Quorum quenching : a study of the inhibition of pseudomonas aeruginosa quorum sensing-controlled biofilm formation and virulence

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Quorum Quenching: A study of the inhibition of *Pseudomonas aeruginosa* quorum sensing-controlled biofilm formation and virulence

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Abstract

Inhibition of bacterial communication by quorum quenching (QQ) has gained popularity for investigating the intricacies involved in biofilm mediated persistent infections. In this context and given the important role quorum sensing (QS) plays in biofilm formation and virulence by *Pseudomonas aeruginosa*, this study utilised QQ to investigate the response of different phenotypes of *P. aeruginosa* under various conditions *in vitro*.

Biofilm formation by three distinct strains of *P. aeruginosa* (non-mucoid, mucoid and heavily mucoid) and subsequent inhibition/ dispersal were explored on abiotic and biotic surfaces. The importance of eDNA and proteins towards biofilm architecture was elucidated with the use of divalent cations. All three strains showed a significant increase in eDNA and protein contend in the presence of $MgCl_2$. Conversely, combination treatment of biofilm formation by *P. aeruginosa* involving tryptophan and erythromycin at sub-inhibitory levels showed a significant decrease in individual components of the biofilm.

Studies were also conducted under stationary and dynamic conditions using a transmission flow-cell which included the use of anthranilate along with tryptophan and *cis*-2-decanoic acid (CDA) to unravel how products of *P. aeruginosa* biochemical pathways affect biofilm formation between phenotypes. These studies showed that the intrinsic dispersal mechanism adopted by *P. aeruginosa* during the course of its biofilm cycle can be exploited to induce dispersion of pre-formed biofilms under *in vitro* conditions.

Static and dynamic flow-cell models showed that the same strain of *P. aeruginosa* can adapt to the requirements and form a biofilm by modulating the individual components of the extracellular polymeric substances (EPS) in order to successfully colonise a surface. Under dynamic conditions, it was observed that anthranilate inversely affects eDNA and pyocyanin production. A decrease in eDNA generally lead to an increase in pyocyanin production. This shows that pseudomonas quinolone signal (PQS), the non-AHL modulated QS system in *P. aeruginosa,* plays an important role in biofilm dispersal as production of pyocyanin is indirectly controlled by the PQS system while tryptophan and anthranilate are precursors to PQS signal synthesis. Static biofilm models showed the importance of hydrophobicity in initial attachment of bacterial cells. Stainless-steel, which is slightly less hydrophilic compared to glass showed a significant increase in bacterial cell attachment to the surface and subsequent biofilm formation. The difference in cell attachment and biofilm formation was found to be greater with the heavily mucoid strain. This signifies the importance of Pel and Psl polysaccharides towards initial attachment as the difference in cell count in the non-mucoid strain was not as great as with the heavily mucoid strain.

Results obtained from QQ studies involving farnesol and tyrosol (fungal QS molecules) confirmed novel QQ activity against *P. aeruginosa* QS system and subsequent biofilm inhibition. It was revealed that farnesol and tyrosol differentially affect the AHL mediated QS system of *P. aeruginosa* and the response of the bacteria towards farnesol and tyrosol was found to be different on abiotic and biotic surfaces when grown in co-culture with epithelial cell lines. This work for the first time reports a comprehensive antagonistic activity of farnesol and tyrosol against AHL mediated QS system of *P. aeruginosa*.

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Author's Declaration

I declare that the present work was carried out in accordance with the guidelines and regulations of the University of Westminster. The work is original except where indicated by special reference within the text.

The submission as a whole or part is not substantially the same as any that I previously or am currently making, whether in published or unpublished form, for a degree, diploma or similar qualification at any University or similar institution.

Until the outcome of the current application to the University of Westminster is known, the work will not be submitted for any such qualification at another University or similar institution.

Any views expressed in this work are those of the author and in no way represent those of the University of Westminster.

Signed: **Date:**

List of abbreviations

- µ - Micro
- µm – Micrometre
- µM – Micromolar
- $3OC_{12}$-HSL – N-3-oxododecanoyl homoserine lactone
- A549 – Adherent lung epithelial carcinoma
- AB – Acid-base
- ACP – Acyl carrier proteins
- AHL – Acylated homoserine lactones
- AI – Autoinducers
- AI-1 – Autoinducer-1
- AI-2 – Autoinducer-2
- AIP – Auto-inducing peptide
- AMPs – Antimicrobial peptides
- AQ – Alkyl quinolones
- ATCC – American type culture collection
- BSA – Bovine serum albumin
- C_4-HSL – N-butanoyl-l-homoserine lactone
- CAP – Cationic antibacterial peptides
- CCE – Crude cell extract
- CDA – cis-2-decanoic acid
- C-di-GMP – Cyclic diguanylate monophosphate
- cDNA – Complementary DNA
- CF – Cystic fibrosis
- CFTR – Cystic fibrosis transmembrane conductance regulator
- CFU – Colony forming units
- CLSM – Confocal laser scanning microscopy
- Cup – Chaperone usher pathway
- CV – Crystal violet
- DFA – Diffusible fatty acid
- DLVO – Derjaguin-Landau-Verwey Overbeek
- DMEM – Dulbecco's modified eagle medium
- DMSO – Dimethyl Sulfoxide
- DNA – Deoxyribonucleic acid
- ECM – Extracellular matrix
- ECR – Elastin Congo red
- eDNA – Extracellular DNA
- EDTA – Ethylene diamine tetra-acetic acid
- EHEC – Enterohaemorragic *E. coli*
- ELISA – Enzyme linked immunosorbent assay
- EM – Erythromycin
- EPI – Efflux pump inhibitor
- EPS – Extracellular polymeric substances
- EtBr – Ethidium bromide
- Far – Farnesol

- FBS – Fetal bovine serum
- gDNA – Genomic DNA
- HaCaT – Human immortalised keratinocyte
- HHQ - 4-hydroxy-2-heptylquinoline
- IIPLC – IIigh performance liquid chromatography
- IL-8 – Interleukin 8
- LB – Luria bertani
- LPS – Lipopolysaccharide
- LPS – Lipopolysaccharides
- LW – Lifshitz-van der Waals
- MATH – Microbial adhesion to hydrocarbons
- MBEC – Minimum biofilm eradication concentration
- MIC – Minimum inhibitory concentration
- mM – Millimolar
- MOI – Multiplicity of infection
- mRNA – Messenger RNA
- MRSA – Methicillin resistant *S. aureus*
- MTT - 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
- NA – Nutrient agar
- NB – Nutrient broth
- NP – Nanoparticles
- OD – Optical density
- OMPs – Outer membrane proteins
- OMV's – Outer membrane vesicles
- PaβN – Phenylalanine-arginine Beta-naphthylamide
- PBS – Phosphate buffered saline
- PBS-T – Phosphate buffered saline with tween 20
- PCR – Polymerase chain reaction
- PDE – Phosphodiesterase
- PDMS – Poly(dimethylsiloxane)
- Pel – Pellicle forming locus
- Pen-strep – Penicillin- Streptomycin
- PET – Polyethylene(terephthalate)
- PGA – Polyglycolide acid
- PIA – Polysaccharide intercellular adhesin
- PLA – Polylactic acid
- PMMA – Poly(methylmethacrylate)
- PMNs – Polymorphonuclear leukocyte
- PNAG – Poly-N-acetyl glucosamine
- PONs – Paraoxonases
- PP – Polypropylene
- PQS – Pseudomonas quinolone signal
- Psl – Polysaccharide synthesis locus
- PTFE – Poly(tetrafluoroethylene)
- PUE – Polyurethane
- qPCR – Quantitative PCR

- QQ – Quorum quenching
- QS – Quorum sensing
- QSI – Quorum sensing inhibitor
- RBHi – Royal Brompton Hospital isolate
- RNA – Ribonucleic acid
- ROS – Reactive oxygen species
- Rpm – Revolutions per minute
- SDS – Sodium dodecyl sulphate
- Tryp – Tryptophan
- Tyr – Tyrosol
- XDLVO – Extended Derjaguin-Landau-Verwey Overbeek
- xg – Times gravity
- γ-PGA - Poly-γ-glutamic acid
- Δ – Delta

Chapter 1 . Introduction

1.1 Bacterial biofilms

1.1.1 History

The continuity of evolution, competition and adaptation has provided bacteria with the ability to survive within extreme and diverse environments and ecological niches. The presence of bacteria has been documented in various habitats ranging from soil, rivers, oceans, arctic glaciers, thermal springs, and deep-sea hydrothermal vents (Pop Ristova *et al*., 2017). The ability of the bacteria to survive under such extreme environments is dictated in part by their ability to form biofilms. Scientific inquiry into biofilms began as early as 1684, when Antoine van Leeuwenhoek, scraped the deposit on his teeth and observed it under his home-made microscope. This was the first documented case of microscopic visualisation of bacteria and unbeknown to Leeuwenhoek, it was also the initial discovery of biofilm (Costerton, 2007). For nearly a century after Leeuwenhoek's contributions to the field of microbiology, there were little interest in biofilms. Biofilms remained unknown to most microbiologists because microbial research primarily focused entirely on successful growth of planktonically grown microbes and their pathogenic properties (Høiby, 2014; Persat *et al*., 2015).

Research conducted by Robert Koch in the late 1880s, pioneered the way microbes were investigated for scientific and medical purposes (Belvins and Bronze, 2010). He pioneered an era of bacterial research in pure free-living planktonic culture. With his work on *Bacillus anthracis*, *Mycobacterium tuberculosis* and *Vibrio cholerae*, Robert Koch ushered in the "golden age" of bacteriology. However, many decades later, in the mid 1930s, ZoBell, Anderson and Henrici observed microcolony formation and dense non-motile bacterial aggregates on glass surfaces submerged in sea water and biofouling in fresh water respectively, which lead to the belief that bacteria in nature, outside the controlled conditions of the laboratory, grew as a community rather than free-living planktonic form. Following the significant discovery of non-motile bacterial aggregates, Costerton in 1980s identified *Pseudomonas aeruginosa* bacterial communities in post-mortem cystic fibrosis lungs as "bacterial glycocalyx" which in his later publications, referred to as "bacterial biofilms". Thus, the term biofilm was coined. Extensive research conducted since the 1980s has determined that biofilms are able to grow and thrive in a myriad of environments and are ubiquitous in nature (Hunter, 2008; Giaouris *et al.,* 2015; Boisvert *et al*., 2016). The

ubiquitous nature of biofilm resonates with the fact that the presence of bacteria has been documented in various habitats as mentioned previously (Pop Ristova *et al*., 2017).

1.1.2 Microbial biofilms

Biofilms are remarkably successful microbial survival structures. A biofilm is a colony of bacteria that has transitioned from a planktonic (free-swimming) state to a fixed, surface attached sessile state (Branda *et al*., 2005). The surface to which the biofilm attaches may be biotic or abiotic. The process of biofilm formation takes place in a number of distinct stages, brought about through differential expression of bacterial genes in response to their environment (O'Toole and Wong., 2016). The constituents of a biofilm vary depending on numerous environmental factors; such as nutrient gradients, oxic and anoxic condition, shear stress, temperature, and pH extremes (Sultana *et al*., 2018; Toyofuku *et al*., 2015; Bogino *et al*., 2013). However, biofilms are generally comprised of living bacteria and macromolecules such as polysaccharides, proteins, glycoproteins, nucleic acids, and phospholipids arranged within an intricate matrix that provides a protective structure as well as a system of channels allowing for the diffusion of water, nutrients and metabolic waste (Chadha, 2014, Hooshangi and Bentley, 2008, Costerton *et al*., 1999).

1.2 Significance of microbial biofilms

Once biofilm has formed on a surface, it is very difficult to remove, as bacteria growing on these structures are typically up to 1000-fold more tolerant to stress factors, including antimicrobial compounds, compared to free floating planktonic counterparts (Potera, 2010). The recalcitrance of biofilms to removal is of great economic concern in industrial, environmental and medical settings. Biofilm fouling is a common occurrence in industrial plants, resulting in release of toxic metabolites and corrosion (Nguyen, Roddick and Fan, 2012). Biofilms cause engineering problems such as impeding heat transfer in tanks and pipes, increased fluid friction, increased corrosion-rate leading to decrease in productivity. Bio-fouling by biofilms is predominantly observed in industrial equipment and installations such as heat exchange units which increase operational costs and maintenance (Goode *et al*., 2013). In the food industry in particular, biofilms formed on raw meat surfaces are a cause for cross contamination as well as contamination of the processing equipment that are in contact with the raw meat (Stellato *et al*., 2016; Parisi *et al*., 2013). Hence food contact

surfaces should be regularly sanitised (Souza *et al.*, 2014). However, the metabolites of biofilms are not always toxic, and beneficial biofilms are sometimes used to degrade and remove organic waste substances, for example from drinking water during sewage treatment and bioremediation in the case of oil spills (Mitra and Mukhopadhyay, 2016). Similarly, in the environment, bioremediation of pollutants such as chlorophenols, azo dyes, herbicides and heavy metals such as nickel, zinc, cadmium, and lead are performed by biofilms formed by *Bacillus* sp. (Kurniawan and Yamamoto, 2012).

The literature suggests that biofilms play an extensive role in transmission of persisting human diseases. Biofilms formed by *V. cholerae* in the gastro intestinal tract in humans are protected from acidic pH and antibiotics, and hence serve as a carrier of faecal-oral mode of transmission of the disease (Hansch, 2012). Similarly, opportunistic biofilm- forming bacteria display their persistence by becoming pathogenic when infecting immune compromised individuals (Kostakioti, Hadjifrangiskou and Hultgren, 2013). Examples of opportunistic infections caused by biofilms are necrotising fasciitis, osteomyelitis, endocarditis and cystic fibrosis (Romling and Balsalobre, 2012). Formation of biofilms on medical devices and subsequent infection lead to the characterisation of new type of infectious diseases by Hall-Stoodley and Stoodley in 2009, termed chronic polymer-associated infections. This is in reference to contamination of medical devices such as urinary catheters, venous catheters, prosthetic heart valves and intra uterine devices. *Klebsiella pneumoniae*, *P. aeruginosa*, *S. aureus* and *Enterococcus* sp. fall under this category (Donlan, 2008). Table 1.1 highlights a range of biofilm forming microorganism associated with indwelling medical device related infections.

Table 1.1 Biofilm forming microorganisms associated with medical device indwelling related infections

Medical devices	Biofilm forming microorganisms
Urinary catheters	*Escherichia coli, Enterobacter aerogenes, Enterococcus faecalis, Proteus vulgaris, Proteus mirabilis, Staphylococcus epidermidis, Klebsiella pneumoniae, P. aeruginosa.*
Intrauterine devices	*Micrococcus* spp, *S. aureus, S. epidermidis, Enterococcus* spp, *Candida albicans, Streptococci* sp.
Central venous catheters	*K. pneumonia, P. aeruginosa, E. faecalis, S. aureus*, coagulase negative staphylococci, *C. albicans*
Prosthetic heart valves	coagulase negative staphylococci, *Enterococcus* sp, *S. aureus*, streptococci
Contact lenses	*E. coli, Fusarium* spp, *P. aeruginosa, Proteus* spp, *S. aureus, S. epidermidis*, *Serratia* sp, *Candida* sp.

However, not all biofilms are a cause for concern and some have a beneficial effect on humans. A degree of symbiosis and commensalism has been observed between microbial biofilms on some biotic surfaces, especially on/ in a healthy human body (Davey and O'toole, 2000; Berlanga and Guerrero, 2016; Rossi *et al*., 2016). For example, the human skin is a host to a wide range of bacterial and fungal biofilms (Grice and Segre, 2011; Brandwein, Steinberg and Meshner, 2016). Another example is the early colonisation of the intestinal mucosa, a hot topic of epigenetic research (Kilian *et al*., 2016; Valdes *et al*., 2018). It has been found that biofilms formed in the gut competitively occupy specific binding sites on intestinal endothelial cells which would otherwise be targets of pathogenic adhesins (Donaldson, Lee and Mazmanian, 2016; Fang, Jin and Hong, 2018). Therein lies the beneficial effect of the biofilm forming gut microbiota. Another benefit is that the gut microbiota also aids in digestion process and metabolism of the host (Conlon and Bird, 2015; Rowland *et al*., 2017).

Bacillus subtilis is often found in the rhizosphere in the area surrounding the plant root system wherein it promotes plant growth and acts as a bio-control agent (Emmert and Handelsman, 1999). Formation of biofilm is essential for the bacterium's ability to act as a bio-control agent (Bais, Fall and Vivanco, 2004). Commercial strains of *B. subtilis* have been marketed as bio-control agents for fungal diseases of crops (Powell and Jutsum, 1993; Shafi, Tian and Ji, 2016). *B. subtilis* is known to produce a wide variety of antibacterial agents that include broad spectrum of lipopeptides, such as surfactin that is a potent bio-surfactant and vital for maintaining aerial structure of the biofilm (Branda *et al*., 2001). Aerial structures or projections of *B. subtilis* biofilm are often indistinguishable from liquid-air interface pellicles, however, they primarily contain sporulating cells (Cairns, Hobley and Stanley-Wall, 2014). These examples highlight a positive aspect of biofilms in bio-remediation, bio-control and waste-water treatment where the role played by biofilms could be further exploited for beneficial impacts (Vlamakis, 2011 and Nzila *et al*., 2016).

Biofilms play an important role in various types of persistent infections, food contamination and bio-fouling in the industrial sector (Van Houdt and Michiels, 2010). Strategies formulated so far seem to be insufficient due to the persistence of biofilms.

1.3 Biofilm development in brief

Development of a bacterial biofilm is a complex process. The process generally involves numerous physiological activities and is decidedly dependant on external environmental factors (e.g. nutrition, shear force, oxygen content) (Toyofuku *et al*., 2015). No matter which models are used to describe biofilm development, the two features microbiologists interested in are always the biofilm cells and extracellular polymeric substances (EPS). The physiology of bacterial cells within a biofilm has been extensively investigated using transcriptomics, proteomics, mutation and reporter gene analyses (Stewart and Franklin, 2008). The common findings so far have been the presence of a heterogeneous population of cells displaying diverse phenotypes and genotypes, distinct metabolic pathways and specific biological activities (Stewart and Franklin, 2008). At the same time, the EPS matrix of the biofilm is also well characterized with the help of methods such as HPLC and specific staining techniques. The EPS composition and concentration ratio vary in different biofilms and consists of a wide variety of polysaccharides, proteins, glycoproteins, glycolipids, as well as extracellular DNA (eDNA) (Flemming, Neu and Wozniak, 2007). Initially it was considered that the components of the extracellular matrix (ECM) served as a "wall" to hinder the penetration of bactericidal agents into biofilms (Stewart, 1996). Since then, this hypothesis has been refuted (Coifu *et al*., 2001; Owlia *et al*., 2014). The heterogeneity in biofilms can greatly facilitate the adaptation of bacterial cells under harmful conditions, which brings many problems (such as persister cell and antibiotic tolerant subpopulation development) for treatment of biofilm- related infectious diseases.

Intensive research conducted over the past two decades has demonstrated that biofilms develop from a variety of bacterial genera and in numerous distinctly different environments and generally exhibit a common pattern of development (Davey and O'toole, 2000). Initially, the planktonic cells attach onto a substratum through inter-molecular interactions such as Van-der-Waals forces or electrochemical attractions (Busscher and van der Mei, 2012; Kendall and Roberts, 2014). This initial stage is reversible, however, swift gene up-regulation upon attachment allows for the production of various irreversible attachment mechanisms which include formation of several types of pili and adhesins (Berne *et al*., 2018 and Kline *et al*., 2009). In the case of *P. aeruginosa*, upon irreversible attachment, the *las* quorum sensing system is activated and genes responsible for the production of EPS are up-regulated (Deep, Chaudhary and Gupta, 2011 and Laverty, Gorman and Gilmore 2014). The EPS envelops the whole biofilm and often accounts for 50-90% of its total organic matter (Martínez and Vadyvaloo, 2014). Bacteria encased within the biofilm replicate rapidly and

form a mature micro-colony. Research conducted by Walters *et al* in 2003 on *P. aeruginosa* biofilm formation demonstrated a specific growth-rate of 0.66 ± 0.04 cells/h in the first 6 h of development compared with a maximum specific growth-rate of 0.8 ± 0.04 cells/h during the exponential growth phase of planktonic cells. However, replication was found to be at a highly decreased rate with an average specific growth-rate of 0.013 ± 0.009 cells/h in the last 24 h of *P. aeruginosa* biofilm development.

Due to the density of bacterial cells within a mature biofilm, steep nutrient and oxygen gradients have been observed (Wessel *et al*., 2014). However, presence of channels and cavities within a mature biofilm facilitate the diffusion of nutrients and oxygen (Wilking *et al*., 2012). The presence of the gradients allows a large amount of genetic variance between cells and is the primary reason for the presence of different mutational and phenotypic variance observed within bacterial species in the biofilms (Steenackers *et al*., 2016; Williamson *et al*., 2012). The importance in genetic variation is crucial in the propagation of secondary infection caused by the bacteria (Arber, 2014). For example, the diversity of genetic variation is highlighted by the presence of non-mucoid, mucoid and heavily mucoid strains of *P. aeruginosa* (Workentine *et al*., 2013). Many studies have documented the genetic variability in *P. aeruginosa* and this is particularly inherent during chronic lower airway infections (Cornforth *et al*., 2018). Mutations in the alginate biosynthesis pathway is often identified as the cause for the variance in mucoid isolates from chronic lower airway infections (Sousa and Pereira, 2014). Due to the presence of environmental heterogeneity, *P. aeruginosa* continues to cultivate and maintain its genetic diversity in order to adapt to the diverse niches present within the lower respiratory tract and this also leads to the formation of numerous sub-populations (Limoli *et al*., 2017). Similar genetic variability and subsequent phenotypic expression has been observed by the presence of motile and non-motile strains (Pires *et al*., 2017). Although *P. aeruginosa* isolates display similar genetic make-up, a number of phenotypes can evolve as a response to the immediate environment (Bhagirath., *et al*., 2016).

1.4 Stages of biofilm development

Development of a biofilm is a complex and dynamic stepwise process involving a combination of genetic and physio-chemical factors (Martinez and Vadyvaloo, 2014). It is well documented that the processes complexity of biofilm formation and development varies from species to species in relation to numerous environmental stimuli, however, the fundamental steps of biofilm formation remain universal as follows: reversible and irreversible attachment of cells to a substratum, formation of micro-colonies, formation of macro-colonies and maturation of the biofilm by rapid production and accumulation of EPS and finally dispersal of cells from within the biofilm (Douterelo *et al.*, 2014). In order for bacterial attachment to surfaces, be it biotic or abiotic, a conditioning layer is required (Whitehead and Verran, 2015). The conditioning molecules generally originate from the surrounding medium and may be comprised of components of the growth medium, cell derived components upon cell lysis (molecular or cellular debris) (Moreira *et al.*, 2017). formation of the conditioning layer may take between a few second to a few minutes, after which bacteria are able to attach to the surface (Lorite *et al.*, 2011).

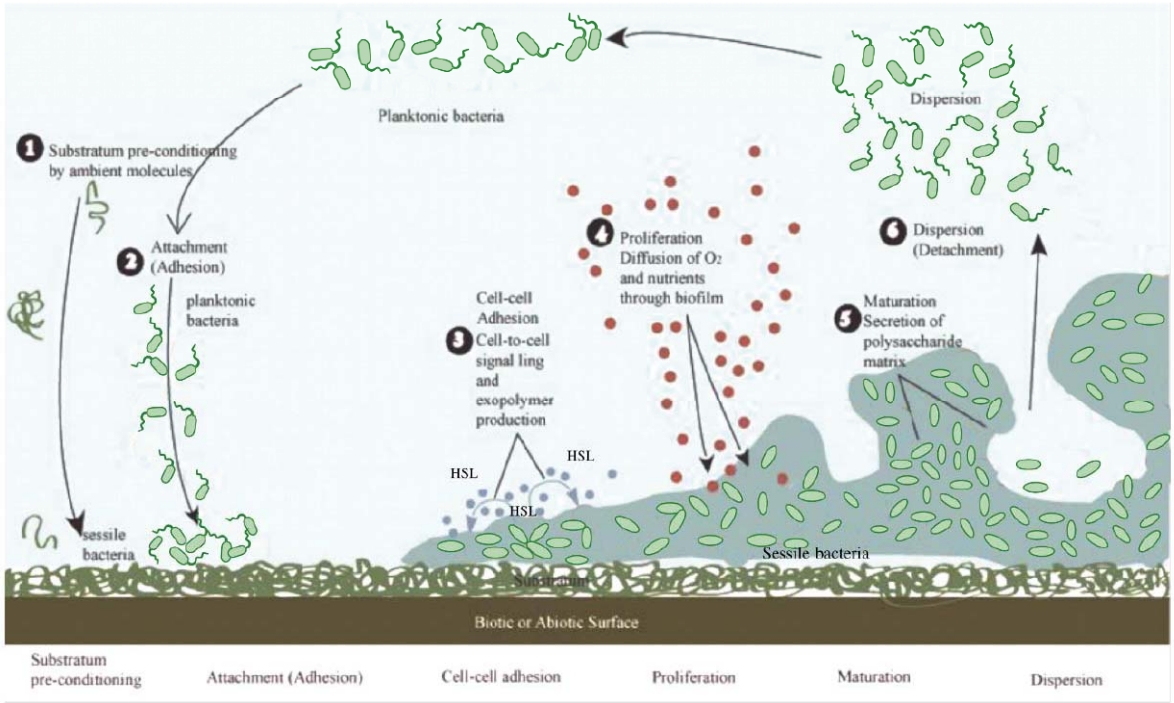

Figure 1.1 Distinct stages of biofilm development. Modified and adapted from Kirmusauglu, 2016

1.4.1 Bacterial attachment to a substratum

Initial attachment occurs when planktonic or motile cells attach to a surface. With the help of pili and/ or flagellar-dependant motility, bacterial cells reach the appropriate surface for attachment (Haiko and Westerlund-Wikström, 2013). In the case of *P. aeruginosa*, type IV pili plays an important role in the initial stages of reversible and irreversible attachment due to its ability to modulate two distinct forms of motility displayed by the bacteria on contact with a surface (Gibiansky *et al*., 2010; Conrad *et al*., 2011). According to O'Toole and Wong (2016), type IV pili aids the bacterial cell to either "crawl" along the surface of the substratum while it's in parallel orientation to the surface and this allows for long distance movement. Whereas, the bacterial cell's ability to "walk" with the aid of type IV pili allows it to stay perpendicular to the surface which allows for bacterial detachment form the surface. These two forms of motility of the bacteria impact the difference between reversible and irreversible attachment. This mode of action of the type IV pili on bacterial motility has been attributed with the levels of cyclic-di-GMP and cyclic AMP which are impacted by the PilY1 protein that is associated with the type IV pili (Kuchma *et al*., 2010; Almblad *et al*., 2015). The figure 1.2, adapted from O'Toole and Wong, 2016 shows how the type IV pili aids in the motility and orientation of the bacterial cells in relation to the surface and how the orientation of the bacterial cells is responsible for reversible and irreversible attachment.

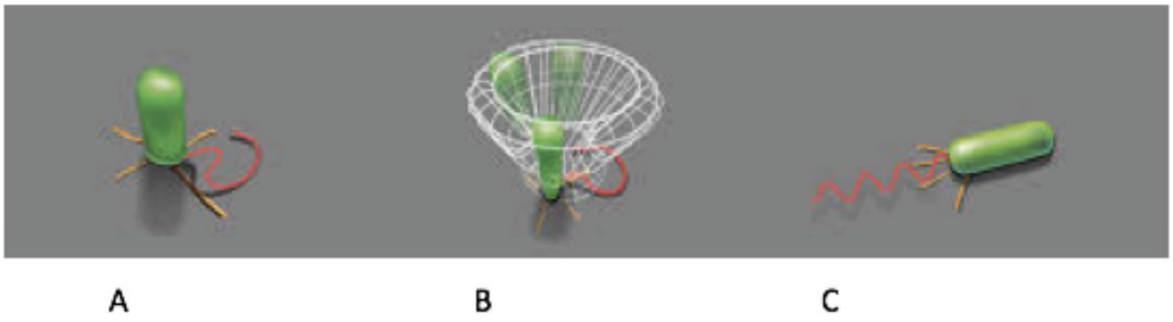

Figure 1.2 In *P. aeruginosa*, Type IV pili (yellow) and flagella (red) play an important role in the transition between the two states of attachment based on the bacterial cell's orientation to the surface. Reversible attachment (A, B), depicted by the perpendicular attachment and "walking" form of motility and irreversible (C) attachment depicted by the "crawling" motility, while parallel to the surface of the substratum. (O'Toole and Wong., 2016).

1.4.1.1 Role of the substratum in microbial attachment

A solid substratum provides numerous opportunities for bacterial cells to attach onto the surface. Firstly, the phenomenon of surface conditioning allows for nutrients to be adsorbed onto the surface of the substratum (Petrova and Sauer, 2012). Second, shear forces and laminar flow velocity of the medium are minimal just over the surface of the liquid-solid interface (Kim *et al*., 2013) which allows for settlement of the bacterial cells onto the surface (Yang *et al*., 2016). Chemical and physical properties of the substratum also influence attachment as a rough surface offers a larger surface area for bacterial attachment (Yuan *et al*., 2017). Finally, the hydrophobicity and the hydrophilicity of the surface plays a crucial role in bacterial attachment (Ploux, Ponche and Anselme, 2012). Earlier studies have shown that bacteria attach to hydrophobic surfaces more readily compared to hydrophilic surfaces (K. Ista *et al*., 1996). It has been reported that hydrophobic properties of microbial surfaces aid in bacterial adhesion to abiotic and biotic surfaces (Heilmann, 2011). Depending on the nature of the surface, the more hydrophobic a bacterial cell is, the stronger it adheres to hydrophobic surfaces whereas, the more hydrophilic a bacterial cell is, it attaches stronger to hydrophilic surfaces (Kochkodan *et al*., 2008).

Physiochemical interactions that balance attractive and repulsive forces are required for bacterial attachment to surfaces. These forces are generally mediated by flagella, pili, proteins and lipopolysaccharides (LPSs) that are found on the outer membrane (Walker *et al*., 2004, Camesano, Natan and Logan, 2000). Numerous interactions between the growth environment, substrate surface and bacterial cell need to be considered when investigating bacterial adhesion (Parent and Velegol, 2004). As mentioned previously, the bacterial cell, general growth environment, and surface properties, such as roughness, chemical structure, ionic strength of culture medium (Abu-Lail and Camesano, 2003), surface charge (Emerson and Casemano, 2004), and hydrophobicity of substrate surfaces (van der Mei, Bos and Busscher, 1998) play a vital role in initial attachment of bacterial cells to a surface (Li and Logan, 1999). Other interfacial interactions such as Lifshitz-van der Waals (LW) and Lewis acid-base (AB) are essential for initial attachment as well. The interfacial interaction mediated by Lifshitz-van der Waals (LW) and Lewis acid-base (AB) represent apolar and polar interactions which is governed by electron-acceptor and electron donor interaction (van Loosdrecht *et al*., 1987). Apart from biofilm formation, attachment of bacterial cells to surfaces is also important for other applications such as development of biomaterials and bioremediation (Tuson and Weibel, 2013).

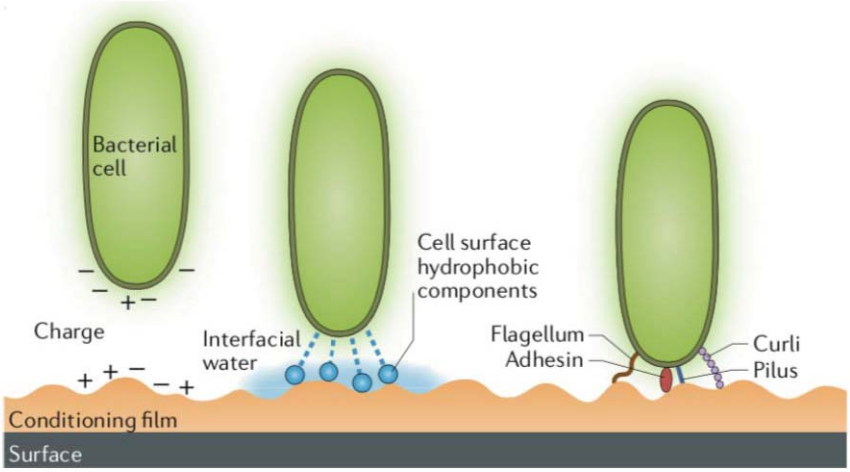

Figure 1.3 Initial interaction between *P. aeruginosa* bacterial cell and a surface. Presence of a conditioning layer aid in distribution of charges and aids in attachment (Berne *et al.*, 2018)

Bacterial attachment is greatly influenced by electrostatic interactions, which can generally be measured by electrophoresis and zeta potential (Halder *et al.*, 2015). The presence of charges groups on bacterial surfaces mediated by carboxyl groups, phosphates, and lipopolysaccharides contribute towards electrostatic forces (Emerson and Camesano, 2004). The Derjaguin-Landau-Verwey Overbeek (DLVO) theory of colloid stability combines the electrostatic and van der Waals forces and describes the energy of interaction as a function of separating distances between the forces. This theory has been used by numerous researchers to interpret the mechanism of bacterial attachment to surfaces (Zhang *et al.*, 2016).

By considering the acid-base interactions that account for the hydrophobicity of surfaces, the DLVO theory has been extended and refered to as XDLVO (Van Oss, 2006). Further additions were made to the two theories by including zeta potential, water contact angles, bacterial cell surface hydrophobicity and surface free energy (Emerson and Camesano, 2004; Hwang *et al.*, 2013). van Loosedrecht *et al* in 1990 showed that hydrophobicity and charge of a bacterium played a role in attachment to surfaces. However, interaction between bacterial cells was not accounted for (Katsikogianni and Missirlis, 2004). Since then, other techniques such as transmission electron microscopy and atomic force microscopy have been adopted to better understand interactions on a microscopic scale (Jucker, Zehnder and Harms, 1998; Vadillo-Rodriguez, 2004).

1.4.2 Reversible microbial attachment

Reversible attachment is considered to be a transitionary phase which allows for bacterial detachment (Li *et al*., 2012). Upon initial contact, bacterial cells adhere to a surface via a single polar flagellum, fimbriae or other adhesins produced by the bacterial cells as explained earlier (Haiko and Westerlund-Wikström, 2013). The reversible attachment is generally initiated as the bacterium overcomes the repulsive electrostatic forces upon first contact (Berne *et al*., 2015). The role of fimbriae is vital during initial attachment due to the presence of numerous hydrophobic amino acid residues. In *P. aeruginosa*, type IV pili is an essential requirement for initial attachment (Melville and Craig, 2013 and Craig, Pique and Tainer, 2004). Studies have shown that type IV pili gene mutants of *P. aeruginosa* were defective in their ability to attach to a substratum. It was hypothesised that the defect in attachment was due to the inability of the bacterial cells lacking the type IV pili, to swim towards and attach to the surface (Persat *et al*., 2015). Subsequent studies proposed that *FilD*, a flagellar cap protein is essential due to its interaction with mucin and may be required for attachment in the lungs of patients with cystic fibrosis (CF) (Janssen *et al*., 2017). Studies conducted by Toutain, Zegans and O'Toole in 2005 showed that *P. aeruginosa* flagella structure contains one motor and two stators, which provide energy for flagellar rotation. However, the absence of flagellar proteins leads to defects in flagellar motility that results in impaired attachment. Yuan and Berg in 2008, showed that presence of a single stator was sufficient for flagellar movement, however, presence of two stators was essential for bacterial attachment. Using beads coated with purified type IV pili, Bucoir *et al* in 2012 demonstrated the importance of type IV pili for maximal binding onto polarised epithelial host cells. In addition to the type IV pili, a chaperone-usher pathway (*Cup*) that synthesises and assembles the fimbriae has been demonstrated to play a vital role in bacterial attachment to abiotic surfaces (Suzuki *et al*., 2015).

1.4.3 Irreversible microbial attachment

Bacteria that are attached irreversibly to a substratum form a monolayer of cells. They usually display reduced motility and flagellar movement and are adhered onto the surface along their axis (Alexandre, 2015). *SadB*, *SadC* and *BifA* protein complexes responsible for swarming motility, were found to play an important role in irreversible attachment of *P. aeruginosa* cells onto a substratum (Petrova and Sauer, 2012). The above-mentioned group of protein complexes can be categorised as diguanylate cyclases (*SadC*) and phosphodiesterases (BifA) (Opoku-Temeng *et al*., 2016). These two classes of proteins play

a vital role in regulating the production and degradation of a secondary messenger molecule known as cyclic diguanylate monophosphate (c-di-GMP) which has been found to regulate the transition from planktonic cells to sessile cells in *P. aeruginosa* (Valentini and Filloux, 2016). According to Ha and O'Toole (2015), the motility of *P. aeruginosa* is reduced as *SadC* begins the synthesis of c-di-GMP which leads to biofilm formation. However, *BifA* degrades c-di-GMP, which in turn increases motility and prevents attachment and reduces biofilm formation (Kuchma *et al*., 2007). *SadB*, on the other hand, aids in irreversible attachment by reduction in flagellar movement and increased polysaccharide production (Verstraeten *et al*., 2008).

Other Gram-negative bacteria such as *Pseudomonas fluorescens* secret a large protein, *LapA* which aids in irreversible attachment to a substratum (Boyd *et al*., 2014). Similarly, *Pseudomonas putida* secretes *LapF* for irreversible attachment (Lahesaare *et al*., 2016). Berne *et al* in 2015 suggested that *P. aeruginosa* secretes a large protein c-di-GMP-regulated protein A, (*CdrA*), which aids in irreversible attachment but does not produce orthologues of *LapA* and *LapF* as seen in other *Pseudomonas* species.

1.4.4 Micro-colony formation and biofilm development

After attachment (reversible and irreversible), bacterial cells proliferate leading to formation of localised micro-colonies. This stage is governed by the increased production of EPS (Janissen *et al*., 2015; Melaugh *et al*., 2016). The bacterial cells (sessile) are then immobilised within the EPS and cell division takes place which leads to a heterogeneous population within the biofilm (Kumar *et al*., 2017). Development of a heterogeneous population within the biofilm leads to formation of micro-gradients of oxygen, nutrients and pH (Lambert *et al*., 2014). According to Lappin-Scott and Costerton (2009), once the biofilm has attained a thickness of 10-25 μm, the microenvironment near the substratum surface becomes anaerobic which results in altered gene expression in comparison to the cells present at the surface of the biofilm.

The architecture of the biofilm is influenced by the division of labour observed due to the presence of sub-populations of cells within the biofilm (Drogos *et al*., 2018). Biofilm cells that were located closer to the substratum showed a lower rate of metabolic activity compared to the cells located near the surface mainly due to the presence of ample supply of oxygen (Zhang *et al*., 2014). Lopez, Vlamakis and Kolter (2010) suggested that the cells

of *P. aeruginosa* located closer to the surface of the biofilm displayed a higher rate of enzymatic activity of an oxygen dependant alkaline phosphatase compared to the cells found closer to the substratum.

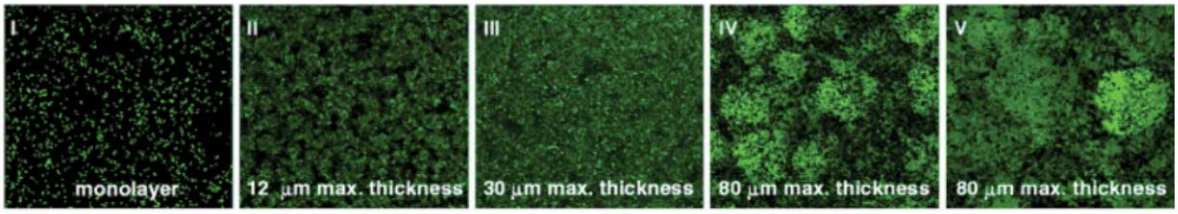

Figure 1.4 *P. aeruginosa* PAO1 confocal images takes at 2 h, 24 h, 48 h, 72 h and 96 h showing the formation of microcolonies and biofilm maturation. Under in vitro conditions, the formation of mushrooms like structures are clearly visible after a period of 72 h (Wagner and Iglewski, 2008).

Numerous publications suggest that biofilms are composed of clustered cells, on this basis Flemming, Neu and Wozniak in 2007 and Flemming and Wingender in 2010 classified the architecture of biofilms as flat, filamentous, cylindrical and mushroom-like. *P. aeruginosa* PAO1 often forms a mushroom-like micro-colonies. However, this is governed by the presence of certain nutrients. When Oglesby-Sherrouse *et al.,* in 2014 inactivated the genes responsible for producing pyoverdine, an iron binding protein, biofilms formed were found to be unstructured and thin even in the presence of exogenously added iron. Increasing the iron content in the medium above a certain threshold was also found to be deleterious and compromised the biofilm architecture in *P. aeruginosa* PAO1 (Yang *et al.*, 2007).

1.4.5 Biofilm dispersal

The final step in the life cycle of a biofilm is the dispersal of bacterial cells. Upon exiting the mature biofilm, planktonic cells flow downstream and attach to a new surface to develop into a new micro-colony and biofilm (Wood, 2014). This stage is primarily triggered by environmental cues and availability of nutrients such as carbon sources, oxygen, nitric oxide (NO) and iron (Uppuluri and Lopez-Ribot, 2016; Fleming and Rumbaugh, 2017). In addition to the dispersal effect of lack of nutrients, certain signalling molecules such as Acyl-homoserine lactones (AHLs), diffusible fatty acid (DFA) and D-amino acids have also been found responsible for initiating dispersal of cells from the biofilm (Krzyzek and Gosciniak, 2018).

As mentioned previously, intracellular levels of c-di-GMP play a vital role in transitioning between planktonic and sessile cells (Valentini and Filloux, 2016). Cole and Lee in 2015 demonstrated that lower concentration of c-di-GMP leads to a switch to planktonic mode to sessile mode in *P. aeruginosa* PA14. Nitric oxide has been observed to catalyse the production of phosphodiesterase (PDE) which leads to a decrease in c-di-GMP concentrations in bacterial cells. This allows the cells to become motile and disperse readily (Hengge *et al*.,2016).

1.5 Extracellular matrix and its components

The extracellular matrix comprising of polymers aids in cell to cell attachment, and cell to substratum attachment while providing a suitable architecture for a three-dimensional structure of the biofilm (Sutherland, 2001). EPS provide a medium for bacterial cells to exist in close proximity which is essential for cell to cell communication to take place which is pre-requisite for biofilm formation and development (Flemming and Wingender, 2010). Apart from providing a platform for bacterial cells to exist within a biofilm, the EPS also plays an important role in protecting the bacterial pathogens from antibiotics, bactericidal agents and also the host immune system. Initially, polysaccharides were considered to be the predominant polymers present in the EPS. However, further studies have revealed other vital components that play a significant role in biofilm architecture (Flemming *et al*, 2016).

1.5.1 Polysaccharides present in the extracellular matrix

Nearly all known bacteria produce extracellular polysaccharides that form the bulk of the biofilm; an exception is *Mycobacterium* spp, which generally produce mycolic acid as the primary component of the extracellular matrix. The polysaccharides produced have been classified into two categories by Wang *et al.,* in 2017 as a) capsular polysaccharides which remain bound to bacterial cells upon extraction from the biofilm, and b) exopolysaccharides which remain in the growth medium after extracting bacterial cells. Differentiating between the two polysaccharides is achievable in shaken liquid culture, however, this is not possible in biofilms as a structured community (Gutiérrez *et al*., 2016).

P. aeruginosa has been extensively used as a model organism to investigate biofilm formation as it produces three distinct kinds of polysaccharides, *Psl*, *Pel* and alginate (Schurr, 2013; Flemming and Wingender, 2010). Jackson *et al.,* in 2004 showed the *Psl* is a

mannose rich polysaccharide, essential for the initial attachment of bacterial cells and biofilm architecture. The role of *Pel* is still not clearly understood but it is thought to contain high levels of glucose (Ha and O'Toole, 2015). Alginate, which is predominantly found in mucoid strains of *P. aeruginosa* is considered to be an anionic polymer, rich in mannuronic acid and glucuronic acid (Limoli, Jones and Wozniak, 2015).

Similarly, *Gluconacetobacter xylinus* was recorded to be the first bacterial species to produce cellulose as its primary exopolysaccharide (Flemming and Wingender, 2010). Since then, cellulose production as a biofilm component has been reported in *Salmonella* Typhimurium (*Slamonella enterica* serovar Typhimurium) and *Escherichia coli* (Yaron and Römling, 2014). Numerous *Staphylococcus* spp. produce polysaccharide intercellular adhesin (*PIA*), encoded by the ica locus. In *S. aureus* the *PIA* has been identified as poly-N-acetyl glucosamine (PNAG) (Brooks and Jefferson, 2014). *E. coli* MG1655 and *Yersinia pestis* have been documented to produce PIA like polysaccharides as well (Steinberg and Kolodkin-Gal, 2015).

Similar to Gram-negative bacteria, Gram-positive bacteria such as *Bacillus subtilis* produce two kinds of polymers EPS and poly-γ-glutamic acid (γ-PGA) (Yu *et al.*, 2016). The production of either of the polymers is dependent on the environment surrounding the bacterial biofilm (Bedrunka and Graumann, 2017). Teichoic acid is an exopolysaccharide produced by *S. epidermidis* (Kiedrowski and Horswill, 2011). Higher concentration of teichoic acid is known to increase the viscosity of the biofilm (Ghafoor, 2014).

Isolates of *P. aeruginosa* from CF patients display a very distinctive character when compared to other infections as well as environmental strains (Huus *et al.*, 2016). A very selective phenotype of alginate overproduction is observed in CF isolates that makes it highly mucoid (Limoli *et al.*, 2017). Similar to other components of the EPS secreted by *P. aeruginosa*, alginate, comprising of polyuronic acids is secreted in greater quantities by CF isolates. This feature is a genetic manifestation of the bacterial strain present in a specific *in vivo* environment, the human lung (Chmiel and Davis, 2003). A study conducted by Folkesson *et al* in 2012 showed that the overproduction of alginate in CF isolates is related to the presence of sublethal levels of hydrogen peroxide which is a product of polymorphonuclear neutrophils. Similarly, a study conducted by Mathee *et al* in 1999 showed that the presence of hydrogen peroxide also leads to non-mucoid strains to produce/ overproduce alginate. Therefore, the production of alginate is an essential process

undertaken by an environmental strain of *P. aeruginosa* to manifest undergo a physiological change into pathogenic behaviour and cause infections in human hosts.

Production of alginate is governed by alginate biosynthesis operon which consists of 12 genes (*algD*) (Ertesvag *et al*., 2017). These genes are also responsible for the production of sugars, polymeric orientation, assembly of polysaccharides. The primary factor involved in upregulation of *algD*, expression is *algU/algT*, which is an alternative sigma factor (Dobrindt, Hacker and Svanborg, 2013). Mutation in *algU* has been found to be the most common cause of mucoid conversion of *P. aeruginosa* in patients suffering from CF (Sousa and Pereira, 2014). *algU* is counter regulated in its activity by a downstream cluster in its immediate vicinity called *mucABCD*, where *mucA* is the cognate anti-sigma factor for *algU* (Damron and Yu, 2010). mucA is an inner membrane protein and its c-terminus interacts with *mucB* and its N-terminus with *algU*. Therefore, inactivation of *mucA* and *mucB* and *mucD* confers mucoidity to *P. aeruginosa* (Okkotsu, Little and Schurr, 2014). Importance of alginate is two-fold in the case of *P. aeruginosa*, apart from providing a safe enclosure for the bacteria, alginate plays an important role in the formation of three-dimensional structure of the mature biofilm (Roy *et al*., 2017).

1.5.2 Proteins present in the extracellular matrix

Proteins form an integral part of the EPS within the biofilm. Apart from aiding in defining the structure of the biofilm, the presence of enzymes secreted by the bacterial cells within the biofilm helps break down of nutrients for metabolism (Zhang *et al*., 2015). Research conducted by Chemani *et al.,* in 2009 showed that *P. aeruginosa* produces two galactose specific lectins, LecA and LecB, which benefit the biofilm architecture. Further work conducted by Fong and Yildiz in 2015 showed that disruption of LecB resulted in complete disruption of the biofilm. As mentioned previously, CdrA plays a vital role in binding with Psl and links Psl to cells (Parsek, 2016). Similarly, type IV pili and Cup fimbriae are essential for the initial stages of biofilm formation by *P. aeruginosa* (Vallet *et al*., 2004). In Gram-positive bacteria, TasA is produced by *B. subtilis* and Bap is produced by *S. aureus*, both of which play a crucial role in biofilm development (Taglialegna *et al*., 2016).

1.5.3 Extracellular DNA present in the extracellular matrix

Previously considered to be a by-product of cell lysis, eDNA has been found to play a vital role in the structural integrity of biofilms formed by Gram-negative as well as Gram-positive bacterial strains (Sugimoto *et al*., 2018). Flemming and WIngender in 2010 described the function of eDNA within the biofilm as a glue-like material which stabilises cell to cell attachment as well as helps maintain the architecture of the biofilm. eDNA has also been found to neutralise the effect of cationic antibacterial peptides (CAP) in-order to protect the bacterial cells (Batoni, Maisetta and Esin, 2016). The presence of eDNA in biofilms is mediated by numerous mechanisms, ranging from prophage-mediated cell death, QS regulated DNA release and secretion by outer membrane vesicles (Wilton *et al*., 2016). The importance of eDNA in early stages of biofilm development by *P. aeruginosa* was documented by Ma *et al.,* in 2009. Upon treating *P. aeruginosa* sessile cells aggregates with DNase prior to microcolony formation, a complete dissolution of the biofilm was observed, however, dissolution was not observed with a mature biofilm with DNase treatment. Spatial work conducted on location of eDNA within the biofilm by Allesen-Holm *et al.,* in 2006 showed that eDNA was concentrated towards the surface of a newly forming biofilm whereas a mature biofilm showed a high concentration of eDNA towards the stalk of the three-dimensional structure. Recently, Magdalena *et al.,* in 2016 proved eDNA to be an important component of motility for *P. aeruginosa* cells. It acts as a trail marker allowing bacterial cells to reorganise within the biofilm to develop the three-dimensional architecture as the biofilm matures (Berne *et al*., 2015).

1.6 Conventional treatment of biofilm-mediated bacterial infections

Conventional treatment of infections follows the use of antibiotics that function by either bacteriostatic or bactericidal effect. Antibiotics such as ceftazidime and vancomycin display an antagonistic effect by either inhibiting peptidoglycan cross-linking and peptidoglycan synthesis respectively, while polymyxins disrupt the cell membrane (Bassetti *et al*., 2013). Other antibiotics such as tobramycin and tetracyclines inhibit protein synthesis at the 30S ribosomal subunit and erythromycin and chloramphenicol inhibit protein synthesis at the 50S ribosomal subunit (Kapoor, Saigal and Elongavan, 2017). Antibiotics such as sulfacetamide and trimethoprim inhibit folic acid synthesis, which is essential for bacteria to synthesise DNA and levofloxacin and rifamycin inhibit the activity of DNA gyrase (required for DNA replication) and RNA synthesis respectively (Fair and Tor, 2014).

Antibiotics are generally derived from fungi and bacteria and/ or are synthetic or semi-synthetic compounds (Wright, Seiple and Myers, 2014). Depending on their host range, the antibiotics can be divided into broad spectrum or narrow spectrum. Flucloxacillin is a narrow spectrum antibiotic primarily used against Gram-positive bacteria, whereas tetracycline is broad-spectrum antibiotic which affects Gram-negative as well as Gram-positive bacteria (Mahamoud *et al.*, 2007). Other examples of broad-spectrum antibiotics include aminoglycosides, carbapenems, quinolones, tetracyclines and ampicillin to name a few and antibiotics such as azithromycin, clarithromycin, erythromycin and vancomycin come under the narrow spectrum category (Sarpong and Miller, 2015; Buckel *et al.*, 2016; Rea *et al.*, 2011). The main challenge faced during antibiotic treatment is the onset of drug resistance, particularly in biofilm forming pathogenic bacteria. This has led to the re-emergence of once considered eradicated microbial diseases and has rendered infections with greater virulence. One of the major causes of resistance is the overuse and misuse of antibiotics (Shallcross and Davies, 2014).

Antibiotic resistance in bacteria can be categorized into two distinct classes, intrinsic and acquired (Munita and Arias, 2016). Intrinsic mode of resistance is generally defined by genes that occur naturally within the chromosome of the bacteria (Nikaido, 2009). For example, β-lactamase production by certain Gram-negative bacteria and the presence of multi drug resistance efflux pump systems like the MexAB OprM. This is an innate ability of the bacteria to resist antagonistic activity of antibiotics through its inherent structural and functional characteristics (Nikaido and Pages, 2012). On the other hand, acquired resistance is known to occur when bacteria attain the ability to resist the antagonistic activity of antibiotics to which is was previously susceptible (Davies and Davies, 2010). Unlike intrinsic resistance, mechanisms of acquired resistance are dictated by mutations in genes that are targeted by the antibiotics, transfer of resistance factors present on plasmids, bacteriophages, transposons, and other genetic material (Domingues, da Silva and Nielsen, 2012). The process of acquired resistance is generally mediated by conjugation with respect to plasmids and transposons, transduction (bacteriophages) and transformation via plasmids, chromosomal DNA and DNA from dead bacterial cells. This process is commonly known as horizontal gene transfer (Roberts and Kreth, 2014; Quebatte *et al.*, 2017). Similarly, formation of biofilm offers a phenotypic mode of resistance where bacterial cells remain protected from antibiotics within their self-secreted EPS matrix (Gunn, Bakaletz and Wozniak, 2016).

1.7 *Pseudomonas aeruginosa*

P. aeruginosa is a mono-flagellated, motile, Gram-negative rod which belongs to the Pseudomonadaceae γ-protobacteria family and is commonly present in the general environment. Due to its ubiquitous nature, the bacterium can colonise numerous environmental niches such as humans (infections and disease), soil, rivers, invertebrates and plants. *P. aeruginosa* is primarily an aerobic bacterium but during oxygen limitation it can thrive by exploiting the nitrate terminal acceptor instead of oxygen for growth (Jensen *et al.*, 2017; Kolpen *et al.*, 2014). In humans, *P. aeruginosa* causes infections and diseases such as wound infections, periodontitis, keratitis and chronic pneumonia during cystic fibrosis (CF), usually in individuals with a compromised immune system as it is an opportunistic pathogen (Gellatly and Hancock, 2013). *P. aeruginosa* PAO1 WT, originally an isolate from a burn wound, has its complete genome sequenced and is one of the most commonly studied organisms in the field of biofilms (Ramos, 2004; Mikkelsen, McMullan and Filloux, 2011). Due to its large genome size, *P. aeruginosa* contains a very high proportion of regulatory genes which contributes towards its ability to adapt and survive within a broad host range and display high intrinsic resistance to a wide range of antibiotics (Strateva and Yordanov, 2009; Chalhoub *et al.*, 2017).

The intrinsic drug resistance feature of *P. aeruginosa* is partially due to the presence of numerous efflux pumps (Dreier and Ruggerone, 2015). The low permeability (12-100 times lower than *E. coli*) of *P. aeruginosa* cell membrane, acts as a selective barrier for the uptake of antibiotics (Breidenstein, de la Fuente-Nunez and Hancock, 2011). The presence of constitutively expressed multidrug efflux pumps in *P. aeruginosa* (MexXY-OprM, MexCD-OprJ and MexEF-OprN) confer the ability to tolerate high doses of antibiotics (Morita, Tomida and Kawamura, 2012). Although the presence of efflux pumps does not provide a high level of resistance, they do reduce susceptibility towards antibiotics (Blanco *et al.*, 2016). The presence of nearly 150 genes, that encode for outer membrane proteins (OMPs), also aids in lowered intra-bacterial antibiotic concentrations (Stover *et al.*, 2000). The clinical relevance of OPMs is clear as they are involved in transport of antibiotics, export of virulence factors and in anchoring the bacterial cells by aiding in adhesion and motility (Bonnington and Kuehn, 2014). In addition to the efflux pumps and OMPs, *P. aeruginosa* produces enzymes such as β-lactamase and aminoglycoside acetyltransferase that target antibiotics used during the treatment of *P. aeruginosa* infection (Garneau-Tsodikova and Labby, 2016).

1.7.1 *P. aeruginosa* and Cystic fibrosis

P. aeruginosa has been recognised as one of the most important pulmonary pathogens and the predominant cause of mortality in cystic fibrosis (CF) patients (Davies, 2002). CF is an autosomal recessive disease and is primarily caused by the dysfunction of a cAMP-regulated chloride channel, CFTR (Cystic fibrosis transmembrane conductance regulator) (Li and Naren, 2010). As CFTR is located on the apical surface of epithelial cells in the respiratory, gastrointestinal, hepatobiliary and reproductive systems, it performs the function of maintaining the basal state fluid balance (Lavelle *et al*., 2016). Lack of CFTR leads to decreased secretion of water which in turn leads to low amount of fluid in the respiratory tissue (Pilewski and Frizzell, 1999). Therefore, patients suffering from CF lack the ability of optimal mucociliary clearance making the respiratory fluid highly viscous (Chmiel and Davis, 2003). This in turn prevents the clearance of pathogens and in the case of *P. aeruginosa* infection, it causes chronic pneumonia in CF patients leading to a high rate of mortality. The lungs of CF patients are known to produce significantly increased levels of interleukin 8 (IL-8) as a marker for inflammation as a result of altered CFTR function. Similarly, other epithelial cells produce IL-8 during inflammation (Zaman *et al*., 2004; Reeves *et al*., 2015).

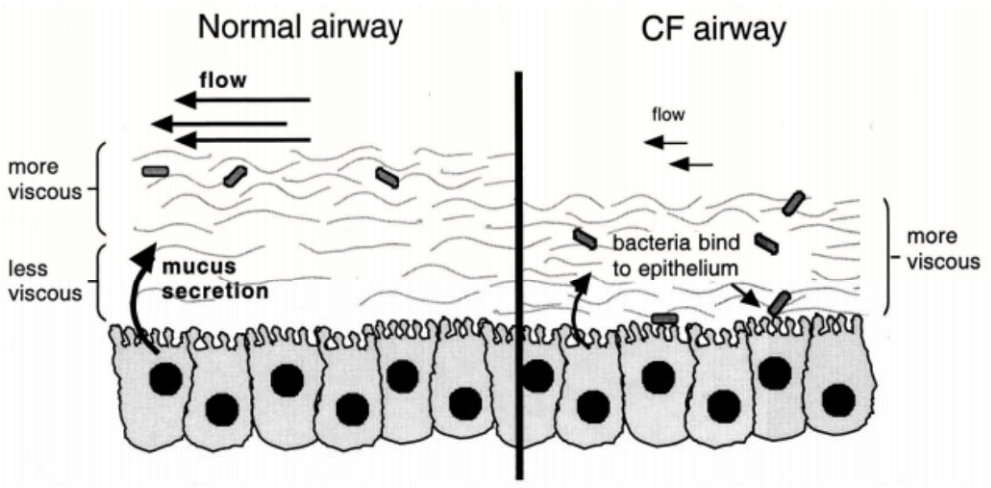

Figure 1.5 Respiratory fluid clearance in normal airway compared with CF airway (Adapted from Lyczak, Cannon and Pier, 2002)

1.7.2 Importance of biofilm formation for *P. aeruginosa* pathogenesis

An important factor contributing toward the pathogenesis of *P. aeruginosa* is its remarkable ability to switch from planktonic mode and biofilm mode of growth which is primarily involved in the onset of chronic infections. Literature suggests that *P. aeruginosa* cells growing within a biofilm are nearly 1000 times more tolerant towards antibiotics compared to their planktonic counterparts (Potera, 2010; Hall and Mah, 2017). Conventionally speaking, cells within the biofilm do not show an increased resistance towards antibiotics. However, they show an increased threshold while measuring the minimum inhibitory concentration (MIC) against the antibiotic of interest when compared to planktonic cells as they are enveloped within the ECM (Lebeaux, Ghio and Beloin, 2014). Several factors contribute towards the protection of bacterial cells within the biofilm. The properties of various antibiotics may play a role as well (Zrelli *et al*., 2013). The basic assumptions regarding the protective role played by the biofilm is that of reduced diffusion of the antibiotic based on the nature of the application as well as the composition of the biofilm (Mah *et al*., 2003).

Tobramycin and gentamicin classified as aminoglycosides, are two of the common antibiotics used against *P. aeruginosa* infections (Krause *et al*., 2015). The cationic aminoglycosides have been found to bind to the anionic polysaccharides (alginate) present in the ECM of *P. aeruginosa* biofilm (Batoni, Maisetta and Esin, 2016). Furthermore, the biofilm may provide a diffusion barrier and contribute towards resistance of sessile cells within the biofilm (Singh *et al*., 2017). Coupling the decreased rate of diffusion of antibiotics within the biofilm and the presence of enzymes (mentioned in chapter 1, section 1.6) that specifically target the antibiotics for rapid degradation, the overall effectiveness of the antibiotics is reduced (Hall and Mah, 2017).

Another feature of *P. aeruginosa* biofilm that plays a role in pathogenesis is the presence of a heterogeneous environment within the biofilm, where nutrients and metabolites are unevenly distributed (Klauck *et al*., 2018). The imbalance of nutrients and metabolites in the form of accumulation of waste products, together with oxygen and nutrient depletion may lead to bacterial cells in different stages of growth and metabolism within the biofilm (Donlan, 2002). The imbalanced environment within the biofilm may cause the bacteria to conform to a non-growing state, exhibiting minimal metabolic activity that may contribute in protection of the cells against antibiotics (Trastoy *et al*., 2018). These cells are categorised as persister cells (Cabral, Wurster and Belenky, 2018). As metabolic activity is a pre-

requisite for antibacterial action, the persister cells remain unhindered and survive, and help restore biofilm population upon reaching favourable growth conditions (Dawson, Intapa and Jabra-Rizk, 2011). Collectively, the presence of heterogeneity within the biofilm in the form of essential nutrients or various stages of cellular growth results in differentiation of the bacterial cells within the biofilm into a protected phenotype (Melaugh *et al*., 2016). The heterogeneity and multicellular nature of the biofilm along with the presence of numerous resistance mechanisms exhibited by biofilm forming cells confer higher level of tolerance towards bactericidal agents. Minimal antibiotic penetration due to formation of biofilm and subsequent protection is dependent on the ability of the cells to form aggregates. Within the biofilm, persister cells rely on sessile cells to propagate, while the sessile cells depend on persister cells to re-establish the community in the event of destruction. These aggregates of cells collectively create an environment conducive to biofilm formation upon reaching a critical cell density. Thus, treating biofilm-related diseases should be approached at a multicellular level rather than combating functions of individual cells. The entire series of events that lead to biofilm formation, starting from free-floating, motile planktonic cells to surface attachment and subsequent biofilm production and pathogenesis in *P. aeruginosa* is mediated by quorum sensing (QS). Therefore, combating biofilm related infections should involve novel antibacterial therapies targeting the QS system in *P. aeruginosa*.

1.8 Regulation of biofilm formation

1.8.1 Quorum sensing mediated bacterial communication

Quorum sensing (QS), which was initially known as autoinduction (Nealson, Platt and Hastings, 1970; Fuqua, Winans and Greenberg, 1994) was first identified in bioluminescent marine bacteria, *Vibrio fischeri* which lives in symbiosis with the bobtail squid, *Euprymna scolopes* (Nealson and Hastings, 1979; Eberhard *et al*., 1981; Ruby and Lee, 1998). The phenomenon of luminescence was found to be cell density dependant (Fuqua, Winans and Greenberg, 1994).

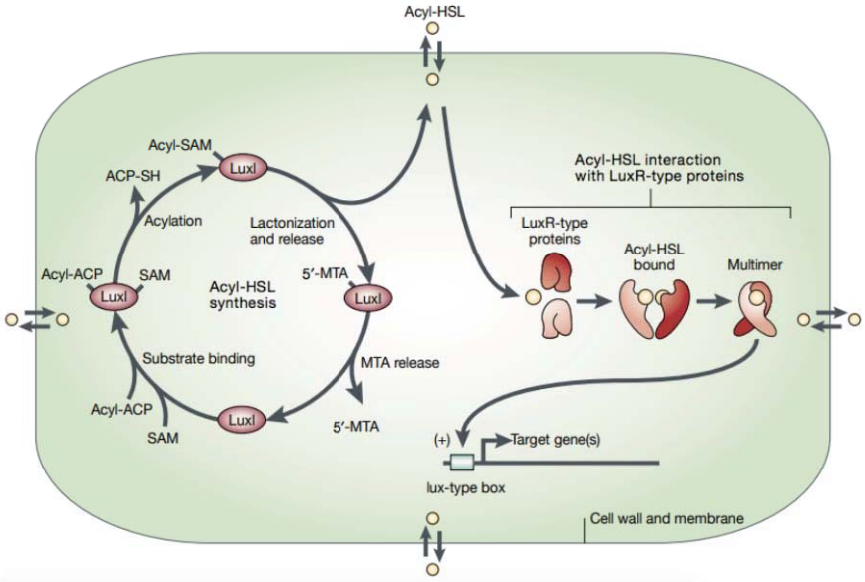

Figure 1.6 AHL dependent QS system displaying the interaction of AHL molecule with LuxR type proteins which induces QS mediated gene regulation. Bioluminescence is a QS mediated phenomenon commonly observed in *V. fischeri* (Fuqua and Greenberg, 2002).

The AHL molecule in *V. fischeri* (3OC6-HSL) is synthesised from S-adenosyl methionine along with an acyl-carrier protein by a synthase encoded by *luxI* gene (Li and Nair, 2012). As the AHL molecule is produced at a basal level by each bacterial cell, over time the concentration of the signal molecule accumulates in the immediate growth environment as the bacteria proliferate (Atkinson and Williams, 2009). Once a certain bacterial cell density is reached, the accumulated AHL signal molecule is recognised and bound by the LuxR sensor protein encoded as *luxR*. This in turn acts as a transcriptional regulator and activates the expression of the *lux* operon (Miyashiro and Ruby, 2012). The R/I protein complex (synthase and sensor proteins) form a positive feedback loop consisting of signal transduction and AHL molecule (Kaplan and Greenberg, 1987; Ng and Bassler, 2009). Once

the LuxR/I protein complex is activated, which in turn activates the Lux operon that turns on bioluminescence (Verma and Miyashiro, 2013).

The initial definition of chemical mediated cell-to-cell communication system to coordinate gene expression within bacterial communities, was given by Fuqua, Winans and Greenberg (1994) who then coined the term quorum sensing. Since then, it has been discovered that the phenomenon of QS is widespread in the microbial world and there are numerous QS systems specific to certain microbial species (Gray and Garey, 2001). Within a bacterial community, individual cells secrete and respond to their species-specific small QS molecules (often referred to as autoinducers) (Rutherford and Bassler, 2012). Research into QS conducted over the past few decades imply that there are a variety of different autoinducers, regardless, the general principles of QS are conserved in a wide range of bacteria (Hense and Schuster, 2015).

Three classes of bacterial QS system have been identified and are categorised based on the type of autoinducer involved and the signal receptors (Zheng and Sintim, 2014). The initial discovery of the LuxR/I QS system in *V. fischeri* (Ruby and Lee, 1998) was followed by the discovery of the presence of the LuxR/I QS system in over 70 species of Gram-negative bacteria (Henke & Bassler, 2004; Miller & Bassler, 2001). A second class of QS system was found to exist in Gram-positive bacteria, where the bacterial communication is based on autoinducers made of modified oligopeptides (Lazazzera and Grossman, 1998). The extracellular oligopeptides produced by Gram-positive bacteria are recognised by a two-component signal transduction protein known as the histidine kinase (Sarwar and Garza, 2016). The transcription of QS mediated genes takes place upon phosphorylation of the histidine kinase response regulator which in turn alters its DNA-binding activity, thereby controlling the change of gene expression (Plate and Marletta, 2013).

The third class of QS system is a partial combination of the Gram-positive and Gram-negative QS systems (Welsh and Blackwell, 2016). A small molecule autoinducer (e.g. AHL) is synthesized and released into the immediate growth environment among Gram-negative bacteria (like the first QS system). However, the sensing of the autoinducer and subsequent control of transcription of QS target genes is done through a two-component signal transduction system similar to the second-class QS system as seen in Gram-positive bacteria (Bassler *et al.*, 1993; Chen *et al.*, 2002). See Figure 1.7. In addition to the above

three QS system, numerous other minor QS system do exist that are highly specific to certain organisms.

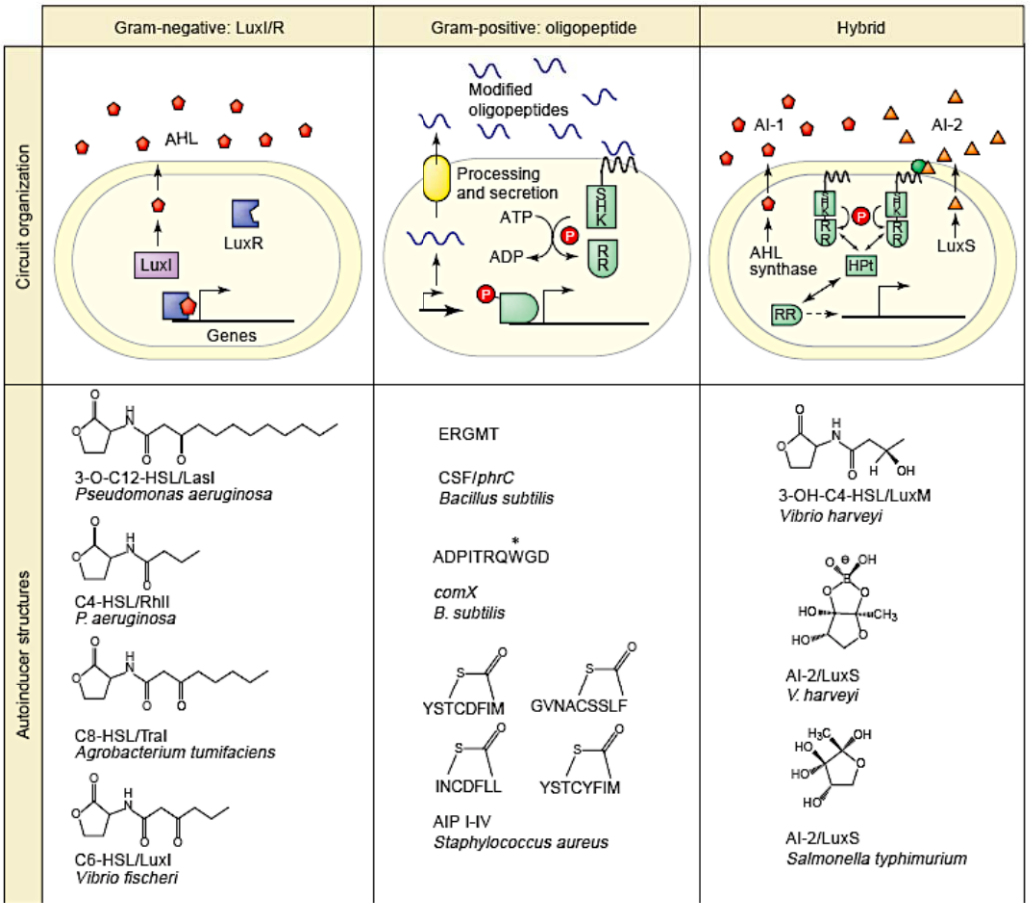

Figure 1.7 Canonical QS systems found in bacteria. The chemical diversity of signals are categorised into three classes as Gram-negative (LuxI/R), Gram-positive (oligopeptides) and hybrid systems that features LuxI/R as well as oligopeptides (Henke and Bassler, 2004).

It is to be noted that certain autoinducer signals are utilised for interspecies interactions (Marques *et al*., 2011). As an example, *luxS* gene which is responsible for the synthesis of autoinducer 2 (AI-2) (Figure1.7 column 3) is not present in *P. aeruginosa*, however, it does respond to AI-2 synthesized by non-pseudomonad bacteria present and isolated from sputum samples from CF patients (Duan *et al*., 2003). In a mixed culture biofilm involving *Burkholderia cepacia and P. aeruginosa*, it was demonstrated by cross feeding assay that even though both the organisms possess atypical AHL synthase and receptor genes, both the organisms responded to AHL produced by each other (Lewenza, Visser and Sokol, 2002).

When considering the role of QS in pathogenic bacteria in developing novel antibacterial therapies, the following features mediated by QS must be noted that make the pathogen robust: host colonisation, adhesion, acid tolerance, biofilm formation, motility, sporulation and various other virulence factors specific to each bacterial species (Rutherford and Bassler,

2012; Obana, Nakamura and Nomura, 2014; Abisado *et al*., 2018). Even though hosts are in constant contact with biofilm forming pathogens, development of chronic infections are rare due to the presence of innate immune system of the host that aids in the defence against infections. However, the host immunity relies on responses induced by presence of microbial threats such as bacterial flagellin or peptidoglycans. Recognition of such microbial threats triggers an immune response that is effective in healthy individuals. Therefore, in an individual with a compromised immune system, the pathogen may overcome the immune response and cause an acute or chronic infection. This is a strategic evolutionary response developed by pathogens to colonise and proliferate for survival. QS plays an extensive role due to its cell density dependent response system. It aids the pathogens in evading the host immune response prior to proliferation.

1.8.2 Quorum sensing in *P. aeruginosa*

The QS of *P. aeruginosa* is one of the most thoroughly researched QS systems amongst QS mediated biofilm forming pathogens (Lee and Zhang, 2014). The QS circuitry is mediated by two distinct classes of signalling molecules with differing chemical structures; N-acyl-homoserine lactones and alkyl quinolones (Chan, Liu and Chang, 2015).

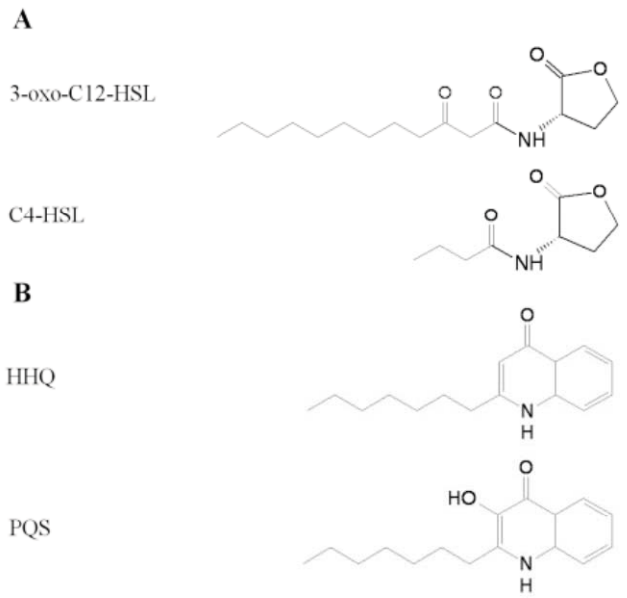

Figure 1.8 *P. aeruginosa* QS molecules and their chemical structures. **A)** represents the acyl-homoserine lactones (Oxododecanoyl-homoserine lactone and Butanoyl-homoserine lactone. **B)** represents the Alkyl quinolones (4-hydroxy-2-heptylquinolone and 2-heptyl-3,4-dihydroxyquinolone) (Pesci *et al*., 1999)

1.8.2.1 Acyl-homoserine lactones (AHLs) as QS signal molecules

AHLs are involved in the Lux type QS system present in *P. aeruginosa* (*las* and *rhl*) (Lee and Zhang, 2014). The signal synthase LasI and the transcriptional regulator LasR are involved in the *las* QS system along with oxododecanoyl-homoserine lactone (3-oxo-C12-HSL) which is the signalling molecule (Dekimpe and Deziel, 2009). Active transport of the signalling molecule (3-oxo-C12-HSL) to the outside of the bacterial cell takes place with the aid of efflux pumps while it also diffuses through the cell membrane at a slower rate (Blanco *et al*., 2016). Regulation of expression of specific genes takes place upon LasR binding to its cognate signal molecule, 3-oxo-C12-HSL. (Papenfort and Bassler, 2016). At physiological levels, LasR binds reversibly to its signal molecule and under *in vitro* conditions, the transcription of genes brought about by LasR-signal binding can be quenched upon removal of the signalling molecule from the growth medium (Sappington *et al*., 2011; Schuster, Urbanowski and Greenberg, 2004).

Similarly, the *rhl* system comprises of RhlI and the RhlR, the signal synthase and the transcriptional regulator respectively (Jensen *et al*., 2006). The signalling molecule produced by RhlI synthase is butanoyl-homoserine lactone (C4-HSL) (Parsek *et al*., 1999). The *las* QS system regulates the *rhl* QS system in a hierarchical manner whereby it interconnects the two (Lee and Zhang, 2014). The LasR and 3-oxo-C12-HSL complex positively regulates the *rhl* QS system (Sun *et al*., 2016). The QS molecule (C4-HSL) of the *rhl* system diffuses rapidly and is not actively transported out of the bacterial cell which is in contrast to the 3-oxo-C12-HSL signalling molecule (Erickson *et al*., 2002). Similar to the LasR and 3-oxo-C12-HSL complex, the complex formed between RhlR and C4-HSL brings about a change in gene expression of specific genes (Medina *et al*, 2003).

Even though both the QS systems produce AHLs, the signalling molecules are very specific to the transcriptional regulator they bind to (Rutherford and Bassler, 2012). A positive feedback loop is created by the LasR and RhlR by amplifying the production of their signalling molecules by activating the expression of their cognate signal synthase (*lasI* and *rhlI*) (Rampioni *et al*., 2007; Churchill and Chen, 2011). Together, the *las* and the *rhl* QS systems regulate the gene expression of over 300 genes in *P. aeruginosa* (Kievit *et al*., 2001; Steindler *et al*., 2009).

1.8.2.2 Alkyl quinolones (AQ) as QS signal molecules

Signalling by the PQS system is mediated by 2-alkyl-4-quinolones (AQ) (Lin *et al.*, 2018). Similar to the Lux QS system, the PQS QS system has a cognate receptor PqsR and is compatible with two signalling molecules, 4-hydroxy-2-heptylquinoline (HHQ) and 2-heptyl-3,4-dihydroxyquinoline (PQS) (Gruber *et al.*, 2016; Dulcey *et al.*, 2013). HHQ and PQS bind to PqsR with low and high affinity respectively and act as ligands and PQS has been found to be more hydrophobic between the two and is mainly associated with membrane vesicles (Hodgkinson *et al.*, 2016; Baker *et al.*, 2017). The PQS QS system regulates nearly 170 gene, many of which are dependent on PqsE (a putative metallo-β-lactamase) and some are co-regulated by the *las* and the *rhl* QS systems (Folch, Deziel and Doucet, 2013).

The PQS system interconnects the *las* and *rhl* QS systems whereby (figure 1.9), the *las* QS system positively regulates the PQS production by activating expression of *pqsR* and *pqsH*, whereas the *rhl* QS system negatively regulates the PQS production (Brouwer *et al.*, 2014; Welsh and Blackwell, 2015) and in turn, PqsR induces the expression of rhlI (C4-HSL) (Maura *et al.*, 2016). A delicate relation between the synthesis of 3-oxo-C12-HSL and C4-HSL (*las* and *rhl* systems) modulates the PQS signalling pathway (Zhang *et al.*, 2017). Along with the thee QS systems (*las*, *rhl* and *pqs*), that allow for temporal expression of virulence genes as a part of a QS response, other environmental factors also do play a role is expression of QS genes as QS signals alone are not sufficient for expression of QS mediates gene expression (Moradali, Ghods and Rehm, 2017).

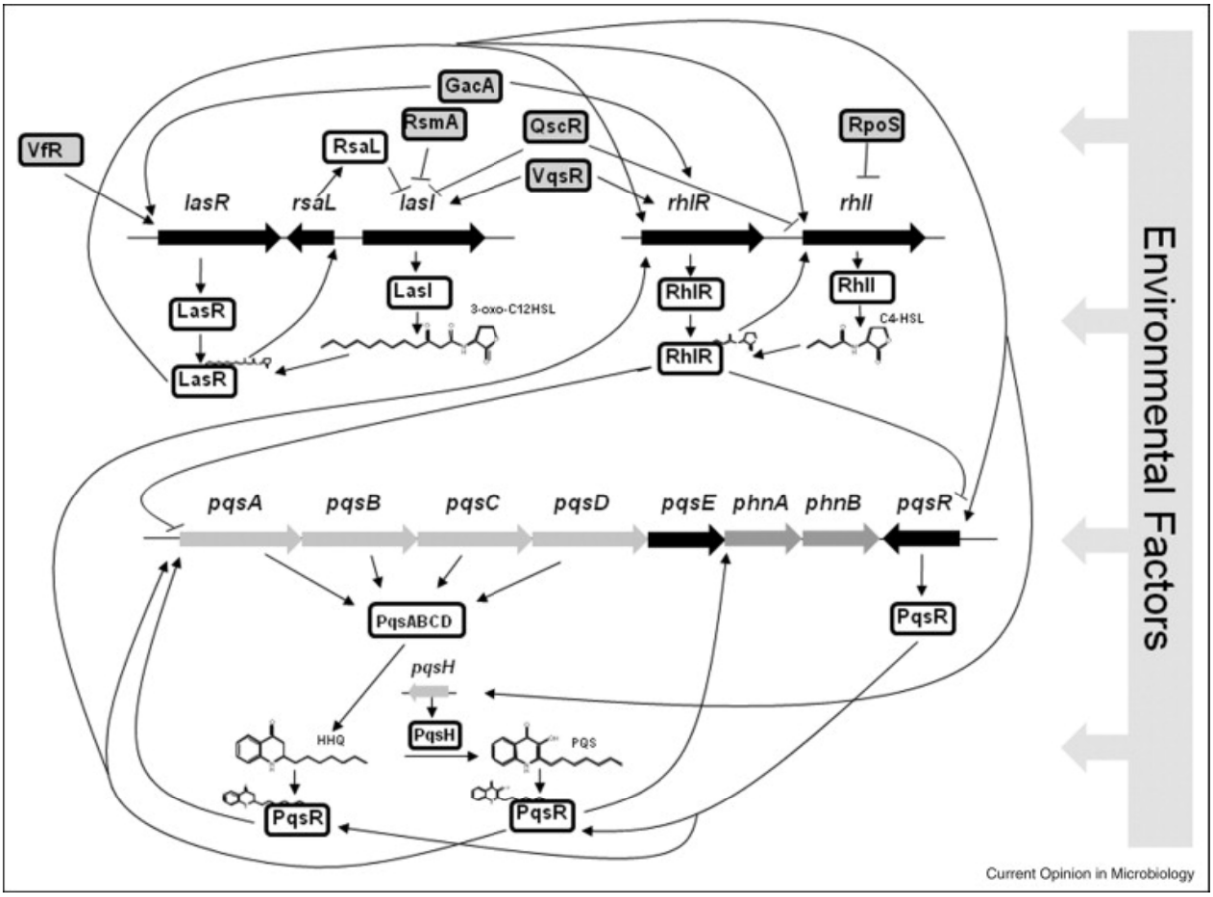

Figure 1.9 Representation of the hierarchical QS system present in *P. aeruginosa* (las, rhl and pqs) adapted from William and Camara, 2009.

The arrows indicate positive and negative transcriptional regulation. The gene lasI encodes a signal synthase that generates 3-oxo-C12, which binds to the transcriptional activator, LasR. This 3-oxo-C12: LasR complex upregulates both the *rhl* and the *pqs* QS systems. The gene *rhlI* encodes a signal synthase that generates C4-HSL, which binds to RhlR. This C4-HSL: RhlR complex negatively regulates the *pqs* system. Genes pqsABCDH, phzAB, and pqsH are involved in the generation of the signal PQS, which binds to PqsR. This PQS: PqsR complex up-regulates the *rhl* QS system.

1.8.3 QS and the expression of virulence factors in *P. aeruginosa*

P. aeruginosa, a pathogenic bacterium is well known for expression of multiple virulence factors (Gellatly and Hancock, 2013). As many of the virulence factors (See table 1.2) are QS regulated, a successful colonisation of the host and subsequent infection is often observed upon attaining a certain cell density (Winstanley, O'Brien and Brockhurst, 2016).

Table 1.2 Some of the QS mediated virulence factors of *P. aeruginosa*

Virulence factor	Gene	Reference
LasA protease	*lasA*	Aybey and Demirkan., 2016
Alkaline protease	*aprA*	Pel *et al.*, 2014
Elastase B	*lasB*	Yu *et al.*, 2014
Catalase	*katA*	Shin, Choi and Cho., 2008
Exotoxin A	*toxA*	Gaines *et al.*, 2007
Cyanide	*hcnAB*	Devi *et al.*, 2011
Chitinase	*chiC*	Folders *et al.*, 2001
Type IV pili	*pilC*	Takhar *et al.*, 2013
Pyocyanin	*phz*	Little *et al.*, 2018
Pyoverdin	*pvd*	Little *et al.*, 2018
Rhamnolipid	*rhlABC*	Bazire and Dufour., 2014
Flagellin protein	*fliC*	Ertugul *et al.*, 2018

Each individual virulence factor plays a role in the pathogenesis of *P. aeruginosa*; for instance, elastase degrades the extracellular matrix proteins of epithelial cells and disrupts the blood vessels (Xu and Shi, 2014), rhamnolipids promote invasion of *P. aeruginosa* by disrupting the host tissue (Alhazmi, 2015), pyocyanin inhibits cellular respiration (Hall *et al.*, 2016). Biofilm formation along with secreted toxins aid *P. aeruginosa* in circumventing the immune response of the host (Moser *et al.*, 2017). The prevalence of certain virulence factors may change with the localisation of the infection in the host tissue by *P. aeruginosa* (Gellatly and Hancock, 2013). Thus, the pathogenesis of *P. aeruginosa* is generally associated with the expression and production of numerous cell-associated as well as extracellular virulence factors mediated by QS.

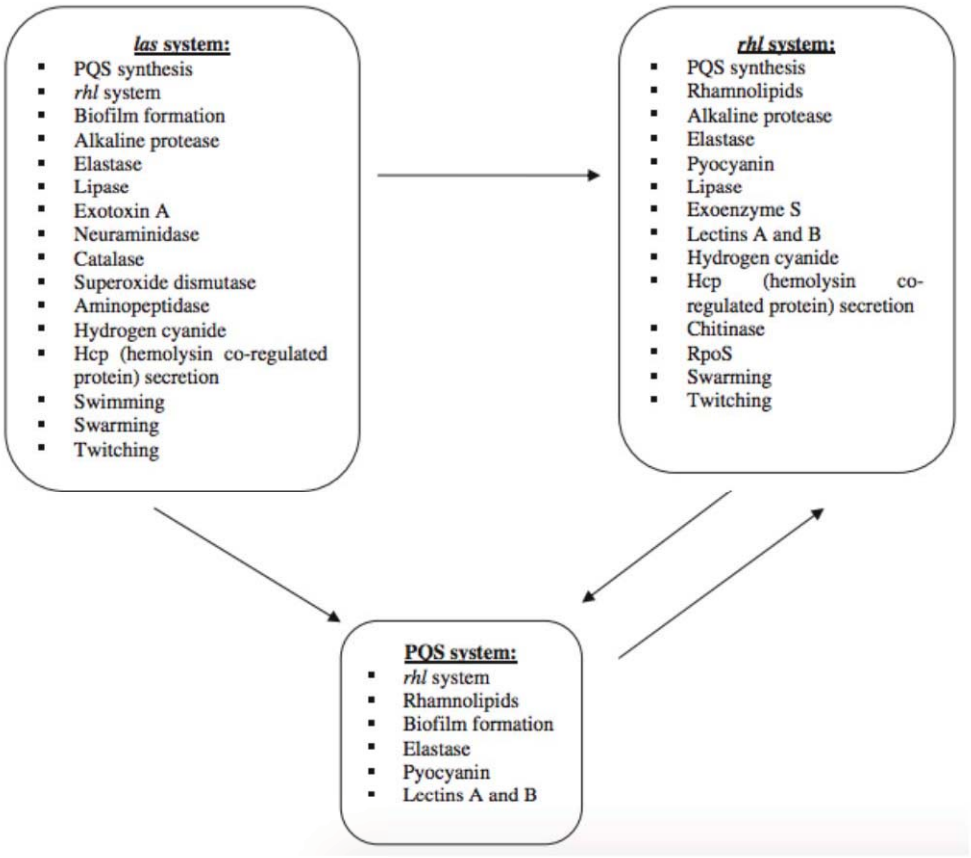

Figure 1.10 Virulence factors of *P. aeruginosa* and the QS system they are dependent on for expression (adapted from van't Wout *et al*., 2015).

1.8.3.1 Commonly studied virulence factors of *P. aeruginosa*

1.8.3.1a Siderophores

In mammalian hosts, *P. aeruginosa* is unable to acquire iron (ferrous Fe^{2+}) freely and iron is necessary for proliferation (Kang *et al*., 2018). Persistent infection by *P. aeruginosa* in the lungs of CF patients is aided by increased concentration of iron in the lungs during infection (Nguyen *et al*., 2014). As pathogenic bacteria cannot obtain iron freely in mammalian hosts, siderophores, iron chelators and transporters are employed to fulfil this function (Michel *et al*., 2005). In *P. aeruginosa*, two siderophores perform the function of accumulating iron, they are; pyochelin and pyoverdine (Minandri *et al*., 2016). Pyoverdine is the primary siderophore in *P. aeruginosa* (Kang *et al*., 2018) and pyoverdine deficient strains of *P. aeruginosa* have been shown to display decreased virulence (Granato *et al*., 2016). Pyochelin displays a lower affinity towards iron, compared to pyoverdine for iron uptake (Brandel *et al*., 2012). Studies conducted by Garcia-Contreras *et al*., 2014 and Ross-

Gillespie *et al*., 2014 showed that the presence of gallium is toxic to *P. aeruginosa* cells as it remains bound to pyoverdine and prevents iron uptake by remaining in the periplasmic space. In such a situation, the iron remains unreduced in its ferric (Fe^{3+}) form which is insoluble and unable to diffuse through cell-surface porins which is essential for absorption by pyoverdine (Cornelis and Dingemans, 2013). The bacterial cells compensate by producing pyocyanin which behaves as a reducing agent and not as an iron chelator (Hunter *et al*., 2013). Pyocyanin, a known virulence factor of *P. aeruginosa* is also a blue-green pigment that gives *P. aeruginosa* cultures its distinct colouration (Granato *et al*., 2016).

1.8.3.1b Pyocyanin

P. aeruginosa is unique in producing pyocyanin, which is a redox-active compound that is permeable through biological membranes (Lau *et al*., 2004a; Miller *et al*., 2015). Pyocyanin is vital to *P. aeruginosa* infections as it inhibits host cell growth and as mentioned previously, plays a role in aiding in iron acquisition (Gellatly and Hancock, 2013; Ballok and O'Toole, 2013). Expression of multidrug efflux pumps (MexGHI-opmD operon) induces the production of pyocyanin and is synthesised by phz and aro pathways (Sakhtah *et al*., 2016). Several studies conducted show that *rhlR* and *pqsR* are required for pyocyanin synthesis and a mutation in either of the genes could result is lack of pyocyanin (Lee and Zhang, 2014). This also goes on to show that pyocyanin synthesis is a QS system regulated virulence factor.

1.8.3.1c QS mediated elastase synthesis by *P. aeruginosa*

Elastase is an essential component of *P. aeruginosa* virulence arsenal. Categorised as a toxin, it's an enzyme belonging to the metalloproteinase family (Michalska and Wolf, 2015). Metalloproteinases are commonly found it snake venom as well (Kang *et al*., 2011) and retain similar function as the virulence factor produced by *P. aeruginosa*. Elastase functions by disrupting/ degrading the elastic lamina of arteries and extracellular matrices of epithelial cells (Lee *et al*., 2011). Synthesis of elastase is governed by the *las* operon (*lasI rhlR*) and PqsR, which makes it predominantly a QS mediated virulence factor of *P. aeruginosa* (Lee *et al*., 2011).

1.8.3.1d Rhamnolipid production by *P. aeruginosa*

A glycolipid-type biosurfactant is produced by *P. aeruginosa* during its late log phase of growth, which commonly contains two rhamnose sugars and is commonly known as rhamnolipid (Cheng *et al*., 2017). As a virulence factor of *P. aeruginosa*, rhamnolipids promote infiltration of the bacteria into the respiratory epithelium and thus invade the tissue (Blume *et al*., 2016). Rhamnolipids are considered a virulence factor of *P. aeruginosa* as they contribute towards swarming motility and biofilm architecture (Boles, Thoendel and Singh, 2005). As vital as rhamnolipids are towards the architecture of the biofilm, they are also involved in detachment of bacterial cell during the biofilm cycle of growth (Petrova and Sauer, 2016). From an industrial perspective, rhamnolipids are one of the most widely used biosurfactants in remediation of oil spills on water and soil (Chen *et al*., 2007).

1.8.3.1e Bacterial motility mediated by QS system of *P. aeruginosa*

The ability to be motile is an essential factor for pathogens as it aids in propagation of the infection (Kohler *et al*., 2000). Rate of mortality is generally commensurate with the motility of *P. aeruginosa* in CF patients (Bhagirath *et al*., 2016). *P. aeruginosa* displays three types of motility; swimming, swarming and twitching and they are assayed under *in vitro* conditions on aqueous agar (0.3% agar), semi-solid (0.5% agar) and solid (1% agar) respectively (Deziel, Comeau and Villemur, 2001). Flagella is essential for swimming and swarming motility whereas, type IV pili is a prerequisite for twitching motility (Bucior, Pielage and Engel, 2012). Swimming motility is required for the development of the biofilm as well as dispersal of the biofilm and is also dependant on rhamnolipid production and hence is governed by the *rhl* QS operon (Silva, Chellamuthu and Boedicker, 2017).

1.8.3.1f Type III secretion system of *P. aeruginosa*

Transfer of toxins from Gram-negative bacteria into the host cells generally takes place through the type III secretion system (Puhar and Sansonetti, 2014). *In vivo* contact with the host cell induces the expression of the type III secretion system in *P. aeruginosa,* which causes apoptosis of the epithelial cells and contributes as virulence factor (Galle, Carpentier and Beyaert, 2012). The mechanistic action of the type III secretion system is a two-step process wherein, the toxins are initially translocated across the inner membrane and then across the outer membrane of the Gram-negative bacterial cell (Pilar and Coombes, 2011). Type III secretion system is encoded by 43 regulated genes that are responsible for the translocation apparatus, type III effectors and regulators in *P. aeruginosa* (Hauser, 2009).

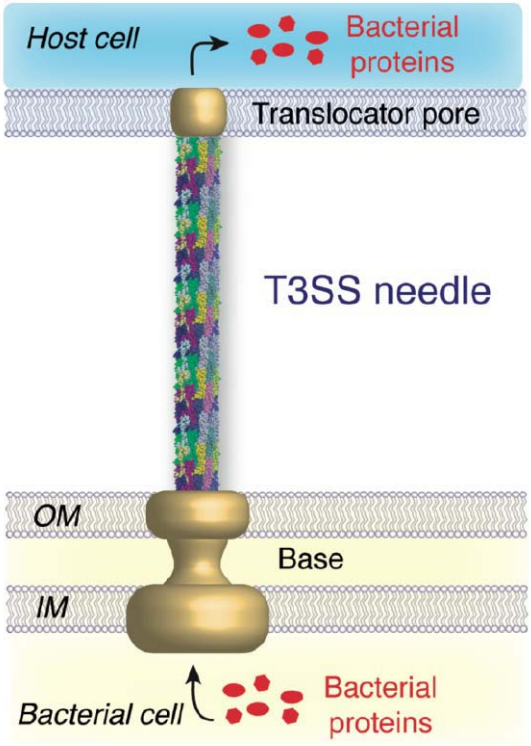

Figure 1.11 A schematic representation of type III secretion system in *P. aeruginosa* (adapted from He, Nomura and Whittam, 2004).

1.9 Conventional strategies used in combating biofilm formation

Over the years, numerous strategies have been formulated to combat biofilms based on the various challenges presented by biofilm formation. In the medical sector, biofilm-related diseases display a chronic and recurrent pattern (Lebeaux, Ghio and Beloin, 2014). Hospital-acquired infections, often referred to as nosocomial infections pose a grave threat as the clinical environment offers the required selective pressure for development of drug resistance (Bebell and Muiru, 2014). As chronic nosocomial infections require specific treatment regimens, conventional antibiotic therapy may fail (Barlow and Nathwani, 2005). Therefore, specific therapeutic regimens targeting biofilm related infections may include systemic antibiotic prophylaxis, antibiotic lock therapy and sterilisation of infection site (Lebeaux, Ghio and Beloin, 2014). A brief summary of various methods categorised based on mechanisms involved in combating biofilm is presented in table 1.3.

Table 1.3 Brief summary of strategies used in combating medical biofilm. (adapted from Mishchenko, 2016)

	Target mechanism	Examples
Biochemical methods		
Prevention	**Affecting bacterial adhesion**	**Surface coating:** Chlorhexidine-silver sulphadiazine, triclosan, antimicrobial peptides (AMPs)
		Attachment mechanism: Mannosides, pilicides, curlicides
		Antibodies neutralizing attachment molecules: anti-Pseudomonas immunoglobulin Y
	Affecting biofilm EPS	**Enzymes:** DNAse, Dispepsin B, lysostaphin
		Chelating agents: Sodium citrate, minocycline-EDTA
		Matrix inhibitors: Allicin
	Signal interference	Halogenated furanones
Eradication	**Signal inhibition**	**Affecting quorum sensing:** Azythromycin, ajoene, RNA III inhibiting peptide
	Disrupting biofilm	**Enzymes:** DNAse, dispepsin B, alginate lyase
		Chelating agents: Metals, tetrasodium-EDTA
		AMPs: cathelicidines, colistin, daptomycin
	Antibacterial	**Conventional antibiotics:** linezolid, rifampicin, fluoroquinolones
Biophysical methods		
Prevention	**Non-invasive sterilization**	Ultraviolet C treatment of surfaces
	Initial attachment	Low-energy surface acoustic waves
	Modified surface topography	Attachment-repelling anodic nanoporous surfaces and micropatterns
Eradication	**Microbubbles**	Ultrasound
	Physical excision	Surgery
Biological methods		
Prevention		**Use of probiotics:** Lactobacillus, Bifidobacteria
Eradication		Bacteriophage therapy

Food spoilage as a result of biofilm formation is common in the food industry. The presence of numerous surfaces and accumulation of microorganisms promotes biofilm formation. Commonly used physical methods to combat biofilm formation in an industrial setting are the use of steam and ultrasonication and certain chemical compounds such as sodium hypochlorite, sodium hydroxide, peracetic acid and hydrogen peroxide are also used (Galie *et al*., 2018). Certain enzymes, bacteriophages, and essential oils are used as well. Table 1.4 highlights the common methods used in combating biofilm formation in the food industry.

Table 1.4 Brief summary of strategies used in combating biofilm in the industrial sector. (Based on Galie *et al*., 2018)

Treatment	Mechanism of action	Examples
Chemical treatments	Cell structure oxidation	NaOCl, peracetic acid, NaOH, H2O2 (sanitisers)
Enzymatic disruption	Biofilm matrix disruption	Cellulases, proteases, glycosidases, Dnases
	Bacterial membrane disruption	Nanoparticles Ag2+, Fe3O4, TiO2, ZnO, CuO, MgO
Steel coating	Bacterial attachment	Repelling surfaces (monolayers, hydrogels, modified topography)
	Bactericidal	Functionalized surfaces (with lisozyme or nisin)
Biosurfactants	Bacterial adhesion	Surfactin, lichenysin
Bacteriophages	Cell lysis	P100
Enzymatic QS inhibitors	Inhibition of QS	Paroxonases
Essential oils	QS and motility inbition	Citral
	Bactericidal	Carvacrol
High hydrostatic pressure	Bactericidal	H_2O
Non-thermal plasma photocatalysis	Bactericidal	UV plus O_2, N_2, O_3, H_2O and He

1.10 Quorum quenching (QQ): A strategic approach to combat QS mediated biofilm infections

In a highly competitive microbial world where bacterial species, with the aid of QS synchronise their behaviour by cell density dependent gene regulation to survive and colonise, it should not come as a surprise when certain other bacterial species stand up to the competition and evolve strategies to overturn the AHL dependent QS mediated survival mechanism. Generally, environmental bacteria adopt such a strategy to compete in a polymicrobial race for survival, the strategy of signal interference mechanism they adopt has come to be recognised as quorum quenching (QQ).

1.10.1 Strategies to interfere with QS

Literature suggests that there are numerous potential strategies aiming to interfere with bacterial QS. These strategies usually come under the following three categories when looking at the QS system of Gram-negative pathogens such as *P. aeruginosa*; targeting the signal generator, the signal molecule and the signal receptor (Jakobsen, Tolker-Neilsen and Givskov, 2017).

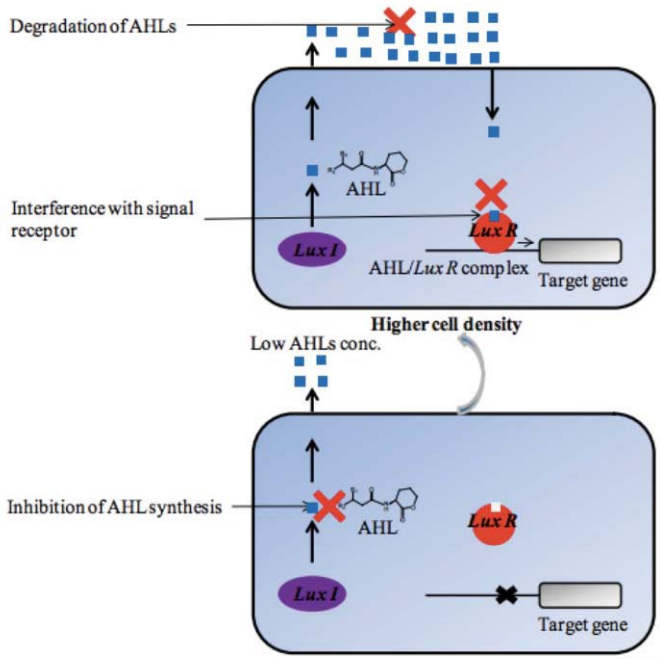

Figure 1.12 QQ of AHL mediates QS systems. 1) Degradation of AHLs, 2) Signal receptor interference and 3) Inhibition of AHL synthesis (Lade, Paul and Kweon, 2014).

Studies conducted on the potential of QQ demonstrate the use of natural substances to interfere with bacterial QS by mimicking bacterial AHL molecules (LaSarre and Federle, 2013). This strategy includes the use of synthetic derivatives that target the QS regulatory proteins (Rutherford and Bassler, 2012). Common examples are halogenated furanones from *Delisea pulchra*, autoinducing peptides (AIP) and AHL analogues (Galloway *et al.*, 2012). These small inhibitors bind to the target receptor by dislodging the original signal molecule from its reporter protein by competitive inhibition (Gopu, Meena and Shetty, 2015).

Furthermore, QQ can be achieved with a great deal of efficiency by hindering the synthesis of QS signalling molecules (Soler *et al.*, 2018). By restricting the fundamental elements or enzymes involved in synthesis of QS molecule, such as acyl carrier protein (ACP) that catalyses the production of acyl side chains, the LuxI homologous protein and S-adenosylmethionine synthase, which is involved in synthesis of AHLs, one can impede the synthesis of QS signal molecules (Ruparell *et al.*, 2016). Studies conducted by Adonizio, Kong and Mathee, 2008 and more recently by Singh, Mishra and Jha, 2017 showed that plants secrete certain secondary metabolites that interfered with the QS system of bacteria found in the rhizosphere. The secondary metabolites do so by mimicking the QS signal molecules. Adonizio, Kong and Mathee, 2008 successfully demonstrated that the secondary metabolites secreted from plants were found to antagonise the QS system of *P. aeruginosa*.

Finally, QQ enzymes play a vital role in inactivating AHL molecules by cleaving the AHL molecule and rendering it unsuitable to aid in triggering a QS meditated reaction (Utari, Vogel and Quax, 2017). These QQ enzymes were found to be stable under various conditions and highly specific towards the QS regulators and were found to function by cleaving the AHL molecules enzymatically without hindering other metabolic processes (Boyer and Wisniewski-Dyé, 2009).

So far, four distinct enzymes for AHL degradation have been proposed; lactonase, acylase, decarboxylase and deaminase (Chen *et al.*, 2013). However, only AHL lactonase and AHL acylase have been thoroughly investigated (Garge and Nerukar, 2016). Initially, AHL lactonase was initially discovered in *Bacillus thuringiensis* and AHL acylase was discovered in *Variovorax paradoxus* (Park *et al.*, 2005), since then numerous other bacterial strains found in plants, soil samples and laboratory cultures have been discovered to synthesise QQ enzymes (Zapata *et al.*, 2017). While AHL-lactonase cleaves the homoserine lactone ring of the AHL molecule in a hydrolytic and reversible manner (Lade, Paul and Kweon, 2014)

rendering the QS molecule redundant, AHL-acylase irreversibly hydrolyses the amide linkage between the acyl chain and the homoserine moiety of the AHL molecule (Zapata *et al*., 2017). While the cleavage performed by AHL-lactonase is reversible by acidification, the homoserine lactone and the corresponding fatty acid released as a result of AHL-acylase cleavage does not exhibit any residual signalling activity (Mukherji and Prabhune, 2015).

As QS signalling systems of microbial pathogens are central regulators of virulence factors, they represent highly attractive targets for developing novel therapeutics to prevent microbial infections. By preventing signal molecules involved in QS, the therapy would aid in combating bacterial pathogenicity rather than bacterial growth and this would be highly applicable in numerous areas of daily life (LaSarre and Federle, 2013). The potency of molecules interfering with the QS signals for therapeutic uses necessitates certain requisite traits for optimal QQ activities they include:

- Low molecular weight
- Activity causing a significant reduction in expression of QS controlled genes
- Inhibitor activity exhibiting a high degree of specificity for QS regulators without toxic side effects on either the bacteria or the eukaryotic host
- Chemical stability and resistance to metabolism and disposal by host organism

QQ compounds can be isolated from natural sources such as plants and fungi (Miao *et al*., 2017). Both plants and fungi have co-existed with QS bacteria for millions of years, so at least some have developed the ability to inhibit microbial QS. Penicillium species produce secondary metabolites with QQ activity (Liu *et al*., 2017). On the other hand, QS can be inhibited synthetically by a) introduction of substitutions in the acyl side-chain without any change in the lactone ring, b) Introduction of substitutions and alterations in the lactone ring with unchanged acyl side-chain, and c) Extensive modifications in both the acyl side-chain and the lactone ring (Churchill and Chen, 2011; Zhang *et al*., 2015).

Prokaryotes are known to release enzymes (as mentioned above) to combat QS system of their niche competitors by degrading the AHL molecules. Similarly, certain eukaryotic cells and mammalian serum contain enzymes such as paraoxanase that degrade AHL molecules (Bar-Rogovsky, Hugenmatter and Tawfik, 2013). *D. pulchra* has been documented to produce halogenated furanones that adversely affect the QS system in *V. fischeri* and *V. harveyi* but do not have an adverse effect against *P. aeruginosa* (Lidor *et al*., 2015). However, synthetically produced furanone - C30 increases *P. aeruginosa* susceptibility towards sodium dodecyl sulphate (SDS) and tobramycin which results in rapid dispersal of

P. aeruginosa biofilm (Chambers and Sauer, 2013). Similarly, naturally occurring chemicals such as; furocoumarins in grapefruit (Koh *et al.*, 2013), clove oil (Kim *et al.*, 2016), cinnamaldehyde (Li *et al.*, 2018), honey, phenols from olive oil and grapes (Singh *et al.*, 2017), garlic (Jakobsen *et al.*, 2017), patulin and penicillic acid (Rasmussen *et al.*, 2005) demonstrate the ability to attenuate QS mediated gene regulation in pathogenic bacteria.

The utilization of QQ as a promising strategy of anti-virulence therapy has been demonstrated *in vitro* and *in vivo* (Tang and Zhang, 2014). AHL mediated QQ mechanism exists in several bacterial species, either to gain a competitive edge or for survival. The application of an enzymatic QQ strategy suggests an alternative non-toxic approach for control of biofilm formation and a reduction in virulence without conferring antibiotic resistance. The external addition of QQ enzymes represents a novel general antibacterial therapy along with beneficial environmental implications. Thus, therapeutics that interfere with AHL-controlled pathogenicity could potentially have longer functional shelf lives than second and third generation antibiotics and this niche in antimicrobial application should be investigated further.

1.11 Mechanisms of dispersal as a mode of combating biofilms

Numerous studies have shown that variation in environmental and growth conditions such as limitation of oxygen, nutrients, variation in pH contributing towards dispersal of biofilm. Flow cell studies conducted on *Shewanella oneidensis* MR-1 by Thromann *et al.*, in 2006 determined that nearly 80% of the biofilm was dispersed within 5 min of halting medium flow due to lack of available oxygen. It was found that glucose, sodium succinate, sodium citrate and sodium glutamate triggered biofilm dispersal under flow condition by Sauer *et al.,* in 2004. Similarly, certain salts, chelating agents, surfactants, nitric oxide and synthetically derived compounds have been found to behave as external stressors that induce biofilm dispersal (Fleming and Rumbaugh, 2017). A study conducted by Chen and Stewart in 2000 showed that EDTA, sodium dodecyl sulphate (SDS), Tween 20 and Triton X-100 caused over 25% reduction of the protein content of biofilms formed by *P. aeruginosa* and *K. pneumoniae* which triggered dispersal. Boles, Thoendel and Singh in 2005 observed the dispersal effect of rhamnolipid, a biosurfactant produced by *P. aeruginosa*, which aided in the detachment of bacterial cells form the biofilm.

Enzymatic biofilm dispersal has been observed in the presence of lysozyme and alginate lyase (Lamppa and Griswold, 2012). Lysozyme is produced as part of the innate immune response while alginate lyase is naturally produced and secreted by *P. aeruginosa* (Ramsey and Wozniak., 2005). Bacteriophages have been engineered that are capable of producing enzymes to induce biofilm dispersal (Drulis-Kawa, Majkowska-Skrobek and Maciejewska, 2015). Two independent studies conducted by Alkawash, Soothill and Schiller in 2006 showed that alginate lyase degraded the alginate present in mucoid biofilms and aided in passage of bacterial cells to the surface of the biofilm to induce dispersal while study conducted by Lu and Collins in 2007 showed an engineered a bacteriophage (T7DspB) was capable of degrading the biofilm while infecting the bacterial cells and subsequent lysis. Presence of genes responsible for chemotaxis, small fatty acid molecule (*cis*-2-decanoic acid) and autoinducing peptide I (AIP-I) have been reported to induce biofilm dispersal by up to 90% (Davies and Marques, 2008).

Given the continuing threat posed by QS mediated biofilm related infections, which are often highly persistent due to a lack of effective medical advances in combating the various challenges posed by Gram-negative pathogens, such as the onset of antimicrobial resistance, there is an unquestionable need for research advances aimed at mitigating the resulting socioeconomic and medical burdens. More recently, there has been a global recognition of the emergence of 'superbugs' and antibiotic resistance, and the subsequent need to investigate the underlying factors giving rise to superbugs, chronic biofilm related infections and antibiotic resistance is gaining momentum. Clearly, the use of conventional antibiotics treatments involving β- lactams and aminoglycosides have their limitations when treating *P. aeruginosa* related chronic infections due to onset of resistance. To this end, investigating the mechanisms of QS and biofilm formation and the effective use of QQ as alternative strategies can be of great potential in combating biofilm related chronic infections.

Aim and objectives

For this study, three strains of *P. aeruginosa* were selected as model bacteria: *P. aeruginosa* NCTC 10662 (non-mucoid), *P. aeruginosa* PAO1 (mucoid) and *P. aeruginosa* RBHi (a CF isolate which is heavily mucoid). The hypothesis that QS mediated formation of biofilm, virulence and subsequent pathogenicity can be curtailed by the use of QQs, either independently or in combination due to their ability to interfere or inhibit the bacterial QS system was explored.

The overall aim of this project was to investigate QS mediated biofilm formation and virulence of *P. aeruginosa* and the disruption/ inhibition of QS through the use of biological and chemical QQs and biofilm dispersal agents. In order to address the overall aim of the project, the following objectives were investigated:

- The impact of medium composition and nutrient content on *P. aeruginosa* biofilm development and architecture.

- The composition, and structure of *P. aeruginosa* biofilm architecture on medically relevant surfaces under static and dynamic conditions.

- Exploiting the QS system of *P. aeruginosa* by adopting an independent as well as synergistic administration of chemical and biological QQs to inhibit biofilm formation and virulence under static and dynamic conditions.

- The effect of fungal QS molecules (farnesol and tyrosol) on *P. aeruginosa* biofilm formation and virulence.

- *P. aeruginosa* biofilm formation in co-culture using A549 and HaCaT cell-lines and secreted virulence factors with and without QQ treatment.

Chapter 2 . Materials and methods

2.1 Materials

Materials used in this study were purchased from Sigma-Aldrich (Dorset, UK), Thermo Fisher Scientific (Invitrogen, Loughborough, UK), Fisher Scientific (Loughborough, UK), Cambridge Biosciences (Cambridge, UK), Biosurface Technologies Ltd (Montana, USA). Bacterial culture media were obtained from Sigma-Aldrich (Dorset, UK), Difco, Oxoid and VWR, UK. All quantitative and qualitative assays were performed using analytical grade reagents. DNA, RNA extraction and purification kits were obtained from Sigma-Aldrich (Dorset, UK). Reagents and kits for qPCR were obtained from Qiagen Ltd (Crawley, UK). PCR master mix (Promega) was used for conventional PCR while primers for PCR and qPCR were obtained from Eurofins Scientific (Ebersberg, Germany).

2.1.1 Bacterial strains

P. aeruginosa PAO1 and *P. aeruginosa* NCTC 10662 were obtained from the University of Westminster, London culture collection. *P. aeruginosa* CF isolate was kindly donated by the culture collection facility at Royal Brompton Hospital, London, UK (referred to as RBHi in this study). *B. licheniformis* NCIMB 8874 was also obtained from the University of Westminster, London UK, culture collection.

2.1.2 Cell culture

A549 pneumocyte cells are human lung carcinoma cells derived from type II alveolar pneumocytes (Kaplan, Ciftci and Kutlu, 2017). They were obtained from American type culture collection (ATCC CCL-185). HaCaT cells are spontaneously immortalised human keratinocyte cell line (Seo *et al*., 2012). The cell-line was obtained from University of Westminster cell culture collection which was a kind donation from the Division of Surgery and Interventional Science, UCL, Royal free Hospital, London, UK.

2.2 Maintenance and growth condition of bacterial strains and cell culture

2.2.1 Media preparation for bacterial cultures

All media used in this study were prepared using deionised water and sterilised at 121 °C for 15 min. Additionally, if the medium required supplementation of sugars, the sugar solution was prepared separately and autoclaved at 110 °C for 10 min. prior to combining with the primary medium under aseptic conditions to obtain the required concentration.

Table 2.1 Media used for bacterial cultures for the duration of the study

Bacterial culture media	Composition/ description
Mueller-Hinton broth	2 g/L beef infusion solids, 1.5 g/L starch, 17.5 g/L casein hydrolysate
Mueller-Hinton agar	2 g/L beef infusion solids, 1.5 g/L starch, 17.5 g/L casein hydrolysate, 15 g/L
Luria Bertani broth	10 g/L sodium chloride, 10 g/L tryptone, 5 g/L yeast extract
Luria Bertani agar	10 g/L sodium chloride, 10 g/L tryptone, 5 g/L yeast extract, 15 g/L agar
M9 minimal medium (5X)	33.9 g/L sodium phosphate, 15 g/L potassium phosphate, 5 g/L ammonium chloride, 2.5 g/L sodium chloride
Nutrient broth	1 g/L meat extract, 2 g/L yeast extract, 5 g/L peptone, 5 g/L sodium chloride
Nutrient agar	1 g/L meat extract, 2 g/L yeast extract, 5 g/L peptone, 5 g/L sodium chloride, 15 g/L agar

2.2.2 Medium for growth and maintenance of *P. aeruginosa* strains

Mueller Hinton broth and agar (Sigma-Aldrich, UK) were used for the maintenance of *P. aeruginosa* strains. Briefly, the medium (Table 2.1) was prepared based on the manufacturer's instructions by dissolving 21 g/L of distilled water to prepare Mueller Hinton broth and 36 g/L to prepare Mueller Hinton agar. To prepare the agar, the solution was heated on a hotplate to fully dissolve the medium prior to autoclaving at 121 °C for 15 min.

Luria-Bertani (LB) broth was prepared according to the manufacturer's instruction (Sigma-Aldrich, UK). Briefly, 25 g/L of the powder was dissolved to prepare LB broth and 40 g/L

of agar powder was used to prepare LB agar as shown in table 2.2. In order to make sure the components of the agar were fully dissolve, the solution was heated and stirred on a hotplate prior to autoclaving both solutions at 121 ºC for 15 min.

M9 minimal medium was prepared according to the manufacturer's instruction. Briefly, 56.4 g/L of M9 minimal medium powder was dissolved in 1L of distilled water to prepare a 5x concentrated stock and autoclaved at 121 ºC for 15 min. The 5x concentrated stock of M9 medium was then diluted to 1x by adding 200 mL of M9 stock to 800 mL of sterile water supplemented with 20% glucose (w/v), 1M magnesium sulphate and 1M calcium chloride. The additional reagents were filter sterilised prior to combing with the M9 stock using 0.22 µm filter (Millipore). The 1x M9 minimal medium was then used for bacterial growth experiments.

Nutrient broth and nutrient agar were prepared according to the manufacturer's instruction (Sigma-Aldrich, UK). Briefly, 28 g/L (broth), and 43 g/L (agar) powder were completely dissolved in 1L of distilled water prior to sterilisation by autoclaving at 121 ºC for 15 min.

2.2.3 Long term storage of bacterial cultures and preparation of working stock

All bacteria were stored as 20% glycerol stocks at -80 ºC. The stocks were prepared by selective growth of a single colony in liquid broth in Mueller-Hinton (*P. aeruginosa*) and Nutrient broth (*B. licheniformis*) to an OD of ~1 (600 nm). A 1:1 ratio (total volume of 1 mL) of bacterial culture in 20% (v/v) glycerol was prepared and vortexed prior to storage at -80 ºC. In order to conduct experiments, a working stock was prepared by taking a loopful of the frozen bacteria to be streaked onto respective agar slants which were then incubated at 37 ºC and 28 ºC respectively for ~18 – 24 h and then stored at 4 ºC. Sub-culturing from the frozen stock was performed every 2 months to maintain the viability and homogeneity of the working stock.

2.2.4 Media preparation for cell culture

Table 2.2 Media used for A549 and HaCaT cell culture and co-culture experiments for the duration of the study

Cell culture and co-culture media	Composition/ description
DMEM (A549)	High glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM) containing phenol red, 10% foetal bovine serum, 1x pen strep.
DMEM (HaCaT)	High glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM) containing phenol red, 10% foetal bovine serum, 1x pen strep.
Co-culture medium (bacterial attachment)	High glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM) without phenol red, containing 10% foetal bovine serum, 2mM L-glutamine.
Co-culture medium (biofilm formation)	High glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM) without phenol red, containing 10% foetal bovine serum, 0.4% arginine.

High glucose Dulbecco's Modified Eagle Medium (DMEM) containing phenol red (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich) and 1x penicillin-streptomycin (Invitrogen) was used. Briefly, FBS and pen-strep were warmed to room temperature from a frozen stock. Once warmed up to room temperature, 55 mL of FBS and 5 mL of pen-strep were added to 500 mL of DMEM under aseptic conditions.

2.2.4.1 A549 and HaCaT cell resurrection from frozen stock

A single vial of each of the cell types were removed from liquid nitrogen storage and warmed to room temperature. The contents of the cryovials were gently transferred into a 15 mL falcon tube. Fresh (5 mL) medium was added and mixed gently using the pipette. The tube was then centrifuged at 1500 rpm for 5 min. The supernatant was discarded, and 10 mL of fresh medium was added to the tube to resuspend the cells. Upon resuspension, the medium containing the cells were transferred to a T75 (Nunc EasyFlask 75 cm^2) tissue culture flask and incubated at 37 ºC with 5% CO_2. Growth medium was changed after 24 h and then every 48 h till a confluency of 85% was achieved.

2.2.4.2 Passaging A549 and HaCaT cells

Briefly, upon reaching 80% confluency, the spent medium was gently aspirated and the T75 (Nunc EasyFlask 75 cm^2) flask was washed with 10 mL of 1 x PBS. Subsequently, 2 mL of Trypsin-EDTA (0.25% BioReagent, Sigma Aldrich) was added to the flasks and incubated at 37 ºC with 5% CO_2 for a period of 5-10 min. After which, 6 mL of fresh DMEM was added to the flask to collect the detached cells and was transferred to a 15 mL falcon tube. The tube containing the cells was centrifuged at 1500 rpm for 5 min. The supernatant was discarded, and the cells were resuspended in 1 mL of fresh DMEM medium. A cell count was performed prior to aliquoting the required number of cells either into 96-well microtiter plates or 6-well tissue culture plates for further experiments.

2.2.4.2 A549 and HaCaT culture

A549 and HaCaT cells were seeded into either 96-well plate at 1 x 10^4 cells per well or 6-well plates (Nunc) at 5 x 10^5 cells per well, unless otherwise indicated. The cells were allowed to adhere overnight in high glucose DMEM (phenol red) supplemented with 10% FBS and 1x pen strep. The plates were incubated at 37ºC at 5% CO_2 for a period of 4 days till they reached ~80% confluency. The wells were then rinsed with 1x PBS to wash away dead cells and phenol red prior co-culture experiments and treatments.

2.2.4.3 Co-culture of *P. aeruginosa* with A549 and HaCaT cell lines

Protocol for the co-culture biofilm model was adopted from Moreau-Marquis *et al.*, 2010 with a few modifications as depicted below:

A) In this study, the static co-culture model involved the growth of A549 cell line (an adherent lung epithelial carcinoma) and HaCaT cell line (human immortalised keratinocyte). The cells were seeded at concentrations of ~10^6 cells per well in a 6-well tissue culture plate in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 µg/mL penicillin, and 50 µg/mL streptomycin. The cells were grown in 1.5 mL of medium per well.
B) The cells were grown at 37º C and at 5% CO_2 – 95% air for a period of 7-10 days to form a confluent monolayer of cells prior to inoculating with bacteria. The medium was changed by gentle aspiration every 2 days.

C) *P. aeruginosa* was grown in 5 mL LB medium for a period of 16-18 h at 37° C on a shaking incubator at 180 rpm. Under these conditions, *P. aeruginosa* cultures attained a cell density of approximately 4.3 x10^9 CFU/ mL. The culture suspension was then centrifuged at 8000 rpm to collect the bacterial cells and the cells were then re-suspended in 1x PBS buffer for further co-culture work.

D) For bacterial inoculation, the A549 and HaCaT growth medium was first removed and the cells were rinsed in 1x PBS buffer. Equal volume of DMEM medium is replaced with DMEM without phenol red and supplemented with 2 mM L-glutamine. The confluent A549 and HaCaT monolayer of cells were inoculated with *P. aeruginosa* at a multiplicity of infection (MOI) roughly calculated to 20:1 in relation to the number of A549 and HaCaT cells initially seeded into the wells.

E) The plates were then incubated for a period of 1-hour at 37° C and at 5% CO_2 – 95% air.

F) Following the 1-hour incubation, the growth medium was replaced with DMEM supplemented with only 0.4% arginine. The plates were then incubated at 37° C and at 5% CO_2 – 95% air for desired time points.

2.3 Buffers, solutions and reagents used in this study

Buffers used in this study were prepared according to manufacturer's instructions. Once all the components of the buffers were fully dissolved, they were either sterilised by autoclaving at 121 °C for 15 min or filter-sterilised using a 0.22 μm membrane filter. Table 2.3 lists the buffers used in this study.

Table 2.3 List of buffers used in this study

Buffers	Composition/ description
10x Phosphate buffered saline (PBS)	80 g/L NaCl, 2 g/L KCl, 14.4 g/L Na_2HPO_4, 2.4 g/L KH_2PO_4
5x TBE buffer	54 g/L Tris base, 27.5 g/L boric acid, 20 mL of 0.5 M EDTA (ph 8.0)
PBS-T	1x PBS, 0.05% Tween-20

Along with the buffers the following solutions (stock) and reagents were used in this study. Physiological (0.9% saline w/v) solution, 2 mg/mL bovine serum albumin stock solution, 2 mg/mL glucose stock solution, 1% (w/v) Congo red, 0.5 mg/mL ethidium bromide, 20% glycerol, 80% glucose, 33% glacial acetic acid, 400 mM $MgSO_4$. $7H_2O$, 400 mM $MnSO_4$. $7H_2O$, 400 mM $ZnSO_4$. $7H_2O$ and 400 mM $MgCl_2$. $6H_2O$.

2.4 Preparation of quorum quenchers, biofilm dispersal agents and antibiotics

Antibiotics, quorum quenchers, biofilm dispersal agents and efflux pump inhibitors used in this study included (Z-)-4-Bromo-5-bromomethylene)-2(5H)-furanone; E, E – farnesol; 2,4-hydroxyphenyl)-ethanol (Tyrosol); *cis*-2-decanoic acid (Cambridge biosciences, Cambridge, UK); methyl anthranilate; D\L tryptophan; Phenylalanine-Arginine Beta-Naphthylamide (PAβN); erythromycin and gentamicin. With the exception of *cis*-2-decanoic acid, all other items were purchased from Sigma-Aldrich (Dorset, UK). Table 2.4 lists the respective solvents and the concentration of stock solutions prepared.

Table 2.4 List of QQs, biofilm dispersal agents and antibiotics used in this study

QQs, dispersal agents and antibiotics	Solvent	Concentration
2(5H)-Furanone	DMSO	500 mM
E, E- Farnesol	Methanol	100 mM
Tyrosol (2,4-(hydroxyphenyl)-ethanol)	Water	100 mM
Cis-2-decenoic acid	DMSO	500 mM
Methyl anthranilate	DMSO	200 mM
D/L - Tryptophan	0.1M HCl	50 mM
Erythromycin	Ethanol	200 ug/mL
Gentamicin	Water	50 mg/mL
PAbN	DMSO	200 mM

All the stock solutions were stored at -20 ºC while the working stock solutions were stored at 4 ºC and renewed every 2 weeks. Working solution for farnesol and tyrosol were prepared on the day of use as they are known to oxidise rapidly at higher temperatures than -20 ºC once in solution.

2.3 Methods

2.3.1 Preparation of bacterial inoculum

P. aeruginosa strains and *B. licheniformis* were sub-cultured into LB broth and NB from the slants and incubated aerobically overnight (16-18 hours) at 37 ºC and 28 ºC at 180 rpm. The absorbance (OD_{600}) of the overnight growth of *P. aeruginosa* was readjusted in sterile LB broth to obtain an equivalence absorption according to 0.5 McFarland standards (~1.5 X 10^8 cells) and used for further biofilm growth. *B. licheniformis* (25 mL) of inoculum was diluted in NB to make up the volume to 250 mL prior to incubation at 28 ºC at 180rpm for a period of 48 h prior to harvesting as crude cell extract.

2.3.2 Preparation of *B. licheniformis* crude cell extract

Inoculum of *B. licheniformis* prepared as mentioned in 2.3.1.1 was sub-cultured in 2 x 250 mL of NB medium and incubated at 28 ºC for 48 hours. After the incubation period, the cells were harvested by centrifugation of the spent broth at 8000 rpm for 5 min. The cell pellets were retained by discarding the supernatant. The cell pellets (~ 0.5 gm each) were re-suspended in 2 x 5 mL of 0.1 M phosphate buffer pH 7. The cell suspensions were then subjected to sonication for 10 min at 70 amplitude, with 10 seconds ON and 10 seconds OFF pulse cycle. Both the sonicated cell suspensions were filtered through 0.22 µm sterile syringe filters. One of the cell suspension samples was freeze dried and re-suspended in 5 mL of 1x phosphate buffer and used for biofilm inhibition studies.

2.3.3 Growth curve and determination of doubling time of *P. aeruginosa*

Growth curves and doubling times were determined based on the increase in optical density at OD_{600} over time, of liquid cultures grown in LB broth. Turbidity (OD_{600}) measurements were made using a Beckman Coulter DU-640B spectrophotometer (Fullerton, CA). Specific growth- rate was calculated from the slope of the natural log of CFU/ mL at respective OD_{600} measurements corresponding to the linear portion of the curve during exponential growth phase, typically between 4 and 10 hours. A ten-fold dilution of each sample was prepared using 0.9% physiological saline and 10 µL was spot plated on LB agar and incubated overnight at 37ºC. Enumerated colonies were expressed as CFU/ mL All experiments were conducted in triplicate.

2.4 Investigation of biofilm formation by *P. aeruginosa*

Numerous *in vitro* models have been suggested to investigate and quantify biofilm formation (Lebeaux *et al*., 2013). Based on all the suggestions, the convenience of using the conventional 96-well microtiter plate biofilm assay is due to its simplicity, versatility and its high-throughput nature making them highly adaptable for varying experimental conditions.

2.4.1 Biofilm formation in 96-well microtiter plates and quantification by crystal violet (CV) staining method

In a 96-well microtiter tray, 200 µL of adjusted inoculum of *P. aeruginosa* was added to all test wells and 200 µL of the appropriate growth medium to control wells. After incubation for a period of 4 h, the wells were gently washed with PBS (Phosphate-buffered saline) to remove unattached planktonic cells and the appropriate amount of medium was re-added for biofilm growth. Under static conditions, the 96-well microtiter plates were incubated at 37 ºC for a period of ~18 h. After incubation for biofilm growth, 96-well trays were inversion washed once with 200 µL of PBS. Biofilm was fixed using heat fixation 60 ºC for 60 min, unless stated otherwise. CV (220 µL volume, 0.1% w/v) was added to each well and allowed to stain for 10 min at room temperature (approximately 22 ºC). Excess stain was rinsed off gently with large amounts of tap water until the rinsing water ran clear, which indicated that all the surplus stain was removed. The wells were then allowed to air-dry for 15 min. A 220 µL volume of 33% glacial acetic acid was added to each well to re-solubilise the CV stain. Glacial acetic acid was left for 5 min and the optical density was determined at 570 nm using VersaMax Tunable microplate reader (VWR).

2.4.2 *P. aeruginosa* biofilm inhibition and dispersal assays

The potential of QQs and biofilm dispersal agents to inhibit and disperse *P. aeruginosa* biofilms as individual treatments and in synergy was investigated using the microtiter plate assay. Further to this, the effect of medium composition involving divalent cations and their effect on *P. aeruginosa* to form biofilm was investigated as well. *P. aeruginosa* NCTC 10662 (non-mucoid), *P. aeruginosa* PAO1 (semi mucoid) and *P. aeruginosa* RBHi (heavily mucoid) were selected for this study due to their phenotypic difference which was represented in their ability to produce biofilms with differing EPS composition, specifically with their level of mucoidy. Quantification of biofilm was performed by the crystal violet assay (refer to 2.4.1).

2.4.2.1 Influence of medium composition on biofilm formation by *P. aeruginosa*

This study was performed to investigate the effect of divalent cations from different sources on biofilm formation by non-mucoid, mucoid and heavily mucoid strains of *P. aeruginosa* The strains were grown in LB broth supplemented with low and high concentration of salts containing divalent cations in the form of $MgSO_4$. $7H_2O$, $MnSO_4$. $7H_2O$, $ZnSO_4$. $7H_2O$ and $MgCl_2$. $6H_2O$. Stock concentration of 400 mM of each of the salts was diluted down to 2 mM and 20 mM into a total volume of 200 µL per well in a 96-well microtiter plate. A control without bacterial inoculum was used for quantification of biofilm biomass. The microtiter plate was incubated at 37 °C for a period of ~18 h. Biofilm formation of the three strains of *P. aeruginosa* was then quantified by the crystal violet assay. Similarly, the experiment was set up in 6-well tissue culture plates with a total volume of 3 mL of growth media. Concentration of salts and medium added per well in the 6-well plates were in ratio to provide the same conditions as the 96-well plate. Biofilm formation in 6-well plates was conducted for EPS extraction and subsequent quantification of components of the biofilm. All experiments were repeated 3 times and done in triplicates for reproducibility.

2.4.2.2 Investigating dispersal effect of D/L tryptophan and erythromycin as individual and combination treatments against *P. aeruginosa* biofilm formation

Experimentation set up was similar to 2.4.2.1. *P. aeruginosa* inocula were grown as mentioned in 2.3.1.1. Stock solution of D/ tryptophan and L/ tryptophan were diluted down to 1 mM, 4 mM and 8 mM into 200 µL total volume in 96-well plates containing *P. aeruginosa* sp. control wells contained the *P. aeruginosa* cells without the treatment.

Similarly, stock solution of erythromycin was diluted down from 50 mg/mL to a sub-inhibitory concentration of 4 µg/ mL as this concentration was found not to have any antagonistic activity against *P. aeruginosa* cells and cell viability. Subsequently, based on the data obtained from the individual effect of D and L tryptophan against *P. aeruginosa* biofilm formation (section 3.4, figure 3.8), combination treatment involving 4 mM D/tryptophan and sub-inhibitory concentration of erythromycin (4 µg/mL) was performed and compared against respective individual treatments and untreated control. All experiments were incubated at 37 ºC for a period of ~18 h. Following this, 6-well plates were set up with similar ratio of medium, bacterial cells and biofilm dispersal agents in a total volume of 3 mL and incubated at 37ºC for 18 h alongside the 96-well microtiter plate assay for EPS extraction and subsequent quantification of EPS components and sessile cells. All experiments were repeated three times and in triplicates for reproducibility.

2.4.2.3 Investigating QQ effect of farnesol, tyrosol and *B. licheniformis* crude cell extract as individual and combination treatments against *P. aeruginosa* biofilm formation and inhibition

Experimentation set up was similar to 2.4.2.1 and 2.4.2.2. *P. aeruginosa* inoculua were grown as mentioned in 2.3.1.1. Sub-inhibitory MICs of farnesol and tyrosol were used independently as well as in combination with CCE. As 100 µg/mL of CCE (chapter 5, figure 5.1) was found to show a significant inhibition of biofilm formation across all strains of *P. aeruginosa*, concentration of 100 µg/mL of CCE was chosen for further combination experiments. The experiments were performed in 96-well plates for biofilm quantification studies while 6-well plates were used for extraction of EPS and subsequent quantification. For the purpose of investigating the synergistic effect of QQ on biofilm architecture and on *P. aeruginosa* biofilms, three concentrations representing the MIC, ½ MIC and ¼ MIC of farnesol, tyrosol and CCE were used. All experiments were performed three times and in triplicates to test for reproducibility.

2.5 Extraction of EPS from *P. aeruginosa* biofilm

Based on work conducted by Liu and Fang, 2002, the protocol for formaldehyde-NaOH method of EPS extraction was adopted for this study with a few modifications. Having compared numerous EPS extraction methods, Liu and Fang, 2002 concluded that the formaldehyde-NaOH process extracted the highest amount of EPS. Formaldehyde fixes the cell by reacting with the amino, hydroxyl, carboxyl and sulfhydryl groups of proteins of the cell membrane and thus prevents cell lysis. The presence of NaOH increases the pH, which results in dissociation of acidic groups in EPS and the repulsion between the negatively charged EPS within the biofilm matrix. This increases the solubility of EPS in water and allows for higher quantity of EPS to be extracted from the biofilm.

The spent medium was gently aspirated, and the wells were washed once with 1x PBS. The attached biofilm was then resuspended in 1x PBS equivalent to the volume of growth medium and transferred to 15 mL falcon tubes. An aliquot (100 μL) was taken out of the suspension for sessile cell enumeration by colony counting. To the remaining solution, 6% (v/v) of formaldehyde was added and incubated at 4°C for 1 h. To the resulting solution, 40% (v/v) of 1M NaOH was added and the tube was further incubated at 4°C for 3 h. Upon which, the resuspended biofilm was centrifuged at 18500 rpm at 4°C for 30 min. The resulting supernatant was filtered through a 0.22 μm filter membrane and further purified using a 3500 Dalton dialysis membrane at 4°C for a period of 24 h. The resulting suspension was then aliquoted for individual biochemical and fluorometric assays.

2.6 Transmission flow-cell and confocal laser scanning microscopy (CSLM)

A dual transmission flow-cell (FC 281-PC) (Fig 2.1), purchased from Biosurface technologies corporation, USA, was used to observe the effect of agents of biofilm dispersal and QQ used in this study on *P. aeruginosa* biofilms. The flow-cell, a microfluidic device, provides a closed system where bacterial biofilm could interact in a hydrodynamic environment. The fluid flow in this device was maintained at the flow-rate required for the experiment. This was done to maximise bacterial attachment and EPS production.

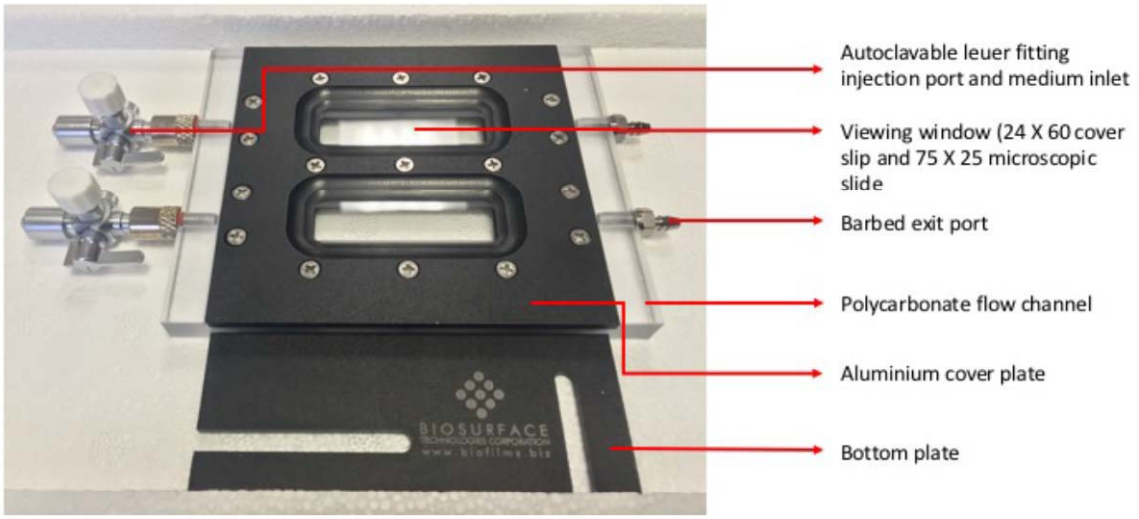

Figure 2.1 Set up of the transmission flow-cell FC 281-PC

2.6.1 Preparation of the transmission flow-cell

The physical set up of the transmission flow-cell was done according to the manufacturer's instructions. The device was then sterilised at 121 °C. Inoculum was prepared as mentioned in section 2.1.3.1. LB broth was prepared separately and sterilised and stored in a medium reservoir. The reservoir was then connected to the transmission flow-cell with the medium flow-rate controlled by a multichannel peristaltic pump. The effluent was directed to a collection bottle. LB broth was changed as required with experimental conditions, which contained specified concentration of test salts, *B. licheniformis* culture extract, farnesol, and tryptophan.

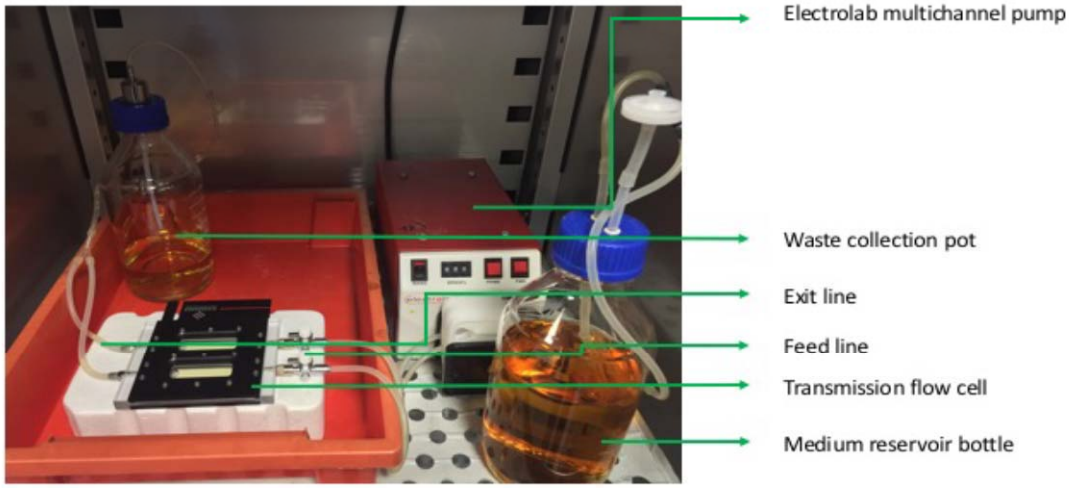

Figure 2.2 Experimental set up containing the flow-cell, the peristaltic pump, medium reservoir and the waste collection pot. The set up was encased in an incubator set at 37 °C

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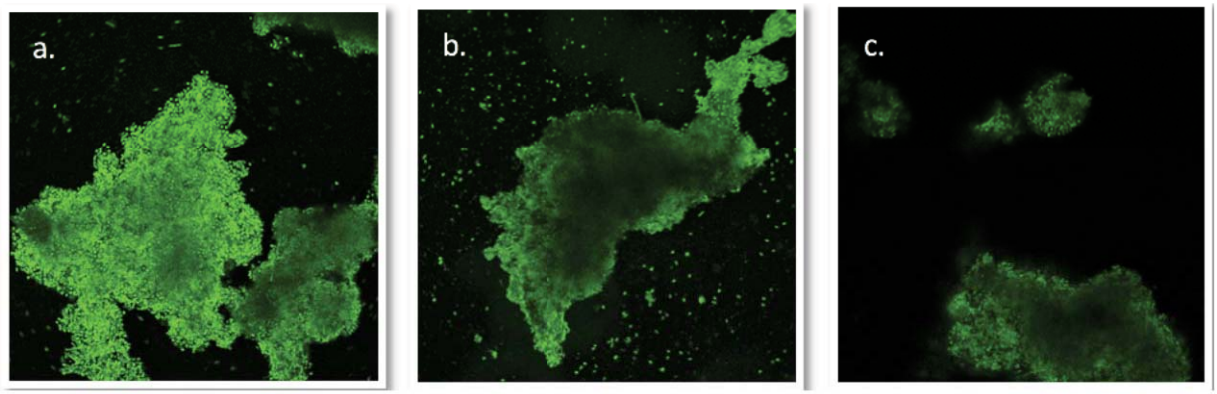
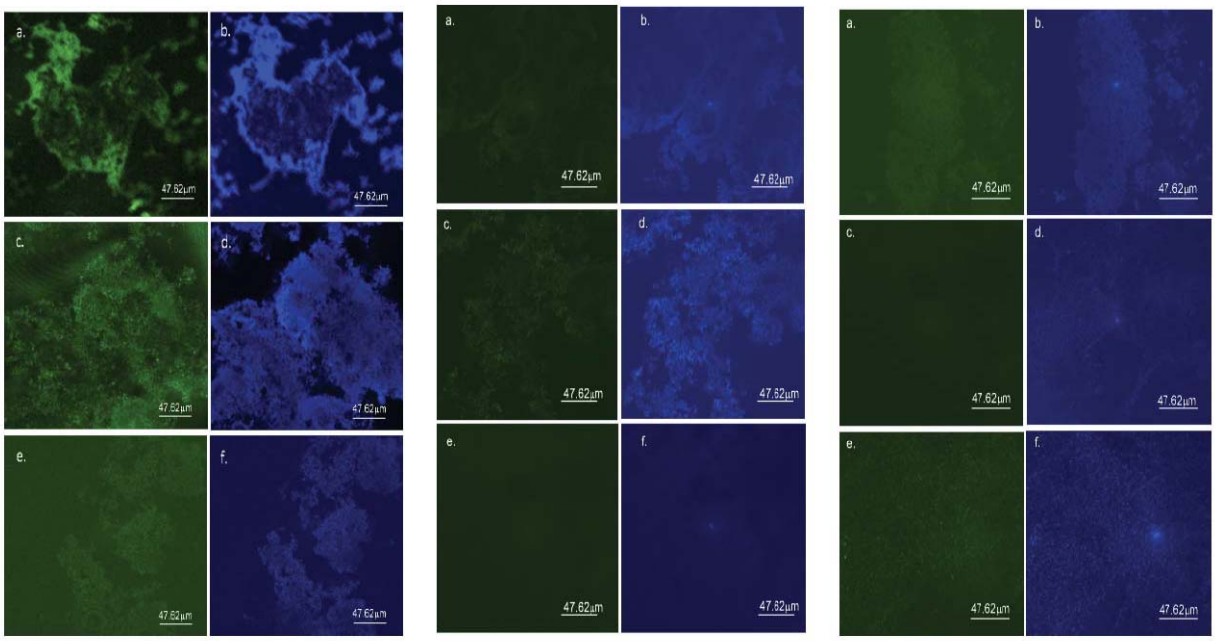
Once LB medium was pumped through the flow-cell, the flow-cell was aseptically inoculated via the injection ports. Upon inoculation, the growth was maintained under static condition for 4 hours to allow for optimal bacterial attachment to the surface of the PDMS surface and the cover slip, held within the flow-cell. Upon attachment, the flow of the medium was started at 150µl/ min and the rate of flow was maintained for the duration of the experiment after which, the slides were carefully extracted from the flow-cell, stained using SYPRO Ruby biofilm matrix stain and Calcofluor White stain and observed under CLSM.

Biofilms of *P. aeruginosa* formed in transmission flow-cells with and without treatment were stained and mounted for confocal laser scanning microscopy (CLSM). Upon completion of the desired time of treatment, the flow-cells were washed with 0.9% saline solution and stained using SYPRO ruby biofilm matrix stain and Calcofluor White stain and incubated at room temperature for 30 min and 15 min respectively under light limiting conditions. The method recommended by the manufacturers was followed. SYPRO ruby biofilm matrix stains (750 µL) loaded into the two windows of the flow-cell. Upon completion of the incubation period, the excess stain was washed away using 1x PBS. Similarly, 0.1% (w/v) Calcofluor White was injected into the flow-cell windows and incubated for a period of 15 min, upon which the excess stain was rinsed with 1x PBS. The stained biofilms were then visualised by a Leica confocal microscope.



P. aeruginosa RBHi *P. aeruginosa* PAO1 *P. aeruginosa* NCTC 10662

Figure 2.3 SYPRO ruby biofilm matrix stain used to visualise the untreated biofilm formation of *P. aeruginosa* as grown on transmission flow-cell. a) attached biofilm *of P. aeruginosa* RBHi, b) attached biofilm of *P. aeruginosa* PAO1 and c) attached biofilm of *P. aeruginosa* NCTC 10662



P. aeruginosa RBHi *P. aeruginosa* PAO1 *P. aeruginosa* NCTC 10662

Figure 2.4 Top to bottom treatment with Tryptophan, CDA and anthranilate respectively for each individual *P. aeruginosa* strain. Biofilm formation of *P. aeruginosa* visualised after staining with SYPRO ruby biofilm matrix stain (green) and Calcofluor White (blue) to depict the protein and carbohydrate content of the biofilm dispersed by the treatment

2.7 Biochemical and fluorometric assays used throughout the study

2.7.1 Analysis of EPS composition of biofilms

2.7.1.1 Quantification of total carbohydrates

The method described by Dubois *et al.*, 1956 has been found to be particularly useful in quantitative determination of small quantities of total sugars. The method was adopted in this study to quantify the total sugars present in the EPS extracted from the biofilm formed by *P. aeruginosa*. Reagents used for the total carbohydrate assay were phenol (80%w/v), and sulphuric acid (95.5% reagent grade). Absorbance was recorded at 490nm. A standard curve was prepared using glucose. A modified version of the phenol-sulphuric acid assay as described by Masuko *et al.*, 2005 was adopted for this study due to the convenience of performing the protocol and also due to the numerous samples that needed to be analysed. The high throughput nature of the assay and use of phenol and sulphuric in limited quantities were desirable.

2.7.1.2 Quantification of alginate

Carbazole reaction mediated quantification of uronic acid content was performed using D-glucuronic acid lactone as standard. A version of the assay based on Bitter and Muir., 1962 and modified by Cesaretti *et al*., 2003 was used. In a 96-well microtiter plate, to 50 µL of the sample, 200 µL of 25 mM sodium tetraborate in sulphuric acid was added. The plate was then incubated in a water bath at 100°C for 15 min. After cooling down to room temperature, 0.125% (w/v) carbazole in ethanol was gently added and incubated at 100°C for 10 min prior to cooling down the plate to room temperature and the plate was read at 550 nm.

2.7.1.3 Quantification of total proteins

Bradford assay is a fast and an accurate method for quantifying total protein content in a given sample. The assay was adopted for quantifying the total protein content in the EPS extracted from the biofilm formed by *P. aeruginosa*. Absorbance for the Bradford assay was recorded at 595 nm. A standard curve was prepared using bovine serum albumin (BSA)

A microtiter plate-based Bradford assay was adopted for this study as modified by Ernst and Zor, 2010. The modifications were based on the Bradford assay that makes use of the coomassie brilliant blue stain. Briefly, 100 µL of the samples were used in each well. To the samples, 100 µL of Bradford's reagent was added to bring the total volume to 200 µL. The plates were incubated at room temperature for 15 min along with a blank which contained only water. After the incubation period, the plates were read at 590 nm. All experiments were performed in triplicates and the experiments were repeated three times for reproducibility.

2.7.1.4 Quantification of eDNA

eDNA content of the biofilms formed by *P. aeruginosa* was quantified by a fluorometric assay using a Qubit. Quantification was carried out based on the manufacturer's instructions provided in the kit for the analysis of dsDNA (BR assay kit). Briefly, 190 µL of Qubit working solution was prepared for each tube. In order to prepare standards, 10 µL of each qubit standard was added to the respective tubes and mixed by vortexing. Similarly, 10 µL of the samples were added to 190 µL of the Qubit working solution. Care was taken for accuracy while pipetting 10 µL of standard or the sample solution. Upon confirming that the total volume of the reaction mix was 200 µL, the tubes were incubated at room temperature

for 2 min. The samples containing the standards were read as "reading standards" and samples were read as "reading samples" in the fluorometer.

2.7.2 Analysis of virulence factors

2.7.2.1 Quantification of elastolytic activity

The elastolytic activity of *P. aeruginosa* was determined using elastin Congo red (ECR) assay as described by Pearson, Pesci and Iglewski, 1997 with modifications describe the modification. Aliquots (100 µL) of biofilm extracts were added to tubes containing 20 mg ECR in 900 µL of ECR buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5). The mixture was then incubated at 37ºC for a period of 3 h and incubated on ice after 0.1 mL of 0.1M EDTA was added. Insoluble ECR was removed by centrifugation and the absorbance of the supernatant was measured at 495 nm.

2.7.2.2 Quantification of pyocyanin

Pyocyanin quantification was performed based on the method by Essar *et al*., 1990 with modifications. Quantification was based on absorbance of pyocyanin at 520 nm under acidic conditions after phase separation using chloroform. Biofilms were resuspended in 1x PBS and 500 µL of the resuspension was extracted with 3 mL of chloroform and re-extracted into 2 mL of 0.2M HCl to provide a pink to red solution. Sample volumes (200 µL) were then transferred to a microtiter plate and the absorbance was measured at 520 nm. The concentration of pyocyanin was expressed as µg/ mL upon multiplying the absorbance at 520 nm with the molar extinction coefficient of 17.072 (Moayedi, Nowroozi and Sepahy, 2017).

2.7.2.3 Quantification of pyoverdine

Spectrofluorometric quantification was performed by measuring the resuspended biofilm filtrate (0.22 µm filter) at 405nm.

2.7.2.4 Quantification of rhamnolipid

Quantification of rhamnolipid was carried out by applying the orcinol reaction as described by Laabei *et al*., 2014 with modifications state the modification. The supernatant was extracted 3 times with 1 mL diethyl ether prior to complete evaporation under vacuum. Upon completion, 0.5 mL of distilled H_2O was added to each of the sample tubes. For the assay 100 µL of samples was taken after resuspension in dH_2O. To the samples, 900 µL of 0.19%

orcinol (diluted in 53% H_2SO_4) was added and incubated at 80ºC for 30 min. After incubation, the samples were cooled to room temperature and the absorbance was measured at 421 nm and the concentration was measured based on the standard curve prepared using rhamnose.

2.8 Motility assays

Since motility status is vital for *P. aeruginosa* to form biofilm, the effect of selected QQ on bacterial motility was tested against three strains of *P. aeruginosa*. Pathogenesis of *P. aeruginosa* and its ability to colonise is dependent on its mobility (Murray *et al*., 2010). In aqueous medium, *P. aeruginosa* is capable of displaying swimming motility which represent the motile behaviour of single cells and is mediated by the bacterial flagella (Inoue *et al*., 2008). Twitching motility is mainly displayed when the bacterium is in contact with a solid or hard surface and is mediated by type IV pili (Deziel, Comeau and Vilemur, 2001). In biofilms, formation of a 3D structure is dependent on binding of the type IV pili to eDNA present in the EPS. Finally, the third form of motility referred to as the swarming motility is dependent on flagellar and type IV pili and is seen in semi-solid medium. Swarming motility is a coordinated behaviour and is primarily mediated by QS (Pamp and Tolker-Nielsen., 2007).

2.8.1 Swimming motility

The capability to swim by *P. aeruginosa* with and without treatment was evaluated on tryptone plates containing 0.3% (w/v) agar, 0.5% (w/v) NaCl and 1% (w/v) tryptone. The desired concentrations of QQ were added to the agar after cooling it down to 50 ºC and immediately before pouring. The plate was then allowed to dry near a flame and inoculated with 1 µL of bacterial culture with the OD readjusted to 0.5 MacFarland standard. The plates were then incubated at 37 ºC for a period of 24 h. The colony diameter was then measured and used for further analysis.

2.8.2 Twitching motility

Twitching motility of *P. aeruginosa* was performed on LB agar plates containing 1% (w/v) agar. Similar to 2.8.1, the LB agar was cooled down to 50ºC prior to the addition of required concentration of QQ prior to pouring. The plates were then allowed to dry. The plates were inoculated by stabbing a single colony of each of the *P. aeruginosa* at the centre of the agar. The plates were incubated at 37 ºC for a period of 24 h prior to recording the colony diameter for further analysis.

2.8.3 Swarming motility

Swarming motility was assayed on modified M9 medium containing 0.4% (w/v) glucose and 0.5% (w/v) agar. Required concentration of QQ were added to the medium after cooling it down to 50 °C prior to pouring. The plates were then incubated with 1 µL of bacterial culture with the OD readjusted to 0.5 MacFarland standard. The plated were then incubated at 37 °C for a period of 24 h prior to measuring the colony diameter for further analysis.

2.9 Evaluation of MexAB-OprM efflux pump activity in the presence of farnesol and tyrosol

2.9.1 Accumulation Assay

A semi-automated fluorometric technique was designed to utilise Ethidium bromide (EtBr) as a substrate during the real-time assessment of the bacterial efflux pump activity. EtBr is commonly used as an efflux pump substrate to detect and measure efflux activity of bacteria. EtBr is essentially a fluorescent probe that emits a weak signal outside of the cell (in aqueous solution), however upon entering periplasm (Gram negative) or cytoplasm (Gram positive) it becomes highly fluorescent. When EtBr is interpolated between nucleic bases of DNA the binding is strong enough to keep EtBr from accessing the efflux pump of the bacterium (Rodrigues *et al.,* 2011). The accumulation of EtBr within the cell is the outcome between cell-wall permeability and efflux system (Rodrigues *et al.,* 2011).

In this study, EtBr movement in *P. aeruginosa* incubated with and without QQ of interest was measured. Cells were grown in 10 mL LB broth, 37°C, 180 rpm until it reached an OD 0.8-1 at 600 nm. The cells were then adjusted to OD of 0.4 at 600 nm by diluting in required volumes of medium and bacterial suspension. Once desired OD (0.4 at 600 nm) was achieved, cells were centrifuged at 3000 rpm (1700 x g), 25°C (room temperature) for 5 min. The supernatant was discarded, and pellet re-suspended in 10 mL PBS. Meanwhile, farnesol and tyrosol working concentrations were prepared as well as known EPI Phenylalanine-arginine β-naphthylamide (Paβn). For the purpose of this assay, ½ MIC and ¼ MIC were used to keep the cells metabolically active and not to kill them. Glucose was used as source of energy, (80% w/v) stock was prepared, and final concentration of each well was 0.4% (w/v). The reaction was run using Fluostar (Optima) plate reader; the readings were taken every1 min at 37°C.

Table 2.5 Concentration of tested EPI against *P. aeruginosa*

	P. aeruginosa		
Compound	MIC µg/mL	½ MIC µg/mL	¼ MIC µg/mL
Farnesol	250	125	62
Tyrosol	250	125	62
Paβn	25	12	6
EtBr	6	3	1.5

Table 2.6 Layout of assay conditions. Set up of conditions was prepared for the *P. aeruginosa* culture in Eppendorf tubes. Samples (95 µL) from each tube were moved to 96-well plate in triplicates, followed by addition of 5 µl of EtBr. Note: It is advisable to add the control set first containing PBS instead of cells and be swift when adding cells and EtBr, as the reaction starts the moment the cells encounter EtBr.

EPI (QQ)	**Far**	**Far**	**Far**	**Tyr**	**Tyr**	**Tyr**	**Paβn**	**Control**
Far 1X	2.5 µL	X	X	X	X	X	X	X
Far ½ X	X	1.25 µL	X	X	X	X	X	X
Far ¼ X	X	X	0.6 µL	X	X	X	X	X
Tyr 1X	X	X	X	2.5 µL	X	X	X	X
Tyr ½ X	X	X	X	X	1.25 µL	X	X	X
Tyr ¼ X	X	X	X	X	X	0.6 µL	X	X
Paβn 1X	X	X	X	X	X	X	2 µL	X
Cells/ PBS	495 µL	495 µL	495 µL	495 µL	495 µL	495 µL	495 µL	495 µL
Glucose 80%	2.5 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µl	2.5 µL	2.5 µL
PBS	x	1.25 µL	1.9 µL	x	1.25 µL	1.9 µl	0.5 µL	2.5 µL
Total	95 µL	95 µL	95 µL	95 µL	95 µL	95 µl	95 µL	95 µL
EtBr ½ X	5 µL	5 µL	5 µL	5 µL	5 µL	5 µl	5 µL	5 µL

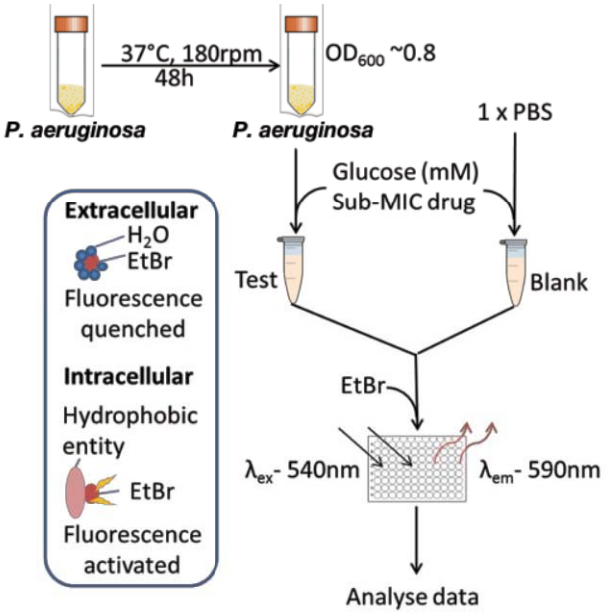


Figure 2.5 Schematic illustration of accumulation assay. Cell culture must be grown until OD of ~0.8-1 at 600 nm to ensure healthy growth and replication, this is followed by adjusting the OD at 600 nm to 0.4 and subsequent addition of compounds of interest with a source of energy, here glucose. Lastly, addition of EtBr used as a substrate for the cells was added to each well separately.

2.9.2 Efflux Pump assay

Reverse efflux relies on similar preparation as for the accumulation assay. *P. aeruginosa* cells were grown in 10 ml LB broth until it reached an OD of 0.8 at 600 nm, then adjusted to OD of 0.4 at 600 nm by adding required volumes of media. Cells were centrifuged at 3000 rpm (1700 xg), 25°C for 5 min. Supernatant was discarded, and pellet was re-suspended in 10 mL PBS. 1 mL of cell suspension was transferred to Eppendorf tube then 2 µl of 500 µg/ml EtBr and either 1.25 µl of farnesol and tyrosol (1 x MIC, ½ x MIC and ¼ x MIC), 1.25 µl of Paβn (1X MIC), was added to corresponding tubes. Cells loaded with EtBr and EPI were placed in the incubator for 1 hour at 37°C, static conditions. Following incubation, tubes were centrifuged at 4°C, 3000 rpm (1700 xg), for 5 min. Centrifugation was performed to remove any remaining EPIs. Lowering temperature is crucial to stop cells from releasing EtBr. Following that, the supernatant was aspirated, and pellet was re-suspended in 1 ml of ice cold PBS. Cells were immediately added to the wells. Meanwhile, 96-well plate was prepared by adding respective volumes of glucose as an energy source along with compounds to be tested and a known EPI as a positive control. Cells were immediately added to the wells. The reaction was run using Fluostar (Optima) plate reader; the readings were taken every 1 min at 37 °C.

2.10 Hydrophobicity assay

The cell surface hydrophobicity of *P. aeruginosa* cells was tested using the MATH test (Das and Kapoor, 2004). The test was conducted on bacterial cells with and without treatment in the presence of farnesol and tyrosol. Mid-log growth phase (~6 h) of *P. aeruginosa* cells were diluted by 50% in LB broth containing the desired concentrations of farnesol, tyrosol and CCE (½ MIC). The control was diluted in LB broth without any treatment. The total working volume was set to 100 mL in conical flasks. The conical flasks were then incubated at 37 ºC for a period of 5 h (300 min). Samples (4 mL) were collected at periods of 30 min intervals and centrifuged at 4700 rpm for 5 min. The pellets were retained, and the supernatants were discarded. The pellets were then washed twice with sterile 1x PBS after which they were resuspended in 3 mL lots of sterile 1x PBS. Absorbance was measured at 540 nm. To the same tube, 0.8 mL of n-hexadecane was added and vortexed for 30 seconds. Once the suspension had separated into an aqueous phase and n-hexadecane phase over a period of 20 min, the aqueous phase was carefully separated, and the absorbance was

measured at 540 nm. The microbial adhesion to hydrocarbons was calculated as a percentage of adherence of n-hexadecane using the following formula.

Percentage adherence = OD_{540} (PBS) – OD_{540} (n-hexadecane)/ OD_{540} (PBS)

2.11 Adherence and internalisation of *P. aeruginosa* cells to A549 and HaCaT cells in co-culture

The adherence and internalisation of *P. aeruginosa* cells to A549 and HaCaT cells were compared as described previously by Carterson *et al.*, (2005). To identify the adherence of bacterial cells, co-culture was carried out as described previously in 2.2.3.5, with and without treatment. Following the required incubation time, the plates containing the A549 and HaCaT cells were washed with 1x PBS and 200 µL of PBS-T was introduced in the wells and incubated for 30 min. Following incubation, the cells were thoroughly aspirated with a micro pipette to ensure dispersion of sessile cells. The medium was then removed, and spot plated for CFU enumeration. Percentage adherence was calculated based on CFU obtained from sessile cell count against the initial CFU of the inoculum used.

Gentamicin exclusion assay was adopted to calculate the internalisation of the bacteria. Co-culture was performed as mentioned in section 2.2.3.5 with and without treatment. Following incubation, the external bacteria were killed using 200 µg/mL of gentamicin for 2 h. Controls of A549 and HaCaT cell free wells were used as controls to show complete killing of external bacteria. After 2 h, gentamicin was washed away, three times, by rinsing with 1x PBS. The A549 and HaCaT cells were then lysed by adding 1% Triton X 100 in PBS for 1 h and the plates were incubated at 37 °C. Following incubation, the medium from the plates were spot-plated to obtain CFU counts. Percentage internalisation was calculated based on the CFU obtained after gentamicin treatment against the initial CFU count of the inoculum.

2.12 Cell viability assay (MTT assay)

Cell viability assay was performed based on the manufacturer's instructions. Briefly, a 12 mM MTT (Vybrant MTT cell viability assay, ThermoFisher scientific) stock solution was prepared by dissolving 10 mg of MTT in 2 mL of sterile 1x PBS by vortexing. The undissolved particles were removed by filtration through a 0.22 µm filter. For the purpose of the assay, the growth medium from the experimental set up from the wells (96-well plate)

was removed and replaced with 100 µL of fresh medium. To the wells, 10 µL of the 12 mM MTT stock solution was added. A negative control was included by adding 10 µL of MTT stock solution to blank wells containing medium alone. After labelling the cells with MTT, 25 µL of the medium was removed and replaced with 50 µL of DMSO with thorough mixing by pipetting. The 96-well plates were then incubated at 37 °C and 5% CO_2 for a period of 3 h. The samples were mixed again prior to reading the plate at 540 nm.

Percentage viability was calculated as the ratio between untreated and treated cell (and in co-culture). The wells without the cells and without treatment were used as the negative controls and the values subtracted from the negative control wells and treatment wells prior to analysis.

2.13 IL-8 ELISA

IL-8 secretion by A549 and HaCaT cells following biofilm formation by *P. aeruginosa*, with and without treatment was performed. Following co-culture and treatment with farnesol and tyrosol in 6-well plates, the supernatant of the co-culture was removed and stored at -80 °C till further use. A sandwich ELISA was performed using the Human IL-8 ELISA kit (Invitrogen). Protocol followed was based on the manufacturer's instruction. Briefly, a 96-well plate pre-coated with monoclonal antibody specific for human IL-8 was used. To the wells 50 µL of samples, including standards of known of IL-8 concentration, respective controls and experimental samples were added. For the first incubation period of the assay, the samples and standards along with 50 µL biotinylated monoclonal antibody (Hu IL-8 Biotin conjugate) were incubated for 90 min at room temperature. The respective well of the 96-well plate were thoroughly aspirated and then washed four times with 1X wash buffer. After washing, 100 µL of 1X Streptavidin-HRP solution was added into the respective well with the exception of blank wells. The plate was then covered and incubated at room temperature for 30 min. After the incubation period, the wells were again thoroughly aspirated and washed four times with 1X wash buffer. Subsequenlty, 100 µL of stabilised chromogen (Tetramethylbenzidine (TMB)) was added and incubated at room temperature for a period of 30 min. After the incubation with the chromogen, 100 µL of stop solution was added and gently mixed by tapping the side of the plate. The absorbance was then recored at 450 nm. Based on the standard curve obtained, the concentration of IL-8 was then calculated.

2.14 Molecular Biology techniques

2.14.1 RNA extraction

RNA extraction from bacterial cells were performed using the Trizol RNA isolation protocol with slight modifications as detailed below. This method was originally described by Chomczynski and Sacchi in 1987.

1) Phase separation

- Bacterial cells from the biofilm were extracted by sonication and collected by centrifugation in 1.5 mL Eppendorf tubes. The supernatant was discarded.
- To the cell pellet, 1 mL of Trizol reagent was added and mixed carefully to obtain a homogenous mixture and left at room temperature for 5 min.
- After 5 min, 200 µL of chloroform/1mL of Trizol was added and gently mixed for 15 seconds and incubated at room temperature for 3 min.
- The samples were then centrifuged at 2000 rpm for 10 min at 2°-4° C.

2) RNA precipitation

- Following centrifugation, three phases were visible inside the tube. The aqueous phase (top) was transferred to a new tube. Care was taken by pipetting gently to not contaminate the solution with other phases.
- To the aqueous phase, 500 µL of isopropanol/ 1 mL of Trizol was added and incubated at room temperature for 10 min. After which the samples were centrifuged at 10000 rpm for 10 min at 2°-4° C.

3) RNA wash and resuspension

- The supernatant from the previous step was carefully removed and washed with 75% EtOH.
- The samples were centrifuges at 5000 rpm for 5 min at 2°-4° C. The supernatant was discarded and the remaining EtOH was allowed to air-dry for 15 min. The tubes were the transferred and placed on 50 ° C heat-block for 5 min.
- The RNA pellet was then re-suspended in 50 µL RNase-free dH_2O at 37 ° C for 15 min by incubation.

4) DNase treatment (using Ambion's DNase treatment kit)

- Buffer (8 µl of 10X DNase I) was added along with 2 µL of DNase I enzyme.
- Samples were vortexed and allowed to incubate at 42° C for 15 min.

5) RNeasy Column purification (using Qiagen's RNeasy protocol)

- A volume of 350µl RLT Buffer (with BME-10µL/ mL) and 250 µL 100% EtOH was loaded to RNeasy column and spun at 13500 rpm for 1 min. The procedure was

repeated twice.

- The column was transferred to a new 2 mL collection tube, 750µL of buffer RPE was added and spun at 13500 rpm for 1 min. The procedure was repeated twice.
- The column was transferred to newly labelled 1.5 mL Eppendorf tubes, RNA suspension was added, and the tubes were incubated at room temperature for 2 min, and spun at 13500 rpm for 2 min. The columns were discarded, and the Eppendorf tubes were transferred to ice.
- Samples were quantified for their RNA content using Nanodrop and subsequently stored at -80ºC. The final preparation of RNA extraction from the samples was found to have a A_{260}/A_{280} ration of ≥ 1.7 prior to subsequent use.

2.14.2 Reverse transcription and cDNA preparation (QuantiTect Reverse Transcription kit by Qiagen)

A) RNA was thawed on ice along with gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT buffer, RT primer mix and RNase-free water. The reagents were gently mixed and centrifuged.

B) Genomic DNA elimination reaction was prepared according to the table below

Table 2.7 Genomic DNA elimination reaction mix

Component	Volume/ reaction
gDNA Wipeout Buffer, 7x	2µl
Template RNA, upto 1µg	Variable
RNase-free water	Variable
Total reaction volume	**14µl**

C) The reaction mix was incubated for 2min at 42 ºC and placed immediately on ice. Incubation was not carried out for more than 10 min.

D) Reverse transcription mix was prepared according to the table below

Table 2.8 Reverse transcription reaction mix

Component	Reaction Volume
Reverse-transcription master mix	
Quantiscript Reverse Transcriptase	1 µl
Quantiscript RT buffer, 5x	4 µl
RT Primer Mix	1 µl
Template RNA	
Entire gDNA elimination reaction (B)	14 µl
Total reaction volume	**20 µl**

E) The reaction mix was then incubated at 42ºC for 15 min.

F) Final incubation was done at 95° C for 3 min to inactivate the Quantiscript Reverse Transcriptase.

G) Samples were used directly for real-time PCR or stored at -20° C for later processing.

2.15 Quantitative real time PCR

SYBR Green real-time PCR was performed in 0.1 mL strip tubes and caps (Qiagen) using Rotor Gene Q (Qiagen) with samples run in duplicate. The reaction was made of 4 µL template (cDNA), 1 µL of diluted forward and reverse primer mix (1/10 dilution), 12.5 µL PowerUp SYBR® Green PCR Master Mix (Applied Biosystems) and 6.5-10.5 µL DEPC H_2O. Controls were run in the PCR set up, where the used cDNA was replaced by H_2O. The conditions for the qPCR were set as follows:

2.15.1 *P. aeruginosa* QS network and virulence factors

Initial denaturation was done at 95°C for 2 min. Subsequently, the amplification program involved 40 cycles of denaturation at 95°C for 15 sec, primer annealing at 55°C for 15 sec and extension at 72 °C for 30 sec. A final extension was performed at 72 °C for 2 min followed by cooling at 4 °C. A dissociation step at 55 °C was used to generate a melting curve with a 1 °C increase every 5 sec till 95 °C to obtain verification of amplified product.

2.15.2 *P. aeruginosa* MexAB-OprM efflux pump

Initial denaturation was done at 95°C for 15 min. Subsequently, the amplification program involved 40 cycles of denaturation at 95°C for 15 sec, primer annealing at 61°C for 15 sec and extension at 71 °C for 20 sec. A dissociation step at 61 °C was used to generate a melting curve with a 1 °C increase every 5 sec till 95 °C to obtain verification of amplified product.

Baseline and threshold were set by the Rotor Gene Q Software with no modifications. Relative expression of the genes was determined using the ΔΔCt method (Livak and Schmittgen, 2001; Sarabhai *et al.*, 2013). The data obtained were normalised against the untreated control and represented as relative fold change in gene expression.

2.16 Primers used for Real-Time quantitative PCR runs

2.16.1 Primers for QS genes in *P. aeruginosa*

Gene	Primer	Nucleotide sequence	Reference
lasI	forward	5' CGTGCTCAAGTGTTCAAGG 3'	Jack *et al*., 2018; Zhu *et al*., 2004
	reverse	5' TACAGTCGGAAAAGCCCAG 3'	
lasR	forward	5' AAGTGGAAAATTGGAGTGGAG 3'	
	reverse	5' GTAGTTGCCGACGACGATGAAG 3'	
rhlI	forward	5' TTCATCCTCCTTTAGTCTTCCC 3'	
	reverse	5' TTCCAGCGATTCAGAGAGC 3'	
rhlR	forward	5' TGCATTTTATCGATCAGGGC 3'	
	reverse	5' CACTTCCTTTTCCAGGACG 3'	

2.16.2 Primers for virulence factors of *P. aeruginosa*

Gene	Primer	Nucleotide sequence	Reference
toxA	forward	5' GGAGCGCAACTATCCCACT 3'	Sabharwal *et al*., 2014; Aghamollaei *et al*., 2014)
	reverse	5' TGGTAGCCGACGAACACATA 3'	
aprA	forward	5' GTCGACCAGGCGGCGGAGCAGATA 3'	
	reverse	5' GCCGAGGCCGCCGTAGAGGATGTC 3'	
rhlAB	forward	5' TCATGGAATTGTCACAACCGC 3'	
	reverse	5' ATACGGCAAAATCATGGCAAC 3'	
lasB	forward	5' TTCTACCCGAAGGACTGATAC 3'	
	reverse	5' AACACCCATGATCGCAAC 3'	

2.16.3 Primers for *P. aeruginosa* MexAB-OprM efflux pump

Gene	Primer	Nucleotide sequence	Reference
MexA	forward	5'ACCTACGAGGCCGACTACCAGA 3'	Pourakbari *et al*., 2015
	reverse	5'GTTGGTCACCAGGGCGCCTTC 3'	
MexB	forward	5'GTGTTCGGCTCGCAGTACTC 3'	
	reverse	5'AACCGTCGGGATTGACCTTG 3'	
OprM	forward	5'CCATGAGCCGCCAACTGTC 3'	
	reverse	5'CCTGGAACGCCGTCTGGAT 3'	

2.16.4 Reference genes and primers for qPCR

Gene	Primer	Nucleotide sequence	Reference
rpsL	forward	5' CCTCGTACATCGGTGGTGAAG 3'	Pourakbari *et al.*, 2015; Jack *et al.*, 2018
	reverse	5' CCCTGCTTACGGTCTTTGACAC 3'	
AmpC	forward	5'GGTGCAGAAGGACCAGGCACAGAT 3'	
	reverse	5'CGATGCTCGGGTTGGAATAGAGGC 3'	

2.17 Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualise the products obtained at the end of the amplification process of the qPCR run. A 1% (w/v) agarose gel was prepared were 1 kb plus ladder (Invitrogen) was used as a size marker. The 1% agarose gel was prepared in TBE buffer (pH 8.5) The composition of 50x TBE buffer is given in table 2.1. PCR products were mixed with 2 µL of 6x blue loading dye (Promega) before loading into the gel wells. The gel was run at 100 V for a period of 40 min (mini-sub cell GT system, Biorad, UK) to allow for the DNA fragments to migrate. After electrophoresis, the gels were stained with ethidium bromide (10 mg/ mL) and visualised on a UV transilluminator (UVITEC, UK). (see appendix D)

2.18 Statistical analysis

All experiments were performed in triplicates. All data for assays performed in this study were statistically analysed using GraphPad Prism to determine *p* values and establish correlation between data sets. All graphs were plotted using GraphPad Prism. $p < 0.05$ was considered significant.

Introduction to results and discussion

The focus of this thesis is to investigate biofilm formation by *P. aeruginosa* and subsequent use of QQ and biofilm dispersal agents to study the composition and efficacy of attenuating bacterial communication to overcome biofilms. The studies include three strains of *P. aeruginosa*, non-mucoid NCTC 10662, mucoid PAO1 and heavily mucoid RBHi (a CF isolate). Various methods were used to study the effect of QQ and biofilm dispersal agents on biofilm formation on abiotic and biotic surfaces as well as observe the changes in biofilm composition and architecture to better understand the process of biofilm formation to develop potential novel therapies.

This section of the thesis is divided into four chapters. Chapter 3 describes the use of various biofilm dispersal agents as individual and combination treatments to overcome biofilm formation as well as investigates the effect of divalent cations as components of growth medium and how they govern biofilm architecture. Chapter 4 focuses on potential QQ and dispersal of *P. aeruginosa* biofilm using metabolites of the biochemical pathway of *P. aeruginosa* as well as a combination treatment of naturally occurring D-amino acid and an antibiotic, erythromycin on dispersing *P. aeruginosa* biofilm. This chapter also includes the use of certain abiotic surfaces were *P. aeruginosa* biofilms are commonly formed. Characterisation of biofilm architecture of *P. aeruginosa* under the influence of potential QQ and biofilm dispersal under static as well as flow conditions by using a transmission flow-cell is included in this chapter. The effect of *B. licheniformis* crude cell extract as a potential QQ containing QQ enzymes along with the use of D-amino acids, farnesol and tyrosol as individual and combination treatments to overcome biofilm formation by *P. aeruginosa* is investigated in chapter 5 along with studies related to the gene expression of *P. aeruginosa* AHL mediated QS system and virulence factors. Chapter 6 includes studies related to co-culture of *P. aeruginosa* biofilms on mammalian epithelial cell lines (A549 and HaCaT) and subsequent inhibition of biofilm formation by farnesol and tyrosol as independent and combination treatments.

Chapter 3 . Influence of medium composition on the characteristics of non-mucoid, mucoid and heavily mucoid biofilms of *P. aeruginosa*

3.1. Introduction

One of the hallmarks of *P. aeruginosa*-related chronic infections is the presence of a mixed population of non-mucoid and mucoid phenotypes of *P. aeruginosa* (Malhotra *et al.*, 2018). The environment of a CF lung is often characterised by presence of reactive oxygen species (ROS) from polymorphonuclear leukocytes (PMNs), high osmolarity, dehydration, nutrient limitation and antibiotics (Becker *et al.*, 2017). Such an environment presents a stressful condition for the growth of *P. aeruginosa* and subsequently influence the production of alginate resulting in a switch in phenotypes between non-mucoid to mucoid (Yang *et al.*, 2012). The mucoid phenotype of *P. aeruginosa* is generally represented by the overproduction of alginate which forms the primary component of the EPS of mucoidal *P. aeruginosa* biofilm. The onset of alginate overproduction correlates with accelerated decline in lung function in CF patients (Fodor *et al.*, 2012). The non-mucoid phenotype present in CF lung is often very low, similar to *P. aeruginosa* environmental strains where production of alginate is very low or non-existent as the algD operon which is responsible for alginate synthesis is repressed (Damron and Goldberg, 2012). A key component of alginate operon (algD) is the alternative sigma factor AlgU which interacts with mucA protein and negatively regulates alginate biosynthesis. Studies conducted have shown that mutations in mucA is the primary cause of conversion to mucoid form (Qiu *et al.*, 2007). Studies conducted by Tart, Wolfgang and Wozniak in 2005 showed an inverse correlation between algU and bacterial motility, where flagella synthesis and functionality were reduced upon expression of algU gene which demonstrates that motility (a form of virulence) of *P. aeruginosa* is dependent on its non-mucoid or mucoid phenotype.

It was suggested that mucoid phenotype of *P. aeruginosa* is highly resistant to antibiotics based on the hypothesis that the biofilm acts as a barrier against the diffusion of antibiotics because of its polyanionic character (Slack and Nichols, 1982). This hypothesis was refuted by studies conducted in 2001 by Ciofu *et al* and again in 2014 by Owlia *et al* which showed that certain antibiotics such as tobramycin can bind to the polysaccharides present in the biofilm produced by *P. aeruginosa*. It was found that non-mucoid strains of *P. aeruginosa* are less susceptible to antibiotics as they are exposed to a relatively high antibiotic selective

pressure compared to mucoid strains during antibiotic treatment. The difference in antibiotic susceptibility between mucoid and non-mucoid strains is because of biofilm formation (Owlia *et al*., 2014). The accumulation of β-lactamase in the superficial layer of the biofilm would inactivate the β-lactam antibiotics before they penetrate deep into the biofilm. Similar results were documented with the use of quinolones and aminoglycosides (Hoiby *et al*., 2010). Thus, the mucoid and non-mucoid phenotypes are present to ensure the survival of the biofilm.

P. aeruginosa biofilms can form under diverse nutrient conditions, ranging from high nutrient availability to almost non-detectable (Paytubi *et al*., 2010). Generally, nutrient rich medium allows for thick and dense biofilm formation when compared to nutrient limiting conditions. Decidedly, biofilm formation and development are also influenced by bacterial growth medium composition, temperature, pH and ionic strength (Donlan, 2002). Understanding environmental factors, interfacial factors involving surface charge and hydrophobicity and physiological factors such as bacterial growth-rate and metabolic activity is vital in evaluating the effect of medium composition on biofilm formation as they govern the initial adhesion mechanism (Krasowska and Sigler, 2014). Divalent cations present in the growth medium can potentially influence bacterial adhesion and biofilm formation through electrostatic interactions and physiology dependent mechanisms (Song and Leff, 2006). Amongst divalent cations, magnesium (Mg^{2+}) plays an important role in numerous biochemical reactions within living cells. Along with divalent cations, the presence of certain aromatic amino acids such as tryptophan, tyrosine and phenylalanine has been known to influence biofilm formation by influencing the non-AHL mediated QS system in *P. aeruginosa*, the PQS system (Tielen *et al*., 2013). It is equally important to consider that bacterial growth medium containing various salts and amino acids may influence the biofilm architecture of non-mucoid, mucoid and heavily mucoid phenotypes of *P. aeruginosa*. Hence, this study looks to investigate the biofilm architecture of non-mucoid, mucoid and heavily mucoid strains of *P. aeruginosa* in the presence of salts and aromatic amino acids in nutritionally rich and limiting environments.

3.2. Results

3.2.1 Growth curves for *P. aeruginosa* strains PAO1, NCTC 10662 and RBHi

Given an appropriate medium, a bacterium will grow until the substrates in the medium are consumed or until the condition of the medium is no longer conducive for growth due to accumulation of toxins and waste. Bacteria grow characteristically through self-replication. This implies that a bacterium is able to produce an identical replicate daughter cells, which have the potential to divide again. Hence, cell numbers will increase exponentially as a function of time in an appropriate medium. With time however, the nutrient is exhausted, and the growth is considerably slower or ceases when the rate of nutrient consumption exceeds the rate of supply. All the *P. aeruginosa* strains in this study showed a typical bacterial growth pattern as shown in Figure 3.1.

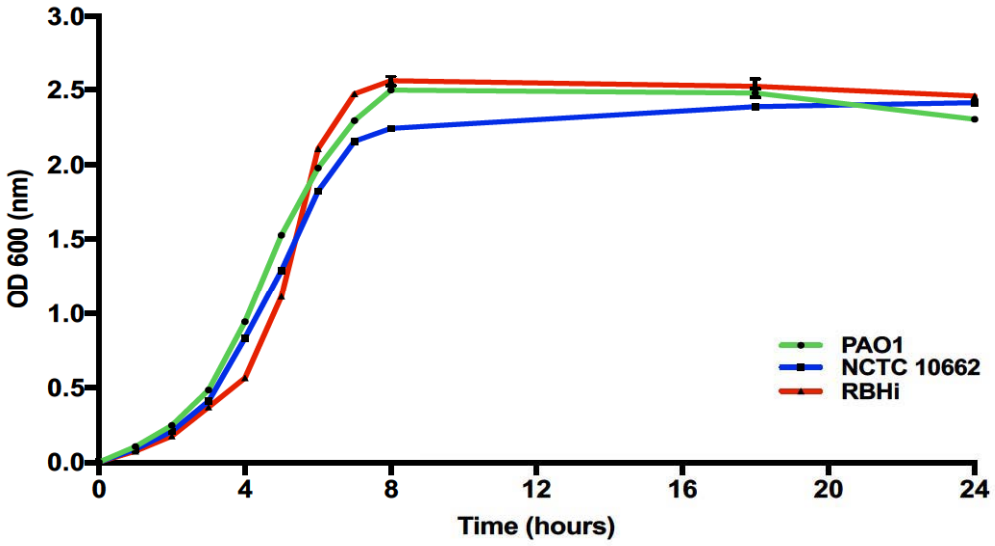


Figure 3.1 Growth curves for *P. aeruginosa* PAO1, *P. aeruginosa* NCTC 10662 and *P. aeruginosa* RBHi in Luria-Bertani broth (n=3)

The absorbance (OD 600 nm) values for the cell suspension was measured against time and prepared in triplicates. The generation time for the three *P. aeruginosa* strains was calculated based on their logarithmic growth obtained from CFU/mL count after plating. Generation time measurements are presented as mean values from three independent growth curves for each culture in Table 3.1.

Table 3.1 Generation time for *P. aeruginosa* strains

P. aeruginosa	Generation time (min)	No (cfu/ mL)	Nt (cfu/ mL)	time (h)
NCTC 10662	43.21	1.80E+05	8.21E+06	4
PAO1	42.95	2.02E+05	9.71E+06	4
RBHi	41.38	2.32E+05	1.29E+07	4

(n=3)

Generation time was calculated based on the following equation:

$$N = \log_{10} Nt - \log_{10} No / 0.301 \quad \text{.....equation 1}$$

N = number of generations, Nt = final cell count, No = initial cell count and 0.301 = $\log_2$ to $\log_{10}$ conversion factor

$$\text{Generation time} = t/N \quad \text{.....equation 2}$$

t = time between samples, N = number of generations

The growth curves demonstrated the different phases (lag, exponential and stationary phase) of growth and allowed for the identification of an absorbance value corresponding to the mid-exponential growth phase. Estimation of bacterial biomass at OD 600 nm at selected time points and their corresponding increases in viable cells (cfu/mL) by colony counting allowed for dilution of bacterial cultures to estimate inoculum sizes for subsequent assays.

3.2.2 Quantitation of *P. aeruginosa* biofilm using the microtiter plate assay

The ability of *P. aeruginosa* strains to form biofilm was determined under optimal conditions of growth for the development of biofilm. Overnight incubation (~18 h) was carried out at 37 °C under aerobic conditions. All strains were grown in nutrient rich LB broth with the pH adjusted to 7.2. The *in vitro* biofilm production was assessed using the microtiter plate assay. Briefly, pure overnight (18 h) growth of individual strains of *P. aeruginosa* were diluted to 0.5 using McFarland standard in LB broth. Aliquots of 200µL were inoculated in triplicates in a flat bottomed 96-well microtiter plate and incubated at 37 °C for a period of 24 h. A negative control of uninoculated LB broth was included in triplicate, each time the study was conducted. The triplicate mean OD 570 nm of the negatives controls were subtracted from the triplicate mean OD 570 nm of the test sample for statistical comparison.

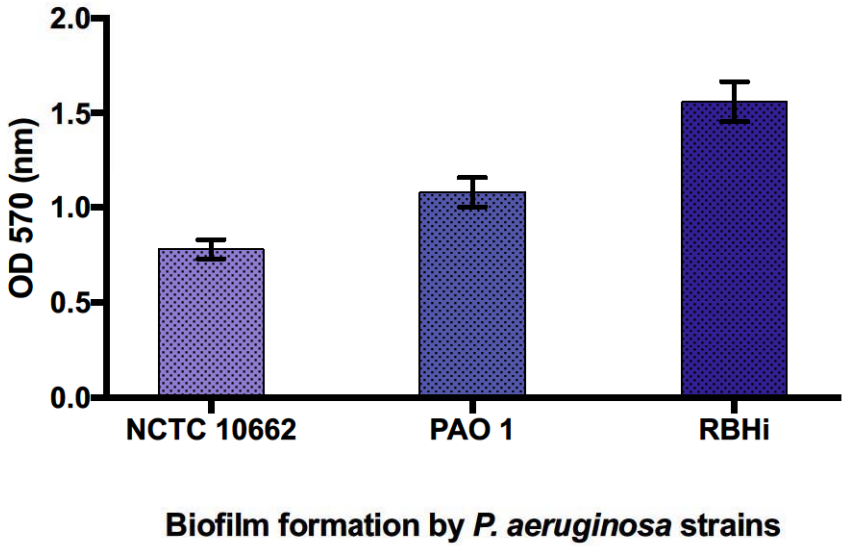


Figure 3.2 Quantitation of biofilm formation by non-mucoid, mucoid and heavily mucoid strains of *P. aeruginosa* in LB medium by crystal violet method. Significant difference was observed when comparing total biofilm of non-mucoid and mucoid ($p < 0.0002$) strains of *P. aeruginosa* (n=9)

The study was conducted in triplicates and as three independent experiments to test for reproducibility (n=9). All the three strains of *P. aeruginosa* formed visible biofilms after an incubation period of 24 h. Based on the quantitative data obtained by the crystal violet assay, the clinical isolate (*P. aeruginosa* RBHi) showed the highest biofilm formation in comparison with non-mucoid *P. aeruginosa* NCTC 10662. In comparison to the non-mucoid strain, both the mucoid and heavily mucoid strains of *P. aeruginosa* (PAO1 and RBHi) showed significantly higher biofilm formation as analysed by Tukey's multiple comparison test.

It is to be noted that the crystal violet assay quantifies the biofilm as a whole including bacterial cells and the EPS.

3.2.3 Production of EPS during biofilm formation by *P. aeruginosa* strains

The production of EPS by non-mucoid and mucoid strains of *P. aeruginosa* was quantified for the presence of total carbohydrates, uronic acid, total proteins and eDNA. A combination of biochemical and fluorometric methods were adopted for EPS quantification. EPS was separated from the bacterial cell as mentioned previously (Chapter 2, section 2.5). Total carbohydrate content of the biofilms was quantified by the phenol-sulphuric acid method, uronic acid quantification was conducted using the carbazole method, protein content was

verified by the Bradford assay and eDNA content of the biofilm was quantified using a fluorimeter (Qubit).

Table 3.2 Composition of EPS from non-mucoid and mucoid strains of *P. aeruginosa*

Strains	Type	Protein (µg)	Total carbohydrate (µg)	Alginate (µg)	eDNA (µg)	Alginate (%)
10662	NM	13.6	232.1	0.05	1.04	0.022
PAO1	M	20.6	919.8	498.2	2.45	54.2
RBHi	HM	49.8	1122.7	856.1	4.87	76.3

NM, non-mucoid; M, mucoid; HM, heavily mucoid. Values represent means of three independent experiments in triplicates. Values represented as "µg" are calculated against 1mg dry weight of the biofilm (n=3).

Based on table 3.2, the difference in the composition of biofilms produced by non-mucoid and mucoid strains of *P. aeruginosa* is clearly evident. The carbazole assay quantified uronic acid content of the biofilm, which is an indicative of the presence of alginate that forms the slime layer in mucoid strains of *P. aeruginosa*. In the heavily mucoid strain (RBHi), the total carbohydrate content comprised of 76.3% of alginate while 54.2% was seen with the mucoid strain (PAO1). A negligible amount of uronic acid was detected in the non-mucoid strain (NCTC 10662) as well. This could be indicative of a metabolite or a by-product from biofilm development that is detected by the carbazole assay. Minimal quantities of alginate were detected from planktonic growth of the mucoid strains, but not from the non-mucoid strain of *P. aeruginosa*. Whitchurch *et al* in 2002 suggested that nucleic acids could react with the carbazole assay for low level false positive detection of alginate in non-mucoid biofilms.

3.3. A study of attached biofilms from *P. aeruginosa* strains reveals diverse effect of divalent ions

Environmental factors such as electrolyte concentrations and medium composition may have important impacts on biofilm formation. Divalent cations such as Mg^{2+}, Zn^{2+} and Ca^{2+} are known to directly influence biofilm formation through their effect on electro-static interactions and indirectly via physiology dependent attachment processes (Song *et al.*, 2006). Stock solutions of 400 mM $MgSO_4$, $ZnSO_4$, $MnSO_4$ and $MgCl_2$ were prepared and filter sterilized prior to serial dilution at the inoculation phase.

Inocula from the three strains of *P. aeruginosa* were diluted to 0.5 McFarland standard for obtaining *P. aeruginosa* biofilm formation and composition in the presence of the salts. The salts were supplemented into LB medium at low (2 mM) and high (20 mM) concentration into 96-well microtiter plate for CV straining, the same ratio of growth medium, salt concentration and inoculum were used at a total volume 3mL in 6-well tissue culture plates for biofilm growth and quantification of biofilm components. All experiments were performed simultaneously to minimise variation of conditions and were performed in triplicates. The plates were incubated at 37 ºC for a period of 24 h prior to analysis.

It has been established that varying environmental ion concentrations impacts *in vitro* biofilm development by *P. aeruginosa* (Palmer, Flint and Brooks, 2007). The goal of this study was to understand the variation in the attached biofilm formation displayed by non-mucoid, mucoid and heavily mucoid phenotypes of *P. aeruginosa* under similar environmental conditions.

Figure 3.3 Non-mucoid strain of *P. aeruginosa* (NCTC 10662) showed a significant increase in biofilm development in the presence of 20mM $ZnSO_4$ (p= 0.001), and low and high concentrations of $MgCl_2$ respectively (p= 0.001). No other salt or concentration had any significance on the ability of the non-mucoid strain to develop a biofilm compared to the untreated control sample. Figure 3.4 Biofilm formation by the mucoid strain of *P. aeruginosa* (PAO1) showed a significant increase in the presence of 20 mM $MgSO_4$ (p= 0.009) and 20mM $ZnSO_4$ (p= 0.001). Low and high concentrations of $MgCl_2$ (p= 0.0001) showed a similar increase in biofilm formation as that of the non-mucoid *P. aeruginosa*. Figure 3.5 Increase in biofilm formation was observed in the presence of 20mM $ZnSO_4$ (p= 0.002) and Low and high concentrations of $MgCl_2$ (p= 0.0001) with the heavily mucoid strain of *P. aeruginosa* (RBHi).

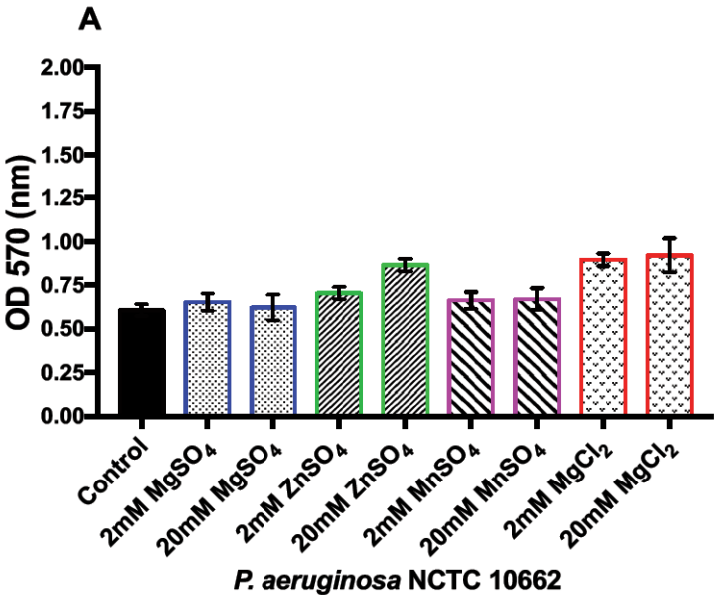


Figure 3.3 Quantification of biofilm formation of *P. aeruginosa* NCTC 10662 under the influence of low and high concentration of $MgSO_4$, $ZnSO_4$, $MnSO_4$ and $MgCl_2$ (n=3)

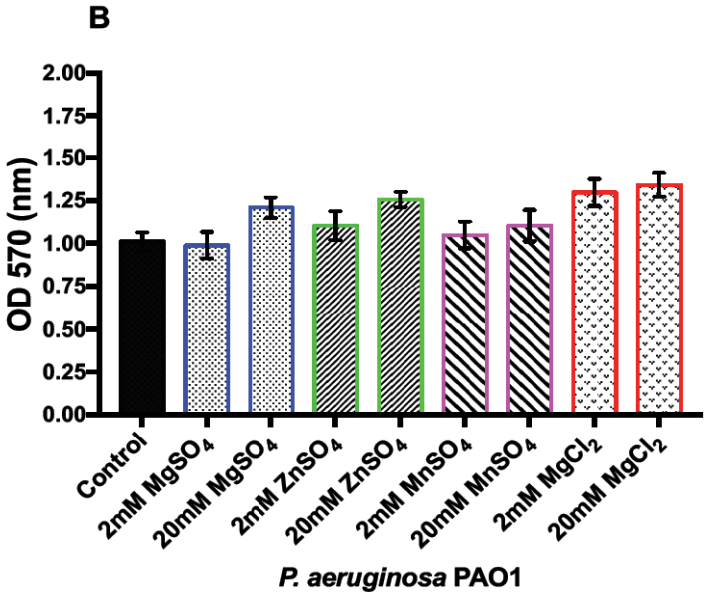


Figure 3.4 Quantification of biofilm formation of *P. aeruginosa* PAO1 under the influence of low and high concentration of $MgSO_4$, $ZnSO_4$, $MnSO_4$ and $MgCl_2$ (n=3)

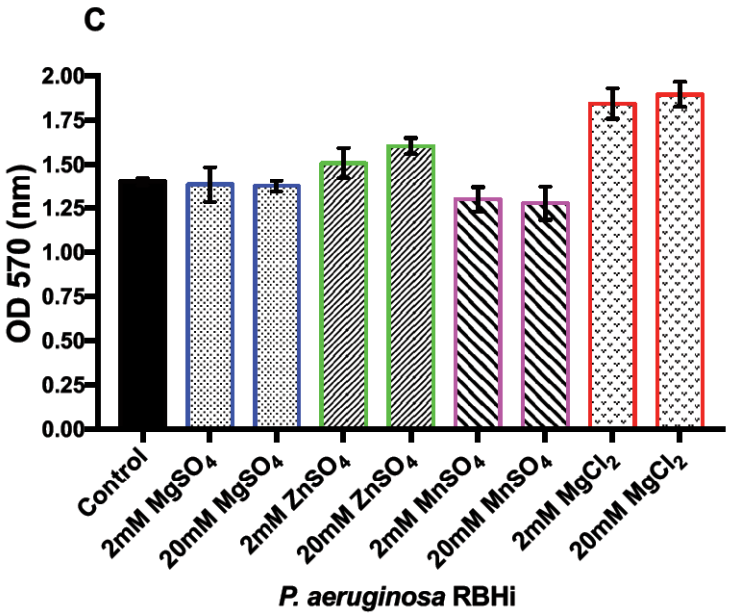


Figure 3.5 Quantification of biofilm formation of *P. aeruginosa* RBHi under the influence of low and high concentration of $MgSO_4$, $ZnSO_4$, $MnSO_4$ and $MgCl_2$ (n=3)

Figure 3.6 Non-mucoid *P. aeruginosa* (NCTC 10662), showed an increase in carbohydrate content of the EPS when treated with low and high concentrations of (2 mM and 20 mM) $MgSO_4$ (p= 0.045). Similar result was observed with $MnSO_4$ (p= 0.0112, p= 0.0042). The highest increase in total carbohydrates was seen with the addition of 2 mM and 20 mM of $MgCl_2$ (p= 0.0001). Figure 3.7 In the case of the mucoid strain (*P. aeruginosa* PAO1), a significant increase was observed only with the addition of (2 mM and 20 mM) $MgCl_2$ (p= 0.024 and 0.034). Figure 3.8 The heavily mucoid strain of *P. aeruginosa* (RBHi) showed similarities between the non-mucoid and the mucoid strains. A significant increase in total carbohydrates was seen with all the salts with the exception of $MnSO_4$. The highest increase was seen with the addition of (2 mM and 20 mM) $MgCl_2$ (p= 0.0001).

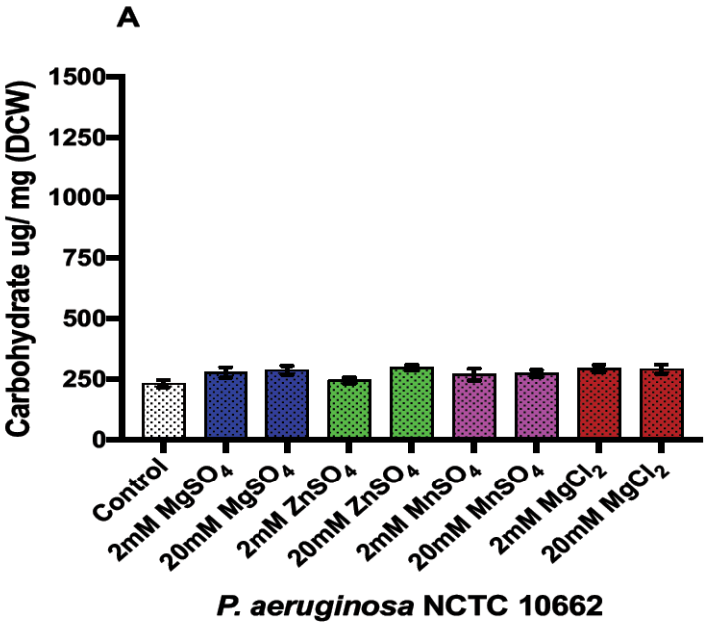


Figure 3.6 Quantification of the carbohydrate fraction of *P. aeruginosa* NCTC 10662 biofilms treated and untreated with salts (n=3)

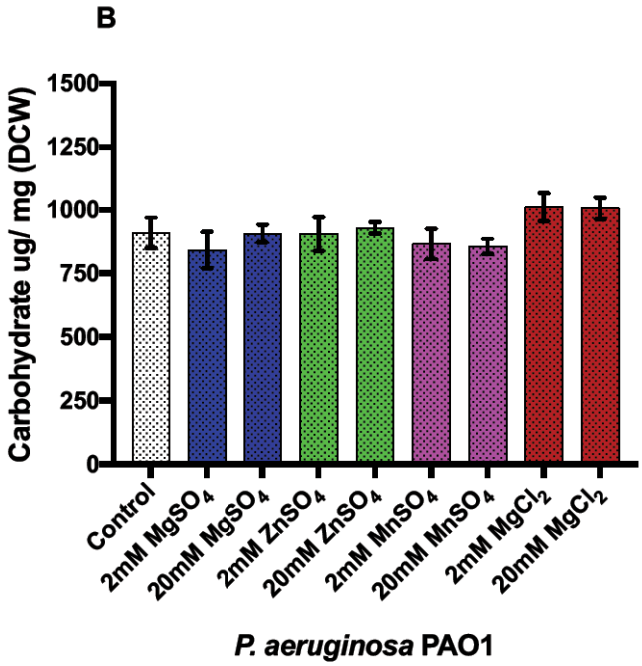


Figure 3.7 Quantification of the carbohydrate fraction of *P. aeruginosa* PAO1 biofilms treated and untreated with salts (n=3)

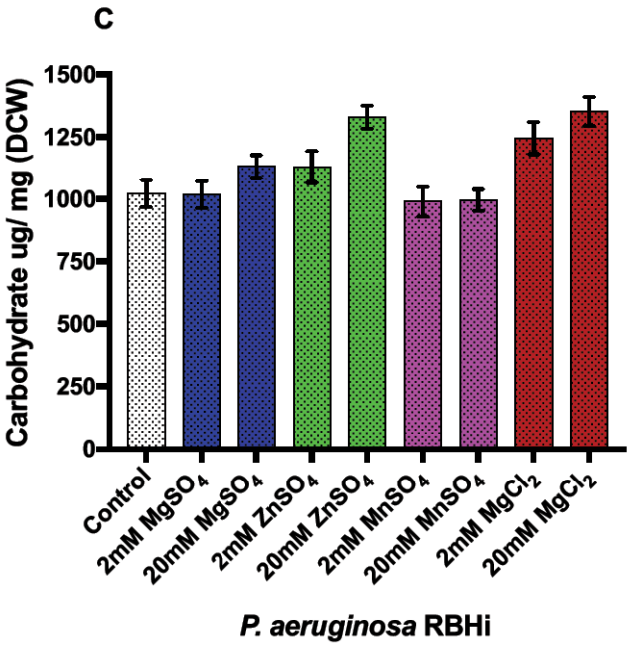


Figure 3.8 Quantification of the carbohydrate fraction of *P. aeruginosa* RBHi biofilms treated and untreated with salts (n=3)

Figure 3.9 Addition of $MgCl_2$ (2 mM and 20 mM) showed a significant increase (p= 0.302 and 0.0022) in protein content of the non-mucoid biofilm. However, no significant effect was observed with the addition of any of the other salts. (Figure 3.10) Protein content in the mucoid strain *P. aeruginosa* PAO1 increased significantly with the addition of (2 mM and 20 mM) $MgCl_2$ (p= 0.0001) and with 20 mM $ZnSO_4$ (p= 0.0048) other unaffected by the other salts when compared to the control experiment. (Figure 3.11) The heavily mucoid strain *P. aeruginosa* RBHi showed a considerable increase in protein content with the addition of (2 mM and 20 mM) $ZnSO_4$ (p= 0.009, 0.0001) and $MgCl_2$ (p= 0.0035, 0.0001) respectively. The change in protein content in the presence of $MgSO_4$ and $MnSO_4$ was not found to be significant by Dunnett's multiple comparison test.

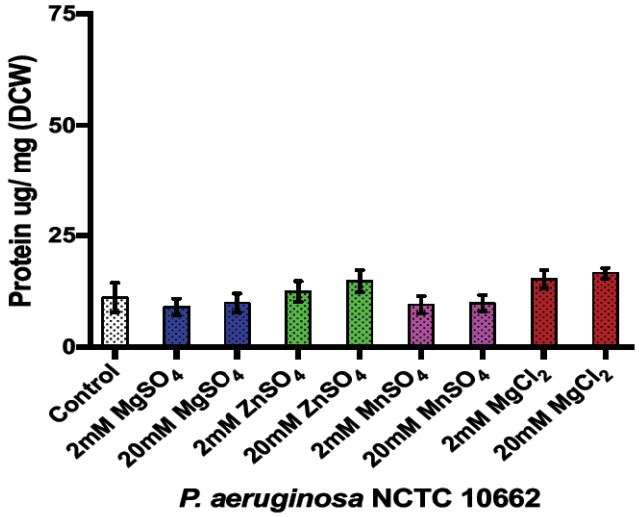


Figure 3.9 Analysis of the protein content of the EPS extracted from biofilms formed by *P. aeruginosa* NCTC 10662 after addition of salts

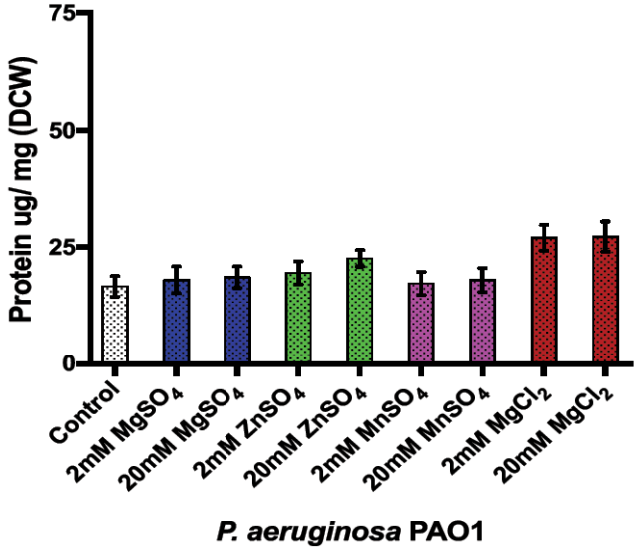


Figure 3.10 Analysis of the protein content of the EPS extracted from biofilms formed by *P. aeruginosa* PAO1 after addition of salts (n=3)

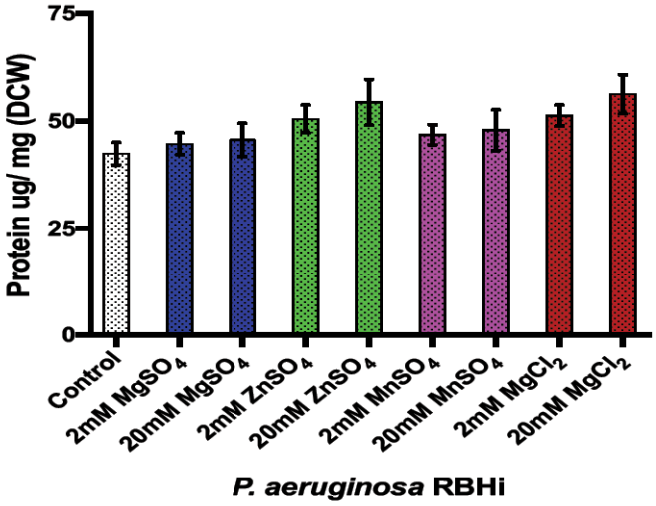


Figure 3.11 Analysis of the protein content of the EPS extracted from biofilms formed by *P. aeruginosa* RBHi after addition of salts (n=3)

Figure 3.12) Compared to the control, a significant increase in eDNA content of the biofilm was observed with all the salt concentrations used. The highest increase was seen with 20 mM $MgCl_2$ (p= 0.0001). Figure 3.13) In the case of the mucoid strain *P. aeruginosa* PAO1, only the magnesium containing salts showed a significant increase in eDNA content, the highest of which was 20 mM $MgCl_2$ (p= 0.0001). Figure 3.14) A significant increase in eDNA content was observed in all salts and concentrations when analysing the EPS of the heavily mucoid strain, *P. aeruginosa* RBHi. Both the high and low concentrations of $MgCl_2$ displayed the highest increase of eDNA compared to the other salts and the control (p= 0.0001).

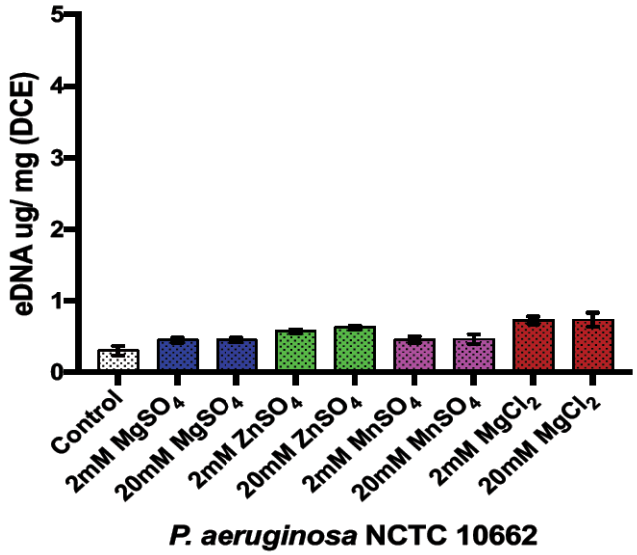


Figure 3.12 Quantitative analysis of eDNA present in the EPS of *P. aeruginosa* NCTC 10662 after addition of salts (n=3)

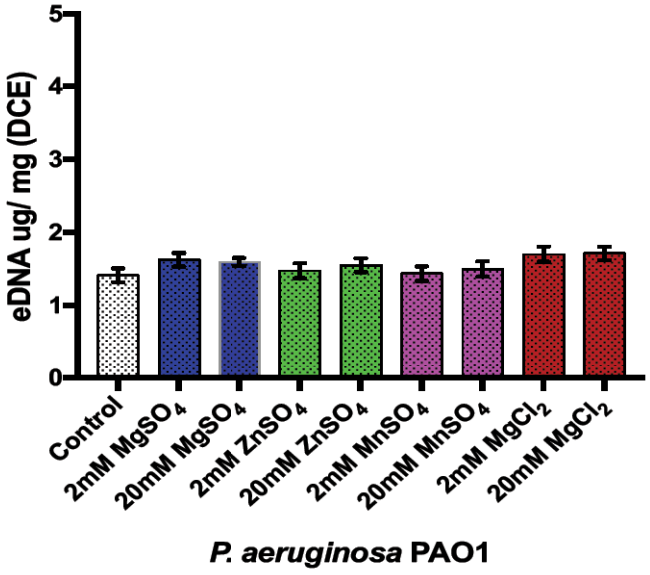


Figure 3.13 Quantitative analysis of eDNA present in the EPS of *P. aeruginosa* PAO1 after addition of salts (n=3)

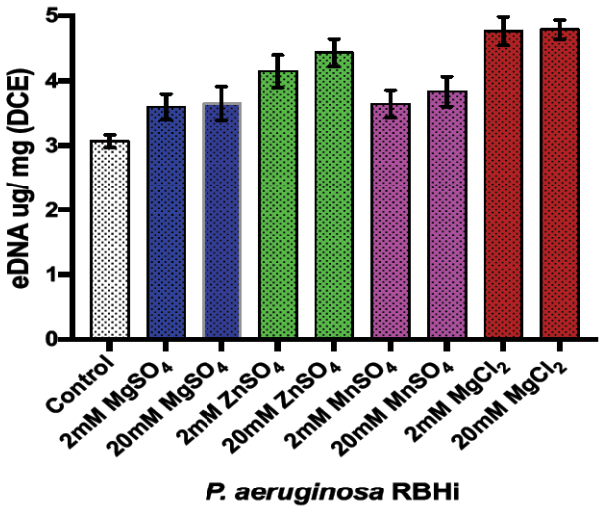


Figure 3.14 Quantitative analysis of eDNA present in the EPS of *P. aeruginosa* RBHi after addition of salts (n=3)

Though *P. aeruginosa* NCTC 10662 produced negligible amounts of alginate (0.0022%), the production was not affected by any of the salts. Figure 3.15 A) A significant increase in alginate was observed with (2 mM and 20 mM) $MgCl_2$ (p=0.0006, 0.0002). Though all the other salts showed an increase in alginate production, the increase was not significant compared to the control. Figure 3.15 B) In the case of the heavily mucoid strain, *P. aeruginosa* RBHi, a significant increase in alginate was observed in all the salts and concentrations apart from $MnSO_4$. The highest increase in alginate was observed in the presence of 20 mM $ZnSO_4$ and 20 mM $MgCl_2$ (p= 0.0001).

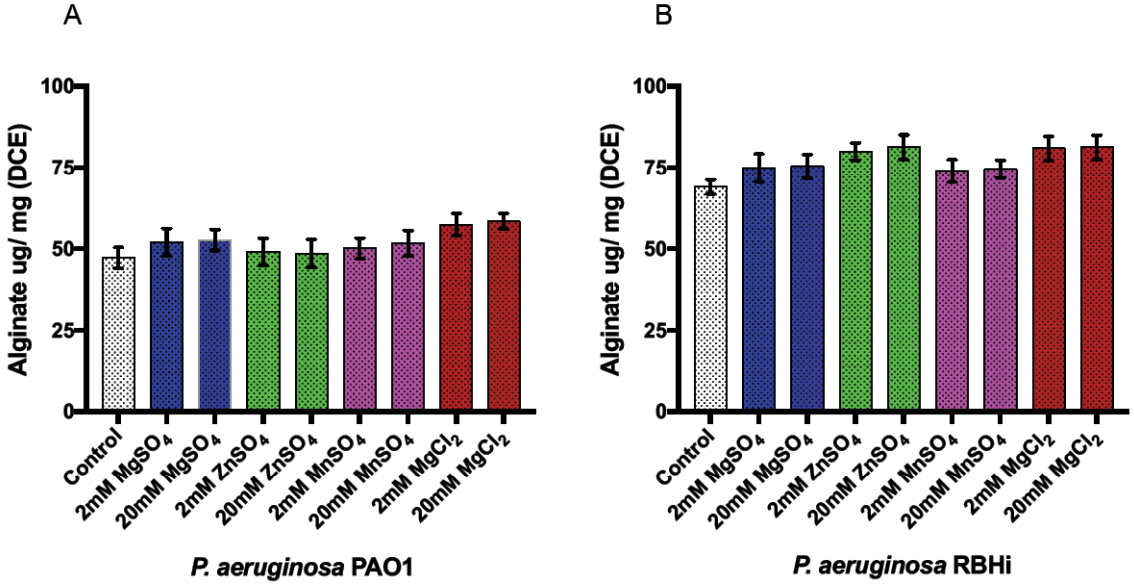


Figure 3.15 Quantification of alginate production by non-mucoid, mucoid and heavily mucoid strains of *P. aeruginosa* treated with salts (n=3)

3.4 Synergistic effect of tryptophan and erythromycin on *P. aeruginosa* biofilm architecture and inhibition

P. aeruginosa express high antibiotic resistance to multiple antibiotics classes and it is difficult to eradicate. Resistance acquired by *P. aeruginosa* is due to chromosomal mutation and acquirement of resistance genes through horizontal gene transfer (Poole, 2011). Scientific literature describes a number of quorum sensing inhibitors already in use to antagonise QS mediated bacterial communication in *P. aeruginosa*. Previous studies have shown that combination amino acids and antibacterial compounds is emerging as novel treatment (Chen *et al*., 2018) to combat the rise in antibiotic resistance. Furthermore, research suggests that macrolides directly affect QS mediated biofilm formation by *P. aeruginosa* (Imperi, Leoni and Visca, 2003). Hence, for the purpose of this study, erythromycin was chosen.

Targeting *P. aeruginosa* biofilm with naturally occurring amino acid such as tryptophan appears to be a highly attractive idea. Numerous studies have shown that certain amino acids are capable of causing biofilm dispersal (Sanchez *et al*. 2013). For the purpose of this study, dextrorotatory (D-) and levorotatory (L-) isoforms of tryptophan have been chosen and three different concentrations (1 mM, 4 mM, 8 mM) selected to determine whether these concentrations have any effect on dispersion of *P. aeruginosa* (NCTC 10662, PAO1 and RBHi) biofilm. Following that, combination of two isoforms of one concentration (the most effective one) of tryptophan and the antibiotic erythromycin against *P. aeruginosa* biofilm was used. The dispersal and inhibition activity of the biofilm dissociation treatment was investigated.

Biofilm formation was significantly reduced in the presence of all concentrations (1mM, 4mM and 8mM) of D and L tryptophan. As shown in figure 3.16 A, B and C, the greatest decrease in biofilm formation was observed with the use of 4 mM and 8mM D- tryptophan (p= 0.0001) amongst all the three strains of *P. aeruginosa*. Therefore, 4mM was selected for further experiments. The greatest biofilm decrease (>70%) was observed with the non-mucoid and mucoid strains, *P. aeruginosa* NCTC 10662 and *P. aeruginosa* PAO1 respectively, with the use of 4 mM D- tryptophan. Figure 3.16 A and B.

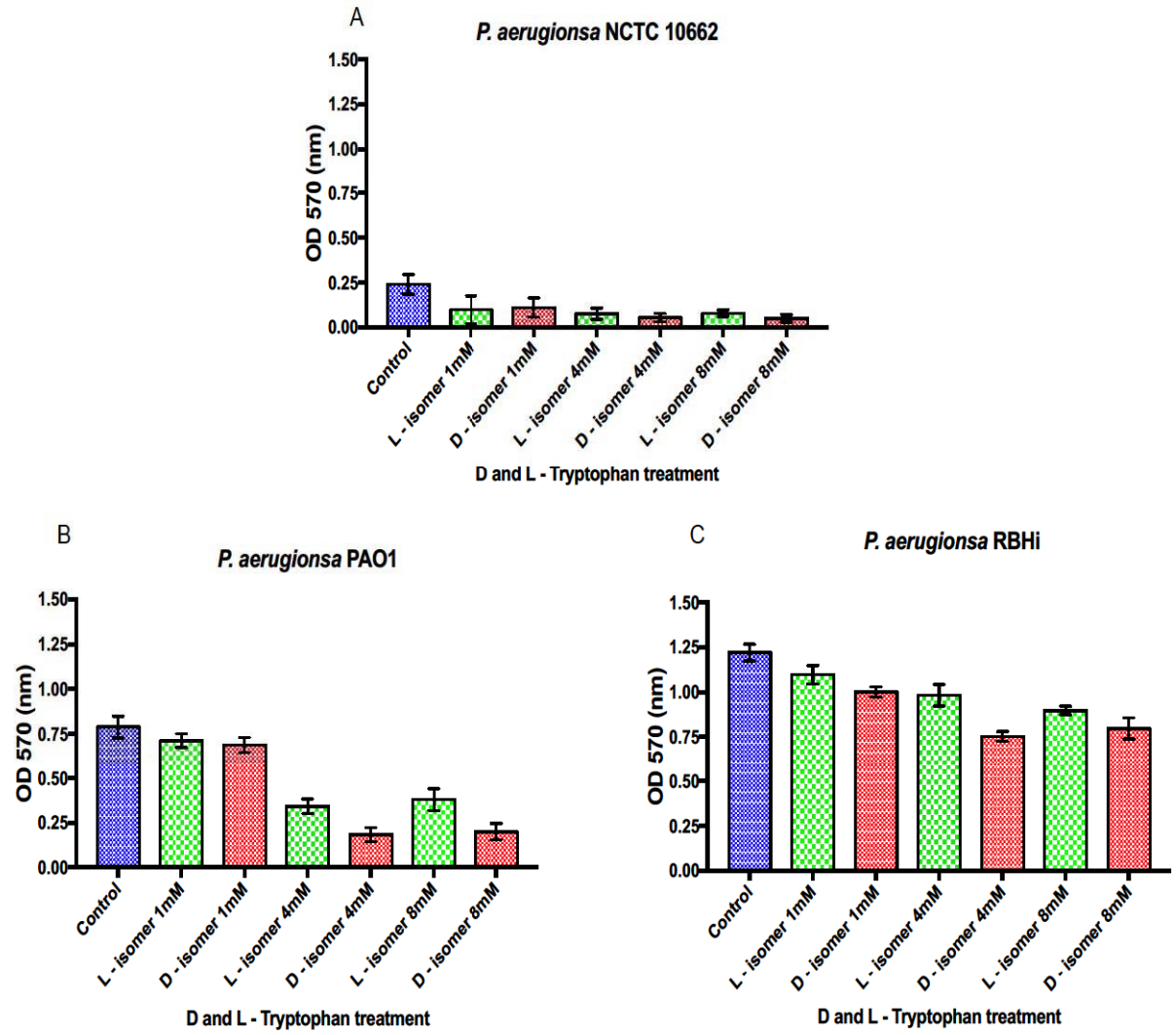


Figure 3.16 Effect of D and L isomeric forms of Tryptophan on biofilm formation by non-mucoid, mucoid and heavily mucoid strains of *P. aeruginosa* (n=24)

Compared to the controls of each strain, (EM) significantly reduced biofilm development with the exception of the non-mucoid strain *P. aeruginosa* NCTC 10662 (p= 0.053) as shown in figure 3.17 A. However, no significant increase or decrease was observed with the CFU/ mL count of planktonic cells of *P. aeruginosa* at the concentration of erythromycin used (4µg/ mL) as shown in figure 3.17 B.

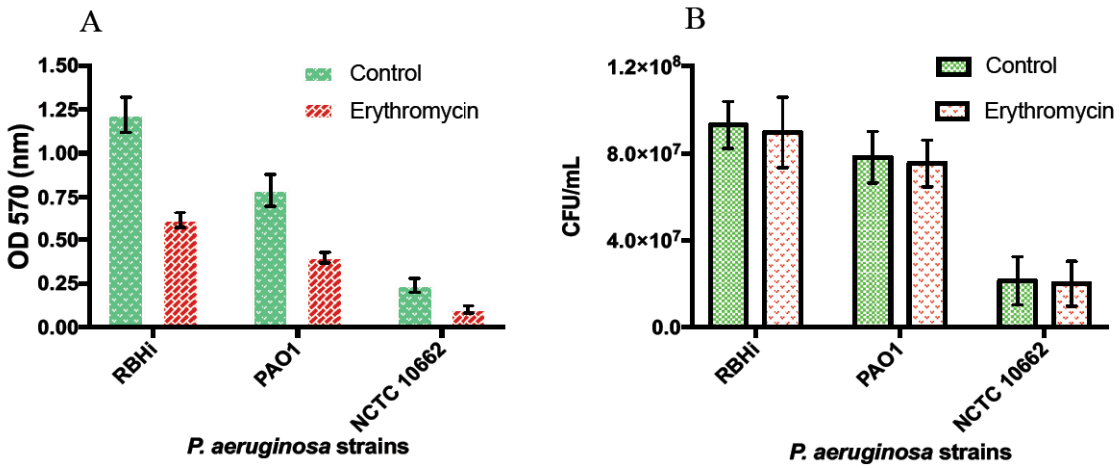


Figure 3.17 Effect of 4µg/ mL of the macrolide, erythromycin (EM) on biofilm formation (A) by non-mucoid, mucoid and heavily mucoid strains of *P. aeruginosa* and the relative CFU/mL count (B) before and after treatment (n=5)

Based on the results obtained from *P. aeruginosa* biofilm inhibition by tryptophan and EM, the synergistic effect of 4 mM D- tryptophan and 4µg/ mL of EM was investigated on biofilm composition and architecture of the three *P. aeruginosa* strains.

Total carbohydrate analysis (figure 3.18 A) of the treatments against all the three strains showed a significant decrease (p= 0.0001) in carbohydrate content when compared to the untreated control. The EPS of the non-mucoid, mucoid and heavily mucoid strains of *P. aeruginosa*, as analysed by two-way ANOVA. However, comparing the difference in total carbohydrate content between individual and synergistic treatment of the non-mucoid strain *P. aeruginosa* NCTC 10662 was found not to be significant (p> 0.5). The decrease in protein and eDNA (figures 3.19 A and B) content of the biofilms formed by *P. aeruginosa* PAO1 and *P. aeruginosa* NCTC 10662 was not found to be significant (p= 0.845, 0.061 and 0.845, 0.137 respectively) when treated with 4 mM of D- tryptophan. All other treatments lead to measurable and significant decrease in proteins and eDNA content of the biofilm. Alginate content (figure 3.18 B) of the EPS was not quantifiable for statistical purpose with the non-mucoid strain *P. aeruginosa* NCTC 10662. However, the mucoid and heavily mucoid strains of *P. aeruginosa* did produce alginate and saw a significant reduction with combination treatment using D- tryptophan and EM. The significance in reduction of alginate using the synergistic treatment was comparable to the individual controls as well as individual treatment with D- tryptophan but was not found to be significant compared to the treatment with antibiotic alone. This goes on to show that sub-inhibitory dose of EM has an antagonistic effect on the production of EPS of mucoid strains of *P. aeruginosa*.

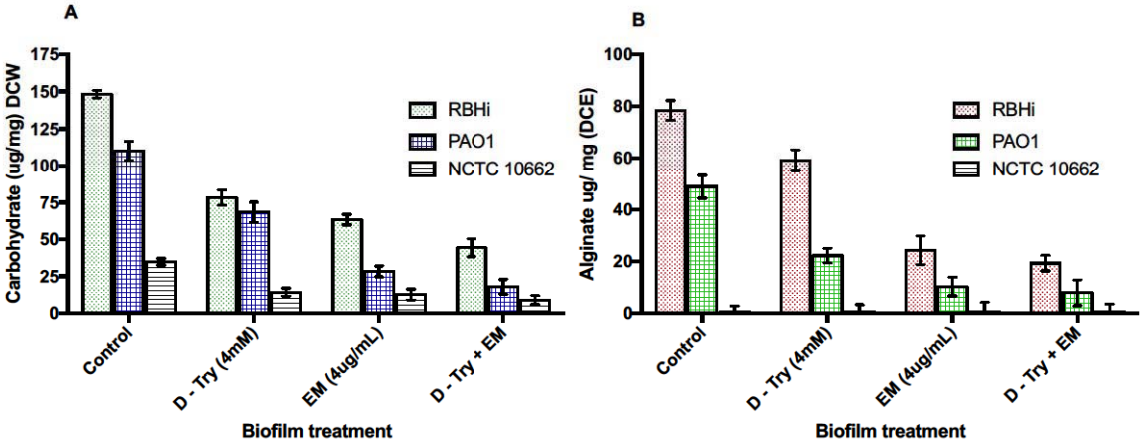


Figure 3.18 A and B Quantification of EPS components (carbohydrate and alginate) of *P. aeruginosa* biofilms after individual and synergistic treatment with 4mM D-tryptophan and 4µg/ mL EM (n=5)

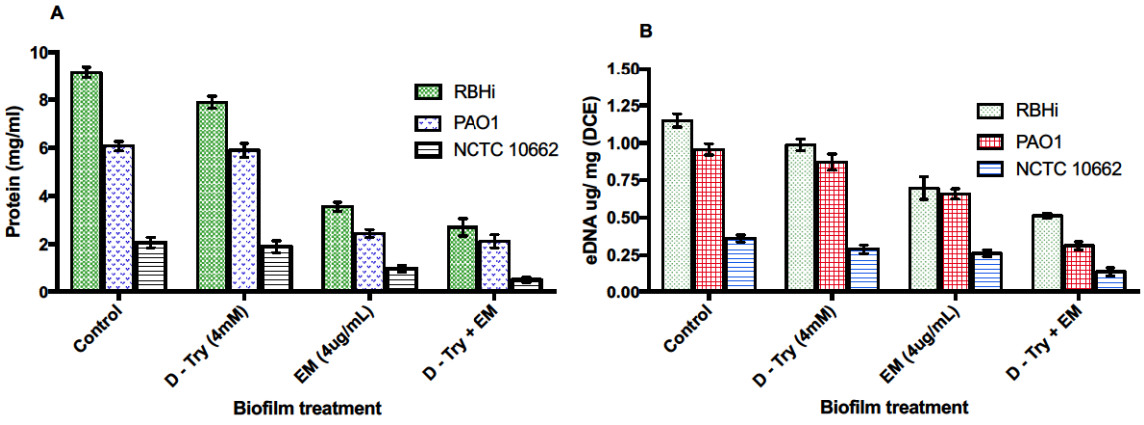


Figure 3.19 A and B Quantification of EPS components (protein and eDNA) of *P. aeruginosa* biofilms after individual and synergistic treatment with 4mM D-tryptophan and 4µg/ mL EM (n=5)

3.5 Discussion

Formation of biofilm is dependent on numerous environmental factors that act as stimuli for regulation of gene expression (Stanley and Lazazzera, 2004). It is reported that *in vitro* use of cations can antagonise biofilm formation by *P. aeruginosa* (Sangani *et al*., 2014; Vincent *et al*., 2014) whereas certain divalent cations contribute towards biofilm formation (Das *et al*., 2014). Electrostatic interactions mediated by cations largely influence biofilm architecture by functioning as cross linkers of the biofilm matrix as they contribute towards the mechanical integrity and stability of the outer membrane of the bacteria affecting the lipopolysaccharides (Garnett and Matthews, 2012). Divalent cations, such as Mg^{2+} and Ca^{2+} have been shown to influence biofilm formation by affecting electrostatic interactions as well as physiology-dependant attachment mechanisms and enzymatic cofactors (Valentini and Filloux, 2016). This study adopts the use of divalent cations to investigate the architecture of non-mucoid, mucoid and heavily mucoid biofilm structure.

As Mg^{2+} has been widely reported in the literature to affect biofilm formation in numerous bacterial species, Zn^{2+} and Mn^{2+} were included in this study as well as to test the effect of other divalent cations along with Mg^{2+}. Based on the results obtained from the effect of low or high concentrations of divalent cations on biofilm architecture of the non-mucoid, mucoid and heavily mucoid phenotypes of *P. aeruginosa*, it is clear that Mg^{2+} ($MgSO_4$ and $MgCl_2$) does in fact show an increase in biofilm formation at low (physiological) and high concentrations amongst all the phenotypes of *P. aeruginosa*. Addition of Mn^{2+} ($MnSO_4$) resulted in similar biofilm production to $MgSO_4$ and the increase in biofilm was not significant. However, the addition of $MgCl_2$ at low and high concentration yielded a significant increase in biofilm formation by all three strains after a period of 24 h (Figure 3.3 A, B and C). Despite the fact that all the salts used contain divalent cations, $MgCl_2$ showed a consistent increase in biofilm formation amongst all the strains of *P. aeruginosa*. Previous studies have found that Mg^{2+} and Mn^{2+} have varying effects on bacterial adhesion and resistance to oxidative stress and pathogenesis respectively (Song and Leff, 2006; Coady *et al*., 2015).

Despite studies referring to the antagonistic effect of ZnO nanoparticles (NP) (Garcia-Lara *et al*., 2015) based oxidative stress induced *P. aeruginosa* biofilm inhibition, other studies have reported increased biofilm formation due to the oxidative stress caused by ZnO-NP (Kumar *et al*., 2011). Therefore, the specific role of ZnO-NP remains unclear (Davies *et al*., 2017). Studies conducted using $ZnCl_2$ have shown to stimulate *P. aeruginosa* biofilm

formation (Marguerettaz *et al.*, 2014). The use of $ZnSO_4$ in this study against the three strains of *P. aeruginosa* showed that Zn^{2+} at high concentration (20 mM) did not have a deleterious effect on biofilm formation but showed a significant increase in biofilm formation in the non-mucoid, mucoid and heavily mucoid strains.

The discrepancy between the effect of the divalent cations from the salts, can be explained by the chemical structure of the salts. All salts are hexa-aqueous complexes;

1) $MgSO_4.\ 7H_2O$ = $[Mg(H_2O)6]^{2+}[SO_4.\ H_2O]$
2) $MnSO_4.\ 7H_2O$ = $[Mn(H_2O)6]^{2+}[SO_4.\ H_2O]$
3) $ZnSO_4.\ 7H_2O$ = $[Zn(H_2O)6]^{2+}[SO_4.\ H_2O]$
4) $MgCl_2.\ 6H_2O$ = $[Mg(H_2O)6]^{2+}$ and 2 Cl^-

As depicted by the chemical structures of the salts, $MgCl_2$ crystals consists of dianions with magnesium coordinated to six water molecules to form a complex and two independent chloride anions, Cl^-. In the case of the other salts, a seventh water molecule is associated with the sulphate anion. As a result of the increased hydration, $MgSO_4$, $MnSO_4$, $ZnSO_4$ maybe more involved in interacting with components of the EPS forming the biofilm rather than affecting bacterial cellular components as in the case of $MgCl_2$ and influencing the bacterial growth rate.

Analysis of the components of the EPS revealed that addition of salts to *P. aeruginosa* NCTC 10662 cultures showed a significant increase in total carbohydrate content compared to the control with the exception of 2 mM $ZnSO_4$, which did show an increase but was statistically not significant. However, in the case of *P. aeruginosa* PAO1, only $MgCl_2$ at low and high concentrations showed an increase in total carbohydrate content. In the case of *P. aeruginosa* RBHi, the heavily mucoid strain, with the exception of low and high concentrations of $MnSO_4$ and low concentration of $MgSO_4$, all the salts showed a significant increase in total carbohydrate content. Increased alginate production was observed in *P. aeruginosa* PAO1 and RBHi, while a significant increase was seen with only low and high concentrations of $MgCl_2$ in PAO1, RBHi showed a significant increase in the presence of low and high concentrations of $ZnSO_4$, $MgCl_2$ and low concentration of $MgSO_4$.

Addition of $MgCl_2$ once again showed a significant increase in protein content for all three strains along with $ZnSO_4$ for RBHi and PAO1 only while $MnSO_4$ did not show a significant increase. Proteins in the EPS serve numerous functions, including enzymes that degrade the

EPS to serve as a source of nutrients (Flemming and Wingender., 2010). Proteins are also present in the form of enzymatic virulence factors as well as interacting with other components of the biofilm to enhance the thermostability of the biofilm (Tielen *et al.*, 2013). Proteins such as lectins and lectin-like proteins serve a structural role in the biofilm by crosslinking exopolysaccharides with bacterial cells and growth surfaces (Fong and Yildiz., 2015). Amyloids are common EPS proteins that function as adhesins and cytotoxins (Otzen and Nielsen., 2008). Structural proteins such as flagella, fimbriae and pili also play a role in *P. aeruginosa* biofilm architecture. Studies have shown that extracellular lipase (LipA) interacts with the alginate component of biofilms formed by *P. aeruginosa* via electrostatic interaction providing a physiological advantage for bacterial cells present within the biofilm (Tielen *et al.*, 2013). Therefore, increase in protein content of the biofilm is consistent with increase in total biofilm as shown by the three strains of *P. aeruginosa* in this study.

The primary function of eDNA within the biofilm is that of facilitate attachment, aggregation and stabilisation of the biofilm (Fong and Yildiz, 2010). Previous studies have shown that DNase treatment of newly formed biofilm results in biofilm dissolution, however mature biofilms resist the action of DNase (Sharma and Singh., 2018). Studied conducted by Mulcahy, Charron-Mazenod and Lewenza, 2008 showed that, due to its highly anionic nature, eDNA functions as a cation chelator and effectively binds to all available divalent cations and in the presence of Ca^{2+} aids in the stabilisation of the bacterial cell wall and promotes ionic cross bridging between bacterial cells. The non-mucoid strain *P. aeruginosa* NCTC 10662 and the heavily mucoid strain RBHi, showed a significant increase in eDNA content of the EPS extracted from the biofilm with the presence of all the salts and concentration. However, *P. aeruginosa* PAO1 did not show a significant increase in eDNA content in the presence of $ZnSO_4$ and $MnSO_4$. However, all strains showed a quantitative increase in eDNA content after treatment with salts compared to their respective control.

Cross bridging effect is a vital mechanism that aids bacterial-substratum attachment and is often used in along with charge neutralisation. Research has reported that divalent cations act as bridging ions by forming cationic bridges between negatively charged bacterial surface and the negatively charged substratum surface (Das *et al.*, 2014). Studies conducted by Rose, Hogg and Shellis in 1993 reported that Ca^{2+} facilitated bacterial association amongst streptococci sp. in the oral cavity by forming polymeric cationic bridges between polymeric molecules individual bacterial cells. Further to this, divalent cations have been

observed to stimulate the formation of thick and dense biofilms with higher mechanical stability (Oknin, Steinberg and Shemesh, 2015).

This phenomenon is clearly documented in this study, as *P. aeruginosa* grown in the presence of Mg^{2+}, Mn^{2+} and Zn^{2+} containing salts. The mechanism of bridging effect is clearly seen when comparing biofilm formation and subsequent analysis of the biofilm between treatment with salts. This also resulted in the biofilm containing a higher quantity of total carbohydrates as well as proteins after extracting the EPS from the biofilm as shown in section 3.3. Increased biofilm production was regularly quantified at various time points when treated with Mg^{2+} in comparison with the control. Studies conducted by Simoni *et al* in 2000 on *Pseudomonas* sp. strain B13 found that the presence of Mg^{2+} ions increased the biomass of the bacterial cells in sand columns when compared with Na^{+} solution with similar ionic strength. This suggests that there is a specific binding of divalent cations to the cell surface, which influences the attachment of bacterial cells to surface of the substratum. In addition, the eDNA sequence of bacterial cells is not always identical to that of genomic DNA, suggesting that DNA may be exported from cells specifically for the purpose of building the EPS matrix (Böckelmann *et al*. 2006).

The use of naturally occurring amino acid as a treatment of infection caused by bacteria is noteworthy research. Amino acids do not express any toxicity to the host and bacteria do not acquire resistance to them. Numerous Gram-positive and Gram-negative bacteria produce D- amino acids such as D- alanine and D-glutamic acid in stationary phase and according to studies, D-amino acid are embedded in the peptidoglycan layer found in cell wall of bacteria. Peptidoglycan enables bacteria to survive in physiochemical environments (Barreteau *et al*., 2008). However, the amino acids are not produced in high volume to supress biofilm formation or cause biofilm dispersion (Kolodkin-Gal *et al.,* 2010). Brandenburg *et al (*2013) argues that some bacteria such as *Bacillus subtilis*, *Staphylococcus aureus* and *P. aeruginosa* biofilms were sensitive to amino acid and caused partial inhibition of biofilm formation. During this experiment, D and L isoforms of tryptophan reacted differently on formed biofilm but not on cell morphology.

With the exception of 1mM (D and L) Tryptophan against *P. aeruginosa* PAO1, all the concentrations used showed a reduction in biofilm development. The lowest and the most common concentration of tryptophan (4 mM D- tryptophan) at was chosen for this study.

Tryptophan mediates/ inhibits biofilm formation in *P. aeruginosa* by modulating the bacterial cell motility. Tryptophan is known to increase flagellar functioning in *P. aeruginosa* (Brandenburg *et al.*, 2013). As a stationary flagellum is required for attachment of bacterial cells to the substratum as the bacteria need to be non-motile for biofilm development. As tryptophan increases bacterial cell motility, it favours detachment of cells from the biofilm, as seen in a natural life cycle of the biofilm (Brandenburg *et al.*, 2013). Possibly, the conversion of sessile cells to planktonic cells is mediated by tryptophan leading to dispersal of biofilm.

The antibiotic chosen for the purpose of this study was erythromycin, due to studies reporting suppression of Gram-negative bacteria virulence factors (Kawamura-Sato *et al.,* 2000) Erythromycin belongs to the antibiotic family of macrolides where its mechanism of action blocks translation by binding to the 50S ribosomal subunit, mostly in Gram- positive organisms. However, *P. aeruginosa* being a Gram- negative is resistant to macrolides. A high MIC (erythromycin) of 512 µg/ml was found to effect *P. aeruginosa* PAO1 in Mueller – Hinton (Morita, Tomida and Kawamura, 2014). Tateda *et al.*, (2001) and Nalca *et al.*, (2006) reported that low dose of another type of macrolides – azithromycin 2 µg/ml had an effect on QS molecules resulting in inhibition of *P. aeruginosa* PAO1 virulence factors.

A study conducted by Tsang *et al.* (2003) suggests that erythromycin in low concentrations, such as the one chosen in this study, 4 µg/ml, affects *P. aeruginosa* cellular morphology and antagonises the QS system of *P. aeruginosa* (Burr *et al.*, 2016). Therefore, this study focused on erythromycin's ability to antagonise the *P. aeruginosa* QS system and subsequent biofilm development. Another study investigating low dose of erythromycin applied to patients suffering from respiratory diseases caused by *P. aeruginosa,* resulted in overall reduction in sputum volume and increased lung function. However, the mechanism of action was not found to be bacteriostatic, bactericidal or anti-inflammatory. The study also found that there were no changes in sputum densities of leukocytes, pro-inflammatory mediators and pathogens. *P. aeruginosa* possess a number of virulence factors such as rhamnolipids, alkaline protease, and elastase (Ballok and O'Toole, 2013).

This study confirmed the synergistic effect of D- tryptophan and erythromycin against *P. aeruginosa* biofilm development and composition. As shown in section 3.4, Figure 3.9, the CFU count of bacterial cells with and without treatment remained similar and there was no significant reduction in CFU after treatment with erythromycin for all three strains. This shows that the bacterial cells remained unaffected by erythromycin. Reduction in total

carbohydrate, proteins, eDNA and alginate was due to interference with QS mediated gene regulation.

In summary, various medium components affect biofilm formation by *P. aeruginosa* in different ways. Divalent cations were found to increase biofilm formation in moderation or significantly ($MgCl_2$) along with changes in quantities of the primary components of the EPS of the biofilm. Though biofilm formation increased with the addition of salts to the medium, this study has provided an insight into how various metallic divalent cations interact with *P. aeruginosa* and affect biofilm formation and composition. Also included in this study was the synergistic effect of an α- amino acid (tryptophan) and macrolide antibiotic, erythromycin on *P. aeruginosa* biofilm development. It is to be noted that isomers of compounds affect *P. aeruginosa* biofilm formation independent of each other as well as erythromycin mediated biofilm reduction without affecting bacterial viability.

Chapter 4 . Effect of QQ and biofilm dispersal agents on *P. aeruginosa* adherence and biofilm formation under static and dynamic conditions

4.1 Introduction

The possibility of contracting biofilm related infections within a hospital environment (nosocomial infections) is greater than in the general environment due to several reasons. Firstly, the patients in hospitals are already afflicted with various kinds of illness, bedridden and weak, with compromised defence which makes them highly susceptible to infections of the skin and the airways. A weakened immune system may not aid in combating infections optimally especially after the natural defence barriers have been rendered ineffective due to physical trauma, surgery and medical devices such as catheters. Secondly, many patients in the hospitals could be carriers of infectious diseases and could become the source to other who may readily become infected by many microbes. These microbes can then be transferred from patient to patient through direct or indirect contact via aerosolization, healthcare officials and medical devices (Mehta *et al*., 2008).

Nosocomial infections are predominantly seen in immunocompromised individuals (eg; CF) and postsurgical complications in patients with implants and biofilm mediated infections in the case of surgical implants is generally difficult to treat and cure. This could lead to a greater adverse effect and impact the quality of life (Khan, Baig and Mehboob., 2017). Commonly recorded areas of biofilm infections are mouth, catheter entry points and medical implants. An increasing body of evidence suggests that it is common to find biofilm contamination on regular equipment in hospitals which generally go undetected (Russotto *et al*., 2015). Biofilm mediated infections of vascular and urinary catheters form the majority of medical device related infections. Alongside the consequences of infections, the presence of biofilm on medical devices may lead to deterioration and loss of functionality. The exoenzymes secreted by bacteria may accelerate material degradation, especially in the case of polymeric biomaterials as it can be utilised as a source of carbon (Zhang *et al*., 2017).

Biomaterials are generally selected for use in the medical industry based on their properties and for biocompatibility, in the case of implants (Prakasam *et al*., 2017). Based on the structural and mechanical requirements, soft or hard materials can be chosen. Commonly used compliable materials are polypropylene (PP), poly(tetrafluoroethylene) (PTFE),

polyethylene(terephthalate) (PET), poly(methylmethacrylate) (PMMA), poly(dimethylsiloxane) (PDMS), polyurethane (PUE), hydrogels and some biodegradable polymers such as polyglycolide acid (PGA), and polylactic acid (PLA) (Dorozhkin, 2011). The advantages of using the compliable materials are the availability of numerous chemical compositions that can be formed into solids, gels, films, fibres and fabrication of shapes and structures (Muller *et al.*, 2017). However, use of said material does pose certain disadvantages related to sterilisation and lack of mechanical properties to use them has orthopaedic implants. To overcome such disadvantages, stainless steel, titanium (and alloys) tantalum or gold are generally used (Ananth *et al.*, 2015).

The numerous materials used in medical devices for various purposes offers biofilm forming bacteria a substratum to attach and colonise. In order to investigate and better understand the interaction between bacteria and the substratum, one must consider the physiochemical properties of the surface with and without conditioning film initiated during reversible attachment phase of biofilm development (Chapter 1: Section 1.3). The conditioning film helps change the physiochemical properties of the substratum surface, facilitating bacterial adhesion.

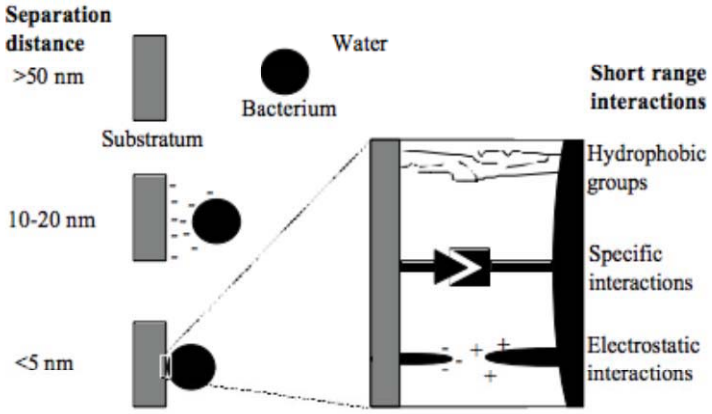


Figure 4.1 Forces of attraction and bacterial adhesion onto substratum (Busscher and Weerkamp, 1987)

Conditioning film usually forms in the presence of an aqueous medium and alteration of the physiochemical properties may promote or even help disperse bacterial attachment. More often than not, it provides a concentrated nutrient source as it is known to contain humic compounds glycoproteins and polysaccharides (Dang and Lovell, 2016). A classic example is the enamel of teeth which is conditioned by albumin, glycoproteins, lipids, phosphoproteins, lysozymes and other salivary components (Donlan, 2002). In the case of biomaterials, various host produced substances such as blood, tears, urine, intravascular fluid

and respiratory secretions characterise the conditioning layer and aid in bacterial attachment (Berne *et al*, 2018).

For the purpose of this study the ability of *P. aeruginosa* to form biofilm on different surfaces in the presence of M9 minimal medium (defined medium) was investigated. The purpose of using M9 minimal medium in this study was to investigate the effect of QQ and biofilm dispersal agents on biofilm formation and virulence factor production. Minimal medium was used to avoid unknown ingredients present in complex medium. The use of dynamic flow conditions for biofilm growth will add to the knowledge on combating/ eradication biofilm forming pathogens and infections.

4.2 Results

4.2.1 Influence of dispersal agents on sessile and planktonic cells of preformed *P. aeruginosa* biofilms in transmission flow cell

Overnight inocula of *P. aeruginosa* strains was grown in LB medium at 37 ºC and the inoculum absorbance was standardised to 0.5 McFarland standard prior to inoculation into the transmission flow-cell system. Upon inoculation, the bacterial cells were allowed to attach for 4 h to form a monolayer biofilm prior to initiating the flow of LB medium at 100µL/ min overnight for the development of the biofilm. After ~16 h, the medium was switched to M9 minimal medium at a flow rate of 150 µL/ min containing the desired concentration of the biofilm dispersal agents. Allowing for initial stabilisation, sample collection started after 2 h for every 2 h period till the end of the experiment.

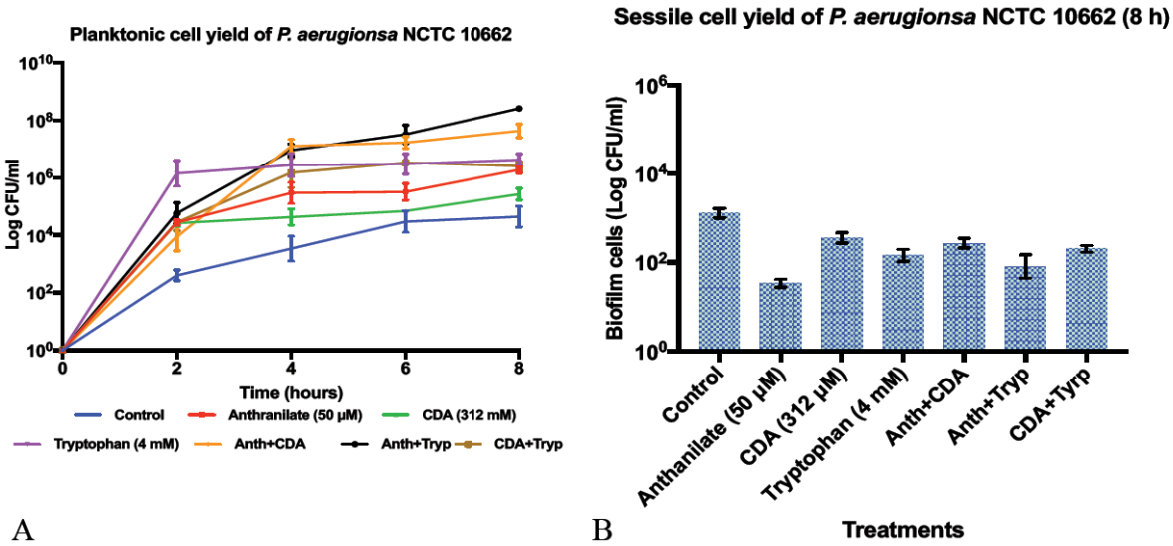
4.2.1.1 Exploiting intrinsic dispersal mechanism of *P. aeruginosa* to inhibit biofilm development on PDMS surfaces

The final stage of a bacterial biofilm cycle involves dispersion where cells break free from the EPS and change from stationary, sessile cell to motile and free-swimming planktonic cells (Flemming *et al*., 2016). Once motile, the planktonic cells flow downstream to attach and colonise a new location. The phenomenon of dispersion can be linked to two scenarios: a) passive dispersion which is dependent on external forces to break open the biofilm, and b) active dispersion where dispersion is mediated by the bacterial cells (Kaplan, 2010).

Tryptophan along with *cis*-2-decenoic acid (CDA) and anthranilate were used as agents of *P. aeruginosa* biofilm dispersal. CDA is a monosaturated fatty acid messenger produced by *P. aeruginosa* known as a diffusible signalling factor (Davies and Marques, 2008) which allows for bacterial communication. Anthranilate is a product of tryptophan catabolism which is an intermediary for Pseudomonas quinolone signal (PQS) which is the non-AHL signalling molecule produced by *P. aeruginosa*.

A 100µL aliquot of the effluent from the flow cell was collected, serially diluted and plated to enumerate the planktonic cell release from the biofilm formed by *P. aeruginosa* NCTC 10662, with and without treatment using dispersal agents. The synergistic effect of the dispersal agents on planktonic cell release was documented as well. In the non-mucoid strain NCTC 10662, all treatments, individual or in synergy induced dispersal of planktonic

cells, greater in number compared to the control at each respective time point. The increase in planktonic cell release was found to be significant (p= 0.001) after the 8 h point (figure 4.2 A). Tryptophan at 4mM showed the highest release of planktonic cells after a period of 2 h (figure 4.2 Λ), the rest were not found to be significant after the 2 h point when the mean values were compared. The highest planktonic count was seen with the combination treatment involving Anthranilate (50 µM) and CDA (312 µM) after 8 h (figure 4.2 B), and the difference in planktonic cell release was found to be significant, while comparing the means of all the other treatments and the control (p= 0.0001). CDA by itself was not as effective compared to its use in synergy with anthranilate and tryptophan. Corresponding cell enumeration of the sessile cells (8 h) showed a reduction in the final cell count with all treatments compared to the control.



A B

Figure 4.2 Cell enumeration by plating showing the planktonic cell (A) vs the sessile cells (B) of *P. aeruginosa* NCTC 10662 with and without treatment with biofilm dispersal agents as specified. Cell enumeration of sessile cells done after 8 h only (n=3)

Upon serially diluting and plating 100µL of *P. aeruginosa* PAO1 biofilm treated with the biofilm dispersal agents, treatment with anthranilate (50 µM) was found to have released the highest (p= 0.001) number of planktonic cells from the biofilm formed by the mucoid strain, *P. aeruginosa* PAO1 (figure 4.3 A). However, the decreased release of planktonic cells from treatment with CDA and combination treatment with anthranilate and CDA suggest that a greater number of sessile cells were present in the biofilm. This statement holds true for the treatment with CDA alone, but in combination with anthranilate, a lower CFU count was seen with sessile cells. A combination treatment with anthranilate and tryptophan showed the least CFU amongst all the extracted sessile cells (p= 0.0001), however it did not show a greater release of planktonic cells (figure 4.3 B).

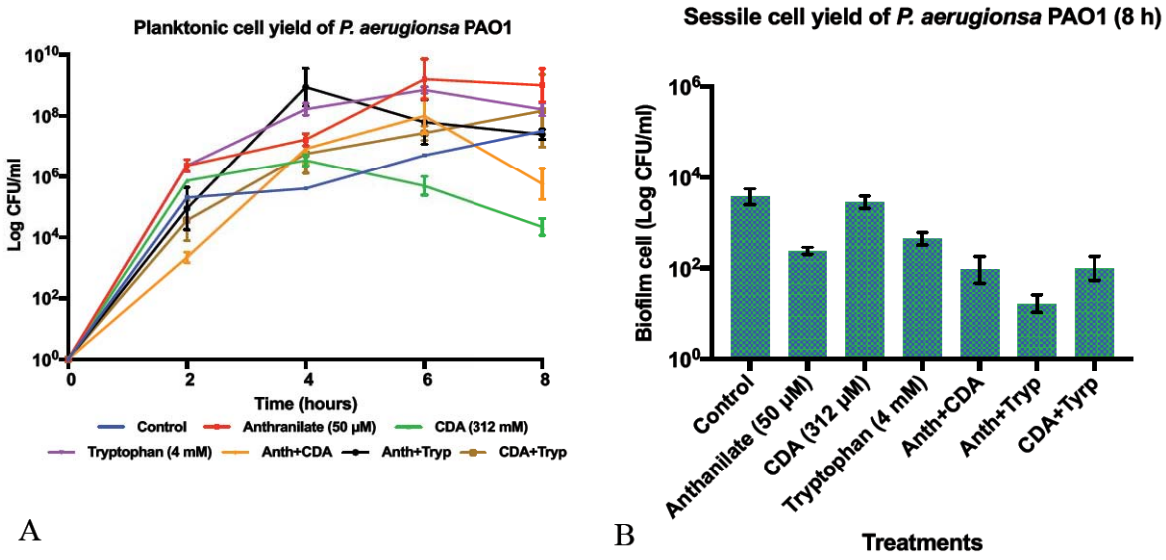


Figure 4.3 Cell enumeration by plating showing the planktonic cell (A) vs the sessile cells (B) of *P. aeruginosa* PAO1 with and without treatment with biofilm dispersal agents as specified. Cell enumeration of sessile cells done after 8 h only (n=3)

In the heavily mucoid strain, *P. aeruginosa* RBHi, dispersal of planktonic cells was affected by anthranilate alone compared to the control (p= 0.026) (figure 4.4 A). However, a reduced CFU count was seen with anthranilate and anthranilate in combination with CDA. Comparing the combination treatment of anthranilate and CDA, a significant difference with CDA treatment (p= 0.0001), however that was not the case when compared to anthranilate treatment for sessile cells (figure 4.4 B).

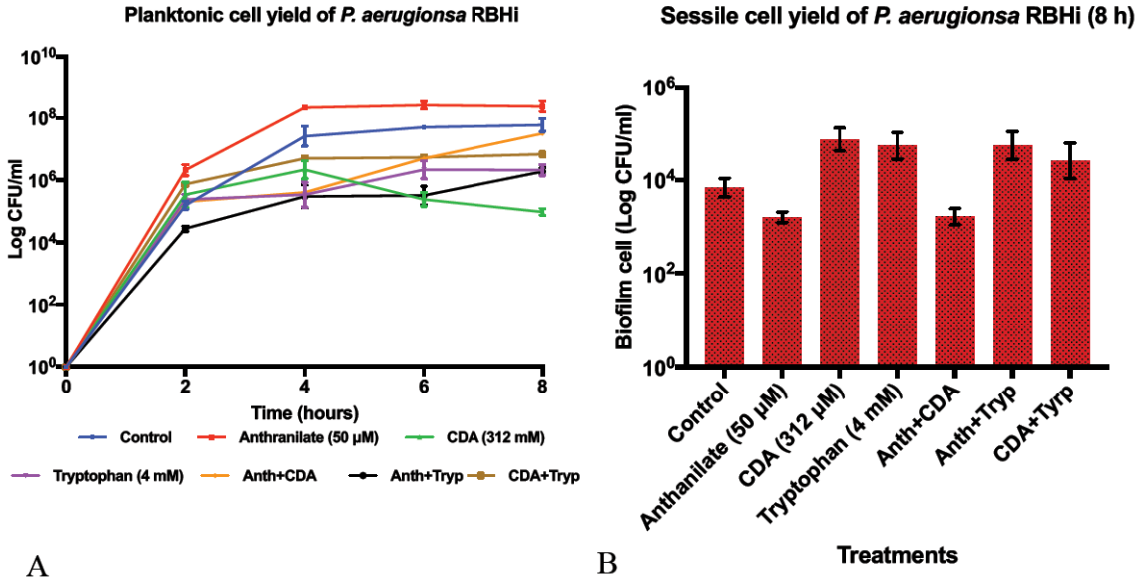


Figure 4.4 Cell enumeration by plating showing the planktonic cell (A) vs the sessile cells (B) of *P. aeruginosa* RBHi with and without treatment with biofilm dispersal agents. Cell enumeration of sessile cells done after 8 h only (n=3)

4.2.2 Analysis of EPS composition of biofilm formed by *P. aeruginosa* in transmission flow cell

After 8 h of biofilm formation, with and without treatment, the flow of medium was stopped and the biofilm from within the flow cell was extracted and the EPS and sessile cells were separated. The primary components of the EPS were then quantified, and the sessile cells were enumerated (Section 4.2.1)

Carbohydrate content of the biofilm formed by *P. aeruginosa* is presented in figure 4.5 A, B and C. Use of anthranilate showed the highest decrease in carbohydrate content amongst all the strains. The decrease was found to be significant ($p = < 0.05$).

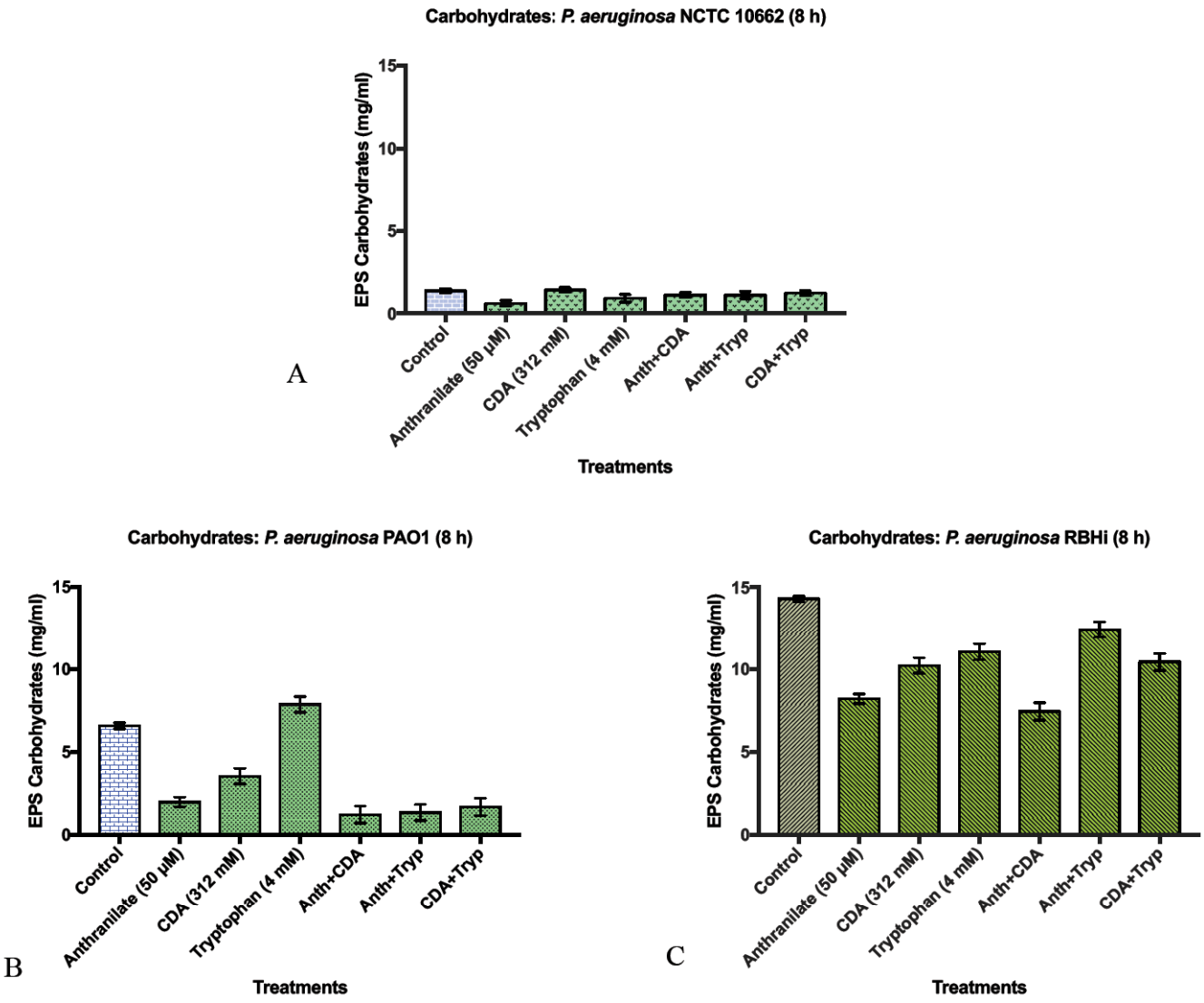


Figure 4.5 Comparison of total carbohydrate content of the biofilm formed by the three strains of *P. aeruginosa* sp after 8 h growth in transmission flow-cell with and without treatment using biofilm dispersal agents (n=5)

In *P. aeruginosa* PAO1, combination treatments showed a decrease in alginate production (p=0.0001) in comparison to the control. Even though the individual treatments did show a reduction in alginate content, the difference was not found to be significant (figure 4.6). However, anthranilate at 50 µM showed a significant decrease (p= 0.0028) in alginate content in the heavily mucoid strain, *P. aeruginosa* RBHi and did the combination treatment involving anthranilate and tryptophan.

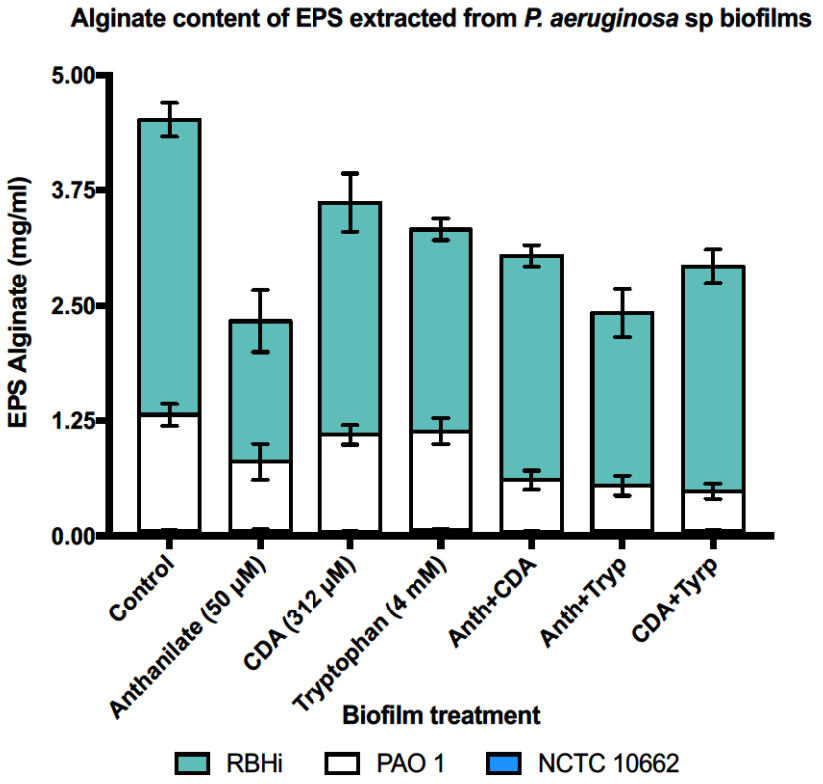


Figure 4.6 Comparison of alginate content of the biofilm formed by the three strains of *P. aeruginosa* after 8 h growth (n=3)

Comparing the eDNA content of all the three *P. aeruginosa* strains against their respective controls, the only significant decrease was observed in *P. aeruginosa* RBHi (figure 4.7 C) with individual treatment involving anthranilate and CDA. Surprisingly, a significant increase in eDNA content was observed in the same strain when treated with tryptophan. A trend of decreasing protein content was seen with all the treatments with the exception of CDA vs *P. aeruginosa* PAO1 (figure 4.7 B), which did show a decrease but not to the extent of the other treatment.

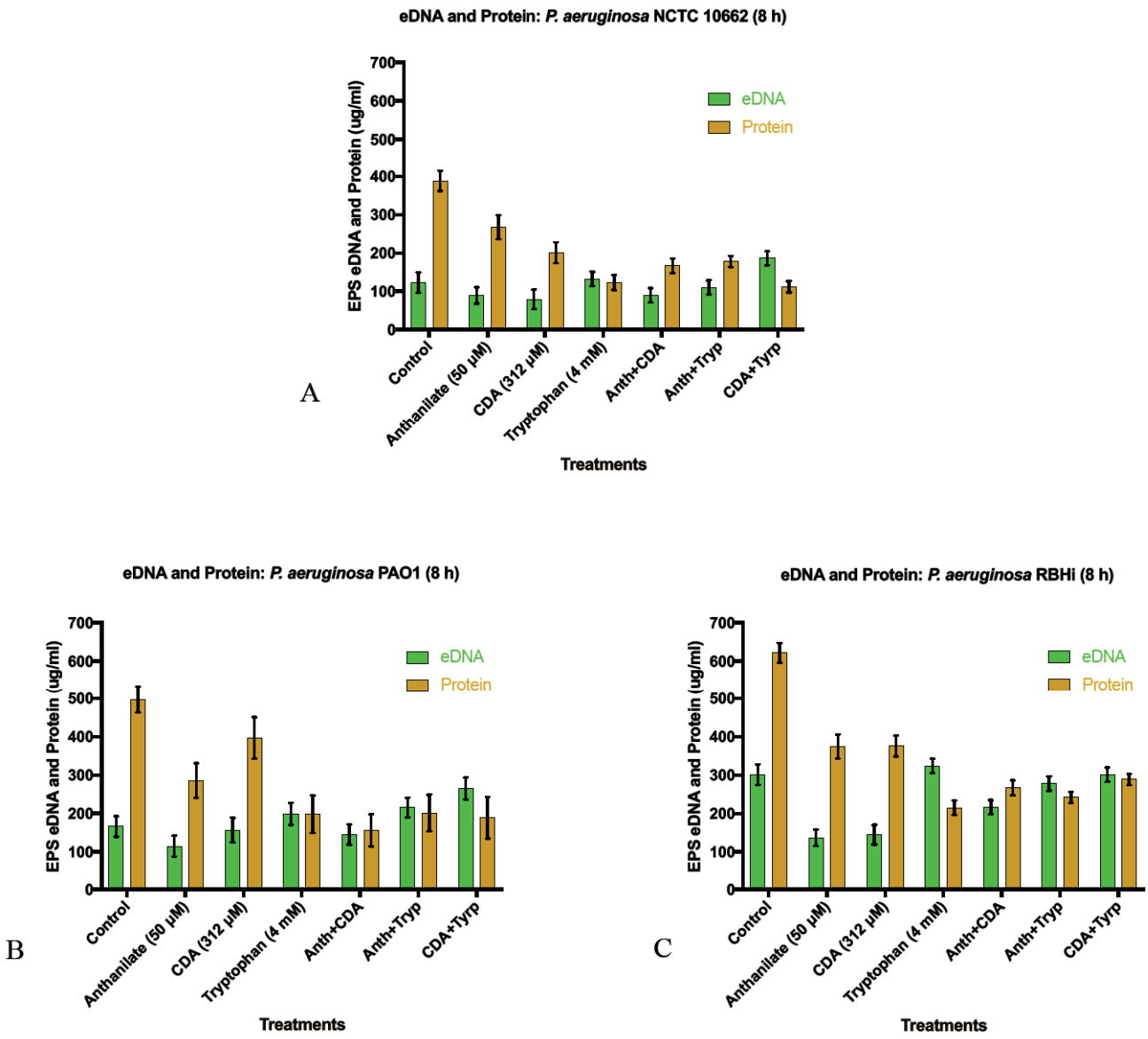


Figure 4.7 Comparison of eDNA and protein content of the biofilm formed by the three strains of *P. aeruginosa* after 8 h growth in transmission flow cell with and without treatment using biofilm dispersal agents (n=5)

The heavily mucoid *P. aeruginosa* RBHi consistently showed higher pyocyanin and rhamnolipid activity (Figure 4.8 A) compared to the other two strains. Compared to the control, no reduction in pyocyanin activity was seen with RBHi with the treatment, while a significant reduction (p=0.0034) in rhamnolipid was seen with 4 mM tryptophan treatment. Treatment with 4 mM tryptophan also showed the highest difference between the ratio of pyocyanin and rhamnolipid by RBHi. In *P. aeruginosa* PAO1 (Figure 4.8 B) the difference in ratio between rhamnolipid and pyocyanin was greater, however none of the changes compared to their respective controls were found to be significant with the expectation of increase in pyocyanin with the combination treatments of anthranilate + tryptophan and anthranilate + CDA.

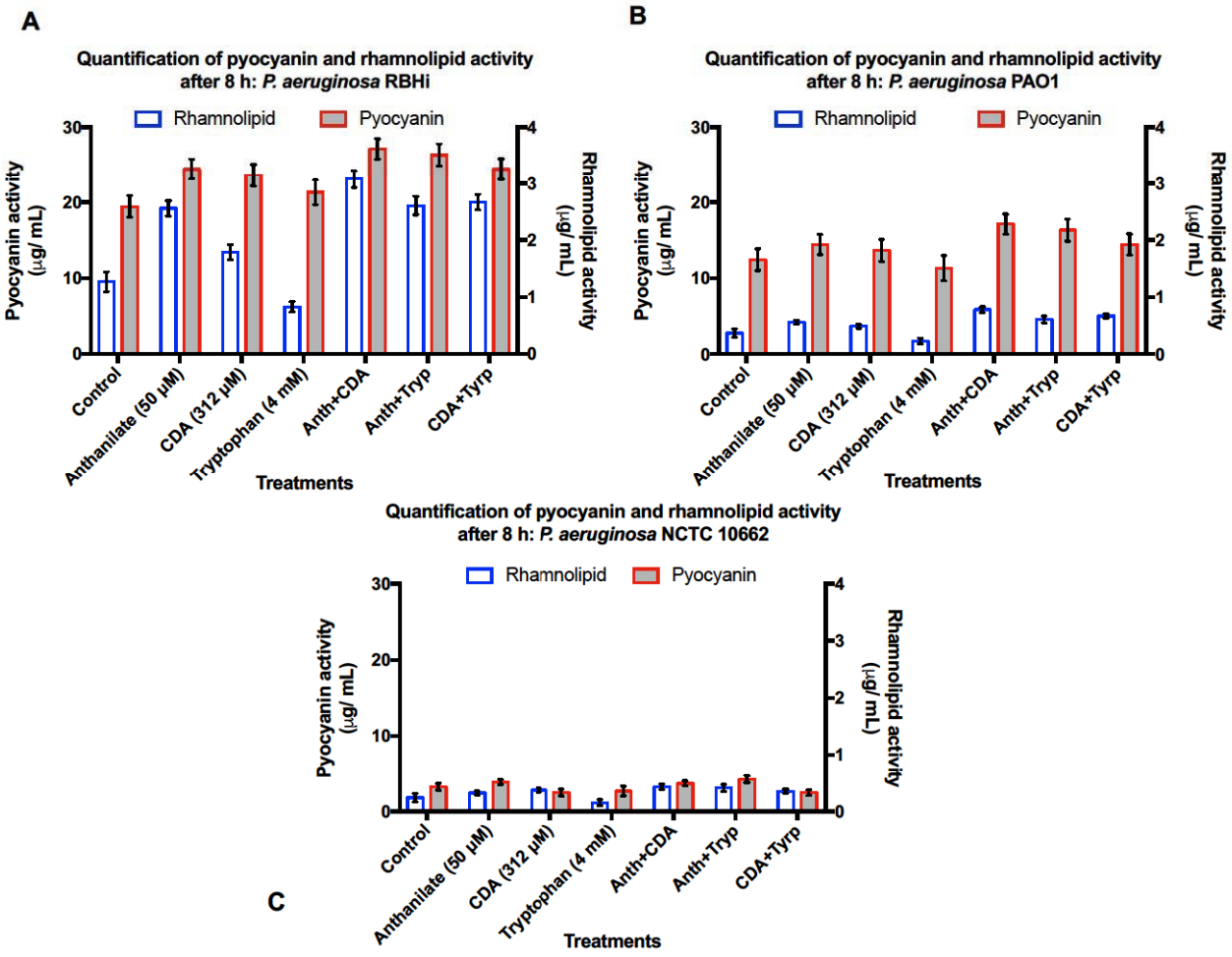


Figure 4.8 Comparison of pyocyanin and rhamnolipid activity in the biofilm formed by the three strains of *P. aeruginosa* after 8 h growth in transmission flow cell with and without treatment using biofilm dispersal agents (n=5)

In the case of *P. aeruginosa* NCTC 10662, the combination treatment with anthranilate + tryptophan showed a significant increase in pyocyanin (p= 0.0084) (Figure 4.8 C) while the increase or decrease in rhamnolipid and pyocyanin with the other treatments was not found to be significant compared to the control.

4.2.2 Influence of dispersal agents on *P. aeruginosa* biofilms architecture in static growth on glass and stainless-steel coupons

Clear microscope glass cover slips (45x15 mm) and 45x15 mm AISI 316L stainless steel coupons were used to study the formation of *P. aeruginosa* biofilms. The surface coupons were extensively cleaned individually with detergent (technical grade), air dried next to a flame and sterilized in an autoclave at 121°C for 15 min. The surface sterilised coupons were then immersed in 95% ethanol and flamed immediately prior to their use.

4.2.2.1 Biofilm formation on glass and stainless-steel surfaces

The results showing the adhesion intensities of *the P. aeruginosa* biofilms on both experimental surfaces; glass and stainless steel, over a time span of 72 h are depicted in figures 4.9 – 4.11. The extent of biofilm formation on the glass surfaces was statistically different from that formed on stainless steel surfaces ($P < 0.01$). Most notably, biofilm formation on stainless-steel was significantly higher than on glass. Statistical analysis of data also verified that optical densities at different time points were significantly different among the two experimental surfaces, highlighting the effect of time on adhering intensities of biofilms, to their respective supporting surfaces ($P < 0.01$). Interestingly, biofilms formed on both glass and stainless steel after 48 h, saw a drop with the combination treatments. The formation of biofilms became increasingly more apparent after 48 h in comparison to the controls, on glass.

Based on the CV assay, ODs for both glass and stainless steel increased at each time point, 24 h, 48 h and 72 h. The highest biofilm formation for both surfaces were observed after an incubation period of 48 h with the exception of the heavily mucoid RBHi strain on stainless-steel, at which the OD for stainless steel was almost four times that of glass. After 72 h, analysis indicated a drop in OD for both surfaces.

Analysis based on two-way ANOVA, treatment with 50 μM anthranilate ($p= 0.0001$) yielded the highest inhibition of *P. aeruginosa* NCTC 10662 biofilm, common to all the time points when grown on glass as a surface (figure 4.9). Multiple comparison test showed that along with anthranilate, CDA was equally effective ($p= 0.0071$) in reducing biofilm formation. Comparing individual treatments against each other, it was found that biofilm inhibition by anthranilate was found to be significant in comparison to tryptophan at 24 h and 48 h only ($p= 0.0349$ and 0.0219 respectively). Although all treatments showed a reduction in biofilm formation after 72 h, comparing individual treatments against each other was not found to be significant.

Biofilm biomass was found to have substantially increased when stainless-steel was used as a surface for biofilm growth when compared to glass. All treatments, when compared to their respective controls showed a significant decrease in biofilm formation ($p= 0.0001$). Comparing individual treatments against each other revealed that biofilm inhibition by tryptophan and anthranilate was not significant at 24 h ($p= 0.794$) and anthranilate and CDA

at 48 h ($p > 1$). All treatments significantly reduced biofilm at 72 h when compared to the control and against each other ($p = 0.0001$).

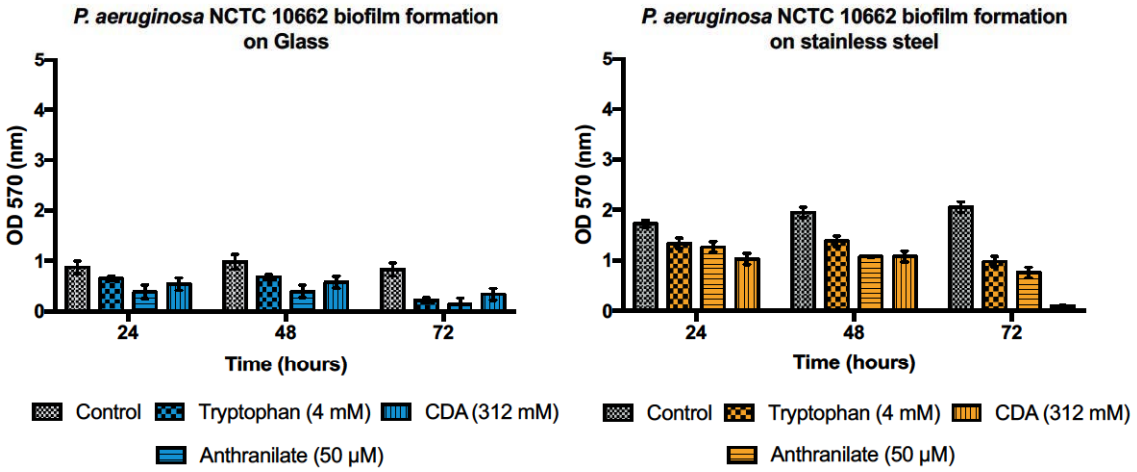


Figure 4.9 *P. aeruginosa* NCTC 10662 biofilm formation on glass and stainless-steel surfaces after 24 h, 48 h and 72 h incubation at 37°C (n=9)

In the case of *P. aeruginosa* PAO1 biofilm formation on glass, only the treatment with CDA was found to have significantly reduced biofilm formation at 24 h ($p = 0.038$) while the others were not found to be significant (figure 4.10). However, all the individual treatments showed significant reduction of biofilm formation at 48 h and 72 h ($p = 0.0001$). When comparing individual treatments, anthranilate showed a greater decrease in biofilm formation compared to tryptophan and was found to be significant ($p = 0.021$).

Once again, a greater biofilm biomass was quantified with the use of stainless-steel as a surface. Though a higher biofilm biomass was observed, all the treatments showed a reduced biofilm formation compared to their respective untreated controls. Anthranilate was found to have the highest inhibitory effect at 24 h, while CDA was more effective at 72 h compared to the other treatments. This followed a trend where CDA is more effective in dispersing 72 h biofilm on stainless-steel surfaces.

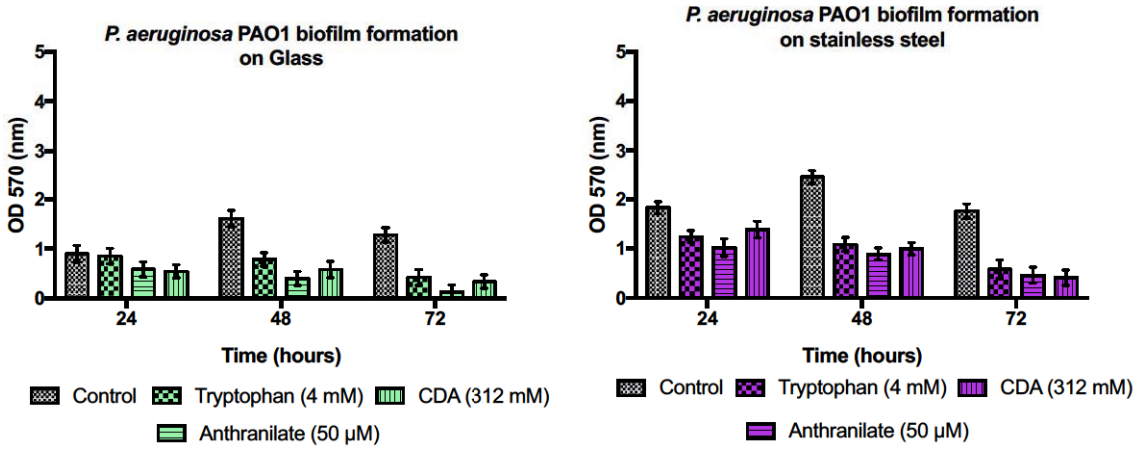


Figure 4.10 *P. aeruginosa* PAO1 biofilm formation on glass and stainless-steel surfaces after 24 h, 48 h and 72 h incubation at 37°C (n=9)

On glass, *P. aeruginosa* RBHi produced the lowest biofilm biomass at all time points. Individual treatments were found to be highly effective in inhibiting biofilm formation on glass (p= 0.0001). Counterintuitively, RBHi also produced the highest biofilm biomass on stainless-steel surface, which was nearly twice of that of NCTC 10662 and PAO1. All individual treatments significantly (p= 0.0001) reduced biofilm formation on stainless-steel by RBHi and anthranilate was found to be the most effective at 72 h. RBHi did not follow the trend which regularly saw significant biofilm reduction at 72 h by CDA.

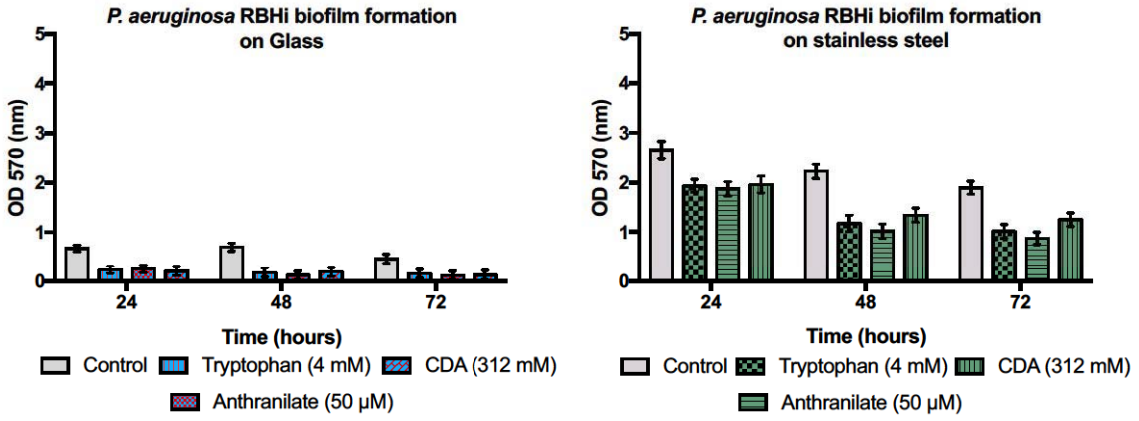


Figure 4.11 *P. aeruginosa* RBHi biofilm formation on glass and stainless-steel surfaces after 24 h, 48 h and 72 h incubation at 37°C (n=9)

Comparison of sessile cells in biofilms grown on glass and stainless-steel (figure 4.12) generally showed that stainless-steel favoured attachment of bacterial cells compared to glass. The heavily mucoid strain, *P. aeruginosa* RBHi showed a greater number of sessile cells in the presence of stainless steel (figure 4.12 F) compared to its growth on glass (figure 4.12 E). RBHi also showed the least sessile cells when grown on glass compared to the other two *P. aeruginosa* strains.

While the sessile cell count decreased over time, untreated PAO1 showed an increase after 48 h and then a decrease (figure 4.12 D). This was not the case with any other surface and treatments for PAO1 and the other strains of *P. aeruginosa*.

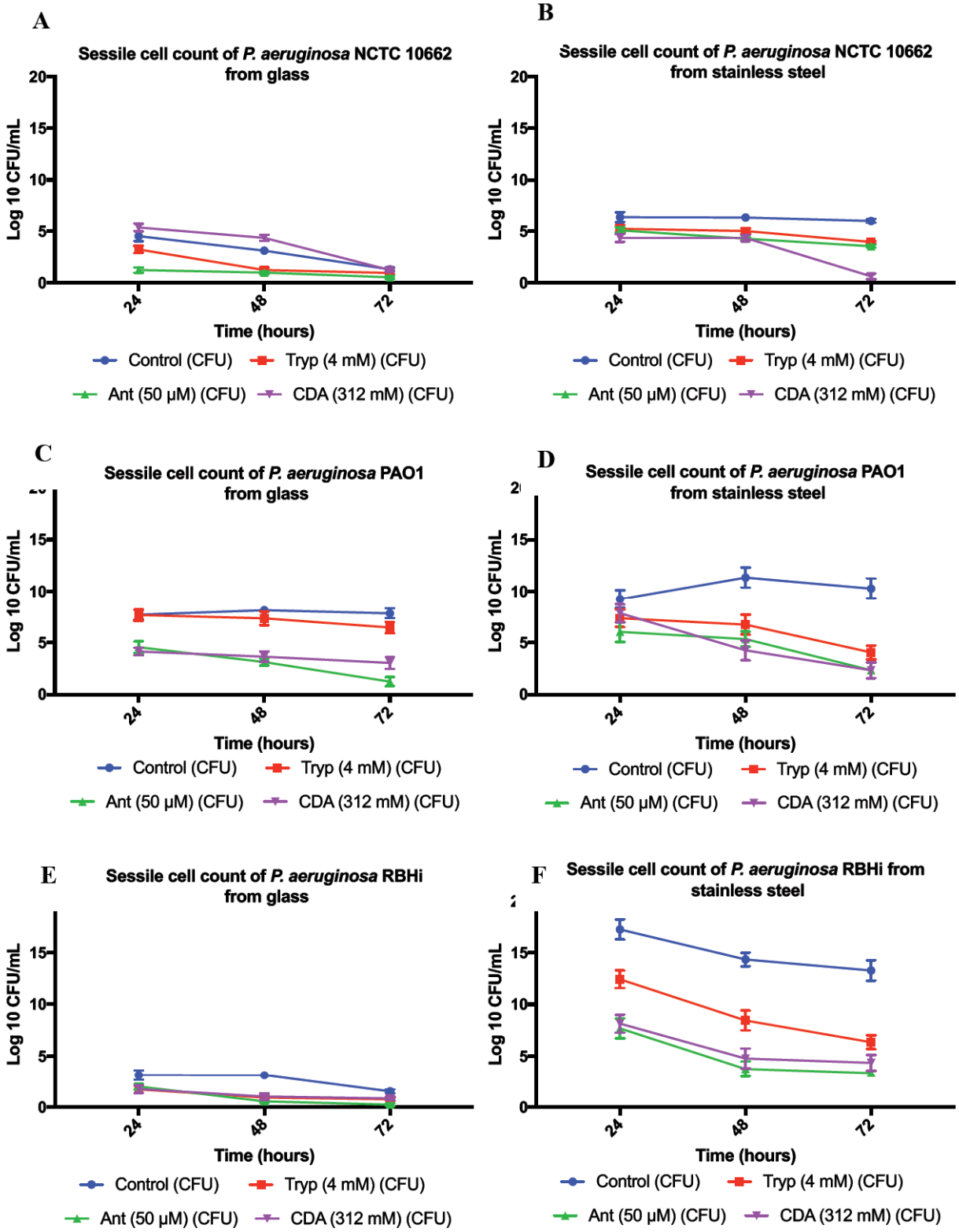


Figure 4.12 Sessile cell enumeration of biofilm formed by *P. aeruginosa* with and without the use of biofilm dispersal agents (n=3)

4.3 Discussion

In this chapter, the process of biofilm formation and architecture by *P. aeruginosa* was investigated under static and dynamic conditions on medically and environmentally relevant surfaces.

An important factor that needs to be established is how different materials act as distinct surfaces for the bacteria to attach onto and build a biofilm upon. In this context, the impact of the properties exhibited by different materials to enhance or diminish biofilm development needs to be considered. Previous studies have delved into the forces that influence the attachment of bacteria to surfaces and how the composition of the material impacts the said forces (Rodrigues, 2011); however, there are very few studies conducted on how to eradicate/ retard/ disperse biofilm development by studying the architecture of biofilms. To this end, this study investigated the development of biofilm from *P. aeruginosa* displaying non-mucoid, mucoid and heavily mucoid phenotypes. The characteristics of biofilm formed by these three strains differs primarily in the production of alginate as an exopolysaccharide. Along with alginate, other polysaccharides such as pel and psl play a role in development of microcolonies upon attachment and biofilm maturation. However, the role of psl is vital towards transitioning from reversible attachment of cells to irreversible attachment (Ghafoor *et al*., 2013). Similarly, protein and eDNA play an essential role in conditioning the surface for bacterial attachment and the structural integrity of the biofilm, once developed (Bogino *et al*., 2013; Sugimoto *et al*., 2018).

While selecting different strains of *P. aeruginosa* and investigating biofilm formation and inhibition, one must keep in mind that in the case of *P. aeruginosa* PAO1, originally obtained as a clinical isolate, it has been passaged numerous times since its initial isolation. A study conducted by Fux *et al*., in 2005 showed that laboratory strains of bacteria generally do not represent the same strain originally isolated. The study also shows that there is a genetic divergence with respect to virulence factors and biofilm formation when repeatedly grown under *in vitro* laboratory conditions. Hence, three phenotypically different strains were selected in this study to assess the impact of biofilm formation, inhibition and dispersal.

Continuing from chapter 3, tryptophan was selected to induce biofilm dispersion along with anthranilate and CDA. They were used as individual treatments as well as in combination. The relevance of selecting these three chemicals to investigate their effect lies in the fact that they play distinct roles in various aspects of bacterial communication (refer to 4.2.1.1). while tryptophan and anthranilate contribute towards the PQS signalling pathway, CDA plays a

role in interkingdom signalling. The commonality between tryptophan, anthranilate and CDA is that they function by modulating motility, enhancing metabolic activity as well as virulence of *P. aeruginosa* (Vital-Lopez, Reifman and Wallqvist, 2015), hence they would aid in understanding *P. aeruginosa* biofilm architecture by means of biofilm dispersal.

The flow-rate under dynamic biofilm development conditions was set to 100 µL/ min considering the total volume of the flow-cell chamber to be 1527.5 mm^3. Over-night flow would then see a complete turnover of the growth medium within the flow cell chamber in ~15.3 h. This would then prevent the build-up of intrinsic biofilm dispersal ability of the bacteria. The flow-rate was then changed to 150 µL/ min with M9 minimal medium containing the dispersal agents as this would aid in investigating the effect of dispersal agents on biofilm eradication over time upon gradual saturation of the growth environment to induce dispersal. As the source of the medium for the control and the treatment in the flow chambers (control and treatment) originated from the same peristaltic pump and sample feed line, the rate of flow of medium was constant through the inlet ports. As the exit ports for the two chambers are independent, recording the volume (data not shown) of eluent collected from the two chambers at each sampling point showed that there was no discrepancy between the two flow cell chambers for biofilm growth. The construction of the flow-cell which included silicone gaskets prevented any leaks from the chambers.

Bacterial cell enumeration of planktonic cells collected over time showed that after 2 h, tryptophan effected the highest recovery of planktonic cells from pre-formed biofilm of *P. aeruginosa* NCTC 10662 compared to the control by a measure of ~4 log counts. As biofilms generally contain a large number of non-motile cells, the increase in CFU after 2 h shows that tryptophan does induce bacterial motility to aid in biofilm disassembly. In the case of *P. aeruginosa* PAO1, the highest planktonic cell yield was obtained with the use of tryptophan and anthranilate as individual treatments in comparison with the control as shown in figure 4.3. However, combination treatment involving anthranilate + CDA and tryptophan + CDA showed a lower planktonic cell yield compared to the control. The use of anthranilate yielded the highest concentration of planktonic cells with *P. aeruginosa* RBHi after a period of 2 h. Combination treatment with anthranilate + tryptophan yielded the lowest compared to the control.

A gradual increase in planktonic cell yield was seen over time for *P. aeruginosa* NCTC 10662 and all treatments yielded a higher planktonic cell count at each time point. This was

not the case with *P. aeruginosa* PAO1 and *P. aeruginosa* RBHi as planktonic cell count dropped below that of the control after a period of 6 h and 4 h respectively. At 8 h, treatment with CDA and combination treatment with anthranilate and tryptophan yielded a lower planktonic cell count compared to the control with *P. aeruginosa* PAO1. With the exception of anthranilate, all treatments yielded a lower planktonic cell yield with *P. aeruginosa* RBHi compared to the control.

Analysing the sessile cell count of *P. aeruginosa* NCTC 10662 at 8 h revealed that biofilm treated with anthranilate had the lowest sessile cell CFU (2 log reduction) compared to the control while all other treatments yielded a log decrease in sessile cell count compared to the control. While in the case of *P. aeruginosa* PAO1, treatment with CDA yielded the lowest planktonic cell count at 8 h compared to the control (~3 log reduction), however, sessile cell count showed that it was similar to the control. The lowest sessile cell CFU was seen with the combination treatment of anthranilate and tryptophan compared to the control as well as the individual treatments. Sessile cell yield of *P. aeruginosa* RBHi showed an increase with all the treatments (~ log increase) with the exception of biofilm treated with anthranilate and combination of anthranilate and CDA, which reduced the sessile cell yield from the biofilm. Overall, biofilms formed by *P. aeruginosa* RBHi yielded more sessile cells compared to the other two strains while *P. aeruginosa* PAO1 yielded more planktonic cells.

Comparing the composition of the biofilm of the three strains, it is evident that the total carbohydrates and alginate produced by *P. aeruginosa* RBHi was greater compared to *P. aeruginosa* PAO1 and NCTC 10662. The use of anthranilate individually or in combination with CDA showed the greatest decrease in total carbohydrate content in *P. aeruginosa* NCTC 10662, PAO1 and RBHi respectively. While anthranilate significantly reduced alginate content in RBHi, the combination treatment involving CDA and tryptophan showed the greatest alginate decrease in PAO1.

Protein content of the biofilms formed by the three strains showed a reduction in total protein content with all the treatments. Combination treatment with CDA and tryptophan was found to be highly effective against reducing total protein content of *P. aeruginosa* NCTC 10662, however, the same combination treatment and tryptophan individually, saw an increase in eDNA content of the biofilm when compared to the control. While a combination of anthranilate and CDA was found to be effective in reducing protein content of the biofilm formed by PAO1, use of tryptophan individually and in combination saw an increase in

eDNA content of the biofilms. Individual treatment involving tryptophan was found to be the most effective against the heavily mucoid strain RBHi in reducing protein content while it was also responsible for an increase in eDNA content of the biofilm compared to the control.

Previous studies conducted have shown the importance of eDNA towards the architecture of biofilms whereby they stabilise cell-to-cell connection and cell to EPS connection (Flemming, Neu and Wozniak, 2007). This is particularly evident in the case of mature biofilm formed by *P. aeruginosa* (Whitchurch *et al*., 2002) when there is a greater abundance of eDNA. eDNA found within the biofilm is generally formed by lysis of bacterial cells which is mediated by the PQS QS system (Allesen-Holm *et al*., 2006). Use of tryptophan individually or in combination could lead to PQS QS mediated lysis of the bacterial subpopulation which could explain the increase in eDNA content of the biofilms as tryptophan is the primary catabolite in the PQS synthesis pathway. Tryptophan, while increasing bacterial motility by increasing flagellar activity (Karatan and Watnick., 2009) may also aid in programmed lysis of a subpopulation of bacterial cells within the biofilm.

P. aeruginosa metabolises tryptophan to anthranilate through the kynurenine pathway (Kurnasov *et al*., 2003) which then becomes a precursor for PQS synthesis and anthranilate needs to be degraded via the TCA cycle for biofilm formation (Costaglioli *et al*., 2011). Hence the exogenous addition of anthranilate (individual or in combination) reduces *P. aeruginosa* biofilm development which is represented by a mean reduction in total carbohydrates, alginate, eDNA and protein content of *P. aeruginosa* biofilms. Anthranilate also has been reported to induce biofilm dispersal by reducing intracellular c-di-GMP levels and modulating the genes involved in EPS production (Kim, Park and Lee., 2015).
c-di-GMP has been reported as a critical component of modulating planktonic and sessile mode of life in *P. aeruginosa*, where increase in intracellular c-di-GMP facilitates sessile mode of life and biofilm formation and decrease in c-di-GMP induces a planktonic mode of life and biofilm dispersal (Kim *et al*., 2018).

Analysis of pyocyanin and rhamnolipid showed that the heavily mucoid strain *P. aeruginosa* RBHi generally produced the highest quantity of pyocyanin and rhamnolipid. While rhamnolipid production in PAO1 and NCTC 10662 were similar. *P. aeruginosa* PAO1, however, did produce significantly larger quantities of pyocyanin compared to NCTC 10662. In *P. aeruginosa* RBHI, treatment with tryptophan saw a reduction in rhamnolipid

production compared to the control while all other treatments showed an increase in rhamnolipid. Pyocyanin content increased greatly in the presence of all the treatments compared to the control. This was the case with PAO1 and NCTC 10662 with the exception of treatment with tryptophan in PAO1 and treatment with CDA and combination treatment with CDA and tryptophan in NCTC 10662.

Pyocyanin production is mediated by the PQS QS system of *P. aeruginosa* which in turn is activated as part of a cascade event involving the *Las* and *Rhl* (AHL mediated) QS systems (Lee and Zhang, 2015). Studies conducted by Pricewhelan, Dietrich and Newman in 2006 have shown that pyocyanin produced by *P. aeruginosa* interacts with molecular oxygen to form reactive oxygen species (ROS) like H_2O_2 which changes the redox balance of host cells inducing cell death. Similarly, a study conducted by Das and Manefield in 2012 showed that H_2O_2 produced by *P. aeruginosa* as a result of pyocyanin coincided with bacterial cell lysis and increase in eDNA release in static planktonic growth of *P. aeruginosa* PAO1 during the late exponential phase of growth. This study shows that the increase in pyocyanin production coincides with increase in eDNA content of *P. aeruginosa* biofilm under dynamic conditions in a flow cell.

The PQS system that mediates the production of pyocyanin is also involved in stress response in *P. aeruginosa* (Haussler and Becker, 2008) during the late logarithmic phase of bacterial growth. Studies have shown that rhamnolipid production is increased under stress conditions (Reis *et al.*, 2011). The stress conditions caused due to production of ROS during the interaction of pyocyanin with molecular oxygen may lead to increased production of rhamnolipid in *P. aeruginosa* biofilms. Though the production of rhamnolipid is governed by the *Rhl* QS circuit in *P. aeruginosa*, studies have shown that due to complexity of QS hierarchy in *P. aeruginosa* QS, the *Rhl* QS system is directly activated by PQS and the biosynthesis of PQS which is driven by *pqsABCDE* operon is activated by PqsR regulator which in turn is activated by the *Las* QS system and repressed by the *Rhl* QS system. This indicated the increased levels of rhamnolipid seen in *P. aeruginosa* biofilm with the treatments compared to the respective controls. The results also show that even though tryptophan and anthranilate are part of the same biosynthetic pathway towards the production of the PQS signalling molecule in *P. aeruginosa*, exogenous addition of anthranilate is evidently more efficient in inducing PQS mediated biofilm dispersal compared to tryptophan.

Results of this study indicated that biofilm forming ability of *P. aeruginosa* was significantly different among the surfaces investigated. The potential of cell adhesion to a surface is attributable to the influence of various surface characteristics such as chemical composition of the material, roughness, physical configuration, wettability and hydrophilicity or hydrophobicity (Katsikogianni and Missirlis, 2004) (Van-Houdt and Michiels, 2010).

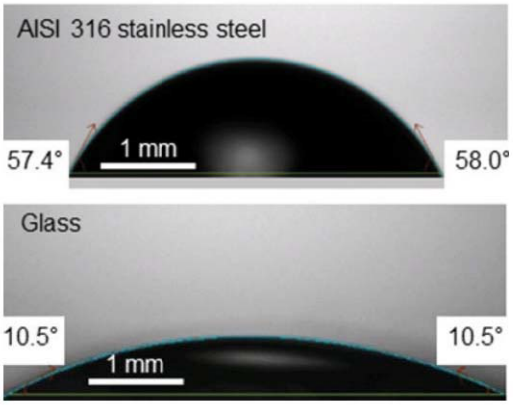


Figure 4.13 Water drop shape and contact angle measurement of AISI 316 stainless steel and glass (Estrada-Martinez, 2017) suggest that neither of the surfaces are hydrophobic in nature as the contact angle is <90º

Nevertheless, biofilm formation was seen on glass as well as stainless steel surfaces. The highest level of biofilm formation was observed after a period of 48 h for all the three strains of *P. aeruginosa* on glass with the exception of biofilms of *P. aeruginosa* NCTC 10662 grown on stainless steel, which showed an increase in biomass at 72 h. Though an increase in biofilm biomass was seen with NCTC 10662 at 72 h, a slight decrease in sessile cells was observed. Therefore, sessile cell count was not found to be indicative of the biofilm biomass when grown on stainless steel. However, a drop in sessile cell count was seen at the same time-point when biofilm was grown on glass.

In static biofilm development of *P. aeruginosa* NCTC 10662 on glass, anthranilate was found to be very effective in reducing biofilm biomass at each of the given time-points when compared to the control. This trend was observed with the mucoid strains of *P. aeruginosa* as well. A significant drop in sessile cell count was seen over time with PAO1, NCTC 10662 and RBHi compared to the control with the use of anthranilate. Of the three strains, PAO1 often showed the highest sessile cell count when grown on glass and the count remained stable throughout the period of 72 h as the decrease in CFU in the control was not found to be significant. Total biofilm biomass of *P. aeruginosa* RBHi on glass was considerably lower than the other two strains while the highest biomass reduction by dispersal agents was also seen with RBHi biofilm on glass when compared with their respective controls.

In comparison with glass, stainless steel generally showed higher biofilm formation. While RBHi showed the lowest biofilm biomass on glass, it showed the highest biofilm biomass on average on stainless steel. Reduction in biofilm formation was seen with all the treatment at all time points with RBHi. There was a decrease in control biofilm biomass over time. After 24 h, all the treatments had reduced biofilm formation, but none were found to be significant when compared against each other. However, Anthranilate and tryptophan were found to be more effective against RBHi at 48 h and 72 h. The untreated biofilm saw an increase in biomass between 24 h to 48 h with PAO1 and then a decrease at 72 h. However, the treatments saw a regular decrease in biofilm biomass over time. This coincided with a drop in sessile cell count for the treatments, while the sessile cell count was representative of biofilm formed in respect to the controls. Anthranilate and CDA yielded the lowest sessile cell count in PAO1 which was the case with RBHi as well. In the case of NCTC 10662, the control growth saw an increase in biofilm biomass over time. However, the use of CDA nearly inhibited biofilm formation completely at 72 h compared to the control. This also coincided with a significant decrease in sessile cell count at 72 h. By this, it possible to postulate that the biofilm dispersal feature of CDA is dependent on alginate content of the biofilm and time. As CDA consistently showed the lowest cell count at 48 h and 72 h with the exception of anthranilate treatment on RBHi when grown on stainless steel. On glass, treatment with CDA consistently showed the lowest sessile cell count at all sampling times against all the strains of *P aeruginosa*.

Although biofilm formation is regulated by a myriad of cofactors, active manipulation of surface material properties could potentially prove to be a better alternative than antimicrobial treatment to reduce adhesion and prevent biofilm formation. Current research has been focusing on production of new classes of materials that are essentially resistant to bacterial attachment of organisms such as *P. aeruginosa*, and *S. aureus* (Hook, *et al*., 2013). However, the discovery of new materials resistant to bacterial attachment is limited by the current poor understanding of bacterial response to materials, for little has been done on the subject. A better understanding of the mechanisms controlling biofilm formation on abiotic surfaces is necessary and should be investigated further.

Chapter 5 The effect of QQ on biofilm formation and virulence factors production by *P. aeruginosa*

5.1 Introduction

It is well known that *P. aeruginosa* produces numerous virulence factors that aid the bacteria in colonisation during an infection. Virulence factors such as mucoid exopolysaccharide production, rhamnolipid, haemolysin, protease, lipopolysaccharides, pili, exoenzyme S and lipase are common tools used by *P. aeruginosa* to invade, adhere and colonise the host. As *P. aeruginosa* carries an arsenal of virulence factors rather than only one (albeit very strong), the presence of numerous virulence factors, therefore makes it a potent pathogen (Schaber *et al*., 2004; Schroeder, Brooks and Brooks, 2017).

As many of the processes involving virulence are modulated by the hierarchical QS circuits documented in *P. aeruginosa* and these traits are shown to be involved in the pathogenicity of the bacteria (Lee and Zhang, 2006), it has been suggested that targeting the QS system of *P. aeruginosa*, thereby interrupting bacterial communication instead of killing the bacteria is an antipathogenic effect and may aid in the fight against biofilm forming pathogens and antibiotic resistance (Barlow and Nathwani, 2005).

The QS system documented in Gram-negative bacteria (*P. aeruginosa*) has three components: 1) Inducer protein that synthesises the QS molecule, 2) the QS signal molecule and 3) the cognate receptor, when bound to the QS molecule induces QS mediated gene expression and regulation (Williams, 2007). This provides three QQ targets to be investigated.

Conventional *P. aeruginosa* antibiotics such as ceftazidime, ciprofloxacin and azithromycin display QQ activity (Skindersoe *et al*., 2008; Swatton *et al*., 2016). However, even though the mechanism is not fully understood, it has been speculated that their QQ activity involves inhibiting bacterial protein synthesis which prevents the expression of the inducer protein from synthesising the QS signal molecules (Reuter, Steinbach and Helms., 2016). Azithromycin is also known to inhibit alginate production in mucoid strains of *P. aeruginosa* (Imperi, Leoni and Visca, 2014). The use of the said antibiotics has been known to induce resistance amongst bacteria, hence they cannot be used as potential QQs.

As AHLs represent the primary QS signal molecules in *P. aeruginosa*, utilising small molecules that mimic AHLs to inhibit QS is a promising strategy. Furanones were first identified as QQs that inhibit by mimicking AHL molecules by attaching to the LasR receptor of *P. aeruginosa*. This interfered with the binding of the AHL molecule, thereby preventing QS mediated gene regulation in *P. aeruginosa* (Manner and Fallarero., 2018). Since the discovery of the role of furanone in inhibition of the QS system, numerous synthetically produced, structurally diverse furanone derivatives have been synthesised (Irie *et al*., 2017). Similarly, an AHL analogue, meta-bromo-thiolactone, competitively inhibits QS in *P. aeruginosa* and prevents biofilm formation and pyocyanin production and protects lung cells against the antagonistic activity of *P. aeruginosa* (O'Loughlin *et al*., 2013).

The most frequently investigated method of QQ is that of degrading the QS signalling molecule (Zhang and Li., 2016). Enzymatic degradation strategy makes use of acylases, oxidoreductases and lactonases to cleave the AHL molecules rendering them inert and incapable of forming a complex with the receptor protein to induce a QS regulated response. Mechanism of action of the three common AHL degrading enzymes is as follows; acylase cleaves the amide bond of AHL irreversibly, which links the acyl chain to the homoserine lactone ring (Roche *et al*., 2004). Alternatively, oxidoreductases catalyse the oxidation or reduction of the acyl side chain thereby modifying the chemical structure of the AHL molecule but do not degrade the AHL molecule (LaSarre and Federle, 2013). A cytochrome derived from *Bacillus megaterium* is known to display similar functions, although further investigation is required on how cytochromes affect AHL activity (Brill *et al*., 2014). Finally, the best studied enzyme is the lactonase which has shown more promise as a potential enzymatic QQ compared to acylase and oxidoreductase. It functions by hydrolysing the lactone ring of the AHL molecule and makes it incapable of binding to its transcriptional regulator making the AHL molecule ineffective (Garge and Nerukar, 2016). Though the mechanism of action of lactonase is reversible by acidification, it is the most studied enzyme as its widely conserved in a range of bacterial species and has variable substrate spectra, unlike acylase and oxidoreductase (Chen *et al*., 2013).

In addition of the mode of action of AHL specific enzymes against QS mediated biofilm formation by *P. aeruginosa*, there are other factors that need to be considered while investigating biofilm inhibition. These include the use of chemicals or molecules that prevent QS signalling and induce dispersal of a pre-formed *P. aeruginosa* biofilms as combating biofilm formation should also consider a scenario of disrupting a pre-formed

biofilm as a therapeutic strategy. While there are numerous environmental and physiological cues for dispersal of biofilm, chemicals produced by microorganism themselves can be adopted into a strategy to induce biofilm dispersal and inhibit QS in *P. aeruginosa*. *Candida albicans* produces two QS molecules, namely farnesol and tyrosol (Kaplan, 2010). Farnesol was shown to inhibit the morphological shift from yeast to hyphae at high cell densities while tyrosol was shown to accelerate the transition from yeast to hyphae in *C. albicans* (Decanis *et al*., 2011). As *P. aeruginosa* and *C. albicans* are known to coexist in numerous nosocomial opportunistic infections (Mear *et al*., 2013), the influence of farnesol and tyrosol as potential QQs may be investigated against *P. aeruginosa* biofilm formation and subsequent virulence production between the non-mucoid and mucoid phenotypes along with other QQs and biofilm dispersal agents.

5.1 Results

5.1.1 Establishing the inhibitory effect of *B. licheniformis* crude cell extract on biofilm formation by *P. aeruginosa*

The crude cell extract from *B. licheniformis* was filter sterilised with a 0.2 µm pore size filter and 100 µL of the extract was spread on NA plates and incubated overnight to ensure no cells were present in the filtrate. The filtrate was then freeze-dried to obtain a pellet which was resuspended in 1x PBS at a concentration of ~500 µg/ mL (stock solution) which was then used for biofilm inhibition assays. Furanone (10 µM) was used as a positive control for *P. aeruginosa* biofilm inhibition.

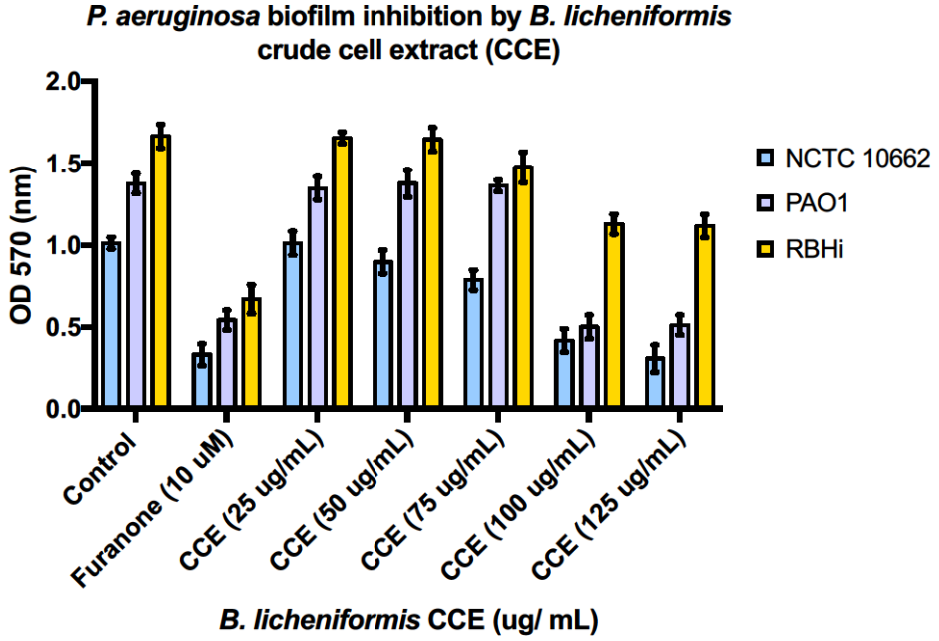


Figure 5.1 Effect of *B. licheniformis* crude cell extract on biofilm formation by *P. aeruginosa* compared against untreated control and 10 µM furanone as positive control (n=5)

B. licheniformis culture extracts were tested for their effect on *P. aeruginosa* biofilm formation at various concentrations (figure 5.1). A significant (p= 0.003) reduction in biofilm formation was observed with 100 µg/mL of *B. licheniformis* CCE, which was found to be common for all the strains of *P. aeruginosa*. Though the inhibition of biofilm formation by all concentration of *B. licheniformis* CCE was not found to be significant in comparison to furanone at 10 µM. The inoculation phase was prepared with dilutions of the culture extracts with the inoculum and medium containing 0.5 McFarland standard of bacterial cells. The concentration of 100 µg/mL of *B. licheniformis* CCE was used for further studies.

5.1.2 Inhibition of *P. aeruginosa* biofilm formation by synergistic activity of combination treatment by potential QQ agents

The synergistic activity of combination treatment involving Farnesol, tyrosol, *B. licheniformis* CCE and tryptophan was investigated against biofilm formation by *P. aeruginosa*. Previously established MIC of farnesol, tyrosol (appendix B) and tryptophan (Chapter 3) were used in this study along with 100 µg/mL of *B. licheniformis* CCE which showed inhibitory effect against all three strains of *P. aeruginosa*. As established previously, farnesol and tyrosol, both QS compound of *C. albicans* do show a significant (p = 0.0001) level of inhibition against biofilm formation by *P. aeruginosa*. Hence, they were selected to be used in combination along with tryptophan and *B. licheniformis* CCE.

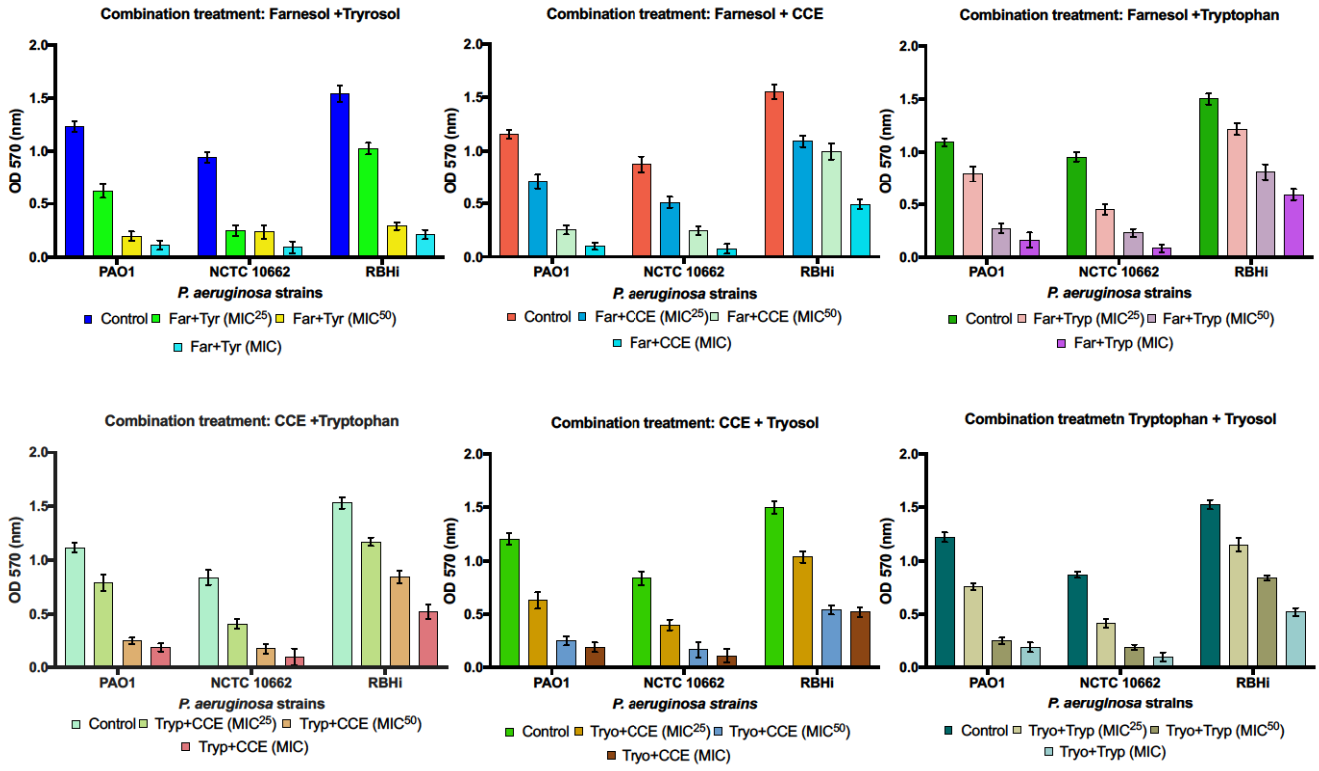


Figure 5.2 Combination treatments showing inhibition of *P. aeruginosa* biofilm formation (MIC, ½ MIC and ¼ MIC concentrations used in synergy) (n=3)

Synergistic effect of combination treatment showed that all the treatments were effective in reducing biofilm formation by *P. aeruginosa* and inhibition of biofilm by all the treatments was found to be significant when compared to the control (p= 0.0001). The combination of farnesol and tyrosol (1/2MIC) was found to be the most effective treatment amongst all the strains of *P. aeruginosa*. Along with the combination of farnesol and tyrosol, *B. licheniformis* CCE and tyrosol combination (1/2MIC) proved effective against the heavily mucoid CF isolate, RBHi. Adhered bacterial cells separated from the biofilms formed with

the two most effective combination treatment were enumerated along with analysing their hydrophobicity. The EPS that was isolated from the biofilms formed with and without the treatment was quantified to analyse the composition.

Table 5.1 EPS composition of *P. aeruginosa* biofilms with and without treatment (n=3)

P. aeruginosa	**NCTC 10662**			**PAO1**			**RBHi**		
	Control	**Far + Tyr ($^{1/2}$MIC)**	**CCE + Tyr ($^{1/2}$MIC)**	**Control**	**Far + Tyr ($^{1/2}$MIC)**	**CCE + Tyr ($^{1/2}$MIC)**	**Control**	**Far + Tyr ($^{1/2}$MIC)**	**CCE + Tyr ($^{1/2}$MIC)**
carbohydrate (µg)	232.1	143.2	153.54	919.8	845.23	867.45	1122.7	1034.5	1087.6
Alginate (µg)	0.067	0.04	0.05	498.2	423.53	465.33	856.1	812.34	823.45
Protein (µg)	13.6	7.34	8.67	20.6	14.65	17.65	49.8	37.34	39.23
eDNA (µg)	1.04	0.98	1.02	2.45	1.078	1.98	4.87	3.78	3.98

As depicted in table 5.1, the most effective treatment was found to be the combination of farnesol and tyrosol which showed a total reduction of ~40% of the carbohydrates (figure 5.3 A) compared to the control, while a ~46% reduction in protein content (figure 5.3 C) and ~6% reduction in eDNA content was seen with the non-mucoid *P. aeruginosa* NCTC 10662. The mucoid and heavily mucoid strains, PAO1 and RBHi showed a decrease of ~8% in total carbohydrates. However, a ~15% decrease in alginate content was seen with PAO1 while the heavily mucoid RBHi showed a ~5% decrease (figure 5.3 B) in total alginate content. In comparison with NCTC 10662 (~46%), the decrease in protein content for PAO1 and RBHi was found to be ~29% and 25% respectively. The highest decrease in eDNA content of the biofilm was seen in PAO1, ~56% while a ~6% and ~23% decrease was seen with NCTC 10662 and RBHi respectively (figure 5.3 D).

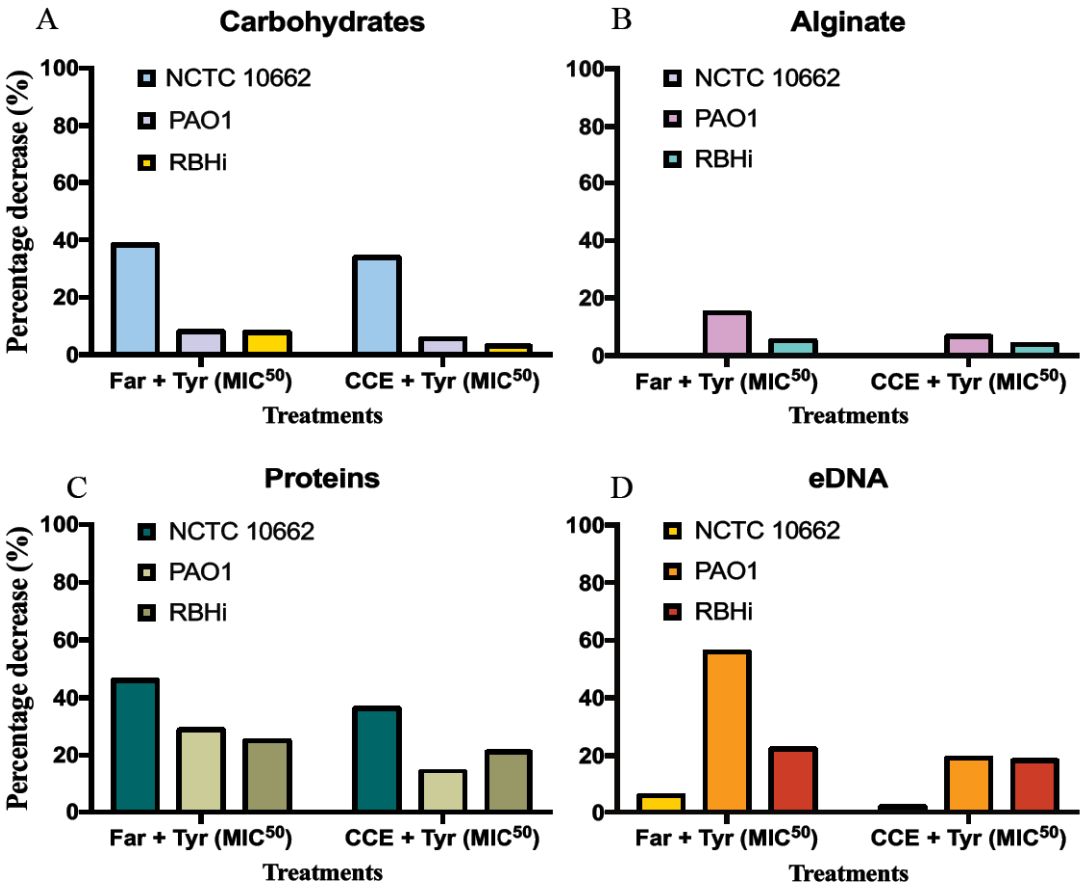


Figure 5.3 Percentage decrease of EPS composition of the non-mucoid, mucoid and heavily mucoid strains of *P. aeruginosa*.

While the combination of CCE and tyrosol induced a significant decrease of the EPS components (figure 5.3), it was found to be quantitatively lower than the overall effect of a combination of farnesol and tyrosol. Also based on figure 5.3 it is clear that the component of the EPS that was decreased significantly as a result of both the combination treatments, amongst all the three strains was the protein content. While PAO1 showed the greatest eDNA decrease amongst all strains and treatment. Between the mucoid strains, decrease of the alginate content of the heavily mucoid strain was less compared to the mucoid strain, while there was a great discrepancy between reduction of eDNA content too, as PAO1 showed a ~56% decrease compared to ~23% from RBHi.

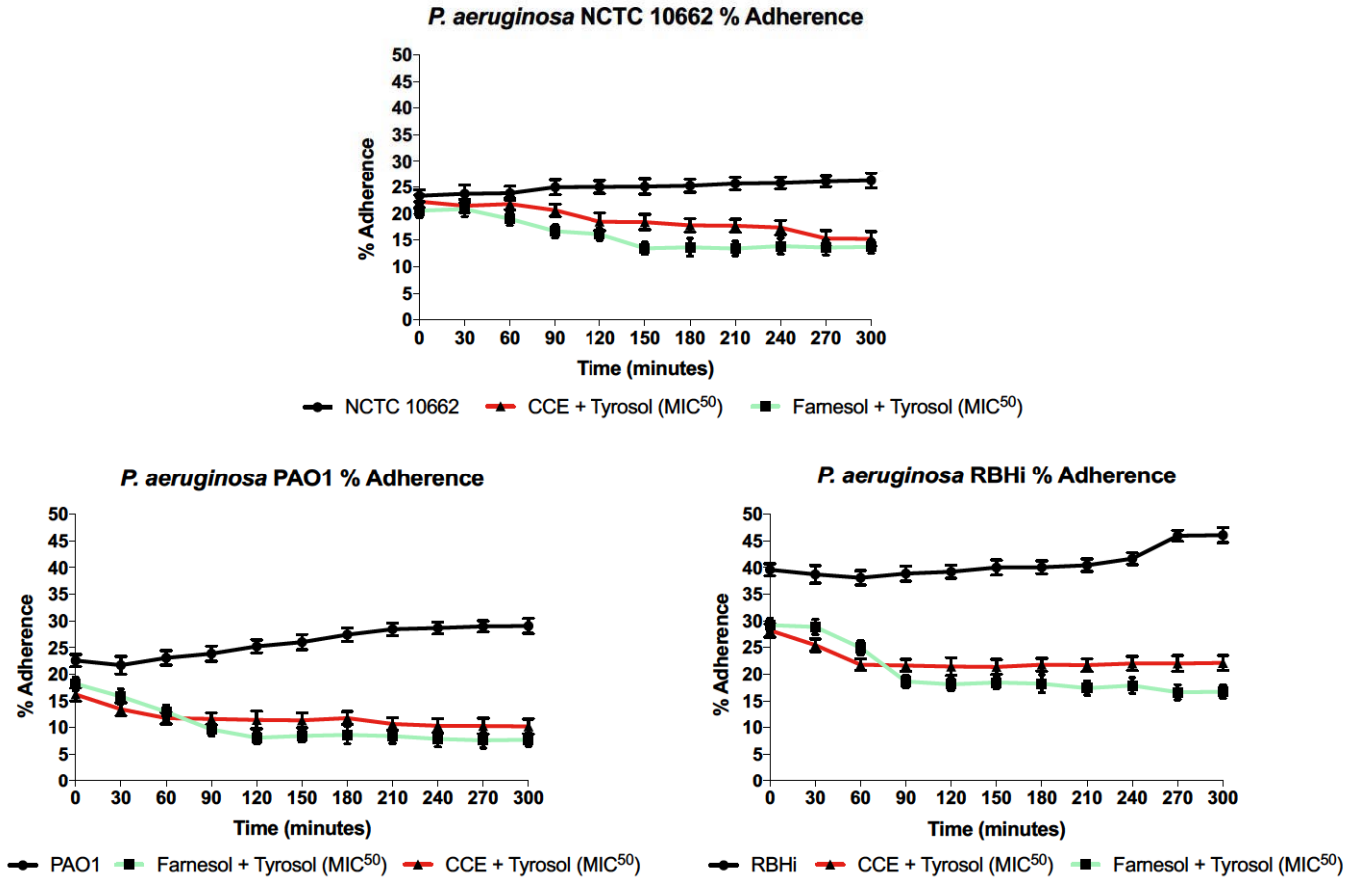


Figure 5.4 Adherence of *P. aeruginosa* cells to n-Hexadecane when grown with and without the presence of QQ. Samples were analysed every 30 minutes for a period of 5 h (n=3)

The MATH assay determines the ability of the bacterial cells to adhere to n-hexadecane, a hydrocarbon. As n-hexadecane has been used frequently in MATH assays compared to other hydrocarbons (eg. p-xylene), it was used in this study as it would allow for better comparison with a greater number of publications. Hydrophobicity of *P. aeruginosa* cells were evaluated based on the adherence cut off values as described by Das and Kapoor., 2004 set as <25%, 25-75%, and >75% which were interpreted as negative, mildly hydrophobic and strongly hydrophobic. A general trend, followed by all the control growth, was a gradual increase in hydrophobicity over time. However, all the strains were found to be mildly hydrophobic and none of the time-points of the assay revealed any cells to be strongly hydrophobic. A significant decrease (p= 0.001) in hydrophobicity (figure 5.4) was seen with both the combination treatments against all the strains and the bacterial cells consistently displayed a greater hydrophilic nature. The increase in hydrophilicity observed during the MATH assay could be specific to planktonic growth as the MATH assay conducted on sessile cells extracted from ~16 h biofilm growth showed a significant increase in hydrophobicity as seen in figure 5.5. However, the highest difference in the rate of decrease in hydrophobicity was seen with *P. aeruginosa* PAO1 with both the combination treatments.

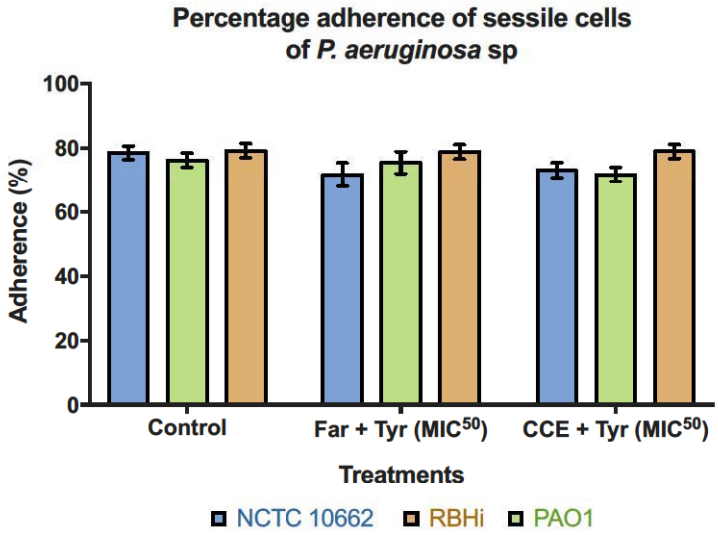


Figure 5.5 Adherence of biofilm sessile cells of *P. aeruginosa* to n-Hexadecane when grown with and without the presence of QQ. Samples were analysed after overnight formation of biofilm (~16 h) (n=5)

Analysing the CFU count of sessile cells extracted from the biofilms formed by *P. aeruginosa* and subsequent treatments showed that the decrease in cell count was not significant (figure 5.6). This is beneficial as utilising QQs to inhibit/ combat biofilm formation should not kill bacterial cells as this may contribute towards development of resistance. In addition to the MATH assay, it is evident that the decrease in hydrophobicity of the planktonic cells over time was inconsequential towards the ability of all the three strains of *P. aeruginosa* to form biofilm. This discrepancy could be due to erroneous measurement of hydrophobicity in planktonic growth as measurements conducted with sessile cells showed a significantly higher percentage of hydrophobicity and adherence and based on the adherence cut off values described by Das and Kapoor, 2004, were found to be strongly hydrophobic.

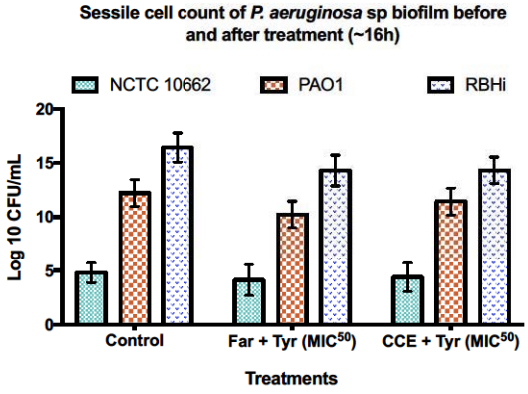


Figure 5.6 Sessile cell enumeration of *P. aeruginosa* cells extracted from biofilms with and without treatments (~16 h) (n=3)

An overall decrease in elastolytic activity was seen with the combination treatments amongst all the three strains (figure 5.7 A). The treatment involving the combination of farnesol and

tyrosol showed the greatest decrease in elastolytic activity involving *P. aeruginosa* RBHi. The difference between the two combination treatments against *P. aeruginosa* RBHi with respect to elastolytic activity was significant (p= 0.0001).

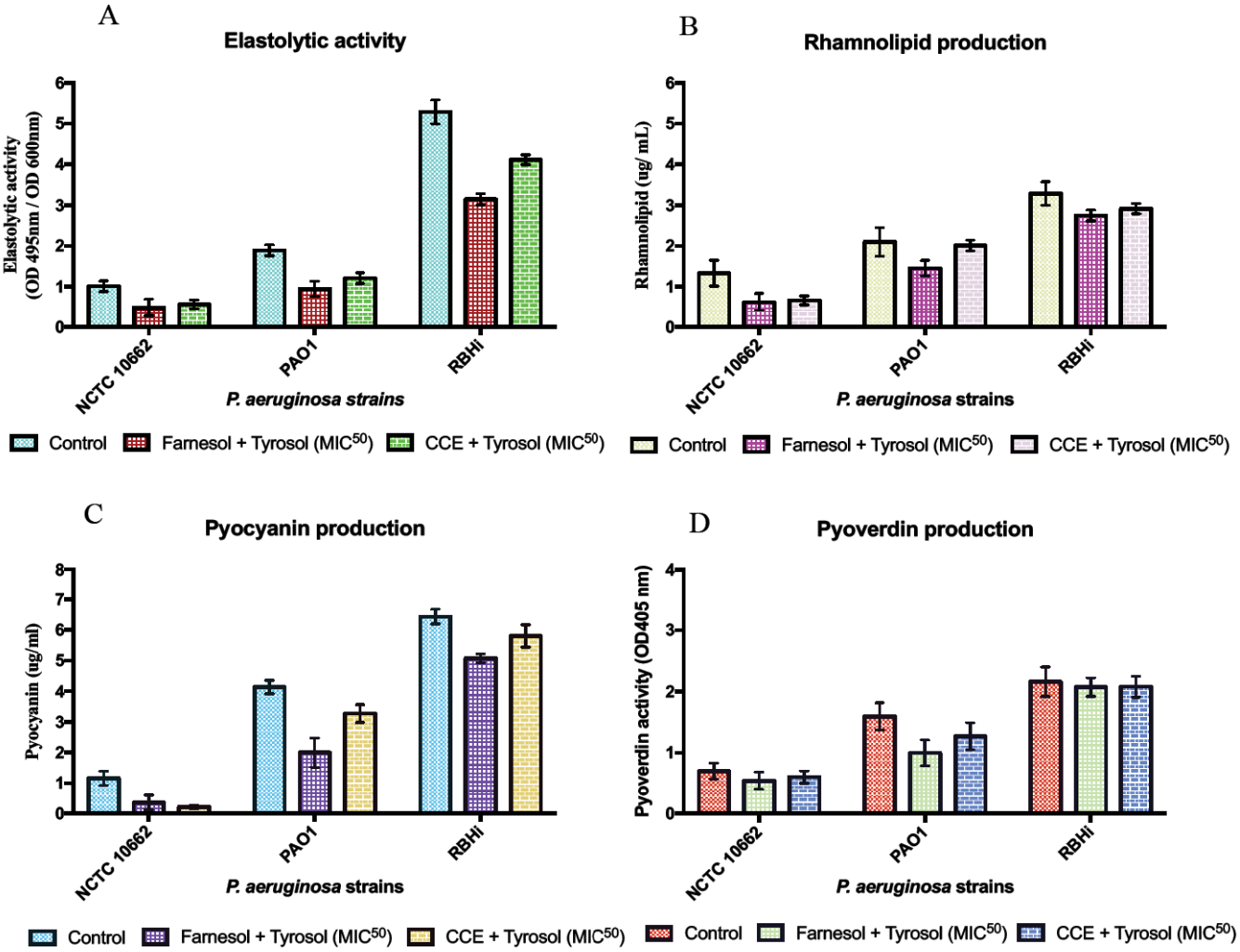


Figure 5.7 Virulence factor quantification from *P. aeruginosa* biofilm before and after treatment (~16 h) (n=5)

Such a significant difference between the treatments was not observed with the other two strains of *P. aeruginosa*. A significant decrease (p= 0.0023) in rhamnolipid production was seen with *P. aeruginosa* NCTC 10662 (figure 5.7 B) with both the combination treatments, this was not the case with the other two strains of *P. aeruginosa*. Only the treatment with farnesol and tyrosol induced a significant reduction in rhamnolipid production in *P. aeruginosa* PAO1 and RBHi.

Both the combination treatments induced a significant decrease (p= 0.0001) in pyocyanin production with *P. aeruginosa* NCTC 10662 and PAO1 (figure 5.7 C) while a significant reduction was seen with only the combination of farnesol and tyrosol in the heavily mucoid RBHi strain. A significant reduction in pyoverdine production (figure 5.7 D) was seen in *P.*

aeruginosa PAO1 with the combination of farnesol and tyrosol only. Neither of the other two strains showed a significant decrease in pyoverdine production.

Accumulation assay performed on the strains of *P. aeruginosa* confirmed that farnesol, and tyrosol at sub-inhibitory concentrations did show similar properties in line with Paβn, a known efflux pump inhibitor (figure 5.8 A, 5.9 A and 5.10 A). Interestingly, the rate of accumulation of the mucoid strains was found to be higher than the non-mucoid strain. This could be attributed to the fact that the mucoid strains display a higher rate of virulence production. The difference in accumulation, can also be attributed to the rate of growth of the strains as NCTC 10662 displayed the slowest generation time of all the three strains and RBHi showed the fastest generation time (Chapter 3, table 3.1). The combination of farnesol and tyrosol showed a greater antagonistic activity against the three strains compared to the combination of CCE and tyrosol.

Compared to the positive control Paβn, both the combination treatments showed a higher rate of inhibition in the non-mucoid strain, NCTC 10662, while both the treatments were similar in their inhibitory effect against PAO1. Only the combination of farnesol and tyrosol showed a greater level of inhibition compared to the positive control. However, all the treatments did show an inhibitory effect towards the functioning of the efflux pumps (figure 5.8 B, 5.9 B and 5.10 B). This is highlighted in the efflux assays. The bacterial cells incubated with EtBr were able to return to their optimal level of functioning upon removal of the combination treatments when compared to the control. The heavily mucoid strain, RBHi showed the highest rate of efflux of EtBr compared to the other two strains when the treatments were removed. This also indicates that the inhibition of the efflux pump achieved through the use of sub-inhibitory concentrations of combination treatments is reversible. As efflux pumps serve as efficient waste management systems under the highly congested conditions within a tightly packed monolayer of cells within biofilms, the inhibition of the efflux pumps would not only promote combating an aspect of drug tolerance but also as a potential treatment for reversing the onset of antimicrobial resistance.

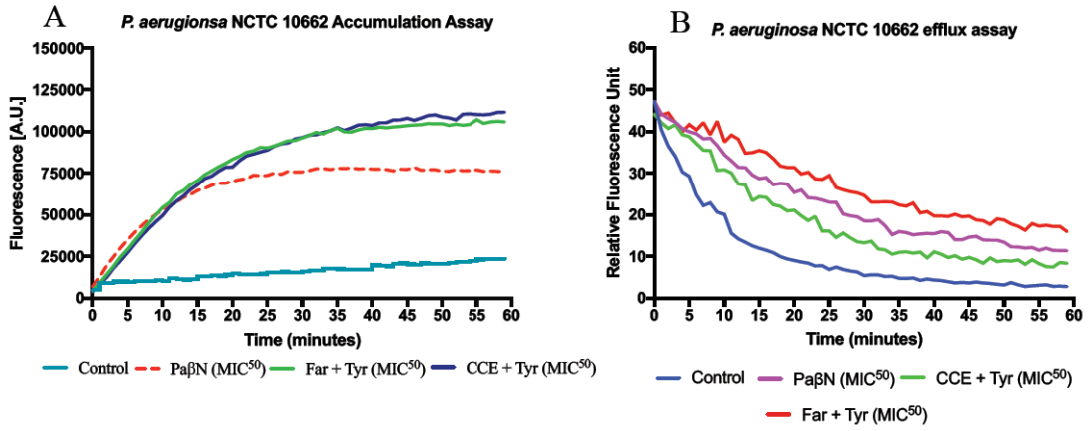


Figure 5.8 Fluorometric accumulation assay and efflux pump activity of *P. aeruginosa* NCTC 10662 at sub-inhibitory concentrations of combination treatments involving farnesol + tyrosol and CCE + tyrosol compared against a positive Paβn and untreated negative control

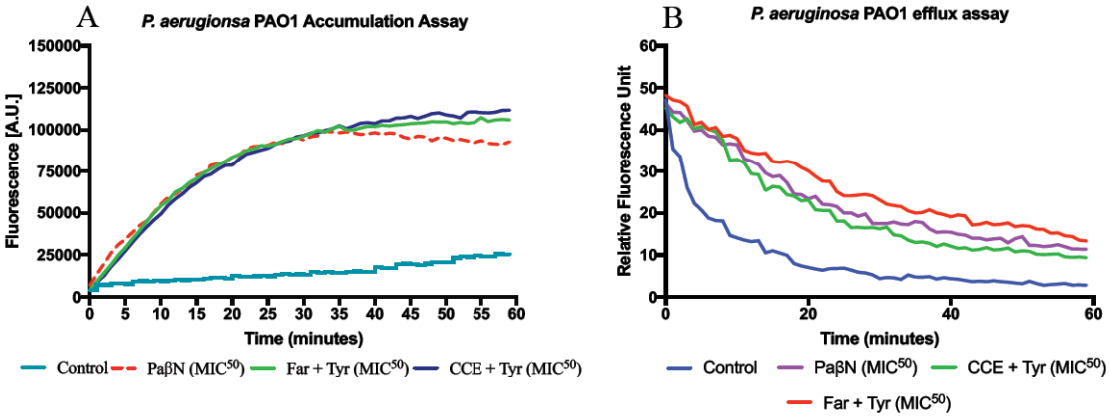


Figure 5.9 Fluorometric accumulation assay and efflux pump activity of *P. aeruginosa* PAO1 at sub-inhibitory concentrations of combination treatments involving farnesol + tyrosol and CCE and tyrosol compared against a positive control Paβn and untreated negative control

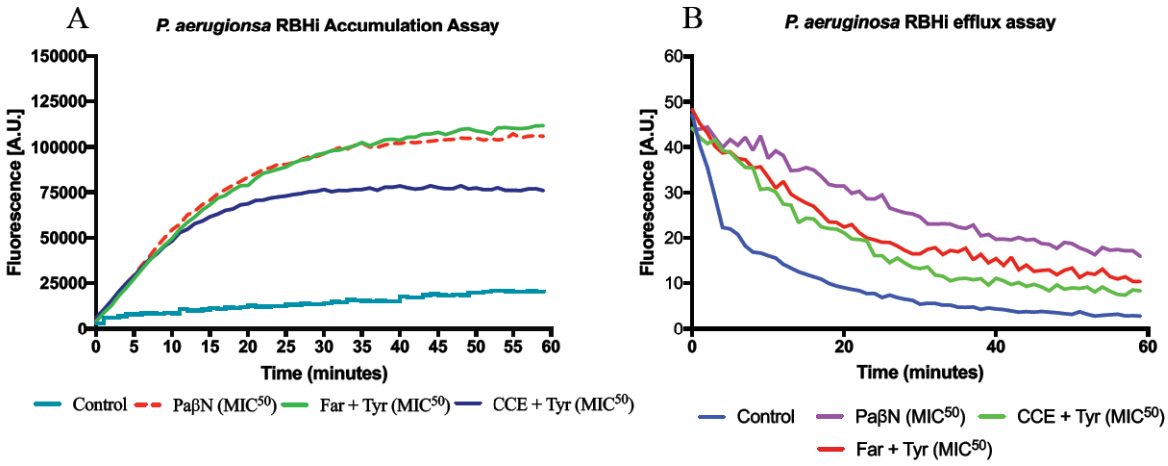


Figure 5.10 Fluorometric accumulation assay and efflux pump activity of *P. aeruginosa* RBHi at sub-inhibitory concentrations of combination treatments involving farnesol + tyrosol and CCE and tyrosol compared against a positive control Paβn and untreated negative control

Expression of MexAB OprM when treated with farnesol and tyrosol independently showed that both individual treatments show a complete downregulation of the MexB inner membrane component (figure 5.11). Both farnesol and tyrosol do in fact down-regulate the expression of the entire efflux system. This is in parallels with the combination studies depicted by the accumulation and efflux assays (figure 5.8, 5.9 and 5.10).

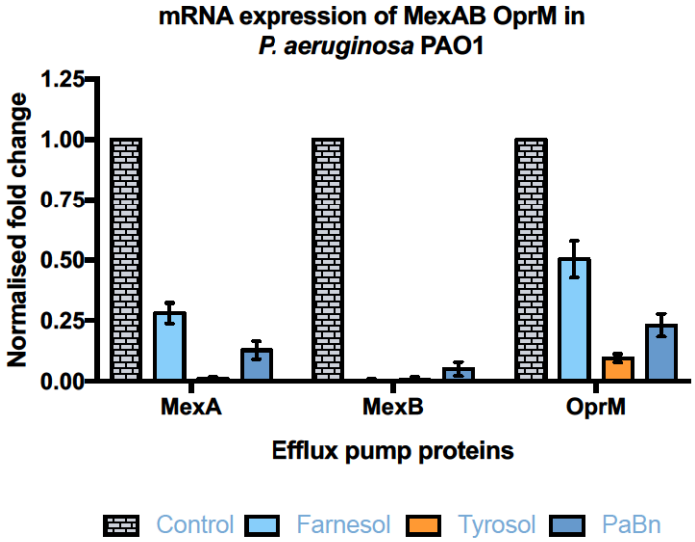


Figure 5.11 mRNA expression of MexAB OprM the multidrug efflux system of *P. aeruginosa*. The fold change for MexA (membrane fusion protein), MexB (inner membrane component) and OprM (outer membrane protein) was determined for *P. aeruginosa* cells grown overnight (~16 h) in the presence and absence of farnesol, tyrosol and Paβn (known inhibitor of efflux pump mechanism). Results are presented as the mean fold change (control standardised to 1.0, represented in grey) with error bars indicating SEM. (n=9)

5.1.3 The effect of farnesol, tyrosol and CCE on motility of *P. aeruginosa*.

Swimming, swarming and twitching motilities were observed for all strains of *P. aeruginosa* for both treated and untreated samples. The motility assays were performed in TSA plates with varying agar concentrations of 0.3%, 0.5% and 1.5% (w/v) respectively. The plates were prepared with addition of the treatments prior to solidifying till the surface was completely dry. The plates were then inoculated with 1μL of bacterial suspension obtained from the mid-exponential phase and then incubated at 37 ºC for a period of ~16 h. The circular zone formed by the bacterial cells migrating away from the point of inoculation was measured in millimetres.

One of the primary factors of initial attachment of bacterial cells to a surface is mediated by the flagellum (Berne *et al.*, 2015). The flagellum allows the bacteria to move in liquid medium and is essential for colonising new surfaces and propagating biofilm mode of growth. The results of the experiment shown in figure 5.12 were found to be statistically significant (p= 0.001) as all strains displayed the ability to swim.

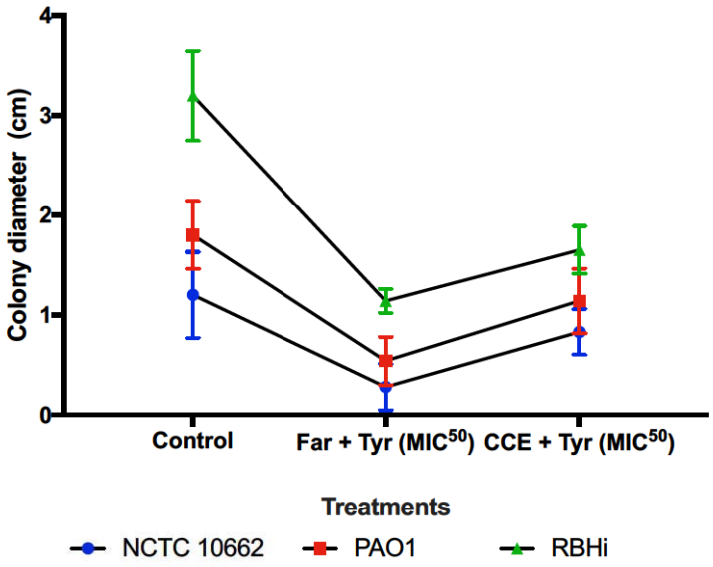


Figure 5.12 Diameter of migration zone formed by *P. aeruginosa* with and without treatment to investigate swimming motility (n=3)

The circular zones measured did reduce with the use of combination treatments. The reduction is the zones was found to be significant (p= 0.0032, 0.0002 and 0.0001) for the combination assay of farnesol and tyrosol when compared to their respective controls. The only treatment that was not found to be significant was the combination of CCE and tyrosol (p= 0.2624) when tested against NCTC 10662.

The ability of the bacteria to swim is a vital virulence factor that is directly related to its ability to form biofilms. As seen in figure 5.12, the *P. aeruginosa* RBHi shows the highest capacity to swim compared to the other two strains. As a trend, this is counterintuitive as lack of motility is essential for attachment of sessile cells prior to biofilm formation. As RBHi and PAO1 regularly display a larger biofilm biomass, this assay shows that the ability to swim and the importance of the flagellum is evident only during the reversible stage of biofilm formation for the purpose of motility and adhesion.

Similar to swimming, the ability to swarm by *P. aeruginosa* is essential as it defines a more coordinated multicellular movement across semi solid surfaces (Overhage *et al.*, 2008).

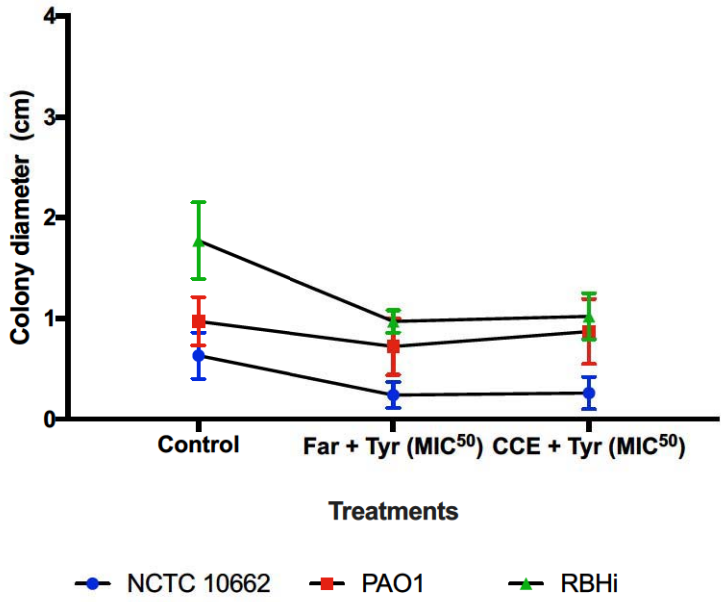


Figure 5.13 Diameter of migration zone formed by *P. aeruginosa* with and without treatment to investigate swarming motility (n=3)

As expected, the CF isolate (RBHi) showed swarming colonies with a larger diameter compared to PAO1 and NCTC 10662. Except in the case of PAO1 and NCTC 10662, the reduction in colony size of RBHi was found to be significantly (p= 0.0029 and 0.0017) reduced with combination treatment of farnesol + tyrosol and CCE + tyrosol respectively.

The third type of motility often associated with *P. aeruginosa* is mediated by type IV pili and is known as twitching motility. Compared to swimming and swarming motilities mediated by the flagellum, twitching motility mediated by type IV pili is at a slower pace and plays a role in biofilm architecture.

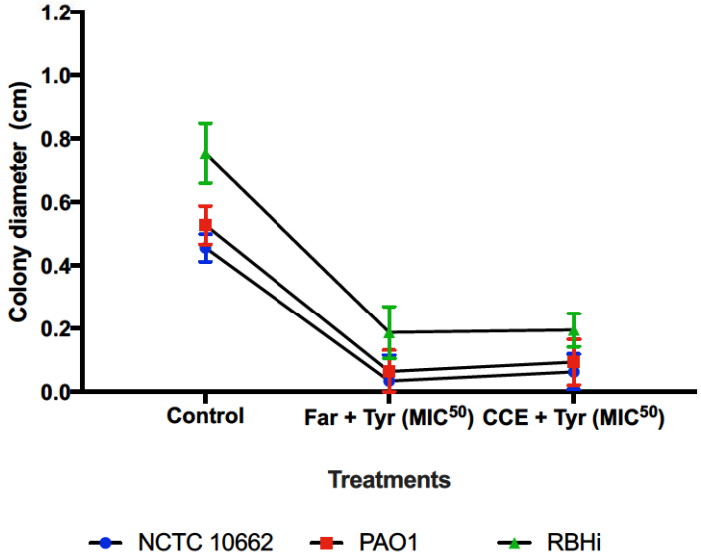


Figure 5.14 Diameter of migration zone formed by *P. aeruginosa* with and without treatment to investigate twitching motility (n=3)

Analysis of twitching motility showed a significant decrease in the colony size upon treatment. Decrease in colony size of all the *P. aeruginosa* strains was significant (p= 0.0001) with combination of farnesol and tyrosol while a significant decrease in colony size was found with the addition of CCE and tyrosol (p= 0.0004). Zone of twitching was significantly decreased in the case of RBHi with both the combination treatments (p= 0.038 and 0.0054 respectively). However, RBHi did show a larger zone of twitching compared to the other two strains.

Studies have shown that type IV pili play a vital role in stabilising interactions between the bacterial cell and abiotic surfaces and cell to cell interaction during initial colonisation (Berne *et al*., 2015) and formation of microcolonies. Therefore, considering the fact that type IV pili directly contributes towards biofilm formation through twitching motility it is understandable for the bacterial strains to exhibit a larger twitching migration zone when untreated compared to the treatment. The decrease in zone diameter in the presence of QQ suggests inhibition of additional factors and adhesins that promote interaction between the bacterial cell and the surface besides type IV pili.

5.1.4 Quantitative PCR and expression levels of QS genes and QS mediated virulence factors of *P. aeruginosa*

Following the treatment with ½ MIC furanone, farnesol and tyrosol, there was a significant reduction in (p= 0.0001) in the relative abundance of RNA for all the LasI protein (figure 5.15 A) from the three strains of *P. aeruginosa*. Comparison between farnesol and tyrosol showed that farnesol had a greater effect of LasI compared to tyrosol. The effect of farnesol and tyrosol on LasI was more pronounced in NCTC 10662 compared to PAO1 and RBHi. This was not the case with LasR. With the exception of RBHi, treatment with farnesol up-regulated the expression of LasR in NCTC 10662 and PAO1. LasR was also up-regulated with tyrosol in PAO1 (figure 5.15 B).

Reduction in RNA for RhlI protein was seen with all the strains wen treated with farnesol and tyrosol (figure 5.15 C). Similar to LasI, NCTC 10662 showed the highest reduction in RNA content for RhlI, while all three strains showed a down-regulation of RhlI when treated with farnesol and tyrosol. With the exception of RBHi when treated with tyrosol, expression

of RhlR (figure 5.15 C) was down-regulated. Although in the case of RBHi, down-regulation of RhlR was marginal when treated with farnesol. Tyrosol up-regulated the expression of RhlR in the case of the heavily mucoid RBHi.

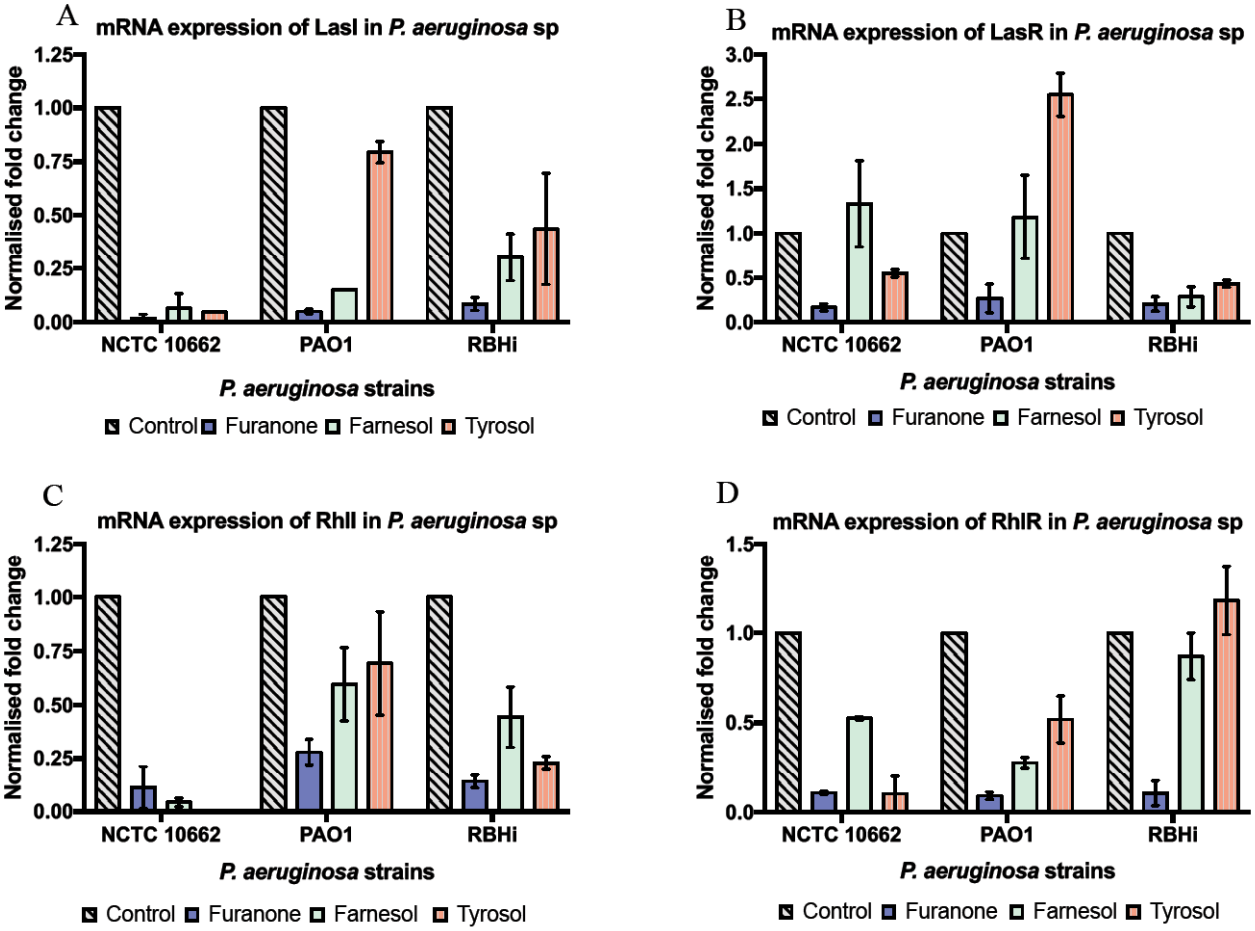


Figure 5.15 mRNA expression of AHL mediated QS circuit in *P. aeruginosa* sessile cells grown with QQ. The fold change of mRNA for LasI, LasR, RhlI and RhlR was determined for *P. aeruginosa* sessile cells extracted from biofilm treated with furanone, farnesol and tyrosol after ~16 h growth. Results are expressed as the mean fold change (control standardised to 1.0) with error bars representing SEM (n=9)

qPCR analysis of expression of genes related to virulence factors of *P. aeruginosa* showed that treatment with farnesol and tyrosol down-regulated the expression of toxA, aprA, rhlAB and LasB when compared to the control as shown in figure 5.16. Farnesol seemed highly effective in down-regulating the genes responsible when compared to tyrosol for all the strains of *P. aeruginosa*. LasB showed the highest down-regulation amongst all the genes analysed with the two treatments.

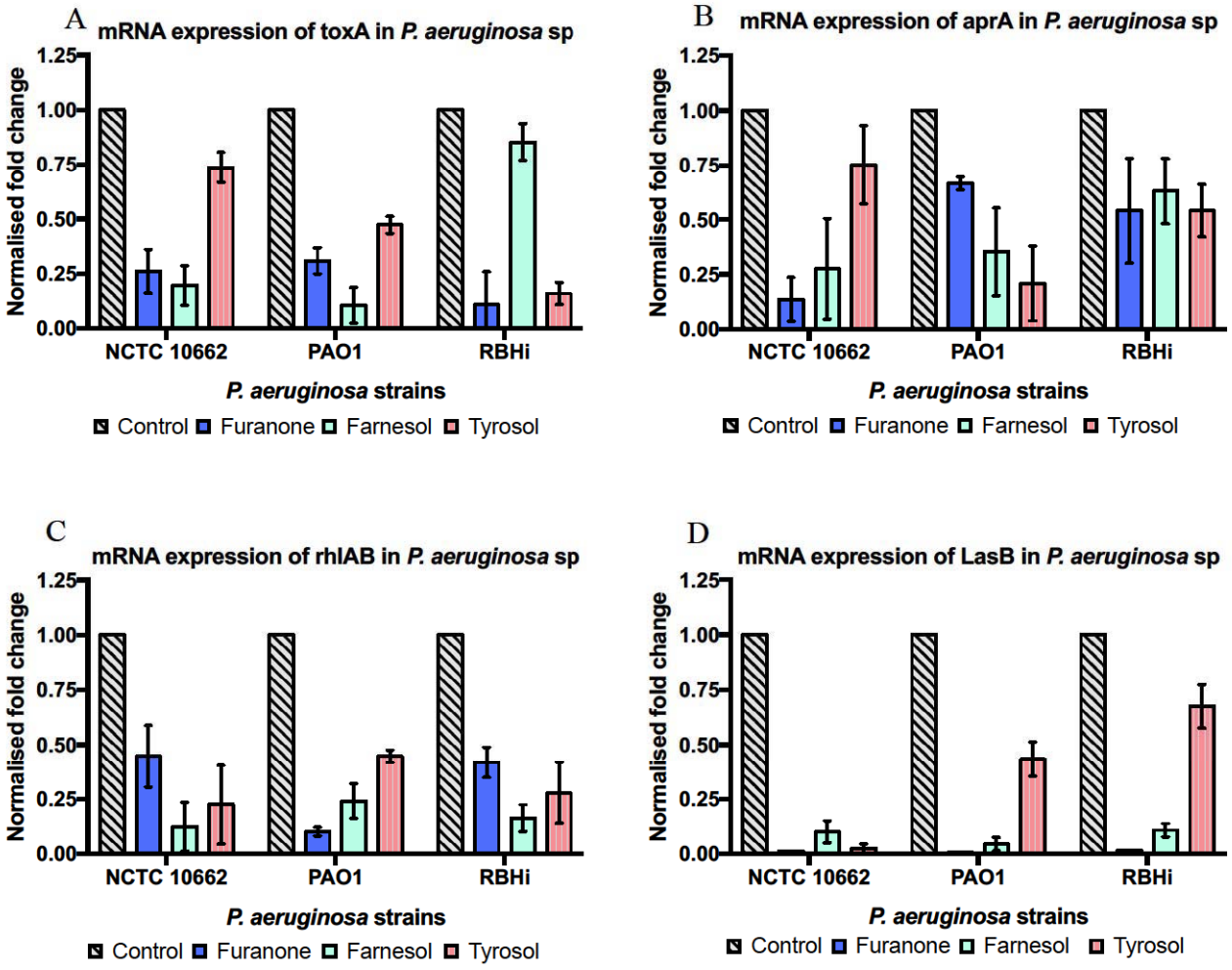


Figure 5.16 mRNA expression of AHL mediated virulence factors of *P. aeruginosa* sessile cells grown with QQs. The fold change of mRNA for toxA, aprA, rhlAB and LasB was determined for *P. aeruginosa* sessile cells extracted from biofilm treated with furanone, farnesol and tyrosol after ~16 h growth. Results are expressed as the mean fold change (control standardised to 1.0) with error bars representing SEM (n=9)

5.2 Discussion

In recent years, QS has been the primary focus of research involving treatment of biofilm mediated chronic infections. It has been well established that QS plays a vital role in development and formation of biofilms which is a recalcitrant mode of growth and aids in the onset of bacterial resistance towards conventional antibiotics (Pletzer, Mansour and Hancock, 2018). This has led to an inexorable rise in formation of superbugs-related infections that are extremely hard to treat due to the dearth of effective therapies (Fernandez, Breidenstein and Hancock, 2011). Therefore, recent research has been focused on elucidating novel therapeutic strategies in order to combat biofilm-related infections by attenuating the ability of cell-to-cell communication by targeting the QS signalling system present in bacteria (Romling and Balsalobre., 2012). This in turn would aid in arresting biofilm formation and biofilm related chronic infections. Novel therapies that target the QS system in pathogenic bacteria could provide the foundation for the development of next generation anti-virulence therapies.

As mentioned in Chapter 1, section 1.10.1, there are three primary strategies that can be adopted to combat biofilm formation by inhibiting QS. The most frequently studied strategies are degradation and modification of QS signals to prevent bacterial communication along with competitive inhibition of the receptor protein of the QS circuit as well as impeding the function of synthase protein responsible for producing the QS molecules (LaSarre and Federle., 2013). In the natural environment, blocking communication of one's ecological niche adversary is essential for survival. This is a promising avenue to explore in order to get a better understanding of the process of social interaction of individual bacterial species or of groups aiming to dictate the niche (Bhagirath *et al.*, 2016). As vital QS is for bacterial coordination and survival, it is also essential for bacteria to interfere with QS of other microbes in order to gain an advantage for survival (Li and Tian., 2012). This naturally existing process of communication interference to gain an advantage over competitors can be exploited to develop novel therapies targeting the QS system of pathogenic bacteria.

The natural environment that *P. aeruginosa* thrives in is also inhabited by other microorganisms competing for the niche. For example, studies conducted by Walker *et al.*, 2004 showed that environmental strains of *P. aeruginosa* can effectively inhabit the rhizosphere and are capable of infecting roots of Arabidopsis and sweet basil and cause plant mortality, while a medically relevant strain can inhabit the lungs or wounds and cause

chronic infections. The environmental niches dominated by *P. aeruginosa* also house other microorganisms, such as *B. licheniformis* in the rhizosphere and *C. albicans* in respiratory diseases (Lore *et al*., 2012). Considering the fact that *P. aeruginosa* is able to thrive in such contrasting environments is a testament to its adaptability.

B. licheniformis present in the rhizosphere is generally considered a plant growth-promoting soil bacterium. Therefore, one of the potential features of *B. licheniformis* is to combat plant pathogens and prevent plant mortality (Gkorezis *et al*., 2016). From this perspective, *P. aeruginosa* and *B. licheniformis* would be in competition to survive in the same niche. This interaction might provide insight into mechanisms that may aid in inhibiting QS mediated pathogenicity of *P. aeruginosa*. Similarly, from a medical perspective, such as in the case of CF patients, *C. albicans* is commonly found in polymicrobial infections with *P. aeruginosa* (Williams, Ranjendran and Ramage, 2016). These two pathogenic microbes interact on a physical level as well as through secreted factors which is primarily antagonistic in nature. *C. albicans*, a dimorphic yeast is known to produce drug resistant biofilms (Morales and Hogan, 2010). It does so, similar to *P. aeruginosa* through the production of QS molecules (farnesol and tyrosol) which allow it to transition from planktonic single cell yeast to hyphal biofilm forming morphology. Farnesol prevents the transition from yeast to hyphal morphology, tyrosol promotes the formation of hyphae and biofilm formation in *C. albicans* (Decanis *et al*., 2011).

As *C. albicans* and *P. aeruginosa* interact on a physical level as well as through secreted factors, the effect of QS molecules secreted by *C. albicans* was tested against biofilm formation by *P. aeruginosa*. Similarly, *B. licheniformis* culture extracts (CCE) was prepared with the prospect of containing AHL-lactonase within the growth culture broth, a known enzymatic QQ against AHL mediated signalling. In theory, the lactonase enzyme hydrolyses the ester bond of the homoserine lactone ring of the AHL molecule. In hydrolysing the lactone bond, lactonase prevents the signalling molecules from binding to their target transcriptional regulators, thereby inhibiting quorum sensing.

Tryptophan was included in this study for its biofilm dispersal ability in conjunction with farnesol, tyrosol and CCE to investigate the synergistic action of combination treatment. Tryptophan was chosen over anthranilate as it is a readily available amino acid for *P. aeruginosa* growth and is found in abundance in CF patients' sputum (Bhagirath *et al*., 2016). The second reason was that eukaryotic cells produce anthranilate from the kynurenine

pathway, similar to *P. aeruginosa,* which aids in dendritic cell activation or T-cell apoptosis, (Bortolotti *et al*., 2016). Therefore, there was a possibility that it would promote PQS signalling in *P. aeruginosa* when used in synergy with other chemicals under *in vivo* conditions. Although metabolites of the kynurenine pathway have not been found to be cytotoxic to mammalian cells (Lovelace *et al*, 2016), it would be challenging to differentiate between the possible interkingdom interaction with the use of exogenous anthranilate as an antagonist against *P. aeruginosa*. Thirdly, as *P. aeruginosa* can metabolise tryptophan and as anthranilate is a by-product of tryptophan catabolism, the use of tryptophan would be better suited to provide an insight into biofilm development and architecture of the three phenotypically different strains of *P. aeruginosa* used in this study rather than an intermediary chemical of the PQS biosynthetic pathway.

The crude cell extract of *B. licheniformis* was found to inhibit *P. aeruginosa* biofilms. It was equally effective against *P. aeruginosa* NCTC 10662 and PAO1. While it did reduce biofilm formation in RBHi, the percentage decrease was not as much as with NCTC 10662 and PAO1. Combination treatments were performed at ¼ MIC, ½ MIC and at MIC of all the selected QQ and compared to the untreated control. Results showed that combination treatment with farnesol + tyrosol and CCE + tyrosol yielded the highest inhibition while all treatments were effective in reducing biofilm formation by all the strains of *P. aeruginosa*. Analysis of the EPS composition of the biofilms formed by the three strains against the two most effective combination treatments showed that the highest reduction was seen in the protein composition of the biofilm of *P. aeruginosa*. The identity of all the proteins in the EPS remains largely unknown however, some of the essential EPS associated proteins have been thoroughly researched. EPS proteins such as CdrA adhesin, which crosslinks Psl polysaccharide and tethers Psl to the bacterial cell wall and provides stability to the biofilm (Borlee *et al*., 2010), OMV's (outer membrane vesicles) are considered to be the predominant proteins present in *P. aeruginosa* EPS (Nevot *et al*., 2015) and more recently ecotin, a serine protease inhibitor has been identified to be present in the EPS as well (Tseng *et al*., 2018). Though numerous enzymes may be present in the form of virulence factors, their quantity would differ based on gene regulation and requirement by the bacterial strain to induce pathogenicity. Therefore, inhibition of the biofilm formed by *P. aeruginosa* can be attributed to the reduction of proteins such as CdrA and OMV which play a role in maintaining the structure of the biofilm. The combination of farnesol and tyrosol was thus very effective in inhibiting biofilm formation by arresting the production of essential proteins of the biofilm. The highest reduction in protein content was observed to be in

P. aeruginosa NCTC 10662 (~40%) with the combination treatment involving farnesol + tyrosol, while a ~30% and 25% reduction in protein content was seen with PAO and RBHi respectively with the same treatment. The combination of CCE + tyrosol showed similar results, with PAO1 showing a higher percentage reduction compared to RBHi while NCTC 10662 showed the highest percentage reduction.

Similarly, the combination of farnesol and tyrosol showed a ~55% reduction (Figure 5.3) in eDNA content of the biofilm formed by *P. aeruginosa* PAO1 and a significant reduction in eDNA in RBHi. With this exception, the component of *P. aeruginosa* biofilm highly affected by the combination treatment remained to be protein. Though the protein content of the biofilms may not be as high in significance as the carbohydrate content, the lack of synthesis of essential proteins may inhibit biofilm formation.

Furthermore, the highest reduction in carbohydrate content (~40%) of *P. aeruginosa* was seen in NCTC 10662 compared to PAO1 and RBHi (~10%). Results (table 5.1) show that NCTC 10662 shows the presence of alginate in negligible quantities. This may also be due to the interference of other EPS materials as a false positive. Nevertheless, the defining feature is the lack of alginate production which in theory shows that the carbohydrate content of NCTC 10662 biofilm is rich in Psl and Pel polysaccharides. As the combination treatments involving farnesol + tyrosol and CCE + tyrosol showed the highest reduction in protein content of the NCTC 10662 biofilm which correspondingly showed the highest percentage reduction in carbohydrate content, it is plausible that farnesol and tyrosol affect the CdrA protein which crosslinks the Psl polysaccharides and bacterial cells to the Psl polysaccharides. The presence of alginate in PAO1 and RBHi strains may overcome this effect to a certain extent as it provides an added structural component for maintaining the biofilm. While the CCE of *B. licheniformis* may contain lactonase of acylases to cleave and prevent QS mediated gene regulation from taking place, farnesol and tyrosol may function by affecting other aspects of gene regulation and development of *P. aeruginosa* biofilms.

Planktonic cultures of *P. aeruginosa* when treated with the combination treatments showed a significant decrease in their levels of adherence (Figure 5.4). A significant decrease was observed with the use of farnesol and tyrosol. Similarly, combination treatment involving CCE and tyrosol decreased the hydrophobicity of the planktonic cells when compared to the control. The untreated planktonic cells were found to show intermediate hydrophobicity but with the combination treatments they lost their hydrophobic nature. The difference in

hydrophobicity of the planktonic cell samples with and without treatment may also suggest that there may have been critical changes in charge across the entire cell envelope. MATH assay conducted on sessile cells after ~16 h of growth showed that all the cells had high levels of hydrophobicity. However, a small but significant drop in hydrophobicity was seen with the combination treatments on NCTC 10662. Enumeration of sessile cells did not show a decrease in any of the *P. aeruginosa* strains, which shows that the treatments can be effective in reducing biofilm development without the onset of resistance.

Quantification of elastolytic activity, rhamnolipid production, pyocyanin and pyoverdine production between the three *P. aeruginosa* strain decreased with the exception of pyoverdine content in RBHi. The highest decrease of virulence factor was the elastolytic activity of the bacterial strains, especially RBHi. While NCTC 10662 and PAO1 showed a significant decrease in pyocyanin production.

Previous studies have shown that MexAB-OprM is one of the most clinically important efflux pumps in *P. aeruginosa* which contributes significantly towards intrinsic resistance towards a wide range of antibiotics (Verchere *et al.*, 2015). This aids in development of chronic infections untreatable by conventional antibiotic therapies. Hence the ability of the combination treatments to inhibit efflux activity was tested. Accumulation and efflux assay (figure 5.10) showed that the combination treatments were effective in inhibiting efflux pump activity when compared to an untreated negative control and a positive control involving a known efflux pump inhibitor Paβn. MexAB-OprM efflux system consists of an inner membrane drug proton antiporter (MexB), periplasmic membrane fusion protein (MexA) and outer membrane channel forming protein (OprM). Relative gene expression level (figure 5.11) of the components of the efflux pumps against farnesol and tyrosol showed a significant (p= 0.0001) reduction in gene expression of the three protein in the presence of farnesol and tyrosol. Paβn was included as a positive control. No expression of MexAB was seen in the presence of tyrosol at all. Down regulation of the MexAB-OprM efflux system will certainly aid in making *P. aeruginosa* more susceptible towards antibiotics.

Motility of *P. aeruginosa* is considered to be a vital virulence factor which makes it highly pathogenic and it is primarily governed by flagellar movement or with the aid of type IV pili (Deziel, Comeau and Villemur., 2001). Motility studies are based on the capacity of the bacterium to migrate from the point of inoculation. In the case of *P. aeruginosa* the assays

asses the functioning of the flagellum and the type IV pili (O'May and Tufenkji, 2011) and the inferences are based on colony size and diameter. Combination treatment involving farnesol and tyrosol inhibited the ability of all the strains to swim significantly while the combination of tyrosol and CCE significantly reduced swimming motility, with the exception of NCTC 10662, which did not show a reduction in colony diameter. This shows that farnesol and tyrosol affect the functioning of the single polar flagellum. Along with swimming, the capacity to swarm was assessed as swarming defines a coordinated behaviour while swimming motility assays ability of individual bacteria to migrate. Thus, the swarming capacity of all the three strains were assessed with the combination treatments and similar to the reduction in colony size observed in the swimming assay, farnesol and tyrosol significantly inhibited the coordinated migration of NCTC 10662, and RBHi, while the colony diameter of the swarm assay was decreased in PAO1, it was not found to be significant. The significant decrease in colony diameter in the twitching assay with the combination treatments suggest an antagonistic effect on the functioning of the type IV pili and adhesin that aid in bacterial attachment.

Gene expression studies, involving the individual effect of farnesol and tyrosol, were conducted along with furanone. A significant down-regulation of both the AHL mediated synthases (LasI and RhlR) was seen against all the three strains (Figure 5.15). However, LasR receptor was upregulated in NCTC 10662 and PAO1 with the use of farnesol and tyrosol showed LasR to be upregulated in PAO1. Similar upregulation of RhlR receptor was observed in RBHi. This shows that farnesol and tyrosol actively reduce the expression of the synthase protein (LasI and RhlI) and prevent the production of 3OC12-HSL and C4-HSL respectively. Significant down-regulation in gene expression was observed with the use of farnesol and tyrosol for exo-proteins toxA, aprA and LasB as well as rhlAB which is responsible for the production of rhamnolipid.

In conclusion, exploiting the production and availability of chemicals from niche competitors of *P. aeruginosa* could be a potential for synergistic treatment of biofilm mediated chronic infections. This study also represents inter-species interactions through the use of *B. licheniformis* CCE and QS signalling molecules of *C. albicans*. Use of tryptophan individually and in combination with farnesol, tyrosol and CCE showed that biofilm development by *P. aeruginosa* can be inhibited and also could lead to an increased susceptibility towards some antibiotics. Farnesol and tyrosol, used in combination and individually attenuate one of the primary intrinsic resistance factors of *P. aeruginosa* in the

form of the MexAB-OprM efflux pump. Through the use of QQ, it has been possible to investigate how different phenotypes of *P. aeruginosa* modulate their gene expression to form biofilms and has given an insight into the varying architecture of *P. aeruginosa* QS mediated biofilm formation and virulence production. Synergistic treatments to overcome biofilm forming pathogens can involve exploiting the ecological niche that they inhabit. Although further research is required to better understand the mechanistic action of the QQ, it is promising to identify several suppressive regulatory effects of *C. albicans* QS signal molecules on AHL mediated *P. aeruginosa* QS network and related virulence factors.

Since the introduction of antibiotics as novel treatment against infections in the 1940s, multi drug resistance traits have become synonymous with biofilm forming opportunistic pathogens that dominate the nosocomial setting (Bryers, 2008). This necessitates the identification and investigation of novel antimicrobial therapies and their mode of action. Exploiting interspecies and interkingdom interactions/ competition as well as naturally occurring products and their use in synergy could provide a potential solution to combat/ eradicate biofilm related chronic infections and drug resistance.

Chapter 6 . Response and virulence regulation of non-mucoid and mucoid biofilms of *P. aeruginosa* to QQs in co-culture with A549 and HaCaT cell lines

6.1 Introduction

The presence of numerous regulatory proteins allows *P. aeruginosa* to adapt and thrive in a variety of environmental niches. A crucial aspect is the diversity of virulence factors produced by *P. aeruginosa* and its ability to form robust biofilms (Moradali, Ghods and Rehm., 2017). Chapter 4 presented an overview of *in vitro P. aeruginosa* biofilm formation on relevant surfaces, however, analysing *P. aeruginosa* biofilm formation and subsequent inhibition of bacterial communication by QQs on a lung epithelial (A549) cell line will provide more insight into the phenomenon of QQ as it would represent an opportunity to analyse the efficacy of QQ in an environment simulating a natural (biotic) surface (epithelial cell line) for bacterial adherence and biofilm formation.

Studies conducted by Moreau-Marquis *et al* in 2010 helped elucidate the interaction between mammalian cells and *P. aeruginosa* biofilms. Since then, a number of cell lines have been used to investigate the host-pathogen interaction model (Mulcahy, Isabella and Lewis., 2014) including CF bronchial epithelial cell lines. Numerous other studies have been conducted to elucidate the host-pathogen interaction and action of antibiotics on biofilm mediated infections, however, there is not enough literature on investigating the effect of QQ on biofilm formation by different phenotypes of *P. aeruginosa* on a biotic surface. This study makes use of the A549 cell line to investigate the variation in biofilm formation and virulence factor secretion between non-mucoid, mucoid and a heavily mucoid CF isolate of *P. aeruginosa* in the presence of QQ.

Apart from biofilm formation during CF infections by *P. aeruginosa*, biofilm formation is observed in infected burn wounds. Therefore, HaCaT cell line, immortalised keratinocyte cells from human skin were used as a second biotic surface for QQ mediated inhibition of biofilm formation and virulence factor production by non-mucoid and mucoid strain of *P. aeruginosa*. As the epithelial cell line within the respiratory system and the keratinocytes on the skin form the first physical barrier of defence, it is more than likely for bacterial attachment, growth and infections. Due to the nature of the A549 and HaCaT cells, biofilm

formation and virulence factor production by *P. aeruginosa* may differ due to differences in host-microbe interactions and the growth environment.

The advantages of using A549 and HaCaT cell lines to study the effect of QQ on *P. aeruginosa* biofilm formation arise from the genetic homogeneity and stability of each individual cell line. As the cells are clonal, there will be minimal variation within the experimental set up, unlike the use of *in vivo* model. The importance of using cell lines lies in their representation of sophisticated reproducible biotic systems. When compared to abiotic surfaces, use of cell lines allows for creating experimental designs that can answer basic mechanical and biochemical questions to aid in the understanding of what occurs in an *in vivo* environment in a controlled *in vitro* set up. This may aid in identifying specific activities of QQ and their effect on specific QS mediated gene expression which promotes bacterial communication and biofilm formation.

6.2 Results

6.2.1 Adherence and internalisation assays involving *P. aeruginosa* on A549 and HaCaT cell line with and without QQ mediated by Farnesol and Tyrosol

For the purpose of the assays, A549 and HaCaT cell were grown to confluency in 6-well tissue culture plates (see chapter 2) prior to bacterial inoculation. The three strains of *P. aeruginosa* were grown overnight in LB medium at 37 ºC. The overnight bacterial growth was centrifuged at 5000 rpm to pellet the cells, and the supernatant containing the spent medium was discarded. The pellet containing the bacterial cells was resuspended in 1x PBS. Based on bacterial cell count and seeded A549 and HaCaT cells, MOI was approximately adjusted to 20:1 and the co-culture was incubated for 1 h with and without treatment involving farnesol, tyrosol and $^{1/2}$MIC combination of the two. After the incubation period, the A549 and HaCaT cells were rinsed and adhered bacteria were quantified by spot plate analysis. The percentage adherence was calculated based on the ratio of adhered bacteria to the number of bacteria in the inoculum. Internalisation assay involving the three strains followed a similar protocol as the adherence assay. Gentamicin at 200 µg/mL was used to eliminate the bacteria in suspension and the A549 and HaCaT cell were lysed open. Quantification of bacterial cells showed that the RBHi isolate of *P. aeruginosa* were internalised by the A548 and HaCaT cell lines significantly more compared to PAO1 and NCTC 10662.

Sub-inhibitory concentration of farnesol (2.5 µM) and tyrosol (1.5 µM) were used in this study. MTT assay was performed on the chosen concentration of farnesol and tyrosol and combination of $^{1/2}$ sub-MIC of each as shown in figure 6.1. The cells were treated after reaching confluency (~3 to 4 day) for a period of 4 h prior to performing the MTT assay. Cellular growth and the MTT assay were carried out in 96 well microtiter plates. The mean of three independent experiment (n= 8) was used to calculate the cell viability

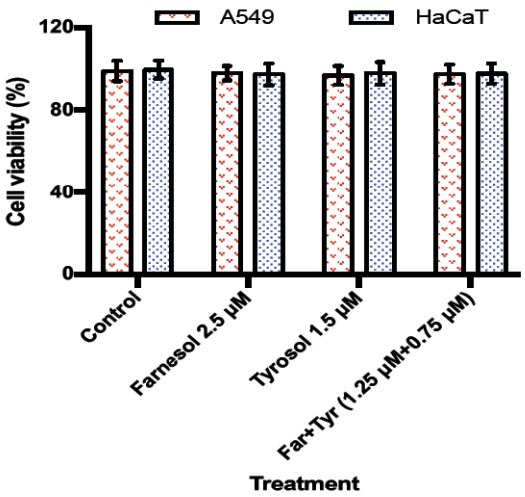


Figure 6.1 Cell viability of A549 and HaCaT displayed as percentage after treatment with farnesol and tyrosol. PBS solution at 1M was used as control (n=5)

A higher percentage of adherence was seen with the A549 cell line compared to the HaCaT cell line (p= 0.002) as shown in figure 6.2 A and B. Neither of the strains showed a stronger affinity to adhere to the mammalian cells when compared to the controls. The addition of farnesol and tyrosol did not have any detectable effect on the ability of the bacterial strains to adhere. The main difference was a higher percentage of adhesion to A549 cells was observed when compared to HaCaT cells. All the treatments, individual and in combination reduced the percentage adherence of bacterial cells to the mammalian cells (p= 0.0001 and 0.0001).

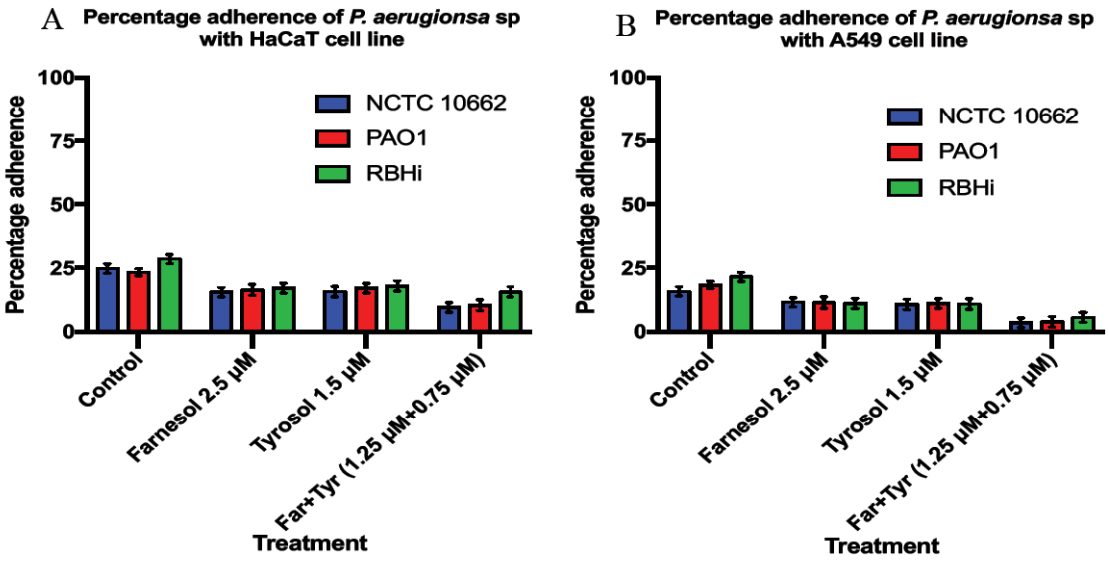


Figure 6.2 Adherence of *P. aeruginosa* cells onto the surface of A549 and HaCaT cell lines. The percentage is based on calculating the adhered CFU vs the initial cell count in the bacterial inoculum (n=5)

Greater amount of adherence from the CF isolate *P. aeruginosa* RBHi was expected. However, it was not the case and difference in adhesion, based on two-way ANOVA showed no significance (p= 0.325). Since the adherence pattern was similar amongst bacterial strains and the mammalian cells, the internalisation percentage was calculated as bacterial internalisation by host tissue has been known to be beneficial for the host as it triggers an immune response to combat the infection during the early stages (Eisenreich *et al.*, 2013).

Surprisingly, the non-mucoid strain *P. aeruginosa* NCTC 10662 showed a higher percentage of internalisation (p= 0.0001) compared to PAO1 and the CF isolate RBHi in co-culture with A549 cell line as shown in Figure 6.3 A and B. Upon treatment with farnesol, tyrosol and a $^{1/2}$ sub-MIC combination of the two, a decrease in attachment was observed amongst all the strains. The highest decrease was seen with the non-mucoid strain NCTC 10662 on the A549 cell line with the combination treatment and individual treatment alike (p= 0.0001).
P. aeruginosa PAO1 showed a significant decrease in attachment with tyrosol (p= 0.0044) and with combination treatment (p=0.0001). However, only the combination treatment of farnesol and tyrosol was able to elicit a significant decrease in bacterial cell internalisation with the CF isolate and A549 cell line. In comparison, a significant decrease in bacterial cell internalisation was observed with tyrosol and in combination with farnesol with just the non-mucoid *P. aeruginosa* NCTC 10662 (p=0.021 and 0.0047 respectively). The mucoid strains followed a similar rate of internalisation against HaCaT as the non-mucoid, however a significant decrease or increase in internalisation was not observed (p= 0.7538).

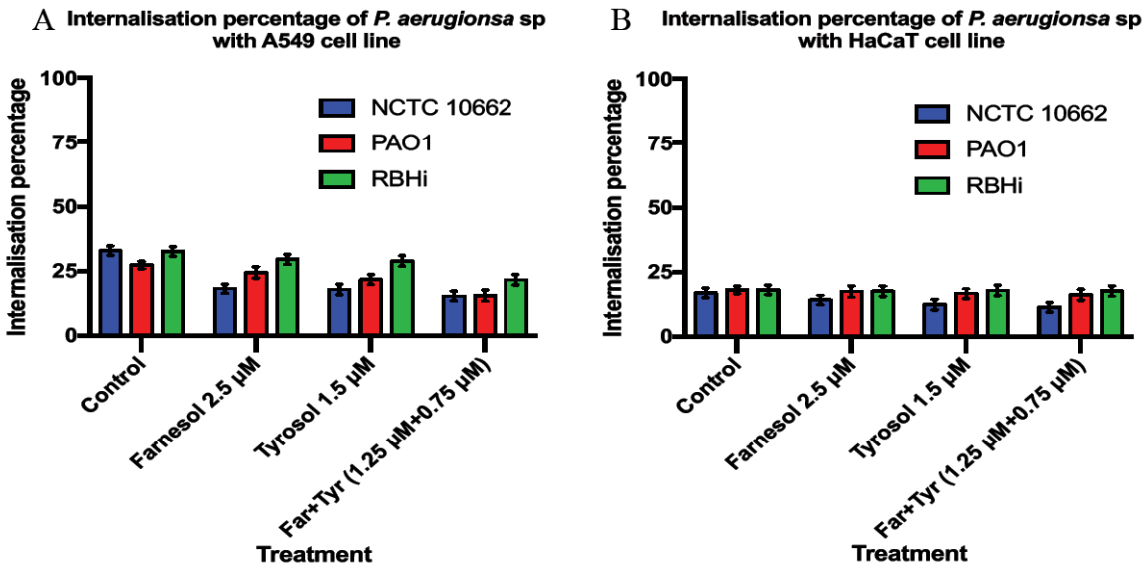


Figure 6.3 Internalisation of *P. aeruginosa* cells into cells of A549 and HaCaT cell line. The percentage is based on calculating the internalised CFU vs the initial cell count in the bacterial inoculum (n=5)

6.2.2 *P. aeruginosa* biofilm formation and virulence factor production on A549 and HaCaT cell lines and subsequent inhibition by farnesol and tyrosol, individually and in combination.

Co-culture experiments were performed in 6-well tissue culture plates and 96-well microtiter plates. To test the effect of farnesol and tyrosol as QQs on *P. aeruginosa* biofilm formation in co-culture with A549 and HaCaT cells, 6-well tissue culture plates and 96-well plates were seeded with the respective cells till confluency.

Time-point inoculation experiments were performed to quantify biofilm formation/ inhibition and virulence factor production by *P. aeruginosa*. As the MIC of farnesol and tyrosol differs greatly between *P. aeruginosa* and mammalian cells (see appendix B), the bacterial cultures were pre-treated with their respective MIC of farnesol and tyrosol and used as inoculum. The co-culture medium contained the MIC of farnesol and tyrosol relevant to A549 and HaCaT cells.

CV assay was performed to quantify biofilm formation of *P. aeruginosa* in co-culture with A549 cell line. In order to prevent over-estimation of quantity of biofilm, the absorbance value obtained with CV staining of the control A549 cells was subtracted from the experimental values prior to analysis. Individual negative control of A549 cells was included in each of the experimental set up undergoing the same treatment without the inclusion of bacterial cells. The same was performed for all biochemical analysis of biofilm components and virulence production. All experiments were performed in triplicates.

Referring to figure 6.4, the first row depicts the individual effect of farnesol, tyrosol and synergistic action of both on *P. aeruginosa* biofilm formation in co-culture with A549 cells. Farnesol and tyrosol, individually and in combination have a reduced effect on total biofilm formation in co-culture when compared to their inhibitory effect on just bacterial biofilm formation. The reduced affect can be attributed to the MIC of farnesol and tyrosol used for co-culture experiments, as A549 cells do not tolerate the MIC of farnesol and tyrosol used for bacterial culture, a significantly lower concentration was used.

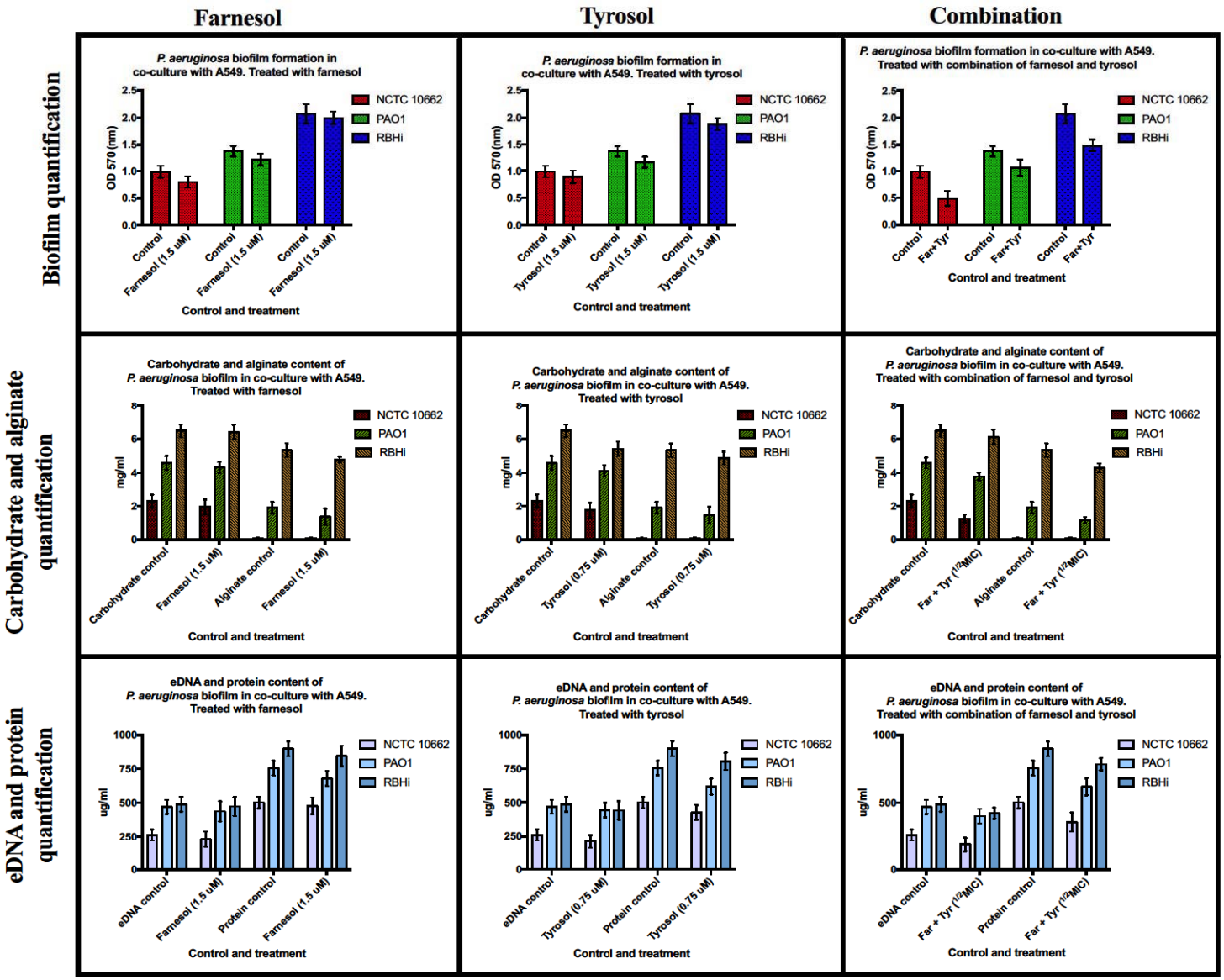


Figure 6.4 Synergistic and individual effect of farnesol and tyrosol on *P. aeruginosa* biofilm formation, architecture and virulence factor production when grown in co-culture with A549 cell line (n=5)

Though the addition of farnesol, tyrosol and combination reduced overall biofilm formation, a significant reduction of biofilm formation was observed with the addition of combination of farnesol and tyrosol (sub-MIC) for the non-mucoid, mucoid and heavily mucoid strains (p= 0.0001, 0.0036 and 0.0001). *P. aeruginosa* RBHi showed ~26% reduction in total biofilm formation, which was the highest amongst all the three strains grown in co-culture. The total carbohydrate content of the biofilms in co-culture did not show a significant decrease with the addition of farnesol for all the three strains. A reduction in alginate content of the EPS produced by *P. aeruginosa* RBHi was seen (p= 0.0012). Conversely, tyrosol showed a reduction in total carbohydrate content with the RBHi strain without any significant reduction in alginate content compared to the treatment with farnesol. The combination treatment saw a significant (p= 0.001) reduction in total carbohydrate content

of NCTC 10662 (~45%) and with PAO1 (p= 0.0101) while the reduction with RBHi was not found to be significant. However, alginate production was greatly reduced in PAO1 and RBHi (p= 0.0002 and 0.0001 respectively). eDNA and protein content on *P. aeruginosa* biofilm remained unaltered with farnesol, however a reduction in protein content was seen with the addition of tyrosol to the co-culutre with PAO1 and RBHi (p= 0.0025 and 0.012 respectively). Combination treatment showed a reduction in eDNA and protein content amongst all the strains of *P. aeruginosa*, however, only the reduction in protein content with the combination treatment was found to be significant by two-way ANOVA (p= 0.0167).

In comparison, co-culture with HaCaT cells showed significantly lower quantity of total biofilm formation via the CV assay across all *P. aeruginosa* strains and treatments (figure 6.5) compared to co-culture with A549 (two-way ANOVA, p=0.0001).

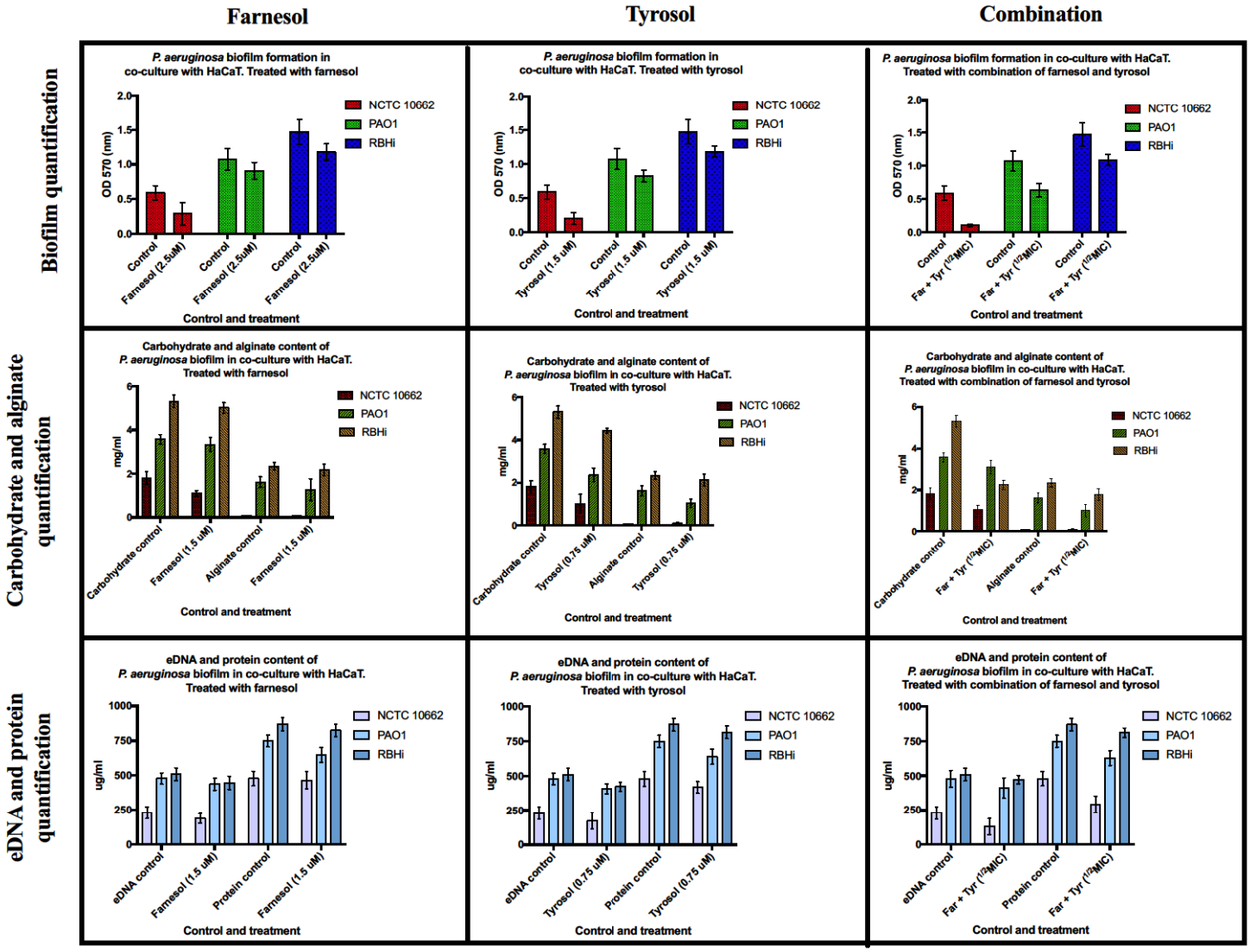


Figure 6.5 Synergistic and individual effect of farnesol and tyrosol on *P. aeruginosa* biofilm formation, architecture and virulence factor production when grown in co-culture with HaCaT cell line (n=5)

Following the trend seen in A549 co-culture, the treatments with farnesol, tyrosol and combination were found to be more effective in inhibiting biofilm formation in co-culture with HaCaT cell line, compared to their respective controls. Nearly, ~75% reduction of biofilm was seen using combination treatment (p= 0.0001) against NCTC 10662 and ~25% reduction was seen with RBHi (p= 0.0002) and ~45% reduction with PAO1 (p= 0.0027). Through farnesol inhibited biofilm formation, it was not found to be significant (p= 0.325). However, inhibition of biofilm formation with tyrosol and combination were found to be significant (p= 0.023 and 0.0002 respectively).

The eDNA and protein content of the biofilm formed by the mucoid strains on HaCaT cell line against all the three treatments remained similar to the control with the exception of NCTC 10662 which seemed to be affected by the combination treatment and showed a ~40% reduction (p= 0.0028) in eDNA content and a ~30% reduction (p= 0.001) in protein content of the biofilm.

A similar trend was observed in the total carbohydrate content of the biofilm. Tyrosol and combination were found to be more effective in reducing total carbohydrate content of the biofilm (p= 0.025 and 0.0001 respectively). The greatest reduction in carbohydrate content was observed in RBHi (~55%) when treated with a combination of farnesol and tyrosol. RBHi was also the only strain to a show a drop (p= 0.034) in alginate content by ~18% with the combination treatment. Comparing the two cell lines, all the phenotypes of *P. aeruginosa* had a stronger affinity towards A549 cell line compared to HaCaT and this was displayed in its ability to form biofilms and secrete EPS. Over all, combination treatment of farnesol and tyrosol was more affective against *P. aeruginosa* biofilm. However, of the individual treatments, tyrosol was more effective.

Virulence factors such as rhamnolipid, elastolytic activity and pyocyanin production were quantified by independent assays. Compared to the untreated control, a reduction in the expression of virulence genes was seen with all three treatments involving farnesol, tyrosol and combination (figure 6.6). Reduction of elastolytic activity and pyocyanin production using farnesol was significant (p= 0.001 and 0.0001 respectively) in *P. aeruginosa* NCTC 10662. However, the decrease in rhamnolipid production was not significant. Use of tyrosol showed a tremendous decrease (p= 0.0001) in rhamnolipid production compared to the treatment with farnesol while no significant decrease was seen in elastolytic activity and

pyocyanin production. However, both individual treatments showed effective inhibition of virulence gene expression of NCTC 10662.

Comparatively in PAO1, all the treatments showed a reduction in elastolytic activity and pyocyanin production (p= 0.0001), while only the combination treatment showed a significant decrease in rhamnolipid production (p= 0.0024), reduction in rhamnolipid production by individual treatments was not significant (p= 0.832). Comparing the individual treatments, tyrosol was more effective in reducing elastolytic activity and pyocyanin production compared to farnesol (p= 0.0001). *P. aeruginosa* RBHi was found to be 3 to 4 times more virulent compared to PAO1 and NCTC 10662 respectively and combination treatment showed a significant decrease (p= 0.0001) in rhamnolipid production and elastolytic activity compared to the individual treatments and the control. Though pyocyanin production was reduced with the combination treatment compared to the control, it was not significant compared to the individual treatments (p= 0.735). Overall, the combination treatment involving farnesol and tyrosol was more effective against the highly virulent RBHi strain when grown in co-culture with A549 cell line.

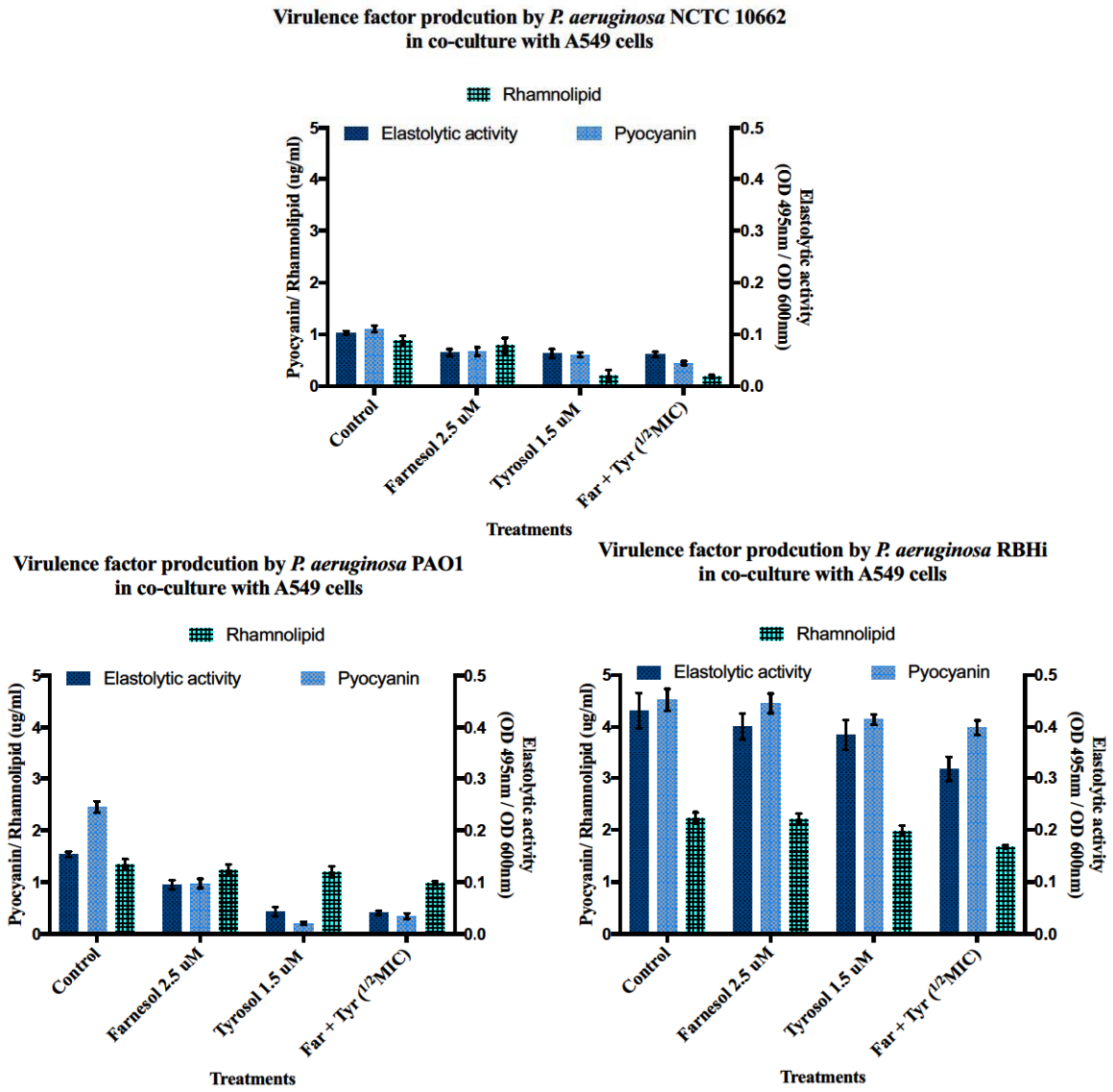


Figure 6.6 Virulence factor production by *P. aeruginosa* strains in co-culture with A549 cell line (n=5)

Quantification and analysis of virulence factors produced by *P. aeruginosa* biofilms grown on HaCaT cell line showed a different profile compared to virulence produced upon co-culture with A549 cell line. In the non-mucoid strain, *P. aeruginosa* NCTC 10662, combination treatment with farnesol and tyrosol was found to be the most effective at reducing the production of virulence factors, however, all treatments reduced the production of virulence factors significantly (p– 0.0032). In *P. aeruginosa* PAO1, elastolytic activity and pyocyanin production were reduced significantly with all treatments (p= 0.0001) however, rhamnolipid production remained at elevated levels even though it did reduce in comparison with the control (p= 0.002). Overall, treatment with tyrosol and in combination was found to be more effective (figure 6.7).

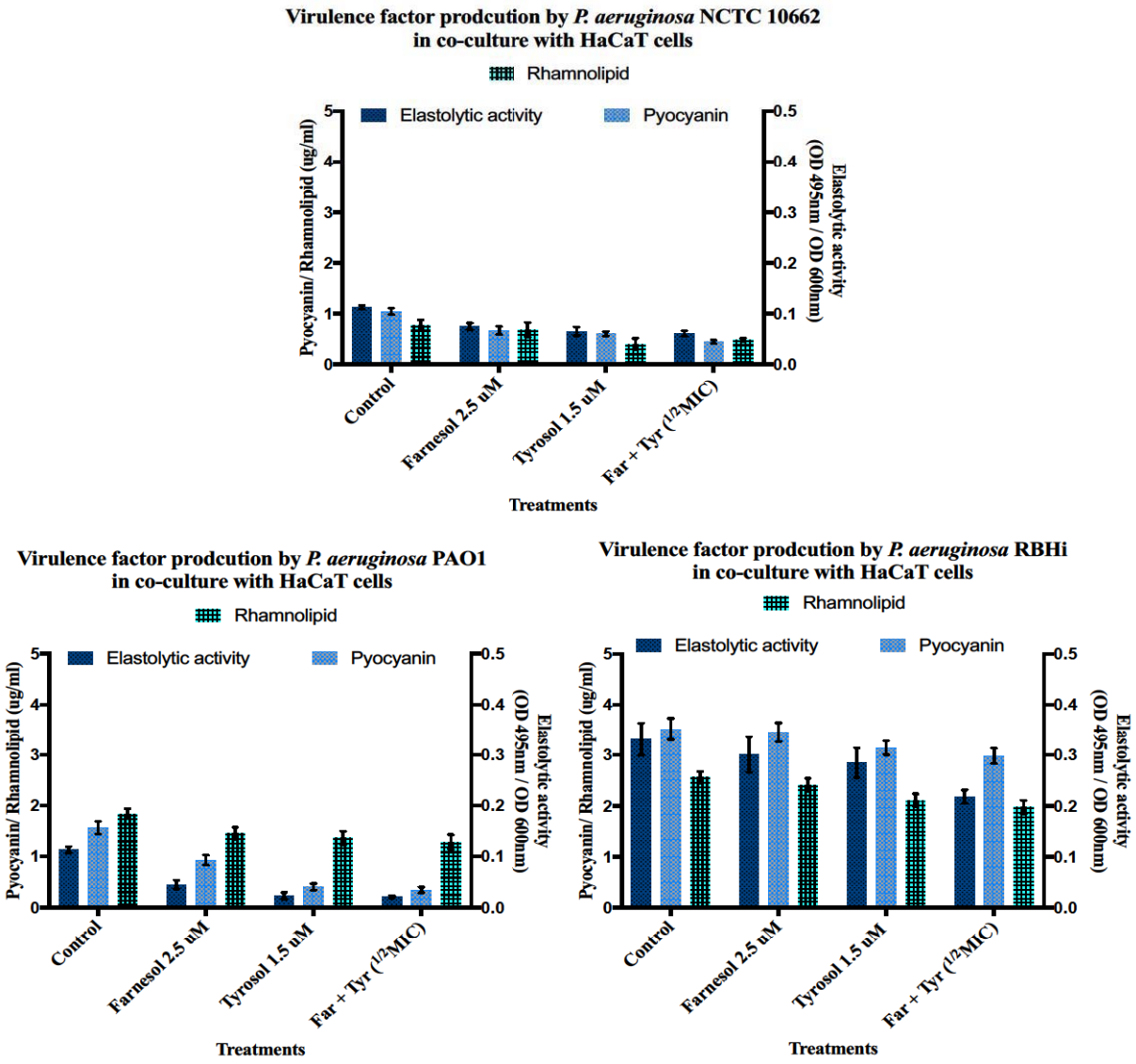


Figure 6.7 Virulence factor production by *P. aeruginosa* strains in co-culture with HaCaT cell line (n=5)

In the CF isolate *P. aeruginosa* RBHi strain, though the reduction in virulence factor production followed a similar trend shown by PAO1, the levels of virulence production was still high. Reduction of rhamnolipid, pyocyanin and elastolytic activity was found to be significant (p= 0.0001) when compared to the untreated control.

Compared to the untreated control, cell viability for both cell lines decreased significantly as expected over a duration of 6 h of growth (figure 6.8). A greater reduction in overall cell viability was seen with HaCaT cells compared to A549 cells. Cell viability for farnesol and tyrosol and in combination showed a small decrease in viability but was not found to be significant (p= 0.532).

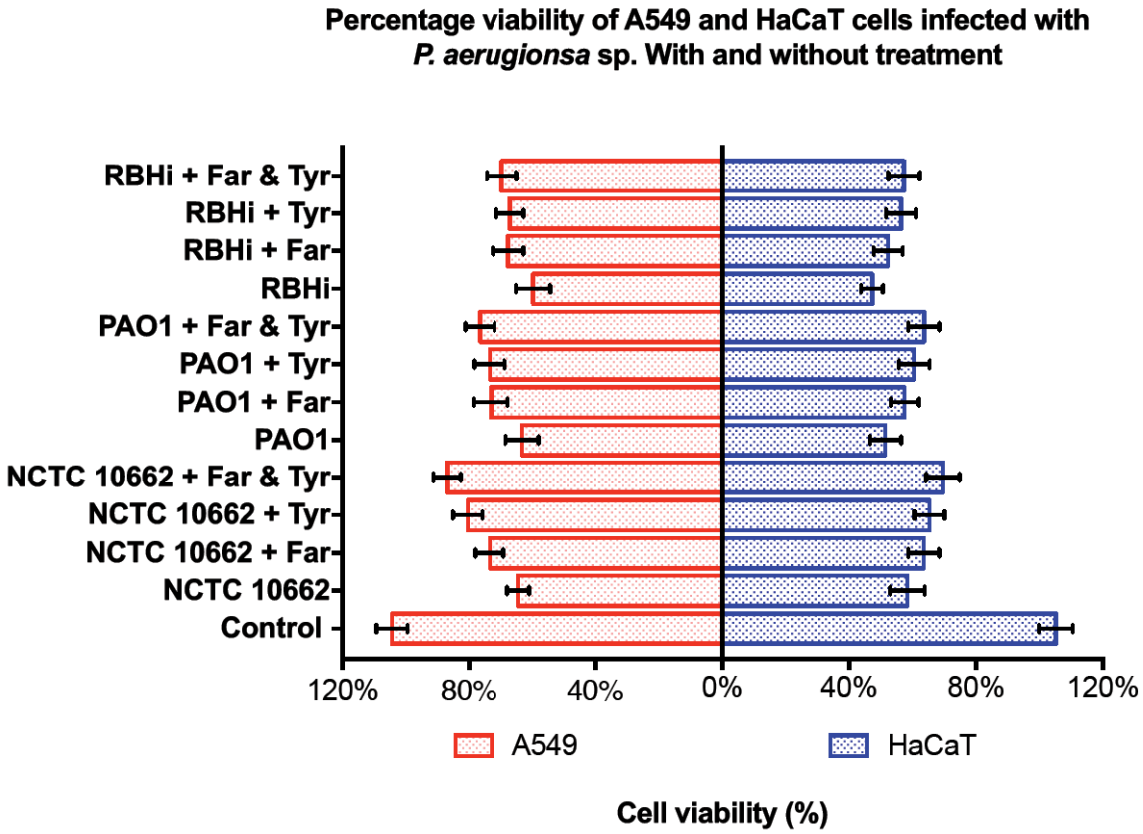


Figure 6.8 Viability of A549 and HaCaT cells in co-culture with bacteria, with treatment using farnesol and tyrosol as QQ and untreated cells based on the MTT assay (n=3)

There is little data comparing IL-8 secretion by two different cell lines when exposed to biofilm formed by *P. aeruginosa*. Chronic lung infections generally favour increased production of IL-8 as an inflammatory response (Montemurro *et al.*, 2012). However, comparative data on IL-8 secretion as an inflammatory response in co-culture with three distinct strains of biofilm forming *P. aeruginosa* is lacking. A549 and HaCaT cells were grown in 96-well plates and exposed to biofilms formed by *P. aeruginosa* NCTC 10662, PAO1 and RBHi. Time-point inoculation studies also included farnesol and tyrosol as individual treatments as well as combination treatment to quantify IL-8 secretion by preventing biofilm formation. From ELISA, it was found that there was an increase in IL-8 secretion by A549 cells, which increased in the presence of biofilm formed by *P. aeruginosa* (figure 6.9). The exogenous addition of farnesol and tyrosol and combination to combat biofilm formation did show a reduced IL-8 secretion. In the case of NCTC 10662, the reduction in IL-8 production when treated with farnesol was found to be significant (p=0.044) while the others were not found to be significant.

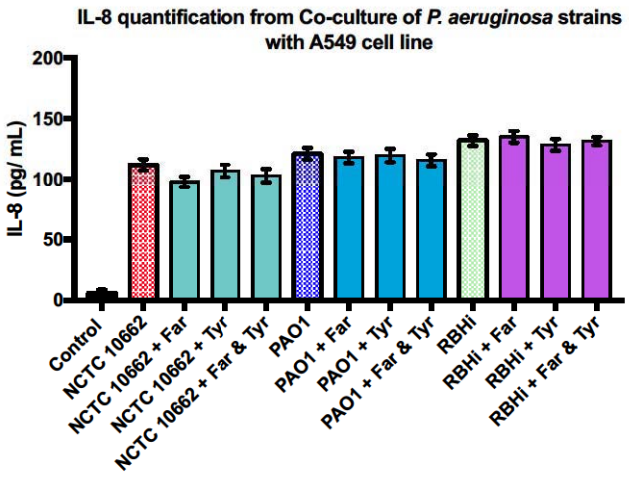


Figure 6.9 *P. aeruginosa* biofilm formation stimulates significantly increased levels of IL-8 release compared to the control. A549 cells when exposed to biofilm formation and treatment with farnesol and tyrosol, secreted IL-8 which was measured by ELISA (n=3)

Comparing the IL-8 secretion by HaCaT cells in in co-culture with *P. aeruginosa* (figure 6.10) showed an increased production of IL-8 when compared to the A549 cell line. However, no reduction of IL-8 was observed in the presence of farnesol, tyrosol or by combination treatment. In fact, addition of farnesol, tyrosol and in combination increased IL-8 secretion. Though none of the increases were found to be significant.

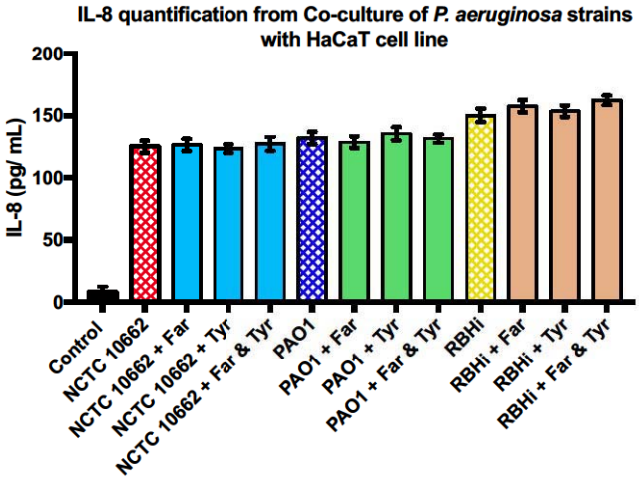


Figure 6.10 *P. aeruginosa* biofilm formation stimulates significantly increased levels of IL-8 release compared to the control. HaCaT cells when exposed to biofilm formation and treatment with farnesol and tyrosol, secreted IL-8 which was measured by ELISA (n=3)

Following farnesol, tyrosol and combination treatment of *P. aeruginosa* PAO1 biofilm in co-culture with A549 and HaCaT cell lines, the LasI/R and RhlI/R genes were significantly up-regulated compared to the control (figure 6.11 A and C). Comparing the two cell-lines, co-culture with HaCaT cell line showed a significantly higher LasI/R and RhlI/R up-regulation compared to co-culture on A549 cell line. While combination of farnesol + tyrosol combination induced a significant up-regulation of LasR and RhlI, individual treatment with

tyrosol was responsible for up-regulation of LasI and RhlR (figure 6.11 A). However, individual treatment with farnesol down-regulated LasI and LasR in co-culture with A549 cell line. Combination treatment with farnesol + tyrosol showed a consistent and significant up-regulation of LasI/R and RhlI/R of PAO1 in co-culture with HaCaT cell line (figure 6.11 C). Individual treatment with farnesol and tyrosol showed significant up-regulation of LasI/R and RhlI/R on HaCaT cell line.

Co-culture of PAO1 with A549 cell line treated with farnesol showed a down-regulation of toxA, aprA, rhlAB and lasB virulence. However, tyrosol and combination treatment showed a tremendous up-regulation of rhlAB in PAO1 co-culture with A549 cell line (figure 6.11 B), while all others were down-regulated with tyrosol and combination treatment. In the case of co-culture with HaCaT cell line (figure 6.11 D), aprA and rhlAB were down-regulated with all the treatments while tyrosol induced an up-regulation of toxA and lasB and combination of farnesol and tyrosol up-regulated toxA.

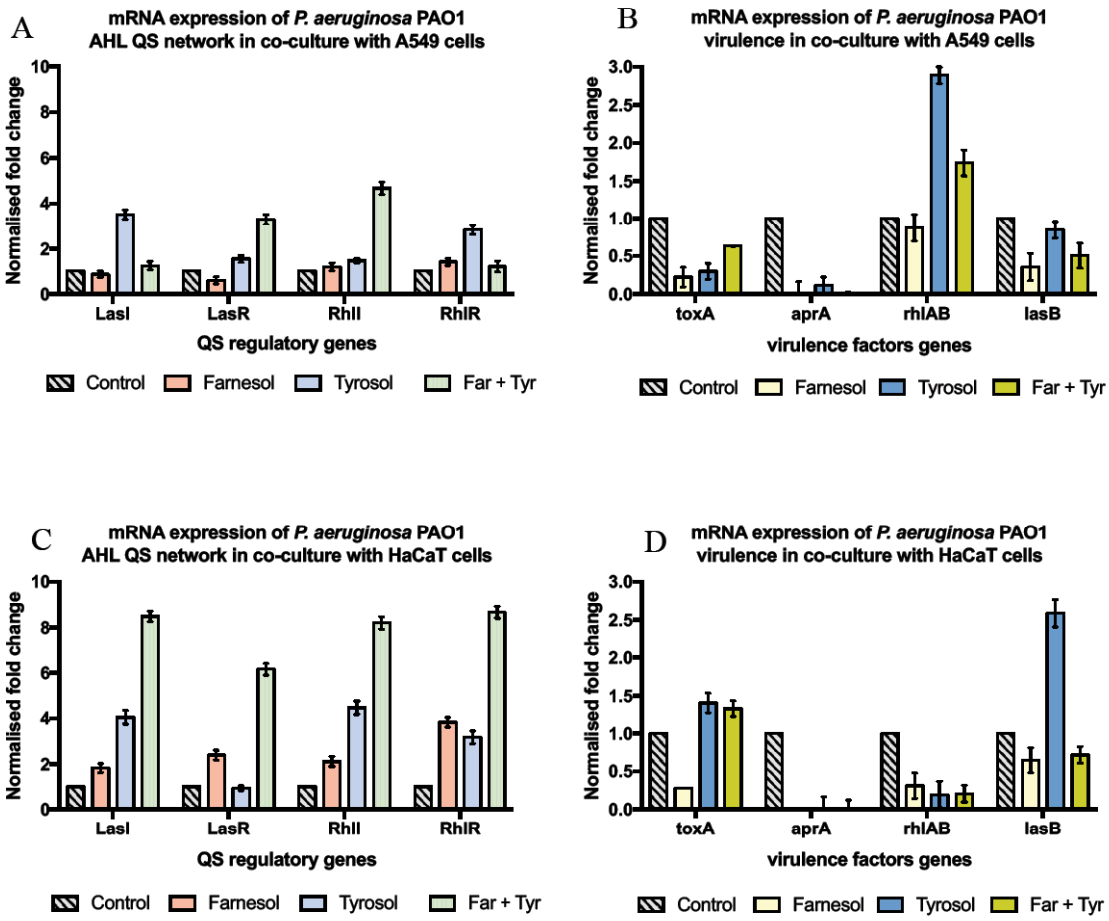


Figure 6.11 mRNA expression of AHL mediated QS circuit and virulence factors *in P. aeruginosa* PAO1 sessile cells grown with QQ. The fold change of mRNA for QS and virulence genes was determined for *P. aeruginosa* PAO1 sessile cells extracted from biofilm treated with furanone, farnesol and tyrosol after ~16 h growth. Results are expressed as the mean fold change (control standardised to 1.0) with error bars representing SEM (n=9)

Gene expression of *P. aeruginosa* NCTC 10662 in co-culture with A549 cell line showed an up-regulation of RhlI and RhlR when treated with tyrosol and combination of farnesol and tyrosol, while farnesol down-regulated RhlI and RhlR (figure 6.12 A). Conversely, farnesol up-regulated LasI and LasR marginally, while treatment with tyrosol and combination down-regulated LasI and LasR. With the exception of up-regulation of rhlAB (combination treatment), all the other virulence factors were down-regulated in co-culture with A549 (figure 6.12 B). Co-culture of NCTC 10662 on HaCaT cell line with treatment showed a consistent down-regulation of LasI/R, RhlI/R and the virulence factors (figure 6.12 C and D).

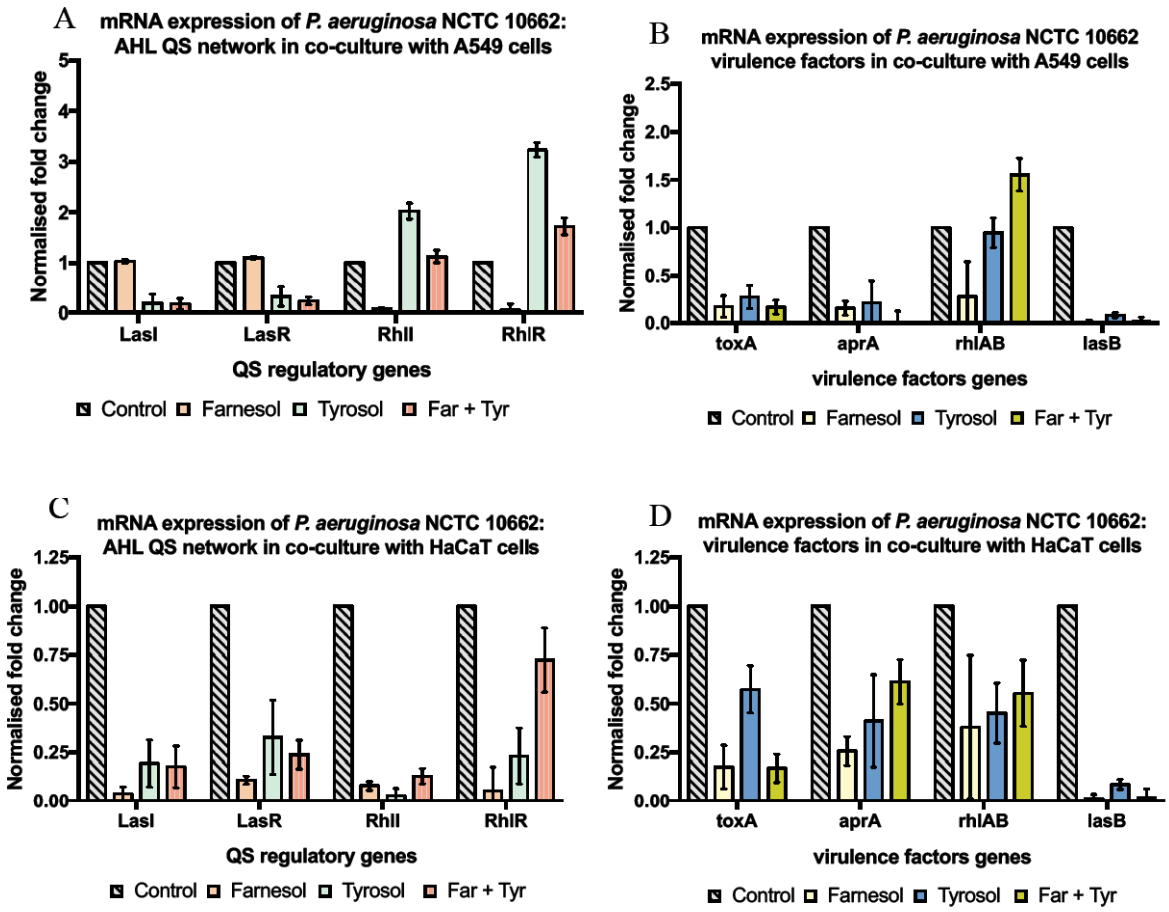


Figure 6.12 mRNA expression of AHL mediated QS circuit in *P. aeruginosa* NCTC 10662 sessile cells grown with QQ. The fold change of mRNA for QS and virulence factors genes was determined for *P. aeruginosa* NCTC 10662 sessile cells extracted from biofilm treated with furanone, farnesol and tyrosol after ~16 h growth. Results are expressed as the mean fold change (control standardised to 1.0) with error bars representing SEM (n=9)

A significant up-regulation of LasI/R and RhlI/R was seen with tyrosol treatment of RBHi co-culture with A549 cell line (figure 6.13 A), while the other treatments showed a pattern of down-regulation. Individual treatment of farnesol and tyrosol showed an up-regulation of toxΛ and rhlΛB, while tyrosol also contributed to the up-regulation of lasB. Combination treatment was responsible for up-regulation of rhlAB as well. aprA remained the only virulence factor that did was down-regulated (figure 6.13 B). With the exception of RhlI, which was up-regulated in co-culture of RBHi and HaCaT, all the other genes were down-regulated (figure 6.13 C and D).

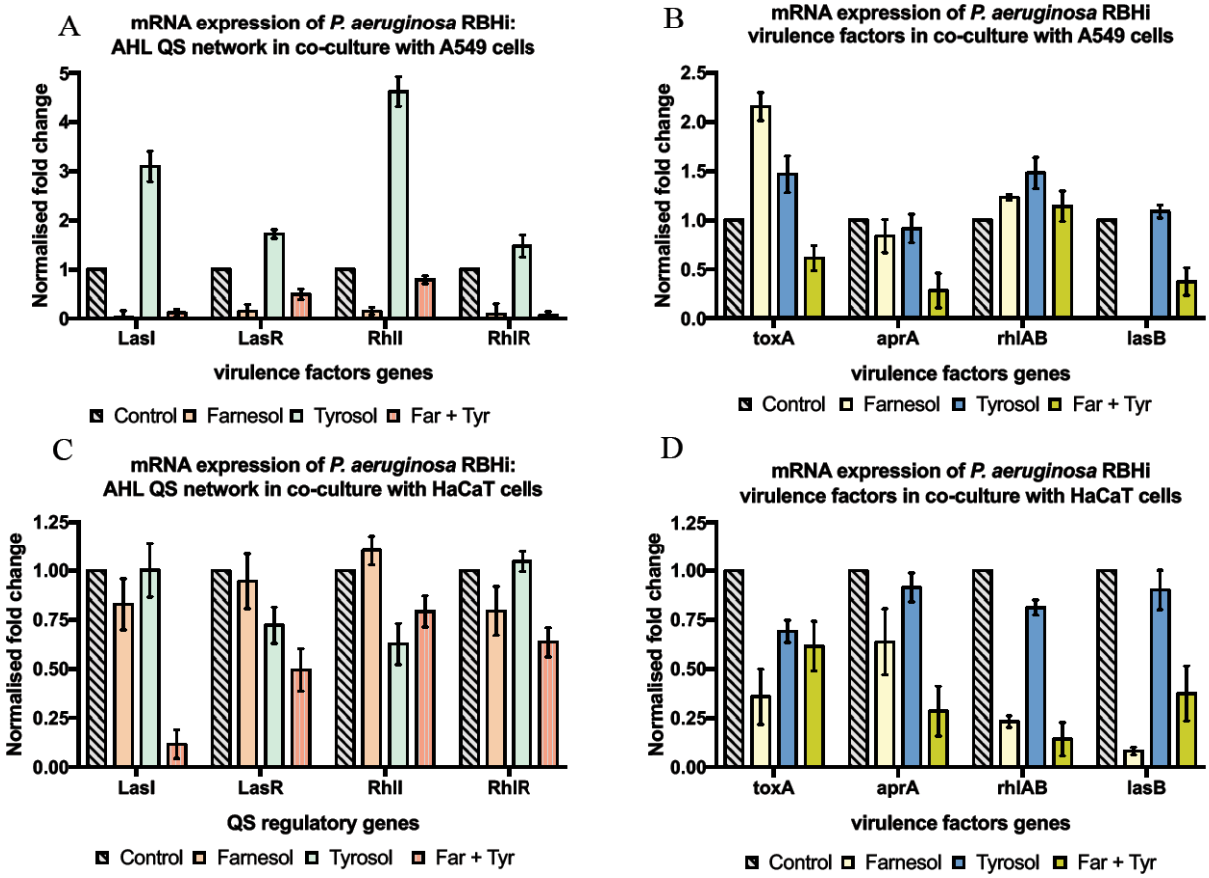


Figure 6.13 mRNA expression of AHL mediated QS circuit in *P. aeruginosa* RBHi sessile cells grown with QQ. The fold change of mRNA for QS and virulence genes was determined for *P. aeruginosa* RBHi sessile cells extracted from biofilm treated with furanone, farnesol and tyrosol after ~16 h growth. Results are expressed as the mean fold change (control standardised to 1.0) with error bars representing SEM (n=9)

6.3 Discussion

Biofilm formation is a survival strategy of *P. aeruginosa* which is critical for development of chronic infections. Analysis of components in this study has revealed that carbohydrates provide much of the attachment and structure for communities while proteins and eDNA play specific role in maintaining the structural integrity of the biofilm. Differing phenotypes of *P. aeruginosa* produce varying levels of EPS to maintain their biofilm structure and the composition of the EPS changes and the regulatory systems respond to QQ based on the growth requirement of the bacterial strain.

Studies have shown that there is a difference in interaction of planktonic cultures and sessile cells of *P. aeruginosa* towards the host tissue (Mulchay, Isabella and Lewis, 2014). However, little is known about the nature of established biofilms *P. aeruginosa* on host tissue. The limitations of the current co-culture models used to the study the interaction is limited by differences in growth profile of bacterial cells and mammalian cells (Ciofu *et al.*, 2001; Magana-Ortiz *et al.*, 2018). The other limitation is that biofilm co-culture investigations are solely focused on a single biotic cell line while the bacterium is capable of forming biofilms on different areas (tissues) of the host. Results obtained in this study show that the behaviour of *P. aeruginosa* differs between strains as well as surfaces. Although research conducted on abiotic surfaces provides relevant *in vitro* data from a pharmacological perspective, it fails to consider the complexities of host-pathogen interaction and subsequent difference in biofilm formation and virulence production. Therefore, this study aims to differentiate the interactions of biofilm formed by different phenotypes of *P. aeruginosa* against two commonly afflicted host tissue (lungs and skin).

Unlike a flow system, static co-culture has some limitation pertaining to depletion of nutrients and accumulation of waste products in the growth culture that cannot be controlled as in a flow-cell co-culture model. It is well established that biofilms form mature 3D structures in a shorter period of time under static condition compared to flow conditions (Musken *et al.*, 2010). Therefore, the time available to study the interaction between biofilm formation and biotic surfaces is limited due to host cell lysis by the virulence factors produced by *P. aeruginosa*. However, one of the advantages of this study is the utilisation of three distinct strains of *P. aeruginosa* along with the lab strain PAO1. This was beneficial in reflecting a range of possibilities of biofilm formation, composition and virulence production. PAO1 is convenient to use and offers a good comparison for initial

characterisation due to the availability of gene and protein expression data for the strain. It also allows for better comparison with previous studies.

Studies have shown that there is an inverse relationship between the production of Psl and alginate (Ghafoor *et al*., 2014), this was reported in chapter 5 while examining the bacterial response and biofilm formation towards combination treatments involving farnesol, tyrosol, tryptophan and CCE. Alginate and Psl are known to form scaffold for the biofilm and maintain its structure (Franklin *et al*., 2011). A similarity in composition between Psl and alginate arises as both contain mannose as subunits (Mann and Wozniak, 2012). Therefore, decreasing amounts of Psl frees up mannose precursors for alginate production (Ma *et al*., 2012). Strains that produce Psl are generally found to overcome hurdles preventing initial attachment to a substratum, as all the three strains used in this study produced significant amounts of biofilms, it is probable that high quantities of Psl were present in the EPS along with the other polymeric substances. As mentioned previously, the initial production of Psl is regulated inversely with alginate production, hence we see lower quantities of biofilm biomass formed by the non-mucoid strain which should primarily be made up of Psl polysaccharides. As alginate is generally produced in larger quantities than Psl, an increase in biofilm biomass is observed over time with the alginate producing strains (Limoli, Jones and Wozniak, 2015). However, the production of alginate differs when comparing biofilm growth on abiotic surfaces compared to biotic surfaces. Figure 6.4 and 6.5 show a significant increase in alginate content of the biofilms grown on biotic surfaces (A549 and HaCaT cell lines) in comparison to alginate content on abiotic surfaces (chapter 4 figure 3.7). However, with individual and combination treatment using farnesol and tyrosol, the total carbohydrate content as well as alginate contend reduced significantly in co-culture with A549 and HaCaT cell lines. Between the two cell lines, biofilms grown on A549 generally showed a greater EPS content.

The difference in gene expression was analysed for each individual strain against individual and combination treatment grown on both the cell lines. Results showed that in PAO1, the QS regulatory genes LasI/R and RhlI/R (figure 6.10) were over-expressed regardless of the treatments on *P. aeruginosa* PAO1 when grown on HaCaT cell lines. However, in the case of A549 cell line, farnesol downregulated the expression of LasR while the others were upregulated. Analysis of virulence showed that rhlAB was upregulated with treatment involving tyrosol and the combination while all the others were downregulated. Treatment involving farnesol only, saw to a reduction in rhlAB activity. In the case of PAO1 biofilm

on HaCaT cells, toxA was upregulated with tyrosol and combination treatment, however, it was downregulated with the use of farnesol alone. Similar trend was observed with LasB, while aprA and rhlAB were downregulated.

In the case of NCTC 10662 biofilms, RhlI/R gene expression was upregulated with tyrosol and combination treatment. While the use of farnesol significantly downregulated RhlI/R gene expression (figure 6.11). However, use of farnesol saw to an upregulation of LasI/R gene expression while tyrosol and combination treatment resulted in downregulation of LasI/R gene expression. When PAO1 biofilm were grown under the same conditions but on HaCaT cells line, all the QS regulatory genes as well as the virulence genes were downregulated.

RBHi showed a significant increase in LasI/R and RhlI/R gene expression. In the presence of tyrosol when grown on A549 cell line while treatment with farnesol and in combination with tyrosol showed downregulation of the genes. Genes responsible for toxA, rhlAB, lasB showed an increase in gene expression with all the treatments with the exception of farnesol against lasB (figure 6.12). However, RBHi growth on HaCaT cell line revealed that farnesol upregulates the expression of RhlI while supressing the expression of RhlR and the inverse was true in the case of tyrosol. In combination, a significant reduction in QS gene expression was observed. Genes responsible for virulence were downregulated as well, very unlike biofilm growth on A549 cell line.

Cell viability assay involving MTT reagent was performed on A549 and HaCaT cells to see if farnesol and tyrosol displayed any antagonistic activity at the concentrations used (figure 6.1). The assay did not show decrease in cell viability of both A549 and HaCaT cells lines. However, MTT assay did reveal a significant decrease in cell viability of both the cell lines when grown in co-culture with *P. aeruginosa*. A greater reduction in overall cell viability was seen with HaCaT cells in comparison with A549. In order to understand the relevance of these findings, assays were performed to identify bacterial adherence pattern to the mammalian cell lines as well as the ability of the bacterial cells to internalise into the epithelial cells.

Bacterial adherence to A549 and HaCaT cell line was determined by spot-plating and by determining the ratio between the number of bacterial cells in the inoculum to the number of bacteria adhered to the mammalian cells. The results showed that the treatments involving

a combination of farnesol and tyrosol prevented bacterial adherence significantly compared to the control. While the percentage of adhered bacteria was not as high as expected, especially with the CF isolate on the A549 cell line, it was promising to see the antagonistic effect of farnesol and tyrosol on reducing adhesion (figure 6.2).

Internalisation of bacterial cells into the epithelium is in a way beneficial for the host early in the infection as it triggers inflammation and an immune response (Lippmann *et al*., 2015). Results indicated a higher percentage of internalisation compared to adherence of *P. aeruginosa* onto A549 and HaCaT cell lines (figure 6.3). Though this process was inhibited in NCTC 10662 by all the treatments, only the combination treatment of farnesol and tyrosol was responsible for a significant reduction in internalisation in A549 cells. NCTC 10662 showed a decrease in internalisation in HaCaT cells too but was not found to be significant. PAO1 and RBHi were affected by the combination treatment when grown on A549 cell line but remained unaffected when grown on HaCaT cell line. This indicates that the potential virulence mechanism of internalisation is more prevalent during initial colonisation compared to adherence.

P. aeruginosa are known to adhere to epithelial cells as well as stimulate internalisation as a survival/ defence mechanism against the host immune response (Lepanto *et al*., 2011). They adhere to the epithelial cells with the aid of their flagellum and pili and enter the cells through receptor mediated endocytosis (Cossart and Helenius., 2014). Studies conducted have shown that the importance of internalisation lies in aiding the survival of the bacteria within the host tissue by evading the killing mechanism of effector cells (Lippmann *et al*., 2015) and to kill the epithelial cells and also to propagate after any antibiotic therapy which aids in re-establishing the infection. The decrease in adherence may be attributed to the secretion of exotoxins through the type III secretion system which disfigures and damages the epithelial cells by causing cytoskeletal rearrangement and results in cell lysis (Galle, Carpentier and Beyaert, 2012).

Bacterial infection mediated by biofilm formation has been found to stimulate to strong IL-8 response (John *et al*., 2010). The production of IL-8 by the epithelial cells is an important indicator of host response and production of IL-8 is generally associated with acute and chronic infections. Treatment with farnesol and tyrosol showed that in co-culture with A549, the production of IL-8 reduced with individual and combination treatments for NCTC 10662 co-culture. While a decrease in IL-8 was observed in PAO1 on A549 cell line, it was not

significant. In the case of HaCaT cells, reduction of IL-8 was not observed under any set of treatments with all the three *P. aeruginosa* strains. However, the basal level of IL-8 secretion for both the cell lines (A549 and HaCaT) was very low in the absence of stimulation along with low levels of apoptosis. This is generally expected of cell lines and were therefore found suitable as mammalian cell models for this study.

There are numerous bacterial secreted factors that stimulate IL-8 production from host tissue (Kobayashi, Malachowa and DeLeo, 2018). However, the presence of flagellin, lipopolysaccharides, and the constant production and release of AHL molecules have identified as the main causes of IL-8 secretion by mammalian cells in the presence of a biofilm related infection (Li and Tian, 2012). The co-culture model set up in this study confirmed that biofilm related infections stimulate increased IL-8 production from epithelial cells (figure 6.9 and 6.10) as seen *in vivo* in chronic infections. However, the use of farnesol and tyrosol did not mitigate the inflammatory response and significant decreases in IL-8 secretion was not observed. This might be due to the static nature of co-culture as well as farnesol and tyrosol being fungal QS molecules. Therefore, they may bring about a similar reaction and increase secretion of IL-8 by epithelial cells. However, at low quantities the did not contribute towards increased production of IL-8. The limitation of this assay to justify the antagonistic effect of farnesol and tyrosol and in combination treatment arises from its static growth model. To verify the effect of farnesol and tyrosol against *P. aeruginosa* biofilm inhibition, this should be performed in a flow model, similar to a microfluidic chip or by employing 3D tissue culture of mammalian cells to create a robust co-culture model.

In conclusion, this study offers the foundation for examination of host-pathogen interaction between phenotypes of the same strain of bacteria on various tissues *P. aeruginosa* might infect. As well as an insight into the differences in responses to environmental stimuli as well as antagonist chemicals towards biofilm formation and composition. There is a need for therapeutic intervention to aid in combating biofilm mediated infections. Therefore, investigating gene expression levels of bacterial communication, virulence production in a host-pathogen model has added to the present knowledge about development of possible novel therapies. This study uncovered key differences in pathogenesis of biofilm formation by three strains of *P. aeruginosa* with differing phenotypes in co-culture with two distinct epithelial cell lines. The future adaptations of this model may better represent *in vivo* conditions of infection to delineate host-pathogen interactions to identify suitable novel therapeutic regimens to combat biofilm formation.

Chapter 7 . General discussion

The ability of *P. aeruginosa* to form biofilms differs among strains and is highlighted in the composition of the EPS and the structure of the biofilm which is generally influenced by growth conditions and availability of nutrients. In addition to external stimuli influencing biofilm formation, the presence or absence of ancillary factors such as flagella, fimbriae production of polysaccharide matrix can influence the development of biofilms and can also define its total biomass (Berne *et al*., 2015). As an opportunistic pathogen, the primary virulence factor of *P. aeruginosa* that is responsible for chronic infections is its ability to form biofilms which in turn is mediated by its ability to coordinate gene expression through cell density dependant process of QS (Sousa *et al*., 2014). Formation of biofilm confers the bacteria with the added ability to withstand conventional antibiotics and become less susceptible to treatment regimens (Lewis, 2001) leading to the onset of antibiotic resistance. The aim of this study was to investigate the structure of biofilm formation by non-mucoid and mucoid strains of *P. aeruginosa* and subsequent inhibition and dispersal of biofilm through the application of QQ and biofilm dispersal agents as individual and synergistic treatments.

The results showed that composition of growth medium influences the structure of the biofilm formed by *P. aeruginosa*. The effect of divalent cations (Mg^{2+}, Zn^{2+} and Mn^{2+}) was investigated on biofilm formation and structure of three strains of *P. aeruginosa* with variation in their ability to secrete extracellular polysaccharide. Along with biofilm structural studies involving the effect of divalent cations, biofilm dispersal effect of erythromycin, a macrolide and an α-amino acid (tryptophan) was conducted. Results from chapter 3 demonstrated the varying degree of biofilm formation and changes in structural composition of the three distinct phenotypes of *P. aeruginosa* depended on the level of water of hydration of metallic salts. Metallic salts with a single anion were able to readily provide a freely available Mg^{2+} divalent cation from a chloride salt when compared to a sulphate salt, which resulted in a shorter doubling time of bacterial growth leading to robust biofilm formation. Effect of Zn^{2+} as a sulphate salts did not show any inhibitory effect against biofilm formation. Numerous publications report the antagonistic effect of ZnO-NP against *P. aeruginosa* biofilm formation, as mentioned in chapter 3. However, this effect might be limited to metal oxide nanoparticles only. A study conducted by Hoseinzadeh, Alikhani and Samarghandi, in 2013, suggested that the bactericidal properties of ZnO-NP arises from increased electrostatic interactions between the bacterial cell surface and the ZnO-NP. The

increased rate of electrostatic interactions results in the production of ROS resulting in cell damage and disruption of metabolic activities. However, the use of $ZnSO_4$ in this study lead to the increase of biofilm formation of *P. aeruginosa*. These finding agree with a study conducted by Marguerettaz *et al* in 2014 which showed that elevated levels of Zn in the lungs of CF patients resulted in an increase in biofilm formation and virulence. The study was conducted *in vitro* with artificial sputum media supplemented with $ZnCl_2$. Therefore, it could be hypothesised that the antagonistic effect of Zn^{2+} is limited to metallic oxide salts.

Bacterial growth medium supplemented with D- tryptophan and in combination with erythromycin inhibited biofilm formation significantly in all the three strains of *P. aeruginosa*. The synergistic use of D- tryptophan and erythromycin yielded the highest biofilm inhibition. Modulation of bacterial cell motility was the primary mode of action of inhibition of biofilm formation by tryptophan as a non-motile state is required for *P. aeruginosa* to form biofilm (Brandenburg *et al*., 2013). Use of macrolides (azithromycin) to treat *P. aeruginosa* infections had been documented in the study conducted by Bala, Kumar and Harjal, 2011 to treat UTI infections. Similarly, there are numerous studies related to the use of erythromycin to treat lower respiratory infections in chronic obstructive pulmonary diseases (Tsang *et al*., 2003; Morita, Tomida and Kawamura, 2013). These studies show that erythromycin affects *P. aeruginosa* infection by perturbing the cell's morphology. However, there was no penetration of erythromycin into the respiratory mucous and airway (Tsang *et al*., 2003). Due to this, the treatment regimen requires long term use of the antibiotic hence, the possibility of onset of antibiotic resistance should be considered. Macrolides have also been documented to inhibit QS in *P. aeruginosa* by inhibiting the expression of small RNAs *rsmY* and *rsmZ* (Perez-Martinez and Haas, 2011). The mode of action of QS inhibition by azithromycin, as explained by Kohler, Dumas and Van-Delden, in 2007, stated that interaction between the antibiotic and ribosomes is vital for inhibiting QS in *P. aeruginosa*. Based on this, the use of tryptophan and erythromycin can be beneficial in overcoming any limitations posed by their individual use to treat *P. aeruginosa* infections. It also provides an opportunity to repurpose the use of the amino acid and the antibiotic as a synergistic application for novel treatment regimen.

Adhesion of bacterial cells is a pre-requisite for colonisation of surfaces by bacteria. Numerous physiochemical interactions between microorganisms and the substratum surface regulate bacterial attachment onto a surface (Tuson, and Weibel, 2013). If the physiochemical properties of a surface prone to bacterial attachment can be controlled, then

eradication of biofilm can be managed (Lewis *et al.*, 2001; Donlan *et al.*, 2002). Surface free energy is known to be essential for initial attachment of bacterial cells (Garrett, Bhakoo and Zhang, 2008) and it is affected by the surface structure, imperfections as well as the conditioning layer of the substratum which is defined by the surrounding medium (Preedy *et al.*, 2014). Also, the difference in surface hydrophobicity plays a crucial role in attachment which is affected by the conditioning layer (Lorite *et al.*, 2011).

Studies conducted under static and dynamic flow condition show how the different phenotypes of *P. aeruginosa* form biofilms differently when grown on the same surface material and treated with different biofilm dispersal agents. Tryptophan, anthranilate and CDA were used for their *P. aeruginosa* biofilm dispersal properties (Vital-Lopez, Reifman and Wallqvist, 2015). Anthranilate was found to modulate bacterial cell behaviour between planktonic and sessile mode of life and which yielded high planktonic cell release from the biofilm and a decreased number of sessile cells in the biofilm under flow conditions. Individual treatment and combination treatments of the biofilms formed by *P. aeruginosa* yielded varying results between the strains which highlighted how different phenotypes of the same bacteria respond differently to the same treatment. The total carbohydrate, alginate, protein and eDNA content of the non-mucoid and mucoid strains differed consistently with untreated growth under flow conditions. The surfaces chosen for this study included PDMS for flow conditions and glass and stainless-steel for static conditions as they represent the common surfaces in a hospital setting were biofilms by pathogens may form and subsequently cause cross contamination and infections. The results involving flow conditions in a transmission flow-cell indicate that the non-AHL QS network of *P. aeruginosa* can be exploited by the use of anthranilate and tryptophan to induce biofilm dispersal and reduction in virulence of *P. aeruginosa* as highlighted in chapter 4.

Under static condition, all the three phenotypes of *P. aeruginosa* formed biofilms with a greater biomass on stainless steel compared to glass. The subsequent inhibition/ dispersal was more effective with the use of CDA under static conditions in dispersing sessile cells at different time points. Based on the results obtained, it is clear the heterogeneity of bacterial cell present within the biofilm pose different profiles of growth and virulence. Therefore, the use of biofilm dispersal agents should accompany modified surfaces to prevent biofilm formation and prevent nosocomial infections.

Chapters 5 and 6 compare the effect of tryptophan, farnesol, tyrosol and *B. licheniformis* cure cell extract as possible QQ agents against *P. aeruginosa* biofilm formation and subsequent inhibition. Also, the inclusion of biotic surfaces in chapter 6 provided an insight into the differing behaviour of *P. aeruginosa* in its ability to form biofilm and the structural composition of the biofilm on biotic and abiotic surfaces. The relative mRNA expression of the QS system and virulence factors of *P. aeruginosa* changed with the use of biotic and abiotic surfaces. Farnesol and tyrosol were found to have antagonistic activity towards the QS regulatory system of *P. aeruginosa* as well as the virulence factors under *in vitro* conditions on abiotic surface. Farnesol and tyrosol were also shown to inhibiting the expression of MexAB-OprM efflux pump system in *P. aeruginosa* which is one of the primary intrinsic drug resistance features of the bacterial cell. This was a promising finding in combating antibiotic resistance commonly observed in Gram-negative pathogens. Overall motility of the three phenotypes of *P. aeruginosa* were affected by the use of farnesol and tyrosol. Though all the treatments significantly reduced the motility of *P. aeruginosa*, the heavily mucoid strain was always found to be the most motile of the three strains.

When biofilms of the three strains of *P. aeruginosa* were grown on A549 and HaCaT epithelial cell lines, they showed different biofilm profiles with and without treatment when compared to abiotic surfaces. The carbohydrate content of the strains increased in the presence of mammalian cells. The use of farnesol and tyrosol individually and in combination showed antagonistic activity against *P. aeruginosa* in co-culture with mammalian cells, however the antagonistic effect of farnesol and tyrosol were not as enhanced as when used in *in vitro* condition with biofilms developed on abiotic surfaces.

Relative mRNA expression showed a general upregulation of the QS genes and virulence factors compared to the control and with treatment when grown on A549 cell lines. However, the gene expression was influenced based on the mammalian cell used. Co-culture of *P. aeruginosa* and HaCaT cell line showed a general decrease in gene expression, especially in the case of *P. aeruginosa* RBHi when treated with farnesol and tyrosol combination.

A clear pattern can be seen in the way the structure of the biofilm and its environment may aid in biofilm development based on changes in gene regulation. This study also shows interspecies interaction based on biotic surface growth and influence of mammalian cells in *P. aeruginosa* biofilm development. Also, as farnesol and tyrosol are QS molecules of *C. albicans*, it is clear that they influence *P. aeruginosa* biofilm formation. As *P. aeruginosa*

and *C. albicans* are generally isolated together from CF patients, it is becoming clearer with this study on the way the bacteria, yeast/fungi and mammalian cells interact with each other during infection and colonisation by microbes.

As it is abundantly clear that CF is generally considered to be polymicrobial in nature (Peters *et al*., 2012; Filkins and O'Toole, 2015) there is a need to understand the structure and development of *P. aeruginosa* biofilm formation by employing a multidimensional approach in order to understand the myriad of variation at play during an actual infection. This study contributes towards that view. Though much work needs to be done to get a better understanding of the way microbes interact with each other as well as mammalian cells during infection, this study highlights the importance of approaching the subject of biofilm inhibition and eradication from a multidimensional perspective.

Chapter 8 . Conclusion

Development of biofilms pose a major threat to the healthcare sector, food processing sector as well as the industrial sector with significant economic implications. Often, there is a lack or shortfall of effective treatment regimens due to the high possibility of failure as biofilms act as a protective shield against antibiotics by raising the minimum dose required for treating biofilm related infections. Research towards development of novel therapies to treat biofilm related infections is essential and the increasing threat of multi drug resistance has urged the scientific community to explore alternatives to conventional antibiotic treatments.

As biofilm formation in *P. aeruginosa* is mediated by the community driven, interconnected hierarchical quorum sensing network, targeting the ability of the pathogen to communicate has emerged as a suitable approach towards attenuating the pathogenicity of *P. aeruginosa*. Rather than killing the pathogen, arresting its ability to communicate is a more viable technique due to biofilm formation. This approach is commonly known as quorum quenching.

This study employed the use of three distinct phenotypes of *P. aeruginosa* to investigate how QS influences biofilm formation and the efficacy of QQs on inhibiting biofilm formation. The studies revealed that though all three strains formed significant amounts of biofilm, their response towards environmental stimuli, growth medium composition, attachment surfaces played a significant role in the structure and architecture of the biofilm. As *in vitro* development of biofilms in microtiter plates under static conditions is not representative of biofilm formation by *P. aeruginosa* in its natural habitat, a transmission flow-cell developed by Biosurface technologies was adapted to demonstrate the capabilities of biofilm dispersal and QQ agents on non-mucoid, mucoid and heavily mucoid strains of *P. aeruginosa*. The resulting data obtained through the use of the transmission flow-cell was the first time the instrument has been used to compare biofilm formation by three strains of *P. aeruginosa* with differing abilities to form biofilms. Thus, the data obtained against each of the strains can be normalised to one equipment rather than collating *in vitro* data from different sources to conclude and understand the mechanism of biofilm formation and subsequent inhibition/ eradication.

This study also provided evidence that repurposing antibiotics and naturally available/ produced products from the general *in vivo* growth environment of *P. aeruginosa* can be beneficial in targeting biofilms dispersal. It also highlighted the importance of the non-AHL

PQS QS system in *P. aeruginosa* and its role in regulating biofilm formation by the three phenotypes of the bacteria. Furthermore, the use of *B. licheniformis* NCIMB 8874 crude cell extract to inhibit biofilm formation in *P. aeruginosa* highlighted the effect of bacterial cross talk in the natural environment. Since there are numerous studies on the presence of AHL-degrading enzymes produced by Bacillus sp., the crude cell extract of *B. licheniformis* was sought for QQ studies. It has been reported that *B. licheniformis* NCIMB 8874 produces ComX pheromone (Esmaeilishirazifard *et al.*, 2017), its own cell density dependant QS molecule. The potential of interaction between ComX pheromone produced by *B. licheniformis* and *P. aeruginosa* QS system should be investigated further. Similarly, the QS molecules of *C. albicans*, farnesol and tyrosol were used in this study. As *C. albicans* and *P. aeruginosa* are two of the primary isolates from the lungs of CF patients, the interaction between farnesol and tyrosol was investigated as potential inhibitors of biofilm formation by *P. aeruginosa* The QQ and biofilm dispersal capacity of the chosen inhibitors was examined as individual and combination treatments under different experimental settings. The synergistic use of combination of QQ proved to be highly beneficial in inhibiting biofilm development.

Through gene expression studies it was revealed that farnesol and tyrosol selectively affect the LasI/R and RhlI/R QS system of *P. aeruginosa* as well as the expression of virulence factors. The antagonistic action of farnesol and tyrosol against efflux pump (MexAB-OprM) mediated intrinsic drug resistance mechanism of *P. aeruginosa* revealed that both the fungal QS molecules play an active role in downregulating the expression of multidrug efflux pump system in *P. aeruginosa*, which makes it more susceptible to antibiotics. While the primary role of farnesol and tyrosol is the modulation of yeast to hyphal change in *C. albicans* to produce biofilm, they display an antagonistic activity against *P. aeruginosa* biofilm formation by targeting the LasI/R and RhlI/R QS regulatory genes.

The study also highlighted the capacity of farnesol and tyrosol to modulate the structure and composition of *P. aeruginosa* biofilms showing direct antagonistic effect against the production of carbohydrates, proteins and eDNA which are the three primary components of *P. aeruginosa* biofilms. It was also shown that combination of *B. licheniformis* crude cell extraction and tyrosol aided in reducing the hydrophobicity of *P. aeruginosa* planktonic cells which in turn would prevent their attachment onto hydrophobic surfaces. However, it was also found to decrease hydrophobicity of only planktonic cells and did not have an effect on sessile cells extracted from the biofilms. This is beneficial for preventing initial attachment.

It was also shown that farnesol and tyrosol affect the motility of *P. aeruginosa* by antagonising the flagellum and pili that govern cellular movement.

Co-culture studies involving A549 and HaCaT cell line showed that the biofilm structure and composition of *P. aeruginosa* is very different than *in vitro* growth on abiotic surfaces. It was also seen that the expression of the QS regulatory system as well as expression of virulence varied with the type of mammalian cells used as biotic surfaces and between strains as well as between QQ treatment involving farnesol and tyrosol. This suggests that developing novel treatments targeting biofilm formation and QS regulatory network should take into consideration that *P. aeruginosa* biofilm formation is dependent on the growth environment and the bacteria is able to adapt very efficiently to overcome any challenges faced.

In summary, this thesis provides evidence of differing architecture of biofilms formed by *P. aeruginosa* and how different phenotypes respond independently towards treatments. The adaptability of the *P. aeruginosa* strains used to the growth environment suggests that a multidimensional approach is required in order to develop effective novel therapies to overcome QS mediated biofilm formation and subsequent infections. Due to the different phenotypes of bacteria found in biofilms, it essential to understand that different phenotypes may respond differently towards treatment, be it novel or conventional.

Chapter 9 . Future work

The use of transmission flow-cell provides an excellent opportunity to compare QS mediated biofilm formation and virulence production by *P. aeruginosa* and also offers the means to test novel QQ and biofilm dispersal agents under dynamic flow conditions. So far comparison of QQs and dispersal agents on inhibiting/dispersing biofilms has been limited to separate investigation of *in vitro* abiotic and biotic surfaces due to the lack of a suitable flow-cell model to incorporate both aspects of the work. The draw-back of working with two separate models is the inability to investigate biofilm formation and virulence characteristic seen during infections and to recreate similar environment under *in vitro* conditions (Caraher *et al*., 2008; Krapp *et al*., 2017). As bacteria adapt to the growth conditions over time, they develop altered sensitivities towards QS molecules resulting in differential expression of genes regulating QS and virulence (Alasil *et al*., 2015; Omar *et al*., 2017), recreating an infection environment under flow conditions in one model involving mammalian cells would provide the opportunity to uncover new mechanisms of virulence and biofilm development which would aid in selecting appropriate QQ treatments. Though the use of A549 and HaCaT cell lines has been ideal for development of new methods of investigating infections and treatment, in the long run the use of primary cell lines isolated from CF patient lungs as well as burn wound cell lines from *P. aeruginosa* biofilm infection would be more representative of the natural environment. Thus, the data collected on biofilm inhibition/dispersal and eradication using QQ and dispersal agents under *in vitro* conditions would be representative of the actual site of infection and would be applicable towards developing treatment regimens. Use of *ex vivo* tissue samples would aid in recreating physiological conditions of the infection sites as well. The benefit of adapting to such a system of investigation is that as they have already been used for assessing planktonic models, they will require few modifications and optimisation studies.

As the growth of the cell lines and co-culture models required the use of DMEM medium, developing and adopting a more physiologically relevant environment with the use of artificial sputum medium for mammalian and bacterial co-culture model would display an altered gene expression (based on chapter 5 and chapter 6 in this study) which would aid in collecting relevant *in vitro* data obtained under *in vivo* conditions for developing strategic treatment regimens. It would also aid in studying the polymicrobial nature of most biofilm-related infections. Finally, use of microfluidics paired with image analysis to investigate the temporal dynamics of biofilm development would be beneficial in attaining further understanding of biofilm formation and inhibition as it would aid in exploring the biofilm

microenvironment and very specific cell-to-cell interactions as well as cell to surface interactions.

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Appendix

A) Standard curves

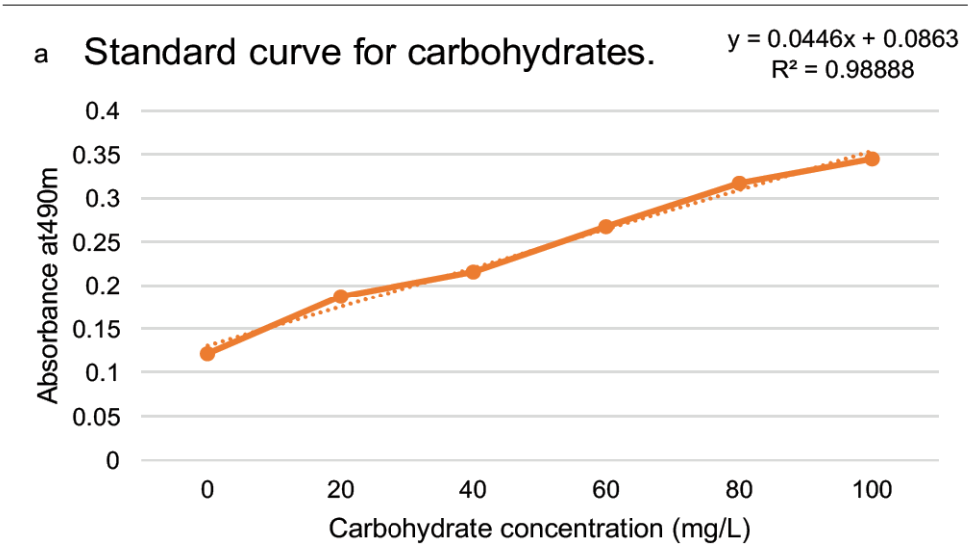

Figure 1: Total carbohydrate assay standard curve

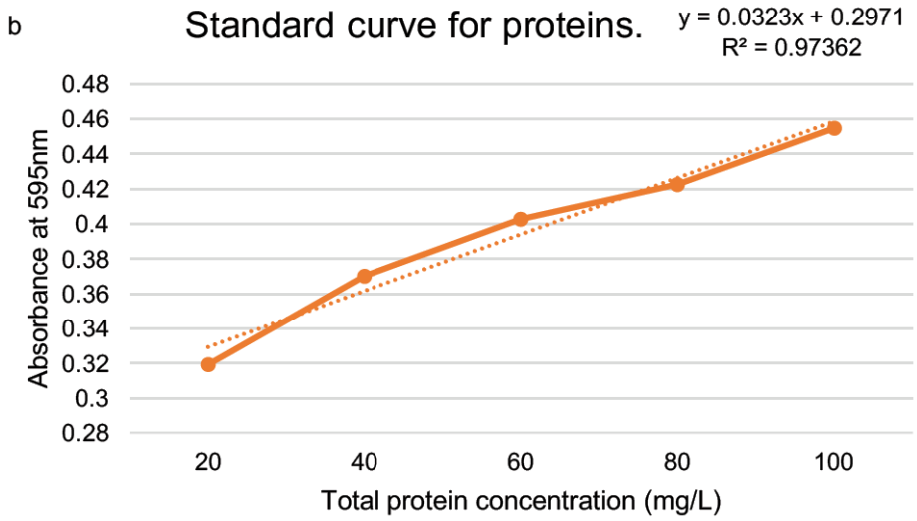

Figure 2: Bradford assay standard curve for protein

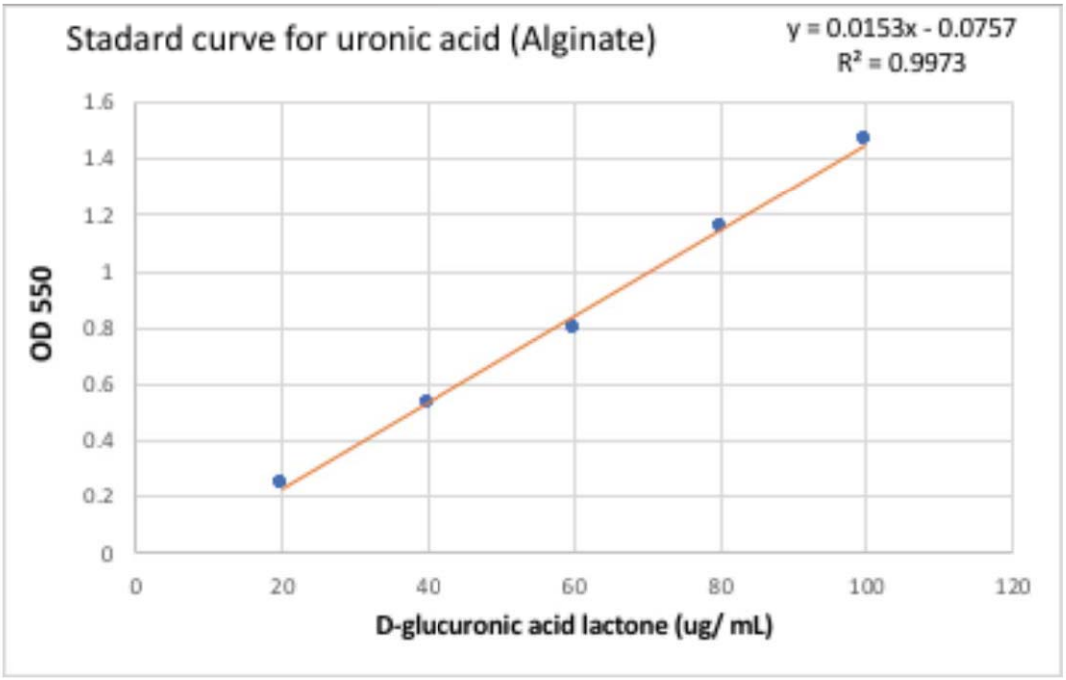

Figure 3: Standard curve for uronic acid assay (Alginate)

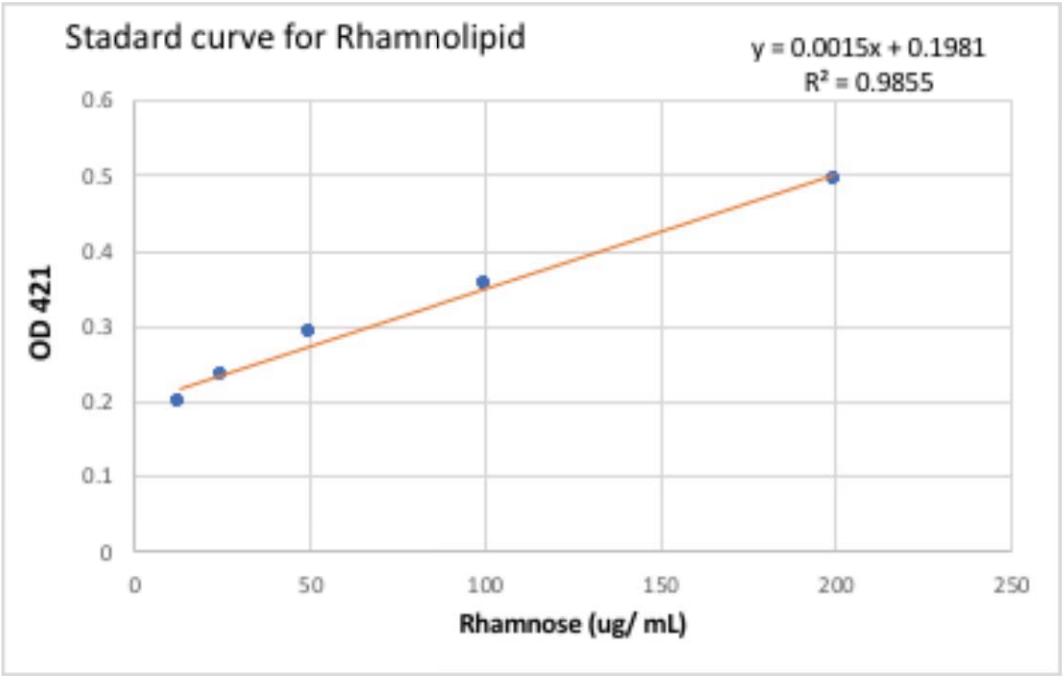

Figure 4: Standard curve for Orcinol acid assay (Rhamnolipid)

B) MIC values for *P. aeruginosa* PAO1

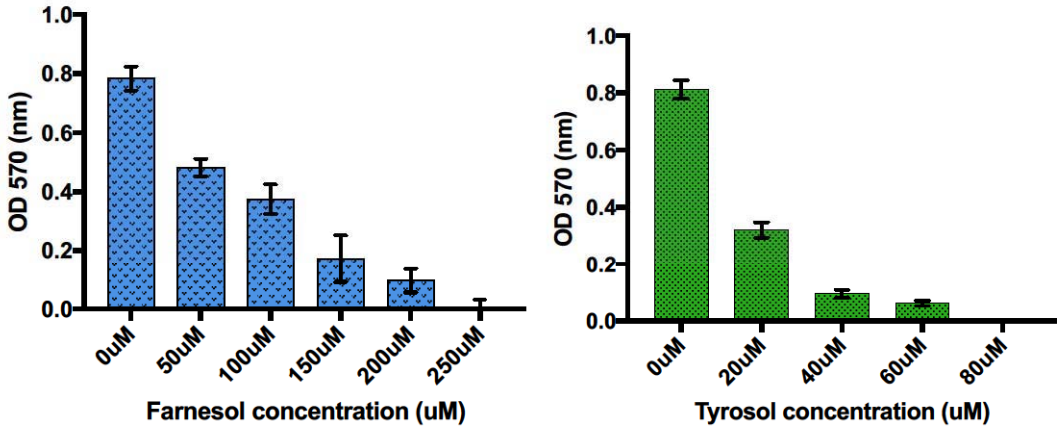

Figure 5: MIC of farnesol and tyrosol

C) MIC of farnesol and tyrosol for A549 and HaCaT cell lines based on the MTT cell viability assay

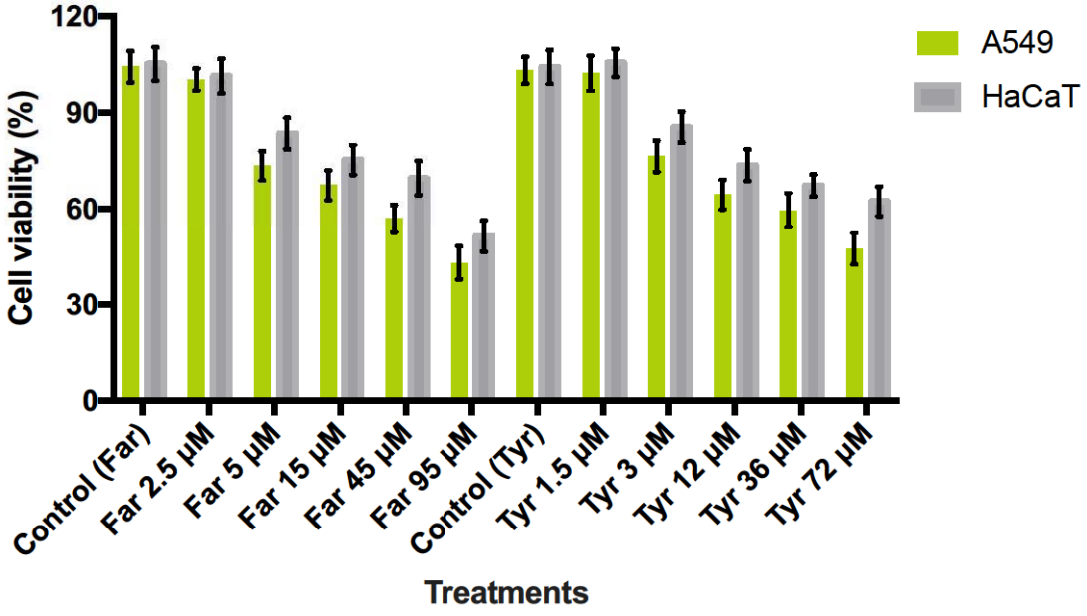

Figure 6: MIC of farnesol and tyrosol used for A549 and HaCaT cell line

C) Template efficiency curves for QS, virulence and efflux pump genes of *P. aeruginosa*

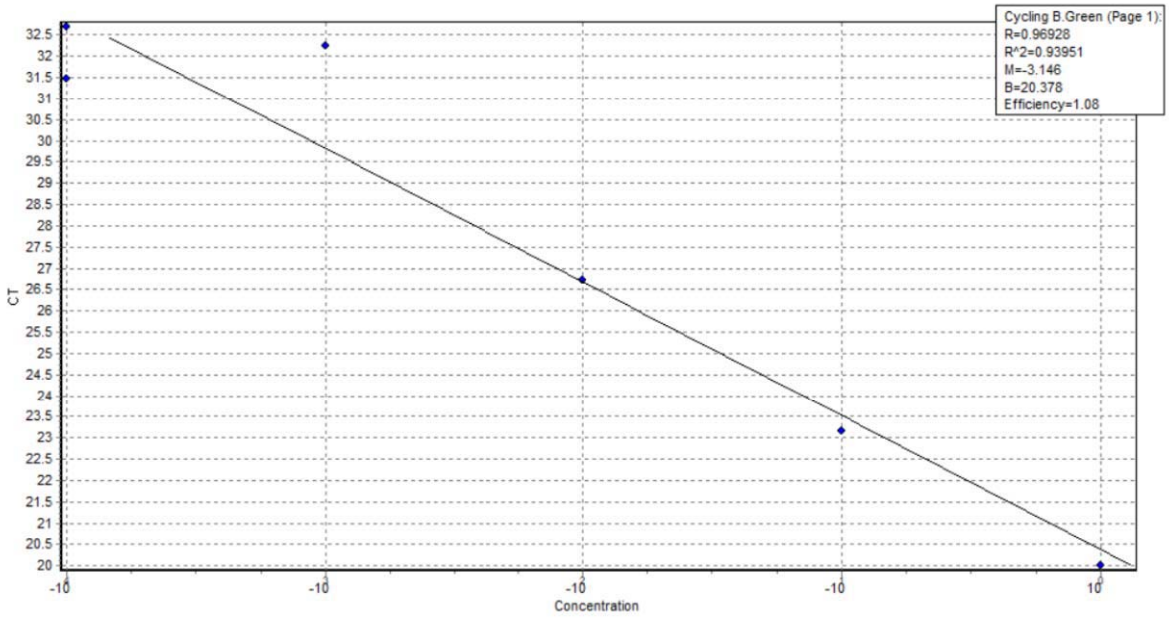

Figure 6: Template efficiency curve (LasI)

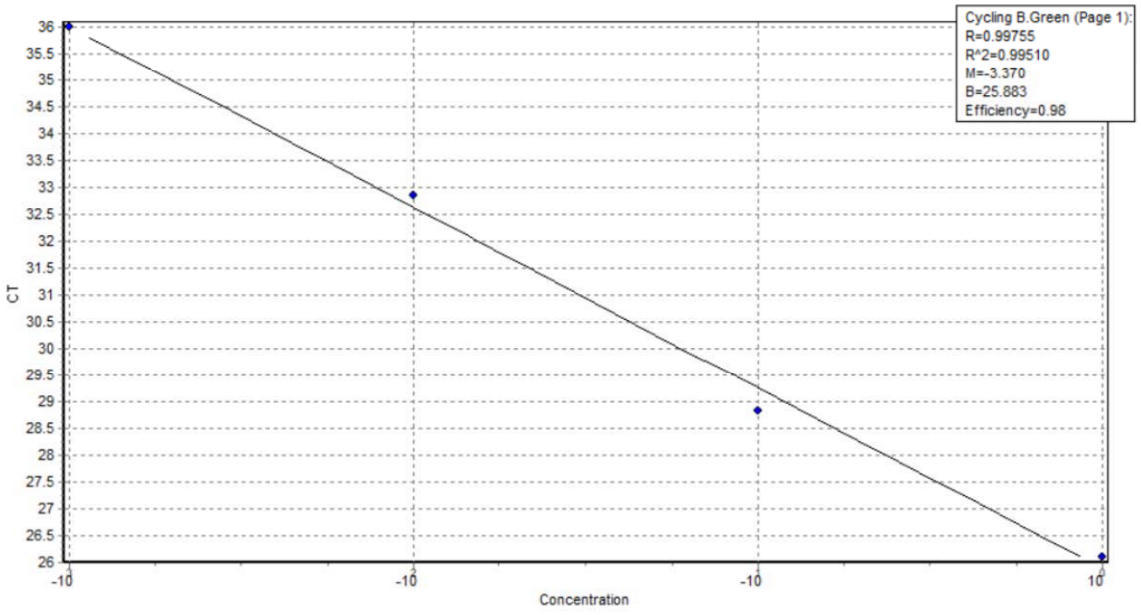

Figure 7: Template efficiency curve (toxA)

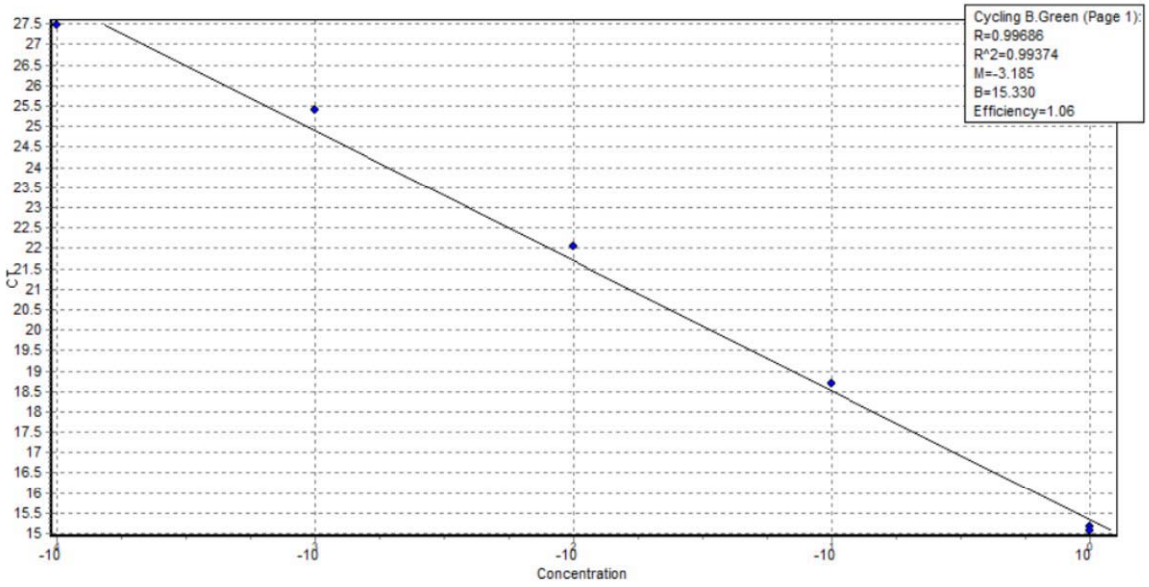

Figure 8: Template efficiency curve (MexA)