The Role of the Microbiome in Cystic Fibrosis

A thesis submitted in partial fulfilment of the

requirements of Manchester Metropolitan University for

the degree of Doctor of Philosophy

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Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

This thesis is being submitted in partial fulfilment of the requirements for the degree of PhD.

This thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by explicit references.

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Abstract

Lung Infection is the leading cause of morbidity and mortality for people with Cystic Fibrosis (CF). Infection is monitored by culture-based microbiology and informs subsequent infection management and treatment. Molecular-based methodology have revealed these infections to be polymicrobial. However, how lung infection microbiota change through time is poorly understood, particularly in mild CF. This thesis investigated longitudinal lung pathogen and microbiota dynamics within respiratory samples derived over several years from a cohort of adult and paediatric CF patients with mild disease.

First, diagnostic bacterial microbiology was compared to targeted quantitative PCR (qPCR). Prevalence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were significantly under-reported by diagnostic microbiology when compared to targeted qPCR. Further, qPCR revealed most patients were chronically infected with key pathogens which was missed by culture. This has wide-reaching implications for the development of chronic infection and clinical management of infection.

Next, bacterial microbiota dynamics through time were investigated. A stable bacterial microbiota was found across adult and paediatric patients, with fluctuations in microbiota composition resulting from changes in acquisition and abundances of transient rare taxa. Furthermore, patients were found to have a tolerance for long-term *S. aureus* infection and the strict anaerobe *Prevotella melaninogenica* appeared to be a key component of infection in mild CF disease.

Finally, temporal dynamics of the underexplored fungal microbiota were investigated to determine key fungal taxa associated with mild disease, along with comparisons to the bacterial microbiota. Patients were dominated by *Candida* taxa

and new correlations between bacterial and fungal taxa were reported, suggesting a protective role for *Candida* in paediatric patients.

Here molecular-based approaches have revealed novel aspects of pathogen and microbiota dynamics of fundamental importance to our understanding of mild CF lung microbiology. This understanding is important as more patients transition to mild disease phenotypes in the era of effective CF transmembrane conductance regulator (CFTR) modulator therapies.

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List of Abbreviations

CF – Cystic Fibrosis

CFTR – Cystic Fibrosis Transmembrane Regulator

NGS – Next Generation Sequencing

BAL – Bronchoalveolar Lavage

QPCR – Quantitative Polymerase Chain Reaction

COPD – Chronic Obstructive Pulmonary Disease

PSA- *Pseudomonas aeruginosa*

SA- *Staphylococcus aureus*

SM- *Stenotrophomonas maltophilia*

AX- *Achromobacter xylosoxidans*

HI- *Haemophilus influenzae*

BCC – *Burkholderia cepacia* complex members

PM- *Prevotella melaninogenica*

LRT – Lower respiratory tract

$\%FEV_1$ – Measurement of lung function: the % of lung capacity that can be expelled in 1 second

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Chapter 1: Introduction

1.1 Cystic Fibrosis Lung Disease

1.1.1 Cystic Fibrosis

Cystic Fibrosis (CF) is a common autosomal recessive genetic disorder of primarily Caucasian populations, with 10,000 and 30,000 children and adults affected in the UK and the US respectively (Cystic Fibrosis Foundation, 2017; Cystic Fibrosis Trust, 2018) and 70,000 worldwide (Bobadilla *et al*., 2002). Mutations of the Cystic fibrosis transmembrane conductance regulator (CFTR) gene causes the removal of the chloride channels in mucosal linings, impairing gastrointestinal functions (production of digestive enzymes, disruption of digestion and absorption), causing pancreatic insufficiency, and a build-up of sticky mucus in the lungs leading to infection and inflammation (Elborn, 2007; Hodges and Conlon, 2019). There have been over a thousand mutations identified to date (Rowntree and Harris, 2003), with the Fdel508 mutation being the most common mutation (50% of European patients are homozygous for this mutation and a further 35-40% are heterozygous) (Amaral *et al*., 2019). Mutations are classified into one of five groups depending on clinical features, and the mechanism by which they impact function (Marson *et al*., 2016). It is important to note that while CFTR mutation is a strong indicator of clinical severity, environmental and further genetic factors known as modifier genes contribute to severity of disease and CF-related disease such as diabetes and liver disease (Knowles and Drumm, 2012).

Lung disease is the most pronounced element of CF, with chronic airway infection and inflammation being the leading cause of mortality and morbidity for the majority of people living with CF (Ahlgren *et al*., 2015a). In the 1950s a child with CF would not be expected to live past the age of 5, whereas current patients can expect to live for more than 40 years (McBennett *et al*., 2021). Continual research and greater

understanding of lung infections in CF and the treatment of commonly encountered bacterial pathogens with targeted antibiotic regimens has greatly improved survival and quality of life (Sherrard *et al*., 2014).

1.1.2 Lung damage in CF lung disease

Lung infection in CF initiates a vicious cycle of inflammation and lung tissue damage and can be found in CF patients as young as 2.6 months of age (Tiddens, 2002). While pulmonary function testing is widely considered the gold standard for assessing disease progress in children and adults alike, lung function does not provide insight into lung structure (de Jong *et al*., 2004). Inflammation in response to lung infection is largely driven by neutrophil-mediated responses, particularly in response to *Pseudomonas aeruginosa*. The resulting high neutrophil elastase levels are highly implicated as causative agent in airway inflammation, mucus hypersecretion and further tissue damage (Wagner *et al*., 2016). Chronic infection increases the incidence of pulmonary exacerbation and disease progression. Exacerbations involve increased sputum production, chest pain, lung function decline and shortness of breath. They are also usually associated with the acquisition of new pathogens (Goss and Burns, 2007). This continued cycle of infection, exacerbation and treatment ultimately contributes to disease progression and lung function decline over time.

1.1.3 The Bacterial microbiota

Despite the knowledge that the lungs are constantly exposed to a multitude of bacteria viruses and fungi from the air we breathe, it was widely thought that the lungs were sterile. Culture-independent methods have since revealed that the lungs harbour a diverse and substantial amount of flora (Dickson *et al*., 2016). The term “microbiome” was first introduced in 2001 (Lederberg and McCray, 2001), and has

since been subject to redefinition, now defined as “the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes, and the surrounding environmental conditions.” (Marchesi and Ravel, 2015).

Recent advances in Next Generation Sequencing (NGS) have enabled researchers and clinicians to expand our understanding of the lung microbiome, and the CF lung microbiome remains to date the best described of the various lung diseases. It was one of the first diseases to fully engage with sequencing, opening new avenues of research, improving antibiotic treatments, and facilitating a deeper understanding of the interactions between host and microbe, and between microbes (Rogers, 2017)

1.1.4 Polymicrobial disease

Koch's postulates have shaped our understanding of medical microbiology, as many important microbial diseases conform to them. These criteria include:

1. The bacterium must be present in all diseased individuals (but should not be isolated in healthy individuals)
2. The bacterium must be isolated from a diseased individual and grown in pure culture
3. The bacterium must cause the specific disease when inoculated into a healthy individual
4. The same bacterium must be re-isolated from the experimentally diseased individual (Nelson *et al.*, 2012).

Clinical treatments still mostly adhere to the concept of “one microbe, one disease”, despite research continually demonstrating that in several diseases, such as CF,

polymicrobial infection is the driving forces behind infection pathogenesis, and that the presence of an opportunistic pathogen does not equal a causative agent (Fredricks and Relman, 1996; Antonelli and Cutler, 2016). Continual antibiotic bombardment of the lungs does not necessarily eradicate the target species, and pathogens such as *P. aeruginosa* can easily become antibiotic resistant, leading to a chronic infection that is almost impossible to completely eradicate (Pang *et al*., 2019). For lung disease, NGS has revealed a complex community of bacteria, fungi, and viruses, and expanded our understanding of exacerbation and accelerated lung function decline. Extensive study into the CF lung over recent years has discovered over 1000 bacterial taxa in connection with the CF lung microbiome (Charlson *et al*., 2012; Zhao *et al*., 2012; Losada *et al*., 2016; Cox *et al*., 2017). While most bacterial species recovered are thought to be relatively harmless, a handful are regularly sought after in clinical samples due to their ability to cause rapid lung function decline, exacerbation, and disease progression (Coutinho *et al*., 2008). In research, taxa can be separated into two categories depending on their prevalence, the “core” and “satellite” microbiome. The core microbiome is made up of species regularly found in samples that may be chronically colonising, whereas the satellite microbiome consists of the transient species that have no long-term presence (van der Gast *et al*., 2011).

As a result of NGS and advanced research techniques, CF lung disease has been redefined as a polymicrobial infection, with two or more pathogens playing key roles in exacerbations and a decrease in lung function. Exploration of polymicrobial infections and interactions have shown that the impact of a community can greatly influence pathogens in a way that monoculture experiments cannot demonstrate. For example, Drosophilia microbiome work has demonstrated the ability of the

"masses" to influence the outcome of an infection, as well as alter the virulence of *P. aeruginosa.* Mixed infection models with *P. aeruginosa* were shown to influence *Pseudomonas'* virulence and the type of immune response mounted against infection (Sibley *et al*., 2008). The interactions between bacteria and fungi within the lung are another key area of investigation. Fungi have yet to be properly investigated despite evidence to show that *P. aeruginosa* infection may prevent fungal infection (Hogan and Kolter, 2002; Sass *et al*., 2019), and that *C. albicans,* a commonly found fungal pathogen, requires a niche within a multispecies community for survival (Jenkinson and Douglas, 2002).

Treatment and our current understanding of the lung microbiome in CF has been largely informed through small population studies with narrow inclusion/exclusion criteria, where focus on specific issues, such as chronic colonisation with specific pathogens or cross-sectional studies comparing community composition (Cuthbertson *et al*., 2020). Varying methodologies and techniques can make it difficult to consolidate the results, particularly as there are no international standard for declaring patients free from "x" pathogen, with several criteria used in international CF centres (Pressler *et al*., 2011). For example, the European Consensus Criteria is based on a paper by Lee *et al*., 2003 (Lee *et al*., 2003). This criterion suggests sampling every 2 months for children and every 3 months for adults, and that any deviation from this would put colonisation categorisation in dispute. This paper also defines 4 categories of infection status based on the previous 12 months of sampling:

- Chronic: more than 50% of samples are culture positive
- Intermittent: 50% or less of samples are culture positive
- Free of infection: No growth over the 12-month period

- Never infected: Never isolated from any samples over lifetime.

However useful in identifying the major pathogenic bacteria this may be, this highlights the need for more consistency between studies. Research is largely driven by cross-sectional studies, which have shown that the lung microbiome is unique to each patient, and that the presence of certain pathogens in the CF lung lead to worse prognosis and decreased lung function (Kerem *et al*., 1990; Kosorok *et al*., 2001; Sagel *et al*., 2009; Price *et al*., 2013). Longitudinal studies are few in number, and many use strict inclusion/exclusion criteria that often reduces the number of participants. Understanding the fluctuations of the microbiome over time may greatly improve our understanding of community composition and interactions. Longitudinal studies have demonstrated the impact of treatments over exacerbation periods (Fodor *et al*., 2012; Cuthbertson *et al*., 2016; Carmody *et al*., 2018), the decline of pulmonary function (Corey *et al*., 1997; Horsley *et al*., 2021) and how the microbiome develops through infancy (Frayman *et al*., 2017; Ahmed *et al*., 2019). From this we know that %FEV_1 (the % of lung capacity that can be expelled in 1 second {Reyes-García, 2019}) progressively declines over time, either gradually or suddenly, however this depends on a multitude of factors including colonisation status, presence of CF-related issues such as diabetes, and frequency of exacerbation. Secondly, the microbiome is unique to each patient, however there are compositional similarities between patients, with genus' such as *Streptococcus, Pseudomonas, Haemophilus, Staphylococcus, Fusobacterium and Prevotella* identified in most patients. Finally, that there is not often a noticeable change in the community composition before, during or after an exacerbation, suggesting that exacerbation may not be a direct result of drastic change to the lung microbiome (Zhao *et al*., 2012; Cuthbertson *et al*., 2016; Whelan *et al*., 2017; Hahn *et al*., 2020).

1.1.5 Current clinical practise

Cultures from throat swabs, bronchi-alveolar lavage (BAL) and expectorated sputum have revealed shared pathogens across the CF patient population. In the clinic, sputum samples given by patients are routinely grown on various agars to determine the presence or absence of these emblematic pathogens, with subsequent treatment according to these results (Cystic Fibrosis Trust, 2010). These tests aim to discover the emblematic pathogens of CF, as outlined by the Cystic Fibrosis Foundation as *Pseudomonas aeruginosa, Haemophilus influenzae, Staphylococcus aureus, Burkholderia cepacia complex* species*, Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* (Cystic Fibrosis Foundation, 2018). Where strains of concern are discovered (new outbreak of *P. aeruginosa,* or the discovery of *Burkholderia cepacia complex* species), samples are sent for targeted sequencing using techniques such as Quantitative Polymerase Chain Reaction (QPCR) for confirmation. This allows hospitals to quickly isolate patients and re-direct their outpatient care to segregated clinics to reduce cross-contamination with non-colonised individuals (Cystic Fibrosis Trust, 2010).

Despite diagnostic microbiology shedding light on the pathogens present in samples, culture-based methods are not perfect and often miss many of the pathogens present in a sample. The notion that we cannot grow the true extent of a polymicrobial sample *in vitro* dates as far back as the 60's, where it was noted that up to 50% of viable pathogens may not be culturable despite a wide range of growth mediums and environments (Socransky *et al.*, 1963). This remains true, and further investigation has shown that some taxa may not grow at all, in a state called "viable but not culturable" (VBNC) (Pasquaroli *et al.*, 2013; Mangiaterra *et al.*, 2018).

Culture-independent measure have become regularly implemented for pathogens of concern such as *Burkholderia* and *P. aeruginosa*, where samples are sent for confirmation. While NGS is more accurate and specific than culture, it is not regularly implemented into routine diagnostics due to cost. There has been debate over the validity of NGS in light of oral contamination, however research has shown through comparison of 16S rRNA gene profiling in both mouthwash and sputum that contamination by the oral microbiome is minimal, and that some “oral” microbiome community members do exist in the lower respiratory tract (LRT) as well (Rogers *et al*., 2006; Lu *et al*., 2020), therefore overlap is to be expected.

1.1.6 Patient classification

To classify patients, there are 3 widely used categories based on %FEV_1 scores, Mild (≥70%), Moderate (40-69%) and Severe (<40%) (Abbott *et al*., 2009). While there is some debate over the use of %FEV_1 scores alone to classify patients, it does serve a purpose in the interim. Additional severity scores have been proposed in the last 10 years or so, including an expanded 5 category scale (including mild-mod, mod-severe categories) (Hafen *et al*., 2008), but the use of %FEV_1 alone is under scrutiny as many models assume a linear decline in %FEV_1 scores, where in reality, %FEV_1 decline is not a constant (Szczesniak *et al*., 2017). A number of measurements alongside FEV_1 scores can be used to improve the categories, including the ability to fully recover from exacerbation and frequency of exacerbation (Hampton *et al*., 2021). The lack of a robust measurement means there is little standardisation across research, with some studies favouring the “mild/moderate/severe” classifications (Cuthbertson *et al*., 2020; Soret *et al*., 2020), and other simply referring to patients as “stable/severe decline” (Bacci *et al*., 2016). Studies in mild lung disease tend to focus on the effect of drugs, how mild lung

function changes to moderate to severe over time, and how pulmonary function testing can be used to predict future lung failure in currently mild patients (Zhao *et al.*, 2012; Fuchs *et al.*, 2014; Paganin *et al.*, 2015; Bacci *et al.*, 2017). The basic microbiology of mild lung disease alone is limited, as studies tend to have groups of patients that cover a range of lung disease states, mild often making up the smallest group of these states.

1.1.7 Mild CF lung disease

As treatment and knowledge progresses, so does the clinical status of patients, with milder CF becoming more and more prevalent. Despite the lack of direct studies on mild phenotypes, we can see patterns and findings from the available literature. Some studies investigating mild lung disease found patients have lower rates of antibiotic resistance genes (Bacci *et al.*, 2017), and that the taxa found in the different spatial regions of the lung between and within patients are not consistent (Hogan *et al.*, 2016). Finally, one study found comparison of the three lung disease states had no significant difference in biodiversity measures (Paganin *et al.*, 2015).

Longitudinal studies have shown that the impact of exacerbations are often undetectable across the patients with milder disease, that diversity does not necessarily change during exacerbation, and that mild patients are generally stable over time (Stressmann *et al.*, 2012; Zhao *et al.*, 2012; Whelan *et al.*, 2017). Exploration into the resilience of stable CF (across mild and moderate phenotypes) has shown that the make-up of the microbiome in stable patients is more resilient to environmental changes and thus able to prevent colonisation with a novel pathogen, which has been previously shown in ecological models (Shade *et al.*, 2012; Bacci *et al.*, 2016).

The majority of research has focussed on *S. aureus* and *P. aeruginosa* as both have been shown to increase hospitalisation, exacerbation and progressive lung function decline (Cystic Fibrosis Trust, 2010), and as such, they have highest clinical significance. From this we can see that stable adult patients were found to have fewer exacerbations while colonised with *S. aureus* than those with *P. aeruginosa*, indicating that *S. aureus* lends itself to a more stable microbiome, and appears to be more abundant in milder/stable patients (Ahlgren *et al*., 2015b; Bacci *et al*., 2016). Frequency of emblematic pathogens in CF are lower in milder patients, as is dominance scores (Cuthbertson *et al*., 2020). Dominance has been repeatedly shown to increase as diversity decreases, and as the microbiome in mild patients have more diversity, this suggests that milder/stable microbiomes aren't typically dominated by any one species. Mild patients were found to have higher similarity between samples in comparison to Moderate/Severe patients, indicating that worse lung function has higher turnover of species, and that milder patients retain more members of the community, which in turn can lend itself to more stability (Hampton *et al*., 2021).

1.1.8 CFTR Modulator therapies

The introduction of CFTR modulating therapies has vastly improved the quality of life for many CF patients. There are currently 4 therapies on the market, Ivacaftor, Symdeko, Orkambi, and recently, Trikafta/Kaftrio (USA/EU branding). These modulators have two mechanisms, either as a Corrector (promoting the correct folding and expression of the CFTR protein) or as a Potentiators (ensuring the CFTR protein works as intended) (Cystic Fibrosis Foundation, 2018). With the exception of Ivacaftor, the remaining therapies are combination drugs, utilising Ivacaftor (a potentiator) in combination with other CFTR modulators (correctors) to promote

proper folding and expression of the CFTR protein and improving function. Long term use of Ivacaftor does have its benefits, with improved survival and reduced lung transplant demand, however recovery after an exacerbation is not improved, and eventually lung function will decline, albeit at a slower rate than normal (Lopes-Pacheco, 2020). Treatment with Orkambi (Ivacaftor/Lumacaftor) was shown to vastly reduce the number of exacerbations even if patients didn't show any improvement in %FEV_1 (McColley *et al*., 2019). Symdeko was approved (Ivacaftor/Tezacaftor) in 2018 and had similar outcomes to Orkambi (Torre and Albericio, 2019). Tezacaftor is a modified version of Lumacaftor (Lopes-Pacheco, 2020), which in combination with Ivacaftor, reduced sweat chloride levels and preserved lung function (Walker *et al*., 2019). These combination drugs have modest improvements in CF patients, providing some benefits, however lung function still declines. The triple therapy Kaftrio (Ivacaftor/Tezacaftor/Elexacaftor) has only been recently approved (2019) and its long-term effects are still being studied. One of the standout issues is that while many of these treatments can be used with patients who have at least one F508del mutation, and the approval of Kaftrio increased the range of patients these drugs were available to, this still leaves many without treatment options (Yi *et al*., 2021).

Studies over the course of introducing CFTR modulators have shown some changes in the microbiology of the lung. One study looking at mild patients with 2 years of Ivacaftor treatment, found that *P. aeruginosa* levels significantly dropped, and *S. aureus* levels were untouched, however it must be noted that this study was based on culture-based evaluation and not NGS methodology (Heltshe *et al*., 2014). Another study on a range of lung disease states found most had a significantly higher %FEV_1 score after treatment, sustained lowered inflammation, and lower

levels of *P. aeruginosa* in the first week of treatment. Persistent infection with *P. aeruginosa* was not prevented, and levels of key pathogens such as *Streptococcus* and *Prevotella* did not change over the course of the treatment (Hisert *et al*., 2017). Reductions in *P. aeruginosa*, and an increase in species thought to lend themselves towards a more stable bacterial community have also been observed (Ronan *et al*., 2018).

While it is important to note the reduction in *P.* aeruginosa, it is consistently observed that eradication is not achieved, and that rebounds are common. Conversely, one study found that total bacterial load and *P. aeruginosa* levels did not change after treatment with Ivacaftor over a 6-month period. There was also no difference noted between the milder patients that required sputum induction and those with more severe lung impairment (Harris *et al*., 2020). Many of these studies focus on diversity scores and prevalence of *P. aeruginosa* through targeted QPCR, and while it is important to identify changes in key pathogens, this leaves many areas in need of further investigation.

Fungal prevalence has also been shown to decline while using CFTR modulators however there are few studies demonstrating this. A significant drop in the prevalence of *Aspergillus spp.* has been previously observed (Heltshe *et al*., 2014; Frost *et al*., 2019), with the latter study finding a reduction from 13% to 4.5% over a 5-year period, and another found a nearly 50% drop in *Aspergillus spp.* rates across both UK and USA patients taking CFTR modulators (Bessonova *et al*., 2018). The impact of CFTR modulators has been investigated with a focus on bacterial taxa, however from these initial results concerning *Aspergillus spp.* it is clear modulators have an impact on all aspects of the microbiome.

1.2 Emblematic bacterial pathogens of Cystic Fibrosis

Over 1000 bacterial species have been associated with the CF lung microbiome (Moran Losada *et al*., 2016) many of which are transient and do not chronically colonise the lung. While most bacterial species recovered are thought to be relatively harmless, there are a handful that are regularly sought after in clinical samples due to their ability to cause rapid lung function decline, exacerbations, and disease progression (Coutinho *et al*., 2008).

1.2.1 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, aerobic rod bacterium, that has an ability to quickly gain antibiotic resistance. As an opportunistic pathogen it is being increasingly discovered in immuno-compromised patients (Wu and Li, 2015), particularly CF lungs, infecting 2 in 3 CF patients before young adulthood (Trust, 2018). With more than 50% of infected patients above 18 years of age (Crull *et al*., 2018), it is recognised as the biggest contributor to morbidity and mortality (Firmida *et al*., 2017). *P. aeruginosa* infections are associated with worse outcomes and a faster decline in lung function, and the impact on airway disease is well documented, however the progression and the initiation of the infection remains poorly understood (Rogers *et al*., 2015). Infection increases as patients get older, however data from the Cystic Fibrosis Trust shows that it reduces in older patients (55+), and generally chronic infection has dropped in comparison to levels in 2008 and 2013 (Trust, 2018) possibly due to increasing number antibiotics specifically for *Pseudomonal* infection.

P. aeruginosa is capable of existing in a mucoid form, allowing attachment to the mucus lining of the lung, and thriving under both aerobic and anaerobic conditions (Worlitzsch *et al*., 2002). Damage to the lung is achieved through exotoxin

production and host-mediated inflammation in response to infection (Davies *et al*., 2006). *P. aeruginosa* uses a range of tactics to avoid detection, for example, it uses a flagellin to manoeuvre itself to its final destination, where upon changing to a mucoid phenotype, removes the flagellin to avoid host detection. This is associated with host-adapted strains in CF and is associated with increased bacterial burden and disease severity (Faure *et al*., 2018).

Treatment for *P. aeruginosa* typically involves aminoglycosides such as Tobramycin, which have been used to great effect when administered via nebuliser (Ratjen *et al*., 2009). Intravenous delivery of these drugs puts patients at risk of nephrotoxic and ototoxic effects, especially at the levels needed to eradicate *P. aeruginosa*, whereas nebulised delivery minimises absorption into the bloodstream and reduces the potential toxicity of the drug (Ramsey *et al*., 1993). Despite the success of these treatments, antibiotic resistance is an ever-present risk for patients. In 2017, the rate of Multi-Drug-Resistance (MDR) for *P. aeruginosa* in the USA had risen to 18.1% (Stefani *et al*., 2017), however infection rates are decreasing amongst the paediatric cohorts, with many reaching adult care without *P. aeruginosa* infection (Cystic Fibrosis Foundation, 2019).

1.2.2 *Haemophilus influenzae*

H. influenzae is a Gram-negative coccobacillus that can survive in both aerobic and anaerobic environments (Wijers *et al*., 2017), and is a common cause of respiratory infection in young CF patients (Hoppe and Sagel, 2019). A frequent coloniser of the lung (14-52%), it can be picked up multiple times over a patient's lifetime (Foweraker and Wat, 2011). There are two types of *H. influenzae,* an encapsulated strain and a non-encapsulated strain. While there is an effective vaccine for the encapsulated type, the non-encapsulated type makes up the majority of infections and is

associated with worse clinical outcomes (Cardines *et al*., 2012; Malfroot *et al.,* 2005). Inside the lung, *H. influenzae* attaches to damaged or non-ciliated mucosal linings (King, 2012), however the lack of an adhesin gene significantly reduces the ability to colonise the airway long-term (Cardines *et al*., 2012). Damage to the airways is caused by factors that damage/inhibit ciliary functions, as well as direct damage to ciliated epithelium (King and Sharma, 2015). It is known to be highly invasive, being found in the epithelium, pulmonary macrophages, and the sub-epithelial layers of the bronchi (Sajjan *et al*., 2001). Ampicillin and amoxicillin are also used to treat *H. influenzae* infections; however the levels of antibiotic resistance are on the rise (Tristram *et al*., 2007; Su *et al.,* 2020).

1.2.3 *Staphylococcus aureus*

S. aureus is a Gram-positive, facultative anaerobe that is part of normal flora for the nasal cavity (Wijers *et al*., 2017), respiratory system and skin (Sakr *et al*., 2018). It is an opportunistic pathogen, and up to 60% of the general population is colonised at any given time, with ~20% thought to be permanent carriers (Etter *et al.,* 2020). It is a notable early coloniser in CF patients of over 6 months of age (Coutinho *et al*., 2008) reducing in frequency as patients age (Cystic Fibrosis Foundation, 2019; Cystic Fibrosis Trust, 2019), where it is typically replaced with *P. aeruginosa* as the most common coloniser (Hubert *et al*., 2013). Due to differing guidelines and treatment plans in various countries, the rates of infection in CF widely vary across the globe, with chronic infections as low as 15% in the UK, to 85% in Armenia (Zolin, 2019). *S. aureus* can persist in a patient for many years, with patients carrying the same clone multiple times despite antibiotic therapy (Kahl, 2010). The impact of *S. aureus* on CF disease progression is poorly understood and the increasing incidence of methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-

resistant *Staphylococcus aureus* (MRSA) is becoming an international issue that must be addressed in all areas of healthcare (Bell and Flume, 2017).

S. aureus is extremely capable of adapting to its environment and can use small-colony-variants (SCVs) to colonise the airways even during the use of long-term macrolides (Kahl *et al*., 2003). There are numerous mechanisms by which *S. aureus* settles in the lung and forms biofilms, but are difficult to study *in vitro* as *S. aureus* isolates lose their biofilms when removed from the lung (Goerke and Wolz, 2010). Expression of *S. aureus* virulence factors are highly regulated by environmental factors (Arvidson and Tegmark, 2001), and the contribution of these virulence factors to chronic infection is poorly understood. SCVs have been suggested as the determining factor in prolonged infection, as they can persist in the epithelium over a long period of time (Proctor *et al*., 2006). *S. aureus* utilises an array of toxins to establish infection, such as pore-forming toxins and superantigens (Oliveira *et al.*, 2018). The *agr* pathway that regulates these has been shown to be down-regulated in some strains, resulting in a more adherent, but less toxic phenotype, which may explain the persistence of *S. aureus* in some patients without active infection (Rumpf *et al.*, 2021).

Long term macrolide treatment has been shown to significantly reduce the incidence of *S. aureus* infection. A Danish centre treated patients with low dose, long term Azithromycin for ten years, and found that it not only controlled and reduced the incidence of *S. aureus* infection, but also that of *S. pneumoniae* and *H. influenzae* (Hansen *et al*., 2009). The most effective treatment for infections is also the most risky, as it lends itself easily to the acquisition of *mecA* resistance, which in turn offers resistance to all available β-lactam based antibiotics (Kahl, 2010).

1.2.4 *Burkholderia cepacia complex*

The *Burkholderia cepacia complex* is a genomovar group of Gram-negative, obligate aerobic bacilli (Farzana *et al*., 2020). There are currently 22 species (Scoffone *et al*., 2017) associated with the group, usually environmental pathogens that are capable of infecting immunocompromised patients. Commonly occurring species differ depending on geographical location, however the commonly encountered pathogens are *B. cepacia, B. cenocepacia, B. multivorans, B. vietnamiensis, and B. dolosa* (Reik *et al*., 2005). *B. cenocepacia* is associated with a severe lung function decline, and with natural resistance to different antibiotic classes, it is incredibly difficult to treat (Scoffone *et al*., 2017). Infection with *Burkholderia cepacia* complex members can vary in presentation, from asymptomatic chronic colonisation, rapid decline and fatal "cepacia syndrome" (Fauroux *et al*., 2004). Due to the severity of disease that can arise from *Burkholderia cepacia* complex infection, detection of an isolate in a clinical sample requires further investigation, and samples are sent for QPCR analysis to confirm results (Cystic Fibrosis Trust, 2010). Infection rates with *Burkholderia* complex members have remain below 10% in the UK since 2004 (Cystic Fibrosis Trust, 2013; 2019), and below 3% in the US from 2003 (Cystic Fibrosis Foundation, 2019).

Burkholderia taxa have a range of characteristics that enable attachment to the lung epithelium, such as surface lipopolysaccharides, cable pili that have been demonstrated to mediate attachment to mucin, and the ability to survive inside epithelial cells (Sajjan *et al*., 2001). *B. cepacia* can take advantage of the downregulated autophagy present in CF patients, by further downregulating autophagy in macrophages it can hide inside the auto phagosomes produced by cells, safe in the knowledge it will not be destroyed (Abdulrahman *et al*., 2011).

Treatment for *Burkholderia* infection typically involves Meropenem, Ceftazidime, and high doses of Tobramycin (Zhou *et al*., 2007).

1.2.5 *Stenotrophomonas maltophilia*

S. maltophilia, is an environmental, Gram-negative bacteria that is known to colonise and infect immune-compromised patients. Due to slow growing colonies, it is often undetected in routine cultures (Wisplinghoff and Seifert, 2010; Wisplinghoff, 2017). *S. maltophilia* is widely considered an emerging pathogen in the CF microbiome, with up to 10% of patients thought to be colonised. The majority of infections are thought to be transient, however *S. maltophilia* is capable of long-term chronic colonisation (Steinkamp *et al*., 2005). This emergence (like other pathogens) is largely thought to be brought on by selective anti-Pseudomonal treatments over recent years, creating niches ready for exploitation. The true extent of infection with this pathogen is not clear, as many papers do not make a note of whether infection is transient or chronic, simply if the sample was positive (Goss *et al*., 2002; Goss *et al*., 2004), with various papers finding roughly similar patterns of incidence in both adults and children (Spicuzza *et al*., 2009; Stanojevic *et al*., 2013).

S. maltophilia is poorly understood, one study found that *S. maltophilia* copes with the lower pH in the lung environment, by expressing stress response genes and colonising less acidic microenvironments in the lung (Gallagher *et al*., 2019). Research into the mechanisms of infection is also limited, however like *P. aeruginosa, S. maltophilia* uses Quorum sensing to control virulence factors such as biofilm formation and motility to initiate infection (Trifonova and Strateva, 2019). There are conflicting studies on the incidence of lung damage and the presence of *S. maltophilia* infection (Karpati *et al*., 1994; Goss *et al*., 2002). In terms of interactions there is very little research, however one study found that where *S.*

maltophilia created a mixed biofilm with *P. aeruginosa,* and used this as a defence against Tobramycin treatment, with the antibiotic only affecting *P. aeruginosa,* causing *P. aeruginosa* to upregulate expression of virulence factors (Pompilio *et al*., 2015). This has serious implications for *in vivo* co-infections. In terms of treatment, *S. maltophilia* has been shown to have resistance to multiple antibiotic classes, including β-lactam antibiotics, macrolides, carbapenems, tetracyclines, and polymyxins (Brooke, 2012). Typically, infection is treated with trimethoprim-sulfamethoxazole, ticarcillin-clavulanic acid, or fluoroquinolone agents (Abbott *et al*., 2011), however the naturally occurring resistance it harbours is a cause for concern.

1.2.6 *Achromobacter xylosoxidans*

A. xylosoxidans is an emerging pathogen, with 16 species and 2 subspecies described in the context of CF. *A. xylosoxidans* is typically associated with increased inflammation and severe lung disease, and repeated exacerbations (Hansen *et al*., 2010). Its true clinical relevance is yet to be determined, as many other *Achromobacter* species *also* colonise the lung (*A. ruhlandii, insuavis* and *dolens* to name a few*)* (Dupont *et al*., 2015). The rates of *A. xylosoxidans* varies across the literature, with rates from 1.1% and 2.3% (Tan *et al*., 2002; Steinkamp *et al*., 2005), to 7.11% (Spain) and 13.33% (Cyprus) (Zolin, 2019). A Gram-negative bacillus, *A. xylosoxidans* is found in a wide range of environments, including hospitals, and is seen to be more prevalent in Adults (Cools *et al*., 2016). It is an opportunistic pathogen that causes a range of diseases in immunocompromised patients, such as bacteraemia and pneumonia. As with other newly emerging pathogens in CF, we are finding new strains already have multiple drug resistance as a result of extensive antibiotic use over the last 30 years or so (Lambiase *et al*., 2011). Treatment for *A. xylosoxidans* infection is becoming increasingly difficult due to the acquisition of

resistance to frequently used drugs such as Tobramycin and Gentamycin, however Meropenem and Imipenem have been shown to remain effective for now (Swenson and Sadikot, 2015).

Studies have brought inconclusive evidence on the role of *A. xylosoxidans* in lung damage. Some studies suggest that perhaps *A. xylosoxidans* is more readily able to colonise more damaged lungs, which is why it is seen in cases with poorer lung function and a decline in health, but more research is needed (De Baets *et al*., 2007; Firmida *et al*., 2016), others hypothesis that biofilm producing strains are more capable of long-term colonisation and tissue induced inflammation (Hansen *et al*., 2010).

1.3 Emerging Pathogens in CF

Research is identifying potential new and emerging pathogens, such as multidrug resistant strains of members of the *Burkholderia cepacia* complex, *Achromobacter* species, *S. maltophilia,* and *Pandoraea spp.* Relative levels of the various member of the *Burkholderia cepacia complex* are rising, with strains previously not seen in the CF lung being isolated. Levels of detected *B. multivorans* are exceeding that of *B. cenocepacia* and patients are found to exhibit genetically distinct strains (LiPuma, 2010). The *Pandoraea* genus was thought to be *Burkholderia* or *Ralstonia,* but is now its' own distinct genus (Coenye *et al*., 2000). With this distinction, *Pandoraea spp.* are now being detected in the CF microbiome and have been observed to contribute towards rapidly declining lung function (Jørgensen *et al*., 2003).

1.3.1 Anaerobes of the CF lung

Anaerobes are undeniably a key component of the LRT microbiome, species within the *Prevotella, Veillonella,* and *Fusobacterium* genera are commonly detected in sputum. Anaerobes are becoming a key subject in CF lung microbiome research, with studies showing higher levels in paediatric patients, higher levels being observed correlated to milder forms of disease and anaerobes demonstrating resistance to exacerbation treatments (Cox *et al*., 2010; Fodor *et al*., 2012; Jiangchao Zhao *et al*., 2012). While these correlations indicated a positive effect on lung health, there is conflicting research on the role of anaerobes such as *Prevotella spp.* in the lung (Carmody *et al*., 2018; Muhlebach *et al*., 2018b; Lamoureux *et al*., 2021b).

1.3.1.1 *Rothia spp.*

Rothia mucilaginosa has been suggested as an emerging pathogen, and while typically found in the oral microbiome, it has been detected in sputum samples alongside other commonly found anaerobes (Bittar and Rolain, 2010). *R. mucilaginosa* has been demonstrated to have an anti-inflammatory properties and has been indicated to have beneficial effects on the CF lung (Rigauts *et al*., 2021). However, there is also evidence to suggest that it could give *P. aeruginosa* a metabolic boost, demonstrating that further work needs to be done to fully understand the role this potential pathogen plays in CF lung disease (Gao *et al*., 2018).

1.3.1.2 *Prevotella spp.*

Prevotella spp. are thought to be common commensals of the upper and lower respiratory microbiome. A group of 28 species, they are obligate anaerobes and difficult to culture from CF sputum samples (Field *et al*., 2010; Sherrard *et al*., 2013).

Prevotella spp. such as *P. melaninogenica, P. nigrescens* and *P. tannerae* are well documented in various lung disease settings, including asthma, chronic obstructive pulmonary disease (COPD), and CF (Hilty *et al*., 2010; Park *et al*., 2014; Larsen, 2017), however it is unknown if the *Prevotella spp.* that resides in the LRT has any impact on disease progression. Previous research demonstrated that a range of *Prevotella* isolates have the potential to shield emblematic pathogens from the effects of antibiotics such as ceftazidime (Sherrard *et al*., 2016), with some studies finding a range of clinical isolates including *P. melaninogenica,* were resistant to commonly used antibiotics such as amoxicillin and tetracycline (Sherrard *et al*., 2013; Lamoureux *et al*., 2021a).

Prevotella nigrescens has been shown to modulate inflammatory responses, an *in* vitro study found *P. nigrescens* induced significantly high levels of IL-6 mediated inflammation and utilised a different inflammatory pathway to *P. aeruginosa,* as well as being able to maintain high IL-6 levels despite knockout assays to prevent this (Bertelsen *et al*., 2020). There is a rising body of evidence to suggest that *Prevotella* is not just a species that found in CF, and that it has some implications on disease progression and the ability of other key species to resist antibiotic therapies and establish chronic colonisation.

1.3.2 *Pandoraea spp*.

A new emerging pathogen, *Pandoraea spp.* is a close relative of the *Burkholderia cepacia complex* and has 11 CF related species, and several genomospecies (Tabatabaei *et al*., 2019). Most worryingly about this species is its resistance to a large amount of antibiotics, including broad spectrum cephalosporin, azithromycin, and aminoglycosides (Fernández-Olmos *et al*., 2012; Lin *et al*., 2019; Coward *et al*., 2020). A study in investigating the prevalence of *Pandoraea spp.* in the UK found

the majority of strains to be *P. apista* and *sputorum,* with some isolates attributed to the newly described *P. fibrosis.* Resistance to commonly used antibiotics was found across all isolates in the study and there were geographical clusters of the same strain (Coward *et al*., 2020). A single centre study recorded a prevalence of 3.3% in paediatric patients over a 2-year period (Panickar and David, 2015), however the true prevalence of this particular taxa is relatively unknown.

1.4 Bacterial interactions.

The polymicrobial nature of the lung microbiome means that bacterial interaction is inevitable and has profound implications on the clinical presentation and progression of CF-related lung disease. Some studies have taken an observational approach to this, observing patients longitudinally, and determining if groups of patients are less likely to become infected with pathogen "x" due to factors such as a pre-existing infection or the medication prescribed to the individuals (Hector *et al*., 2016; Granchelli *et al*., 2018). Other approaches to determine bacterial interactions take place *in vitro*, with studies using cultures to determine antagonistic effects between key taxa such as *P. aeruginosa* and *S. aureus* (Machan *et al*., 1992; Fugère *et al*., 2014; Pompilio *et al*., 2015). These studies have shown that polymicrobial infections of the lung are typically facilitated by biofilm formation, to promote chronic infection, resist antibiotic therapies and maintain infection (Høiby, 2002; Tré-Hardy *et al*., 2009).

1.4.1 *P. aeruginosa* and *S. aureus* interactions.

Two of the most widely recognised emblematic pathogens, *S. aureus* and *P. aeruginosa,* have been demonstrated to result in decreased lung function and increased exacerbation when co-infecting (Limoli *et al*., 2016). Early detection and

eradication is a key focal point for many studies (Taccetti *et al*., 2005; Schelstraete *et al*., 2013; Héry-Arnaud *et al*., 2017; Blanchard *et al*., 2018), however little is known about whether interaction with other taxa increases the potential for *P. aeruginosa* to become more virulent or to acquire antibiotic resistance genes. There is conflicting research on chronic colonisation with *S. aureus*, as milder patients were shown to tolerate chronic infection, with steady infection suggested to contribute to a more stable microbiome (Ahlgren *et al*., 2015b; Bacci *et al*., 2016), however many studies associate chronic colonisation with worsening lung function and faster decline (Wolter *et al*., 2013; Junge *et al*., 2016).

Co-infection of *S. aureus* and *P. aeruginosa* has been demonstrated to have wider effects on the lung microbiome, and co-infection is closely monitored. Studies have demonstrated that interactions between these taxa are linked to worse disease outcomes, and that they contribute in their individual ways as well as a combined force (Hotterbeekx *et al*., 2017). Coinfection may be brought on earlier due to the heavy antibiotic regimes patients undergo, with paediatric patients receiving prophylactic Flucloxacillin treatment shown to have earlier detection of *P. aeruginosa* (Hurley *et al*., 2018). Co-infection is associated with a notable increase in inflammatory markers, and increased mortality during the first 10 years of diagnosis (Sagel *et al*., 2009). Interestingly, both species have been shown to influence the ability of the host to acquire the other, with mouse models and *in vitro* testing demonstrating the ability of *P. aeruginosa* to out-compete *S. aureus* for colonisation, as well as limit biofilm formation, which may explain why patients shift from being *Staphylococcus* dominant to *Pseudomonas* dominant as they age (Baldan *et al*., 2014; Fugère *et al*., 2014). Studies looking at pathogens cultured from patients over time have shown that *S. aureus* may limit the acquisition of *P.*

aeruginosa for some patients, and *P. aeruginosa* may aid the acquisition of *S. aureus* for others (Granchelli *et al*., 2018), however one study found that infection with *S. aureus* on top of chronic infection with *P. aeruginosa* may contribute towards preserving lung function (Hector *et al*., 2016). While it is clear that the 2 pathogens are capable of mutually co-existing in the lung, the dynamics of their relationship varies so much that further work needs to be done to fully understand it.

1.4.1.1 Virulence influence

Co-infection of *S. aureus* with chronic *P. aeruginosa* has been demonstrated to increase *S. aureus's* ability to become mucoid and increase its nuclease activity, thereby increasing IL-6 inflammatory markers and mucus viscosity in the lung (Wieneke *et al*., 2021). *P. aeruginosa* has also been shown to use *S. aureus* as an iron source, and produce an anti-staphylococcal agent called 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO) (Machan *et al*., 1992). A study investigating the effect of both pathogens on each other in regard to antibiotics, showed the production of HQNO by *P. aeruginosa* can decrease the sensitivity of *S. aureus* to antibiotics such as streptomycin and tobramycin (Hoffman *et al*., 2006). *P. aeruginosa* is also able to affect the ability of *S. aureus* to form biofilms *in vitro*, using Quorum sensing and HQNO, however this is strain dependent (Fugère *et al*., 2014).

1.4.1.2 Other interactions

Interactions between *Burkholderia* and other residential pathogens have been poorly studied, with focus mainly on the interaction between *Burkholderia* and *P. aeruginosa.* The two pathogens are thought to grow in mixed biofilm structures in the lung, though this has only been observed *in vitro* (Schwab *et al*., 2014). It was thought that pre-existing infection with *P. aeruginosa* increases the risk of co-infection with *Burkholderia* (Whiteford *et al*., 1995; Mc Closkey *et al*., 1998), however

with research now considering the complexity of polymicrobial infection, this is now being re-evaluated. Further study has shown that *P. aeruginosa* can form biofilms with *Burkholderia complex* members when competing for space, and there is evidence that *P. aeruginosa* can contribute to *B. cepacia* survival by inhibiting the host response (O'Brien and Fothergill, 2017). A paper by Hector *et al*., (Hector *et al*., 2016) found that multiple different pathogens co-infecting with *P. aeruginosa* resulted in a significant loss of lung function. While understanding the composition of the bacterial microbiota is useful, understanding the interactions and the roles of these interactions have in the progression of lung disease is imperative.

1.5 The Fungal microbiota

Fungal spores make up ≥50,000 spores per m^3 of air during their peak season, and are a constant in our microbiomes, present in both health and disease (Nguyen *et al*., 2015). As a result, the human lung is constantly exposed to large quantities of fungal spores throughout the year. Fungal spores are easily inhaled, and the warm and moist environment of the lung is perfect for colonisation (Chotirmall and McElvaney, 2014). Fungi can be dimorphic or polymorphic, taking on various forms depending on the environment, such as yeasts, fungal masses, multicellular filaments and molds (Goughenour and Rappleye, 2016). Of the ~100,000 known species of fungus, only a handful are capable of causing disease in humans, with mucosal and skin surfaces being their main targets (Iliev and Underhill, 2013).

1.5.1 The fungal microbiota in CF Lung Disease

Less than 0.1% of the microbiome is comprised of eukaryotes and protozoans (Qin *et al*., 2010). Over recent years, there has been a rise in interest into this largely ignored part of our microbiome, with studies showing that the intestine holds a

distinct population of fungi, with potentially as much diversity as its prokaryotic counterpart (Iliev *et al*., 2012; Hoffmann *et al*., 2013). The fungal microbiota has also been shown to hold the same amount of spatial variation across the lung as its bacterial counterpart, further demonstrating the complexity of this understudied area (Hogan *et al*., 2016). Despite these findings, the human lung fungal microbiota remains understudied when compared to the bacterial microbiota, with many fungal studies focussed on a particular taxon, such as *Candida albicans* or *Aspergillus fumigatus*. While these known pathogens are important, our understanding of fungi in the CF lung remains poor. Robust framework is in place for a variety of CF bacterial pathogens, for example the segregation of patients with certain infection statuses. This framework is not there for fungal infection (Magee *et al*., 2021), and it is unclear if the absence or presence of certain bacterial pathogens provide a niche for opportunistic fungi. Patients could also be responsible for their own fungal infections. It is known that poor nebuliser hygiene can lead to reinfection and bacterial contamination, with one study finding positive fungal isolates on nebuliser mouthpieces, 18% of which being *Aspergillus spp.* isolates (Peckham *et al*., 2016).

1.5.2 The role of fungi in CF

Fungi are frequently isolated from the lungs, however their role in population dynamics and what taxa make up the fungal community is poorly researched. CF patients are at higher risk of developing fungal disease, especially from filamentous fungi such as *Candida* and *Aspergillus*. Thick mucus provides a colonisation niche and some studies have even shown that standard CF treatments such as Tobramycin can encourage *Aspergillus* colonisation (Chotirmall and McElvaney, 2014; Garczewska, 2016). Like the bacterial microbiome, diversity reduces in the presence of disease (Krause *et al*., 2016; Tipton *et al*., 2017), and species such as

Candida and *Aspergillus* have been associated with pulmonary exacerbations (Soret *et al*., 2020), both of which have become emblematic pathogens for the fungal biome.

1.6 Emblematic fungal pathogens of Cystic Fibrosis

1.6.1 *Candida spp.*

Candida albicans is an opportunistic pathogenic yeast, that colonises the mucosal barrier of the lung (Richardson *et al*., 2019), and is found in up to 70% of healthy adults (Huffnagle and Noverr, 2013). The role of *Candida spp.* in the lung is disputed as despite being frequently isolated from lavages and the LRT samples and broad spectrum antibiotic use shown to increase prevalence, invasive infection is not frequently reported ((Iliev and Underhill, 2013; Krause *et al*., 2016). Five forms of *Candida* have been identified and associate with CF (Kim *et al*., 2015), but the majority of research focusses on *Candida albicans*. *C.albicans* is responsible for 95% of *Candida* based infection, with the remainder caused by *C. glabreta, C. parapsilosis* and *C. krusei. C. dubliniensis* (Chotirmall *et al*., 2010a). *C. dubliniensis* is also recovered from sputum samples and is known to provide false positives due to its similar characteristics to *C. albicans* (Sebti *et al*., 2001). While the true cause of pulmonary candidiasis is still to be investigated, mouse models and patient cases have demonstrated that long term antibiotic use is a common factor in facilitating *Candida's* ability to colonise (Noverr *et al*., 2004; Shweihat *et al*., 2015). There is also evidence to suggest that candidemia can be caused by other strains of *Candida* and not *C. albicans* as previously thought (Dutta and Palazzi, 2011).

Clearance of *C. albicans* infection involves the recruitment of neutrophils and alveolar macrophages, and one study found in mouse studies that alveolar macrophages could cause more harm than good, with macrophage depleted mice surviving longer than wild type models (Kubota *et al*., 2001). Despite this, *C. albicans* could also be a beneficial member of the fungal microbiota, as it is thought to play a role in regulating fungal levels and how the host responds to fungal colonisation (Huffnagle and Noverr, 2013). Research into the interaction of *C. albicans* with the bacterial microbiota is conflicting, with links to poorer lung function and worse outcomes, however it may play a role in the development of the lung microbiome, and may coordinate with persistent bacterial residents to form "climax communities" which appear to be more resistant to antibiotic use (Nguyen *et al*., 2015). Interestingly, the presence of *Candida spp.* in the gut has been shown to influence alveolar macrophage responses in the lung and contribute to allergic airway disease (Kolwijck and van de Veerdonk, 2014). *C. albicans* infection is treated with a short course of Fluconazole (Chotirmall *et al*., 2010), Caspofungin, Micafungin or Itraconazole (Limper *et al*., 2011).

1.6.2 *Aspergillus spp*

Aspergillus spp. is a soil mold ubiquitous to the environment and is easily cleared by alveolar macrophages in immunocompetent individuals. *A. fumigatus* is the most commonly encountered filamentous fungi colonizing patients with CF, with colonisation rates between 10% and 57% (Amin *et al*., 2010; Rougeron *et al*., 2014) and a further 10.9% of colonised patients developing allergic bronchopulmonary aspergillosis (ABPA) (Kim *et al*., 2015). Other species of *Aspergillus* that are known to cause disease are *A. flavus, A. niger, A. terreus, A. clavatus* and *A. nidulans* (van

de Veerdonk *et al*., 2017), which have correlations to increased hospitalisation, exacerbations, and a significant decrease in lung function (Amin *et al*., 2010).

Aspergillus prevalence is poorly reported, as many papers focus solely on patients with established *A. fumigatus* infection or ABPA, with one study concluding that there was “significant statistical heterogeneity and evidence of publication bias” (Maturu and Agarwal, 2015). (Armstead *et al*., 2014) collated the prevalence as reported by registry data across various countries, showing that prevalence is higher in patients in their late teens/early adulthood, and that there is an increase towards later life (55+).

1.6.3 *Exophiala dermatitidis*

E. dermatitidis is a ubiquitous, melanised, yeast like fungus. It is commonly found in soils and dead plant material, taking the form of grey/black colonies with a wool like appearance when grown on sabourand agar, and there has been an increase in infections in recent years (Suzuki *et al*., 2012). In 2004 isolation rates from CF patients for this fungus stood between 1.8 and 15.7% depending on geographical location (Horré *et al*., 2004b) and it has been identified as a potential pathogen. A cross-sectional study covering the Dutch Registry in 2020 found that *E. dermatitidis* prevalence was at around 2%, with patients more likely to be older and carry more severe genotypes (de Jong *et al*., 2020). *E. dermatitidis* is seen as a relatively harmless coloniser of the lung, however it can cause lung infection and fungal pneumonia (Kusenbach *et al*., 1992). Very little is known about its interactions with the host and other residential taxa (both bacterial and fungal) and its potential impact on disease progression requires further research.

1.6.4 *Scedosporium apiospermum*

S. apiospermum is a well-known human pathogen capable of causing respiratory illness in immunocompromised patients (Williamson *et al*., 2001; Guignard *et al*., 2008). It can be easily mistaken for *A. fumigatus* when analysed by microscopic morphology alone and can cause disease with symptoms that mimic *A. fumigatus* infection. *S. apiospermum* has a reported frequency of between 0.7%-9% amongst patients and is the second most commonly isolated filamentous fungi in CF (Cimon *et al*., 2000; Sedlacek *et al*., 2015). Treatment of *Scedosporium* infection is typically achieved with voriconazole, however it has been shown to readily re-infect despite maintaining susceptibility to the typical treatments (Goldman *et al*., 2016).

1.6.5 *Malassezia spp.*

Members of the *Malassezia spp.* are frequently found in fungal lung studies and are reported across multiple studies (Delhaes *et al*., 2012; Willger *et al*., 2014; Willger *et al*., 2014). They are the dominant pathogen in upper airway studies, however their prevalence in the lower airways appears to be much lower (Lee *et al*., 2020). *Malassezia spp.* is a known commensal of the human body, particularly of the skin (Theelen *et al*., 2018). There are 18 recognised species, thought to make up nearly 90% of the skin microbiome (Ianiri and Heitman, 2020) and are currently recognised as emerging infectious pathogens (Delhaes *et al*., 2012), with one study suggesting they could be responsible for pulmonary exacerbation in CF (Soret *et al*., 2020). Much of the research into *Malassezia* has been regarding skin conditions, however, this species is known to inhabit the CF lung, with studies demonstrating it can represent a significant portion of the fungal microbiota, and that severe phenotypes appear to have less (Nagano *et al*., 2010; Delhaes *et al*., 2012; Willger *et al*., 2014).

Despite this, little has been done to investigate the fluctuations over time or during treatment, and the impact it may have on disease progression is unknown.

1.7 Fungal impact on disease.

As with bacterial pathogens, chronic colonisation with fungal pathogens invariably results in a decline in lung function and increased pulmonary exacerbation. Few studies have been completed on the long-term implications of fungal infection, with *Candida spp.* and *A. fumigatus* infection at the forefront of these studies. Colonisation with *A. fumigatus* with ABPA is typically associated with a significant decrease in lung function and increased exacerbations (Baxter *et al*., 2013; Fillaux *et al*., 2014), and as such is a key investigative focus. Interestingly, a study on *Aspergillus spp.* in the COPD lung found that infection did lead to worse lung function, but that even when patients were sensitised, there was no significant decline (Bafadhel *et al*., 2014). This suggests that infection alone may not be sufficient to cause severe decline in some patients, or that there are other mechanisms or interactions at play that we don't fully understand. This is echoed by a several studies investigating CF lung function and chronic *A. fumigatus* infection that found no significant differences in lung function between infected and non-infected groups (Milla *et al*., 1996; de Vrankrijker *et al*., 2011).

A 16-year study in Sweden using culture to detect fungi in 133 patients found that lung function decline was associated with the persistent *C. albicans* and *Aspergillus.* Infection with *C. dubliniensis* was also associated with lung function decline (Al Shakirchi *et al*., 2020). Another 6-year study (also utilising culture detection), found that *C. albicans* infection significantly reduced %FEV_1 over time (Gileles-Hillel *et al*., 2015). It is clear that fungi have the capacity to significantly impact patient health,

however with conflicting data on the mechanisms used and the impact on lung function, this remains to be fully understood.

1.8 Fungal-Bacterial interactions

Attempts to find connections, correlations, and relationships between the fungal and bacterial components of the microbiome are far and few between. In lieu of longitudinal research on patients, research has been mainly focussed on cell: cell interactions *in vitro*, exploring how virulence, nutrient and niche competition are conducted (for example: (Hogan and Kolter, 2002) (McAlester *et al*., 2008; Reece *et al*., 2018)). There are a limited number of longitudinal studies with both bacterial and fungal biomes explored and compared, however from the few available, we do know that both communities experience a loss of diversity in exacerbation (Soret *et al*., 2020).

Very few correlations between bacterial and fungal communities have been found and reported correlations have little evidence to support them, for example the appearance of *C. albicans* alongside *P. aeruginosa* is repeatedly reported, however like *P. aeruginosa, C. albicans* is a chronically colonising pathogen, and as such this finding may be co-incidental (Delhaes *et al*., 2012). Studies outside of CF have investigated the bacterial and fungal microbiota, with one study finding no correlations between *Candida spp.* and the LRT microbiome in intensive care unit patients (Krause *et al*., 2016). Another study determined a rise in oral *Candida spp.* was linked to a rise in the relative abundance of saccharolytic species from the genera *Streptococcus*, *Lactobacillus*, and *Scardovia* (Kraneveld *et al*., 2012). Some studies on the bacterial and fungal oral microbiota in HIV patients have shown correlations between commonly occurring CF taxa (Mukherjee *et al*., 2014),

however whether these correlations occur in CF is yet to be determined. The genus' *Malassezia, Naganishia* and *Coniochaeta* are found in both the LRT and the sinuses and a study on sinus infections found that there are multiple correlations between these taxa and their bacterial counterparts that are also found in the LRT (Mackenzie *et al*., 2019). While these studies are often focussed on the oral biome, the upper and lower respiratory tract have some degree of overlap and therefore any relationships established here may also be apparent in other areas of the respiratory system.

1.8.1 Bacterial interactions with *Candida spp.*

Candida albicans is the most commonly isolated opportunistic fungal pathogen in the CF lung, with several studies demonstrating its interactions with the bacterial microbiota. *P. aeruginosa* has been shown to secrete selective molecules to kill *C. albicans.* A co-culture study demonstrated that *P. aeruginosa* had no interest in the yeast form of *C. albicans* however when presented with the filamentous form of *C. albicans,* it secreted selective molecules with the intent to kill (Hogan and Kolter, 2002). *C. albicans* was also reported to have a protective role in preserving lung function in a 10-year study, provided that that patient did not carry *P. aeruginosa* (Hector *et al*., 2016). *In vitro* testing on interactions between *P. aeruginosa* and *C. albicans* found that *C. albicans* “hides” from *P. aeruginosa* in a yeast form until *P. aeruginosa* stops producing cell signalling molecules, then switches to a filamentous form that could facilitate invasive growth (McAlester *et al*., 2008). This could indicate that *P. aeruginosa* could be acting in a protective role to ensure its niche, and by extension protecting the lung from fungal colonisation. *S. aureus* has been shown to preferentially attach to *C. albicans* and use it to enhance infection severity in mouse models (Harriott and Noverr, 2009), and *Burkholderia cenocepacia* has also

been shown to inhibit filament formation and thereby prevent *C. albicans* from switching form and colonising the space (Boon *et al*., 2008). These interactions demonstrate the complex nature of polymicrobial infections and communities, and better understanding will invariably aid in developing better treatments and monitoring.

1.8.2 Bacterial interactions with *Aspergillus spp.*

Aspergillus fumigatus rates have been shown to increase with age, antibiotic and steroid use, geography and colonisation with *S. maltophilia* (Amin *et al*., 2010). *Aspergillus* is frequently co-isolated with *P. aeruginosa* (Sass *et al*., 2019), and has associations with *S. aureus,* with repeated *Staphylococcus* treatments increasing the incidence of *Aspergillus* infection (Hong *et al*., 2018). *S. aureus* has been demonstrated to inhibit hyphal growth in *A. fumigatus,* and co-colonisation of *Aspergillus* and *P. aeruginosa* demonstrates a mutually antagonistic effect (Reece *et al*., 2018). *P. aeruginosa* has also been shown to inhibit *A. fumigatus* growth through a range of mechanisms, including the production of phenazine, which generates reactive oxygen species. Other secreted molecules alter *A. fumigatus'* cell wall structure, and *P. aeruginosa* has been demonstrated to starve *A.* fumigatus by storing and hiding iron (Keown *et al*., 2020).

1.9 Viruses in Cystic Fibrosis

Alongside bacterial and fungal infection, viruses are known to cause frequent acute infections. Human rhinovirus, Influenza types and A and B, and parainfluenza are commonly found in CF, hospitalising up to 50% of patients (Kiedrowski *et al.,* 2018), with seasonal variations found across the year (Banjar *et al.*, 2020). Viral infection presents with similar symptoms to bacterial and fungal infection, such as reduced

lung function, increased antibiotic use and hospital admission (Frickmann *et al.,* 2012). Viral infection causes significant tissue damage over the course of infection, pre-disposing patients to concurrent exacerbation due to pathogens such as *P. aeruginosa* and *S. aureus* (Armstrong *et al.*, 1998). Due to a lack of surveillance, many exacerbations caused by viruses are still attributed to bacterial infection, and while identification is becoming more routine, studies present conflicting results in terms of prevalence and severity of exacerbation (Meyer *et al.,* 2020).

1.10 Gaps in knowledge and thesis aims

1.10.1 The bacterial microbiota

While the bacterial microbiota has been under intense scrutiny for some time, there are some areas of research that are under-developed or ignored and could provide crucial understanding of the impact of the entire community on health. Research has put a significant amount of focus on the key disease-causing pathogens such as *P. aeruginosa* and *S. aureus,* and while undoubtedly useful, the role and impact of minor taxa, transient members of the community, and commensals are largely ignored, despite evidence to show their potential to impact on clinical outcomes.

1.10.1.1 Longitudinal analysis in mild patients

While longitudinal studies are useful, many focus on specific disease phenotypes, infection status or progression of disease (with a focus on moderate and declining disease states). Advancements in treatments and new CFTR modulation therapy is anticipated to gradually move patients towards more stable and milder CF phenotypes. Longitudinal patterns in milder states are not as clearly defined, and while there have been some attempts to define the “stable” microbiome, we have

yet to properly characterise it. With this in mind it is important that we also understand the milder forms of the disease in order to maintain these states.

1.10.1.2 Non-pathogenic taxa in the lung

While much of the focus has been rightfully on the key pathogens, there are notable fluctuations in other non-pathogenic taxa that may be key to disease progression. For example, *Prevotella* spp. is a commonly occurring member of the lung microbiome, who's precise role in CF lung is yet to be fully explored. It has been linked to milder disease and stable lung function, as well as the regulation of inflammatory responses, however higher loads have also been associated with higher inflammation of the airways (Larsen *et al*., 2012; Segal *et al*., 2013; Larsen, 2017). *Rothia mucilaginosa* is another commensal of the lung that has been suggested as a newly emerging CF pathogen (Guss *et al*., 2011). It is associated with anti-inflammatory mechanisms and has been found to have strains unique to CF (Lim *et al*., 2013). These taxa are often chronic colonisers and are considered commensals; however, whether they are friend or foe has yet to be determined.

1.10.2 The fungal microbiota

1.10.2.1 Fungal primer issues

One of the main issues across studies regarding the fungal microbiota is the lack of standardisation in terms of detection methods (Schwarz *et al*., 2018). A significant proportion of the large-scale fungal microbiota studies have utilised culture methods (for example: (Sudfeld *et al*., 2010; Güngör *et al*., 2013; de Dios Caballero *et al*., 2016; Engel *et al*., 2019; Al Shakirchi *et al*., 2020)), which are capable of picking up a range of fungal taxa, however VNBC (Viable but Non-Culturable) strains will not be picked up. NGS methods can detect a wider range of taxa, including the VBNC taxa in samples (Boers *et al*., 2019), however the ITS region is less conserved in

fungus than the 16S region in bacteria, meaning that there is more potential primer targets. This results in the potential exclusion of various taxa or phyla depending on which primer set is used (Tipton *et al*., 2017).

While there is an overlap between taxa discovered by both culture-dependent and –independent methods, there are some important differences that must be investigated. For example, *Candida* species are routinely found and easily detected by both methods, however potentially pathogenic taxa such as *Aspergillus* are routinely picked up by culture, but not by NGS depending on what primer targets are used (Nagano *et al*., 2010; Op De Beeck *et al*., 2014). Furthermore, if a primer target is used to ensure *Aspergillus* is detected alongside other taxa in samples, this may invariably result in another taxa being removed from detection due to primer bias. Analysing samples with a range of NGS primer targets and/or a range of targeted QPCR probes would provide a complete picture of the fungal community in a sample but the cost involved in just one of these analytical techniques prevents this from becoming a reality. The nature of the ITS region means that detection down to species level is sometimes unattainable. Choosing a primer target that includes the flanking regions of the ITS gene improves the depth to which taxa can be identified as these regions are slower to evolve and therefore more conserved than the ITS itself (Raja *et al*., 2017; Heeger *et al*., 2019).

While research into the fungal microbiota has good intentions, the lack of standardisation and research put into finding the best targets for identifying fungi in airways samples means that the data available is difficult to consolidate. There is also a large disparity in the reporting of patient demographics.

1.10.2.2 Composition of the fungal microbiota

While several studies have exclusively looked at the fungal aspect of the lung microbiome, the research available does little more than identify key pathogens and make a note of their abundance within a defined population. Fungi are poorly investigated in many areas of microbiome research, with CF being no exception. There are very few studies examining the longitudinal fluctuations of the fungal microbiota (Nguyen *et al*., 2015), and as *P. aeruginosa* is the poster boy of the bacterial world, *Candida spp.* and *Aspergillus spp.* take centre stage in the fungal world. While the importance of both is noted, much of the focus in CF research is set squarely on bacterial communities. As a result, while we know of the commonly occurring taxa, the exact frequency and role in disease is poorly understood, as well as how the community fluctuates over time and in response to treatment and exacerbation.

1.10.3 Clinical detection methods: is it time to switch it up?

The detection methods of pathogens in clinical samples has remained relatively unchanged for some time, despite advances in molecular detection methods becoming more widespread. Despite this, molecular methods are only employed when pathogens of particular interest are found during culture-based examination to confirm detection. While many studies have taken samples from the CF community and have explored the microbiome using molecular-based methods, there is little in the way of comparing the efficiency and efficacy of these methods against the detection methods used in clinic to inform treatments. We know that molecular methods are more sensitive and accurate than culture-based methods, and that detection procedures are often not sufficient to detect some medically important taxa, further impressing the importance of improving our methodology

(Pashley, 2014). Patients are regularly segregated based on clinical colonisation status, if pathogens have been missed in routine assessment, community transmission could be higher as a result, switching to a more accurate detection method could dramatically reduce this.

1.10.4 Fungal: Bacterial interactions.

While there have been some attempts to marry the bacterial and fungal microbiotas together, little has been discovered. As highlighted in section 1.8, some correlations have been noted between some fungal bacterial taxa, however these are not backed up by any other studies. It is inevitable that these two communities interact and communicate with each other, raising many questions such as; do certain bacterial taxa promote colonisation by certain fungus? Do certain bacterial taxa provide protection against certain fungi, and vice versa? Does the presence of certain fungi protect against lung function decline? Without further study and more importantly, longitudinal study, these questions remain unanswered.

1.10.5 Aims

The aim of this thesis is to investigate the bacterial and fungal components of the microbiome over a longitudinal dataset. The bacterial microbiota is well established in literature, however more work needs to be completed in longitudinal studies, particularly with mild patients and larger datasets. While clinical practice is centred around culture-based techniques, it is clear that the use of molecular-based methods in regular sampling may provide more accurate treatments, reduce pathogen spread across local CF populations and improve patient outcomes. In my first chapter I have explored the efficacy of culture-dependent methods, as routinely used in clinic, to accurately detect key CF pathogens compared to a targeted culture-independent approach. I have also explored the whole bacterial microbiota

in the same patients using Next Generation Sequencing, to observe the fluctuations and patterns over a 3-year period in mild adult and paediatric patients.

The fungal microbiota is relatively untouched and while efforts have been made to establish the key taxa, lack of standardisation and the absence of a robust primer set for fungal analysis has made this area of research difficult. For my final chapter, samples used in the bacterial microbiota study were used to further investigate the incidence and composition of fungi over a wide range of patients. The aim of this was to establish if the primer set fITS7/ITS4 was suitable for analysis of the fungal microbiota in the LRT, and to investigate the composition and fluctuations over the 3-year period.

Chapter 2: Methods and Materials.

2.1 Patient demographics and sampling

2.1.1 Patient recruitment

Patients from two different CF centres were recruited as part of a longitudinal study of adults and children with CF (Horsley *et al*., 2021). Adults were recruited from the Manchester Adult CF Centre, and children from the Royal Manchester Children's Hospital. Inclusion criteria required patients to be at least five years old, considered free of chronic *P. aeruginosa* infection as defined by the Leeds criteria (Lee *et al*., 2003), and have a %FEV_1 of >50% predicted at study entry (Table 2.1). Patients or parents/guardians provided written informed consent and children provided assent. This study was reviewed and approved by the NHS Research Ethic Committee North West, Lancaster (Ref 14/NW/1195).

2.1.2 Sample collection

Patients were assessed at their usual clinic appointments by their regular clinical team and included routine and emergency visits (Paediatric sampling: table 2.2 and Adult sampling: table 2.3). Molecular microbiology samples were transported to the lab within three hours and stored at -80°C prior to DNA extraction and PCR (Cuthbertson *et al*., 2014; Cuthbertson *et al*., 2015). Sputum samples were mixed and weighed prior to splitting and storage. To assess longitudinal microbiology, only patients with a minimum of six respiratory samples with full diagnostic microbiology data were included.

Table 2.1 Patient demographics for the adult and paediatric cohorts

Patient no.	No. samples	Mean age	Sex	Mean predicted $\%FEV_1$	Mild/ Moderate[a]	Genotype[b]	Pancreatic sufficiency	Longterm Azithromycin
				Paediatric				
201	8	13.9	F	84.4	Mild	2	N	N
203	9	8.0	F	100.2	Mild	1	N	Y
206	6	11.0	F	91.2	Mild	3	N	N
207	7	7.8	F	95.7	Mild	3	N	Y
212	6	10.7	F	83.9	Mild	1	N	N
213	7	7.9	M	82.3	Mild	1	N	Y
216	8	10.6	F	117.8	Mild	3	N	N
217	8	9.5	M	100.4	Mild	2	N	Y
218	6	12.2	M	89.6	Mild	1	N	Y
219	6	9.5	M	98.7	Mild	2	N	Y
223	6	9.9	M	110	Mild	2	Y	Y
225	6	13.5	F	91.6	Mild	1	N	Y
228	8	7.7	M	99.9	Mild	2	N	N
233	6	16.7	M	71.2	Mild	1	N	Y
235	7	16.1	M	86.8	Mild	1	N	Y
240	8	12.6	F	60.6	Moderate	1	N	Y
241	6	9.9	M	90.9	Mild	2	Y	N
242	8	7.9	F	74.7	Mild	2	N	Y
245	8	7.1	M	92	Mild	1	N	Y
246	8	10.9	F	91	Mild	3	Y	Y

				Adult				
101	20	24.2	M	64.2	Moderate	3	Y	Y
103	8	23.4	M	79.7	Mild	2	Y	Y
104	7	29.2	M	77.9	Mild	3	Y	Y
106	1	20.8	M	57.2	Moderate	1	N	Y
108	12	36.8	M	60.7	Moderate	1	N	Y
110	9	30.6	F	120	Mild	1	N	N
112	7	25.6	M	93.2	Mild	1	Y	Y
113	14	22.8	F	61.6	Moderate	2	N	Y
114	8	22.5	M	87.1	Mild	1	N	Y
115	7	22.4	F	47.9	Moderate	1	N	Y
116	7	25.8	M	99.6	Mild	3	Y	Y
118	10	27.3	F	73.8	Mild	2	N	Y
119	10	21.6	F	47.9	Moderate	3	N	Y
120	11	20.7	M	78.6	Mild	1	N	Y
121	6	29.7	M	92.9	Mild	2	Y	Y
132	7	24.7	F	105.7	Mild	2	N	Y
133	6	30.6	M	80.8	Mild	2	N	N
135	6	30.2	F	83.1	Mild	3	Y	N
140	12	19.2	M	47.3	Moderate	2	Y	Y
147	11	21.8	F	71.4	Mild	2	N	Y

[a] Mild Lung disease is defined as an %FEV_1 score of 70% and above, Moderate as between 40-69%.

[b] Genotypes are categorised as follows: 1=Homozygous Fdel508, 2=Heterozygous Fdel508, 3=Other and 4=Unknown.

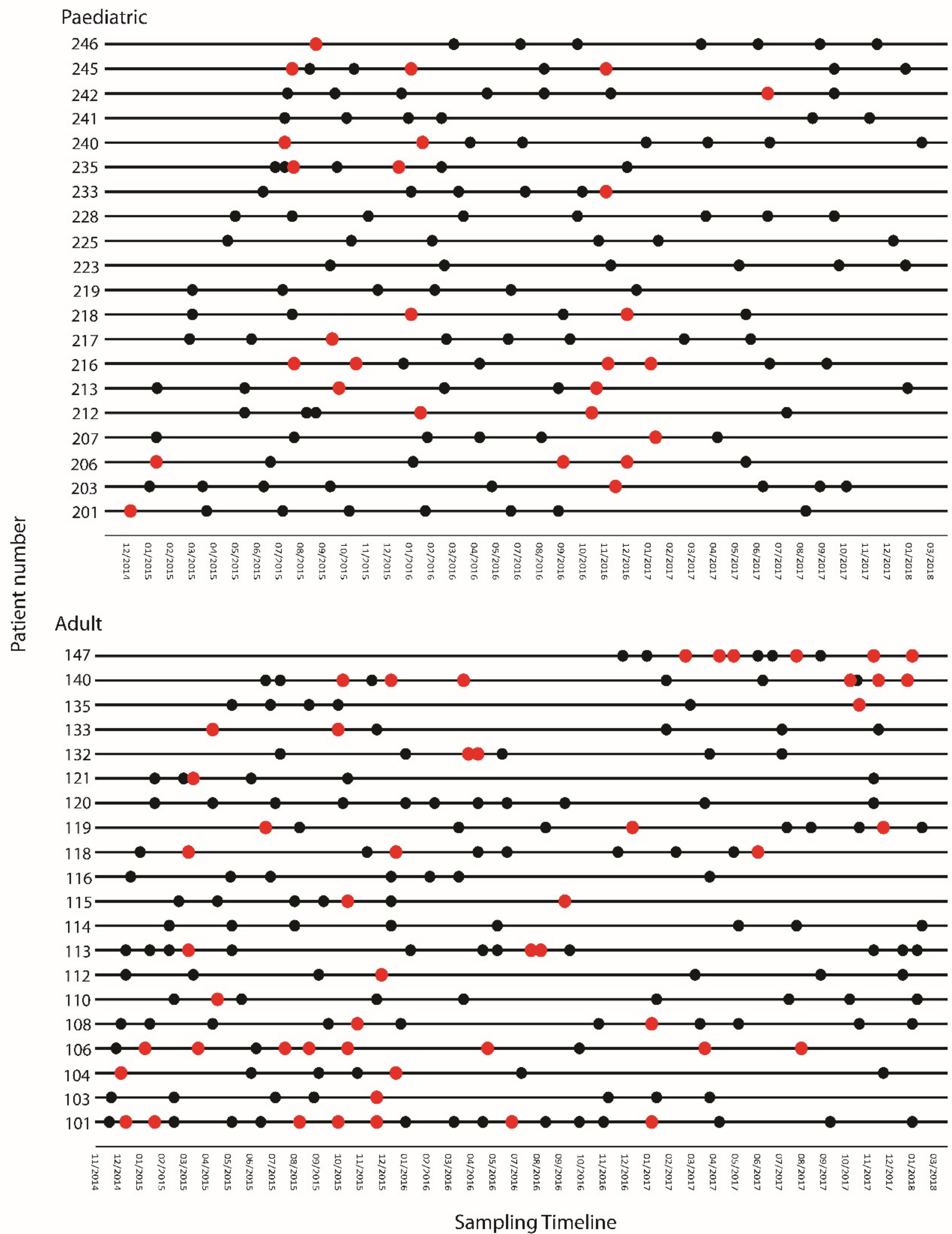

Figure 2.2 Sampling time line for all patients. The top graph illustrates the paediatric sampling, and the bottom graph the adult patients. Samples in black were taken during clinically stable periods, and smaples in red were taken at time of exacerbation.

2.2 Diagnostic microbiology

Diagnostic culture-based bacterial microbiology data for all samples were provided by the microbiology service within Manchester University NHS Foundation Trust who perform microbiology testing for the participating centres, in line with international guidance and standards (Smyth *et al*., 2014; Public Health England, 2015). Full mycology was also provided by the Trust, which performs mycology for participating centres according to full ISO and accreditation from the Mycology Reference Centre (Manchester University NHS Foundation Trust, 2020)

2.3 Sample preparation

Sputum was washed in and equal volume of sterile PBS (i.e. 500 mg sputum in 500 µl PBS) to minimise oral contaminants introduced during collection (Rogers *et al*., 2006). Sputum-PBS mixtures were centrifuged for 5 minutes at 4109 x *g*. The supernatant was discarded, and the pellet was resuspended in fresh PBS up to 500µl. Swabs were placed in a 1.5 ml microcentrifuge tube and trimmed to fit. A total of 500µl PBS was added and the swab soaked for 10 minutes, after which the swab was squeezed with sterile tweezers to recover the maximum volume of bacterial suspension.

2.4 DNA Extraction

DNA was extracted based on a protocol previously described in Rogers *et al.* (2003). Washed sputum and swab recovered bacterial suspension was transferred to a clean microcentrifuge tube containing 300µl Guanidium Thiocyanate-EDTA-Sarkosyl (Guanidine thiocyanate [Merck, Dorset, UK], EDTA [Thermo Fisher, UK], Sarkosyl [Merck, Dorset, UK], and 0.2 (±0.1) g 0.5mm glass beads (Merck, Dorset, UK). All

samples underwent a bead-beating (mechanical lysis) step on a Retsch Mixer Mill MM 400 (Retsch, Dusseldorf, Germany) for two cycles of 30 seconds at 17.5 m/s. Following the mechanical lysis, the samples were subjected to thermal lysis steps through incubation at 80°C for 3 minutes, rapid cooling and storage at -20°C for 5 minutes. Centrifugation for 10 minutes at 13,000 x *g* allowed the supernatant to be transferred to a new microcentrifuge tube containing 141µl 5M NaCl (final concentration 0.5 mM) (Fisher Scientific, UK) and 374µl PEG 6000 (final concentration of 15%) (Merck, Dorset, UK), which was mixed and left to incubate for 15 minutes at 4°C to allow for the precipitation of DNA during a further centrifugation step at 13,000 x *g* for 5 minutes and the supernatant discarded. The pellet was resuspended in 500µl sterile water to which 500µl Phenol (Thermo Fisher, UK) was added for an initial clean-up step. The sample was mixed, prior to centrifugation at 13,000 x *g* for 5 minutes, and the top (organic) layer was then transferred into a new microcentrifuge tube and mixed with an equivalent volume of phenol: chloroform (1:1) was added (Chloroform, Thermo Fisher, UK). Samples were centrifuged at 13,000 x *g* for 5 minutes and the organic layer containing purified DNA was transferred to a new microcentrifuge tube, where 500µl of Isopropanol (Fisher Scientific, UK): 5M ammonium acetate (10:1 v/v) and 1µl of linear polyacrylamide (5mg/ml, Thermo Fisher, UK) was added. The sample was mixed and stored at -20°C for an hour, pelleted at 13,000 *x g* for 10 minutes and the supernatant discarded. The pellet was twice washed in 500µl 70% ethanol (Fisher Scientific, UK) and left to dry. The dried pellet was then resuspended in 50µl sterile water and stored at -20°C until needed.

2.5 Positive controls

To confirm the identification of taxa using molecular methods, emblematic bacterial and fungal strains found in Cystic Fibrosis were used as positive controls throughout the sequencing process and qPCR protocols to ensure correct amplification of target genes, and to ensure correct identification of key strains. All strains have been determined as clinically relevant in the literature (Elborn, 2016; Cystic Fibrosis Trust, 2019; Cystic Fibrosis Foundation, 2020). Bacterial strains were grown on Lysogeny Broth (LB) agar (Thermo Fisher, UK) for 48h at 37°C, and fungal strains were grown on Potato Dextrose (PD) Agar (Merck, Dorset, UK) for 72h at varying temperatures depending on strain (Table 2.4). Colonies established on agar were then transferred to broth (LB for bacterial, PD for fungal) and grown overnight. Bacterial cultures were grown for 24h at 37°C with shaking at 100 rpm, fungal cultures were grown over 3 days at varying temperatures depending on strain (Table 2.4).

Liquid cultures were then centrifuged at 3000 rpm for 10 minutes and the supernatant removed. All centrifugation steps were performed at room temperature unless otherwise stated. The pellet was resuspended in 500µl of sterile Phosphate Buffered Saline (PBS) (PBS tablets, [Sigma-Aldrich, UK]) ready for extraction. Where bacterial strains were used for qPCR, the pellet was resuspended in 1ml of sterile PBS and grown in tenfold dilutions on LB agar overnight at 37°C. The remaining solution was used immediately for DNA extraction.

Table 2.2 Bacterial and Fungal strains used for sequencing confirmation and QPCR standards. Pure Bacterial strains were acquired from Public Health England Culture Collection (PHE). Pure Fungal strains were acquired from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) and PHE.

Strain	Source	Use	Growth temperature (°C)	Confirmed by sequencing?
		Bacterial Strains		
Pseudomonas aeruginosa	PHE	Sequencing control/ qPCR standard	37	Y
Staphylococcus aureus	PHE	Sequencing control/ qPCR standard	37	Y
Haemophilus influenzae	PHE	Sequencing control	37	Y
Burkholderia multivorans	PHE	Sequencing control	37	Y
Burkholderia cenocepacia	PHE	Sequencing control	37	
Stenotrophomonas maltophilia	PHE	Sequencing control	35	Y
Pandoraea sputorum	PHE	Sequencing control	37	Y
Ralstonia mannitolytica	PHE	Sequencing control	30	N
		Fungal strains		
Candida albicans	PHE	Sequencing control	37	Y
Aspergillus fumigatus	DSMZ	Sequencing control	30	N
Saccharomyces cerevisiae	DSMZ	Sequencing control	25	Y
Scedosporium apiospermum	PHE	Sequencing control	32	N
Rhodotorula mucilaginosa	DSMZ	Sequencing control	25	Y
Zymo gut control	Cambridge Bioscience	Sequencing control	N/A	Y

2.6 PCR for sequencing

Amplification of DNA was achieved using a 2-step protocol and Illumina tag primers (Illumina, 2014). Bacterial sequencing targeted the V5-V6 region of the 16S rRNA gene, using forward primer 16SIAF (5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTG GATTAGATACCCTGGTA) and reverse primer 16SIAR (5'-AGACGTGTGCTCTTCC GATCTCGACRRCCATGCANCACCT). For Fungal sequencing, the fITS7f and ITS4r (Ihrmark *et al*., 2012) (5'- ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCCTCC GCTTATTGATATGC and 5'- AGACGTGTGCTCTTCCGATCTGTGARTCATCGAATC TTTG, forward and reverse, respectively) were used for the first step PCR (Invitrogen, UK). Samples were processed in a 50µl reaction comprising of: 0.5µl Q5 Taq Polymerase, 10µl 5x reaction buffer (New England Biolabs, Massachusetts, USA), 1µl dNTPs (Invitrogen, UK), 0.5µl each of forward and reverse primer @100uM and 35.5µl molecular grade water (Thermo Fisher, UK). Bacterial PCR underwent a 25 cycle PCR, while Fungal PCR underwent a 35 cycle PCR (Table 2.5). This resulted in a 550bp amplicon for 16S rRNA gene and 350–425bp amplicon for Internal Transcribed Spacer (ITS). PCR products were verified with 2% w/v TAE (Tris-acetate-EDTA) agarose gel electrophoresis containing Sybersafe gel stain (Invitrogen, UK; 1:10,000 dilution in 1x TAE buffer). Gels were viewed using the LI-COR Odyssey (LI-COR, LI-COR Biosciences, Ltd. UK).

First step PCR products were cleaned using the Zymo ZR-96 DNA clean up kit (Cambridge Bioscience, Cambridge, UK), and were then given a unique barcode (Table 2.6). Samples were processed in a 50µl reaction comprising of 10µl 5x Reaction Buffer, 1µl dNTPs, 5µl of Barcode array, 0.5µl Q5 Taq Polymerase, 20µl of

Table 2.3 PCR conditions for 1st and 2nd step PCR.

Initial amplification of bacterial and fungal have different cycle numbers to maximise the amount of fungal DNA captured in the first stage.

PCR 1 (Initial amplification)			
	Stage	**Temperature**	**Time**
	Denaturation	98°c	30sec
25 cycles for Bacteria, **35 cycles for Fungi.**	Annealing	98°c	10sec
	Amplification	50°c	10sec
	Elongation	72°c	20sec
	Elongation	72°c	10mins
PCR 2 (Barcoding)			
	Stage	**Temperature**	**Time**
	Denaturation	98°c	30sec
10 cycles	Annealing	98°c	10sec
	Amplification	62°c	20sec
	Elongation	72°c	30sec
	Elongation	72°c	2mins

Table 2.4 Barcode sequences used to identify samples.

Barcodes were diluted to a final concentration of 10uM, and made into four plates of unique sequence mixes labelled A-D:

A: F.SA and R.SA B: F.SB and R.SA
C: F.SB and R.SB D: F.SA and R.SB

Barcode Name	Sequence	Barcode Name	Sequence
ITR.SA701	CAAGCAGAAGACGGCATACGAGATAACTCTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SA501	AATGATACGGCGACCACCGAGATCTACACATCGTACGACACTCTTTCCCTACACGACG
ITR.SA702	CAAGCAGAAGACGGCATACGAGATACTATGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SA502	AATGATACGGCGACCACCGAGATCTACACACTATCTGACACTCTTTCCCTACACGACG
ITR.SA703	CAAGCAGAAGACGGCATACGAGATAGTAGCGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SA503	AATGATACGGCGACCACCGAGATCTACACTAGCGAGTACACTCTTTCCCTACACGACG
ITR.SA704	CAAGCAGAAGACGGCATACGAGATCAGTGAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SA504	AATGATACGGCGACCACCGAGATCTACACCTGCGTGTACACTCTTTCCCTACACGACG
ITR.SA705	CAAGCAGAAGACGGCATACGAGATCGTACTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SA505	AATGATACGGCGACCACCGAGATCTACACTCATCGAGACACTCTTTCCCTACACGACG
ITR.SA706	CAAGCAGAAGACGGCATACGAGATCTACGCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SA506	AATGATACGGCGACCACCGAGATCTACACCGTGAGTGACACTCTTTCCCTACACGACG
ITR.SA707	CAAGCAGAAGACGGCATACGAGATGGAGACTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SA507	AATGATACGGCGACCACCGAGATCTACACGGATATCTACACTCTTTCCCTACACGACG
ITR.SA708	CAAGCAGAAGACGGCATACGAGATGTCGCTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SA508	AATGATACGGCGACCACCGAGATCTACACGACACCGTACACTCTTTCCCTACACGACG
ITR.SA709	CAAGCAGAAGACGGCATACGAGATGTCGTAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SB501	AATGATACGGCGACCACCGAGATCTACACCTACTATAACACTCTTTCCCTACACGACG
ITR.SA710	CAAGCAGAAGACGGCATACGAGATTAGCAGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SB502	AATGATACGGCGACCACCGAGATCTACACCGTTACTAACACTCTTTCCCTACACGACG
ITR.SA711	CAAGCAGAAGACGGCATACGAGATTCATAGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SB503	AATGATACGGCGACCACCGAGATCTACACAGAGTCACACACTCTTTCCCTACACGACG
ITR.SA712	CAAGCAGAAGACGGCATACGAGATTCGCTATAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SB504	AATGATACGGCGACCACCGAGATCTACACTACGAGACACACTCTTTCCCTACACGACG
ITR.SB701	CAAGCAGAAGACGGCATACGAGATAAGTCGAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SB505	AATGATACGGCGACCACCGAGATCTACACACGTCTCGACACTCTTTCCCTACACGACG
ITR.SB702	CAAGCAGAAGACGGCATACGAGATATACTTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SB506	AATGATACGGCGACCACCGAGATCTACACTCGACGAGACACTCTTTCCCTACACGACG
ITR.SB703	CAAGCAGAAGACGGCATACGAGATAGCTGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SB507	AATGATACGGCGACCACCGAGATCTACACGATCGTGTACACTCTTTCCCTACACGACG
ITR.SB704	CAAGCAGAAGACGGCATACGAGATCATAGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ITF.SB508	AATGATACGGCGACCACCGAGATCTACACGTCAGATAACACTCTTTCCCTACACGACG
ITR.SB705	CAAGCAGAAGACGGCATACGAGATCGTAGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		
ITR.SB706	CAAGCAGAAGACGGCATACGAGATCTCGTTACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		
ITR.SB707	CAAGCAGAAGACGGCATACGAGATGCGCACGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		
ITR.SB708	CAAGCAGAAGACGGCATACGAGATGGTACTATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		
ITR.SB709	CAAGCAGAAGACGGCATACGAGATGTATACGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		
ITR.SB710	CAAGCAGAAGACGGCATACGAGATTACGAGCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		
ITR.SB711	CAAGCAGAAGACGGCATACGAGATTCAGCGTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		
ITR.SB712	CAAGCAGAAGACGGCATACGAGATTCGCTACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		

purified DNA, and 13.5µl molecular grade water. Barcoded samples were then cleaned using the SequalPrep Normalisation Plate Kit (Invitrogen, UK). The cleaned library was then pooled and vacuum condensed using a Genevac miVac DNA (SP Industries, Philadelphia, USA) to a final volume of 50µl. The final library was then stored at -20°c until needed.

2.7 Sequencing

The library was processed in accordance with the MiSeq Denature and Dilute Guide (Illumina, 2019). The library was normalised to 4nM, and 5 µl was denatured with 5 µl 1nM NaOH for 5 minutes at room temperature and 5 minutes at 95°C. Ice-cold HT1 buffer (Illumina, Cambridge, UK) (900 µl) was immediately added and the resulting 20pM library was stored on ice. The final library was comprised of the denatured library (ranging from 8-12pM depending on library), and a 5% (bacterial) or 10% (fungal) PhiX spike.

2.8 Quantitative PCR

Targeted quantitative PCR (qPCR) was carried out for *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Standard curves were created using pure strains grown in potato dextrose broth (Merck, Dorset, UK) and serially diluted before extraction to provide known quantities of DNA for a given CFU/ml. These were then used as a positive control and a standard for each plate of samples to ensure technical reproducibility. Primers and probes for *P. aeruginosa* were chosen from (Feizabadi *et al*., 2010; Rogers *et al*., 2010), and primers/probes for *S. aureus* were chosen from (Alarcón *et al*., 2006) (Table 2.7) and samples were processed in a 20 µl reaction (Table 2.8) using TaqMan Gene Expression Mastermix (Thermo fisher, UK).

Table 2.5 qPCR Primers and Probes

Target species	Target gene	Sequence		References
P. aeruginosa	*OprL*	Forward	CGAGTACAACATGGCTCTGG	(Feizabadi *et al.*, 2010); (Geraint B. Rogers *et al.*, 2014)
		Reverse	ACCGGACGCTCTTTACCATA	
		Probe	FAM–CCTGCAGCACCAGGTAGCGC-TAMRA	
S. aureus	*nuc*	Forward	CGCTACTAGTTGCTTAGTGTTAACTTTAGTTG	(Alarcón *et al.*, 2006)
		Reverse	TGCACTATATACTGTTGGATCTTCAGAA	
		Probe	FAM-TGCATCACAAACAGATAACGGCGTAAATAGAAG-TAMRA	

Table 2.6 qPCR protocols

<table>
<tr><th colspan="3">*P. aeruginosa* (oprL) FAM-TAMRA – 117bp</th><th colspan="3">*S. aureus* (*nuc*) FAM-TAMRA – 124bp</th></tr>
<tr><td colspan="6">**Mastermix**</td></tr>
<tr><td colspan="2">Forward primer (100uM)</td><td>0.2µl</td><td colspan="2">Forward primer (100uM)</td><td>0.06µl</td></tr>
<tr><td colspan="2">Reverse primer (100uM)</td><td>0.2µl</td><td colspan="2">Reverse primer (100uM)</td><td>0.06µl</td></tr>
<tr><td colspan="2">Probe (100uM)</td><td>0.2µl</td><td colspan="2">Probe (100uM)</td><td>0.02µl</td></tr>
<tr><td colspan="2">DNA</td><td>2µl</td><td colspan="2">DNA</td><td>2µl</td></tr>
<tr><td colspan="2">Mastermix</td><td>20µl</td><td colspan="2">Mastermix</td><td>10µl</td></tr>
<tr><td colspan="2">Water</td><td>17.4µl</td><td colspan="2">Water</td><td>7.86µl</td></tr>
<tr><td colspan="6"></td></tr>
<tr><td colspan="6">**QPCR Protocol**</td></tr>
</table>

<table>
<tr><td colspan="2">Stage</td><td>Time</td><td>Temperature</td><td colspan="2">Stage</td><td>Time</td><td>Temperatu re</td></tr>
<tr><td colspan="2">Initial Denaturation</td><td>10 min</td><td>95ºc</td><td colspan="2">Initial Denaturation</td><td>10 min</td><td>95ºc</td></tr>
<tr><td rowspan="3">40 cycles</td><td>Denaturation</td><td>10 sec</td><td>95ºc</td><td rowspan="3">40 cycles</td><td>Denaturation</td><td>30 sec</td><td>95ºc</td></tr>
<tr><td>Annealing</td><td>30 sec</td><td>58ºc</td><td>Annealing</td><td>30 sec</td><td>60ºc</td></tr>
<tr><td>Elongation</td><td>1 min</td><td>72ºc</td><td>Elongation</td><td>1 min</td><td>72ºc</td></tr>
</table>

2.9 Sequencing and Data analysis

2.9.1 Bacterial sequencing analysis

Analysis of raw sequence data was performed through the DADA2 pipeline (Callahan *et al*., 2016) using R (R Core Team, 2020). For bacterial data, an Operational Taxon Unit (OTU) table was generated by trimming raw sequence files to remove erroneous, poor-quality reads, and chimeras. All sequences were putatively assigned species level identification using an appropriate database, first by using the GTDB (Chaumeil *et al*., 2019) database, then any remaining non-assigned sequences were then ran manually through BLAST (Altschul *et al*., 1990) to ensure all possible sequences were identified. Multiples assigned to the same genus and species were then condensed to create the final OTU. Given the varying length of the sequences analysed, these identifies should be considered putative. A sequence match of 97% or more when ran through the databases was required for identification.

2.9.2 Fungal sequencing analysis

Due to the less conserved nature of fungal DNA, the raw sequences were subject to further processing to extract useful data. Reads were processed using the Dada2 package to determine the presence of the primer sequences, then transferred to the Cutadapt (Martin, 2011) programme in Ubuntu (Version: 20.04). Cutadapt searches through raw sequences and finds the target primer sequences in all orientations, and cuts them from the sequence, leaving only the relevant data behind. Cutadapt also removed sequences shorter than 100bp. The processed reads were then transferred back to the Dada2 pipeline and processed to quality check the reads and remove chimeras in the same way the bacterial reads were. Final assignment

of taxonomy was achieved using the UNITE database. Like with the bacterial assignment, a 95% or more match was required for assignment.

Contamination of extraction controls required careful examination to determine impact on the results. First, all reads present in the blanks but not the samples were removed, and the true reads condensed. The remaining reads present in both the blanks and the samples were then analysed using a Wilcoxon signed rank test, to determine if the reads in the process blanks effected the true reads in the samples. Those that had a significant result ($p=<0.05$, where the sample reads were significantly higher than the control) were kept and those without were removed from analysis.

For both sets of data, any identified sequences not thought likely to be found in humans were removed from the dataset.

2.9.3 Statistical analysis

Statistical analysis was performed in Microsoft Excel using the XLSTAT package (Data Analysis and Statistical Solution for Microsoft Excel, Addinsoft, France) and PAST (ver. 3.26) (Hammer-Muntz *et al*., 2001). Unknown bacterial and fungal OTUs will be referred to as taxa, assigned OTU's will be referred to as species. Details of data processing and statistical tests used throughout the study are detailed below:

2.9.3.1 Distribution abundance relationships (DAR) and partitioning

The application of ecological measures of persistence and abundance has revealed patterns in microbiome studies in terms of spatial abundance distribution over time. This measure, when applied to temporal data, can illustrate community structure and highlight the chronically colonising and intermittent taxa in the samples available (Magurran and Henderson, 2003; van der Gast *et al*., 2011). For this study,

partitioning of the microbiome into chronic and intermittent taxa was based on the infection status criteria set out by Lee *et al*., (Lee *et al*., 2003). In this criteria, if more than 50% of samples are positive over a 12-month period the infection is chronic, and where 50% or less are positive, the infection is intermittent. This criteria was applied to the 3-year period rather than the 12-month period, in order to determine which taxa are persistent members of the community in each patient.

2.9.3.2 Diversity Measures

To determine the diversity, community composition, and similarity between samples, several analyses were performed on the raw OTU tables for all samples. Performed in PAST (Paleontological Statistics Software Package for Education {Hammer *et al.,* 2001}); alpha diversity, SIMPER analysis and Bray Curtis/Sorenson Dice dissimilarity measures were extrapolated from the data.

2.9.3.3 Alpha diversity

To determine the diversity of the samples in comparison with each other and to observed how diversity fluctuated over time, Fisher's alpha scores were determined for each sample. In order to determine how samples differ to each other over time, diversity is a key measure. Fishers' alpha is particularly useful in this setting as it is not influenced by sample size and provides a simple measure of the distribution of species within a sample (Schulte *et al*., 2005; Thukral, 2017).

2.9.3.4 Community composition (SIMPER)

Community composition and the relative contributions taxa play in the differences between patients is an important measure of which species actively contribute towards inter-patient variability. Using PAST, SIMPER analysis was conducted on the raw OTU tables to measure the relative contributions of taxa between adult and paediatric patients, as well as between patients within those groups. SIMPER is a

useful analysis in determining dissimilarity between groups based on Bray-Curtis dissimilarity, and the relative contribution of individual taxa (Clarke and Ainsworth, 1993). However useful this may be in extracting over-arching trends from data and identifying taxa that mark the differences between groups, SIMPER may produce false positives, as it is unable to determine what may be the result of high intra-sample variability (Warton *et al*., 2012). Due to this, any significant results produced by SIMPER were confirmed by T-test. For example, a taxon thought to be causing significant difference between groups may be due to intra-patient variability rather than a true difference, mean values for all patients will be taken and compared using a T-test to determine if SIMPER was influenced by high read numbers. It could be that the group with the higher read number has several patients that have a significant amount of "x" taxa, where others don't. This measure was used to identify potential key taxa, and any significant results were investigated further to confirm their role in dissimilarity between groups.

2.9.3.5 Community change between samples.

To assess compositional changes between samples, levels of turnover between samples was measured using the method described by Brown and Kodric-Brown (Brown and Kodric-Brown, 1977). Turnover was calculated as $t=b+c/S_1+S_2$. Where b=the number of unique taxa present in the first sample; c=the number of unique taxa in the second sample; S_1=the total taxa in the first sample; and S_2 the total taxa in the second sample. Using the turnover measure, we were able to show how much of the previous microbiota composition was retained through time, across the whole microbiota, the chronic and intermittently colonising taxa.

To further explore these differences, samples were manually compared to each other sequentially and the loss, gain and retention of individual taxa was noted. This

allowed exploration of the community composition that lead to the turnover values seen in the previous analysis.

2.9.3.6 Correlations

To determine if measures such as diversity or turnover were correlated with clinical measures of health, or to determine correlations between taxa, mean values of each measure to be tested were generated per patient and compared against each other using Spearman's Rank correlation in PAST. Spearman's was employed in this analysis as it is a non-parametric technique that is unaffected by the distribution of the samples (Gauthier, 2001).

2.9.3.7 Comparisons between groups.

To compare measures between groups, for example between adult and paediatric patients; mean values of each measure, per patient, were compared using a Welch's T-test. Non-parametric testing was used across the these, as while both groups are equal in size (20 adult and 20 paediatric patients), the data extrapolated from them are from non-parametric sources (Ling *et al*., 2021).

Chapter 3: Microbial culture underestimates lung pathogen detection and infection status in Cystic Fibrosis

This Chapter is provisionally accepted in the journal *Microbiology Spectrum*. The chapter is presented in submitted manuscript format.

Gavillet, H., Hatfield, L., Rivett, D., Jones, A., Maitra, A., Horsley, A., van der Gast, C. *Greater than anticipated levels of chronic lung infection found in paediatric and adult cystic fibrosis patients*. Microbiol Spectr, 2022. In review.

3.1 Introduction

Cystic fibrosis (CF) is characterised by chronic airway infection and consequent inflammation (Pillarisetti *et al*., 2011; Elborn, 2016). This starts early in life, most commonly with *Staphylococcus aureus* and *Haemophilus influenzae* infection. As time progresses, CF microbiology evolves and becomes increasingly dominated by a small number of Gram-negative pathogens rarely encountered in immunocompetent hosts. Chief amongst these is *Pseudomonas aeruginosa*, which increases in prevalence in adolescence and is cultured from sputum in over half of all adult CF patients (Cystic Fibrosis Foundation, 2019; Cystic Fibrosis Trust, 2019). Other important pathogens isolated in CF include *Achromobacter* spp.*, Stenotrophomonas maltophilia,* and members of the *Burkholderia cepacia* complex.

Clinical diagnosis of microbial infection relies on microbiological culture as the gold standard for pathogen detection (Public Health England, 2015). Regular microbiological surveillance throughout the life of a CF patient is considered best practice (Smyth *et al*., 2014). This is used to guide targeted treatment, provides an indication of the effectiveness of treatment against pre-existing infection, and enables discovery of recently acquired infections to allow timely eradication (Pattison *et al*., 2013; Smyth *et al*., 2014). Following diagnosis of acute pulmonary exacerbation, further regular surveillance for the duration of treatment is recommended to direct antibiotic therapy (Smyth *et al*., 2014). CF patients are typically categorised by infection type and attend segregated outpatient clinics to prevent cross-infection between patient cohorts colonised with, for example, *P. aeruginosa* and *B. cepacia* complex members (Doe *et al*., 2010; Smyth *et al*., 2014).

Over the last decade, culture-independent molecular microbiology techniques have become increasingly investigated. These methods are more sensitive than culture and are able to identify infections which are harder to detect using traditional

microbiological culture alone (Stewart, 2012). As such, molecular-based approaches like quantitative polymerase chain reaction (QPCR) have been increasingly proposed as alternatives to culture (Pattison *et al*., 2013; Burns and Rolain, 2014). QPCR is well suited to this application, offering targeted organism identification and quantification with inherent high-target specificity and sensitivity, and can be performed on small amounts of material (as found in cough swabs) (Pattison *et al*., 2013). Despite many cross-sectional studies reporting on improved sensitivity using QPCR, little progress has been made in establishing such molecular techniques in clinical care, and there are few longitudinal studies (see Supplementary materials and Table S3.1).

In the current study, respiratory samples from paediatric and adult CF patients were prospectively collected over a period of up to three years. These were all patients believed to be free of chronic *Pseudomonas* infection by culture prior to start of study. The aim of the study was to longitudinally compare the diagnostic gold standard of microbiological culture with QPCR for detection of two prominent pathogens of concern, *P. aeruginosa* and *S. aureus*; both of which are deemed to be readily detected by culture (Public Health England, 2015). Additionally, we assessed the impact of the resulting disparity in detection methods on clinical classification. The clinical implications for CF and for pathogen detection and surveillance more broadly are discussed.

3.2 Materials and methods

3.2.1 Study and patient sampling

Patients were recruited as part of a longitudinal observational study of adults and children with CF cared for at two different CF centres (Horsley *et al*., 2021). Adults were recruited from the Manchester Adult CF Centre, and children from the Royal

Manchester Children's Hospital. Patients were required to be at least five years old, with a %FEV_1 of >50% predicted at study entry. Patients were eligible if considered free of chronic infection with *Pseudomonas aeruginosa* using diagnostic culture approaches and as defined by the Leeds criteria (Lee *et al*., 2003). Patients or parents/guardians provided written informed consent and children provided assent. This study was reviewed and approved by the NHS Research Ethic Committee North West, Lancaster (Ref 14/NW/1195).

Patients were assessed at their usual clinic appointments by their regular clinical team and included routine and emergency visits. Spirometry was performed by the usual clinical team. Normal ranges for spirometry were those from the Global Lung Initiative (Quanjer *et al*., 2012). At study entry, 85% of adults (17/20) were on long-term azithromycin treatment, while one patient (patient 110) was receiving long-term colistin and the two remaining patients (133 & 135) were not prescribed any long-term antibiotics. Seventy percent of paediatric patients (14/20) were also on long-term azithromycin, the remaining six (201, 206, 212, 216, 228 & 241) were not prescribed any long-term antibiotics. Sputum or cough swab samples were taken at each clinic visit for diagnostic and molecular microbiology. Molecular microbiology samples were transported to the lab within three hours and stored at -80°C prior to DNA extraction and PCR (Cuthbertson *et al*., 2014; Cuthbertson *et al*., 2015). Sputum samples were mixed and weighed prior to splitting and storage. To assess longitudinal microbiology, only patients with a minimum of six respiratory samples along with accompanying full diagnostic microbiology data were included (Figure 3.1 and Table 3.1).

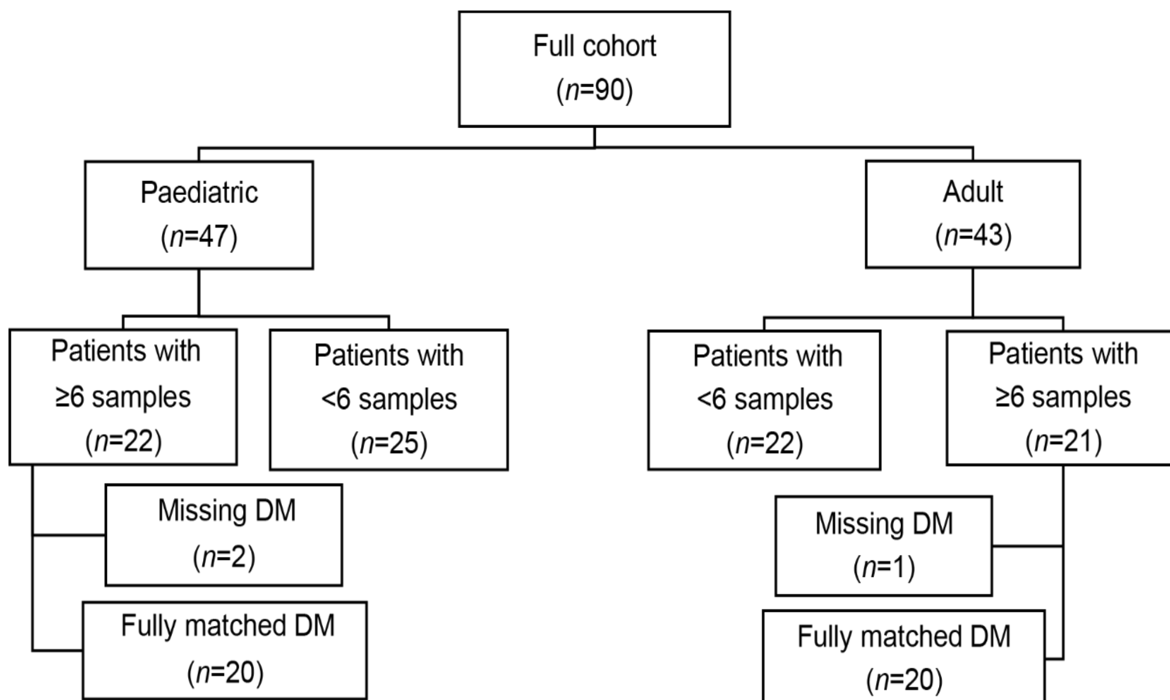

Figure 3.1 Flow diagram detailing patient selection process. Only patients who contributed ≥6 samples with contemporaneous diagnostic microbiology data (DM) were included in the final analyses. Using a modification of the Leeds criteria, patients were deemed to be chronically or intermittently colonised with a given pathogen if >50% or ≤50% of samples, respectively, over the three-year study period were positive by diagnostic microbiology or targeted QPCR. A minimum of ≥6 samples was chosen as less samples would have increased the likelihood of misclassifying infection status.

Table 3.3 Summary of clinical characteristics for all patients

	Paediatric	Adult	All patients
Number of patients	20	20	40
Mean number of samples per patient (±SD)	7.0 (1.0)	9.4(3.5)	8.2 (2.8)
Number of sample types (sputum/swab)	81/58	152/36	233/94
Sex (female/male)	10/10	8/12	18/22
Mean age (±SD) [a]	10.5 (2.7)	25.1 (4.6)	18.9 (8.1)
Mean %FEV_1 (±SD)	91.0 (15.3)	73.0 (19.5)	80.6 (19.9)
Pancreatic insufficiency (sufficient/insufficient)	3/17	8/12	11/29
CF related diabetes (Y/N)	0/20	1/19	1/39
CFTR genotype [b]			
Phe508del homozygous	9	7	16
Phe508del heterozygous	7	8	15
Non-Phe508del	4	5	9

SD denotes standard deviation of the mean. [a] Based on age at time of first sample for each patient. [b] CFTR genotype - cystic fibrosis transmembrane conductance regulator genotype. Homozygous Phe508del, two copies of the Phe508del gene mutation. Heterozygous Phe508del, single copy of this mutation plus another mutation.

3.2.2 Diagnostic microbiology

Diagnostic culture-based microbiology data was provided by the microbiology service within Manchester University NHS Foundation Trust who perform microbiology testing for the participating centres, in line with international guidance and standards (Smyth *et al*., 2014; Public Health England, 2015).

3.2.3 DNA extraction and QPCR

Nucleic acid extraction was performed on sputum and cough swab samples as previously described, with a modification for the latter sample type (Rogers *et al*., 2003). As an alternative to the wash stage for sputum, cough swabs were saturated in sterile phosphate buffer solution for 5 minutes, then squeezed with sterile tweezers to extract as much material as possible. The resulting solution was then introduced at the bead beating stage and the protocol continued as normal as for the sputum samples thereafter. QPCR was performed using targeted primers for *P. aeruginosa* (Feizabadi *et al*., 2010; Rogers *et al*., 2010) and *S. aureus* (Alarcón *et al*., 2006) (Table S3.2). In brief, samples were run on a Bio-Rad CFX connect machine (Bio-Rad, Deeside, UK). A blank consisting of the master mix, probes, primers and water was run on each plate. Pure strains of each pathogen were run on each plate as both a positive control and as a standard. Both *P. aeruginosa* and *S. aureus* QPCR were run using Taqman Gene Expression MasterMix (Applied Biosystems, Warrington, UK). Additional detail on methods and primers is provided in the Supplementary materials.

3.2.4 Data analysis

For longitudinal analysis, this was restricted to patients who contributed ≥6 samples with contemporaneous diagnostic microbiology data (Figure 3.1). Using a modification of the Leeds criteria (Lee *et al*., 2003), patients were deemed to be

chronically or intermittently colonised with a given pathogen if >50% or ≤50% of samples, respectively, over the three-year study period were positive by diagnostic microbiology or targeted QPCR. A minimum of six samples was chosen as less samples would have increased the likelihood of misclassifying infection status. Summary statistics, including means and standard deviations (SD), were calculated using XLSTAT v2018.1 (Addinsoft, Paris, France). Welch's *t*-test was employed due to unequal sample sizes and variance in the data. Welch's test statistic (*t*), approximated degrees of freedom (*df*), and significance (*P*) were calculated in XLSTAT. Significance was set at $P < 0.05$. Pathogen targeted culture and QPCR data has been deposited at figshare.com under https://doi.org/10.6084/m9.figshare.14483394.v2

3.3 Results

Twenty paediatric and twenty adult patients contributed 327 respiratory samples over the three-year study period, with a mean ± SD of 8.2 (± 2.8) samples per patient (minimum 6 samples, maximum 20) (Table S3.3 and table S3.4). Overall, 233 (71%) of samples were sputum, the remainder (*n* = 94 being cough swabs taken by clinical staff, with rates of cough swab higher in children compared to adults (42% vs 19%). Clinical characteristics of all patients are summarised in Table 3.1. Overall, this was a population with mild CF lung disease, with a mean %FEV_1 of 80.6% predicted.

Pathogen detection by culture and QPCR approaches was compared across the patients included in the study (Figure 3.2). In all instances, culture significantly underestimated pathogen detection. For *P. aeruginosa*, the mean percentage of

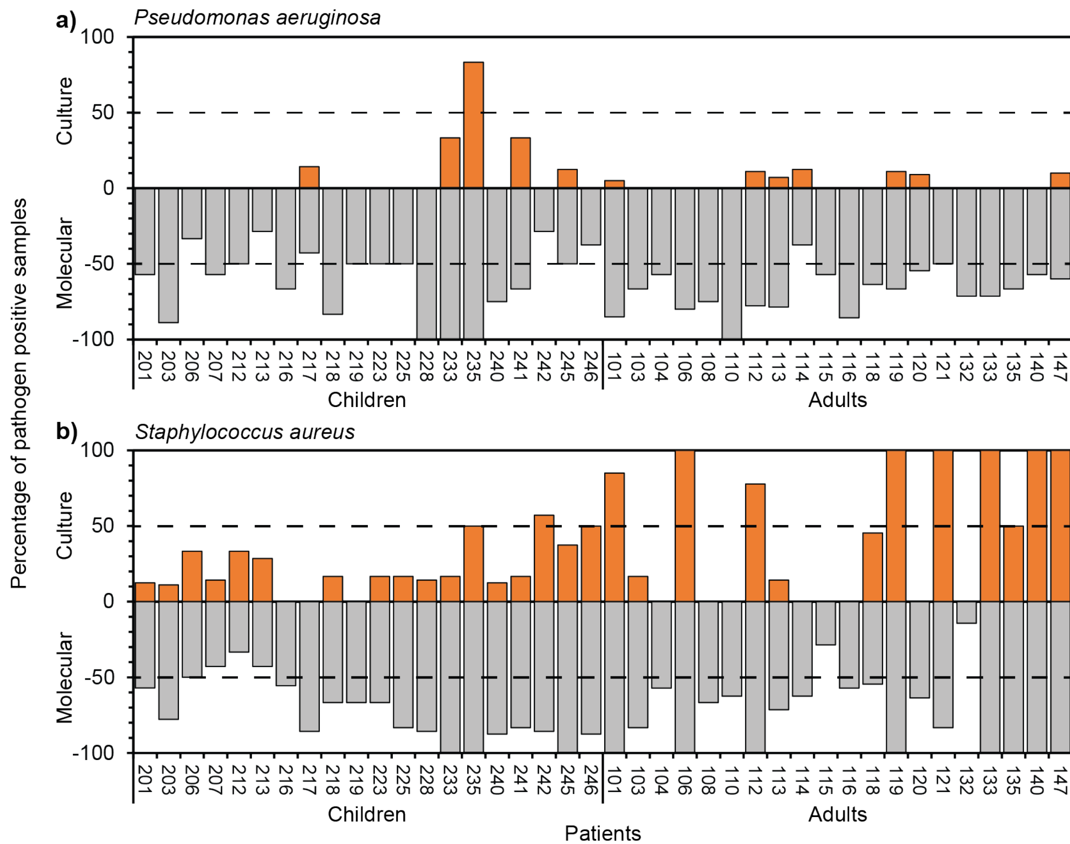

Figure 3.2 Pathogen detection by conventional culture and molecular-based approaches in paediatric and adult cystic fibrosis patients. Given for each patient, is the percentage of respiratory samples over the three-year study duration that were culture (orange) or QPCR (grey) positive for (a) *Pseudomonas aeruginosa* and (b) *Staphylococcus aureus*. In each instance, the dashed line denotes the threshold for chronic (>50%) or intermittent (≤50%) colonisation. Numbers on the x axis represent individual patient study numbers.

pathogen detection (±SD) across all paediatric patients by culture was 8.8 ± 20.4% compared to 60.9 ± 23.4% by QPCR (Welch's *t*-test, t =-7.51, df = 37.3, P <0.001). For *P. aeruginosa* in adults, the means were 3.3 ± 4.8% and 68.5 ± 15.7% by culture and QPCR, respectively (t = -17.79, df = 22.5, P <0.001). For *S. aureus* in paediatric patients the mean detection by culture and QPCR were 21.6 ± 16.8% and 72.2 ± 20.8%, respectively (t =-8.45, df = 36.4, P <0.001). Within adult patients, *S. aureus* mean detection was 44.5 ± 45.3 and 75.1 ± 25.9 by culture and QPCR, respectively (t = -2.62, df = 30.2, P = 0.01).

More specifically, *P. aeruginosa* and *S. aureus* were detected by culture on at least one occasion in 12 (30%) and 29 (73%) of the patients, respectively. Conversely, both pathogens were detected in all 40 patients on at least one occasion by QPCR (Figure 3.2). For *P. aeruginosa*, culture determined only one patient to be chronically infected and a further 11 intermittently infected (Figures 3.2 & 3.3). That increased to 28 chronically infected and 12 intermittently infected with *P. aeruginosa* when using QPCR to identify presence of each taxa. For *S. aureus,* nine patients were deemed to be chronically infected by culture compared to 34 by QPCR. Change in bacterial infection status was seen in both the adult and paediatric patients. Overall, *P. aeruginosa* and *S. aureus* infection status changed for 36 and 27 out of all patients, respectively. In no cases did molecular identification lead to a patient being allocated to a negative or intermittent infection category when culture placed them as intermittent or chronically infected (Figure 3.3).

There was only a single instance of a greater percentage of pathogen positive samples detected by culture than by QPCR; specifically, *S. aureus* in patient 121 (Figure 3.2b). That patient was however still deemed to be chronically colonised with *S. aureus* irrespective of method of detection. Out of the 18 samples culture

positive for *P. aeruginosa* samples, only five of those did not have corresponding detection by QPCR. For *S. aureus*, from the 121 culture positive samples, eight did not have matching molecular detection. Pathogen abundance was assessed to ascertain whether it contributed to the underestimation of pathogen detection by culture; whereby low bacterial cell abundances could contribute to false negatives by culture. However, abundance derived by QPCR for both pathogens was relatively high across all samples (Figure S3.1). The mean abundance (± SD) for *P. aeruginosa* in paediatric and adult patients was $7.53 \times 10^{10} \pm 2.37 \times 10^{10}$ colony forming units (CFU) ml^{-1} equivalents and $1.50 \times 10^{10} \pm 6.59 \times 10^{9}$ CFU ml^{-1} equivalents, respectively. For *S. aureus*, mean abundances were $8.33 \times 10^{9} \pm 3.01 \times 10$ and $2.63 \times 10^{9} \pm 7.89 \times 10^{8}$ CFU ml^{-1} equivalents for paediatric and adult patients, respectively. Similarly, the influence of respiratory sample type was assessed, with an expectation that cough swabs would have lower pathogen abundances when compared to sputum as a source sample. In all instances, there was no significant differences in pathogen abundances when derived from sputum or cough swab samples (Figure S3.2).

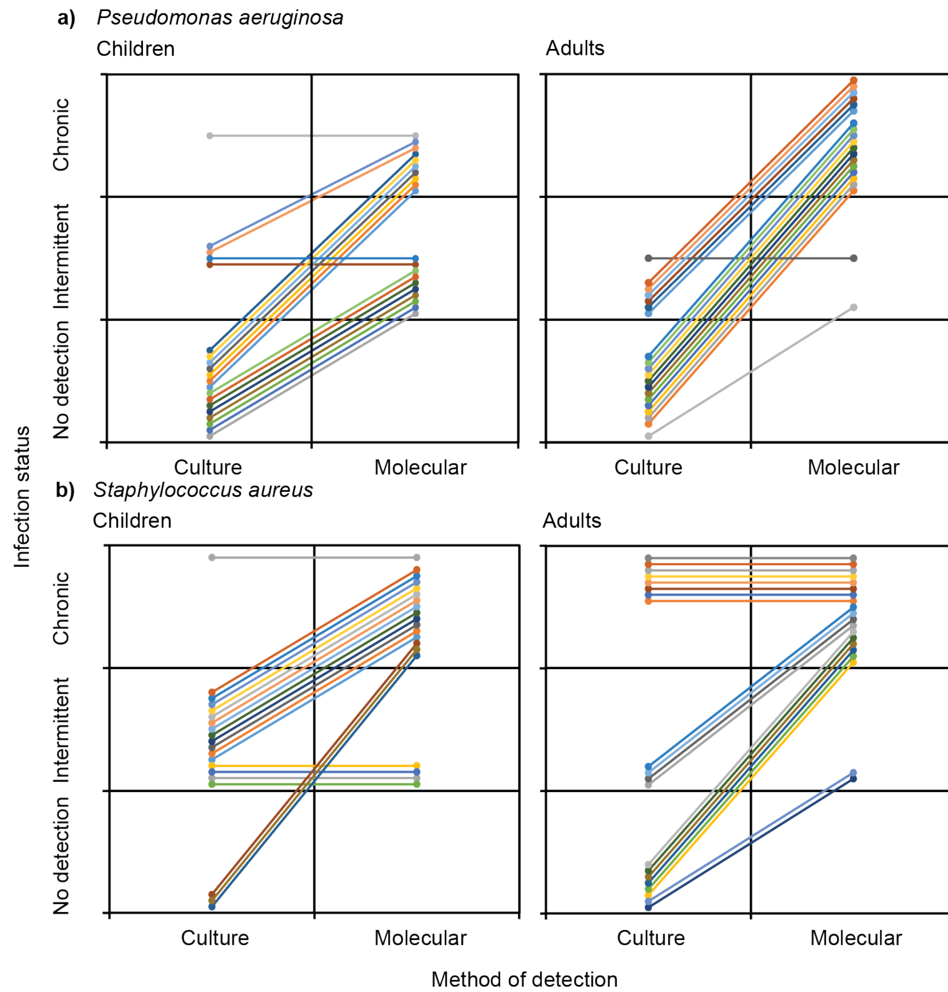

Figure 3.3 Changes in pathogen infection status from diagnostic culture to molecular-based detection in paediatric and adult patients. Given are changes in infection status for (a) Pseudomonas aeruginosa and (b) Staphylococcus aureus in each of the children and adult CF patients. In each instance, coloured lines represent individual patients.

3.4 Discussion

In this study we have compared the detection of key pathogens on repeated samples from the same cohort of CF children and adults over several years. Since the patients were considered free of chronic *Pseudomonas* infection by culture, we have focused on the identification of this pathogen along with *S. aureus* in sputum and cough swabs collected at the same time as diagnostic microbiology. Both pathogens are broadly accepted as readily detectable by culture-based diagnostic microbiology (Public Health England, 2015; 2020). Strikingly, detection of both *P. aeruginosa* and *S. aureus* was significantly lower by culture, which in many instances did not detect either pathogen despite these being found repeatedly by targeted QPCR (Figure 3.2). Consequently, the infection status of patients was also misclassified in the majority of instances (Figure 3.3), with implications for understanding of infection in CF and clinical management of emergent infections.

Current infection surveillance approaches in CF are guided by classical aerobic culture-based diagnostic microbiology (Smyth *et al*., 2014; Public Health England, 2015), and these data are collated across CF centres and used to inform national annual CF patient registry reports (Cystic Fibrosis Foundation, 2019; Cystic Fibrosis Trust, 2019). Based on these culture-based microbiology results, patients are also cohorted into groups and segregated to avoid cross infection (Doe *et al*., 2010; Smyth *et al*., 2014). In this study, patients were specifically drawn from *Pseudomonas*-free clinics, containing patients with no new growth of *P. aeruginosa* for at least 12 months. Although it was accepted that some would subsequently become infected by this pathogen over the three-year study period, culture indicated all patients were free of chronic infection throughout the study, with only one exception (Figure 3.2). Conversely, QPCR indicated that 28 (70%) of all patients

were chronically infected with *P. aeruginosa*, and the remainder intermittently infected. Based on culture-based reporting alone, none of the paediatric and adult patients would have been redirected into *P. aeruginosa* based outpatient clinics.

In the case of *Pseudomonas* in particular, this has implications for the treatments that patients are offered. Eradication treatments, targeted oral or intravenous therapies for exacerbations, and long term inhaled anti-pseudomonal antibiotics are not prescribed unless *P. aeruginosa* is isolated on culture. Our data would indicate that this significantly underestimates the true prevalence of chronic infection. Suboptimal antimicrobial therapy could further contribute to continued infection, and an increase likelihood of developing antimicrobial resistance or transitioning to chronic infection with time (Flume *et al*., 2018). Importantly, underestimation or non-detection may miss the critical period in early *P. aeruginosa* infection typically used as an ideal timeframe for targeted eradication therapy to prevent establishment of chronic infection (Høiby *et al*., 2005).

Over the last ten years or so, molecular microbiology techniques, have challenged our fundamental understanding of CF airway pathogen epidemiology (Zhao *et al*., 2012). Previous literature focused on QPCR in CF is summarised in the online supplement and Table S3.1. In support of our findings, a CF lung microbiome study of 297 patients attending 13 CF centres in Europe and the USA, representing a broad cross-section of respiratory disease, found *P. aeruginosa* in all patients and *S. aureus* in the majority of those patients (93%) (Cuthbertson *et al*., 2020). There have been surprisingly few studies though that have addressed this issue in longitudinal sampling. Notably, in a study of 80 Danish CF patients, whole genome sequencing approaches and positive culture history of *P. aeruginosa* infections demonstrated that the same clone type could persist from cultures taken before and

after a judged *Pseudomonas*-free eradication period in many of those patients (Bartell *et al*., 2020). This suggested that the original *Pseudomonas* strain had not been cleared, and was missed by routine culture-based surveillance, as we have shown. Similar to the current study, Héry-Arnaud *et al*. looked at patients apparently free of *Pseudomonas* and compared QPCR with culture. They showed that QPCR would often become positive before culture and showed high rates of molecular identification of *Pseudomonas* in those in whom it was never cultured (Héry-Arnaud *et al*., 2017). Results were presented in aggregate however, and the impact on classification of microbiological status of individual patients was not reported.

Although methodological strengths and weaknesses of molecular and culture detection methods have been reviewed by others (Pattison *et al*., 2013; Burns and Rolain, 2014), it is important to consider potential factors that might contribute to the observed disparity between the sets of outcomes. For instance, low pathogen abundance within a sample or low abundance resulting from different respiratory sample types (e.g. cough swab vs. sputum) could be factors leading to misdetection by culture (Pattison *et al*., 2013; Burns and Rolain, 2014). However, given the relatively high abundances observed for both pathogens by QPCR across all patients this is unlikely to have been a major factor here (Figures S3.1 & S3.2). It is also possible that artefacts of cell death were amplified by QPCR, creating false positives. Again, this seems unlikely to be a major or constant factor given the observed abundances across all patients over the 3-year study period. Viable but not culturable (VBNC) status may explain to some degree why these readily culturable pathogens were undetected or underestimated (Pasquaroli *et al*., 2013; Héry-Arnaud *et al*., 2017; Mangiaterra *et al*., 2018). VBNC is believed to be a protective state in response to some form of environmental stressor, including

changes from aerobic or anaerobic conditions, prevalence of energy and nutrient sources, or as a defence mechanism against antibiotics. Pertinent to this study, the ability of antibiotics to induce a VBNC state have been proposed from studies of *S. aureus* and *P. aeruginosa*, which may also support pathogen survival to extended and repeated antibiotic interventions (Pasquaroli *et al*., 2013; Mangiaterra *et al*., 2018). The clinical impact of bacteria in such states on either current or future health remains to be established. In our study, there were a few instances where there was detection by culture but not by QPCR. However, it is possible for culture to misidentify pathogens and generate false positives. For example, other *Staphylococcus* species can be mistaken for *S. aureus* in routine testing (Notarnicola *et al*., 1985; Public Health England, 2020). A previous study by Kateete (*et al.,* 2010) found that even the most robust testing methods provided false positives in even coagulase negative staphylococci, demonstrating the need for more accurate detection methods.

3.5 Conclusion

Microbiological surveillance to detect pathogens is the foundation of CF clinical care and treatment. Our longitudinal data substantially builds on previous observations to demonstrate that the diagnostic gold standard of microbiological detection by culture significantly underestimates prevalence (Table S3.1). Crucially, we have further shown how classification of a patient's infection status would change using findings from QPCR in comparison with culture even for what are believed to be readily culturable pathogens in paediatric and adult patients. While there may be methodological factors contributing to these observations, it remains that our findings have implications for understanding the evolution of infection and the

clinical care of patients. This issue will become even more important in CF patients treated with highly effective CFTR modulators. These treatments often lead to dramatic reductions in sputum volumes, increasing our reliance on culture of other specimens than sputum, such as cough swabs, with known reduced sensitivity in detection of pathogens. Thus, molecular detection may have an increasing potential role for detection of potential CF pathogens, but there is a need to establish its relevance in comparison with current culture-based techniques (Hisert *et al*., 2017). These findings also have implications beyond CF, since microbiological detection and surveillance are critical to guide therapeutics in other airways diseases. As targeted molecular identification becomes quicker and cheaper, it is likely to become increasingly deployed in clinical scenarios. Subsequently, treatment approaches developed in response to conventional clinical microbiology need re-evaluation to deal with these more sensitive techniques.

3.6 Author contributions and Funding

CvdG, AH, and HG conceived the study. HG, LH, and DR performed sample processing and analysis. HG, DR, and CvdG performed data and statistical analysis. AH, AM, and AJ were responsible for sample collection, clinical care records and documentation. HG, AH, and CvdG verified the underlying data. HG, AH, and CvdG were responsible for the creation of the original draft of the manuscript. All authors contributed to the development of the final manuscript. CvdG and AH are guarantors of this work. All authors read and approved the final manuscript.

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3.7 Acknowledgements and Declaration of Competing Interest

The authors would like to thank the patients who took part in this and the clinical teams who supported this work.

All authors declare support from the CF Trust. AH reports personal fees for advisory services (Mylan Pharmaceuticals) and educational and presentation activities (Vertex Pharmaceuticals).

3.9 Supplementary materials

3.9.1 Literature Review

PubMed was searched for studies on cystic fibrosis (CF) published from database inception to August 30th, 2021, with the search terms “cystic fibrosis” AND “pathogen” AND “culture” AND “QPCR”, with no language restrictions (Table S1). Studies that did not use QPCR to target specific pathogens, that only used 16S rRNA gene targeted QPCR, only used next generation sequencing (NGS) approaches, or other detection methods such as dot blots were not considered in this literature review. Of the 21 papers that met the above criteria (Table S1), eight were cross-sectional only studies [1, 2, 5, 11, 12, 13, 19, 20]. Of the remaining 13 papers that did incorporate some form of longitudinal sampling into their studies, ten studies pooled all samples for grouped analysis, irrespective of patients and time points that samples were taken from [3, 4, 6, 7, 9, 10, 16, 17, 18, 21].

The three remaining studies did explicitly track individual patients over time, but with caveats [8, 14, 15]. One study [14], tracked individual patients but with a focus on the effectiveness of tracking *Pseudomonas aeruginosa* infection before it became apparent in routine clinical culture, and analysed data through pooled samples for a grouped average time to detection. A second study [15], focused on the impact of Ivacaftor on *P. aeruginosa* (PSA) along with the general microbiome via NGS. However, the analyses combined all patients as a group, discussing *P. aeruginosa* based results as an average of the group. The final paper [8], focused on similar issues to our study, i.e. whether QPCR would be more sensitive than conventional culture in the detection of PSA in individual patients through time. Comparison of targeted QPCR and culture was based chiefly on cough swab samples taken from only paediatric patients (*n* = 33) over eight months from 2007; mean number of

samples per patient (± SD) = 4·2 ± 1·4, minimum = 1 and maximum = 7. The study found an increased detection of *P. aeruginosa* when using QPCR. Although pathogen detection was plotted for individual patients, data was analysed only as a group. Finally, none of the studies explicitly investigated the impact that more sensitive detection methods would have on infections status or clinical outcomes.

Table S3.1 Literature review comparing current study with results from PubMed search.

	No. patients	Paediatric	Adult	No. samples (sample type)	No. centres	Target pathogen [a]	QPCR primer target(s) [b]	Comparison to matched culture [c]	Study type	Duration (months)	Were individuals tracked?	Analysis (Grouped or Individual) [d]	Key takeaway points	Explicit clinical impact	Citation
	40	20	20	328 (234 sputum, 94 swabs)	2	PSA, SA	*PSA: oprL, SA: nuc*	Yes	Longitudinal	42 months	Yes	Individual	Comparison of culture data to QPCR, impact on clinical status for individual patients	Yes	This Study
1	Not clearly specified	n/a	n/a	200 (175 sputum, 21 swabs and 4 BAL)	1	PSA	*oprL/ gyrB/ ETA*	Yes	Cross-sectional	No	n/a	Grouped	Combinations of primer targets will improve PSA detection	No	Qin X, 2003
2	8 patients	n/a	n/a	8 (sputum)	1	PSA	*oprL*	Yes	Cross-sectional	No	n/a	Grouped	Comparison of pre-treatments for samples to improve detection rates	No	Deschaught 2009 [2]
3	397	n/a	n/a	Unknown mix of sputum, nasopharyngeal or throat swab	7	PSA	*oprL*	Yes	Longitudinal	ca. 16 months	No	Grouped	Culture and QPCR work just as well as each other, the predictive value of QPCR may be quite limited	No	Deschaught 2010 [3]
4	33	33	0	103 (sputum)	1	PSA	*oprL*	Yes	Longitudinal	ca. 30 months	No	Grouped	QPCR can detect PSA before culture	No	Billard-Pomares 2010 [4]
5	Not clearly specified	n/a	n/a	n/a	Unknown	*Streptococcus spp.*	*cpn60*	No	Cross-sectional	No	n/a	Grouped	QPCR development	No	A B Olson 2010 [5]
6	16	n/a	n/a	159 (85 Sputum, 47 swabs and 27 saliva)	1	Various CF pathogens	*PSA: PSD7F, SA:STPYF, HI:* unknown,	Yes	Longitudinal	ca. 12 months	No	Grouped	Comparison of various pathogen primer targets	No	Zemanick 2010 [6]

7	183	183	0	2099 (851 sputum and 1248 throat swabs)	1	PSA	*gyrB/ algD*	Yes	Longitudinal	ca. 29 months	No	Grouped	QPCR can detect PSA before culture	No	Logan 2010 [7]
8	186	186	0	542 (42 sputum and 500 swabs	1	PSA	*Pa23*	Yes	Longitudinal	ca. 19 months	Yes	Grouped	QPCR is more sensitive than culture in PSA detection	Stated clinical impact needs assessing but doesn't go beyond that	McCulloch 2011 [8]
9	230	74	156	459 (293 sputum, 162 swab and 4 BAL)	2	PSA	*gyrB*	Yes	Longitudinal	ca. 30 months	1 patient tracked.	Grouped	Analysis of PSA levels during exacerbation & stability, with optimised method. No difference found in disease states	Introduction of QPCR may have implications on better segregation strategies/could help patients be free of PSA for longer.	Fothergill 2013 [9]
10	34	n/a	n/a	46 (sputum)	3	PSA	*oprL/ gyrB/ ecfX*	Yes	Longitudinal	ca. 38 months	No	Grouped	Prevention of false negatives using 2-step confirmatory QPCR	No	Le Gall 2013 [10]
11	9	0	9	13 samples	1	PSA	*oprL* and *ClpX*	No	Cross-sectional	No	n/a	Grouped	Validation of 2 PSA reference genes in CF sputum	No	Costaglioli 2014 [11]
12	65	65	0	87 (45 sputum and 42 swabs)	1	SA	*femA*	Yes	Cross-sectional	No	n/a	Grouped	Method comparison for sample processing.	No	Johnson 2016 [12]
13	Not clearly specified	n/a	n/a	15 (11 sputum and 4 BAL)	Unknown	NTM	*atpE*	Yes	Cross-sectional	No	n/a	Grouped	Comparison of sample processing methods for better NTM detection.	No	Caverly 2016 [13]
14	96	96	0	707 (sputum)	1	PSA	*oprL/ gyrB/ ecfX*	Yes	Longitudinal	ca. 42 months	Yes	Grouped	QPCR can detect PSA before culture	No	Héry-Arnaud 2017 [14]
15	12	0	12	Not clearly specified	1	PSA, *Streptococcus, Prevotella*	*PSA: gyrB, SA: nuc*	Yes	Longitudinal	ca. 34 months	Yes	Grouped	Ivacaftor briefly impacts PSA levels, but PSA somewhat recovers over time.	No	Hisert 2017 [15]
16	64	n/a	n/a	379 (141 throat swabs and	1	PSA	*oprL/ ecfX/ gyrB*	Yes	Longitudinal	Not clearly specified	No	Grouped	PSA abundance in swabs/sputum compared to culture. Swabs may be	No	Boutin 2018 [16]

				238 sputum)									beneficial for early detection.		
17	47	47	0	312 (sputum)	1	PSA	*oprL*	Yes	Longitudinal	ca. 17 months	No	Grouped	Time taken for PSA positive culture to be determined vs. QPCR	No	Blanchard 2018 [17]
18	Not clearly specified	n/a	n/a	88 (sputum)	1	PSA	*ecfX*	Yes	Longitudinal	ca. 20 months	No	Grouped	Comparison of sample storage, DNAse treatments and QPCR targets.	No	Mangiaterra 2018 [18]
19	9	0	9	16 (sputum)	1	SM	Custom primers for *Strenotrophomonas* strains	Yes	Cross-sectional	No	n/a	Grouped	QPCR development	Identification of SM in clinic allows for earlier detection, may reduce prevalence of other emblematic pathogens through correct treatment.	Fraser 2019 [19]
20	78	78	0	78 (BAL)	7	PSA, HI, SA, SM	Not clearly specified	Yes	Cross-sectional	No	n/a	Grouped	Correlation of bacterial densiy/ presence of pathogens to inflammation, mucus plugging etc.	No	Taylor 2020 [20]
21	14	n/a	n/a	28 (sputum)	1	PSA	*oprL*	No	Longitudinal	ca. 12 months	Yes	Grouped	Does the prevalence of specific pathogens decrease with Ivacaftor treatment.	No	Einarsson 2021 [21]

[a] PSA: *Pseudomonas aeruginosa,* SA: *Staphylococcus aureus,* NTM: *Non-tuberculosis mycobacteria,* SM: *Streptococcus maltophilia, HI* : *Haemophilus influenzae*

[b] Papers using 16S rRNA, NGS or other methods such as dot blots were not considered in this literature review

[c] Must have matched culture records for each sample

[e] Explicitly followed individually through time, or grouped (all samples irrespective of longitudinal/cross-sectional origin, or combined for analysis)

3.9.2 QPCR primers and conditions

It was not within the scope of the current study to design and assess new primers for targeted QPCR against *P. aeruginosa* and *S. aureus,* as many effective primer sets have been developed and evaluated over at least the last two decades; for example, see Table S1 for *P. aeruginosa* based primers. Instead the approach here was to use primer sets already established as highly effective.

3.9.2.1 *Pseudomonas aeruginosa – oprL*

While there are several targets for *P. aeruginosa* amplification, studies vary in their usage. Since development in the late 1990s, the *oprL* gene target has consistently shown good sensitivity and specificity across multiple studies [22]. Multiple studies have utilised the *oprL* gene to identify *P. aeruginosa* in CF sputum and swabs [2-4, 10, 11, 14, 16, 17, 21]. While some studies have further characterised the *P. aeruginosa* positive samples using confirmative QPCR for the *gyrB/ecfX* gene targets [10, 14, 16], the sensitivity and specificity of *oprL* alone (100% and 70-75% respectively) [16, 22] was sufficient for the current study, particularly in light of the further expense needed for confirmation with other gene targets. For this study, the average r^2 (efficiency) value was 0.97 across 5 runs (min: 0.96, max: 0.99). The r^2 in this study sat between 0.99 and 0.96 for all five runs.

3.9.2.2 *Staphylococcus aureus – nuc*

The *nuc* gene used for *S. aureus* detection was developed 1992 [23]. The gene is highly specific for *S. aureus* and is not found in other key staphylococcal strains [24]. The *nuc* gene is a well-established *S. aureus* QPCR primer, with a sensitivity and specificity of around 98% and 100% respectively [25-28]. For this study, the average r^2 (efficiency) value for this target was 0.97 over 4 runs (min: 0.94, max: 1).

Table S3.2 Overview of QPCR primers, probes, and parameters for each pathogen.

Pseudomonas aeruginosa **(oprL) FAM-TAMRA – 117bp**			
Mastermix			
Forward primer (100µM): CGAGTACAACATGGCTCTGG		0·2µl	
Reverse primer (100µM) ACCGGACGCTCTTTACCATA		0·2µl	
Probe (100µM) FAM–CCTGCAGCACCAGGTAGCGC-TAMRA		0·2µl	
DNA		2µl	
Taqman Gene expression Mastermix		20µl	
Water		17·4µl	
QPCR Protocol			
Stage		Time	Temperature (ºc)
	Initial Denaturation	10 minutes	95
40 cycles	Denaturation	10 seconds	95
	Annealing	30 seconds	58
	Elongation	1 minute	72
Staphylococcus aureus **(nuc) FAM-TAMRA – 124bp**			
Mastermix			
Forward primer (100µM) CGCTACTAGTTGCTTAGTGTTAACTTTAGTTG		0·06µl	
Reverse primer (100µM) TGCACTATATACTGTTGGATCTTCAGAA		0·06µl	
Probe (100µm) FAM-TGCATCACAAACAGATAACGGCGTAAATAGAAG-TAMRA		0·02µl	
DNA		2µl	
Taqman Gene expression Mastermix		10µl	
Water		7·86µl	
QPCR Protocol			
Stage		Time	Temperature (ºc)
	Initial Denaturation	10 minutes	95
40 cycles	Denaturation	30 seconds	95
	Annealing	30 seconds	60
	Elongation	1 minute	72

Table S3.3 Sampling for paediatric patients, samples shaded in grey were taken during an exacerbation

Paediatric									
201	09/12/14	31/03/15	21/07/15	27/10/15	16/02/16	21/06/16	30/08/16	29/08/17	
203	06/01/15	25/03/15	23/06/15	29/09/15	24/05/16	22/11/16	27/06/17	19/09/17	28/10/17
206	16/01/15	03/07/15	29/01/16	06/09/16	09/12/16	02/06/17			
207	16/01/15	07/08/15	19/02/16	06/05/16	05/08/16	20/01/17	21/04/17		
212	26/05/15	25/08/15	08/09/15	09/02/16	18/10/16	01/08/17			
213	17/01/15	26/05/15	12/10/15	15/03/16	30/08/16	25/10/16	26/01/18		
216	07/08/15	06/11/15	15/01/16	06/05/16	11/11/16	13/01/17	07/07/17	29/09/17	
217	06/03/15	05/06/15	02/10/15	18/03/16	17/06/16	16/09/16	03/03/17	09/06/17	
218	10/03/15	04/08/15	26/01/16	06/09/16	09/12/16	02/06/17			
219	10/03/15	21/07/15	08/12/15	01/03/16	21/06/16	23/12/16			
223	29/09/15	15/03/16	15/11/16	23/05/17	17/10/17	23/01/18			
225	01/05/15	30/10/15	26/02/16	28/10/16	24/01/17	05/01/18			
228	12/05/15	04/08/15	24/11/15	12/04/16	27/09/16	04/04/17	04/07/17	10/10/17	
233	22/06/15	26/01/16	05/04/16	12/07/16	04/10/16	08/11/16			
235	10/07/15	24/07/15	06/08/15	09/10/15	08/01/16	11/03/16	09/12/16		
240	24/07/15	12/02/16	22/04/16	08/07/16	06/01/17	07/04/17	07/07/17	16/02/18	
241	24/07/15	23/10/15	22/01/16	11/03/16	08/09/17	01/12/17			
242	28/07/15	06/10/15	12/01/16	17/05/16	09/08/16	15/11/16	04/07/17	10/10/17	
245	04/08/15	30/08/15	03/11/15	26/01/16	09/08/16	08/11/16	10/10/17	23/01/18	
246	08/09/15	29/03/16	05/07/16	27/09/16	28/03/17	20/06/17	19/09/17	12/12/17	

Table S3.4 Sampling for adult patients, samples shaded in grey were taken during an exacerbation

Adult																				
101	21/11/14	15/12/14	26/01/15	23/02/15	18/05/15	29/06/15	24/08/15	19/10/15	14/12/15	25/01/16	04/04/16	16/05/16	27/06/16	15/08/16	03/10/16	07/11/16	16/01/17	24/04/17	02/10/17	29/01/18
103	24/11/14	23/02/15	20/07/15	14/09/15	14/12/15	14/11/16	23/01/17	10/04/17												
104	08/12/14	15/06/15	21/09/15	16/11/15	11/01/16	11/07/16	18/12/17													
106	01/12/14	12/01/15	30/03/15	22/06/15	03/08/15	07/09/15	02/11/15	23/05/16	03/10/16	03/04/17	21/08/17									
108	08/12/14	19/01/15	20/04/15	05/10/15	16/11/15	18/01/16	31/10/16	16/01/17	27/03/17	22/05/17	13/11/17	29/01/18								
110	23/02/15	27/04/15	01/06/15	14/12/15	18/04/16	23/01/17	03/08/17	30/10/17	05/02/18											
112	15/12/14	23/03/15	21/09/15	21/12/15	20/03/17	18/09/17	15/01/18													
113	15/12/14	19/01/15	16/02/15	16/03/15	18/05/15	01/02/16	16/05/16	06/06/16	25/07/16	08/08/16	19/09/16	04/12/17	15/01/18	05/02/18						
114	16/02/15	18/05/15	17/08/15	04/01/16	06/06/16	22/05/17	14/08/17	12/02/18												
115	02/03/15	27/04/15	17/08/15	28/09/15	02/11/15	04/01/16	12/09/16													
116	22/12/14	16/05/15	13/07/15	04/01/16	29/02/16	11/04/16	10/04/17													
118	05/01/15	16/03/15	30/11/15	11/01/16	09/05/16	20/06/16	28/11/16	20/02/17	15/05/17	19/06/17										
119	06/07/15	24/08/15	11/04/16	15/08/16	19/12/16	31/07/17	04/09/17	13/11/17	18/12/17	12/02/18										
120	26/01/15	20/04/15	20/07/15	26/10/15	25/01/16	07/03/16	09/05/16	20/06/16	12/09/16	03/04/17	04/12/17									
121	26/01/15	09/03/15	23/03/15	15/06/15	02/11/15	04/12/17														
132	27/07/15	25/01/16	25/04/16	09/05/16	13/06/16	10/04/17	24/07/17													
133	20/04/15	19/10/15	14/12/15	06/02/17	24/07/17	11/12/17														
135	18/05/15	13/07/15	07/09/15	19/10/15	13/03/17	13/11/17														
140	06/07/15	27/07/15	26/10/15	07/12/15	04/01/16	18/04/16	06/02/17	26/06/17	31/10/17	10/11/17	11/12/17	22/01/18								
147	05/12/16	09/01/17	06/03/17	24/04/17	15/05/17	19/06/17	10/07/17	14/08/17	18/09/17	04/12/17	29/01/18									

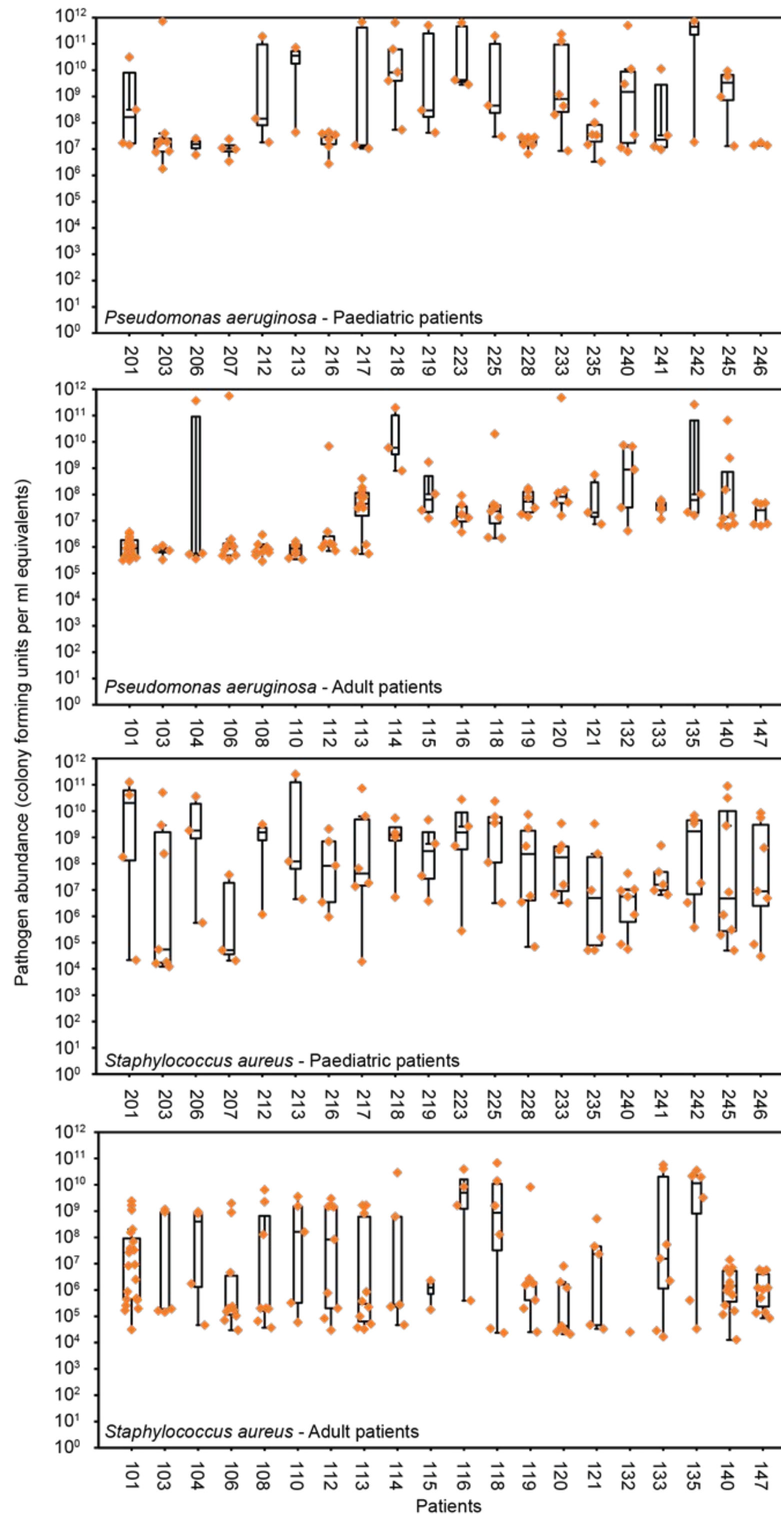

Figure S3.1 Pathogen abundance within paediatric and adult patients. Boxplots show 25–75th interquartile (IQR) range with whiskers showing 1·5 times IQR. Orange diamonds indicate individual samples for a given patient. For *P. aeruginosa*, the minimum and maximum abundances were $1{\cdot}77 \times 10^{6}$ and $9{\cdot}41 \times 10^{11}$ colony forming units (CFU) ml^{-1} equivalents, respectively, in paediatric patients (mean ± SD, $7{\cdot}53 \times 10^{10} \pm 2{\cdot}37 \times 10^{10}$), and ranged from $2{\cdot}85 \times 10^{5}$ to $5{\cdot}53 \times 10^{11}$ CFU ml^{-1}

equivalents in adult patients (mean ± SD, $1{\cdot}50 \times 10^{10} \pm 6{\cdot}59 \times 10^{9}$). For *S. aureus*, abundance ranged from $1{\cdot}21 \times 10^{4}$ to $2{\cdot}50 \times 10^{11}$ colony forming units (CFU) ml^{-1} equivalents in paediatric patients (mean ± SD, $8{\cdot}33 \times 10^{9} \pm 3{\cdot}01 \times 10^{9}$), and from $1{\cdot}27 \times 10^{4}$ to $6{\cdot}81 \times 10^{10}$ CFU ml^{-1} equivalents in adult patients (mean ± SD, $2{\cdot}63 \times 10^{9} \pm 7{\cdot}89 \times 10^{8}$).

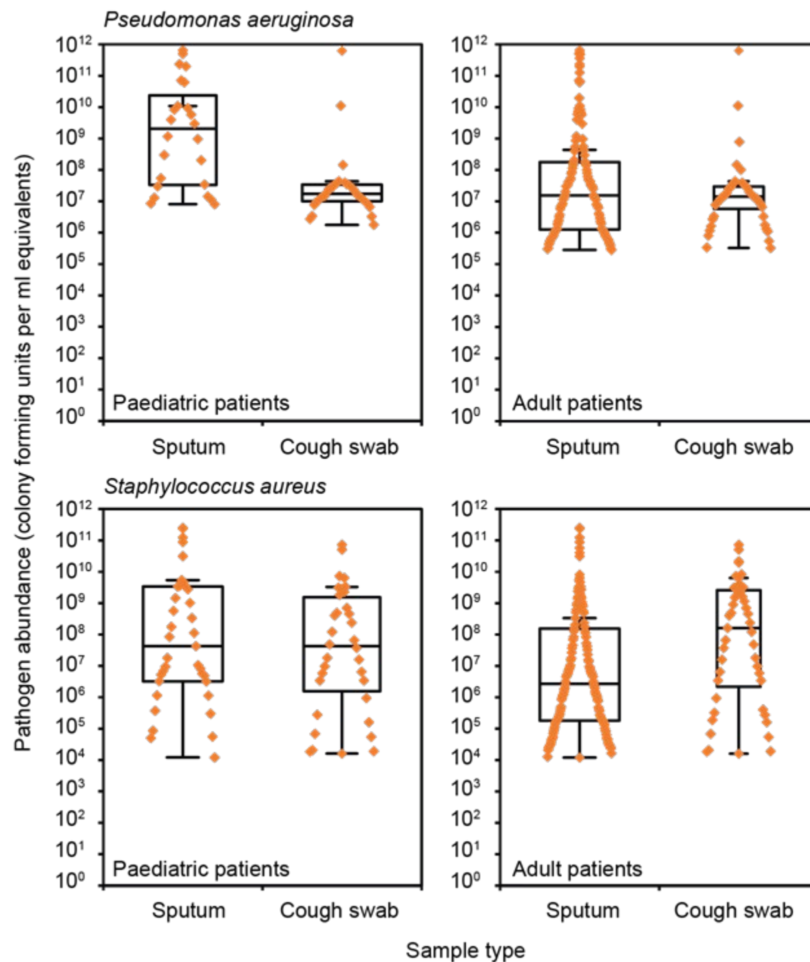

Figure S3.2 Comparison of pathogen abundance within paediatric and adult patients by underpinning respiratory sample type. Boxplots show 25–75th interquartile (IQR) range with whiskers showing 1·5 times IQR. Orange diamonds indicate individual samples for a given patient. For P. aeruginosa in paediatric patients, mean abundance from sputum and swab, respectively, was 7·93x1010 ± 1·86x1011 (n = 24) and 5·38x1010 ± 2·14x1011 CFU ml-1 equivalents (n = 34); Welch's t-test, t = 0·47, df = 53·4, P = 0·64. In adults, mean P. aeruginosa abundance from sputum and cough swabs was 2·87x1010 ± 1·13x1011 (n = 123) and 3·82x1010 ± 1·82x1011 CFU ml-1 equivalents (n = 48), respectively; t = -0·33, df = 61·4, P = 0·74. For S. aureus in paediatric patients, sputum sample based mean abundance (± SD) was 2·05x1010 ± 5·95x1010 CFU ml-1 equivalents (n = 37) and from cough swabs was 4·50x109 ± 1·49x1010 CFU ml-1 equivalents (n = 34); Welch's t-test, t = 1·58, df = 40·8, P = 0·12. In adult patients, mean S. aureus abundance from sputum and cough swabs was 5·17x109 ± 3·02x1010 (n = 157) and 4·57x109 ± 1·31x1010 CFU ml-1 equivalents (n = 47), respectively; t = 0·37, df = 176·9, P = 0·71.

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Chapter 4: Longitudinal dynamics of the bacterial microbiota in CF patients with mild disease

4.1 Introduction

4.1.1 CF research

Research into the polymicrobial nature of the lung has contributed to improving care and treatment of people with CF, and much of the work is largely focused on cross-sectional studies following severe and declining patients (Cox *et al*., 2010; van der Gast *et al*., 2011; Coburn *et al*., 2015; Cuthbertson *et al*., 2020). Cross-sectional studies have been incredibly useful in demonstrating the impact of infection status and antibiotic treatments on clinical factors, however they can only provide a glimpse into a single point in time, and do little to investigate the fluctuating nature of the microbiome, and the mechanisms by which the impact on health occurs. To address these issues, longitudinal studies are becoming more prevalent, and are demonstrating key aspects of the lung microbiome (Abbott *et al*., 2015; Metzger *et al*., 2021). As such, the fluctuations during and after treatments, the resilience of the microbiome to exacerbation events and the use of antibiotics is now being better understood (Zhao *et al*., 2012; Cuthbertson *et al*., 2016; Whelan *et al*., 2017; Hahn *et al*., 2020; Raghuvanshi *et al*., 2020). Many of these studies, however, have focused on severe disease or the transition to severe disease and the associations with the lung microbiota. Another facet of these studies is the analysis of the microbiome as a whole; partitioning into chronic and intermittently colonising taxa allows researchers to understand the contributions of transient taxa alongside the chronically colonising taxa (van der Gast *et al*., 2011).

4.1.2 Key CF taxa

The presence of some key chronically infecting pathogens are often associated with worsening health and progression of CF lung disease. The CF Trust and CF Foundation maintain patient data registries monitoring commonly occurring pathogens known to be capable of causing severe lung function decline and

worsening outcomes in patients (Cystic Fibrosis Foundation, 2019; Cystic Fibrosis Trust, 2019). These pathogens include *P. aeruginosa, S. aureus, H. influenzae,* the *B. cepacia complex* members, and *Non-tuberculosis mycobacterium*. The exact roles of emerging pathogens such as *S. maltophilia* and *A. xylosoxidans* remains unknown (De Baets *et al*., 2007; Waters *et al*., 2011; Cools *et al*., 2016; Esposito *et al*., 2017). Chronic infection with these pathogens are typically taken as an indication that worsening health will soon follow, however whether this is true for all patients is yet to be determined, particularly in those with milder phenotypes.

4.1.3 The introduction of CF modulator therapy

CFTR modulators have provided significant therapeutic benefit to many patients with CF, and have been shown to decrease hospital visits, improve survival rates, reduce rates of pulmonary exacerbation, and lower the demand for lung transplant (Lopes-Pacheco, 2020). Patients taking CFTR modulators have been shown to have lower inflammation levels and a reduction in *P. aeruginosa* infection, however, persistent infection is not prevented (Hisert *et al*., 2017). *S. aureus* has been found in patients taking CFTR modulators at a similar rate to those who do not, suggesting that the use of modulators does not impact on the acquisition of *S. aureus* (Heltshe *et al*., 2014). The similarities between the CFTR modulator treated patients and mild patients are extremely pertinent, thus further study of mild patients may lend itself to understand and predict infection outcomes following CFTR modulator treatment.

4.1.4 Mild CF lung disease

Longitudinal studies on mild patients are limited and suggest that mild lung disease is defined by fewer exacerbations, better lung function and a stable bacterial microbiota (Zhao *et al*., 2012; Hampton *et al*., 2021). Mild patients tend to be included in studies to increase the range of disease states for comparison and are not often studied alone (e.g. (Zhao *et al*., 2012; Whelan *et al*., 2017; Metzger *et al*.,

2021)). With the data being produced as a result of treatment with these modulators, milder lung disease is looking to become the “new normal” for many patients. With the few papers available on mild CF, research suggests that the mild lung microbiome is more stable than more severe phenotypes, (Zhao *et al*., 2012; Whelan *et al*., 2017), and that *S. aureus* is often a chronic coloniser (Bacci *et al*., 2016; Zhao *et al*., 2020). If mild lung disease is to be the predominant phenotype for patients on CFTR modulators, more research needs to be conducted in order to understand the dynamics of the mild microbiome and how this could impact clinical outcomes.

4.1.5 Aims and objectives

It is hoped that CFTR modulators will encourage CF patients towards a mild disease state and reduce the number of patients with severe lung disease (Heijerman *et al*., 2019; Middleton *et al*., 2019; Sosinski *et al*., 2021). In anticipation of this, more research must be done to better inform practice and treatment. In this chapter, I investigated the composition and key features of the mild bacterial microbiota in both adults and paediatric patients over a period of three years. Samples analysed represented 20 adult and 20 paediatric patients. The aims and objectives of this study were to determine if mild disease lends itself to a more “stable” lung microbiome, by examining the diversity and composition of the microbiome, as well as turnover, the loss and gain of species and the prevalence of emblematic CF pathogens.

4.2 Materials and Methods

Methods are outlined in Chapter 2 “Materials and Methods”. Patient details can be seen in Table 2.1. The cohort was made up of 20 adult and 20 paediatric patients, all with stable mild (19 paediatric and 13 adult) or moderate (1 paediatric and 7 adult) CF as determined by their predicted $\%FEV_1$. All patients provided a minimum of 6 samples over a 3-year period as detailed in Chapter 2; Table 2.2 and 2.3.

4.3 Results

To characterise, compare, and elucidate long-term changes, the prevalence of key CF pathogens and differences in community composition, dynamics and diversity was compared between adult and paediatric patients.

4.3.1 Presence and prevalence of key CF pathogens

Chronic colonisation was defined in accordance with a modified version of the Leeds criteria for *P. aeruginosa* infection (Lee *et al*., 2003), where if more than 50% of samples were positive across the 3-year period, this was defined as a chronic infection, and if ≤50% of samples were positive this was an intermittent infection. Prevalence of emblematic pathogens was considerably lower than expected in our patients. Of the 20 adult patients, 15 (75%) were chronically colonised with at least 1 emblematic pathogen, with *P. aeruginosa* being the most commonly occurring chronic pathogen, infecting 11 patients (Figure 4.1). Out of the 20 paediatric patients, only 4 were chronically colonised with at least one emblematic pathogen, and again, *P. aeruginosa* was the most commonly occurring pathogen, infecting 4 patients chronically and 12 intermittently (Figure 4.2). For both groups the second most commonly encountered pathogen was *S. aureus,* with chronic colonisation in 9 adult patients (45%) and 3 paediatric patients (15%).

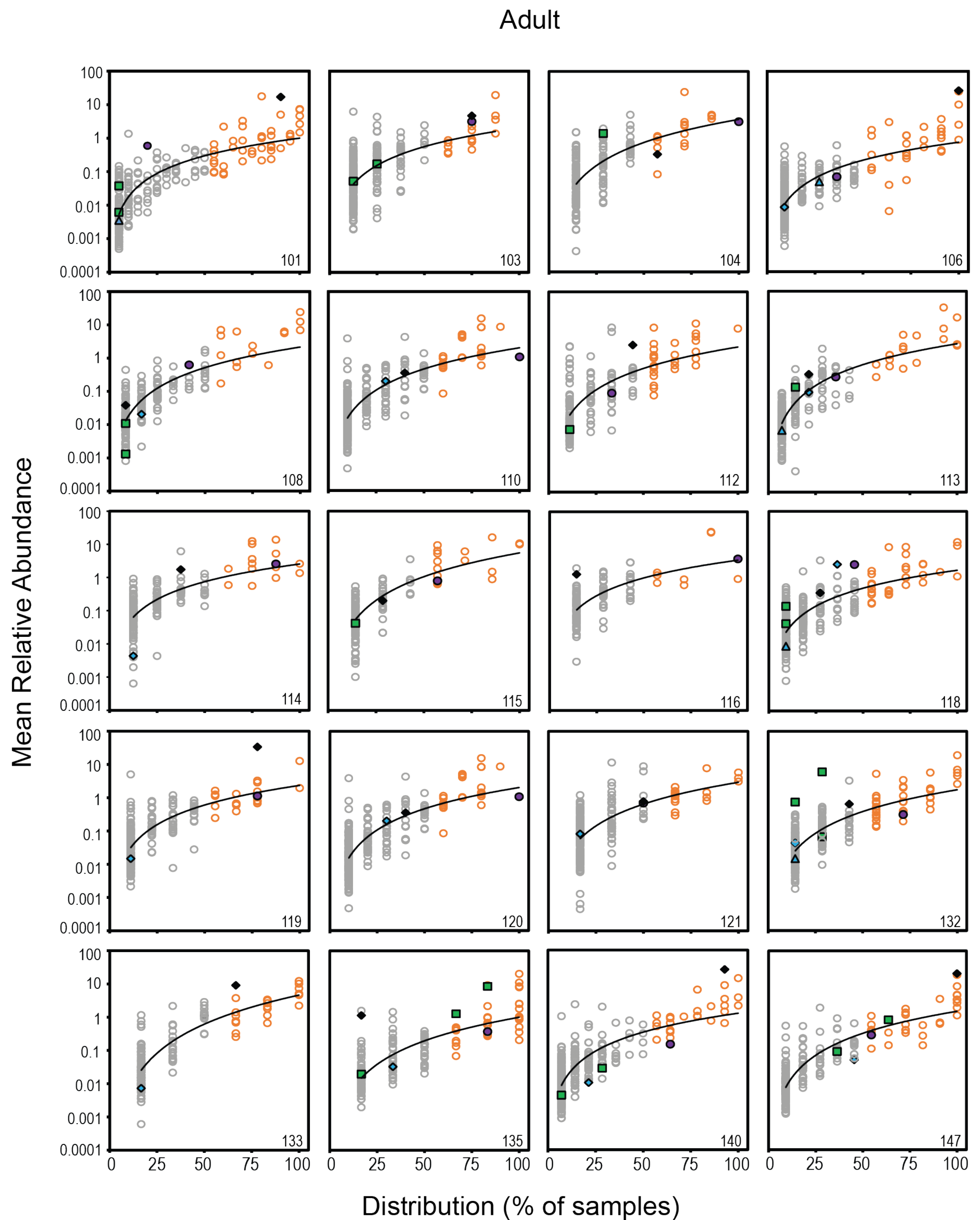

Figure 4.4 Temporal distribution and relative abundance of bacterial taxa across all adult patients. Emblematic pathogens are highlighted as follows: *P. aeruginosa* (purple circle), *S. aureus* (black diamond), *H. influenzae* (light blue triangle), *B. cepacia* complex *members* (green square) and *S. maltophilia* (blue diamond). Chronically infecting taxa are defined as species present in more than 50% of samples (orange circles), and intermittently infecting taxa (50% or less) as grey circle. *B. cepacia* complex members are highlighted as individuals due to read depth not being sufficient.

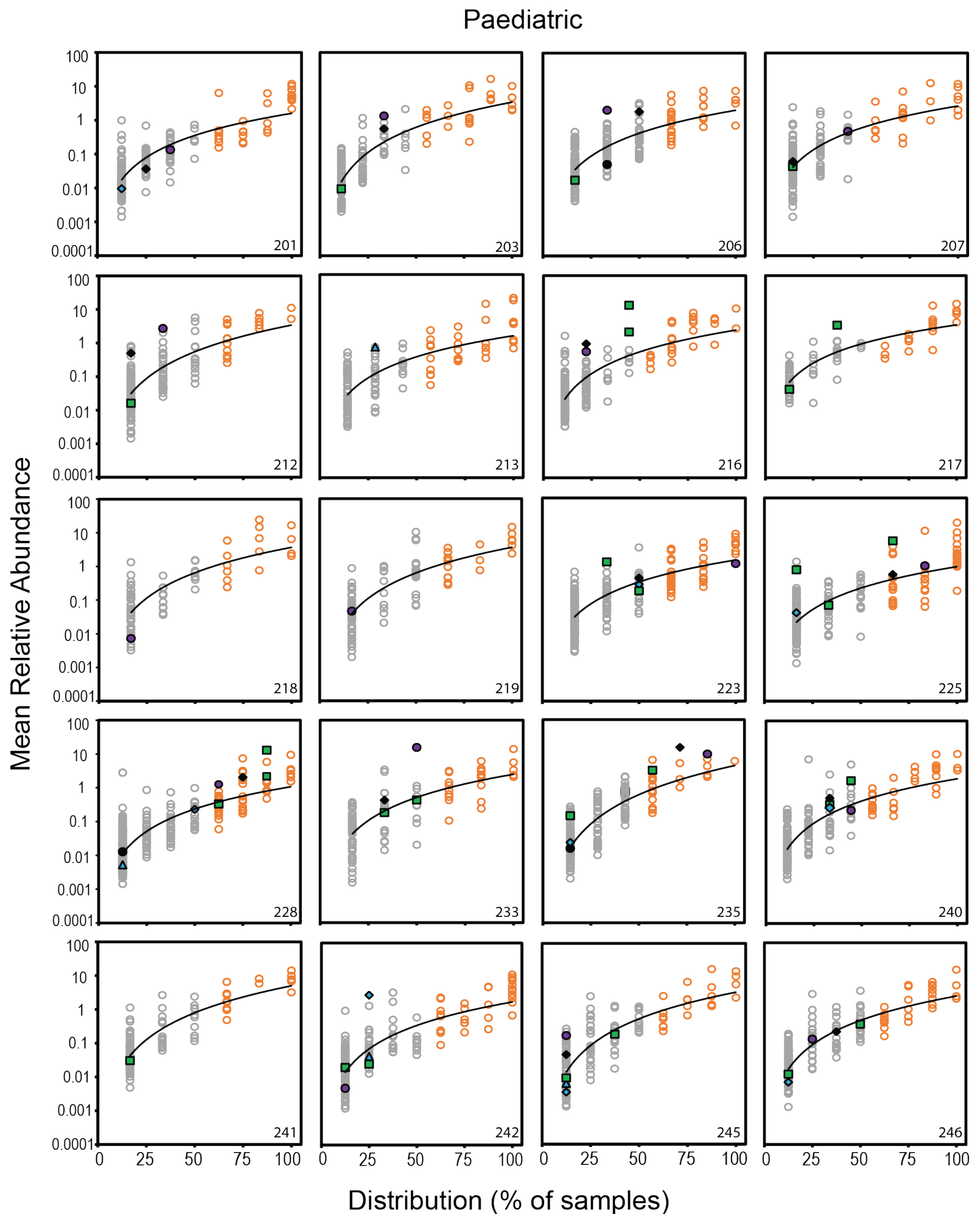

Figure 4.5 Temporal distribution and relative abundance of bacterial taxa across all paediatric patients. Emblematic pathogens are highlighted as follows: *P. aeruginosa* (purple circle), *S. aureus* (black diamond), *H. influenzae* (light blue triangle), *B. cepacia* complex *members* (green square) and *S. maltophilia* (blue diamond). Chronically infecting taxa are defined as species present in more than 50% of samples (orange circles), and intermittently infecting taxa (50% or less) as grey circle. *B. cepacia* complex members are highlighted as individuals due to read depth not being sufficient.

Intermittent colonisation, particularly with emblematic pathogens was frequently observed across all patients. *S. maltophilia* was a common intermittent coloniser in 13 adults, followed interestingly, by *S. aureus,* with 10 adults having an intermittent infection. Likewise, *P. aeruginosa* and *B. cepacia* complex members were frequent intermittent colonisers in 12 and 10 paediatric patients, respectively.

4.3.2 Diversity

Regarding diversity, there were clear differences between the general, chronic, and intermittent taxa (Figure 4.3). Comparing Fishers alpha scores between adult and paediatric cohorts, there were no significant differences between general microbiome, chronic or intermittent colonisers (T-test; microbiome: t=2.039, DF=31, p=0.729, chronic taxa: t=2.024, DF=38, p=0.13 and intermittent taxa: t=2.04, DF=30, p=0.54 respectively). Diversity appears to have its own individual mark on each patient with no distinct pattern or uniformity between patients, more so between paediatric patients than adults. Diversity scores were typically low for the chronic taxa but raised for the intermittent taxa. Mean diversity scores (± standard deviation throughout) for the general microbiome, chronic or intermittently colonising taxa were similar between paediatric and adult patients (6.6 ± 2.1/ 6.4 ± 1.3 for the general microbiome, 2.6 ± 0.8/ 2.2 ± 0.9 for the chronic taxa and 4.4 ± 1.7/ 4.7 ± 0.9 for the intermittent taxa [all paediatric/adult respectively]).

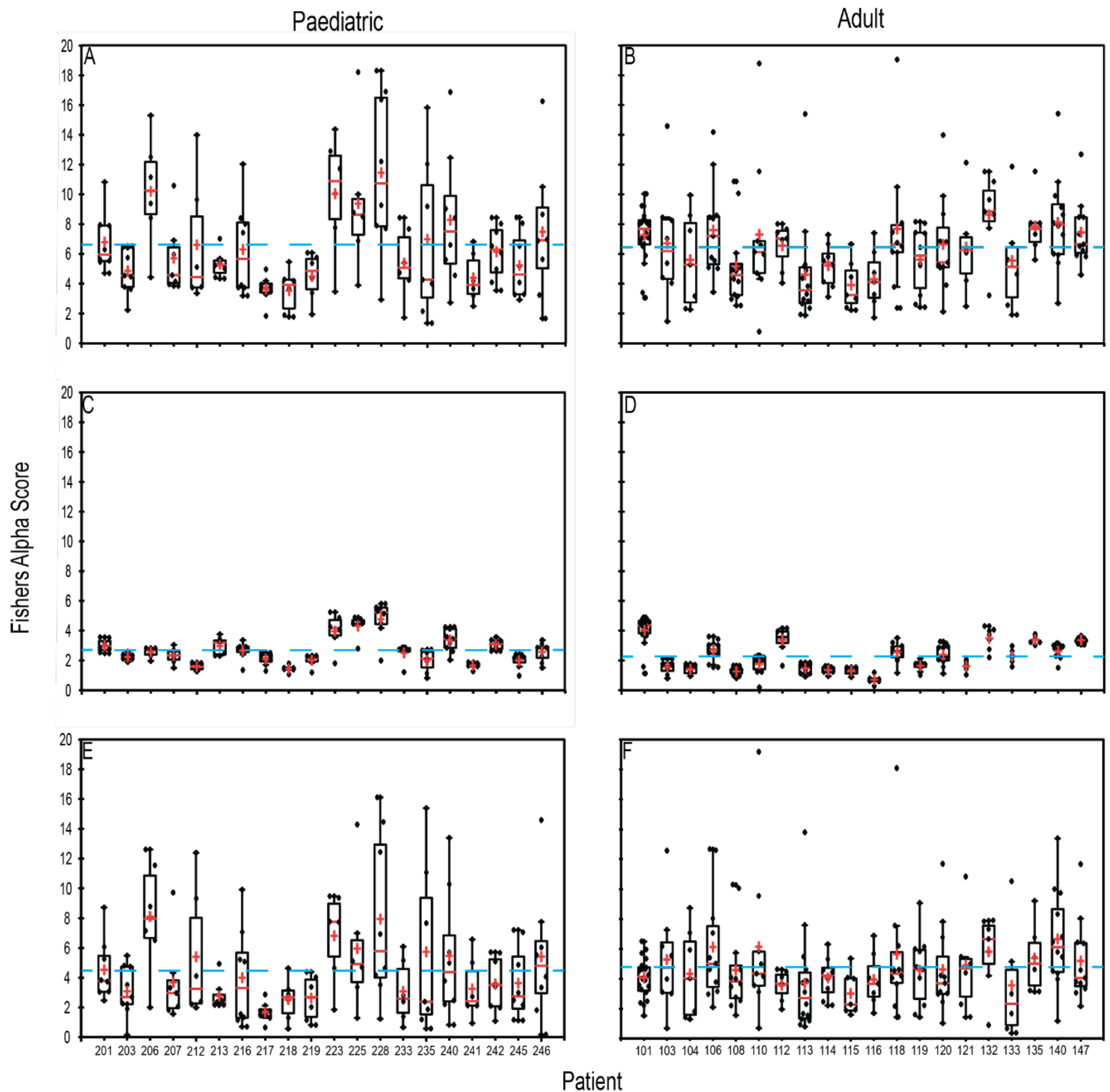

Figure 4.6 Fishers alpha diversity across the whole bacterial microbiota, the chronically colonising and intermittently colonising taxa. Panels A and B illustrate the diversity across the general microbiota, panels C and D the chronically colonising taxa, while E and F illustrate the intermittently colonising taxa. Mean diversity is represented by the blue dashed line.

4.3.3 Differences in Microbiome composition

SIMPER analysis of all taxa found an overall mean dissimilarity of 84.33%, 76% dissimilarity between paediatric patients and 83.37% dissimilarity between adult patients, demonstrating the unique nature of the microbiome to each patient. Taking the 50% cut-off from the chronic and intermittent colonisation criteria and applying this to our SIMPER results, only 14 taxa contributed to the top 50% between cohorts, and *S. aureus* was the only emblematic pathogen to make a significant contribution (6.9%). This was driven by the adult cohort, as *S. aureus* was the top contributor between patients with a contribution of 9.6% (out of 11 species). Between paediatric patients, *S. aureus* only contributed 1.07% (bottom 50%). The only emblematic pathogen in the top 50% for paediatric patients was the *B. cepacia* complex (grouped for this analysis) with a contribution of 3.3% (out of 12 top contributing taxa).

Mean OTU read numbers between adults and paediatric patients showed that adults had significantly higher prevalence of *P. melaninogenica* (T-test: t=2.07, DF=22.7, p=0.002)*, S. aureus* (T-test: t=2.09, DF=19.1, p=0.031), *and P. puraquae* (T-test: t=2.08, DF=20, p=<0.001). A chronic taxon in all but one adult patient, *P. melaninogenica* makes up significantly more of the microbiome in adults, making up on average 10.3% (± 6.65) of the adult microbiome, but only 6.4% (± 4.6) of the paediatric microbiome (T-test: t=2.033, DF=33.8, p=0.043). *P. melaninogenica* also had a positive relationship trend (however not significant) with $\%FEV_1$ in adults (Spearman's rank correlation: Rho= 0.69, p=0.09).

4.3.3.1 Chronic and Intermittent taxa

Partitioning the microbiome into its chronic and intermittent taxa, the differences between the adult and paediatric chronic taxa were driven by nine taxa, with the highest contributions from *P. melaninogenica* and *S. aureus* (10.3% and 8.3%

respectively). Adults had significantly higher levels of *P. melaninogenica* and *S. aureus* (T-test; *P. melaninogenica:* t=2.07, DF=22.7, p=0.002, *S. aureus: t*=2.092, DF=19.1, p=0.033). No other emblematic pathogen contributed to the top 50%. When analysing the intermittent taxa however, four emblematic pathogens contributed to the top 50% of differences between adult and paediatric patients, namely *P. aeruginosa* (1.8%), *Burkholderia spp.* (1.4%), *S. aureus* (0.8%), and *S. maltophilia* (0.6%).

4.3.3.2 Turnover between samples

To investigate the fluctuations in community composition between samples, turnover was calculated for each patient. Rates of turnover appeared to be largely unique to each patient; however, there were overarching trends that can be elucidated from the data (Figure 4.4). Mean % turnover was significantly lower in paediatric patients across the general microbiome (T-test: t=2.027, DF=36.47, p=0.025), however the overall trends for the general microbiome in both groups was driven by the high turnover in the intermittent taxa (mean % turnover: 86.27% ± 6.95% and 86.6% ± 7.23%, adult and paediatric patients respectively). Mean % turnover in the chronic taxa was relatively low in comparison, (25.5% ± 7.1% and 22.3% ± 5.8%, adult and paediatric patient's respectively). Between adult paediatric patients, no significant differences were found in the chronic or intermittent taxa (T-test; Chronic taxa: t=2.027, DF= 36.5, p=0.146, and intermittent taxa: t=2.024, DF= 37.9, p=0.887).

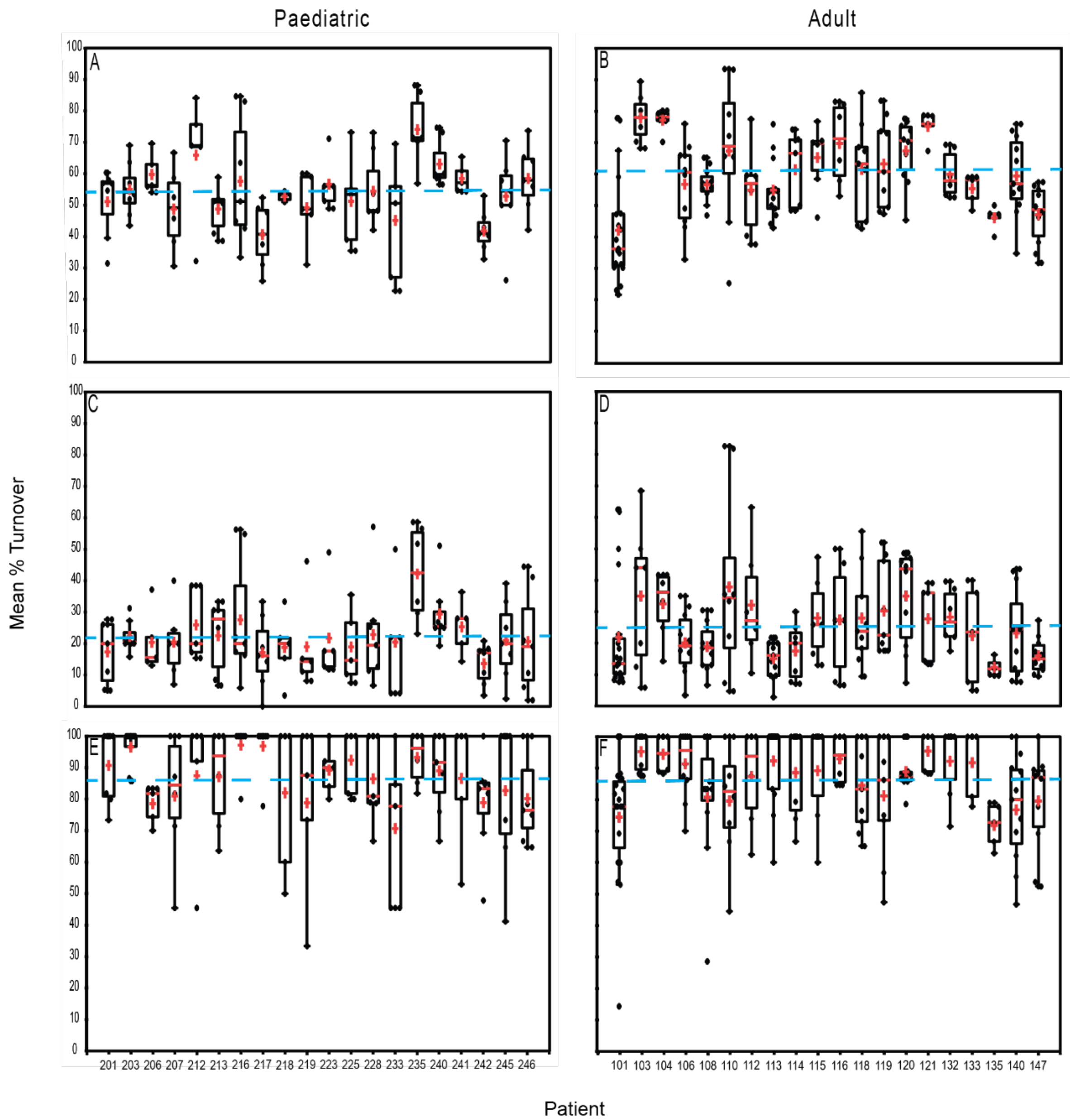

Figure 4.7 Mean turnover for individual patients. The first column denotes the paediatric patients, while the second denotes the adult patients. Panels A and B show turnover for the whole microbiome, panels C and D show turnover for the chronically colonising taxa, and panels E and F show turnover for the intermittently colonising taxa. Mean % turnover is represented by the dashed blue line.

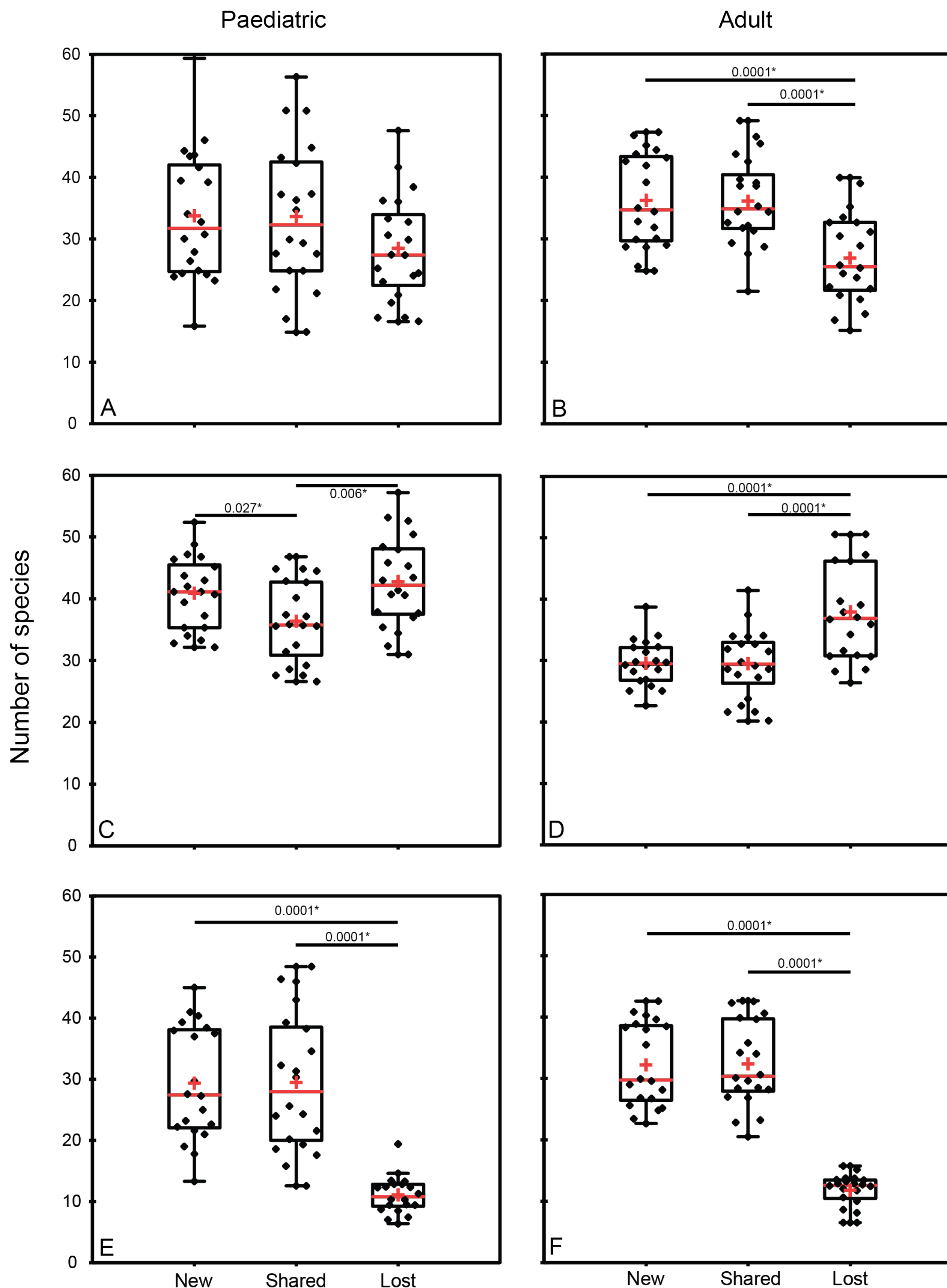

Figure 4.8 Mean loss, gain and shared species between samples across all patients. The left column denotes paediatric patients, and adults to the right. Panels A and B show the rates in the general microbiome, panels C and D the chronically colonising taxa and panels E and F the intermittently colonising taxa. Red crosses denote the mean for each category.

Turnover had no correlation with %FEV_1 in either adult or paediatric patients across the overall microbiome, chronic or intermittent taxa (Spearman's rank correlation: Adult microbiome [Rho= 0.042, p=0.385], chronic [Rho= 0.077, p=0.237] and Intermittent taxa [Rho= 0.059, p=0.299]; Paediatric microbiome [Rho= 0.001, p=0.911] chronic [Rho=0.00, p=0.947] and intermittent taxa [Rho= 0.126, p=0.125]).

4.3.5.2 Community composition and similarity

To understand these results, the actual loss, gain and sharing of species was also calculated (Figure 4.5). The loss of species was significantly lower in the intermittent taxa for both adults and paediatric patients compared to the general microbiome (T-test, adult; t=2.06, DF=23, p=<0.001, and paediatric patients; t=2.07, DF=24, p=<0.001). Acquisition of new species in adult patients was significantly lower in the chronic taxa than the intermittent taxa when compared to the general microbiome, demonstrating that introduction of new species is driven by the transient taxa (T=test; general compared to chronic, t=2.04, DF=28, p=0.001; general compared to intermittent, t=2.02, DF=38, p=0.08). For paediatric patients, there were no significant differences between the overall microbiome and the chronic or intermittent taxa in terms of new species, suggesting that the microbiome is still in a state of flux, and has not established itself as of yet.

4.4. Discussion

Research on CF lung disease has focussed on the prevention of chronic colonisation with several key pathogens, such as *P. aeruginosa* and *S. aureus*, with their prevalence (McCulloch *et al*., 2011; Junge *et al*., 2016; Crull *et al*., 2018), interactions (Hoffman *et al*., 2006; Baldan *et al*., 2014; Ahlgren *et al*., 2015b) and infection mechanisms (Arvidson and Tegmark, 2001; Goerke and Wolz, 2010;

Curran *et al*., 2018) rigorously investigated. Shifts in clinical outcomes, hospitalisations and microbiome composition are anticipated in the wake of new and more effective CFTR modulators. It is suggested that these milder phenotypes brought on by CFTR treatment experience chronic *S. aureus* infection and lower but consistent levels of *P. aeruginosa* (Ahlgren *et al*., 2015b; Bacci *et al*., 2016; Hisert *et al*., 2017; Ronan *et al*., 2018; Zhao *et al*., 2020) with fewer exacerbations. The mild microbiome has not been subject to the same scrutiny as severe or declining patients, but the few studies available do show a "stable" microbiome, with a tolerance for long term *S. aureus* infection (Ahlgren *et al*., 2015b; Hogan *et al*., 2016; Junge *et al*., 2016). The concordance between CFTR modulator outcomes and mild phenotypes in CF indicates that CFTR modulators promote a milder disease state, and investigation of the mild microbiome could allow for better quality of care.

4.4.1 Presence and prevalence of Emblematic Pathogens

All patients were either chronically or intermittently colonised with *P. aeruginosa* and *S. aureus*, with 55% of the adult patients chronically colonised with *P. aeruginosa*. This is consistent with current knowledge, in that up to 50% of adults across the UK have a *P. aeruginosa* infection (Cystic Fibrosis Trust, 2019). Likewise, *S. aureus* was a persistent infection across all patients, in agreement with previous mild studies and CFTR modulation outcomes. There was at least one chronically colonising key CF pathogen in 75% of all adults, demonstrating that regardless of how stable or mild a patient is, they are still likely to have a chronically colonising CF pathogen. The remaining key CF pathogens were seen as intermittent infections across all patients, with the most common transient infections caused by *S. maltophilia* and *Burkholderia spp.*

The frequency and severity of infection with *S. aureus* and *P. aeruginosa,* combined with the absence of observable lung function decline, brings into question how the

microbiome tolerates these infections without consequence. Studies have found that as patients age, the risk of adverse events as a result of *S. aureus* infection declines, and that infection with *S. aureus* before transplant is a marker of better survival post-transplant (Ahlgren *et al.*, 2015). This brings the role of *S. aureus* into question, as well as community interactions and whether the strain of *S. aureus* is a key determining factor into the type of “infection” seen in patients. Several hypotheses can be drawn, such as whether the composition of the remaining chronic colonisers are providing protection or reducing the virulence of these key pathogens, or if the key pathogens are simply a less virulent strain. Another possibility is the external influence of medication; no patient was on long-term anti-pseudomonal medication over the 3-year period, whereas any indication of *P. aeruginosa* infection typically results in a course of anti-*pseudomonal* drugs. In response to treatment, bacteria typically seek out ways to become more resilient and as a result, can become more virulent. A study by (Quinn *et al*., 2016), demonstrated that *P.* aeruginosa can be readily detected by both culture- and molecular-based methods, however that this taxon may not be actively growing or producing invasive metabolites in the sample, and as such may not be the cause of infection simply by presence alone. Without the pressure to acquire more survival mechanisms, perhaps the strains of *P. aeruginosa* in these patients remained in a less virulent state.

4.4.2 Impact of key CF pathogens

SIMPER analysis of the bacterial microbiota revealed that despite being a chronic coloniser in many patients, *P. aeruginosa* was not a key component in the overall composition. Conversely, *S. aureus* was a key taxon in the difference between adult and paediatric microbiota composition, however there was a notable amount of intra-patient variability in the adult cohort that demonstrates that while *S. aureus* is

more likely to be present in adult samples, this is not observed across all individuals. The notable finding in this analysis was that the key contributor to overall composition was the anaerobe *P. melaninogenica*. *Prevotella spp.* has been noted as more prevalent in some studies on milder patients (Muhlebach *et al*., 2018a; Lamoureux *et al*., 2021b), and its role in CF lung disease is conflicting, with some studies showing anti-inflammatory properties, but also providing other taxa protection from antibiotic treatments (Field *et al*., 2010; Sherrard *et al*., 2013). The conflicting research into the role of anaerobes demonstrates the multiple roles these taxa can play in communities, however the data presented here suggests that *P. melaninogenica* may be a key component of the mild lung, however more work needs to be done to fully understand its role.

4.4.3 Diversity and Community composition.

Stability in terms of composition is difficult to define, as intra-patient variability is such that no two microbiomes are the same. Instead, stability must be measured in a different way. Clear definitions of a “stable” microbiome need to be established in order to utilise this term across CF research. Previous research has described the mild microbiome as “stable”. These definitions are based on lower variation in community composition (Françoise and Héry-Arnaud, 2020; Hampton *et al*., 2021). It is clear from our data that the chronically colonising taxa are “stable” under those definitions, whereas the intermittent taxa are undergoing constant fluctuations and change. The main driver of change in diversity measures across all patients were the intermittent taxa, and there were no differences between adults and paediatric patients in terms of diversity and turnover in any partitioning of the microbiome. Interestingly, the driving force behind the high levels of turnover in the intermittent taxa was due to acquisition of new species and shifts in abundance rather than a complete remodelling of the community.

4.5 Conclusion

CFTR modulators have the potential to push many CF patients into a milder phenotype and improve quality of life for many. As such, it is imperative that we better understand the mild CF lung in order to maintain quality of care. This data shows that “stable” cannot be applied to the whole microbiome, and that correct application of this term needs to be agreed upon for use across CF research.

Key CF pathogens were found across all patients, however the presence of persistent *P. aeruginosa* or *S. aureus* infection did not amount to worsening lung function over the 3 years. In this, we can say our results agree with previous studies, in that these infections are well tolerated and do not cause significant loss of lung function. This further brings into question the true impact of being infected with these potential pathogens, and the role of the surrounding microbiome in keeping these pathogens in check. Anaerobes were a significant factor in the composition of the adult microbiota and had a significant presence across both paediatric and adult cohorts. This data suggests that anaerobes such as *P. melaninogenica* may be crucial in maintaining a mild phenotype and can promote and maintain a “healthy” microbiome.

These results confirm some previous work into mild CF and raise new questions about the dynamics between taxa in the microbiome, the true impact of harsh antibiotic therapies and what is defined as a “stable” microbiome.

Chapter 5: Longitudinal dynamics of the fungal microbiota in CF patients with mild disease

5.1 Introduction

Despite making up less than 0.1% of the human microbiome (Qin *et al*., 2010), the fungal microbiota of the lung has become of increasing interest in recent years. The rise of antifungal resistance and a growing understanding that fungi may play a bigger role than previously expected, has meant it is difficult to ignore the fungal component of the CF lung any longer (Underhill and Iliev, 2014; Wiederhold, 2017). Detection of fungi in clinic is conducted much the same as for bacteria, where clinical mycology utilises culture-based detection methods to determine the presence of yeasts and molds (Kozel and Wickes, 2014). Research into the fungal microbiota has followed suit, with many studies utilising culture to detect fungi in patients (de Dios Caballero *et al*., 2016; Ziesing *et al*., 2016; Al Shakirchi *et al*., 2020), however NGS methods are being increasingly used due to the sensitivity and efficiency that culture cannot achieve (Nagano *et al*., 2010; Cuthbertson *et al*., 2021). As such, much of our knowledge on fungal prevalence and efficiency of treatment in CF is based on cultural detection methods.

Comparative analyses of the bacterial and fungal biota in the CF lung are rare, however the studies that are available have found that there does not appear to be many significant correlations between the two (Kramer *et al*., 2015; Krause *et al*., 2016). Microbiota based studies have found that *Candida albicans* often co-occurs with *P. aeruginosa*, however this may simply be due to it being likely to chronically infect, rather than as a true finding (Delhaes *et al*., 2012; Güngör *et al*., 2013; Krause *et al*., 2016). Some *in vitro* studies have tried to determine connections between the bacterial and fungal biota, with *P. aeruginosa* being shown to reduce the ability of *Aspergillus fumigatus* to grow and form biofilms, and conversely *A. fumigatus* has been shown to inhibit *P. aeruginosa* (Reece *et al*., 2018; Sass *et al*., 2019). Similarly, *P. aeruginosa* has been demonstrated to effect *C. albicans* growth in a strain

dependent manner (McAlester *et al*., 2008), and can encourage *C. albicans* to form a biofilm (Kasetty *et al*., 2021). While it is important to demonstrate how these taxa interact, it is unknown if these observed effects occur *in vivo*. Studies solely focusing on fungi is far and few between with inconsistent detection methods (Masoud-Landgraf *et al*., 2013). Focus on patients with explicit fungal disease and relatively few attempts to find correlations with the bacterial microbiota makes data increasingly difficult to consolidate.

The role of fungi in CF lung disease is largely unknown, however taxa such as *Candida* and *Aspergillus* have been associated with pulmonary exacerbations (Soret *et al*., 2020), both of which have become emblematic pathogens in CF, alongside *Exophiala dermatitidis, Scedosporium spp. and Malassezei spp* (Horré *et al*., 2004a; Pihet *et al*., 2009; Delhaes *et al*., 2012). Despite several taxa being implicated as important in CF lung disease, *A. fumigatus* is typically the only tracked fungi across CF patient registries (Cystic Fibrosis Trust, 2019; Cystic Fibrosis Foundation, 2020). *Aspergillus spp.* is known to cause a severe allergic reaction (Allergic Bronchopulmonary Aspergillosis or ABPA), and as such has been subject to scrutiny to determine causes and inform treatment (de Vrankrijker *et al*., 2011; Fillaux *et al*., 2014; Maturu and Agarwal, 2015). While these studies highlight the need for further investigation into fungal infection and the appropriate treatments, it is often difficult to determine a fungal cause to infection, in that key bacterial pathogens must be ruled out before treatment can be administered, and these bacterial pathogens can reside in patients without causing infection. Furthermore, there is no current defined diagnostic criteria to determine fungal cause of infection in CF (Schwarz *et al*., 2019; Magee *et al*., 2021).

Research into the fungal microbiota is largely made up of cross-sectional studies, to determine prevalence and confirm detection of specific taxa (Burns and Rolain,

2014; Coron *et al*., 2018). Several studies have followed patients longitudinally and identified fungi in CF patients, however only a few have compared the trends over time (Ziesing *et al*., 2016; Engel *et al*., 2019; Viñado *et al*., 2021). These longitudinal studies have indicated that the prevalence of key taxa fluctuate over time in the same way their bacterial counterparts do, however the key changes in diversity, turnover, and the role of transient taxa, if any, remains poorly understood. Further research is needed to truly demonstrate the fungal microbiota over time, particularly as many published studies have focused on clinically relevant taxa with transient taxa often not reported or considered.

Molecular based methods such as Next Generation Sequencing (NGS) approaches and targeted QPCR are more sensitive and specific than culture-based methods (Stewart, 2012), however in terms of fungal detection, there are issues that need to be resolved. Chiefly amongst these is the use of multiple target regions for Internal Transcribed Spacer (ITS) amplification, with different combinations of targets producing differing specificity and sensitivity, as well as bias towards different phyla and sequence lengths (Bellemain *et al*., 2010; Tipton *et al*., 2017). This lack of uniformity between studies has resulted in a wide range of results that vary depending on the primer pairs used and makes consolidating data difficult to achieve.

The aims of this chapter were to investigate the various aspects of the fungal microbiota over a longitudinal dataset. Samples used in Chapter 4 were further investigated, to determine the prevalence of key fungal taxa in mild CF, the composition and dynamics of the fungal microbiome, and the suitability of primers targeting the ITS2 region for human microbiome work.

5.2 Methods

Fungal analysis was performed on the same DNA extracts used in Chapter 4: “Longitudinal dynamics of the bacterial microbiota in CF patients with mild disease”. ITS sequencing was performed as detailed in Chapter 2, Section 2.6.

5.3 Results

5.3.1 Primers and detection limits

The OTU table generated contained 143 taxa, with a mean of 3.65 (± a standard deviation of the mean = 2.22) taxa per sample. Mean richness in adult patients was 3.27 ± 1.90, and paediatric richness (4.08 ± 2.46) was found to be significantly higher in comparison (T-test, t=2.02, DF=36.02, p=0.017). A total of 25,861 reads were generated, with a mean of 53.4 (± 106.9) reads per sample. Overall, 331 samples were used, comprising of 99 cough swabs and 232 sputum samples. The mean number of detected taxa in sputum samples was 3.22 ± 2.18, and for cough swabs, 3.57 ± 2.61. There was no significant difference between sample types in terms of the relative abundance of taxa (T-test, t=0.244, DF=159, p=0.244).

Pure strains of frequently reported fungal pathogens for CF were sequenced alongside samples to ensure correct identification and to also determine if the primers would detect key taxa. These strains were *Rhodotolura mucilaginosa, Candida albicans, Aspergillus fumigatus, and Scedosporium apiospermum* (Chapter 2: Table 2.4). Sequencing confirmed correct identification of *C. albicans*, and *R. mucilaginosa. S. apiospermum.* While *A. fumigatus* was not confirmed through sequencing, it was detected in sample cultures.

5.3.2 Prevalence of emblematic pathogens

5.3.2.1 Culture based detection of Fungi

Diagnostic mycology for all samples mainly detected the presence of *Candida spp, C. albicans, A. fumigatus* and unidentified fungi (Figure 5.1). Interestingly *C. albicans* was widely detected across the paediatric cohort but only in 1 adult patient by culture, however 18 paediatric and all adult patients were considered chronically colonised by *C. albicans* by NGS, using the modified Leeds criteria employed in Chapter 3 to define chronic and intermittent infection. Similarly, *Candida* species were found in 7 paediatric patients and only 3 adult patients by culture but detected at least once in every patient by NGS. Culture also found 3 paediatric and 4 adults to be intermittently infected with *A. fumigatus,* with a further 3 adults chronically colonised, however NGS only detected intermittent infection in 1 adult and 1 paediatric patient. The detection of "other" taxa (not deemed clinically relevant) in samples were grouped together for this analysis, with culture only finding other yeasts and mold in 4 paediatric and 8 adults. NGS found "other" taxa in all paediatric patients and 17 adult patients at varying levels, with 12 paediatric and 5 adult patients having transient taxa in over 50% of their samples, whereas the remaining patients had very little or no transient taxa.

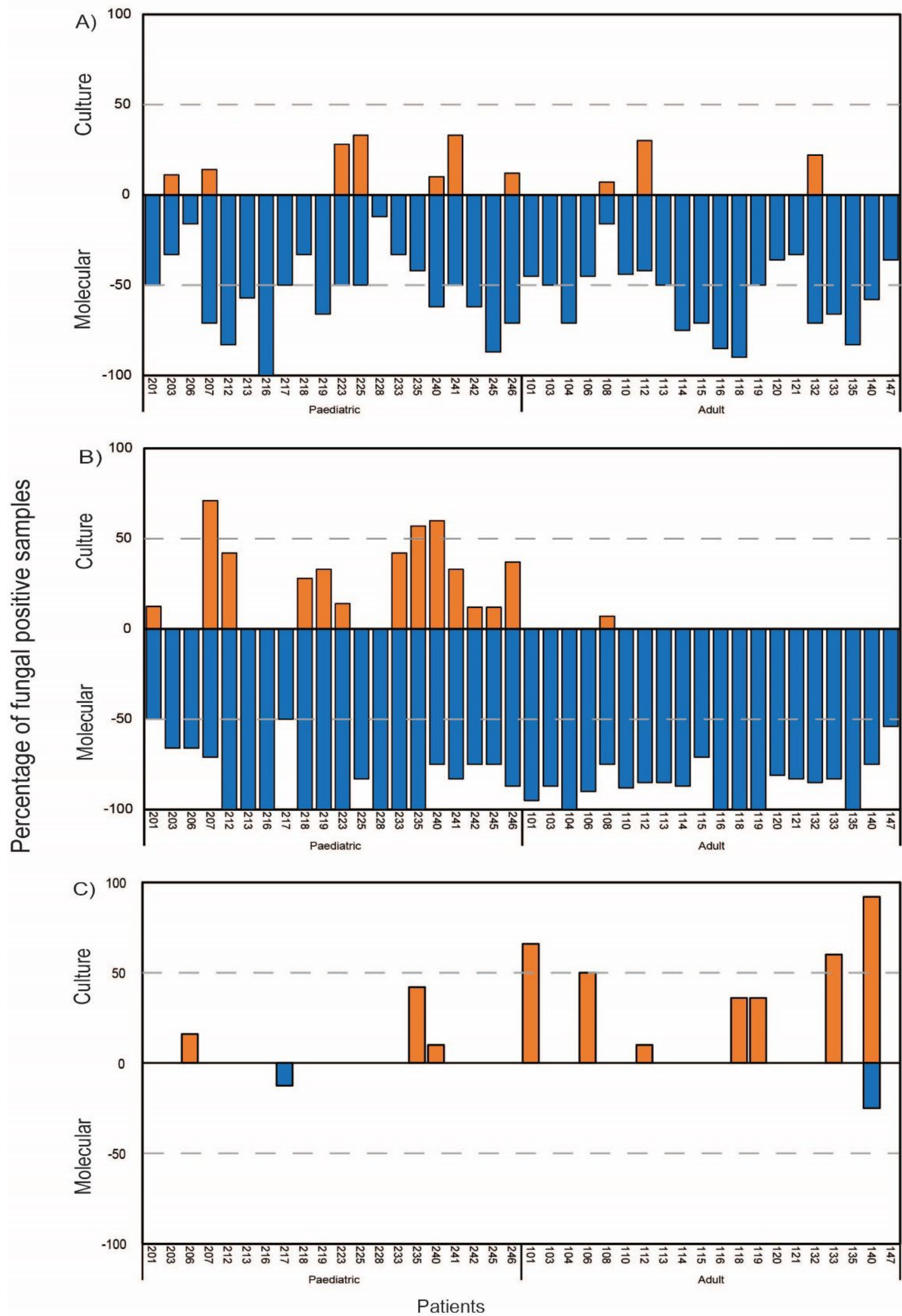

Figure 5.9 Percentage detection of Candida spp. (A), Candida albicans (B), and Aspergillus fumigatus (C) across the three-year period by culture-dependent methods (orange) and Molecular methods (NGS- blue bars) in all patients. The 50% threshold for chronic (>50%) and intermittent colonisation (≤50%) is marked with a dashed line in accordance with the modified Leeds criteria as set out in Chapter 4. Individual patient numbers are marked on the x-axis.

5.3.2.2 Molecular detection of Fungi

Prevalence of fungi was low across all patients, with the majority of patients having only one chronic taxon, and 2 paediatric patients having no chronically colonising pathogens (mean chronically colonising taxa: 1.5 ± 0.72 across all samples) (Figures 5.2 and 5.3). *C. albicans* was the most common chronically colonising pathogen, and a chronic coloniser in all adults and all but 2 paediatric patients, where it was intermittently infecting. For the remaining notable taxa, *E. dermatitidis* and *R. mucilaginosa* chronically infected one adult patient each, whereas *R. mucilaginosa* chronically colonised 8 paediatric patients, and *E. dermatitidis* was found to be an intermittent coloniser across the paediatric cohort. Most transient taxa were unique to each individual, with very few being shared between patients. A total of 34 taxa were found in adult patients and not paediatric, and a further 31 taxa were found in paediatric patients but not adult.

5.3.3 Relative abundance of *Candida* taxa

For the majority of patients, the fungal microbiota was mostly made up of *Candida spp.* however the rates of this were quite dynamic (Figure 5.4). *Candida* was the dominant taxa for most patients, with *C. albicans* representing a mean of 54% ± 20.9% of the total taxa in adults and a significantly lower proportion of 39% ± 22.9% in paediatric patients (T-test, $t=2.02$, $DF= 37.7$, $p = 0.046$), with a further 13% ± 11.1% and 11% ± 9.2% represented by the remaining *Candida* taxa in adult and paediatric patients respectively. A total of 14 *Candida* taxa were found across paediatric patients, and 11 in adults. Four *Candida* taxa were unique to paediatric patients (*C. glaebosa, C. quercitrusa, C. saopaulonensis,* and *C. sojae*), whereas only *C. norvegica* was unique to adults. Shared *Candida* taxa included *C. africana, C. albicans, C. boidinii,* C. *dubliniensis, C. glabrata, C. metapsilosis, C. orthopsilosis, C. parapsilosis, C. sake,* and *C. tropicalis.*

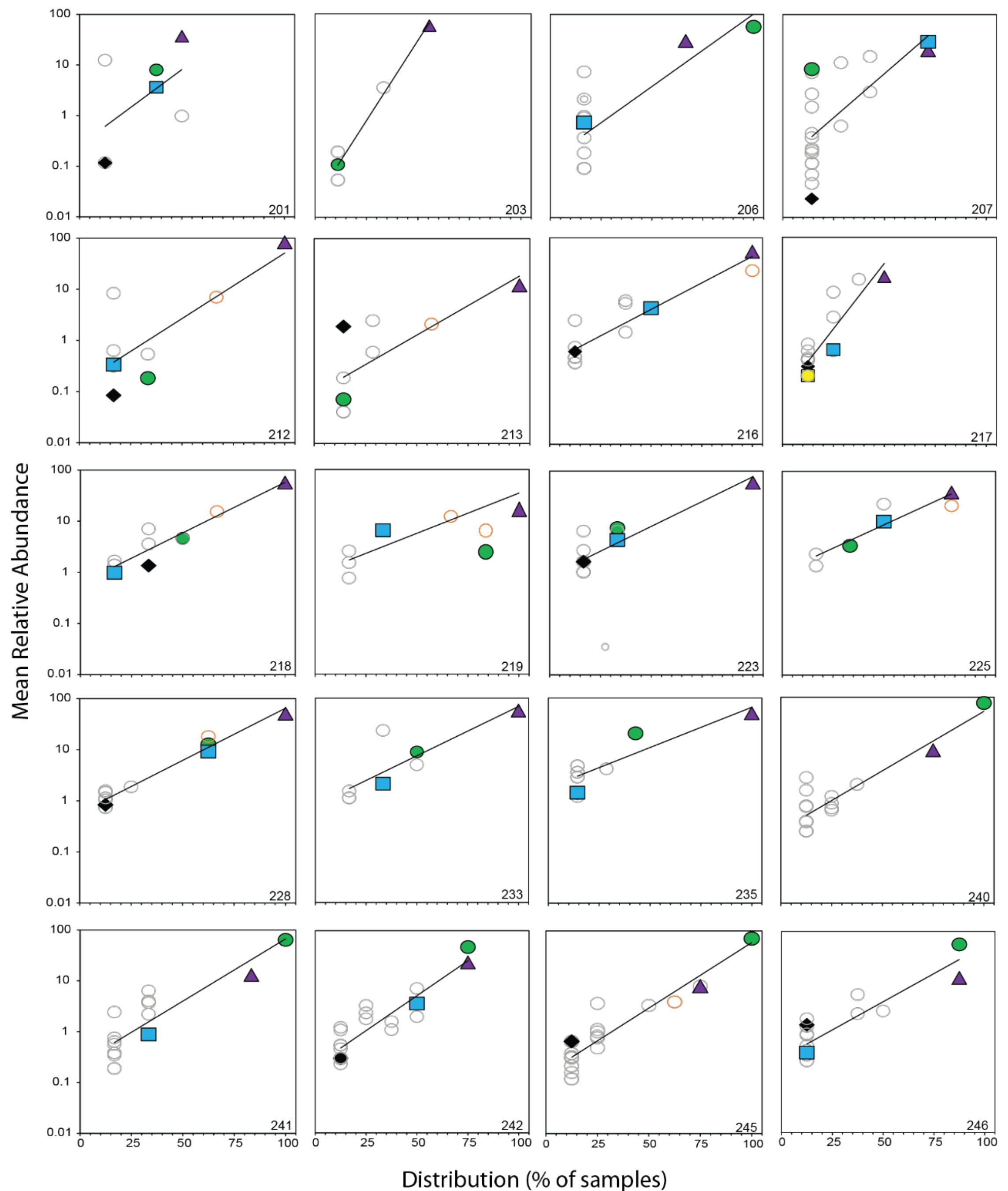

Figure 5.10 Temporal distribution and relative abundance of fungal taxa across all paediatric patients. Emblematic pathogens are highlighted as follows: *C. albicans* (purple triangle), *E. dermatitidis* (black diamond), *R. mucilaginosa* (green circle), *S. cerevisiae* (blue square) and *A. fumigatus* (yellow square). Chronically infecting taxa are defined as species present in more than 50% of samples (orange circles), and intermittently infecting taxa (50% or less) as grey circle.

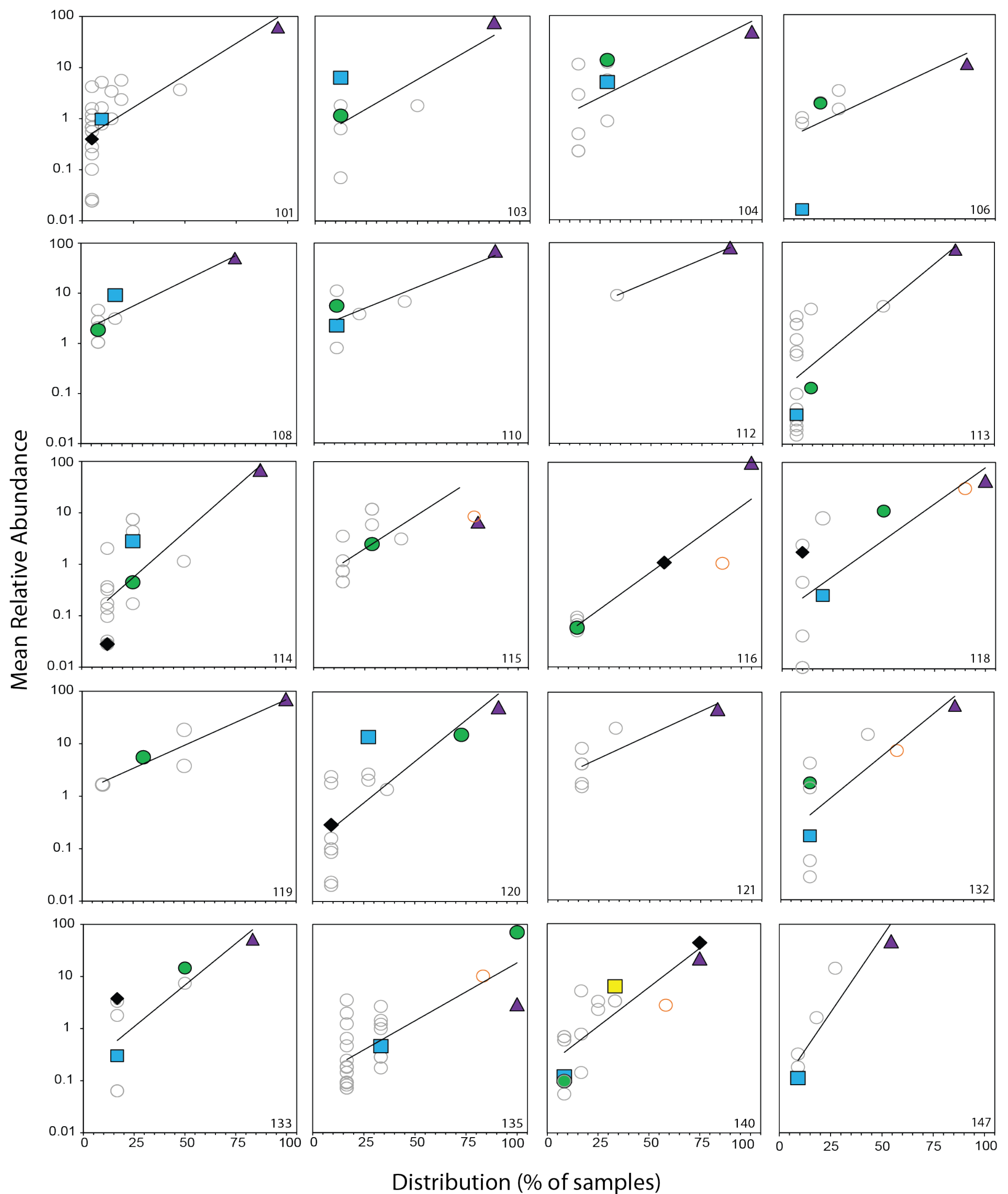

Figure 5.1 Temporal distribution and relative abundance of fungal taxa across all adult patients. Emblematic pathogens are highlighted as follows: *C. albicans* (purple triangle), *E. dermatitidis* (black diamond), *R. mucilaginosa* (green circle), *S. cerevisiae* (blue square) and *A. fumigatus* (yellow square). Chronically infecting taxa are defined as being present in more than 50% of samples (orange circles), and intermittently infecting taxa (50% or less) as grey circle.

5.3.4 Relative abundance of other key taxa

R. mucilaginosa was found across most patients, with 19 paediatric and 16 adult patients having at least one positive sample, however chronic infection was more common in paediatric patients, with 8 patients chronically colonised compared to 2 adult patients. Mean abundance was also significantly higher in paediatric patients, (21.7% (± 25.5%) compared to 7.2% (± 15.2%) in adult patients (T-test, $t=2.03$, $DF=31.02$, $p=0.042$). *R. mucilaginosa* was the dominant taxa in 1 adult and 6 paediatric patients. *S. cerevisiae* was transiently present in 18 paediatric and 16 adult patients, representing 2.6% (± 2.6%) and 1.7% (± 3.12%) of the total taxa in paediatric and adult patients respectively. *E. dermatitidis* was more prevalent in paediatric patients (11 paediatric compared to 7 adults), and 1 adult patient was dominated by *E. dermatitidis*. A notable CF pathogen, *A. fumigatus*, was not confirmed by its control sample, however, was positively identified in 4 samples, 3 of which for 1 adult and the remaining sample for a paediatric patient. For all remaining taxa, three adults were found to have no other taxa beyond the key emblematic pathogens, and out of all 331 samples, 29 had no positive fungal presence, 18 of which were adult samples. Paediatric patients had a significantly higher abundance of transient taxa (mean abundance of 3.14 ± 1.21 taxa per sample, compared to 2.1 ± 0.87 per sample in adult patients, T-test, $t=2.03$, $DF=34.4$, $p=0.009$). Paediatric patients also had a higher prevalence of transient taxa within samples, with an average of 1.31 (± 2.19) taxa per sample compared to 0.89 (± 1.09) for adult patients.

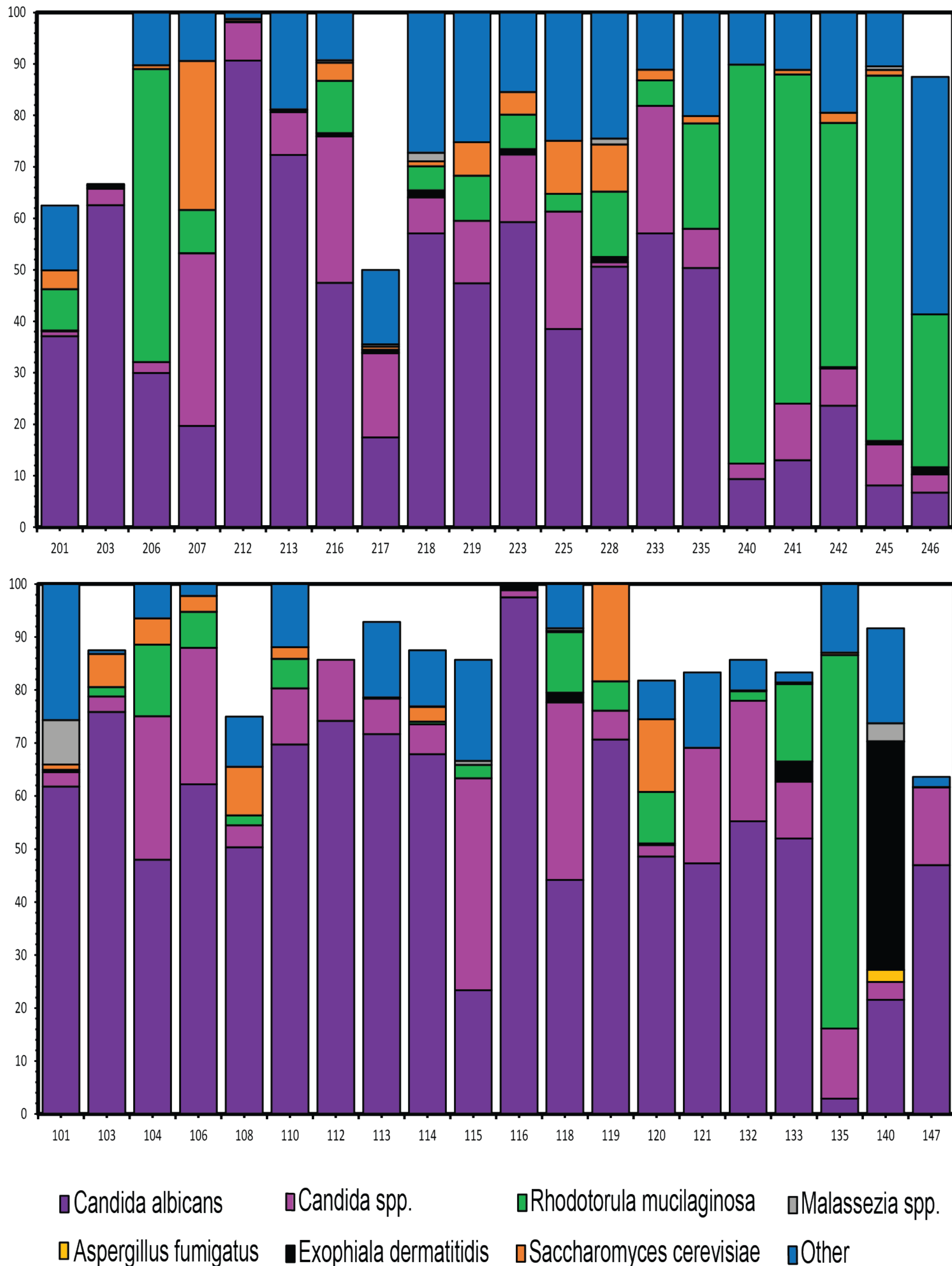

Figure 5.12 Mean percentage abundance of key taxa for each patient over the 3-year period. Patients without 100% coverage had samples with fungi detected. For example, patient 217 had detectable fungi in half of provided samples. Key taxa are highlighted, with non-clinically relevant and transient taxa grouped as “other”. C. albicans has been separated from the other Candida taxa to demonstrate impact.

5.3.5 Diversity and Turnover measures

5.3.5.1 Diversity

Fishers alpha scores across all partitions of the fungal microbiota were low (Figure 5.5). The overall diversity for paediatric patients was 1.9 ± 0.8, significantly higher than the adult patients (1.21 ± 0.5, T-test; t=2.03, DF=32.3, p=0.007). There was no significant difference between the chronically colonising taxa in adult (0.5 ± 0.19) and paediatric patients (0.36 ± 0.04) (T-test, t=2.05, DF=26, p=0.06), however paediatric patients had a significantly higher diversity in their intermittently colonising taxa (Paediatric: 1.4 ± 1.4, Adult: 0.7 ± 0.25, T-test: t=2.057, DF=25.6, p=0.02). The intermittent taxa in both cohorts had significantly higher diversity than the chronic taxa, indicating that the intermittent taxa were the driving force behind the general microbiome trends (Paediatric: T-test, t=2.06, DF=24.2, p=0.007. Adult: t=2.05, DF= 25, p=0.008).

5.3.5.2 Turnover of Fungal taxa

Measurement of turnover between samples showed that changes between samples were driven almost predominately by the intermittent taxa, particularly in paediatric patients (Figure 5.6). There was no significant difference between adult and paediatric patients in terms of the general microbiome, chronic or intermittently colonising taxa (T-test; general microbiome: t=2.026, DF=37, p=0.91, chronic taxa: t=2.026, DF=38, p=0.32, and intermittent taxa: t=2.028, DF=36, p=0.57). The rates of turnover in the intermittent taxa were significantly higher than that of the chronically colonising taxa, demonstrating their impact on the trends seen in the general microbiome (T-test; paediatric: t=2.028, DF=36, p=<0.001, and adult patients: t=2.024, DF=38, p=<0.001).

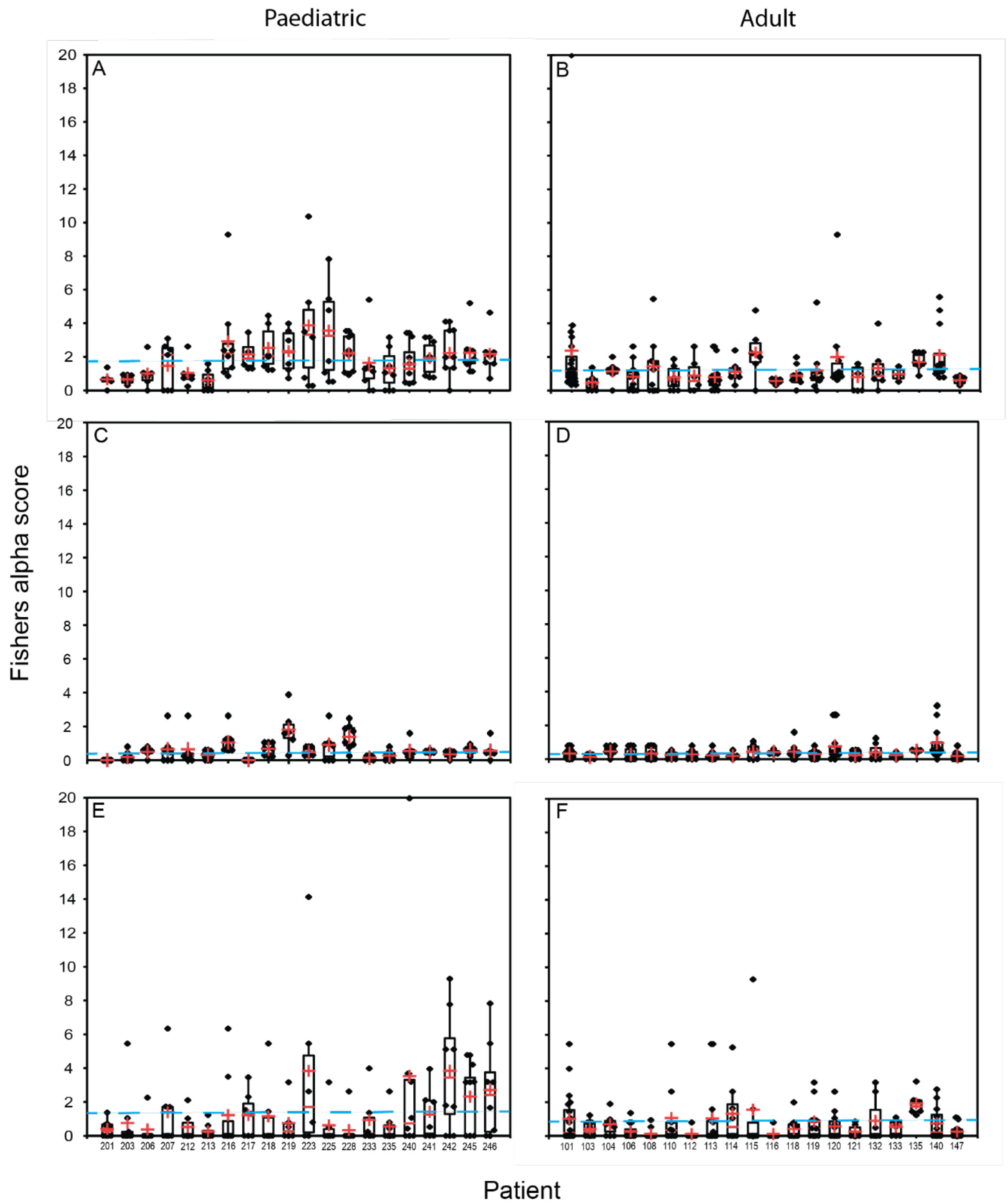

Figure 5.1 Fungal diversity in the various partitions of the microbiome. Panels A and B illustrate the diversity across the general microbiome, panels C and D the chronically colonising taxa, while E and F illustrate the intermittently colonising taxa. Mean diversity is represented by the blue dashed line.

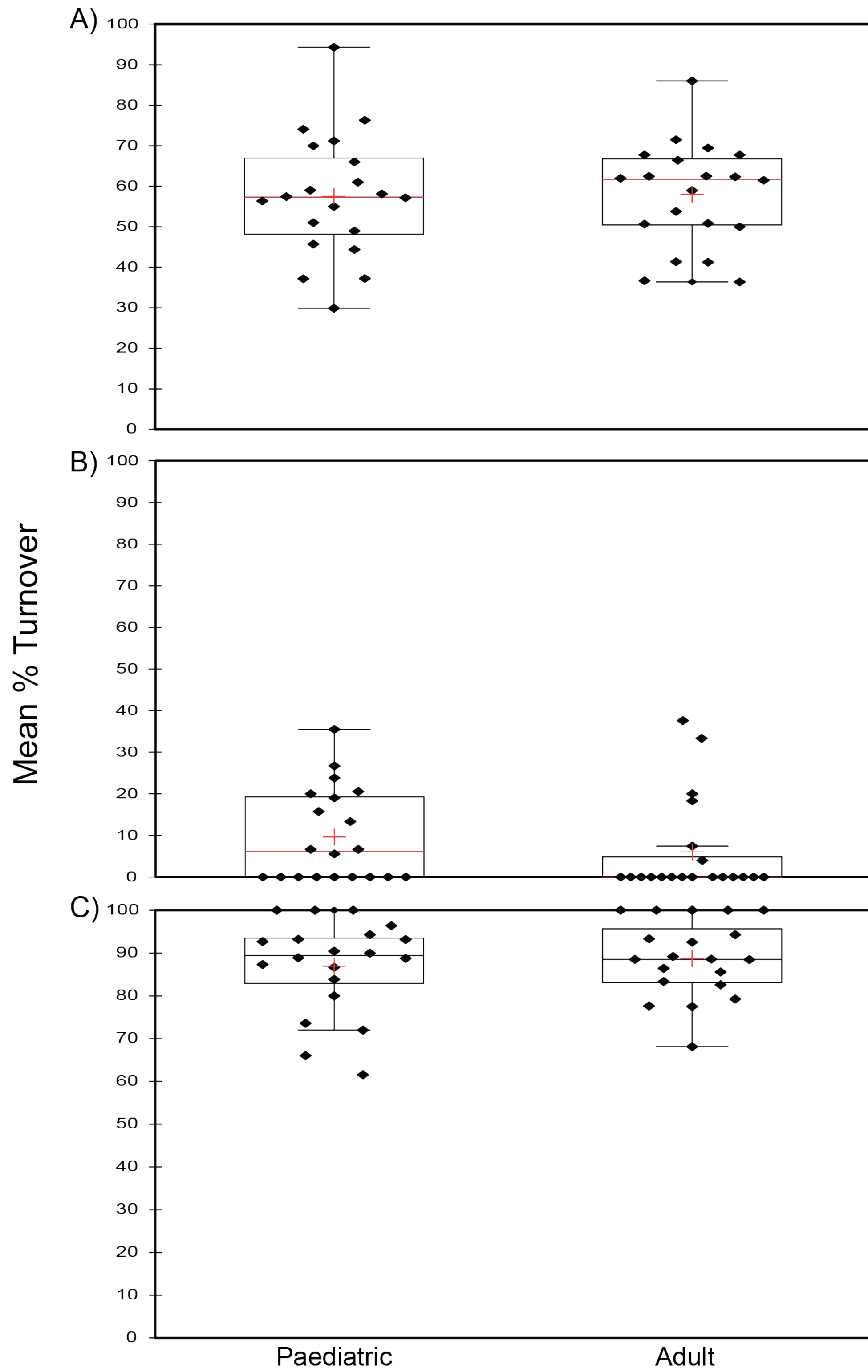

Figure 5.13 Mean % turnover across both patient cohorts. Panel A denotes changes in the overall microbiome, Panel B the core taxa and Panel C the satellite taxa. Red crosses denote the mean for each group.

5.2.5 Correlation with clinical measures

Correlation between diversity scores and $\%FEV_1$ showed a strong positive relationship in paediatric patients, but a strong negative relationship in adult patients (Spearman's Rank correlation; paediatric: Rho: 0.442, p=0.0509, adult: Rho: -0.403, p=0.07). Infection with *Candida spp.* has been previously shown to contribute towards $\%FEV_1$ decline (Chotirmall *et al*., 2010b). In this study, *C. albicans* had a non-significant but slightly positive relationship with $\%FEV_1$ in adult patients, and a non-significant but slightly negative relationship in paediatric patients (Spearman's Rank correlation; Adult: Rho: 0.2, p=0.38, Paediatric: Rho: -0.11, p=0.65). Only one adult was on a long term anti-fungal treatment, Itraconazole, typically used to treat infection with *Candida* or *Aspergillus* (Potter, 2005), unfortunately this was not sufficient for a comparison between patients on antifungals and those who were not.

5.3.6 Comparison with the bacterial microbiota.

5.3.6.1 Correlations between fungi and bacteria.

To find relationships between the bacterial and fungal microbiota, data from this study was compared to the bacterial NGS data from Chapter 4. The average number of fungal taxa recovered from samples was significantly lower than the bacterial taxa, with a mean of 3.45 fungal taxa (± 1.38) and 50.5 bacterial taxa (±13.06) per sample (T-test, t=2.02, DF=38, p=0.0001). There was also no correlation between the general fungal and bacterial diversity scores across all patients (Spearman's Rank correlation, RHO: 0.037, p=0.81).

Key taxa from both bacterial and fungal datasets were compared to determine if there were any correlations (Figure 5.7). In this study a significantly positive relationship between *Burkholderia* and *R. mucilaginosa* was found in both adult and paediatric patients (adult: Rho:0.96, p=0.0001, and paediatric: Rho:0.96, p=<0.001),

and a significantly positive relationship between *Burkholderia* and S*. cerevisiae* was found in adult patients (Rho:0.84, p=0.0001). Interestingly, paediatric patients had significant negative correlations between *C. albicans* and both *Burkholderia* and *S. maltophilia* (*Burkholderia*; Rho: -0.59, p=0.006, and *S. maltophilia*; Rho: -0.45, p=0.045).

5.3.6.2 *Candida* and *Pseudomonas*

Previous studies have found that co-colonisation with both *Candida* and *P. aeruginosa* may be associated with progressive lung function decline (Chotirmall *et al*., 2010b; Gileles-Hillel *et al*., 2015). In this study, *C. albicans* had a positive but not significant relationship with *P. aeruginosa* (Figure 5.7), and all but 4 patients (1 adult and 3 paediatric patients) were co-colonised at some point over the three-year period, with the 4 patients colonised only by *C. albicans*. All but two paediatric patients were chronically colonised with *C. albicans,* and 15 patients, 11 adult and 4 paediatric were chronically colonised with both taxa. There was no significant difference in %FEV_1 between those chronically co-colonised and those with only chronic *C. albicans* (T-test, t=2.05, DF=26.3, p=0.667).

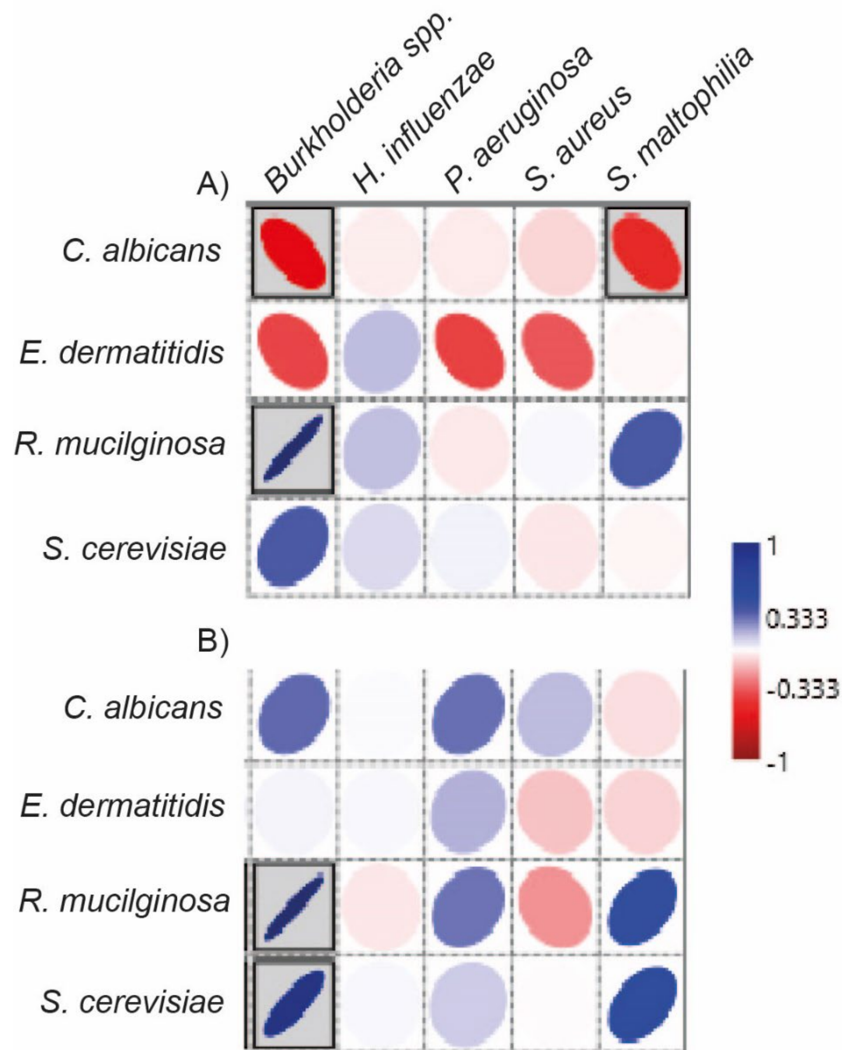

Figure 5.14 Correlations between key fungal and bacterial taxa across 20 adult and 20 paediatric patients. Panel A denotes correlations in the paediatric cohort, and Panel B the adult cohort. A. fumigatus and A. xylosoxidans have been excluded from analysis due to insufficient read depth. Direction of ellipses denote the nature of the relationship, and significant relationships (p=<0.005) are highlighted with a bold outline.

5.4 Discussion

The fungal microbiota of the CF lung is relatively understudied, despite recent evidence that fungal taxa may play a key role in clinical outcomes such as exacerbation and decreases in lung function (Weaver *et al*., 2019). Studies specifically focused on the fungal element of the microbiome and how it could impact on health and disease progression have been far and few between, however these studies have shown that there are key tax associated with CF, some of which are detrimental to health and that prevalence across patients is variable (Bakare *et al*., 2003; R. Amin *et al*., 2010; Nguyen *et al*., 2015). Comparisons to the bacterial microbiota are also limited, with studies finding little correlation between the two (Delhaes *et al*., 2012; Willger *et al*., 2014; Kramer *et al*., 2015; Hector *et al*., 2016). There are relatively few longitudinal studies particularly on milder CF patients and as such our understanding of long-term fluctuations in composition is limited. In addition, issues with primer design for fungal sequencing makes data across studies difficult to consolidate.

5.4.1 Detection methods

Analysing the fungal microbiota through NGS methods is considerably more sensitive and accurate than culture methods, however it is not without limitations. The fungal target region for most primers, the ITS (Internal transcribed spacer) is not as conserved as its bacterial counterpart, the 16S region, and as such, different primer targets have their own unique biases and detection limits (Heeger *et al*., 2019). In this paper, the primer pair ITS7 and ITS4 were chosen for analysis (Ihrmark *et al*., 2012). This primer set is designed to reduce discrimination against long amplicons, reduce chimera formation and to maximise the range of taxa that can be amplified. While the target region, ITS2, comes with its own set of limitations (a bias toward the *Ascomycetes* phyla and a reduction in *Basidiomycetes*

representation (Bellemain *et al*., 2010)), it is considered beneficial for human microbiome studies, as *Saccharomyces* is better represented by this target region, however this still leaves several clades poorly represented (Bellemain *et al*., 2010; Monard *et al*., 2013; Mbareche *et al*., 2020). Despite this, this primer set should provide a more diverse representation of the taxa in a given sample.

Control samples were used throughout both bacterial and fungal studies to ensure correct identification and amplification of key taxa. In this study, several notable fungal pathogens were not sequenced in the control set, namely *S. apiospermum* and *A. fumigatus.* This would initially suggest that the primer pair chosen were not suitable for the detection of these two taxa. In spite of this, *A. fumigatus* was positively identified in samples. There are several possibilities as to why the control sample failed. Firstly, culture has been shown to readily detect *Aspergillus spp.* in samples in a way that NGS methods fail to do, and rates of detection across molecular-based studies are highly variable (Nagano *et al*., 2010; Nielsen *et al*., 2014; Cuthbertson *et al*., 2021), as one study found that a variety of pan-fungal primers were not enough in many cases to detect various strains of *Aspergillus* (J. Zhao *et al*., 2001). Secondly, there is debate around the preparation of samples for fungal analysis, with some methods shown to not be suitable for efficiently breaking up the cell wall in fungal cells, leading to inaccurate detection (Baxter *et al*., 2011; Garcia-Rubio *et al*., 2020). Finally, the pure cultures set up for control samples may not have had sufficient DNA to be sequenced.

5.4.2 Comparison of detection methods

Culture has been repeatedly demonstrated to lack the specificity and sensitivity that NGS detection methods can offer (Stewart, 2012), with one study finding that culture-based methods failed to detect 60% of the taxa in fungal communities that molecular methods could (Delhaes *et al*., 2012). Despite this, many fungal

microbiota studies still utilise culture-based methods (e.g., (Bakare *et al*., 2003; Sudfeld *et al*., 2010; Güngör *et al*., 2013; Parize *et al*., 2014; Hector *et al*., 2016; Coron *et al*., 2018; Patel *et al*., 2021)). In Chapter 3, diagnostic microbiology was compared to QPCR results for the detection of *P. aeruginosa* and *S. aureus*, with culture shown to have a significantly lower sensitivity and specificity. The diagnostic microbiology for these samples included the mycological analysis and as such these were compared to the fungal NGS results. Culture was able to determine colonisation of some samples with *Candida spp.* and more specifically, *Candida albicans,* however even though the molecular methods utilised in this study aren't as specific as QPCR, culture was still unable to demonstrate the true breadth of *Candida* colonisation in these samples. C. *albicans* was readily detected by culture in paediatric patients but only in 1 adult patient, whereas NGS determined that all adults were chronically colonised.

A. fumigatus was readily discoverable by culture across adults and paediatric patients, however NGS was only able to determine an *A. fumigatus* presence in one adult patient, but not to the extent that culture found. As previously discussed, there are a several reasons as to why this could occur, and further investigation is needed to explore the disparity between culture and molecular detection limits for some fungal taxa. If we were to take the culture results as the correct prevalence of *A. fumigatus* for this cohort however, it would be at the lower end of reported prevalence rates, with 25% of patients positive. Another explanator for the lower results could be due to the mild phenotype of the patients in this study. Moderate and severe patients have been shown to have higher levels with more advanced lung disease. In one study, *A. fumigatus* was found in 70+% of samples in moderate and declining patients (Cuthbertson *et al*., 2021) and another found that 57% of severe patients in their study cohort were *A. fumigatus* positive (Saunders *et al*.,

2016). It could be stipulated that an absence of *A. fumigatus* is an indicator of health, however it has been noted in several studies that there was a correlation between the presence of a chronic *A. fumigatus* infection in declining patients and the presence of other pathogens known to accelerate lung function decline, such as *P. aeruginosa* and *S. aureus* (Mastella *et al*., 2000; Saunders *et al*., 2016). One of these studies queried whether this trend was as a result of the aggressive treatment of bacterial infection (such as with *P. aeruginosa*), which in turn could facilitate the introduction of fungal disease (Bargon *et al*., 1999). This brings into question the impact of bacterial: fungal interactions and the need for robust fungal disease diagnostics.

5.4.3 Molecular detection of fungi

Prevalence of fungi across all samples was relatively low, with a mean of 3.65 ± 2.22 taxa per sample. Studies utilising NGS for fungal detection have found a range of taxa in samples, ranging from 2 or 3 taxa per sample, to 20 or more per sample (Willger *et al*., 2014; Kramer *et al*., 2015; Cuthbertson *et al*., 2021). In this study, patients were dominated by *Candida,* with several paediatric patients dominated by *R. mucilaginosa*. Moderate to high levels of *C. albicans* is frequently reported by the literature, with prevalence reported across studies as low as 36.2% and as high as 77% or 93% (Muthig *et al*., 2010; Ziesing *et al*., 2016; Magee *et al*., 2021). *Candida* is often thought to be a commensal taxa across the human microbiome (Romo and Kumamoto, 2020), however it is capable of invasive candidiasis (Chotirmall *et al*., 2010), and has been associated with lung function decline (Navarro *et al*., 2001; Chotirmall *et al*., 2010b). The various strains of *Candida* seen in this study demonstrates the ability of the broader *Candida* family to colonise in CF, particularly with several taxa seen to only colonise either adults or paediatric patients.

Malassezia spp. is a CF associated taxa that is often not reported in studies, despite the potential to be a dominant taxa and recognition as an emerging pathogen (Delhaes *et al*., 2012; Willger *et al*., 2014; Kim *et al*., 2015). *Malassezia* is largely under-reported due to its inability to be cultured without specific medium (Willger *et al*., 2014), further demonstrating the need for more specific detection methods. Levels of *R. mucilaginosa* have been mentioned in relatively few studies, typically those exploring the full fungal microbiota, with levels reported to be around 1-3% (Nagano *et al*., 2010; Güngör *et al*., 2013; Ziesing *et al*., 2016), and is typically thought of as a rare fungal taxa in CF (Ziesing *et al*., 2016). The levels seen in this study are unusually high in comparison, with 87% of patients testing positive. The studies referenced here do not provide much detail on the status of the patients involved in the study, so it is difficult to determine if the rates reported are attributed to mild or declining patients. From this study however, rates of colonisation by this taxa are markedly higher in paediatric patients, suggesting that this may be a taxa more commonly seen in childhood.

5.4.4 Diversity and turnover between samples.

Diversity was low across all samples, however the main driver of diversity scores in both cohorts was the intermittent taxa. With an average of one or two taxa per patient, the diversity scores for chronically colonising taxa were extremely low, and while the intermittent taxa scores were not much higher, they were the driving force behind the trends for diversity in much the same way as their bacterial counterparts. Intermittent diversity was significantly higher in paediatric patients than adult patients, suggesting that like the bacterial microbiota, the paediatric fungal microbiota goes through a period of change in childhood and then “settles” in adulthood.

Turnover between samples further demonstrated this trend, the transient taxa almost completely changed between sampling with a mean % turnover of 87.8% across both cohorts. For the majority of adult patients, the chronically colonising taxa did not change between sampling, with 14 patients retaining the same chronic taxa across the 3 years, and 9 paediatric patients also maintaining the same chronic taxa. Unlike the bacterial microbiota, the fungal community appears to be made up of one or two "lifetime" taxa and the remaining community changes rapidly through time. Whether patients retain the same strain of these chronic taxa requires further investigation.

5.4.5 Correlations with clinical outcomes.

While the diversity of the fungal microbiota showed an extremely strong relationship with %FEV_1 outcomes it was not a significant relationship. It must be noted however, that this relationship was positive in paediatric patients and negative in adult patients, an interesting trend that would require further investigation as to why the diversity of the fungal microbiota appears to better lung function in paediatric patients but worsening lung function in adults. The role of *Candida* in the CF lung is yet to be fully understood, particularly with emerging strains such as *C. dubliniensis* being linked to worsening lung function (Al Shakirchi *et al*., 2020). In this study *C. albicans* had a no significant relationships with lung function, despite being a chronic coloniser for most patients.

5.4.6 Correlations with the bacterial microbiota.

Comparisons with the bacterial microbiota are few, however from the data available, it appears that there are few direct correlations between the two groups, with some studies finding significant correlations between *Candida spp.* and *Rothia spp.* in the lower respiratory tract of intensive care unit patients (Krause *et al*., 2016), however very few significant correlations have been found in the context of CF (Kramer *et*

al., 2015; Soret *et al*., 2020). In this study, fungal taxa recovered from samples were significantly lower than bacteria taxa, as demonstrated in previous studies (Kramer *et al*., 2015). Studies investigating the relationships between key taxa such as *Candida* and *P. aeruginosa* mainly take place as *in vitro* studies (Brand *et al*., 2008; McAlester *et al*., 2008), and have determined that aggressive anti-*pseudomonal* treatment may pre-dispose patients to infection with *C. albicans* (Chotirmall *et al*., 2010b; Gileles-Hillel *et al*., 2015). In this study, *C. albicans* had no significant correlations with any of the key bacterial taxa in adult patients, however had a significant negative relationship with *Burkholderia* in paediatric patients, implicating that the presence of *Candida* may play a protective role against *Burkholderia cepacia* complex members in childhood. Significant positive relationships were found between *S. cerevisiae* and *Burkholderia* in adult patients, and both adult and paediatric patients were found to have a significantly positive relationship between *R. mucilaginosa* and *Burkholderia*. As of writing, these correlations have not been published previously, and will require further investigation.

Previous research has found associations between co-colonisation with *Candida* and *P. aeruginosa* and a decline in lung function (Chotirmall *et al*., 2010b; Gileles-Hillel *et al*., 2015). These studies involved patients with moderate CF, and conclusions vary on the ability of both taxa to influence lung function outcomes. In this study the were no differences in %FEV_1 scores between those with co-colonisation and those with chronic *C. albicans* only, suggesting that milder CF is more tolerant of co-colonisation with both taxa.

5.5 Conclusion

Research into the fungal microbiota is still in its infancy, particularly in the context of CF. This study has further demonstrated some trends previously seen in research and presented new data for further consideration. Much of the work in current literature is facilitated by culture-based detection methods. Here we demonstrate that culture-based methods cannot detect as many taxa in the way NGS methods can, as has been demonstrated in bacterial work. In agreement with previous studies, the fungal microbiota was mostly composed of *Candida* taxa, alongside frequently reported taxa such as *E. dermatitidis* and *Malassezia spp.* The use of an alternative primer set produced some interesting results, with higher levels of taxa typically thought to be rare in CF, such as *R. mucilaginosa.* It has also added to the body of work showing disparity between molecular- and culture-based detection of *Aspergillus spp*.

This study is one of the first to explore the dynamics of the fungal microbiota over time, demonstrating that the driving force behind fungal diversity is due to the intermittently colonising taxa. The results presented here also suggest that patients, particularly adults, may maintain the same chronically colonising taxa over many years, typically *Candida*. Like previous studies, no major correlations were found between the fungal microbiota and lung function, however a significant positive relationship between *Burkholderia cepacia* complex members and *R. mucilaginosa* and *S. cerevisiae* was found, which has not been reported previously.

In order to understand the impact of the fungal microbiota in CF lung disease, much more work needs to be done. The role of fungi is poorly understood, and while some correlations with worsening lung function have been established, it must be noted that many studies find these correlations in the presence of known pathogens such as *P. aeruginosa* and *S. aureus.* Determining whether fungi are the direct cause of

lung function decline or simply an exacerbator to an already established infection is crucial. Molecular detection methods have proven to be more accurate and more sensitive across all aspects of research, however the nature of the fungal ITS region has resulted in multiple targets and little concordance between studies, particularly in relation to *Aspergillus spp,* with this study being no exception.

Chapter 6: General Discussion

6.1 Introduction

Microbiome studies and the use of Next Generation Sequencing (NGS) have enabled researchers to explore the otherwise hidden microbial communities inside the body, and have revolutionised our understanding of infection, disease mechanisms and subsequent treatments (Feigelman *et al*., 2017; Françoise and Héry-Arnaud, 2020). Due to the results of these types of studies, CF lung infection has since been redefined as a polymicrobial disorder, whereby a multitude of pathogens are responsible for lung function decline (Keating *et al*., 2017; S. Esposito *et al*., 2019), and in some cases, possibly protecting lung function (Acosta *et al*., 2018). Despite the growing number of studies on the microbiome in CF lung disease, there are several gaps in knowledge, particularly regarding community interactions and composition over time. In bacterial studies, much attention has been given to key CF pathogens such as *Pseudomonas aeruginosa, Staphylococcus aureus* and *Haemophilus influenzae,* and the few longitudinal studies available tend to focus on the dynamics surrounding exacerbation events (Price *et al*., 2013; Dickson *et al*., 2014; Cuthbertson *et al*., 2016). Features of mild CF lung disease phenotypes such as community composition over time and the role of anaerobes need to be explored, because of the anticipation that CFTR modulation therapy will encourage milder phenotypes of the disease. Current methods of pathogen detection in clinic are useful but lack the sensitivity and specificity that molecular methods can offer, and clinical diagnoses may be missing key pathogens as a result (Deschaght *et al*., 2009).

The issues in CF bacterial microbiota work are also reflected in the CF fungal lung microbiology; much of the work utilises culture-based methods, and longitudinal analysis is few and far between. To date, work into the fungal microbiota focusses on the key pathogens, such as *Candida spp.* and *Aspergillus spp.* (Amin *et al*., 2010;

Chotirmall *et al*., 2010; de Vrankrijker *et al*., 2011; Al Shakirchi *et al*., 2020), and while important, the composition and longitudinal fluctuations of the fungal microbiota remain poorly understood. Further research will not only expand our understanding of the overall microbiome, but also could help inform clinical practise, particularly as currently there is no official framework in place to diagnose fungal infection (Magee *et al*., 2021). Further understanding of the composition of the fungal biota, the interactions with its bacterial counterpart, and mechanisms of infection would help to inform and improve treatment.

The aim of this thesis was to address some of these pertinent issues. I have investigated the disparity between clinical diagnostic microbiology and targeted molecular-based methods (Chapter 3) and explored the composition and key features of the bacterial and fungal microbiota (Chapters 4 and 5, respectively) in a cohort of 20 mild adult and 20 mild paediatric patients over a 3-year period. This thesis also tested the suitability of an alternative set of fungal primers for human microbiome studies.

6.2 Comparison of culture- and molecular-based detection methods

In Chapter 3, the disparity between clinical diagnostic microbiology and targeted molecular-based detection methods was investigated. While research has mostly transitioned to NGS and targeted sequencing approaches to identify pathogens, clinical diagnostic microbiology relies on the detection of key pathogens through culture-based methods (Hogardt *et al*., 2009; Burns and Rolain, 2014). While useful, issues such as viable but not culturable (VBNC) taxa (Pasquaroli *et al*., 2013; Mangiaterra *et al*., 2018), user bias and lack of precision often mean taxa are missed from analysis and that true levels of infection are misreported (Kerem *et al*., 2005).

Comparing diagnostic microbiology to targeted QPCR for *P. aeruginosa* and *S. aureus* further demonstrated that the levels of infection were severely under-reported by culture-based methods, and that this has over-arching implications on the diagnosis of patients and their subsequent care. Comparing these two detection methods for two key pathogens (*P. aeruginosa* and *S. aureus*), this study demonstrated the scale at which *P. aeruginosa* was routinely missed from diagnostic microbiology, and as such those patients will have attended clinic for those without *P. aeruginosa* infection, possibly infecting other members of the community. A similar trend was observed for *S. aureus* and will more than likely be a repeated pattern across the cohort, not just limited to *P. aeruginosa* and *S. aureus,* as explored here.

Disparity in detection methods is not limited to the bacterial microbiota. In Chapter 5 the differences between diagnostic mycology and NGS was explored, further demonstrating the limits of culture-based detection methods. Unlike bacterial microbiota work, many studies into the fungal microbiota still utilise culture-based methods (for example: (Bakare *et al*., 2003; Sudfeld *et al*., 2010; Güngör *et al*., 2013; Parize *et al*., 2014; Hector *et al*., 2016; Coron *et al*., 2018; Patel *et al*., 2021)), and as such new research with molecular-based methods are presenting data that is in disagreement. Molecular methods are still more effective than culture in regard to fungal detection. More than 60% of taxa in fungal communities detected by molecular methods have been shown to not be detected by culture-based methods (Delhaes *et al*., 2012). The major issue in transitioning from culture methods to molecular methods for fungal detection is the lack of a robust fungal primer set. This disparity was demonstrated in Chapter 5, whereby levels of key taxa such as *Candida albicans* were under-reported across both cohorts, and in comparison to NGS, the general detection of yeasts and molds were significantly lower. A major

issue in the detection of several pathogens such as *A. fumigatus* was highlighted in the comparison, as several studies have also found that the detection of these taxa by molecular methods is variable (Zhao *et al*., 2001; Nagano *et al*., 2010; Nielsen *et al*., 2014; Cuthbertson *et al*., 2021), with this study as no exception.

6.3 The bacterial microbiota in mild CF

With new and improved therapies, the way CF is managed and treated is potentially on the cusp of a paradigm shift. Ever more effective CFTR modulators are vastly improving patient outcomes, reducing hospitalisations, exacerbations and improving overall health (Bessonova *et al*., 2018; McColley *et al*., 2019). These treatments have the potential to move CF patients into milder phenotypes, and as such further research is required to fully understand this phenotype. Moreover, the temporal dynamics of the bacterial microbiota in mild CF is an understudied area, with milder patients typically used as a comparator group (e.g., (Acosta *et al*., 2018; Cuthbertson *et al*., 2020; Heirali *et al*., 2020)). More research into long-term patterns and community dynamics combined with prospective use of NGS and targeted QPCR could allow researchers and clinicians to accurately determine the prevalence and persistence of key pathogens in the lung, and in turn, prescribe better treatment plans (Pattison *et al*., 2013; Burns and Rolain, 2014).

6.3.1 Chronic colonisation in mild CF

With CFTR modulators anticipated to move patients towards a milder phenotype, it is imperative that research works towards better understanding the mild microbiome. In Chapter 4, longitudinal analysis of mild adult and paediatric patients across 3 years were investigated, and the prevalence of key bacterial taxa in the CF lung, as well as the fluctuations in community composition over time, was determined. Taxa were partitioned into either chronically or intermittently colonising

groups, in accordance with a modified version of the Leeds criteria as used in Chapter 3 (Lee *et al*., 2003).

This study was in agreement with previous work indicating that *S. aureus* is a marker of a milder phenotypes (Ahlgren *et al*., 2015b; Bacci *et al*., 2016), and found that long-term colonisation with *P. aeruginosa* (typically associated with a rapid decline in lung function) was tolerated by all patients, with no observable decline in lung function. This could be as a result of the mechanisms controlling toxin expression (as previously discussed in Chapter 1, section 1.2.3), whereby some clones may downregulate toxin expression in order to promote a more persistent phenotype. The presence of *P. melaninogenica* and the community composition agreed with previous work whereby the make-up of the mild microbiome could be key in tolerating *P. aeruginosa* infections. Chronically colonising non-pathogenic taxa could aid in "controlling" dangerous pathogens through down-regulation of virulence, and actively prevent the initiation of severe infection. Further work needs to be done, particularly in light of previous research that demonstrated an increase in any given pathogen may not necessarily occur before exacerbation (Stressmann *et al*., 2011), showing that infection may not necessarily be predated by a sudden increase in bacterial load.

6.3.2 Longitudinal community composition.

Previous work has demonstrated the stable nature of the bacterial microbiota in mild CF (Hampton *et al*., 2021), however these conclusions have been brought about in reference to the entire microbiota. Partitioning the microbiota into chronic and intermittently colonising taxa, Chapter 4 was able to demonstrate that the stable component of the microbiota was the chronically colonising taxa, with the intermittent taxa undergoing constant flux. Further investigation into the dynamics of these groups demonstrated that while the intermittent taxa had high levels of

turnover, this was not due to a complete remodelling of the community, rather a shift in composition and abundance. This has not been previously reported and provides an insight into the community dynamics over time.

6.4 The fungal microbiota in mild CF

The fungal microbiota in the context of CF is poorly understood. While several cross-sectional studies have revealed common taxa across the population, the long-term community dynamics and role in infection remains vastly understudied. Issues such as a lack of robust primers for fungal detection have resulted in a wide range of prevalence rates and reported taxa, and as such there is not only disparity between culture- and molecular-based datasets, but also between molecular-based datasets.

6.4.1 Community composition

Chapter 5 focussed on the composition of the fungal microbiota in mild patients. Previous work has shown that a range of patient phenotypes carry multiple fungal taxa at any time (de Dios Caballero *et al*., 2016; Cuthbertson *et al*., 2021). This chapter demonstrated previously reported elements of the fungal microbiota and presented new and un-reported trends. In agreement with previous studies *Candida* taxa represented the largest section of the microbiota, with other frequently reported taxa such as *Malassezia spp.* and *E. dermatitidis* found across the cohort. Interestingly, prevalence of *R. mucilaginosa* was exceptionally higher than previously reported, possibly due to the mild nature of the cohort or the use of alternative primers.

6.4.2 Comparison of the bacterial and fungal microbiota

Comparisons of the bacterial and fungal components of the microbiome are far and few between (Kramer *et al*., 2015; Krause *et al*., 2016), and as such little is known about their interactions. The NGS data for key bacterial and fungal taxa generated

in Chapters 4 and 5 were compared to identify any relationships. Significant positive relationships between *Burkholderia cepacia* complex members and *R. mucilaginosa* were found in both adult and paediatric cohorts, a previously unreported correlation. Similarly, a significant negative correlation between *C. albicans* and both *Burkholderia cepacia* complex members and *S. maltophilia* were reported, suggesting a protective role for *C. albicans* in the lung.

6.5 Caveats and study limitations

6.5.1 Study population

The same cohort was used across all three research chapters, and while comprised of both adult and paediatric patients, it was still comparatively small where other studies are considered. Despite this, the groups were evenly sized and there was representation for most CFTR genetics across both groups, with 16 patients F508del homozygous, 15 patients F508del heterozygous and 9 patients representing the other CFTR genotypes. The study could have been further improved with a declining group for comparison, and unfortunately while there were several adult patients that were classed as moderate phenotypes (n = 7), there were insufficient numbers to justify a mild/moderate comparison.

6.5.2 NGS detection of fungal taxa.

Molecular methods are by and large a more effective method for the detection of bacteria and fungi in a given sample, however the detection of fungal taxa is hampered with issues from primer choice to database choice, and as such this has a profound impact on the results presented by all studies.

6.5.2.1 Primer choice

The variability of the ITS region has been demonstrated to be less conserved than the 16S utilised for bacterial sequencing (Heeger *et al*., 2019). As such, different primer targets result in different biases and ranges of detectable taxa. The ITS region as targeted in this study, comes with its own set of biases, with less *Basidiomycota* represented, and a preference for *Ascomycetes* (Bellemain *et al*., 2010; Monard *et al*., 2013; Mbareche *et al*., 2020). The primers chosen for this study were designed to reduce long-amplicon bias, and chimera formation (Ihrmark *et al*., 2012), and should provide a more even representation of taxa in a given sample. By choosing an alternative primer for the study in Chapter 5, this study has expanded on the range of primers used for CF microbiome studies, the results of which have challenged some previous knowledge of certain taxa.

6.5.2.2 Fungal databases

Fungal databases are not as complete as bacterial ones, and there are large swathes of “cryptic” taxa (unknown taxa that are morphologically and phenotypically identical to known taxa, with minute alterations to their genetic code. NGS has the potential to begin identifying these, but the workload is huge). These are often grouped with known taxa and could be unintentionally swaying results (Arastehfar *et al*., 2018; Sato *et al*., 2020). There are multiple gene banks available for fungal identification, however while some are collaborative and derive information from other databases, many are standalone, focussing on specific strains, fungal families, or taxa found in certain environments (Jayasiri *et al*., 2015). While focussed databases can be useful, centralised, collaborative databases are essential for identifying fungi regardless of origin.

6.6 Future work

6.6.1 Detection methods

Multiple studies have demonstrated the ability of molecular-based detection methods to reveal the true levels of taxa in samples (Chapter 3: Table S1, (Fadi Bittar *et al*., 2008; Pattison *et al*., 2013)), however these studies have focussed on key taxa such as *P. aeruginosa* and *S. aureus,* and very little has been done to explore the impact these detection methods have on the patients themselves. Similar studies exploring the true rates of colonisation for other key taxa, both bacterial and fungal, will add to the growing body of evidence and the argument for switching to more accurate detection methods in clinic. Accurate infection diagnostics could prevent patients attending the wrong clinics and reduce community spread, and thereby reduce infection rates. A study following patients longitudinally, whereby one group is assessed using standard diagnostic microbiology and the other a molecular based method such as QPCR, could demonstrate the impact on patients, the treatment they receive and the overall monitoring of infection across clinics.

6.6.2 Longitudinal bacterial and fungal microbiota studies

While effective CFTR modulators are only now becoming widely available, they have not only demonstrated their ability to reduce levels of key taxa, but also to improve patient outcomes. Further research is required to fully understand the impact these drugs have on the CF lung microbiome, and the long-term changes from pre-treatment onwards. New CFTR modulators are anticipated to promote milder phenotypes, and this must be confirmed through long-term studies following patients along their treatment journey. Understanding the long-term dynamics of the CFTR modulated lung will help improve treatments and inform future CFTR modulator research.

Early reports have shown dramatic influence on key CF pathogens, both bacterial and fungal, however the majority of focus has been attributed to the former. Concurrent research into the impact of CFTR modulators on both the bacterial and fungal microbiota could help to unravel the complex relationships between them, and how treatment impacts their communities, and by extension, the patient. As well as the changes within patients over time, these studies could also be compared to a group of stable patients with no CFTR modulator treatment to determine similarities or differences. While CFTR modulators are beneficial to many patients, there are still groups that are not eligible for this treatment, these patients could still indirectly benefit from CFTR modulators through investigation of treated patients.

6.6.3 Robust fungal detection methods

The main stumbling block for fungal detection is the lack of a robust primer sets to robustly and routinely recover fungal taxa in the CF lung. Each target region comes with its own bias against certain clades or fragment lengths, and as such there is no universal primer in the way that bacterial research has. While several studies have utilised molecular-based detection methods effectively, the difference in primer targets used means that some results may not be comparable. A “one size fits all” primer may not be possible; however it may be possible to determine a target region (or combination of target regions) that mediates these issues, providing a range of cover across all (or most of the clinically relevant) clades, and possibly further investigation with targeted methods for specific taxa.

The development of a robust detection method is essential, particularly for human microbiome studies, as accurate detection of taxa can inform treatment, determine more accurate prevalence and aid understanding of infection. This need is reflected in the conflicting detection data for taxa such as *A. fumigatus,* a taxon that is known

to cause invasive disease, which studies have found variable detection rates depending on the primer targets utilised.

6.6.4 The impact of Viruses

While bacterial infection is robustly studied, the impact of the fungal and viral components of the microbiota is poorly understood. Both areas lack robust routine testing and while bacterial infection is the main focal point in CF disease progression, it is likely that viruses play their own roles, and further study is warranted into their potential impact. Viral prevalence is poorly reported, and symptoms manifest in a similar fashion to bacterial infection. As such are these are often under-reported. Investigation into the prevalence of viral infection alongside bacterial and fungal surveillance may provide a more complete picture of infection and the impact on disease progression, as well as the interactions between the various communities in the lung.

6.7 Conclusion

Molecular-based detection methods have revealed a complex network of taxa in the CF lung and have raised new questions as to the nature of these taxa, their role in invasive disease, their interactions and contribution to lung function decline. This thesis has addressed some of the gaps in knowledge, particularly the longitudinal nature of the bacterial microbiota in mild patients, and the composition of the fungal microbiota. This work presents data in agreement with previous studies, such as the long-term toleration of key taxa such as *S. aureus* in the mild CF lung, and the domination of *Candida* taxa in the fungal microbiota. It also presents new data for consideration, such as the fluctuations of the intermittent taxa in the bacterial

microbiota, the interactions between the key bacterial and fungal taxa, and the use of an alternative fungal primer target.

The future of CF, in light of new therapies such as CFTR modulators, is looking positive, and investigation into the CFTR modulated microbiome as a whole will provide crucial data on the role of the microbiome in the pathogenesis of CF lung disease.

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