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SEPTEMBER 26, 2018

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**Metagenomic Characterisation of the Cystic Fibrosis Airway  
Microbial Community in *Pseudomonas aeruginosa*  
Colonised and Non-Colonised Paediatric Patients**

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A thesis submitted towards the degree of M.Sc. (Research)

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## Summary

Cystic fibrosis (CF) airway disease is characterised by a repeated cycle of persistent infection and inflammation. Morbidity and mortality are primarily caused by airway infections and associated inflammation. Antimicrobial therapy forms the cornerstone of CF care and this traditionally has been reliant on culture. There are a few well known easily cultivable CF pathogens of which *Pseudomonas aeruginosa* is the most commonly isolated and often associated with exacerbations and disease progression. With the development of advanced sequencing technologies we now know that the CF airways is host to a diverse microbial community. This study focused on thoroughly characterising the CF airway microbial community in patients with and without *P. aeruginosa* colonisation employing traditional culturing methods and the most advanced cutting edge sequencing technologies.

We collected 105 sputum samples from 29 adolescents and children ( $\leq 19$  yrs) with CF attending Tallaght Adelaide and Meath Hospital between November 2016 and November 2017. Thorough culturing of these samples was performed employing several different culture media under appropriate environmental conditions. The DNA was also isolated from a portion of these samples and Ribosomal Intergenic Spacer Analysis (RISA) and Whole Genome Shotgun Sequencing (WGSS) performed on a subset of samples.

Our culture detection methods were compared to two types of molecular techniques. WGSS was far superior in identification of diverse bacterial species present in our CF sputa samples, with an average of 12 species detected by WGSS versus only 2.5 by our culturing methods. Culture methods and molecular methods were comparable for detecting *P. aeruginosa*, which were also comparable to national prevalence for patients of the same age. RISA was carried out to assess its applicability as an efficient and economical molecular diagnostic tool and to estimate diversity. We found this method was ambiguous as a measure of diversity and could not be reliably used to identify species in such diverse biological specimens. However paired with culture results, RISA could serve as a useful guide in identifying pathogenic species and giving rough estimates of diversity.

This is the first time WGSS has been employed to characterise the CF airway community in a population of CF patients. Employing WGSS we can be confident in our species level taxonomic assignment. A considerable proportion of our top 25 most dominant species were anaerobes which were often in higher abundance than common CF pathogens also detected such as *Haemophilus influenzae*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia* and *P. aeruginosa*. We observed a significantly higher

microbiome diversity in samples dominantly colonised with *S. aureus* suggesting *S. aureus* may be part of the healthier airway microbiome and may support growth of a diverse community. This has implications on the controversial role of antistaphylococcal prophylaxis for children with CF. *Prevotella melaninogenica* was also observed to be associated with higher microbiome diversity but to a lesser degree. For the first time we report that dominance of *S. maltophilia* is linked to reduced microbiome diversity reinforcing its pathogenic role in CF. Paired with our finding that lower bacterial diversity is associated with severe deterioration in FEV<sub>1</sub> in children and adolescents with CF, the influence of dominant pathogens on microbiome diversity likely has clinical significance. Perhaps investigating the impact of other CF bacteria on microbiome diversity could begin to shed light on which species have positive or negative effects in CF.

Some patients in our study had a stable microbiome over time while others exhibited considerable changes in their microbiome over time. The CF airway microbiome is sensitive to disruption by outgrowth of pathogenic species such as *P. aeruginosa*. We noted a significantly dissimilar composition and metabolic capability of *P. aeruginosa* colonised and non-colonised CF microbiomes. *P. aeruginosa* colonisation was found to be positively associated with *Bordetella* species and *Achromobacter xylosoxidans* and negatively associated with *S. maltophilia*. *P. aeruginosa* colonised communities were noted to have significantly increased activity in metabolic pathways involved in growth in hypoxic environments, quorum sensing and polymixin resistance; all useful in the CF lung. Interestingly we found that the number of recent antibiotics prescribed was positively correlated with *P. aeruginosa* abundance but negatively correlated with several other Gram negative anaerobic species. This corroborates previous findings that antibiotics can have little impact on the target organisms but negatively impact the remainder of the microbiome.

The use of WGSS in this study allowed us to identify and monitor pathogenic strains persisting in the CF airway. A number of pathogenic sequence types (ST) of *S. aureus*, including the methicillin resistant ST5, were found to be chronically colonizing several patients. One *S. aureus* strain was observed to persist in some patients revealing a potential transmissible strain. Pathogenic strains of *P. aeruginosa* and *S. maltophilia* were also identified to persist in certain patients in our study.

Overall, this study has contributed several significant and novel findings to the field of microbiome research in CF. It has been the first of its kind to use WGSS to fully characterise the microbiome of *P. aeruginosa* colonised and non-colonised paediatric CF

patients. A practical assessment of the applicability of the RISA technique and a reinforcement of previously reported, though perhaps underappreciated findings are presented in this work. Higher microbial diversity in *S. aureus* colonized patients compared to *S. aureus* non-colonised patients has been observed, the opposite of which was noted in *S. maltophilia* colonised patients.

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## List of Abbreviations

4-PBA	Sodium-4-phenylbutyrate
AB	<i>Acinetobacter baumannii</i>
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
AMNCH	Adelaide and Meath incorporating the National Children's Hospital
AMP	Anti-microbial peptide
ANOVA	Analysis of Variance
ASF	Airway surface fluid
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
Bcc	<i>Burkholderia cepacia</i> complex
BMI	Body mass index
bp	Base pair
CAMP	Cationic Anti Microbial Peptides
CaCC	Ca <sup>2+</sup> -activated Cl <sup>-</sup> channels
CF	Cystic fibrosis
CFE	Cystic fibrosis Foundation
CFRD	Cystic fibrosis related diabetes
CFRI	Cystic fibrosis Registry of Ireland
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CO <sub>2</sub>	Carbon Dioxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide
FEV <sub>1</sub>	Forced expiratory volume in one second
GRE	Glycopeptide-resistant <i>Enterococci</i>
GRSA	Glycopeptide-resistant <i>Staphylococcus aureus</i>
Has	Heme assimilation system
HBE	Human bronchial epithelial cells
HI	<i>Haemophilus influenzae</i>
IL	Interleukin
IRT	Immunoreactive trypsinogen
ITS1	Internal transcribed spacer region 1
LB	Luria Bertani
LPS	Lipopolysaccharide
MDR	Multi-Drug Resistant
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NGS	Next Generation Sequencing
OD	Optical density
OTU	Operational taxonomical unit
PA	<i>Pseudomonas aeruginosa</i>
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCoA	Principal coordinate analysis
PFGE	Pulse Field Gel Electrophoresis

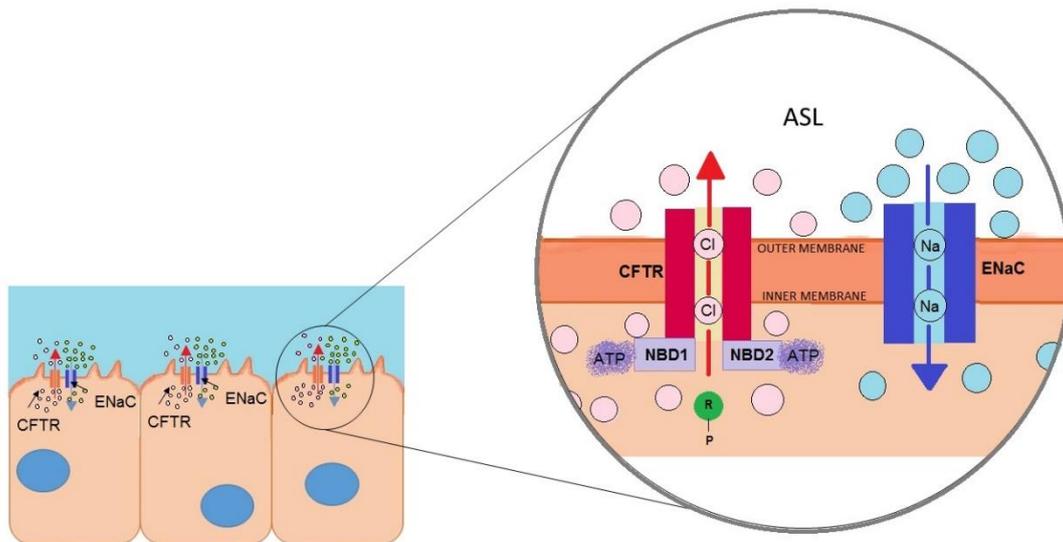
PGM	Personal Genome Machine
Phu	<i>Pseudomonas</i> utilization systems
PLC	Phospholipase C
PRR	Pattern recognition receptor
PTC	Premature termination codon
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
QS	Quorum sensing
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Real time polymerase chain reaction
RISA	Ribosomal Intergenic Spacer Analysis
SA	<i>Staphylococcus aureus</i>
sBHI	Supplemented Brain Heart Infusion
SCV	Small colony variant
SM	<i>Stenotrophomonas maltophilia</i>
SOLiD	Sequencing by Oligonucleotide Ligation and Detection
ST	Sequence type
TLR	Toll like receptor
TNF- $\alpha$	Tumour necrosis factor-alpha
WGSS	Whole Genome Shotgun Sequencing
ZOI	Zone of inhibition

**Chapter 1**  
**Introduction**

## 1.1 Cystic Fibrosis

Cystic Fibrosis (CF) is the most common inherited life-shortening disease in Caucasians, brought about by a mutation in the CF Transmembrane Conductance Regulator (CFTR) gene on human chromosome 7. Prevalence in Ireland is particularly high, with the most recent registered number of patients standing at 1266 according to the 2016 Cystic Fibrosis Registry Ireland (CFRI) Annual Report (CFRI 2016). This condition was recognised as far back as the 1500s with medical texts linking salty skin and pancreatic damage with death in childhood. The condition was first described as a specific disease in 1938 by the American pathologist, Dorothy Andersen, who called the disease “cystic fibrosis of the pancreas”, stemming from her autopsy findings from children dying of malnutrition as a result of CF-related coeliac disease (Andersen 1938). As morbidity and mortality in CF are often a result of pulmonary infections (Gibson et al. 2003), the disease was often thought to only affect the lung. However it is a multi-organ disease with effects on the pancreas, sweat glands, gall bladder, intestines as well as the respiratory tract and the vas deferens (Oppenheimer EH 1975; Scriver & Fujiwara 1992).

With advances in sequencing technologies, came the identification of mutations in the CFTR gene as the cause of this disease. The first studies were published in 1985, noting that the CFTR protein was either rendered ineffective or absent in those with CF (Welsh et al. 1986; Tsui et al. 1985; Riordan et al. 1989; Rommens et al. 1989). The wild-type form of the CFTR protein is 1480 amino acids in length, has N- and C-terminal extensions of approximately 80 and 30 nucleotide residues respectively and is complete with an additional regulatory (R) domain which gates the channel through phosphorylation (Fig. 1.1 - (Riordan et al. 1989; Welsh et al. 1986)). Phosphorylation of the R domain occurs through cAMP-dependent protein kinase (PKA), which is required for channel gating by ATP. Uniquely, CFTR is the only ATP-binding cassette transporter known to be an ion channel, and the only ligand-gated channel which consumes its ligand (ATP) in the gating process (Hwang & Kirk 2013; Hunt et al. 2013). This membrane protein functions by binding and hydrolysing ATP through utilisation of its two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs) (Gadsby et al. 2006; Cant et al. 2014; Riordan 2005). The CFTR gene and in turn the CFTR membrane protein play a vital role in regulating the airway surface fluid (ASF) in the lungs by mediating the translocation of negatively charged chloride ions out of epithelial cells. Similarly, positively charged



**Figure 1.1:** Functional CFTR and ENaC channels in lung epithelial cells. In lung epithelial cells, chloride and sodium ions translocate across the CFTR and ENaC channels, respectively. In this active CFTR channel,  $\text{Cl}^-$  is transported at a higher rate than  $\text{Na}^+$  transport. This hydration enables mucociliary clearance of the airway and protects against infection in non-CF patients.

sodium ions translocate across the epithelial sodium channel (ENaC) which occurs in epithelial cells of the lung and pancreas (Ahmed et al. 2003) (Fig.1.1). Chloride ion secretion is significantly reduced or absent when CFTR is mutated, resulting in sodium hyperabsorption due to ENaC dysregulation (Stutts et al. 1995; Stutts et al. 1997). The CFTR protein normally functions to maintain an osmotic balance between the absorption of sodium ions into a cell and control of chloride concentration within the cell. In the absence of this essential ion flux, the ASF becomes viscous and mucociliary clearance is impaired (Voynow & Rubin 2009). The subsequent build-up of this mucus creates an ideal colonising environment for opportunistic pathogens.

Up to 95% of morbidity and mortality in CF is a result of airway infections and the associated inflammation (Zhao et al. 2012). Whether the lungs of people with CF are intrinsically inflamed from birth or become inflamed upon first exposure to microbes has been highly debated (Khan et al. 1995; Birrer et al. 1994; Balough et al. 1995). The patient's immune response to bacterial infections materialises in the form of inflammation, often resulting in parenchymal damage and airway obstruction. Upon recognition of pathogen-associated molecular patterns (PAMPs) by pathogen recognition receptors (PRRs) of circulating alveolar macrophages and lung epithelial cells, interleukin-6 (IL-6) and IL-8

production is induced (Bonfield et al. 1995), leading to mass infiltration of neutrophils (Conese et al. 2003). A cascade of chemokines, cytokines and activation of lymphocytes is triggered which elicits further inflammation (Karin et al. 2006). Neutrophils work to clear pathogens by secretion of proteases, antimicrobial proteins like neutrophil elastase (NE) and neutrophil extracellular traps (NETs), killing bacteria though causing epithelial cell damage in the process (Sørensen & Borregaard 2016). Alveolar macrophages and neutrophils can phagocytose bacterial cells and kill them internally within the phagolysosome (Richard G. Painter et al. 2006). The inflammatory response to these pathogens ultimately causes irreversible lung damage and impairs clearance of mucus thus hindering lung function (Voynow & Rubin 2009). Inflammation in the CF airway is evidently very complex (Pillarisetti et al. 2011; Sagel et al. 2012; Elizur et al. 2008) and is only briefly covered in this thesis as it is not the focus of this work.

## **1.2 Classes of CFTR mutations**

There are >2000 CFTR mutation variants identified to date (re3data.org 2017) which are classified based on their effect on the CFTR protein (Rowntree & Harris 2003) (Table 1.1). The  $\Delta F508$  is the most frequently identified mutation in CF patients globally and 91.6% of Irish CF patients having at least one copy of this mutation (CFRI 2016). This class II mutation causes a deletion of three base pairs, leading to the loss of the phenylalanine amino acid in the 508<sup>th</sup> position of the CFTR protein, thus failing to produce sufficient functional protein at the membrane of the cell (McKone et al. 2003; MP Rogan, DA Stoltz 2011). The CFTR protein product from this mutation is known to be retained in the endoplasmic reticulum (ER) and thus it is prevented from reaching the cell membrane (Lukacs et al. 1994). Clinical symptoms often vary between patients, however homozygous  $\Delta F508$  patients suffer with more severe disease than heterozygotes (Santis et al. 1990; Kerem et al. 1990). G551D is the most prevalent class III mutation in Ireland (Table 1.1), affecting a greater proportion of the Irish CF community than affected in other countries (Accurso et al. 2010; Van Goor et al. 2009; CFRI 2016). Among the CF population in Ireland 15.2% were found to have had at least one copy of G551D (Harrison et al. 2013; Barry et al. 2014). Novel CFTR mutations are still being discovered, with some of the most recent being a nonsense mutation (c.2777 T>A (p.L926X)) in exon 17 and a heterozygous missense mutation (c.3119 T>A (p.L1040H)) (Mohseni et al. 2016).

**Table 1.1:** Details of Class I to IV Mutations in CF

<b>Class of Mutation</b>	<b>Molecular Defects</b>	<b>Functional Consequences</b>	<b>Examples</b>
I	Premature stop codons (PTC) Nonsense, frameshift & large deletions Defective protein synthesis	CFTR protein not expressed or truncated CFTR protein	W1282X R553X G542X E60X
II	In-frame deletions and missense mutations Disrupted CFTR proteins folding and trafficking	Lack of transport/ residual transport of misfolded CFTR to surface. Premature CFTR degradation.	ΔF508 N1303K A559T G480C
III	Substitution of amino acids Missense mutations CFTR channel no longer opens in response to agonists	Lessened/ complete lack of CFTR channel opening	G551D G551S G1349D G85E
IV	Missense mutations CFTR protein structure changes	Malformed CFTR pore restricts movement of Cl <sup>-</sup>	R117H R334W R347P R792G
V	Missense mutations Alternative splicing mRNA processing is disrupted	Greatly reduced CFTR protein synthesis - less protein at cell surface	2789+5G→A 3272-26A→G A455E P574H
VI	Mature CFTR protein is unstable due to mutations	Membrane stability of CFTR and ion flux are greatly reduced	1811+1.6 kb A>G

### 1.2.1 Diagnosing CF

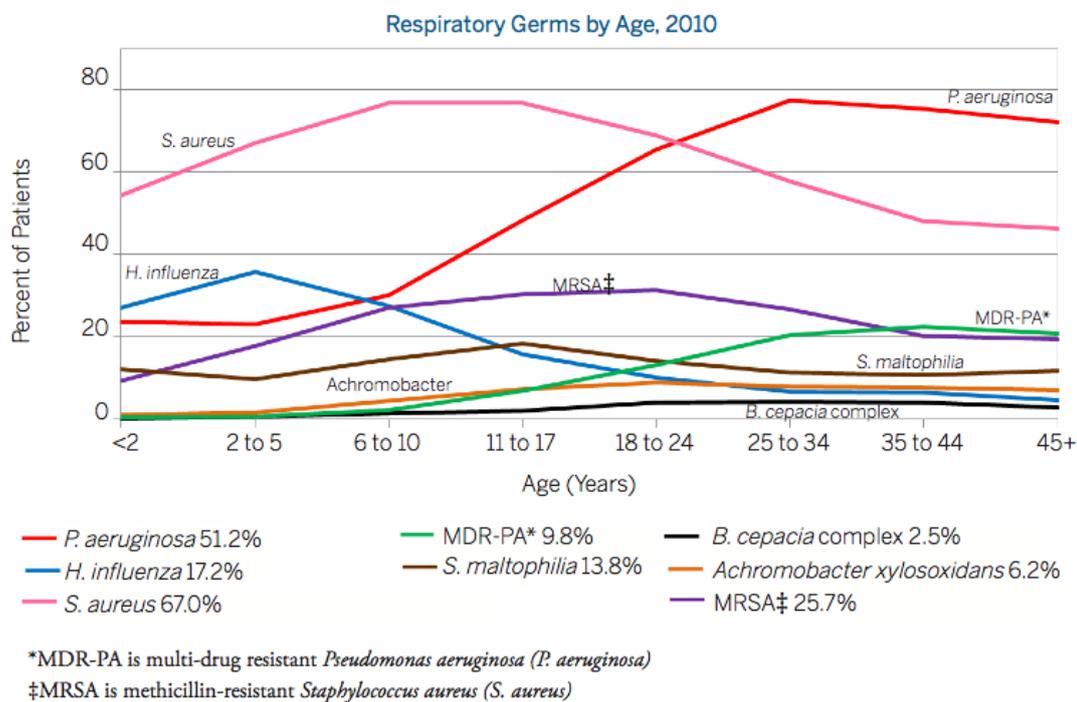
CF can be diagnosed using the sweat test, the nasal potential difference (PD) test and molecular identification of CFTR mutations. The sweat test measures the concentration of electrolytes on the skin, a procedure which was first established in 1959 and remains the standard procedure to initiate confirmative diagnosis of CF. Patients with dysfunctional or absent CFTR have increased chloride ions in their sweat, making it salty. To account for false positives, further tests are required to give a definitive diagnosis. CFTR mutations can be identified through sequencing, with current screening panels identifying 90% of CFTR mutations. Specific CF phenotypes have also been identified through haplotype analysis of CFTR mutations (Cordovado et al. 2012). In Ireland, screening for CF was introduced in 2011 as part of the National New-born Bloodspot Screening Programme, commonly known

as the heel prick test. Upon an atypical sweat test result, an immunoreactive trypsinogen (IRT) assay is carried out on a blood spot from the heel prick sample. Trypsinogen, a pancreatic enzyme precursor, is elevated in the blood of new-borns with CF due to blockages in the pancreatic ducts. Heterozygous carriers of a CFTR mutation can also exhibit raised IRT, therefore this assay alone cannot be used for diagnosis (Kumar & Clark 2012). To confirm diagnosis the sample is screened for CFTR mutations. If two mutations are present, the patient most likely has CF while if just one mutation occurs, a second sweat test is performed. A second positive result would warrant further DNA analysis to identify the second CFTR allele mutation (Flume et al. 2007).

On occasion CF is only tested for after clinical manifestations of the disease have started to appear. Such manifestations may include chronic sinopulmonary disease, chronic cough and sputum production, acute salt depletion, gastrointestinal and nutritional abnormalities, chronic metabolic alkalosis and occasionally genital abnormalities in males resulting in obstructive azoospermia (Rosenstein & Cutting 1998). Other clinical presentations of CF include abnormalities in chest radiographs, nasal polyps, gastroesophageal reflux disease (GERD), constipation, pancreatic insufficiency, meconium ileus (MI), distal intestinal obstruction syndrome (DIOS), chronic forms of pancreatitis and hepatic disease as well as failure to thrive (FTT) (Rosenstein & Cutting 1998).

### **1.3 Microbiology of the CF airway**

Airway infections cause the burden of morbidity and mortality (Gibson et al. 2003) in CF and diagnosis of these infections forms the cornerstone of CF care. Diagnosis is largely based on the ability to culture these microorganisms from CF airway samples. The antibiotic resistance profiles of the cultured bacteria then informs the therapeutic approach. Some of the most common bacteria isolated from the airways of people with CF are *S. aureus*, *P. aeruginosa*, *H. influenzae*, *S. maltophilia* and *A. baumannii*. Cultivation data has revealed a general pattern of succession with *S. aureus* and *H. influenzae* colonisation common in childhood, while *P. aeruginosa*, *S. maltophilia* and/or *Burkholderia spp.* are more common in adults (Harrison 2007; Pettigrew et al. 2008) (Fig. 1.2). *P. aeruginosa* becomes more prevalent with age with approximately 80% of adults with CF colonised with *P. aeruginosa* (Döring et al. 2000).

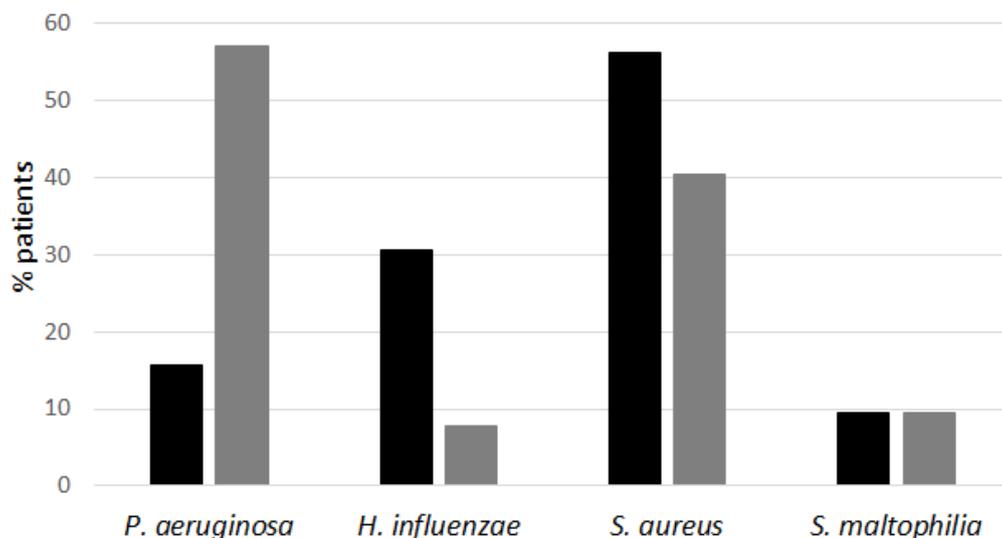


**Figure 1.2:** The 2010 Annual data report from the Cystic Fibrosis Foundation Patient Registry. Percentage abundances of top bacterial pathogens and their prevalence over time in CF patient’s airway microbiome

In order to treat chronic Gram negative bacterial biofilms, such as those of *P. aeruginosa*, azithromycin has proven effective in the past (Mulet et al. 2009; Gillis & Iglewski 2004). This macrolide has demonstrated not only antimicrobial activities, but also anti-inflammatory (Amsden 2005) and more recently, immune-modulatory capabilities (Cramer et al. 2017; Kovaleva et al. 2012) in CF patients. New inhaled antibiotics are being proposed for the treatment of both *P. aeruginosa* and *S. maltophilia* (Geller et al. 2011; Konstan et al. 2011). Tobramycin inhalation powder (TIP) has been compared to tobramycin inhalation solution (TIS) recently, with TIS proving to achieve higher sputum concentration, though with less clinical benefits than TIP (Konstan et al. 2011). Clinical trials to determine the use of TIP against *Bcc.* and *S. maltophilia* in the CF airway are planned. Inhaled aztreonam and the second generation fluoroquinolone, levofloxacin, are also proving effective against *P. aeruginosa* with levofloxacin showing additional anti-inflammatory effects (Geller et al. 2011; Tsivkovskii et al. 2011).

### 1.3.1 *P. aeruginosa* in CF

*P. aeruginosa* is a Gram negative bacteria that commonly colonises the CF airways in the first decade of life (Döring et al. 2000; Pitt et al. 2003). There is a chronology to *P. aeruginosa* colonisation in CF in which the prevalence in children's airways are lower (2.8% to 97.5% (Lebecque et al. 2006; Burns et al. 2001)) than adults (50% to >80% (Lebecque et al. 2006; FitzSimmons 1993)). A study reported that 29% of children with CF have acquired *P. aeruginosa* in the first 6 months of their life (Li et al. 2005). While another study reported the prevalence to be 72.5% in the first three years of life using culture-dependent techniques. This prevalence rate increased to 97.5% when combined culture and serological testing was implemented (Burns et al. 2001). *P. aeruginosa* infection is known to transition from initial sporadic colonisation to an intermittent infection, before becoming established as a chronic colonisation (Smith et al. 2006; Rajan et al. 2000; Vogne et al. 2004; Bacci et al. 2017). By adulthood up to 80% of patients are chronically colonised with *P. aeruginosa* (McCallum et al. 2001). Reported prevalence differs depending on the detection method used (culture-based or molecular detection), the age and disease status of the patient cohort and the country of the study. In Ireland an average of 15.6% of children aged  $\leq 19$  years were colonized with *P. aeruginosa* in 2016, while chronicity with *P. aeruginosa* was recorded to affect an average of 9.8% of paediatric patients (Fig. 1.4) (CFRI 2016). The Irish adult CF (>19 yrs) *P. aeruginosa* prevalence was reported to be 56.98%, while chronic infections affected 41.14% of adult CF patients (CFRI 2016).



**Figure 1.3:** Irish population prevalence rates for bacterial pathogens in children and adults. This data is based on CFRI data. An average of prevalences for all child age-groups  $\leq 19$  years (black bars) and all adult age-groups >19 years (grey bars) were taken (CFRI 2016). *A. baumannii* was not included as the Irish prevalence of this bacteria has not been documented

Once a CF patient becomes chronically colonised with *P. aeruginosa* it is very difficult to eradicate (Langton Hewer & Smyth 2014) and has been observed to mark the beginning of significant lung function decline (Pamukcu et al. 1995). Chronic *P. aeruginosa* colonisation is considered a major limiting factor in patient survival (Koch 2002; Li et al. 2005), with current estimates of 10 years reduced life expectancy in chronically colonised patients (Kosorok et al. 2001). For these reasons the development of chronic *P. aeruginosa* colonisation is recognized as an important milestone in the progression of CF disease. In order to establish this chronic colonisation in the CF airway, *P. aeruginosa* must face and invade diverse physiological environments and microbial communities, which requires extensive microevolution and genetic adaptation.

Early *P. aeruginosa* colonisation is usually intermittent with periods of clinical stability and culture negativity in between exacerbations with this bacteria. *P. aeruginosa* isolates are normally non-mucoid during early and intermittent infection (Penketh et al. 1983). However *P. aeruginosa* can genetically adapt to persist in the CF airway by transitioning to a mucoid phenotype (Govan & Nelson 1992). *P. aeruginosa* in its environmental, non-mucoid and thus more motile state, can initially penetrate further into thickened mucus and migrate into hypoxic mucus zones, growth then occurs in a nitrate-dependent fashion, which is a slightly slower growth rate than usual conditions (Worlitzsch et al. 2002). This physiological environment is thought to apply a selective pressure, which induces a mucoidy phenotype. Conversion of *P. aeruginosa* to mucoidy growth and loss of motility are often seen as a response to stress (Deretic et al. 1994). Biofilm formation is promoted with this change to mucoidy and is advantageous in this environment, thus increasing the fitness advantage of *P. aeruginosa*. The gene known to be responsible for transition to mucoidy is *mucA*, though some studies have noted that during establishment of chronic infection loss of function mutations occur in several genes, only one of which is *mucA* (Smith et al. 2006). A study found two patients which were only colonized with non-mucoid strains of *P. aeruginosa* consistently over several years, supporting the hypothesis that other adaptive features were important in persistence and establishment of chronic infection (Jelsbak et al. 2007). Secretion of phenazines and siderophores in addition to formation of biofilms have been known to result in poorer clinical outcomes (Jimenez et al. 2012). It has been observed that many strains of *P. aeruginosa* actually reduce their expression of virulence factors and extracellular toxins in chronic infections (Smith et al. 2006). This phenomenon has not been observed universally in disease progression in patients with *P. aeruginosa* and thus could be a strain specific trend.

*P. aeruginosa* has many virulence factors that help it invade and evade the host immune system. Pyocyanin is one of the four main classes of phenazines secreted by *P. aeruginosa* and is known to exert host inflammatory responses (Gee W. Lau et al. 2004; Fothergill et al. 2007). Phenazines are secondary metabolites produced by many bacteria and pyocyanin acts as an iron reducing agent in the lung microbiome (Pierson & Pierson 2010). A study using animal CF models also identified an increase in pyocyanin levels during exacerbations which tended to lead to more virulent infections mediated by tissue damage and necrosis during *P. aeruginosa* lung infection (Gee W Lau et al. 2004). A recent study investigating the three phenazines, pyoverdine, pyocyanin and LasA protease, concluded that high-virulence sub-populations of *P. aeruginosa* can persist in CF patients with chronic infections (O'Brien et al. 2017). This result, along with results of other studies (Fothergill et al. 2007; Fothergill et al. 2010), suggest that pyocyanin over-producing (OP) phenotypes can often be found in combination with underproducing phenotypes within the CF lung. A potential explanation for this is that pyocyanin OP phenotypes may retain the ability to transmit between patients, though further work is needed in this area (O'Brien et al. 2017). With a better understanding as to whether bacterial community composition has a bearing on *P. aeruginosa* pyocyanin production, it may be possible to minimise the extent of clinical symptoms which present during CF-associated exacerbations (O'Brien & Fothergill 2017). Loss of function (LOF) genetic adaptations occur in virulence-factor related genes in *P. aeruginosa* (Smith et al. 2006), namely the primary regulator of quorum sensing (QS) *lasR* (J P Pearson et al. 1994; Schuster et al. 2003). In the early stages of infection *P. aeruginosa* utilizes QS to determine high cell density and subsequently induces an upregulation of virulence factor-related gene expression (Bjarnsholt et al. 2010). A functional *lasR* is required for *P. aeruginosa* to exist in a biofilm in an airway infection, meaning that selection to mutate *lasR* results in a loss of *P. aeruginosa* virulence in established bacterial infections (Smith et al. 2006). Smith *et al.* have shown that virulence factors are selected against in chronic infections perhaps for evasion of the host immune system. The human immune system targets cells which express numerous virulence factors, thus for *P. aeruginosa* to survive, there is a selection pressure to reduce virulence factor expression (Hollsing et al. 1987). A range of virulence factors and their regulators have been found to be mutated including quorum sensing related genes, genes associated with iron acquisition, phenazine biosynthesis, multidrug efflux, twitching motility and others were all noted to incur mutations in the genome of *P. aeruginosa* to maximize survival and progression to chronic infection (Marvig et al. 2015).

Over half of *P. aeruginosa* colonised adults and children with CF were found to harbour a clonal strain of this pathogen in a study carried out by O'Carroll *et al.* (O'Carroll *et al.* 2004). No strong evidence for a clinically detrimental effect was associated with clonal strains of *P. aeruginosa*, though the degree of virulence of a particular clonal strain was deemed likely to be strain specific. However, there has been evidence supporting the potential transmission of clonal strains (Armstrong *et al.* 2003), thus supporting a movement toward separation of *P. aeruginosa* positive and negative patient groups in the clinical setting. Furthermore ongoing monitoring should be put in place to screen for *P. aeruginosa* superinfection (O'Carroll *et al.* 2004). These recommendations came about by work from Cheng *et al.* which identified 55 of 65 paediatric CF patients to harbour the exact same epidemic strain (Liverpool Epidemic Strain) of beta-lactam resistant *P. aeruginosa* (Cheng *et al.* 1996). While another study examining hypermutable strains of *P. aeruginosa* actually found no evidence of inter-patient transmission (Oliver *et al.* 2000). It was notable that the hypermutable (mutator) strain in this study persisted in a patient over a number of years. This was reasoned to be a result of an accumulation of adaptive mutations to survive in the CF lung environment which became fixed, resulting in abundant populations of the mutator genotype (Taddei *et al.* 1997; Oliver *et al.* 2000). Further studies like these are required in light of the recent WHO publication of *P. aeruginosa* as one of the top 3 most critical bacterial species for which new antibiotics are urgently needed (Tacconelli *et al.* 2017). The appearance of *P. aeruginosa* on this global priority list is of serious concern to immunocompromised CF patients.

Genomic changes in strains of *P. aeruginosa* infecting recently colonized patients show significant resemblance to environmental *P. aeruginosa* strains, while strains identified in long-term, often chronically colonized patients appear to be genetically distinct from environmental strains, likely as a result of accumulated adaptive mutations (Jelsbak *et al.* 2007). However, the selective pressures causing genetic adaptations in the *P. aeruginosa* genome are not limited to physiological conditions. It has become apparent through the literature reviewed here, that *P. aeruginosa* has considerable interaction with the microbial communities present in CF airways, and thus likely also adapts in response to the microbial communities that it encounters (Jelsbak *et al.* 2007; Smith *et al.* 2006).

Antibiotic sensitive strains of *P. aeruginosa* infections are usually treated with beta-lactams, for example ceftazidime, meropenem or an anti-pseudomonal penicillin combined with tobramycin or colistin (CF TRUST 2009). Colistin can often be reserved for treatment of more resistant strains of *P. aeruginosa*, as is often the case in chronic infection (Defez

et al. 2004). MDR *P. aeruginosa* has been shown to arise due to exposure to antibiotics (Mohr et al. 2004), in particular beta-lactams (Wolter & Lister 2013) and fluoroquinolones (Gasink et al. 2006). CF patients attending the same clinic can show colonisation by identical strains, indicative of colonisation from the same source or patient-to-patient spread (McCallum et al. 2001). Furthermore, acquisition of MDR strains of *P. aeruginosa* may also occur by cross-infection or ‘super-infection’, meaning spread of *P. aeruginosa* between patients already colonised by different strains of this bacteria (McCallum et al. 2001). There has been an observed rise of antibiotic resistance mechanisms in severe patients (Bacci et al. 2017), likely in response to prolonged treatment with antibiotic therapies, demonstrating the rapid adaptability of the bacterial microbiome to the dynamic environment of the CF airway. Antibiotic resistance mechanisms have been detected in *P. aeruginosa*, for example a common mutational target in chronic CF airway infection is the multidrug efflux gene *mexZ* (Sobel et al. 2003). This is a negative regulator of *mexX* and *mexY* which are components of a multidrug-efflux pump which contribute to intrinsic and acquired aminoglycoside resistance (Vogne et al. 2004; Sobel et al. 2003). LOF mutations in *mexZ* increase the expression of *mexX* and *mexY*. This is more common in CF patients who are frequently treated with antibiotics, as selection can occur for multidrug-efflux pumps and their regulators. Deletion of *mexXY* has been shown to enhance susceptibilities to aminoglycosides in some *P. aeruginosa* isolates (Sobel et al. 2003). The identification of LOF mutations in genes like *lasR* and *mexZ* may offer future potential to therapeutic developments.

### 1.3.2 *S. aureus* in CF

*S. aureus* is the most common Gram positive bacteria detected in people with CF (Goss & Muhlebach 2011). This prominent pathogen has been commonly isolated in the respiratory tract of children and adults with CF for decades, with the earliest descriptions of microbial infections in CF focused on this species (Di Sant’agnese & Andersen 1946). The prevalence of methicillin-susceptible *S. aureus* (MSSA) in children with CF ( $\leq 19$  years old) in 2016 in Ireland was approximately 56.3% (Fig. 1.4) (CFRI 2016), which lies between prevalence in the UK and US, at 31.5% and ~62%, respectively (Razvi et al. 2009; CF Trust 2016). An average of 37.6% of children with CF were recorded to be chronically colonized with *S. aureus*, a trend which is seen to reverse with age as only 22.1% of adults were chronically colonised (CFRI 2016). It should be noted that *S. aureus* prophylaxis is common practice

in children in many Irish CF centres and this may influence the chronology of prevalence rates. Currently there is no consensus on anti-staphylococcal antibiotic prophylaxis use in young children with CF in Ireland, with each clinic differing in their approach. Prophylaxis is currently recommended from infancy in the United Kingdom (Lo et al. 2011; CF Trust 2011) but advised against in the United States (Lahiri et al. 2016). There has been an increase in studies attempting to determine the benefit of anti-staphylococcal prophylaxis (Smyth & Walters 2001; Smyth & Walters 2003) however there are contradicting outcomes. One trial reported no significant differences in clinical outcome between the placebo group and a trial group treated with cephalexin (5-7 year course), however it was noted that children treated with *S. aureus* prophylaxis were more likely to become positive for *P. aeruginosa* (Stutman et al. 2002). On the contrary, other work has proven effective clearance of *S. aureus* and improved lung function upon anti-staphylococcal treatment (McCaffery et al. 1999). Narrow spectrum antibiotics such as flucloxacillin can be used as prophylactic drugs and dose can be increased if required. There has been a rising prevalence of MSSA and methicillin-resistant *S. aureus* (MRSA) in CF in recent years which has been shown to cause similar lung function decline and inflammation as *P. aeruginosa* (Sagel et al. 2009; Gangell et al. 2011). MRSA infection can contribute to morbidity in CF patients, with an infected paediatric cohort showing impaired growth and an increased requirement for intravenous antibiotics compared to a non-MRSA infected cohort (Miall et al. 2001). MRSA is resistant to  $\beta$ -lactams and often aminoglycosides and macrolides (Leski et al. 1999), thus it is recommended that tetracyclines and clindamycin are used, with glycopeptides and linezolid advised for more severe infections (CF TRUST 2009).

### 1.3.3 *H. influenzae* in CF

*H. influenzae* is among the top three most common bacterial genera of Gram negative bacteria to affect paediatric CF patients. Upon formation of biofilms, this bacteria exhibits reduced susceptibility to antibiotics, and thus can become chronic and cause eventual lung damage (Starner et al. 2006). Approximately 30.6% of children (aged  $\leq 19$  years) were colonized with this bacteria in Ireland in 2016 (CFRI 2016). *H. influenzae* is more prevalent in childhood than adulthood, with the prevalence for this bacteria dropping to an average of 7.9% in adults over 19 years of age (Fig. 1.4) (CFRI 2016). Cardines *et al.* found the majority of *H. influenzae* strains in their CF cohort to be nonencapsulated or nontypeable (NTHi), with many different genotypes identified (Cardines et al. 2012). Genes involved

in biofilm production and adhesion were persistent in *H. influenzae* colonising clones, though it was unclear if this was due to perpetual colonisation with the same clone of *H. influenzae* or a result of sequential colonisation with different clones (Cardines et al. 2012). Colonisation with *H. influenzae* is generally treated with antibiotics such as co-amoxiclav or doxycycline for two to four weeks, to which most strains of *H. influenzae* are susceptible (CF TRUST 2009). Resistances include azithromycin, amoxicillin and macrolides, while resistance to imipenem may be on the increase, though some resistant strains still remain susceptible to ampicillin (Giufrè et al. 2011; CF TRUST 2009).

#### **1.3.4 *S. maltophilia* in CF**

*S. maltophilia* is a Gram negative, MDR bacteria which was first isolated in 1943, and has begun to be recognised as an emerging global opportunistic pathogen (Hugh & Leifson 1963; Brooke 2012). This bacteria has a prevalence of approximately 9.5% in children with CF in Ireland (CFRI 2016), with similar prevalence in adult patients (Fig. 1.4), though some studies have found an increased prevalence in older patients (Ballesteros et al. 1995). There has been contradictory evidence for an association between *S. maltophilia* colonisation and a decline in lung function, with some research finding CF patients positive for *S. maltophilia* having to have worse lung function than *S. maltophilia* negative patients at the time of FEV<sub>1</sub> testing (Goss et al. 2004). Confounding factors and lack of a distinction between chronic and intermittently colonised patients with *S. maltophilia* likely contributed to the resulting non-significant association in this study. Another study measured the serum antibody levels to whole cell *S. maltophilia* and *S. maltophilia* flagellae in chronically infected CF patients (Waters et al. 2011). A specific immunostimulatory response involving IL-8 and TNF-alpha production and higher levels of *S. maltophilia* antibodies were identified in patients with chronic *S. maltophilia* colonisation, compared to intermittently or non-*S. maltophilia* colonised patients (Waters et al. 2011). This was also associated with worse lung function and thus could be said to represent true *S. maltophilia* infection (Waters et al. 2011). Treatment of *S. maltophilia* is often based on co-trimoxazole due to its resistance to anti-pseudomonal drugs (CF TRUST 2009). Though one study identified high rates of co-trimoxazole resistance *in vivo* and suggested doxycycline and a high concentration of colistin to be the most active against *S. maltophilia*, despite multidrug resistance becoming a common occurrence (San Gabriel et al. 2004).

### 1.3.5 *A. baumannii* in CF

*Acinetobacter* species are becoming recognized as emerging pathogenic Gram negative genera in CF, with one recent study identifying colonisation in 9% of samples from CF patients (Fernández-Olmos et al. 2012). *A. baumannii* is phenotypically indistinguishable from those closely related species *A. calcoaceticus*, *A. pittii* and *A. nosocomialis* in routine laboratory technologies, thus the term Acb complex now covers *A. calcoaceticus*, *A. baumannii*, *A. seifertii* and *A. dijksboorniae*.

Recently over 50 different strains of *Acinetobacter* species were identified from CF sputum samples, many of which were MDR (Rocha et al. 2017). MDR carbapenemase-resistant strains of *A. baumannii* accounted for approximately 28% of all isolated *A. baumannii* strains in this study. These MDR strains may represent lineages which are a source of resistance genes with potential to spread to other pathogens in the CF airway (Rocha et al. 2017). The current recommendation for treatment of *A. baumannii* infections is carbapenems, though a rising resistance has been documented (Karageorgopoulos & Falagas 2008).

### 1.3.6 Emerging CF pathogens

Many other microorganisms are commonly found in the lung of CF patients including bacterial genera of *Streptococci* (Coburn et al. 2015), *Klebsiellae* (Ramsey et al. 1991), *Achromobacter* (Liu et al. 2002) and more rarely *Burkholderia* (Chaparro et al. 2001). Additionally fungi and yeast are commonly isolated including *Aspergillus* (Nunley et al. 1998) and *Candida* species (Muthig et al. 2010).

A study into emerging CF pathogens identified the Gram negative bacterial genera of *Klebsiellae* species to be present in 17% of samples tested (n =36/213) (Steinkamp et al. 2005). However, there have been mixed reports as to whether these species are early colonizers of the CF airways or merely represent transient colonizers (Rosenfeld et al. 2001; Ramsey et al. 1991). Longitudinal and multi-centre research is required to track the prevalence of this organism in paediatric CF populations. The *Burkholderia cepacia* complex (Bcc) is a collection of phenotypically similar bacteria, which differ by genotype and are divided into approximately nine species. Bcc has been recognised to infect the lungs of patients with CF on rare occasion, though can then result in chronic infection, or even ‘cepacia syndrome’ which leads to a serious decline in lung function and can exhibit invasive disease (Mahenthiralingam et al. 2005).

A plethora of studies have demonstrated the detrimental effects of infection with *Aspergillus* species (Amin et al. 2010; Schønheyder et al. 2009). A 2010 study identified chronic *A. fumigatus* infection in 16% of patients and noted they had significantly lower FEV<sub>1</sub>% predicted (Amin et al. 2010). Other studies have reported prevalences of 52.6% and 56.7% in adult patients (Burgel et al. 2012; Paugam et al. 2010). Reported prevalence of this fungus tends to vary greatly between studies (Nagano et al. 2007; Burns et al. 1998) depending on the method of detection used. According to the CFRI in 2016, 9% of CF patients aged ≤19 years were colonised with *A. fumigatus* and 13.6% of adults (CFRI 2016). A study carried out in Ireland identified 30% of patients grew *Aspergillus* species in sputum cultures and itraconazole treatment of these patients improved lung function suggesting a pathogenic role for *A. fumigatus* in CF (Chotirmall et al. 2008). Treatment of *A. fumigatus* includes prophylaxis using posaconazole (Karthaus 2011) or treatment upon identification of infection with azoles such as itraconazole and voriconazole (Dupont 1990; Walsh et al. 2002; Coughlan et al. 2012).

*Candida albicans* is the most frequently isolated *Candida* species in CF and has an association with decreased FEV<sub>1</sub> and increased hospital-treated exacerbations in patients has been reported (Chotirmall et al. 2010). It is likely that frequent use of antibiotics and steroids predispose CF patients to colonisation with this species. Reported prevalence vary with reports of 60.7% (Chotirmall et al. 2010) and 78.3% (Valenza et al. 2008), while one study isolated *C. albicans* from the respiratory tract in 93% of CF patients (Muthig et al. 2010). Options for treatment of *C. albicans* infections include fluconazole and amphotericin (CF TRUST 2009).

#### **1.4 The CF airway microbiome**

Traditionally, bacterial identification and detection were reliant upon selective and differential cultivation techniques. These practices could not account for fastidious bacteria which were difficult to culture or present in low concentrations (Bittar & Rolain 2010; Zemanick et al. 2010). In fact by their very nature, diagnostic culturing media are designed to select out the common CF bacteria while prohibiting the growth of others. In the past two decades culture independent microbial identification approaches have revolutionised CF airway microbiology. It is now well accepted that the CF airway is host to a diverse community of bacteria (Sibley et al. 2006), fungi (Nagano et al. 2007; Burns et al. 1998) and viruses (Frickmann et al. 2012; Burns et al. 2012). The ‘microbiome’ has been defined

as “the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes, and the surrounding environmental conditions” (Marchesi & Ravel 2015). The CF lung microbiome is now known to harbour polymicrobial communities, with a range of previously unidentified bacterial species emerging in the wake of molecular technology developments (Chmiel et al. 2014). Many molecular based studies have been published providing a considerably more comprehensive view of the microbiology of CF airways than was traditionally considered using culture-based techniques (Rogers et al. 2010; Robinson et al. 2010).

Early molecular studies heavily depended on clone hybridization or fingerprinting methods such as terminal restriction fragment length polymorphism (T-RFLP) (Rogers et al. 2004), temporal temperature gradient electrophoresis (TTGE) (Kolak et al. 2003) and length heterogeneity PCR (LH-PCR) (Rogers et al. 2004; Belkum et al. 2000). Identification of a diverse range of bacterial species in these studies were hindered due to limited sequence data, though it was possible to identify DNA fragments consistent with classic CF-associated pathogens and even some obligate anaerobic bacteria (Dunbar et al. 2000; Rogers et al. 2003). Genera identification progressed with the advent of 16S rRNA gene cloning and Sanger sequencing, as previously under-identified genera such as *Streptococcus* and unidentified genera *Prevotella* and *Veillonella* were now being shown to not only be common in the CF airway but highly abundant (Lane et al. 1985; Bittar et al. 2008). The high abundance of these genera mean that caution should be taken with antibiotic exposure in these patients. The antibiotic treatment aimed at a single pathogenic species has potential to diminish microbial diversity of multiple genera whose abundance often far outweigh that of a pathogenic bacteria (Tunney et al. 2008).

The core microbiota of both adult and paediatric CF patients were recently defined by Coburn *et al.* (Coburn et al. 2015), where it became apparent that bacterial genera with highest prevalence also had the largest relative abundance in the patient microbiome. The genera listed in Table 1.2 are ordered in decreasing abundance, with *Streptococcus* found to be the most abundant genera in paediatric and adult CF lung microbiota (Coburn et al. 2015). Among these highly abundant genera, the majority are considered non-classical CF pathogens and are mostly anaerobes. The common CF bacteria, *Staphylococcus*, *Pseudomonas* and *Stenotrophomonas* were less abundant in paediatric patient microbiota but are considered core members of adult CF microbiota (Surette 2014; Lynch & Bruce 2013). The relative abundances of these genera vary considerably depending on patient age, different areas of the lung and individual differences between patients (Parkins & Floto

2015). Van der Gast *et al* conducted research on adult CF patients, identifying majorly similar core microbiota as paediatric patients (Table 1.2), though with differences in community diversity and evenness (van der Gast et al. 2011).

The CF airway microbiome has been shown to be associated with patient age and concurrently, disease stage. The early acquisition and succession of colonisation in CF airway has been confirmed by metagenomic studies (Muhlebach et al. 2018). Using 16S rRNA sequencing (Si-Seq) in addition to culture analysis Coburn *et al.* concluded that age was significantly correlated with decreased alpha-diversity (Coburn et al. 2015), while other studies have shown the risk of acquiring pathogenic organisms increased with age (Cox et al. 2010; Zhao et al. 2015). Changes in community diversity and lung function were noted to somewhat plateau at the age of 25 (Coburn et al. 2015). For example, a significant increase in *Pseudomonas* and *Burkholderia* dominance was observed in patients of this age (~25 years), which coincided with the lowest sputum sample diversity and lowest lung function recorded in patients of all ages (Coburn et al. 2015). Children have higher absolute richness in their airway microbiome, in addition to greater phylogenetic diversity and evenness (Klepac-Ceraj et al. 2010). The reducing community complexity in adult patients is suggested to be a result of frequent disturbances to the ecological environment of the lungs of CF patients, with frequent use of antibiotics and *P. aeruginosa* invasion as patients age (Klepac-Ceraj et al. 2010). From an ecological standpoint reduced diversity is associated with a weaker microbial community with more potential for invasion by a pathogenic species. Through longitudinal studies of fluctuations in microbiome community composition, it was noted that patterns of colonisation before an exacerbation were dependent on the stage of disease the patient was in (Carmody et al. 2018). Thus it was concluded that the age and therefore the clinical state of the patient was highly important in these studies. A study examining changes in the microbiome of the CF lung before and during exacerbations of undefined cause, found changes were largely dependent on the community composition and diversity at baseline, with particular genera such as *Pseudomonas* and *Gemella* acting as important drivers of change in the microbial community at exacerbation (Carmody et al. 2013). In fact, particular dominant pathogens in the microbiome, such as *P. aeruginosa*, were found to decrease with the onset of symptoms of a pulmonary exacerbation (Carmody et al. 2013). The majority of these samples had a dominant pathogen at baseline, including *Pseudomonas*, *Haemophilus* and *Streptococcus* among others. However neither the dominant pathogen nor the antibiotics administered to a patient were found to have any significant effect on bacterial density at

exacerbation. These factors may be more important to change of the community diversity than bacterial density during exacerbation (Carmody et al. 2013). Microbial community composition was noted to be highly similar in patients during an exacerbation and when these patients were clinically stable in another study, thus suggesting that exacerbation may represent an intrapulmonary spread of infection rather than a change in composition of the microbial community (Fodor et al. 2012). In support of this, Cuthbertson *et al.* also identified a compositional resistance in the CF microbiota during exacerbation (Cuthbertson et al. 2016). An investigation into the causes and consequences of pulmonary exacerbations in CF patients from a hospital ICU and Pulmonary Department supported the findings of Carmody *et al.* They determined that infection was the most frequent cause of severe exacerbation and compared bacterial pathogens in CF sputum samples before exacerbation and during exacerbation in these patients (Ellaffi et al. 2005). The results revealed identification of several dominant bacterial pathogens which had previously not been identified in these patients, including *S. aureus*, *S. maltophilia*, *E. coli*, *H. influenzae* and *A. xylosoxidans*. Furthermore, a review of some characteristics of these patients when in a stable pre-exacerbation condition revealed interesting insights in that 90% of these patients were colonised with *P. aeruginosa*, 67% colonised with *S. aureus* and 10% with *B. cepacia*. Thus, colonisation with these pathogenic bacteria could be considered as a risk factor for pulmonary exacerbation (Ellaffi et al. 2005). Carmody and colleagues reported an increased community diversity upon CF exacerbation in what they referred to as the climax-attack model (Carmody et al. 2018; Conrad et al. 2013). The climax or baseline microbiota consisting of typical CF pathogens and the attack/ exacerbation community being dominated by anaerobes such as *Prevotella*, *Veillonella*, *Fusobacterium* and microaerophilic *Streptococcus*. Overall these studies show there are unexpected differences between the stable/baseline and exacerbation CF airway microbiome.

**Table 1.2:** The most common genera identified from the CF airway using molecular approaches

<b>Genera</b>	<b>Identification Methods Used on CF Airway Samples</b>	<b>Reference/s</b>	<b>Type of Technology Applied in Referenced Study</b>
<i>Streptococcus</i>	16S rRNA Sequencing, PhyloChip, 454 pyrosequencing, 454 GS FLX Titanium pyrosequencing, T-RFLP, RISA	(Christopher D Sibley et al. 2008)	Targetted amplicon (16S rRNA)
<i>Rothia</i>			
<i>Prevotella</i>			
<i>Actinomyces</i>		(Coburn et al. 2015)	Targetted amplicon (16S rRNA)
<i>Veillonella</i>			
<i>Gemella</i>		(Stressmann et al. 2012)	Targetted amplicon (T-RFLP)
<i>Haemophilus</i>		(van der Gast et al. 2011)	Targetted amplicon (16S rRNA)
<i>Neisseria</i>		(Cox et al. 2010)	Targetted amplicon (16S rRNA)
<i>Staphylococcus</i>		(Lim et al. 2014)	Metagenomic (shotgun sequencing)
<i>Pseudomonas</i>		(Lim, Schmieder, Haynes, Willner, et al. 2013)	Metagenomic and metatranscriptomic
<i>Stenotrophomonas</i>		(Flight et al. 2015)	Targetted amplicon (RISA)
<i>Achromobacter</i>			

The frequent use of antibiotics to treat infections with some of these bacterial species is likely to shape the host's airway microbiome. Optimising treatment regimes so that specific pathogenic species are efficiently targeted with reduced damage to beneficial community diversity is a great importance and requires further research to become a reality. It has been speculated that prolonged and frequent antibiotic use in CF patients may be a primary driver for the observed reduction in diversity as patients age, or as disease status worsens (Chmiel et al. 2014; Klepac-Ceraj et al. 2010). This resulting reduced diversity coincides with a single dominant species in the CF microbiome, while bacterial abundance remains relatively constant. Although there are options for the treatment of these Gram negative bacteria, there is very little known about the impact of antibiotics in polymicrobial pulmonary exacerbations (Chmiel et al. 2014). Some studies have shown that antibiotics impact the microbial community structure (Zhao et al. 2012) while others have shown little influence of antibiotics on the overall diversity and community composition (Fodor et al. 2012). Fodor *et al.* detected a small decrease in community richness after treatment with antibiotics including ceftazidime, meropenem, ciprofloxacin, though this did not reach significance (Fodor et al. 2012). A resilient microbial community composition after

antibiotic treatment for pulmonary exacerbation was also found by Cuthbertson *et al.* (Cuthbertson et al. 2016). Carmody et al. reported directly contrasting results with a reduced community diversity during antibiotic therapy (Carmody et al. 2018). It has also been shown that antibiotic therapy directed towards *P. aeruginosa* had little effect on *P. aeruginosa* bioburden but did significantly reduce the relative abundance of anaerobic species in the CF airway microbiome (Carmody et al. 2018). Repeated exposure to antibiotics most likely has an impact on the microbial community structure however whether this is transient or over time has long term effects is still not known.

### **1.5 *P. aeruginosa* and the CF lung microbiome**

Hierarchical clustering of 16S rRNA microarray samples was observed in patients which had been treated with antibiotics and were positive for *P. aeruginosa* colonisation, while non-colonised patients also on antibiotics formed another distinct cluster (Klepac-Ceraj et al. 2010). Furthermore, it was found that beta-diversity was significantly smaller between non-*P. aeruginosa* colonised communities than between *P. aeruginosa* colonised communities (Klepac-Ceraj et al. 2010). Thus, microbial communities dominated by *P. aeruginosa* are more variable while *P. aeruginosa*-negative communities are more similar to each other (Klepac-Ceraj et al. 2010). Antibiotic exposure was controlled for in this study therefore this effect in *P. aeruginosa* positive communities can be linked directly to *P. aeruginosa* colonisation. In agreement, Renwick *et al.* reported that paediatric CF patients with dissimilar *P. aeruginosa* culture bioburden also had overall significantly dissimilar microbial community structure in terms of evenness ( $p=0.011$ ) and richness ( $p = 0.009$ ) (Renwick et al. 2014). A detailed understanding of how *P. aeruginosa* colonisation changes the surrounding microbiome is still not known and studies in this area are limited.

#### **1.5.1. Polymicrobial interactions with *P. aeruginosa* in the CF lung**

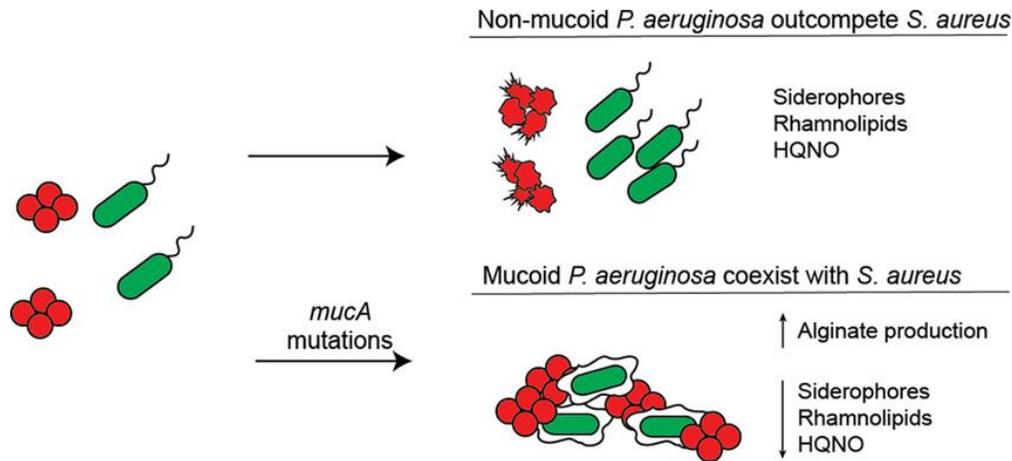
A plethora of studies have confirmed the presence of a diverse range of microbes in samples taken from the CF lung suggesting that coinfections involving different species of bacteria probably represent the norm in the CF lung (Harrison 2007; Bittar et al. 2008; Christopher D Sibley et al. 2008; Rogers et al. 2010). Some studies have begun to enlighten our understanding of the polymicrobial interactions taking place among these bacteria, for example recent work carried out by Korgaonkar *et al.* has revealed that *P. aeruginosa* can respond directly to cell wall fragments from Gram positive bacteria by increasing

production of multiple extracellular factors, including pyocyanin (Korgaonkar et al. 2013; Korgaonkar & Whiteley 2011). This data confirms that multispecies interactions can contribute to alterations in *P. aeruginosa* pyocyanin production (Gee W Lau et al. 2004). Pages-Monteiro *et al.* performed meta-taxogenomic analyses on CF samples in order to define the core CF microbial community at the genus level. The results of this sequencing were suggestive of a preferential affiliation between *P. aeruginosa* and some genera such as *Stenotrophomonas* and negative associations with others such as *Haemophilus*, *Burkholderia* and *Neisseria*. However, not enough is known of the specific pathways and interactions occurring between *P. aeruginosa* and other core pathogens.

#### **1.5.1.1 *P. aeruginosa* and *S. aureus* interactions**

Employing a co-culturing technique, one study revealed that *P. aeruginosa* actually acts as a driving force in transitioning *S. aureus* from aerobic respiration to fermentative metabolism (Filkins et al. 2015). Analysis of RNA-seq on coculture assay mixtures made it possible to identify that this transition was dependent on two factors, 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), a component of the *Pseudomonas* quinolone signal (PQS) system, and siderophores produced by *P. aeruginosa*. HQNO has been noted in other studies to be present in CF patients colonised with *P. aeruginosa* but not in uninfected patients, further suggesting that HQNO-mediated interactions have potential to influence disease progression by facilitating a *P. aeruginosa*-dominated microbiome (Smith et al. 2006; Filkins et al. 2015). It was noted that *S. aureus* produces lactate that can be consumed by *P. aeruginosa*, supporting the idea that these bacteria may be able to coexist for a time. However as time progresses *P. aeruginosa* reduces the viability and presence of *S. aureus* in a HQNO and siderophore dependent manner (Filkins et al. 2015). *P. aeruginosa* isolates from mono-infected patients were found to be more competitive with *S. aureus* than *P. aeruginosa* isolates from co-infected patients (Limoli et al. 2017) suggesting a degree of adaptation to coexistence. Therefore *S. aureus* and *P. aeruginosa* may initially coexist in mutual or commensal relationships in the CF airway but as disease progresses and the host environment becomes more aggressive *P. aeruginosa* may out-compete *S. aureus* for resources (Fig. 1.4). This theory would support these findings and the historical culture data (Fig 1.2). *P. aeruginosa* may also be able to displace *S. aureus* through its manipulation of innate host immunity. *P. aeruginosa* has been shown to induce the production of a *S. aureus* killing phospholipase, sPLA2-IIA, by bronchial epithelial cells (Qu & Lehrer 1998) which subsequently enhances the growth of *P. aeruginosa*.

Targeting Gram positive bacteria, sPLA2-IIA is thought to be the most potent antibacterial enzyme in mammals (Nevalainen et al. 2008). This suggests that interactions between *P. aeruginosa* and the host can play a significant role in determining the dynamics of the bacterial communities present (Pernet et al. 2014).



**Figure 1.4:** A model of co-existing *P. aeruginosa* and *S. aureus* in biofilm production. *P. aeruginosa* can outcompete *S. aureus* when it is in a non-mucoid state, this occurs as it kills *S. aureus* through siderophores, rhamnolipids and a host of other antimicrobial agents. Upon mutations in the *P. aeruginosa mucA* gene, an overproduction of the polysaccharide alginate occurs, resulting in reduced expression of genes associated with siderophore, HQNO, and rhamnolipid synthesis. Thus, impairing the ability of *P. aeruginosa* to outcompete *S. aureus*, resulting in coexistence of these species in the CF lung. Figure extracted from Limoli et al. 2017

*P. aeruginosa* uses exopolysaccharides (EPS) to form biofilms with *S. aureus* (Periasamy et al. 2015). The EPS alginates, *Pel* and *Psl*, are produced by *P. aeruginosa* and are likely to be involved in biofilm formation and the close association of biofilms with *S. aureus* (Limoli et al. 2017). *Psl* in particular is vital for allowing *P. aeruginosa* to form a single species biofilm on top of *S. aureus* (Colvin et al. 2012). Accordingly, the specific type of extracellular polysaccharide produced by *P. aeruginosa* can significantly affect the architecture of the biofilm and the ability of these two species to interact closely (Chew et al. 2014).

*P. aeruginosa* iron acquisition can be influenced by social interactions (Oglesby et al. 2008). It has been known to overcome iron restriction in the CF airway by producing iron-chelating siderophores which can acquire otherwise sequestered ferric iron (Meyer et al. 1997). Siderophores are thus considered to be virulence factors as they enable bacterial growth enhancement. Non-iron producing bacteria can exploit producers to gain a fitness advantage. An example of this is how *P. aeruginosa* can sequester iron from damaged host

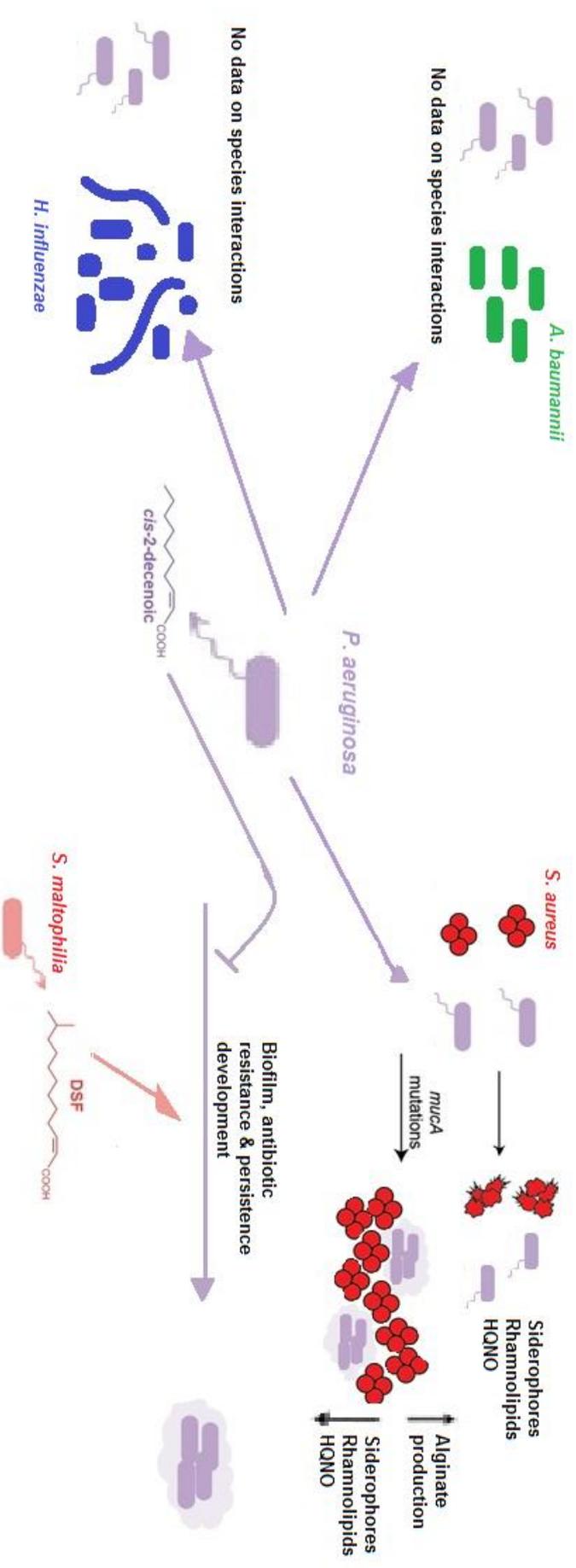
cells using Haem Assimilation System (Has) and Pseudomonas Haem Uptake (Phu) (Cornelissen & Sparling 1994; Finkelstein et al. 1983). Tognon *et al.* examined the evolution of *P. aeruginosa* in the presence of *S. aureus*, by sequencing the ancestor *P. aeruginosa* genome compared to that after 150 generations of co-colonisations with *S. aureus* (Tognon et al. 2017). *P. aeruginosa* upregulated siderophore production in response to *S. aureus*, which acted as an iron competitor in this experiment. *P. aeruginosa* can lyse *S. aureus* cells to access iron, the benefit of which would depend on the degree of competition between the two strains. If a broad range of species are in competition for iron, then iron limitation hinders the ability of *P. aeruginosa* to successfully form biofilms (Pierson & Pierson 2010). Inactivation mutations of virulence-associated lipopolysaccharide (LPS) were noted to be a direct consequence of *S. aureus* imposed selection *in vitro* (Tognon et al. 2017). It was noted that this adaptation also conferred enhanced resistance to beta-lactam antibiotics, though this evolution took place in their absence. Mutations in the *wsp* signalling system of *P. aeruginosa* were also identified. These were assumed to occur as a response to reduced oxygen in growth conditions. In support of the clinical applicability of these results, both LPS and *wsp* mutants were observed in CF clinical isolates (Tognon et al. 2017).

#### **1.5.1.2 *P. aeruginosa* and *S. maltophilia* interactions**

*S. maltophilia* has been observed to co-colonise patients with *P. aeruginosa* (Wainwright et al. 2009) and more recently, has been proven to modulate the virulence of *P. aeruginosa* when in a mixed biofilm (Pompilio et al. 2015). Pompilio *et al.* revealed that *P. aeruginosa* reduces the growth of *S. maltophilia* in a cell-cell contact dependent manner. Concurrently, the adherence of *P. aeruginosa* to CF bronchial epithelial cells was reduced by *S. maltophilia*, which was speculated to be a result of reduced swarming motility in *P. aeruginosa*. They also showed that *P. aeruginosa* overexpressed *aprA*, coding for protease production, and *algD*, coding for alginate production. Quorum sensing related *rhlR* and *lasI* genes were downregulated under the same physiologically representative conditions (Pompilio et al. 2015). Furthermore, *S. maltophilia* was protected against tobramycin activity when in a mixed biofilm with *P. aeruginosa*, with speculation that the induced alginate expression in *P. aeruginosa* could be the reason for this. A reciprocal interference may be at play between *S. maltophilia* and *P. aeruginosa* in the CF lung, with a possible outcome where *S. maltophilia* stimulates the virulence of *P. aeruginosa*, leading to

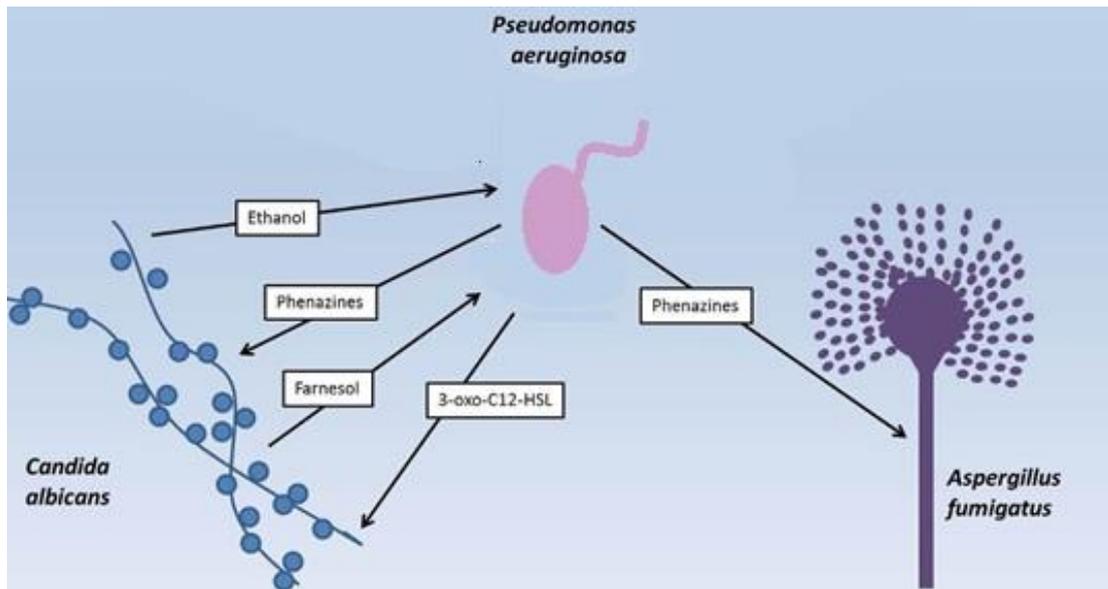
pulmonary exacerbations in the patient (Pompilio et al. 2015). De Vidipó *et al.* added to our knowledge about the interaction of these species through incubation of host human bronchial epithelial cells with *P. aeruginosa* prior to incubation with a CF *S. maltophilia* isolate (De Vidipó et al. 2001). They found that pre-exposure to *P. aeruginosa* did not alter the adhesion of *S. maltophilia* to the host cells, indicating that these two bacteria were not competing for the same host cell receptors (De Vidipó et al. 2001; Brooke 2012). However, when these bacteria were co-incubated with host cells, there was a significant reduction in *S. maltophilia* cell adherence, implying that secreted products from *P. aeruginosa* can impair the adherence of *S. maltophilia* to bronchial epithelial cells (De Vidipó et al. 2001). This study was complementary to Pompilio *et al.* as each group examined the effect of one of these pathogens on the capability of the other to adhere to human bronchial epithelial cells with contrasting findings.

A comprehensive study undertaken by Ryan *et al.* examined the interaction between these two bacteria with particular focus on signalling via the *S. maltophilia* diffusible signal factor (DSF) (Ryan et al. 2008) (Fig. 1.5). *S. maltophilia* DSF was observed to affect polymyxin tolerance and induce filamentous biofilm formation in *P. aeruginosa*. The *rpfF* gene encodes the DSF synthase RpfF in *S. maltophilia* (Huedo et al. 2015). Upon co-culturing an *rpfF* mutant *S. maltophilia* with *P. aeruginosa*, the biofilm architecture of *P. aeruginosa* returned to its appearance in monoculture, therefore the *rpfF* gene appeared to be essential in the production of DSF (Ryan et al. 2008). A deeper understanding of the DSF system and its effects on biofilm architecture may have future clinical significance for the treatment of polymicrobial interactions between *S. maltophilia* and *P. aeruginosa*.



**Figure 1.5:** Summary of known and unknown interactions between *P. aeruginosa* and other bacterial species discussed. Detailed knowledge of the interactions of *P. aeruginosa* with *A. baumannii* and with *H. influenzae* is currently lacking. The DSF compound produced by *S. maltophilia* is shown here to play an important role in *P. aeruginosa* biofilm development. Meanwhile siderophores, rhamnolipids, HONO and the changes observed with *muca* mutations all play an important role in the interaction between *P. aeruginosa* and *S. aureus*.

Upon thorough examination of the literature to date, there appears to be no significant research into the interactions between *P. aeruginosa* and *A. baumannii* or *H. influenzae*, thus making this area a worthwhile avenue of future research. There is literature on cross-Kingdom interactions of *A. fumigatus* and *P. aeruginosa* (Reece et al. 2017; Sass et al. 2018), *P. aeruginosa* and *Candida* (Holcombe et al. 2010; McAlester et al. 2008), among others (Fig. 1.6), however these are outside the scope of this thesis.



**Figure 1.6:** Summary of cross kingdom interactions between *P. aeruginosa* and other microbial inhabitants of the CF lung. Arrows illustrate the direction of the interaction with interactions driven by iron acquisition omitted. This is because siderophores have the ability to shape interactions which is likely to be a result of indirect effects of iron limitation. This figure was adapted from O'Brien & Fothergill 2017.

The studies presented above provide evidence that inter-species and cross-Kingdom interactions can influence not only pathogen virulence but also host responses. The impact of these interactions in CF airway disease are yet mostly unexplored.

## 1.6 Non-antimicrobial therapies for CF

The cornerstone of CF care is antimicrobial therapy for airway infections, examples of which were described throughout section 1.3. CF currently remains an incurable disease, however, in recent years there have been many promising advancements. Developments have been broadly focused, from new antimicrobials aimed at treating problematic chronic infections, to those directed at correcting mucus transport imbalances in these patients.

CFTR potentiators and correctors have brought about better quality of life in many patients to date. ORKAMBI® (Lumacaftor/Ivacaftor) medication is the first of its kind to target complex protein defects in patients greater than 6 years of age, who are homozygous

for the  $\Delta F508$  CFTR mutation. Lumacaftor functions to improve the conformational stability of the protein, and thus increases the processing and trafficking of the mature CFTR protein to the surface of the cell. While Ivacaftor simultaneously works as a CFTR potentiator by facilitating an increased chloride transport. This drug has shown increases in patient FEV<sub>1</sub> and reductions in the rate of pulmonary exacerbations in patients (Wainwright et al. 2015). KALYDECO® (Ivacaftor) is available to patients of 2 years of age and is primarily aimed at those with a G551D mutation. This drug increases the action of defective CFTR protein and has been shown to increase lung function in patients. Risk of pulmonary exacerbations, weight and concentration of chloride in sweat were also improved with use of Ivacaftor (Accurso et al. 2010).

Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels (CaCCs) are known to regulate membrane potential in various cells (Schroeder et al. 2008; Sanders et al. 2006) and have been suggested as a drug target for CF patients. The relationship between CaCC and CFTR is an interesting one, as both channels induce stimulation-dependent fluid secretion, though do so by different intracellular pathways (Berg et al. 2012).

There is a plethora of other research on CF therapeutic options, though most can not be discussed in detail in this thesis. Histone deacetylase inhibitors, class II targeted drugs such as Miglustat (Lubamba et al. 2009), drugs allowing protein translation in sequences containing missense mutations such as Ataluren (PTC124) (Sermet-Gaudelus et al. 2010), and flavonoids such as Genistein (Al-Nakkash et al. 2001) or sodium 4-phenylbutyrate (Rubenstein et al. 1997) have each proved effective treatment options in CF patients with different mutations, as do many other exciting studies (Norez et al. 2009; Noël et al. 2008; Norez et al. 2006; Dormer et al. 2005; Kerem et al. 2008; Lubamba et al. 2012; Esther et al. 2017; Narayanaswamy et al. 2017; van Eijk et al. 2017; Tängdén 2014).

## **1.7 Aims of this project**

To date no studies have fully characterised the microbiome of CF patients colonized with *P. aeruginosa* and compared them to those patients not colonized with *P. aeruginosa*. Studies sequencing the 16S rRNA region have provided valuable insight into the genera present in the CF airway and have begun to elucidate the importance of the airway microbiome in CF disease. However these 16S rRNA sequencing techniques are limited in their discriminatory ability and can only identify bacteria to the genera level with confidence. The aim of this study was to employ culturing techniques alongside two

molecular techniques, RISA and WGSS, to fully characterize the CF airway microbiome of *P. aeruginosa* positive and negative communities. Employing detailed analysis of the WGSS data we aimed to explore the metabolic capabilities, strain-level composition and resistome of these *P. aeruginosa* positive and negative communities in order to identify key changes in the microbiome during *P. aeruginosa* colonisation. In addition we hoped to identify symbiotic and/or antagonistic species within the *P. aeruginosa* positive microbiomes in order to explore the potential for future exploitation of these relationships.

## **Chapter 2**

**A description of the paediatric CF airway microbial community  
employing culture and Ribosomal Intergenic Spacer Analysis**

## 2.1 Introduction

Traditional microbial culture analysis is often considered insufficient in terms of accurate representation of the complete microbiome (Lleo et al. 2005; Rudkjøbing et al. 2016). Some microbes grow more successfully than others when plated on agar which is not necessarily indicative of their true *in vivo* relative abundance. Additionally, certain pathogenic bacteria can be present in very small concentrations in the sputum of a patient before becoming established infections and therefore may not be noticeable upon analysis of agar culture plates. This could result in a false negative result for a pathogenic bacteria and delay treatment for a patient at the beginning of an infection. For this reason it is key to introduce efficient and accurate molecular identification techniques to identify emerging CF pathogens and known pathogens at early stages of infection.

RISA utilizes the hypervariable spacer region between bacterial 16S and 23S genes in the bacterial genome, in order to identify different bacterial species from one another (García-Martínez et al. 1999; Selenska-Pobell et al. 2001). This involves PCR amplification of the intergenic transcribed spacer (ITS) region, and subsequent fingerprinting of the resulting amplification products. The ITS region exhibits interspecies variability in sequence and in length offering a culture-independent approach to bacterial species identification. The RISA technique utilises the variable length of the ITS amplicon to predict species identification. This is primarily a means of measuring diversity and could not be considered an absolute method of bacterial pathogen identification (Flight et al. 2015). Intergenic spacer analysis has been described as highly reproducible, robust and efficient, thus offering an attractive microbial fingerprinting alternative to traditional culture techniques (van Dorst et al. 2014). RISA results have been shown to be consistent with results from 454 pyrosequencing analysis with regard to microbial community richness and diversity measures (van Dorst et al. 2014). RISA has also been employed by research groups examining CF sputum samples (Pages-Monteiro et al. 2017; Flight et al. 2015; Nazaret et al. 2009; Baxter et al. 2013). Pages-Monteiro *et al.* performed RISA on the ITS region of bacterial DNA in CF sputum samples and further metagenomics analysis of the V5 to V6 *rrs* (16S rDNA). They found that RISA profiles of CF sputum samples clustered based on *P. aeruginosa* positivity, indicating that this had a strong influence on the microbiome in the CF lung. However their results were confounded by age and antibiotic exposure (Pages-Monteiro et al. 2017). RISA proved useful in dissociating data from some confounding factors and estimating diversity (Pages-Monteiro et al. 2017),

though more longitudinal work is required to fully evaluate the benefit of this type of bacterial community typing approach. A 2015 study profiled adult CF patient sputum samples using traditional culture, RISA and 16S rRNA pyrosequencing to evaluate if RISA PCR could be used as a routine diagnostic tool in a medical laboratory setting (Flight et al. 2015). In approximately 12% of cases RISA had displayed a dominant ITS amplicon for a pathogen which was not detected by culture. This was further confirmed by 16S rRNA gene sequencing. They concluded that RISA could be used to rapidly identify dominant pathogens and for estimating overall diversity but could not be used as the sole identifier for pathogenic bacterial detection (Flight et al. 2015). Baxter *et al.* found RISA useful in categorising CF patient samples into diverse or pathogen-dominated groups (Baxter et al. 2013), while another study evaluated RISA and High Performance Liquid Chromatography (HPLC) for detection and monitoring of common microbial colonisers of the CF airway. They found that a specific *P. aeruginosa* RISA-HPLC peak was identifiable in the profiles generated from these paediatric patients. RISA peaks identified bacterial species which were not detected using culture (Nazaret et al. 2009). There have also been studies employing RISA as a bacterial fingerprinting technique which extend further than the CF field (Borneman & Triplett 1997; García-Martínez et al. 1999). These studies demonstrated that RISA was useful to give approximate measures of diversity, though revealed other molecular approaches of microbial diagnostics to be more accurate. In this study the applicability of the RISA technique in identifying key CF pathogens from patient samples and assessing overall community diversity was examined.

## **2.2 Materials and methods**

### **2.2.1 Culture and maintenance of bacterial reference strains**

Reference strains (*P. aeruginosa* – PA27853, *H. influenzae* – HI8468, *S. aureus* – SA25923, *S. maltophilia* – SM17666 and *A.baumannii* (MALDI ToF confirmed clinical isolate used) were obtained from the medical microbiology laboratories in AMNCH. Strains were stored on Microorganism Preservation Beads (Protect – TSC Ltd.) at -80°C and 2 to 3 beads of each strain cultured onto Colombia blood agar plates (Fannin/Oxoid) when needed. Each strain was sub-cultured twice prior to use in any experiments.

### **2.2.2 Sample collection**

Sputum samples (n=105) were collected between November 2016 and November 2017 from the microbiology diagnostic laboratory in the Adelaide and Meath, incorporating the National Children's Hospital (AMNCH) in Tallaght as part of standard patient care. Ethics for the use of these samples in research was granted by the Tallaght/St James's ethics committee (reference code 2006/37/06).

### **2.2.3 Immediate sample processing**

Samples were treated with 1:1 Sputasol (Oxoid) and incubated shaking at 35°C for 15-20 min to homogenise sputum. Samples were subsequently vortexed for 30 sec and split into two 1.5ml microcentrifuge tubes. Tubes were labelled with the 4-digit identifier of the sample hospital number (Fig. 2.1 A and B). One tube was used for culture-dependent analysis and the other for culture-independent analysis (section 2.4). Initially both tubes were centrifuged at 9500 x g at 4°C for 10 min and the supernatants pooled and transferred to fresh 1.5ml microcentrifuge tubes. Supernatants were aliquoted and frozen at -80°C for future use (section 2.4). RNALater was added to the pellet of one tube to bring the solution to a total volume of 1ml. The pellet was resuspended and this solution was left at 4°C overnight and then stored at -80°C for future culture independent analysis (Fig. 2.1. H & I). The pellet of the second tube was resuspended in Phosphate Buffered Saline (PBS) (Sigma), to a total volume of 1ml and used for culture-dependent analysis on the day of collection.

#### 2.2.4 Culture-dependent processing of samples

A serial dilution ( $10^0$  to  $10^{-3}$ ) was performed in sterile PBS in a class II safety cabinet and 20 $\mu$ l of each dilution was plated, in triplicate on to each of the agars (Table 2.1 and Fig. 2.1. E). Plates were then incubated in the environmental conditions and durations specified in Table 2.1. Following incubation, the numbers of colonies present on each plate was counted and used to calculate the value of the Colony Forming Units (CFU) per millilitre on each respective plate using the calculation below.

$$\frac{\# \text{ colonies} \times \text{dilution factor}}{\text{volume of culture on plate (ml)}}$$

As each dilution was run in triplicate for each media, the CFU/ml values were averaged for each dilution factor. The mean of all dilution factor averages was then calculated to get a single CFU/ml value for each bacteria in each patient sputum sample.

*P. aeruginosa* was initially tested for on cefrimide agar (Fannin) though subsequent tests were used to confirm the identification of this bacteria, as will be discussed in section 2.4. *S. aureus* was tested for initially using *S. aureus* agar (SAID agar) (Biomerieux) and subsequently CHROMID® *S. aureus* Elite agar (SAIDE) from the same manufacturer. Chocolate agar with bacitracin (Fannin) was used to test for the presence of *H. influenzae* in these samples in an anaerobic environment. *S. maltophilia* was identified using a Vancomycin, Imipenem & Amphotericin B Agar (VIA agar) according to instructions and products set out by Kerr et al (Kerr et al. 1996). CHROMagar™ Acinetobacter was used to identify the presence of any *Acinetobacter* species in patient sputum samples. CHROMagar™ Orientation was used to identify the species listed in Table 2.1 based on their phenotypic manifestation on a plate of CHROMagar™. All agars were made according to manufacturer's instructions.

**Table 2.1:** Agars used for approximate identification of listed bacteria

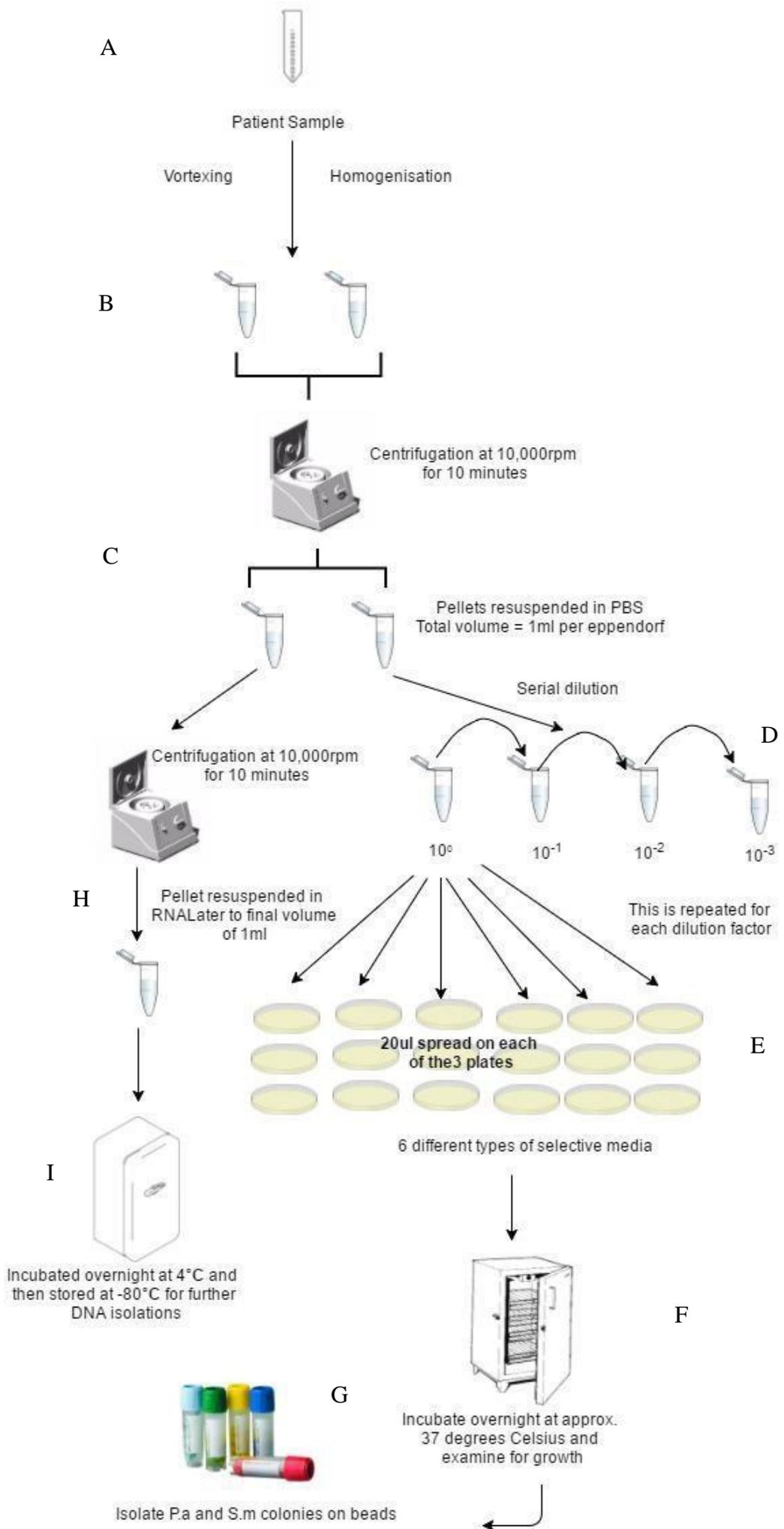
Bacteria	Selective Agar	Oxygenation	Time (h)	Temp
<i>P. aeruginosa</i>	Cetrimide (Fannin)	Aerobic	24	37°C
<i>S. aureus</i>	CHROMID® <i>S. aureus</i> Elite agar (SAIDE) and <i>S. aureus</i> agar (SAID) – (Biomerieux)	Aerobic	24	37°C
<i>H. influenzae</i>	Chocolate agar with Bacitracin (Fannin)	Anaerobic	24	37°C
<i>S. maltophilia</i>	Vancomycin, Imipenem & Amphotericin B Agar (VIA agar)	Aerobic	48	37°C
<i>A. baumannii</i>	CHROMagar™ Acinetobacter	Aerobic	48	37°C
<i>E. coli</i> , <i>Enterococcus</i> , <i>Klebsiella</i> , <i>Enterobacter</i> , <i>Citrobacter</i> , <i>Proteus</i> , <i>Pseudomonas</i> , <i>S. aureus</i> , <i>S. saprophyticus</i>	CHROMagar™ Orientation	Aerobic	24	37°C

### 2.2.5 Medical microbiology diagnostic laboratory techniques

All samples were plated on Colombia sheep blood agar (Fannin), chocolate agar (Oxoid) and cetrimide agar (Fannin) for the specific identification *P. aeruginosa*. Upon examination of these plates an oxidase test (section 2.3.1) was performed on any suspected *P. aeruginosa* (oxidase +), *S. maltophilia* (oxidase +) and *H. influenzae* (oxidase -) colonies. Colonies that were query *Staphylococci* were treated with a Pastorex test to indicate whether it was *S. aureus* or an alternative *Staphylococcus* species (section 2.3.4). Ultimately bacterial species identification was determined using the MALDI-ToF (section 2.3.3) and antimicrobial sensitivity determined employing the VITEK system.

### 2.2.6 Isolation and archiving of *P. aeruginosa* and *S. maltophilia* isolates

If growth was observed on cetrimide or VIA agar, colonies were counted and subsequently individual colonies were preserved in cryovials of Microorganism Preservation Beads (Protect – TSC Ltd.) (Fig. 2.1 G). Between 3 and 5 individual *P. aeruginosa* colonies were picked with inoculating loops for each patient sample and stored in cryovials. These vials were then placed at -80°C.



**Figure 2.1:** Flow diagram of sample processing. Samples were processed beginning at (A) and splitting at point (C), to either (D) or (H). Both pathways from (D) to (G) and (H) to (I) were carried out on different portions of all samples

## **2.3 Identification techniques for *P. aeruginosa* and *S. aureus***

### **2.3.1 The oxidase test**

The oxidase test detects the presence of oxidising enzymes which are only produced by certain Gram-negative organisms such as *Pseudomonas* bacteria. A negative reaction excludes all types of *Pseudomonas*, while a positive reaction indicates that an organism belongs to the *Pseudomonas* group (Wahba & Darrell 1965). Oxidase positive bacteria possess an iron containing haemo-protein called cytochrome oxidase or indophenol oxidase. The oxidase test reagent acts as an electron acceptor for the enzyme oxidase and thus indophenol blue forms from the oxidised reagent.

A 1% solution of N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (Sigma-Aldrich) was made up in a total volume of 3.5ml of sterile distilled water and used immediately, with any remaining solution discarded. A sterile cotton swab was dipped into this prepared solution, with excess solution removed at the neck of the bijoux. The swab was then touched off a suspect colony and observed for a colour change to blue/dark purple colour over the next 10 sec. If a colour change was observed this meant that oxidase production was occurring, thus this colony could be *P. aeruginosa*. Any oxidase negative colonies were noted but not included in MALDI-ToF confirmation.

### **2.3.2 Thermotolerance**

Isolates that passed the oxidase test were then tested for their ability to grow at 42°C to distinguish between *P. aeruginosa* and *P. putida*. *P. aeruginosa* is capable of growth at 42°C while *P. putida* is not (Oberhofer 1981).

### **2.3.3 MALDI-TOF confirmation**

Isolates that were found to be oxidase positive and grew at 42°C were further identified using Matrix-assisted laser desorption/ionization - time of flight (MALDI-ToF). MALDI-ToF is a procedure which provides a unique mass spectral fingerprint of the ribosomal proteins of microorganisms using the intrinsic property of mass spectrometry to detect the mass-to-charge ratio (m/z) of a bioanalyte. (Hillenkamp et al. 1991). Bacterial ribosomal

proteins from cell extracts were profiled using MALDI-ToF in order to identify specific *Pseudomonas* species. Suspect isolates were picked from the surface of the agar and inoculated onto the MALDI slide. Extraction fluid was placed on the slide and the slide put on the MALDI-ToF (Vitek).

#### **2.3.4 PASTOREX™ STAPH-PLUS test for *S. aureus***

This test was used to confirm identification of *S. aureus* from other *Staphylococcus* species. This test is based on identification of capsular polysaccharides and detection of free coagulase production (Karakawa et al. 1985). Gram positive cocci and catalase positive colonies were tested using the PASTOREX™ STAPH-PLUS test following manufacturer's guidelines. A positive reaction for *S. aureus* was identifiable by formation of aggregates only with the reagent test. This was visible to the naked eye under normal lighting within 30 seconds of beginning the card rotation. A negative reaction would not produce any aggregates and retain a milky appearance.

### **2.4 Culture-independent Analysis**

#### **2.4.1 DNA extraction from RNAL samples**

The RNALater treated pellets were thawed on ice and subsequently centrifuged at 9500 x g for 10 min. The pellets were then resuspended in 200µl molecular grade water and transferred to a sterile screw capped tube containing 0.3g sterile acid washed glass beads (Sigma-Aldrich) and were bead beaten for 180 sec at 5.5 m/sec to release the bacterial DNA. These tubes were then centrifuged at 8000 x g for 60 sec and the supernatant was transferred to 1.5ml sterile centrifuge tubes. Beads were then washed with 100µl molecular grade water, centrifuged at 8000 x g for 60 sec and the supernatants pooled. A 200µl volume of binding buffer and 40µl proteinase K were added and incubated at 70°C for 10 minutes. Following incubation, Roche high pure PCR template preparation kit (Roche) instructions were followed. The DNA was eluted in 100µl elution buffer and stored at -20°C in 20µl aliquots. For each batch of extractions, a negative extraction control was performed where the protocol was carried out from start to finish on 200µl molecular grade water. DNA was also isolated from the reference strains listed in section 2.2.1.

### **2.4.2 Qubit fluorometer DNA quantification**

The Qubit working solution was made up using buffer and reagents supplied in the dsDNA HS (high sensitivity) Assay Kit (Life Technologies) for the number of samples that were required to be quantified in a given run. The volume of working solution was calculated by accounting 199µl of buffer for each sample and two standards. Standards and samples were prepared according to manufacturer's instructions and the Qubit Fluorometer was calibrated and subsequently used to quantify the DNA in each sample.

### **2.4.3 Ribosomal Intergenic Spacer Analysis - Polymerase Chain Reaction**

The RISA-PCR Master Mix was prepared taking into account the number of reactions required. This included the number of samples being run, plus an additional positive and negative control and one additional reaction to account for any pipetting error. The negative control used in this process were the same 'blanks' used in the DNA extraction step described in section 2.4.1 as part of the quality control measures in this study. DNA from *P. aeruginosa*, *S. aureus*, *H. influenzae*, *S. maltophilia* and *A. baumannii* were used as positive controls and the negative extraction control were included in all experiments. Reagents required for each reaction were added to a final volume of 25µl, including nuclease free H<sub>2</sub>O (Severn Biotech Ltd), 10X PCR buffer, 5X Q-solution, 10mM dNTPs, 10pmol/µl primers (Eurofins Genomics) and 2U *Taq* polymerase (Qiagen Ltd). Unless otherwise specified, all reagents were supplied by Qiagen Ltd. The PCR primers used for the amplification of the bacterial ITS region were as follows; 1406 forward primer 5'-TGYACACACCGCCCGT-3' and 23S reverse primer 5'-GGGTTBCCCCATTCRG-3' (Fisher & Triplett 1999). The RISA-PCR was run in a thermal cycler with the programmed PCR conditions as follows; 95°C for 5 min (initial denaturation) and then 35 cycles of 95°C for 1 min (denaturation), 54°C for 30 sec (annealing) and 72°C for 1 min (extension). A subsequent final extension of 5 min at 72°C was completed, followed by a final infinite hold at 4°C (Flight et al. 2015). All PCR amplicons were then run on a 1.5% agarose gel incorporating 0.01% SYBR Safe dye (Biosciences) alongside a 2-log DNA ladder (New England Biolabs Inc.). The gel was then visualised under UV light using the Omega Lum G (aplegen) UV cabinet.

#### **2.4.4 Microfluidic separation and cluster analysis of RISA-PCR profiles**

Samples which gave positive PCR products from section 2.4.3 were subsequently quantified and sized on the Agilent Bioanalyzer (Agilent Technologies UK Ltd., Cheshire, United Kingdom) using a DNA 7500 chip (Agilent) according to the manufacturer's instructions. Bioanalyzer profiles were imported to Bionumerics software (Applied Maths, Gent, Belgium) for analysis. A dedicated script was provided to Cardiff University by Applied Maths to convert BioAnalyzer profiles to a format compatible with Bionumerics. This Bionumerics software was used to perform cluster analysis of the RISA profiles. A dendrogram of all samples was constructed using unweighted-pair group method using average linkages (UPGMA) and similarity calculated using Dice coefficient (Fig. 2.5). Tree branches were coloured by patient (Fig. 2.5). The sizes of specific RISA amplicons determined using the Bioanalyzer were compared to specific bacteria in the In Silico PCR database (<http://insilico.ehu.es/PCR>) (Bikandi et al. 2004).

## **2.5 Results**

### **2.5.1 Patient Demographics**

A total of 105 sputum samples from 29 children with CF ( $\leq 19$  years) attending Tallaght University Hospital between November 2016 and November 2017 were collected and processed (Table 2.2). This cohort consisted of 41.4% males and 58.6% females, with an overall prevalence of 20.7% *P. aeruginosa*. Significant differences between genders were notable for BMI and FEV<sub>1</sub>% predicted (Unpaired t-test:  $p = 0.0369$  and  $p = 0.0014$ , respectively). An even distribution of  $\Delta F508$  homozygosity and heterozygosity was recorded in the male cohort, while a greater proportion (70.6%) of females were homozygous for the  $\Delta F508$  CFTR mutation. Information on antibiotic usage was only recorded for the sequenced samples, and did not extend to all samples. The antibiotics usage at the time of sampling and in the 2 months prior to sample collection were recorded for the 48 sequenced samples, which is further discussed in chapter 3 (pages 86-88).

**Table 2.2:** Demographics and clinical data of patient population

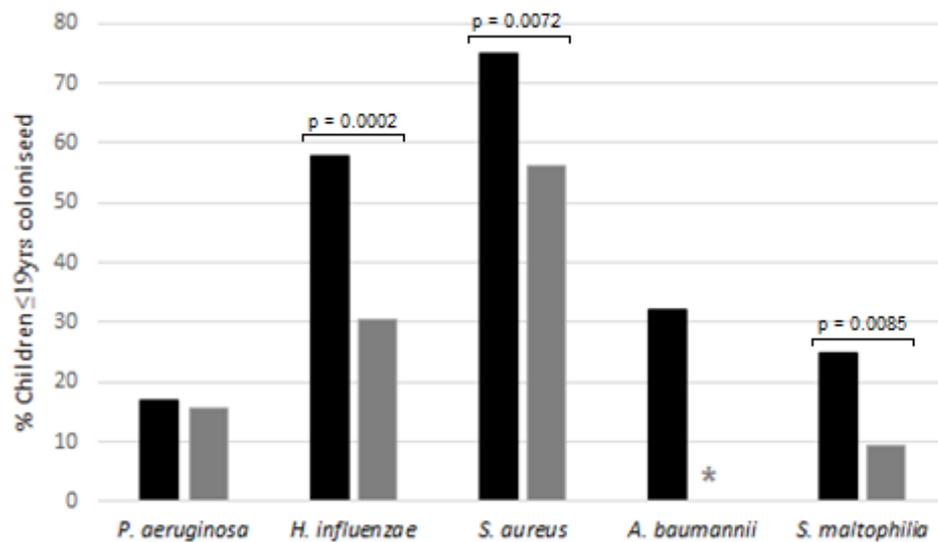
	Male	Female	Total	P-values
N (%)	12 (41.4%)	17 (58.6%)	29	0.2935
<i>P. aeruginosa</i> positive	4/12 (33.33%)	2/17 (11.76%)	6/29 (20.7%)	0.1981
Median Age (range)	15 (6 - 19)	13 (6 - 19)	13 (6 - 19)	0.3794
Median BMI z-scores (range)	0.376 (-0.319 – 0.532)	-0.533 (-1.438 - -0.188)**	-0.263 (-1.438 – 0.532)	0.0369*
FEV <sub>1</sub> % predicted median (range)	94.5% (59 – 116%)	59% (28 – 110%)	81% (28 – 116)	0.0014*
CFTR Genotype				
ΔF508/ΔF508	50% (6/12)	70.6% (12/17)	62% (18/29)	0.4384
ΔF508/other	50% (6/12)	29.4% (5/17)	38% (11/29)	0.4384

\* *significant difference*

\*\* *missing data*

### 2.5.2 Bacterial prevalence in the paediatric CF population

The prevalence of particular pathogenic species of bacteria can vary significantly depending on the age of a patient (Muhlebach et al. 2018). In this study *S. aureus* was the most frequently identified bacteria in patients (83%, 87/105), followed by *H. influenzae* (56%, 59/105), *S. maltophilia* (32%, 34/105), *A. baumannii* (30%, 32/105), and *P. aeruginosa* (16%, 17/105) in reducing order of prevalence. These prevalences differ significantly from prevalence recorded in people with CF ≤19years by the CFRI (CFRI 2016) (Fig. 2.2). Higher prevalence of *S. aureus* (t-test:  $p = 0.0072$ ), *H. influenzae* ( $p = 0.0002$ ) and *S. maltophilia* ( $p = 0.0085$ ) were recorded in this study. There was no difference between prevalence of *P. aeruginosa* in this study and the CFRI data.

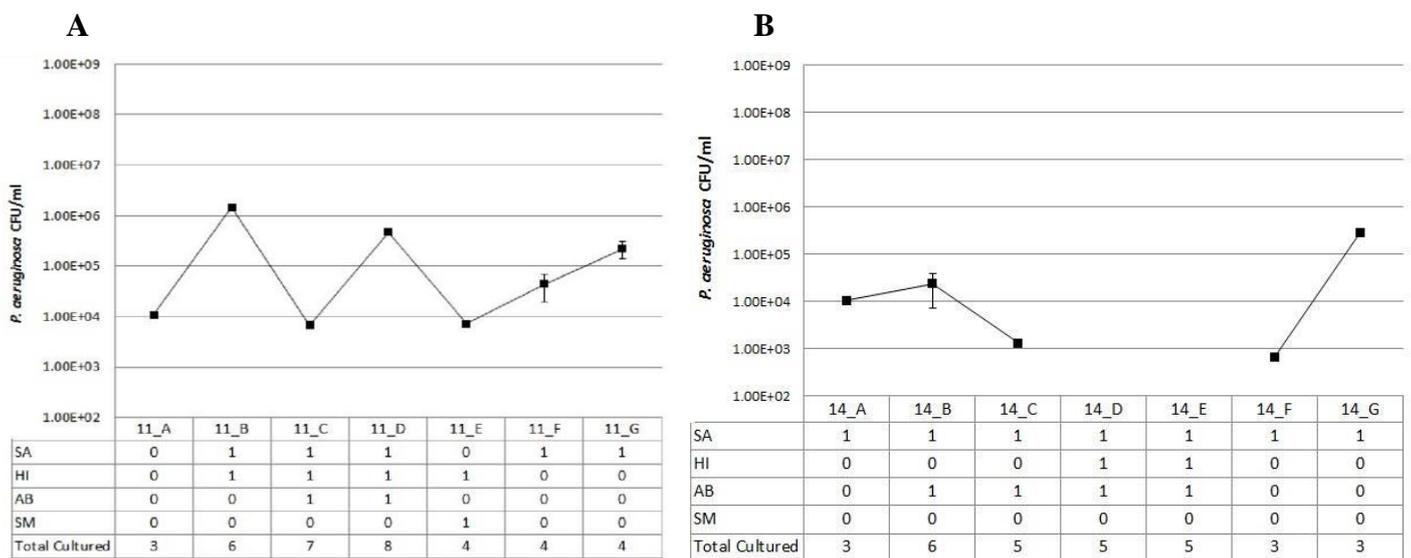


**Figure 2.2:** Prevalence of bacterial species in this study versus the CFRI 2016 data. Study prevalence (black bars) vs. CFRI prevalence (grey bars). \* no data on *A. baumannii* published for the Irish paediatric population. Irish population data was extracted from CFRI 2016 Annual Report (CFRI 2016).

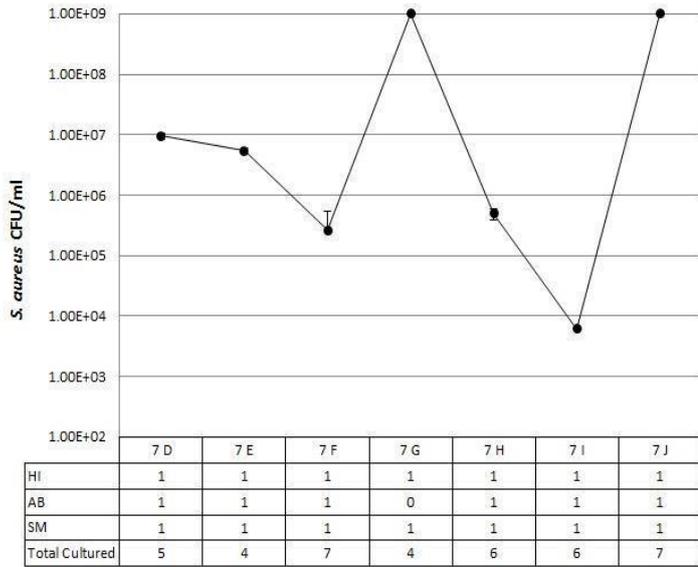
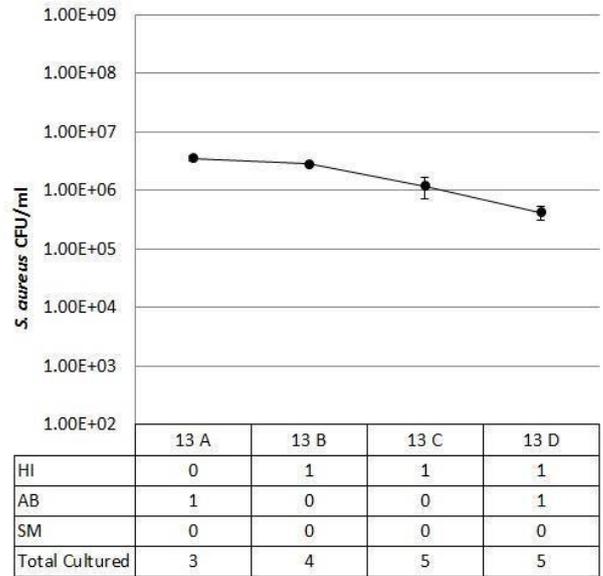
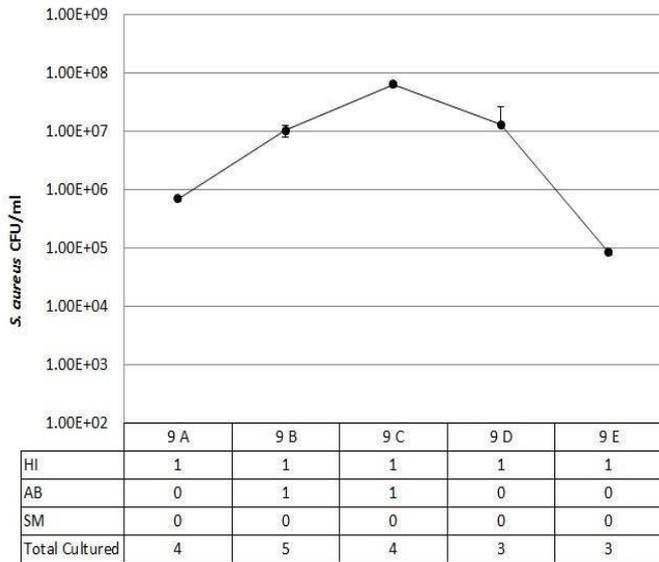
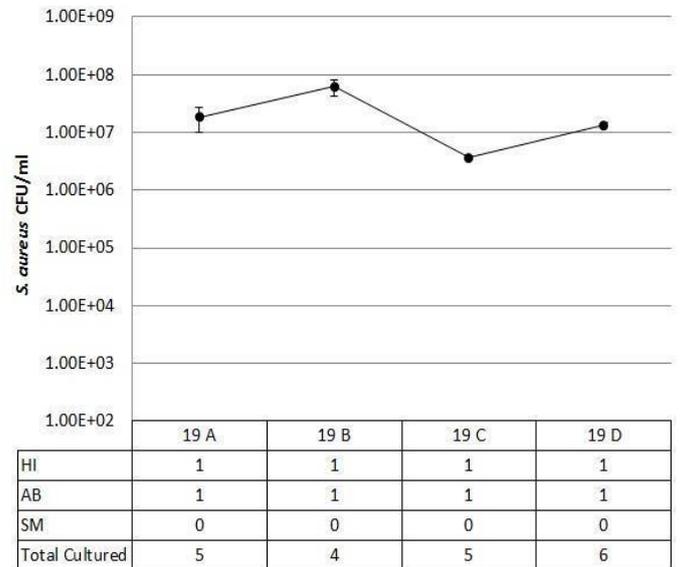
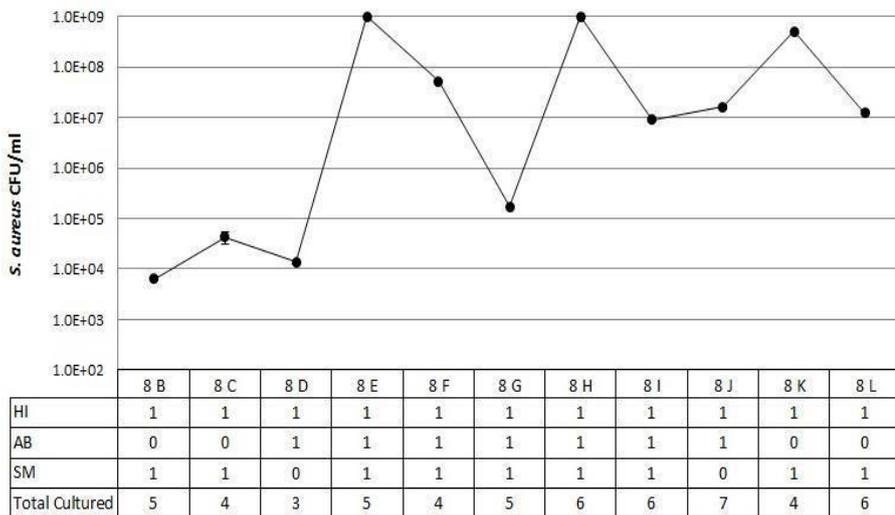
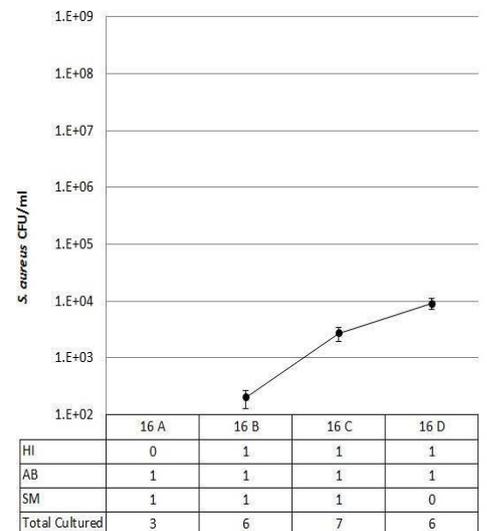
### 2.5.3 Microbial profiling of *P. aeruginosa* dominant and *S. aureus* dominant communities using culture-dependent methodologies

The following species were identified by culture in this study; *P. aeruginosa*, *P. putida*, *P. stutzeri*, *H. influenzae*, *H. parainfluenzae*, *S. aureus* and *Staphylococcal* species, *A. baumannii*, *S. maltophilia*, *Escherichia coli*, *Citrobacter spp*, *Enterococcus spp*, *K. oxytoca*, *Proteus mirabilis*, *S. saprophyticus*, *Moraxella catarrhalis*, *Acinetobacter haemolyticus*, *Morganella morganii*, *Brevundimonas diminuta*, *St. pneumoniae*, *St. pyogenes*, *St. mitis*, *Chryseobacterium gleum*, *Enterobacter cloacae*, *St. multivorum*, *M. abscessus*, *S. epidermidis*, *A. denitrificans* and *A. xylosoxidans*. Patients were classified as persistently colonised with *P. aeruginosa* (Fig. 2.3) or with *S. aureus* (Fig. 2.4) by applying the Leeds criteria (Lee et al. 2003), defining persistence as positivity for the bacterial species in  $\geq 50\%$  samples over the one-year period. Two patients were persistently colonised with *P. aeruginosa* (Fig 2.3 A & B). Bioburden of *P. aeruginosa* fluctuated considerably over time in both patients (from 0 to  $>1 \times 10^6$  CFU/ml of sputum). At all time-points these patients also cultured other bacteria (ranging from 3 to 8) and at most time points both patients cultured other known CF bacteria. Note that both *P. aeruginosa* persistent patients were also persistently colonised with *S. aureus*. A further 4 patients were positive for *P. aeruginosa* but could not be described as persistently or intermittently colonised.

Overall 23 patients were known to be positive for *S. aureus* at one time point during the study. Nine patients were persistently colonised with *S. aureus* (Fig 2.3 and Fig 2.4). Six of the patients persistently colonised with *S. aureus* while negative for *P. aeruginosa* are represented in Fig 2.4. Patients 7 (Fig. 2.4 A) and 8 (Fig. 2.4 E) had fluctuating levels of *S. aureus* (ranging from  $<1 \times 10^4$  to  $>1 \times 10^9$  CFU/ml). Patients 13 (Fig. 2.4 B) and 19 (Fig. 2.4 D) had consistently high levels of *S. aureus* over time (ranging  $>1 \times 10^6$  to  $<1 \times 10^7$  CFU/ml). Patient 9 (Fig. 2.4 C) shows a steady increase followed by a decrease in *S. aureus* levels, ranging from  $>1 \times 10^4$  to  $<1 \times 10^8$  CFU/ml. Finally, patient 16 (Fig. 2.4 F) shows a steady increase in *S. aureus* colonisation, rising from  $>1 \times 10^2$  to  $<1 \times 10^4$ . At all time-points *S. aureus* positive patients cultured other bacteria (range 3 to 7) and at most time-points *S. aureus* positive patient groups cultured other known CF bacteria. 86% (61/71) were also positive for *H. influenzae*. A minimum of 3 bacterial species were identified by culture analysis in 100% of patient samples represented here (Fig 2.3 & 2.4), though the overall minimum of total cultured species was 1 (Fig. 2.5).



**Figure 2.3:** Culture-based microbial profile and bioburden of *P. aeruginosa* (CFU/ml) in CF sputum samples. (A) Persistently and (B) intermittently *P. aeruginosa* colonised CF patients. Linked tables represent other common CF bacteria present (1) and absent (0) in each sample; SA – *S. aureus*, HI – *H. influenzae*, AB – *A. baumannii*, SM – *S. maltophilia*. Total cultured refers to the total number of bacterial species identified by culture in each sample, inclusive of those detailed in graphs/tables. Serial dilutions and CFU counts were carried out in triplicate. Error bars represent standard deviations.

**A****B****C****D****E****F**

**Figure 2.4:** Culture-based microbial profile and bioburden of *S. aureus* (CFU/ml) in CF sputum samples. Linked tables represent other common CF bacteria present (1) and absent (0) in each sample; HI – *H. influenzae*, AB – *A. baumannii*, SM – *S. maltophilia*. Total cultured refers to the total number of bacterial species identified by culture in each sample, inclusive of those detailed in graphs/tables. Serial dilutions and CFU counts were carried out in triplicate. Error bars represent standard deviations.

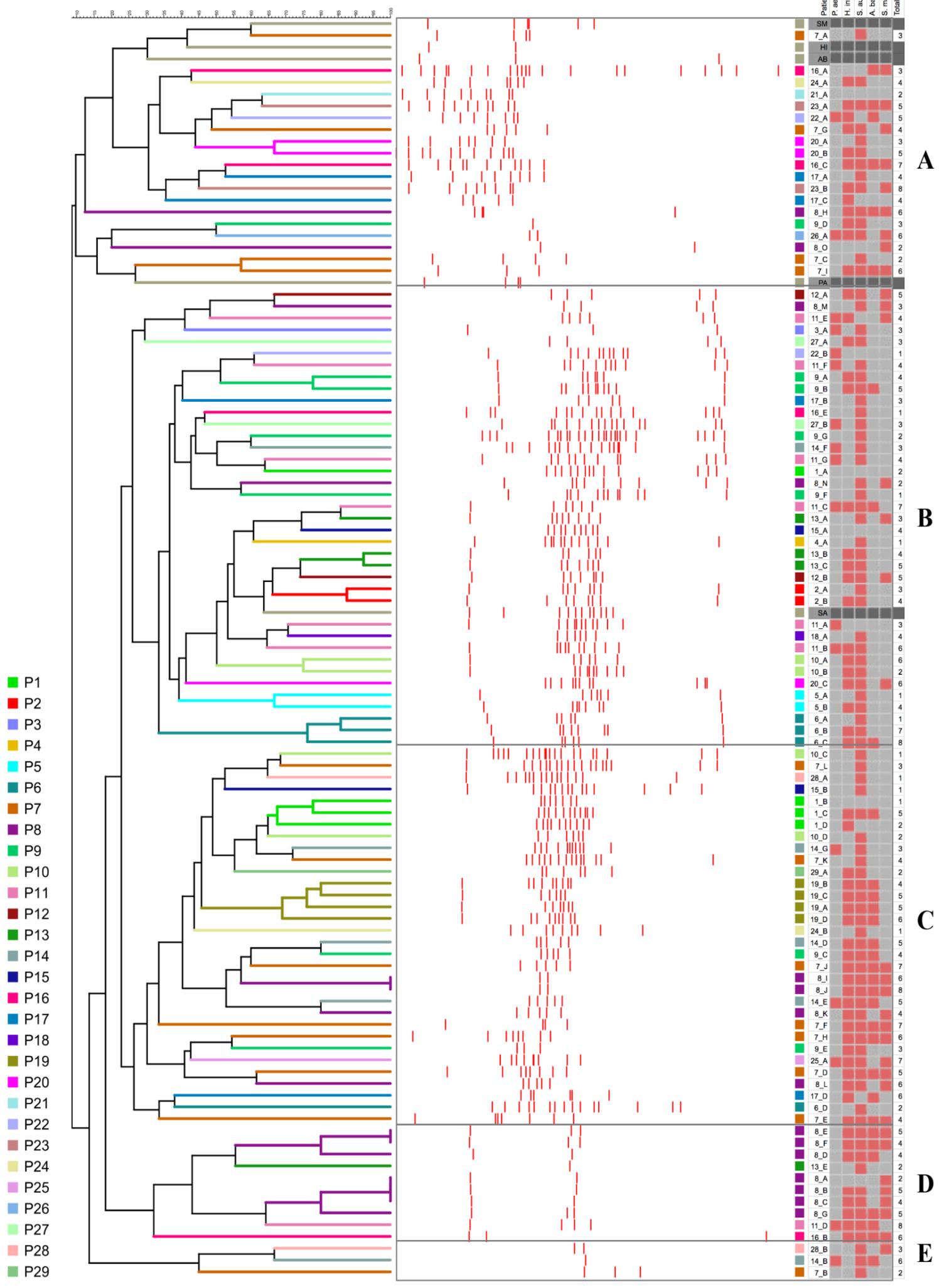
#### 2.5.4 RISA profiling of the CF airway microbial community

The cophenetic correlation was measured at each branch of the dendrogram (Fig. 2.5 and table 2.3) and ranged from 50% to 100%, as an estimate of faithfulness of each sub-cluster in the dendrogram using Bionumerics software. Groups A to E have been designated based on abundance of bands (range 1 - 24) present in RISA profiles. No significant association was discernible between the number of bands and any bacterial species positivity. Group A comprised 19 samples with relatively large numbers ( $\leq 21$ ) of high molecular weight (MW) bands. Four of the 5 reference strains delineated into group A. The total number of cultured bacteria in these samples is also relatively high with an average of 4 species (2-8) and only 2 samples in this group were *P. aeruginosa* positive. The majority of samples in group A were dominated with *H. influenzae* (63% of group A) and *S. aureus* (74% of group A). Group B comprised 38 samples with higher numbers of amplicons/ bands (4-24) and a wider distribution of band sizes. The majority of *P. aeruginosa* positive samples (~59%) were in this group. The *S. aureus* reference strain clustered in this group along with 38% *S. aureus* colonised samples, while just 18% of this group were positive for *S. maltophilia*. The richness of these samples was quite variable, with an average of ~3.5 species (1-8) total cultured bacteria. Thirty-two samples were in Group C with large numbers of bands (2-20) of mostly high MW. Approximately 31% of *S. aureus* positive samples, ~38% of *H. influenzae* positive samples and 50% *A. baumannii* samples fell into Group C. This group is notable for being more diverse by culture analysis with 4 total cultured bacteria on average (1-7). Group D had a total of 10 samples and had low numbers of bands (1-4) despite being more diverse by culture analysis with ~5 total cultured bacteria on average (2-8). This group appeared to be dominated by samples from *S. maltophilia* positive patient 8. Group E consisted of a cluster of 3 samples with the lowest number of amplicon bands (1-3). These samples were consistently *S. aureus* culture positive and just one sample was *P. aeruginosa* culture positive. Total numbers of bacteria cultured ranged from 2 to 6 in this group. The number of bands did not always correlate with complexity of microbial community detected by culture. Group A had multiple samples from patient 20 (3) which

clustered together, and patient 7 (3) which did not. Group B had multiple samples from patient 9 (4), patient 6 (3) and patient 13 (3) among others. Group C had multiple samples from patient 7 (7) and patient 19 (4), patient 8 (4) and patient 1 (3). Group D was dominated by samples from patient 8 (7), which clustered together in this group. Group E did not show multiple samples from any patient. Patients such as patient 8, 6 and 5 were notable to cluster together within particular groups.

**Table 2.3:** Summary of RISA-generated dendrogram

<b>Group</b>	<b>Total samples</b>	<b>Total patients</b>	<b>Average band number (range)</b>	<b>Average number of cultured bacteria (range)</b>	<b>Most commonly cultured bacteria</b>
A	19	11	6 (1-21)	4 (2-8)	<i>S. aureus</i>
B	38	19	12 (4-24)	4 (1-8)	<i>P. aeruginosa</i>
C	32	14	9 (2-20)	4 (1-7)	<i>A. baumannii</i>
D	10	4	2.5 (1-4)	5 (2-8)	<i>S. maltophilia</i>
E	3	3	2 (1-3)	4 (2-6)	<i>S. aureus</i>



**Figure 2.5:** Dendrogram generated from RISA profiles of n=102 samples. Branches colour coded based on individual patients, RISA bands generated using Bionumerics software are displayed at the end of each branch. Colour chart shows patient and sample identifier (ID) and culture positivity (red = positive, grey = negative) for *P. aeruginosa*, *H.influenzae*, *S. aureus*, *A. baumannii* and *S. maltophilia*. Total cultured values are displayed for the total number of bacterial species isolated from the sample in question. Reference strains are coloured in dark grey.

### 2.5.5 Discussion

The prevalence of the bacterial species investigated in this study were significantly higher in our sample cohort compared to that reported by the CFRI for paediatric patients of the same age (Fig. 2.2). These differences were found to be significant in each of the bacterial species *H. influenzae*, *S. aureus* and *S. maltophilia*, with the exception of *P. aeruginosa* which reported similar prevalence. We employed very stringent culturing techniques in this study which could explain our higher detection rate. Our methodology involved triplicate plating of serial dilutions of homogenized sputum on a variety of selective and differential agars and further confirmation of cultured isolates by MALDI-ToF (Vitek). Standard diagnostic procedure is to dip a swab into homogenised sputum and spread this on agar. Therefore our methodology would sample a higher proportion of specimen making it more likely to pick up bacteria at lower bioburdens. Triplicate plating of serial dilutions is not feasible in a busy medical laboratory. Our detected prevalence of *P. aeruginosa* was comparable to CFRI data perhaps because this bacterial species is a well known CF pathogen therefore protocols are in place in medical laboratories to detect this specific pathogen. There are issues with the use of registry databases, such as the lack of standardised methodology across Irish hospitals. Some medical laboratories are dependent on culture while others use molecular means of pathogen identification. Thus, registry data is reliant entirely on the quality of data generated in different hospitals and can not account for false positive or negative results. Our recorded prevalences were within the reported range in the literature for these bacterial species in paediatric CF patients (Burns et al. 2001; Razvi et al. 2009; Cardines et al. 2012; Goss et al. 2004; CF Trust 2016).

We performed rigorous culture-based identification of bacteria in CF sputum samples and detected 28 bacterial species overall. Patient samples had a minimum of 1 and maximum of 8 bacterial species in their sputum at any one time therefore the polymicrobial nature of the CF airway can be described using culture based methods. Fluctuations in abundance of *P. aeruginosa* were notable in persistently colonised patients (Fig. 2.3 A) and well known CF bacteria such as *S. aureus*, *S. maltophilia* and *H. influenzae* commonly co-colonised with *P. aeruginosa*. Richness was not significantly different in *P. aeruginosa*

colonised patients compared to non-colonised patients with persistent *S. aureus* colonisation (Fig. 2.3 B). Our culture results support evidence of a highly diverse microbial community in children with CF with several known CF pathogens capable of co-colonising the airways (Harris et al. 2007; Tunney et al. 2008; Cox et al. 2010). The co-isolation of *H. influenzae* with *S. aureus* colonised paediatric patients has been reported by several other studies (Armstrong et al. 1995; Harris et al. 2007; Emerson et al. 2002). Some patients with consistently high abundance of *S. aureus* (e.g. patient 13 and 19 - Fig. 2.4 B & D) were found to be consistently clear of *S. maltophilia* colonisation. This could be suggestive of a potential protective effect of highly abundant *S. aureus*, supported by Stutman *et al.* in which children receiving antistaphylococcal prophylaxis had a higher likelihood of becoming colonised with more detrimental pathogens such as *P. aeruginosa* (Stutman et al. 2002). Bacteria less commonly associated with CF were also identified by culture such as *Moraxella catarrhalis*, *Acinetobacter haemolyticus*, *Morganella morganii* and *Brevundimonas diminuta* among others, suggesting that culture techniques can provide a wider view of the CF airway microbiome if developed and optimised.

Our dendrogram appeared to cluster based on individual patients microbiome. RISA failed to cluster samples based on their dominant pathogen with *P. aeruginosa* positive samples scattered throughout the 5 clusters. Samples with higher numbers of CF pathogens and overall higher diversity generally clustered into groups C and D. In a number of cases samples from the same patient clustered together (e.g. Group D - patient 8 samples 8\_A, 8\_B, 8\_C, 8\_G, Group C - patient 1 samples 1\_C, 1\_D and 1\_B and patient 19 samples 19\_A, 19\_B, 19\_C, 19\_D). This would suggest that some patients' microbiomes are similar over time which has been shown by sequencing studies (Zhao et al. 2012). In contrast to Harris *et al.*'s study, our *P. aeruginosa* cluster did not branch closely to *S. maltophilia* in our tree. A *Proteobacteria* group including *P. aeruginosa*, *S. maltophilia* and *H. influenzae* branched together in their work carried out on paediatric BALF samples (Harris et al. 2007). We performed RISA on CF sputum samples while Harris *et al.* prepared rRNA gene libraries with specific bacterial PCR amplicons and screened the clones produced using RFLP. Therefore difference in methodologies could explain these differing results. It should be noted that lower numbers of RISA bands were not necessarily linked to lower total bacteria cultured. Culture-based richness values of samples with few bands remained equivalent to samples in other groups with larger numbers of bands. However samples with higher numbers of common CF pathogens did cluster into groups C and D. This suggests that RISA would have limited suitability for estimating diversity in microbiome studies

which is in contrast to others (Flight et al. 2015; Pages-Monteiro et al. 2017) but comparable to some (Harris et al. 2007). An additional restriction of RISA was that certain species such as *S. aureus* could produce multiple bands after ITS PCR (<http://insilico.ehu.es/PCR>) (Bikandi et al. 2004). This is notable in the reference strains in our dendrogram where *S. aureus* has 11 bands, *S. maltophilia* has 5 bands, *P. aeruginosa* has 3 bands, *A. baumannii* has 3 bands and *H. influenzae* has just 2 bands. If the number of ITS amplicon bands is different for different species and one species doesn't always produce 1 band then RISA would struggle to equate numbers of bands to number of species in the microbiome. RISA has been found to be imprecise in accurately identifying particular species of bacteria (Hewson & Fuhrman 2006; Danovaro et al. 2006). Nonetheless, certain species such as *P. aeruginosa* can often be indicated based on RISA peaks (Nazaret et al. 2009). Flight *et al.* carried out RISA to profile CF sputa samples and subsequently generated a dendrogram which was segregated into eNFGN dominated or *P. aeruginosa* dominated samples which did not concur with the structure of our RISA dendrogram (Flight et al. 2015). We saw clustering based on the patient-specific samples which is more in concordance with the dendrogram by Harris *et al.* (Harris et al. 2007).

Pages-Monteiro *et al.* analysed CF sputa with similar methodology to generate a RISA dendrogram (Pages-Monteiro et al. 2017). Three defined clusters were identifiable (Ip, Iip and IIIp) in their work, in which Ip and IIIp showed lower RISA diversity and higher prevalence of *P. aeruginosa* positive samples ( $\geq 93\%$ ) and Iip consisted of a lower *P. aeruginosa* positive sample prevalence (24.2%). Significantly different to our dendrogram, both adult and child samples were used with a subsequent finding that their *P. aeruginosa* positive cohort of samples clustered based on age. Thus, the fact that our study was focused only on paediatric patients could mean that *P. aeruginosa* infection in children has not altered the microbiome as extensively as in adult patients. An alternative explanation could be that the microbiome differences that exist in the microbiomes of these cohorts are simply not detectable through RISA. In similarity to the tree generated from our study, the dendrogram clustering based on RISA structures presented by Pages-Monteiro *et al.* could not be entirely dissociated from confounding factors such as age and antibiotic exposure (Pages-Monteiro et al. 2017). A clear limitation of the RISA method is the lack of species specificity in detection, although this technique has the advantage of quickly suggesting the extent of diversity in a patient sample which may be useful in a clinical setting.

## **Chapter 3**

### **Whole genome shotgun sequencing of the paediatric CF airway microbial community**

### 3.1 Introduction

The advances in molecular techniques to identify the presence of pathogenic microbial species has revolutionised clinical microbiology in recent decades (Cox et al. 2013). Studies in the CF airway microbiome have utilised metagenomics (Tunney et al. 2008; Filkins et al. 2012) including 16S rDNA sequencing (Pages-Monteiro et al. 2017) and more recently WGSS (Hauser et al. 2014), in an attempt to fully characterise the CF airway microbiome. Beyond the bacterial communities, there are also diverse viral (Willner et al. 2009) and fungal (Willger et al. 2014) communities in the CF airway.

WGSS can now be performed using Next-Generation Sequencing (NGS) technology, which has largely replaced the Sanger dideoxy chain termination sequencing method (Mardis 2008; Mardis 2011). NGS systems are now largely represented by instruments from three companies; Life Sciences, Roche and Illumina. The common systems on offer include SOLiD/Ion Torrent PGM (Life Sciences), GS FLX Titanium and GS Junior (Roche) and the Genome Analyser, HiSeq, MiSeq and most recently NextSeq (Illumina). The methodology of these sequencers involve three core steps, library preparation using random fragmentation of DNA, followed by ligation using custom adaptors and finally the library is amplified using clonal amplification PCR (Balasubramanian 2011). NGS technologies are advantageous over Sanger sequencing for a number of reasons, including automatic in-library preparation of samples, faster running time for longer and more accurate reads and less expensive per million bases sequenced (Lin et al. 2012). However, there is a higher cost still associated with WGSS compared to that of other sequencing methods (Dunne et al. 2012) as a result of the higher cost of the instrument. Sanger sequencing has some advantages in that run time can be as short as 20 minutes to 3 hours, no secondary device is required and it generates high read quality and long read length (Lin et al. 2012). Sanger sequencing inefficiently handles large genomes, genomes with high GC content or bacterial genomes which made it an inappropriate option for this study. A thorough review on the innovative technology that is involved in NGS methods is beyond the constraints of this thesis, though comprehensive reviews of this topic are available (Mardis 2011; Shendure & Ji 2008; Schadt et al. 2010; Su et al. 2011). Many studies have used NGS of specifically the 16S rRNA region (variable regions 1 to 6) to characterise the CF airway microbiome. This has been the most common genetic marker for studying bacterial phylogeny and taxonomy for several reasons (Janda & Abbott 2007); the 16S rRNA gene is present in almost all bacteria, 16S rRNA region is large enough for

informatics analyses (Patel 2001) and the consistent function of the gene over time means that sequence changes can be used as a measure of evolution (Janda & Abbott 2007). 16S rRNA gene sequencing is relatively easy to perform and can provide confident genus level identification, in addition to increased species identifications compared to conventional methods (Patel 2001). However, this method is not foolproof in terms of species identification. Results from several studies revealed genus identification in most cases (>90% of cases) though significantly less species identification (65-85% of cases) (Drancourt et al. 2000; Mignard & Flandrois 2006; Woo et al. 2003). Taxonomic assignment to the species level can be less reliable using 16S rRNA sequencing because a lack of sequences deposited in nucleotide databases, nomenclature problems or species sharing very similar or identical 16 rRNA sequences. Furthermore, strain types for many species may not accurately reflect the entire genomic composition of nomenclatures deposited in the database. Results can differ depending on the variable region of the 16S rRNA region amplified prior to sequencing (Chakravorty et al. 2007). Ultimately species level identification with 16S rRNA sequencing has low phylogenetic power and reduced discriminatory power at the genera level (Bosshard et al. 2006; Mignard & Flandrois 2006). For these reasons our objective was to employ WGSS to our CF samples in this study. WGSS has been found to enhance identification of bacterial species with improved accuracy and increased detection of microbial diversity (Ranjan et al. 2016). Furthermore, with WGSS much more information about the bacteria can be gleaned through analysis of antibiotic resistance mechanisms contributing to the resistome and investigation of metabolic pathways specific to particular species. Information on strain types of particular species can also give valuable insights, particularly in bacterial complexes which have more than one genomovar (DNA group) existing within a species which cannot be separated phenotypically, such as *Pseudomonas stutzeri* (18 genomovars) (Janda & Abbott 2007).

WGSS has been applied to CF patient samples in relatively few studies to date and only one study regarding the CF airway microbiome. Hauser *et al.* was among the first to move away from the more common 16S rRNA sequencing to study the CF respiratory microbiome (Hauser et al. 2014). They investigated the performance of PCR-based metagenomics, stringent culturing and WGSS for microbial community identification from 2 CF sputum samples (Hauser et al. 2014). WGSS provided a more accurate qualitative and quantitative assessment of the microbial composition of each sample. WGSS identified *Haemophilus*, *Staphylococcus* and *Streptococcal* species, in addition to low amounts of anaerobes such as *Veillonella*, *Prevotella* and *Fusobacterium* and aerobes/ facultative

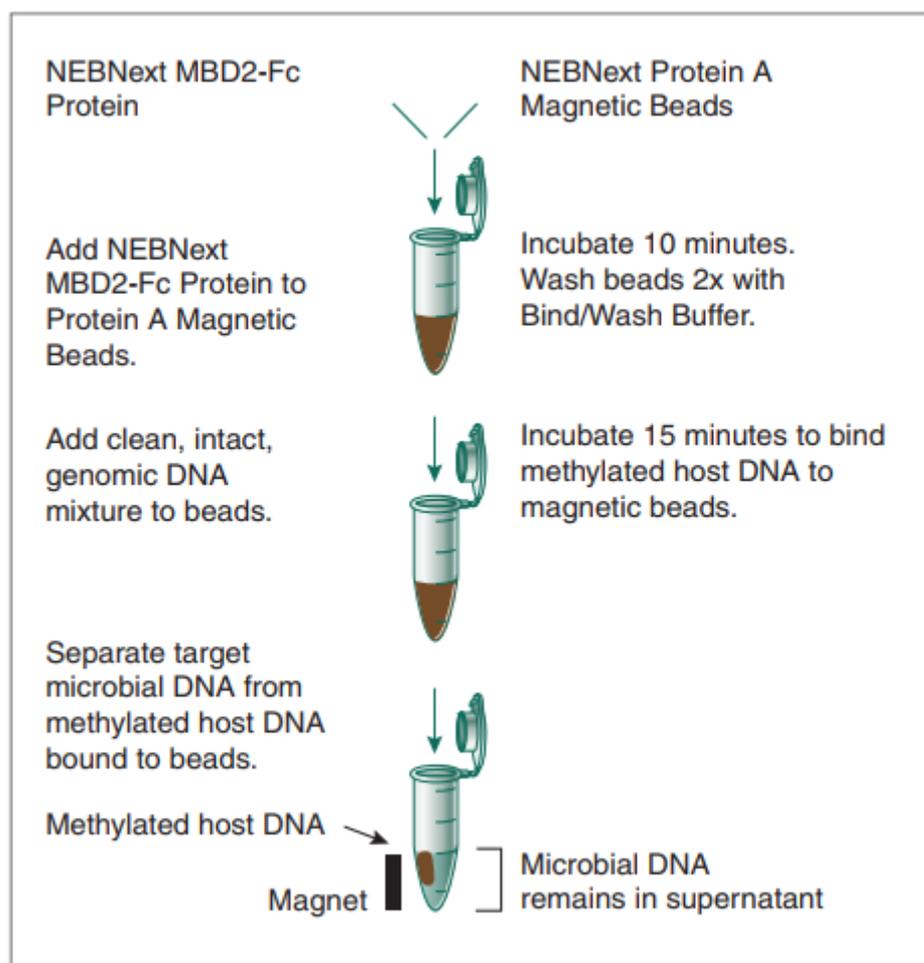
anaerobes like *Gemella*, *Moraxella* and *Granulicatella*. Some of these genera would have required deep sequencing of PCR products or very specific culture conditions to have been identified using the other methodologies (Hauser et al. 2014). It has been speculated that if WGSS were to be employed as a routine diagnostic tool with CF sputum samples, there would be a much broader range of bacterial species and sub-species identified (Dunne et al. 2012). Upon identification of such species in the CF airway, the functional and clinical relevance of these bacteria could begin to be determined with the aim of elucidating their importance in the lung microbiome.

It has been said that “*one of the most powerful applications of NGS has been its role in understanding the complex biodiversity of the human microbiome*” (Dunne et al. 2012). Already, WGSS has aided unanticipated advances in pathogen diagnostics and detection of virulence and antibiotic resistance genes in common CF bacteria (Sharma et al. 2014). This has helped in beginning to comprehend the underlying mechanisms of genomic changes in strains of species which survive in the CF airway. However much more research is needed, especially studies utilising WGSS for greater understanding of the complexities of the CF airway microbiome.

## 3.2 Materials and Methods

### 3.2.1 Microbial DNA enrichment

WGSS was performed on a subset of 48 samples (n=11 patients) which comprised 12/48 *P. aeruginosa* culture positive samples from 6/11 patients, 36/48 *P. aeruginosa* culture negative samples from 5/11 patients and 36/48 consecutive samples from 10/11 patients. The microbial DNA of each sample was enriched by separation of methylated host DNA. This was achieved using a magnetic bead-based technology to bind CpG-methylated host DNA (NEBNext® Microbiome DNA Enrichment Kit (NEB)) (Feehery & Pradhan 2009). MBD2-Fc protein (NEB Kit) was first bound to the magnetic beads. The volume of prepared beads was determined by adding 1µl of MBD2-Fc-bound magnetic beads for every 6.25ng of input DNA (as per manufacturer's instructions).



**Figure 3.1:** CpG-Methylated DNA Separation Workflow (Extracted from *New England BioLabs Inc.* NEBNext Microbiome DNA Enrichment Kit Manual)

Extracted DNA samples were mixed with the appropriate amount of magnetic beads in a LoBind Eppendorf tube (Sigma-Aldrich). These tubes were then placed on a magnetic

block to pellet the beads. Host (human) DNA is heavily methylated and so will bind to the beads while non-CpG-methylated (microbial) DNA will be in the supernatant (Feehery et al. 2013) (Fig 3.1). Microbial DNA was recovered by transferring the supernatant to a fresh 1.5ml tube. The microbial DNA was then purified by ethanol precipitation, as described in section 3.2.2. To isolate the captured human DNA, manufacturer's instructions were adhered to and these samples then underwent ethanol precipitation for purification.

### **3.2.2 Ethanol precipitation**

To each sample tube 2.5 volumes of absolute ethanol were added, incubated for 10 mins on ice and centrifuged at 16000 x g for 30 sec. The residual ethanol was removed and the pellet was allowed to air dry. A quantity in the range of 15 - 40µl of TE buffer was then added to resuspend the pellet. This volume was calculated depending on the final eluted volume of each sample (section 3.2.1). The sample was then stored at -20°C for downstream analysis.

### **3.2.3 DNA library preparation**

This procedure was carried out in Teagasc Food and Research Centre, Fermoy, Co. Cork with the help of Dr. Fiona Crispie. Samples were first diluted to 0.2ng/ml before adding 10µl Tagment DNA Buffer and 5µl Amplicon Tagment Mix (Nextera DNA Library Preparation Kit (Illumina®)) to 5µl of DNA. PCR was performed under the following conditions; 55°C for 7 minutes, hold at 10°C. This step used the Nextera transposome to tagment gDNA, in a process which fragments the DNA and then tags it with adapter sequences in a single step.

A neutralizing tagment buffer (Illumina®) was first added in order to amplify the tagged DNA. Nextera PCR Mastermix was then added to each sample, the tubes centrifuged and PCR programme performed with the following cycling parameters; 3 min at 72°C, 30 sec at 95°C, followed by 12 cycles of 95°C for 10 sec, 55°C for 30 sec and 72°C for 30 sec. Finally, one cycle of 72°C for 5 min, followed by infinite hold at 10°C. These steps functioned to add index adapters to both ends of the DNA which enabled dual-indexed sequencing of pooled libraries on Illumina® platforms. Each sample was barcoded with these index adapters so that they all had a unique pair of indices to generate sequencing-ready fragments.

### **3.2.4 Library clean-up**

PCR products were centrifuged before being transferred to 1.5ml tubes. A 30 $\mu$ l volume of 0.6x AMPure XP beads (Beckman Coulter) was then added. Each sample was then vortexed to mix and incubated at room temperature for 5 min. The tubes were then placed on a magnetic stand for approximately 2 min until the liquid became clear. The supernatant was then removed and discarded from each tube. The samples were washed twice with 80% ethanol before being allowed to air dry and subsequently resuspended in 52.5 $\mu$ l resuspension buffer (Illumina). The contents of the tubes were mixed again by vortexing and replaced on a magnetic stand, before the clear supernatant was transferred to fresh, labelled PCR tubes. This yielded a purer library and provided a size selection step that removed short library fragments. An Agilent High Sensitivity DNA chip (Agilent) was run on the Agilent Technology 2100 Bioanalyzer according to manufacturer's instructions. Typically libraries show fragment sizes between ~250 to 1000 base pairs, thus a broad range of libraries can be sequenced.

### **3.2.5 Quality control**

The libraries were quantified using the Qubit dsDNA High Sensitivity Assay (ThermoFisher). The molarity of each library was calculated based on the average fragment size, determined by the Agilent bioanalyzer trace. Every sample has differential fragmentation thus the molarity needed to be calculated for each sample library individually. All sample libraries were then pooled at an equimolar concentration of 2nM before performing the qPCR Assay as described in section 3.2.6.

### **3.2.6 Quantification of final pool by qPCR**

The final pool was quantified using a KAPA Library Quantification Kit (KAPA Biosystems) for Illumina® platforms. The fully prepared indexed DNA library and the DNA standards supplied were diluted 1 in 20,000 as required for the 300 cycle NextSeq run. To each well of a 96 well plate the following was added; 12 $\mu$ l of 10X Primer Premix (2X KAPA SYBR® FAST qPCR Master Mix – this is an antibody-mediated hot-start DNA polymerase formulation), 4 $\mu$ l of the diluted DNA library or the DNA standard and brought to a final volume of 20 $\mu$ l per well with molecular grade water. All DNA standards and library dilutions were assayed in triplicate, as were the no-template controls (NTCs). qPCR

cycling took place under the following conditions; 95°C for 5 min followed by 35 cycles of 95°C for 30 sec and 60 °C for 45 sec.

The average Cq score (this is the ‘quantification cycle’ or ‘cycle threshold’ as per the MIQE Guidelines (Bustin et al. 2009)) for each DNA standard was then plotted against  $\log_{10}$ (concentration in pM) to create a standard curve. The  $\Delta Cq$  for all pairs of DNA standards was checked and confirmed to be within the range from 3.1 - 3.6 as specified. A sufficiently high reaction efficiency was confirmed by calculating  $R^2$  to be  $>0.99$ . Size-adjustment calculations were performed and concentrations of the diluted library samples were calculated against the standard curve using absolute quantification. KAPA Library Quantification Kit for Illumina® platforms (KAPA Biosystems) manufacturer’s instructions were then strictly adhered to. To confirm the reliability of the concentrations calculated by this assay, the  $\Delta Cq$  for the dilutions of the sample library and the DNA control was calculated. For the dilutions performed here, a  $\Delta Cq$  value of 1.0 was expected, though  $\pm 0.1$  to this value were considered acceptable. The average Cq score was then converted to pmol by using the generated standard curve. The average size-adjusted concentration of each library was calculated (in nM) of each undiluted library. The control was then calculated from each of the dilutions that were assayed. The calculated concentration of the control was found to be within 10% of the expected result, meaning that no significant errors occurred when carrying out this assay.

### **3.2.7 Denaturation and dilution of libraries for NextSeq 500 system**

All reagents were defrosted and diluted as per NextSeq 500 System (Illumina®) Guide instructions. The DNA library was firstly denatured with freshly prepared 0.2N NaOH solution. For a DNA library at a starting concentration of 2nM, 10 $\mu$ l of the library was combined with 10 $\mu$ l of NaOH. This solution was vortexed and briefly incubated for 5 min at room temperature before adding the same volume (10 $\mu$ l in this case) of Tris-HCl at pH7. This ensured that the NaOH was fully hydrolysed in the final solution. Subsequently, the libraries were diluted to 20pM with prechilled Hybridization Buffer (HT1). This 20pM solution was then diluted to the loading concentration of 1.8pM. The diluted and denatured library was then chilled on ice. PhiX (Illumina®) was added to this solution as a control. PhiX is a phage-derived adapter-ligated library which has a very well characterised genome and offers benefit to the sequencing and alignment in Illumina® sequencing runs.

### 3.2.8 Loading DNA libraries

To perform a sequencing run on the NextSeq 500, a reagent cartridge was carefully defrosted in a room temperature water bath for approximately 1 hour. The flow cell and reagent cartridge were then prepared as per manufacturer's instructions, using previously calculated dilutions of our DNA libraries, as outlined in section 3.2.7.

The library was then loaded into the reagent cartridge in the reservoir outlined as per manufacturer's guidelines. Upon loading of the reagent cartridge and flow cell, the run parameters and automatic check results were reviewed, and the 300 cycle run was started. It was then monitored from BaseSpace as it proceeded.

### 3.2.9 Bioinformatic analysis of sequencing reads

All reads found to be from human sources were removed from the raw (fastq) sequencing files by employing the NCBI Best Match Tagger (BMTagger) (<ftp://ftp.ncbi.nlm.nih.gov/pub/agarwala/bmtagger/>). SAMtools (Li et al. 2009) were used to convert the raw metagenomics files to bam files (<http://samtools.sourceforge.net/>). This set of tools handles alignments in the bam format, and exports to the SAM (Sequence Alignment/Map) format. Duplicate reads were then removed using Picard Tools, a set of tools commonly used for high-throughput sequencing (HTS) data and formats such as bam or SAM (<https://github.com/broadinstitute/picard>). Both SAMtools and Picard Tools were employed to remove low quality reads. The remaining reads were filtered to 105 bp, with low quality reads (scoring < Q30) being discarded. The resulting bam files were converted to fastq files with the "fastq-dump" option from the NCBI SRA Toolkit (<https://github.com/ncbi/sratoolkit>). These were subsequently converted to fasta files with IDBA-UD (Peng et al. 2012), using the "fq2fa" option.

In order to analyse microbial pathways HUMAnN2 was used (Abubucker et al. 2012) (<http://huttenhower.sph.harvard.edu/humann2>), which measured the abundance of UniRef clusters (UniProt Reference Clusters) (Suzek et al. 2015). This is completed through alignment of sequences against the ChocoPhlan database. This is a pangenome database comprised of approximately 4000 bacterial pangenomes. MetaPhlan2 (Truong et al. 2015) was used to perform species-level analysis, while strain-level analysis was performed using Pangenome-based phylogenomic analysis (PanPhlAn) (Scholz et al. 2016). PanPhlAn aligns reads against the pangenome database, which allowed functional characterisation of strains (<http://segatalab.cibio.unitn.it/tools/panphlan/>). Reconstruction of multi-

locus sequence types (MLST) was carried out using MetaMLST (Zolfo et al. 2017). MetaMLST is a means of reconstructing MLST loci of microorganisms present in the microbiome of a sample from metagenomics data (<https://bitbucket.org/CibioCM/metamlst>). The heatmap generated for the top 25 most abundant species was constructed using hclust2 (<https://bitbucket.org/nsegata/hclust2>) and samples were clustered based on Euclidean distance. Resistome analysis was performed by aligning reads against the MEGARes antimicrobial resistance (AMR) database (Lakin et al. 2017) using Bowtie 2 (Langmead & Salzberg 2012). The identity and abundance of AMR genes was determined using ResistomeAnalyzer (<https://github.com/cdeanj/resistomeanalyzer>).

### **3.2.10. Statistical analyses**

The statistical analyses applied to the resulting sequencing data was performed in R-3.2.2 (Team RC 2014). In order to calculate Bray-Curtis based multidimensional scaling (MDS) analysis, and for alpha diversity analysis, the vegan package (version 2.3.0 was used (Oksanen et al. 2007)). In vegan, the adonis function was employed for permutational analysis of variance analysis (PERMANOVA). The one-way ANOVA test was used as a non-parametric means of detecting if samples originated from the same distribution and identifying significant differences between them. The resulting p-values were tested with the Benjamini-Hochberg procedure to reduce the false discovery rate. Subsequently, the HMISC package (version 3.16.0) (Harrell & Dupont 2003) was used for correlation computational analyses. Data was graphically represented using the ggplot2 package (version 2.2.1) (Wickham 2016). *P. aeruginosa* was correlated to the relative abundances of all other species detected with MetaPhlan2 and the number of antibiotics using the Spearman method.

## **3.3 Results**

### **3.3.1 Clinical and demographic data for sub-population of patient samples sequenced**

Samples included in the sequencing run were from a subset of 12 *P. aeruginosa* positive samples and 36 negative samples from a total of 11 patients (Table 3.1). There was no significant difference in gender, age range, BMI or FEV<sub>1</sub> between *P. aeruginosa* positive

and *P. aeruginosa* negative cohorts. An even distribution of delF508 homozygosity and heterozygosity were also recorded in these cohorts.

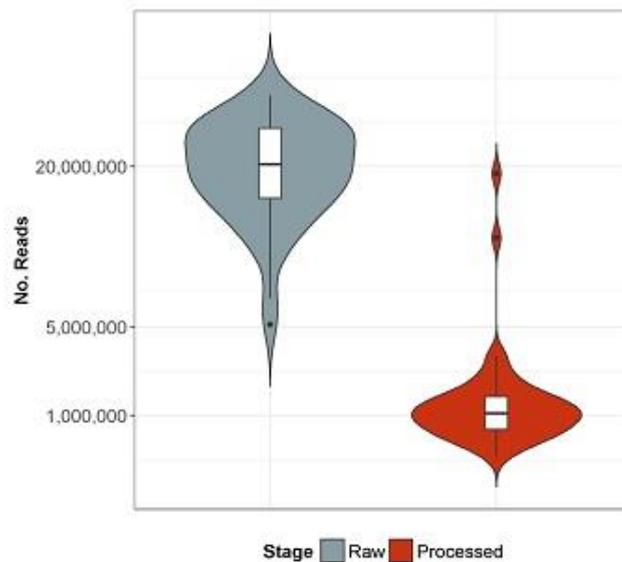
During the course of this study, 40% (2/5 patients) of *P. aeruginosa* positive patients showed no change in *P. aeruginosa* colonisation status and were persistently colonised with this bacteria. Conversely, 40% (2/5 patients) of *P. aeruginosa* positive patients demonstrated a change in colonisation status over the course of this study, in which some initial samples were *P. aeruginosa* negative and later became *P. aeruginosa* positive. One patient was unknown to be persistently or intermittently colonised.

**Table 3.1:** Patient demographics of sequenced samples (n=48)

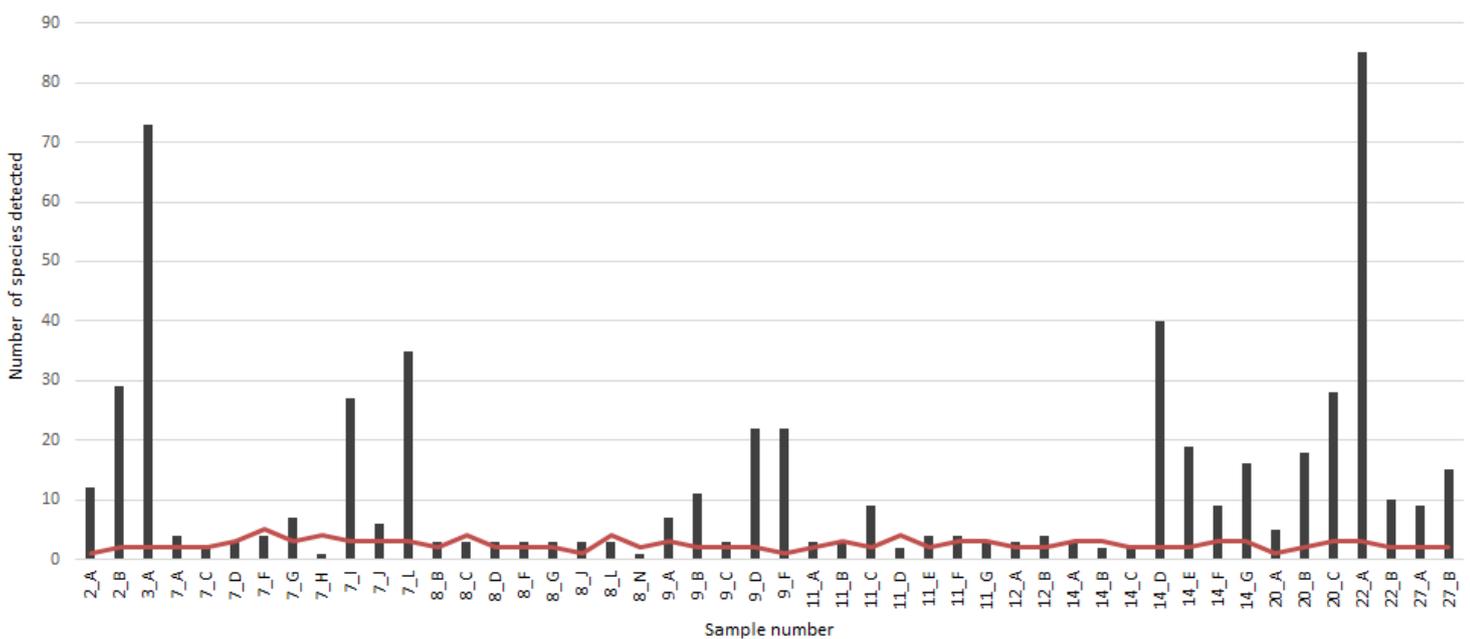
	<i>P. aeruginosa</i> positive	<i>P. aeruginosa</i> negative	Total (All patients)	P-values
N (%)	5/11 (45.45%)	6/11 (54.55%)	11	>0.9999
Male	4/5 (80%)	2/6 (33.33%)	6/11 (54.55%)	0.2063
Median Age (range)	17 (6-19)	11.5 (8-19)	16 (6-19)	0.4347
Median BMI z- scores (range)	-0.0416 (-3.2 – 0.897)	-0.147 (-1.95 – 1.55)	-0.0963 (-3.2 – 1.55)	0.3331
FEV <sub>1</sub> % predicted median (range)	59 (37 - 87)	64.5 (28-94)	59% (28 – 94)	0.9845
CFTR Genotype				
delF508/delF508	80% (4/5)	83.3% (5/6)	81.8% (9/11)	>0.9999
delF508/other	20% (1/5)	16.67% (1/6)	18.2% (2/11)	>0.9999

### 3.3.2. Sequencing quality and processing

There was a large reduction in read number after processing the raw reads (Fig. 3.2). This was mainly due to large numbers of human reads found in our samples despite the inclusion of a microbial DNA enrichment step (section 3.2.1). BMTagger was used to remove human reads (section 3.2.9). NextSeq generated an average of 19,985,506 raw reads (range 5,155,594 – 29,839,978) per sample and an average of 1,776,458 processed reads (185,162 – 19,123,414) (Fig. 3.2). Following alignment of processed reads to the default MetaPhlAn2 marker gene database an average of 12 bacterial species were detected per



**Figure 3.2:** Quality of reads generated from the NextSeq run. The number of reads before (grey) and after (red) quality filtering (raw versus processed) and how these were distributed.

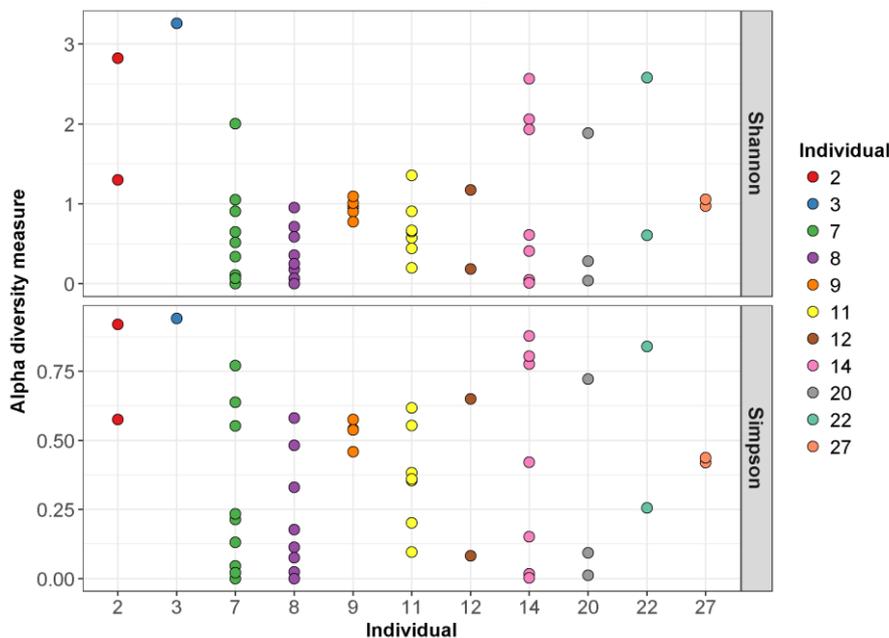


**Figure 3.3:** Number of species detected (richness) in each sample as determined with WGSS data (black bars) and culture data (red line)

sample (range 1 - 85) (Fig. 3.3). This was higher than the average number of bacterial species detected by culture of 2.5 (1-8) (Fig. 3.3).

### 3.3.3 The intra-patient (alpha) diversity of the CF airway samples

The microbial diversity of the CF airway differs considerably within the same patient over one year (Fig 3.4). All patients are represented by multiple samples, with the exception of patient 3, for which just one sample was collected. Shannon's diversity index differs to Simpson's index in that it accounts for both abundance and evenness of the species present in a sample. Simpson's diversity index gives more weight to common or dominant species, meaning the presence of few rare species will not affect diversity values. Diversity fluctuated greatly within most patients, particularly patients 7, 8, 11 and 14. Samples from these patients had a wide distribution of Simpson's diversity, implying the presence of primarily a few dominant species in some samples and a diverse range of species in others. Patients 9 and 27 showed little change in diversity over time as measured by Simpson and Shannon diversity indices. Both patients appeared to have even communities with Shannon indices around 1, however there were low numbers of samples collected for these patients. Some patients were difficult to categorise due to small sample numbers (e.g. patient 2 & 3). Individual samples from each patient were collected between 1 and 5 months apart. These differences in sample collection timepoints are a limitation of this study. Two

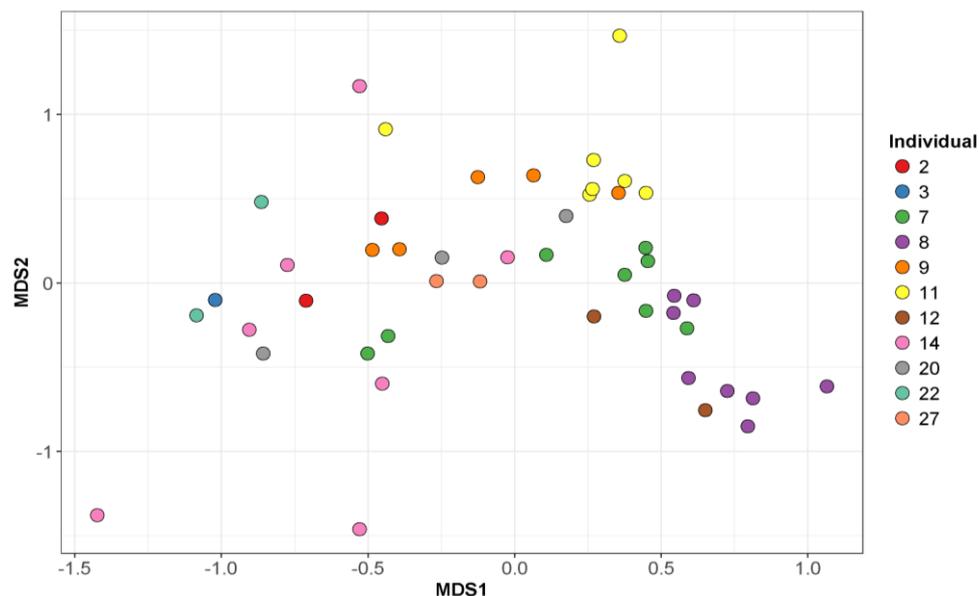


**Figure 3.4:** Alpha diversity of sequenced patient samples, coloured according to patient.

samples represent patient 27 (Fig. 3.4) which were collected within one month. This may represent a stable microbiome for this patient. Two samples also represent patient 2 (Fig.3.4), which were collected 3 months apart and therefore may reflect a change in microbiome complexity over time. Thus, patients where multiple samples have been collected over a one year period in this study (e.g. patient 7, 8, 11 and 14) give a more accurate representation of microbiome diversity change over time in our paediatric cohort.

### 3.3.4 Inter-patient (Beta) diversity of the CF airway samples

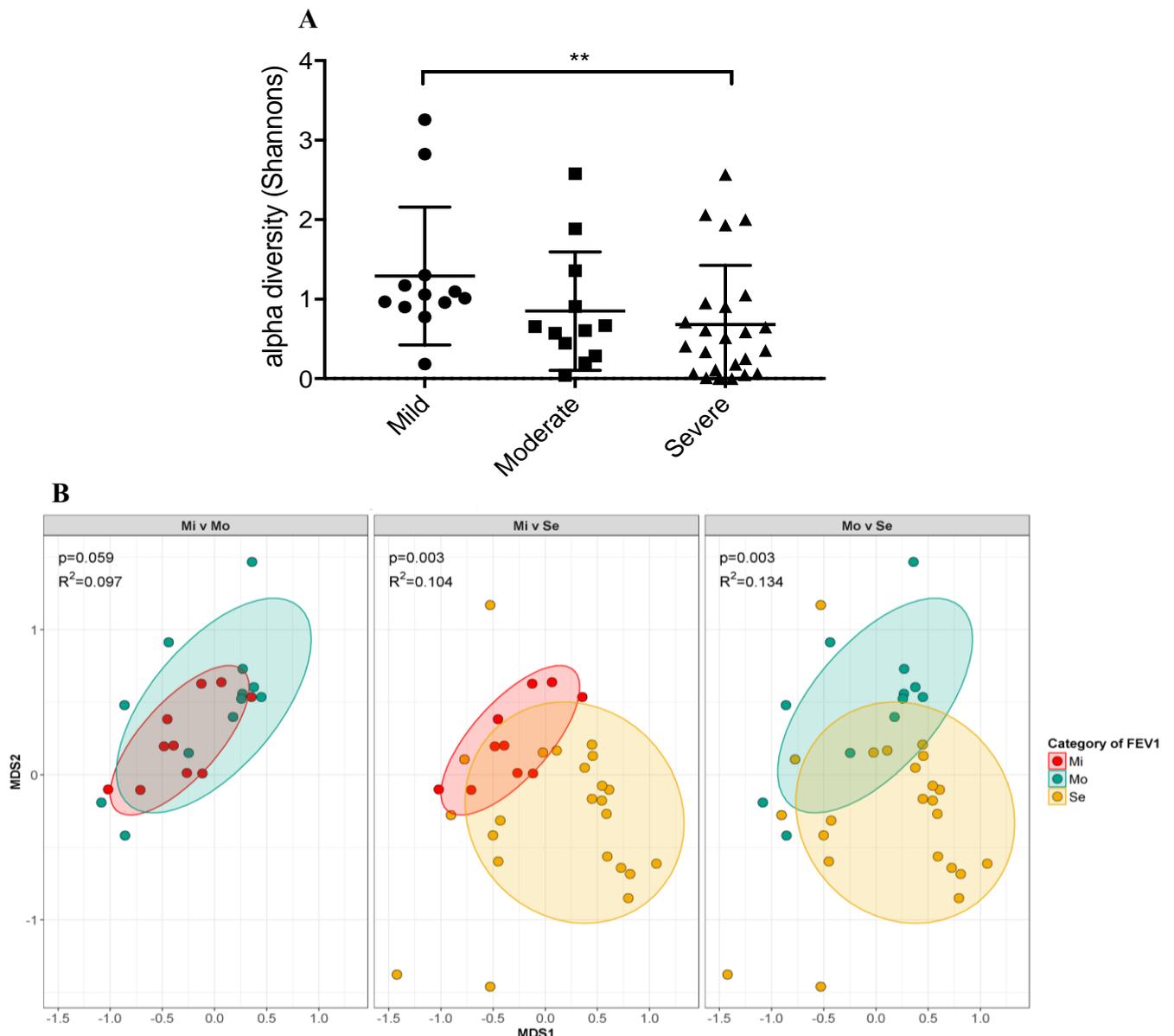
The microbial diversity of the CF airway differs considerably from patient-to-patient but for the most part remains similar within a patient over time. Bray-Curtis MDS was used to plot beta-diversity between all samples in the study. In some cases samples from the same patient tend to cluster together. Therefore some patient samples maintain a similar microbial community over time (Fig. 3.5). The composition of the airway microbial community of patients 7, 8 and 11 remained relatively similar over time as demonstrated by their close proximity on the MDS plot. Two samples from patient 7 and two samples from patient 11 diverge from the main cluster. Other patients' samples were widely distributed by Bray-Curtis MDS meaning their microbiome changed considerably over time. Patient 14 samples can be found widely dispersed across the MDS plot and so have quite dissimilar microbial composition (Fig. 3.5).



**Figure 3.5:** Bray-Curtis Multidimensional Scaling (MDS) plot of MetaPhlAn2 output of 48 consecutive samples from 11 patients. Patient samples are colour coded according to the legend (right).

### 3.3.5 The CF airway microbiome and lung function (FEV<sub>1</sub>)

Lower microbiome diversity was associated with reduced lung function (FEV<sub>1</sub> % predicted) (Fig. 3.6). Patients were categorised into mild deteriorating lung function (72% – 89% FEV<sub>1</sub>), moderate deterioration (48% – 59% FEV<sub>1</sub>) and severe deterioration (28% - 37% FEV<sub>1</sub>) as per previously published studies (Quanjer et al. 2012; Flume et al. 2007). Patient FEV<sub>1</sub> measures were taken at the time of sample collection and these determined which category of deterioration the patient sample belonged to. Patients with severely deteriorating lung function had significantly lower microbiome diversity (Kruskal-Wallis with Dunn's multiple comparisons,  $p=0.012$ ) (Fig 3.6 A). The MDS plot revealed significant clustering of patients based on lung function category (Fig 3.6B). Patients with mild lung function deterioration had the lowest beta-diversity. Patients in the moderate category had greater beta-diversity and this was trending towards significantly different from the mild group (Adonis;  $p=0.059$ ) (Fig 3.6B). The severe category had the largest beta-diversity of all groups and this was significantly different from both mild and moderate categories (Adonis;  $p=0.003$ ). Alpha and Beta diversity were not associated with any other clinical or demographic factors measured in this study, such as *P. aeruginosa* positivity or antibiotic exposure at the time of sample collection

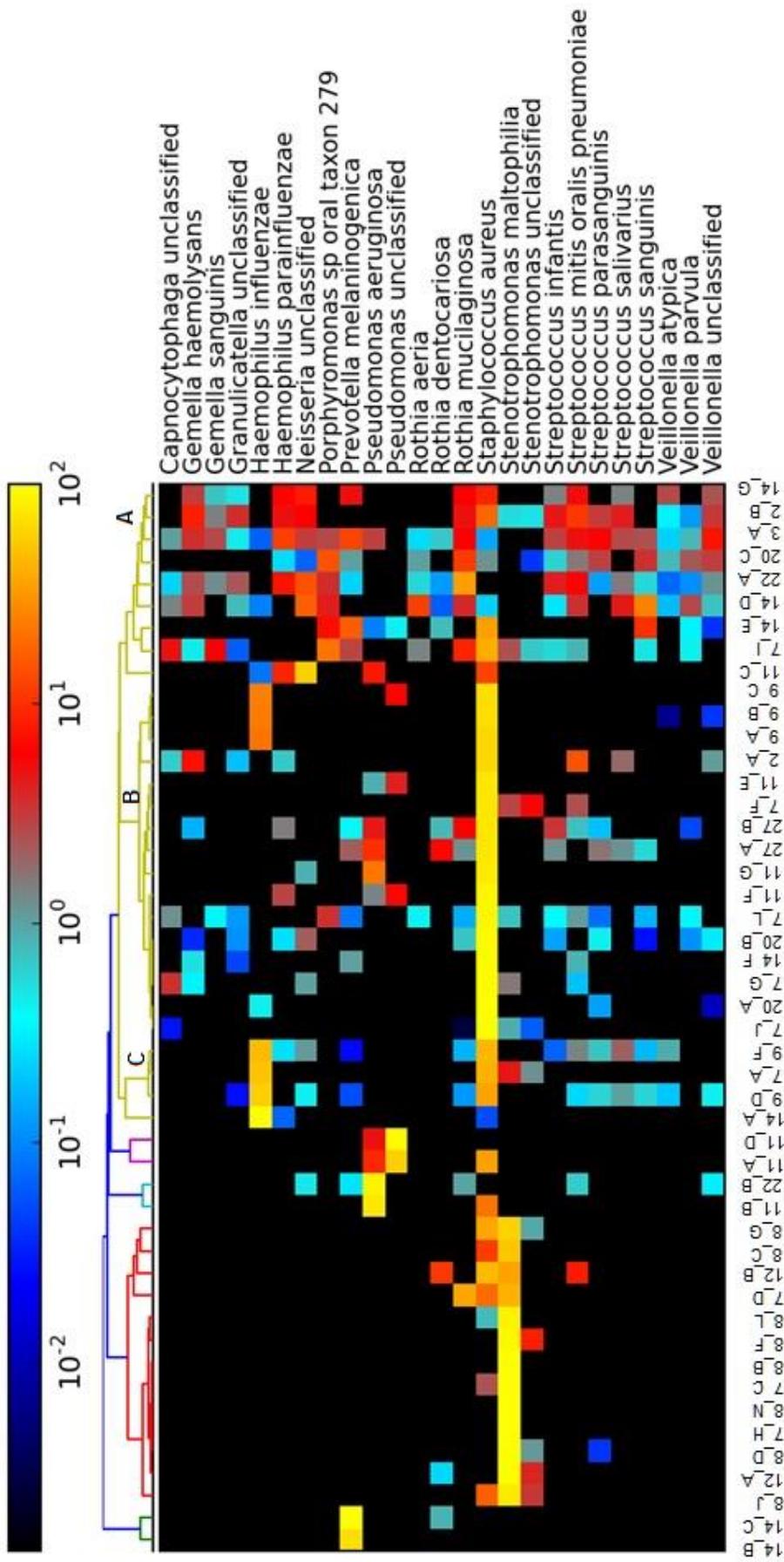


**Figure 3.6:** Diversity of CF airway microbiome in patients with mild (Mi), moderate (Mo) and severe (Se) deterioration in lung function. A) Alpha diversity as measured by Shannon’s diversity index of samples categorized based on level of lung function; Normal ( $FEV_1 >90\%$ ) Mild ( $FEV_1 70\% - 89\%$ ), moderate ( $FEV_1 40\% - 69\%$ ) and severe ( $FEV_1 <40\%$ ) (Quanjer et al. 2012; Flume et al. 2007). B) Bray-Curtis MDS plot of all samples coloured by lung function category. \*\*  $p=0.012$ . Pairwise comparisons of mild vs moderate  $FEV_1$  lung function, mild vs severe and moderate vs severe are represented. Ellipses represent significant clustering as determined by the Adonis function in PERMANOVA.

### 3.3.6 Impacts of dominant bacterial species on the microbiome

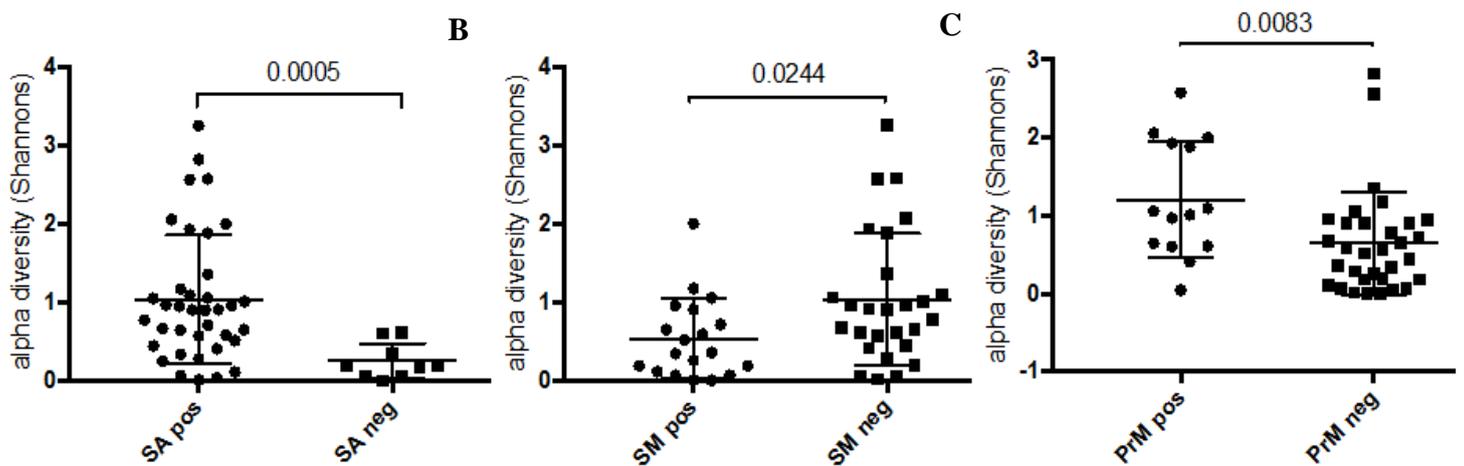
The top 25 abundant species comprise a mixture of the ‘usual suspects’ in paediatric CF for example *P. aeruginosa*, *S. aureus*, *H. influenzae*, *H. parainfluenzae*, *Streptococcal spp* and *S. maltophilia*, alongside emerging CF bacterial genera *Gemella (Ge)*, *Granulicatella*

(*Gr*), *Rothia*, *Porphyromonas* (*Po*), *Prevotella* (*Pr*) and several *Veillonella* species (Fig. 3.7). A variety of non-*pneumoniae* *Streptococcus* (*St*) were identified among the top 25 most abundant species including *St. salivarius*, *St. infantis*, *St. parasanguinis* and *St. sanguinis*. An unclassified *Capnocytophaga* and *Neisseria* species were also in the top 25 abundant species. Samples clustered based on the dominant species in the sample, however this result should be interpreted with caution due to our small sample size. In general samples in the yellow cluster had high numbers of other dominant bacterial species, demonstrating a more even community. Within the yellow cluster three sub-clusters can be seen (A, B and C), all of which showed positivity for *S. aureus* colonisation with the exception of one sample (22\_A). Sub-cluster A shows the highest number of dominant species with *S. aureus* being either absent or present but generally less dominant or as dominant as other species. In sub-cluster B *S. aureus* has clear dominance with reduced presence of other dominant species. Sub-cluster C samples were dominated by *S. aureus* and *H. influenzae* with fewer other dominant species than sub-cluster A. Samples in the purple cluster were dominantly colonised with *Pseudomonas* unclassified and *P. aeruginosa*. This cluster comprises two samples, one of which appears to be co-colonised with *S. aureus*. No other dominant bacteria are present in these samples. Samples in the blue cluster were dominated by *P. aeruginosa* and the unclassified *P. aeruginosa* species is not present. Samples in the red cluster were dominated by *S. maltophilia*. Approximately half (53.8%) of these samples were also colonised with *S. aureus* in mostly similar abundance. These samples also had reduced numbers of other dominant species. Some of the species noted to co-colonise with *P. aeruginosa* also co-colonise with *S. maltophilia*, these include *S. mitis oralis pneumoniae*, *R. mucilaginosa* and *R. dentocariosa*. An unclassified *Stenotrophomonas* species is also notable in this cluster. Samples in the green cluster were dominated by *Prevotella melaninogenica* and these samples had the most uneven microbial communities with only one other dominant species, *Rothia dentocariosa*, detected in these samples. *Pr. melaninogenica* was present in lower abundance in other samples. Patient 11 samples are consistently colonised with *S. aureus* and *P. aeruginosa* with intermittent colonisation with *H. influenzae*, *H. parainfluenzae* and an unclassified species of *Neisseria*. Another patient of interest in this group is patient 8, which makes up a large proportion of the red *S. maltophilia*-dominated cluster. All samples from this patient occur within this cluster and exhibit markedly lower numbers of dominant bacterial species than other samples not colonised with *Stenotrophomonas*.



**Figure 3.7:** Heatmap and dendrogram of the top 25 most abundant species across all patient samples using MetaPhlAn2. This heatmap represents copies per million (CPM) with black indicating absence and yellow indicating highest abundance. Dendrogram colour-coding denotes clustering, based on patient samples (top) and species (left).

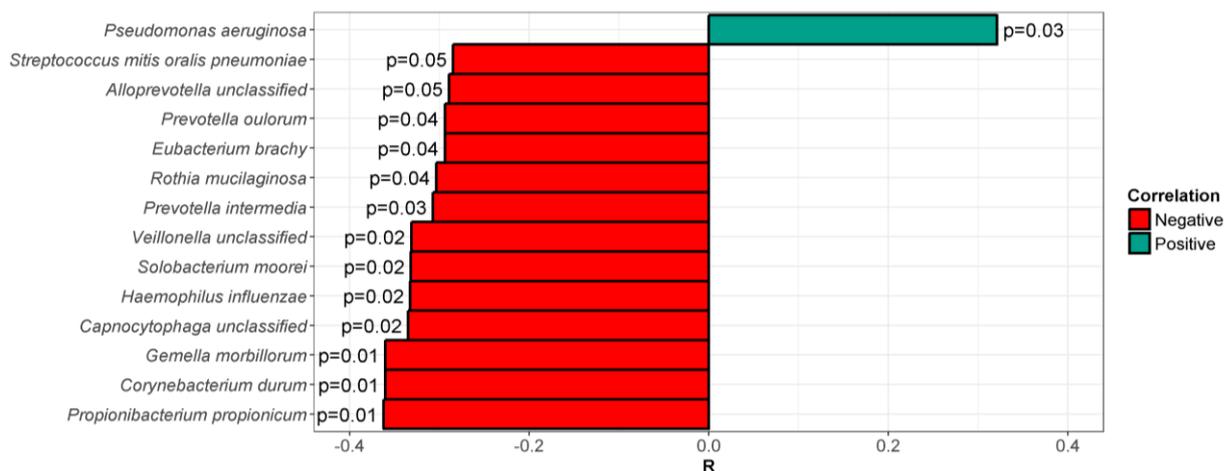
In agreement with the above heatmap, significant differences in the alpha diversity of samples were detected upon colonisation with some bacterial pathogens (Fig. 3.8). Samples colonised with *S. aureus* had significantly higher Shannon's alpha diversity measures than non-colonised samples (Mann-Whitney test;  $p=0.0005$ ) (Fig 3.8A). *Pr. melaninogenica* colonised samples had significantly higher Shannon's alpha diversity than non-colonised samples ( $p = 0.0083$ ) (Fig 3.8C). The opposite trend was detected in samples colonised with *S. maltophilia* which had lower microbial diversity ( $p = 0.0244$ ) (Fig 3.8B).



**Figure 3.8:** Shannon's alpha diversity differences between colonized and non-colonized samples with the dominant bacterial species *S. aureus* (SA), *S. maltophilia* (SM) and *Pr. melaninogenica* (PrM). Significance was determined using a two-tailed Mann-Whitney test.

### 3.3.7 Impact of antibiotics on the microbiome

The number of antibiotics a patient was on in the 2 months prior to sample collection was significantly correlated to abundance of a number of species (Fig. 3.9) as measured by the Spearman method. *P. aeruginosa* had a positive correlation ( $p = 0.03$ ) with antibiotic exposure. All other bacteria listed were negatively correlated with antibiotic exposure and interestingly are all anaerobes/ facultative anaerobes.



**Figure 3.9:** Correlation between antibiotics given in previous two months and species abundance in our population.

The resistance profile of the microbiome over time is relatively consistent in some patients (Fig. 3.10: patient 7, 9 & 20) while in other patients each sample differs (Fig. 3.10: patient 2, 11 & 14). Resistance mechanisms against trimethoprim, glycopeptides and cationic antimicrobial peptides were not common in our sample cohort. This contrasted with highly common resistance mechanisms such as multi-drug resistance and resistance to aminoglycosides. Some patients' samples were found to have a high frequency of antibiotic resistance mechanisms. Patient 7 and patient 9 showed high occurrences of these mechanisms, with the exception of some samples (e.g. 7\_F and 9\_C). Patient 8 and patient 12 were found to possess fewer resistance genes in the majority of their samples. Both resistance mechanisms towards aminoglycosides and multidrug resistance were present in most samples while trimethoprim resistance genes were consistently absent in these 2 patients.



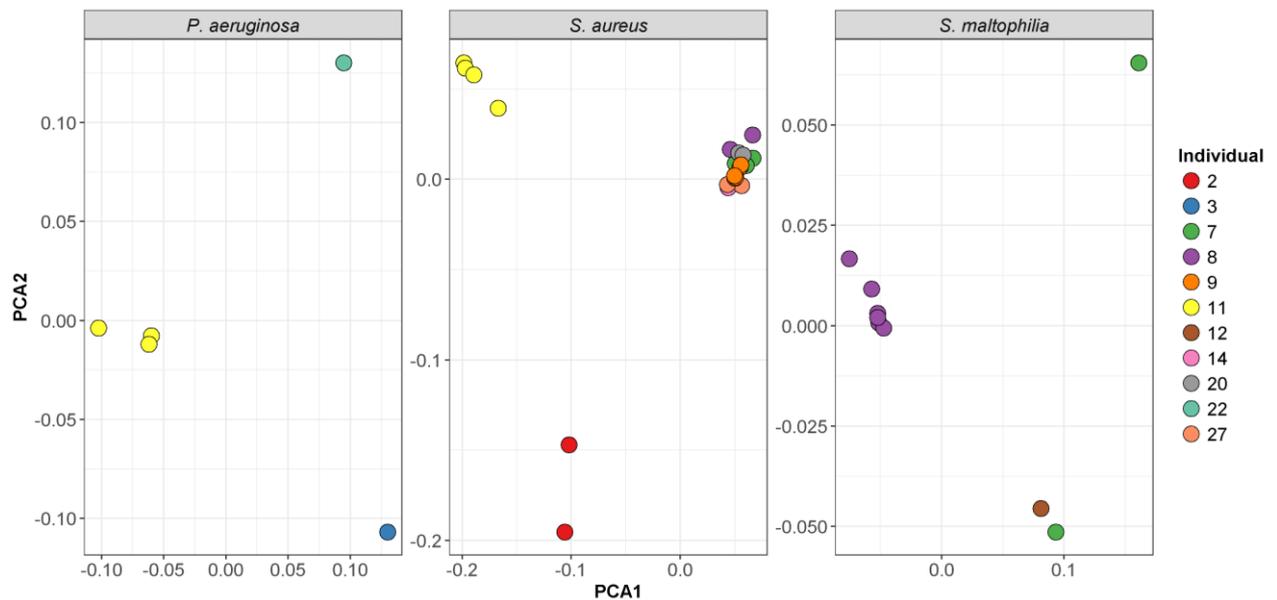
### 3.3.8 Patients colonised with pathogenic strain types tended to be colonised with the same strain type over time

Eleven pathogenic sequence types (ST) were identified in 23 samples with  $\geq 98.52\%$  confidence using MetaMLST analysis. Nine patients had at least 1 pathogenic ST present (Table 3.2). *S. aureus* STs appeared most frequently and had the largest variety of pathogenic STs (n=8). These consisted of ST5, ST15, ST845, ST100001, ST100002, ST100003, ST100004 and ST100005. ST5 was the most common (43%; 6/14 *S. aureus* STs) and all ST5 strains were shown to have methicillin resistance genes by MetaMLST analysis. In some samples more than 1 species' pathogenic ST was identified; for example *S. aureus* and *S. maltophilia* STs were both detected in sample 7\_J. Furthermore, some patients (e.g. patient 7, 8 and 9) carry more potentially pathogenic STs than others. Patient 8 harboured 3 pathogenic STs of *S. maltophilia* (ST100001, ST100002 & ST4) contrasting with other longitudinal patient samples which consist of STs from several species (e.g. patient 7 & 9).

**Table 3.2:** Pathogenic sequence types detected using MetaMLST

Species	Sample	Individual	Sequence Type (ST)
<i>Haemophilus</i>	7_A	7	100001
<i>Staphylococcus aureus</i>	7_G	7	5
<i>Staphylococcus aureus</i>	7_I	7	100001
<i>Staphylococcus aureus</i>	7_J	7	5
<i>Stenotrophomonas maltophilia</i>	7_J	7	5
<i>Staphylococcus aureus</i>	7_L	7	5
<i>Stenotrophomonas maltophilia</i>	8_B	8	4
<i>Stenotrophomonas maltophilia</i>	8_D	8	4
<i>Stenotrophomonas maltophilia</i>	8_F	8	100001
<i>Stenotrophomonas maltophilia</i>	8_G	8	4
<i>Stenotrophomonas maltophilia</i>	8_J	8	100002
<i>Staphylococcus aureus</i>	9_A	9	100003
<i>Streptococcus pyogenes</i>	9_A	9	100001
<i>Haemophilus</i>	9_B	9	100002
<i>Staphylococcus aureus</i>	9_B	9	100004
<i>Haemophilus</i>	9_D	9	100003
<i>Staphylococcus aureus</i>	9_D	9	100005
<i>Haemophilus</i>	9_F	9	100004
<i>Staphylococcus aureus</i>	9_F	9	5
<i>Staphylococcus aureus</i>	11_G	11	100002
<i>Stenotrophomonas maltophilia</i>	12_A	12	5
<i>Haemophilus</i>	14_A	14	368
<i>Staphylococcus aureus</i>	14_E	14	15
<i>Staphylococcus aureus</i>	14_F	14	15
<i>Staphylococcus aureus</i>	20_A	20	5
<i>Staphylococcus aureus</i>	20_B	20	5
<i>Pseudomonas aeruginosa</i>	22_B	22	313
<i>Staphylococcus aureus</i>	27_B	27	845

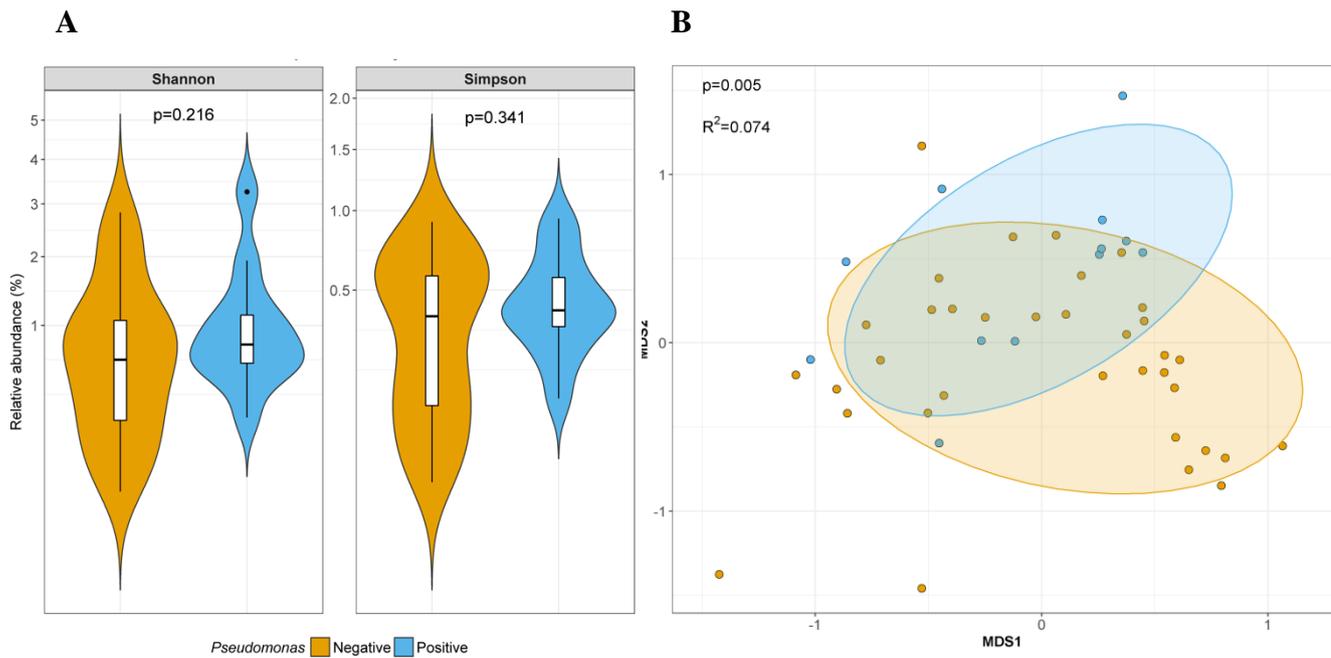
PanPhlAn was used to generate a PCA plot of pathogenic strains detected in our patient samples based on the overall genetic composition of these strains (Fig 3.11). The proximity of samples in this plot implies highly similar genetic composition or the same pathogenic strains in multiple samples. Patient 3, 11 and 22 were detected to each have distinct pathogenic *P. aeruginosa* strains using PanPhlAn analysis (PA01, Nhmuc & DK1, SCV and B136-33). Patient 11 had the same pathogenic *P. aeruginosa* Nhmuc strain in 2 consecutive samples (GCF\_001900265, 81.2% identity) and a very similar DK1 strain in a third sample (GCF\_900069025, 81.4% identity). Several patients' samples (7, 8, 9, 20 and 27) had the same pathogenic *S. aureus* FDAARGOS\_16 strain (GCF\_001019355, 81.5-95.6% identity) in consecutive samples. Four consecutive samples from patient 11 harboured the same pathogenic FDAARGOS\_29 strain of *S. aureus* (GCF\_001018965, 75.7-85.6% identity). Two consecutive samples from patient 2 harboured the same *S. aureus* NRS 143 strain (GCF\_001018975, 84.1-85.8% identity). Overall a large proportion (~64% of samples; 7/11 patients) of patient samples had pathogenic *S. aureus* strains. Samples from patient 7 and 8 had pathogenic strains of both *S. aureus* and *S. maltophilia*, though only patient 8 showed the same pathogenic *S. maltophilia* K279a strain in all consecutive samples (GCF\_000072485, 68.3-73.1% identity). Patient 12 was found to harbour a pathogenic strain of *S. maltophilia* which appeared to be a highly similar strain to one found in patient 7. Patient 11 had pathogenic *P. aeruginosa* and *S. aureus* strains over multiple samples.



**Figure 3.11:** Dissimilarity in genetic composition of pathogenic strains of *P. aeruginosa*, *S. aureus*, *S. maltophilia* based on PanPhlAn output. PCA plots of genetic distance of pathogenic strains. Samples are colour coded according to patient key.

### 3.3.9 Children with CF positive for *P. aeruginosa* have distinct airway microbial communities from those negative for *P. aeruginosa*

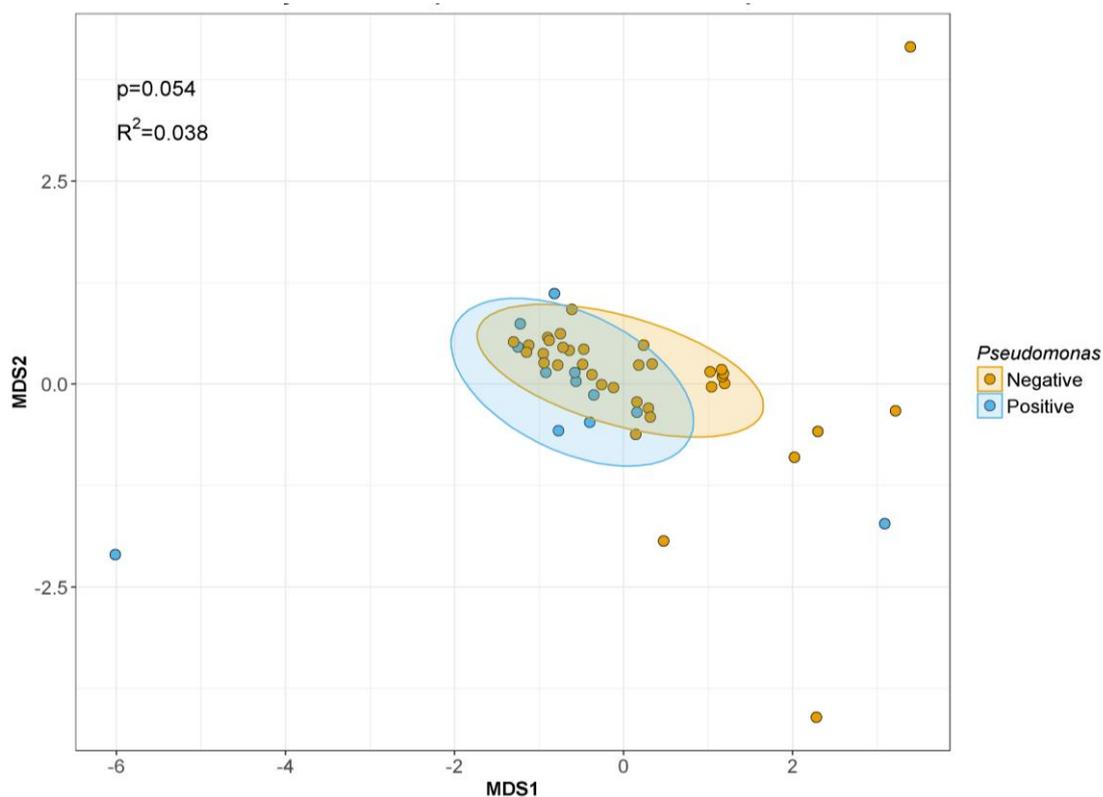
A total of 12 *P. aeruginosa* positive samples from 5 patients were identified by WGSS sequencing (Fig 3.12) and 36 samples from 6 patients were grouped into the *P. aeruginosa* negative cohort. There was no difference in alpha diversity between the *P. aeruginosa* negative and positive cohorts (Fig. 3.12 A). However the microbial communities positive for *P. aeruginosa* have distinct composition from that of *P. aeruginosa* negative communities (Fig 3.12 B). Patient samples positive for *P. aeruginosa* cluster together and this cluster was significantly dissimilar from the *P. aeruginosa* negative sample cluster (Adonis,  $p=0.005$ ,  $R^2=0.074$ ).



**Figure 3.12:** Diversity of *P. aeruginosa* positive and negative microbial communities. A) Alpha diversity as measured by Shannon and Simpsons diversity indices and B) Beta diversity dissimilarity Bray-Curtis Multidimensional Scaling (MDS). Ellipses represent significant clustering (80% CI) of *P. aeruginosa* positive (blue) and negative (orange) samples as determined by the Adonis function in PERMANOVA.

### 3.3.10. *P. aeruginosa* positive microbial communities harbour distinct metabolic capabilities to *P. aeruginosa* negative communities in the CF airway

HUMAN2 was used to detect the presence and abundance of metabolic pathways in microbial communities from WGSS data (Abubucker et al. 2012). The microbial communities positive for *P. aeruginosa* harboured significantly dissimilar metabolic functions to *P. aeruginosa* negative microbial communities (Adonis,  $p = 0.54$ ,  $r^2 = 0.038$ ) suggesting that *P. aeruginosa* brings distinct metabolic functions to the microbial community (Fig. 3.13).



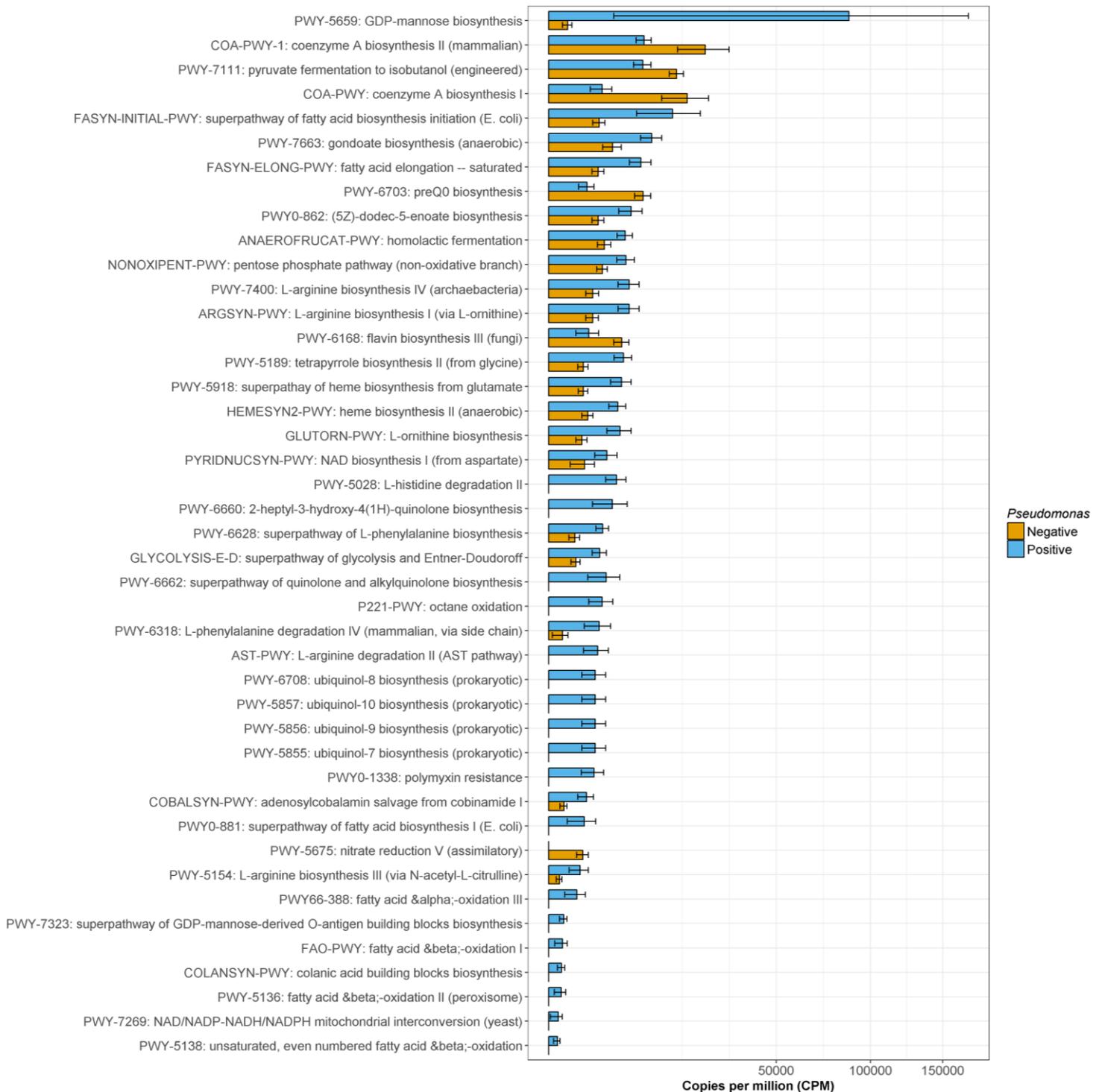
**Figure 3.13:** Bray-Curtis MDS plot based on HUMAnN2 output for cohorts of *P. aeruginosa* positive and negative samples. Dissimilarity is plotted based on the metabolic pathways of *P. aeruginosa* positive (blue) and *P. aeruginosa* negative (orange) groups.

Several metabolic pathways were significantly altered between *P. aeruginosa* positive and negative microbial communities (Fig. 3.14) (One way ANOVA,  $p < 0.05$ ). There were 18 pathways specific to the *P. aeruginosa* positive communities of which 5 were related to fatty acid biosynthesis or oxidation and 4 involved in ubiquinol biosynthesis. Other pathways specific to *P. aeruginosa* communities were amino acid degradation - L-Histidine degradation II, L-arginine degradation II (AST pathway), *P. aeruginosa* Quinolone Signalling (PQS) (2-heptyl-3-hydroxy-4(1H)-quinolone biosynthesis and superpathway of quinolone and alkylquinolone biosynthesis), antigen synthesis (superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis and colanic acid building blocks biosynthesis) and those involved in cellular metabolic processes.

Nineteen pathways were increased in *P. aeruginosa* positive samples compared to *P. aeruginosa* negative samples (Fig. 3.14). Some of these pathways were similar to those only seen in *P. aeruginosa* positive samples (6 amino acid biosynthesis or degradation pathways, 4 fatty acid biosynthesis or oxidation pathways) while other pathways related to

glycolysis and in diverse metabolic processes. The GDP-mannose biosynthesis pathway had the highest abundance (87,000 CPM) in *P. aeruginosa* positive samples.

A group of six pathways were increased in *P. aeruginosa* negative samples. Three of these were noted to be involved in glycolysis (coenzyme A biosynthesis II (mammalian), pyruvate fermentation to isobutanol (engineered) and coenzyme A biosynthesis I) while the others were involved in diverse processes such as Flavin biosynthesis and nitrate reduction. In fact nitrate reduction V was only present in the *P. aeruginosa* negative communities.



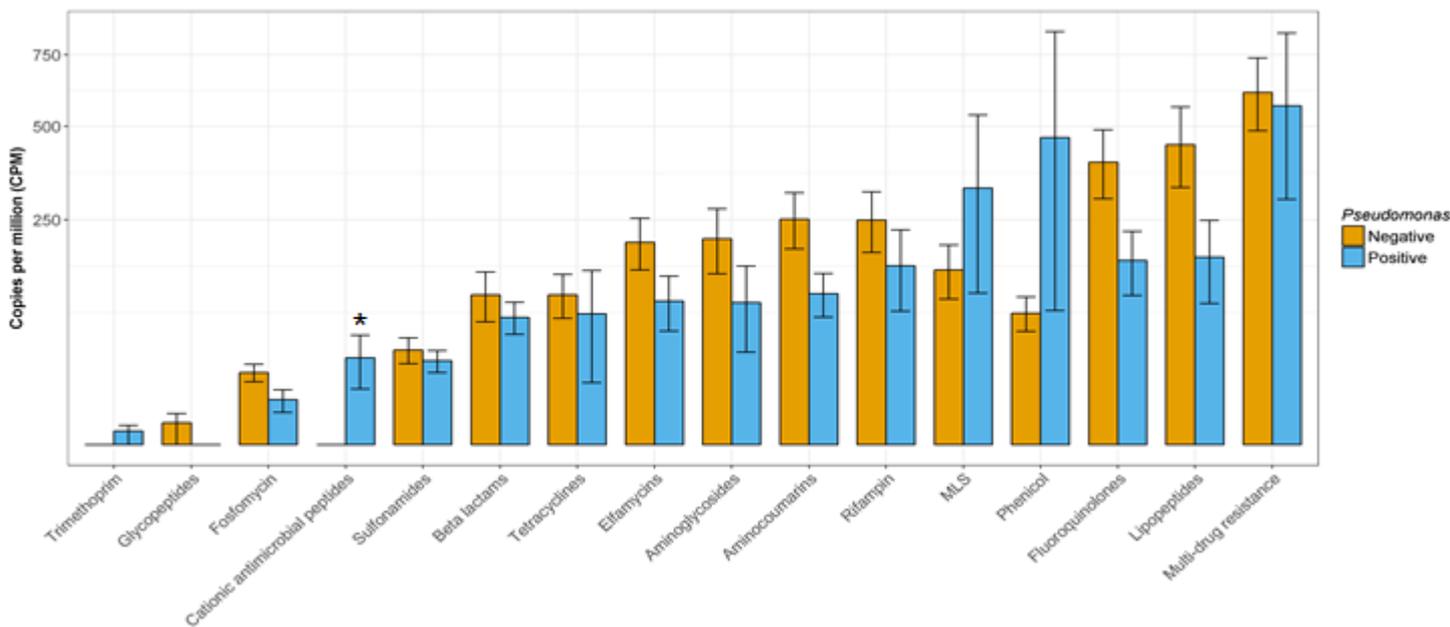
**Figure 3.14:** MetaCyc metabolic pathways of significantly differential abundance (CPM) between *P. aeruginosa* positive and negative microbial communities. All pathways presented are significantly altered between *P. aeruginosa* positive (blue) and negative (orange) microbial communities.

**Table 3.3:** Summary of identified metabolic pathways in *Pseudomonas* positive and negative groups

Pathway	In PA+ group only (n=18)	↑ in PA+ group (n=19)	↑ in PA- group (n=6)	Relevance
Fatty acid biosynthesis or oxidation	Y	Y	N	PA01 genes involved in lipid synthesis. SCFAs may be used as a nutrient source. SCFAs have role in inflammation.
Ubiquinol biosynthesis	Y	N	N	Aids survival in hypoxic and microaerobic environments. Exacerbating CF airway hypoxia (Alvarez-Ortega & Harwood 2007).
Amino acid biosynthesis or degradation	Y	Y	N	Possible advantageous use as energy source
L-histidine/L-arginine degradation	Y	N	N	Degradation required to use amino acids as energy source
PQS	Y	N	N	Interbacterial signalling molecule of <i>P. aeruginosa</i> (Pezzoni et al. 2015)
Antigen synthesis	Y	N	N	Component of OM in Gram negative bacteria
Cellular processes	Y	N	N	Vital to bacterial cellular survival
Glycolysis	N	Y	Y	Vital release of energy used for many processes. Different pathways involved in glycolysis were increased in PA+ groups than other glycolysis related pathways increased in the PA- group.
Flavin biosynthesis	N	N	Y	Extracellular electron transfer in biofilms
Nitrate reduction	N	N	Y	Conversion of nitrogen to biochemically useful form. Nitric oxide interacts with oxygen which subsequently harms airway epithelium in CF patients (Marozkina & Gaston 2011) .

### 3.3.11. Antibiotic resistance mechanisms in *P. aeruginosa* negative and positive samples

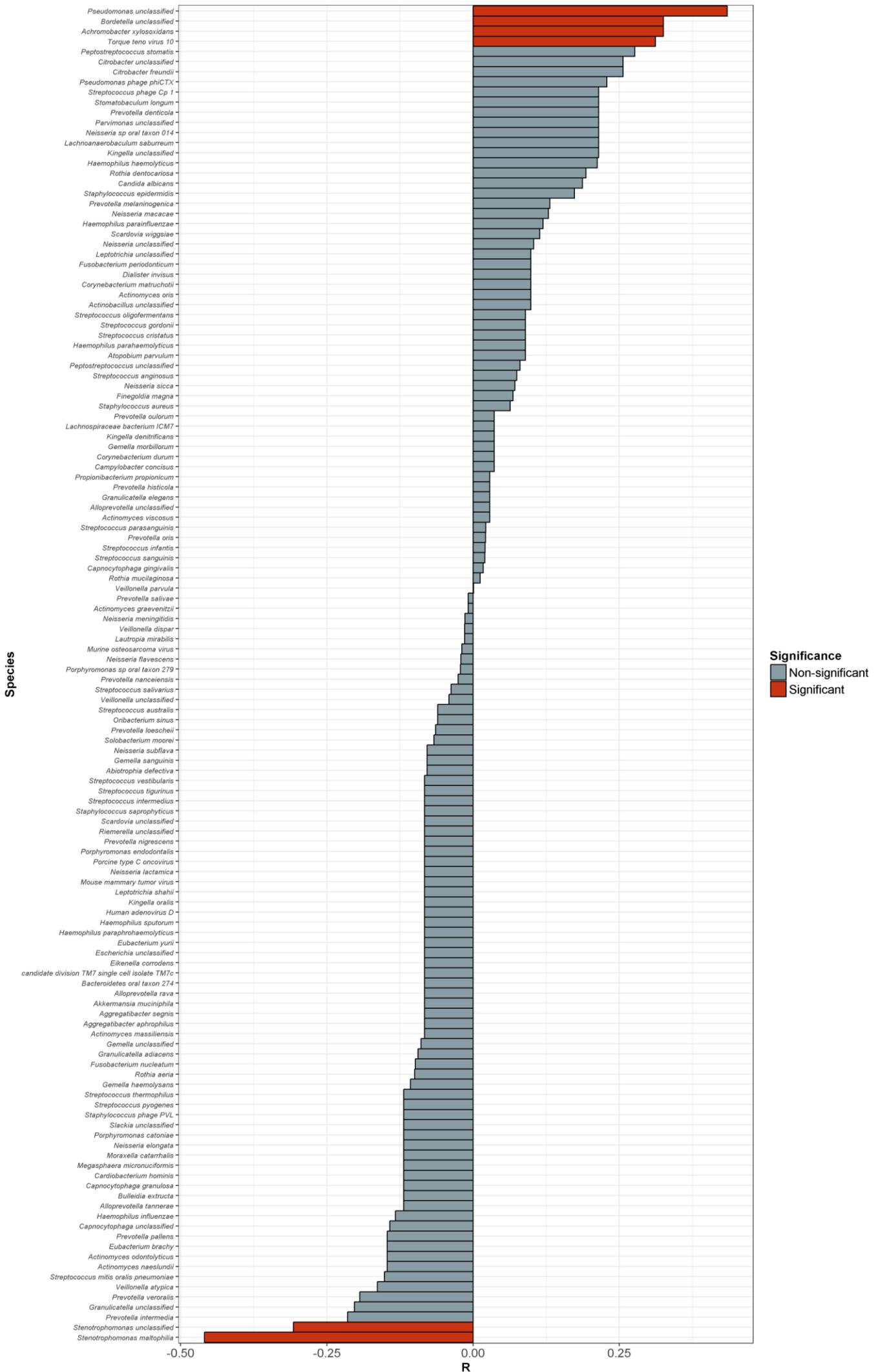
Antibiotic resistance gene classes for trimethoprim and cationic antimicrobial peptides were only present in patient samples which were positive for *P. aeruginosa* ( $p = 0.083$ ,  $p < 0.001$  respectively) (Fig. 3.15). Only cationic antimicrobial peptide genes were significantly differently abundant between *P. aeruginosa* positive and negative groups (One way ANOVA,  $p < 0.05$ ) with these genes only being detected in *P. aeruginosa* positive samples. All other antibiotic resistance genes were more abundant in samples negative for *P. aeruginosa* (Fig 3.15) however this was not statistically significant. Multi-drug resistance (MDR) mechanisms were the greatest of any antibiotic resistance classes in both *P. aeruginosa* negative and positive samples.



**Figure 3.15:** Difference in abundance (CPM) of antibiotic resistance mechanisms between *P. aeruginosa* positive (blue) and negative (orange) sample groups as detected by ResistomeAnalyzer.

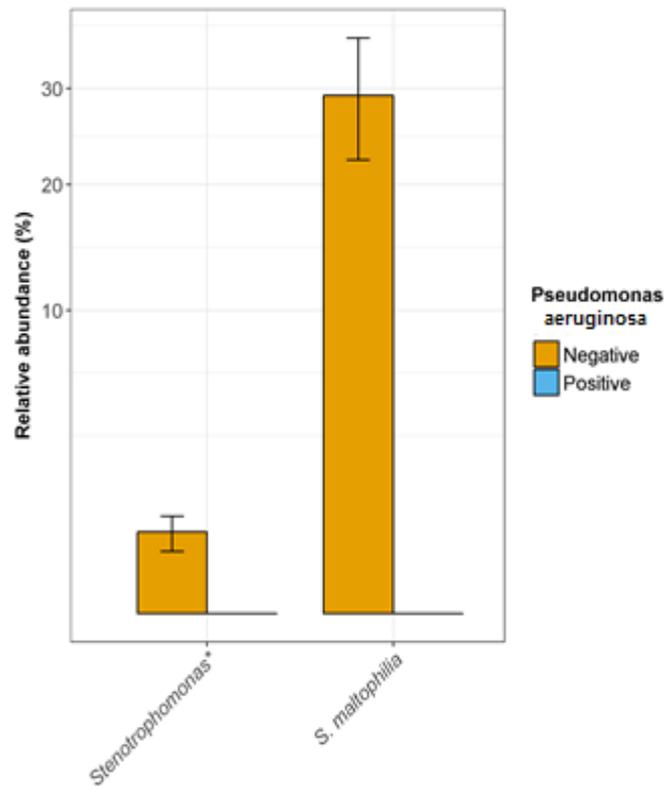
### 3.3.12. Identifying potential competitive and mutual relationships between *P. aeruginosa* and other species within the CF airway microbiome

The HMISC package (version 3.16.0) (Harrell & Dupont 2003) was employed to correlate species relative abundance to identify potential positive and negative relationships between *P. aeruginosa* and other CF airway species. An unclassified *Pseudomonas*, unclassified *Bordetella*, *Achromobacter xylosoxidans* and Torque teno virus 10 were positively correlated with *P. aeruginosa* (Fig 3.16).



**Figure 3.16:** Correlations between *P. aeruginosa* and all other species identified in sequenced patient samples. Red bars - significant

An unclassified *Stenotrophomonas* species and *S. maltophilia* were negatively correlated with *P. aeruginosa*. In fact *P. aeruginosa* was absent from all 20 samples positive for *S. maltophilia* (Fig 3.17). Several other species were identified to have somewhat positive or negative correlations with *P. aeruginosa* but none of these were significant.



**Figure 3.17:** The relative abundance of *Stenotrophomonas* species in *P. aeruginosa* positive and negative cohorts. Sample cohorts are colour coded according to their positivity (blue) or negativity (orange) for *P. aeruginosa*.

### 3.4 Discussion

In total 128 bacterial species were detected by WGSS across our samples with an average of 12 species per sample. WGSS detected many more bacterial species per sample and identified an overall wider variety of bacteria than culture. Bacterial richness detected by WGSS in this study was lower than other studies on children with CF using 16S rRNA sequencing (Cox et al. 2010; Bittar et al. 2008) but was similar to numbers reported in adult CF patients in a study using 16S rRNA T-RFLP profiling (Rogers et al. 2004). Studies have suggested 16S rRNA sequencing might overestimate bacterial diversity to varying degrees depending on the variable region focused on and the Phylum identified (Sun et al. 2013). Therefore WGSS may give a more reserved and exact measure of CF airway microbiome richness.

There was considerable intra-patient variability in the diversity of the CF airway microbiome of our patient population, substantiating evidence for a dynamic and evolving microbiome in children and adolescents with CF (Surette 2014; Zemanick et al. 2011). Alpha diversity was measured in both Shannon and Simpson indices to give a complete picture of the diversity (evenness and richness) within samples from an individual patient. Some patients showed little variation in diversity over consecutive samples collected. This may be suggestive of patients in a clinically stable state, as described by Zhao *et al.* in which stable airway microbiome diversity of adults with CF (age 18-30) was linked to a mild lung disease phenotype (~1% change in FEV<sub>1</sub>% predicted per year) over ~ 8-9 years (Zhao et al. 2012)). Other patients in our study showed highly variable diversity in their airway microbiome over time, perhaps indicating a less stable clinical course. Kramer *et al.* suggested that continuous treatment with antibiotics over time may have been a factor contributing to high individuality of microbial communities in their adult CF cohort (Kramer et al. 2015). Our findings suggest that these patterns of stable and disrupted microbiomes may be evident earlier in life and over short-term monitoring periods which would be diagnostically useful. Renwick *et al.* reported that disruptions in the lower CF airway microbiome are evident early in life (Renwick et al. 2014) however further research on larger patient numbers is required to specifically link stable and unstable microbiome diversity to disease outcomes and to investigate their use in predicting disease status.

Interpatient variability has been shown to exceed inpatient variability in consecutive CF airway samples, implying that individual patients have distinct microbiome compositions. Stressmann *et al.* found that variability was higher between adult CF patients

than within samples from individuals taken over 1 year (Stressmann et al. 2012). A number of other studies have shown that individual CF patients have distinct airway microbial communities (Price et al. 2013; Cox et al. 2010; Zhao et al. 2012s). Beta diversity dissimilarity between samples in our study supports the individuality of the CF airway microbiome with samples generally clustering based on patient identity. These findings highlight the need for a more personalised approach to CF care as a ‘one size fits all’ approach to antimicrobial treatment of CF patients can no longer be considered favourable. This can only become a reality when the cost and time of sequencing strategies decrease.

An association between microbiome community diversity and lung function was notable in our patient cohort (Fig. 3.6). An inverse relationship between lung function deterioration and microbial community richness has been identified in CF patients in previous work (Stressmann et al. 2012; Paganin et al. 2015; Zhao et al. 2012; Bacci et al. 2017). Stressmann *et al.* characterised CF microbial communities in adults using T-RFLP and measured community change correlated to lung function measures (FEV<sub>1</sub> and FEV<sub>6</sub>) (Stressmann et al. 2012). Paganin *et al.* also highlighted this association and implicated specific species, *S. pneumoniae* and *R. mucilaginosa*, with a severe decline in FEV<sub>1</sub> in adults with CF (Paganin et al. 2015). Bacci *et al.* carried out similar work using 16S rRNA sequencing and were also able to make this association between lower microbial diversity and decline in FEV<sub>1</sub> (Bacci et al. 2017). For the first time we have made this association of lower airway community diversity and reduced lung function in a young CF population indicating that reduced diversity early in life may indicate early disease progression.

No significant associations were made between our patient microbiome community diversity and other clinical and demographic factors. Age has previously been associated with microbiome diversity in CF (Cox et al. 2010; Muhlebach et al. 2018) however we did not observe this association, perhaps due to the young age and limited age range of our cohort. There are contradicting findings on the influence of antibiotics on the CF airway microbiome. Zhao *et al.* identified antibiotic exposure as the primary driver in decreases of microbial diversity (Zhao et al. 2012). Additionally, research from Klepac-Ceraj *et al.* and Daniels *et al.* supported a negative correlation between antibiotic exposure and community diversity (Klepac-Ceraj et al. 2010; Daniels et al. 2013). Klepac-Ceraj *et al.* examined cumulative antibiotic exposure over a patients’ lifetime and could thus identify a clear impact. However, Fodor *et al.* could only detect a minimal reduction in species richness with overall microbial community composition remaining unchanged by antibiotic exposure in adult CF patients (Fodor et al. 2012). The reason for this may be a short term

monitoring approach differing to that of Klepac-Ceraj. In the absence of cumulative antibiotic data, it can appear that the microbiome is only transiently perturbed by antibiotics during treatment. We found no association between number of antibiotics in the previous 2 months and diversity, though a limitation of our study is that we did not account for the cumulative number of antibiotics taken in our patients' lifetime. Furthermore, patients in our study are young and have likely been exposed to less antibiotics than older CF patients, which is another possible reason for no association detected here.

The dominant species present in a patients sample was found to be the major influencing factor for phylogenetic clustering. Among the top 25 abundant species detected were a number of the well-known and well documented CF bacteria including *P. aeruginosa*, *S. aureus*, *S. maltophilia* and *H. influenzae*. Other less documented bacteria in the top 25 abundant species included unclassified species of *Neisseria*, *Granulicatella*, *Capnocytophaga* and *Veillonella*. This is not the first time these genera have been identified in the CF microbiome. Guss *et al.* identified *Neisseria*, *Granulicatella*, *Capnocytophaga* and *Veillonella* using 16S rRNA pyrosequencing (Guss *et al.* 2011). The *Granulicatella* genus has only been previously identified in CF airway sputum samples of a paediatric patient by Armougom and colleagues using 16S rDNA sequencing (Armougom *et al.* 2009) however an unclassified species within the *Granulicatella* genus was detected in 22% of our patient samples in relatively high abundance suggesting it is not a rare coloniser of the CF airway. This species was also only detected in the more diverse, *S. aureus* positive phylogenetic cluster (Fig. 3.7) suggesting it may be a member of a healthier airway community. *Granulicatella* has not been well researched in the context of CF, though it was considered a potential protective genera in periodontium microbiome studies (Zorina *et al.* 2014). *Ge. haemolysans*, *St. sanguinis*, *St. parasanguinis*, *St. mitis*, *St. oralis*, *St. pneumoniae*, *St. salivarius*, *V. atypica*, *R. mucilaginosa*, *R. dentocariosa* and *Pr. melaninogenica* have all been identified to the species level in CF patient airway samples in previous work using 16S rRNA sequencing (Guss *et al.* 2011; Tunney *et al.* 2008; Harris *et al.* 2007; Bittar *et al.* 2008) and here we validate these findings using the more stringent and sensitive WGSS. *Ge. haemolysans* was first identified to species level in CF patients by Bittar *et al.* (Bittar *et al.* 2008). This species has not been shown to be pathogenic in CF though has been reported as an etiologic pathogen causing lung abscess (Soyza *et al.* 2000). All *Streptococci* species detected in our top 25 most dominant species have been suggested to play an important role in patient stability and increasing CF microbiome diversity

(Filkins et al. 2012) and interestingly these species all fall within the *S. aureus* dominant and more even cluster in our study (Fig. 2.5).

*V. atypica* has been identified in CF airway samples in multiple studies (Tunney et al. 2008; Bittar et al. 2008; Harris et al. 2007), though to our knowledge there is no research on this specific species as a human pathogen. *R. mucilaginosa* has been reported in similar prevalence and abundance as *P. aeruginosa* in the CF airway microbiome and recent studies have begun to highlight its importance as an opportunistic pathogen (Lim, Schmieder, Haynes, Furlan, et al. 2013). *R. dentocariosa* has not been examined for potential pathogenicity in CF, however has been reported to cause bacteremia (Salamon & Prag 2002) and endocarditis (Boudewijns et al. 2003) in humans. Several members of our top 25 most dominant species have been much less frequently identified in paediatric CF patients. These include *Ge. sanguinis*, *St. infantis* and *V. parvula*, while the species *R. aeria* and *Po. species oral taxon 279* to our knowledge have never been detected from CF airway samples. *Ge. sanguinis* and *St. infantis* were detected in a study focused on the use of McKay agar in isolating the *Streptococcus milleri* group in CF patients (Sibley et al. 2010). *Ge. sanguinis* has not been deemed a pathogenic species in CF to date, though has been implicated in prosthetic joint infections (Leung et al. 2011) and endocarditis (FitzGerald et al. 2006). *St. infantis* is considered part of commensal microbiota in the adult upper respiratory tract and is not known to be pathogenic (Bek-Thomsen et al. 2008). *V. parvula* has been described as a strict anaerobe in CF (Brook & Fink 1983), with studies linking this species to bacteremia (Fisher & Denison 1996) and meningitis (Bhatti & Frank 2000), though there is a lack of data regarding its pathogenicity in CF. Although *R. aeria* has not been reported before in the context of the CF airway microbiome, acute *R. aeria* infection has been reported in a case of bronchitis (Michon et al. 2010) implying potential for pathogenicity in the CF airway. *Po. species oral taxon 279* encompasses KUFDS01<sup>T</sup> and *Porphyromonas* oral clones CW034 and DP023 according to the Human Oral Microbiome Database ([www.homd.org](http://www.homd.org))(Dewhirst et al. 2010). This species has been identified in human saliva but never before in the CF lung (Yamashita et al. 2015). There is currently no data on the potential pathogenicity of this species. The detection of *Po. species oral taxon 279* and *R. aeria* in the CF airway is a novel finding of our study and calls for further research on the potential protective and/ or pathogenic roles of these species in CF airway disease. The majority of these rarely detected and never before detected bacterial species were present in the diverse, mostly *S. aureus* positive phylogenetic cluster (Fig 3.7) suggesting many of these species are present in the healthier, more diverse CF airway.

Samples with high abundance of *Pr. melaninogenica* had the most uneven microbiome in our cohort (Fig. 3.7). Patients colonised with *Pr. melaninogenica* had significantly higher alpha-diversity (Fig. 3.8) suggesting this bacteria may promote diversity. This bacteria has been identified in sputum and BALF of adult patients using 16S rRNA sequencing (Tunney et al. 2008), in BALF from children with CF (Harris et al. 2007) and from sputum using T-RFLP (Rogers et al. 2004). In contrast to this finding, *Pr. melaninogenica* has been recently shown to have pathogenic potential in CF (Carlson et al. 2017). CF epithelial cell lines (IB3) infected with *Pr. melaninogenica* produced similar levels of inflammatory cytokines to cells infected with other CF pathogens. Furthermore, *Pr. melaninogenica* was found to harbour hemolysin genes such as *phxA* (Carlson et al. 2017). It has also been suggested that the pathogenicity of *Prevotella* genera in CF has to do with their ability to produce AI-2, thus facilitating the modulation of virulence factor gene expression of *P. aeruginosa*. (Field et al. 2010). In light of our findings of high alpha diversity in paediatric patients with this bacteria, it may be reasoned that *Pr. melaninogenica* can become more pathogenic in later stages of this disease as patients age and eventually have a negative effect on diversity.

Here samples dominated by *S. maltophilia* were less diverse (Fig 3.7 & Fig. 3.8). It has been shown previously that dominant colonisation with a pathogenic species is associated with reduced diversity of the CF airway microbiome, though never before with regard to *S. maltophilia* (Klepac-Ceraj et al. 2010; Cox et al. 2010). In fact, a reduction of community diversity due to *P. aeruginosa* colonisation was not detected in our work. This lack of association between *P. aeruginosa* was likely a result of the young age of our cohort, as chronic colonisation with this pathogen is known to occur more frequently in adult patients (Cox et al. 2010; Pittman et al. 2010; Klepac-Ceraj et al. 2010). Here we highlight *S. maltophilia* as an important pathogen in children that causes significantly reduced microbiome diversity from a young age. Patients with severe lung function deterioration had significantly lower microbiome diversity than patients with mild lung function deterioration (Fig 3.6). Our findings in corroboration with other groups, support the hypothesis that uneven and low diversity microbial communities dominated by pathogenic species are associated with poor lung function. We show that diverse communities are associated with milder lung function deterioration. The maintenance of diversity and overall evenness in *S. aureus* dominant samples suggests that *S. aureus* may not be as detrimental to the surrounding microbiome as other pathogenic species. Ecologically more diverse microbial communities are healthier and more resilient to transient colonisation by

pathogens (Magalhães et al. 2016). *S. aureus* colonisation in CF has also been shown to have no significant clinical impact on disease when compared to those on antistaphylococcal prophylaxis (Stutman et al. 2002). *S. aureus* prophylaxis guidelines differ with some countries endorsing prophylaxis, some not recommending it and others, including Ireland, having no specific guidelines (Lo et al. 2011; Lahiri et al. 2016; Smyth & Walters 2003). Our findings suggest that treatment of *S. aureus* dominant patients may not be the best course of action as these patients have more diverse microbiomes and better lung function. Antibiotics have been shown to have limited effect on the more dominant pathogens while decreasing the abundance of other species in the microbiome both in this study and others (Daniels et al. 2013) while cumulative antibiotic exposure has been shown to reduce diversity (Klepac-Ceraj et al. 2010). Therefore *S. aureus* prophylaxis could reduce overall microbiome diversity and make patients vulnerable to invasion by pathogenic species, as shown with *P. aeruginosa* (Stutman et al. 2002) and more likely to have impaired lung function.

The majority of the top 25 abundant species we detected in this study were anaerobic bacteria. Tunney *et al.* reported the top 14 anaerobic genera in the sputum of adult CF patients (Tunney et al. 2008), 6 of which (*Veillonella*, *Gemella*, *Prevotella*, *Rothia*, *Staphylococcus* and *Streptococcus*) were also identified in our top 25 dominant species in children. Another study revealed an abundance of obligate anaerobic bacteria in a predominantly adult CF population (Worlitzsch et al. 2009). They identified the same anaerobic bacteria as Tunney *et al.* with additional detection of *Capnocytophaga*, a genus also found to be common in our patient samples. Bittar *et al.* detected anaerobic bacterial species such as *Ge. haemolysans*, *M. catarrhalis*, *A. xylosoxidans* and other typical CF pathogens in their CF adult and paediatric cohort (Bittar et al. 2008). We have used WGSS in this study and can therefore confirm the presence of the bacterial species detected in other studies by 16S rRNA sequencing with greater confidence and identified species never before associated with the CF airway. The confirmation of 16S rRNA sequencing identified bacteria by WGSS endorses the validity of employing 16S rRNA sequencing for microbiome studies.

As *P. aeruginosa* is the most commonly isolated pathogen in CF (Pitt et al. 2003) and also causes significant disease progression (Szaff et al. 1983; Burns et al. 2001; Klepac-Ceraj et al. 2010; O'Brien et al. 2017), we compared the airway microbiomes of CF patients positive and negative for *P. aeruginosa*. Significantly different beta diversity between these groups was detected which supports previous findings that patients positive for *P.*

*aeruginosa* have distinct microbial communities and dissimilar community composition to patients not colonised with *P. aeruginosa* (Klepac-Ceraj et al. 2010; Sagel et al. 2009; Pittman et al. 2010; Burns et al. 2001). It is now generally accepted that the CF airway harbours a diverse community of bacteria (Cox et al. 2010; Guss et al. 2011) and it is likely that *P. aeruginosa* interacts with many of these bacteria in the CF airway. Employing the Spearman method and MetaPhlAn2 in order to identify potential competitive and mutual relationships within the CF airway microbiome we observed that an unclassified *Pseudomonas*, *Bordetella* and *A. xylosoxidans* had significantly positive associations with *P. aeruginosa* (Fig. 3.16). It is likely that the unclassified *Pseudomonas* species are positively correlated with *P. aeruginosa* as a result of a highly similar genetic composition of these species allowing them to occupy similar environmental niches in the CF lung. To our knowledge, no previous work in CF has identified a positive correlation with *P. aeruginosa* and unclassified species of *Pseudomonas*. Current literature on co-isolation of *P. aeruginosa* and *Bordetella* species is significantly lacking. One of the few studies focused on *Bordetella* species in CF has noted that it is likely significantly under-identified and often mistaken for other species in culture results (Spilker et al. 2008). There is also a paucity in current research regarding *A. xylosoxidans* and *P. aeruginosa* co-infection in CF. However, De Baets *et al.* observed that *A. xylosoxidans* was found to be present in 87.5% of *P. aeruginosa* positive patients (7/8) (De Baets et al. 2007) which corroborate our findings. *A. xylosoxidans* is a bacteria that primarily causes infection in the lungs (Tan et al. 2002) and is an emerging pathogen in CF (Lambiase et al. 2011). Further studies are needed to investigate the potential interaction between *P. aeruginosa* and *A. xylosoxidans*. A strong negative correlation between *P. aeruginosa* and *S. maltophilia* and *Stenotrophomonas* species was detected in our samples (Fig. 3.14 & Fig. 3.15). This finding is contradictory to work carried out by Pompilio *et al.* and Pages-Monteiro *et al.* in which *P. aeruginosa* was noted to be positively correlated with *S. maltophilia* (Pompilio et al. 2015; Pages-Monteiro et al. 2017). Our result is comparable with findings from work by De Vidipo *et al.* in which *P. aeruginosa* was noted to have negative effects on the growth rate and epithelial cell adherence of *S. maltophilia* (De Vidipó et al. 2001). Other than these studies there is little known about the interaction between *P. aeruginosa* and *S. maltophilia*.

We employed HUMAnN2 and the ChocPhlAn database to determine the differences in functional capabilities between *P. aeruginosa* positive and negative microbial communities. Metabolic pathways present in the *P. aeruginosa* positive

microbiomes were significantly different to those in the *P. aeruginosa* negative microbiomes suggesting these communities can behave differently. The specific pathways of difference between *P. aeruginosa* negative and positive communities were identified and they are discussed in 3 groups.

(1) Pathways only detected in *P. aeruginosa* positive microbiomes.

Five of these pathways were found to be involved in fatty acid biosynthesis and oxidation. Fatty acid biosynthesis genes were found to be important in *P. aeruginosa* by Oberhardt *et al.* which determined 6% of PAO1 genes were involved in lipid synthesis (Oberhardt *et al.* 2008). Short chain fatty acids (SCFA) produced by *P. aeruginosa* in particular have been thoroughly researched and identified to play an important role in modulating inflammation and can affect bacterial growth in the CF airway microbiome (Ghorbani *et al.* 2015). A study undertaken by Ghorbani *et al.* went about analysing the involvement of *P. aeruginosa* in CF patient SCFA concentrations before and after an exacerbation requiring antibiotic treatment. The mean levels of SCFAs were observed to be lower after antibiotic treatment, compared to clinically stable patients. This small decrease in SCFA levels post-antibiotic treatment may suggest that *P. aeruginosa* was contributing to the SCFA levels in CF sputa, though further research is required to confirm this (Ghorbani *et al.* 2015). Through analysing sputum neutrophil count, it was confirmed that SCFAs act on the host to aggravate inflammatory responses in the CF lung. Their work led them to conclude that SCFAs can also act as a nutrient source when oxidised by colonising bacteria, thus increasing their growth in the hypoxic cystic fibrosis lung environment (Ghorbani *et al.* 2015). *P. aeruginosa* has been found to have the ability to use fatty acids and phosphatidylcholine (PC) as a carbon source and furthermore uses type IV pili to twitch toward phospholipids and LCFAs in order to optimise its nutrient acquisition (Abd *et al.* 2008; Matz *et al.* 2008). Thus it is not surprising that both fatty acid biosynthesis and oxidation pathways were both increased in *P. aeruginosa* dominated communities, supported by a plethora of studies which show this pathogen to cause inflammation and exacerbations in CF (Penketh *et al.* 1983; Jelsbak *et al.* 2007; Pittman *et al.* 2010; Boutin *et al.* 2017).

Four ubiquinol biosynthesis pathways (ubiquinol-7, ubiquinol-8, ubiquinol-9 and ubiquinol-10 biosynthesis) were more prevalent in the *P. aeruginosa* positive microbiomes. Ubiquinol is used by enzymes incorporated in aerobic terminal oxidases as an electron donor in a pathway identified in *P. aeruginosa*. This is thought to aid its survival in the CF lung (Comolli & Donohue 2004; Alvarez-Ortega & Harwood 2007; Kawakami *et al.* 2009;

Williams et al. 2006). The aerobic terminal oxidase that uses the ubiquinol is dependent on the oxygen affinity that is required, thus facilitating growth of *P. aeruginosa* in a range of hypoxic environments. The different compound names (i.e. ubiquinol-7, ubiquinol-8, etc.) arise from the number of carbons in the side chain (Shepherd et al. 1996). In order for *P. aeruginosa* to adapt to the hypoxic environment of the CF lung, it is expected to find ubiquinol biosynthesis pathways increased in *P. aeruginosa* positive communities.

Two other pathways only detected in *P. aeruginosa* positive patient samples were involved in biosynthesis of quinolones which are known to be produced by many bacterial species. Some quinolones can have antimicrobial properties or act as quorum sensing (QS) molecules. In *P. aeruginosa*, biosynthesis of virulence factors and the generation of biofilms are under QS control. A pathway to produce PQS molecule 2-heptyl-3-hydroxy-4-quinolone was found in this study (Fig. 3.14), which is responsible for the regulation of numerous virulence factors (Deziel et al. 2004; Wade et al. 2005). The association of this pathway with PQS and control of virulence makes its increase in *P. aeruginosa* positive communities unsurprising. Other pathways only expressed in *P. aeruginosa* positive patient samples were alpha-amino acid (L-histidine and L-arginine) degradation. *P. aeruginosa* is known to break down and utilise these alpha amino acids as energy sources (Mercenier et al. 1980; Newell & Lessie 1970) and other work has confirmed *P. aeruginosa* genes are involved in amino acid catabolism (Oberhardt et al. 2008). It is possible that the ability of *P. aeruginosa* to utilise amino acids as an energy source provides an advantage to outcompete other bacterial species. Antigen synthesis such as the superpathway of GDP-mannose derived O-antigen building blocks biosynthesis and colanic acid building blocks biosynthesis (M-antigen) were present in *P. aeruginosa* positive communities. The O-antigen has been identified to vary in different isolates of *P. aeruginosa* (Raymond et al. 2002). Colanic acid is a carbohydrate-rich substance (Friedman & Kolter 2003) which has been associated with bacterial biofilm formation, and thus is not surprising that this pathway was increased in *P. aeruginosa*, as this species is known to form biofilms to during chronic infection (Costerton et al. 1999; Sutherland 2001). A polymixin resistance pathway was also only present in *P. aeruginosa* positive communities. Polymixins are a group of cationic polypeptide antibiotics which target Gram negative LPS bacterial membranes and are often considered a last resort for treatment of *P. aeruginosa* (Falagas et al. 2005). This corroborates our finding that only *P. aeruginosa* positive samples had genes for cationic polypeptide antibiotic resistance. There has been a notable rise in polymixin resistance in clinical isolates of *P. aeruginosa* over recent years (Owusu-Anim & Kwon 2012) and the

presence of this resistance pathway in all *P. aeruginosa* positive microbial communities is concerning.

(2) Pathways increased in *P. aeruginosa* positive samples.

Six pathways involved in amino acid biosynthesis and degradation and 4 pathways involved in fatty acid biosynthesis and degradation were increased in *P. aeruginosa* positive communities. Amino acid biosynthesis, such as the L-arginine biosynthesis pathways has been identified to be regulated by the *argR* gene in *P. aeruginosa* (Park et al. 1997). Though Cunin *et al.* described these arginine genes to be evident in several species of bacteria and biosynthesis of arginine was found to be a precursor of many metabolic components (Cunin et al. 1986). The most significantly increased pathway in this group was the GDP-mannose biosynthesis pathway. GDP- $\alpha$ -D-mannose is an important intermediate in carbohydrate metabolism and glycoconjugate biosynthesis (Wagner et al. 2009). Glycoproteins are known to play a major part in cell-cell signalling (Garami & Ilg 2001) thus it is unsurprising that this pathway is abundant in *P. aeruginosa* positive communities, as it is likely key for interactions between bacterial species. Some pathways in this group are involved in heme biosynthesis which supports literature regarding the importance of iron in the survival of bacterial species in the microbiome (J P Pearson et al. 1994).

(3) Pathways only in *P. aeruginosa* negative patient samples.

These 6 pathways were generally confined to the glycolysis-related utilisation of glucose and others key to bacterial cellular metabolism such as PreQ<sub>10</sub> biosynthesis, which is a deazapurine base found throughout biology (McCarty et al. 2009). We can confirm that colonisation with *P. aeruginosa* significantly impacts the structure of metabolic pathways in the CF airway microbiome. This finding was made possible through the deep sequencing data gleaned from WGSS and would not be possible with other identification techniques such as 16S rRNA sequencing or T-RFLP. With few studies employing WGSS in CF microbiome investigations, we believe this is the first time the impact of *P. aeruginosa* colonisation on the community metabolic pathway structure has been reported on. We have shown that pathways involved in fatty acid biosynthesis and oxidation, amino acid biosynthesis and degradation, glycolysis and other pathways, are altered in *P. aeruginosa* positive microbial communities. In particular we have shown the additional metabolic capabilities that *P. aeruginosa* brings to the microbiome such as ubiquinol biosynthesis, PQS molecule synthesis, antigen biosynthesis and polymixin resistance. These are significant in *P. aeruginosa* for a range of reasons. Ubiquinol biosynthesis is likely to be

key to the adaptation of *P. aeruginosa* allowing its survival in hypoxic environments, while QS has been revealed to be key to the growth and virulence of *P. aeruginosa* (Hentzer et al. 2003; Wagner et al. 2003).

A strong positive correlation was identified between *P. aeruginosa* and antibiotic exposure, which may suggest that *P. aeruginosa* is not negatively impacted by antibiotic treatment or could be simply reflective of *P. aeruginosa* positive CF patients receiving more antibiotics. Daniels *et al.* showed that the relative abundances of *P. aeruginosa* and non-*Pseudomonas* species increased during and after antibiotic treatment aimed at treating *P. aeruginosa*. They found the mean relative abundance of *P. aeruginosa* to increase after antibiotic therapy, while the mean abundances on non-pseudomonal species decreased (Daniels et al. 2013). An increased resistance to antibiotics in *P. aeruginosa* positive communities was ruled out in this study with only cationic antimicrobial resistance genes in significantly higher abundance in the *P. aeruginosa* positive samples (Fig. 3.15). Exposure to antibiotics in the previous 2 months was negatively correlated to 13 anaerobes and facultative anaerobes (Fig. 3.9), namely unclassified species of *Alloprevotella*, *Veillonella* and *Capnocytophaga* and the following specific species; *St. mitis*, *St. oralis*, *St. pneumoniae*, *Pr. oulorum*, *Eubacterium brachy*, *R. mucilaginoso*, *P. intermedia*, *Solobacterium moorei*, *H. influenzae*, *Ge. morbillorum*, *Corynebacterium durum* and *Pp. proprionicum*. In this study we aligned reads against MEGARes AMR database to identify resistance genes in the microbial communities of our patients and detected multiple resistance genes in almost all patient samples (Fig 3.10). CF patients colonised with *P. aeruginosa* are exposed to repeated high dose antibiotics from birth and the ability of bacteria to persist in the CF airways provides a suitable environment for natural selection of resistant phenotypes. The majority of samples in our cohort showed positivity for MDR genes and aminoglycoside genes. Lim *et al* examined the impact of antibiotic resistance genes in the microbiome in a small cohort of CF patients over time (Lim et al. 2014). They found more than half of the antibiotic resistance genes identified were associated with efflux-mediated resistance and genes encoding beta-lactamase were the most abundant. There was a considerable number of resistance mechanisms detected in our relatively young CF cohort suggesting acquisition of resistance in the CF airway microbiome occurs early in life. Cationic antimicrobial peptide (CAMP) resistance was the only class of antibiotic resistance significantly higher in *P. aeruginosa* positive communities (Fig. 3.13). CAMPs are important players of the innate immune system of vertebrates, invertebrates and plants, as they are active against bacteria, viruses and fungi (Hale & Hancock 2007).

They are known to bind DNA which may be the mechanism by which they can disrupt otherwise stable *P. aeruginosa* biofilm structures (Otvos 2005; Lobo et al. 2003). Research has been carried out into *P. aeruginosa* resistance to cationic antimicrobial peptides (Owusu-Anim & Kwon 2012; Barrow & Kwon 2009) with some studies focused on the PhoP-PhoQ two component regulatory system (Macfarlane et al. 2000). This system controls the PmrA-PmrB regulator (Gunn & Miller 1996; Soncini & Groisman 1996), which is known to be responsible for alterations in the structure of the outer membrane LPS. Mutations affecting this regulator can lead to polymixin B resistance (Gunn et al. 1998; Helander et al. 1994) as seen in the metabolic pathways in some of our *P. aeruginosa* positive samples. Furthermore, using gene deletion strains to examine the operon of the PhoP-PhoQ two component regulatory system, it was found to influence the resistance of both cationic antimicrobial peptides and aminoglycoside antibiotics (Macfarlane et al. 2000; Zhang et al. 2001; Sánchez & Martínez 2010). MDR peptides were the most abundant of any antibiotic resistance classes in both *P. aeruginosa* positive and *P. aeruginosa* negative sample groups (Fig. 3.7). Emergence of *P. aeruginosa* MDR has been recognised by several studies (Falagas et al. 2005; Aloush et al. 2006; Alonso et al. 1999; Obritsch et al. 2005) where it was thought likely that constant exposure to antibiotics has acted as a selective pressure resulting in an MDR phenotype. This MDR phenotype is not specific to *P. aeruginosa*, as several gram negative bacteria such as *Acinetobacter* species, *Klebsiella* and *Enterobacteria* MDR isolates have also been isolated (Falagas et al. 2005). For this reason polymixin antibiotics have been used in attempt to treat these infections. Polymixin E (Colistin) is now frequently used as a therapeutic option (Reed et al. 2001). To our knowledge, no other studies have thoroughly examined differences in antibiotic resistance genes between *P. aeruginosa* colonised and non-colonised patients.

Employing MetaMLST several pathogenic STs were identified in the microbial communities of patients in this study (Table 3.2). There were 8 different pathogenic *S. aureus* STs identified. *S. aureus* ST5 was found in 3 patients and all ST5 strains detected here were shown to have methicillin-resistance genes. ST5 is considered an MRSA genotype (Rossney et al. 2007) though there is currently no literature on this strain persisting in CF patients. MRSA infection in children with CF has been associated with significantly worse height standard deviation scores and worse chest X-ray scores at infection onset, though no significant effect was determined on overall lung function decline (Miall et al. 2001). Four different pathogenic *S. aureus* STs were found in sequential samples from patient 9, confirming the presence of multiple pathogenic strains

of *S. aureus* within one patient. Some patients were co-colonised with pathogenic STs of different species at the same time. It was interesting to see persistence of a pathogenic *S. maltophilia* (ST4 - K279a) strain in samples from patient 8. A study employing ribotyping and PFGE have previously identified the persistence of undefined strains of *S. maltophilia* in CF patients over periods of >6 months (Valdezate et al. 2001). Similarly, Di Bonaventura and colleagues identified strains of *S. maltophilia* (OBGTC9 and -10) which persisted in two CF patients for several years (Di Bonaventura et al. 2007). MetaMLST has been shown to have a higher false negative rate in detection of pathogenic STs when compared with other tools (Walsh et al. 2017) therefore we employed the PanPhlAn tool to compliment the MetaMLST findings and identify further pathogenic strains. Patients colonised with pathogenic strains tended to be colonised with the same strain over time. Three distinct pathogenic strains of *P. aeruginosa* were detected and 1 patient had the same strain in 3 consecutive samples. The same pathogenic *S. aureus* strain was isolated from multiple patients, suggesting a common source of infection or possibly patient-to-patient transfer of this strain. Highly transmissible strains of *S. aureus* have been identified in CF patients and their families, which were found to persistent in these individuals for up to 3 to 19 months after initial isolation of the strain (Goerke et al. 2000). There is potential that the FDAARGOS\_16 *S. aureus* identified in our samples is similarly transmissible, which would explain its persistence and presence in multiple patients. This explanation would corroborate findings in other work. Prunier *et al.* found that a high proportion of hypermutator strains of *S. aureus* caused adaptation of strains in colonised patients (Prunier et al. 2003), while methicillin-susceptible *S. aureus* strains were persistently identified in a CF patient airway using PFGE of of *SmaI* macrorestriction fragments (Branger et al. 1996). Other persistent pathogenic strains of *S. aureus* in patient 11 and 2 show evidence that multiple strains of *S. aureus* can colonise CF patients which has been shown by several studies (Kahl et al. 1998; Gales et al. 2006; Prunier et al. 2003). A highly variable alpha diversity was notable in patient 7 and 8 which may be explained by the *S. aureus* and *S. maltophilia* pathogenic strains detected in these patient samples. The microbiomes of these patients could therefore be subject to frequent and significant change with loss and gain of pathogenic strains from these species, suggesting these patients may be clearing infections. In this study, we support research demonstrating persistent colonisation of the CF airway by pathogenic strains of *P. aeruginosa*, *S. aureus* and *S. maltophilia*. Further research would be required to identify potential differences in clinical status of patients with higher

frequency of colonisation with pathogenic strains as this has not been carried out in CF patients to date.

We have found considerable differences in the microbiome of *P. aeruginosa* colonised and non-colonised patients. Our novel findings between these groups included differences in metabolic pathways, antimicrobial resistance gene composition and bacterial community composition between these cohorts. With the information that has been gleaned from this work, both antagonistic and synergistic relationships between species of pathogenic bacteria could be exploited for future therapeutic development. Caution should be taken when administering antibiotics and prophylactic therapy to patients. We have shown a significant impact of antibiotics on several Gram negative species with little impact on the persistent pathogen, *P. aeruginosa* and we have noted a higher alpha diversity in dominantly *S. aureus* colonised patients' samples.

## **Chapter 4**

### **Discussion**

## 4.1 Discussion

The overall objective of this study was to address the current lack of characterisation of the airway microbiome community of *P. aeruginosa* colonised and non-colonised paediatric CF patients. Species-level characterisation of these patient microbiomes were achieved through employing stringent microbial culture techniques and two forms of molecular identification (RISA and WGSS). This is the first time that WGSS has been employed to characterise the airway microbiome in a population of children and adolescents with CF. Here we have linked early airway microbiome compositions (both alpha and beta-diversity) to lung function (FEV<sub>1</sub>). WGSS made it possible to explore differences in metabolic pathways, antibiotic resistance mechanisms and strain-level composition of CF airway microbial communities colonised and not colonised with *P. aeruginosa*. We identified species with potential antagonistic and synergistic relationships with *P. aeruginosa* which have promising potential for exploitation in future research.

Microbial airway infections cause up to 95% of morbidity and mortality in CF (Zhao et al. 2012) meaning accurate diagnosis and antimicrobial therapy forms the cornerstone of CF care. Most diagnostic laboratories still rely heavily on traditional culture based technologies. This method is useful in medical laboratories as it is fast, cheap and easy to carry out. The core disadvantage of this approach is inaccuracy due to high rates of false negative results (Lleo et al. 2005). Some species are known to grow on agar better than others which can misrepresent the dominant bacteria in a patients' microbiome. Some microorganisms are so fastidious that we are unable to culture them in the laboratory. Additionally bacteria in low concentrations may be missed using culture methods, thus going unidentified until they become more dominant in the microbiome, by which time a patients' infection has become established. Despite these known failures in culture-based diagnostics, these are the results that populate CF registries. Registry analyses have advantages in facilitating large scale studies. The annual CFRI reports provide valuable information on the prevalence of pathogenic bacteria in the Irish CF population and these can be linked to lung function decline and other clinical outcomes across broad CF populations. Kerem *et al.* carried out a study using the ECFS patient registry and determined significant links between factors such as *P. aeruginosa* colonisation, pancreatic status and BMI on FEV<sub>1</sub> % predicted (Kerem et al. 2014). However there is currently a lack of standardised methodology in Irish hospitals, where some are reliant on culture techniques alone while others have mass spectrometry and other molecular methods of confirming bacterial identification. Thus, registry data is at the mercy of the quality and

reliability of data reported by different hospitals. In this study we reported a significantly higher prevalence of common CF bacteria than the registry of paediatric CF patients nationwide (CFRI 2016). Although it is possible that our centre has higher levels of these organisms, we hypothesize that prevalence of *S. aureus*, *H. influenzae*, *A. baumannii* and *S. maltophilia* are currently underestimated in the Irish CF population. *P. aeruginosa* was an exception to this trend, perhaps as a result of microbiology laboratories being more aware and having protocols in place to rule out infection with this pathogen. Although many diagnostic labs in Ireland are improving the technology used to identify microorganisms more efforts need to be made to move away from traditional culture-based diagnostic techniques and toward more accurate molecular identification methods. These progressions carry with them financial burdens and upskilling and training of staff.

The move towards employing molecular based techniques in research has led to the discovery of diverse communities of microorganisms in the CF airway (Guss et al. 2011; Tunney et al. 2008; Bittar et al. 2008; Armougom et al. 2009), mostly at genus level confidence due to the use of 16S rRNA sequencing for almost all studies to date. Overall we determined that RISA profiling of CF patient sputum samples had advantages in terms of affordability and fast results, though was limited by unreliable species identification and often ambiguously represented diversity. The total numbers of species detected using our stringent culture techniques compared with the considerably higher numbers of species we detected using WGSS highlighted the shortcomings of microbial culture techniques for species identification. Evidence of patient-specific clustering was mirrored on both our RISA and WGSS generated dendrograms. An example of this could be seen in samples from patient 8, which mostly clustered together in our RISA dendrogram and similarly clustered in WGSS MDS plots. This was also the case for patient 11 (Fig. 2.5 - group B) and patient 7 (Fig. 2.5 - group C) and furthermore patient 14, which did not show obvious clustering in either our RISA dendrogram or our WGSS plots. For this reason RISA gave a reasonably good estimate of beta-diversity in our samples.

We used WGSS which gave us high confidence in the species level taxonomic assignment. A high abundance of other species were detected through WGSS which were not detected through culture methods. We identified species such as *Capnocytophaga*, *Granulicatella* and *Porphyromonas*, among other dominant Gram negative bacteria which had not been the subject of research in CF to date. The significance of these anaerobic bacteria in the microbiome is not yet certain as there is a paucity of research confirming pathogenicity of several of these species in CF. We have identified 2 bacteria never before

associated with the CF airway, namely *R. aeria* and *Porphyromonas species oral taxon 279*. Some of our identified species have been reported as commensal human microbiota including *St. infantis*, *St. mitis*, *St. oralis*, *St. pneumoniae*, *St. parasanguinis*, *St. sanguinis*, *St. salivarius* (Filkins et al. 2012) and *Granulicatella* (Zorina et al. 2014), while others have no record of human pathogenicity such as *V. atypica* and *Porphyromonas*. Additionally, several species that have been identified in CF before, though have never been noted to be pathogenic in this disease were detected, including *R. aeria*, *R. dentocariosa*, *Ge. sanguinis* and *Ge. haemolysans* among others. The lack of association of these anaerobes with pathogenicity or disease progression suggest that some of the bacteria we have identified may represent normal or ‘good’ bacteria in the CF lung microbiome. If this is the case, these bacteria have been shown here to successfully colonise and persist in the hypoxic CF lung environment and thus could hold potential for exploitation in development of a probiotic therapy. Promising research has already begun to be carried out in this field with Weiss and colleagues reporting a reduction of pulmonary exacerbations in CF patients when on a course of probiotics (Weiss et al. 2010). This is likely a result of the immune system becoming educated by the good bacteria in the gut. The gut-lung axis has been identified as an important aspect for consideration in respiratory disease (Marsland et al. 2015). A study on paediatric CF patients’ respiratory and intestinal microbiota revealed that colonisation patterns in the gut of a patient were determinants for the development of respiratory tract microbiota (Madan et al. 2012). Establishment of a strong community diversity early in the life of a CF patient through means of probiotics could help prevent or delay colonisation with more detrimental bacteria such as *P. aeruginosa*.

In view of this comparison of culture methods with RISA and WGSS molecular methods of microbial identification, the optimal approach to achieve precision medicine in paediatric CF patients is questioned. The data collected in this study calls for a combined approach, using both molecular and culture means of identification as a baseline for microbial identification. Ideally molecular methods are routinely used to confirm identification of microorganisms grown by culture. Patient treatment should begin to focus on elucidating the full picture of the CF lung microbiome in order to make educated choices in a clinical setting. If this were implemented in a paediatric cohort of CF patients, early attempts could be made to maintain higher levels of microbiome complexity as these patients age.

A higher microbiome complexity was determined in *S. aureus* dominant samples. We hypothesize that *S. aureus* may support the retention of bacterial diversity in the CF

airway community. Therefore the use of antistaphylococcal prophylaxis in paediatric CF patients may not be beneficial and may make patients vulnerable to colonisation with more detrimental pathogens such as *P. aeruginosa* (Stutman et al. 2002). Large scale longitudinal studies are required to confirm our findings and prove our hypothesis. WGSS allowed identification of specific species associated with a reduction in community diversity. For the first time *S. maltophilia* colonisation was associated with low diversity of other species. Despite *S. maltophilia* being a well known CF pathogen, this is the first report of a negative impact of *S. maltophilia* on diversity in the microbiome of paediatric CF patients to our knowledge. We suggest that further research on this species and its interaction with other members of the CF microbial community would be pertinent to patient care.

It is now generally accepted that the CF airway is host to a diverse community of microorganisms and these co-colonising species are likely to interact in the airways with potentially positive and negative influences on inflammation and disease progression. It has been suggested that the microbial composition of the airway microbiome is a “*product of dynamic conditions in which infection is initiated when species with pathogenic potential find suitable partners and conditions in the community to increase in abundance*” (Jenkinson & Lamont 2005). For instance some anaerobic species can degrade mucins in CF mucus (Flynn et al. 2016). With the hypoxic nature of the CF airway this provides ideal environmental conditions for anaerobes to grow and become abundant in the CF airway. Hypoxic environmental conditions has been shown to promote the growth of *P. aeruginosa* (Yoon et al. 2002) and other anaerobic bacterial species (C. D. Sibley et al. 2008). Interplay, competition and antagonism between species in the microbiome are likely to drive shifts in species dominance and thus potentially infection. Mechanisms by which *P. aeruginosa* outcompetes and interacts with other bacterial species have been discussed above. *P. aeruginosa* can produce SCFAs such as butyrate (Moss & Dees 1976). Ghorbani *et al.* recently reported that SCFAs can control the growth of *P. aeruginosa* in a concentration and pH-dependent manner. This group also observed that SCFAs can induce inflammatory responses in CFBEs and on colonising bacteria to increase their growth under certain conditions such as those present in the hypoxic cystic fibrosis lung (Ghorbani et al. 2015). *P. aeruginosa* may compete with other bacterial species in the CF microbiome for iron by producing pyocyanin (Pierson & Pierson 2010). As a phenazine-producing bacteria in the form of pyocyanin, it is associated with morbidity and mortality in CF patients (Courtney et al. 2007; Murray et al. 2007). This has been highlighted in other studies which have demonstrated that *P. aeruginosa* produced phenazines are negatively correlated with

microbial complexity and have a negative effect on overall CF disease state. *S. aureus* can metabolise anaerobically when co-colonised with *P. aeruginosa* and become less viable in the CF lung environment (Filkins et al. 2015). *P. aeruginosa* has been identified to interact with other species in the CF microbiome, including bacterial, yeast and fungal species by other groups (Periasamy et al. 2015; Reece et al. 2017; Sass et al. 2018; Holcombe et al. 2010). We detected a significantly negative association between *P. aeruginosa* and *S. maltophilia*. De Vidipó *et al.* have shown *P. aeruginosa* negatively affects the growth and cellular adherence of *S. maltophilia* to lung epithelial cells (De Vidipó et al. 2001). These species may be competitive (occupy the same nutrient niches) or antagonistic and further studies are required in this area. A significant positive association was identified between *P. aeruginosa* and *A. xylooxidans* and an unclassified species of *Bordetella*. No studies have been published investigating the interactions between *A. xylooxidans* or *Bordetella* with *P. aeruginosa* to date. Future research into the interactions of *P. aeruginosa* and other CF airway microbiome bacterial species may provide great insight into the behaviour of *P. aeruginosa* in these mixed communities. Moreover, this could hold potential for the development of novel therapeutic targets and approaches.

Some patients in our study had a unique or stable microbiome over time while others exhibited considerable changes in their microbiome over time. The extent of change was dependent on the patient, supporting potential benefit of a more personalised approach to an individual's treatment. The individuality of a patients' samples was highlighted through clustering of patient-specific samples in the phylogenetic trees presented (Fig. 2.5 and Fig. 3.7), indicating a somewhat similar microbiome composition in samples from the same patient. The interpatient microbiome diversity presented in this work reflects the need for antimicrobial therapy to be specific to the microbial community of the patient. This can only become a reality for clinicians upon introduction of efficient and accurate microbiome sequencing techniques which should become standard with the declining cost of sequencing and furthermore when we begin to understand how the microbiome is linked to clear clinical outcomes. There are few studies looking into the causality of the microbiome in lung disease progression and this is an area which needs to expand in the future. One study beginning to link microbiota and clinical disease status was recently carried out on paediatric CF patients (Frayman et al. 2017). This study measured clinical markers of inflammation such as IL-8 and NE in addition to microbiome composition. Dominant species in patient airway microbiota was associated with higher airway inflammation in their work, though no significant link to pulmonary function was determined in these young

patients. With information on both microbiome composition and clinical status of a patient, a more personalised therapeutic approach could be tailored to individual needs and translate into a more sophisticated treatment strategy than is currently in place.

We determined an association of low microbiome diversity with worse FEV<sub>1</sub> scores, in a young CF patient population. Others have described this association mainly in older patient populations (Stressmann et al. 2012; Bacci et al. 2017; Paganin et al. 2015). Stressmann *et al.* measured community change in adult CF patients, which correlated to lung function measures (FEV<sub>1</sub> and FEV<sub>6</sub>) (Stressmann et al. 2012). While Paganin and colleagues identified similar association in their CF cohort, though could additionally implicate species of *S. pneumoniae* and *R. mucilaginosa*, with a severe decline in FEV<sub>1</sub> in adults with CF (Paganin et al. 2015). Bacci *et al* recently supported this finding using 16S rRNA sequencing techniques (Bacci et al. 2017). This substantiates the importance of linking microbiome data to clinical factors, as the pulmonary function of our paediatric patients were shown to be degraded with lower microbiome diversity measures. Clinically this translates to a significant impact of the microbiome on lung deterioration of these patients from an early age.

Antibiotics have been shown to have contradictory impacts on the CF airway microbiome as some studies have identified antibiotics as the primary cause of reduced microbiome complexity (Zhao et al. 2012), while Fodor *et al.* detected only a minimal reduction in species diversity and reported an overall unchanged microbiome after antibiotic exposure (Fodor et al. 2012). While antibiotics were not linked to overall microbiome diversity in this study, we identified a significant positive association between *P. aeruginosa* and the number of antibiotics given in the two months prior to sample collection. Daniels *et al.* identified a similar trend where an increase in administration of antibiotics to combat *P. aeruginosa* was inefficient at reducing its abundance and frequently resulted in a decrease in other Gram negative bacteria, excluding *P. aeruginosa* (Daniels et al. 2013). We also saw a decrease in non-pseudomonas Gram negatives which were largely anaerobes. It is possible that our data is supportive of this finding, however a limitation of our study was an insufficiency of clinical data to determine if prescribed antibiotics were intended to treat *P. aeruginosa* infection. The conclusions drawn from the data presented in this work require verification through a much larger scale study. Small sample size is a limiting factor in this research.

*P. aeruginosa* colonisation was found to significantly impact on microbial community composition in this work, supporting findings of several other studies (Klepac-

Ceraj et al. 2010b; Zemanick et al. 2011; Burns et al. 2001). A number of *in vitro* studies discussed above highlight how *P. aeruginosa* may interact with other species in the CF airway microbiome. We compared the microbiomes of *P. aeruginosa* colonised and non-colonised CF patients to investigate the impact of *P. aeruginosa* on the CF microbiome *in vivo*. CAMPs resistance mechanisms were more abundant in *P. aeruginosa* positive microbiomes and a polymixin resistance metabolic pathway was found exclusively in *P. aeruginosa* positive microbiomes. Polymixin resistance was noted to influence the efficacy of CAMPs and aminoglycosides. This has become increasingly identified in *P. aeruginosa* isolates in recent years (Owusu-Anim & Kwon 2012) and could explain the increased relative abundance of *P. aeruginosa* in patients receiving more antibiotics. Our work has been one of the first to identify a significant difference in overall metabolic pathway structures between cohorts of *P. aeruginosa* colonised and non-colonised paediatric patients. Several metabolic pathways were found to be significantly increased in *P. aeruginosa* positive microbiomes using MetaCyc and many of these pathways play a role in survival in harsh environment like the CF airway. These included ubiquinol biosynthesis pathways for survival in low oxygen concentration environments (Alvarez-Ortega & Harwood 2007), PQS molecule biosynthesis for quorum sensing (Wade et al. 2005) and virulence (J. P. Pearson et al. 1994) and polymixin resistance (Falagas et al. 2005).

According to Leed's criteria, chronic bacterial infection is defined as more than 50% of the prior 12 months reported as culture positive for a pathogenic species, often *P. aeruginosa* (Lee et al. 2003). Chronic colonisation with *P. aeruginosa* is a major milestone in CF disease progression. Colonisation with *P. aeruginosa* has been shown to significantly reduce lung function (Pamukcu et al. 1995) and reduces life expectancy by approximately 10 years (Kosorok et al. 2001). We have used WGSS and METAMLST/ PanPhlAn to identify pathogenic strains of *P. aeruginosa*, *S. aureus* and *S. maltophilia* persisting in CF patients. The pathogenic methicillin resistant ST5 was found in 3 patients. The MRSA strain ST5 has been identified in other studies on MRSA (Ko et al. 2005; Francois et al. 2008; Enright 2003) few of which were reported in CF, though this strain of MRSA was described as the most frequent hospital clone in the USA (Champion et al. 2014). PanPhlAn analyses of sequencing data revealed a majority of samples to harbour a pathogenic strain of *S. aureus*, which is not surprising given the high prevalence of *S. aureus* in our culture data and WGSS identification of dominant species. Three unique strains of *S. aureus* were detected in multiple samples from certain patients, one of which was persistent in several patients of our cohort. This chronic infection with one particular

pathogenic strain of *S. aureus* in several patients suggested it was possibly a highly transmissible and persistent strain of *S. aureus* similar to that described by Goerke *et al.* in CF patients (Goerke *et al.* 2000). An alternative explanation for this finding was a common source of acquisition of this strain of *S. aureus*, as described by Molina *et al.* in CF patients colonised with an identical clone of hospital-acquired MRSA (Molina *et al.* 2008). Three *P. aeruginosa* strains were detected which appeared to be patient-specific. This was suggestive of environmental acquisition and unlikely that any of these were highly transmissible strains. This is a contrasting finding to more recent reports of highly transmissible strains of *P. aeruginosa* which have occasionally been associated with increased antibiotic resistance (Fothergill *et al.* 2012). We identified a persistent *S. maltophilia* strain in multiple samples from patient 8, with two other less persistent strains evident in patient 7 and 12 which is supportive of other work identifying potential for strains of *S. maltophilia* to persist (Valdezate *et al.* 2001; Di Bonaventura *et al.* 2007). Patient 7 and 8 samples had pathogenic strains of both *S. aureus* and *S. maltophilia* which was supportive of their presence in our culture results. Our finding of a high alpha diversity in these patients' samples were also consistent with these results. Patient 11, 22 and 3 also concurred with our culture data, where each of these were categorised as *P. aeruginosa* positive patients and were found here to harbour pathogenic *P. aeruginosa* strains. It was possible to conclude that specific strains of each of the pathogens *P. aeruginosa*, *S. aureus* and *S. maltophilia* were capable of persisting in the microbiome of a patient over time. Future research should be carried out into potential exploitation of mechanisms allowing the persistence of these strains with a view to enhancement of 'good bacteria' which could outcompete or delay colonisation with pathogens in CF patients. The benefit of this type (WGSS) of metagenomic sequencing of CF sputum samples was highlighted in this study as we gained a more complete picture of the metabolic capabilities, resistome and pathogenic sequence types in the CF airway microbiome.

## **4.2 Future work**

This study is subject to certain caveats, primarily an uneven distribution of *P. aeruginosa* positive to *P. aeruginosa* negative group sample sizes. If this study were conducted without time constraints, our sample collection period would be extended over several years, in order to gain a true longitudinal insight to the evolution of the paediatric CF microbiome. Although our population size was a restriction, this is the largest study carried out to date

incorporating WGSS to characterise the CF airway microbiome. Statistically significant results were achieved when measuring numerous outcomes, however caution should be taken with interpretation of these results when small patient numbers were used. Although we report significant novel findings we believe further analyses on larger patient cohorts is necessary to corroborate these results. Further, in the absence of funding constraints WGSS could be performed on all samples in our dataset, though current WGSS sequencing costs made this an unreasonable prospect at this time. With more time, it would have been ideal to review historical records of each patient in order to determine if we had caught an instance of first colonisation with *P. aeruginosa*. Other limitations of the work presented here included our incomplete data regarding clinical stability of the patient. It must be noted that a patient during exacerbation or pre-exacerbation may have different microbiome community composition than they would at baseline (Carmody et al. 2018). Furthermore, an ideal strategy would be to age-stratify patients to compare the first decade of life with adolescence and early adulthood, in order to follow the evolution and impact of *P. aeruginosa* over time. Another limitation in this study was our inability to directly link microbiome structural changes to clinical status of a patient as we were limited to annual FEV<sub>1</sub> measurements, as opposed to sample-specific FEV<sub>1</sub> measurements. Additional measurements of disease status such as CT scores, serum CRP, biomarkers for lung tissue breakdown and lung clearance index would have been ideal to categorise patients into disease cohorts.

In light of the significant differences in prevalence between our study and national averages, an interesting route of further research could be concerned with geographical differences in CF airway bacterial prevalence. Furthermore intra-hospital differences within one geographical region could be performed to determine whether this finding is due to insufficient diagnostic techniques, or a significant contribution of geographical location on the prevalence of particular species.

Our study has repeatedly proven the potential benefit of sequencing data to guide clinicians in an educated treatment regime. For this reason, a clinical-based study using metagenomic sequencing analyses of patient sputum samples to guide treatment strategies would be a very interesting avenue of research. In particular, we have revealed a species-level identification of several Gram-negative bacteria which dominantly colonise paediatric CF airways. It would be interesting to examine the prevalence of these emerging species in a larger paediatric cohort, in order to determine the significance of this finding. Given this data, further studies could be initiated to examine synergistic or antagonistic relationships

among these bacteria and between these bacteria with more common CF pathogens such *S. aureus* or *S. maltophilia*.

With our evidence of the negative effect of *S. maltophilia* on community diversity, further work needs to be done to establish the virulence of these bacteria. Furthermore, other species of significant interest to future research have been identified through correlation analyses with *P. aeruginosa*. Significant positive associations were identified with both *A. xylosoxidans* and *Bordetella* species with *P. aeruginosa*, making these species particularly attractive areas of future research. Similarly *S. maltophilia* was detected to have a significantly negative correlation with *P. aeruginosa*, implying an antagonistic relationship which also needs to be fully elucidated.

In light of the numerous and complex metabolic pathways found to be differentially distributed between our *P. aeruginosa* positive and negative patient sample groups, a thorough investigation could be conducted to understand the molecular basis of these pathways and examine the possibility of manipulating the metabolic pathways of *P. aeruginosa* in order to reduce its pathogenicity. Overall, this study has made significant contributions to the field of research focused on the effects of *P. aeruginosa* on the CF airway microbiome. It is hoped that from this work, there will be initiation of further research based on some of the key results described here. We have highlighted that colonisation with *P. aeruginosa* in early life has significant and detrimental effects on the CF airway microbiome. Previously unrecognised relationships between *P. aeruginosa* and other bacterial species have been identified in this work. Particular species of interest which impacted microbiome diversity were described in this work such as *S. maltophilia* associated with reduced diversity and *S. aureus* which was associated with stable diversity. Overall, a significant insight into complexities of the CF airway microbiome of paediatric patients has been achieved in this work.

## Professional Presentations

Trinity Translational Medicine Institute Annual Scientific Conference 2018

Trinity College GSU Research Showcase, Trinity Biomedical Science Institute 2018

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