Comparison of the microbiological profile of sputum and percutaneous endoscopy gastrostomy fed gastric juice aspirate in Cystic Fibrosis patients: descriptive evidence of a potential aerodigestive microbiome.

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Abstract

Gastro-Oesophageal Reflux (GOR) occurs in Cystic Fibrosis and is associated with deteriorating lung function. The hypothesis of this project is that the CF gastric and lung microbiome are related suggesting potential bidirectional transmission of pathogens through swallowing and aspiration of gastric contents. Gastric pepsin and bile might impact upon the lung microbiome and potentially exacerbate pulmonary disease.

Paired gastric and sputum cultures were obtained from 18 adult CF patients receiving percutaneous endoscopic gastrostomy (PEG) feeding. Non-CF gastric juice samples were obtained from 14 patients without known lung disease through endoscopy.

Bacterial and fungal isolates were identified by culture and next generation sequencing (NGS) of the 16S rRNA gene. The impact of pepsin, pH and bile acids on the growth and behaviour of *Pseudomonas aeruginosa* (*PA*) were tested.

Culture-based and molecular-based approach demonstrated that the bacterial species present in CF gastric juice were different compared to the control group (non-CF patients). A high rate of pathogenic bacteria and organisms such as *PA* and Non-Tuberculosis Mycobacterium (NTM) were isolated from CF gastric juice samples and PEG tubes. Identical strains of *PA* and *NTM* in sputum and gastric juice from the same patient were isolated. Gastric juice samples and the PEG tube of 3 patient were positive for *PA* or NTM and had no *PA* or NTM present in the their sputum samples. This suggests that *PA* can survive acid environments in the presence of pepsin and bile acids. The hostile gastric environment may have a negative effect upon *PA* growth and induce drug resistant biofilm formation.

In conclusion, the stomach is a potential microbiological niche where organisms relevant to CF pathophysiology can survive particularly for biofilm *PA* and NTM. This may be influenced by CF related gastrointestinal pathophysiology, antibiotic therapy and acid suppression.

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This thesis is based on research performed at the Institute for Cell and Molecular Biosciences, Newcastle University. I performed all of the work and analysis of the results with the exception of the NGS (performed by Dr Chris Stewart and Dr Andrew Nelson at Northumbria University) and VNTR analysis in Chapters 3 and 4. Mrs Audrey Perry performed clinical microbiology for non-CF gastric juice at the Freeman Hospital Microbiology department. Medical staff at the Royal Victoria Infirmity hospital (RVI) chest clinic performed lung function and CF patient phenotyping.

Publications

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Abbreviations

ABPA	Allergic bronchopulmonary aspergillosis
API	Analytical profile index
ASL	Airway surface liquid
ANOVA	Analysis of variance
BA	Bile acid
BAL	Bronchioalveolar lavage
BMI	Body mass index
BOS	Bronchiolitis obliterans syndrome
CA	Cholic acid
cAMP	Cyclic adenosine monophosphate
CDCA	Chenodeoxycholic acids
CF	Cystic Fibrosis
CFF	Cystic Fibrosis Foundation
CFTR	Cystic Fibrosis Transmembrane Regulator
CLED	Cysteine Lactose Electrolyte Deficient
COPD	Chronic Obstructive Pulmonary Disease
DCA	Doxycholic acid
DGGE	Denaturing gradient gel electrophoresis
DH2O	Deionised water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ENa$^+$C	Epithelial Na^+ channel
EOR	Extra-oesophageal reflux
EPSs	Extracellular polymeric substances
FAA	fastidious anaerobic agar
FEV_1	Forced expiratory volume in 1 second
FISH	Fluorescence in situ hybridization
FVC	Forced Vital Capacity
GA	Gastric acid
gDNA	Genomic deoxyribonucleic acid
GJ	Gastric juice
GI	Gastro-intestinal

GOR	Gastro-oesophageal reflux
GORD	Gastro-oesophageal reflux disease
H^+	Hydrogen ion
HCL	Hydrochloric acid
HI	Heamophilus influenza
HIF-1α	Hypoxia-inducible factor-1α
IFN-γ	interferon-γ
IL	Interleukin
K^+	Potassium ion
LOS	Lower Oesophageal Sphincter
MABSC	M. abscessus complex
MAC	Mycobacterium avium complex
MALDI-TOF	Matrix Assisted Laser Desorption/ Ionization-Time of Flight
MEDLINE	Medical Literature Analysis and Retrieval System Online
MEF50	Maximal Expiratory Flow at 50%
MMEF	Maximum mean expiratory flow
MRSA	Methicillin-resistant *S. aureus*
MUC5B	Mucin-5B
MUC5AC	Mucin-5AC
n	Number
NGT	Nasogastric tube
NTM	*Nontuberculous mycobacteria*
OD	Optical Density
OTU	Operational taxonomic unit
PA	*Pseudomonas aeruginosa*
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	percutaneous endoscopic gastrostomy
PEG-s	PEG conditioned saline
pH	potential of Hydrogen
PLS-DA	partial least squares discriminant analysis
PPI	Proton Pump Inhibitor
PQS	pseudomonas quinolone signal
QS	quorum-systems

RAPD	Random Amplified Polymorphic DNA
RSI	Reflux Symptom Index
RVI	Royal Victoria Infirmary
SA	Staphylococcus aureus
SD	Standard deviation
SDS	Sodium dodecyl sulphate
16S rRNA	16S Ribosomal Ribonucleic acid
sp	Species (species of a particular known genus but of unknown species)
spp	Species (plural) (more than one unknown species of known genus)
TLOSR	transient lower oesophageal sphincter relaxation
TNF-α	Tumour necrosis factor alpha
TSB	Tryptone Soy Broth
UK	United Kingdom
USA	United States of America
VNTR	Variable number tandem repeat
V4	Variable region 4
WGS	Whole genome sequencing
WHO	World Health Organisation

Chapter 1.0: General introduction

1.1 Cystic fibrosis

Cystic fibrosis (CF) is an inherited disease acquired through autosomal recessive inheritance, and is seen in the UK in almost 1 live birth for each 2,500 (Collins, 1992). CF impacts upon multiple systems of the body, with effects on the liver and pancreas, respiratory and gastro-intestinal tracts, as well as reproductive and musculoskeletal systems.

CF develops as a result of a faulty gene causing a thick layer of mucus to form within the respiratory passages and the pancreas (Riordan *et al.*, 1989). The gene involved is the CFTR gene, encoding Cystic Fibrosis Transmembrane Conductance Regulator (Gregory *et al.*, 1990).

The genotype of *CFTR* gene is highly variable with more than 1600 mutations have been determined already. Yet elucidating the effect of mutations upon function and their relationship to symptom severity is limited to only a few, high-occurrence genotypes (De Boeck *et al.*, 2014). *CFTR* mutations can be categorised according to the mutation's effect on function. There are four groups: i) failure to manufacture proteins; ii) inadequate protein processing; iii) faulty regulation; iv) protein misfolding due to the imperfect synthesis of phenylalanine at position 508 (CFTRΔF508 mutation). As a consequence of the latter, the misfolded protein undergoes proteosomal degradation and there is an absence of CFTR transporters at the apical surface of the membrane (Dequeker *et al.*, 2009; Fanen *et al.*, 2014). The CFTRΔF508 mutation is commonly found in at least one copy of *CFTR* in most patients; symptoms are severest in those patients in whom the mutation is present in both copies of the *CFTR* gene (Alfonso-Sánchez *et al.*, 2010).

CFTR is a membrane protein and channel within cells of the epithelium, managing bicarbonate and chloride ions exchange in cells. Where both genes for CFTR have the mutation (recessive mutation), functional CFTR protein will not be produced, leading the individual to develop CF (Derichs, 2013). Further, the fault in this gene leads unusually thick mucus to be generated within the pancreas, and bile ducts, the lungs and the digestive system. These factors lead to clogging of the lumens for the organs involved, which causes the morbidities associated with CF. Within the lungs, respiratory system defence is compromised in CF, and long-term

infections result, with most CF fatalities being a result of either sepsis following repeated lung infection or from respiratory failure (Emerson *et al.*, 2002).

Prior to the 1970s, the median life expectancy with CF was just 8 years, with many patients dying as infants. However, there has been considerable progress in increasing longevity for CF patients since that time, and in those born on or after 1990, the current predictions show that 90% will live to at least 40 years (Reid *et al.*, 2011). A large proportion of this progress has been brought about by centralising care from multiple disciplines for patients of all ages, plus widespread screening for neonates (Lim *et al.*, 2013), as well as developing strategies for managing nutritional status and lung disease.

1.2 Primary Complications of Cystic Fibrosis

Numerous complications are caused by CF, with the central impact being seen in the glands of the submucosa and the respiratory passages (Gibson *et al.*, 2003). The airway epithelium normally expresses CFTR and this assists in regulating the level of chloride present.

In CF airways, the absence of functional CFTR protein in the apical plasma membrane causes epithelial Na^+ channel (ENaC) hyperactivity, and the resulting excessive Na^+ absorption contributes to airway surface liquid (ASL) dehydration, mucus stasis, and bacterial infections (Althaus, 2013). and thus the level of chloride within cells rises, while levels outside the cells fall (Lubamba *et al.*, 2012). The result of this difference is that water moves in greater volumes into the epithelial cells of the air passages, drying out the mucus, which coats the surfaces of these passages.

This mucus therefore becomes thicker and less easily moved, impairing the flow of air and interfering with the capacity for respiration (Boucher, 2004). The thicker mucus layer additionally provides suitable conditions for bacteria growth, allowing infection. Because of the effects of the altered mucus layer, the reduction in water content on the surfaces of the airways is seen as the occurrence which initiates lung disease for CF patients (Reeves *et al.*, 2012).

The mucus within normally functioning lungs mainly contains mucins, and these may be from cellular secretions or be attached to the cellular membranes. MUC5B and MUC5AC are generally the principal mucins and are overproduced in CF lung secretions especially following exacerbations (Kirkham *et al.*, 2002; Henke *et al.*, 2007). Further, while the thicker mucus seen in CF is primarily attributed to the lowered volumes of water outside cells, another contributing

factor appears to be a greater quantity of anionic polyelectrolytes, which is partly made up of DNA from bacterial invaders, as well as that from inflammatory cells on lysis and enhanced mucosal cell turnover (Tang *et al.*, 2005). The mucus which accumulates in the air passages becomes detached from the cilia after some time, meaning that mucociliary transport is impeded, and it is this issue which mainly leads to morbidity of the lungs and eventually death (Sibley *et al.*, 2008; Reeves *et al.*, 2012).

As described above, one result of the mucus in the lungs of CF patients being thickened is that bacterial infection is promoted (Boucher, 2004). New-borns acquire the first bacterial infections within a short time and the poor mucociliary transport in CF in a depleted ASL volume acts against the airways being cleared. Further, antimicrobial peptides of the air passages are unable to cope with the demands placed on them, and infection becomes established, and primary inflammatory response occurs over an extended period, as seen in chronic infections (Puchelle *et al.*, 2002). This inflammatory response involves the generation of mediators for inflammation where the infection is located, and neutrophils are recruited to destroy the infectious agents (Gibson *et al.*, 2003). However, processes of inflammation within the respiratory passages are ineffective in removing recurring infection in the CF patient, and inflammatory responses both become excessive in comparison to the level of infection, and are ongoing due to the inability to eliminate that infection (Chmiel and Davis, 2003b). The result of this ongoing inflammatory process is more harm being caused to the respiratory passage, with airway remodelling (structural changes that occur in both the large and the small airways) (Hilliard *et al.*, 2007). Viruses may also cause harm to the epithelial layer (Banner *et al.*, 2009). A main factor in deaths among CF patients is progressive pulmonary disease (Sagel *et al.*, 2007).

1.3 CF lung microbiome

Lung failure as a consequence of chronic airway infection is responsible for more than 90% of CF's morbidity and mortality (LiPuma, 2010; Barry *et al.*, 2015). However, as more airway infections are successfully treated, more CF patients are living longer but they face extrapulmonary challenges (Quon and Aitken, 2012). CF-related diabetes, arthropathy, chronic kidney disease, depression and osteoporosis are typical extrapulmonary diseases encountered (Quon and Aitken, 2012). In many of these diseases, the human microbiome has been identified as having a role (Shanahan, 2013), indicating that the microbiome and environment in CF patients is a factor that needs to be understood in greater detail.

The upper airways, particularly the oral cavity, are home to endogenous microbiota as well as being an important interface location for interacting with the environment (Zaura *et al.*, 2009; Dewhirst *et al.*, 2010), making this location the body's most varied microbiome. In healthy individuals, the lower airways are, in effect, sterile as aerosolised microbes transported into the lungs encounter the innate immune system, which rapidly and effectively neutralise the threat (Cui *et al.*, 2014).

Investigations into the CF microbiome are increasing. Yet efforts to directly compare microbiomes between studies is frustrated by variations in the extraction protocols, bioinformatics techniques and sequencing technologies employed in the different studies (Willner *et al.*, 2012; Yuan *et al.*, 2012). Furthermore, the DNA recovered for culture-independent approaches can skew results as the thick mucus in the airway of CF patients can accumulate a significant quantity of DNA of dead cells (Filkins and O'Toole, 2015). The quantity of *Pseudomonas aeruginosa (PA)* DNA, for example, does not reliably correspond to the quantity of the viable pathogen determined by cell counts (Rogers *et al.*, 2010a). This indicates that the existing methodology is limited in its ability to present a current state of the CF microbiome. Furthermore, different DNA extraction protocols favour particular microbes, with some organisms being underrepresented (Salonen *et al.*, 2010), which may explain the discrepancies between studies of the relative quantities of microbes. In spite of methodological differences between the various studies, it has been possible to deduce the main microbes of the CF microbiome.

Streptococcus, Prevotella, Veillonella, Rothia, Actinomyces, Granulicatella, Fusobacterium, Neisseria and *Atopobium* are the microbial genera most often found in the CF microbiome (Hampton et al., 2014; Mahenthiralingam, 2014; Surette, 2014). Indeed, Finding obligate anaerobes (e.g. *Prevotella* species) that are ubiquitous in upper airways appear to form major components of the lower airway microbiome in CF and other disease states (e.g. chronic obstructive pulmonary disease (COPD) (Mahenthiralingam, 2014; Surette, 2014). The evidence from some studies is that there is a correlation between CF disease severity and a decline in the diversity of the microbiome (Filkins *et al.*, 2012; Zhao *et al.*, 2012). In a small longitudinal study comprised of six patients providing sputum samples over an 8-year period that employed Shannon diversity to determine microbiome stability, the microbiomes of the three patients who suffered from disease progression were less diverse than those microbiomes of patients whose disease status was stable (Zhao *et al.*, 2012).

Genetic defects, modified genes, nutrition, treatment, environmental exposure, as well as primary CF pathogens contribute to CF, making it a heterogeneous disease (Cutting, 2015). The microbiomes of each individual are likely to be unique. A positive correlation between *PA* and reduced volume of air on forced expiration for one second (FEV1), serum C-reactive protein and neutrophil elastase in sputum was detected in the analysis undertaken by Zemanick *et al.* (2013)that explored the relationship between inflammatory markers and microbiome profiles of 21 patients. The researchers findings support the conclusion that *PA* plays a prime role in the morbidity of CF airway disease (Zemanick *et al.*, 2013).

1.3.1 Role of microbiome in modulating immune response

The human immune system is sophisticated and discerning, able to discriminate between harmful, pathogenic microbes and beneficial commensal species. Investigations into gastrointestinal microbiota reveal that there is a positive relationship between reduced microbial diversity and chronic inflammatory disease, mirroring the findings observed for CF airway disease (Walker *et al.*, 2011; Lynch and Bruce, 2013). The implications of this observation is that colonisation resistance, which is the gut's ability to resist invading pathogenic microbes, and immune homeostasis is founded on suitable colonisation of the mucosa. Disruption to the microbiota may result in persistent inflammatory responses, especially where there is a loss of diversity and an increase in the number of particular immunogenic species (Beck *et al.*, 2012; Lynch and Bruce, 2013).

The integral role of the microbiome to a host's health means that disruptions to the microbiota can affect the host's health and wellbeing (Cho and Blaser, 2012); the implications of this are significant when taking antibiotic therapy as a standard feature of CF management. Paediatric studies highlight the sizeable effects of antibiotics on gastrointestinal microbiota (Zemanick *et al.*, 2011). From the faecal microbiota of 11 infants obtained throughout their first year of life, researchers found the bacterial burden was significantly reduced as a consequence of antimicrobial therapy (Palmer *et al.*, 2007). Yet within a few weeks, the microbial community was re-established and was comparable to the pre-treatment microbiota. However, as Palmer *et al.* (2007) emphasised, particular species that had been abundant prior to the antimicrobial intervention were absent immediately after administering the therapy and remained absent throughout the duration of the study, which in some instances was up to one year.

Similar results were obtained in the decade-long study by Zhao *et al.* (2012) that analysed samples collected from CF patients. The researchers observed re-establishment of the colonies

following significant antimicrobial-instigated disruption (Zhao *et al.*, 2012). These findings are also similar to those of Cox *et al.* (2010), who showed that patients who presented declining microbial diversity experienced progressive disease states. On the basis of these observations and consistent with other severe inflammatory disease, it is reasonable to speculate that over time, the microbial community loses the benefits effects that diversity confers due to repeated antimicrobial interventions to manage pulmonary function in CF patients (Cox *et al.*, 2010; Zhao *et al.*, 2012).

1.4 Infections and CF

The respiratory passages in the CF patient are vulnerable to infection from a broad range of agents. While in initial stages, infections often arise from agents including *Haemophilus influenza* (*HI*) and *Staphylococcus aureus* (*SA*), subsequently, bacterial pathogens infecting the airways opportunistically may include *Burkholderia* species (spp), and *Pseudomonas aeruginosa* (*PA*). There have been numerous research studies to date exploring the changes in infectious agents in the respiratory system over time in CF, considering this within the individual and on a population scale, and charting new pathogenic agents appearing.

Before antibiotic drugs were made available to doctors, and at a time when CF patients did not survive until adulthood, *SA* was the most commonly found infectious agent in the CF airway, and data from the registry of the USA indicates that it is still the most frequently seen in the child and teenage CF populations (Rosenfeld *et al.*, 2001). *SA* can be found as a commensal on relation to the skin and is often identified in samples from healthy individuals' anterior nares (Wertheim *et al.*, 2005). Thus, the organism enjoys a widespread reservoir across the population and is well placed to infect an individual with CF. Further, there is research evidence that where a CF child is infected with *SA*, their immediate family have a high likelihood of carrying an identical *SA* strain within the nasal passages, and this gives a shared exposure pathway for acquiring the infection (Stone *et al.*, 2009).

Life prolonging advances in CF management and the development of successful treatment for *SA* through antibiotics means that different agents of infection for the respiratory system have appeared. Mearns *et al.* (1972) studied alterations in the picture of CF respiratory pathway microbiology in one clinic in infants under one across a time from 1950 to 1971, and found a fall from 86% to 30% in *SA* infections occurring between the periods 1950-1957 and 1969-1971. In general, across the clinic, *SA* was isolated in 45% of patients in the first period and in 12% in the second, while *PA* was seen to rise from 3% of cases to 28%. Further, those with the

most serious disease in the lungs were identified as showing the greatest reduction in *SA* and rise in *PA*. Considering this finding, the authors hypothesised that rather than this change being causal; *PA* could be considered a marker for higher levels of disease.

A later study looked at data from 1985 to 2005 for adults attending one facility (Millar *et al.*, 2009). In this study, *PA* was the most frequently isolated pathogen in the samples from patients, and this occurred in between 77% and 82% consistently within the 20-year span. Further, *SA* was isolated in between 54% and 47%. Meanwhile, a fall was seen over the period in *Aspergillus*, *Burkholderia cepacia* complex and *Haemophilus influenzae*, in contrast with rises in both *Stenotrophomonas maltophilia* and methicillin-resistant *S. aureus* (MRSA), with incidence of the latter reaching 4% from just 1% previously.

A recent research area related to infections in CF is the examination of the part played by anaerobic microbes, which are not subject to routine culturing and therefore had not previously been a focus for investigation. It has been found that such bacteria have a major presence in sputum taken from CF patients. Worlitzsch *et al.* (2009) found 35 separate anaerobic species and up to four per sputum sample in a study of 114 CF patients. It was further found that 58% of these microbes within samples collected during exacerbation displayed *in vivo* resistance to the antibiotic drugs being taken. While it has been suggested that the anaerobic bacteria found here were sample contaminants from the mouth and pharynx, the large number of these bacteria present in the lavage fluids of the bronchi and alveoli oppose this hypothesis (Tunney *et al.*, 2008).

The studies described above relate to individual facilities only, but with the creation of databases at country level for CF, it is now possible to consider the agents involved in airway infection in CF across greater numbers of patients. *PA* has been shown to be the most frequent pathogenic bacterium in the adult CF population of both the USA and the United Kingdom, In the UK data, it was found in 2009 that 67% of 28-31 year-old CF patients carried this infection. Similar data is seen for the USA, albeit with far greater incidence of infection with MRSA, at 23.7% of the total CF population of all ages (Buzzetti *et al.*, 2009; Razvi *et al.*, 2009).

1.4.1 Pseudomonas aeruginosa (PA)

P aeruginosa (*PA)* is a bacterial microbe, which is a common environmental organism, has a rod-like form and is gram negative. The species is an opportunistic pathogen, with illness only occurring from *PA* in the airways where the patient has compromised immunity, such as in

those where chronic disease of the lungs is already established, e.g. CF, or in cases of pneumonia linked to ventilators (Oliver *et al.*, 2000).

When anti-staphylococcal drugs began to be used, infection rates reduced in paediatric CF patients, but *PA* then became the primary infective agent of the respiratory passages (Gilligan, 1991), and the pathogen grows to reach its greatest prevalence in CF patients of approximately 30 years of age. Identifying the mechanisms through which the defensive systems of the epithelial layer of the lung and *PA* interact represents a significant research focus in CF (Hoffman *et al.*, 2005). Further, this infection elicits an especially strong response from the immune system, while also being good for resisting those responses (Campodónico *et al.*, 2008)

P. aeruginosa's effectiveness as an infective agent seems to be partially because it forms a biofilm, which is 'a structured community of bacterial cells' which generates self-enclosing polymeric matrix and can attach itself to surfaces (Prakash *et al.*, 2003). Such biofilms were first identified in the 1970s, and subsequently, the theory has been put forward biofilm state is mechanism for bacteria survival (Watnick and Kolter, 2000).

For CF patients, the initial isolation of *PA* is a clinically important event, as the patient's age when it is first seen is predictive for overall life expectancy (Emerson *et al.*, 2002), with timely and intensive response with antibiotic drugs to initial cultures of *PA* also seeming to enhance the functioning of the lungs (Emerson *et al.*, 2002). Extended application of antibiotics to eradicate *PA* has been considered to be effective when viewed through serum *PA* antibody titres as well as sputum or throat-swab sample cultures (Taccetti *et al.*, 2005; Treggiari *et al.*, 2007). However, these approaches are not considered sensitive markers for *PA* detection (Farrell and Govan, 2006). Supporting evidence for the capability to eradicate the bacteria, however is seen in strain analysis comparison between initial and subsequent isolations (Munck *et al.*, 2001). In one study, the strains seen second were not the same as those isolated initially in 14 patients from 19. However, this also suggests that in over one quarter of cases, the organism was likely not to have been eradicated.

Planktonic and biofilm states display major differences, with growth being slower for bacteria in a biofilm and resistance to antibiotic treatment being far stronger in this condition (Hill and Larsen, 2005). In *PA* biofilm, up-regulation and/or down-regulation of genes expression is over 6 times greater than the planktonic state, affecting a minimum of eight hundred genes and representing more than half of the genome of *PA* (Sauer *et al.*, 2002). Singh *et al.* (2000) made the discovery of biofilm of *PA* in patients with CF, through identifying quorum sensing molecules (QS molecules), which allow bacteria to communicate with each other to identify

how many others are present in their immediate environment, enabling conversion into biofilm mode to occur at the most suitable moment (Kjelleberg and Molin, 2002). *PA* is not the only species to demonstrate biofilm capability in the respiratory pathways of CF patients, with *HI* (Starner *et al.*, 2006) as well as *SA* (Leid *et al.*, 2002), in addition to a number of fungal organisms being identified (Douglas, 2003; Lynch and Robertson, 2008). Considering development of new therapies to address this, the use of molecular-level blocking agents to prevent quorum sensing from occurring effectively may present an alternative to antibiotics alone (Wu *et al.*, 2004).

1.4.1.1 P. aeruginosa biofilm formation

Certain microbes are capable of gathering with others to create a biofilm, generally encased within a matrix made up of EPSs or extracellular polymeric substances. EPSs represent different classes of macromolecules such as polysaccharides, proteins, nucleic acids, lipids and other polymeric compounds presented in the interior and surroundings of various microbial aggregates (Wingender *et al.*, 2012).

The point where previously planktonic, single-celled *PA* transitions phenotypically to form biofilm is considered a key transformation point within the progression of lung disease for CF patients. While *PA* infection for such patients is considered to first occur from the environment with planktonic forms, after an unspecified period, this community converts to a biofilm formation, and once this occurs, it is suggested that *PA* is more challenging to eliminate and may chronically infect the lungs.

1.4.1.2 PA Biofilm formation through modelling in vitro

PA biofilm forms *in vitro* through five identifiable stages (Sauer *et al.*, 2002):

Stage 0: Planktonic

The planktonic form is motile and is seen in the wider environment.

Stage 1: Reversible attachment

In the reversible attachment phase, *PA* cells attach to surfaces in a temporary manner. This is achieved via the cell-pole, with mediation from the pili and flagella of the bacterium. far less attachment levels are seen for those cells with mutations in pili or flagella (Bucior *et al.*, 2012).

Stage 2: Irreversible attachment

Irreversible or permanent attachment of *PA* to surfaces occurs through re-orientation in order to use the longitudinal cell axis, with the cells losing their motility and a clustering process beginning, and as observed by Sauer et al., 2002, quorum-systems (QS) were activated at this stage. QS are the mechanism whereby an individual bacterium produces small diffusible molecules that can be detected by surrounding organisms. This system is used by *PA* for cell–cell communication (Pesci *et al.*, 1997; Waters and Bassler, 2005).

In the irreversible attachment phase, EPSs begin to form, comprising extracellular DNA, proteins and polysaccharides. These form a matrix in which the bacteria are enmeshed, and this is one characteristic of biofilm which is seen to both strengthen its structure and render the bacteria within it phenotypically more resistant (Sutherland, 2001; Branda *et al.*, 2005)

Stage 3: First Maturation Stage

In the initial maturation stage, layering or clustering of cells occurs to a depth of greater than 10μm, with dramatic upregulation of protein expression. The proteins affected include Arc proteins, which are activity-regulated cytoskeleton-associated proteins, and these are considered to drive anaerobic activity in the microbes (Verhoogt *et al.*, 1992; Sauer *et al.*, 2002) with the implication that when in biofilm formations, bacteria lack sufficient oxygen to carry out activities aerobically.

Stage 4: Second Maturation Stage

The second maturation stage is when the layer of clustered cells is at its thickest, as much as 100μm, with cells differing most radically in phenotype from those in their planktonic form: over half of identified proteins have altered regulation during this stage (Sauer et al., 2002). The expression of proteins here also differs markedly from the previous stage of maturation, and this has been suggested to be linked to the large proportion of bacteria within an environment in which oxygen is absent or reduced. Additionally, clusters of cells detach themselves from the attachment surface at this time (Sauer et al., 2002).

Stage 5: Dispersion

The last developmental stage is dispersion, whereupon certain microbes attain motility, swimming to exit the clusters as channels and pores open up (Sauer et al., 2002). The assumption is that this is in order to access higher nutrient levels available out with clusters. During this stage, there is downregulation in certain proteins, with microbes appearing similar to the planktonic form in comparison to those from the second maturation phase.

1.4.1.3 PA Biofilm in Vivo

It is interesting to consider whether the 5-stage model developed by Sauer for biofilm production translates into the complexity of the conditions within the respiratory passages of the CF patient. In studying models of chronic infection, Worlitzsch et al. (2002) put forward the idea that biofilms are formed within intraluminal spaces as opposed to on the surface of the epithelium. Their work showed, via immune-localisation approaches on explanted lungs, and by use of electron microscopy, that *PA* microbial cells were not attached to epithelial air passage cells (Worlitzsch *et al.*, 2002). More than 95 % of the bacteria were found within the lumen of the air passage, at a distance of between 5 and 17μm from the surface of the cells, with colonies structured spherically, while the remainder were located 2 to 5μm away from the surface of the cell. *PA* microbes also appeared to prefer to bind to mucus as opposed to the epithelial air passage cells. Bacterial colonies in spheres within the intra-luminal area were found to be lacking in oxygen, as also noted by Sauer in terms of protein upregulation to allow anaerobic activity. Further, *PA* subjected to hypoxia was found to create raised levels of alginate, which is a significant constituent of biofilm extrapolysacharide matrix. It was also found that the mucus that adhered to the surface of the CF respiratory passage showed a gradient of hypoxia. This gradient was suggested to promote alginate generation in *PA* and biofilm formation (Worlitzsch et al., 2002).

1.5 CF complications of the gastrointestinal system

CF is most known for its impact on the lungs, in the modern era as disease in this area is responsible for a large proportion of the mortality and morbidity of the condition. However, there is a rising appreciation of complications of the gastro-intestinal tract on CF morbidity, which has arisen in part due to the greater longevity of CF patients in recent years. Certain complications of CF also occur in healthy individuals, including constipation and gastroesophageal reflux (Meyerholz *et al.*, 2010). However, these occur more frequently in CF patients and require particular attention to diagnosing and managing these where the individual has CF(Borowitz *et al.*, 2005).

Another CF complication of the gastrointestinal system is exocrine pancreatic insufficiency. CF patents have a high protein concentration in the pancreatic secretions that participate in the duct lumina causing pancreatic obstruction and damage (Wilschanski and Novak, 2013).

In this condition, enzymes for digestion are produced in inadequate quantities and this leads to difficulty in absorbing nutrients from food. Steatorrhea (fat in stool) and failure to thrive may also be seen (Kopelman *et al.*, 1988; Kopelman, 1991; Ramsey *et al.*, 1992; Riordan, 1993). Further, obstructive syndromes frequently arise in the intestines, such as meconium ileus in new-borns, as well as distal ileal obstruction syndrome in later childhood (Davis, 2006). Gastro-oesophageal reflux (GOR) is well-established as a challenging complication of CF (Mokhlesi *et al.*, 2001).

As this thesis is intended to study the role of GOR in lung infection in CF, the next section will give an introduction of the GOR in CF patients.

1.6 Gastro Oesophageal Reflux (GOR)

1.6.1 Introduction

Gastro-oesophageal reflux, or GOR, refers to the physiological mechanism in which contents from the stomach pass back through the stomach's entrance and reach the oesophagus (Sifrim *et al.*, 2004; Herbella and Patti, 2010). According to Zerbib *et al.* (2005), examination of the oesophagus in 72 healthy adults for 24 hours identified instances of reflux which varied between acidic reflux, in which pH was lower than 4, weaker acid reflux with pH of between 4 and 6, gases and liquid. It is clear from the findings that stomach contents are refluxed frequently among those with no identified problems.

Where reflux reaches proximally to the top of the oesophagus and past the oesophagus to the larynx, pharynx or nose this is known as EOR, or extra-oesophageal reflux (Vakil, 2010). This phenomenon has been linked to the occurrence of various problems of the respiratory system, giving symptoms such as post nasal drip, coughing, continual throat-clearing, sore throat, tight chest and wheeze (Button *et al.*, 2005). Decayed teeth have been linked with EOR, as has otitis media, and refluxate has been found in the respiratory passageways (Barron *et al.*, 2003; Blondeau *et al.*, 2008a).

Reflux becomes pathological in nature where symptoms arise from it, including heartburn and harm to the mucosa of the oesophagus. This is then termed as the condition of gastro-oesophageal reflux disease, or GORD. GORD has been described by the Montreal Consensus Group as a disorder seen where gastric contents are refluxed causing complication or symptoms which cause disturbance (Vakil *et al.*, 2006). GORD thus encompasses a number of

complications which harm the oesophageal tissues to differing extents and in different ways. Further, GORD has varying symptoms, often including regurgitation of acid and heartburn (Vakil *et al.*, 2006), but also thoracic pain, water brash and dysphagia (Ronkainen *et al.*, 2006).

From surveys among the population, countries of the West have high rates of symptoms of acid reflux, with heartburn prevalent in between 29 and 44 per cent of the population and from 10 to 20 per cent reporting experiencing heartburn each week (Kennedy and Jones, 2000). This estimation is founded on the idea that such symptoms indicate GORD, but using objectively assessed indicators of oesophageal damage, such as Barrett's oesophagus and endoscopic oesophagitis, which are objective markers of reflux-induced oesophageal injury, some patients do not always have heartburn. When data was systematically reviewed, GORD appeared at between 10-20% of the population in the West, while in Asian countries this was just 5% (Dent *et al.*, 2005). Further, survey results based on the US population found that from the sample, 6 per cent stated that they had heartburn over two times each week, while for acid regurgitation this was 3 %. (Camilleri *et al.*, 2005). In terms of GORD diagnosis, Vakil *et al.* (2006) concluded that mild symptom display on over two days each week or moderate to severe symptoms occurring more frequently than once weekly was a sign of pathologic acid reflux which caused problems, while also concluding that the troublesome nature or otherwise of symptoms should in the end be decided by the patient.

GORD can also be associated with asthma and chronic coughing, and also linked to complications of the lungs (Farrokhi and Vaezi, 2007). Further possible symptoms are throat clearing, being hoarse, postnasal drip, epigastric burn, and nausea (Irwin *et al.*, 1989; Ruigomez *et al.*, 2008).

1.6.2 CF Patients and GOR

There is evidence that GOR is greater for CF patients than for healthy individuals (Ledson *et al.*, 1998a; Button *et al.*, 2005), with GOR which reaches a pathological level estimated at from 35 to 81% for CF populations (Ledson *et al.*, 1998a; Brodzicki *et al.*, 2002b; Blondeau *et al.*, 2008a). Further, symptoms of EOR are demonstrated to have affected 94 per cent of those with CF (Blondeau *et al.*, 2008a). Refluxate has the potential to restrict lung function in micro-aspiration as well as spasmodic reflex in the glottis and bronchi (Blondeau *et al.*, 2010).

There are two theories proposed to account for the symptoms arising from the association between GOR and respiratory disease; these are the reflex theory and reflux theory (Figure 1.1)

(Praud, 2010). According to the reflex theory, the vagus nerve is stimulated by gastric reflux in the oesophagus, leading to bronchospasms (Ates and Vaezi, 2013). On the other hand, the reflux theory postulates that gastric refluxate is aspirated, which causes the airways to become damaged and inflamed (Bulmer *et al.*, 2010).

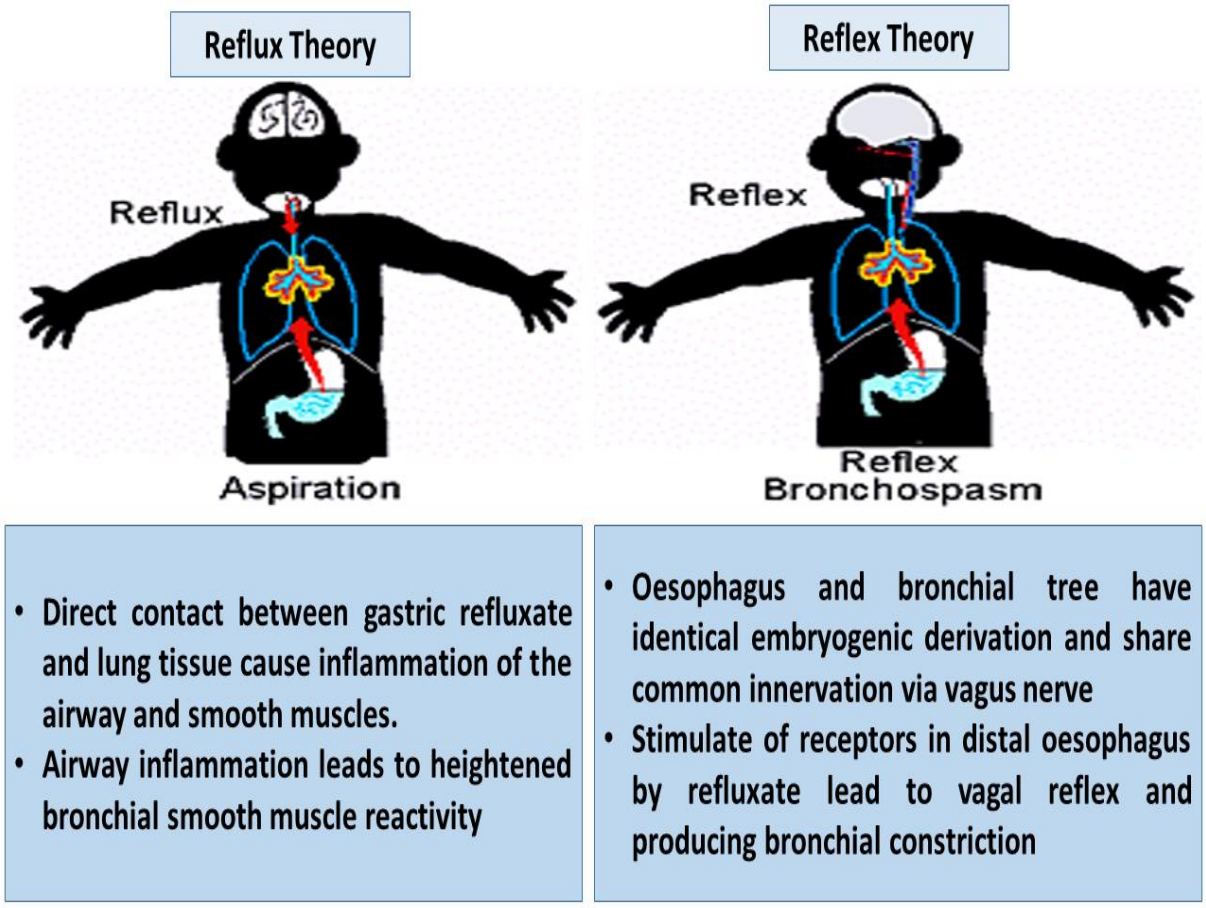

Figure 1-1: GOR and pulmonary symptom in CF patients

1.6.2.1 GOR Mechanisms for CF patients

The mechanisms governing the link between GORD and CF have presented a challenge to researchers: especially as this link does not seem influenced by surroundings, mutation, gender or age (Pauwels *et al.*, 2011). It is probable however; those multiple factors are involved in this association.

Increased frequency of transient lower oesophageal sphincter relaxation (TLOSR) seem to have significance in linking GORD to CF. In a study which involved 14 juvenile CF patients (Cucchiara *et al.*, 1991), CF patients were reported as more likely to have TLOSR as the factor

leading to instances of GOR than from elevated intragastric pressure or lowered steady-state basal lower oesophageal sphincter (LOS) pressure.

The reasons for LOS relaxations were hypothesised as coming from stomach or pharynx stimulation (Cucchiara *et al.*, 1991). Later studies support the contributory role of TLOSRs (Button *et al.*, 2005; Katkin and Schultz, 2010), although reduced basal tone of the LOS, as well as higher pressure within the abdomen might also be responsible (Katkin and Schultz, 2010). Prolonged gastric emptying is reported in 5-17 year-old CF patients, while in case of exocrine pancreatic insufficiency, this period is shorter than normal (Cucchiara *et al.*, 1996).

Primary lung disorders, including poorer pulmonary function caused by accumulated intraluminal secretion, as well as bronchiolitis and chronic destruction of the wall of the respiratory passage, are reported as also being contributory elements (Blondeau *et al.*, 2010). It is also possible that bronchopulmonary dysplasia or infections of the airways such as frequently seen in childhood may lead to GORD (Cucchiara *et al.*, 1991). Further, the suggestion has been made that GOR itself is a contributory factor to these issues of the lung as a result of the oesophagus becoming more acidic, which can lead to a reflexive constriction of the bronchi induced by chemoreceptors (Ledson *et al.*, 1998b). Certain drugs, including alpha-adrenergics, might also be a factor in developing GORD (Katkin and Schultz, 2010), as well as changes to diet, as adopting a diet which is high in fat and protein increase the GOR (Button *et al.*, 2005).

Sporadic cough and wheeze present as frequent CF symptoms, and each creates greater pressure in the abdomen (Cucchiara *et al.*, 1991). Some research reported lower frequency of greater intragastric pressure as a causal factor for GOR than for lower LOS pressure (Cucchiara *et al.*, 1996). Despite this, certain researchers prefer to categorise acid reflux as a primary rather than secondary cough-related occurrence (Blondeau *et al.*, 2010).

A further contributory element in childhood CF GORD is suggested to be postural drainage. This procedure involves percussive strikes delivered manually or mechanically with the aim of encouraging thickened mucus to come loose and be expelled (Button *et al.*, 1998), and is a highly successful approach to mucus removal with benefits for survival. Notwithstanding this, some research has found that this procedure, and in particular where the head is held downward, may make reflux worse.

1.6.3 Gastric Reflux contents

The principal elements of threat within the gastric juices include bile salts, pepsin, acid, pancreatic proteolytic enzymes and, especially in those individuals taking PPIs, microbial pathogens (Pearson and Parikh, 2011a). The stomach converts food ingested into a liquid form by use of pepsin, acid and regular, forceful muscular contraction (Marieb and Hoehn, 2007). Within the mucosal stomach lining, cells form gastric pits, each constructed from a number of glands, and secrete gastric components (Figure 1.2).

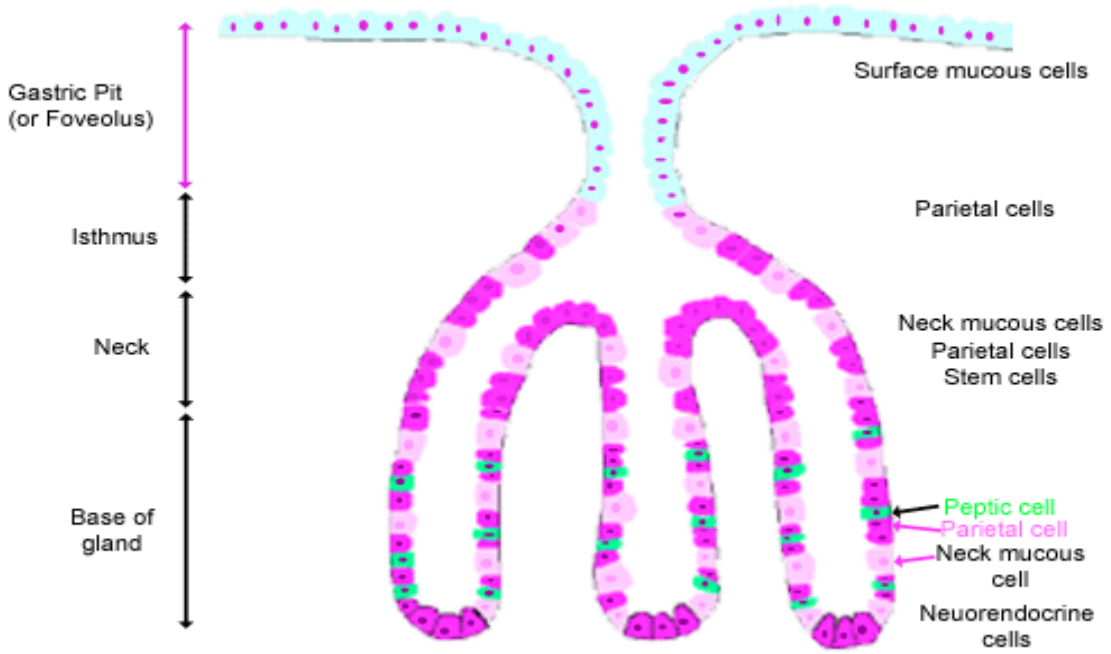

Figure 1-2: Structure of the gastric gland. Mucosal epithelium of the fundus and the body of the stomach form a deep glandular compartment called the gastric gland that is responsible for gastric juice synthesis and secretion, gastric acid secretes from parietal cells in the isthmus, base and the neck of the glands, pepsinogen releases from peptic cells that are found in the base of gastric glands http://www.histology.leeds.ac.uk/digestive/stomach.php.

The pH of the stomach is maintained at between 2 and 4 by secretions of hydrochloric acid (HCl) from the gastric parietal cells within the glands (see Figure 1.1). This acidic environment provides defence against certain pathogenic organisms as well as activating a proteolytic enzyme, pepsinogen released from the peptic stomach cells to pepsin (Owen, 1986).

On the activation of pepsin, it is stable in its molecular structure and can operate in a pH environment as high as 6.0. Mucus is the main substance to be secreted by the cells of the

epithelium surface, and this accumulates as a 2-part layer of protection for the stomach to prevent harm from acid or pepsin (Allen and Flemström, 2005).

The epithelial surfaces of the larynx and oesophagus are demonstrated to be at little risk of damage when pH is 4, but pepsin causes damage to these areas. Thus, where pH is 4 or over, only refluxed material containing pepsin would cause harm (Bulmer *et al.*, 2010). At lower pH levels, acid does not harm the stomach but damages oesophageal tissues (Dvorak *et al.*, 2007).

Refluxate containing pancreatic enzymes or alkaline bile (pH=8) from the duodenum is produced in duodenogastric reflux, in which relaxation of the pyloric sphincter occurs, allowing the stomach to be exposed to duodenal fluid. Bile salts, or BSs, work in the small intestine to allow lipids to be digested and absorbed, The high level of cytotoxicity of BSs leads to their association with malignant occurrence in the gastrointestinal tract (Baptissart *et al.*, 2013). The liver's hepatocyte cells generate BSs, which are modified steroids and sparingly soluble in water. Their synthesis is achieved through cholesterol being catabolised to form chenodeoxycholic acid and cholic acid, CDCA and CA respectively, which make up the primary bile acids. When these enter the colon, they are deconjugated into free bile acids under the action of bacterial enzymes, and these bile acids can then be changed to become secondary bile acids, this modification, mainly 7α-dehydroxylation, converting cholic acid to deoxycholic acid (DCA) and chenodeoxycholic acid to lithocholic acid. According to (Pearson and Parikh, 2011b), fasting gastric juice of patients undergoing routine upper GI endoscopy ranged from 10 to 10 000 mmol／L.

There is evidence of damage being caused to the mucosa of the oesophagus by bile acids in individuals with Barrett's oesophagus, owing to the linking of the condition to cyclooxygenase-2 expression upregulation, as well as functions of cell proliferation. When the oesophageal epithelium is exposed to bile acids, DNA has been demonstrated to become damaged (Jolly et al., 2004).

In one cross-sectional study D'Ovidio *et al.* (2005), bile salts were found in raised concentration within bronchoalveolar lavage (BAL) in 120 patients who had recently undergone transplant, and they were particularly concentrated in those suffering from early onset bronchiolitis obliterans syndrome (BOS). Further, it was reported that bile acids in the bronchoalveloar lavage (BAL) were linked to interleukin 8, alveolar neutrophilia and positive bacterial and fungal cultures. For those with CF, estimates for aspirated bile have been given as including up to 80 per cent of patients (Blondeau *et al.*, 2008a).

Bile acids can arrive in the lungs via aspirated content from the gastro-intestinal area, or may be taken directly from the blood. Aspirated bile in pig lungs generated serious chemical pneumonitis (Porembka *et al.*, 1993). Further, bile acids injected into the intratracheal area is reported in a rabbit model to cause severe pulmonary oedema (Brown, 1967). A study in rabbits, where taurocholic acid was introduced intratracheally, reported atelectasis hyaline membrane formation and eosinophilic substance accumulation within intra-alveolar areas, as detected with microscopy. The study team hypothesised that there may be impairment of the function of surfactants due to bile acids (Kaneko *et al.*, 1990). Aspirated bile acid may lead to serious harm to the lungs (Zecca *et al.*, 2008).

1.6.4 GOR and pulmonary disease pathogenesis in CF

A number of studies identify a possible association between GOR and exacerbations in lung disease as a contributory factor for greater morbidity as well as poorer life quality (Euler *et al.*, 1979; Berquist *et al.*, 1981). In fact, a variety of pulmonary disorders have been strongly linked to GOR, aspiration to the lungs and exacerbated damage to the lungs (Tobin *et al.*, 1998). This is true of severe lung damage following transplant (Davis *et al.*, 2003; Hadjiliadis *et al.*, 2003; Blondeau *et al.*, 2008b), as well as for ventilator associated pneumonia (Collard *et al.*, 2003).

CF patients suffering from GOR are reported to have reduced pulmonary function in comparison to other CF patients, as assessed through continuing decreased pulmonary function as viewed by the FEV_1 (Navarro *et al.*, 2001; Palm *et al.*, 2012).

A Dutch study conducted longitudinally with CF children showed results to indicate that GOR was linked to lowered lung function (van der Doef *et al.*, 2009). There is no clear picture as yet of the extent to which aspiration and GOR are harmful, but these results imply a role of refluxed material in decreasing pulmonary function as possibly a primary effect through refluxate harming epithelial tissues or as a secondary factor in greater exacerbations of lung disease with a link to GOR. It is significant to note that GOR has been associated with CF infections and decreased pulmonary function (Vos *et al.*, 2008). GOR is also linked in several studies to *PA* and *SA* infection (van der Doef *et al.*, 2009; Palm *et al.*, 2012), and aspirated bile acid is emphasised as a factor in greater risk of *PA* following lung transplant (Vos *et al.*, 2008) although the mechanism for GOR as a causal factor in infections of the lungs is poorly understood.

Stomach colonisation by pathological bacteria in CF patients is established through research (Atherton and White, 1978) and may be a factor in pulmonary infections where pathogen-laden

stomach contents are refluxed and aspirated into the lungs. Thus, the stomach has an identified function in pathogenesis of the lung through hosting pathogenic bacteria (Atherton and White, 1978; Bonten *et al.*, 1997). Bacteria from the stomach and airways were found to match, with a large proportion of the microbes identified being found in the gastrointestinal tract before their identification in the air passages (Round and Mazmanian, 2009; Madan *et al.*, 2012). .

Individuals with CF are especially vulnerable to potential two-way pathogenic transfer from stomach to upper airways and vice versa because of the prevalence of gastric reflux in this group (Rogers *et al.*, 2010b). Research conducted in 2006 correlated bacterial colonisation in the gastric juices and the oropharynx for patients in old age fed through NGT, with the implication being that bacteria were transmitted in two directions (Segal *et al.*, 2006).

1.6.5 Aspirated Bile and Pulmonary Infection in CF

Bacteria in CF airways seem to grow very slowly or be in stationary phase, probably due to the surrounding conditions of the mucus environment with no or very little oxygen (Worlitzsch *et al.*, 2002), possibly forming biofilms (Singh *et al.*, 2000). Such biofilms are not susceptible to antibiotic drugs, and cells that are sessile rather than planktonic have greater resistance (Nickel *et al.*, 1985; Spoering and Lewis, 2001; Prince, 2002). Therefore, the challenges found in resolving chronic *PA* infection for CF patients might be partially due to the application of treatment approaches that are based on poorly matched testing of susceptibility, as the bacterial growth within the air passage may be in a different form. In fact, a more suitable predictor for sensitivity to antibiotic treatment could be the susceptibility of laboratory-grown biofilm of microaerophilic microbes in stationary state.

Research carried out retrospectively with juvenile CF patients showed an association between aspirated bile and *PA* colonies in the respiratory passage (van der Doef *et al.*, 2009; Palm *et al.*, 2012), as did a study related to lung transplantation (Vos *et al.*, 2008). Bile aspirated due to GOR is now seen as a major complication of CF, and for respiratory diseases more broadly.

In recent *in vitro* studies, it has been found that bovine bile at physiological concentration (0.03%-0.3%) can lead to *PA* and a number of pathogenic species to take up biofilm modality on a chronic basis (Reen *et al.*, 2012). In addition, there is evidence that the host's molecular targets are modulated by the bile acid to suppress hypoxia-inducible factor-1α (HIF-1α), which is the immune and hypoxic responses' key regulator (Legendre *et al.*, 2014), providing

persuasive evidence that the role of bile aspiration to the lung might represent a significant determinant in the host in initiating pathogens taking biofilm state.

Recently, a number of studies have specifically focused on the microbiome specific to CF and the differences between this and the microflora in healthy control (Cox *et al.*, 2010; Sibley *et al.*, 2011; Sibley and Surette, 2011; Blainey *et al.*, 2012). The variables which influence the microbiome in CF are not established, (Reen *et al.*, 2012) but research points to aspiration of bile as a possible factor in altering microflora, and in particular regarding certain pathogenic species coming to dominate and create chronic infection.

A further study by the same group (Reen *et al.*, 2014) looked at bile aspiration and its influence on the pulmonary microflora of patients with respiratory disease. Profiling techniques were applied to 25 samples of sputum provided by juvenile patients with CF to determine whether bile acids were present using high-resolution liquid chromatography-mass spectrometry. Five samples were then isolated from patients with bile aspiration and 5 from those without. The samples were subjected to pyrosequencing and denaturing gradient gel electrophoresis (DGGE) and it was found that sputum from samples with bile aspiration had a comparatively narrow range of microflora in comparison to the other samples. Furthermore, in samples without bile aspiration, the predominant genera found were those seen in normal lungs, while CF-associated types of microbial life principally defined those with bile.

1.7 Diversity of microbes within the stomach

Where stomach function is normal, the majority of pathogenic microbes find survival impossible in the stomach, dues to the acidity levels of pH less than 2 caused by the hydrochloric acid in this environment, and bacteria such as *Lactobacillus*, *Neisseria* and *Streptococcus*, come principally from foodstuffs or transient oral populations (Giannella *et al.*, 1972). However, where stomach acidity fails to reach a pH of less than 4, survival within the gastric organ is possible for a greater diversity of species to proliferate. The pH of the stomach may become raised through lowered production of stomach acid, or through externally derived acid suppressors or antacid blocking secretions or neutralising acidity, meaning that stomach loses a significant defensive strategy to prevent pathogenic microbes from surviving in the stomach (Smith, 2003).

The majority of CF patients suffer from exocrine pancreatic insufficiency and receive medication to replace the pancreatic enzymes. Inhibiting gastric acid or GA may form part of treatment, using histamine-2 receptor antagonists or proton pump inhibitors, if inadequate

levels of fat are absorbed even with correct doses of pancreatic enzyme replacement (Walkowiak *et al.*, 2005; Littlewood *et al.*, 2006). Treatments to inhibit gastric acid secretion are also used in CF patients with signs of GORD. PPIs or proton pump inhibitors prevent acid from being secreted through blocking H^+/K^+ ATPase of the gastric parietal cell, and a transporter, which acts as a proton pump, and plays a role in the final step in the cell's acid secreting activity. There are a number of proton pump-inhibiting compound, such as Omeprazole, Lansoprazole, Esomeprazole, Dexlansoprazole, Raberprazole and Pantoprazole (Orenstein *et al.*, 1999).

There is a report of microbial flora within the stomach increasing from 0.47×10^6 under a pH of 2 to 5.13×10^6 cfu/ml under a pH of 6 on treatment with PPI medication (Goddard and Spiller, 1996). When compared to healthy individuals, greater quantities of gram negative microbes were isolated in the gastric juices of patients in intensive care who were being treated with acid suppressors (Du Moulin *et al.*, 1982). It has been demonstrated that PPI medicines change the microflora of the stomach and lead to rises in overgrowth of bacteria: these mainly come from the mouth and in reduced acid conditions can live within the stomach (Williams and McColl, 2006).

Across a range of non-CF populations, a link has been made to associate gastric acid inhibition medications with higher risk of lung infection: for instance, patients in critical care who are given histamine-2 antagonists to prevent stress ulcers are at greater risk of developing pneumonia (Mallow *et al.*, 2004). Further, children with GORD and adults on gastric acid inhibiting medication are both more likely to develop pneumonia in the community (Laheij *et al.*, 2004; Canani *et al.*, 2006).

CF patients being treated with gastric acid inhibitors for GORD or for malabsorption of fats were targeted in a longitudinal study with 218 paediatric patients with CF considering microbial colonisation and the functioning of the lungs (Forced expiratory volume in 1 second FEV_1, Forced vital capacity FVC, Maximum expiratory flow at 50% of the largest FVC (MEF_{50}), maximum mean expiratory flow ($MMEF_{25-75}$)). No effect was reported on lung function or bacterial culture for acquiring or colonisation by *SA* and *PA* in patient taken GA inhibition for fat malabsorption, with no difference compared to patients with no history of GA inhibition (van der Doef *et al.*, 2009). The findings support and expand upon a previous study which found no difference in FVC, MEF50 and FEV_1 for 14 CF patients who had taken GA inhibitors for 12 months to counter malabsorption of fat (Zapletal *et al.*, 1987), and this indicated a potentially positive impact on obstruction of the peripheral respiratory passages. Thus, there is no

contraindication for CF patients in taking GA inhibiting medications to promote absorption of fat, and further, there may be a positive impact for certain areas of decreased lung function (van der Doef *et al.*, 2009).

However, CF individuals taking GA inhibitors to treat GORD showed decreases in lung function (van der Doef *et al.*, 2009), as also seen in a previous cohort research project of cross-sectional design (Stringer *et al.*, 1988). In terms of variation between FVC and FEV_1, for CF patients, those diagnosed with GORD initially acquired *SA* and *PA* sooner. It is suggested that hyperinflation and obstruction to airways more likely stem from GORD rather than the gastric acid inhibitors, as the treatment did not impact upon lung function for those taking it for fat absorption issues (van der Doef *et al.*, 2009).

Further, aspirating gastric acid has been demonstrated in mice to support *PA* in attaching to the epithelium of the respiratory passage (Mitsushima *et al.*, 2002). Mitsushima *et al.* (2002) sought to establish whether aspirated acid plays a role in *PA*'s ability to adhere to surfaces. Microbial numbers surviving within the tissues of the lungs were assessed following the introduction of HCl alone, *PA* alone or the two in combination in the intratracheal area. A scanning electron microscope was used to assess how far bacteria had attached to the epithelial wall of the trachea following aspiration of acid. Introduction of 50 µl 10^{-1} Molar hydrochloric acid together with *PA* at lower than lethal dose gave a large increase in quantities of *PA* in the tissues of the lungs, lowering survival rates. Further, it was noted that those mice receiving hydrochloric acid showed a much higher rate of epithelial adhesion of *PA* in comparison to saline. The findings suggest that damage was inflicted to the epithelium and *PA* was then able to adhere further to it epithelium, causing subjects to develop bacterial pneumonia. Based on this, greater ability for bacterial pathogens to adhere to epithelial tissue, which has been damaged by acid, could provide a cause for bacterial pneumonia leading to death in humans with aspirated gastric juices.

The bacteria inside the stomach may be transferred to the lungs via GOR and this might underlie reports of greater pneumonia prevalence for patients in intensive care who received PPIs (Tryba and Cook, 1995; Torres *et al.*, 1996). For those taking PPI, the pH of the stomach contents refluxed is higher, with the likelihood of higher bacterial concentration and greater levels of endotoxins in comparison to those not on the medication, and this may cause a more pronounced inflammatory response where aspiration to the lungs occurs (Pauwels *et al.*, 2013).

1.8 Gastrostomy feeding in CF patients

The energy requirements associated with chronic respiratory infection, anorexia, inadequate dietary intake, maldigestion and malabsorption are the main causes that children with CF often fail to thrive (Borowitz *et al.*, 2009; Matel and Milla, 2009). Malnutrition remains stubbornly common amongst CF patients, in spite of advances in treatment (Matel and Milla, 2009). Energy-rich foods and fluids may be prescribed to boost nutrition; other techniques to promote nutrition include oral supplements, pancreatic enzyme replacement therapy (PERT) and enteral feeding via gastrostomy (Anthony *et al.*, 1999; White *et al.*, 2009)

In CF, enteral tube feeding through percutaneous gastrostomy tube (PEG) may become necessary to ensure adequate nutrition essential for optimal lung function to be attained (Borowitz *et al.*, 2005). Although this is a long-standing procedure that has been used for more than three decades, there have not been any large-scale clinical trials evaluating the effectiveness of the procedure in CF patients. However, evidence of improved pulmonary function and weight gain is provided by small, single-centre retrospective studies (Best *et al.*, 2011; Vandeleur *et al.*, 2013; White *et al.*, 2013; Woestenenk *et al.*, 2013).

An advantage of PEG is that once inserted it is easy to manage outside of the hospital setting, such as at home (Novotny *et al.*, 2009; Wang *et al.*, 2014). It is possible to leave PEG tubes in situ for long periods, though in time they do deteriorate or may be accidently removed by the patient, so need to be replaced (Wang *et al.*, 2014).

1.8.1 PEG tube infection

Using the random amplified polymorphic DNA (RAPD) technique, Dautle *et al.* (2003) analysed PEG tube microbiota. Biofilms that that developed on 18 gastronomy devices were collected from CF patients ranging from 6 months to 17 years. The mean time that the PEG tubes had been in place was 20 months (range 3–47 months). There were diverse species present, including *enterococci*, *staphylococci*, *E. coli*, *Lactobacilli*, *Candida*, *Pseudomonas* and *Bacilli* (Dautle *et al.*, 2003).

Culturing methods were used to evaluate PEG patients' gastric and duodenal microbiota as well as of their PEG tube surfaces. The prevalence of some types of infection was increased in those patients who had antibiotic therapy before the PEG tube was placed; they also had lower mortality rates (Nicholson *et al.*, 2000). The predominant species isolated were *Candida*,

Enterobacteria, Lactobacilli, Staphylococci and *Streptococci* (Graeme *et al.*, 2005a). The evidence suggests that the density of the colonies in the stomach and duodena of EN patients was not influenced by gastric pH; however, the composition of microbiota was affected. *Bifidobacterium, Klebsiella and Staphylococcus* species were detected but only in aspirate where the pH was above 3. Patients, who during a hospital stay had received antibiotic treatment, presented *Candida, E. coli* and *Staphylococci* in aspirate.

Smith *et al.* (2011) characterised the microbial colonies of the gastric mucosa in eight PEG patients by using real-time polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH). Compared to controls, the mean levels of *Enterobacteria* and *Staphylococci* were considerably greater in PEG patients. However, the PEG patients also had lower levels of the pro-inflammatory cytokines IL-1α, IL-6 and TNF-α. Microbial biofilms containing pathogenic species contaminate PEG tubes, potentially resulting in several infections including peristomal infection and presenting the risk of sepsis (Blomberg *et al.*, 2012). Because antibiotics alone are unable to resolve the pathogens in the biofilm, to cure the infection and prevent re-infection, the PEG may need to be removed.

1.9 Overall aim of the thesis

In light of the previous discussion, the current research project was created to explore the effect of gastroesophageal reflux on the lung microbiome. I suggested here that bacteria in the stomachs of those treated with PPIs grow excessively, with an upstream impact upon the microbial content in the lungs and oropharynx due to full column reflux. Moreover, this project explores the possibility that bile derived from GOR might impact upon the microbial picture within the lungs and in this way exacerbate pulmonary disease seen in patient with GOR.

Chapter 2.0: Methods

2.1 Ethical approval

Research Approval was obtained from Newcastle and North Tyneside Research Ethics Committee (UK) to perform research on samples collected as part of the HPB (Hepatopancreatobiliary) groups biobank based in Newcastle University. All study participants provided written informed consent prior to initiation of the study. All methods were carried out in accordance with relevant guidelines.

2.2 Recruitment of patients

The research sample cohort comprised of 18 adults with (mean age 26.7 ±5.7, with unknown gender) with CF in a state of clinical stability who had undergone PEG (percutaneous endoscopic gastrostomy) feeding and who had a routine outpatient appointment with the Royal Victoria Infirmary (RVI) chest clinic. Patients were given information concerning the research project and time to read this via nursing staff, and then asked for their consent to participate. After agreeing to participate, a nurse read the consent form aloud to patients, ticking off points as agreement was given. Following this, patients and nurses signed, printed their names and dated the documents. Copies of all consent forms were held by the research centre and each participant held a consent form and an information document. Recruitment of patients was done according to the inclusion criteria, which meant that participant had to be; an adult (> 18 years old), diagnosed with CF, clinically stable, and in attendance at an RVI appointment with PEG Tube feeding. Those who had not provided sputum and those less than 18 years old with oral feeding were excluded.

2.3 CF patients included in this study

There were 270 CF patients attending the regional CF Centre at the time of the study, and among these only 18 were adult, stable CF patients receiving PEG tube feeding and were therefore included in this study. CF patients receiving PEG feeding represented an important opportunity to directly sample gastric juice, with less potential for contamination with oropharyngeal commensals.

From the study population of 18 individuals, 5 replaced PEG tubes, 31 samples of gastric juice and 31 sputum samples were gathered. In addition, 16 complete Reflux Symptomatic Index (RSI) questionnaires were collected (Figure 2.1).

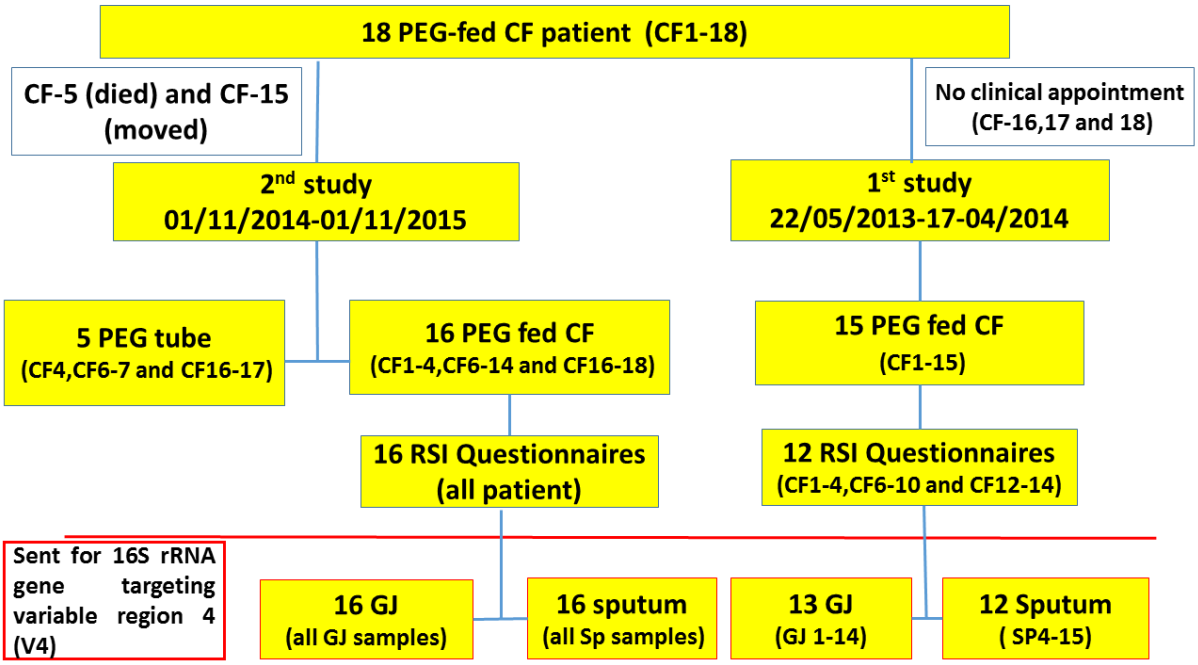

Figure 2-1: Summary of patient recruitment. 15 patients were included in the first study (Chapter 3) and 16 patients were included in the second (Chapter 4) and third studies (Chapter 5). CF: cystic fibrosis, GJ: gastric juice, PEG: percutaneous gastrostomy tubes.

2.4 Patient characteristics and clinical details for CF patients

Suitably qualified personnel took measurements of forced expiratory volume in 1 second (FEV_1) during patients' regular appointments. Mr Alan Anderson, a specialist CF nurse, collected and passed on this data to the researcher, as approved by Dr. Stephen Bourke, Respiratory Consultant at the RVI. Moreover, Mr Alan Anderson gathered patient age, sex, BMI, long-term antibiotic status and CFTR genotypes, as well as whether the patient was on acid suppression medication or not from participants' records. Clinical details of the patients are presented in Table 2.1.

Table 2.1: Demographic data for CF patients

Patient	Genetics	Age	RSI score	PPI yes/no	Gastric Juice pH	FEV1 (%)	BMI	IV (days /year)	Long-term antibiotic
CF-1	F508del/F508del	26	17	Yes	6	2.0 L (52%)	19.9	22	Azith, Inh Coli and Inh Tob
CF-2	F508del/F508del	27	20	Yes	2	1.7 L (42%)	23.2	70	Azith, Fluclox and Inh coli
CF-3	F508del/F508del	20	25	Ranitidin	3	0.8 L (26%)	19.5	28	Azith, Fluclox and Inh Coli
CF-4	F508del/F508del	24	36	Yes	6	0.76 L (28%)	19	154	Azith and Inh Coli
CF-5	F508del/F508del	41	NA [died]	Yes	5.5	0.45 L (18%)	18.2	65	Azith and Inh Tob
CF-6	F508del/F508del	31	16	Yes	6	0.5 L (12%)	19.1	70	Azith and Inh Coli
CF-7	F508del/ R117H	22	16	Yes	3	2.7 L (66%)	16.4	14	Fluclox
CF-8	I507del/ Arg560Lys	18	13	Yes	2	3.5 L (88%)	19.4	37	Fluclox, Inh Coli and Inh Tob
CF-9	F508del/R117H	30	14	Yes	6	1.55 L (46%)	17.8	56	Fluclox and Inh Coli
CF-10	F508del/F508del	25	17	Yes	2	1.7 L (38%)	15.9	98	Azith, Fluclox and Inh Tob
CF-11	F508del/G542X	32	NA [PEG removed]	Yes	2	1.15 L (36%)	19.4	112	Azith and Inh Coli
CF-12	F508del/F508del	30	19	Yes	6	1.2 L (29%)	19.8	115	Azith, Fluclox and Inh Coli
CF-13	F508del/G542X	24	15	Yes	2	1.65 (36%)	15.24	197	Azith, Inh Coli and Inh Tob
CF-14	F508del/ Arg851Ter	23	22	Yes	6	2.3 (59%)	20.2	56	Azith
CF15	G542X/G551D	22	NA [moved country]	Yes	2	0.85 L (28%)	18	42	Azith and Inh Coli
CF-16	F508del/F508del	25	24	Yes	4	0.85 L (29%)	17.3	84	Azith Inh Colistin and Fluclox
CF-17	F508del/Arg851Ter	25	8	Yes	3	2.1 L (54%)	20.1	56	Doxycycline and Azith
CF-18	F508del/Ile507del	36	NA	Yes	4	0.72 L (17%)	21.9	112	Inh Colistin

Azith = oral azithromycin long-term. Fluclox = oral flucloxacillin long-term. Inh Coli = inhaled colistin (nebulised or inhaler). Inh Tob = inhaled tobramycin (nebulised or inhaler). RSI score= Reflux symptom index score, 12 or less is normal. NA= not available. IV days /year= Number of days per year that CF patient treated with intravenous antibiotics. Gender is not known for patients.

2.5 Non-CF patients included in this study

Fourteen patients without CF undergoing routine upper gastro-intestinal endoscopy performed according to British Society of Gastroenterology guidelines were also included in this study as a control group. Gastric juice only was collected from those patients. Sputum was not collected from the non-CF patients (Table 2.2).

Table 2.2: Demographic data for the non-CF patients

Patient No	Age (year)	Background disease	PPI yes/no*	Gastric juice pH
1	75	Oesophagitis	yes	2.4
2	56	Oesophagitis and Pyloroplasty	yes	6.6
3	65	Barrett's Oesophagus and Hiatus Hernia	no	4.8
4	59	Hiatus Hernia	yes	2
5	45	Oesophagitis and Hiatus Hernia	n/a	1.4
6	42	Gastritis and Hiatus Hernia	yes	5.5
7	58	Oesophagitis and Hiatus Hernia	yes	4
8	80	Not known	n/a	4.7
9	50	Gastric ulcer	yes	8.4
10	78	Gastritis and Hiatus Hernia	n/a	1.6
11	73	Barrett's Oesophagus	yes	5.1
12	55	Not known	n/a	5.2
13	68	Duodenal ulcer	n/a	6
14	65	Gastritis	n/a	1.7

All patients were off PPI or any other acid suppression medication 2 weeks before the endoscopy procedure (n=14). Gender is not known for patients.

2.6 Collection of samples

2.6.1 Collecting gastric juice from CF patients (CFGJ)

After a fast from the previous night, a total of 31 samples of gastric juice were taken from 18 individuals through aspiration and collection via the PEG tube, carried out by specialist nursing staff in the RVI cystic fibrosis clinic. The procedure for sampling via aspiration was as follows. First, the individual is positioned on their side, before injecting between 3 and 5 ml saline to the tube via a large volume syringe. The patient is shaken gently between 3 and 5 times, aspirating between one and three minutes later.

In this way, 31 samples of gastric juice (GJ) were gathered from 18 individuals with CF. For 13 of the participants (CF1-4 and CF 6-14), aspiration was used to take two samples of GJ with a gap of 6-12 months. Meanwhile, for the other 5, only one sample was taken (CF5 and CF15-18). This was due to participants not attending a second appointment within the time of study (CF16-18), moving from the regional clinic (CF15), or dying (CF-5).

2.6.2 Sputum collection from CF patient (CFS)

Voluntary expectorated (non-induce) sputum samples were requested from each patient. On entering the RVI, nurses gave out a sputum pot and participants were asked to provide a sample of sputum via expectoration, with time allowed in a consulting room for this. Normal contamination protocols for the clinic were used.

31 spontaneously expectorated sputum samples from the same 18 CF patients were also obtained and collected into sterile cups. 2 sputum samples aspirated from 13 CF patients on 2 different occasions with 6-12 months in between. Only one sputum sample was aspirated from the remaining 5 patients, because either the patient had died (CF-5), moved (CF-15) or not attended a second appointment at the time of the study (CF16-18).

2.6.3 Collection of non-CF gastric juice samples (GJ)

Collection of gastric juice samples was conducted with fourteen participants who did not have CF and were attending the hospital for a clinical endoscopic examination of the upper gastro-intestinal tract to identify a range of diseases of this area. The procedure was carried out after approximately eight hours' fasting. Suction was applied via the endoscope to collect the fluid. As per guidance from the British Society of Gastroenterology. Participants were given either 5 mg midazolam or xylocaine prior to aspirating gastric juice from the stomach and gathering it into a trap (Pennine Healthcare, UK). This group of participants were not asked to provide sputum samples.

2.6.4 Collection of replaced PEG tubes from CF patients

Five participants with CF who were fed via PEG provided a total of 5 PEG tubes which were being replaced, with no need for endoscopy or anaesthetic. Manual traction was gently applied

to the portion of the PEG tubing visible externally, and the apparatus was, complete with collapsible internal bumper, removed through the PEG hole. Where PEG tubes had a balloon retainer, these were completely deflated prior to removing the tube. Removals were undertaken by dieticians at the RVI.

2.7 Transferring samples to the Freeman Hospital

The general rule as applied to samples of biological origin is that accuracy and reliability are best served by processing as soon as possible after collection. Thus, the Health Protection Agency's Standard Operating Procedures (Advisory Committee on Dangerous Pathogens, 2005), advises rapid transport and processing, with refrigeration allowable for only between two and three hours before significant pathogen content is lost. Thus, PEG tubes and samples of sputum and gastric juice underwent immediate transfer by the researcher to the Freeman Hospital's Department of Microbiology in not more than 3 hours from being provided (SOP Index S19, Version 2, Sir William Leech Centre, Freeman Hospital, Newcastle upon Tyne, UK).

2.8 Processing of sputum and gastric juice samples

As the gastric juice samples arrived, their pH was measured with strip indicators (Scientific Laboratories, UK). Samples of sputum were delivered to a category 3 cabinet, where saliva and the sputum plug underwent separation before a matching volume of sputasol, containing sputolysin (Dithiothreitol (DTT) at a concentration of 0.2% in DH_2O (de-ionised water) was mixed with the sputum. This dissolves the mucus by cleavage of disulphide bonds. For this, the sample was generally shaken for between one and three minutes at ambient temperature in a vortex mixer to homogenise the sample, as checked by eye.

2.9 Microbial study

2.9.1 Microbial study of samples of gastric juice and sputum

Samples of gastric juice from CF and non-CF participants, as well as samples of sputum from CF patients, underwent processing and culturing within the Freeman Hospital's Department of

Microbiology, following the hospital's accredited safety and sterility protocols and the guidelines put forward by the Health Protection Agency. This was supervised by Mrs Audrey Perry, clinical scientist.

Separate plating took place for 10 µl undiluted gastric juice and 10 µl homogenised sputum. The media used were those routinely employed for anaerobic and aerobic microorganisms as well as yeasts. These media were: Columbia blood agar with 5% supplementation of horse blood, Burkholderia selective agar, chocolate agar with supplementation of 70 mg/L bactericine, fastidious anaerobic agar (FAA), sabouraud's agar and cysteine lactose electrolyte deficient agar (CLED). For plating of the sputum samples, a category 3 cabinet was used since they may contain tubercle bacilli. Incubation of the plates was then done following routine protocols.

Each plate was assessed every 24 hours to check for signs of microbes growing, and separate colony varieties were counted. Presumptive isolates which emerged and all different morphological forms of these were subject to sub-culturing, done by placing sterile wires on colonies before using these to inoculate agar culture plates and incubating for 24 hours at 37°C. Microbes were identified using the resultant subcultures, which were immediately stored in 10% glycerol at -80°C.

The standard culture plates, specific incubation conditions and intended selected bacteria are detailed in Table 2.3

Table 2.3: Cystic fibrosis Sputum Samples Culture Protocol, Microbiology Department at the Freeman Hospital.

Standard media	**Incubation**			**Cultures read**	**Target organisms**
	Temp (°C)	**Atmosphere**	**Time**		
Columbia blood agar	35-37	5-10% CO_2	24-48h	Daily	*Streptococcus pneumoniae* *Moraxella catarrhalis* *Staphylococcus aureus* Other organism in pure growth
Chocolate agar with Bacitracin	35-37	5-10% CO_2	24-48h	Daily	*Haemophilus spp* Other gram negative bacteria
Cysteine Lactose Electrolyte Deficient	35-37	Air	24-48h	Daily	*Enterobacteriaceae* *Staphylococcus* spp, *Enterococci* spp, *Pseudomonas* spp
Burkholderia Cepacia agar	30	Air	5 days	Daily 10-day Terminal Read	*Burkholderia cepacia* *Atypical Mycobacteria*
Sabourauds agar	35	Air	24-48h,up to 5 days	Daily	*Candida spp* *Aspergillus spp* Other fungi
Fastidious Anaerobic agar	35-37	anaerobic	5 days	10-day Terminal Read	A primary isolation medium capable of growing most clinically significant anaerobes.

2.9.2 Microbial study of PEG tubes

On retrieval from a participant, the PEG tube was put quickly into a sterile bag for immediate transfer to the Freeman Hospital.

The inner and outer parts of the PEG tube were divided using an aseptic technique into small pieces and vigorously washed with 3ml saline, yielding a PEG conditioned saline (PEG-s).

2.10 Organism identification by MALDI-TOF MS

The Microbiology Department of the Freeman Hospital principally identified bacteria using MALDI-TOF: Matrix Assisted Laser Desorption/ Ionization-Time of Flight (Bruker Daltonics, UK). Where results were not completely clear, identification also relied on features seen on microscopy and on gram-stain and where necessary, appropriate analytical profile index (API) kits which is a commercial miniaturized biochemical test panels that cover a significant number of clinically important groups of bacteria (bioMérieux, UK) (Blauwendraat *et al.*, 2012)..

MALDI-TOF MS has come into routine use across a number of laboratories of clinical microbiology as a much faster route to identification than previous approaches. Most cultured colonies can be accurately and quickly identified via this method (Seng *et al.*, 2013), and evaluation of the technique has been undertaken for pathogenic organisms in CF (Desai *et al.*, 2012). The approach is based on the ability to create a distinct peptidic spectrum based on each different isolate, and use of database comparison to identify the microbe. Desai et al. (2012) have recently shown that when using a microbial database without supplementing this with strains which were specifically related to CF, 92% of microbes isolated in 24 samples from CF patients could be identified with 100% agreement at the level of both genus and species, while for 98% this was true for genus. Among those microbes which could only be identified at the level of genus were *Acinetobacter* spp, *Achromobacter xylosoxidans* and *Ralstonia pickettii*, while *Burkholderia multivorans* is the sole microbe among the *B. cepacia* complex identifiable on a reliable basis through MALDI-TOF MS, making this method unsuitable for identification of this group, which, along with such organisms as non-aeruginosa *Pseudomonas*, frequently need to be identified via sequence-based approaches.

MALDI-TOF MS is also suitable to quickly and accurately identify a range of non-bacterial organisms, such as in the case of CF-sample filamentous fungi, as shown by Del Chierico *et al.* (2012). Further, mycobacterial identification via MALDI-TOF MS has been partially investigated, with El Khechine *et al.* (2011) describing a protocol for extracting protein in an optimised manner and creating a database for mycobacteria to allow the majority of mycobacterial isolates grown in solid media to be identified. While this approach is not currently routinely employed, it has the potential to increase speed and accuracy in identifying such pathogenic organisms in CF. A further development is that due to the scope for comprehensive analysis via MALDI-TOF MS, it may be possible to identify potentially emergent or new microbes in CF (Bittar *et al.*, 2010).

2.10.1 Mycobacterium and Pseudomonas aeruginosa strain identification

Mycobacteria was identified by rpoB, sodA and hsp65 gene sequencing and strain typed using variable number tandem repeat (VNTR), Colindale, UK (Harris *et al.*, 2012). All isolates of *PA* were typed via VNTR profiling (Turton *et al.*, 2010)

2.11 DNA extraction

Microbial genomic deoxyribonucleic acid (gDNA) extraction from sputum and gastric juice samples was carried out at Freeman Hospital in a category 3 facility using a MOBIO PowerSoil™ DNA Isolation Kit (http://www.mobio.com/images/custom/file/protocol/12888.pdf). To the PowerBead Tubes provided in the kit, 0.25g of the sample was added before being gently vortexed. The PowerBead Tube contains buffers that begin to dissolve bacterial cells and protect nucleic acids from degradation.

Sixty µL of solution C1 which was then pipetted and mixed into the PowerBead tubes by inverting it several times. Solution C1 contain Sodium dodecyl sulphate (SDS) which is an anionic detergent that breaks down the fatty acid and lipid in the cell membrane. PowerBead Tubes were secured horizontally and vortexed at maximum speed for 10 minutes. Following this, the PowerBead tubes were then centrifuged at room temperature for 1 minute at 10,000g after which the supernatant was transferred to a clean sterile 2 mL collection tube (provided).

250 µL of solution C2, which contains reagent to precipitate non-DNA organic and non-organic cell material, was then added to the supernatant. This supernatant was then vortexed for 5 seconds and incubated for 5 minutes at 4°C before being centrifuged at 10,000g at room temperature for 1 minute, to form a pellet.

After that, 600 µL of supernatant was transferred to a sterile 2 mL collection tube containing 200 µL of solution C3, which is a second reagent to help precipitate any remaining non-DNA cellular material, and then vortexed and incubated at 4°C for 5 minutes followed by centrifugation at 10,000g at room temperature for 1 minute. Again, transfer of the supernatant to a clean 2 mL collection tube was performed before the addition of 1.2 mL of solution C4, which is a highly concentrated salt solution to help the binding of DNA to the spin filters.

Approximately 650 µL was then loaded onto a spin filter and centrifuged at 10,000g for 1 minute at room temperature after the flow through liquid was discarded. This spin filter process was repeated twice more. 500 µL of solution C5, which is an ethanol wash solution used to clean the DNA bound to the silica membrane of spin filter, was added before centrifugation at 10,000g for 1 minute at room temperature, after which the flow through liquid was discarded from the 2 mL collection tube. Centrifugation at 10,000g was carried out again for 1 minute at room temperature for the effective removal of any residual ethanol wash solution.

After the removal of solution C5, the spin filter was placed into a clean sterile 2 mL collection tube and 100 µL of Solution C6, which is an elution buffer, to help to make sure that the entire membrane is wetted, was added to the centre of the filter membrane, releasing the previously bound gDNA from the silica spin filter membrane. The final step was to centrifuge the 2 mL collection tube at 10,000g for 1 minute at room temperature after which the released gDNA (50 µL in volume) was collected. The spin filter was discarded and the extracted gDNA was stored at -80°C.

2.12 Identification of extra oesophageal reflux symptoms (EOR)

The questionnaire instrument used to evaluate symptoms of EOR and GOR symptoms was the RSI or Reflux Symptom Index questionnaire (for RSI: see Appendix1). This instrument was created originally for the evaluation of laryngopharyngeal reflux (Belafsky *et al.*, 2002); however, it later received validation as a means of evaluating EOR, through its use in a number of such projects. The survey contains multiple questions concerning EOR signs and symptoms, such as throat-clearing, being hoarse, problems with swallowing, cough, postnasal drip, symptoms of GOR, Globus and breathing problems. Questions used a 6-point scale with a score of 0-5, with 0 meaning that no issue is reported and 5 indicating an issue of the highest severity. The values obtained were added together. Symptomatic participants were defined by a score of more than 12 as a total, while those below this were counted as not showing EOR symptoms (Belafsky *et al.*, 2002).

The published validation procedure for this questionnaire involved 25 individuals diagnosed with laryngopharyngeal reflux, compared to an identical number of healthy participants who were matched in terms of sex and age. The RSI scoring was done pre- and post- proton pump inhibitor application. Scoring before treatment for the laryngopharyngeal reflux group gave a median scoring of 21, while after the proton pump inhibitors, a median of 13 was obtained. For

the normal group the value was 13, which led to this being taken as the limit of normal symptomatic range. Thus, this questionnaire was considered validated for research into EOR and GOR for cohorts undergoing operations to reduce reflux (Robertson *et al.*, 2012).

2.13 Molecular based studies

Extracted DNA was sent to Northumbria University, where Dr Chris Stewart and Dr Andrew Nelson, post-doctoral fellows, performed the molecular analysis of the extracted DNA.
Bacterial were profiled using 16S rRNA gene targeting variable region 4 (V4) based on the Schloss wet-lab MiSeq SOP (http://www.mothur.org/wiki/MiSeq_SOP). Processing of raw fastq data took place via version 1.31.2 of Mothur, in line with guidance given by MiSeq SOP (Kozich *et al.*, 2013). Chimeric sequences were detected by Chimera.uchime and removed from downstream analysis. Alignment was generated via the Silva database (Schloss *et al.*, 2011). A cutoff of 70 (maximum average error allowed) was applied to assign sequences to the trainset_ 9_032012 resulting in 2,228,291 reads. All sequences were deposited in Metagenomics-Rapid Annotation using Subsystem Technology MG-RAST under the accession numbers 4603845.3 - 4603893.3. The server provides the annotation of sequence fragments, their phylogenetic classification, functional classification of samples, and comparison between different metagenomes.

2.14 Statistical analysis

Analysis of the NGS profiles were performed by Dr Cristopher Stewart and Dr Andrew Nelson (Chapter 3 and 4) and conducted by multivariate partial least squares discriminant analysis (PLS-DA) (SIMCA 13.0 software, Stockholm, Sweden) (Eriksson *et al.*, 2006b). PLS-DA uses assigned variables to interrogate data for maximum variance. To check data was adhering to multivariate normalities, Hotelling's T2 tolerance limits were calculated and set at 0.95.

Shannon diversity index was also calculated used the following;

$$H' = -\Sigma (p_i \log[p_i])$$

Individual species' relative intensity logs (Pi) were multiplied by relative intensities from all species across every sample, as (pilog[pi]). Values for individual lanes were added together and then multiplied by -1 (-Σ).

Chapter 3.0: Microbiological profiles of sputum and gastric juice aspirates in cystic fibrosis patients

3.1 Introduction

Among individuals of Caucasian ethnicity, the most frequently occurring condition passed on through recessive inheritance is cystic fibrosis (CF) (Gibson *et al.*, 2003). This condition occurs as a result of a mutation in the cystic fibrosis transmembrane conductance regulator gene (CFTR). CFTR is found on the seventh chromosome, on its long arm (7q31.2), and mutation in CFTR causes disturbance to cAMP-regulated chloride channel activity. This leads to negative effects across several of the body's systems, significantly including lowered enzyme production in the pancreas, impaired nutrition and growth, and chronic disease in the lungs (Rosenecker, 2000).

The majority of early deaths and morbidity linked with CF arise as a result of infection in the lungs which leads to inflammatory processes and ultimately chronic disease of the lungs (Chmiel and Davis, 2003a; Murray *et al.*, 2007). In terms of bacteria infecting the CF lung, the primary phyla involved are Firmicutes (e.g. *SA*) and Proteobacteria (including *Achromobacter xylosoxidans*, *Burkholderia cepacia*, *HI*, *PA* and *Sternotrophomonas maltophilia*) (LiPuma, 2010). Additionally, the development of chronic lung disease in CF is also linked to fungi such as moulds and yeasts, as well as non-tuberculosis mycobacteria (e.g. *M. abscessus)*. Current work involving molecular approaches to identifying microbes has supplemented the long-established culturing techniques, enhancing the ability to identify a range of organisms. This means that increasing numbers of microbes are being identified as linked to lung disease in CF (Rogers *et al.*, 2006; Klepac-Ceraj *et al.*, 2010b).

Pseudomonas aeruginosa (*PA)* can infect lungs recurrently and persistently, and, particularly for CF patients in adulthood, this pathogen is a significant source of infections (Govan and Deretic, 1996). There is evidence to suggest that 8 in 10 adults and 6 in 10 children with CF carry a chronic infection of this pathogen, and thus it constitutes a significant risk to both groups (Gilligan and Kiska, 2006).

There is evidence that patients who are treated at specialist CF facilities show better survival rates, and effective care for lung disease is seen as a primary goal in CF. The advantages offered by specialist CF facilities however are linked to care given across multiple disciplines, with significance also attributed to treatment of gastro-intestinal issues. These issues include gastro-

oesophageal reflux (GOR), which occurs at a >50% prevalence rate in CF patients (Blondeau *et al.*, 2008a). A number of causes for GOR in CF are suggested in the literature. These include a greater transient relaxation of the lower oesophageal sphincter (LOS), lower LOS pressure, delay in emptying of the stomach and the possibility of physiotherapeutic interventions and coughing creating a higher gradient of pressure in the abdomino-thoracic region (Pauwels *et al.*, 2011).

GOR is particularly significant in CF research in terms of the link between material from the stomach being aspirated and decreased function in the lungs, as suggested by evidence that those CF patients who experience GOR tend to have poorer lung function than those who do not (Navarro *et al.*, 2001). Further, treatments to prevent acid reflux are linked to improved lung function and reduced symptoms of respiratory ill-health, while surgical intervention to reduce acid reflux has been shown to reduce exacerbation of CF significantly and to reduce the rate of deterioration in lung function (Sheikh *et al.*, 2013). Conversely, although there is little literature in this area, there is some evidence to suggest that taking the proton pump inhibitor (PPI) esomeprazole (against a placebo) could be linked with CF exacerbations starting earlier and occurring more often (DiMango *et al.*, 2014), suggesting that the acidity is not the only aspect of refluxate which has impact on the airways.

The aero-digestive microbiome in non-CF contexts is already the focus of attention in both gastro-intestinal and respiratory medical fields, with a number of researchers identifying the stomach's microbial reservoir as significant in an intensive care context for the acquisition of nosocomial pneumonia (Du Moulin *et al.*, 1982). Research focusing on older individuals without CF additionally found a link between those bacteria present in the airways and in the gastric area, and report the existence of large numbers of gastric microbes before these appear in the airways (Madan *et al.*, 2012). Findings from research also point to an association between colonies of bacteria developing in the stomach and lower respiratory tract with individuals in intensive care units who receive food through nasogastric tubes (Segal *et al.*, 2006).

There is yet no comprehensive picture of the microbes present in the gastric juice of CF individuals, nor of the possibility of the stomach hosting a reservoir of pathogens identified as responsible for colonising the lungs. In light of this, a study was conducted in which I examined microbiological features of both the gastric juice and the sputum of CF patients, hypothesising that there would be some concordance between microbes found in the two.

3.2 Methods

3.2.1 Samples of sputum and gastric juice from CF patients

It was possible to take direct samples of gastric juice from PEG-fed CF patients while minimising the risk that the samples would be contaminated with commensals from the mouth and pharynx as detailed in Chapter 2. In brief, fifteen PEG-fed study participants were recruited from those attending the clinic between 22nd May 2013 and 17th April 2014. The participants were numbered CF-1 to CF-15, in sequence, and this number comprised 83% of the total number of PEG-fed CF individuals living locally. No exclusions or targeted inclusions of patients in the relevant population were made.

To provide a control, samples of gastric juice were collected from fourteen non-CF individuals receiving gastro-intestinal endoscopy as a routine intervention. The procedure was carried out in line with the guidance given by the British Society of Gastroenterology as described in Chapter 2. This control group were not asked to provide sputum samples.

Microbiological culture of both sputum and gastric juice samples took place in line with methodology adopted as standard in UK practice. Each type of sample underwent DNA extraction, and the resulting DNA underwent molecular profiling at the University of Northumbria. The 16S rRNA gene targeting variable region 4 (V4), in line with the Schloss wet-lab MiSeq SOP, was used to profile the bacteria present. CF individuals were given an assessment for extra-oesophageal reflux (EOR) symptoms through Reflux Symptoms Index (RSI) scoring, with any result of 12 or less categorised as not showing symptoms of EOR.

Dr Chris Stewart, a postdoctoral fellow of the University of Northumbria, undertook analysis of the NGS profile through multivariate partial least squares discriminant analysis (PLS-DA) (SIMCA 13.0, Stockholm, Sweden) (Eriksson *et al.*, 2006a). This technique examines data through set variables for maximum variance. Data was checked to find whether it was in line with multivariate norms by calculating Hotelling's T2 tolerance limits, which were identified as 0.95.

3.3 Results

3.3.1 CF Patients

The study involved fifteen adults with CF, who were invited to take part via routine appointments at the chest clinic at the RVI in Newcastle upon Tyne. The ages of the patients ranged from 18 to 41, with a median age of 25 (Figure 3.1 A), and BMI ranged between 15 and 23, with a median of 19.1 (Figure 3.1 B). The severity level of cystic fibrosis lung disease for the group was in line with the population parameters, with a median forced expiratory volume in 1 second (FEV_1) of 1.55L (39%), ranging from 0.45 to 3.5L (from 12% to 88%) (Figure 3.1 C). The patient group therefore included people with moderate to severe airflow obstruction.

Long-term antibiotic use was recorded at a mean average of 65 days per year, ranging from 14 to 197 days (Figure 3.1 D). All of the participants took treatment to suppress gastric acid (either PPI or H2-antagonist), in line with common clinical practice in CF.

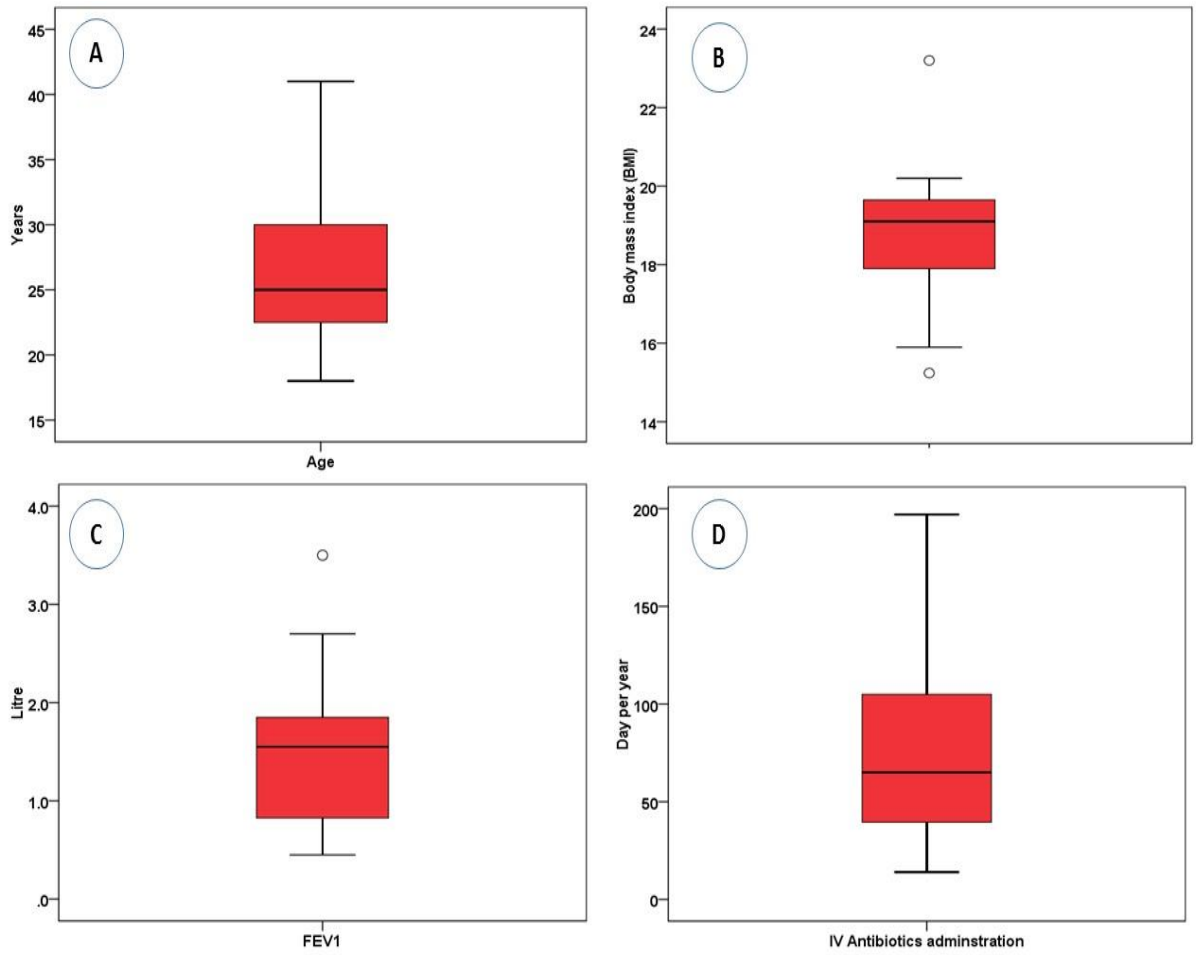

Figure 3-1: Demographic characteristic of CF Patients (n=15). A) Age of CF patients (range 18-41, median 25 years). B) Patient Body Mass Index (kg/m^2) (median 19.10, range 15-23). C) FEV_1 in CF patients included in this study (median = 1.55L (39%), range 0.45-3.5L (12%-88%). D) CF patients intravenous (IV) antibiotic status (average number of days in which patients receive IV antibiotics per year) (median 65 day/year, range 14-197). The small circles represent the outliers.

3.3.2 CF group: symptoms of extra-oesophageal reflux

EOR symptom assessment and scoring was accessed for 12 out of 15 CF patients, as shown in Table 2.1, and with scores of over 12, all 12 individuals were scored as showing symptoms, ranging from 13 to 36 with a median score of 17, as shown in Figure 3.2.

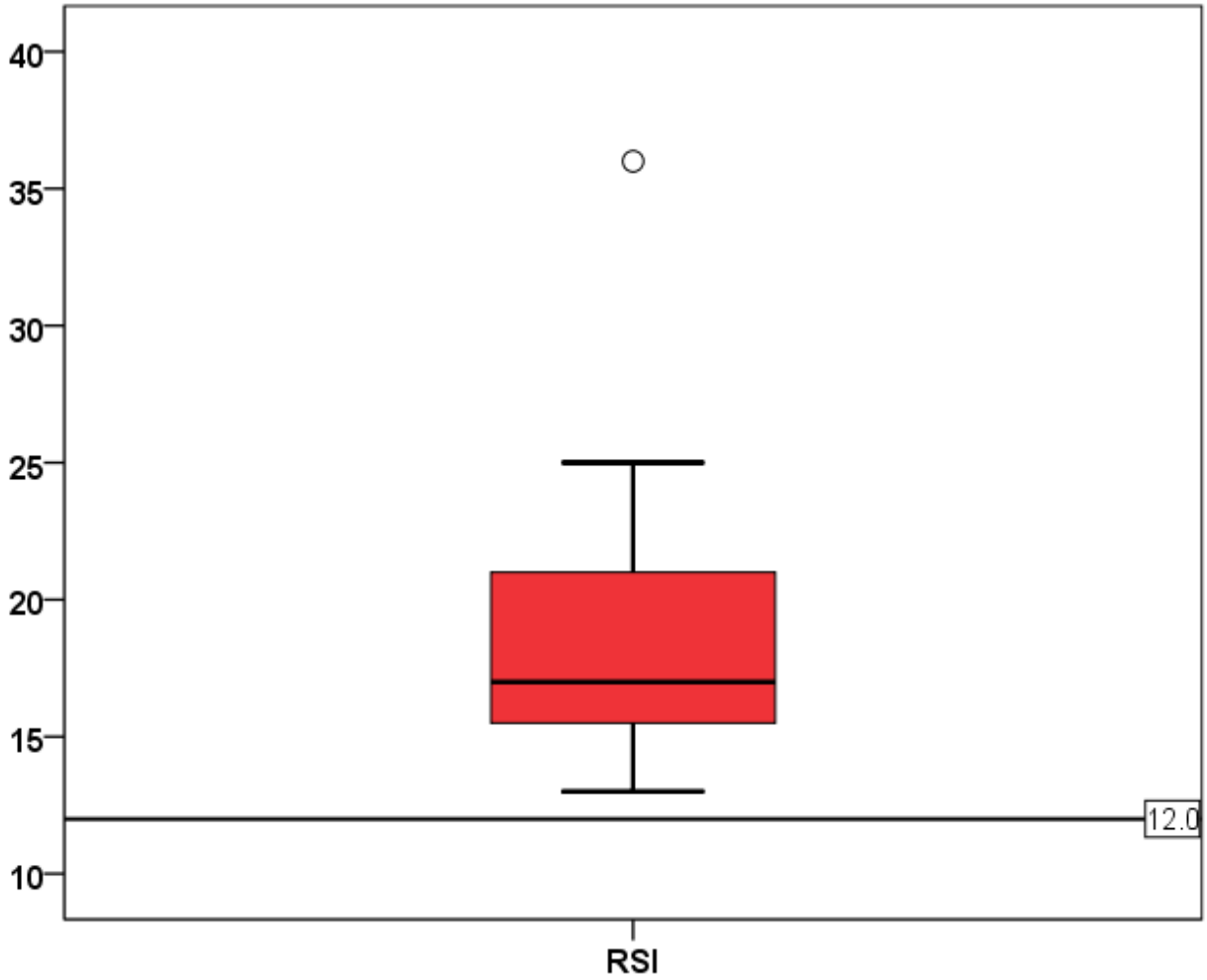

Figure 3-2: EOR symptom status as scored using the Reflux Symptom Index (RSI). The highest score possible on the scale is 45, with scores of 12 or less considered symptom-free, as shown by the line across the chart. Patients were all scored as having EOR symptoms, with scores ranging from 13 to 36, and a median score of 17. The small circle represent the outlier.

3.3.3 Microbial culture

When samples were cultured, positive findings resulted for fungi and/or bacterial microbes across all of both types of sample (sputum and gastric juice). Further, while one sample (CF-8 gastric juice sample) led to isolation of a single species, *Candida albicans*, each of the remaining samples led to isolation of more than a single organism. In 9 out of 14 samples of gastric juice from participants without CF, organisms were isolated.

The microbes most commonly identified in both gastric and sputum CF samples were *Candida* spp, *Streptococcus* spp, *PA and* S*taphylococcus* spp. In the gastric juice samples from participants without CF, the most common microbes isolated were *Streptococcus* spp, found in four of the samples, *Lactobacillus* spp were found, in two of the samples, and *Staphylococcus* spp, in two of the samples (Tables 3.1, 3.2 and 3.3 show the microbial content of CF gastric juice samples, CF sputum samples and gastric juice samples from participants without CF).

Table 3.1: CF gastric juice culture results

Gastric juice 1	*Candida albicans, Streptococcus mitis, Pseudomonas aeruginosa* and *Corynebacterium* sp
Gastric juice 2	*Candida kruzei* and *Aspergillus fumigatus*
Gastric juice 3	*Candida albicans* and *Staphylococcus hominis*
Gastric juice 4	*Candida* sp, *Streptococcus mitis, Staphylococcus haemolyticus, Neisseria sp, Brevundimonas* sp and *Delftia acidominus*
Gastric juice 5	*Candida albicans, Candida glabrata ,Candida kruzei, Pseudomonas aeruginosa , Achromobacter xylosoxidans, Lactobacillus fermentum* and *Alpha haemolytic streptococcus*
Gastric juice 6	*Candida albicans, Candida parasilosis, Candida kruzei* and *Streptococcus parasanguinis*
Gastric juice 7	*Candida glabrata* and *Candida albicans*
Gastric juice 8	*Candida albicans*
Gastric juice 9	*Candida glabrata, Candida albicans, Candida krusei* and *Lactobacillus* sp
Gastric juice 10	*Candida krusei, Acinetobacter* sp, *Lactobacilli gasseri* and *Micrococcus luteus*
Gastric juice 11	*Candida albicans, Candida parapsilosis , Candida glabrata* and *Staphylococcus hominis*
Gastric juice 12	*Candida albicans, Candida glabrata, Propionibacterium acnes* and *Pseudomonas aeruginosa*
Gastric juice 13	*Candida glabrata, Candida albicans* and *Candida krusei*
Gastric juice 14	*Candida albicans, Candida glabrata, Enterococcus faecium* and *Klebsiella pneumoniae*
Gastric juice 15	*Candida lusitania, Candida parapsilosis, Pseudomonas aeruginosa* and *Lactobacillus gasseri*

Table 3.2: Sputum sample microbiology result

Sputum 1	*Candida albicans , Pseudomonas aeruginosa* and *Streptococcus mitis*
Sputum 2	*Streptococcus oralis, Achromobacter* sp and *Aspergillus fumigatus*
Sputum 3	*Rothia mucilaginosa, Streptococcus mitis, Aspergillus fumigatus* and *Citrobacter koseri*
Sputum 4	*Candida albicans, Candida* spp*, Capnocytophaga sputigena, Staphylococcus aureus, Streptococcus mitis, Streptococcus parasanguinis, Aspergillus fumigatus,* and *Mycobacterium abscessus* subsp. *abscessus*
Sputum 5	*Candida albicans, Pseudomonas aeruginosa* and *Achromobacter xylosoxidans*
Sputum 6	*Candida albicans, Achromobacter xylosoxidans* and *Pseudomonas aeruginosa*
Sputum 7	*Rothia dentocariosa, Rothia mucilaginosa, Rothia aeria, Haemophilus parainfluenzae, Streptococcus mitis, Neisseria mucosa* and *Mycobacterium abscessus* subsp. *abscessus*
Sputum 8	*Neisseria flavescens , Streptococcus salivarius, Actinomyces graevenitzii Pseudomonas aeruginosa, Rothia mucilaginosa* and *Stenotrophomonas maltophilia*
Sputum 9	*Pseudomonas aeruginosa* and *Aspergillus fumigatus*
Sputum 10	*Pseudomonas aeruginosa, Alpha haemolytic streptococcus* and *Burkholderia multivorans*
Sputum 11	*Candida albicans, Streptococcus mitis, Rothia mucilaginosa,* and *Pseudomonas aeruginosa*
Sputum 12	*Candida albicans, Pseudomonas aeruginosa, Streptococcus parasanquinis* and *Staphylococcus epidermidis*
Sputum 13	*Candida glabrata, Candida albicans, Candida parapsilosis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus faecium*
Sputum 14	*Candida parapsilosis, Staphylococcus aureus, Streptococcus salivarius, Neisseria* spp*, Streptococcus salivarius, Actinomyces odontolyticus, Pseudomonas aeruginosa, Haemophilus parainfluenzae* and *Exophiala dermatitidis*
Sputum 15	*Candida dubliniensis, Streptococcus gordonii* and *Pseudomonas aeruginosa*

Table 3.3: Non-CF gastric juice microbiology results

Gastric juice 1	No growth
Gastric juice 2	*Streptococcus angionosis, Alpha haemolytic Streptococcus* and *Corynebacterium* spp
Gastric juice 3	*Serratia liquefaciens, Rahnella aquatilis* and *Neisseria* sp
Gastric juice 4	No growth
Gastric juice 5	No growth
Gastric juice 6	*Candida albicans, Coagulase negative Staphyloccocus* and *Acinetobacter junni*
Gastric juice 7	No growth
Gastric juice 8	*Candida albicans, Alpha haemolytic streptococcus* and *Acinetobacter lwoffii*
Gastric juice 9	*Alpha haemolytic streptococcus*
Gastric juice 10	*Candida albicans*
Gastric juice 11	*Proteus mirabilus* and *kelbsella ozaenae*
Gastric juice 12	*Corynebacterium* spp and *Coagulase neqative staphyloccous*
Gastric juice 13	*Pseudomonas aeruginosa* and *Alpha haemolytic streptococcus*
Gastric juice 14	No growth

3.3.3.1 Findings from gastric juice sample culture

Bacteria were identified within eleven out of fifteen samples of CF gastric juice, with the isolation of high numbers denoted by analysis of the number of colony forming units at >10^4 cfu ml^{-1} gastric juice. Seven gastric juice samples provided more than a single bacterial species. Thus, the mean number of bacterial species was 1.6 per patient. However, if there is acknowledgement of both mucoidal and non-mucoidal morphotypes of *Pseudomonas*, a mean of 1.73 species per sample was reached. *Streptococcus spp*, *Lactobacillus* and *Pseudomonas aeruginosa* were the most commonly isolated bacterial species, each being found in four of the fifteen samples, while *Staphylococcus spp* was also common, found in three of the fifteen.

All fifteen of the samples of CF gastric juice samples contained fungi, with all but one containing more than a single species: the mean number of fungal species was two. From these, the most commonly found were *Candida*, which were found in all fifteen samples and were thus the most frequently found organism overall (see Figure 3.3).

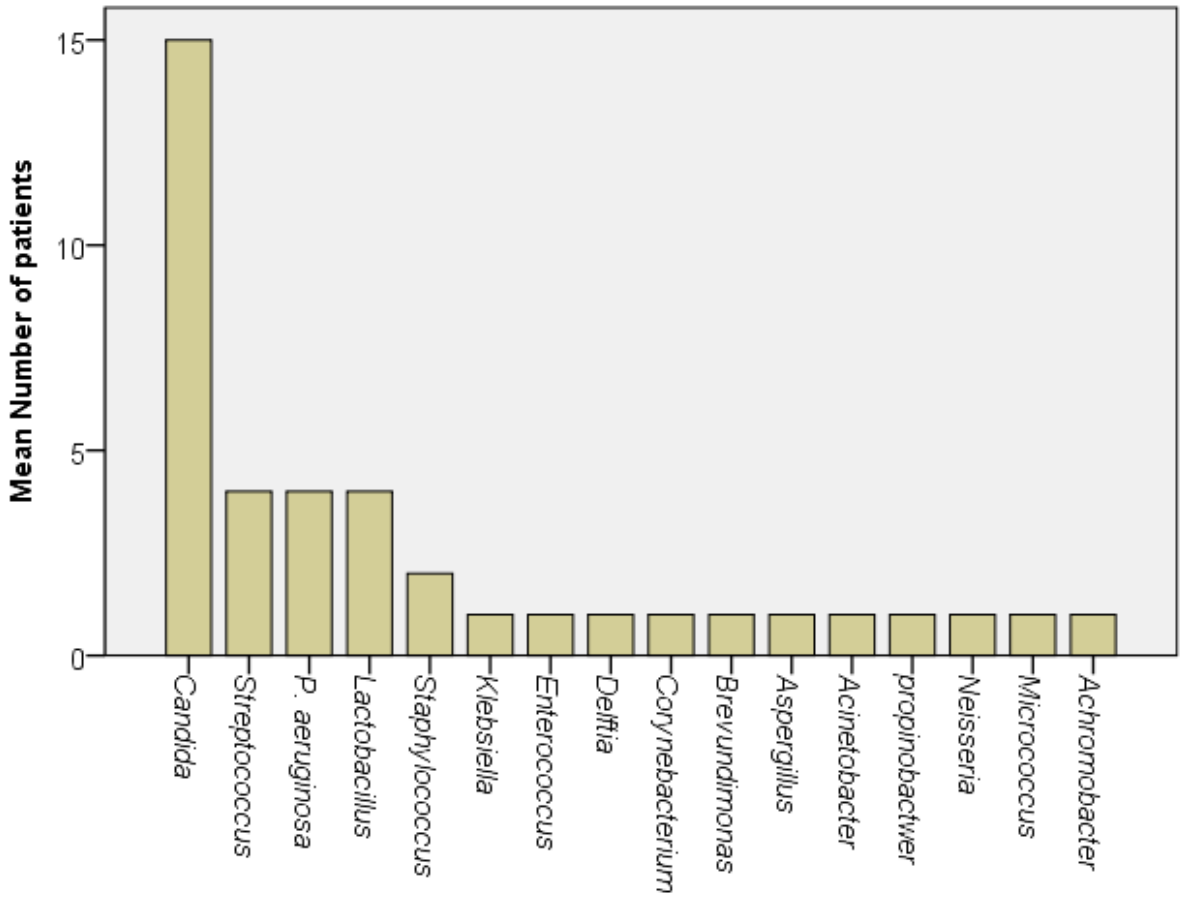

Figure 3-3: Results of gastric juice sample culture for 15 PEG-fed CF patients. The x-axis represents the bacterial and fungal species. The y-axis represent number of patients.

3.3.3.2 Findings from sputum sample culture

Fifteen CF sputum samples given by different patients were cultured and in each sample, bacteria species were found and isolated in high numbers, at $>10^4$ cfu ml^{-1} sputum. Further, in fourteen of the samples, between 2 and 5 taxa of bacteria were found, with the number of species per patient reaching a mean of 3.33, or 3.6 if identifying varied pseudomonas morphotypes as species. Meanwhile, twelve of the fifteen samples contained fungal species, and two samples contained more than a single fungal species. The mean number of fungal species per patient was 1.

Identification of the bacteria isolated revealed 17 genera. The most commonly isolated bacteria were as follows: eleven out of fifteen samples contained *Streptococcus* spp, eleven contained *Pseudomonas aeruginosa*, four contained *Staphylococcus* spp, four contained *Achrombacter* spp, and four contained *Rothia* spp (Figure 3.4).

The most commonly identified species was *P. aeruginosa*, and furthermore, six out of eleven of the samples testing positively for *P. aeruginosa* contained both non-mucoidal and mucoidal samples, with the remaining five containing a single phenotype of this bacteria.

Fungi present in sputum samples were also identified using microbial cultures, with eleven of fifteen samples containing fungi. Of these, nine contained *Candida* spp (of which seven contained *C. albicans)* and four contained *Aspergillus fumigatus*.

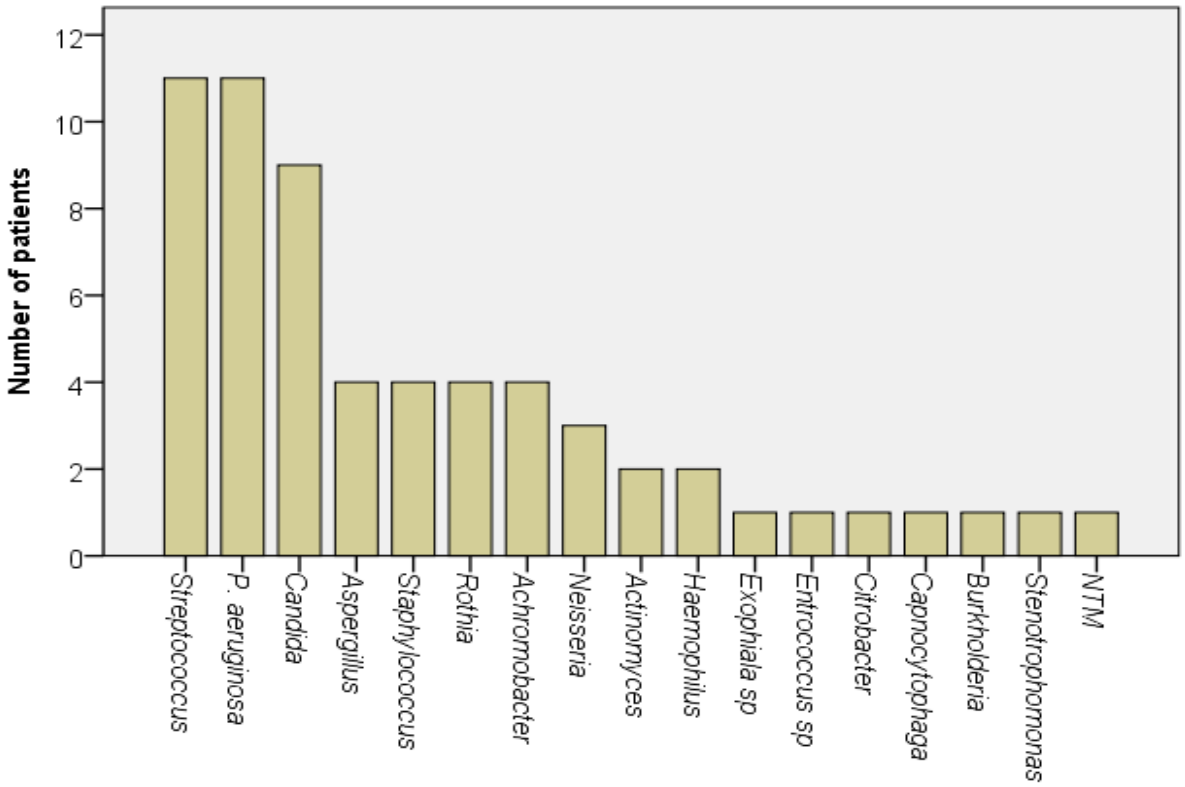

Figure 3-4: Results of sputum samples culture for 15 PEG-fed CF patients. The x-axis represents the bacterial and fungal species. The y-axis represent number of patients.

3.3.3.3 Culture findings for non-CF samples of gastric juice

Bacteria were identified in eight non-CF samples of gastric juice out of fourteen. Seven samples contained more than a single bacterial species which could be isolated, with a mean number of 2.2 per patient. Four contained *Streptococcus* spp, two contained *Lactobacillus* spp and two S*taphylococcus* spp, with these being the species which were isolated most often. One out of fifteen samples led to an isolate of *PA*, and this subject had neither CF nor any lung disease. *Candida* spp isolates were identified in just three out of fourteen of the non-CF samples of gastric juice (Figure 3.5). Comparing the microbial profile of CFGJ (n=15) and non-CFGJ (n=14) is represented in figure 3.6.

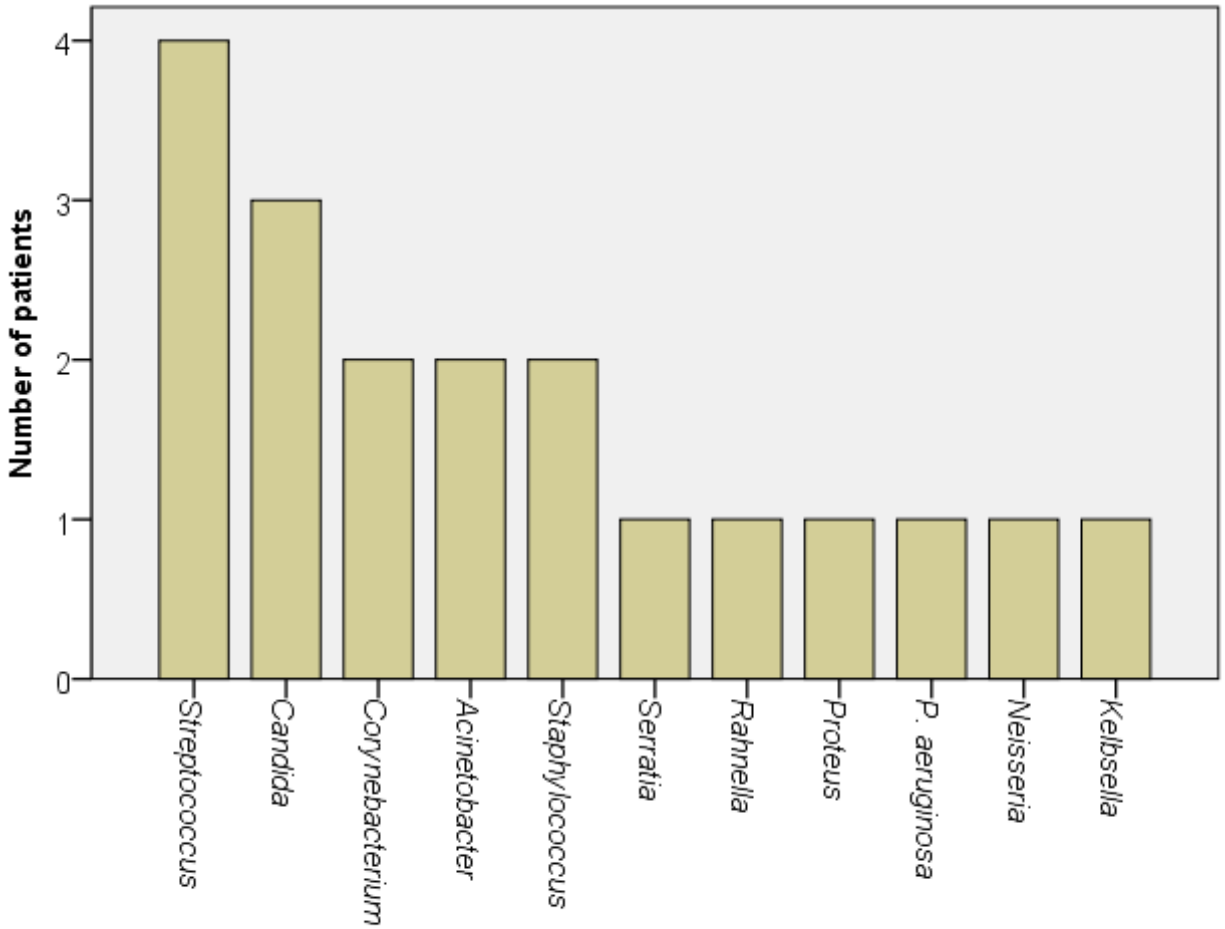

Figure 3-5: Results of non-CF gastric juice samples culture. The x-axis represents the bacterial and fungal species. The y-axis represent number of patients.

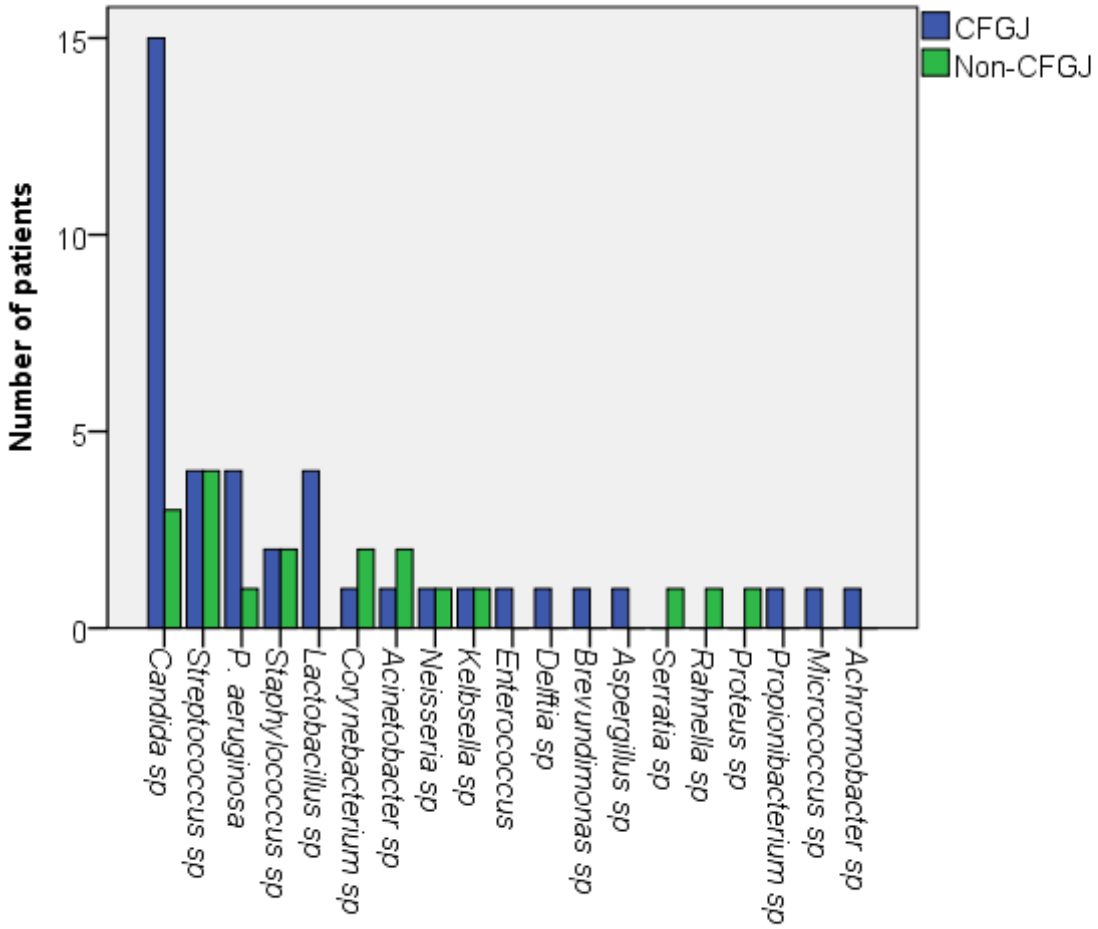

Figure 3-6: Comparing the microbial profile of CFGJ (n=15) and non-CFGJ (n=14).The most commonly identified bacterial species in CF gastric juice were found to be *PA* (4/15), *Lactobacillus* spp (4/15) and *Streptococcus* (4/15). In non-CF gastric juice, the most commonly identified species were *Streptococcus* spp (4/14), *Lactobacillus* spp (2/14) and *Staphylococcus* spp (2/14). *Candida* spp were isolated from all CFGJ samples and 3 out of 14 of non-CF GJ samples.

3.3.4 CF gastric juice vs sputum samples culture results

Comparison of the profiles of the microflora in each gastric and sputum CF sample revealed that in nine individuals in the CF group, one or a number of bacterial or fungal pathogens were found in both gastric juice and sputum. Five demonstrated one or more taxa of bacteria across the two types of sample and four displayed one or a number of fungal pathogens in common. Bacteria which were identified in both sample types from a single patient comprised: *PA*, in four cases; *Streptococcus* spp in two cases; and *Achromobacter* spp, in two cases. Meanwhile, for the remaining six in the group, while isolates of microbial pathogens were found in both sample types, there were no species found across both samples for the same patient (Figure 3.7).

For each of the 15 CF participants, fungal pathogens could be isolated for one of their samples, and for eight patients, *Candida* spp. was common between the gastric juice and sputum samples. A single participant was found to have *Aspergillus* spp. in both samples. Figure 3.8 illustrates microorganisms found in sputum as compared to those in gastric juice samples.

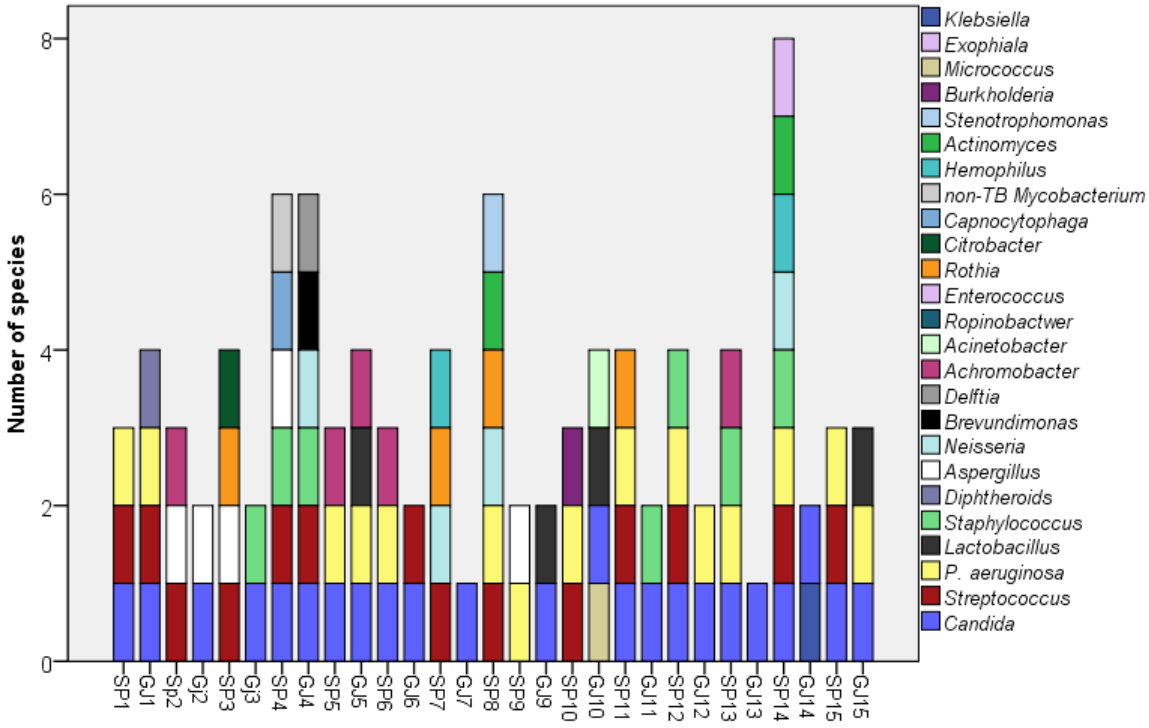

Figure 3-7: Microbial species isolated from each CF gastric juice and sputum sample. The x-axis represents the sample. The y-axis represents number of bacteria and/or fungal species isolated from a particular sample.

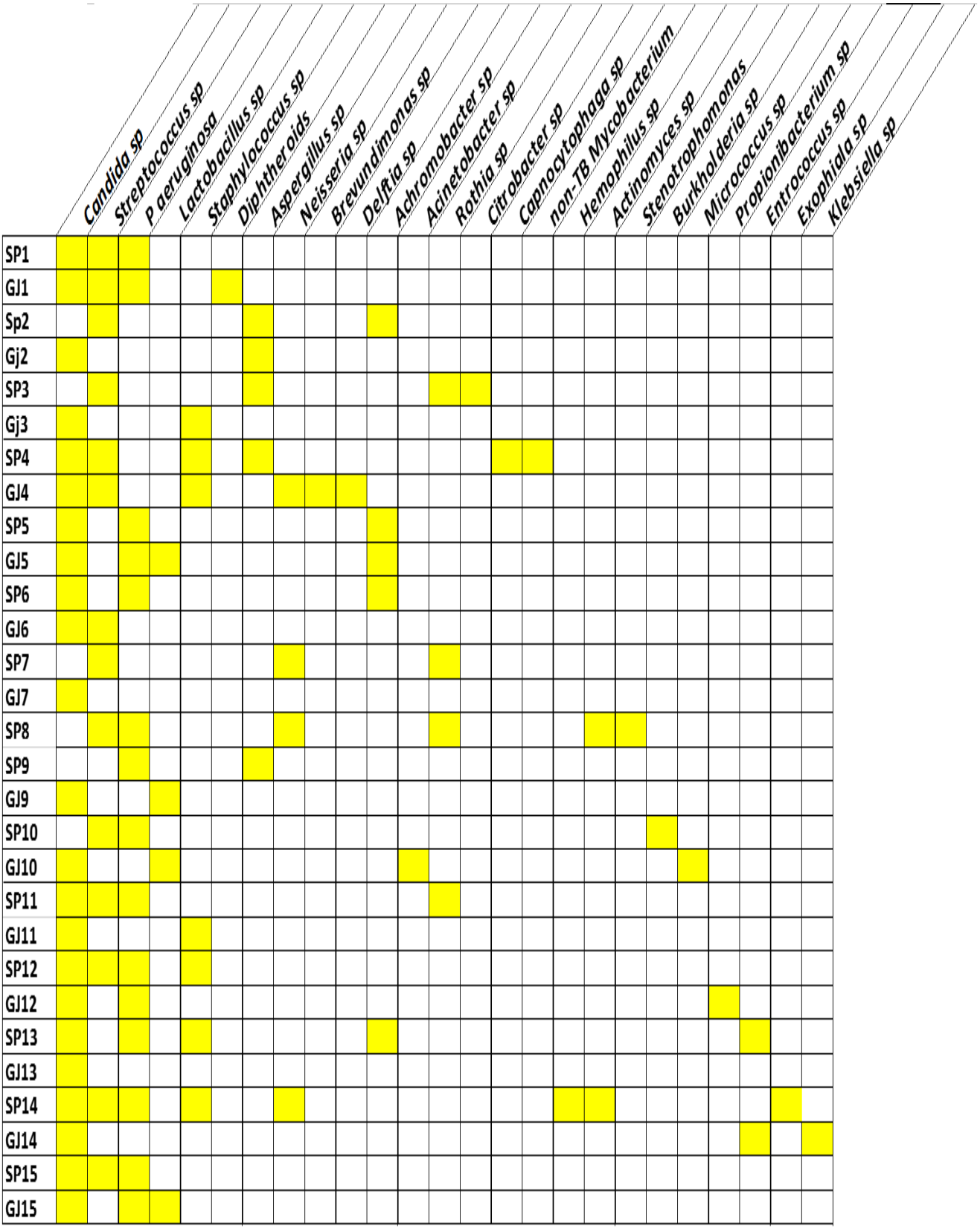

Figure 3-8: Microorganisms found in sputum as compared to those in gastric juice samples for all samples (n=15).

3.3.5 Pseudomonas aeruginosa

Identification of *PA* was made for eleven sputum samples out of fifteen: 73% of the samples (CF-1, CF-5, CF-6 and CF8-15). The pathogen was also found in four samples of CF gastric juice (26%) (CF-1, CF-5, CF-12 and CF-15). Both CF-1 and CF-12 contained non-mucoidal and mucoidal types of *PA* across each type of sample. Meanwhile, CF-5 sputum contained both *PA* forms, while the gastric sample had just mucoidal *PA*. CF- 15 contained the mucoidal type of *PA* only but across both sputa and gastric juice.

Patients with *PA* had a median age of 28.5, ranging from 18 to 41, while those without had a median age of 22, ranging from 16 to 27. In terms of FEV_1, those with *PA* showed a median score of 1.25L, ranging from 0.45 to 3.5L), those without *PA* also had a median of 1.25L, but ranged from 0.76 to 2.7L.

3.3.5.1 Molecular characterisation of matching PA in sputum and gastric juice of CF patients

VNTR (variable number tandem repeat) analysis applied to *PA* revealed identical strains between sputa and gastric juice for three out of four of the CF individuals in which *PA* was found across both sample types. For the fourth individual in this group (CF-1), while matching strains were identified, there were also additional and different *PA* strains in the sputum sample only.

3.3.6 Next Generation Sequencing

Next generation sequencing analysis was applied to 14 samples of non-CF gastric juice using 16S rRNA gene targeting variable region 4 (V4) (GJ 1-14), 13 samples of CF gastric juice (CF-GJ 1, 2, 4-12, 14, 15) and to 12 samples of CF sputum (CFS 4-15) to investigate the microbiome.

Greater diversity was found in non-CF against CF gastric juice samples and against CF samples of sputum (see Figure 3.9A). There was an abundance of proteobacteria for sputum and gastric juice samples in the CF group, with 74% and 72% relative abundance, while there was greater abundance (48%) for *Firmicutes* in gastric juice from non-CF patients.

Non-CF samples of gastric juice displayed considerably greater average Shannon diversity indices (*H'*) as opposed to CF gastric samples (*P* = 0.002) or CF sputum samples (*P* = <0.001). Both sputum and gastric juice CF samples had an *H'* of P = 0.93, as seen in Figure 3.9B.

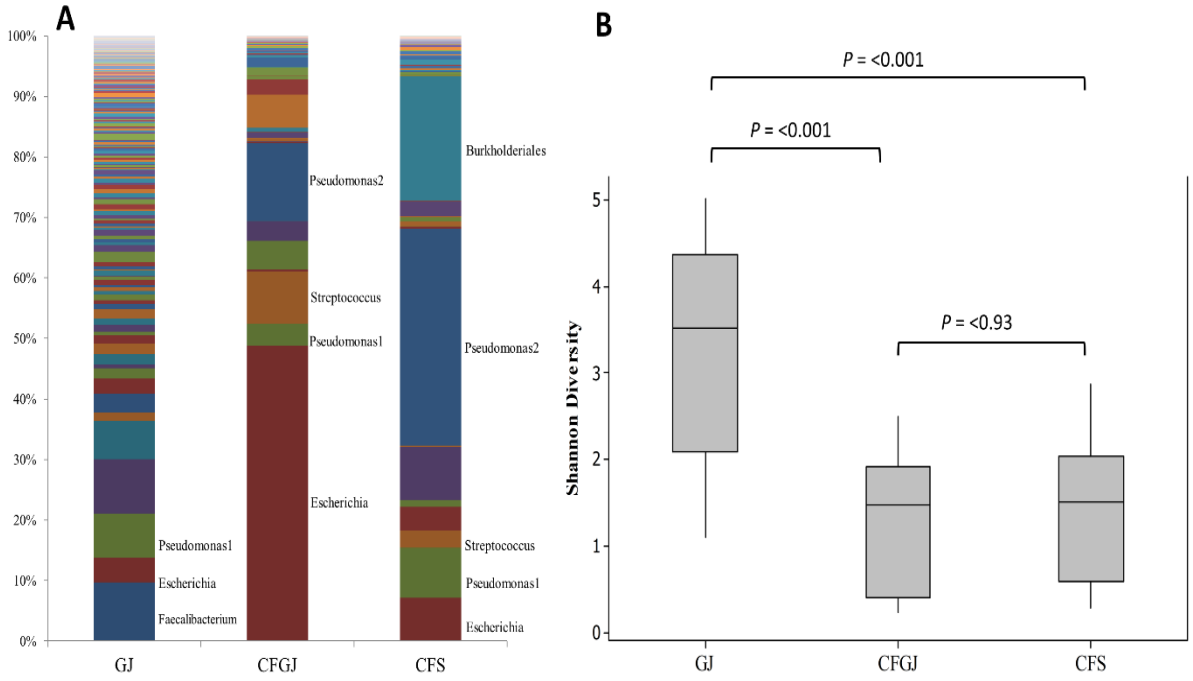

Figure 3-9: (A) Bar plot comparing abundance between different operational taxonomic units (OTUs) in non-CF gastric juice (GJ) (n=14), CF gastric juice (CFGJ) (n=13), and sputum samples (CFS) (n=12). (B) Shannon Diversity Index for CF samples (CFGJ and CFS) as well as non-CF GJ.

Using PLS-DA across each sample, it is seen that in CF patients, sputum and gastric juice samples were comparable, and this differed from samples of gastric juice from non-CF patients, as shown in Figure 3.10. Further, samples from CF patients were relatively lower in terms of average Shannon diversity and this clustered close to the PLS-DA plot origin, in contrast with the highly diverse gastric juice samples from non-CF patients.

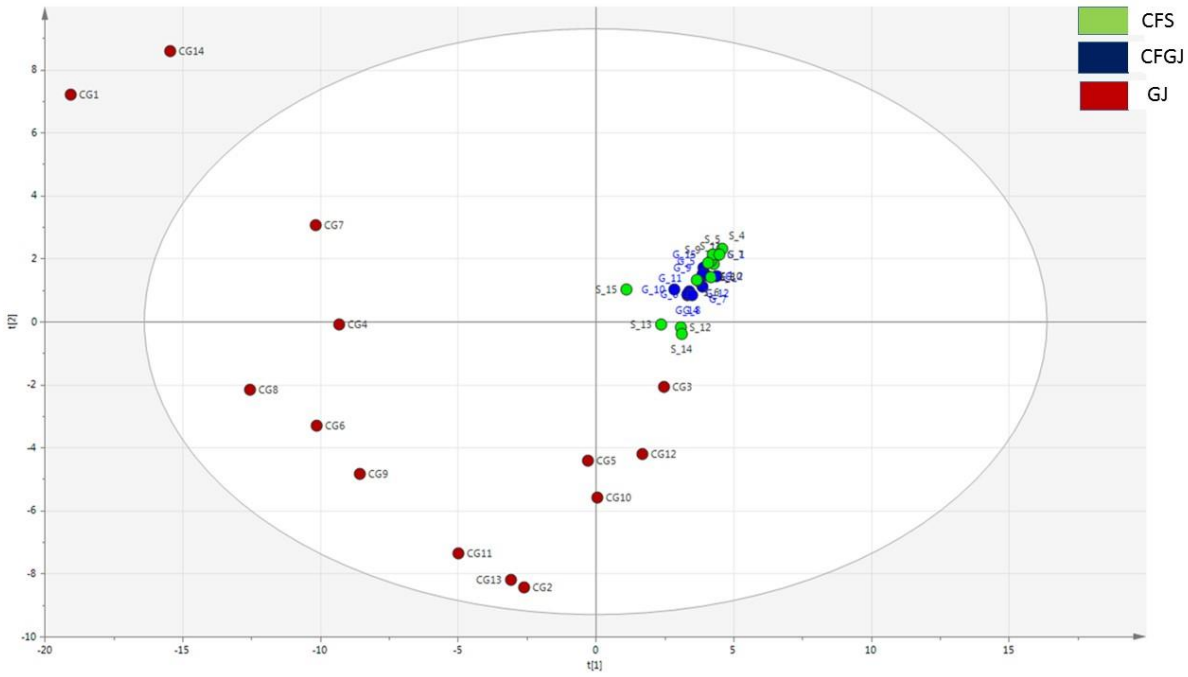

Figure 3-10: Partial least square decrement analysis (PLS-DA) score scatter plot for each of the samples. GJ=non-CF gastric juice; CFGJ=CF gastric juice; CFS=sputum sample. Percentages of variance are given by the axes. Correlation is seen between CF sputum and gastric juice samples, while gastric juice from the controls show greater variance and are markedly different from CF sample clusters.

Analysing matched samples of sputum and gastric juice from a single CF patient revealed a tendency for these to group together, as shown in Figure 3.11.

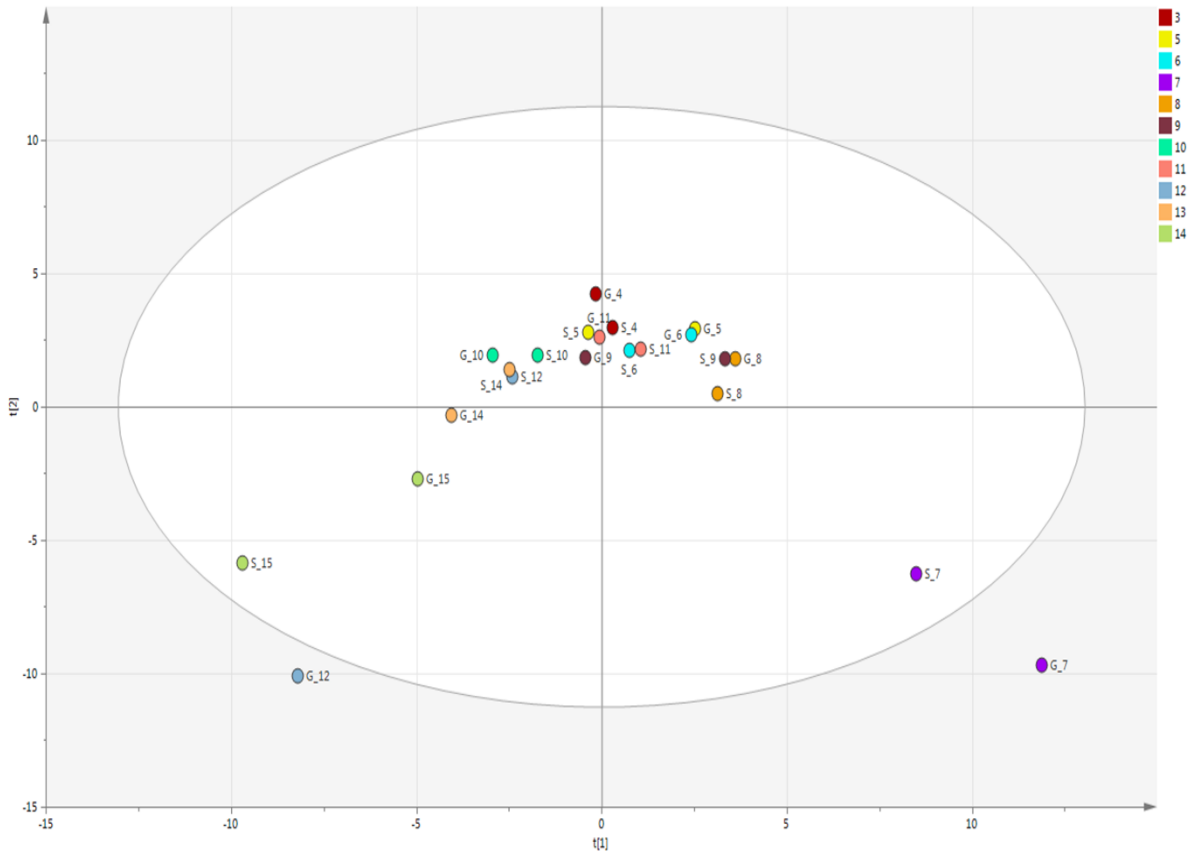

Figure 3-11 Partial least square decrement analysis (PLS-DA) score scatter plot. Coloured circles represent individual samples, and coloured groupings represent individual patients with CF. In samples of gastric juice and sputum from a single patient with CF, there is generally clustering of the solid ellipse indicate Hotelling's (T) range (confidence of 95%).

3.4 Discussion

This research project is, to the best of my knowledge, the first to make direct comparisons between the microflora of the air passages and those of gastric juice for the same CF individual using PEG feeding. Both the gastric and respiratory passages were found to be colonised with microbes significant to lung disease in CF patients and in some cases these microbes were identical. The fact that both systems sometimes contained *PA* of the type which can constitute a biofilm suggests that a reservoir of this bacterium may be present in the stomach, and that this may have a role in cystic fibrosis pathophysiology (Dickson *et al.,* 2014).

The study's findings revealed marked differences between gastric juices taken from adults with CF and those who did not have the condition. Molecular profiling of gastric juice samples through next generation sequencing revealed that bacteria were significantly less abundant in the CF samples. These samples typically contained *Faecalibacterium, Bacteroides* and levels of *Pseudomonas* which were greater than that found in the control samples (Cho and Blaser, 2012). Meanwhile, the samples from individuals without CF were found to be in line with established data on the microbiome of the stomach in healthy individuals (Bik *et al.*, 2006).

For each CF case in which *PA* was identified in both sputum and gastric juice samples, VNTR analysis showed that the microbes were identical in genetic terms: a result which would be extremely unusual if these colonisations had occurred from different or random sources. The genetic make-up of *PA* is highly diverse across CF patients who have recently been chronically infected with or are occasionally infected with the pathogen (Jelsbak *et al.*, 2007). This implies that strains of *PA* are acquired by CF individuals from varied sources in their surroundings (Burns *et al.,* 2001). In those with intermittent infection, it is possible for the lungs to be recolonised after the previous infection has been eliminated through inhalation of antibiotics, and in most cases the genotype of the two infections differs, which is indicative of different sources for the infection. However, in around one quarter of individuals with a recurring *PA* infection, the genotype was identified as identical to the previous infection, and this points to two possibilities: the *PA* has survived but could not be detected; or there has been reinfection from the same, unidentified source in the patient's surroundings (Jelsbak *et al.,* 2007). Findings from methodical longitudinal studies of eradication of CF lung infections reveal that subjects can experience recolonization by an identical strain of *PA* even where a number of years have passed without *PA* being detected in the sputum (Johansson *et al.*, 2014). The current study's findings may provide support for the proposal that the stomach can host a continuing population

of bacteria not eradicated by the inhalation of antibiotics as conducted in current protocols for best practice infection interventions (Mogayzel Jr *et al.*, 2014).

Taking a sample of gastric juice via the PEG tube meant that it could not be contaminated by microbes from the air passages and the upper part of the gastro-intestinal tract, as may be a concern when using alternative methods, including endoscopy. The data obtained from PEG tube sampling provides clear evidence that bacterial organisms are capable of existing within the stomach and may form a reservoir which harbours pathogenic microbes of types which are significant in CF lung disease. There is a broad body of evidence indicating aspiration and reflux occurring in individuals with CF (Brodzicki *et al.*, 2002a). The CF group in the current study were EOR symptomatic in excess of normal symptoms. Thus, the findings support the potential for aspiration of microbes significant in the development of CF lung disease.

The data also suggest the possibility of *PA* found in the gastric juices deriving from sputum coughed up from the lungs which then enters the stomach through swallowing. In one sample from the study, there was *PA* of the same strain in both gastric juice and sputum samples, but also a different strain of *PA* in the sputum. Further, in one participant in the non-CF group, who was not suffering from lung disease, *PA* was isolated from the gastric juice. The findings suggest that while it is possible for *PA* in the stomach to have been swallowed on the clearing of mucus from the airways in lung disease, the microbe may come from a range of sources and pathways, in which micro-aspiration in *PA* of the lung is one potential route.

Microbiological continuity across the aerodigestive passages of healthy individuals has been reported through methodologies other than culture, which may support the notion of frequent microaspiration even among the healthy population (Bassis *et al.*, 2015). Migration between respiratory and gastric areas is further supported by evidence linking *PA* infection of the lower airways and GOR in paediatric CF patients (Palm *et al.*, 2012). A recent study conducted with paediatric patients suffering from chronic cough and where gastrointestinal endoscopy and bronchoscopy were undertaken revealed that from microbes most numerous in gastric fluid samples, eight were also found in large numbers within the lung. This was presented as supportive of the transfer of microbes between the gastrointestinal compartment and lungs which was not dependent on the microflora of the oropharyngeal tract (Rosen *et al.*, 2015).

Further, research in lung transplant patients with CF in terms of molecular epidemiology found that the transplanted tissue became colonised with identical *PA* to that isolated from the lungs prior to transplant (Walter *et al.*, 1997), and this is attributed to reinfection from sinus and upper respiratory airways (Nunley *et al.*, 1998). Additionally however, risk of recolonization may also

be present from *PA* aspirated from the gastrointestinal tract after the transplant (Krishnan *et al.*, 2013). Further, this migration of pathogens may occur in both directions from oropharynx to stomach and vice versa (Segal *et al.*, 2006). In the case of lung transplants, there is also support for the notion that GOR can play a causal role in cases of Bronchiolitis Obliterans Syndrome (BOS) (Kinnier *et al.*, 2016). Surgical intervention with anti-reflux fundoplication has been linked with enhanced function of the allograft in lung transplantation (Cantu Iii *et al.*, 2004; Griffin *et al.*, 2013).

Using molecular techniques, it has been shown that there is a highly diverse microbiome in the normal stomach, with features closely related to those of the typical lower gastrointestinal microbiome (Bik *et al.*, 2006; Andersson *et al.*, 2008). Meanwhile, in the respiratory passages of CF patients, lowered diversity has been found in the microbiome (Zhao *et al.*, 2012). It is possible that this is in line with the findings of my research, which found a less diverse gastric microbiome in the CF group in comparison to controls when assessed using molecular techniques. Both the gastric and sputum samples in the CF group were less diverse in comparison with the gastric juice from patients without CF. Multivariate discriminant analysis and molecular identification demonstrated clustering for CF samples of gastric juice, in which they could be distinguished from samples from the controls. In addition, the profiles of sputum and gastric juice samples from the same CF individual were comparable. Considering these findings, the possibility emerges that there is an association between the particular microflora of the gastric juices and sputum for some individuals with CF. This makes the case for additional research in this area, to assess whether the profile correlation within gastric juice and sputum samples observed in this study could stem from a shared source for colonisation e.g. in the oro-pharynx and/or sinuses.

This study is novel but has a limitation related to the design adopted and the context of the project. CF gastric juice samples were collected only from those fed by a PEG tube, and although more than 80% of accessible individuals meeting these criteria participated, this meant that the findings were from a particular and small cohort. Patients were not excluded on the basis of either antibiotic use level or disease stage. It should also be noted that in previous work, our group has achieved isolation of *PA* for the gastric juice samples of CF individuals who did not undergo PEG feeding, and for whom samples were obtained with an endoscope (Krishnan *et al.*, 2013). This earlier research identified molecularly identical *Pa* in samples of broncho-alveolar lavage (BAL), as well as sputum samples and gastric juice obtained through endoscopy. This provides support for the notion that it is not only in PEG-fed CF patients such

as those in the current study that bacteria significant in the pathophysiological processes of CF may exist in a gastric reservoir (Krishnan *et al.*, 2013).

Further, we note that the use of antibiotics on a long term basis, as well as Proton Pump Inhibitor treatment in the group studied, may significantly influence the microflora identified in the CF gastric juice samples obtained. There is growing recognition of an association between PPI use and a changed microbiome of the stomach, lungs and oro-pharynx (Rosen *et al.*, 2015). At present, the significance of this is being widely discussed, and this study hopefully contributes to this discussion (Jones *et al.*, 2016).

The use of histamine-2 receptor antagonists or proton pump inhibitors is common to reduce gastric acid for CF individuals suffering from a continuing inadequate ability to absorb fats even where pancreatic enzyme replacement is being given at sufficient levels (Littlewood *et al.*, 2006), Further, CF patients are also given such medications where they are diagnosed with gastroesophageal reflux disease. In fact, most individuals with CF in North America take drugs to suppress gastric acid (Com *et al.*, 2014), and in the current study, every one of the CF subjects took acid suppressive medication, in line with practice at the clinic where the research took place. Standard treatment for CF patients at the present time also includes antibiotics as a principle intervention. In light of this, it is suggested that a significant aspect for future research is the need to study the impact of both acid suppressive and antibiotic drugs for the CF aero-digestive microbiome, despite the research challenges presents. It is hoped that the findings of the research project presented here can contribute to these efforts.

To summarise, the study reveals new evidence linking the microflora of gastric juices and sputum samples for individuals with CF. The findings point to a potentially significant source of lung infection in CF patients, and a possible *PA* reservoir. Thus, the microbiome of the stomach may be partly made up of microbes transferred from the lungs through the patient coughing or expectorating and then swallowing. In addition to this, it has been shown that potentially it may also be possible in CF sufferers with reflux for microbes from the stomach to be aspirated and transferred into the lungs. In conclusion, an ‘aerodigestive microbiome’ is suggested as a possible factor in the pathophysiological processes of cystic fibrosis, and in light of this, as currently, treatment to eliminate infection with *PA* takes no account of the gastric population, this factor may be significant to consider for the future development of CF interventions.

Chapter 4.0: Study 2 gastric juice, sputum, and PEG tube microbiology in CF patients

4.1 Introduction

Pauwels *et al.* (2012) and Wu (2008) both found that a higher prevalence of respiratory disease, which contributes to higher morbidity and lower quality of life, could be linked to acid- and non-acid reflux. Navarro *et al.* (2001) found a strong relationship between gastro-oesophageal reflux (GOR), pulmonary aspiration, and increased lung damage for a variety of lung conditions, ranging from advanced lung damage after lung transplantation (Vos *et al.*, 2008) to ventilator-induced pneumonia (Wu *et al.*, 2009). Most importantly, Palm *et al.* (2012) found a connection between gastroesophageal reflux (GOR), infection and reduced lung function in CF patients.

In the Caucasian population, CF is the most common inherited life-threatening disease and according to Murray *et al.* (2007), the primary cause of death in CF patients is chronic respiratory infection. With over half of CF patients experiencing GOR, this appears to be a common problem faced in CF (Blondeau *et al.*, 2008b). GOR typically presents when the stomach's contents leak into the oesophageal tract leading to heartburn, and other symptoms.

A study by van der Doef *et al.* (2009) found a link between reduced lung function and earlier acquisition of *PA* and *Staphylococcus aureus* (*SA*), two key contributing pathogens to CF lung disease. A retrospective study by Palm and colleagues identified a higher prevalence of *PA* in the lungs of children experiencing acid and non-acid reflux (measured by oesophageal impedance tracings) in comparison to children who did not have reflux (Palm *et al.*, 2012). Rosen *et al.* (2011) used standard culture techniques to further demonstrate how lung disease can be caused by full column, non-acid reflux, suggesting that non-acidic, bacterial-laden gastric contents could be refluxed and aspirated into the lung.

The possible ways in which GOR causes respiratory symptoms, infections, or lower lung function in CF are not clearly known. Intermittent aspiration of acidic stomach contents as a result of reflux into the proximal oesophagus, especially while the patient is in the supine position, may produce a vicious circle of inflammation, infection and lung disease progression in CF (Robinson and DiMango, 2014). It is further suggested by Carpagnano *et al.* (2006) that, when exposed to gastric acid, afferent receptors in the oesophageal mucosa trigger reactions that travel through motor neurons to the respiratory muscles and tracheobronchial tree, thus

causing coughing, bronchospasms, and perhaps even a neurogenic increase in inflammation of the neutrophilic airway. Hamamoto *et al.* (1997) showed in animal tests that acute oesophageal acid instillation occurring in under 1 minute is enough to cause airway inflammation in a neurogenic tachykinin-mediated pathway. Pulmonary function may therefore be damaged by refluxed gastric contents through microaspiration, reflex bronchospasm, or increased inflammation of the airway.

Stomach colonisation by pathological bacteria in CF patients has been established through previous research (Atherton and White, 1978). Such colonization could contribute to lung infections, because the pathogen-rich stomach contents could be refluxed and inhaled into the lungs. As the stomach hosts pathogenic bacteria, it therefore has an established potential in the pathogenesis of lung diseases (Atherton and White, 1978; Bonten *et al.,* 1997). Identical matching microorganisms were found in both the stomach and the airway in studies by Madan *et al.* (2012) and Round and Mazmanian (2009) and recently by our studies (Al-Momani *et al.*, 2016) presented in Chapter 3.

Rogers *et al.* (2010b) point out that the presence of gastric reflux in CF patients, makes them particularly susceptible to the transfer of pathogens from stomach to upper airways and vice versa. A 2006 study by Segal *et al.* (2006), found a relationship between bacterial growth in the gastric juices and the airways of elderly non-CF patients fed via nasogastric tube (NGT), which suggested that microorganisms were transmitted in two directions.

The possibility that an aerodigestive microbiome exists in CF is presented in Chapter 3. This may be significant because my results outlined how identical matching microorganisms (including biofilm forming strains of *PA*) which were notorious contributors to CF pulmonary disease were present in both the digestive tract and the airways (Al-Momani *et al.*, 2016).

I used culture and molecular testing methods, which both identified similarities between bacteria present in sputum and gastric juice specimens taken from the same CF patients, thus highlighting the necessity for more research into the role of the ' aerodigestive microbiome' in CF disease and its possible role in the pathophysiology of CF (Segal *et al.*, 2006; Al-Momani *et al.*, 2016). Culture isolation techniques have also been used before by Bassis *et al.* (2015) to examine microorganisms in the aerodigestive tract, which indicated that many healthy individuals may also experience microaspiration.

This chapter is a continuation of my investigation into the association between gastric colonisation and potential pathogen transmission to the air passages or lungs via aspirated refluxed stomach contents for patients with CF. It extends the work in the previous chapter by looking at repeated samples taken over time in a cohort of CF patients. This work is therefore able for the first time to my knowledge to evaluate the variability of the newly described aerodigestive microbiome.

4.2 Methods (CF gastric juice and sputum samples included in this study)

In this study, samples were collected six months after the first study (Chapter 3). All available patients fed via PEG were included, which meant that repeated sample data were collected in some patients.

In the prior study (Chapter 3), a total of 18 PEG-fed CF patients were available (CF-1 to CF-18), and only 15 of the available patients were included (CF-1 to CF-15). At the start of this study, however, only 16 patients (CF 1-4, CF 6-14 and CF 16-18) were available because one patient had emigrated (CF-15) and one had passed away (CF-5).

Thirteen patients (CF1-4 and CF6-14) were therefore common to both studies (Chapter 3 and Chapter 4), generating repeated samples. Three new patients were also involved in the study (CF16-18). Along with gastric juice and sputum samples from the 16 CF individuals, replaced PEG tubes were also taken from five patients (CF4, CF 6-7 and CF 16-17). I obtained a Reflux Symptoms Index (RSI) score for fourteen CF patients (CF1-4, CF 6-10, CF12-14 and CF16-17). The same non-CF patients used in Chapter 3 represented a control group of non-CF gastric juice.

The methods outlined in Chapter 2 were used to analyse gastric juice and sputum specimens with DNA isolated from gastric juice and sputum. Subsequent molecular analysis of the microbial profile was conducted by Dr Andy Nelson at Northumbria University using the 16S RNA pyrosequencing technique. This was the same methodology used to generate the aerodigestive microbiome data in Chapter 3. Bland-Altman plots were used to test the repeatability of the diversity across the repeated sample (gastric juice and sputum samples) (Bland and Altman, 1986).

4.3 Results

4.3.1 CF Patients characteristics

The patients in this investigation, with a median age of 25 (range 18-36 year) (Figure 4.1A) demonstrated moderate to severe CF lung disease, which is common amongst the PEG-fed CF population (median FEV_1, 1.6L (37% predicted) range 0.5-3.5L (12%-88%) (Figure 4.1B). They also showed low BMI (median 19.2, range 15.2-23.2) (Figure 4.1C) and were long-term users of antibiotics (using them on average 70 days/year, range 14-197) (Figure 4.1D) and acid suppressants.

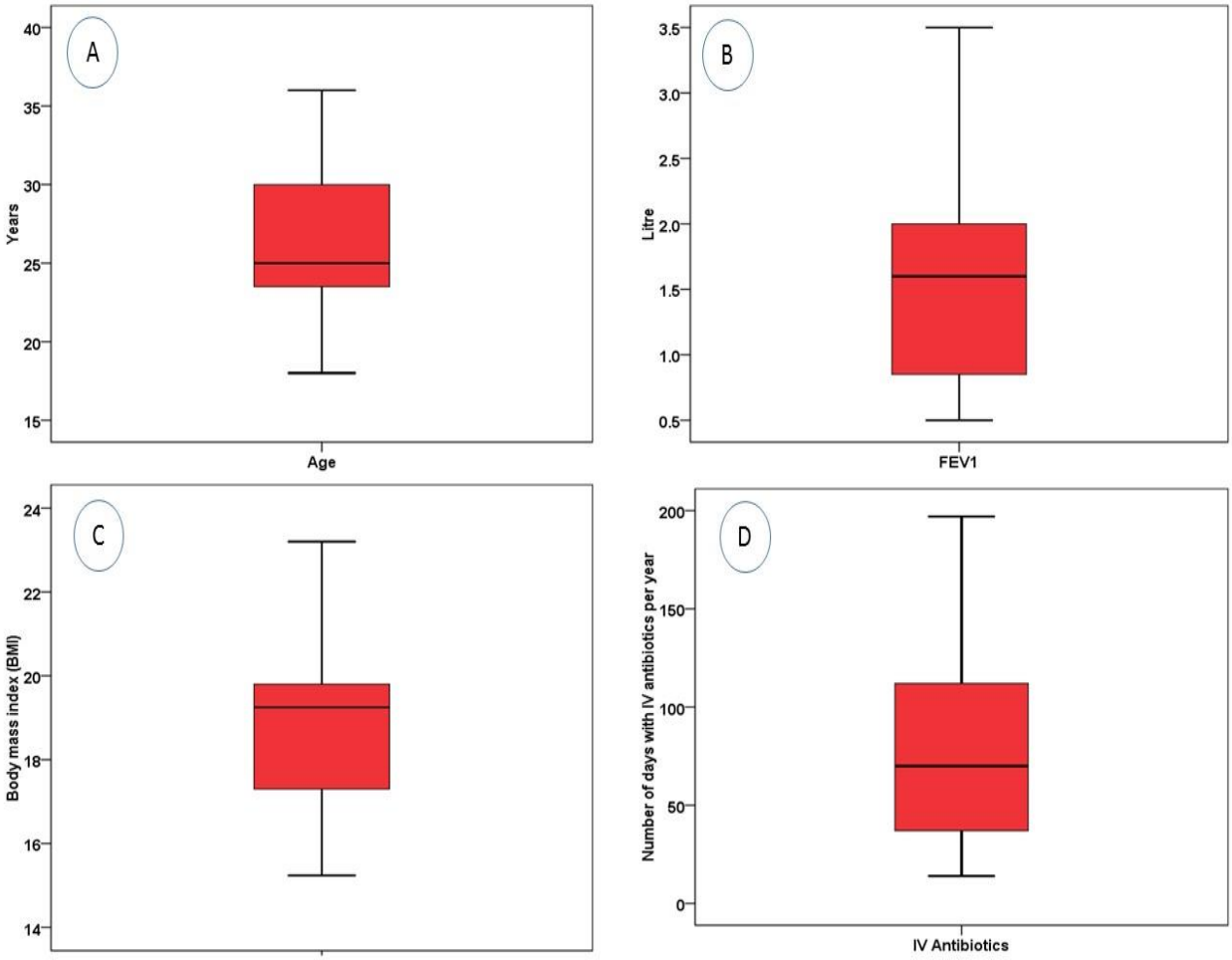

Figure 4-1: Demographic characteristic of CF Patients included in this study (n=16). A) Patients age (years) (range 18-36, median 25). B) FEV_1) median FEV_1, 1.6L (37% predicted) range 0.5-3.5L (12%-88%). C) BMI (median 19.2, range (15.2-23.2). D) The average days per year of IV antibiotic usage for CF patients (median 70, range 14-197).

4.3.2 Symptoms of extraoesophageal reflux in CF patients

I obtained extraoesophageal reflux symptom scores for fourteen of the sixteen CF patients. Thirteen patients showed symptoms of EOR, obtaining an RSI score of more than twelve (median RSI score 17 (range 13-36)). One patient, who scored eight, was shown to be EOR non-symptomatic (Figure 4.2).

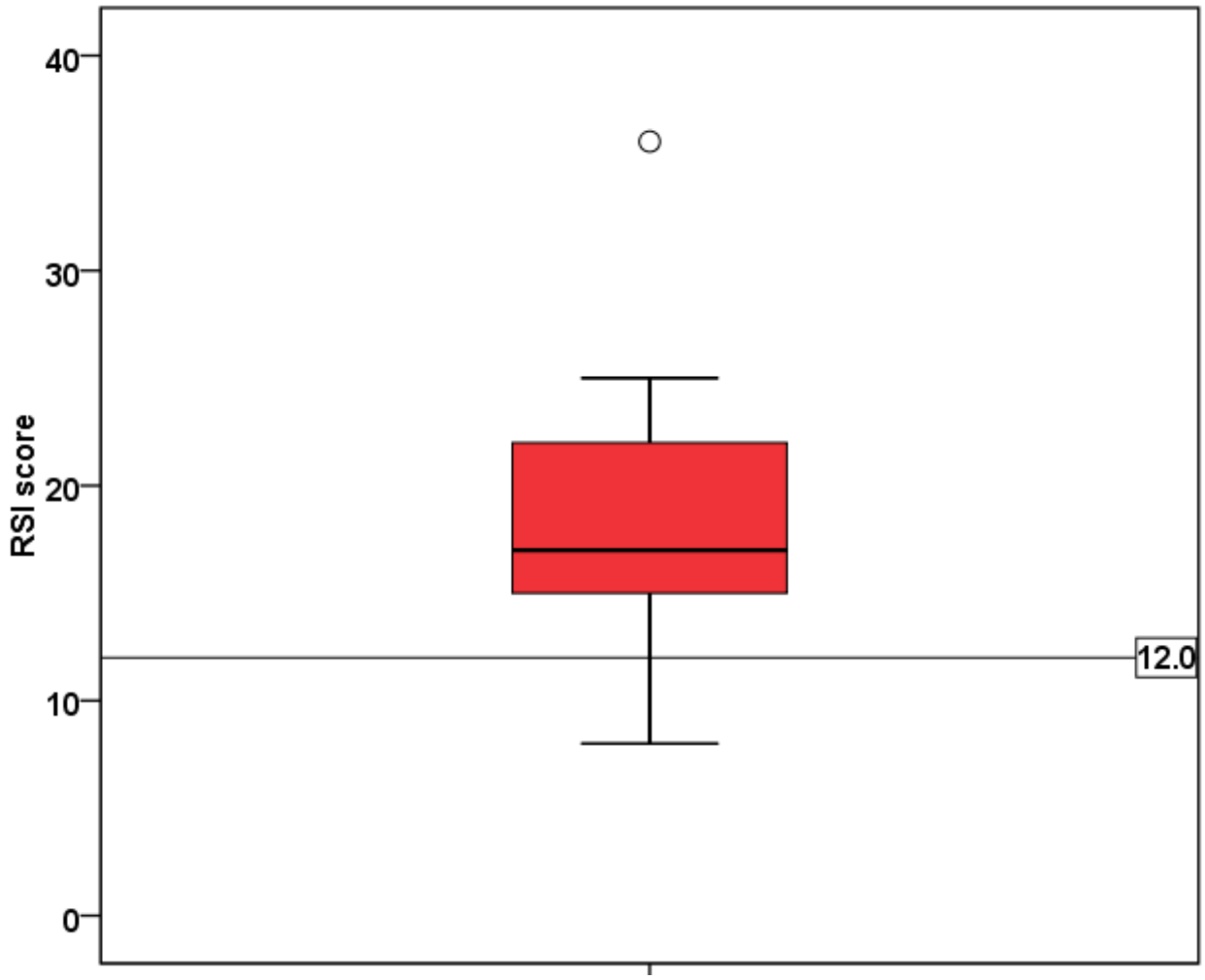

Figure 4-2: RSI score for CF patients (n=14, median 17, range 8-36). Normal score of 12 or less represented by black line. The small circle represents an outlier.

4.3.3 Microbial analysis

Similar to the previous chapter, All CF gastric juice and sputum specimens tested positive for bacteria and/or fungi in routine microbial analysis. The most commonly identified bacteria in both sputum and gastric juice samples were again *Streptococcus* spp, *Non-Tb mycobacterium (NTM)* and *PA*. Tables 4.1and 4.2 show the culture findings for CF gastric juice and sputum. Figures 4.3 and 4.4 show the number of bacterial species isolated from gastric juice and sputum samples.

Table 4.1: CF gastric juice culture results

Gastric juice 1	*Candida glabrata, Enterococcus faecium, Lactobacillus paracasi, Saccharomyces cerevisiae* and *Mycobacterium abscessus* subsp *massiliense*
Gastric juice 2	*Candida lustania* and *Candida krusei*
Gastric juice 3	*Candida albicans* and *Mycobacterium abscessus* subsp *massiliense*
Gastric juice 4	*Candida albicans, Candida krusie* and *Lactobacillius fermentum.*
Gastric juice 6	*Streptococcus mitis, Lactobacillus rhamnosus, Saccharomyces cerevisiae, Achromobacter xylosoxidans* and *Pseudomonas aeruginosa*
Gastric juice 7	*Candida albicans, Candida glabrata* and *Lactobacillius plantarum*
Gastric juice 8	*Candida albicans* and *Lactobacillus gasseri*
Gastric juice 9	*Candida krusei, Candida glabrata* and *Pseudomonas aeruginosa*
Gastric juice 10	*Candida albicans, Candida dubliniensis* and *Lactobacillius gasseri*
Gastric juice 11	*Candida glabrata* and *Staphylococcus hominis*
Gastric juice 12	*Candida albicans, Candida parapsilosis, Hafnia alvei, Enterococcus faecalis, Serratia fonticola* and *Raoultella sp*
Gastric juice 13	*Candida albicans, Candida glabrata* and *Candida krusei*
Gastric juice 14	*Candida albicans, Candida glabrata* and *Mycobacterium abscessus* subsp m*assiliense*
Gastric juice 16	*Candida albicans* Streptococcus *salivarius , Streptococcus mitis, Rothia mucilaginosa, Achromobacter xylosoxidans, Pseudomonas aeruginosa* and *Neisseria sp*
Gastric juice 17	*Candida glabrata* and *Exophiala dermatitidis*
Gastric juice 18	*Candida albicans, Escherichia coli* and *Pseudomonas aeruginosa*

Table 4.2: Sputum sample microbiology results

Sputum 1	*Candida albicans, Streptococcus anginosus, Streptococcus mitis* and *Rothia dentocariosa*
Sputum 2	*Aspergillus fumigatus, Streptococcus mitis, Streptococcus parasanguinis, Streptococcus salivarius, Rothia mucilaginosa* and *Achromobacter xylosoxidans*
Sputum 3	*Streptococcus oralis, Rothia mucilaginosa Pseudomonas aeruginosa* and *Mycobacterium abscessus* subsp *massiliense*
Sputum 4	*Candida albicans, Aspergillus fumigatus, Rothia mucilaginosa, Rothia dentocariosa, Streptococcus mitis, Streptococcus sanguinis, Streptococcus oralis* and *Mycobacterium abscessus* subsp. *abscessus*
Sputum 6	*Candida albicans, Rothia mucilaginosa, Rothia dentocariosa, Streptococcus mitis* and *Streptococcus oralis*
Sputum 7	*Candida albicans, Streptococcus mitis, Rothia mucilaginosa, Neisseria meningitidis* and *Haemophilus influenzae*
Sputum 8	*Streptococcus mitis* and *Rothia mucilaginosa*
Sputum 9	*Streptococcus oralis, Rothia, Haemophilus parainfluenzae* and *Pseudomonas aeruginosa*
Sputum 10	*Rothia mucilaginosa, Streptococcus sanguinis, Streptococcus mitis, Pseudomonas aeruginosa, Burkholderia multivorans, Actinomyces oris, Penicillium* and *Mycobacterium abscessus* subsp *massiliense*
Sputum 11	*Candida albicans, Streptococcus mitis* and *Rothia mucilaginosa*
Sputum 12	*Pseudomonas aeruginosa*
Sputum 13	*Candida albicans, Achromobacter* xyosoxidans and *Pseudomonas aeruginosa*
Sputum 14	*Streptococcus mitis, Stenotrophomonas maltophilia, Staphylococcus aureus, Cardiobacterium hominis, maltophilia, Veillonella parvula* and *Mycobacterium abscessus* subsp *massiliense*
Sputum 16	*Streptococcus oralis, Streptococcus mitis, Streptococcus sanguinis, Rothia dentocariosa, Rothia aeria, Klebsiell oxytoca, Neisseria flavescens, Achromobacter xyxlosoxidans* and *Pseudomonas aeruginosa*
Sputum 17	*Staphylococcus warneri, Neisseria sp, Streptococcus parasanguinis, Stenotrophomonas maltophilia* and *Exophiala dermatitidis*
Sputum 18	*Aspergillus fumigatus, Escherichia coli* and *Pseudomonas aeruginosa*

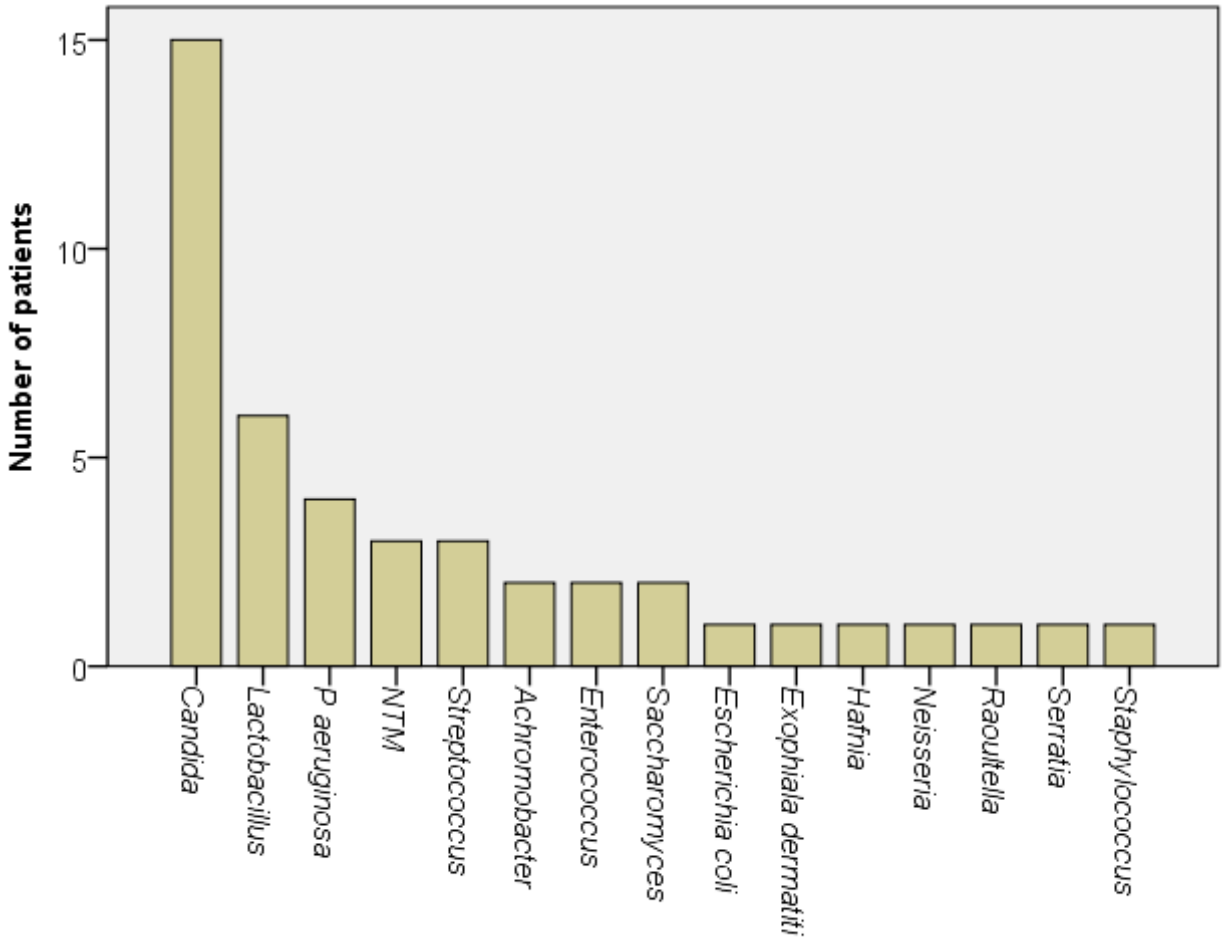

Figure 4-3: Gastric juice sample cultivation results for 16 CF patients with PEG feeding tubes. *Streptococcus* spp (3/16), *Lactobacillus* (6/16), *PA* (4/16) and NTM (3/16) were the most commonly identified bacteria in the gastric juices. The most frequently identified fungal microorganism in gastric juice specimens was the *Candida* species (15/16 samples).

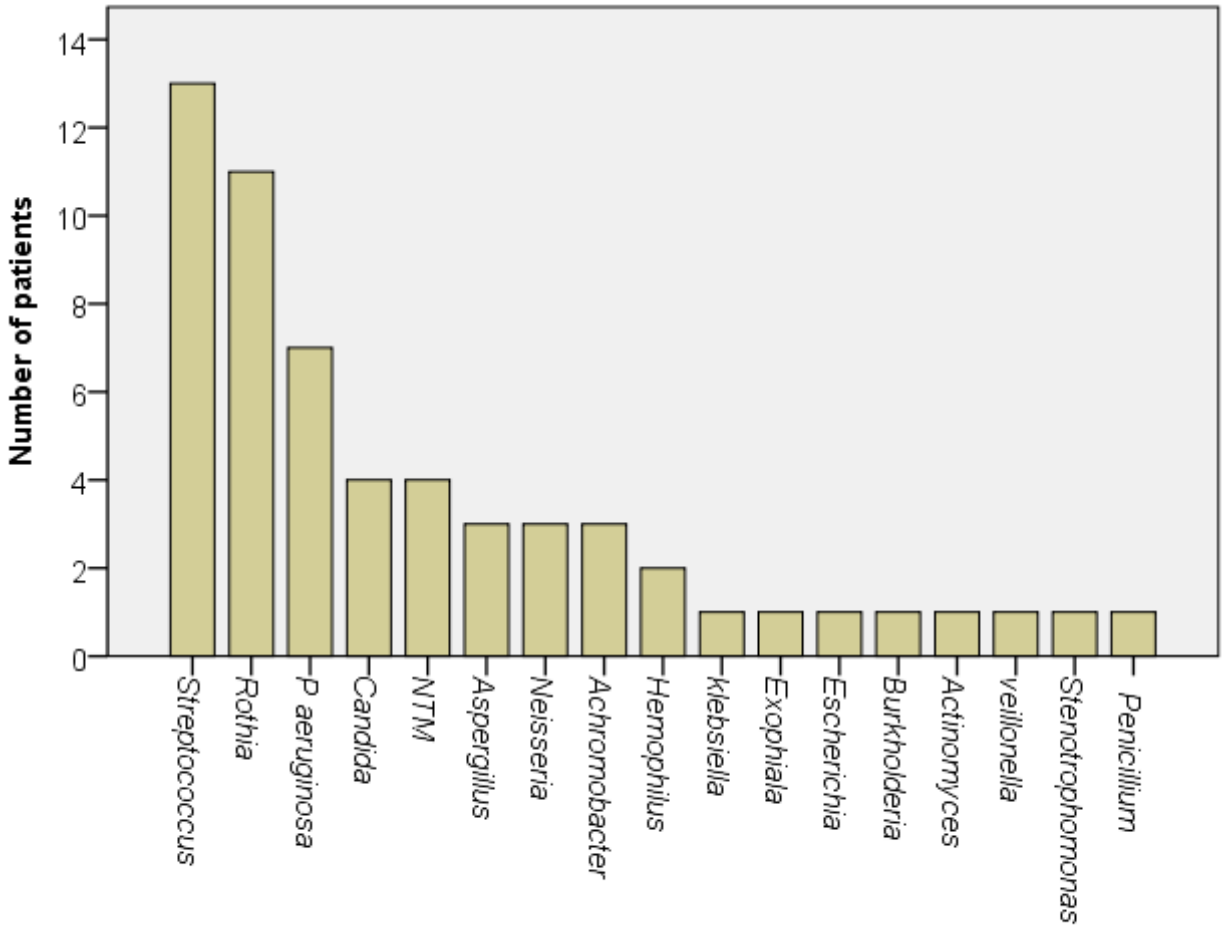

Figure 4-4: Sputum sample cultivation results in 16 CF patients with PEG feeding tubes. *Streptococcus* spp being present in 13/16 samples, *Rothia spp* in 11/16 samples, *PA* in 7/16 samples, *NTM* in 4/15 samples, and *Achromobacter* spp in 3/16 samples. The most frequently identified fungal microorganism was the *Candida* species (4/16 samples).

4.3.4 Comparing the microbial profile of CF gastric juice (CFGJ) CF sputum samples, (CFS) and non-CF gastric juice sample (non-CFGJ)

A difference in the bacterial profile of the CF samples (gastric juice and sputum) and non CF gastric juices was evident when comparing the samples. There was a similarity in the microbial profile between CF gastric juice and sputum in the level of prevalence of *PA* and NTM (Figure 4.5). Figure 4.6 show the microbial profile of the samples taken from CF patients. Similar to the previous finding in Chapter 3, there was a common bacterial species isolated in both the gastric juice and sputum sample from the same patient. Three CF patient (CF-9, CF-16 and CF-18) had identical *PA* strains in both sputum and gastric juice samples, which was established using VNTR. Interestingly, one patient's sample (CF-6), contained *PA* in the gastric juice but not in their sputum. A clear difference was found in microbial profiles between CF gastric juice and non-CF gastric juice (Figure 4.7)

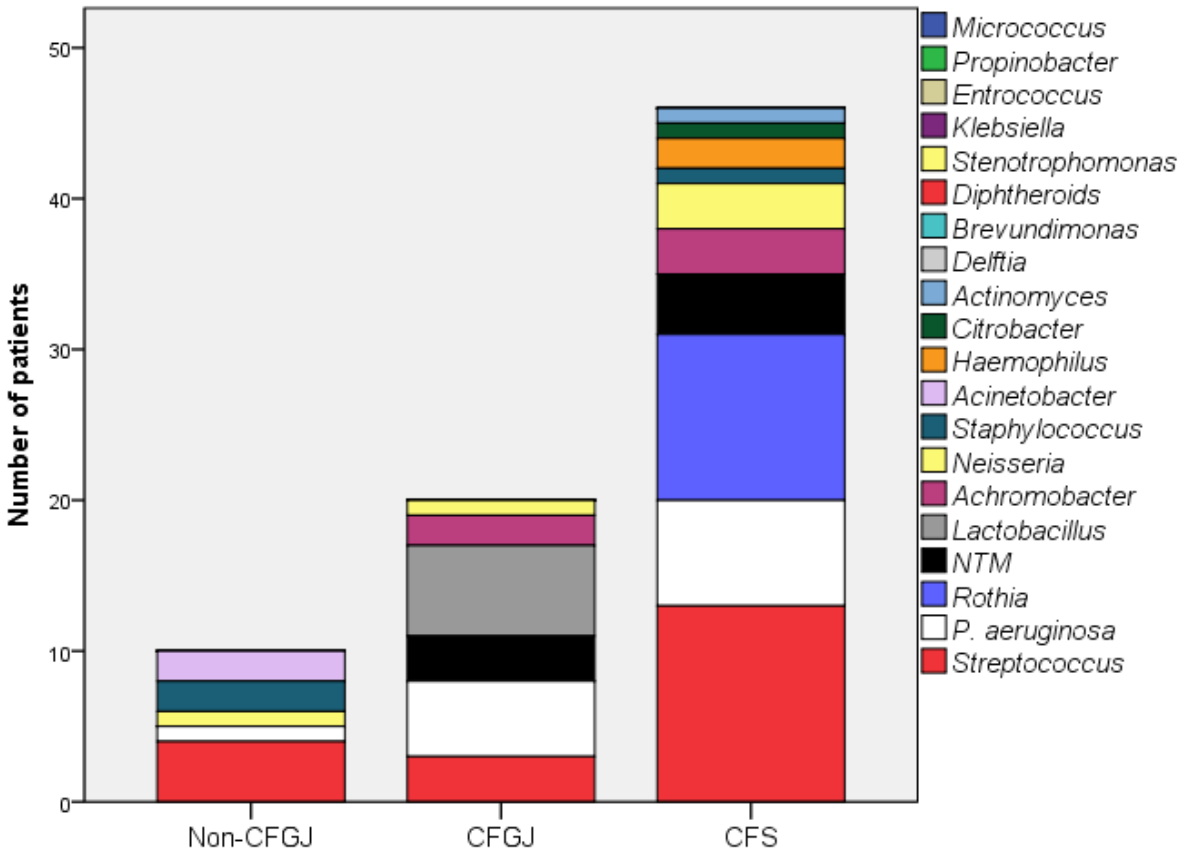

Figure 4-5: Bacterial species isolated from CF gastric juice samples (CFGJ) (n=16), CF sputum samples (CFS) (n=16) and non-CF gastric juice samples (Non-CFGJ) (n=14). The x-axis represents the type of samples. The y–axis represents the number of patients with a particular bacterial species.

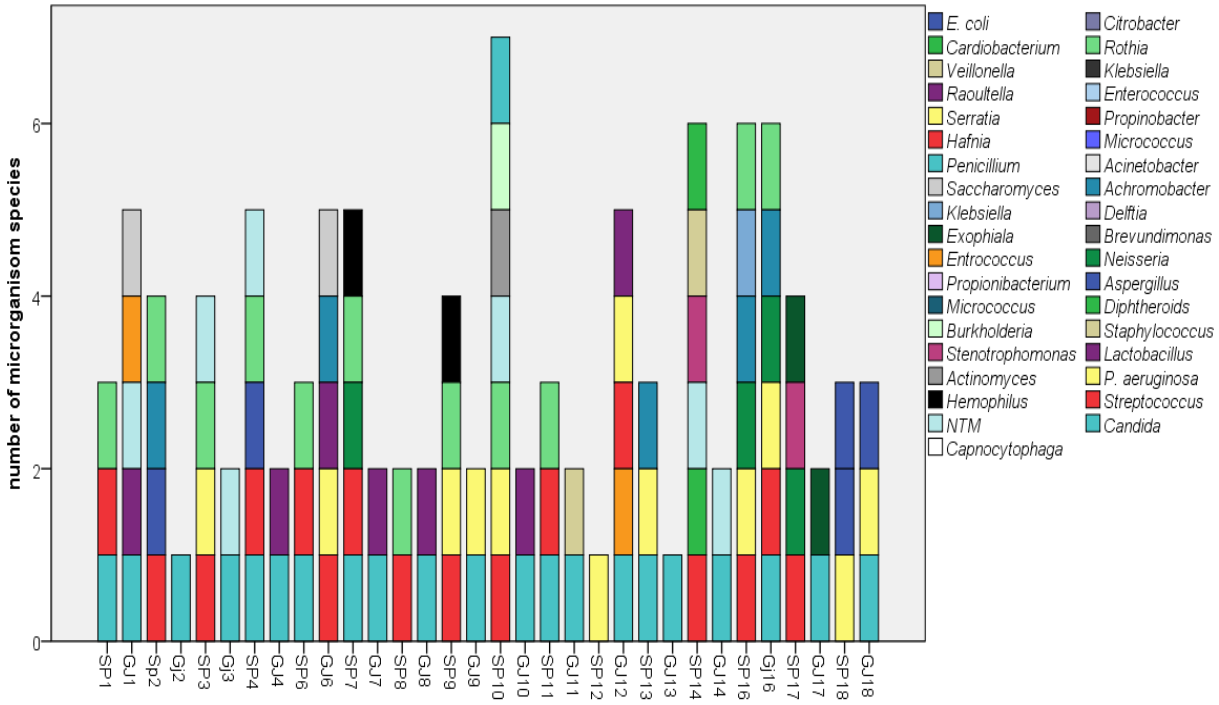

Figure 4-6: Microbial profile for CFGJ and CFS; each bar represent one sample. The bacterial strains common to both sputum and gastric juice included *PA* (3/11 patients), *Streptococcus* spp (2/11 patients) and *NTM* (2/11 patients).

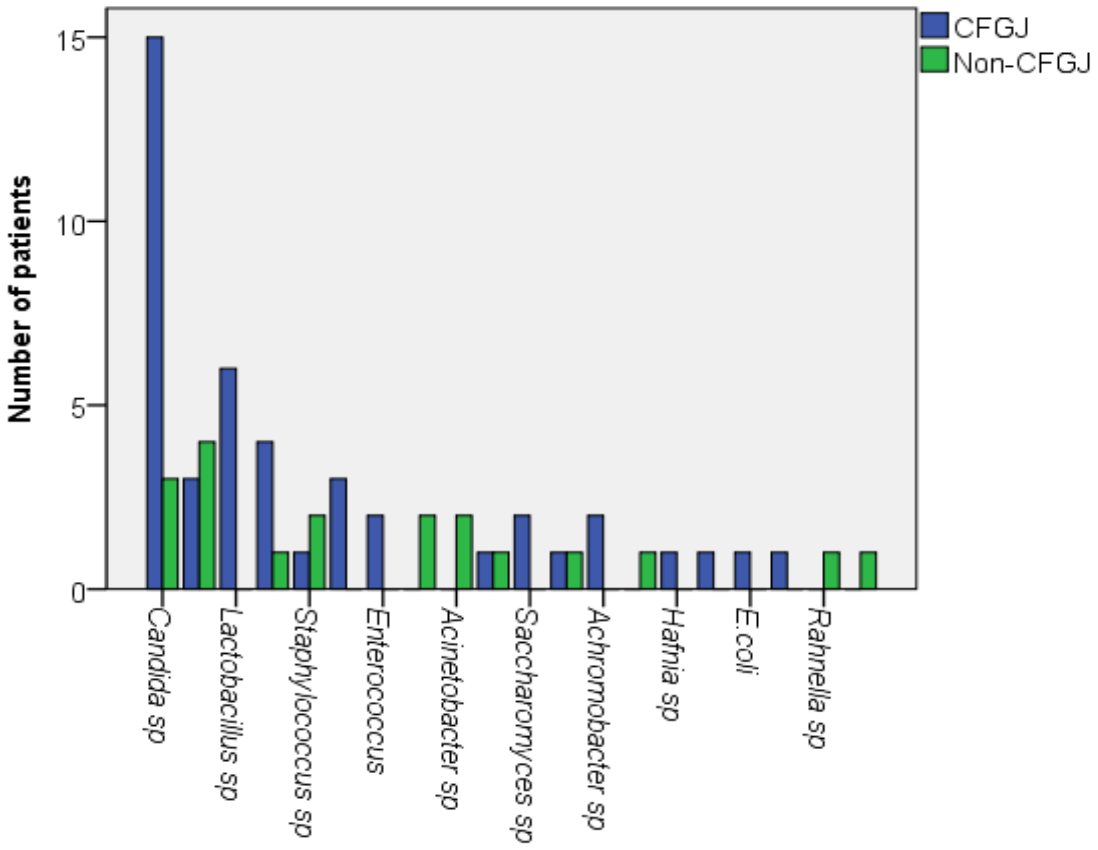

Figure 4-7: Microbial profile of CFGJ (n=16) and non-CFGJ (n=14). The most commonly identified bacterial species in CF gastric juice were found to be *PA* (4/16), *Lactobacillus* spp (6/16) and NTM (3/16). In non-CF gastric juice, the most commonly identified species were *Streptococcus* spp (4/14), *Lactobacillus* spp (2/14) and *Staphylococcus* spp (2/14). *Candida* spp were isolated from 15 out of 16 and 3 out of 14 of CFGJ and non-CF GJ, respectively.

4.3.5 Molecular based microbial analysis

NGS sequencing analysis performed by Dr Andrew Nelson (Northumbria University) show that there were marked differences between the amount and types of microorganisms present in CF samples and non-CF samples, according to both Alpha diversity and Shannon diversity index analysis. There were no major differences found amongst CF samples (Figure 4.8). This data was in broad agreement with that in Chapter 3.

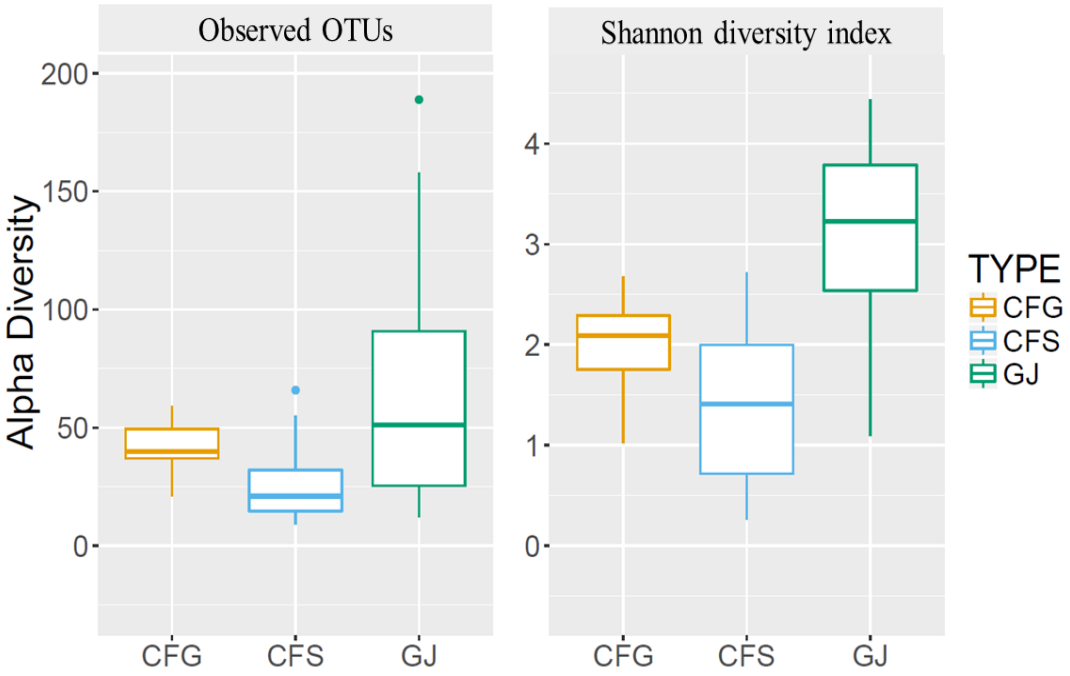

Figure 4-8: Alpha diversity (left) and Shannon diversity index (right) for cystic fibrosis gastric juice samples (CFG), CF sputum samples (CFS) and non-CF gastric juice samples (GJ). There was a significant difference between CF samples (CFG and CFS) and non-CF gastric juice sample in both alpha and Shannon diversity index. No significant difference was detected between CF samples (CFG and CFS) in both indices.

The major difference amongst CF samples was the prevalence of *PA*. There was a higher prevalence of *Bacteroides* and *Faecalibacterium* in the gastric juice samples of the non-CF control group (Figure 4.9).

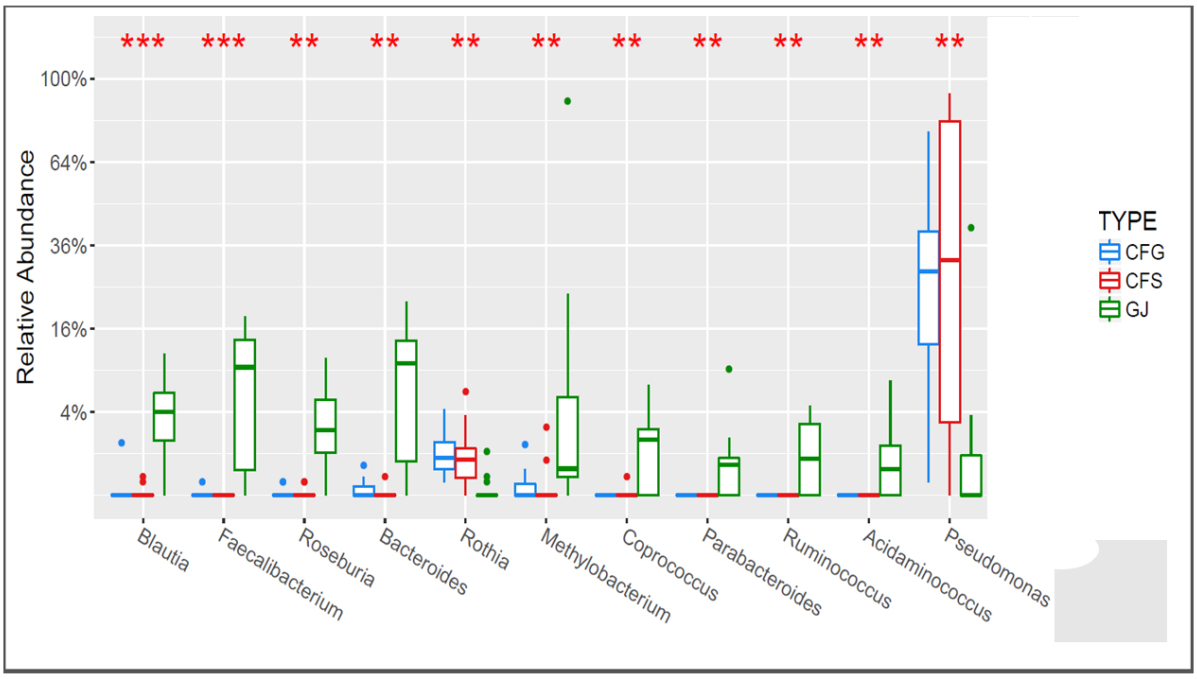

Figure 4-9: The difference between cystic fibrosis gastric juice (CFG), sputum (CFS) and non-CF gastric juice (GJ) in term of the most abundant bacteria genera. The x-axis represents the most abundant genera in both CF samples and non-CF samples. The y-axis represents the relative abundance of each OUT. ** P value <.05. *** P value <0.001.

4.3.6 Comparing repeated gastric juice and sputum samples.

13 patients were sampled on two separate occasions (T1 and T2) six months apart (CF1-4, CF6-13 and CF-16), bacterial species were isolated from 9 gastric juice samples in T1 and from 12 gastric juice samples on T2, the most frequent bacterial species isolated from gastric juice were *Streptococcus* spp (6/13) and *PA* (3/13) at T1 and *Lactobacillus* spp (5/13), *Streptococcus* spp (2/13) and *PA* (2/13) at T2.
Fungal sp were isolated from all gastric juice samples at both time points (T1 and T2) and the most frequent fungal species were *Candida* spp (isolated from nearly all samples).

In CF sputum samples, bacterial species were isolated from all sputum samples at the 2 time points (T1 and T2). The most frequent bacterial species isolated were S*treptococcus sp* (11/13) and *PA* (11/13) at T1 and *Streptococcus spp* (12/13) and *Rothia* spp (11/13) and *PA* (6/13) at T2.
Comparing the sputum sample microbial profile between T1 and T2 showed that *Streptococcus* spp were common in 10 sputum sample at T1 and T2, followed by *PA* which was common in 5 sputum samples.

4.3.7 Comparing repeated gastric juice and sputum samples using a molecular based approach

Using Alpha diversity and the Shannon diversity index, I observed biological variability between the two patient sampling time points but with no statistically significant difference found between the samples taken at different times. The CF sputum tended to have higher diversity in the second testing, but gastric juice stayed almost the same. There seems therefore to be a relatively stable profile of microbes in gastric juice compared to the increasing variation present in the sputum samples (Figure 4.10). Bland and Altman plots showed that the repeated gastric juice and sputum samples were not significantly different at the two time points (Figures 4.11 and 4.12). However, greater fluctuations in the Shannon diversity index were detected in sputum sample compared to gastric juice samples.

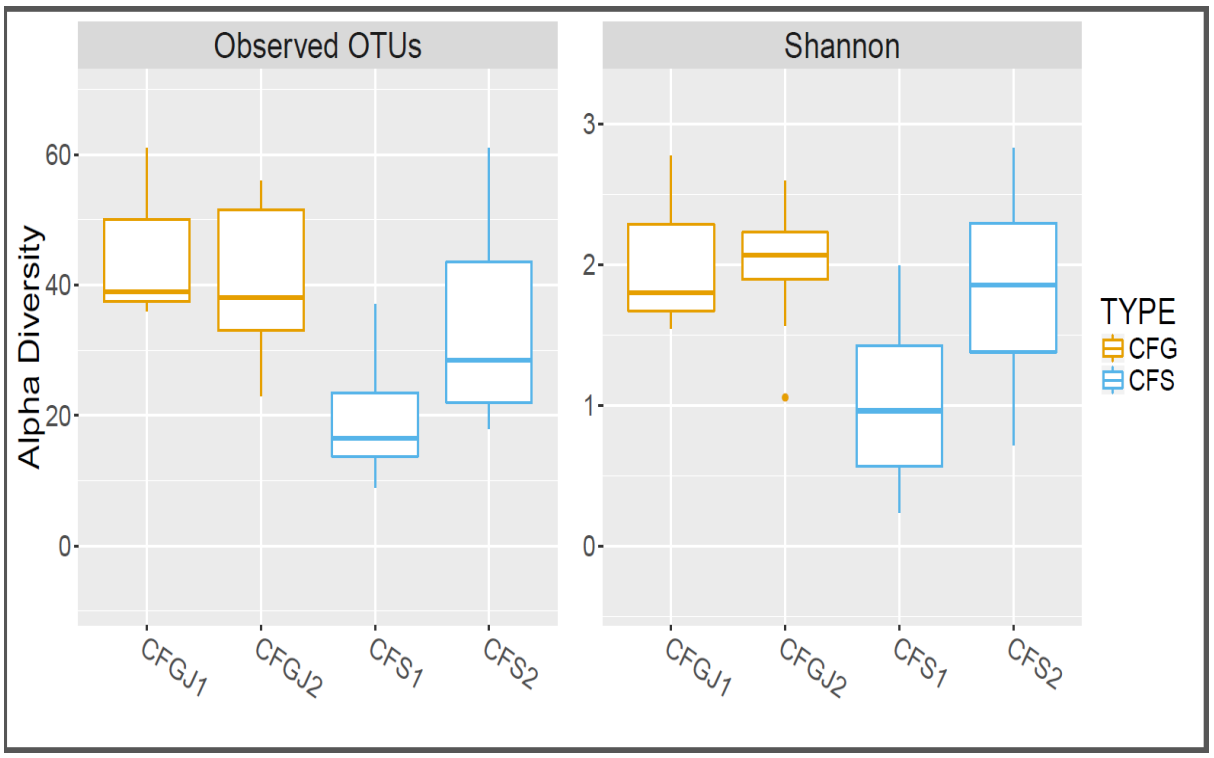

Figure 4-10: Alpha diversity (left) and Shannon diversity index (right) of repeated CF gastric juice and CF sputum samples at the two time points (T1 and T2).

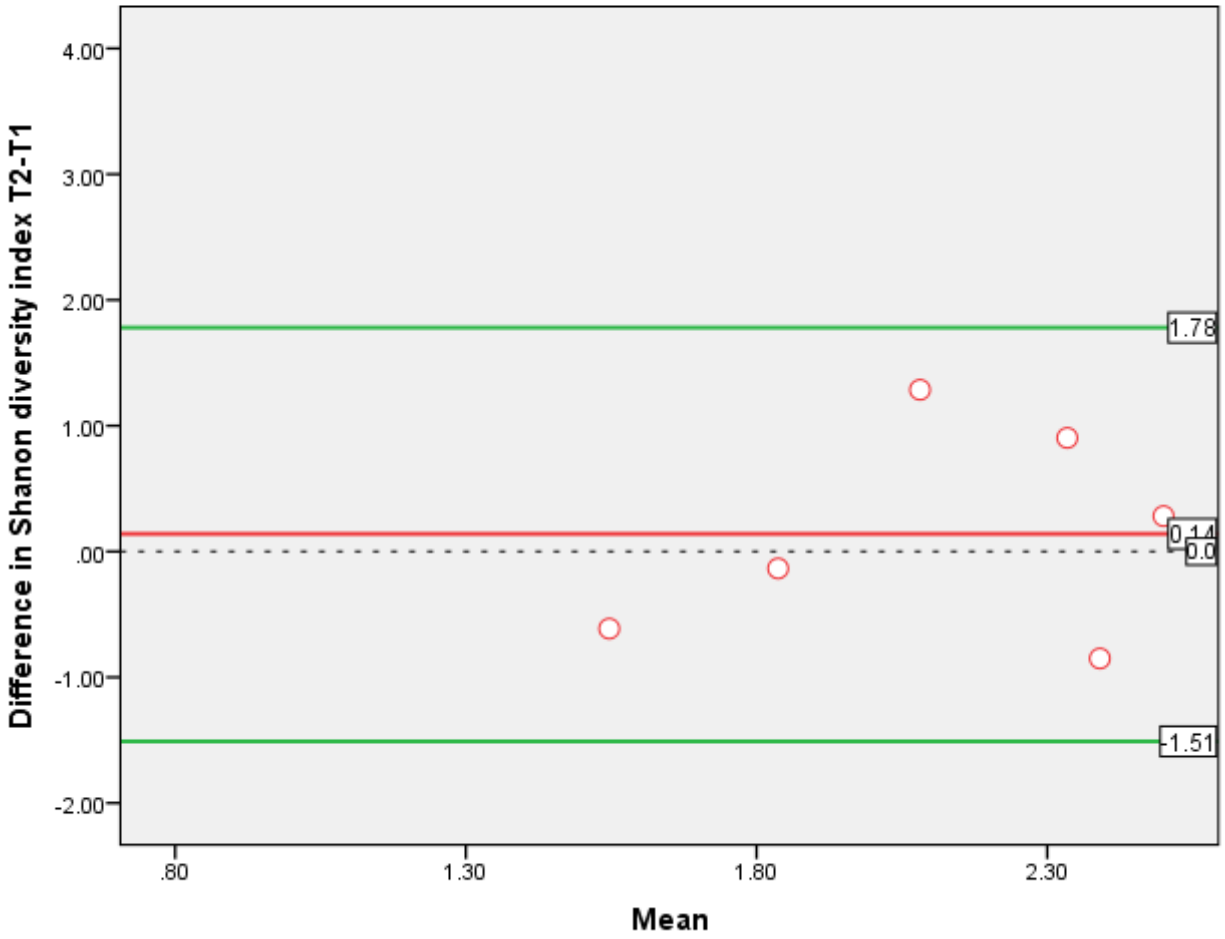

Figure 4-11: Bland Altman plot of the Shannon diversity index of gastric juice samples (n= 6) from CF patients at 2 time points, T1 and T2. The mean of the Shannon diversity of each subject (x-axis) is plotted against the differences between T1 and T2 (y-axis). Mean difference between T1 and T2 = 0.14 (range -1.15-1.78).

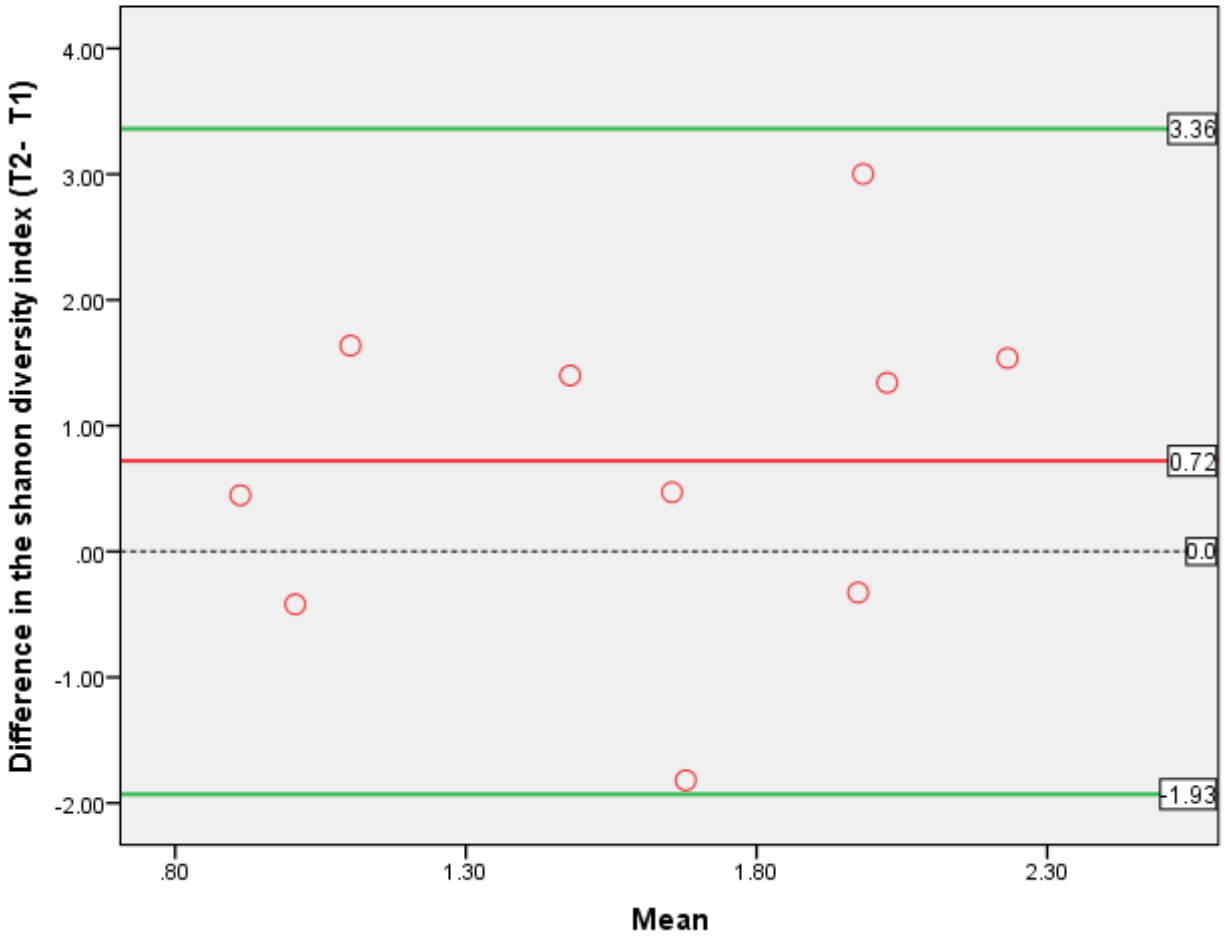

Figure 4-12: Bland Altman plot of the Shannon diversity index sputum samples (n=10) from CF patient at 2 time points T1 and T2. The mean of the Shannon diversity of each subject (x-axis) is plotted against the differences between T1 and T2 (y-axis). Mean difference between T1 and T2 = 0.72 (range -1.93-3.36).

4.3.8 PEG tube microbiology study

PEG tubes from five patients were obtained (CF4, 6, 7, 16, 17), and subsequently bacterial species were found on all collected PEG tubes, with the most common strains being *NTM* (2) *PA* (2) *Lactobacillus* spp (2) and *Enterococcus spp* (2). Four PEG tubes contained *Candida* fungal species (Figure 4.13)

Two of the collected PEG tubes contained *PA* (CF-6 and CF-7). The PEG tube of patient number 6 (CF-6) contained an identical strain of *PA* to that found previously in their gastric juice and sputum specimens. In contrast, the tube of patient number 7 (CF-7), which was positive for *PA*, had no *PA* present in the earlier samples (Table 4.3). NTM was a common and important finding in the microbial profile isolated from PEG tubes, and this will be explored in more detail in Chapter 5.

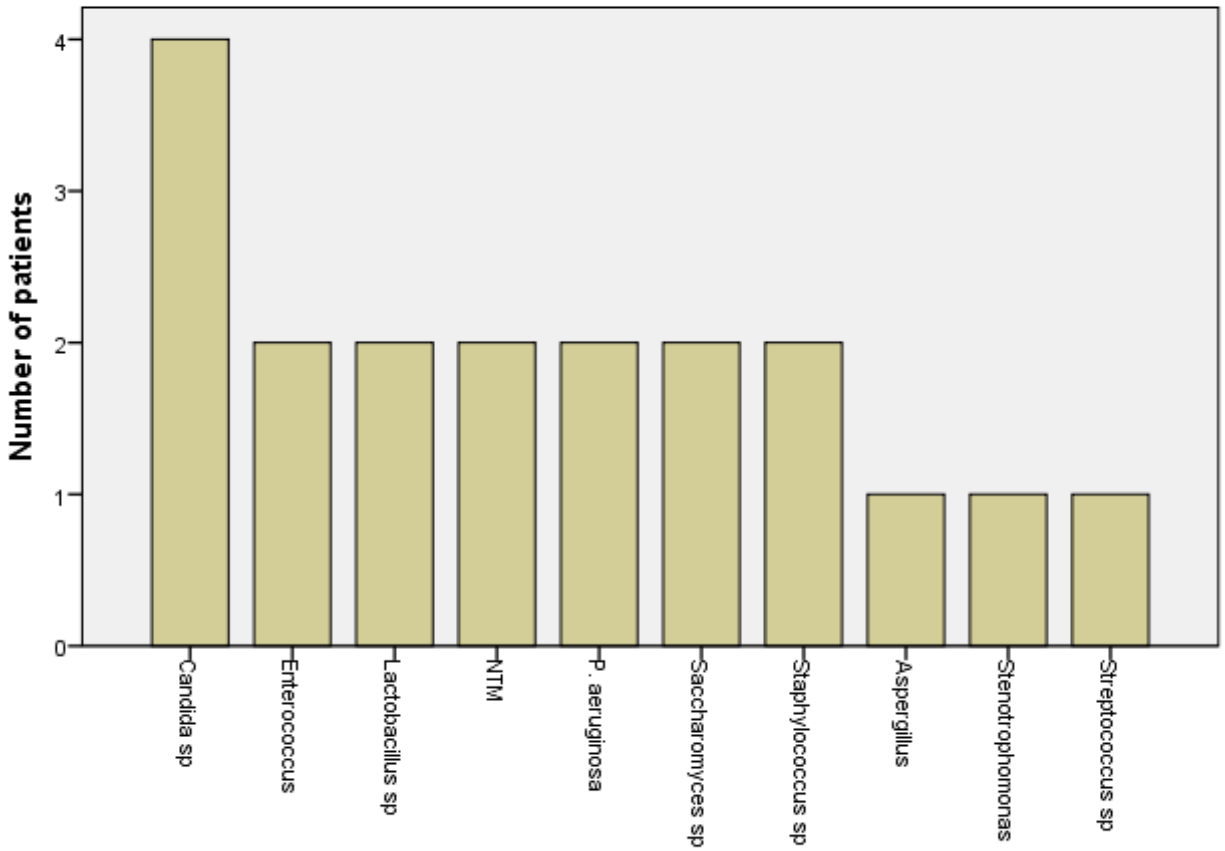

Figure 4-13: PEG tube microbiology results (n=5).

Table 4.3: PEG-fed tube culture results

PEG 4	*Candida albicans, Candida krusie, Candida glabrata Candida parapsilosis Lactobacillius fermentum, Enterococcus faecium* and *Mycobacterium abscessus sub sp abscessus*
PEG 6	*Candida albicans, Candida parapsilosis, Saccharomyces cerevisiae, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*
PEG 7	*Enterobacter cloacae, Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*
PEG 16	*Candida albicans, Streptococcus oralis, Mycobacterium abscessus sub sp abscessus, Mycobacterium bolletii* and *Aspergillus fumigatus*
PEG 17	*Candida glabrata, Candida parapsilosis, Staphylococcus epidermidis* and *Lactobacillus paracasei*

4.4 Discussion

Chronic suppurative lung disease is the most common cause of death in CF patients. The connection between the airways and the stomach has been proposed to be a source for CF lung infection (Rogers *et al.*, 2006; Al-Momani *et al.*, 2016), highlighting the need for further research.

The microbes colonizing the sputum and gastric juices of PEG-fed CF patients were compared in this investigation using repeated samples from the same patients described in Chapter 3. I identified microbial species in the sputum and gastric juice samples of CF patients, in addition to gastric juice samples from non-CF patients, by using a combination of conventional culture-based and culture independent methods. I was also able to collect some patients' replaced PEG tubes.

Similar to the finding of the previous chapter, this study, which includes some repeated samples, demonstrated using culture-based and molecular-based approached that the bacterial species present in CF gastric juice were different compared to the control group (non-CF patients). A high rate of bacterial species such as *PA* and NTM that can potentially cause lung infection were isolated from CF gastric juice samples and the collected PEG tubes. Thus, these finding highlight the possibility that the stomach compartment may store CF-related pathogens. These pathogens could subsequently be aspirated during reflux.

In the previous chapter, 4 identical strains of *PA* in sputum and gastric juice from the same patient were isolated. In this study, different patients (n=3) had identical strains of *PA* common in their sputum and gastric juice. Moreover, two patients had identical strain of *M. massiliense* in their sputum and gastric juice and this will be explored in more detail in chapter 5. On the whole, this points towards a possible link between microbes found in the sputum and gastric juice in some CF patients. Rosen *et al.* (2015), Palm *et al.* (2012) and Rosen *et al.* (2011) highlighted the microflora exchange between the stomach and the lungs and the connection between reflux and an increased chance of positive bronchioaleveolar lavage and *PA* cultures in cystic fibrosis patients.

CF patients included in this study rely on PEG to maintain nutrition. A number of natural defence mechanisms are compromised in PEG tube-fed patients. Graeme *et al.* (2005b) explains that a lack of sensory stimuli associated with eating reduces saliva production and peristalsis, while decreased swallowing causes an increase in gastric pH. The overall result is a higher

predisposition to microbial colonization in the stomach. PEG tubes have been proved to be contaminated with *Candida* spp, and this fungal microorganism could also cause the tube to deteriorate, according to Gottlieb *et al.* (1994). Similar to my findings, Dautle *et al.* (2002) isolated *Enterococci* spp, *Staphylococci* spp, *Lactobacilli* spp, *Candida* spp and *PA* on the PEG tube of non-CF child patients, while Rolston *et al.* (2011) discovered that on the tubes of PEG -fed cancer patients, *PA* bacteria were amongst the most frequently identified organisms. Microbiology of the non-CF gastric juice samples (control group) also suggested that *PA* can be found in the gastric juices of non-CF patients when endoscopically tested (Al-Momani *et al.*, 2016). Likewise, *PA* was found in samples of patients with gastritis in a study by Monstein *et al.* (2000), thus suggesting that our findings are not exclusive to PEG fed CF patients and can be generalised to other non-CF patients with PEG tube feeding.

There was agreements in the repeatability of the gastric juice and sputum samples at the 2 time points, with a degree of expected variability inherent to biological samples taken at different time points and there was no significant difference in Shannon diversity index using Bland Altman plots. It was of interest, however that more diversity was noticed in sputum samples at T2 compared to T1, although it is statistically insignificant.

This is the first study which has assessed the repeatability of estimating the Shannon diversity index in the sputum samples and gastric juice samples of CF patients. Loss of diversity is often cited as a key finding in the CF lung microbiome and increase in diversity has been noted with disease-modifying therapy in CF (Zeybel *et al.*, 2016). The present description of variability in diversity that can occur inherently due to repeated biological sampling therefore provides novel and useful data, e.g. for sample size estimation for studies aiming to modulate the microbiome.

The findings presented in this chapter support the findings of the previous chapter that the stomach of CF patients hosts pathogenic bacteria which could be refluxed up to the extraoesophageal compartment and aspirated. This hypothesis was strengthened by the finding that a gastric juice sample (CF-6) and the PEG tube of patient number 7 (CF-7) were positive for *PA* and had no *PA* present in the earlier sputum samples suggesting that bacteria species survive in the gastric compartments independent from respiratory sources.

Chapter 5.0: Nontuberculous mycobacteria in percutaneous endoscopic gastrostomy fed cystic fibrosis patients

5.1 Introduction

Along with the increasing survival rates amongst CF patients over the last two decades, a number of new pathogens are also emerging which tend not to be seen in younger CF patients and which have not been previously seen in CF (Leung and Olivier, 2013). One of these microbes is non-tuberculous mycobacteria (NTM), of which 140 species are believed to currently described according to Gillespie (2006). There has been a dramatic increase in the number of NTM species identified in recent years, and not all are believed to be pathological in humans. Nonetheless, Gillespie (2006) and Jordan *et al.* (2007) point out that classification of NTM strains has improved, along with the ability to separate closely related strains (e.g. *Mycobacteria chelonae* and *Mycobacteria abscessus*) due to advancements in microbiological sequencing methods which have evolved.

NTM are aerobic, stationary organisms that can be seen by using acid-fast alcohol stains (Murray *et al.*, 2015). Their cell wall, which is hydrophobic and rich in lipids, is significantly thicker than the cell walls of most other bacteria, according to Murray et al, (2015). NTM species are resistant to heavy metals, disinfectants, and antibiotics due to the thickness and properties of their cell wall (Gillespie, 2006; Murray *et al.*, 2015).

NTM are environmental microbes that are present all over the world, especially in soil and water (including domestic water supplies). Although the highest concentrations of NTM are found in soil and water sources, the mycobacteria are actually present in all environments. They are thought to be able to form biofilms (Schulze-Röbbecke *et al.*, 1992), and Falkinham Iii (2007) described how such formation contributes to disinfectant and antibiotic resistance. Furthermore, many such organisms are acid resistant (pH<3) (Bodmer *et al.*, 2000).

In light of these typical NTM characteristics, it follows that drinking water, household plumbing, marshes and peat rich soils act as NTM reservoir (Falkinham, 2013). Phillips and Von Reyn (2001) pointed out that water systems in hospitals, haemodialysis centres, and dental practices contain particularly high levels of NTM colonization. Falkinham Iii (2011) suggested that, given the organisms' tendency for biofilm formation, it is important to include biofilms in samples being tested for NTM colonization

5.1.1 Epidemiology of non-tuberculous mycobacteria in cystic fibrosis patients

In both Europe and North America, NTM are being discovered more and more frequently in the sputum of CF patients adults and children (Floto *et al.*, 2016), The prevalence of NTM in CF patients has been measured over the years, and has ranged from 1.3% in the first recorded study by Smith *et al.* (1984) to 32.7% in a 2005 study of CF patients over 40 years old by (Rodman *et al.*, 2005). The two biggest documented investigations were conducted by Esther *et al.* (2010), who studied 1216 CF patients, and Roux *et al.* (2009) who studied 1582 CF patients. These studies found a 13.7% and 6.6% rate of NTM-positive specimens respectively. Salsgiver *et al.* (2016) points out that recent examination of registry data from the US Cystic Fibrosis Foundation (CFF) has discovered a 12% prevalence rate for NTM-positive specimens.

5.1.2 Important NTM species in CF

The most frequently identified species of NTM in CF patients are the slow- growing *Mycobacterium avium complex* (MAC) (including *M. avium, M. intracellulare and M.chimaera*). According to Olivier *et al.* (2003), these strains are found in as many as 72% of NTM-positive sputum samples. The second most frequent species in CF patients are the fast-growing *M. abscessus complex* (MABSC) (including the subspecies *M. abscessus subsp abscessus* (*M. a. abscessus*), *M. a. bolletii* and *M. a. massiliense)* (Adékambi *et al.*, 2004; Adékambi *et al.*, 2006). *M. simiae, M. kansasii* and *M. fortuitum* have been identified by Floto *et al.* (2016) as being less prevalent species of NTM. The prevalence of different strains differs not only between, but also within, countries, suggesting geographical influences on the prevalence of NTM.

5.1.3 Risk factors for NTM infection

CF patients with positive NTM sputum samples were found, in a large multicentre prevalence study in North America, to be older people with higher FEV_1 and lower incidence of *PA* pulmonary infection than patients with negative NTM sputum samples, according to Olivier *et al.* (2003).

However, it has also been found that NTM is associated with lower FEV_1, haemoptysis and higher prevalence of *PA* (Levy *et al.*, 2008). The strain of mycobacterial species involved here

may explain this discrepancy. Although MAC is the most common mycobacterial pathogen found throughout America, Israel and Europe, MABSC is more prevalent. Renna *et al.* (2011) point out that in a large UK study of CF patients, elevated rates of NTM infection were found to be influenced by continuous use of azithromycin medication.

The two final factors that have been found to influence NTM infection in CF patients are the increased use of steroids and allergic bronchopulmonary aspergillosis (ABPA) (Mussaffi *et al.*, 2005). In ABPA, altered immune response, in particular the T-helper 2 mediated up-regulation of interleukin-4 (IL-4) and down-regulation of interferon-γ (IFN-γ), is likely to provide an ideal environment for mycobacteria to thrive (Hernandez *et al.*, 2005).

5.1.4 Mode of transmission

The mode of transmission of NTM to humans has not been defined. Person-to-person transmission has not been convincingly demonstrated. Further, although animals may serve as a reservoir, animal to human transmission is not thought to occur (Biet *et al.*, 2005).

On the other hand, it is possible that the infection can be transferred by sharing drinking water systems with animals (Kankya *et al.*, 2011). The repeated use of surgical equipment on patients is thought to be responsible for NTM infections. Although the exact source of infection often remains unidentified, the instruments generally become infected through poor cleaning standards, or from the water used in the disinfection process (Olivier *et al.*, 1996; Quittell, 2004). Although the exact cause of NTM infection is unknown, it is probable, based on the environmental source of NTM, that the bacteria is contracted via ingestion, inhalation, or implantation (Wolinsky, 1995).

Pulmonary NTM disease is thought to be the result of aerosolization of tiny droplets entering the alveoli. Bathroom showers have been identified as fundamental breeding grounds for aerosolized NTM (Falkinham, 2003; Falkinham *et al.*, 2008). Furthermore, a cohort case study conducted by Dirac *et al.* (2012) explored the use of aerosols in the home and discovered that using a spray bottle for watering plants was the only aerosol activity found to increase the chances of NTM-related lung disease.

An elevated concentration of NTM can be found in potting soils, and especially peat-rich soils, and it is possible that soil-generated dust produces particles small enough to get into the alveoli (De Groote *et al.*, 2006). NTM colonization and nosocomial outbreaks of disease have been

caused by contamination of hospital water supplies, medical equipment (such as bronchoscopes and endoscopes) and contaminated dialysis solutions. Skin abscesses, pulmonary alveoli, meningitis rashes, and surgical sites are all described as areas affected by NTM infection (Phillips and Von Reyn, 2001).

In CF patients, in spite of thorough isolation, a high potential NTM transmission rate amongst CF patients was identified in a recent study, specifically with regard to the *M. abscessus strain*. (Bryant *et al.*, 2013) speculated that the disease could be contracted indirectly, via fomite contamination or aerosols used during physiotherapy and spirometry testing. The investigation also showed that the disease could be passed on by patients who tested negatively for culture positive sputa, meaning that an extremely low level of inoculum might be enough to cause infection. This evidence is important for ongoing debates that promote the need for rigorous preventative measures (Bryant *et al.*, 2013).

5.1.5 NTM pulmonary infection: implications for treatment and lung transplantation

The treatment of NTM lung infections is clinically problematic. The range of potential side effects of anti-NTM drugs must be taken into account, along with any interaction that such drugs may have with the patient's CF medication. Wallace Jr *et al.* (2012) suggest that these issues are of particular importance to CF patient's with liver disease. The often lengthy treatment process and the patients other existing conditions must therefore be taken into account when deciding the length and toxicity of anti-mycobacterial treatment. An overview of a patient's current treatment course is often conducted before starting treatment for NTM. (Johnson and Odell, 2014) assert that the prognosis of untreated NTM infection is difficult to establish, due to a lack of relevant data regarding untreated NTM infections.

NTM infection is present in a large amount of CF patients being considered for a lung transplant. Approximately twenty percent of CF patients showed NTM infection before transplantation and around 14% tested positive for NTM pulmonary infection after transplantation (Brown, 2010). The patients' own remaining lungs (above the area of the anastomosis) were considered to be the source for post-transplantation NTM.

Although NTM disease after lung transplantation can be treated, life threatening disseminated infection can be caused by the MABSC strain (Gilljam *et al.*, 2010); thus, many transplant

centres regard active MABSC pulmonary disease to be serious enough to warrant deferral of an impending transplant.

5.1.6 Gastroesophageal reflux, acid suppression and NTM *pulmonary disease*

There has not been an in-depth study investigating the connection between GORD and NTM lung diseases. Nevertheless, a connection has been discovered between NTM lung disease and gastroesophageal disorders in previous research (Varghese *et al.*, 1988; Griffith *et al.*, 1993; Hadjiliadls *et al.*, 1999).

Hadjiliadls *et al.* (1999) conducted a MEDLINE search of English language publications from 1966 to 1997 to assess the relationship between NTM lung infection and oesophageal disorders, and revealed that twenty cases of NTM lung disease in patients with oesophageal disorders. Griffith et al (1993) reported that six percent (10 of 154) of patients with NTM lung disease caused by quick-growing strains such as *M. abscessus* or *M. fortuitum* had a gastroesophageal disorder causing chronic vomiting.

Fifty-eight patients with *M. avium complex* (MAC) lung disease were investigated by Thomson *et al.* (2007), who found that GORD was more frequently diagnosed among patients with MAC lung disease than among age-matched controls (44% compared to 28%; p=0.019). Thomson *et al.* (2007) suggested that, as patients with MAC were more frequently prescribed acid suppressive therapy, acid suppression may heighten the risk of contracting NTM lung disease because gastric fluid promotes the growth and survival of NTM.

In a 24 hours pH observation study on fifty eight patients with 2 types of NTM lung disease (MAC and *M. abscessus*), Koh *et al.* (2007) found that 26% tested positive for GORD. The airways disease on tomography scan was more widespread in those with GORD, and they were more likely to demonstrate acid-fast bacilli on sputum smear (80% versus 44%; p= 0.033).

It is therefore uncertain whether GORD symptoms are just more common among coughing patients, whether GORD only plays a part in causing airways disease, which then makes the patient susceptible to NTM disease, or whether reflux alone heightens the risk of contracting NTM lung disease, perhaps by supplying NTM to the airways. De Groote and Huitt (2006) and Field and Cowie (2006) have explained how these reports have led to many authorities declaring GORD as a key factor in predisposing patients to NTM lung disease and subsequently advising

careful questioning of patients with pulmonary disease caused by rapid-growing NTM bacterial strains regarding any symptoms that indicate recurrent GOR and aspiration.

Although some research has indicated a prevalence of GORD in patients with NTM lung disease, the nature of this relationship is unclear. It is possible that GORD could be responsible for the exposure of the pulmonary parenchyma to refluxed gastric acid, which could cause the development or progression of NTM lung disease. On the other hand, GORD could be a secondary phenomenon. Changes in lung function may make NTM lung disease patients more susceptible to irregular reflux, along with frequent coughing which exerts increased pressure on the diaphragm. The increased intake of acid suppressants could cause weakly acidic or non-acidic reflux into the oesophagus, which may actually create an environment conducive to growing NTM bacteria.

I have previously outlined results that demonstrate a relationship between the bacteria in gastric juices and CF lung pathophysiology in patients who are fed by percutaneous endoscopic gastrostomy (PEG) tubes. I consequently investigated whether gastric juice and PEG tubes from people with CF may act as an under-estimated reservoir of NTM colonization (Al-Momani *et al.*, 2016).

The investigation aim in this chapter was to provide new insights into the mycobacterial profiles of sputum, gastric juice and PEG tubes taken from the same CF patients.

5.2 Materials and methods

This investigation used samples of gastric juice and sputum from 16 PEG-fed CF patients (CF1-4, CF6-14 and CF16-18) as used in Chapter 4. PEG tubes were taken from 5 patients either after these were routinely renewed or when they were removed (CF-4, CF 6-7 and CF 16-17). pH strips were used to test the acidity of gastric juices. Dithiothreitol was used to homogenize sputum samples. A PEG-conditioned saline mixture (PEG-s) was created when the internal and external parts of the PEG tubes were separated into small sections and thoroughly washed with 3ml of saline mixture. Microbiological analysis of gastric juice, sputum samples and PEG-s was conducted using nationally recognized techniques, as presented in Chapter 2.

Matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, UK) and in some cases suitable Analytical Profile Index (API) kits (BioMérieux UK) were used to identify microorganisms (Blauwendraat *et al.*, 2012). RpoB, sodA and hsp65 gene sequencing techniques were used to determine mycobacterium species, and the strain of the bacteria was subsequently established using variable number tandem repeat (VNTR), Colindale, UK (Harris *et al.*, 2012).

The Reflux Symptoms Index (RSI) score was used to identify symptoms of extraoesophageal reflux (EOR) and if the score was 12 or less, the reflux was considered to be not EOR symptomatic (Belafsky *et al.*, 2002).The reflux symptom index relies on patients reporting their symptoms and is used to assess laryngeal symptoms secondary to reflux, including coughing.

Differences in demographic and clinical variables in CF patients with and without NTM infection were compared using univariate analysis. Comparisons were performed using a two-tailed unpaired T-test. All data were evaluated using SPSS for Windows, version 22; statistically significant results were considered if p-values equal to or less than 0.05.

5.3 Results

5.3.1 CF Patients characteristic

The CF patients included in this studied display moderate to severe pulmonary disease, which is consistent with PEG feeding. The median FEV_1 was 1.6L (37%) range 0.5-2.7L (17%-88%), and participants were being treated with long-term use of antibiotics and acid suppression medicine. Symptom scores for extraoesophageal reflux were obtained for fourteen out of sixteen CF patients, thirteen of whom were clearly shown to be EOR symptomatic with an RSI score >12; median RSI score 17 (range 13-36), even though they had been using PPI or Ranitidine. One patient did not display symptoms of EOR, resulting in an RSI score of 8.

5.3.2 Microbiological results

One of the most frequent bacteria detected in both sputum and gastric juice were *Non-Tb mycobacterium* (NTM), as shown in Figure 5.1. The most commonly identified NTM strains were *M. massiliense* and *M. abscessus sub sp abscessus*

The samples from seven of the sixteen (43%) CF individuals contained NTM. Among those 7 patients, five patients' sputum tested positive for NTM, three contained the strain *M. massiliense* (CF-3, CF-10, CF-14) and two the *M. abscessus sub sp abscessus* strain (CF-4 and CF-7). Two of the five NTM positive sputum specimens contained an identical strain of NTM established by VNTR in their gastric juice, and one contained a strain identical to that found on their removed PEG tube (which was the *M. abscessus sub sp abscessus* (CF-4).

Two patients who were sputum sample negative for *NTM* (CF-1 and CF-16) were found to have either *M. massiliense* in their gastric juice (CF-1) or *M. abscessus sub sp abscessus* and *M. bolletii* in their PEG tube (CF-16). NTM were isolated for the first time from CF-1 and CF -16 from a non-lung, gastric site (Table 5.1).

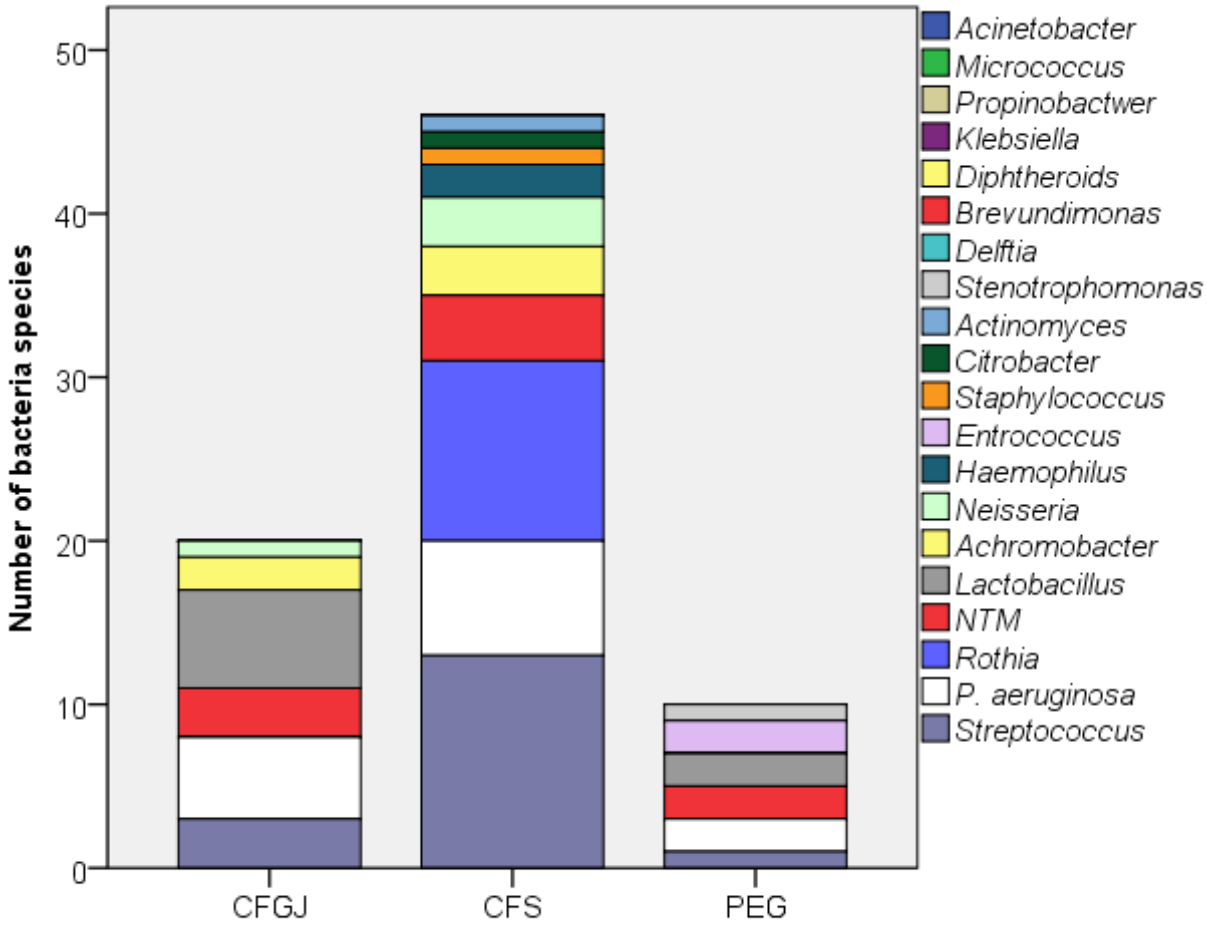

Figure 5-1: Culture results of gastric juice samples (n=16), sputum samples (n=16) and PEG-s (n=5).

Table 5.1: Patient details related to chronic colonisation of NTM

	Gastric juice	**Sputum**	**PEG**	**NTM status**
CF-1	*M. massiliense*	No NTM isolated	No PEG	No previous NTM
CF-3	*M. massiliense*	*M. massiliense*	No PEG	+ve for *M. massiliense* since 6-12-2006
CF-4	No NTM isolated	*M. abscessus sub sp abscessus*	*M. abscessus sub sp abscessus*	+ve for *M. abscessus* 10-7-2008
CF-7	No NTM isolated	*M. abscessus sub sp abscessus*	No NTM isolated	+ve for *M. abscessus* 9-1-2009
CF-10	No NTM isolated	*M. massiliense*	No PEG	No previous NTM
CF-14	*M. massiliense*	*M. massiliense*	No PEG	Grew *M. chelonae* once only
CF-16	No NTM isolated	No NTM isolated	*M. abscessus sub sp abscessus* *M. bolletii*	No previous NTM

Patients who tested positive for NTM were younger (median 24, range 20-26) than those who tested negative (median 30, range 18-32) with almost similar FEV_1 measures (Figure 5.2 A and 5.2 B), and had a lower BMI (mean 18.3 (SD 1.75) vs 19.1(SD 2.4) (Figure 5.2 C). The average annual IV antibiotics intake for NTM positive patients was lower (median 56, range 14-154) than that of NTM negative patients (median 70, range 37-160) (Figure 5.2 D). None of these differences between NTM positive and negative patients was statistically significant.

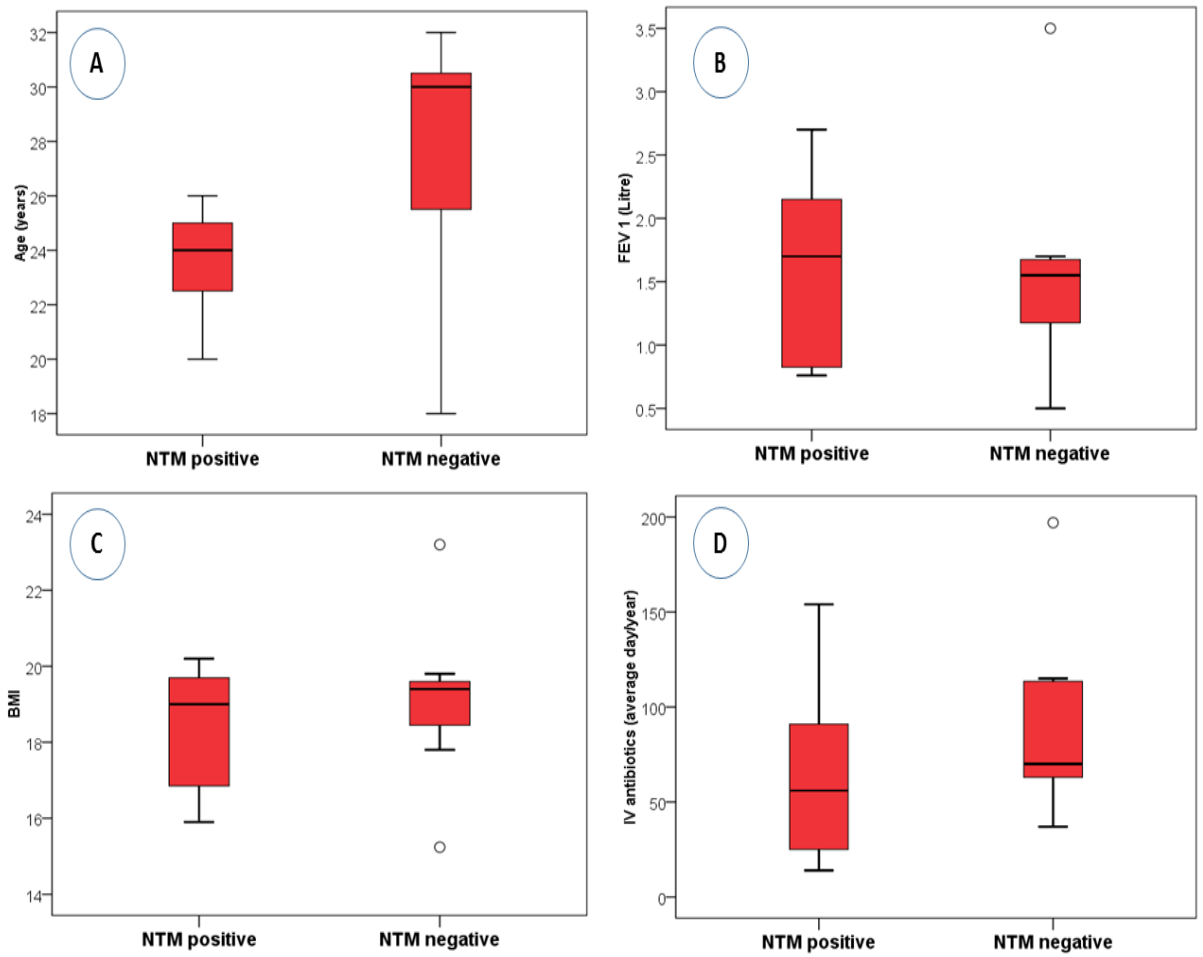

Figure 5-2: Difference between NTM positive (n=7) versus negative (n=9) patients according to: The age (A), FEV_1 (B), BMI (C) and The average IV antibiotics (number of days per year) use (D). The small circles are outliers.

NTM-positive patients had higher scores on the RSI (median 22, range 16-36) in comparison to NTM-negative patients (median 15, range 8-20) but this was not statistically significant (p=0.224). The RSI questionnaire demonstrated a range of scores from 2-5 for the coughing domain score (5 was the highest possible score) (Figure 5.3).

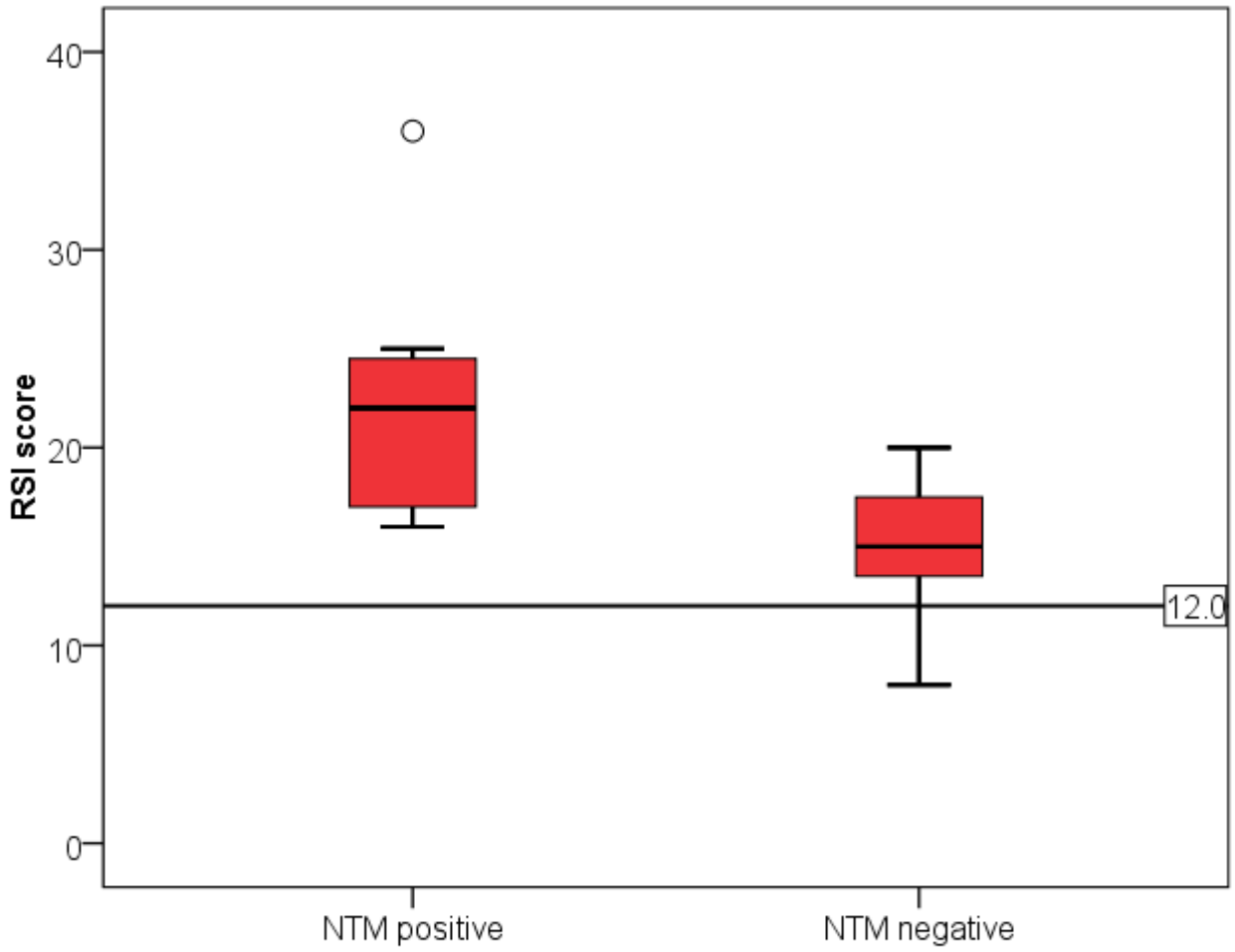

Figure 5-3: The RSI scores of NTM positive (n=7) and NTM negative patients (n=9). The small circle is outlier

5.4 Discussion

This investigation is the first of its kind to demonstrate that NTM, including *M. abscessus,* as well as being found in sputum, is also present in the gastric juice and PEG tubes of CF patients. Patients with NTM- negative sputa showed NTM in their PEG and gastric juice. This was the first time that NTM had been identified in those patients despite frequent sputum sample analysis during follow up of those patients in our CF centre.

NTM has reportedly had outbreaks in individuals without CF, which Wallace Jr *et al.* (1998) report have been due to situations where soft tissue and bone has become infected after contaminated equipment has been used during operations. Griffith *et al.* (2007) explain that, although the use of disinfected operating equipment is thought to cause NTM outbreaks, presumably because of insufficient cleaning or from the water used in the disinfection process, the precise source of the outbreaks remains unknown.

A popular technique used to enhance feeding in CF patients is the use of a gastrostomy tube, which can be inserted either endoscopically, surgically, or fluoroscopically. Transmission of NTM infections via foreign bodies has been reported (Trupiano *et al.*, 2001). It is believed that the existence of a foreign body enables colonisation by making host barrier defence mechanisms weaker (Linmans *et al.*, 2008). According to Chiu *et al.* (2010), there has been a reported instance of *M. abscessus*-caused perigastrostomy infection in an immunocompetent patient.

Gastrostomy tube feeding has previously been considered a risk factor for developing tuberculosis (Snider, 1985). I am not aware of any available information concerning whether a gastrostomy is a reserve for NTM in CF patients. The previous chapters demonstrated that gastric juice is an under-acknowledged source of CF-related microorganisms including *PA* and NTM (Al-Momani *et al.*, 2016). In this part of the study, I therefore examined the sputum, gastric juice and PEG tubes from the same CF patients for the existence of NTM. The combined samples from our PEG fed study demonstrated a 43% prevalence of NTM, this is a high rate in comparison to the 15.7% prevalence found in the latest study of CF patients in a regional institution which used exactly the same culture methodology (Preece *et al.*, 2016) and the 12% found in another recent study (Adjemian *et al.*, 2014). These results may simply demonstrate that our patients have more severe CF, but the possibility that a PEG feed tube may increase the chances of CF patients developing NTM infection must also be considered.

NTM are environmental pathogens found across the world, with soil and water, including home water supplies, being the main breeding grounds for such bacteria. As far as I am aware, there is no evidence suggesting that animal-to-human or human-to-human transmission can pass on this infection. Nevertheless, it is thought to be possible that cross-infection can occur between CF patients, which has been supported by indirect studies combining whole genome sequencing and in-depth epidemiology (Bryant *et al.*, 2013). Ricketts *et al.* (2014) have recently investigated a case of suspected human-human transmission of *M. kansasii* between a married couple living in East London, where genetically identical bacteria were found in both partners.

The data in this chapter showed that NTM was present in the gastric compartment and was grown both from gastric juice and also PEG tubes. The removal of the PEG tubes was conducted as part of the patients' care routine and not because they were thought to be infected. The present findings support the idea that NTM organisms are resistant to atypical environments including high temperature and low pH (Bodmer *et al.*, 2000). This indicates an under acknowledged and new possibility that the stomach acts as a potential reserve of active NTM in PEG-fed individuals with CF. It could be hypothesised that this is part of the reason why treatment is unable to eradicate NTM in some patients.

A connection between NTM colonization in the gastric juice and sputum of some CF patients was established in this investigation. NTM was found in the gastric juice and sputum specimens of two patients, and furthermore, molecular methods identified genetically identically strains of NTM present in CF patients' gastric juice and sputum.

These results might therefore demonstrate that bacteria such as NTM could be passed from the lungs to the stomach via coughing and swallowing. Furthermore, NTM was also found in the PEGs and gastric juice of patients who tested negative for NTM in their sputa, and this study was the first of its kind to isolate NTM in gastric samples of two patients. These findings highlight the possible risk of transferring the infection to the lung from the stomach via consistent reflux and aspiration. This should be taken into account and further examined in CF. There are prior studies that point to a relationship between NTM lung disease (including diseases caused by *M. abscessus)* and gastroesophageal disorders, such as that of Koh *et al.* (2007). Reflux and aspiration problems are known to be particularly prevalent in CF, according to Brodzicki *et al.* (2002c).

The fact that there is higher reported prevalence of GORD in non-CF patients with pulmonary *Mycobacterium avium complex* (MAC) than in the general population may thus be significant

(Koh *et al.*, 2007; Thomson *et al.*, 2007). Additionally, a connection between the regular use of acid-suppressive drugs and an increased chance of developing MAC pulmonary disease has been discovered. This is significant for CF patients, who commonly use such drugs, A recent suggestion put forward by Floto *et al.* (2016) for treating NTM emphasised the potential risk of using PPI. Thus, I believe that these findings demonstrate a requirement for further research into the possible role that GOR and acid suppression medication play in the development of NTM in CF patients.

A series of nine questions make up the validated RSI questionnaire instrument used in this study, which is designed to assess extra oesophageal reflux and coughing. All of the patients in our group who had gastric NTM also gained a high RSI domain score for cough.

Fennelly *et al.* (2012) explained how one cough can produce a large amount of bioaerosols, which may contribute to the contraction of infections. Once breathed in, bioaerosols with a critical size range of 1–5 μm have a greater chance of reaching the alveoli than those of more than 5 μm (Wurie *et al.*, 2016). The Mycobacterium bacillus is 0.2–0.5 μm wide and 2–4 μm long, and thus it is possible that bioaerosols measuring 1–5 μm may carry this pathogen, allowing this infection to enter the alveolar macrophages in distal portions of the lungs, according to Wurie *et al.* (2016). Halstrom *et al.* (2015) point out that the inhalation of aerosols from environmental sources such as jacuzzi and showers is known to be a major method of contracting NTM, thus causing lung disease. Since mycobacteria have very hydrophobic cell walls, it is possible that they aerosolise more easily than other bacteria (Halstrom *et al.*, 2015).

It is crucial to be able to identify NTM, especially in CF patients, since NTM are resistant to traditional antibiotics and anti-tuberculosis medication. If identified early, susceptibility testing can be conducted promptly and suitable treatment for PEG management can be established. As far as I am aware, this investigation is the first of its kind to demonstrate how gastric juice and PEG-tube insertion are possible reservoir for NTM infection in CF patients, thus this is a strength of this study. A weakness, however, is that the size of the study group was modest in comparison to those used in prior influential investigations on NTM in CF patients. The placement of the PEG allowed the collection of gastric juice without needing commensal sampling from the upper airways; however, this limits the results to PEG fed CF patients only. Thus, I believe that this study should lead to further investigation using a wider range of patients in multi-centre approaches. In conclusion, I emphasise the need for such further research, due to rising clinical concerns about the growing prevalence of NTM infection in CF patients.

Chapter 6.0: *Pseudomonas aeruginosa* survival in acidic environment

6.1 Introduction

While the stomach was historically considered to be a hostile environment, which did not allow microbial pathogens to survive, it is increasingly recognised that in fact, certain microbes have developed the ability to withstand extreme conditions, surviving in the face of strong detergent, high pH levels, digestive enzymes, and host immune reactions (Sachs *et al.*, 2000). Further, the presence of certain environmental factors in the stomach can trigger upregulation, downregulation or activation of genes which allow the organism to adapt to conditions in their specific location within a host organism (Tamplin, 2005). Over the past twenty years, it has been established that certain microorganism use differential genes expression to allow them to adapt to moderately acid environments (Booth *et al.*, 2002; Cotter and Hill, 2003).

According to the findings presented in Chapters 3 and 4, I isolated microbial organisms in gastric juice samples taken from both non-CF (aspirated through upper GI endoscopy) and CF subjects (through aspiration via PEG tubes), identifying the presence of *PA*, among other genera. These findings indicated the significance of the stomach in providing a potential reservoir for viable pathogenic organisms, and particularly *PA* as the principle pathogenic species in CF lung disease (Al-Momani *et al.*, 2016). *PA* is the major pathogen in the cystic fibrosis (CF) lung. The pathogen is highly prevalent and initial acquisition in most cases leads on to infection on a chronic basis. In light of this, this chapter seeks to determine how far *PA* is capable of subsisting within the stomach, through experimental work to understand what impact the pepsin in gastric juice and its pH level has on the ability of *PA* to grow.

6.1.1 Gastric juice

The main components of gastric juice are water, hydrochloric acid, electrolytes, enzymes, intrinsic factor and mucus (Hall, 2015). The parietal cells secrete hydrochloric acid, which creates an acidity of pH 2 for gastric juice (Hall, 2015). Meanwhile, peptic cells secrete pepsinogen, which turns to pepsin under the action of the hydrochloric acid. Pepsin works to break down both secondary and tertiary protein structures, in preparation for further enzymatic digestion when food reaches the small intestine (Hall, 2015). The peptic cells also secrete gastric lipase, and this acts by breaking down medium- and short-chain triglycerides (Hall, 2015).

Gastric juice also contains amylase, which enters the stomach in saliva mixed with the incoming food. While this enzyme acts on carbohydrate, once it reaches the stomach it is only active for a short time before it stops working in the acid conditions (Hall, 2015). The parietal cells secrete intrinsic factor, which is required for vitamin B12 absorption: a substance which is vital to both the proper functioning of the nervous system and to produce blood cells. Neck cells and surface mucosal cells in the stomach secrete mucus, which provides a protection to the lining of the stomach from the low pH and acts as a diffusion barrier to pepsin (Hall, 2015).

6.1.2 Gastric juice as a bactericide

It is considered that the stomach acts as a barrier for bacteria due to a bactericidal action from pepsin and HCl, with little assistance from the remaining gastric juice components (Wilder-Smith and Merki, 1992). Further, the action of the two relevant components in this regard is not easily divisible, given that the action of the enzyme is reliant on the acid conditions provided by the HC1. Moreover, gastric acid is found across mammalian, reptilian, amphibian and piscine (related to fish) species, suggesting that this characteristic has been favoured in natural selection (Koelz, 1992). The roles identified for gastric acid are: denaturing protein and activation of pepsinogen; enhancing the ability to absorb iron and calcium from food; and preventing microbial pathogens from entering the intestines (Chu and Schubert, 2013).

The parietal cells secrete hydrochloric acid into the compartment of the stomach. Acid is produced by the proton pump, or H^+,K^+-ATPase, through the replacement of luminal K^+ with cytoplasmic H^+ (Driel and Callaghan, 1995; Chu and Schubert, 2013). Histamine is the primary stimulus for gastric acid to be secreted, and this histamine is secreted by enterochromaffin-like cells reacting to gastrin as a stimulus (Lindström *et al.*, 2001).

Gastric acid is held to be the principal component in gastric juice with a bactericidal function, with little contribution being observed from the remaining components which make up this liquid (Wilder-Smith and Merki, 1992). Further, a large body of research points to the failure to secrete gastric acid effectively as creating conditions which allow various infections to occur (Martinsen *et al.*, 2005). On the other hand, there is also research evidence indicating that pH conditions of less than 2 are needed in order to kill bacterial microbes, and this condition is seldom reached for a sustained duration in the stomach. This is particularly so when eating, and this is the time when the stomach receives the largest numbers of bacterial organisms (Dressman *et al.*, 1990). When investigating pH fluctuations in individuals consuming the diet, pHs of 5.5

have been recorded while eating, only decreasing to 1.5 between three and four hours post-meal, assuming that average time for the stomach to empty (3 hours) is taken (Coldewey et al., 2007). These fluctuations in pH may allow pathogenic bacteria the chance to breach the protective barrier of the stomach.

There appears to be a key role for gastric acid in action to prevent bacteria from overgrowing both in the stomach and the upper section of the small intestines (Stockbruegger, 1985; Howden and Hunt, 1987), and this is supported by studies finding a high bacteria count in the intragastric area for patients who had been treated with proton pump inhibiting drugs or histamine-2 receptor antagonists (Williams and McColl, 2006; Herzig *et al.*, 2009) .

Qualitatively altered microflora in the stomach for those who experience hypochlorhydria (low level of gastric acid) appear reliant on how longstanding and how severe the condition is (Williams, 2001; Martinsen *et al.*, 2005). Older research into stomach contents in chronically ahydrochloric individuals revealed a high frequency of coliforms, while more recent studies with individuals being treated with acid inhibitors and who had experienced hypo- or a-hydrochloria for comparatively less time, found principally gram-positive microbial overgrowth, such as might be seen in the oropharyngeal and mouth areas (Dellipiani and Girdwood, 1964; Williams, 2001).

Pepsin is considered the most significant enzymatic secretion in the stomach. This proteolytic enzyme is generated firstly as pepsinogen which the peptic cells secrete, before hydrochloric acid converts it to pepsin (Hall, 2015). Generally, the gastric juices contain from 0.5 to 1 mg/ml-1 of pepsin (Balan *et al.*, 1996). Research investigating how pepsin was impacted by acidity levels found that it was active up to 70% of maximum levels in pH 4.5 conditions (Johnston *et al.*, 2007). Meanwhile, for pepsin to stop being active altogether as a result of non-reversible protein denaturation, a pH of 5.5 or more is required (Pearson and Parikh, 2011a).

6.2 Methods

6.2.1 Materials

All chemicals and reagents were obtained from Sigma-Aldrich, Poole, Dorset, UK, unless otherwise specified. To reach pH targets ranging from 2.5 to 7.4, 1 Molar HC1 was added to PBS, and this then received porcine pepsin to create a concentration of between 0.5 and 1.5 mg ml^{-1}.

6.2.2 Strains of bacteria and cultures used.

Clinical scientist Mrs Audrey Perry of the Freeman Hospital, Newcastle upon Tyne NHS Trust and University of Northumbria supplied four separate *PA* strains. The strains were S27, S33, S34 and PA14. S33 and S34 were isolated from 2 gastric juice samples in our patient cohorts. These are not referenced in any paper but were chosen as they were known biofilm producers from a previous study (Chapter 4). Isolate reference PA14 and S27 are from the International PA panel (De Soyza *et al.*, 2013). Columbia blood agar was then used for subculturing the strains, and the subcultures were incubated at 37°C in the presence of oxygen until the next day.

6.2.3 Establishing effects from pepsin, human gastric juices and acidity level.

Following incubation, the *PA* cultures were used to produce standardised suspensions of the microbes. They were suspended in 2ml PBS, and a densitometer was used to reach 0.5 McFarland standard comparable density ($1.5 * 10^8$ cfu/ml).

Inoculum 10ul was pre-incubated with 1 ml solution containing the test substances, with 20 µl being taken at durations of 0 minutes, 5, 30, 60 and 120 minutes. These underwent dilution in 1.98ml PBS with a 7.4. pH. 50 µl of the neutralised suspension of bacteria was taken for plating in blood agar, and this was done in triplicate, to give about 50-60 *PA* colonies. Viable counts were determined after culturing at 37°C for 24 h under aerobic conditions.

6.2.4 Statistical analysis.

Data were statistically analysed via analysis of variance (ANOVA), values of P equal or less than 0.05 were regarded as having statistical significance.

6.3 Result

6.3.1 Acid-tolerance experiments

Experimental work investigating tolerance of acidity for *PA* in PBS used pH conditions of 7.4 as a control with which to compare the results. When pH levels were 2.5, none of the strains of *PA* tested in the PBS survived incubation after the 15-minute point, while when pH was increased to 3, up to three-quarters of bacteria were no longer viable by the same point, and none survived to 30 minutes. When tested at a pH of 3.5, approximately 55% of *PA* samples had died by the 30-minute point, rising to 80% at 1 hour and between 90 and 95% at 2 hours. Once pH had been raised to 4 however, just 13% of the bacteria had died at the 2-hour point, and at the higher pH levels of 5 and 6, any difference with the numbers in the control sample was found to be statistically insignificant (see Figure 6.1).

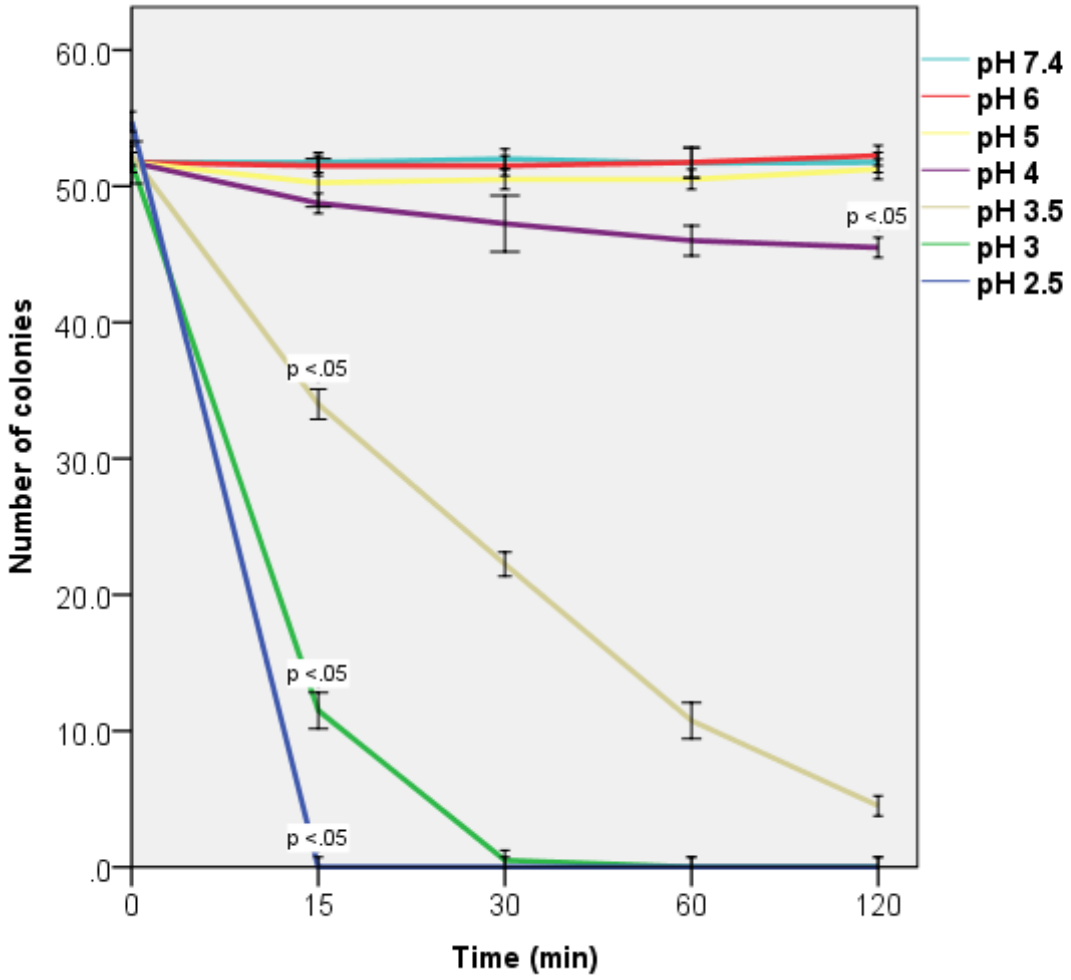

Figure 6-1: Effect of different pH level (2.5-7.4) on subsequent growth of *PA*. Compared to the *PA* survival at pH 7.4 (control), there was a statistical significant drop in the number of *PA* colonies at pH 2.5-4, while there was no significant difference in the number of colonies at pH >4. At 15 minutes, there was a significant decrease in the number of colonies at pH 2.5 and pH 3 compared to the control (pH 7.4) (T-value = 37.60, P-Value = 0.000, DF = 4) and pH 3.5 (T-value = 20.79, P-value = 0.000, DF = 5). At 30 minutes, there were statistically significant decreases in the number of colonies compared to the control (pH 7.4) at pH 3 and 3.5 (T-value = 103.00, P-value = 0.000, DF = 5). At 60 minutes, there were statistically significant decreases in the number of colonies at pH 3 and pH 3.5 (T-value = 41.96, P-palue = 0.000, DF = 4). At 120 minutes, there were statistically significant decreases in the number of colonies compared to the control (pH 7.4) at pH 3.5 (T-value = 64.81 P-value = 0.000 DF = 5) and pH 4 (T-value = 13.17, P-value = 0.000, DF = 5).

6.3.2 Effects of pepsin

Incubating *PA* in PBS containing porcine pepsin at various levels (0.5, 1 and 1.5 mg ml^{-1}) and a range of pH levels demonstrated that all 4 *PA* strains were sensitive to proteolysis. At a pH level of 3, the samples displayed a large decrease in viable microbes (number of colonies), and the majority had died by 15 minutes incubation, as shown in Figure 6.2. There was also large loss of bacteria where pH was 3.5, and at 1 and 1.5 mg of porcine pepsin, all had been killed by 60 minutes incubation, whereas for 0.5 mg, this had been achieved by 120 minutes, as shown in Figure 6.3.

For the samples incubated at a pH of 4, porcine pepsin had a demonstrable impact on *PA* strains by the 15-minute incubation point. Approximately 15% had died at 15 minutes where pepsin was present, and 30% at the 120-minute point, while in the non-pepsin sample, just 12% had died at this point, as shown in Figure 6.4. There was a small but insignificant effect of pepsin with pH 5-7.4 (Figure 6.5).

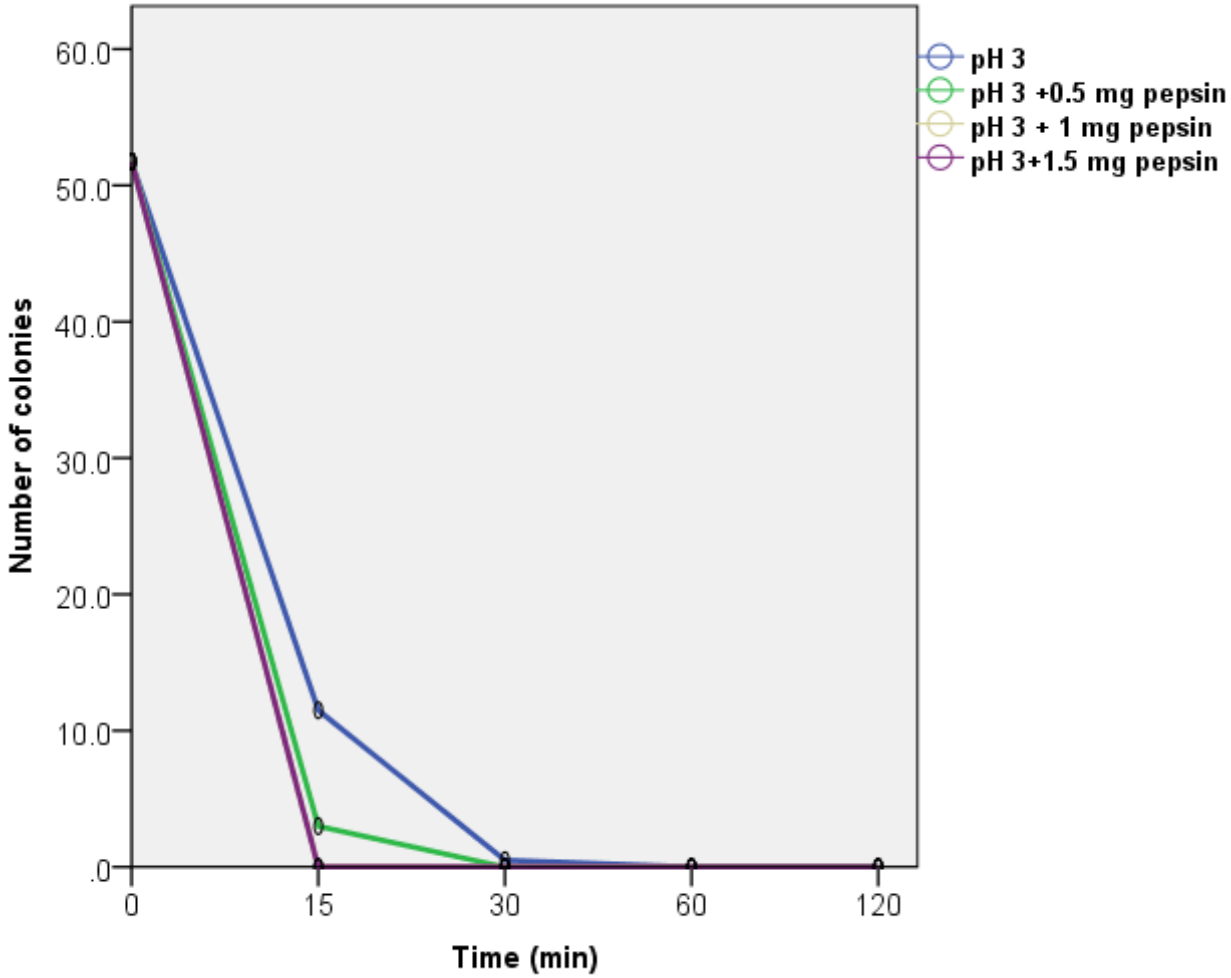

Figure 6-2: Effect of pH and pepsin (0.5-1.5 mg/ml) on *PA* viability as indicated by colony counts. There was a significant drop in the number of colonies when pepsin (at concentrations of 0.5-1.5mg ml^{-1}) added to PBS with pH 3 (T-value = 27.71, P-value = 0.000, DF = 6).

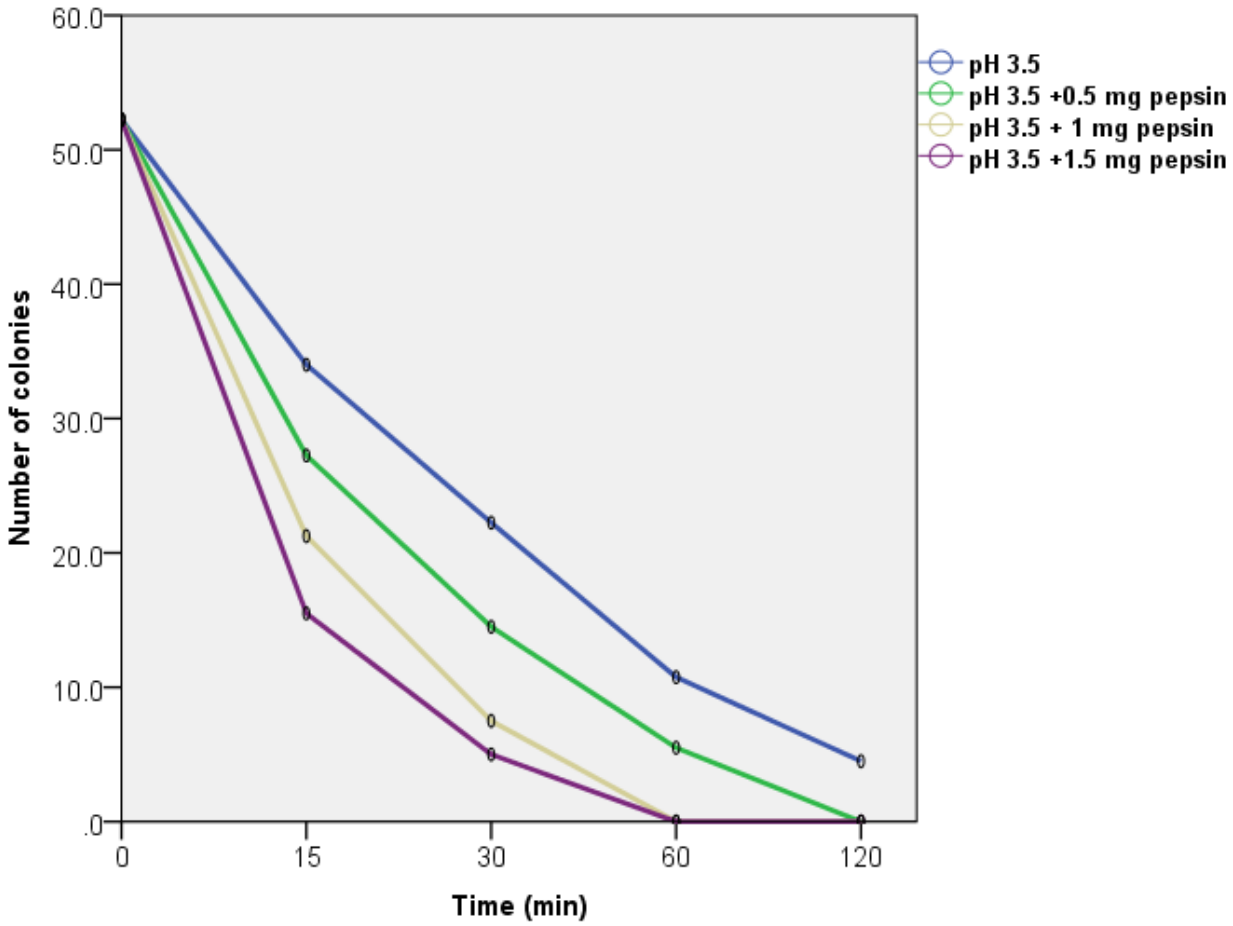

Figure 6-3: Effect of pH 3.5 and pepsin (0.5-1.5 mg/ml) on *PA* viability as indicated by colony counts. There was a statistically significant drop in the number of colonies when pepsin was added to the PBS at all pepsin concentrations used (except at 120 minutes at 0.5 mg/ml). At 15 minutes, there was a statistically significant drop in the number of colonies at all pepsin concentrations compared to the samples without pepsin (0.5 mg: T-value = 11.55, P-value = 0.007, DF = 2), 1mg: T-value = 21.89, P-value = 0.002, DF = 2) and 1.5mg: T-value = 31.88, P-value = 0.001, DF = 2). At 30 minutes, there were statistically significant drops in colony counts at all pepsin concentrations compared to the number of colonies without pepsin (0.5mg: T-value = 13.01, P-value = 0.006, DF = 2). 1 mg: T-value = 43.96, P-value = 0.000, DF = 3) and1.5 mg: T-value = 28.70, P-value = 0.001, DF = 2). At 60 minutes, there was statistically significant decrease in the number of colonies at pepsin concentration 0.5 mg (T-value = 41.05, P-value = 0.001, DF = 2). There was no statistically significant difference in colony counts with 0.5mg/ml pepsin compared to the number of colonies without pepsin at 120 minutes.

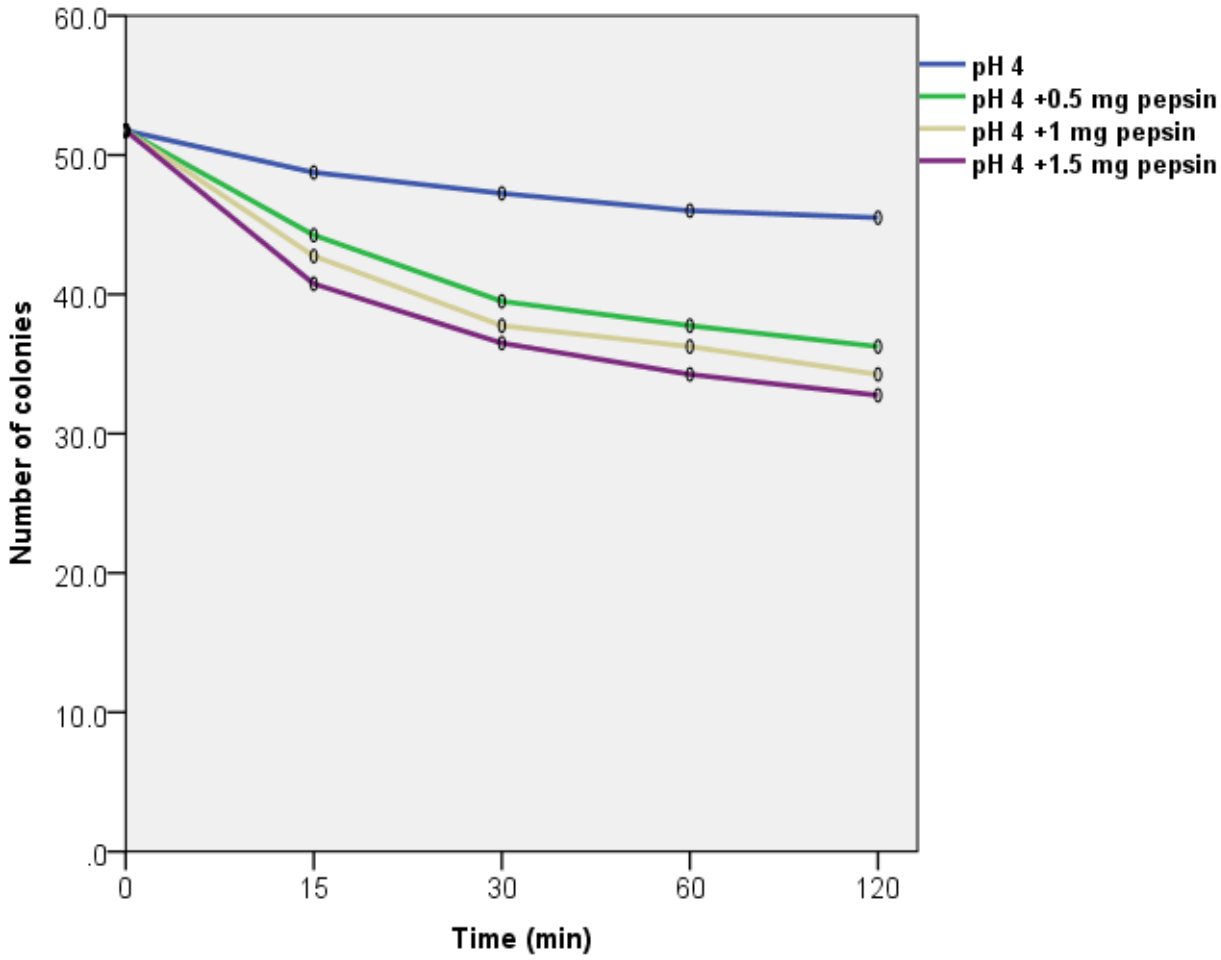

Figure 6-4: Effect of pH 4 and pepsin (0.5-1.5 mg/ml) on *PA* viability, as indicated by colony counts. There was a statistically significant drop in the number of colonies when pepsin was added to the PBS at all concentrations and at all time points. At 15 minutes, there was a significant decrease in the number of colonies when was pepsin added at all concentrations compared to incubating the *PA* without pepsin (0.5mg/ml: T-value = 10.28, P-value = 0.009, DF = 2). 1mg: T-value = 14.01, P-value = 0.005, DF = 2, at 1.5mg: T-value = 18.43 P-value = 0.003, DF = 2). At 30 minutes (0.5mg: T-value = 25.09, P-value = 0.002 , DF = 2, at 1mg/ml :T-value = 58.18, P-value = 0.000, DF = 4, at 1.5mg: T-value = 34.74 P-value = 0.001, DF = 2). At 60 minutes (0.5mg: T-value = 13.93, P-value = 0.005, DF = 2). 1mg/ml: T-value = 16.09, P-value = 0.004, DF = 2, at 1.5 mg/ml (T-value = 19.41, P-value = 0.003, DF = 2). At 120 minutes (0.5mg/ml: T-value = 27.33, P-value = 0.000, DF = 3), at 1mg/ml: T-value = 33.27, P-Value = 0.000, DF = 3). At 1.5 mg/ml: T-value = 40.85, P-value = 0.001, DF = 2).

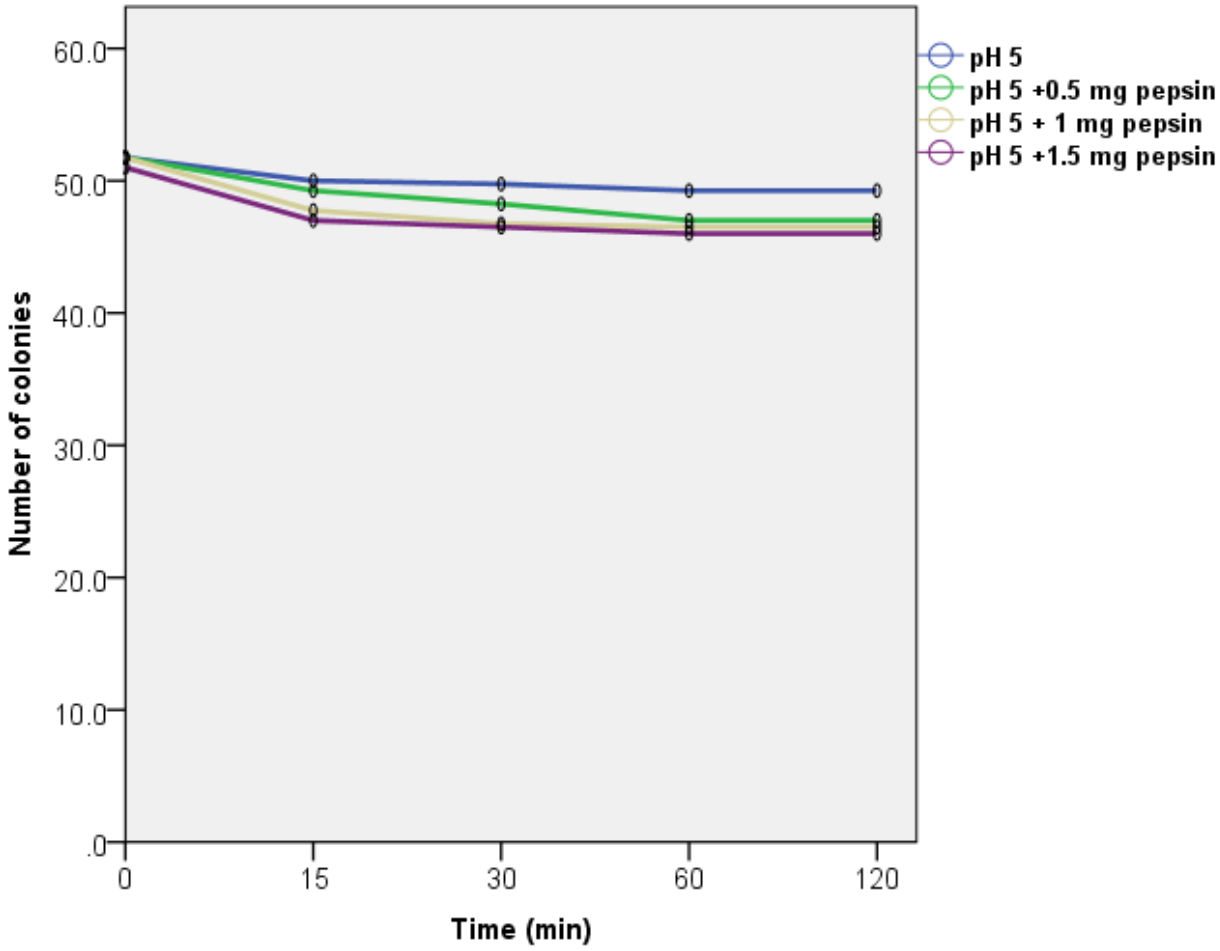

Figure 6-5: Effect of pH 5 and pepsin (0.5-1.5 mg/ml) on *PA* viability as indicated by colony counts. There was no statistically significant drop in colony counts when pepsin was added to the PBS at any concentration used.

6.4 Discussion

The work described here demonstrates a significant bactericidal impact for *PA* in acidic conditions at or lower than pH 3, while between pH 3.5 and 4, this impact was less, and at higher pH levels, the impact was small. It is also shown that where pepsin exists alongside acid conditions, *PA* are more effectively destroyed. Within this, it is interesting to note that pepsin's effect on *PA* had an association with acidity levels, as the action of pepsin was seen between pH 3 and pH 4, but was far less in evidence at higher pH levels, as proteolysis was reduced.

While research into acidity levels and their impact on bacterial organisms is limited, previous work demonstrates an association between pH survival in *Serratia marcescens,* in which 90% attrition was achieved before 30 minutes' exposure in conditions of pH 2, but required a minimum of 1 hour at pH 3, while colonies were unaffected by acidity of pH 4-7 (Borriello *et al.*, 1985; Waterman and Small, 1998). This is similar to the results of this experiment, which finds that survival within the stomach is possible for *PA* at a pH level of >3.5, and further, that with the action of pepsin, approximately 2 hours were required for PA to be destroyed at acidity levels of pH 3.5.

Thus, the effectiveness of the stomach's bacterial barrier function relies upon pH remaining low for a minimum of between 15 and 30 minutes. At the same time, food entering the stomach, and bringing bacteria with it, lowers the acidity of gastric contents considerably, bringing pH to between 3 and 4.5. Further, pathogens which are bound to components of the food in the stomach find some protection from acidity in this way (Rosina, 1982), Pepsin concentration is a second essential element in bactericidal activity in the stomach, with effectiveness much more notable at levels >1.0 mg ml^{-1} compared with 0.5 mg ml^{-1} in my data. Further, within a live host, the number of minutes taken to destroy *PA* is a significant consideration, and it has been demonstrated here that pepsin may require 120 minutes to achieve this in conditions of pH 3.5 and at a concentration of 0.5 mg ml^{-1}. By contrast, at the same acidity but with a pepsin concentration of 1.0 mg ml^{-1}, just 60 minutes is required.

Gram-positive bacterial organisms possess acid resistance through a range of strategies. Frequently, such solutions involve forming biofilms and altering density, as seen in *Streptococcus mutans* (Cotter and Hill, 2003). *Listeria monocytogenes* also becomes more acid resistant at a different density, and in biofilms on stainless steel has been seen to be less affected by acetic acid (Oh and Marshall, 1996). In light of this, a biofilm formation of *PA* might have greater resistance to low pH levels than seen in the experiment described in this chapter. In

previous chapters, isolation of biofilm-forming *PA* was achieved in samples of gastric juice at a pH of 3.

While this experiment is unique to my knowledge in investigating the impact of acidity levels on *PA*, earlier work concerning *E. coli* O157: H7 demonstrated that it can tolerate acidity of pH 3-3.5 during its exponential growth phase (Arnold and Kaspar, 1995). It was further shown that stationary-phase or biofilm phase gram-negative microbes showed greater tolerance to acidity (Arnold and Kaspar, 1995). Bacteria undergo physical adaptation on entering the stationary stage to allow them to resist stressors during this comparatively inactive time (Angelichio *et al.*, 2004).

As already discussed, the majority of individuals with CF are treated by gastric acid suppression, frequently in response to symptoms of GOR, which has a high prevalence in this patient group. Moreover, CF patients experiencing malnutrition often take acid inhibition medication such as proton pump inhibitors, based on the logic that this might enhance pancreatic enzyme replacement therapy, which has been the mainstay of the modern CF patient care that has led to better life expectancy in those with CF (Proesmans and De Boeck, 2003; Littlewood *et al.*, 2006). According to the CF Foundation Patient Registry Report (CFFPR) 2011, from CF patients in the USA, 70.1% were being treated with medication to block gastric acid, while Com *et al.* (2014) report up to 100% of patients being treated for certain treatment centres. Based on the findings of the current experiment, the survival of *PA* is possible within the gastric juices of CF individuals being treated with acid suppressants.

The pH levels I have found in CF gastric juice (Chapters 3 and 4) are consistent with the stomach providing an environmental niche that could allow *PA* to survive. The pH range in the CF patients was between 2 and 6, with 50% having gastric juice with pH equal to or more than 4. Further, a number of other variables can act to promote hypochlorhydric conditions, where pH is between 4 and 7, and achlorohydric conditions, at a pH of 7. Among these factors are malnutrition and atrophic gastritis (Tennant *et al.*, 2008; Agréus *et al.*, 2012). The results reported here therefore do not exclusively apply to patients being treated to inhibit gastric acid.

To conclude, this experimental chapter demonstrates the inability of *PA* to remain viable at pH levels of 3 or below, and shows that where pepsin is present, *PA* is destroyed more quickly as long as pH is between 3 and 4. Pepsin's effect on *PA* is affected by its concentration and also by acidity, although where conditions are only moderately acidic, survival is possible for *PA.* Raising pH within the gastric lumen has the effect of removing the stomach's bactericidal

barrier and therefore, protection from *PA* colonisation. Proton pump inhibitors could change the gastric microflora and promote overgrowth of microbial organisms, chiefly meaning that bacteria found in the mouth can then persist within the stomach, instead of being destroyed by gastric acid (Williams and McColl, 2006).

Chapter 7.0: Effect of bile acids on *P. aeruginosa* growth and behaviour

7.1 Introduction

Individuals with cystic fibrosis produce thickened mucus in the lungs as a result of impaired epithelial ion transport, and this greatly increases vulnerability to lung infection by pathogenic microbes and leads to impaired lung function (Jayaraman *et al.*, 2001). The principle pathogenic agent linked to CF is *PA*, and chronic infections have proven ineradicable once established in this group of patients, despite advances in treatments to combat microbial infection (Goss and Burns, 2007). The reason for this appears to be mainly that such microbes are capable of behavioural alterations during chronic colonisation, becoming more antibiotic resistant, producing lower toxin levels and moving to a biofilm phase (Moreau-Marquis *et al.*, 2008). In this situation, the chronically *PA* infected CF lung leads to progressive and fatal loss of lung function (Moreau-Marquis *et al.*, 2008). These alterations seen in chronic infections of *PA* are different to mechanisms that occur during acute colonisation, in which *PA* may lead to pneumonia, overcoming the protective mechanisms of the lung and spreading through the blood.

The leaking of the gastric contents into the oesophageal tract leads to GOR. Refluxed gastric content frequently contains bile, which includes bile salts, conjugated bile acids and bile acids, which has previously entered the stomach via the pylorus in a further reflux process from the duodenum to the stomach (duodeno-gastric reflux). It is a physiological phenomenon, and can occur both on an empty stomach and in subjects who have eaten (Pearson and Parikh, 2011b). In samples from participants receiving routine endoscopy of the upper GI, gastric juices were found to contain between 0.01 and 10 mmol/l (median = 0.055 mmol/l), measured through an enzymatic approach utilising 3α-hydroxydehydrogenase (Pearson and Parikh, 2011b; Ali *et al.*, 2013).

Bile refluxing into the oesophagus in CF patients, as found in as many as 4 in 5 patients, occurs mainly due to delay in emptying of the stomach, damage to the lower oesophagus sphincter, and on occasion due to physiotherapeutic treatment of the chest (Blondeau *et al.*, 2010). In fact, it is possible that aspirated bile reflux is even more prevalent than this, as where this occurs without symptoms; it will remain unidentified by clinicians. Thus, bile acids (BAs) transferred through GOR have been observed within both sputum and bronchoalveolar lavage (BAL), with

concentration levels measured at between 0.4 μM (Pauwels *et al.*, 2012) and 32 μM (D'Ovidio *et al.*, 2005).

A study by D'Ovidio *et al.* (2005) reported an association between higher concentrations of BAL bile acids and interleukin 8, alveolar neutrophilia and the presence of fungi and bacterial organisms. Further, evidence linking aspirated bile with the presence of *PA* in the airways has been observed in lung transplant recipients (Vos *et al.*, 2008), as well as in research conducted retrospectively with juvenile CF patients (Palm *et al.*, 2012). There is therefore a possibility presented that bile aspirated through GOR might have an impact on the microbiome of the lungs, and be a factor in the association between GOR and worsened lung disease.

Recently, a number of researchers have presented evidence of a microbiome which is distinctive to CF patients, differing from those without CF (Sibley and Surette, 2011; Blainey *et al.*, 2012). Although it is not yet clear what leads this differentiation in microbiome to exist, research proposes a possible role for aspiration of bile in this: especially with regard to microbial diversity and chronic infection (Reen *et al.*, 2012).

I have previously isolated *PA* from gastric juice and sputum from CF patients and these were sometimes identical strains at the molecular level. The presence of *PA* in the stomach or in the airway may be influenced by bile acids, whether highly concentrated within the gastric lumen or more diffusely within the respiratory passages. The objective of the current experimental work is to evaluate the potential influence of bile acid presence within the two locations on *PA*, as the main infective agent which increases mortality and morbidity for patients with CF.

7.2 Methods

7.2.1 Materials

All chemicals and reagents were obtained from Sigma-Aldrich, Poole, Dorset, UK unless otherwise specified. Four bile acids present in humans, namely; lithocholic acid (LCA), cholic acid (CA), doxycholic acid (DCA) and chenodeoxycholic acid (CDCA) were used. The addition of bile salts to Tryptone Soy Broth (TSB) was made at two concentration scales: from 0.3 mmol/l - 20 mmol/l, in line with gastric concentrations, and from 9.4 µmol /l - 150 µmol/l, as typical of BAL in CF patients.

7.2.3 PA strains and culture conditions

Similar to previous chapter (Chapter 6), four *PA* strains were used in this experiment. The strains were S27, S33, S34 and PA14. S33 and S34 were isolated from 2 gastric juice samples in our patient cohorts. These are not referenced in any paper but were chosen as they were known biofilm producers from a previous study (Chapter 4). Isolate reference PA14 and S27 are from the International PA panel (De Soyza *et al.*, 2013). Subculturing of the *PA* was conducted on Columbia blood agar, and cultures underwent aerobic incubation at 37°C for 24 hours.

7.2.4 Biofilm assay: microtiter-plate test

For the biofilm assay (Figure 7.1), cultures of the four *PA* strains were used to produce standardised suspensions. This was done using Tryptone Soy Broth (TSB), using 250µl aliquots for each suspension, to which was added 20 µl aliqouts of the bacteria in suspension with TSB and bile acids, leaving 270µl in each well in the microtiter well. Each test was performed in triplicate. In addition, positive and negative control wells were used, with positives containing *PA* and TSB with the omission of bile salts, and negatives containing TSB only.

This preparation was followed by aerobic incubation at 37°C for 24 hours, before aspirating the wells and washing each in sterile physiological saline (250µl) 3 times. Vigorous shaking of the plates was carried out for the purpose of removing any bacteria which had not adhered. Those microbes which were left underwent fixing using 99% methanol, at 200 µl for each of the wells.

The plates were left for 15 minutes, before emptying them and letting them dry. Five minutes' staining was then carried out for each plate, using 2% Hucker crystal violet, suitable for Gram-stain use, at 2 ml for each of the wells. Excess staining material was removed through rinsing plates with flowing water from the tap. The plates were then allowed to dry once more, and resolubilisation of the cell-bound dye was done using 33% (v/v) glacial acetic acid, at 160 µl for each of the wells.

Optical density (OD) measurement was carried out for all the wells via an automated reader, the ICN Flow Titertek Multiscan Plus. Readings were taken at three stages: firstly, prior to incubating the samples (OD 600nm); secondly, post-incubation for growth assessment (OD 600 nm); and finally, once the biofilm assay had been completed (OD 570nm). The ratio selected was 570/600, for normalisation of the measure of biofilm formed against growth of bacteria.

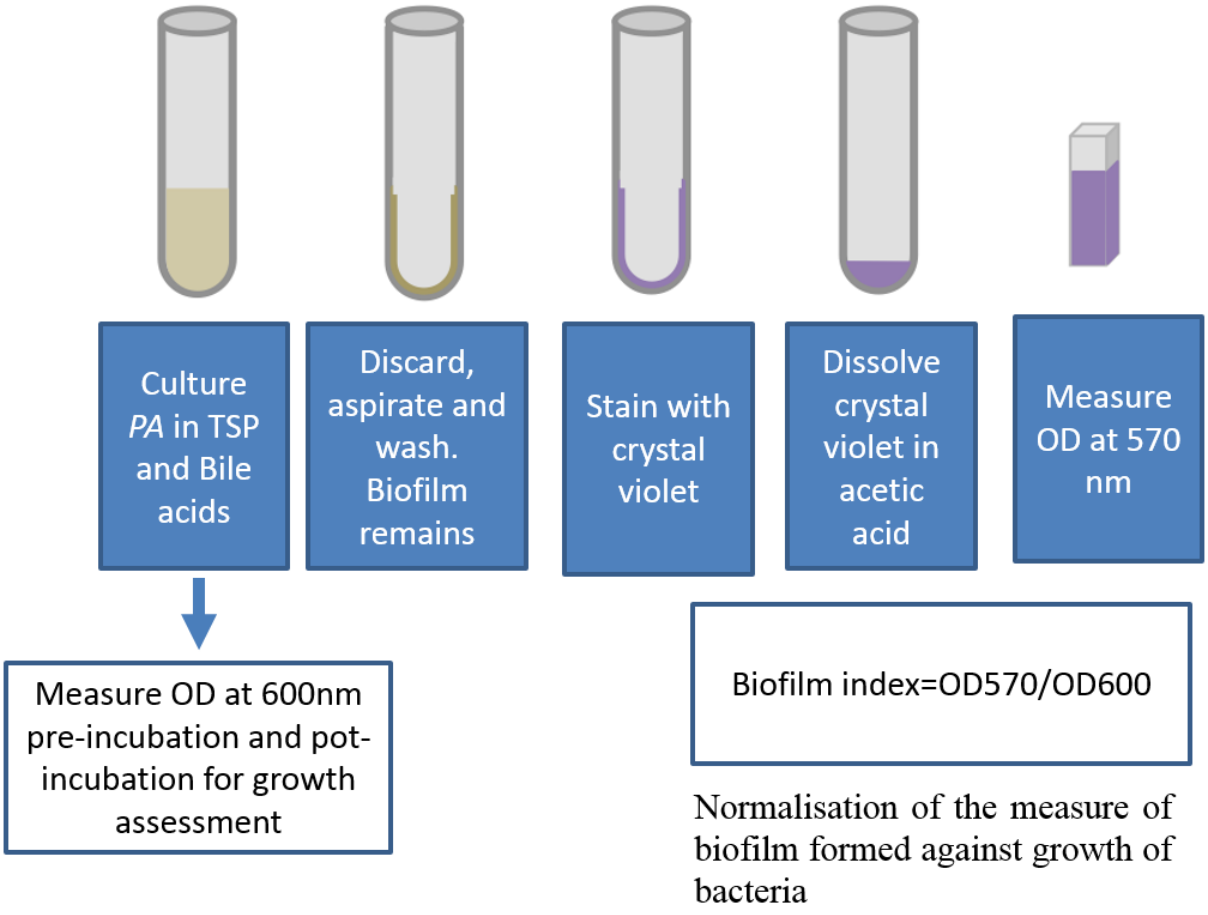

Figure 7-1: Biofilm microtiter-plate test assay.

7.2.5 Statistical analysis.

ANOVA was used to examine statistical variations across the microtiter-plate test data for samples containing bile salts and those which did not. The Tukey test was employed for comparison of OD measurements from microtiter-plate testing for samples containing bile and those which did not. Statistical significance was assumed for all findings with a p value equal to or smaller than 0.05.

7.3 Results

7.3.1 Impact of bile acids on PA growth at concentrations from 0.3mmol/l - 20mmol/l

Incubation of *PA* in the presence of four different bile acids in concentrations from 0.3 to 20 mmol/l had a negative impact on *PA* growth. Lithocholic acid reduced growth strongly in two of the strains of *PA* (S33 and S34) with a 1.25 mmol/l concentration or above, while the remaining two strains were impacted when the concentration reached 5 or above (see Figure 7.1). Incubation with DCA reduced growth significantly for two of the strains (S33 and S34), at the lowest concentration of 0.3 mmol/l, while for the remaining two strains, growth was reduced significantly when DCA concentrations were over 1.25 mmol/l (see Figure 7.2). Both CDCA (see Figure 7.3) and CA (see Figure 7.4) concentrations of over 2.5mmol/l and 5 mmol/l reduced significantly the growth of *PA*, respectively. There was variability among different strains in term of bile tolerance.

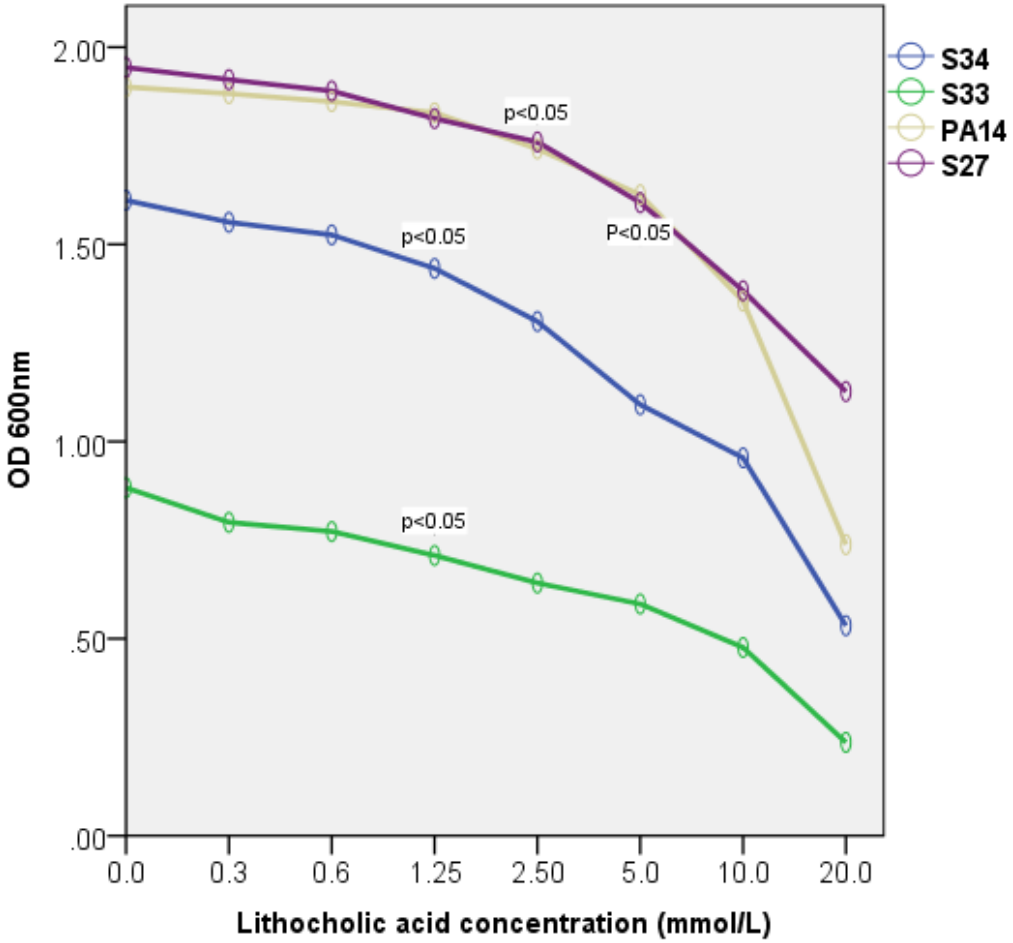

Figure 7-2: Impact of lithocholic acid on *PA* growth. Lithocholic acid concentration is shown on the x-axis, and OD measurement at 600nm is given on the y-axis. P-values indicate concentrations at which the growth of *PA* was significantly reduced (see Appendix 2A). Overall, a statistically significant decrease in growth emerged across the four strains at 10 mmol/l and above.

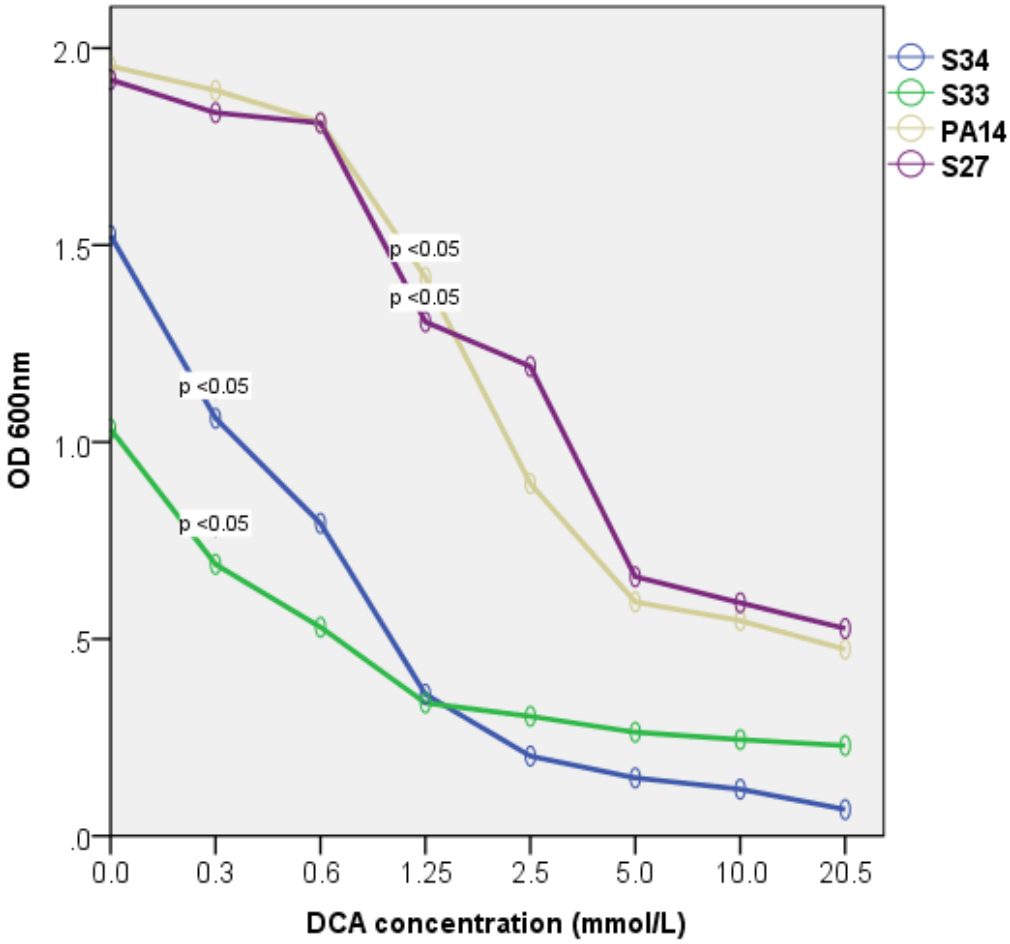

Figure 7-3: Impact of DCA on *PA* growth. DCA concentration is shown on the x-axis, and OD measurement at 600nm is given on the y-axis. P-values indicate concentrations at which the growth of *PA* was significantly reduced (see Appendix 2B). Overall, statistically significant decrease in growth emerged across the four strains at 1.25 mmol/l and above.

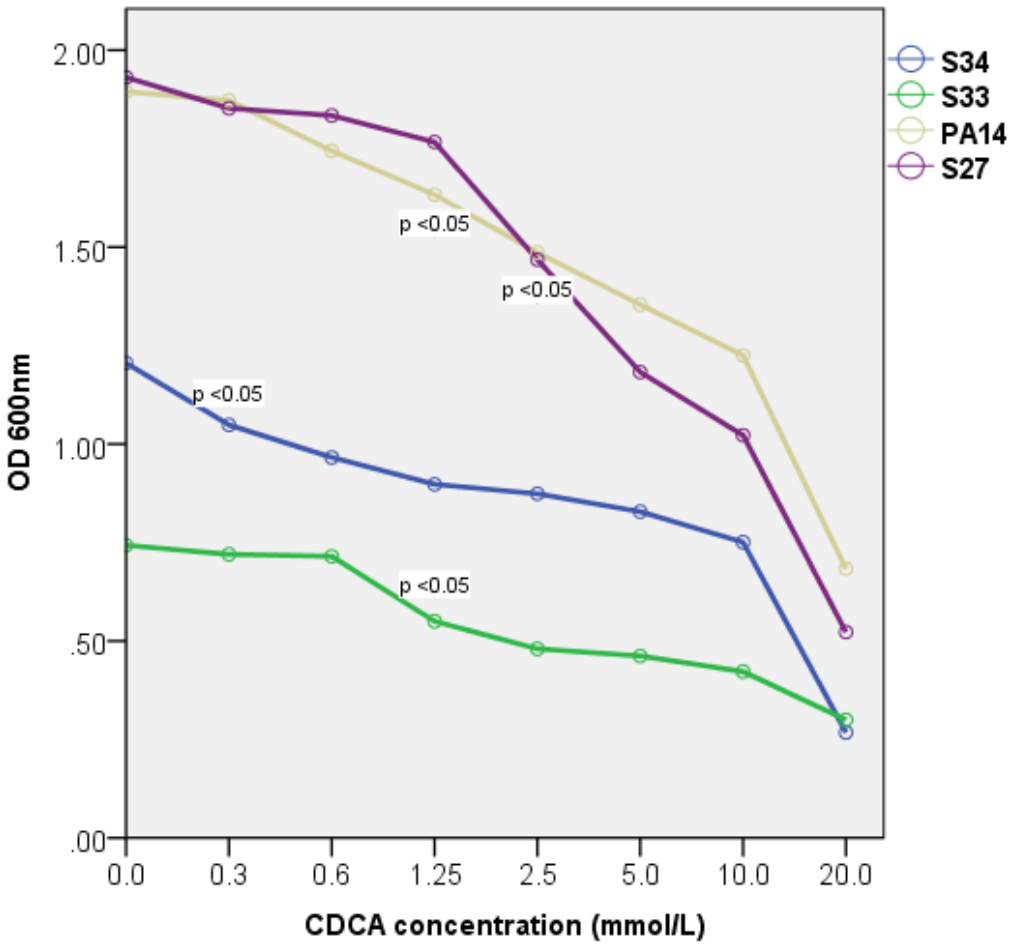

Figure 7-4: Impact of CDCA on *PA* growth. CDCA concentration is shown on the x-axis, and OD measurement at 600nm is given on the y-axis. P-values indicate concentrations at which the growth of *PA* was significantly reduced (see Appendix 2C). Overall, statistically significant growth decreases emerged across the four strains at 2.5 mmol/l and above.

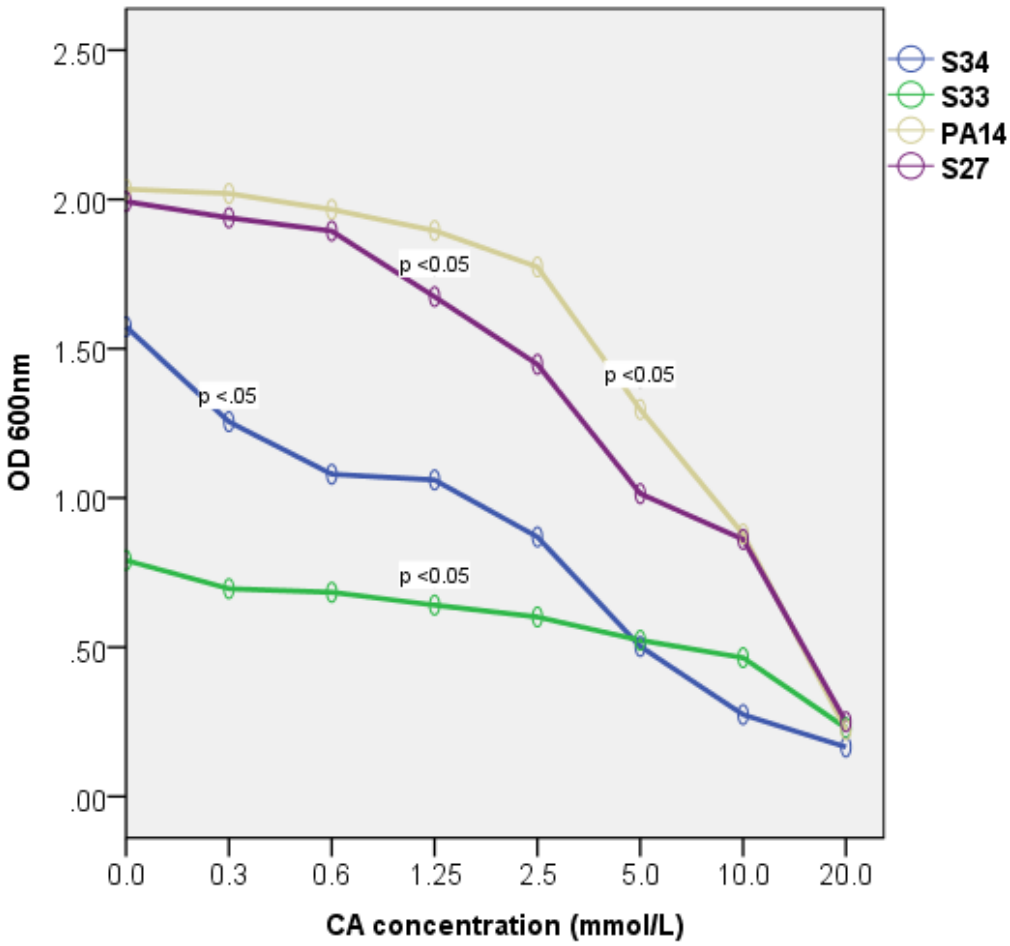

Figure 7-5: Impact of CA on *PA* growth. CA concentration is shown on the x-axis, and OD measurement at 600nm is given on the y-axis. P-values indicate concentrations at which the growth of *PA* was significantly reduced (see Appendix 2D). For all strains, statistically significant growth decreases emerged across the four strains at 5 mmol/l and above.

7.3.2 Impact of bile acids on PA biofilm formation at concentrations from 0.3 mmol/l – 20 mmol/l

Increased formation of biofilm was shown for each strain on incubation at varying bile acids concentrations. Further, statistically significant increases in biofilm formation were observed at 20 mmol/l for all bile acids (Figure 7.5 -7.8).

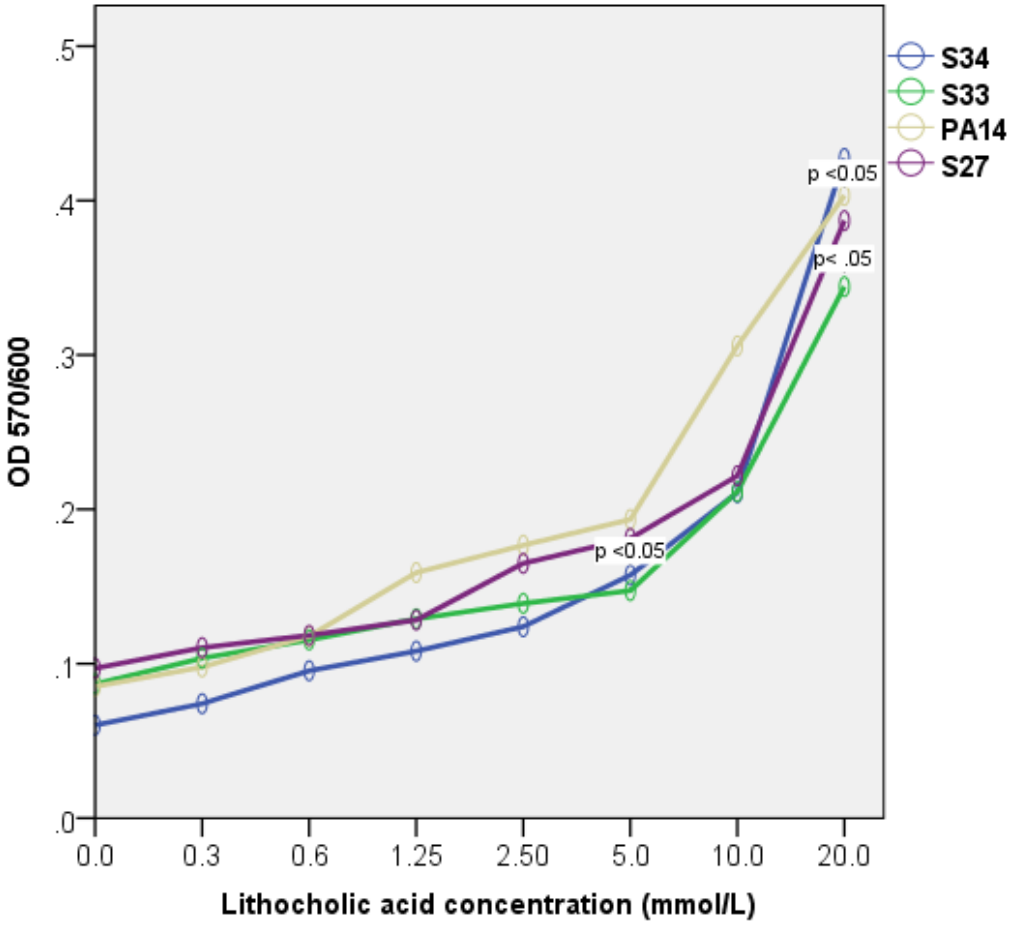

Figure 7-6: Impact of lithocholic acid on *PA* biofilm formation for concentrations of 0-20mmol/l. Lithocholic acid concentration is shown on the x-axis, and OD measurement at 570/600nm is given on the y-axis. P-values indicate concentrations at which the rise in biofilm formation becomes significant in statistical terms (see Appendix 2E). For *PA* S34 strain, there was statistically significant increase in biofilm formation at 5 mmol/L of Lithicholic acid concentration. A statistically significant increase in biofilm formation emerged at 20 mmol/l for *PA* S33 and PA14 strains. There was no significant increase in biofilm formation at any concentrations for the S27 strain.

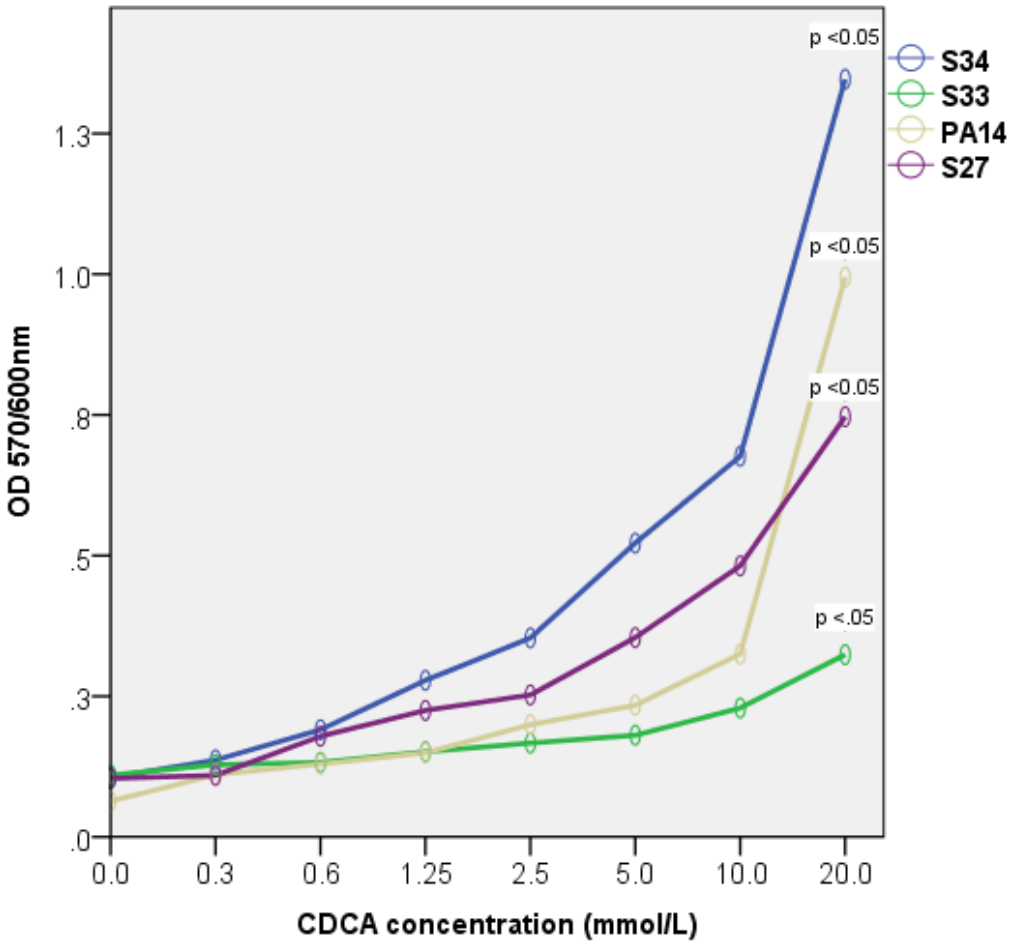

Figure 7-7: Impact of CDCA on *PA* biofilm formation for concentrations of 0-20mmol/l. CDCA concentration is shown on the x-axis, and OD measurement at 570/600nm is given on the y-axis. P-values indicate concentrations at which the rise in biofilm formation becomes significant in statistical terms (see Appendix 2F). For all strains, a statistically significant increase in biofilm formation emerged at 20 mmol/l.

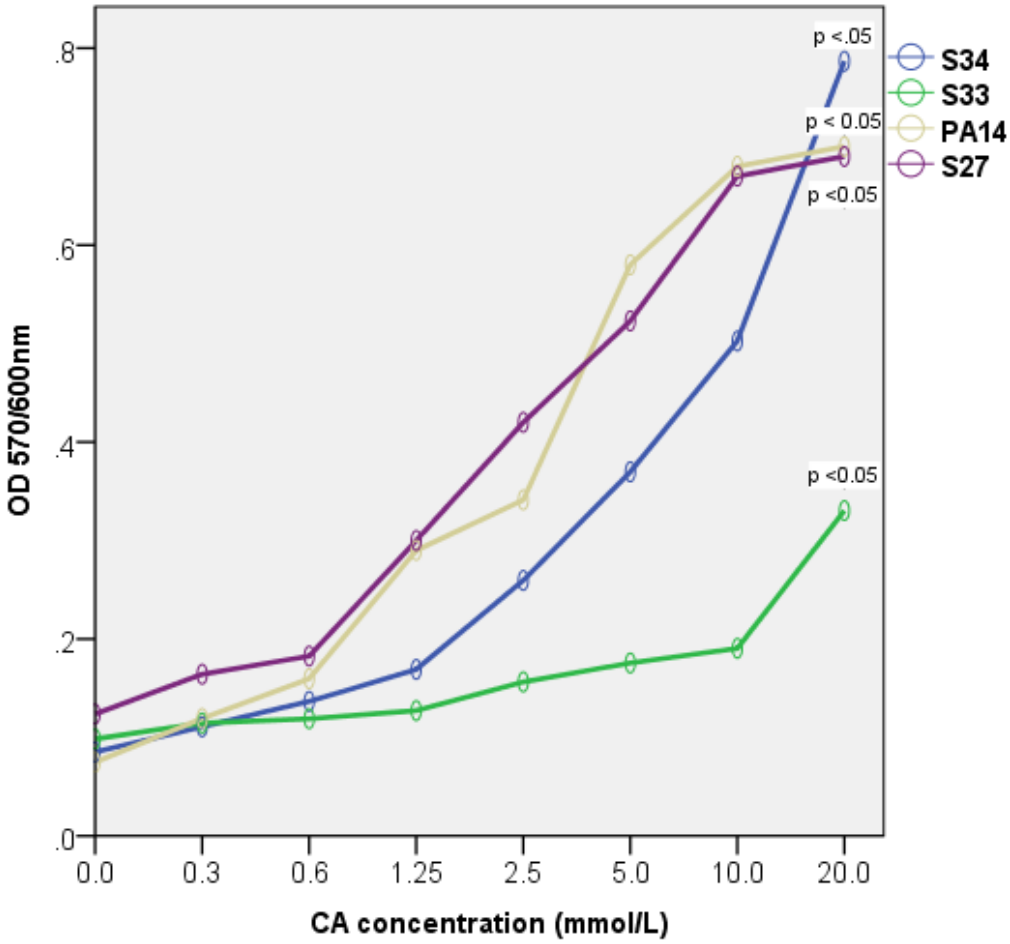

Figure 7-8: Impact of CA on *PA* biofilm formation for concentrations of 0-20mmol/l. CA concentration is shown on the x-axis, and OD measurement at 570/600nm is given on the y-axis. P-values indicate concentrations at which the rise in biofilm formation becomes significant in statistical terms (see Appendix 2G). Statistically significant growth in biofilm formation emerged at 20 mmol/l for all strains.

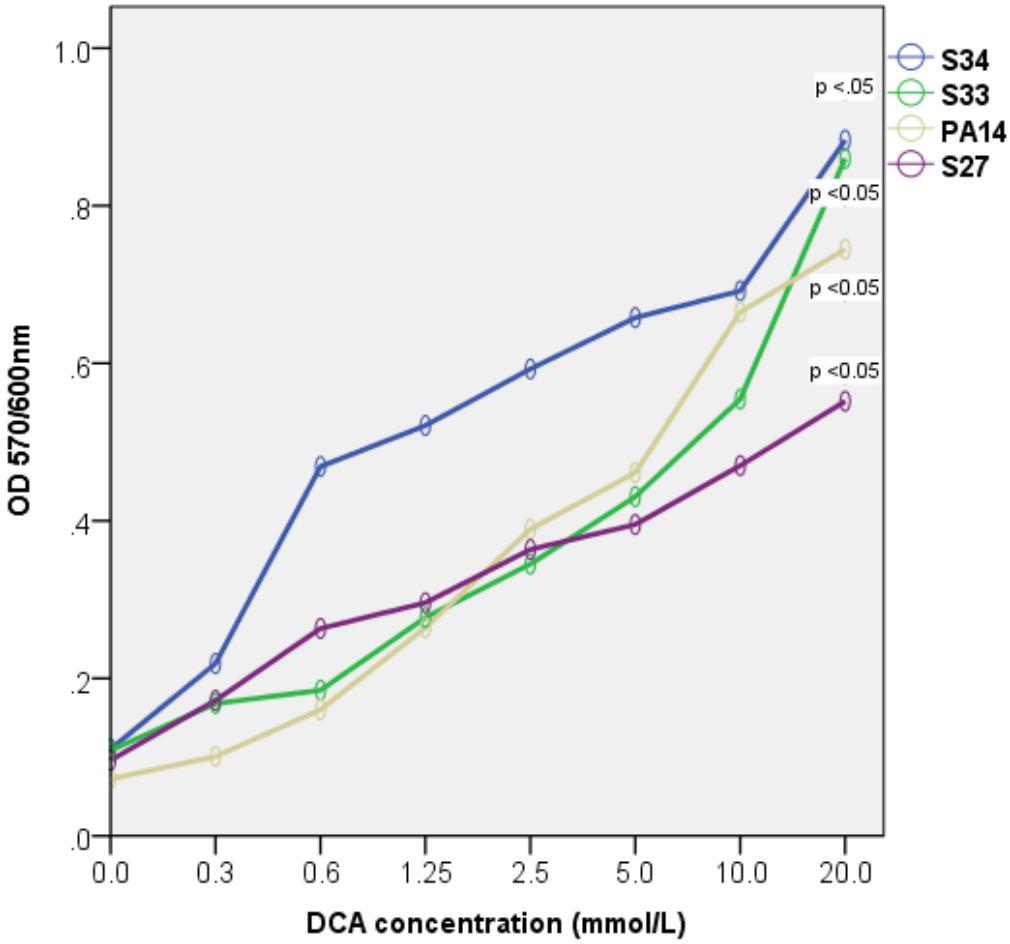

Figure 7-9: Impact of DCA on *PA* biofilm formation for concentrations of 0-20mmol/l. DCA concentration is shown on the x-axis, and OD measurement at 570/600nm is given on the y-axis. P-values indicate concentrations at which the rise in biofilm formation becomes significant in statistical terms (see Appendix 2H). Overall, statistically significant growth in biofilm formation emerged at 20 mmol/l for all strains.

7.3.3 Impact of bile acids concentrations from 9.4 µmol/l to 150 µmol/l on PA growth and biofilm formation

When *PA* was incubated alongside bile acids at similar concentrations to those observed in refluxed material (9.4 µmol/l to 150 µmol/l). *PA* growth was not altered in a significant way (Figure 7.9-7.12). On the other hand, there was significant increase in biofilm formation after incubating *PA* with different concentration of bile acids (Figure 7.13-7.16). There was variability in the strain response to bile acids in terms of biofilm formation.

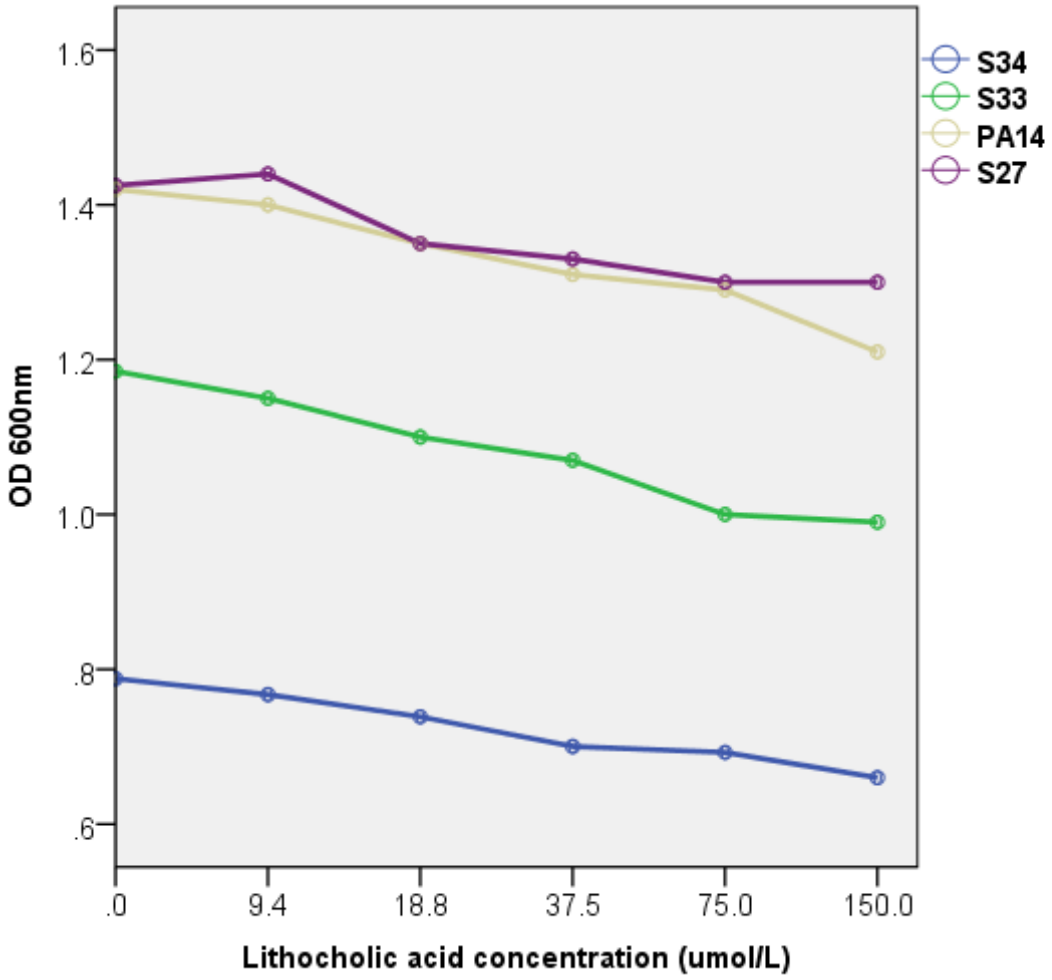

Figure 7-10: Impact of lithocholic acid on *PA* growth for concentrations of 0-150 µmol/l. Lithocholic acid concentration is shown on the x-axis, and OD measurement at 600nm is given on the y-axis. None of the four strains of *PA* showed statistically significantly reduced growth in comparison with the control (without lithocholic acid).

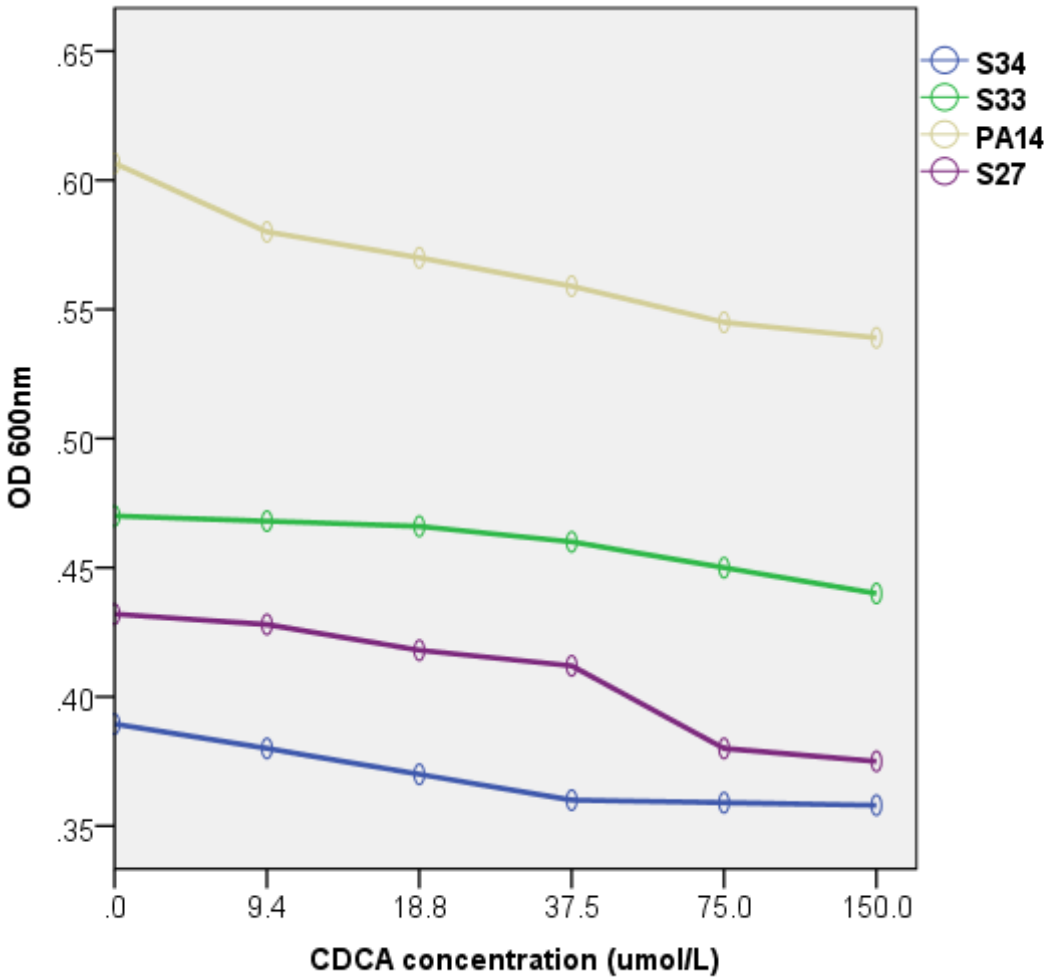

Figure 7-11: Impact of CDCA on *PA* growth for concentrations of 0-150 µmol/l. CDCA concentration is shown on the x-axis, and OD measurement at 600nm is given on the y-axis. None of the four strains of *PA* showed statistically significantly reduced growth in comparison with the control (without CDCA).

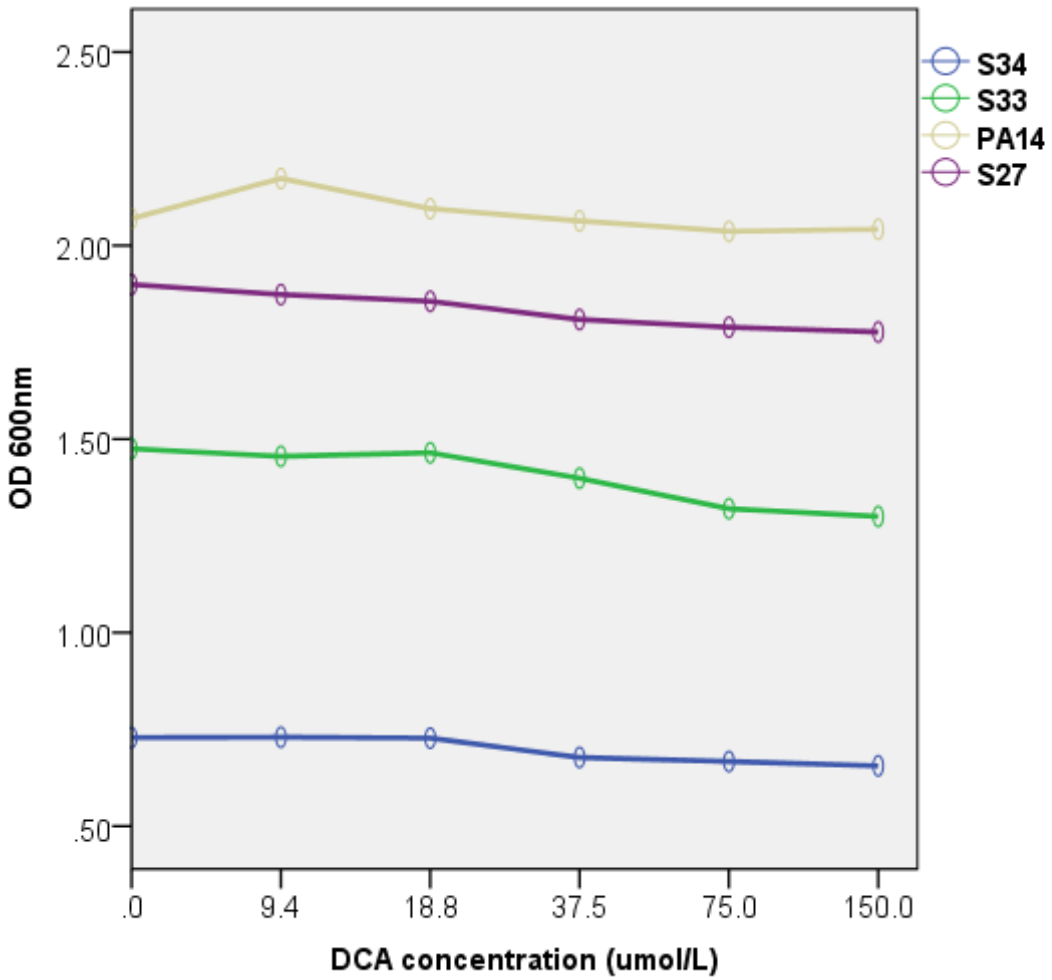

Figure 7-12: Impact of DCA on *PA* growth for concentrations of 0-150 µmol/l. DCA concentration is shown on the x-axis, and OD measurement at 600nm is given on the y-axis. None of the four strains of *PA* showed statistically significantly reduced growth in comparison with the control (without DCA).

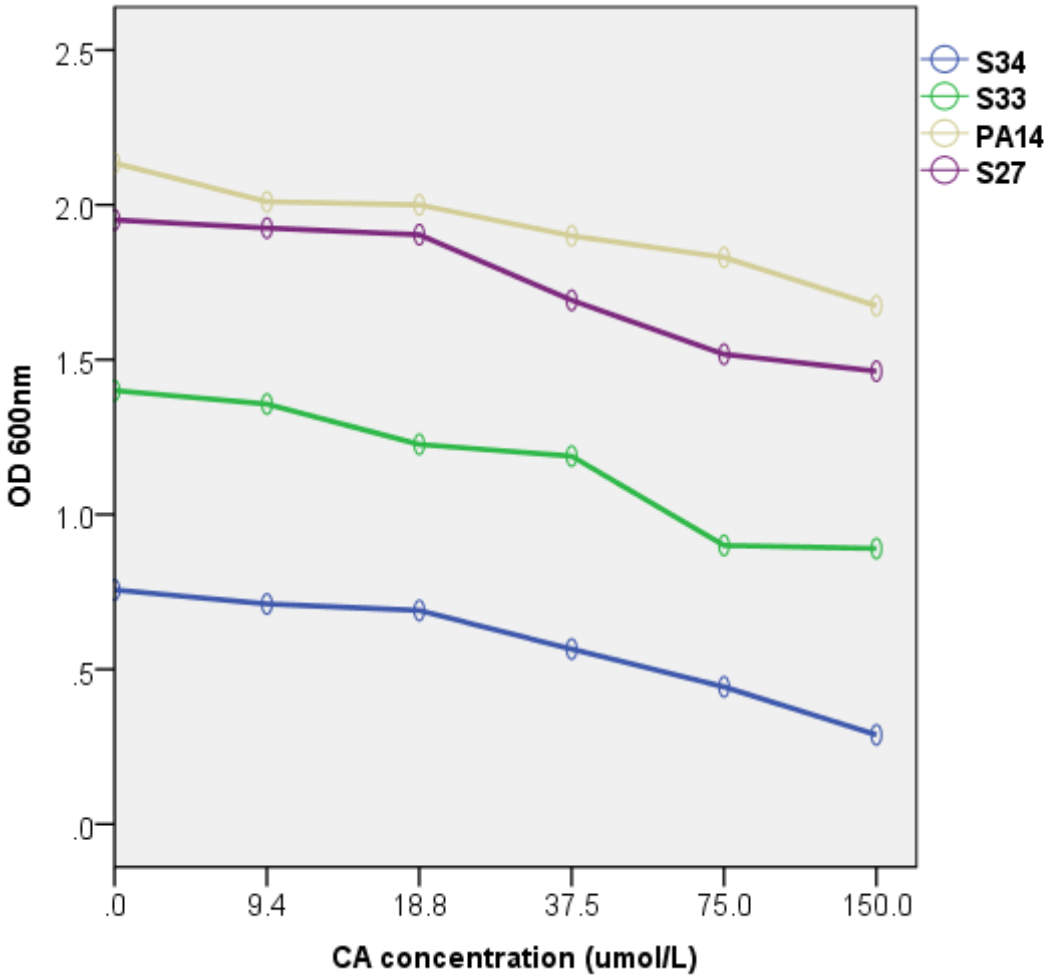

Figure 7-13: Impact of CA on *PA* growth for concentrations of 0-150 µmol/l. CA concentration is shown on the x-axis, and OD measurement at 600nm is given on the y-axis. None of the four strains of *PA* showed statistically significantly reduced growth in comparison with the control (without CA).

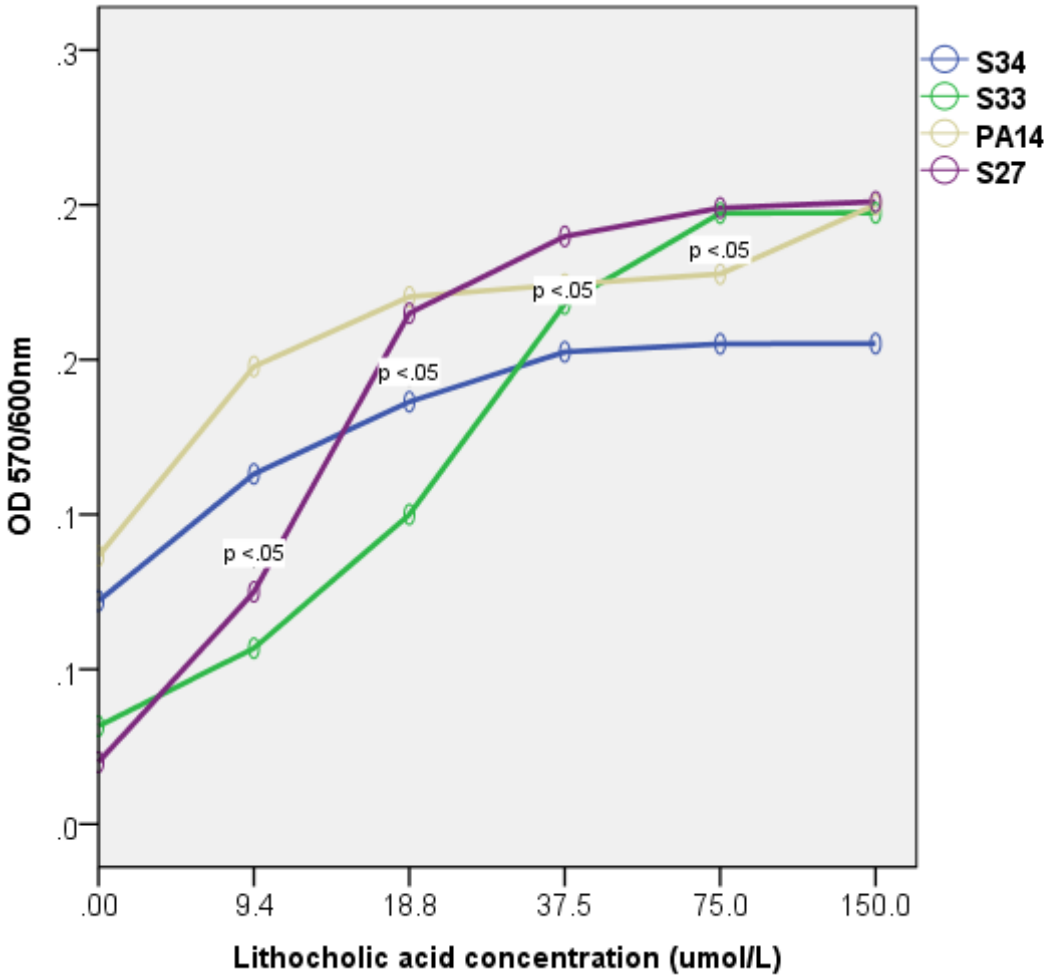

Figure 7-14: Impact of lithocholic acid on *PA* biofilm formation for concentrations of 0-150 µmol/l. Lithocholic acid concentration is shown on the x-axis, and OD measurement at 570/600nm is given on the y-axis. P-values indicate concentrations at which the rise in biofilm formation becomes significant in statistical terms (see Appendix 3A). For all strains, statistically significant growth in biofilm formation emerged at 75 µmol/l.

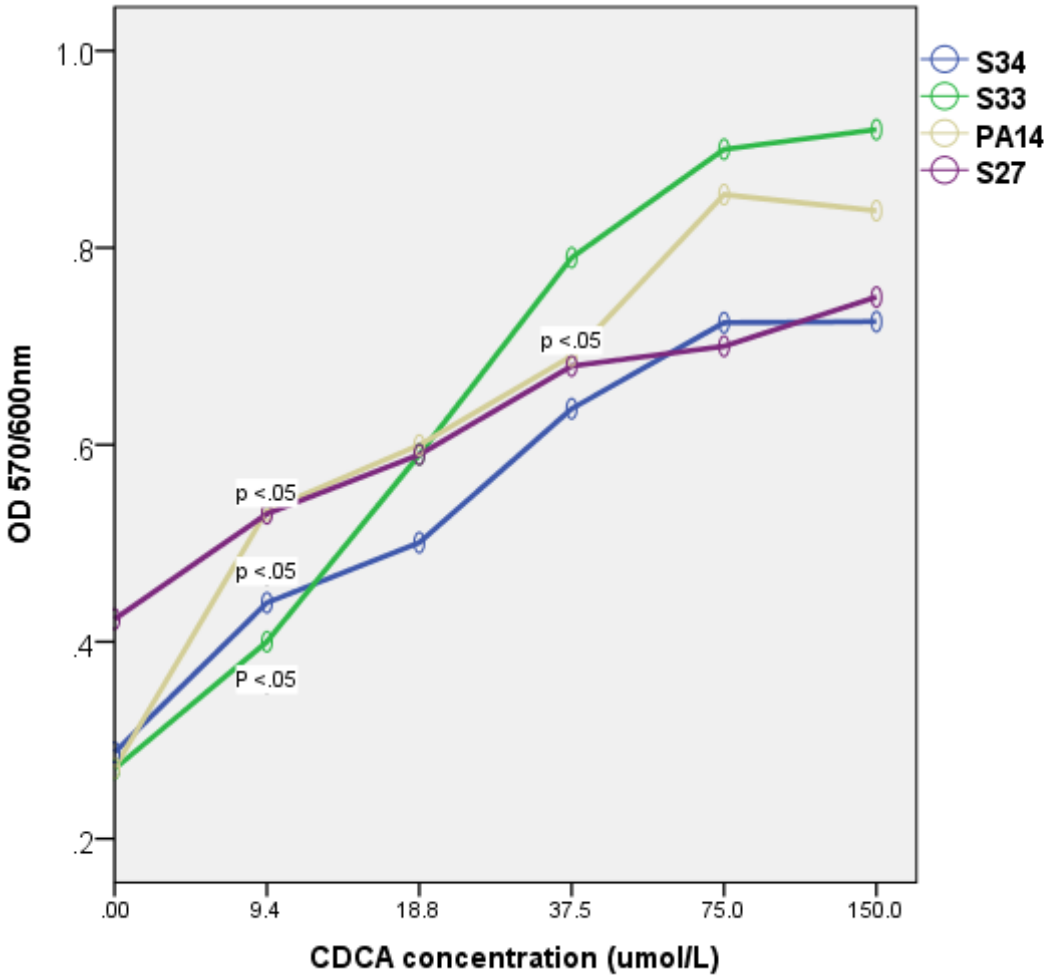

Figure 7-15: Impact of CDCA on *PA* biofilm formation for concentrations of 0-150 µmol/l. CDCA concentration is shown on the x-axis, and OD measurement at 570/600nm is given on the y-axis. P-values indicate concentrations at which the rise in biofilm formation becomes significant in statistical terms (see Appendix 3B). Statistically significant growth in biofilm formation emerged at 9.4 µmol/l among 3 *PA* strains and in the other one at 37.5 µmol/l.

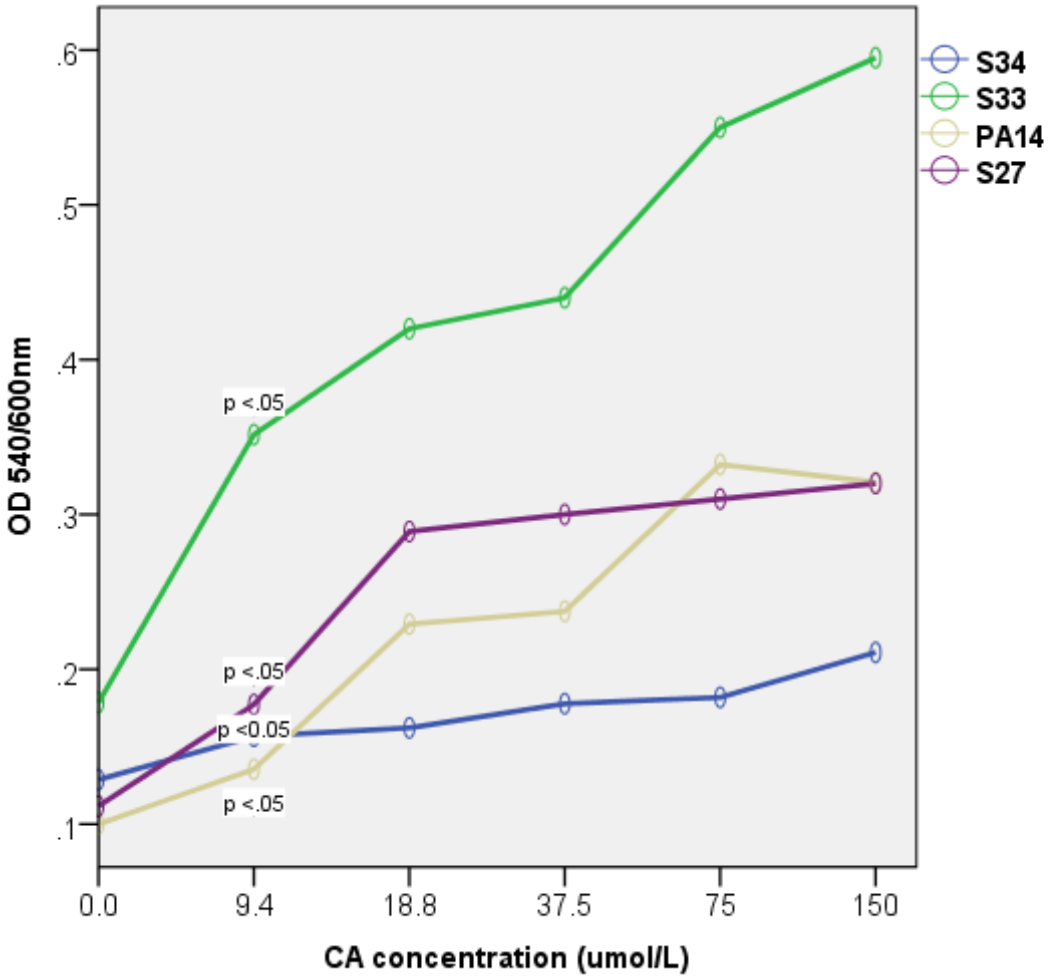

Figure 7-16: Impact of CA on *PA* biofilm formation for concentrations of 0-150 µmol/l. CA concentration is shown on the x-axis, and OD measurement at 570/600nm is given on the y-axis. P-values indicate concentrations at which the rise in biofilm formation becomes significant in statistical terms (see Appendix 3C). Statistically significant growth in biofilm formation emerged at 9.4 µmol/l for all strains.

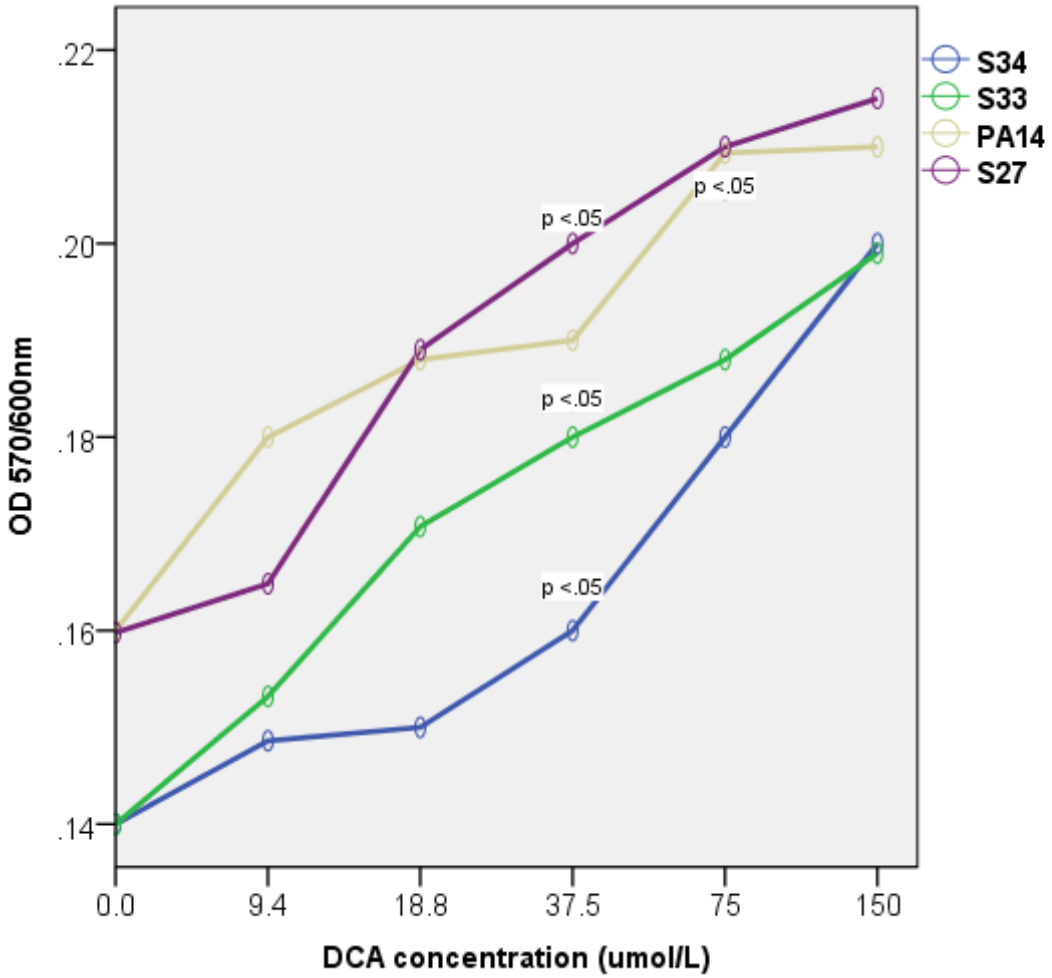

Figure 7-17: Impact of DCA on *PA* biofilm formation for concentrations of 0-150 µmol/l. DCA concentration is shown on the x-axis, and OD measurement at 570/600nm is given on the y-axis. P-values indicate concentrations at which the rise in biofilm formation becomes significant in statistical terms (see Appendix 3D). Statistically significant growth in biofilm formation emerged at 37.5 µmol/l for 3 strains, while for the other strain this occurred at 75 µmol/l.

7.4 Discussion

The findings from this experiment demonstrate a potential negative impact from bile on *PA* growth when at concentrations similar to those in the stomach. At the same time, bile acids induced alteration in the behaviour of *PA*, with a tendency emerging to form a biofilm and with variability between strains in response to different bile acids at different concentrations.

Meanwhile, at a concentration analogous to that contained in material refluxed from the stomach into the oesophagus and aspirated into the lungs, *PA* growth was unchanged, but a rise in biofilm formation was observed. The findings give weight to the proposal that the presence of bile acids within the lungs is a significant environmental contributory factor in heightened morbidity and gradual decrease in lung function for those suffering from CF respiratory disease (Reen *et al.*, 2012; Reen *et al.*, 2016). The results support the findings of previous research in suggesting that interventions to stop bile acid transfer to the lung might be useful in reducing the likelihood of inflammatory processes and chronic infections, with obvious advantages for the life quality and health status of patients (Reen *et al.*, 2016).

Previous work reports that a bovine bile concentration of 0.1-1 mmol ml^{-1} causes certain pathogenic microbes, including *PA*, to move into a chronic infective mode (Reen *et al.*, 2012). My previous work achieved isolation of *PA* as well as other bacterial species from samples of gastric juice taken from individuals with cystic fibrosis, and the findings from this experiment showed that bile acids exposure such as occurs within the stomach pushed bacteria to adopt a biofilm form, in which they have more antibiotic resistance (Stewart and Costerton, 2001). This finding may be particularly important if such bacteria may enter the lungs following GOR-related reflux and aspiration.

This research found an increase in the formation of *PA* biofilm with bile acids, and this is in line with previous research reporting that *PA* were less motile, lost their flagella and produced pseudomonas quinolone signal (PQS) in the presence of bovine bile salts (Reen *et al.*, 2012). The creation of PQS is demonstrated in previous work to be involved in the formation of biofilm by *PA* (Yang *et al.*, 2009). Further, (Reen *et al.*, 2012) report that a reduction in the formation of biofilm by *S. maltophilia* and *S. aureus* after exposure to bile acids could have importance, as lung microflora in CF progresses from *S. aureus* as the primary pathogen to the predominance of *PA* in the CF population aged more than 3 years old (Goss and Burns, 2007).

The results may have importance considering the prevalent nature of aspirated bile due to GOR in the CF population, as these findings contribute to evidence to associate GOR with the presence of *PA* in the airways. It is proposed in this study, as in others, that higher rates of *PA* lung infections in those CF individuals diagnosed with GOR might be as a result of these bacteria being aspirated into the lungs (Palm *et al.*, 2012). The evidence from this research points to the possibility that the bile acts to direct these microbes towards a biofilm mode of life. Based on this, there is potentially a link between the bile action on bacterial behaviour and the fact that *PA*, and potentially other infective agents, are more prevalent in individuals diagnosed with GOR. Under these circumstances, *PA* and other pathogenic bacteria would benefit from the protective effects of forming biofilms, allowing them to persist in the lungs and form a chronic type of infection.

In summary, bile acids at concentrations similar to the concentration present in gastric juice affect the growth of *PA*, while the concentration of bile in BAL enhances the formation of biofilm in *PA*. This finding may have important implications clinically, because once chronic *PA* infection arises, it becomes difficult to eliminate the infection, and the focus of care shifts instead to quality of life and managing lung function decline. It is considered that *PA* in the stomach, which is converted to biofilm by the effect of high bile acid levels in the stomach, is refluxed into the airways, where bile acid concentrations fall to a low level but still maintain *PA* in their biofilm behaviour. Also, *PA* entering the lung from sources other than the stomach may be converted to biofilm by the effect of the low level of bile acid presented in BAL.

Knowledge of the mechanism through which bile aspiration leads to chronic inflammatory states and infections could present a strong channel for developing new therapeutic interventions to treat chronic disease of the respiratory system. The gold standard intervention for reflux leading to aspiration is the surgical procedure known as the laparoscopic Nissen fundoplication (Sheikh *et al.*, 2013). This intervention carries significant risk, and for this reason, different approaches are needed which can focus on removal of the trigger of aspirated bile for the introduction of chronic-type infections. Some of these for example focus on the origin of the aspirations, seeking to prevent material being aspirated through GORD: pro-kinetic macrolides are one example of this, minimising episodes of aspiration, as well as also targeting pathogenic microbes in the respiratory passages (Bradley, 2001). Another possibility is treatment with bile acid sequestrants to remove the impact of bile aspiration on the host and on pathogens. This might be inhaled and thus cause titration of bile acids present in the lungs. The third possibility that emerges is greater understanding of the way in which bile acids

interact with host and pathogen at a molecular level, leading to focused, individualised treatments. To pursue this avenue, large-scale studies of patients and systems would be needed to create a comprehensive picture of the impact of bile on the pathophysiological processes of respiratory diseases.

Chapter 8.0: General discussion

Gastro-oesophageal reflux (GOR) is highly prevalent in CF patients (Chen *et al.*, 2010; Armstrong, 2005). GOR diminishes the quality of life (Ronkainen *et al.*, 2013; Holt *et al.*, 2013) and is associated with reduced lung function and increased lung infections in CF patients (Vos *et al.*, 2008). Indeed, investigations have established links between GOR and *PA* and *SA* infection in CF patients (van der Doef *et al.*, 2009; Palm *et al.*, 2012).

For CF patients, *PA* is a particularly persistent pathogen, able to elude immune responses, making it all but impossible to eliminate once an infection becomes chronic (Goss and Burns, 2007). There is a behavioural trend of drug-resistant *PA*, where *PA* forms impenetrable biofilms that persist, albeit with a reduction in toxin production (Moreau-Marquis *et al.*, 2008). This persistence behaviour of *PA* in a chronic infection differs appreciably to that of the same pathogen in an acute infection. In that instance, *PA* can trigger pneumonia, overcome lung defence mechanisms and circulate in the blood. The risks associated with aspirating bile acid raising the potential of *PA* lung infection following lung transplant have been stressed by Vos *et al.* (2008). Studies by Reen *et al.* (2012) revealed that bovine bile influenced biofilm formation of respiratory pathogens, including *PA*, enabling them to persist in a chronic infection. Our group has shown that bile acids are detectable in the lower airway in advanced CF lung disease and persist after lung transplantation (Aseeri *et al.*, 2012; Brodlie *et al.*, 2015)

A similar association has been proposed between gastroesophageal disorders and non-tuberculosis mycobacteria (NTM) (Varghese *et al.*, 1988; Griffith *et al.*, 1993; Hadjiliadls *et al.*, 1999). NTM have emerged recently but a progressive trend suggests that they are increasingly important (Leung and Olivier, 2013). *M. abscessus complex* (MABSC), of which there are three subspecies (subsp) (*M. abscessus subsp abscessus*, *M. abscessus subsp massiliense*, and *M. abscessus subsp bolletii*) is the prime NTM species present in CF patients (Adékambi and Drancourt, 2004; Gillespie, 2006).

It is not yet clear how GOR can result in lung infection; however, there is evidence that in intensive care settings, a gastric reservoir may be associated with increased risk of nosocomial pneumonia (Du Moulin *et al.*, 1982). Furthermore, studies of infant, non-CF patients indicate that the incidence of bacteria is initially greater in the gut but then become present in the respiratory tract (Madan *et al.*, 2012). This finding is in agreement with other study results for ICU patients fed using nasogastric tubes, which show a relationship between gastric bacteria and those colonising the lower respiratory tract (Segal *et al.*, 2006).

This research project aims to investigate the effect of GOR on the lung microbiome. Furthermore, it explores the relationship between the gastric and lung microbes in individual CF patients. I propose that stomach bacteria grow excessively and exert an effect upon the microbial content of the lungs and oropharynx through full column reflux. The possibility is also considered that pulmonary disease may be exacerbated by bile present in the stomach or GOR through duodenal reflux, influencing the behaviour of stomach and lung microbes. Bile acids were shown to drive *PA* into a biofilm phylotype according to Reen *et al.* (2012)

The microorganisms from the airways and gastric secretions of the same PEG-fed patients were collected and then compared using molecular and conventional approaches. As Chapters 3 and 4 describe, populations of bacteria known to be relevant to CF lung disease, including biofilm-forming strains of *PA* and NTM, were recovered from the airways and digestive tract. This finding strengthens the hypothesis that the stomach is a bacterial reservoir that contributes to the pathophysiology of CF (Dickson *et al.*, 2014).

A striking finding was that in routine cultures, *PA* and NTM formed the majority constituent pathogens in CF gastric secretion. It was of particular interest that genetic analysis revealed that where *PA* and NTM were consistent in CF sputum and gastric secretion, the strains were identical. This finding suggests that in some CF patients there may be a link between the microbes that colonise sputum and those that inhabit the gut.

Whilst the stomach is frequently credited with being an effective antimicrobial barrier, it is apparent that it is not as robust a mechanism as originally presumed. In this study, 100% of the gastric fluid samples (n = 31) collected from CF patients via the PEG tube contained bacterial and or fungal species. At 65%, (n = 9) the incidence in non-CF was still appreciable. It is well recognised that some pathogens have evolved mechanisms that enable the organism to survive extreme environmental conditions, such as digestive enzymes, extreme temperatures, fluctuating pH, harsh detergents and microbial competition as well as the host immune response (Sachs *et al.*, 2000). Not only can pathogens survive these environmental states, but their gene expression behaviour can change in response to them, up- or down-regulating specific genes to promote their adaptation to the particular microenvironment (Tamplin, 2005).

This study verifies that *PA* is capable of surviving acid environments. At pH 3.5, it took about 120 minutes for *PA* to be killed in the presence of pepsin. A previous study has shown that *E.coli* in stationary phase are more acid tolerant compared to the growth phase (Arnold and Kaspar, 1995). Zhu *et al.* (2006) found that gram-negative bacteria were more resistant in their

stationary phase or growing in a biofilm than in other phases. When in the presence of an environmental stressor, bacteria reaching a stationary phase activate several stress response systems, which protect the bacteria (Angelichio *et al.,* 2004). Consequently, *PA* biofilm in swallowed sputum may persist in the stomach for a long time and present a long-standing endogenous source of infection to the patients in this study. My data also indicates that bile acids in gastric juice might also drive the formation of new biofilm forms of *PA.* In addition, the presence of the PEG tube may promote attachment and survival of the *PA* biofilm in the stomach. A common treatment practise for CF patients in North America is to administer gastric acid suppressants (Culhane *et al.,* 2013). The CF patients in this study were also given acid suppressing therapy, raising the gastric pH, which may have enhanced the *PA*'s ability to survive in the stomach (Yang *et al.,* 2013).

The effect of bile salts on *PA* in the stomach and airways was also explored. The results indicate that bile salt concentrations comparable to the concentration that may be found in the stomach have a negative effect upon *PA* growth. On the other hand, this exposure to bile salts at concentrations comparable to ones detected in GOR induced changes that promoted the formation of drug resistant-biofilm formation. This finding strongly supports the supposition that bile acids in the lungs play a significant role in the morbidity of those suffering from respiratory diseases (Mertens *et al.,* 2011). In light of this evidence, it is appropriate to re-evaluate clinical practises that have the potential to transfer bile acids to the lungs in an effort to improve patient health.

NTM was a common and important finding in the microbial profile isolated from gastric juice samples and PEG tubes themselves. This study has shown for the first time to my knowledge that NTM, including *M. abscessus,* can be isolated from the gastric juice and PEG tubes of patients with CF in addition to sputum (Al-Momani *et al.,* 2017). NTM were isolated from the PEG and gastric juice of patients with NTM negative sputa. NTM were therefore identified for the first time from a gastric sample in these patients, despite rigorous previous sputum based surveillance in a tertiary laboratory, with a specialised interest in NTM culture methodology (Preece *et al.,* 2016).

Reports of NTM outbreaks in non-CF patients have been attributed to contaminated surgical equipment (Wallace Jr *et al.,* 1998). In patients with CF, gastrostomy tubes are frequently used to support feeding; placement of the tube can be achieved surgically, endoscopically or fluoroscopically guided through the skin into the stomach. NTM infections arising from foreign bodies, such as indwelling medical devices, have received increased interest (Trupiano *et al.,*

2001); they are thought to weaken the host's barrier defence mechanisms, enabling microbial colonisation (Linmans *et al.*, 2008). In a rare incident, *M. abscessus* was identified as causing perigastrostomy infection in an immunocompromised patient (Chiu *et al.*, 2010). The role of PEG in NTM warrants further investigation.

Coughing and swallowing sputum is a potential mechanism for transferring pathogenic organisms, including *PA* and NTM, from the lung to the gut. Although the results presented in this thesis do not reject this hypothesis, here, NTM was recovered from the PEG and gastric secretions of patients whose sputa were NTM-negative. A gastric sample represented the first time that NTM was collected from two patients. Also in this study, *PA* was obtained for the first time from the PEG tube of a CF patient who did not exhibit *PA* in the sputum. These results indicate the possibility of pathogen transfer from the stomach to the lung through recurrent reflux and aspiration; this warrants further investigation.

Data collected through an RSI questionnaire showed that CF patients enrolled in this study experienced many symptoms associated with reflux leaving the oesophagus, including coughing. Coughing is an effective form of transmission for infectious diseases, as a single cough produces significant quantities of bioaerosols (Fennelly *et al.*, 2012). Pulmonary disease can arise from NTM being transmitted through environmental sources of infected aerosols, such as showers and hot tubs (Halstrom *et al.*, 2015). Thus, gastric refluxate coughed into the environment presents a source of NTM that can be passed between patients.

Despite rigorous separation between CF patients, the potential rate of NTM transmission, particularly *M. abscessus*, between CF patients is high (Floto *et al.*, 2016). A case suspected human-to-human transmission of *M. kansasii* is described by Ricketts *et al.* (2014); genetically identical organisms were collected from a husband and wife. Transmission of NTM was proposed to occur through indirect means, such as fomites or aerosol spread caused during spirometry testing and physiotherapy (Bryant *et al.*, 2013). Bryant *et al.* (2013) further proposes that infection may be transmitted by low quantities of microorganism, stressing the importance of adopting and adhering to protocols that prevent transmission.

I found that microbiome of the gastric and sputum samples in the CF group were less diverse in comparison with the gastric juice from patients without CF. Similar to this finding, a number of studies have associated a loss of bacterial diversity with CF disease (Ott *et al.*, 2004; Fujimura *et al.*, 2010). Further evidence is provided by Erb-Downward *et al.* (2011), who examined the lung microbiomes of healthy smokers (those with no signs of disease or reduced

lung function) and patients with COPD. The researchers found that there was a correlation between reduced lung function and reduced diversity of the pulmonary microbiome; indeed the predominant species was *PA*. Comparable results have been collected in studies exploring the differences in bacterial communities in CF patients in different age groups (Cox *et al.*, 2010; Klepac-Ceraj *et al.*, 2010a). Adult patients exhibited less bacterial diversity than what was detected in their younger counterparts (Cox *et al.*, 2010).

Innate and adaptive immune responses are stimulated and shaped by the microbiota; therefore, allergic responses may be influenced by microbiota and may be implicated in other respiratory conditions such as allergic asthma and COPD (Starkey *et al.*, 2013; Starkey *et al.*, 2014). A number of studies have determined that the risk of early-onset childhood asthma is enhanced by childhood antibiotic therapy disturbing the normal microbiome (Ong *et al.*, 2014). The variety of species contributing to the microbiome appears to be more influential than the extent of the microbial load.

Research shows that there are distinct temporal and biogeographical differences in the diversity of microbes and their relative abundance in patients with COPD (Sze *et al.*, 2012; Millares *et al.*, 2014).

The greater emphysema and immune cell infiltration found in COPD patients are attributed, in part, to the loss of diversity of the respiratory microbiome (Sze *et al.*, 2012).

Looking at exacerbated COPD, Wang *et al.* (2016) identified different phenotypic patterns (e.g. bacterial or eosinophilic) that corresponded with particular microbiome profiles; these profiles were consistent at genus and phylum levels. The Proteobacteria phyla (chiefly *Haemophilus spp.*) were most evident in bacterial exacerbation; on the other hand, in eosinophilic exacerbations, Firmicutes predominated. The conclusion from this is that in some individuals, there may be an association between the composition of lung microbiota and acute exacerbations, which may determine the inflammatory responses of the host (particularly IL-8).

There is evidence that healthy immune responses are enhanced by particular microbes, and that chronic inflammatory lung diseases such as asthma, CF and COPD are compounded by microbial dysbiosis (Cho and Blaser, 2012). Although the concept needs fuller exploration, it is thought that the communication between different mucosal barriers, such as the gut and the lungs, is facilitated by patrolling immune cells and resident microbes (Shukla *et al.*, 2017). At present, major non-communicable lung disease-treatments aim only to alleviate symptoms and

are limited in capability in terms of completely preventing or treating the disease. Armed with a deeper understanding of the role of the microbiome in terms of pathophysiology and inflammation, together with greater insight into its relationship and genetic-risk factors, it may be possible to devise superior treatments for chronic lung conditions (Shukla *et al.*, 2017).

This knowledge may also lead to the development of novel therapeutic interventions. Currently, the effects of existing therapies on the overall microbiome and implications to disease severity and progression are not well characterised. There is potential to modify the microbiome through diet, probiotics, transfer of selected bacteria or faeces may support the existing therapies or be effective treatments on their own. However, on every level, more research is required to determine role and effectiveness of individual and combined treatments on the microbiome.

8.1 Conclusion and future directions

This thesis shows that the stomach is a potential microbiological niche where organisms relevant to CF pathophysiology can survive. In particular, the stomach is a potential reservoir for biofilm *PA* and NTM, which appear to enhance by acid suppression therapy. This under-recognised phenomenon is of particular significance to CF patients who are vulnerable to reflux and aspiration. *PA* is a resilient pathogen which has demonstrated its ability to persist in acidic environments. It is affected by bile salts in the stomach and in reflux, causing the pathogen to adopt biofilm-forming behaviours, which enable it to persist. This study also revealed an association between the microbiome of sputum and gastric juice in CF patients. *PA* eliminating therapies presently do not incorporate the pathogen's gastric niche, yet the 'aerodigestive microbiome' may be of particular importance for CF pathophysiology and have therapeutic implications. It has been demonstrated that CF patients may become infected with NTM from gastric secretions or PEG-tubes. This is particularly concerning as NTM are resistant to anti-tuberculosis drugs and standard antibiotics and presents a mortal risk to CF patients in particular. Early identification may facilitate prompt susceptibility testing, suitable therapy and PEG management.

One drawback of this study was the small sample size. Although PEG enables access to gastric secretions without potential contamination from sampling the upper airways, the number of CF patients fed by PEG, were limited. Future research could be to expand the sample size by extending the study to several CF centres. Further investigation into the effect on different microbes by various components of gastric reflux is also warranted, as this study was restricted to evaluating bile on *PA.* As the drug resistance and prevalence of NTM of *PA* increase, the need for more data as it relates to CF becomes more urgent.

According to this thesis, Bile salts contribute to *PA* biofilms forming, which emphasises the need to devise new antimicrobial molecules or therapeutic interventions that are effective against biofilm *PA* (Chatterjee *et al.*, 2016). Potential treatments that is worthy of further investigation are bacteriophage-based therapies, as several bacteriophages have been identified that are effective against *PA* (Fu *et al.*, 2010; Hraiech *et al.*, 2015). Several of these bacteriophages have demonstrated their efficiency in destroying *PA* strains collected from CF patients (Alemayehu *et al.*, 2012).

The advantage that phage therapy offers over conventional antibiotic therapy is that the bacteriophages are able to penetrate the surface of the biofilm where they replicate, resulting in

an increased concentration of phage close to the infection location (Wright *et al.*, 2009). Growth of the biofilm bacteria is inhibited by the alginase synthesised by the bacteriophages that depolymerise *PA*'s alginic acid capsule, disrupting the growth of the bacteria in the biofilm (Harper *et al.*, 2014).

A significant benefit of using phages for therapeutic purposes is that they can be genetically modified to minimise the host's inflammatory system responses (Soothill, 2013). This is important because the destruction of bacteria brought about by lytic phages and numerous antibiotics, results in the release of endotoxins and other cell wall constituents into the blood stream, initiating systemic immune responses similar to septicaemia. This is known as the Jarisch-Herxheimer reaction (Guerrier and D'Ortenzio, 2013).

Whilst evidence of the effectiveness of using phages to treat *PA* has been demonstrated in several *in vitro* and pre-clinical studies, thus far, research supported by human clinical trials is limited.

In this thesis, 16S rRNA gene targeting variable region 4 (V4) was used to profile bacteria.

This method is limited by the annotation being based on the presumed association of the 16S rRNA gene with taxa, which is defined as an operational taxonomic unit (OTU) (Langille *et al.*, 2013). Typically, OTUs are imprecise at the species level, but are able to analyse at phyla or genera levels (Ranjan *et al.*, 2016). Furthermore, OTUs are used to predict specific gene sequences, which are rarely sequenced directly. Yet, because bacteria transfer genes horizontally between species, of which there can be numerous strains in the environment, a comprehensive understanding of the microbiome is limited without directly identifying the genes (Poretsky *et al.*, 2014) (Konstantinidis and Stackebrandt, 2013).

Whole genome sequencing (WGS) presents an alternative to 16S rRNA amplicon sequencing; WGS uses random primers to sequences overlapping sections of a genome (Ranjan *et al.*, 2016). This technique enables the accurate definition of taxa at the species level, which is a considerable advantage. It is worth noting that the databases used by the 16S and WGS methods to classify taxa are different. Drawbacks of WGS are that it demands greater in-depth analysis of the data and it is more expensive than the 16S method (Kuczynski *et al.*, 2012; Luo *et al.*, 2014). Moreover, fully characterising the genes in a bacterial taxon may require sequencing a high-coverage genome (Sims *et al.*, 2014).

9. Bibliography

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10. Appendix

10.1 Appendix 1: The reflux symptom index (RSI) questionnaire

Within the last **month,** how did the following problems affect you?
(0-5 rating scale with 0 = No problem and 5 = Severe)

1. Hoarseness or a problem with your voice	0 1 2 3 4 5
2. Clearing your throat	0 1 2 3 4 5
3. Excess throat mucous or postnasal drip	0 1 2 3 4 5
4. Difficulty swallowing food, liquids or pills	0 1 2 3 4 5
5. Coughing after you ate or after lying down	0 1 2 3 4 5
6. Breathing difficulties or choking episodes	0 1 2 3 4 5
7. Troublesome or annoying cough	0 1 2 3 4 5
8. Sensations or something sticking in your throat	0 1 2 3 4 5
9. Heart burn, chest pain, indigestion, or acid coming up	0 1 2 3 4 5

10.2 Appendix 2: The ANOVA test result for the effect of different concentration of bile acids on growth and biofilm formation among 4 different strains of *PA*. C1 represent the control with PA strains incubated without adding bile acids.C2-C8 represent different concentration ofbile acids added to the TSB (C2=0.3 mmol/L,C3=0.6 mmol/L, C4=1.25 mmol/L, C5=2.5 mmol/L, C6=5 mmol/L mmol/L, C7=10 mmol/L and C8 =20mmol/L)

Appendix 2A: Effect of lithocholic acid on *PA* strains growth

Impact of lithocholic acid on *PA* strain S33 growth

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.0557	0.0577	(-0.2245, 0.1131)	-0.96	0.870
C3 - C1	-0.0877	0.0577	(-0.2565, 0.0811)	-1.52	0.518
C4 - C1	-0.1727	0.0577	(-0.3415, -0.0039)	-2.99	0.044
C5 - C1	-0.3073	0.0577	(-0.4761, -0.1385)	-5.32	0.000
C6 - C1	-0.5183	0.0577	(-0.6871, -0.3495)	-8.98	0.000
C7 - C1	-0.6530	0.0577	(-0.8218, -0.4842)	-11.31	0.000
C8 - C1	-1.0787	0.0577	(-1.2475, -0.9099)	-18.68	0.000

Impact of lithocholic acid on PA strain S43 growth

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.0877	0.0424	(-0.2116, 0.0362)	-2.07	0.234
C3 - C1	-0.1110	0.0424	(-0.2349, 0.0129)	-2.62	0.089
C4 - C1	-0.1717	0.0424	(-0.2956, -0.0478)	-4.05	0.005
C5 - C1	-0.2417	0.0424	(-0.3656, -0.1178)	-5.70	0.000
C6 - C1	-0.2947	0.0424	(-0.4186, -0.1708)	-6.95	0.000
C7 - C1	-0.4050	0.0424	(-0.5289, -0.2811)	-9.56	0.000
C8 - C1	-0.6457	0.0424	(-0.7696, -0.5218)	-15.24	0.000

Impact of lithocholic acid on PA strain *PA14* growth.

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.0167	0.0488	(-0.1593, 0.1259)	-0.34	0.999
C3 - C1	-0.0370	0.0488	(-0.1796, 0.1056)	-0.76	0.953
C4 - C1	-0.0653	0.0488	(-0.2079, 0.0773)	-1.34	0.637
C5 - C1	-0.1570	0.0488	(-0.2996, -0.0144)	-3.22	0.028
C6 - C1	-0.2733	0.0488	(-0.4159, -0.1307)	-5.60	0.000
C7 - C1	-0.5433	0.0488	(-0.6859, -0.4007)	-11.14	0.000
C8 - C1	-1.1603	0.0488	(-1.3029, -1.0177)	-23.79	0.000

Impact of lithocholic acid on PA strain S27 growth.

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.031	0.126	(-0.399, 0.337)	-0.25	1.000
C3 - C1	-0.060	0.126	(-0.428, 0.308)	-0.47	0.996
C4 - C1	-0.130	0.126	(-0.498, 0.238)	-1.03	0.835
C5 - C1	-0.189	0.126	(-0.557, 0.199)	-1.70	0.049
C6 - C1	-0.343	0.126	(-0.711, 0.045)	-2.93	0.033
C7 - C1	-0.566	0.126	(-0.934, -0.198)	-4.50	0.002

Appendix 2B: Effect of DCA on *PA* strains growth

Effect of DCA on *PA* S33 growth

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.4640	0.0461	(-0.5987, -0.3293)	-10.07	0.000
C3 - C1	-0.7307	0.0461	(-0.8653, -0.5960)	-15.86	0.000
C4 - C1	-1.1647	0.0461	(-1.2993, -1.0300)	-25.29	0.000
C5 - C1	-1.3217	0.0461	(-1.4563, -1.1870)	-28.69	0.000
C6 - C1	-1.3770	0.0461	(-1.5117, -1.2423)	-29.90	0.000
C7 - C1	-1.4057	0.0461	(-1.5403, -1.2710)	-30.52	0.000
C8 - C1	-1.4573	0.0461	(-1.5920, -1.3227)	-31.64	0.000

Effect of DCA on *PA* S34 growth.

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.3433	0.0462	(-0.4784, -0.2083)	-7.43	0.000
C3 - C1	-0.5030	0.0462	(-0.6380, -0.3680)	-10.89	0.000
C4 - C1	-0.6953	0.0462	(-0.8304, -0.5603)	-15.06	0.000
C5 - C1	-0.7293	0.0462	(-0.8644, -0.5943)	-15.79	0.000
C6 - C1	-0.7697	0.0462	(-0.9047, -0.6346)	-16.67	0.000
C7 - C1	-0.7883	0.0462	(-0.9234, -0.6533)	-17.07	0.000
C8 - C1	-0.8037	0.0462	(-0.9387, -0.6686)	-17.40	0.000

Effect of DCA on *PA* S14 growth

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.061	0.118	(-0.405, 0.283)	-0.52	0.993
C3 - C1	-0.142	0.118	(-0.486, 0.202)	-1.21	0.725
C4 - C1	-0.536	0.118	(-0.880, -0.192)	-4.56	0.002
C5 - C1	-1.060	0.118	(-1.404, -0.716)	-9.01	0.000
C6 - C1	-1.360	0.118	(-1.704, -1.016)	-11.56	0.000
C7 - C1	-1.407	0.118	(-1.751, -1.063)	-11.96	0.000
C8 - C1	-1.480	0.118	(-1.824, -1.136)	-12.58	0.000

Effect of DCA on *PA* S27 growth.

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.0837	0.0812	(-0.3211, 0.1538)	-1.03	0.835
C3 - C1	-0.1097	0.0812	(-0.3471, 0.1278)	-1.35	0.629
C4 - C1	-0.6147	0.0812	(-0.8521, -0.3772)	-7.57	0.000
C5 - C1	-0.7277	0.0812	(-0.9651, -0.4902)	-8.96	0.000
C6 - C1	-1.2617	0.0812	(-1.4991, -1.0242)	-15.53	0.000
C7 - C1	-1.3277	0.0812	(-1.5651, -1.0902)	-16.35	0.000
C8 - C1	-1.3930	0.0812	(-1.6305, -1.1555)	-17.15	0.000

Appendix 2C: Effect of CDCA on *PA* strains growth

Effect of CDCA on *PA* S33 growth.

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.0380	0.0274	(-0.1180, 0.0420)	-1.39	0.603
C3 - C1	-0.0620	0.0274	(-0.1420, 0.0180)	-2.27	0.168
C4 - C1	-0.1040	0.0274	(-0.1840, -0.0240)	-3.80	0.009
C5 - C1	-0.1383	0.0274	(-0.2183, -0.0583)	-5.06	0.001
C6 - C1	-0.1663	0.0274	(-0.2463, -0.0863)	-6.08	0.000
C7 - C1	-0.2060	0.0274	(-0.2860, -0.1260)	-7.53	0.000
C8 - C1	-0.3957	0.0274	(-0.4757, -0.3157)	-14.46	0.000

Effect of CDCA on *PA* S34 growth.

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.1570	0.0408	(-0.2764, -0.0376)	-3.85	0.008
C3 - C1	-0.2397	0.0408	(-0.3590, -0.1203)	-5.87	0.000
C4 - C1	-0.3080	0.0408	(-0.4274, -0.1886)	-7.54	0.000
C5 - C1	-0.3320	0.0408	(-0.4514, -0.2126)	-8.13	0.000
C6 - C1	-0.3773	0.0408	(-0.4967, -0.2580)	-9.24	0.000
C7 - C1	-0.4550	0.0408	(-0.5744, -0.3356)	-11.14	0.000
C8 - C1	-0.9380	0.0408	(-1.0574, -0.8186)	-22.97	0.000

Effect of CDCA on *PA* S14 growth.

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.0223	0.0885	(-0.2810, 0.2363)	-0.25	1.000
C3 - C1	-0.1500	0.0885	(-0.4086, 0.1086)	-1.70	0.410
C4 - C1	-0.2613	0.0885	(-0.5200, -0.0027)	-2.95	0.047
C5 - C1	-0.4083	0.0885	(-0.6670, -0.1497)	-4.62	0.002
C6 - C1	-0.5403	0.0885	(-0.7990, -0.2817)	-6.11	0.000
C7 - C1	-0.6693	0.0885	(-0.9280, -0.4107)	-7.57	0.000
C8 - C1	-1.2110	0.0885	(-1.4696, -0.9524)	-13.69	0.000

Effect of CDCA on *PA* S27 growth.

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.0787	0.0769	(-0.3035, 0.1462)	-1.02	0.839
C3 - C1	-0.0970	0.0769	(-0.3219, 0.1279)	-1.26	0.690
C4 - C1	-0.1643	0.0769	(-0.3892, 0.0605)	-2.14	0.209
C5 - C1	-0.4630	0.0769	(-0.6879, -0.2381)	-6.02	0.000
C6 - C1	-0.7483	0.0769	(-0.9732, -0.5235)	-9.73	0.000
C7 - C1	-0.9087	0.0769	(-1.1335, -0.6838)	-11.81	0.000
C8 - C1	-1.4083	0.0769	(-1.6332, -1.1835)	-18.31	0.000

Appendix 2D: Effect of CA on *PA* strains growth

Effect of CA on *PA* S33 growth.

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.0950	0.0433	(-0.2216, 0.0316)	-2.19	0.190
C3 - C1	-0.1070	0.0433	(-0.2336, 0.0196)	-2.47	0.117
C4 - C1	-0.1500	0.0433	(-0.2766, -0.0234)	-3.46	0.017
C5 - C1	-0.1897	0.0433	(-0.3163, -0.0631)	-4.38	0.003
C6 - C1	-0.2680	0.0433	(-0.3946, -0.1414)	-6.19	0.000
C7 - C1	-0.3260	0.0433	(-0.4526, -0.1994)	-7.53	0.000
C8 - C1	-0.5613	0.0433	(-0.6879, -0.4347)	-12.97	0.000

Effect of CA on *PA* S34 growth.

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.3180	0.0658	(-0.5104, -0.1256)	-4.83	0.001
C3 - C1	-0.4940	0.0658	(-0.6864, -0.3016)	-7.51	0.000
C4 - C1	-0.5130	0.0658	(-0.7054, -0.3206)	-7.80	0.000
C5 - C1	-0.7040	0.0658	(-0.8964, -0.5116)	-10.70	0.000
C6 - C1	-1.0710	0.0658	(-1.2634, -0.8786)	-16.28	0.000
C7 - C1	-1.2993	0.0658	(-1.4917, -1.1070)	-19.75	0.000
C8 - C1	-1.4077	0.0658	(-1.6000, -1.2153)	-21.40	0.000

Effect of CA on *PA* S14 growth.

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.015	0.147	(-0.444, 0.414)	-0.10	1.000
C3 - C1	-0.068	0.147	(-0.497, 0.361)	-0.47	0.996
C4 - C1	-0.139	0.147	(-0.568, 0.290)	-0.94	0.880
C5 - C1	-0.261	0.147	(-0.690, 0.168)	-1.78	0.366
C6 - C1	-0.738	0.147	(-1.167, -0.309)	-5.03	0.001
C7 - C1	-1.155	0.147	(-1.584, -0.726)	-7.87	0.000
C8 - C1	-1.811	0.147	(-2.240, -1.382)	-12.34	0.000

Effect of CA on *PA* S27 growth.

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.0543	0.0790	(-0.2855, 0.1768)	-0.69	0.970
C3 - C1	-0.0987	0.0790	(-0.3298, 0.1325)	-1.25	0.698
C4 - C1	-0.3187	0.0790	(-0.5498, -0.0875)	-4.03	0.005
C5 - C1	-0.5450	0.0790	(-0.7761, -0.3139)	-6.89	0.000
C6 - C1	-0.9783	0.0790	(-1.2095, -0.7472)	-12.38	0.000
C7 - C1	-1.1313	0.0790	(-1.3625, -0.9002)	-14.31	0.000
C8 - C1	-1.7427	0.0790	(-1.9738, -1.5115)	-22.05	0.000

Appendix 2E: Effect of lithocholic acid on *PA* strains biofilm formation

Impact of lithocholic acid on *PA* S33 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.0183	0.0427	(-0.1065, 0.1431)	0.43	0.998
C3 - C1	0.0001	0.0427	(-0.1247, 0.1249)	0.00	1.000
C4 - C1	-0.0207	0.0427	(-0.1455, 0.1041)	-0.49	0.995
C5 - C1	0.0149	0.0427	(-0.1099, 0.1397)	0.35	0.999
C6 - C1	0.0505	0.0427	(-0.0743, 0.1753)	1.18	0.742
C7 - C1	0.0873	0.0427	(-0.0375, 0.2121)	2.05	0.242
C8 - C1	0.2248	0.0427	(0.1000, 0.3496)	5.27	0.000

Impact of lithocholic acid on *PA* S34 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.121	0.187	(-0.620, 0.379)	-0.64	0.982
C3 - C1	-0.192	0.187	(-0.692, 0.308)	-1.02	0.843
C4 - C1	-0.280	0.187	(-0.780, 0.220)	-1.49	0.514
C5 - C1	-0.425	0.187	(-0.924, 0.075)	-2.27	0.128
C6 - C1	-0.764	0.187	(-1.263, -0.264)	-4.08	0.001
C7 - C1	-0.978	0.187	(-1.478, -0.478)	-5.22	0.000
C8 - C1	-1.381	0.187	(-1.880, -0.881)	-7.37	0.000

Impact of lithocholic acid on *PA* S14 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.040	0.314	(-0.842, 0.923)	0.13	1.000
C3 - C1	0.081	0.314	(-0.801, 0.964)	0.26	1.000
C4 - C1	0.027	0.314	(-0.855, 0.910)	0.09	1.000
C5 - C1	0.003	0.314	(-0.879, 0.886)	0.01	1.000
C6 - C1	0.131	0.314	(-0.752, 1.013)	0.42	0.998
C7 - C1	0.102	0.314	(-0.781, 0.984)	0.32	1.000
C8 - C1	1.374	0.314	(0.491, 2.257)	4.38	0.001

Impact of lithocholic acid on *PA S27* biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.084	0.181	(-0.595, 0.426)	-0.47	0.997
C3 - C1	-0.096	0.181	(-0.606, 0.415)	-0.53	0.993
C4 - C1	-0.110	0.181	(-0.620, 0.401)	-0.61	0.986
C5 - C1	-0.091	0.181	(-0.601, 0.420)	-0.50	0.995
C6 - C1	-0.021	0.181	(-0.531, 0.490)	-0.11	1.000
C7 - C1	-0.023	0.181	(-0.534, 0.488)	-0.13	1.000
C8 - C1	0.356	0.181	(-0.155, 0.866)	1.96	0.263

Appendix 2F: Effect of CDCA on *PA* strains biofilm formation

Impact of CDCA on *PA* S33 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.089	0.606	(-1.617, 1.795)	0.15	1.000
C3 - C1	0.311	0.606	(-1.395, 2.017)	0.51	0.994
C4 - C1	0.819	0.606	(-0.887, 2.525)	1.35	0.625
C5 - C1	0.471	0.606	(-1.235, 2.177)	0.78	0.949
C6 - C1	0.329	0.606	(-1.377, 2.035)	0.54	0.992
C7 - C1	0.364	0.606	(-1.342, 2.070)	0.60	0.986
C8 - C1	1.683	0.606	(-0.023, 3.389)	2.78	0.049

Impact of CDCA on *PA* S34 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.018	0.208	(-0.603, 0.567)	-0.09	1.000
C3 - C1	0.022	0.208	(-0.563, 0.607)	0.11	1.000
C4 - C1	0.780	0.208	(0.195, 1.365)	1.04	0.833
C5 - C1	0.292	0.208	(-0.293, 0.878)	1.41	0.586
C6 - C1	0.143	0.208	(-0.443, 0.728)	0.69	0.972
C7 - C1	0.182	0.208	(-0.403, 0.768)	0.88	0.913
C8 - C1	0.216	0.208	(-0.370, 1.801)	3.75	0.004

Impact of CDCA on *PA* S14 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.000	0.130	(-0.367, 0.367)	0.00	1.000
C3 - C1	0.057	0.130	(-0.309, 0.424)	0.44	0.998
C4 - C1	0.264	0.130	(-0.102, 0.631)	2.03	0.234
C5 - C1	0.354	0.130	(-0.013, 0.721)	2.72	0.062
C6 - C1	0.244	0.130	(-0.122, 0.611)	1.88	0.302
C7 - C1	0.233	0.130	(-0.134, 0.599)	1.79	0.348
C8 - C1	0.560	0.130	(0.193, 0.927)	4.30	0.002

Impact of lithocholic acid on *PA* S27 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.0199	0.0936	(-0.2436, 0.2834)	0.21	1.000
C3 - C1	0.1360	0.0936	(-0.1275, 0.3995)	1.45	0.554
C4 - C1	0.3231	0.0936	(0.0596, 0.5866)	3.45	0.012
C5 - C1	0.1623	0.0936	(-0.1012, 0.4257)	1.73	0.377
C6 - C1	0.1766	0.0936	(-0.0868, 0.4401)	1.89	0.296
C7 - C1	0.2657	0.0936	(0.0023, 0.4992)	2.54	0.092
C8 - C1	0.2362	0.0936	(-0.0272, 0.5297)	2.82	0.047

Appendix 2G: Effect of CA on *PA* strains biofilm formation

Impact of CA on *PA* S33 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.042	0.199	(-0.518, 0.601)	0.21	1.000
C3 - C1	0.046	0.199	(-0.513, 0.606)	0.23	1.000
C4 - C1	0.288	0.199	(-0.271, 0.848)	1.45	0.556
C5 - C1	0.183	0.199	(-0.376, 0.743)	0.92	0.893
C6 - C1	0.308	0.199	(-0.252, 0.867)	1.55	0.491
C7 - C1	0.296	0.199	(-0.263, 0.856)	1.49	0.529
C8 - C1	0.997	0.199	(0.437, 1.556)	5.01	0.000

Impact of CA on *PA* S34 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.0008	0.0422	(-0.1195, 0.1179)	-0.02	1.000
C3 - C1	-0.0174	0.0422	(-0.1361, 0.1014)	-0.41	0.998
C4 - C1	-0.0002	0.0422	(-0.1189, 0.1185)	-0.00	1.000
C5 - C1	0.0235	0.0422	(-0.0952, 0.1422)	0.56	0.991
C6 - C1	0.0258	0.0422	(-0.0929, 0.1445)	0.61	0.985
C7 - C1	0.0332	0.0422	(-0.0855, 0.1519)	0.79	0.946
C8 - C1	0.1580	0.0422	(0.0393, 0.2767)	3.75	0.006

Impact of CA on *PA* PA14 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.012	0.101	(-0.258, 0.281)	0.11	1.000
C3 - C1	0.001	0.101	(-0.269, 0.270)	0.01	1.000
C4 - C1	0.116	0.101	(-0.154, 0.385)	1.14	0.767
C5 - C1	0.150	0.101	(-0.119, 0.419)	1.48	0.521
C6 - C1	0.281	0.101	(0.012, 0.551)	2.78	0.057
C7 - C1	0.155	0.101	(-0.114, 0.425)	1.53	0.485
C8 - C1	0.591	0.101	(0.322, 0.861)	5.84	0.000

Impact of CA on *PA* S27 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.026	0.218	(-0.588, 0.640)	0.12	1.000
C3 - C1	-0.042	0.218	(-0.656, 0.573)	-0.19	1.000
C4 - C1	0.021	0.218	(-0.593, 0.635)	0.10	1.000
C5 - C1	0.165	0.218	(-0.449, 0.779)	0.76	0.955
C6 - C1	0.213	0.218	(-0.401, 0.828)	0.98	0.866
C7 - C1	0.035	0.218	(-0.579, 0.650)	0.16	1.000
C8 - C1	0.595	0.218	(-0.020, 1.209)	2.72	0.061

Appendix 2H: Effect of DCA on *PA* strains biofilm formation

Impact of CA on *PA* S33 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.011	0.115	(-0.313, 0.335)	0.09	1.000
C3 - C1	0.042	0.115	(-0.282, 0.366)	0.36	0.999
C4 - C1	-0.000	0.115	(-0.324, 0.324)	-0.00	1.000
C5 - C1	0.131	0.115	(-0.193, 0.455)	1.14	0.771
C6 - C1	0.508	0.115	(0.184, 0.833)	1.41	0.651
C7 - C1	0.123	0.115	(-0.201, 0.447)	1.07	0.812
C8 - C1	0.360	0.115	(-0.036, 0.684)	4.13	0.025

Impact of CA on *PA* S34 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	-0.0073	0.0318	(-0.0969, 0.0822)	-0.23	1.000
C3 - C1	0.0055	0.0318	(-0.0840, 0.0951)	0.17	1.000
C4 - C1	0.0010	0.0318	(-0.0886, 0.0905)	0.03	1.000
C5 - C1	-0.0199	0.0318	(-0.1094, 0.0697)	-0.62	0.983
C6 - C1	0.0275	0.0318	(-0.0621, 0.1170)	0.86	0.919
C7 - C1	0.0302	0.0318	(-0.0594, 0.1197)	0.95	0.880
C8 - C1	0.2111	0.0318	(0.1215, 0.3006)	6.63	0.000

Impact of CA on *PA* S14 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.005	0.609	(-1.709, 1.719)	0.01	1.000
C3 - C1	0.018	0.609	(-1.696, 1.732)	0.03	1.000
C4 - C1	0.169	0.609	(-1.545, 1.883)	0.28	1.000
C5 - C1	0.492	0.609	(-1.222, 2.206)	0.81	0.939
C6 - C1	2.007	0.609	(0.293, 3.721)	1.30	0.588
C7 - C1	1.325	0.609	(-0.389, 3.039)	2.18	0.180
C8 - C1	0.855	0.609	(-0.859, 4.569)	3.40	0.017

Impact of CA on *PA* S27 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.005	0.609	(-1.709, 1.719)	0.01	1.000
C3 - C1	0.018	0.609	(-1.696, 1.732)	0.03	1.000
C4 - C1	0.169	0.609	(-1.545, 1.883)	0.28	1.000
C5 - C1	0.492	0.609	(-1.222, 2.206)	0.81	0.939
C6 - C1	1.507	0.609	(0.293, 3.321)	2.90	0.087
C7 - C1	1.325	0.609	(-0.389, 3.039)	2.18	0.180
C8 - C1	0.855	0.609	(-0.859, 2.569)	1.40	0.588

Appendix 3: The ANOVA test result for the effect of different concentration of bile acids on biofilm formation among 4 different strains of *PA*. C1 represent the control with *PA* strains incubated without adding bile acids.C2-C6 represent different concentration of bile acids added to the TSB (C2=9.4 µmol/L,C3=18.8 µmol/L, C4=37.5 µmol/L, C5=75 µmol/L and C6=150 µmol/L)

Appendix 3A: Effect of lithocholic acid on *PA* strains biofilm formation

Impact of lithocholic acid on *PA* S33 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.0410	0.0198	(-0.0254, 0.1075)	2.07	0.360
C3 - C1	0.0644	0.0198	(-0.0020, 0.1309)	3.26	0.059
C4 - C1	0.0805	0.0198	(0.0140, 0.1469)	4.07	0.015
C5 - C1	0.0831	0.0198	(0.0167, 0.1496)	4.20	0.012
C6 - C1	0.0831	0.0198	(0.0167, 0.1496)	4.20	0.012

Impact of lithocholic acid on *PA* S34 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.0252	0.0357	(-0.0948, 0.1451)	0.70	0.97830
C3 - C1	0.1536	0.0357	(0.0336, 0.2736)	4.30	0.010
C4 - C1	0.1637	0.0357	(0.0437, 0.2837)	4.58	0.006
C5 - C1	0.1657	0.0357	(0.0457, 0.2857)	4.64	0.006
C6 - C1	0.1657	0.0357	(0.0457, 0.2857)	4.64	0.006

Impact of lithocholic acid on *PA* S14 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.0612	0.0264	(-0.0276, 0.1499)	2.31	0.260
C3 - C1	0.0837	0.0264	(-0.0051, 0.1724)	3.17	0.069
C4 - C1	0.0876	0.0264	(-0.0011, 0.1764)	3.32	0.054
C5 - C1	0.0910	0.0264	(0.0023, 0.1797)	3.44	0.043
C6 - C1	0.1555	0.0264	(0.0667, 0.2442)	5.88	0.001

Impact of lithocholic acid on *PA* S27 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.1455	0.0432	(0.0004, 0.2906)	3.37	0.049
C3 - C1	0.1690	0.0432	(0.0239, 0.3141)	3.91	0.020
C4 - C1	0.1699	0.0432	(0.0248, 0.3150)	3.93	0.019
C5 - C1	0.1990	0.0432	(0.0539, 0.3441)	4.61	0.006
C6 - C1	0.1883	0.0432	(0.0432, 0.3334)	4.36	0.009

Appendix 3B: Effect of CDCA on *PA* strains biofilm formation

Impact of CDCA on *PA* S33 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.1520	0.0658	(0.0087, 0.2953)	2.31	0.039
C3 - C1	0.2130	0.0658	(0.0697, 0.3563)	3.24	0.007
C4 - C1	0.3490	0.0658	(0.2057, 0.4923)	5.31	0.000
C5 - C1	0.4363	0.0658	(0.2931, 0.5796)	6.64	0.000
C6 - C1	0.4970	0.0658	(0.3537, 0.6403)	7.56	0.000

Impact of CDCA on *PA* S34 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.2630	0.0312	(0.1950, 0.3310)	8.42	0.000
C3 - C1	0.5783	0.0312	(0.5103, 0.6464)	18.52	0.000
C4 - C1	0.6420	0.0312	(0.5740, 0.7100)	20.56	0.000
C5 - C1	0.6270	0.0312	(0.5590, 0.6950)	20.08	0.000
C6 - C1	0.5730	0.0312	(0.5050, 0.6410)	18.35	0.000

Impact of CDCA on *PA* PA14 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.262	0.199	(-0.317, 0.840)	1.31	0.574
C3 - C1	0.328	0.199	(-0.250, 0.906)	1.65	0.379
C4 - C1	0.581	0.199	(0.002, 1.159)	2.91	0.049
C5 - C1	0.624	0.199	(0.045, 1.202)	3.13	0.044
C6 - C1	0.566	0.199	(-0.012, 1.144)	2.84	0.040

Impact of CDCA on *PA* S27 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.2863	0.0310	(0.1963, 0.3763)	9.23	0.000
C3 - C1	0.2953	0.0310	(0.2053, 0.3853)	9.52	0.000
C4 - C1	0.5583	0.0310	(0.4683, 0.6483)	18.00	0.000
C5 - C1	0.5893	0.0310	(0.4993, 0.6793)	19.00	0.000
C6 - C1	0.6013	0.0310	(0.5113, 0.6913)	19.39	0.000

Appendix 3C: Effect of CDCA on *PA* strains biofilm formation

Impact of CA on *PA* S33 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.02967	0.00206	(0.02368, 0.03566)	14.37	0.000
C3 - C1	0.03617	0.00206	(0.03018, 0.04216)	17.52	0.000
C4 - C1	0.04970	0.00206	(0.04371, 0.05569)	24.07	0.000
C5 - C1	0.05507	0.00206	(0.04908, 0.06106)	26.67	0.000
C6 - C1	0.08440	0.00206	(0.07841, 0.09039)	40.88	0.000

Impact of CA on *PA* S34 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.08003	0.00454	(0.06687, 0.09320)	17.64	0.000
C3 - C1	0.14640	0.00454	(0.13324, 0.15956)	32.26	0.000
C4 - C1	0.17183	0.00454	(0.15867, 0.18500)	37.87	0.000
C5 - C1	0.17483	0.00454	(0.16167, 0.18800)	38.53	0.000
C6 - C1	0.21817	0.00454	(0.20500, 0.23133)	48.08	0.000

Impact of CA on PA S14 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.02217	0.00352	(0.01197, 0.03237)	6.31	0.000
C3 - C1	0.11883	0.00352	(0.10863, 0.12903)	33.80	0.000
C4 - C1	0.12680	0.00352	(0.11660, 0.13700)	36.07	0.000
C5 - C1	0.22210	0.00352	(0.21190, 0.23230)	63.17	0.000
C6 - C1	0.22483	0.00352	(0.21463, 0.23503)	63.95	0.000

Impact of CA on *PA* S27 biofilm formation

difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.0579	0.0158	(0.0142, 0.1015)	3.66	0.008
C3 - C1	0.1646	0.0158	(0.1210, 0.2083)	10.42	0.000
C4 - C1	0.1828	0.0158	(0.1392, 0.2264)	11.57	0.000
C5 - C1	0.2140	0.0158	(0.1704, 0.2577)	13.55	0.000
C6 - C1	0.2252	0.0158	(0.1816, 0.2688)	14.25	0.000

Appendix 3D: Effect of CDCA on *PA* strains biofilm formation

Impact of CA on *PA* S33 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.00820	0.00569	(-0.00832, 0.02472)	1.44	0.494
C3 - C1	0.01000	0.00569	(-0.00652, 0.02652)	1.76	0.324
C4 - C1	0.02333	0.00569	(0.00682, 0.03985)	4.10	0.006
C5 - C1	0.04567	0.00569	(0.02915, 0.06218)	8.02	0.000
C6 - C1	0.05338	0.00569	(0.03687, 0.06990)	9.38	0.000

Impact of CA on *PA* S34 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.00867	0.00924	(-0.01815, 0.03549)	0.94	0.812
C3 - C1	0.02100	0.00924	(-0.00582, 0.04782)	2.27	0.146
C4 - C1	0.03567	0.00924	(0.00885, 0.06249)	3.86	0.009
C5 - C1	0.04400	0.00924	(0.01718, 0.07082)	4.76	0.002
C6 - C1	0.04867	0.00924	(0.02185, 0.07549)	5.26	0.001

Impact of CA on *PA* PA14 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.0230	0.0102	(-0.0065, 0.0525)	2.26	0.149
C3 - C1	0.0207	0.0102	(-0.0089, 0.0502)	2.03	0.215
C4 - C1	0.0190	0.0102	(-0.0105, 0.0485)	1.87	0.276
C5 - C1	0.0431	0.0102	(0.0136, 0.0726)	4.24	0.005
C6 - C1	0.0448	0.0102	(0.0152, 0.0743)	4.40	0.004

Impact of CA on *PA* S27 biofilm formation

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
C2 - C1	0.0093	0.0236	(-0.0420, 0.0607)	0.40	0.699
C3 - C1	0.0287	0.0236	(-0.0226, 0.0801)	1.22	0.246
C4 - C1	0.0801	0.0236	(0.0287, 0.1314)	3.40	0.005
C5 - C1	0.0901	0.0236	(0.0387, 0.1414)	3.82	0.002
C6 - C1	0.0884	0.0236	(0.0370, 0.1398)	3.75	0.003