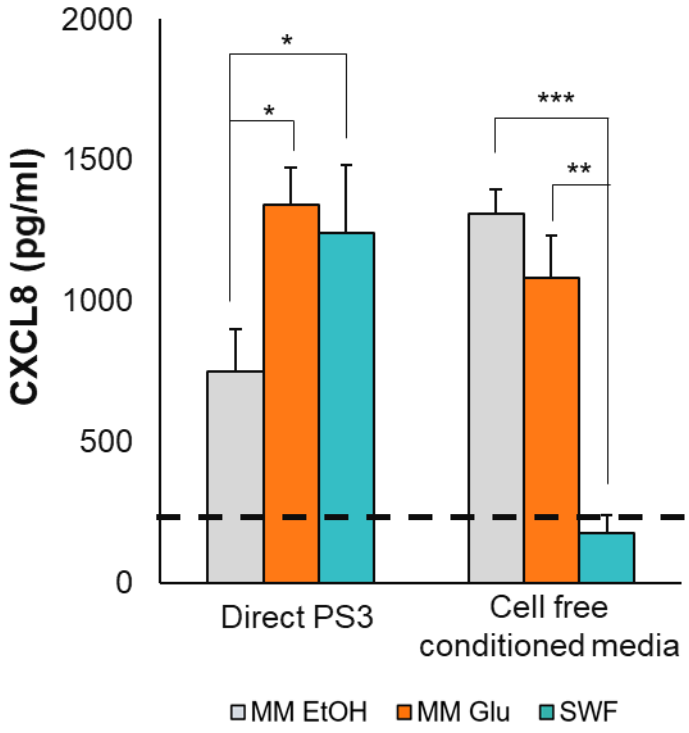

Figure 4.1 CXCL8 secretion from cultured keratinocytes following direct exposure for 2 hours to live PS3 or the corresponding cell free conditioned media from 24 hour cultures. CXCL8 secretion was quantified by ELISA from samples taken 8 hours after a 2 hour exposure to the bacteria or cell free conditioned media. Control treated cells (HBSS only) are indicated by the dashed line. Data are expressed as mean (+) standard deviation (n=2). A two way ANOVA with Tukey's post hoc showed significances as indicated on the graph (*p ≤0.05, **p ≤0.01, ***p ≤ 0.001).

4.2.2 CXCL8 stimulation from live PS3 and cell free conditioned media from 80 hour cultures

Direct exposure to PS3 from 80 hour cultures grown in MM EtOH and MM Glu produced a minimal stimulation of CXCL8, with secretion being similar to control (HBSS only) (figure 4.2). By contrast PS3 grown in SWF produced a 5 fold increase in CXCL8 secretion compared to both MM EtOH and MM Glu ($p \leq 0.001$) (figure 4.2)

The corresponding cell free conditioned media from MM EtOH and MM Glu grown for 80 hours both produced a greater stimulation of CXCL8 secretion compared to the direct exposure of the cells to PS3. For MM Glu this was around 10 fold greater and for MM EtOH around 7 fold. The CXCL8 secretion stimulated by cell free conditioned media from MM Glu was significantly greater than for MM EtOH ($p \leq 0.05$). CXCL8 secretion from SWF cell free conditioned media was not measured due to lack of viable cells as shown in figure 3.17.

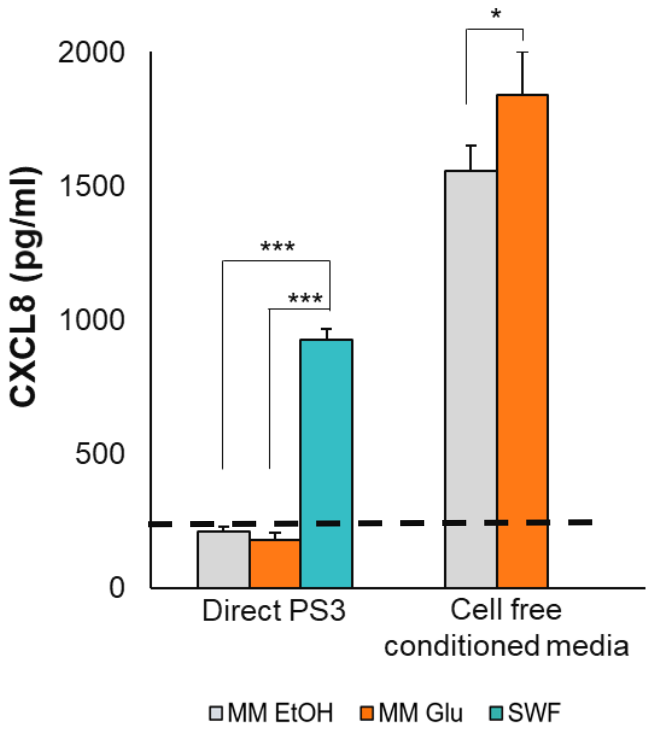

Figure 4.2 CXCL8 secretion from cultured keratinocytes following direct exposure to live PS3 or the corresponding cell free conditioned media from 80 hour cultures. CXCL8 secretion was quantified by ELISA 8 hours after a 2 hour exposure to bacteria or cell free conditioned media. Control treated cells (HBSS only) are indicated by the dashed line. Data are expressed as mean (+) standard deviation (n=2). A two way ANOVA with Tukey's post hoc showed significances as indicated on the graph (*p ≤0.05, ***p ≤ 0.001).

4.3 CXCL8 secretion from *Pseudomonas* cell free conditioned media only.

Once it was established that *Pseudomonas* cell free conditioned media stimulated CXCL8 secretion from keratinocytes more consistently than direct exposure to live *Pseudomonas* further experiments were conducted using cell free conditioned media only. Keratinocytes were exposed to cell free conditioned media diluted in complete DMEM from different culture conditions for a longer time period of 4 hours, the supernatant from the cells were then collected 24 hours after initial exposure and CXCL8 secretion was quantified by ELISA.

4.3.1 CXCL8 secretion from *Pseudomonas* cell free conditioned media from 24 hour cultures

Exposure to PS3 cell free conditioned media grown for 24 hours stimulated CXCL8 secretion in all of the conditions and dilutions tested (figure 4.3)

PS3 conditioned MM EtOH and MM Glu, resulted in the greatest CXCL8 secretion of around 2000pg/ml when keratinocytes were exposed to the 1:2 dilution, with both conditions being significantly higher than CXCL8 secretion from SWF conditioned media at the same dilution ($p \leq 0.0001$) (figure 4.3).

At the 1:5 dilution, the stimulation of CXCL8 secretion was around a 5 fold less compared to 1:2 in all conditions, however the secretion of CXCL8 was still significantly lower for SWF compared to MM EtOH ($p \leq 0.01$) and MM Glu ($p \leq 0.0001$).

At a 1:10 dilution there was a further decrease in CXCL8 secretion, however for MM EtOH and SWF conditioned media CXCL8 secretion was similar to a 1:5 dilution (figure 4.3). With similar significances occurring between MM EtOH and SWF ($p \leq 0.01$) and MM Glu and SWF ($p \leq 0.001$).

Cell free conditioned media from PF grown for 24 hours showed a small CXCL8 secretion compared to controls at a 1:2 dilution (figure 4.3). No significant differences were seen between conditions for PF conditioned media.

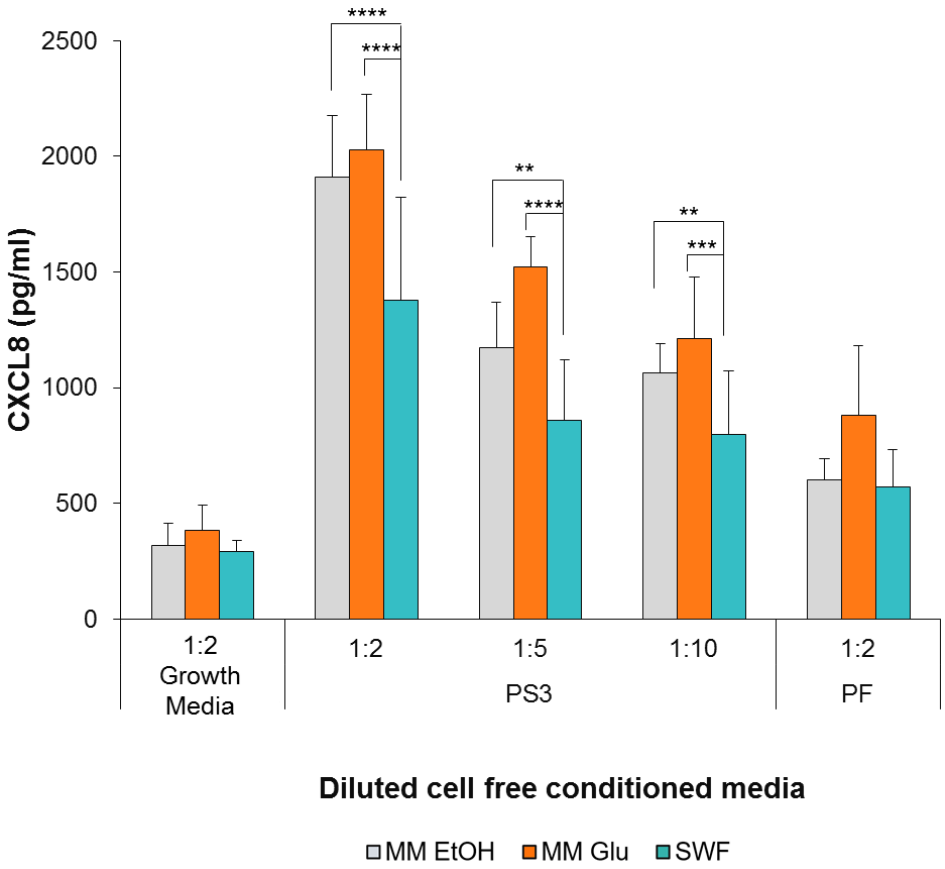

Figure 4.3 CXCL8 secretion from cultured keratinocytes exposed for 4 hours to diluted cell free conditioned media from *Pseudomonas* cultures grown for 24 hours. CXCL8 secretion was quantified from supernatants collected 24 hours after initial 4 hour exposure. Data are expressed as mean (+) SEM (n=3, 2). A two way ANOVA with Tukey's post hoc showed significances as indicated on the graph (**$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

4.3.2 CXCL8 secretion from keratinocytes *Pseudomonas* cell free conditioned media from 80 hour cultures.

Similar to cell free conditioned media from 24 hour cultures, PS3 cell free conditioned media grown for 80 hours stimulated CXCL8 secretion in a similar manner to the 24 hour cultures (figure 4.3 and 4.4). At the 1:2 dilution PS3 grown in MM Glu stimulated 1500 (pg/ml) CXCL8 secretion, this was significantly greater than MM EtOH ($p \leq 0.0001$) (figure 4.4) but this was because no CXCL8 secretion occured in response to the supernatant due to the lack of viable cells as shown in figure 3.11. At a 1:5 dilution, CXCL8 secretion from MM EtOH was 1613 (pg/ml), which was greater than MM Glu, however not significant (figure 4.4). At a 1:10 dilution, there was a slight decrease seen in CXCL8 secretion compared to a 1:5 dilution for both conditions with MM EtOH PS3 conditioned media producing around 1300 (pg/ml) CXCL8 secretion, which was significantly greater than MM Glu conditioned media (1000 (pg/ml) ($p \leq 0.05$). (figure 4.4)

Cell free conditioned media from PF conditioned cultures grown for 80 hours at the 1:2 dilution, produced similar results to the 24 hour cultures (figure 4.4). Again there was no significant differences between the different conditions.

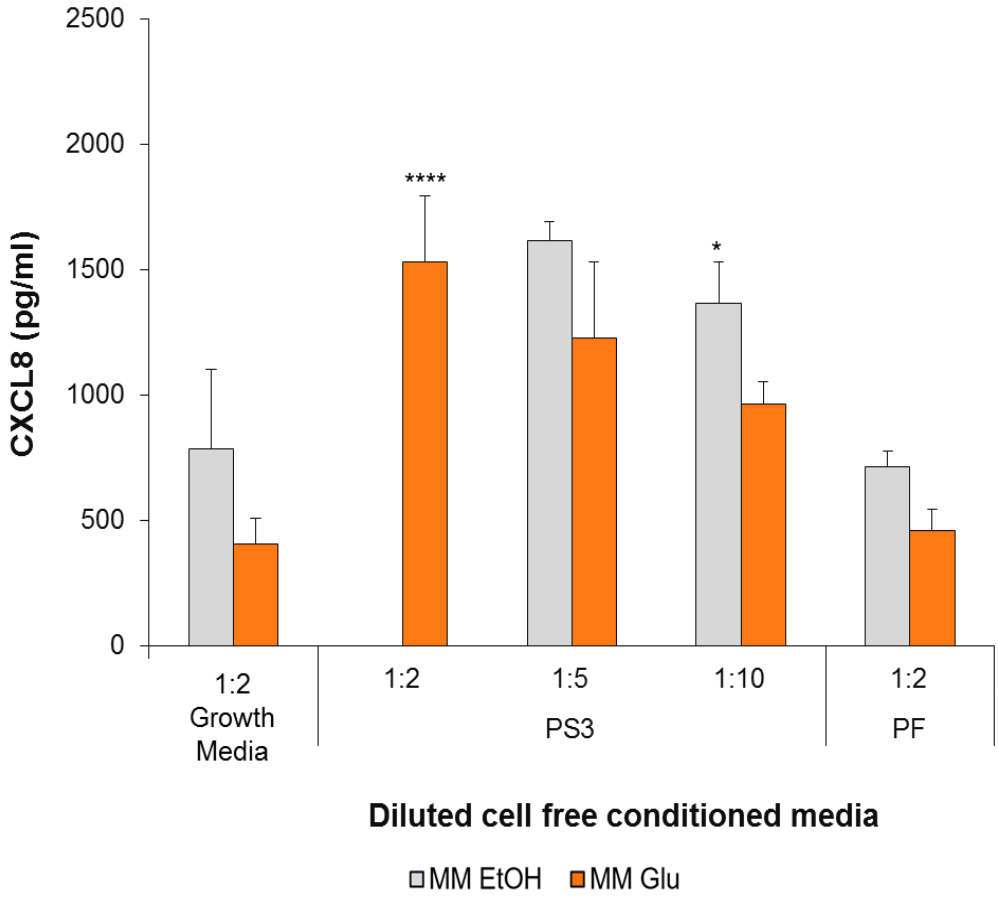

Figure 4.4 CXCL8 secretion from cultured keratinocytes exposed for 4 hours to diluted cell free conditioned media from *Pseudomonas* cultures grown for 80 hours. CXCL8 secretion was quantified from supernatants collected 24 hours after initial 4 hour exposure. Data are expressed as mean (+) SEM (n=3, 2). A two way ANOVA with Bonferroni post hoc showed significances as indicated on the graph (*p ≤0.05, ****p ≤0.0001).

4.3.3 PS3 grown in simulated wound fluid produced varied CXCL8 secretion from keratinocytes.

PS3 grown in SWF gave varied results on CXCL8 secretion from cultured keratinocytes. For the 1:2 dilution, 5624 pg/ml of CXCL8 was produced in 1 experiment on average but only 377 pg/ml in another (figure 4.5) For the third experiment CXCL8 was not measured due to lack of viable cells (figure 3.12).

At the 1:5 and 1:10 dilutions for PS3 conditioned media, there was a lower variability but the 1st experiment consistently produced a higher CXCL8 secretion than the second two, this lead to overall large variation within the data (figure 4.5). A similar variation also occurred in the MTS data (figure 3.12).

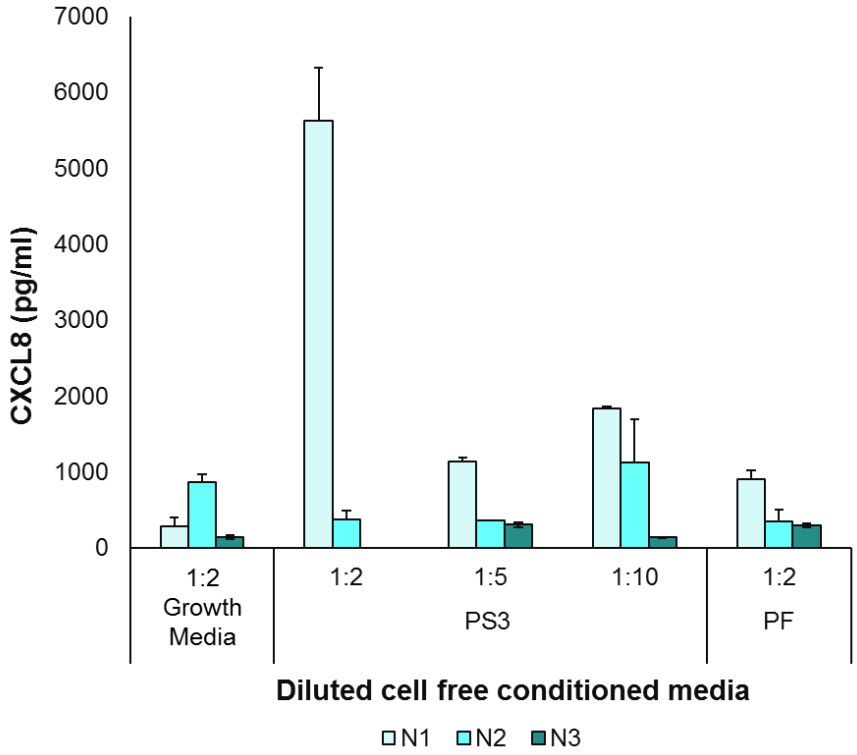

Figure 4.5 CXCL8 secretion from cultured keratinocytes exposed for 4 hours to diluted cell free conditioned media from *Pseudomonas* cultures grown for 80 hour in SWF. CXCL8 secretion was quantified from supernatants collected 24 hours after initial 4 hour exposure. Data are expressed as mean (+) standard deviation of 2 technical replicates. The different bars represent individual experiments.

4.3.4 Comparison of the effect of bacterial time in culture on CXCL8 secretion from cultured keratinocytes exposed to supernatants from ethanol or glucose grown PS3.

To directly compare the effects of the cell free conditioned media from 24 and 80 hour cultures on CXCL8 secretion, ELISA data from 24 and 80 hour cultures of PS3 grown in MM EtOH or MM Glu were plotted together (figure 4.6).

Due to lack of cell viability in keratinocytes treated with a 1:2 dilution of PS3 grown in MM EtOH for 80 hours as indicated by the MTS data (figure 3.11), there was no CXCL8 secretion (figure 4.6). For a 1:2 dilution of MM Glu 24 and 80 hour cultures there was no significant difference in CXCL8 secretion.

At a 1:5 dilution, supernatants from MM EtOH cultures grown for 80 hours produced a greater CXCL8 secretion compared to 24 hour cultures (figure 4.6) By contrast, cell free conditioned media from MM Glu cultures stimulated more CXLC8 secretion from 24 hours compared to 80 hour cultures. At a 1:5 dilution the differences in CXCL8 secretion within MM EtOH and MM Glu grown cultures was not significant (figure 4.6).

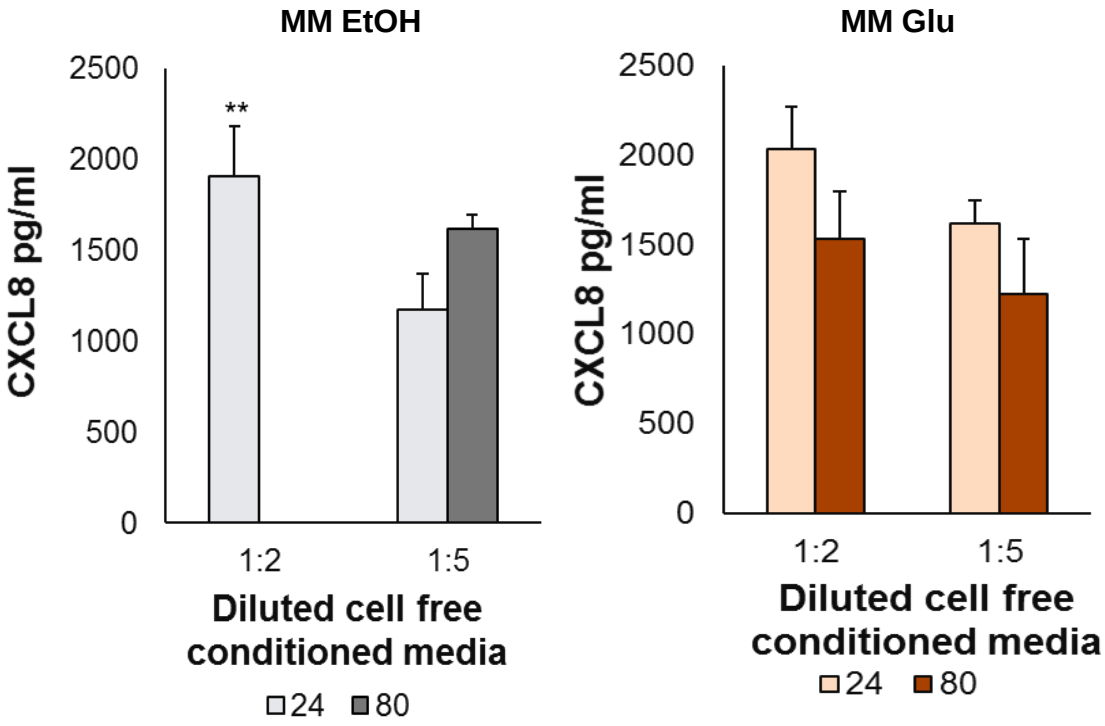

Figure 4.6 CXCL8 secretion from keratinocytes challenged with 24 and 80 hours PS3 cell free conditioned media from cultures grown in MM EtOH or MM Glu. The keratinocytes were exposed for 4 hours and CXCL8 secretion was quantified 24 hours after initial 4 hour exposure. Data is expressed as mean (+) SEM (n=3, 2). A T-test showed significances between groups as indicated on the graph (** $p < 0.01$).

4.4 Expression of MAMP receptors from cultured keratinocytes

To investigate immune responses initiated by PS3 further flow cytometry was used to quantify the expression of the MAMP receptors TLR 2 and 4 and the associated co-receptor CD14 in response to cell free conditioned media from PS3 cultures grown in the different growth conditions for 24 and 80 hours.

Keratinocytes were seeded in 24 well plates and exposed for 4 hours to diluted cell free conditioned media from 24 hour cultures at a 1:2 dilution or for 80 hour cultures a 1:5 dilution was used, both were diluted in complete cell culture media. Non-conditioned growth media (diluted 1:2 in DMEM) was used as control.

4.4.1 Expression of MAMP receptors from cultured keratinocytes following exposure to PS3 cell free conditioned media grown for 24 and 80 hours.

All the MAMP receptors tested were expressed by cultured keratinocytes in control conditions with the exception of TLR 4 that had minimal expression when exposed to diluted non-conditioned SWF (figure 4.7).

For MM EtOH, there was little differences between control cells and cell free conditioned media from 24 hour cultures, with cell counts and levels of fluorescence being very similar (figure 4.7), media from 80 hour cultures produced similar counts and fluorescence for CD-14, but showed lower counts for TLR 2 and 4 compared to both control cell exposed to 24 hour conditioned media (figure 4.7).

For MM Glu, differences were observed between the control and 24 hour cell free conditioned media for TLR 2 and 4 with an increase in fluorescence for TLR 2 and increase in the number of fluorescent cells for TLR 4. A very small increase in the expression of CD-14 also occurred (figure 4.7) In contrast, 80 hour MM Glu cultures produced a lower expression of TLR 2, TLR 4 and CD-14 expression compared to cells treated with media from 24 hour cultures, with both cell counts and fluorescence levels being similar to that of the control (figure 4.7).

For SWF cultures, an increase in expression of both TLR 2 and 4 and CD14 occured, with the amount of fluorescence and the number of fluorescent cells being increased compared to control (figure 4.7). For 24 and 80 hour cultures the expression of TLR 2 and 4 was increased compared to control, however the number of fluorescent cells was only higher following with treatment with 24 hour cell free SWF media (figure 4.7). For CD-14 expression, 80 hour cell free conditioned media treated cells showed similar level of expression to controls (figure 4.7).

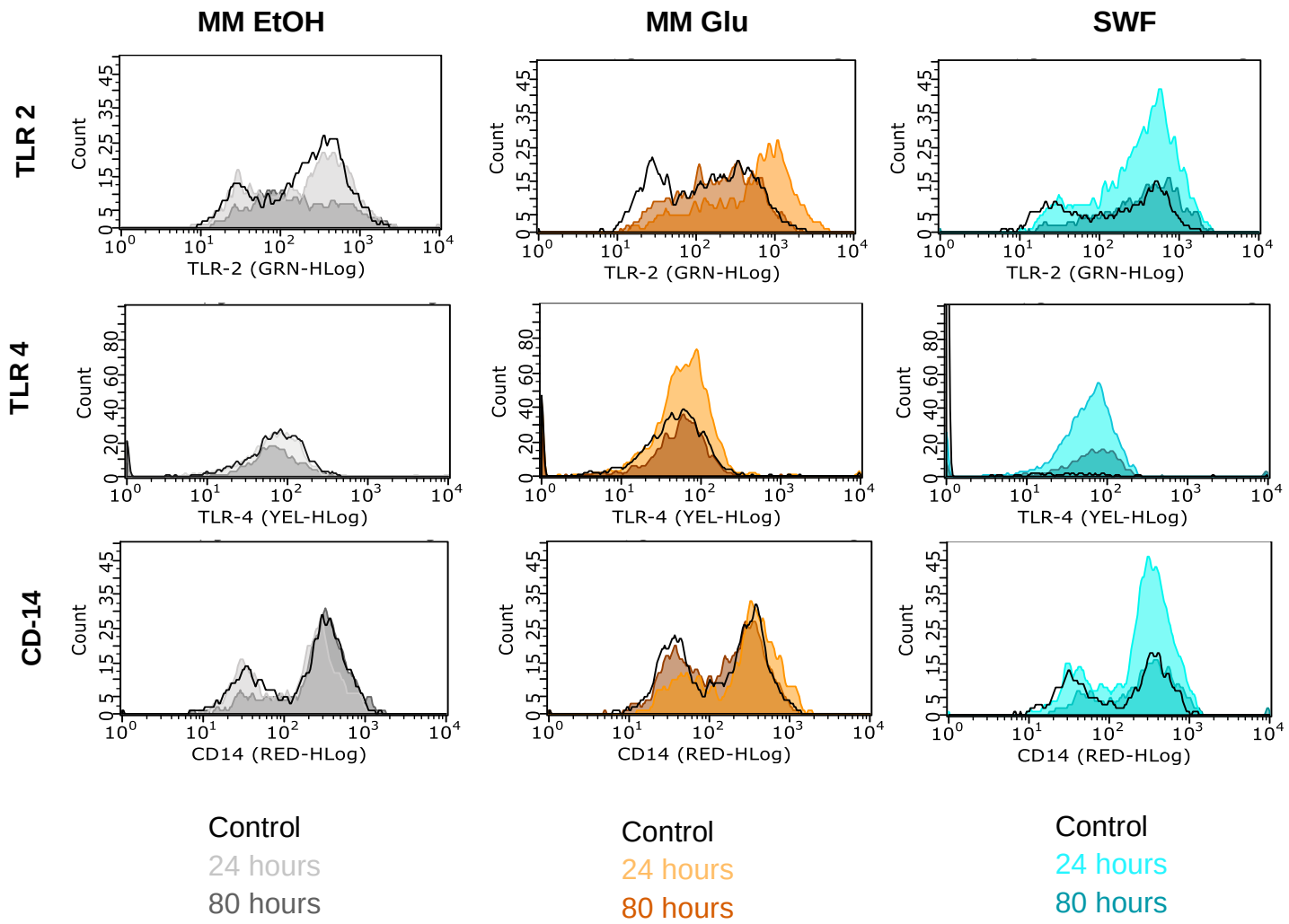

Figure 4.7 Expression of toll like receptors 2, 4 and CD-14 in cultured keratinocytes following exposure to PS3 cell free conditioned media. Keratinocytes were exposed for 4 hours to diluted cell free conditioned media grown for 24 or 80 hours from each of the different growth conditions as indicated. The keratinocytes were then lifted, stained and analysed by flow cytometry.

In order to directly compare the effects of bacteria grown in different media on receptor expression, the data was replotted so the effects of bacteria grown for the same time in different media could be more easily compared (figure 4.8). The pattern of expression was similar for both TLR's and CD-14 for both 24 and 80 cultures (figure 4.8).

For 24 hour cultures, cell free media from both MM Glu and SWF increased the level of fluorescence for both TLR's and CD-14 compared to MM EtOH (figure 4.8). SWF treated cells produced an increased number of fluorescent cells for TLR 2 and CD-14 compared to MM Glu and MM EtOH treatments whereas for TLR 4 MM Glu treated cells showed a small increase compared to SWF and around a 4 fold increase compared to MM EtOH (figure 4.8).

For 80 hour cultures, cell free conditioned media from MM Glu, produced a small increase in the number of fluorescent cells for TLR 2 and 4 compared to MM EtOH and SWF, however the total fluorescence was the same for each condition (figure 4.8). For CD-14 expression, MM Glu and MM EtOH conditioned media produced a similar level of expression with the number of fluorescing cells being around 2 fold higher than SWF treated cells (figure 4.8).

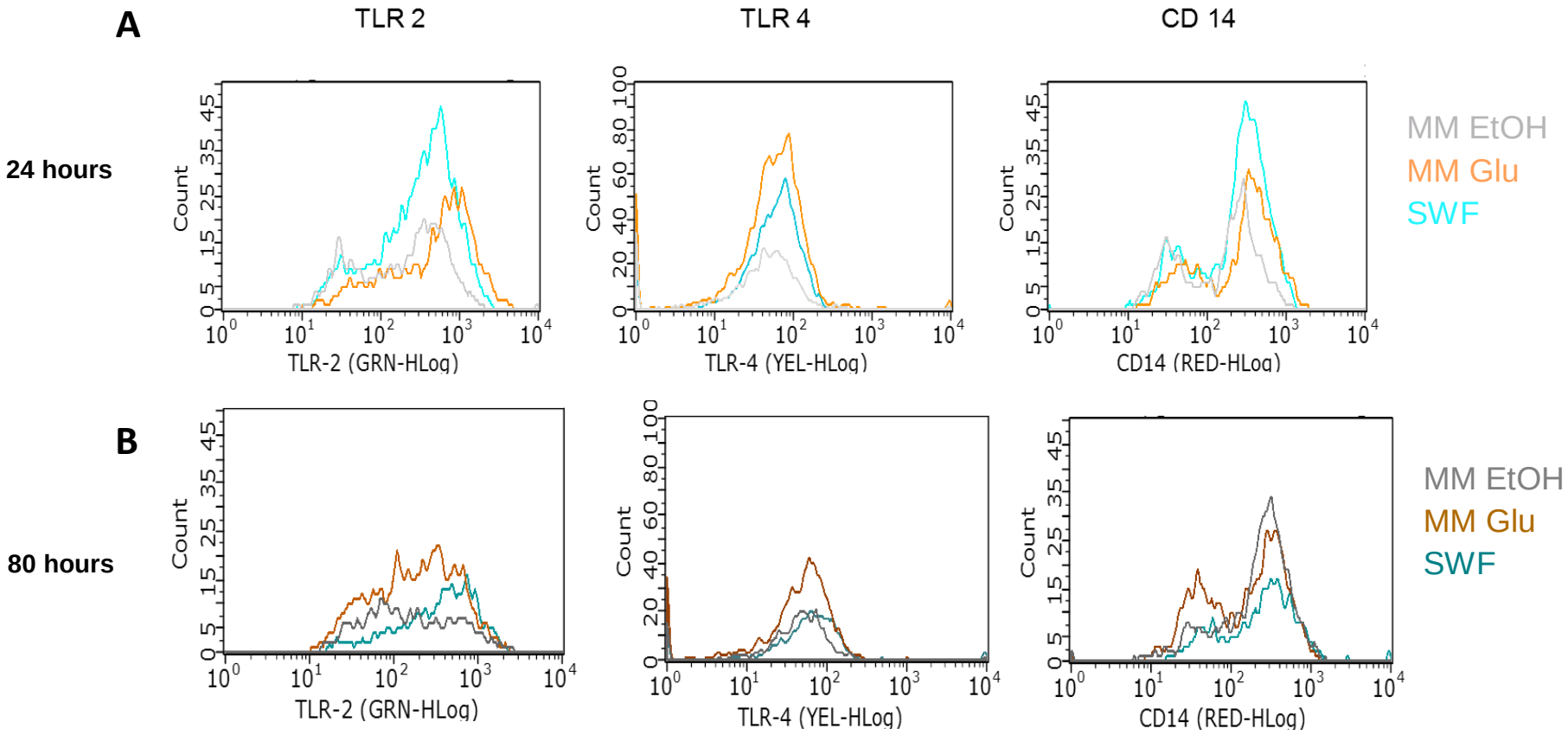

Figure 4.8 Expression of toll like receptors 2, 4 and CD-14 in cultured keratinocytes following exposure to PS3 cell free conditioned media grown for 24 hours (A) or 80 hours (B) for each of the different growth conditions. Keratinocytes were exposed to the cell free conditioned media from 24 or 80 hour cultures for 4 hours before being lifted, stained and analysed by flow cytometry.

4.5 CXCL8 secretion from ex-vivo skin models.

Although informative, simple monolayers of HaCaT cells is a very reductive model of skin physiology. For this reason it was investigated whether cells would act in a similar manner when assembled onto a dermal scaffold, which produce a more representative 3D model of human skin. Models were constructed and incubated for 2 weeks to allow development of stratified layers before being challenged with *Pseudomonas* cell free conditioned media. Similar to the experiments on monolayers, the skin models were exposed to cell free conditioned media for 4 hours and samples were collected 24 hours after initial exposure and CXCL8 was quantified by ELISA.

4.5.1 CXCL8 stimulation from skin models challenged with *Pseudomonas fluorescens* grown for 80 hours.

Cell free conditioned media from PF grown for 80 hours was used as a control and investigated in initial experiments. Models challenged with diluted (1:2) cell free conditioned media from PF 80 hour cultures produced little CXCL8 secretion of 584pg/ml for ethanol grown PF and 163 pg/ml for glucose grown PF, with levels of CXCL8 secretion being similar to control (non-conditioned diluted media) (figure 4.9). Significant differences were observed between MM EtOH and MM Glu in both control and conditioned media treated cells ($p \leq 0.05$) (figure 4.9).

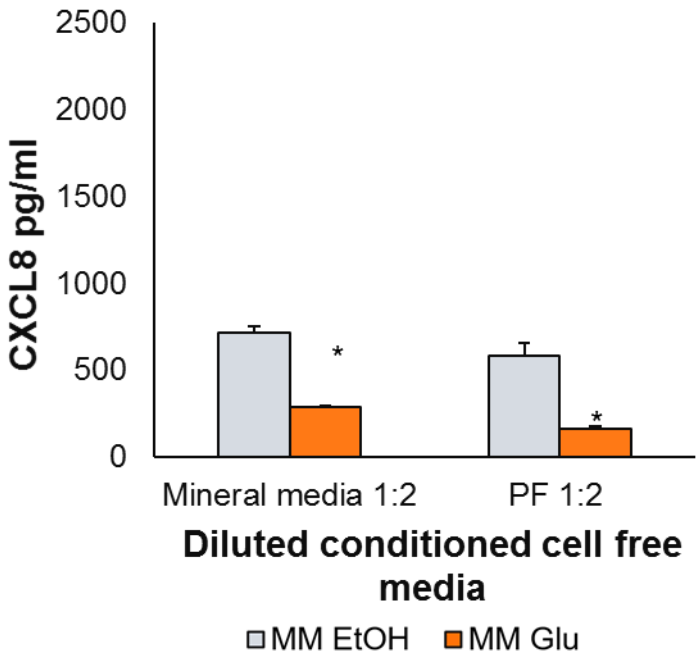

Figure 4.9 CXCL8 secretion from ex vivo skin models following exposure for 4 hours to *Pseudomonas fluorescens* cell free conditioned media. Ex vivo skin models were exposed to diluted cell free conditioned media from PF cultures grown for 80 hours. CXCL8 secretion was quantified from supernatants collected 24 hours after initial 4 hour exposure**.** Data are expressed as mean (+) standard deviation of 1 experiment (n=2). A T-test showed significances between groups as indicated on the graph (* $p \leq 0.05$).

4.5.2 CXCL8 secretion from ex vivo skin models challenged with PS3 grown for 24 and 80 hours.

Once effects of laboratory reference strain PF had been established and minimal CXCL8 secretion was observed further experiments were conducted with PS3 grown for 24 and 80 hours.

Exposure to PS3 grown for 24 and 80 hours showed increased CXCL8 secretion, with both 24 and 80 hour cell free conditioned media producing a 2 fold increase to control (figure 4.10). CXCL8 secretion data is similar to that for the cells grown as a monolayer for PS3 24 hour conditioned media at a 1:2 dilution (figure 4.4 and figure 4.10), in addition PS3 grown in MM EtOH for 80 hours also stimulated CXCL8 secretion. No significant differences were observed between conditions (figure 4.10) or within conditions (data not shown).

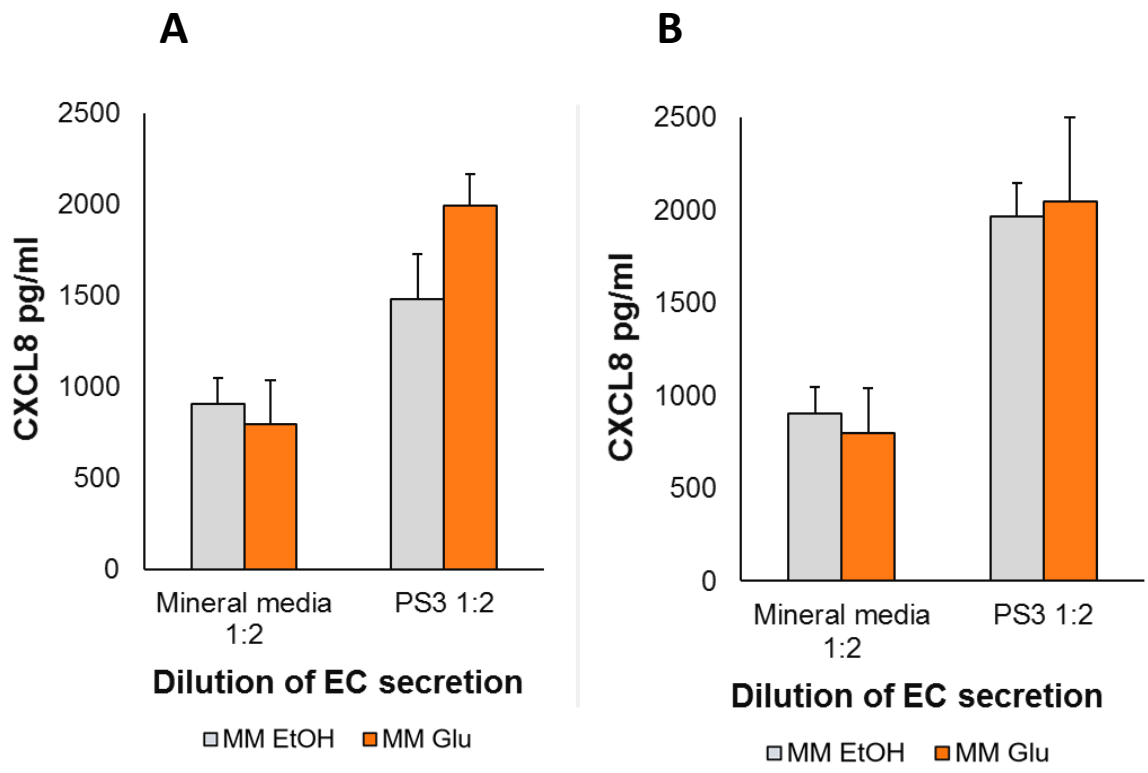

Figure 4.10 CXCL8 secretion from ex vivo skin models following exposure to PS3 cell free conditioned media. Ex vivo skin models were exposed to cell free conditioned media diluted 1:2 in complete DMEM from PS3 cultures grown for 24 hours (A) or 80 hours (B). CXCL8 secretion was quantified from supernatants collected 24 hours after initial 4 hour exposure**.** Data are expressed as mean (+/-) standard deviation of 1 experiment (n=2).

4.6 Summary and key findings

The results in this chapter show that PS3 can illicit CXCL8 secretion from cultured keratinocytes, and that cell free conditioned media containing secreted factors can generally produce a greater secretion compared to exposure of live bacteria. Keratinocytes exposed to cell free conditioned media from PS3 grown for 24 hours in MM EtOH and MM Glu showed greater CXCL8 secretion compared to those exposed to SWF. For 80 hour cultures, cells exposed to MM EtOH produced greater CXCL8 secretion compared to MM Glu, with the exception of the 1:2 dilution, which was not measured due to lack of viable cells. SWF treated cells showed varied results in CXCL8 secretion with the biggest variation seen at the 1:2 dilution, however viability data shown in the previous chapter also showed large variation. Following direct comparison of 24 and 80 hour cultures, looking at the 1:5 dilution, within the different growth conditions it showed PS3 grown in MM EtOH for 80 hours could produce greater CXCL8 secretion, however for PS3 grown in MM Glu, 24 hour conditioned media produced the greater a greater CXCL8 secretion. From the flow cytometry data investigating the expression of selected MAMP receptors, cell free conditioned media from PS3 grown for 24 or 80 hours had no effect on the change in expression in any of the MAMP receptors, with expression levels being similar to control. For MM Glu and SWF, following exposure to cell free conditioned media from PS3 grown for 24 hours, there was an increase in expression and/or number of cells emitting the signal for all 3 MAMP receptors. For 80 hour cultures cell free conditioned media from MM Glu cultures showed no differences compared to control, however for SWF 80 hour cultures, cell free conditioned media showed increases in expression for TLR2 and increase in cells omitting a signal for TLR4.

For the ex-vivo skin model experiments, CXCL8 secretion was increased following exposure to PS3 compared to non-conditioned media and PF cell free conditioned media however, no significant differences were observed between growth conditions.

Chapter 5
Investigation of cellular models of wound healing.

5.1 Introduction

Wound healing is a conserved, intricate process by which the skin repairs itself after injury. There are many in vitro models that are currently employed to study wound healing including single cell monolayer models and ex-vivo models (Sami et al., 2019).

Single cell monolayers provide a quick and cost effective way to investigate wound healing, with one of the most common assays being the scratch assay which provides an easy and low cost effective method to mimic cell proliferation and migration in response to various agents (Liang et al., 2007). Monolayers of cells are 'scratched' to create an artificial gap and are then monitored over a period of time to investigate how cells proliferate and migrate to achieve closure thus returning to the original monolayer state (Chen, 2012). Keratinocytes and skin fibroblast cells make suitable cell types for the scratch assay due to their ability to collectively migrate in a process known as 'sheet migration' (Grada et al., 2017).

Once cell proliferation and migration have been established within the scratch assay model, other more complex skin models can be employed such as three dimensional ex-vivo skin models, which have been in development since 1981 (Bell et al., 1981). These can provide a more relevant physiological model of human skin, and potentially provide a more realistic approach to studying tissue repair (MacNeil et al., 2011). Similar to the scratch assay, models are artificially damaged to provide an area to allow cell migration and proliferation which can then be measured often via histology staining.

As mentioned in previous chapters, *Pseudomonas aeruginosa* is a persistent coloniser of wounds where colonisation can lead to impaired healing (Goldufsky et al., 2015). In addition to this there is debate about the role of microorganisms in wounds and

whether they can be used as a tool to pre-determine infectivity and healing (Bowler et al., 2001). Therefore it is important to understand the cellular mechanisms in response to *Pseudomonas* in order to address the management and treatment of chronic wounds.

5.2 Consistency check of scratch methodology used for cultured keratinocytes

Initially experiments were performed to determine that scratches of similar sizes could be made consistently. A total of 30 scratches were made using an aspirator tip and were photographed at time 0, the total area of the scratches was measured using ImageJ software. The median area of the scratches was 10.6 x 10^5, with an interquartile range of 22 x 10^4 (figure 5.1).

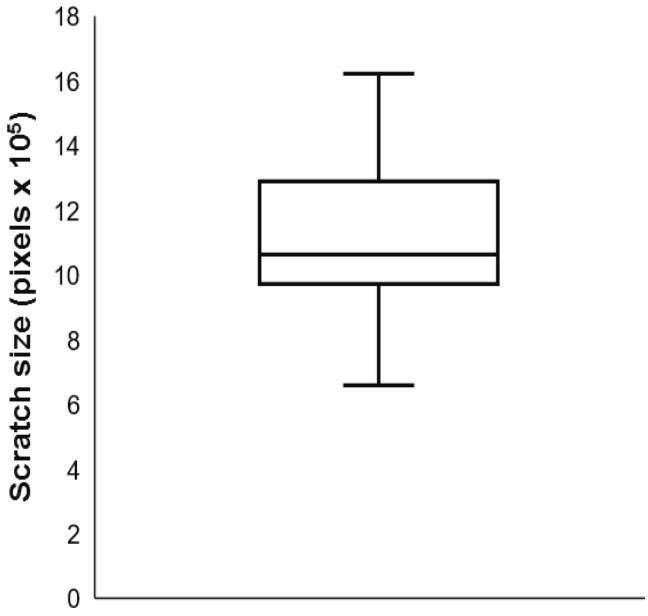

Figure 5.1 Scratch sizes in cultured keratinocytes. Initial sizes, in pixels, of 30 scratches made in cultured keratinocytes using an aspirator tip.

5.3 Determination of scratch closure from cultured keratinocytes exposed to growth media only

In order to establish the effects of growth media, cells were scratched and challenged with diluted growth media (1:2 in complete cell culture media) for 4 or 6 hours. Wells were photographed every 24 hours for up to 5 days and the size of the scratches was measured using ImageJ software. There was some variation in the amount of closure in some conditions at day 1 following a 4 hour exposure times with scratches exposed to SWF showing the slowest initial closure of 17% which was significantly less than MM Glu ($p \leq 0.05$) and significantly less than that of than that of MM EtOH media and control ($p \leq 0.0001$) (figure 5.2). In addition to this MM Glu treated cells were also produced significantly lower closure compared to MM EtOH treated cells and control ($p \leq 0.001$) At day 2, scratch closure still remained significantly slower for SWF and MM Glu treated cells compared to control ($p \leq 0.01$, $p \leq 0.0001$), however at day 3 SWF and MM Glu treated cells showed closure to be the same as MM EtOH and control with all conditions reaching almost full scratch closure (figure 5.3). Similar patterns were observed following a 6 hour exposure, where SWF and MM Glu treated cells showed significantly less closure than that of the control ($p \leq 0.01$) ($p \leq 0.0001$) at day 1, at day 2 however SWF treated cells showed closure to be similar to that of MM EtOH treated cells and control cells, but MM Glu still remained significantly slower compared to the other conditions and control ($p \leq 0.05$, $p \leq 0.01$). At day 3 each condition, showed similar closure to control, with each condition producing almost 100% closure.

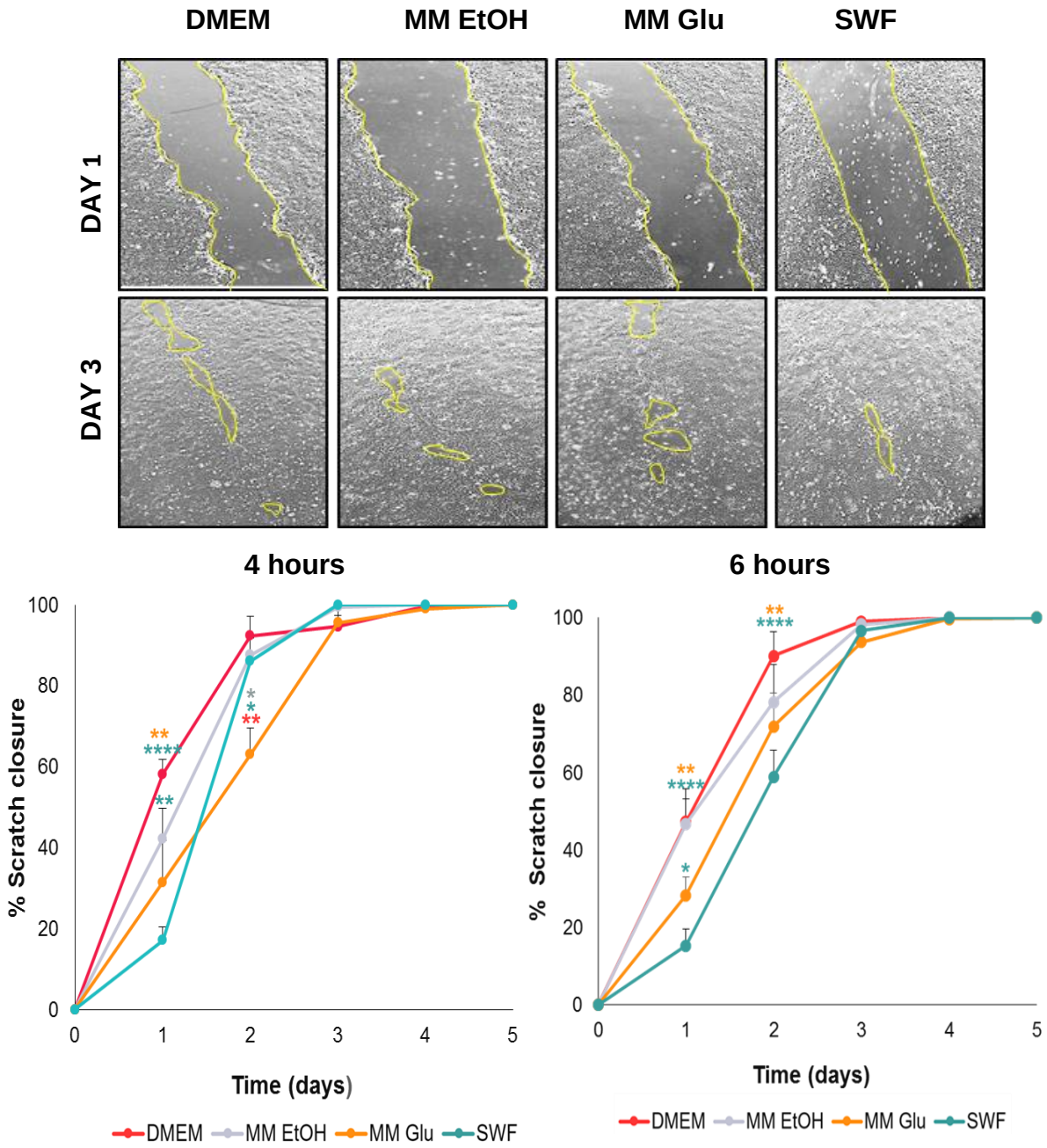

Figure 5.2 Representative scratch assay images and corresponding mean percentage scratch closure from cultured keratinocytes exposed to diluted growth media. Keratinocyte monolayers were scratched and exposed to non-conditioned growth media diluted 1:2 in complete cell culture media for 4 or 6 hours, controls (cell culture media only) are indicated by the red line. Data is mean (+) SEM of 3 independent experiments (n=2). A two way ANOVA with Tukey's post hoc showed significances between groups graphs * p≤0.05, ** p ≤0.01, **** p≤ 0.0001), colour of stars indicates the treatment where significance lies. Keratinocyte images are representative of 4 hour exposure only.

5.4 Effect of *Pseudomonas* cell free conditioned media on keratinocyte scratch assays.

Once effects of the different bacterial media on the scratch assays had been the determined the effects of exposure to *Pseudomonas* cell free conditioned media for grown in different conditions was investigated. The same method was used for the experiments with the non-conditioned media described above with both PS3 and PF conditioned media with a 4 or 6 hour exposure time.

5.4.1 Determination of scratch healing after exposure to *Pseudomonas* cell free conditioned media from 24 hour cultures.

Cell free conditioned media from *Pseudomonas* cultures grown for 24 hours in any of conditions did not have a significant effect on scratch closure from any of the conditions.

Scratches exposed to PS3 cell free conditioned media for 4 hours, showed scratch closure to be the same as control with all conditions showing over 50% reduction in scratch size by day 2 (figure 5.3) and almost all conditions showing full scratch closure by day 4, with SWF grown PS3 taking slightly longer to fully close, occurring in 5 days (figure 5.3). No significant differences were observed between any conditions at any of the time points for PS3 cell free conditioned media. For the longer exposure time of 6 hours to PS3 cell free conditioned media showed a slower rate of scratch closure with scratches only reaching 50% closure at day 2 for each condition. At day 4 full scratch closure had only occurred for keratinocytes exposed to PS3 grown in MM Glu, and after 5 days, full scratch closure had not occurred for PS3 cell free conditioned media from MM EtOH or SWF cultures (figure 5.3).

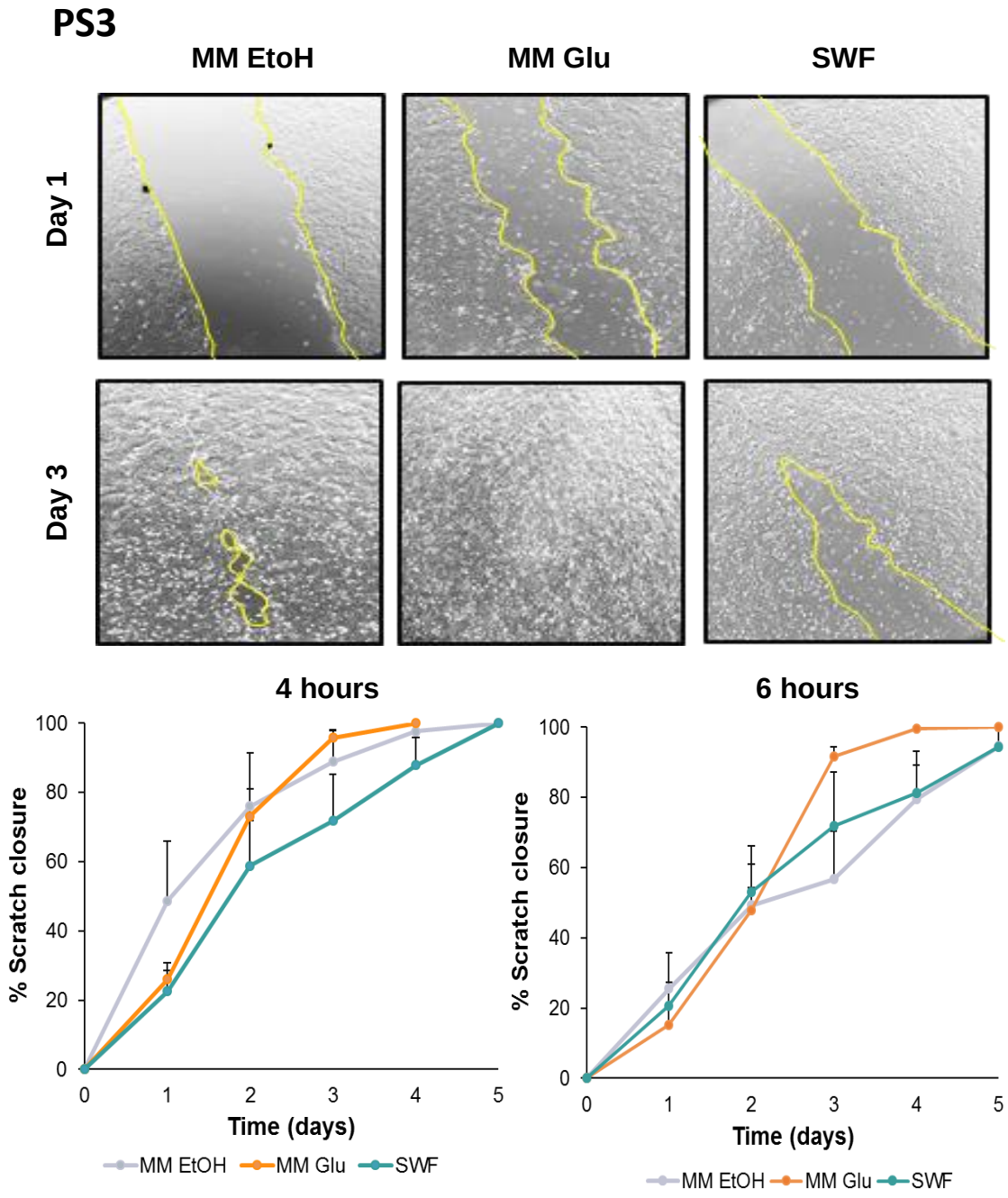

Figure 5.3 Representative images of scratches and corresponding mean scratch closure over time from cultured keratinocytes exposed to PS3 grown for 24 hours. Scratches were exposed to cell free conditioned media from PS3 grown for 24 hours and diluted 1:2 in complete cell culture media for 4 or 6 hours. Scratches were photographed and closure determined every 24 hours over 5 days. Data is mean (+) SEM of 3 independent experiments (n=2). A two way ANOVA showed no significant differences between groups. Keratinocyte images are representative of 4 hour exposure only.

For scratches exposed to PF cell free conditioned media for 4 hours, MM EtOH and SWF showed very similar scratch closure throughout each time point reaching full closure at day 3 (figure 5.4). For both these conditions scratch closure was significantly greater than that for MM Glu at days 1 ($p \leq 0.001$, $p \leq 0.01$), days 2 and 3 ($p \leq 0.05$), by day 4 there was no difference as scratch closure was almost complete for keratinocytes exposed to MM Glu (figure 5.4).

In comparison 6 hour exposure times showed an increase in scratch closure for SWF conditioned media with scratches reaching full closure after 2 days (figure 5.4). This was significantly quicker than that for MM Glu and MM EtOH conditioned media at both days 1 and 2 ($p \leq 0.0001$). For the 6 hour exposure to MM Glu conditioned media, scratch closure occurred at the same rate as after a 4 hour exposure (figure 5.4). For cells treated with MM EtOH cell free conditioned media scratch closure was slower for cells exposed for 6 hours, taking an extra 24 hours to achieve full healing compared to a 4 hour exposure. Full scratch closure following a 6 hour treatment with either MM EtOH or MM Glu occurred after 4 days (figure 5.4).

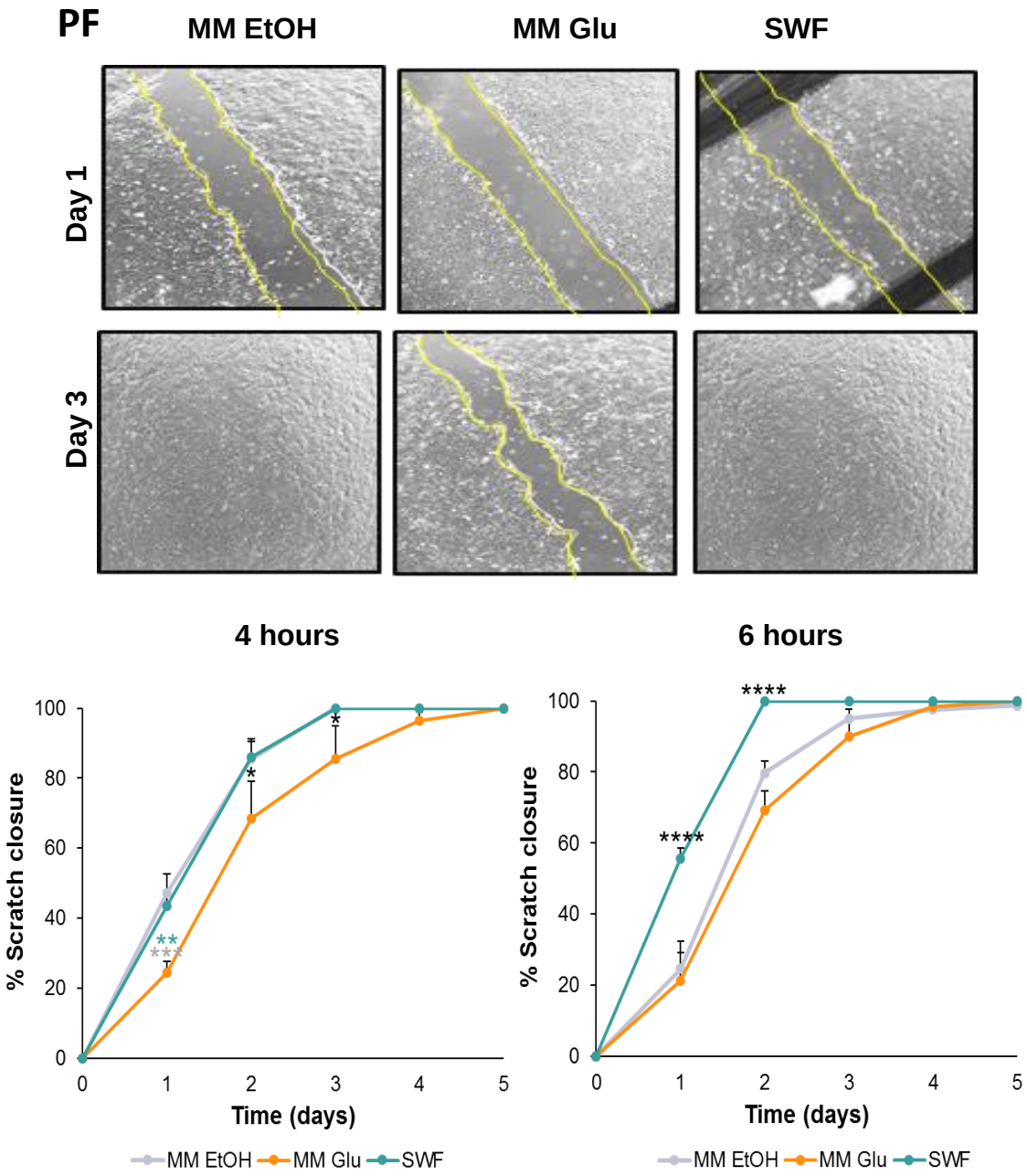

Figure 5.4 Representative images of scratches and corresponding scratch closure over time from cultured keratinocytes exposed to PF grown for 24 hours. Scratches were exposed to cell free conditioned media from PF grown for 24 hours and diluted 1:2 in complete DMEM for 4 and 6 hours. Scratches were photographed and closure determined every 24 hours up to 5 days. A two way ANOVA with Tukey's post hoc showed significance between groups (*p ≤0.05, ** ≤0.0001), colour of stars indicates the treatment where significance lies, black stars show significance to both the other treatments. Keratinocyte images are representative of 4 hour exposure only.

5.4.1.1 PF grown in SWF for 24 hours showed accelerated closure to above control.

Once scratch closure from keratinocytes exposed to 24 hour conditioned media had been determined, data were replotted together to investigate whether any significant differences in scratch closure were seen between conditioned media and the corresponding control of non-conditioned media. Cell free conditioned media from PF cultures grown in SWF for 24 hours was the only condition to show significantly greater closure compared to non-conditioned media which was observed following both a 4 and 6 hour exposure time (figure 5.5). Following a 4 and 6 hour exposure conditioned media produced significantly greater closure at day 1 compared to control ($p \leq 0.0001$), with conditioned media producing around a 2 fold increase following 4 hour exposure and a 3 fold increase following a 6 hour exposure (figure 5.5). At day 2, following a 4 hour exposure, again conditioned media produced significantly greater closure compared to control, showing 20% greater closure ($p \leq 0.0001$) (figure 5.5). Following a 6 hour exposure at day 2, conditioned media showed slightly greater closure reaching 100% however this was not significant compared to cells treated with non-conditioned media (figure 5.5).

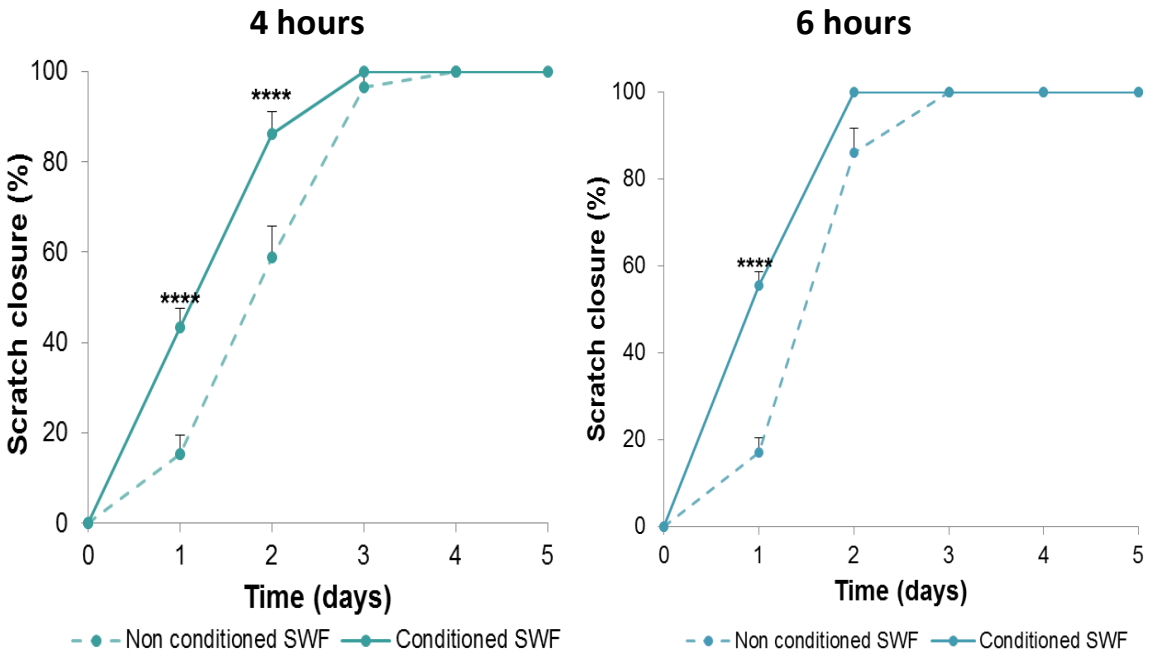

Figure 5.5 Mean scratch closure from keratinocytes exposed to conditioned media from PF cultures grown in SWF for 24 hours compared to non-conditioned SWF. Scratches were exposed to conditioned or non-conditioned SWF diluted 1:2 in complete cell culture media for 4 or 6 hours scratches were photographed and closure determined every 24 hours over 5 days. A two way ANOVA with Bonferroni post hoc showed significant differences between groups (**** ≤0.0001).

5.4.2 Determination of scratch healing from treatment with conditioned media taken from *Pseudomonas* 80 hour cultures

For SWF and MM Glu cultured grown for 80 hours the effect of a 4 hour exposure to the cell free media on the rate of scratch healing was similar to that for 24 hour cultures with around 50% closure by day 2 (figure 5.3 and figure 5.6) Scratches exposed to MM Glu showed further steady scratch closure reaching over 80% closure by day 3, which was significantly greater than SWF ($p < 0.05$) which showed slower closure over days 3-5, with full scratch closure not occurring after 5 days (figure 5.6).

Scratches exposed for 4 hours to PS3 grown in MM EtOH for 80 hour showed significantly slower closure compared to MM Glu and SWF (figure 5.6). The Initial scratch closure was slow with only 20% scratch closure achieved by day 2 which was significantly less than for the other two conditions ($p \leq 0.0001$ to SWF, $p \leq 0.001$ to MM Glu) (figure 5.6). By day 4 scratch closure was only 50%, again significantly less than the other two conditions. ($p \leq 0.01$ to SWF, $p \leq 0.0001$ to MM Glu). By day 5 full closure had not occurred, with closure not being above 80% (figure 5.6).

Following a 6 hour exposure to MM Glu initial scratch closure was similar to that for a 4 hour exposure (figure 5.6). For SWF there was a slightly faster scratch closure reaching 60% by day 2, however, similar to 4 hour exposure, closure slowed between days 3-5, but full scratch closure occurred by day 5 (figure 5.6). For scratches exposed to PS3 grown in MM EtOH, scratch closure was slowed further compared to 4 hour exposures, with significant differences seen between MM EtOH and MM Glu and SWF at all time points (figure 5.6) ($p \leq 0.0001$). There was actually a small increase in scratch size at day 1 leading to no overall scratch closure at day 2 and little closure occurred for all time points, with less than 50% closure occurring by day 5 (figure 5.6).

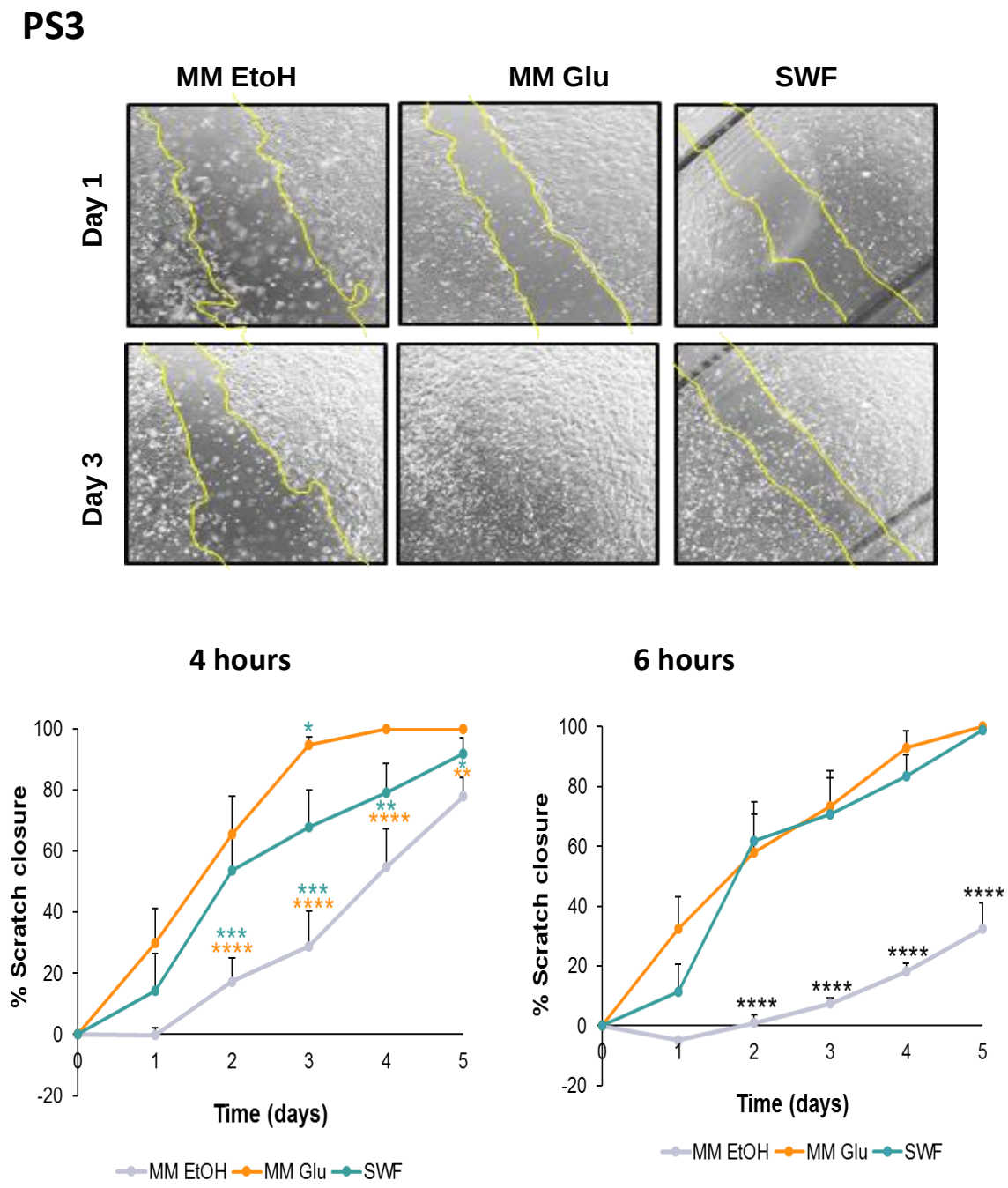

Figure 5.6 Representative images of scratches and corresponding mean scratch closure over time from cultured keratinocytes exposed to PS3 grown for 80 hours. Scratches were exposed to cell free conditioned media from PS3 grown for 80 hours and diluted 1:2 in complete cell culture media for 4 and 6 hours, scratches were photographed and closure determined every 24 hours over 5 days. A two way ANOVA with Tukey's post hoc showed significant differences between groups (*p ≤0.05, ** p ≤0.01, ***p ≤0.001 **** ≤0.0001), colour of stars indicates the condition where significance lies, black stars show significance to both of the other conditions. Keratinocyte images are representative of 4 hour exposure only.

PF cell free conditioned media had relatively little effect on scratch healing. After a 4 hours exposure to MM Glu or MM EtOH there was similar scratch closure reaching around 80% after 2 days with MM Glu fully closing by day 3. For MM EtOH, closure was slower after day 2 with full scratch closure not occurring until day 5 (figure 5.7). For both conditions the initial scratch closure was significantly faster than that for SWF, with the amount of closure being significantly less at days 1 ($p \leq 0.001$, $p \leq 0.05$) 2 ($p \leq 0.01$, $p \leq 0.0001$) and 3 ($p \leq 0.05$, $p \leq 0.01$), however by day 5 full scratch closure had occurred (figure 5.7).

For 6 hour exposure times, MM Glu produced a similar scratch closure to that of 4 hour exposure, with full closure occurring after 3 days (figure 5.7). For MM EtOH and SWF scratch closure was slower than that for 4 hour exposure with SWF being significantly less than MM Glu at day 1 ($p \leq 0.01$) and less than both MM Glu and MM EtOH ($p \leq 0.01$) at day 2 (figure 5.7). At day 3 MM EtOH and SWF showed similar closure (around 60%), and both were more than 90% closed by day 5 (figure 5.7).

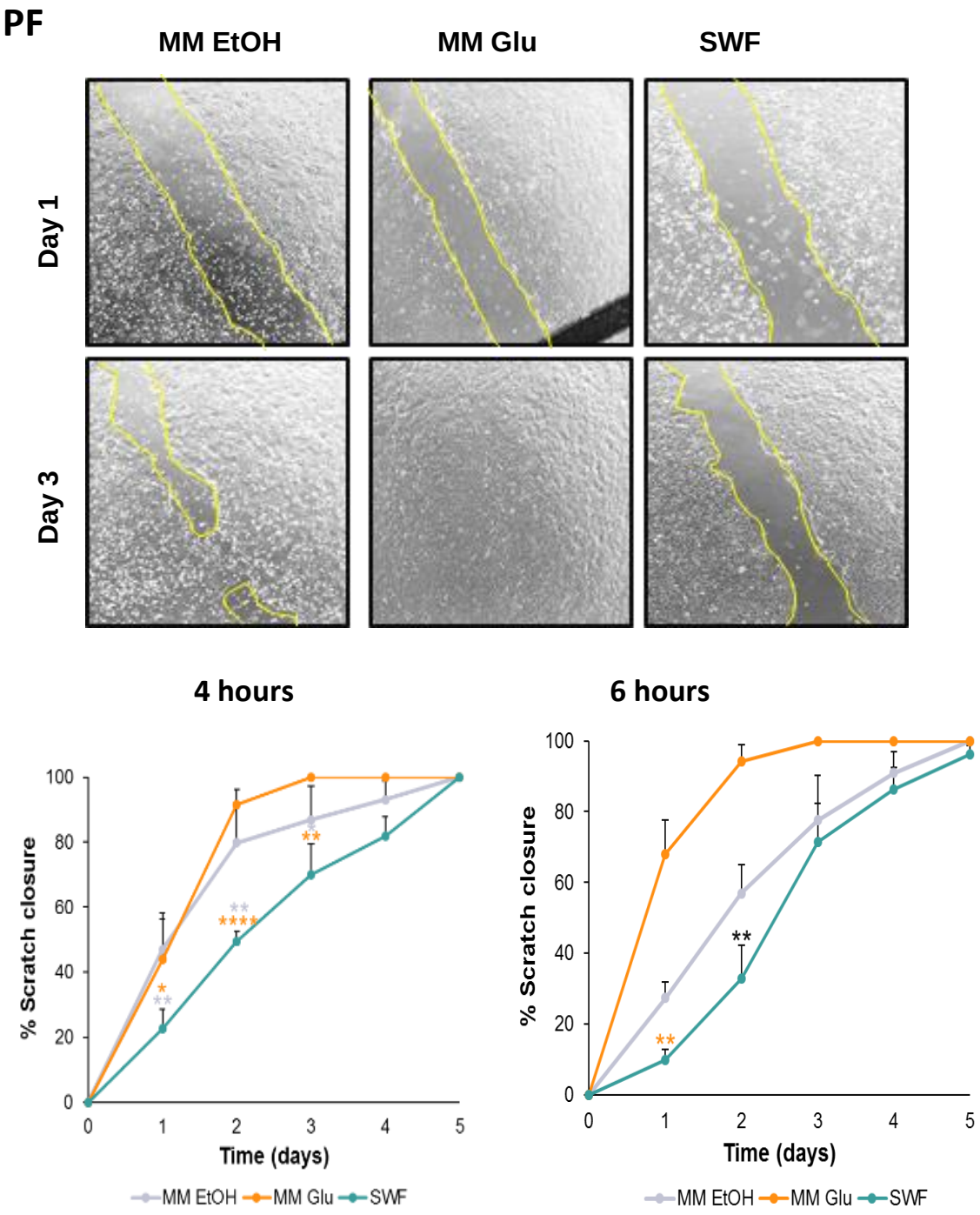

Figure 5.7 Representative images of scratches and corresponding scratch closure over time from cultured keratinocytes exposed for 4 or 6 hours to PF conditioned media grown for 80 hours. Scratches were exposed to cell free conditioned media from PF grown for 80 hours and diluted 1:2 in complete culture media for 4 or 6 hours. Scratches were photographed and closure determined every 24 hours up to 5 days. A two way ANOVA with Tukey's post hoc showed significance between groups (*p ≤0.05, **p ≤0.01, **** ≤0.0001), colour of stars indicates the condition where significance lies, black stars show significance to both of the other conditions. Keratinocyte images are representative of 4 hour exposure only.

5.4.2.1 PF grown in MM Glu for 80 hours showed accelerated closure to above control.

Similar to section 5.4.1.1, once scratch closure from keratinocytes exposed to 80 hour conditioned media had been determined, data were replotted together to investigate whether any significant differences in scratch closure were seen between conditioned media and the corresponding control of non-conditioned media. Only cell free conditioned media from PF cultures grown in MM Glu for 80 hours showed accelerated closure above control, which differed from 24 hour cultures. The accelerated scratch closure was seen following a 4 and 6 hour exposure, with around a 20% increase in closure seen at days 1 and 2 following a 4 hour exposure, however these differences were not significant (figure 5.8). Greater differences between conditioned and non-conditioned were observed following a 6 hour exposure with conditioned media showing a 40% increase in closure at day 1 and a 30% increase at day 2 however these were also not significant (figure 5.8). At day 3, minimal differences were seen between conditioned and non-conditioned media with both cell exposed to both treatments showing almost 100% closure (figure 5.8)

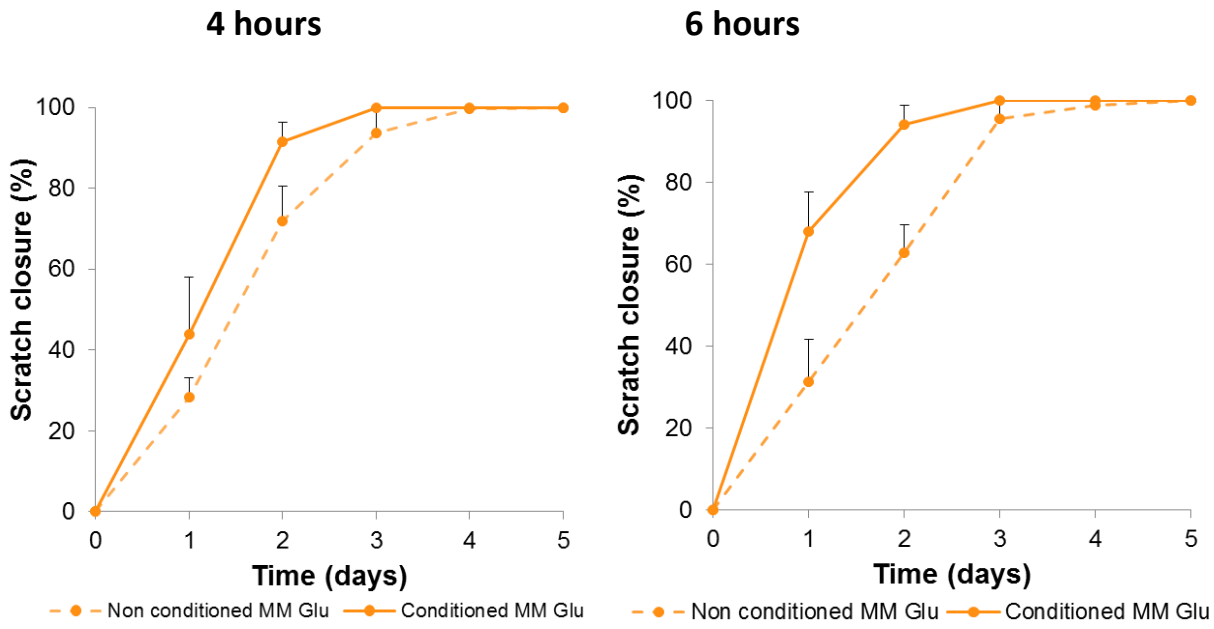

Figure 5.8 Mean scratch closure from keratinocytes exposed to conditioned media from PF cultures grown in MM Glu for 80 hours compared to non-conditioned media. Scratches were exposed to conditioned or non-conditioned SWF diluted 1:2 in complete cell culture media for 4 or 6 hours scratches were photographed and closure determined every 24 hours over 5 days. A two way ANOVA with Bonferroni post hoc showed significant differences between groups (**** ≤0.0001).

5.4.3 Comparison of the effects of PS3 bacterial culture time on scratch healing

To compare the effects of the cell free conditioned media from 24 and 80 hour cultures on scratch closure, scratch assay data from 24 and 80 hour cultures of PS3 grown in MM EtOH, MM Glu or SWF were plotted together (figure 5.9). This clearly shows that for PS3 grown in MM EtOH there is a significant difference between bacterial culture time and scratch closure conditioned media from 24 hour cultures producing more rapid and complete scratch closure compared to 80 hour cultures with significant differences seen at each time point with the biggest significant differences seen at days 2 and 3 ($p \leq 0.0001$) (figure 5.9). For scratches treated with media from 24 hour cultures there was 75% closure at day 2, by comparison for 80 hour cultures it was almost 4 fold less at 20% (figure 5.9). These differences were not observed for MM Glu or for SWF conditioned media for which scratch closure in response to 24 and 80 hour cultures was the same throughout the experiment (figure 5.9).

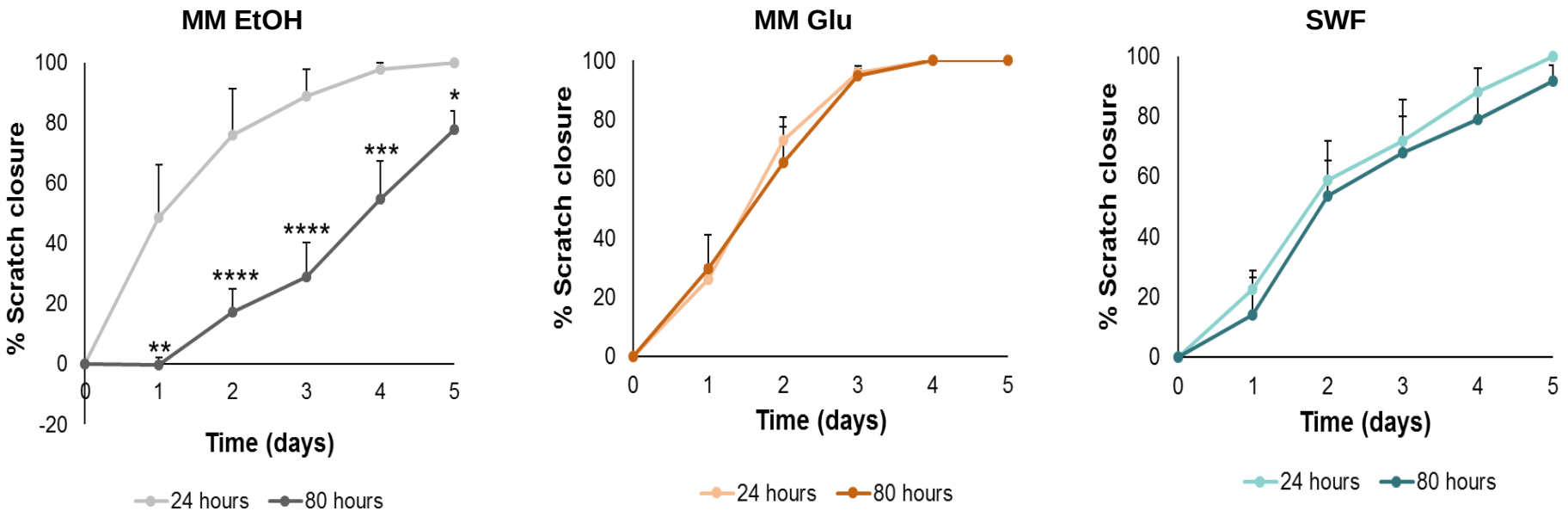

Figure 5.9 Comparison of scratch closure from keratinocytes exposed for 4 hours to PS3 grown for 24 or 80 hours in each of the different bacterial culture conditions. Scratches were exposed to cell free conditioned media diluted 1:2 in complete cell culture media for 4 hours, scratches were then photographed everyday with scratch closure determined every 24 hours over 5 days. Data is expressed as mean (+) SEM of 3 independent experiments (n=2). A two way ANOVA with Tukey's post hoc showed significance as indicated on the graphs (*p ≤0.05 **p ≤0.01, ***p ≤0.001, ****p ≤0.0001).

5.4.4 Scratch closure in keratinocytes exposed 1:5 and 1:10 dilutions of PS3 80 hour conditioned media

Having established that a 1:2 dilution of PS3 grown for 80 hours in MM EtOH clear had a significant detrimental effect on the rate of scratch closure, the effect of 1:5 and 1:10 dilution of conditioned media was investigated (figure 5.10).

A 1:5 dilution of PS3 conditioned MM EtOH, reduced scratch closure compared to MM Glu conditioned media, but was similar to SWF conditioned media (figure 5.10).

At the 1:10 dilution, minimal differences were observed between conditions with each condition showing similar scratch closure throughout each of the different time points (figure 5.10). Small differences in scratch closure were seen at day 3 between MM EtOH/SWF and MM Glu however these were not significant.

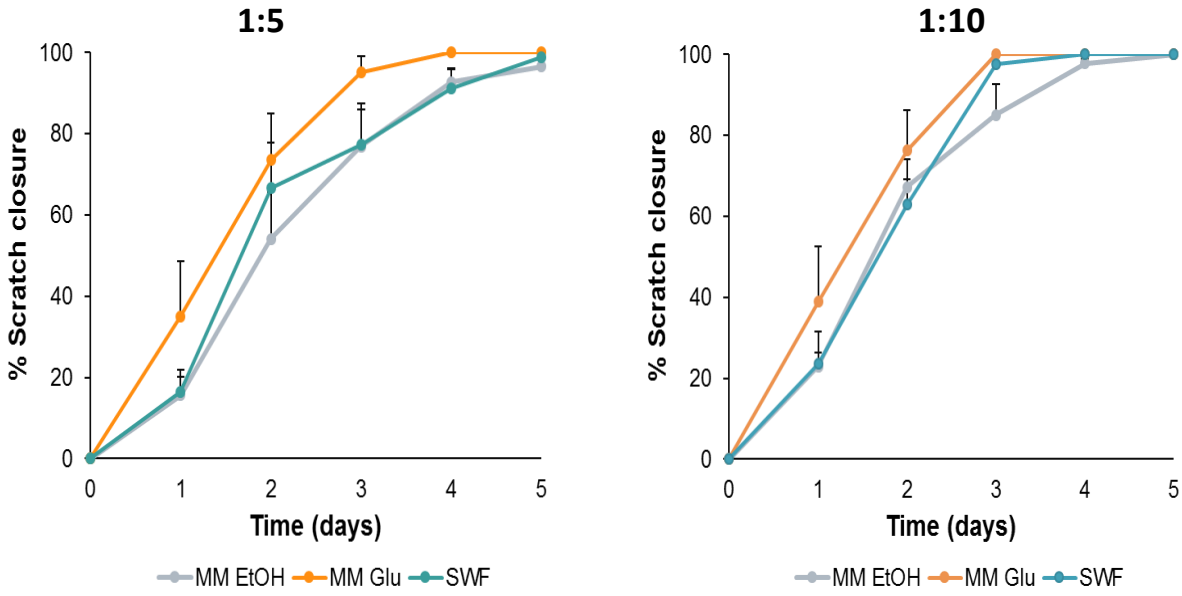

Figure 5.10 Scratch closure from keratinocytes exposed for 4 hours to cell free conditioned media from each of the different growth conditions diluted 1:5 or 1:10 in complete cell culture media. Keratinocytes were exposed for 4 hours and then photographed every 24 hours for up to 5 days, with scratch closure determined every 24 hours. Data are mean (+) SEM of three independent experiments (n=2).

5.4.5 Size of scratch had no direct effect on scratch closure in experiments.

To determine that size of scratch did not play a role in determining scratch closure and that effects seen were down to the presence of secreted factors within the cell free conditioned media, initial scratch area were collated at the end of the experiment and was plotted against the closure time

Initial scratch size did not have an effect on scratch closure on keratinocytes, with scratches of a similar initial size taking different lengths of time for full scratch closure to occur (figure 5.11).

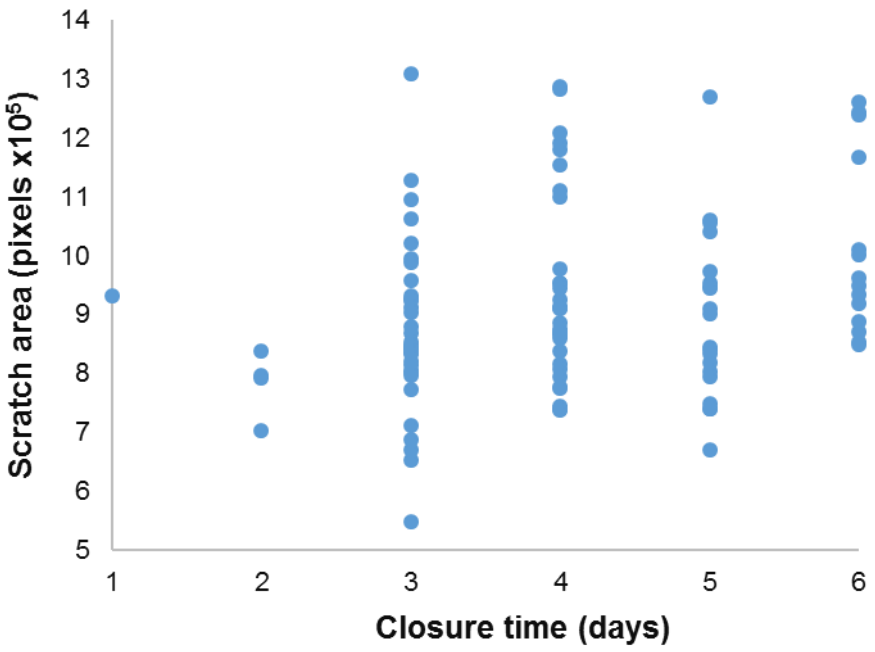

Figure 5.11 Initial size of size of scratches made in HaCaT cell monolayers and time taken to achieve full scratch closure. Initial scratch sizes of keratinocytes exposed to cell free conditioned media from PS3 and PF for each growth condition were plotted against time taken for full scratch closure to occur. Each data point represents 1 scratch.

5.5 Determination of scratch closure from keratinocytes exposed to live *Pseudomonas*

Once effects of cell free conditioned media from the different growth conditions had been assessed, scratches were exposed to live PS3 that had been grown in MM EtOH or MM Glu for 24 and 80 hours. The cultures were then centrifuged at 4000rpm for 12 minutes, and the bacteria re-suspended at the same concentration in HBSS. A ten-fold serial dilution was prepared and scratched monolayers were exposed for 4 hours to PS3 at an MOI of 1 and 10. Following exposure, bacteria were removed and complete cell culture media supplemented with antibiotics (1μg/ml) was placed on the cells. Due to the use of live bacteria, experiments were performed for a shorter time period of 3 days. Cells were photographed using an EVOX XL microscope and scratch closure was determined every 24 hours.

5.5.1 Scratch closure from keratinocytes exposed to PS3 at an MOI of 1 and 10 from 24 hour cultures.

Scratches exposed to live PS3 from 24 hour cultures did not generally affect scratch closure in keratinocytes in culture. Scratches exposed to an MOI of 1 from PS3 grown in MM EtOH had a similar scratch closure to control over all 3 days (figure 5.12) For MM Glu grown bacteria scratch closure was generally overall slower, only reaching around 80% closure at day 3, however initial scratch closure at day 1 was greater than control and MM EtOH (figure 5.12). In comparison to the effect of cell free conditioned media shown (figure 5.3), scratch closure from keratinocytes exposed to live PS3 was around 20% greater compared to MM EtOH, by contrast for MM Glu the healing from exposure to live bacteria was around 20% slower compared to cell free conditioned media (figure 5.3 and figure 5.12).

When bacterial numbers were increased to an MOI of 10, the relative scratch closure was reversed, with MM Glu grown PS3 being similar to control with both being 100% healed by day 3 (figure 5.12). By comparison for PS3 grown in MM EtOH, only 80% scratch closure occurred by day 3 which was significantly less than that for MM Glu ($p \leq 0.01$), but initially, at day 1, scratch closure was significantly greater than that for MM Glu and control ($p \leq 0.001$) (figure 5.12). These differences in response to bacteria from the different growth conditions are more similar to that for cell free conditioned media (figure 5.3).

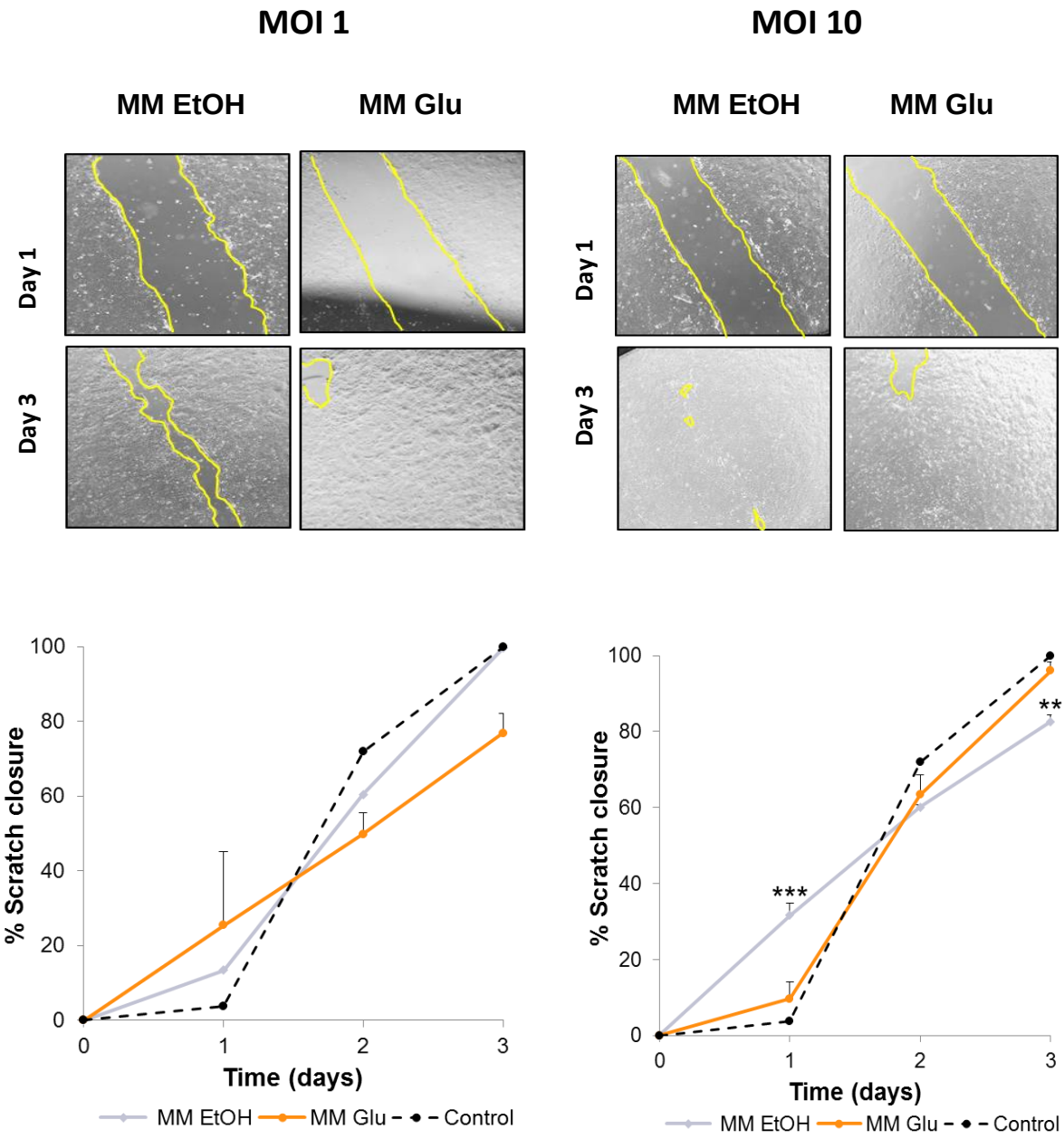

Figure 5.12 Keratinocyte images and corresponding mean scratch closure from keratinocytes exposed for 4 hours to live PS3 grown for 24 hours. Keratinocytes were exposed for 4 hours to PS3 at an of MOI 1 or 10 grown in MM EtOH or MM Glu, control (HBSS only) data are indicated by the dashed line. Data are expressed as mean (+) standard deviation of 1 experiment (n=2). A two way ANOVA with Bonferroni post hoc test showed significant differences as indicated on the graph ** ($p \leq 0.01$, ***$p \leq 0.001$).

5.5.2 Scratch closure from keratinocytes exposed to PS3 at an MOI of 1 and 10 from 80 hour cultures.

Similar to 24 hour cultures, live PS3 grown for 80 hours did not have a detrimental effect on scratch closure. For scratches exposed to an MOI of 1 PS3 grown in MM EtOH and MM Glu showed similar closure throughout each of the time points, with initial scratch closure being above control (figure 5.13). Full scratch closure was seen from MM EtOH conditioned PS3 by day 3, with MM Glu grown PS3 showing slightly lower but not significant scratch closure, reaching 80% closure (figure 5.13).

When scratches were exposed to a higher bacterial number at an MOI of 10, similar to MOI 1, PS3 grown in both conditions showed similar scratch closure throughout each of the time points, however at day 3, a decrease in scratch closure was seen from PS3 grown in MM EtOH compared to an MOI of 1 with scratch closure reaching 80% (figure 5.13). For MM Glu the increase in bacterial number had minimal effect with scratch closure being similar to closure seen when keratinocytes were challenged with an MOI of 1 (figure 5.13).

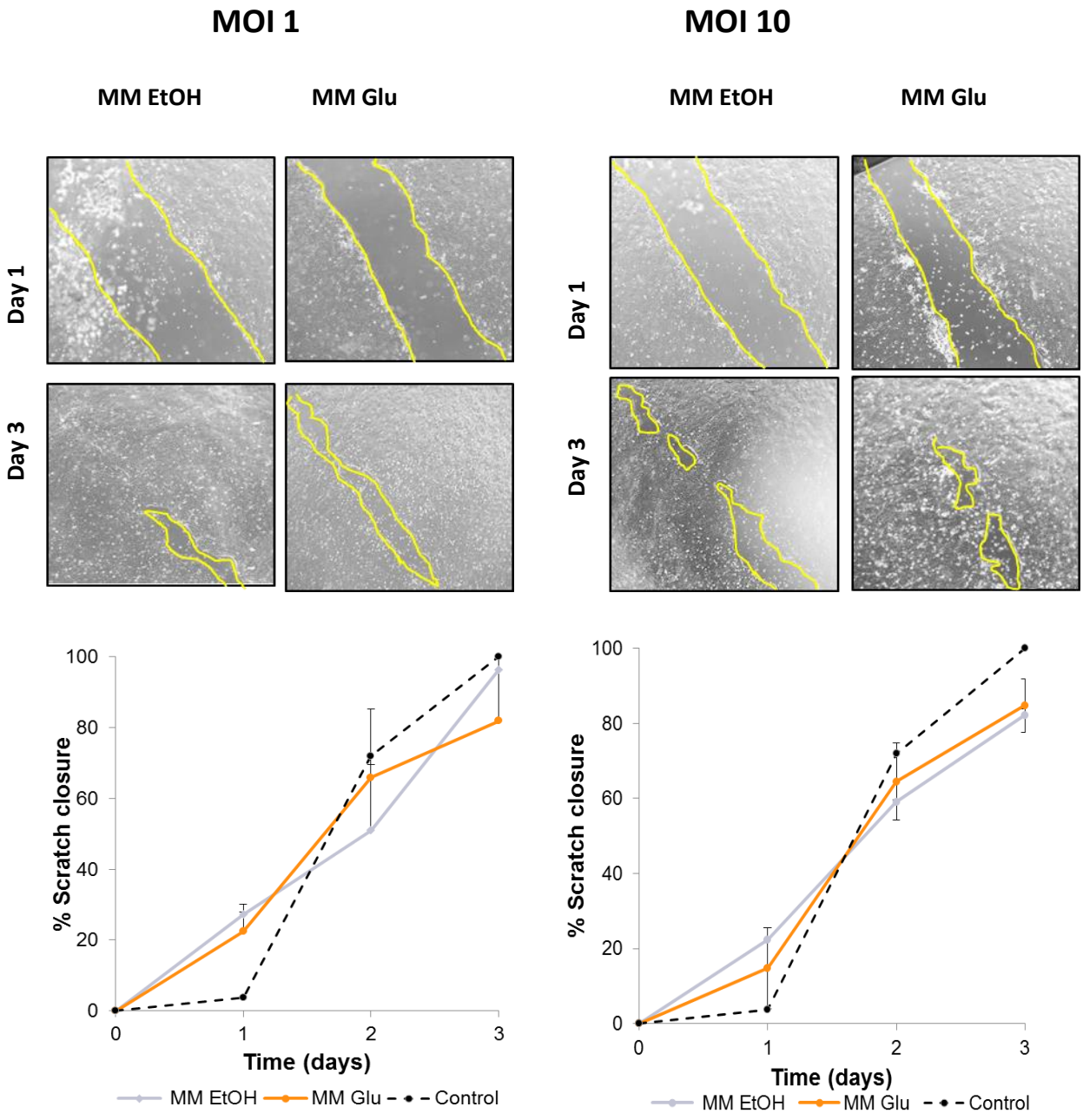

Figure 5.13 Keratinocyte images and mean corresponding scratch closure data from keratinocytes exposed for 4 hours to live PS3 grown for 80 hours. Keratinocytes were exposed to PS3 grown in MM EtOH and MM Glu at an MOI of 1 or 10 for 4 hours control (HBSS only) are indicated by the dashed line. Data are expressed as mean (+) standard deviation of 1 experiment (n=2). A two way ANOVA showed no significance between groups.

5.5 Non-conditioned media showed minimal scratch closure using a migration assay.

Once effects of cell free conditioned media had been established on proliferation and migration of cells into the scratched area of the keratinocytes, preliminary experiments were performed to investigate whether the role of cell migration on scratch recovery could be measured. To do this, once cells had reached confluence they were serum starved by replacing the normal growth media with DMEM supplemented with 1% FBS. Cells were then exposed to non-conditioned growth media only (diluted 1:2 in DMEM with 1% FBS) for 4 hours, the media was then removed and media containing 1% FBS was placed back on the cells.

Minimal scratch closure was seen from cells treated with DMEM (1% FBS) with only 20% scratch closure seen after 3 days (figure 5.14). In addition to this, non-conditioned growth media, showed a decrease in scratch closure from both MM EtOH and MM Glu following day 2 (figure 5.14). As minimal scratch closure occurred in control conditions these experiments were not continued.

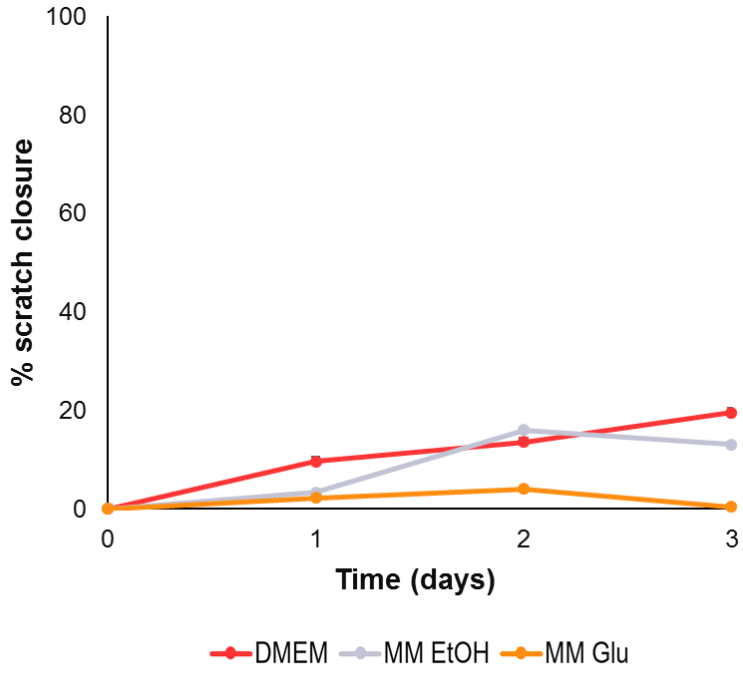

Figure 5.14 Scratch closure from keratinocytes exposed to growth media only during a migration assay. Keratinocytes were serum starved and exposed to non-conditioned growth media (diluted in DMEM supplemented with 1% FBS) for 4 hours, controls (DMEM with 1% FBS) are indicted by the red line. Data are expressed as mean of 1 experiment.

5.6 Ex vivo models of wound healing

Having established that PS3 and their secreted products had an effect on a model of wound healing using HaCaT monolayers, further experiments were performed using 3D reconstructed dermal models. The models were assembled and grown for 2 weeks with rings placed on the growing surface. Following a 2 week incubation the inner portion of the ring was treated with dispase (2mg/ml) for 4 hours in order to disrupt the basement membrane, to create an artificial wound. The rings were then removed and the models were exposed to cell free conditioned media (diluted 1:2 in complete cell culture media) for 4 hours before being washed and then incubated for a further 2 weeks. The models were then prepared for hematoxylin and eosin staining before being imaged.

5.6.1 PS3 conditioned media shows disrupted healing of wounds within ex vivo models

Models treated with non-conditioned media showed re-epithelisation of the ‘wound’ with a thin layer of HaCaT and fibroblast cells being visible all across top of the epidermis on the skin section (figure 5.15) indicating that growth media alone had no effect on the re-epithelisation of the wound. In addition to the epidermis, the dermis has ordered, stratified layers, indicative of undamaged skin (figure 5.15).

For models exposed to PS3 conditioned media grown for 24 hours in MM EtOH or MM Glu minimal re-epithelisation compared to control occurred with no difference evident between the two conditions (figure 5.15). In addition dermal layers of the models exposed to cell free conditioned media from PS3 grown for 24 hours also appear damaged with stratified layers not being visible in places and appearing thin. This erosion of the dermal layers was particularly evident in the models treated with MM EtOH indicating potential damage of the dermis (figure 5.15).

Dermal models exposed to cell free conditioned MM EtOH grown for 80 hours, also showed minimal re-epithelisation of the wound with the epidermal layer almost being non-existent (figure 5.15). However the dermis exposed to MM Glu conditioned media appeared to be unaffected having neat and stratified layers similar to the control (figure 5.15). In contrast the dermis models exposed to cell free conditioned media from MM EtOH was damaged with minimal neat layers visible and the overall appearance looking fragile.

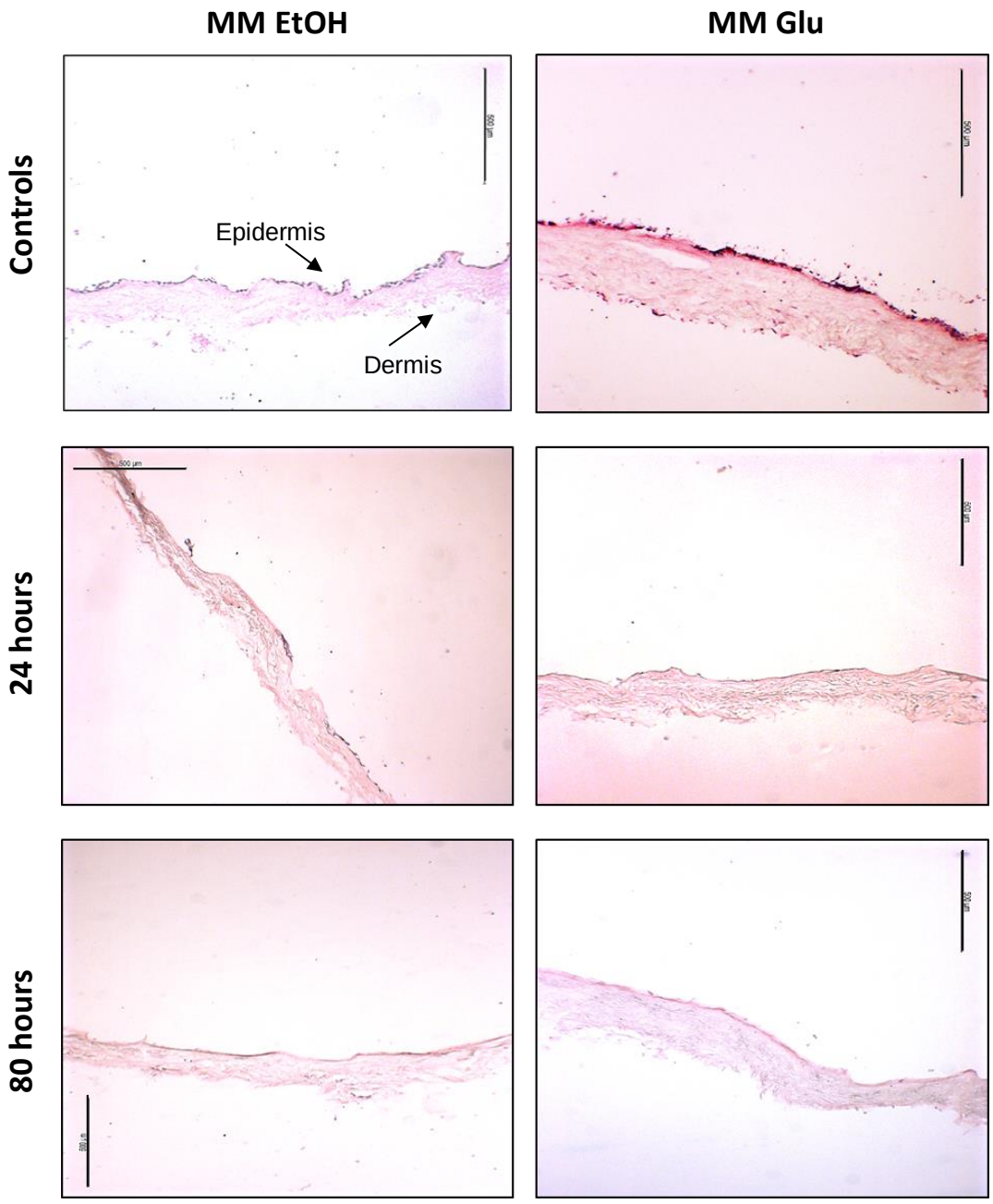

Figure 5.15 Ex-vivo skin models exposed to non-conditioned and conditioned growth media. Models were wounded and were exposed for 4 hours to conditioned growth media (diluted 1:2 in complete cell culture media) from PS3 cultures grown for 24 or 80 hours. Culture time is indicated on the left side of the panel, and the condition is indicated above the panel. Models were incubated for 2 weeks to allow re-epithelisation of the wound to occur. Models were sectioned, stained and imaged to show a cross section of the artificial wound created on the ex-vivo model. Controls (growth media only diluted 1:2 in complete DMEM) were used and shown on the top row of the panel above to provide comparison between both conditioned and non-conditioned media to determine if re-epithelialisation had occurred in each of the different experimental conditions. Formation of new epithelia is visible for the control conditions and is indicated by the presence of a dark purple layer above the dermis.

5.7 Summary

The results in this chapter show that the scratch assay was a suitable methodology to study wound healing, with closure being measurable over time from all conditions and initial scratch sizes being relatively consistent. All scratches showed almost full closure with the exception of those treated with cell free conditioned media from PS3 grown for 80 hours in MM EtOH, which produced significantly slower closure 5 days following a 4 hour exposure and an even bigger significant decrease in closure following a 6 hour exposure. Cells exposed to PF conditioned media from cultures grown in SWF for 24 hours showed significantly greater closure above control following both 4 and 6 hour exposures which was not observed for any other 24 hour cultures, in addition PF cell free conditioned media from MM Glu cultures grown for 80 hours also produced faster closure compared to control however differences were not significant. The exposure of cells to live PS3 had little effect on scratch closure, with results being similar to control, some variances were seen however these were not significant, as well there was little difference in scratch closure compared to the corresponding cell free conditioned media. The effects of cell migration only could not be studied due to the lack of closure seen from non-conditioned bacterial growth media. Further experiments using ex-vivo skin models showed differences between conditioned and non-conditioned media, with re-epithelisation occurring on the artificial wound created. For conditioned media there were minimal differences observed between culture conditions or time in culture, with all conditioned media producing no re-epithelisation and, in most cases, dermal damage also occurred.

5.8 General Summary of results

Taken all together, the results of this study show that culture condition can have an effect on *Pseudomonas* and these differences can have an effect on the HaCaT cell line. In order to make comparisons of these effects easier and to act as a reference point, a summary table of the results found in this study has been created for both conditioned media (appendix 4) and live bacteria (appendix 5). Due to the lack of adverse effects seen from PF, PS3 data is summarised only.

Chapter 6
Discussion

6.1 Use of cultured keratinocytes to study *Pseudomonas* host-pathogen interactions within the skin.

In-vitro models to study host-pathogen interactions in the skin, despite being reductive, can still provide relevant information regarding early defence mechanisms employed by the host (Barrila et al., 2018). Indeed whilst these are limited due to their inability to replicate normal physiological architecture (Brohem et al., 2011) *in vitro* models such as single layer cell lines can provide a cost-effective and convenient method to study many biological processes. In addition to this cell lines provide a consistent population of cells allowing more reproducible results to be obtained (Kaur and Dufour, 2012). As keratinocytes account for around 90% of the epidermis and often form the skins first line of defence against toxic substances as well as initiating skin immune responses (Colombo et al., 2017) this study utilised the human keratinocyte cell line HaCaT as an *in vitro* model of the host to study early host-pathogen interactions within the skin.

The interactions between host and pathogen are important in providing insight into the infection, treatment and prevention of many diseases (Chattopadhyay et al., 2018). Host-pathogen relationships are complex and can result in a variety of cellular responses, with the outcome often dictated by the host's response to the pathogen (Wollert et al., 2012). Whilst it is assumed host responses are activated as a defence mechanism, it is possible that the pathways activated may be of benefit to the pathogen (Ichikawa et al., 2000). Currently, host pathogen-interactions often focus on the use of live bacteria to study host responses and *Pseudomonas* host-pathogen interactions have been well established using the

lung as a host model, due to its ability to cause chronic infections in the lungs of cystic fibrosis patients (Tang et al., 2014, Bhagirath et al., 2016). However considering *Pseudomonas aeruginosa* is nosocomial pathogen and involved in a variety of mild skin disorders (Wu et al., 2011) as well as a frequent coloniser of wounds (Morrison and Wenzel, 1984) it is surprising that little work has been carried out using the skin or skin cells as a host model. In addition, recently the role of bacterial secretions in mediating interactions with host cells independent of live bacteria has become an area of emerging interest.

6.2 Production of virulence factors from *Pseudomonas*

Pseudomonas aeruginosa expresses a variety of virulence factors that are associated with its ability to cause disease. (Lau et al., 2004). Virulence factors can be both cell attached and secreted, with each having the ability to target host cells via specific mechanism, some of which aid in direct toxicity and some interfere with host responses to allow evasion of defence mechanisms initiated by the host.

6.2.1 Biofilm production

Pseudomonas has been recognised as being a biofilm producer as well as having the ability to form pellicles, which are specific biofilms that form only at air-liquid interfaces (Armitano et al., 2014, Friedman and Kolter, 2004). Currently three EPS polymers produced by *Pseudomonas;* alginate, Psl and Pel have been identified in the production of mature biofilms (Ghafoor et al., 2011). There are many factors that can influence *Pseudomonas* biofilm formation and regulation, including growth conditions quorum sensing and EPS production (Palmer et al., 2007, De Kievit, 2009). In addition quorum sensing has been implicated in influencing EPS production of *Pseudomonas* however the extent of this is unknown (Frederick et al., 2011).

In this study PS3 was identified as being a pellicle producer as pellicles were visible after 24 hours in all 3 conditions, with MM EtOH and SWF showing slightly thicker pellicles than MM Glu. In addition to this there was a difference in the expression of different biofilm genes, with greater expression of Psl genes in MM Glu and similar expression of associated Pel genes in MM Glu indicating that

within different environments, *Pseudomonas* can alter expression of genes involved in early biofilm formation (Musken et al., 2010).

After 80 hour incubation, pellicles continued to appear thicker and better established in tubes with MM EtOH or SWF in comparison to tubes containing MM Glu, which began to show disintegration of the biofilm. The increased production of biofilm seen in MM EtOH tubes is supported by other findings which showed low concentrations of ethanol stimulated biofilm production in *Pseudomonas* (Tashiro et al., 2014) and could promote colonisation of plastic surfaces (Chen et al., 2014). In addition, there was a greater expression of Psl associated genes MM EtOH after 80 hours compared to 24 hour cultures providing a further explanation for the continued structurally strong biofilm visible in MM EtOH tubes at 80 hours. By comparison, expression of most Psl associated genes were decreased in MM Glu cultures, specific Psl genes A, B and D have been identified as being crucial for biofilm maintenance and structure (Wei and Ma, 2013) expression of these were decreased after 80 hours and this, most likely contributed to the break breakup of the biofilm. Minimal differences in genes involved in Alg and Pel polysaccharide were seen between culture conditions at both 24 and 80 hours, however unlike Psl, these polysaccharides are not vital can be dispensable in biofilm formation (Baker et al., 2016, Vasseur et al., 2005).

Whilst no transcriptomic data was available for SWF cultures in this study, there was a presence of green pigmentation in SWF tubes, which was identified as pyoverdine based on observations from other research (Meyer, 2000). Pyoverdines are strong iron scavengers, which play a role in biofilm formation (Banin et al., 2005). Under iron limiting conditions, which would be likely after 80

hours, pyoverdine production is increased, thus contributing further to the already established biofilm.

PF was identified as being a non-pellicle producer with no visible pellicle after 24 or 80 hours. However, previous data on biofilm formation from PF is mixed with some studies showing biofilms forming after 10 hours from PF (O'Toole and Kolter, 1998) whereas some have observed thin PF pellicles after 7 days (Rhodes, 1959) and others have shown PF to only form biofilms at lower temperatures of 10ºC and showed that PF attachment to surfaces is much weaker at 30ºC (Rossi et al., 2016) a feature that is essential for biofilm formation.

6.2.2 Pyocyanin production

Pyocyanin is responsible for the production of the blue green pigment and has been identified as a secreted virulence factor involved in supressing inflammatory responses of hosts (Allen et al., 2005) and in quorum sensing (Jayaseelan et al., 2014). In this study pyocyanin was detected from MM EtOH and SWF 24 hour cultures but not MM Glu cultures, indicating that mechanisms involved in pyocyanin production are increased by the presence of ethanol or FBS (Katri and Gilboa-Garber, 2007). Following a further incubation of 80 hours, pyocyanin was detected in cell free conditioned supernatants at a higher absorbance compared to 24 hour cultures, however it has been suggested that pyocyanin production is increased in response to a decrease of nutrients such as sources of carbon (Whooley and McLoughlin, 1982)

No pyocyanin was detected from any of the PF cultures indicating that PF is not capable of producing pyocyanin, whilst PF is capable of producing similar colour pigments pyocyanin has not been identified in PF (Scales et al., 2014).

6.2.3 Protease production

Proteases are also recognised as an important secreted virulence factor that that are responsible for causing damage to host cells by degrading proteins and modulating the host immune response (Milesi Galdino et al., 2017, Oldak and Trafny, 2005). Three main proteases have been identified as being common to most *Pseudomonas* species; alkaline protease, elastase and collagenase (Morihara and Tsuzuki, 1977).

Proteases were quantified by using skim milk agar plates to measure the breakdown of casein which is a common property of the main proteases identified (Sokol et al., 1979). After a 24 hour incubation period protease production was greater in 24 hour cultures compared to 80 hour cultures indicating increased protease production in 24 hour cultures. Due to more available nutrients from the original growth media at 24 hours compared to 80 hours it has been suggested that *Pseudomonas aeruginosa* will continue to secrete enzymes to continue gaining nutrients from the environment (Cezairliyan and Ausubel, 2017). After 48 hour incubation, bacteria from 80 hour cultures had greater protease activity compared to 24 hour cultures, suggesting there was not full activation of protease activity in the initial 24 hour incubation. As extracellular secretions, including proteases, are often dependent on quorum sensing (Park et al., 2014) any disruption to this often inhibits secretions (Cezairliyan and Ausubel, 2017). Protease production could not be detected in cell free conditioned media from any of the conditions suggesting lack of active protease in the conditioned supernatant, this is in contrast to other research which has shown protease activity from the supernatants from *Pseudomonas aeruginosa* cultures, however

supernatants were tested after shorted culture times of 18 hours (Casilag et al., 2015)

No differences in the amount of protease produced by the bacteria was seen between the different culture conditions at any time point, however due to the amount of protease produced and the number of bacteria used, the assay quickly became saturated. A refinement of the methodology used would be needed to further investigate the role of growth culture condition on protease production, with the need for a lower initial bacterial number and/or a different size petri dish with a larger surface area.

No protease production was found from PF in this study, however some proteases have been found in other PF strains isolated from raw cow's milk (Alves et al., 2016, Koka and Weimer, 2000).

6.2.4 Hemolysin production

Hemolysins are a secreted exotoxin implicated in the virulence and pathogenesis of *Pseudomonas aeruginosa* burn infections (Bnyan and Ahmed, 2013) as well as ocular and lung infections (Johnson and Boese-Marrazzo, 1980). Two main hemolysins have been identified from *Pseudomonas aeruginosa*, phospholipase C and a rhamnolipid (Van Delden and Iglewski, 1998).

A small amount of hemolysin activity was detected after a 120 hour (5 day) incubation on an agar plate from 24 hour cultures, indicating 24 hour cultures produce a very low concentration of hemolysin. Hemolysin production was greater from 80 hour cultures, with clear zones visible on the plates after 48 hour incubation these continued to develop for up to 120 hours, these findings are

supported by Altenbern (1965) who found that hemolysin production was minimal from 24 hour cultures, but increased in 72 hour cultures. After 120 hour plate incubation of 80 hour cultures, PS3 grown in MM EtOH showed significantly greater hemolysin production compared to MM Glu and SWF this is supported by other research which shows in the presence of ethanol, hemolytic phospholipase C production was increased (Katri and Gilboa-Garber, 2007). An additional observation of the clear zones present on the plates was the appearance of a green colour indicative of partial hemolysis of blood cells caused by alpha hemolysin production (Buxton, 2005). No hemolysin production was observed from cell free conditioned media however it has been observed that hemolysin secretion does not occur in broth cultures (Berk, 1962).

No hemolysin activity was detected in PF cultures however hemolysis production is not common in PF and only a few rare clinical isolates have been identified as having the ability to produce hemolysins (Sperandio et al., 2010).

6.2.5 Quorum sensing on *Pseudomonas* virulence

Similar to other Gram negative bacteria, the expression of virulence factors is controlled by QS mechanisms (Papenfort and Bassler, 2016, Smith and Iglewski, 2003). There are 4 main intracellular communication signals that contribute to the QS network of *Pseudomonas; las, rhl, PQS and IQS* (Lee and Zhang, 2015) and most signalling networks have been implicated in the control of various virulence factors (Mattmann and Blackwell, 2010). In this study, whilst there was not always a clear difference in the production of different virulence factors between culture condition, the production of virulence factors was increased in all conditions after 80 hours. Considering QS occurs in response to the increased numbers of

bacteria, it shows that PS3 can utilise quorum sensing mechanisms to change the production of virulence factors. In addition, as QS changes in response to the microenvironment (Lee and Zhang, 2015) with some subtle changes for specific virulence factors occurring in response to the different culture conditions, indicating that there could be a change in the QS mechanisms between conditions. Transcriptomic data of PS3 showed that PS3 expressed the main genes involved in each of the different signalling networks that contribute to overall quorum sensing, showing that PS3 can utilise all four communication systems.

It was shown that PF is not able to produce the virulence factors that were investigated in this study, which potentially shows that QS mechanisms are not involved in the regulation of gene expression, with QS systems of PF identified as being involved in antibiotic production and colonisation of roots of plants (Martins et al., 2014).

Differences in the expression of genes between culture conditions indicate that *Pseudomonas aeruginosa* can change quorum sensing mechanisms in response to the environment, these culture condition specific effects have been reported previously (Winzer et al., 2002). The change in quorum sensing mechanisms can provide a partial explanation for the differences seen in virulence factors that were quantified.

6.3 Interaction of *Pseudomonas* on cultured keratinocytes

As discussed previously, *Pseudomonas* can cause a wide variety of skin infections where it can interact with host skin cells by the production of virulence and immunogenic factors to mediate the host responses. To aid with the promotion of colonisation and infections *Pseudomonas* can secrete OMVs which via the dissemination and delivery of vesicles aid in the interaction of immunogenic factors directly with host cells. The delivery of these factors though OMVs or via direct secretion into the environment allows interaction with host cells without the need for the direct presence of the live bacterium. Secretion of *Pseudomonas* OMVs can be influenced by the bacterial microenvironment which has also been found in other Gram negative bacteria (Yu et al., 2018). In addition, the presence of host stress mediators in the bacterial environment has been found to increase vesicle production by *Pseudomonas* (Macdonald and Kuehn, 2013) and increase the expression of virulence factors as controlled by QS mechanisms (Moradali et al., 2017). Other research has also shown that OMV production can be influenced by the bacterial microenvironment, with different culture conditions found to affect the protein composition of OMVs in plant pathogens (Sidhu et al., 2008). Changes in extracellular secretions caused by the bacterial micro-environment may have an effect on the cytotoxicity and immune responses of human skin cells in vivo.

6.3.1 Cytotoxic effects of *Pseudomonas* on cultured keratinocytes

In this study the cytotoxic effects of cell free conditioned media from *Pseudomonas* cultures on HaCaT cells was assessed by the use of the MTS assay, which provides a quantitative measurement of mitochondrial metabolic rate (Wang et al., 2010). Cell metabolic activity assays are often employed to investigate the potential effects of biological agents on cell proliferation or cell cytotoxicity (Rampersad, 2012) as they can provide an indirect measurement of the quantity of viable cells.

No detrimental effects were observed in cells exposed to growth media only, indicating any changes in biomass upon exposure to conditioned media are due to secreted products from the bacterial cultures. Cells exposed to non-conditioned media or cell free conditioned media from 24 hour cultures showed up to 40% increased metabolic activity compared to control cells treated with normal cell culture media, with no differences seen between conditions or *Pseudomonas* strains suggesting there is no toxicity of *Pseudomonas* conditioned media from PS3 or PF after a 24 hour growth period. The increase in metabolic activity indicates increase in the number or metabolic activity of the cultured keratinocytes, which could be in response to a low level of cellular stress – caused by exposing the cells to diluted growth media itself rather than the effects of cell free conditioned media. It has previously been reported that the partial activation of cell stress pathways can increase the rate of cell metabolism without causing cellular damage (Fulda et al., 2010) a process which involves the activation of mitochondria (Picard et al., 2018). These findings are supported by Marano et al. (2015) which showed an increase in metabolic activity in normal

epidermal keratinocytes following exposure to *Pseudomonas aeruginosa* conditioned media grown for 24 hours.

6.3.2 Toxicity of PS3 conditioned media from 80 hour cultures grown in mineral media with ethanol or simulated wound fluid on cultured keratinocytes

A total loss of viable cells occurred consistently in keratinocytes exposed to a 1:2 dilution of cell free conditioned media taken from PS3 cultures grown for 80 hours in MM EtOH, that did not occur with MM Glu conditioned media or for PF cultures grown in any of the media for 80 hours. This indicates that after 80 hours in culture there is a difference in toxicity of the extracellular factors secreted by the bacteria grown in different conditions but only for PS3, which may be caused by the alteration of the production of cell associated virulence factors of the parent bacterium or altered production of OMVs both of which can be caused by the bacterial microenvironment. The loss of viable cells could be due to a greater concentration of toxic secretions in the media causing a high level of cell stress which cannot be overcome by the cell resulting in cell death (Krampe and Al-Rubeai, 2010). Studies on cultured bronchial epithelial cells showed *Pseudomonas aeruginosa* secretions caused splicing of several proteins involved in endoplasmic reticulum function, resulting in prolonged endoplasmic reticulum stress (van 't Wout et al., 2015) which could provide an explanation for the loss of viable cells.

SWF cell free conditioned media produced a variation in HaCaT viability, indicating variability in the toxicity of secretions from PS3 80 hour cultures, this could be due to inconsistencies with the growth media as SWF is a mix of MRD and FBS, as serum is a natural product, variation between batches of FBS, even

from the same manufacturer, is possible (Zheng et al., 2006). Variation in the toxicity is likely to be due to *Pseudomonas* responding to the availability of specific nutrients in the FBS. In addition to this, serum contains haemoglobin and thus large concentrations of iron which can be scavenged by pyoverdines which have been identified as having potential to be directly toxic to cells (Kirienko et al., 2019) and the production of these can be increased in response to iron limiting conditions (Granato and Kümmerli, 2017), which would be likely after 80 hour culture times.

6.3.3 Effects of live PS3 on keratinocyte viability

Keratinocytes exposed to live PS3 did not show any decrease in metabolic activity, indicating that live PS3 do not initiate toxic effects in the HaCaT cell line over a short exposure time of 2 hours. Transcriptome analysis of PS3 (Akbar et al., 2015) showed no ExsA expression, a gene thought to be involved in cytotoxicity (Evans et al., 2002, Cowell et al., 2000). When exposure times of cell free conditioned media were reduced to match those of live PS3, a similar pattern of loss of metabolically active cells occurred to that of 4 hours, indicating that the extracellular products from PS3 cultures can mediate cytotoxicity and cause loss of viable cells even over a shorter exposure time. These data indicate that it is factors secreted from PS3 that are responsible for its acute toxicity, this is important as these could penetrate more easily beyond the site of infection and thus results in the development of a chronic would. It also provides some justification for the use of cell free cultures to investigate the interactions of PS3 with host cells.

6.4 Attachment and invasion of *Pseudomonas aeruginosa*

Attachment of *Pseudomonas* to host cells is one of the first steps in establishing the infection process as well as being important in biofilm formation (Siryaporn et al., 2014) once attached, *Pseudomonas* can interact with host cells to modulate signalling pathways to allow entry into the host cell (Bucior et al., 2012). Invasion into the host cell is important in initiating infection, by internalising bacteria into the host cells and thus giving protection from host defence mechanisms, it also facilitates the penetration of infecting bacteria into basal layers of the tissue and the blood stream (Esen et al., 2001). Much of the work investigating the internalisation and attachment of bacteria into keratinocytes has focused on *Staphylococcus aureus* due to its prevalence as a skin and wound pathogen (Kintarak et al., 2004, Edwards et al., 2011) however, there has been little work has investigating *Pseudomonas aeruginosa* attachment and invasion of keratinocytes.

6.4.1 *Pseudomonas aeruginosa* attachment and invasion into host cells

The most striking feature of the interaction of PS3 with HaCaT cells is the relatively low level of attachment and internalisation. However, this is consistent with the lack of cytotoxicity which occurred in response to exposure to live PS3 grown in any of the culture conditions tested here.

PS3 grow for 24 hours in MM Glu showed the highest attachment and invasion but for 80 hour cultures it was MM EtOH, however both showed generally a very low level of attachment and invasion with no increase occurring with greater

concentration of bacterial number indicating bacterial number does not directly influence the *Pseudomonas* virulence that is involved in attachment. This is supported by other research which found MOI had no effect on the relative virulence of *Pseudomonas* upon attachment to different surfaces (Siryaporn et al., 2014). In addition there was also a very low level of invasion of *Pseudomonas* into HaCaT cells, with no invasion above 0.1 bacteria per cell, this is in contrast to other data which have shown *Pseudomonas* invasion to be as high as 1×10^6 per 100 HaCaT cell (Hosseinidoust et al., 2013) however the methodology used OD of planktonic cultures to normalise for the numbers of bacteria added to the cultures so the level of invasion may be exaggerated. In addition to this in this study it was found that the invasion of *Staphylococcus epidermidis* into keratinocytes was higher compared to *Pseudomonas* suggesting the presence of a skin commensal which is part of the normal skin microbiome and of benefit to skin cells, (Sabaté Brescó et al., 2017) has greater internalisation in HaCaT than the potentially pathogenic *Pseudomonas* species. This surprising result indicates that the internalisation of *Pseudomonas aeruginosa* is not important in mediating its effects on keratinocytes and is supported by the data reported here that live bacteria have less acute effects on the cells compared to the cell free secretions.

Considering that there was a change in the amount of bacterial attachment between growth conditions and it is hypothesised that the expression of virulence factors involved in mediating this can increase bacterial attachment to host cells (Kline et al., 2009), it indicates that *Pseudomonas* can alter the expression of virulence factors in response to changes in the environment. The transcriptomic data showed that after 24 hours in culture, there was increased expression of fleQ and a high expression of pilA in MM Glu cultures and at 80 hours there was

increased expression of pilA coupled with high expression of fleQ which indicates that both flagella and pili are required for *Pseudomonas* attachment (Conrad et al., 2011).

Interestingly at 24 hours, whilst MM Glu showed increased attachment, it was SWF cultures that showed increased invasion indicating PS3 grown in MM Glu has a low ability to invade host cells whereas at 80 hours, MM EtOH showed the greatest attachment to keratinocytes. Attachment to host cells is important in initial colonisation of *Pseudomonas aeruginosa* infections, considering that after 80 hours MM EtOH showed the greatest attachment and invasion in the keratinocytes.

As it was shown that the greatest stress response was produced by the cell free secretions from *Pseudomonas aeruginosa* it was hypothesized that the conditioned media might produce a cellular response that would increase the level of attachment and/or internalisation of bacteria. In addition, the removal of extracellular virulence factors that was done here might explain the very low amount of bacterial interaction with the cells due to the resuspension in HBSS. To test this, attachment and invasion assays were repeated with cells that had been pre-treated with diluted cell free culture media that would contain the secreted virulence factors. However, the addition of extracellular products had little effect on cell attachment and invasion and had a reduced effect on PS3 attachment and invasion at 24 hours, particularly for PS3 grown in MM EtOH possibly indicating that EC secretions may be priming cell receptors in advance of attachment and invasion (Metruccio et al., 2016) which leads to greater cellular defence thus decreasing attachment and invasion of the *Pseudomonas* itself.

The low level of attachment and invasion suggests either this specific stain of *Pseudomonas* does not normally invade cells (at least in culture) or that it stimulates a mechanism in HaCaT cells that resist attachment and invasion. This could include the production of anti-microbial peptides (AMPs) which are produced by host cells and are active against a wide range a Gram negative bacteria (Wanke et al., 2011). The production of AMPs is reported to be upregulated in vitro in response to pro-inflammatory cytokines or bacteria (Schröder and Harder, 2006). In keratinocytes, β-defensins and cathelicidins constitute the major classes of AMPs as well and have been well studied (Braff et al., 2005).

6.5 Initiation of immune responses

MAMP receptors, also known as toll like receptors are a highly conserved family of type 1 transmembrane proteins involved in the recognition of recognition of ligands present on invading microbes. *Pseudomonas* possess many MAMPs that can bind to and activate TLRs on a variety of host cells, in addition to being cell attached MAMPs can be extracellular often associated with outer membrane vesicles (Koeppen et al., 2016). In this study quantification of the pro-inflammatory cytokine CXCL8 was chosen as a measurement of TLR activation and initiation of innate immune responses by HaCaT cells in response to *Pseudomonas* and cell free conditioned media as keratinocytes have been shown to increase CXCL8 expression in response to inflammatory stimuli (Jiang et al., 2012).

6.5.1 Mediation of CXCL8 secretion in response to live PS3

Cell attached immunogenic factors of *Pseudomonas* include flagellin, pili and LPS which can interact with TLR 2 and 5 to induce CXCL8 release from host cells (Gellatly and Hancock, 2013, Venza et al., 2009). In this study exposure of keratinocytes to live PS3 grown for 24 hours could illicit CXCL8 secretion which was similar to that of corresponding cell free conditioned media, with the exception of PS3 grown in SWF where CXCL8 secretion from cell free conditioned media was minimal. These results indicate that cell attached appendages can produce CXCL8 secretion from HaCaT cells.

By contrast exposure to live PS3 grown for 80 hours produced lower CXCL8 secretion compared to 24 hour cultures, with PS3 from MM EtOH and MM Glu showing minimal CXCL8 secretion. The decrease in CXCL8 secretion from live PS3 between 24 and 80 hours media indicates a change in the potential of immune-stimulatory activity between the two culture times. It has been suggested that a decrease in available nutrients encountered by pathogens can lead to decreased ability to stimulate immune response, thus leading to increased risk of colonisation at the site of infection and increased harm caused by the pathogen (Pike et al., 2019). In addition to this at 80 hours, greater CXCL8 secretion was seen in response to cell free conditioned media indicating that the initiation of host immune responses is mediated by the presence of extracellular factors such as pyocyanins, which have been found to induce CXCL8 secretion in a concentration dependent manner from airway epithelial cells (Denning et al., 1998).

As previously discussed PS3 grown in MM EtOH after 80 hours showed increased expression of genes involved in flagellum and pili synthesis, but CXCL8 secretion was low at 80 hours indicating a possible dampening of the host immune response, possibly in response to the lack of nutrients as described above (Pike et al., 2019). In addition, whilst the expression of genes controlling flagella were increased, the amount of the TLR5 ligand flagellin on the cell surface may have been low as shedding of flagellin is possible, in which rhamnolipids (a hemolysin) have been identified (Gerstel et al., 2009) the production of which were increased in MM EtOH 80 hour cultures in this study.

6.5.2 *Pseudomonas* extracellular products modulation of CXCL8 secretion

Pseudomonas is known to secrete extracellular factors that can modulate host immune responses in order to help *Pseudomonas* invade and disseminate into host cells and tissues. In addition, in common with other Gram negative bacteria it will actively secrete outer membrane vesicles that carry immunogenic and virulent factors derived from the original pathogen (Bitto et al., 2018) and these will not only occupy the site of infection, they can also penetrate systemically.

In this study it was shown that PS3 could stimulate CXCL8 secretion from keratinocytes challenged with diluted cell free conditioned media from 24 hour cultures, with MM EtOH and MM Glu conditioned media producing significantly more CXCL8 than SWF across each dilution, indicating that secreted factors present in conditioned media can stimulate CXCL8 secretion from cultured keratinocytes. Following incubation for 80 hours, there was an increase in CXCL8 secretion from keratinocytes exposed to PS3 MM EtOH secretions in comparison

to 24 hour cultures and to MM Glu, with the exception of the 1:2 dilution where no CXCL8 could be measured, suggesting an increase in immunogenic factors in the MM EtOH conditioned media. SWF produced varied CXCL8 secretion, with high levels of CXCL8 secretion from keratinocytes indicative of the presence of immunogenic factors inducing a large immune and stress response, compared to low level CXCL8 secretion or unmeasurable CXCL8 secretion due to lack of viable cells as discussed in section 6.3.2. The variation seen in CXCL8 secretion is most likely due to variation of nutrients available in the growth media influencing the immunogenicity of vesicles and secreted extracellular factors. This is supported by Leidal et al. (2001) who found small differences in CXCL8 secretion from alveolar cell line A549 thought to be mediated by the presence of different factors present from *Pseudomonas aeruginosa* grown under different growth conditions, one of which was identified as being pyocyanin. In addition it is possible with the longer bacterial culture time there is an increase in the numbers of bacteria dying and subsequent lysis of cells could increase the concentration of immunogenic factors in the culture media.

PF showed much lower CXCL8 secretion at both 24 and 80 hours in comparison to PS3, indicating the lack of immunogenic factors within the conditioned media and no change of additional immunogenic factors over time within the conditioned media, most likely due to not being a human pathogen (Scales et al., 2014). In this study, PF was identified as being non- virulent due to the lack of positive results found in biofilm formation and production of pyocyanin, proteases and hemolysins and as such the OMVs produced by PF may not contain any virulent or immunogenic factors. As MAMPs are highly conserved between species and MAMPs presented to the keratinocytes are both from *Pseudomonas* species, it

indicates that host cells have a mechanism to recognise secreted MAMPs in the absence of the pathogenic bacteria itself. This is thought to be mediated by the recognition of virulence factors from pathogenic bacteria, which PRRs recognise as danger signals such as LPS which can interact with TLR4 (Srinivasan, 2010) which provides an explanation to the differences seen in this study, as LPS is likely to be present in the conditioned media and can interact with TLR to induce CXCL8 secretion from the host cell . Additionally, it has been shown that primary keratinocytes produced different CXCL8 secretion to flagellin isolated from two different bacterial species, which is also likely to be present in the conditioned media and can interact with TLR5 to induce CXCL8 secretion. (Kollisch et al., 2005).

Similar results were obtained within the ex-vivo skin models, which were used to represent more of a realistic model of human skin compared to keratinocyte cell lines alone with PS3 stimulating a higher CXCL8 secretion compared to PF showing that HaCaT cells can act in a similar manner when assembled onto a dermal scaffold which provided more of a 'keratinocyte like' environment. However, no differences in CXCL8 secretion were observed between PS3 grown in MM EtOH and MM Glu for 24 or 80 hours, which may be due to the increased sensitivity of the model used, due to the requirement of additional nutrients in the media and the cadaver origin of the dermis.

6.5.3 PS3 conditioned media and expression of MAMP receptors

Considering PS3 conditioned media stimulated CXCL8 secretion from HaCaT cells and differences in the amount of CXCL8 secreted was evident between growth conditions and time in culture it was hypothesised that there could be a

change in the expression of MAMP receptors on cultured keratinocytes in response to PS3 conditioned media as it is known that in some conditions stimulation of MAMP receptors leads to modification of their expression.

6.5.4 Toll like receptors in HaCaT cells

HaCaT cells have been identified by real time PCR (RT-PCR) to express MAMP receptors (also known as toll like receptors) with a similar pattern of expression to normal human keratinocytes (Kollisch et al., 2005). In this study, changes of expression of TLR 2 and 4 were investigated in response to *Pseudomonas* conditioned media. In addition the expression cluster of differentiation 14 (CD - 14), a co-receptor involved in LPS recognition (Halmer et al., 2015) was also investigated. All of these receptors have previously been reported to be expressed in HaCaT cells (Song et al., 2002, Pivarcsi et al., 2004).

Each of the TLRs investigated in this study can recognise specific MAMPs, which have been identified in *Pseudomonas* OMV's (Jan, 2017), due to cells been exposed to cell free media only, any changes in expression would most likely be mediated through the presence of MAMPs on OMVs.
The activation of TLR 4 could occur as LPS is present on OMVs and *Pseudomonas* can continuously shed LPS in its normal growth cycle (Kell and Pretorius, 2015) The role of LPS in activating TLR 2 and 4 has been disputed, with research showing that LPS activation of TLRs in normal epidermal keratinocytes is mostly mediated by TLR2 (Kawai et al., 2002) however in contrast other research has shown that LPS stimulates both TLR 4 and CD-14 and that activation of both of these resulted in CXCL8 secretion (Song et al., 2002).

In addition to LPS, TLR2 has been reported to be activated by PGN (Medzhitov, 2007) resulting in an increase in CXCL8 secretion from TLR 2 in normal human keratinocytes when challenged with PGN from *Staphylococcus aureus* (Mempel et al., 2003). However in contrast, other work has found low NF-κB activation upon stimulation with PGN from *Pseudomonas aeruginosa* in the human embryonic kidney cell line (HEK293T) (Travassos et al., 2004) indicating that TLR 2 activation by PGN may only be mediated by Gram positive PGN.

As well as being a co-receptor involved in LPS recognition, activation of CD-14 is involved in the activation of a range of toll like receptors to facilitate innate immune responses (Zanoni and Granucci, 2013), specifically CD14 interacts with TLR 7 and 9 to induce inflammatory responses in mice (Baumann et al., 2010).

There were some subtle changes in the expression of TLR 2, 4 and CD-14 receptors in HaCaT cells exposed to cell free conditioned media from some of the growth conditions found in this study, which are thought to be mediated by the presence of different MAMP ligands within the extracellular secretions from PS3. This suggests that the bacterial microenvironment may be able to alter the composition of certain MAMPs, which is supported by Pier (2007) who found variability in the lipid A structure of LPS depending on the culture environment of *Pseudomonas aeruginosa* which could influence TLR 4 mediated responses. Whilst there were some differences observed, due to lack of clarity seen in the histograms, it is difficult to draw conclusions regarding increased expression of these receptors mediated by the presence of the extracellular secretions from PS3 and more research is needed to investigate the role of other families of pattern recognition receptors and their role in mediating an immune response in keratinocytes.

6.5.5 Other pattern recognition receptors in HaCaT cells

Whilst this study focused primarily on the role of TLR's in HaCaT cells, there are other families of pattern recognitions receptors, as mentioned in section 1.2.1, and more generally other cell receptors which can interact with immunogenic and virulence factors of *Pseudomonas* which can mediate the host response and can potentially provide more insight into the biological effects on the HaCaT cells observed in this study.

Factors involved in the production of *Pseudomonas* biofilms can interact with mannose receptors belonging to the family of c-type lectin receptors, which have been found to be expressed on normal human keratinocytes (Szolnoky et al., 2001) with particular attention being paid to those where Psl is the predominant carbohydrate, due to Psl being rich in mannose. Psl from *Pseudomonas aeruginosa* biofilms was found to bind with mannose receptors present on dendritic cells which facilitated in their attachment, in addition dendritic cells treated with high molecular weight fractions taken from *Pseudomonas* biofilms showed higher levels of pro-inflammatory cytokine TGF alpha production (Singh et al., 2020). Additionally, other studies have found binding of macrophages to *Pseudomonas aeruginosa* biofilms via mannose receptors was increased when there was increased Psl expression (Rahman et al., 2015). In this study, genes involved in Psl production were increased in MM EtOH cultures after 80 hours which may have interacted with mannose receptors on the HaCaT cells in culture and may have been involved in the attachment to HaCaT cells as well as the generation of CXCL8 secretion.

In addition to mannose receptors, *Pseudomonas* can interact with NOD like receptors which have been identified as being involved in intracellular pattern recognition and mediation of immune responses in keratinocytes (Tervaniemi et al., 2016). The main NOD like receptors, NOD1 and 2 are involved in the recognition of peptidoglycan from Gram negative bacteria including *Pseudomonas aeruginosa* thought to be delivered by OMVs or through secretion systems and are involved in activation of NF- κB (Alhazmi, 2018) (Oviedo-Boyso et al., 2014) which can then act as a transcription factor for pro-inflammatory cytokines such as CXCL8, which may have contributed to CXCL8 secretion in this study. Additionally, in macrophages, other NOD like receptors have been found to be involved in the modulation of cell death pathways and pro-inflammatory cytokines in response to internalised flagellin from *Pseudomonas aeruginosa* (Franchi et al., 2009). Whilst this has been observed in macrophages, it is possible the HaCaT cells used in this study may share NOD like receptor signalling mechanisms which may have contributed to the loss of viable cells seen from some conditions.

As discussed here, *Pseudomonas* can interact with other host cell receptors which can recognise immunogenic and virulent factors presented both extra and intracellularly. These interactions can stimulate a variety of host responses including activation of cell death pathways and generation of innate immune responses. Additional experiments investigating the role of these receptors in keratinocytes would be beneficial to this work as it may provide more of insight into the observations made in this study and could provide more information regarding the role of the bacterial microenvironment on extracellular secretions and their effects within host cells.

6.6 Scratch assays as a model of wound healing

Cultured cell monolayers provide a quick and cost effective way to investigate wound healing, with one of the most common assays being the scratch assay which provides an easy method to mimic cell proliferation and migration in response to various agents (Liang et al., 2007). Whilst the scratch assay is a reductive model of wound healing, they can provide valuable information about the effects of biological and chemical agents on the ability of cell proliferation and migration, without the use of more sophisticated models or animal models. The ability to create a cell free area within the scratch assay can be done by two main methods; damaging the cell monolayer by biological equipment such as a pipette tip or the use of silicon inserts where cells are seeded around the insert which is then removed upon confluency (Poujade et al., 2007). Whilst the use of an insert can create a consistent cell free area, inserts can be expensive and there is no physical damage to the cell monolayer, in this study damaging of the cell monolayer was chosen as it allows cells to undergo damage and disrupt cell interactions near to the scratch border, mimicking damage that would be encountered in a real-life setting (Hulkower and Herber, 2011). Validation of the method was also performed to ensure that the initial size and small variations in producing the scratch did not affect the rate of closure, because the precise method used to produce the scratch can itself cause alterations to the environment of the cells which can impede cell migration and produce variable healing (Cormier et al., 2015).

6.6.1 *Pseudomonas* effects on wound healing

Much of the work focusing on *Pseudomonas* and wound healing has focused on the role of *Pseudomonas* virulence factors involved in attachment and the formation of biofilms in chronic wounds which have been associated with a detrimental effect on healing (Wolcott et al., 2008) However, there is little information regarding *Pseudomonas* virulence mechanisms involved in early colonisation of wounds or in acute infections, in addition the role of secreted extracellular products and their role in epithelial repair in early infection stages are poorly understood. In this study the effects of *Pseudomonas* secreted products on scratch closure in culture was assessed.

There are many factors that have been associated with promoting or attenuating wound healing including host contact with secreted products from *Pseudomonas* (Wang et al., 2018) as well as components of host defence mechanisms that can be mediated by *Pseudomonas* these include TLR activation and CXCL8 secretion (Chen and DiPietro, 2017). These have been discussed, along with their role in mediating responses from keratinocytes above however now the potential role of these factors in promoting or attenuating scratch closure will be considered.

6.6.1.1 Effects of *Pseudomonas* extracellular products on wound healing

As previously discussed specific secreted products have been identified in PS3 cultures by being directly measured specifically these were pyocyanins, proteases and hemolysins. Research investigating wound repair in airway epithelial cell lines found there was a decrease in cellular migration and proliferation in airway epithelial cells when cells were exposed to excreted

products from *Pseudomonas* (Ruffin et al., 2016) which could explain why, in this study, almost all scratches showed slower closure compared to those treated with non-conditioned media. For the virulence factors detected here only pyocyanins were found in the cell free media with proteases and hemolysins only being measured in cultures with live bacteria not in the cell free supernatant alone. Pyocyanins have been found to impair the wound healing process in normal skin fibroblasts where cells challenged with pyocyanin from *Pseudomonas aeruginosa* showed cell enlargement and began to induce early cellular senescence (Muller, 2006). Interestingly whilst proteases and hemolysins were only measured from live bacteria, and there were some significant differences seen in hemolysin production after 80 hours between conditions, scratches exposed to live bacteria grown for 80 hours did not show any differences in scratch closure indicating that hemolysin production did not play a role in closure in this model. However hemolysins and proteases have been identified as being important virulence factors in initiating other *Pseudomonas* infections such as in the lungs and corneas (Van Delden and Iglewski, 1998) rather than in skin infections and it has been found that clinical strains of *Pseudomonas aeruginosa* isolated from chronic wounds were deficient in protease and rhamnolipid production (Morgan et al., 2019). In addition, other research has found that proteases produced by *Pseudomonas* can degrade proteins found on skin fibroblasts and can inhibit their growth (Koziel and Potempa, 2013) so potentially in a more realistic model the effects of hemolysin and proteases from live bacteria could be more profound.

For PS3 exposed scratches, MM EtOH 80 hour conditioned media produced the greatest decrease in scratch closure, however, surprisingly there was not a clear reduction of cell viability that occurred when cells exposed to the same media

was analysed with a MTS assay. This is possibly due to the higher cell density and confluency used for the scratch assay. Interestingly, this culture condition did not consistently produce the greatest amount of each of the virulence factors measured which suggests it may be a combination of factors that affect scratch closure, or may be caused by other extracellular factors such as toxins that were not investigated in this study, for example exotoxin A, which has been found to delay wound healing in mice (Heggers et al., 1992).

Whilst overall there was delayed healing from scratches exposed to PS3 excreted products, for PF, some conditions produced faster scratch closure compared to control. In this context, it is noteworthy that PF did not produce detectable amounts of any of the virulence factors tested nor was there a large increase in cell proliferation measured by the MTS assay. These data indicate that in the presence of secreted products from a non-virulent strain of *Pseudomonas*, scratch closure may be accelerated and might indicate that in some conditions commensal bacteria could be beneficial to the wound healing process.

6.6.1.2 Effect of TLR activation and CXCL8 secretion on wound healing

As previously discussed cell free conditioned media can stimulate some TLRs in cultured keratinocytes and previous research has shown that activation of TLRs and their stimulatory molecules, could either improve or impair the healing of a wound depending on the manner and timing in which the inflammatory responses were initiated (Dasu and Isseroff, 2012). The TLRs investigated in this study were TLR 2, 4 and the co-stimulatory receptor CD-14, with CXCL8 secretion also quantified as a measure of the initiation of immune response. Differences in the cellular responses on the exposure to media from different conditions and culture

times occurred which could explain the subtle differences seen between scratch closure.

It is thought that TLR activation is likely to play a role in influencing wound repair due to damage that occurs as a result of wounding and the likelihood that there will be microbial contamination (Chen and DiPietro, 2017). Some research has shown that the activation of TLRs can positively influence wound healing, such as the activation of intracellular TLRs such as TLR 3 and TLR 9 by their respective ligands (Dasu and Isseroff, 2012). For the extra-cellular TLRs and co receptors highlighted in this study, TLR4 has been shown to influence early wound healing with increased expression of TLR 4 in keratinocytes leading to increased cytokine release from keratinocytes from 6 hours to up to 3 days post wounding (Chen, 2012). However other research has found that TLR 4 can negatively impact wound healing, particularly in response to a thermal injury (Breslin et al., 2008). Research investigating models of wound healing using knock out mice has found that TLR 2 and TLR 4 deficient mice had decreased wound healing compared to wild type mice (Suga et al., 2014). However, in a similar model using wounded diabetic mice it was shown that activation of TLR 2 and TLR 4 by their ligands resulted in accelerated healing compared to control wild type animals (Dasu et al., 2010).

Stimulation of TLR's investigated in this study is likely to be mainly through the presence of LPS in the cell free conditioned media however the reported role of LPS itself on wound healing is mixed. Research has shown that LPS can positively impact wound healing by stimulating epidermal growth factor receptors (EGFR) which can promote wound healing (Koff et al., 2006) however this was

only found when airway epithelial cells were challenged with low concentrations of LPS, with high concentrations of LPS found to be detrimental to healing.

Interestingly, whilst some bacterial growth conditions tested here produced an increased expression of the TLRs investigated, an effect that is probably mediated by the presence of LPS, it was only PF conditioned media treated cells that showed accelerated healing above control indicating that LPS present in the PS3 conditioned media does not accelerate wound healing above control. In addition, whilst there was increased expression of TLR 2, 4 and CD-14 expression from SWF and MM Glu 24 hour cultures there was no significant differences in scratch closure between these conditions and for MM EtOH. There was no change in the expression of TLR 2, 4 or CD-14 in response to PS3 grown in MM EtOH for 24 or 80 hours thus the lack of scratch closure from the 80 hour media cannot be attributed a change in expression of each of the TLRs as full closure was seen from the control.

6.6.1.3 Cytokine secretion and wound healing

As previously discussed exposure of keratinocytes to cell free conditioned media from PS3 can stimulate significant secretion of the pro-inflammatory cytokines. Imbalances in cytokine release during wound healing can cause alterations to the normal wound healing process and result in ineffective healing. CXCL8 has been identified as having an important role in the progression of wound healing, particularly in the initial inflammatory stages (Ridiandries et al., 2018) however there is little information regarding the role of CXCL8 in cutaneous wound healing or the effects of CXCL8 on keratinocytes (Jiang et al., 2012).

Research carried out on the comparison of tissue biopsies from patients with burns found CXLC8 secretion to be significantly higher in unhealed wounds compared to healed wounds and to normal skin and in cell line experiments performed within the same study, exposure of epidermal keratinocytes to high concentrations of CXLC8 (100ng/ml) caused a decrease in cell replication compared to control. At low concentrations (1ng/ml) exposure of keratinocytes to CXCL8 showed an increase in proliferation (Iocono et al., 2000) which indicates that at low concentrations, CXCL8 secretion may positively impact wound closure.

By contrast, other research has shown that tissues isolated from chronic wounds had a low expression of CXCL8 receptors, compared to acute wounds as assessed by mRNA analysis, however these differences were not significant. In cell line experiments using HaCaT cells, exposure to recombinant human CXCL8 produced increased cell migration over a period of 6 hours post wounding compared to control (Jiang et al., 2012) .

In this study it was shown that whilst CXLC8 secretion was high from keratinocytes exposed to cell free conditioned media from PS3 grown for 24 hours, it was only PF conditioned media that showed wound closure faster than control indicating that low concentrations of CXCL8 can potentially increase wound healing as supported by findings described above (Iocono et al., 2000) however as this was only seen for SWF cultures, not MM EtOH cultures despite CXCL8 secretion being similar, it indicates that potentially there may be other pro-inflammatory cytokines involved that were not investigated in this study such as CXCL1, which are important in keratinocyte migration (Raja et al., 2007).

For PS3, at 24 hours both MM Glu and MM EtOH produced a larger CXCL8 secretion than SWF cultures however in the scratch assay there were no significant differences in closure between these conditions. For live PS3, CXCL8 secretion from keratinocytes was generally lower than that produced by cell free conditioned media, particularly from 80 hour cultures of MM EtOH and MM Glu, with CXLC8 secretion being similar to that of the control. Scratches exposed to live PS3 from these conditions, showed an initial slightly faster closure compared to HBSS only resulting in a greater healing at the end of day 3, showing that whilst the presence of cell stress may have had an initial stimulatory effect on the cells, this is not mediated by CXCL8.

Because of the more complex physiology of the ex-vivo skin model and the different mechanisms required for 'wounding' this model was generally more sensitive than the cell monolayers as some of the subtle differences between conditions previously observed did not occur. However differences in the re-epithelisation between conditioned media and non-conditioned were observed showing that PS3 conditioned media can cause impaired wound healing in a more-realistic skin model, even though differences between the different PS3 growth conditions were not apparent.

It has been previously reported that exposure of HaCaT cells to CXCL8 increased cell migration (Jiang et al., 2012). This was not found in this study, however here the focus was on the role of EC secretions and scratch closure rather than the direct application of CXCL8 to HaCaT cells. Whilst CXCL8 was secreted from HaCaT cells exposed to most conditions tested here, the presence of immunogenic and virulent factors in the EC secretions and the reduced FBS in the media resulted in no migration, with controls also showing minimal migration.

In addition, much of the research in this area has focused on the stimulation of migration in the scratch assay and investigated the promotion of scratch closure rather than its attenuation and as such, images are often obtained up to 12 hours after wounding (Cormier et al., 2015, Justus et al., 2014) meaning potentially any increase in migration in this study was not seen due to scratches being left for extended periods in low serum conditions.

6.7 Future work

One of the major limitations of this work was the use of only one clinical isolate of *Pseudomonas aeruginosa,* due to limitations in obtaining other clinical isolates from wound bandages. Due to this, the clinical relevance of results obtained in this study may be limited in their applications. To overcome this it would be beneficial to firstly investigate the role of growth conditions in another clinical isolate of *Pseudomonas aeruginosa* and then to continue this further with another clinical isolate of a different species of bacteria such as strains of *E.coli*. In addition to this limitation, infections that occur within the skin are often multi-species infections (Yadav et al., 2017, Percival and Bowler, 2004) so the limitations of challenging keratinocytes in culture with bacterial products from one species may not be realistic in mimicking what would occur in a more real life setting.

Another limitation of this work is the reductive nature of the models used to study skin host immune responses. Whilst this work focused on the use of monolayers of HaCaT cells as a model of skin, some key experiments were performed using ex-vivo skin models however these are still reductive in terms of mimicking a real-life infection setting and due associated costs and extensive timings needed to

develop stratified skin models more long term experiments were needed in this study, particularly in terms of wound healing.

6.8 Overall summary and key findings

Pseudomonas is a highly versatile pathogen that is associated with human skin infections and can significantly impact the pathogenesis of disease, particularly those strains that commonly reside in clinical environments due to their opportunistic nature and increased resistance to both antibiotics and biocidal compounds. The versatility of *Pseudomonas* allows it to undergo genetic and phenotypic changes to adapt to different environmental conditions (Moradali et al., 2017) the investigation of which was the main focus of this study. In this study the effects of growth condition on two *Pseudomonas* strains (a clinical isolate, PS3, and laboratory reference strain, PF) were investigated both directly through the quantification of select virulence factors and through the effects of secreted factors from the bacteria on the human skin cell line HaCaT.

PS3 grown in ethanol showed increased biofilm, pyocyanin and hemolysin production, particularly after 80 hours in culture which indicates that ethanol can alter bacterial cell physiology, as supported by Smith et al. (2004). The effect of ethanol was not observed in the laboratory reference strain which indicates that ethanol alone cannot promote the production of virulence factors, rather that virulence mechanisms may need to-pre-exist.

The alteration of bacterial cell physiology by ethanol was also further evident in the cell line experiments where full cytotoxicity occurred consistently following 80 hour culture times and in the scratch assay, where PS3 grown in MM EtOH for 80 hours produced the biggest delay in scratch closure, which indicates an

increase in virulent extracellular factors causing cell stress, which did not occur in response to bacteria grown in other conditions. When CXCL8 secretion was quantified from HaCaT cells, both the 1:5 and 1:10 dilution produced greater CXCL8 secretion compared to glucose and ethanol 24 hour cultures, indicating that in addition to virulence factors, immunogenic factors may also be altered by exposure of the bacteria to ethanol. Despite the high CXCL8 secretion investigation into the expression of MAMP receptors in the keratinocytes showed no change in any of the receptors selected in this study indicating this effect is probably mediated via a different MAMP receptor. The decrease in CXCL8 secretion seen in response to PS3 grown in MM Glu from 80 hour cultures, which was also correlated with a decreased MAMP expression, indicates that production of immunogenic factors is decreased in this condition, possibly due to the reduction in the concentration of glucose in the media.

The results of this study indicate that bacterial culture conditions can have an effect on the bacterial cell attached and secreted virulence and immunogenic factors associated in *Pseudomonas aeruginosa*, with time in culture also having an effect with these differences primarily occurring PS3 that were grown in ethanol conditioned media for 80 hours. Considering the striking effects seen from these conditions including the increased production of certain virulence factors in conjunction with the high level of toxicity in the HaCaT cells and longer healing times in the scratch assay, it indicates that prolonged exposure to ethanol within the bacterial microenvironment can alter microbial physiology through the production of pathogenic and virulent factors, particularly within clinical isolates, as PS3 was originally isolated from a discarded wound dressing, and these effects were not seen in the *fluorescens* strain. As *Pseudomonas aeruginosa* is

becoming increasingly recognised as an opportunistic pathogen that is known to reside in clinical environments with increasing antibiotic resistance found within the species. Additionally, there is increasing wide spread use of ethanol based products, particularly for disinfection of the hands which can then be transferred to high contact areas such as door handles and call buttons it is plausible that these opportunistic bacteria are being exposed to ethanol within their environment over prolonged periods of time. Taking into account the findings of this study, the improper use of ethanol-based products within clinical environments may potentially be leading to the new emergence of antibiotic and biocide resistant bacteria.

Chapter 7
References

ABDALLAH, F., MIJOUIN, L. & PICHON, C. 2017. Skin Immune Landscape: Inside and Outside the Organism %J Mediators of Inflammation. 2017**,** 17.

ABISADO, R. G., BENOMAR, S., KLAUS, J. R., DANDEKAR, A. A. & CHANDLER, J. R. 2018. Bacterial Quorum Sensing and Microbial Community Interactions. *mBio,* 9**,** e02331-17.

AKBAR, S. 2016. *Gram Negative Bacterial Biofilm Formation and Characterisation of Extracellular Polymeric Substances.* Doctor of Philosophy, University of Huddersfield.

AKBAR, S., ROUT, S. P. & HUMPHREYS, P. N. 2015. Draft Genome Sequences of Pseudomonas aeruginosa Strain PS3 and Citrobacter freundii Strain SA79 Obtained from a Wound Dressing-Associated Biofilm. *Genome announcements,* 3**,** e00561-15.

AL-WRAFY, F., BRZOZOWSKA, E., GORSKA, S. & GAMIAN, A. 2017. Pathogenic factors of Pseudomonas aeruginosa - the role of biofilm in pathogenicity and as a target for phage therapy. *Postepy Hig Med Dosw (Online),* 71**,** 78-91.

AL TAMEEMI, W., DUNNILL, C., HUSSAIN, O., KOMEN, M. M., VAN DEN HURK, C. J., COLLETT, A. & GEORGOPOULOS, N. T. 2014. Use of in vitro human keratinocyte models to study the effect of cooling on chemotherapy drug-induced cytotoxicity. *Toxicoloy in Vitro,* 28**,** 1366-1376.

ALHAZMI, A. 2018. NOD-like receptor(s) and host immune responses with Pseudomonas aeruginosa infection. *Inflammation Research,* 67**,** 479-493.

ALLEN, L., DOCKRELL, D. H., PATTERY, T., LEE, D. G., CORNELIS, P., HELLEWELL, P. G. & WHYTE, M. K. 2005. Pyocyanin production by Pseudomonas aeruginosa induces neutrophil apoptosis and impairs neutrophil-mediated host defenses in vivo. *J Immunol,* 174**,** 3643-9.

ALTENBERN, R. A. 1965. FORMATION OF HEMOLYSIN BY STRAINS OF PSEUDOMONAS AERUGINOSA. *Canadian Journal of Microbiology,* 12**,** 231-241.

ALVES, M. P., SALGADO, R. L., ELLER, M. R., VIDIGAL, P. M. P. & FERNANDES DE CARVALHO, A. 2016. Characterization of a heat-resistant extracellular protease from Pseudomonas fluorescens 07A shows that low temperature treatments are more effective in deactivating its proteolytic activity. *Journal of Dairy Science,* 99**,** 7842-7851.

AMARANTE-MENDES, G. P., ADJEMIAN, S., BRANCO, L. M., ZANETTI, L. C., WEINLICH, R. & BORTOLUCI, K. R. 2018. Pattern Recognition Receptors and the Host Cell Death Molecular Machinery. *Frontiers in Immunology,* 9.

ANAND, D. & CHAUDHURI, A. 2016. Bacterial outer membrane vesicles: New insights and applications. *Molecular Membrane Biology,* 33**,** 125-137.

ANDONOVA, M. & URUMOVA, V. 2013. Immune surveillance mechanisms of the skin against the stealth infection strategy of Pseudomonas aeruginosa-review. *Comp Immunol Microbiol Infect Dis,* 36**,** 433-48.

ANTUNES, L. C., FERREIRA, R. B., BUCKNER, M. M. & FINLAY, B. B. 2010. Quorum sensing in bacterial virulence. *Microbiology,* 156**,** 2271-82.

ARMITANO, J., MEJEAN, V. & JOURLIN-CASTELLI, C. 2014. Gram-negative bacteria can also form pellicles. *Environ Microbiol Rep,* 6**,** 534-44.

ARPAIA, N. & BARTON, G. M. 2013. The impact of Toll-like receptors on bacterial virulence strategies. *Curr Opin Microbiol,* 16**,** 17-22.

ASHRAFI, M., HAGUE, A., BAGUNEID, M., ALONSO-RASGADO, T. & BAYAT, A. 2018. 9 - Wound healing and cutaneous scarring models of the human skin. *In:* MARQUES, A. P., PIRRACO, R. P., CERQUEIRA, M. T. & REIS, R. L. (eds.) *Skin Tissue Models.* Boston: Academic Press.

BAKER, B. S., OVIGNE, J. M., POWLES, A. V., CORCORAN, S. & FRY, L. 2003. Normal keratinocytes express Toll-like receptors (TLRs) 1, 2 and 5: modulation of TLR expression in chronic plaque psoriasis. *British Journal of Dermatology,* 148**,** 670-679.

BAKER, P., HILL, P. J., SNARR, B. D., ALNABELSEYA, N., PESTRAK, M. J., LEE, M. J., JENNINGS, L. K., TAM, J., MELNYK, R. A., PARSEK, M. R., SHEPPARD, D. C., WOZNIAK, D. J. & HOWELL, P. L. 2016. Exopolysaccharide biosynthetic glycoside hydrolases can be utilized to disrupt and prevent Pseudomonas aeruginosa biofilms. *Sci Adv,* 2**,** e1501632.

BALLOK, A. E. & O'TOOLE, G. A. 2013. Pouring salt on a wound: Pseudomonas aeruginosa virulence factors alter Na+ and Cl- flux in the lung. *J Bacteriol,* 195**,** 4013-9.

BANIN, E., VASIL, M. L. & GREENBERG, E. P. 2005. Iron and Pseudomonas aeruginosa biofilm formation. *Proc Natl Acad Sci U S A,* 102**,** 11076-81.

BARONI, A., BUOMMINO, E., DE GREGORIO, V., RUOCCO, E., RUOCCO, V. & WOLF, R. 2012. Structure and function of the epidermis related to barrier properties. *Clin Dermatol,* 30**,** 257-62.

BARRÉ-SINOUSSI, F. & MONTAGUTELLI, X. 2015. Animal models are essential to biological research: issues and perspectives. *Future science OA,* 1**,** FSO63-FSO63.

BARRILA, J., CRABBÉ, A., YANG, J., FRANCO, K., NYDAM, S. D., FORSYTH, R. J., DAVIS, R. R., GANGARAJU, S., OTT, C. M., COYNE, C. B., BISSELL, M. J. & NICKERSON, C. A. 2018. Modeling Host-Pathogen Interactions in the Context of the Microenvironment: Three-Dimensional Cell Culture Comes of Age. *Infection and Immunity,* 86**,** e00282-18.

BARTON, G. M. & KAGAN, J. C. 2009. A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nat Rev Immunol,* 9**,** 535-42.

BASSETTI, M., VENA, A., CROXATTO, A., RIGHI, E. & GUERY, B. 2018. How to manage Pseudomonas aeruginosa infections. *Drugs in context,* 7**,** 212527-212527.

BAUMANN, C. L., ASPALTER, I. M., SHARIF, O., PICHLMAIR, A., BLUML, S., GREBIEN, F., BRUCKNER, M., PASIERBEK, P., AUMAYR, K., PLANYAVSKY, M., BENNETT, K. L., COLINGE, J., KNAPP, S. & SUPERTI-FURGA, G. 2010. CD14 is a coreceptor of Toll-like receptors 7 and 9. *J Exp Med,* 207**,** 2689-701.

BECEIRO, A., TOMAS, M. & BOU, G. 2013. Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clin Microbiol Rev,* 26**,** 185-230.

BELL, E., EHRLICH, H. P., BUTTLE, D. J. & NAKATSUJI, T. 1981. Living tissue formed in vitro and accepted as skin-equivalent tissue of full thickness. *Science,* 211**,** 1052.

BERK, R. S. 1962. Production and characterization of Pseudomon as aeruginosa hemolysin. *J Bacteriol,* 84**,** 1041-8.

BERNARD, F. X., MOREL, F., CAMUS, M., PEDRETTI, N., BARRAULT, C., GARNIER, J. & LECRON, J. C. 2012. Keratinocytes under Fire of Proinflammatory Cytokines: Bona Fide Innate Immune Cells Involved in the Physiopathology of Chronic Atopic Dermatitis and Psoriasis. *J Allergy (Cairo),* 2012**,** 718725.

BEVERIDGE, T. J. 1999. Structures of gram-negative cell walls and their derived membrane vesicles. *J Bacteriol,* 181**,** 4725-33.

BHAGIRATH, A. Y., LI, Y., SOMAYAJULA, D., DADASHI, M., BADR, S. & DUAN, K. 2016. Cystic fibrosis lung environment and Pseudomonas aeruginosa infection. *BMC pulmonary medicine,* 16**,** 174-174.

BITTO, N. J., BAKER, P. J., DOWLING, J. K., WRAY-MCCANN, G., DE PAOLI, A., TRAN, L. S., LEUNG, P. L., STACEY, K. J., MANSELL, A., MASTERS, S. L. & FERRERO, R. L. 2018. Membrane vesicles from Pseudomonas aeruginosa activate the noncanonical inflammasome through caspase-5 in human monocytes. *Immunol Cell Biol,* 96**,** 1120-1130.

BNYAN, I. & AHMED, H. 2013. Effect of some factors on extracellular hemolysin filtrate from bacterial Pseudomonas aeruginosa isolated from burn infection in Hilla city. *Research in Pharmacy,* 3**,** 26-32.

BOCHEŃSKA, K., SMOLIŃSKA, E., MOSKOT, M., JAKÓBKIEWICZ-BANECKA, J. & GABIG-CIMIŃSKA, M. 2017. Models in the Research Process of Psoriasis. *International journal of molecular sciences,* 18**,** 2514.

BODEY, G. P., BOLIVAR, R., FAINSTEIN, V. & JADEJA, L. 1983. Infections Caused by Pseudomonas aeruginosa. *Clinical Infectious Diseases,* 5**,** 279-313.

BOELSMA, E., VERHOEVEN, M. C. & PONEC, M. 1999. Reconstruction of a human skin equivalent using a spontaneously transformed keratinocyte cell line (HaCaT). *J Invest Dermatol,* 112**,** 489-98.

BOMBERGER, J. M., MACEACHRAN, D. P., COUTERMARSH, B. A., YE, S., O'TOOLE, G. A. & STANTON, B. A. 2009. Long-Distance Delivery of Bacterial Virulence Factors by Pseudomonas aeruginosa Outer Membrane Vesicles. *PLOS Pathogens,* 5**,** e1000382.

BORISH, L. C. & STEINKE, J. W. 2003. 2. Cytokines and chemokines. *J Allergy Clin Immunol,* 111**,** S460-75.

BOTOS, I., SEGAL, D. M. & DAVIES, D. R. 2011. The structural biology of Toll-like receptors. *Structure,* 19**,** 447-59.

BOWLER, P. G., DUERDEN, B. I. & ARMSTRONG, D. G. 2001. Wound microbiology and associated approaches to wound management. *Clin Microbiol Rev,* 14**,** 244-69.

BOYCE, J. M. 2007. Environmental contamination makes an important contribution to hospital infection. *Journal of Hospital Infection,* 65**,** 50-54.

BRAFF, M. H., BARDAN, A., NIZET, V. & GALLO, R. L. 2005. Cutaneous Defense Mechanisms by Antimicrobial Peptides. *Journal of Investigative Dermatology,* 125**,** 9-13.

BRAT, D. J., BELLAIL, A. C. & VAN MEIR, E. G. 2005. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro Oncol,* 7**,** 122-33.

BRESLIN, J. W., WU, M. H., GUO, M., REYNOSO, R. & YUAN, S. Y. 2008. TOLL-LIKE RECEPTOR 4 CONTRIBUTES TO MICROVASCULAR INFLAMMATION AND BARRIER DYSFUNCTION IN THERMAL INJURY. *Shock,* 29**,** 349-355.

BROHEM, C. A., CARDEAL, L. B., TIAGO, M., SOENGAS, M. S., BARROS, S. B. & MARIA-ENGLER, S. S. 2011. Artificial skin in perspective: concepts and applications. *Pigment Cell Melanoma Res,* 24**,** 35-50.

BUCIOR, I., PIELAGE, J. F. & ENGEL, J. N. 2012. Pseudomonas aeruginosa Pili and Flagella Mediate Distinct Binding and Signaling Events at the Apical and Basolateral Surface of Airway Epithelium. *PLOS Pathogens,* 8**,** e1002616.

BUXTON, R. 2005. BLOOD AGAR PLATES AND HEMOLYSIS PROTOCOLS. *American Society for Microbiology*.

CA, #XF1, EDO-DORANTES, L., CA, #XF1 & EDO-AYALA, M. 2019. Skin Acute Wound Healing: A Comprehensive Review %J International Journal of Inflammation. 2019**,** 15.

CAPITA, R., VICENTE-VELASCO, M., RODRÍGUEZ-MELCÓN, C., GARCÍA-FERNÁNDEZ, C., CARBALLO, J. & ALONSO-CALLEJA, C. 2019. Effect of low doses of biocides on the antimicrobial resistance and the biofilms of Cronobacter sakazakii and Yersinia enterocolitica. *Scientific Reports,* 9**,** 15905.

CASADEVALL, A. & PIROFSKI, L. A. 1999. Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infect Immun,* 67**,** 3703-13.

CASILAG, F., LORENZ, A., KRUEGER, J., KLAWONN, F., WEISS, S. & HÄUSSLER, S. 2015. The LasB Elastase of Pseudomonas aeruginosa Acts in Concert with Alkaline Protease AprA To Prevent Flagellin-Mediated Immune Recognition. *Infection and immunity,* 84**,** 162-171.

CAVAILLON, J. M. 2001. Pro- versus anti-inflammatory cytokines: myth or reality. *Cell Mol Biol (Noisy-le-grand),* 47**,** 695-702.

CEZAIRLIYAN, B. & AUSUBEL, F. M. 2017. Investment in secreted enzymes during nutrient-limited growth is utility dependent. *Proceedings of the National Academy of Sciences,* 114**,** E7796.

CHANDEL, H. S., PANDEY, S. P., SHUKLA, D., LALSARE, K., SELVARAJ, S. K., JHA, M. K. & SAHA, B. 2014. Toll-like receptors and CD40 modulate each other's expression affecting Leishmania major infection. *Clinical & Experimental Immunology,* 176**,** 283-290.

CHANG, C. Y. 2017. Surface Sensing for Biofilm Formation in Pseudomonas aeruginosa. *Front Microbiol,* 8**,** 2671.

CHATTOPADHYAY, P. K., ROEDERER, M. & BOLTON, D. L. 2018. A deadly dance: the choreography of host–pathogen interactions, as revealed by single-cell technologies. *Nature Communications,* 9**,** 4638.

CHEN, A. I., DOLBEN, E. F., OKEGBE, C., HARTY, C. E., GOLUB, Y., THAO, S., HA, D. G., WILLGER, S. D., O'TOOLE, G. A., HARWOOD, C. S., DIETRICH, L. E. & HOGAN, D. A. 2014. Candida albicans ethanol stimulates Pseudomonas aeruginosa WspR-controlled biofilm formation as part of a cyclic relationship involving phenazines. *PLoS Pathog,* 10**,** e1004480.

CHEN, H., WUBBOLTS, R. W., HAAGSMAN, H. P. & VELDHUIZEN, E. J. A. 2018. Inhibition and Eradication of Pseudomonas aeruginosa Biofilms by Host Defence Peptides. *Scientific Reports,* 8**,** 10446.

CHEN, L. & DIPIETRO, L. A. 2017. Toll-Like Receptor Function in Acute Wounds. *Advances in wound care,* 6**,** 344-355.

CHEN, Y. 2012. Scratch Wound Healing Assay. *Bio-protocol,* 2**,** e100.

CHOI, D. S., KIM, D. K., CHOI, S. J., LEE, J., CHOI, J. P., RHO, S., PARK, S. H., KIM, Y. K., HWANG, D. & GHO, Y. S. 2011. Proteomic analysis of outer membrane vesicles derived from Pseudomonas aeruginosa. *Proteomics,* 11**,** 3424-9.

CICCHESE, J. M., EVANS, S., HULT, C., JOSLYN, L. R., WESSLER, T., MILLAR, J. A., MARINO, S., CILFONE, N. A., MATTILA, J. T., LINDERMAN, J. J. & KIRSCHNER, D. E. 2018. Dynamic balance of pro- and anti-inflammatory signals controls disease and limits pathology. *Immunol Rev,* 285**,** 147-167.

CLARK, E. C., PATEL, S. D., CHADWICK, P. R., WARHURST, G., CURRY, A. & CARLSON, G. L. 2003. Glutamine deprivation facilitates tumour necrosis factor induced bacterial translocation in Caco-2 cells by depletion of enterocyte fuel substrate. *Gut,* 52**,** 224-230.

COATES, M., BLANCHARD, S. & MACLEOD, A. S. 2018. Innate antimicrobial immunity in the skin: A protective barrier against bacteria, viruses, and fungi. *PLoS Pathog,* 14**,** e1007353.

COLOMBO, I., SANGIOVANNI, E., MAGGIO, R., MATTOZZI, C., ZAVA, S., CORBETT, Y., FUMAGALLI, M., CARLINO, C., CORSETTO, P. A., SCACCABAROZZI, D., CALVIERI, S., GISMONDI, A., TARAMELLI, D., DELL, #X & AGLI, M. 2017. HaCaT Cells as a Reliable In Vitro Differentiation Model to Dissect the Inflammatory/Repair Response of Human Keratinocytes %J Mediators of Inflammation. 2017**,** 12.

CONRAD, J. C., GIBIANSKY, M. L., JIN, F., GORDON, V. D., MOTTO, D. A., MATHEWSON, M. A., STOPKA, W. G., ZELASKO, D. C., SHROUT, J. D. & WONG, G. C. 2011. Flagella and pili-mediated near-surface single-cell motility mechanisms in P. aeruginosa. *Biophys J,* 100**,** 1608-16.

COOKE, A. C., NELLO, A. V., ERNST, R. K. & SCHERTZER, J. W. 2019. Analysis of Pseudomonas aeruginosa biofilm membrane vesicles supports multiple mechanisms of biogenesis. *PloS one,* 14**,** e0212275-e0212275.

CORMIER, N., YEO, A., FIORENTINO, E. & PAXSON, J. 2015. Optimization of the Wound Scratch Assay to Detect Changes in Murine Mesenchymal Stromal Cell Migration After Damage by Soluble Cigarette Smoke Extract. *Journal of visualized experiments : JoVE***,** e53414-e53414.

CORZO-LEÓN, D. E., MUNRO, C. A. & MACCALLUM, D. M. 2019. An ex vivo Human Skin Model to Study Superficial Fungal Infections. *Frontiers in Microbiology,* 10.

COWELL, B. A., CHEN, D. Y., FRANK, D. W., VALLIS, A. J. & FLEISZIG, S. M. J. 2000. ExoT of Cytotoxic Pseudomonas aeruginosaPrevents Uptake by Corneal Epithelial Cells. *Infection and Immunity,* 68**,** 403.

CROSS, S., GON, G., MORRISON, E., AFSANA, K., ALI, S. M., MANJANG, T., MANNEH, L., RAHMAN, A., SAXENA, D., VORA, K. & GRAHAM, W. J. 2019. An invisible workforce: the neglected role of cleaners in patient safety on maternity units. *Global health action,* 12**,** 1480085-1480085.

DABBOUE, H., BUILLES, N., FROUIN, #XC9, RIC, SCOTT, D., RAMOS, J. & MARTI-MESTRES, G. 2015. Assessing the Impact of Mechanical Damage on Full-Thickness Porcine and Human Skin Using an In Vitro Approach %J BioMed Research International. 2015**,** 10.

DANCER, S. J. 1999. Mopping up hospital infection. *J Hosp Infect,* 43**,** 85-100.

DANCER, S. J. 2014. Controlling hospital-acquired infection: focus on the role of the environment and new technologies for decontamination. *Clin Microbiol Rev,* 27**,** 665-90.

DASU, M. R., DEVARAJ, S., PARK, S. & JIALAL, I. 2010. Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects. *Diabetes Care,* 33**,** 861-8.

DASU, M. R. & ISSEROFF, R. 2012. Toll-Like Receptors in Wound Healing: Location, Accessibility, and Timing. *J Invest Dermatol,* 132**,** 1955-1958.

DE KIEVIT, T. R. 2009. Quorum sensing in Pseudomonas aeruginosa biofilms. *Environmental Microbiology,* 11**,** 279-288.

DENNING, G. M., WOLLENWEBER, L. A., RAILSBACK, M. A., COX, C. D., STOLL, L. L. & BRITIGAN, B. E. 1998. Pseudomonas pyocyanin increases interleukin-8 expression by human airway epithelial cells. *Infect Immun,* 66**,** 5777-84.

DENYER, S. P. & STEWART, G. S. A. B. 1998. Mechanisms of action of disinfectants. *International Biodeterioration & Biodegradation,* 41**,** 261-268.

DEYRIEUX, A. F. & WILSON, V. G. 2007. In vitro culture conditions to study keratinocyte differentiation using the HaCaT cell line. *Cytotechnology,* 54**,** 77-83.

DIGGLE, S. P., GRIFFIN, A. S., CAMPBELL, G. S. & WEST, S. A. 2007. Cooperation and conflict in quorum-sensing bacterial populations. *Nature,* 450**,** 411-4.

DINARELLO, C. A. 2000. Proinflammatory cytokines. *Chest,* 118**,** 503-508.

DONLAN, R. M. 2002. Biofilms: microbial life on surfaces. *Emerg Infect Dis,* 8**,** 881-90.

DONNARUMMA, G., BUOMMINO, E., FUSCO, A., PAOLETTI, I., AURICCHIO, L. & TUFANO, M. A. 2010. Effect of temperature on the shift of Pseudomonas fluorescens from an environmental microorganism to a potential human pathogen. *Int J Immunopathol Pharmacol,* 23**,** 227-34.

DOXEY, A. C., MANSFIELD, M. J. & LOBB, B. 2019. Exploring the Evolution of Virulence Factors through Bioinformatic Data Mining. *mSystems,* 4**,** e00162-19.

EDWARDS, A. M., POTTER, U., MEENAN, N. A. G., POTTS, J. R. & MASSEY, R. C. 2011. Staphylococcus aureus keratinocyte invasion is dependent upon multiple high-affinity fibronectin-binding repeats within FnBPA. *PloS one,* 6**,** e18899-e18899.

EL ZOWALATY, M. E., AL THANI, A. A., WEBSTER, T. J., EL ZOWALATY, A. E., SCHWEIZER, H. P., NASRALLAH, G. K., MAREI, H. E. & ASHOUR, H. M. 2015. Pseudomonas aeruginosa: arsenal of resistance mechanisms, decades of changing resistance profiles, and future antimicrobial therapies. *Future Microbiol,* 10**,** 1683-706.

ELIAS, S. & BANIN, E. 2012. Multi-species biofilms: living with friendly neighbors. *FEMS Microbiol Rev,* 36**,** 990-1004.

ELLIS, T. N., LEIMAN, S. A. & KUEHN, M. J. 2010. Naturally produced outer membrane vesicles from Pseudomonas aeruginosa elicit a potent innate immune response via combined sensing of both lipopolysaccharide and protein components. *Infect Immun,* 78**,** 3822-31.

ENOCH, S. & LEAPER, D. J. 2005. Basic science of wound healing. *Surgery - Oxford International Edition,* 23**,** 37-42.

ENOCH, S. & PRICE, P. 2004. Cellular, molecular and biochemical differences in the pathophysiology of healing between acute wounds, chronic wounds and wounds in the aged. *World Wide Wounds,* 2005.

ESEN, M., GRASSMÉ, H., RIETHMÜLLER, J., RIEHLE, A., FASSBENDER, K. & GULBINS, E. 2001. Invasion of human epithelial cells by Pseudomonas aeruginosa involves src-like tyrosine kinases p60Src and p59Fyn. *Infection and immunity,* 69**,** 281-287.

EVANS, D. J., KUO, T. C., KWONG, M., VAN, R. & FLEISZIG, S. M. J. 2002. Mutation of csk, encoding the C-terminal Src kinase, reduces Pseudomonas aeruginosa internalization by mammalian cells and enhances bacterial cytotoxicity. *Microbial Pathogenesis,* 33**,** 135-143.

FAZELI, H., AKBARI, R., MOGHIM, S., NARIMANI, T., ARABESTANI, M. R. & GHODDOUSI, A. R. 2012. Pseudomonas aeruginosa infections in patients, hospital means, and personnel's specimens. *J Res Med Sci,* 17**,** 332-7.

FELEKE, T., ESHETIE, S., DAGNEW, M., ENDRIS, M., ABEBE, W., TIRUNEH, M. & MOGES, F. 2018. Multidrug-resistant bacterial isolates from patients suspected of nosocomial infections at the University of Gondar Comprehensive Specialized Hospital, Northwest Ethiopia. *BMC Research Notes,* 11**,** 602.

FLEMMING, H.-C., NEU, T. R. & WOZNIAK, D. J. 2007. The EPS matrix: the "house of biofilm cells". *Journal of bacteriology,* 189**,** 7945-7947.

FORBES, S., DOBSON, C. B., HUMPHREYS, G. J. & MCBAIN, A. J. 2014. Transient and sustained bacterial adaptation following repeated sublethal exposure to microbicides and a novel human antimicrobial peptide. *Antimicrobial agents and chemotherapy,* 58**,** 5809-5817.

FRANCHI, L., WARNER, N., VIANI, K. & NUÑEZ, G. 2009. Function of Nod-like receptors in microbial recognition and host defense. *Immunological reviews,* 227**,** 106-128.

FREDERICK, M. R., KUTTLER, C., HENSE, B. A. & EBERL, H. J. 2011. A mathematical model of quorum sensing regulated EPS production in biofilm communities. *Theoretical biology & medical modelling,* 8**,** 8-8.

FRIEDMAN, L. & KOLTER, R. 2004. Genes involved in matrix formation in Pseudomonas aeruginosa PA14 biofilms. *Mol Microbiol,* 51**,** 675-90.

FRYKBERG, R. G. & BANKS, J. 2015. Challenges in the Treatment of Chronic Wounds. *Advances in wound care,* 4**,** 560-582.

FULDA, S., GORMAN, A. M., HORI, O. & SAMALI, A. 2010. Cellular Stress Responses: Cell Survival and Cell Death. *International Journal of Cell Biology,* 2010**,** 23.

GALLE, M., CARPENTIER, I. & BEYAERT, R. 2012. Structure and Function of the Type III Secretion System of Pseudomonas aeruginosa. *Current protein & peptide science,* 13.

GANESHAN, G. & ARTHIKALA, M. K. 2005. Pseudomonas fluorescens, a potential bacterial antagonist to control plant diseases. *Journal of Plant Interactions,* 1**,** 123-134.

GAY, N. J., GANGLOFF, M. & WEBER, A. N. R. 2006. Toll-like receptors as molecular switches. *Nature Reviews Immunology,* 6**,** 693-698.

GEISINGER, E. & ISBERG, R. R. 2017. Interplay Between Antibiotic Resistance and Virulence During Disease Promoted by Multidrug-Resistant Bacteria. *J Infect Dis,* 215**,** S9-s17.

GELLATLY, S. L. & HANCOCK, R. E. 2013. Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. *Pathog Dis,* 67**,** 159-73.

GERSTEL, U., CZAPP, M., BARTELS, J. & SCHRÖDER, J.-M. 2009. Rhamnolipid-induced shedding of flagellin from Pseudomonas aeruginosa provokes hBD-2 and IL-8 response in human keratinocytes. *Cellular Microbiology,* 11**,** 842-853.

GHAFOOR, A., HAY, I. D. & REHM, B. H. 2011. Role of exopolysaccharides in Pseudomonas aeruginosa biofilm formation and architecture. *Applied and environmental microbiology,* 77**,** 5238-5246.

GHANEM, B. & HADDADIN, R. N. 2018. Multiple drug resistance and biocide resistance in Escherichia coli environmental isolates from hospital and household settings. *Antimicrobial Resistance & Infection Control,* 7**,** 47.
GILLITZER, R. & GOEBELER, M. 2001. Chemokines in cutaneous wound healing. *J Leukoc Biol,* 69**,** 513-21.
GOLDUFSKY, J., WOOD, S. J., JAYARAMAN, V., MAJDOBEH, O., CHEN, L., QIN, S., ZHANG, C., DIPIETRO, L. A. & SHAFIKHANI, S. H. 2015. Pseudomonas aeruginosa uses T3SS to inhibit diabetic wound healing. *Wound Repair Regen,* 23**,** 557-64.
GOMILA, M., PEÑA, A., MULET, M., LALUCAT, J. & GARCÍA-VALDÉS, E. 2015. Phylogenomics and systematics in Pseudomonas. *Frontiers in microbiology,* 6**,** 214-214.
GRADA, A., OTERO-VINAS, M., PRIETO-CASTRILLO, F., OBAGI, Z. & FALANGA, V. 2017. Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay. *J Invest Dermatol,* 137**,** e11-e16.
GRANATO, E. T. & KÜMMERLI, R. 2017. The path to re-evolve cooperation is constrained in Pseudomonas aeruginosa. *BMC evolutionary biology,* 17**,** 214-214.
GROSS, H. & LOPER, J. E. 2009. Genomics of secondary metabolite production by Pseudomonas spp. *Natural Product Reports,* 26**,** 1408-1446.
GUEST, J. F., AYOUB, N., MCILWRAITH, T., UCHEGBU, I., GERRISH, A., WEIDLICH, D., VOWDEN, K. & VOWDEN, P. 2017. Health economic burden that different wound types impose on the UK's National Health Service. *Int Wound J,* 14**,** 322-330.
GUO, S. & DIPIETRO, L. A. 2010. Factors affecting wound healing. *J Dent Res,* 89**,** 219-29.
GUTTMAN-YASSKY, E., ZHOU, L. & KRUEGER, J. G. 2019. The skin as an immune organ: Tolerance versus effector responses and applications to food allergy and hypersensitivity reactions. *Journal of Allergy and Clinical Immunology,* 144**,** 362-374.
GUY, J.-B., ESPENEL, S., VALLARD, A., BATTISTON-MONTAGNE, P., WOZNY, A.-S., ARDAIL, D., ALPHONSE, G., RANCOULE, C., RODRIGUEZ-LAFRASSE, C. & MAGNE, N. 2017. Evaluation of the Cell Invasion and Migration Process: A Comparison of the Video Microscope-based Scratch Wound Assay and the Boyden Chamber Assay. *Journal of visualized experiments : JoVE***,** 56337.
HAAS, D. & DEFAGO, G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol,* 3**,** 307-19.
HALMER, R., DAVIES, L., LIU, Y., FASSBENDER, K. & WALTER, S. 2015. The Innate Immune Receptor CD14 Mediates Lymphocyte Migration in EAE. *Cellular Physiology and Biochemistry,* 37**,** 269-275.
HAN, G. & CEILLEY, R. 2017. Chronic Wound Healing: A Review of Current Management and Treatments. *Adv Ther,* 34**,** 599-610.
HANFT, J. & SMITH, B. 2005. How To Differentiate Between Infected Wounds And Colonized Wounds. *Podiatry Today,* 18**,** 85-90.
HAQUE, M., SARTELLI, M., MCKIMM, J. & ABU BAKAR, M. 2018. Health care-associated infections - an overview. *Infection and drug resistance,* 11**,** 2321-2333.
HARI, A., FLACH, T. L., SHI, Y. & MYDLARSKI, P. R. 2010. Toll-like receptors: role in dermatological disease. *Mediators of inflammation,* 2010**,** 437246-437246.
HARRIS, A. D., JACKSON, S. S., ROBINSON, G., PINELES, L., LEEKHA, S., THOM, K. A., WANG, Y., DOLL, M., PETTIGREW, M. M. & JOHNSON, J. K. 2016. Pseudomonas aeruginosa Colonization in the Intensive Care Unit: Prevalence, Risk Factors, and Clinical Outcomes. *Infection control and hospital epidemiology,* 37**,** 544-548.
HEGGERS, J. P., HAYDON, S., KO, F., HAYWARD, P. G., CARP, S. & ROBSON, M. C. 1992. Pseudomonas aeruginosa exotoxin A: its role in retardation of wound healing: the 1992 Lindberg Award. *J Burn Care Rehabil,* 13**,** 512-8.

HENLY, E. L., DOWLING, J. A. R., MAINGAY, J. B., LACEY, M. M., SMITH, T. J. & FORBES, S. 2019. Biocide Exposure Induces Changes in Susceptibility, Pathogenicity, and Biofilm Formation in Uropathogenic Escherichia coli. *Antimicrob Agents Chemother,* 63.

HICKMAN, J. W. & HARWOOD, C. S. 2008. Identification of FleQ from Pseudomonas aeruginosa as a c-di-GMP-responsive transcription factor. *Mol Microbiol,* 69**,** 376-89.

HIROBE, T. 2014. Keratinocytes regulate the function of melanocytes. *Dermatologica Sinica,* 32**,** 200-204.

HOEBE, K., JANSSEN, E. & BEUTLER, B. 2004. The interface between innate and adaptive immunity. *Nature Immunology,* 5**,** 971-974.

HOH, A. & MAIER, K. Comparative Cytotoxicity Test with Human Keratinocytes, HaCaT Cells, and Skin Fibroblasts to Investigate Skin-Irritating Substances. 1993 Berlin, Heidelberg. Springer Berlin Heidelberg, 341-347.

HOLM, A. & VIKSTROM, E. 2014. Quorum sensing communication between bacteria and human cells: signals, targets, and functions. *Front Plant Sci,* 5**,** 309.

HOSSEINIDOUST, Z., VAN DE VEN, T. G. & TUFENKJI, N. 2013. Evolution of Pseudomonas aeruginosa virulence as a result of phage predation. *Appl Environ Microbiol,* 79**,** 6110-6.

HUET, A. A., RAYGADA, J. L., MENDIRATTA, K., SEO, S. M. & KAATZ, G. W. 2008. Multidrug efflux pump overexpression in Staphylococcus aureus after single and multiple in vitro exposures to biocides and dyes. *Microbiology,* 154**,** 3144-53.

HULKOWER, K. I. & HERBER, R. L. 2011. Cell migration and invasion assays as tools for drug discovery. *Pharmaceutics,* 3**,** 107-24.

HURLEY, M. N., CÁMARA, M. & SMYTH, A. R. 2012. Novel approaches to the treatment of Pseudomonas aeruginosa infections in cystic fibrosis. *European Respiratory Journal,* 40**,** 1014.

ICHIKAWA, J. K., NORRIS, A., BANGERA, M. G., GEISS, G. K., VAN, T WOUT, A. B., BUMGARNER, R. E. & LORY, S. 2000. Interaction of Pseudomonas aeruginosa with epithelial cells: Identification of differentially regulated genes by expression microarray analysis of human cDNAs. *Proceedings of the National Academy of Sciences,* 97**,** 9659.

IOCONO, J. A., COLLERAN, K. R., REMICK, D. G., GILLESPIE, B. W., EHRLICH, H. P. & GARNER, W. L. 2000. Interleukin-8 levels and activity in delayed-healing human thermal wounds. *Wound Repair Regen,* 8**,** 216-25.

JAN, A. T. 2017. Outer Membrane Vesicles (OMVs) of Gram-negative Bacteria: A Perspective Update. *Front Microbiol,* 8**,** 1053.

JANEWAY, C. A., JR. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol,* 54 Pt 1**,** 1-13.

JANG, J.-H., SHIN, H. W., LEE, J. M., LEE, H.-W., KIM, E.-C. & PARK, S. H. 2015. An Overview of Pathogen Recognition Receptors for Innate Immunity in Dental Pulp. *Mediators of inflammation,* 2015**,** 794143-794143.

JÄRBRINK, K., NI, G., SÖNNERGREN, H., SCHMIDTCHEN, A., PANG, C., BAJPAI, R. & CAR, J. 2017. The humanistic and economic burden of chronic wounds: a protocol for a systematic review. *Systematic reviews,* 6**,** 15-15.

JAYASEELAN, S., RAMASWAMY, D. & DHARMARAJ, S. 2014. Pyocyanin: production, applications, challenges and new insights. *World J Microbiol Biotechnol,* 30**,** 1159-68.

JIANG, W. G., SANDERS, A. J., RUGE, F. & HARDING, K. G. 2012. Influence of interleukin-8 (IL-8) and IL-8 receptors on the migration of human keratinocytes, the role of PLC-γ and potential clinical implications. *Experimental and therapeutic medicine,* 3**,** 231-236.

JOHNSON, M. K. & BOESE-MARRAZZO, D. 1980. Production and properties of heat-stable extracellular hemolysin from Pseudomonas aeruginosa. *Infection and immunity,* 29**,** 1028-1033.

JUNDI, K. & GREENE, C. M. 2015. Transcription of Interleukin-8: How Altered Regulation Can Affect Cystic Fibrosis Lung Disease. *Biomolecules,* 5**,** 1386-98.
JUSTUS, C. R., LEFFLER, N., RUIZ-ECHEVARRIA, M. & YANG, L. V. 2014. In vitro cell migration and invasion assays. *J Vis Exp*.
KANITAKIS, J. 2002. Anatomy, histology and immunohistochemistry of normal human skin. *Eur J Dermatol,* 12**,** 390-9; quiz 400-1.
KATRI, N. & GILBOA-GARBER, N. 2007. Ethanol effects on Pseudomonas aeruginosa lectin, protease, hemolysin, pyocyanin, autoinducer, and phosphatase levels depending on medium composition and choline presence. *Curr Microbiol,* 54**,** 296-301.
KAUR, G. & DUFOUR, J. M. 2012. Cell lines: Valuable tools or useless artifacts. *Spermatogenesis,* 2**,** 1-5.
KAWAI, K., SHIMURA, H., MINAGAWA, M., ITO, A., TOMIYAMA, K. & ITO, M. 2002. Expression of functional Toll-like receptor 2 on human epidermal keratinocytes. *Journal of Dermatological Science,* 30**,** 185-194.
KAWAI, T. & AKIRA, S. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity,* 34**,** 637-50.
KAWASAKI, T. & KAWAI, T. 2014. Toll-like receptor signaling pathways. *Front Immunol,* 5**,** 461.
KAWASUMI, A., SAGAWA, N., HAYASHI, S., YOKOYAMA, H. & TAMURA, K. 2012. Wound Healing in Mammals and Amphibians: Toward Limb Regeneration in Mammals. *Current topics in microbiology and immunology,* 367.
KEARNS, D. B. 2010. A field guide to bacterial swarming motility. *Nat Rev Microbiol,* 8**,** 634-44.
KELL, D. B. & PRETORIUS, E. 2015. On the translocation of bacteria and their lipopolysaccharides between blood and peripheral locations in chronic, inflammatory diseases: the central roles of LPS and LPS-induced cell death. *Integr Biol (Camb),* 7**,** 1339-77.
KEMÉNY, L., MICHEL, G., DOBOZY, A. & RUZICKA, T. 1994. Cytokine system as potential target for antipsoriatic therapy. *Experimental Dermatology,* 3**,** 1-8.
KERR, K. G. & SNELLING, A. M. 2009. Pseudomonas aeruginosa: a formidable and ever-present adversary. *J Hosp Infect,* 73**,** 338-44.
KI, V. & ROTSTEIN, C. 2008. Bacterial skin and soft tissue infections in adults: A review of their epidemiology, pathogenesis, diagnosis, treatment and site of care. *The Canadian journal of infectious diseases & medical microbiology = Journal canadien des maladies infectieuses et de la microbiologie medicale,* 19**,** 173-184.
KINTARAK, S., WHAWELL, S. A., SPEIGHT, P. M., PACKER, S. & NAIR, S. P. 2004. Internalization of Staphylococcus aureus by human keratinocytes. *Infection and immunity,* 72**,** 5668-5675.
KIRIENKO, D. R., KANG, D. & KIRIENKO, N. V. 2019. Novel Pyoverdine Inhibitors Mitigate Pseudomonas aeruginosa Pathogenesis. *Frontiers in Microbiology,* 9.
KLIMENTOVA, J. & STULIK, J. 2015. Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiol Res,* 170**,** 1-9.
KLINE, K. A., FALKER, S., DAHLBERG, S., NORMARK, S. & HENRIQUES-NORMARK, B. 2009. Bacterial adhesins in host-microbe interactions. *Cell Host Microbe,* 5**,** 580-92.
KOEPPEN, K., HAMPTON, T. H., JAREK, M., SCHARFE, M., GERBER, S. A., MIELCARZ, D. W., DEMERS, E. G., DOLBEN, E. L., HAMMOND, J. H., HOGAN, D. A. & STANTON, B. A. 2016. A Novel Mechanism of Host-Pathogen Interaction through sRNA in Bacterial Outer Membrane Vesicles. *PLOS Pathogens,* 12**,** e1005672.
KOFF, J. L., SHAO, M. X., KIM, S., UEKI, I. F. & NADEL, J. A. 2006. Pseudomonas lipopolysaccharide accelerates wound repair via activation of a novel epithelial cell signaling cascade. *J Immunol,* 177**,** 8693-700.
KOKA, R. & WEIMER, B. C. 2000. Isolation and characterization of a protease from Pseudomonas fluorescens RO98. *J Appl Microbiol,* 89**,** 280-8.

KOLLISCH, G., KALALI, B. N., VOELCKER, V., WALLICH, R., BEHRENDT, H., RING, J., BAUER, S., JAKOB, T., MEMPEL, M. & OLLERT, M. 2005. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. *Immunology,* 114**,** 531-541.

KOO, H., ALLAN, R. N., HOWLIN, R. P., STOODLEY, P. & HALL-STOODLEY, L. 2017. Targeting microbial biofilms: current and prospective therapeutic strategies. *Nat Rev Microbiol,* 15**,** 740-755.

KOSTAKIOTI, M., HADJIFRANGISKOU, M. & HULTGREN, S. J. 2013. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harb Perspect Med,* 3**,** a010306.

KOZIEL, J. & POTEMPA, J. 2013. Protease-armed bacteria in the skin. *Cell and tissue research,* 351**,** 325-337.

KRAMPE, B. & AL-RUBEAI, M. 2010. Cell death in mammalian cell culture: molecular mechanisms and cell line engineering strategies. *Cytotechnology,* 62**,** 175-88.

KUEHN, M. J. & KESTY, N. C. 2005. Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes Dev,* 19**,** 2645-55.

KUIPERS, M. E., HOKKE, C. H., SMITS, H. H. & NOLTE-'T HOEN, E. N. M. 2018. Pathogen-Derived Extracellular Vesicle-Associated Molecules That Affect the Host Immune System: An Overview. *Frontiers in microbiology,* 9**,** 2182-2182.

KULP, A. & KUEHN, M. J. 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu Rev Microbiol,* 64**,** 163-84.

KUMAGAI, Y. & AKIRA, S. 2010. Identification and functions of pattern-recognition receptors. *J Allergy Clin Immunol,* 125**,** 985-92.

LABAUVE, A. E. & WARGO, M. J. 2012. Growth and laboratory maintenance of Pseudomonas aeruginosa. *Curr Protoc Microbiol,* Chapter 6**,** Unit 6E.1.

LAND, W. G. 2015. The Role of Damage-Associated Molecular Patterns in Human Diseases: Part I - Promoting inflammation and immunity. *Sultan Qaboos University medical journal,* 15**,** e9-e21.

LANDEN, N. X., LI, D. & STAHLE, M. 2016. Transition from inflammation to proliferation: a critical step during wound healing. *Cell Mol Life Sci,* 73**,** 3861-85.

LAU, G. W., HASSETT, D. J. & BRITIGAN, B. E. 2005. Modulation of lung epithelial functions by Pseudomonas aeruginosa. *Trends in Microbiology,* 13**,** 389-397.

LAU, G. W., HASSETT, D. J., RAN, H. & KONG, F. 2004. The role of pyocyanin in Pseudomonas aeruginosa infection. *Trends in Molecular Medicine,* 10**,** 599-606.

LAVOIE, E. G., WANGDI, T. & KAZMIERCZAK, B. I. 2011. Innate immune responses to Pseudomonas aeruginosa infection. *Microbes Infect,* 13**,** 1133-45.

LEAS, B. F., SULLIVAN, N., HAN, J. H., PEGUES, D. A., KACZMAREK, J. L. & UMSCHEID, C. A. 2015. AHRQ Comparative Effectiveness Technical Briefs. *Environmental Cleaning for the Prevention of Healthcare-Associated Infections.* Rockville (MD): Agency for Healthcare Research and Quality (US).

LEBRE, M. C., VAN DER AAR, A. M., VAN BAARSEN, L., VAN CAPEL, T. M., SCHUITEMAKER, J. H., KAPSENBERG, M. L. & DE JONG, E. C. 2007. Human Keratinocytes Express Functional Toll-like Receptor 3,4,5,and 9. *Journal of Investigative Dermatology,* 127**,** 331-341.

LEE, B. L. & BARTON, G. M. 2014. Trafficking of endosomal Toll-like receptors. *Trends Cell Biol,* 24**,** 360-9.

LEE, J. & ZHANG, L. 2015. The hierarchy quorum sensing network in Pseudomonas aeruginosa. *Protein Cell,* 6**,** 26-41.

LEIDAL, K. G., MUNSON, K. L. & DENNING, G. M. 2001. Small Molecular Weight Secretory Factors from Pseudomonas aeruginosaHave Opposite Effects on IL-8 and RANTES Expression by Human Airway Epithelial Cells. *American Journal of Respiratory Cell and Molecular Biology,* 25**,** 186-195.

LIANG, C. C., PARK, A. Y. & GUAN, J. L. 2007. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc,* 2**,** 329-33.
LIMOLI, D. H., JONES, C. J. & WOZNIAK, D. J. 2015. Bacterial Extracellular Polysaccharides in Biofilm Formation and Function. *Microbiol Spectr,* 3.
LIN, L. & ZHANG, J. 2017. Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. *BMC Immunology,* 18**,** 2.
LINEBACK, C. B., NKEMNGONG, C. A., WU, S. T., LI, X., TESKA, P. J. & OLIVER, H. F. 2018. Hydrogen peroxide and sodium hypochlorite disinfectants are more effective against Staphylococcus aureus and Pseudomonas aeruginosa biofilms than quaternary ammonium compounds. *Antimicrobial Resistance & Infection Control,* 7**,** 154.
LISTER, P. D., WOLTER, D. J. & HANSON, N. D. 2009. Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clinical microbiology reviews,* 22**,** 582-610.
MACDONALD, I. A. & KUEHN, M. J. 2013. Stress-induced outer membrane vesicle production by Pseudomonas aeruginosa. *J Bacteriol,* 195**,** 2971-81.
MACNEIL, S., SHEPHERD, J. & SMITH, L. 2011. Production of tissue-engineered skin and oral mucosa for clinical and experimental use. *Methods Mol Biol,* 695**,** 129-53.
MAILLARD, J.-Y. 2005. Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems. *Therapeutics and clinical risk management,* 1**,** 307-320.
MARANO, J. R., JANE WALLACE, H., WIJERATNE, D., WILLIAM FEAR, M., SAN WONG, H. & O'HANDLEY, R. 2015. Secreted biofilm factors adversely affect cellular wound healing responses in vitro. *Scientific reports,* 5**,** 13296-13296.
MARTINS, M. L., PINTO, U. M., RIEDEL, K., VANETTI, M. C. D., MANTOVANI, H. C. & DE ARAÚJO, E. F. 2014. Lack of AHL-based quorum sensing in Pseudomonas fluorescens isolated from milk. *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology],* 45**,** 1039-1046.
MATSUI, T. & AMAGAI, M. 2015. Dissecting the formation, structure and barrier function of the stratum corneum. *International Immunology,* 27**,** 269-280.
MATTMANN, M. E. & BLACKWELL, H. E. 2010. Small molecules that modulate quorum sensing and control virulence in Pseudomonas aeruginosa. *The Journal of organic chemistry,* 75**,** 6737-6746.
MCDONNELL, G. & RUSSELL, A. D. 1999. Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev,* 12**,** 147-79.
MCGUIRE, V. A. & ARTHUR, J. S. 2015. Subverting Toll-Like Receptor Signaling by Bacterial Pathogens. *Front Immunol,* 6**,** 607.
MCKINNON, K. M. 2018. Flow Cytometry: An Overview. *Current protocols in immunology,* 120**,** 5.1.1-5.1.11.
MEDZHITOV, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature,* 449**,** 819-26.
MEMPEL, M., VOELCKER, V., KOLLISCH, G., PLANK, C., RAD, R., GERHARD, M., SCHNOPP, C., FRAUNBERGER, P., WALLI, A. K., RING, J., ABECK, D. & OLLERT, M. 2003. Toll-like receptor expression in human keratinocytes: nuclear factor kappaB controlled gene activation by Staphylococcus aureus is toll-like receptor 2 but not toll-like receptor 4 or platelet activating factor receptor dependent. *J Invest Dermatol,* 121**,** 1389-96.
MENON, G. K. 2002. New insights into skin structure: scratching the surface. *Adv Drug Deliv Rev,* 54 Suppl 1**,** S3-17.
METRUCCIO, M. M., EVANS, D. J., GABRIEL, M. M., KADURUGAMUWA, J. L. & FLEISZIG, S. M. 2016. Pseudomonas aeruginosa Outer Membrane Vesicles Triggered by Human Mucosal Fluid and Lysozyme Can Prime Host Tissue Surfaces for Bacterial Adhesion. *Front Microbiol,* 7**,** 871.

MEYER, J. M. 2000. Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent Pseudomonas species. *Arch Microbiol,* 174**,** 135-42.
MILESI GALDINO, A. C., BRANQUINHA, M., SANTOS, A. & VIGANOR, L. 2017. Pseudomonas aeruginosa and Its Arsenal of Proteases: Weapons to Battle the Host.
MILLER, L. S. 2008. Toll-Like Receptors in Skin. *Advances in Dermatology,* 24**,** 71-87.
MILLER, L. S. & MODLIN, R. L. 2007a. Human Keratinocyte Toll-like Receptors Promote Distinct Immune Responses. *Journal of Investigative Dermatology,* 127**,** 262-263.
MILLER, L. S. & MODLIN, R. L. 2007b. Toll-like receptors in the skin. *Seminars in Immunopathology,* 29**,** 15-26.
MILLER, M. B. & BASSLER, B. L. 2001. Quorum sensing in bacteria. *Annu Rev Microbiol,* 55**,** 165-99.
MOGENSEN, T. H. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev,* 22**,** 240-73, Table of Contents.
MORADALI, M. F., GHODS, S. & REHM, B. H. A. 2017. Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. *Frontiers in cellular and infection microbiology,* 7**,** 39-39.
MORETTI, J. & BLANDER, J. M. 2014. Insights into phagocytosis-coupled activation of pattern recognition receptors and inflammasomes. *Curr Opin Immunol,* 26**,** 100-10.
MORGAN, S. J., LIPPMAN, S. I., BAUTISTA, G. E., HARRISON, J. J., HARDING, C. L., GALLAGHER, L. A., CHENG, A.-C., SIEHNEL, R., RAVISHANKAR, S., USUI, M. L., OLERUD, J. E., FLECKMAN, P., WOLCOTT, R. D., MANOIL, C. & SINGH, P. K. 2019. Bacterial fitness in chronic wounds appears to be mediated by the capacity for high-density growth, not virulence or biofilm functions. *PLOS Pathogens,* 15**,** e1007511.
MORIHARA, K. & TSUZUKI, H. 1977. Production of protease and elastase by Pseudomonas aeruginosa strains isolated from patients. *Infect Immun,* 15**,** 679-85.
MORRISON, A. J., JR. & WENZEL, R. P. 1984. Epidemiology of infections due to Pseudomonas aeruginosa. *Rev Infect Dis,* 6 Suppl 3**,** S627-42.
MULCAHY, L. A., PINK, R. C. & CARTER, D. R. 2014. Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles,* 3.
MULLER, M. 2006. Premature cellular senescence induced by pyocyanin, a redox-active Pseudomonas aeruginosa toxin. *Free Radic Biol Med,* 41**,** 1670-7.
MUSKEN, M., DI FIORE, S., DOTSCH, A., FISCHER, R. & HAUSSLER, S. 2010. Genetic determinants of Pseudomonas aeruginosa biofilm establishment. *Microbiology,* 156**,** 431-41.
NEGUT, I., GRUMEZESCU, V. & GRUMEZESCU, A. M. 2018. Treatment Strategies for Infected Wounds. *Molecules,* 23.
NESTLE, F. O., DI MEGLIO, P., QIN, J. Z. & NICKOLOFF, B. J. 2009. Skin immune sentinels in health and disease. *Nat Rev Immunol,* 9**,** 679-91.
NEWMAN, M. A., SUNDELIN, T., NIELSEN, J. T. & ERBS, G. 2013. MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front Plant Sci,* 4**,** 139.
NGUYEN, A. V. & SOULIKA, A. M. 2019. The Dynamics of the Skin's Immune System. *Int J Mol Sci,* 20.
NHS 2009. The Revised Healthcare Cleaning Manual.
NICE. 2012. *Healthcare-associated infections: prevention and control in primary and community care* [Online]. [Accessed].
O'DONOGHUE, E. J. & KRACHLER, A. M. 2016. Mechanisms of outer membrane vesicle entry into host cells. *Cell Microbiol,* 18**,** 1508-1517.
O'TOOLE, G. A. & KOLTER, R. 1998. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol,* 28**,** 449-61.

OLDAK, E. & TRAFNY, E. A. 2005. Secretion of proteases by Pseudomonas aeruginosa biofilms exposed to ciprofloxacin. *Antimicrob Agents Chemother,* 49**,** 3281-8.
OLIVEIRA-NASCIMENTO, L., MASSARI, P. & WETZLER, L. M. 2012. The Role of TLR2 in Infection and Immunity. *Front Immunol,* 3**,** 79.
ORENCH-RIVERA, N. & KUEHN, M. J. 2016. Environmentally controlled bacterial vesicle-mediated export. *Cell Microbiol,* 18**,** 1525-1536.
OTTER, J. & GELLATLY, T. 2018. Environmental decontamination 1: what is it and why is it important? *Nursing Times,* 114**,** 32-34.
OVIEDO-BOYSO, J., BRAVO-PATIÑO, A. & BAIZABAL-AGUIRRE, V. M. 2014. Collaborative action of Toll-like and NOD-like receptors as modulators of the inflammatory response to pathogenic bacteria. *Mediators of inflammation,* 2014**,** 432785-432785.
OZEN, A. I. & USSERY, D. W. 2012. Defining the Pseudomonas genus: where do we draw the line with Azotobacter? *Microb Ecol,* 63**,** 239-48.
PACHORI, P., GOTHALWAL, R. & GANDHI, P. 2019. Emergence of antibiotic resistance Pseudomonas aeruginosa in intensive care unit; a critical review. *Genes & diseases,* 6**,** 109-119.
PALM, N. W. & MEDZHITOV, R. 2009. Pattern recognition receptors and control of adaptive immunity. *Immunol Rev,* 227**,** 221-33.
PALMER, J., FLINT, S. & BROOKS, J. 2007. Bacterial cell attachment, the beginning of a biofilm. *J Ind Microbiol Biotechnol,* 34**,** 577-88.
PAN, X., YANG, Y. & ZHANG, J.-R. 2014. Molecular basis of host specificity in human pathogenic bacteria. *Emerg Microbes Infect,* 3**,** e23.
PAPENFORT, K. & BASSLER, B. L. 2016. Quorum sensing signal-response systems in Gram-negative bacteria. *Nat Rev Microbiol,* 14**,** 576-88.
PARK, S.-J., KIM, S.-K., SO, Y.-I., PARK, H.-Y., LI, X.-H., YEOM, D. H., LEE, M.-N., LEE, B.-L. & LEE, J.-H. 2014. Protease IV, a quorum sensing-dependent protease of Pseudomonas aeruginosa modulates insect innate immunity. *Molecular Microbiology,* 94**,** 1298-1314.
PASPARAKIS, M., HAASE, I. & NESTLE, F. O. 2014. Mechanisms regulating skin immunity and inflammation. *Nat Rev Immunol,* 14**,** 289-301.
PASTAR, I., LIANG, L., SAWAYA, A. P., WIKRAMANAYAKE, T. C., GLINOS, G. D., DRAKULICH, S., CHEN, V., STOJADINOVIC, O., DAVIS, S. C. & TOMIC-CANIC, M. 2018. 10 - Preclinical models for wound-healing studies. *In:* MARQUES, A. P., PIRRACO, R. P., CERQUEIRA, M. T. & REIS, R. L. (eds.) *Skin Tissue Models.* Boston: Academic Press.
PAULSEN, I. T., PRESS, C. M., RAVEL, J., KOBAYASHI, D. Y., MYERS, G. S. A., MAVRODI, D. V., DEBOY, R. T., SESHADRI, R., REN, Q., MADUPU, R., DODSON, R. J., DURKIN, A. S., BRINKAC, L. M., DAUGHERTY, S. C., SULLIVAN, S. A., ROSOVITZ, M. J., GWINN, M. L., ZHOU, L., SCHNEIDER, D. J., CARTINHOUR, S. W., NELSON, W. C., WEIDMAN, J., WATKINS, K., TRAN, K., KHOURI, H., PIERSON, E. A., PIERSON III, L. S., THOMASHOW, L. S. & LOPER, J. E. 2005. Complete genome sequence of the plant commensal Pseudomonas fluorescens Pf-5. *Nat Biotech,* 23**,** 873-878.
PEARSON, J. P., PESCI, E. C. & IGLEWSKI, B. H. 1997. Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *Journal of bacteriology,* 179**,** 5756-5767.
PENDSEY, S. P. 2010. Understanding diabetic foot. *International journal of diabetes in developing countries,* 30**,** 75-79.
PERCIVAL, S. & BOWLER, P. 2004. Biofilms and. their potential role in wound healing. *Wounds: a compendium of clinical research and practice,* 16**,** 234-240.
PERCIVAL, S. L., SULEMAN, L., VUOTTO, C. & DONELLI, G. 2015. Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control. *J Med Microbiol,* 64**,** 323-34.

PERSAT, A., INCLAN, Y. F., ENGEL, J. N., STONE, H. A. & GITAI, Z. 2015. Type IV pili mechanochemically regulate virulence factors in Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences of the United States of America,* 112**,** 7563-7568.

PETERS, A., OTTER, J., MOLDOVAN, A., PARNEIX, P., VOSS, A. & PITTET, D. 2018. Keeping hospitals clean and safe without breaking the bank; summary of the Healthcare Cleaning Forum 2018. *Antimicrobial Resistance & Infection Control,* 7**,** 132.

PICARD, M., MCEWEN, B. S., EPEL, E. S. & SANDI, C. 2018. An energetic view of stress: Focus on mitochondria. *Frontiers in Neuroendocrinology,* 49**,** 72-85.

PIER, G. B. 2007. Pseudomonas aeruginosa lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. *International journal of medical microbiology : IJMM,* 297**,** 277-295.

PIKE, V. L., LYTHGOE, K. A. & KING, K. C. 2019. On the diverse and opposing effects of nutrition on pathogen virulence. *Proceedings. Biological sciences,* 286**,** 20191220-20191220.

PIKTEL, E., WNOROWSKA, U., CIEŚLUK, M., DEPTULA, P., POGODA, K., MISZTALEWSKA-TURKOWICZ, I., PAPROCKA, P., NIEMIROWICZ-LASKOWSKA, K., WILCZEWSKA, A. Z., JANMEY, P. A. & BUCKI, R. 2019. Inhibition of inflammatory response in human keratinocytes by magnetic nanoparticles functionalized with PBP10 peptide derived from the PIP2-binding site of human plasma gelsolin. *Journal of Nanobiotechnology,* 17**,** 22.

PIVARCSI, A., BODAI, L., RÉTHI, B., KENDERESSY-SZABÓ, A., KORECK, A., SZÉLL, M., BEER, Z., BATA-CSÖRGOŐ, Z., MAGÓCSI, M., RAJNAVÖLGYI, É., DOBOZY, A. & KEMÉNY, L. 2003. Expression and function of Toll-like receptors 2 and 4 in human keratinocytes. *International Immunology,* 15**,** 721-730.

PIVARCSI, A., KORECK, A., BODAI, L., SZÉLL, M., SZEG, C., BELSŐ, N., KENDERESSY-SZABÓ, A., BATA-CSÖRGŐ, Z., DOBOZY, A. & KEMÉNY, L. 2004. Differentiation-regulated expression of Toll-like receptors 2 and 4 in HaCaT keratinocytes. *Archives of Dermatological Research,* 296**,** 120-124.

POHAR, J., LAINŠČEK, D., IVIČAK-KOCJAN, K., CAJNKO, M.-M., JERALA, R. & BENČINA, M. 2017. Short single-stranded DNA degradation products augment the activation of Toll-like receptor 9. *Nature Communications,* 8**,** 15363.

POUJADE, M., GRASLAND-MONGRAIN, E., HERTZOG, A., JOUANNEAU, J., CHAVRIER, P., LADOUX, B., BUGUIN, A. & SILBERZAN, P. 2007. Collective migration of an epithelial monolayer in response to a model wound. *Proceedings of the National Academy of Sciences,* 104**,** 15988-15993.

RADA, B. & LETO, T. L. 2013. Pyocyanin effects on respiratory epithelium: relevance in Pseudomonas aeruginosa airway infections. *Trends Microbiol,* 21**,** 73-81.

RAHMAN, T., ALSHAHRANI, M., IRIE, Y., CÁMARA, M. & MARTÍNEZ-POMARES, L. 2015. WS10.7 The role of C-type lectins in the recognition of Pseudomonas aeruginosa. *Journal of Cystic Fibrosis,* 14**,** S21.

RAJA, SIVAMANI, K., GARCIA, M. S. & ISSEROFF, R. R. 2007. Wound re-epithelialization: modulating keratinocyte migration in wound healing. *Front Biosci,* 12**,** 2849-68.

RAKITA, A., NIKOLIĆ, N., MILDNER, M., MATIASEK, J. & ELBE-BÜRGER, A. 2020. Re-epithelialization and immune cell behaviour in an ex vivo human skin model. *Scientific Reports,* 10**,** 1.

RAMPERSAD, S. N. 2012. Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. *Sensors (Basel, Switzerland),* 12**,** 12347-12360.

RASAMIRAVAKA, T., LABTANI, Q., DUEZ, P. & EL JAZIRI, M. 2015. The Formation of Biofilms by Pseudomonas aeruginosa: A Review of the Natural and Synthetic Compounds Interfering with Control Mechanisms %J BioMed Research International. 2015**,** 17.

RHODES, M. 1959. The Characterization of *Pseudomonas fluorescens*. *Journal of General Microbiology,* 21**,** 221-263.
RIDIANDRIES, A., TAN, J. T. M. & BURSILL, C. A. 2018. The Role of Chemokines in Wound Healing. *International journal of molecular sciences,* 19**,** 3217.
RITTIE, L. 2016. Cellular mechanisms of skin repair in humans and other mammals. *J Cell Commun Signal,* 10**,** 103-20.
RODULFO, H., ARCIA, A., HERNANDEZ, A., MICHELLI, E., MARTINEZ, D. D. V., GUZMAN, M., SHARMA, A. & DONATO, M. 2019. Virulence factors and integrons are associated with MDR and XDR phenotypes in nosocomial strains of Pseudomonas aeruginosa in a Venezuelan university hospital. *Rev Inst Med Trop Sao Paulo,* 61**,** e20.
ROSSI, C., CHAVES-LÓPEZ, C., SERIO, A., GOFFREDO, E., GOGA, B. T. C. & PAPARELLA, A. 2016. Influence of Incubation Conditions on Biofilm Formation by Pseudomonas Fluorescens Isolated from Dairy Products and Dairy Manufacturing Plants. *Italian journal of food safety,* 5**,** 5793-5793.
RUFFIN, M., BILODEAU, C., MAILLE, E., LAFAYETTE, S. L., MCKAY, G. A., TRINH, N. T., BEAUDOIN, T., DESROSIERS, M. Y., ROUSSEAU, S., NGUYEN, D. & BROCHIERO, E. 2016. Quorum-sensing inhibition abrogates the deleterious impact of Pseudomonas aeruginosa on airway epithelial repair. *Faseb j,* 30**,** 3011-25.
RUFFIN, M. & BROCHIERO, E. 2019. Repair Process Impairment by Pseudomonas aeruginosa in Epithelial Tissues: Major Features and Potential Therapeutic Avenues. *Frontiers in cellular and infection microbiology,* 9**,** 182-182.
RUSSOTTO, V., CORTEGIANI, A., RAINERI, S. M. & GIARRATANO, A. 2015. Bacterial contamination of inanimate surfaces and equipment in the intensive care unit. *Journal of Intensive Care,* 3**,** 54.
RUTALA, W. A. & WEBER, D. J. 2004. Disinfection and Sterilization in Health Care Facilities: What Clinicians Need to Know. *Clinical Infectious Diseases,* 39**,** 702-709.
RUTHERFORD, S. T. & BASSLER, B. L. 2012. Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb Perspect Med,* 2.
RYDER, C., BYRD, M. & WOZNIAK, D. J. 2007. Role of polysaccharides in Pseudomonas aeruginosa biofilm development. *Curr Opin Microbiol,* 10**,** 644-8.
SABATÉ BRESCÓ, M., HARRIS, L. G., THOMPSON, K., STANIC, B., MORGENSTERN, M., O'MAHONY, L., RICHARDS, R. G. & MORIARTY, T. F. 2017. Pathogenic Mechanisms and Host Interactions in Staphylococcus epidermidis Device-Related Infection. *Frontiers in microbiology,* 8**,** 1401-1401.
SADIKOT, R. T., BLACKWELL, T. S., CHRISTMAN, J. W. & PRINCE, A. S. 2005. Pathogen–Host Interactions in Pseudomonas aeruginosa Pneumonia. *American Journal of Respiratory and Critical Care Medicine,* 171**,** 1209-1223.
SAITO, H., INOUE, K., DITAI, J., WANUME, B., ABESO, J., BALYEJUSSA, J. & WEEKS, A. 2017. Alcohol-based hand rub and incidence of healthcare associated infections in a rural regional referral and teaching hospital in Uganda ('WardGel' study). *Antimicrobial resistance and infection control,* 6**,** 129-129.
SAKA, K., AA, A., OBASA, T., RA, R. & OSHODI, A. 2017. Bacterial Contamination of Hospital Surfaces According to Material Make, Last Time of Contact and Last Time of Cleaning/Disinfection. *Journal of Bacteriology & Parasitology,* 08.
SALMON, J. K., ARMSTRONG, C. A. & ANSEL, J. C. 1994. The skin as an immune organ. *Western Jounal of Medicine,* 160**,** 146-152.
SAMI, D. G., HEIBA, H. H. & ABDELLATIF, A. 2019. Wound healing models: A systematic review of animal and non-animal models. *Wound Medicine,* 24**,** 8-17.
SAUDER, D. N. 1990. The role of epidermal cytokines in inflammatory skin diseases. *J Invest Dermatol,* 95**,** 27s-28s.

SAUER, K. & CAMPER, A. K. 2001. Characterization of Phenotypic Changes inPseudomonas putida in Response to Surface-Associated Growth. *Journal of Bacteriology,* 183**,** 6579-6589.

SCALES, B. S., DICKSON, R. P., LIPUMA, J. J. & HUFFNAGLE, G. B. 2014. Microbiology, Genomics, and Clinical Significance of the Pseudomonas fluorescens Species Complex, an Unappreciated Colonizer of Humans. *Clinical Microbiology Reviews,* 27**,** 927-948.

SCHABER, J. A., TRIFFO, W. J., SUH, S. J., OLIVER, J. W., HASTERT, M. C., GRISWOLD, J. A., AUER, M., HAMOOD, A. N. & RUMBAUGH, K. P. 2007. Pseudomonas aeruginosa forms biofilms in acute infection independent of cell-to-cell signaling. *Infect Immun,* 75**,** 3715-21.

SCHAIBLE, B., RODRIGUEZ, J., GARCIA, A., VON KRIEGSHEIM, A., MCCLEAN, S., HICKEY, C., KEOGH, C. E., BROWN, E., SCHAFFER, K., BROQUET, A. & TAYLOR, C. T. 2017. Hypoxia Reduces the Pathogenicity of Pseudomonas aeruginosa by Decreasing the Expression of Multiple Virulence Factors. *The Journal of Infectious Diseases,* 215**,** 1459-1467.

SCHRÖDER, J. M. & HARDER, J. 2006. Antimicrobial skin peptides and proteins. *Cellular and Molecular Life Sciences CMLS,* 63**,** 469-486.

SCHWECHHEIMER, C. & KUEHN, M. J. 2015. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol,* 13**,** 605-19.

SEN, C. K. 2019. Human Wounds and Its Burden: An Updated Compendium of Estimates. *Adv Wound Care (New Rochelle),* 8**,** 39-48.

SIDHU, V. K., VORHOLTER, F. J., NIEHAUS, K. & WATT, S. A. 2008. Analysis of outer membrane vesicle associated proteins isolated from the plant pathogenic bacterium Xanthomonas campestris pv. campestris. *BMC Microbiol,* 8**,** 87.

SILBY, M. W., CERDEÑO-TÁRRAGA, A. M., VERNIKOS, G. S., GIDDENS, S. R., JACKSON, R. W., PRESTON, G. M., ZHANG, X.-X., MOON, C. D., GEHRIG, S. M., GODFREY, S. A., KNIGHT, C. G., MALONE, J. G., ROBINSON, Z., SPIERS, A. J., HARRIS, S., CHALLIS, G. L., YAXLEY, A. M., HARRIS, D., SEEGER, K., MURPHY, L., RUTTER, S., SQUARES, R., QUAIL, M. A., SAUNDERS, E., MAVROMATIS, K., BRETTIN, T. S., BENTLEY, S. D., HOTHERSALL, J., STEPHENS, E., THOMAS, C. M., PARKHILL, J., LEVY, S. B., RAINEY, P. B. & THOMSON, N. R. 2009. Genomic and genetic analyses of diversity and plant interactions of Pseudomonas fluorescens. *Genome Biology,* 10**,** R51.

SINGH, S., ALMUHANNA, Y., ALSHAHRANI, M. Y., LOWMAN, D., RICE, P. J., GELL, C., MA, Z., GRAVES, B., JACKSON, D., LEE, K., KELKAR, R., KORANTENG, J., MITCHELL, D., SILVA, A. D., HUSSAIN, F., YILMAZ, G., MASTROTTO, F., IRIE, Y., WILLIAMS, P., WILLIAMS, D., CAMARA, M. & MARTINEZ-POMARES, L. 2020. Pseudomonas aeruginosa biofilms display carbohydrate ligands for CD206 and CD209 that interfere with their receptor function. *bioRxiv***,** 2020.04.20.051292.

SIRYAPORN, A., KUCHMA, S. L., O'TOOLE, G. A. & GITAI, Z. 2014. Surface attachment induces Pseudomonas aeruginosa virulence. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** 16860-16865.

SMITH, M. G., DES ETAGES, S. G. & SNYDER, M. 2004. Microbial synergy via an ethanol-triggered pathway. *Mol Cell Biol,* 24**,** 3874-84.

SMITH, R. S. & IGLEWSKI, B. H. 2003. P. aeruginosa quorum-sensing systems and virulence. *Curr Opin Microbiol,* 6**,** 56-60.

SMITH, W. D., BARDIN, E., CAMERON, L., EDMONDSON, C. L., FARRANT, K. V., MARTIN, I., MURPHY, R. A., SOREN, O., TURNBULL, A. R., WIERRE-GORE, N., ALTON, E. W., BUNDY, J. G., BUSH, A., CONNETT, G. J., FAUST, S. N., FILLOUX, A., FREEMONT, P. S., JONES, A. L., TAKATS, Z., WEBB, J. S., WILLIAMS, H. D. & DAVIES, J. C. 2017. Current and future therapies for Pseudomonas aeruginosa infection in patients with cystic fibrosis. *FEMS Microbiology Letters,* 364.

SMITS, J. P. H., NIEHUES, H., RIKKEN, G., VAN VLIJMEN-WILLEMS, I., VAN DE ZANDE, G., ZEEUWEN, P., SCHALKWIJK, J. & VAN DEN BOGAARD, E. H. 2017. Immortalized N/TERT keratinocytes as an alternative cell source in 3D human epidermal models. *Sci Rep,* 7**,** 11838.

SOKOL, C. L. & LUSTER, A. D. 2015. The chemokine system in innate immunity. *Cold Spring Harb Perspect Biol,* 7.

SOKOL, P., OHMAN, D. & IGLEWSKI, B. 1979. A More Sensitive Plate Assay for Detection of Protease Production by Pseudomonas aeruginosa. *Journal of clinical microbiology,* 9**,** 538-40.

SONG, P. I., PARK, Y. M., ABRAHAM, T., HARTEN, B., ZIVONY, A., NEPARIDZE, N., ARMSTRONG, C. A. & ANSEL, J. C. 2002. Human keratinocytes express functional CD14 and toll-like receptor 4. *J Invest Dermatol,* 119**,** 424-32.

SPERANDIO, D., ROSSIGNOL, G., GUERILLON, J., CONNIL, N., ORANGE, N., FEUILLOLEY, M. G. J. & MERIEAU, A. 2010. Cell-associated hemolysis activity in the clinical strain of Pseudomonas fluorescens MFN1032. *BMC microbiology,* 10**,** 124-124.

SRINIVASAN, N. 2010. Telling apart friend from foe: discriminating between commensals and pathogens at mucosal sites. *Innate Immunity,* 16**,** 391-404.

STREILEIN, J. W. 1983. Skin-associated lymphoid tissues (SALT): origins and functions. *J Invest Dermatol,* 80 Suppl**,** 12s-16s.

SUGA, H., RENNERT, R. C., RODRIGUES, M., SORKIN, M., GLOTZBACH, J. P., JANUSZYK, M., FUJIWARA, T., LONGAKER, M. T. & GURTNER, G. C. 2014. Tracking the elusive fibrocyte: identification and characterization of collagen-producing hematopoietic lineage cells during murine wound healing. *Stem Cells,* 32**,** 1347-60.

SURESH, R. & MOSSER, D. M. 2013. Pattern recognition receptors in innate immunity, host defense, and immunopathology. *Adv Physiol Educ,* 37**,** 284-91.

SZOLNOKY, G., BATA-CSÖRGÖ, Z., KENDERESSY, A. S., KISS, M., PIVARCSI, A., NOVÁK, Z., NAGY NEWMAN, K., MICHEL, G., RUZICKA, T., MARÓDI, L., DOBOZY, A. & KEMÉNY, L. 2001. A Mannose-Binding Receptor is Expressed on Human Keratinocytes and Mediates Killing of Candida albicans. *Journal of Investigative Dermatology,* 117**,** 205-213.

TAKEDA, K. & AKIRA, S. 2005. Toll-like receptors in innate immunity. *International Immunology,* 17**,** 1-14.

TANG, A. C., TURVEY, S. E., ALVES, M. P., REGAMEY, N., TÜMMLER, B. & HARTL, D. 2014. Current concepts: host–pathogen interactions in cystic fibrosis airways disease. *European Respiratory Review,* 23**,** 320.

TANIMURA, N. & MIYAKE, K. 2014. Toll Like Receptors. *Glycoscience: Biology and Medicine***,** 707-712.

TASHIRO, Y., INAGAKI, A., ONO, K., INABA, T., YAWATA, Y., UCHIYAMA, H. & NOMURA, N. 2014. Low concentrations of ethanol stimulate biofilm and pellicle formation in Pseudomonas aeruginosa. *Biosci Biotechnol Biochem,* 78**,** 178-81.

TERVANIEMI, M. H., KATAYAMA, S., SKOOG, T., SIITONEN, H. A., VUOLA, J., NUUTILA, K., SORMUNEN, R., JOHNSSON, A., LINNARSSON, S., SUOMELA, S., KANKURI, E., KERE, J. & ELOMAA, O. 2016. NOD-like receptor signaling and inflammasome-related pathways are highlighted in psoriatic epidermis. *Scientific reports,* 6**,** 22745-22745.

THOMAS, M. S. & WIGNESHWERARAJ, S. 2014. Regulation of virulence gene expression. *Virulence,* 5**,** 832-4.

TRAN, V. B., FLEISZIG, S. M. J., EVANS, D. J. & RADKE, C. J. 2011. Dynamics of Flagellum- and Pilus-Mediated Association of Pseudomonas aeruginosa with Contact Lens Surfaces. *Applied and Environmental Microbiology,* 77**,** 3644-3652.

TRAVASSOS, L. H., GIRARDIN, S. E., PHILPOTT, D. J., BLANOT, D., NAHORI, M. A., WERTS, C. & BONECA, I. G. 2004. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. *EMBO Rep,* 5**,** 1000-6.

TRIPPE, K., MCPHAIL, K., ARMSTRONG, D., AZEVEDO, M. & BANOWETZ, G. 2013. Pseudomonas fluorescens SBW25 produces furanomycin, a non-proteinogenic amino acid with selective antimicrobial properties. *BMC microbiology,* 13**,** 111-111.

TURNER, K. H., EVERETT, J., TRIVEDI, U., RUMBAUGH, K. P. & WHITELEY, M. 2014. Requirements for Pseudomonas aeruginosa Acute Burn and Chronic Surgical Wound Infection. *PLOS Genetics,* 10**,** e1004518.

UD-DIN, S. & BAYAT, A. 2017. Non-animal models of wound healing in cutaneous repair: In silico, in vitro, ex vivo, and in vivo models of wounds and scars in human skin. *Wound Repair Regen,* 25**,** 164-176.

VALINS, W., AMINI, S. & BERMAN, B. 2010. The Expression of Toll-like Receptors in Dermatological Diseases and the Therapeutic Effect of Current and Newer Topical Toll-like Receptor Modulators. *The Journal of clinical and aesthetic dermatology,* 3**,** 20-29.

VAN 'T WOUT, E. F., VAN SCHADEWIJK, A., VAN BOXTEL, R., DALTON, L. E., CLARKE, H. J., TOMMASSEN, J., MARCINIAK, S. J. & HIEMSTRA, P. S. 2015. Virulence Factors of Pseudomonas aeruginosa Induce Both the Unfolded Protein and Integrated Stress Responses in Airway Epithelial Cells. *PLoS Pathog,* 11**,** e1004946.

VAN DELDEN, C. & IGLEWSKI, B. H. 1998. Cell-to-cell signaling and Pseudomonas aeruginosa infections. *Emerg Infect Dis,* 4**,** 551-60.

VASIL, M. L. 1986. Pseudomonas aeruginosa: biology, mechanisms of virulence, epidemiology. *J Pediatr,* 108**,** 800-5.

VASSEUR, P., VALLET-GELY, I., SOSCIA, C., GENIN, S. & FILLOUX, A. 2005. The pel genes of the Pseudomonas aeruginosa PAK strain are involved at early and late stages of biofilm formation. *Microbiology,* 151**,** 985-997.

VENTURI, V. 2006. Regulation of quorum sensing in Pseudomonas. *FEMS Microbiology Reviews,* 30**,** 274-291.

VENZA, I., CUCINOTTA, M., VISALLI, M., DE GRAZIE, G., OLIVA, S. & TETI, D. 2009. Pseudomonas aeruginosa Induces Interleukin-8 (IL-8) Gene Expression in Human Conjunctiva through the Recruitment of Both RelA and CCAAT/Enhancer-binding Protein β to the IL-8 Promoter. *Journal of Biological Chemistry,* 284**,** 4191-4199.

VOULHOUX, R., FILLOUX, A. & SCHALK, I. J. 2006. Pyoverdine-Mediated Iron Uptake in Pseudomonas aeruginosa: the Tat System Is Required for PvdN but Not for FpvA Transport. *Journal of Bacteriology,* 188**,** 3317.

WALKER, J. & MOORE, G. 2015. Pseudomonas aeruginosa in hospital water systems: biofilms, guidelines, and practicalities. *Journal of Hospital Infection,* 89**,** 324-327.

WANG, G., LI, Z., LI, T., WANG, S., ZHANG, L., ZHANG, L. & TANG, P. 2018. Negative-Pressure Wound Therapy in a Pseudomonas aeruginosa Infection Model. *BioMed Research International,* 2018**,** 11.

WANG, P., HENNING, S. M. & HEBER, D. 2010. Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. *PLoS One,* 5**,** e10202.

WANKE, I., STEFFEN, H., CHRIST, C., KRISMER, B., GOTZ, F., PESCHEL, A., SCHALLER, M. & SCHITTEK, B. 2011. Skin commensals amplify the innate immune response to pathogens by activation of distinct signaling pathways. *J Invest Dermatol,* 131**,** 382-90.

WEBER, D. J., RUTALA, W. A., MILLER, M. B., HUSLAGE, K. & SICKBERT-BENNETT, E. 2010. Role of hospital surfaces in the transmission of emerging health care-associated pathogens: norovirus, Clostridium difficile, and Acinetobacter species. *Am J Infect Control,* 38**,** S25-33.

WEI, Q. & MA, L. Z. 2013. Biofilm matrix and its regulation in Pseudomonas aeruginosa. *Int J Mol Sci,* 14**,** 20983-1005.

WHO 2011. Report on the Burden of Endemic Health Care-Associated Infection Worldwide. *Clean Care is Safer Care* World Health Organisation.

WHOOLEY, M. A. & MCLOUGHLIN, A. J. 1982. The regulation of pyocyanin production in Pseudomonas aeruginosa. *European journal of applied microbiology and biotechnology,* 15**,** 161-166.
WILSON, R. & DOWLING, R. B. 1998. Pseudomonas aeruginosa and other related species. *Thorax,* 53**,** 213.
WINSOR, G. L., GRIFFITHS, E. J., LO, R., DHILLON, B. K., SHAY, J. A. & BRINKMAN, F. S. 2016. Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. *Nucleic Acids Res,* 44**,** D646-53.
WINZER, K., HARDIE, K. R. & WILLIAMS, P. 2002. Bacterial cell-to-cell communication: sorry, can't talk now — gone to lunch! *Current Opinion in Microbiology,* 5**,** 216-222.
WOLCOTT, R. D., RHOADS, D. D. & DOWD, S. E. 2008. Biofilms and chronic wound inflammation. *J Wound Care,* 17**,** 333-41.
WOLLERT, T., ROLLENHAGEN, C., LANGFORD, G. M. & SUNDSTROM, P. 2012. Human oral keratinocytes: a model system to analyze host-pathogen interactions. *Methods Mol Biol,* 845**,** 289-302.
WONG, V. W., SORKIN, M., GLOTZBACH, J. P., LONGAKER, M. T. & GURTNER, G. C. 2011. Surgical Approaches to Create Murine Models of Human Wound Healing. *Journal of Biomedicine and Biotechnology,* 2011**,** 969618.
WU, D. C., CHAN, W. W., METELITSA, A. I., FIORILLO, L. & LIN, A. N. 2011. Pseudomonas skin infection: clinical features, epidemiology, and management. *Am J Clin Dermatol,* 12**,** 157-69.
YADAV, M. K., CHAE, S. W., GO, Y. Y., IM, G. J. & SONG, J. J. 2017. In vitro Multi-Species Biofilms of Methicillin-Resistant Staphylococcus aureus and Pseudomonas aeruginosa and Their Host Interaction during In vivo Colonization of an Otitis Media Rat Model. *Front Cell Infect Microbiol,* 7**,** 125.
YU, Y.-J., WANG, X.-H. & FAN, G.-C. 2018. Versatile effects of bacterium-released membrane vesicles on mammalian cells and infectious/inflammatory diseases. *Acta Pharmacologica Sinica,* 39**,** 514-533.
ZANONI, I. & GRANUCCI, F. 2013. Role of CD14 in host protection against infections and in metabolism regulation. *Frontiers in cellular and infection microbiology,* 3**,** 32-32.
ZHANG, J.-M. & AN, J. 2007. Cytokines, inflammation, and pain. *International anesthesiology clinics,* 45**,** 27-37.
ZHAO, G., USUI, M. L., LIPPMAN, S. I., JAMES, G. A., STEWART, P. S., FLECKMAN, P. & OLERUD, J. E. 2013. Biofilms and Inflammation in Chronic Wounds. *Adv Wound Care (New Rochelle),* 2**,** 389-399.
ZHENG, X., BAKER, H., HANCOCK, W. S., FAWAZ, F., MCCAMAN, M. & PUNGOR, E., JR. 2006. Proteomic analysis for the assessment of different lots of fetal bovine serum as a raw material for cell culture. Part IV. Application of proteomics to the manufacture of biological drugs. *Biotechnol Prog,* 22**,** 1294-300.

Chapter 8
Appendix

Appendix 1

	Reagent	Manufacturer	Catalogue Number
Cell culture	Dulbeco's Modified Eagles Medium	Sigma	D6546-6X500ml
	FBS 500ml	Sigma	F7524-500ML
	L-Glutamine 200nM solution	Sigma	G7513.100 ml
	EDTA 500g	Sigma	E9884
	PBS	Sigma	P5493-1L
	Trypsin-EDTA (0.25%)	Sigma	T4174-20ml
	Aquaguard	Generon	AQ-250-50L-1
	Cell Titer	Promega	G3581
	6 well plates	Sarstedt	83.392
	24 well plates	Sarstedt	83.3922
	96 well plates	Sarstedt	83.3924
Bacterial culture	Tryptone Soy agar	LabM	NCM0020B
	Maximum recovery diluent	LabM	NCM0085B
ELISA	Human IL-8/CXCL8 DuoSet ELISA 1 kit	RnD	DY208-05
	Ancillary Duo Set Reagent Kit 2	RnD	DY008
Flow cytometry	CD282 (TLR2)	Biolegend	309706
	CD284 (TLR4)	Biolegend	312806
	CD14	Biolegend	301824
	ICF	Millipore	4200-0140
	FACS clean	Millipore	4200-1420
Ex-vivo skin models	Cadaver dermis	Euroskin bank	
	Fibroblast growth medium supplement	Promocell	C-39315
	Recombinant TGF alpha	RnD	239-A-100
	Dispase I	Sigma	D4818-2MG Dispase I
	Transwell 'bucket' inserts	Thermo Fisher	10380291
	Neg50	Thermo Fisher	6502B

Table 8.1 Table of reagents. Table of reagents used in each of the experimental conditions in this study with details of supplier and product code.

Appendix 2

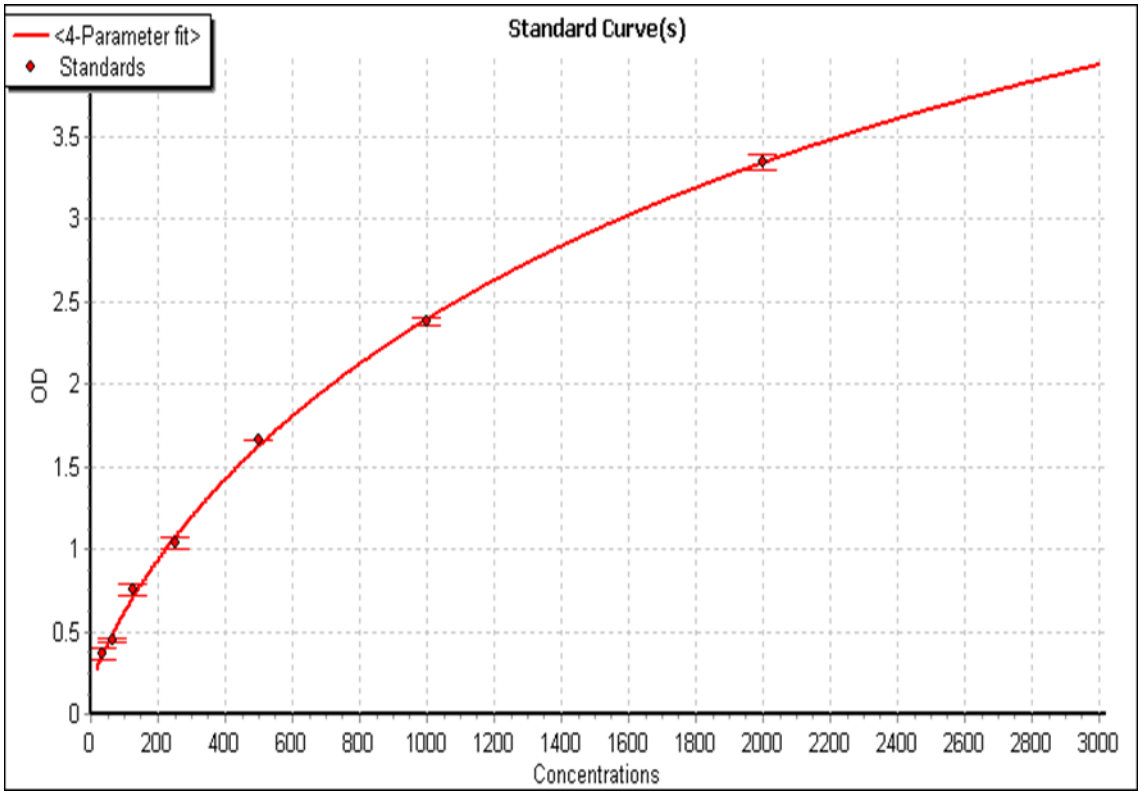

Figure 8.1 Standard curve. Standard curve of known concentrations of CXCL8 constructed using OPTIMA software used to determine the concentration of CXCL8 secretion in samples collected from cells that were challenged with diluted *Pseudomonas* cell free conditioned media.

Appendix 3

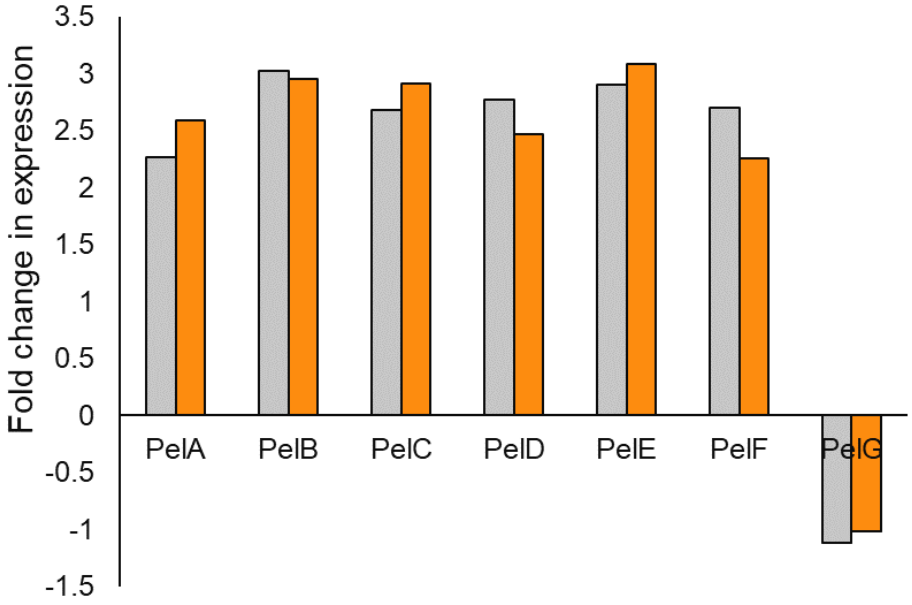

Figure 8.2 Fold change in the expression values of Pel genes of PS3. Transcriptome analysis showing the fold change of the expression of genes involved in Pel (Pel A-G) production from PS3 cultures grown in MM EtOH and MM Glu between 24 and 80 hour culture times.

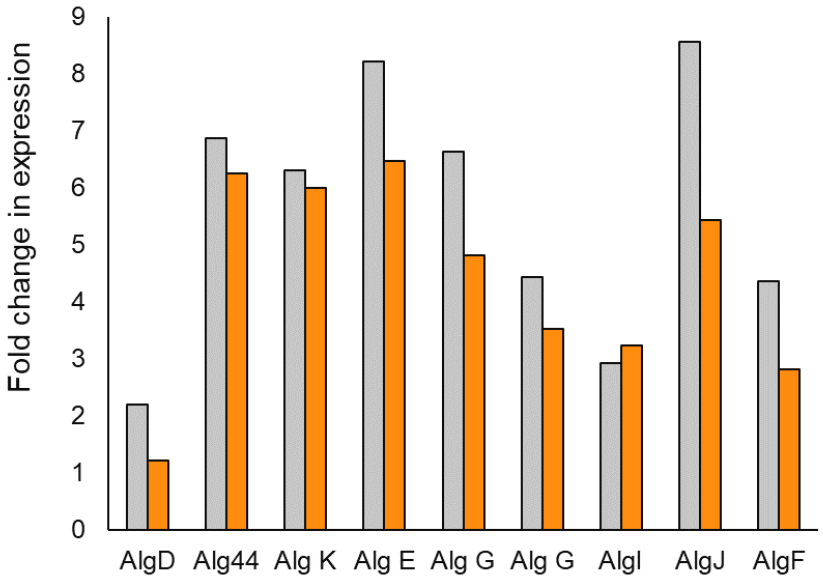

Figure 8.3 Fold change in the expression values of Alg genes of PS3. Transcriptome analysis showing the fold change of the expression of genes involved in Alg production from PS3 cultures grown in MM EtOH and MM Glu between 24 and 80 hour culture times.

Appendix 4

	Secreted products					
Time in culture	**24 hours**			**80 hours**		
Growth media	**MM EtOH**	**MM GLU**	**SWF**	**MM EtOH**	**MM GLU**	**SWF**
Loss of metabolically acitve HaCaT cells	-	-		+ ****	-	varied
Pyocyanin production	+	+	+	+****	+****	+
Protease production 48 hour incubation	-	-	-	-	-	-
Hemolysin production 120 hour incubation	-	-	-	-	-	-
CXCL8 (pg/ml) above control in HaCaT 1:2 dilution	+ ****	+ ****	+	-	+	varied
CXCL8 (pg/ml) above control in HaCaT 1:5 dilution	+ **	+ ****	+	+	+	varied
CXCL8 (pg/ml) above control in HaCaT 1:10 dilution	+ **	+ ***	+	+ *	+	varied
Change in expression of TLR 2	-	+	+	-	-	+
Change in expression of TLR 4	-	+	+	-	-	+
Change in expression of CD-14	-	+	+	-	-	-
CXCL8 (pg/ml) above control in ex-vivo skin model	+	+	n/a	+	+	n/a
50% scratch closure by day 3 after 4 hour exposure	+	+	+	- *** *	+ *	+
100% scratch closure by day 5 after 4 hour exposure	-	+	-	- * **	+	+
50% scratch closure by day 3 after 6 hour exposure	+	+	+	- ****	+	+
100% scratch closure by day 5 after 6 hour exposure	+	+	+	- ****	+	+

Table 8.2 The effects of growth condition on conditioned media from PS3. A summary of the results gained in this study from experiments that investigated the role of conditioned media from PS3 grown in MM EtOH, MM Glu and SWF for 24 and 80 hours. Significance levels are indicated by * colour of stars indicates the treatment where significance lies, black stars show significance to both the other treatments.

Appendix 5

	Live bacteria					
Time in culture	**24 hours**			**80 hours**		
Growth media	**MM EtOH**	**MM GLU**	**SWF**	**MM EtOH**	**MM GLU**	**SWF**
Loss of metabolically acitve HaCaT cells	-	-	-	-	-	-
Biofilm formed	+	+	+	+	-	+
Protease production 24 hour incubation	+	+	+	-	-	-
Protease production 48 hour incubation	+	+	+	+	+	+
Hemolysin production 48 hour incubation	-	-	-	+	+	+
Hemolysin production 120 hour incubation	+	+	+	+ ** *	+	+
Attachment/invasion above 1 bacteria per cell at *MOI 1* *MOI 100*	 + +	 + ** + *	 - +	 + **** +****	 - -	 - +
Attachment/invasion above 1 bacteria per cell + pretreatment at *MOI 1* *MOI 100*	 - +	 + * + ** *	 - +	 + +	 - -	 - +
Invasion above 0.05 bacteria per cell at *MOI 1* *MOI 100*	 - -	 - -	 - +	 + + **	 - -	 - -
Invasion above 0.05 bacteria per cell + pretreatment at *MOI 1* *MOI 100*	 - -	 - -	 - +	 + -	 - -	 - -
CXCL8 (pg/ml) above control	+	+	+	+	+	+
50% scratch closure by day 3 following exposure to MOI 1	+	+	+	+	+	+
50% scratch closure by day 3 following exposure to MOI 10	+	+	+	+	+	+

Table 8.3 The effects of growth condition on live bacteria from PS3. A summary of the results gained in this study from experiments that investigated the role live bacteria from PS3 grown in MM EtOH, MM Glu and SWF for 24 and 80 hours. Significance levels are indicated by * colour of stars indicates the treatment where significance lies, black stars show significance to both the other treatments.