The Effects of Naturally-Derived Plant Compounds on *Pseudomonas aeruginosa* Pathogenicity

A thesis presented to

The Faculty of Graduate Studies

of

Lakehead University

by

KARINA KACHUR

In partial fulfilment of the requirements

for the degree of

Masters of Science in Biology

July 8, 2014

© Karina Kachur, 2014

Abstract

Pseudomonas aeruginosa has risen as one of the most resistant bacterial pathogens affecting people worldwide. Inefficacy of current antimicrobial treatments to relieve *P. aeruginosa* infections demands the need for novel antimicrobial therapies. Natural products such as the aqueous and alcoholic extracts of North American ginseng, as well as thymol and carvacrol, compounds purified from thyme and oregano, have previously displayed antibacterial properties against *P. aeruginosa*. In this study we examined the effect of these compounds on *P. aeruginosa* pathogenicity by measuring pathogenic markers (ie virulence factor secretion, biofilm formation) after PAO1 and Cystic Fibrosis clinical isolates were treated with the natural compounds. Thymol and carvacrol were able to attenuate the growth of *P. aeruginosa* clinical isolates in nutrient and minimal media however PAO1growth was only attenuated in the nutrient media. The alcoholic ginseng extract displayed no growth inhibitory action towards any strain tested; however the aqueous ginseng extract attenuated growth of all strains at concentrations of 8.0-7.0% w/v and 3.0-2.0% w/v in the minimal and nutrient media respectively. Sub-inhibitory concentrations of the aqueous ginseng extract increased growth rate and bacterial adherence, but decreased the production of many virulence factors, while the alcoholic ginseng treatment increases secretion of select virulence factors. Microarray analysis revealed decreases in virulence factors with sub-inhibitory aqueous ginseng extract exposure. Treatment with sub-inhibitory concentrations of thymol or carvacrol reduced pigment, lipase, and protease secretion after exposure to thymol or carvacrol in a strain specific manner; however bacterial motility was negatively affected in all strains with

treatment of either compound. Microarray the thymol 6 h and carvacrol 24 h treatments displayed enhanced expression of pyoverdine and pyochelin synthesis genes. Enrichment of the ribosomal pathway was substantial after the 24 h carvacrol and aqueous extract exposures, findings which require further investigation. Overall reduction in pathogenesis was most evident with the aqueous ginseng extract however thymol and carvacrol were most effective at inhibiting bacterial growth. Further chemical analysis of the alcoholic and aqueous ginseng extracts may reveal specific extract constituents with potent antibacterial properties. Analysis of potential synergy between the tested plant components and current antibiotics of *P. aeruginosa* may provide a greater appreciation for their use in antibacterial therapies.

Lay Summary

Faculty and students in the Department of Biology are bound together by a common interest in explaining the diversity of life, the fit between form and function, and the distribution and abundance of organisms. The research undertaken in this thesis aimed to better our understanding of the function of bacterial pathogen *Pseudomonas aeruginosa* under the influence of naturally derived antibacterial compounds. Through the assessment of virulence factor production, bacterial growth, and expression ananlysis we were able to determine the efficacy of these naturally produced compounds to inhibit *P. aeruginosa* pathogenicity. Further exploration of larger samples sizes from varying populations would help to better our understanding of the potential for these naturally produced compounds to act as therapeutic agents against *P. aeruginosa* and possibly other bacterial pathogens.

Acknowledgements

The last two years have been an incredible journey and a time of tremendous growth. My time spent at NOSM in the lab and out in the scientific community have imparted on me a deeper appreciation and resepct for the importance of research. To my fellow aspiring researchers, NOSM lab assistants, and faculty thank you for all your words of encouragement and guidance during my time here. Working with all of you has been a joyous experience and I wish every one of you the best of luck for the future.

To Dr. Nix, thank you for your continued guidance over the past two years in regards to microbiology, science, and life in general. Thank you to Joshua Choi for your assistance in optimizing my RNA extraction experiments, to Dr. Lees for assisting me in my RNA quality and quantity analysis, and to Mr. Carter at the London Regional Genomics Centre for his continued assistance and knowledge with the microarray analysis.

To my family and friends I cannot thank you enough for your support over the last two years. Your encouragement has motivated me and your company has always lifted my spirits.

To my committee members, Dr. Leung and Dr. Malek, thank you for your continual support throughout my Masters program. You have always made yourself available if I'v ever needed guidance which I appreciate very much. Finally, I'd like to thank my supervisor Dr. Zach Suntres. Thank you for trusting me enough to allow me independence with this project. I've learnt so much from this opportunity and I have you to thank for all of it. Your encouragement and leadership have helped me to reach beyond my own expectations. Thank you for the opportunities you've provided me with to share

my research, they have been invaluable experiences I will take with me in my future endevours.

Abbreviations

AI	Autoinducer
ATP	Adenosine triphosphate
cDNA	Complementary DNA
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CGH	Chlorohexidine digluconate
CI	Clinical isolate
DNA	Deoxyribonucleic acid
ERk	Extracellular signal-regulated kinases
Exo	Exotoxins
GO	Gene ontology
HI	*Hemophilius influenza*
Ig	Immunoglobulin
IFN	Interferon
IL	Interluekin
IV	Intravenous
JNK	c-Jun N-terminal kinases
KEGG	Kyoto Excyclopedia of Genes and Genomes
LPS	Lipopolysaccharide
M9	Minimal salts media
MAPK	Mitogen activated protein kinases
MHCA	Mueller-Hinton cation adjusted broth

MIC	Minimum inhibitory concentration
mm	millimeter
MMR	Mis-match repair
NF-κB	Nuclear factor kappa B transcription factor
OD	Optical density
p38	mitrogen activated protein kinase p38
PAI-1	Plasminogen activator inhibitor-1
PI3K	phosphoinositide-3-kinase
PM	peritoneal macrophages
PMNL	polmorphonuclear leukocytes
PPD	protopanaxadiol
PPT	protopanaxatriol
PQS	Pseudomonas quinolone signalling
QS	Quorum sensing
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	*Staphlococcus aureus*
sscDNA	Single stranded complimentary DNA
TCA	Citric acid cycle
TNF	Tumor necrosis factor

Table of Contents

Abstract i

Lay Summary iii

Acknowledgements iv

Abbreviations vi

List of Figures and Tables 1

Chapter I. Thesis Overview 3

1.1 *Pseudomonas aeruginosa* Characteristics 3

1.2 *Pseudomonas aeruginosa* and Nosocomial Infections 4

1.3 Cystic Fibrosis 4

1.3.1 Pathogenicity of Cystic Fibrosis 5

1.4 *P.aeruginosa* Pathogenicity 7

1.4.1 Quorum Sensing 7

1.4.2 Proteases, Elastases, and Lipases-Virulence Factors 8

1.4.3 Excreted Pigments 9

1.4.4 Exotoxins 11

1.4.5 Attachment Mechanisms: Flagella and IV Pili 12

1.5 Biofilm Development 13

1.6 Antibiotic Resistance Mechanisms 14

1.6.1 Permeability and Catabolic Enzyme Expression 14

1.6.2 Expression of Multiple Efflux Pump Systems 15

1.6.3 Biofilm Mode of Growth 15

1.6.4 Highly Mutable Genome and Acquired Resistance 17

1.7 Treatment of *P.aeruginosa* Lung Infections 17

1.7.1 Early/Acute Infection 18

1.7.2 Chronic Infections 18

1.8 Novel Treatment Options: Naturally Derived Antibiotic Compounds 19

Thesis Objectives 21

Chapter II. The Effects of North American Ginseng (*Panax quinquefolius*) Aqueous and Alcoholic Extracts towards *P.aeruginosa* Pathogenicity 23

2.1 Introduction 23
2.1.1 Ginseng: An Herbaceous Plant 23
2.1.2 Ginseng as a Natural Medicine 23
2.1.3 Active Components of Ginseng 24
2.1.4 P*anax quinquefolius* and Bacterial Infections 25
2.1.5 Rationale for Future Research 29
2.2 Methodology 30
2.2.1 Bacterial Strains and Media 30
2.2.2 Ginseng Extract Preparation 32
2.2.3 Minimum Inhibitory Concentration Determination 32
2.2.4 Growth Curve Analysis 34
2.2.5 Bacterial Adherence Assay 35
2.2.6 Virulence Factor Production 37
2.2.7 Motility Assay 39
2.2.8 Genomic Expression Analysis 40
2.2.9 Microarray Processing 42
2.2.10 Statistics 43
2.3 Results 44
2.3.1 Minimum Inhibitory Concentration of Aqueous or Alcoholic Ginseng Extracts 44
2.3.2 Effect of Aqueous or Alcoholic Ginseng Treatment on Bacterial Growth 45
2.3.3 Effect of Aqueous or Alcoholic Ginseng Treatment on Preventing Bacterial Attachment 48
2.3.4 Effects of Ginseng Treatment on Preformed Biofilms 50
2.3.5 Effect of Ginseng Extract Treatment on *P.aeruginosa* Pigment Production 52
2.3.6 The Outcome of Aqueous or Alcoholic Ginseng Treatment on Bacterial Protease and Lipase Production 54
2.3.7 Influence of Aqueous and Alcoholic Ginseng Treatment on *P.aeruginosa* Motility 57
2.3.8 Effects of Aqueous Ginseng Treatment on *P. aeruginosa* Gene Expression 59
2.3.9 Gene-Ontology Enrichment Analysis of *P. aeruginosa* Treated with Aqueous Ginseng Extract 72

2.3.10 Pathway Enrichment Analysis of *P. aeruinosa* treated with Aqueous Ginseng Extract 78
2.4 Discussion 80
Chapter III. The Effect of Thymol or Carvacrol on *P. aeruginosa* Pathogenicity. 99
3.1 Introduction 99
3.1.1 Sources of Thymol and Carvacrol 99
3.1.2 Chemical Structure and Function 99
3.1.2 Antimicrobial Properties and Mechanisms 100
3.1.3 Current Development of Thymol and Carvacrol Uses 102
3.1.4 Rationale for Further Exploration of the Antibacterial Properties of Thymol and Carvacrol 104
3.2 Methodology 105
3.2.1 Bacterial Strains and Media 105
3.2.2 Compound Solution Preparation 105
3.2.3 Minimum Inhibitory Concentration Determination 105
3.2.4 Growth Curve Analysis 105
3.2.5 Bacterial Adherence Assays 106
3.2.6 Virulence Factor Production 106
3.2.7 Motility Assays 106
3.2.8 Genomic Expression Analysis 106
3.2.9 Microarray Processing 107
3.2.10 Statistics 107
3.3 Results 108
3.3.1 Minimum Inhibitory Concentration of Thymol or Carvacrol 108
3.3.2 Effect of Thymol or Carvacrol Treatment on Bacterial Growth 110
3.3.3 Effect of Thymol or Carvacrol Treatment on Preventing Bacterial Attachment 113
3.3.4 Effect of Thymol or Carvacrol Treatment on Preformed Biofilms 115
3.3.5 Effect of Thymol or Carvacrol Treatment on *P. aeruginosa* Pigment Production 117
3.3.6 Outcome of Thymol or Carvacrol Treatment on Bacterial Lipase and Protease Production 119

3.3.7 Influence of Thymol or Carvacrol Treatment on *P. aeruginosa* Motility 122
3.3.8 Effects of Thymol and Carvacrol Treatment on *P. aeruginosa* Gene Expression 124
3.3.9 Gene-Ontology Enrichment Analysis of *P. aeruginosa* Treated with Thymol or Carvacrol 134
3.3.10 Pathway Enrichment Analysis of *P. aeruginosa* Treated with Thymol or Carvacrol 146
3.4 Discussion 148
Conclusion 161
References 162

List of Figures and Tables

Figure 1.1: Growth curve analysis in the presence of the aqueous ginseng extract……...46

Figure 1.2: Growth curve analysis in the presence of the alcoholic ginseng extract…....47

Figure 1.3: The effect of aqueous and alcoholic ginseng treatment on the prevention of *P. aeruginosa* adherence…………………………………………………………....49

Figure 1.4: The effect of aqueous and alcoholic ginseng treatment on 48 h *P. aeruginosa* biofilms……………………………………………………………………………..51

Figure 1.5: The effect of aqueous and alcoholic ginseng extract treatment on the production of the pigment pyoverdine…………………………………………………..53

Figure 1.6: Protease production in the presence of aqueous or alcoholic ginseng extract……………………………………………………………………………………55

Figure 1.7: Lipase production in the presence of aqueous or alcoholic ginseng extract...56

Figure 1.8: Swimming motility in the presence of aqueous or alcoholic ginseng extract……………………………………………………………………………………58

Figure 2.1: Chemical Structure of thymol and carvacrol………………………………100

Figure 2.2: Growth curve analysis in the presence of the sub-inhibitory concentrations of thymol……………………………………………………………………………..111

Figure 2.3: Growth curve analysis in the presence of the sub-inhibitory concentrations of carvacrol……………………………………………………………………….....112

Figure 2.4: The effect of thymol or carvacrol treatment on the prevention of *P. aeruginosa* adherence…………………………………………………………..…..114

Figure 2.5: The effect of thymol or carvacrol treatment on 48 h *P. aeruginosa* biofilms………………………………………………………………………………116

Figure 2.6: The effect of thymol or carvacrol treatment on the production of the pigment pyoverdine……………………………………………………………………………118

Figure 2.7: Protease production in the presence of thymol or carvacrol……………….120

Figure 2.8: Lipase production in the presence of thymol or carvacrol…………………121

Figure 2.9: Swimming motility in the presence of thymol or carvacrol……………….123

Table 1.1: Minimum inhibitory concentrations (MIC) of *P. aeruginosa* treated with either aqueous or alcoholic ginseng extracts…………………………………………………....44

Table 1.2: Significant gene fold changes after exposure to the aqueous ginseng extract………………………………………………………………………………......62

Table 1.3: Gene ontology (GO) enrichment analysis of *P. aeruginosa* after exposure to the aqueous ginseng extract……………………………………………………..….73

Table 1.4: Pathway enrichment analysis of *P. aeruginosa* after exposure to the aqueous ginseng extract……………………………………………...………………....79

Table 2.1: Minimum inhibitory concentrations (MIC) of *P. aeruginosa* treated with either aqueous or alcoholic ginseng extracts……………………………………………..109

Table 2.2: Significant gene fold changes after exposure to thymol or carvacrol……....126

Table 2.3: Gene ontology (GO) enrichment analysis of *P. aeruginosa* after exposure to thymol or carvacrol……...……………………………………………………...136

Table 2.4: Pathway enrichment analysis of *P. aeruginosa* after exposure to thymol or carvacrol…………………………………………………...…………....147

Chapter I. Thesis Overview

1.1 *Pseudomonas aeruginosa* Characteristics

Infections and disease caused by virulent bacteria are a prominent health concern across the world. The adaptability of bacteria to a variety of environments, host defence mechanisms, and antibiotic treatments has led to persistent infections with limited available antibiotic therapies. Of prominence is the opportunistic pathogen *Pseudomonas aeruginosa*, a highly mutable and antibiotic resistant bacterium which is a leading cause of nosocomial infections and chronic lung infections.

P. aeruginosa is a gram negative, facultative anaerobic, motile, rod-shaped bacterium. A environmentally ubiquitous organism, *P. aeruginosa* thrives in both terrestrial and aquatic environments adapting to variations of nutrient supplies, atmospheric oxygen concentration, salinity, and temperature (Hardalo and Edberg 1997, Worlitzsch, Tarran et al. 2002). *P. aeruginosa's* adaptability is largely attributed to its genome size of 6.3 million base pairs, larger than another other bacterial genome sequenced to date (Stover, Pham et al. 2000). *P. aeruginosa's* genome is comprised of approximately 10% of regulatory genes (Stover, Pham et al. 2000, Schuster and Greenberg 2006), many of which are thought to function as gene expression regulators allowing *P. aeruginosa* great control and manipulation of its genome increasing the bacteria's adaptability to a broad range of conditions (Schuster and Greenberg 2006).

The large genome size of *P. aeruginosa* is attributed to an increase in genetic diversity, displayed through the overwhelmingly diverse number of paralogous groups found within the genome and not genome organization (Stover, Pham et al. 2000). Each

paralogous group encodes genes with specific functions such as outer membrane proteins, quorum sensing compounds, excreted enzymes and toxins, and virulence factors.The ability of *P. aeruginosa* to orchestrate this immense repetoir of genes has allowed it to thrive as an opportunistic pathogen.

1.2 *Pseudomonas aeruginosa* and Nosocomial Infections

Alongside *Escherichia coli* and *Staphylococcus aureus*, *P. aeruginosa* is a leading cause of nosocomial infections (Schaberg, Culver et al. 1991). *P. aeruginosa* has been reported to cause 12% of nosocomial urinary tract infections, 10% of bacteremias, 8% of wound infections from surgery, and 16% of hospital acquired pneumonia (Van Delden and Iglewski 1998). Those patients with compromised immune systems, such as those with burn wounds, transplants, cancer, and cystic fibrosis (CF) are most susceptible to infections caused by this pathogen (Bodey, Bolivar et al. 1983, Barbieri and Sun 2004). The cause of infection can often be traced back to contaminated respirators, surgical tools, faucets, and nebulizers for antibiotic administration (Bodey, Bolivar et al. 1983). Often the most detrimental infections caused by *P. aeruginosa* are in patients with cystic fibrosis.

1.3 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder and the most common genetic disorder within Caucasian populations (Wood, Boat et al. 1976, Tummler and Kiewitz 1999). The disease is characterized by mutations to the cystic fibrosis transmembrane conductor (CFTR) gene which is responsible for the proper synthesis of a transmembrane cAMP regulated channel which transports ions, primarily chloride, across

the membranes of exocrine epithelial cells [B (Tummler and Kiewitz 1999, Lubamba, Dhooghe et al. 2012). The most prominent mutation accounting for approximately 90% of all cases leading to CF is the ΔF508 mutation (Riordan 2008, Lubamba, Dhooghe et al. 2012) in which a phenylalanine residue is deleted at the 508 position leading to improper folding of the protein and its early degradation (Lubamba, Dhooghe et al. 2012).

Improper passage of ions and the subsequent movement of water into the epithelial cells from improper CFTR function, leads to an dehydrated environment which favours the build up of viscous mucus (Bye, Ewig et al. 1994, Lubamba, Dhooghe et al. 2012, Lukacs and Verkman 2012). Improper ciliary clearance of the mucous leads to airway obstruction in the peripheral airways, causing stress on the respiration of the individual (Bye, Ewig et al. 1994). Continual airway obstruction creates a state of constant exaggerated inflammation and an excessive recruitment of pro-inflammatory cytokines, neutrophils, and macrophages (Elizur, Cannon et al. 2008) . The environment created within the airways of CF individuals creates circumstances favouring bacterial infection (Govan and Deretic 1996). Bacterial colonization starts a viscious cycle of infection, inflammation, and destruction of the airway epithelium due toproduction of host and bacterial proteases and neutrophil elastase (Birrer, McElvaney et al. 1994). This cycle instigates lung deterioration, respiratory failure, and in many cases death (Govan and Deretic 1996).

1.3.1 Pathogenicity of Cystic Fibrosis

Bacterial lung infections begin early in life for CF individuals starting with infections of *Staphlococcus aureus* (SA) and non-encapsulated *Hemophilus influenzae*

(HI), which dominate for the first two years of life (Abman, Ogle et al. 1991). Prior to, and during these initial infections, the inflammatory response starts and an overabundance of pro-inflammatory cytokines, neutrophils, and phagocytic cells are signalled to the lungs (Lyczak, Cannon et al. 2002). The innate over inflammation is exaggerated by the early bacterial infections by SA, which produces multiple virulence factors (Cohen 1986). Production of bacterial virulence factors, in conjunction with the host cell produced elastase and proteases, causes severe lung tissue damage which further weakens the host cell's ability to ward off future bacterial infection (Bye, Ewig et al. 1994).

Due to the efficacy of current anti-*Staphlococcal* treatments, primary infections caused by SA are kept under control, and seldom lead to long term chronic infections. Implementation of beta-lactam antibiotics including oxacillin and nafcillin, and long term prophylaxis with tetracyclins and cephalosporins has kept the majority of SA lung infections at bay (Michel 1988). Improvements in overall patient health are rarely seen following SA eradication (Lyczak, Cannon et al. 2002), and can in part be explained by the phenomena of continual SA antibiotic administration increasing the risk of acquiring more detrimental infections caused by *P. aeruginosa* (Bauernfeind, Emminger et al. 1987, Ratjen, Comes et al. 2001).

After the first two years of life, infections caused by *P. aeruginosa* begin to dominate the lungs of patients with cystic fibrosis (Abman, Ogle et al. 1991). Infections caused by *P. aeruginosa* can be acquired through hospital settings and social contact; however most often clinical isolates suggest an origin from the environment (Burns, Gibson et al. 2001). Due to defective phagocytosis mechanisms and muscocillary

clearance of airway mucus, once present within the lungs, *P. aeruginosa* infections rapidly escalate. Chronic *P. aeruginosa* infection, often characterized by mucoid phenotype and biofilm formation, are difficult to fully eradicate with current chemotherapy options (Gilligan 1991). As infection with *P. aeruginosa* increases lung damage worsens, which can lead to a state of complete lung deterioration (Gilligan 1991, Bye, Ewig et al. 1994, Govan and Deretic 1996).

1.4 *P.aeruginosa* Pathogenicity

P. aeruginosa produces a wide variety of virulence factors, such as proteases, elastases, lipases, and pigments, which aid the bacteria in overcoming the host's immune responses and out-competing other pathogens. *P. aeruginosa* also expresses various cell-associated virulence factors such as pili, flagella, and lipopolysaccharide (Wagner and Iglewski 2008). The overproduction of extracellular polysaccharides, such as alginate, has also been shown to increase *P.aeruginosa's* pathogenicity by promoting mucoid phenotype conversion (Stapper, Narasimhan et al. 2004).The synthesis of many *P. aeruginosa* virulence factors is largely under the control of quorum sensing, a density-dependent signalling method employed by various bacterial species (Schuster, Sexton et al. 2013).

1.4.1 Quorum Sensing

Quorum sensing (QS) is a signalling system which employs cell density dependent autoinduced chemical signals to direct and coordinate mechanisms such as biofilm formation and virulence factor secretion (Fuqua, Winans et al. 1994, Schuster,

Sexton et al. 2013). This communication process is controlled through the production of self-synthesized molecules called autoinducers (AI) (Miller and Bassler 2001). In *P. aeruginosa* the autoinducer molecules are acyl-homoserine lactones (AHLs) which are synthesized and secreted under the control of the *las* and *rhl* pathways (Schuster and Greenberg 2006). The LasR-LasI pathway functions to send out signals via the production of the AHL 3-oxo-dodecaconyl-homoserine lactone (3OC12-HSL), and controls the function of the downstream RhlR-RhlI pathway, which sends signals via the production of the AHL butanoyl-homoserine lactone (C4-HSL) (Schuster, Sexton et al. 2013).

Over 300 genes within the *P. aeruginosa* genome are under the control of these two QS pathways including many virulence factors (Schuster, Lostroh et al. 2003, Wagner, Bushnell et al. 2003). Previous studies implementing animal model hosts have demonstrated the importance of QS control in virulence factor production for the establishment and persistence of *P. aeruginosa* infections (Rumbaugh, Griswold et al. 1999, Pearson, Feldman et al. 2000, Wu, Song et al. 2001). The population dependence of QS signalling additionally promotes the organization and development of bacterial biofilms (Schuster, Sexton et al. 2013).

1.4.2 Proteases, Elastases, and Lipases-Virulence Factors

Exoenzymes are those enzymes secreted by eukaryotic and prokaryotic cells which function extracellularly. *P. aeruginosa* produces a variety of exoenzymes including several elastase, protease, and lipase enzymes (Van Delden and Iglewski 1998, Wang, Tu et al. 2013). The alkaline proteases of *P. aeruginosa* are not well studied, but

play a role in tissue invasion and the progression of systemic infections (Van Delden and Iglewski 1998). However, the protease enzymes involved in the breakdown of elastin have been well characterized.

LasA and LasB are the main elastase exoenzymes produced by *P. aeruginosa* (Galloway 1991). Combined, these enzymes break down lung epithelial tissue through elastin, collagen, and fibrin destruction leading to haemorrhages in the lungs during invasive infections (Heck, Morihara et al. 1986, Galloway 1991, Van Delden and Iglewski 1998). LasB has also been shown to disrupt host defence mechanisms through the inactivation of human immunoglobulins G and A, and the disruption of α-1-protinease inhibitor (Morihara, Tsuzuki et al. 1979, Heck, Alarcon et al. 1990, Hong and Ghebrehiwet 1992).

P. aeruginosa also produces various lipase and hemolysin enzymes, such as phospholipase C and rhamnolipid, which together act to break down lipid and lecithin molecules aiding in tissue invasion (Van Delden and Iglewski 1998). The decomposition of lung surfactant phospholipids is believed to play a critical role in the increased rates of lung collapse and airway closure associated with lung infections. The ability of ciliary transport to properly clear lung surfactant is also negatively impacted by the presence of rhamnolipids from *P. aeruginosa* (Read, Roberts et al. 1992).

1.4.3 Excreted Pigments

P. aeruginosa also produces multiple pigments which are implicated in the defence against phagocytosis and competing bacteria, as well as playing major roles in micro-nutrient acquisition. Pyoverydine is the name given to a group of green fluorescent

pigments excreted by *Pseudomonas* (Visca, Imperi et al. 2007). More than 50 different forms of pyoverdine have been determined, all sharing a conserved fluorescent dihydroxyquinoline chromophore with both an acyl and peptide side chain (Budzikiewicz 2004). In combination with pyoverdine, *P. aeruginosa* also produces a second chromophore pigment named pyochelin, which is involved in iron sequestration for growth requirements (Cox, Rinehart et al. 1981). Iron is an essential nutrient of *P. aeruginosa* and pyoverdine and pyochelin are the main molecules used to acquire this nutrient (Visca, Imperi et al. 2007). Pyoverdine has also been linked to the regulation of virulence factor production and quorum sensing signalling through its involvement in various iron-dependent pathways (Lamont, Beare et al. 2002, Beare, For et al. 2003).

Pyocyanin, a blue pigment with a tricyclic phenazine structure is another important virulence factor of *P. aeruginosa* (Wilson, Sykes et al. 1988). The redox- cycling activity of this pigment produces reactive oxygen species (ROS) which inhibit catalase enzymes, disrupt the balance of reduced/oxidized glutathione in epithelial cells, and cause severe tissue damage (Lau, Hassett et al. 2004). In addition, pyocyanin displays inhibitory effects towards epithelium ciliary beating, (Wilson, Sykes et al. 1988)(Wilson et al., 1988)(Wilson et al., 1988) nitric oxide production in macrophages and endothelial cells, increases superoxide production, disrupts α-1 protease inhibitors, and increases the rate of apoptosis in neutrophils (Wilson, Sykes et al. 1988, Shellito, Nelson et al. 1992, Muller and Sorrell 1997, Britigan, Railsback et al. 1999, Usher, Lawson et al. 2002). Immune modulation by pyocyanin production stimulates the release of proinflammatory cytokine IL-8 while repressing IL-2 (Nutman, Berger et al. 1987, Denning, Wollenweber et al. 1998). In animal models pyocyanin has been linked to an increase in neutrophil

influx accompanied by an increase in bronchoconstriction (Forteza, Lauredo et al. 1994, Lauredo, Sabater et al. 1998).

1.4.4 Exotoxins

A collection of exotoxins are also produced by *P. aeruginosa*, which act to damage foreign cells and/or disrupt proper cell metabolism. Exotoxins ExoS, ExoT, ExoU, and ExoY are all transferred via type III secretion, a process in which metabolites are transferred from the bacteria to the eukaryotic cell via a contact dependent mechanism (Blocker, Jouihri et al. 2001). All of the type III secreted exotoxins of *P. aeruginosa* aid in pathogenecitiy by inhibiting proper function of the host cell's innate immune response (Barbieri and Sun 2004). ExoU is a phospholipase toxin primarily involved in the cytotoxicity of macrophages and epithelial cells, and fibroblasts (Finck-Barbancon, Goranson et al. 1997, Sato, Frank et al. 2003). ExoY is an adenlyate cyclase which increases the expression of cAMP in eurkaryotic cells causing actin cytoskeleton disorder (Yahr, Barbieri et al. 1996). ExoS and ExoT are structurally similar toxins both containing N-terminal region which functions to disrupt actin cytoskeleton organization through activation of Rho GTPases and a C-terminal ADP-ribosyltransferase domain which functions primarily to disrupt host protein synthesis (Barbieri and Sun 2004). ExoT's primary pathogenic mechanism is through phagocytosis inhibition, while ExoS greatly increases host cell apoptosis (Kaufman, Jia et al. 2000, Barbieri and Sun 2004).

P. aeruginosa also secretes the exotoxin ExoA, a toxin similar in structure and mechanism to the diphtheria toxin of *Corynebacterium diptheriae* and the cholera toxin of *Vibrio cholera* (Popoff 2005). Unlike the previously discussed toxins, ExoA is

secreted through a type II secretion system (Cianciotto 2005). This mono-ADP ribosyltransferase toxin increases bacterial virulence by causing cell death to eukaryotic cells through protein synthesis inhibition by blocking elongation factor 2 (eEF2) (Van Ness, Howard et al. 1980, Corda and Di Girolamo 2003).

1.4.5 Attachment Mechanisms: Flagella and IV Pili

Attachment of bacteria to host cells is critical for the initiation of infection (Ramos, Rumbo et al. 2004). The majority of these interactions are mediated by bacterial surface structures involved in attachment and motility. The singular unipolar flagella of *P. aeruginosa* generate the main motility force of the organism, but also aid in the initial stages of lung infections by propelling the bacteria through mucosa of the host organism, increasing the bacteria's ability to reach and adhere to lung epithelial cells (Ottemann and Miller 1997). Although flagella aid in bacterial infectivity, monomers of the flagellar structure can also be detected by the host's immune cells (Hayashi, Smith et al. 2001).

In conjunction with flagella, *P. aeruginosa* also produces surface adhesion structures termed type IV pili (Strom and Lory 1993). Type IV pili aid in recognition of other bacterial cells, attachment to eukaryotic cells, and some forms of bacterial motility such as twitching (Amako and Umeda 1982, Mattick 2002). In various tissue types it has been shown that pilated bacteria have higher pathogenicity over their non-pilated counterparts (Strom and Lory 1993). Expression of type IV pili and flagella greatly enhances *P.aeruginosa* biofilm formation (O'Toole and Kolter 1998, Stoodley, Sauer et al. 2002).

1.5 Biofilm Development

One of the main mechanisms which have made *P. aeruginosa* a successful opportunistic pathogen is the bacteria's ability to thrive in a biofilm mode of growth. Biofilms are aggregations of microbial cells forming unified communities, which are attached to various abiotic and biotic surfaces (O'Toole, Gibbs et al. 2000). Chronic lung infections of patients with CF are dominated by *P. aeruginosa* biofilms.

The initiation of biofilm development is thought to be triggered by environmental stimuli, however each bacterial species reacts differently to environmental changes and some, not unlike *P. aeruginosa*, can develop biofilms under a wide range of growth conditions (O'Toole and Kolter 1998, O'Toole and Kolter 1998). Interactions between bacterial surface structures such as pili, flagella, lipopolysaccharides (LPS), and alginate, with host cells or secretions such as mucin, initiate the attachment process and primary biofilm production (Saiman, Ishimoto et al. 1990, Simpson, Ramphal et al. 1992). Surface adhesion of *P. aeruginosa* reduces flagellar expression, but increases type IV pili expression causing increased twitching motility during initial biofilm development (Bradley 1980). Twitching motility allows bacteria to spread along the surface, eventually producing large microcolonies.

Initial surface attachment prompts a shift in gene expression within adhered bacteria that is carried out largely under QS control (Davies, Parsek et al. 1998). QS control is so critical to biofilm formation that *lasI* mutants of *P. aeruginosa* can adhere to surfaces, but never develop mature biofilm architecture (Davies, Parsek et al. 1998). Proper QS signalling has also been linked to the increased biocide resistance found in

bacterial biofilms when compared to their planktonic counterparts (O'Toole, Gibbs et al. 2000). *P. aeruginosa* with mutated *lasI* display a reduced biocide resistance, irrespective of exopolysaccharide production, which has been shown to increase biofilm resistance to antibiotic treatment due to the impediment of the antibiotics through the biofilm (Nickel, Ruseska et al. 1985, Walters, Roe et al. 2003).

1.6 Antibiotic Resistance Mechanisms

Antibiotic administration is the main therapy available to treat bacterial infections. Although generally antibiotics do an efficient job of halting and eradicating bacterial infections, years of exposure to such therapies has resulted in an increased resistance in many bacterial species. *P. aeruginosa* is a particularly problematic bacterium to control through antibiotic therapy due to a great intrinsic resistance to many forms of antibiotics and effortlessly acquired resistance to many others.

1.6.1 Permeability and Catabolic Enzyme Expression

The ability of an antibiotic drug to permeate into the target bacteria is often crucial to its efficacy as a therapeutic agent. *P. aeruginosa's* seemingly impermeable outer membranes act as barriers to the impediment of some forms of antibiotics such as the hydrophobic macrolids (Normark and Normark 2002). *P. aeruginosa's* outer membrane is scattered with porins, which can allow passage of some antibiotics into the cells (Benz and Hancock 1981), however increases in permeability to antibiotics are not typical with increased porin expression. It is hypothesized that *P. aeruginosa* regulates the functionality and size of the porins in correspondence to antibiotic treatment (Livermore 2002). Previous studies have shown disrupting the membrane of bacteria

increases the permeability of antibiotics, therefore impermeability is an important first line of defence against antibiotics (Li, Zhang et al. 2000).

The expression of catabolic enzymes by bacterial cells can lead to the breakdown or reduction in efficacy of antibiotics. It is well documented that *P. aeruginosa* is capable of producing β-lactamase enzymes when treated with drugs such as cephalothin and ampicillin (Livermore 1995). Other enzymes secreted by *P. aeruginosa* have been characterized, which modify aminoglycoside enzymes reducing their efficacy leading to resistance (Livermore 1995).

1.6.2 Expression of Multiple Efflux Pump Systems

The presence and increased expression of efflux pump systems plays a substaintial role in *P. aeruginosa's* resistance. *P. aeruginosa* expresses four separate multidrug efllux systems; MexAB-OprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN. Upregulation of the MexAB-OprM system leads to the resistance towards flouroquinolones, penicillins, cephalosporins and certain forms of meropenem drugs excluding imipenem (Li, Nikaido et al. 1995, Masuda, Gotoh et al. 1999, Masuda, Sakagawa et al. 2000). Increased expression of the MexXY-OprM efflux system decreases aminoglycoside efficacy (Livermore 2002). Finally, the increased expression of either MexCD-oprJ or MexEF-OprN decreases the susceptibility of *P. aeruignosa* to flouroquinolones and various forms of β-lactam antibiotics (Livermore 2002).

1.6.3 Biofilm Mode of Growth

Increased resistance to antibiotic therapy is seen when *P. aeruginosa* adopts a biofilm mode of growth. In these instances, bacteria form a matrix fluid composed of extracellular polysaccharides, proteins, extra-cellular DNA, and water which holds cells together and protects the bacteria from environmenal stresses (Ma, Conover et al. 2009, Mann and Wozniak 2012). During severe chronic infections of CF patients, *P. aeruginosa* adopts a mucoid phenotype caused predominantly from a mutation to the mucA gene which controls the regulation of synthesis genes for(Damron and Goldberg 2012)(Damron and Goldberg, 2012) alginate (Damron and Goldberg 2012), a linear polysaccharide composed of D-mannuronate and L-guluronic acid (May, Shinabarger et al. 1991). When overexpressed by *P. aeruginosa*, alginate has been shown to reduce the ability of host phagocytic cells to engulf bacteria and to decrease the penetration of antibiotics (Govan and Fyfe 1978, Slack and Nichols 1981). Impediment to antibiotic penetration varies between antibiotics and is alginate concentration dependent, therefore not all strains expressing alginate show greater resistance to antibiotic therapy (Thomassen, Demko et al. 1979, Demko and Thomassen 1980, Gordon, Hodges et al. 1988).

Resistance found in *P. aeruginosa* biofilms may in part be attributed to differential metabolic phases of bacteria within the biofilm. It is well established that within biofilms lie small populations of persister cells which are generally multi-drug resistant and not metabolically active (Lewis 2005). Under the conditions of biofilm growth, genomic expression changes occur, and some forms of physiological resistance may arise that are not found in planktonic growing cells (Whiteley, Bangera et al. 2001).

1.6.4 Highly Mutable Genome and Acquired Resistance

Along with intrinsic resistance, *P. aeruginosa* can also acquire resistances through genomic mutations and horizontal gene transfer from one bacterium to another. Spontaneous point mutations can cause the deregulation of various efflux pump, enzyme, and porin expression genes (Lister, Wolter et al. 2009). The acquisition of many such mutations over time can lead to strains which are multidrug resistant (Lee, Mao et al. 2000, Livermore 2002). Mutations in DNA repair protein genes and can lead to a large increase in the rate of acquired mutations and the adoption of a mutator phenotype. In *P. aeruginosa*, the mutator phenotype often occurs due to mutations to the mis-match repair (MMR) genes mutS, mutL, and uvrD (Mena, Smith et al. 2008). Environmental and nutritional restrictions, such as those found in chronic state infections of *P. aeruginosa* in CF patients, where an abundance of ROS are produced, may favour mutator phenotypes increasing the bacteria's antibiotic resistance with time (Ciofu, Riis et al. 2005, Mena, Smith et al. 2008).

1.7 Treatment of *P.aeruginosa* Lung Infections

The treatment of *P. aeruginosa* lung infections depends heavily on the state of the infection, severity of the infection, and characteristics of the patient's medical condition. Antibiotics can be administered in several different forms such as oral, aerosolized, and intravenous injections (IV) (Rogers, Hoffman et al. 2011). Patients suffering from *P. aeruginosa* lung infections are often given long term daily and continual administration of multiple antibiotics (Mesaros, Nordmann et al. 2007). Bacterial eradication in chronic

infections is highly unlikely, and therefore treatment success is often measured by lung function, not bacterial clearance (Flume, Mogayzel et al. 2009).

1.7.1 Early/Acute Infection

In situations of acute *P. aeruginosa* infection in patients with CF, early administration of antibiotics is able to eradicate the infection if treated within the first 12 weeks (Hansen, Pressler et al. 2008). During early infection the form and type of administered antibiotics is not specific and a wide range of treatment options have been shown to be successful (Rogers, Hoffman et al. 2011). Administered therapies of aerosolized tobramycin with or without oral ciprofloxacin, as well as colistin with oral ciprofloxacin are both effective for the treatment of early *P. aeruginosa* infections (Valerius, Koch et al. 1991, Ratjen, Doring et al. 2001, Mayer-Hamblett, Burns et al. 2010).

1.7.2 Chronic Infections

Unlike acute and early infections, the treatment of chronic *P. aeruginosa* infections is based on lung maintenance and quick recovery from acute exacerbations in the hopes of limiting further therapy (Rogers, Hoffman et al. 2011). Treatment of chronic infections is often a daily regime of multiple antibiotics for extended periods of time.Typical maintenance therapy consists daily inhaled antibiotics and oral azithromycin treatment which is paired with shorter sequences of IV administered antibiotics during periods of acute exascerbations (Frederiksen, Koch et al. 1997). Most often more than one IV antibiotic is given to those patients colonized with *P.aeruginosa* in order to slow the build-up of resistance, and possibly enhance treatment through drug synergy

(Chernish and Aaron 2003, Gibson, Emerson et al. 2003). Typical course length for IV administered antibiotics is 10-14 days; however there is no firm evidence to support the efficiency of this time frame (Rogers, Hoffman et al. 2011).

1.8 Novel Treatment Options: Naturally Derived Antibiotic Compounds

With the rising of antibiotic resistance towards many therapies used for the treatment of *P. aeruginosa* infection, an increasing need for novel therapies and the development of new antibiotics is gaining prominence. Due to the necessity to fend off antibiotic infections, plants produce many adaptogenic compounds which act as natural antibiotics. Often these compounds are readily accessible from the plant and display low toxicity to eukaryotic cells.

Oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) are two examples of common herbaceous plants which contain the natural antibiotic compounds, thymol and carvacrol, which can be extracted from the leaves of both plants and have been shown to display antibiotic properties to a variety of gram negative and gram positive bacteria (Burt 2004). Similarly, species of the herbaceous plant ginseng (*Panax ginseng, Panax quinquefolius*) have also shown antibacterial properties (Song, Kong et al. 2010, Alipour, Omri et al. 2011). In conjunction with the ability to inhibit or reduce bacterial growth, treatment of *P. aeruginosa* with various ginseng components decreases the production of many virulence factors, and has displayed the ability to reduce mature biofilms (Song, Kong et al. 2010, Alipour, Omri et al. 2011, Wu, Lee et al. 2011). Further research into the use of these plant components as natural antibiotics or therapy adjuvants would assist in the elucidation of their efficacy as future treatment options. In this thesis, the effects of

thymol, carvacrol, and multiple extracts of the North American ginseng species, *Panax quinquefolius*, were monitored towards *P. aeruginosa* pathogenecity through the measurement of secreted virulence factors, bacterial adherence and biofilm formation, and whole-genome expression analysis after exposure to the plant treatments.

Thesis Objectives

Recognizing the fact that plants produce many adaptogenic compounds which act as natural antibiotics, the antibacterial effects of the aqueous and alcoholic extracts from *Panax quinquefolius* as well as those of thymol or carvacrol from thyme or oregano, respectively, were examined against *Pseudomonas aeruginosa in vitro*.

Objective 1. To further elucidate the effect of *Panax quinquefolius* alcoholic and aqueous extract exposure to *Pseudomonas aeruginosa* pathogenicity. Through the assessment of growth inhibition and growth rate in the presence of either extract the bacteriostatic effects will be quantified. Secretion of virulence factors such as LasB elastase, lipase, and pyoverdine after exposure to either extract will be measured to provide further evidence of the effect on *P. aeruginosa* pathogenicity. Additionally the analysis of bacterial adherence and biofilm disruption will be monitored in the presence of either extract. The effect of treatment on the regulation of bacterial genes will be assessed by microarray analysis of the laboratory strain PAO1 after exposure to the aqueous ginseng extract for 6 and 24 h.

Objective 2. To explore the effects of Thymol or Carvacrol exposure on *Pseudomonas aeruginosa* pathogenicity. Through the assessment of growth inhibition and growth rate in the presence of either compound, the bacteriostatic capabilities of both thymol and carvacrol will be measured. Secretion of virulence factors, such as LasB elastase, lipase, and pyoverdine after exposure to either compound, will be analyzed to provide further evidence of the effect on *P. aeruginosa* pathogenicity. Additionally the

analysis of bacterial adherence and biofilm disruption will be monitored in the presence of either compound. The effect of treatment on the regulation of bacterial genes will be assessed by microarray analysis of the laboratory strain PAO1 after exposure to thymol for 6 and 24 h and carvacrol for 24 h.

Chapter II. The Effects of North American Ginseng (*Panax quinquefolius*) Aqueous and Alcoholic Extracts towards *P.aeruginosa* Pathogenicity

2.1 Introduction

2.1.1 Ginseng: An Herbaceous Plant

The perennial herbaceous plant ginseng belongs to the family Araliaceae and genus *Panax*. Physical characteristics shared by the handful of species identified include dark green leaves, small ruby berries, and ample fleshy roots (Lemmon, Sham et al. 2012). Geographically ginseng grows throughout North America, Northeast and central China and much of Korea (Attele, Wu et al. 1999, Cruse-Sanders and Hamrick 2004). In many parts of the world ginseng was traditionally used and still is utilized as an essential component of herbal medicine.

2.1.2 Ginseng as a Natural Medicine

For thousands of years the root of ginseng has been used in traditional Asian medicine (Attele, Wu et al. 1999, Wang and Yuan 2008). The effects of traditional ginseng treatment are alluded to include restorative, anti-aging, stress reduction, and immune stimulation, and are often claimed to be very powerful and all-healing (Wang and Yuan 2008, Lu, Yao et al. 2009)]. Several species of ginseng have been researched, however two prominent species used in herbal supplements today are the Asian *Panax ginseng* and the North American *Panx quinquefolius* (Jia and Zhao 2009).

2.1.3 Active Components of Ginseng

Ginseing contains many active components, but much of the research conducted to date focuses on the polysaccharides and ginsenosides of ginseng to which most of the medicinal properties of the herb have been attributed (Lu, Yao et al. 2009). Structural analysis of ginsenosides and ginseng polysaccharides has brought about a greater understanding of the specific medicinal properties of these compounds; however futher analysis is still needed.

Ginsenosides

Ginsenosides are a classification of compounds produced by ginseng which all share a common dammarane-type backbone with a triterpenoid saponin structure (Qi, Wang et al. 2011). Ginsenosides are produced in many parts of the plant, but are most often extracted from the roots where they are found in the highest concentration (Wang, Wang et al. 2005). Large varieties of ginsenosides exist due to the attachment of various sugar moieties and the configuration, and abundance of such functional groups (Fuzzati 2004). Classification based on the placement of sugar residues is used to group these compounds into two major types; protopanaxadiols (PPD) and protopanaxatriols (PPT) (Yoshikawa, Murakami et al. 1998, Qi, Wang et al. 2011).

The quantity and type of ginsenosides present in ginseng differs between species, but is influenced heavily by growth conditions and plant age (Yoshikawa, Murakami et al. 1998, Wang, Wang et al. 2005, Qi, Wang et al. 2011). Typically it has been found that *P.quinquefolius* has a higher concentration of ginsenosides than *P.ginseng* (Qi, Wang et al. 2011). The most common ginsenosides found in *P.quinquefolius* are Re, Rb_1, and Rd

(Wang, Wang et al. 2005, Qi, Wang et al. 2011). The variety of ginsenosides found in *P.quinquefolius* is further expanded by alterations to structure through enzymatic action, intestinal bacteria, and the metabolic processes of animals (Tawab, Bahr et al. 2003, Hasegawa 2004). With so many different structural orchestrations, it is not surprising that ginenosides have been shown to display a wide range of effects such as; anti-inflammatory, anti-bacterial, and immune modulation (Song, Johansen et al. 1997, Qi, Wang et al. 2011).

Polysaccharides

Ginsenosides are not the only reactive compounds of ginseng, purified acidic polysaccharides have displayed a wide range of medicinal properties. Early studies using ginseng polysaccharides have shown antiadhesive effects with bacterial pathogens, as well as being potent immune modulating compounds (Lee, Park et al. 2004, Ivanova, Han et al. 2006). Structural homology between ginseng polysaccharides and ligands or surface markers is believed to play a large role in how these compounds may interfere with bacterial adherence or disrupt immune responses (Lee, Shim et al. 2006). Of particular interest is ginsan, a pectin-like acidic polysaccharide purified from *P.ginseng*, which displays an array of immune modulatory properties and bacterial growth inhibitory effects (Lee, Park et al. 2004, Ahn, Choi et al. 2006, Ivanova, Han et al. 2006, Lee, Shim et al. 2006).

2.1.4 P*anax quinquefolius* and Bacterial Infections

Pathogenic bacteria still prominent causes of infection and disease today, with resistance to current antibiotic therapies becoming a growing concern. The introduction

of new pharmaceuticals to replace current antibiotics or to work in synergy with current treatments, are required to better handle bacterial infections. With a long history of medicinal strength, research into the use of ginseng has revealed several antibacterial properties that have great potential for further development.

Growth Inhibition

Treatments which reduce or inhibit the growth of pathogenic bacteria help greatly to relieve bacterial infections. The aqueous extract of *P. quinquefolius* has been found to inhibit growth of *Pseudomonas aeruginosa* (Alipour, Omri et al. 2011). Both the laboratory strain PAO1 and clinical isolates from sputum samples of CF patients were completly inhibited by treatment of 2.5% *P. quinquefolius* aqueous extract. Interestingly, findings also demonstrated that levels below the minimum inhibitory concentration increased the growth rate of *P. aeruginosa*, suggesting a concentration dependent effect of growth inhibition. Similarily the aqueous extract of *P. ginseng* was also shown to decrease the growth rate of *P. aeruginosa* at concentrations of 5.0% w/v, but also marginally increased growth at lower concentrations (Song, Kong et al. 2010).

Anti-adhesive Properties and Biofilm Production

Although much of the anti-adhesive studies focused on ginseng utilize compounds of *P. ginseng*, the anti-adhesive and biofilm disrupting qualities of *P. quinquefolius* extracts have been briefly explored. Previous research has shown treatment of *P. aeruignosa* with the aqueous extract of *P. quinquefolius* disrupts mature biofilm formation (Alipour, Omri et al. 2011). In general, exposure to the aqueous extract from

either ginseng species reduces the viability of the cells within *P. aeruginosa* biofilms leading to biofilm dispersal (Alipour, Omri et al. 2011, Wu, Lee et al. 2011)

Disruption of Quorum Sensing Control

The treatment of *P. aeruginosa* with *P. ginseng* aqueous extract has resulted in decreased levels of two acyl-homoserine lactones in culture supernatant suggesting a decrease in QS communication (Song, Kong et al. 2010). Additionally, QS controlled metabolites such as LasA and LasB proteases were reduced by *P. ginseng* aqueous extract treatment (Song, Kong et al. 2010). Similar reductions in QS controlled lipase enzymes and pigments pyoverdine and pyocyanin, were reduced by treatment with the aqueous extract *P. quinquefolius* suggesting this extract may also directly affect QS signalling (Alipour, Omri et al. 2011). A reduction in quorum sensing control and overall virulence factor production would greatly decrease the bacteria's rate of infectivity. However, the production of alginate, which is QS controlled, was also shown to increase with *P. ginseng* aqueous extract treatment (Song, Kong et al. 2010).These findings suggest the timing of ginseng therapy will be crucial to its effectiveness as an antibacterial therapy (Song, Kong et al. 2010).

Immune Modulation

Treatment which increases bacterial clearance through the modulation of host immune responses has the potential to relieve patients of severe bacterial infections. Previous studies have shown animals pre-treated with *P. ginseng* aqueous extract had delayed onset of *Listeria monocytogenes* infection (Kim, Germolec et al. 1990). Similar results were displayed with *P. aeruginosa* infections in healthy and athymic mice, which

had reduced lung pathology, bacterial titers, and mast cell counts after *P. ginseng* treatment (Song, Johansen et al. 1997, Song, Johansen et al. 1997, Song, Wu et al. 2002). Pre-treatment with ginsan has also showed reduced bacterial titers and increase survival in mice challenged with experimental sepsis of various bacterial species (Ahn, Choi et al. 2006).

Increases in bacterial clearance through ginseng treatment are attributed to multiple findings indicating increased phagocytic activity of peritoneal macrophages (PM) and polymorphonuclear leukocytes (PMNL) (Shin, Song et al. 2002, Ahn, Choi et al. 2006). Measurement of cytokine release after ginsan pre-treatment revealed decreased production of inflammatory cytokines TNF-α, IFN-γ, IL-2, IL-8, IL-6 and IL-1β, as well as modulation of mitogen-activated protein kinases (MAPK) and JNK1/2 signalling pathways, and reduced NF-κβ activation (Ahn, Choi et al. 2006). Decreased phosphorylation of ERk1/2 and reduced NF-κβ activation were also found in an experimental model of *Helicobacter pylori* gastric cell infection pre-treated with the aqueous extract of *P. ginseng* (Park, Yeo et al. 2005).

Contrasting the findings of Ahn JY et al 2006, increased production of inflammatory cytokines and overall immune response shifts have been detailed in multiple other studies. Up-regulation of TNF-α, IFN-γ, IL-6, and IL-1β have been found with both ginsan and polysaccharide extracts of *P. quinquefolius* in *in vitro* experiments (Assinewe, Amason et al. 2002, Shin, Song et al. 2002, Lemmon, Sham et al. 2012). Lemmon HR et al (2012) also found modulation to MAPK, ERK1/2, PI3K, p38, and NF-κβ pathways through ginseng treatment in *in vitro* studies leading to a shift from a Th2-Th1 immune response.

Animal model studies have also showed evidence for a shift from a primarily Th2 type response to a type Th1 response with ginseng treatment (Song, Johansen et al. 1997, Song, Johansen et al. 1997, Smolina, Solov'eva et al. 2001, Song, Wu et al. 2002). Athymic rats with *P. aeruginosa* infections treated with *P. ginseng* aqueous extract exhibited reduced rates of IgM and IgA antibodies, which is consistent with a decrease in the humoral immune response (Song, Johansen et al. 1997). Mice with fully functioning immune systems subjected to *P. aeruginosa* infection also displayed decreased production of IgG countered by increased release of IgG2a and a shift to a more chronic inflammatory state (Song, Johansen et al. 1997). Reduced bacterial infectivity and increased bacterial clearance are prominent during Th1 immune responses, therefore treatment which encourages this shift could increase bacterial clearance (Johansen, Hougen et al. 1995, Moser, Johansen et al. 1997)

2.1.5 Rationale for Future Research

This study sought to improve our understanding of how *P. aeruginosa* is affected by treatment with ginseng, utilizing both an aqueous and alcoholic extract of *Panax quinquefolius*. The production of various virulence factors, bacterial adherence, and biofilm formation were all monitored in the presence of either extract to delineate the effect of *P. quinquefolius* on *P .aeruginosa* pathogenicity. To further our understanding, a genomic array of *P.aeruginosa* after exposure to *P. quinquefolius* was also performed to elucidate the gene expressional changes caused by the treatment.

2.2 Methodology

2.2.1 Bacterial Strains and Media

Bacterial Strains

Pseudomonas aeruginosa PAO1 nonmucoid laboratory strain was generously donated by Dr. M. Ulanova (Northern Ontario School of Medicine). Clinical isolates PA3 (mucoid) and PA4 (non-mucoid) were collected from sputum samples of regular patients at the Cystic Fibrosis Clinic in Sudbury, Ontario. Canada. Isolates were further purified and strain identity was confirmed by the Clinical Microbiology Laboratory of Memorial Hospital in Sudbury, Ontario, Canada.

Media Composition

All experiments were conducted in supplemented M9 salts media (Sigma-Aldrich, Oakville, Ont., Canada) with the exception of the minimum inhibitory concentration analysis which was performed` in both supplement M9 salts media (M9) and Mueller-Hinton Cation Adjusted media (MHCA) (Sigma-Aldrich). M9 salts media was supplemented with 0.4% w/v glucose (Fisher-Scientific Inc., Ottawa, Ont., Canada), 0.0005% w/v Thiamine (Sigma-Aldrich), 2 mM $MgSO_4$ (Sigma-Aldrich), and 1 mM $CaCl_2$ (Fisher-Scientific Inc.). M9 supplemented media was stored at 4 ˚C while MHCA was stored at room temperature. Agar plates were made with either broth by the addition of 1.5% w/v agar (Fisher-Scientific Inc.).

Bacterial Storage

From original freezer stocks of *P. aeruginosa* strains, 50 µl was used to inoculate 5 ml of either M9 or MHCA broth. Cultures were incubated for 24 h at 37 ˚C and 200 rpm shaking. Secondary freezer stocks were prepared by combining 500 µl of overnight culture with 500 µl of 50% glycerol solution (Sigma-Aldrich). Freezer stocks were stored in 2 ml cyro tubes at -80 ˚C. Fridge storage plates (4 ˚C) were streaked from secondary freezer stocks onto M9 or MHCA plates and incubated inverted at 37 ˚C for 24 h. For storage, plates were wrapped in parafilm and stored inverted in the fridge for no longer than 14 days.

Bacterial Enumeration

Bacterial strains were enumerated via the drop plate method in both types of media. An isolated colony from a M9 agar plate was used to inoculate 5 ml of MHCA broth media and was incubated for 24 h at 37 ˚C and 200 rpm. Bacterial cultures were further diluted 1:5 into MHCA media and re-incubated at 37 ˚C for 6 h. Using a 96 well plate a 10x dilution series was performed beginning with an optical density (OD) reading at 625 nm = 0.100±0.005 adjusted culture. From each well of the dilution series, six 10 µl aliquots were plated onto MHCA agar plates and incubated for 20-24 h at 37 ˚C. Bacterial colonies were counted, averaged, and back calculated to the adjusted 0.100±0.005 OD_{625nm} culture. All experiments were repeated in duplicate three times. A desired bacterial concentration of $1x10^8$ CFU/ml was achieved through adjustment of the culture based on the 0.100±0.005 OD_{625nm} colony counts and subsequently re-plating and

counting in duplicate three times. All experiments were repeated with at an optical density measurement at 600 nm as well. Integrity of results was verified through repetition using a 10 ml volume for the 10x dilution series.

2.2.2 Ginseng Extract Preparation

Plant Cultivation and Extract Purification

The aqueous and alcoholic ginseng extracts were prepared as previously described by Lemmon et al 2012. Characterization of each extract through high performance liquid chromatography was preformed by Azike et al 2011.

Extract Solution Preparation

Both aqueous and alcoholic extract powder was reconstituted directly into either type of media to the twice the desired testing concentration. Solutions were vortexed on high for ten seconds until extract was completely dissolved. Extract solutions were centrifuged at 12000 rpm for 10 minutes to pellet out residual insoluble components. Supernatants were collected and centrifuged a second time for 10 minutes at 12000 rpm. Solutions were sterilized by filteration through 0.2 µ pipette-or bottle top-filter. Extract preparations were used immediately or stored at 4 ˚C for no more than 6 h. For preparations of motility experiments alcoholic and aqueous extract solutions in M9 media were centrifuged for 30 minutes at 4500 xg twice before solutions were filter sterilized and stored.

2.2.3 Minimum Inhibitory Concentration Determination

Bacterial cultures were grown by first streaking each bacterial strain onto MHCA agar plates and incubating for 24 h at 37 ˚C. From each plate a single colony was used to inoculate 5ml of MHCA broth which was incubated for 24 h at 37 ˚C with 200 rpm shaking. Overnight cultures were then diluted into 5ml of fresh MHCA broth and re-inoculated for 6 h at 37 ˚C and 200 rpm.

Antimicrobial effects of the aqueous and alcoholic ginseng extracts were assessed through the determination of the minimum inhibitory concentration (MIC) using a microdilution method (Wiegand, Hilpert et al. 2008). Solutions of 10% w/v alcoholic and aqueous extract were prepared in MHCA broth as previously described. Dilutions from 10%-1%w/v were prepared from the original stock solution and plated in 50 µl volumes into wells 1-10 of a flat bottom 96 well plate. Positive and negative growth control wells with untreated media were plated in 50 µl and 100 µl volumes into wells 11 and 12, respectively.

After the 6hr incubation, bacterial cultures were removed and bacteria were diluted to $1x10^8$ CFU/ml according to OD_{625nm}. This culture solution was then further diluted 1:100 in untreated MHCA broth before 50 µls were added into treatment and positive control wells for a final bacterial concentration of $5x10^5$ CFU/ml. Final concentrations of both extracts were from 5%-0.5% w/v. Plates were wrapped in parafilm and incubated for 24 h at 37 ˚C. After incubation the MIC concentration was determined as the lowest concentration at which no visual bacterial growth was detected. All experiments were repeated three times with two replicates.

Identical experiments were repeated in M9 media with the following changes. Bacterial enumeration was based on optical density readings at 600 nm rather than 625 nm. Concentration of both aqueous and alcoholic extract was increased to final treatment concentrations of 10%-1% w/v. Due to a darkening of the alcoholic extract during incubation at higher concentrations, three 10 µl aliquots were removed from each well and plated onto M9 agar plates. Plates were incubated for 24 h at 37 ˚C and visual assessment of bacterial growth by the formation of colonies was used to verify the visual MIC assessment.

2.2.4 Growth Curve Analysis

Bacterial growth in the presence of the alcoholic and aqueous ginseng extract was assessed over 48 h. Bacterial cultures were produced by the inoculation of 5ml of M9 broth with a single colony of a M9 agar streak plate. Broth cultures were incubated for 24hrs at 37 ˚C and 150 rpm. After incubation 1ml was withdrawn and diluted into 4ml of M9 media and re-incubated for an additional 4hrs at 37 ˚C and 150 rpm. Both extracts were diluted into M9 media at 2x the 0.5 MIC concentration and further diluted to produce a 2x 0.25 MIC concentration. For each treatment, 5ml of treatment media was added into a 125 ml erlenmeyer flask containing 4ml of untreated M9 media. Bacterial cultures were diluted to a concentration of $1x10^8$ CFU/ml in M9 media and 1ml of diluted culture was added to the 125 ml erlenmeyer flasks. Control flasks containing 9ml and 10ml of untreated M9 media were used as positive and negative controls respectively.

Flasks were covered with tinfoil and incubated at 37 ˚C with 150 rpm shaking. At the following time points of 0, 6, 12, 24, and 48 h 1ml of culture were withdrawn from

the flasks and the absorbance was measured on a spectrophotometer at 600 nm. Due to the base absorbance of the alcoholic and aqueous extract treatments at 600 nm, 1 ml of each concentration of un-inoculated extract was pre-measured at 600 nm for each time point, and the value was subtracted from the absorbance values measured during the experiment for the corresponding treatments and time points. Growth curves were repeated in triplicate.

2.2.5 Bacterial Adherence Assay

Bacterial attachment was measured through the growth of the bacteria in the presence of the alcoholic and aqueous extracts, and secondly through the treatment of 48 h attached bacteria with the alcoholic and aqueous extracts. In both experiments the alcoholic and aqueous extracts were prepared at 2x the 0.5 MIC concentration and further diluted in M9 media to produce a 2x 0.25 MIC concentration. Bacterial cultures were started from a single colony inoculation into 5ml of M9 broth with incubation for 24 h at 37 ˚C and 175-200 rpm for both experiments.

Adherence Prevention Assay

After the initial culture incubation, cultures were diluted 1:5 in untreated M9 media and re-incubated for an additional 4 h at 37 ˚C and 200-225 rpm. In triplicate wells of a flat bottom 96 well plate, 100 µl of each concentration of test extract was added for each strain. Positive and negative control wells containing 100 µl and 200 µl of untreated M9 media respectively were also plated in triplicate for each strain of bacteria. Wells containing 100 µl of either concentration of extract and 100 µl of untreated M9 media

were also plated in order to determine the base adherence of the extracts without bacterial inoculation.

After the second culture incubation, all strains were diluted to an OD_{600nm} equal to $1x10^8$ CFU/ml and 1ml of this dilution was further diluted 1:5 into M9 media. The 5ml diluted cultures were vortexed for 5 seconds at low speed and 100 µl was added to treatment and positive control wells. Plates were wrapped in parafilm and incubated for 24 h at 37 ˚C.

After incubation, plates were washed three times with 200 µl of distilled water and allowed to dry in the biosafety cabinet for 30 minutes. Wells were stained with 200 µl of 0.1% crystal violet for 10 minutes. Stain was aspirated from the plates and plates were again washed three times with 200 µl of distilled water and allowed to dry for 30 minutes. Stain was solubilized with 200 µl of absolute ethanol (Commercial Alcohols, Brampton, Ont., Canada). Into a new flat bottom 96 well plate, 150 µl from each well was transferred and the absorbance was measured with an automated plate reader at 600 nm. Triplicate wells were averaged and treatments were expressed as a percentage of the control values. Base adherence of the alcoholic and aqueous extract were averaged and subtracted from the values of the corresponding treatment wells.

Biofilm Disruption Assay

Post 24 h incubation, original cultures were further diluted 1:5 in M9 media and incubated for an additional 4hrs at 37 ˚C and 200-225 rpm shaking. Six wells of 100 µl of M9 media were plated for each strain as pre-treatment wells. Triplicate wells containing 100 µl and 200 µl of M9 media were also plated as positive and negative controls for

each strain. After the second incubation, cultures were diluted to an OD_{600} equal to $1x10^8$ CFU/ml and further diluted 1:5 in M9 media. Adjusted bacterial cultures were plated into pre-treatment and positive control wells in 100 µl volumes. Plates were wrapped in parafilm and incubated for 48 h at 37 ˚C.

After incubation, culture was carefully removed from all wells. Wells were washed three times with 200 µl of distilled water to remove un-adhered bacteria. Triplicate pre-treatment wells were then filled with 200 µl of either2.0 MIC or 1.0 MIC extract solutions. Untreated medium in 200 µl volumes was added into both the negative and positive control wells. Wells containing 200 µl of each concentration of treatment were also plated as base treatment adherence controls. Plates were wrapped in parafilm and incubated for 24 h at 37 ˚C. Following the treatment incubation, plates were removed and wells were washed, stained, and analyzed as previously described in ***2.2.5*** **Bacterial Adherence Assay,** ***Adherence Prevention Assay***.

2.2.6 Virulence Factor Production

Growth Conditions and Aliquot Storage

Bacterial cultures were started from a colony inoculation into 5 ml of M9 media and incubated for 24 h at 37 ˚C and 150 rpm shaking. After initial incubation, cultures were diluted 1:5 into M9 media and incubated a second time for 4 h at the same parameters. Extracts were prepared at 5x the 0.5 MIC and 0.25 MIC values and diluted into 9 ml of M9 media in a 125 ml erlymer flask. After the second incubation, cultures were diluted to a concentration of $1x10^8$ CFU/ml at OD_{600nm} and 1 ml was added to each

treatment and positive control flask containing 9 ml of untreated M9 media. Flasks were covered in aluminum foil and incubated at 37 ˚C and 150 rpm for 24 h.

After treatment incubation, 1ml of culture was removed and immediately the absorbance at 600 nm was measured to provide a growth control value. The rest of the culture was transferred to a 15 ml tube and centrifuged at 4000 xg for 30 minutes. The supernatant was removed from the pellet and filtered through a 0.2 μ synringe or bottle top filter. Four 1 ml aliquots were transferred to sterile 2 ml cryogenic vials (Fisher-Scientific Inc.), labelled, and immediately stored at -20 ˚C.

Pyoverdine Measurement

Immediately following the storage of the supernatant samples pyoverdine absorbance was measured in a spectrophotometer (Genesys 10uv, Thermo Scientific). 1ml aliquots from each sample were measured directly at 405 nm. A 1 ml aliquot of untreated M9 media was used to blank the spectrophotometer and the absorbance of 1 ml of each treatment concentration was also measured and subtracted from the corresponding supernatant sample absorbance readings. Experiments were repeated three times with duplicate and values were normalized by the growth control absorbance at OD_{600nm} and represented as a percentage of the control.

LasA Protease Assay

Petri dishes containing 1% skim milk powder and 1.5% agar were prepared, and a well was cut in the center with a sterilized glass pipette. Freezer supernatant aliquots were thawed to room temperature and 50 μl was added to the well of duplicate plates. Plates

were incubated for 24 h at 37 ˚C. Protease activity was assessed through the measurement of the zone of clearance using a ruler (mm). Treatments were expressed as a percentage of the control. Experiments were repeated three times with duplicate plates for each trial.

Lipase Production Assay

In 15 ml glass test tubes the following volume of solutions were combined; 2.3 ml of 0.05 M tris buffer (pH 7.6) (Fisher Scientific Inc.), 100 µl of both 0.1 M $CaCl_2$ and 10% tween 20 in tris buffer (pH 7.6) (Fisher Scientific Inc., Sigma-Aldrich), and 0.5 ml of supernatant sample. Test tubes were incubated at 37 ˚C with 150 rpm shaking for 4 h. Negative controls were created containing 0.5 ml of M9 media in place of 0.5 ml of supernatant. After incubation, the absorbance of each treatment and controls were measured at 400 nm, blanking with 1 ml of M9 media. The absorbance of the negative control was subtracted from all treatments and positive controls, and all values were normalized to the original growth control absorbance at 600 nm. Base absorbance measurements of un-inoculated extract treatment absorbance at 400 nm were also subtracted from the corresponding treatment values. Data were expressed as a percentage of the control and all experiments were repeated three times with duplicate measurements.

2.2.7 Motility Assay

Bacterial cultures grown from plate inoculation in 5 ml of M9 media were incubated for 24 h at 37 ˚C and 150 rpm shaking. Overnight cultures were then diluted 1:5 in M9 media and incubated again at the same parameters for 4 h. M9 plates were prepared containing 0.3% agarose and aqueous or alcoholic extract at 0.25 MIC and

0.125 MIC concentrations. When incubation was complete, bacterial cultures were diluted to an absorbance at 600 nm equal to $1x10^8$ CFU/ml in M9 media. At the center of the plate and piercing into the media, 1 µl of bacterial culture was inoculated. Plates were incubated inverted at 32 ˚C for 18 h (PAO1) or 36 h (clinical isolates). Using a ruler, the diameter of the distance traveled from the point of inoculation was recorded (mm). All data are expressed as a percentage of the control, and all experiments were repeated three times with duplicate plates.

2.2.8 Genomic Expression Analysis

Treatment Parameters

Inoculation from an agar M9 plate into 5 ml of M9 media was used to create a 24 h culture of PAO1 grown at 37 ˚C and 150 rpm shaking. Overnight culture was diluted 1:5 into fresh M9 media and allowed to incubate for an addition 4 h at the same parameters. During the second incubation a solution of the aqueous extract was prepared in M9 media at 5x the desired concentration of 0.25 MIC. After the second incubation, PAO1 culture was diluted to an absorbance at 600 nm equivalent to $1x10^8$ CFU/ml. To a 125 ml erlymer flask, 1 ml of diluted bacterial culture was added to 9 ml of 0.25 MIC aqueous extract treatment in M9 media. Flasks were covered with aluminum foil and incubated for 6 h or 24 h at 37 ˚C and 150 rpm shaking. Positive control flasks containing untreated media were also analyzed. Experiments were repeated three times.

RNA Isolation

From the 24 h treatment and control flasks, 2 ml of culture was centrifuged at 10000 xg for 2 minutes in 2 ml Eppendorf tubes. Pellets were re-suspended in 500 µl of M9 media and combined to form one culture aliquot. Combined culture aliquots were centrifuged and washed two additional times with 1ml volumes of M9 media. Final culture pellets were re-suspended to a total volume of 2 ml with M9 media and optical density of the culture was measured at 600 nm From the absorbance measurement the culture samples were diluted to the appropriate amount (~2 ml @ OD_{600nm} = 1.2) for RNA extraction. Samples were transferred to RNase free 2 ml centrifuge tubes and RNA was extracted using the Bio-Rad RNeasy Mini Kit as per manufacturer's instructions with the following exceptions. Lysozyme was added into the reaction tube at volumes of 150 µl not 100 µl. RNA was eluted with nuclease free water (Sigma-Aldrich) instead of elution solution. Final extraction samples were aliquoted and stored at -20 ˚C before shipment.

RNA Quality and Quantity Validation

RNA integrity, concentration, and quantity were assessed through the Experion RNA StdSens analysis kit (Bio-Rad) using the Experion Automated Electrophoresis Station (Bio-Rad). In brief, denatured RNA samples and ladder were added in 1 µl volumes to a microfluidic StdSens chip. The microfluid chip has a network of channels that when primed with the provided gel solution permitted the detection, staining, and data collection of the samples through the measurement of the 16S and 23S RNA peaks. Only RNA samples which met the quality, quantity and concentration requirements of the array analysis were further subjected to absorbance analysis.

The purity of the extracted RNA samples was assessed through the absorbance ratio at 260 nm/230 nm. A 1 µl aliquot of RNA sample was measured at 260 nm and 230 nm on the BioTek Powerwave XS microplate reader using a Take3 Multi-Volume plate. A reference blank was set with 1 µl of nuclease free water (Sigma-Aldrich). Only samples with a 260 nm/230 nm ratio of ≥ 1.5 were approved for shipment and further analysis.

2.2.9 Microarray Processing

All sample labelling and GeneChip processing was completed at the London regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada).

RNA Integrity Analysis

RNA sample quality was assessed using the Aligent 2100 Bioanlyzer (Aligent Technologies Inc., Palo Alto, CA) as well as the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA). RNA samples were only labelled and hybridized to a Genechip if the sample was not degraded and the quality was high.

cDNA Synthesis and GeneChip Hybridization

From 10 µl of total RNA, single stranded complementary DNA (sscDNA) was prepared according to the Affymetrix GeneChip Expression Analysis Technical manual, Chapter 4 – Prokaryotic Target Peparation (Affymetrix, Santa Clara, CA). Initially, using random primers, RNA was converted to cDNA and subsequently fragmented and end labelled. For 16 h at 50 ˚C the cDNA cocktail was hybridized to a *P. aeruginosa* Genome array. All liquid handling steps were performed by a GeneChip Fluidics Station 450 and

GeneChips were scanned with a GeneChip Scanner 3000 7G (Affymetrix) using Command Console v3.2.4.

Data Analysis

An initial data quality assessment was performed via the Affymetrix Expression Console. Probe level data (.CEL files) were generated using the Affymetrix Command Console v3.2.4. and summarized to the gene level data provided in Partek Genomics Suite v6.6 (Partek, St. Louis, MO) using the RMA algorithm (Irizarry, Hobbs et al. 2003). Both the fold-change and p-value of each gene was calculated using Partek's multi-way ANOVA. Using a 2-fold change of expression as a significant change, gene lists were created containing only those genes with a ± 2-fold change. Such lists were then analyzed for enriched Gene Ontology (GO) terms and KEGG Pathways using a Fisher's Exact test to determine significance.

2.2.10 Statistics

Data were displayed as averaged values of 3 independent experiments (n=3) ± SEM and data analysis was performed through Graph Pad Prism v5.01. Statistical significance was determined through a one-way ANOVA (repeated measures ANOVA for growth curves) test with a p-value of < 0.05 considered significant when compared to untreated controls. ANOVA analysis was followed by a Tukey's Post-hoc analysis to determine between which groups the significant difference(s) occurred. All data were presented as a percent of the untreated control with the exception of the MIC and growth curve experiments. All array data were analyzed as previously described in **2.2.9 Microarray Processing, *Data Analysis***.

2.3 Results

2.3.1 Minimum Inhibitory Concentration of Aqueous or Alcoholic Ginseng Extracts

Through a microdilution assay the minimum inhibitory concentration of both the aqueous and alcoholic ginseng extract was determined for the laboratory PAO1 strain and two clinical isolates. Bacteria were cultured and tested in both MHCA and M9 supplemented media for MIC determination. It was found that the aqueous extract was able to inhibit bacterial growth of all strains at 3.0-2.0% w/v and 8.0-7.0% w/v in MHCA and M9 media respectively (Table 1.1 A, Table 1.1 B). In either media, bacterial growth was not inhibited by the presence of the alcoholic extract at any concentration.

Table 1.1: Minimum inhibitory concentrations (MIC) of *P. aeruginosa* treated with either aqueous or alcoholic ginseng extracts. MIC values were determined through microdilution series of either extract in 96 well plates, denoting the MIC value by visual growth assessment. Experiments were repeated three times (n=3) in duplicate in either both Mueller-Hinton cation adjusted broth (MHCA) (Table 1.1) and M9 supplemented media (Table 1.2)

Table 1.1 A Minimum inhibitory concentration of ginseng extract treatment in MHCA media

Strain	Aqueous Ginseng Extract (%w/v)	Alcoholic Ginseng Extract (%w/v)
PAO1	3.0	>5.0
Mucoid Isolate	2.5	>5.0
Non-mucoid Isolate	2.0	>5.0

Table 1.1 B Minimum inhibitory concentration of ginseng extract treatment in M9 media

Strain	Aqueous Ginseng Extract (%w/v)	Alcoholic Ginseng Extract (%w/v)
PAO1	8.0 - 7.0	>10.0
Mucoid Isolate	7.0	>10.0
Non-mucoid Isolate	8.0 - 7.0	>10.0

2.3.2 Effect of Aqueous or Alcoholic Ginseng Treatment on Bacterial Growth

Growth of *P. aeruginosa* in the presence of the aqueous ginseng extract resulted in a significant increase in growth rate for all strains when exposed at a 0.5 MIC concentration (Figure 1.1). Treatment of the bacteria with the alcoholic extract displayed mixed results with no significant changes to growth rate in comparison to untreated controls. Both PAO1 and the mucoid clinical isolate grew slower at both concentrations after 24 and 48 h of incubation (Figure 1.2 A, B). Both clinical isolates grew faster than the control with the 0.5 MIC concentration treatment during the first six hours (Figure 1.2: B, C).

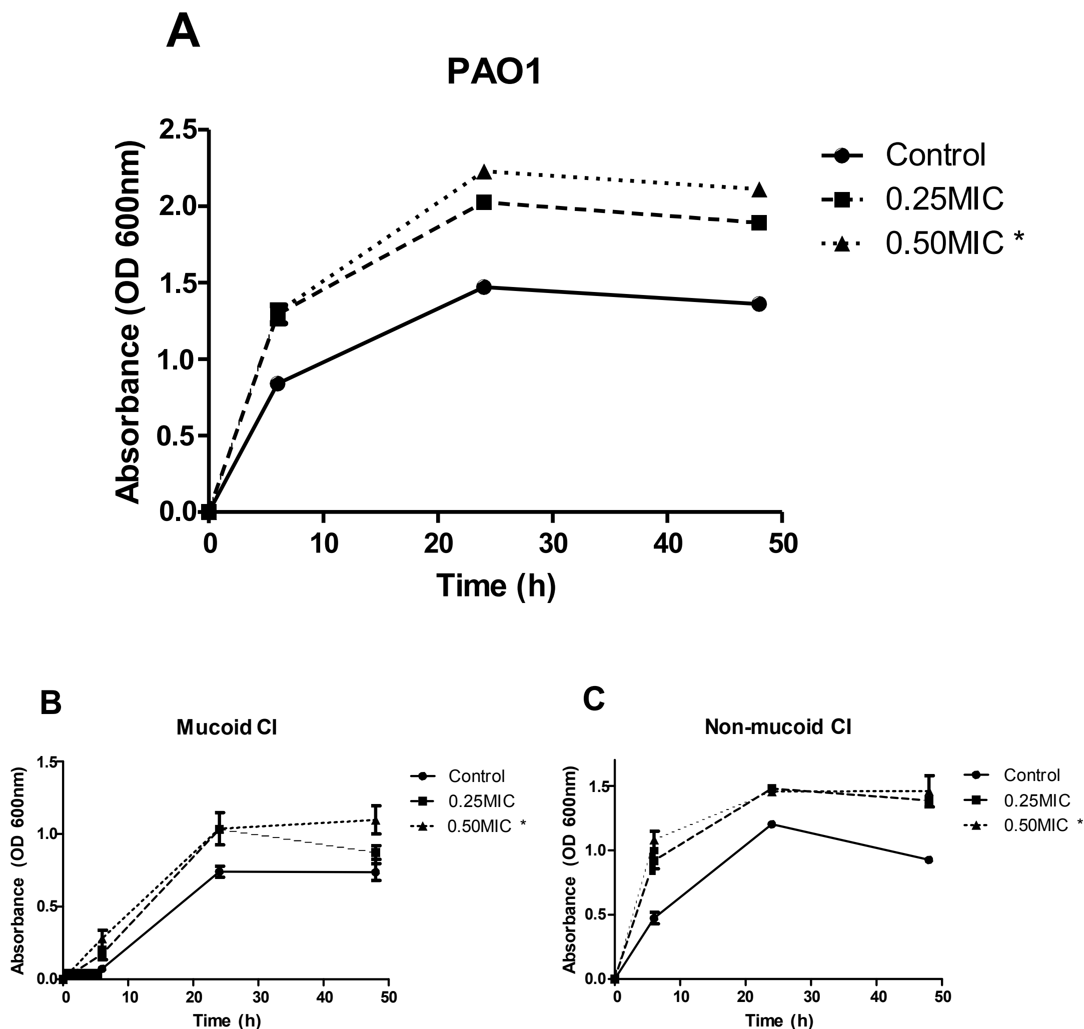

Figure 1.1: Growth curve analysis in the presence of the aqueous ginseng extract. Growth in the presence of sub-inhibitory concentrations of the aqueous ginseng extract was assessed for 48 h at 37 ˚C with 150 rpm shaking. After 0, 6, 24, and 48 h of incubation, 1ml of culture was removed and the absorbance at 600 nm was measured. Values normalized to the time 0 absorbance and adjusted based on background aqueous extract absorbance at 600 nm for each time point was used both for the statistical interpretation and presentation of results. Experiments were repeated three times (n=3) and data is represented as an average ± SEM. Statistical comparisons to the untreated control for each treatment were done through a repeated-measures ANOVA and subsequent Tukey's post-hoc test (p-value < 0.05=*).

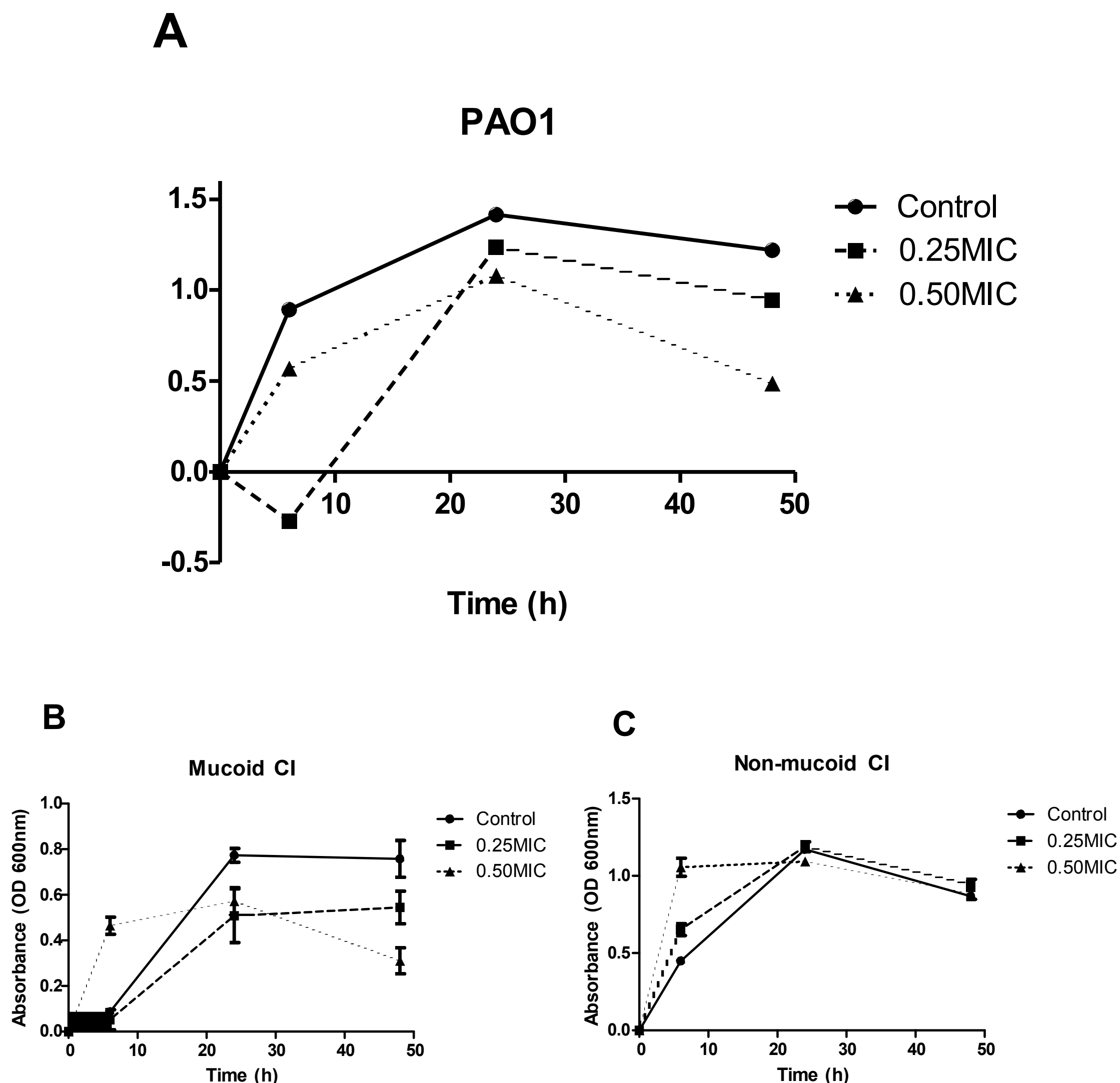

Figure 1.2: Growth curve analysis in the presence of the alcoholic ginseng extract. Growth in the presence of the sub-inhibitory concentrations of the alcoholic ginseng extract was assessed for 48 h at 37 ˚C with 150 rpm shaking. After 0, 6, 24, and 48 h of incubation, 1ml of culture was removed and the absorbance at 600 nm was measured. Values normalized to the time 0 absorbance and adjusted based on background alcoholic extract absorbance at 600 nm for each time point were used for the statistical interpretation of the data, however raw values normalized to time point zero were used for results presentation. Experiments were repeated three times (n=3) and data are represented as an average ± SEM. Statistical comparisons to the untreated control for each treatment were done through a repeated- measures ANOVA and subsequent Tukey's post-hoc test.

2.3.3 Effect of Aqueous or Alcoholic Ginseng Treatment on Preventing Bacterial Attachment

The adherence ability of *P. aeruginosa* in the presence of either ginseng extract was assessed in 96 well flat bottom plates. PAO1 adherence decreased slightly with aqueous extract exposure at 0.25 MIC concentration, but significactly increased adherence with a 0.50 MIC concentration exposure was found (Figure 1.3 A) with similar results found with both clinical isolates as well (Figure 1.3: B, C). Adherence in the presence of the alcoholic extract for either clinical isolate caused a significant reduction, however PAO1 was unaffected (Figure 1.3). Reduced adherence of the clinical isolates lower than the adherence found for the alcoholic extract controls leading to negative adherence values when normalized for the alcoholic control (Figure 1.2: B, C).

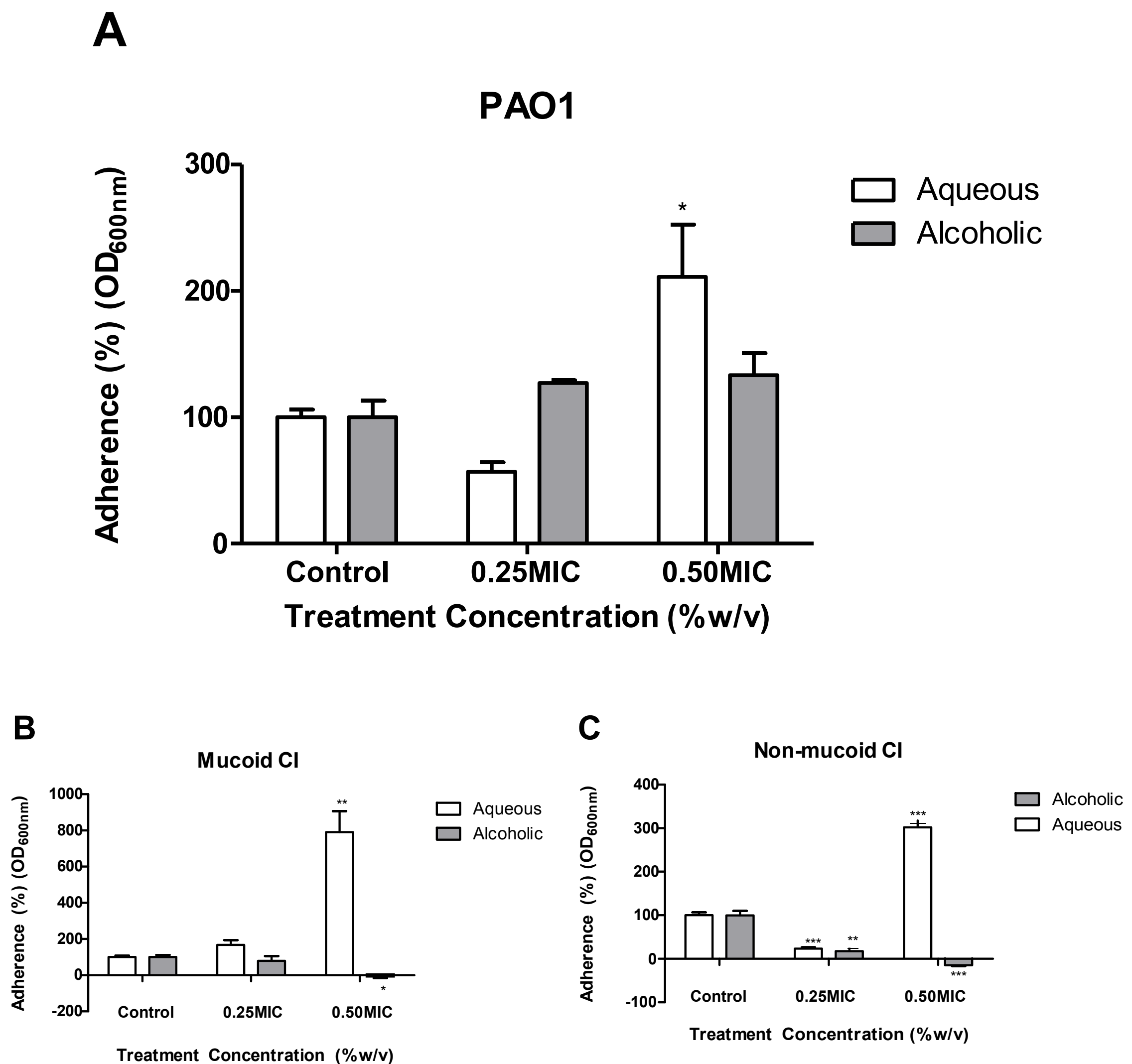

Figure 1.3: The effect of aqueous and alcoholic ginseng treatment on the prevention of *P. aeruginosa* adherence. Bacteria were grown in the presence of 0.25 MIC and 0.50 MIC concentrations of either extract in 96 well flat bottom plates for 24 h at 37 ˚C. Post incubation, plates were washed with distilled water and adhered bacteria were stained with a 0.1% crystal violet stain and solubilized stain was measured at an absorbance of 600 nm. Experiments were repeated three times in triplicate (n=3) and expressed as an average percentage of the untreated control ± SEM. Statistical comparisons to the untreated control for each treatment was done through a one-way ANOVA and subsequent Tukey's post-hoc test (p-value < 0.05= *, p-value < 0.01= **, p-value < 0.001= ***).

2.3.4 Effects of Ginseng Treatment on Preformed Biofilms

The effect of ginseng extract treatment on 48 h old preformed biofilms was assessed by measuring the rate of adherence after 24 h of ginseng treatment. With regards to PAO1, the alcoholic extract had no effect on the adherence of the preformed biofilms (Figure 1.4 A). The aqueous extract significantly increased PAO1 adherence at a 1.0 MIC concentration, however reduced adherence at the 2.0 MIC concentration (Figure 1.4: A). For either clinical bacterial isolate the aqueous extract decreased the adherence of the preformed biofilms at both concentrations (Figure 1.4: B, C). Treatment of the clinical isolates with the alcoholic extract showed varied results. The adherence of the mucoid clinical isolate was significantly reduced at both concentrations (Figure 1.4: B), while the non-mucoid clinical isolate showed reduced adherence only at the 1.0 MIC concentration (Figure 1.4: C).

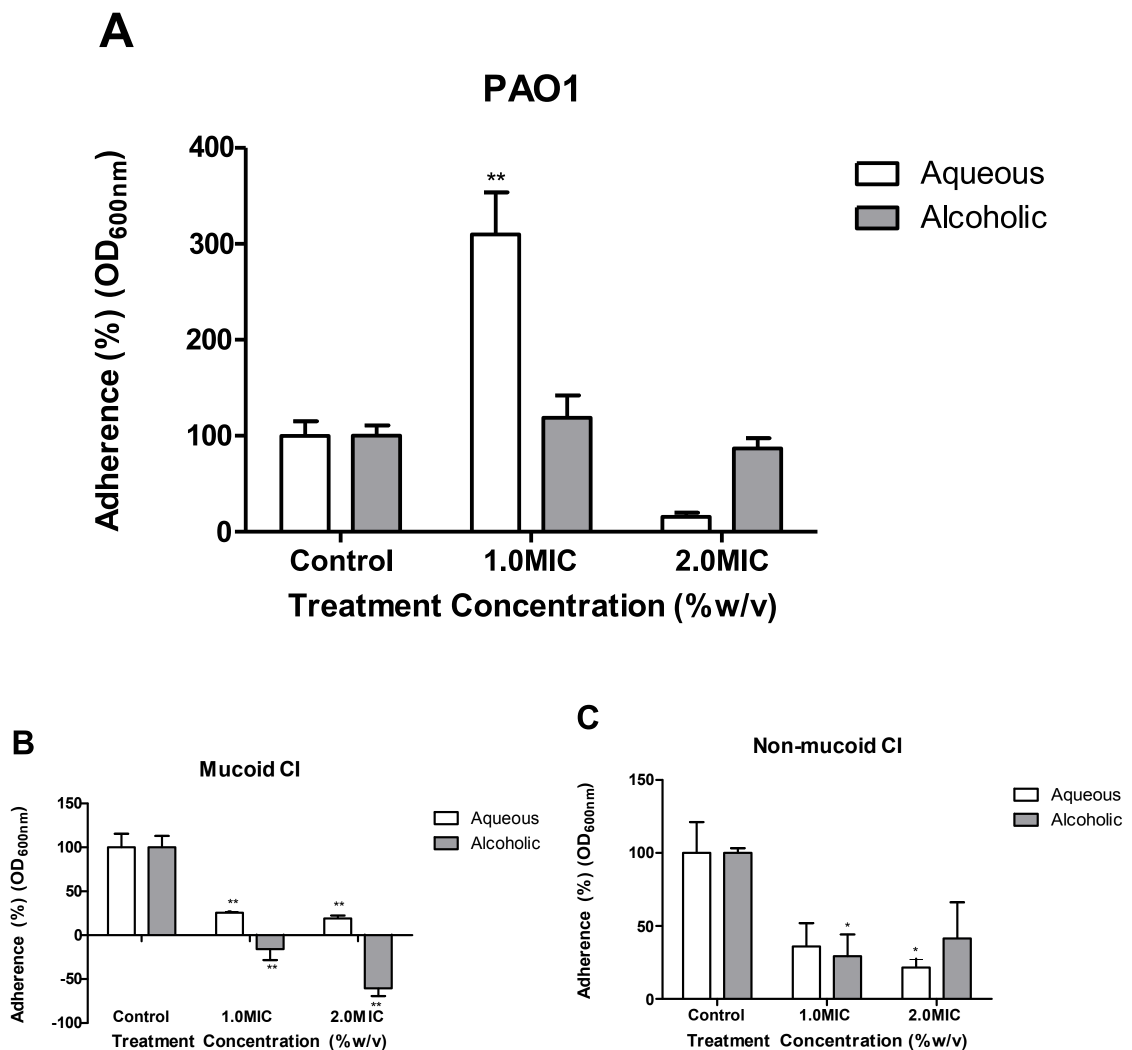

Figure 1.4: The effect of aqueous and alcoholic ginseng treatment on 48 h *P. aeruginosa* biofilms. Bacteria were allowed to adhere to a 96 well flat bottom plates for 48 h at 37 ˚C. Post incubation, wells were washed with distilled water and bacteria were treated with either extract at 1.0 MIC and 2.0 MIC concentrations for 24 h at 37 ˚C. After treatment, wells were washed with distilled water and adhered bacteria were stained with a 0.1% crystal violet stain and solubilized stain was measured at an absorbance of 600 nm. Experiments were repeated three times in triplicate and expressed as an average percentage of the untreated control ± SEM. Statistical comparisons to the untreated control for each treatment was done through a one-way ANOVA and subsequent Tukey's post-hoc test (p-value < 0.05= * and p-value < 0.01= **).

2.3.5 Effect of Ginseng Extract Treatment on *P.aeruginosa* Pigment Production

The absorbance of pyoverdine at 405 nm was measured from culture supernatants treated with sub-inhibitory concentrations of either ginseng extract. Significant decreases in pyoverdine with both 0.5 MIC and 0.25 MIC concentrations of either aqueous or alcoholic extract were measured for PAO1 (Figure 1.5 A). The mucoid clinical isolate showed similar results to PAO1, with even greater decrease in pyoverdine found with the aqueous ginseng treatment (Figure 1.5 B). The non-mucoid clinical isolate displayed the same results for the aqueous ginseng treatment as the other two strains; however the alcoholic ginseng treatment significantly increased the expression of pyoverdine at either concentration (Figure 1.5 C).

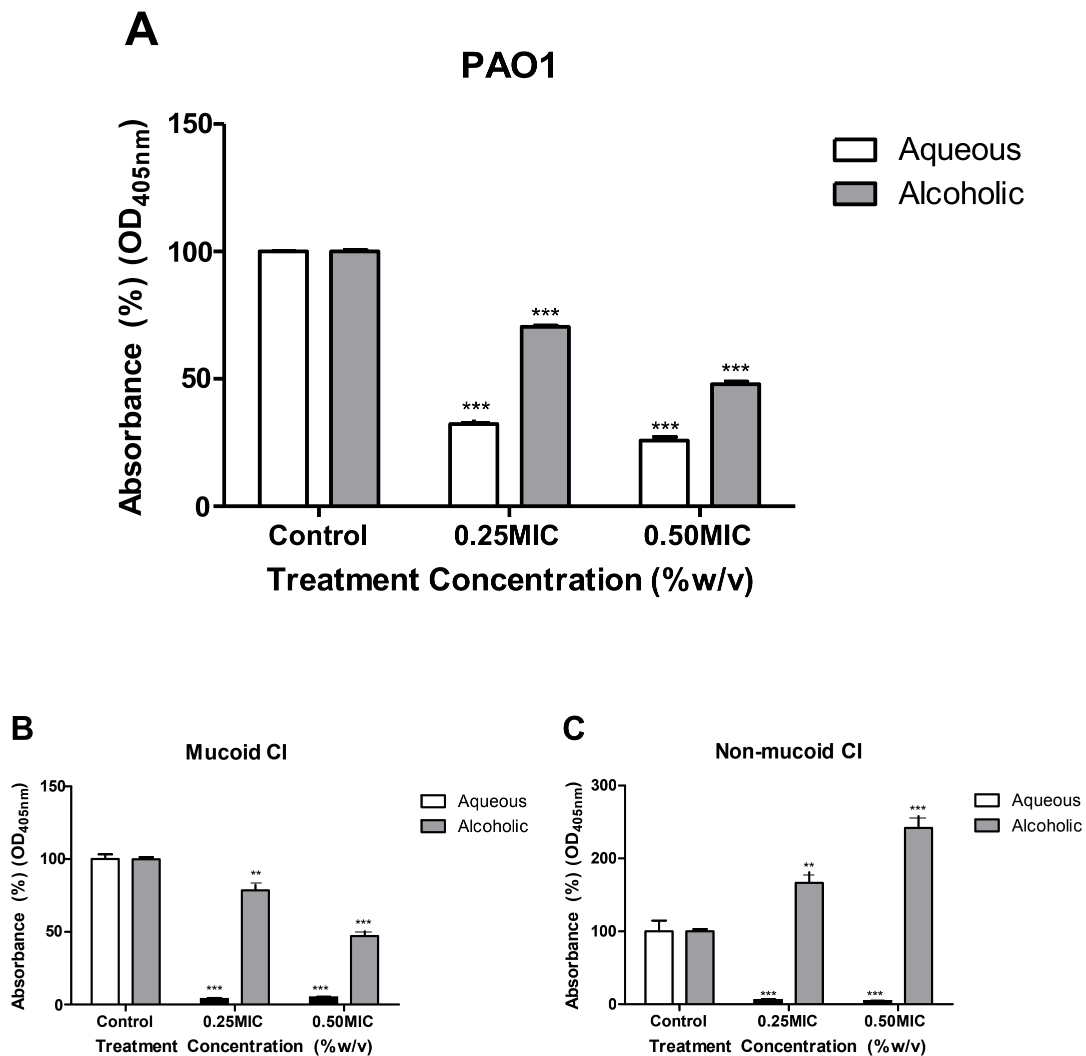

Figure 1.5: The effect of aqueous and alcoholic ginseng extract treatment on the production of the pigment pyoverdine. Bacterial cultures of were prepared and incubated in the presence of 0.25 MIC and 0.5 MIC concentrations of either extract for 24 h at 37 ˚C & 150 rpm. Cultures were then centrifuged to remove cells, filtered, and 1ml of supernatant was assayed in a spectrometer at 405 nm. Data represents averaged mean values of three independent experiments preformed in duplicate, and expressed as a percentage of untreated controls ± SEM. Statistical significance was determined through one-way ANOVA analysis followed by Tukey's post-hoc test to determine significance relative to the untreated controls (p-value < 0.01= ** and p-value < 0.001= ***).

2.3.6 The Outcome of Aqueous or Alcoholic Ginseng Treatment on Bacterial Protease and Lipase Production

The production and secretion of proteases into the bacterial culture media was measured by the zone of clearance formed on a milk agar plate. Neither the aqueous nor the alcoholic extract had a significant effect towards the measured protease production in PAO1 (Figure 1.6: A). Statistically significant changes were found for both extract treatments using the clinical isolates, with the alcoholic ginseng treatment some what increasing the measured protease in both isolates (Figure 1.6: B, C). Exposure to the aqueous extract significantly reduced or prevented the protease production in both clinical isolates. Protease production in the non-mucoid isolate treated with aqueous extract was reduced to an undetectable amount (Figure 1.5: C), as was the 0.25 MIC treated mucoid isolate (Figure 1.5: B).

The synthesis and production of lipase was measured in treated culture supernatants using a tween 20 hydrolysis. For all strains tested, growth in the presence of the alcoholic ginseng extract caused increases in the measurement of lipase (Figure 1.7). In contrast, growth of all strains in the presence of the aqueous ginseng extract caused significant decreases in the lipase production by the bacteria (Figure 1.7), with the greatest reductions found in both clinical isolates.

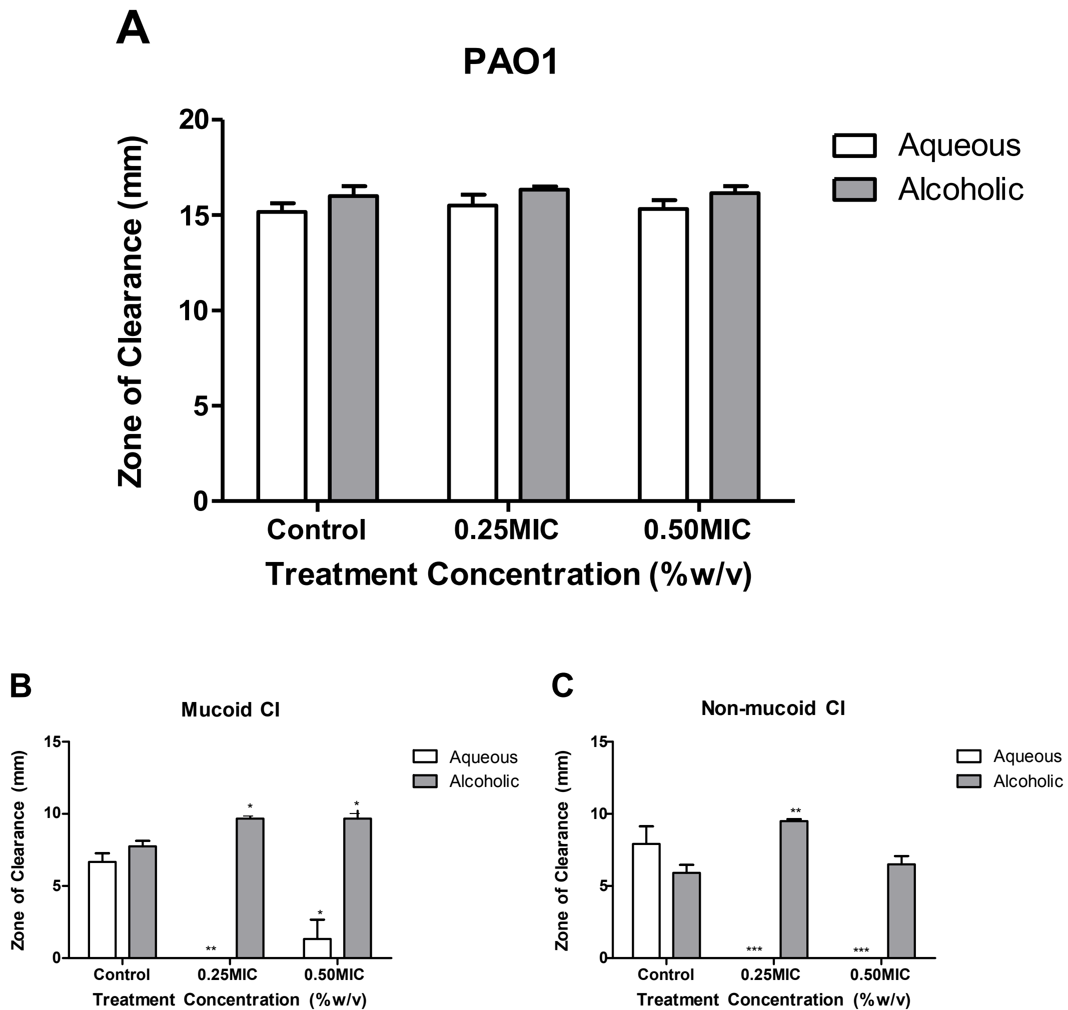

Figure 1.6: Protease production in the presence of aqueous or alcoholic ginseng extract. Bacterial cultures were prepared and incubated in the presence of 0.25 MIC and 0.5 MIC concentrations of either extract for 24 h at 37 ˚C & 150 rpm. Cultures were then centrifuged to remove cells and remaining supernatants were filter sterilized. To a single center well of a 1% skim milk plate (1.5% agar), 50 μl of filtered supernatant was added and plates were incubated at 37 ˚C for 24 h. The zone of clearance around the inoculation well was measured with a ruler (mm). Data are represented as the average of three separate experiments conducted in duplicate ± SEM. Statistical significance was determined through one-way ANOVA analysis followed by Tukey's post-hoc test to determine significance relative to the untreated controls (p-value<0.05= * and p-value < 0.01= **).

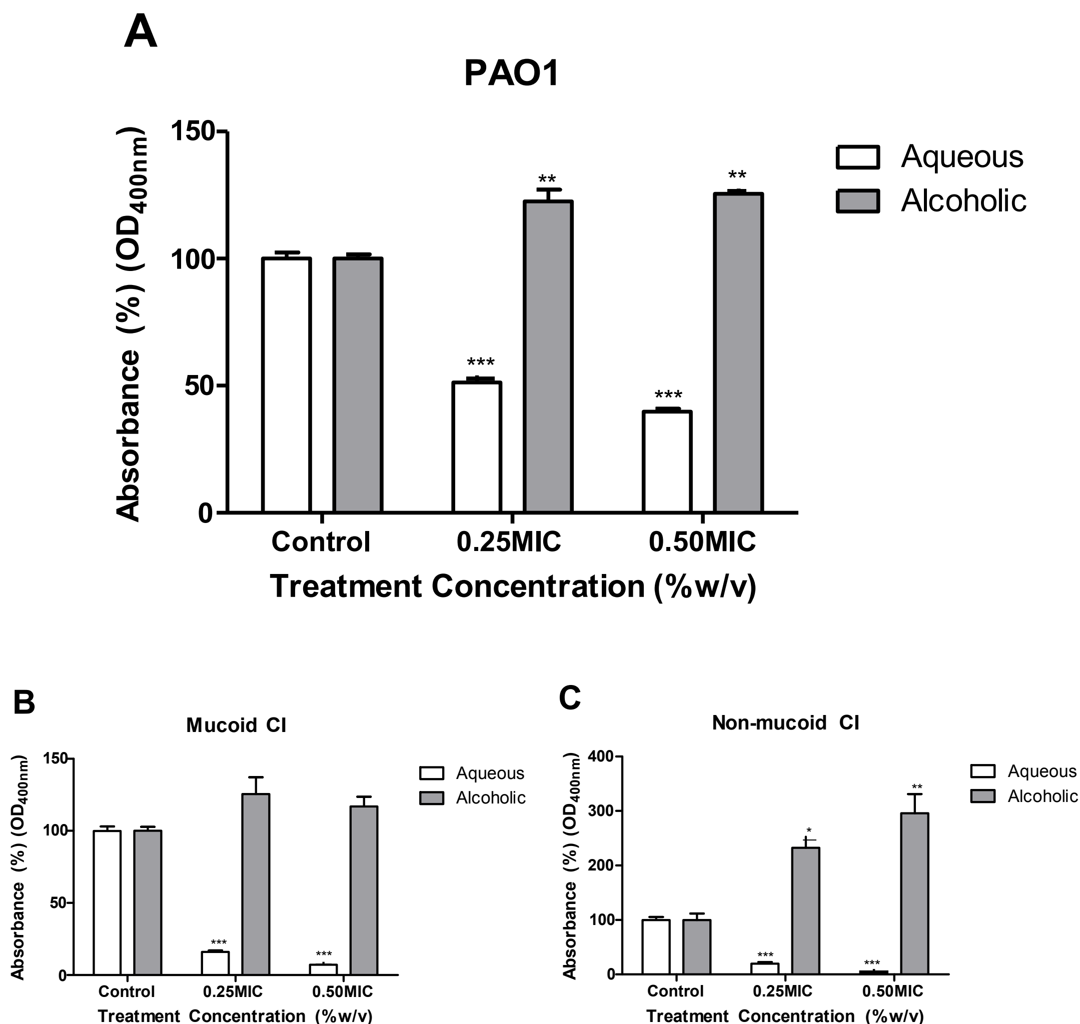

Figure 1.7: Lipase production in the presence of aqueous or alcoholic ginseng extract. Bacterial cultures were prepared and incubated in the presence of 0.25 MIC and 0.5 MIC concentrations of either extract for 24 h at 37 ˚C & 150 rpm. Cultures were then centrifuged to remove cells and remaining supernatants were filter sterilized. To a glass test tube, 2.3 ml of 0.50 M tris buffer (pH 7.6), 0.1 ml 0.1 M $CaCl_2$ in tris buffer (pH 7.6), 0.1 ml tween 20 in tris buffer (pH 7.6), and 0.5 ml filtered supernatant were combined and incubated at 37 ˚C & 150 rpm for 4 h. After incubation, 1 ml was removed and measured in a spectrophotometer at 400 nm. Data represents the mean value of three independent experiments preformed in duplicate and expressed as a percentage of the control ± SEM. Statistical significance was determined through one-way ANOVA analysis followed by Tukey's post-hoc test to determine significance relative to the untreated controls (p-value<0.05= *, p-value < 0.01= **, and p-value < 0.001= ***).

2.3.7 Influence of Aqueous and Alcoholic Ginseng Treatment on *P.aeruginosa* Motility

Flagellum-mediated motility of *P .aeruginosa* was measured through the point inoculation of plates containing 0.125 MIC and 0.25 MIC concentrations of either ginseng extract, and the measurement of the distance travelled from the point of inoculation. Motility trends were similar for both the mucoid clinical isolate and the PAO1 laboratory strain which both showed statistically significant decreases in motility in the presence of either ginseng extract (Figure 1.8: A, B). Results were less conclusive for the non-mucoid isolate, which showed no significant change in motility with either treatment (Figure 1.8: C).

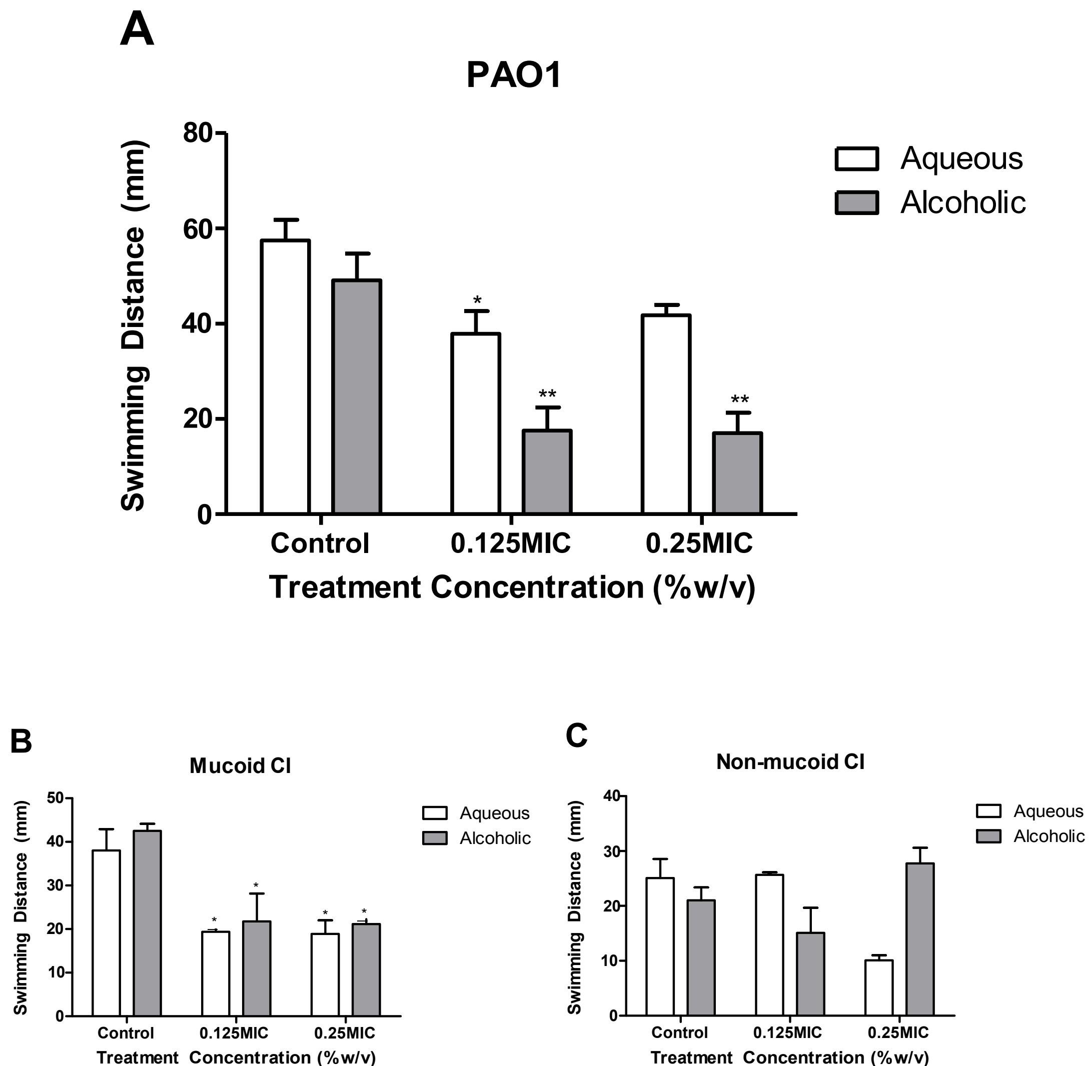

Figure 1.8: Motility in the presence of aqueous and alcoholic ginseng extracts. Plates comprising 0.125 MIC and 0.25 MIC concentrations of either aqueous or alcoholic ginseng extracts, and 0.3% agarose were point inoculated with bacterial cultures. Plates were incubated at 37 ˚C for 12 h (A) or 36 h (B & C). Distance travelled through the media was measured with a ruler (mm) after incubation. Data are represented as the average of three separate experiments conducted in duplicate ± SEM. Statistical significance was determined through one-way ANOVA analysis followed by Tukey's post-hoc test to determine significance relative to the untreated controls (p-value<0.05= * and p-value < 0.01= **).

2.3.8 Effects of Aqueous Ginseng Treatment on *P. aeruginosa* Gene Expression

Gene expression changes with aqueous extract exposure after 6 h and 24 h was assessed through microarray analysis. Overall, deviations in expression from the control grown in untreated media were far more varied after the 6 h aqueous exposure than after the 24 h exposure. Significant changes (relative to controls at either 6 h or 24 h) are presented in Table 1.2.

Many genes involved in the metabolism of varying organic compounds were increased in expression with the 6 h aqueous treatment (Table 1.2 A). Multiple genes involved in the citrate cycle (TCA cycle) (*acnB, acnA, sdhB, fdnG*),), nitrogen metabolism (*nirS, nosZ, norB, norC, fdnH, napA, napB, nosD, ccpR, nirC, nirM, nirN, glnA),* propanoate metabolism (*prpB, prpC, prpD),* and glyoxylate and dicarboxylate metabolism (*glcD, fdnG*) increased in expression. Multiple genes involved in heme biosynthesis (*nirJ, nirL, nirF*) also increased RNA transcripts the after aqueous extract exposure for 6hrs.

Several genes displayed decreased expression with the 6 h aqueous treatment. Among these genes involved in phenazine syntheses were; *phzC1/phzC2, phzE1/phzE2, and phzF1/phzF2* which decreased in expression of -3.05, -3.91, and -4.91 fold respectively. Similarily, transcripts for biosynthetic proteins for two other pigments produced by *P.aeruginosa*, pyochelin (*pchG, pchB, pchC*) and pyoverdine (*pvdE, pvdJ, pvdL, pvdH, pvdA, pvdD*) decreased. Two genes (*fptA, fpvA*) involved in pigment iron transport into the cell and *pchA* and *pchB* (genes involved in the biosynthesis of

pyochelin precursor salicylate) also decreased expression after the 6 h aqueous extract exposure.

Other virulence associated genes decreased expression after the 6 h aqueous extract treatment. Translocator protein genes (*popD and popB*) as well as a type III export protein (*pscE*) decreased in expression. Virulence factors ExoT and ExoS showed decreases in expression of -2.16 and -3.45 respectively after 6 h aqueous exposure. Gene *rhlA* rhamnolipidtransferase gene and *cupA1* fimbraie gene were both similarly decreased. Finally, several genes involved in oxidative phosphorylation and oxygen-dependent carbon metabolism were reduced after the 6 h aqueous extract treatment.

Treatment of the bacteria with the aqueous extract for 24 h showed a much different gene expression profile. Increased expression of iron-associated genes for flavohemoprotein (*fhp*), ferreodoxin (*fxd2*), ferripyochelin receptor (*fptA*), and *TonB* were all found after 24 h aqueous extract treatment. Three genes encoding cysteine desulferase (*iscA, iscS, iscU*) and two related chaperon proteins (*hscA, hscB*) were upregulated.

Exposure of the bacteria to the aqueous extract for 24 h caused significant increases in multiple genes involved in the synthesis (*accB, accC*) and metabolism (*fabA, fabB)* of fatty acids, and nitrogen and carbon metabolism (*antA*, *antB*). A regulatory gene, *cbrB*, involved in the orchestration of multiple metabolic pathways was also increased after the 24 h aqueous extract incubation.

The great majority of those genes with a significant increase in expression after the 24 h treatment are involved in ribosomal structure and function. 10 genes involved in 50s ribosomal proteins, 7 in 30s ribosomal proteins and a ribosome modulation factor

were all increased following 24 h aqueous extract treatment. Transcription termination factor (*rho),* ribonuclease protein (*rnpA*), and ribonuclear modulation factor (*rmf*) transcripts were all also increased. Also significantly increased were a flagellar capping protein (*fliD*), a transcriptional regulator (*anr*), and a negative regulator of the lasl QS system (*rsaL*). A single trasncriptioal regulator (*dnr*) was minimally decreased with the 24 h aqueous treatment. Overall, far fewer genes were affected by the 24 h aqueous treatment than the 6hr treatment.

Table 1.2: Significant gene expression changes after exposure to the aqueous ginseng extract. RNA isolated from bacteria grown in the presence of the aqueous ginseng extract for 6 or 24 h at a 1.5% w/v concentration was analysed through the Affymetrix *Pseudomonas aeruginosa* GeneChip array. Partek multi-way ANOVA analysis was performed to assess significant differences in the expression of genes when compared to RNA from untreated controls. Only genes displaying increased or decreased expression of a ≥2.0 fold change were considered significant.

Table 1.2 A: Significant gene expression changes with 6 h aqueous ginseng treatment (n=3) when compared to 6 h control (n=3)

Probeset ID	Gene Symbol	Gene Title	p-value	Fold Change
PA0519_nirS_at	nirS	nitrite reductase precursor	3.78E-11	13.289
PA0518_nirM_at	nirM	cytochrome c-551 precursor	9.00E-10	9.87022
PA0263_hcpC_s_at	hcpA / hcpB / hcpC	secreted protein Hcp	3.78E-06	6.94621
PA2338_at	---	---	2.81E-05	6.06149
PA0517_nirC_at	nirC	probable c-type cytochrome precursor	6.08E-09	6.03595
PA3392_nosZ_at	nosZ	nitrous-oxide reductase precursor	1.16E-07	5.54854
PA1657_at	---	---	2.20E-05	4.85483
PA1556_at	---	---	0.0114198	4.57164
PA4133_at	---	---	1.83E-10	4.53765
PA0795_prpC_at	prpC	citrate synthase 2	0.000143425	4.23113
PA4587_ccpR_at	ccpR	cytochrome c551 peroxidase precursor	6.73E-06	4.19885
PA0516_nirF_at	nirF	heme d1 biosynthesis protein NirF	1.30E-09	4.06298
PA1658_at	---	---	8.93E-05	3.97869
PA5100_hutU_at	hutU	urocanase	2.37E-09	3.95938
PA1555_at	---	---	0.00399444	3.94427
PA0887_acsA_at	acsA	acetyl-coenzyme A synthetase	4.87E-05	3.87622
PA0918_at	---	---	1.53E-07	3.5942
PA3922_at	---	---	1.93E-06	3.42735
PA3570_mmsA_at	mmsA	methylmalonate-semialdehyde dehydrogenase	0.00115913	3.42713
PA2015_at	gnyD	Citronelloyl-CoA dehydrogenase, GnyD	0.000785982	3.4167
PA3920_at	---	---	5.22E-06	3.33524
PA4366_sodB_at	sodB	superoxide dismutase	0.00016931	3.30562

			8	
PA4430_at	---	---	0.000336696	3.27291
PA1551_at	---	---	0.000666065	3.23781
PA2265_at	---	---	1.36E-07	3.15793
PA0796_prpB_at	prpB	carboxyphosphonoenolpyruvate phosphonomutase	0.0026867	3.14881
PA1659_at	---	---	1.29E-05	3.14021
PA2340_at	---	---	3.64E-05	3.11368
PA5355_glcD_at	glcD	glycolate oxidase subunit GlcD	2.48E-05	3.10712
PA0524_norB_at	norB	nitric-oxide reductase subunit B	3.50E-11	3.09339
PA2016_at	gnyR	Regulatory gene of gnyRDBHAL cluster, GnyR	0.000238715	3.07871
PA0523_norC_at	norC	nitric-oxide reductase subunit C	0.000286474	3.07407
PA2014_at	gnyB	beta subunit of geranoyl-CoA carboxylase, GnyB	0.000223558	3.06297
PA4812_fdnG_at	fdnG	formate dehydrogenase-O, major subunit	0.000653142	3.04728
PA2264_at	---	---	6.55E-08	3.04089
PA1562_acnA_at	acnA	aconitate hydratase 1	0.000297492	3.01044
PA2013_at	gnyH	gamma-carboxygeranoyl-CoA hydratase, GnyH	0.00295466	2.95937
PA0514_nirL_at	nirL	heme d1 biosynthesis protein NirL	2.09E-07	2.95519
PA0515_at	---	---	1.09E-07	2.91782
PA0958_oprD_at	oprD	Basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor	0.00500756	2.87338
PA0511_nirJ_at	nirJ	heme d1 biosynthesis protein NirJ	3.54E-06	2.86918
PA4431_at	---	---	0.000549334	2.85503
PA1174_napA_at	napA	periplasmic nitrate reductase protein NapA	2.76E-06	2.85168
PA1173_napB_at	napB	cytochrome c-type protein NapB precursor	9.87E-08	2.83419
PA1984_s_at	---	---	0.000374376	2.78421
PA3235_at	---	---	0.00193917	2.71325
PA2339_at	---	---	0.000371102	2.69236
PA1666_at	---	---	6.85E-05	2.63224

PA2341_at	---	---	1.93E-05	2.61542
PA1667_at	---	---	2.01E-08	2.59932
PA2554_at	---	---	0.00256784	2.59254
PA4578_at	---	---	0.00382733	2.57081
PA0510_at	---	---	0.0011197	2.57062
PA1288_at	---	---	0.00438429	2.56853
PA0513_at	---	---	5.13E-09	2.54968
PA5429_aspA_at	aspA	aspartate ammonia-lyase	5.02E-05	2.5308
PA4500_at	---	---	0.016752	2.51823
PA0794_at	---	---	0.000408102	2.48682
PA0291_oprE_at	oprE	Anaerobically-induced outer membrane porin OprE precursor	0.00888121	2.48605
PA3393_nosD_at	nosD	NosD protein	8.44E-05	2.46591
PA3923_at	---	---	4.98E-08	2.45018
PA0132_at	---	---	4.55E-05	2.37414
PA3188_at	---	---	0.00429773	2.36035
PA4236_katA_at	katA	catalase	0.00978967	2.34582
PA2646_nuoK_at	nuoK	NADH dehydrogenase I chain K	0.000283154	2.32897
PA0509_nirN_at	nirN	probable c-type cytochrome	6.55E-05	2.32641
PA4811_fdnH_at	fdnH	nitrate-inducible formate dehydrogenase, beta subunit	1.71E-05	2.30058
PA4131_at	---	---	0.00204047	2.30044
PA3524_gloA1_at	gloA1	lactoylglutathione lyase	7.55E-05	2.29328
PA3189_at	---	---	0.00398028	2.27678
PA5461_at	---	---	0.0258161	2.26625
PA4661_at	pagL	Lipid A 3-O-deacylase	0.00101846	2.25616
PA1584_sdhB_at	sdhB	succinate dehydrogenase (B subunit)	0.020709	2.25449
PA2112_at	---	---	9.12E-06	2.21727
PA3234_at	---	---	0.000322336	2.2153
PA2249_bkdB_at	bkdB	branched-chain alpha-keto acid dehydrogenase (lipoamide component)	0.0018092	2.21266
PA1787_acnB_at	acnB	aconitate hydratase 2	0.0156434	2.20273
PA5119_glnA_at	glnA	glutamine synthetase	0.0111685	2.19902
PA4429_at	---	---	0.00183448	2.1969
PA5266_at	---	---	3.28E-06	2.18529
PA5490_cc4_at	cc4	cytochrome c4 precursor	0.000245296	2.18296

PA3187_at	---	---	0.0280127	2.1781
PA0526_at	---	---	9.21E-06	2.17524
PA5153_at	---	---	0.000356252	2.16278
PA1074_braC_at	braC	branched-chain amino acid transport protein BraC	0.0016183	2.14659
PA3519_at	---	---	0.00118905	2.13953
PA1665_at	---	---	2.38E-06	2.1369
PA1195_at	---	---	1.42E-07	2.13571
PA2648_nuoM_at	nuoM	NADH dehydrogenase I chain M	0.00167441	2.13213
PA2482_at	---	---	0.000115694	2.12997
PA0512_at	---	---	5.40E-06	2.11553
PA2126_at	---	---	1.96E-05	2.11198
PA3569_mmsB_at	mmsB	3-hydroxyisobutyrate dehydrogenase	0.00672201	2.09497
PA2553_at	---	---	0.0228512	2.08739
PA3190_at	---	---	0.0115915	2.08506
PA5099_at	---	---	0.000889048	2.06348
PA2128_at	cupA1	fimbrial subunit CupA1	0.000601258	2.04294
PA3038_at	---	---	0.0101208	2.03553
PA1511_at	---	---	2.80E-05	2.01785
PA5167_at	---	---	7.78E-05	2.01087
PA1550_at	---	---	0.00456837	2.00806
PA1718_pscE_at	pscE	type III export protein PscE	0.00762587	-2.01391
PA5027_at	---	---	0.0125507	-2.01742
PA4916_at	---	---	0.01634	-2.01773
PA3531_bfrB_at	bfrB	bacterioferritin	0.0257969	-2.03242
PA0211_mdcD_at	mdcD	malonate decarboxylase beta subunit	0.00120954	-2.04803
PA2404_at	---	---	0.00950382	-2.05336
PA1914_at	---	---	9.20E-05	-2.05828
PA0534_at	---	---	0.0161202	-2.07925
PA2034_at	---	---	0.00272246	-2.11222
PA3690_at	---	---	0.0191532	-2.11784
PA3479_rhlA_at	rhlA	rhamnosyltransferase chain A	0.0133196	-2.12425
PA1876_at	---	---	0.00013079	-2.13998
PA3842_at	---	---	0.00430214	-2.15196
PA1318_cyoB_at	cyoB	cytochrome o ubiquinol oxidase subunit I	0.0165511	-2.15297
PA4467_at	---	---	0.00899147	-2.15659

PA1001_phnA_at	phnA	anthranilate synthase component I	0.00190017	-2.16079
PA0044_exoT_at	exoT	exoenzyme T	3.03E-05	-2.16469
PA1707_pcrH_at	pcrH	regulatory protein PcrH	0.000123882	-2.17814
PA4142_at	---	---	0.00031226	-2.21003
PA4917_at	---	---	0.000157097	-2.21668
PA0792_prpD_at	prpD	propionate catabolic protein PrpD	0.00662303	-2.26371
PA1875_at	---	---	3.61E-06	-2.26537
PA4306_at	---	---	3.35E-05	-2.2731
PA3584_glpD_at	glpD	glycerol-3-phosphate dehydrogenase	0.019293	-2.28816
PA2501_at	---	---	0.0140032	-2.33496
PA5436_at	---	---	0.00559478	-2.40766
PA2405_at	---	---	0.000746154	-2.41224
PA2033_at	---	---	0.0126758	-2.44053
PA3195_gapA_at	gapA	glyceraldehyde 3-phosphate dehydrogenase	5.01E-05	-2.45955
PA1874_at	---	---	3.37E-06	-2.47945
PA2452_at	---	---	0.0151902	-2.68475
PA1196_at	---	---	0.0131888	-2.7019
PA4211_g_at	phzB1 / phzB2	probable phenazine biosynthesis protein	0.00169332	-2.73589
PA0210_mdcC_at	mdcC	malonate decarboxylase delta subunit	0.000340578	-2.80477
PA2389_at	---	---	0.00206801	-2.81264
PA0208_mdcA_at	mdcA	malonate decarboxylase alpha subunit	0.00299533	-2.82689
PA2398_fpvA_at	fpvA	ferripyoverdine receptor	0.0404226	-2.89529
PA2433_at	---	---	2.46E-05	-2.91184
PA2427_at	---	---	0.00280955	-3.00129
PA0209_at	---	---	0.000134477	-3.00585
PA1901_s_at	phzC1 / phzC2	phenazine biosynthesis protein PhzC	1.68E-05	-3.05613
PA0122_at	---	---	0.000908487	-3.05739
PA4229_pchC_at	pchC	pyochelin biosynthetic protein PchC	0.00037407	-3.07568
PA4217_at	phzS	flavin-containing	0.00099136	-3.1247

		monooxygenase		
PA1709_popD_at	popD	Translocator outer membrane protein PopD precursor	6.43E-06	-3.23647
PA4226_pchE_at	pchE	dihydroaeruginoic acid synthetase	0.00109602	-3.3663
PA2397_pvdE_at	pvdE	pyoverdine biosynthesis protein PvdE	0.0322414	-3.3833
PA1317_cyoA_at	cyoA	cytochrome o ubiquinol oxidase subunit II	4.56E-06	-3.42533
PA3841_exoS_at	exoS	exoenzyme S	1.50E-06	-3.45172
PA4175_at	prpL	Pvds-regulated endoprotease, lysyl class	1.03E-06	-3.50964
PA3361_at	lecB	fucose-binding lectin PA-IIL	0.00200522	-3.5439
PA0315_at	---	---	3.98E-06	-3.54514
PA3181_at	---	---	8.85E-07	-3.7041
PA2394_at	pvdN	PvdN	0.0020704	-3.80565
PA2386_pvdA_at	pvdA	L-ornithine N5-oxygenase	0.0111724	-3.82158
PA1903_s_at	phzE1 / phzE2	phenazine biosynthesis protein PhzE	5.75E-06	-3.91308
PA2393_at	---	---	0.00549147	-4.12925
PA4221_fptA_at	fptA	Fe(III)-pyochelin outer membrane receptor precursor	0.00567876	-4.19199
PA2411_at	---	---	2.42E-05	-4.34107
PA1708_popB_at	popB	translocator protein PopB	1.67E-08	-4.53525
PA2402_at	---	---	1.34E-05	-4.55376
PA2413_at	pvdH	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH	0.00025913	-4.76076
PA4470_fumC1_at	fumC1	fumarate hydratase	0.0092648	-4.76918
PA1904_s_at	phzF1 / phzF2	probable phenazine biosynthesis protein	1.12E-06	-4.91159
PA1245_at	---	---	9.59E-08	-4.92912
PA3407_hasAp_at	hasAp	heme acquisition protein HasAp	4.70E-06	-5.00349
PA4218_at	---	---	2.53E-05	-5.29589
PA4222_at	---	---	1.58E-08	-5.3744
PA3183_zwf_at	zwf	glucose-6-phosphate 1-dehydrogenase	2.45E-08	-5.40556
PA4223_at	---	---	4.66E-09	-5.54624
PA2399_pvdD_at	pvdD	pyoverdine synthetase D	1.45E-06	-5.64402

PA4469_at	---	---	0.000395749	-5.79569
PA2401_at	---	---	4.08E-05	-5.89781
PA3182_at	pgl	6-phosphogluconolactonase	2.70E-08	-6.10826
PA4231_pchA_at	pchA	salicylate biosynthesis isochorismate synthase	1.08E-10	-6.19586
PA2412_at	---	---	0.000490037	-6.20121
PA1905_s_at	phzG2	probable pyridoxamine 5'-phosphate oxidase	5.39E-09	-6.58124
PA4468_sodM_at	sodM	superoxide dismutase	4.73E-06	-6.60205
PA2424_at	pvdL	PvdL	4.78E-09	-7.04124
PA4141_at	---	---	0.000194111	-7.70085
PA2400_at	pvdJ	PvdJ	4.64E-06	-8.01899
PA4225_pchF_at	pchF	pyochelin synthetase	4.22E-07	-8.11832
PA4224_at	pchG	pyochelin biosynthetic protein PchG	5.00E-07	-8.92838
PA4230_pchB_at	pchB	salicylate biosynthesis protein PchB	1.87E-09	-11.6901

Table 1.2 B: Significant gene expression changes with 24 h aqueous ginseng treatment (n=3) when compared to 24 h control (n=3)

Probeset ID	Gene Symbol	Gene Title	p-value	Fold Change
PA2664_fhp_at	fhp	flavohemoprotein	1.03E-08	17.8077
PA3813_iscU_at	iscU	probable iron-binding protein IscU	6.30E-05	11.7175
PA2663_at	---	---	1.71E-07	11.6566
PA3815_at	---	---	7.29E-07	7.77983
PA3361_at	lecB	fucose-binding lectin PA-IIL	3.53E-05	7.52668
PA3811_hscB_at	hscB	heat shock protein HscB	1.42E-05	7.20502
PA1847_at	---	---	0.000109092	6.46437
PA3812_iscA_at	iscA	probable iron-binding protein IscA	0.000157939	6.41803
PA2662_at	---	---	2.62E-05	6.27684
PA3814_iscS_at	iscS	L-cysteine desulfurase (pyridoxal phosphate-dependent)	0.000857719	5.96988
PA3584_glpD_at	glpD	glycerol-3-phosphate dehydrogenase	0.000241755	4.71812

PA5531_tonB_at	tonB	TonB protein	0.000944246	4.37658
PA0179_at	---	---	0.000814828	3.75166
PA4257_rpsC_at	rpsC	30S ribosomal protein S3	0.0327932	3.56291
PA1178_oprH_at	oprH	PhoP/Q and low Mg2+ inducible outer membrane protein H1 precursor	0.00307541	3.42063
PA4241_rpsM_at	rpsM	30S ribosomal protein S13	0.0294963	3.34495
PA3049_rmf_at	rmf	ribosome modulation factor	0.00018426	3.29575
PA1610_fabA_at	fabA	beta-hydroxydecanoyl-ACP dehydrase	0.00897408	3.2276
PA1431_rsaL_at	rsaL	regulatory protein RsaL	0.00865849	3.10445
PA4273_rplA_at	rplA	50S ribosomal protein L1	0.028352	3.04697
PA4258_rplV_at	rplV	50S ribosomal protein L22	0.0179047	3.01569
PA3656_rpsB_at	rpsB	30S ribosomal protein S2	0.0432387	2.9873
PA2513_antB_at	antB	anthranilate dioxygenase small subunit	0.000230907	2.96269
PA2840_at	---	---	0.00750856	2.78178
PA0456_at	---	---	0.0394716	2.78129
PA5030_at	---	---	0.000219311	2.71799
PA4221_fptA_at	fptA	Fe(III)-pyochelin outer membrane receptor precursor	0.0397513	2.6925
PA4259_rpsS_at	rpsS	30S ribosomal protein S19	0.0164566	2.69021
PA3930_cioA_at	cioA	cyanide insensitive terminal oxidase	0.00931686	2.6884
PA5570_rpmH_at	rpmH	50S ribosomal protein L34	0.02924	2.68469
PA3190_at	---	---	0.00181828	2.656
PA3808_at	---	---	0.00237087	2.63236
PA4256_rplP_at	rplP	50S ribosomal protein L16	0.0430538	2.6301
PA4935_rpsF_at	rpsF	30S ribosomal protein S6	0.0363297	2.59759
PA1774_at	---	---	0.000793488	2.59662
PA4262_rplD_at	rplD	50S ribosomal protein L4	0.0390145	2.56978
PA1609_fabB_a	fabB	beta-ketoacyl-ACP synthase I	0.00053737	2.55104

t			3	
PA4253_rplN_at	rplN	50S ribosomal protein L14	0.025885	2.55052
PA4847_accB_at	accB	biotin carboxyl carrier protein (BCCP)	0.0233972	2.54003
PA1775_at	---	---	0.000254331	2.5127
PA0505_at	---	---	0.00173712	2.47089
PA4843_at	---	---	0.000219815	2.46765
PA0122_at	---	---	0.00430983	2.46544
PA4261_rplW_at	rplW	50S ribosomal protein L23	0.0417189	2.46124
PA3809_fdx2_at	fdx2	ferredoxin [2Fe-2S]	0.00216273	2.45535
PA4244_rplO_at	rplO	50S ribosomal protein L15	0.0259151	2.45125
PA3533_at	---	---	0.0309253	2.43241
PA4726_at	cbrB	two-component response regulator CbrB	0.000824545	2.42429
PA4944_at	---	---	0.00888775	2.41197
PA4251_rplE_at	rplE	50S ribosomal protein L5	0.0395769	2.40326
PA4751_ftsH_at	ftsH	cell division protein FtsH	0.00332574	2.38314
PA0579_rpsU_at	rpsU	30S ribosomal protein S21	0.0105975	2.35959
PA3349_at	---	---	0.000335839	2.3489
PA0176_at	---	---	0.000854131	2.32553
PA2512_antA_at	antA	anthranilate dioxygenase large subunit	0.000196959	2.27617
PA2788_at	---	---	0.00227802	2.25694
PA2642_nuoG_at	nuoG	NADH dehydrogenase I chain G	0.00905427	2.25505
PA5374_betI_at	betI	transcriptional regulator BetI	0.00592773	2.24552
PA5569_rnpA_at	rnpA	ribonuclease P protein component	0.0230047	2.23946
PA1544_anr_at	anr	transcriptional regulator Anr	0.0124458	2.23672
PA1777_oprF_at	oprF	Major porin and structural outer membrane porin OprF precursor	0.0167288	2.22495
PA3009_at	---	---	0.000871116	2.21905
PA2604_at	---	---	0.0388175	2.2067
PA3530_at	---	---	0.00108567	2.19214
PA2622_cspD_	cspD	cold-shock protein CspD	0.00458832	2.17943

at				
PA2638_nuoB_at	nuoB	NADH dehydrogenase I chain B	0.0466691	2.14697
PA4563_rpsT_at	rpsT	30S ribosomal protein S20	0.0203625	2.14338
PA3810_hscA_at	hscA	heat shock protein HscA	0.00128999	2.14236
PA4848_accC_at	accC	biotin carboxylase	0.0260101	2.13322
PA0839_at	---	---	0.00253363	2.13078
PA2621_at	---	---	0.00128594	2.12855
PA1094_fliD_at	fliD	flagellar capping protein FliD	0.0456645	2.11167
PA3031_at	---	---	0.0373232	2.09272
PA4515_at	---	---	0.00513476	2.0829
PA5438_at	---	---	0.00257018	2.04864
PA3581_glpF_at	glpF	glycerol uptake facilitator protein	0.0124227	2.04369
PA2741_rplT_at	rplT	50S ribosomal protein L20	0.0470199	2.03743
PA1093_at	---	---	0.0293185	2.02944
PA1584_sdhB_at	sdhB	succinate dehydrogenase (B subunit)	0.0404479	2.01949
PA2897_at	---	---	0.00282981	2.01785
PA3326_at	---	---	0.00313689	2.01496
PA5239_rho_at	rho	transcription termination factor Rho	0.0488799	2.0044
PA0527_dnr_at	dnr	transcriptional regulator Dnr	0.0471663	-2.00957
PA1789_at	---	---	0.0277343	-2.12231
PA5232_at	---	---	0.0116958	-2.17726
PA1673_at	---	---	0.0121	-2.36053
PA5427_adhA_at	adhA	alcohol dehydrogenase	0.037982	-2.65738
PA5475_at	---	---	0.00407286	-2.7331
PA3572_at	---	---	0.00437332	-2.77132
PA1557_at	---	---	0.0416549	-3.22679
PA1546_hemN_at	hemN	oxygen-independent coproporphyrinogen III oxidase	0.00643591	-3.23242
PA3309_at	---	---	0.00385956	-3.29413
PA4067_oprG_at	oprG	Outer membrane protein OprG precursor	0.00220388	-3.59858
PA5170_arcD_at	arcD	arginine/ornithine antiporter	0.00636456	-4.70364

2.3.9 Gene-Ontology Enrichment Analysis of *P. aeruginosa* Treated with Aqueous Ginseng Extract

Gene ontology analysis of the 6 h aqueous ginseng extract treatment revealed that a large fraction of genes related to iron and heme binding, iron homeostasis, siderophore transmembrane transport activity, and iron-sulfur clustering were significantly affected by the treatment (Table 1.3 A). Most significantly of these were those genes with the common function of heme binding with an enrichment score of 17.795 with 42.30% of the genes present significantly affected by the 6 h aqueous extract treatment. Other gene groups enriched by the 6 h treatment include; oxidation and reduction processes, glucose metabolism, ATP synthesis coupled electron transport, fermentation, NADH dehydrogenase activity, and peroxidase activity.

After 24 h of aqueous ginseng treatment iron binding, transport, and iron-sulfur cluster assembly and binding were still among the most greatly affected groups of genes (Table 1.3 B). Of these, iron-sulfur cluster assembly had the greatest enrichment of 12.95 with 100% of the genes in the pathway affected significantly. Gene groups involved in ribosomal structure, RNA binding, translation, and translation regulation were all enriched with the 24 h treatment. Genes involved in RNA binding and ribosomal structure, displayed enrichment scores of 21.35 and 29.54 respectively. Multiple gene groups involved in amino acid transport, nucleic acid binding, and DNA-template transcription and termination were significantly affected by the 24 h aqueous treatment as well. Additionally, gene groups involved with fatty acid synthesis and metabolism, lipid metabolism, and carbohydrate derivative metabolism and synthesis showed significant enrichment after 24 h treatment.

Table 1.3: Gene ontology (GO) enrichment analysis of *P. aeruginosa* after exposure to the aqueous ginseng extract. From the sample of genes deemed significantly increased by a factor of ≥2.0 fold change, genes were grouped based on similar GO functions and differences in overall group enrichment compared to the control was performed through a Fisher's Exact Test. Only those gene groups with an enrichment p-value > 0.05 are displayed.

Table 1.3 A: GO-enrichmen of 6 h aqueous ginseng treatment (n=3) compared to 6 h control (n=3)

Function	Type	Enrichment Score	Enrichment p-value	% genes in group that are present
heme binding	molecular function	17.795	1.86943E-08	42.3077
oxidation-reduction process	biological process	15.6217	1.64276E-07	13.4529
iron ion binding	molecular function	11.1763	1.40023E-05	26.3158
extracellular region	cellular component	10.505	2.73999E-05	36.8421
antibiotic biosynthetic process	biological process	9.48148	0.000076251	50
glucose metabolic process	biological process	6.61556	0.00133937	60
siderophore transmembrane transporter activity	molecular function	5.96136	0.0025764	50
ATP synthesis coupled electron transport	biological process	5.96136	0.0025764	50
peroxidase activity	molecular function	5.44061	0.00433683	42.8571
ACP phosphopantetheine attachment site binding involved in fatty acid biosynthetic process	molecular function	4.81687	0.00809209	66.6667
glucose-6-phosphate dehydrogenase activity	molecular function	4.81687	0.00809209	66.6667
chorismate metabolic process	biological process	4.81687	0.00809209	66.6667
glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity	molecular function	4.81687	0.00809209	66.6667
iron ion homeostasis	biological process	4.81687	0.00809209	66.6667
copper ion binding	molecular function	4.64261	0.00963249	33.3333
iron-sulfur cluster binding	molecular	4.4674	0.0114771	14.5833

	function			
fumarate hydratase activity	molecular function	4.15875	0.0156271	50
glutamate-ammonia ligase activity	molecular function	4.15875	0.0156271	50
glycerol-3-phosphate metabolic process	biological process	4.15875	0.0156271	50
glutamine biosynthetic process	biological process	4.15875	0.0156271	50
molybdenum ion binding	molecular function	4.15875	0.0156271	50
fermentation	biological process	4.15875	0.0156271	50
isoleucine catabolic process	biological process	3.68281	0.0251522	40
leucine catabolic process	biological process	3.68281	0.0251522	40
valine catabolic process	biological process	3.68281	0.0251522	40
oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	molecular function	3.36837	0.0344459	21.4286
NADH dehydrogenase (quinone) activity	molecular function	3.36837	0.0344459	21.4286
glycerol metabolic process	biological process	3.31208	0.0364402	33.3333
hydrolase activity, hydrolyzing O-glycosyl compounds	molecular function	3.01021	0.0492815	28.5714

Table 1.3 B: GO-enrichment of 24 h aqueous ginseng treatment (n=3) compared to 24 h control (n=3)

Function	Type	Enrichment Score	Enrichment p-value	% genes in group that are present
structural constituent of ribosome	molecular function	29.547	1.47191E-13	33.3333
RNA binding	molecular function	21.3596	5.29206E-10	23.2877
intracellular	cellular compone nt	20.2277	1.64146E-09	23.5294
translation	biological process	19.0256	5.46098E-09	18.9474

cellular protein metabolic process	biological process	13.2523	1.75629E-06	12.1212
iron-sulfur cluster assembly	biological process	12.9548	2.36476E-06	100
iron-sulfur cluster binding	molecular function	9.54333	7.16781E-05	18.75
fatty acid metabolic process	biological process	8.19847	0.000275075	31.25
iron ion binding	molecular function	7.48839	0.000559543	18.4211
regulation of translation	biological process	7.445	0.000584359	60
glycerol metabolic process	biological process	6.78082	0.00113534	50
siderophore transmembrane transporter activity	molecular function	6.78082	0.00113534	50
2 iron, 2 sulfur cluster binding	molecular function	5.57408	0.00379497	23.5294
glucose-6-phosphate dehydrogenase activity	molecular function	5.3698	0.00465506	66.6667
fatty acid biosynthetic process	biological process	5.14664	0.00581894	21.0526
cellular aromatic compound metabolic process	biological process	5.10453	0.0060692	30
oxidoreductase activity, acting on NAD(P)H	molecular function	5.10453	0.0060692	30
biotin carboxylase activity	molecular function	4.70284	0.0090695	50
glycerol-3-phosphate metabolic process	biological process	4.70284	0.0090695	50
DNA-templated transcription, termination	biological process	4.70284	0.0090695	50
protein catabolic process	biological process	4.70284	0.0090695	50
carbohydrate derivative metabolic process	biological process	4.70284	0.0090695	50
carbohydrate derivative biosynthetic process	biological process	4.70284	0.0090695	50
organic substance biosynthetic process	biological process	4.70284	0.0090695	50
organophosphate metabolic process	biological process	4.70284	0.0090695	50
phosphorus metabolic process	biological process	4.70284	0.0090695	50
phosphate-containing compound metabolic process	biological process	4.70284	0.0090695	50

organophosphate biosynthetic process	biological process	4.70284	0.0090695	50
biosynthetic process	biological process	4.70284	0.0090695	50
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	molecular function	4.21812	0.0147263	40
organic cyclic compound metabolic process	biological process	4.21812	0.0147263	40
cellular metabolic process	biological process	4.21812	0.0147263	40
small molecule metabolic process	biological process	4.21812	0.0147263	40
single-organism process	biological process	4.21812	0.0147263	40
single-organism metabolic process	biological process	4.21812	0.0147263	40
single-organism cellular process	biological process	4.21812	0.0147263	40
cellular aromatic compound metabolic process	biological process	4.21812	0.0147263	40
organic substance metabolic process	biological process	4.21812	0.0147263	40
metabolic process	biological process	4.21812	0.0147263	40
cellular process	biological process	4.21812	0.0147263	40
NADH dehydrogenase (quinone) activity	molecular function	4.10963	0.0164139	21.4286
amino acid transmembrane transporter activity	molecular function	3.83869	0.0215217	33.3333
ATP synthesis coupled electron transport	biological process	3.83869	0.0215217	33.3333
biological_process	biological process	3.83869	0.0215217	33.3333
dioxygenase activity	molecular function	3.73595	0.0238506	18.75
nucleic acid binding	molecular function	3.63346	0.0264245	13.7931
amino acid transmembrane transport	biological process	3.52818	0.0293583	28.5714
cellular lipid metabolic process	biological process	3.29219	0.0371722	10.6383
3-oxoacyl-[acyl-carrier-protein] synthase activity	molecular function	3.26638	0.0381443	25
arginine catabolic process	biological	3.26638	0.0381443	25

	process			
metalloendopeptidase activity	molecular function	3.04087	0.0477934	22.2222
cell adhesion	biological process	3.04087	0.0477934	22.2222

2.3.10 Pathway Enrichment Analysis of *P. aeruinosa* treated with Aqueous Ginseng Extract

Pathway enrichment analysis provided further elucidation of the effects of both 6 h and 24 h aqueous ginseng treatment of *P. aeruginosa* (Table 1.4). With the 6 h treatment, the pathway most enriched was that of the biosynthesis of siderophore group nonribosomal peptides. This pathway had an enrichment score of 13.29 with 83.33% of the genes within the pathway significantly altered in expression. Microbial metabolism in diverse environments pathway was enriched by a factor of 9.18, however only 12.02% of the genes in this pathway were significantly altered. Other pathways significantly enriched through 6hr treatment include nitrogen, propanoate, glyoxlate and dicarboxylate metabolism, carbon metabolism, the citrate acid cycle, and oxidative phosphorylation.

After 24 h of aqueous ginseng treatment far fewer pathways were significantly affected. The most prominently effected pathway was that of the ribosome with an enrichment score of 31.24 with 32.07% of the pathway genes significantly affected. Fatty acid biosynthesis and metabolism were the next two pathways with the greatest enrichment with scores of 6.89 and 5.66 respectively. Aminobenzoate degradation and oxidative phosphorylation were also significantly enriched with the 24 h treatment.

Table 1.4: Pathway enrichment analysis of *P. aeruginosa* after exposure to the aqueous ginseng extract. From the sample of genes deemed significantly increased by a factor of ≥2.0 fold change, genes were grouped based similarity in the pathways which they function through KEGG pathway analysis. Differences in pathway enrichment compared to the control were performed through a Fisher's Exact Test. Only those pathways with an enrichment p-value > 0.05 are displayed.

Table 1.4 A: Pathway analysis of 6 h aqueous ginseng treatment (n=3) compared to 6 h control (n=3)

Pathway Name	Enrichment Score	Enrichment p-value	% genes in pathway that are present
Biosynthesis of siderophore group nonribosomal peptides	13.2934	1.68559E-06	83.3333
Microbial metabolism in diverse environments	9.1831	0.000102761	12.0253
Nitrogen metabolism	9.13212	0.000108136	29.1667
Propanoate metabolism	9.13059	0.000108302	35.2941
Glyoxylate and dicarboxylate metabolism	8.56927	0.000189852	26.9231
Carbon metabolism	6.6063	0.00135182	13.9241
Citrate cycle (TCA cycle)	3.78648	0.0226752	18.1818
Valine, leucine and isoleucine degradation	3.67554	0.0253358	23.0769
Oxidative phosphorylation	3.20965	0.0403709	13.1579

Table 1.4 B: Pathway analysis of 24 h aqueous ginseng treatment (n=3) compared to 24 h control (n=3)

Pathway Name	Enrichment Score	Enrichment p-value	% genes in pathway that are present
Ribosome	31.2444	2.69603E-14	32.0755
Fatty acid biosynthesis	6.89769	0.00101012	28.5714
Fatty acid metabolism	5.66985	0.00344839	21.0526
Aminobenzoate degradation	3.51728	0.0296802	25
Oxidative phosphorylation	3.19965	0.0407764	10.5263

2.4 Discussion

Ginseng is globally recognized as the “king” of medicinal plants displaying a plethora of medicinal uses including anti-bacterial properties (Cruse-Sanders and Hamrick 2004). *Panax quinquefolius* is the native ginseng species of North America and has been less intensly studied for its medicinal properties than the Asian *P. ginseng*. Published research on the medicinal properties of *P. quinquefolius* has shown various anti-bacterial mechanisms against opportunistic pathogen *P. aeruginosa* (Alipour, Omri et al. 2011). To further elucidate the efficacy of extracts from *P. quinquefolius* against *P. aeruginosa* pathogenicity, the effects of both the aqueous and alcoholic extracts of the plant were investigated against bacterial growth, biofilm formation, virulence factor production, and bacterial genetic expression.

To analyze the bacteriostatic effects of either ginseng extract towards *P. aeruginosa*, bacteria were subjected to MIC assays. Results from the MIC experiments revealed an inhibitory effect against *P. aeruginosa* when treated with the aqueous ginseng extract in either MHCA broth or M9 broth (Table 1.1.1, Table 1.1.2). Values for growth inhibition were lower in the MHCA (2.0-3.0% w/v) broth than in the M9 (7.0-8.0% w/v). Differences in the MIC concentrations found for each media may be attributed to the rate/amount of specific components dissolving in the media, or interactions of extract components with the media component(s) such as the ginsenosides of each extract (Rios, Recio et al. 1988, Burt 2004).

MIC experiments with the alcoholic ginseng extract presented no visible growth attenuation of any strain of *P. aeruginosa* for concentrations up to 5.0% w/v in MHCA

and 10.0% w/v in M9 media (Table 1.1). To our knowledge, no data are available on the antibacterial effects of *P. quinquefolius* alcoholic extract. The higher MIC values of the alcoholic extract might be attributed to its composition; it is largely composed of ginsenosides which have been reported to have weak growth inhibitory action against other species of pathogenic bacteria (Sung and Lee 2008).

Growth curve analysis of the bacteria in the presence of subinhibitory concentrations of either ginseng extract revealed growth stimulatory effects of the aqueous ginseng extract, while the alcoholic extract displayed mild inhibitory action (Figure 1.1, 1.2). For all strains significant increases in growth were found in the presence of the aqueous extract at a 0.5 MIC concentration, while growth was only minimally increased with a 0.25 MIC concentration. In regards to pathogenicity, increased growth rate could increase *P. aeruginosa* survival within a host. Similar increase in growth rate with aqueous extract treatment of *P. aeruginosa* has been previously described by Alipour et al (2011). In constrast to the treatment with the aqueous extract, the alcoholic extract reduced growth of both the mucoid clinical isolate and PAO1, with minimal effect towards the non-mucoid clinical isolate. Experimentation with specific components of either extract would improve our understanding of which component(s) influence growth rate.

The explanation(s) regarding the stimulatory effects of aqueous extract on bacterial growth are not clear at the present time. Gene expression analysis (Table 1.2) showed that 6 h of aqueous extract treatment, increased expression of propanoate (prpB, prpC, prpD), glycoxylate and dicarboxylate metabolism (glcD, fdnG), TCA cycle (acnB, acnA, sdnB, fdnG), and most significantly nitrogen metabolism (glnA, fdnH, nirN, nirM,

nirC, ccpR, nosD, napB, napA, norB, norC, nosZ, nirS) which are all involved in energy metabolism. Heme biosynthesis genes (nirJ, nirL, nirF) specific for cytochrome c d_1, the electron donor for nitrite reductase (nirS), showed comparable increases in expression. Increases in biochemical components of bacterial metabolism may be indicative of the bacteria preparing for increases in growth rate by producing necessary metabolites and ATP.

After 24 h of exposure to the aqueous extract, genes involved in both nitrogen and carbon (*antA, antB)* metabolism were increased in expression, as well as genes involved in the synthesis (*accB, accC*) and metabolism (*fabA, faB)* of fatty acids (Table 1.2 B). Interestingly the regulatory protein encoded by the *cbrB*, which when expressed allows for the utilization of multiple carbon and nitrogen sources simultaneously also displayed increased expression. Increases in such a wide variety of energy metabolism pathways after both the 6 h and 24 h incubations may partially explain the increased growth associated with the aqueous extract treatment. In regards to an infectious state, increased growth would result in a more aggressive infection.

P. aeruginosa is classified as a facultative anaerobic organism and often depends heavily on anaerobic metabolism during states of chronic infection in CF patients where anaerobic biofilms dominate (Yoon, Hennigan et al. 2002). However, several genes involved in nitrogen metabolism were decreased after the 24 h incubation. Gene *dnr*, an anaerobic transcription regulator of denitrification enzyme production (Arai et al., 1997), such as hemN and hemF heme biosynthesis genes, was down regulated after the 24 h incubation. A reduction in the anaerobically induced *hemeN* was similarly decreased after the 24hr (Rompf, Hungerer et al. 1998). The control of *dnr* is under transcriptional

regulator *anr* (Arai, Kodama et al. 1997), was significantly increased after 24 h. Gene *anr* controls for the regulation of hydrogen cyanide biosynthesis, and arginine deiminase and denitrification pathways (Zimmermann, Reimmann et al. 1991, Ye, Haas et al. 1995). As previously discussed, genes involved in the denitrifying pathway were increased after 6 h of incubation, but not the 24 h incubation with the aqueous extract. Support for increased denitrification during the exponential growth phase was found through increased expression of flavohemoglobin, *fhp*, after 24 h of incubation with the aqueous extract. *fhp* is involved in the detoxification of nitric oxide from within cells in the presence of oxygen (Arai, Hayashi et al. 2005). This suggests anaerobic nitrogen metabolism was most heavily expressed during the exponential growth phase (6 h incubation), but reduced in expression once the bacteria entered the stationary phase (24 h incubation).

Gene ontology (GO) and pathway enrichment analysis from the microarray data mimic the findings detailed for genes involved in metabolism (Table 1.3). Pathways analysis for the 6 h incubation showed enrichment of nitrogen, propanoate, glycoxylate and dicarboxylate, and carbon metabolism. Enrichment in pathways of the TCA cycle, valine, leucine, and isoleucine degradation, oxidative phosphorylation, and microbial metabolism in diverse environments were also significantly affected. After 24 h of incubation fatty acid biosynthesis and metabolism, oxidative phosphorylation, and aminobenozate degradation pathways were all enriched, which correlates directly to the up-regulated genes previously described.

Although the pathway analysis displayed enrichment of the oxidative phosphorylation pathway at both incubation times, genes within the pathway were both significantly increased and decreased therefore it is unlikely that as an entire pathway

oxidative phosphorylation was increased. Rather a shift to anaerobic metabolism after the 6 h incubation is evident. Treatment with the aqueous extract may limit *P. aeruginosa's* ability to utilize oxygen forcing the bacteria to increase the expression of alternative pathways for energy production. Anaerobic metabolism is often utilized by *P. aeruginosa* during chronic infections when a biofilm mode of growth is adopted (Yoon, Hennigan et al. 2002). Increased anaerobic metabolism from aqueous extract exposure may encourage a transition towards biofilm formation, adding to *P. aeruginosa's* pathogenicitiy.

Biofilm formation is a prominent mechanism through which *P. aeruginosa* becomes resistant to antibiotic therapies (Costerton, Stewart et al. 1999, Hoiby, Krogh Johansen et al. 2001). Compounds which reduce bacterial surface adherence or disrupt preformed biofilms have potential to both increase the clearance of chronic *P. aeruginosa* infections and increase the efficacy of available antibiotics. When grown in the presence of the aqueous extract, PAO1 adherence was significantly increased with a 0.5 MIC concentration treatment, but minimally decreased with a 0.25 MIC concentration treatment (Figure 1.3 A). Increases in adherence with aqueous ginseng extract treatment have been previously described for the PAO1 strain (Alipour, Omri et al. 2011). The alcoholic ginseng extract also increased adherence of PAO1, however the effects were not as great as with the aqueous extract.

Adherence of either clinical isolate in the presence of the aqueous extract displayed increased attachment at a 0.50 MIC concentration (Figure 1.3: B, C). Results from the alcoholic ginseng extract treatment display significant reduction in bacterial attachment for either strain. Adherence of either clinical isolate in the presence of the alcoholic extract was below the background adherence of the extract alone. A possible

explanation of this observation could be the alcoholic extracts reduces bacterial adherence by binding directly to the bacteria preventing either from further binding to the surface. There is no information available with regards to the effects of either ginseng extract towards clinical strains of *P. aeruginosa* beyond growth inhibition, and this is the first report addressing such interactions. Further research implementing a larger sample size of clinical isolates is necessary to support the findings of this study.

Biofilm disruption was monitored through 48 h old biofilms treated for 24 h with either ginseng extract at an inhibitory (MIC) or 2x inhibitory concentrations (Figure 1.4). Increases in biofilm adherence of PAO1 were found with the aqueous extract treatment at the MIC concentration, suggesting that the adoption of biofilm growth increased the bacteria's resistance to the treatment. An increase in biofilm disruption was found with the treatment at 2x the inhibitory concentration and may be attributed to the toxic effects of the extract towards the bacteria. Viability was not assessed in our study -and therefore bacterial cells not adhering may or may not have been viable. Previously, Alipour et al (2011) concluded that the higher concentrations of the aqueous extract did have a toxic effect towards preformed biofilms of *P. aeruginosa,* and the sloughing of dead bacterial cells may be responsible for the decreased in adhesion. The aqueous extracts of both *P. quinquefolius* and *P. ginseng* contain a mixture of compounds including polysaccharides. Purified polysaccharide extracts from *P. ginseng* have displayed reduced bacterial adherence of *Helicobacter pylori* to epithelial and gastric cells, as well as human erythrocytes (Belogortseva, Yoon et al. 2000, Lee, Park et al. 2004). Future studies utilizing purified polysaccharide fractions from *P. quinquefolius* may reveal specific fractions with similar anti-adhesive properties.

In regards to the clinical isolates, both were significantly decreased in adherence with either concentration of aqueous extract treatment (Figure 1.4: B, C). Increased resistance to treatment may not have been seen in the clinical isolates due to the slower rates of growth, or because biofilm formation was still naïve. Further analysis of mature biofilms of clinical isolates may reveal different results due to more robust biofilm architecture. Evidence to date suggests the adoption of a mucoid phenotype provides advantageous characteristics towards biofilm formation and added immune and antibiotic resistance (Hentzer, Teitzel et al. 2001, Hoiby, Krogh Johansen et al. 2001) In our experiments, there was no evidence of the mucoid phenotype providing any increases in adherence or biofilm resistance to either ginseng extract. It is uncertain if this lack of enhanced adherence or resistance is relavent to the mucoid phenotype. Supplementary studies on clinical isolates with large sample sizes may result in alternative findings.

Preformed biofilms of the clinical isolates treated with the alcoholic extracts displayed substantial decreases in adherence, below the background adherence of the extract alone for the mucoid isolate. As previously hypothesized, reduction below the extract absorbance may be due to a physical interaction between the extract and bacteria preventing either from binding to the surface. Reduced biofilm adherence of the mucoid strain may also be due to reduced growth in the presence of sub-inhibitory concentrations of the alcoholic extract. The 2x MIC treatment may have been toxic towards the bacteria (Figure 1.4: B, C), which may have contributed to the reduced adherence at this concentration of treatment.

The ability of the aqueous extract to inhibit preformed biofilms is substantiated by data obtained from the gene array analysis which displayed decreased *lecB* expression

with 6 and 24 h aqueous treatments, as well as increased expression of a fimbrae (cupA1) and flagellar (fliD) gene after 6 and 24 h of exposure respectively. Gene *lecB* encodes a surface sugar- binding lectin of *P. aeruginosa* (Sonawane, Jyot et al. 2006) which plays a role in airway epithelial cell adhesion (Alverdy, Holbrook et al. 2000), formation of biofilms (Tielker, Hacker et al. 2005), and cell injury (Laughlin, Musch et al. 2000). Decreased *lecB* expression may be involved in the decreased biofilm formation found with the aqueous extract treatment. *lecB* is also involved in pilus biogenesis and previous studies have shown that blocking expression of *lecB* reduces twitching motility (Sonawane, Jyot et al. 2006) Pilus production is critical for early bacterial adherence and biofilm formation (Strom and Lory 1993, O'Toole and Kolter 1998).

In addition to IV pili surface fimbrae and flagella also aid in bacterial adherence to various substrates (Arora, Ritchings et al. 1998, Vallet, Olson et al. 2001). As stated only a single gene for the synthesis of either surface structure was increased with aqueous extract treatment. Full production of either structure involved several genes, and therefore it is unlikely that increased expression of only a single gene had a great effect on complete formation of *P. aeruginosa* fimbrae or flagella.

Direct analysis of flagellar swimming motility was measured on 0.3% agarose plates infused with the alcoholic and aqueous ginseng extract. Against PAO1, both compounds were capable of reducing bacterial motility, although the reduction was greater for the alcoholic extract (Figure 1.8 A). Previous studies by Alipour et al (2011) show a reduction in PAO1 motility when exposed to aqueous ginseng extract. Similar reductions in motility with either extract was also observed in the mucoid clinical isolate (Figure 1.8 B), however neither extract caused significant effects towards the motility of

the non-mucoid isolate (Figure 1.8 C). Differences in the effect against the clinical isolates will require further exploration to determine if mucoidy plays a role in the differential outcomes. Regardless, reduced flagellar associated motility at an early stage of infection could greatly impede the advancement of the infection. The potential of these ginseng extracts to disrupt *P. aeruginosa* swimming motility in clinical isolates should be further explored in order to build on the results of Alipour et al (2011) which studied all three forms of motility against PAO1 in the presence of the aqueous extract. Findings from this study revealed decreases in both swimming and swarming motility, but an increase in twitching motility in the presence of the aqueous ginseng extract.

Exoenzymes which act as virulence factors are an important part of *P. aeruginosa's* pathogenicity. Exotoxins S, T, U, and Y are all secreted through type III (Lee, Smith et al. 2005) secretion systems, whereas exotoxin A is secreted through type II systems (Pavlovskis 1972). Multiple genes involved in the type III secretory system were significantly decreased after the 6 h exposure to the aqueous extract (Table 1.2 A). Gene *pscE*, a needle assembly protein was reduced in expression, along with two key translocator protein genes, *popB* and *popA* (Frithz-Lindsten, Holmstrom et al. 1998). Reduction in these genes may be linked to decreases in the expression of ExoS and ExoT found after the 6 h incubation with the aqueous extract.The production and excretion of ExoT and ExoS play a significant role during *P. aeruginosa* infections by disrupting hot cell protein synthesis increasing apoptosis (Barbieri and Sun 2004)and inhibiting phagocytosis (Kaufman, Jia et al. 2000). Decreases in the production of these enzymes with treatment could reduce bacterial virulence during an infection, particularly during early stages when inhibiting phagocytosis is essential in infection establishment.

Rhamnolipids, an additional important virulence factor that was not measured directly from *P. aeruginosa* supernatants, was found to be reduced in expression following the 6 h incubation (Table 1.2 A). Rhamnolipids lyse PMNs (Jensen, Bjarnsholt et al. 2007) and macrophages derived from monocytes through their enzymatic activity (McClure and Schiller 1992).The *rhlA* gene, which was decreased after the 6 h exposure, encodes an essential rhamnosyltransferase (Van Gennip, Christensen et al. 2009). Without expression of this gene, rhamnolipid production does not comense and therefore the protection from PMNs and macrophages is disabled (Van Gennip, Christensen et al. 2009). Combined reductions of rhamnolipids and ExoT and ExoS would create a situation in which *P. aeruginosa* is disabled from protecting its self from the host immune system and subsequent phagocytosis.

Also secreted by *P. aeruginosa* are numerous protease, lipase, and elastase enzymes which act to break down proteins, lipids, and elastin respectively (Van Delden and Iglewski 1998, Wang, Tu et al. 2013). In this study extracellular protease was measured in the supernatant of *P. aeruginosa* cultures grown in the presence of the either ginseng extract. Neither extract at sub-inhibitory concentrations caused a significant effect on protease production of PAO1 (Figure 1.6 A), findings similar to those reported previously by Alipour et al (2011). Utilizing an aqueous extract of *P. ginseng*, Song et al (2010) measured complete attenuation of LasB protease, a protease which specifically breaks down elastin, with treatment at a concentration of 5.0% w/v. It is possible that higher concentrations of the aqueous extract may display inhibitory actions towards LasB protease production in PAO1 as well.

Interestingly, the aqueous extract caused drastic decreases in the protease production of both clinical isolates at sub-inhibitory concentrations (Figure 1.6: B, C).While exposure to the alcoholic extract caused significant increases in protease production at sub-inhibitory concentrations. Such results suggest a fundamental difference in how these extracts are affecting the clinical strains in comparison to the laboratory PAO1 (Figure 1.6 A). Possible differences might arise in the permeability of the clinical isolates in comparison to the laboratory strain which may allow components of either extract past the cellular membrane affecting transport and other processes of protease synthesis. In terms of pathogenicity, the ability of the aqueous extract to reduce the production of proteases in the clinical isolates would decrease protein cleaving resulting in a reduction of host tissue damage. Further exploration into the effect on LasA and LasB elastase production would provide a more unified interpretation of protease function in the presence of these ginseng extracts, as these enzymes work in conjunction with one another to catabolize elastin (Heck, Morihara et al. 1986, Galloway 1991). Decreases in the production of both elastases with aqueous extract treatment could greatly decrease the bacteria's pathogenic capabilities against the host. Previous data collected by Song et al (2010) demonstrated decreases in both enzymes with treatment of the aqueous extract of *P. ginseng* suggesting that LasA may also be decreased with *P .quinquefolius* aqueous extract exposure.

P. aeruginosa also secretes lipases which hydrolyse triacylglycerol (Gupta, Gupta et al. 2004). Lipase production in the presense of the aqueous extract caused significant decreases, while the alcoholic extract caused significant increases in measured lipase for all strains (Figure 1.7). Decreased production of lipases with the aqueous extract

treatment would decrease *P. aeruignosa's* pathogenicity. The production of lipase by PAO1 in the presence of the aqueous was analyzed previously by Alipour et al (2011), who found similar decreases in lipase production. Alipour et al (2011) showed reduced concentrations also decreased lipase production, though to a lesser degree. An additional study monitoring the production lipase enzymes in a larger sample of clinical isolates may reveal that lower concentrations of the aqueous extract, which do not significantly increase growth, can significantly decrease lipase production. To be applicable for use as treatments against *P. aeruginosa* infections, rigorous testing with large populations of clinical isolates will need to be performed to determine the correct concentration at which to utilize these extracts for treatment purposes.

As with the production of excreted protease enzymes, the alcoholic extract caused increased production of the lipase enzymes, especially for the non-mucoid isolate (Figure 1.7 C). Increases in the production of these virulence factors with treatment could greatly increase the severity of the bacterial infection. Therefore, as important as concentration is to treatment efficacy the timing of treatment administration will also play a role in treatment efficacy. Administration early on in infection when virulence factors are being produced in large quantities could further increase the production of such virulence factors leading to an increase in infection progression, while treatment in a later chronic phase may be beneficial, as displayed by the decreased attachment and biofilm disruption caused by the alcoholic ginseng treatment discussed previously.

Virulence factor production is largely under QS control. Previous research conducted by Song et al (2010) demonstrated reduced levels of two acyl-homoserine lactones involved in quorum sensing after exposure to the aqueous extract of *P.ginseng*.

No such reductions in acyl-homoserine lactones were detected in the aqueous extract induced gene expression analysis (Table 1.2); however negative regulator of *lasl* RNA increased in expression after 24 h of incubation . Increases in *rsaL* expression can cause decreases in expression of all LasR PAI-1 related virulence factors (de Kievit, Seed et al. 1999), which include lasA and lasB, alkaline protease (aprA) , as well as exotoxin A (toxA) (Gambello and Iglewski 1991, Gambello, Kaye et al. 1993). No expression decreases in any of the PAI-1 virulence genes listed were found after the 6 or 24 h incubation, which suggests the increase in *rsaL* expression, was not high enough to create a significant effect.

Other secreted factors of *P. aeruginosa* include a variety of pigments. Of these pyoverdine, a fluorescent green pigment, is a molecule involved both in iron aquistion and iron-dependent pathways which affect the production of other virulence factors (Lamont, Beare et al. 2002, Beare, For et al. 2003). Reduced production of pyoverdine decreases the bacteria's ability to sequester iron, a crucial step in colonization and the initial development of infection (Cox and Adams 1985).

Treatment of *P. aeruginosa* with sub-inhibitory concentrations of the aqueous ginseng extract caused significant decreases in pyoverdine production. (Figure1.5). Our data corroborates the findings Alipour et al 2011, who showed lower concentrations still caused significant decreases in pyoverdine levels in PAO1. Diminishing the production and release of pyoverdine could slow the bacteria's ability to sequester iron, leading to reduced growth (Martinez, Delgado-Iribarren et al. 1990). These sub-inhibitory concentrations of aqueous extract increased bacterial growth, however the metabolic growth requirements within a host are limited compared to a monitored lab setting,

especially iron which is bound to iron sequestering molecules and heme proteins as a form of host defence mechanism (Litwin and Calderwood 1993). Therefore, although aqueous extract treatment increases growth rate of the bacteria in the presence of sufficient iron availability, it may act negatively towards the bacteria in an environment with reduced iron where increased pyoverdine expression is necessary for adequate iron acquisition.

Reductions in pyoverdine were also found for PAO1 and the mucoid clinical isolate treated with the alcoholic ginseng extract (Figure 1.5: A, B). Contrastingly, treatment of the non-mucoid isolate caused significant increases in the pyoverdine measured (Figure 1.5 C). Inconsistencies in the outcome of pyoverdine production between the clinical isolates suggest that the mechanism through which this extract affects individual bacterial strains may vary. Different forms of pyoverdine exist due to structural variations with the greatest differences occurring between strains of *P. aeruginosa* (Meyer, Stintzi et al. 1997). Reduction of one particular form of pyoverdine during an interaction with either extract may not correlate with reductions of all forms of the pigment and therefore not all strains may display reduced pyoverdine. Utilizing a larger sample size of clinical and laboratory strains of bacteria would produce data with more significance.

The effects of aqueous ginseng extract on pyoverdine levels are further correlated with the data collected from the microarray analysis. Several genes involved in pyoverdine synthesis were significantly decreased after treatment for 6 h with the aqueous extract (Table 1.2 A). These genes include; *pvdD*, a peptide synthase (Merriman, Merriman et al. 1995), *pvdA*, an L-Ornithine N^5-oxigenase (Visca, Ciervo et

al. 1994), *pvdE, pvdJ*, and *pvdL*, peptide synthases (McMorran, Merriman et al. 1996, Lamont and Martin 2003), *pvdH*, an aminotransferase (Vandenende, Vlasschaert et al. 2004), and *fvdA* a ferric-pyoverdine outer membrane receptor (Poole, Neshat et al. 1993). The products of these genes are synthesized without the utilization of ribosomes as demonstrated by enrichment of the biosynthetic pathway for siderophore group nonribosomal peptides after the 6 h aqueous extract incubation (Table 1.4 A).

Several genes involved in the synthesis of pyochelin, another siderophore pigment of *P. aeruginosa*, were also reduced after the 6 h aqueous extract treatment (Table 1.2 A). Genes *pchA* and *pchB* encode isocrosimate synthase (Serino, Reimmann et al. 1995) and isochorismate pyruvate –lyase/chrosimate mutase respectively (Gaille, Kast et al. 2002). These genes are essential for the production of salicylate, a precursor of pyochelin (Serino, Reimmann et al. 1995). The gene *pchC* product is a thioesterase, which aids in the optimal production of pyochelin (Reimmann, Patel et al. 2004). The surface pyochelin receptor encoded by the *fptA* gene was also reduced in expression (Heinrichs and Poole 1996). Decreased expression of these essential pyochelin biosynthesis genes after aqueous extract exposure suggests similar decreases in the direct measurement pyochelin would likely be found. After 24 h of incubation with the aqueous extract the ftpA gene expression increased, which may reflect a time dependent suppression of pyochelin with the extract exposure (Table 1.2 B).

Additionally, after the 24 h incubation period with the aqueous extract, three genes, *iscS*, *iscA*, and *iscU,* expression increased. These encode cysteine desulfurase and two scaffold proteins, which are required during the formation of Fe-S clusters (Mihara and Esaki 2002). The expression of ferredoxin, *fdx2*, a protein which binds iron and

sulphur (2Fe-2S) was also increased (Mihara and Esaki 2002). Two heat shock proteins (*hscA/hscB*) which act as chaperons to iscA/iscU during iron-sulphur cluster formation also increased (Vickery and Cupp-Vickery 2007). Iron-sulphur clusters are used in the synthesis of various important biological molecules such as biotin and thiamine (Birch, Fuhrmann et al. 1995, Lauhon and Kambampati 2000). Increased expression in biosynthesis genes involved in Fe-S cluster formation paired with decreases in siderophore biosynthesis genes suggests changes to iron availability and use. Increases in Fe-S clustering (Table 1.2B, 1.3B) may indicate compensation by the bacteria in order to continue regular metabolic functioning with decreased iron acquisition abilities after exposure to the aqueous extract.

In addition to the siderophore pigments *P. aeruginosa* also produces pyocyanin, a phenazine pigment which acts as a virulence factor for the bacteria causing host immune-modulation and tissue damage due to enhanced production of ROS (Wilson, Sykes et al. 1988, Lau, Hassett et al. 2004). Expression analysis suggests decreases in the production of this pigment after 6 h exposure to the aqueous extract with decreased expression of 9 phenazine biosynthesis genes; *phzG2*, *phzF1/phzF2*, *phzE1/phzE2*, *phzC1/phzC2*, and *phzB1/phzB2* (Table 1.2 A). These genes belong to the *phzA1B1C1D1E1F1G1/phzA2B2C2D2E2F2G2* operons which produce the compound phenazine-1-carboxylic acid, an essential pyocyanin precursor (Mavrodi, Bonsall et al. 2001). Therefore reduction in the aforementioned genes could lead to a reduction in pyocyanin. A previous study demonstrated a reduction of pyocyanin with aqueous ginseng extract treatment against PAO1 (Alipour et al 2011), however no evidence has been produced for clinical isolates or for the effect with alcoholic ginseng treatment.

The final major alterations to *P. aeruginosa* after exposure to the aqueous extract involved ribosomal structure assembly and function. After the 24 h incubation with the aqueous extract, 10 genes involved in 50S ribosomal structure and 7 genes involved in 30S ribosomal structure were significantly increased (Table 1.2 B). Genes for a ribonuclease protein (*rnpA*), a transcription terminator (*rho*), and a ribosome modulation factor gene (*rmf*) were similarly expressed at elevated levels. These findings were reflected in the GO- and pathway enrichment analysis (Tables 1.3 B, 1.4 B). GO-enrichment presented enrichment of genes involved in structural constituents of ribosomes, RNA binding, translation, regulation of translation, DNA templated transcription, termination, and nucleic acid binding. Pathways analysis suggested enrichment of the ribosome pathway with a score of 31.24 and ~32% of the genes in the pathway affected.

Increases in ribosomal assembly, and subsequent translation and transcription suggest increased turnover of proteins. Such changes normally occur when bacteria enter the transition from exponential to stationary growth (Kolter, Siegele et al. 1993). As previously discussed evidence of a shift in metabolism was evident in the expression analysis between the 6 h and 24 h incubation, which would involve large changes in protein requirements. At 24 h, the aqueous extract treatment stimulated significantly increased growth compared to control, which could also contribute to the increases in protein turnover emphasized in the analysis of the 24 h aqueous incubation treatments.

In conclusion, the treatment of *P. aeruginosa* with the aqueous extract increased growth rates and displayed a shift to anaerobic metabolism state during the exponential growth phase. Increased growth was accompanied by increased attachment of the bacteria

in the presence of the aqueous extract. However, treatment of preformed biofilms with the aqueous extract diminished bacterial adherence which could greatly reduce the bacteria's virulence in chronic infections. Aqueous extract exposure also reduced ExoT and ExoS, rhamnolipid, and lipase expression, as well as LasB protease production in both clinical strains. Increased expression of a *lasl* negative regulator and decreased expression of type III secretion system genes were revealed through the microarray analysis. Significant reduction in both pyoverdine and pyochelin synthesis were resulted from aqueous extract treatment. Evidence for the reduction in pyocyanin synthesis was also shown through the microarray analysis. Motility of PAO1 was negatively impacted by the presence of the aqueous extract, while results for the clinical isolates were inconclusive. Incubation with the aqueous extract for 24 h caused significant up-regulation of ribosomal pathways when compared to the control, causes may include increased growth rate, transition to stationary growth phase, and interactions of the extract and the bacteria which are presently unknown.

Results from the treatment of the *P. aeruginosa* with the alcoholic extract lead to mixed results regarding growth, with minimal decreases in growth rate observed. Reduced growth may partially account for the decreased adherence of the clinical isolates in the presence of the alcoholic extract, as well as the reduced biofilm adherence revealed when 48 h old clinical isolate biofilms were treated with the extract. Treatment with the alcoholic extract increased protease production in the clinical strains, while not affecting PAO1. Increases in lipase production were found for all strains. Both pyoverdine secretion and bacterial swimming motility by *P. aeruginosa* after alcohol extract exposure showed strain specific results.

In general, it seems that the aqueous extract is the more promising extract of ginseng for further development and research. Continued exploration into the components of either extract may allow for use of specific extract components as targeted therapies. Concentrations used in this study were too high for use in a clinical application, however further research into the may reveal potent components with beneficial attributes which are effective at lower concentrations. The use of ginseng for medicinal purposes may still provide an economical and safe choice for *P. aeruginosa* infection therapy.

Chapter III. The Effect of Thymol or Carvacrol on *P. aeruginosa* Pathogenicity.

3.1 Introduction

3.1.1 Sources of Thymol and Carvacrol

Thymol and carvacrol, phenolic compound isomers (Figure 2.1), represent the main constituents of the essential oil of herbaceous plants thyme and oregano, respectively (Burt 2004, Nostro, Sudano Roccaro et al. 2007). Both plants also contain smaller percentages of the other isomer (Bagamboula, Uyttendaele et al. 2004, Burt 2004). Concentrations of each isomer vary depending on the species, climate, process of extraction, and seasonal variations (Santoyo, Cavero et al. 2006, Oussalah, Caillet et al. 2007). Distillation and purification of thymol and carvacrol can be achieved directly from plant essential oil or a purified form can be purchased from various chemical manufacturing companies (Sigma Aldrich, Fisher Scientific).

3.1.2 Chemical Structure and Function

Thymol and carvacrol are monoterpenoid compounds (Trombetta, Castelli et al. 2005), with a structure of a single phenolic ring formed from the bonding of two isoprene molecules with three functional group substituents (Figure 2.1). In both structures the hydroxyl group is attached at the C-1 position. In thymol (a) the methyl group is attached at the C-2 position and the isopropyl group is attached at the C-5 position, while in carvacrol (b) the methyl group is attached at the C-5 position and the isopropyl group is attached at the C-2 position.

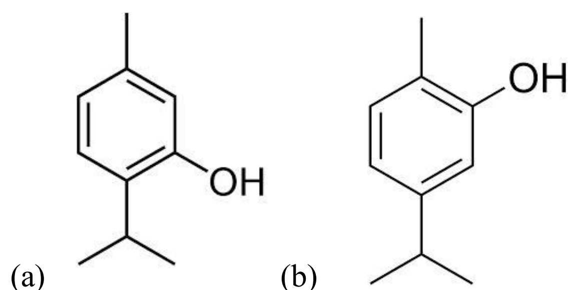

Figure 2.1: **Chemical structure of thymol (a) and carvacrol (b)**

These compounds are produced as part of the plant secondary metabolism (Oussalah, Caillet et al. 2007). Typically these compounds concentrate in specific organs such as the leaves and fruit, with the amount and composition differing between organs (Oussalah, Caillet et al. 2007). The function of these compounds within the plants is mainly as defense mechanisms against bacterial, viral, and fungal infections (Regnault-Roger, Hamraoui et al. 1993). High concentrations of these compounds may also act as deterrents to herbivores due to their strong flavour and aroma (Trombetta, Castelli et al. 2005). Most of the antibacterial action of thymol and carvacrol essential oil is imparted by thymol and carvacrol, which are two of the most effective antimicrobial plant compounds known today (Sivropoulou, Papanikolaou et al. 1996, Nevas, Korhonen et al. 2004).

3.1.2 Antimicrobial Properties and Mechanisms

Growth Inhibition

Thymol and carvacrol have both been shown to be potent antibiotic agents against a wide range of bacteria (Helander, Alakomi et al. 1998, Walsh, Maillard et al. 2003, Oussalah, Caillet et al. 2007). Treatment with either compound has been shown to inhibit

the growth of pathogenic bacteria such as *Staphlococcus aureus*, *Esherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes*, and *Shigella sonnei* (Helander, Alakomi et al. 1998, Bagamboula, Uyttendaele et al. 2004, Trombetta, Castelli et al. 2005, Oussalah, Caillet et al. 2007). Concentrations below the MIC can reduce the growth rate of the bacterial species, even those which were not completly attenuated by higher concentrations such as *P. aeruginosa* (Walsh, Maillard et al. 2003, Nazer, Kobilinsky et al. 2005, Oussalah, Caillet et al. 2007).

The mechanism through which these compounds inhibit and reduce growth is attributed to their effects on the bacterial cell membrane, as both thymol and carvacrol are potent outer membrane damaging compounds (Helander, Alakomi et al. 1998). Due to the hydrophobic nature of thymol and carvacrol, these compounds have the ability to integrate into the bacterial cell membranes, causing disruption and disturbance to normal membrane function (Lambert, Skandamis et al. 2001, Gill and Holley 2006) leading to increased permeability of ATP and an increased release of components from within the cells (Helander, Alakomi et al. 1998, Walsh, Maillard et al. 2003). The ability to disrupt bacterial cell membranes causing cell instability and lysis is a recognized mechanism through which phenolic compounds act as antimicrobial agents (Lambert and Hammond 1973).

The ability of phenolic compounds to penetrate through bacterial membranes is highly dependent on multiple factors such as: net charge of the membrane surface, the ability to travel through aqueous medium, lipid composition of the membrane, and the presence and concentration of lipopolysaccharide (LPS) (Trombetta, Castelli et al. 2005). Although increasing membrane permeability has been shown to be a main mechanism of

thymol and carvacrol action, the ability of these compounds to pass into the cells suggests intracellular targets may also be linked to the antimicrobial properties (Denyer and Stewart 1998, Ultee, Kets et al. 1999, Trombetta, Saija et al. 2002). Further exploration into the interactions of thymol and carvacrol with intracellular bodies may reveal novel mechanisms through which these compounds act as antimicrobials.

Biofilm Reduction

When bacteria adopt a biofilm mode of growth an increase in resistance towards antibiotics and host defense mechanisms is found (Nickel, Ruseska et al. 1985, O'Toole, Gibbs et al. 2000, Walters, Roe et al. 2003, Lewis 2005). Treatments which can decrease this resistance or continue to be effective against biofilm bacteria would greatly improve treatment of chronic state infections. Using *Staphylococcus aureus* and *Staphlococcus epidermis* as model organisms, the ability of these bacteria to form biofilms in the presence of thymol or carvacrol, as well as the effect of either treatment on established biofilms was monitored (Nostro, Sudano Roccaro et al. 2007). Bacteria grown in the presence of subinhibitory concentrations of the two compounds displayed a reduced capability to form biofilms, with a greater effect observed at higher concentrations (Nostro, Sudano Roccaro et al. 2007). Decreasing established biofilms through treatment with thymol or carvacrol has the potential to increase the efficacy of current antibiotics. Future research with gram-negative species, such as *P. aeruginosa* would improve our understanding of thymol and carvacrol effects on bacterial biofilms.

3.1.3 Current Development of Thymol and Carvacrol Uses

Although interest in thymol and carvacrol as antimicrobial agents has heightened recently, both compounds have been used commercially for many years. Their potent fragrance and flavour have made thymol and carvacrol common food and beverage additives (Cowan 1999, Oussalah, Caillet et al. 2007). The food production industry is one of the largest industries in the world. Every year food spoilage due to fungal, viral, and bacterial infection leads to large economic losses for the industry, but more importantly leads to thousands of cases of food borne illness worldwide (Burt 2004). Natural products which could be added directly to food or into the packing of food products are appealing options for methods of reducing food spoilage and potential food borne illness (Nazer, Kobilinsky et al. 2005). Research continues into the possibility of directly applying thymol/carvacrol to various food products, or incorporating either compound into plastic packaging to deter the growth of mircoorganisms and to further preserve perishable products (Bagamboula, Uyttendaele et al. 2004, Nazer, Kobilinsky et al. 2005, Castillo, Perez-Alfonso et al. 2014).

Although commercially available antibiotics based on thymol or carvacrol has not yet been developed, health products have incorporated thymol into their formulations. Thymol has been used in dental applications such as mouthwash and dental sealers for years (Marsh 1992, Cowan 1999, Ouhayoun 2003). Incorporation of thymol into these medicinal products was based on research which revealed the ability of this compound to inhibit the growth of common oral pathogens (Botelho, Bezerra Filho et al. 2007) Continued investigation into the the antibacterial properties of thymol and/or carvacrol has the potential for further incorporation into future medical and health related products.

3.1.4 Rationale for Further Exploration of the Antibacterial Properties of Thymol and Carvacrol

. In this study, both thymol and carvacrol were assessed in their abilities to inhibit *P. aeruginosa* growth, and also their effects on the production of bacterial virulence factors. The ability of these compounds to disrupt biofilm formation and inhibit bacterial adherence were also monitored. Finally, to benefit our understanding of drug interactions with bacterial expression, microarray analysis of *P. aeruginosa* in the presences of either compound was also analyzed.

3.2 Methodology

3.2.1 Bacterial Strains and Media

The same bacterial strains noted in Chapter 2 were used for all experiments in Chapter 3. Media composition and bacterial sample storage were prepared and performed as outlined in Chapter 2.

3.2.2 Compound Solution Preparation

Both thymol and carvacrol (Sigma-Aldrich) were dissolved into absolute ethanol (Sigma-Aldrich) prior todilution into either media. For all experiments stock solutions of either compound were made at 200x the desired test concentration and diluted into media, so that the final testing solutions was ≤0.9% (v/v) absolute ethanol, which showed no significant effect on growth rate of the bacteria (data not shown). Solutions were filter sterilized with a 0.2 µ syringe filter and stored at 4 ˚C until needed or used immediately.

3.2.3 Minimum Inhibitory Concentration Determination

Minimum inhibitory concentration (MIC) was determined as specified in Chapter 2 with the following changes. Thymol and Carvacrol were originally tested against all three strains of bacteria at concentration dilutions from 2000 µg/ml – 200 µg/ml. Final results were taken from the treatment of PAO1 and the mucoid clinical isolate with 2000 µg/ml-200 µg/ml and the non-mucoid isolate with 1000 µg/ml-100 µg/ml.

3.2.4 Growth Curve Analysis

Growth curve analysis was monitored as specified in Chapter 2 with the following additions. Flasks containing 0.45% (v/v) absolute ethanol in M9 media was prepared as vehicle controls.

3.2.5 Bacterial Adherence Assays

Attachment assays were performed as detailed in Chapter 2 with the following addition. Triplicate vehicle control wells were plated containing 0.45% (v/v) absolute ethanol for each trial in both assays.

3.2.6 Virulence Factor Production

Virulence factor production was measured as outlined in Chapter 2 with minor addition of a vehicle control flasks containing M9 media supplemented with 0.45% (v/v) abolsute ethanol.

3.2.7 Motility Assays

Motility experiments were performed as stated in Chapter 2 with the addition of plates containing 0.45% (v/v) absolute ethanol produced as vehicle controls.

3.2.8 Genomic Expression Analysis

Expression analysis was performed and analyzed as outlined in Chapter 2 with the following exemptions. Thymol at a 0.5 MIC concentration was repeated for 3 trials at both 6 h and 24 h of incubation. Carvacrol treatment was only repeated twice at 0.5 MIC concentration for 24 h.

3.2.9 Microarray Processing

Microarray processing and data analysis was completed as specified in Chapter 2.

3.2.10 Statistics

Statistical analysis was performed as outlined in Chapter 2 with the additions of vehicle control data compared to untreated controls to determine the presence of statistical differences.

3.3 Results

3.3.1 Minimum Inhibitory Concentration of Thymol or Carvacrol

Through microdilutions of both thymol and carvacrol and visual growth assessment, the MIC concentration of either compound was determined. Both compounds were unable to inhibit growth of PAO1 up to concentrations of 2000 μg/ml in the M9 media (Table 2.1B). For both compounds in the M9 media, the mucoid clinical isolate showed greater tolerance with MIC values higher than that of the non-mucoid isolate. The mucoid isolate had MIC values of 1200 μg/ml and 1000 μg/ml for thymol and carvacrol respectively (Table 2.1B). The non-mucoid isolate gave MIC values of 600 μg/ml and 800 μg/ml for thymol and carvacrol, respectively, in the M9 media (Table 2.1 B). Treatment in the MHCA media reduced MIC values for all strains, however general trends between strains remained (Table 2.1A).

Table 2.1: Minimum inhibitory concentrations (MIC) of *P. aeruginosa* treated with thymol or carvacrol. MIC values were determined through microdilution series of either extract in 96 well plates, denoting the MIC value by visual growth assessment. Experiments were repeated three times (n=3) in duplicate in either both Mueller-Hinton cation adjusted broth (MHCA) (Table 2.1 A) and M9 supplemented media (Table 2.1 B)

Table 2.1A Minimum inhibitory concentration of thymol or carvacrol in MHCA media

Strain	Thymol (μg/ml)	Carvacrol (μg/ml)
PAO1	1200-1050	1200-1050
Mucoid Isolate	150	450
Non-mucoid Isolate	150	300

Table 2.1 B Minimum inhibitory concentration of thymol or carvacrol in M9 media

Strain	Thymol (μg/ml)	Carvacrol (μg/ml)
PAO1	>2000	>2000
Mucoid Isolate	1200	1000
Non-mucoid Isolate	600	800

3.3.2 Effect of Thymol or Carvacrol Treatment on Bacterial Growth

Growth of *P. aeruginosa* in the presence of either thymol or carvacrol was assessed for 48 h and no statistically significant influence on growth rate were found for either treatment on any of the strains tested (Figure 2.2, 2.3).

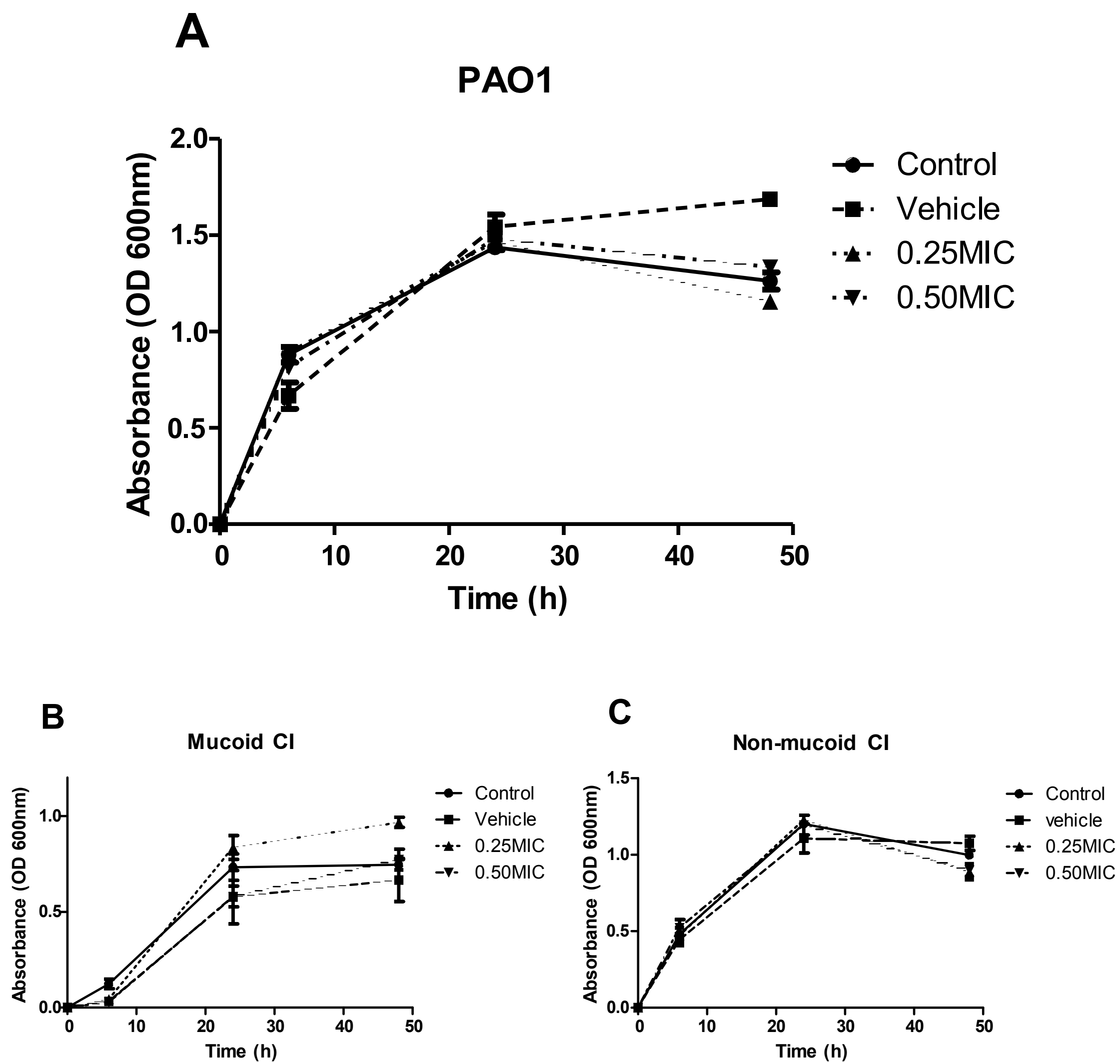

Figure 2.2: Growth curve analysis in the presence of the sub-inhibitory concentrations of thymol. Growth in the presence of sub-inhibitory concentrations of thymol was assessed for 48 h at 37 ˚C with 150 rpm shaking. After 0, 6, 24, and 48 h of incubation, 1ml of culture was removed and the absorbance at 600 nm was measured. All values were normalized to the time 0 absorbance. Experiments were repeated three times (n=3) and data is represented as an average ± SEM. Statistical comparisons to the untreated control for each treatment was done through a repeated-measures ANOVA and subsequent Tukey's post-hoc test.

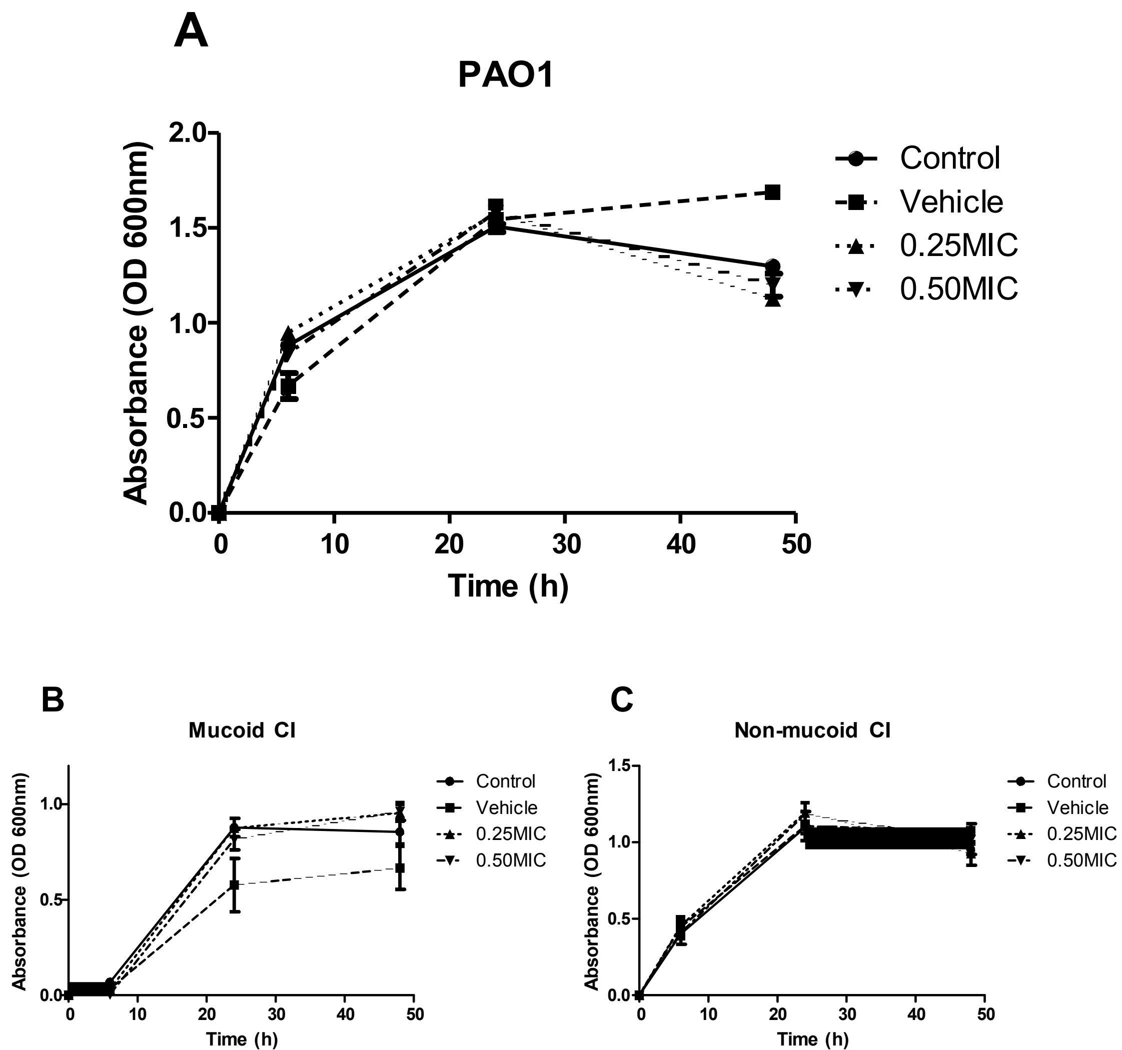

Figure 2.3: Growth curve analysis in the presence of sub-inhibitory oncentrations of carvacrol. Growth in the presence of sub-inhibitory concentrations of carvacrol was assessed for 48 h at 37 ˚C with 150 rpm shaking. After 0, 6, 24, and 48 h of incubation, 1 ml of culture was removed and the absorbance at 600 nm was measured. All values were normalized to the time 0 absorbance. Experiments were repeated three times (n=3) and data is represented as an average ± SEM. Statistical comparisons to the untreated control for each treatment was done through a repeated-measures ANOVA and subsequent Tukey's post-hoc test.

3.3.3 Effect of Thymol or Carvacrol Treatment on Preventing Bacterial Attachment

The assessment of bacterial adherence in the presence of either thymol or carvacrol was measured by crystal violet staining of the adhered bacteria. For both PAO1 and the non-mucoid clinical isolates, no significant differences in adherence were measured under any treatment (Figure 2.4: A, C). No significant differences were measured between bacteria grown in the untreated controls or ethanol vehicle controls for either strain as well. A significant reduction in bacterial adherence was measured for the mucoid clinical isolate challenged with carvacrol at either concentration (Figure 2.4: B), while a significance increase was found with the ethanol vehicle alone.

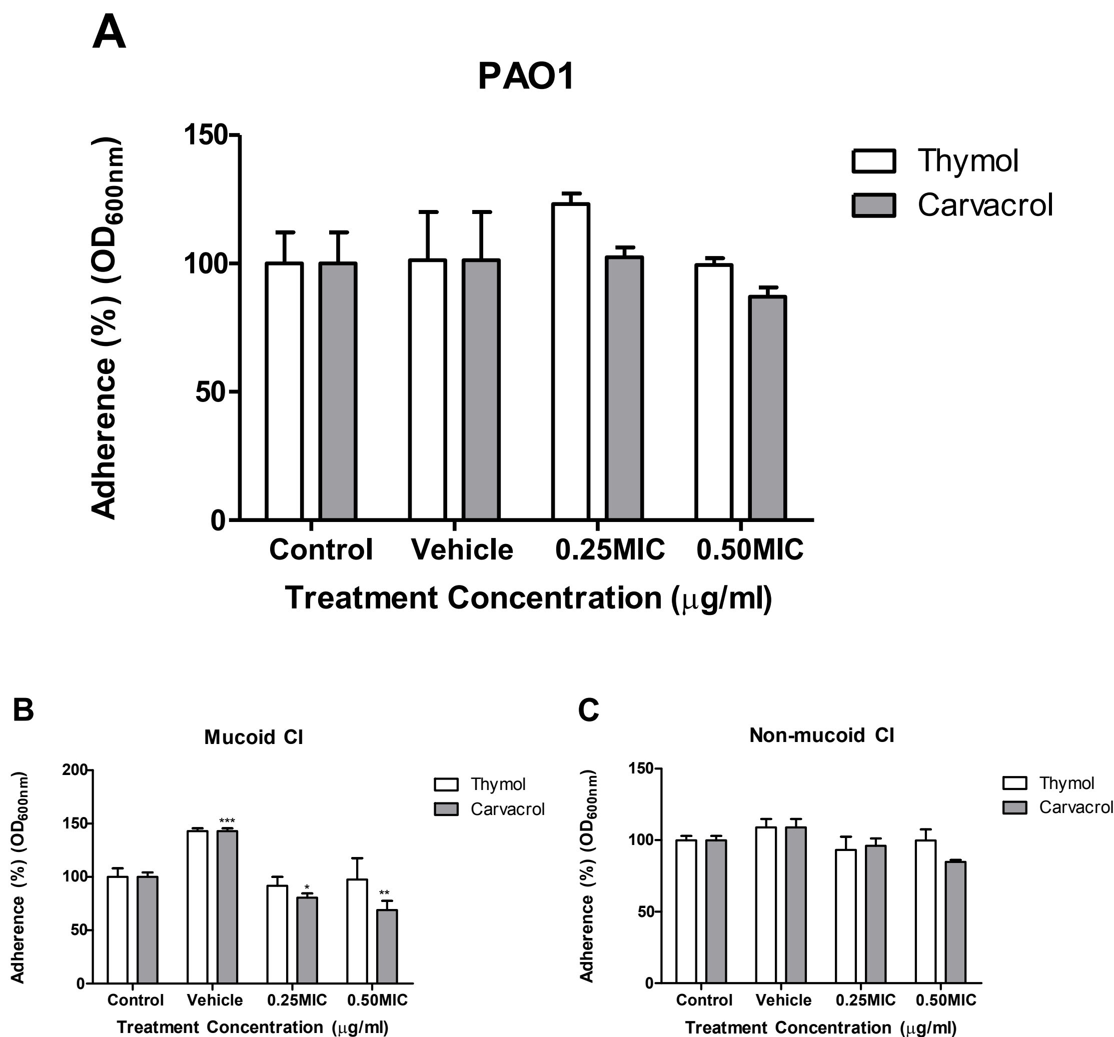

Figure 2.4: The effect of thymol and carvacrol treatment on the prevention of *P. aeruginosa* adherence. Bacteria were grown in the presence of 0.25 MIC and 0.50 MIC concentrations of either compound in 96 well flat bottom plates for 24 h at 37 ˚C. Post incubation, plates were washed with distilled water and adhered bacteria were stained with a 0.1% crystal violet stain and solubilized stain was measured at an absorbance of 600 nm. Average adherence of the ethanol vehicle is also shown as a percentage of the untreated control ± SEM (n=3). Statistical comparisons to the untreated control for each treatment were performed through an ANOVA analysis and Tukey's post hoc test (p-value < 0.05=*, p-value < 0.01=**).

3.3.4 Effect of Thymol or Carvacrol Treatment on Preformed Biofilms

The treatment of 48 h old biofilms with thymol or carvacrol at 1.0 MIC and 2.0 MIC concentrations was assessed and measured through crystal violet stain absorbance at 600 nm. Treatment of the mucoid clinical isolate with either compound at both concentrations caused significant decreases in biofilm adherence (Figure 2.5: B). The increased biofilm adherence of the mucoid isolate ethanol vehicle control was again observed (Figure 2.5: B). Both the laboratory PAO1 and non-mucoid strain showed no significant changes with either treatment (Figure 2.5: A, C), as well as no differences between the vehicle control and untreated control.

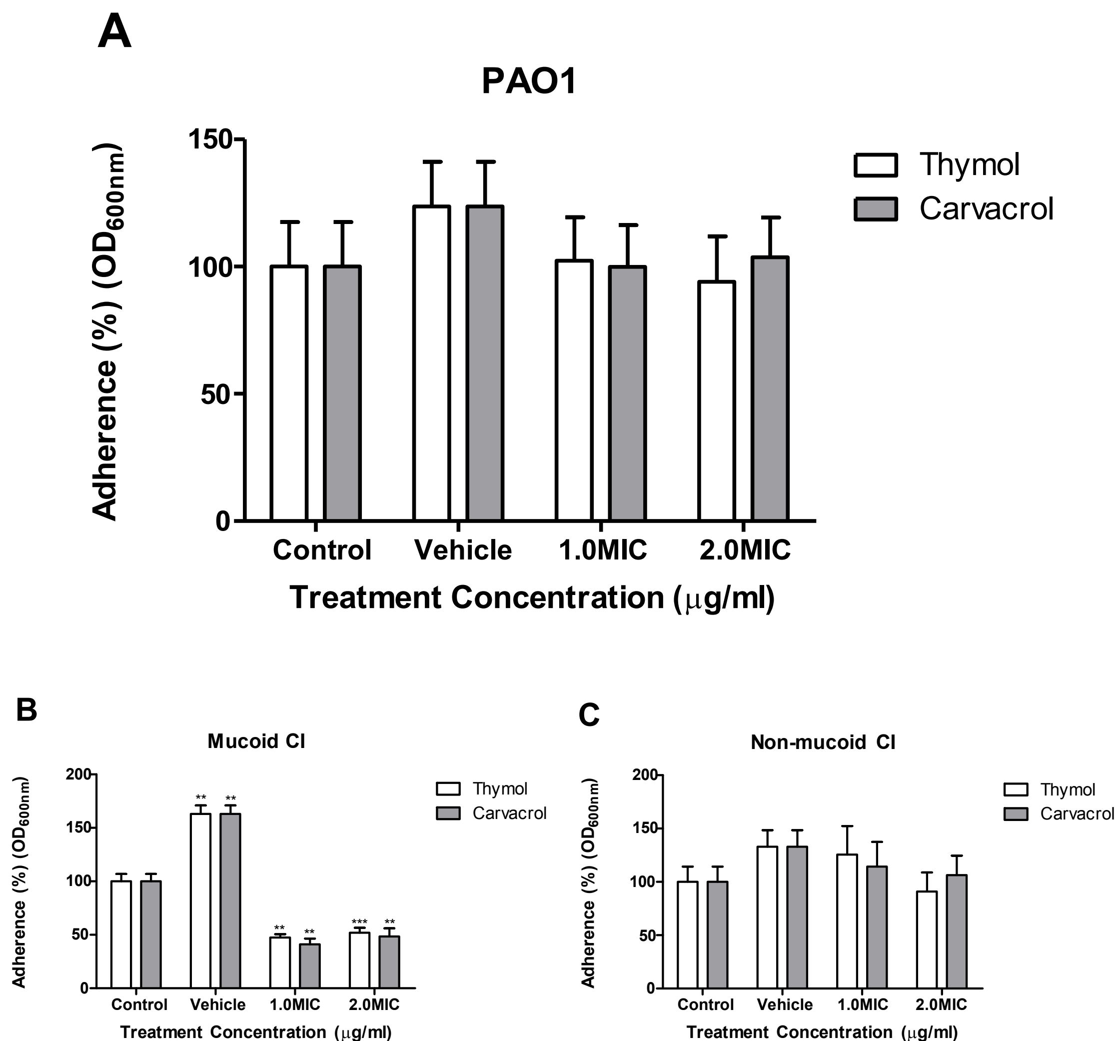

Figure 2.5: The effect of thymol and carvacrol treatment on 48 h *P. aeruginosa* biofilms. Bacteria were allowed to adhere to a 96 well flat bottom plates for 48 h at 37 ˚C. Post incubation, wells were washed with distilled water and bacteria were treated with either compound at 1.0 MIC and 2.0 MIC concentrations for 24 h at 37 ˚C. After treatment, wells were washed with distilled water and adhered bacteria were stained with a 0.1% crystal violet stain and solubilized stain was measured at an absorbance of 600 nm. Experiments were repeated three times in triplicate and expressed as an average percentage of the untreated control ± SEM. Average adherence of the ethanol vehicle is also displayed as a percentage of the untreated control ± SEM (n=3). Statistical comparisons to the untreated control for each treatment was done through a one-way ANOVA and subsequent Tukey's post-hoc test (p-value < 0.05= *, p-value < 0.01= **, p-value < 0.001= ***).

3.3.5 Effect of Thymol or Carvacrol Treatment on *P. aeruginosa* Pigment Production

Pyoverdine production by *P. aeruginosa* was measured by an absorbance reading of the supernatant of cultures grown in the presence of either compound at sub-inhibitory concentrations. Either treatment had very minimal effects towards the production of pyoverdine in PAO1 (Figure 2.6: A). The mucoid isolate displayed significant decreases in pyoverdine measurement with treatment of either compounds at both concentrations (Figure 2.6: B). Like the PAO1 strain, no difference between untreated controls and vehicle controls were found for the mucoid isolate. In contrast, the non-mucoid isolate displayed decreases in pyoverdine with the vehicle control and treatment of either compound at 0.50 MIC concentrations (Figure 2.6: C). Treatment of the non-mucoid strain with 0.25 MIC concentrations caused a significant decrease in pyoverdine with thymol exposure, but had no significant effect with carvacrol exposure (Figure 2.6: C).

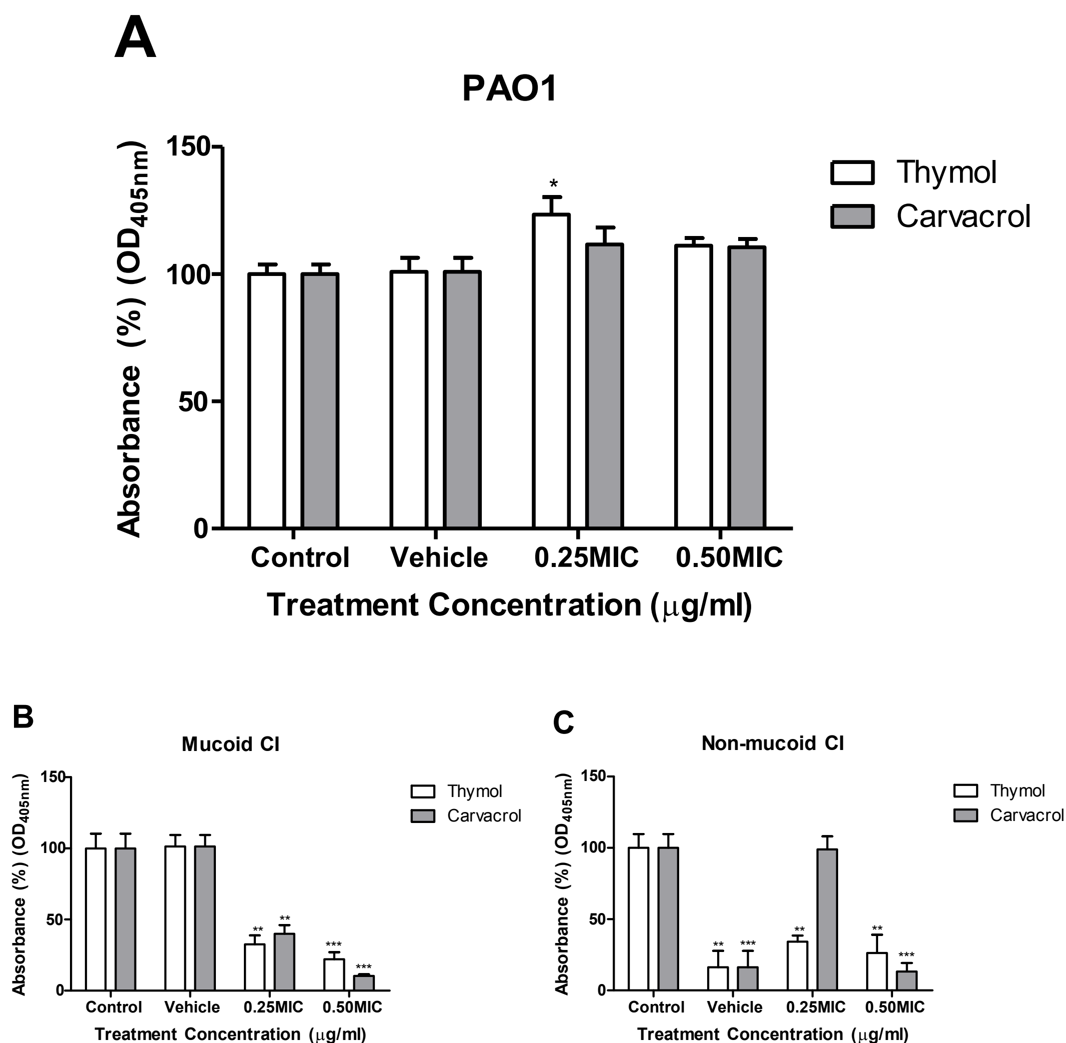

Figure 2.6: The effect of thymol and carvacrol treatment on the production of the pigment pyoverdine. Bacterial cultures were prepared and incubated in the presence of 0.25 MIC and 0.5 MIC concentrations of either compound for 24 h at 37 ˚C & 150rpm. Cultures were then centrifuged to remove cells, filtered, and 1 ml of supernatant was assayed in a spectrometer at 405 nm. Data represents averaged mean values of three independent experiments preformed in duplicate, and expressed as a percentage of untreated controls ± SEM. Average absorbance of the ethanol vehicle is also displayed as a percentage of the untreated control ± SEM (n=3). Statistical significance was determined through one-way ANOVA analysis followed by Tukey's post-hoc test to determine significance relative to the untreated controls (p-value <0.05= *, p-value < 0.01= **, and p-value < 0.01= ***).

3.3.6 Outcome of Thymol or Carvacrol Treatment on Bacterial Lipase and Protease Production

Milk agar plate hydrolysis was used to measure the production and secretion of protease in cultures grown in the presence of either thymol or carvacrol. Significant decreases in protease measurement were found for PAO1 when grown in the presence of either compound at a 0.5 MIC concentration (Figure 2.7: A). Less definitive results were found for the mucoid clinical isolate in which neither treatment had a significant effect on the protease production (Figure 2.7: B). The greatest effect of treatment was found towards the non-mucoid strain, for which both the vehicle control and 0.50 MIC concentration of either compound reduced protease production to a level below measurement (Figure 2.7: C).

Lipase Tween 20 hydrolysis assays was used to measure the production of such enzyme under the influence of thymol or carvacrol treatment. Treatment of PAO1 with either compound at sub-inhibitory concentrations caused significant increases in the lipase production (Figure 2.8: A). In contrast, reductions in lipase were found for the mucoid clinical isolate when subjected to either treatment at both concentrations, as well as with the vehicle control (Figure 2.8: B). Results for the non-mucoid isolate displayed decreases with vehicle control for both treatments, increases with carvacrol treatment at 0.25 MIC concentration, and decreases with carvacrol and thymol exposure at 0.50 MIC and 0.25 MIC concentrations respectively (Figure 2.8: C).

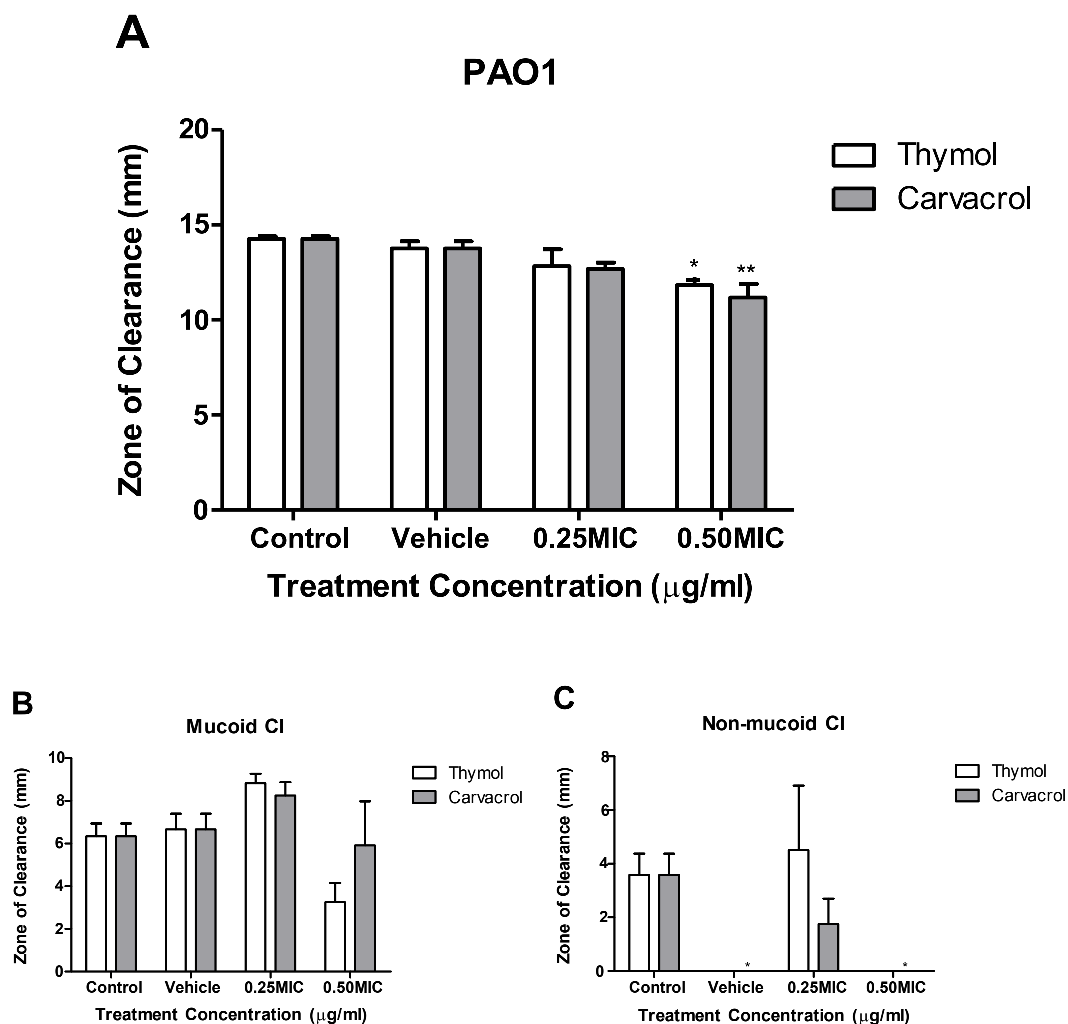

Figure 2.7: Protease production in the presence of thymol or carvacrol. Bacterial cultures were prepared and incubated in the presence of 0.25 MIC and 0.5 MIC concentrations of either compound for 24 h at 37 ˚C & 150 rpm. Cultures were then centrifuged to remove cells and remaining supernatants were filter sterilized. To a single center well of a 1% skim milk plate (1.5% agar), 50 µl of filtered supernatant was added and plates were incubated at 37 ˚C for 24 h. The zone of clearance around the inoculation well was measured with a ruler (mm). Data is represented as the average of three separate experiments conducted in duplicate ± SEM. Average zone of clearance of the ethanol vehicle is also displayed as a percentage of the untreated control ± SEM (n=3). Statistical significance was determined through one-way ANOVA analysis followed by Tukey's post-hoc test to determine significance relative to the untreated controls (p-value<0.05= * and p-value < 0.01= **).

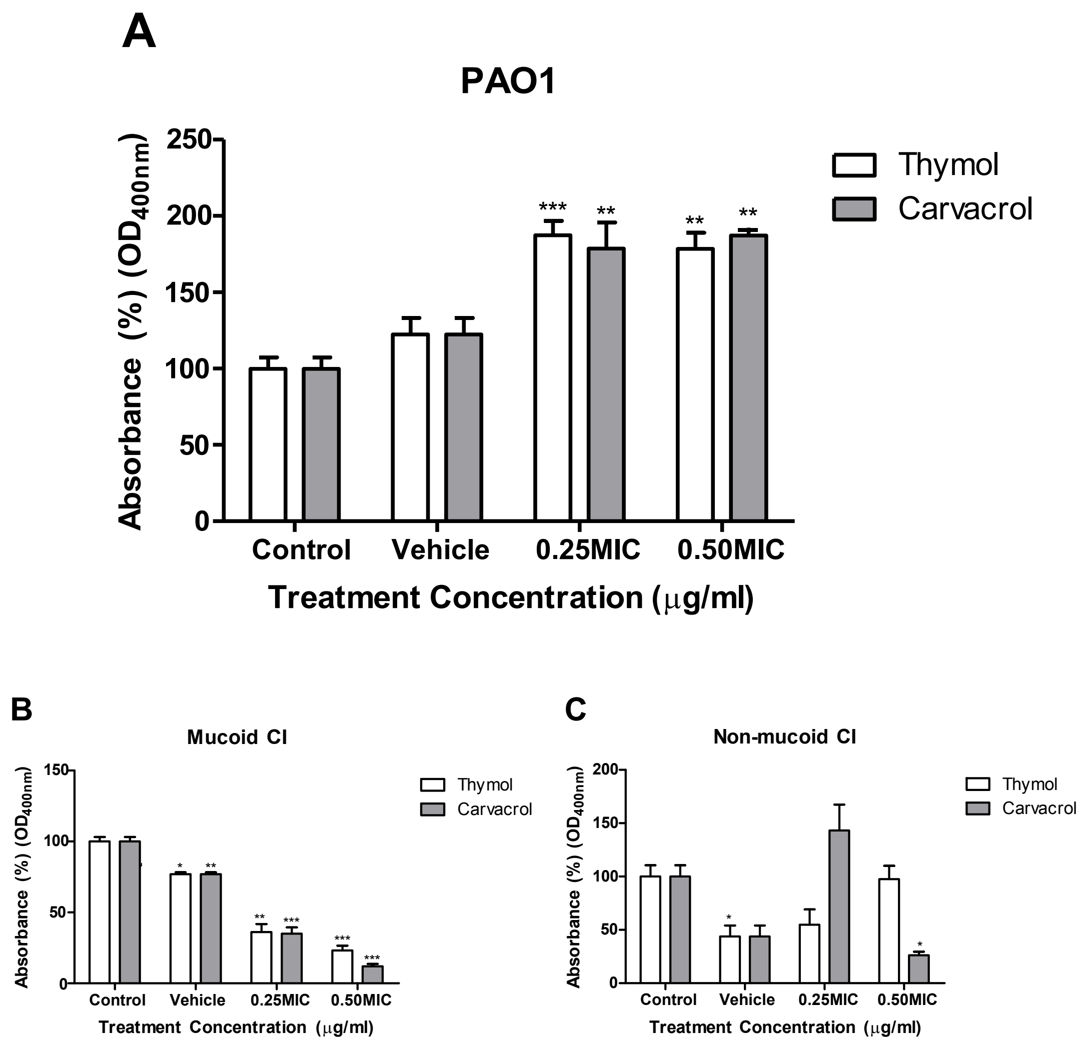

Figure 2.8: Lipase production in the presence of thymol or carvacrol. Bacterial cultures were prepared and incubated in the presence of 0.25 MIC and 0.5 MIC concentrations of either compound for 24 h at 37 ˚C & 150 rpm. Cultures were then centrifuged to remove cells and remaining supernatants were filter sterilized. To a glass test tube, 2.3 ml of 0.50 M tris buffer (pH 7.6), 0.1 ml 0.1 M $CaCl_2$ in tris buffer (pH 7.6), 0.1 ml tween 20 in tris buffer (pH 7.6), and 0.5 ml filtered supernatant were combined and incubated at 37 ˚C & 150 rpm for 4 h. After incubation, 1 ml was removed and measured in a spectrophotometer at 400 nm. Data represents the mean value of three independent experiments preformed in duplicate and expressed as a percentage of the control ± SEM. Average absorbance of the ethanol vehicle is also displayed as a percentage of the untreated control ± SEM (n=3). Statistical significance was determined through one-way ANOVA analysis and Tukey's post hoc test (p-value < 0.05=*, p-value < 0.01=**, p-value < 0.001=***).

3.3.7 Influence of Thymol or Carvacrol Treatment on *P. aeruginosa* Motility

The addition of either compound to media plates containing 0.3% agarose was used to determine the motility of bacteria in the presence of thymol or carvacrol. Significant decreases in motility with either treatment at sub-inhibitory concentrations, and with the vehicle control, were observed with PAO1 (Figure 2.9: A). Interestingly, the vehicle control did not cause statistically significant decreases in swimming motility for either clinical isolate. Significant decreases in motility with treatment of either thymol or carvacrol at a 0.50 MIC concentration was found for both clinical strains (Figure 2.9: B, C). At the 0.25 MIC concentration thymol significantly decreases motility in the mucoid isolate, while carvacrol at the same concentration caused decreases in the non-mucoid isolate (Figure 2.9: B, C).

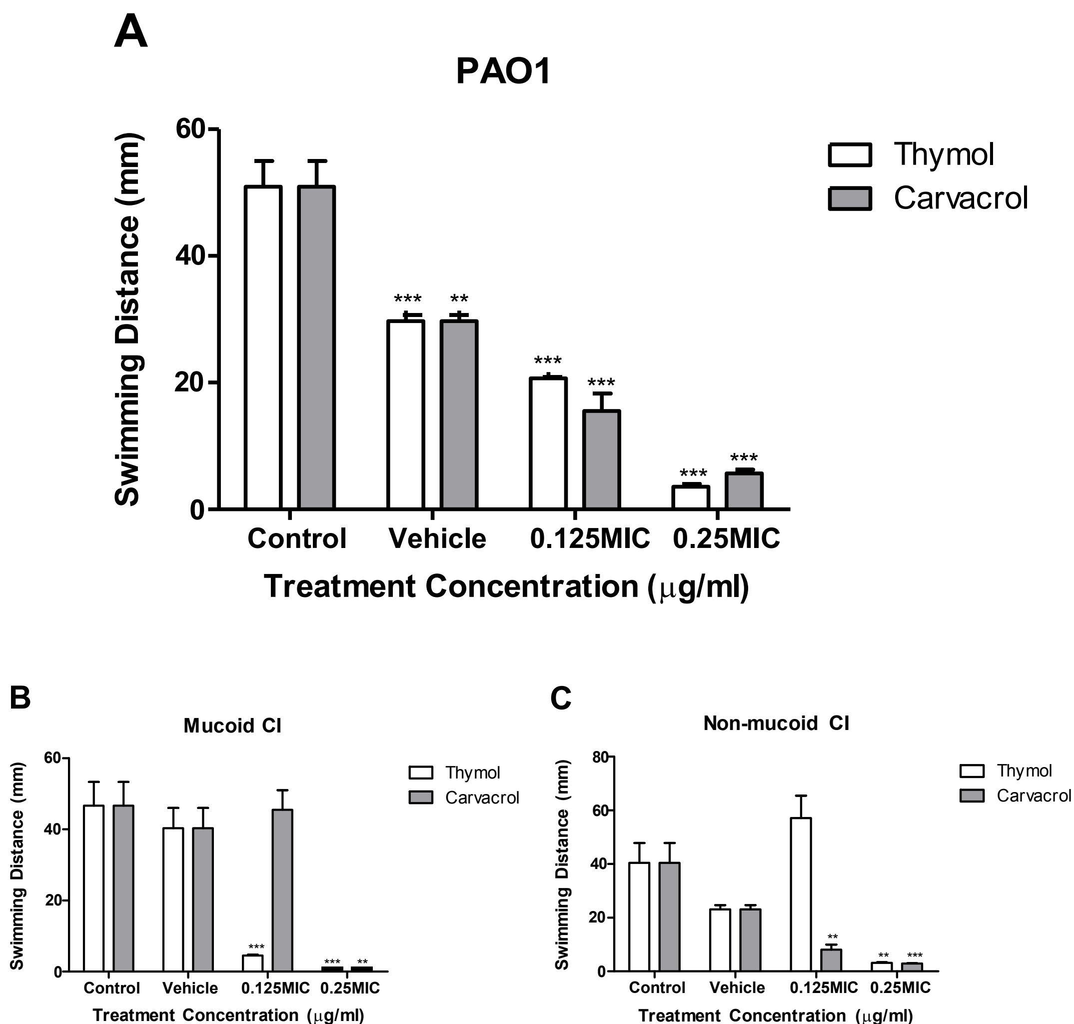

Figure 2.9: Motility in the presence of thymol and carvacrol. Plates comprising 0.125 MIC and 0.25 MIC concentrations of either thymol or carvacrol and 0.3% agarose were point inoculated with bacterial cultures. Plates were incubated at 37 ˚C for 12 h (A) or 36 h (B, C). Distance travelled through the media was measured after incubation with a ruler (mm). Data is represented as the average of three separate experiments conducted in duplicate ± SEM. Average distance travelled of the ethanol vehicle is also displayed as a percentage of the untreated control ± SEM (n=3). Statistical significance was determined through one-way ANOVA analysis followed by Tukey's post-hoc test to determine significance relative to the untreated controls (p-value < 0.01= ** and p-value < 0.001= ***).

3.3.8 Effects of Thymol and Carvacrol Treatment on *P. aeruginosa* Gene Expression

Gene expression changes from the microarray analysis of the laboratory PAO1 strain revealed significant expressional changes with thymol treatment at both 6 h and 24 h, as well as carvacrol treatment at 24 h. Treatment of the bacteria for 24 h with carvacrol had the greatest influence on bacterial gene expression, while the 24 h treatment of thymol displayed the least. The 6 h thymol exposure caused the greatest population of genes to decrease their expression, many of which are involved in virulence factor production.

With the 6 h thymol treatment a pair of genes involved in pyochelin biosynthesis (*pchC, pchD*) was significantly upregulated (Table 2.2 A). Additionaly, four genes involved in pyoverdine biosynthesis (*pvdA, pvdO, pvdH*, and *pvdS*) were likewise upregulated after 6 h exposure to thymol (Table 2.2 A). As seen with the 6hr aqueous treatment multiple genes involved in iron binding (*hitA)*, and heme acquisition (*TonB, phuR*) were significantly upregulated with the 6hr thymol treatment.

Of these genes down regulated by the 6 h thymol treatment, 4 genes involved in the biosynthesis of phenazine were down regulated in expression. The virulence factor elastase gene *lasB* was also negatively affected by exposure to thymol for 6 h. Additionally, the 6 h thymol treatment reduced the expression of chitin-binding protein precursor gene *cbpD* and fucose-binding lectin gene *lecB*, two genes involved in bacterial adherence during host interactions (Table 2.2 A). Treatment of *P. aeruginosa* for 24 h with thymol only induced significant changes to three genes. Notably, bacterioferritin

gene *bfrB* and a low Mg^{2+} inducible outer membrane protein precursor gene *oprH* were significantly upregulated (Table 2.2 B).

In contrast to the 24 h thymol treatment, 96 genes were significantly affected by the treatment of *P. aeruginosa* with carvacrol for 24 h (Table 2.2 C). The only down regulated gene with known function was the *narH* gene which codes for a respiratory nitrate reductase protein involved in nitrogen metabolism. Significantly upregulated were 18 genes encoding 50S ribosome structure components and 9 encoding 30S ribosome structure components. Multiple genes involved in protein synthesis and folding (*fusA1, rpoA*) were also significantly upregulated after 24 h treatment (Table 2.2 C). Several genes involved in iron acquisition and storage (*bfrB, pchA, pchF, pchG, pvdH, pvdJ, pvdD,* and *pvdL*) were found to be upregulated with the 24 h carvacrol treatment, of which bacterioferritin was upregulated the most. Two genes involved in bacterial flagella production, *fliF* a flagella M-ring outer membrane protein precursor and *fliD* a flagellar capping protein were both increased with the 24 h carvacrol treatment (Table 2.2 C). Comparisons of the carvacrol 24 h treatment and the thymol 24 h treatment mainly displayed increased changes to gene expression in the carvacrol over thymol treatment. The 24 h thymol treatment had a greater expression than 24 h carvacrol treatment for only a single gene, *fis*, a gene coding for a DNA-binding protein.

Table 2.2: Significant gene fold changes after exposure to thymol or carvacrol. RNA isolated from bacteria grown in the presence of the either thymol for 6 or 24 h and carvacrol for 24 h at a 500 µg/ml concentration was analysed through the Affymetrix *Pseudomonas aeruginosa* GeneChip array. Partek multi-way ANOVA analysis was performed to assess significant differences in the expression of genes when compared to RNA from untreated controls. Only genes displaying increased or decreased expression of a ≥2.0 fold change were considered significant.

Table 2.2 A: Significant gene expression changes with 6 h thymol treatment (n=3) when compared to 6 h control (n=3)

Probeset ID	Gene Symbol	Gene Title	p-value	Fold Change
PA4228_pchD_at	pchD	pyochelin biosynthesis protein PchD	0.00580222	3.43891
PA1983_exaB_at	exaB	cytochrome c550	0.0133731	3.20906
PA2386_pvdA_at	pvdA	L-ornithine N5-oxygenase	0.0278372	3.07509
PA4570_at	---	---	0.0107843	3.00146
PA4761_dnaK_at	dnaK	DnaK protein	0.00818894	2.7996
PA4385_groEL_at	groEL	GroEL protein	0.0125542	2.74187
PA4229_pchC_at	pchC	pyochelin biosynthetic protein PchC	0.000986176	2.71412
PA2452_at	---	---	0.014839	2.69637
PA4710_at	phuR	Haem/Haemoglobin uptake outer membrane receptor PhuR precursor	0.00803246	2.68771
PA4471_at	---	---	0.0432388	2.67239
PA4386_groES_at	groES	GroES protein	0.0153828	2.61186
PA2426_pvdS_at	pvdS	sigma factor PvdS	0.0264479	2.44968
PA5559_atpE_at	atpE	atp synthase C chain	0.0464143	2.41982
PA4370_at	icmP	Insulin-cleaving metalloproteinase outer membrane protein precursor	0.0218232	2.3783
PA4469_at	---	---	0.0428466	2.30334
PA2395_at	pvdO	PvdO	0.0139955	2.3019
PA4762_grpE_at	grpE	heat shock protein GrpE	0.00116947	2.28582

PA0070_at	---	---	0.00163887	2.24532
PA5531_tonB_at	tonB	TonB protein	0.0442122	2.16712
PA2413_at	pvdH	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH	0.0288235	2.16508
PA1982_exaA_at	exaA	quinoprotein alcohol dehydrogenase	0.0132604	2.15835
PA0085_at	---	---	5.27E-05	2.15296
PA3720_at	---	---	0.00441056	2.13393
PA0083_at	---	---	0.00159987	2.13082
PA1718_pscE_at	pscE	type III export protein PscE	0.00474154	2.12743
PA1719_pscF_at	pscF	type III export protein PscF	0.000936218	2.11241
PA3785_at	---	---	0.00143257	2.09986
PA1984_s_at	---	---	0.00440302	2.09617
PA4687_hitA_at	hitA	ferric iron-binding periplasmic protein HitA	0.00747976	2.08649
PA0905_csrA_at	rsmA	RsmA, regulator of secondary metabolites	0.0120737	2.05157
PA1151_imm2_at	imm2	pyocin S2 immunity protein	0.0392814	2.0374
PA3570_mmsA_at	mmsA	methylmalonate-semialdehyde dehydrogenase	0.0331027	2.03153
PA4467_at	---	---	0.0147382	2.02195
PA0400_at	---	---	0.027226	2.0114
PA0179_at	---	---	0.0407447	-2.00065
PA1789_at	---	---	0.0396622	-2.00244
PA4306_at	---	---	0.000153219	-2.01375
PA2069_at	---	---	0.00411611	-2.02902

PA3724_lasB_at	lasB	elastase LasB	0.0170161	-2.05425
PA1914_at	---	---	9.35E-05	-2.05565
PA0852_cpbD_at	cbpD	chitin-binding protein CbpD precursor	0.00847351	-2.06209
PA5475_at	---	---	0.0259862	-2.06671
PA5232_at	---	---	0.0155643	-2.09279
PA1876_at	---	---	0.000155644	-2.10955
PA1874_at	---	---	1.14E-05	-2.24606
PA3309_at	---	---	0.0326791	-2.25338
PA4211_g_at	phzB1 / phzB2	probable phenazine biosynthesis protein /// probable phenazine biosynthesis protein	0.00578668	-2.3212
PA5027_at	---	---	0.00320747	-2.39642
PA2788_at	---	---	0.000756878	-2.56115
PA1901_s_at	phzC1 / phzC2	phenazine biosynthesis protein PhzC /// phenazine biosynthesis protein PhzC	5.28E-05	-2.70654
PA1746_at	---	---	0.0123516	-2.73028
PA1903_s_at	phzE1 / phzE2	phenazine biosynthesis protein PhzE /// phenazine biosynthesis protein PhzE	5.07E-05	-3.01012
PA4352_at	---	---	0.00525222	-3.48927
PA1561_aer_at	aer	aerotaxis receptor Aer	0.00065044	-

			5	3.69243
PA4141_at	---	---	0.00497458	-3.83298
PA0122_at	---	---	0.00015714	-3.94713
PA4217_at	phzS	flavin-containing monooxygenase	0.000203297	-3.95694
PA1904_s_at	phzF1 / phzF2	probable phenazine biosynthesis protein /// probable phenazine biosynthesis protein	4.09E-06	-4.11428
PA1905_s_at	phzG2	probable pyridoxamine 5'-phosphate oxidase	6.19E-08	-4.65341
PA3361_at	lecB	fucose-binding lectin PA-IIL	0.000188763	-5.41996

Table 2.2 B: Significant gene expression changes with 24 h thymol treatment (n=3) when compared to 24 h control (n=3)

Probeset ID	Gene Symbol	Gene Title	p-value	Fold Change
PA3531_bfrB_at	bfrB	bacterioferritin	0.00217643	2.92487
PA0122_at	---	---	0.00487607	2.42434
PA1178_oprH_at	oprH	PhoP/Q and low Mg2+ inducible outer membrane protein H1 precursor	0.0468215	2.10623

Table 2.2 C: Significant gene expression changes with 24 h carvacrol treatment (n=2) when compared to 24 h control (n=3)

Probeset ID	Gene Symbol	Gene Title	p-value	Fold Change
PA4272_rplJ_at	rplJ	50S ribosomal protein L10	0.002359	7.2286
PA4257_rpsC_at	rpsC	30S ribosomal protein S3	0.00793631	6.44369
PA4141_at	---	---	0.00116285	6.31333

PA4258_rplV_at	rplV	50S ribosomal protein L22	0.00143535	6.26675
PA4273_rplA_at	rplA	50S ribosomal protein L1	0.00363265	5.97563
PA4260_rplB_at	rplB	50S ribosomal protein L2	0.00220167	5.5979
PA1592_i_at	---	---	0.0311538	5.4882
PA4251_rplE_at	rplE	50S ribosomal protein L5	0.00212694	5.13973
PA4256_rplP_at	rplP	50S ribosomal protein L16	0.00537229	4.99501
PA3531_bfrB_at	bfrB	bacterioferritin	0.000212493	4.95174
PA4250_rpsN_at	rpsN	30S ribosomal protein S14	0.00743626	4.89287
PA4274_rplK_at	rplK	50S ribosomal protein L11	0.0129355	4.72881
PA4220_i_at	---	---	0.0101216	4.66334
PA2840_at	---	---	0.000950531	4.65135
PA4262_rplD_at	rplD	50S ribosomal protein L4	0.00559478	4.58926
PA4240_rpsK_at	rpsK	30S ribosomal protein S11	0.00744515	4.48088
PA3337_rfaD_at	rfaD	ADP-L-glycero-D-mannoheptose 6-epimerase	0.000929575	4.43828
PA0999_fabH1_at	pqsD	3-oxoacyl-[acyl-carrier-protein] synthase III	5.11E-05	4.31167
PA5427_adhA_at	adhA	alcohol dehydrogenase	0.00989624	4.16936
PA2976_rne_at	rne	ribonuclease E	0.000145238	4.15669
PA3692_at	---	---	0.00111357	4.10974
PA4933_at	---	---	0.00715716	4.01871
PA3309_at	---	---	0.00299713	3.98713
PA4263_rplC_at	rplC	50S ribosomal protein L3	0.00416207	3.86823
PA4352_at	---	---	0.00697342	3.80267
PA4254_rpsQ_at	rpsQ	30S ribosomal protein S17	0.0207891	3.72529
PA4244_rplO_at	rplO	50S ribosomal protein L15	0.00663231	3.61965

PA4266_fusA1_at	fusA1	elongation factor G	0.0383887	3.57759
PA4261_rplW_at	rplW	50S ribosomal protein L23	0.0139266	3.54646
PA0998_at	pqsC	Homologous to beta-keto-acyl-acyl-carrier protein synthase	0.000273278	3.44921
PA3691_at	---	---	0.0052331	3.44203
PA2853_oprI_at	oprI	Outer membrane lipoprotein OprI precursor	0.000717423	3.32257
PA4259_rpsS_at	rpsS	30S ribosomal protein S19	0.0112981	3.27166
PA4264_rpsJ_at	rpsJ	30S ribosomal protein S10	0.0184413	3.23883
PA2400_at	pvdJ	PvdJ	0.00270135	3.15716
PA0836_at	---	---	0.000831564	3.08133
PA4245_rpmD_at	rpmD	50S ribosomal protein L30	0.0328426	3.04076
PA4248_rplF_at	rplF	50S ribosomal protein L6	0.0283239	3.01815
PA4247_rplR_at	rplR	50S ribosomal protein L18	0.0304193	3.01383
PA0997_at	pqsB	Homologous to beta-keto-acyl-acyl-carrier protein synthase	0.000259802	3.00659
PA2412_at	---	---	0.0283858	2.97826
PA0962_at	---	---	0.036133	2.96012
PA2753_at	---	---	0.00190587	2.95547
PA4222_at	---	---	1.00E-05	2.89901
PA2399_pvdD_at	pvdD	pyoverdine synthetase D	0.000518693	2.89833
PA4238_rpoA_at	rpoA	DNA-directed RNA polymerase alpha chain	0.0299189	2.83997
PA2411_at	---	---	0.00137807	2.83418
PA4464_ptsN_at	ptsN	nitrogen regulatory IIA protein	0.0024572	2.8122
PA1094_fliD_at	fliD	flagellar capping protein FliD	0.0172256	2.80643
PA0763_mucA_at	mucA	anti-sigma factor MucA	0.0109717	2.75116
PA1777_oprF_at	oprF	Major porin and structural outer	0.00895102	2.7191

		membrane porin OprF precursor		5
PA4935_rpsF_at	rpsF	30S ribosomal protein S6	0.0497944	2.6889
PA4253_rplN_at	rplN	50S ribosomal protein L14	0.0363175	2.64221
PA2248_bkdA2_at	bkdA2	2-oxoisovalerate dehydrogenase (beta subunit)	0.00366905	2.62187
PA2413_at	pvdH	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH	0.0196846	2.54585
PA4246_rpsE_at	rpsE	30S ribosomal protein S5	0.0365724	2.51244
PA4249_rpsH_at	rpsH	30S ribosomal protein S8	0.00609997	2.49779
PA1245_at	---	---	0.000117862	2.49736
PA2742_rpmI_at	rpmI	50S ribosomal protein L35	0.0489776	2.4707
PA4577_at	---	---	0.0244596	2.46462
PA2427_at	---	---	0.0201159	2.42431
PA4225_pchF_at	pchF	pyochelin synthetase	0.00416639	2.39697
PA5232_at	---	---	0.0130006	2.34797
PA1561_aer_at	aer	aerotaxis receptor Aer	0.0249822	2.29486
PA2741_rplT_at	rplT	50S ribosomal protein L20	0.0415698	2.27039
PA2401_at	---	---	0.0289641	2.23561
PA0996_at	pqsA	probable coenzyme A ligase	0.000166273	2.22887
PA5027_at	---	---	0.0113877	2.22195
PA4224_at	pchG	pyochelin biosynthetic protein PchG	0.0106176	2.2201
PA4237_rplQ_at	rplQ	50S ribosomal protein L17	0.0450652	2.19956
PA4223_at	---	---	9.18E-05	2.19113
PA1789_at	---	---	0.0416973	2.1536
PA3572_at	---	---	0.0416658	2.11297
PA2250_lpdV_at	lpdV	lipoamide dehydrogenase-Val	0.0257921	2.10617
PA4231_pchA_a	pchA	salicylate biosynthesis	1.52E-05	2.0964

t		isochorismate synthase		3
PA2146_i_at	---	---	0.0447527	2.07759
PA0345_at	---	---	0.0166682	2.03953
PA3788_at	---	---	0.0101504	2.03616
PA2402_at	---	---	0.0142658	2.03131
PA1101_fliF_at	fliF	Flagella M-ring outer membrane protein precursor	0.00690666	2.00969
PA2424_at	pvdL	PvdL	0.000812802	2.00868
PA0740_at	---	---	0.0474676	-2.00874
PA4638_at	---	---	0.0275454	-2.01273
PA3874_narH_at	narH	respiratory nitrate reductase beta chain	0.0104611	-2.03265
PA0445_s_at	---	---	0.0298365	-2.0611
PA4033_at	---	---	0.0417178	-2.12048
PA2029_i_at	---	---	0.0134619	-2.37368
PA3600_at	---	---	0.01666	-2.40183
PA1942_at	---	---	0.0121489	-2.47494
PA3577_i_at	---	---	0.00269391	-2.52335
PA4877_at	---	---	0.00759684	-2.54596
PA5250_at	---	---	0.0346362	-2.719

3.3.9 Gene-Ontology Enrichment Analysis of *P. aeruginosa* Treated with Thymol or Carvacrol

Gene ontology (GO) enrichment analysis of *P. aeruginosa* with 6 h and 24 h thymol treatment and 24 h carvacrol treatment revealed significant changes to various groups of expressed genes (Table 2.3). Six hour thymol exposure caused a significant enrichment in transcripts of 10 gene groups, of which the most enriched gene group was that of antibiotic biosynthetic processes which were enriched by a factor of 15.26 with 50% of the genes in the group significantly affected. Other major gene groups affected include those involved in the extracellular region and those dealing with siderophore transmembrane transporter activity, whose transcripts were enriched by a factor of 5.61 and 5.49 respectively. In addition genes groups related to DNA transcription and initiation, and protein folding and transport were enriched after the 6 h treatment of thymol.

GO-enrichment analysis of the 24 h thymol treatment resulted in less reliable results. With only three genes showing significant up-regulation ($\geq \pm 2$ fold change) the ability to group genes based on similarities in function is reduced. Therefore although many gene groups display enrichment scores of significance (Table 2.3 B), the true significance of the data is only based on a very small number of genes indicated by the low percentage of genes responding and significantly altered by the treatment in each group. Therefore data presented in Table 2.3 B should be taken as inconclusive. In relation to the three genes which were significantly upregulated with the 24 h thymol treatment GO-enrichment analysis showed increased enrichment for iron-sulfur cluster

assembly, siderophore transmembrane transporter activity, response to antibiotics, and type II protein secretion system.

With the 24 h carvacrol GO-enrichment analysis several gene groups relating to ribosome structure and function were enriched (Table 2.3 C). Of particular importance, gene groups relating to the structural constituent of ribosomes, translation, and RNA binding were given enrichment scores of 67.62, 51.49, and 49.86 respectively. The percentage of genes present in each of threse groupings ranged from approximately 30-52%. Gene groups of ribonuclease activity, regulation of translation, and ribosome biogenesis were similarly enriched with 24 h carvacrol treatment. Enrichment of intracellular and cellular protein metabolic genes was also found with scores of 35.82 and 34.44. Protein binding, cell adhesion, and fatty acid biosynthesis gene groups were also enriched with the 24 h carvacrol treatment.

Table 2.3: Gene ontology (GO) enrichment analysis of *P. aeruginosa* after exposure to the thymol or carvacrol. From genes deemed significantly increased by a factor of ≥2.0 fold change, genes were grouped based on similar GO functions and differences in overall group enrichment compared to the control was performed through a Fisher's Exact Test. Only those gene groups with an enrichment p-value > 0.05 are displayed.

Table 2.3 A: GO-enrichment of 6 h thymol treatment (n=3) compared to 6 h control (n=3)

Function	Type	Enrichment Score	Enrichment p-value	% genes in group that are present
antibiotic biosynthetic process	biological process	15.2649	2.34713E-07	50
extracellular region	cellular component	5.61263	0.00365145	15.7895
siderophore transmembrane transporter activity	molecular function	5.49565	0.00410459	33.3333
oxidoreductase activity, acting on CH-OH group of donors	molecular function	4.89265	0.00750153	25
metalloendopeptidase activity	molecular function	4.6519	0.00954348	22.2222
DNA-templated transcription, initiation	biological process	4.6519	0.00954348	22.2222
ATP biosynthetic process	biological process	4.43931	0.0118041	20
oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	molecular function	3.77724	0.0228858	14.2857
protein folding	biological process	3.59544	0.0274485	7.69231
protein transport	biological process	3.11241	0.0444937	6.38298

Table 2.3 B: GO-enrichment of 24 h thymol treatment (n=3) compared to 24 h control (n=3)

Function	Type	Enrichment Score	Enrichment p-value	% genes in group that are present
ACP phosphopantetheine attachment site binding involved	molecular function	5.04199	0.00646086	33.3333

in fatty acid biosynthetic process				
alkaline phosphatase activity	molecular function	5.04199	0.00646086	33.3333
chorismate mutase activity	molecular function	5.04199	0.00646086	33.3333
cysteine synthase activity	molecular function	5.04199	0.00646086	33.3333
glucose-6-phosphate dehydrogenase activity	molecular function	5.04199	0.00646086	33.3333
glutamate-cysteine ligase activity	molecular function	5.04199	0.00646086	33.3333
phosphoribosylglycinamide formyltransferase activity	molecular function	5.04199	0.00646086	33.3333
phosphorylase activity	molecular function	5.04199	0.00646086	33.3333
protein kinase activity	molecular function	5.04199	0.00646086	33.3333
protein tyrosine phosphatase activity	molecular function	5.04199	0.00646086	33.3333
shikimate 3-dehydrogenase (NADP+) activity	molecular function	5.04199	0.00646086	33.3333
spermidine synthase activity	molecular function	5.04199	0.00646086	33.3333
inorganic phosphate transmembrane transporter activity	molecular function	5.04199	0.00646086	33.3333
sugar:hydrogen symporter activity	molecular function	5.04199	0.00646086	33.3333
extracellular space	cellular component	5.04199	0.00646086	33.3333
transposition, DNA-mediated	biological process	5.04199	0.00646086	33.3333
transcription initiation from RNA polymerase II promoter	biological process	5.04199	0.00646086	33.3333
protein dephosphorylation	biological process	5.04199	0.00646086	33.3333
L-serine biosynthetic process	biological process	5.04199	0.00646086	33.3333
heme-copper terminal oxidase activity	molecular function	5.04199	0.00646086	33.3333
DNA integration	biological process	5.04199	0.00646086	33.3333
sulfate transmembrane transporter activity	molecular function	5.04199	0.00646086	33.3333
polyamine-transporting ATPase activity	molecular function	5.04199	0.00646086	33.3333

organic phosphonate transport	biological process	5.04199	0.00646086	33.3333
carbohydrate biosynthetic process	biological process	5.04199	0.00646086	33.3333
oxidoreductase activity, acting on the aldehyde or oxo group of donors, disulfide as acceptor	molecular function	5.04199	0.00646086	33.3333
sodium:dicarboxylate symporter activity	molecular function	5.04199	0.00646086	33.3333
metal ion transport	biological process	5.04199	0.00646086	33.3333
type III protein secretion system complex	cellular component	5.04199	0.00646086	33.3333
adenyl nucleotide binding	molecular function	5.04199	0.00646086	33.3333
peptidyl-tyrosine dephosphorylation	biological process	5.04199	0.00646086	33.3333
'de novo' CTP biosynthetic process	biological process	5.04199	0.00646086	33.3333
ATP metabolic process	biological process	5.04199	0.00646086	33.3333
chorismate metabolic process	biological process	5.04199	0.00646086	33.3333
protein insertion into membrane	biological process	5.04199	0.00646086	33.3333
polysaccharide biosynthetic process	biological process	4.75503	0.00860828	25
translation initiation factor activity	molecular function	4.75503	0.00860828	25
aminomethyltransferase activity	molecular function	4.75503	0.00860828	25
biotin carboxylase activity	molecular function	4.75503	0.00860828	25
fumarate hydratase activity	molecular function	4.75503	0.00860828	25
glutamate-ammonia ligase activity	molecular function	4.75503	0.00860828	25
protein serine/threonine kinase activity	molecular function	4.75503	0.00860828	25
amino sugar metabolic process	biological process	4.75503	0.00860828	25
glycerol-3-phosphate metabolic process	biological process	4.75503	0.00860828	25
DNA-templated transcription, termination	biological process	4.75503	0.00860828	25
translational termination	biological	4.75503	0.0086082	25

	process		8	
protein complex assembly	biological process	4.75503	0.0086082 8	25
cysteine biosynthetic process from serine	biological process	4.75503	0.0086082 8	25
glutamate biosynthetic process	biological process	4.75503	0.0086082 8	25
glutamine biosynthetic process	biological process	4.75503	0.0086082 8	25
glutathione biosynthetic process	biological process	4.75503	0.0086082 8	25
iron-sulfur cluster assembly	biological process	4.75503	0.0086082 8	25
acetyltransferase activity	molecular function	4.75503	0.0086082 8	25
oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	molecular function	4.75503	0.0086082 8	25
molybdenum ion binding	molecular function	4.75503	0.0086082 8	25
protein catabolic process	biological process	4.75503	0.0086082 8	25
carbohydrate binding	molecular function	4.75503	0.0086082 8	25
cell division site	cellular component	4.75503	0.0086082 8	25
precorrin-2 dehydrogenase activity	molecular function	4.75503	0.0086082 8	25
extracellular polysaccharide biosynthetic process	biological process	4.75503	0.0086082 8	25
carbohydrate derivative metabolic process	biological process	4.75503	0.0086082 8	25
carbohydrate derivative biosynthetic process	biological process	4.75503	0.0086082 8	25
organic substance biosynthetic process	biological process	4.75503	0.0086082 8	25
organophosphate metabolic process	biological process	4.75503	0.0086082 8	25
phosphorus metabolic process	biological process	4.75503	0.0086082 8	25
phosphate-containing compound metabolic process	biological process	4.75503	0.0086082 8	25
organophosphate biosynthetic process	biological process	4.75503	0.0086082 8	25

biosynthetic process	biological process	4.75503	0.00860828	25
aminoacyl-tRNA editing activity	molecular function	4.53261	0.0107526	20
aminopeptidase activity	molecular function	4.53261	0.0107526	20
serine-type endopeptidase activity	molecular function	4.53261	0.0107526	20
ribonuclease activity	molecular function	4.53261	0.0107526	20
glucose metabolic process	biological process	4.53261	0.0107526	20
translational initiation	biological process	4.53261	0.0107526	20
regulation of translation	biological process	4.53261	0.0107526	20
dephosphorylation	biological process	4.53261	0.0107526	20
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	molecular function	4.53261	0.0107526	20
rRNA methylation	biological process	4.53261	0.0107526	20
ribosome biogenesis	biological process	4.53261	0.0107526	20
Gram-negative-bacterium-type cell outer membrane assembly	biological process	4.53261	0.0107526	20
organic cyclic compound metabolic process	biological process	4.53261	0.0107526	20
cellular metabolic process	biological process	4.53261	0.0107526	20
small molecule metabolic process	biological process	4.53261	0.0107526	20
single-organism process	biological process	4.53261	0.0107526	20
single-organism metabolic process	biological process	4.53261	0.0107526	20
single-organism cellular process	biological process	4.53261	0.0107526	20
cellular aromatic compound metabolic process	biological process	4.53261	0.0107526	20
organic substance metabolic process	biological process	4.53261	0.0107526	20
metabolic process	biological process	4.53261	0.0107526	20
cellular process	biological	4.53261	0.0107526	20

	process			
pseudouridine synthesis	biological process	4.35101	0.0128938	16.6667
glycerol metabolic process	biological process	4.35101	0.0128938	16.6667
regulation of translational fidelity	biological process	4.35101	0.0128938	16.6667
amino acid transmembrane transporter activity	molecular function	4.35101	0.0128938	16.6667
siderophore transmembrane transporter activity	molecular function	4.35101	0.0128938	16.6667
tetrapyrrole biosynthetic process	biological process	4.35101	0.0128938	16.6667
ATP synthesis coupled electron transport	biological process	4.35101	0.0128938	16.6667
pilus organization	biological process	4.35101	0.0128938	16.6667
biological_process	biological process	4.35101	0.0128938	16.6667
amino acid transmembrane transport	biological process	4.19757	0.015032	14.2857
ATP-dependent DNA helicase activity	molecular function	4.19757	0.015032	14.2857
hydrolase activity, hydrolyzing O-glycosyl compounds	molecular function	4.19757	0.015032	14.2857
peroxidase activity	molecular function	4.19757	0.015032	14.2857
protein histidine kinase activity	molecular function	4.19757	0.015032	14.2857
tRNA modification	biological process	4.19757	0.015032	14.2857
protein disulfide oxidoreductase activity	molecular function	4.19757	0.015032	14.2857
lipid catabolic process	biological process	4.19757	0.015032	14.2857
nickel cation binding	molecular function	4.19757	0.015032	14.2857
hydroxymethyl-, formyl- and related transferase activity	molecular function	4.19757	0.015032	14.2857
cell wall organization	biological process	4.19757	0.015032	14.2857
3-oxoacyl-[acyl-carrier-protein] synthase activity	molecular function	4.06477	0.017167	12.5
arginine catabolic process	biological process	4.06477	0.017167	12.5
glycine catabolic process	biological	4.06477	0.017167	12.5

	process			
oxidoreductase activity, acting on CH-OH group of donors	molecular function	4.06477	0.017167	12.5
oxidoreductase activity, acting on the CH-CH group of donors	molecular function	4.06477	0.017167	12.5
transferase activity, transferring pentosyl groups	molecular function	4.06477	0.017167	12.5
metalloendopeptidase activity	molecular function	3.9477	0.019299	11.1111
copper ion binding	molecular function	3.9477	0.019299	11.1111
protein binding	molecular function	3.9477	0.019299	11.1111
DNA-templated transcription, initiation	biological process	3.9477	0.019299	11.1111
cell adhesion	biological process	3.9477	0.019299	11.1111
ATPase activity, coupled to transmembrane movement of substances	molecular function	3.9477	0.019299	11.1111
pyrimidine nucleotide biosynthetic process	biological process	3.84307	0.0214278	10
rRNA processing	biological process	3.84307	0.0214278	10
histidine metabolic process	biological process	3.84307	0.0214278	10
tyrosine biosynthetic process	biological process	3.84307	0.0214278	10
cellular aromatic compound metabolic process	biological process	3.84307	0.0214278	10
ubiquinone biosynthetic process	biological process	3.84307	0.0214278	10
ATP biosynthetic process	biological process	3.84307	0.0214278	10
oxidoreductase activity, acting on NAD(P)H	molecular function	3.84307	0.0214278	10
phosphotransferase activity, alcohol group as acceptor	molecular function	3.84307	0.0214278	10
antibiotic biosynthetic process	biological process	3.84307	0.0214278	10
cytochrome complex assembly	biological process	3.84307	0.0214278	10
protein secretion by the type III secretion system	biological process	3.84307	0.0214278	10
protein peptidyl-prolyl isomerization	biological process	3.74848	0.0235536	9.09091

peptidyl-prolyl cis-trans isomerase activity	molecular function	3.74848	0.0235536	9.09091
exonuclease activity	molecular function	3.74848	0.0235536	9.09091
cellular protein modification process	biological process	3.74848	0.0235536	9.09091
type II protein secretion system complex	cellular component	3.74848	0.0235536	9.09091
cellular metabolic process	biological process	3.74848	0.0235536	9.09091
Mo-molybdopterin cofactor biosynthetic process	biological process	3.66219	0.0256763	8.33333
arginine biosynthetic process	biological process	3.58286	0.027796	7.69231
coenzyme binding	molecular function	3.58286	0.027796	7.69231
histidine biosynthetic process	biological process	3.50948	0.0299125	7.14286
glutamine metabolic process	biological process	3.50948	0.0299125	7.14286
oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	molecular function	3.50948	0.0299125	7.14286
transferase activity, transferring acyl groups other than amino-acyl groups	molecular function	3.50948	0.0299125	7.14286
cell redox homeostasis	biological process	3.50948	0.0299125	7.14286
NADH dehydrogenase (quinone) activity	molecular function	3.50948	0.0299125	7.14286
purine nucleotide metabolic process	biological process	3.44121	0.032026	6.66667
manganese ion binding	molecular function	3.44121	0.032026	6.66667
fatty acid metabolic process	biological process	3.37739	0.0341364	6.25
dioxygenase activity	molecular function	3.37739	0.0341364	6.25
tryptophan biosynthetic process	biological process	3.31749	0.0362438	5.88235
oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	molecular function	3.31749	0.0362438	5.88235
2 iron, 2 sulfur cluster binding	molecular function	3.31749	0.0362438	5.88235

phosphorelay sensor kinase activity	molecular function	3.26105	0.038348	5.55556
signal transduction by phosphorylation	biological process	3.26105	0.038348	5.55556
NADP binding	molecular function	3.26105	0.038348	5.55556
extracellular region	cellular component	3.20771	0.0404492	5.26316
fatty acid biosynthetic process	biological process	3.20771	0.0404492	5.26316
arginine metabolic process	biological process	3.15714	0.0425474	5
response to antibiotic	biological process	3.15714	0.0425474	5
DNA replication	biological process	3.06327	0.0467345	4.54545
DNA recombination	biological process	3.01955	0.0488234	4.34783
sequence-specific DNA binding	molecular function	3.01955	0.0488234	4.34783

Table 2.3 C: GO-enrichment of 24 h carvacrol treatment (n=2) compared to 24 h control (n=3)

Function	Type	Enrichment Score	Enrichment p-value	% genes in group that are present
structural constituent of ribosome	molecular function	67.621	4.29134E-30	51.8519
translation	biological process	51.4926	4.33576E-23	30.5263
RNA binding	molecular function	49.8652	2.20702E-22	35.6164
intracellular	cellular component	35.8216	2.77262E-16	30.8824
cellular protein metabolic process	biological process	34.4494	1.09346E-15	17.5758
3-oxoacyl-[acyl-carrier-protein] synthase activity	molecular function	6.13909	0.00215688	37.5
glucose-6-phosphate dehydrogenase activity	molecular function	5.59569	0.00371384	66.6667
ribonuclease activity	molecular function	4.43816	0.0118177	40
regulation of translation	biological process	4.43816	0.0118177	40

ribosome biogenesis	biological process	4.43816	0.0118177	40
transferase activity, transferring acyl groups other than amino-acyl groups	molecular function	4.42021	0.0120317	21.4286
fatty acid biosynthetic process	biological process	3.5676	0.0282235	15.7895
protein binding	molecular function	3.24942	0.0387968	22.2222
cell adhesion	biological process	3.24942	0.0387968	22.2222
ubiquinone biosynthetic process	biological process	3.04917	0.0473981	20

3.3.10 Pathway Enrichment Analysis of *P. aeruginosa* Treated with Thymol or Carvacrol

Further analysis looking at pathway enrichment revealed three significant pathways affected by the 6 h thymol treatment. These pathways in order of ascending enrichment were RNA degradation, propanoate metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis (Table 2.4 A). For all three pathways low percentages of genes involved were statistically altered in expression. Due to the very minimal number of genes significantly affected by the 24 h thymol exposure, collectively no single pathway was affected by this treatment.

After 24 h, carvacrol treatment significantly enriched two pathways, ribosome and siderophore biosynthesis (Table 2.4 C). In the ribosomal pathway, 50% of the genes were significantly affected with a pathway enrichment score of 60.32. Compared to the 24 h thymol treatment, carvacrol treatment enriched both the ribosome pathway and that of valine, leucine, and isoleucine degradation.

Table 2.4: Pathway enrichment analysis of *P. aeruginosa* after exposure to thymol or carvacrol. From the sample of genes deemed significantly increased by a factor of ≥2.0 fold change, genes were grouped based similarity in the pathways which they function through KEGG pathway analysis. Calculation of differences in pathway enrichment compared to the control was performed through a Fisher's Exact Test. Only those pathways with an enrichment p-value > 0.05 are displayed.

Table 2.4 A: Pathway analysis of 6 h thymol treatment (n=3) compared to 6 h control (n=3)

Pathway Name	Enrichment Score	Enrichment p-value	% genes in pathway that are present
RNA degradation	4.5173	0.0109185	16.6667
Propanoate metabolism	3.83369	0.0216296	11.7647
Phenylalanine, tyrosine and tryptophan biosynthesis	3.26017	0.038382	8.69565
Inositol phosphate metabolism	2.90756	0.0546088	25

Table 2.4 B: Pathway analysis of 24 h carvacrol treatment (n=2) compared to 24 h control (n=3)

Pathway Name	Enrichment Score	Enrichmen t p-value	% genes in pathway that are present
Ribosome	60.3255	6.32351E-27	50.9434
Biosynthesis of siderophore group nonribosomal peptides	6.475	0.0015415	50

3.4 Discussion

Isometric phenolic compounds thymol and carvacrol extracted from either thyme or oregano have shown antibacterial properties to various species of bacteria (Helander, Alakomi et al. 1998, Walsh, Maillard et al. 2003, Oussalah, Caillet et al. 2007). In this study the antibacterial properties and effects of thymol and carvacrol towards the pathogenicity of *P. aeruginosa* were assessed by examining their effects against the laboratory strain PAO1and two clinical isolates. MIC experiments revealed bacteriostatic effects towards the clinical *P. aeruginosa* isolates, but not towards PAO1 when tested in M9 minimal salts media. Our data are consistent with those reported by others, where concentrations up to 1000 µg/ml did not attenuate growth (Walsh, Maillard et al. 2003). Concentrations of up to 3 mM and 3-5 mM have been reported as MIC concentrations for carvacrol and thymol respectively, against *Escherichia coli* (Helander, Alakomi et al. 1998, Trombetta, Castelli et al. 2005). In our study, such high concentrations were not assessed, but it is reasonable to suggest that concentrations at this high level may have inhibited the growth of PAO1. However, in a neutral medium such as the MHCA, bacteriostatic effects of either compound were seen towards PAO1.

Inhibitory growth action towards both clinical isolates was displayed for thymol and carvacrol. The mucoid clinical isolate growth was inhibited at 1200 µg/ml and 1000 µg/ml in M9 media and 150 µg/ml and 450 µg/ml in MHCA media for thymol and carvacrol respectively. Inhibitory concentrations were lower for the non-mucoid strain for either compound in MHCA and M9 media. It is difficult to decide whether if this is strain specific effect or an effect of the mucoid phenotype. Thymol and carvacrol act as antimicrobial agents through their ability to insert into the cell membrane causing

disruption, which leads to cell leakage and lysis at higher concentrations (Trombetta, Castelli et al. 2005). Speculation regarding the role of the mucoid phenotype in the efficacy of thymol or carvacrol as antibiotics will need to be further explored with larger sample sizes of clinical isolates.

Growth curve analysis revealed little effect of either compound on the growth rate of *P. aeruginosa* at sub-inhibitory concentrations. Minimal attenuation towards bacterial growth suggests these compounds would not be very effective in aiding the clearance of bacterial infections if administered at low concentrations. However, reducing bacterial growth is only one mechanism for alleviating infection, reduction of key virulence factors or adherence in the presence of either compound would be beneficial mechanism(s) of action of a novel antibiotic.

Pathway analysis for the 6 h thymol incubation displayed enrichment of the energy metabolism pathway ofpropanoate metabolism; however, only ~12% of the genes in this pathway were affected, therefore it is unlikely that larger overall changes in this pathway occurred with treatment (Table 2.2 A). Increased expression in two genes involved in ethanol oxidation, *exaA* and *exaB* were recorded (Gorisch 2003), and likely reflect the dilution of either compound into ethanol before dilution into the media. Day-long thymol incubation showed very little expressional differences related to energy metabolism in the bacteria. Expression analysis was not completed for a 6 h incubation with carvacrol, however few genes of interest related to bacterial metabolism were effected after 24 h of incubation. Two genes primarily involved in the metabolism of valine, leucine, and isoleucine were significantly upregulated. Gene *bkdA2* encodes two subunits of the branched chain keto-acid dehydrogenase, an enzyme in the metabolism of

valine, leucine, and isoleucine (Debarbouille, Gardan et al. 1999), while *lpdV* which encodes a lipamide dehydrogenase which works in concert with the *bkd* operon to which *bkdA2* also belongs (Madhusudhan, Lorenz et al. 1993).

In the presence of either thymol or carvacrol, reduced adherence was only demonstrated for the mucoid clinical isolate (Figure 2.4). Preformed biofilms were treated with suprainhibitory concentrations of either compound; showing reductions in biofilm formation only in the mucoid clinical isolate (Figure 2.5). As suggested earlier, it cannot be stated whether these findings are mucoid phenotype dependent without further studies implementing larger sample sizes.

Previous studies monitoring the effects of thymol and carvacrol on biofilm formation and bacterial adherence have revealed inconsistent results. With gram-positive bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, reduced bacterial adherence and preformed biofilm reduction with thymol or carvacrol treatment have been found (Nostro, Sudano Roccaro et al. 2007). Carvacrol treatment has also been shown to decrease naïve and mature biofilm formation of dual-species biofilms composed of *S. aureus* and *Salmonella enterica* Serovar Typhimuirum (Knowles, Roller et al. 2005). Dual-species biofilms subjected to a mixture of thymol and eugenol, another naturally produced plant phenol, displayed no significant changes in biofilm arichitecture (Lebert, Leroy et al. 2007). Thymol has also been tested against planktonic and biofilm cultures of *S. epidermidis* alone and in combination with chlorohexidine digluconate (CHG), a common skin antisepsis antimicrobial (Karpanen, Worthington et al. 2008). Decreased growth of planktonic and biofilm *S. epidermidis* was found with only thymol treatment, however no synergistic effects were found in combination with CGH

(Karpanen, Worthington et al. 2008). Carvacrol administered in polymeric nanocapsules disrupted preformed *S. epidermidis* biofilms allowing further penetration of the particles to the core of the biofilms, an area hard to reach with traditional antibiotics (Iannitelli, Grande et al. 2011). Further research into the mechanism of how thymol and carvacrol affect bacterial surface interactions would provide a more complete picture of ability of these compounds to reduce bacterial biofilm formation, and could enhance treatment of *P. aeruginosa* biofilms.

Bacterial adherence to abiotic surfaces is mediated by cell-surface structures, such as LecB encoded by the *lecB* gene which was decreased in expression after incubation with thymol for 6 h (Table 2.2 A). The absence of *lecB* expression negatively effects pili biogenesis (Sonawane, Jyot et al. 2006). As detailed in Chapter 2, mutants of *lecB* lost twitching motility (Sonawane, Jyot et al. 2006), due to *lecB* mutants displaying reduced levels of the pilJ gene, an essential gene in the *pilGHIJK* operon for pili biogenesis (Kearns, Robinson et al. 2001). Decreasing lectin production or completely repressing the expression of *lecB* through treatment has potential to reduce *P.aeruginosa's* pathogenicity.

Conversion to a mucoid phenotype is a detrimental change during *P. aeruginosa* chronic infections. After the 24 h incubation with carvacrol the *mucA* gene, which negatively regulates the genes encoding alginate production and subsequent conversion to mucoid phenotype, was significantly increased (Table 2.2 C) (Martin, Schurr et al. 1993). Increased expression of *mucA* represses alginate production and conversion to the mucoid phenotype. If reduced of mucoid conversion through carvacrol treatment occurred, this

treatment has potential to greatly reduce *P. aeruginosa* pathogenicity during chronic infections.

Incubation in the presence of carvacrol for 24 h increased expression of two genes encoding flagellar structural components (Table 2.2 C). Gene *fliF* codes for a precursor to the M-ring outer membrane protein (Thomas, Morgan et al. 2001), and *fliD* encodes a flagellar capping protein (Arora, Ritchings et al. 1998). As discussed in Chapter 2, *fliD* has been shown experimentally to be involved in mucin adhesion, an important feature of infectious lung pathogens (Arora, Ritchings et al. 1998). The full assembly and regulation of flagellar synthesis involves many more genes than the two found to be increase with the carvacrol incubation (Arora, Ritchings et al. 1998, Thomas, Morgan et al. 2001). Therefore it is unlikely that significant production increases in functional flagella would occur from exposure to carvacrol for 24 h.

In fact, experimental data from this study demonstrated decreased flagellar swimming motility for PAO1 when treated with either thymol or carvacrol at sub-inhibitory concentrations (Figure 2.9 A). The ethanol vehicle also displayed significantly decreased motility in comparison to the control for the PAO1 strain, but only minimally reduced motility for the non-mucoid strain and produced no effect against the mucoid strain. The clinical isolates showed significantly reduced motility with both compounds at subinhibitory concentrations (Figure 2.9: B, C). Had higher concentrations been tested, complete attenuation of motility may have been seen with either compound treatment, an attribute which could significantly decrease initial bacterial establishment during infection.

Two studies with *E. coli* found reduced motility with exposure of the bacteria to carvacrol. Reduced motility in the study conducted by Gill & Holley (2006) deemed *E.coli* subjected to carvacrol at a concentration of 10 mM non-motile, and those treated at 5 mM as reduced in motility. Protein analysis by Burt SA et al (2007) revealed significant decreases in flagellum to the point of extinguishing motility when *E.coli* was treated with carvacrol at 1mM overnight. Interestingly, these authros also noted that the decrease in motility was both concentration and time dependent (in agreement with Gill & Holley (2006).

The importance of excreted enzymes towards bacterial virulence is critical. In this study the measurement of secreted protease and lipase was taken from culture supernatants grown in the presence of subinhibitory concentrations of either thymol or carvacrol. Growth in the presence of either compound significantly reduced PAO1 protease production at a 0.5 MIC concentration (Figure 2.7 A). Expression analysis after 6 h of growth in the presence of thymol also displayed decreased expression of the *lasB* gene which encodes a *P. aeruginosa* elastase (Table 2.2 A) (Hentzer, Wu et al. 2003). Expression analysis after only 6 h of incubation with carvacrol may also have displayed similar decreases in *lasB* gene expression.

No significant changes in secreted protease were measured for the mucoid clinical isolate (Figure 2.7 B) however complete attenuation of protease production was displayed for the non-mucoid clinical isolate with 0.5 MIC treatment of thymol or carvacrol, and the vehicle control (Figure 2.7 C). Interestingly, the 0.25 MIC concentration for both thymol and carvacrol had a rescue effect on the protease production in the non-mucoid isolate. It is unclear why the vehicle control created such attenuation, and why it only

affected the non-mucoid clinical isolate. It is possible that this outcome is strain specific, and not at all indicative of what would be seen for a larger sample size of clinical isolates.

Lipase production, assessed through a tween 20 hydrolysis reaction, showed carvacrol and thymol treatment resulted in significant increases in lipase production for PAO1 (Figure 2.8 A). Contrastingly, the mucoid isolate, displayed decreases with either treatment as well as with the vehicle control (Figure 2.8 B). Results from the non-mucoid isolate were inconclusive with only carvacrol at a 0.5 MIC concentration showing any significant attenuation however the vehicle control significantly decreased the measured lipase secretion (Figure 2.8 C). Unlike with the protease assay, no expressional evidence was found for an increase in lipase associated genes in the microarray analysis.

The regulation of global signalling systems, like qurorum sensing, can also cause changes in the expression of virulence associated factors without altering the gene expression of the synthesis genes involved. An increased expression after 6 h of incubation with thymol was found for *rsmA*, a global translation regulator which when overexpression significantly reduces excreted metabolites such as proteases, elastase, hydrogen cyanide and pyocyanin (Table 2.2 A) (Pessi, Williams et al. 2001). Expression of *rsmA* has a negative effect on quorum sensing by down regulating the production of N-acylhomoserine lactones (Pessi, Williams et al. 2001). Expessional increases in *rsmA* after the 6 h incubation may be in part responsible for the decrease in LasB observed in our study. Gene *rsmA* affects translation greastest during the late exponential phase of growth (Pessi, Williams et al. 2001) anda therefore no expressional changes were found with the carvacrol treatment.

Although *rsmA* was not affected by the 24 h incubation with carvacrol, four genes involved in the Pseudomonas quinolone signalling (PQS) system were significantly upregulated after carvacrol exposure (Table 2.2 C). The PQS system is an intermediate system between *las* and *rhl* quorum sensing pathways, and is involved in the synthesis of secondary metabolites (Pesci, Milbank et al. 1999), such as elastase B and pyocyanin (McKnight, Iglewski et al. 2000, Gallagher, McKnight et al. 2002). The genes upregulated with treatment were the *psqABCD* operon. Gene *psqA* activates anthranilite while *psqBCD* produce long-chain hydrocarbons which then react with anthranilite during the biosynthetic pathway of PQS (Calfee, Coleman et al. 2001). Increasesd expression of these genes did not correlate with increased production of secondary metabolites controlled by the PQS pathway due to the additional control of the *rhl* quorum sensing pathways and various other control systems for the synthesis of these secondary metabolites.

The production of pigments which aid both in iron acquisition and cytoxicity are important virulence factors of *P. aeruginosa*. Through absorbance readings of cultures supernants grown in the presence of either thymol or carvacrol, the production of pyoverdine was assessed. Towards PAO1 very little change was found with treatment of either compound except an increase in pyoveredine was found with the 0.25 MIC concentration treatment of thymol (Figure 2.6 A). The mucoid isolate displayed significant reductions in pyoverdine for either compound. The vehicle control was again found to only affect the non-mucoid isolate with a significant decrease that of measured for the 0.5 MIC concentration of either extract on the non-mucoid isoalte. The 0.25 MIC concentration of carvacrol treatment displayed a rescue effect of pyoverdine production

for the non-mucoid isolate but as stated it is unclear why these results are only found in the non-mucoid isolate.

Genes involved in both pyoverdine and pyochelin biosynthesis were significantly increased with thymol or carvacrol exposure after 6 h and 24 h respectively (Table 2.2: A, C). The 24 h carvacrol incubation increased *pvdH*, *pvdJ*, *pvdD*, and *pvdL* (pyoverdine) as well as *pchA*, *pchF*, and *pchG* (pyochelin). The 6 h thymol incubation increased *pvdA*, *pvdS*, *pvdO*, and *pvdH* (pyvoerdine) as well as *pchD* and *pchC* (pyochelin) were significantly increased. As detailed in Chapter 2, both pyoverdine and pycohelin are siderophore pigments synthesized and secreted by *P. aeruginosa* as primary iron acquisition molecules (Budzikiewicz 1993, Lamont, Beare et al. 2002). Increased expression of synthesis genes for both siderophore pigments suggests treatment with thymol or carvacrol disruptes the bacteria's ability to sequester iron, or by unknown mechanisms triggers the increased expression and synthesis of genes for either pigment. Overall increases in pyoverdine production were not directly measured for the PAO1 as only a few genes involved in the synthesis pathways were increased in expression. Increased expression of siderophore pigments during an infection could increase *P. aeruginosa's* iron sequestering capabilities which could lead to growth stability in an otherwise growth restrictive environment. Pyoverdine is involved in various other iron-dependent signalling pathways within the cell, and therefore increase production of pyoverdine could have multiple final effects (Lamont, Beare et al. 2002).

Increased expression of bacterioferritin (*bfrB*), an iron storage protein, was also significantly increased after the 24 h carvacrol exposure (Table 2.2 C) (Burrowes, Baysse et al. 2006). In constrast, the 24 h thymol exposure reduced *bfrB* expression (Table 2.2

B). Differences in the expression of *bfrB* after the exposure to either compound may be indicative of differences in a time dependent effect of the compounds towards the bacteria or in how either compound effects bacterial iron allocation.

After exposure to thymol for 6 h, three additionaly genes involved in iron acquisition were also increased in expression (Table 2.2 A). Gene *tonB*, an essential protein involved in the internalization of siderophore pigments and heme utilization (Takase, Nitanai et al. 2000), *phuR* a heme/hemeoglobin outer membrane receptor protein (Ochsner, Johnson et al. 2000), and *hitA* a ferric-binding protein involved in perplasmic transfer were all increased in expression (Sanders, Cope et al. 1994). The protein encoded by *phuR* is involved in the TonB dependent heme uptake system which involves several other genes in the *phuSTUVW* (Ochsner, Johnson et al. 2000). Increased expression of multiple iron acquisition genes, paired with the increase in pyoverdine and pyochelin synthesis suggest an increased need for iron acquisition after treatment with thymol or carvacrol. Stress from the treatment of the bacteria with either compound may be triggering a response in the bacteria to focus on growth requirements, and therefore iron sequestering mechanisms would be engaged.

An additional virulence associated pigment of *P. aeruginosa* is pyocyanin.As addressed in Chapter 2, pyocyanin synthesis involves the operons *phzA1B1C1D1E1F1G1*/*phzA2B2C2D2E2F2G2* and three modifying enzymes *phzM*, *phzS*, and *phzH* (Mavrodi, Bonsall et al. 2001). Incubation with thymol for 6 h decreased the expression of *phzB1/phzB2*, *phzC1/phzC2*, *phzE1/phzE2*, *phzF1/phzF2*, *phzG2*, *phzS* (Table 2.2 A). Reduction of an important virulence factor such as pyocyanin could decrease *P. aeruginosa's* pathogenic potential during an infection.

Finally, as seen with the 24 h aqueous extract exposure, 24 h of exposure to carvacrol caused significant upregulations to genes involved in ribosomal structure and function (Table 2.2 C). Pathway analysis revealed an enrichment of 60.32 with just over 50% of the genes in the pathway significantly affected (Table 2.4 B). Overall 18 genes encoding ribosomal 50S proteins and 9 genes encoding ribosomal 30S proteins were significantly up-regulated. In combination with this, both elongation factor G (*fusA1*) and a DNA directed RNA polymerase (*rpoA*) were both increased in expression (Gil, Silva et al. 2004). As described in Chapter 2, increases in the ribosome pathway may be indicative of a great deal of protein turnover in the cell. Recycling of unnecessary proteins to create metabolites for the assembly of new proteins conserves the need to acquire raw materials which is an important strategy in situations, such as infections, where extracellular materials may be limited. No increases in ribosomal structure or function were found after the 24 h exposure to thymol, which suggests a fundamental difference in the way carvacrol and thymol affect and the growth of *P. aeruginosa.*Such differences may also be indicative of differences in growth-phase dependent effects of either compound against *P. aeruinogsa.*

Although many similarities were found between the two compound treatements effects towards *P. aeruignosa* growth, metabolism, and virulence, fundamental similarities and differences were detected. Both compounds were capable of reducing *P. aerguinosa* growth to the point of inhibition for all strains dependent on the media used. Although adherence in the presence of either compound did not alter PAO1 or the non-mucoid strain response, however the mucoid clinical isolate adherence was reduced. This provides evidence for the possibility of either compound to be effective against clinical

isolates in regards to biofilm establishment. These results were corroborated for preformed biofilms treated with either compound. Pyoverdine and pyochelin synthesis genes were both found to increase in expression with treatment of either compound, however pyoverdine directly measured in culture supernatants displayed strain specific results. Increased expression of siderophore pigment synthesis genes may be indicative of future increases in pigment production, suggesting an increasing need for iron acquisition. Decreased expression of phenazine synthesis genes were also found for the 6hr thymol incubation, although it is possible that decreases with carvacrol may also have been found if RNA had been assessed after only 6 h.

Differences between the effects of thymol and carvacrol for both protease and lipase production were minimal. Decreases in protease expression were found for both PAO1 and the non-mucoid isolate; however inconclusive results were obtained for the mucoid isolate. Lipid hydrolysis assays displayed increased amounts of lipase with either treatment for PAO1, while significant decreases were found for the mucoid isolate, and inconclusive results were presented in the non-mucoid isolate. Swimming motility was fairly universally decreased in all strains after the exposure to either compound.

The greatest difference between the two compounds was found with respect to the ribosomal pathway enrichment after the 24 h carvacrol treatment. This suggests a fundamental difference in either the timing of thymol's or carvacrol's action on *P. aeruginosa*, or a difference in the mechanism in which they affect the bacteria. Further exploration into this area may produce a better understanding of why such dramatic increases in ribosomal activity were found.

In regards to pathogenicity, there is potential for either compound to work as a growth inhibiting treatment. However, it would likely be most effective when bacteria are growing planktonically and therefore is not well suited for chronic state infections of *P. aeruginosa*. Mixed effects regarding virulence factor production suggest that side effects of thymol or carvacrol treatment may cause more harm than good in complicated infection situations, however only two clinical isolates were analyzed in this study. To make a firm assessment of the efficacy of either compound as a therapeutic agent towards *P. aeruginosa* infections, larger sample sizes and repeated standardized testing would provide more concrete evidence for the adoption of these compounds as future antibacterial therapies.

Conclusion

Evidence acquired during this in vitro analysis of *P. aeruginosa* pathogenicity in the presence of multiple plant extracts revealed the ability of naturally derived compounds to inhibit bacterial virulence and growth in multiple ways. Reduction of bacterial growth, biofilm formation, bacterial virulence factor secretion and synthesis, and bacterial motility after exposure to the aqueous ginseng extract of *Panax quinquefolius* provide evidence and encouragement for further development of this extract. Although such intriguing results were not found with the alcoholic ginseng extract, development of purified compounds from the extract may produce compounds with more promising antibacterial properties. Due to the low concentrations needed to inhibit bacterial growth with either thymol or carvacrol, both show potential for future development as antibacterial treatments. Continued exploration into the efficacy of these plant derived components against other species of bacteria, in synergy with current antibacterial drugs and each other, as well as further purification and chemical analysis will undoubtedly help to define the proper dosage and medicinal use for each of these compounds.

References

Abman, S. H., J. W. Ogle, R. J. Harbeck, N. Butler-Simon, K. B. Hammond and F. J. Accurso (1991). "Early bacteriologic, immunologic, and clinical courses of young infants with cystic fibrosis identified by neonatal screening." J Pediatr **119**(2): 211-217.

Ahn, J. Y., I. S. Choi, J. Y. Shim, E. K. Yun, Y. S. Yun, G. Jeong and J. Y. Song (2006). "The immunomodulator ginsan induces resistance to experimental sepsis by inhibiting Toll-like receptor-mediated inflammatory signals." Eur J Immunol **36**(1): 37-45.

Alipour, M., A. Omri and Z. E. Suntres (2011). "Ginseng aqueous extract attenuates the production of virulence factors, stimulates twitching and adhesion, and eradicates biofilms of Pseudomonas aeruginosa." Can J Physiol Pharmacol **89**(6): 419-427.

Alverdy, J., C. Holbrook, F. Rocha, L. Seiden, R. L. Wu, M. Musch, E. Chang, D. Ohman and S. Suh (2000). "Gut-derived sepsis occurs when the right pathogen with the right virulence genes meets the right host: evidence for in vivo virulence expression in Pseudomonas aeruginosa." Ann Surg **232**(4): 480-489.

Amako, K. and A. Umeda (1982). "Flagellation of Pseudomonas aeruginosa during the cell division cycle." Microbiol Immunol **26**(2): 113-117.

Arai, H., M. Hayashi, A. Kuroi, M. Ishii and Y. Igarashi (2005). "Transcriptional regulation of the flavohemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of Pseudomonas aeruginosa." J Bacteriol **187**(12): 3960-3968.

Arai, H., T. Kodama and Y. Igarashi (1997). "Cascade regulation of the two CRP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in Pseudomonas aeruginosa." Mol Microbiol **25**(6): 1141-1148.

Arora, S. K., B. W. Ritchings, E. C. Almira, S. Lory and R. Ramphal (1998). "The Pseudomonas aeruginosa flagellar cap protein, FliD, is responsible for mucin adhesion." Infect Immun **66**(3): 1000-1007.

Assinewe, V. A., J. T. Amason, A. Aubry, J. Mullin and I. Lemaire (2002). "Extractable polysaccharides of Panax quinquefolius L. (North American ginseng) root stimulate TNFalpha production by alveolar macrophages." Phytomedicine **9**(5): 398-404.

Attele, A. S., J. A. Wu and C. S. Yuan (1999). "Ginseng pharmacology: multiple constituents and multiple actions." Biochem Pharmacol **58**(11): 1685-1693.

Bagamboula, C. F., M. Uyttendaele and J. Debevere (2004). "Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards Shigella sonnei and S-flexneri." Food Microbiology **21**(1): 33-42.

Barbieri, J. T. and J. Sun (2004). "Pseudomonas aeruginosa ExoS and ExoT." Rev Physiol Biochem Pharmacol **152**: 79-92.

Bauernfeind, A., G. Emminger, G. Horl, B. Lorbeer, B. Przyklenk and C. Weisslein-Pfister (1987). "Selective pressure of antistaphylococcal chemotherapeutics in favour of Pseudomonas aeruginosa in cystic fibrosis." Infection **15**(6): 469-470.

Beare, P. A., R. J. For, L. W. Martin and I. L. Lamont (2003). "Siderophore-mediated cell signalling in Pseudomonas aeruginosa: divergent pathways regulate virulence factor production and siderophore receptor synthesis." Mol Microbiol **47**(1): 195-207.

Belogortseva, N. I., J. Y. Yoon and K. H. Kim (2000). "Inhibition of Helicobacter pylori hemagglutination by polysaccharide fractions from roots of Panax ginseng." Planta Med **66**(3): 217-220.

Benz, R. and R. E. Hancock (1981). "Properties of the large ion-permeable pores formed from protein F of Pseudomonas aeruginosa in lipid bilayer membranes." Biochim Biophys Acta **646**(2): 298-308.

Birch, O. M., M. Fuhrmann and N. M. Shaw (1995). "Biotin synthase from Escherichia coli, an investigation of the low molecular weight and protein components required for activity in vitro." J Biol Chem **270**(32): 19158-19165.

Birrer, P., N. G. McElvaney, A. Rudeberg, C. W. Sommer, S. Liechti-Gallati, R. Kraemer, R. Hubbard and R. G. Crystal (1994). "Protease-antiprotease imbalance in the lungs of children with cystic fibrosis." Am J Respir Crit Care Med **150**(1): 207-213.

Blocker, A., N. Jouihri, E. Larquet, P. Gounon, F. Ebel, C. Parsot, P. Sansonetti and A. Allaoui (2001). "Structure and composition of the Shigella flexneri "needle complex", a part of its type III secreton." Mol Microbiol **39**(3): 652-663.

Bodey, G. P., R. Bolivar, V. Fainstein and L. Jadeja (1983). "Infections caused by Pseudomonas aeruginosa." Rev Infect Dis **5**(2): 279-313.

Botelho, M. A., J. G. Bezerra Filho, L. L. Correa, S. G. Fonseca, D. Montenegro, R. Gapski, G. A. Brito and J. Heukelbach (2007). "Effect of a novel essential oil mouthrinse without alcohol on gingivitis: a double-blinded randomized controlled trial." J Appl Oral Sci **15**(3): 175-180.

Bradley, D. E. (1980). "A function of Pseudomonas aeruginosa PAO polar pili: twitching motility." Can J Microbiol **26**(2): 146-154.

Britigan, B. E., M. A. Railsback and C. D. Cox (1999). "The Pseudomonas aeruginosa secretory product pyocyanin inactivates alpha1 protease inhibitor: implications for the pathogenesis of cystic fibrosis lung disease." Infect Immun **67**(3): 1207-1212.

Budzikiewicz, H. (1993). "Secondary metabolites from fluorescent pseudomonads." FEMS Microbiol Rev **10**(3-4): 209-228.

Budzikiewicz, H. (2004). "Siderophores of the Pseudomonadaceae sensu stricto (fluorescent and non-fluorescent Pseudomonas spp.)." Fortschr Chem Org Naturst **87**: 81-237.

Burns, J. L., R. L. Gibson, S. McNamara, D. Yim, J. Emerson, M. Rosenfeld, P. Hiatt, K. McCoy, R. Castile, A. L. Smith and B. W. Ramsey (2001). "Longitudinal assessment of Pseudomonas aeruginosa in young children with cystic fibrosis." J Infect Dis **183**(3): 444-452.

Burrowes, E., C. Baysse, C. Adams and F. O'Gara (2006). "Influence of the regulatory protein RsmA on cellular functions in Pseudomonas aeruginosa PAO1, as revealed by transcriptome analysis." Microbiology **152**(Pt 2): 405-418.

Burt, S. (2004). "Essential oils: their antibacterial properties and potential applications in foods--a review." Int J Food Microbiol **94**(3): 223-253.

Bye, M. R., J. M. Ewig and L. M. Quittell (1994). "Cystic fibrosis." Lung **172**(5): 251-270.

Calfee, M. W., J. P. Coleman and E. C. Pesci (2001). "Interference with Pseudomonas quinolone signal synthesis inhibits virulence factor expression by Pseudomonas aeruginosa." Proc Natl Acad Sci U S A **98**(20): 11633-11637.

Castillo, S., C. O. Perez-Alfonso, D. Martinez-Romero, F. Guillen, M. Serrano and D. Valero (2014). "The essential oils thymol and carvacrol applied in the packing lines avoid lemon spoilage and maintain quality during storage." Food Control **35**(1): 132-136.

Chernish, R. N. and S. D. Aaron (2003). "Approach to resistant gram-negative bacterial pulmonary infections in patients with cystic fibrosis." Curr Opin Pulm Med **9**(6): 509-515.

Cianciotto, N. P. (2005). "Type II secretion: a protein secretion system for all seasons." Trends Microbiol **13**(12): 581-588.

Ciofu, O., B. Riis, T. Pressler, H. E. Poulsen and N. Hoiby (2005). "Occurrence of hypermutable Pseudomonas aeruginosa in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation." Antimicrob Agents Chemother **49**(6): 2276-2282.

Cohen, M. L. (1986). "Staphylococcus aureus: biology, mechanisms of virulence, epidemiology." J Pediatr **108**(5 Pt 2): 796-799.

Corda, D. and M. Di Girolamo (2003). "Functional aspects of protein mono-ADP-ribosylation." EMBO J **22**(9): 1953-1958.

Costerton, J. W., P. S. Stewart and E. P. Greenberg (1999). "Bacterial biofilms: a common cause of persistent infections." Science **284**(5418): 1318-1322.

Cowan, M. M. (1999). "Plant products as antimicrobial agents." Clin Microbiol Rev **12**(4): 564-582.

Cox, C. D. and P. Adams (1985). "Siderophore activity of pyoverdin for Pseudomonas aeruginosa." Infect Immun **48**(1): 130-138.

Cox, C. D., K. L. Rinehart, Jr., M. L. Moore and J. C. Cook, Jr. (1981). "Pyochelin: novel structure of an iron-chelating growth promoter for Pseudomonas aeruginosa." Proc Natl Acad Sci U S A **78**(7): 4256-4260.

Cruse-Sanders, J. M. and J. L. Hamrick (2004). "Genetic diversity in harvested and protected populations of wild American ginseng, Panax quinquefolius L. (Araliaceae)." Am J Bot **91**(4): 540-548.

Damron, F. H. and J. B. Goldberg (2012). "Proteolytic regulation of alginate overproduction in Pseudomonas aeruginosa." Mol Microbiol **84**(4): 595-607.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton and E. P. Greenberg (1998). "The involvement of cell-to-cell signals in the development of a bacterial biofilm." Science **280**(5361): 295-298.

de Kievit, T., P. C. Seed, J. Nezezon, L. Passador and B. H. Iglewski (1999). "RsaL, a novel repressor of virulence gene expression in Pseudomonas aeruginosa." J Bacteriol **181**(7): 2175-2184.

Debarbouille, M., R. Gardan, M. Arnaud and G. Rapoport (1999). "Role of bkdR, a transcriptional activator of the sigL-dependent isoleucine and valine degradation pathway in Bacillus subtilis." J Bacteriol **181**(7): 2059-2066.

Demko, C. A. and M. J. Thomassen (1980). "EFFECT OF MUCOID PROPERTY ON ANTIBIOTIC SUSCEPTIBILITY OF PSEUDOMONAS-AERUGINOSA." Current Microbiology **4**(2): 69-73.

Denning, G. M., L. A. Wollenweber, M. A. Railsback, C. D. Cox, L. L. Stoll and B. E. Britigan (1998). "Pseudomonas pyocyanin increases interleukin-8 expression by human airway epithelial cells." Infect Immun **66**(12): 5777-5784.

Denyer, S. P. and G. Stewart (1998). "Mechanisms of action of disinfectants." International Biodeterioration & Biodegradation **41**(3-4): 261-268.

Elizur, A., C. L. Cannon and T. W. Ferkol (2008). "Airway inflammation in cystic fibrosis." Chest **133**(2): 489-495.

Finck-Barbancon, V., J. Goranson, L. Zhu, T. Sawa, J. P. Wiener-Kronish, S. M. Fleiszig, C. Wu, L. Mende-Mueller and D. W. Frank (1997). "ExoU expression by Pseudomonas aeruginosa correlates with acute cytotoxicity and epithelial injury." Mol Microbiol **25**(3): 547-557.

Flume, P. A., P. J. Mogayzel, Jr., K. A. Robinson, C. H. Goss, R. L. Rosenblatt, R. J. Kuhn, B. C. Marshall and C. Clinical Practice Guidelines for Pulmonary Therapies (2009). "Cystic fibrosis pulmonary guidelines: treatment of pulmonary exacerbations." Am J Respir Crit Care Med **180**(9): 802-808.

Forteza, R., I. T. Lauredo, R. Burch and W. M. Abraham (1994). "Extracellular metabolites of Pseudomonas aeruginosa produce bronchoconstriction by different mechanisms." Am J Respir Crit Care Med **149**(3 Pt 1): 687-693.

Frederiksen, B., C. Koch and N. Hoiby (1997). "Antibiotic treatment of initial colonization with Pseudomonas aeruginosa postpones chronic infection and prevents deterioration of pulmonary function in cystic fibrosis." Pediatr Pulmonol **23**(5): 330-335.

Frithz-Lindsten, E., A. Holmstrom, L. Jacobsson, M. Soltani, J. Olsson, R. Rosqvist and A. Forsberg (1998). "Functional conservation of the effector protein translocators PopB/YopB and PopD/YopD of Pseudomonas aeruginosa and Yersinia pseudotuberculosis." Mol Microbiol **29**(5): 1155-1165.

Fuqua, W. C., S. C. Winans and E. P. Greenberg (1994). "Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators." J Bacteriol **176**(2): 269-275.

Fuzzati, N. (2004). "Analysis methods of ginsenosides." Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences **812**(1-2): 119-133.

Gaille, C., P. Kast and D. Haas (2002). "Salicylate biosynthesis in Pseudomonas aeruginosa. Purification and characterization of PchB, a novel bifunctional enzyme displaying isochorismate pyruvate-lyase and chorismate mutase activities." J Biol Chem **277**(24): 21768-21775.

Gallagher, L. A., S. L. McKnight, M. S. Kuznetsova, E. C. Pesci and C. Manoil (2002). "Functions required for extracellular quinolone signaling by Pseudomonas aeruginosa." J Bacteriol **184**(23): 6472-6480.

Galloway, D. R. (1991). "Pseudomonas aeruginosa elastase and elastolysis revisited: recent developments." Mol Microbiol **5**(10): 2315-2321.

Gambello, M. J. and B. H. Iglewski (1991). "Cloning and characterization of the Pseudomonas aeruginosa lasR gene, a transcriptional activator of elastase expression." J Bacteriol **173**(9): 3000-3009.

Gambello, M. J., S. Kaye and B. H. Iglewski (1993). "LasR of Pseudomonas aeruginosa is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression." Infect Immun **61**(4): 1180-1184.

Gibson, R. L., J. Emerson, S. McNamara, J. L. Burns, M. Rosenfeld, A. Yunker, N. Hamblett, F. Accurso, M. Dovey, P. Hiatt, M. W. Konstan, R. Moss, G. Retsch-Bogart, J. Wagener, D. Waltz, R. Wilmott, P. L. Zeitlin, B. Ramsey and G. Cystic Fibrosis Therapeutics Development Network Study (2003). "Significant microbiological effect of inhaled tobramycin in young children with cystic fibrosis." Am J Respir Crit Care Med **167**(6): 841-849.

Gil, R., F. J. Silva, J. Pereto and A. Moya (2004). "Determination of the core of a minimal bacterial gene set." Microbiol Mol Biol Rev **68**(3): 518-537, table of contents.

Gill, A. O. and R. A. Holley (2006). "Disruption of Escherichia coli, Listeria monocytogenes and Lactobacillus sakei cellular membranes by plant oil aromatics." Int J Food Microbiol **108**(1): 1-9.

Gilligan, P. H. (1991). "Microbiology of airway disease in patients with cystic fibrosis." Clin Microbiol Rev **4**(1): 35-51.

Gordon, C. A., N. A. Hodges and C. Marriott (1988). "Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis-derived Pseudomonas aeruginosa." J Antimicrob Chemother **22**(5): 667-674.

Gorisch, H. (2003). "The ethanol oxidation system and its regulation in Pseudomonas aeruginosa." Biochim Biophys Acta **1647**(1-2): 98-102.

Govan, J. R. and V. Deretic (1996). "Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia." Microbiol Rev **60**(3): 539-574.

Govan, J. R. and J. A. Fyfe (1978). "Mucoid Pseudomonas aeruginosa and cystic fibrosis: resistance of the mucoid from to carbenicillin, flucloxacillin and tobramycin and the isolation of mucoid variants in vitro." J Antimicrob Chemother **4**(3): 233-240.

Gupta, R., N. Gupta and P. Rathi (2004). "Bacterial lipases: an overview of production, purification and biochemical properties." Appl Microbiol Biotechnol **64**(6): 763-781.

Hansen, C. R., T. Pressler and N. Hoiby (2008). "Early aggressive eradication therapy for intermittent Pseudomonas aeruginosa airway colonization in cystic fibrosis patients: 15 years experience." J Cyst Fibros **7**(6): 523-530.

Hardalo, C. and S. C. Edberg (1997). "Pseudomonas aeruginosa: assessment of risk from drinking water." Crit Rev Microbiol **23**(1): 47-75.

Hasegawa, H. (2004). "Proof of the mysterious efficacy of ginseng: Basic and clinical trials: Metabolic activation of ginsenoside: Deglycosylation by intestinal bacteria and esterification with fatty acid." Journal of Pharmacological Sciences **95**(2): 153-157.

Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill and A. Aderem (2001). "The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5." Nature **410**(6832): 1099-1103.

Heck, L. W., P. G. Alarcon, R. M. Kulhavy, K. Morihara, M. W. Russell and J. F. Mestecky (1990). "Degradation of IgA proteins by Pseudomonas aeruginosa elastase." J Immunol **144**(6): 2253-2257.

Heck, L. W., K. Morihara, W. B. McRae and E. J. Miller (1986). "Specific cleavage of human type III and IV collagens by Pseudomonas aeruginosa elastase." Infect Immun **51**(1): 115-118.

Heinrichs, D. E. and K. Poole (1996). "PchR, a regulator of ferripyochelin receptor gene (fptA) expression in Pseudomonas aeruginosa, functions both as an activator and as a repressor." J Bacteriol **178**(9): 2586-2592.

Helander, I. M., H. L. Alakomi, K. Latva-Kala, T. Mattila-Sandholm, I. Pol, E. J. Smid, L. G. M. Gorris and A. von Wright (1998). "Characterization of the action of selected essential oil components on gram-negative bacteria." Journal of Agricultural and Food Chemistry **46**(9): 3590-3595.

Hentzer, M., G. M. Teitzel, G. J. Balzer, A. Heydorn, S. Molin, M. Givskov and M. R. Parsek (2001). "Alginate overproduction affects Pseudomonas aeruginosa biofilm structure and function." J Bacteriol **183**(18): 5395-5401.

Hentzer, M., H. Wu, J. B. Andersen, K. Riedel, T. B. Rasmussen, N. Bagge, N. Kumar, M. A. Schembri, Z. Song, P. Kristoffersen, M. Manefield, J. W. Costerton, S. Molin, L. Eberl, P. Steinberg, S. Kjelleberg, N. Hoiby and M. Givskov (2003). "Attenuation of Pseudomonas aeruginosa virulence by quorum sensing inhibitors." EMBO J **22**(15): 3803-3815.

Hoiby, N., H. Krogh Johansen, C. Moser, Z. Song, O. Ciofu and A. Kharazmi (2001). "Pseudomonas aeruginosa and the in vitro and in vivo biofilm mode of growth." Microbes Infect **3**(1): 23-35.

Hong, Y. Q. and B. Ghebrehiwet (1992). "Effect of Pseudomonas aeruginosa elastase and alkaline protease on serum complement and isolated components C1q and C3." Clin Immunol Immunopathol **62**(2): 133-138.

Iannitelli, A., R. Grande, A. Di Stefano, M. Di Giulio, P. Sozio, L. J. Bessa, S. Laserra, C. Paolini, F. Protasi and L. Cellini (2011). "Potential Antibacterial Activity of Carvacrol-Loaded Poly(DL-lactide-co-glycolide) (PLGA) Nanoparticles against Microbial Biofilm." Int J Mol Sci **12**(8): 5039-5051.

Irizarry, R. A., B. Hobbs, F. Collin, Y. D. Beazer-Barclay, K. J. Antonellis, U. Scherf and T. P. Speed (2003). "Exploration, normalization, and summaries of high density oligonucleotide array probe level data." Biostatistics **4**(2): 249-264.

Ivanova, T., Y. Han, H. J. Son, Y. S. Yun and J. Y. Song (2006). "Antimutagenic effect of polysaccharide ginsan extracted from Panax ginseng." Food Chem Toxicol **44**(4): 517-521.

Jensen, P. O., T. Bjarnsholt, R. Phipps, T. B. Rasmussen, H. Calum, L. Christoffersen, C. Moser, P. Williams, T. Pressler, M. Givskov and N. Hoiby (2007). "Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by Pseudomonas aeruginosa." Microbiology **153**(Pt 5): 1329-1338.

Jia, L. and Y. Q. Zhao (2009). "Current Evaluation of the Millennium Phytomedicine-Ginseng (I): Etymology, Pharmacognosy, Phytochemistry, Market and Regulations." Current Medicinal Chemistry **16**(19): 2475-2484.

Johansen, H. K., H. P. Hougen, S. J. Cryz, Jr., J. Rygaard and N. Hoiby (1995). "Vaccination promotes TH1-like inflammation and survival in chronic Pseudomonas aeruginosa pneumonia in rats." Am J Respir Crit Care Med **152**(4 Pt 1): 1337-1346.

Karpanen, T. J., T. Worthington, E. R. Hendry, B. R. Conway and P. A. Lambert (2008). "Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of Staphylococcus epidermidis." J Antimicrob Chemother **62**(5): 1031-1036.

Kaufman, M. R., J. Jia, L. Zeng, U. Ha, M. Chow and S. Jin (2000). "Pseudomonas aeruginosa mediated apoptosis requires the ADP-ribosylating activity of exoS." Microbiology **146 (Pt 10)**: 2531-2541.

Kearns, D. B., J. Robinson and L. J. Shimkets (2001). "Pseudomonas aeruginosa exhibits directed twitching motility up phosphatidylethanolamine gradients." J Bacteriol **183**(2): 763-767.

Kim, J. Y., D. R. Germolec and M. I. Luster (1990). "Panax ginseng as a potential immunomodulator: studies in mice." Immunopharmacol Immunotoxicol **12**(2): 257-276.

Knowles, J. R., S. Roller, D. B. Murray and A. S. Naidu (2005). "Antimicrobial action of carvacrol at different stages of dual-species biofilm development by Staphylococcus aureus and Salmonella enterica serovar Typhimurium." Appl Environ Microbiol **71**(2): 797-803.

Kolter, R., D. A. Siegele and A. Tormo (1993). "The stationary phase of the bacterial life cycle." Annu Rev Microbiol **47**: 855-874.

Lambert, P. A. and S. M. Hammond (1973). "Potassium fluxes, first indications of membrane damage in micro-organisms." Biochem Biophys Res Commun **54**(2): 796-799.

Lambert, R. J. W., P. N. Skandamis, P. J. Coote and G. J. E. Nychas (2001). "A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol." Journal of Applied Microbiology **91**(3): 453-462.

Lamont, I. L., P. A. Beare, U. Ochsner, A. I. Vasil and M. L. Vasil (2002). "Siderophore-mediated signaling regulates virulence factor production in Pseudomonasaeruginosa." Proc Natl Acad Sci U S A **99**(10): 7072-7077.

Lamont, I. L. and L. W. Martin (2003). "Identification and characterization of novel pyoverdine synthesis genes in Pseudomonas aeruginosa." Microbiology **149**(Pt 4): 833-842.

Lau, G. W., D. J. Hassett, H. Ran and F. Kong (2004). "The role of pyocyanin in Pseudomonas aeruginosa infection." Trends Mol Med **10**(12): 599-606.

Laughlin, R. S., M. W. Musch, C. J. Hollbrook, F. M. Rocha, E. B. Chang and J. C. Alverdy (2000). "The key role of Pseudomonas aeruginosa PA-I lectin on experimental gut-derived sepsis." Ann Surg **232**(1): 133-142.

Lauhon, C. T. and R. Kambampati (2000). "The iscS gene in Escherichia coli is required for the biosynthesis of 4-thiouridine, thiamin, and NAD." J Biol Chem **275**(26): 20096-20103.

Lauredo, I. T., J. R. Sabater, A. Ahmed, Y. Botvinnikova and W. M. Abraham (1998). "Mechanism of pyocyanin- and 1-hydroxyphenazine-induced lung neutrophilia in sheep airways." J Appl Physiol (1985) **85**(6): 2298-2304.

Lebert, I., S. Leroy and R. Talon (2007). "Effect of industrial and natural biocides on spoilage, pathogenic and technological strains grown in biofilm." Food Microbiol **24**(3): 281-287.

Lee, A., W. Mao, M. S. Warren, A. Mistry, K. Hoshino, R. Okumura, H. Ishida and O. Lomovskaya (2000). "Interplay between efflux pumps may provide either additive or multiplicative effects on drug resistance." J Bacteriol **182**(11): 3142-3150.

Lee, J. H., E. K. Park, C. S. Uhm, M. S. Chung and K. H. Kim (2004). "Inhibition of Helicobacter pylori adhesion to human gastric adenocarcinoma epithelial cells by acidic polysaccharides from Artemisia capillaris and Panax ginseng." Planta Med **70**(7): 615-619.

Lee, J. H., J. S. Shim, J. S. Lee, M. K. Kim, M. S. Chung and K. H. Kim (2006). "Pectin-like acidic polysaccharide from Panax ginseng with selective antiadhesive activity against pathogenic bacteria." Carbohydr Res **341**(9): 1154-1163.

Lee, V. T., R. S. Smith, B. Tummler and S. Lory (2005). "Activities of Pseudomonas aeruginosa effectors secreted by the Type III secretion system in vitro and during infection." Infect Immun **73**(3): 1695-1705.

Lemmon, H. R., J. Sham, L. A. Chau and J. Madrenas (2012). "High molecular weight polysaccharides are key immunomodulators in North American ginseng extracts: characterization of the ginseng genetic signature in primary human immune cells." J Ethnopharmacol **142**(1): 1-13.

Lewis, K. (2005). "Persister cells and the riddle of biofilm survival." Biochemistry (Mosc) **70**(2): 267-274.

Li, X. Z., H. Nikaido and K. Poole (1995). "Role of mexA-mexB-oprM in antibiotic efflux in Pseudomonas aeruginosa." Antimicrob Agents Chemother **39**(9): 1948-1953.

Li, X. Z., L. Zhang and K. Poole (2000). "Interplay between the MexA-MexB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of Pseudomonas aeruginosa." J Antimicrob Chemother **45**(4): 433-436.

Lister, P. D., D. J. Wolter and N. D. Hanson (2009). "Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms." Clin Microbiol Rev **22**(4): 582-610.

Litwin, C. M. and S. B. Calderwood (1993). "Role of iron in regulation of virulence genes." Clin Microbiol Rev **6**(2): 137-149.

Livermore, D. M. (1995). "beta-Lactamases in laboratory and clinical resistance." Clin Microbiol Rev **8**(4): 557-584.

Livermore, D. M. (2002). "Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: our worst nightmare?" Clin Infect Dis **34**(5): 634-640.

Lu, J. M., Q. Yao and C. Chen (2009). "Ginseng compounds: an update on their molecular mechanisms and medical applications." Curr Vasc Pharmacol **7**(3): 293-302.

Lubamba, B., B. Dhooghe, S. Noel and T. Leal (2012). "Cystic fibrosis: insight into CFTR pathophysiology and pharmacotherapy." Clin Biochem **45**(15): 1132-1144.

Lukacs, G. L. and A. S. Verkman (2012). "CFTR: folding, misfolding and correcting the DeltaF508 conformational defect." Trends Mol Med **18**(2): 81-91.

Lyczak, J. B., C. L. Cannon and G. B. Pier (2002). "Lung infections associated with cystic fibrosis." Clin Microbiol Rev **15**(2): 194-222.

Ma, L., M. Conover, H. Lu, M. R. Parsek, K. Bayles and D. J. Wozniak (2009). "Assembly and development of the Pseudomonas aeruginosa biofilm matrix." PLoS Pathog **5**(3): e1000354.

Madhusudhan, K. T., D. Lorenz and J. R. Sokatch (1993). "The bkdR gene of Pseudomonas putida is required for expression of the bkd operon and encodes a protein related to Lrp of Escherichia coli." J Bacteriol **175**(13): 3934-3940.

Mann, E. E. and D. J. Wozniak (2012). "Pseudomonas biofilm matrix composition and niche biology." FEMS Microbiol Rev **36**(4): 893-916.

Marsh, P. D. (1992). "Microbiological aspects of the chemical control of plaque and gingivitis." J Dent Res **71**(7): 1431-1438.

Martin, D. W., M. J. Schurr, M. H. Mudd, J. R. Govan, B. W. Holloway and V. Deretic (1993). "Mechanism of conversion to mucoidy in Pseudomonas aeruginosa infecting cystic fibrosis patients." Proc Natl Acad Sci U S A **90**(18): 8377-8381.

Martinez, J. L., A. Delgado-Iribarren and F. Baquero (1990). "Mechanisms of iron acquisition and bacterial virulence." FEMS Microbiol Rev **6**(1): 45-56.

Masuda, N., N. Gotoh, C. Ishii, E. Sakagawa, S. Ohya and T. Nishino (1999). "Interplay between chromosomal beta-lactamase and the MexAB-OprM efflux system in intrinsic resistance to beta-lactams in Pseudomonas aeruginosa." Antimicrob Agents Chemother **43**(2): 400-402.

Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, H. Tsujimoto and T. Nishino (2000). "Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in Pseudomonas aeruginosa." Antimicrob Agents Chemother **44**(12): 3322-3327.

Mattick, J. S. (2002). "Type IV pili and twitching motility." Annu Rev Microbiol **56**: 289-314.

Mavrodi, D. V., R. F. Bonsall, S. M. Delaney, M. J. Soule, G. Phillips and L. S. Thomashow (2001). "Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from Pseudomonas aeruginosa PAO1." J Bacteriol **183**(21): 6454-6465.

May, T. B., D. Shinabarger, R. Maharaj, J. Kato, L. Chu, J. D. DeVault, S. Roychoudhury, N. A. Zielinski, A. Berry, R. K. Rothmel and et al. (1991). "Alginate synthesis by Pseudomonas aeruginosa: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients." Clin Microbiol Rev **4**(2): 191-206.

Mayer-Hamblett, N., J. L. Burns, U. Khan, G. Retsch-Bogart, M. Treggiari and B. W. Ramsey (2010). "PREDICTORS OF PSEUDOMONAS AERUGINOSA RECURRENCE IN CYSTIC FIBROSIS: RESULTS FROM THE EPIC TRIAL." Pediatric Pulmonology: 326-327.

McClure, C. D. and N. L. Schiller (1992). "Effects of Pseudomonas aeruginosa rhamnolipids on human monocyte-derived macrophages." J Leukoc Biol **51**(2): 97-102.

McKnight, S. L., B. H. Iglewski and E. C. Pesci (2000). "The Pseudomonas quinolone signal regulates rhl quorum sensing in Pseudomonas aeruginosa." J Bacteriol **182**(10): 2702-2708.

McMorran, B. J., M. E. Merriman, I. T. Rombel and I. L. Lamont (1996). "Characterisation of the pvdE gene which is required for pyoverdine synthesis in Pseudomonas aeruginosa." Gene **176**(1-2): 55-59.

Mena, A., E. E. Smith, J. L. Burns, D. P. Speert, S. M. Moskowitz, J. L. Perez and A. Oliver (2008). "Genetic adaptation of Pseudomonas aeruginosa to the airways of cystic fibrosis patients is catalyzed by hypermutation." J Bacteriol **190**(24): 7910-7917.

Merriman, T. R., M. E. Merriman and I. L. Lamont (1995). "Nucleotide sequence of pvdD, a pyoverdine biosynthetic gene from Pseudomonas aeruginosa: PvdD has similarity to peptide synthetases." J Bacteriol **177**(1): 252-258.

Mesaros, N., P. Nordmann, P. Plesiat, M. Roussel-Delvallez, J. Van Eldere, Y. Glupczynski, Y. Van Laethem, F. Jacobs, P. Lebecque, A. Malfroot, P. M. Tulkens and F. Van Bambeke (2007). "Pseudomonas aeruginosa: resistance and therapeutic options at the turn of the new millennium." Clin Microbiol Infect **13**(6): 560-578.

Meyer, J. M., A. Stintzi, D. De Vos, P. Cornelis, R. Tappe, K. Taraz and H. Budzikiewicz (1997). "Use of siderophores to type pseudomonads: the three Pseudomonas aeruginosa pyoverdine systems." Microbiology **143 (Pt 1)**: 35-43.

Michel, B. C. (1988). "Antibacterial therapy in cystic fibrosis. A review of the literature published between 1980 and February 1987." Chest **94**(2 Suppl): 129S-140S.

Mihara, H. and N. Esaki (2002). "Bacterial cysteine desulfurases: their function and mechanisms." Appl Microbiol Biotechnol **60**(1-2): 12-23.

Miller, M. B. and B. L. Bassler (2001). "Quorum sensing in bacteria." Annu Rev Microbiol **55**: 165-199.

Morihara, K., H. Tsuzuki and K. Oda (1979). "Protease and elastase of Pseudomonas aeruginosa: inactivation of human plasma alpha 1-proteinase inhibitor." Infect Immun **24**(1): 188-193.

Moser, C., H. K. Johansen, Z. Song, H. P. Hougen, J. Rygaard and N. Hoiby (1997). "Chronic Pseudomonas aeruginosa lung infection is more severe in Th2 responding BALB/c mice compared to Th1 responding C3H/HeN mice." APMIS **105**(11): 838-842.

Muller, M. and T. C. Sorrell (1997). "Modulation of neutrophil superoxide response and intracellular diacylglyceride levels by the bacterial pigment pyocyanin." Infect Immun **65**(6): 2483-2487.

Nazer, A. I., A. Kobilinsky, J. L. Tholozan and F. Dubois-Brissonnet (2005). "Combinations of food antimicrobials at low levels to inhibit the growth of Salmonella sv. Typhimurium: a synergistic effect?" Food Microbiology **22**(5): 391-398.

Nevas, M., A. R. Korhonen, M. Lindstrom, P. Turkki and H. Korkeala (2004). "Antibacterial efficiency of Finnish spice essential oils against pathogenic and spoilage bacteria." J Food Prot **67**(1): 199-202.

Nickel, J. C., I. Ruseska, J. B. Wright and J. W. Costerton (1985). "Tobramycin resistance of Pseudomonas aeruginosa cells growing as a biofilm on urinary catheter material." Antimicrob Agents Chemother **27**(4): 619-624.

Normark, B. H. and S. Normark (2002). "Evolution and spread of antibiotic resistance." J Intern Med **252**(2): 91-106.

Nostro, A., A. Sudano Roccaro, G. Bisignano, A. Marino, M. A. Cannatelli, F. C. Pizzimenti, P. L. Cioni, F. Procopio and A. R. Blanco (2007). "Effects of oregano, carvacrol and thymol on Staphylococcus aureus and Staphylococcus epidermidis biofilms." J Med Microbiol **56**(Pt 4): 519-523.

Nutman, J., M. Berger, P. A. Chase, D. G. Dearborn, K. M. Miller, R. L. Waller and R. U. Sorensen (1987). "Studies on the mechanism of T cell inhibition by the Pseudomonas aeruginosa phenazine pigment pyocyanine." J Immunol **138**(10): 3481-3487.

O'Toole, G. A., K. A. Gibbs, P. W. Hager, P. V. Phibbs, Jr. and R. Kolter (2000). "The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by Pseudomonas aeruginosa." J Bacteriol **182**(2): 425-431.

O'Toole, G. A. and R. Kolter (1998). "Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development." Mol Microbiol **30**(2): 295-304.

O'Toole, G. A. and R. Kolter (1998). "Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis." Mol Microbiol **28**(3): 449-461.

Ochsner, U. A., Z. Johnson and M. L. Vasil (2000). "Genetics and regulation of two distinct haem-uptake systems, phu and has, in Pseudomonas aeruginosa." Microbiology **146 (Pt 1)**: 185-198.

Ottemann, K. M. and J. F. Miller (1997). "Roles for motility in bacterial-host interactions." Mol Microbiol **24**(6): 1109-1117.

Ouhayoun, J. P. (2003). "Penetrating the plaque biofilm: impact of essential oil mouthwash." J Clin Periodontol **30 Suppl 5**: 10-12.

Oussalah, M., S. Caillet, L. Saucier and M. Lacroix (2007). "Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: E-coli O157 : H7, Salmonella Typhimurium, Staphylococcus aureus and Listeria monocytogenes." Food Control **18**(5): 414-420.

Park, S., M. Yeo, J. H. Jin, K. M. Lee, J. Y. Jung, R. Choue, S. W. Cho and K. B. Hahm (2005). "Rescue of Helicobacter pylori-induced cytotoxicity by red ginseng." Dig Dis Sci **50**(7): 1218-1227.

Pavlovskis, O. R. (1972). "Pseudomonas aeruginosa exotoxin: effect on cellular and mitochondrial respiration." J Infect Dis **126**(1): 48-53.

Pearson, J. P., M. Feldman, B. H. Iglewski and A. Prince (2000). "Pseudomonas aeruginosa cell-to-cell signaling is required for virulence in a model of acute pulmonary infection." Infect Immun **68**(7): 4331-4334.

Pesci, E. C., J. B. Milbank, J. P. Pearson, S. McKnight, A. S. Kende, E. P. Greenberg and B. H. Iglewski (1999). "Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa." Proc Natl Acad Sci U S A **96**(20): 11229-11234.

Pessi, G., F. Williams, Z. Hindle, K. Heurlier, M. T. Holden, M. Camara, D. Haas and P. Williams (2001). "The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in Pseudomonas aeruginosa." J Bacteriol **183**(22): 6676-6683.

Poole, K., S. Neshat, K. Krebes and D. E. Heinrichs (1993). "Cloning and nucleotide sequence analysis of the ferripyoverdine receptor gene fpvA of Pseudomonas aeruginosa." J Bacteriol **175**(15): 4597-4604.

Popoff, M. R. (2005). "Bacterial exotoxins." Contrib Microbiol **12**: 28-54.

Qi, L. W., C. Z. Wang and C. S. Yuan (2011). "Ginsenosides from American ginseng: Chemical and pharmacological diversity." Phytochemistry **72**(8): 689-699.

Ramos, H. C., M. Rumbo and J. C. Sirard (2004). "Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa." Trends Microbiol **12**(11): 509-517.

Ratjen, F., G. Comes, K. Paul, H. G. Posselt, T. O. Wagner, K. Harms and F. German Board of the European Registry for Cystic (2001). "Effect of continuous antistaphylococcal therapy on the rate of P. aeruginosa acquisition in patients with cystic fibrosis." Pediatr Pulmonol **31**(1): 13-16.

Ratjen, F., G. Doring and W. H. Nikolaizik (2001). "Effect of inhaled tobramycin on early Pseudomonas aeruginosa colonisation in patients with cystic fibrosis." Lancet **358**(9286): 983-984.

Read, R. C., P. Roberts, N. Munro, A. Rutman, A. Hastie, T. Shryock, R. Hall, W. McDonald-Gibson, V. Lund, G. Taylor and et al. (1992). "Effect of Pseudomonas aeruginosa rhamnolipids on mucociliary transport and ciliary beating." J Appl Physiol (1985) **72**(6): 2271-2277.

Regnault-Roger, C., A. Hamraoui, M. Holeman, E. Theron and R. Pinel (1993). "Insecticidal effect of essential oils from mediterranean plants uponAcanthoscelides Obtectus Say (Coleoptera, Bruchidae), a pest of kidney bean (Phaseolus vulgaris L.)." J Chem Ecol **19**(6): 1233-1244.

Reimmann, C., H. M. Patel, C. T. Walsh and D. Haas (2004). "PchC thioesterase optimizes nonribosomal biosynthesis of the peptide siderophore pyochelin in Pseudomonas aeruginosa." J Bacteriol **186**(19): 6367-6373.

Riordan, J. R. (2008). "CFTR function and prospects for therapy." Annu Rev Biochem **77**: 701-726.

Rios, J. L., M. C. Recio and A. Villar (1988). "Screening methods for natural products with antimicrobial activity: a review of the literature." J Ethnopharmacol **23**(2-3): 127-149.

Rogers, G. B., L. R. Hoffman and G. Doring (2011). "Novel concepts in evaluating antimicrobial therapy for bacterial lung infections in patients with cystic fibrosis." J Cyst Fibros **10**(6): 387-400.

Rompf, A., C. Hungerer, T. Hoffmann, M. Lindenmeyer, U. Romling, U. Gross, M. O. Doss, H. Arai, Y. Igarashi and D. Jahn (1998). "Regulation of Pseudomonas aeruginosa hemF and hemN by the dual action of the redox response regulators Anr and Dnr." Mol Microbiol **29**(4): 985-997.

Rumbaugh, K. P., J. A. Griswold, B. H. Iglewski and A. N. Hamood (1999). "Contribution of quorum sensing to the virulence of Pseudomonas aeruginosa in burn wound infections." Infect Immun **67**(11): 5854-5862.

Saiman, L., K. Ishimoto, S. Lory and A. Prince (1990). "The effect of piliation and exoproduct expression on the adherence of Pseudomonas aeruginosa to respiratory epithelial monolayers." J Infect Dis **161**(3): 541-548.

Sanders, J. D., L. D. Cope and E. J. Hansen (1994). "Identification of a locus involved in the utilization of iron by Haemophilus influenzae." Infect Immun **62**(10): 4515-4525.

Santoyo, S., S. Cavero, L. Jaime, E. Ibanez, F. J. Senorans and G. Reglero (2006). "Supercritical carbon dioxide extraction of compounds with antimicrobial activity from Origanum vulgare L.: determination of optimal extraction parameters." J Food Prot **69**(2): 369-375.

Sato, H., D. W. Frank, C. J. Hillard, J. B. Feix, R. R. Pankhaniya, K. Moriyama, V. Finck-Barbancon, A. Buchaklian, M. Lei, R. M. Long, J. Wiener-Kronish and T. Sawa (2003). "The mechanism of action of the Pseudomonas aeruginosa-encoded type III cytotoxin, ExoU." EMBO J **22**(12): 2959-2969.

Schaberg, D. R., D. H. Culver and R. P. Gaynes (1991). "Major trends in the microbial etiology of nosocomial infection." Am J Med **91**(3B): 72S-75S.

Schuster, M. and E. P. Greenberg (2006). "A network of networks: quorum-sensing gene regulation in Pseudomonas aeruginosa." Int J Med Microbiol **296**(2-3): 73-81.

Schuster, M., C. P. Lostroh, T. Ogi and E. P. Greenberg (2003). "Identification, timing, and signal specificity of Pseudomonas aeruginosa quorum-controlled genes: a transcriptome analysis." J Bacteriol **185**(7): 2066-2079.

Schuster, M., D. J. Sexton, S. P. Diggle and E. P. Greenberg (2013). "Acyl-homoserine lactone quorum sensing: from evolution to application." Annu Rev Microbiol **67**: 43-63.

Serino, L., C. Reimmann, H. Baur, M. Beyeler, P. Visca and D. Haas (1995). "Structural genes for salicylate biosynthesis from chorismate in Pseudomonas aeruginosa." Mol Gen Genet **249**(2): 217-228.

Shellito, J., S. Nelson and R. U. Sorensen (1992). "Effect of pyocyanine, a pigment of Pseudomonas aeruginosa, on production of reactive nitrogen intermediates by murine alveolar macrophages." Infect Immun **60**(9): 3913-3915.

Shin, J. Y., J. Y. Song, Y. S. Yun, H. O. Yang, D. K. Rhee and S. Pyo (2002). "Immunostimulating effects of acidic polysaccharides extract of Panax ginseng on macrophage function." Immunopharmacol Immunotoxicol **24**(3): 469-482.

Simpson, D. A., R. Ramphal and S. Lory (1992). "Genetic analysis of Pseudomonas aeruginosa adherence: distinct genetic loci control attachment to epithelial cells and mucins." Infect Immun **60**(9): 3771-3779.

Sivropoulou, A., E. Papanikolaou, C. Nikolaou, S. Kokkini, T. Lanaras and M. Arsenakis (1996). "Antimicrobial and cytotoxic activities of Origanum essential oils." Journal of Agricultural and Food Chemistry **44**(5): 1202-1205.

Slack, M. P. and W. W. Nichols (1981). "The penetration of antibiotics through sodium alginate and through the exopolysaccharide of a mucoid strain of Pseudomonas aeruginosa." Lancet **2**(8245): 502-503.

Smolina, T. P., T. F. Solov'eva and N. N. Besednova (2001). "[Immunotropic activity of panaxans--bioglycans isolated from ginseng]." Antibiot Khimioter **46**(7): 19-22.

Sonawane, A., J. Jyot and R. Ramphal (2006). "Pseudomonas aeruginosa LecB is involved in pilus biogenesis and protease IV activity but not in adhesion to respiratory mucins." Infect Immun **74**(12): 7035-7039.

Song, Z., H. K. Johansen, V. Faber, C. Moser, A. Kharazmi, J. Rygaard and N. Hoiby (1997). "Ginseng treatment reduces bacterial load and lung pathology in chronic Pseudomonas aeruginosa pneumonia in rats." Antimicrob Agents Chemother **41**(5): 961-964.

Song, Z., K. F. Kong, H. Wu, N. Maricic, B. Ramalingam, H. Priestap, L. Schneper, J. M. Quirke, N. Hoiby and K. Mathee (2010). "Panax ginseng has anti-infective activity against opportunistic pathogen Pseudomonas aeruginosa by inhibiting quorum sensing, a bacterial communication process critical for establishing infection." Phytomedicine **17**(13): 1040-1046.

Song, Z., H. Wu, K. Mathee, N. Hoiby and A. Kharazmi (2002). "Gerimax ginseng regulates both humoral and cellular immunity during chronic Pseudomonas aeruginosa lung infection." J Altern Complement Med **8**(4): 459-466.

Song, Z. J., H. K. Johansen, V. Faber and N. Hoiby (1997). "Ginseng treatment enhances bacterial clearance and decreases lung pathology in athymic rats with chronic P. aeruginosa pneumonia." APMIS **105**(6): 438-444.

Stapper, A. P., G. Narasimhan, D. E. Ohman, J. Barakat, M. Hentzer, S. Molin, A. Kharazmi, N. Hoiby and K. Mathee (2004). "Alginate production affects Pseudomonas aeruginosa biofilm development and architecture, but is not essential for biofilm formation." J Med Microbiol **53**(Pt 7): 679-690.

Stoodley, P., K. Sauer, D. G. Davies and J. W. Costerton (2002). "Biofilms as complex differentiated communities." Annu Rev Microbiol **56**: 187-209.

Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory and M. V. Olson (2000). "Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen." Nature **406**(6799): 959-964.

Strom, M. S. and S. Lory (1993). "Structure-function and biogenesis of the type IV pili." Annu Rev Microbiol **47**: 565-596.

Sung, W. S. and D. G. Lee (2008). "The combination effect of Korean red ginseng saponins with kanamycin and cefotaxime against methicillin-resistant Staphylococcus aureus." Biol Pharm Bull **31**(8): 1614-1617.

Takase, H., H. Nitanai, K. Hoshino and T. Otani (2000). "Requirement of the Pseudomonas aeruginosa tonB gene for high-affinity iron acquisition and infection." Infect Immun **68**(8): 4498-4504.

Tawab, M. A., U. Bahr, M. Karas, M. Wurglics and M. Schubert-Zsilavecz (2003). "Degradation of ginsenosides in humans after oral administration." Drug Metabolism and Disposition **31**(8): 1065-1071.

Thomas, D., D. G. Morgan and D. J. DeRosier (2001). "Structures of bacterial flagellar motors from two FliF-FliG gene fusion mutants." J Bacteriol **183**(21): 6404-6412.

Thomassen, M. J., C. A. Demko, B. Boxerbaum, R. C. Stern and P. J. Kuchenbrod (1979). "Multiple of isolates of Pseudomonas aeruginosa with differing antimicrobial susceptibility patterns from patients with cystic fibrosis." J Infect Dis **140**(6): 873-880.

Tielker, D., S. Hacker, R. Loris, M. Strathmann, J. Wingender, S. Wilhelm, F. Rosenau and K. E. Jaeger (2005). "Pseudomonas aeruginosa lectin LecB is located in the outer membrane and is involved in biofilm formation." Microbiology **151**(Pt 5): 1313-1323.

Trombetta, D., F. Castelli, M. G. Sarpietro, V. Venuti, M. Cristani, C. Daniele, A. Saija, G. Mazzanti and G. Bisignano (2005). "Mechanisms of antibacterial action of three monoterpenes." Antimicrob Agents Chemother **49**(6): 2474-2478.

Trombetta, D., A. Saija, G. Bisignano, S. Arena, S. Caruso, G. Mazzanti, N. Uccella and F. Castelli (2002). "Study on the mechanisms of the antibacterial action of some plant alpha,beta-unsaturated aldehydes." Lett Appl Microbiol **35**(4): 285-290.

Tummler, B. and C. Kiewitz (1999). "Cystic fibrosis: an inherited susceptibility to bacterial respiratory infections." Mol Med Today **5**(8): 351-358.

Ultee, A., E. P. Kets and E. J. Smid (1999). "Mechanisms of action of carvacrol on the food-borne pathogen Bacillus cereus." Appl Environ Microbiol **65**(10): 4606-4610.

Usher, L. R., R. A. Lawson, I. Geary, C. J. Taylor, C. D. Bingle, G. W. Taylor and M. K. Whyte (2002). "Induction of neutrophil apoptosis by the Pseudomonas aeruginosa exotoxin pyocyanin: a potential mechanism of persistent infection." J Immunol **168**(4): 1861-1868.

Valerius, N. H., C. Koch and N. Hoiby (1991). "PREVENTION OF CHRONIC PSEUDOMONAS-AERUGINOSA COLONIZATION IN CYSTIC-FIBROSIS BY EARLY TREATMENT." Lancet **338**(8769): 725-726.

Vallet, I., J. W. Olson, S. Lory, A. Lazdunski and A. Filloux (2001). "The chaperone/usher pathways of Pseudomonas aeruginosa: identification of fimbrial gene clusters (cup) and their involvement in biofilm formation." Proc Natl Acad Sci U S A **98**(12): 6911-6916.

Van Delden, C. and B. H. Iglewski (1998). "Cell-to-cell signaling and Pseudomonas aeruginosa infections." Emerg Infect Dis **4**(4): 551-560.

Van Gennip, M., L. D. Christensen, M. Alhede, R. Phipps, P. O. Jensen, L. Christophersen, S. J. Pamp, C. Moser, P. J. Mikkelsen, A. Y. Koh, T. Tolker-Nielsen, G. B. Pier, N. Hoiby, M. Givskov and T. Bjarnsholt (2009). "Inactivation of the rhlA gene in Pseudomonas aeruginosa prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes." APMIS **117**(7): 537-546.

Van Ness, B. G., J. B. Howard and J. W. Bodley (1980). "ADP-ribosylation of elongation factor 2 by diphtheria toxin. NMR spectra and proposed structures of ribosyl-diphthamide and its hydrolysis products." J Biol Chem **255**(22): 10710-10716.

Vandenende, C. S., M. Vlasschaert and S. Y. Seah (2004). "Functional characterization of an aminotransferase required for pyoverdine siderophore biosynthesis in Pseudomonas aeruginosa PAO1." J Bacteriol **186**(17): 5596-5602.

Vickery, L. E. and J. R. Cupp-Vickery (2007). "Molecular chaperones HscA/Ssq1 and HscB/Jac1 and their roles in iron-sulfur protein maturation." Crit Rev Biochem Mol Biol **42**(2): 95-111.

Visca, P., A. Ciervo and N. Orsi (1994). "Cloning and nucleotide sequence of the pvdA gene encoding the pyoverdin biosynthetic enzyme L-ornithine N5-oxygenase in Pseudomonas aeruginosa." J Bacteriol **176**(4): 1128-1140.

Visca, P., F. Imperi and I. L. Lamont (2007). "Pyoverdine siderophores: from biogenesis to biosignificance." Trends Microbiol **15**(1): 22-30.

Wagner, V. E., D. Bushnell, L. Passador, A. I. Brooks and B. H. Iglewski (2003). "Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: effects of growth phase and environment." J Bacteriol **185**(7): 2080-2095.

Wagner, V. E. and B. H. Iglewski (2008). "P. aeruginosa Biofilms in CF Infection." Clin Rev Allergy Immunol **35**(3): 124-134.

Walsh, S. E., J. Y. Maillard, A. D. Russell, C. E. Catrenich, D. L. Charbonneau and R. G. Bartolo (2003). "Activity and mechanisms of action of selected biocidal agents on Gram-positive and -negative bacteria." J Appl Microbiol **94**(2): 240-247.

Walters, M. C., 3rd, F. Roe, A. Bugnicourt, M. J. Franklin and P. S. Stewart (2003). "Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin." Antimicrob Agents Chemother **47**(1): 317-323.

Wang, A., C. Z. Wang, J. A. Wu, J. Osinski and C. S. Yuan (2005). "Determination of major ginsenosides in Panax quinquefolius (American ginseng) using high-performance liquid chromatography." Phytochem Anal **16**(4): 272-277.

Wang, C. Z. and C. S. Yuan (2008). "Potential role of ginseng in the treatment of colorectal cancer." Am J Chin Med **36**(6): 1019-1028.

Wang, C. Z. and C. S. Yuan (2008). "Potential Role of Ginseng in the Treatment of Colorectal Cancer." American Journal of Chinese Medicine **36**(6): 1019-1028.

Wang, H., F. Tu, Z. Gui, X. Lu and W. Chu (2013). "Antibiotic resistance profiles and quorum sensing-dependent virulence factors in clinical isolates of pseudomonas aeruginosa." Indian J Microbiol **53**(2): 163-167.

Whiteley, M., M. G. Bangera, R. E. Bumgarner, M. R. Parsek, G. M. Teitzel, S. Lory and E. P. Greenberg (2001). "Gene expression in Pseudomonas aeruginosa biofilms." Nature **413**(6858): 860-864.

Wiegand, I., K. Hilpert and R. E. Hancock (2008). "Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances." Nat Protoc **3**(2): 163-175.

Wilson, R., D. A. Sykes, D. Watson, A. Rutman, G. W. Taylor and P. J. Cole (1988). "Measurement of Pseudomonas aeruginosa phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium." Infect Immun **56**(9): 2515-2517.

Wood, R. E., T. F. Boat and C. F. Doershuk (1976). "Cystic fibrosis." Am Rev Respir Dis **113**(6): 833-878.

Worlitzsch, D., R. Tarran, M. Ulrich, U. Schwab, A. Cekici, K. C. Meyer, P. Birrer, G. Bellon, J. Berger, T. Weiss, K. Botzenhart, J. R. Yankaskas, S. Randell, R. C. Boucher and G. Doring (2002). "Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients." J Clin Invest **109**(3): 317-325.

Wu, H., B. Lee, L. Yang, H. Z. Wang, M. Givskov, S. Molin, N. Hoiby and Z. J. Song (2011). "Effects of ginseng on Pseudomonas aeruginosa motility and biofilm formation." Fems Immunology and Medical Microbiology **62**(1): 49-56.

Wu, H., Z. Song, M. Givskov, G. Doring, D. Worlitzsch, K. Mathee, J. Rygaard and N. Hoiby (2001). "Pseudomonas aeruginosa mutations in lasI and rhlI quorum sensing systems result in milder chronic lung infection." Microbiology **147**(Pt 5): 1105-1113.

Yahr, T. L., J. T. Barbieri and D. W. Frank (1996). "Genetic relationship between the 53- and 49-kilodalton forms of exoenzyme S from Pseudomonas aeruginosa." J Bacteriol **178**(5): 1412-1419.

Ye, R. W., D. Haas, J. O. Ka, V. Krishnapillai, A. Zimmermann, C. Baird and J. M. Tiedje (1995). "Anaerobic activation of the entire denitrification pathway in Pseudomonas aeruginosa requires Anr, an analog of Fnr." J Bacteriol **177**(12): 3606-3609.

Yoon, S. S., R. F. Hennigan, G. M. Hilliard, U. A. Ochsner, K. Parvatiyar, M. C. Kamani, H. L. Allen, T. R. DeKievit, P. R. Gardner, U. Schwab, J. J. Rowe, B. H. Iglewski, T. R. McDermott, R. P. Mason, D. J. Wozniak, R. E. Hancock, M. R. Parsek, T. L. Noah, R. C. Boucher and D. J. Hassett (2002). "Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis." Dev Cell **3**(4): 593-603.

Yoshikawa, M., T. Murakami, K. Yashiro, J. Yamahara, H. Matsuda, R. Saijoh and O. Tanaka (1998). "Bioactive saponins and glycosides. XI. Structures of new dammarane-type triterpene oligoglycosides, quinquenosides I, II, III, IV, and V, from American ginseng, the roots of Panax quinquefolium L." Chemical & Pharmaceutical Bulletin **46**(4): 647-654.

Zimmermann, A., C. Reimmann, M. Galimand and D. Haas (1991). "Anaerobic growth and cyanide synthesis of Pseudomonas aeruginosa depend on anr, a regulatory gene homologous with fnr of Escherichia coli." Mol Microbiol **5**(6): 1483-1490.