Improved methods for the detection of cystic fibrosis pathogens

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PhD 2022

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A thesis submitted in partial fulfilment of the requirements of Manchester Metropolitan University for the degree of Doctor of Philosophy

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Manchester Metropolitan University

2022

COVID-19 statement

In March 2020, the UK went into lockdown due to the COVID-19 pandemic. For 4 months the university laboratories shut, and all staff and students were told to work from home. In March 2020, I had just cracked the majority of my qPCR assays and was excited to apply them to the respiratory samples from the two clinical studies. However, because of the COVID-19 pandemic, this had to wait. When the laboratories re-opened in July 2020, space was limited due to social distancing and a booking system was put in place for lab space. Although I had limited access to the laboratory, office spaces in the university were still unavailable due to a work from home order from the government. With the help of an unpaid extension, I was able to collect qPCR and microbiota data on the respiratory samples from the two clinical studies. The writing of this thesis was mainly done from home whilst working a part-time job.

Abstract

Lung infection is the leading cause of morbidity and mortality in people with cystic fibrosis (CF). Routine microbiological surveillance is crucial to guide effective antimicrobial therapies. Culture-based methods are currently used for identification and surveillance of CF pathogens in respiratory samples. However, molecular methods such as quantitative PCR (qPCR) and microbiota sequencing can be more sensitive and informative. In this thesis, qPCR assays were optimised for the detection and enumeration of CF pathogens, and then applied along with microbiota sequencing to two clinical studies.

During the coronavirus pandemic in-person clinics were reduced, highlighting the need for remote microbiological surveillance. In a postal study, microbiology of fresh and posted samples from adult patients were assessed. Key pathogens were still detected in postal samples with culture (*A. xylosoxidans* 85%, *B. cepacia* complex 100%, *P. aeruginosa* 85%, *S. aureus* 87%, *S. maltophilia* 57%) and qPCR (*A. xylosoxidans* 70%, *B. cepacia* complex 95%, *P. aeruginosa* 87%, *S. aureus* 82%, *S. maltophilia* 65%). No overall significant difference was seen in pathogen abundances ($P>0.05$ for all targeted pathogens) between fresh and posted samples, however, there was variation in pathogen abundances and microbiota compositions in individual samples. This indicated posted samples could be utilised for remote microbiological surveillance, but with caution.

CF lung disease is characterised by periods of worsening symptoms, known as pulmonary exacerbations, which contribute to progressive loss of lung function. In an exacerbation study, microbiology was retrospectively assessed in sputum samples at start and end of antibiotic treatment for exacerbation, and at follow up. Pathogen targeted qPCR and microbiota sequencing indicated no significant difference ($P>0.05$) in the presence or abundances of CF pathogens or the bacterial microbiota between samples irrespective of antibiotic therapy. Prospective application of pathogen targeted qPCR could be a useful approach to inform effective treatment for exacerbation, allowing changes in antibiotic regimen to be implemented during treatment.

The work presented in this thesis highlights the utility of pathogen targeted qPCR and microbiota sequencing in providing rapid and accurate representation of the CF lung bacteria. Based on this, it is possible to recommend prospective use of such molecular approaches for pathogen detection and surveillance to enhance management of CF lung infections.

Acknowledgements

Firstly, I would like to thank my supervisors; Prof Christopher van der Gast and Dr Damian Rivett. Thank you for giving me this opportunity. Your help and support throughout the whole of this PhD has been greatly appreciated. Thank you for all your help with PAST and R, two complete unknowns for me, I really appreciate your patience.

I would like to thank the CF Trust for part funding this PhD. I would also like to thank all the staff who provided me with samples and metadata to allow me to do this research and all the people with CF who donated samples, without you I would not have been able to do any of this research.

I would like to thank the friends I have made at MMU; Helen, Xenia, Nathalie, Michelle and Ryan, for your friendship and support. Thank you for keeping me smiling and encouraging me to never give up. Also, thanks for every hot chocolate Tuesday, brunch club and afternoon chat around the radiator. I am glad you guys were along for the journey. Also thank you to my friends; Tom, Carl, Daniel and Chris, for the laughs and for putting up with my moaning over the past 4 years.

A huge thank you to my family for keeping me sane during the covid pandemic by walking for what feels like thousands of miles. A special thank you to my Mum and Dad for all your support; emotionally and financially. I would never have got here if it wasn't for both of you.

Finally, to my partner, Sam, thank you times a million. For the past 12 years, you have supported me unwaveringly and encouraged me through every high and low of this PhD. Thank you for everything.

Presentations

Work from this thesis was accepted for a poster presentation at European Cystic Fibrosis Society Conference 2020. However, the conference was cancelled and the abstract published in the Journal of Cystic Fibrosis. 'Hatfield, L., Bianco, B., Gavillet, H., Burns, P., Rivett, D., Jones, A., van der Gast, C. and Horsley, A. (2020) 'P088 Determining the effect of postage on the recovery of cystic fibrosis pathogens from respiratory samples.' *Journal of Cystic Fibrosis*, 19, p. S80.'

Part of Chapter 4 was presented at North American Cystic Fibrosis Conference 2020 in the form of a virtual poster entitled 'Determining the effect of postage on the recovery of cystic fibrosis pathogens from respiratory samples'. The abstract was published in Paediatric Pulmonology. 'Hatfield, L., Bianco, B., Gavillet, H., Burns, P., Rivett, D. W., Horsley, A. R., Jones, A. M. and van der Gast, C. J. (2020) 'Determining the effect of postage on the recovery of cystic fibrosis pathogens from respiratory samples.' Paediatric pulmonology, 55(S2) p. 166.'

Methods optimised in Chapter 2 were used and published ahead of print in Microbiology Spectrum: Gavillet, H., Hatfield, L., Rivett, D., Jones, A., Maitra, A., Horsley, A. and van der Gast, C Bacterial culture underestimates lung pathogen detection and infection status in cystic fibrosis https://doi.org/10.1128/spectrum.00419-22

Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviation	Definition
+ve	Positive
A. xylosoxidans	*Achromobacter xylosoxidans*
AMP	Adenosine monophosphate
ANOSIM	Analysis of similarity
ASV	Amplicon sequence variant
B. cenocepacia	*Burkholderia cenocepacia*
BAL	Bronchoalveolar lavage
Bcc	*Burkholderia cepacia* complex
BHQ	Black hole quencher
BLAST	Basic Local Alignment Search Tool
bp	Basepairs
CF	Cystic Fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
e.g.	For example
EDTA	Ethylenediaminetetraacetic acid
EMA	Ethidium monoazide
FAM	6-carboxyfluorescein
FEV	Forced expiratory volume
GES	Guanidinium thiocyanate-EDTA-Sarkosyl
GTDB	Genome Taxonomy Database
H. influenzae	*Haemophilus influenzae*
HEX	Hexachloro-fluorescein
I:A	Isopropanol: ammonium acetate
ITS	Internal transcribed spacer
LPA	Linear polyacrylamide
LRTI	Lower respiratory tract infection
MALDI-TOF-MS	Matrix-assisted laser desorption/ionisation – time of flight – mass spectrometry
min	Minutes
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin susceptible *Staphylococcus aureus*
n.d.	No date
N/A	Not available
NaCl	Sodium chloride
NGS	Next generation sequencing
NHS	National Health Service
NICE	National Institute for Health and Care Excellence

Abbreviation	Definition
No. of	Number of
NTHi	Non-serotypeable *Haemophilus influenzae*
NTM	Non-tuberculous mycobacterium
P. aeruginosa	*Pseudomonas aeruginosa*
P:C	Phenol:Chloroform
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMA	Propidium monoazide
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcription
S. aureus	*Staphylococcus aureus*
S. maltophilia	*Stenotrophomonas maltophilia*
S. pneumoniae	*Streptococcus pneumoniae*
SCV	Small colony variant
sec	Seconds
SIMPER	Similarity percentage analysis
SpC3	Spacer C3
spp	Multiple species
TAE	Tris base/acetic acid/EDTA
TAMRA	Carboxytetramethylrhodamine
TP	TaqMan Probe
T-RFLP	Terminal restriction fragment length polymorphism
UK	United Kingdom
US	United States
USA	United States of America
UV	Ultraviolet
VBNC	Viable but not culturable
-ve	Negative

Chapter 1 Introduction

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive genetic disorder, affecting more than 70,000 people worldwide (Cystic Fibrosis Foundation, n.d.). Clinical manifestations include lung infection (Lyczak *et al.*, 2002), pancreatic insufficiency (Singh and Schwarzenberg, 2017), gastrointestinal complications (Ooi and Durie, 2016; Marsh *et al.*, 2021), male infertility (Ahmad *et al.*, 2013) and diabetes (Kayani *et al.*, 2018). People with CF have a lower life expectancy than the healthy population; the median age of people with CF who died in 2020 in the UK was 36 (Cystic Fibrosis Trust, 2021) compared to 82.3-85.8 for the whole population of the UK from 2018-2020 (Office for National Statistics, 2020).

A person with CF has mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (O'Sullivan and Freedman, 2009). Over 2,000 different mutations have been identified in the CFTR gene which are grouped into several classifications based on their effect on the function (Ratjen, 2007). The most common mutation is known as ΔF508 and is the result of a deletion of phenylalanine at position 508 of the gene which leads to a misfolding of the CFTR protein during translation (Lukacs and Verkman, 2012). In 2020, at least one copy of ΔF508 was identified in 89% of patients in the UK (Cystic Fibrosis Trust, 2021) and 85.8% of patients in the US (Cystic Fibrosis Foundation, 2021). Research has previously focused on predicting the severity of disease from the genetic mutation. However, patients with the ΔF508 mutation display a wide variation in the rate of development and severity of lung disease (McKone *et al.*, 2006).

The CFTR protein is a cyclic-AMP-regulated chloride transport channel found in the membrane of epithelial cells (Lukacs and Verkman, 2012). The protein maintains an osmotic balance by regulating chloride secretion and sodium absorption across epithelial cells (Saint-Criq and Gray, 2017). Dysfunctional CFTR proteins lead to dehydration and thickened secretions in organs with an epithelial cell lining; particularly evident in the production of thick mucus in the lungs. CF affects multiple organs but mainly the respiratory and digestive systems. Pulmonary infections are the main cause of mortality and morbidity in people with CF (Cystic Fibrosis Foundation, 2017; Orenti *et al.*, 2018). The lungs are constantly exposed to microorganisms from the air and in healthy individuals, microorganisms are expelled from the lungs by the mucociliary escalator system. In a functional system, microorganisms are trapped in mucus and are transported by cilia to the nasopharynx where they are swallowed or expectorated (Bhagirath *et al.*, 2016). In the lungs of CF patients, thick mucus accumulates due to dehydration of the cell surfaces,

reducing the efficacy of the mucociliary escalator system. High volumes of thick mucus cause microorganisms to become trapped but cilia are unable to move the mucus. An inability to clear mucus and the continual production of mucus by goblet cells results in the formation of plaques and plugs (Boucher, 2007). These plugs obstruct the airways and allows the colonisation of microorganisms in the warm, moist environment (Harris *et al.*, 2007).

The life expectancy of people with CF has improved over time due to major advances in disease management and earlier detection of the disease. These advances include better nutritional information, earlier detection of pathogens (and therefore appropriate of antibiotics for lung infection), enzyme replacement therapy and CFTR modulators. CFTR modulator compounds are a relatively recent discovery and only four have been approved for use in the US and UK so far for people with specific CF mutations (Lopes-Pacheco, 2020). A small number of studies have shown CFTR modulators decrease the prevalence and acquisition of key CF pathogens (Singh *et al.*, 2019; Volkova *et al.*, 2020). More than 2,000 CFTR mutations have been identified so it has been suggested a multi-pronged approach may be required to target the many defective functions (Awatade *et al.*, 2018). Although CFTR modulators show promise in reducing pathogen prevalence, the current CFTR modulators do not work for all CFTR mutations.

1.2 Key pathogens in the cystic fibrosis airways

CF is a multisystemic disease, however, chronic lung infection is the leading cause of death in people with CF. Lung disease in CF patients begins early in life and inflammation, impaired mucociliary clearance and chronic infection lead to a decline in lung function interspersed with acute pulmonary exacerbations (Flume *et al.*, 2009). Chronic lung infections are usually managed using long term antibiotic therapies (Cystic Fibrosis Trust Antibiotic Group, 2009).

CF patients are routinely screened for the presence of key CF pathogens in respiratory samples to start treatment as early as possible. These key microorganisms include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans, Burkholderia cepacia* complex and *Aspergillus fumigatus*. Understandably, a large proportion of CF pulmonary disease research is focused on *P. aeruginosa* due to its presence in the lungs of over 40% of adult CF patients (Cystic Fibrosis Foundation, 2019; Cystic Fibrosis Trust, 2019). Despite this, research is also being conducted to determine the relevance of other microorganisms detected in the CF lung related to the progression and severity of CF lung disease (Jørgensen *et al.*, 2003; Jones *et al.*, 2004; Hansen *et al.*, 2010; Waters *et al.*, 2013). The CF Trust in the UK and CF Foundation in the USA recognise the importance of key CF pathogens and monitor their prevalence. The prevalence of the key pathogens in the USA in 2018 is shown in Figure 1.1. It has been shown there is generally an increase in the prevalence of *P. aeruginosa* and a decrease in *S. aureus* as people with CF age (Figure 1.1) (Cox *et al.*, 2010; Cystic Fibrosis Foundation, 2019; Cystic Fibrosis Trust, 2019).

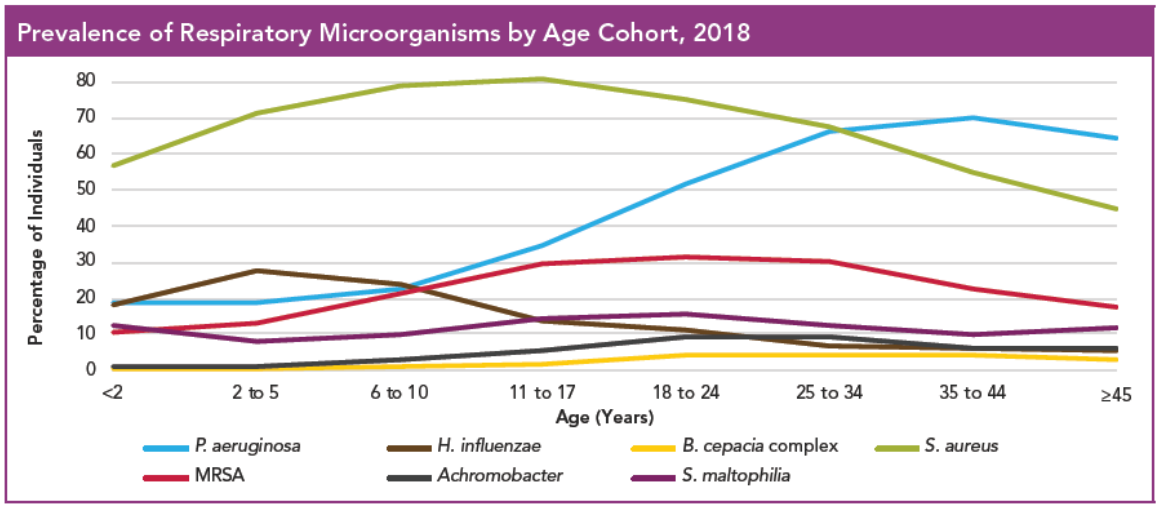

Figure 1.1 Prevalence of pathogens commonly isolated from CF patients in the USA in 2018 as recorded by the Cystic Fibrosis Foundation (2019).

1.2.1 *Pseudomonas aeruginosa*

P. aeruginosa is the primary pathogen in CF and was isolated from 43.2% of patients in the USA and 43.84% of patients in Europe in 2019 (Cystic Fibrosis Foundation, 2019; Orenti *et al.*, 2021). Initial *P. aeruginosa* infection of the CF lung is usually by motile and prototrophic strains, susceptible to a variety of antibiotics. Early detection and aggressive treatment to eradicate *P. aeruginosa* is important before patients develop chronic infection (Hansen *et al.*, 2008; Rivas Caldas *et al.*, 2015). Once a patient develops a chronic infection, *P. aeruginosa* exhibits phenotypic changes including loss of pigmentation, mucoid cells, auxotrophy, biofilm formation, expression of virulence factors and antibiotic resistance (Miller *et al.*, 2003; Goss and Burns, 2007; Moreau-Marquis *et al.*, 2008; Anuj *et al.*, 2009). Many of these changes lead to further epithelial cell damage and increased inflammation within the lungs (Béatrice *et al.*, 2005; Bhagirath *et al.*, 2016; Malhotra *et al.*, 2019). *P. aeruginosa* biofilms provide the cells within the biofilm increased tolerance to antibiotics and the body's immune defences (Aaron *et al.*, 2002). This contributes to chronic infection and chronic inflammation around biofilms (Høiby *et al.*, 2017). These phenotypic changes also cause identification using routine culture and treatment of infection difficult.

P. aeruginosa is the most commonly studied microorganism typically found in the CF lung. Therefore, the relationship between *P. aeruginosa* and CF lung disease is well understood and alternatives to the culture-based methods used to detect it have been developed (Table 1.2).

1.2.2 *Staphylococcus aureus*

S. aureus is the most prevalent pathogen in paediatric CF patients and still has a high prevalence in adult CF patients (Figure 1.1) (Cox *et al.*, 2010; Cystic Fibrosis Foundation, 2019). *S. aureus* is a commensal of the human skin and is often associated with wound infections. It has been shown that *S. aureus* is linked to a decline in lung function but its role in the progression of CF lung disease is not fully understood. *S. aureus* also exhibits phenotypic diversity including methicillin resistance, multidrug resistance, small colony variants and biofilm formation. The *mecA* gene causes methicillin resistance in *Staphylococcus* species (Fang and Hedin, 2003) and methicillin resistant *S. aureus* (MRSA) has increased in prevalence in the lungs of CF patients (Dasenbrook *et al.*, 2010; Cystic Fibrosis Foundation, 2020). It is important to distinguish MRSA from methicillin susceptible *S. aureus* (MSSA) to direct antibiotic therapy.

1.2.3 Other notable CF pathogens

The *B. cepacia* complex is comprised of at least 17 closely related species, originally mistaken as part of the *Pseudomonas* genus (LiPuma, 2010). These species are usually isolated from environmental sources such as soil and water (Bergmark *et al.*, 2012). However, not long after their discovery in soil they were also shown to be human pathogens (LiPuma, 2010). Although 16 out of the 17 *B. cepacia* complex species have been isolated from the lungs of CF patients, some species are more frequently isolated than others. The most common species isolated from CF lungs is *Burkholderia multivorans* followed by *Burkholderia cenocepacia* (Reik *et al.*, 2005; LiPuma, 2010; Kenna *et al.*, 2017). The prevalence of all *B. cepacia* complex species in CF patients is low relative to other species (Figure 1.1). However, it is a serious pathogen for CF patients, as infection with these species often leads to severe pneumonia, bacteraemia, sepsis and death (Jones *et al.*, 2004). During routine culture, other species often found in the CF lung as part of the microbiota (such as *Ralstonia* spp. and *Pandoraea* spp.) and other pathogens (such as *Achromobacter* spp.) are mistaken for *B. cepacia* complex species (Devanga Ragupathi and Veeraraghavan, 2019). This can lead to misdiagnosis and an incorrect treatment regimen. Species in the *B. cepacia* complex are also resistant to many antibiotics and disinfectants resulting in very few treatment options (Wright *et al.*, 2010). Genotyping has shown that patient-to-patient transmission of *B. cepacia* complex species in CF is common and hospitals often segregate *B. cepacia* complex infected patients (LiPuma, 2010). However, segregation may cause psychological issues by excluding people from attending support groups (Duff, 2002). The nature of *B. cepacia* complex members means rapid, accurate identification and correct segregation of colonised patients is of high importance in CF clinics.

S. maltophilia is widely found in aqueous environments and has increasingly being isolated from hospital equipment (Green and Jones, 2015). *S. maltophilia* is capable of forming biofilms (Pompilio *et al.*, 2010) and can produce multiple beta-lactamase enzymes (Cantón *et al.*, 2003). Both these factors contribute to antibiotic resistance in *S. maltophilia* including resistance to fluoroquinolones and penicillins (Leng *et al.*, 2017). The role of *S. maltophilia* infections in CF patients is unclear but it has been shown to decrease lung function and chronic infection is associated with increased risk of requiring a lung transplant or death (Goss *et al.*, 2004; Waters *et al.*, 2013; Barsky *et al.*, 2017). *S. maltophilia* is often isolated with *P. aeruginosa* and is capable of altering the virulence and development of antibiotic resistance in *P. aeruginosa* (Pompilio *et al.*, 2015).

A. xylosoxidans causes infection in immunocompromised patients and there has been an increased frequency of nosocomial outbreaks in CF patients (Lambiase *et al.*, 2011). In CF patients, multidrug resistant strains are often isolated, possibly due to the antibiotics administered for other isolated pathogens (Saiman *et al.*, 2001; Lambiase *et al.*, 2011). It is unclear the effect of *A. xylosoxidans* on the clinical outcome of CF patients (Hansen *et al.*, 2010; Lambiase *et al.*, 2011), but it is often misidentified as a *Pseudomonas* species using phenotypic identification leading to the use of incorrect antibiotic therapies (Saiman *et al.*, 2001). Other *Achromobacter* species have also been isolated from CF patients including *Achromobacter ruhlandii* (Spilker *et al.*, 2013), *Achromobacter insuavis* and *Achromobacter dolens* (Coward *et al.*, 2016) with unknown roles in CF lung infection.

H. influenzae is a common commensal of the upper respiratory tract in children and adults but can cause lower respiratory infections in immunocompromised people. There are multiple strains of *H. influenzae* and non-serotypeable *H. influenzae* (NTHi) is the most common isolated from CF as well as Chronic Obstructive Pulmonary Disease (COPD) and bronchiectasis (King, 2012; Price *et al.*, 2017). It is more prevalent in paediatric CF patients than adult CF patients (Figure 1.1) (Rosenfeld *et al.*, 2001). Other species of *Haemophilus* are also isolated from the lungs of CF patients, but these are considered to be non-pathogenic. Correct identification of *H. influenzae* is difficult as it displays a similar phenotype to other *Haemophilus* species (Murphy *et al.*, 2007).

A. fumigatus is one of the most common fungus detected in the CF lung with other *Aspergillus* species, such as *A. flavus*, *A. niger* and *A. terreus,* isolated less frequently (Delfino *et al.*, 2019). The prevalence of *Aspergillus* species in CF patients in the UK is monitored by the CF Trust (Cystic Fibrosis Trust, 2021). However, the CF Foundation do not note its prevalence in annual reports but do report cases of allergic bronchopulmonary aspergillosis (Cystic Fibrosis Foundation, 2021). In recent years, there has been an increase in the isolation of anti-fungal drug resistant *A. fumigatus*, which has been linked to high mortality rates (Guegan *et al.*, 2018b). Pulmonary aspergillosis is difficult to diagnose as often patients do not have symptoms. It is often diagnosed by the detection of *Aspergillus* species in respiratory samples.

1.3 Cystic fibrosis airway microbiota

In the 19th century, Robert Koch and Friedrich Loeffler defined criteria to describe the relationship between a microbe and a disease. Koch's postulates state that a microorganism must be found in all diseased individuals and should not be found in healthy individuals, the microorganism must be isolated and grown in pure culture, the microorganism should cause the disease when introduced into a healthy individual and must then be re-isolated from the experimental host (Nelson *et al.*, 2012). This led to the concept of 'one microbe, one disease'. However, multiple infectious diseases have been shown to be polymicrobial where several microorganisms that are not always culturable are involved in a single disease. Determining which diseases were incorrectly identified as monomicrobial is important for treatment (Vonaesch *et al.*, 2018).

Originally, it was thought that CF lung infections were caused by a small number of culturable pathogens, namely *S. aureus* in childhood and *P. aeruginosa* and *Burkholderia cepacia* complex in adults. However, culture-independent molecular studies have shown the lungs of CF patients have a high diversity of microorganisms including facultative and obligate anaerobes (Rogers *et al.*, 2004; Harris *et al.*, 2007; Fodor *et al.*, 2012; Parkins and Floto, 2015). Originally it was thought these 'other' bacterial species were contaminants from the upper respiratory tract but they have since been shown to be part of the CF lung microbiota (Rogers *et al.*, 2006). Although many potential pathogens have been identified from the CF lung, it is still unclear the role of these 'other' microorganisms in CF lung disease (Parkins and Floto, 2015). Several studies have shown the community of species colonising the CF lung differs between individuals (Stressmann *et al.*, 2011a, 2012; van der Gast *et al.*, 2011) and a reduction in richness and diversity is associated with older patients and individuals with decreased lung function (Cox *et al.*, 2010; Klepac-Ceraj *et al.*, 2010; van der Gast *et al.*, 2011; Zhao *et al.*, 2012; Cuthbertson *et al.*, 2020).

Microbiota research has led to the identification of bacterial species in the CF lung not typically targeted with culture-based methods such as *Rothia, Provetella* and *Veillonella* species (Tunney *et al.*, 2008; Zemanick *et al.*, 2013, 2017; Paganin *et al.*, 2015). The role of these taxa in CF lung disease is unclear. However, a small number of studies have associated *Rothia mucilaginosa* with a decline in lung function in people with CF (Paganin *et al.*, 2015) whereas Zemanick *et al.* (2013) and Zemanick *et al.* (2017) have shown an association between *Veillonella* and *Prevotella* taxa and decreased airway inflammation in people with CF.

1.4 Pulmonary exacerbations & treatment

CF patients often suffer from acute pulmonary exacerbations defined as a period of worsening symptoms which often cause a decline in lung function that does not recover following treatment. However, there is no standard clinical definition or criteria to define a pulmonary exacerbation. The diagnosis of an exacerbation is based on a range of symptoms including increased sputum production, shortness of breath, chest pain, decreased exercise tolerance, weight loss, increased cough and a decline in lung function (measured as FEV1% the ratio of forced expiratory volume in the first second (FEV1) to the forced vital capacity of the lungs (FVC)) (Goss and Burns, 2007; Cuthbertson *et al.*, 2016) and often relies on patient-reported outcome scores (Rogers *et al.*, 2011b). These scores are often unreliable as they are based on an individual's opinion. It has been suggested that identifying markers for pulmonary exacerbation could lead to more accurate diagnosis and improved therapies (Cuthbertson *et al.*, 2016).

Pulmonary exacerbations may be caused by a number of factors, but no single cause has been identified. These factors include an increase in bacterial load, the acquisition of a new bacterial strain or species, the expansion of the existing bacterial population into different areas of the lung, interactions between members of the microbial community, expression of bacterial virulence factors, viral infections, and environmental factors such as pollution (Rogers *et al.*, 2011b). Some research has indicated that exacerbations are caused by an increase in bacterial load, as shown for COPD (Garcha *et al.*, 2012), or the acquisition of a new bacterial species (Deschaght *et al.*, 2013). It was thought if bacterial load increase could be monitored and new species detected, an exacerbation could be treated prior to the onset of symptoms. However, molecular research has shown no significant changes in bacterial load prior to the onset of exacerbation (Stressmann *et al.*, 2011b; Price *et al.*, 2013) and no correlation between the acquisition of a new microbial community member and exacerbation (Aaron *et al.*, 2004; Fodor *et al.*, 2012). Although bacterial load has not been shown to cause exacerbation, antibiotics are still used to manage exacerbations. Previous studies have shown that patients appear to feel better and improve clinically after antibiotic treatment (Fodor *et al.*, 2012; Deschaght *et al.*, 2013; Smith *et al.*, 2014). It has also been suggested that exacerbations could be caused by the acquisition of a new genotype/phenotype (Sethi *et al.*, 2002). However, this hypothesis was based on research conducted on exacerbations in COPD patients and the data may not be transferrable to CF. Aaron *et al.* (2004) showed the genotype of *P. aeruginosa* in CF patients during

exacerbation was the same as during clinical stability. It has now been suggested that exacerbations are not caused by an increase in bacterial load or the acquisition of a new genotype but by a spatial alteration in the CF bacterial community where bacteria spread to a previously uninfected part of the airways (Fodor *et al.*, 2012).

1.4.1 Treatment of pulmonary exacerbations

Antibiotics are used for the treatment of lung disease in CF patients including maintenance therapy, treatment of exacerbations and treatment of infection with a new pathogen (Rogers *et al.*, 2010a). Maintenance antibiotic therapy has been shown to reduce the frequency of exacerbations but also effects untargeted bacteria in CF lungs (Nelson *et al.*, 2020).

When a patient is diagnosed with a pulmonary exacerbation, the identification of bacteria and their antibiotic susceptibility profiles from a previous respiratory sample can be used to develop an antibiotic treatment strategy. However, antibiotics are often chosen based on previous success for a patient, severity of disease, their cost, availability and toxicity (Rogers *et al.*, 2011a). To treat exacerbations, typically two antibiotics are administered for 10-14 days. To reduce the likelihood of antibiotic resistance developing, combinations of antibiotics are often taken with different modes of action (Cystic Fibrosis Trust Antibiotic Group, 2009).

The antibiotics prescribed are targeted to reduce specific bacterial species isolated from respiratory samples include beta-lactams, penicillins, cephalosporins, aminoglycosides and quinolones (Cystic Fibrosis Trust Antibiotic Group, 2009). These antibiotics are often also broad-spectrum and are given orally or intravenously. They have a range of mild to severe side effects as well as also affecting microorganisms in non-target organs (Cystic Fibrosis Trust Antibiotic Group, 2009). This, among other issues, can lead to an unbalanced gut microbiome causing more discomfort for the patient as well as an increased risk of renal failure (Cystic Fibrosis Trust Antibiotic Group, 2009).

The prescription of antibiotics can also vary between countries. While colistin is regularly prescribed in Europe, tobramycin is recommended in the US (Taccetti *et al.*, 2021). In lower-income countries, the choice of antibiotics can be limited due to the cost. For example, in South Africa, tobramycin is the only inhaled antibiotic available but is too expensive for the majority of patients (estimated to be accessible to roughly 10% of CF patients with health insurance). This has led to the investigation of alternative 'off-label' antibiotics such as

nebulising gentamicin solution available for intravenous administration (Van Stormbroek *et al.*, 2019).

Previous studies have indicated that during antibiotic therapy a significant decrease in *P. aeruginosa* abundance is seen within the first week of treatment (Deschaght *et al.*, 2013; Smith *et al.*, 2014). Therefore, antibiotics taken after the first week may be ineffective, a financial burden and lead to more discomfort for the patient. More research is required to determine the effect of antibiotics on other key CF pathogens within the first week of treatment.

1.5 Respiratory sampling for informing treatment

It is recommended that respiratory samples are taken frequently at routine check-ups (Cystic Fibrosis Trust, 2010). This informs clinicians on the acquisition of a new pathogen or development of antimicrobial resistance. The CF Trust recommends various sample types to analyse the microbiology of the CF lung including; spontaneous and induced sputum, cough swabs, cough plates and bronchoalveolar lavage (BAL) (Cystic Fibrosis Trust, 2010).

Spontaneous sputum samples are most commonly used for the detection of lung microbiology in people with CF and are provided by coughing hard to bring up sputum from the lungs. However, some people, particularly paediatrics and those with milder disease, are unable to expectorate sputum. Therefore, induced sputum, cough swabs and BAL are often utilised for these patients. To induce sputum, a nebuliser is used to administer a saline solution into the lungs to loosen the sputum before it is expectorated. Cough swabs and plates are commonly used for paediatrics as they are less invasive than induced sputum production or BAL sampling. To obtain a cough swab, a sterile swab is held at the back of the throat while the patient coughs. A cough plate is obtained by the patient coughing directly on to a bacterial agar plate. Research has shown that cough swabs and plates do not accurately represent the colonisation of the respiratory tract compared to sputum (Equi *et al.*, 2001; Cystic Fibrosis Trust, 2010; Fenn *et al.*, 2022). However, sometimes they are the only option for obtaining a sample from paediatric patients. BAL is considered the gold standard for the acquisition of respiratory samples, but the procedure is invasive and requires anaesthesia. Therefore, induced sputum is most frequently used in adult patients who cannot expectorate as the microbiological results have been shown to be comparable to BAL and spontaneous sputum (Henig *et al.*, 2001; Sagel *et al.*, 2001; Eyns *et al.*, 2018).

1.6 Culture-based detection methods

Culture-based methods are considered the gold standard for the detection of bacteria from infections in the clinical setting including pulmonary infections in CF patients. The CF Trust recommends the use of culture-based methods on respiratory samples (further detail in section 1.5) for the identification and susceptibility profiling of pathogens causing respiratory infection to inform antibiotic treatment. Standard culture methods are standardised across laboratories and include using selective media, different incubation temperatures and biochemical tests to isolate and identify species (Cystic Fibrosis Trust, 2010).

However, a previous quality assessment of laboratories in Europe providing diagnostic microbiology services to CF centres found issues with the current standard diagnostic methods (Hogardt *et al.*, 2009). Whilst common pathogens such as *P. aeruginosa* and *S. aureus* were correctly identified, uncommon and newly emerging pathogens were often misidentified or not detected at all, such as *B. cepacia* complex members and *A. xylosoxidans*. The report by Hogardt *et al.* (2009) was compiled in 2007 and 2008, thus some of the bacterial species which were newly emerging at the time are now targeted. However, some of the issues with correct identification may still be relevant. The assessment also used single isolates or a mixture of two to three isolates which is not a true representation of the polymicrobial nature of the CF lung. Therefore, it would be expected the performance of the laboratories in identifying species from real respiratory samples would be poorer.

Due to the polymicrobial nature of the CF lung and the presence of slow-growing strains and the phenotypical diversity exhibited by some pathogens, standard culture methods may not be appropriate to assess the microorganisms present. Some species such as *P. aeruginosa* are fast growing and can outgrow other species that may be present in low numbers (van Belkum *et al.*, 2000). This causes the species present in low numbers to be missed during identification or an under estimation of their load. Some species such as *P. aeruginosa* and *S. aureus* also demonstrate large phenotypic diversity including low pigmentation and formation of slow growing, small colony variants (SCVs), which leads to the misidentification of these species (Wellinghausen *et al.*, 2005; Le Gall *et al.*, 2013; Hery-Arnaud *et al.*, 2017). SCVs are small, nonpigmented colonies that have been shown to be more resistant to antibiotics than wild-type strains (Schneider *et al.*, 2008). *P. aeruginosa* and *S. aureus* are also capable of forming biofilms which can induce the development of

viable but not culturable (VBNC) cells (Moreau-Marquis *et al.*, 2008; Pasquaroli *et al.*, 2013). VBNC cells have low metabolic activity and, in some instances, a strengthened cell wall (Li *et al.*, 2014). The low metabolic activity has been attributed to why these cells are unculturable, meaning they are missed during routine culture-based diagnosis (Table 1.1). These traits alongside the extracellular matrix produced in a biofilm can also lead to unsuccessful antibiotic treatment (Aaron *et al.*, 2002). Antibiotic resistance in biofilms has shown to be linked to slow or incomplete penetration of the extracellular matrix by the antibiotic (Mah and O'Toole, 2001) and the presence of VBNC cells that have low metabolic activity (Li *et al.*, 2014). Some species are often mistaken for other species because of similar growth patterns and phenotypes. For example, *Pandorea* and *Ralstonia* which have been identified in the CF lung using sequencing techniques, are capable of growing on *B. cepacia* complex selective agar leading to the misidentification of these for *B. cepacia* complex species (Green and Jones, 2015).

As well as potential misidentification, there is another disadvantage to culture-based identification: the time from taking the sample to receiving results can take several days (Table 1.1) (Rogers *et al.*, 2004; Pattison *et al.*, 2013). As it is important to start antibiotic therapy as soon as possible, therapy decisions are usually based on the results from a previous sample taken up to 3 months ago because of the delay in receiving results from fresh samples (Cystic Fibrosis Trust Antibiotic Group, 2009).

Culture-based methods can also be used for quantification but is not usually carried out during the routine microbiology culture of respiratory samples (Cystic Fibrosis Trust, 2010). These methods are not always an accurate depiction of the bacterial load in respiratory samples due to the presence of slow growing bacteria, SCVs and VBNC cells (Rogers *et al.*, 2011b).

Standard culture methods are advantageous for determining the antibiotic susceptibility profiles of isolated bacteria. Molecular methods can be used to detect specific resistance genes but only a small selection of resistance gene assays have been approved and not all resistance genes have been identified (Pattison *et al.*, 2013). Although some resistance genes can be determined using molecular methods, susceptibility profiles to common antibiotics cannot. Therefore, culture-based susceptibility testing is recommended over molecular-based methods.

1.7 Molecular based detection methods

Molecular based techniques have been highlighted as quicker, more sensitive methods for bacterial detection in the clinical setting (Rogers *et al.*, 2009). Rentschler *et al.* (2021) compared the advantages and disadvantages of culture-based methods and molecular based methods (Table 1.1). Molecular based detection methods target molecules specific to the bacterial genus or strain. The most common detection method is PCR which detects and amplifies targeted nucleic acid sequences but other methods such as matrix-assisted laser desorption/ionisation – time of flight – mass spectrometry (MALDI-TOF-MS), which targets proteins, have also been developed. To understand the wider microbiota within the CF lung, sequencing techniques, such as high-throughput targeted gene sequencing, have been used on DNA extracted from respiratory samples without the need to isolate species.

Table 1.1 Summary of techniques currently used for the diagnosis of bacterial infections. (Source: [adapted from] Rentschler *et al.*, 2021 :12)

Method	Pathogen Identification (ID)	Time	RD	AST	Advantages and Disadvantages
Cell culture	Growth based; all culturable bacteria	24–72 h cultivation + 18–24 h for biochemical ID	-	√	+ Cost-effective + Good specificity – Long turnaround times – Lacking sensitivity – Prone to errors in workflow – Difficulties with fastidious organisms – Unculturable organisms not detectable
PCR-analysis and real-time PCR	Sequence dependent amplification of bacterial genes > pathogen-specific	One to several hours	√	-	+ No cultivation + Good performance – Expensive – A priori knowledge on suspected pathogens necessary – Turnaround time – High-end instrumentation
Next-generation sequencing	Simultaneous sequencing of billions of nucleic acid fragments contained in heterogenous samples > identification on subspecies or strain level based on SNPs	14–20 h	√	-	+ Primer independent + Identification without a priori knowledge or suspicion + Faster adaption to new resistance mechanisms – Complex workflow with experimental pitfalls and biases – High overall error rate – Differentiation between colonization and infection critical
MALDI-TOF; Direct sample testing	Generated mass spectrum of molecular sample composition compared to spectral database containing spectra from pure colonies (pre-cultivation); Cell enrichment followed by specific isolation	2–50 h	(√)	(√)	+ Automatable + Low costs per test + Fast analysis – Pre-cultivation necessary – Several resistance mechanisms not detectable – Identification of subspecies limited – Polymicrobial analysis difficult + No pre-cultivation – A priori knowledge necessary

RD – Resistance determination, AST – Antimicrobial susceptibility testing

1.7.1 MALDI-TOF-MS

MALDI-TOF-MS is currently available in many UK clinical laboratories for the identification of bacterial species and has been trialled for the detection of key pathogens from CF lungs (Degand *et al.*, 2008; Desai *et al.*, 2012). It identifies specific proteins within bacterial cells and compares the peptide signature to a database. This method is more reliable than identification that relies on phenotypic characteristics and has been successfully used to identify bacteria recovered from CF patients (Degand *et al.*, 2008). However, by relying on the isolation of bacteria from samples, MALDI-TOF-MS can still take days to identify

bacterial species (Burns and Rolain, 2014) (Table 1.1) and is still subject to the problem of some species outgrowing other species encountered in culture-based detection. It is also unable to quantify bacteria in samples.

1.7.2 PCR

PCR is commonly used for the identification of bacterial species in samples, however, it is not common in the CF clinical laboratory. PCR is used to target specific DNA sequences which can be used for identification of genus and/or species or to detect the presence of specific functional genes. This method is quicker, more accurate and sensitive than standard culture methods for identification (Jaffe *et al.*, 2001) and there is no need to isolate bacteria using culture before PCR is performed (Table 1.1). The CF Trust only recommends the use of PCR when a *B. cepacia* complex member has been isolated so the species can be accurately determined (Cystic Fibrosis Trust, 2010). Although useful for the identification of bacterial species and subspecies, there is a greater cost associated with using PCR and antibiotic susceptibility profiling cannot be accurately conducted.

Quantitative real-time PCR (qPCR) can be used to identify and quantify bacterial species within a variety of clinical samples without the use of culture (Espy *et al.*, 2006; Zemanick *et al.*, 2010). It is especially useful in determining the reduction of bacterial load in the lung of CF patients during antibiotic treatment (Price *et al.*, 2013; Reid *et al.*, 2013; Zemanick *et al.*, 2013; Stokell *et al.*, 2015).

qPCR is similar to conventional PCR as DNA is amplified using gene specific primers. However, in qPCR a fluorescent probe or dye that bind to double stranded DNA is added. The most common dye used is SYBR Green which binds to any double stranded DNA. Probes, however, are designed to be specific to the target gene. When using either probe or dye, during the PCR reaction, the amount of fluorescence increases as the amount of amplified DNA increases (Espy *et al.*, 2006). The qPCR machine measures the fluorescence throughout the PCR reaction and creates an amplification curve. The concentration of DNA within the original sample can be estimated using the cycle number (Ct or Cq value) at which the fluorescence crosses a threshold. Samples with a higher DNA concentration pass the threshold sooner and therefore have a lower Ct value than those with a lower DNA concentration. A standard curve can be created using known quantities of bacteria to estimate the number of colony forming units (CFU) per ml in a sample (Bustin *et al.*, 2009).

The use of qPCR to identify and quantify CF pathogens in respiratory samples has multiple advantages over the currently used culture-based methods. The main advantage is that nucleic acids are extracted directly from the respiratory sample, so bacteria do not need to be isolated prior to identification. Bacterial species are often misidentified using culture methods due to sharing similar phenotypic traits with other species (Rogers *et al.*, 2011b). This technique decreases the likelihood of misidentification as it targets the DNA of the bacteria. This technique also means anaerobic species, VBNC cells and species at low abundances are not missed. This technique is rapid, sensitive and could be carried out using automated platforms (Rogers *et al.*, 2011b; Rocchetti *et al.*, 2018). As with PCR, qPCR is also more expensive than culture-based methods and susceptibility profiling is not possible. However, the cost of this method may be outweighed by its accuracy and sensitivity meaning optimal antibiotics could be prescribed quickly reducing the financial burden and bed capacity of the healthcare system.

1.7.2.1 Previously developed quantitative PCR assays

qPCR methods have been developed to allow the identification and enumeration of key CF lung pathogens in a range of sample types; the studies have targeted multiple genes using qPCR with varying degrees of success (Table 1.2). A literature search also highlighted that more studies have developed, or utilised, qPCR targeted at *P. aeruginosa* than any other key CF pathogen.

Multiple studies determined the specificity and sensitivity of the developed qPCR in comparison to culture-based methods. The sensitivity and specificity were high (>80%) for the majority of these studies. However, some developed methods required further development to be comparable to culture-based methods. This may be due to the choice of target gene or primers. Sensitivity is defined as the percentage of samples that had the pathogen detected using culture-based methods that the qPCR assay also defined as positive for the pathogen. Whereas specificity refers to the detection of the target pathogen rather than nonspecific targets (Bustin *et al.*, 2009). Lower sensitivities (<80%) have been linked to the target gene being a single-copy gene (Louie *et al.*, 2002). It has also been suggested that a lower than 100% specificity may be due to qPCR detecting DNA from non-viable cells; although this can be remedied with the use of propidium monoazide (PMA), a DNA chelator, to exclude DNA from amplification during qPCR (discussed further in 1.7.4). Hery-Arnaud *et al.* (2017) and Fothergill *et al.* (2013) showed qPCR was capable of detecting lower numbers of bacterial cells than culture-based methods causing a >100%

specificity when targeting *P. aeruginosa*. Hery-Arnaud *et al.* (2017) also showed patients who gave *P. aeruginosa* culture negative and PCR positive samples became culture positive within 8 months indicating that culture was unable to detect *P. aeruginosa* when PCR could.

Previous research has shown the potential of qPCR for the detection of key CF pathogens. However, the use of qPCR to enumerate the pathogens in the lungs has not been as thoroughly researched. Curran *et al.* (2007), Zemanick *et al.* (2010) and Garcha *et al.* (2012) have shown that qPCR is more sensitive than culture-based methods for the enumeration of pathogens in respiratory samples.

Table 1.2 Literature review of quantitative PCR studies.

Target pathogen	Target gene	Sample environment/ type	Sample size	qPCR probe/ type	Specificity (%)	Sensitivity (%)	Detection limit (CFU/ml or gene copies)	Reference
A. xylosoxidans	*blaOxa-114*	CF lung/ respiratory samples	402	TP/ Multiplex	97	78	10^2 CFU/ ml	(Rocchetti *et al.*, 2018)
Aspergillus spp.	mitochondrial gene	Lung/ BAL	64	TP/ Single	81	83	10^2 conidia/ ml	(Fréalle *et al.*, 2009)
B. multivorans	*recA*	Lung epithelial cells/ lab strain	N/A	SYBR/ Single	N/A	N/A	200 CFU	(Wright *et al.*, 2010)
B. cepacia complex	*16S rRNA*	CF lung/ sputum	136	SYBE/ RT Single	N/A	N/A	N/A	(Reid *et al.*, 2013)
B. cepacia complex	*16S rRNA*	CF lung/ respiratory samples	402	TP/ Multiplex	99	80	10^2 CFU/ml	(Rocchetti *et al.*, 2018)
B. cepacia complex	conserved 12-kDa protein	CF lung/ sputum	130	SYBR/ Single	N/A	N/A	N/A	(Stokell *et al.*, 2015)
Haemophilus haemolyticus and *H. influenzae*	*hypD, siaT* and *fucP*	Culture/ clinical isolates	143	TP/ Triplex	N/A	N/A	10 copies (*fucP* and *siaT*), 100 copies (*hypD*)	(Price *et al.*, 2017)
H. influenzae	*23S rRNA*	CF lung/ sputum	136	SYBR/ RT Single	N/A	N/A	N/A	(Reid *et al.*, 2013)

Target pathogen	Target gene	Sample environment/ type	Sample size	qPCR probe/ type	Specificity (%)	Sensitivity (%)	Detection limit (CFU/ml or gene copies)	Reference
H. influenzae	*bexA*	Suspected meningitis patients/ cerebrospinal fluid specimens	111	TP/ Single	88	93	200 genome copies/ reaction	(Wang *et al.*, 2011)
H. influenzae	*fucP* and *hpd*	Otitis media/ nasopharyngeal swabs and middle ear effusions	80	TP/ Duplex	100	100	6 copies	(de Gier *et al.*, 2016)
H. influenzae	*glpQ*	Nose/ nasal swabs	111	TP/ Single	N/A	N/A	$6.5x10^2$ cells/ml	(Smith-Vaughan *et al.*, 2006)
H. influenzae	*Hel*	Non-CF bronchiectasis lung/ sputum	107	TP/ Single	N/A	100	N/A	(Rogers *et al.*, 2014)
H. influenzae	*hpd*	Indigenous children with acute otitis media/ naso-pharyngeal and ear discharge swabs	58	TP/ Single	N/A	N/A	N/A	(Marsh *et al.*, 2012)
H. influenzae	*hpd#3*	Suspected meningitis patients/ cerebrospinal fluid specimens	111	TP/ Single	87	93	40 genome copies/ reaction	(Wang *et al.*, 2011)
H. influenzae	*fucK*	Suspected LRTI patients/ sputum	62	TP/ Single	N/A	N/A	N/A	(van den Munckhof *et al.*, 2019)
H. influenzae	N/A	CF lung/ swabs, sputum, saliva	122	SYBR/ Single	60	65	8.1 copies/ reaction	(Zemanick *et al.*, 2010)

Target pathogen	Target gene	Sample environment/ type	Sample size	qPCR probe/ type	Specificity (%)	Sensitivity (%)	Detection limit (CFU/ml or gene copies)	Reference
H. influenzae	P4 lipoprotein	COPD lung/ sputum	373	TP/ Multiplex	N/A	N/A	105 CFU/ml	(Garcha *et al.*, 2012)
H. influenzae	P6	LRTI lung/ BAL	187	TP/ Multiplex	65	90	3-30 copies	(Alarcón *et al.*, 2006)
H. influenzae	P6 outer membrane protein	CF lung/ sputum	5	SYBR/ RT Single	N/A	N/A	N/A	(Nelson *et al.*, 2010)
Haemophilus spp.	P6	COPD lung/ sputum	30	SYBR/ Nested (2 step)	57	100	654 copy/ml	(Curran *et al.*, 2007)
P. aeruginosa	*16S rRNA*	CF lung/ sputum	5	SYBR/ RT Single	N/A	N/A	N/A	(Nelson *et al.*, 2010)
P. aeruginosa	*16S rRNA*	CF lung/ sputum	136	SYBR/ RT Single	N/A	N/A	N/A	(Reid *et al.*, 2013)
P. aeruginosa	*16S rRNA*	CF lung/ clinical isolates	88	TP/ Single	100	98.1	N/A	(Wellinghausen *et al.*, 2005)
P. aeruginosa	*16S rRNA*	CF lung/ swabs, sputum, saliva	122	SYBR/ Single	88	82	N/A	(Zemanick *et al.*, 2010)
P. aeruginosa	*23S rDNA*	CF lung/ swabs, sputum	542	TP/ Single	92	100	85 CFU/ml	(McCulloch *et al.*, 2011)
P. aeruginosa	*23S rDNA*	CF lung/ sputum	130	SYBR/ Single	N/A	N/A	N/A	(Stokell *et al.*, 2015)
P. aeruginosa	*algD*	Culture/ CF clinical isolates	200	SYBR/ Single	100	89	N/A	(Qin *et al.*, 2003)
P. aeruginosa	*algD* and *gyrB*	CF lung/ swabs, sputum	1837	TP/ Multiplex	92	94	10 copies/ reaction	(Logan *et al.*, 2010)

Target pathogen	Target gene	Sample environment/ type	Sample size	qPCR probe/ type	Specificity (%)	Sensitivity (%)	Detection limit (CFU/ml or gene copies)	Reference
P. aeruginosa	*ecfX*	CF lung/ sputum	88	SYBR/ Single	81	100	70cells/ml	(Mangiaterra *et al.*, 2018)
P. aeruginosa	*ETA*	Culture/ CF clinical isolates	200	SYBR/ Single	100	96	N/A	(Qin *et al.*, 2003)
P. aeruginosa	*gyrB*	CF lung/ swabs, sputum, BAL	459	TP/ Single	74	100	47 copies/ reaction	(Fothergill *et al.*, 2013)
P. aeruginosa	*gyrB*	CF lung/ swabs, sputum, BAL	459	SYBR/ Single	72	100	5800 copies/ reaction	(Fothergill *et al.*, 2013)
P. aeruginosa	*gyrB*	Clinical specimens/ sputum, urine, faeces	152	SYBR/ Single	98.4	100	0.39 CFU/5µl	(Motoshima *et al.*, 2008)
P. aeruginosa	*gyrB*	Culture/ lab strain	1	SYBR/ Single	N/A	N/A	N/A	(Tavernier and Coenye, 2015)
P. aeruginosa	*gyrB* and *ecfX*	CF lung/ saliva, sputum	14	TP/ Single	100	100	10^3 CFU/ml	(Rivas Caldas *et al.*, 2015)
P. aeruginosa	*gyrB* and *ecfX*	CF lung/ sputum	46	TP/ Multiplex	90	100	730 CFU/ml	(Le Gall *et al.*, 2013)
P. aeruginosa	*oprL*	CF lung/ sputum, nasopharyngeal aspirations	754	Biodetect kit/ Single	93	100	N/A	(Bassani *et al.*, 2013)
P. aeruginosa	*oprL*	Rat lung/ homogenised lung	76	TP/ RT Single	N/A	N/A	10^3 cells/ml	(Béatrice *et al.*, 2005)
P. aeruginosa	*oprL*	CF lung/ sputum	103	TP/ Single	70	N/A	150 CFU/ml	(Billard-Pomares *et al.*, 2011)
P. aeruginosa	*oprL*	CF lung/ sputum	312	TP/ Single	71.5	93.5	N/A	(Blanchard *et al.*, 2018)

Target pathogen	Target gene	Sample environment/ type	Sample size	qPCR probe/ type	Specificity (%)	Sensitivity (%)	Detection limit (CFU/ml or gene copies)	Reference
P. aeruginosa	*oprL*	CF lung/ sputum, throat swabs	379	TP/ Single	81.1	94.3	N/A	(Boutin *et al.*, 2018)
P. aeruginosa	*oprL*	CF lung/ sputum	8	TP and SYBR/ Single	N/A	N/A	N/A	(Deschaght *et al.*, 2009)
P. aeruginosa	*oprL*	CF lung/ nasopharyngeal and throat swabs, sputum	852	TP/ Single	85	90	N/A	(Deschaght *et al.*, 2010)
P. aeruginosa	*oprL*	CF lung/ sputum	27	TP/ Single	N/A	N/A	N/A	(Deschaght *et al.*, 2013)
P. aeruginosa	*oprL*	Pneumonia lung/ BAL, sputum	110	TP/ Single	98.85	100	10^3 CFU/ml	(Feizabadi *et al.*, 2010)
P. aeruginosa	*oprL*	Culture/ clinical isolates	58	SYBR/ Single	N/A	N/A	10 CFU/ml	(Jaffe *et al.*, 2001)
P. aeruginosa	*oprL*	CF lung/ sputum	46	TP/ Single	73	100	10 CFU/ml	(Le Gall *et al.*, 2013)
P. aeruginosa	*oprL*	CF lung/ sputum	15	TP/ Single	N/A	N/A	N/A	(Nguyen *et al.*, 2016)
P. aeruginosa	*oprL*	Burn wound/ skin biopsy samples	21	TP/ Single	N/A	N/A	10^3 CFU/g	(Pirnay *et al.*, 2000)
P. aeruginosa	*oprL*	Culture/ CF clinical isolates	200	SYBR/ Single	99	99	N/A	(Qin *et al.*, 2003)
P. aeruginosa	*oprL*	CF lung/ saliva, sputum	20	TP/ Single	N/A	N/A	N/A	(Rivas Caldas *et al.*, 2015)
P. aeruginosa	*oprL*	Non-CF bronchiectasis lung/ sputum	107	TP/ Single	N/A	96.9	N/A	(Rogers *et al.*, 2014)

Target pathogen	Target gene	Sample environment/ type	Sample size	qPCR probe/ type	Specificity (%)	Sensitivity (%)	Detection limit (CFU/ml or gene copies)	Reference
P. aeruginosa	*oprL, gyrB* and *ecfX*	CF lung/ sputum	707	TP/ 2 step	86.3	94.3	N/A	(Hery-Arnaud *et al.*, 2017)
P. aeruginosa	*regA*	CF lung/ respiratory samples	402	TP/ Multiplex	99	97	10^2 CFU/ml	(Rocchetti *et al.*, 2018)
P. aeruginosa	*regA*	CF lung/ sputum	28	TP/ Single	N/A	N/A	N/A	(Rogers *et al.*, 2010b)
P. aeruginosa	*regA*	CF lung/ sputum	30	TP/ Single	N/A	N/A	N/A	(Rogers *et al.*, 2013a)
P. aeruginosa	*regA*	CF lung/ sputum	132	TP/ Single	N/A	N/A	N/A	(Stressmann *et al.*, 2011b)
P. aeruginosa	*rplU*	CF lung/ sputum	19	SYBR/ Single	N/A	N/A	25 gene copies	(Filkins *et al.*, 2012)
P. aeruginosa	*rplU*	CF lung/ sputum	46	SYBR/ Single	N/A	N/A	N/A	(Price *et al.*, 2013)
S. aureus	*16S rRNA*	CF lung/ swabs, sputum	87	SYBR/ Single	100	58.1	$1x10^3$ CFU/ml	(Johnson *et al.*, 2016)
S. aureus	*16S rRNA*	CF lung/ swabs, sputum, saliva	122	SYBR/ Single	98	46	N/A	(Zemanick *et al.*, 2010)
S. aureus	DNA gyrase	CF lung/ sputum	136	SYBR/ RT Single	N/A	N/A	N/A	(Reid *et al.*, 2013)
S. aureus	*femA*	CF lung/ saliva, sputum	87	TP/ Single	100	80.6	$1x10^3$ CFU/ml	(Johnson *et al.*, 2016)
S. aureus	*nuc, mecA* and *lukS*-PV	Suspected LRTI patients/ sputum	62	TP/ Multiplex	N/A	N/A	N/A	(van den Munckhof *et al.*, 2019)
S. aureus	*mecA*	Culture/ clinical and reference isolates	289	SYBR/ Duplex	100	N/A	N/A	(Fang and Hedin, 2003)

Target pathogen	Target gene	Sample environment/ type	Sample size	qPCR probe/ type	Specificity (%)	Sensitivity (%)	Detection limit (CFU/ml or gene copies)	Reference
S. aureus	*nuc*	COPD lung/ sputum	30	SYBR/ Nested (2 step)	43	100	6570 copy/ml	(Curran *et al.*, 2007)
S. aureus	*nuc*	Culture/ clinical and reference isolates	289	SYBR/ Single	89.6	93.3	1-10 CFU/ml	(Fang and Hedin, 2003)
S. maltophilia	*23S rRNA*	CF lung/ clinical isolates	100	SYBR/ Single	N/A	N/A	N/A	(Gallo *et al.*, 2013)
S. maltophilia	*23S rRNA*	Biofilm/ clinical strain	17	TP/ Single	N/A	N/A	N/A	(Melloul *et al.*, 2016)
S. maltophilia	*23S rRNA*	Biofilm/ clinical strain	9	TP/ Single	N/A	N/A	N/A	(Melloul *et al.*, 2018)
S. maltophilia	*metB*	CF lung/ respiratory samples	402	TP/ Multiplex	85	96	10^3 CFU/ml	(Rocchetti *et al.*, 2018)

1.7.3 Microbiota composition analysis

Techniques such as Terminal Restriction Fragment Length Polymorphism (T-RFLP) have allowed the determination of microbial communities in polymicrobial samples. T-RFLP was a common technique used to profile bacterial communities in CF sputum samples (Rogers *et al.*, 2005, 2006; Sibley *et al.*, 2008; Paganin *et al.*, 2015). Despite its usefulness, the use of T-RFLP has been replaced with next generation sequencing due to its poor resolution (Rogers and Bruce, 2010).

Next generation sequencing (NGS) has become a more popular method for the detection and identification of bacterial species and taxa in the microbiota. In shotgun metagenomic sequencing, all genomic DNA present in the sample is sequenced whereas targeted sequencing can utilise targeted primers, such as 16S rRNA or internal transcribed spacer (ITS) situated in the rRNA subunit genes, to target conserved sequences in bacteria and fungi, respectively. Similar to PCR, NGS is capable of highlighting the presence of species that may be present in low abundance as well as VBNC cells and unculturable species. Targeted sequencing such as Roche 454 pyrosequencing and Illumina sequencing have previously been used to identify members of the CF lung microbiota (Bittar *et al.*, 2008; Delhaes *et al.*, 2012; Fodor *et al.*, 2012; Zhao *et al.*, 2012). Although 454 pyrosequencing provided longer read lengths, Illumina-based sequencing now provides a larger throughput with lower error rates (Loman *et al.*, 2012; Jünemann *et al.*, 2013). Operational taxonomical units (OTUs) have been used to assign taxa to sequences from targeted sequencing methods. OTUs are clusters of sequences with a sequence identity of 97% which reduces the size of the 16S rRNA data. By clustering sequences, the impact of sequence errors is reduced on downstream analysis. However, as sequencing platforms such as Illumina have improved error rates during sequencing, amplicon sequence variants (ASVs) can now be commonly used. ASVs can accurately be defined as 'true' sequences (rather than an erroneous sequence) with one nucleotide difference between separate ASVs. The use of ASVs has allowed the identification of sequences down to strain level (Chiarello *et al.*, 2022).

High-throughput targeted gene sequencing has increasingly been used to study the lung microbiota of people with CF including changes during disease progression (Stokell *et al.*, 2015; Cuthbertson *et al.*, 2020) and at the onset of and during an exacerbation (Carmody *et al.*, 2013, 2015; Cuthbertson *et al.*, 2016). NGS can also be used in clinical trial studies to determine changes in the microbiota following antibiotic treatment, not just targeted

bacterial species (Fodor *et al.*, 2012; Price *et al.*, 2013; Smith *et al.*, 2014; Cuthbertson *et al.*, 2016; Nelson *et al.*, 2020).

NGS is an important tool for bacterial identification. However, in some cases the ability to identify bacteria to the species and subspecies level is limited. One such case is species of the *B. cepacia* complex where targeting genes specific to the complex are more discriminatory (Bittar and Rolain, 2010). NGS is currently only used in a research context and is not used in the clinical laboratory.

The cost of NGS and the need for experienced technicians limits its use in the clinical laboratory (Table 1.1). Large volumes of data are produced from NGS, the bioinformatics required to process the output data into bacterial species and the advancing and changing nature of NGS means experienced technicians and continuous training are required (Pattison *et al.*, 2013). Its use in the clinical laboratory would be invaluable in terms of providing a complete picture for informing timely and appropriate antibiotic selection.

1.7.4 Propidium monoazide

Critics of the use of DNA-based techniques, such as NGS based approaches and qPCR, for the detection of microorganisms in clinical samples have highlighted that these methods are unable to distinguish DNA present in extant cells from extracellular DNA or DNA from dead or damaged cells (Nocker and Camper, 2006; Rogers *et al.*, 2008, 2010b, 2013a). Clearance of DNA from lysed cells is reduced in the lungs of CF patients so qPCR can overestimate the amount of bacteria present (Stressmann *et al.*, 2011b).

DNA binding dyes that are unable to permeate cell membranes have been shown to overcome this problem. Ethidium monoazide (EMA) was first suggested for use in microscopy, flow cytometry and then PCR. EMA intercalates with DNA when exposed to light and can enter cells with permeable cell membranes. Once chelated, the DNA becomes insoluble and is lost during DNA extraction (Nocker and Camper, 2006). Although EMA looked promising, it was shown to penetrate the viable cells of *Escherichia coli* as well as other bacterial species (Nocker and Camper, 2006). This would lead to viable bacterial cells not being detected using PCR and the underestimation of those species in samples.

Nocker *et al.* (2006) therefore tested an alternative to EMA called propidium monoazide (PMA) which is derived from propidium iodide. Propidium iodide has been extensively used to stain dead cells when distinguishing live and dead cells during microscopy (ThermoFisher, n.d.). Several studies have now shown that PMA is a more selective

alternative to EMA (Rogers *et al.*, 2008, 2010b, 2013a; Tavernier and Coenye, 2015; Nguyen *et al.*, 2016). PMA covalently binds to extracellular DNA (due to its inability to permeate cell membranes, DNA from live cells is not affected) and inhibits amplification during PCR. It is now common practice to use PMA prior to DNA extraction for NGS (Stressmann *et al.*, 2012; Daniels *et al.*, 2013; Cuthbertson *et al.*, 2014, 2015, 2016, 2020; Stokell *et al.*, 2015; Nguyen *et al.*, 2016) but is not as common prior to qPCR (Stressmann *et al.*, 2011b; Deschaght *et al.*, 2013; Stokell *et al.*, 2015).

1.8 Aims & Objectives

Current culture-based methods utilised in standard diagnostic tests for the detection of CF pathogens in respiratory samples do not fully portray the CF lung. The current standard diagnostic methods for the detection of CF pathogens involve utilising selective media and biochemical tests. The presence of different phenotypes and the fast growth of some bacterial species has led to bacterial species being misidentified or missed using culture-based methods. These methods also take two to four days to identify targeted species. Molecular based methods are quicker and more accurate at identifying bacterial species than the standard culture-based methods. Molecular methods such as PCR can be utilised to target specific bacterial species whereas NGS techniques can be used to investigate the bacterial microbiota.

The aim of this research was to determine the usefulness of qPCR for the enumeration of pathogens and high-throughput gene targeted sequencing to determine the bacterial microbiota in respiratory samples from CF patients. To do this the following objectives were drawn:

- To optimise previously published qPCR assays targeting known CF pathogens to be utilised in further studies (Chapter 3).
- To determine the effects of postage on the microbiology of respiratory samples by investigating the abundance of pathogens by qPCR (Chapter 4) and the bacterial microbiota by gene targeted sequencing (Chapter 5).
- To determine the effects of antibiotic treatment for an exacerbation on the abundance of CF pathogens by qPCR (Chapter 6) and the bacterial microbiota by gene targeted sequencing (Chapter 7).

Chapter 2 Materials and Methods

2.1 Bacterial isolates

Bacterial isolates from cystic fibrosis patients were used to optimise the qPCR protocols, as standards during qPCR analysis and as controls during high-throughput targeted gene sequencing. Bacterial isolates were isolated from CF patients at Wythenshawe Hospital, Manchester and gifted from Public Health England (Table 2.1). The lab strain PA01 of *Pseudomonas aeruginosa* was used and a strain of *Haemophilus influenzae* was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Table 2.1 Bacterial isolates used in this study.

Organism	Source	Isolate No.
Achromobacter xylosoxidans	PHE	90305
Burkholderia cenocepacia	PHE	90537
Haemophilus influenzae	DSMZ	DSM 24049
Pseudomonas aeruginosa	MMU	PA01
Staphylococcus aureus	PHE	91388
Stenotrophomonas maltophilia	PHE	90335

DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures), MMU – Manchester Metropolitan University, PHE – Public Health England

Bacterial isolates were cultured on nutrient agar (Oxoid, Hampshire, UK) or nutrient broth (Oxoid, Hampshire, UK) under aerobic conditions except *Haemophilus influenzae,* which was cultured on chocolate agar (EO Labs, Bonnybridge, Scotland, UK). All isolates except *Burkholderia cenocepacia* were incubated at 37 °C for 24 hours. *Burkholderia cenocepacia* was incubated at 30 °C for 48 hours.

2.2 Clinical samples

Spontaneous expectorated sputum from patients with CF attending the Manchester CF Centre, UK and Southampton University Hospital, UK were used in this study. Ethical approval and patient consent were gained before the collection of samples (NHS REC numbers: 15/NW/0409 and 08/H0502/126, respectively). Samples collected at Southampton University Hospital were collected in sterile containers and stored at -80 °C prior to processing. Samples collected at Manchester CF Centre were split and half sent in the post (further details in Chapter 4).

2.3 DNA extraction

2.3.1 Sputum wash

Sputum samples were washed with sterile phosphate-buffered saline (PBS). Sputum (500 µl) was mixed with 5 ml of PBS in a sterile centrifuge tube. If samples contained less than 500 µl, 100 µl was mixed with 1 ml of PBS. After mixing by vortex for a couple of seconds, samples were centrifuged at 4000 rpm for 5 min. The supernatant was removed and the sputum re-suspended in 500 µl of PBS.

2.3.2 PMA treatment

PMA was obtained in solid form from Cambridge Bioscience (Cambridge, UK) and suspended in 20% dimethyl sulfoxide (DMSO) (Fisher Scientific, Loughborough, UK) to make a 20 mM working stock solution which was stored in the dark at -20 °C. A previously described protocol for DNA cross-linking using PMA with slight modification was used (Rogers *et al.*, 2008; Cuthbertson, 2014). Washed sputum was added to amber Eppendorf micro-centrifuge tubes (SLS, Wilford, UK) with PMA to a final concentration of 50 µM (as per manufacturer's instructions). Samples were mixed by vortex for 10 sec prior to incubation at room temperature for 30 min, mixing by vortex for 10 sec every 15 min. After incubation, samples were transferred to clear micro-centrifuge tubes and place in an LED light box for 15 min. Samples were then stored at -20 °C prior to further processing.

2.3.3 DNA extraction

The DNA from grown bacterial isolates and PMA treated sputum samples were extracted using a previously developed phenol-chloroform DNA extraction method with slight modifications (Rogers, van der Gast, *et al.*, 2013). This is summarised in Figure 2.1. Sputum was split into batches for DNA extraction and 500 µl of sterile PBS was extracted in parallel for each batch to act as a negative control. DNA extraction was also conducted on overnight cultures of bacterial isolates (Table 2.1) to serve as positive controls.

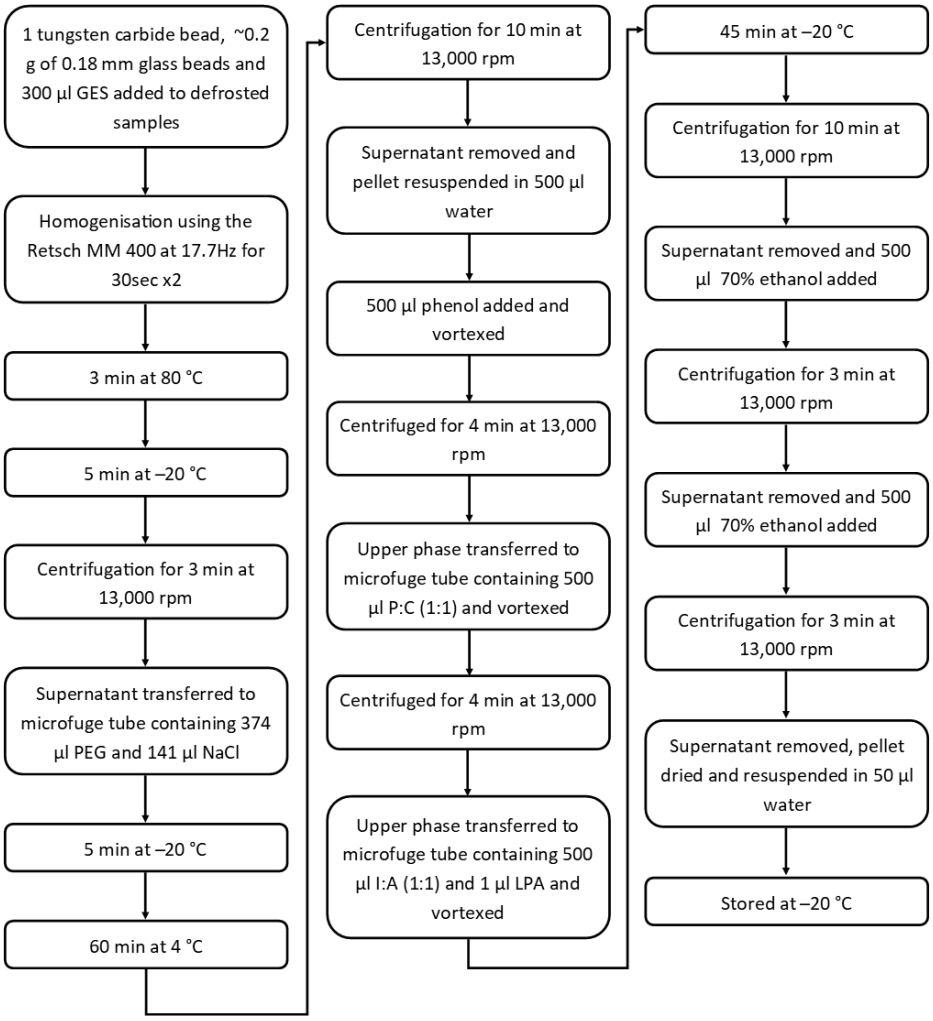

Figure 2.1 Workflow diagram for DNA extraction of washed and PMA treated sputum samples.

GES: Guanidinium thiocyanate-EDTA-Sarkosyl (Sigma Aldrich, Dorset, UK, Fisher Scientific, Loughborough, UK, Sigma, UK), PEG: Polyethylene glycol (Merck, UK), NaCl: Sodium chloride (Fisher Scientific, Loughborough, UK), P:C: Phenol:Chloroform (Sigma Aldrich, Dorset, UK, Sigma Aldrich, Dorset, UK), I:A: Isopropanol: ammonium acetate (Fisher Scientific, Loughborough, UK, Fisons Scientific Equipment, Glasgow, UK), LPA: Linear polyacrylamide (Alfa Aeser, Heysham, UK).

2.4 DNA sequencing

2.4.1 Sequencing

The bacterial community was assessed by amplicon-based sequencing of the V5-6 region of the 16S rRNA gene. A 2-step PCR was used to prepare samples for MiSeq sequencing. The first step of the PCR added Illumina adapters (Supplementary Table 1) (obtained from Invitrogen, Paisley, UK). Each PCR reaction contained 10 µl 5x Q5 Buffer (New England Biolabs, Hitchin, UK), 0.5 µl Q5 High-fidelity polymerase (New England Biolabs, Hitchin, UK), 1 µl 10 mM mixed dNTPs (New England Biolabs, Hitchin, UK) and 1 µM of each primer in a total volume of 50 µl.

The following parameters were used; 98 °C for 30 sec, 35x (98 °C for 10 sec, 50 °C for 10 sec & 72 °C for 20 sec) and 72 °C for 10 min. The presence of a product was confirmed using gel electrophoresis (further detailed in 3.2.2).

Following the first step PCR, the ZR-96 DNA clean up kit (Cambridge Bioscience, Cambridge, UK) was used according to the protocol to purify the PCR products. The second step PCR was used to add individual Illumina barcodes (Supplementary Table 2). Each PCR reaction contained 10 µl 5x Q5 Buffer (New England Biolabs, Hitchin, UK), 0.5 µl Q5 High-fidelity polymerase (New England Biolabs, Hitchin, UK), 1 µl 10 mM mixed dNTPs (New England Biolabs, Hitchin, UK), 0.25 µM of each primer and 20 µL of cleaned PCR product in a total volume of 50 µl.

The following parameters were used; 98°C for 30 sec, 10x (98°C for 10 sec, 62°C for 20 sec & 72°C for 30 sec) and 72°C for 2 min. The presence of a product was confirmed using gel electrophoresis (further detailed in 3.2.2).

PCR products were normalised to 25 ng using the Invitrogen SequelPrep Normalisation Kit (Invitrogen, Paisley, UK) before being pooled. The pooled samples were then concentrated to a volume of 100 µl using the MiVac concentrator DNA-2305-A00 (Fisher Scientific, Loughborough, UK). The Qubit dsDNA HS assay kit (Invitrogen, Paisley, UK) was used to quantify the condensed library using the Qubit 3 fluorometer (Invitrogen, Paisley, UK) before dilution to 4 nM. The library and a phiX control were denatured and diluted to 20 pM following the Illumina guide (Illumina Inc, 2019). The 20 pM library was diluted in HT1 with a 10% 20 pM phiX spike to a concentration of 10 pM. Samples were sequenced on the Illumina MiSeq platform using the Illumina MiSeq V3 600 cycle reagent kit (Illumina Cambridge Ltd, Cambridge, UK).

2.4.2 Sequencing analysis

The R package dada2 was used to remove primer sequences, quality filter and trim, join paired-end reads and remove chimeras (Callahan *et al.*, 2016). The R code for preparing the sequences and assigning taxa can be found online (Callahan, n.d.). Forward and reverse reads were truncated at 200bp and 175bp, respectively. The Silva (Quast *et al.*, 2013) and Genome Taxonomy Database (GTDB) (Alishum, 2019) databases was trialled to taxonomically assign sequences. The GTDB assigned a greater number of sequences so was used. Any unassigned amplicon sequence variant (ASV) were manually assigned using the Basic Local Alignment Search Tool (BLAST) nucleotide database (National Center for Biotechnology Information (NCBI), n.d.) and matched appropriately based on query coverage.

2.5 Statistical analysis

2.5.1 Presence/absence data

Generalised linear models on presence/absence data and odd ratios were performed and calculated using the package lmerTest and lme4 in R version 3.6.2 (Bates *et al.*, 2015; Lam *et al.*, 2015; Kuznetsova *et al.*, 2017; Singh *et al.*, 2019).

2.5.2 Comparison of CFU/ml

Data were assessed against the assumptions for parametric statistics using Shapiro-Wilk's test for normality and Bartlett's and Levene's tests for homogeneity of variance using the car package in R (Fox and Weisberg, 2019). For those data that conformed to the assumptions, paired t-tests were used to determine significant differences between the means of two groups. For those that failed to meet the assumptions, a Wilcoxon rank sum test was undertaken.

Mixed effect models were used to compare the CFU/ml equivalents, as determined by qPCR, of samples at the start and end of treatment and at follow up using the package lmerTest in R version 3.6.2 (Kuznetsova *et al.*, 2017). There is high variability in the bacterial microbiota between CF patients (Rogers *et al.*, 2005; Harris *et al.*, 2007; van der Gast *et al.*, 2011). Mixed effects models allow this variation to be considered as a random effect while the CFU/ml was compared between groups as a fixed effect (Cuthbertson *et al.*, 2016). Values of R^2 were calculated using the package MuMIn in R version 3.6.2 (Bartoń, 2020).

2.5.3 Diversity

Measures of taxa richness, Fisher's alpha index of diversity and Berger-Parker index of dominance were calculated in PAST (PAleontological STatistics) package (Hammer *et al.*, 2001). Richness is defined as the number of taxa present in each sample. Fisher's alpha index of diversity is independent of sample size when sequence reads per sample >1000 so is ideal for comparing communities of different sizes (Magurran, 2004). Berger-Parker index of dominance compares the number of individuals in the dominant taxa to the total number of individuals. This measures the numerical importance of the most abundant taxa.

2.5.4 Similarity

PAST was used to calculate Sørensen and Bray-Curtis measures of similarity. The community composition of samples could be compared using Sørensen and Bray-Curtis. Sørensen similarity is a presence/absence-based measure of similarity between two data

sets (Magurran and McGill, 2011). Whereas Bray-Curtis takes into account both the number of shared species and the abundance of the shared species (Magurran, 2004).

Analysis of similarity (ANOSIM) was used to determine if two or more groups had significantly different community composition. ANOSIM was carried out using the package vegan in R (Jari Oksanen *et al.*, 2020) on Sørensen and Bray-Curtis measures of similarity. ANOSIM generates two values; the test statistic (R) and the probability (P) that two groups are significantly different. P values indicate if the communities of the two groups are significantly different. ANOSIM R values scale from +1 to -1 and indicate if similar samples are found within the group. +1 indicates similar samples are within the same group, 0 indicates similarities have no relationship with the group and -1 indicates similar samples are outside of the group (Hedin *et al.*, 2016).

Similarity percentage analysis (SIMPER) was calculated using the package vegan in R to determine the contribution of identified species on the overall Bray-Curtis similarity between two groups (Clarke, 1993).

Chapter 3 Optimisation of qPCR for the detection of key CF lung pathogens

3.1 Introduction

Frequently taking respiratory samples from people with cystic fibrosis (CF) is recommended to screen for known bacteria (Cystic Fibrosis Trust, 2010; Smyth *et al.*, 2014). In clinically stable patients, check-ups are recommended every 3-6 months (National Institute for Health and Care Excellence (NICE), 2017). When a pulmonary exacerbation is diagnosed, samples are taken more frequently during treatment to inform antibiotic therapy (Smyth *et al.*, 2014). Screening for known pathogens frequently informs targeted, timely treatment and can indicate the effectiveness of treatment. Accurate and rapid identification of pathogens is important as patients at outpatient clinics are often segregated by the presence of pathogens to prevent cross-infection (Kerem *et al.*, 2005).

Upon acquisition, samples are processed using culture-based techniques, which are considered the gold standard, including the use of selective agars and biochemical tests to determine the pathogens present and their susceptibility profiles (Cystic Fibrosis Trust, 2010; Public Health England, 2019). A small number of culturable pathogenic bacteria are associated with CF lung infections including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, members of the *Burkholderia cepacia* complex, *Haemophilus influenzae*, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* (Cystic Fibrosis Foundation, 2019). These key pathogens are associated with driving the inflammatory response and decreased lung function in people with cystic fibrosis (Elborn, 2008). During an exacerbation, antibiotic therapy is deemed successful if the respiratory sample is negative for the previously detected pathogen or if the patients' symptoms improve. A culture-negative sample may be misleading to clinicians as the pathogen may have reduced to below the detectable limit of culture-based methods. Culture-based methods and culture-independent methods could also be used to quantify the bacterial species before, during and after antibiotic treatment but are not routinely conducted. This could allow clinicians to take a different approach to therapy part way through a treatment regime.

Culture-based methods of detection are beneficial for the detection of CF pathogens because of the cheap cost, standardised methods and the ability to determine susceptibility profiles. However, the reliability of culture-based methods for CF pathogen detection has been questioned (Bittar *et al.*, 2008; Burns and Rolain, 2014). Culture-based methods take days to yield results and may fail to detect slow-growing organisms or misidentify small colony variants, which could lead to the microbial burden being underestimated. Alternatively, culture-independent techniques that use DNA from samples, such as qPCR,

can be used as they are not effected by phenotypic differences or competition from other bacterial species (van Belkum *et al.*, 2000), can detect pathogens present in low numbers and can yield results more rapidly (Burns and Rolain, 2014).

The use of qPCR to monitor changes in the bacterial densities of key CF pathogens in respiratory samples is important for monitoring the effectiveness of antibiotic therapy in a clinical setting as well as in clinical trials. Other studies have previously developed qPCR assays for the detection of key CF pathogens in a variety of sample types including sputum (Table 1.2). The majority of previous studies focused on one pathogen rather than the need to develop a suite of qPCR assays to detect multiple pathogens. A small number of studies have used multiple qPCR assays to detect and enumerate key CF pathogens in children with acute otitis media (Marsh *et al.*, 2012), people with lower respiratory tract infections (van den Munckhof *et al.*, 2019) and Chronic Obstructive Pulmonary Disease (COPD) (Curran *et al.*, 2007; Garcha *et al.*, 2012) and CF (Zemanick *et al.*, 2010; Reid *et al.*, 2013; Rocchetti *et al.*, 2018) respiratory samples. Zemanick *et al.* (2010) showed that qPCR assays targeting *P. aeruginosa, S. aureus, H. influenzae* and five anaerobes in oropharyngeal and sputum samples were reliable and reproducible. Reid *et al.* (2013) used RT-qPCR assays to determine changes in the bacterial load of *P. aeruginosa, H. influenzae*, *Streptococcus pneumoniae*, *B. cepacia* complex and *Aspergillus fumigatus* during pulmonary exacerbation. The study also tried to develop a Reverse transcription (RT)-qPCR assay for *S. aureus* without success. Reverse transcription qPCR is used to generate cDNA from RNA which has the advantage of only amplifying genes from viable cells. However, RNA rapidly degrades and requires the addition of a preservation agent when the sample is given. Rocchetti *et al.* (2018) optimised a multiplex qPCR assay on the BD MAX system to detect the presence of A*. xylosoxidans, B. cepacia* complex*, P. aeruginosa* and *S. maltophilia*. The BD MAX system performs DNA extraction and qPCR on one instrument in clinical settings. An all-in-one automated system is useful in the clinical setting and reduces the risk of user error. However, in comparison to a qPCR machine costing ~£26,000 (Bio-Rad CFX96), the BD MAX system is expensive at ~£138,000 (correct July 2021).

In addition to the option of utilising RT-qPCR, researchers must also decide whether to use SYBR Green or probe based qPCR assays. SYBR Green binds to any double stranded DNA (dsDNA) in a sample and fluoresces. The more dsDNA present in the sample, the larger the fluorescent signal detected by the qPCR machine (ThermoFisher Scientific, n.d.; Espy *et al.*, 2006; Tajadini *et al.*, 2014). Probe-based qPCR assays utilise a probe consisting of a

fluorescent reporter dye and a quencher attached to a specific DNA sequence. When the probe is intact the dye and the quencher are in close proximity and the quencher dye quenches the fluorescent emissions from the dye. When the DNA sequence anneals to the target sequence on DNA present in the sample, the fluorescent dye is cleaved which separates the dye from its quencher probe. This leads to an increase in fluorescence (ThermoFisher Scientific, n.d.; Espy *et al.*, 2006; Tajadini *et al.*, 2014). Probe-based qPCR assays are more specific than SYBR Green based qPCR assays due to the specific DNA sequence. However, SYBR Green is cheaper and can be shared by multiple researchers making it more appealing in research settings (Tajadini *et al.*, 2014).

This study aimed to optimise qPCR protocols for the detection and enumeration of the key pathogens monitored by the CF Foundation without the need for reverse transcription or a relatively expensive piece of equipment such as the BD MAX. This study did not use an automated system, such as the BD MAX, which reduces the risk of user error. However, the BD MAX is expensive and therefore unlikely to be implemented into a clinical research laboratory. An array of qPCR assays have previously been developed targeting different genes to enumerate CF pathogens (Table 1.2). Primers and probes were selected from the literature and protocols were optimised using cultured bacterial isolates (Table 2.1) by altering the reaction mixes and qPCR conditions. The limit of detection and qPCR efficacy of each protocol was determined.

3.2 Methods

3.2.1 Bacterial standards

Bacterial strains (Chapter 2, Table 2.1) were grown from frozen stocks in 10 ml of nutrient broth for 24 hours at 37 °C whilst shaking at 100 rpm except *Burkholderia cenocepacia*. *B. cenocepacia* was incubated at 30 °C for 48 hours whilst shaking at 100 rpm. After incubation, bacterial cultures were centrifuged at 4000 rpm for 5 min to pellet the bacterial cells. The supernatant was removed, and the pellet resuspended in 500 µL of PBS. The DNA was extracted from 500 µL of the resuspended cells as described in Chapter 2 section 2.3.3. The CFU/ml of the grown isolates was determined using the Miles and Misra technique (Miles *et al.*, 1938).

3.2.2 Confirmation PCR

Before qPCR could be used to enumerate pathogens in CF respiratory samples, the primers were first optimised for PCR and qPCR with pure bacterial isolates. Primers were selected from previously published PCR and qPCR assays (Table 1.2). Confirmation PCR was performed to determine the correct size amplicon was amplified from pure bacterial isolates using published parameters. The primers used are shown in (Table 3.1) and were obtained from Life Technologies Ltd (Paisley, UK) and prepared as per the manufacturer's instructions.

Table 3.1 Primers and probes used for this study.

Target	Primer/probe Sequence (5' to 3')	Expected product size	Reference
16S rRNA (*A. xylosoxidans*)	F; GCAGGAAAGAAACGTCGCGGGT R; ATTTCACATCTTTCTTTCCG P; N/A	163bp	(Liu *et al.*, 2002)
recA (*B. cepacia* complex)	F; TGACCGCCGAGAAGAGCAA R; CTCTTCTTCGTCCATCGCCTC P; N/A	1,043bp	(Mahenthiralingam *et al.*, 2000)
16S rRNA (*B. cepacia* complex)	F; AGRGTTYGATYMTGGCTCAG R; CCGRCTGTATTAGAGCCA P; N/A	463bp	(Bauernfeind *et al.*, 1999; Reid *et al.*, 2013)
16S rRNA (*B. cepacia* complex)	F; TCCGGAAAGAAAWCCTTGGY R; AATGCAGTTCCCAGGTTGAG P; N/A	196bp	(Rocchetti *et al.*, 2018)
fucP & hpd (*H. influenzae*)	F; GCCGCTTCTGAGGCTGG R; AACGACATTACCAATCCGATGG P; FAM-TCCATTACTGTTTGAAATAC-BHQ1 F; GGTTAAATATGCCGATGGTGTTG R; TGCATCTTTACGCACGGTGTA P; HEX-TTGTGTACACTCCGT /BHQ1/ GGTAAAAGAACTTGCAC-SpC3	68 151	(de Gier *et al.*, 2016)
oprL (*P. aeruginosa*)	F; CGAGTACAACATGGCTCTGG R; ACCGGACGCTCTTTACCATA P; FAM–CCTGCAGCACCAGGTAGCGC-TAMRA	117bp	(Feizabadi *et al.*, 2010; Rogers *et al.*, 2014)
nuc (*S. aureus*)	F; CGCTACTAGTTGCTTAGTGTTAACTTTAGTTG R; TGCACTATATACTGTTGGATCTTCAGAA P; FAM-TGCATCACAAACAGATAACGGCGTAAATAGAAG-TAMRA	124bp	(Alarcón *et al.*, 2006)
23S rRNA (*S. maltophilia*)	F; GCTGGATTGGTTCTAGGAAAACGC R; ACGCAGTCACTCCTTGCG P; N/A	278bp	(Gallo *et al.*, 2013)

F – forward primer, R – reverse primer, P – probe, N/A – not applicable due to the use of SYBR Green, FAM – 6-carboxyfluorescein, BHQ1 – Black hole quencher, TAMRA – Carboxytetramethylrhodamine, SpC3 – Spacer C3

The Hain Lifescience Q-cycler 96 PCR machine (Hain Lifescience GmbH Nehren, Germany), Q5 High Fidelity 2x Master Mix (New England Biolabs UK Ltd, Hitchin, UK) and nuclease free water (Qiagen, Manchester, UK) were used for each reaction to a total volume of 25 µL or 20 µL depending on the target/primers. The following PCR parameters were used for all reactions with alterations to the annealing temperature and cycle number; 95 °C for 10 min, y cycles of (95 °C for 15 sec, z °C for 30 sec & 72 °C for 1 min) followed by extension at 72 °C for 10 min where y and z are to be determined during optimisation. A negative control was also run with each experiment consisting of the same reaction mix with nuclease free water instead of DNA template.

Following PCR, a 1.5% agarose (Fisher Scientific UK, Loughborough, UK) gel prepared with 1x Tris base/acetic acid/EDTA (TAE) buffer with the addition of SYBR safe (Invitrogen, Paisley, UK) to visualise PCR products alongside a 1 Kb plus DNA ladder (Invitrogen, Paisley, UK). Electrophoresis was carried out at 120V for 60 min. Bands were visualised using the Li-COR Odyssey Fc (LI-COR Biosciences UK Ltd, Cambridge, UK).

3.2.3 Quantitative PCR

Following PCR confirmation, qPCR was carried out using the published parameters. If these conditions were not optimal for qPCR, optimisation was carried out by altering the annealing temperature, number of cycles and primer and probe concentrations (Table 3.2).

Real-time qPCR amplifications were performed using the Bio-Rad CFX connect (Bio-Rad Laboratories Ltd, Watford, UK) machine. For SYBR Green qPCR, SYBR Green PCR MasterMix from Applied Biosystems (Paisley, UK) was used. For TaqMan qPCR, TaqMan Gene Expression MasterMix from Applied Biosystems (Paisley, UK) was used. TaqMan probes were obtained from Eurofins Genomics UK Ltd (Wolverhampton, UK) and prepared as per the manufacturer's instructions.

Firstly, the published parameters were used with SYBR Green for qPCR. This allowed the visualisation of melt peak/s. The annealing temperature that gave a strong single melt peak was then chosen. The cycle number was chosen based on the cycle number at which no Cq value was seen in the negative control, but a Cq value was still seen in the positive control.

Table 3.2 PCR conditions tested during optimisation of primers.

Primer set	Primer concentrations (nM)	Cycles	Annealing temperature (°C)
16S rRNA (*A. xylosoxidans*)	200, 250, 400, 1000	30, 35	56
recA (*B. cepacia* complex)	20,000	30	58, 60, 62, 64, 66
16S rRNA (*B. cepacia* complex)	10, 37.5, 50, 100, 500, 1000	40	54
16S rRNA (*B. cepacia* complex)	2000, 1000, 500, 250	42	54, 56, 58, 60, 62, 64
hpd & fucP (*H. influenzae*)	200, 400, 500, 800, 1000	40	60
oprL (*P. aeruginosa*)	200, 400, 500	35, 40, 45	50, 52, 54, 56, 58, 60, 62, 64, 68, 70
nuc (*S. aureus*)	200, 300, 400	30, 35, v40	46, 50, 52, 54, 56, 60
23S rRNA (*S. maltophilia*)	200, 1000	30, 35	52, 54, 55, 56, 58, 60, 68

3.2.3.1 *Pseudomonas aeruginosa* quantitative PCR

For the *P. aeruginosa oprL* primers, Rogers *et al.* (2014) conducted qPCR using 500 nM of each primer, 45 cycles and an annealing temperature of 58 °C. However, Feizabadi *et al.* (2010) used 200 nM of each primer, 35 cycles and an annealing temperature of 60 °C. The PCR was optimised by testing different primer concentrations, primer annealing temperatures and number of cycles (Table 3.2).

3.2.3.2 *Staphylococcus aureus* quantitative PCR

For the *S. aureus nuc* primers, Alarcón, Vicedo and Aznar (2006) conducted qPCR using 300 nM of each primer, 30 cycles and an annealing temperature of 60 °C. The qPCR was optimised by testing different primer concentrations, primer annealing temperatures and number of cycles (Table 3.2).

3.2.3.3 *Burkholderia cepacia* complex quantitative PCR

To target the *B. cepacia* complex *recA* gene, BCR1 and BCR2 primers described by Mahenthiralingam *et al.* (2000) were optimised for PCR and then trialled using SYBR Green qPCR. However, the SYBR Green qPCR showed two peaks on the melt curves. This implied that two products were produced during qPCR. The DNA amplicon of the qPCR should be 1,043bp. It is hypothesised that the large amplicon size led to inefficient amplification. Optimisation of the qPCR was attempted by trialling extended annealing (45 sec, 1 min) and extension (1 min, 1 min 30 sec) times and multiple annealing temperatures (Table 3.2).

These primers could not be optimised and a different set of primers that had previously been used for qPCR were trialled.

The *B. cepacia* complex primers targeting the *16S rRNA* gene published by Reid *et al.* (2013) did not require optimising for PCR. The PCR was conducted using 500 nM of each primer, 40 cycles and an annealing temperature of 54 °C as described by Reid *et al.* (2013). However, SYBR Green qPCR showed two peaks on the melt curves. It was believed that the latter peak was caused by the SYBR binding to the DNA extract and the earlier peak was caused by SYBR binding to primer dimers. Therefore, multiple concentrations of primers were trialled (Table 3.2). However, a balance between no primer dimers and no DNA amplification could not be found. Therefore, a third set of primers was trialled.

For the third set of primers trialled, Rocchetti *et al.*, (2018) conducted PCR using 1800 nM of each primer targeting the *16S rRNA* gene, 42 cycles and an annealing temperature of 60 °C. The PCR was optimised by testing multiple primer concentrations and primer annealing temperatures (Table 3.2).

3.2.3.4 *Stenotrophomonas maltophilia* quantitative PCR

For the *S. maltophilia 23S rRNA* primers, Gallo *et al.* (2013) conducted PCR using 1000nM of each primer, 30 cycles and an annealing temperature of 68 °C. The qPCR was optimised by testing different primer concentrations (200 nM, 1000 nM), primer annealing temperatures and number of cycles (Table 3.2).

3.2.3.5 *Achromobacter xylosoxidans* quantitative PCR

For the *A. xylosoxidans 16S rRNA* primers, Liu *et al.* (2002) conducted PCR using 1000 nM of each primer, 30 cycles and an annealing temperature of 56 °C. The qPCR was optimised by testing different primer concentrations and number of cycles (Table 3.2).

3.2.3.6 *Haemophilus influenzae* quantitative PCR

For the *H. influenzae hpd* primers, de Gier *et al.* (2016) conducted qPCR using 1000 nM of each primer, 40 cycles and an annealing temperature of 60 °C. The qPCR was optimised by testing different primer concentrations (Table 3.2).

For the *H. influenzae fucP* primers, de Gier *et al.* (2016) conducted qPCR using 1000 nM of each primer, 40 cycles and an annealing temperature of 60 °C. The qPCR was optimised by testing different primer concentrations (Table 3.2).

3.3 Results

3.3.1 PCR

DNA was extracted from bacterial isolates (Chapter 2, Section 2.1) as described in Chapter 2 section 2.3 and PCR used to amplify target sequences of DNA. Gel electrophoresis was used to determine if the PCR primers had amplified the correct targets in the pure culture strains. For all assays, the products were the expected size (Table 3.1) (Figure 3.1)

A ten-fold serial dilution of the original bacterial culture was used to determine the number of colonies present. A ten-fold serial dilution of the DNA extracted from the original bacterial culture was conducted. These dilutions were used to optimise the qPCR conditions and create a standard curve for qPCR.

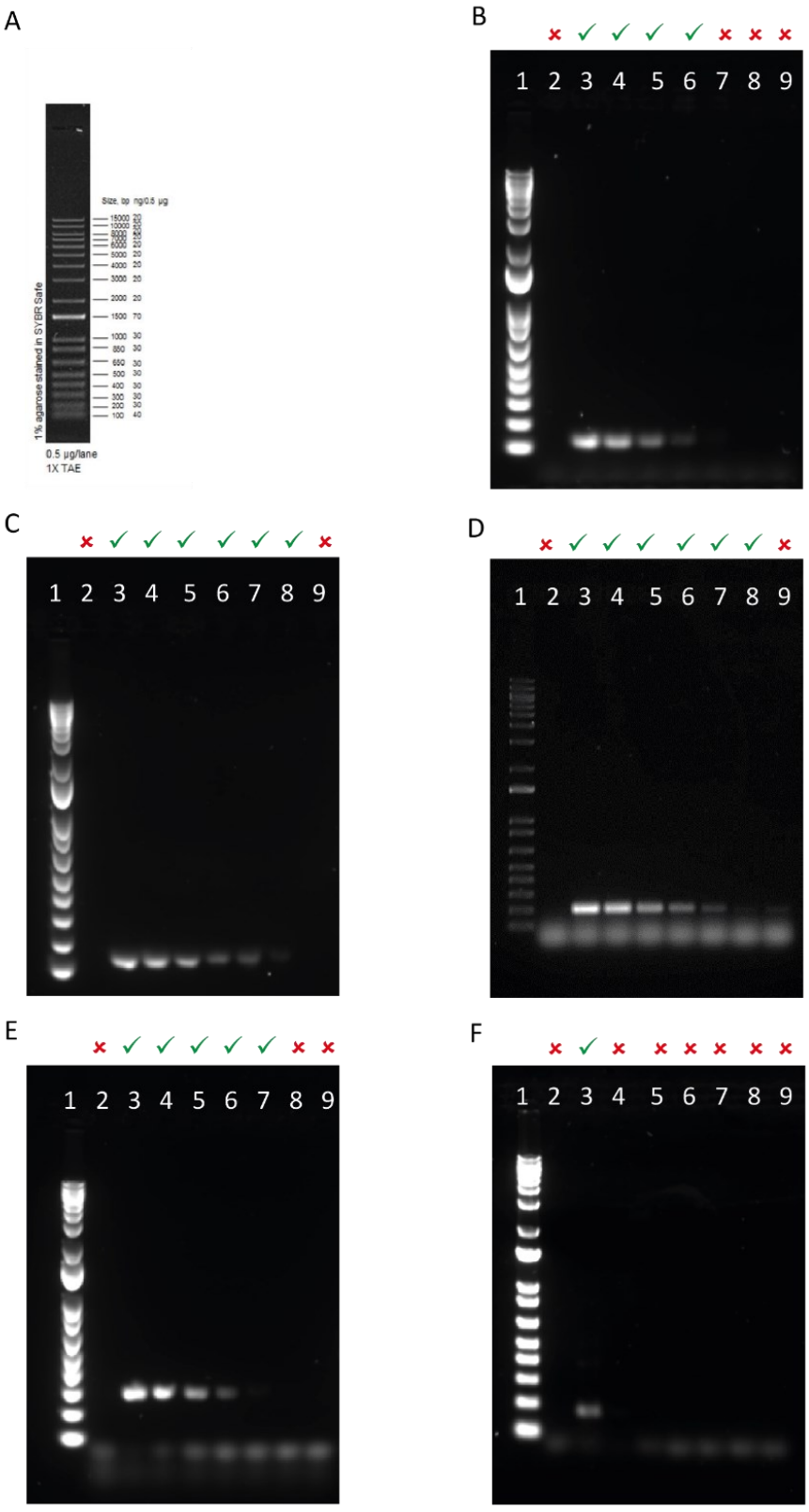

Figure 3.1 Gel electrophoresis to determine the amplification of DNA during *P. aeruginosa* 117bp (B), *S. aureus* 124bp (C), *B. cepacia* complex 196bp (D), *S. maltophilia* 278bp (E) and *A. xylosoxidans* 163bp (F) PCR. Fragment sizes of the Invitrogen 1Kb plus ladder (A) (Source: (ThermoFisher Scientific, n.d.):online). Lane 1: 1Kb plus ladder (Invitrogen, Paisley, UK), lane 2: negative control, lane 3: undiluted DNA (~10ng), lane 4-9: serial dilution of DNA.

3.3.2 Optimising quantitative PCR

To optimise the qPCR conditions, the alterations described in section 3.2.3 were trialled individually. SYBR Green was used to allow the creation of a melt peak curve. Melt curves confirmed that a single product was made during qPCR (see Figure 3.2 for an example). Melt peak curves, in combination with amplification curves, were used to optimise *B. cepacia* complex 16S rRNA qPCR. As shown in Figure 3.3, by carrying out a temperature gradient of annealing temperatures, amplification curve with the lowest Cq value and expected curve shape could be used to determine the optimal annealing temperature for the *B. cepacia* complex qPCR reaction (60 °C).

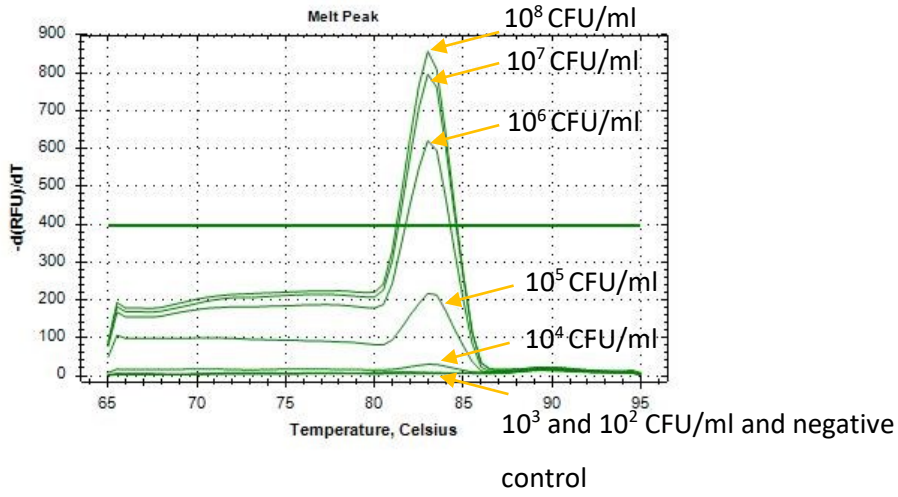

Figure 3.2 Melt peak curve of *A. xylosoxidans* qPCR using *A. xylosoxidans* standards (10^8 - 10^2 CFU/ml).

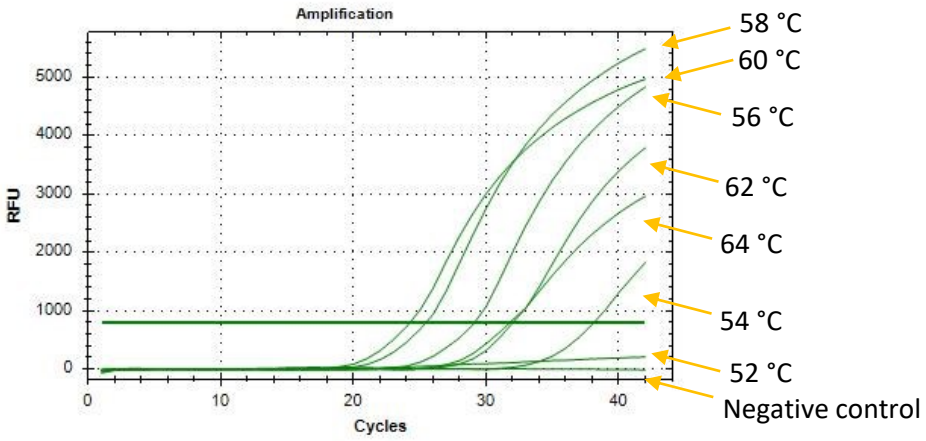

Figure 3.3 Amplification curves of *B. cenocepacia* (3x10^9 CFU/ml) using *B. cepacia* complex primers conducted at different annealing temperatures.

3.3.3 Determining the limit of detection of quantitative PCR assays

The limit of detection for each qPCR assay was determined by running qPCR with a dilution series of DNA. DNA was extracted from a known concentration of bacterial cells and a series of 10-fold serial dilutions created (Section 3.3.1). The highest dilution of DNA there was a Cq value for using the qPCR assays was considered the lower limit. The qPCR amplification curves for *S. aureus* serial dilutions are shown in Figure 3.4 as an example. The concentration of bacterial cells the dilution of DNA corresponded to was considered the limit of detection. Guidelines have suggested the Cq values of detected DNA should be <40 and to accurately determine the limit of detection negative controls should have a Cq value lower than the Cq of the lowest concentration of DNA (Bustin *et al.*, 2009). These guidelines were considered and the limit of detection for the qPCR assays were as follows; *P. aeruginosa orpL* 10^5 CFU/ml, *S. aureus nuc* 10^6 CFU/ml, *B. cepacia* complex *16S rRNA* 10^6 CFU/ml, *S. maltophilia 23S rRNA* 10^6 CFU/ml and *A. xylosoxidans 16S rRNA* 10^6 CFU/ml.

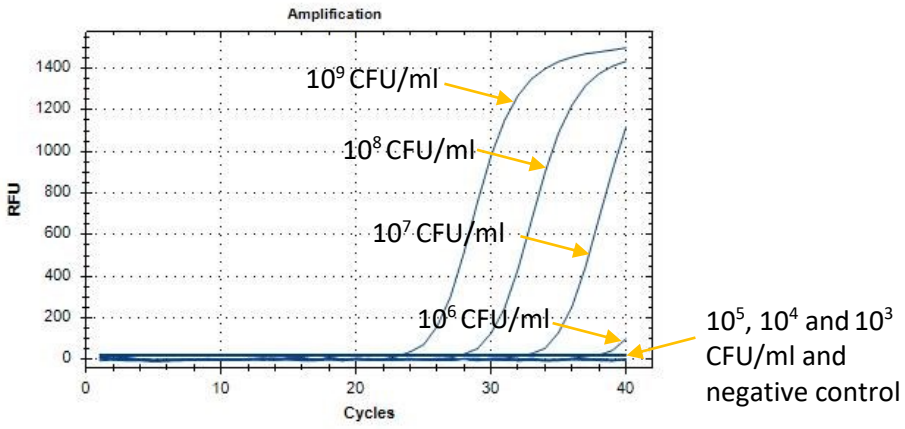

Figure 3.4 Amplification of *S. aureus* serial dilutions (10^9-10^3 CFU/ml) to determine limit of detection.

The efficiency of each set of primers to amplify PCR products using qPCR was determined by creating a standard curve (Figure 3.5) and calculated using Equation 1.

Equation 1:

$$\text{qPCR efficiency} = (10^{\left(\frac{-1}{slope}\right)})-1$$

The optimal range for qPCR efficiency is 90-110%. The PCR efficiency of the qPCR assays were as follows; *P. aeruginosa* 94%, *S. aureus* 90%, *B. cepacia* complex 102%, *S. maltophilia* 104%, *A. xylosoxidans* 93.4%.

To determine the bacterial density of each pathogen in sputum samples, a standard curve was created by carrying out the qPCR assays on the standards on the same plate as the

DNA extracted sputum samples. A standard curve created for a *S. aureus* qPCR assay is shown in Figure 3.5. A standard curve was created for each run and the Cq values of the sputum samples were compared to this to determine the CFU/ml equivalents of the pathogen in each sample.

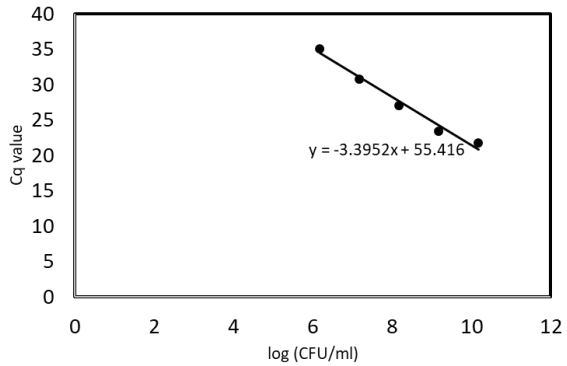

Figure 3.5 Standard curve for *S. aureus* qPCR assay created using a serial dilution (10^{10}-10^{6}) of *S. aureus* DNA extracted from pure culture.

3.3.4 Optimised quantitative PCR conditions

The determined optimised qPCR conditions are summarised in Table 3.3. These conditions were used throughout the rest of the study to quantify bacterial species in CF sputum samples. The *B. cepacia* complex primers described by Mahenthiralingam *et al.* (2000) and Reid *et al.* (2013) and trialled in this study could not be optimised. Therefore, the primers described by Rocchetti *et al.*, (2018) for the detection of *B. cepacia* complex were used in further studies.

Table 3.3 Optimised qPCR conditions for the quantification of key CF pathogens.

Target	Reaction Mix	qPCR parameters
oprL (*P. aeruginosa*)	500 nM forward primer 500 nM reverse primer 500 nM probe 2 µl (~10ng) template DNA 20 µl 2X MasterMix 17.4 µl nuclease free water	95 °C for 10 min, 40x (95°C for 10 sec, 58 °C for 30 sec & 72°C for 1 min)
nuc (*S. aureus*)	300 nM forward primer 300 nM reverse primer 100 nM probe 2 µl (~10ng) template DNA 10 µl 2X MasterMix 7.86 µl nuclease free water	95 °C for 10 min, 40x (95 °C for 30 sec, 60 °C for 30 sec & 72°C for 1 min)
16S rRNA (*B. cepacia complex*)	250 nM forward primer 250 nM reverse primer 2 µl (~10ng) template DNA 10 µl 2X MasterMix 7.9 µl nuclease free water	95 °C for 10 min, 42x (95 °C for 15 sec, 60 °C for 30 sec & 72 °C for 1 min) & melt curve
23S rRNA (*S. maltophilia*)	1000 nM forward primer 1000 nM reverse primer 2 µl (~10ng) template DNA 10 µl 2X MasterMix 7.6 µl nuclease free water	95 °C for 10 min, 40x (95 °C for 30 sec, 68 °C for 30 sec & 72°C for 1 min) & melt curve
16S rRNA (*A. xylosoxidans*)	400 nM forward primer 400 nM reverse primer 2 µl (~10ng) template DNA 10 µl 2X MasterMix 7.84 µl nuclease free water	95 °C for 10 min, 35x (95 °C for 30 sec, 56 °C for 1 min & 72 °C for 1 min) & melt curve

3.3.5 *H. influenzae* quantitative PCR

The *hpd* and *fucP* primers targeting *H. influenzae* were previously used in a duplex qPCR reaction (de Gier *et al.*, 2016). The primers were first optimised for PCR individually before being optimised for qPCR individually. The intention was to then combine the two primers and probes in one reaction. However, the *fucP* primers could not be successfully optimised for SYBR or TaqMan qPCR. The *hpd* primers were optimised using SYBR qPCR before being optimised for TaqMan qPCR. A small selection of DNA extractions from sputum samples were then run with the optimised SYBR qPCR assay alongside the *H. influenzae* standards. Although the standards amplified as expected, the sputum samples caused multiple melt curve peaks. Therefore, it was presumed that any results from SYBR or TaqMan qPCR assays would not be an accurate representation of *H. influenzae* present in sputum samples.

3.4 Discussion

Cystic fibrosis patients are regularly screened for the presence of pathogens in their lungs by providing multiple respiratory samples throughout the year (Cystic Fibrosis Trust, 2010). Pulmonary exacerbations occur frequently in CF patients and are managed with antibiotics. It is important to determine if antibiotic treatment is working so samples are taken more often during an exacerbation.

The current guidance is to culture key CF pathogens from sputum, BAL or cough swab samples provided in clinic. The culture-based methods employed by the clinical laboratory determine the presence or absence of the targeted pathogens. However, different phenotypes and overgrowth of some species make it difficult to accurately identify pathogens using culture-based methods (van Belkum *et al.*, 2000). The detection limit of culture-based methods may also cause samples to appear negative for a pathogen when the pathogen may still be present in low numbers.

Molecular methods such as qPCR could be used as an alternative to culture-based methods for the detection and quantification of key CF pathogens. By developing targeted qPCR assays, key CF pathogens can be monitored during antibiotic treatment to assist in treatment strategies.

The aim of this study was to optimise previously published qPCR assays to enumerate key CF lung pathogens from pure culture and in clinical samples. Key CF pathogens were identified as those monitored by the Cystic Fibrosis Foundation (Cystic Fibrosis Foundation, 2019).

To target known CF pathogens, primers and probes that had previously been published were chosen from the literature (Table 1.2). If available, qPCR assays utilising TaqMan probes were chosen. When probe-based assays had not been developed, SYBR based assays were selected. TaqMan assays were preferred over SYBR assays due to their higher specificity (Espy *et al.*, 2006).

To meet the aim, the published methods for each set of primers were first trialled before alterations were made to determine the optimal annealing temperature, cycle number and primer concentration. A standard curve made from diluted DNA extracted from a pure culture of known concentration was used to optimise and discover the limit of detection of qPCR assays.

As the qPCR assays had previously been published, they had previously been assessed for their specificity to the target species. However, a PCR reaction was carried out with a dilution series of DNA from a pure culture of a known target species (Section 3.3.1). This was to determine the primers bound to the correct target and to start optimisation with cheaper reagents than those required for qPCR.

The efficiency of each qPCR reaction still needed to be determined as the reagents and qPCR machine used were different from those published. A qPCR efficiency of 100% indicates that the qPCR product doubles after each cycle (Bustin *et al.*, 2009). The optimal range for qPCR efficiency is 90-110%. All assays fell within this range after optimisation (Section 3.3.3).

The *hpd* and *fucP* primers targeting *H. influenzae* were previously used in a duplex qPCR reaction (de Gier *et al.*, 2016). The primers were first optimised for PCR individually before being optimised for qPCR individually. The strategy was to then combine the two primers and probes in one reaction. However, the *fucP* primers could not be successfully optimised for TaqMan or SYBR qPCR. The *hpd* primers were optimised using SYBR qPCR before being optimised for TaqMan qPCR. Although the *H. influenzae* qPCR assay targeting the *hpd* gene showed promise with standards made of pure bacterial isolates, it did not perform well with clinical samples. The *hpd* assay was shown to amplify the correct sized target (151bp) and had a qPCR efficiency of 86% (data not shown). However, when a small number of sputum samples were run, multiple melt peak curves were produced.

This could be due to a number of factors that were not explored. The most common cause of multiple melt peak curves is primer dimers. However, these were not seen in the standards or the negative control. Therefore, it is unlikely they were present in the reaction with sputum samples. However, gel electrophoresis could be used to determine if this was the case. Another, more likely, cause would be the presence of multiple bacterial species in the samples that the primers had attached to. de Gier *et al.* (2016) stated the primers were tested against 144 isolates including relations to *H. influenzae* (*Haemophilus haemolyticus*, *Haemophilus parainfluenzae, Haemophilus parahaemolyticus*) that may be found in CF sputum samples and found the *hpd* assay to be 100% specific. It is possible a strain not tested by de Gier *et al.* (2016) was present in the CF sputum tested in this study. When Primer-BLAST (National Center for Biotechnology Information (NCBI), n.d.) was used to determine the specificity of the primers, a strain of *Haemophilus aegyptius* was an exact match for the primers and members of the *Aggregatibacter* genus had one to three

mismatches with the primers. Therefore, these species may have been amplified instead of *H. influenzae.*

Due to time constraints, developing a *H. influenzae* qPCR assay was not pursued further. If more time and resources had been available, it would have been possible to trial other previously published primers (Table 1.2) to potentially optimise them for qPCR. As this was not possible and the primers tested could not be optimised, *H. influenzae* was not targeted in any of the further studies.

Once optimised, the qPCR assays could then be utilised to detect and enumerate key CF pathogens in respiratory samples. This indicates that if these methods were to be utilised by other researchers or in a clinical laboratory which have different qPCR equipment or master mix available, further optimisation would be required. This study has shown that it is possible to optimise previously published assays using different equipment and reagents. There are some barriers to implementing qPCR as a diagnostic tool in clinical laboratories such as the training of laboratory personnel and the cost of new equipment (Pattison *et al.*, 2013; Rentschler *et al.*, 2021). Laboratories that provide diagnostic microbiology services to CF clinics already have PCR protocols in place for the accurate identification of *B. cepacia* complex members (Cystic Fibrosis Trust, 2010). The implementation of qPCR, therefore, would only require some extra training for PCR experienced staff. The cost of training staff and the initial outlay for equipment and the ongoing cost of reagents would deter the implementation of qPCR. However, a cost analysis may find using qPCR will decrease the use of partially effective and ineffective antibiotic therapies which will offset the higher cost of qPCR (Rentschler *et al.*, 2021).

3.5 Conclusion

Previously published qPCR assays used to quantify known CF pathogens were optimised by altering the annealing temperature, number of cycles and primer concentrations within the assays. The results showed the *A. xylosoxidans, B. cepacia* complex, *P. aeruginosa, S. aureus* and *S. maltophilia* assays were suitable for the quantification of their specific targets when a pure bacterial isolate was used. Unfortunately, the *H. influenzae* qPCR assay could not be optimised for use with DNA extractions from sputum samples and was not used in further research. The enumeration of bacterial pathogens using qPCR is important to further understand the abundance of key pathogens in CF lung disease during stable periods and exacerbation. The optimised protocols for qPCR assays targeting key CF pathogens were then used for the detection and enumeration of pathogens in CF sputum samples. The assays could also be utilised by other researchers with minor optimisation for other qPCR machines.

Chapter 4 Effect of postage on the prevalence and quantification of CF pathogens in sputum samples

4.1 Introduction

In the light of the recent COVID-19 pandemic, hospitals have put more emphasis on the rising number of patients with COVID-19 and reduced the number of outpatient clinics. As CF patients are extremely vulnerable to COVID-19 they were advised to 'shield' by staying at home and minimising contact with others. This meant face-to face appointments were minimised and consultations were often conducted over telephone or video calls (Davies, 2020; National Institute for Health and Care Excellence (NICE), 2020). Throughout the pandemic, it was possible for clinicians to monitor lung function remotely as patients could carry out spirometry at home (Cox *et al.*, 2012). This was not possible for diagnostic microbiology samples. Some CF centres took respiratory cultures outside of the hospital setting and had them delivered to the laboratory for analysis (Cystic Fibrosis Trust, 2020). However, there was no evidence to show whether the culture results from these samples would be altered due to the time in, and environment of, transit.

Respiratory samples are frequently taken from people with CF to screen for known bacteria. This informs treatment during periods of pulmonary exacerbations. Sputum samples are usually taken from adults and cough swabs are usually used for children due to their lack of spontaneous sputum production (further details of respiratory sampling can be found in section 1.5). Routine reviews are carried out every 3-6 months and samples are provided as part of this (National Institute for Health and Care Excellence (NICE), 2017). Upon acquisition, samples are processed using culture-based techniques including the use of selective agars and biochemical tests to determine the pathogens present and their susceptibility profiles (Cystic Fibrosis Trust, 2010).

For convenience to the patient, it has been suggested that patients could provide a sample at home and post it to the clinic for culture (Lenhart-Pendergrass *et al.*, 2021; Moore *et al.*, 2021). Posting samples would reduce the need for patients to go into clinics, reducing their contact with other CF patients where they could be cross-infected (de Biase *et al.*, 2020). It would also be convenient for patients in remote locations or those who struggle logistically, physically, or economically to visit clinics frequently. This would include patients who live in places where CF is uncommon and specific CF clinics are sparse (Walicka-Serzysko *et al.*, 2018).

In 2010, the Cystic Fibrosis Trust used previously published work to conclude that 'The results from the culture of respiratory samples from people with CF submitted to the laboratory by post should be interpreted with caution.' (Cystic Fibrosis Trust, 2010). This

conclusion was made from two studies; one analysed the detection of *Haemophilus influenzae* and pneumococci in posted CF samples (May and Delves, 1964) and the other determined bacterial isolation rates in posted bronchiectasis samples (Pye *et al.*, 2008). The study by Pye *et al.* (2008) showed posting bronchiectasis samples had no overall effect on bacterial numbers when culture-based methods were used to quantify bacteria. The study by May and Delves (1964) showed that isolation rates of *H. influenzae* and pneumococci decreased when sputum samples were posted. The CF Trust drew conclusions based on a study from over 50 years ago and another, more recent, study on bronchiectasis samples. Two other studies conducted over 20 years ago, determined there was no effect on the ability to isolate *H. influenzae, Pseudomonas aeruginosa* or *Staphylococcus aureus* (Bilton *et al.*, 1995) or isolation and density of *P. aeruginosa* and *S. aureus* (Hoppe *et al.*, 1997) from CF sputum samples after postage. It is, therefore, important that up to date research is conducted to determine the detection rates of all recognised CF pathogens in posted samples to allow the CF Trust to make a more informed decision.

The Cystic Fibrosis Trust stated that there is 'insufficient evidence to judge whether refrigeration of samples produces better microbiological results than keeping samples at room temperature' (Cystic Fibrosis Trust, 2010). Ideally, patients would be able to send respiratory samples in the standard postage system. However, the CF Trust also stated that respiratory samples should be transported at 4°C if possible, as recommended by the Health Protection Agency National Standard Method (Public Health England, 2019) and The American Society for Microbiology Cumulative Techniques and Procedures in Clinical Microbiology (Miller *et al.*, 2018). Studies have shown that storing samples at 4°C for 48 hours gives comparable results to sputum samples analysed when fresh (Gould *et al.*, 1996). However, it would be more expensive and would require patients to utilise a courier to send samples at 4°C.

Previous, more recent, studies have analysed, using culture-based methods and molecular methods, whether the bacterial density of specific bacteria is changed when sputum samples are stored differently; specifically at room temperature for 24 hours compared to fresh, refrigerated and frozen samples (Pye *et al.*, 2008; Murray *et al.*, 2010; Nelson *et al.*, 2010). These studies showed storing samples at room temperature effected the bacterial density of samples compared to fresh samples and samples refrigerated or frozen immediately.

There is a lack of clear consensus on whether posting CF sputum samples alters the detection and enumeration of key CF pathogens. Previous research has determined that the detection and enumeration of some pathogens are unaffected by postage (Bilton *et al.*, 1995; Hoppe *et al.*, 1997; Pye *et al.*, 2008) but are effected by storage at room temperature for 24 hours (Pye *et al.*, 2008; Murray *et al.*, 2010; Nelson *et al.*, 2010).

This study aimed to determine if the postal of CF respiratory samples from home to clinic would alter the microbiology of the sample. The study compared the cultured pathogens and the quantification of pathogens using qPCR from fresh samples with posted samples. Results from previous studies suggest that qPCR will be more sensitive than culture for the detection of key CF pathogens and posted samples will have an altered bacterial density compared to fresh samples.

4.2 Methods

4.2.1 Patient samples

A total of 180 spontaneously expectorated sputum samples were collected from people with CF attending the Manchester Adult CF Centre between 27th April 2018 and 15th July 2019. Written informed consent was obtained from participants and ethical approval was granted from the Manchester Research Ethics Committee (NHS REC number: 15/NW/0409). Participants were administered a range of oral, intravenous and nebulised antibiotics at time of sampling. Eighty-seven samples were excluded from the study because the sample did not weigh enough to split (82) or metadata was incomplete (5).

Sputum samples were transported to the on-site laboratory where they were weighed and mixed before being split into two aliquots (1 & 2). One aliquot was sent by standard Royal Mail post back to the laboratory (2). Both aliquots were then further divided into two; one was frozen for molecular analysis (1b & 2b) and the other was processed using standard culture-based diagnostic methods (1a & 2a) (Figure 4.1).

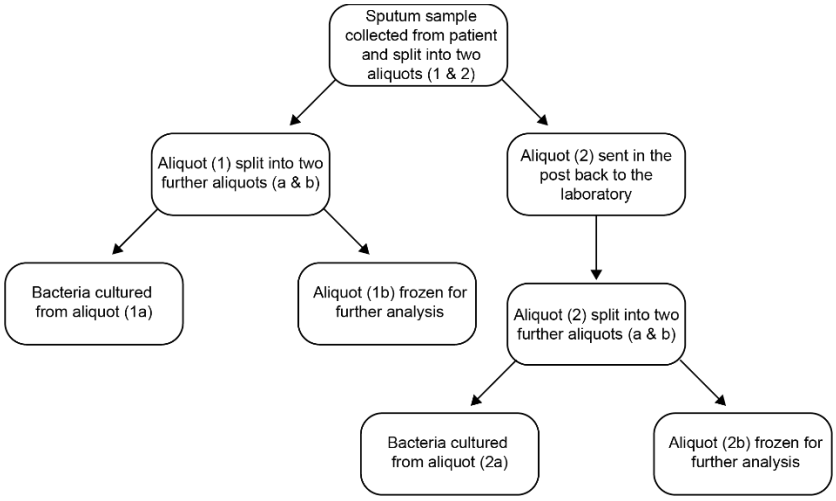

Figure 4.1 Workflow diagram from sample taken to sample processing.

4.2.2 Sample processing

Aliquots (1a & 2a) taken for culture were processed using routine culture techniques (Cystic Fibrosis Trust, 2010) by staff in the clinical microbiology laboratory at Wythenshawe Hospital, Manchester, UK. Aliquots taken and frozen at -80 °C for further analysis (1b & 2b) (Figure 4.1) were transported on ice to Manchester Metropolitan University, Manchester, UK. These aliquots were defrosted, washed and treated with PMA to exclude extracellular DNA and DNA from dead cells. DNA was then extracted as described previously (Chapter 2,

Section 2.3). qPCR was carried out to quantify the CF pathogens *A. xylosoxidans, B. cepacia* complex, *P. aeruginosa, S. aureus* and *S. maltophilia* in samples as described in Chapter 3.

4.2.3 Statistical analysis

Generalised linear models on presence/absence data and odd ratios were performed and calculated using the package lmerTest in R (Lam *et al.*, 2015; Singh *et al.*, 2019). Data were assessed against the assumptions for parametric statistics using Shapiro-Wilk's test for normality and Bartlett's and Levene's tests for homogeneity of variance using the car package in R (Fox and Weisberg, 2019). For those data that conformed to the assumptions, a paired t-test were used to determine significant differences between the means of groups. For those that failed to meet the assumptions, a Wilcoxon rank sum test was undertaken.

4.3 Results

4.3.1 Clinical characteristics of patients

Sputum samples (n=93) were obtained from people with CF (n=73) attending the Manchester Adult CF Centre, Wythenshawe Hospital, Manchester, UK (Table 4.1).

Table 4.1 Clinical characteristics of study participants.

Number of patients	73
Number of paired samples*	93
Patients with two sets of paired samples*	20
Sex of patients (M:F)	45:28
Mean age (years) at first or only sample (±SD)	32.1 (± 11.1)
Minimum and maximum age (years)	19 to 70
CFTR Genotype:	
Homozygous F508del	35
Heterozygous F508del	30
Non-F508del	8
Mean %FEV1 at sampling (±SD)	43.8 (± 16.3)
Patients on CFTR modulator therapy	12 (16.4%)

*Paired samples refers to samples where a fresh and posted aliquot were available for analysis.

4.3.2 Prevalence of CF pathogens using culture-based techniques

Standard culture methods were used to determine the prevalence of key CF pathogens *A. xylosoxidans, B. cepacia complex, P. aeruginosa, S. aureus* and *S. maltophilia* (Cystic Fibrosis Foundation, 2019) in fresh and posted sputum samples at the clinical microbiology laboratory. The total number of samples with each pathogen present in either, or both, the fresh and posted samples was determined. Of the emblematic CF pathogens targeted during culture-based detection, *Achromobacter xylosoxidans* was detected in 14% (overall: 13/93, fresh and posted samples (F&P): 11/93, fresh but not posted samples (F): 1/93, posted but not fresh samples (P): 1/93), *B. cepacia* complex members were detected in 20% (overall: 19/93, F&P: 19/93, F: 0/93, P: 0/93), *P. aeruginosa* was detected in 65% (overall: 60/93, F&P: 51/93, F: 5/93, P: 4/93), *S. aureus* was detected in 32% (overall: 30/93, F&P: 26/93, F: 2/93, P: 2/93) and *Stenotrophomonas maltophilia* was detected in 8% (overall: 7/93, F&P: 4/93, F: 0/93, P: 3/93) of samples.

In the majority of positive samples, pathogens were detected in both fresh and posted samples (blue bars, Figure 4.2). In some instances, the pathogens were detected in either the fresh (orange bars, Figure 4.2) or the posted (yellow bars Figure 4.2) sample but not the other. Odd ratios were used to determine there was no significant difference between

the presence of any of the key pathogens in fresh and posted samples. There was no difference (odds ratio of 1) in the chance of *A. xylosoxidans* (95% confidence interval (CI) 0.056-17.906), *B. cepacia* complex (95% CI N/A; suggesting perfect correlation) and *S. aureus* (95% CI 0.132- 7.603) being present in the fresh compared to in the posted samples. However, *S. maltophilia* was 4.5 times more likely (95% CI 0.337-60.159) to be present in the posted sample than in the fresh sample and *P. aeruginosa* was 1.273 (95% CI 0.325-4.991) more likely to be present in the fresh sample than in the posted sample.

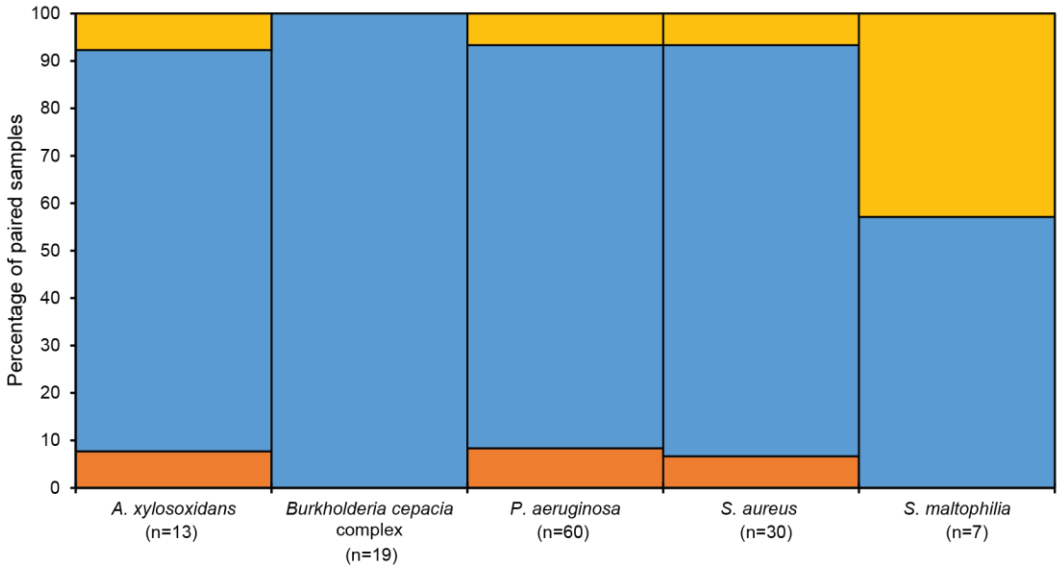

Figure 4.2 CF pathogens present in fresh and posted samples determined by culture-based techniques. Blue bars indicate the percentage of positive samples with the pathogen detected in both fresh and posted samples. Orange bars indicate the percentage of positive samples with the pathogen detected in the fresh sample but not the posted sample. Yellow bars indicate the percentage of positive samples with the pathogen detected in the posted samples but not the fresh sample. n is the number of paired samples in which culture was positive in either fresh or posted.

4.3.3 Prevalence of CF pathogens using quantitative PCR

qPCR assays were used to determine the prevalence of key CF pathogens in fresh and posted sputum samples using the optimised methods in Chapter 3. The total number of samples with each pathogen present in either, or both, the fresh and posted samples was determined. Of the emblematic CF pathogens targeted during qPCR, *A. xylosoxidans* was detected in 70% (overall: 65/93, fresh and posted samples (F&P): 33/93, fresh but not posted samples (F): 15/93, posted but not fresh samples (P): 17/93), *B. cepacia* complex members were detected in 95% (overall: 84/88, F&P: 59/93, F: 14/93, P: 11/93), *P. aeruginosa* was detected in 87% (overall: 81/93, F&P: 68/93, F: 6/93, P: 7/93), *S. aureus*

was detected in 82% (overall: 76/93, F&P: 45/93, F: 16/93, P: 15/93) and *S. maltophilia* was detected in 66% (overall: 57/87, F&P: 22/93, F: 20/93, P: 15/93) of samples.

In the majority of positive samples, pathogens were detected in both fresh and posted samples (blue bars, Figure 4.3). In some instances, the pathogens were detected in either the fresh (orange bars, Figure 4.3) or the posted (yellow bars, Figure 4.3) sample but not the other. Odd ratios were used to determine that there was no significant difference between the presence of any of the targeted pathogens in fresh and posted samples. *A. xylosoxidans* and *P. aeruginosa* were more likely to be present in the posted sample than in the fresh sample by 1.181 (95% CI 0.531-2.626) and 1.182 (95% CI 0.379-3.685) times, respectively. *B. cepacia complex, S. aureus* and *S. maltophilia* were 1.327 (95% CI 0.564-3.121), 1.084 (95% CI 0.492-2.388) and 1.404 (0.624-3.155) times more likely to be present in the fresh sample than in the posted sample.

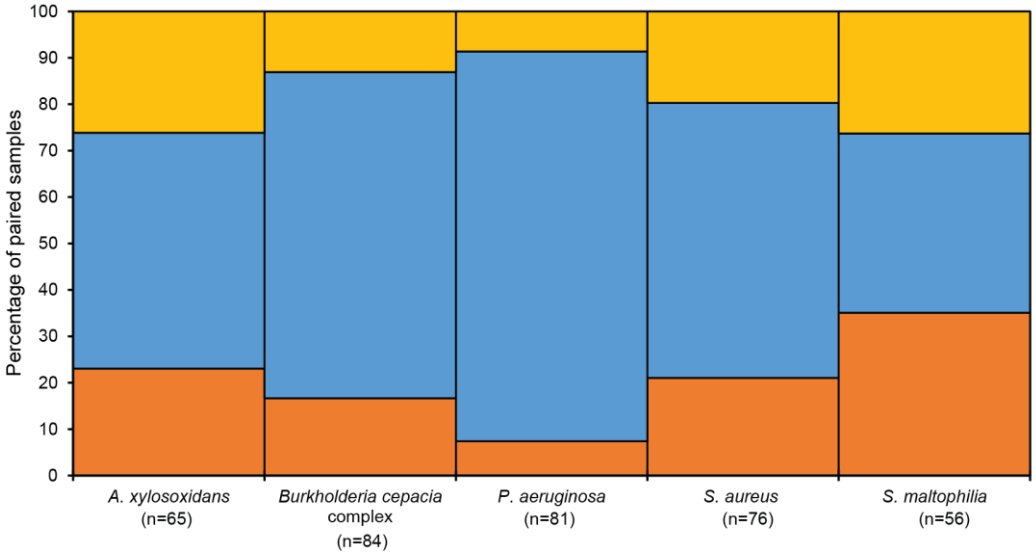

Figure 4.3 CF pathogens present in fresh and posted samples determined by qPCR. Blue bars indicate the percentage of positive samples with the pathogen detected in both fresh and posted samples. Orange bars indicate the percentage of positive samples with the pathogen detected in the fresh sample but not the posted sample. Yellow bars indicate the percentage of positive samples with the pathogen detected in the posted sample but not the fresh sample. n is the number of paired samples in which qPCR was positive in either fresh or posted.

In comparison to culture-based methods, qPCR detected targeted pathogens in a larger percentage of samples; *A. xylosoxidans* (14% culture vs 71% qPCR), *B. cepacia* complex (20% vs 95%), *P. aeruginosa* (65% vs 87%), *S. aureus* (32% vs 82%) and *S. maltophilia* (8% vs 64%).

To further investigate the difference in results seen between culture and qPCR, the culture data and qPCR data was compared irrespective of if the sample was fresh or posted. In 4/5 pathogens targeted, qPCR detected the pathogen in samples when culture did not, more frequently than qPCR and culture were in agreement (Figure 4.4). Odds ratios were calculated to determine all targeted pathogens were more likely to be detected by qPCR than by culture (Table 4.2).

Table 4.2 Odds ratios comparing the detection of bacterial species by qPCR and by culture.

Bacterial species	Odds ratio	95% Confidence interval
A. xylosoxidans	104.805	30.427-360.994
B. cepacia complex	117.402	40.620-339.319
P. aeruginosa	9.843	4.052-23.914
S. aureus	14.929	7.361-30.279
S. maltophilia	522.696	257.709-1060.152

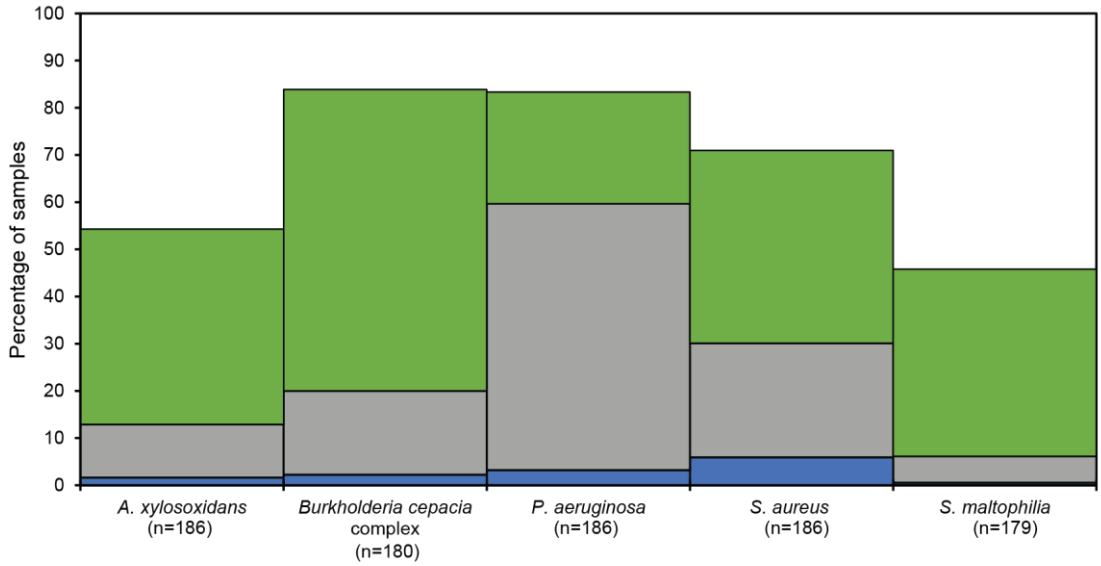

Figure 4.4 Comparison of the detection of CF pathogens by culture and qPCR in all samples. Grey bars indicate the percentage of samples with the pathogen detected using both culture and qPCR. Blue bars indicate the percentage of samples with the pathogen detected by culture but not by qPCR. Green bars indicate the percentage of samples with the pathogen detected by qPCR but not by culture. n is the total number of fresh and posted samples qPCR was applied to.

4.3.4 Quantification of CF pathogens using quantitative PCR

The CFU/ml equivalents of *A. xylosoxidans, B. cepacia* complex, *P. aeruginosa, S. aureus* and *S. maltophilia* in fresh and posted samples were determined using specific qPCR assays (Figure 4.5). The log change between fresh and posted samples was also calculated.

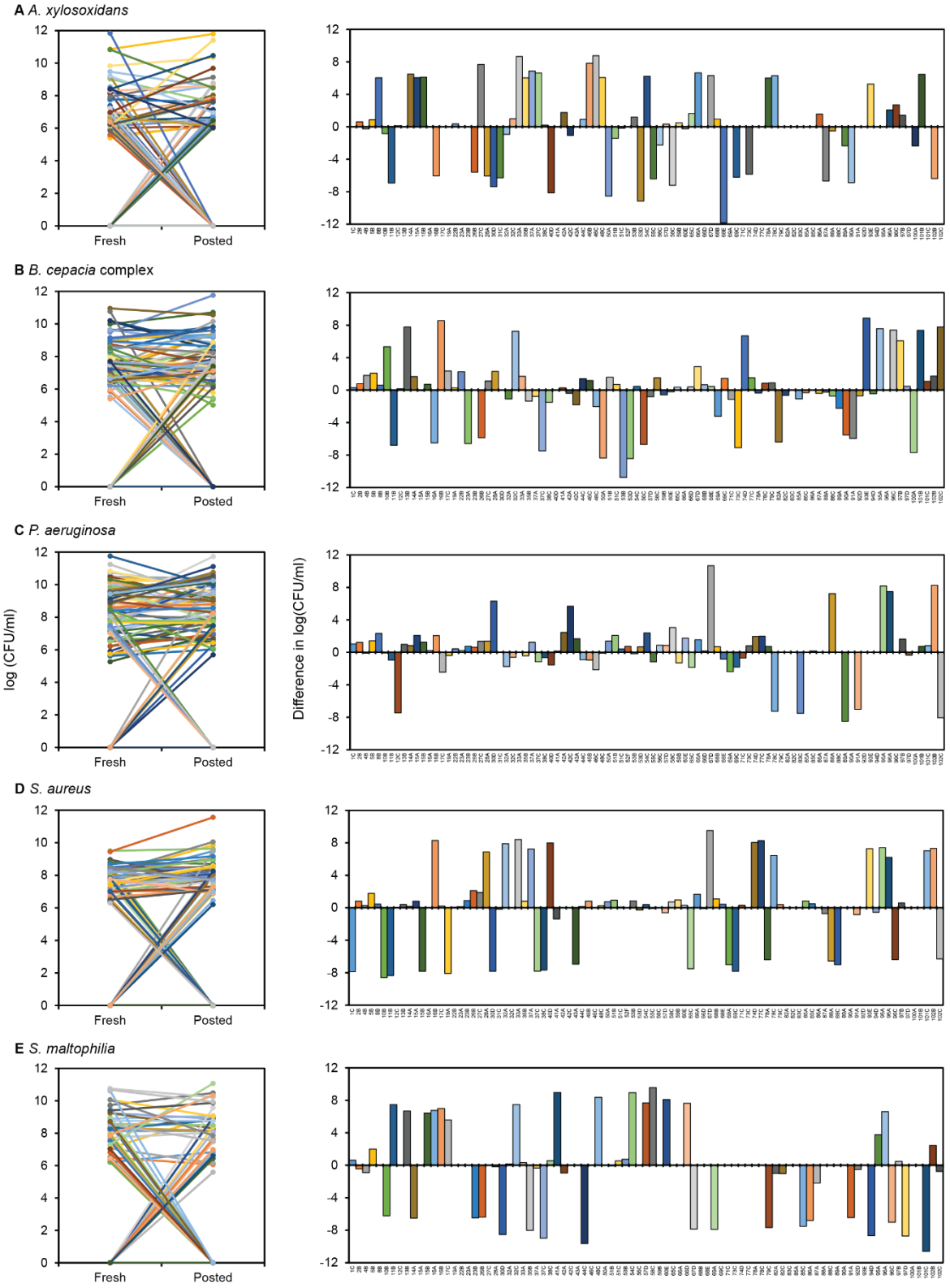

Figure 4.5 CFU/ml equivalents and change in CFU/ml equivalents (log_{10} scale) of (A) *A. xylosoxidans* (n=93), (B) *B. cepacia* complex (n=88), (C) *P. aeruginosa* (n=93), (D) *S. aureus* (n=93) and (E) *S. maltophilia* (n=87) determined by qPCR in fresh and posted samples. n is the number of paired samples qPCR was applied to. Change in CFU/ml equivalents is shown as the difference between the log (CFU/ml) of fresh and posted samples.

Results from the t-tests showed there was no significant difference in CFU/ml equivalents between fresh and posted samples (*A. xylosoxidans* P=0.572 T-test statistic (t)=-0.409, *B. cepacia* complex P=0.928 t=0.090, *P. aeruginosa* P=0.282 t=-1.083, *S. aureus* P=0.881 t= -0.150, *S. maltophilia* P=0.606 t= 0.519). There was no overall trend in the increase or decrease of specific CF pathogens (Figure 4.5). However, there was a higher percentage of samples with a higher CFU/ml in the posted sample than in the fresh sample (all pathogens except *S. maltophilia*) (Table 4.3).

Table 4.3 Samples with a change in CFU/ml equivalents (determined by qPCR) before and after postage.

Pathogen	**Samples with an increase in CFU/ml between fresh and posted**	**Samples with a decrease in CFU/ml between fresh and posted**
A. xylosoxidans	36/93 (38.7%)	28/93 (30.1%)
B. cepacia complex	46/88 (52.3%)	38/88 (43.2%)
P. aeruginosa	49/93 (52.7%)	32/93 (34.4%)
S. aureus	49/93 (52.7%)	26/93 (28%)
S. maltophilia	27/87 (31%)	29/87 (33.3%)

x/y(z%) x=number of paired samples with an increase or decrease in CFU/ml from fresh to posted, y=total number of paired samples qPCR was applied to, z=percentage of samples with an increase or decrease in CFU/ml from fresh to posted

4.4 Discussion

It has been suggested that if patients could provide respiratory samples at home and post them to the clinic for analysis it would be advantageous for the management of lung disease in CF patients. Reducing the need for CF patients to go into clinic would reduce the likelihood of cross-infection from other CF patients (de Biase *et al.*, 2020) and the potential to contract other infections such as COVID-19 (Davies, 2020) as well as being convenient for patients in remote locations.

The aim of this study was to determine if posting sputum samples using the standard postal system affected the detection of key CF pathogens and their bacterial densities. This study showed that the detection of key CF pathogens in posted samples does not significantly differ from fresh samples when using standard culture-based methods. The detection of *B. cepacia* complex, *P. aeruginosa* and *S. aureus* were also not altered when samples were posted when qPCR was used. However, the bacterial density determined by qPCR of CF pathogens was affected by posting samples.

It has previously been shown that storing sputum samples at room temperature for 24 hours increases the bacterial density of known pathogens compared to fresh samples and samples stored at 4°C identified using culture-based methods and molecular methods (Pye *et al.*, 2008; Murray *et al.*, 2010; Nelson *et al.*, 2010). It was therefore hypothesised that bacterial densities in sputum samples would be altered following postage.

The prevalence of key CF pathogens was determined in fresh and posted samples using standard culture techniques and qPCR. Using culture methods, the results showed little change in the prevalence of CF pathogens between fresh and posted samples except for *S. maltophilia* (Figure 4.2). Previous studies showed little change in the prevalence of *P. aeruginosa, S. aureus* and other bacterial species not included in this study (*H. influenzae*, *Branhamella cararrhalis* (now in the genus *Moraxella*)) when culture-based methods were used (Bilton *et al.*, 1995; Hoppe *et al.*, 1997; Pye *et al.*, 2008) whereas another study showed a decrease in the isolation of *H. influenzae* and pneumococci when samples were posted (May and Delves, 1964). The qPCR results indicated that a smaller proportion of samples showed no change in prevalence between fresh and posted samples compared to culture results.

QPCR did show a larger percentage of samples had CF pathogens present than when culture-based methods were used (Figure 4.2, Figure 4.3).When fresh and posted samples

were taken as individual samples and results from culture and qPCR compared, all pathogens were more likely to be detected by qPCR than by culture (Figure 4.4). In this study, all samples were treated with PMA to exclude any extracellular DNA from dead bacterial cells. qPCR assays would, therefore, only amplify DNA from live cells and only live cells would be cultured during culture-based detection. Therefore, it would be expected that the culture and qPCR results would show a similar trend. Culture-based methods consistently correctly identify *P. aeruginosa* and *S. aureus,* however, their use to identify other CF pathogens is unreliable (Hogardt *et al.*, 2009). However, previous studies have also shown a difference between culture and qPCR detection when detecting *P. aeruginosa* (Xu *et al.*, 2004; Deschaght *et al.*, 2010; Hery-Arnaud *et al.*, 2017), *H. influenzae* (Nelson *et al.*, 2010; data not shown) and *H. influenzae, Streptococcus pneumoniae* and *B. cepacia* complex (Reid *et al.*, 2013). Hery-Arnaud *et al.* (2017) also showed qPCR can detect *P. aeruginosa* 8 months before culture-based methods. Another explanation for the discrepancy between culture and qPCR results is that pathogens may have been misidentified when culture-based methods were used due to the presence of altered phenotypes including viable but not culturable cells which qPCR would detect (Le Gall *et al.*, 2013; Hery-Arnaud *et al.*, 2017; Mangiaterra *et al.*, 2018). Pathogens could have also been present in low numbers and hidden by the growth of other species in those samples that were culture negative but qPCR positive as previously suggested (Deschaght *et al.*, 2010; Le Gall *et al.*, 2013; Hery-Arnaud *et al.*, 2017).

In the present study, qPCR also showed that for a proportion of samples the pathogens were not detected when samples returned from the post (*A. xylosoxidans* 16%, *B. cepacia* complex members 16%, *P. aeruginosa* 7%*, S. aureus* 17% and *S. maltophilia* 20%) and in other samples pathogens were not detected before postage but were detected after (*A. xylosoxidans* 18%, *B. cepacia* complex members 13%, *P. aeruginosa* 8%*, S. aureus* 16% and *S. maltophilia* 17%) (Figure 4.3). It is possible the pathogens were present in the samples but in numbers lower than the limit of detection for the qPCR. Due to the polymicrobial nature of CF lung disease, bacterial species, such as *P. aeruginosa*, in the sample could also outcompete each other while in the post (Nelson *et al.*, 2010). May and Delves (1964) also suggested an overall lower pH in posted samples compared to fresh samples may contribute to the isolation rates of some bacterial species. Previous research has shown different pathogens alter the pH in different ways (Sánchez-Clemente *et al.*, 2018) and further investigation would need to be carried out to determine how the growth of CF lung bacterial species effects the pH of samples sent through the post.

The bacterial density of key CF pathogens were determined in samples before and after postage using qPCR. The results showed that there was no overall trend in changes in bacterial density when comparing samples before and after postage (Figure 4.5, Table 4.3). *A. xylosoxidans*, *B. cepacia* complex, *P. aeruginosa* and *S. aureus* increased after postage in a higher percentage of samples than decreased. *S. maltophilia* decreased after postage in a higher percentage of samples than increased.

Murray *et al.* (2010) showed a small but significant increase in *P. aeruginosa* density after storage for 24 and 48 hours when culture-based methods were used. Nelson *et al.* (2010) used qPCR to also show a significant increase in *P. aeruginosa* density after storage for 24 hours at room temperature compared to storage at 4°C as well as a decrease in *H. influenzae*. Pye *et al.* (2008) showed no significant difference in bacterial density when bronchiectasis samples were posted or stored at room temperature for 24 hours when *P. aeruginosa*, *H. influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* were monitored using culture-based methods. However, the sample size was small in this study (n=13 for *P. aeruginosa* and *H. influenzae* for posted samples and n=15 and n=11 for *P. aeruginosa* and *H. influenzae*, respectively, for samples kept at room temperature). Hoppe *et al.* (1997) also showed no significant difference in the bacterial density of *P. aeruginosa* and *S. aureus* isolates between fresh and posted CF sputum samples when standard culture methods were used.

Previous studies (Hoppe *et al.*, 1997; Pye *et al.*, 2008; Murray *et al.*, 2010) used mean values of CFU/ml which could explain why no significant change was seen. Alternatively, in this study the results were shown as individual samples which showed pathogens increased in some patients and decreased in others after postage. Both Murray *et al.* (2010) and Nelson *et al.* (2010) showed an increase in *P. aeruginosa* after storage at room temperature. However, both studies used a small sample size, n=10 and n=5, respectively, and Murray *et al.* (2010) selected patients that were known to be colonised with *P. aeruginosa*.

Previous studies have shown either a significant increase in *P. aeruginosa* density after being stored at room temperature for 24 and 48 hours or no change in density. One study used standard culture methods to show no change in the density of *S. aureus* isolates after postage. However, no other studies could be found that determined the effects of postage or storage at room temperature for >24 hours on the density of *A. xylosoxidans, B. cepacia* complex members or *S. maltophilia* in sputum samples.

4.5 Conclusion

This study showed that the presence of key CF pathogens in posted samples does not significantly differ from fresh samples when using standard culture-based methods. The prevalence of *B. cepacia* complex, *P. aeruginosa* and *S. aureus* were also not significantly altered when samples were posted when qPCR was used. However, the bacterial density of CF pathogens is affected by posting samples. Therefore, quantification results from posted samples could be an inaccurate representation of bacterial densities in the lungs of CF patients. Therefore, posting samples would not be recommended for samples used for quantification of key CF pathogens which could be used to determine the effectiveness of antibiotics or to inform antibiotic strategies. However, the use of standard culture-based methods on posted samples could be recommended for the detection of key CF pathogens.

Chapter 5 Effect of postage on the bacterial microbiota of CF sputum samples

5.1 Introduction

Lung infections in CF patients were once thought to be caused by a small number of culturable pathogens, specifically *Staphylococcus aureus* in childhood and *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex members in adulthood (Cystic Fibrosis Foundation, 2019). It has since been established, using culture independent methods, that the CF lung has a resident microbiota that alters with age and disease progression (Cox *et al.*, 2010; Klepac-Ceraj *et al.*, 2010; Parkins and Floto, 2015).

Next generation sequencing is being increasingly used to understand how the microbiota changes over time (Fodor *et al.*, 2012), during exacerbation (Carmody *et al.*, 2013, 2015; Cuthbertson *et al.*, 2016) and its relationship with lung function (Cuthbertson *et al.*, 2020) across multiple CF centres. During clinical studies, such as those into the effectiveness of antimicrobial interventions, it would be advantageous for patients to provide samples more frequently than the normal recommendations for monitoring (Hansen *et al.*, 2005; Cuthbertson *et al.*, 2016). More frequent sampling would highlight the effectiveness of antimicrobial treatments earlier in the regimen; however, it would require patients to attend clinics more frequently. If these samples could be provided at home and posted to the clinic or research institution, it would reduce the risk of infection to patients from attending the clinic in person and be more convenient for patients providing multiple samples in a short time period. In multicentre clinical studies, samples are often shipped from the CF centre to a central processing laboratory or research institute. It is, therefore, important to understand if samples shipped at room temperature accurately represent the microbiota of the fresh sample.

Previous research has shown storing sputum samples at room temperature for 24 hours and posting samples has negligible effect on the pathogens cultured from the samples (Bilton *et al.*, 1995; Pye *et al.*, 2008). However, the bacterial density of pathogens determined by quantitative culture in samples stored at room temperature (Murray *et al.*, 2010; Nelson *et al.*, 2010) differed from fresh samples but was not effected by postage (Bilton *et al.*, 1995; Pye *et al.*, 2008). In Chapter 4, it was determined that the bacterial density of *Achromobacter xylosoxidans*, *B. cepacia* complex, *P. aeruginosa*, *S. aureus* and *Stenotrophomonas maltophilia* were altered after postage (Figure 4.5). A change in the bacterial density of the key CF pathogens after postage indicates that the community composition of the microbiota within the samples may also change during postage.

Previous studies have conflicting conclusions; one study showed no effect on the bacterial community after storage at room temperature for up to 4 weeks (Zhao *et al.*, 2011) whereas others showed a significant alteration in bacterial community after 12 (Cuthbertson *et al.*, 2014) and 24 hours (Nelson *et al.*, 2010) at room temperature. Zhao *et al.* (2011) split a single sputum sample into multiple sub-samples and tested the effect of temperature and time on community composition using pyrosequencing. The study reported no significant difference in community composition after storage at room temperature for 1, 2 and 4 weeks compared to the fresh sub-sample. However, as the study used one sputum sample split into 23 sub-samples, any conclusions on the effect of room temperature on the bacterial composition of all sputum samples are difficult to make. Cuthbertson *et al.* (2014) used pyrosequencing to show that bacterial community composition was altered after 1 hour at room temperature. However, alterations were not significant until after 12 hours at room temperature. Therefore, they suggested freezing samples within 12 hours to minimise alterations to the bacterial community. This study also split the community into common and rare species based on their abundance across the samples. This allowed them to observe that storage at room temperature had a greater effect on the rare species. Nelson *et al.* (2010) split five sputum samples in two before refrigerating one sub-sample and storing the other at room temperature for 24 hours. Denaturing gradient gel electrophoresis analysis was used to show a significant difference in the bacterial community between samples stored at room temperature for 24 hours and samples stored at 4 °C.

The presented study aimed to determine if the bacterial microbiota would be affected when respiratory samples were sent through the post. This study determined the bacterial taxa present in samples before and after postage using high-throughput gene targeted sequencing. The bacterial taxa were partitioned into core and satellite taxa in fresh and posted samples. The bacterial richness, diversity, dominance and dissimilarity of the microbiota, core and satellite taxa were compared from fresh and posted samples.

The bacterial community was partitioned as previous studies investigating the microbiota of CF lung samples have shown splitting the microbiota into common, core taxa and rarer, satellite taxa can reveal more clearly the relationships between sample treatment (e.g. storage conditions, antibiotic treatment, DNA chelator treatment, patient lung function, etc) and the abundance of species distributed across samples (van der Gast *et al.*, 2011, 2014; Rogers *et al.*, 2013a; Cuthbertson *et al.*, 2014, 2016, 2020; Soret *et al.*, 2020).

5.2 Methods

5.2.1 Patient samples and processing

Samples and DNA extraction from samples was previously described in Chapter 4 section 4.2.2.

5.2.2 DNA sequencing

The bacterial microbiota from fresh and posted samples were sequenced by targeting the V5-V6 region of the 16S rRNA gene using the Illumina MiSeq platform and sequence analysis performed as previously described (Chapter 2, Section 2.4). Due to lack of sequences, one sample (17C) was excluded from analysis. Sequences were taxonomically assigned. However, due to the length of sequences, species identities should be considered putative.

5.2.3 Statistical analysis

The microbiota of fresh and posted samples were partitioned into core and satellite taxa using a distribution abundance relationship (Magurran and Henderson, 2003). Core taxa was defined as those taxa that appeared in 75% or more samples and all others were considered to be satellite taxa (van der Gast *et al.*, 2011).

Generalised linear models on presence/absence data and odd ratios were performed and calculated using the package lmer in R version 3.6.2 (Lam *et al.*, 2015; Singh *et al.*, 2019). PAST was used to calculate Fisher's alpha index of diversity, Berger-Parker index of dominance, Sørensen and Bray-Curtis indices of dissimilarity as described previously (Chapter 2, Sections 2.5.3 and 2.5.4) (Hammer *et al.*, 2001). Data were assessed against the assumptions for parametric statistics using Shapiro-Wilk's test for normality and Bartlett's and Levene's tests for homogeneity of variance using the car package in R (Fox and Weisberg, 2019). For those data that conformed to the assumptions, paired t-tests were used to determine significant differences between the means of groups. For those that failed to meet the assumptions, a Wilcoxon rank sum test was undertaken. ANOSIM and SIMPER were performed and calculated using the package vegan in R as described previously (Chapter 2, Section 2.5.4)(Clarke, 1993).

5.2.4 Combining quantitative PCR and sequencing data

Targeted gene sequencing putatively identified multiple sequence variants for key CF pathogens. The qPCR data described in Chapter 4 was presumed to be the total quantity of each CF pathogen. The number of reads for each variant identified as a CF pathogen in

individual samples was converted into a percentage of the total for each pathogen. The percentage of reads for each variant was then used to calculate the CFU/ml equivalents of each variant.

5.3 Results

5.3.1 Clinical characteristics of patients

Sputum samples collected for the previous study (Chapter 4) were used in this study excluding one sample from patient 17 that lacked sequence reads.

Table 5.1 Clinical characteristics of patients within the study.

Number of patients	72
Number of paired samples	92
Patients with two sets of paired samples	20
Sex of patients (M:F)	45:27
Mean age (years) at first or only sample (±SD)	31.9 (± 10.96)
Minimum and maximum age (years)	19 to 70
CFTR Genotype:	
Homozygous F508del	35
Heterozygous F508del	29
Non-F508del	8
Mean %FEV1 at sampling (±SD)	43.8 (± 16.4)
Patients on CFTR modulator therapy	12 (16.4%)

5.3.2 Partitioning of core and satellite taxa

Bacterial taxa were partitioned into core and satellite taxa based on their distribution and abundance in fresh and posted samples (Figure 5.1). In fresh samples, 5 core taxa and 544 satellite taxa were identified. In posted samples, 8 core taxa and 575 satellite taxa were identified. The core or satellite status of key CF pathogens was determined, and all pathogens had the same status in fresh and posted samples. *B. cepacia* complex, *P. aeruginosa, S. aureus* and *S. maltophilia* were all classified as core taxa, while *A. xylosoxidans* was part of the satellite taxa (Figure 5.1, orange circles).

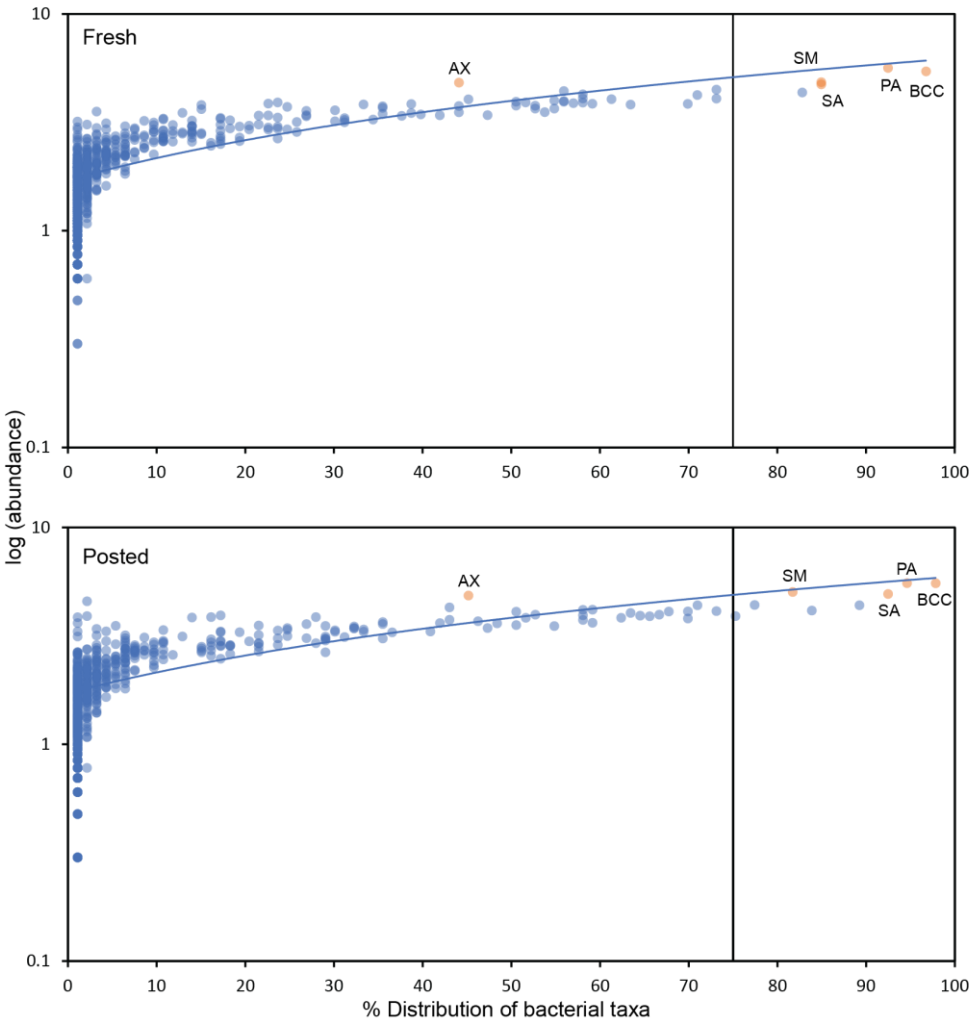

Figure 5.1 Distribution-abundance relationship of bacterial taxa in fresh and posted samples. Given is the percentage of samples in which each bacterial taxon was observed (% distribution) against the abundance (log_{10} scale) across those samples. Core taxa were defined as those which fell in the upper quartile (right of the vertical line), all other taxa that fell below this were considered to be satellite. Key CF pathogens are highlighted in orange and marked as follows; AX *A. xylosoxidans*, BCC *B. cepacia* complex, PA *P. aeruginosa*, SA *S. aureus*, SM *S. maltophilia*. (Fresh: r^2=0.48, $F_{(1,754)}$=682.6, P<0.001, Posted: r^2=0.44, $F_{(1,754)}$ = 604, P<0.001)

5.3.3 Prevalence of CF pathogens using high-throughput sequencing

High-throughput 16S rRNA gene targeted sequencing was used to determine the prevalence of key CF pathogens (Cystic Fibrosis Foundation, 2019) in fresh and posted sputum samples. The total number of samples with each pathogen present in either, or both, the fresh and posted samples was determined. Of the key CF pathogens identified, *B. cepacia* complex members were detected in 100% (92/92), *S. aureus* was detected in 99% (91/92), *P. aeruginosa* was detected in 97% (89/92), *S. maltophilia* was detected in 94% (86/92) of samples and *A. xylosoxidans* was detected in 61% (56/92).

In the majority of positive samples, pathogens were detected in both fresh and posted samples (blue bars, Figure 5.2). In some instances, the pathogens were detected in either the fresh (orange bars, Figure 5.2) or the posted (yellow bars, Figure 5.2) sample but not the other. Odds ratios were used to determine there was no significant difference between the detection of key pathogens in fresh and posted samples. *B. cepacia* complex*, P. aeruginosa* and *S. aureus* were more likely to be present in the posted sample than in the fresh sample by 1.517 (95% CI 0.247-9.296), 2.047 (95% CI 0.365-11.471) and 2.152 (95% CI 0.771-6.009) times, respectively. *A. xylosoxidans* and *S. maltophilia* were more likely to be present in the fresh sample than in the posted sample by 1.093 (95% CI 0.478-2.503) and 1.956 (95% CI 0.740-5.169) times, respectively.

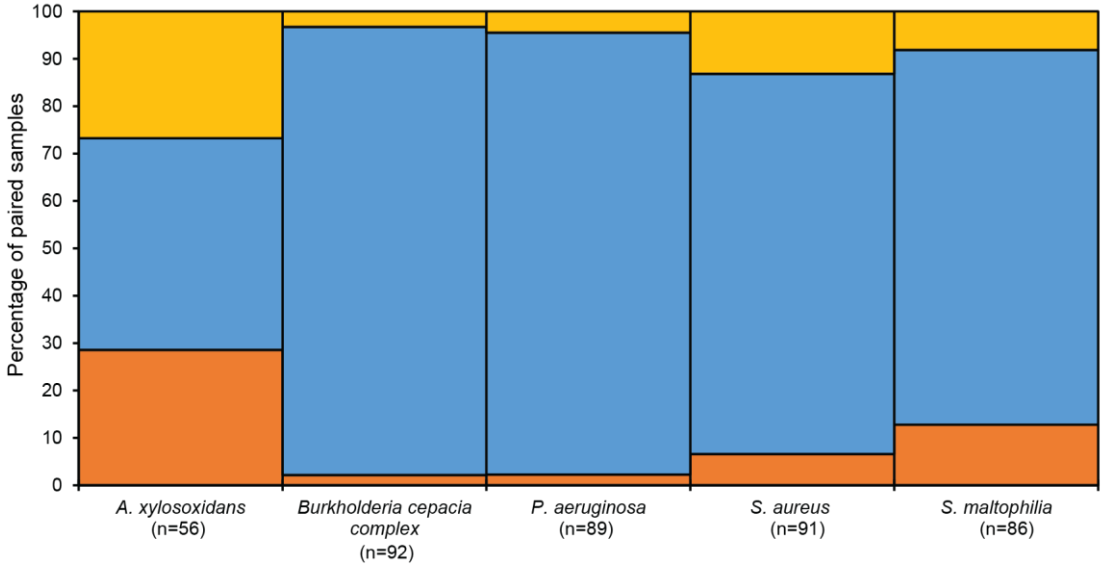

Figure 5.2 CF pathogens present in fresh and posted samples identified by targeted gene sequencing. Blue bars indicate the percentage of positive samples with the pathogen identified in both fresh and posted samples. Orange bars indicate the percentage of positive samples with the pathogen detected in the fresh samples but not the posted sample. Yellow bars indicate the percentage of positive samples with the pathogen detected in the posted samples but not the fresh sample. n is the number of paired samples in which targeted gene sequencing was positive in either fresh or posted.

In comparison to culture-based methods (Chapter 4, Figure 4.2) and qPCR (Chapter 4, Figure 4.3), microbiota sequencing identified pathogens in a larger percentage of samples except for *A. xylosoxidans*; *A. xylosoxidans* (culture 14% vs qPCR 70% vs sequencing 61.3%), *B. cepacia* complex (20% vs 95% vs 100%), *P. aeruginosa* (65% vs 87% vs 96.8%), *S. aureus* (32% vs 82% vs 98.9%) and *S. maltophilia* (8% vs 64% vs 93.5%).

5.3.4 Microbiota analysis

Characteristics of the broader bacterial microbiota were investigated for differences before and after postage using high-throughput sequencing. Specifically, taxa richness, diversity and dominance were calculated and assessed. The similarity of fresh and posted samples was also assessed.

5.3.4.1 Taxa richness

Taxa richness is the number of different bacterial taxa in a given sample. Taxa richness in fresh and posted samples is shown (Figure 5.3) and the percentage change in taxa richness between fresh to posted samples was calculated (Figure 5.4). The mean percentage change in taxa richness from fresh to posted was 45.6% (SD ±191.7) in the microbiota taxa, 63.4% (SD ±55.7) in the core taxa and 83.8% in the satellite taxa (SD ±439.2). The mean change in taxa richness was positive, however, there was no overall trend in taxa richness change in the microbiota as 50% of samples increased and 48% of samples decreased from fresh to posted. A paired Wilcoxon test indicated there was a significant difference ($P<0.0001$, Wilcoxon test statistic (Z)=-7.997) between the richness within the core taxa of fresh samples compared to posted samples as 87% of samples increased in taxa richness from fresh to posted. Richness in the microbiota ($P=0.515$, $Z=-0.340$) and satellite ($P=0.7$, $Z=-0.739$) taxa was not significantly different between fresh and posted samples.

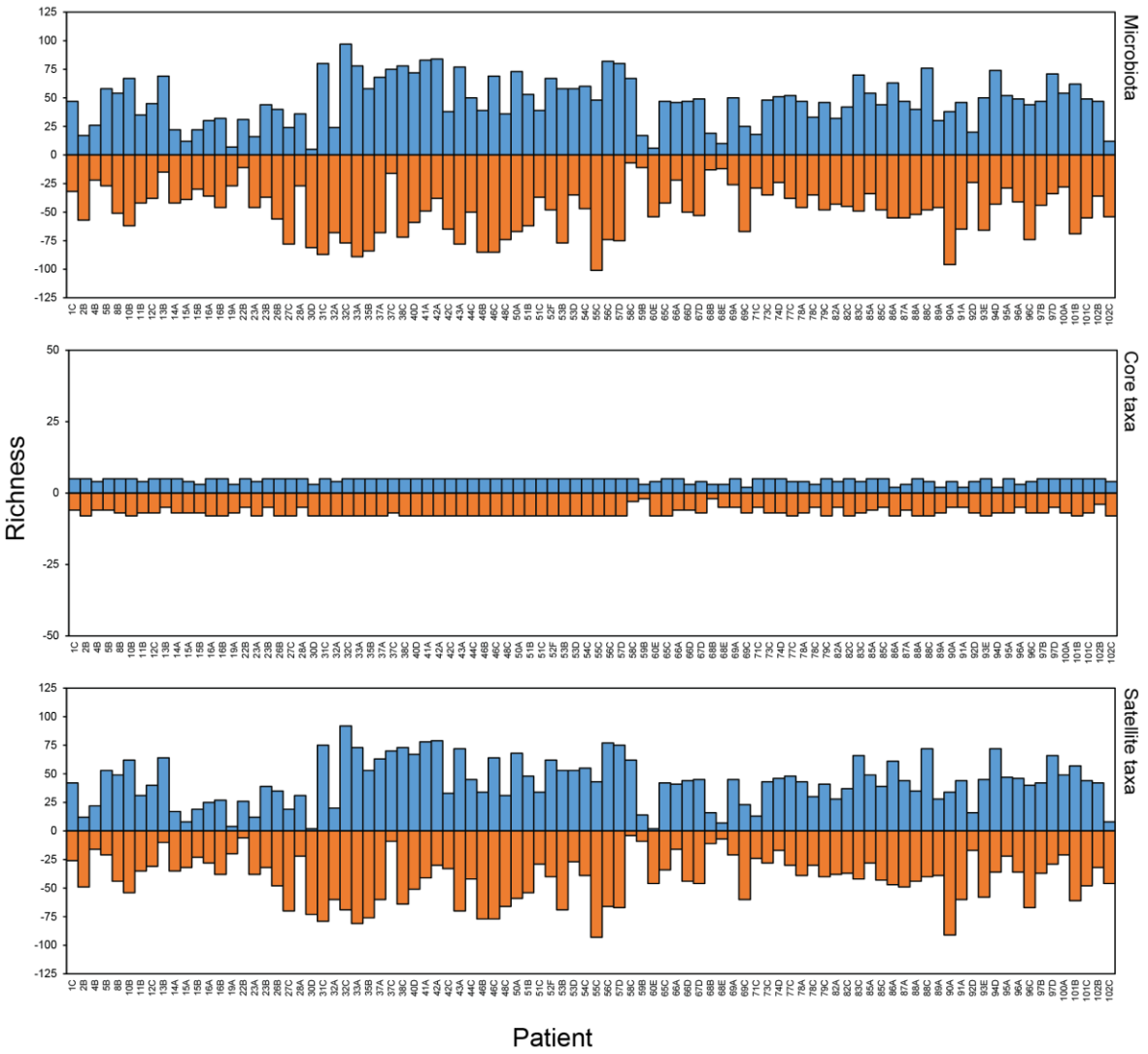

Figure 5.3 Richness in microbiota, core taxa and satellite taxa in fresh and posted samples. Fresh samples are shown in blue and posted samples are inverted and shown in orange.

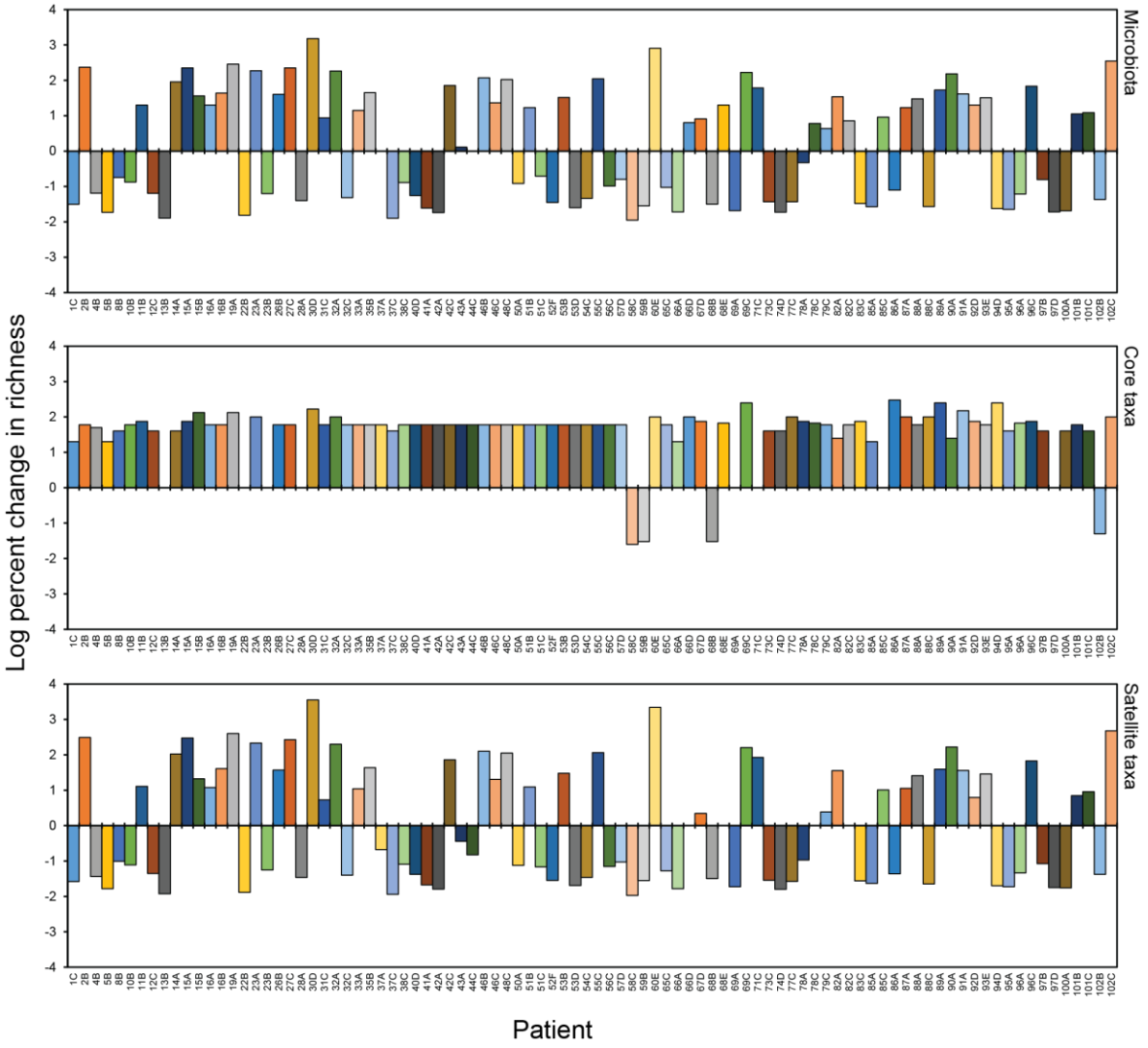

Figure 5.4 Richness change (log_{10} scale) in microbiota, core taxa and satellite taxa between fresh and posted samples. Positive values indicate an increase from fresh to posted and negative values indicate a decrease from fresh to posted.

5.3.4.2 Diversity

Diversity in fresh and posted samples is shown (Figure 5.5) and the percentage change in diversity between fresh and posted samples was assessed (Figure 5.6). The mean percentage change in diversity from fresh to posted was 58.7% (SD ±281.1) in the microbiota taxa, 69.5% (SD ±60.1) in the core taxa and 109.4% in the satellite taxa (SD ±731.4). The mean change in diversity was positive, however, there was no overall trend in diversity change in the microbiota as 51% of samples increased and 49% of samples decreased from fresh to posted. A paired Wilcoxon test indicated there was a significant difference ($P<0.0001$, $Z=-7.996$) between the dominance within the core taxa of fresh samples compared to posted samples as 91% of samples increased in diversity from fresh to posted. Diversity in the microbiota ($P=0.618$, $Z=-0.251$) and satellite ($P=0.774$, $Z=-0.706$) taxa was not significantly different between fresh and posted samples.

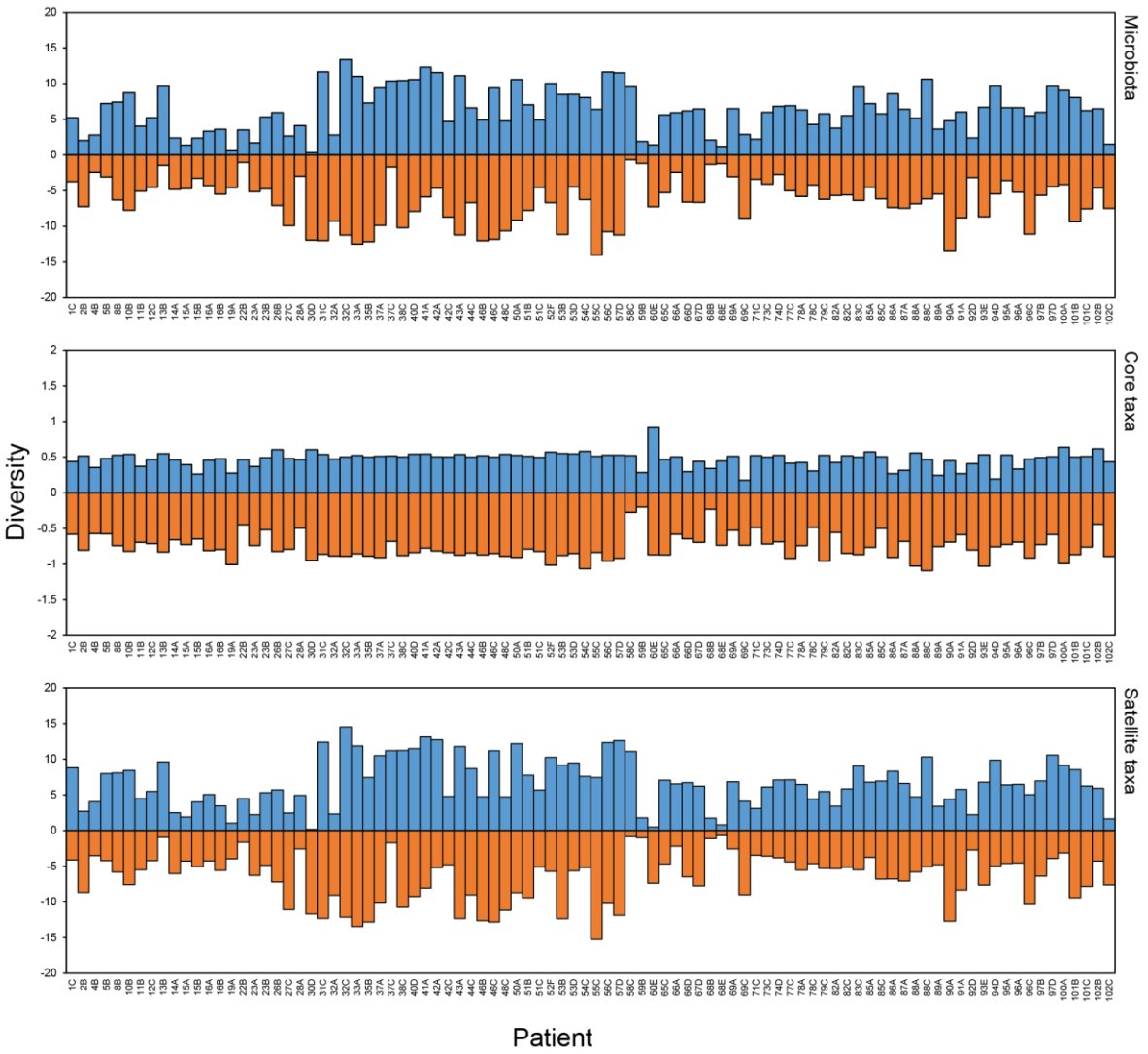

Figure 5.5 Diversity in microbiota, core taxa and satellite taxa in fresh and posted samples. Fresh samples are shown in blue and posted samples are inverted and shown in orange.

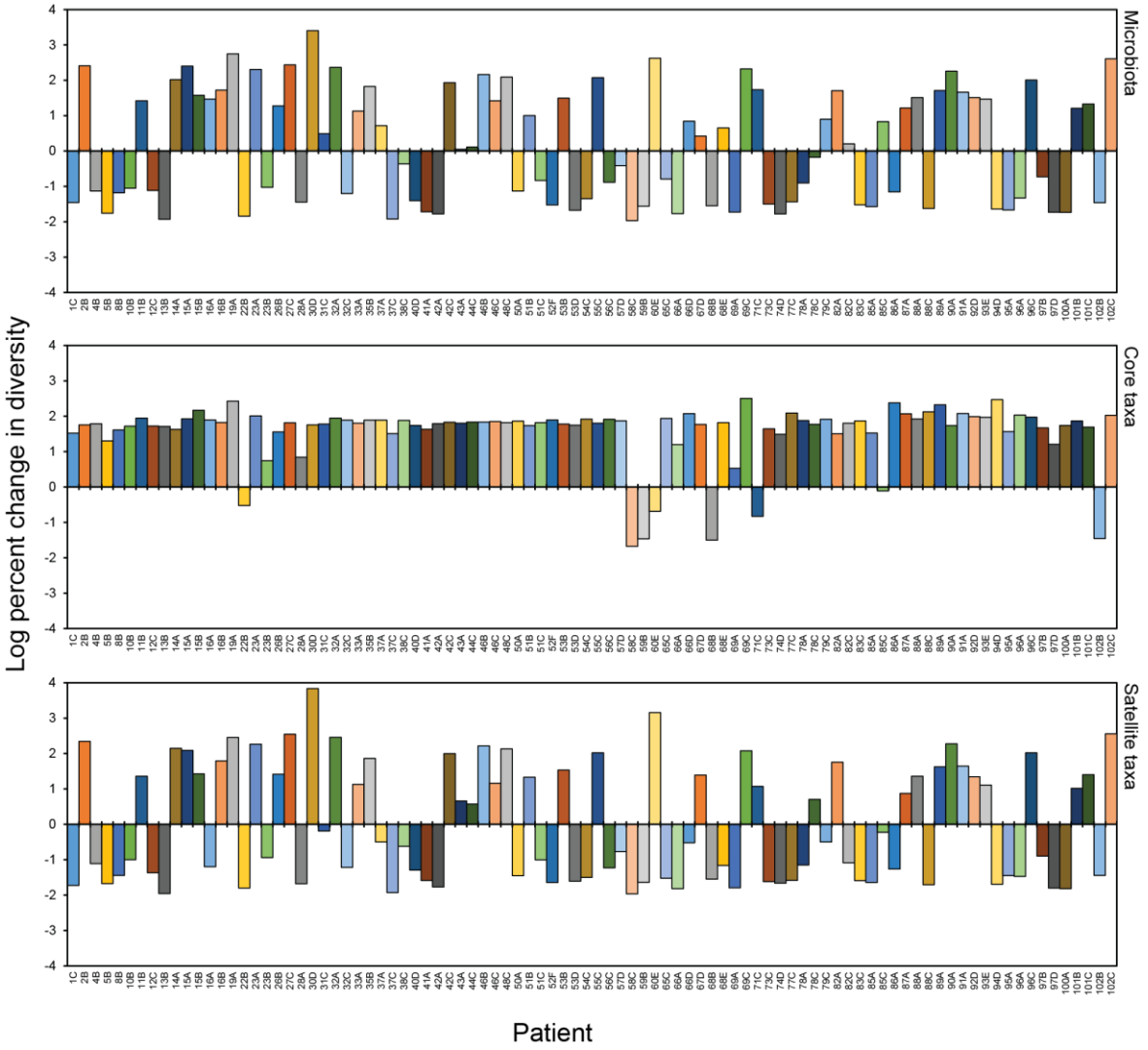

Figure 5.6 Diversity change (log_{10} scale) in microbiota, core taxa and satellite taxa between fresh and posted samples. Positive values indicate an increase from fresh to posted and negative values indicate a decrease from fresh to posted.

5.3.4.3 Dominance

Dominance in fresh and posted samples is shown (Figure 5.7) and the percentage change in dominance between fresh and posted samples was assessed (Figure 5.8). The mean percentage change in dominance from fresh to posted was 53.3% (SD ±87.4) in the microbiota taxa, -5.7% (SD ±39) in the core taxa and 42.4% in the satellite taxa (SD ±126). The mean change in dominance was positive, however, there was no overall trend in dominance change in the microbiota as 45% of samples increased and 53% of samples decreased from fresh to posted. A paired Wilcoxon test indicated there was a significant difference ($P<0.01$, $Z=-3.078$) between the dominance within the core taxa of fresh samples compared to posted samples as 58% of samples decreased in dominance from fresh to posted. Dominance in the microbiota ($P=0.649$, $Z=-0.878$) and satellite ($P=0.854$, T-test

statistic (t)=-0.184) taxa was not significantly different between fresh and posted samples when Wilcoxon and t-tests were carried out, respectively.

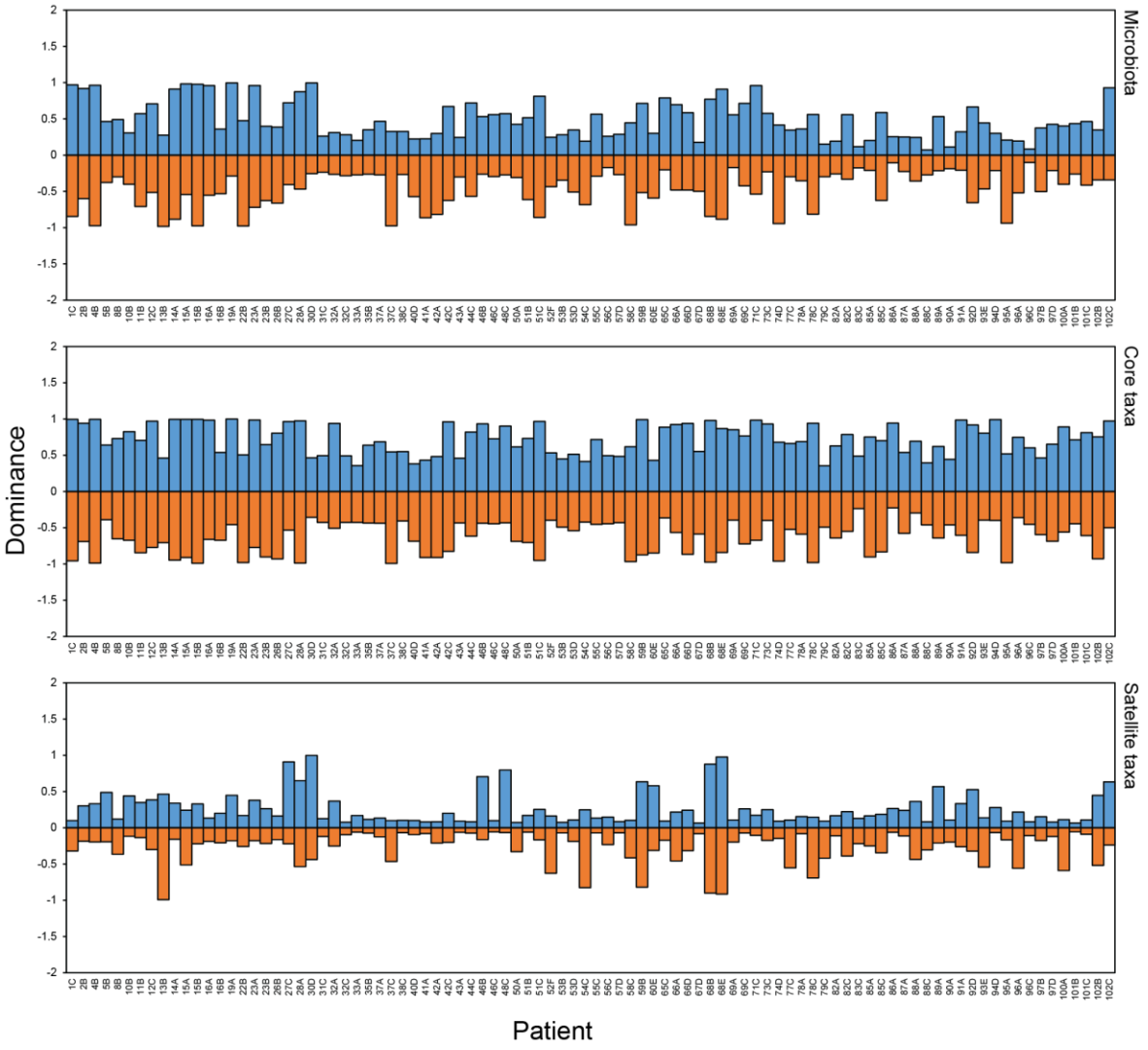

Figure 5.7 Dominance in microbiota, core taxa and satellite taxa in fresh and posted samples. Fresh samples are shown in blue and posted samples are inverted and shown in orange.

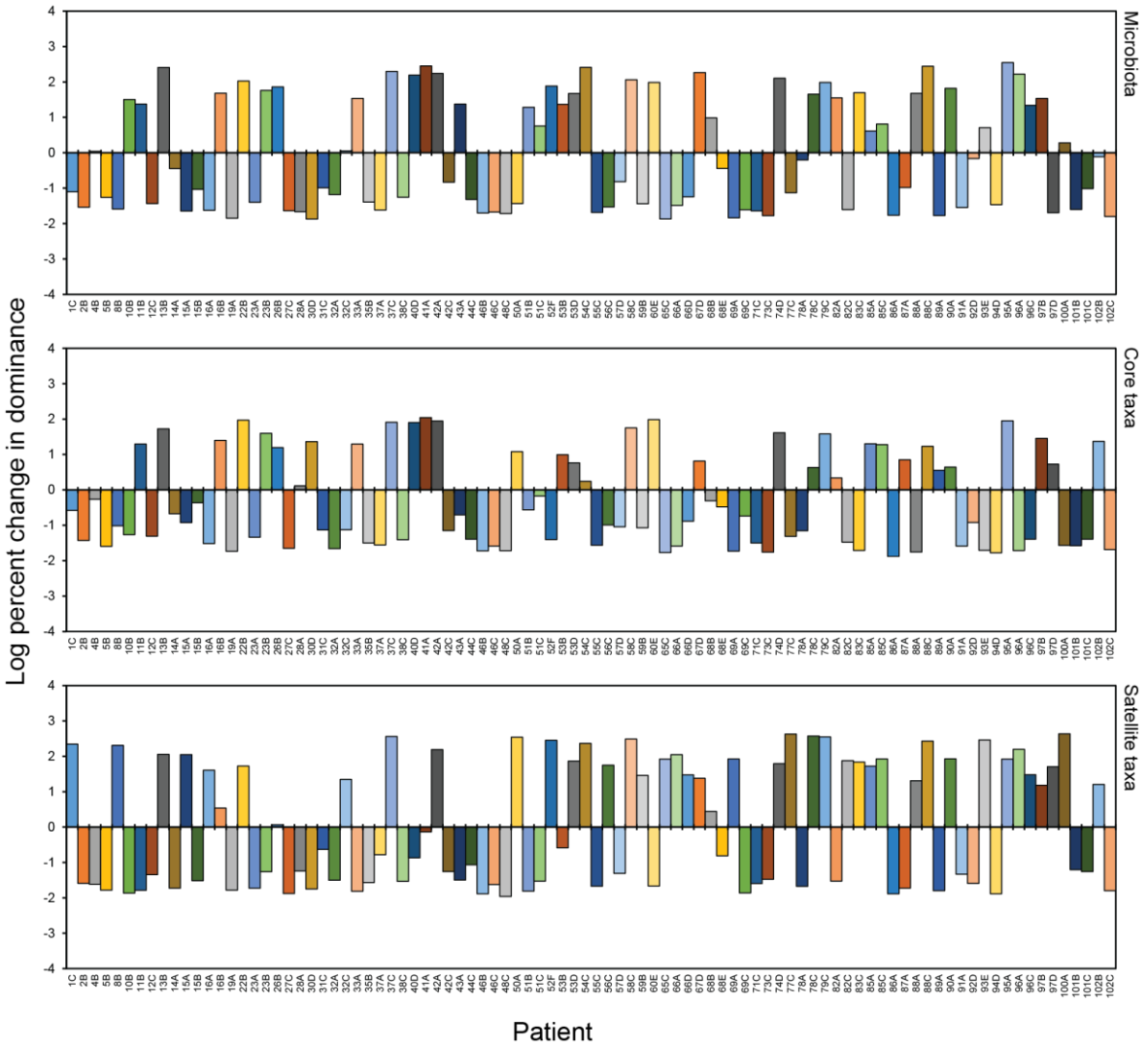

Figure 5.8 Dominance change (log_{10} scale) in microbiota, core taxa and satellite taxa between fresh and posted samples. Positive values indicate an increase from fresh to posted and negative values indicate a decrease from fresh to posted.

5.3.4.4 Dissimilarity

Sørensen and Bray-Curtis indices of dissimilarity were used to determine changes in the microbiota, core and satellite taxa in fresh and posted samples.

5.3.4.5 Sørensen Dissimilarity

Sørensen dissimilarity-based analysis showed the mean dissimilarity of the microbiota, core taxa and satellite taxa between fresh and posted samples was 55% (SD ±13.9), 28% (SD ±13.4) and 63% (SD ±14.9), respectively (Figure 5.9). ANOSIM showed the microbiota of the fresh samples were not significantly different to the posted samples (P=0.076, ANOSIM test statistic €=0.009). However, the core and satellite taxa communities of the fresh samples were significantly different to the posted samples (Core; P<0.001, R=0.593, Satellite; P<0.001, R=0.058).

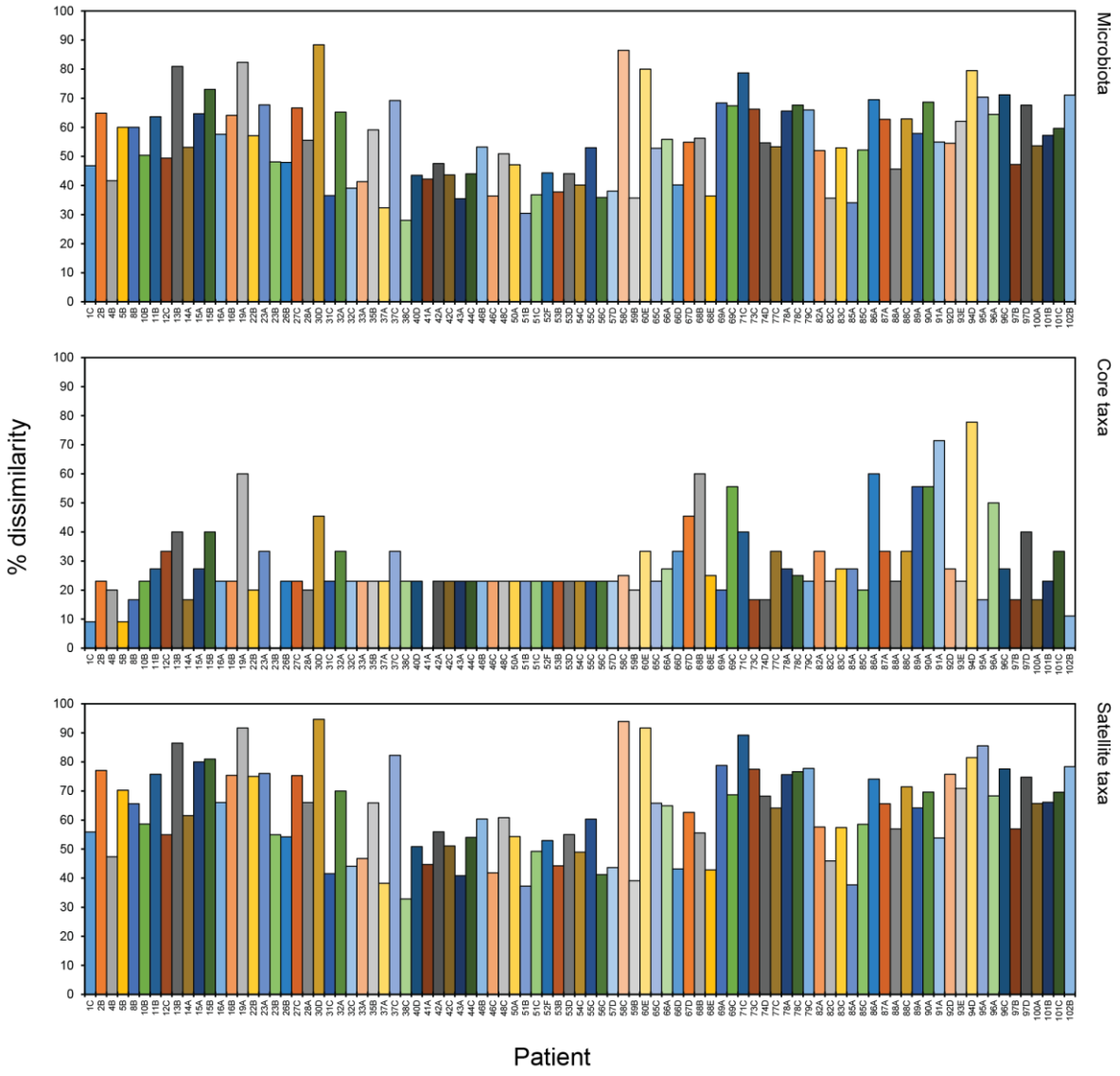

Figure 5.9 Sørensen measure of dissimilarity of microbiota, core, and satellite taxa between fresh and posted samples.

5.3.4.6 Bray-Curtis Dissimilarity

Bray-Curtis dissimilarity-based analysis showed the mean dissimilarity of the microbiota, core taxa and satellite taxa between fresh and posted samples was 53% (SD ±22.9), 58% (SD ±26.5) and 30% (SD ±21.1), respectively (Figure 5.10). ANOSIM showed the microbiota of the fresh samples were not significantly different to the posted samples ($P=0.396$, $R<0.001$). However, the core and satellite taxa communities of the fresh samples were significantly different to the posted samples (Core; $P=0.004$, $R=0.027$, Satellite; $P<0.001$, $R=0.037$).

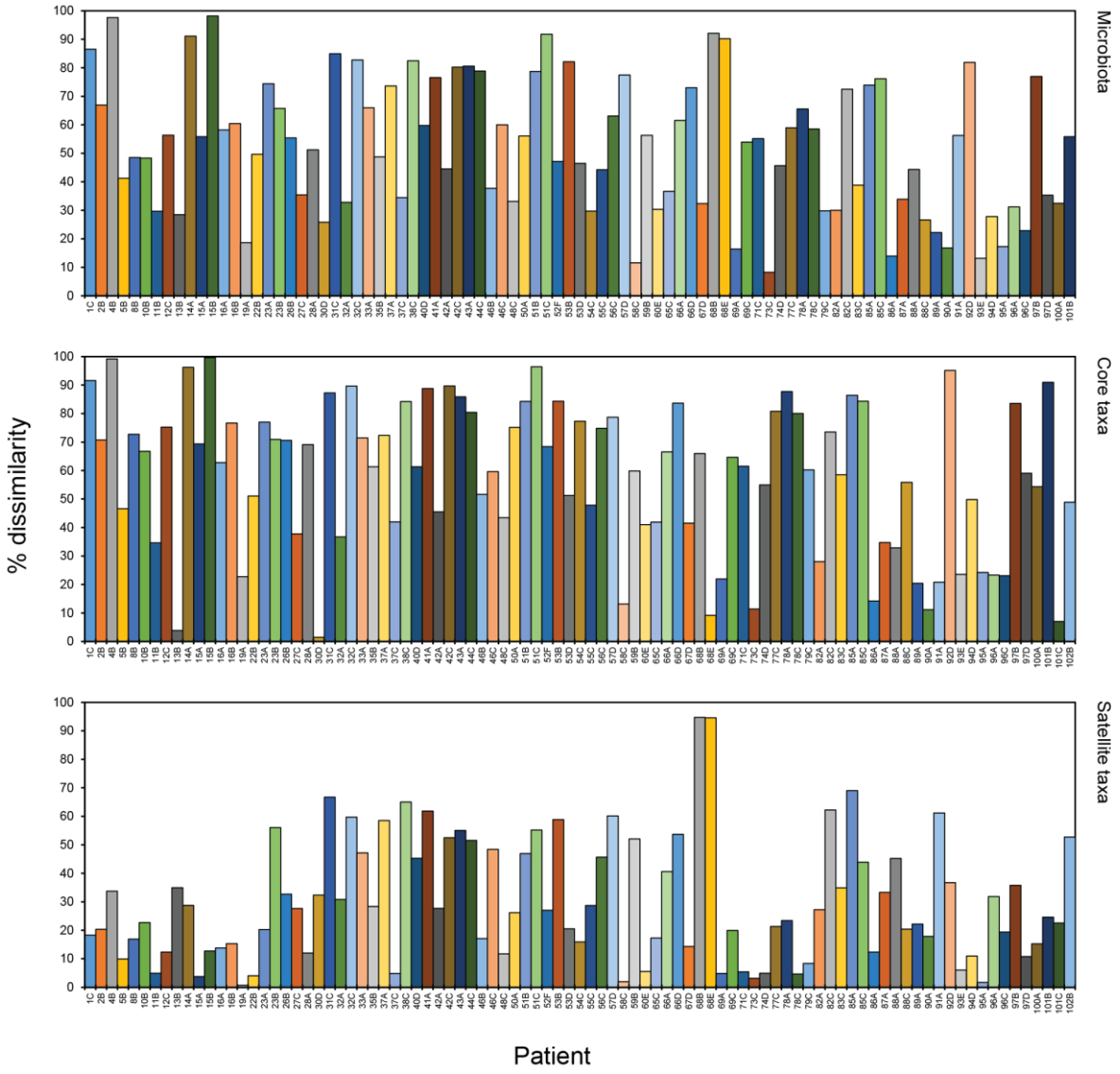

Figure 5.10 Bray-Curtis measure of dissimilarity of microbiota, core and satellite taxa between fresh and posted samples.

To determine the specific taxa that were responsible for the Bray-Curtis dissimilarity of the community between fresh and posted samples SIMPER analysis was used. *P. aeruginosa* was found to have the greatest contribution to the dissimilarity of the community between fresh and posted samples in the whole microbiota (Table 5.2). The CF Foundation monitors the prevalence of *Achromobacter* species, *B. cepacia* complex, *Haemophilus influenzae, P. aeruginosa, S. aureus* and *S. maltophilia* (Chapter 1, Figure 1.1). Five of the six monitored pathogens were identified in this patient cohort and those five were also shown to have the largest contributions to dissimilarity between fresh and posted samples (Table 5.2). *H. influenzae* was not identified by high-throughput gene sequencing in any of the patients in this study. Other *Haemophilus* species were identified but none had a greater than 1% contribution on the dissimilarity between fresh and posted samples.

SIMPER also showed *P. aeruginosa* had a higher mean relative abundance in fresh samples than in posted samples. Whereas *A. xylosoxidans*, *B. cepacia* complex, *S. aureus* and *S. maltophilia* all had higher mean relative abundances in posted samples than in fresh samples.

Table 5.2 Similarity of percentage (SIMPER) analysis of microbiota dissimilarity between fresh and posted samples for taxa with a greater than 1% contribution.

		Mean abundance	
Taxa	**% contribution to dissimilarity**	**Fresh**	**Posted**
*Pseudomonas aeruginosa**	20.646	**28.226**	**21.005**
Burkholderia cepacia complex*	15.304	**17.919**	**20.262**
*Stenotrophomonas maltophilia**	8.310	**6.278**	**8.235**
*Achromobacter xylosoxidans**	6.049	4.546	4.767
*Staphylococcus aureus**	4.902	**4.478**	**5.365**
Prevotella scopos	2.192	2.054	1.463
Streptococcus anginosus	1.828	2.303	1.042
Rothia mucilaginosa	1.781	1.380	1.927
Sphingomonas hengshuiensis	1.447	1.501	**1.834**
Variovorax paradoxus	1.347	**1.774**	**1.688**
Streptococcus infantis	1.129	0.964	1.136

Core taxa in fresh and posted samples are highlighted in bold. Key CF pathogens are indicated by an asterisk. The list of taxa is not exhaustive.

5.3.5 Abundance of pathogen sequence variants

The targeted gene sequencing used in this study was not able to distinguish strains of each species. However, multiple sequence variants identified as the same species were seen after processing. Therefore, this study suggests that sequence variants could be used as a proxy for strains. The qPCR data described in Chapter 4 was combined with the targeted gene sequencing data to determine the CFU/ml equivalent of each sequence variant identified as a CF pathogen in fresh and posted samples (Figure 5.11). Targeted gene sequencing identified one *A. xylosoxidans* variant (Figure 5.11, D). Multiple variants were identified as *P. aeruginosa* (34), *B. cepacia* complex (23), *S. maltophilia* (18) and *S. aureus* (4) (Figure 5.11, A, B, C, E).

Variants identified as *B. cepacia* complex members could not be confidently assigned a species. Therefore, the multiple variants identified are most likely multiple species within the complex.

The majority of samples in which the pathogens were detected were dominated by one ASV variant (defined as >75% of sequences were one variant) (Figure 5.11, shown in blue)

(*A. xylosoxidans* 100%, *B. cepacia* complex: 96%, *P. aeruginosa* 90%, *S. aureus* 83% and *S. maltophilia* 19%). In some samples, the pathogens were detected by sequencing but were present in numbers below the threshold of qPCR and were therefore not detected by qPCR (*A. xylosoxidans* 37%, *B. cepacia* complex: 17%, *P. aeruginosa* 22%, *S. aureus* 38% and *S. maltophilia* 50%).

In numerous samples, one or more variants were detected by sequencing and qPCR in the fresh sample but not the posted sample and vice versa; *P. aeruginosa* 57% (fresh +ve, posted -ve) and 50% (fresh -ve, posted +ve), *B. cepacia* complex 47% and 52%, *S. maltophilia* 31% and 20%, *A. xylosoxidans* 14% and 9%, *S. aureus* 18% and 23%.

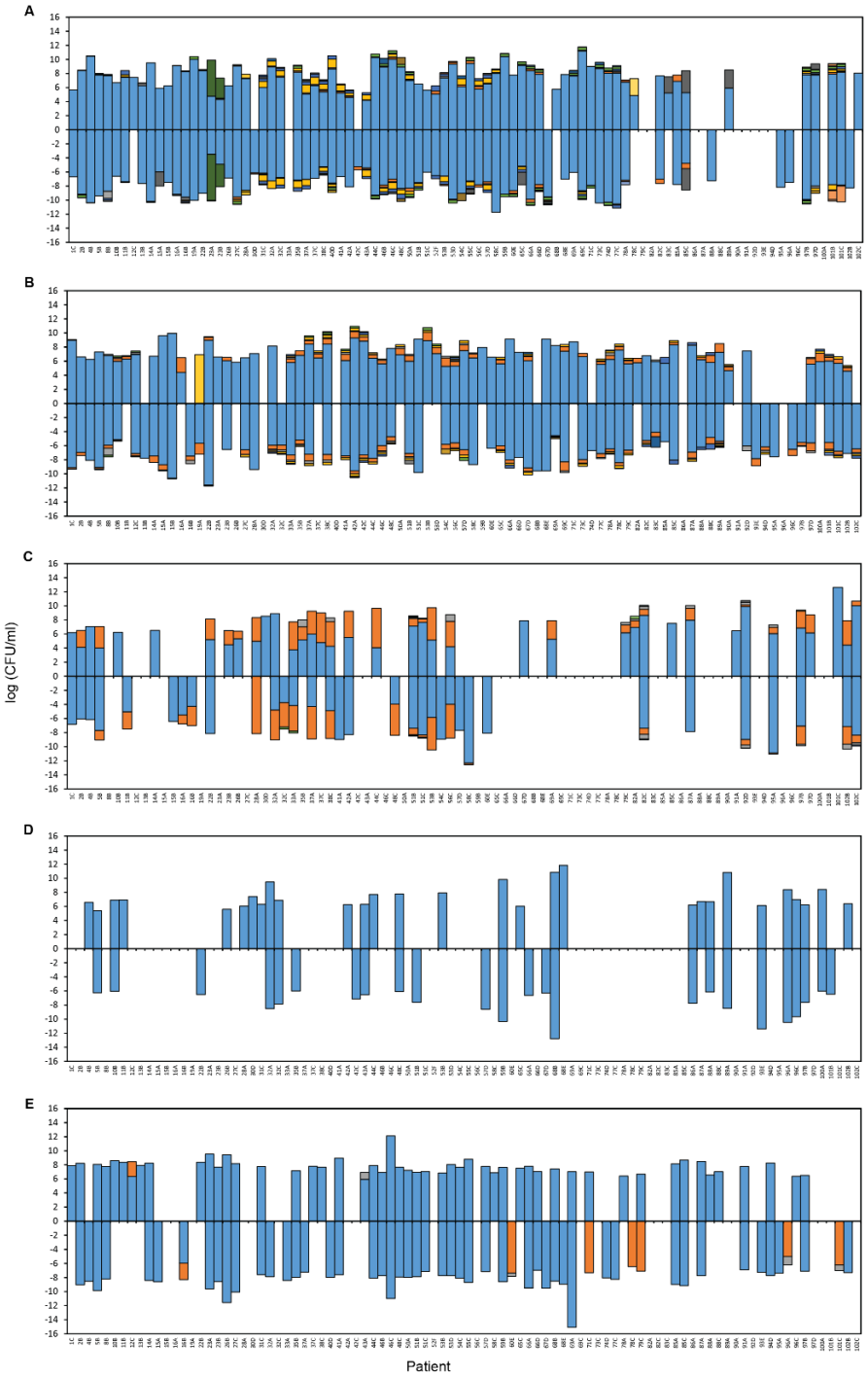

Figure 5.11 CFU/ml equivalent (log_{10} scale) of variants identified in fresh and posted samples as (A) *P. aeruginosa* (n=92), (B) *B. cepacia* complex (n=87), (C) *S. maltophilia* (n=86), (D) *A. xylosoxidans* (n=92) and (E) *S. aureus* (n=92) by microbiota sequencing. Different variants are shown in different colours for each pathogen. Fresh samples are shown above and posted samples have been inverted and are shown below the x axis. n is the total number of paired samples qPCR was applied to.

5.4 Discussion

The aim of this study was to determine how bacterial microbiota composition and structure within sputum samples was affected by postage in the standard UK postal system. The use of high-throughput gene sequencing to research the effects of exacerbation and treatment on the lung microbiota is increasing (Fodor *et al.*, 2012; Carmody *et al.*, 2013, 2015; Cuthbertson *et al.*, 2016, 2020). Patients usually provide samples for clinical studies at routine clinic appointments. However, if samples could be provided at home and posted to the clinic or research institution, more frequent samples could be taken without an increased risk of infection to patients attending clinics in person or an increase in appointments needed, reducing the healthcare burden for the patient, clinic, and clinicians.

This study showed that key CF pathogens identified by microbiota sequencing were more likely to be present in the fresh (*A. xylosoxidans* and *S. maltophilia*) or posted sample (*B. cepacia* complex*, P. aeruginosa* and *S. aureus*) (Figure 5.2). However, confidence intervals were large for each pathogen indicating no overall trend could be concluded. It is possible that this large range is due to sample variability and may be caused by interactions between different bacterial species present in samples. Microbiota sequencing also showed a larger percentage of samples with key CF pathogens present than culture-based methods and qPCR (excluding *A. xylosoxidans* which qPCR determined a higher percentage of samples had present). As previously discussed, qPCR and culture-based methods have detection limits. Namely, qPCR can only detect pathogens when the abundance is above a specific threshold and culture-based methods are limited by the presence of VBNC cells and other phenotypic changes that complicate correct identification. These results suggest both culture-based methods and, to a lesser extent, qPCR do not show a complete representation of the CF pathogens present.

In this study, high-throughput gene sequencing was used to identify bacterial taxa within the microbiota of fresh and posted sputum samples. The effect of posting samples on the microbiota composition was assessed. Bacterial richness, diversity and dominance of the microbiota were not significantly altered when samples were posted (Figures 5.4, 5.6 and 5.8). The Sørensen and Bray-Curtis dissimilarities of the microbiota between fresh and posted samples were non-significant (Figures 5.9 and 5.10). Richness and diversity were shown to increase in a higher proportion of samples after postage whereas dominance was shown to decrease in a higher proportion of samples after postage. Zhao *et al.* (2011) split a single CF sputum sample and used pyrosequencing to show there was no effect on the

bacterial community composition when sub-samples were stored at room temperature for 1,2 and 4 weeks compared to the fresh sub-sample. Cuthbertson *et al.* (2014) used pyrosequencing to analyse the microbiota of PMA-treated sputum samples stored at room temperature for 1-72 hours. They found that significant changes in bacterial community composition occurred after 12 hours of storage at room temperature and suggested room temperature masks intersample variability and is less favourable for visualising organisms with lower 16S rRNA GC content such as *H. influenzae* and *S. aureus*. Nelson *et al.* (2010) used DGGE to show a significant difference in bacterial community profiling in sub-samples stored for 24 hours at room temperature compared to sub-samples stored at 4 °C. However, these studies used a much smaller sample size than in the current study; Zhao *et al.* (2011) 1, Cuthbertson *et al.* (2014) 8 and Nelson *et al.* (2010) 5.

Previous studies have demonstrated partitioning the whole microbiota into core and satellite taxa groups reveals important aspects of community diversity and distributions that are missed when looking at the microbiota as a whole (van der Gast *et al.*, 2011). Cuthbertson *et al.* (2014) split the microbiota of samples into common and rare taxa to show storage at room temperature had a larger effect on the rare taxa than the common taxa. The present study showed that by partitioning the microbiota into core and satellite taxa, mean changes in richness, diversity and dominance were greater in satellite taxa than in core taxa (Figures 5.4, 5.6 and 5.8). This suggests that the satellite taxa are more likely to be altered when samples are posted. However, richness and diversity within the core taxa were significantly increased between fresh and posted samples and dominance within the core taxa significantly decreased between fresh and posted samples. This suggests that satellite taxa are more likely to undergo large positive and negative changes in richness, diversity and dominance when samples are posted. However, the core taxa group is more likely to significantly increase in richness and diversity and decrease in dominance.

Sørensen analysis showed a greater dissimilarity of identified taxa between fresh and posted samples in satellite taxa compared to core taxa (Figure 5.9). However, when abundance was taken into consideration, Bray-Curtis analysis showed a greater dissimilarity of taxa and abundance between fresh and posted in samples in core taxa compared to satellite taxa (Figure 5.10). This suggests that the individual taxa present in the satellite taxa altered from fresh to posted samples, but the abundance was more altered in the core taxa although no positive or negative correlation was seen.

SIMPER analysis was used to determine which identified taxa had the largest contribution to Bray-Curtis dissimilarity between fresh and posted samples. These were identified as the five key CF pathogens previously targeted by qPCR; *P. aeruginosa, B. cepacia* complex*, S. maltophilia, A. xylosoxidans* and *S. aureus* (Table 5.2). With the exception of *A. xylosoxidans*, these taxa were also part of the core taxa. Cuthbertson *et al.* (2020) also previously identified *P. aeruginosa, B. cepacia* complex*, S. maltophilia* and *S. aureus* as members of the core taxa and *A. xylosoxidans* as part of the satellite taxa in their patient population. No *Haemophilus* species contributed more than 1% to the dissimilarity between fresh and posted samples and *H. influenzae* was not identified in any of the samples in this study. *H. influenzae* is more prevalent in the lungs of children with CF (Cystic Fibrosis Foundation, 2019) and as this study only recruited adults with CF, a low prevalence of *H. influenzae* was expected.

Other bacterial species were also shown to contribute largely to the Bray-Curtis dissimilarity observed including *Prevotella, Rothia, Streptococcus* and *Variovorax* species. The role of these taxa in CF lung disease is unclear. *Rothia mucilaginosa* and *Streptococcus pneumoniae* have been associated with a worse lung function in people with CF (Paganin *et al.*, 2015) whereas *Prevotella* and *Streptococcus* taxa have been associated with decreased airway inflammation in people with CF (Zemanick *et al.*, 2013, 2017). *Prevotella, Rothia* and *Streptococcus* are facultative or obligate anaerobes (Tunney *et al.*, 2008; Field *et al.*, 2010; Lim *et al.*, 2013). Therefore, an alteration in these species is unsurprising as the oxygen concentration in the postage container is altered as oxygen is used by aerobic species present in the microbiota. Previous research has shown an decrease in anaerobic species during the first 48 hours of storage at room temperature followed by an increase in the following 24 hours (Cuthbertson *et al.*, 2014). However, these timings will depend on the volume ratio of sample and air in the sample container and the bacterial species and load within the sample. Further analysis would be required to determine the optimum sample weight, container size and time in the post to avoid the over- or underestimation of anaerobic species during CF research if posted samples were to be used.

Multiple sequence variants were identified as each CF pathogen (expect *A. xylosoxidans*) which indicated multiple strains of each taxon were detected. Sequencing data was combined with the qPCR data from Chapter 4 to show the CFU/ml of each variant in individual samples. Variants were detected in the fresh sample but not the posted and vice

versa in numerous samples (Figure 5.11). This indicates that the conditions of the fresh and posted samples were suited to different strains (or species of the *B. cepacia* complex).

During exacerbation and antibiotic intervention studies, it is often important to determine the antibiotic resistance profile of the pathogens present/ being targeted. An individual can have different strains of the same species with different antibiotic susceptibilities and phenotypes present (Cantón *et al.*, 2003; Kahl *et al.*, 2003; Foweraker *et al.*, 2005; Leitão *et al.*, 2008; Clark *et al.*, 2015). Therefore, it is important that strains present in the fresh sample are also present in the posted sample if any genotype, phenotype, or antibiotic susceptibility testing is required.

5.5 Conclusion

This study showed that the overall lung microbiota, identified by high-throughput targeted gene sequencing, is not significantly altered when sputum samples are posted. However, positive and negative changes between fresh and posted aliquots were seen in individual samples. The microbiota was split into core and satellite taxa and the majority of CF pathogens were identified as core taxa. It was also shown that the richness, diversity and dominance of the core taxa was significantly altered when samples were posted. The overall microbiota of the fresh samples was not significantly dissimilar to the microbiota of the posted samples. However, the core and satellite taxa are significantly dissimilar. The microbiota dissimilarity between fresh and posted samples is driven by the main CF pathogens; *P. aeruginosa, B. cepacia* complex members*, S. maltophilia, A. xylosoxidans* and *S. aureus*. Multiple strains of CF pathogens were identified by targeted gene sequencing. It was shown that the presence and abundance of these strains were altered during postage. Therefore, the use of posted respiratory samples to determine the CF lung microbiota should be utilised with caution.

Chapter 6 Pathogen assessment during antibiotic therapy for pulmonary exacerbation using qPCR

6.1 Introduction

Lung disease in CF patients begins early in life and inflammation, impaired mucociliary clearance and chronic infection lead to a decline in lung function interspersed with acute pulmonary exacerbations (Flume *et al.*, 2009). There is no clinical consensus for the definition of a pulmonary exacerbation and diagnosis is based on a range of clinical symptoms including increased sputum production, shortness of breath, chest pain, increased cough and a decline in lung function (Goss and Burns, 2007; Cuthbertson *et al.*, 2016). The cause of exacerbations is unknown, however, multiple possible causes have been postulated including an increase in bacterial load, the acquisition of a new bacterial strain or species and environmental factors (Rogers *et al.*, 2011b). Although the cause of exacerbations is unknown, known pathogens have been isolated from respiratory samples during exacerbations so antibiotic regimens have been focused on supressing the growth of these pathogens during an exacerbation. The frequency of pulmonary exacerbations has been linked to progressive lung function decline so it is important to treat infections effectively (Goss and Burns, 2007; De Boer *et al.*, 2011).

Once a person with CF has been assessed to be experiencing a pulmonary exacerbation they are admitted to hospital and typically receive enhanced antibiotic therapy for approximately 10-14 days. To direct antibiotic therapy, bacteria isolated from a respiratory sample are identified and their antibiotic susceptibility profiles assessed using culture-based techniques including the use of selective agars and biochemical tests (Cystic Fibrosis Trust, 2010). However, previous studies have shown no or a weak association between the outcome of antibiotic therapy and the susceptibility of *Pseudomonas aeruginosa* to antibiotics by culture (Smith *et al.*, 2003; Parkins *et al.*, 2012). Therefore, it is important to track the effectiveness of antibiotic therapy by monitoring symptoms, bacterial load, and susceptibility of pathogens throughout therapy and alter the antibiotics, if necessary, rather than rely on the results of susceptibility testing.

Culture-based techniques only provide the clinician with a presence/absence qualitative result against a suite of known pathogens (Cystic Fibrosis Trust Antibiotic Group, 2009). The effectiveness of exacerbation treatment is determined by a respiratory sample being negative for the target pathogen by culture. However, qPCR could be used to quantify the bacterial species present before, during and after treatment (Deschaght *et al.*, 2013). This would indicate if antibiotic treatment was working by, for example, demonstratable reduction in pathogen load over the course of treatment for exacerbation, allowing

clinicians to alter the treatment to optimise therapy before the end of the course of antibiotics.

Culture-based methods have previously been used to show antibiotics significantly reduce the bacterial load of *P. aeruginosa* (Blumer *et al.*, 2005; Tunney *et al.*, 2011; Deschaght *et al.*, 2013; Lam *et al.*, 2015), methicillin-sensitive *Staphylococcus aureus* (MSSA) (Lam *et al.*, 2015) and *Burkholderia cepacia* complex members (Blumer *et al.*, 2005) between the start and end of treatment for an exacerbation. However, when the culture-independent method Terminal Restriction Fragment Length Polymorphism (T-RFLP) was used a relative increase in *P. aeruginosa* was found during *Pseudomonas*-targeted antibiotic treatment (Daniels *et al.*, 2013). However, the absolute numbers of *P. aeruginosa* and non-*Pseudomonas* species both decreased suggesting the range of antibiotics administered not only decreased *Pseudomonas* but also, to a greater extent, non-*Pseudomonas* species. Non-*Pseudomonas* species decreased to a larger extent than *P. aeruginosa*, therefore an overall relative increase of *P. aeruginosa* was seen. It is, therefore, difficult to conclude the effect of antibiotic treatment on *P. aeruginosa* from this study but it is clear that *Pseudomonas*-targeted antibiotics have a negative effect on non-*Pseudomonas* species.

Previous research utilising qPCR to quantify *P. aeruginosa* (Deschaght *et al.*, 2013; Price *et al.*, 2013; Reid *et al.*, 2013; Zemanick *et al.*, 2013; Stokell *et al.*, 2015) and other common bacterial species (Reid *et al.*, 2013; Stokell *et al.*, 2015) in CF sputum samples before and after antibiotic treatment for an exacerbation have contradicted each other. For example Price *et al.* (2013) showed no significant difference in the abundance of *P. aeruginosa* between samples taken at the start and end of treatment for an exacerbation. However, Reid *et al.* (2013) and Zemanick *et al.* (2013) showed a significant decrease in *P. aeruginosa* between the start and end of antibiotic treatment. Reid *et al.* (2013) also showed a significant decrease in *Haemophilus influenzae* and *Streptococcus pneumoniae* following treatment with a range of antibiotics.

Previous culture-independent research has mainly focused on changes in *P. aeruginosa* (Deschaght *et al.*, 2013; Price *et al.*, 2013; Reid *et al.*, 2013; Zemanick *et al.*, 2013; Stokell *et al.*, 2015) with a single study also focusing on *H. influenzae* and *S. pneumoniae* (Reid *et al.*, 2013) and another study also focused on *B. cepacia* complex (Stokell *et al.*, 2015). It is unclear from these studies if the abundance of *P. aeruginosa* is affected by antibiotic treatment as the studies reported different findings. It is also important to determine the effect of antibiotic treatment on other key CF pathogens.

This study aimed to establish whether antibiotic therapy for an exacerbation alters the density of key CF pathogens. This chapter determined whether known pathogens could be quantified during treatment for exacerbation using the previously developed species-specific qPCR assays. Sputum samples were obtained from CF patients at the beginning, end, and following a range of antibiotic treatment regimens for an exacerbation and the density of pathogens determined from each sample.

6.2 Methods

6.2.1 Patient samples

A total of 59 spontaneously expectorated sputum samples were collected from 23 people with CF attending Southampton University Hospital, UK between 4th November 2013 and 14th March 2014 and stored at -80 °C until transport to Manchester Metropolitan University on ice. Written informed consent was obtained from participants and ethical approval was granted by the NHS Research Ethics Committee (NHS REC number: 08/H0502/126). Participants were administered a range of oral, intravenous and nebulised antibiotics. Samples were taken at the beginning of antibiotic treatment, when the antibiotic treatment was finished and a week to 3 weeks after antibiotic treatment was stopped. Nine people who did not provide a sample at the start of treatment, end of treatment and at follow up were excluded from this study (Table 6.1).

6.2.2 Sample processing

Sputum samples were washed, treated with PMA and DNA extracted as described in Chapter 2 in section 2.3. qPCR was used for the quantification of CF pathogens in the sputum samples using the previously optimised conditions described in Chapter 3.

6.2.3 Statistical analysis

Generalised linear models on presence/absence data and odd ratios were performed and calculated using the package lmerTest in R version 3.6.2 (Lam *et al.*, 2015; Singh *et al.*, 2019).

Mixed effects models to compare the CFU/ml, as determined by qPCR, in samples at the start and end of treatment and at the follow up were performed and calculated using the package lmerTest in R (Cuthbertson *et al.*, 2016; Kuznetsova *et al.*, 2017).

6.3 Results

6.3.1 Clinical characteristics of patients

Sputum samples (n=42) were obtained from people with CF (n=14) attending Southampton University Hospital, Southampton, UK (Table 6.1, Table 6.2).

Table 6.1 Summary of clinical characteristics of patients included in the study.

Number of patients	14
Sex of patients (M:F)	11:3
Mean age (years) at first or only sample (±SD)	25.6 (± 7.9)
Minimum and maximum age (years)	18 to 46
CFTR Genotype:	
Homozygous F508del	8
Heterozygous F508del	5
Unknown	1
Mean %FEV1 at date closest to first sample (±SD)	49.7 (±15.3)
Antibiotic target:	
Gram negative bacteria	12
Staphylococci	5
Broad spectrum	9

Table 6.2 Clinical characteristics of individuals included in the study.

Subject	Age	Gender	Genotype 1	Genotype 2	FEV_1 (litres)	FEV_1 %	Prescribed antibiotics
1	30	M	unknown	unknown	1.41	36	Ceftazidime, tobramycin
2	25	F	DF508	G542X	NA	NA	Ceftazidime, flucloxacillin, tobramycin
3	39	M	DF508	DF508	2.34	61	Colistrimethate, meropenem
4	24	F	DF508	G542X	1.7	48	Colomycin, meropenem
5	26	M	DF508	DF508	1.67	43	Aztreonam, colomycin, tobramycin
6	21	M	DF508	621+1G>T	2.79	59	Ciprofloxacin
7	29	M	DF508	DF508	2.59	73	Colistin, meropenem, teicoplanin
8	20	F	DF508	DF508	2.03	65	Aztreonam, flucloxacillin
9	20	M	DF508	unknown	3.25	77	Co-trimoxazole, meropenem, tobramycin
10	18	M	DF508	DF508	4.06	26	Ciprofloxacin, tetracycline
11	21	M	DF508	G542X	1.52	39	Meropenem, tobramycin
12	21	M	DF508	DF508	1.59	34	Amikacin, ceftazidime, fosfomycin
13	46	M	DF508	DF508	1.79	47	Colomycin, vancomycin
14	18	M	DF508	DF508	1.46	38	Flucloxacillin, moxifloxacin, timetin

FEV_1 is shown from the date closest to the first sample. FEV_1 - forced expiratory volume in one second. NA – not available.

6.3.2 Prevalence of CF pathogens using quantitative PCR

The prevalence of *Achromobacter xylosoxidans, B. cepacia* complex, *P. aeruginosa, S. aureus* and *Stenotrophomonas maltophilia* in sputum samples were determined using specific qPCR assays (Figure 6.1) at three time points: 1) at the start of treatment for an exacerbation, 2) when the treatment was finished and 3) a week to 3 weeks after the end of treatment. Odds ratios were used to determine *P. aeruginosa* and *S. maltophilia* were more likely to be present in the sample at the start of treatment than at the end by 17 (95% confidence interval (CI) 0.738-391.699) and 1.750 (95% CI 0.215-14.223) times,

respectively. Whereas *A. xylosoxidans*, *B. cepacia* complex and *S. aureus* were more likely to be present in the sample at the end of treatment than at the start by 4 (95% CI 0.363-44.111), 2.4 (95% CI 0.165-34.934) and 1.371 (95% CI 0.288-6.535), respectively.

P. aeruginosa and *S. maltophilia* were also more likely to be present at the start of treatment than in the follow up sample by 2.667 (95% CI 0.361-19.711) and 1.750 (95% CI 0.215-14.223) times, respectively. Whereas *A. xylosoxidans*, *B. cepacia* complex and *S. aureus* were more likely to be present in the follow up sample than present at the start of treatment by 1.8 (95% CI 0.21-15.406), 3.2 (95% CI 0.540-18.982) and 1.333 (95% CI 0.301-5.912) times, respectively.

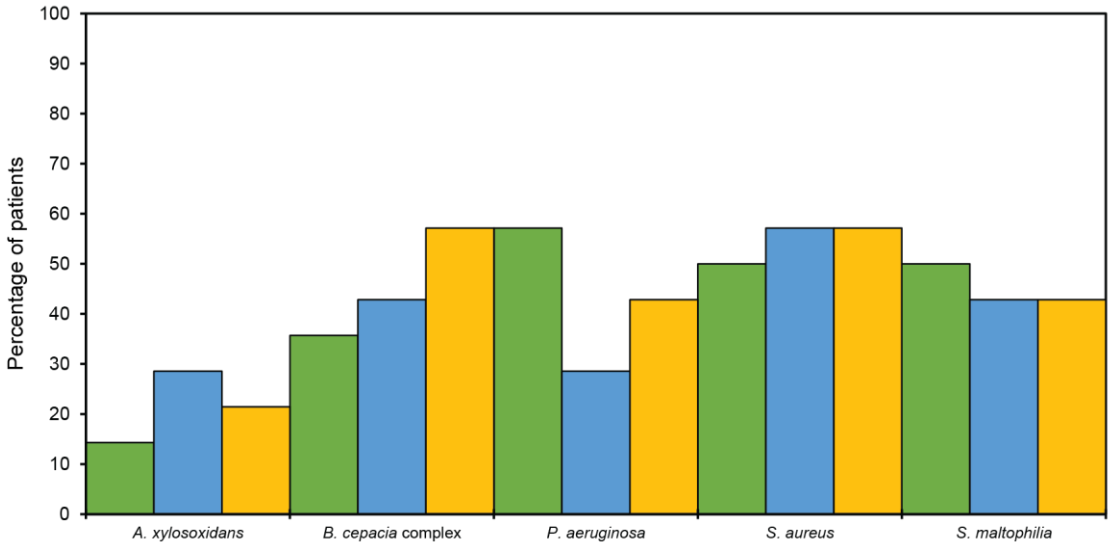

Figure 6.1 CF pathogens present in samples at the start and end of treatment and at follow up determined by qPCR. Percentage of patients positive for each pathogen at the start (green) and end (blue) of treatment and at follow up (yellow).

For a number of patients that had the pathogen present at the start of antibiotic treatment, the pathogen fell below the threshold of qPCR detection at the end of treatment (Table 6.3) then at follow up the pathogen returned to levels over the detection threshold (except *A. xylosoxidans*) (Table 6.3).

Table 6.3 Pathogen positive patients at the start of treatment with a change in pathogen status at the end of treatment and at follow up.

Pathogen	No. of patients pathogen +ve at start and -ve at end of treatment	No. of patients pathogen +ve at start, -ve at end of treatment and +ve at follow up
A. xylosoxidans	2/2 (100%)	0/2 (0%)
B. cepacia complex	1/5 (20%)	1/1 (100%)
P. aeruginosa	4/8 (50%)	2/4 (50%)
S. aureus	5/7 (71%)	4/5 (80%)
S. maltophilia	3/7 (43%)	3/3 (100%)

+ve positive and -ve negative by qPCR

6.3.3 Quantification of CF pathogens using quantitative PCR

The CFU/ml equivalents of key CF pathogens in sputum samples were determined using specific qPCR assays (Figure 6.2) at three time points: 1) at the start of treatment for an exacerbation, 2) when the treatment was finished and 3) a week to 3 weeks after the end of treatment.

When samples from the start of treatment were compared with samples at the end of treatment, a mixed effect model showed no significant difference in the abundance (CFU/ml equivalents) of key CF pathogens (*A. xylosoxidans* (P=0.189, R^2=0.074, ANOVA test statistic (F)=0.924)*, B. cepacia* complex (P=0.6145, R^2=0.048, F=0.807), *P. aeruginosa* (P=0.0522, R^2=0.181, F=2.159)*, S. aureus* (P=0.7002, R^2=0.009, F=0.181) and *S. maltophilia* (P=0.707, R^2=0.022, F=0.078)).

When only samples that had the pathogen detected at the start of treatment were compared to the paired sample at the end of treatment CF pathogens were shown to have decreased in CFU/ml equivalents; 100% (2/2) *A. xylosoxidans* (P=0.033, R^2=0.711, F=6.155), 20% (1/5) for *B. cepacia* complex (P=0.554, R^2=0.203, F=1.788), 87.5% (7/8) for *P. aeruginosa* (P=0.019, R^2=0.324, F=3.912), 85.7% (6/7) for *S. aureus* (P<0.01, R^2=0.351, F=5.407), 71.4% (5/7) for *S. maltophilia* (P=0.125, R^2=0.166, F=1.358).

Finally, when samples taken at the start of treatment were compared to samples taken at follow up (i.e. excluding end of treatment samples) no significant difference in CFU/ml equivalents was seen (*A. xylosoxidans* (P=0.583, R^2=0.074, ANOVA test statistic (F)=0.924)*, B. cepacia* complex (P=0.216, R^2=0.048, F=0.807), *P. aeruginosa* (P=0.418, R^2=0.181, F=2.159)*, S. aureus* (P= 0.556, R^2=0.009, F=0.181) and *S. maltophilia* (P=0.786, R^2=0.022, F=0.078)). When only samples that had the pathogen detected at the start of treatment

were compared to samples taken at follow up, the CFU/ml equivalents returned to the same or higher CFU/ml in the follow up sample; 0% (2/2) for *A. xylosoxidans* (P=0.033, R^2=0.711, F=6.155), 20% (1/5) for *B. cepacia* complex (P=0.084, R^2=0.203, F=1.788), 37.5% (3/8) *P. aeruginosa* (P=0.044, R^2=0.324, F=3.912), 42.9% (3/7) *S. aureus* (P=0.073, R^2=0.351, F=5.407) and 57.1% (4/7) *S. maltophilia* (P=0.332, R^2=0.166, F=1.358).

When patients were considered individually, antibiotic treatment affected the key CF pathogens detected differently. In 13 out of 14 patients, when one or more pathogens decreased during antibiotic treatment, one or more other pathogens increased. In 10 out of 14 patients, one or more pathogens decreased to below the limit of detection of the qPCR assay after 2 weeks of antibiotic treatment and then increased above the level of detection after a further 1 to 3 weeks in 6 out of the 10 patients (Figure 6.2). When patients were grouped by antibiotic type, no trends were seen between the target of the antibiotics given and an increase or decrease in specific pathogens.

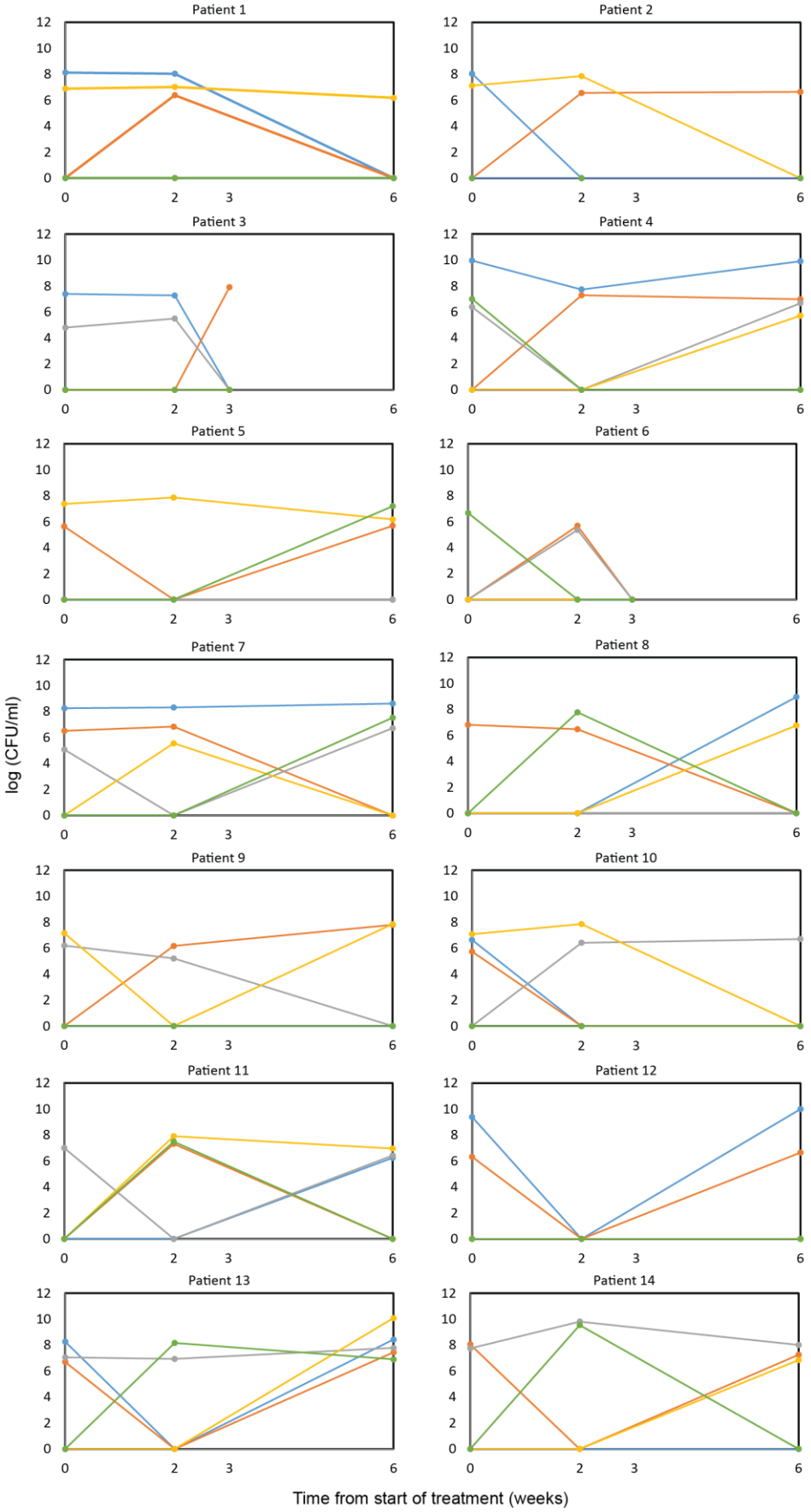

Figure 6.2 CFU/ml equivalents (log_{10} scale) of *A. xylosoxidans* (green), *B. cepacia* complex (yellow), *P. aeruginosa* (blue), *S. aureus* (orange) and *S. maltophilia* (grey) determined by qPCR in samples taken at the beginning, end and at follow up of exacerbation treatment for a pulmonary exacerbation.

6.4 Discussion

When people with CF are diagnosed with an exacerbation, clinicians prescribe a range of antibiotics to reduce pathogens in the lung which could have led to the worsening of symptoms. Respiratory samples are usually taken to identify key pathogens present in the lung. Culture-based methods are used to identify and determine the antibiotic susceptibility profile of the pathogens to inform antibiotic treatment strategies (Cystic Fibrosis Trust, 2010). However, current guidance does not employ enumerating pathogens during exacerbation or treatment. This would be beneficial as the effectiveness of the antibiotic treatment could be monitored during the treatment rather than waiting to see if symptoms improve. The aim of this study was to determine if changes in bacterial density of CF pathogens in sputum samples before and after antibiotic treatment could be monitored using qPCR. This study used previously optimised species-specific qPCR assays to determine the prevalence and quantification of key CF pathogens.

The results showed *A. xylosoxidans*, *B. cepacia* complex and *S. aureus* were present in a higher percentage of patients at the end of the treatment period than at the beginning whereas *P. aeruginosa* and *S. maltophilia* was present in fewer patients at the end of the treatment period than at the beginning (Figure 6.1). It also showed that in a high percentage of patients that were positive at the start of treatment, *A. xylosoxidans*, *P. aeruginosa, S. aureus* and *S. maltophilia* decreased below the limit of detection of qPCR after two weeks of antibiotic treatment (Table 6.3). However, after 1 to 3 weeks after the end of treatment, the decrease was not sustained, and many pathogens increased above the limit of detection (Table 6.3).

This study showed no significant difference in the CFU/ml equivalents of the targeted CF pathogens between the start and end of treatment. This study had a relatively small sample size (n=14) and the change in *P. aeruginosa* density was approaching significance (p=0.052), with a larger sample size it may have been significant as previous studies have shown when using culture-based methods (Blumer *et al.*, 2005; Tunney *et al.*, 2011; Deschaght *et al.*, 2013) (n=121, 26 and 27, respectively) and qPCR (Deschaght *et al.*, 2013; Reid *et al.*, 2013; Zemanick *et al.*, 2013) (n=27, 15 and 21, respectively). This study also showed no significant difference in the CFU/ml equivalents of the targeted pathogens in samples taken at the start of treatment and at follow up.

Previous studies have shown conflicting findings when culture-based methods were used to quantify pathogens at the start and end of treatment. Previous studies are in agreement

that *P. aeruginosa* decreased from the start to the end of antibiotic treatment (Blumer *et al.*, 2005; Tunney *et al.*, 2011; Deschaght *et al.*, 2013; Lam *et al.*, 2015). However, when other CF pathogens were detected; one study showed a decrease in *B. cepacia* complex members from the start of treatment to the end (Blumer *et al.*, 2005) whereas another study found no significant difference in any of the non-*Pseudomonas* detected species including *B. cepacia* complex (Tunney *et al.*, 2011) and another found no significant difference in the abundance of *B. cepacia* complex (Stokell *et al.*, 2015). Only one study was found that used culture-based methods to determine *P. aeruginosa* density returned to near baseline values in the follow up sample (Lam *et al.*, 2015).

When culture-independent methods were used to determine the density of key CF pathogens in samples at the start and end of treatment and at follow up studies do not support each other. A study utilising T-RFLP analysis to identify bacterial species showed an increase in the relative abundance of *P. aeruginosa* (Daniels *et al.*, 2013). However, this study used T-RFLP analysis to identify bacterial species and their relative abundances in samples before, during and after antibiotic treatment for an exacerbation. The study showed absolute numbers of both *P. aeruginosa* and non-pseudomonal species decreased from before to after treatment but as non-pseudomonal species decreased to a greater extent, the relative abundance of *P. aeruginosa* increased. Therefore, T-RFLP does not accurately depict changes in the abundance of *P. aeruginosa* during antibiotic treatment. In contrast, other studies found a significant decrease in *P. aeruginosa* from the start to the end of treatment with qPCR methods (Deschaght *et al.*, 2013; Reid *et al.*, 2013; Zemanick *et al.*, 2013; Stokell *et al.*, 2015). However, another study showed no significant difference in *P. aeruginosa* abundance, quantified by qPCR, between exacerbation and treatment samples or between exacerbation and recovery samples (Price *et al.*, 2013).

Previous research indicated there would be no difference in the density of CF pathogens, specifically *P. aeruginosa*, between samples at the start of treatment and at follow up in the present study (Price *et al.*, 2013; Lam *et al.*, 2015; Stokell *et al.*, 2015). There was no significant difference in CFU/ml equivalents for any targeted pathogen between the start and follow up or between the start and end of treatment for an exacerbation. This indicates CF pathogens were not eradicated (or significantly reduced) during treatment. Therefore, one to three weeks after treatment, when follow-up samples were taken, pathogens are likely to have returned to the levels present in samples at the start of treatment.

Although the difference in *P. aeruginosa* density between the start and end of exacerbation treatment was close to significant, no other targeted species was significantly different. It was expected that *P. aeruginosa* density would significantly decrease between the start and end of antibiotic treatment as previous research has shown (Blumer *et al.*, 2005; Tunney *et al.*, 2011; Reid *et al.*, 2013; Zemanick *et al.*, 2013; Stokell *et al.*, 2015). The lack of significant difference between the start and end of antibiotic treatment in this study could be due to a number of reasons. Previous studies have indicated it could be due to the relatively small sample size used in the study and not all the participants were positive for *P. aeruginosa* at the start of treatment (8/14 (57%) participants were *P. aeruginosa* positive) unlike in the previous studies (Blumer *et al.* (2005);89%, Tunney *et al.* (2011);62%, Reid *et al.* (2013);100%, Zemanick *et al.* (2013); 81%, Deschaght *et al.* (2013);100%). Whilst it was not tested in the present study, previous work has indicated the lack of significance could also be due to highly antibiotic resistant strains of CF pathogens. Susceptibility profiling on respiratory samples is undertaken to inform antibiotic therapies. However, this analysis does not include testing biofilms which have been shown to be more resistant than planktonic bacteria (Singh *et al.*, 2000; Aaron *et al.*, 2002). Therefore, the current methods of susceptibility testing may not give an accurate representation of the susceptibility of isolates.

It has previously been shown that qPCR and other DNA based techniques of identification cannot distinguish between DNA from viable cells and extracellular DNA from non-viable cells. In research utilising qPCR to quantify bacterial cells during antibiotic treatment it is especially important to exclude DNA from non-viable cells from analysis. The use of propidium monoazide (PMA) has previously been showed to effectively bind to extracellular DNA to inhibit amplification during PCR. This study, Stokell *et al.* (2015) and Deschaght *et al.* (2013) used PMA prior to DNA extraction whereas Price *et al.* (2013), Zemanick *et al.* (2013) and Reid *et al.* (2013) did not. Antibiotic treatment leads to an increase in non-viable cells and it is possible not excluding them from analysis is the reason Price *et al.* (2013) found no significant difference between samples at the start and end of treatment. It is also likely that Zemanick *et al.* (2013) and Reid *et al.* (2013) may have also overestimated the abundance of *P. aeruginosa* in samples during treatment. Therefore, the use of PMA would have strengthened their argument that *P. aeruginosa* decreased during antibiotic treatment.

The previous publications also utilised statistical methods to determine changes in pathogen load between samples that did not take into account the variation between patients (Blumer *et al.*, 2005; Tunney *et al.*, 2011; Daniels *et al.*, 2013; Price *et al.*, 2013; Reid *et al.*, 2013; Zemanick *et al.*, 2013; Lam *et al.*, 2015). By using a mixed effect model this study was able to account for patient variation as a random effect during statistical analysis (Section 6.2.3).

When patients were analysed individually, it was shown that in some patients one or more pathogens decreased from the start to the end of treatment while other pathogens increased. Cuthbertson *et al.* (2016) observed an increase in the proportional abundance of *P. aeruginosa* in recovery samples during treatment for an exacerbation. It was suggested that antibiotic treatment led to a reduction in the abundance of other species allowing *P. aeruginosa* to fill the niche. It is possible, during this study, as one pathogen decreased, another pathogen increased due to selective pressures causing the development of antibiotic resistance or could simply be the mechanisms of action of the antibiotic did not affect that bacterial species. Previous research has also detected bacterial species in samples during treatment that were not present at the start of treatment (Lam *et al.*, 2015) and antibiotic treatment had a significant effect on non-targeted bacterial species (Daniels *et al.*, 2013). These studies suggest administered antibiotics could also affect other members of the resident lung microbiota and the microbiota as a whole.

6.5 Conclusion

This study showed no significant difference in the density of key CF pathogens between the start and end of treatment for an exacerbation or between the start of treatment and at follow up. Individual results showed a decrease in one pathogen and an increase in another pathogen from the start to the end of treatment in some patients. This indicates that, although no overall effect is seen, antibiotics may be affecting one pathogen which leads to an opportunity for another pathogen to increase in density. This, along with results from previous research, suggests that monitoring pathogen load throughout antibiotic treatment could direct optimised treatment that is missed with the current pathogen presence/absence reporting. The presented results pose the question; if antibiotics are not having an overall significant effect on the density of key CF pathogens after two weeks, are they affecting the bacterial microbiota as a whole?

Chapter 7 Effects of antibiotic therapy for pulmonary exacerbation on CF lung microbiota

7.1 Introduction

Lung disease in CF is interspersed with pulmonary exacerbations that are treated with antibiotic therapies. To direct antibiotic therapies, bacteria are isolated from respiratory samples and identified using selective agars and biochemical tests (Cystic Fibrosis Trust, 2010). This method focuses on the presence or absence of known CF pathogens and their susceptibility profiles. Treatment is then usually directed towards the identified known CF pathogens such as *Pseudomonas aeruginosa, Staphylococcus aureus* and *Burkholderia cepacia* complex members.

The use of culture-independent methods has shown that CF lung disease is polymicrobial with a resident microbiome (Rogers *et al.*, 2004; Harris *et al.*, 2007; Fodor *et al.*, 2012; Parkins and Floto, 2015). Microbiota sequencing techniques have previously been utilised to show an association between the lung microbiota and age, CFTR mutation and lung function in CF (Cox *et al.*, 2010; Klepac-Ceraj *et al.*, 2010; van der Gast *et al.*, 2011, 2014; Zhao *et al.*, 2012; Cuthbertson *et al.*, 2020). Specifically, *Pseudomonadaceae* and *Burkholderiaceae* were associated with older patients and *Pasteurellaceae* and *Mycobacteriaceae* were associated with younger patients (Cox *et al.*, 2010; van der Gast *et al.*, 2014).

There is no consensus for the cause of exacerbations (Rogers *et al.*, 2011b) and the bacterial density of key CF pathogens was not significantly altered between the start and end of antibiotic treatment in the previous chapter(Chapter 6). However, patients report feeling better and clinical symptoms improve after antibiotic treatment for an exacerbation (Fodor *et al.*, 2012; Deschaght *et al.*, 2013; Smith *et al.*, 2014). Therefore, it has previously been investigated whether the bacterial microbiota is altered during antibiotic treatment for an exacerbation (Fodor *et al.*, 2012; Price *et al.*, 2013; Smith *et al.*, 2014; Stokell *et al.*, 2015; Cuthbertson *et al.*, 2016; Nelson *et al.*, 2020).

Although antibiotics are usually targeted to specific pathogens, they are also often broad spectrum against gram-positive bacteria, gram-negative bacteria or both. Therefore, it is likely that such treatment, especially broad-spectrum antibiotics, will affect the lung microbiota as a whole. During antibiotic treatment, a decrease in the target pathogen/s may lead to an increase in other species, as seen in Chapter 6, which may have similar antagonistic effects or antibiotic treatment may also decrease other susceptible bacterial species leading to an increase in lung function (Döring *et al.*, 2012).

Previously, the bacterial community composition has shown to be unchanged from the start to the end of treatment for an exacerbation (Tunney *et al.*, 2011; Fodor *et al.*, 2012; Cuthbertson *et al.*, 2016; Sherrard *et al.*, 2019). However, Fodor *et al.* (2012) indicated a small decrease in species richness during antibiotic treatment was attributed to changes in a small number of taxa in low abundance. Cuthbertson *et al.* (2016) also highlighted a significant alteration in the composition of the core and rare taxa but not in the microbiota as a whole.

Other research has also shown a decrease in the richness of bacterial taxa during antibiotic treatment for an exacerbation (Daniels *et al.*, 2013; Stokell *et al.*, 2015; Nelson *et al.*, 2020). Nelson *et al.* (2020) also determined the richness returned to similar levels in follow up samples as seen in baseline samples.

Previous research has shown contrasting changes in diversity before and after exacerbation treatment (Zhao *et al.*, 2012; Price *et al.*, 2013; Smith *et al.*, 2014; Stokell *et al.*, 2015; Sherrard *et al.*, 2019). Price *et al.* (2013), Stokell *et al.* (2015) and Sherrard *et al.* (2019) revealed no significant change in bacterial diversity in samples taken at the start and end of exacerbation treatment or at follow up. However, Zhao *et al.* (2012) and Smith *et al.* (2014) showed a decrease and an increase, respectively, in bacterial diversity in samples taken at the start of an exacerbation and samples taken during antibiotic treatment.

Previous studies have not been able to meet a consensus on the effects of antibiotic treatment on the bacterial microbiota of the CF lung during or following treatment which may be due to differences in sampling or microbiota sequencing techniques. Previous studies (Tunney *et al.*, 2011; Daniels *et al.*, 2013; Sherrard *et al.*, 2019) have utilised techniques that have since been shown to be unrepresentative for the detection of the bacterial microbiota of the CF lung (Engebretson and Moyer, 2003; Rogers and Bruce, 2010) so the results of these studies may not be accurate. In addition, Zhao *et al.* (2012) used samples that were unmatched (i.e. samples were not taken at the start and end of the same exacerbation) and patients participating in the study by Nelson *et al.* (2020) were not exacerbating at the time of sampling. In the study by Daniels *et al.* (2013), samples were not taken before antibiotic treatment had started which also makes comparisons difficult as Smith *et al.* (2014) has shown that antibiotic treatment can alter the microbiota after 3-4 days of treatment. Although, a large percentage of these studies also included follow up samples (Daniels *et al.*, 2013; Price *et al.*, 2013; Stokell *et al.*, 2015; Cuthbertson *et al.*, 2016), the period of time for samples to be categorised as follow up were not consistent

(1-10 weeks) and samples categorised as follow up in some studies would have been categorised as baseline in other studies because of the large timeframe allowed (Price *et al.*, 2013; Stokell *et al.*, 2015). To establish if antibiotic treatment for an exacerbation has an effect, and if so what effect, on the bacterial microbiota and the common and rare taxa within the microbiota, further research is required.

This research obtained samples from CF patients at the start, end and following antibiotic treatment for an exacerbation. In the previous chapter, it was shown that in this cohort, antibiotic treatment for an exacerbation did not significantly alter the bacterial load of key CF pathogens quantified by targeted qPCR. The aim of this study was to determine if antibiotic treatment for an exacerbation alters the microbiota using 16S rRNA targeted high-throughput sequencing.

7.2 Materials and Methods

7.2.1 Patient samples and processing

Samples and DNA extraction from samples was previously described in Chapter 6 sections 6.2.1 and 6.2.2 with the addition of three patients who provided samples at the start and end of antibiotic treatment but not follow up.

7.2.2 DNA sequencing

The bacterial microbiota in all sputum samples were sequenced using the Illumina MiSeq platform and sequence analysis performed as previously described (Chapter 2, Section 2.4). Sequences were taxonomically assigned. However, due to the length of sequences, species identities should be considered putative.

7.2.3 Statistical analysis

Generalised linear models on presence/absence data and odd ratios were performed and calculated using the package lmer in R version 3.6.2 (Lam *et al.*, 2015; Singh *et al.*, 2019).

The microbiota of samples was partitioned into common and rare taxa. Common taxa were defined as those taxa that accounted for more than 1% of the relative abundance in each sample (Magurran and Henderson, 2003).

PAST was used to calculate taxa richness, Fisher's alpha index of diversity, Berger-Parker index of dominance, Sørensen and Bray-Curtis indices of dissimilarity as described previously (Chapter 2, Sections 2.5.3 and 2.5.4) (Hammer *et al.*, 2001).

To compare the richness, diversity and dominance of samples at the start and end of treatment and at the follow-up, mixed effects models were performed using package lmer in R version 3.6.2 (Kuznetsova *et al.*, 2017). Mixed effects models contain both fixed and random effects (Cuthbertson *et al.*, 2016). This allowed for variation between patients to be taken into account when determining if richness, diversity or dominance significantly changed from the start of treatment to the end of treatment and from the start of treatment to the follow up.

7.2.4 Combining quantitative PCR and sequencing data

Targeted gene sequencing putatively identified multiple sequence variants for key CF pathogens. The qPCR data described in Chapter 6 was presumed to be the total quantity of each CF pathogen. The number of reads for each variant identified as a CF pathogen in individual samples was converted into a percentage of the total for each pathogen. The

percentage of reads for each variant was then used to calculate the CFU/ml equivalent of each variant.

7.3 Results

7.3.1 Clinical characteristics of patients

Sputum samples (n=48) were obtained from CF patients (n=17) attending Southampton University Hospital, Southampton, UK (Table 7.1).

Table 7.1 Summary of clinical characteristics of patients included in the study.

Number of patients	17
Sex of patients (M:F)	14:3
Mean age (years) at first or only sample (±SD)	25.4 (± 7.2)
Minimum and maximum age (years)	18 to 46
CFTR Genotype:	
Homozygous F508del	11
Heterozygous F508del	5
Unknown	1
Mean %FEV1 at date closest to first sample (±SD)	54.6 (±18)
Antibiotic target:	
Gram negative bacteria	13
Staphylococci	5
Broad spectrum	11

7.3.2 Prevalence of CF pathogens using high-throughput sequencing

High-throughput gene targeted sequencing was used to determine the prevalence of key CF pathogens (Cystic Fibrosis Foundation, 2019) in samples at the start and end of treatment for an exacerbation and at follow up (Figure 7.1). Odds ratios were used to determine there was no difference, an odds ratio of 1, in the chance of *P. aeruginosa* (95% confidence interval (CI) N/A; indicating perfect correlation) being present in samples at the start than in the samples at the end of treatment. *B. cepacia* complex, *S. aureus* and *Stenotrophomonas maltophilia* were shown to be 2.4 (95% CI 0.165-34.934), 7.857 (95% CI 0.752-82.13) and 3.333 (95% CI 0.362-3.424), respectively, times more likely to be present in samples at the end of treatment than at the start. However, *Achromobacter xylosoxidans* was 25 (95% CI 0.341-1831.738) times more likely to be present in samples at the start of treatment than samples at the end of treatment.

Odds ratio determined there was no difference (odds ratio of 1) in the chance of *A. xylosoxidans* (95% CI 0.063-15.982) and *P. aeruginosa* (95% CI N/A; indicating perfect correlation) being present in the samples at the start of treatment than in the follow up samples. *B. cepacia* complex, *S. aureus* and *S. maltophilia* were more likely to be present in the follow up sample than present at the start of treatment by 2.667 (95% CI 0.434-16.391), 5.4 (95% CI 0.778-37.504) and 4 (95% CI 0.563-28.397) times, respectively.

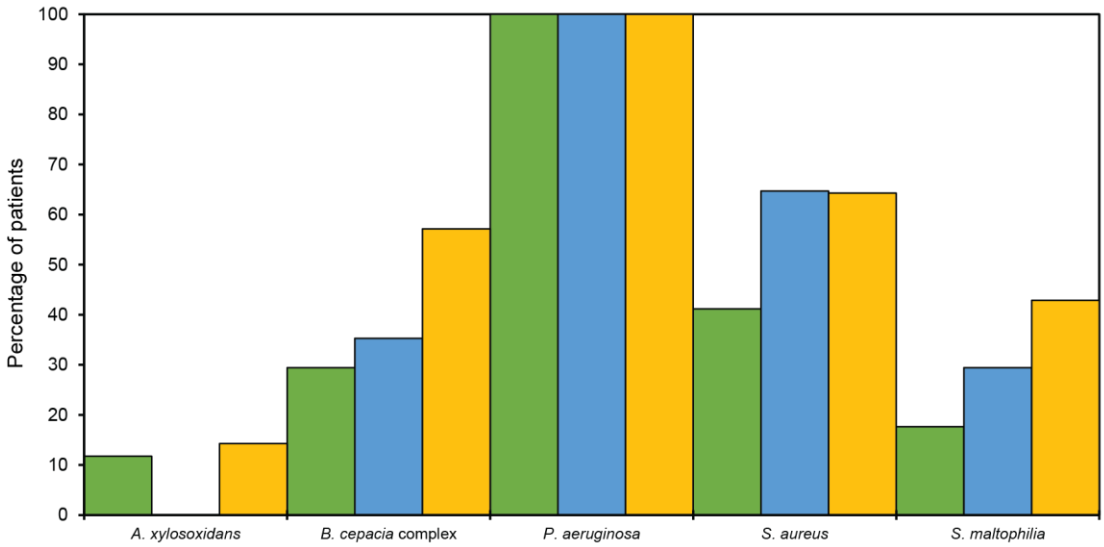

Figure 7.1 CF pathogens present in samples at the start and end of treatment and at follow up determined by targeted gene sequencing. Percentage of patients positive for each pathogen at the start (green) (n=17) and end (blue) (n=17) of treatment and at follow up (yellow) (n=14). n indicates the number of patients with samples in each group. Three patients provided only start and end samples and did not provide a follow up sample.

7.3.3 Microbiota analysis

High throughput sequencing was used to identify the microbiota in each patient at the start of treatment for an exacerbation, when the treatment finished two weeks later and a week to three weeks after the end of treatment. The microbiota was split into common and rare taxa and the taxa richness, diversity and dominance are also shown.

7.3.3.1 Taxa richness

Taxa richness at the start and end of treatment and at the follow up was determined (Figure 7.2). Taxa richness of the microbiota increased from the start to the end of antibiotic treatment in 59% of patients (10/17) and decreased in 35% of patients (6/17) (Figure 7.3). Mixed effect models were used to investigate how taxa richness changed between the start and end of treatment and the start of treatment and follow up. These models take into account the fixed (start, end and follow up sample) and random effects (patient) to allow the variation between patients to be accounted for during statistical analysis (Cuthbertson *et al.*, 2016). These models showed there was a significant difference between the richness within the common taxa at the start of antibiotic treatment and at the follow up (P=0.039, R^2=0.085, test statistic (F)=2.371). There was no significant difference in richness within the microbiota (P=0.102, R^2=0.058, F=1.663), common (P=0.515, R^2=0.085, F=2.371) and rare (P=0.093, R^2=0.074, F=2.133) taxa at the start of antibiotic treatment and the end of

treatment. There was also no significant difference in richness within the microbiota (P=0.891, R^2=0.058, F=1.663) and rare (P=0.832, R^2=0.074, F=2.133) taxa at the start of antibiotic treatment and at the follow up.

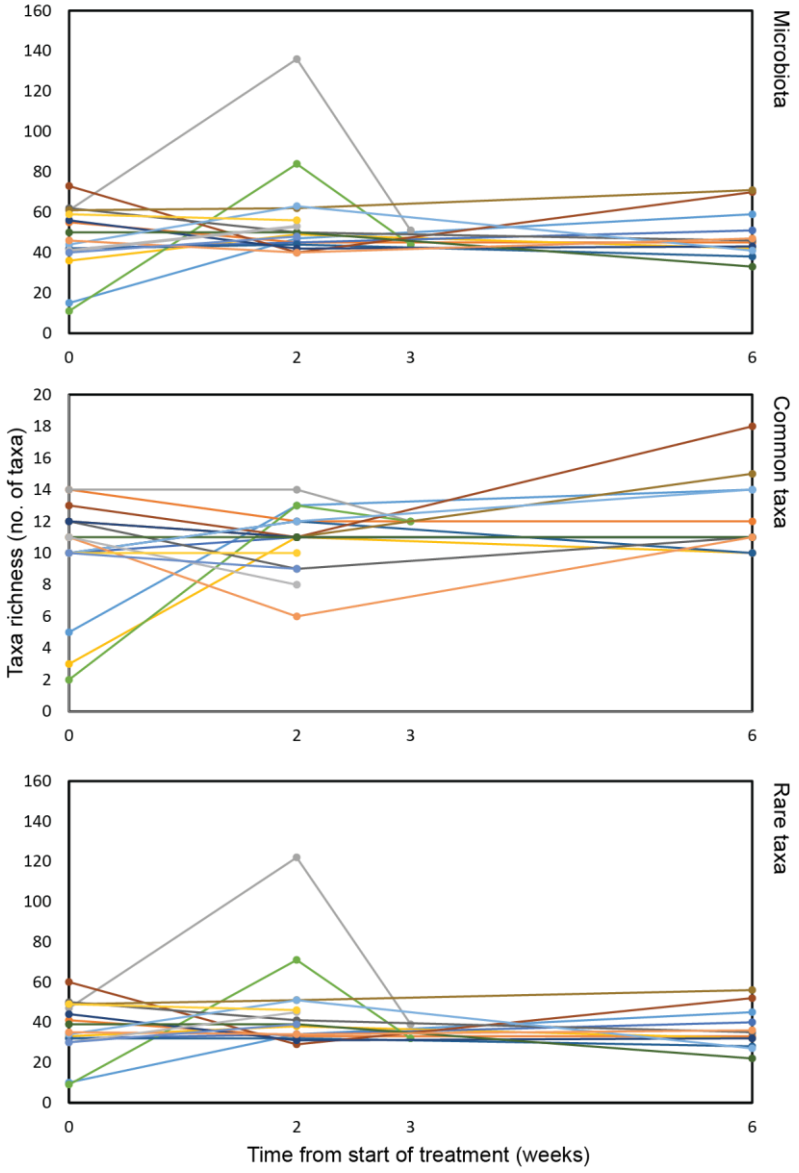

Figure 7.2 Taxa richness in microbiota, common, and rare taxa in samples at the start of treatment, at the end of treatment and at follow up. Each colour represents an individual patient.

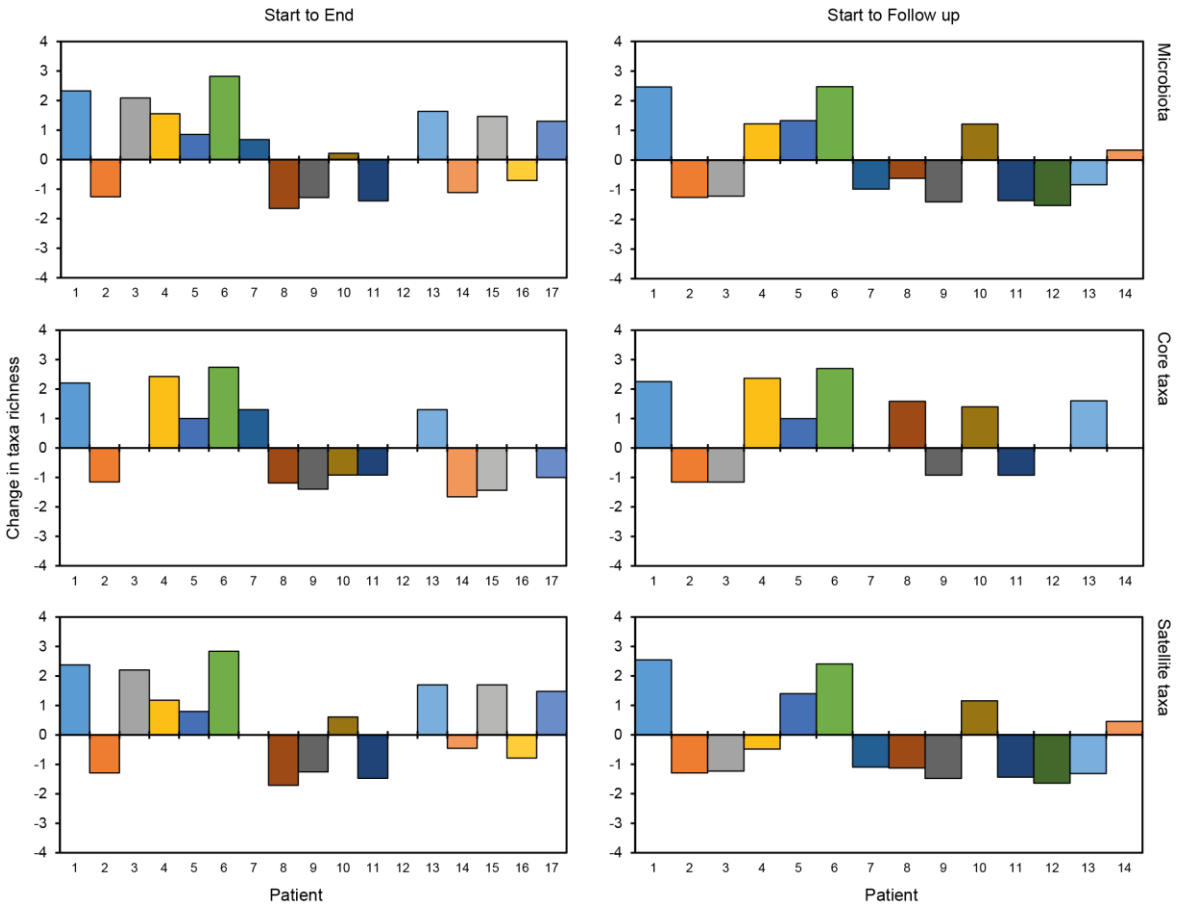

Figure 7.3 Taxa richness change (log_{10} scale) in microbiota, common, and rare taxa between samples at the start and end of treatment (n=17) and between samples at the start of treatment and at follow up (n=14). n indicates the number of patients with samples in each group. Three patients provided only start and end samples and did not provide a follow up sample.

7.3.3.2 Diversity

Fisher's alpha measure of diversity at the start and end of treatment and at the follow up was determined (Figure 7.4). Diversity of the microbiota increased from the start to the end of antibiotic treatment in 59% of patients (10/17) and decreased in 41% of patients (7/17) (Figure 7.5). There was a significant difference between the diversity within the common taxa at the start of antibiotic treatment and at the follow up (P=0.035, R^2=0.091, F=2.541). There was no significant difference in diversity within the microbiota (P=0.128, R^2=0.051, F=1.407), common (P=0.641, R^2=0.091, F=2.541) and rare (P=0.117, R^2=0.068, F=1.949) taxa at the start of antibiotic treatment and the end of treatment. There was also no significant difference in diversity within the microbiota (P=0.87, R^2=0.051, F=1.949) and rare (P=0.786, R^2=0.068, F=1.949) taxa at the start of antibiotic treatment and at the follow up.

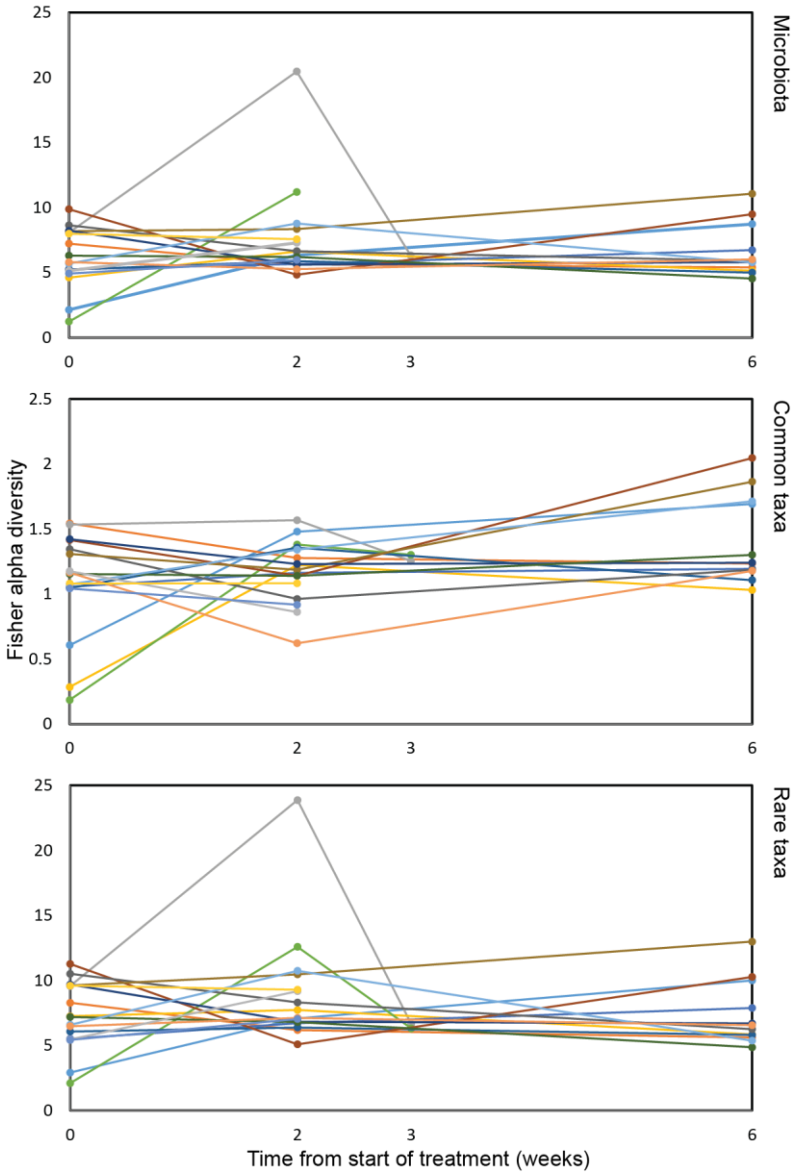

Figure 7.4 Diversity in microbiota, common, and rare taxa in samples at the start of treatment, at the end of treatment and at follow up. Each colour represents an individual patient.

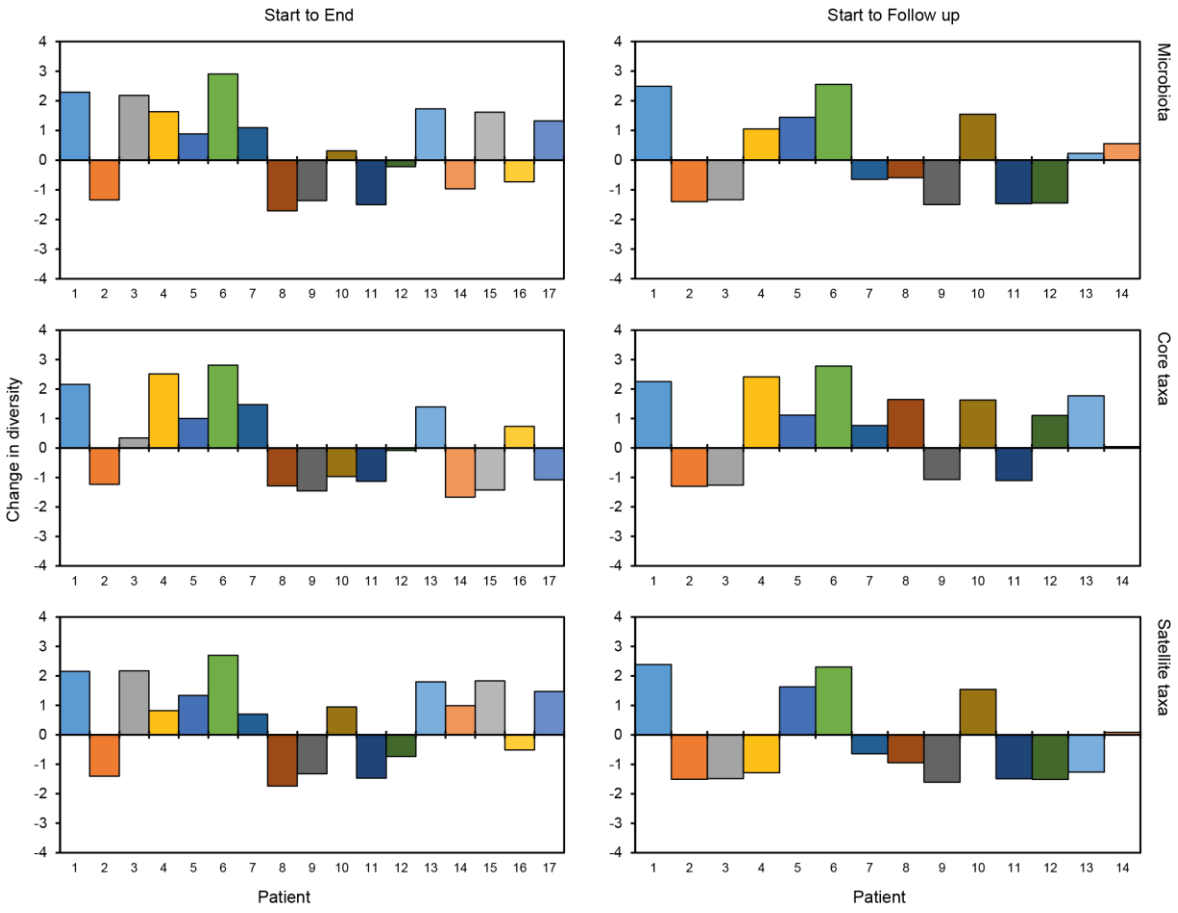

Figure 7.5 Diversity change (log_{10} scale) in microbiota, common and rare taxa between samples at the start and end of treatment (n=17) and between samples at the start of treatment and at follow up (n=14).n indicates the number of patients with samples in each group. Three patients provided only start and end samples and did not provide a follow up sample.

7.3.3.3 Dominance

Dominance, defined as the numerical importance of the most abundant taxa, at the start and end of treatment and at the follow up was determined (Figure 7.6). Dominance within the microbiota increased from the start to the end of antibiotic treatment in 47% of patients (8/17) and decreased in 53% of patients (9/17) (Figure 7.7). There was no significant difference in dominance within the microbiota (P=0.256, R^2=0.053, F=1.43), common (P=0.304, R^2= 0.048, F=1.299) and rare (P=0.207, R^2=0.039, F=1.047) taxa at the start of antibiotic treatment and the end of treatment. There was also no significant difference in dominance within the microbiota (P=0.165, R^2=0.053, F=1.43), common (P=0.155, R^2= 0.048, F=1.299) and rare (P=0.365, R^2=0.039, F=1.047) taxa at the start of antibiotic treatment and at the follow up.

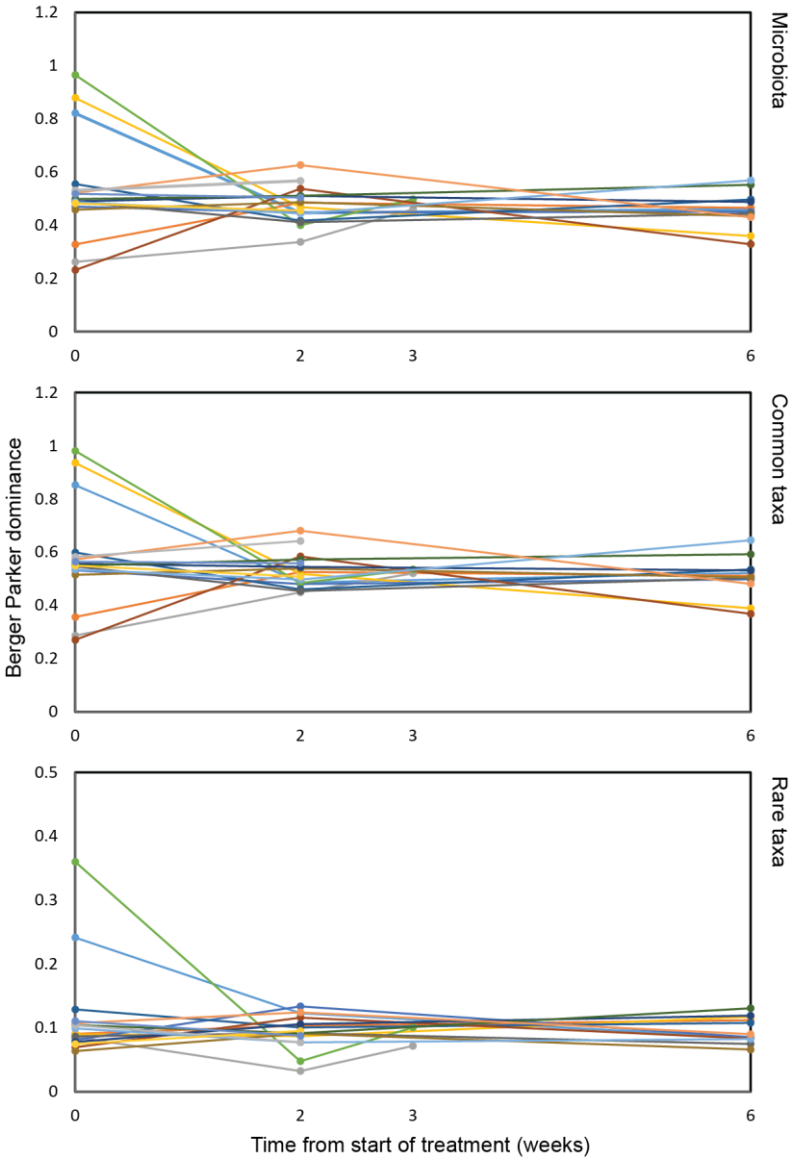

Figure 7.6 Dominance in microbiota, common, and rare taxa in samples at the start of treatment, at the end of treatment and at follow up. Each colour represents an individual patient.

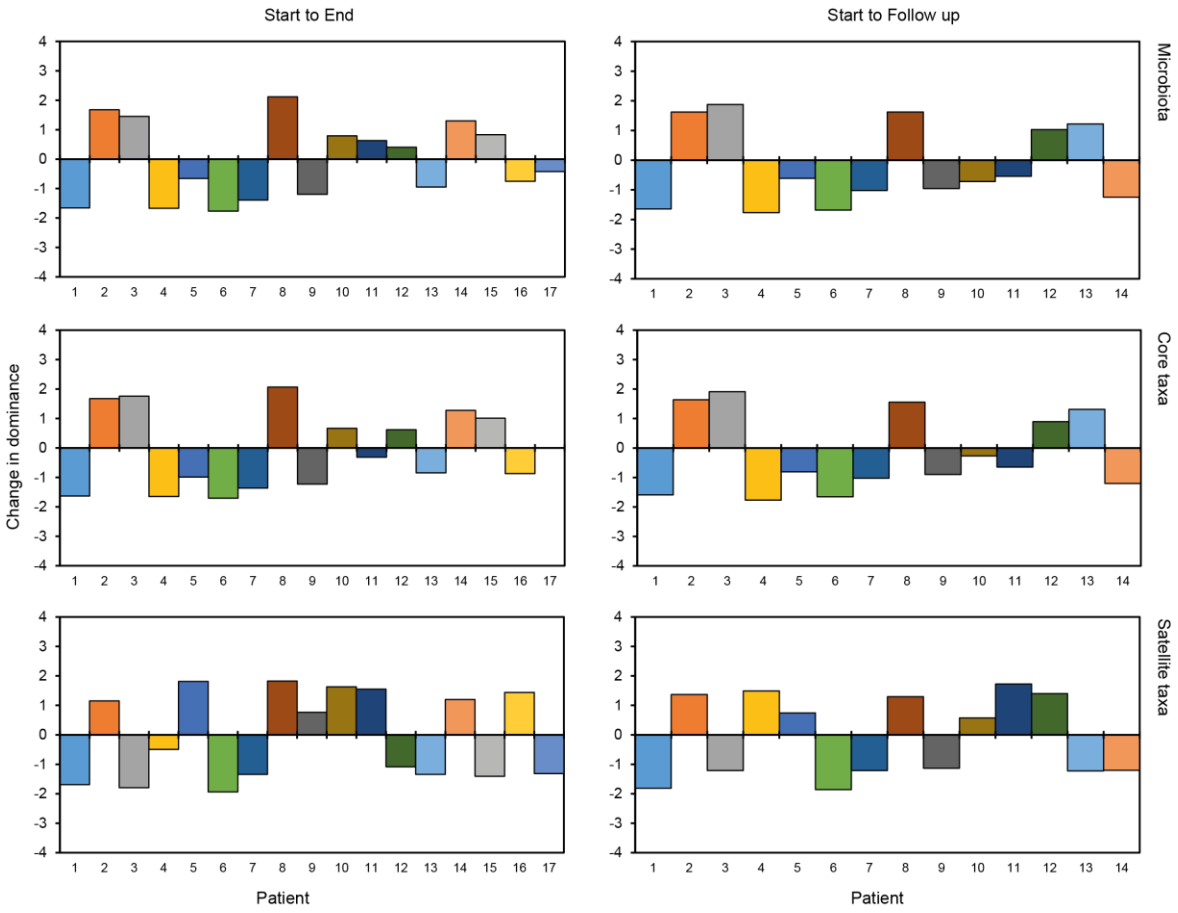

Figure 7.7 Dominance change (log_{10} scale) in microbiota, common, and rare taxa between samples at the start and end of treatment (n=17) and between samples at the start of treatment and at follow up (n=14). n indicates the number of patients with samples in each group. Three patients provided only start and end samples and did not provide a follow up sample.

7.3.3.4 Sørensen Dissimilarity

Sørensen dissimilarity-based analysis showed the mean dissimilarity of the microbiota, common and rare taxa between samples at the start and end of treatment was 41% (SD ±16), 27.5% (SD ±17.9) and 65.3% (SD ±12.4), respectively (Figure 7.8). ANOSIM showed no significant difference between the start and end of treatment in the microbiota (P=0.362, ANOSIM test statistic (R)=0.005), common (P=0.094, R=0.027) or rare taxa (P=0.32, R=0.008). The mean dissimilarity of the microbiota, common and rare taxa between samples at the start of treatment and at follow up was also assessed; 42.5% (SD ±9.4), 33.6% (SD ±21.1) and 67.8% (SD ±10.9), respectively (Figure 7.8). ANOSIM showed no significant difference between the start of treatment and follow up in the microbiota (P=0.598, R=-0.009), common (P=0.502, R=-0.004) or rare taxa (P=0.720, R=-0.022).

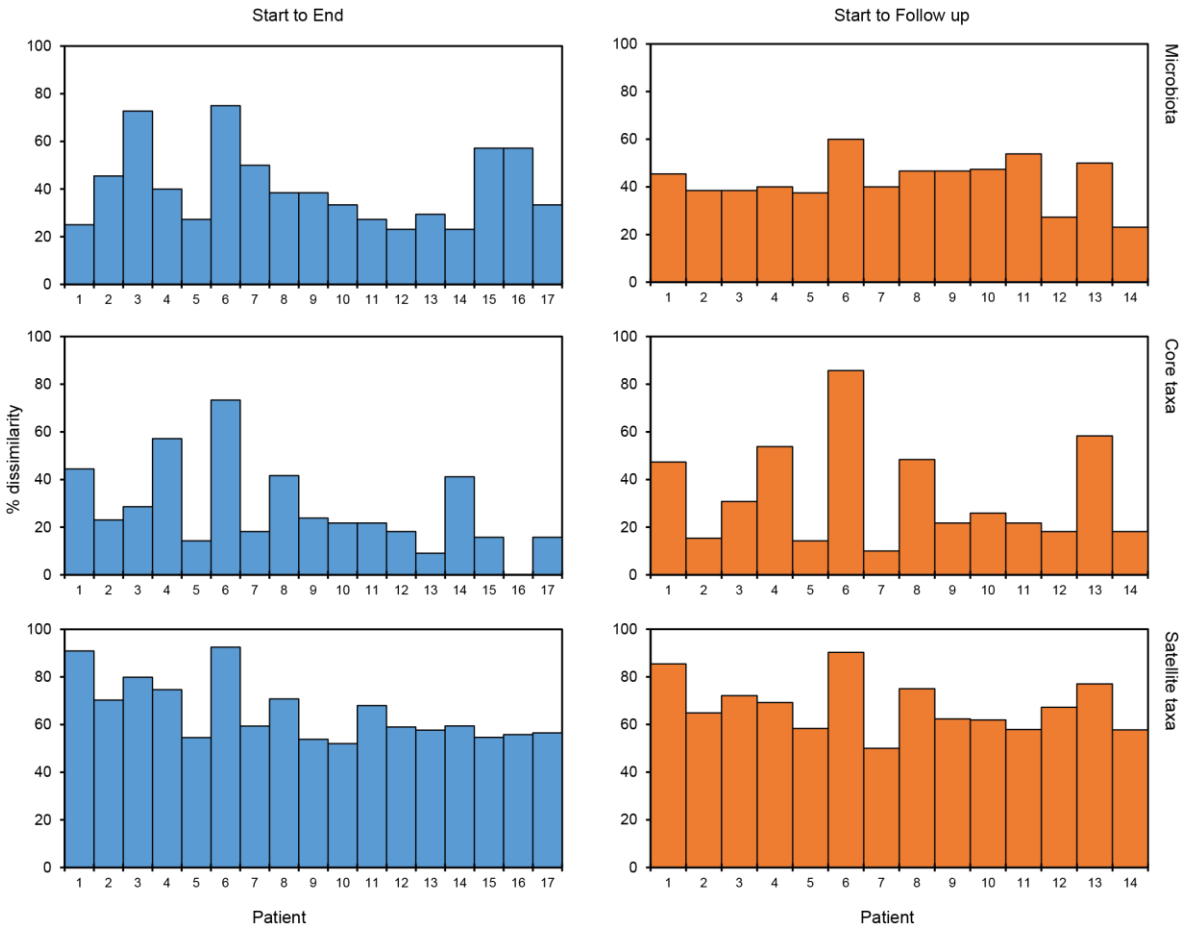

Figure 7.8 Sørensen measure of dissimilarity of microbiota, common and rare taxa between samples at the start and end of treatment (blue) (n=17) and between samples at the start of treatment and follow up (orange) (n=14). n indicates the number of patients with samples in each group. Three patients provided only start and end samples and did not provide a follow up sample.

7.3.3.5 Bray-Curtis Dissimilarity

Similar to Sørensen, Bray-Curtis dissimilarity-based analysis also compares the presence and absence of bacterial taxa in two data sets. In addition, Bray-Curtis also takes into consideration the abundance of shared species. Bray-Curtis dissimilarity-based analysis showed the mean dissimilarity of the microbiota, common taxa and rare taxa between samples at the start and end of treatment was 36.6% (SD ±29.3), 34.9% (SD ±31.3) and 72.1% (SD ±13.1), respectively (Figure 7.9). ANOSIM showed no significant difference between the start and end of treatment in the microbiota (P=0.276, ANOSIM test statistic (R)=0.007), common (P=0.265, R=0.008) or rare taxa (P=0.18, R=0.02). The mean dissimilarity of the microbiota, common and rare taxa between samples at the start of treatment and at follow up was also assessed; 39% (SD ±28.8), 37.8% (SD ±30.7) and 72.2% (SD ±12.6), respectively (Figure 7.9). ANOSIM showed no significant difference between the

start of treatment and follow up in the microbiota (P=0.427, R=-0.003), common (P=0.37, R=-0.0006) or rare taxa (P=0.879, R=-0.033).

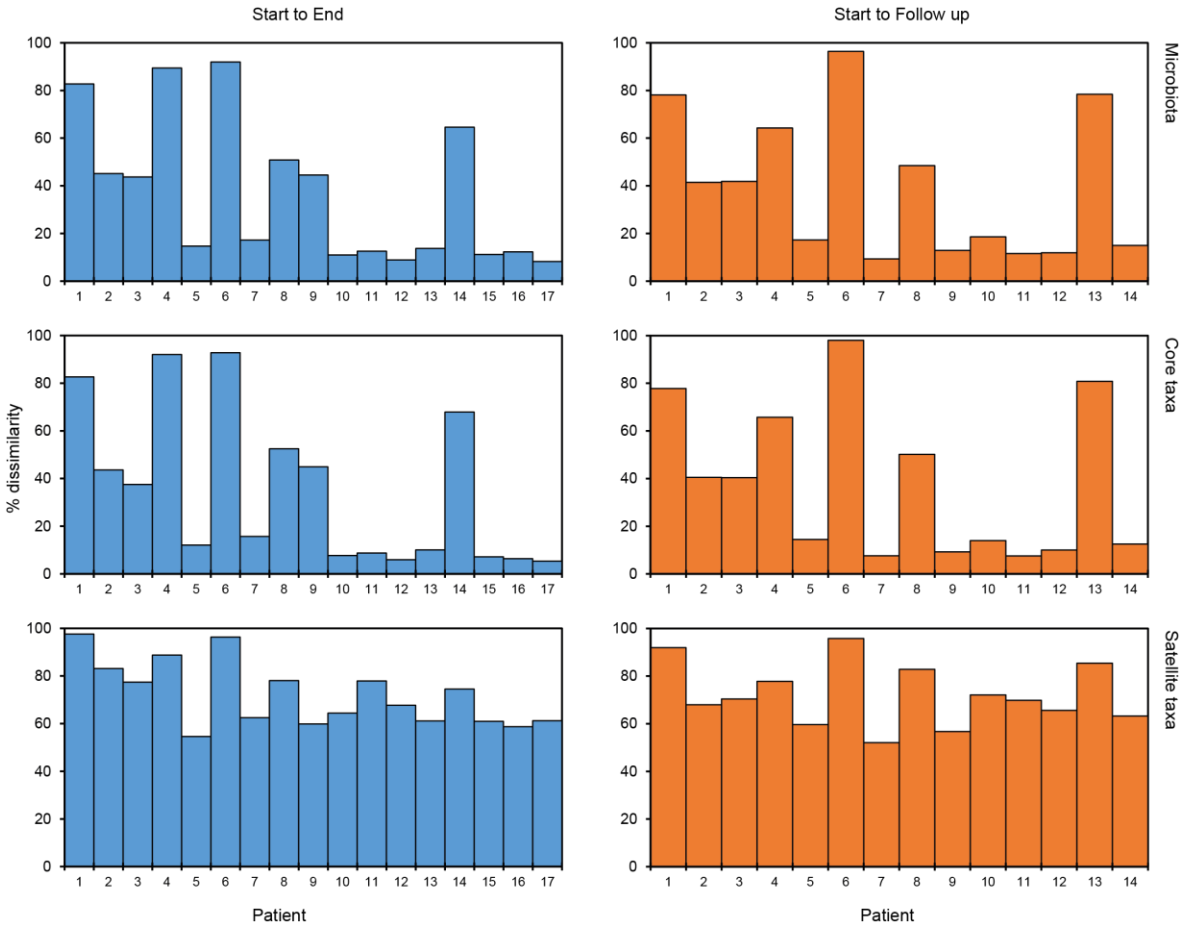

Figure 7.9 Bray-Curtis measure of dissimilarity of microbiota, common and rare taxa between samples at the start and end of treatment (blue) and between samples at the start of treatment and follow up (orange). n indicates the number of patients with samples in each group. Three patients provided only start and end samples and did not provide a follow up sample.

Following on from Bray-Curtis dissimilarity analysis, similarity percentage (SIMPER) analysis was used to determine the contribution of specific taxa to the community dissimilarity between the start and end of treatment and the start of treatment and follow up. *P. aeruginosa* was found to have the greatest and second greatest effect on the dissimilarity of the community between the start and end of treatment (Table 7.2, A) and the start of treatment and follow up (Table 7.2, B), respectively, in the whole microbiota. The CF Foundation monitors the prevalence of *Achromobacter* species, *B. cepacia* complex, *Haemophilus influenzae, P. aeruginosa, S. aureus* and *S. maltophilia* (Chapter 1, Figure 1.1) which were all identified in this patient cohort. Of the six pathogens, five were shown to

have the largest contributions to the dissimilarity in the entire microbiota of end of treatment and follow up samples from samples at the start of treatment (Table 7.2).

SIMPER analysis also showed *P. aeruginosa, H. influenzae* and *S. aureus* had a higher mean relative abundance in samples at the start of treatment compared to at the end of treatment (Table 7.2, A) and compared to follow up samples (Table 7.2, B). Whereas *S. maltophilia* and *B. cepacia* complex had a higher mean relative abundance at the end of treatment and in follow up samples than at the start of treatment.

Table 7.2 Similarity of percentage (SIMPER) analysis of microbiota dissimilarity between samples at the start and end of treatment (A) and between samples at the start of treatment and samples at follow up (B) for taxa with a greater than 1% contribution to the dissimilarity.

A

		Mean abundance	
Species	**% contribution to dissimilarity**	**Start**	**End**
Prevotella melaninogenica	20.997	36.800	42.520
*Pseudomonas aeruginosa**	16.972	13.801	1.418
*Haemophilus influenzae**	9.312	5.231	2.499
Atopobium parvulum	5.046	8.131	10.052
*Stenotrophomonas maltophilia**	4.413	0.066	3.452
Veillonella dispar	3.536	5.106	6.174
Streptococcus salivarius	3.161	5.708	6.606
Escherichia coli	2.713	0.026	2.132
Streptococcus oralis	2.559	1.623	1.020
Porphyromonas pasteri	2.260	1.772	0.130
Streptococcus parasanguinis	2.236	3.352	3.272
Prevotella salivae	1.396	1.675	2.185
Actinomyces graevenitzii	1.380	2.039	2.431
*Staphylococcus aureus**	1.316	0.914	0.241
Rothia mucilaginosa	1.174	1.317	1.551
Schaalia odontolytica	1.138	1.651	2.108
Moraxella osloensis	1.126	0.711	0.218

Key CF pathogens are indicated by an asterisk. The list of taxa is not exhaustive.

B

Species	% contribution to dissimilarity	Mean abundance	
		Start	Follow up
*Pseudomonas aeruginosa**	23.289	15.127	7.679
Prevotella melaninogenica	21.257	34.785	42.646
*Haemophilus influenzae**	8.891	7.070	0.075
Atopobium parvulum	5.031	8.017	10.360
Veillonella dispar	3.579	4.844	6.112
Streptococcus salivarius	3.096	5.743	6.379
Streptococcus oralis	2.804	1.923	0.986
Streptococcus parasanguinis	2.710	3.473	3.454
Porphyromonas pasteri	2.264	1.695	0.300
*Staphylococcus aureus**	1.879	1.170	0.483
Rothia mucilaginosa	1.410	1.300	1.631
Actinomyces graevenitzii	1.321	2.038	2.514
Prevotella salivae	1.269	1.632	2.095
*B. cepacia complex**	1.113	0.094	0.827
Schaalia odontolytica	1.037	1.562	1.852

Key CF pathogens are indicated by an asterisk. The list of taxa is not exhaustive.

7.3.4 Abundance of pathogen sequence variants

The targeted gene sequencing used in this study was not able to distinguish strains of each species. However, multiple sequence variants identified as the same species were seen after processing. This study suggested that sequence variants could be used as a proxy for strains. The qPCR data described in Chapter 6 was combined with the targeted gene sequencing data to determine the CFU/ml equivalent of each sequence variant identified as a CF pathogen in samples at the start and end of treatment and follow up (Figure 7.10). One variant of *S. aureus* and *A. xylosoxidans* was detected in qPCR positive samples. Multiple variants were identified as *P. aeruginosa* (13), *S. maltophilia* (5) and *B. cepacia* complex (2) in qPCR positive samples.

When qPCR detected the pathogens, the sample was often dominated by one variant (Figure 7.10, shown in blue). When other variants were detected at the start of treatment by sequencing and qPCR, the variant was often not detected at the end of treatment (*P. aeruginosa*; patients 2, 3 and 4) or at the follow up (*P. aeruginosa*; patients 2, 3 and 4). Other variants were also detected in the end of treatment or follow up samples but not at the start of treatment in some cases (end +ve, start -ve: *P. aeruginosa*; patients 3 and 16, *S. maltophilia*; patients 6 and 14 and *B. cepacia* complex; patient 5 and follow-up +ve, start -ve: *P. aeruginosa*; patients 8 and 13 and *B. cepacia* complex; patient 5) (Figure 7.10).

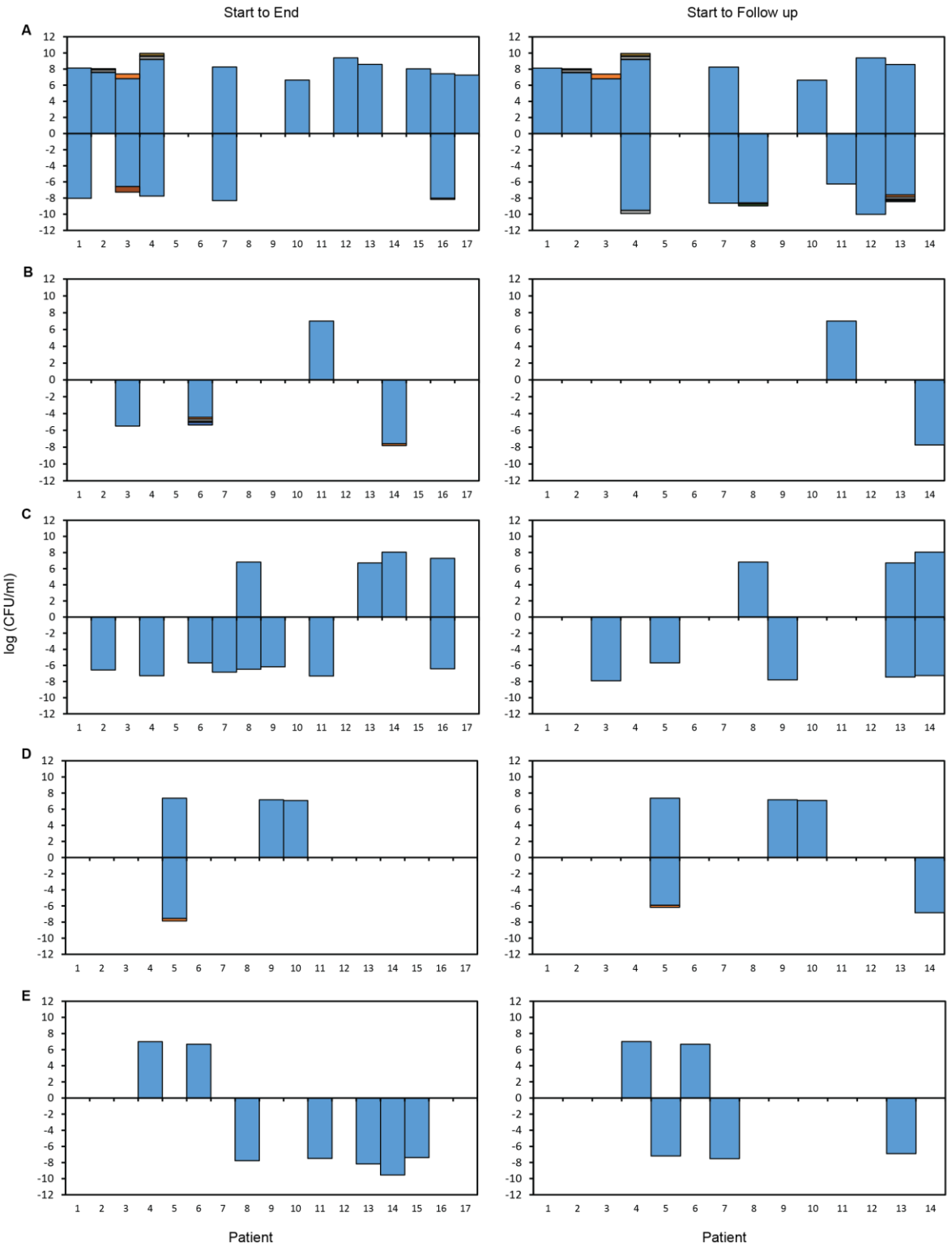

Figure 7.10 CFU/ml equivalents (log_{10} scale) of sequence variants identified in samples at the start and end of treatment and at the start of treatment and at follow up by microbiota sequencing as (A) *P. aeruginosa*, (B) *S. maltophilia*, (C) *S. aureus*, (D) *B. cepacia* complex and (E) *A. xylosoxidans.* Different variants are shown in different colours for each pathogen. Samples at the start of treatment are shown above the x axis and samples at the end of treatment and at follow up have been inverted and are shown below the x axis.

7.4 Discussion

The aim of this study was to determine how the CF lung bacterial composition and structure within CF sputum samples was affected after antibiotic treatment for an exacerbation. Targeted gene sequencing was used to define the bacterial microbiota in sputum samples taken at the start and end of antibiotic treatment and at follow up, 1 to 3 weeks after the end of treatment.

This study showed that key CF pathogens identified by microbiota sequencing were more likely to be present at the end of treatment (*B. cepacia* complex*, S. aureus* and *S. maltophilia*) or at follow up (*B. cepacia* complex, *S. aureus* and *S. maltophilia*) when compared to the start of treatment (Figure 7.1). *A. xylosoxidans* was more likely to be present at the start of treatment than at the end but no difference was determined between samples at the start of treatment and at follow up. *P. aeruginosa* was identified in all samples by microbiota sequencing so no differences were seen. However, confidence intervals were large for each pathogen (excluding *P. aeruginosa*) indicating no overall trend could be concluded. This could be due to variability in the pathogens present at the start of treatment and the antibiotic treatments given.

In this study, high-throughput gene targeted sequencing was used to identify bacterial taxa within the microbiota of samples at the start and end of treatment and at follow up. The effect of treatment on the microbiota composition was assessed. Bacterial richness, diversity and dominance of the microbiota were not significantly altered after two weeks of antibiotic treatment or at follow up one to three weeks later (Figures 7.3, 7.5 and 7.7). The Sørensen and Bray-Curtis dissimilarities of the microbiota at the start and end of treatment and at follow up were also not significant (Figures 7.8 and 7.9). Although not significant, richness and diversity were shown to increase in a higher proportion of samples and dominance was shown to decrease in a higher proportion of samples between the start and end of treatment.

Previous studies have also shown no significant difference in the bacterial community composition of samples taken at the start and end of antibiotic treatment when T-RFLP (Tunney *et al.* ,2011), extended culture (Sherrard *et al.,* 2019) and pyrosequencing (Fodor *et al.*, 2012; Cuthbertson *et al.*, 2016) were used. Sherrard *et al.* (2019) also determined no significant difference in the bacterial taxa richness, diversity or dominance between samples taken at the start of treatment and at follow up in seven patients. Follow up samples were taken 1-10 months after the completion of antibiotics which make

comparisons between this study and the presented study difficult. Fodor *et al.* (2012) used pyrosequencing to find similar microbial community composition and structure in CF samples during exacerbation and when patients were clinically stable. The stable CF samples were taken prior to exacerbation, post-exacerbation and between two exacerbations which makes the results difficult to compare to the present studies follow up samples. The study also showed a decrease in species richness (not significant, p=0.06) during antibiotic treatment which was driven by taxa in low abundance. It was suggested that this could be due to widespread antibiotic resistance in the most abundant species such as *P. aeruginosa* and *B. cepacia* complex members. Cuthbertson *et al.* (2016) also found no alteration in the composition of the whole microbiota between CF samples at the start of treatment and samples taken during treatment and during recovery. However, the composition of the core and satellite taxa significant differed between samples the start of and during treatment. The study also found the composition of the satellite taxa significant differed between samples at the start of treatment and samples taken during recovery. When the microbiota was split into common and rare taxa in the present study, no significant difference was seen between samples at the start of treatment and samples at the end of treatment or samples at the start of treatment and at follow up (Figure 7.9).

Although the studies mentioned found no difference in the community composition of samples taken at the start and end of treatment, other studies have shown an alteration in the bacterial richness of samples (Daniels *et al.*, 2013; Stokell *et al.*, 2015; Nelson *et al.*, 2020). Daniels *et al.* (2013) used T-RFLP to determine bacterial taxa richness significantly decreased in bacterial richness between pre- and post-treatment samples. Nelson *et al.* (2020) used gene targeted sequencing to also show a significant decrease in community richness between CF samples at the start of a month-long tobramycin treatment regime and after a week of treatment. The study also showed baseline samples were significantly different to samples taken one month after the end of the completion of treatment. However, this study trialled a tobramycin maintenance course on patients who were not exacerbating. This study may not be a true representation of the effects of antibiotic treatment for an exacerbation. Stokell *et al.* (2015) utilised targeted gene sequencing to determine taxa richness decreased between CF samples taken <30 days before treatment and during treatment. No significant difference was seen between samples taken <30 days before treatment and samples taken during recovery taken <30 days after an exacerbation. However, there was a significant difference between stable samples and samples taken during recovery. This study followed one person with CF over 1063 days (just under 3

years). Although sampling was more frequent than other studies (twice weekly), conclusions cannot be made from one person for all people with CF.

When the abundance of the bacterial taxa in the community composition was taken into consideration, previous research is not in agreement as to how the diversity of the microbiota is altered between the start and end of treatment and at follow up (Zhao *et al.*, 2012; Price *et al.*, 2013; Smith *et al.*, 2014; Stokell *et al.*, 2015; Sherrard *et al.*, 2019). Price *et al.* (2013), Stokell *et al.* (2015) and Sherrard *et al.* (2019) showed no significant change in bacterial diversity between samples taken at the start and end of treatment and at follow up when extended culture, pyrosequencing and targeted gene sequencing were used, respectively. Price *et al.* (2013) and Stokell *et al.* (2015) also found no significant difference in the bacterial diversity of samples at baseline and during recovery. This lack of difference was seen irrespective of the magnitude of change in the exacerbation or treatment samples. However, recovery samples taken by Price *et al.* (2013) and Stokell *et al.* (2015) were taken at the next routine appointment and up to 30 days after treatment, respectively, making comparisons difficult. Smith *et al.* (2014) used pyrosequencing to indicate bacterial diversity increased during the first 3-4 days of treatment for an exacerbation and then returned to pre-treatment diversity after 8-10 days of treatment. It is possible that by only sampling at the end of the treatment course the present study missed an increase in bacterial diversity at the beginning of treatment. In contrast to the previously mentioned studies, Zhao *et al.* (2012) utilised pyrosequencing to detect a decrease in bacterial diversity between samples taken at the start of an exacerbation and samples taken during antibiotic treatment and between samples taken at the start of an exacerbation and samples taken during the recovery period. This study included six patients (two of which did not have an exacerbation during the study period) that were sampled regularly for eight years. 'Exacerbation' (n=17), 'treatment' (n=28) and 'recovery' (n=21) samples were not matched i.e., samples were not taken at the start of exacerbation and then during the treatment for that exacerbation. Exacerbation samples were any samples taken where an exacerbation had been diagnosed but treatment had not yet started whereas treatment samples were samples taken at any point during treatment for an exacerbation. It is therefore difficult to compare the results of this study to the present study as samples were matched and taken at the start and end of treatment rather than any time in a treatment period.

SIMPER analysis was used to determine which of the identified taxa had the largest contribution to Bray-Curtis dissimilarity between samples at the start and end of treatment (Table 7.2, A) and samples at the start of treatment and follow up (Table 7.2, B). Five of the six key CF lung pathogens monitored by the CF Foundation were identified as being among the largest contributors; P. aeruginosa, *H. influenzae, S. maltophilia, S. aureus* and *B. cepacia* complex. *P. aeruginosa* was shown to be the second largest and largest contributor of dissimilarity between samples at the start and end of treatment and samples at the start of treatment and at follow up, respectively. Cuthbertson *et al.* (2016) also showed *P. aeruginosa* contributed the most to the dissimilarity between baseline, exacerbation, treatment and recovery samples. Interestingly, *Prevotella, Rothia, Veillonella* and *Streptococcus* species were also identified as contributing largely to the difference between samples taken at the start, end and follow up of antibiotic treatment. The role of these taxa in CF lung disease is unclear. Paganin *et al.* (2015) associated *Rothia mucilaginosa* and *Streptococcus pneumoniae* with a worse lung function in people with CF whereas Zemanick *et al.* (2013) and Zemanick *et al.* (2017) showed an association between *Veillonella* and *Prevotella* taxa and *Streptococcus* and *Prevotella* taxa, respectively, and decreased airway inflammation in people with CF. The present study found no significant change in the relative abundance of *Prevotella* or *Veillonella* between the start and end of antibiotic treatment which is in concordance with the study by Zemanick *et al.* (2013) which also found no significant difference between early and late treatment periods.

Multiple sequence variants were identified as key CF pathogens which indicates multiple strains of each taxa were detected. Sequencing data was combined with qPCR data from Chapter 6 to determine the CFU/ml of each variant in individual samples. Variants were detected at the start of treatment by sequencing and qPCR, the variant was often not detected at the end of treatment or at the follow up or vice versa (Figure 7.10). It is important to know if multiple strains of the same species are present before the start of antibiotic treatment as different strains often exhibit different antibiotic susceptibilities and phenotypes (Cantón *et al.*, 2003; Kahl *et al.*, 2003; Foweraker *et al.*, 2005; Leitão *et al.*, 2008; Clark *et al.*, 2015). This could lead to the incorrect conclusion that treatment has decreased the target pathogen when one stain could have replaced another strain with an altered phenotype.

7.5 Conclusion

This study showed that key CF pathogens (*B. cepacia* complex, *S. aureus* and *S. maltophilia*) were more likely to be detected in samples at the end of antibiotic treatment and at follow up than at the start of treatment and *P. aeruginosa* was detected in all samples. This indicates antibiotic treatment did not eradicate *P. aeruginosa* and possibly led to an increase in the prevalence or acquisition of other CF pathogens. Importantly, this suggests that by determining the microbiota throughout antibiotic treatment the prevalence of all key pathogens could be monitored and could be used to direct optimised treatment that presence/absence reporting at the start of treatment wouldn't be able to.

This study also showed that the overall lung microbiota, identified by targeted gene sequencing is not significantly altered after two weeks of antibiotic treatment for an exacerbation or after a further one to three weeks after the end of treatment. Any dissimilarity between samples was driven by, amongst other taxa, the main CF pathogens (except *A. xylosoxidans*). In the future, it may be important to investigate the role of the bacterial taxa not previously identified as key CF pathogens during exacerbation and treatment for an exacerbation.

In conclusion, antibiotic treatment in this cohort did not affect the overall microbial community of sputum after two weeks of treatment or at follow up. However, in the future, the microbiota could be followed throughout antibiotic treatment to direct antibiotic treatment.

Chapter 8 Discussion and Future Work

Cystic fibrosis (CF) is a multisystemic disease effecting the mucosal membranes of multiple organs. Despite advances in CFTR modulator therapies and antibiotic treatment, respiratory infections are still the leading cause of morbidity and mortality for CF patients (Cystic Fibrosis Foundation, 2019). Lung infection begins early in life and inflammation, impaired mucociliary clearance and chronic infection lead to a decline in lung function interspersed with periods of pulmonary exacerbation (Flume *et al.*, 2009). To guide antibiotic treatment during a pulmonary exacerbation and to monitor the progression of lung disease, respiratory samples are taken at regular intervals.

Currently, culture-based methods are the gold standard for the identification of targeted pathogens in respiratory samples. However, culture-independent methods, such as qPCR and microbiota sequencing, have expanded our understanding of lung disease in cystic fibrosis through the quantification of bacteria and determining changes in the bacterial microbiota (Rogers *et al.*, 2003; Döring *et al.*, 2012; Dickson *et al.*, 2013).

For convenience and to reduce the risk of cross-infection from other patients, it has been suggested that patients could provide samples at home and post them to the clinic for analysis (Moore *et al.*, 2021). During the COVID-19 pandemic, CF patients, deemed extremely vulnerable to the virus, were advised to 'shield' at home. This led to an increase in consultations being conducted over telephone or video calls (Davies, 2020; National Institute for Health and Care Excellence (NICE), 2020). The lung function of patients could be monitored at home with patients carrying out spirometry measures themselves (Cox *et al.*, 2012). However, this was not possible for diagnostic microbiology samples. Some CF centres had arranged for respiratory samples to be given at home and transported to the laboratory for diagnostic testing (Cystic Fibrosis Trust, 2020; Moore *et al.*, 2021). However, little research has been conducted to show if diagnostic results from these samples would be altered.

The aim of this research was to utilise qPCR to determine the bacterial load of known CF pathogens and utilise high-throughput gene targeted sequencing to determine the bacterial microbiota in respiratory samples from CF patients. To meet this aim, firstly previously published qPCR assays were optimised for the quantification of key CF pathogens. The qPCR assays were then used to enumerate pathogens in two studies. Gene targeted sequencing was also used to determine the microbiota of samples in the two studies. The first study investigated the effects of posting sputum samples on the density of key CF pathogens and the bacterial microbiota. The second study investigated changes

in the density of key CF pathogens and the bacterial microbiota composition between samples at the start, end and follow up of antibiotic treatment for a pulmonary exacerbation.

8.1 Optimisation of quantitative PCR assays targeting key CF pathogens

To optimise qPCR assays to quantify key CF pathogens, previously published assays were selected. The published methods were trialled before changes to the annealing temperature, cycle number and primer concentration of the PCR reaction were altered. qPCR assays to quantify *A. xylosoxidans, B. cepacia complex, P. aeruginosa, S. aureus* and *S. maltophilia* were optimised using pure bacterial cultures.

In previous studies, the most common CF pathogen targeted was *P. aeruginosa* and the most common gene targeted during qPCR for the detection of *P. aeruginosa* in respiratory samples was *oprL* (17 studies) (Table 1.2). Other common gene targets were *ecfX* (4 studies), *gyrB* (7 studies) and *regA* (4 studies). The primers and probe optimised in this study were designed by Feizabadi *et al.* (2010) and shown to have a specificity of 98.85% and a sensitivity of 100%. The assay has previously been used for the detection of *P. aeruginosa* in 110 bronchoalveolar lavage samples and 11 sputum samples from people with pneumonia (Feizabadi *et al.*, 2010), 107 sputum samples from people with bronchiectasis (Rogers *et al.,* 2014) and 94 sputum samples from people with CF (Peleg *et al.,* 2018).

To quantify *A. xylosoxidans*, one qPCR assay was identified in the literature which was used in a multiplex PCR (Rocchetti *et al.*, 2018). This study showed the assay had a specificity and sensitivity of 97% and 78%, respectively. However, the study tested the specificity of the assay using 52 microorganisms of which two were *Achromobacter* species. Liu *et al.* (2002) developed a PCR assay that had a specificity and sensitivity of 97% and 100%, respectively. This study tested the PCR assay against 149 isolates including 48 *A. xylosoxidans* isolates. Therefore, these primers were optimised for a SYBR qPCR assay.

To quantify *B. cepacia* complex, three qPCR assays were trialled targeting the *recA* gene and the *16S rRNA* gene. The first primers to be trialled targeted a large region of the *recA* gene and had previously only been used in PCR (Mahenthiralingam *et al.*, 2000). It is therefore likely they did not work as the product size was too large (1043bp) for qPCR (Debode *et al.*, 2017). Secondly, primers targeting the *16S rRNA* gene were trialled (Reid *et al.*, 2013). These primers had previously been used for PCR (Bauernfeind *et al.*, 1999) and were optimised by the authors for SYBR-based qPCR. When the primers were trialled in this study, possible primer dimers were seen as a second peak on melt curves. It is possible the study also found primer dimers and accounted for this in their analysis. The third and final set of primers trialled in this study also targeted the *16S rRNA* gene (Rocchetti *et al.*, 2018).

The primers had previously been used in a multiplex qPCR applied to CF respiratory samples for the detection of *B. cepacia* complex members. As previously mentioned, the study tested the specificity of the assay using 52 microorganisms, however unlike *Achromobacter*, nine *Burkholderia* species were tested.

To quantify *S. aureus*, a qPCR assay developed by Alarcón *et al.* (2006) targeting the *nuc* gene was optimised. Previous studies have used *nuc* targeted qPCR assays to detect *S. aureus* in respiratory samples from patients with COPD (Curran *et al.*, 2007) and suspected lower respiratory tract infections (van den Munckhof *et al.*, 2019). The primers from these studies were not chosen because Curran *et al.* (2007) used a nested/2 step qPCR and van den Munckhof *et al.* (2019) had not published the study before the assay was optimised.

To quantify *S. maltophilia,* a qPCR assay developed by Gallo *et al.* (2013) targeting the *23S rRNA* gene was optimised. Another study had previously targeted the *metB* gene during a multiplex qPCR (Rocchetti *et al.*, 2018). However, the study only tested the specificity of the assay with 52 microorganisms one of which was a lab strain of *S. maltophilia*. Gallo *et al.* (2013), however, used three lab strains of *S. maltophilia* and one hundred clinical isolates of *S. maltophilia*.

Due to time and financial constraints a qPCR assay targeting *H. influenzae* was not successfully optimised. All of the optimised assays had efficiencies within the optimal range. Once optimised, qPCR assays were used to quantify key CF pathogens in sputum samples before and after postage and at the start, end and follow up of treatment for an exacerbation.

In this study, qPCR assays were optimised for the detection and enumeration of bacterial pathogens identified by the CF Foundation as key to CF lung disease. It would also be of interest to optimise qPCR assays which target key fungal pathogens such as *Aspergillus* and *Candida* species (Hauser *et al.*, 2011; Cuthbertson *et al.*, 2021; Cystic Fibrosis Trust, 2021). The increase in microbiota sequencing research has also led to the discovery of other bacterial species of interest such as *Prevotella*, *Veillonella* and *Rothia* species which were shown in this study (Table 5.2 and Table 7.2) and other research (Zemanick *et al.*, 2015, 2017; Laguna *et al.*, 2016; Cuthbertson *et al.*, 2020) to be highly prevalent in CF respiratory samples. As further research is conducted into the role of these taxa in CF lung disease, qPCR assays targeting them may also become vital.

The optimised qPCR methods could also be utilised for the detection and enumeration of pathogens in other lung diseases such as COPD (Shimizu *et al.*, 2015), severe asthma (Zhang *et al.*, 2012) and pneumonia (Jones, 2010; Shindo *et al.*, 2013) as these diseases have pathogens in common. Using the methods of optimisation in Chapter 3, further qPCR assays could be optimised which target other common lung pathogens in COPD, severe asthma, and pneumonia such as *Streptococcus pneumonia*, *Moraxella catarrhalis* and *Streptococcus pyogenes.*

8.2 Comparison of culture, quantitative PCR and gene targeted sequencing

The optimised qPCR assays were used to enumerate key CF pathogens and 16S rRNA targeted gene sequencing was used to investigate the bacterial microbiota of sputum samples from two studies: one study investigated the effect of postage, and the other study investigated the effect of antibiotic treatment for an exacerbation. The prevalence of key CF pathogens in samples before and after postage were determined by standard culture methods, qPCR and gene targeted sequencing. The prevalence of key CF pathogens in samples at the start and end of antibiotic treatment and at follow up were determined by qPCR assays and gene targeted sequencing. The prevalence results from each method were compared. In the samples before and after postage, qPCR identified the CF pathogens in a higher percentage of samples than standard culture methods. Microbiota sequencing putatively identified all the CF pathogens except *A. xylosoxidans* in a higher percentage of samples than standard culture or qPCR. In samples at the start and end of antibiotic treatment and at follow up, microbiota sequencing putatively identified *P. aeruginosa* and *S. aureus* in a higher percentage of samples than qPCR. Whereas *A. xylosoxidans*, *B. cepacia* complex and *S. maltophilia* were detected in a higher percentage of samples by qPCR.

Previous research has shown qPCR had a similar sensitivity compared to standard culture for the detection of key CF pathogens in CF sputum samples (Deschaght *et al.*, 2009; Zemanick *et al.*, 2010; Nguyen *et al.*, 2016; Nelson *et al.*, 2020). In contrast, other studies have shown qPCR is able to detect *P. aeruginosa* in CF samples earlier than culture-based methods (Deschaght *et al.*, 2010; Hery-Arnaud *et al.*, 2017) and is capable of detecting *P. aeruginosa* and *S. aureus* more frequently than culture-based methods in intermittent and chronically colonised people with CF (Gavillet *et al.*, 2022). qPCR has also been shown to be more sensitive for the detection of *H. influenzae*, *S. pneumoniae* and *Moraxella catarrhalis* (Curran *et al.*, 2007; Garcha *et al.*, 2012) and *S. aureus* (Curran *et al.*, 2007) in COPD respiratory samples. The use of sequencing has been shown to be closely aligned to standard culture-based methods (Fodor *et al.*, 2012; Nelson *et al.*, 2020) for the detection of *P. aeruginosa*, *S. aureus* and *B. cepacia* complex. In contrast, another study found sequence analysis was more likely than culture-based methods to detect *Achromobacter*, *Burkholderia*, *Haemophilus*, *Stenotrophomonas* and *Streptococcus* genus whereas culture was more likely to detect *Staphylococcus* genus (Mahboubi *et al.*, 2016).

Results from previous research and this study suggests that culture-based methods and, to a lesser extent, qPCR and microbiota sequencing do not completely represent the CF

pathogens present. This suggests that, although culture-independent methods detected CF pathogens in more samples than standard culture methods, neither is perfect at detecting the presence of key CF pathogens and all methods should be used to create a clear representation of pathogens present in the lung of CF patients. By utilising culture, qPCR and microbiota sequencing to determine the effect of postage on sputum samples, a clear representation of the effects on the key CF pathogens and microbiota could be concluded.

In clinical settings, culture-based methods are considered the gold standard for the detection of bacteria from respiratory samples. The present research showed qPCR and sequencing identified key CF pathogens in respiratory samples more frequently than culture-based methods. However, culture-based methods are still useful for understanding the antibiotic susceptibility of pathogens within respiratory samples. qPCR takes less time to identify and enumerate targeted pathogens than the standard culture-based methods and was shown to detect pathogens in a higher percentage of samples in this study and previous studies (Deschaght *et al.*, 2010; Hery-Arnaud *et al.*, 2017). Therefore, the implementation of qPCR in the clinical laboratory could provide a quicker identification of pathogens from samples which would allow prompt, appropriate antibiotic therapy to be administered.

To implement the use of qPCR in the clinical laboratory it would need to be recommended by the CF Trust (UK), CF Foundation (US) and European Cystic Fibrosis Society (Europe) (Flume *et al.*, 2009; Cystic Fibrosis Trust, 2010; Saiman *et al.*, 2014). Further studies will be needed, including a prospective antibiotic study, to fully reveal the usefulness of qPCR in the clinic before it will be accepted as an integral part of antibiotic treatment.

The present study and previous research (Mahboubi *et al.*, 2016) has also shown gene targeted sequencing is more likely to detect key CF pathogens in respiratory samples than culture-based methods. The current cost of sequencing and the requirement of highly skilled technicians to carry out sequencing means that it is not currently an option for use in clinical laboratories as a standard diagnostic tool.

8.3 Effect of postage on the detection of key CF pathogens and the bacterial microbiota

Previous research indicated that postage would have no effect on the detection of key CF pathogens when culture-based methods were used (Bilton *et al.*, 1995; Hoppe *et al.*, 1997; Pye *et al.*, 2008). However, research using qPCR to enumerate pathogens in fresh samples and samples stored at room temperature for 24 hours indicated that a significant increase in the density of *P. aeruginosa* was likely to be found in posted samples (Murray *et al.*, 2010; Nelson *et al.*, 2010).

In Chapter 4, this research showed no significant difference between the prevalence of CF pathogens in fresh and posted samples using culture-based methods and qPCR. However, qPCR detected pathogens in a higher percentage of samples than culture-based methods. Previous studies have also shown a difference between culture and qPCR detection when detecting *P. aeruginosa, H. influenzae, S. pneumoniae* and *B. cepacia* complex (Xu *et al.*, 2004; Deschaght *et al.*, 2010; Nelson *et al.*, 2010; Reid *et al.*, 2013; Hery-Arnaud *et al.*, 2017). It has been suggested that the discrepancy between culture and molecular methods for the detection of CF pathogens could be caused by culture-based methods frequently misidentifying bacterial species due to phenotypic changes or species being masked by the overgrowth of other species (Deschaght *et al.*, 2010; Le Gall *et al.*, 2013; Hery-Arnaud *et al.*, 2017; Mangiaterra *et al.*, 2018).

The results presented in Chapter 4 also determined no significant difference in the density of key CF pathogens between fresh and posted samples using species specific qPCR assays. When the patient samples are analysed individually it is clear there is no trend in the increase or decrease of key CF pathogens between fresh and posted samples, however, the density is affected. It is possible these results differ from those previously published because previous studies (Murray *et al.*, 2010; Nelson *et al.*, 2010) compared fresh samples and samples stored at room temperature for 24 hours whereas the presented research sent samples in the standard UK postal system where the environment (temperature, movement) is not predictable. Previous research that posted CF sputum samples was conducted in Germany through spring and summer (Hoppe *et al.*, 1997) and another study that analysed bronchiectasis sputum samples was conducted in the UK but did not indicate when samples were posted (Pye *et al.*, 2008). Both studies found no significant difference in the prevalence or abundance of bacterial pathogens between fresh and posted samples. The research presented in Chapter 4 and Chapter 5 was conducted in the UK from July 2018

to July 2019 which allowed postage in all seasons to be represented. Therefore, this study has added to the current published studies by posting samples throughout the year, exposing samples to the postal service during all weather conditions. The samples in this study were posted in the UK and further research would be required to determine the effect of postage in countries with different climates.

High-throughput gene targeted sequencing was used to determine the bacterial microbiota of samples before and after postage in Chapter 5. Since the development of next generation sequencing techniques there has been an increase in research into the lung microbiota of CF patients and its relationship with age (Cox *et al.*, 2010; Klepac-Ceraj *et al.*, 2010), pulmonary exacerbation (Carmody *et al.*, 2013, 2015; Cuthbertson *et al.*, 2016) and lung function (Cuthbertson *et al.*, 2020). It has previously been suggested that it would be advantageous if patients could provide respiratory samples more frequently during clinical studies than the normal recommendations for monitoring (Hansen *et al.*, 2005; Cuthbertson *et al.*, 2016).

Microbiota sequencing identified bacterial taxa in samples before and after postage and the prevalence of key CF pathogens were also identified. No overall significant difference in the prevalence of any of the key CF pathogens between fresh and posted samples was seen. This is consistent with the results from culture-based methods and qPCR assessments (Chapter 4). When compared to standard culture methods and qPCR, microbiota sequencing showed a larger percentage of all samples had key CF pathogens present (except *A. xylosoxidans*). There was no significant difference in the bacterial richness, diversity and dominance in the microbiota of fresh and posted samples. Sørensen and Bray-Curtis dissimilarities of the microbiota in fresh and posted samples were also not significant. Previous research showed no effect on the community composition (Zhao *et al.*, 2011) and significant changes in community composition (Nelson *et al.*, 2010; Cuthbertson *et al.*, 2014) between fresh samples and samples stored at room temperature for various lengths of time. As previously mentioned, the presented results contradict previous studies potentially because those studies utilised culture-based methods or stored samples at room temperature for 24 hours, whereas the environment of the postage system is not predictable, or those studies had relatively small sample sizes compared to this study.

Previous studies have demonstrated partitioning the whole microbiota into core and satellite taxa groups reveals aspects of the community that were missed when looking at the whole microbiota (van der Gast *et al.*, 2011; Cuthbertson *et al.*, 2014). When this

strategy was employed in this study, it showed a greater change in the richness, diversity and dominance of the satellite taxa but a significant change in the core taxa. Sørensen analysis, which measured the dissimilarity in the presence of taxa between fresh and posted samples, showed a larger dissimilarity in the satellite taxa than the core taxa. However, when Bray-Curtis analysis was used, which is similar to Sørensen but also takes into account the abundance of taxa, there was a larger dissimilarity in the core taxa than the satellite taxa. This indicated that although no significant difference was seen in the whole bacterial community between fresh and posted samples, when the taxa were partitioned into core and satellite taxa groups, a difference was seen. This is important if, in the future, samples were posted to be used in microbiota research as the posted samples would not be true representations of fresh samples.

This result adds to the current research as no previous studies have been conducted into the detection or enumeration of key CF pathogens other than *P. aeruginosa* in posted CF respiratory samples. No previous studies had also determined if posting samples would influence the bacterial microbiota.

As a pilot study, this study indicated that postage would have a limited effect on cultured key CF pathogens. However, this studied only utilised the UK Royal Mail postal system in Manchester, UK. Shipping innovations such as Safebox (UK) (Royal Mail, n.d.), FedEx Clinical Pak (US) (FedEx, n.d.) and UPS Healthcare (Worldwide) (UPS Healthcare™, n.d.) or the use of drones in more remote locations (Amukele *et al.*, 2015; Ochieng *et al.*, 2020) may provide a more convenient and efficient way of posting respiratory samples from patients' homes to the lab. Specialist services such as these may be quicker but also incur a larger cost to the healthcare provider. Postal systems in other countries and those provided to remote location should also be tested, including standard international shipping, as the time and conditions could be different from the one studied. This would be of particular interest to clinicians whose patients wish to travel and/or work abroad. In the present study, samples were provided by patients and handled and sent in the post by clinic staff. If patients were to provide and post samples themselves from home, a delay in postage may occur as patients will send samples through the post when it is convenient to them rather than immediately after sample collection. It is important a further clinical study is conducted to access the microbiology of samples provided and posted by patients at home. Another limitation of this study is that it only analysed sputum samples. Some patients are not able to produce sputum consistently so other respiratory samples such as cough swabs would

need to be tested. This research also utilised samples taken when patients were clinically stable. To recommend the postage of respiratory samples to the clinic samples should be tested from every clinical stage through a pulmonary exacerbation (baseline, exacerbation, treatment and recovery) (Zhao *et al.*, 2012; Price *et al.*, 2013; Cuthbertson *et al.*, 2016).The present study did not include a mock community when posting, rather only patient samples were utilised. A mock community containing known concentrations of chosen bacterial species could also have been included (Fouhy *et al.*, 2016). This would have allowed the analysis of the effects of postage on a known bacterial community to further support the effects seen in the patient samples. In the present study, sputum samples were sent in the post before being analysed using culture-based and molecular techniques. The addition of DNA/RNA shield or the use of Flinders Technology Associates (FTA) cards should also be considered when samples are provided and posted. DNA/RNA shield is a medium that can be added directly to various human and environmental samples for the preservation of DNA during storage and transportation (Cambridge Bioscience, n.d.). Although not widely used for the storage and transportation of sputum, FTA cards have previously been used to preserve DNA at room temperature for the bacterial identification of pathogens (Rajendram *et al.*, 2006) from food (Kim *et al.*, 2017) and human respiratory samples (Guio *et al.*, 2006; Nuchprayoon *et al.*, 2007). It is possible these cards or the addition of DNA/RNA shield would be useful for the DNA based identification of pathogens. However, in a clinical setting culture-based methods are still used for identification and the inactivation of organisms on FTA cards or by DNA/RNA shield means they would not be useful for clinics.

8.4 Effects of antibiotic therapy for pulmonary exacerbation on key CF pathogens and bacterial microbiota

Lung infection is the leading cause of mortality and morbidity in people with cystic fibrosis and is interspersed with acute pulmonary exacerbations (Flume *et al.*, 2009). The frequency of pulmonary exacerbations has been linked to declining lung function which has made accurate and rapid treatment important (De Boer *et al.*, 2011). There is no clinical definition for an exacerbation and diagnosis is based on a range of clinical symptoms (Goss and Burns, 2007; Cuthbertson *et al.*, 2016). The cause of exacerbations is unknown; however, pathogens have been isolated from respiratory samples during exacerbations suggesting a link.

Culture-independent methods such as qPCR and high-throughput gene targeted sequencing do not require the isolation of bacteria for identification and are, therefore, quicker than culture-based methods and not affected by phenotypic changes such as small colony variants and low pigmentation.

Using the optimised species-specific qPCR assays (Chapter 3) key CF pathogens were quantified in samples taken at the start, end and follow up of antibiotic treatment for an exacerbation. Previous research utilising culture-based methods and qPCR suggested that *P. aeruginosa* would decrease between the start and end of treatment (Blumer *et al.*, 2005; Tunney *et al.*, 2011; Deschaght *et al.*, 2013; Reid *et al.*, 2013; Zemanick *et al.*, 2013; Lam *et al.*, 2015). Research into the effect of antibiotic treatment on non-*Pseudomonas* species was lacking (Blumer *et al.*, 2005; Tunney *et al.*, 2011; Reid *et al.*, 2013; Lam *et al.*, 2015).

This study showed no significant difference in the CFU/ml equivalents of the targeted pathogens between samples at the start and end of treatment or at follow up (Chapter 4). The results also indicated that in some individual patients as the abundance of one or more pathogens decreased, the abundance of other pathogens increased. This suggests that, although a high proportion of antibiotics were broad spectrum, some pathogens were susceptible to the antibiotics and decreased whereas as others were not susceptible and increased in abundance to fill the niche left by other species (Daniels *et al.*, 2013; Cuthbertson *et al.*, 2016). This indicates it may be useful for more frequent samples to be taken during antibiotic treatment so antibiotic therapies could be altered. It also indicates antibiotic therapies may also alter the presence and abundance of other bacterial species present in the microbiota of the lungs (Daniels *et al.*, 2013).

To understand if antibiotic therapies were also affecting the lung microbiota samples, high-throughput gene targeted sequencing was used to determine the bacterial microbiota of samples taken at the start, end and follow up of antibiotic treatment for an exacerbation (Chapter 5). Previous research utilising T-RFLP and pyrosequencing suggested that the microbial composition of the microbiota would not be significantly altered between the start and end of antibiotic treatment for an exacerbation (Tunney *et al.*, 2011; Fodor *et al.*, 2012; Cuthbertson *et al.*, 2016). Both a significant decrease (Daniels *et al.*, 2013) and increase (Cuthbertson *et al.*, 2016) in bacterial taxa richness have previously been identified between samples at the start and end of treatment for an exacerbation. The diversity of the microbiota has also been shown to decrease (Zhao *et al.*, 2012) and increase (Smith *et al.*, 2014) between samples taken at the start of treatment and during treatment. However, Sherrard *et al.* (2019) and Price *et al.* (2013) found no significant difference between samples at the start and end of treatment or at follow up when extended culture and pyrosequencing were used, respectively.

Both extended culture and T-RFLP are not ideal to determine the bacterial community of samples. Extended culture still requires the use of selective agars and biochemical tests to identify bacterial species. This technique often means uncommon species are misidentified or not cultured at all and the relative abundances cannot be accurately determined (Rogers and Bruce, 2010). Although an improvement on extended culture, T-RFLP has also been shown to lack depth when used to determine the bacterial community of complex microbial populations (Engebretson and Moyer, 2003). There has, therefore, been a larger focus on the use of next generation sequencing, such as pyrosequencing and high throughput sequencing, for the identification of bacterial communities.

When the bacterial microbiota was analysed in samples taken at the start and end of antibiotic treatment and at follow up in this study, no significant difference in the bacterial richness, diversity or dominance of the microbiota was found (Chapter 5). There was also no significant dissimilarity between the microbiota at the start and end of treatment or at follow up. The results from this research are consistent with previous research that indicated the CF lung microbiota demonstrated resilience to antibiotic therapies (Fodor *et al.*, 2012; Cuthbertson *et al.*, 2016; Sherrard *et al.*, 2019). The reason for this resilience may be due to recolonisation of bacteria from the upper respiratory tract (Fodor *et al.*, 2012) or antibiotic resistance (Sherrard *et al.*, 2014).

Previous studies and the present study were retrospective, determining the prevalence and abundance of pathogens and the bacterial community composition of samples taken before and after antibiotic treatment (Tunney *et al.*, 2011; Fodor *et al.*, 2012; Daniels *et al.*, 2013; Price *et al.*, 2013; Reid *et al.*, 2013; Zemanick *et al.*, 2013; Lam *et al.*, 2015; Stokell *et al.*, 2015; Cuthbertson *et al.*, 2016; Sherrard *et al.*, 2019). However, Smith *et al.* (2014) showed an alteration in the bacterial community in the first 3-4 days of antibiotic treatment which returned after 8-10 days. Deschaght *et al.* (2013) also showed a significant decrease in *P. aeruginosa* during the first week of antibiotic treatment when culture and qPCR was used. The results from these studies highlight the importance of carrying out a prospective study where samples are taken more frequently, such as daily, and culture, qPCR and microbiota sequencing informs the antibiotic regimen which could be altered to increase the chances of successfully managing exacerbations.

It is possible that no significant difference was seen in pathogen load or microbiota composition between samples at the start and end of treatment because of antibiotic resistance which is frequently developed in CF pathogens (Sherrard *et al.*, 2014). Previous research has shown that *P. aeruginosa, S. aureus* and *Haemophilus* species isolated from CF patients are more likely to be resistant to antibiotics than isolates from non-CF patients (Phaff *et al.*, 2006; Rao *et al.*, 2012). Previous research has also shown that different antibiotic susceptibility profiles can be found amongst isolates from the same sputum sample (Foweraker *et al.*, 2005). These could be the reasons another study showed pulmonary exacerbations commonly fail to respond to initial antibiotic treatment (Parkins *et al.*, 2012).

Due to its usefulness in susceptibility testing (Burns and Rolain, 2014), culture-based methods should still be used but this study has indicated the importance of using culture-independent methods to detect and enumerate key CF pathogens during antibiotic treatment for an exacerbation. Although no significant difference was seen between samples at the start and end of treatment and at follow up, in a high percentage of samples when one pathogen decreased in density, another increased. When microbiota sequencing was employed, the microbiota of individual samples increased and decreased in richness, diversity, and dominance. However, no overall trend in difference was seen from the start to the end of treatment. It would still be useful to utilise microbiota sequencing during treatment for an exacerbation as more is discovered about the role of the microbiota in lung infection.

The use of 16S rRNA targeted gene sequencing has limitations which have tried to be overcome by this study and other studies. These include the effective lysis of bacterial cells, contaminating DNA, the inability to differentiate between live and dead cells and low taxonomic resolution (Wintzingerode *et al.*, 1997). In this study, and developed by previous studies (Rogers *et al.*, 2013b), during DNA extraction cells were mechanically and chemically lysed and a negative control of PBS was also processed with each batch of samples to effectively lyse cells and account for any contaminating DNA, respectively. The addition of PMA prior to DNA extraction allowed the amplification of DNA from only live cells (previously discussed in section 1.7.4). This method of sequencing allows the user to select a target within the 16S rRNA gene. The 16S rRNA gene consists of conserved regions interspersed with variable regions. Targeted sequencing is usually carried out targeting one or more of the variable regions usually spanning 300-500 bp. Some bacterial species share similar 16S rRNA sequences in the variable regions including *Burkholderia* spp. and *Mycobacterium* spp. which can lead to low taxonomic resolution (Church *et al.*, 2020; Muhamad Rizal *et al.*, 2020). The development of longer read technologies will allow the identification of species down to genus and species level (discussed further in section 8.5).

A limitation of this study includes a lack of culture-based methods applied to samples. This would have allowed the comparison of gold standard culture-based methods with the results obtained from qPCR and bacterial microbiota sequencing. Another limitation was that measures of lung function were not available at each sample timepoint. Measures of lung function including $\%FEV_1$ would have allowed an assessment of the success of antibiotic treatment in terms of lung function improvement (Parkins *et al.*, 2012). Another limitation was that a relatively small number of patients were included in the study and they were not all administered the same antibiotic combinations. This makes comparisons and conclusions difficult to make. A multi-centre study would have been more ideal as a larger number of patients could be studied and the effect of different antibiotic combinations could be analysed. To further improve the study, samples could have been taken at more regular intervals, such as daily, to better track the effectiveness of treatment throughout the whole course. However, this would be time consuming for the clinician collecting samples and would possibly exclude patients who are unable or not willing to expectorate sputum that frequently.

8.5 Future work

In this study, a qPCR assay targeting *H. influenzae* could not be optimised (Chapter 3). As one of the pathogens tracked by the Cystic Fibrosis Foundation (Cystic Fibrosis Foundation, 2019), it is important that a sensitive and specific assay is developed. Previously, de Gier developed an assay targeting *H. influenzae* in nasopharyngeal swabs and middle ear effusions with 100% sensitivity and 100% specificity. However, this study was unable to optimise the assay to detect a *H. influenzae* isolate (Chapter 3). Therefore, further work is required to either optimise the assay or develop a new assay for the detection of *H. influenzae* in CF respiratory samples.

qPCR and targeted gene sequencing were utilised in this study to enumerate pathogens and assess the bacterial microbiota in sputum samples before and after postage. Sputum is not freely expectorated by all people with CF, especially paediatrics, therefore it would be important to study the effects of postage on alternative respiratory samples such as cough swabs, induced sputum and bronchoalveolar lavage (BAL) (Cystic Fibrosis Trust, 2010).

It is recommended respiratory samples are taken every three months and at the onset of an exacerbation. This study indicated more frequent sampling during antibiotic treatment for an exacerbation may allow for the alteration of antibiotics during therapy. Antibiotic therapies can be provided in clinic or at home (Cystic Fibrosis Trust Antibiotic Group, 2009). Therefore, if more frequent sampling was carried out it may be more convenient for people to send samples from home straight to the clinical laboratory. Previous studies have assessed the effects of room temperature storage and/or postage on respiratory samples taken during periods of clinical stability (Bilton *et al.*, 1995; Hoppe *et al.*, 1997; Pye *et al.*, 2008; Murray *et al.*, 2010). A single study utilised samples taken during antibiotic treatment to assess the effects of storage at room temperature on respiratory samples (Cuthbertson *et al.*, 2016). Therefore, further research should be conducted to assess the effect of postage on samples taken at every clinical stage including throughout antibiotic treatment.

Previous studies have shown an alteration in the abundance of *P. aeruginosa* and the microbial diversity of respiratory samples in the first 3-4 days of antibiotic treatment which returned to pre-treatment levels at 8-10 days (Smith *et al.*, 2014). This, along with the research presented here, indicates that a prospective study where samples were taken daily during treatment for an exacerbation would be useful. This study would allow a

change in antibiotic regimen if the current regimen was shown to not be having the desired effect.

The present study utilised qPCR to enumerate key CF pathogens at the start, end and follow up of antibiotic treatment for an exacerbation. In the future, qPCR assays could also be developed to identify antibiotic resistance genes present in samples to guide antibiotic treatment. Previous research has shown multiple phenotypes of *P. aeruginosa* with multiple antibiotic susceptibility profiles can be present in one sputum sample (Foweraker *et al.*, 2005; Clark *et al.*, 2015). The use of qPCR would allow the expression levels of specific antibiotic resistance genes to be assessed allowing more appropriate antibiotic selection. Antibiotic resistance mechanisms have been determined in some of the key CF pathogens. Assays have been developed to target *mecA*, the gene responsible for methicillin resistance in *Staphylococcus* species (Francois *et al.*, 2003) and *ampC* and *mexX,* genes linked to *P. aeruginosa* antibiotic resistance (Martin *et al.*, 2018) as well as a multiplex kit targeting 87 antibiotic resistance genes (Qiagen, n.d.; Hahn *et al.*, 2018). As well as qPCR, metagenomic sequencing could be utilised to identify antibiotic resistance genes in CF respiratory samples (Bacci *et al.*, 2020). However, the use of metagenomic sequencing is currently restricted to the research environment due to cost and time constraints.

This study and previous research has demonstrated that during treatment for a pulmonary exacerbation whilst there was a reduction in abundance of some bacterial species, other bacterial species increased in abundance (Chapter 6) (Daniels *et al.*, 2013; Lam *et al.*, 2015). It would be of interest to use a larger patient cohort alongside metagenomics to determine if functional genes remain consistent during antibiotic treatment (Lim *et al.*, 2014; Quinn *et al.*, 2016; Nelson *et al.*, 2020) and if, therefore, 'replacement' species are fulfilling the same roles within the microbiome.

High-throughput gene targeted sequencing was utilised in the present study to identify bacterial taxa within the microbiota of CF respiratory samples (Chapter 5 and Chapter 7). However, the identification of bacterial species using this method is putative due to the length of the amplicon. This meant members of the *Burkholderia cepacia* complex could not be distinguished from each other. The CF Trust has noted the importance of identifying species and strains within the complex due to their ability to cause epidemics (Mahenthiralingam *et al.*, 2001; Govan *et al.*, 2007; Cystic Fibrosis Trust, 2010). This method was available in house at the time the study was conducted. In the future, it may be possible to use a sequencing method that targets a larger amplicon of the *16S rRNA* gene or the

whole *16S rRNA* gene such as the Nanopore GridION™ (Nanopore, n.d.). In the present study, sequence variants were used as a proxy for strains which was also putative. The use of a targeted sequencing method such as the Nanopore sequencing or whole genome shotgun sequencing may also allow strains to be distinguished from each other more accurately (Feigelman *et al.*, 2017).

As well as a bacterial microbiota, the CF lung has been shown to have a fungal microbiota (Nagano *et al.*, 2010; Nguyen *et al.*, 2016; Cuthbertson *et al.*, 2021). Previous studies have shown a link between the incidences of fungal infection and antibiotic usage (Burns *et al.*, 1999) and the presence of fungal species and reduced lung function and increase in exacerbations (Chotirmall *et al.*, 2010; Delhaes *et al.*, 2012; Fillaux *et al.*, 2012; Reece *et al.*, 2017; Soret *et al.*, 2020) in CF. Little research has been conducted into the effects of antibiotic treatment for an exacerbation on the fungal microbiota; one study compared the fungal microbiota pre and post antibacterial treatment and found no significant difference in the microbiota (Willger *et al.*, 2014). Although, no direct effect would be expected; it is possible interactions between fungal species and bacterial species may be affected leading to a disruption in the microbiota (Amin *et al.*, 2010; Peleg *et al.*, 2010; Melloul *et al.*, 2016; Reece *et al.*, 2017, 2018). Further research is required to understand the effects of antibiotic treatment on the fungal microbiota and its role in exacerbation recovery. This research could involve the utilisation of previously developed qPCR assays to enumerate targeted fungal species (Khot *et al.*, 2008; Fréalle *et al.*, 2009; Reid *et al.*, 2013; Nguyen *et al.*, 2016; Guegan *et al.*, 2018a; Rocchetti *et al.*, 2018) and ITS gene targeted high throughput sequencing to investigate fungal microbiota (Nguyen *et al.*, 2016; Cuthbertson *et al.*, 2021) changes during and following antibiotic therapy.

The bacterial density of key CF pathogens and the bacterial microbiota have shown not to be significantly altered prior to an exacerbation (Stressmann *et al.*, 2011b; Carmody *et al.*, 2013, 2015; Price *et al.*, 2013) or between samples taken at the start and end of treatment except *P. aeruginosa* (Chapter 6 and Chapter 7) (Tunney *et al.*, 2011; Fodor *et al.*, 2012; Stokell *et al.*, 2015; Cuthbertson *et al.*, 2016; Sherrard *et al.*, 2019). Despite this, patients do clinically improve after antibiotic treatment (Fodor *et al.*, 2012; Deschaght *et al.*, 2013; Smith *et al.*, 2014) so it may be beneficial to build on previous research and, within one study with a large cohort of patients from multiple CF centres, analyse the prevalence and abundance of CF pathogens, the microbiome including sequence variants and functional

genes (Bacci *et al.*, 2020) and the host response (Zemanick *et al.*, 2013) during antibiotic treatment to fully understand the clinical improvement seen during antibiotic treatment.

8.6 Conclusion

This research investigated changes in the bacterial load of key CF pathogens and the bacterial microbiota in CF respiratory samples by employing species-specific qPCR assays and high throughput 16S rRNA targeted gene sequencing as an alternative to traditional diagnostic microbiology.

The following conclusions can be drawn from this research:

- Published qPCR assays require optimisation before use.
- Overall, there was no significant difference in the density of key CF pathogens between fresh and posted samples. However, densities increased and decreased in individual samples.
- No significant difference in the overall bacterial microbiota was seen between fresh and posted samples. However, the core taxa were significantly altered, driven by key CF pathogens.
- Determining the presence and abundance of key CF pathogens and the bacterial microbiota of the CF lung from CF respiratory samples that have been posted should be utilised with caution.
- No significant difference in the density of key CF pathogens was found between samples at the start and end of antibiotic treatment and at follow up.
- No significant difference in the bacterial microbiota of sputum samples was seen between samples at the start and end of antibiotic treatment and at follow up.
- Key CF pathogens and the bacterial microbiota of the CF lung at the start and end of antibiotic treatment for an exacerbation and at follow up are resilient and to investigate this further sampling should be done as treatment is ongoing.

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Supplementary materials

Supplementary Table 1 Illumina adaptors used in this study.

Primer	Sequence
16S Illumina adaptor forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGATTAGATACCCTGGTA
16S Illumina adaptor reverse	AGACGTGTGCTCTTCCGATCTCGACRRCCARGCANCACCT

Supplementary Table 2 Illumina sequencing barcodes.

Name	Full length
ITF.SA501	AATGATACGGCGACCACCGAGATCTACACATCGTACGACACTCTTTCCCTACACG ACG
ITF.SA502	AATGATACGGCGACCACCGAGATCTACACACTATCTGACACTCTTTCCCTACACG ACG
ITF.SA503	AATGATACGGCGACCACCGAGATCTACACTAGCGAGTACACTCTTTCCCTACACG ACG
ITF.SA504	AATGATACGGCGACCACCGAGATCTACACCTGCGTGTACACTCTTTCCCTACACG ACG
ITF.SA505	AATGATACGGCGACCACCGAGATCTACACTCATCGAGACACTCTTTCCCTACACG ACG
ITF.SA506	AATGATACGGCGACCACCGAGATCTACACCGTGAGTGACACTCTTTCCCTACACG ACG
ITF.SA507	AATGATACGGCGACCACCGAGATCTACACGGATATCTACACTCTTTCCCTACACG ACG
ITF.SA508	AATGATACGGCGACCACCGAGATCTACACGACACCGTACACTCTTTCCCTACACG ACG
ITF.SB501	AATGATACGGCGACCACCGAGATCTACACCTACTATAACACTCTTTCCCTACACG ACG
ITF.SB502	AATGATACGGCGACCACCGAGATCTACACCGTTACTAACACTCTTTCCCTACACG ACG
ITF.SB503	AATGATACGGCGACCACCGAGATCTACACAGAGTCACACACTCTTTCCCTACACG ACG
ITF.SB504	AATGATACGGCGACCACCGAGATCTACACTACGAGACACACTCTTTCCCTACACG ACG
ITF.SB505	AATGATACGGCGACCACCGAGATCTACACACGTCTCGACACTCTTTCCCTACACG ACG
ITF.SB506	AATGATACGGCGACCACCGAGATCTACACTCGACGAGACACTCTTTCCCTACACG ACG
ITF.SB507	AATGATACGGCGACCACCGAGATCTACACGATCGTGTACACTCTTTCCCTACACG ACG
ITF.SB508	AATGATACGGCGACCACCGAGATCTACACGTCAGATAACACTCTTTCCCTACACG ACG
ITR.SA701	CAAGCAGAAGACGGCATACGAGATAACTCTCGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT
ITR.SA702	CAAGCAGAAGACGGCATACGAGATACTATGTCGTGACTGGAGTTCAGACGTGTG CTCTTCCGATCT

Name	Full length
ITR.SA703	CAAGCAGAAGACGGCATACGAGATAGTAGCGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA704	CAAGCAGAAGACGGCATACGAGATCAGTGAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA705	CAAGCAGAAGACGGCATACGAGATCGTACTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA706	CAAGCAGAAGACGGCATACGAGATCTACGCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA707	CAAGCAGAAGACGGCATACGAGATGGAGACTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA708	CAAGCAGAAGACGGCATACGAGATGTCGCTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA709	CAAGCAGAAGACGGCATACGAGATGTCGTAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA710	CAAGCAGAAGACGGCATACGAGATTAGCAGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA711	CAAGCAGAAGACGGCATACGAGATTCATAGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA712	CAAGCAGAAGACGGCATACGAGATTCGCTATAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB701	CAAGCAGAAGACGGCATACGAGATAAGTCGAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB702	CAAGCAGAAGACGGCATACGAGATATACTTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB703	CAAGCAGAAGACGGCATACGAGATAGCTGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB704	CAAGCAGAAGACGGCATACGAGATCATAGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB705	CAAGCAGAAGACGGCATACGAGATCGTAGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB706	CAAGCAGAAGACGGCATACGAGATCTCGTTACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB707	CAAGCAGAAGACGGCATACGAGATGCGCACGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB708	CAAGCAGAAGACGGCATACGAGATGGTACTATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB709	CAAGCAGAAGACGGCATACGAGATGTATACGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB710	CAAGCAGAAGACGGCATACGAGATTACGAGCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB711	CAAGCAGAAGACGGCATACGAGATTCAGCGTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB712	CAAGCAGAAGACGGCATACGAGATTCGCTACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT