**THE PRESENCE OF HONEY TRIGGERS INHIBITION OF *PSEUDOMONAS*
AERUGINOSA VIRULENCE AND METABOLISM**

BY

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ABSTRACT

Honey is an ancient remedy with a high potency against drug-resistant bacteria, which has gained renewed interest in naturopathic medicine for its beneficial effect in treatment and prevention of wound infections. The exact antibacterial property and mode of action of honey is still unknown, however in recent years, there has been various studies focusing on the effect of honey on bacterial gene regulation. With the focus of current literature being at the molecular level, the first aim of this study was to examine the effect of honey at the cell level and its influence on the metabolism of *P. aeruginosa* biofilm. The second objective of this study was to test the influence of the combination of the iron chelating agent (EDTA) and honey on biofilm metabolism. *P. aeruginosa* metabolism in this study was analyzed through (i) siderophore excretion and (ii) monitoring of the biofilm CO₂ respiration rate with a Carbon dioxide Evolution Measurement System (CEMS). The results obtained indicate that honey reduces biofilm metabolism and inhibits siderophore production, while the combination of honey and EDTA has a greater impact on biofilm metabolism, which influences *P. aeruginosa* iron homeostasis, inhibits siderophore production, and increases bacterial recovery time after exposure. However, when provided at concentrations lower than its inhibitory concentration, honey is used as a nutrient source for biofilm development. The results obtained illustrated the importance of the environmental conditions on biofilm metabolism, as the biofilm response varied with minor changes in the composition of their media. In summary, this study showed that biofilm cells shut down their metabolism in the presence of honey, which also inhibits bacterial siderophore production and can play an importance role on the virulence of *P. aeruginosa*.

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I would like to start with this quote that I always admired and remembered since childhood:

“Ali Ibn Abi-Taleb stated to Komaini:

O komaini! Knowledge is better than wealth, since knowledge, is your protector, but you should protect your wealth. If you give from your wealth, it would reduce, but if you give from your knowledge, you would gain more. The position you obtain with wealth might be lost one day. O komaini! , Learning knowledge gives you rewards, and with knowledge human beings (male and female) will know god better, which will help them praise him better. After death you will be remembered (for the knowledge that will be passed on to the next generations). Knowledge is sovereign, and wealth is obedient. (Nahj Al-balagheh, Wisdom 147:2).”

I would like to thank god for giving me the opportunity to pursue my graduate studies and work with fabulous professors Dr. Gideon Wolfaardt, Dr. Otini Kroukamp and great lab members during my graduate studies. I consider myself to be very fortunate of having such great supervisors that guided me throughout my studies and helped me to learn, observe and tackle problems and gaps in scientific research. They have not only taught me scientific principles but also great life lessons that I will carry on through the years.

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LIST OF ABBREVIATIONS

AH	Artificial honey
AHL	Acylhomoserine lactone
A _w	Activity of water
CEMS	Carbon dioxide evolution measurement system
CF	Cystic fibrosis
CH	Clover honey
CRBI	Catheter-related blood stream infections
CRI	Catheter related infections
CSE1034	Ceftriaxone and sulbactam
DI	Distilled water
DFO	Deferoxamine
DSX	Defrasirox
EDTA	Ethylenediamine tetraacetic acid disodium
HAIs	Healthcare-associated Infections
Hb	Hemoglobin
Lf	Lactoferrin
LPS	Lipopolysaccharide
MDR	Multidrug resistance
MH	Manuka honey
MRSA	Methicillin-resistance <i>Staphylococcus aureus</i>
MSSA	Methicillin-sensitive <i>Staphylococcus aureus</i>
NRPS	Non-ribosomal peptide synthetases
QS	Quorum sensing
PVD	Pyoverdine
PA	<i>Pseudomonas aeruginosa</i>
Pch	Pyochelin

Tf	Transferrin
TSA	Tryptic Soy Broth Agar
TSB	Tryptic Soy Broth
UTIs	Urinary tract infections

CHAPTER 1

Introduction and Objectives

Medicinal properties of honey

Honey is an ancient remedy used for treatment of infected wounds, which was reintroduced into modern medicine during the 21st century (Al-Waili et al., 2005; Al-Waili et al., 2011; Israili, 2013). The beneficial use and consumption of honey date back to 5500 BC, and the nutritional and medicinal quality of this compound has been documented in Hindu, Greek, Roman, Jewish, Christian, Islamic and other faiths (Israili, 2013). Health benefits of honey have been reported in a variety of conditions including treatment of skin (Fidaleo et al., 2010; Israili, 2013), eye disease, respiratory diseases (Sio et al., 2006), urinary diseases (Al-Wali, 2005), gastrointestinal diseases, skin ulcers, psoriasis, and eczema (Daglia et al., 2013; Al-Wali et al., 2003; Allen et al., 1991). Honey is known to inhibit reactive oxygen species (Mesaik et al., 2008; Erejuwa, 2012), and inhibit quorum sensing (QS) molecules in a biofilm (Lee et al., 2011; Truchado et al., 2009- A; Truchado et al., 2009- B). Studies have also shown that honey exhibits a broad spectrum of antibacterial activities that can inhibit growth of more than 80 species of pathogenic bacteria including methicillin-resistant *S. aureus* (Jervis-Bardy, 2011; Maddock et al., 2012; Stover et al., 2000; Truchado et al., 2009), methicillin-resistant *Staphylococcus epidermis* (Truchado et al., 2009), *Pseudomonas aeruginosa* (Singh et al., 2000; Schalk and Guillon, 2012) and *Escherichia coli* to mention a few.

Disease, wounds and biofilm resistance

Bacterial cells in the form of biofilms which is defined as establishment of microorganisms as communities (Abdallah, 2011), can be up to a thousand times more resistant to antibiotics and disinfectants when compared to planktonic cells (Mah et al., 2003). The formation of *P. aeruginosa* biofilm is the main cause of the intrinsic resistance of this bacterium to several antibiotics. The main reasons for an increase in antibiotic resistance in biofilms are low antibiotic

penetration in the EPS, expression of various efflux pumps and expression of various antibiotic inactivating enzymes (Storz and Hengee-Aronis, 2000), all of which can result in increased resistance of the bacterium to antibiotics.

For more than half of a century antibiotics have been credited as the foundation of many infectious disease therapies (Sekhri, 2013). The commercial use and availability of manufactured antibiotics date back to the 1950's when scientists and medical practitioners started to realize the beneficial use of antibiotics for treatment of infection, and its power of reducing the rate of human mortality. However, only two decades after the start of antibiotic production, scientists realized that bacterial species are capable of forming resistance to most available antibiotics and overcoming the innovation of modern medicine (Gould and Van-Der-Meer, 2007; Gregoire et al., 2010). The term “antimicrobial resistance” of microorganisms was then defined as the ability of microorganisms to resist the growth inhibitory or killing activity of any anti-microbial beyond the normal susceptibility (Verrases et al., 2013; McDonnell and Russell, 1999).

As stated above, microorganisms use one or a variety of mechanisms to overcome the presence of antibiotics, for example, enzymatic degradation or modification of antibiotics (Verrases et al., 2013). Enzymatic modification of antibiotics is one of the main concerns in treatment of Gram-negative bacteria and is commonly seen in the presence of aminoglycosides or β -lactam antibiotics (Verrases et al., 2013). Gram-negative bacteria are capable of producing β -lactamase enzymes that hydrolyze the β -lactam ring (Levin et al., 1984) or acetyltransferases, nucleotidyltransferases and phosphotransferases that are known to degrade aminoglycosides (Zichichi et al., 2000).

However, studies have shown that the nature of biofilm structure attribute to inherited resistance to antimicrobial agents, whether these are antibiotics, disinfectants or germicides (Donlan and Costerton, 2002). This indicates that exposure of biofilms to high doses or new antibiotics will not necessarily solve the problem of antibiotic resistance.

Due to an increase of antimicrobial resistance to the present synthetic antibiotics, the need for novel therapies has generated renewed interest in natural products exhibiting antibacterial properties, especially when studies have also shown that antibiotic consumption is not without risk to humans (Alandejani et al., 2009).

This has led many researchers to study the effect of medicinal plants for treatment of infection. Although this route has not solved the current problem of antibiotic resistance it has shown some promising results compared to synthetic antibiotics. Many of these traditional medicines have shown to have anti-QS activity. QS is a molecular crosstalk that bacteria use for cell-to-cell communication. Inhibition of this molecule results in loss of the signal and can interfere with biofilm formation and development (Brackman et al., 2009). For example a study by Adonizo et al., has shown that *Conocarpus erectus*, *Chamaesyce hypericifolia*, *Callistemon viminalis*, *Bucida buceras*, *Tetrazygia bicolor*, and *Quercus virginiana* extracts have anti-QS properties and can be used to treat *P. aeruginosa* infection (Adonizo et al., 2008). Traditionally these plants were used to treat respiratory and skin infections.

The underlying reasons attributed to the antimicrobial action of honey were related to high osmolality, acidity, sugar content, viscosity and presence of enzymes. However, the antimicrobial mechanisms of honey have shown to vary among different floral source, which depends on factors such as environmental condition, climate and pollen source of the plant (Azeredo et al., 2003; Maddocks and Jenkins, 2013). Studies have shown that hydrogen peroxide present in almost all types of honey, with the exception of manuka honey, is capable of inhibiting microbial growth and stimulates production of new capillaries in wound tissue (Tur et al., 1995; Bang et al., 2003). In the host system, honey has been shown to stimulate inflammatory cytokines from monocytic cells, and act as an antioxidant at the site of infection. In addition to this, other cationic antimicrobial peptides, such as bee defesin-1, which has been identified in honey are known to have a broad acting contribution to the antimicrobial properties of honey (Maddocks and Jenkins, 2013). In other honeys, such as manuka, bee defesin-1 is modified and changed to phytochemical methylglyoxal (MGO), which is the main active ingredient contributing to the antimicrobial activity of manuka honey (Maddocks and Jenkins, 2013).

Possibility of combination therapies

With an increase in antibiotic resistance to the current available antibiotics, research has shifted to look at a variety of alternative routes to treat infections. One of the recent routes that received interested is combination therapy of honey with antibiotics (Muller et al., 2013). This route of treatment with honey was first tested in 1998, between Indian honey and multidrug resistance

(MDR) bacterial isolates (Karavil et al., 1998). Since then many studies have considered the combination effect of honey and different classes of antibiotics such as gentamicin, amikacin, oxacillin, tetracycline, rifampicin, imipenem and mupirocin (Jenkins and Cooper, 2012; Muller et al., 2013; Karavil et al., 1998). One of the advantages of using this route of therapy is the improved effectiveness of this treatment in the killing of antibiotic resistant bacteria. To date, no bacterial resistance to combination therapy has been reported.

Objectives

The central objective of this research was to test the effect of honey in inhibiting bacterial metabolism. This research is novel with respect to testing the effect of honey on bacterial metabolism through a real-time analysis. Therefore, the goal was to investigate the metabolic response of *P. aeruginosa* biofilms to different concentrations of honey. The research presented in this thesis is divided into two parts, each detailing the research conducted to address a specific objective. Chapters three and four address the two objectives of this study, which are as follows:

Objective 1. To determine the influence of honey on bacterial metabolism through two indirect methods: CO₂ production and siderophore secretion in a real time system.

Objective 2. To test the combined effect of honey and ethylenediamine tetraacetic acid disodium (EDTA) on biofilm metabolism (through CO₂ and pyoverdine secretion).

CHAPTER 2

Literature Review

Honey has been used as a food source as well as for medicinal purposes since ancient times. Recent publications on the beneficial uses of honey for medicinal purposes have rekindled interest in the use of honey as a naturopathic source for treatment of infections such as burns and wounds (Cooper et al., 2010). Especially when in vivo studies have confirmed that 'no bacterial resistance' is formed through the use of this remedy for treatment of infections (Cooper et al., 2010; Lerrer et al., 2007). Numerous studies have confirmed that honey contains both antibacterial and antifungal properties, and is capable of reducing inflammation, stimulating epithelialization and tissue growth, proliferating of phagocytes, B and T lymphocytes (Fidaleo et al., 2010; Israili, 2013; Daglia et al., 2013; Al-Wali et al., 2003; Sio et al., 2006; Jervis-Bardy, 2011; Maddocks et al., 2012; Allen et al., 1991). It is known that honey from varying botanical sources have different levels of antibacterial activity (Israili, 2013).

Honey on its own is not an antibiotic, but its complex mixture of sugars, enzymes and other substances afford these unique biocidal characteristics to honey (Merckoll et al., 2009).

General honey antimicrobial action benefits the host

Studies have evaluated the activity of different types of honey against planktonic and biofilm cultures, concluding that the addition of 5% - 11% honey to media inhibits growth of planktonic cells, and 60-90% honey can inhibit growth of biofilms (Merckoll et al., 2009; Schalk and Guillon, 2012; Singh et al., 2000; Truchado et al., 2009; Van der Strate et al., 2001). No studies have identified the exact antimicrobial mechanism of honey, although various reported factors that contribute to the antimicrobial properties of honey include: the presence of a high sugar content (~80%), minerals and vitamins (Vasil et al., 1991; Singh et al., 2000); its acidity (pH 3.2 - 6); production of hydrogen peroxide due to glucose oxidase (Ahmas et al., 2008; White et al., 1963); osmotic effects, antioxidant contents and stimulation of immunity neutrophils (Ahmed et al., 2011).

A study performed by Truchado et al. (2009) illustrated that chestnut honey could inhibit the production of N-Acyl-L-homoserine lactone (AHL) in a biofilm (Verrases et al., 2013). Also,

in their previous studies, different unifloral honey could inhibit the QS of *Chromobacterium violaceum* (Wahjudi et al., 2013). In addition, through genetic screening of *E. coli*, Lee et al. 2011 have shown that low concentrations of honey in growth media significantly repress genes responsible for motility, QS signals and virulence factors (Adonizio et al., 2008; Wiehlmann et al., 2007). Moreover, other studies have also confirmed that the presence of honey in growth media affects dextran (polysaccharides of glucose) production of *S. mutans*, which in turn affects formation of biofilm and adherence to hard surfaces such as glass and teeth (Fjallman, 2000; Badet and Quero, 2011). Additionally, the vast amount of research performed on different aspects of honey has shown the benefits of using this substance for the treatment of wounds and burns (Blair et al., 2009; Carnwath et al., 2014).

Out of the vast range of available honey, the honey that is mainly used for medicinal purposes and available in various commercial wound dressing is Manuka honey that originates from the manuka tree (*Leptospermum scoparium*), which is mainly found in New Zealand (Israili, 2013). One of the known advantages of manuka honey, is the fact that no bacterial resistance was observed after exposure of the sub-inhibitory concentration of honey to *E. coli*, multidrug-resistant *Staphylococcus aureus* (MRSA), *P. aeruginosa* and *S. epidermidis* (Lusby et al., 2005; Cooper et al., 2010; Müller et al., 2013). In vitro studies have indicated that the antibacterial activity of manuka honey is associated with a high concentration of methylglyoxal (MGO), which is normally derived from the conversion of dihydroxyacetone (Wright and Romano, 2006; Mavric et al., 2008). MGO is a α -dicarbonyl compound and has been widely used in many food products and beverages (Yanaihara et al., 2000; Da Silva Ferreira et al., 2007). A number of investigators suggested that MGO is the main compound that increases the antibacterial properties of honey (Yeterian et al., 2010), whereas other studies reject this phenomenon. Most manuka honey marketed for medicinal purposes has a potency rating based on “Unique Manuka Factor” (UMF) which ranges between 10 - 20 UMF (equivalent to 10 - 20% of phenol activity against bacteria). UMF activity is unrelated to the content of hydrogen peroxide in manuka, as studies have shown that manuka and clover honey have non-peroxide activity (Allen et al., 1991; Carnwath et al., 2013; Majtan et al., 2013), and UMF is primarily based on the *S. aureus* inhibition test (Yeterian et al., 2010).

Honey induce bacterial killing

Although the exact antimicrobial killing effect of honey is still not known, studies at the gene level have attributed this effect to down regulation of important genes, which can mainly be influenced by the actual composition of honey with regards to high acidity, osmolality, and other components (Al-Waili and Saloom, 1999; Al-Waili and Noori, 2004; Alvarez-Suarez et al., 2010). The killing effect of honey has been reported at different concentrations between 25% - 100% (Al-Waili et al., 2013; Israili, 2013), which vary among different bacterial species. Studies on honey have tested the effect of this compound on Gram-positive bacteria such as MRSA, moreover genetic analysis of honey on gram positive and Gram-negative bacteria has shown that treatment of honey on Gram-negative bacteria was as successful as Gram-positive bacteria (Al-Waili and Saloom, 1999; Cooper et al., 2002). A large amount of studies have focused on the effect of honey on *S. aureus* and antibiotic-resistant strains. *S. aureus* isolates have been shown to have a high sensitivity to the presence of honey. In addition, no microbial resistance to honey has been reported to date (Lusby et al., 2005; Cooper et al., 2010; Müller et al., 2013), which could be due to multiple properties of honey presenting too many different pressures for resistance to occur (Maddocks and Jenkins, 2013).

Inhibition effect of honey

Numerous studies have determined the minimum inhibitory concentration (MIC) of honey against different bacterial isolates. MIC studies are mainly used for identifying the minimum concentration that inhibits bacterial growth. In the case of honey, MIC values change among different types and strains of bacterium. The lowest MIC value recorded for manuka honey was at a concentration between 2% to 4% (w/v) honey that inhibited a total of 58 isolates of *S. aureus* (Cooper et al., 1999). Although sensitivity amongst bacterial species was different, the sensitivity amongst stains of the same species are consistent (Maddocks and Jenkins, 2013). In addition, the inhibitory action of honey varies among species and between planktonic cells and biofilms. Earlier studies have shown that honey not only is capable of inhibiting growth of individual cells, but can initiate reduction of biomass in *E. coli* biofilm (Lee et al., 2013).

Honey is known to target different parts of the cell. For example, proteomic studies on MRSA have shown that manuka honey is capable of inhibiting production of universal stress

protein (UspA) up to 16- fold (Jenkins and Cooper, 2011), where this effect causes the bacterium to lose its ability of surviving in the presence of cellular and metabolic stress. A similar response was observed in the case of *E. coli*, where a total of 2% of its genome was up-regulated and 1% was down regulated. Moreover, transcriptome analyses have indicated that down regulated genes in *E. coli* were all associated with stress response and protein synthesis (Blair et al., 2009). Honey has not only been shown to inhibit gene regulation, but also influences bacterial surface adhesion in *Streptococcus mutans* (Nassar et al., 2012) and *S. pyrogenes* (Maddocks et al., 2012), and can prevent biofilm formation by *P. aeruginosa*, *S. aureus* and *E. coli*.

Honey decreases virulence

The use of honey as an anti-virulent remedy for treatment of infection has also gained interest, and a few recent studies have indicated the inhibitory role of honey on bacterial virulence. The effect of honey on bacterial virulence was tested when researchers confirmed the inhibitory action of honey on the expression of quorum sensing molecules, as these molecules are known to regulate the production of virulence factors. Studies have shown that the concentration of honey that inhibits bacterial quorum sensing and virulence is normally below their MIC, indicating the sensitivity of bacterial virulence to their environment (Wang et al., 2012; Maddocks and Jenkins, 2013; Lee et al., 2011). Microarray and qRT-PCR analysis, have shown that honey mediates inhibition of curli genes, AI-2 genes in quorum sensing, and virulence genes of *E. coli* O157:H7 (Lee et al., 2011). Similarly, other studies have shown that honey is capable of impairing *las* and *rhl* genes and transcriptional regulator MvfR in *P. aeruginosa* which are all associated with quorum sensing and regulatory networks that control virulence of this organism (Wang et al., 2012; Maddocks and Jenkins, 2013).

Biofilm vs planktonic cells

Biofilm formation is a developmental progression that is regulated through various processes, and relies on the controlled expression of a collection of biofilm-specific genes (Bjarnsholt et al., 2010). Previous studies have shown that production of the extracellular polymeric matrix (EPS) by bacteria is one of the defining features of biofilm formation (Parsek and Tolker-Nielsen, 2008). These matrices, which are composed of mainly exopolysaccharides, proteins, lipids, surfactants, DNA and ions, shield the outer layer of a biofilm and act as a

protective barrier against toxic substances (O'Toole and Ghannoum, 2004; Karatan and Watnick, 2009; Francis, 2008). The extracellular matrices of bacterial biofilms contain an interwoven structure of negatively charged and neutral polysaccharides that may prevent penetration of antimicrobial agents through ionic or electrostatic interactions (Ingle et al., 2008).

The presence of these matrices plays an important role in the structural scaffold of the biofilm, which also facilitates cell to cell communication (Parsek and Tolker-Nielsen, 2008; O'Toole and Ghannoum, 2004; Francis, 2008). Therefore, the formation of *P. aeruginosa* biofilm is the main cause of the intrinsic resistance of this bacteria to several antibiotics. Notable potential reasons for an increase in antibiotic resistance in biofilms is due to low antibiotic penetration in the EPS, expression of various efflux pumps and expression of various antibiotic inactivating enzymes (Storz and Hengge-Aronis, 2000).

Bacterial defense mechanisms are known to be major contributors to prolonged or acute infections. Recent studies have shown that these defense mechanisms arise due to the formation of biofilms. Biofilm formation by bacteria was noticed back in the early 1970's when J.W. Costerton and K.J. Cheng were studying bacterial cultures with transmission electron microscopy and noted the difference in bacterial morphology of an old culture versus fresh (planktonic) cultures (Abdallah, 2011). This led to the birth of the term 'biofilm' in microbiology.

Biofilm formation is a multistage process that starts with an initial reversible attachment of free floating microbial cells to a surface followed by a maturation stage which includes cell replication, proliferation and production of an outer shell made of exopolysaccharide around the community (Coenye and Nelis, 2010). The establishment of bacteria in the context of biofilm is known to increase the defense mechanisms of bacteria in harsh and unfavorable environments, which is one of the underlying reasons for the increased resistance to toxic substances such as biocides or antibiotics (Harrison et al., 2007; Perez et al., 2010). Biofilm formation does not only increase the defense mechanisms of bacteria in the presence of antibiotics; it also helps microorganisms to establish and maintain a long-term relationship with each other, through physical and chemical interaction of cells (O'Toole and Ghannoum, 2004). Moreover, research has confirmed that bacterial resistance is higher in a biofilm, compared to planktonic (individual) cells (Barrett and Stanberry, 2009; Harrison et al., 2007). The formation of biofilm provides an advantage for the bacterial cells by reducing the penetration of antibiotic within the community,

decreasing space for bacterial communication through quorum sensing and increasing the chance of gene transfer; all of which contribute to the increasing of bacterial resistance to antibiotics. Although this form of mechanism is advantageous to microorganisms, studies have shown that biofilm formation is a serious threat to the public health systems; almost 60% of microbial infections are as a result of this strategy (Barrett and Stanberry, 2009; Harrison et al., 2007; Spoering and Lewis, 2001).

Bacterial differences between Gram-positive and Gram-negative bacteria

Bacterial species are characterized into two broad groups that are mainly differentiated based on their structure and composition of their cell wall, which plays an important role in growth and survival of the organism in all non-isotonic environments. All bacterial species contain peptidoglycan, a substance composed of polysaccharide chains interlinked with short peptides, which contributes as a part of the bacterial cell wall (Al-Waili and Saloom, 1999). The amount of peptidoglycan in the bacteria separates the organisms into two distinct groups, Gram-positive and Gram-negative bacteria. Peptidoglycan in Gram-positive bacteria is thicker than Gram-negative species, which benefits the bacteria by reducing the passage of some groups of antimicrobial agents into the cell (Roberts et al., 2012). However, Gram-negative bacteria have a different shield that gives them an extra protection compared to Gram-positive and that is the presence of outer and inner cell membrane separated by periplasmic space (Mercholl et al., 2009).

A large portion of research on the effect of honey was performed mainly on Gram-positive bacteria. Therefore the need for research on understanding the effect of honey and the microbial mode of action on Gram-negative species needs further investigation.

Pseudomonas aeruginosa

P. aeruginosa has been recently categorized as one the most dangerous multidrug-resistant (MDR) bacteria, responsible for causing healthcare-associated infections (HAIs) (Hirdon et al., 2008; Rice, 2008; Macor, 2011). In the US alone, the annual cost for treatment of infection as a result of MDR species ranged between USD \$15 million to \$30 billion a year (2008 dollars), whereby according to the U.S department of Healthcare and Human Services about 13% of severe HAIs are as a result of *P. aeruginosa* infection (Frieden, 2013; Richand et al., 2001; Niederkom

and Kaplan, 2007). Treatment of this species has become very difficult due to the adaptation and increase of antibiotic resistance to the available antibiotics.

P. aeruginosa is a Gram-negative rod shaped bacterium commonly found in the soil, fresh water environments, around the sinks, showers and hospital environments (Levin et al., 1984; Zichichi et al., 2000). This bacterium was first isolated by Carle Gessard in 1882 from wound infections of soldiers (Gessard, 1925). Later in 1894, Walter Migula was the first to propose the genus of *Pseudomonas* as a Gram-negative rod shape bacterium (Pexi et al., 2009). *P. aeruginosa* is a ubiquitous bacteria and is known to cause various types of infections in humans, animals and plants (Wiehlmann et al., 2007; Rahme et al., 2000; Elrod and Braun, 1942).

P. aeruginosa is responsible for causing up to 4% of Catheter Related Infections (CRI), 19% of respiratory infections and 17% of urinary tract infection but also causes a variety of chronic and acute infections such as, pneumonia, surgical wound infections, blood stream infections, infections of neutropenic patients, burn wound infections, chronic infections of respiratory pathways (including panbronchiolitis, bronchiectasis), and cystic fibrosis (Wisplinghoff et al., 2004; Wright et al., 2006; Koch and Hoiby, 1993; Bjarnsholt and Givskov, 2007). In the United States, approximately 80,000 Catheter-Related Blood Stream Infections (CRBI) occur annually at a substantial cost, both in terms of morbidity and financial resources expended for treatment of CRI (Grady et al., 2011). The annual treatment of CRI can cost up to \$2.3 billion dollars (Sadfar, 2011), out of which \$50 to \$350 million dollars is spent on the treatment of Urinary tract infections (UTI) (Barrett and Stanberry, 2009).

One of the most classical examples of biofilm formation which has threatened and caused an increase of chronic infections in cystic fibrosis (CF) patients is the colonization and biofilm formation of *P. aeruginosa* in lungs, which causes a decline in pulmonary function by influencing oxygen availability in lungs (Evans et al., 1996; Kerem et al., 1990).

Virulence factors in gram negative bacteria

P. aeruginosa is known to produce a wide range of virulence factors such as exoprotease elastase (Bjarnsholt, and Givskov, 2007; Woods et al., 1982), ADP ribosylating enzymes (Vasil et al., 1989; Bjarnsholt, and Givskov, 2007); lipopolysaccharides (Tang et al., 1996; Bjarnsholt, and Givskov, 2007); hemolytic phosphatase (Vasil et al., 1991); lipases; phospholipases; exotoxin A;

pyocyanin and siderophores (iron-chelating compounds) such as pyochelin and pyoverdine (Cox, 1982; Cornelis, 2010). The presence of these virulence factors is known to cause extensive tissue damage. Studies have shown that the production of many virulence factors are controlled by expression of small molecular weight molecules known as quorum sensing molecules (Cornelis and Dingemans, 2013). In *P. aeruginosa*, virulence factors are expressed differently depending on the environmental signals and metabolic factors (Bjarnsholt et al., 2010). It is known that *P. aeruginosa* uses an estimated 468 transcriptional regulators to control expression of different virulence factors (Bjarnsholt et al., 2010; Stover et al., 2000).

In addition, one of the most unique characteristics of *P. aeruginosa* is the production of pyocyanin, a green water-soluble pigment, which is an indication of *P. aeruginosa* colonization of the host or the environment (Stephen and Hawkey, 2006). The study by Stephen and Hawkey has indicated that pyocyanin has antibiotic activity against other bacteria and fungi, which provides an advantage over competing bacteria occupying the same niche (Stephen and Hawkey, 2006).

Communication is the degree of agreement between the message sent and the meaning understood by the receiver. In the microbial world, bacterial communication or quorum sensing (QS) involves synthesis, excretion, and detection of low molecular weight molecules called autoinducers (AI), which are known to have a significant advantage for the microorganism. In *P. aeruginosa* species, QS involves production of N-acyl-homoserine lactones (AHL) molecules which are mediated by the expression of *las* and *rhl* genes (Annapoorani et al., 2012; Davies et al., 1998; Jung, 2011). Previous studies have confirmed the role of AHL molecules in controlling the expression of various sets of genes that are responsible for biofilm formation (Shrout et al., 2006), conjugation (McLean et al., 1997), pigment production (Pessi et al., 2001), swarming motility (Shrout et al., 2006), and expression of many virulence factors such as exoproteases, hemolysins, exotoxin A, exoenzymes, pyoverdine and pyocyanin (Pearson et al., 2000; Hentzer et al., 2003). It is known that AHL molecules are regulated with an increase in cell population which then results in activation of regulator proteins LasR and RhlR, responsible for the regulation of virulence genes (Clutterbuck et al., 2007). A study by Clutterbuck et al., has shown that irreversible attachment of cells activated the *rhlR-rhlI* gene, which activates C4-HSL and plays a role in biofilm development (Clutterbuck et al., 2007).

Iron

Iron is the most abundant element on earth and comprises 5% of the earth's crust (Heli et al., 2011). It is an essential nutrient and is crucial for the biochemical function of all living species (Weinberg, 2009). Iron can exist in two oxidation states, Fe^{2+} and Fe^{3+} . It is known that Fe^{3+} (ferric iron) has a low solubility and dominates in oxygenated environments (Andrews et al., 2003). At physiological pH 7, the concentration of bioavailable Fe^{3+} in the environment is about 10^{-9} M. However this concentration decreases inside the host to 10^{-18} M (Brandel et al., 2012). Therefore soluble iron cannot get into the cell via diffusion as the concentration of iron in the cytoplasm of bacteria is 10^{-6} M (Braun et al., 2013). Conversely, the soluble Fe^{2+} is mainly found in anaerobic environments or in environments with low pH (Andrews et al., 2003). The solubility of ferric iron can be calculated by the product constant $K_{\text{sol}} = [\text{Fe}^{3+}] [\text{OH}^-]^3$; $[\text{Fe}^{3+}] = (10^{-38}) / [\text{OH}^-]^3$, which indicates that at pH 3, 5, and 7, the concentration of ferric ion is approximately 10^{-5} , 10^{-11} , and 10^{-7} M (Yoder and Kisaalita, 2011; Neilands et al., 1987).

A normal human body contains 40 - 50 mg/kg of iron. It is known that iron plays an important role in mammalian metabolism such as oxygen transport (Heli et al., 2011), the electron transport chain (cytochrome C) (de Ungria et al., 2000; Heli et al., 2011), oxidation-reduction (alcohol dehydrogenases, dihydroorotate dehydrogenase, cytochrome oxidase) (Heli et al., 2011), glycolysis (Oxele et al., 1999; Racker and Krimsky, 1947), pentose shunt (Dhur et al., 1989; Heli et al., 2011), proteolysis (Rana and Meares, 1991), production of new erythroid cells (Heli et al., 2011) and many more.

Iron Uptake System in *P. aeruginosa*

Bacterial pathogens are challenged with the problem of acquiring iron in the host since iron is sequestered by circulating proteins such as transferrin and lactoferrin, or is bound to heme molecules (Finkelstein et al., 1983; Cornelis and Dingemans, 2013). The uptake of iron by microorganisms varies, depending on the type of bacterium and the environment (Donlan and Costerton, 2002). *P. aeruginosa* uses four different strategies to acquire iron from the environment such as inside a host (Donlan and Costerton, 2002; Ratledge and Dover, 2000; Cornelis, 2010; Jakubovics and Jenkinson, 2001; Poole and McKay, 2003; Drake and Gulick, 2011).

These strategies include (Figure 2.1.):

- (i) Production of high-affinity iron uptake molecules called siderophores that chelate extracellular Fe^{3+}
- (ii) Direct contact of the bacterium with the source of iron containing proteins such as transferrin, lactoferrin or heme proteins
- (iii) Uptake of xenosiderophores that are not produced by the bacterium itself
- (iv) and use of Feo transport system.

Overall, depending upon the type of infection, *P. aeruginosa* adapts itself to the environment to fulfill the need for iron from the host (Cornelis and Dingemans, 2013).

FIGURE 2. 1 IRON TRANSPORT SYSTEM IN GRAM NEGATIVE BACTERIUM

Transport of ferric iron complex across the outer membrane to the periplasm occurs through binding of the ferric complex to the membrane receptors (denoted as R). The ferric iron complex is transported from the periplasm to the cytoplasm through ATP-binding cassette transporter (denoted as ABC) (Figure modified from Braun et al., 2013).

The *P. aeruginosa* Siderophore mediated Fe^{3+} uptake system

Siderophores are low molecular weight molecules produced and excreted from the bacterium to chelate iron from the environment. Siderophore production is known to play an important role in the virulence of pathogenic *Pseudomonas*. Consistent with being a virulence factor, siderophores are mainly important for acquisition of iron within the host. It is known that

siderophores have specific affinity for Fe^{3+} iron. All Gram-negative bacteria such as fluorescent pseudomonads transport iron across the outer membrane to the cytoplasmic membrane in an independent manner (Braun et al., 2013). The outer membrane of pseudomonads contains a TonB-dependent receptor complex, which is a large porin with α -barrel shaped membrane protein that gates the entry of compounds inside the cell and is responsible for recognition of iron-load complexes (Cornelis, 2010). Regardless of whether iron is bound to siderophore or heme protein, the iron ligand binds tightly to the transporter and a change in conformation of the binding site causes the bound iron to enter the periplasm. This occurs through the N-terminal end of the TonB-receptor located in the outer membrane (Figure 2.2).

FIGURE 2. 2 IRON UPTAKE SYSTEM IN *P. AERUGINOSA* BACTERIUM VIA SIDEROPHORE OR HEME.

Iron-Siderophore complex is recognized by the outer membrane TonB-dependent receptor, which is a gated porin and is in contact with TonB. TonB complex contains ExbB and ExbD, which transmit the energy from the proton-motive-force to open the gate where iron-siderophore complex can be transported to the periplasm. (Figure adopted from Cornelis, 2010).

The TonB receptor is a part of the TonB complex that is located inside the periplasmic space and bacterial cytoplasmic membrane of the cell. TonB is also connected to ExbB and ExbD proteins, which are responsible to transmit energy of the protein motive force and allows the opening, closing, and transport of ferric complex into the periplasm (Cornelis, 2010; Postle and Larsen, 2007). Once the iron is transported inside the periplasm of the cell, the complex will be

transported into the cytoplasm through ATP-binding cassette transporter (ABC) receptors located on the cytoplasmic membrane (Figure 2.3). The ABC transporter consists of the transmembrane permease which separates the siderophore and iron complex and reduces Fe^{3+} to Fe^{2+} (Heli et al., 2011). This occurs through the redox system and the proposed reaction can be summarized as follows:

FIGURE 2. 3 SIDEROPHORE TRANSPORT SYSTEM ACROSS GRAM NEGATIVE MEMBRANES

In Gram-negative bacteria, siderophore complex is transported into the cell through the TonB complex, located in the periplasm of the cell. One end of the TonB complex is attached to the outer membrane transport receptor and the other end is connected to the cytoplasmic receptor of the cell. TonB complex located on the cytoplasmic membrane controls the uptake of iron-siderophore complex, through providing the energy required for opening of the outer membrane complex. Once the iron-siderophore complex is transported into the periplasm it is then transported to the cytoplasm through ABC transporter. The ABC transporter consist of the transmembrane permease to which to which an ATPase is attached in the cytoplasm. Separation of siderophore and iron complex occurs via the permease which reduces Fe^{3+} to Fe^{2+} . (Adopted from Braun et al., 2013).

Research on siderophore-mediated iron acquisition systems of *Pseudomonas* has mainly focused on fluorescent pseudomonads, however other studies have shown that non-fluorescent strains such as *Burkholderia* are capable of producing siderophores (Poole and McKay, 2003). One of the striking characteristics of *Pseudomonas* is its ability to acquire iron through other bacterial siderophores (for example aerobactin, enterobactin cepabactin) (Poole and McKay, 2003;

Schalk and Guillon, 2012; Liu and Shokrani, 1978), fungal siderophores (Poole and McKay, 2003; Poole et al., 1990), synthetic chelators (Poole and McKay, 2003; Hohnadel and Meyer, 1988) and natural occurring chelators (Poole and McKay, 2003; Meyer and Hohnadel, 1992; Harding and Royt, 1990). *P. aeruginosa* is capable of synthesizing two well-studied siderophores, pyoverdine (also referred to as PVD) and pyochelin (also referred to as Pch). Both of these iron mediated siderophores have an outer membrane transporter protein with a high affinity for ferric iron in the range of nanomolar concentrations (Cox, 1980; Brandel et al., 2012). It is important to note that siderophores are different from one another, and this difference arises based on the way that the iron is complexed. Based on these differences, siderophores are categorized as phenolate-, catecholate-, hydroxamate-, carboxylate-, or mixed type of siderophores (Cornelis and Dingemans, 2013).

***P. aeruginosa* Siderophore—Pyochelin**

Pyochelin is a hydroxyphenyl-thiazoliny-thiazolidin molecule and is known to be a member of hydrophobic siderophores (Brandel et al., 2012; Ankenbauer et al., 1985). Due to its hydrophobicity, this molecule is known to be poorly soluble in aqueous media (Brandel et al., 2012). Pyochelin is known to have a very low affinity for Fe^{3+} , however it is capable of releasing iron from transferrin under acidic conditions. Previous studies have shown that the pyochelin molecule is capable of chelating other metallic ions, such as Zn (II), Cu (II), Co(II), Mo (VI) and Ni (II) (Brandel et al., 2012; Cox et al., 1981; Visca et al., 1992), as well as non-biologically relevant cations (Brandel et al., 2012; Hayen and Volmer, 2006). Pyochelin biosynthesis involves a different cascade of production than pyoverdine. Recent studies have shown that *P. aeruginosa* first produces pyochelin and switches to pyoverdine production when the iron concentration is extremely low in the environment (Dumas et al., 2013; Cornelis and Dingemans, 2013). Studies have shown that in chronic infections, such as lungs of CF patients, pyochelin production can play a significant role in causing tissue damage (Cornelis and Dingemans, 2013). This effect is mainly due to the redox cycle that pyochelin-iron complexes possess in the presence of pyocyanin, another extracellular compound produced by *P. aeruginosa* (Cornelis and Dingemans, 2013; Coffman et al., 1990; Britigan et al., 1992; Britigan et al., 1997).

***P. aeruginosa* Siderophore—Pyoverdine**

Pyoverdine, also known as pseudobactin, is a mixed siderophore that contains a conserved chromophore and a peptide chain (Cornelis and Dingemans, 2013; Meyer, 2000). These siderophores are mainly secreted from *P. putida*, *P. syringae*, *P. aeruginosa* and *P. fluorescence* species in low iron environments (Cornelis and Dingemans, 2013; Meyer, 2000; Gross and Loper, 2009; Hannauer et al., 2012). Pyoverdine is a fluorescent, water-soluble molecule (Musthafa et al., 2010) that binds strongly to Fe^{3+} , and this results in fluorescent quenching of this molecule (Yoder and Kisaalita, 2008, 2011; Ongela et al., 2002). The length of peptide chains in pyoverdine can vary among different bacteria, however studies have shown that the chromophore region is conserved among species (Escaich, 2010). The chromophore region of pyoverdine contains a dihydroxyquinoline (fluorescent) chromophore linked to a small dicarboxylic acid and is unique in producing a green-yellow fluorescent emission upon exposure to UV light (Drake and Gulick, 2011).

The process of pyoverdine biosynthesis begins in the cytoplasm and ends in the periplasm of the cell, where it is then secreted to the outside of the cell (Hoegy et al., 2005; Schalk and Guillon, 2012). The biosynthesis of pyoverdine involves the expression of a variety of enzymes specifically non-ribosomal peptide synthetases (NRPSs), which are responsible for the synthesis of both the chromophore backbone and peptide moiety of pyoverdine (Schalk and Guillon, 2012). Briskot et al., (1989) suggested that assembly of the PVD backbone in PA01 strain starts in the cytoplasm through the PvdL, which is the only common PVD-synthesis NRPS (Briskot et al., 1989; Mossialos et al., 2002; Gross and Loper, 2009). PvdL enzyme is responsible for many things such as forming the structure of cytoplasmic PVD precursor (Briskot et al., 1989; Hannauer et al., 2012), coupling coenzymes A and fatty acids (Drake and Gulick, 2011), building pyoverdine backbone, followed by formation of a tetrahydropyrimidin ring (precursor of the dihydroxyquinoline chromophore) (Briskot et al., 1989; Mossialos et al., 2002), and catalyses in the attachment of the first amino acid of the peptide moiety of PVDI (Briskot et al., 1989). PVD synthesis is then followed by the progression of cytoplasmic enzymes PvdI, PvdJ, and PvdD. The cytoplasmic PVDI precursor has an unformed chromophore, and a myristoleic chain that is bound to the peptide backbone (Glu), which is responsible for attaching the cytoplasmic PVDI precursor bound to the inner membrane (Briskot et al., 1989; Hannauer et al., 2012). As shown in Figure

2.4., the non-fluorescent PVDI precursor is transported across the inner membrane into the periplasm by the export ABC transporter pump, PvdE (Briskot et al., 1989; Yeterian et al., 2010). In the periplasm, the PVD precursor (also known as ferribactin) goes through a maturation process of PVDI chromophore that happens by condensation of Try and Dab by periplasmic acylase PvdQ (Yeterian et al., 2010). This enzyme was first recognized as a periplasmic quorum quenching enzyme that was responsible for cleaving of acyl homoserine lactone of AHL molecule (Briskot et al., 1989; Sio et al., 2006). This role was also confirmed in an in vitro study with *Caenorhabditis elegans* where addition of PvdQ quenched the signaling molecule or quorum sensing molecule (Papaioannou et al., 2009). In another study Wahjudi et al. (2013) showed the beneficial effect of combining PvdQ enzyme and antibiotic as an inhaler for treatment of *P. aeruginosa* infection of lungs (Wahjudi et al., 2013).

FIGURE 2. 4 PYOVERDINE BIOSYNTHESIS IN *P. AERUGINOSA*.

PVD precursor backbone is assembled in the cytoplasm by four enzymes (PvdL, PvdI, PvdJ and PvdD), which is then transferred to the periplasm. The cytoplasmic PVD precursor has unformed chromophore and a myristic acid chain that keeps the cytoplasmic PVD precursor bound to the inner membrane. Once the PVD precursor is transported across the inner membrane through the ABC transporter, periplasmic acylase PvdQ changes the fatty acid of PVD. With the help of PvdN, PvdQ, and PvdP, the chromophore of PVD is formed through condensation of tyr and Dab. (Refer to text for more details) (Adopted from Isabelle and Guillon, 2012).

Recent studies by Yeterian et al. suggested that newly synthesized siderophores with a formed chromophore are stored in the periplasm (Briskot et al., 1989; Yeterian et al., 2010). It is known that these newly synthesized PVDI are secreted to the extracellular environment through PvdRT-OpmQ efflux system (Yeterian et al., 2010). In *P. aeruginosa* the extracellular PVDI-Fe complex crosses the outer membrane through two specific membrane transporters FpvA (Briskot et al., 1989) and FpvB (Briskot et al., 1989; Ghysels et al., 2004) that are activated through TonB machinery (Briskot et al., 1989, Postle and Larsen, 2007). The release of iron from PVDI occurs through a reduction mechanism that involves no chemical modification of the siderophore. In addition to chelating iron, pyoverdine is known to be responsible for chelating a variety of metals such as Al^{3+} , Cd^{2+} , Co^{2+} , Cu^{2+} , Ga^{3+} , Mn^{2+} , Ni^{2+} and Zn^{2+} (Briskot et al., 1989; Braud et al., 2009).

As stated earlier, pyoverdine binds iron with a high affinity. In addition to its role as a siderophore, it can also act as a signaling molecule that triggers the production of other extracellular virulence factors, such as exotoxin A and protease PrpL (Cornelis and Dingemans, 2013; Lamont et al., 2002; Cornelis, 2010). In addition this siderophore has shown to be involved in the establishment of biofilms (Cornelis and Dingemans, 2013; Banin et al., 2005; Patriquin et al., 2008; Heli et al., 2011; Martin et al., 2011).

Human Siderophyins

The second common method that bacterial species use to acquire iron within the host is through protein receptors that bind to lactoferrin (Lf), transferrin (Tf), or hemoglobin (Hb). Transferrin and lactoferrin proteins are common constituents of plasma or mucosal secretions and contribute to host defense mechanisms by acting as an iron storage and reducing the availability of iron during an infection (Britigan et al., 1993). Studies have shown that some bacterial species are able to overcome the presence of these proteins through production of specific enzymes. For example the production of elastase enzyme by *Pseudomonas* species has been shown to cleave iron bound to transferrin protein and enable PVD siderophore to chelate the iron (Ankenbauer et al., 1985; Britigan et al., 1993).

Heme uptake system in *P. aeruginosa*

Many Gram-negative bacteria including *Pseudomonas* have the ability to uptake iron from the environment through their Heme uptake system, which is a process that involves regulation and synthesis of proteins through Has and Phu systems (Cornelis and Dingemans, 2013; Ponka, 1997). Heme is always extracted from hemoproteins such as hemoglobin or hemopexin that contain bound iron (Cornelis and Dingemans, 2013). The Has system is known to regulate production of hemophore (shown in Figure 2.2.), which involves binding and transferring of a heme molecule to the cytoplasm of bacterium. Once the heme-hemophore complex is transferred to the periplasm, it is then bound to a protein that transports the complex across the ABC transporter. In the cytoplasm, a heme complex is degraded to form biliverdin, CO and Fe^{2+} (Cornelis and Dingemans, 2013; Bhakta and Wilks, 2006; Barker et al., 2012; O'Neill et al., 2012).

Lactoferrin

Lactoferrin is an 80kD iron-binding glycoprotein, produced by mucosal epithelial cells in various mammalian species and is known to be responsible for transport of iron in blood serum, which also plays an important role in the defense mechanism of the body (Yeterian et al., 2010; Adlerova et al., 2008). Lactoferrin is mainly found in sites of infection and inflammation, especially in mucosal secretions such as tears (González-Chávez et al., 2009), saliva, semen (Jensen et al., 1986), vaginal fluids (Buckett et al., 1997), nasal and bronchial secretion (Pillay et al., 2001), bile, gastrointestinal fluids and urine (Harada et al., 1999). This glycoprotein is also known to regulate the concentration of unbound iron, prevent the formation of hydroxyl radicals (rf), and retain metal over a wide pH range (Abergel et al., 2006). Lactoferrin is the second most abundant protein found in milk (Abergel et al., 2006; Pan et al., 2010; Murata et al., 2013).

Lactoferrin is a well-known protein responsible for sequestering iron from the site of infection, and is also able to cause cell lysis during bacterial, viral, or fungal infection by interacting with anionic molecules on the surface of cells (Abergel et al., 2006). This protein has an α -helix and β -pleated sheet on each of its lobes that can bind two metal ions such as Fe^{3+} , Fe^{2+} , Cu^{2+} , Zn^{2+} , and Mn^{2+} (Abergel et al., 2006; Croguennec et al., 2012). Studies by Ellison et al., have shown that siderophyins, specifically lactoferrin damage the external membrane of Gram-negative bacteria by binding to lipopolysaccharide (LPS) of the bacterial cell wall and causing LPS to

release from the cell wall thereby increasing the membrane's permeability and damaging the bacterial membrane (Ellison et al., 1988; Abergel et al., 2006; Van der Strate et al., 2001).

During a microbial infection inside the host, iron levels within the host can be depressed due to the presence of defense proteins. This happens due to the proteins secreted mainly in mucosal glands such as lipocalins that are responsible for taking away the catechol siderophores that are produced by pathogenic bacteria (Yeterian, et al., 2010). However, this is not true for all siderophores, as some siderophores such as pyoverdine (Weinberg, 2009) and petrobactin synthesized by *Bacillus anthracis* (Fluckinger et al., 2004) resist capture of lipocalins and can promote bacterial growth. Studies performed by Xiao and Kisaalita., have shown that lactoferrin has a high affinity for iron similar to transferrin, however, this affinity increases in low pH environments, and unlike transferrin, lactoferrin does not release the bound iron at low pH (Xiao and Kisaalita, 1997).

External administration of lactoferrin is known to be effective in reducing kidney infections (Xiao and Kisaalita, 1997; Yanaihara et al., 2000), can stimulate the proliferation of endometrium stroma cells (Yanaihara et al., 2000; Cornish et al., 2004), stimulate osteoblast proliferation, reduce apoptosis of osteoblasts (Yanaihara et al., 2000; Damiens et al., 1999), and halt the growth of human mammary gland carcinoma (Yanaihara et al., 2000; Bhimani et al., 1999). In other studies lactoferrin has shown to prevent biofilm formation and stimulate twitching motility by binding to LPS and disrupting bacterial membrane (Singh et al., 2002).

Nutrients & carbon

Biofilm formation is not specific to bacterial species but is also seen in fungi, algae and other microorganisms (Miller et al., 2013). As mentioned earlier biofilm formation occurs through a complex interplay between different factors such as cell migration and proliferation, molecular signals, and more importantly, nutrient availability (Parsek and Tolker-Nielsen, 2008). Several studies have shown the importance of nutrient sources on initiating biofilm formation and biofilm structure (Parsek and Tolker-Nielsen, 2008). For example, depending upon the carbon source, *P. aeruginosa* biofilms have been shown to have a different structural scaffold.

Klausen et al., have shown that *P. aeruginosa* biofilm forms flat shaped biofilms when citrate was used as the carbon source (Klausen et al., 2003). In their study, time lapse microscopy

indicated that *P. aeruginosa* pilA mutant (strain that was deficient in biogenesis of type IV pili) was unable to migrate as efficiently in citrate medium as opposed to glucose medium (Parsek and Tolker-Nielsen, 2008; Klausen et al., 2003) showing the influence of carbon source on surface migration of the bacteria or type IV pili activation (Parsek and Tolker-Nielsen, 2008). In addition, the study showed that carbon source influenced microcolony formation and biofilm development. Biofilms grown in the presence of glucose seem to form a mushroom-shaped structure whereas bacteria form a flat-shaped structure when citrate is present in their medium (Parsek and Tolker-Nielsen, 2008; Klausen et al., 2003). In another study Ponraj et al. demonstrated the influence of carbon source such as glucose on regulation and synthesis of bacterial siderophores (pyoverdine) (Ponraj et al., 2013) which will be discussed in the later chapters.

Nutrient source is not only important for biofilm development but can have a dual function within the biofilm itself. For example, it is noted that carbon sources such as citrate have a dual function and can be used in the TCA cycle or act as an iron chelator and mediate iron uptake in *Pseudomonas* strains (Marshall et al., 2009; Hohnadel and Meyer, 1988). In addition to carbon source, micronutrients such as phosphorus, nitrogen, and iron have been shown to have an important effect on biofilm formation and structure (Parsek and Tolker-Nielsen, 2008). In addition to the nutrient availability, optimal temperature, physiological pH, water availability, cell migration and proliferation, and molecular signals contribute to biofilm formation (Ponraj et al., 2013).

Bacterial species all possess a versatile metabolism that allows them to use different compounds as a source of energy. Under different environmental conditions bacterial species are capable of either selecting and catabolizing carbon sources or assimilating specific compounds that afford them with the most efficient growth. The collection of regulatory processes that controls bacterial selection for specific carbon had been named as catabolite repression (CCR) or catabolite repression control (CRC), which was first studied in *Escherichia coli* through hierarchical assimilation of sugars (Magasanik, 1970).

CCR is known to be responsible for inhibiting expression of pathways for non-preferred compounds as well as generating important reorganization of metabolism that requires activation of several genes through a process known as Carbon Catabolite Activation (CCA). Both CCA and CCR are part of the common term of Carbon Catabolite Control (CCC). In addition, molecular

analysis has shown that the global regulation process of CCC not only affects specific catabolic pathways, but is also responsible for impacting important aspects of cell physiology and their interaction with the environment. For example, genetic analysis of pathogenic bacteria has shown that catabolite repression is capable of controlling expression of several genes such as those responsible for bacterial virulence (Gorke and Stulke, 2008). However, the preferred carbon source in the CCC pathway of *Pseudomonas* is known to be different from *E. coli* and *B. subtilis*. The preferred carbon source of *E. coli* and *B. subtilis* is glucose, whereas *Pseudomonas* has shown to prefer some organic acids or amino acids as opposed to glucose. For example, in the presence of succinate and glucose, *Pseudomonas* has shown to repress expression of enzymes responsible for glucose catabolism until succinate is fully consumed (Collier et al., 1996). However it is important to note that the sequential hierarchy in *Pseudomonas* is still not fully understood but it is known that this species prefers some organic acids and amino acids to hydrocarbons, and glucose is lies within this hierarchy (Hester et al., 2000, Morales et al., 2004).

Environment and available nutrients are the factors that influence bacterial metabolic differences and facilitate a different life style or strategy to compete within a host. For example *Pseudomonas* species living in soil mainly uptake nutrients and compounds from decomposition as opposed to *Pseudomonas* that is associated to plant roots that can feed on organic compounds excreted by the roots.

Closing remarks

Several studies have reported the effectiveness of honey as a remedy against *P. aeruginosa* (Molan, 2006; Henriques et al., 2011). However this remedy was mainly effective in the presence of high concentrations of honey, or in combination with antibiotics. The beneficial use of ingestion of honey and milk has shown that this combination helps in enhancing the immune system of an individual. Although the exact mechanism of this combination is still unknown, it is proposed that the presence of lactoferrin (an iron chelator) in milk could promote the beneficial health effect of this combination.

Lactoferrin, a mammalian protein, is the principle component of iron-withholding defense systems in the human body. This protein has shown to have a high affinity for binding metal and is capable of retaining iron molecules at the site of infection. The advantage of lactoferrin over

other proteins such as transferrin, is its ability to withhold the bound metal in a relatively low pH environment (Weinberg, 2001). Both in vivo and in vitro studies indicated the antibacterial activity of lactoferrin by producing an iron-deficient environment, which limits the bacterial growth (Ellison et al., 1988) and promotes eradication of the bacterium at the site of infection. In addition, a recent study has shown the beneficial effect of using lactoferrin/xylitol hydrogel against pathogenic *P. aeruginosa* (Ammons et al., 2010).

Several studies have confirmed the bactericidal nature of iron chelators or honey against many bacterial species. However, the combined effect of these two and their influence on bacterial metabolism is not known. Therefore, this study investigates the combined effect of an iron chelator and honey on a pathogenic bacterium, and evaluates the potential use of such combination as a novel therapy for treatment of infections associated with *P. aeruginosa*.

CHAPTER 3

The effect of honey on bacterial metabolism as measured by CO₂ production and siderophore secretion in a real time system.

INTRODUCTION

Honey, one of the oldest known sweeteners and health food sources, has been widely accepted throughout history for its uses as a drug and an ointment (Bogdanov et al., 2008). The consumption of honey dates back to 5500 BC, and the nutritional and medicinal quality of this compound has been documented in Hindu, Greek, Roman, Jewish, Christian, Islamic and other scripts (Israili, 2013). The medicinal use of honey waned following the discovery of penicillin and the antibiotic revolution, but this remedy has been re-introduced into modern medicine during the 21st century (Al-Waili et al., 2005; Al-Waili et al., 2013; Israili, 2013). Honey is known to possess antimicrobial, antiviral, anti-parasitic, anti-inflammatory, antioxidant, anti-mutagenic and antitumor effects (Bogdanov et al., 2008). Health benefits of this compound have been reported in a variety of conditions including the treatment of skin diseases (Fidaleo et al., 2010; Israili, 2013), eye diseases, respiratory diseases (Sio et al., 2006), urinary diseases (Al-Wali, 2005), gastrointestinal diseases, skin ulcers, psoriasis, and eczema (Daglia et al., 2013; Al-Wali et al., 2003; Allen et al., 1991). Continued studies have shown that honey exhibits a broad spectrum of antibacterial activities and can inhibit growth of more than 80 species of pathogenic bacteria (Molan, 1992; Blair and Carter, 2005; Lu et al., 2013). Honey affords three main advantages for an infected host; killing of pathogenic bacteria, inhibition of pathogen growth and reduction of excreted virulence factors. Therefore, the first objective of this study is to investigate the influence of honey on these three areas.

In any clinical situation, successful treatment of an infection mainly refers to eradication of the pathogen from the site of infection (Harris and Misiewicz, 2002). Eradication is defined as complete removal of the pathogen. For example in the case of *Helicobacter pylori* infection, eradication is referred to as a negative test for the bacterium four weeks after the treatment (Harris and Misiewicz, 2002). Clinical studies have shown that due to bacterial antibiotic tolerance it is

often difficult or impossible to eradicate bacteria with antibiotics (Costerton et al., 1999). However, clinical studies have shown that continuous treatment of infection with honey may lead to complete bacterial eradication from the site of infection (Al-Waili and Saloom, 1999; Al-Waili and Noori, 2004). In addition, no microbial resistance to honey has been reported to date (Lusby et al., 2005; Cooper et al., 2010; Müller et al., 2013), which could be due to multiple properties of honey presenting too many different pressures for resistance to develop (Maddocks and Jenkins, 2013). Honey is mainly composed of carbohydrates, proteins, enzymes, minerals, amino acids, trace elements, vitamins, aroma compounds and polyphenols (Alvarez-Suarez et al., 2010). The general mechanism of antimicrobial action of honey is attributed to various factors including its high osmolality (a_w : 0.6), acidity (pH 3.4 – 6.1), production of hydrogen peroxide, and the presence of polyphenolic compounds and flavonoids (Maddocks and Jenkins, 2013). These antimicrobial mechanisms are known to vary among the botanical origin where the honey was harvested. With the exception of manuka honey, almost all honey produces different levels of hydrogen peroxide as a result of the presence of glucose oxidase in it (Bang et al., 2003). Hydrogen peroxide is an effective antimicrobial agent that is capable of inhibiting microbial growth and stimulates production of new capillaries in wound tissue (Tur et al., 1995; Bang et al., 2003). In addition to the above mechanisms, cationic antimicrobial peptides, such as bee defesin-1 is a broad acting antimicrobial found in honey (Maddocks and Jenkins, 2013). In some honeys, especially manuka, bee defesin-1 is modified and changed to the phytochemical methylglyoxal (MGO), which is known to be the main active ingredient contributing to the antimicrobial activity of manuka honey (Maddocks and Jenkins, 2013).

Appropriate antimicrobial therapy has shown that growth inhibition of pathogenic bacteria is beneficial to the host, as it hinders bacterial duplication, giving the defense mechanism of the body an opportunity to get rid of the organism (Schaechter et al., 2007). Different types of honey possess different inhibitory concentrations. This is normally determined through bacterial minimum inhibitory concentration (MIC), which is defined as the lowest concentration of an antiseptic compound that completely inhibits multiplication (Medoff et al., 1971; Lambert and Pearson, 2000). MIC is normally measured against bacterial planktonic or free floating cells. Honey is useful against different types and strains of bacteria, but also against planktonic bacteria and biofilms (cluster of cells embedded in communities) (Mercholl et al., 2009). MIC varies among bacterial species as honey's mechanism of action against bacteria is known to vary. Honey affects

microbial physiology and metabolism through influencing their gene regulation. For example, studies on Gram-positive bacteria such as MRSA and methicillin-sensitive *S. aureus* (MSSA) demonstrated that honey interrupts the cell cycle and arrests cell division (Henriques et al., 2010). In addition to this, honey is capable of influencing the universal stress proteins in MRSA (Jenkins et al., 2011) and inhibits bacterial attachment (Badet and Quero, 2011). In Gram-negative bacteria, treatment with honey resulted in different cell morphology (Henriques et al., 2011) and cell lysis (Roberts et al., 2012).

Although the majority of studies on honey have tested its effects on Gram-positive bacteria, molecular analysis on Gram-positive and Gram-negative bacteria has shown that honey is equally effective against both types of bacteria (Al-Waili and Saloom, 1999; Cooper et al., 2002). Gram-negative bacteria, such as *P. aeruginosa* possess a thinner peptidoglycan membrane enclosed in between the outer and inner membrane of the cell. The presence of inner and outer cell wall in Gram-negatives reduces the passage of antimicrobial agents to the target site within the cell. However, treatment of these bacteria with honey reduces the expression of OprF, which influences structural stability within the cell envelope of the bacteria (Roberts et al., 2012; Maddocks and Jenkins, 2013).

Genetic studies confirmed that honey inhibits genes associated with virulence factor regulation. Biofilm formation and expression of virulence factors are profound factors that initiate host colonization by pathogenic bacteria (Maddocks and Jenkins, 2013). Therefore, studies are beginning to focus on remedies that possess anti-virulent characteristics as a new paradigm for antimicrobial therapy (Cegelski et al., 2008; Clatworthy et al., 2007; Rasko and Spernadio, 2010; Escaich, 2008). Anti-virulent therapies are normally evaluated through inhibition of quorum sensing or bacterial signaling molecules which is known to be associated with adhesion to surfaces, biofilm formation and stimulation of virulence (Maddocks and Jenkins, 2013). Treatment of biofilms with honey inhibits quorum-sensing genes (Maddocks and Jenkins, 2013). In a recent study, Lee et al., demonstrated that honey represses quorum sensing genes (AI-2 importers) and virulence genes (LEE genes) in *E. coli* O157:H7 (Lee et al., 2011). Similarly, other studies have shown that honey is capable of impairing *las* and *rhl* genes in *P. aeruginosa* which is associated with quorum sensing and regulatory networks that control virulence factors (Wang et al., 2012; Maddocks and Jenkins, 2013). From the many known virulence factors, siderophores such as PVD

are regulated by quorum sensing in pathogenic bacteria (Maddocks and Jenkins, 2013). Siderophores are iron-chelating molecules that are responsible for acquisition of iron in the host environment. Pyoverdine and pyochelin are two well-studied siderophores utilized by *P. aeruginosa* and are needed for colonization and subsequent progression of infection. Pyoverdine secretion in the environment occurs through an energy dependent mechanism in the bacterium (Braun et al., 2013). Recent studies have shown that honey possesses the capability to influence bacterial siderophore production (Lee et al., 2012; Kronda et al., 2013), which indicates the applicability of honey as a novel therapy.

The proposed concept of using honey as an anti-virulent compound is still under investigation, as the beneficial use of this compound has been mainly studied at the genetic level, for example the regulation of quorum sensing genes. It is rare to identify an antimicrobial compound that possesses antimicrobial properties that target the microorganism at different levels such as extracellular, cellular and molecular. Even though the effect of honey at all these levels has been studied individually, its combined effect in a real time system has not been studied. Moreover, a large body of research on the effects of honey on pathogenic bacteria has focused on the effect of honey at the genetic level, and the MIC or minimum bactericidal concentration (MBC) of different types of honey. Although both MIC and MBC are necessary to ascertain the effects of an antimicrobial agent, they suffer from being endpoint measurements and are limited in their ability to measure the viability of a population over time (Goring and Mims, 2012). Therefore, this measured how honey influences biofilm behavior and metabolism in real-time, including the effect on biofilm killing, growth inhibition and virulence secretion. Biofilm metabolism allows one to distinguish the usual response of the bacteria to an antimicrobial compound, as it portrays microbial ability to live, function and replicate in an appropriate chemical milieu (Jurtshuk, 1996). Moreover, bacterial metabolic response to antimicrobial agents is known to be similar among most bacterial cells, regardless of species (Kluyver and Niel, 1956). This study therefore aimed to provide a deeper insight into the effect of honey on bacterial behavior and response. *Pseudomonas aeruginosa* biofilm response was monitored against exposure to three different types of honey (manuka, clover and artificial honey), through real time monitoring of carbon dioxide production and siderophore secretion.

MATERIAL AND METHODS

Bacterial strain and growth conditions.

Pseudomonas aeruginosa strain PAO1 (ATCC® 39324™) was used in all experiments. Freeze cultures of this organism were made in 40% glycerol and kept at -80°C. The organism was cultivated at room temperature on AB defined medium agar plates (2.20 mmol/L KH₂HPO₄, 1.51 mmol/L (NH₄)₂SO₄, 3.37 mmol/L Na₂HPO₄, 179 mmol/L NaCl, 4.2 mmol/L NaNO₃, 0.01 mmol/L MgCl₂·6H₂O, 0.01 mmol/L CaCl₂·2H₂O, 0.001 mmol/L FeCl₃, 1 mmol/L Na-citrate·2H₂O).

Growth conditions in AB defined Medium. The overnight cultures for both static and flow systems were prepared by growing PAO1 in 10ml broth medium containing AB defined media with 10mM sodium citrate or 10mM glucose in a test tube which was incubated for 24 h at 37°C in a shaking incubator at 250 rpm. *P. aeruginosa* biofilms were grown at 25°C and typically reached stable growth conditions after 48 hours of incubation.

Growth conditions in Tryptic Soy Broth. The overnight cultures for both static and flow systems (see below) were prepared by growing PAO1 in 10ml tryptic soy broth (TSB) at a concentration of 3 g/L in 20 ml test tubes, for 24 h at 37°C and at 250 rpm. *P. aeruginosa* biofilms were grown in 0.03 g/L TSB under continuous flow (described below). Stable growth phase of PAO1 biofilms in TSB medium was after 24 hours of incubation.

Honey

Unpasteurized commercially available Manuka honey (MH) (Loblaws™, Canada), consisting of a blend of Australian and New Zealand honey from bees feeding on *Leptospermum scoparium* plants, was used and compared to clover and artificial honey.

The Canadian Clover honey ‘Wild country Clover honey™’ (Wild Country Ltd, Canada) was chosen since a study performed by Brudzynski (2006) illustrated that this honey possesses high levels of antimicrobial action.

Artificial honey (AH) was prepared as described by Wilkinson and Cavangah (2005), in which 33.5% glucose, 40.5% fructose, 7.5% maltose, and 1.5% sucrose were added to 18% distilled water (DI), autoclaved and stored at room temperature until needed.

In biofilm experiments, the manuka, clover and artificial honey was added to 0.03 g/L TSB at concentrations of either 5% or 30% (w/v), to which steady state biofilms were exposed for 2.5 hour or 24 hours at 25°C.

To assess the number of cells detached from the biofilm prior, during and post treatment, effluent was collected at the different time intervals and the total CFU of detached cells from the biofilm were enumerated by plating on TSA agar.

Agar Diffusion Test

In order to determine if PAO1 planktonic cells grow in the presence of honey, 100 µL aliquots of 10^7 CFU/ mL PAO1 overnight cultures were spread plated in triplicate onto tryptic soy agar (TSA) plates with 5%, 15%, 30% and 50 % (w/v) manuka honey, and incubated at 37°C for 24 h.

Biofilm growth and metabolism analysis

Biofilms were grown on the inside of a silicone tube in a carbon dioxide evolution measurement system (CEMS) as described by Kroukamp and Wolfaardt (2009). In brief, the CEMS consists of a one meter long, sterile silicone tube with an inner diameter of 1 mm and encased in an outer Tygon tube, which was connected to a compressed CO₂-free air supply (TOC grade, CO₂ <0.5 ppm, CO < 0.5 ppm, O₂= 20-22% and THS <0.1 ppm, Linde, Canada). The silicone tubes were sterilized with 10% (vol/vol) bleach for two hours and rinsed with autoclaved dH₂O for 24 hours. The metabolic activity in the biofilms in CEMS was measured from the diffusion of CO₂ through the silicone tubing to the Tygon tube which was analyzed and recorded by the LI-820 CO₂ analyzer (LI-COR Bioscience, Lincoln, N, USA). This system allowed the monitoring of metabolic activity in real-time under various environmental conditions. The silicone tubes were rinsed with sterile growth (AB defined or TSB) medium for 30 min prior to inoculation, followed by incubation at 25 °C. The flow rate of the system was maintained with a Watson Marlow 205S multichannel peristaltic pump, with a rate of 14.5 ml. h⁻¹, which resulted in a dilution rate of 4h⁻¹. For inoculation, the medium flow was halted and the silicone tubes were injected with 1ml of PAO1 overnight culture. The medium flow was resumed after 30 min, to allow bacterial adherence to the silicone walls.

LED Light Analysis Biofilm system (LLABs)

Biofilm cell density and PVD production was studied in a LED Light analysis Biofilm system (LLABs) developed in our laboratory, which uses a photo-diode light sensor that measures the amount of optical input of light that passes a medium and is received by the detector (figure M1). In this system, LLABs is placed on one region of the silicon tube and the detector measures the transfer of light from the light source that passes through the silicone tubing. The silicone tubes were rinsed with sterile growth (AB defined or TSB) medium for 30 min prior to inoculation, followed by incubation at room temperature. The flow rate of the system was maintained with a Watson Marlow 205S multichannel peristaltic pump, with a rate of $14.5 \text{ ml} \cdot \text{h}^{-1}$. For inoculation, the medium flow was halted and the silicone tubes were injected with 1 mL of PAO1 overnight culture. The medium flow was resumed after 30 min, to allow bacterial adherence to the silicone walls.

FIGURE 3. LED ANALYSIS BIOFILM SYSTEM (LLABs)

Siderophore- pyoverdine (PVD) spectrum measurement

Pyoverdine was measured by collecting effluent samples in 4 mL cuvettes. The presence of the fluorescent chromophore group of pyoverdine produces a characteristic pattern of excitation and emission which allows the qualitative measurement of PVD by measuring fluorescence of excitation at 405 nm and emission at 450 nm (Meyer and Abdallah, 1978; Cox and Adams, 1985; Visca et al., 1992; Dao et al., 1999). In this study, a Perkin-Elmer LS-5B luminescent spectrometer (Oak Brook, Illinois) was used to measure PVD. Low ($0.01 \mu\text{mol/L}$) and high ($10 \mu\text{mol/L}$) iron was used to compare siderophore production in *P. aeruginosa*.

Growth and qualitative estimates of pyoverdine production were recorded at 20 hour intervals prior and after the exposure to the antimicrobial compound. During the antimicrobial exposure, PVD

fluorescence was recorded at one hour intervals for the first 12 hours of exposure. Luminometer measurements were typically from a dilution of 1:100 or 1:1000 depending on the level of PVD fluorescence in the sample.

Data Analysis

Bacterial growth, biofilm formation and PVD relation to cell number were determined using one-way analysis of variance. Student's t test analysis was used to assess bacterial viability at different concentrations of honey.

RESULTS

During biofilm development pyoverdine-secretion was related to cell biomass increase (figure 3.1) as determined with the light transmittance analyzer (LLABs). The CO₂ measurements were used as an indication of actively respiring cells in the biofilm and similarly during biofilm development, pyoverdine secretion was related to increases in CO₂ production (figure 3.2).

When biofilms were exposed to 5% honey, biofilm cell growth increased rapidly while pyoverdine secretion levels dropped to around 19% of pre-exposure levels (figure 3.3). Both metabolism and pyoverdine secretion returned to pre-exposure levels when the honey was removed from the system. A decrease in CO₂ production can be indicative of either inhibition of metabolism or cell death. Biofilm exposure to 30% honey caused an immediate decrease in CO₂ production to around 33% of pre-exposure levels with a concomitant decrease in pyoverdine secretion (figure 3.4). The inability of *P. aeruginosa* cells to multiply while being exposed to 30% honey was confirmed when planktonically grown *P. aeruginosa* PAO1 was exposed to 30% (w/v) or higher concentration of honey on agar plates. Planktonically grown *P. aeruginosa* cells exposed for 24 hours to 30% and 50% honey did not grow on agar plates which provides evidence that bacterial killing occurred or severe growth inhibition that lasted longer than 24 hours after exposure.

The biofilm always recovered after 30% honey exposure for 2.5 hours in terms of metabolism and pyoverdine secretion but exposing the biofilm for 24 hours compared to 2.5 hours resulted in longer recovery times (figure 3.4 and 3.5).

Single sugars (data not shown), (glucose, etc) or sugar combinations, as described in the Methods section, simulating artificial honey, caused comparable responses in biofilm metabolism and pyoverdine secretion when compared to honey (figure 3.5 A and B). Changing the nutrient medium concentration from 0.03 g·L⁻¹ to 0.003 g·L⁻¹ TSB, resulted in biofilm behavior that mimicked exposure to 30% honey (figure 3.5C).

Manuka honey at 30% resulted in longer recovery times for recovery of biofilm metabolism and pyoverdine secretion when compared to the same concentration of clover honey. However, removal of honey from the system was followed by full biofilm recovery within 24 hours after exposure (results not shown).

FIGURE 3. 1. REAL TIME ANALYSIS OF PYOVERDINE SECRETION IN *P. AERUGINOSA* BIOFILM.

Pyoverdine (PVD) secretion (— blue) from *P. aeruginosa* culture on the inside of Tygon tubing was monitored during biofilm development, by the amount of light received to the LED analyzer system- LLABs system. The data shown is the inverse of the amount of light received by the analyzer (— Grey).

FIGURE 3. 2. REAL-TIME ANALYSIS OF PYOVERDINE SECRETION OF *P. AERUGINOSA* BIOFILM IN CEMS.

P. aeruginosa biofilm growth was analyzed through CO₂ production in the Carbon Dioxide Evolution Measurement System (CEMS) (— Green). Pyoverdine (PVD) secretion into the effluent was concurrently measured (— Blue).

FIGURE 3.3. EFFECT OF 5 % (W/V) HONEY ON *P. AERUGINOSA* SIDEROPHORE PRODUCTION AND BIOFILM METABOLISM.

Stationary phase *P. aeruginosa* biofilm (— Black) incubated in CEMS at 25°C, exposed to 5% (g/L) manuka honey, clover honey, and artificial honey for 24 h. PVD secretion in the effluent was measured (— Red, Orange and Green) respectively.

FIGURE 3. 4. EFFECT OF 24 HOUR EXPOSURE OF 30% (W/V) HONEY ON *P. AERUGINOSA* SIDEROPHORE PRODUCTION AND BIOFILM METABOLISM.

Stationary phase *P. aeruginosa* biofilm (— Black) incubated in CEMS at 25°C, exposed to 30% (w/v) (A) Manuka honey and (B) Clover honey for 24 h. PVD secretion in the effluent labeled (— Orange).

FIGURE 3. 5. EFFECT OF 2.5 HOUR EXPOSURE OF 30% (w/v) HONEY ON *P. AERUGINOSA* SIDEROPHORE PRODUCTION AND BIOFILM METABOLISM.

Stationary phase *P.aeruginosa* biofilm (— Black) incubated in CEMS at 25°C, exposed to 30% (w/v) (A) Clover honey, (B) artificial honey (C), and 0.003 g·L⁻¹ tryptic soy broth medium for 2.5 h. PVD secretion in the effluent (—red, green and yellow).

DISCUSSION

Honey is an ancient remedy that gained renewed interest in modern medicine in the late 1950's. This remedy has been previously used as preventative topical treatment for nosocomial infections (A-Jabari, 2005). Honey is primarily composed of various types of saccharides (Siddiqui, 1971; Roberts et al., 2012), enzymes (Bogdanov et al., 2008), and compounds that result in its antibacterial characteristics. Amongst all known types of honey studied, manuka honey is popular for its high antimicrobial properties in comparison to other types of honey (A-Jabari, 2005). An antimicrobial effect may benefit the infected host by killing or inhibiting the pathogen's metabolism and growth, or by reducing the production and excretion of pathogenic virulence factors. The exact antimicrobial action of honey is still not known, however, numerous studies demonstrated the sub-inhibitory effect of manuka honey and how it influences *P. aeruginosa* morphology (Henriques et al., 2011; Lu et al., 2013), promotes cell lysis (Roberts et al., 2012), impairs quorum sensing genes (thereby reducing expression of genes associated with virulence factors) (Wang et al., 2012) and inhibits bacterial growth (Roberts et al., 2012). A proper understanding of any antimicrobial compound's mechanisms of action will aid in developing appropriate application/dosage regimes and more effective combination therapies. This research was therefore designed to study bacterial biofilm responses, in particular metabolic response and siderophore secretion, upon exposure to honey to provide additional insight into the current literature's genetic and morphological knowledge. The increase in microbial resistance to current available antibiotics has necessitated the search for alternative ways of inhibiting pathogenic progression within the host and the development and administration of anti-virulent compounds (Cegelski et al., 2008).

***Pseudomonas aeruginosa* as model organism**

P. aeruginosa is one of the most prevalent pathogens responsible for nosocomial infections, leading to morbidity and mortality of patients, highlighting the urgency for novel therapies to combat this species. This organism was chosen as a model Gram-negative opportunistic pathogen for its ability to secrete numerous virulence factors of which we measured the siderophore pyoverdine. The production of pyoverdine in a mouse pathogenesis model enhanced bacterial infection and virulence (Meyer et al., 1996). It is known from literature that *P.*

aeruginosa possesses a strong ability to resist antimicrobial agents such as honey (Mandal et al., 2010).

The ability to accurately determine whether microbial cells are alive or dead (or something in between) at a particular time is not that well established (Davey, 2011). After any antibacterial exposure we can usually tell, in hindsight, if all bacteria were killed but if regrowth occurs at all it could either have been from a small percentage of survivors (while all other were killed) or the entire inoculum that went into a state of dormancy, followed by repair, restoration of growth (division) and metabolism once the stress has been removed. We measured CO₂ as an indication of actively metabolizing cells and therefore our results cannot distinguish between dead or dormant cells. From the perspective of an infected host, however, it could be argued that both dead and/or dormant pathogens would allow host defensive strategies to overcome the infection. The measurement of pyoverdine (before, during and after exposure to honey) as a virulence factor was also central to this study. Through the CEMS (measuring the amount of CO₂ produced from the biofilm as an indicator of biofilm growth) and LLABs (records the amount of transmitted light as an indication of biofilm biomass increase) systems, two independent real-time methods demonstrated the relationship between pyoverdine production and cell density.

Response to 5% honey

Pyoverdine secretion can be influenced at a gene regulation level. It is known that honey can affect bacterial genetic regulatory systems, for example the quorum sensing pathway (e.g. impairing quorum sensing by reducing the expression of *las* and *rhl* genes) which results in a reduction of gene expression associated with virulence factors (e.g. pyoverdine) (Starkey et al., 2012). These results were confirmed when reduced virulence factor production was demonstrated by exposing *P. aeruginosa* to 5% (w/v) manuka honey (Krona et al., 2013). It is also known that pyoverdine secretion and uptake in *P. aeruginosa* occurs through an ATP dependent manner and is thus a metabolically influenced pathway. The TonB complex is located on the inside of the periplasmic membrane and is controlled by the ExbB protein complex, which is responsible for transmitting energy of the protein motive force (Cornelis, 2010; Postle and Larsen, 2007). On the inner side of the cell membrane the ferric iron complex is transported to the cytoplasm of the cell through an ATP-binding cassette transporter (ABC) receptor (Heli et al., 2011).

At 5% honey exposure we observed an immediate decrease in pyoverdine excretion levels (figure 3.3). The 5% honey also caused an increase in metabolism that is consistent with an increase in biomass (cell growth). From what is known currently, the reduced pyoverdine excretion can therefore be attributed to regulation at the gene level (and not the absence of available ATP) as ATP regeneration should have occurred at sufficient levels to sustain prolonged (around 24 hours) active cell mass increase. Metabolism and pyoverdine production was restored to pre-exposure levels immediately after honey was removed from the system.

Honey at 5% apparently functioned as a nutrient (carbon) source as evidenced by the increase in biomass during exposure. These observations were previously reported in studies by Roberts et al. (2012) where *P. aeruginosa* growth was increased when manuka honey was diluted below the inhibitory levels.

Response to 30% honey

Upon 30% honey exposure, biofilm metabolism and pyoverdine excretion decreased immediately (figure 3.4). As mentioned before, a decrease in metabolism in our system can be interpreted as either cell killing and/or cell dormancy. The non-zero CO₂ levels during the 30% exposure together with CO₂ recovery curves (indicating regrowth after removal of the honey) suggested that a certain percentage of the cells remained alive and metabolically active (even during the entire 24 hours of exposure). Planktonic overnight *P. aeruginosa* cultures plated on agar containing different concentrations of honey showed that the cells were viable at concentrations of 5% and 15% (w/v) but could not grow on plates containing 30%, 50% (w/v) or more manuka honey.

Comparing honey with sugars

The biofilm responses in terms of changes in metabolic rate and pyoverdine excretion were similar when sugars (singularly or in combination) were added as compared to honey addition to equivalent final concentrations. Wang et al., has shown that sugar content in honey is responsible for inhibition of the QS pathway (Wang et al., 2012), which is known to be an important pathway for regulation of biofilm virulence factors (Labbate et al., 2004; Wen and Burn, 2004; Irie and Parsek, 2008).

Biofilm response to 30% honey was mimicked under starvation conditions

The biofilm response (in terms of changes in metabolism and pyoverdine excretion) to 30% honey was mimicked when the biofilm was exposed to starvation conditions (10 fold reduction in nutrient concentration). Similarity of this response to nutrient starvation condition can be explained through the bacterial stringent response, in which the biofilm undergoes a series of physiological and phenotypic changes that enables survival of some of the cells (Gilbert et al., 1990; Harder and Dijkhuizen, 1983; Azevedo et al., 2012). Studies have shown that metabolic states of the bacterium differ under nutrient starvation conditions. It is thought that this condition causes a rapid decrease in the rate of protein, RNA and cell wall synthesis and growth (Svitil et al., 1993; Van Delden et al., 2001). Therefore, the biofilm response to 30% (w/v) honey can be explained through the stringent response, where bacterial metabolism is shut down in harsh environments.

Differences in honeys

Different honeys (clover, manuka and artificial) resulted in qualitatively similar biofilm responses. However, exposure to 30% manuka honey seemed to have a residual effect resulting in more time needed for recovery (restoration of metabolism and pyoverdine excretion to pre-exposure levels). Planktonic *P. aeruginosa* cells that were exposed to 30% manuka honey also did not grow on agar plates 24 hours after plating (while cells incubated with 5% and 15% manuka honey for 24 hours did grow).

All three types of honey at 30% (w/v) were capable of inhibiting biofilm metabolism. Decreases in biofilm metabolism occurred almost instantaneously with exposure to honey, but all biofilms recovered their metabolic activity within 24 hours after exposure.

Pyoverdine secretion was inhibited in the presence of all three types of honey (manuka, clover, artificial) and both concentrations (5% and 30%) tested. Additional experiments with glucose, sucrose and maltose (data not shown) further indicated that saccharides play an inhibitory role on siderophore production. However, while pyoverdine secretion recovered in all experiments after the removal of honey, the recovery rate both in terms of PVD and CO₂ was faster after exposure to artificial honey compared to clover and manuka honey. The recovery of cell metabolism after removal of exposure to honey was also delayed in the case of manuka honey.

The benefit of manuka honey as antimicrobial treatment compared to clover or artificial honey therefore seems to be limited to an increased duration of biofilm metabolic inhibition that might provide extra time to an infected host to launch a defense.

No formation of resistance to honey was observed when the same biofilms were repeatedly exposed to honey, as measured in terms of metabolic response and pyoverdine excretion. These results support those presented by Cooper et al., (2010) and Lerrer et al., (2007).

Honey will be beneficial to the host from a few perspectives, firstly, with reduced pathogenic virulence, the host defense system is able to initiate bacterial killing by the presence of iron chelating agents. Secondly, honey's inhibitory action on the biofilm will benefit the host defense mechanism as it inhibits the increase of pathogenic cells. Thirdly, Lerrer et al. (2007) have shown that the inhibitory action of honey can play a significant role in preventing establishment of *P. aeruginosa* biofilms by blocking bacterial sugar binding proteins.

The results presented here provide insights into honey as a remedy when used at appropriate concentrations and for sufficient durations to result in decreased bacterial metabolism. The choice of using a rich growth medium in this study was asymmetrical to the host blood. Through real-time non-destructive metabolic measurements used in this study we were able to gain insight into the antimicrobial mechanism of honey, indicating that dosage can play an important role in treatment of infections. For example in the case of 5% manuka honey, the results gave a broad perspective of the effect of honey on the biofilm metabolism where pyoverdine was decreased, whereas growth and metabolism was increased during exposure. As an application this result might speak into dosage regimes because this means that failure to ensure high honey concentrations throughout treatment may harm the host by feeding the pathogens and providing them an opportunity to increase.

CHAPTER 4

The combined effect of honey and ethylenediamine tetraacetic acid disodium (EDTA) on biofilm metabolism

INTRODUCTION

This chapter takes a step forward and examines the potential of honey as a combination therapy. In the previous chapter, it was demonstrated that honey inhibits *P. aeruginosa* virulence and metabolism. This chapter will look at biofilm response when honey is used under conditions simulating combination therapy.

Combination antibiotic therapy or polytherapy is based on the rationale of ensuring that pathogenic species are treated by more than one drug at a time, which will target one or more parts of the cell and therefore reduces the chance of formation of resistance (Cohen, 1992). Studies have shown that antibiotic combination therapy improves efficiency of treatment of infection by reducing dosage of antibiotics. Given the difficulty of treating multi-resistance bacteria, combination therapy has been shown to improve the process of treatment (Muller et al., 2013; Karayil et al., 1998).

Within the past decade, the use of honey combined with antibiotics on Gram-positive species such as MRSA has been studied extensively. In a recent study, MRSA growth was inhibited two fold when 5% (w/v) manuka honey was combined with mupirocin, imipenem, tetracycline, rifampicin, and tazobactam antibiotics (Jenkins and Cooper, 2012; Karayil et al., 1998). Moreover, synergistic interaction between manuka honey and oxacillin has shown to reverse oxacillin resistance in MRSA, and result in down regulation of important genes in this species (Jenkins and Cooper, 2012). In addition, a similar study on *S. aureus* species has shown that no antibiotic resistance formed when rifampicin was combined with sub-inhibitory concentrations of manuka, whereas the use of this antibiotic alone resulted in emergence of resistance (Muller et al., 2013). Moreover, the synergistic effect of honey and antibiotics has been

also observed in Gram-negative species, when 5% manuka honey was combined with aminoglycoside antibiotics (Jenkins and Cooper, 2012).

As described in chapter 2, iron is a vital element that is essential for normal functioning and survival of all living organisms, and bacteria are not exceptions, especially as this element is known to play a role in the in vivo development of *P. aeruginosa* pathogenesis, biofilm formation and the defense against oxidative stress within its host (Vasil and Ochsner, 1999; Kim et al., 2003; Ratledge and Dover, 2000). Biofilm formation is strongly dependent on iron availability; therefore studies have indicated that chelating iron can be a promising new therapy for treatment of *P. aeruginosa* biofilms. Similar to the synergistic effect of honey and antibiotics, studies have reported that the combination of iron chelating agents with antibiotics initiates killing and biofilm dispersal (Moreau-Marquis et al., 2009).

This study investigated biofilm response in terms of metabolism and pyoverdine secretion to the combined effect of honey and an iron-chelating agent. The application of this study reflects biofilm response to the host system at the site of infection. In order to simulate application of combination therapy as occurring in wounds, a rich growth medium was used in this study, as is recommended for testing bacterial contaminants in cosmetics (Curry et al., 1993), food (Cunnif, 1995; US food and drug administration, 1995), and clinical applications (Murray et al., 1995).

MATERIAL AND METHODS

Bacterial strain and growth conditions.

Pseudomonas aeruginosa strain PAO1 (ATCC[®] 39324[™]) was used in all experiments, as described in chapter 3.

Growth conditions in AB defined Media. The overnight cultures for flow systems were prepared by growing PAO1 in 10ml broth medium containing AB defined medium supplemented with 10mM glucose in a test tube which was incubated for 24 h at 37°C in a shaking incubator at 250 rpm. *P. aeruginosa* biofilms were grown at 25°C.

Growth conditions in Tryptic Soy Broth. The overnight cultures for flow systems (see below) were prepared by growing PAO1 in 10ml tryptic soy broth (TSB) at a concentration of 3 g/L in 20 ml

test tubes, for 24 h at 37°C and at 250 rpm. *P. aeruginosa* biofilms were grown in 0.03 g/L TSB under continuous flow (as described in chapter 3).

Effect of iron depletion

The influence of iron on pyoverdine production by *P. aeruginosa* biofilms were studied by inoculating PAO1 in CEMS in defined medium containing 0.001 mM•L⁻¹ iron, and 1mM•L⁻¹ glucose as the primary carbon source. To illustrate the influence of iron on an established biofilm, the defined medium was switched to a medium depleted of iron for up to 24 hours. Pyoverdine secretion in the effluent was monitored continuously throughout and after the exposure.

The effect of iron on *P. aeruginosa* growth was studied through inoculating PAO1 in defined media void of iron. The biofilm was then exposed to 30% (w/v) manuka honey in defined medium containing 1mM glucose as the carbon source for 24 hours.

Effect of iron chelation

In order to assess the influence of an iron-chelating agent on biofilm metabolism and pyoverdine secretion, EDTA was chosen for further study. The CEMS system was used to monitor biofilm growth; once biofilms reached a stationary phase, 0.001 mM or 1mM EDTA was added to AB defined medium for 24 h under continuous flow (as described in chapter 3).

Combined effect of manuka honey and EDTA

P. aeruginosa was inoculated in CEMS with TSA as the growth medium. At stationary phase, biofilms were exposed to the media containing honey and EDTA for 24 hours. Combined treatment was studied using 5% and 30 % (w/v) manuka honey with 1mM EDTA both in AB defined growth media and TSB.

RESULTS AND DISCUSSION

Role of pyoverdine in biofilms

In the present study, available iron in the growth medium was manipulated firstly by introducing an iron chelating agent and secondly by omitting iron from the growth medium. In the presence of an iron-chelating agent such as lactoferrin, *P. aeruginosa* is known to secrete pyoverdine to chelate iron from the environment (Cornelis and Dingemans, 2013). However, the results in this study showed that the addition of EDTA to *P. aeruginosa* biofilm caused a decrease in biofilm metabolism and an initial decrease in pyoverdine secretion (figure 4.1). An increase in pyoverdine secretion during EDTA exposure occurred after the initial drop. It is known that EDTA can initiate biofilm dispersal by disrupting extrapolymeric substance stability (Banin et al., 2006; Raad et al., 2003). However we did not observe an excessive sloughing of biofilm biomass upon EDTA introduction and the rapid increase in metabolism after EDTA removal suggests that the biofilm remained intact but was hindered in its ability to function at full capacity, as seen by the lowered metabolism.

In a low iron environment, *P. aeruginosa* biofilms secrete pyoverdine to chelate iron. However, in the case of depleting iron from the media, there was no effect on the amount of pyoverdine secreted (figure 4.2 A). Switching from a medium containing iron to an iron depleted media resulted in a slight increase in biofilm metabolic activity while pyoverdine secretion dropped to slightly below the pre-exposure level (figure 4.2 B). Moreover, with addition of iron back into the medium, biofilm metabolism returned to pre-exposure level while pyoverdine secretion was not altered (figure 4.2 C). The results indicate that pyoverdine secretion is not directly related to iron concentration in the growth medium. Studies have also shown that pyoverdine synthesis can be highly influenced under different carbon conditions (Ponraj et al., 2013).

In addition, this study has shown that *P. aeruginosa* metabolic activity is low in an iron-depleted medium (figure 4.3 A compared to B). Upon 30% honey exposure, *P. aeruginosa* metabolism and pyoverdine secretion decreased immediately. It was demonstrated that *P. aeruginosa* was metabolically active throughout exposure and the biofilm metabolism continuously increased during exposure whereas pyoverdine secretion remained low (figure 4.3 A). Moreover, switching back to an iron depleted medium, biofilm metabolism increased even

further while pyoverdine secretion remained low and gradually increased in the biofilm. This response indicates that pyoverdine secretion is used for something other than only iron sequestration.

The response of *P. aeruginosa* biofilm to a combination of 30% manuka honey and EDTA in defined growth medium resulted in an immediate decrease in biofilm metabolic activity and pyoverdine secretion (figure 4.4) although the biofilm remained metabolically active throughout the exposure. Metabolism and pyoverdine secretion were restored when growth was switched back to the pre-exposure medium (figure 4.4). This unexpected response of *P. aeruginosa* indicates that a combination of drugs may have a reverse effect on biofilm activity, as our results have shown that the biofilm remained metabolically active throughout combination therapy but were metabolic inactive when exposed to honey alone (figure 3.4).

Combination effect of honey and an iron chelator in rich growth medium

In the previous chapter, honey at 5% was shown to function as a nutrient (carbon) source for the biofilm, as it resulted in an increase in *P. aeruginosa* biomass during exposure. However, combination of 5% (w/v) honey and EDTA did not have this effect and was shown to cause an immediate decrease both in biofilm metabolism and pyoverdine secretion (figure 4.5 A). This effect was diminished at the end of exposure where biofilm metabolic activity was returned to pre-exposure levels.

At 30% honey and EDTA exposure, a similar response to 5% honey and EDTA was observed; the only difference being that biofilm metabolic activity and pyoverdine secretion was delayed and did not recover immediately (figure 4.5 B). As mentioned earlier in chapter 3, a decrease in metabolism in the CEMS can be interpreted as either cell killing and/or cell dormancy. However, biofilm metabolic activity remained low for up to 24 hours after honey and EDTA was removed from the system (figure 4.5 B), indicating that presence of chelator and honey could have an important effect on *P. aeruginosa* biofilms.

FIGURE 4. 1. EFFECT OF EDTA ON *P. AERUGINOSA* CO₂ PRODUCTION AND SIDEROPHORE SECRETION.

P. aeruginosa biofilm incubated in defined medium containing 0.001 mM iron and 1mM glucose in CEMS at 25°C, exposed to 1 mM EDTA for 6 h.

FIGURE 4. 2. EFFECT OF IRON DEPLETED ENVIRONMENT ON *P. AERUGINOSA* METABOLISM.

(A) Mature *P. aeruginosa* biofilm (— Black) incubated in defined media containing 0.001 mM iron and 1mM glucose in the CEMS at 25°C, exposed to media depleted of iron for 24 h. (B) exposure of biofilm to an iron depleted medium. (C) Switch from an iron depleted medium to the pre-exposure medium. Pyoverdine did not change when the medium, was switched back to a defined medium containing iron. The changes in the medium was sensed by the species and resulted in an increase in CO₂ production as well as pyoverdine secretion. Results indicate that absence of iron in the environment did not have a significant effect on an established mature biofilm.

FIGURE 4. 3. EFFECT OF HONEY ON *P. AERUGINOSA* BIOFILM UNDER IRON DEPLETED CONDITIONS.

Mature *P. aeruginosa* biofilm (— Black) incubated in defined growth medium containing 1mM glucose which was void of iron was exposed to 30 % manuka honey for 20 h (A). The presence of honey caused an increase in CO₂ production during the exposure indicating biofilm activity. Biofilm CO₂ production was increased after removal of honey from the growth medium. The level of CO₂ production in (A) was compared to (B) which the same culture was inoculated in growth media containing 0.001 mM iron and not exposed to honey.

FIGURE 4. 4. THE COMBINED EFFECT OF 30% HONEY AND EDTA ON *P. AERUGINOSA* IN A DEFINED GROWTH MEDIUM.

Stationary phase *P. aeruginosa* biofilm (— Black) incubated in CEMS at 25°C in defined growth media was exposed to 30% (w/v) Manuka honey and 1 mM EDTA for 24 h. After 24 h treatment, media was switched to pre-exposure growth medium.

FIGURE 4. 5. THE COMBINED EFFECT OF HONEY AND EDTA ON *P. AERUGINOSA* IN RICH GROWTH MEDIUM.

Stationary phase *P. aeruginosa* biofilm (— Black) incubated in rich growth media (0.3 g·L⁻¹ tryptic soy broth) in CEMS at 25°C was exposed to (A) 5% (w/v) Manuka honey and 1 mM EDTA (B) 30% (w/v) Manuka honey containing 1 mM EDTA for 24 h. After 24 h treatment, media was switched to pre-exposure growth medium.

CHAPTER 5

CONCLUSION AND FUTURE WORK

The findings presented in this research will contribute to a better understanding of honey as a natural remedy and its abilities to counter bacterial metabolism and virulence. Through non-destructive metabolic measurement it was possible to explore, in real-time, the effect of honey on bacterial metabolism both in terms of response to exposure as well as recovery under different environmental conditions. As, described in chapter 3, it was noted that the antimicrobial effect of honey is highly dependent on the dosage used and can have a significant role in biofilm inhibition and therefore potentially also in the treatment of infections. For example in the case of 5% manuka honey, the results gave a broad perspective of the effect of honey on the biofilm metabolism where pyoverdine was decreased, whereas growth and metabolism was increased during exposure. When applied at 30%, manuka honey caused a decrease in both pyoverdine secretion and metabolism. In an applied sense, this result might speak into dosage regimes because this means that failure to ensure high honey concentrations throughout treatment may harm the host by feeding the pathogens and providing them an opportunity to increase.

In addition, the results obtained from defined growth medium indicated that pyoverdine production in *P. aeruginosa* was unrelated to iron availability. This is an indication that pyoverdine is possibly produced for other purposes in the biofilm, which requires further investigation. Biofilm behavior in response to combination treatment showed surprising results. For example, treatment of honey and EDTA in rich growth medium decreased biofilm metabolic activity and increased biofilm recovery time whereas, similar treatment in defined medium did not have such effects.

Studies on honey and its role on biofilm metabolism require further research. Although biofilm metabolic activity was measured with the CEMS system in this project, it is proposed that the effect of honey on biofilm metabolism should be also tested in the presence of compounds that target bacterial metabolism. Colistin is a good example of such a compound that is responsible for targeting cells with low metabolic active. It is hypothesized that at 5% honey the presence of colistin will inhibit metabolism, which can be determined at different concentrations of honey.

This will also demonstrate if exposure to honey can initiate formation of dormant cells. In *P. aeruginosa*, colistin has proven to be effective against inactive cells in the deep layers of biofilm, induce expression of *pmr*-LPS modification system and inhibition of MexAB-OprM efflux pump (Harsmen et al., 2010; Pamp et al., 2008). Overall, the application of understanding the mechanism of honey that influence metabolism will help in better design of therapies.

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