Non-Tuberculosis Mycobacteria Infection and The Wider Lung Microbiota in Cystic Fibrosis

Michelle H. Hardman

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Michelle H. Hardman

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

Department of Life Science

Faculty of Science and Engineering

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Declaration of originality

I declare that this thesis and the material presented therein are entirely my own original work, except where appropriately referenced. All experiments were conducted by myself except where explicitly stated.

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COVID-19 Statement

In March 2020, the United Kingdom implemented a nationwide lockdown in response to the COVID-19 pandemic. As a result, university laboratories were temporarily closed, and all staff and students were instructed to work remotely from home. This presented significant challenges to my research project, as it coincided with crucial stages of sample collection, ethics approval, and strain acquisition.

Shortly before the lockdown was implemented, I had just received my initial cohort of samples from University Hospital Southampton. I was also in the process of securing ethics approval for samples from the United States and acquiring essential strains from the Health and Safety Executive in Buxton, UK. Unfortunately, due to the pandemic, my samples had to be stored, and progress on ethics approval, travel to Vermont, and strain acquisition came to a halt. These unforeseen circumstances posed potential risks to the future of my project.

During the lockdown period, I focused my efforts on developing an alternative method of sample collection that would allow my project to move forward. However, the implementation of this alternative method had to be postponed until restrictions began to ease. When the laboratories finally reopened in July 2020, limited space was available due to social distancing requirements, and a booking system was implemented to ensure fair access to lab facilities.

Despite the challenges posed by the pandemic, I made every effort to mitigate its impact on my project. Restarting sample acquisition proved to be a slow process, and ethics approval had to be reassessed. Additionally, I had to allocate time away from the lab to personally collect samples from the United States and the HSE Buxton. Regrettably, these unforeseen circumstances, combined with the limited resources and time available, resulted in the inability to complete certain components of the original research plan. Consequently, it became necessary to prioritize and allocate less time to each section of the study than originally intended.

With the aid of an unpaid extension, I managed to successfully collect, analyse, and interpret my data, ultimately completing my thesis.

Abstract

Non-tuberculosis mycobacteria (NTM) infections in people with cystic fibrosis (PWCF) pose significant challenges and can have detrimental effects on patient prognosis. This study aims to investigate the impact of NTM positivity on the background microbiome in PWCF, focusing on the culture-positive cohorts for *Mycobacterium avium* complex and *Mycobacterium abscessus* complex. Additionally, the relationship between genetic modulator status and alterations in microbial composition was examined. The study employed optimised detection methods using the Illumina MiSeq platform for NTM complexes in low-diversity samples. Furthermore, the study investigated the regulation of gene expression during CF exacerbation states and explored the effect of NTM positivity on patient factors, such as the percent predicted forced expiratory volume in one second (%FEV_1).

The study's findings demonstrate the optimization and improvement of NTM complex detection in low diversity samples, enabling a comprehensive evaluation of the effects of NTM positivity on the broader lung microbiota. The results indicate that the presence of NTM complexes does indeed alter the diversity and the composition of the lung microbiota in CF patients, including those taking genetic modulators. Moreover, patient factors, including NTM culture status, age, and exacerbation phases, were found to influence %FEV_1. Additionally, the study observed correlations between the regulation of certain virulence factors and the patient factors: lung function, C-reactive protein levels and neutrophil counts.

These findings contribute to the understanding of NTM infections in CF patients and highlight the need for further research in this area. The knowledge gained from this study has implications for improving diagnostics, treatment strategies, and patient management, ultimately aiming to enhance outcomes and prolong the lives of CF patients.

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Thank you to my family for your support, Mum, Frans, and Rick thanks for being there and supporting me in your own ways. Martyn, Julie, and the rest of the Pilling family you guys are amazing thank you for always making me feel welcome and making me a brew. Esther, Hannah, and Nan P. Thank you for the chats, food, and general silliness.

To Jonny and Stephen, I should hopefully get my sense of humour back now!! I'm looking forward to our next adventure.

Finally, to my dad Ian Hardman, without you I wouldn't be the person I am today. You taught me to be tenacious and compassionate. You are truly missed.

You are all amazing and thank you for everything!

Presentations

Part of Chapter 7 was presented as a poster entitled “The regulation of non-tuberculosis mycobacteria virulence genes in cystic fibrosis patients undergoing exacerbation treatment” at the Colorado Mycobacteria Conference: Focus on Non- tuberculosis Mycobacteria.

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Abbreviation List

αMβ2	alpha-M beta-2
%FEV_1	percent forced expiratory volume in the first second.
%PF	Per-cent passing filter
µl	Microlitre
ABPA	Allergic bronchopulmonary aspergillosis
ACTs	Airway clearance techniques
AFB	Acid fast bacilli
ANOSIM	Analysis of similarities
ASV	Amplicon Sequence Variants
(ATS/IDSA)	The American Thoracic Society and The Infectious Diseases Society of America
BAL	Bronchial lavage
BCC	*Burkholderia cepacia* complex
BLAST	Basic Local Alignment Search tool
bp	Base pairs
cDNA	Complementary DNA
cDNA	Copy DNA
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CNS	Central Nervous System
COPD	Chronic obstructive pulmonary disease
CPA	Chronic pulmonary aspergillosis
CR3	Complement receptor 3
CV	Coefficient of Variation
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleoside triphosphate
ECFS	European Cystic Fibrosis Society
eDNA	Environmental DNA
EDTA	Ethylenediaminetetraacetic Acid
EMA	European Medicines Agency

ESBLs	Extended-spectrum β-lactamases
Gbp	Giga base pairs
GPL	glycopeptidolipids
HGT	Horizontal Gene Transfer
HP	Hypersensitivity pneumonitis
HPLC	High Performance Liquid Chromatography
HRCT	High-resolution computed tomography
ICNP	International Code of Nomenclature of Prokaryotes (ICNP)
IL	Interleukin
IL-8	Interleukin-8
IL-17	Interleukin-17
IRB	Institutional Review Board
ITS	Internal Transcribed Spacer
IV	Intravenous
I variant	Intermediate variant
L	Litre
LRT	Lower respiratory tract
NMDS	Non-Metric Multidimensional Scaling
MABS	*Mycobacterium abscessus*
MABSC	*Mycobacterium abscessus* complex
MAC	*Mycobacterium avium* complex
Mac-1	Macrophage-1 antigen
MAH	*Mycobacterium avium* subspecies *hominissuis*
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MB	*Mycobacterium bovis*
Mbp	Mega Base pairs
MDS	Metric Multidimensional Scaling
MFG	*Mycobacterium fortuitum group*
MgCl2+	Magnesium chloride
MKC	*Mycobacterium kansasii Complex*
ml	Millilitre
ML	*Mycobacterium leprae*

MMU	Manchester Metropolitan University
mRNA	Messenger RNA
MRSA	Methicillin resistant *Staphylococcus aureus*
MTB	*Mycobacterium tuberculosis*
MWF	Metal Working Fluids
NBD	nucleotide binding domains
NGS	Next generation sequencing
NTM	Non-tuberculosis *Mycobacterium*
NTM-PD	Non-tuberculosis *Mycobacterium* pulmonary disease
OTU	unique operational taxonomic units
PAH	polycyclic aromatic hydrocarbons
PAP	pulmonary alveolar proteinosis
PCR	Polymerase chain reaction
PDP	Peritoneal dialysis peritonitis
PEG	Polyethylene glycol
PERMANOVA	Permutational multivariate analysis of variance
PERT	pancreatic enzyme replacement therapy
PFT	Pulmonary Function Test
PI	Pancreatic insufficiency
PMA	propidium monoazide
PRA	PCR Restriction Analysis
PWCF	People with cystic fibrosis
qPCR	Quantitative polymerase chain reaction
R	Regulatory domain
REC	Research Ethics Committee
RGM	Rapid growing mycobacteria
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
R variant	Rough variant
SBS	Sequencing by synthesis

SD	Standard deviation
SGM	Slow growing mycobacteria
SIMPER	Similarity Percentage analysis
S variant	Smooth variant
Ta	Annealing temperature
TAE buffer	tris base/ acetic acid/ Ethylenediaminetetraacetic Acid (EDTA)
TB	Tuberculosis
T_h	T helper cells
Tm	primer melting temperature
TNF-α	Tumour necrosis factor alpha
T-RFLP	Terminal restriction fragment length polymorphism
UHS	University Hospital Southampton, NHS trust
URT	Upper respiratory tract
UVM	University of Vermont
WGS	Whole genome sequencing
ΔF508	Delta F508 mutation

Chapter 1: Introduction

1 Introduction

Non-tuberculosis mycobacteria (NTM) are ubiquitous environmental organisms that can cause chronic pulmonary infection in cystic fibrosis. People with CF (PWCF) infected with NTMs are more likely to develop severe lung disease and experience complications than those in the general population (Floto et al., 2016; The Cystic Fibrosis Trust, 2017; National Jewish Health, 2023).

This introduction aims to give a comprehensive overview of the current knowledge about the impact of NTM pulmonary disease in PWCF. It also highlights any areas where further research is needed.

1.1 What is Cystic Fibrosis?

Cystic fibrosis (CF) is a multi-systemic genetic disorder affecting more than 70,000 people worldwide (The Cystic Fibrosis Trust, 2019). This autosomal recessive disease causes a mutation that affects the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Figure 1.1) (Riordan et al., 1989). The CFTR is a cyclic AMP (cAMP)-activated chloride (Anderson et al., 1991; Crick et al., 2010) and bicarbonate channel (Quinton, 2008) and is responsible for the interchange of cellular salt and water (Quinton, 1990). Mutations result in an accumulation of thick, sticky, mucus in several major organ systems, which leads to a variety of symptoms affecting the whole body. Due to the nature of the mucus created it can cause blockages in the pancreas, which manifests as constipation, bowel obstruction (meconium ileus in new-borns) and jaundice (Cystic Fibrosis Foundation, 2017). These symptoms result in malnutrition and eventual organ destruction (Wilschanski and Novak, 2013). The mucus also has major effects on the respiratory system, which progressively reduces lung function. Patients diagnosed with CF show several pulmonary symptoms: recurrent chest infections, coughing, trouble breathing and wheezing. CF patients are also likely to develop related conditions such as bronchiectasis, diabetes, osteoporosis, and infertility (The Cystic Fibrosis Trust, 2019).

1.1.1 CFTR Mutation and Protein

The CFTR gene is found on chromosome 7q31.2 (Rowntree and Harris, 2003), consisting of 27 exons and encoding a mature transcript of 6.129bp that is translated into a 1480 amino acid protein of around 168kDa (Ellsworth et al., 2000), which functions as a chloride channel (Riordan et al., 1989). According to the CF trust over 2000 CF gene

mutations have been identified (The Cystic Fibrosis Trust, 2019). The mutations have been classified into five groups, each group affecting the CFTR channel or protein in different ways (Figure 1.1). These mutations can be located anywhere throughout the gene's entire coding region and promoter region. There are sections of the gene where mutation is more common: nucleotide binding domains (NBD) and regulatory domains (R). These areas are functionally and structurally important domains within the protein (Rowntree and Harris, 2003). This study focuses on PWCF who are heterozygous for the ΔF508 mutation.

The most common CFTR gene mutation is F508del which accounts for 70% of chromosomes in CF patients (Kerem et al., 1989; Rowntree and Harris, 2003). Caused by a deletion of the F508 residue, the mutation creates a temperature-sensitive misfold in the protein, stopping it from reaching the plasma membrane, resulting in protein destruction (Morito et al., 2008; Okiyoneda et al., 2010; Stefano et al., 2014) and diminished channel gating (Dalemans et al., 1991). As a result of this diminished gating, CF is often identified initially by a sweat chloride test and then via molecular screening (Rosenstein et al., 1998).

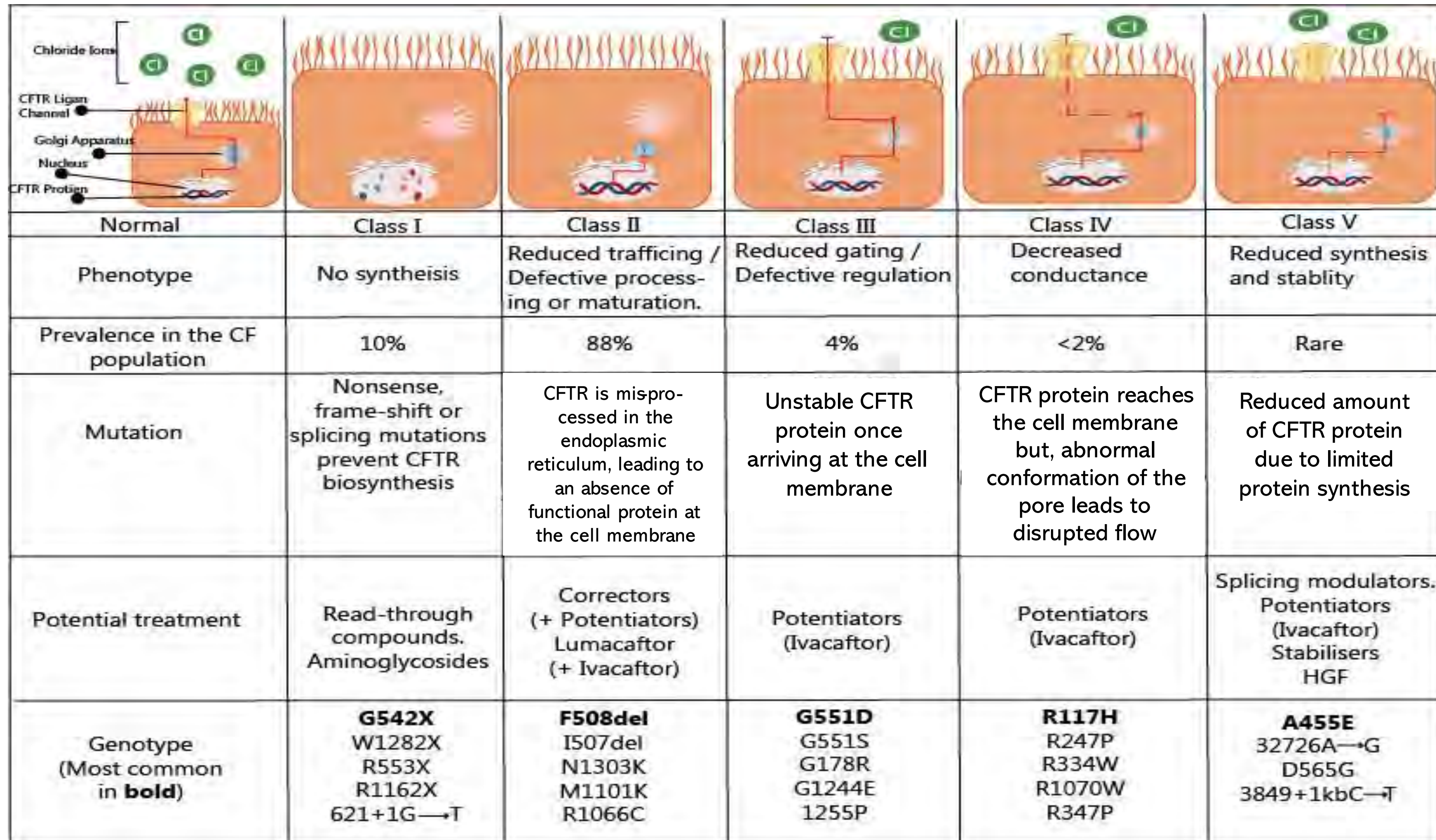
1.1.2 Cystic Fibrosis and Associated Airway Infection.

CF-associated airway disease due to chronic infection is the leading cause of morbidity and mortality in CF patients (The Cystic Fibrosis Trust, 2019). The bacteria in the surrounding environment is constantly inhaled into the lungs, and in healthy people, is not a threat. For people with CF, where the CFTR mutations cause dehydration of the peri-ciliary layer in the lungs preventing the patient from clearing the mucus efficiently (Matsui et al., 1998), a build-up of dehydrated mucus acts as a reservoir for particles containing; microorganisms, dust, and pollen (Foweraker, 2009). This accumulation can result in progressive reduction in lung function and eventual respiratory collapse (Flume et al., 2009). This decline can present as CF-associated bronchiectasis, where the bronchial lumen is permanently and irregularly widened, increasing the chance of bacterial colonisation and further damage (Chalmers et al., 2015; Schäfer et al., 2018). The constant colonisation of the airway by bacteria is due to impaired mucociliary clearance and the failure of neutrophil opsonophagocytic killing (King et al., 2006).

Patients showing progressive decline also have periods of "pulmonary exacerbations"; periods of worsening symptoms identify these phases. The Indications and severity of

exacerbations vary from patient to patient (Flume et al., 2009). These episodes could be caused by a range of influences including, an increased bacterial load, a new bacterial strain or species has been acquired, migration of the bacterial population to a new site within the lung, microbe/microbe interaction, amplified expression of bacterial virulence factors, a viral infection, or environmental factors; yet no single cause has been identified (Rogers et al., 2011). Patients experiencing frequent exacerbation events (>2 yearly) are at increased risk of death or need a lung transplant; increased monitoring should be implemented (De Boer et al., 2011). Response to treatment can, however, be poor and infection can reduce patient eligibility for lung transplantation, which is often life-extending (NHS, 2018).

CFTR Mutations and Modulators



Normal	Class I	Class II	Class III	Class IV	Class V
Phenotype	No syntheisis	Reduced trafficing / Defective processing or maturation.	Reduced gating / Defective regulation	Decreased conductance	Reduced synthesis and stablity
Prevalence in the CF population	10%	88%	4%	<2%	Rare
Mutation	Nonsense, frame-shift or splicing mutations prevent CFTR biosynthesis	CFTR is mis-processed in the endoplasmic reticulum, leading to an absence of functional protein at the cell membrane	Unstable CFTR protein once arriving at the cell membrane	CFTR protein reaches the cell membrane but, abnormal conformation of the pore leads to disrupted flow	Reduced amount of CFTR protein due to limited protein synthesis
Potential treatment	Read-through compounds. Aminoglycosides	Correctors (+ Potentiators) Lumacaftor (+ Ivacaftor)	Potentiators (Ivacaftor)	Potentiators (Ivacaftor)	Splicing modulators. Potentiators (Ivacaftor) Stabilisers HGF
Genotype (Most common in **bold**)	**G542X** W1282X R553X R1162X 621+1G⟶T	**F508del** I507del N1303K M1101K R1066C	**G551D** G551S G178R G1244E 1255P	**R117H** R247P R334W R1070W R347P	**A455E** 32726A⟶G D565G 3849+1kbC⟶T

Figure 1. 1 The classification of CFTR mutations including the phenotype, prevalence in the CF population, mutation, treatment, and genotype. This diagram highlights the CFTR classifications. The top left-hand box shows a schematic of the cell function with no CFTR mutation, and the subsequent boxes show how the mutation acts with their corresponding classification. Adapted from (Barrio, 2015) (Koivula et al., 2016) (Marunaka, 2017).

1.1.3 Polymicrobial Disease

Traditionally, it was believed that one microbe contributed to one disease (monomicrobial). This was due to Koch's postulates, which were the criteria used to describe the relationship between a microbe and a disease, developed by Robert Koch and Friedrich Loeffler in the 19th century. Koch's postulates criteria include: (1) the microorganism must exist in diseased but not in healthy individuals, (2) The microorganism must be cultured from the diseased individual, and exposure of a healthy individual with the cultured microorganism must acquire the illness, (3) The microorganism must be re-isolated from the diseased individual and paired to the primary microorganism (Nelson et al., 2012). With advances in molecular biology, it has been revealed that this is not universal. Several infectious diseases are shaped by a mixture of microorganisms and viruses (Smith, 1982). This is known as "polymicrobial disease" (Brogden, 2002).

Previously, CF-associated airway infection was thought to be caused by a small group of pathogens (*Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenzae and Burkholderia cepacia*). Sputum samples were screened for these pathogens using culture techniques on selective media introducing a culture bias and only supporting a fraction of the bacterial species present (Dunbar et al., 1997; Rogers et al., 2004). Advances in culture-independent molecular techniques have exposed the high diversity of microorganisms residing in the lungs of CF patients, including facultative and obligate anaerobes and other fastidious bacteria (Rogers et al., 2004; Lynch and Bruce, 2013). These advances have encouraged research in this area and the characterisation of the CF lung microbiome has revealed more than 1000 bacterial species residing there, which varies greatly between individuals (Dickson et al., 2016). Whilst most are classified as commensal, some emerging pathogens have been identified (Parkins and Floto, 2015; Thornton et al., 2022).

The CF lung is polymicrobial therefore co-infection or bacterial interaction is expected and can significantly affect the progression of CF lung disease; studies in this area have found that the disease caused by co-infection is accelerated by biofilm formation, which promotes antibiotic resistance, increased virulence, and maintenance of infection. Precise mechanisms are still poorly understood (Bernardy et al., 2022), and the normal flora is not regularly examined for any interaction with pathogens (Høiby, 2002; Peters et al., 2012).

1.1.4 Recognised Cystic Fibrosis Pathogens.

Even though CF lung infection is known to be polymicrobial (Rogers et al., 2004), patients are still regularly screened for the presence of key pathogens in an effort to stem severe infection, which could lead to increased lung damage (Cystic Fibrosis Foundation, 2017; The Cystic Fibrosis Trust, 2019). The treatment regimen is often single pathogen focused (Section 1.1.6).

1.1.4.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, motile, rod-shaped, and the pathogen most isolated and studied from CF patients' sputum samples (Foweraker, 2009). It is a prevalent coloniser linked to chronic lung infection and decline in more than 50% of cases (Vonberg and Gastmeier, 2005) and is predominant during periods of exacerbation (Foweraker, 2009). Often found in sinks, drains and the surrounding environment (Davies, 2002), it utilises several carbon and nitrogen sources and is a facultative anaerobe. Its broad genetic diversity allows it to persevere in various environments, contributing to its pathogenesis (Wagner and Iglewski, 2008).

Pseudomonas aeruginosa has a range of virulence factors to enable host invasion and defend against other bacteria. These factors include secreted factors and cell-associated factors. One of the more studied virulence factors is *P. aeruginosa' s* ability to form small biofilm aggregates during long-term colonisation of CF airways, ensuring its survival (Singh et al., 2000; Rossi et al., 2021). *P. aeruginosa* can also quickly gain antibiotic resistance and resist all clinically relevant classes of antibiotics (Pitt, 2003). Lung damage during *P. aeruginosa* infection is caused by the excretion of endotoxins and host-mediated inflammation response. It can evade the host immune response using numerous methods, such as flagellin and phenotypic changes (Gupta et al., 1994; Deshpande and Zou, 2020).

1.1.4.2 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, cocci-shaped bacterium and another common CF pathogen. It is an opportunistic pathogen and resides as part of the normal flora of the skin and respiratory system (Sakwinska et al., 2009). *S. aureus* is often isolated from bronchial lavage (BAL) samples of young infants with CF (Stutman et al., 2002; Foweraker, 2009). It is a primary coloniser and can cause epithelial damage, encouraging other pathogens like *P. aeruginosa* to attach (Lyczak et al., 2002). Increased lower airway

inflammation in the presence of both *P. aeruginosa* and *S. aureus* has been found in children with CF (Sagel et al., 2009). These pathogens may work in unison during infection states (Sagel et al., 2009) or benefit each other through co-infection (DeLeon et al., 2014). *S. aureus* is highly adaptable to a high-stress environment like the CF lung and exhibits survival mechanisms similar to *P. aeruginosa* (Goerke and Wolz, 2010).

Methicillin-resistant *S. aureus* (MRSA) is also an organism of note due to its antimicrobial resistance. An increase in prevalence has been seen in CF populations (Belarski and Pettit, 2020). MRSA is a nosocomial pathogen of major concern due to its difficulty to treat (Coutinho et al., 2008).

1.1.4.3 *Burkholderia cepacia complex*

Burkholderia cepacia complex (BCC) is a complex comprising of more than 20 named species of *Burkholderia,* including *B. cepacia* and *B. multivorans*, which are the two most common *Burkholderia* species isolated from CF lungs (LiPuma, 2010). Thought to be acquired from the environment, clinical and environmental strains are extremely closely related (Baldwin et al., 2007). BCC is highly adaptive and resistant to antimicrobials; most species contain a modified lipopolysaccharide that enables innate polymyxin resistance (Rhodes and Schweizer, 2016).

BCC is the causal agent of *cepacia* syndrome, a severe combination of necrotizing pneumonia, bacteraemia and advancing respiratory failure (Scoffone et al., 2017). During periods of acute exacerbation, chronic infection with BCC may occur, and it has been connected to a decline in lung function in CF patients (Coutinho et al., 2008).

1.1.4.4 *Aspergillus fumigatus*

Aspergillus fumigatus is the fungi most isolated from patients with lung infections (Li et al., 2019), including those associated with CF-associated lung infections (Magee et al., 2021). Spores are found in air and water, originating from rotting vegetation. It causes allergic bronchopulmonary aspergillosis (ABPA) and chronic pulmonary aspergillosis (CPA) and is associated with airway damage and lung function decline (Foweraker, 2009; Burgel et al., 2016). A contributing factor to the rise of *A. fumigatus* is that the antibiotics given to control CF lung infection do not contain anti-fungal agents; therefore, they could create a niche for this organism to thrive.

1.1.5 Current Clinical Practice for Microbial Identification in CF Lung Infection

During routine appointments, PWCF are screened for new pathogen infections and antibiotic resistance profiles. Detection of new infections is achieved by analysing various sample types: spontaneous and induced sputum, cough swabs and plates and BAL. These samples are then cultured on various selective agars to determine the presence or absence of the key CF pathogens (*S. aureus, P. aeruginosa, and B. cepacia*). Treatment, if needed, is then decided depending on the culture results (The Cystic Fibrosis Trust, 2009).

The current clinical practice uses culture-based detection methods as a gold standard for respiratory samples due to the standardisation of culture-based methods across clinical laboratories (The Cystic Fibrosis Trust, 2009). However, issues have arisen during the quality assessment of the current standard diagnostic methods used in the laboratories that provide analytical microbiology services to CF centres (Hogardt et al., 2009). While conventional CF pathogens like *P. aeruginosa* and *S. aureus* were accurately recognized, less common and recently identified pathogens were frequently misidentified or undetected, including members of the *B. cepacia* complex and *A. xylosoxidans*. These traditional methods also overlook the slow-growing taxa that may be present in low numbers, with faster-growing species like *P. aeruginosa* outgrowing them and becoming overrepresented (ven Belkum et al., 2000). The report authored by Hogardt et al. (2009), assembled in 2007 and 2008, stated some of the bacterial species that were emerging at that time are now the focus of detection efforts.

The polymicrobial nature of the CF lung is the main reason for culture-dependent methods being labelled as less reliable than initially thought (Rogers et al., 2004). Misidentification and non-detection are major causes of concern clinically, as this has implications for patient treatment. However, another disadvantage of culture-based techniques is the slow turnover of sampling to results, which can sometimes take several days (Rogers et al., 2004). Slow turnover of clinical results delays treatment, prolonging infection and impacting the patient (Chmiel et al., 2014).

However, there are still advantages to traditional culture methods. It accurately determines specific antibiotic sensitivity profiles of specific strains (Burns and Rolain, 2014). It allows the isolation of live organisms for research; compared to newer molecular methods, it is more cost-effective and widely available to use in multiple settings (Bonnet

et al., 2020). When examining the polymicrobial nature of CF lung infection, alternative methods may yield a better understanding of the complex nature of these microbiomes.

1.1.6 Antibiotic Therapy for Typical CF Lung Infection

The Cystic Fibrosis Trust's antibiotic treatment guidance (2009) states that antibiotic therapy for CF lung infection aims to prevent, eliminate, or control respiratory infections, focusing mainly on treating *P. aeruginosa*. The trust deems antibiotic usage in these situations a major reason for the decline in respiratory morbidity and increased longevity of CF patients in the last few decades (The Cystic Fibrosis Trust, 2009).

Antibiotic prophylaxis with flucloxacillin is used to reduce the incidence of *S. aureus* infection, inhibit the onset of bronchiectasis, and prevent further bacterial infection during acute viral illness (The Cystic Fibrosis Trust, 2009; Smyth and Rosenfeld, 2017). This narrow spectrum β-lactam targets the cell wall in Gram-positive organisms (Waxman and Strominger, 1983). This treatment is recommended in the UK for up to 3 years old (Smyth and Rosenfeld, 2017), however, there has been some apprehension about the long-term effects regarding the treatment of *P. aeruginosa* infection and doubt of the clinical benefit, leading to this recommendation not being used in the United States (Mogayzel et al., 2014; Smyth and Rosenfeld, 2017; Hurley et al., 2018). Antibiotic prophylaxis is initiated when the patient acquires a viral infection to reduce the risk of secondary infection with other known CF pathogens. Patient samples would be cultured to indicate what organism is present and treated accordingly. If the secondary infection is *P. aeruginosa*, ciprofloxacin is prescribed to prevent further deterioration. This course of treatment lasts until the patient has returned to a healthy state, regardless of whether this takes more than three weeks (The Cystic Fibrosis Trust, 2009). Ciprofloxacin is a fluoroquinolone that targets Gram-negative bacteria; it limits DNA replication by inhibiting DNA topoisomerase and DNA-gyrase (Campoli-Richards et al., 1988).

The elimination antibiotic strategy is used depending on the patient's age, organism, and the severity of the exacerbation. When displaying mild symptoms, oral antibiotics are given, which can be a combination depending on what organisms are present. If the infection is severe, intravenous (IV) antibiotics are given (Lyczak et al., 2002). When a newly developed *P. aeruginosa* infection is detected, the treatment regimen is given as a combination of oral, inhaled and IV antibiotics due to the increased risk of death

(Mogayzel et al., 2014). Nevertheless, there is no agreement on the best preparations, dosages, or length of treatment (The Cystic Fibrosis Trust, 2009).

In some cases, inhaled and IV antibiotics are administered to control infection. They are primarily used to control chronic *P. aeruginosa* infection and aim to preserve lung function and reduce the need for additional IV treatments (Ryan et al., 2003). The treatment schedule consists of twice-a-day inhalation of colistin or tobramycin administered on alternate months. With a minimum duration of six months but 12 months, treatment may be required depending on the individual (The Cystic Fibrosis Trust, 2009). Colistin disrupts the Gram-negative cell membrane by disturbing the molecules that usually stabilise the lipopolysaccharide resulting in increased membrane permeability (Falagas and Kasiakou, 2005). Tobramycin is an aminoglycoside and works on Gram-negative bacteria by targeting the 30S ribosomal subunit, disrupting protein production and resulting in cell death (Davis, 1987).

The recommendations laid out by the CF Trust demonstrate that keeping lung infection in CF under control is a delicate and complex undertaking. The eradication or reduction of one organism could lead to a secondary infection or proliferation by another. This creates a constant cycle of infection and treatment.

1.1.7 CFTR Modulators:

CFTR modulators are relatively new to the CF treatment regimen. They can enrich or repair the expression, function, and stability of a faulty CFTR gene. They are only effective against certain mutations (Figure 1.1) and have been classified into five main categories depending on their effects: potentiators, correctors, stabilisers, read-through agents and amplifiers (Lopes-Pacheco et al., 2016).

Potentiators are compounds that repair or improve the CFTR channel opening, allowing increased CFTR-dependent anion conductance (Lopes-Pacheco et al., 2016; Lopes-Pacheco, 2020). Clinical use is limited, however, as these drugs showed probable modulation of multiple signalling pathways and physiological functions (Illek and Fischer, 1998).

Stabilisers secure the CFTR channel at the plasma membrane and decrease protein degradation rate, correcting the instability of class VI mutants (Lopes-Pacheco et al., 2016). Correctors enhance the conformational stability of CFTR which enhances protein

folding and rescues the trafficking of the mature CFTR to the plasma membrane, this treatment is beneficial to people with the ΔF508 (Pedemonte et al., 2005; Quon and Rowe, 2016). Amplifiers enhance the activity of other modulators by providing more protein substrate, but they can act independently by stabilising the CFTR mRNA enhancing translation of the CFTR protein (Dukovski et al., 2020). Patients with class I mutations benefit from read-through agents as this molecule rescues the protein synthesis by allowing continued protein translation in the presence of a premature stop codon (Lopes-Pacheco et al., 2016).

There are four types of modulators currently available for patient use:

Ivacaftor (Kalydeco®) (Vertex Pharmaceuticals, 2012) is for use in patients six years and older (USA) and patients over the age of 4 months (UK) (Vertex Pharmaceuticals, 2012; Donaldson et al., 2018). Trials have shown that partially restored CFTR activity in patients with at least one class III gating mutation (Figure 1.1). It increases the ion function of activated cell surface CFTR. The compound induces channel opening via a mechanism that does not require ATP binding and hydrolysis. So, the patient has improved airway clearance via the decrease in mucus plugging and better hydration of the airway surface (Van Goor et al., 2009; Vertex Pharmaceuticals, 2012).

Lumacaftor/Ivacaftor (Orkambi®) is a combination of a potentiator and a corrector, which is suitable for patients who are homozygous for the class II mutation F508del (Vertex Pharmaceuticals, 2021). Correctors help the CFTR protein form the right shape so it can travel to the cell surface. The potentiator will enable the transport of the protein (Van Goor et al., 2011).

Tezacaftor/ Ivacaftor (Symdeko®) functions in the same way that lumacaftor does. It changes the proteins' structure to ensure it can travel and stay at the cell surface for longer. It is again used in patients who are homozygotes for F508del mutation but can also be used in patients with single copies of residual function mutations such as A455E, D110E or R117c or Splice mutations 711+3A-> G or E831X (Donaldson et al., 2018; Vertex Pharmaceuticals, 2021).

Triple combination therapy Kaftrio® (UK) or Trikafta® (USA). A combination of Tezacaftor, Ivacaftor and Elexacaftor, which is for patients who are 12 years and over and

homozygous for F508del or have one copy of F508del and one minimal function mutation (Vertex Pharmaceuticals, 2021).

With the introduction of CFTR modulators, the outlook for PWCF is extremely positive. All the modulators have been shown to reduce the symptoms of CF. Patients with more acute impairment of lung function also saw an improvement of sweat chloride concentrations, per cent predicted forced expiratory volume in 1 second (%FEV_1), less frequent *P. aeruginosa* detection, reduced exacerbation periods and slower deterioration of lung function (Ramsey et al., 2011; Barry et al., 2014; Veit et al., 2020). Even with the introduction of these new drugs, around 20% of patients (Fajac and Sermet, 2021) who do not have the mutation associated with the modulator are still not eligible, are still at risk from chronic lung infection and will eventually need a lung transplant. There is currently no research examining the impacts of triple combination therapy on CF-associated lung infection and their effects on the microbiome (Allen et al., 2023).

1.2 The Human Microbiome and the Microbiota

The human microbiome is the diverse collection of microorganisms that reside internally and within externally upon a person. This community of organisms plays a crucial role in maintaining human health, influencing various physiological processes, immune function, and contributing to the development of certain diseases (Turnbaugh et al., 2007; Ursell et al., 2012; Belkaid and Timothy, 2014; Wang et al., 2017).

The microbiota refers to the microbial communities related to specific tissues or organs of the host, and the microbiome is the total genome of this community in humans; this is the total sum of all microorganisms inhabiting a person at a particular time (Ursell et al., 2012; Shukla et al., 2017). The motive of a microorganism within the microbiota may be commensal, mutualistic, or pathogenic, and its role may be influenced by external factors or the introduction of a new resident (Ding and Schloss, 2014; Ogunrinola et al., 2020). The composition of the microbiome can change depending on healthy and diseased states. In many disease states, or during "dysbiosis" (Hooks and O'Malley, 2017), the microbiome is dominated by specific species and has less diversity than in healthy states. (Cuthbertson, 2014; Amon and Sanderson, 2017; Cuthbertson et al., 2020). The functional role of the microbiome is mostly unknown (Gao et al., 2023). Research has shown that it is integral to maintaining physiological balance within the body, actively contributing to

digestion, metabolism, and immune system regulation (Belkaid and Timothy, 2014). This study is focused on the respiratory microbiota.

1.2.1.1 The Lungs

The lungs are constantly exposed to transient bacteria, and potential pathogens, through their normal function and exposure to the air (O'Dwyer et al., 2016). Previously, it was thought that the lungs were a sterile environment where no bacteria could grow, However, since the arrival of culture-independent techniques and their application to the CF lung, evidence has suggested this is untrue (Dickson et al., 2016). Early studies found a large, diverse community of microorganisms in the CF lung (Rogers et al., 2003; Rogers et al., 2004).

1.2.1.2 The Respiratory Microbiome

The upper respiratory tract (URT) comprises the nasal and oral cavities, pharynx, and larynx. It is colonised by an extensive range of microbial species immediately after birth (Goldenberg et al., 2000). Its composition is dependent on if the delivery is via vaginal or caesarean, this establishment continues radically during the first year of life in conjunction with the maturation of the immune system (Dominguez-Bello et al., 2010; Kumpitsch et al., 2019). It experiences ecological succession throughout a person’s life, where the microbiota changes over time due to changing situations (Biesbroek et al., 2014; Schenck et al., 2016).

The potential habitats present in the URT are very different; the microbiota present will be unique. From early years, the URT can be colonised by numerous pathogenic bacteria such as *Streptococcus pneumoniae*, *S. aureus* and *Haemophilus influenzae*, all present in an asymptomatic state (Schenck et al., 2016). However, this can vary further depending on the specific area within the URT. For example, the nasal cavity in healthy humans was found to be comprised of the phylum of Bacteroidetes, Firmicutes and Proteobacteria including the genera *Bifidobacterium, Corynebacterium, Staphylococcus, Streptococcus, Dolosigranulum* and *Moraxella* (Bassis et al., 2014).

Meanwhile, the oral cavity houses a range of surfaces, leading to a wide range of microbes. The oral cavity is mainly colonised in different ratios by obligate anaerobes and aerobes that are from the *Streptococcus, Actinomyces, Veillonella* and *Neisseria* genera (Segata et al., 2012; Sharma et al., 2018). Studies of the pharynx and larynx microbiota

found that the area was dominated by *Streptococcus, Prevotella, Fusobacterium* and *Rothia* genus' (Gong et al., 2013).

The lower respiratory tract (LRT) comprises the trachea, bronchial tubes, and the lungs. As previously mentioned, the LRT was believed to be sterile but has been found to host a distinct microbiota (Dickson et al., 2016). Bacteria enter the LRT via the URT through mucosal dispersion (the split and distribution of organisms via the mucosa) and micro-aspiration, therefore sharing some similarities in composition with the URT (Bassis et al., 2015; Man et al., 2017). When comparing the URT and LRT, some research has found that the LRT is less densely populated (Rao et al., 2021).

Research using culture-independent molecular techniques found taxa from the *Actinobacteria, Proteobacteria, Bacteroidetes* and *Firmicutes* as the prevalent phyla residing in the LRT (Pulvirenti et al., 2019). In healthy individuals, the LRT microbiota is like the oropharyngeal microbiota and is dominated by taxa of the *Firmicutes, Bacteroidetes*, and *Proteobacteria* phyla (He et al., 2022). The lower airways contain approximately 100 different taxa, including *Streptococcus, Veillonella*, and *Prevotella* (Hilty et al., 2010; Bassis et al., 2015; Cookson et al., 2018; Pettigrew et al., 2020). However, the actual species present in the microbiome could be influenced by internal and external factors like host lifestyle, chronic disease, and diet (Hou et al., 2022).

The exact function of the lung microbiome is unclear. However, it has been hypothesized that it has an essential role in modulation of host inflammation, immune response (O'Dwyer et al., 2016) and maintaining homeostasis (Wang et al., 2017).

1.2.1.3 The Influence of the Microbiota on Immune System Function and Host Inflammation

The microbiome has been shown to modulate the host immune system, influencing innate and adaptive responses (O'Dwyer et al., 2016). The changes in the microbiome composition over a person's lifetime can affect the regulation of immunoglobulins and innate immune responses (Natalini et al., 2023). These changes could also create a predisposition to some respiratory conditions in later life (Thorsen et al., 2019). Changes in the microbiome could be influenced by external environmental factors which trigger exacerbations, airway inflammation and damage, which in turn could affect the severity of respiratory infections (Goss et al., 2004; Thornton et al., 2022).

he mucosal surfaces of the lungs must regulate the bacterial burden to ensure adequate clearance to reduce the risk of disease; the mechanisms to remove unwanted microbes from the LRT include mucociliary clearance, cough, and a combination of innate and adaptive immune responses (Natalini et al., 2023). Micro-aspiration models show that microbial clearance occurs at different rates depending on the microbial taxa present (Wu et al., 2021; Natalini et al., 2023). A study using mouse models showed that when the oral commensals *Prevotella melaninogenica, Veillonella parvula* and *Streptococcus mitus* were detected in the LRT, it resulted in the activation of CD4+ and CD8+ T cells, enlistment of interluken-17 (IL-17) producing T cells, and other counter-regulatory immunological responses (Wu et al., 2021). Other studies found in the LRT of humans that *Prevotella*, *Rothia* and *Veillonella* enrich showed a positive correlation with T helper 17 cells(T_H17 cytokines and lung neutrophils (Segal et al., 2016)—giving some insight into how the immune system of the lung responds to transient microbe populations (Natalini et al., 2023).

In pulmonary diseases such as asthma, chronic obstructive pulmonary disorder (COPD), bronchiectasis and CF, where inflammation plays a major role (Barnes, 2008; Petrocheilou et al., 2022), the composition of the microbiota and interaction with the host can be specific to the disease (Caverly et al., 2019). Neutrophilic asthma is characterised by high levels of neutrophils in the lungs, frequent exacerbations, and airflow obstruction (Crisford et al., 2021). *H. influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae* are frequently isolated from the lung tissue of these patients (Korppi, 2010) as well as those suffering from COPD (Beasley et al., 2012) and bronchiectasis (Yagi et al., 2021). In asthmatics, significant associations were found between neutrophilic recruitment due to T_H17 inflammatory markers in the presence of Proteobacteria (Huang et al., 2015). Whereas in COPD, a multicentre study examining sputum samples showed associations between the microbiota composition and the type of inflammation. When *Haemophilus* dominated the sputum, it showed increased levels of IL-1β and tumour necrosis factor (TNF) (Wang et al., 2021), resulting in increased airway inflammation (Tufvesson et al., 2015). Another study consolidated this work, reporting that when *P. aeruginosa* and *H. influenzae* are dominant, there were higher levels of IL-8 and IL-1β, which coincided with impaired lung function (Rogers et al., 2014).

In CF patients, *P. aeruginosa, S. aure*us and *H. influenaze* are the most commonly isolated microbial species (Bhagirath et al., 2016), with *P. aeruginosa* becoming more dominant as

the lung function declines (Malhotra et al., 2019). As the disease progresses, the CF microbiota exhibits immunomodulatory activity, which could alter the host's inflammatory response (Thornton et al., 2022). Whilst some *Prevotella* spp. Have been implicated in positive regulation of host immune cells, members of this genus have differing roles during pathogenesis ; *P. intermedia* has shown an ability to enhance disease progression by the secretion of cytotoxic extracellular toxins, which stimulate an influx of macrophages and neutrophils to the area (Ulrich et al., 2010). Further, some *Prevotella* isolates that can produce extended-spectrum β-lactamases (ESBLs) were able to shield *P. aeruginosa* from treatment and contribute to prolonged CF lung infection (Sherrard et al., 2016).

There is some commonality between the diseases; studies have found that members of the Proteobacteria phylum are more frequently isolated from individuals suffering from these diseases in the advanced stages when compared to healthy controls (Sze et al., 2015; Teo et al., 2015; Zemanick et al., 2017). Some anaerobic bacteria, *Prevotella* and *Veillonella* species, have been found more abundantly during host inflammatory response. However, their significance is unclear due to their detection in milder and healthy phenotypes (Horn et al., 2022).

Comprehending the intricacies of the lung microbiome and its interactions provides insights into how it contributes to immune system development, responsiveness, and defence against pathogens. Leading to enhanced clinical diagnostics and developing therapies that augment the microbiome, resulting in improved patient outcomes.

1.3 Culture Independent Microbial Identification

Advances in molecular-based techniques for detecting bacteria in a clinical setting have been highlighted as faster and more sensitive (Rogers et al., 2009) and have led to reassessing the microbes customarily linked with CF lung infection (Pattison et al., 2013). Molecular detection techniques target molecules specific to the bacterial genus or strain. They often involve the separation of PCR amplicons by exploiting variations in the DNA sequences of genes associated with functional or phylogenetic significance (Muyzer et al., 1993; Blackwood et al., 2003).

1.3.1 Bacterial 16S rRNA Gene

The 16S rRNA gene plays a vital role in bacterial phylogenetics species delineation and is crucial in microbiome studies (Johnson et al., 2019). This gene is the most utilised target

in various PCR-based methods (Janda and Abbott, 2007). It is widely used because it is highly genetically, functionally conserved, and detectable in all bacterial species, enabling the detection of fastidious microbes with no evidence of horizontal gene transfer (HGT). Despite this genetic conservation, the 16S rRNA gene has regions of variability, enabling the recognition of different taxa (Clarridge, 2004). The gene is approximately 1500bp and has nine hypervariable regions (V1 to V9) interspaced with conserved regions (Woese, 1987). However, it has limitations. The 16S rRNA gene is relatively short in length, which can impede its ability to identify bacteria down to the species level, especially when examining different species within the same genus (Mignard and Flandrois, 2006; Srinivasan et al., 2015); therefore, when studying polymicrobial systems where bacterial complexes may be present when analysing results, this must be considered.

Methods using the 16S rRNA gene, like Terminal restriction fragment length polymorphism (T-RFLP), next-generation sequencing (NGS) and others, have increased our understanding of microbial diversity in CF populations (Rogers et al., 2004; Rogers et al., 2006; Sibley et al., 2011; Pattison et al., 2013).

1.3.1.1 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) is a culture-independent, high-throughput method of obtaining a genetic fingerprint of the composition of a microbial community (Schütte et al., 2008). It has been used to study the microbiome in various settings including, the natural environment (Katsivela et al., 2005; Noll et al., 2005; Hullar et al., 2006) and the human environment (Sakamoto et al., 2003; Andoh et al., 2007; Dekio et al., 2007; Brugger et al., 2012). It has been used to study the CF microbiome, where it provided a fingerprint of the most abundant community members (Rogers et al., 2006; Rogers et al., 2010) and can be used to show shifts in the community composition in relationship to treatment (Fodor et al., 2012; Lynch and Bruce, 2013). However, this technology, whilst still useful, is being taken over by NGS, which provides more in-depth information and better resolution in terms of microbial diversity (Prakash et al., 2014).

1.3.1.2 Next Generation Sequencing

Medical microbiology research was transformed with the introduction of NGS, also known as high throughput sequencing. It revolutionised the way genetic information was acquired and analysed. This rapidly evolving technology enables the parallel sequencing

of millions of DNA fragments, allowing the examination of the complex relationships in the microbiome independent of traditional culture techniques (Hodkinson and Grice, 2015; Feigelman et al., 2017; Wensel et al., 2022). It has two main techniques depending on the type of research being conducted. Whole genome shotgun metagenomic sequencing (WGS) targets all genomic DNA present in the samples (Quince et al., 2017) and targeted or amplicon sequencing utilises specific primers that target specific regions of DNA, for example the 16S rRNA gene (Fouhy et al., 2016).

1.3.1.2.1 Whole Genome Sequencing

Whole genome sequencing allows the untargeted sequencing of all microbial genes in a sample, giving a detailed mechanistic view of microbial communities (Quince et al., 2017). This technique is efficient in metagenomic studies: assembly, community, and functional profiling and (Pérez-Cobas et al., 2020) it enables the evaluation of bacterial abundance and diversity within various environments (Khachatryan et al., 2020). WGS provides excellent microbial resolution precision and captures a wide range of bacterial genera. It can also distinguish assumed functional genes that are not detectable through amplicon sequencing (Brumfield et al., 2020). However, it yields massive amounts of data, which can be complicated by incomplete coverage, short read lengths, and intrinsic sequencing errors (Ma et al., 2014). Another consideration is that WGS is more expensive than amplicon sequencing and requires large-volume data storage capabilities and extensive specialist data analysis (Sims et al., 2014).

1.3.1.2.2 Amplicon Sequencing

Numerous studies using amplicon sequencing use primers that target the 16S rRNA gene in bacteria (Kim et al., 2011; Fadrosh et al., 2014; Ranjan et al., 2016; Fadeev et al., 2021). Amplicon sequencing is the most widely used microbiome sequencing approach and was used on large-scale projects like the Human Microbiome Project (Turnbaugh et al., 2007; Huttenhower et al., 2012; Ranjan et al., 2016).The 16S rRNA gene, when targeted in amplicon sequencing, yields thousands of short sequences aligned and classified down to genus or species level using publicly available databases. The data is then analysed as a population survey that can detail a myriad of information, including total bacterial abundance, relative abundance, community structure and diversity (Dickson et al., 2016).

Though widely used, targeting the 16S rRNA gene for researching the microbiome has its limitations. The classification of taxa relies on the presumed link between the 16S rRNA

gene and a taxonomic group defined as an operational taxonomic unit (OTU) (Edgar, 2018). Usually, OTUs are investigated at the phyla or genera level, which lacks precision than at the species level (Franzén et al., 2015). Furthermore, individual genes are not sequenced directly but are instead assumed based on the OTU. Due to indirect assumptions of genetic information, and the potential for horizontal gene transfer, the lack of direct gene identification limits the understanding of the microbiome (Poretsky et al., 2014; Ranjan et al., 2016). This study uses amplicon sequencing.

1.4 The Emerging Non-tuberculosis mycobacteria

In some CF populations, non-tuberculosis mycobacteria (NTM) have been detected (Floto and Haworth, 2015). NTM are mycobacteria that do not cause tuberculosis or leprosy. They are also known as mycobacteria other than tuberculosis (MOTT), atypical mycobacteria, anonymous mycobacteria, or environmental mycobacteria (Johnson, 2013). Thought to be acquired from the environment, fomites, or water sources, these aerobic, non-motile, lipid-rich organisms are intrinsically resistant to antibiotics (Chapter 1, Section 1.4.10), disinfectants, heavy metals (Jarlier and Nikaido, 1994) and are known to form biofilms as an additional defence against antimicrobials (Schulze-Robbecke et al., 1992).

1.4.1 Taxonomy of NTM

Mycobacteria belong to the order *Actinomycetaceae,* family of *Mycobacteriaceae* and the genus *Mycobacterium* (Rastogi et al., 2001). It has defining characteristics that make it easy to differentiate from other genera like the mycolic acid membrane (Section 1.4.5). In 1954, Timpe and Runyon reported a systematic program for the classification of NTM as an attempt to establish and collate common, clinical, and differential characteristics of NTM (Wayne et al., 1957). This classification is still used and considers the growth rate and chromogen production when grouping NTM (Nogueira et al., 2021). Currently, 197 species and 14 sub-species have been identified and can be sub-divided by growth rate (Forbes, 2017; Parrish, 2019).

Rapid growing mycobacteria (RGM) principally based on growth in seven days or less and include the major taxa are the *Mycobacterium fortuitum* group and the *M. abscessus* complex (MABSC) involving three subspecies: *M. abscessus subsp. abscessus*, *M. massiliense* and *M. bolletii*. The lesser groups are the *M. smegmatis, M. mucogenicum* and the *M. magertitense/ wolinskyi* (Brown-Elliott and Philley, 2017).

The slow-growing mycobacteria (SGM), which take seven days or more to grow, can be further subdivided by pigmentation: Group I: Pigmentation when exposed to light (photochromogenic), Group II: always pigmentated (scotochromogenic), Group III: No/weak/late pigmenting regardless of light exposure (non-photochromogenic) (Turenne, 2019). The major taxa of this group are *M. avium* complex (MAC), which consists of the main species *M. avium, M. chimaera and M. intracellulare* (Hwang et al., 2017), *M. kansasii, M. malmoense* and *M. simiae* (Falkinham, 2009).

1.4.1.1 Recent Proposed Changes to NTM Taxonomy

In 2018, it was proposed to revise the taxonomy of NTM. This proposal would redistribute 150 species of the *Mycobacterium* genus into five new and different genera: *Mycobacteroides, Mycolicibacter, Mycolicibacterium,* and *Mycolicibacillus.* This proposal was based on the theory of synapomorphies, which classifies genera based on the detection of ancestral molecular markers (Gupta et al., 2018); the International Code of Nomenclature of Prokaryotes (ICNP) validity published the changes (Parte et al., 2020; Oren and Garrity, 2021). This proposal has sparked debate surrounding the necessity of revising the taxonomy, with most arguments against the reclassification and for the reconstitution of the previous classification as the revised naming was found to provide no benefits to the clinical field of mycobacteriology and had the potential to cause confusion to researchers and clinicians (Tortoli et al., 2019; Meehan et al., 2021; Moore and Millar, 2022). This study uses the genus *Mycobacterium*.

1.4.2 NTM Pathogenicity in Humans

The prevalence of NTM infection is growing in the UK, USA, and Europe, with cases overtaking *M. tuberculosis* (Brode et al., 2014; Jarchow-MacDonald et al., 2023). The most common NTMs in human infections include *M. avium* complex (MAC), *M. kansasii*, and *M. xenopi* among the SGM. *M. abscessus* complex (MABSC) and *M. chelonae* among the RGM (Griffith et al., 2007). These opportunistic pathogens can cause disease in both immunocompromised and competent groups and are associated with significant morbidity and mortality (Floto et al., 2016; Haworth et al., 2017; To et al., 2020). In humans, NTM infection can manifest as four unique clinical conditions: (1) Skin and soft tissue infections (SSTI), (2) Disseminated disease, (3) Superficial lymphadenitis, and (4) pulmonary disease with symptoms often being non-distinctive and resembling other diseases such as TB, creating a challenge in accurate diagnostics (Griffith et al., 2007; Lipman et al., 2020; To et al., 2020). Diagnosis requires a combination of clinical,

radiographic, and microbiological criteria (Griffith et al., 2007; Stout et al., 2016; Daley et al., 2020). Treatment often involves a combination of three antibiotics: rifampin, ethambutol with a macrolide or fluoroquinolone (Griffith et al., 2007; Daley et al., 2020), dependant on species antimicrobial sensitivities (Griffith et al., 2007)(Table 1.1/1.2)(Section1.4.10).

1.4.2.1 *Mycobacterium avium* Complex

Mycobacterium avium complex (MAC) is part of the SGM and is an environmental *Mycobacterium* often isolated from soil, water, birds, and livestock (Falkinham, 2013). This complex includes *M. avium*, *M. intracellulare* and *M. chimera* (Moravkova et al., 2008; To et al., 2020), all of which have a complex and diverse genetic makeup, with multiple subspecies and genotypes (Shin and Shin, 2021). Recent genetic sequencing, genotypic and phenotypic tests have identified additional species present within MAC, all of which are SGM, non-chromogenic in group III of the Runyon classification (Hwang et al., 2017). Presently, MAC consists of 10 species: *M. avium, M. intracellulare, M. chimera, M. colombiense, M. marseillense, M. timonense, M. boucherdurhonense, M. vulneris, M. arosiense* and *M. youngenesne*. (Daley, 2017). Four subspecies make up *M. avium*: *M. avium* subsp. *paratuberculosis, M. avium* subsp. *avium, M. avium* subsp. *silvaticum* and *M. avium* subsp. *hominissuis* (Thorel et al., 1990; Turenne et al., 2006; Daley, 2017). Among all NTM, MAC is the most common cause of pulmonary disease in humans, particularly in individuals with underlying lung diseases such as bronchiectasis, CF, or COPD (Griffith et al., 2007; Diel et al., 2018).

1.4.2.2 *Mycobacterium abscessus* Complex

One of the most clinically relevant RGMs is the *Mycobacterium abscessus* complex (MABSC). The detection and isolation of MABSC have been increasing globally (Cristancho-Rojas et al., 2023), which is of great concern as it is associated with significant morbidity and mortality rates, particularly in immunocompromised individuals and those with underlying pulmonary diseases (Griffith et al., 2007; Lee et al., 2015). MABSC is ubiquitous in the surrounding environment, aquatic, and hospital settings (Falkinham, 2009), and it is responsible for various human infections; some are like the SGM, including SSTI (Dumic and Lutwick, 2021), disseminated infections and pulmonary infection (Lee et al., 2015). In some rare cases, MABSC has also been isolated from central nervous system (CNS) infections (Lamb et al., 2019) and ocular infections (Chu et al., 2015). MABSC comprises three closely related sub-species, including *M. abscessus* subsp. *abscessus, M.*

abscessus subsp. *massiliense*, and *M. abscessus* subsp. *bolletii*, some of which are multi-drug resistant, and all have different antibiotic susceptibility patterns, depending on the strain (Lee et al., 2015; Adekambi et al., 2017). With some *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* strains showing higher levels of clarithromycin resistance than *M. abscessus* subsp. *massiliense* (Mougari et al., 2016; Sukmongkolchai et al., 2023).

1.4.3 Epidemiology and Clinical Relevance:

The epidemiology data of NTM is varied depending on the global region, but in all the prevalence of NTM infection is showing a clear increasing trend. In some areas, information about the epidemiology of NTM infection is limited due to non-mandatory reporting of NTM disease, although there is an increasing prevalence (Thornton et al., 2021). This increase is likely due to multiple factors including growing populations, more frequent extreme weather, improvement of detection, enhanced NTM virulence, altered host-pathogen interactions, and use of medical immunosuppression (Huttenhower et al., 2012; Prevots and Marras, 2015; Donohue, 2021; Thornton et al., 2021; Mejia-Chew et al., 2023). Another element affecting NTM epidemiology data is the type of clinical infection reported.

Whilst NTMs can cause infection in extrapulmonary sites, this is not an area of active surveillance in most areas worldwide, with only some countries actively reporting cases (Wentworth et al., 2013; Winthrop et al., 2017; Shih et al., 2018). However, studies actively examining this area found that over six years, the annual prevalence of extrapulmonary NTM infection was stable. SSTIs showed increased cases, but this data still had high variability due to location, patient factors, and associated differences in environments (Shih et al., 2018; Ricotta et al., 2021). Another study also found that among 334 infections, 50% were caused by MAC, 22.8% were caused by MABSC or *M. chelonae,* 9.6% were caused by *M. fortuitum* and 6.3% *by M. marinum.* Indicating that RGM is most associated with SSTIs, with MAC causing the most disseminated lymph node infections, and most outbreaks are likely isolated cases (Henkle et al., 2017).

NTM pulmonary disease (NTM-PD) is the most common type of NTM infection globally and accounts for 80-90% of all NTM-associated diseases (Prevots and Marras, 2015; Chindam et al., 2021; Saptawati et al., 2022; Mejia-Chew et al., 2023). MAC and MABSC are associated with around 90% of the total reported cases of NTM-PD (Olivier et al., 2012; Schiff et al., 2019; Lipman et al., 2020).

1.4.3.1 The UK and Europe

The epidemiology of NTM-PD in the UK and Europe is not thoroughly understood, as it is not a notifiable disease in these regions (Van Der Werf et al., 2014). However, several studies conducted within these regions have provided some insight. Research carried out in Denmark from 2000 to 2010 (Andréjak et al., 2010), Scotland from 1997 to 2008 (Russell et al., 2014), Germany in 2014 (Ringshausen et al., 2016), and the UK from 2006 to 2016 (Schildkraut et al., 2021)have estimated the annual prevalence rates to be 2.43, 2.44, 3.3, and 6.4 cases per 100,000 people per year, respectively. Another study estimated the annual prevalence of all NTM-PD in Europe at 6.2 per 100,000 of the population (Schildkraut et al., 2020). In the UK, a recent study showed an eight-fold increase in NTM culture-positive isolates between 1995 and 2012, from 0.9 per 100,000 of the population to 7.6 per 100,000 (Lipman et al., 2020).

Concerning the prevalence of NTM species in Europe, this varies between areas, but the distribution is not a clear north/south split. MAC is the predominant species overall but with higher isolation rates in the north (44%) than in the south (31%) (Van Der Werf et al., 2014; Wassilew et al., 2016; Honda et al., 2018). In most European countries, *M. xenopi* (representing <0.01% of isolates), followed by *M. kansasii* and *M. malmoense*, is more frequently encountered in NTM-PD than MABSC in non-CF populations. When examined by location and compared to MAC, *M. xenopi* was more commonly isolated in Hungary (49/16%), *M. kansasii* in Poland (35/23%) and Slovakia (26/28%). RGM species were more common in the UK (44/22%) and Greece (46/36%) (Hoefsloot et al., 2013; Wassilew et al., 2016; Cowman et al., 2018; Honda et al., 2018).

1.4.3.2 North America

In the USA, the annual prevalence of all NTM diseases was estimated to be 6.1 per 100,000 of the population (Grigg et al., 2023). With incidence rising from 2.4 per 100,000 of the population in the 1980s to 15.2 per 100,000 of the population in 2013 in the US (Donohue, 2018). However, data showed different incidence rates depending on the state and the monitoring systems (Grigg et al., 2023). Like Europe, NTM-PD is the most common type of disease detected in the USA, with almost 80% of cases caused by MAC, then *M. kansasii* (Kendall and Winthrop, 2013; Honda et al., 2018; Zweijpfenning et al., 2018). Some of the highest levels of NTM-PD were detected in California, Hawai'i, New York, Louisiana, Pennsylvania, and Florida (Adjemian et al., 2012; Honda et al., 2018), and

in the southeastern states, RGMs were most commonly recovered (Stout et al., 2016; Honda et al., 2018; Zweijpfenning et al., 2018).

1.4.3.3 Asia

The epidemiological data for this region is again variable, with the lack of reporting from most areas. However, some data from specific countries can estimate the prevalence within Asia (Ratnatunga et al., 2020; Zhou et al., 2022). MABSC is common in Asia and Oceania, unlike Europe, North America, and South America (Hoefsloot et al., 2013).

A population-based Chinese study showed an increase in NTM isolation rate from 3 to 8.5% from 2008 to 2012 (Wu et al., 2014; Ratnatunga et al., 2020) and a national survey of NTM-PD in China based on acid-fast (AFB) positive sputum samples collected in 2013 found that of 4917 mycobacterial isolates cultured, 6.4% were confirmed as NTM (Liu et al., 2021). Another study showed a trend in higher isolation of *M. intracellulare* (a member of the MAC) in areas with higher latitudes when compared to areas with lower latitudes where *M. chelonae* was recovered more frequently (Yu et al., 2016). Taiwan showed similar data with higher cases of NTM due to MAC recovered in the north and MABSC in the south (Huang et al., 2017; Honda et al., 2018).

In Japan, data has shown a rise in NTM infection and related mortality from 1994 to 2010 (Morimoto et al., 2014). The annual prevalence of NTM-PD in 2016 was estimated at 24.9 per 100,000 of the population in Japan (Schildkraut et al., 2020). MAC was the most frequently isolated NTM (81%) from respiratory specimens between 1971 and 2007, followed by *M. kansasii* (10%) (Simons et al., 2011). Okinawa showed MABSC as the predominant NTM isolated (Hoefsloot et al., 2013). Studies in South Korea showed a 62% increase in NTM lung disease from 2002 to 2008, with a marked increase in MABS infection (Park et al., 2010) and between 2010 and 2021, the annual prevalence of NTM diseases in South Korea increased from 11.4 to 56.7 cases per 100,000 population (Kim et al., 2022).

In the regions of Asia where there are some rare studies, NTM isolation rates in India reported to range from 0.5 to 8.6% (Jain et al., 2014). The prevalence of NTM-PD varied from 0.7% to 34% in India (Maurya et al., 2015), with MABSC, *M. fortuitum* (27.5%), and *M. intracellulare* (20.9%) most commonly isolated from clinical samples (Desikan et al., 2017). In a study conducted in Singapore, 13 different NTM species were isolated from a

cohort of 485 patients. The most frequently isolated NTM was MABSC (38.4%), followed by *M. fortuitum* (16.6%) and MAC (16.3%) (Lim et al., 2018).

1.4.3.4 Australia

Australia, particularly Queensland, has a unique epidemiological perspective on NTM due to its mandatory reporting scheme for NTM infections (Thomson et al., 2013; Chou et al., 2014). This reporting has shown that NTM-PD in this region is estimated between 1.0-1.8 per 100,000 population (Chou et al., 2014). MAC has been reported as the most prevalent NTM in Queensland, with *M. intracellulare* making up almost 80% of the MAC isolates (Hoefsloot et al., 2013). MABSC is also regularly recovered in southern Australia (Hoefsloot et al., 2013; Wassilew et al., 2016; Honda et al., 2018).

1.4.3.5 Africa

Like other regions, NTM disease reporting is non-mandatory in Africa. There have been some studies examining the frequency of NTM-PD, with research in this area often being split between two types of populations: those suspected of having pulmonary TB and those with chronic pulmonary TB (Prevots and Marras, 2015).

A review examining these groups in different African countries found that in the suspected TB group between 2007-2009, 45.1% of patients in Western Kenyan hospitals (*n =10)* tested positive for mycobacteria and the patients with positive cultures 95% had TB and 4.2 had NTM (Nyamogoba et al., 2012). In Nigeria, between 2010 and 2011, there were 1603 suspected TB cases; 28% had mycobacteria identified, with 85% being positive for TB and 15% having NTM (Aliyu et al., 2013). A final study conducted in a rural area of Eastern Uganda focusing on paediatric patients found that out of 2200 participants, 0.36% had TB, and 4.3% were positive for NTM (Asiimwe et al., 2013; Prevots and Marras, 2015). In the chronic TB group between 2007 -2008, a study conducted in Burkina Faso showed that from a total of 63 cases, 60.3% were positive for TB and 20.6% were positive for NTM. Similarly, in a study in Mali, where 61 patients were investigated, 18% tested positive for only NTM (Maiga et al., 2012; Prevots and Marras, 2015). A large proportion of both groups were predicted to have NTM-PD, but the studies reviewed by Prevots and Marras (2015) lacked adequate details regarding the proportion of patients who fulfilled the criteria for NTM disease.

A systematic review and meta-analysis of 37 studies showed that the overall prevalence of NTM-PD samples in sub-Saharan Africa was 7.5%. However, this study again found that

the lack of population details made estimating the prevalence difficult (Okoi et al., 2017; Hamed and Tillotson, 2023). The review by Okoi et al. (2017) found that in the suspected TB group, the isolation rate of NTM varies from 1.7% in Kenya to 39.1% in Nigeria. Ethiopia, South Africa, and Zambia show further variation with 2.7-15.1% isolation rates. In the chronic TB group, patients in Kenya had an overall prevalence of 42.4%; in South Africa and Zambia, the prevalence ranged from 59.3 to 100% (Adekambi et al., 2017; Okoi et al., 2017).

In Zambia, MAC, MABSC, and *M. nebraskense* were the most frequently isolated NTM species from clinical samples (Monde et al., 2018), whereas in Ghana, *M. fortuitum* (Pokam et al., 2022) and *M. intracellulare* (Otchere et al., 2017) were the species mostly isolated from human respiratory samples. In South Africa, 40% of all mycobacteria cultured were MAC (Hoefsloot et al., 2013). While there are some comprehensive studies in this area, it is hard to determine an overall prevalent NTM species due to regional variability.

1.4.4 Populations at Risk

NTM-PD is primarily associated with structural lung diseases and immunological disorders. Patients with chronic lung diseases such as CF, bronchiectasis, COPD, and primary ciliary dyskinesia are at a significantly increased risk of developing NTM-PD (Cassidy et al., 2009; Cowman et al., 2019; Sharma and Upadhyay, 2020). Patients with pre-existing chronic lung disease (like COPD) (Erasmus et al., 1999) or a history of smoking (Kwon and Koh, 2016) often exhibit fibrocavitory radiological presentation (Martinez et al., 2007), which shows rapid disease progression (Kwon and Koh, 2016) which can be indistinguishable from active TB infection (Erasmus et al., 1999; Martinez et al., 2007). This presentation is most often associated with *M. kansasii, M. malmoense* and *M. xenopi* infection. MAC can be associated with this presentation but is also present in different manifestations (Matveychuk et al., 2012; Zweijpfenning et al., 2017; Vande Weygaerde et al., 2019). This disease is distinguished by cavities found primarily in the upper lobe. The cavities may be small with associated centrilobular nodules; in some cases, pleural thickening is also present. The presence of cavities is a factor due to disease progression and a predictor of treatment failure (Zweijpfenning et al., 2017; Musaddaq and Cleverley, 2020).

Patients testing positive for NTM with no known pre-existing lung disease often show nodular-bronchiectatic radiological presentation. The most common aetiological agents are MAC (Dhillon and Watanakunakorn, 2000; Rao et al., 2016) and *M. kansasii* (Musaddaq and Cleverley, 2020). The disease is characterised by bronchiectasis, bronchial wall thickening, multiple nodules and mucus plugging with occasional focal consolidation (Martinez et al., 2007; Musaddaq and Cleverley, 2020). The disease progresses slowly (Moon et al., 2019) and can be found in females with the long and lean ectomorph body type who are post-menopausal. This group have a higher level of thoracic skeletal abnormalities (Kartalija et al., 2013; Cowman et al., 2019); this phenotype has been given the term "Lady Windermere's syndrome" (Reich and Johnson, 1992) and has led to speculation of hormonal changes playing a role in NTM disease susceptibility (Kartalija et al., 2013; Mirsaeidi and Sadikot, 2015).

Mycobacterium immunogen and MAC have been found to cause hypersensitivity pneumonitis (HP) in immunocompetent people with regular exposure to metalworking fluids (MWF) or hot tubs. HP is an interstitial lung disease associated with a hypersensitive immune response to inhaled antigenic agents, causing inflammation of the alveoli and distal bronchioles (Gordon et al., 2006; Chandra et al., 2013). Radiograph examination often shows a typical image but can show small, scattered, rounded and reticulonodular opacities and dense regions of infection (Musaddaq and Cleverley, 2020).

In addition to the risk factors categorising NTM radiological presentation, other factors are worth considering, such as low body mass index (BMI) in immunocompetent patients (Song et al., 2021) and vitamin D deficiency can impair the immune response to mycobacteria (Faverio et al., 2016). Comorbidities such as gastroesophageal reflux disease (GORD), diabetes, and chronic kidney disease can further increase the risk of NTM-PD (Lipman et al., 2020).

Extrapulmonary NTM disease can occur in various sites outside the lungs, including the skin, lymph nodes, and disseminated disease. The risk factors for extrapulmonary NTM disease are somewhat different from those for NTM-PD (Sharma and Upadhyay, 2020) and include Immunosuppression, autoimmune disorders, immunodeficiency, trauma, and recent healthcare exposures involving injections or surgery, which can also increase the risk of infections (Griffith et al., 2007; Sharma and Upadhyay, 2020; Ricotta et al., 2021).

Soft skin tissue infection (SSTI) these infections typically occurs in individuals who have compromised immune systems, underlying medical conditions, or damaged skin barriers caused by surgical procedures (Xu et al., 2019), trauma (Hsiao et al., 2011), or burns (Boyer et al., 2010). The infection can also involve subcutaneous tissues and muscles and manifest as cellulitis, abscesses, or ulceration (Akram and Attia, 2023). Most NTM SSTI infections are associated with the RGM group with MABSC (Gonzalez-Santiago and Drage, 2015), *M. chelonae* (Akram et al., 2023) and *M. fortuitum* (Gutierrez and Somoskovi, 2014). The SGM *M. marinum* has been known to cause "Fish-tank" granuloma (Swift and Cohen, 1962), where Patients often exhibit groups of nodules, ulcers, or wart-like plaques. These can spread red or pink lumps from the arms or legs towards the body's centre (Wu et al., 2012). *M. ulcerans* is known to cause the Buruli ulcer (Yotsu et al., 2018). This infection starts as a cutaneous lesion that necrotises and progresses to painless ulcers. The World Health Organization (WHO) deemed this infection a neglected tropical disease that is treatable with the antibiotics rifampin and clarithromycin (Omansen et al., 2019).

From these primary sites of infection, NTM infection can spread throughout the body; disseminated infection occurs when localised infection spreads from its original area to the rest of the body or other organ systems. This disease progression creates an issue regarding containing and treating the disease (Ingram et al., 1993). Most opportunistic NTM species can cause disseminated disease (Griffith et al., 2007) and infection mainly occurs in individuals with advanced HIV disease or other forms of severe immunosuppression (Chai et al., 2022) associated with low CD4+ lymphocyte counts and impaired granulocyte function (Nightingale et al., 1992). The infection can cause multiple erythematous-violaceous nodules on the body, which can be inflamed, ulcerated or open wounds (Brown-Elliott et al., 2001). Disseminated *M. chelonae* can also cause extreme illness in patients suffering from peritoneal dialysis peritonitis (PDP) (Hayat et al., 2022). PDP is the inflammation of the inner lining of the abdomen which occurs in patients undergoing peritoneal dialysis (Salzer, 2018); when infection with *M. chelonae* occurs, it often displays symptoms that are indistinguishable from other bacterial peritonitis infections (Hayat et al., 2022).

Lymphadenitis occurs when one or more lymph nodes become infected due to disseminated disease (Gosche and Vick, 2006). MAC is the NTM most frequently isolated from children suffering from cervical lymphadenitis (Akram and Attia, 2023).

lymphadenitis mainly affects young children and HIV-infected individuals, causing painless, firm, and enlarged lymph nodes. (Griffith et al., 2007; Tebruegge et al., 2016). The disease is usually self-limiting in immunocompetent individuals, but in immunocompromised individuals, it can be chronic and require antibiotic treatment (Griffith et al., 2007).

1.4.5 NTM Physiology

The mycobacteria cell wall (Figure 1.2) is intricate. It has different layers each with their own roles. Firstly, a capsule is present which is composed of polysaccharides with an abundance of α-glucan followed by arabinomannan and mannan (Rastogi et al., 1986). In the inner part of the capsule lipids have been found, which possibly interact with mycolic acids (Ortalo-Magné et al., 1996). The role of the capsule is thought to initiate the first stages of infection and the polysaccharides are understood to be virulence factors (Wittkowski et al., 2007) In *M. tuberculosis* it has been determined that the capsule has several roles including the prevention of desiccation, attachment to host cells and resistance to host immunity by the down regulation of inflammatory cytokines (Kalscheuer et al., 2019). These traits, however, are not necessarily shared throughout the *Mycobacterium* genus as specific capsular composition has been shown to differ between species. Production of extracellular material (ECM) also shows variances. In *M. kansasii* high levels of carbohydrates were detected in the ECM and surface exposed materials (SXM). Whereas *M. avium, M. gastri, M. phlei* and *M. smegmatis* produce smaller amounts of ECM that are not relative to the SXM composition. This reflects some of the differences between the species such as growth rate or pathogenicity (Lemassu et al., 1996).

The cell wall is composed of the outer membrane, arabinogalactan, and peptidoglycan layers. It has a cell-wall skeleton that has a range of non-covalently linked lipids, glycolipids and proteins that occur here are in smaller amounts than found in the capsule. The skeleton defines the shape of the cell due to its existence as a large macromolecule (Daffe and Draper, 1998). The waxy outer membrane also known as the "Mycomembrane" due to its large quantity of mycolic acids (Hoffmann et al., 2008; Zuber et al., 2008). Mycolic acids are high molecular weight, long chain fatty acids with a β-hydroxyl-α-alkyl branched structure (Stodola et al., 1938). Found in two basic forms: covalently bound to the cell wall and loosely associated to a variety of carbohydrate containing molecules (Barry et al., 1998). Its presence contributes to the conservation of

the cell wall structure, resistance to chemical damage, low permeability to hydrophobic antibiotic substances, inhibits dehydration and the ability to resist the hosts macrophage phagosome (Barry and Mdluli, 1996). Small differences in mycolic acid composition have been found between species, which could again contribute to the major differences between them (Barry et al., 1998; Watanabe et al., 2001). The mycomembrane is attached to arabinogalactan which is covalently connected to the peptidoglycan via phosphodiester bonds (Chiaradia et al., 2017). This section also houses free lipids: cord factor- trehalose dimycolate and a range of phospholipids and glycolipids (Bayan et al., 2003) The cord factor is fundamental in the pathogenesis of Mycobacteria and plays a role in the formation of granulomas, originally thought to only be found in *M. tuberculosis*(Silva et al., 1985). This formation has also been demonstrated in some morphotypes of *M. abscessus* (Sánchez-Chardi et al., 2011) and *M. marinum* (Staropoli and Branda, 2008). Arabinogalactan situated in the periplasm is mostly composed of arabinose and galactose constitutes. Its structure can be further divided into three separate segments: the linker unit, galactan and arabinan. Its role is to add structure to the cell in unison with the peptidoglycan layer in which it is attached (McNeil et al., 1990) The peptidoglycan's knitted structure gives rigidity to the cell, providing the ability to resist osmotic pressure preserving cell structure, like *Escherichia coli* (Heijenoort, 2001). The peptidoglycan forms the base layer of the mAGP complex (Figure 1.2) which is comprised of interchanging N-acetylglucosamine (GlcNAc) and N-acetyl-muramic acid (MurNAc) residues linked in a β(1→4) formation (Lederer et al., 1975).

Also residing in the periplasm are the lipomannan (LM), lipoarabinomannan (LAM) and the mannose capped lipoarabinomannan (Man-LAM) (Besra and Brennan, 1997). Present in a few *Mycobacteria* species: MAC, *M. kansasii, M. fortuitum, M. chelonae* and *M. smegmatis* (Nigou et al., 2003). Non-covalently attached to the inner phospholipid membrane. Vital for cell wall stability and a virulence factor of some mycobacteria species (Besra and Brennan, 1997; Fukuda et al., 2013). LM and phosphatidyl-myo-inostiol mannosides (PIMs) are the precursors of LAM. LAM modulates the immune response through inducing the secretion of cytokines; interleukin (IL)-8, IL-12, tumour necrosis factor-α (TNF) and apoptosis. Man-LAM are responsible for the integration in to host plasma membranes and a range of macrophage and cytokine modulation roles (Nigou et al., 2003).

The *Mycobacteria* cell wall is complex and provides the organism with a range of functions. Cell wall stability being the main function which helps to increase the organism's resistance to antimicrobials and environmental pressures. Its role in pathogenesis and host immune response stimulation also enables these bacteria to survive.

1.4.6 Mechanisms of infection

NTMs are opportunistic pathogens found in nearly all natural and manufactured systems. Sources of transmission are from the environment by ingestion, inhalation, or direct skin contact with Inhalation of aerosols created from plumbing systems, swimming pools and other water or dust sources is the most likely route of transmission (Falkinham, 2009; 2013). Human-to-human transmission is also possible, with several studies examining the CF population confirmed this via molecular detection methods examining relatedness between strains. One of the factors that enable mycobacteria to gain entry to a host is its mycolic acid membrane (Figure 1.2), which provides it with a high level of hydrophobicity; this mechanism helps the NTM to become an aerosol and attach to surfaces (Falkinham, 2009; Faria et al., 2015).

The *Mycobacterium* infection persists by using tactics to evade and capture the host immune system, enabling disease progression (Habtamu et al., 2022). The survivability of mycobacteria within the host is highly dependent on their ability to reside in and avoid the engulfment of host macrophages (Echeverria-Valencia et al., 2018), however, there are marked differences between the SGM and RGM groups (To et al., 2020). Forming biofilms and NTM-mediated inhibition of inflammatory cytokine production promotes colonisation and epithelial invasion (Honda et al., 2015). NTM can attach to bronchial and intestinal epithelial cells via the fibronectin-attachment protein (FAP) (Honda et al., 2015) or the hemagglutinin binding protein (Menozzi et al., 1998) found on the surface of MAC. The FAP interacts with the host fibronectin and creates a connection binding to integrin receptors located on the surface of mucosal cells. Once entry into the cell has occurred, the *Mycobacterium* undergoes a phenotypic change, increasing its ability to invade macrophages (McGarvey and Bermudez, 2002; Honda et al., 2015). *M. kansasii* can encode the type-VII secretion system ESX-1 locus, involved in the secretion of several effectors like EsxA (ESAT-6) and EsxB (CFP-10), through its ESX conserved components, called Ecc proteins (Wang et al., 2015; Tagini et al., 2021). ESX-1-secreted proteins are thought to modulate the activation of macrophages, allowing phagosomal escape and

induce necrosis or apoptosis (Gröschel et al., 2016; Tagini et al., 2021). Additionally, some *in vivo* experiments showed *M. kansasii' s* ability to disrupt the phagosomal membranes of hosts (Wang et al., 2015) and translocate to the cytosol as means of escape (Houben et al., 2012).

The RGM MABSC can exist in two different morphotypes: a smooth (S) variant and a rough (R) variant. It can transition between the morphotypes to ensure the best chance of survival (Jönsson et al., 2007; Rüger et al., 2014). This transition is mediated by the presence/absence of surface-associated glycopeptidolipids (GPL). This existence or non-existence considerably influences the physiological and physiopathological abilities of MABSC. The GPL have various functions, including biofilm formation, host-cell interaction and intramacrophage trafficking (Howard et al., 2006; Medjahed et al., 2010; Gutiérrez et al., 2018).

The S variant is characterised by a smooth colony morphology, with GPL and is the colonising form (Ryan and Byrd, 2018). It can produce mature biofilms and exhibits significant motility when cultured on soft agar (Howard et al., 2006; Le Moigne et al., 2019). It colonises host internal surfaces at high loads and tends to be the more dominant morphotype during infection (Hortle et al., 2019). It shows higher survival rates than the R variant due to early granuloma formation and engagement of T-cells. The S variant can survive within a macrophage phagosome for extended periods, which displays chronic and persistent infection (Bernut et al., 2014; Hortle et al., 2019). The S variant switches to the R variant in response to changes in environmental conditions and gains heightened virulence characteristics (Howard et al., 2006; Ryan and Byrd, 2018).

A rough colony morphology characterises the highly virulent R variant, has absent GPL, cannot form biofilms, and is associated with aggressive disease (Catherinot et al., 2009; Clary et al., 2018). The R variant infection penetrates host organs, causing an inflammatory immune response, forming a necrotic granuloma within 14 days (Kam et al., 2022). This phenotype also showed similar virulence factors to MTB with the formation of corded invasive microcolonies enabling persistent infection (Byrd and Lyons, 1999; Clary et al., 2018; Ryan and Byrd, 2018). Both morphotypes have been implicated in various diseases (Clary et al., 2018; Kam et al., 2022).

MABSC is known to modulate host immune responses, triggering a proinflammatory response that contributes to chronic inflammation and tissue damage (Kim et al., 2020). It

is also able to acquire resistance to antibiotics through various mechanisms, including intrinsic resistance, efflux pumps, and acquired resistance genes (Luthra et al., 2018)

Beyond the MAC and MABSC other NTMs are important as well for example: *Mycobacterium fortuitum* has a unique ability to grow in high NaCl concentrations of up to 8% and has unique genes, which enhances its survivability in various environments. This study also found that more host-adapted *Mycobacterium,* such as MTB or MAC, are less tolerant to high NaCl concentrations, suggesting that this niche may contribute to *M. fortuitum' s* pathogenicity (Asmar et al., 2016). Like other NTMs, *M. fortuitum* can survive under a range of stressors via the formation of biofilms. These biofilms increasingly resist routinely used biocides such as chlorohexidine acetate, H_2O_2, bleach, glutaraldehyde, and others (Bartos et al., 2004; Sharma et al., 2023). These biofilms are known to form under nutrient-dense and poor conditions and showed enhanced resistance to the amikacin, gatifloxacin and tobramycin antibiotics. The treatment of the biofilm with DNase showed a reduction in its mass, suggesting that environmental DNA (eDNA) plays a role in maintaining the structure of the biofilm (Aung et al., 2016). Forming biofilms enables the *M. fortuitum* to cause healthcare-associated infections, allowing it to thrive on medical equipment and biomaterials (Sharma et al., 2023).

Further research into *M. fortuitum*' s pathogenesis showed that *M. fortuitum* species with higher levels of porins are associated with faster growth, allowing it to increase its numbers and virulence. Nevertheless, the increase in porins is a double-edged sword for *M. fortuitum* as it has also shown increased susceptibility to some antibiotics (Sharbati et al., 2009). *M. fortuitum*, MABSC and MTB share the ability to form extracellular cords, increasing virulence by enabling intracellular macrophage survival, extracellular replication and the formation of granulomas and abscesses for host immune system evasion (Bernut et al., 2014; Kalsum et al., 2017; Cornejo-Granados et al., 2021).

The combination of these factors makes *Mycobacterium* genus an effective opportunistic pathogen contributing to its emergence.

1.4.7 Diagnosis of NTM-PD

The non-specific clinical presentation of NTM infections complicates the diagnostic process. Symptoms of NTM-PD often mimic those of other respiratory conditions, making it difficult to identify based on clinical presentation alone (Gopalaswamy et al., 2020;

Musaddaq and Cleverley, 2020). Moreover, the difficulty in growing NTMs from biological specimens adds another layer of complexity (Baldwin et al., 2019).

The current recommended diagnostic protocol for identifying NTM and classifying patients as having NTM-PD was set out in 2007 by The American Thoracic Society and The Infectious Diseases Society of America (ATS/IDSA) and updated in 2020. It focuses on adult patients who do not have CF or HIV (Griffith et al., 2007; Daley et al., 2020).

The guidelines state three minimum evaluation criteria for patients to undergo:

1. Chest radiograph or a high-resolution computed tomography (HRCT) scan of the chest if there is no cavitation.
2. Three or more respiratory samples to have acid-fast bacilli (AFB) analysis.
3. Elimination of the possibility of similar infections like TB

Following an initial consultation, If NTM-PD is suspected, the doctor will perform a physical exam and measure spirometry. Then, a microbiological investigation and chest imaging are requested to determine any NTM positivity and structural changes to the lung tissue (Griffith et al., 2007; Van Ingen, 2013). The patient must produce at least two culture-positive sputum samples and/or one positive sample obtained from bronchoalveolar lavage (BAL) or lung biopsy; this is done either before or alongside chest imaging. A specialist referral is recommended if the radiograph or HRCT shows nodular or cavitary opacities on the image (Griffith et al., 2007; Parada et al., 2016; Ryu et al., 2016).

This process is often lengthy and has several limitations. Microbial investigation must be performed on early morning samples produced on different days; if the patient cannot spontaneously produce sputum, then sputum induction is recommended (Griffith et al., 2007; Daley et al., 2020). This procedure is done in the clinic (Pizzichini et al., 2002). The specimen must be then decontaminated to reduce the chance of contamination or overgrowth from other microorganisms, and this must be done with care as to minimise the accidental removal of the NTM (Kent, 1985; Griffith et al., 2007; Pfyffer and Palicova, 2011). The sample is then stained for AFB using the auramine-rhodamine fluorochrome staining technique (Griffith et al., 2007). The dye binds to the mycolic acid and fluoresces under UV light (Wanger et al., 2017). If needed, the Ziehl-Neelson or Kinyoun methods are also acceptable but less sensitive (Peterson et al., 1999; Griffith et al., 2007). Notably, these staining techniques cannot differentiate between rapid- RGM and SGM, and a

negative smear does not necessarily denote a negative sample (Griffith et al., 2007). The sample is then cultured on solid and liquid media and, depending on the NTM present, can take up to 12 weeks if an SGM is suspected (Griffith et al., 2007; Daley et al., 2020). Just establishing if an NTM is present takes a long time, significantly delaying treatment and could lead to increased severity of the disease (van der Laan et al., 2022).

Meanwhile, in the UK, as of March 2023, the reported average waiting time for CT and other imaging tests is at least <4 weeks from the date the referral was received (NHS England, 2023). Additionally, when examining radiographic or HRCT images, it can be challenging to differentiate NTM-PD from TB due to the similar symptoms produced, requiring an experienced practitioner, and confirming other diagnostic processes (Ryu et al., 2016; Yan et al., 2022). Using radiographic imaging also exposes patients to additional radiation risks (Ryu et al., 2016).

Following chest imaging and initial microbiological investigation, there is still a need to identify the NTM species responsible for infection; this is imperative as NTM species have different antibiotic susceptibility profiles (Brown-Elliott et al., 2012). Species identification is initially done phenotypically and biochemically via growth rate and pigmentation following isolation. However, these traditional methods often omit the newer species of NTM, and it is recommended that molecular methods be used in conjunction with these tests (Griffith et al., 2007).

1.4.8 Molecular Methods Currently Used in NTM Species Identification

Chemotaxonomic testing: This method aims to identify NTM based on their mycolic acid composition. Testing is achieved using High-Performance Liquid Chromatography (HPLC) (Butler and Guthertz, 2001). HPLC is a rapid, reproducible method used routinely in many laboratories. However, in some cases, this method has failed to differentiate species within the same taxonomic group (Butler and Guthertz, 2001; Griffith et al., 2007; Conte et al., 2015). HPLC is expensive and requires specially trained personnel to operate (Butler and Guthertz, 2001; Conte et al., 2015) and is recommended to be used in conjunction with other identification methods like PCR (Griffith et al., 2007; Conte et al., 2015).

Genotypic identification of NTM: This identification technique targets the 16S rRNA gene using commercially available molecular probes. This method can be carried out on solid or liquid culture, and identification can be achieved within 2 hours. It is highly specific but can only be used on some NTM species (Griffith et al., 2007). While easy to perform, this

method can lack discriminatory power, leading to potential misidentification (Daley et al., 2020).

PCR- restriction enzyme analysis (PRA): Identifies NTM via the amplification of the heat shock protein 65 gene (*hsp*65), which is then analysed by restriction digest (Telenti et al., 1993). This method does not require viable organisms and can detect a range of NTM species that are not detectable by phenotypic or chemotaxonomic techniques; however, this method is used mainly in reference laboratories and not in clinical settings (Griffith et al., 2007).

Gene sequencing: It can detect NTM in a sample down to the sub-species level but is only accessible to those with sequencing facilities. This method has rapidly evolved since the previous ATS guidelines were released in 2007 (Daley et al., 2020). Several newer target genes are described as suitable for NTM detection: 16S rRNA, *hsp*65, *rpo*B and ITS. However, the 16S rRNA gene has been shown to lack discriminatory power and should be used in conjunction with the other targets mentioned (Adékambi et al., 2003; McNabb et al., 2004; De Zwaan et al., 2014; Daley et al., 2020).

While rapid, the newer molecular methods of NTM species detection are often expensive, making them unattainable for healthcare providers in lower-income countries. These techniques are often not offered until initial radiography or positive culture has been provided (Griffith et al., 2007; Daley et al., 2020).

1.4.9 Why are NTMs important in CF?

The impact on the health of PWCF colonised with NTMs is variable; like other lung conditions, the severity is dependent on the species of NTM present. The clinically meaningful MAC and MABSC are the most consistently isolated NTM in CF (Olivier et al., 2012; Skolnik et al., 2016; Sharma and Upadhyay, 2020). Detection of NTM groups is increasing in CF sputum samples; MAC is more commonly identified in patients residing in North America (Floto et al., 2016), and MABSC is more commonly isolated in the UK (The Cystic Fibrosis Trust, 2009). Studies conducted during low MABSC infection found genetically exclusive isolates from specific patients Indicating that the strains were picked up independently from the environment and not passed between patients (Bryant et al., 2016). Other research speculates that infectious clones have emerged and spread into patient groups through transmission events, possibly via cross-infection from fomites (Bryant et al., 2013).

Geographical location is not the only factor in the type of NTM acquired; age also plays a part. The occurrence of NTM infection has increased in tandem with the life expectancy of CF patients (Cullen et al., 2000), and its emergence tends to be more common in adults (Viviani et al., 2016). Incidence increased from 10% in children aged ten years to over 30% of adults aged 40 and above, with more than 50% of adults diagnosed to be female. Adults aged 25 and above were found to have MAC isolated more routinely from sputum cultures. MABSC was common across all age groups, particularly in adolescents aged 11-15 (Roux et al., 2009; Floto et al., 2016). There has been a noticeable increase in NTM prevalence in respiratory samples from CF patients, making NTM a rising concern for patients living with CF (Caverly et al., 2016; Floto et al., 2016).

Colonisation with NTM in PWCF can be transient, and active disease may not always develop (Skolnik et al., 2016). However, some patients who exhibit active NTM-PD have also shown a marked decline in lung function and increased episodes of exacerbation (Martiniano et al., 2014; Caverly et al., 2021). Patients positive for NTM-PD are contraindicated for lung transplant due to NTM's natural resistance to antimicrobial therapeutics (Leard et al., 2021). MAC lung infection is often associated with less aggressive disease and better pulmonary function than MABSC, which is associated with rapid lung function decline and poorer patient outcomes. Detection and treatment of NTM infection in CF is essential to stem disease progression and prevent further damage to the respiratory system (Qvist et al., 2016; Skolnik et al., 2016). Identifying the role of NTM and how it influences CF-associated lung infection could provide insight into the pathogenesis and management of the disease. However, studies in this area are limited (Thornton et al., 2021).

1.4.10 NTM Treatment

Treatment for NTM is often a prolonged course of multiple hydrophilic antibiotics: macrolides, ethambutol, aminoglycosides, and rifampicin. When treating patients with CF, careful considerations must be made: Is the patient currently on antibiotic therapy for prophylaxis? Drug - drug interactions? (Section 1.4): Effects on the patient? What NTM complex is present? What are the NTM sensitivities and the lung's health? (Lambert, 2002).

According to NHS guidelines 2019/2020, the treatment of MAC is with the combination of three oral antibiotics (Table 1.1) (macrolides, rifampicin, aminoglycosides, or

ethambutol). If there is culture conversion the treatment will continue for 12 months. It also recommends azithromycin as the macrolide of choice due to fewer interactions (Floto et al., 2016; Alexander et al., 2020).

MABSC, which is usually multidrug resistant, is treated in two phases (Table 1.2): I. induction phase, which lasts 3 weeks, and intravenous antibiotics are administered. II. Consolidation is carried out over 18-24 months and includes 4 medications that are a combination of oral and nebulised treatment. The dosage and type of antibiotics are dependent on the sensitivities of the complex (Floto et al., 2016; Alexander et al., 2020).

Macrolides are commonly used for infection control and general CF management due to anti-inflammatory and immunomodulatory activity (Binder et al., 2013). They are often used to treat Gram-positive bacterial infections but come in a variety of structures treating a range of infections (Dinos, 2017). Macrolides bind to the large ribosomal subunit, inhibiting protein synthesis and stopping cell growth. Some small-scale studies found that long-term macrolide treatment for CF management reduced the occurrence of NTM infection (Coolen et al., 2015). However, some incidence of macrolide resistance in MAC has been reported in some populations (Morimoto et al., 2016). As for MABSC, it has commonly expressed macrolide resistance, which is caused by the methylation of the *erm41* gene at times of exposure (Skolnik et al., 2016). This could have serious consequences in the management of NTM in patients with CF.

Ethambutol works by stopping the mycolic acid transfer into the *Mycobacterium* cell wall. It is often prescribed as a combination with a macrolide to inhibit the acquirement of resistance. This treatment is considered the most effective for MAC infections (Field and Cowie, 2003). MABSC is naturally resistant to ethambutol due to the variant nucleotides within the ethambutol resistance-determining regions (ERDR) located in the embCAB operon (Nessar et al., 2012). Ethambutol must be used with caution due to its links with ocular toxicity, which can manifest as blurred vision, impaired red-green colour discrimination and peripheral visual field defects. It is recommended that patients under this regimen have regular vision testing (Floto et al., 2016).

Rifampicin is part of the usual MAC treatment schedule; it is often prescribed with ethambutol and macrolides. Once rifampicin enters the cell, it binds to the β-subunit or *rpoB* (DNA-dependent RNA polymerase), forming a stable complex which suppresses chain formation in RNA synthesis, preventing cell growth (Brunton et al., 2011). For the

treatment of MABSC, it has been shown that *M. abscessus* creates enzymes that can alter rifampicin resulting in its inactivation. ADP-ribosyl transferase and a monooxygenase could be linked to *M. abscesses* resistance to rifampicin (Nessar et al., 2012).

Aminoglycoside exhibits activity against Gram-negative bacteria and is often given with other antibiotics to treat MAC infections. MABSC shows inherent resistance due to the presence of enzymes that transfer acetyl or phosphate residues, leaving this class of antibiotics inactive (Nessar et al., 2012). The use of aminoglycosides is often recommended in patients with severe cavity disease (caused by aspergillosis or bronchiectasis) to improve patient outcomes (Skolnik et al., 2016).

The combination of these drugs each with a range of targets will have a major effect on the patient's natural microbiome and possibly give rise to other known CF pathogens.

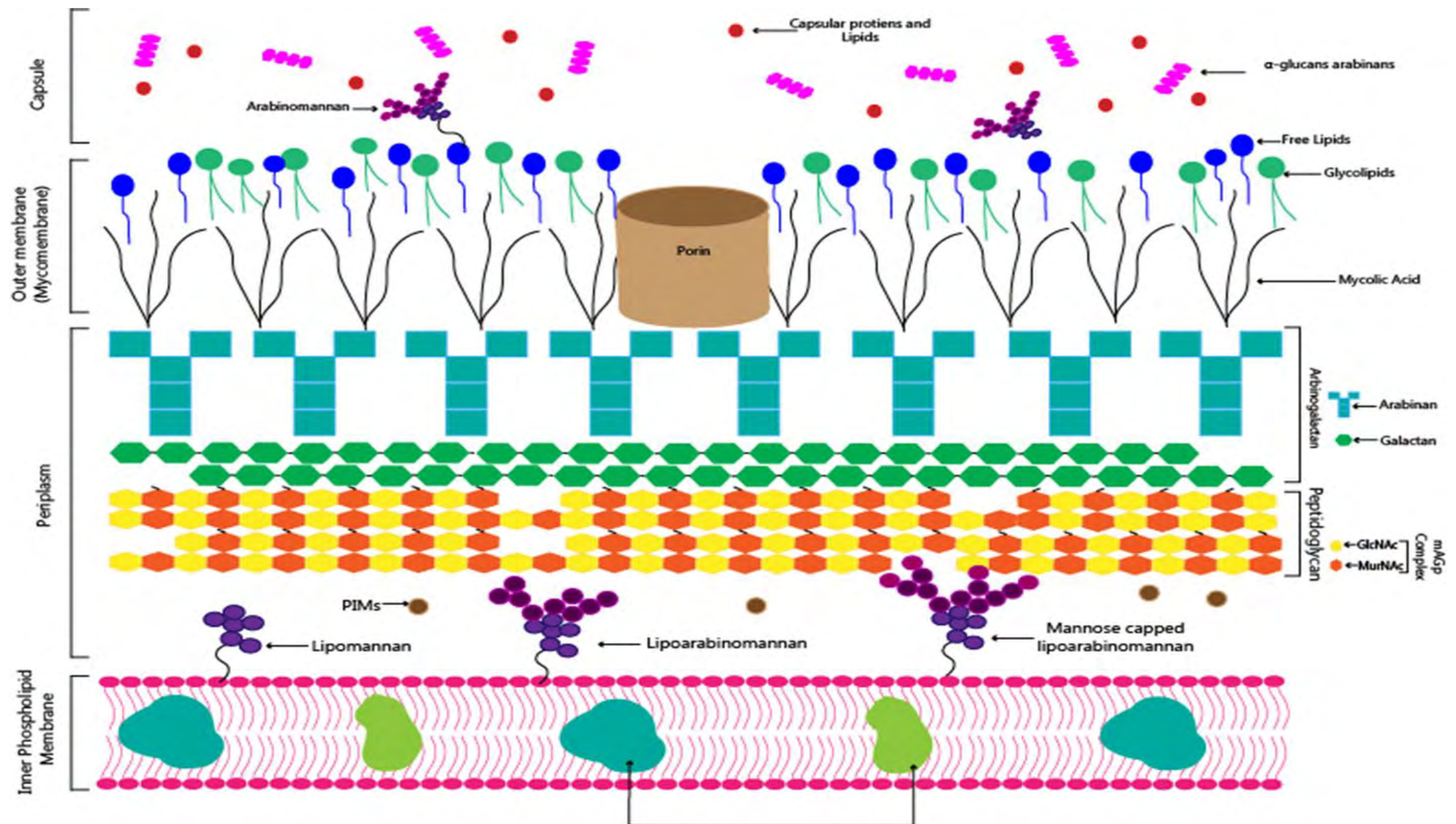


Figure 1. 2 Schematic of the Mycobacteria cell wall, a detailed view of the layers and structures making up the cell wall in *mycobacterium*. The diagram illustrates the mycolic acid layer in the outer membrane with the glycolipids and free lipids attached. It also shows the structure of the inner phospholipid membrane (Chapter 1, section 1.4.5). Adapted from (Crick et al., 2010; Kleinnijenhuis et al., 2011; Bhat et al., 2017).

Table 1. 1 MAC Antibiotic Treatment Schedule

NTM	Susceptibility testing	Treatment prescribed	Treatment interval	Treatment duration
MAC non-severe pulmonary disease	Clarithromycin and amikacin. Macrolide resistant strains should be tested against a wider range of antibiotics.	Rifampicin 600mg, ethambutol 25mg/kg and clarithromycin 500mg or azithromycin 1g divided into two doses	Three times a week	12 months minimum after culture conversion
Severe MAC pulmonary disease		Rifampicin 600mg and ethambutol 15mg/kg and Azithromycin 250mg or clarithromycin 500mg. Consider amikacin intravenous or nebulised	Daily except clarithromycin which is twice a day.	
Clarithromycin MAC-pulmonary disease		Rifampicin 600mg and ethambutol 15mg/kg and Isoniazid 300mg (pyridoxine 10mg) or Moxifloxacin 400mg. Consider IV or nebulised amikacin.	Daily	
M. kansasii Rifampicin sensitive	Rifampicin. Rifampicin resistant strains should be tested against a wider range of antibiotics	Rifampicin, ethambutol and isoniazid or a macrolide.	Daily	
M. kansasii rifampicin resistant		Consider sensitivities when prescribing. Three drug regimens should be used	Daily	
M. malmoense		Rifampicin, ethambutol, and macrolide. IV or nebulised aminoglycoside should be considered	Daily	
M. xenopi		Four drug regimens where tolerated. Rifampicin, ethambutol, and a macrolide together whit quinolone or isoniazid. IV or nebulised aminoglycoside for severe disease		

MAC antibiotic treatment schedule. This table highlights the treatment schedule for *Mycobacterium avium* complex (MAC), and other common non tuberculosis mycobacteria species found in pulmonary infection. It states the antibiotic sensitivities where known. The dosage given under the treatment schedule, the treatment interval and the total duration of the treatment adapted from (Haworth et al., 2017)

Table 1. 2 MABSC Antibiotic Treatment Schedule

MABSC	Sensitivities	Oral	Intravenous	Nebulised	Treatment duration
Initial phase. IV treatment duration is dependent on infection severity	Clarithromycin sensitive	4 weeks clarithromycin or azithromycin	4 weeks Amikacin, Tigecycline, Where tolerated imipenem	Amikacin in place of IV where admiration is impractical or contraindicated.	Minimum of 12 months post culture conversion. However. Culture conversion failure may benefit from a long term suppressive antibiotic regimen.
	Macrolide resistance		4 Weeks amikacin, tigecycline and where tolerated imipenem		
	Amikacin resistant (16S rRNA mutation)		Amikacin substitute	Amikacin substitute	
Continuation phase	Clarithromycin sensitive	Macrolide combined with one to three of the following clofazimine, linezolid, minocycline or doxycycline , moxifloxacin, or ciprofloxacin and co-trimoxazole		Amikacin	
	Macrolide resistant	Two to four combined guided on sensitivities: clofazimine, linezolid, minocycline or doxycycline, moxifloxacin, or ciprofloxacin and co-trimoxazole		Amikacin	
	Amikacin-resistant (16S rRNA mutation)			Amikacin substitute	

MABSC antibiotic treatment schedule. This table highlights the treatment schedule for *Mycobacterium abscessus* complex (MABSC). It states the antibiotic sensitivity profiles and the recommended treatment and administration route with the total treatment duration. Adapted from (Haworth et al., 2017).

1.4.11 NTMs and Their Role in the CF Microbiota

The existence of NTMs and their relationship with other CF pathogens and the diversity of the CF microbiome has not been a central research focus, despite evidence showing that CF lung infection is polymicrobial (Rogers et al., 2006; Maughan et al., 2012; Rogers et al., 2013; Cuthbertson et al., 2020). Previous studies examining the interaction between NTM populations and NTM pulmonary disease in CF microbiomes are sparse. The specific role of NTM in the microbiota is an area which has not been studied yet.

Caverly et al. (2021) examined CF sputum samples collected 3.5 years prior and up to 6 months following an NTM infection. The study found a positive correlation between NTM-PD and taxa from the *Pseudomonas, Streptococcus, Veillonella, Prevotella and Rothia* genera, indicating that patients positive for these bacteria are more likely to be diagnosed with NTM-PD and have long-lasting infections. This study showed differences between the clustering of taxa in patients positive for NTM infection and those who are not, suggesting that NTM could alter the microbiota composition. In patients with transient NTM infection compared to those with persistent infection (Caverly et al., 2021).

Other research examining metabolic profile differences in sputum associated with NTM infection in CF found an increased concentration in 2-methyl citrate/homocitrate and selected ceramides. These biomarkers are thought to influence microbial growth and competition during exposure. Furthermore, these metabolites can also affect the host-microbe interactions by potentially influencing the immune response, which could alter the microbiota composition (Breen et al., 2022).

A review by Gannon and Darch (2021) discussed the impact of the emerging pathogens NTMs and *Aspergillus fumigatus* in CF populations. Focusing on understanding the current research surrounding their interactions with established microbial communities. The review found No research focusing on NTM and *A. fumigatus* interactions. However, it highlighted that both organisms have been linked to poor health outcomes in CF patients and that there is a link to NTM-positive cultures and NTM-PD with the presence of *A. fumigatus* and patients positive for ABPA are more likely to produce an NTM-positive culture (Floto et al., 2016; Viviani et al., 2016; Gannon and Darch, 2021). The review also emphasised the relationship between *P. aeruginosa* and MABSC in biofilms previously studied by Rodriguez-Sevilla et al. (2018). The research did postulate that *P. aeruginosa* biofilm could shield the NTM from detection and increase its resistance to antimicrobials

(Rodriguez-Sevilla et al., 2018; Gannon and Darch, 2021). The same review also exposed the need for research in the area of how NTM positivity could affect microbiome host-microbe interactions and the limited understanding of how microbial communities are assembled within the CF lung (Gannon and Darch, 2021).

Interestingly asymptomatic patients who were positive for MAC showed that *P. aeruginosa* was absent in 16S sequencing results. However, they found no differences in composition regarding other microbial flora present, implying that the presence of MAC may affect the composition of the microbiota, explicitly concerning *P. aeruginosa* (Iwasaki et al., 2021). Conversely, the study by Rodriguez-Sevilla et al. (2018) showed that MABSC and *P. aeruginosa* had a negative relationship, with *P. aeruginosa* having higher population numbers in biofilms than MABSC in CF populations (Rodriguez-Sevilla et al., 2018).

There is a commonality between the studies regarding the taxa present in NTM-positive samples; all the studies mentioned found taxa from the *Prevotella, Streptococcus* and *Neisseria* genera (Yamasaki et al., 2015; Sulaiman et al., 2018; Philley et al., 2019; Iwasaki et al., 2021). However, there are differences between the overall taxa present, suggesting a unique bacterial community residing within each patient. There is a commonality between studies regarding the taxa present in NTM-positive samples; all the studies mentioned found taxa from the *Prevotella, Streptococcus* and *Neisseria* genera (Yamasaki et al., 2015; Sulaiman et al., 2018; Philley et al., 2019; Iwasaki et al., 2021). However, whilst there are differences between the overall taxa present evidence is repeatedly reported that the presence, and type, of NTMs significantly alters the composition of the microbiome (Sulaiman et al., 2018; Thornton et al., 2021; Kim et al., 2023).

1.5 Knowledge gaps

This introduction has highlighted that some NTMs can cause persistent and aggressive infections in a wide group of people (Floto and Haworth, 2015; Schiff et al., 2019; Caverly et al., 2021). They cause the most significant harm in immunocompromised individuals such as PWCF (Viviani et al., 2016). Delays in diagnosis can lead to prolonged exposure to the disease, can cause further damage to the patient and have implications on their future health (Griffith et al., 2007; Martiniano et al., 2014; Floto et al., 2016; Ryu et al., 2016; Daley et al., 2020; Daley and Winthrop, 2020). Once diagnosis and treatment are acquired, eradication is difficult because some NTM species are intrinsically resistant to antibiotics, making them notoriously hard to treat resulting in a course of aggressive antibiotics (Jeon et al., 2009; Griffith et al., 2015; Griffith, 2019; Daley et al., 2020), which could enable other opportunistic pathogens to incite disease, leading to a cycle of reinfection, ultimately impacting patient health leading to poor outcomes.

There is the need for research in various areas to improve the clinical outcomes of patients suffering from NTM-PD.

The current diagnostic protocol for NTM-PD in both CF and generally lacks efficiency regarding the time it takes from the onset of symptoms to treatment. A faster-targeted approach would reduce the time diagnosis takes ultimately improving patient outcomes.

The change in microbiota composition in the presence of NTM species is an area where research is lacking, especially in CF, where patients are extremely prone to lung infection. Knowing how NTMs influence the presence of other canonical CF pathogens would be insightful to understanding disease progression in CF-associated airway disease. This knowledge could also be applied to help understand how the presence of NTMs affects other disease systems, such as COPD or bronchiectasis. This understanding could influence treatment practices, leading to improved treatment regimens.

Recently, a new area of study has emerged that is still in its infancy, the interplay between NTM colonisation and the changes made to the microbiota by CFTR modulators. Very little is understood about the associations and interactions that may be occurring. What is known is that modulators effectively alter the pulmonary environment, which could lead to a shift in the organisms present (Ricotta et al., 2022) (Yi et al., 2021). The introduction of modulators may also impact the current methods of diagnosis, with patients on modulator therapy producing less spontaneous sputum, leading to NTMs, and

other potential pathogens, to go undetected (Rogers et al., 2020). To mitigate this, there is a clear need for more efficient, faster diagnostics. Understanding how the presence of NTMs in the microbiota changes the microbial community in patients taking CFTR modulators and those who are not can lead to understanding the changes in lung function and disease severity. It is also insightful to investigate these changes in relationship to other patient demographic data such as gender, age, and CF genotype, which provide the bigger picture when assessing the importance of NTM in CF-associated airway disease.

Moreover, observing how the presence of NTMs in the microbiota can change the microbial community in patients taking CFTR modulators and those who are not will lead to a better understanding the changes in lung function and disease severity. It is also insightful to investigate these changes in relationship to other patient demographic data such as gender, age, and CF genotype, which provide the bigger picture when assessing the importance of NTM in CF-associated airway disease.

There needs to be more understanding of the interplay between host immune response and the regulation, repair, efflux, and protection during NTM exacerbation states and how the regulation of such responses affects lung function and immune regulation. Investigating this enables further understanding of the pathogenesis of some NTM species, which could also lead to the development of new drug targets and treatment strategies. Ultimately improving patient outcomes and leading to further understanding of NTM disease progression.

1.6 Study Aims

Non-tuberculosis mycobacteria (NTM) can cause serious pulmonary infection in people with cystic fibrosis (CF). The detection, diagnosis, and treatment of NTM pulmonary disease (NTM-PD) is a long process which can lead to prolonged disease and poor patient outcomes. Therefore, there is a need for more precise and efficient methods of NTM detection.

Further studying the microbiome there is little known about the changes in its composition in the presence of NTM species; understanding how NTMs can influence the surrounding microbiome in patients with CF can enable further insight into the community dynamics and disease progression. There are numerous knowledge gaps within the research area of NTM-effects on the microbiome and NTM disease pathogenesis, which this study seeks to address.

The Aims of This Study Are:

1. Improve molecular based NTM complex detection in low microbial diversity samples for high throughput sequencing, whilst examining the wider lung microbiome (Chapter 3).
2. Understand how NTM infection affects the composition of the lung microbiota when compared to matched non-NTM CF controls (Chapter 4/5/6)
3. Assess the effects of CFTR modulators on NTM complexes and the composition of microbiota as a result of treatment (Chapter 5)
4. Assess the effects of patient factors (age, gender, genotype) and their relationship to NTM culture status on %$FEV_{1.}$ (Chapter 6)
5. Evaluate the regulation of some NTM virulence genes during CF exacerbation states (Chapter 7)

I declare that all the work in this thesis and the material presented therein are entirely my own work, except where appropriately referenced and noted. All experiments were conducted by me except where explicitly stated.

Chapter 2: Core Methods

2 Introduction

This core methods chapter introduces the general methodology used throughout this thesis. It is a precursor to the method optimisation and troubleshooting in Chapter 3: Optimisation of Amplicon Sequencing for the Detection of NTM in Microbiota. It also includes information on how sample collection changed during the COVID-19 pandemic (Section 2.1.1).

2.1 Patient Recruitment

Patients were recruited from the University Southampton Hospital (UHS) NHS Trust, Cystic Fibrosis Centre, UK, and The University of Vermont (UVM), USA (Table 2.1/2.2). Inclusion criteria required patients to be adults over 18 years old, culture positive for non-tuberculosis *mycobacteria* (NTM) infection, with a mixture of exacerbation states (stable, exacerbation, on treatment) at study enrolment. Patients provided written consent. This study was reviewed and approved by the National Research Ethics Committee (A), Southampton and southwest Hampshire, UK, and assigned the Research Ethics Committee (REC) reference number: 08/H0602/126, and by The University of Vermont Institutional Review Board in the USA under the Institutional Review Board (IRB) number 00000485.

2.1.1 Sample Collection

Prior to March 16th, 2020, participants were assessed, and samples were collected in clinic during routine appointments by the regular cystic fibrosis team. Collected specimens were stored at -80°C while waiting for transport to the laboratory at Manchester Metropolitan University (MMU). The samples were then stored at -80°C until processing. Due to the COVID-19 pandemic, after March 16th, 2020, samples were collected using a postal pack (Section 2.1.1.1).

2.1.1.1 Postal Pack

A postal pack was designed and assembled (Figure 2.1). This pack allowed the collection of sputum or cough swabs from patients without them having to visit a CF clinic.

The pack was sent directly to the UH S CF unit and was distributed to the study participants. UVM set up and issued their packs based on this design. The pack included a questionnaire to capture patient demographic data, a user guide detailing the correct way

to provide a sample, whether they are producing sputum or not, and a self-addressed prepaid envelope addressed directly to MMU.

The pack had two sample collection methods (Figure 2.2), sputum collection into the red-topped Falcon tube (Thermo Fisher, UK) (Figure 2.2A). In the cough swab collection, participants were to cough onto the swab and return it to the tube (Figure 2.2B). Once the specimen was collected, DNA/RNA shield™ (Zymo Research Corporation, USA) (Figure 2.2B) was added to the sample. The sample was then safe to transport.

Before being distributed to the hospital the packs underwent a trial to ensure they could be returned easily through the postal service. A volunteer followed both methods (sputum and swab collection) and returned both packs following instructions in the user guide. The packs were received at the correct address without any breakages or leaks; the trial was successful. The volunteer noted that information regarding cleaning the surrounding area would be helpful in the information provided, as would the inclusion of a small bag to capture any leaks if they occurred. The feedback given by the volunteer was actioned, the pack was finalised, sent out to UHS and UVM, and distributed to eligible CF patients (Table 2.1/2.2). Upon receipt of the packs, the samples were stored at -80°C for future use, and the completed patient questionnaire was used to compile the meta-data for the patients (Supplementary Materials 11.1/11.2).

Figure 2. 1 Respiratory sample collection postal pack. Containing (from left to right) collection tubes, clear bag for sample return, self-addressed prepaid envelope, patient questionnaire, and a user guide. These packs were allocated by the CF clinic and returned to MMU via the Royal Mail.

Figure 2. 2 The Sample collection containers. **(A)** Red topped Falcon tube for sputum collection **(B)** DNA/RNA shield™ for inactivation of pathogens **(C)** Self-contained cough swab. These containers were used for participants to send their samples back to MMU.

2.1.2 Sample Allocation

The participant samples collected via the hospital CF clinics and via the postal pack (Table 2.1/2.2) were assigned to different chapters in this thesis depending on certain criteria (Figure 2.3). The all samples were included in Chapter 4, except those participants on CFTR modulator therapy, and Chapter 5 only included participants undergoing CFTR modulator therapy. Chapter 6 included all patient samples with recent $\%FEV_1$ readings. Chapter 7 only included sputum samples and those patients which had exacerbation status, $\%FEV_1$ readings, C-reactive protein and neutrophil counts provided by UHS.

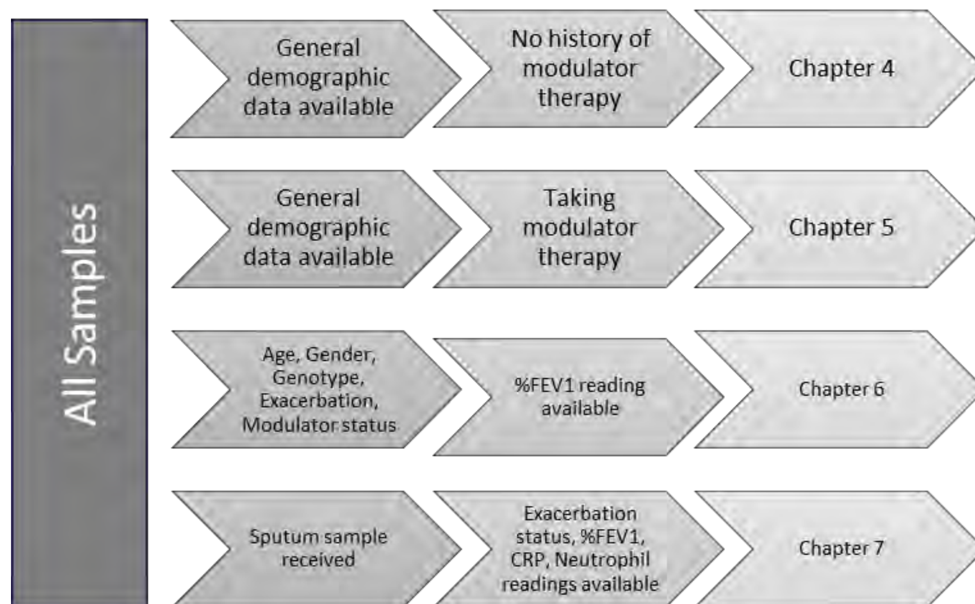


Figure 2. 3 Flow chart illustrating how the samples were allocated to each chapter in the study. Chapters 4 and 5 included samples with all demographic data. Chapter 4 examined samples from participants not taking CFTR modulator therapy. Chapter 5 examined samples from participants who were taking CFTR modulator therapy. Chapter 6 included all patients regardless of CFTR modulator status. Chapter 7 included patients that produced sputum and their clinical data included C-reactive protein and neutrophil counts.

Table 2. 1 Participant Demographics of The Entire Cohort Used in This Thesis

[a] Origin	[b] Sample ID	[C] Collection method	Age	Column1	Type of Sample	[D] Genotype	[e]%FEV_1	Exacerbation	NTM	[f] NTM Type	Modulator	Chapter
UHS	13UH1s	Clinic	33	Male	Sputum	Heterozygous	36	Stable	Positive	MABSC	Ivacaftor	5/6
UHS	16UH1s	Clinic	28	Male	Sputum	Heterozygous	86	Exacerbation	Positive	MABSC	Ivacaftor	5/6/7
UHS	1SHS2S	Postal	39	Female	Sputum	Heterozygous	73	Exacerbation	Positive	MAC	Ivacaftor	5/6
UHS	1UH1s	Clinic	28	Female	Sputum	Heterozygous	27	Exacerbation	Positive	MABSC	Ivacaftor	5/6/7
UHS	25SHS2S	Postal	28	Female	Sputum	Heterozygous	72	Stable	Positive	MAC	Ivacaftor	5/6
UHS	8UH1s	Clinic	28	Female	Sputum	Homozygous	27	Exacerbation	Positive	MABSC	Ivacaftor	5/6/7
UHS	12SHS2S	Postal	32	Female	Sputum	Homozygous	45	Stable	Positive	Other	Kaftrio	5/6
UHS	14SHS2S	Postal	22	Female	Swab	Heterozygous	75	Stable	Positive	MAC	Kaftrio	5/6
UHS	2CUVS	Clinic	29	Female	Sputum	Homozygous	22	Exacerbation	Positive	MAC	Kaftrio	5/6
UHS	2UVS	Clinic	29	Female	Sputum	Homozygous	22	Exacerbation	Positive	MAC	Kaftrio	5/6
UHS	11SHS2S	Postal	39	Female	Sputum	Heterozygous	73	Stable	Negative	Negative	Symkevi	5/6
UHS	15UH1s	Clinic	29	Female	Sputum	Homozygous	22	Exacerbation	Positive	MABSC	Symkevi	5/6/7
UHS	23UH1S	Clinic	30	Female	Sputum	Homozygous	85	Exacerbation	Positive	MABSC	Symkevi	5/6
UHS	24SHS2S	Postal	39	Female	Sputum	Heterozygous	73	Exacerbation	Positive	Other	Symkevi	5/6
UHS	24UH1S	Clinic	37	Female	Sputum	Heterozygous	71	Stable	Positive	Other	Symkevi	5/6/7
UHS	107AS	Clinic	27	Female	Sputum	Homozygous	49	Exacerbation	Positive	Other		4/6

UHS	107BBS	Clinic	26	Male	Sputum	Heterozygous	68	Stable	Positive	Other	4/6
UHS	107BS	Clinic	20	Female	Sputum	Homozygous	88	Stable	Positive	Other	4/6
UHS	107CS	Clinic	23	Male	Sputum	Homozygous	50	Exacerbation	Positive	Other	4/6
UHS	10UH1s	Clinic	30	Female	Sputum	Homozygous	40	Exacerbation	Positive	MABSC	4/6/7
UHS	11UH1S	Clinic	37	Female	Sputum	Heterozygous	68	Stable	Positive	MABSC	4/6
UHS	12UH1	Clinic	31	Female	Sputum	Homozygous	57	Exacerbation	Positive	MAC	4/6/7
UHS	148BS	Clinic	25	Male	Sputum	Heterozygous	49	Exacerbation	Positive	MAC	4/6
UHS	14UHS1	Clinic	21	Female	Sputum	Heterozygous	95	Stable	Positive	MABSC	4/6
UHS	153S	Clinic	25	Male	Sputum	Homozygous	86	Exacerbation	Negative	Negative	4/6
UHS	164AS	Clinic	56	Female	Sputum	Heterozygous	51	Stable	Negative	Negative	4/6
UHS	164BS	Clinic	22	Male	Sputum	Homozygous	82	Exacerbation	Negative	Negative	4/6
UHS	179S	Clinic	27	Female	Sputum	Homozygous	63	Exacerbation	Negative	Negative	4/6
UHS	17UH1s	Clinic	30	Female	Sputum	Homozygous	56	Exacerbation	Negative	Negative	4/6/7
UHS	186S	Clinic	28	Male	Sputum	Heterozygous	104	Exacerbation	Positive	MAC	4/6
UHS	187AS	Clinic	23	Female	Sputum	Homozygous	94	Exacerbation	Positive	MAC	4/6
UHS	187CS	Clinic	28	Male	Sputum	Heterozygous	45	Stable	Positive	MAC	4/6
UHS	188AS	Clinic	26	Male	Sputum	Heterozygous	45	Stable	Positive	MAC	4/6
UHS	188BS	Clinic	22	Female	Sputum	Heterozygous	45	Exacerbation	Positive	MAC	4/6
UHS	18UH1s	Clinic	31	Female	Sputum	Homozygous	52	Stable	Positive	MABSC	4/6
UHS	190CCS	Clinic	25	Female	Sputum	Heterozygous	52	Exacerbation	Positive	MAC	4/6
UHS	190CS	Clinic	19	Female	Sputum	Heterozygous	49	Stable	Positive	MAC	4/6
UHS	197S	Clinic	25	Female	Sputum	Heterozygous	106	Stable	Negative	Negative	4/6
UHS	199S	Clinic	56	Male	Sputum	Homozygous	88	Stable	Negative	Negative	4/6
UHS	19UH1s	Clinic	20	Female	Sputum	Heterozygous	51	Exacerbation	Positive	MAC	4/6/7
UHS	203AS	Clinic	23	Female	Swab	Heterozygous	40	Stable	Positive	MAC	4/6
UHS	203BBS	Clinic	20	Female	Sputum	Heterozygous	40	Exacerbation	Positive	MAC	4/6
UHS	203BS	Clinic	20	Male	Sputum	Homozygous	95	Exacerbation	Positive	MAC	4/6

UHS	204AS	Clinic	20	Female	Sputum	Homozygous	63	Exacerbation	Negative	Negative		4/6
UHS	204BS	Clinic	19	Female	Sputum	Homozygous	32	Exacerbation	Negative	Negative		4/6
UHS	204CS	Clinic	20	Female	Sputum	Heterozygous	112	Stable	Negative	Negative		4/6
UHS	205AS	Clinic	21	Female	Sputum	Heterozygous	88	Stable	Positive	MAC		4/6
UHS	20AS	Clinic	29	Female	Sputum	Heterozygous	52	Exacerbation	Negative	Negative		4/6
UHS	211S	Clinic	19	Male	Sputum	Heterozygous	35	Stable	Negative	Negative		4/6
UHS	213S	Clinic	18	Male	Sputum	Heterozygous	52	Exacerbation	Positive	MAC		4/6
UHS	21UH1s	Clinic	19	Male	Sputum	Homozygous	97	Exacerbation	Negative	Negative		4/6
UHS	25UH1s	Clinic	27	Female	Sputum	Homozygous	66	Exacerbation	Negative	Negative		4/6/7
UHS	2UH1s	Clinic	33	Female	Sputum	Heterozygous	47	Stable	Positive	MABSC		4/6
UHS	3UH1s	Clinic	30	Female	Sputum	Homozygous	84	Stable	Positive	MABSC		4/6/7
UHS	46S	Clinic	29	Female	Sputum	Homozygous	59	Exacerbation	Negative	Negative		4/6
UHS	4SHS2S	Postal	23	Female	Sputum	Heterozygous	45	Stable	Positive	MABSC		4/6
UHS	54AS	Clinic	22	Female	Sputum	Unknown	45	Stable	Negative	Negative		4/6
UHS	54BS	Clinic	28	Male	Sputum	Homozygous	91	Exacerbation	Negative	Negative		4/6
UHS	54CS	Clinic	24	Female	Sputum	Homozygous	78	Stable	Negative	Negative		4/6
UHS	55S	Clinic	29	Female	Sputum	Heterozygous	23	Stable	Negative	Negative		4/6
UHS	5UH1s	Clinic	21	Female	Sputum	Heterozygous	99	Stable	Positive	MABSC		4/6/7
UHS	6AS	Clinic	30	Female	Sputum	Heterozygous	41	Exacerbation	Positive	MAC		4/6
UHS	6CS	Clinic	27	Female	Sputum	Homozygous	91	Stable	Positive	MAC		4/6
UHS	73BS	Clinic	25	Male	Sputum	Heterozygous	55	Stable	Positive	MAC		4/6
UHS	81S	Clinic	26	Female	Sputum	Heterozygous	59	Exacerbation	Positive	MAC		4/6
UHS	9BUH1s	Clinic	33	Female	Sputum	Heterozygous	39	Stable	Positive	MABSC		4/6
UHS	9SHS2S	Postal	33	Female	Sputum	Homozygous	55	Stable	Positive	MABSC		4/6
UHS	9UH1S	Clinic	28	Female	Sputum	Homozygous	27	Exacerbation	Positive	MABSC		4/6
UVM	36UVS	Postal	53	Male	Swab	Heterozygous	104	Stable	Negative	MABSC	Ivacaftor	5/6

UVM	11UVS	Postal	33	Female	Swab	Homozygous	50	Stable	Negative	Negative	Kaftrio	5/6
UVM	12UVS	Postal	28	Male	Swab	Homozygous	97	Stable	Negative	Negative	Kaftrio	5/6
UVM	13UVS	Postal	33	Female	Swab	Homozygous	59	Stable	Negative	Negative	Kaftrio	5/6
UVM	14BUVS	Postal	36	Male	Sputum	Heterozygous	51	Exacerbation	Negative	Negative	Kaftrio	5/6
UVM	14UVS	Postal	37	Male	Sputum	Heterozygous	52	Exacerbation	Negative	Negative	Kaftrio	5/6
UVM	15UVS	Postal	30	Female	Swab	Homozygous	44	Stable	Negative	Negative	Kaftrio	5/6
UVM	16BUVS	Postal	35	Female	Swab	Homozygous	71	Exacerbation	Negative	Negative	Kaftrio	5/6
UVM	16UVS	Postal	36	Female	Swab	Homozygous	72	Exacerbation	Negative	Negative	Kaftrio	5/6
UVM	17UVS	Postal	35	Female	Swab	Homozygous	101	Stable	Negative	Negative	Kaftrio	5/6
UVM	18UVS	Postal	23	Female	Swab	Heterozygous	83	Exacerbation	Negative	Negative	Kaftrio	5/6
UVM	19UVS	Postal	23	Male	Swab	Homozygous	76	Stable	Negative	Negative	Kaftrio	5/6
UVM	21UVS	Postal	32	Female	Sputum	Homozygous	95	Exacerbation	Negative	Negative	Kaftrio	5/6
UVM	22SHS2S	Postal	28	Female	Swab	Homozygous	99	Stable	Negative	Other	Kaftrio	5/6
UVM	22UVS	Postal	28	Female	Swab	Homozygous	99	Stable	Negative	Negative	Kaftrio	5/6
UVM	23CUVS	Postal	27	Male	Swab	Homozygous	25	Stable	Positive	MAC	Kaftrio	5/6
UVM	23UVS	Postal	27	Male	Swab	Homozygous	25	Stable	Positive	MAC	Kaftrio	5/6
UVM	25UVS	Postal	47	Female	Swab	Heterozygous	93	Stable	Positive	MAC	Kaftrio	5/6
UVM	26UVS	Postal	25	Female	Swab	Heterozygous	94	Stable	Negative	Negative	Kaftrio	5/6
UVM	27UVS	Postal	31	Male	Swab	Homozygous	25	Stable	Negative	Negative	Kaftrio	5/6
UVM	28UVS	Postal	38	Female	Swab	Homozygous	100	Stable	Negative	Negative	Kaftrio	5/6
UVM	33UVS	Postal	29	Male	Swab	Homozygous	64	Stable	Negative	Negative	Kaftrio	5/6
UVM	34UVS	Postal	41	Female	Swab	Homozygous	80	Stable	Positive	MAC	Kaftrio	5/6

UVM	4UVS	Postal	34	Male	Sputum	Homozygous	75	Stable	Positive	Other	Kaftrio	5/6
UVM	5UVS	Postal	31	Male	Sputum	Homozygous	41	Stable	Negative	Negative	Kaftrio	5/6
UVM	6UVS	Postal	26	Male	Swab	Homozygous	80	Stable	Negative	Negative	Kaftrio	5/6
UVM	9UVS	Postal	30	Male	Swab	Homozygous	145	Stable	Negative	Negative	Kaftrio	5/6
UVM	22UH1S	Postal	44	Female	Sputum	Homozygous	28	Exacerbation	Positive	MAC	Symkevi	5/6/7
UVM	24CUVS	Postal	24	Female	Swab	Homozygous	74	Stable	Positive	MAC	Symkevi	5/6
UVM	24UVS	Postal	24	Female	Swab	Homozygous	74	Stable	Positive	MAC	Symkevi	5/6
UVM	1UVS	Postal	31	Female	Sputum	Heterozygous	37	Stable	Negative	Negative		4/6
UVM	30UVS	Postal	50	Male	Swab	Homozygous	56	Stable	Negative	Negative		4/6
UVM	3UVS	Postal	23	Male	Sputum	Homozygous	80	Stable	Negative	Negative		4/6

This table contains the participant demographics for the entire thesis.
[a] Origin of the sample UHS: University Hospital Southampton, UVM: University of Vermont.
[b] Sample ID: The sample identifier as assigned by the hospital
[c] Collection method: Clinic: if the sample was collected in the clinic, pre-COVID 19 pandemic or via the postal-pack method which is denoted as Postal.
[D] CFTR genotype- CF transmembrane conductance regulator genotype. Homozygous ΔF508, two copies of the ΔF508 gene mutation, ΔF508 heterozygous, single copy of ΔF508 gene mutation and another mutation. Other, Other mutation no ΔF508 mutation, Unknown; data not supplied.
[e] Percent predicted forced expiratory volume in 1 second (%FEV_1) as recorded by the clinic on the participants last visit
[f] NTM type- Samples culture positive for *M. avium* complex (MAC), *M. abscessus* complex (MABSC), Other NTM.
Patient modulator status at time of sampling.
Chapter what chapter the sample was used in.

*Participant demographic information was collected by the participating CF clinic.

Table 2. 2 Summary of Participant Demographics for The Whole Thesis Cohort

Origin [a]		**Collection Method** [b]		**Type of Sample** [c]	
UHS	70	Clinic	60	Sputum	74
UVM	31	Postal	41	Cough Swab	27
Exacerbation status		**NTM Status**		**NTM Type** [f]	
Exacerbation	44	Positive	56	MAC	29
Stable	57	Negative	45	MABSC	18
				Other	9
Age		**FEV_1 (%)** [e]		**Gender**	
Mean	30.4 (SD± 10.14)	Mean	64.74 (SD± 25.15)	Female	69
Median	28	Median	59	Male	32
Range(Min/Max)	18/72	Range(Min/Max)	22/145		
Modulator Status [g]		**Genotype** [d]		**% of samples used in each chapter**	
None	54	Homozygous	56	Chapter 4	54.46%
Symkevi	8	Heterozygous	45	Chapter 5	44.55%
Ivacaftor	8			Chapter 6	100%
Kaftrio	31			Chapter 7	15.84%
				Total number of samples (*n*)	101

A summary of demographic totals corresponding to Table 2.1.
[a] Origin of the sample UHS: University Hospital Southampton, UVM: University of Vermont.
[b] Collection method: Clinic: if the sample was collected in the clinic, pre-COVID 19 pandemic or via the postal-pack method which is denoted as Postal.
[C] Type of sample provided Sputum or Cough Swab.
[D] CFTR genotype- CF transmembrane conductance regulator genotype. Homozygous ΔF508, two copies of the ΔF508 gene mutation, ΔF508 heterozygous, single copy of ΔF508 gene mutation and another mutation.
[e] Percent predicted forced expiratory volume in 1 second (%FEV1).
[f] NTM type- Samples culture positive for *M. avium* complex (MAC), *M. abscessus* complex (MABSC), Other NTM.
[G] Patient modulator status at time of sampling.
SD denotes standard deviation of the mean.
** The participant demographic data was collected by the corresponding CF clinic.

2.1.3 Bacterial Isolates

Bacterial isolates were used in this study to optimise the polymerase chain reaction (PCR) stages of the next generation-sequencing (NGS) library preparation and create the mock community used as controls. The strains were obtained from numerous sources (Table 2.3). All non-NTM isolates were cultured using aseptic technique on both brain heart infusion (BHI) broth and agar (Oxoid, Hampshire, UK) and nutrient agar and nutrient broth (Oxoid, Hampshire, UK). NTM isolates were cultured using aseptic technique on Middlebrook agar with ODC supplement (Thermo Fisher, UK) and Middlebrook broth with AODC supplement and glycerol (Thermo Fisher, UK).

Table 2. 3 Bacterial Strains Used Throughout the Thesis

Organism	Source	Isolate number	Study /Use	Growth Temperature (°C)	Media used	Incubation time
Pseudomonas aeruginosa	MMU	PA01	All	37°C	Nutrient agar/broth Brain heart infusion agar/broth	24 hours
Staphylococcus aureus	PHE	91388	All	37°C	Nutrient agar/broth Brain heart infusion agar/broth	24 hours
Stenotrophomonas maltophilia	PHE	90335	All	37°C	Nutrient agar/broth Brain heart infusion agar/broth	24 hours
Burkholderia cenocepacia	PHE	90537	All	30°C	Nutrient agar/broth Brain heart infusion agar/broth	48 hours
Burkholderia multivorans	PHE	13010	All	37°C	Nutrient agar/broth Brain heart infusion agar/broth	24 hours

Haemophilus influenzae	DSMZ	DSM24049	CF	37°C, $C0_2$ incubator	Chocolate agar	24 hours
Mycobacterium abscessus	DSMZ	44196	All	28°C	Middlebrook agar with ODAC supplement and Middlebrook broth with ADC supplement	7 days
Mycobacterium malmoense	DSMZ	44163	All	37°C	Middlebrook agar with ODAC supplement and Middlebrook broth with ADC supplement	21 days
Mycobacterium kansasii	DSMZ	44162	All	37°C	Middlebrook agar with ODAC supplement and Middlebrook broth with ADC supplement	7 days
Mycobacterium intracellulare	DSMZ	43223	All	37°C	Middlebrook agar with ODAC supplement and	14 days

					Middlebrook broth with ADC supplement	
Mycobacterium simiae	DSMZ	44165	All	37°C	Middlebrook agar with ODAC supplement and Middlebrook broth with ADC supplement	14 days
Mycobacterium chelonae	DSMZ	43804	All	37°C	Middlebrook agar with ODAC supplement and Middlebrook broth with ADC supplement	14 days
Mycobacterium xenopi	DSMZ	43995	All	45°C	Middlebrook agar with ODAC supplement and Middlebrook broth with ADC supplement	14 days
Mycobacterium asiaticum	DSMZ	44297	All	37°C	Middlebrook agar with ODAC supplement and	28 days

					Middlebrook broth with ADC supplement	
Mycobacterium peregrinum	DSMZ	43271	All	37°C	Middlebrook agar with ODAC supplement and Middlebrook broth with ADC supplement	14 days
Mycobacterium phlei	MMU	ATCC 11728	All	37°C	Middlebrook agar with ODAC supplement and Middlebrook broth with ADC supplement. Nutrient agar/broth Brain heart infusion agar/broth	3 days
Mycobacterium avium	HSE Buxton	NCTC 13034	All	37°C	Middlebrook agar with ODAC supplement and Middlebrook broth with ADC supplement	28 days

Mycobacterium terrae	HSE Buxton	NCTC 10856	All	37°C	Middlebrook agar with ODAC supplement and Middlebrook broth with ADC supplement	21 days
Mycobacterium immunogenum	HSE Buxton	ATCC 700506	All	37°C	Middlebrook agar with ODAC supplement and Middlebrook broth with ADC supplement	21 days
Mycobacterium fortuitum	HSE Buxton	ATCC 6841	All	37°C	Middlebrook agar with ODAC supplement and Middlebrook broth with ADC supplement	14 days
Acinetobacter baumannii	HSE Buxton	NCTC 13301	MWF	37°C	Nutrient agar/broth Brain heart infusion agar/broth	24 hours
Pseudomonas pseudoalcaligenes	HSE Buxton	NCTC 10860	MWF	30°C	Nutrient agar/broth	48hours

					Brain heart infusion agar/broth	
Citrobacter freundii	HSE Buxton	NCTC 6071	MWF	37°C Facultative anaerobe	Nutrient agar/broth Brain heart infusion agar/broth	24 hours
Propionibacterium acnes	HSE Buxton	NCTC 737	MWF	37°C Fastidious anaerobe	Nutrient agar/broth Brain heart infusion agar/broth	48 hours
Alcaligenes faecalis	HSE Buxton	NCTC 655	MWF	37°C Facultative anaerobe	Nutrient agar/broth Brain heart infusion agar/broth	24 hours

Bacterial strains used in this study. Detailing the source, including Manchester Metropolitan University (MMU), Public Health England (PHE), Health And safety executive Buxton (HSE Buxton) and Deutshe Sammlung von Mikroorganismen und Zellkulturen (DMSZ). Study: CF all CF based studies in this thesis and MWF the Metal Working Fluid based studies. Shows the designated strain number and growth requirements.

2.1.4 Mock Community Preparation

The strains acquired (Table 2.3) were used to create a mock community that acted as a positive control for NGS and accounted for sequencing bias. The strains were selected due to their resemblance to the CF (Chapters 4 to 6) and MWF (Chapter 3) microbiomes. The strains were cultured, and DNA extractions performed (Section 2.1.4). The strains were then quantified using the Qubit fluorometer (Thermo Fisher, UK) and pooled in equal quantities.

When conducting microbiome community analysis research, the inclusion of a mock community as a positive control helps validate the techniques used in the study by providing a reference microbiome enabling accurate identification of microbial taxa detected in experimental samples (Bokulich et al., 2020). The mock community also serves as mitigation for sequencing bias by allowing for correction of any distortion that may be introduced during the sequencing process, during the DNA extraction process or when preparing the sequencing library (Pollock et al., 2018; Nearing et al., 2021). The introduction of biases may have significant implications on downstream analysis and could give a false overview of the microbial communities being studied (Hugerth and Andersson, 2017; Pollock et al., 2018; Nearing et al., 2021).

2.1.5 Sample Preparation

Prior to DNA extraction, patient sputum and MWF samples were prepared in a class II cabinet. The samples were centrifuged for 10 minutes at room temperature with a speed of 1107xg, the supernatant was discarded, and the pellet was resuspended in 900µl of phosphate buffered saline (PBS) and centrifuged again under the same conditions for 5 minutes, supernatant was discarded, and the pellet resuspended in 500µl of PBS (Rogers et al., 2006).

2.1.5.1 DNA Cross Linking

To effectively analyse microbial community dynamics and understand how any changes affect the community, discrimination between live and dead cells is important. This is done by introducing a DNA binding dye which attaches to cells with compromised membranes therefore considered dead. Propidium monoazide (PMA) is an intercalating dye that can be covalently crosslinked to DNA inhibiting amplification by polymerase chain reaction (PCR), this excludes the dead/damaged cells from further analysis as they

could create a bias in the final sequencing analysis (Nocker et al., 2007; Rogers et al., 2013).

As previously described by Rogers et al. (2008); Cuthbertson (2014). In brief 500µl of washed sputum was transferred into a 1.5ml amber micro-centrifuge tube (Sigma Aldrich, UK) and 1.25µl of PMA (Biotium, USA) was added to each tube, then incubated for 15 minutes at room temperature. The mixture was then transferred into a clear micro-centrifuge tube then added to an LED lightbox for a further 15 minutes.

2.1.5.2 Bead-beating and DNA/RNA Extraction

500µl of sputum was added to a capped 1.5ml microcentrifuge tube which were previously prepared with one tungsten carbide bead and glass beads (Merck, Dorset, UK) filled up to the 0.5ml mark. Then 400µl of DNA/RNA lysis buffer (Zymo research, USA) was added. The microcentrifuge tubes were inserted into a FastPrep-24 homogeniser (MP Biomedicals, Loughborough, UK) and processed for 30 seconds in two bursts.

Nucleic acid extraction was performed following manufacturer's instructions using Quick-DNA/RNA miniprep kit (Zymo-research, USA). DNA was then stored at -20˚C for future use and RNA was stored at -80˚C.

2.1.6 DNA Sequencing

The microbiome of the samples was assessed by 2 step amplicon-based sequencing using the Illumina MiSeq system (Figure 2.3). The sequencing targets both the 16S rRNA gene for the detection of the bacterial microbiome and a specific mycobacteria gene to detect specific mycobacteria complexes. The mycobacterial gene sequencing underwent a series of optimisation steps (Chapter 3).

2.1.6.1 Amplicon PCR

First step amplicon PCR was achieved using phased primer sets. This stage's purpose is to add the Illumina adapters and introduce a random base to increase diversity as described by Wu et al. (2015); Naik et al. (2020). This was done in two batches; the first batch targeted the V4-V5 Regions of the 16S rRNA gene. The V4-V5 region was chosen as a target due to its higher specificity of genera detection (Erb-Downward et al., 2011; Morris et al., 2013; Mendez et al., 2019) in comparison to the V3-V4, region which has better taxonomic coverage and is often the preferred choice for lung microbiome studies (Bokulich et al., 2020; Gao et al., 2023; López-Aladid et al., 2023).

Each PCR 25µl reaction consisted of 12.5µl 2x Q5 Master Mix (New England Biolabs, Hitchin, UK), 2µl (0.2µM) of phased primer pool (Invitrogen, Paisley, UK) (further detailed in Chapter 3) and 8.5µl of ultra-pure, molecular grade water (Cytiva, Fisher Scientific UK Ltd.).

The following parameters were used: 95°C for 3 minutes, followed by 30 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, ending with one cycle at 72°C for 5 minutes.

The second batch of amplicon PCR targeted a specific mycobacteria gene (*hsp*65 or *rpoB*) and the reaction consisted of 12.5µl 2x Q5 Master Mix (New England Biolabs, Hitchin, UK), 1µl (0.1µM) of phased primer pool (Invitrogen, Paisley, UK), 1.5µl of 12% molecular grade dimethyl sulfoxide (Thermo Fisher, UK) and 8.5µl of ultra-pure, molecular grade water (Cytiva, Fisher Scientific UK Ltd).

The following parameters were used: 94°C for 3 minutes followed by 45 cycles at 94°C for 60 seconds, 60°C for 60 seconds and 72°C for 60 seconds, ending with one cycle at 72°C for 10 minutes (Gebert et al., 2018). Both PCR reactions were confirmed by gel electrophoresis (Section 2.1.5.2).

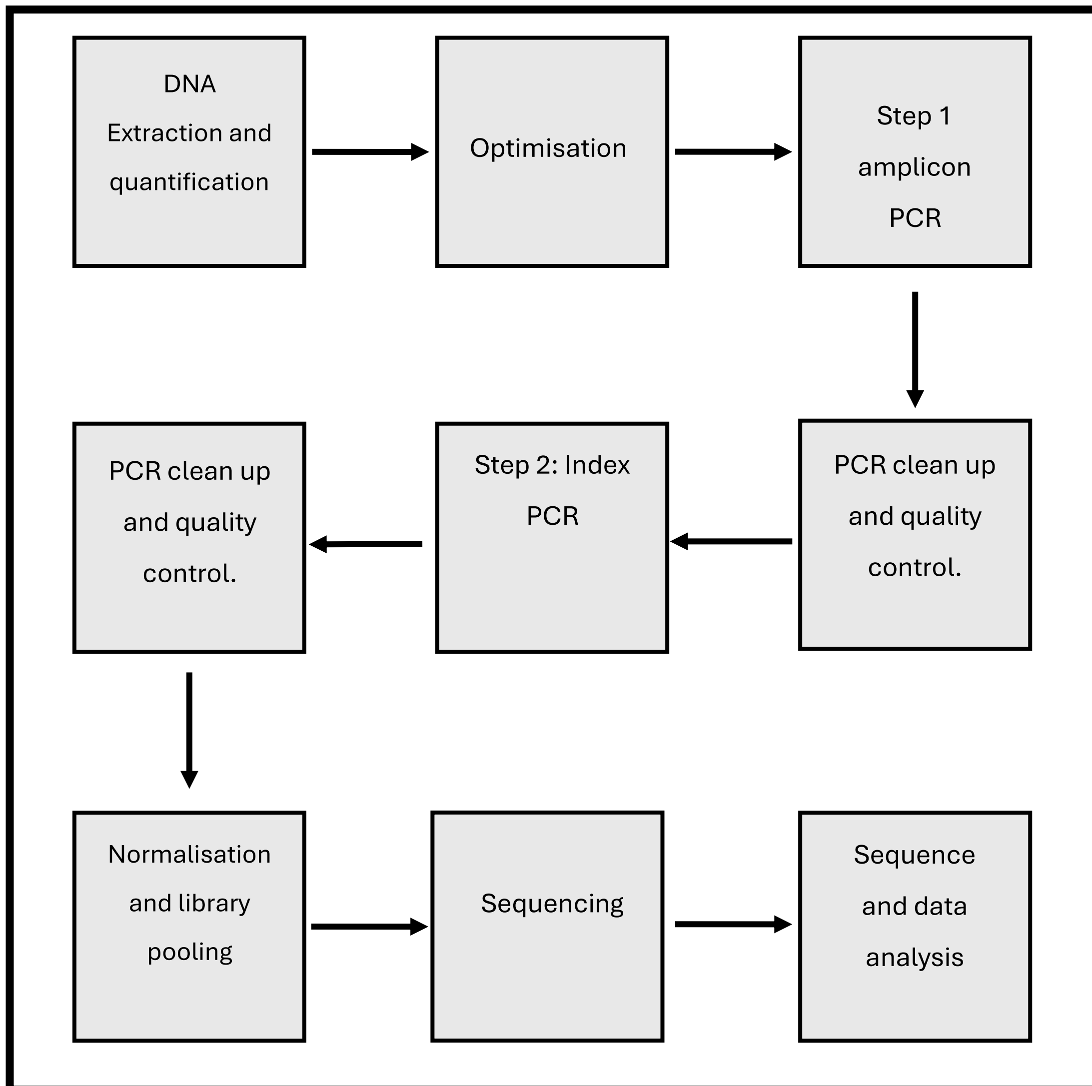


Figure 2. 4 Workflow diagram of DNA sequencing, illustrating the steps taken to generate the data used in this study.

2.1.6.2 Gel Electrophoresis

On completion of the PCR, a 2% agarose (Thermo Fisher, UK) gel was prepared with 1x TAE buffer (tris base/ acetic acid/ Ethylenediaminetetraacetic Acid (EDTA)) with the addition of SYBR safe DNA Gel stain (Invitrogen, Paisley, UK). Electrophoresis was carried out at 100V for 60 minutes and then visualised using the Bio-Rad ChemiDoc Transilluminator (Bio-Rad Laboratories, Inc. UK).

2.1.6.3 PCR Clean Up

Following successful amplicon amplification, AMpure XP PCR purification beads (Beckman Coulter Life Sciences, USA) were used to remove any PCR fragments of 350bp and below, following the manufacturer's instructions. This was to ensure the removal of primer dimer or any other unwanted fragments that may have formed. The purified PCR product was then quantified using the Qubit fluorometer (Thermo Fisher, UK).

2.1.6.4 Index PCR

The second step PCR was to add the Illumina sequencing adapters to each sample to ensure adhesion to the MiSeq flow cell. Each 25µl PCR reaction consisted of 12.5µl, 2x Q5 Master Mix (New England Biolabs, Hitchin, UK), 1.25mM of each primer, 5µl cleaned PCR product and 2.5µl of ultra-pure molecular grade water (Cytiva, Fisher Scientific UK Ltd).

The following parameters were used: 95°C for 3 minutes, followed by 20 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, ending with one cycle at 72°C for 5 minutes. The fragment was confirmed using gel electrophoresis and then purified (Sections 2.1.5.2/2.1.5.3) and reconfirmed using the Agilent Bioanalyzer (Agilent, USA) (Illumina, 2013).

2.1.6.5 Normalisation and Sequencing

Purified PCR products were quantified and normalised to a 1nM library and processed as stated in the MiSeq Denature and Dilute guide (Illumina, 2013; 2019); 5µl of the library was denatured with 0.2N NaOH for 5 minutes at room temperature and transferred to a heat block at 95°C for 5 minutes. 990µl of pre-chilled HT1 buffer was added to the denatured library, and this created the 10pM denatured library, which was stored on ice. The final library consisted of the denatured library of 7pM and a 30% PhiX spike. The samples were sequenced on the Illumina MiSeq platform using the Illumina MiSeq V3 600 cycle reagent kit (Illumina Cambridge Ltd, Cambridge, UK).

2.1.6.6 Sequencing Analysis

FastQ files were downloaded from Illumina base-space. The analysis of raw sequence data was then performed through the DADA2 pipeline, which focuses on processing 16S rRNA gene amplicon data and correcting for errors which may be introduced during sequencing; it then uses this information to infer what bacteria are present and in what proportion in the sample (Callahan et al., 2016). DADA2 also removes primer sequences, quality filters, trims, merges pair end reads and removes chimeras (Callahan et al., 2016) using R version 4.2.2 (R Core Team, 2023). Forward and reverse reads were truncated at 200bp and 175bp, respectively. This truncation was based on the quality scores provided by the DADA2 pipeline and aimed to remove low-quality bases and retain the required overlap of the paired ends reads for merging downstream (Callahan et al., 2016). The Silva Database version 138.1 release (Quast et al., 2013) was used to assign taxonomy to the sequences. Any unassigned amplicon sequence variants (ASV) found were manually assigned using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) nucleotide database (Sayers et al., 2022) and matched with sequences based on a minimum of 95% query coverage, with the lowest possible e-value and a minimum of 95% identity cutoff. Multiple sequences assigned to the same ASV were condensed into OTU for statistical analysis. Given the varying lengths of the sequences analysed, these identities should be considered putative.

2.1.7 Statistical analysis

The statistical analysis for the thesis can be found in each chapter under the statistical analysis section.

Chapter 3: Optimisation of Amplicon Sequencing for the Detection of NTM in Microbiota

3 Introduction

Non-tuberculosis mycobacteria (NTM) are emerging pathogens that can affect the cystic fibrosis population and can be present during acute pulmonary exacerbations (Floto and Haworth, 2015). They are abundant in the environment and have 197 species in their genera, with some species being very genetically closely related (Wilson et al., 2001). Certain NTM's are intrinsically resistant to some antimicrobials (Section 1.4.10), making them very difficult to treat (Lambert, 2002; Skolnik et al., 2016). Therefore, species identification is essential (Tortoli, 2012; Daley et al., 2020). There is a need for rapid diagnosis and species detection to enable the correct treatment to be prescribed. This chapter aims to optimise a method to examine the entire microbiome and investigate what NTM species are present independently of the whole microbiome.

3.1 Hypersensitivity pneumonitis

People with CF are not the only group at risk from NTM infection. This risk extends to people suffering from a range of respiratory disorders such as COPD, non-CF bronchiectasis, asthma, and hypersensitivity pneumonitis. Hypersensitivity pneumonitis (HP) is an immune response to the inhalation of metalworking fluids (MWF), also known as machine operator's lung. MWFs are complete mineral oil or synthetic-based lubricants used to manufacture of car parts and other metal machinery (Mirer, 2010). Respiratory allergies and HP have been associated with exposure to MWF, resulting in inflammation of lung tissue in some cases, *Mycobacterium immunogenum* is one of the main aetiological agents in MWF-associated HP (Tillie-Leblond et al., 2011). Symptoms often include shortness of breath, fever, and joint pains. Repeated exposure to the irritant can cause fibrosis of the lung (Walters et al., 2019).

3.1.1 Mycobacterium immunogenum

Mycobacterium immunogenum is part of the RGM group of mycobacteria. It is often recovered during water-borne nosocomial outbreaks, pseudo-outbreaks and MWF associated HP. It is closely related to MABS, exhibiting similar clinical and microbiological features, which can lead to misidentification between the two species (Wilson et al., 2001). Biochemical and molecular methods are best used for differentiation between them (Li et al., 2009), as misdiagnosis can affect treatment regimens because the two species exhibit different resistances to biocides and antibiotic treatments (Griffith et al.,

2007). The treatment regimen of MABSC was discussed in (Section 1.4.10). *M. immunogenum* has exhibited resistance to disinfectants such as glutaraldehyde (Falkinham, 2009), and the antibiotics β-lactam and aminoglycoside (Griffith et al., 2007). Mycobacteria infections are increasing in CF populations, with *Mycobacterium avium complex (M*AC) and MABS being the main culprits (Section 1.4). The detection of *M. immunogenum* is rare in this population but it has been detected (Plongla et al., 2017).

3.2 Current Specimen Processing and Culture.

Routine screening of NTMs in CF is performed annually in the UK and the USA on all individuals producing sputum (Haworth et al., 2017). In both CF and non-CF populations, the detection and diagnosis of NTM is considered specialist, and positive patients must have isolates referred to a mycobacteriology reference laboratory to undergo sub-speciation and strain typing (Griffith et al., 2007; The Cystic Fibrosis Trust, 2009). Current practices in diagnosing NTM infection in patients rely on at least two culture-positive sputum samples and/or one positive sample obtained from bronchial Lavage (BAL) or lung biopsy before referral to a specialist clinician (Griffith et al., 2007; Parada et al., 2016; Ryu et al., 2016).

Once a specialist referral has been obtained, the presence of NTM lung disease must be established; the patient is required to produce three early morning sputum samples on different days over one week, or a clinician can induce sputum (Griffith et al., 2007; Daley et al., 2020). These samples are subjected to acid-fast bacilli (AFB) staining using the Ziehl-Neelson or Kinyoun staining technique, which can penetrate the mycolic acid membrane (Section1.4.3), differentiating them from other bacterial taxa (Talbot and Raffa, 2015). This technique cannot differentiate between rapid-growing mycobacteria (RGM) and slow-growing mycobacteria (SGM) and a negative smear does not denote a negative sample (Griffith et al., 2007).

The samples are then decontaminated to reduce the presence of other bacterial strains, which may out-compete the NTM during culture (Peres et al., 2009). Specimens are cultured on both liquid and solid media, with Löwenstein-Jensen broth being the most sensitive for NTM detection (Idigoras et al., 2000). However, Middlebrook 7H10 and 7H11 are recommended by the Clinical Laboratory Standards Institute (CLSI); they have also recommended incubation temperatures of 36°C for SGM and 28°C for RGM (Clinical and Laboratory Standards Institute, 2018; Daley et al., 2020). Incubation time varies widely

depending on the species; RGM tends to grow within seven days of subculture, and SGMs can take two to three weeks, with some needing incubation for at least eight to twelve weeks (Griffith et al., 2007), then an additional one to two weeks for antibiotic susceptibility testing (Deggim-Messmer et al., 2016).

Where patients have been AFB or culture negative, and NTM infection is still suspected, samples undergo molecular detection. A *Mycobacterium* genus-specific assay is used alongside sequencing to differentiate *Mycobacterium Tuberculosis* (MTB) from NTM infection. The test is an adapted version of the Cobas Amplicor MTB test (Peter-Getzlaff et al., 2008), which uses a genus-specific capture probe enabling NTM to be identified by PCR-mediated sequencing (Peter-Getzlaff et al., 2010).

3.2.1 Matrix-assisted Laser Desorption Ionization-time of Flight (MALDI-TOF)

Matrix-assisted Laser Desorption Ionization-time of Flight (MALDI-TOF) is a mass spectrometry method that detects the protein mass pattern extracted from whole bacteria. It focuses on the signals derived from ribosomal or other abundant proteins (Sauer and Kliem, 2010). This is an effective method however, the need to culture or enhance the sample is very time consuming when dealing with NTMs and their mycolic membrane and low number of ribosomes poses a challenge in terms of extraction techniques. So, MALDI-TOF is most effective when used in combination with line-probe assays or sequencing technologies (Alcaide et al., 2018; Kalaiarasan et al., 2020).

3.2.2 Probe-based Identification

Line probe assays are a rapid technique based on PCR for the detection of mycobacteria. Most tests are streamlined for the detection of MTB. They are quick and easy to run but lack sensitivity when used for NTM and can lead to the misidentification of species (Daley et al., 2020). These assays are, however, very effective when used in conjunction with sequencing practices (Kalaiarasan et al., 2020).

3.2.3 Culture-Independent Techniques

The current diagnostic protocol for NTM-PD, or any NTM infection, heavily relies on initial culture. If patients are positive for an NTM, SGM culture results cannot be acquired in less than two weeks and, in some cases, a maximum of 12 weeks (Griffith et al., 2007; Daley et al., 2020). Though there are some molecular identification methods (Section 3.2) within the NTM diagnostic repertoire, these are not issued without a positive culture and are used for the detection of *Mycobacterium tuberculosis* DNA or genetic mutations

associated with drug resistance (Griffith et al., 2007; Daley et al., 2020; Public Health England, 2020). One of the main areas of improvement for improving patient outcomes would be reducing the time it takes from initial consultation to diagnosis and treatment.

Advances in molecular-based techniques for detecting bacteria in a clinical setting have been highlighted as faster and more sensitive than culture-based techniques (Rogers et al., 2009). Molecular detection techniques target molecules specific to the bacterial genus or strain. They often involve the separation of PCR amplicons by exploiting variations in the DNA sequences of genes associated with functional or phylogenetic significance (Muyzer et al., 1993; Blackwood et al., 2003). If culture-independent techniques were utilised fully, the time it takes to get a NTM diagnosis would be significantly reduced, and sensitivity would be increased (Cowman et al., 2019).

3.2.4 Next Generation Sequencing

Medical microbiology research was transformed with the introduction of NGS, also known as high throughput sequencing. It revolutionised the way genetic information was acquired and analysed. This rapidly evolving technology enables the parallel sequencing of millions of DNA fragments, allowing the examination of the complex relationships in the microbiome independent of traditional culture techniques (Hodkinson and Grice, 2015; Feigelman et al., 2017; Wensel et al., 2022). It has two main techniques depending on the type of research being conducted. Whole genome shotgun metagenomic sequencing (WGS) targets all genomic DNA present in the samples (Quince et al., 2017) and targeted or amplicon sequencing utilises specific primers that target specific regions of DNA (Fouhy et al., 2016).

3.2.4.1 NGS Platforms

There is a wide range of NGS platforms available for research use, each using different sequencing methodologies (some mentioned below), but all are usable for amplicon and WGS sequencing:

- Ion Torrent PGM (Life Technologies): Uses semiconductor sequencing technology, a type of sequencing by synthesis. This sequencing method is based on the detection of hydrogen ions, which are released during DNA polymerisation (Budczies et al., 2014).
- Roche 454 GS FLX+ 9 (Support discontinued 2016. May still be used) Uses pyrosequencing technology, another type of sequencing by synthesis.

Pyrosequencing is a DNA sequencing method that synthesises a complementary strand and detects light emitted from the enzymatic conversion of released pyrophosphate into ATP, providing a real-time readout of the DNA sequence (Ronaghi et al., 1998).

- Pacific bioscience RSSI: It uses single molecule real-time (SMRT) sequencing. A single DNA polymerase enzyme is attached at the bottom of a zero-mode waveguide with a single molecule of DNA as a template (Rhoads and Au, 2015)
- Illumina MiSeq: Sequencing by synthesis. This method involves synthesising a complementary DNA strand and detecting each base as it is incorporated in real-time (Illumina, 2017). This study uses the Illumina MiSeq Platform.

While all the platforms mentioned are more than capable of both WGS and amplicon sequencing, there are some differences in the data generated (Allali et al., 2017). The different platforms have variations in sequencing depth, read length and quality. Further downstream analysis has found that using different bioinformatic pipelines can lead to differences in results and taxonomic abundance (Fouhy et al., 2016; Allali et al., 2017; Pollock et al., 2018). The differences in data generated by different sequencing platforms and bioinformatic pipelines mean that direct comparisons between studies using different methods may not be straightforward. Variations in sequencing depth, read length, and quality can lead to differences in the detection and quantification of microbial species, impacting the conclusions drawn from the data. Therefore, it is crucial to consider these factors when comparing study results.

3.2.5 Whole Genome Sequencing

This method allows the untargeted sequencing of all microbial genes in a sample, giving a detailed mechanistic view of microbial communities (Quince et al., 2017) (Table 3.1). This technique is efficient in metagenomic studies: assembly, community, and functional profiling and (Pérez-Cobas et al., 2020) it enables the evaluation of bacterial abundance and diversity within various environments (Khachatryan et al., 2020). WGS provides excellent microbial resolution precision and captures a wide range of bacterial genera. It can also distinguish assumed functional genes that are not detectable through amplicon sequencing (Brumfield et al., 2020). However, it yields massive amounts of data, which can be complicated by incomplete coverage, short read lengths, and intrinsic sequencing errors (Ma et al., 2014). Another consideration is that WGS is more expensive than

amplicon sequencing and requires large-volume data storage capabilities and extensive specialist data analysis (Sims et al., 2014).

3.2.6 Amplicon Sequencing

Numerous studies using amplicon sequencing use primers that target the 16S rRNA gene in bacteria (Kim et al., 2011; Fadrosh et al., 2014; Ranjan et al., 2016; Fadeev et al., 2021). Amplicon sequencing (Table 3.1) is the most widely used microbiome sequencing approach and was used on large-scale projects like the Human Microbiome Project (Turnbaugh et al., 2007; Huttenhower et al., 2012; Ranjan et al., 2016). The 16S rRNA gene, when targeted in amplicon sequencing, yields thousands of short sequences aligned and classified down to genus or species level using publicly available databases. The data is then analysed as a population survey that can detail a myriad of information, including total bacterial abundance, relative abundance, community structure and/or diversity (Dickson et al., 2016).

Though widely used, targeting the 16S rRNA gene for researching the microbiome is not without its limitations. The classification relies on the presumed link between the 16S rRNA gene and a taxonomic group defined as an operational taxonomic unit (OTU) (Edgar, 2018). Usually, OTUs are investigated at the phyla or genera level, which lacks precision than at the species level (Franzén et al., 2015). Furthermore, individual genes are not sequenced directly but are instead assumed based on the OTUs. Because of horizontal gene transfer and multiple bacterial strains, the lack of direct gene identification limits the understanding of the microbiome (Poretsky et al., 2014; Ranjan et al., 2016).

However, one of the strengths of amplicon sequencing is the ability to target different areas of the genome by changing the target amplicon (De Filippis et al., 2017; Kim and Shin, 2018); this has a direct impact on NTM-based detection methods by targeting genes that are conserved in NTMs (Shin et al., 2006). It also benefits from being cheaper and quicker and yielding fewer complex data (Ranjan et al., 2016). This study uses amplicon sequencing.

Table 3. 1 Comparison of Sequencing Methods

	Amplicon Sequencing	Whole Genome Sequencing (WGS)
Coverage	Targets specific regions of the genome (around 100x) (Medini et al., 2008)	Targets the whole genome (around 30x to 50x) (Sims et al., 2014)
Cost (Estimated)	£13 per library (96 libraries/run)	£2250 to £3750/ run (Illumina, 2024)
Speed	Up to 35 hours (Illumina, 2013)	Up to 72 hours (Balloux et al., 2018)
Data complexity	6-9 GB	105 GB (Liu et al., 2021)
Resolution	Lower due to target specificity. Does not detect variations outside of the target area	Higher due to covering the entire genome (Ranjan et al., 2016)
Applications	Population genetics studies, Disease Research, microbial phylogeny and taxonomy, clinical diagnostics	Disease research, drug trials and pharmacogenomics, and oncology research (Buermans and den Dunnen, 2014)
Bias	Potential bias from PCR amplification and primer selection	Potential bias from library preparation and certain genomic regions (Pinard et al., 2006)

3.2.6.1 Low Diversity Samples

One of the issues working with samples acquired from diseased patients can be the lack of microbial diversity present (Alam et al., 2020; Bouquet et al., 2020; Fenn et al., 2022). Patients with CF have been found to have less diverse taxa in their lung microbiome as their age and disease progresses (Cuthbertson et al., 2020; Metzger et al., 2021). Those patients with HP also have less microbial diversity than healthy subjects (Invernizzi et al., 2021). Low diversity samples can apply to environmental settings where microbial growth is selective (Wintzingerode et al., 1997), meaning that due to specific environmental conditions, only some microbial species can thrive, therefore creating an environment where there is little difference in the species present (Nguyen et al., 2020). MWF is a selective environment because these fluids are made up of complex mixtures of industrial chemicals. Making it selective to only some types of bacteria, making it a low-diversity environment (Thompson and van der Gast, 2010; Koch, 2014).

Using NGS to examine the microbiome in disease or environmental settings is one of its main applications (Turnbaugh et al., 2007; Panthee et al., 2022). However, when examining low-diversity samples, some issues are faced. NGS platforms like the Illumina MiSeq require equal proportions of DNA bases (ACGT) at each position in a sequencing library to ensure effective template generation (Krueger et al., 2011; Illumina, 2022). Low-diversity libraries that lack an adequate proportion of bases have lower yields and quality scores than libraries with higher nucleotide diversity (Kozich et al., 2013; Illumina, 2022). This is due to issues with cluster identification, where the fluorescent signal becomes saturated when the same base nucleotide is being incorporated repeatedly (Kozich et al., 2013). NTMs have a high GC content of 61 to 71% (Pereira et al., 2020), which could potentially create issues with over-clustering during sequencing (Chen et al., 2013). When NTMs are present within a low-diversity community, the base diversity is potentially lower due to the high GC content.

3.2.6.2 Amplicon Sequencing of NTMs in The Microbiome

There has been previous research using amplicon sequencing to target NTMs within the microbiome, with studies using 16S rRNA (Caverly et al., 2016; Amha et al., 2017; Sulaiman et al., 2018; Philley et al., 2019) or NTM-specific targets *hsp*65 (heat shock protein 65) (Cowman et al., 2018; Yang et al., 2024), *rpo*B (β-subunit of RNA polymerase) (Manjeese et al., 2017). Some studies sequenced multiple targets separately, such as 16S rRNA v4 and hsp65 (Gebert et al., 2018), 16S rRNA and *rpo*B (Shen et al., 2022), *hsp*65 and

*rpo*B (Clarke et al., 2022). None have addressed the low base diversity, multiplex 16S rRNA, or a mycobacterium-specific target.

3.2.6.3 16S rRNA Gene Amplicon Sequencing for Detection of NTM

Some studies examining the NTM-PD microbiome using 16S rRNA gene sequencing found that the 16S rRNA gene sequencing is not sensitive enough to accurately identify NTM in airway samples that are culture positive (Caverly et al., 2016; Sulaiman et al., 2018). It is, however, helpful in identifying the presence of NTM in clinical (Philley et al., 2019) and environmental samples (Marshall et al., 2023). Whilst using the 16S rRNA gene for the identification of NTM species within environmental and clinical samples lacks in accuracy (Klindworth et al., 2013; Caverly et al., 2016; Sulaiman et al., 2018), it is still crucial in Identifying the different taxa present in polymicrobial clinical and environmental samples to enable a thorough evaluation of the microbial communities present (Clarridge, 2004; Ames et al., 2017; Winand et al., 2019).

3.2.6.4 *Mycobacterium* Heat-shock Protein 65 Gene

Most bacteria have a single copy of the *hsp65* gene in their genome, which means that it is not easily transferable from one bacterium to another; it also has highly conserved primary structures and, therefore, is suitable for phylogenetic studies (Kim et al., 2005). It can identify various species and is widely used to identify mycobacteria (Telenti et al., 1993).

Research examining the detection power of the *hsp*65 gene for NTMs found that it was able to identify MABSC, *M. chelonae, M. fortuitum* and *M. peregrinum* which are all from the RGM group (Ringuet et al., 1999; Pascapurnama et al., 2023), unlike 16S rRNA gene target which could not provide a clear distinction between the RGM species (Ringuet et al., 1999). When used for examining NTMS from the SGM group, research found that the *hsp*65 gene target was able to detect SGM species (Ong et al., 2010; Kim and Shin, 2018), sensitive enough to detect subspecies of MAC (Turenne et al., 2006; Johansen et al., 2009) and distinguish between MAC and *M. intracellulare* (Turenne et al., 2006; Maleki et al., 2017; Cowman, 2018). A possible downside to this approach is the inability to examine the entire bacterial microbiome, as the targeted sequencing of *hsp*65 is specific to NTMs only.

3.2.6.5 *Mycobacterium* β-subunit of RNA Polymerase

The *rpoB* gene has been used in numerous studies to identify Mycobacteria (Lee et al., 2000; De Zwaan et al., 2014; Manjeese et al., 2017). It encodes the β-subunit of RNA polymerase (Honoré et al., 1993). It is relatively conserved in the *Mycobacterium* genus and is often used to identify rifampicin resistance (Kim et al., 1999).

Studies examining the discriminatory power of the *rpo*B gene for NTM detection found it was capable of accurately identifying clinically significant NTM species: *M. kansasii, M. xenopi,* MAC, *M. intracellulare* , *M. chimera* (De Zwaan et al., 2014) *M. fortuitum* (Dastranj et al., 2018; Hashemzadeh et al., 2023), MABSC and *M. simiae (*Dastranj et al., 2018). *rpoB* can also identify NTM subspecies in MAC; *M. avium* subsp. *avium* (Haig et al., 2018).

3.2.7 Summary

Single target amplicon sequencing targeting the 16S rRNA gene lacks the ability to distinguish between some of the more genetically related groups like the *Mycobacterium abscessus* (MABSC) and *Mycobacterium chelonae* complexes (Van Ingen et al., 2010; Morais et al., 2022). Other targets have been used to identify NTMs, such as the heat-shock protein (*hsp65)* and β-subunit of RNA polymerase(*rpoB),* which are more direct; these targets used in conjunction with the 16S rRNA gene provide the best results and allow identification of subspecies (Van Ingen et al., 2010; Daley et al., 2020).

3.3 Chapter Aims and Objectives.

Diagnostic practices lack sensitivity and speed. This can result in a slower access to treatment for the patient, potentially exposing them to increased damage from the infection.

Gene sequencing is a fast and accurate way to distinguish pathogens and identify non-tuberculosis mycobacteria (NTMs). However, next-generation sequencing (NGS) using the Illumina MiSeq has its limitations; when examining low diversity samples like CF, metal working fluids (MWF) or NTMs that have high levels of GC content (Pereira et al., 2020), the MiSeq can struggle with over clustering leading to reduced quality and output ultimately affecting downstream analysis (Kozich et al., 2013)

This chapter aims to optimise and maximise amplicon sequencing using the Illumina MiSeq system, enabling the detection of the total microbiota and specific NTM complexes in low-diversity samples in one single multiplex assay. This was achieved by:

i. Determine appropriate targets for the whole microbiome and NTM-specific species for NGS sequencing using the Illumina MiSeq system.
ii. Optimise Illumina library preparation where needed.
iii. Improve Illumina cluster generation in low-diversity samples by using phased primer sets.
iv. Sequence low-diversity samples using phased primers and multiple libraries in a single assay.
v. Determine the success of multi-sequencing targets using phased primer sets by measuring against a mock community.

3.4 Methods

The protocols in this chapter were optimised using cultured bacterial isolates to confirm amplification (Table 2.1), then using MWF samples, which served as an experimental low diversity NTM positive sequencing library. The successful optimisation was then adopted for sequencing CF samples (Chapters 4 /5/6).

3.4.1 Metal Working Fluids

The samples used for MWF sequencing were collected and donated by the Health and Safety Executive in Buxton, England. The samples are from random sites where MWF are used with suspected or confirmed presence of NTM. They consist of wipes and synthetic/semi-synthetic oils with and without biocides. MWF possess a low-diversity microbiome (Murat et al., 2012) which allowed the optimisation of Illumina cluster generation.

3.4.2 Sequencing Target Selection

Prior to any optimisation, the amplicon target must be selected, and the targets must be of similar size, between 300-600bp in length (Profaizer et al., 2015). If the sizes vary excessively, it may cause inaccuracies in sequencing and clustering issues; it may not amplify efficiently and result in lower sequencing coverage (Illumina, 2016). This chapter focused on two target genes, *hsp65* and *rpoB*, alongside the 16S rRNA gene to achieve the most accurate representation of mycobacteria diversity within the samples.

3.4.2.1 Bacterial 16S rRNA Gene

The 16S rRNA gene was used as the primary amplicon target. The 16S rRNA gene fulfils the criteria of a phylogenetically important gene because it is highly genetically, functionally conserved, and detectable in all bacterial species, with no evidence of horizontal gene transfer (HGT). Despite this genetic conservation, the 16S rRNA gene has regions of variability enabling the distinguishing of different taxa (Clarridge, 2004). The V4 and V5 regions span the nucleotide position of 515 to 926 with a corresponding fragment size between 400-450bp in length (Klindworth et al., 2013). It has a high coverage of bacteria and archaea (Quince et al., 2011) and able to detect a higher amount of ASVs than other 16S rRNA regions (Fadeev et al., 2021). The V4-V5 region has been used successfully in other lung microbiome studies (Sze et al., 2015; Li et al., 2019; Ramsheh et al., 2021), providing higher specificity of detection than the V1 to V3 regions, which are often favoured for the better taxonomic coverage (Mendez et al., 2019). It was amplified

using the 515Y and 926R primer pair (Parada et al., 2016). After the amplicon has undergone library preparation its size can increase to between 500-600bp (Caporaso et al., 2012). Once the 16S rRNA library is prepared, it is used as a control and to capture background microbiota.

3.4.2.2 *Mycobacterium* Heat-shock Protein 65 Gene

The heat-shock protein 65 *(hsp65)* gene for mycobacteria specificity was selected as it contains highly conserved primary structures (Kim et al., 2005). It can identify a range of species and is widely used for the identification of mycobacteria. Its fragment size is around 441bp in length and amplified using the Tb11 and Tb12 primer pair (Telenti et al., 1993) prior to library preparation and between 550-650bp after, which matches it to the 16S rRNA amplicon (Illumina, 2013).

3.4.2.3 *Mycobacterium* β-subunit of RNA Polymerase

The final amplicon target is the *rpoB* gene, which has been used in numerous studies for the identification of Mycobacteria (Lee et al., 2000; De Zwaan et al., 2014; Manjeese et al., 2017). It encodes the β-subunit of RNA polymerase (Honoré et al., 1993). The expected amplicon size is 392bp, which is amplified using the *rpoB* FW and *rpoB* RV primer pairs (Manjeese et al., 2017). Between 420-500bp after sequencing library preparation, which is still compatible with the 16S rRNA and *hsp65* amplicons.

3.4.3 Bacterial Culture and Amplicon Confirmation

Bacterial Strains were cultured, incubated and DNA extracted as described earlier (Section 2.1.1). PCR was used to amplify the target genes using the 16S rRNA (Figure 3.2). Initially, the 16S rRNA gene was used to confirm successful extraction, with *hsp65* and *rpoB* gene targets amplified to assess their accuracy and efficacy (Table 3.1). Successful amplification was confirmed via gel electrophoresis (Section 2.5.1.2).

3.4.3.1 Optimisation and Troubleshooting

To get the best downstream result, the bacterial strains were subjected to several optimisation steps applied to the *hsp65* target. Once the ideal settings were found, they were applied to experimental samples as part of the Illumina sequencing library preparation. The following methods were used in this optimisation.

3.4.3.1.1 Primer Concentration Gradient

To find the best concentration of primers, with minimal primer dimer and specific target amplification, a primer concentration gradient was carried out using polymerase chain reaction (PCR). Phased primers were pooled from a working stock of 10µM. The pool was then serially diluted to 10 pmol concentration. Then a, 12.5µl master-mix was prepared for each dilution with 6.25µl 2x BioMix™ Red *Taq* DNA polymerase (Scientific Laboratory Supplies Limited, Nottingham, UK) and 4.75 µl of ultra-pure molecular grade water (Cytiva, Fisher Scientific UK Ltd). Then it was amplified using DNA extracted from a bacterial strain and confirmed via gel electrophoresis (Section 2.5.1).

3.4.3.1.2 Annealing Temperature Gradient

Correct annealing temperature (Ta) in PCR is essential to ensure the correct target is amplified. If the Ta is too low, then there is a risk of amplification of nonspecific DNA fragments; if it is too high, then it reduces yield and/or the quality is lowered due to poor attachment of the primer to the target strand (Rychlik et al., 1990).

To determine the best Ta for the primers, a gradient PCR was performed on both step one amplicon PCR and step 2 index PCR. A 12.5µl master-mix was prepared (Section 3.4.4.1) with 2µl (1.9µM) of pooled primer and 0.5µl (12.5ng/ml) of bacterial strain DNA for the template. This was amplified on a thermocycler (PCRmax, UK) using the following parameters: 95°C for 3 minutes, proceeded by 30 cycles at 95°C for 30 seconds, then the gradient annealing stage which is set between 51.5°C to 65.5°C, each well increases in increments of 1.25°C and 72°C for 30 seconds ending with one cycle at 72°C for 5 minutes. Confirmation of amplification and optimal Ta was established via agarose gel electrophoresis (Section 2.5.1.1).

3.4.3.1.3 Magnesium Chloride Concentration Gradient.

Another factor that can influence the PCR reaction is the concentration of magnesium Chloride ($MgCl_2^+$); this affects the melting of the DNA and the attachment of the primers, and the *Taq* polymerase utilises it for activity (Schmidt et al., 2014). A $MgCl_2^+$ gradient was carried out. Three master-mixes were prepared and amplified as described before (Section 3.6.6) with the addition of: No additional $MgCl_2^+$ (2.5mM), 3mM $MgCl_2^+$, 3.5mM $MgCl_2^+$. Confirmation of amplification and optimal $MgCl_2^+$ concentration was established via agarose gel electrophoresis (Section 2.5.1.1).

3.4.3.1.4 Dimethyl Sulfoxide Gradient

In some cases, a PCR additive such as DMSO is used to encourage specific primer binding and reduce the formation of secondary structures during amplification. It is beneficial in templates with high GC content (Hardjasa et al., 2010), such as *mycobacteria*. The DMSO gradient was carried out and prepared as described previously (Section 3.6.6) but with the addition of 0% to 18% DMSO in separate master-mixes. Confirmation of amplification and optimal DMSO concentration was established via agarose gel electrophoresis (Section 2.5.1.1).

3.4.4 MiSeq and Low Sequence Diversity Samples

Although the MiSeq is a powerful tool for sequencing-based studies when working with low diversity libraries or unbalanced structures in DNA sequences found in sample sets like CF sputum or MWF, it can have issues with cluster identification (Krueger et al., 2011; Naik et al., 2020). This issue is due to the fluorescent signal becoming saturated when the same nucleotide is repeatedly being incorporated during sequencing, resulting in over-clustering leading to errors in base calling and reduced sequence quality, ultimately affecting downstream analysis (Kozich et al., 2013). To mitigate the effects of over-clustering, it is suggested to adjust the concentration of the sequencing library; diluting the library can reduce the density of clusters on the flow cell. Achieving optimal cluster density is a very fine balance. Under clustering can produce high-quality data but gives lower data output (Illumina, 2016).

3.4.4.1 Library Preparation Step One: Amplicon PCR/ Phased Primers

To address the MiSeq' s issues with over-clustering in low diversity samples, a phased primer (Figure 3.1) approach was adopted (Wu et al., 2015); artificial base diversity was increased by adding between zero and four random bases between the Illumina adapter overhang and the target sequence primer, this is used at the step one amplicon PCR library preparation stage (Chapter 2, Section 2.5.1).

Figure 3. 1 Schematic of phased primer sets. Primer name in bold above first line of code with the forward primer on the right and the reverse primer on the left. Black text indicates Illumina adapter overhang, red N represents random bases up to 4 and the purple code is the specific amplicon primer, the red arrow indicates the direction of amplification (5‘to 3’). **(A)** Total background microbiome targeting the 16S rRNA gene using v4 (515Y) and V5 (926R) region primers. **(B)** *hsp*65 mycobacteria specific target. **(C)** *rpo*B mycobacteria specific target.

3.4.4.2 Library Preparation Step Two: Index PCR

Once the optimal parameters were established for the 1st step amplicon PCR, the Ta gradient was applied to the 2nd stage index PCR to ensure that the previous optimisation did not have any detrimental effect on this stage of the workflow. Then, the Index PCR stage of the library preparation was executed (Section 2.1.5.4).

3.4.4.3 MiSeq Sequencing

The three libraries (16S rRNA, *hsp*65 and *rpo*B) were prepared individually, normalised, and then combined into one library (Section 2.5.4). The pooled library was denatured and ran on two v2 nano cartridges (300/500 cycles, 6pM 20% PhiX) as a validity check prior to being sequenced on the Illumina MiSeq (7pM and a 30% PhiX) using the v3 600-cycle reagent kit (Illumina Cambridge Ltd, Cambridge, UK). The results underwent sequencing analysis. (Section 2.1.5.6). To ensure the library is at optimum quality and has not degraded, a new library is prepared prior to each experiment; this is particularly important for reproducibility and consistency. As the samples are freshly prepared, the downstream results are of the best quality.

3.4.4.4 MiSeq Run Metrics

The initial success of the sequencing experiments was measured on the Illumina MiSeq built in quality control metrics which are average %Q30 score, percent of clusters passing the filter (%PF), and the Yield of each run.

The %Q30 score estimates the probability of error. Higher scores represent lower probability of error. For sufficient data quality and reliability, the score needs to be ≥70%. The %PF represents the cluster density, if the density is high then the %PF will be low. The %PF should be ≥80%, a lower score could indicate issues in library preparation and quantification. Finally, the Mega base pairs (Mbp) yield varies depending on the chemistry used, the v2 nano 300 cycle reagent kit can yield up to 300Mbp, v2 nano 500 cycle reagent kit can yield up to 500Mbp and v3 600 cycle reagent kit can yield up to 15 giga base pairs (Gbp) (Illumina, 2014; 2022).

3.4.4.5 Sequence Analysis and Determination of NTM Target Efficiency

During sequence analysis using R version 4.2.2 (R Core Team, 2023) and DADA2 package (Callahan et al., 2016) which aligned the sequences to The Silva Database version; 138.1 release (Quast et al., 2013)(Section 2.1.5.6), it was noted which primer pair was the most robust in terms of identification of NTM species by assessing the following criteria:

Inspection of quality reads: To determine which primer set produced the better-quality reads.

1. How many reads passed the filtering stage.
2. Number of successful merged paired end reads.
3. Percent of chimeric sequences
4. Number and range of taxa assigned to the Silva database (Quast et al., 2013)
5. Number of taxa assigned manually using BLAST
6. Assessment against the mock community for reference

Once the DADA2 part of the sequence analysis was complete any unassigned amplicon sequence variants (ASV) where manually assigned using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). For *rpo*B and *hsp*65 the *Mycobacteriales* (taxid:85007) nucleotide database (Sayers et al., 2022) was used and matched with sequences based on a minimum of 95% query coverage, with the lowest possible e-value and a minimum of 95% identity cutoff. Multiple sequences assigned to the same ASV were condensed into OTU for statistical analysis. Given the length of the sequences analysed, these identities should be considered putative.

3.5 Results

3.5.1 Bacterial Culture and Amplicon Confirmation

The confirmation of the desired amplicons for each assay was determined by PCR and visualised using agarose gel electrophoresis. Confirmation of the 16S rRNA amplicon (Figure 3.2) shows clear bands around the 500bp mark in all samples excluding the negative control (lane 11) which has no visible band. *hsp65* confirmation (Figure 3.3) shows bands between the 400bp and 500bp marker which confirms the correct fragment has been amplified, some mycobacteria strains showing very faint bands or smearing, the negative and bacterial controls all show a negative result (lanes 18-20). Some primer dimer is clearly visible around the ≤100bp marker. Finally, *rpoB* confirmation (Figure 3.4) shows most of the mycobacteria strains have successfully been amplified and the desired band is in the correct place ≤400bp marker. The strains in lanes 8 to 10 show no bands and primer dimer is present at the bottom of the gel around the 100bp mark. Both the mycobacteria primer sets showed non-specific binding and smearing in some samples.

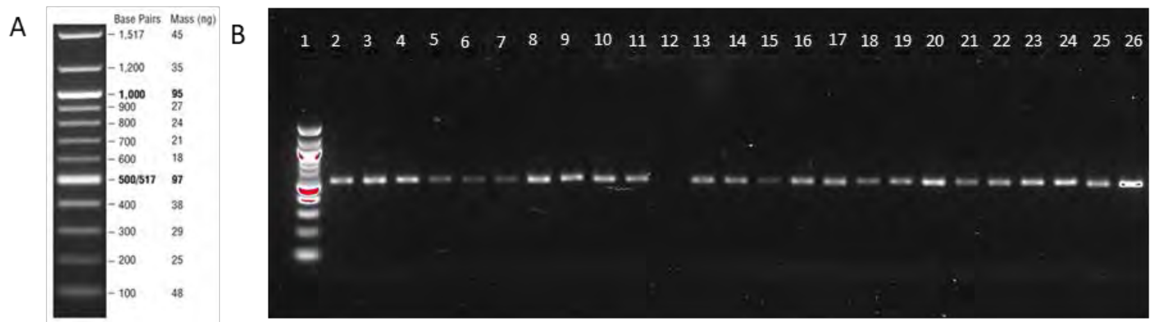


Figure 3. 2 Confirmation of desired amplicon via PCR. **(A)** PCR fragment sizes in the 100bp ladder from New England Biolabs **(B)** fragment confirmation of ± 412bp on all bacterial and mycobacteria strains. In lanes left to right: 1. 100bp ladder, 2. *P. aeruginosa,* 3. *S. aureus,* 4. *S. maltophilia,* 5. *B. cepacia,* 6. *B. multivorans,* 7. *H. influenzae,* 8. *M. avium,* 9. *M. abscessus,* 10. *M. malmoense,* 11. *M. fortuitum,* 12. Negative control , 13. *M. kansasii, 14. M. intracellulare, 16. M. simiae, 17. M. chelonae,* 18. *M. xenopi,* 19. *M. asiaticum,* 20. *M. peregrinum,* 21. *M. phlei,* 22. *M. bovis,* 23. *M. immunogenum,* 24. *A. baumannii,* 25. *P. pseudoalcaligenes,* 26. *C. freundii*

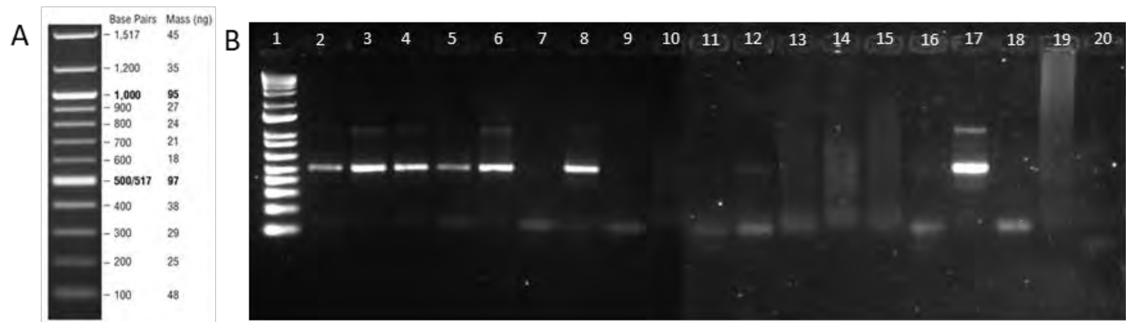


Figure 3. 3 Confirmation of desired *hsp*65 amplicon via PCR. **(A)** PCR fragment sizes in the 100bp ladder from New England Biolabs **(B)** *hsp*65 fragment confirmation ± 439bp on all mycobacteria and some bacterial strains from left to right lane 1. 100bp ladder, 2. *M. avium,* 3. *M. avium,* 4. *M. abscessus,* 5. *M. peregrinum,* 6. *M. fortuitum,* 7. *M. asiaticum,* 8. *M. malmoense,* 9. *M. simiae,* 10. *M. terrae,* 11. *M. kansasii,* 12. *M. abscessus* ,13. *M. phlei,* 14. *M. immunogenum,* 15. *M. xenopi,* 16. *M. chelonae,* 17. *M. intracellulare,* 18. *S. aureus,* 19. *P. aeruginosa,* 20. Negative control

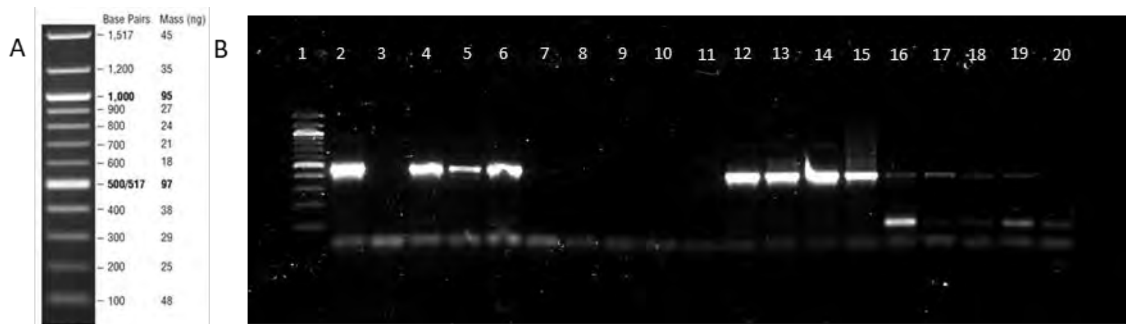


Figure 3. 4 Confirmation of desired *rpo*B amplicon via PCR. **(A)** PCR fragment sizes in the 100bp ladder from New England Biolabs **(B)** fragment confirmation of ± 392bp on all mycobacteria and some bacterial strains. In lanes left to right: 1. 100bp ladder, 2. *M. kansasii,* 3. *M. abscessus,* 4. *M. xenopi,* 5. *M. avium,* 6. *M. terrae,* 7. *M. immunogenum,* 8. *M. bovis,* 9. *M. phlei,* 10. *M. fortuitum,* 11. *M. chelonae,* 12. *M. peregrinum,* 13. *M. asiaticum,* 14. *M. malmoense,* 15. *M. simiae* 16. CF mock community, 17. MWF mock, 18. sample 203a, 19. sample 14shs1, 20. Negative control

3.5.2 Library Preparation Step One: Amplicon PCR

Once the correct amplicon was determined the first stage of the library preparation was conducted using the phased primer sets. The 16S rRNA step one (Figure 3.5) showed good amplification in all the experimental samples (Lanes 2-88), NTM strains (Lanes 89-92) and mock community (Lane 93) and no amplification in the negative controls (Lanes 94-100).

The *rpo*B gel (Figure 3.6) also showed good amplification in most of the experimental samples (Lanes 2-88), the lanes without any amplification could be due to the sample being NTM negative. The NTM strains have amplified (Lanes 89-92) as well as the mock community (Lane 93). No negative controls showed any bands (Lanes 94-100).

The *hsp65* gel (Figure 3.7) showed smearing across all lanes including the strains (Lanes 89-92, Mock community (Lane 93) and negative controls (94-100). Because of this this target needed to undergo a arrange of optimisation to eliminate the smearing and make it a viable target for this experiment.

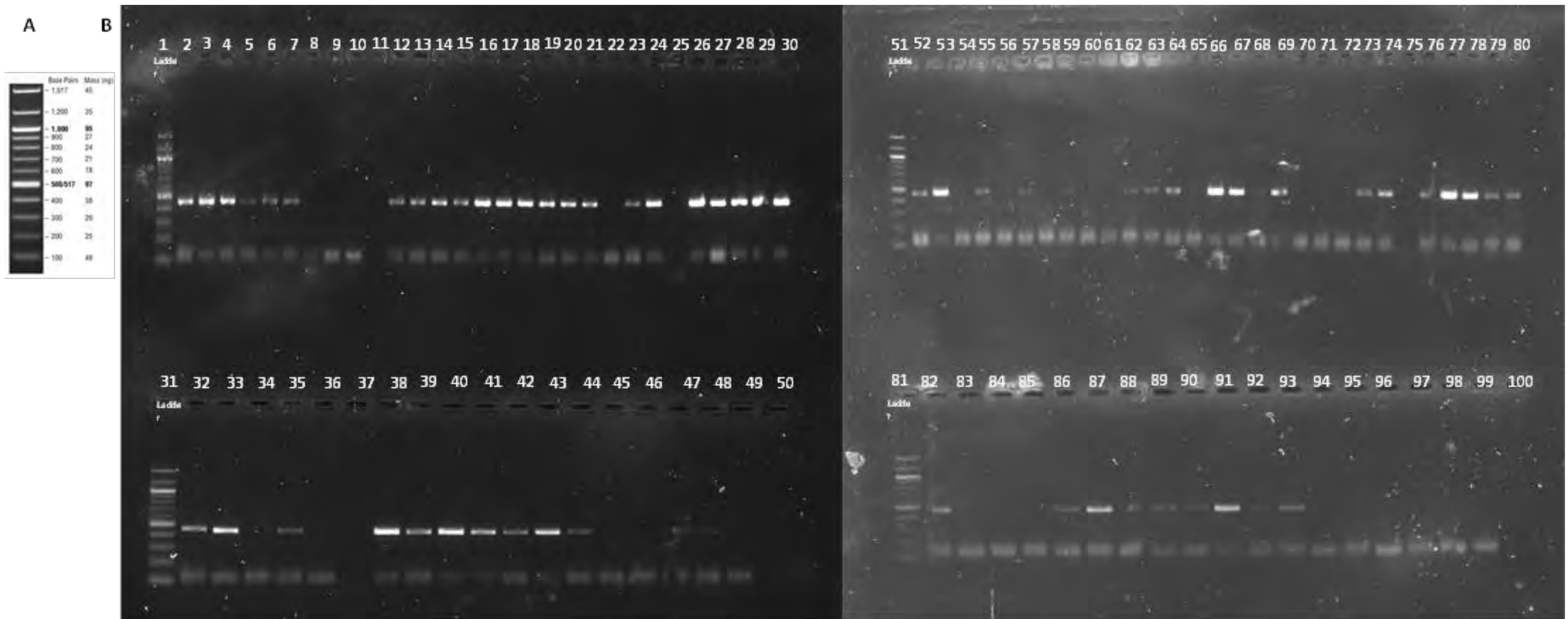


Figure 3. 5 The 16S rRNA gene target amplicon PCR with the complete MWF sample set **(A)** PCR fragment sizes in the 100bp ladder from New England Biolabs **(B)** 16S rRNA gene target amplicon PCR. Lanes 1, 31, 51 and 81 are the DNA ladder. Lanes 2-89 are experimental samples. Lane 89 is *M. avium*, 90. *M. abscessus*, 91. *M. immunogenum*, 92. *M. bovis*, 93. Mock Community, 94-100 Negative control.

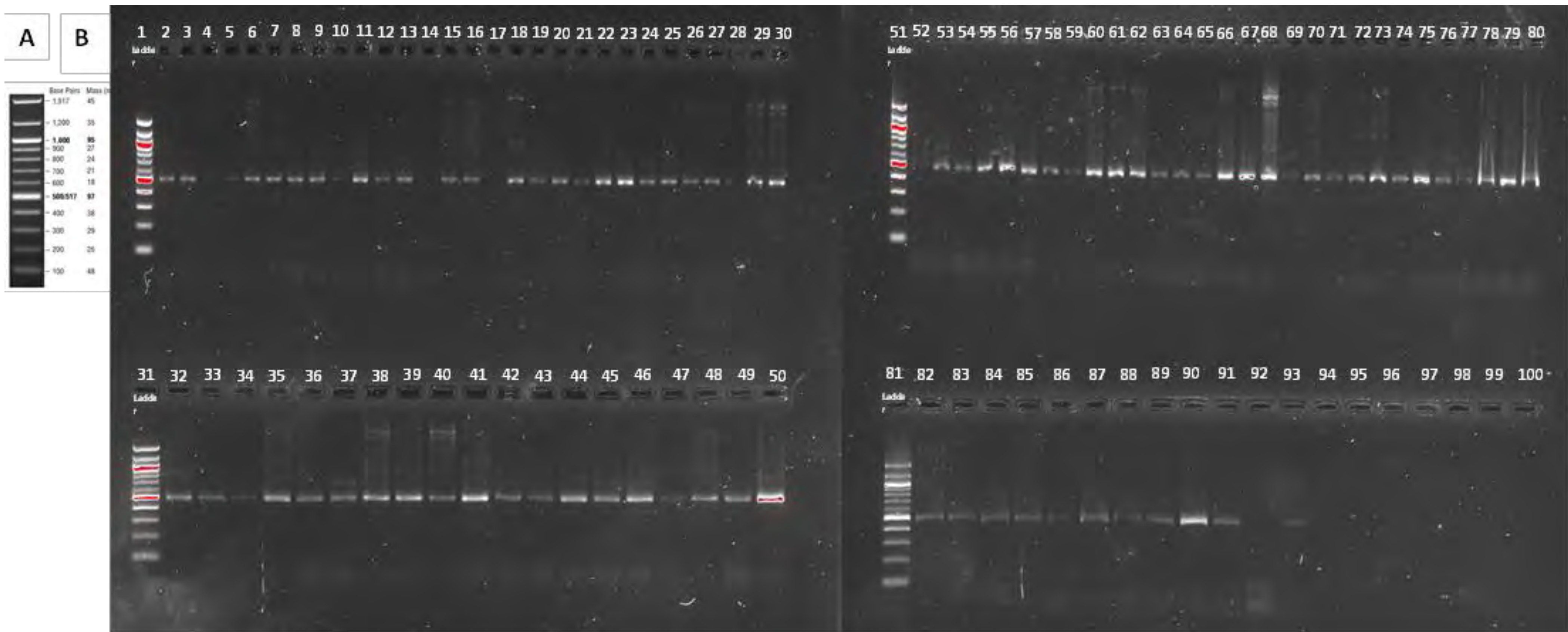


Figure 3. 6 The *rpo*B gene target amplicon PCR with the complete MWF sample set **(A)** PCR fragment sizes in the 100bp ladder from New England Biolabs **(B)** *rpo*B gene target amplicon PCR. Lanes 1, 31, 51 and 81 are the DNA ladder. Lanes 2-89 are experimental samples. Lane 89 *is M. avium*, 90. *M. abscessus*, 91. *M. immunogenum*, 92. *M. bovis*, 93. Mock Community, 94-100 Negative control.

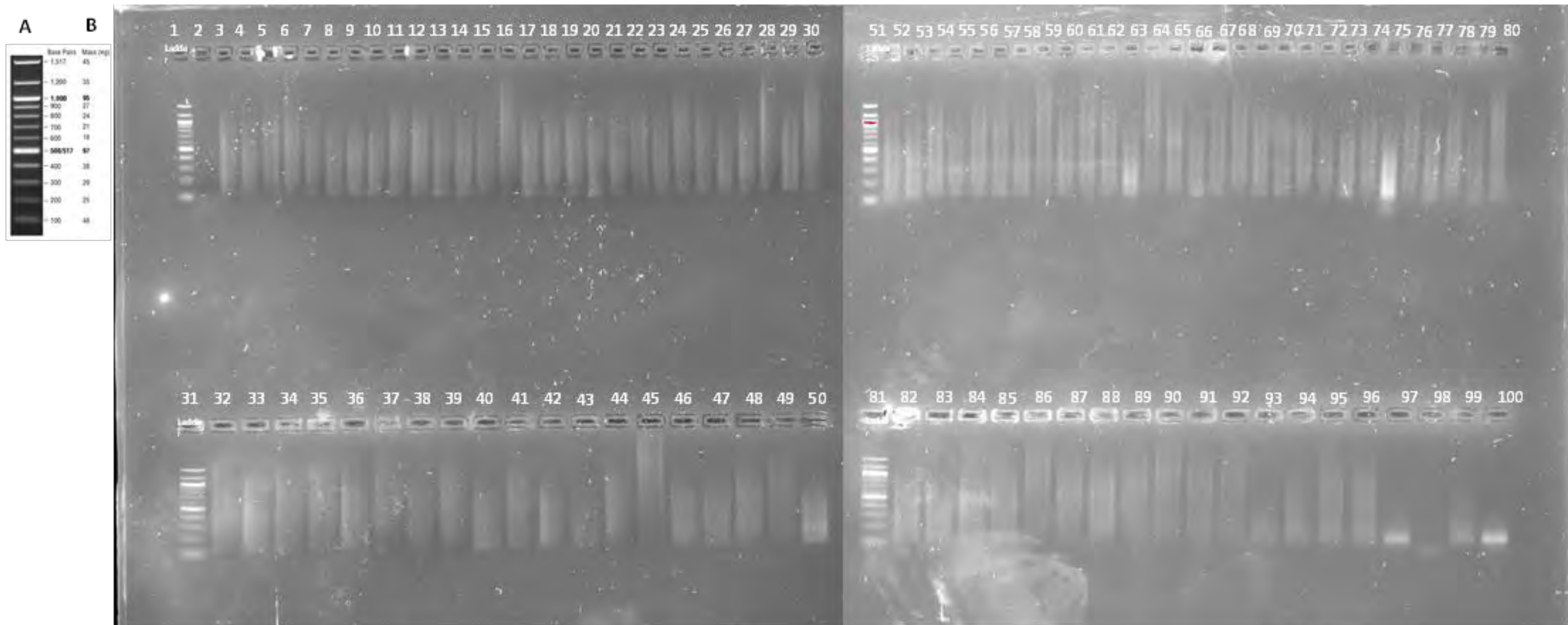


Figure 3. 7 The *hsp*65 gene target amplicon PCR with the complete MWF sample set **(A)** PCR fragment sizes in the 100bp ladder from New England Biolabs **(B)** *hsp*65 gene target amplicon PCR Lanes 1, 31, 51 and 81 are the DNA ladder. Lanes 2-89 are experimental samples. Lane 89 is M. avium, 90. *M. abscessus*, 91. *M. immunogenum*, 92. *M. bovis*, 93. Mock Community, 94-100 Negative control.

3.5.3 *hsp65* Primer Troubleshooting and Optimisation.

Once amplification of the correct fragment was confirmed via gel electrophoresis optimisation began. The 16S rRNA (Figures 3.5 and 3.5) and *rpoB* primers showed good clear target amplification using both the non-phased and phased primer sets (Figure 3.4 and 3.6). The following troubleshooting stages only applied to the *hsp65* mycobacteria specific target which showed smearing and undesired fragment attachment (Figures 3.3 and 3.7).

3.5.3.1 Pooled Primer Concentration Gradient

The *hsp65* pooled phased primer gradients (Figures 3.8) showed amplification in only the mycobacteria strains. As expected, the three controls *P. aeruginosa, S. aureus*, and no template controls had no amplification. The first dilution showed good amplification in both repeats but, a lot of smearing was present and primer dimer. The second dilution showed no amplification in both repeats. The third showed similar results as the first dilution with large amounts of primer dimer but with bright clear bands at the expected size. The second repeat in the 10^{-3} dilution showed less amplification with only the *M. avium* showing a clear band. The other strains showed smearing in their lanes. The 10^{-4} dilution showed clear bands in the *M. abscessus* and *M. avium* lanes, less smearing in all the lanes and less visible primer dimer making this the best dilution of the phased pooled primers for the *hsp65* test.

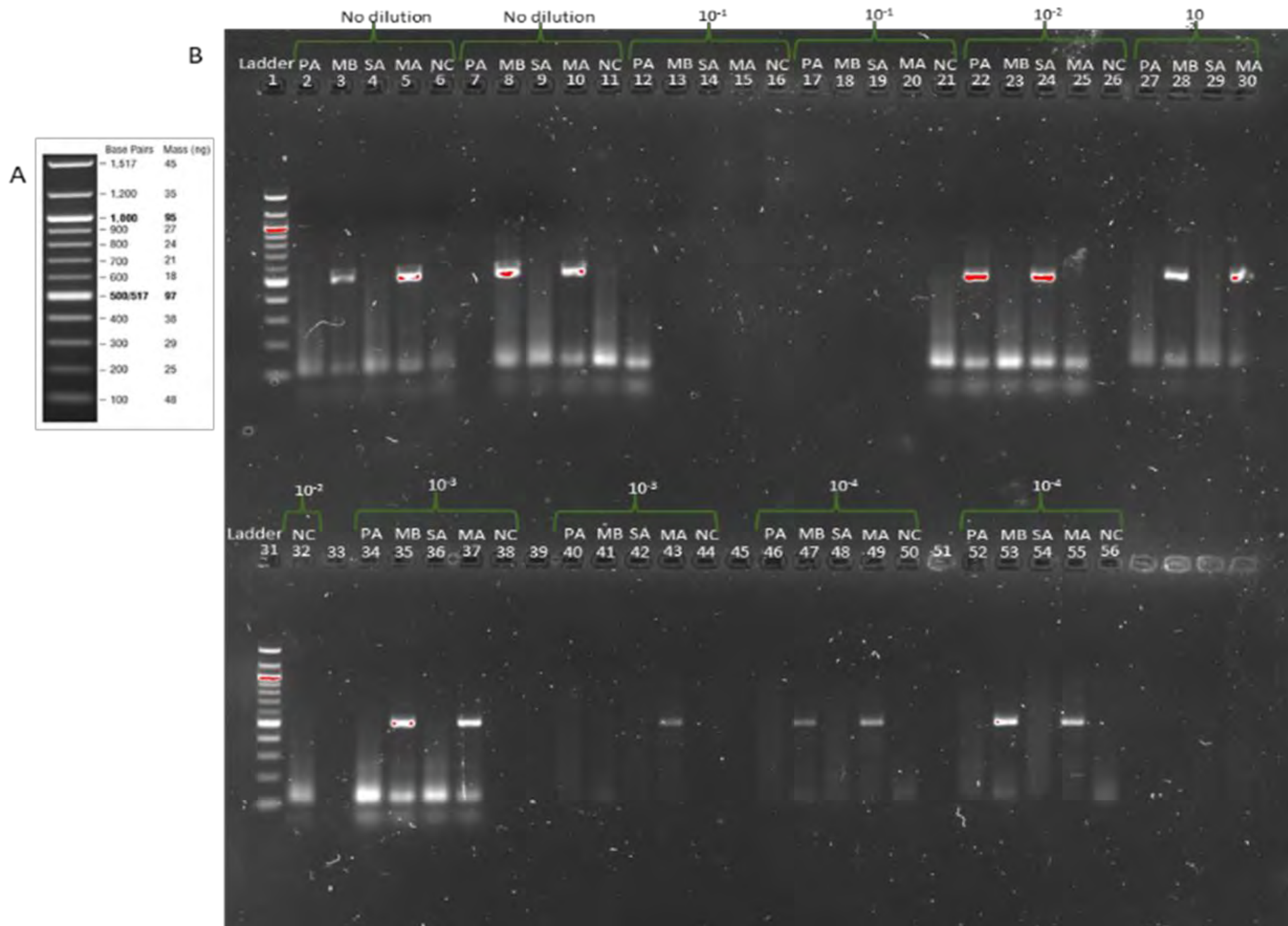


Figure 3. 8 *hsp65* phased primer concentration gradient. Lanes 1 and 31 is the 100bp Ladder from New England Biolabs. Lanes 2 onwards shows the primer dilution factor indicated by the green arrow ranging from no dilution to $10^{-4.}$ The gradient was conducted on the same four strains for each dilution as indicated by the initials under the green arrow. Abbreviation PA (*P. aeruginosa) MB (M. abscessus), SA (S. aureus), (MA) M. avium and* NC (Negative control). Lanes 33,39,45 and 51 are empty.

3.5.3.2 Annealing Temperature Gradient

The *hsp65* Ta gradient (Figure 3.9) shows amplification in both *M. avium* and *M. abscessus*. *M. avium* (lanes 2-13) shows amplification in some Ta with 60.25°C (lane 9) being the best Ta for this strain. *M. abscessus* (lanes 15-27) showed amplification across the gradient and an adequate level in the corresponding Ta (60.25°C, Lane 22). Therefore, 60.25°C is deemed the best Ta for this primer set allowing amplification of two main *Mycobacterium* species. There is however a large amount of smearing in both strains across the lanes.

3.5.3.3 Magnesium Chloride Concentration Gradient

The *hsp65* $MgCl_2^+$ gradient (Figure 3.10) showed no advantage to using increased $MgCl_2^+$. The master mix with no additional $MgCl_2^+$ with a concentration of 2.5mM showed the clearest bands in the most samples. There is still a noticeable amount of smearing in all the lanes of the gel with no clear reduction in any of the samples.

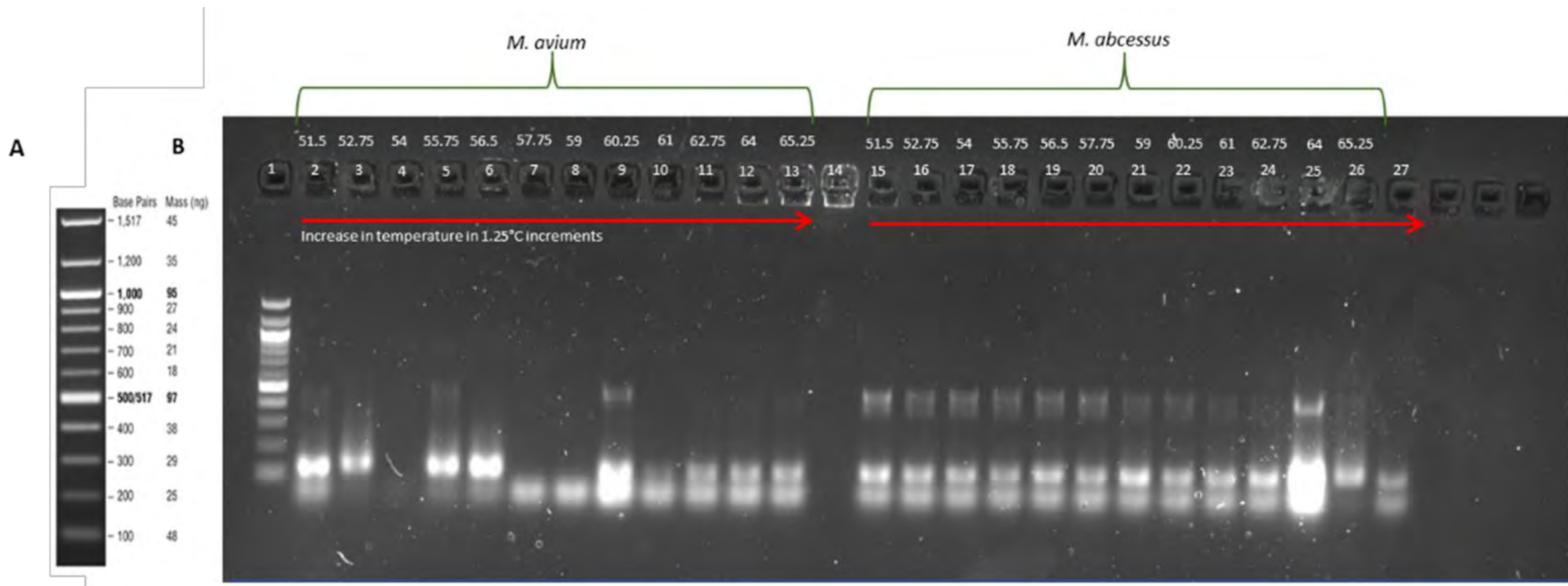


Figure 3. 9 *hsp65* annealing temperature gradient. **(A)** 100bp ladder for reference. **(B)** Annealing gradient green brackets at the top show which strain *M. avium* wells 2-13, *M. abscessus* wells 15-27. The temperature in white corresponds to the well which increases by 1.25°C increments as illustrated by the red arrow below the wells.

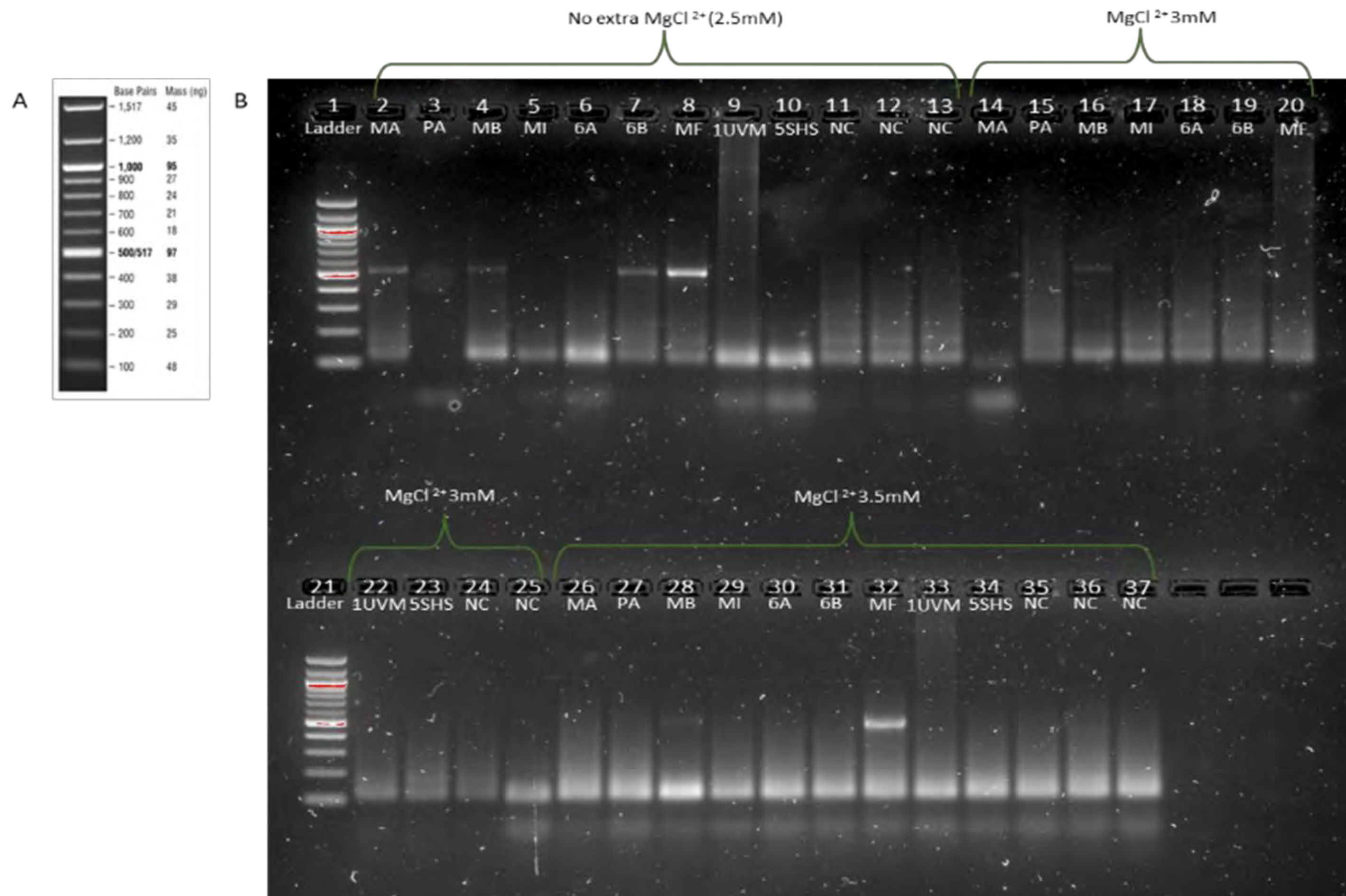


Figure 3. 10 *hsp65* $MgCl_2^+$ gradient **(A)** 100bp ladder for reference. **(B)** $MgCl_2^+$ gradient. Green brackets show concentrations of $MgCl_2^+$, numbers indicate lane number and initials show strains/samples designation; MA – *M. avium*, PA- *P. aeruginosa*, MB- *M. abscessus*, MI- *M. immunogenum*, 6A and 6B sample numbers, MF- *M. fortuitum*, 1 UVM and 5SHS sample numbers, NC-negative controls.

3.5.3.4 Dimethyl Sulfoxide Gradient

The DMSO gradient with low concentrations (Figure 3.11) shows amplification but large amounts of smearing present. The gradient with the larger percentage of DMSO shows much clearer bands where amplification is present. The smearing is still present in the lower concentrations (0-10%) but the band present in the 12 and 14% is clear and the smearing is visibly reduced.

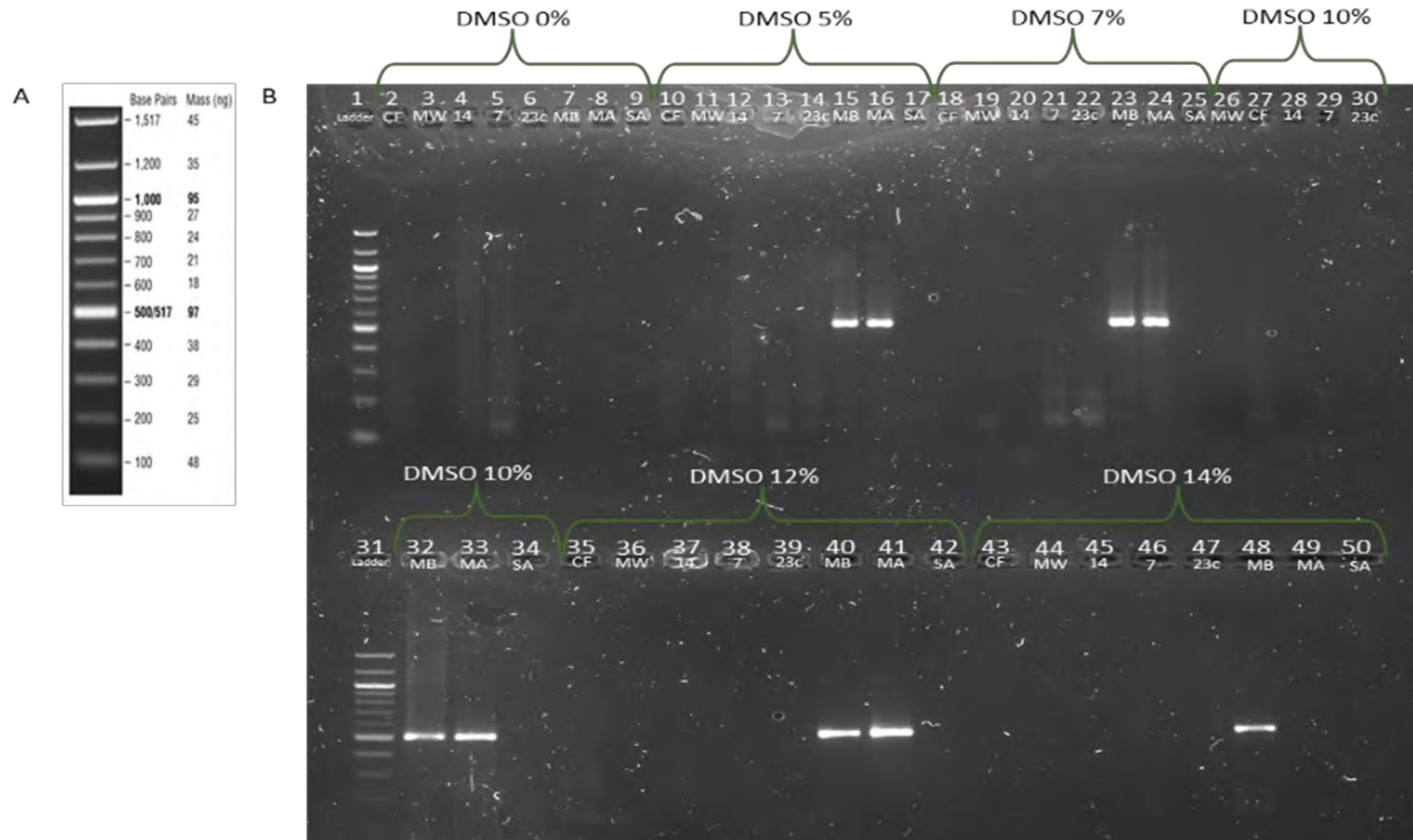


Figure 3. 11 *hsp65* DMSO gradient **(A)** 100bp ladder for reference. **(B)** DMSO gradient. Green brackets show percentage concentrations, numbers indicate lane number and initials show strains/samples designation; MA – *M. avium*, PA- *P. aeruginosa*, MB- *M. abscessus*, MI- M. *immunogenum*, 6A and 6B sample numbers, MF- *M. fortuitum*, 1 UVM and 5SHS sample numbers, NC-negative controls.

3.5.4 Index PCR

The *hsp65* troubleshooting was successful (Figure 3.12) and the step one amplicon PCR was also effective on the 16S rRNA (Figure 3.5) and *rpoB* (Figure 3.6) targets enabling continuation of the library preparation of all three targets.

Index PCR worked well on the MWF experimental samples with all 3 primer sets, confirmed via gel electrophoresis and by Bioanalyzer (Agilent, USA) (Figure 3.5/3.6/3.12). The *hsp65* bioanalyzer trace (Figure 3.13B), shows a lot of noise surrounding the main fragment peak at 620bp. This could be due to residual smearing that was still present after the troubleshooting and PCR clean up (Chapter 2) steps. *rpoB* (Figure 3.13C) has some smaller fragments between 100-300bp which are likely to be primer dimer, followed by a main peak at around 550bp. The 16S rRNA trace (Figure 3.13D) is clean with one prominent peak around the 600bp mark.

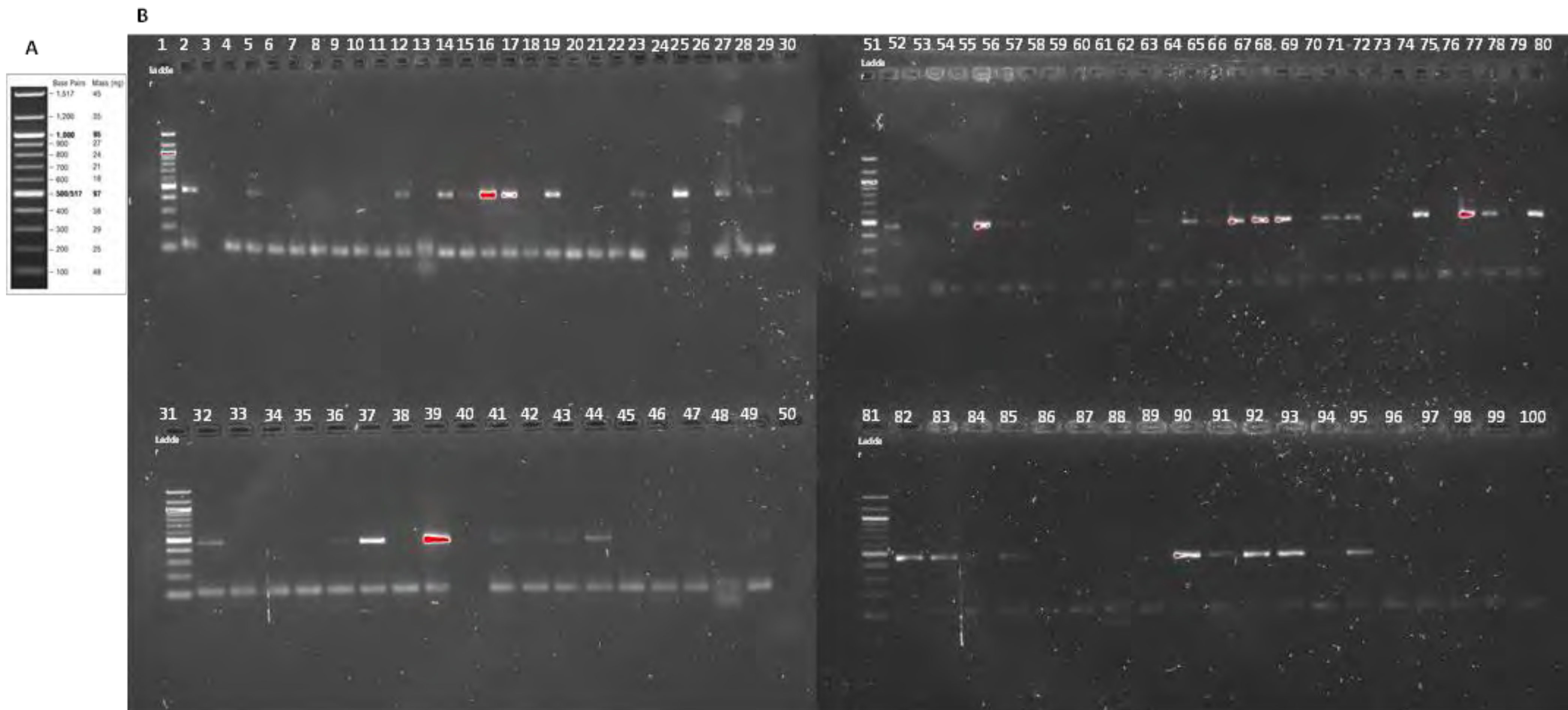
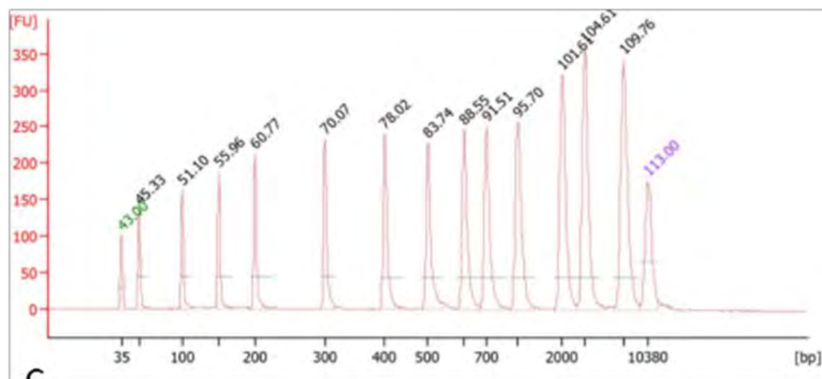
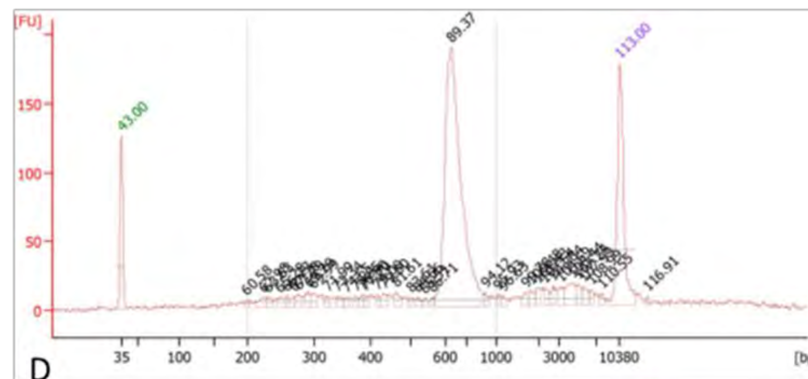


Figure 3. 12 The *hsp*65 gene target amplicon PCR after optimisation with the complete MWF sample set **(A)** PCR fragment sizes in the 100bp ladder from New England Biolabs **(B)** *hsp*65 gene target amplicon Lanes 1, 31, 51 and 81 are the DNA ladder. Lanes 2-89 are experimental samples. Lane 90 is *M. avium*, 91. *M. abscessus*, 92. *M. immunogenum*, 93. *M. Bovis*, 94/95. Mock Community, 96-100 Negative control

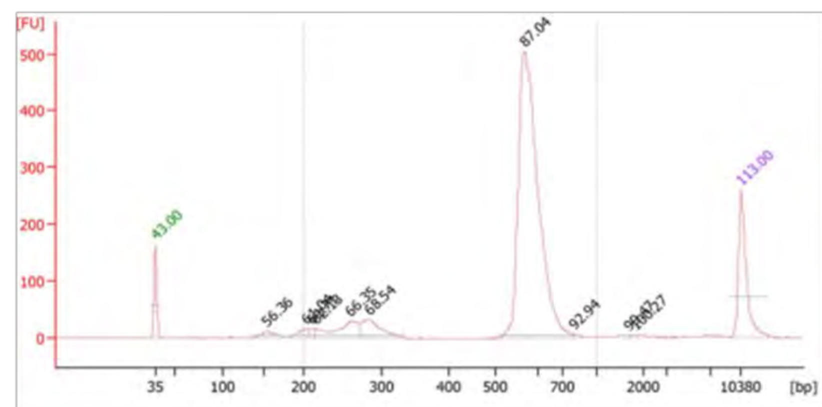
A



B



C



D

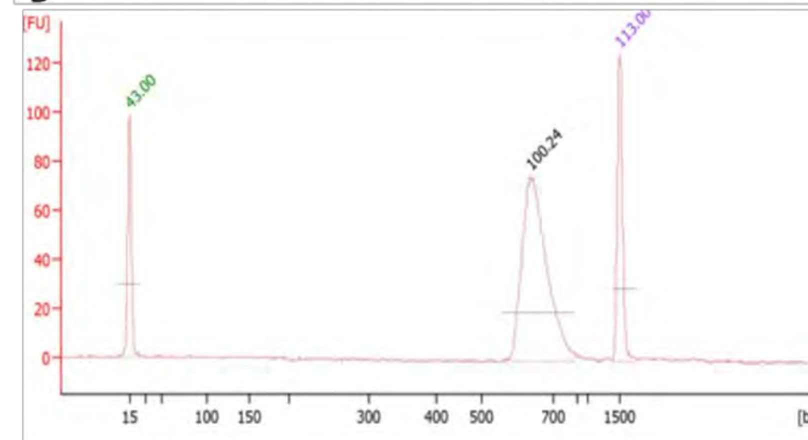


Figure 3. 13 Step 2 index PCR bioanalyzer traces on *M. avium* showing the final fragment sizes before sequencing of the 3 primer sets. **(A)** Ladder, **(B)** *hsp65,* **(C)** *rpoB* **(D)** 16S rRNA

3.5.5 MiSeq Quality Control Metrics.

The initial metrics (Table 3.2) for the v2 500 cycle cartridges had higher MiSeq run metrics compared to the v3 cartridge which showed the lower run metrics.

Table 3. 2 MiSeq Quality Control Metrics

Run ID	[a] %PF	[b] %Q30	[c] Actual yield	[d] Potential yield
v2 500 cycle, PhiX 30%	89.23	77.92	523.17 Mbp	500Mbp
v3 600, PhiX, 30%	88.01	68.28	11.33Gbp	15Gbp

MiSeq quality control metrics: [a] %PF is the percentage of cluster density, [b] %Q30 score estimates the probability of error. [c] The actual yield in base pairs (bp) of the completed sequencing run. [d] The potential maximum of the sequencing run as described by Illumina (2013)

3.5.6 16S rRNA Gene Sequencing Results

The 16S rRNA DADA2 filtering metrics (Table 3.3) showed the v2 500 Nano cartridge had 84% reads passing the filter and the v3 had 9%. It also showed that the v2 500 and v3 cartridges a high percentage of successful merged reads, 85% and 71% respectively, but with higher percent of chimeric sequences (20% and 24%), a range of taxa was assigned to a minimum of genus level. The mock community comparison for the 16S rRNA gene (Figure 3.14) showed that this gene failed to detect all the NTM and bacterial taxa in the mock community. The v2 500 cartridge (Purple bar) shows detection of *S. aureus*, *P. aeruginosa*, and *M. abscessus* which were expected to be seen in the mock community. However, it also shows that the 16S rRNA gene detected *Streptococcus cristatus, Pseudoflavonifractor phocaeensis, Delftia tsuruhatensis* and *Achromobacter marpltensis* which were not part of the mock community composition.

Table 3. 3 16S rRNA gene DADA2 Filtering Metrics

Run ID	Target	[a] Reads passing filter			[b] Successful merged reads	[c]himeric sequences
v2 500 cycle, PhiX 30%	16S rRNA gene	In: 241045	Out: 84 %	Lost: 15%	85%	20%
v3 600, PhiX, 30%	16S rRNA gene	In: 12709198	Out: 9%	Lost 90%	71%	24%

16S rRNA gene target DADA2 filtering metrics: [a] the reads passing filter indicate the reads that were of good quality according to DADA2s algorithm. Forward and reverse reads were truncated at 200bp and 175bp, respectively. This truncation was based on the quality scores provided by the DADA2 pipeline and aimed to remove low quality bases and retain the required overlap of the paired ends reads for merging downstream. [b] Successful merged reads is the percentage of forward and reverse reads that are successfully paired and merged together. [c] %Chimeric sequences is the percentage of sequences that DADA2 donated as chimeric (a sequence that is a hybrid of two or more different sequences)

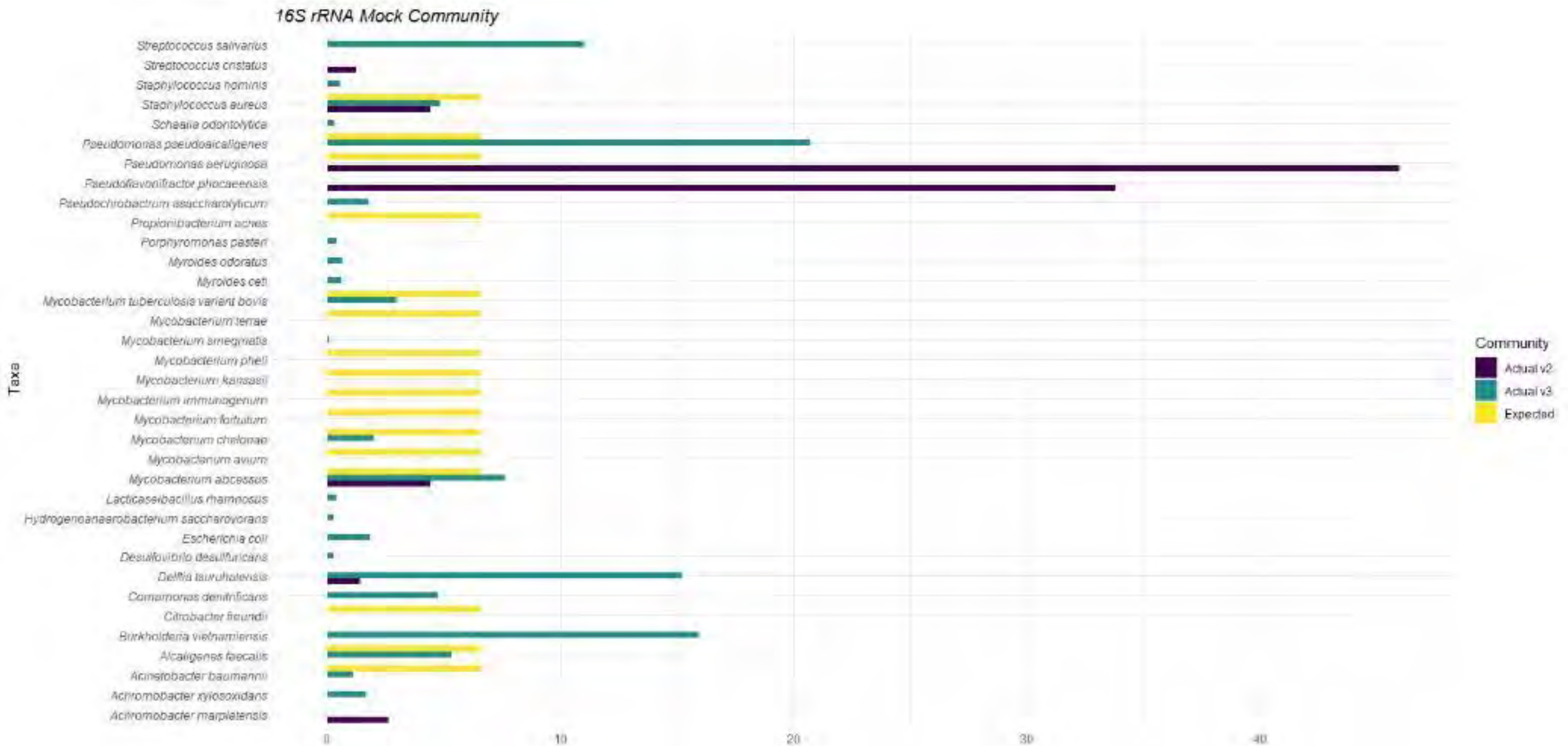


Figure 3. 14 The 16S rRNA Mock community. The left-hand side shows the taxon names, and the bars represent taxa expected (yellow) to be detected against the actual from the v2 (dark purple) and v3 (teal) sequencing results. The plot shows a range of different bacterial taxa detected and expected by the gene sequencing. This gene has the most expected taxa then the mycobacteria specific genes.

3.5.7 *hsp6*5 Sequencing Results

The *hsp65* DADA2 filtering metrics (Table 3.3), shows the v2 nano 500 cycle had the highest number of reads passing the quality control filter (56%) in comparison to the v3 (55%). It showed that the v2 500 cycle had 89% of pair end reads merged and 0% of chimeric sequences and successfully assigned 13 NTM OTUs. The v3 cartridge had 55% of reads passing the filter and a higher percentage of successful merged reads (94%), with 4 NTM OTU taxa assigned (Figure 3.15).

The mock community comparison for the *hsp65* gene (Figure 3.15) showed that this gene did not detect any non-NTM taxa in the mock community and the v2 (Purple bar) detected 2 out of 9 NTM taxa present in the mock community and found *M. gadium*. The v3 detected ~33% NTM taxa present in the mock community and detected *M. austroafricanum* which was not part of the mock community.

Table 3. 4 *hsp*65 DADA2 Filtering Metrics

Run ID	Target	Reads passing filter			Successful merged reads	%Chimeric sequences
v2 500 cycle, PhiX 30%	*hsp*65 gene	140390	56 %	43 %	89%	0%
v3 600, PhiX, 30%	*hsp*65 gene		55%	44%	94%	1%

*hsp*65 gene target DADA2 filtering metrics: [a] the reads passing filter indicate the reads that were of good quality according to DADA2s algorithm. Forward and reverse reads were truncated at 200bp and 175bp, respectively. This truncation was based on the quality scores provided by the DADA2 pipeline and aimed to remove low quality bases and retain the required overlap of the paired ends reads for merging downstream. [b] Successful merged reads is the percentage of forward and reverse reads that are successfully paired and merged together. [c] %Chimeric sequences is the percentage of sequences that DADA2 donated as chimeric (a sequence that is a hybrid of two or more different sequences)

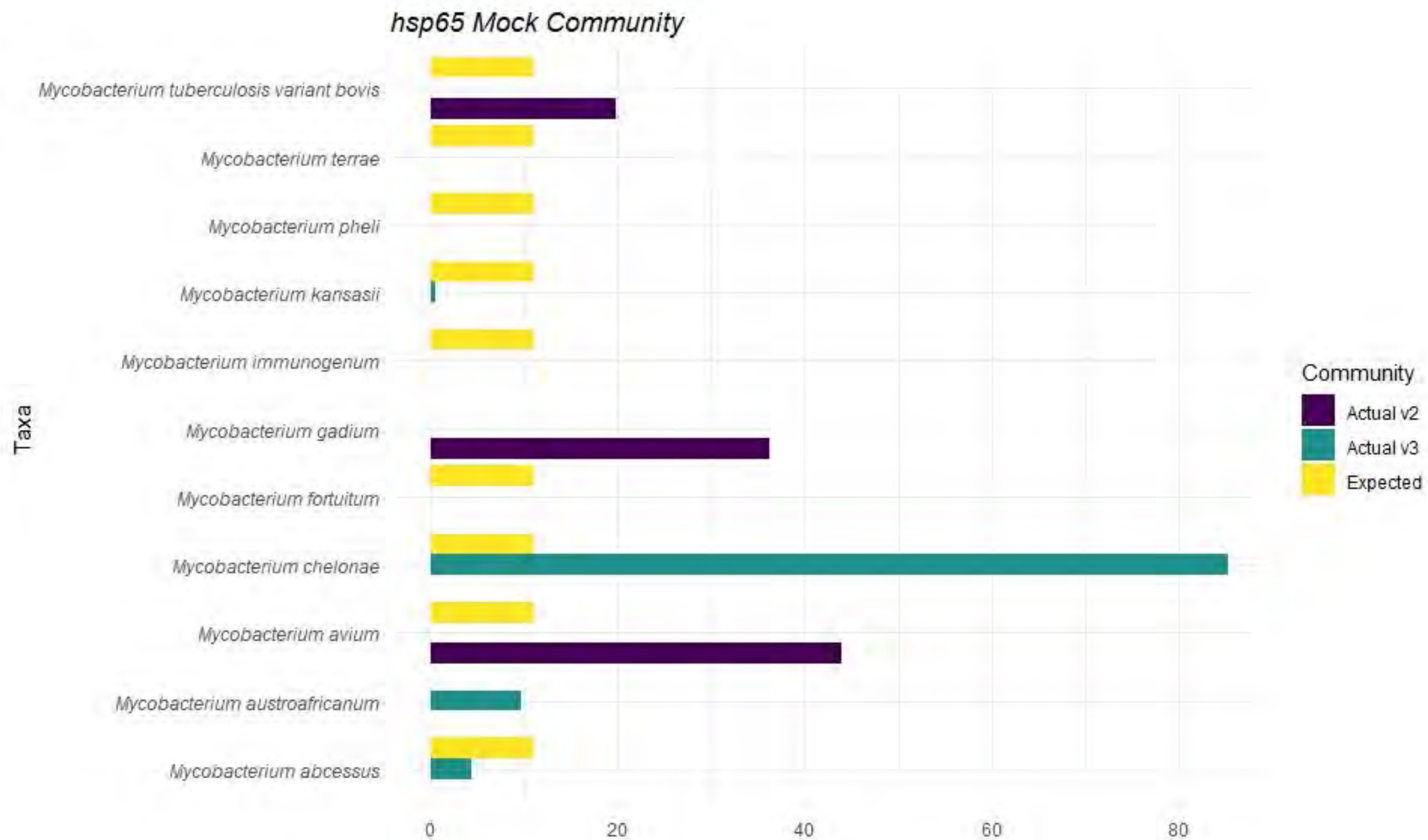


Figure 3. 15 The *hsp65* Mock community. The left-hand side shows the Taxon names, and the bars represent taxa expected (yellow) to be detected against the actual from the v2 (dark purple) and v3 (teal) sequencing results.

3.5.8 *rpo*B Gene Sequencing Results

The *rpoB* DADA2 filtering metrics (Table 3.4), shows the v2 nano 500 cycle had the highest number of reads passing the quality control filter (56%). It showed that the v2 500 cycle had 67% of pair end reads merged and 0% of chimeric sequences and successfully assigned 13 NTM OTUs. The v3 cartridge had 46% of reads passing the filter and a higher percentage of successful merged reads (70%), with 17 NTM OTU taxa assigned (Figure 3.16).

The mock community comparison for the *rpo*B gene (Figure 3.16) showed that this gene did not detect any non-NTM taxa in the mock community and the v2 (Purple bar) detected ~77% NTM taxa present in the mock community. The v3 also detected 7 out of the 9 NTM taxa present in the mock community.

Table 3. 5 *rpo*B DADA2 Filtering Metrics

Run ID	Target	Reads passing filter			Successful merged reads	%Chimeric sequences
v2 500 cycle, PhiX 30%	*rpo*B gene	In: 499556	Out 56 %	Lost: 43%	67%	0%
v3 600, PhiX, 30%	*rpo*B gene	In: 2933315	Out: 46 %	Lost: 53%	70%	1%

*rpo*B gene target DADA2 filtering metrics: [a] the reads passing filter indicate the reads that were of good quality according to DADA2s algorithm. Forward and reverse reads were truncated at 200bp and 175bp, respectively. This truncation was based on the quality scores provided by the DADA2 pipeline and aimed to remove low quality bases and retain the required overlap of the paired ends reads for merging downstream. [b] Successful merged reads is the percentage of forward and reverse reads that are successfully paired and merged together. [c] %Chimeric sequences is the percentage of sequences that DADA2 donated as chimeric (a sequence that is a hybrid of two or more different sequences)

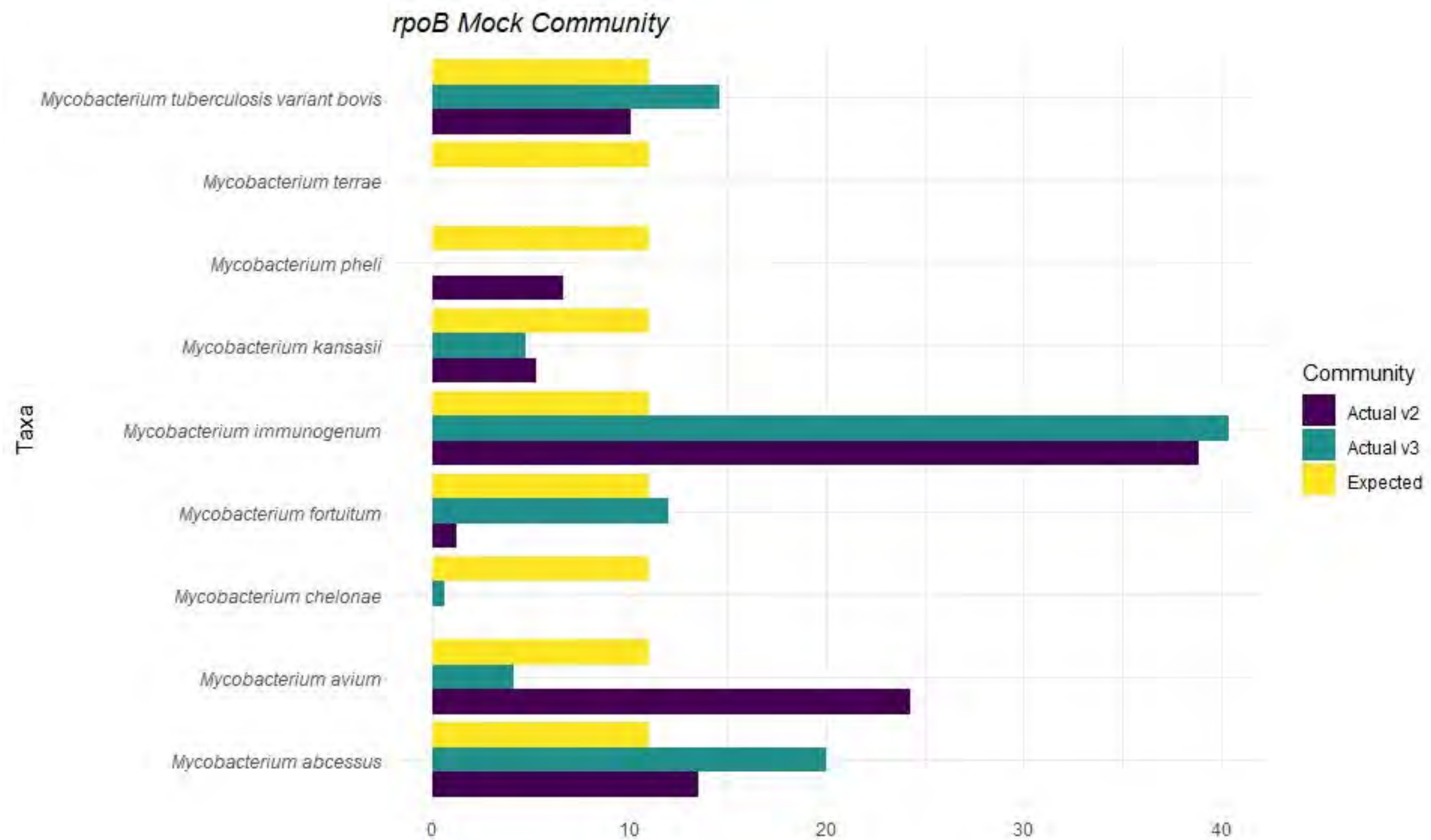


Figure 3. 16 The *rpoB* Mock community. The left-hand side shows the Taxon names, and the bars represent taxa expected (yellow) to be detected against the actual from the v2 (dark purple) and v3 (teal) sequencing results.

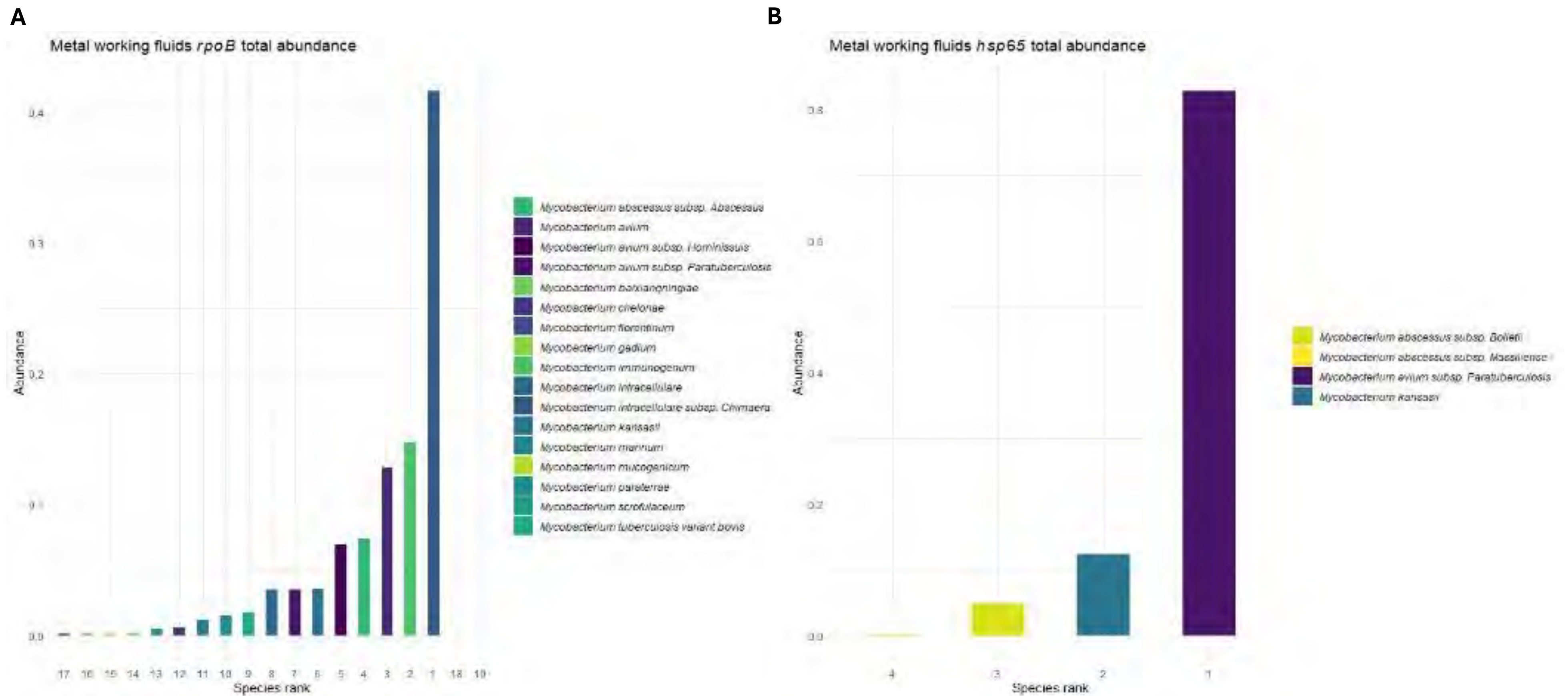


Figure 3. 17 The *rpoB* and *hsp*65 total abundance. The coloured bars correspond to the taxa listed in the legend. The y axis shows the abundance, and the x axis is the rank of the species detected. **(A)** The total community abundance detected by the *rpoB* and v3 reagent kit. **(B)** The *hsp*65 total abundance using the v3 reagent kit.

3.6 Discussion

Detection of NTMs is challenging, with the detection of specific species even more difficult to achieve. Molecular methods are generally more sensitive and less time consuming than traditional culture-based techniques. But these techniques can be costly and difficult to implement into practice (Griffith et al., 2007; Ryu et al., 2016).

The aim of this chapter was to optimise amplicon sequencing on the Illumina MiSeq system and increase the detection of NTMs in low diversity sample sets. To meet this aim primers for 16S rRNA, *hsp65* and *rpoB* gene targets were selected from the literature and underwent several optimisation steps. The selected primers were adapted with Illumina overhang adapters and phased random bases (Figure 3.1) as an attempt to increase the final base diversity to prevent over clustering during sequencing.

Previous studies examining NTMs using NGS mostly used a single sequencing target approach such as 16S rRNA V4 region (Caverly et al., 2016), *hsp*65 target (Cowman, 2018) or *rpo*B target (Manjeese et al., 2017; Nasiri et al., 2017). Other research used multiple targets but sequenced them separately (Gebert et al., 2018), 16S rRNA and *rpo*B (Shen et al., 2022), *hsp*65 and *rpo*B (Clarke et al., 2022). However, I have failed to find any other studies multiplexing 16S rRNA and an NTM specific target using phased primer sets.

3.6.1 Fragment Confirmation and Step One Amplicon PCR

Firstly, the primers were assessed to confirm the correct amplicon was being amplified (Section 3.5.2). This was to ensure that all the targets were the expected size in bp, and further optimisation steps could be taken. The correct amplicon was amplified in all three targets (Section 3.6.1). Then the MWF samples were prepared for sequencing using the phased primer sets (Figure 3.1).

The 16S rRNA and *rpoB* gene targets (Figures 3.5 and 3.6) showed the PCR had worked and the correct fragment was amplified with no fragments in the negative controls. Since the 16S rRNA gene is used widely (Clarridge, 2004) and the confirmation gel electrophoresis image (Figure 3.2) was clear no further optimisation was done on this target. Similarly, for the *rpoB* target the amplicon gel (Figure 3.4) also showed clear bands and minimal amount of smearing although some primer dimer and some smaller amplicon fragments around 200bp were amplified. This was of no concern because the

PCR clean up using Ampure XP beads (Beckman Coulter Life Sciences, USA) (Chapter 2) removes fragments of >200bp.

The *hsp65* target confirmation gel (Figure 3.3) showed multiple different size fragments, the correct fragment at 439bp, a large fragment around 900bp and some smaller primer dimer fragments when applied to the MWF samples the confirmation gel showed failed PCR with large amounts of smearing in almost all lanes (Figure 3.7). The possibility of template degradation and reagent contamination was low since all library preparation was done in the same way using the same freshly prepared reagents, new plasticware and template. Therefore, it signified to be an issue with primer/target binding.

3.6.1.1 *hsp65* Optimisation

Due to the smearing the *hsp65* primer target underwent a range of optimisation stages (Section 3.5.4) before being prepared for downstream sequencing.

A primer concentration gradient (Figure 3.8) was performed to determine the optimal primer concentration of the pooled phased primer sets and to check that the primer was still specific to NTM species. The ideal concentration for the gradient was 10^{-4} dilution due to the least amount of smearing and visible primer-dimer with both RGM (*M. abscessus*) and SGM (*M. avium*) NTM species being amplified successfully. This was then followed by a temperature gradient (Figure 3.9) to find the best Ta for the pooled primers on both NTM species this was determined to be 60.25°C as this showed amplification in NTM targets. Another attempt to reduce the smearing and increase binding specificity was changing the concentration of $MgCl_2^+$ (Figure 3.10). The concentration of $MgCl_2^+$ made no difference to the amplification or the smearing with higher concentrations showing large amounts of smearing so, this variable remained unadjusted (neat master mix 2.5mM). The final optimisation stage was a DMSO gradient (Figure 3.11). This showed that an added 12% DMSO to the master mix reduced the smearing and unwanted amplicon amplification significantly.

Once the optimisation was completed the new parameters (Pooled primer concentration 10^{-4}, Ta of 60.25, and 12% DMSO) were used on the first stage amplicon PCR using the *hsp*65 target (Figure 3.12). This showed clearer bands in most of the gel lanes, the lanes without a band could be NTM negative. The successful reduction of the template smearing in the *hsp65* target meant that this could be used in the downstream sequencing and evaluated against the *rpoB* target for NTM detection.

3.6.2 Mock Community and Taxa Assignment

The 16S rRNA target's role was to amplify the entire microbiome to give an overview of what taxa are resident at the time of sampling. When compared to the mock community (Figure 3.14) it showed the widest range of taxa including expected bacterial *species P. pseudoalcaligenes, P. aeruginosa, S. aureus, A. faecalis* and *A. baumanii.* It did detect a range of species not included in the mock community *S. salivarius, S. cristatus, S. hominis, P. phocaeensis, E. coli* and others. This could be due to the 16S rRNA primers being unable to distinguish between certain species due to genetic relatedness (Kim et al., 2011; Fouhy et al., 2016), the wrong taxa being assigned in the sequence analysis stage (Schloss and Westcott, 2011) or DNA extraction kit / environmental contaminants (Sheik et al., 2018). In terms of NTM species detected from the mock community the 16S rRNA gene only managed to detect *M. abscessus, M. chelonae* and *M. bovis*, but it did detect *M. smegmatis* and which can be hard to distinguish from other NTM species (Falkinham, 1996).

Several studies using the 16S rRNA gene as a target in conjunction with a NTM specific gene found the 16S rRNA gene lacked in discriminatory power when identifying closely related sequences (Maleki et al., 2017; Gebert et al., 2018). This suggests that the 16S rRNA gene should be used to capture the wider microbiota and an NTM specific target should be used to detect the NTMs present in the sample. One study found that the rate of NTM species level identification for 16S rRNA was 71.13%, which was lower than that of *hsp*65 (86.79%) and *rpo*B (81.55%) (Kim and Shin, 2018). Another study found that when targeting the 16S rRNA V4 region only 53% of NTM sequences where detected in NTM culture positive samples (Clarridge, 2004; Caverly et al., 2016). However, the 16S rRNA gene is an excellent target for a broad range of bacterial taxa (Clarridge, 2004) and should not be disregarded when designing studies looking at specific complexes like NTMs. The capture of the wider microbiome is important to understand how the communities that reside within it interact.

The *rpoB* assigned the widest range of NTM OTUs (Figure 3.16) and detected both RGM and SGMs, including *M. abscessus and M. immunogenum* which are not only expected in this type of sample but previously confirmed by inhouse sequencing done at HSE Buxton (data not shown). It was also successful in detecting NTMs down to sub species level. When examining the *rpoB* detection regarding the mock community it was found that it was successful in detecting most of the NTM species expected except *M. terrae.* These

results are similar to another study examining the ability of the *rpoB* primers to detect a range of NTM species and succeeded in using PCR amplification-restriction analysis (PRA) to detect several NTMs down to sub-species level, with the exclusion of *M. terrae* (Kim et al., 2001).

Other work found that *rpo*B gene target for sequencing could differentiate between the species and subspecies in the *M. avium* complex; *M. avium, M. avium* subsp *paratuberculosis, and M. intracellulare* (Haig et al., 2018). All of which were detected by the *rpo*B gene in the MWF (Figure 3.17). The study by De Zwaan et al. (2014) found that *rpo*B correctly identified 88.5% of NTM isolates to species level when compared to 16S rRNA gene target which correctly identified 85.7% of NTMs to species level. Concluding that *rpoB* gene sequencing had more discriminatory power then 16S rRNA and that it showed promising results (De Zwaan et al., 2014), similar to results reported in this thesis. However, Kim and Shin (2018) found the *rpo*B target 5.44% less effective at NTM identification then the *hsp*65 target (Kim and Shin, 2018) with a more recent study has proposed the use of other gene targets as confirmation to the use of *rpoB* to increase accuracy and validity of the targets and further discriminate between closely related species (Khosravi et al., 2022). Though these results are very favourable in the use of *rpo*B for NTM detection, none of the studies (De Zwaan et al., 2014; Haig et al., 2018; Kim and Shin, 2018) mentioned or found the need to multiplex or add phasing to the primers to increase diversity.

The *hsp65* gene target showed the least number of taxa assigned (Figure 3.17), however it was very specific and able to detect *M. abscessus* subspecies (*subsp. Bolletii, Massiliense*) and *M. avium subsp. Paratuberculosis.* When examining the mock community, it performed the worse detecting only five expected species (*M. avium, M. abscessus, M. kansasii* and *M. bovis*, *M. chelonae). M. austroafricanum and M. gadium* were also detected. This could be due the primer failing to distinguish between species due to genetic relatedness (Wilson et al., 2001) or the additional species being present as part of complexes (Jia Khor et al., 2021). In this study these primers had to undergo a large amount of optimisation which could have affected its downstream efficiency. This target is widely used in NTM research, and other studies have confirmed its effectiveness in a range of settings (Telenti et al., 1993; Maleki et al., 2017; Cowman et al., 2018; Gebert et al., 2018).

Cowman et al. (2018) targeted the *hsp*65 gene in sputum samples from NTM positive COPD and bronchiectasis patients using a mock community (16 type strains) as a positive control. The study successfully detected all species within the mock community with very little variability between the expected proportion of each species detected. However, *M. xenopi* and *M. tuberculosis* were found to be overrepresented. There was no mention of additional optimisation steps on the *hsp*65 primer. The sequencing of patient samples detected 21 NTM species/subspecies (Cowman et al., 2018), a much higher detection rate than the current study which only detected 5 different species. Similarly to the NTM detected in this study Cowman et al. (2018), found that *M. avium* was the most detected species within the sample group, and was able to detect *M. abscessus* subsp. *bolletii, M. kansasii* but, did not detect *M. abscessus* subsp. *Massiliense* or *M. avium.* Subsp. *Paratuberculosis* (Figure 3.17).

A study that used both the 16S rRNA and *hsp*65 gene to detect NTM species from water sources found the 16S rRNA lacking in differentiational ability and the *hsp*65 gene was able to distinguish the closely related species (Maleki et al., 2017). There was significant differences in experimental design between the Maleki et al. (2017) work and this chapter. Maleki and colleagues used an *M. tuberculosis* strain as a positive control not a mock community, no further optimisation was done on the target primers, and the studies purpose was to detect NTM in hospital water systems. It did however succeed in detecting 12 different species of NTM, only one species (*M. kansasii*) matched the species detected in our study (Figure 3.17), and 6 species (*M. abscessus, M. fortuitum, M. simiae, M. fortuitum, M. peregrinum, M. kansasii*) matched Cowman et al. (2018). This could be due to the samples being derived from water systems and not MWF.

Research examining the ecological distribution of NTM in showerhead biofilms used a combination of 16S rRNA and *hsp*65 gene sequencing and like other studies determined that 16S rRNA alone lacked resolution for closely related species (Gebert et al., 2018). Again, there are differences in the experimental design between the Gebert et al. (2018) study, the previously mentioned studies (Maleki et al., 2017; Cowman et al., 2018) and in this chapter. Gerbert and colleagues had no mention of a positive sequencing control except for a “DNA extraction kit control” (Gebert et al., 2018). Their study did find 34 phylogenetically defined mycobacterial clades which were mostly related to *M. gordonae* a species detected in the two previously mentioned studies (Maleki et al., 2017; Cowman et al., 2018) but not detected in this chapter. Gebert et al. (2018) did however, detect

MAC, *M. chelonae, M. gadium, MABSC,* and *M. fortuitum* all which were successfully found in the mock community of this study, however, there was no mention of classification down to sub species level.

Whilst all the chosen targets had their limitations, they all successfully identified some bacterial taxa within the mock communities. This approach is useful in terms of multiplexing different targets on one sequencing experiment. The use of phased primers and multiple gene targets may have increased the clustering diversity allowing low diversity libraries to be sequenced simultaneously without, much need for adjustment in downstream data analysis steps. But further comparative testing should be done.

The results are beneficial as it reduces the need to run multiple MiSeq cartridges, reducing cost and time. It also, identified NTM species down to subspecies level in some instances increasing the knowledge of NTM complexes present in the sample sets. The 16S rRNA provided detailed background microbiome sequencing data whilst acting as a control and reducing bias toward NTM. The *rpoB* showed the most diverse NTM detection picking up the core pathogens and other lesser NTM species. The *hsp65* performed well in the metrics and filtering stages it did not provide however the depth of NTM species expected from these samples. In terms of application the choice of primers is heavily dependent on the expected species/sub-species in the study.

3.6.3 Sequencing

The three targets were prepared for sequencing. A final essential quality control step was done on the Agilent Bioanalyzer (Figure 3.13), to ensure that all the amplicon fragments are of similar size. This data is used for the normalisation of each library. This showed that all three libraries had the correct size but, the *hsp65* trace (Figure 3.13B) showed a lot of noise around the base of the peak, this was probably due to residual smearing that couldn't be removed through PCR clean up and optimisation steps. A possible solution to this would be adding an additional PCR clean-up step, but this could reduce the target DNA yield for sequencing (Aigrain et al., 2016), which could skew downstream results. Another possible solution would be to excise the DNA band from an agarose gel. This would ensure the correct fragment size was selected but does not guarantee removal of the noise and DNA loss is again an issue (Aigrain et al., 2016; Abraham et al., 2017).The *rpoB* trace (Figure 3.13C) also showed a small amount of noise, this could be unspecific binding.

The v2 500 cycle reagent kit which had all three targeted libraries showed 89.23%PF, 77.92% Q30 score and a yield 523.17Mbp. This was overall a successful run and two of three libraries produced high percentages of merged reads in the DADA2 pipeline (Table 3.2 and 3.3). 16S rRNA showed 85% merged reads and 20% chimeric, the *rpoB* target performed the worse out of the three libraries with only 21% merged reads but, the chimeric value was low (1%). The *hsp65* showed good metrics with 89% reads merged and a low chimeric value (1%). Because this final quality control step was successful the multiplexed library sequencing was done on a final v3 600 cycle reagent kit.

The final v3 run was overall a success the initial metrics (Table 3.1) showed 88% PF, the Q30% score was a little under the threshold (68.28%) possibly because the v3 runs for longer and has a higher capacity (Illumina, 2017), and with 11.33Gbp out of a potential of 15Gbp the yield was good considering this is an experimental run with a very low diversity sample set. For the DADA2 filtering (Tables 3.1 and 3.2) all three targets performed well. The 16S rRNA target had 71% successful merged reads with 24% chimeras, the *rpoB* target performed similarly with 70% successfully merged reads but with 0% chimeric sequences and finally the *hsp65* performed highest on DADA2 metrics with 94% paired reads and 1% chimeras. However, for the purpose of this study and for testing polymicrobial samples *rpo*B used in conjunction with 16S rRNA would capture a wider range of species. Therefore, the *hsp*65 target was not used in subsequent chapters.

3.7 Study limitations

The *hsp65* primers had to undergo a large amount of optimisation, this could have affected its downstream capabilities resulting in a less optimal primer. Therefore, when considering which target to use a robust selection process should be used.

This study was limited by non-standardised data bases for the analysis of *rpo*B and *hsp*65 sequences. The database used was NCBI BLAST, which while useful for 16S rRNA sequencing proved difficult when accurately assigning taxa via the *rpo*B or *hsp*65 data. To alleviate this issue only sequences with <99% were used. The limitations of using a targeted amplicon such as *rpo*B or *hsp*65 is the partial coverage within the genus, as this study has shown both primer sets succeeded in detecting NTMs within both the mock community and the experimental samples but differed in the taxa identified.

3.8 Conclusion

The multiplex amplicon sequencing of 16S rRNA and *rpo*B using phased primers is an innovative method for detecting non-tuberculous mycobacteria (NTM) species and the background microbiome concurrently. This approach holds the potential to significantly reduce the time required for NTM detection in clinical samples, which could lead to quicker and more targeted treatment, ultimately improving patient outcomes.

However, to fully realize the benefits of this method, further streamlining is required. The next stage of this research involved applying the phased 16S rRNA and *rpo*B multiplex amplicon sequencing approach to clinical samples from cystic fibrosis (CF) patients who are positive for NTM infection, but omitting the hsp65 as the coverage was not robust enough. The goal is to assess the composition of the microbiota in these patients and explore the potential impact of genetic modulators on the lung microbiome in NTM-positive CF patients.

By gaining a better understanding of the microbiota composition in NTM-positive CF patients, this research has the potential to identify new targets for treatment and improve the clinical management of this disease. By highlighting the species present in the wider microbiome and within the NTM complexes themselves. The aim is to provide more personalized and effective treatment options for CF patients with NTM infections, which could lead to improved patient outcomes and a better quality of life.

Chapter 4: NTM Communities in Adult CF Lung Infection

4 Introduction

Pathogens such as *Mycobacterium avium* and other non-tuberculosis mycobacteria (NTM) complexes have been found as aetiological agents of cystic fibrosis (CF) associated lung infection. Their presence is related to greater decline in lung function and faster progression of pulmonary disease in comparison to those infected with canonical CF pathogens (Esther et al., 2010; Martiniano et al., 2014). Treatment and diagnosis is often an lengthy process (Floto et al., 2016; Daley et al., 2020) and the current protocols for NTM pulmonary disease (NTM-PD) detection and diagnosis, neglects to examine the wider lung microbiome which could have effects on disease progression, patient treatment regimens and overall patient health.

4.1 The CF Lung Microbiome in Associated Airway Disease

In non-CF lungs the microbiota consists of taxa from the *Streptococcus, Prevotella, Fusobacterium, Veillonella, Porphyromonas, Haemophilus* and *Neisseria* genera (Marsland and Gollwitzer, 2014; Françoise and Héry-Arnaud, 2020). In the past CF associated airway disease was believed to be monomicrobial, but with the introduction of culture independent techniques it has been found to be polymicrobial (Rogers et al., 2004).

The CF pulmonary microbiome has over 1000 bacterial species detected (Losada et al., 2016), with taxa from the *Rothia, Gemella, Granulicatella, Atopobium and Actinomycetes* being present (Françoise and Héry-Arnaud, 2020). It has notably less anaerobic taxa than the non-CF pulmonary microbiome (Lamoureux et al., 2019), which could possibly be due to the increased mucus and reduced airway clearance caused, by the mutation of cystic fibrosis transmembrane conductance regulator (CFTR) gene (Bhagirath et al., 2016).

During states of infection and lung function (FEV_1) decline, research has shown that the CF airways are low in microbial diversity and dominated by canonical CF pathogens (*Pseudomonas aeruginosa, Staphylococcus aureus, Burkholderia cepacia complex* (BCC), *Haemophilus influenzae, Stenotrophomonas maltophilia*, and *Achromobacter xylosoxidans*) (Cuthbertson et al., 2020), and emerging pathogens like NTMs are becoming increasingly detected in CF populations, which is of growing concern (Floto and Haworth, 2015).

4.2 NTM Pulmonary Disease

The recent estimated global prevalence of NTM infection in people with CF (PWCF) is approximately 7.9%, with *Mycobacterium abscessus* complex (MABSC) infection estimated at 4.1% and *M. avium* at 3.7% (Prieto et al., 2023). This is variable as geographic location has an influence on NTM surveillance with policies of non-mandatory reporting of NTM detection in some countries (Thornton et al., 2021). In 2018 NTM prevalence was increasing by 5% annually in the US CF population driven mainly by *M. avium* Complex (MAC) infection (Adjemian et al., 2018) and with a 2.5% rise over a 5-year period in the UK (Gardner et al., 2019) with *M. abscessus* (MABS) being the predominant species detected (Sokhi et al., 2021).

People with CF (PWCF) colonised by NTM do not always have active disease (Skolnik et al., 2016). However, those patients where colonisation progresses into active disease have shown a significant reduction in FEV_1 and increased frequency of exacerbations (Martiniano et al., 2014; Caverly et al., 2021). Patients positive for NTM pulmonary disease (NTM-PD) are ineligible for lung transplantation, due to the intrinsic antimicrobial properties of some NTM species (Leard et al., 2021). The severity of NTM-PD is highly dependent on the type of NTM acquired; MAC infection often exhibits less aggressive disease and better patient outcomes when compared to MABSC (Haworth et al., 2017). Therefore, the accurate and timely diagnosis of the type if NTM causing the disease is essential to stop disease and prevent further damage to the pulmonary system (Qvist et al., 2016; Skolnik et al., 2016).

4.2.1 NTM and the CF Microbiome

The presence of NTMs, and their association with other CF pathogens and the diversity of the CF microbiome has not been a major research focus, despite evidence that lung infection in CF is unquestionably polymicrobial in nature (Rogers et al., 2006; Maughan et al., 2012; Cuthbertson et al., 2020; Frey et al., 2022). Previous studies examining the interplay between NTM populations and NTM-PD in CF microbiomes are sparse, however, there is some limited research into NTM – microbiome associations in other pulmonary disorders that can situate this work.

Macovei et al. (2015) found that NTMs, including opportunistic pathogens, were present in healthy participants and that *Streptococcaceae* and *Staphlyococcaceae* constituted a significant proportion of the microbiota. Yamasaki et al. (2015) discovered that patients

positive for NTM had a microbiota predominantly composed of *Prevotella, Streptococcus*, *Neisseria,* and *Pseudomonas*, and that the incidence of anaerobes was higher in patients diagnosed with NTM infection. This suggests that anaerobes may play a role in the pathogenesis of NTM disease. However, due to the non-specific nature of 16S rRNA gene sequencing (Klindworth et al., 2013), NTM complexes are often underrepresented (Shin et al., 2006) and incorrectly identified (Clarridge, 2004; Morais et al., 2022).

There have been other studies examining the composition of the microbiota in the presence of NTM with most suggesting a unique bacterial community residing within each patient (Macovei et al., 2015; Yamasaki et al., 2015; Sulaiman et al., 2018). This, though, has not been a research focus in relationship to NTM complexes and CF associated lung infection. Understanding the impact NTM presence has on the composition of the microbiome is an important area of research. Knowing this will enable the understanding of NTMs influence on other pathogens and could potentially provide information regarding CF lung disease progression in relationship to the microbiome.

4.3 Chapter Aims and Objectives

This chapter aimed to identify NTM complexes in CF sputum samples and examine any difference in composition of the wider lung microbiota, in patients' culture positive or negative for NTM, this was achieved by:

1. Sequencing the 16S rRNA gene to gain an overview of the background microbiota in CF sputum samples.
2. Sequencing the *rpo*B gene to gain an insight into the specific mycobacteria complexes present in the CF sputum samples.
3. Analysis of the microbiota composition in patients' culture positive or negative for NTM to determine any differences.
4. Assessment of the NTMs present in patients' culture positive or negative for NTM to determine any differences.
5. Assement of the NTM complexes present in culture positive patients.

4.4 Methods

Methods are outlined in Chapter 2 "Core Methods" and detailed in Chapter 3 "Optimisation of Amplicon Sequencing for the Detection of NTM in Microbiota."

4.4.1 Participant Samples

Participants were recruited as part of a longitudinal and cross-sectional study of adults as described in Chapter 2. Patients who were culture positive at the time of sampling were denoted as "NTM positive", those who have no history of positive NTM culture were "NTM negative". Patients are sub-grouped according to their culture status designated by the hospital, including their "NTM type" status which is the species of NTM they were culture positive at the time (*M. avium* complex (MAC), *M. abscessus* complex (MABSC) and Other). Culture of patient samples was done and confirmed by University Hospital Southampton (UHS) and The University of Vermont (UVM). Only patients not on modulator therapy at the time of sampling were included in this analysis (Table 4.1/4.2).

4.4.2 Sample Preparation and DNA Extraction

Prior to DNA extraction, patient sputum samples were prepared in a class II cabinet. The samples were centrifuged for 10 minutes at room temperature with a speed of 1107xg, the supernatant was discarded, and the pellet was resuspended in 900µl of phosphate buffered saline (PBS) and centrifuged again under the same conditions for 5 minutes, supernatant was discarded, and the pellet resuspended in 500µl of PBS (Rogers et al., 2006). The DNA in the samples were cross linked using 1.25µl of PMA (Biotium, USA) (Section 2.1.5.1).

500µl of sputum was added to capped 1.5ml microcentrifuge tubes with 1 tungsten carbide bead and glass beads (Merck, Dorset, UK) filled up to the 0.5ml mark, 400µl of DNA/RNA lysis buffer (Zymo research, USA) was added. The samples were then mechanically homogenised. Nucleic acid extraction was performed following manufacturer's instructions using Quick-DNA/RNA miniprep kit (Zymo-research, USA). DNA was then stored at -20˚C for future use and RNA was stored at -80˚C.

4.4.3 DNA Sequencing

The microbiome of the samples was assessed by 2 step amplicon-based sequencing using the Illumina MiSeq system.

4.4.3.1 Amplicon PCR

First step amplicon PCR was done in two batches the first batch targeted the V4-V5 Regions of the 16S rRNA gene. Each PCR 25µl reaction consisted of 12.5µl 2x Q5 Master Mix (New England Biolabs, Hitchin, UK), 2µl (0.2µM) of phased primer pool (Invitrogen, Paisley, UK) (further detailed in Chapter 3) and 8.5µl of ultra-pure, molecular grade water (Cytiva, Fisher Scientific UK Ltd.).

The following parameters were used; 95°C for 3 minutes proceeded by 30 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds ending with one cycle at 72°C for 5 minutes.

The second set of amplicon PCR targeted the *rpo*B gene and the reaction consisted of 12.5µl 2x Q5 Master Mix (New England Biolabs, Hitchin, UK), 1µl (0.1µM) of phased primer pool (Invitrogen, Paisley, UK) 12% molecular grade dimethyl sulfoxide (Thermo Fisher, UK) and 8.5µl of ultra-pure, molecular grade water (Cytiva, Fisher Scientific UK Ltd).

The following parameters were used; 94°C for 3 minutes proceeded by 45 cycles at 94°C for 60 seconds, 60°C for 60 seconds and 72°C for 60 seconds ending with one cycle at 72°C for 10 minutes (Gebert et al., 2018) Both PCR reactions were confirmed by gel electrophoresis (Section 2.5.1.1). PCR was confirmed via gel electrophoresis and prepared for the next stage using AMpure XP PCR purification beads (Beckman Coulter Life Sciences, USA) were used to remove any PCR fragments of 350bp and below, following manufacturer's instructions.

4.4.3.2 Index PCR

Each 25µl PCR reaction consisted of 12.5µl, 2x Q5 Master Mix (New England Biolabs, Hitchin, UK), 1.25mM of each primer, 5µl cleaned PCR product and 2.5µl of ultra-pure molecular grade water (Cytiva, Fisher Scientific UK Ltd).

The following parameters were used; 95°C for 3 minutes followed by 20 cycles at 95°C for 30 second, 55°C for 30 seconds and 72°C for 30 seconds ending with one cycle at 72°C for 5 minutes. The fragment was confirmed using gel electrophoresis and then purified as described in section 2.5.2. and reconfirmed using the Agilent Bioanalyzer (Agilent, USA) (Illumina, 2013).

4.4.3.3 Normalisation and Sequencing

Purified PCR products were quantified then normalised to a 1nM library and processed as stated in the MiSeq Denature and Dilute guide (Illumina, 2013; 2019), 5µl of the library was denatured with 0.2N NaOH for 5 minutes at room temperature and transferred to a heat block at 95°C for 5 minutes. 990µl of pre-chilled HT1 buffer was added to the denatured library, and this created the 10pM denatured library which was stored on ice. The final library consisted of the denatured library of 7pM and a 30% PhiX spike. The samples were sequenced on the Illumina MiSeq platform using the Illumina MiSeq V3 600 cycle reagent kit (Illumina Cambridge Ltd, Cambridge, UK).

4.4.3.4 Sequencing Analysis

FastQ files were downloaded from Illumina base-space. The analysis of raw sequence data was then performed through the DADA2 pipeline (Callahan et al., 2016), Using R, version 4.2.2 (R Core Team, 2023). Forward and reverse reads were truncated at 200bp and 175bp, respectively. This truncation was based on the quality scores provided by the DADA2 pipeline and aimed to remove low quality bases and retain the required overlap of the paired ends reads for merging downstream (Callahan et al., 2016). The Silva Database version 138.1 release (Quast et al., 2013) was used to assign taxonomy to the sequences. Any unassigned amplicon sequence variants (ASV) where manually assigned using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) nucleotide database (Sayers et al., 2022) and matched with sequences based on a minimum of 95% query coverage, with the lowest possible e-value and a minimum of 95% identity cutoff. Multiple sequences assigned to the same ASV were condensed into OTU for statistical analysis. Any chloroplasts, mitochondria or environmental contaminants were manually removed following sequence assignment. Given the varying length of the sequences analysed, these identities should be considered putative.

Sequencing depth was assessed using rarefaction, this analysis adjusts for differences in library sizes across samples to aid comparisons in alpha diversity. It allows the determination of adequate sequencing depth in the samples is enough to capture the sample diversity (Willis, 2019; Schloss, 2024) .

4.4.3.4.1 NTM Culture Status and the Microbiota

Bacterial taxa richness, diversity, and community composition within the microbiota sampled from NTM positive and negative patients was assessed by 16S rRNA gene

sequencing. The differences between the microbiota composition regarding the NTM type (MAC, MABS and Other) was also examined.

4.4.4 Statistical Analysis

The raw OTU tables obtained through the DADA2 pipeline (Callahan et al., 2016) were analysed to evaluate the diversity, community composition, and similarity among samples.

To gauge the richness and evenness of the bacterial community each sample, was examined for changes in several measures. These included the Fisher's alpha index of diversity (Fisher's alpha), Berger-Parker dominance, and Bray-Curtis/Sørensen similarity measures. The significance of the alpha diversity measures was determined using the Kruskal-Wallis analysis, as outlined by Yadolah. (2008) and the similarity was tested for significance using Bray-Curtis based analysis of similarities (ANOSIM) with Bonferroni correction Cuthbertson et al. (2020).

4.4.4.1 Fisher's Alpha Diversity Index

To assess diversity within samples when compared to each other, and to measure the changes of diversity over time Fisher's alpha was used. This measures the richness of taxa independent of sample size when sequence reads are >1000 per sample. It is robust enough to use when samples are different sizes, comparing communities with different total abundances or when there are a different number of reads (Magurran, 2004; 2021). It is also less sensitive to rare species, which in some instances can influence the diversity estimate (Beck and Schwanghart, 2010). Fishers Alpha provides a simple measure of the distribution of species within a sample (Magurran, 2004; 2021) .

4.4.4.2 Berger-Parker index of Dominance

To assess the dominant taxa to the total number of individuals Berger-Parker index of dominance was used. This assesses the numerical importance of the most abundant taxa within the microbiota (Magurran, 2004).

4.4.4.3 Similarity

Similarity was measured using the Sørensen and Bray-Curtis measures. Sørensen measures the similarity between two data sets assessing presence/absence. Bray-Curtis looks at both the number of shared species and the abundance of the shared species (Magurran, 2004; Magurran and McGill, 2011). Both tests are not affected by the total

species or richness of the communities being tested (Hao et al., 2019). Statistical significance was measured using Bray-Curtis based analysis of similarities (ANOSIM) with Bonferroni correction to determine which taxa contributed most to the compositional changes between the groups (Armstrong, 2014). ANOSIM was used because it is robust to handle large variations between groups and can detect significance among groups (Armstrong, 2014). A similarity of percentages (SIMPER) was used as described by (Clarke, 1993; Cuthbertson et al., 2020; Khomich et al., 2021).

In addition to the similarity indices Non-Metric Multidimensional Scaling (NMDS) was used to enable clearer visualisation of the differences between groups. This technique uses the Bray-Curtis dissimilarity measure and does not make assumptions about the data (Bakker, 2024) The results were assessed for statistical significance using Permutational Multivariate Analysis of Variance (PERMANOVA) this test was used because it allows the testing of the associated microbial composition with covariates of interests, it also partitions within groups and between group distances to allow assessment of the grouping factors (Zhu et al., 2021).

Table 4. 1 Participant Demographics for this Chapter

Origin	Sample ID	Collection Method	Age	Gender	Type of Sample	Genotype	%FEV$_1$	Exacerbation	NTM	NTM Type
UHS	179S	Clinic	27	Female	Sputum	Homozygous	63	Exacerbation	Negative	Negative
UHS	20AS	Clinic	29	Female	Sputum	Heterozygous	52	Exacerbation	Negative	Negative
UHS	46S	Clinic	29	Female	Sputum	Homozygous	59	Exacerbation	Negative	Negative
UHS	54AS	Clinic	22	Female	Sputum	Unknown	45	Stable	Negative	Negative
UHS	153S	Clinic	25	Male	Sputum	Homozygous	86	Exacerbation	Negative	Negative
UHS	164BS	Clinic	22	Male	Sputum	Homozygous	82	Exacerbation	Negative	Negative
UHS	211S	Clinic	19	Male	Sputum	Heterozygous	35	Stable	Negative	Negative
UHS	17UH1s	Clinic	30	Female	Sputum	Homozygous	56	Exacerbation	Negative	Negative
UHS	164AS	Clinic	56	Female	Sputum	Heterozygous	51	Stable	Negative	Negative
UHS	204BS	Clinic	19	Female	Sputum	Homozygous	32	Exacerbation	Negative	Negative
UHS	197S	Clinic	25	Female	Sputum	Heterozygous	106	Stable	Negative	Negative
UHS	204CS	Clinic	20	Female	Sputum	Heterozygous	112	Stable	Negative	Negative

UVM	1UVS	Postal	31	Female	Sputum	Heterozygous	37	Stable	Negative	Negative
UHS	204AS	Clinic	20	Female	Sputum	Homozygous	63	Exacerbation	Negative	Negative
UHS	54CS	Clinic	24	Female	Sputum	Homozygous	78	Stable	Negative	Negative
UHS	25UH1s	Clinic	27	Female	Sputum	Homozygous	66	Exacerbation	Negative	Negative
UHS	55S	Clinic	29	Female	Sputum	Heterozygous	23	Stable	Negative	Negative
UHS	199S	Clinic	56	Male	Sputum	Homozygous	88	Stable	Negative	Negative
UHS	21UH1s	Clinic	19	Male	Sputum	Homozygous	97	Exacerbation	Negative	Negative
UVM	30UVS	Postal	50	Male	Swab	Homozygous	56	Stable	Negative	Negative
UVM	3UVS	Postal	23	Male	Sputum	Homozygous	80	Stable	Negative	Negative
UHS	54BS	Clinic	28	Male	Sputum	Homozygous	91	Exacerbation	Negative	Negative
UHS	10UH1s	Clinic	30	Female	Sputum	Homozygous	40	Exacerbation	Positive	MABSC
UHS	11UH1S	Clinic	37	Female	Sputum	Heterozygous	68	Stable	Positive	MABSC
UHS	14UHS1	Clinic	21	Female	Sputum	Heterozygous	95	Stable	Positive	MABSC
UHS	2UH1s	Clinic	33	Female	Sputum	Heterozygous	47	Stable	Positive	MABSC
UHS	3UH1s	Clinic	30	Female	Sputum	Homozygous	84	Stable	Positive	MABSC

UHS	9SHS2S	Postal	33	Female	Sputum	Homozygous	55	Stable	Positive	MABSC
UHS	5UH1s	Clinic	21	Female	Sputum	Heterozygous	99	Stable	Positive	MABSC
UHS	9UH1S	Clinic	28	Female	Sputum	Homozygous	27	Exacerbation	Positive	MABSC
UHS	9BUH1s	Clinic	33	Female	Sputum	Heterozygous	39	Stable	Positive	MABSC
UHS	18UH1s	Clinic	31	Female	Sputum	Homozygous	52	Stable	Positive	MABSC
UHS	4SHS2S	Postal	23	Female	Sputum	Heterozygous	45	Stable	Positive	MABSC
UHS	187AS	Clinic	23	Female	Sputum	Homozygous	94	Exacerbation	Positive	MAC
UHS	190CCS	Clinic	25	Female	Sputum	Heterozygous	52	Exacerbation	Positive	MAC
UHS	190CS	Clinic	19	Female	Sputum	Heterozygous	49	Stable	Positive	MAC
UHS	6CS	Clinic	27	Female	Sputum	Homozygous	91	Stable	Positive	MAC
UHS	203BBS	Clinic	20	Female	Sputum	Heterozygous	40	Exacerbation	Positive	MAC
UHS	6AS	Clinic	30	Female	Sputum	Heterozygous	41	Exacerbation	Positive	MAC
UHS	188BS	Clinic	22	Female	Sputum	Heterozygous	45	Exacerbation	Positive	MAC
UHS	187CS	Clinic	28	Male	Sputum	Heterozygous	45	Stable	Positive	MAC
UHS	148BS	Clinic	25	Male	Sputum	Heterozygous	49	Exacerbation	Positive	MAC

UHS	203BS	Clinic	20	Male	Sputum	Homozygous	95	Exacerbation	Positive	MAC
UHS	12UH1	Clinic	31	Female	Sputum	Homozygous	57	Exacerbation	Positive	MAC
UHS	19UH1s	Clinic	20	Female	Sputum	Heterozygous	51	Exacerbation	Positive	MAC
UHS	188AS	Clinic	26	Male	Sputum	Heterozygous	45	Stable	Positive	MAC
UHS	205AS	Clinic	21	Female	Sputum	Heterozygous	88	Stable	Positive	MAC
UHS	81S	Clinic	26	Female	Sputum	Heterozygous	59	Exacerbation	Positive	MAC
UHS	186S	Clinic	28	Male	Sputum	Heterozygous	104	Exacerbation	Positive	MAC
UHS	213S	Clinic	18	Male	Sputum	Heterozygous	52	Exacerbation	Positive	MAC
UHS	73BS	Clinic	25	Male	Sputum	Heterozygous	55	Stable	Positive	MAC
UHS	203AS	Clinic	23	Female	Swab	Heterozygous	40	Stable	Positive	MAC
UHS	107BS	Clinic	20	Female	Sputum	Homozygous	88	Stable	Positive	Other
UHS	107BBS	Clinic	26	Male	Sputum	Heterozygous	68	Stable	Positive	Other
UHS	107AS	Clinic	27	Female	Sputum	Homozygous	49	Exacerbation	Positive	Other
UHS	107CS	Clinic	23	Male	Sputum	Homozygous	50	Exacerbation	Positive	Other

Participant demographics for the samples used in this chapter. No participants on modulator therapy are included in this chapter
Origin of the sample UHS: University Hospital Southampton, UVM: University of Vermont.
[b] Collection method: Clinic: if the sample was collected in the clinic, pre-COVID 19 pandemic or via the postal-pack method which is denoted as Postal.
[C] Type of sample provided Sputum or Cough Swab.
[D] CFTR genotype- CF transmembrane conductance regulator genotype. Homozygous ΔF508, two copies of the ΔF508 gene mutation, ΔF508 heterozygous, single copy of ΔF508 gene mutation and another mutation.
[e] Percent predicted forced expiratory volume in 1 second (%FEV1).
[f] NTM type- Samples culture positive for *M. avium* complex (MAC), *M. abscessus* complex (MABSC), Other NTM.

Table 4. 2 Summary of Participant Demographics used in this Chapter

Demographics	NTM positive	NTM negative
Number of patients (*n= 55)*	34	21
Collected in clinic	32	19
Collected by postal pack	2	2
Age (years) at the time of sampling		
Mean	26(SD±4.62)	28 (SD±11.8)
Median	26	27.5
Range (min/max)	18-37	19-64
Gender		
Female	24	13
Male	10	8
CFTR Genotype [a]		
ΔF508 homozygous	15	15
ΔF508 heterozygous	19	6
Other	0	0
NTM Type [b]		-
MAC	20	-
MABS	10	-
Other NTM	4	-
Exacerbation status		
Stable/ recovering	11	8
Exacerbation	22	14
None	1	0
Mean predicted %FEV1	61(SD±23.5)	68(SD±21.81)

SD denotes standard deviation of the mean. [a] CFTR genotype- CF transmembrane conductance regulator genotype. Homozygous ΔF508, two copies of the ΔF508 gene mutation, ΔF508 heterozygous, single copy of ΔF508 gene mutation and another mutation. [b] NTM type- Samples culture positive for *M. avium* (MAC), *M. abscessus* (MABS), Other NTM.

4.5 Results

4.5.1 Sequencing Analysis

One Illumina MiSeq v3 600 cycle reagent kit was used for the data generated for the 16S rRNA gene sequencing and the rpo*B* gene sequencing used in this chapter and subsequent chapters (Chapters 5/6). The internal quantity control provided by the Miseq showed that the sequencing of the targets were successful with the run having an average %Q30 of 68.45%, 88.27% passing filter (%PF) and a total yield of 13.81 Gbp.

The 16S rRNA gene sequencing yielded a total of 5,797,585 bacterial raw sequence reads. The internal filtering and quality control steps performed by DADA2 (Callahan et al., 2016) found 15.3% chimeric sequences and successfully merged 77% of sequences.

In this chapter 54 of the samples (Table 4.1/4.2), were used (mean ± 1 standard deviation throughout (mean = 101177±97284.3 SD, sequence reads per sample) (Figure 4.1), were assigned to a taxonomic classification (Figure 4.2). Reads were distributed unevenly across the samples (minimum of 2290 and a maximum of 4,925,75 reads). In total 152 bacterial OTUs, were assigned after manual removal of chloroplasts, mitochondrial, environmental contaminants, and any sequences unassigned to a genus.

The rarefaction analysis (Figure 4.3) (*n*=55) provided a very mixed picture regarding the sequencing depth and species abundance. Each curve saturates at different points, indicating an unique species distribution in each sample. The steep rise of the curve, and rapid plateau suggests high initial species diversity that tapers off, implying a few species dominate with less common species. The curves with shorter length indicate less sequencing depth.

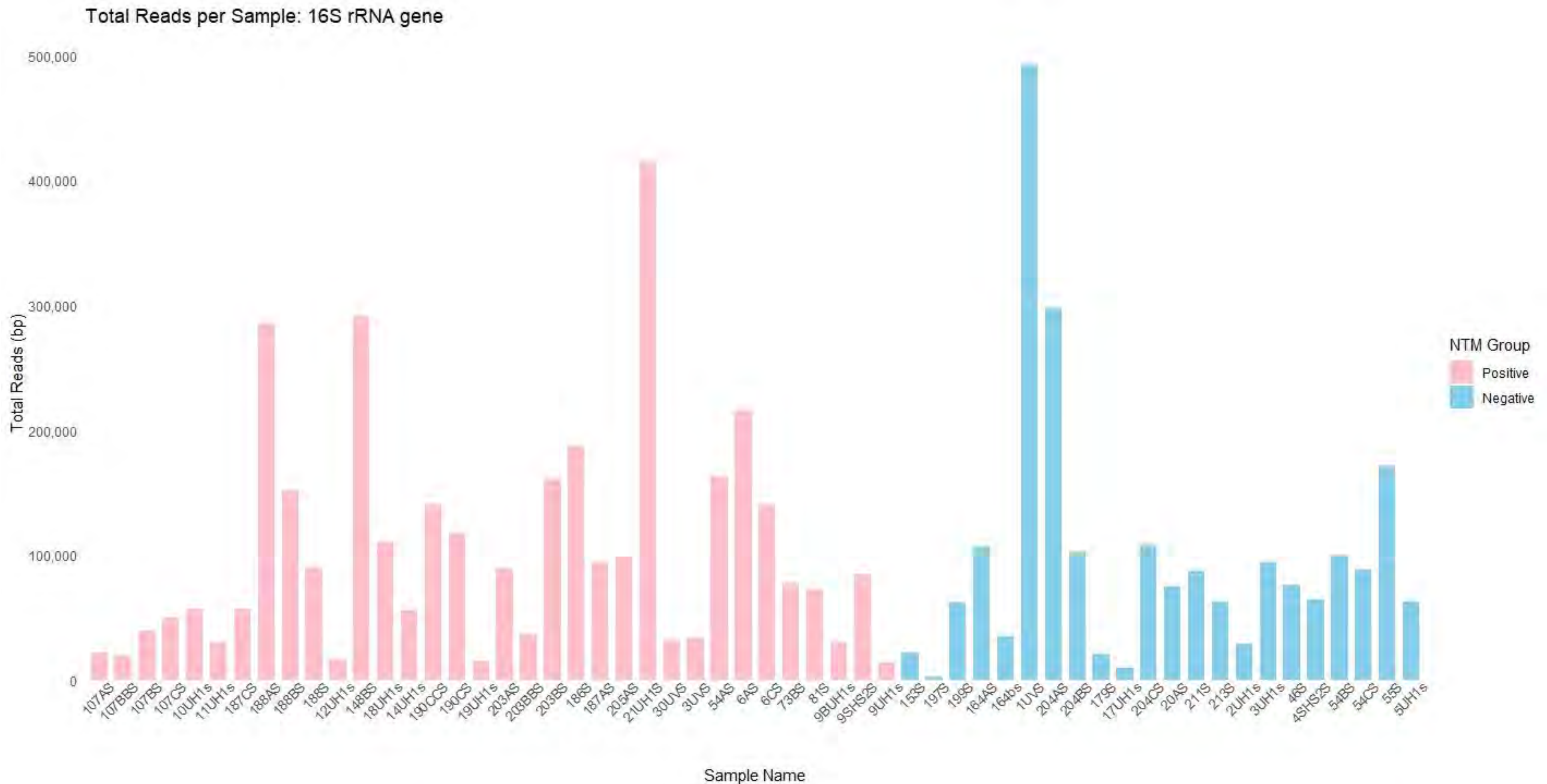


Figure 4. 1 16S rRNA gene target total reads used per-sample after built-in DADA2 filtering. Split into NTM Positive (Pink on the left) and NTM Negative groups (Blue on the right) Showed an uneven distribution of reads across the samples in both groups (mean= 101177±97284.30, Range; min =2290, max=492575) . Sample names on the x- axis are the sample names assigned by the clinic.

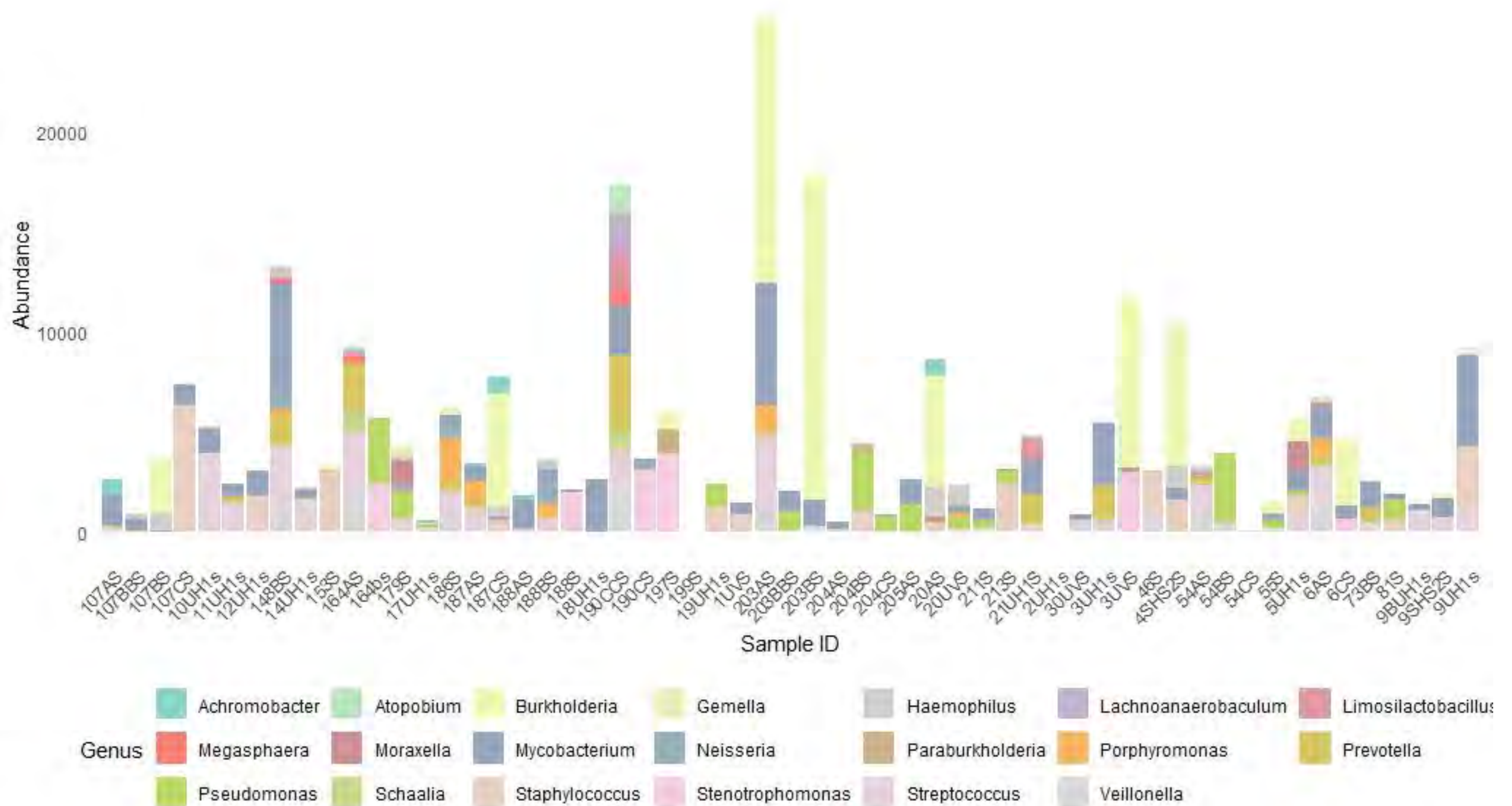


Figure 4. 2 : Total abundance of the 20 most abundant genus derived from the 16S rRNA gene sequencing. Different colours donate the different genus in correspondence to the sample ID. Only the first 20 are used as there was a large variation of genus amongst the samples. Given the length of the sequences analysed, these identities should be considered putative.

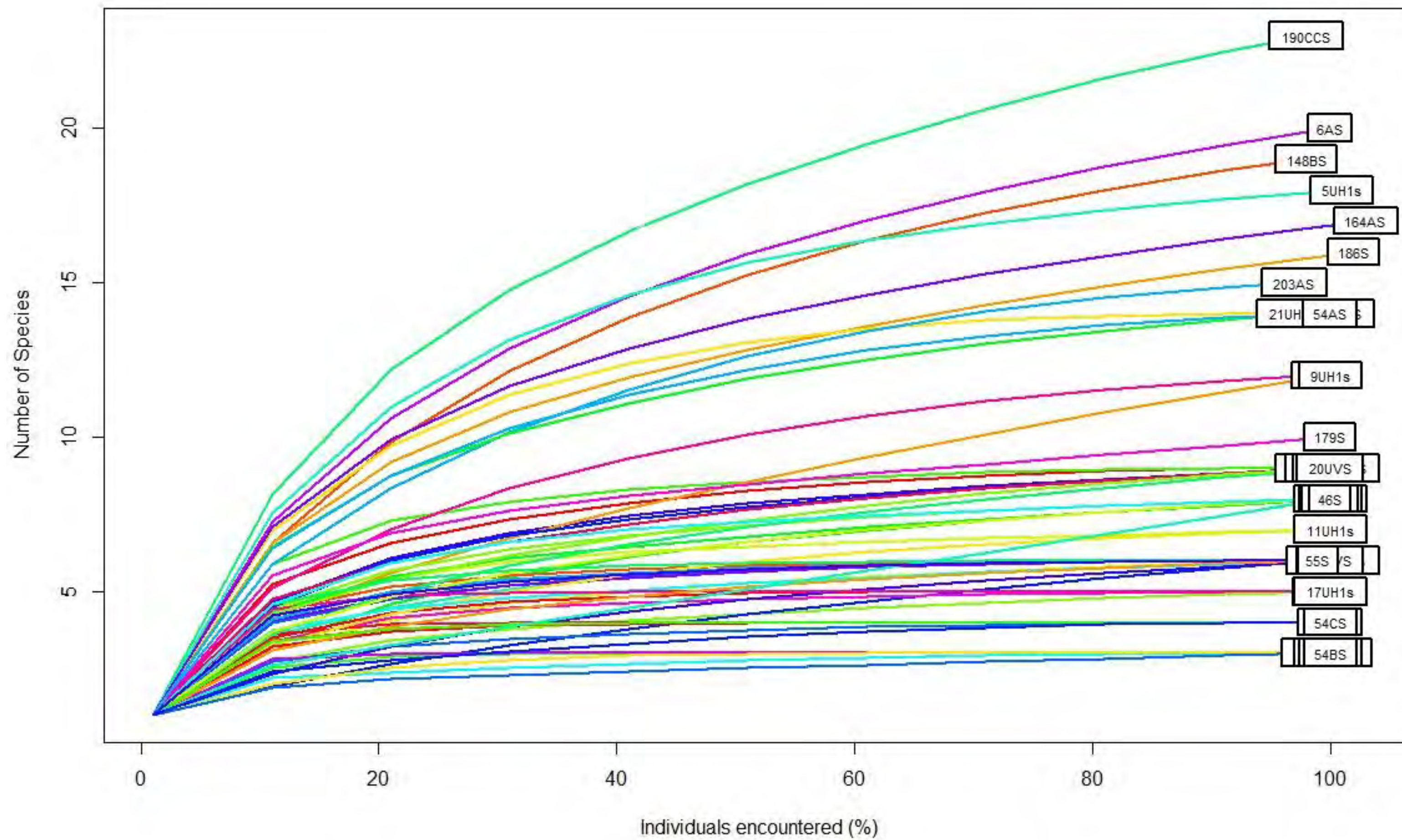


Figure 4. 3 : Rarefaction curve of the 16S rRNA gene sequencing reads after filtering, quality control and removal of unwanted sequences. Each curve represents a patient sample with the sample ID. The curve shows the percentage of individual species detected corresponding to the number.

4.5.2 NTM Group and the Microbiota

4.5.2.1 Microbiome Diversity and Composition

In each sample, the diversity and composition of the total microbiota taxa (unique operational taxonomic units [OTUs]) were evaluated by calculating the richness (number of species), Fisher's alpha index of diversity (which measures both species richness and evenness in the community), and the Berger-Parker index of dominance (measuring the relative abundance of the dominant taxa in the community) (Magurran and McGill, 2011). This analysis standardizes the species richness based on the common number of individual samples across all communities, it is also able to adjust for library size (Sanders, 1968; Shimadzu, 2018; Willis, 2019).

For richness, whilst the NTM positive group (*n* = 34) had a higher taxa richness (OTU) (mean= 9.88 ±6.38 SD) than the negative group (*n* = 21, mean =7.61±4.57 SD), these results showed no statistical significance (Kruskal-Wallis test: X^2 = 1.75, p = 0.18) (Figure 4.4A). This was mirrored by the Fisher's alpha diversity index (Figure 4.4B) which showed the NTM positive group (mean=1.18±0.75 SD), had a higher range of diversity than the negative group (mean 0.94±0.56 SD), the results showed no statistical difference (Kruskal-Wallis test: H= 1.26, p =0.26) between groups. But the Berger-Parker index of dominance (Figure 4.4C) showed that the NTM positive group (mean 0.48±0.19) and the negative group (mean 0.49±0.21 SD), were very similar, suggesting that both groups have dominant taxa but again showed no statistical significance (Kruskal-Wallis test: H= 0, p =1).

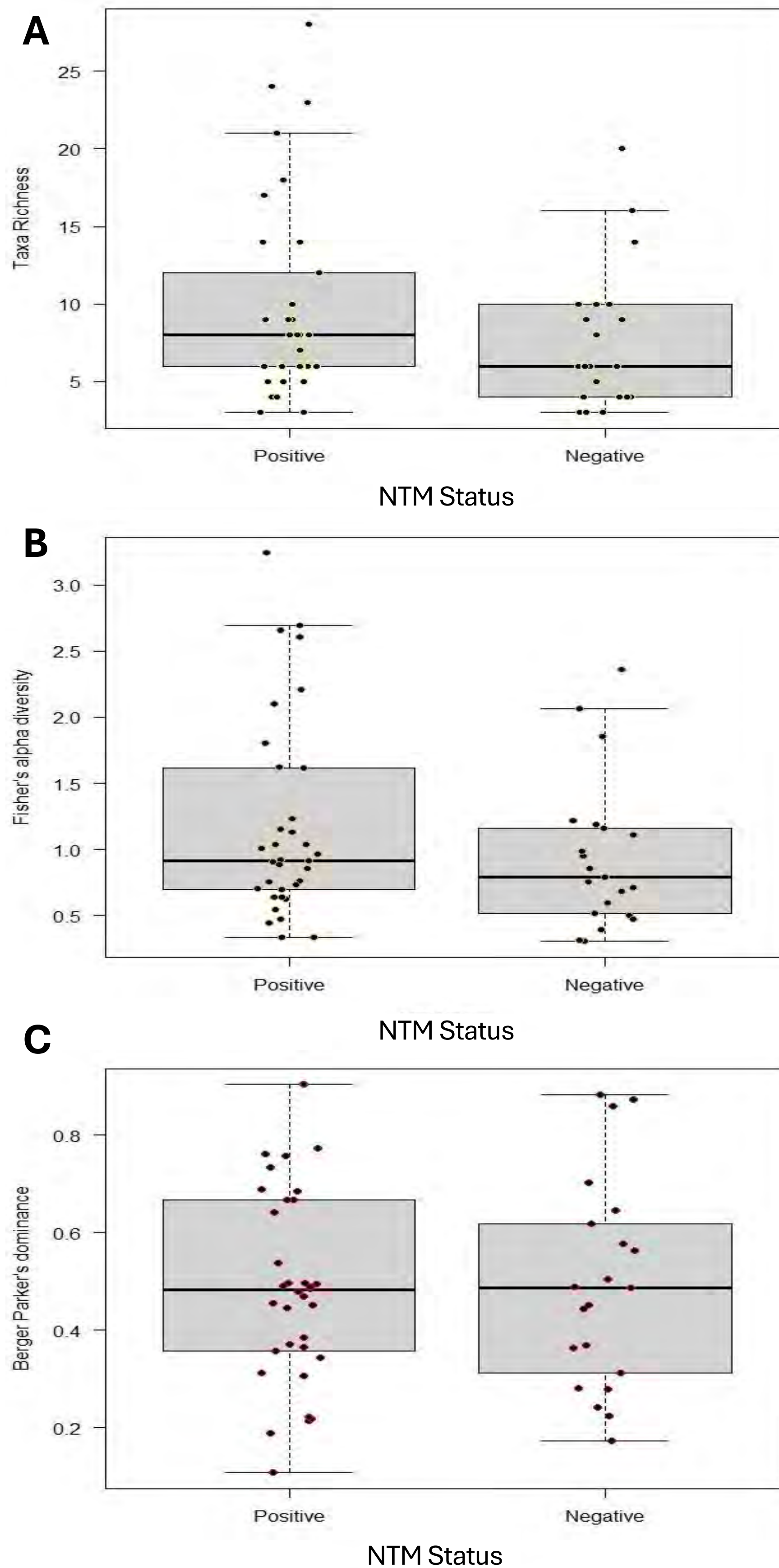


Figure 4. 4 Microbiota diversity and composition of the NTM positive group (OTU) (*n* = 34) and negative group (*n* = 21). Both taxa richness (**A**) NTM positive and Fisher's alpha index of diversity (**B**) was found to be higher in the NTM positive group than the NTM negative group. The Berger Parker index of Dominance (**C**) were very similar but was slightly higher in the NTM negative group compared to the NTM positive group. All the differences were found to be statistically not significant (p>0.05).

4.5.2.2 Total Microbiota Similarity

Differences in microbiota composition between the NTM positive and NTM negative groups were determined using the Bray-Curtis (incorporating abundance) and Sørensen index of similarity (using absence/presence only) (as shown in Figure 4.5). Statistical significance was measured using Bray-Curtis based Analysis of similarities (ANOSIM) with Bonferroni correction.

Both measures of similarity showed there was little compositonal similarity between the groups. The Bray-Curtis similarity (Figure 4.5A) shows the NTM-positive group (mean= 0.098±0.14 SD), Negative group (mean= 0.105, ±0.16 SD), and between groups (mean= 0.12±0.23 SD), had very little similarities in compostion. The Sørensen (Figure 4.5B) similarity-based analysis shows very little similarites between the groups. The NTM-positive group (mean= 0.14±0.12 SD), Negative group (mean= 0.13±0.128 SD) and the between groups (mean= 0.12±0.12 SD). These results were statistically significant (Bray-Curtis based ANOSIM: p= 0.0002, R = 0.14) and remained significant after applying a Bonferroni correction for multiple comparisons (p=0.0002). The combined results indicate very low similarity between groups indicating major differences in microbiota composition.

The same analysis was also conducted on the data with all the NTM taxa removed (Figure 4.6) to assess whether their presence is the contributing factor of the similarity. The Bray-Curtis similarity (Figure 4.6A) shows the NTM-positive group (mean= 0.093±0.15 SD), Negative group (mean=0.11, ±0.17 SD), and between groups (mean= 0.09±0.16 SD). The Sørensen (Figure 4.6B) similarity-based analysis shows the NTM-positive group (mean= 0.12±0.14 SD), Negative group (mean= 0.13±0.136 SD) and the between groups (mean= 0.124±0.14 SD). These results were statistically non-significant (Bray-Curtis based ANOSIM: p= 0.182, R = 0.031) and remained non-significant after applying a Bonferroni correction for multiple comparisons (p=0.179). With the NTMs removed the differences between the groups become statistically non-significant. This suggests that the presence of NTM taxa contributes to the observed differences in microbiota composition between the groups, this is further supported by the minute changes in the averages.

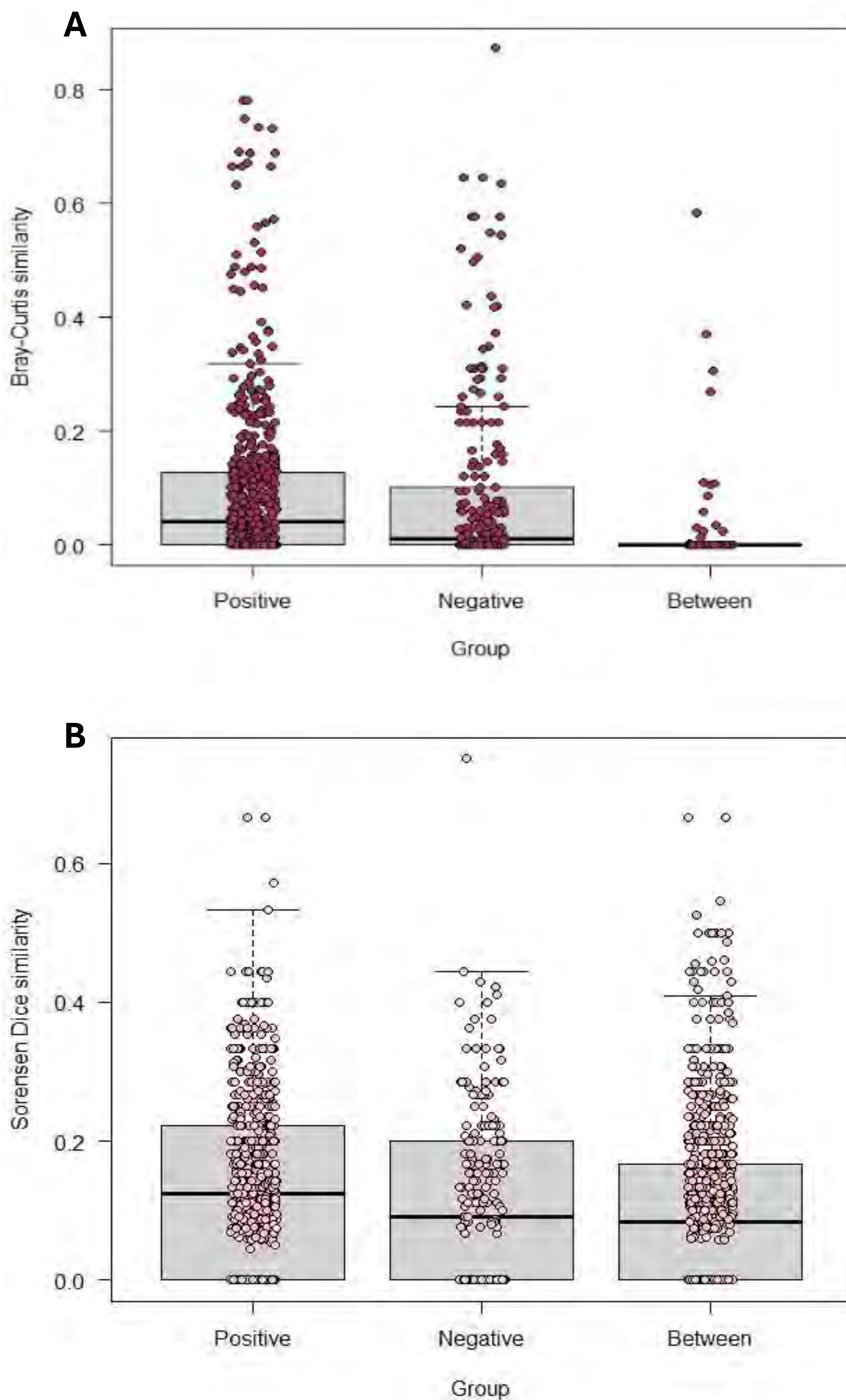


Figure 4. 5 Similarity Indices of the NTM positive, negative and between groups examining the whole microbiota. The Bray-Curtis measure of similarity (**A**) and Sørensen index of similarity (**B**) both show a very small ammount of similarity between groups These results are statistically significant (p = 0.0002, R = 0.14), Bonferroni correction for multiple comparisons (0.0002).

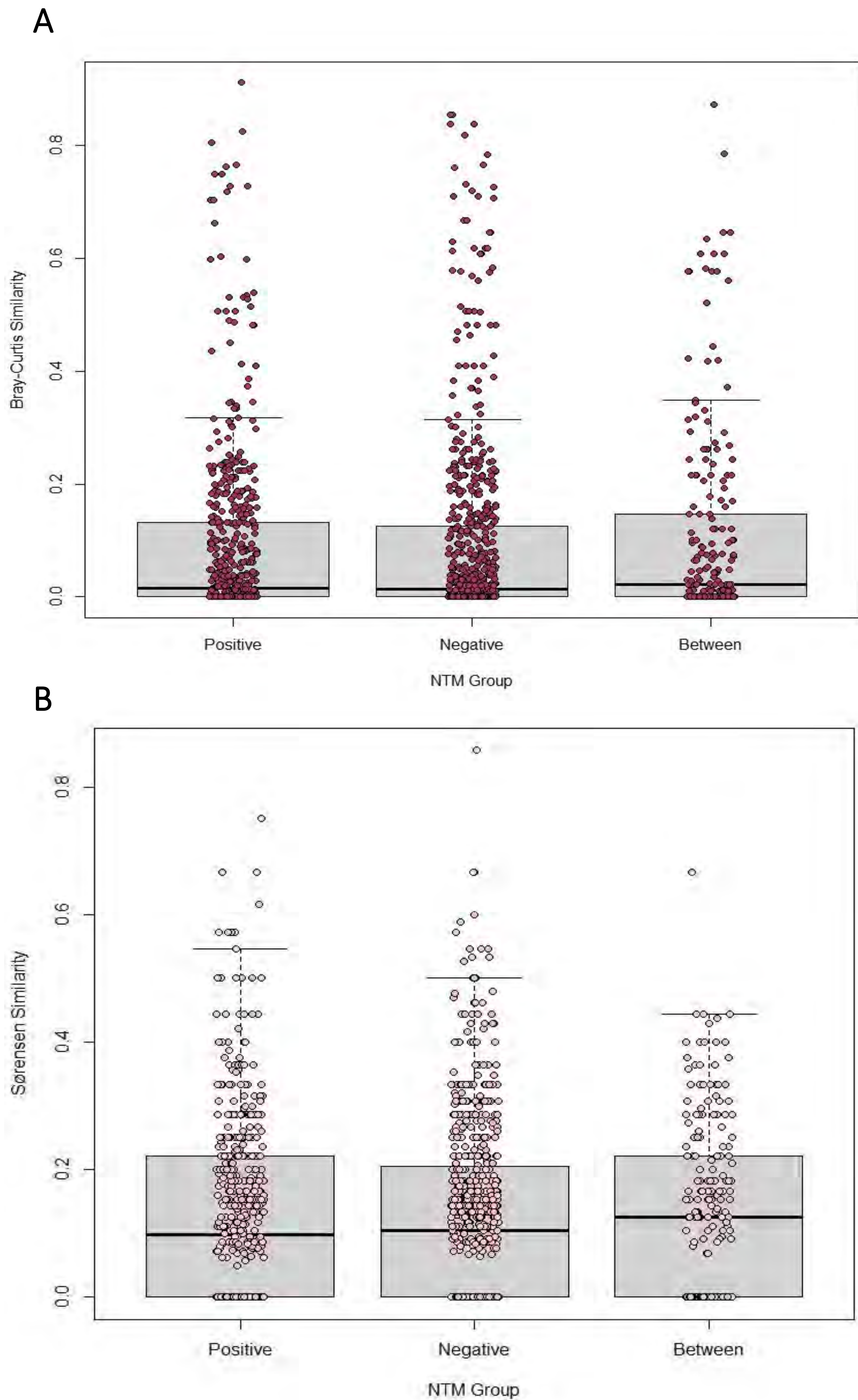


Figure 4. 6 Similarity Indices of the NTM positive, negative and between groups examining the whole microbiota with the NTM taxa removed. The Bray-Curtis measure of similarity (**A**) and Sørensen index of similarity (**B**) both show a very small ammount of similarity between groups These results are statistically non-significant Bray-Curtis based ANOSIM: p= 0.182, R = 0.031.

4.5.2.3 Non-Metric Multidimensional Scaling

To further visualise the patterns of similarity between the groups a Non-Metric Multidimensional Scaling (NMDS) plot was generated (Figure 4.7). The plot including the NTM taxa (Figure 4.7A) shows some overlap between the groups but is again very small. The statistical significance was assessed using Permutational Multivariate Analysis of Variance (PERMANOVA, also known as ADONIS). The test showed statistical significance between the groups ($p=0.006$, $R^2= 0.6666$, $Df= 36.0$). When examining the NDMS plot (Figure 4.7B) with the NTM taxa removed the plot showed closer linked groups but the results were non-significant ($p=0.093$, $R^2= 0.6667$, $Df= 36.0$). This analysis suggests that the NTM taxa are contributing to the differences in composition between the groups which corresponds to the similarity analysis (Section 4.5.2.2) done previously.

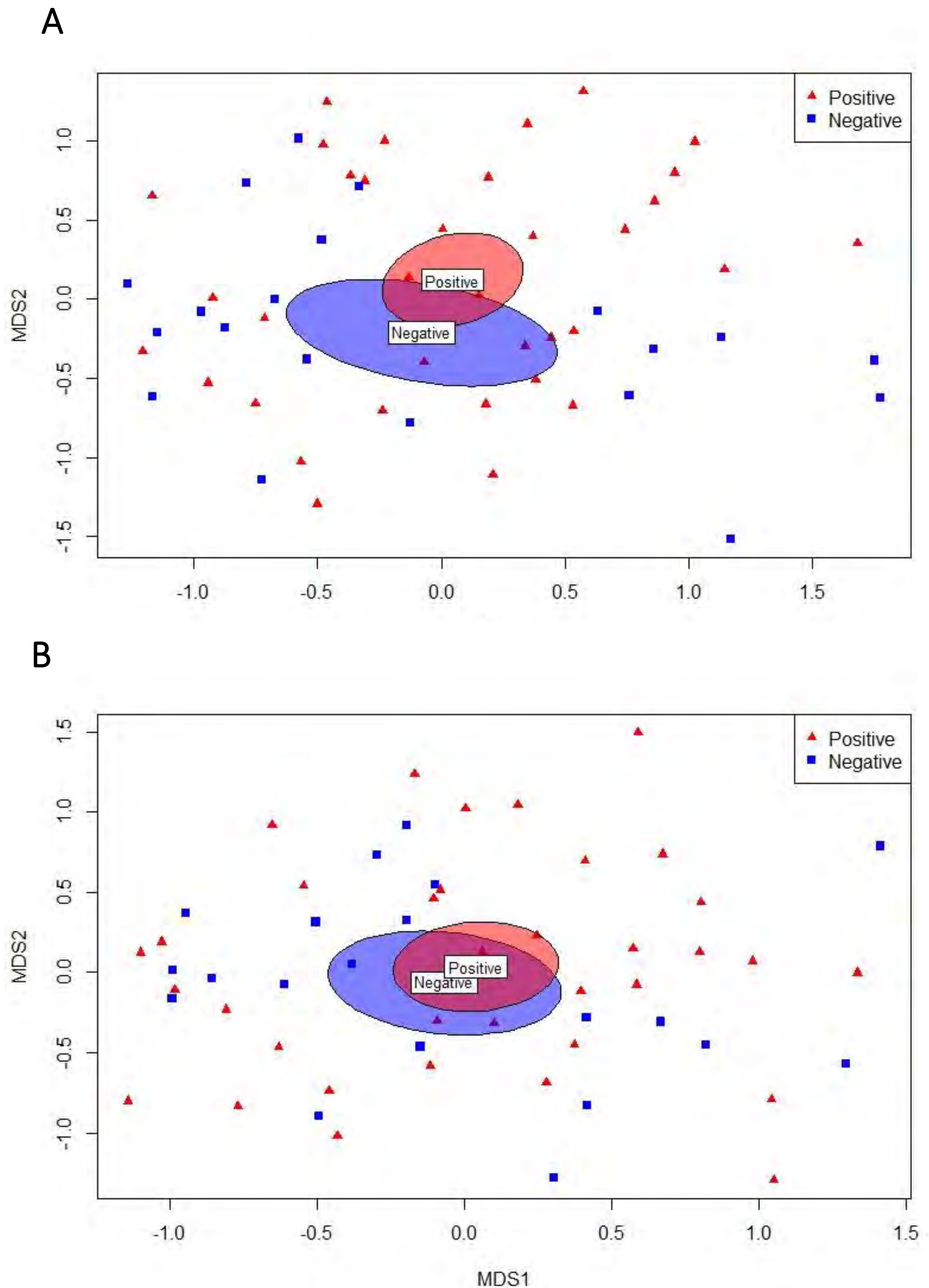


Figure 4. 7 NMDS plot showing how the groups are distributed by similarity. The overlap of the coloured ellipsis indicates the area where the groups share similarity. The red triangles are representing the NTM positive group, and the blue squares are representing the NTM negative group. (**A**) NMDS of the 16S rRNA sequencing of the whole microbiome and the NTM taxa. (**B)** NMDS of the 16S rRNA sequencing of the whole microbiome without the NTM taxa included.

4.5.2.4 Similarity Percentages Analyses

To identify the driver of dissimilarity between the NTM positive and negative groups, a Similarity Percentages (SIMPER) analysis (focuses on species contributing to the dissimilarity between groups) (Table 4.3/Figure 4.8A) was conducted. It shows the main drivers of dissimilarity between the two groups are *Pseudomonas aeruginosa* (12.13%), *Burkholderia multivorans* (21.86%), *Staphylococcus aureus* (28.19%) and *M. avium* (37.12%) contributing to the overall dissimilarity between the groups. The analysis also shows that the relative abundance (Figure 4.8A) is reflective of this in the positive group, in the negative group it shows that *P. aeruginosa* (mean=20.2%), *B. multivorans* (mean= 9.15%), and *S. aureus* (mean= 5.31 %) are ranked highest in relative abundance.

The SIMPER analysis was also conducted on the same groups but with the NTM taxa omitted from the examination (Table 4.4/Figure 4.8B). It shows the main drivers of dissimilarity between the two groups are *Streptococcus salivarius* (39.51%), *S. aureus* (32.97%), *B. multivorans* (24.32%), and *P. aeruginosa* (13.74%), contributing to the overall dissimilarity between the groups. With the NTM taxa removed the main drivers of difference shifted which could be indicative that the presence of the NTMs in the analysis drives the similarity between the groups.

Table 4. 3 Similarity Percentage Analysis (SIMPER) 16S rRNA gene Sequencing with NTM species Included

Taxon	Contrib. %	Cumulative %	Mean Relative abundance	
			Positive	Negative
Burkholderia multivorans	9.73	9.73	**11.9**	**9.15**
Mycobacterium avium	**4.416**	**14.146**	**8.13**	**0**
Staphylococcus aureus	**6.327**	**20.473**	**8.08**	**5.31**
Streptococcus salivarius	**4.52**	24.993	**7.45**	1.66
Pseudomonas aeruginosa	12.13	**37.123**	**6.47**	**20.2**
Mycobacterium abscessus	3.482	**40.60**5	**6.3**	**0.245**
Mycobacterium chelonae	2.844	**43.449**	5.24	0
Staphylococcus succinus	**2.855**	46.304	**4.14**	1.57
Stenotrophomonas maltophilia	3.154	**49.458**	3.19	**2.99**
Streptococcus toyakuensis	1.72	51.178	3.17	0
Haemophilus influenzae	**2.821**	**53.999**	**2.35**	**3.41**
Porphyromonas pasteri	**1.391**	**55.39**	**2.18**	0.498
Stenotrophomonas pavanii	2.541	57.931	1.73	3.2
Streptococcus symci	2.882	60.813	1.66	**4.51**
Neisseria mucosa	1.847	62.66	1.64	2.17
Achromobacter xylosoxidans	1.626	64.286	1.56	1.67
Mycobacterium kansasii	0.8399	65.1259	1.55	0
Veillonella nakazawae	**3.27**	**68.3959**	**1.51**	5.6
Burkholderia cepacia	**0.535**	**68.9309**	**0.985**	**0**
Mycobacterium intracellulare	2.784	**71.7149**	0.918	**4.39**

Bold text denotes species of interest regarding NTM and CF pathogens. Also given is within category mean percent abundance for taxa, Percentage contribution of each taxon to the average dissimilarity between the two groups being compared. Taxa with higher values in this column contribute more to the overall dissimilarity between the groups. Percentage cumulative contribution of each taxon to the average dissimilarity between the two groups. The taxa are typically listed in descending order of their contribution. The list of species is not exhaustive, so cumulative percent does not sum to 100%. Given the length of the ribosomal sequences analysed, species identities should be considered putative. (Full analysis: Supplementary Materials 11.3)

Table 4.4 Similarity Percentage (SIMPER) analysis 16S rRNA gene sequencing on NTM positi and negative groups without the presence of NTM taxa.

Taxon	Contrib. %	Cumulative %	Mean Relative abundance	
			Positive	Negative
Burkholderia multivorans	**10.58**	10.58	**13.8**	**8.7**
Streptococcus salivarius	6.538	17.118	11.1	1.59
Pseudomonas aeruginosa	**13.74**	30.858	**9.61**	**20.8**
Staphylococcus aureus	**8.648**	39.506	**7.58**	**10.6**
Staphylococcus succinus	3.734	43.24	5.78	1.5
Neisseria mucosa	3.433	46.673	4.63	2.06
Streptococcus toyakuensis	2.323	48.996	4.21	0
Achromobacter xylosoxidans	2.995	51.991	4.1	1.59
Haemophilus influenzae	3.466	55.457	3.69	3.25
Stenotrophomonas maltophilia	3.304	58.761	3.51	2.85
Porphyromonas pasteri	1.653	60.414	2.64	0.477
Veillonella nakazawae	4.22	64.634	2.29	6.84
Streptococcus symci	3.336	67.97	2.11	4.92
Stenotrophomonas pavanii	2.6	70.57	1.91	3.04
Prevotella salivae	1.631	72.201	1.39	1.9
Burkholderia cepacia	0.7294	72.9304	1.32	0
Prevotella melaninogenica	0.6751	73.6055	1.22	0
Prevotella vespertina	0.6304	74.2359	1.14	0
Peribacillus simplex	0.548	74.7839	0.993	0
Veillonella parvula	3.029	77.8129	0.838	4.81

Bold text denotes species of interest regarding CF pathogens. Also given is within category mean percent abundance for taxa, Percentage contribution of each taxon to the average dissimilarity between the two groups being compared. Taxa with higher values in this column contribute more to the overall dissimilarity between the groups. Percentage cumulative contribution of each taxon to the average dissimilarity between the two groups. The taxa are typically listed in descending order of their contribution. The list of species is not exhaustive, so cumulative percent does not sum to 100%. Given the length of the ribosomal sequences analysed, species identities should be considered putative. (Full analysis: Supplementary Materials 11.4)

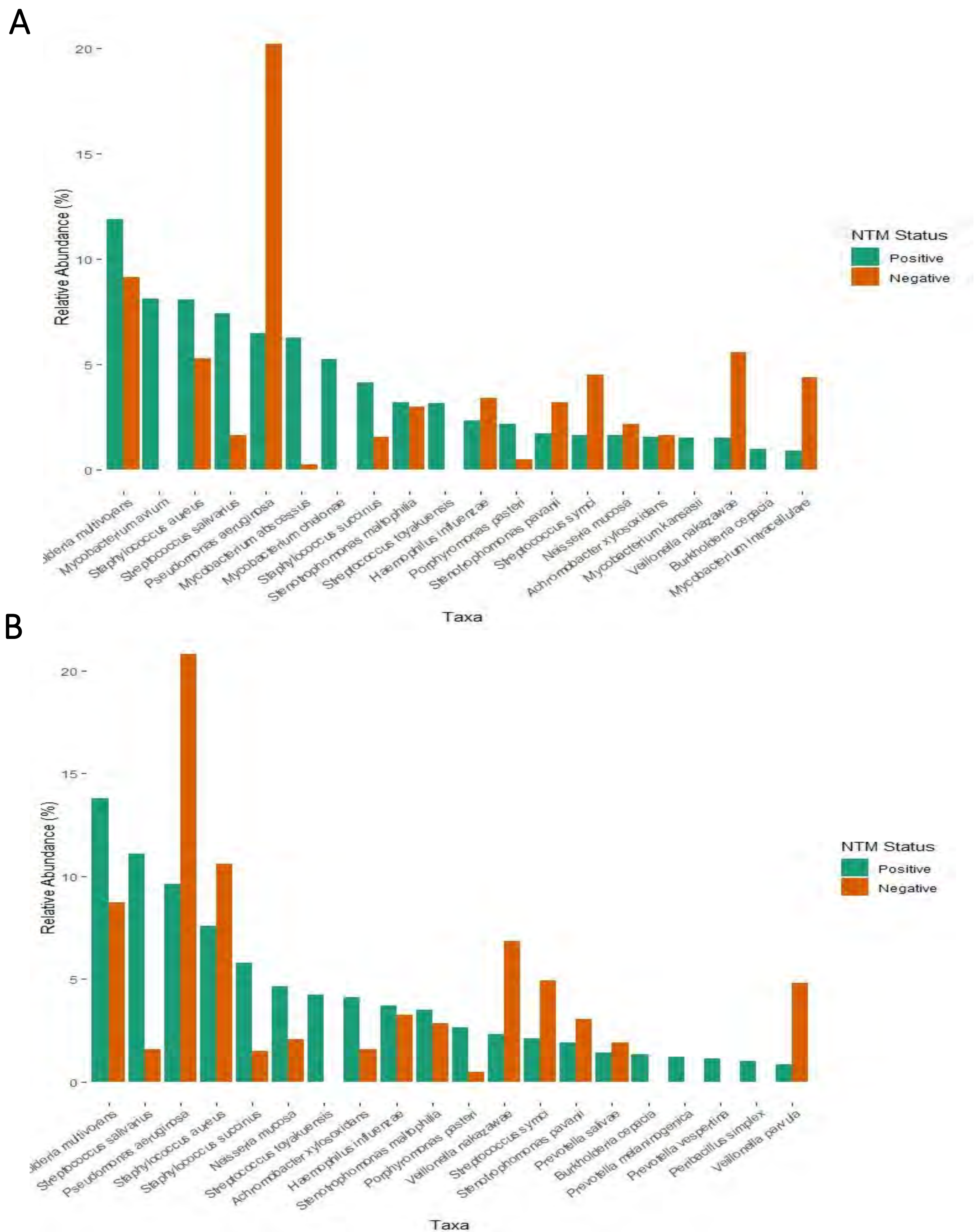


Figure 4. 8 The relative abundance as calculated by the SIMPER analysis (Table 4.3/4.4) Shows the mean relative abundance for each species detected within the NTM positive and NTM negative groups. (**A**) The mean relative abundance of the 16S rRNA sequencing analysis with NTM taxa included. (**B**) The mean relative abundance of the 16S rRNA sequencing without the NTM taxa included. Given the length of the ribosomal sequences analysed, species identities should be considered putative.

4.5.2.5 Rank-abundance

The rank-abundance curves (Figure 4.9) for the NTM positive and negative groups demonstrate the abundance of taxa in proportion to the sample group. *B. multivorans* was the most abundant in both groups, and *M. avium* was ranked second in the NTM positive group (A), followed by *S. aureus, Mycobacterium chelonae*, and *M. abscessus*. Notably*, P. aeruginosa* was ranked lower than *S. aureus* and the *Mycobacterium* detected in this group. In the NTM negative group (B), after *B. multivorans, P. aeruginosa* was ranked second, followed by *S. aureus and Stenotrophomonas maltophilia*. These results are consistent with the SIMPER analysis (Table 4.3/ Figure 4.8A), indicating *B. multivorans* as the prominent taxa in the NTM positive group, while *P. aeruginosa* is dominant in the NTM negative group.

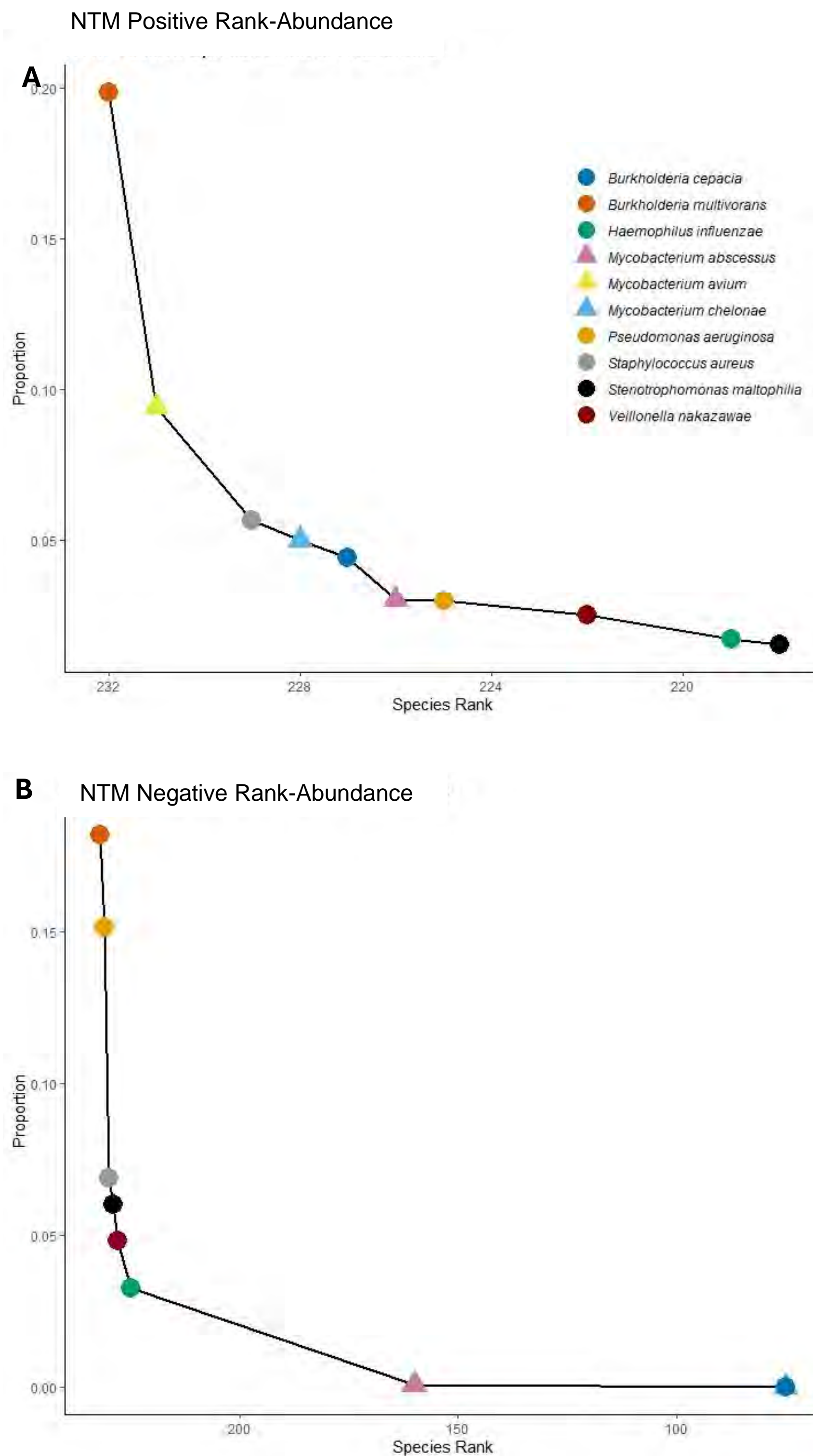


Figure 4. 9 Rank-abundance curves for the NTM positive (**A**) and NTM negative groups (**B**). Only taxa and NTM species of interest are highlighted by coloured shape. The figure shows the hierarchy of the dominant taxa with *B. multivorans* ranked the highest in both groups. (**A**) Shows *M. avium* second followed by *S. aureus, M. chelonae*, *M. abscessus*, *P. aeruginosa, H. influenzae* and *S. maltophilia*. (**B**) Following on from *B. multivorans* is *P. aeruginosa*, *S. aureus, S. maltophilia, H. influenzae* and *M. abscessus*.

4.5.3 NTM Type and the Microbiota

The following section examines the data generated by the 16S rRNA gene sequencing focusing on the microbiome composition in patients' culture positive for MAC (*n* = 20), MABS (*n* = 10), Other NTM type (*n* = 4).

4.5.3.1 NTM Type Microbiome Diversity and Composition

The 16S rRNA microbiome sequencing revealed a higher number of detected OTUs (Richness) in the MAC group compared to the other groups (Figure 4.10A). The MAC group (*n* = 20, mean =11.25±7.44 SD), while the MABSC group (*n* = 10, mean= 8.6±4.37 SD), and the Other NTM group (*n* = 4, mean= 6.25±2.0 SD). However, the results showed no statistical significance between the richness of the groups, (Kruskal-Wallis test: H= 3.25, p = 0.31), which like the previous section (Section 4.5.2.1), it corresponded to the Fisher's alpha diversity index (Figure 4.10B). Where the MAC group (mean 1.34±0.87 SD), had a higher range of diversity in comparison to the other groups (MABSC, 1.02±0.52, Other NTM group, 0.75±0.24, (Kruskal-Wallis test: H = 2.84, p =0.41). Again, the Berger-Parker index of dominance (Figure 4.10C) showed very similar values among the groups (MAC, mean 0.45±0.21. MABSC, mean 0.51±0.17 Other NTM group, mean 0.54±0.09), This signifies the groups have dominant taxa. But the results were non-significant (Kruskal-Wallis test: H = 1.96, p = 0.58).

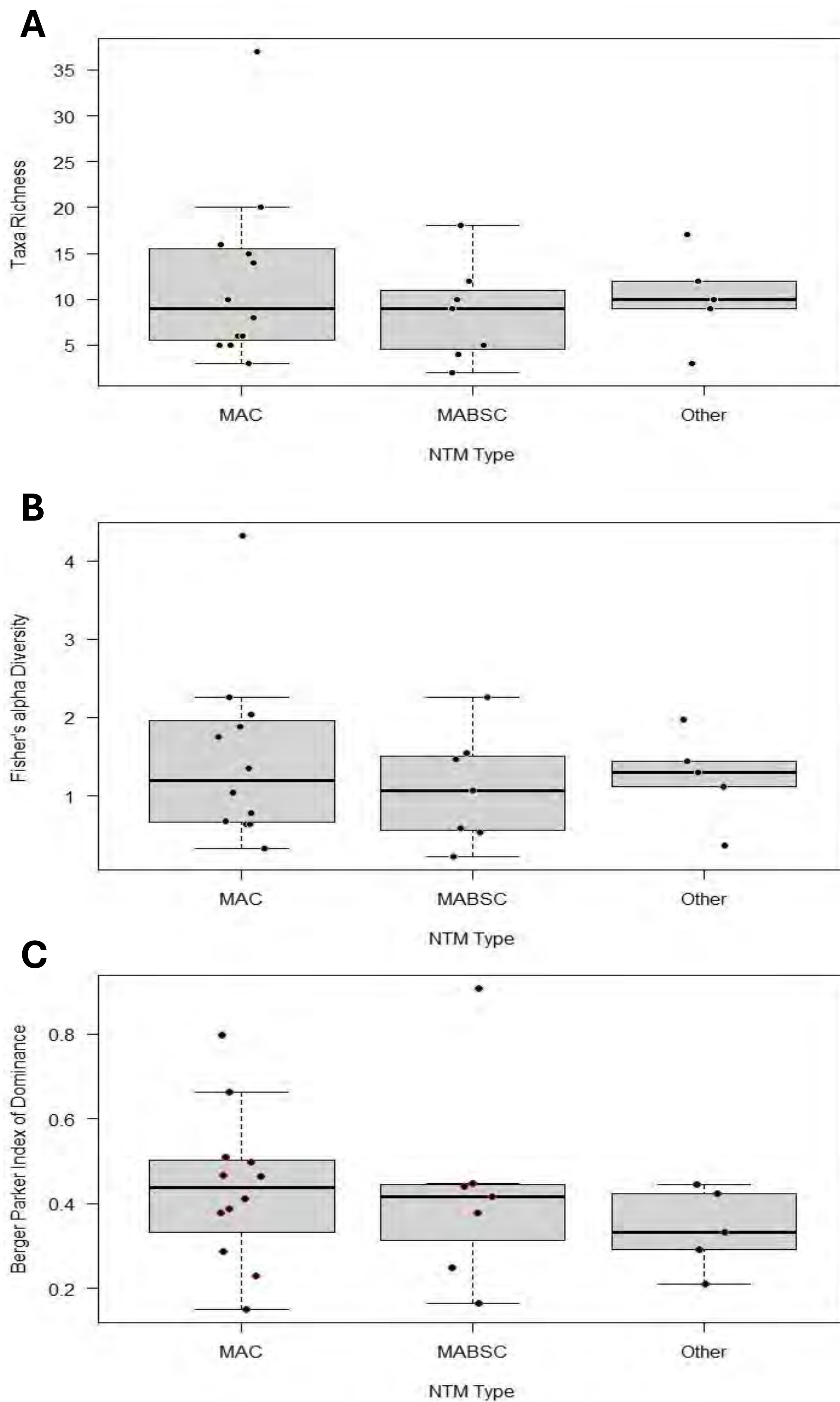


Figure 4. 10 NTM type Microbiome diversity and composition MAC – *M. avium* complex (*n*= 20), MABSC -*M. abscessus* complex (*n* = 10), Other – other NTM present (*n*= 4). Showing the Taxa richness (**A**). Fishers' alpha diversity(**B**). Berger Parker Dominance(**C**) All the differences were found to be statistically not significant (p>0.05).

4.5.3.2 NTM Type Similarity

To determine the change in microbiota between the NTM types (MAC, MABSC and Other NTM) the Bray-Curtis (abundance) and Sørensen (absence/presence) indices of similarity were used (Figure 4.11/Table 4.5) and statistical significance was measured using Bray-Curtis based ANOSIM with Bonferroni correction.

The Bray- Curtis similarity (Figure 4.11A/Table 4.5A), between the MAC group, MABSC and Other NTM groups; results shows there is similairty between the groups, this shows where more similarity was in the MABSC and Other NTM groups when compared to the MAC group. The Sørensen index of similarity (Figure 4.5B/Table 4.3) in the NTM type data also shows similarity in the groups. These results showed significant difference ($p = 0.0001$, $R = 0.17$) which remained significant in the MAC vs MABSC group after applying a Bonferroni correction for multiple comparisons (Table 4.5A).

The analysis conducted on the data with the NTM taxa omitted (Figure 4.12/Table 4.5B), revealed all the groups tested are non-significant with and without the applied Bonferroni correction, when compared to the analysis with the NTMs included (Figure 4.12/Table 4.5A). This was the case in both the Bray- Curtis similarity (Figure 4.12A), and the Sørensen index of similarity anlaysis (Figure 4.12B). As in the previous section (Section 4.5.2.5) this is indicative of the NTM taxa are contributing to the differences in composition between the groups.

Table 4. 4 Statistical Analysis for the Similarity of NTM types including (A) and excluding (B) NTM taxa

A

NTM type [a]	Bray-Curtis mean	Sørensen mean	Bonferroni p value
MAC	0.12± 0.15	0.15± 0.13	
MAC vs MABSC	0.06± 0.16	0.11± 0.11	0.0006
MAC VS Other NTM	0.09± 0.16	0.14± 0.14	0.1422
MABSC	0.06± 0.10	0.11± 0.11	
MABSC vs other	0.11± 0.11	0.14± 0.13	1
Other NTM	0.08± 0.11	0.22± 0.14	

B

NTM type [a]	Bray-Curtis mean	Sørensen mean	Bonferroni p value
MAC	0.09± 0.16	0.12± 0.14	
MAC vs MABSC	0.08±0.13	0.11± 0.12	1
MAC VS Other NTM	0.09± 0.16	0.15± 0.16	1
MABSC	0.14± 0.20	0.13± 0.12	
MABSC vs other	0.08± 0.17	0.12± 0.15	0.332
Other NTM	0.10± 0.15	0.27± 0.21	

Table (**A**) the statistical analysis for the NTM type with NTM taxa included. Table (**B**) the statistical analysis for the NTM type with the NTM taxa removed. Mean, ± 1 standard deviation throughout. [a] NTM type- Samples culture positive for MAC (*n* = 20), MABSC (*n* = 10), Other NTM type (*n* = 4) Statistical significance was measured using Bray-Curtis based ANOSIM with Bonferroni correction. A similarity of 1 indicates groups are identical whereas, a similarity of 0 signifies no matching species.

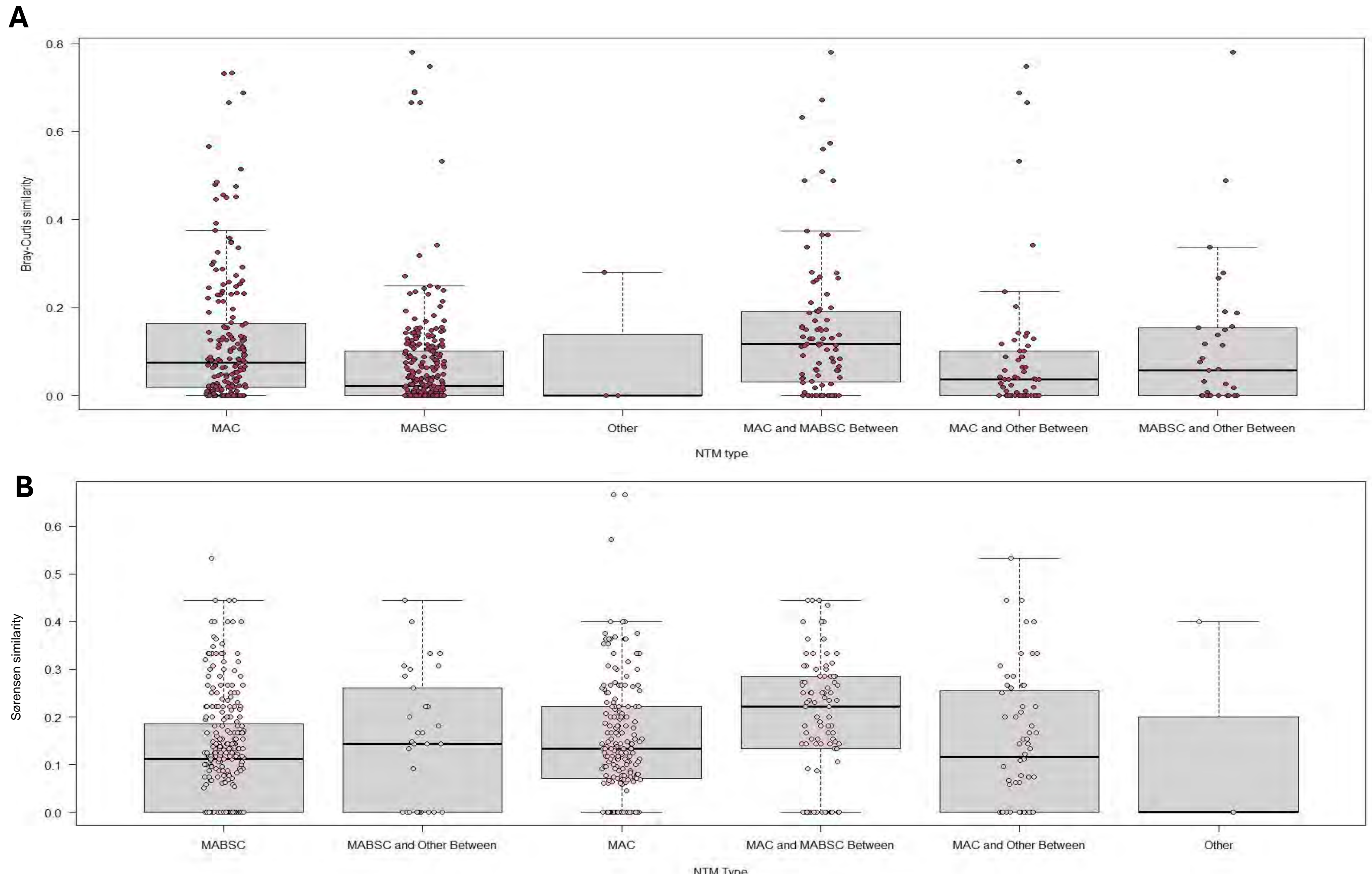
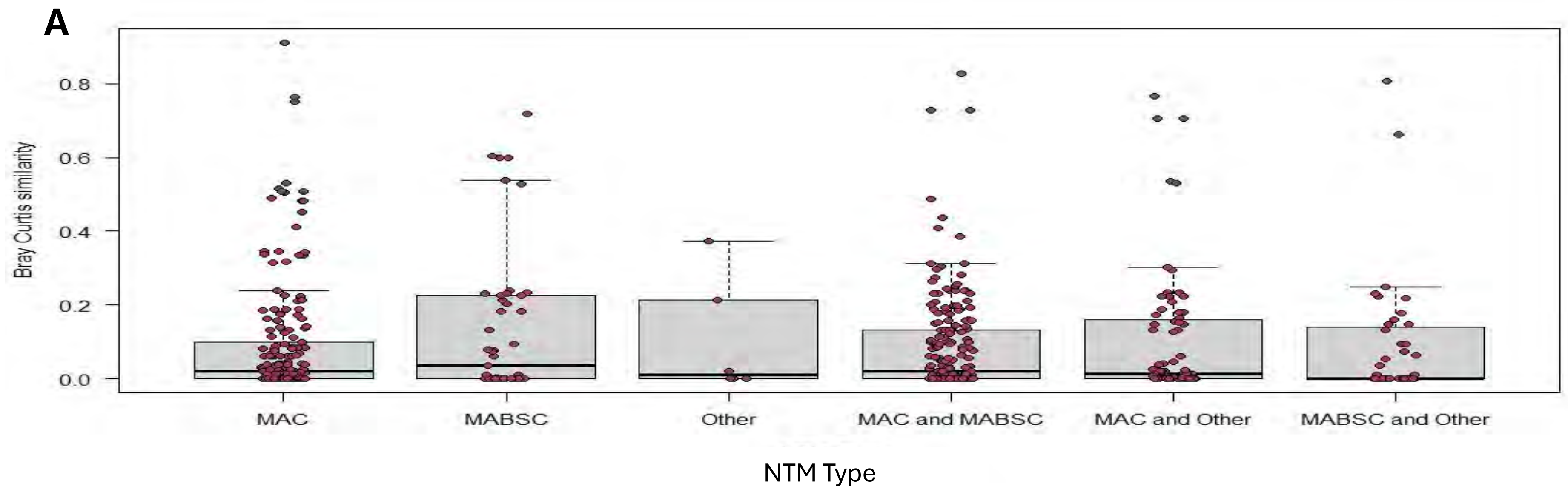


Figure 4. 11 Similarity index of the NTM types with NTM taxa included. The samples are culture positive for MAC (n = 20), MABSC (n = 10), Other NTM type (n = 4). Using Bray-Curtis's measure of similarity(**A**) Sørensen similarity(**B**). Means and corrected p values are given in Table 4.5.



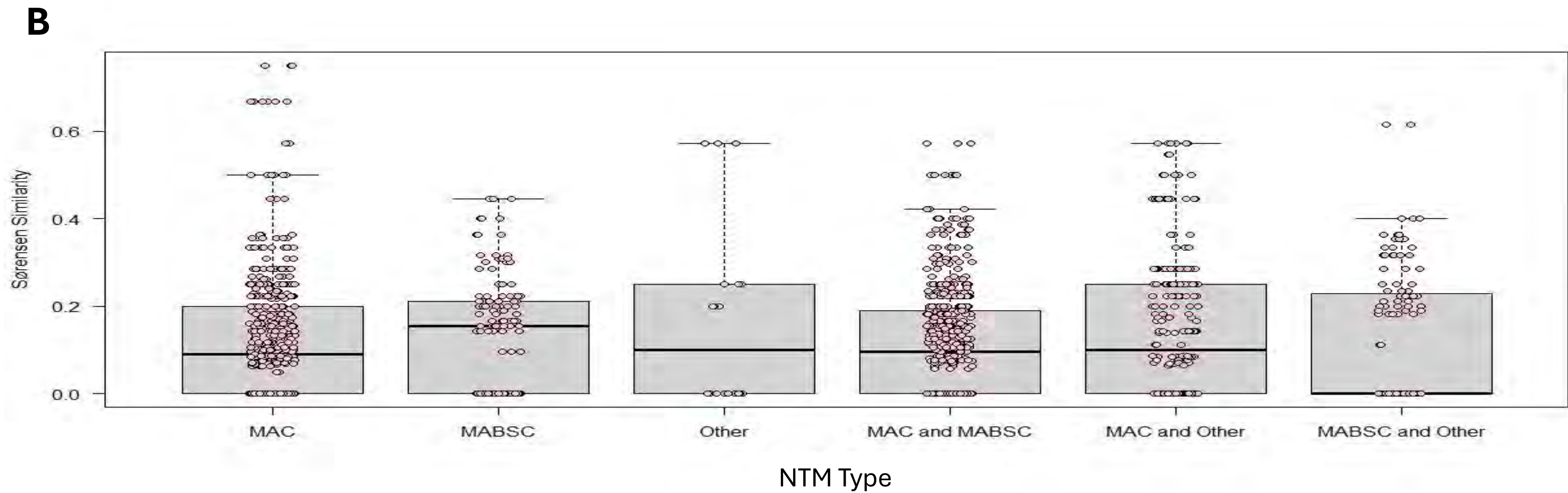


Figure 4. 12 Similarity index of the NTM types with NTM taxa excluded. The samples are culture positive for MAC (*n* = 20), MABSC (*n* = 10), Other NTM type (*n* = 4). Using Bray-Curtis's measure of similarity(**A**) Sørensen similarity(**B**). Means and corrected p values are given in Table 4.5

4.5.3.3 Non-Metric Multidimensional Scaling

To illustrate the patterns of similarities between the groups an NDMS plot was created (Figure 4.13). The plot included the NTM taxa (Figure 4.13A) showed some negligible overlap between the MABSC and Other groups, showing some small similarity. No overlap between the MAC, MABSC and other groups were visible indicating distinct groups. The statistical significance was assessed using PERMANOVA, with statistical significance between the groups ($p=0.001$, $R^2= 0.6667$, $Df= 22.67$).

The second plot (Figure 4.13B) shows the distribution of the groups with the NTM taxa removed. It shows some degree of similarity between the MAC and MABSC groups which are both clearly separated from the other group. The PERMANOVA revealed significance between the groups ($p= 0.049$, $R^2= 0.6667$, $Df= 22.67$)

This analysis indicating that the presence of the NTM taxa are contributing to the similarity of the groups.

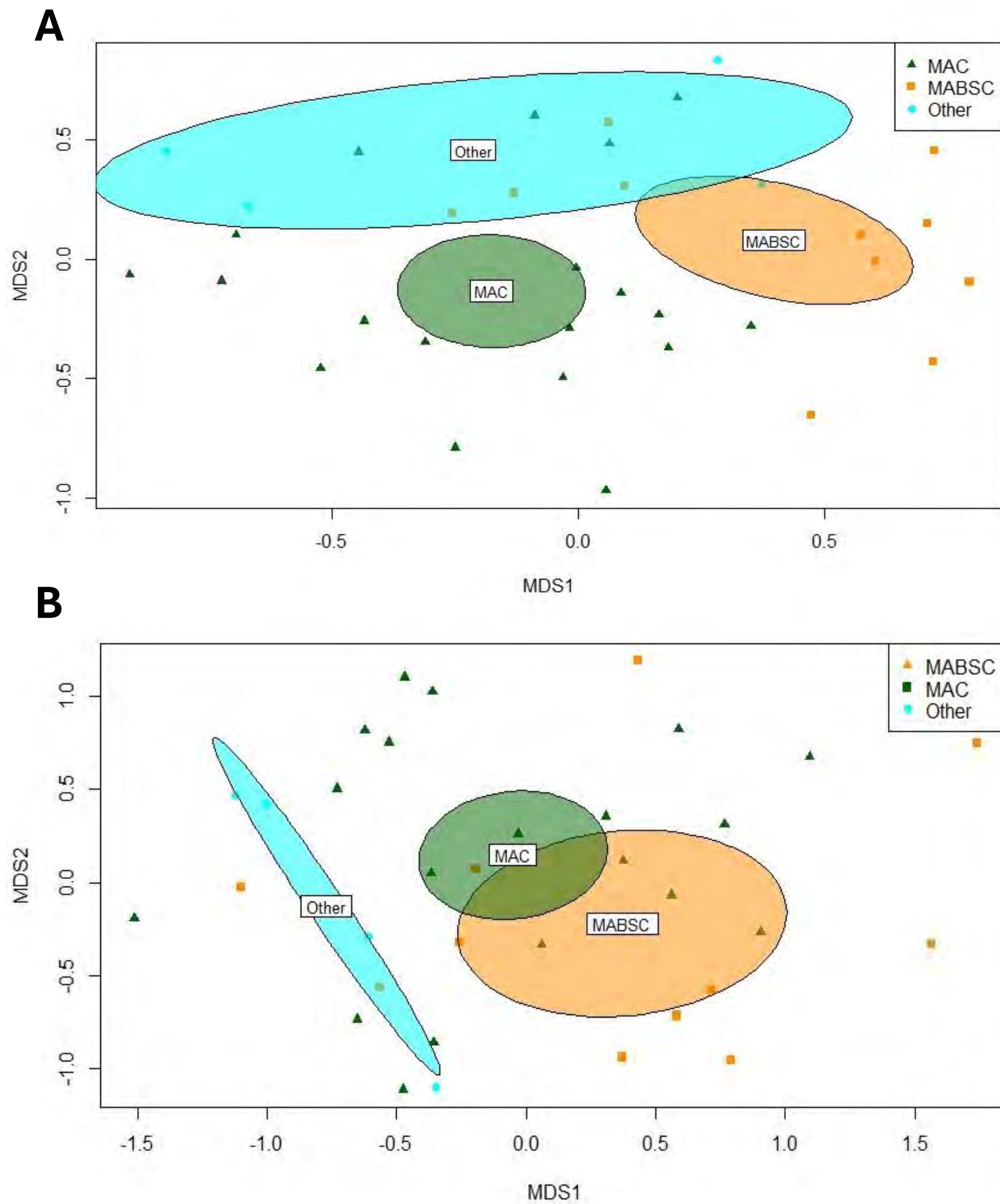


Figure 4. 13 NMDS plot showing how the groups are distributed by similarity. The overlap of the coloured ellipsis indicates the area where the groups share similarity. The orange triangles are representing the MABSC group, the green squares for the MAC group and the blue squares are representing the Other group. (A) NMDS of the 16S rRNA sequencing of the whole microbiome split into NTM types(B) NDMS of the 16S rRNA sequencing of the whole microbiome split into NTM types without the NTM taxa included

4.5.3.4 NTM type SIMPER

To determine the driver of dissimilarity for the NTM types, a SIMPER analysis (Table 4.6/ Table 4.7) was performed. Initially this analysis was undertaken with the NTM taxa included (Table 4.6/Figure 4.14A). When comparing MAC (*n* = 20) and MABSC (*n* = 10), *S. salivarius* and *B. multivorans* are the taxa contributing to dissimilarity between the groups accounting for most of the dissimilarity (9.9% and 9.95% respectively), followed by the two main NTM groups *M. avium* (7.45%) and *M. abscessus* (7.32%). *M. avium* has the highest mean relative abundance (14%) in the MAC group and *S. salivarius* the highest in the MABSC group (18.6%). The Other NTM group showed similarities to both groups with *B. multivorans* having the highest mean relative abundance (18.6%).

The analysis with the NTM taxa removed (Table 4.7/ Figure 4.14B) showed in the MAC group *P. aeruginosa* (14.2%), *B. multivorans* (12.7%) and *S. succinus* (7.79%) had the highest mean relative abundance. Conversely the MABSC group showed a different arrangement of mean relative abundance with *S. salivarius* (32.5%), *B. multivorans* (13.1%) and *Neisseria mucosa* (11.2%). Furthermore, the other group again showed differences in hierarchical mean relative abundance with *B. multivorans* (21.3%), *S. aureus* (20.8%) and *H. influenzae* (20.4%) as the highest ranked species.

When comparing the two analyses *B. multivorans* is consistently ranked high in all the groups with and without NTM taxa present. *S. salivarius* is also consistently ranked in the MABSC groups as is *S. aureus* in the other group. This information coincides with the similarity measures preformed previously (Section 4.5.3.2) Which indicates that there is very little similarity between each group.

Table 4. 5 Similarity Percentage Analysis (SIMPER) NTM Types with NTM Taxa Included.

Taxon	Contrib. %	Cumulative %	Mean Relative abundance		
			MAC	MABSC	Other
Burkholderia multivorans	11.36	11.36	12.2	8.92	24.8
Mycobacterium abscessus	8.659	20.02	0.302	15	16.3
Streptococcus salivarius	8.446	28.46	2.82	18.6	0
Mycobacterium chelonae	7.243	35.71	0.0238	16.6	0
Mycobacterium avium	6.813	42.52	14	0	1.25
Staphylococcus aureus	5.904	48.42	4.68	4.51	17.9
Pseudomonas aeruginosa	5.47	53.89	10.7	0.376	2.93
Streptococcus toyakuensis	3.727	57.62	1.49	7.38	0
Staphylococcus succinus	3.661	61.28	5.95	1.36	3.68
Haemophilus influenzae	3.23	64.51	0.924	1.67	15.1
Stenotrophomonas maltophilia	2.696	67.21	5.59	0	0
Porphyromonas pasteri	1.839	69.05	3.81	0	0
Achromobacter xylosoxidans	1.669	70.71	1.3	2.6	0
Streptococcus symci	1.488	72.2	1.94	1.74	0
Stenotrophomonas pavanii	1.459	73.66	3.02	0	0
Neisseria mucosa	1.434	75.09	2.32	1.01	0
Veillonella nakazawae	1.358	76.45	1.48	2.11	0
Mycobacterium kansasii	1.306	77.76	2.71	0	0
Mycobacterium malmoense	0.9652	78.72	0	2.21	0

Bold text denotes species of interest regarding NTM and CF pathogens. Also given is within category mean percent abundance for taxa, Percentage contribution of each taxon to the average dissimilarity between the two groups being compared. Taxa with higher values in this column contribute more to the overall dissimilarity between the groups. Percentage cumulative contribution of each taxon to the average dissimilarity between the two groups. The taxa are typically listed in descending order of their contribution. The list of species is not exhaustive, so cumulative percent does not sum to 100%. Given the length of the ribosomal sequences analysed, species identities should be considered putative. (Full analysis: Supplementary Materials 11.5)

Table 4. 6 Similarity Percentage Analysis (SIMPER) NTM Type without NTM Taxa

Taxon	Contrib. %	Cumulative %	Mean Relative abundance		
			MAC	MABSC	Other
Burkholderia multivorans	12.84	26.25	12.7	13.1	21.3
Staphylococcus aureus	8.215	34.47	5.05	7.61	20.8
Haemophilus influenzae	5.388	53.16	1.04	2.45	20.4
Achromobacter xylosoxidans	5.134	58.29	3.24	0	17.8
Pseudomonas aeruginosa	7.736	42.2	14.2	0.468	6.12
Staphylococcus pasteuri	0.9977	83.38	0.161	0.512	3.54
Staphylococcus succinus	4.815	63.11	7.79	2.24	3.19
Staphylococcus hominis	0.7147	86.6	0.447	0.109	2.34
Mogibacterium diversum	0.3858	90.91	0.0515	0	1.71
Brevibacterium otitidis	0.2774	92.91	0	0	1.3
Oligella urethralis	0.3907	90.52	0.00883	0.472	1
Paracoccus angustae	0.05785	99.44	0	0	0.271
Prauserella isguenensis	0.5849	87.89	1.01	0.232	0.167
Streptococcus salivarius	13.41	13.41	4.09	32.5	0
Neisseria mucosa	5.567	47.77	2.71	11.2	0
Streptococcus toyakuensis	3.575	66.68	5.7	2.59	0
Stenotrophomonas maltophilia	2.76	69.44	5.69	0	0
Veillonella nakazawae	2.238	71.68	1.87	4.29	0
Porphyromonas pasteri	2.072	73.75	4.27	0	0

Bold text denotes species of interest regarding NTM and CF pathogens. Also given is within category mean percent abundance for taxa, Percentage contribution of each taxon to the average dissimilarity between the two groups being compared. Taxa with higher values in this column contribute more to the overall dissimilarity between the groups. Percentage cumulative contribution of each taxon to the average dissimilarity between the two groups. The taxa are typically listed in descending order of their contribution. The list of species is not exhaustive, so cumulative percent does not sum to 100%. Given the length of the ribosomal sequences analysed, species identities should be considered putative. (Full analysis: Supplementary Materials 11.6)

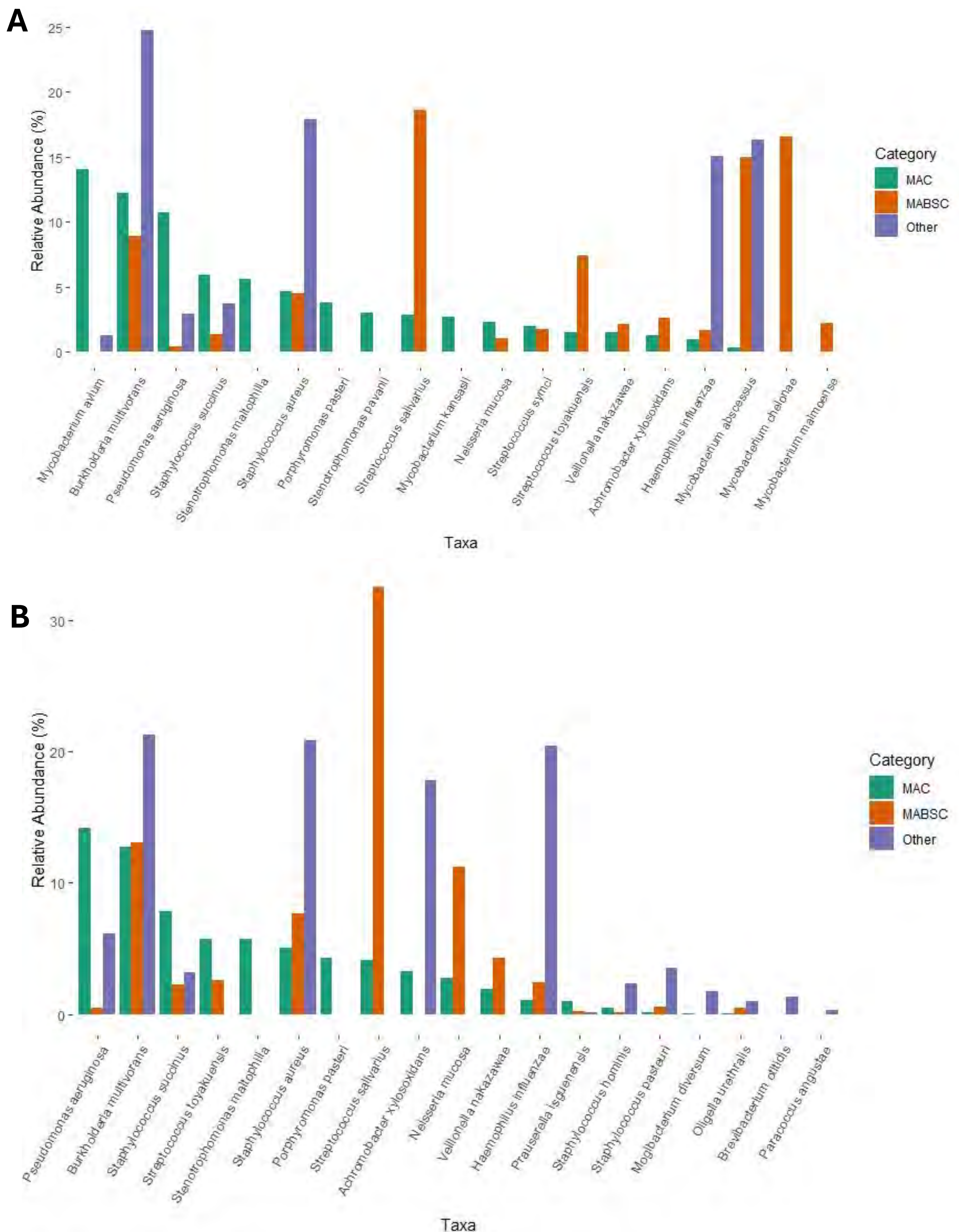


Figure 4. 14 The relative abundance as calculated by the SIMPER analysis (Table 4.6/4.7) Shows the mean relative abundance for each species detected within the NTM type groups MAC, MABSC and Other. (A) The mean relative abundance of the 16S rRNA sequencing analysis with NTM taxa included. (B) The mean relative abundance of the 16S rRNA sequencing without the NTM taxa included. Given the length of the ribosomal sequences analysed, species identities should be considered putative.

4.5.3.5 Rank Abundance Analyses

NTM type Ranked Abundance showed hierarchy of taxa present dependant on what NTM the sample was culture positive for. For those samples which were MAC positive (Figure 4.15A) *B. multivorans* had the highest abundance followed by MAC, *P. aeruginosa* and *S. aureus.* For those with MABSC and *M. chelonae* present alongside MAC these NTMs where ranked lowest out of the notable taxa reinforcing the results of the SIMPER analysis (Table 4.4). Samples which were MABSC positive (Figure 4.15 B) showed a difference in abundance hierarchy. Here, *B. multivorans* was highest followed by *M. abscessus* and *M. chelonae.* Finally, samples with other NTMs (Figure 4.15C) displayed another different ranking with *S. aureus* being the highest followed by *B. multivorans* then *M. chelonae.*

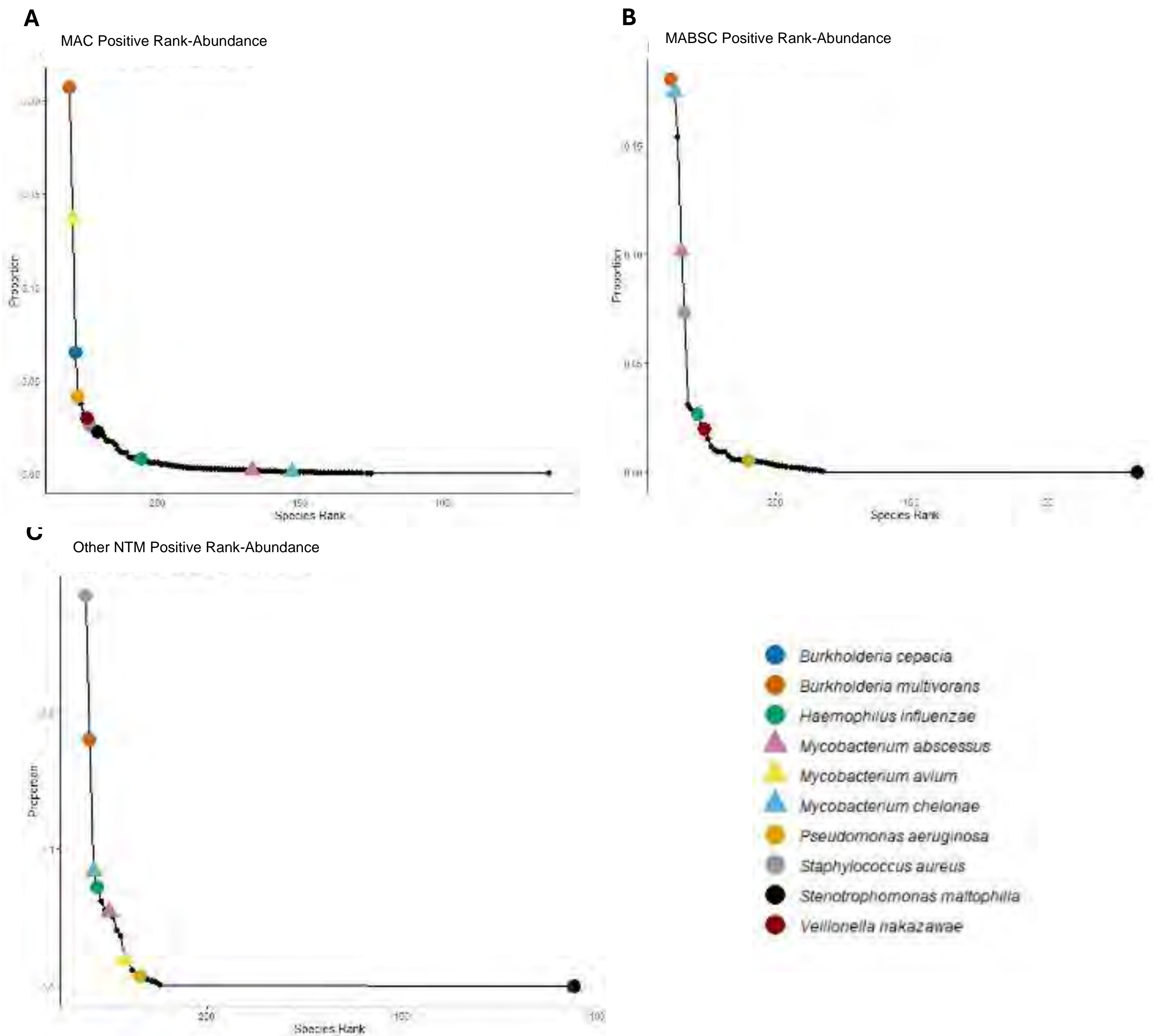


Figure 4. 15 The rank-abundance of the NTM type. Only taxa and NTM species of interest are highlighted by coloured shape. (**A**) MAC Positive Ranked Abundance shows *B. multivorans* as the highest ranked followed by *M. avium* and *B. cepacia* (**B**) MABSC Positive Ranked Abundance, Shows *B. multivorans* followed closely by *M. chelonae* then *M. abscessus* (**C**) Other NTM Positive Ranked Abundance shows *S. aureus* as the highest ranked than *B. multivorans and M. chelonae.*

4.5.4 NTM Complexes

4.5.4.1 Sequencing Analysis

The *rpo*B gene sequencing used in this chapter and subsequent chapters (Chapters 5/6), yielded a total of 3,998,441 mycobacterial raw sequences. The internal filtering and quality control steps performed by DADA2 (Callahan et al., 2016) found 0.63% chimeric sequences and successfully merged 49.1% of sequences.

In this Chapter 54 of the samples (Table 4.1/4.2), were used (mean = 778.123±2194.30 SD sequences per sample) (Figure 4.16), were assigned to a taxonomic classification. Reads were distributed unevenly across the samples (minimum of 168 and a maximum of 16,450 reads). In total 17 mycobacterial OTUs (total sequences; 5,555,80) were assigned after manual removal of chloroplasts, mitochondrial, environmental contaminants, and any sequences unassigned to a genus.

The rarefaction analysis (Figure 4.17) (*n*=55) provided a very mixed picture regarding the sequencing depth and species abundance. Each curve saturates at different points, indicating an unique species distribution in each sample. The steep rise of the curve, and rapid plateau suggests high initial species diversity that tapers off, implying a few species dominate with less common species. The curves with shorter length indicate less sequencing depth.

The richness, diversity, and community composition for samples culture positive for MAC, MABSC and other NTM acquired from CF patients was assessed by *rpoB* gene sequencing targeting only *Mycobacteria* species and sub-species that may make up the individual NTM complexes.

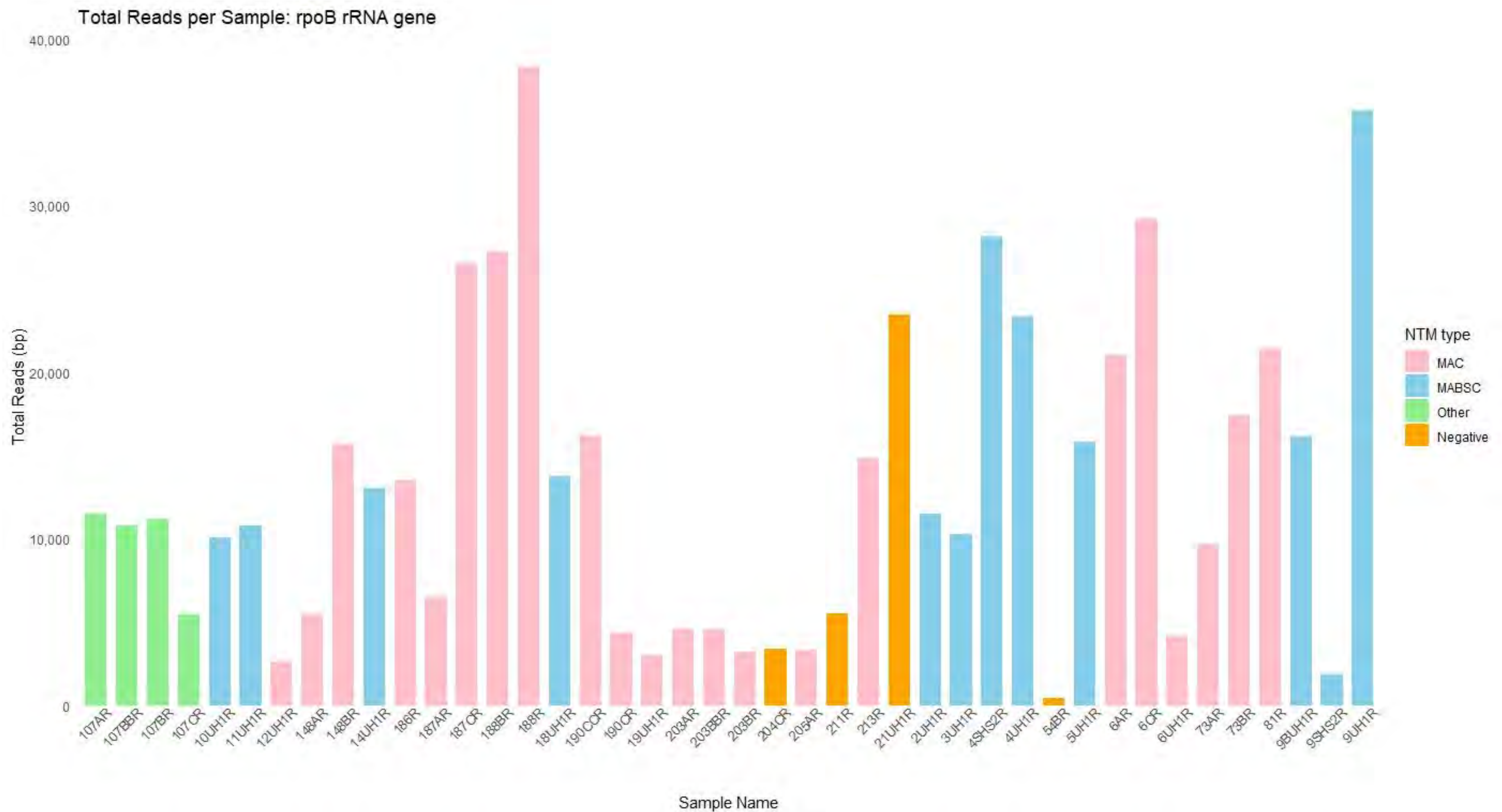


Figure 4. 16 *rpo*B gene target total reads used per-sample after built-in DADA2 filtering. Split into NTM types distinguished by the colours on the legend. Showed an uneven distribution of reads across the samples in both groups (mean= 101177±97284.30, Range; min =2290, max=492575) . Sample names on the x- axis are the sample names assigned by the clinic.

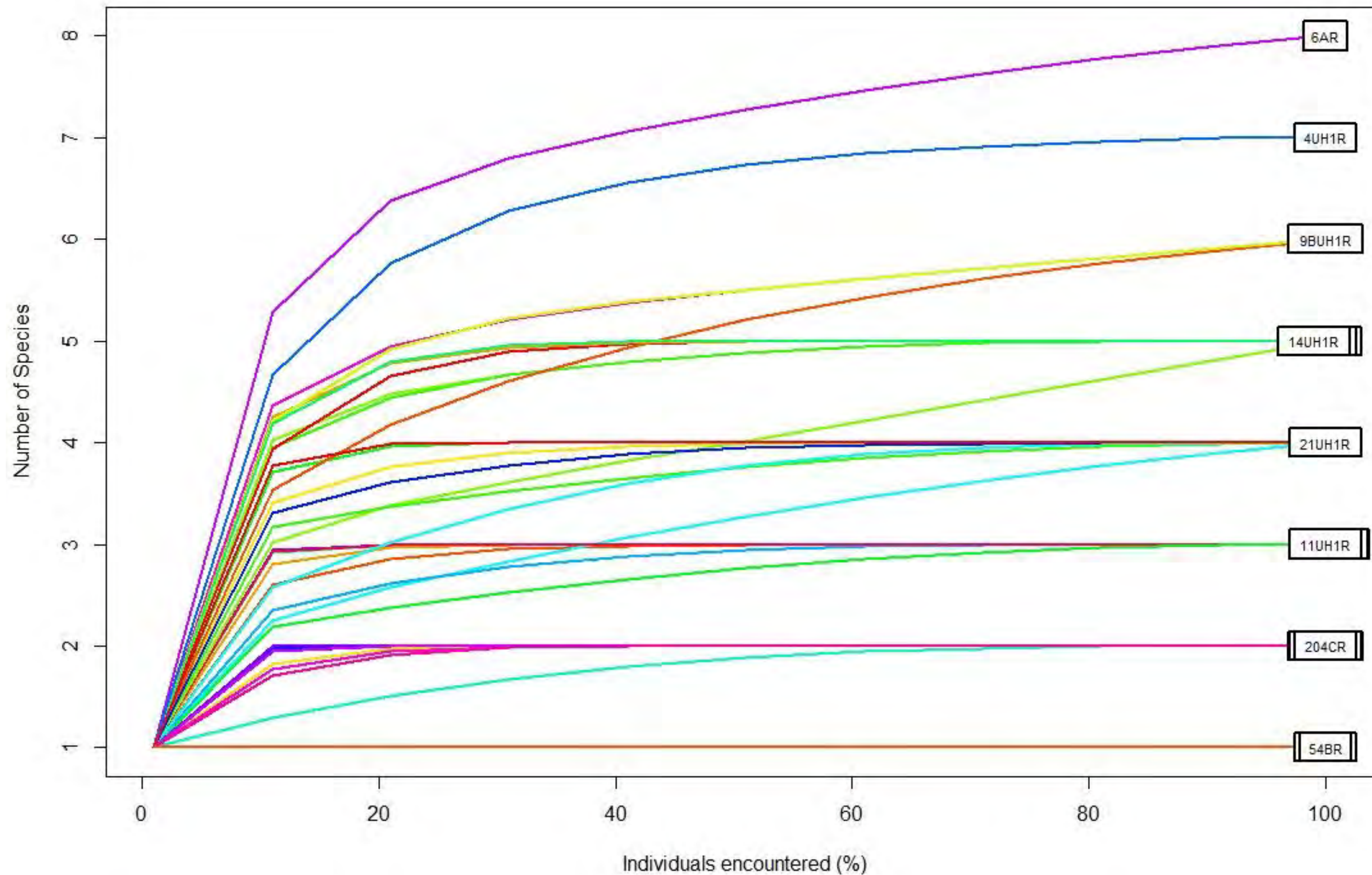


Figure 4. 17 Rarefaction curve of the *rpo*B gene sequencing reads after filtering, quality control and removal of unwanted sequences. Each curve represents a patient sample with the sample ID. The curve shows the percentage of individual species detected corresponding to the number of species in each sample.

4.5.4.2 NTM Complex Diversity and Composition

Taxa richness within each NTM complex (4.16A) showed that the MABSC (n= 10,mean= 4.16±1.69 SD) had a higher number of detected NTM species and subspecies on average than the MAC (*n*=20, mean= 3.63±1.81 SD), Other NTM (*n=4,* mean= 2.25±0.5 SD). Some NTMs were detected in the NTM negative samples and therefore have been included in this part of the analysis. NTM negative groups (*n=4,* mean= 2.0±1.41 SD), these results were non-significant (Kruskal-Wallis test: H= 7.45, p =0.058), which suggested that there was some evidence for a difference in taxa richness among the three culture positive groups, but it is not statistically strong. Again, The Fisher's alpha diversity index (Figure 4.18B) was complementary to this result showing that the group MABSC (mean= 0.39±0.16 SD) had a higher range of diversity than the other three groups, The mean diversity index was in the MAC group (mean=0.35±0.17 SD), Other NTM group (mean=0.21±0.04 SD) and in the Negative group (mean=0.194±0.12 SD). These results are significant (Kruskal-Wallis test: H= 8.61, p =0.03). The Berger-Parker Index of dominance (Figure 4.18C) again indicated dominant taxa within the four groups (MAC, mean 0.59±0.22 SD, MABSC mean= 0.55±0.20 SD, Other NTM group, mean= 0.72±016 SD, Negative groups mean= 0.82±0.28 SD) but again showed no significance (Kruskal-Wallis test: H= 4.99, p =0.17).

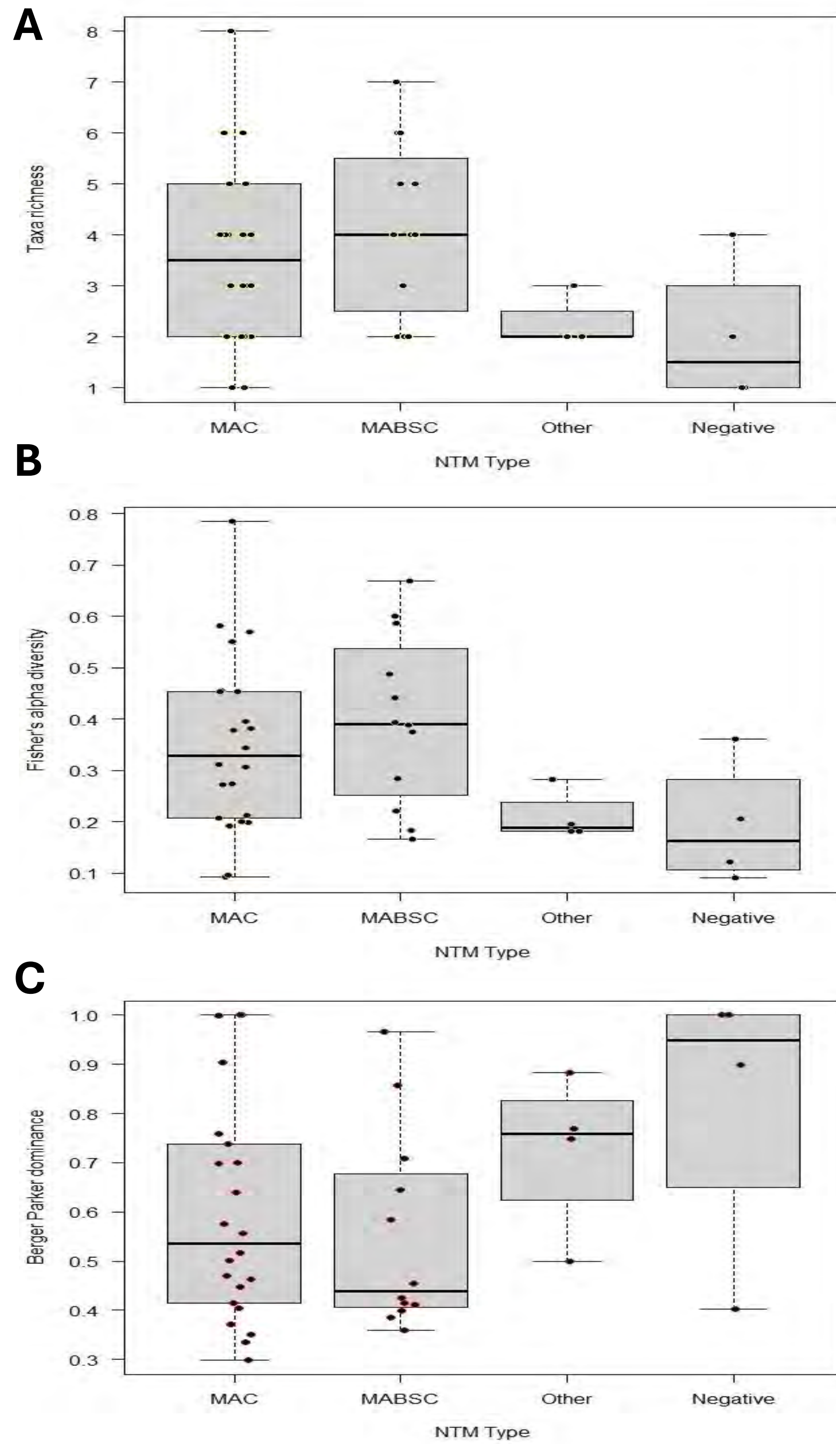


Figure 4. 18 NTM complex alpha diversity and composition of CF patients' culture positive for NTM. Taxa richness (**A**) MAC (*n*=20), MABSC (*n*= 10), Other (*n*= 4) and NTM negative groups (*n*=4) OTUs. Fisher's alpha diversity (**B**). Berger Parker index of Dominance (**C**)

4.5.4.3 NTM Complex Similarity

To determine any differences in species/sub-species between the NTM complexes the Bray-Curtis (Figure 4.19A) and Sørensen index (Figure 4.19A) of similarity were used and statistical significance was measured using Bray-Curtis based ANOSIM with Bonferroni correction.

The Bray- Curtis (Figure 4.19A/Table 4.8) and Sørensen index (Figure 4.19B/Table 4.8) both show that whilst there is a degree similarity within the MAC, MABSC, Other NTM and Negative groups the similarity between each group it is extremely low, this data is statisically significant ($p = 0.0001$, $R = 0.95$) and remained significant once the Bonferroni correction was applied (Table 4.8) This shows that the NTM complexes are distinct from each other and contain different taxa.

Table 4. 7 Statistical Analysis for the Similarity of NTM types

NTM Type [a]	Bray-Curtis mean	Sørensen index mean	Bonferroni p value
MAC	0.51±0.21	0.59±018	
MAC vs MABS	0.005±0.01	0.03±0.07	0.0001
MAC VS other	0.0005±0.001	0.05±0.12	0.0002
MAC vs Negative	0.09±0.131	0.16±0.18	0.0001
MABSC	0.40±0.20	0.67±0.21	
MABSC vs other	0.05±0.09	0.16±0.20	0.0007
MABSC vs Negative	0.006±0.02	0.01±0.05	0.0008
Other	0.63±0.18	0.61±0.10	
Other vs Negative	0.0002±0.0008	0.02±0.10	0.029
Negative	0.02±0.07	0.06±0.15	

Statistical analysis for the NTM complexes similarity indices. Mean, ± 1 standard deviation throughout. [a] NTM type- Samples culture positive for MAC (n = 20), MABSC (n = 10), Other NTM type (n = 4) Statistical significance was measured using Bray-Curtis based ANOSIM with Bonferroni correction. A similarity of 1 indicates groups are identical whereas, a similarity of 0 signifies no matching species.

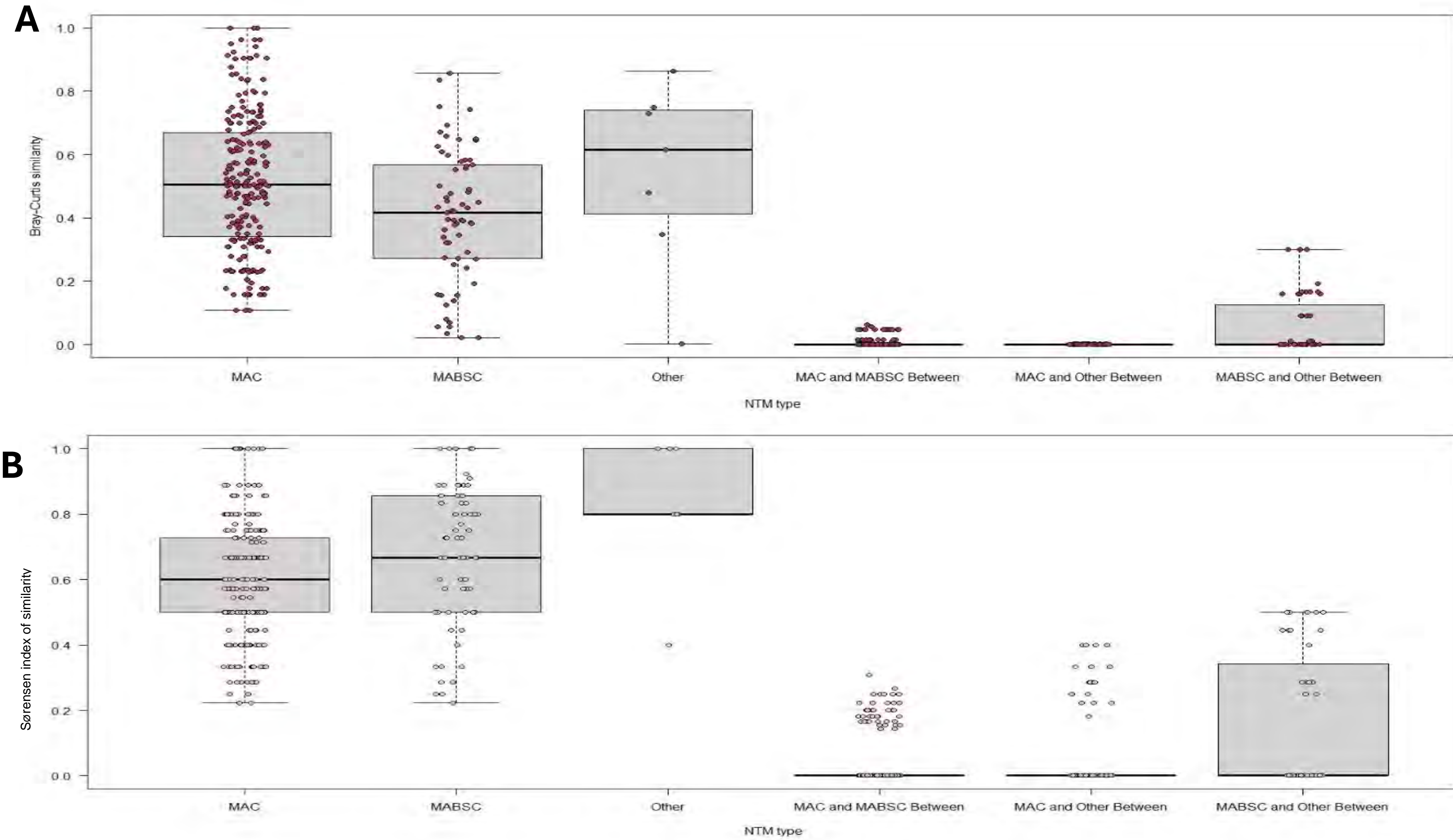


Figure 4. 19 NTM Complexes Similarity index of the NTM types which are samples culture positive for MAC (*n* = 20), MABSC (*n* = 10), Other NTM type (*n* = 4), Negative (*n*=4). Using Bray-Curtis's measure of similarity(**A**) Sørensen (**B**). Means and corrected p values are given in Table 4.5.

4.5.4.4 Non-Metric Multidimensional Scaling

The patterns of similarities between the groups was visualised using an NDMS plot (Figure 4.20). The plot shows the similarities between the NTM complexes as detected by the *rpo*B sequencing. It highlights the large distances between the MAC, MABSC and other groups. The large ellipsis generated by the Negative group indicates the wide amount of variation within the group, when compared to the other groups that have smaller ellipsis, suggesting less variation amongst the group. The data was found to be significant as assessed by PERMANOVA (p=0.001, R^2= 0.6667, Df= 22.67).

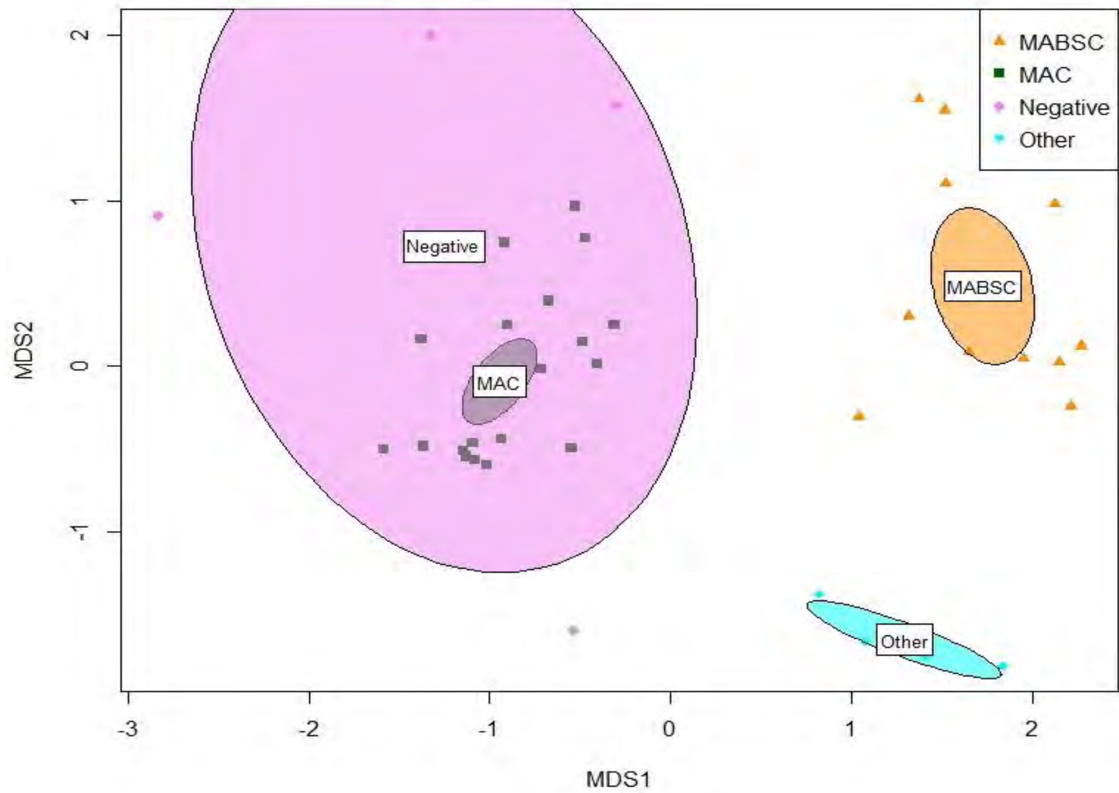


Figure 4. 20 NMDS plot showing how the groups are distributed by similarity. The overlap of the coloured ellipsis indicates the area where the groups share similarity. The large purple negative area covers the MAC distribution indicating that there is substantial overlap in there two groups. Whereas the orange MABSC and blue other group are distinct from the other groups.

4.5.4.5 NTM SIMPER Analysis

To determine the main dissimilarities for the NTM complexes, a SIMPER analysis (Table 4.9/Figure 4.21) was performed. The overall dissimilarity was 98.05% which followed the previous Bray-Curtis and Sørensen analysis (Figure 4.17) indicating the NTM complexes were distinct from each other. The analysis (Table 4.9/Figure 4.19) shows that *M. avium* and *M. abscessus* were the most abundant taxa, contributing 22.61% and 12.75% of the abundance, respectively. Other taxa, such as *M. chelonae subsp. gwanakae, M. avium subsp. hominissuis*, *and M. abscessus subsp. bolletii,* also had significant contributions to the overall dissimilarity.

Table 4. 8 Similarity Percentage Analysis (SIMPER) for NTM Complexes

Taxon	Contrib. %	Cumulative %	Mean Relative abundance			
			MAC	MABSC	Other	Neg
M. avium	22.61	22.61	53.5	0.401	0	0
M. abscessus	12.75	35.36	0.0709	35.4	0	0
M. chelonae subsp. gwana kae	9.853	45.21	0	2.89	59.1	0
M. avium subsp. hominissuis	9.823	55.03	16.4	0	0.0803	44.9
M. abscessus subsp. bolletii	8.722	63.76	0.0411	24.2	0	0
M. abscessus subsp. massiliense	7.191	70.95	0	20	0	0
M. chelonae	7.143	78.09	0	3.18	40.9	0
M. chimera	6.574	84.67	6.64	0	0	50
M. avium subsp. Paratuberculosis	4.872	89.54	11.5	0	0	0
M. abscessus subsp. abscessus	4.12	93.66	0	11.4	0	0
M. intracellulare	4.034	97.69	9.51	0	0	0
M. kansasii	0.8973	98.59	2.12	0	0	0
M. gadium	0.4602	99.05	0.121	0	0	5.09
M. xenopi	0.4586	99.51	0.0269	1.25	0	0
M. malmoense	0.4278	99.93	0	1.19	0	0
M. asiaticum	0.04218	99.98	0.0994	0	0	0

Given is within category mean percent abundance for taxa, Percentage contribution of each taxon to the average dissimilarity between the groups being compared. Taxa with higher values in this column contribute more to the overall dissimilarity between the groups. Percentage cumulative contribution of each taxon to the average dissimilarity between the two groups. The taxa are typically listed in descending order of their contribution.

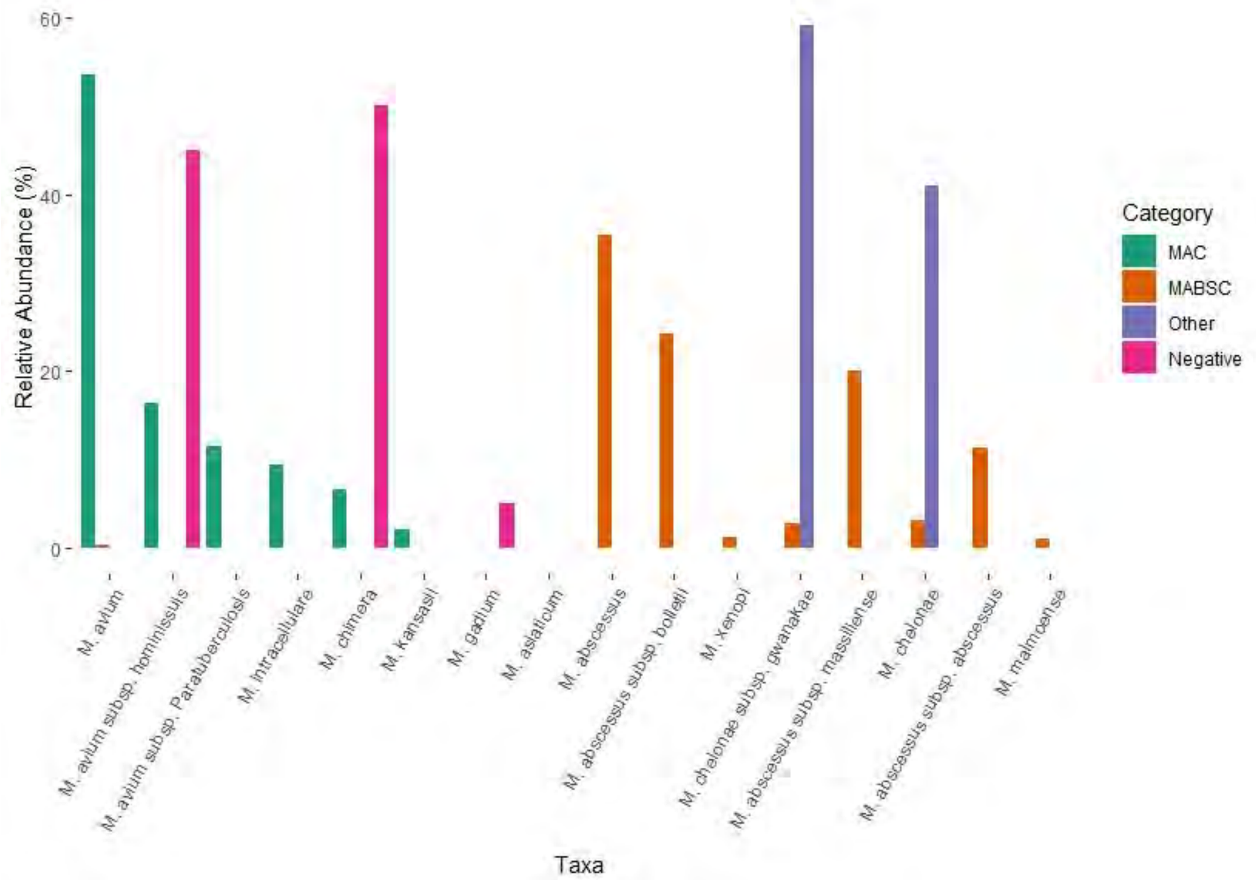


Figure 4. 21 The relative abundance as calculated by the SIMPER analysis (Table 4.9). Shows the mean relative abundance for each species detected within the NTM type groups MAC, MABSC and Other as detected by *rpo*B gene sequencing.

4.5.4.6 Rank-Abundance

The NTM complex Rank-Abundance (Figure 4.22) Shows the hierarchy of the taxa present dependant on what NTM the sample was culture positive for as determined by *rpo*B sequencing. This further, reinforces the SIMPER (Table 4.4) and Similarity analysis (Section 4.5.4.3) showing that within the complexes the taxa present are mostly unique. The MAC Positive group (Figure 4.22A). *M. avium* is greatest followed by *M. avium paratuberculosis* then *M. intracellulare* these are all known members of the MAC. The MABSC positive (Figure 4.22B) shows the species highest proportional abundance in this group are those associated with MABSC. *M. abscessus* ranked the highest followed by the *M. abscessus* subsp *abscessus*, *M. abscessus* subsp. *Bolletii* and *M. abscessus massiliense*. The other NTM positive group (Figure 4.22C) consisted of *M. chelonae* and *M. chelonae*. Subsp. *gwanake* and *M. avium* subsp. *hominissuis*. The NTM negative group (Figure 4.22D) shows that *M. chimera* and *M. xenopi* are the most abundant in this group.

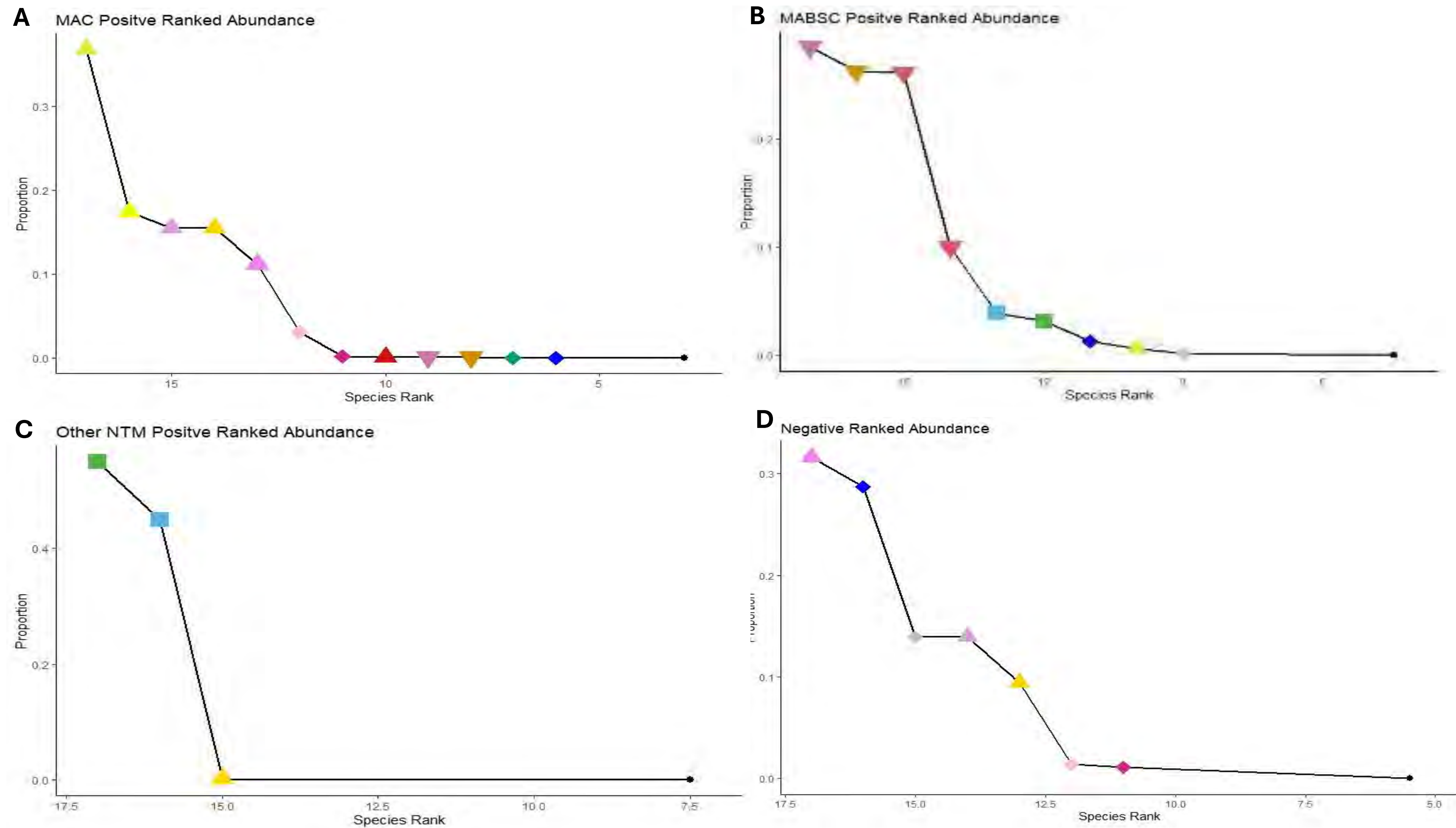


Figure 4. 22 The rank-abundance of the NTM complexes as determined by *rpoB* sequencing. NTM species are highlighted by coloured shape. (**A**) MAC Positive Rank-abundance (*n=20)* Shows members of the *M. avium* complex as the most abundant, (**B**) MABSC Positive Rank-abundance (*n*= 10), showing members of the *M. abscessus* complex as the most abundant (**C**) Other NTM Positive Rank-abundance, (*n*=4) showing members of the *M. chelonae* complex as the most abundant (**D**) Negative Rank-abundance (*n*=4) shows no complex as abundant but is dominated by *M. abscessus*.

4.6 Discussion

In CF, the function of mucociliary clearance is impaired reducing the ability to effectively clear microbial colonisers leading to CF associated respiratory infection (LiPuma, 2010). CF associated airway infection is often characterized by recurrent infection, and lung function decline which leads to eventual respiratory collapse (Flume et al., 2009). With the use of culture independent techniques, it has become clear that the respiratory system in PWCF is the host to a large community of microorganisms many of which are not identified by routine clinical screening (Rogers et al., 2009; Thornton et al., 2022).

Understanding the lung microbiome influence on diseases is essential to understanding disease progression (Gilbert et al., 2018; Li et al., 2024). Research has shown when the microbiota has low diversity and high species dominance patients have poorer clinical outcomes in relationship to lung function (LiPuma, 2012; Cuthbertson et al., 2020; Metzger et al., 2021). Especially when the dominant organisms are opportunistic pathogens such as *P. aeruginosa* (Boutin et al., 2017), BCC members (Coburn et al., 2015) or NTMs (Floto and Haworth, 2015; Park et al., 2016).

The influence of NTMs on the wider CF lung microbiota is poorly understood despite evidence that NTM infection has effects on clinical outcomes, lung function and is present during exacerbation states (Floto et al., 2016). The few studies on this to date have focused on the prevalence of NTMs in CF patients (Floto and Haworth, 2015; Lipman et al., 2020), and the clinical impact of NTM infection (Bryant et al., 2013), rather than the specific effects on the characteristics of the CF lung microbiota.

This chapter was designed to investigate the variations in the CF microbiota between CF patients who are culture positive or negative for NTM infection, through the application of 16S rRNA gene sequencing. Additionally, it aimed to explore the differences in microbiota composition amongst NTM culture positive patients infected with different types of NTMs such as MAC, MABSC, and other NTMs, also using 16S rRNA gene sequencing. Moreover, it aimed to analyse the NTM complexes' composition in these groups by analysing NTM-specific sequencing data obtained by sequencing the *rpo*B gene.

4.6.1 Sequencing Analysis

The raw fastQ files used in this chapter where sequenced along with the data used in subsequent chapters, targeting both the 16S rRNA gene and the *rpo*B gene. The following section refers to the sequencing analysis with all these files. The initial quality control done internally by the Illumina MiSeq showed a positive start with an average %Q30 score of 68.45 and a 88.27%PF.

The 16S rRNA gene fastQ files were quality controlled internally by the DADA2 algorithm (Callahan et al., 2016), the internal filtering found 15.3% chimeric sequences within the data which were removed. However, this figure is quite high and could indicate that the data may not accurately represent the original microbial community (Smyth et al., 2010). The DADA2 package also successfully merged 77% of the remaining sequences. The 23% unsuccessful merged sequences could be due to large amounts of sequencing errors or lack of overlap as a result of error trimming (Dacey and Chain, 2021). The *rpo*B gene sequencing (Section 4.5.4.1) showed a lower level of chimeras (0.63%) but, only merged 49.1% of sequences.

These issues could have arisen due to several factors starting from initial sample quality. If the initial DNA extraction was poor quality, suffered degradation or acquired DNAse contamination. This could have led to excessive or unexpected signal and poor-quality data (Laurence et al., 2014; Salter et al., 2014; Qi et al., 2021). Another possible cause could be the library preparation step where the introduction of PCR artifacts which become chimeras are most likely to occur (Schloss et al., 2011), the estimated rate of chimerism is generally between 5 to 45% (Haas et al., 2011; Schloss et al., 2011). These chimeras are formed when incomplete PCR products become primers and amplify fragments (Haas et al., 2011). Library preparation for amplicon sequencing requires two rounds of PCR to prepare the target strand for attachment to the flow cell (Illumina, 2013), which doubles the chance of chimeras forming. The formation of PCR artefacts are not the only errors that can be introduced during the library preparation stage; polymerases used for PCR amplification can introduce errors during the process and has error rates of 1 substitution per105-106 bases (Cline et al., 1996; Schloss et al., 2011; Potapov and Ong, 2017). Errors can also be added during the sequencing process (Stoler and Nekrutenko, 2021). The Illumina MiSeq which is used in this study has an 0.1% chance of adding an erroneous base (Illumina, 2016), and runs with high error rates are usually

due to over clustering issues which can affect the ability of the bases to be read correctly (Section 3.2.6.2) (Kozich et al., 2013; Illumina, 2016).

However, the chance of over clustering was mitigated by adding phased primers to the target sequences (Section 3.2.6.2), and the internal MiSeq quality control showed no evidence of this with the internal quality control showing good scores, (Section 4.5.1). Therefore, the loss of data could possibly be due to the large amount of chimeras introduced in the PCR stages of the library preparation which could have been further influenced by the addition of the ambiguous bases in the phased primers, or poor-quality starting material.

Despite the issues highlighted by the internal quality control provided by the DADA2 pipeline (Callahan et al., 2016) the analysis of the fastQ files was carried out. The 54 samples (Table 4.1/4.2), which were examined in this chapter showed large amounts of variation in the quantity of reads assigned to each sample (Figure 4.1/4.16). The discrepancies were further highlighted by the rarefaction curve (Figure 4.3/4.17) which showed a very wide range of sequence depth achieved by the samples but all the samples analysed did reach plateau indicating that there is sufficient sequencing depth per sample (Sanders, 1968; Kleine Bardenhorst et al., 2022; Xia, 2023). However, there is some controversy surrounding the use of rarefaction for NGS based microbiome studies. The method of rarefaction involves some random subsampling of the original data to find a common sequencing depth which can cause loss of data (Willis, 2019; Chiarello et al., 2022). This stresses a possible issue with the data quality in this study as large amounts of information was lost at each filtering stage. Conversely, the distribution of a range of genera per sample in the 16S rRNA gene data (Figure 4.2) whilst also variable did show that even samples with lower read numbers there was in most cases more than one taxon present.

4.6.2 Presence of NTMs in the Microbiota

This study showed that patient's culture positive for NTM when compared to those culture negative showed no statistical significance in the alpha diversity tests (Section 4.5.2.1/Figure 4.4). The lack of significance shown here could be due to numerous reasons, issues with the data as highlighted in the previous section (Section 4.6.1), leading to false positives or negatives in the results, which could influence the statistical significance of the data (Banerjee et al., 2009). This is plausible due to detection of NTM

species in some negative samples (n=3) but, it is possible that these samples were positive and went undetected in clinical settings, potentially contributing to the non-significance shown here.

When microbiota similarities were examined (Section 4.5.2.2/4.5.2.3), they demonstrated clear and significant differences in the composition of the microbiota, with NTM positive patients having lower levels of *P. aeruginosa* and higher levels of *B. multivorans* and *S. aureus* than those who were NTM negative (Table 4.3/Figure 4.5/4.8A), these results were significant. When reanalysed with the NTM taxa removed the similarity results (Figure 4.5), were non-significant and the hierarchy of taxa as reported by the SIMPER analyses (Table 4.3/Figure 4.8B), remained the same with the taxa ranked below the NTMs taking their place, this is indicative that the presence of the NTMs in the community drives the similarity between the groups. This was further confirmed by the NMDS plot (Figure 4.7), which clearly illustrates the distance and the large variation between the groups with and without NTMs included. The NMDS analysis did find significance when tested in relationship to the presence of NTMs (Figure 4.7A) but, showed non significance when tested in their absence (Figure 4.7B).

The presence of NTM in the microbiota shows they have an influence on the structure of the microbiome. The results are consistent with a previous study that found CF patients positive for NTM had higher rates of *S. aureus* and lower rates of *P. aeruginosa* (Olivier et al., 2003). Why the presence of NTM reduces the rate of *P. aeruginosa* is unknown, however, patients on aminoglycoside treatment such as amikacin for NTM infection (Raaijmakers et al., 2021) may have higher populations of *S. aureus* and lower *P. aeruginosa* due to its activity against Gram-negative bacteria (Ratjen et al., 2009). *B. multivorans* is intrinsically resistant to some aminoglycosides (Moore et al., 2001) which may indicate the high levels detected in this study is due to this resistance, as some of the patients in this study could possibly have been undergoing NTM-PD treatment at the time of sampling. The increase in a member of the *Burkholderia cepacia* complex (BCC) like, *B. multivorans* is important as it has been associated with poor clinical outcomes such as accelerated pulmonary decline leading to increased chance of morbidity and mortality (Courtney et al., 2004) and possible respiratory failure caused by progressive pneumonia known as *cepacia* syndrome (Scoffone et al., 2017). This initial analysis is vital in confirming the presence and absence of NTM infection in CF sputum samples and could also be used to inform treatment strategies for Gram negative pathogens present. Due to

the nature of NTM species and their differences to treatment sensitivities (Griffith et al., 2007; Daley et al., 2020), however, a simple positive/negative diagnosis may not be an adequate way to inform treatment. Therefore, investigation into the type of NTM present within an infection community would be more informative for treatment regimens.

4.6.3 The Type of NTM Present in the Microbiome.

Like the NTM positive and negative group analysis (Section 4.5.2), the NTM type analysis (Section 4.5.3) showed no statistical significance in the alpha diversity tests (Figure 4.10), conversely to the NTM group examination (Section 4.5.2) the negative samples were omitted from the analysis in this section, therefore the lack of statistical significance could be due to other factors like inadequate sequencing depth (Cameron et al., 2021) or low sample numbers (Ferdous et al., 2022).

When the NTM types were evaluated for similarity with the NTM taxa included in the analysis (Section 4.5.3.2) the results showed statistical significance and some small resemblances between the groups. Most notably is that *B. multivorans* is ranked high in abundance in all three groups, followed by an NTM, with *P. aeruginosa* being ranked relatively low (Figure 4.14A/4.15). However, in relation to similarities in the microbiota and the type of NTM the Bray-Curtis and Sørensen analysis (Figure 4.12) displayed very little in terms similarity between the groups, this was further confirmed by the SIMPER analysis (Table 4.6) and the NMDS plot (Figure 4.13A) which shows significance and a clear separation between the groups except for a slight overlap between the MABSC and other group. The plot also highlights the large variation within the other group illustrated by the large ellipsis on the plot.

When the similarity was reassessed with the NTM taxa omitted the plots showed a similar theme as the NTM group analysis (Section 4.5.2.2) the Bray-Curtis and Sørensen tests were no longer significant (Figure 4.12). On the other hand, the NMDS plot (Figure 4.13B) showed significance and differences in the distance between the groups, with MAC and MABSC showing slight overlap and the other group being isolated. The SIMPER analysis also showed a slight shift in hierarchy when the NTM taxa were removed (Table 4.6/Figure 4.14B) with *P. aeruginosa* ranked highest in the MAC group, followed by *B. multivorans* and *S. succinus*. In the MABSC group the arrangement also altered with *S. salivarius*, *B. multivorans*, and *Neisseria mucosa* being the highest ranked. The other NTM group continued this trend and showed. *B. multivorans*, *S. aureus* and *H. influenzae* as the

highest ranked species however, *B. multivorans* remained relatively highly ranked in all the groups. As before when examining the NTM groups the data shows that NTM influence the similarity of the groups. Which could largely be due to the presence or absence of NTM species within the samples. Also, as mentioned previously, treatment for NTM type will have different effects on the microbiota present further driving the differences found. MAC lung infection is typically treated with a combination of antibiotics which include a macrolide, rifampicin, and ethambutol whereas MABSC is treated according to its antibiotic sensitivity profile as it can be resistant to a range of antibiotics (Griffith et al., 2007; Laudone et al., 2021). These results provide a positive step toward targeted prescribing by taking into consideration the NTM type and the other CF pathogens present during lung infection. As culture independent NTM detection methods become more sensitive, less time could be taken applying antimicrobial susceptibility testing speeding up the time it takes from diagnosis to treatment.

4.6.4 The Composition of NTM Complexes.

The composition of the NTM complexes was determined using *rpo*B gene sequencing specifically designed to only target NTM species and subspecies. This was used to examine the differences and measure the abundance of species/subspecies within the NTM complexes.

The alpha diversity analysis for the NTM complexes follows the previous trend of non-significance but, the Fishers alpha diversity (Figure 4.17B) did show significance which means that there is a difference in diversity between the groups. The similarity indices (Table 4.8/Figure 4.18) showed very little similarity between each group but was statistically significant. This was further confirmed by the NMDS analysis which showed each group was spread away from each other except for the NTM negative group and the MAC group which showed some overlap. The NTM negative group however, had a large ellipsis indicating variation between the species found in the group. This is mostly due to the presence/absence of NTM species which make up the specific complexes this is confirmed by the SIMPER analysis (Table 4.8/Figure 4.20) and the ranked abundance curve (Figure 4.21). The MAC, MABSC and Other NTM groups were predominantly composed of complex specific NTM species/subspecies, however, these were not in equal quantities.

Some of the NTM species residing within complexes found in this study (Table 4.9/Figure 4.20/4.21) have been identified as drivers of infection in various types of infections, and with varying degrees of infectivity in animals and humans (Hamilton et al., 2017; Eslami et al., 2019; Azar et al., 2020). The species most highly detected in the MAC group was *M. avium*, this result showed similarities to another study examining the distribution of MAC complex members in relation to their clinical outcomes. Finding *M. avium* isolated most frequently and *M. chimera* the least in patients culture positive for MAC. Whilst the study had some similarities in the distribution of the MAC complex members it did not report the presence of any subspecies (Azar et al., 2020). However, if the MAC subspecies were removed from the analysis in the current study a change in abundance could have been seen and it is likely that the distribution of MAC complex members would match what found Azar et al. (2020). Another fact to take into consideration regarding the abundance of *M. avium* found in the study is that patients positive for *M. avium* often experience less aggressive disease progression when compared to other NTM species and the infection could go undetected until routine screening is carried out (Daley and Winthrop, 2020).

M. avium is often grouped together with *M. intracellulare* due to their similarities in clinical manifestation (Maesaki et al., 1993; Field and Cowie, 2003; Griffith et al., 2007; Koh et al., 2012). But M*. intracellulare* has often been linked to poorer response to treatment when compared to *M. avium* (Koh et al., 2012). Patients with higher levels of *M. intracellulare* experienced more aggressive disease (Koh et al., 2012; Azar et al., 2020), which could encourage them to seek diagnosis sooner than if they are experiencing milder symptoms. Notably, *M. intracellulare* was found in lower abundance than *M. avium* in this study.

Another NTM detected in the MAC group was *M. avium* subsp. *Paratuberculosis* which has been known to cause Johne's disease in livestock (Grant, 2005; Waddell et al., 2008; Eslami et al., 2019). It has been detected in human patients suffering from inflammatory bowel disease (Naser et al., 2014) and some autoimmune diseases such as insulin dependent diabetes mellitus (Paccagnini et al., 2009; Eslami et al., 2019). Even though there is no direct study linking this subspecies to CF, it has been linked to patients with diabetes (Paccagnini et al., 2009) which is a common co-morbidity in PWCF (Granados et al., 2019). So, there is a possibility that the patients within the cohort that also have diabetes may be more likely to have high amounts of positivity for *M. avium* subsp.

Paratuberculosis, driving the infection within their MAC complexes. However, the diabetic status of the study participants was not included in the data provided by the clinics.

The final subspecies ranked highly in the MAC group is *M. avium* subsp*. hominissuis* which has been isolated from humans and pigs (Tirkkonen et al., 2010). In humans it has been found to cause pulmonary infection (Stout and Hamilton, 2021), and disseminated disease (Despierres et al., 2012). There has been research surrounding the virulence of *M. avium* subsp. *hominissuis* (Dragset et al., 2019; Abukhalid et al., 2021), which highlights its efficiency in its ability to proliferate within macrophages to evade host elimination strategies (Haug et al., 2013; Dragset et al., 2019). This could possibly indicate that its presence in abundance in this study could be that this subspecies is active at this time. But this is speculation, and its presence in this study is unknown and can only be estimated that it is an intrinsic part of the MAC complex and has roles in virulence. All the species and subspecies in the MAC have been found to target a range of hosts and apply different levels of pathogenicity (Shin and Shin, 2021).

The MABSC group (Table 4.9/Figure 4.20/4.21) was predominantly made up of MABSC species and subspecies. However, the contribution and role of each subspecies is unclear in terms of disease (Minias et al., 2020). This study found *M. abscesses* as the highest in abundance in this group followed by *M. abscessus* subsp*. bolletii* then, *M. abscessus* subsp. *massiliense*. These findings are not consistent with the current literature which reports that *M. abscessus* subsp. *bolletii* is usually rarer in comparison to the other two species detected (Lee et al., 2015; Minias et al., 2020). However, this is regarding as instance as an infectious agent not regarding its abundance within the MABSC itself. Research regarding the distribution of the species and subspecies within the MABSC itself is lacking, therefore the expected distribution of the species and subspecies found in this study is incomparable. The findings made here are still extremely important as there has been a lot of work examining the antimicrobial susceptibility profiles within MABSC with most studies finding significant variability between the species and subspecies that make up the complex. With some species showing higher levels of resistance to some antibiotics than others (Nie et al., 2014; Mougari et al., 2016; Guo et al., 2020). All three MABC members show high levels of resistance to ciprofloxacin, doxycycline, moxifloxacin, and tobramycin. With *M. abscessus* showing the highest levels of resistance to clarithromycin and amikacin in comparison to *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense* (Sukmongkolchai et al., 2023), these levels seem to be

variable. With a different study showed that resistance levels in amikacin, linezolid, cefoxitin, and imipenem were low in MABSC members and high in doxycycline, ciprofloxacin, moxifloxacin, cotrimoxazole, tobramycin, and clarithromycin (Ruedas-López et al., 2023). This highlights the need for the ability to decipher what species and subspecies are making up the MABSC complex during an infection will enable the targeted treatment of the aetiological agent.

The other NTM group showed that the NTMs present were *M. chelonae* and *M. chelonae* subsp. *gwanakae* which were the most abundant, a small amount of *M. avium* subsp. *hominissuis* also was detected. As with the MABSC group there is no literature regarding the distribution of *M. chelone* and its subspecies in CF lung infection so no direct comparisons could be made. The detection of the *M. avium* subspecies is most likely due to miss identification at the taxa assignment stage (Jia et al., 2022), or PCR artefacts (Schloss et al., 2011) or due to transient infection (Azar et al., 2020). These suggestions could also be extended to the NTMs detected in the negative group.

This analysis (Section 4.5.4.2) did find some instances of possible co-infection between the MAC and MABSC groups, but the levels were very low (Table 4.9). Whilst relatively uncommon there has been some studies looking at MAC and MABS co-infection in lung disease such as COPD and mostly in older female patients. One study found that patients infected with MAC and MABS at the same time had less rates of sputum culture conversion than those infected with MAC and *M. massiliense*. The same study also found a strong association of co-infection with nodular bronchietatic NTM lung disease (Shin et al., 2018). Another similar study examined patients positive with MABS infection over an 8-year period who were diagnosed with additional MAC infection during or prior to MABS infection, they found that patients experiencing co-infection also had increased exacerbation than those just infected MABSC (Furuta et al., 2016). These studies show that the co-infection of multiple NTM complexes is possible, yet to be detected in the CF population. Again, highlighting the need to distinguish between NTM species and their complexes to enable accurate diagnostics leading to quicker treatments.

4.7 Conclusion and Further Work

Overall, the layered evaluation of the NTM and the CF microbiome conducted in this chapter has shown that the detection of NTM in CF sputum samples is more complex than just a positive or negative diagnosis. The presence of these microorganisms influences the possibility for other pathogens to gain an advantage such as members of BCC which can have detrimental outcomes for patients. The determination of the species/ subspecies that make up the NTM complexes is important in informing treatment and increasing the knowledge in understanding how NTM complex composition influences disease states such as CF exacerbation.

The possibility of NTM treatment creating a niche for members of BCC is extremely important due to the negative clinical outcomes associated with its infection. This, however, is an area that needs more research to further determine the exact factors involved in the creation of this niche.

The understanding of the configuration of NTM complex members is a very under studied area and this work further highlights the gap in the research. It does however, put further focus on the need for precision and quicker detection of NTM in patient samples. Which ultimately leads to faster treatment and increased positive outcomes in patient care.

This work has shown the potential for further studies examining the changes of NTM complexes and the microbiota in CF patients undergoing modulator therapy, this is discussed in Chapter 5. It has also shown that further understanding the effects of patient factors on microbiota composition is important, this is examined in Chapter 6.

Chapter 5: The Effects of CFTR Modulators on NTM Communities and The Wider Lung Microbiome

5 Introduction

Cystic fibrosis(CF) is a genetic disease associated with low life expectancy and lifelong treatment (National Jewish Health, 2023). People with CF (PWCF) are at high risk of developing chronic lung infection, which is often recurrent, leading to eventual respiratory collapse (Ciofu et al., 2013). However, recent advances in treatment have seen the induction of CF transmembrane conductance regulators (CFTR) modulators, which can alter the lungs to resemble those without CF, leading to increased life expectancy, and more favourable clinical outcomes (Ramsey et al., 2011; Burgener and Moss, 2018).

Patients on modulator therapy are, however, sill at risk from chronic infection by opportunistic pathogens including NTM (Ricotta et al., 2021; Ricotta et al., 2022; Saluzzo et al., 2022). Disease caused by NTMs can lead to more rapid lung function decline and faster progression of pulmonary disease compared to those infected with canonical CF pathogens (Esther et al., 2010; Martiniano et al., 2014). The treatment and diagnosis of NTM-pulmonary disease (NTM-PD) is a lengthy process which can lead to prolonged infection and poor patient outcomes. Moreover, the current methods fail to acknowledge the broader lung microbiome, which could influence disease progression, treatment plans and overall patient health (Griffith et al., 2007; Floto et al., 2016; Daley et al., 2020).

This chapter aims to investigate NTM's effect on the broader CF lung microbiome in CF-associated airway disease in patients taking CFTR modulator therapy.

5.1 Advances in CF treatment

Advances in CF treatment have led to a significant increase in the life expectancy of CF patients. In the 1960s, CF patients had a median survival age of 10 years (National Jewish Health, 2023), while patients born in 2021 had a median predicted survival age of approximately 53.3 years (Sokhi et al., 2021).

This increase in life expectancy is attributed to several factors including improved antibiotic therapy, airway clearance techniques, nutritional support, and more recently the introduction of CF transmembrane conductance regulators (CFTR) modulators (Sokhi et al., 2021; McBennett et al., 2022; National Jewish Health, 2023). CFTR modulators have shown remarkable results in clinical trials, with some studies demonstrating improvement

in lung function, reduced exacerbations, and improved quality of life (Ramsey et al., 2011; Burgener and Moss, 2018). These results have led to regulatory approval in, the USA, Europe, and UK of several CFTR modulators (Vertex Pharmaceuticals Incorporated, 2012; 2015; 2019; 2021). There are currently four CFTR modulators on the market:

Ivacaftor (Kalydeco®) (Vertex Pharmaceuticals, 2012), is indicated for use by patients who are at least 6 years old in the United States and over 4 months old in the United Kingdom (Vertex Pharmaceuticals, 2012; Donaldson et al., 2018). Ivacaftor has been shown to partially restore CFTR activity in patients with at least one class III gating mutation and it increases the ion function of activated cell surface CFTR proteins. The compound induces channel opening via a mechanism that does not require ATP binding and hydrolysis. As a result, patients experience improved airway clearance through the decrease in mucus plugging and better hydration of the airway surface (Van Goor et al., 2009; Vertex Pharmaceuticals, 2012). Clinical trials of Ivacaftor have shown significant improvements in lung function, with some studies reporting an increase in %FEV_1 by up to 10%. Ivacaftor has also been shown to reduce the frequency of pulmonary exacerbations, improve weight gain, and reduce the need for other CF medications (Ramsey et al., 2011).

Symkevi® (Tezacaftor/Ivacaftor) Changes the proteins' structure to ensure it can travel and stay at the cells' surface for longer. Tezacaftor works by helping the CFTR protein to fold correctly and reach the cell surface (Donaldson et al., 2018) while Ivacaftor improves the function of the CFTR protein once it reaches the cell surface (Ramsey et al., 2011). Together, these medications work to improve the transport of salt and water in and out of cells, leading to improved lung function and a reduced frequency of pulmonary exacerbations (Taylor-Cousar et al., 2017). It is used in patients' homozygous for ΔF508 mutation but, can also be used in patients with single copies of residual function mutations such as A455E, D110E or R117c or Splice mutations 711+3A-> G or E831X (Donaldson et al., 2018; Vertex Pharmaceuticals, 2021) It was approved by the European Medicines Agency (EMA) in 2018 (European Medicines Agency, 2018).

Orkambi® (Lumacaftor/Ivacaftor) is a combination of a potentiator and a corrector which is suitable for patients who are homozygous for the class II mutation ΔF508 (Vertex (Vertex Pharmaceuticals, 2021). Correctors help the CFTR protein form the right shape, so it can travel to the cell surface. The potentiator enables transport of the protein (Van Goor et al., 2011).

Kaftrio® (Elexacaftor/Tezacaftor/Ivacaftor) a medication approved by the US FDA in 2020 for the treatment of CF, it is a combination of three CFTR modulators - Elexacaftor, Tezacaftor, and Ivacaftor. This medication is specifically indicated for patients aged 12 years and older who have at least one copy of the ΔF508 mutation in the CFTR gene, which is the most common mutation in people with CF (Vertex Pharmaceuticals Incorporated, 2021).

Elexacaftor is a novel CFTR modulator that works by improving the stability and function of the CFTR protein at the cell surface, while Tezacaftor and Ivacaftor work together to improve the processing and function of the CFTR protein once it reaches the cell surface (Shaughnessy et al., 2021).

Clinical trials of Kaftrio have demonstrated significant improvements in lung function, with some studies reporting an increase in %FEV_1 by up to 13.8% after four weeks which was maintained for 24 weeks. It has also been shown to reduce the frequency of pulmonary exacerbations, improve quality of life, and reduce the need for other CF medications (Vertex Pharmaceuticals Incorporated, 2021; 2023)

CFTR modulators are relatively new to the CF treatment regimen. They can enrich or repair the expression, function, and stability of a faulty CFTR gene. However, they are only affective against certain mutations and treatment is lifelong (Lopes-Pacheco et al., 2016).

5.1.1 Pre-Modulator Therapies

Before the introduction of modulator therapy treatment for CF treatments were only able to help PWCF manage symptoms (Pettit and Fellner, 2014). PWCF can face a range of complications due to their condition and often need an arsenal of preventative measures or treatments to reduce and control symptoms and complications; patients have regular intervention from health care providers to monitor their health for any changes (The Cystic Fibrosis Trust, 2019). Treatment is often tailored to patient genotype as some medications target specific mutations (Ramsey et al., 2011). Traditionally PWCF are prescribed antibiotics, steroids and anti-mucolytics for the treatment and prevention of chest infections. These patients are also advised about physical airway clearing techniques, diet, and exercise (NHS, 2018). However, a persistent cycle of disease and treatment occurs, eventually leading to decreased quality of life and the potential need for lung transplantation (Charman et al., 2020).

5.2 CFTR Modulators and The CF Lung Microbiome

CFTR modulators have a variety of effects on the microbiome of PWCF, including changes in bacterial load and composition. Recent studies have suggested that some CFTR modulators may have direct antimicrobial effects (Davies and Martin, 2018).

For instance, ivacaftor has antibacterial properties possibly due to a quinoline ring in its chemical structure, it showed selective activity against Gram positive bacteria such as *Staphylococcus aureus* and a weak effect on the Gram-negative *Pseudomonas aeruginosa in vitro* (Reznikov et al., 2014). It also exhibited positive interactions with other antibiotics such as ciprofloxacin (Reznikov et al., 2014) and reduced *P. aeruginosa* bacterial load in chronically infected CF patients, but infection endured (Hisert et al., 2017). A recent study found that CFTR modulator therapy is associated with decreased risk of and less frequent positive NTM culture and concluded that NTM patients could have reduced treatment burden in PWCF over time (Ricotta et al., 2022). Though, a commonly used treatment for *Mycobacterium avium* complex (MAC) rifampicin has shown severe negative effects *in vitro* when used with ivacaftor (Guimbellot et al., 2018), which may exclude patients positive for MAC from modulator treatment and remaining on pre-modulator therapies (Section 5.1.1). It is also worth noting that bacterial community structure can be regulated by the local microenvironment and any treatment that can change this environment can also modify the microbiota, leading to a potential change in microbiota community dynamics and composition (Héry-Arnaud et al., 2019; Yi et al., 2021). However, in the context of NTM microbiota community dynamics and the changes made to the microbiota by CFTR modulators, this is a relatively new area of research and not much is known about the associations and interactions that may be occurring. This highlights the need for further investigation into this topic.

5.3 Chapter Aims and Objectives

The aim of this chapter is to examine the composition of the lung microbiota in CF patients who are undergoing modulator therapy and discover any differences in composition between those who are culture positive or negative for NTM. This was achieved by:

1. Generation of 16S rRNA and *rpo*B data via next generation sequencing.
2. Evaluation of 16S rRNA microbial community composition in relationship to modulator therapy.
3. Comparison of the microbiota in NTM positive and negative patents taking modulator therapy
4. Assessment of NTM complexes and their composition in relationship to modulator therapy.

5.4 Methods

Methods are outlined in Chapter 2 "Core Methods" and detailed in Chapter 3 "Optimisation of Amplicon Sequencing for the Detection of NTM in Microbiota."

5.4.1 Participant Samples

Participants were recruited as part of a longitudinal and cross-sectional study of adults as described in Chapter 2. Patients who were culture positive at the time of sampling were denoted as "NTM positive", those who have no history of positive NTM culture were "NTM negative". Patients are sub-grouped according to their culture status designated by the hospital, including their "NTM type" status which is the species of NTM they were culture positive at the time (*M. avium* complex (MAC), *M. abscessus* complex (MABSC) and Other). Culture of patient samples was done and confirmed by University Hospital Southampton (UHS) and The University of Vermont (UVM) microbiology departments. Only patients on modulator therapy at the time of sampling were included in this analysis (Table 5.1/5.2).

5.4.2 Sample Preparation and DNA Extraction

Prior to DNA extraction, patient sputum samples were prepared in a class II cabinet. The samples were centrifuged for 10 minutes at room temperature with a speed of 1107xg, the supernatant was discarded, and the pellet was resuspended in 900µl of phosphate buffered saline (PBS) and centrifuged again under the same conditions for 5 minutes, supernatant was discarded, and the pellet resuspended in 500µl of PBS (Rogers et al., 2006). The DNA in the samples were cross linked using 1.25µl of PMA (Biotium, USA) (Section 2.1.5.1).

500µl of sputum was added to capped 1.5ml microcentrifuge tubes with 1 tungsten carbide bead and glass beads (Merck, Dorset, UK) filled up to the 0.5ml mark, 400µl of DNA/RNA lysis buffer (Zymo research, USA) was added. The samples were then mechanically homogenised. Nucleic acid extraction was performed following manufacturer's instructions using Quick-DNA/RNA miniprep kit (Zymo-research, USA). DNA was then stored at -20˚C for future use and RNA was stored at -80˚C.

Nucleic acid extraction was performed following manufacturer's instructions using Quick-DNA/RNA miniprep kit (Zymo-research, USA). DNA was then stored at -20˚C for future use and RNA was stored at -80˚C.

5.4.3 DNA Sequencing

The microbiome of the samples was assessed by 2 step amplicon-based sequencing using the Illumina MiSeq system. First step amplicon PCR was done in two batches the first batch targeted the V4-V5 Regions of the 16S rRNA gene. Each PCR 25µl reaction consisted of 12.5µl 2x Q5 Master Mix (New England Biolabs, Hitchin, UK), 2µl (0.2µM) of phased primer pool (Invitrogen, Paisley, UK) (further detailed in Chapter 3) and 8.5µl of ultra-pure, molecular grade water (Cytiva, Fisher Scientific UK Ltd.).

The following parameters were used; 95°C for 3 minutes proceeded by 30 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds ending with one cycle at 72°C for 5 minutes.

The second batch of amplicon PCR targeted the *rpo*B gene and the reaction consisted of 12.5µl 2x Q5 Master Mix (New England Biolabs, Hitchin, UK), 1µl (0.1µM) of phased primer pool (Invitrogen, Paisley, UK) 12% molecular grade dimethyl sulfoxide (Thermo Fisher, UK) and 8.5µl of ultra-pure, molecular grade water (Cytiva, Fisher Scientific UK Ltd).

The following parameters were used; 94°C for 3 minutes proceeded by 45 cycles at 94°C for 60 seconds, 60°C for 60 seconds and 72°C for 60 seconds ending with one cycle at 72°C for 10 minutes (Gebert et al., 2018) Both PCR reactions were confirmed by gel electrophoresis (Section 2.5.1.1). PCR was confirmed via gel electrophoresis and prepared for the next stage using AMpure XP PCR purification beads (Beckman Coulter Life Sciences, USA) were used to remove any PCR fragments of 350bp and below, following manufacturer's instructions.

5.4.3.1 Index PCR

Each 25µl PCR reaction consisted of 12.5µl, 2x Q5 Master Mix (New England Biolabs, Hitchin, UK), 1.25mM of each primer, 5µl cleaned PCR product and 2.5µl of ultra-pure molecular grade water (Cytiva, Fisher Scientific UK Ltd).

The following parameters were used; 95°C for 3 minutes followed by 20 cycles at 95°C for 30 second, 55°C for 30 seconds and 72°C for 30 seconds ending with one cycle at 72°C for 5 minutes. The fragment was confirmed using gel electrophoresis and then purified as described in section 2.5.2. and reconfirmed using the Agilent Bioanalyzer (Agilent, USA) (Illumina, 2013).

5.4.3.2 Normalisation and Sequencing

Purified PCR products were quantified then normalised to a 1nM library and processed as stated in the MiSeq Denature and Dilute guide (Illumina, 2013; 2019), 5µl of the library was denatured with 0.2N NaOH for 5 minutes at room temperature and transferred to a heat block at 95°C for 5 minutes. 990µl of pre-chilled HT1 buffer was added to the denatured library, and this created the 10pM denatured library which was stored on ice. The final library consisted of the denatured library of 7pM and a 30% PhiX spike. The samples were sequenced on the Illumina MiSeq platform using the Illumina MiSeq V3 600 cycle reagent kit (Illumina Cambridge Ltd, Cambridge, UK).

5.4.3.3 Sequencing Analysis

FastQ files were downloaded from Illumina base-space. The analysis of raw sequence data was then performed through the DADA2 pipeline (Callahan et al., 2016), Using R version 4.2.2 (R Core Team, 2023). Forward and reverse reads were truncated at 200bp and 175bp, respectively. This truncation was based on the quality scores provided by the DADA2 pipeline and aimed to remove low quality bases and retain the required overlap of the paired ends reads for merging downstream (Callahan et al., 2016). The Silva Database version 138.1 release (Quast et al., 2013) was used to assign taxonomy to the sequences. Any unassigned amplicon sequence variants (ASV) where manually assigned using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) nucleotide database (Sayers et al., 2022) and matched with sequences based on a minimum of 95% query coverage, with the lowest possible e-value and a minimum of 95% identity cutoff. Multiple sequences assigned to the same ASV were condensed into OTU for statistical analysis. Any chloroplasts, mitochondria or environmental contaminants were manually removed following sequence assignment. Given the varying length of the sequences analysed, these identities should be considered putative.

Sequencing depth was assessed using rarefaction, this analysis adjusts for differences in library sizes across samples to aid comparisons in alpha diversity. It allows the determination of adequate sequencing depth in the samples is enough to capture the sample diversity (Willis, 2019; Schloss, 2024) .

5.4.3.4 Statistical Analysis

The raw OTU tables obtained through the DADA2 pipeline (Callahan et al., 2016) were analysed to evaluate the diversity, community composition, and similarity among samples.

To gauge the richness and evenness of the bacterial community each sample, was examined for changes in several measures. These included the Fisher's alpha index of diversity (Fisher's alpha), Berger-Parker dominance, and Bray-Curtis/Sørensen similarity measures. The significance of the alpha diversity measures was determined using the Kruskal-Wallis analysis, as outlined by Yadolah. (2008) and the similarity was tested for significance using Bray-Curtis based analysis of similarities (ANOSIM) with Bonferroni correction Cuthbertson et al. (2020).

5.4.3.4.1 Fisher's Alpha Diversity Index

To assess diversity within samples when compared to each other, and to measure the changes of diversity over time Fisher's alpha was used. This measures the richness of taxa independent of sample size when sequence reads are >1000 per sample. It is robust enough to use when samples are different sizes, comparing communities with different total abundances or when there are a different number of reads (Magurran, 2004; 2021). It is also less sensitive to rare species, which in some instances can influence the diversity estimate (Beck and Schwanghart, 2010). Fishers Alpha provides a simple measure of the distribution of species within a sample (Magurran, 2004; 2021) .

5.4.3.4.2 Berger-Parker index of Dominance

To assess the dominant taxa to the total number of individuals Berger-Parker index of dominance was used. This assesses the numerical importance of the most abundant taxa within the microbiota (Magurran, 2004).

5.4.3.4.3 Similarity

Similarity was measured using the Sørensen and Bray-Curtis measures. Sørensen measures the similarity between two data sets assessing presence/absence. Bray-Curtis looks at both the number of shared species and the abundance of the shared species (Magurran, 2004; Magurran and McGill, 2011). Both tests are not affected by the total species or richness of the communities being tested (Hao et al., 2019). Statistical significance was measured using Bray-Curtis based analysis of similarities (ANOSIM) with Bonferroni correction to determine which taxa contributed most to the compositional

changes between the groups (Armstrong, 2014). ANOSIM was used because it is able to handle large variations between groups and is able to detect significance among groups (Armstrong, 2014). A similarity of percentages (SIMPER) was used as described by (Clarke, 1993; Cuthbertson et al., 2020; Khomich et al., 2021).

In addition to the similarity indices a Multidimensional Scaling plot (MDS) was used to assess the pairwise distances amongst the groups. MDS was done in place of Non-Metric Multidimensional Scaling (NMDS) in this chapter due to its direct linear approach which is more effective for dealing with smaller datasets (Hout et al., 2013). This technique uses the Bray-Curtis dissimilarity measure and does not make assumptions about the data (Bakker, 2024) The results were assessed for statistical significance using Permutational Multivariate Analysis of Variance (PERMANOVA, also known as ADONIS) this test was used because it allows the testing of the associated microbial composition with covariates of interests, it also partitions within groups and between group distances to allow assessment of the grouping factors (Zhu et al., 2021).

Table 5. 1 Participant Demographics used in this Chapter

Origin	Sample ID	Collection method	Age	Gender	Type of Sample	Genotype	%FEV_1	Exacerbation	NTM	NTM Type	Modulator
UVM	36UVS	Postal	53	Male	Swab	Heterozygous	104	Stable	Negative	MABSC	Ivacaftor
UVM	22SHS2S	Postal	28	Female	Swab	Homozygous	99	Stable	Negative	Other	Kaftrio
UVM	11UVS	Postal	33	Female	Swab	Homozygous	50	Stable	Negative	Negative	Kaftrio
UVM	12UVS	Postal	28	Male	Swab	Homozygous	97	Stable	Negative	Negative	Kaftrio
UVM	13UVS	Postal	33	Female	Swab	Homozygous	59	Stable	Negative	Negative	Kaftrio
UVM	14BUVS	Postal	36	Male	Sputum	Heterozygous	51	Exacerbation	Negative	Negative	Kaftrio
UVM	14UVS	Postal	37	Male	Sputum	Heterozygous	52	Exacerbation	Negative	Negative	Kaftrio
UVM	15UVS	Postal	30	Female	Swab	Homozygous	44	Stable	Negative	Negative	Kaftrio
UVM	16BUVS	Postal	35	Female	Swab	Homozygous	71	Exacerbation	Negative	Negative	Kaftrio
UVM	16UVS	Postal	36	Female	Swab	Homozygous	72	Exacerbation	Negative	Negative	Kaftrio
UVM	17UVS	Postal	35	Female	Swab	Homozygous	101	Stable	Negative	Negative	Kaftrio
UVM	18UVS	Postal	23	Female	Swab	Heterozygous	83	Exacerbation	Negative	Negative	Kaftrio

UVM	19UVS	Postal	23	Male	Swab	Homozygous	76	Stable	Negative	Negative	Kaftrio
UVM	21UVS	Postal	32	Female	Sputum	Homozygous	95	Exacerbation	Negative	Negative	Kaftrio
UVM	22UVS	Postal	28	Female	Swab	Homozygous	99	Stable	Negative	Negative	Kaftrio
UVM	26UVS	Postal	25	Female	Swab	Heterozygous	94	Stable	Negative	Negative	Kaftrio
UVM	27UVS	Postal	31	Male	Swab	Homozygous	25	Stable	Negative	Negative	Kaftrio
UVM	28UVS	Postal	38	Female	Swab	Homozygous	100	Stable	Negative	Negative	Kaftrio
UVM	33UVS	Postal	29	Male	Swab	Homozygous	64	Stable	Negative	Negative	Kaftrio
UVM	5UVS	Postal	31	Male	Sputum	Homozygous	41	Stable	Negative	Negative	Kaftrio
UVM	6UVS	Postal	26	Male	Swab	Homozygous	80	Stable	Negative	Negative	Kaftrio
UVM	9UVS	Postal	30	Male	Swab	Homozygous	145	Stable	Negative	Negative	Kaftrio
UHS	11SHS2S	Postal	39	Female	Sputum	Heterozygous	73	Stable	Negative	Negative	Symkevi
UHS	14SHS2S	Postal	22	Female	Swab	Heterozygous	75	Stable	Positive	MAC	Kaftrio
UVM	23CUVS	Postal	27	Male	Swab	Homozygous	25	Stable	Positive	MAC	Kaftrio
UVM	23UVS	Postal	27	Male	Swab	Homozygous	25	Stable	Positive	MAC	Kaftrio
UVM	25UVS	Postal	47	Female	Swab	Heterozygous	93	Stable	Positive	MAC	Kaftrio

UHS	2CUVS	Clinic	29	Female	Sputum	Homozygous	22	Exacerbation	Positive	MAC	Kaftrio
UHS	2UVS	Clinic	29	Female	Sputum	Homozygous	22	Exacerbation	Positive	MAC	Kaftrio
UVM	34UVS	Postal	41	Female	Swab	Homozygous	80	Stable	Positive	MAC	Kaftrio
UVM	22UH1S	Postal	44	Female	Sputum	Homozygous	28	Exacerbation	Positive	MAC	Symkevi
UVM	24CUVS	Postal	24	Female	Swab	Homozygous	74	Stable	Positive	MAC	Symkevi
UVM	24UVS	Postal	24	Female	Swab	Homozygous	74	Stable	Positive	MAC	Symkevi
UHS	1SHS2S	Postal	39	Female	Sputum	Heterozygous	73	Exacerbation	Positive	MAC	Ivacaftor
UHS	25SHS2S	Postal	28	Female	Sputum	Heterozygous	72	Stable	Positive	MAC	Ivacaftor
UHS	13UH1s	Clinic	33	Male	Sputum	Heterozygous	36	Stable	Positive	MABSC	Ivacaftor
UHS	15UH1s	Clinic	29	Female	Sputum	Homozygous	22	Exacerbation	Positive	MABSC	Symkevi
UHS	23UH1S	Clinic	30	Female	Sputum	Homozygous	85	Exacerbation	Positive	MABSC	Symkevi
UHS	1UH1s	Clinic	28	Female	Sputum	Heterozygous	27	Exacerbation	Positive	MABSC	Ivacaftor
UHS	16UH1s	Clinic	28	Male	Sputum	Heterozygous	86	Exacerbation	Positive	MABSC	Ivacaftor
UHS	8UH1s	Clinic	28	Female	Sputum	Homozygous	27	Exacerbation	Positive	MABSC	Ivacaftor
UHS	12SHS2S	Postal	32	Female	Sputum	Homozygous	45	Stable	Positive	Other	Kaftrio

UVM	4UVS	Postal	34	Male	Sputum	Homozygous	75	Stable	Positive	Other	Kaftrio
UHS	24SHS2S	Postal	39	Female	Sputum	Heterozygous	73	Exacerbation	Positive	Other	Symkevi
UHS	24UH1S	Clinic	37	Female	Sputum	Heterozygous	71	Stable	Positive	Other	Symkevi

Participant demographics for the samples used in this chapter. Only participants on modulator therapy are included in this chapter.
Origin of the sample UHS: University Hospital Southampton, UVM: University of Vermont.
[b] Collection method: Clinic: if the sample was collected in the clinic, pre-COVID 19 pandemic or via the postal-pack method which is denoted as Postal.
[C] Type of sample provided Sputum or Cough Swab.
[D] CFTR genotype- CF transmembrane conductance regulator genotype. Homozygous ΔF508, two copies of the ΔF508 gene mutation, ΔF508 heterozygous, single copy of ΔF508 gene mutation and another mutation.
[e] Percent predicted forced expiratory volume in 1 second (%FEV1).
[f] NTM type- Samples culture positive for *M. avium* complex (MAC), *M. abscessus* complex (MABSC), Other NTM.

Table 5. 2 Participant Demographic Summary for this Chapter

Demographics	NTM positive	NTM negative
Number of patients (*n*=45)	22	23
Clinic	9	0
Postal	13	23
Age(years)		
Mean	31 (SD±6.725)	32(SD±4.76)
Median	29	31
Range (min/max)	22-47	23-53
Gender		
Female	17	13
Male	5	10
CFTR Genotype [a]		
ΔF508 homozygous	14	18
ΔF508 heterozygous	8	5
CFTR Modulator		
Ivacaftor ®	6	1
Symkevi®	5	1
Kaftrio®	10	21
NTM Type [b]		-
MAC	12	-
MABSC	6	-
Other	4	-
Exacerbation status		
Stable/ recovering	12	17
Exacerbation	10	6
Mean predicted %FEV1	55(SD±26.24)	67(SD±27.11)

SD denotes standard deviation of the mean. [a] CFTR genotype- CF transmembrane conductance regulator genotype. Homozygous ΔF508, two copies of the ΔF508 gene mutation, ΔF508 heterozygous, single copy of ΔF508 gene mutation and another mutation. [b] NTM type- Samples culture positive for *M. avium* (MAC), *M. abscessus* (MABS), Other NTM.

5.5 Results

5.5.1 Sequencing analysis

One Illumina MiSeq v3 600 cycle reagent kit was used for the data generated for the 16S rRNA gene sequencing and the rpo*B* gene sequencing used in this chapter and chapters 4/6. The internal quantity control provided by the Miseq showed that the sequencing of the targets were successful with the run having an average %Q30 of 68.45%, 88.27% passing filter (%PF) and a total yield of 13.81 Gbp.

The 16S rRNA gene sequencing yielded a total of 5797585 bacterial raw sequences. The internal filtering and quality control steps performed by DADA2 (Callahan et al., 2016) found 15.3% chimeric sequences and successfully merged 77% of sequences.

In this chapter 45 of the samples (Table 5.1/5.2), were used (mean = 36.87±298.53 SD sequences per sample) (Figure 5.1), were assigned to a taxonomic classification. Reads were distributed unevenly across the samples (range of minimum 18 and a maximum of 9841 reads). In total 152 bacterial OTUs, were assigned after manual removal of chloroplasts, mitochondrial, environmental contaminants, and any sequences unassigned to a genus.

The rarefaction analysis (Figure 5.2) (*n*=45) provided a very mixed picture regarding the sequencing depth and species abundance. Each curve saturates at different points, indicating an unique species distribution in each sample. The steep rise of the curve, and rapid plateau suggests high initial species diversity that tapers off, implying a few species dominate with less common species.

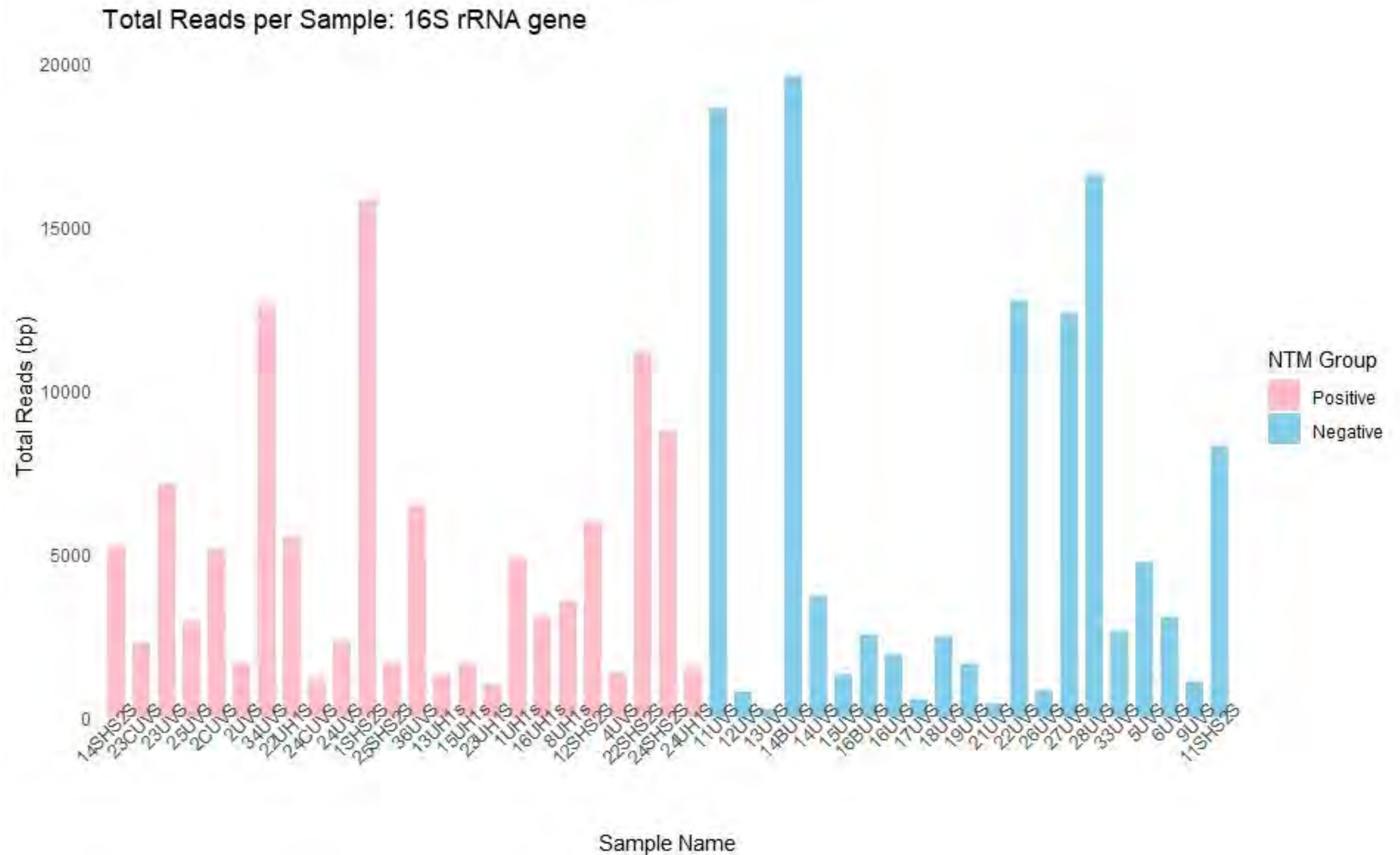


Figure 5.1 16S rRNA gene target total reads used per-sample after built-in DADA2 filtering. Split into NTM Positive (Pink on the left) and NTM Negative groups (Blue on the right) Showed an uneven distribution of reads across the samples in both groups (mean = 36.87±298.53, Range; min =18, max=9841) . Sample names on the x- axis are the sample names assigned by the clinic.

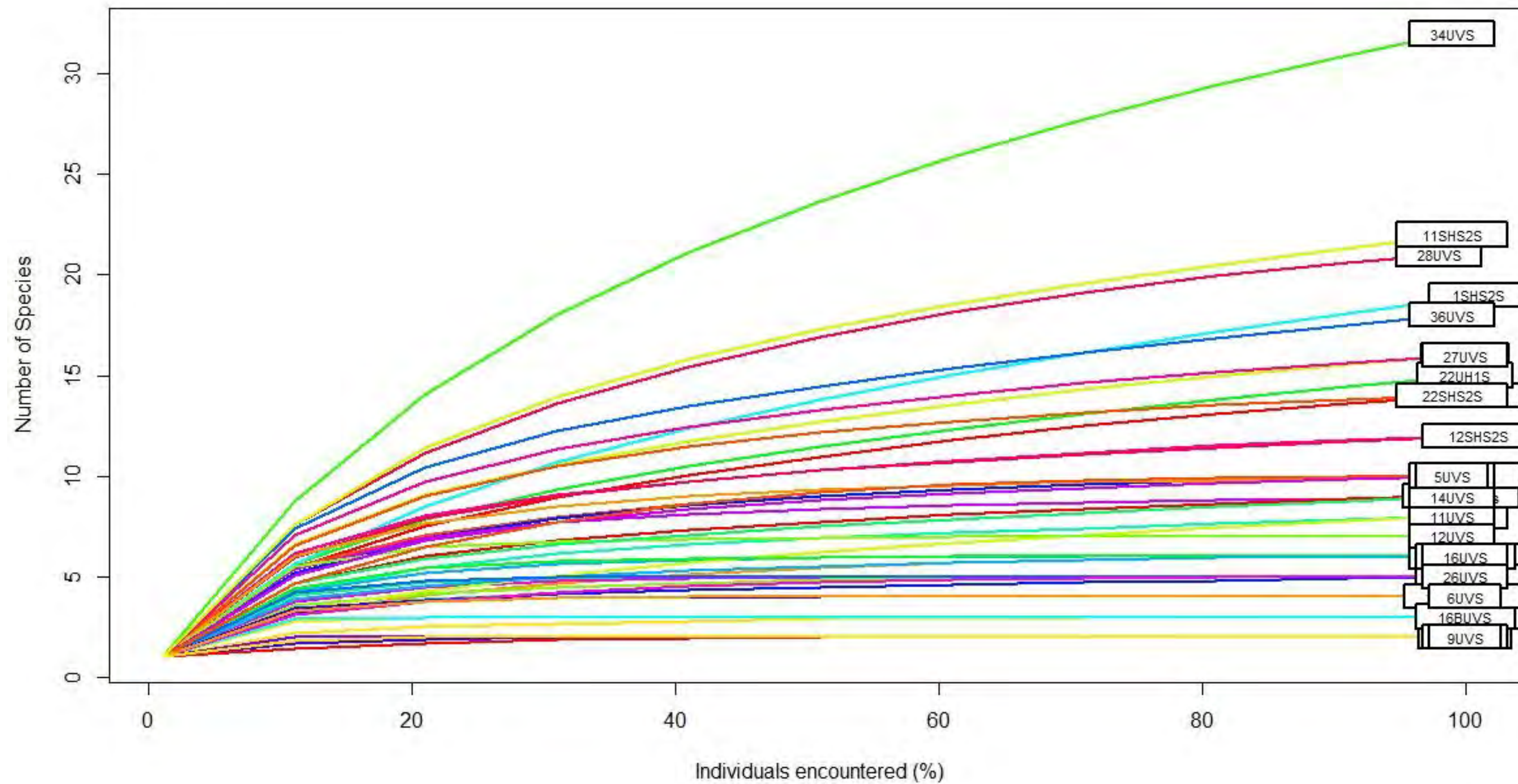


Figure 5.2 Rarefaction curve of the 16S rRNA gene sequencing reads after filtering, quality control and removal of unwanted sequences. Each curve represents a patient sample with the sample ID. The curve shows the percentage of individual species detected corresponding to the number of species in each sample.

5.5.2 NTM Group and the Microbiota

5.5.2.1 Microbiota Diversity and Composition

Whilst the NTM positive group (*n* = 22) had a higher taxa richness, (mean ± standard deviation of the mean throughout), (mean= 10.6±7.59 SD) than the negative group (*n* = 23, mean=8.14±7.49 SD) the results showed non-significance (Kruskal-Wallis test: H= 2.89, p =0.08) (Figure 5.3A). This result was echoed with the Fishers' alpha diversity (Kruskal-Wallis test: H= 3.18, p= 0.07) with the NTM positive group (mean= 1.31±0.89 SD) having a higher range of diversity than the negative group (mean=0.99±0.89 SD) (Figure 5.3B). When investigating the Berger-Parker diversity metric, there was no significant (Kruskal-Wallis test: H= 2.18, p = 0.13) difference between the NTM positive (mean= 0.41±0.18 SD) or negative (mean= 0.46±0.18 SD) groups. This indicated there was no difference in dominance of the taxa present(Figure 5.3C).

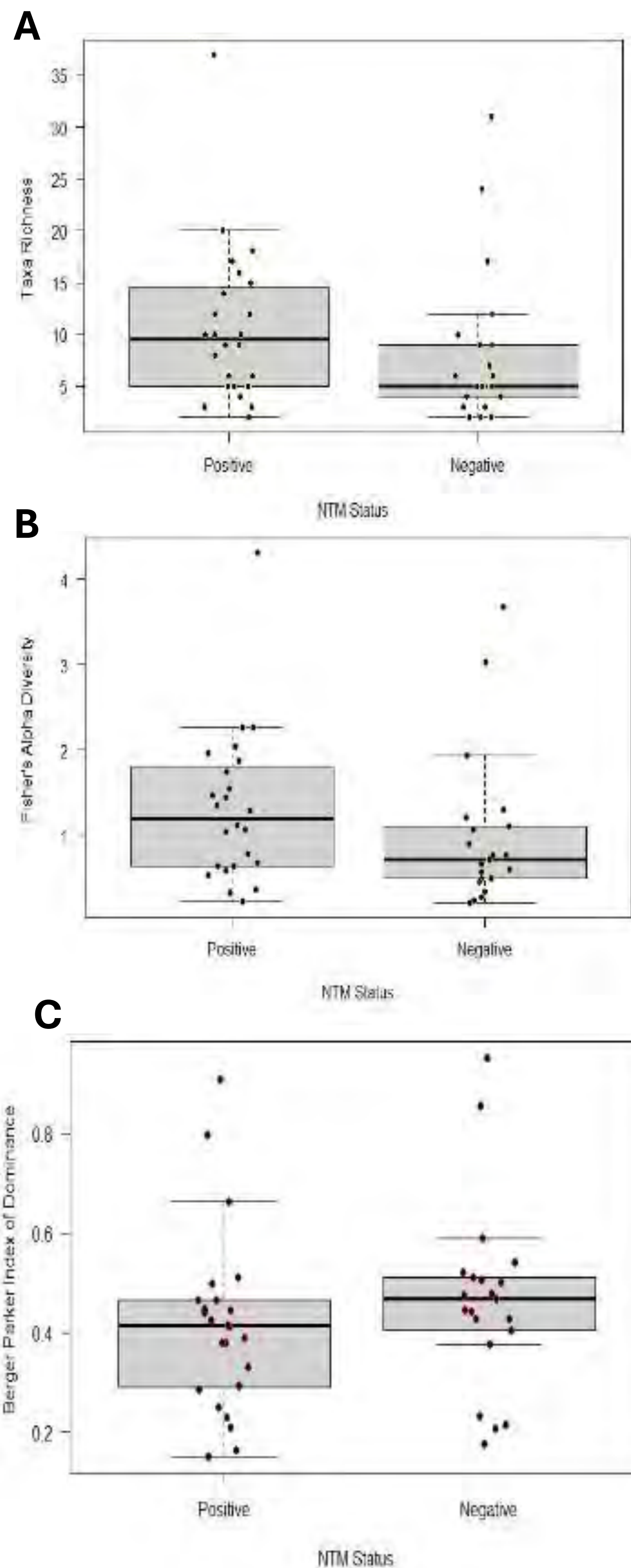


Figure 5.3 16S rRNA Microbiota diversity and composition (OTU) of NTM positive (n = 22) and NTM negative (n = 23) groups for patients on modulator therapy. Both Taxa richness (**A**) and Fisher's alpha (**B**) were found to be higher in the NTM positive group than the negative group. Conversely, the Berger-Parker index of dominance (**C**) was higher in the NTM negative group compared to the positive group.

5.5.2.2 Total Microbiota Similarity

To determine the change in microbiota between the NTM positive and NTM negative groups the Bray-Curtis (incorporating abundance) and Sørensen (absence/presence only) indices of similarity were used (Figure 5.4) and statistical significance was measured using Bray-Curtis based analysis of similarities (ANOSIM) with Bonferroni correction.

The Bray-Curtis similarity index (Figure 5.4A) for the NTM-positive group (mean= 0.11±0.13 SD), did show some likeness between the samples but, most of the samples in this group were unique. The negative group (mean= 0.07±0.12 SD), showed a similar trend, with very few samples sharing taxa. The similarity between the two groups (mean= 0.06±0.10 SD), showed a minimal degree of similarity indicating that there is less shared taxa between the samples of each group then there is within the groups.

The Sørensen coefficient showed the same trend as the Bray-Curits analysis. The NTM-positive group (mean= 0.13± 0.12 SD), negative (mean= 0.09± 0.13 SD), and between groups (mean= 0.07± 0.09 SD) indicating that whilst there is some shared taxa, the composition of the samples within the groups and between the groups are unalike. These results showed a significant difference between the NTM postive and negative groups (Bray-Curtis based ANOSIM: R = 0.16, p = 0.0001) which remained significant after applying a Bonferroni correction for multiple comparisons (p = 0.0003).

The same analysis was also conducted on the data with all the NTM taxa removed (Figure 5.5) to assess whether their presence is the contributing factor of the similarity. The Bray-Curtis similarity (Figure 5.5A), showed a slighlty higher degree of similarity within samples and between groups but remain distinct even without the presence of the NTMs. The NTM-positive group (mean= 0.085±0.132 SD), Negative group (mean= 0.084, ±0.133 SD), and between groups (mean= 0.077±0.13 SD).

The Sørensen (Figure 5.5B) similarity-based analysis contiuned the trend set by the Bray-Curtis analysis where there is slightly more similarity now the NTM taxa have been removed , however, there is only marginal similarity. The NTM-positive group (mean= 0.109±0.121 SD), Negative group (mean= 0.112±0.147 SD) and the between groups (mean= 0.086±0.113 SD). The results were significant (Bray-Curtis based ANOSIM: p= 0.0268, R = 0.0667) and remained significant after applying a Bonferroni correction for multiple comparisons (p=0.0264). The remaining significance indicates that the NTM

contribute to the differences in the microbiota but may not be the only contributing factor.

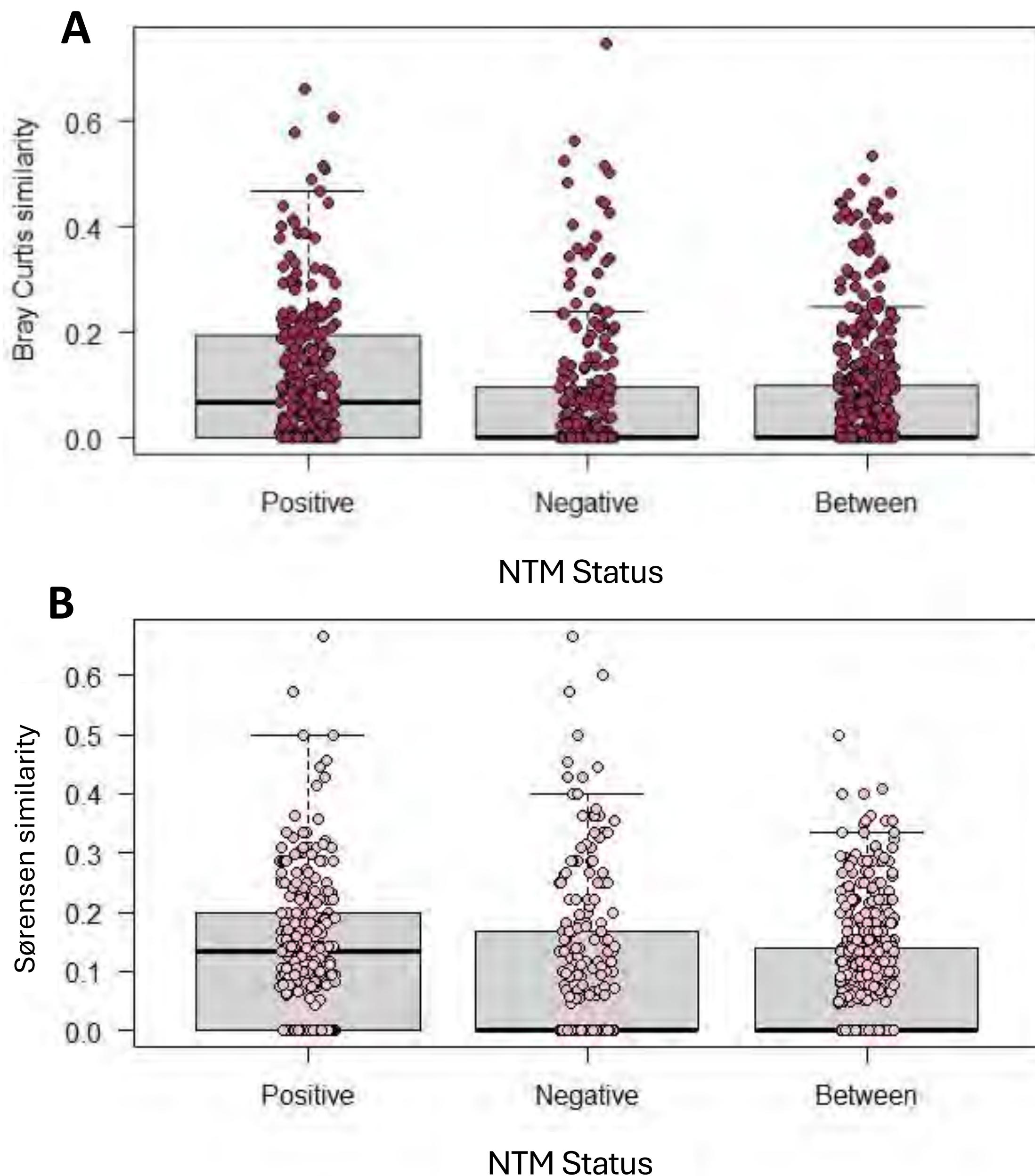


Figure 5.4 Similarity Indices of the NTM positive, negative and between groups examining the whole microbiota via 16S rRNA gene sequencing. The Bray-Curtis measure of similarity (**A**) and Sørensen similarity (**B**) both show a very small ammount of similarity between groups These results are statistically significant (Bray-Curtis based ANOSIM: R = 0.16, p = 0.0001), Bonferroni correction for multiple comparisons (p = 0.0003).

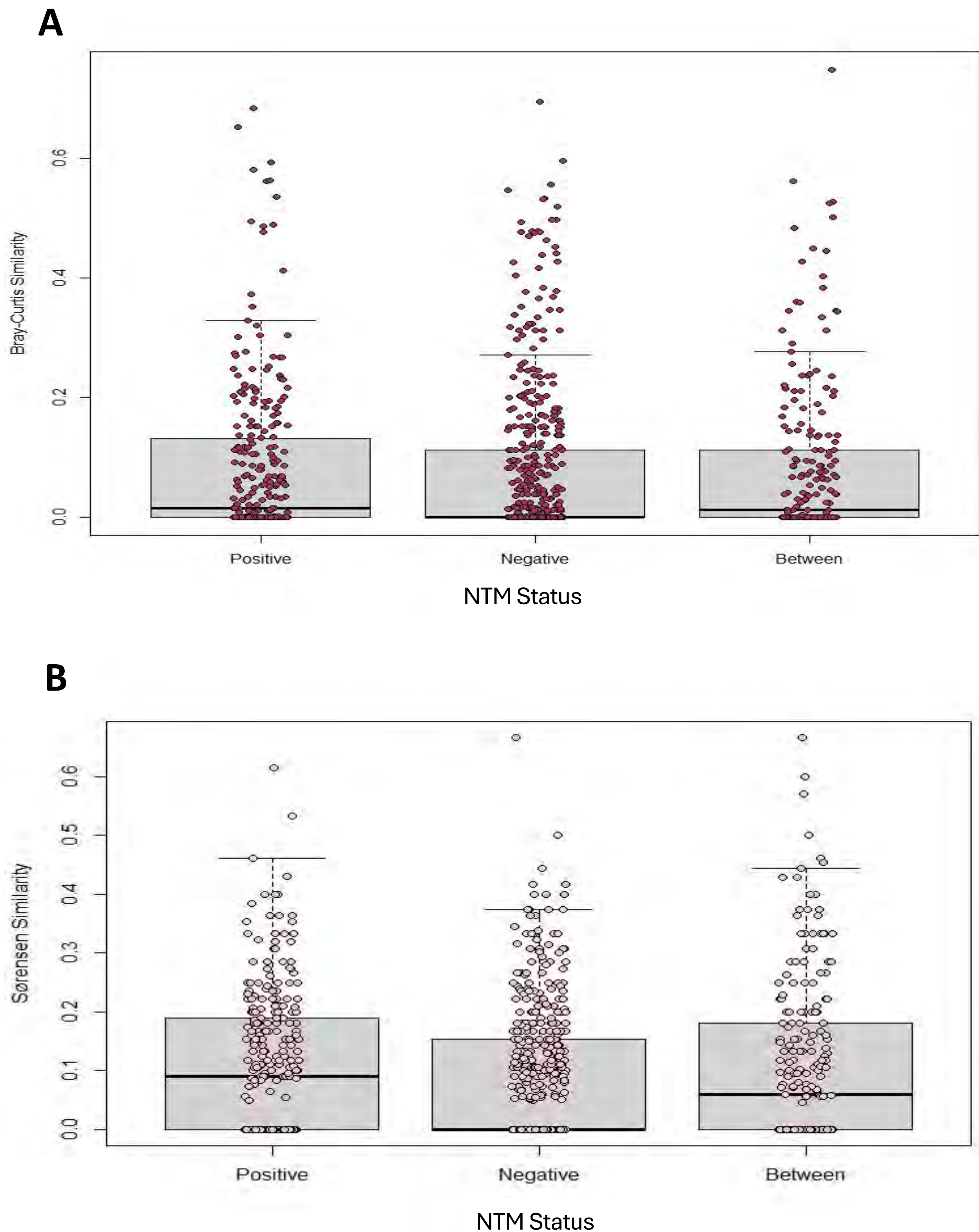
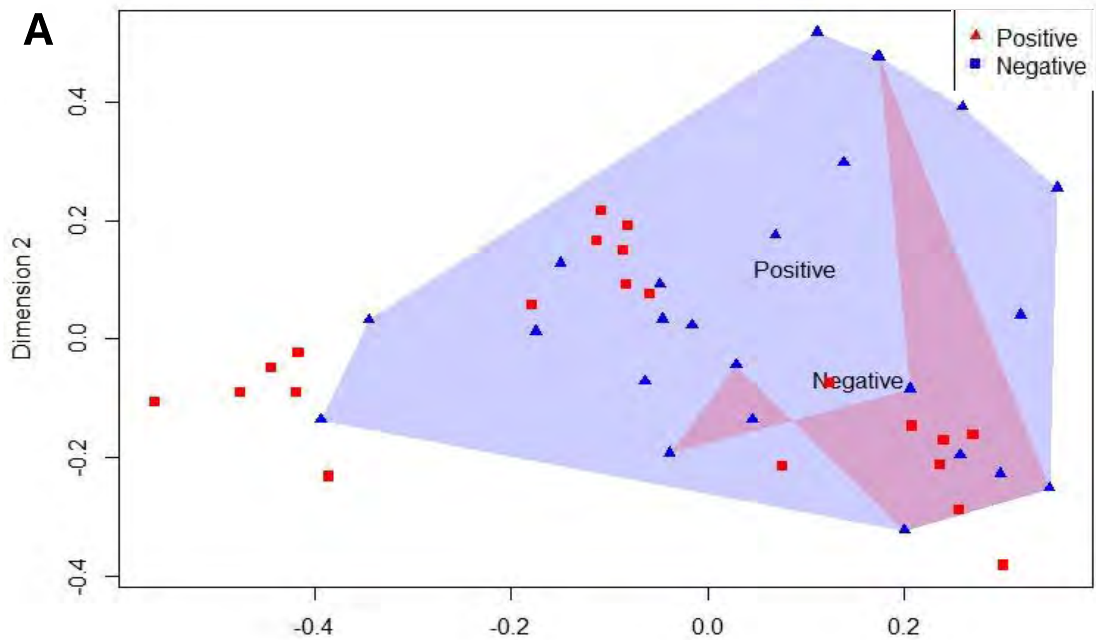


Figure 5.5 Similarity Indices of the NTM positive, negative and between groups examining the whole microbiota via 16S rRNA gene sequencing. The Bray-Curtis measure of similarity (A) and Sørensen similarity (B) both show a very small amount of similarity between groups These results are statistically significant (Bray-Curtis based ANOSIM: p= 0.0268, R = 0.0667), Bonferroni correction for multiple comparisons (p=0.0264).

5.5.2.3 Metric Multidimensional Scaling

To illustrate the patterns of similarities between the groups an MDS plot was created (Figure 5.6). The plot including the NTM taxa (Figure 5.6A), does show some similarities between the groups, like the similarity analysis (Section 5.5.2.2). But most of the samples are distinct from each other. The statistical significance was assessed using PERMANOVA. The test showed statistical significance between the groups (p=0.001, R^2= 0.6667, Df= 29.33).

The second plot (Figure 5.6B) shows the distribution of the groups with the NTM taxa removed. It shows a degree of similarity between the groups than with the NTM included, which is consistent with the similarity analysis done previously. The PERMANOVA revealed non-significance between the groups (p= 0.076, R^2= 0.6667, Df= 29.33), which indicates that the NTM are a contributing factor between the groups.



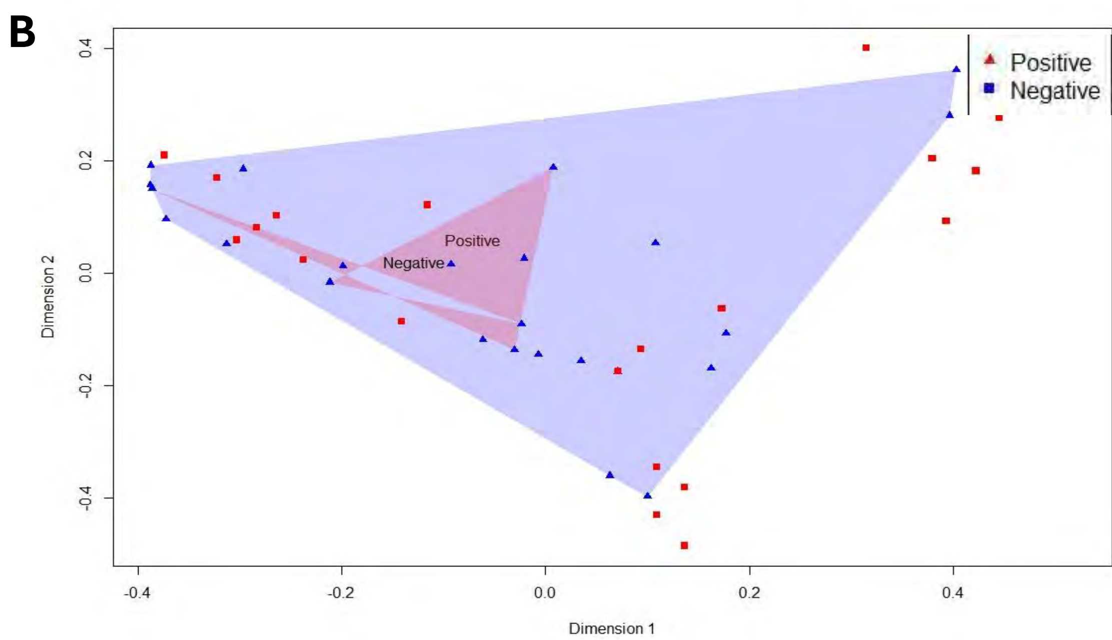


Figure 5.6 MDS plot showing the similarities between the samples. The red triangles represent the positive group, and the blue squares the negative group. The shaded area represents areas of predominance in each category. The closer together the more similar the samples are. (A) Is MDS plotted with the NTM taxa included. (B) Is the MDS analysis plotted with the NTM taxa omitted.

5.5.2.4 Total Microbiota SIMPER and Rank Abundance

To identify the main drivers of dissimilarity between the NTM positive and negative groups, a Similarity Percentage (SIMPER) analysis (focuses on species contributing to the similarity between samples) (Table 5.3/Figure 5.7) was conducted. It shows the main drivers of dissimilarity between the two groups are *M. avium (*16.19%), *Streptococcus salivarius (*7.05%), and *Staphylococcus aureus (8.44*%). The plotted relative abundance (Figure 5.7A), further highlights this hierarchy but, it includes a wider range of taxa.

The rank-abundance (the relative abundance of different species in a community) curves (Figure 5.8) further demonstrated that *M. avium,* is the most abundant species in the Positive group (Figure 5.8 A) followed by *S. aureus* and *S. salivarius* while *S. aureus* and *S. salivarius* are the most abundant in the NTM negative group (Figure 5.8B). Which is synonymous with the SIMPER analyses (Table 5.3) and the relative abundance plot (Figure 5.7A)

The analysis with the NTM taxa removed (Table 5.4/ Figure 5.74B) there was a shift in the order of abundance in the SIMPER analysis. It showed the main drivers of dissimilarity between the two groups are *Staphylococcus aureus (9.25*%). *Streptococcus salivarius (*8.86%), and *Streptococcus symci* (5.63%). Notably the canonical CF pathogens *S. aureus, P. aeruginosa and B. multivorans* are ranked reasonably lower in the relative abundance of the positive group when compared to the negative group.

Table 5. 3 Similarity Percentage Analysis (SIMPER) 16S rRNA gene sequencing of NTM Groups with NTM species Included

Taxon	Contrib. %	Cumulative %	Mean relative abundance	
			Positive	Negative
Mycobacterium avium	7.746	8.444	14.5	0.0379
Streptococcus salivarius	7.059	16.19	8.31	9.53
Mycobacterium abscessus	3.343	23.25	5.99	0.49
Streptococcus symci	4.367	27.62	5.85	3.52
Veillonella nakazawae	2.873	34.49	4.78	1.42
Staphylococcus aureus	8.444	37.84	4.72	13.6
Streptococcus toyakuensis	3.374	44.23	4.69	2.13
Mycobacterium chelonae	1.998	47.1	3.74	0
Mycobacterium asiaticum	1.886	53.89	3.53	0
Staphylococcus succinus	3.056	55.78	2.8	3.36
Mycobacterium kansasii	1.199	70.97	2.24	0

Bold text denotes species of interest regarding NTM and CF pathogens. Also given is within category mean percent abundance for taxa, Percentage contribution of each taxon to the average dissimilarity between the two groups being compared. Taxa with higher values in this column contribute more to the overall dissimilarity between the groups. Percentage cumulative contribution of each taxon to the average dissimilarity between the two groups. The taxa are typically listed in descending order of their contribution. The list of species is not exhaustive, so cumulative percent does not sum to 100%. Given the ribosomal sequences analysed, species identities should be considered putative. (Full analysis: Supplementary Materials 11.7)

Table 5. 4 Similarity Percentage Analysis (SIMPER) 16S rRNA gene Sequencing of NTM Groups with NTM species excluded

Taxon	Contrib. %	Cumulative %	Mean relative abundance	
			Positive	Negative
Streptococcus salivarius	8.868	9.295	12.1	9.77
Streptococcus symci	5.631	18.16	8.28	3.53
Veillonella nakazawae	4.469	23.79	7.76	1.42
Streptococcus toyakuensis	4.522	29.37	6.86	2.13
Staphylococcus aureus	9.295	33.89	6.56	13.6
Pseudomonas aeruginosa	5.574	38.36	5.2	6.1
Streptococcus pseudopneumoniae	3.327	41.87	4.77	1.79
Variovorax guangxiensis	2.257	48.58	4.17	0
Staphylococcus succinus	3.509	51.55	3.61	3.36
Burkholderia multivorans	2.969	56.27	2.98	2.87

Bold text denotes species of interest regarding NTM and CF pathogens. Also given is within category mean percent abundance for taxa, Percentage contribution of each taxon to the average dissimilarity between the two groups being compared. Taxa with higher values in this column contribute more to the overall dissimilarity between the groups. Percentage cumulative contribution of each taxon to the average dissimilarity between the two groups. The taxa are typically listed in descending order of their contribution. The list of species is not exhaustive, so cumulative percent does not sum to 100%. Given the ribosomal sequences analysed, species identities should be considered putative. (Full analysis: Supplementary Materials 11.8)

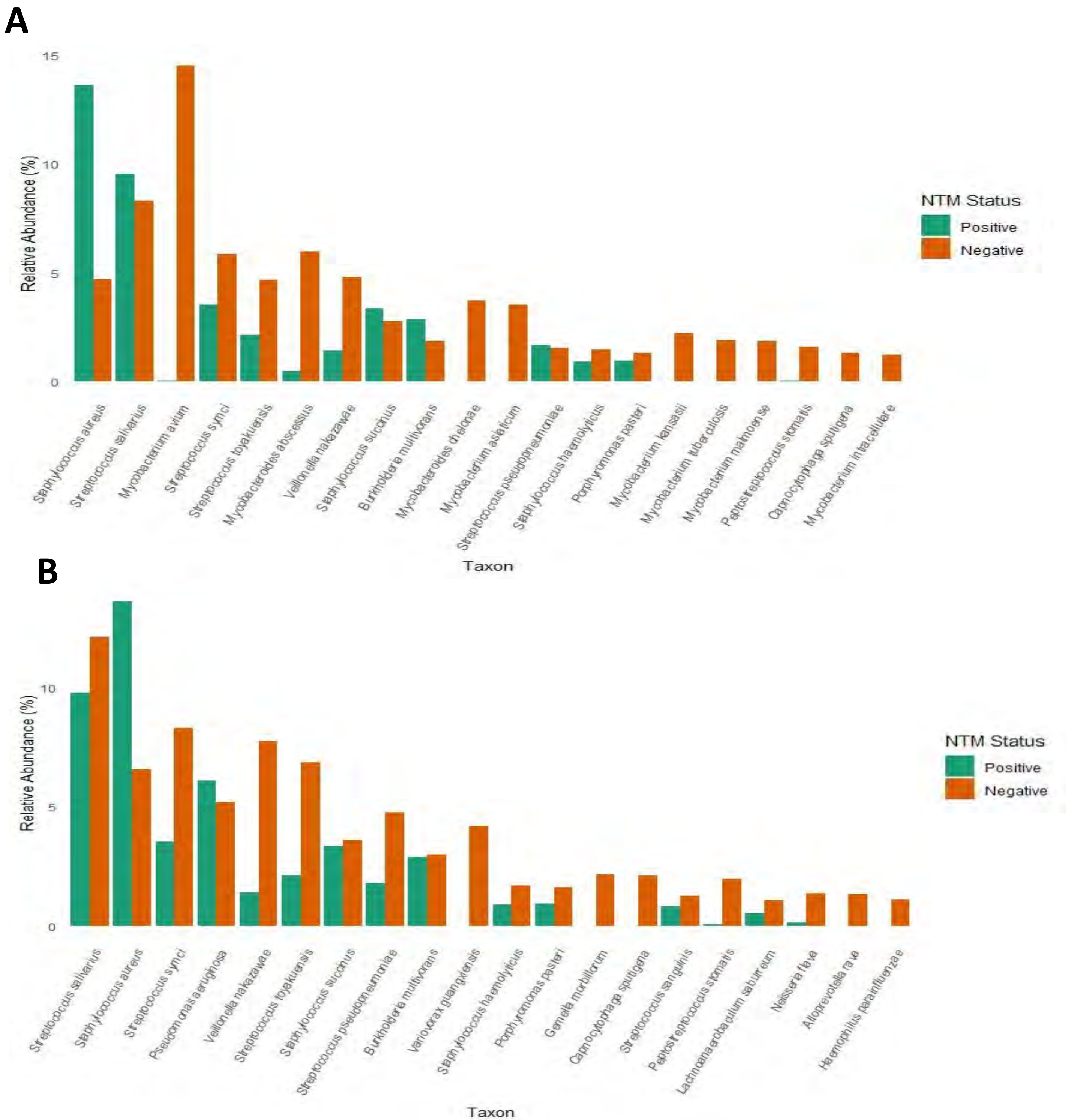


Figure 5.7 The relative abundance as calculated by the SIMPER analysis (Table 5.3/5.4) Shows the mean relative abundance for each species detected within the NTM positive and NTM negative groups. (**A**) The mean relative abundance of the 16S rRNA sequencing analysis with NTM taxa included. (**B**) The mean relative abundance of the 16S rRNA sequencing without the NTM taxa included. Given the length of the ribosomal sequences analysed, species identities should be considered putative.

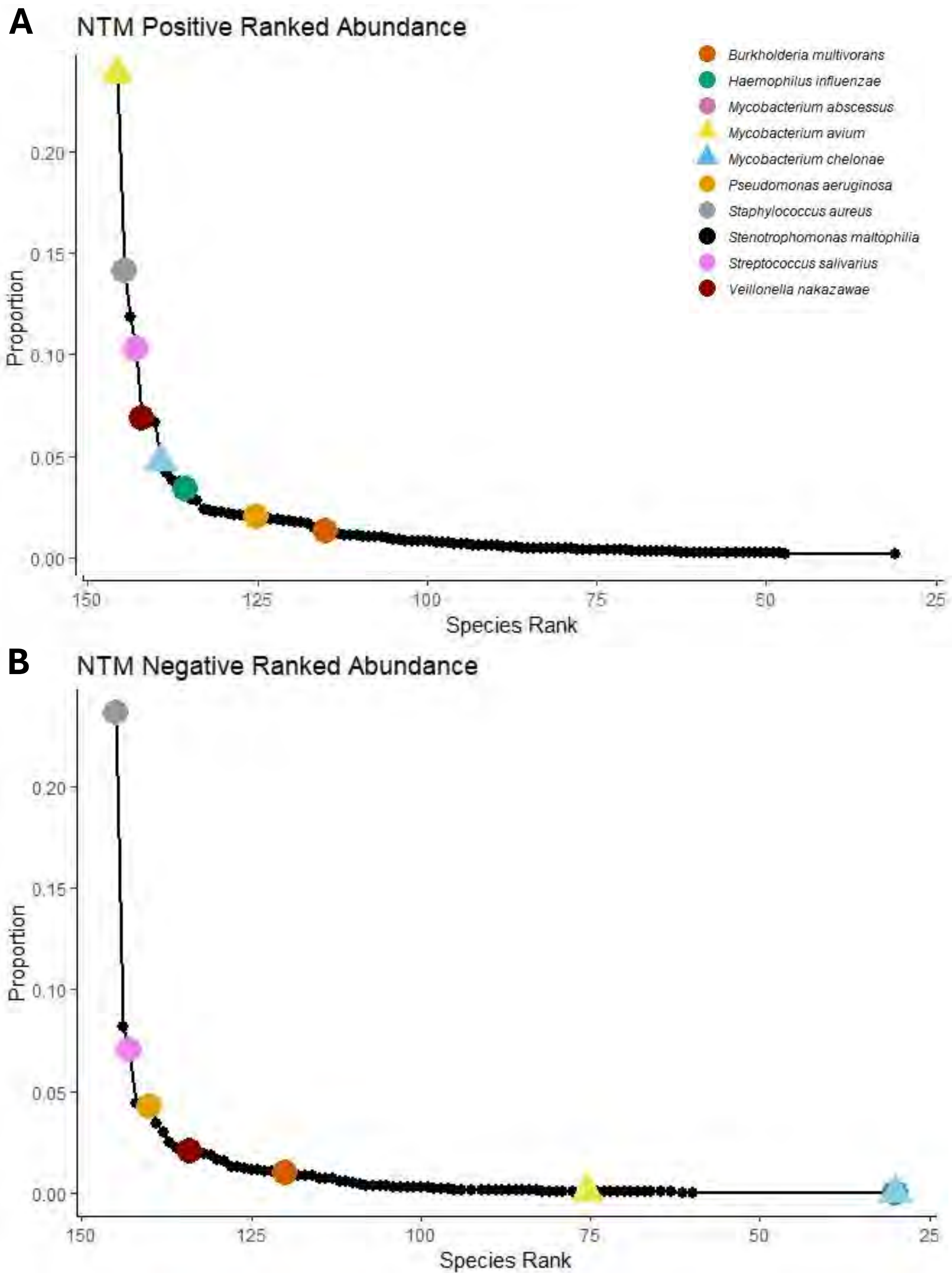


Figure 5.8 Rank-abundance curves. Only taxa and NTM species of interest are highlighted by coloured shapes which are plotted in terms of hierarchy abundance within the NTM status group. NTM positive (**A**) shows MAC second followed by *S. aureus* as the highest ranked in abundance and NTM negative groups (**B**) Shows *S. aureus* and *S. salivarius* are the most abundant.

5.5.3 NTM Type and Microbiota.

The following section examines the data generated by the 16S rRNA gene sequencing focusing on the microbiota composition in patients undergoing CFTR modulator therapy and then spilt into MAC (*n* = 12), MABSC (*n* = 6), and other NTM type (*n* = 4) positive groups.

5.5.3.1 NTM Type Microbiota Diversity and Composition

Whilst the MAC (*n* = 12) had a higher taxa (OTU) richness (mean=12.08±9.48) than the MABSC group (*n* = 6, mean= 8.57±5.47 SD) and Other NTM group (*n*= 4, mean= 10.20±5.0 SD) These results showed no statistical significance (Kruskal-Wallis test: H= 0.63, p =0.72) (Figure 5.9A). Like the previous section the Fishers' alpha diversity reflected the taxa richness and showed that the MAC group (mean=1.47±1.09 SD) had a higher range of diversity than the MABSC group, (mean=1.09, ±0.71 SD) and Other NTM group (mean=1.23±0.58 SD). These results showed no statistical significance (Kruskal-Wallis test: H= 0.48, p= 0.78) (Figure 5.9B). Finally, the Berger-Parker index of dominance showed that the MAC group (mean=0.43±0.17 SD), the MABSC (mean=0.42±0.23 SD) and Other NTM group (mean=0.34±0.09 SD) have dominant taxa but, again the results showed no statistical significance (Kruskal-Wallis test: H= 1.34, p =0.51) (Figure 5.9C). These results indicate that whilst there were some differences between the groups, the type of NTM does not affect the alpha diversity of the samples.

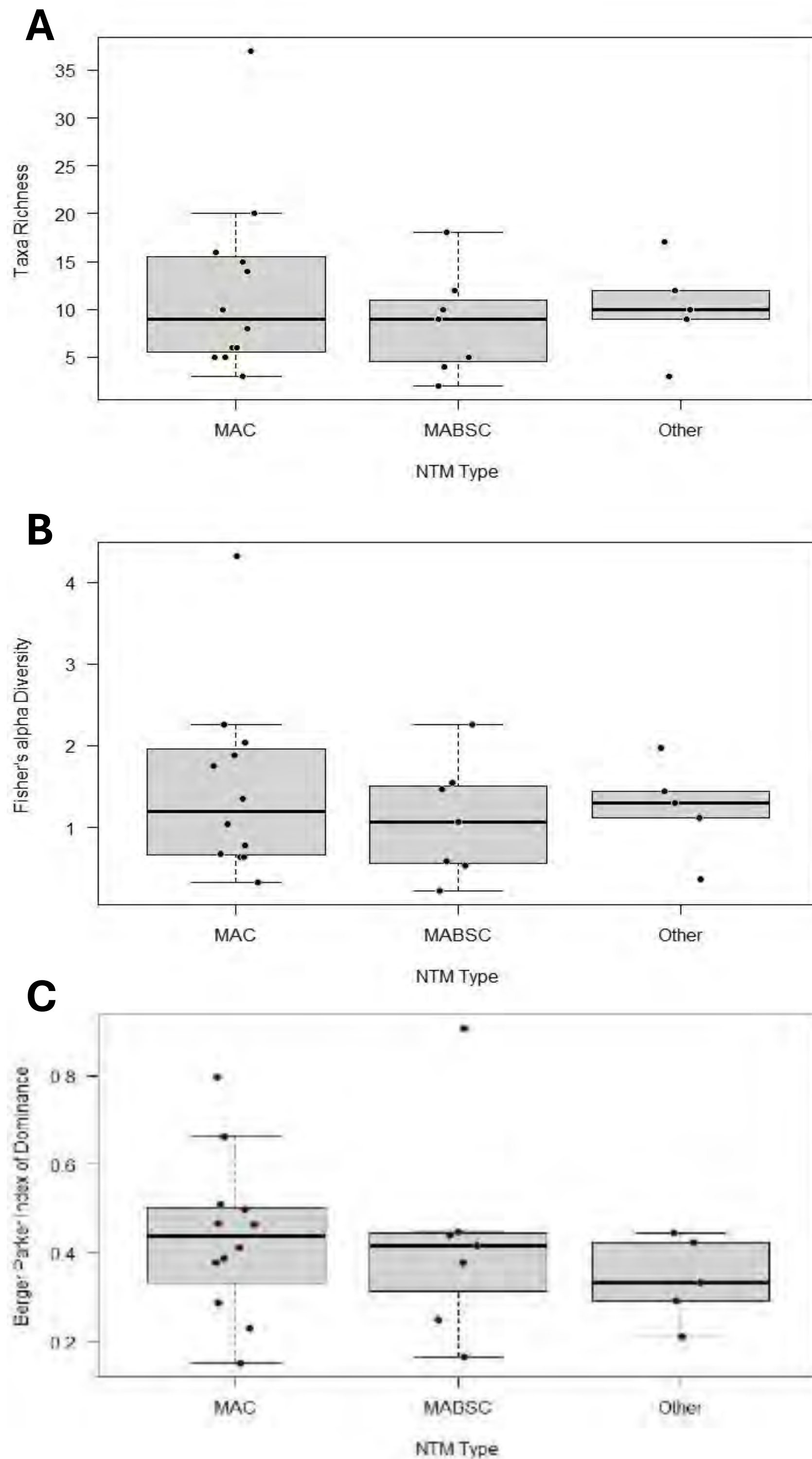


Figure 5.9 16S rRNA Microbiota diversity and composition of samples NTM positive for MAC – *M. avium* complex, MABSC -*M. abscessus* complex, Other – other NTM present. From patients on modulator therapy. Taxa richness (**A**) MAC group (*n* = 12) MABSC group (*n* = 6), and Other NTM group (*n*= 5). Fishers' Alpha diversity (**B**) showed that the MAC group had a higher range of diversity than the MABSC group and Other NTM group. The Berger-Parker index of dominance(**C**) showed that the MAC group, the MABSC and Other NTM group all had dominant taxa.

5.5.3.2 NTM Type Similarity

To determine the change in microbiota between the NTM types (MAC, MABSC and Other NTM) the Bray-Curtis (abundance) and Sørensen (absence/presence) indices of similarity were used (Figure 5.10/Table 5.5) and statistical significance was measured using Bray-Curtis based ANOSIM with Bonferroni correction.

The Bray- Curtis similarity (Figure 5.10A/Table 5.5A) between the MAC group, MABSC and Other NTM groups shows there is very low similarity between the groups. The Sørensen analysis (Figure 5.10B/Table 5.5A) in the NTM type data also shows little similarity in the groups, indicating a distinct community within the groups. These results showed significant difference (Bray-Curtis based ANOSIM: $R = 0.19$, $p=0.001$) which remained significant in all groups after applying a Bonferroni correction for multiple comparisons (Table 5.5A).

To understand the role of the NTM taxa in these differences the same analysis was conducted without the NTM taxa (Figure 5.11/Table 5.5B). The analysis showed that without the NTMs the groups became more similar. However, the statistical tests revealed all the groups tested are non-significant with and without the applied Bonferroni correction, when compared to the analysis with the NTMs included (Figure 5.11/Table 5.5A). This was the case in both the Bray- Curtis similarity (Figure 4.11A), and the Sørensen index of similarity anlaysis (Figure 4.11B). This indicates that the NTM taxa are a key feature of the differences between the groups.

Table 5. 5 Statistical Analysis for Similarity Indices of NTM Groups with and without NTM taxa.

A

NTM type [a]	Bray-Curtis mean	Sørensen mean	Bonferroni p value
MAC	0.23±0.15	0.21±0.12	
MAC vs MABSC	0.08±0.11	0.10±0.10	0.0005
MAC VS other	0.08±0.09	0.09±0.08	0.0019
MABSC	0.10±0.10	0.19±0.16	
MABSC vs other	0.08±0.10	0.11±0.10	0.0234
Other	0.07±0.08	0.11±0.09	

B

NTM type [a]	Bray-Curtis mean	Sørensen mean	Bonferroni p value
MAC	0.113±0.144	0.149±0.131	
MAC vs MABSC	0.097±0.147	0.112±0.124	0.561
MAC VS other	0.076±0.131	0.082±0.103	0.253
MABSC	0.056±0.076	0.118±0.125	
MABSC vs other	0.051±0.094	0.077±0.105	0.726
Other	0.039±0.069	0.064±0.090	

The statistical analysis for the similarity indices with the NTM taxa included (**A**). Table (**B**) is the statistical analysis for the similarity indices without the NTM Taxa included. Mean, ± 1 standard deviation throughout. [a] NTM type- Samples culture positive for MAC (*n* = 12), MABSC (*n* = 6), Other NTM type (*n* = 4) Statistical significance was measured using Bray-Curtis based ANOSIM with Bonferroni correction. A similarity of 1 indicates groups are identical whereas, a similarity of 0 signifies no matching species.

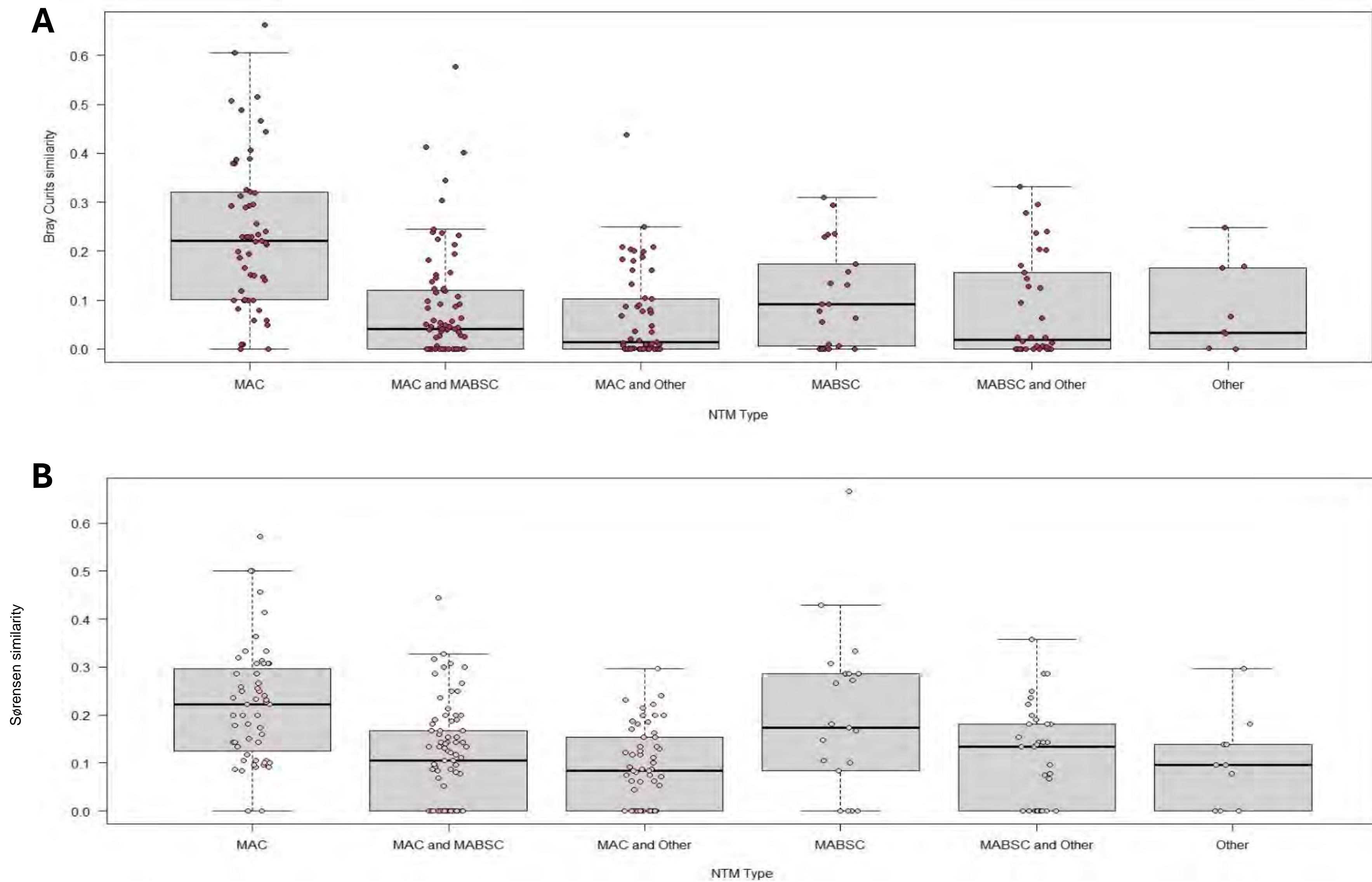


Figure 5.10 Similarity indices for the NTM type data with the NTM taxa included. Shows the distribution of the samples in terms of similarity, with 0 indicating no similar species are present and 1 indicating identical groups (**A)** The Bray Curtis indices of similarity (**B**) The Sørensen index of similarity. The corresponding statistical analysis Table 5.5.

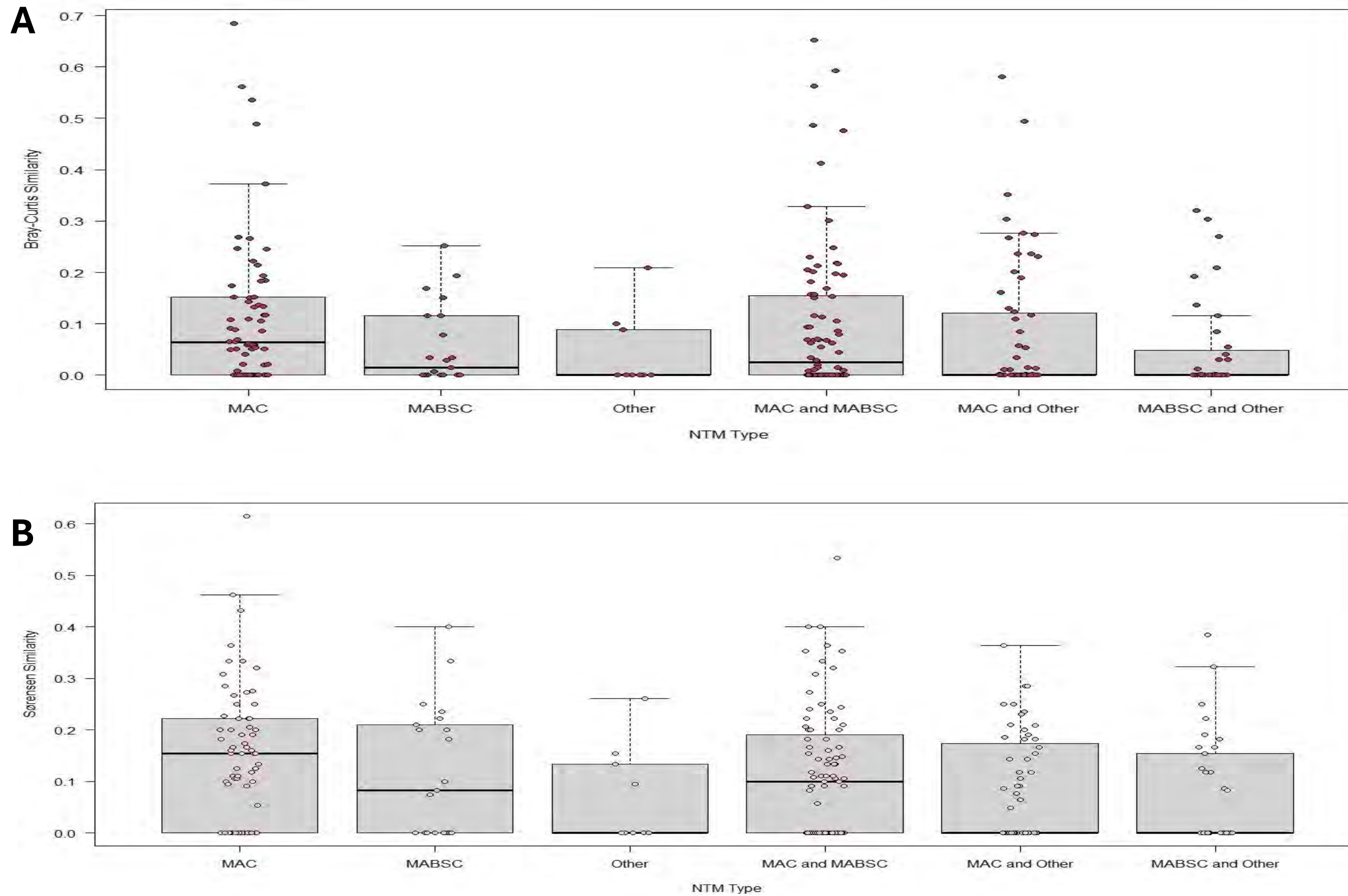


Figure 5.11 Similarity indices for the NTM type data with the NTM taxa omitted. Shows the distribution of the samples in terms of similarity, with 0 indicating no similar species are present and 1 indicating identical groups (**A)** The Bray Curtis indices of similarity (**B**) The Sørensen index of similarity. The corresponding statistical analysis Table 5.5.

5.5.3.3 Metric Multidimensional Scaling

To illustrate the patterns of similarities between the groups an MDS plot was created (Figure 5.12). The plot including the NTM taxa (Figure 5.12A), showed some similarities between the groups which keeps in trend with the similarity indices (Section 5.5.3.3), but most of the samples are distinct from each other. The statistical significance was assessed using PERMANOVA. The test showed statistical significance between the groups (p=0.001, R^2= 0.6667, Df= 29.33).

The second plot (Figure 5.12B) shows the distribution of the groups with the NTM taxa removed. It shows some degree of similarity between the groups, which is consistent with the similarity analysis done previously. The PERMANOVA revealed non significance between the groups (p= 0.26, R^2= 0.6667, Df= 29.33), which keeps on trend with the similarity indices which indicates that the NTM are a driving the differences within the groups.

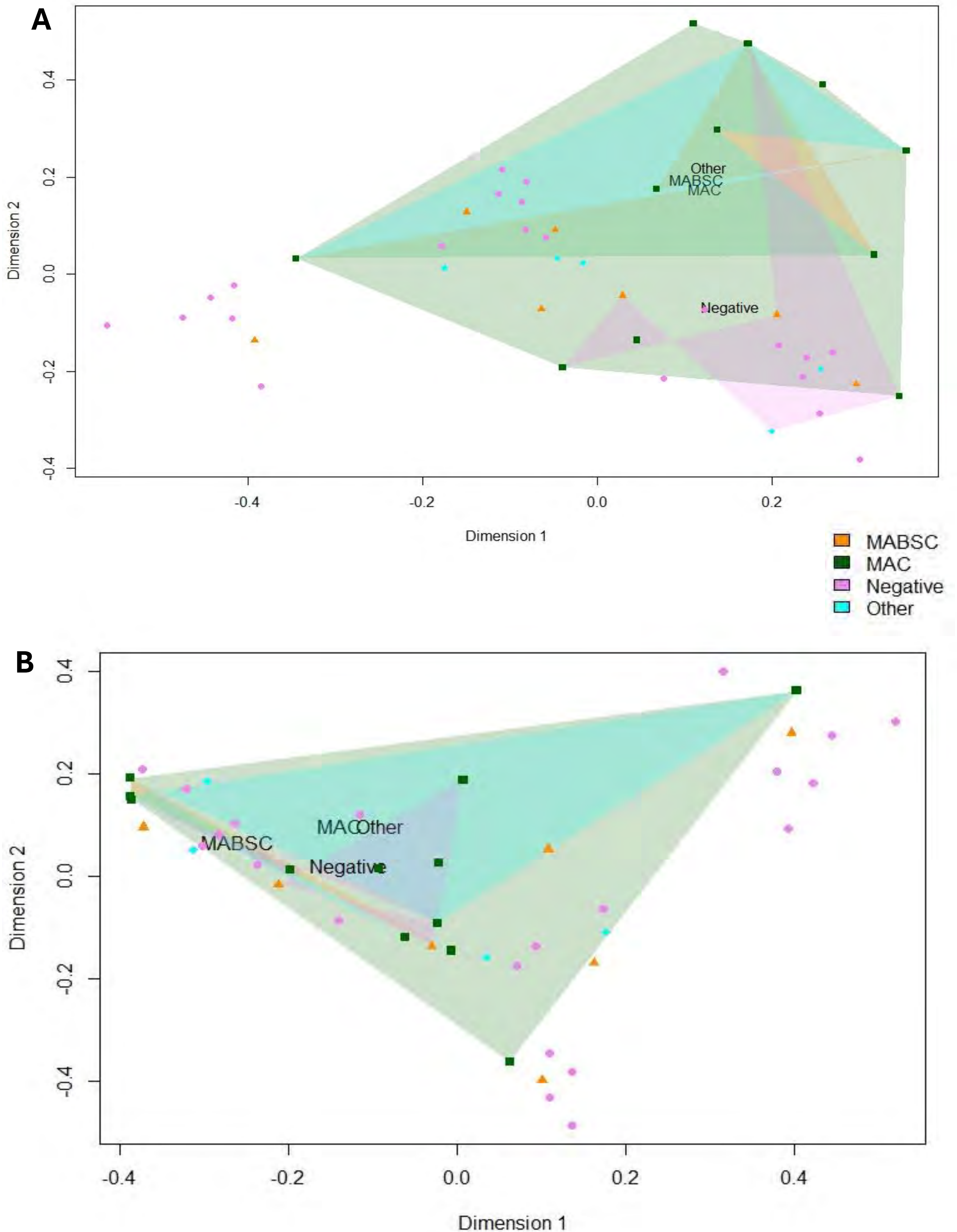


Figure 5.12 MDS plot showing the similarities between the samples. The coloured points represent the different NTM type groups. The shaded area represents areas of predominance in each category. The closer together the more similar the samples are. (**A**) Is MDS plotted with the NTM taxa included. (**B**) Is the MDS analysis plotted with the NTM taxa omitted.

5.5.3.4 NTM Type Based SIMPER and Abundance Analysis

A Similarity Percentage (SIMPER) analysis (Table 5.6/Figure 5.13A) was conducted to establish the main contributors of dissimilarity between the groups. It showed *M. avium* (16.19%), *S. aureus* (7.37%) and *S. salivarius (*6.985%) as the top three contributors. The taxa with the highest relative abundance in the MAC group is *M. avium (29%)*, followed by *S. salivarius (9.38%)* and *Streptococcus symci (8.95%)*. The first CF taxa of interest is *S. aureus* (5.09%), then *M. kansasii* (3.76%), with *P. aeruginosa* only having a relative abundance of 1.23% in this group. The Rank-abundance curve for the MAC group (Figure 5.14A) shows a slightly different picture with *M. avium* and *S. salivarius* as the highest in abundance followed by *Veillonella nakazawae* and *S. aureus* but again *P. aeruginosa* is ranked low in terms of abundance.

Conversely the ranked abundance (Figure 5.14B) in the MABSC group showed a different distribution of taxa within the groups. The SIMPER analysis (Table 5.3) showed *M. abscessus* (18.7%), *M. chelonae* (10.7%) and *V. nakazawae* (8.94%) as the three highest taxa in terms of relative abundance with the CF taxa of interest, *S. aureus* (7.45%) and *P. aeruginosa* (1.58%) scoring lower. The Rank-abundance curve (Figure 5.6B) again showing a slightly different order of ranking with *M. abscessus* as the highest, then *S. aureus* and *V. nakazawae* with *P. aeruginosa* ranked lower in comparison.

Finally, the Other NTM groups relative abundance as determined by the SIMPER analysis (Table 5.6) shows *M. asiaticum* (13.5%), *M. malmoense* (8.97%) and *Staphylococcus succinus* (8.49%) as the highest in relative abundance with both *S. aureus* and *P. aeruginosa* being absent from this group. The rank-abundance curve (Figure 5.14C) showed *Neisseria flava* as the highest ranked species followed by; *M. asiaticum* as the highest ranked species of interest followed by *S. salivarius* and *Haemophilus parainfluenaze*. This supports the previous section (Section 5.5.3.2) in suggesting the NTM type and its microbiota is unique.

The analysis omitting the NTM taxa (Table 5.7/Figure 13.B) showed that the CF pathogen was *S. aureus* (7.9%) in the MAC groups which was ranked below three other *Streptococcus* species. The MABSC group showed a different picture with *P. aeruginosa* (14.6%), second to *Veillonella nakazawae*(16.1%) and the Other NTM group showing very low levels of CF pathogens with *Haemophilus parainfluenaze* (3.87%) appearing quite low in terms of relative abundance. This further enhances the information provided by the

similarity and MDS plots which have revealed that the groups are quite distinct from each other with some similarities which are mostly being influenced by the presence of the NTMs.

Table 5. 6 Similarity Percentage Analysis (SIMPER) 16S rRNA gene Sequencing of NTM Types with NTM species Included

Taxon	Contrib. %	Cumulative %	Mean relative abundance		
			MAC	MABSC	Other
Mycobacterium avium	8.995	8.995	29	0	0
Staphylococcus aureus	7.37	16.37	5.09	7.45	0
Streptococcus salivarius	6.985	23.35	9.38	7.42	6.98
Streptococcus symci	4.593	27.94	8.95	0	6.63
Mycobacterium abscessus	4.288	32.23	0.303	18.7	1.9
Streptococcus toyakuensis	3.599	35.83	4.93	7.62	0
Veillonella nakazawae	3.236	39.07	2.91	8.94	3.43
Staphylococcus succinus	3.183	42.25	0	3.55	8.49
Pseudomonas aeruginosa	2.85	45.1	1.23	1.58	0
Mycobacterium chelonae	2.626	47.73	0	10.7	2.99
Mycobacterium asiaticum	2.498	50.22	1.43	0	13.5

SIMPER analysis showing the first 10 taxa. Bold text denotes species of interest regarding NTM and CF pathogens. Also given is within category mean percent abundance for taxa, Percentage contribution of each taxon to the average dissimilarity between the two groups being compared. Taxa with higher values in this column contribute more to the overall dissimilarity between the groups. Percentage cumulative contribution of each taxon to the average dissimilarity between the two groups. The taxa are typically listed in descending order of their contribution. The list of species is not exhaustive, so cumulative percent does not sum to 100%. Given the length of the ribosomal sequences analysed, species identities should be considered putative. (Full analysis Supplementary materials 11.9)

Table 5. 7 Similarity Percentage Analysis (SIMPER) 16S rRNA gene Sequencing of NTM Types with NTM species Omitted

Taxon	**Contrib. %**	**Cumulative %**	**Mean relative abundance**		
			MAC	MABSC	Other
Streptococcus salivarius	9.726	9.726	15.2	9.01	8.9
Streptococcus symci	7.466	17.19	12.4	0	9.88
Veillonella nakazawae	7.325	24.52	4.52	16.1	3.88
Streptococcus toyakuensis	6.153	30.67	7.86	10.1	0
Staphylococcus aureus	**6.013**	**36.68**	**7.9**	**8.96**	**0**
Pseudomonas aeruginosa	**5.947**	**42.63**	**1.88**	**14.6**	**0**
Variovorax guangxiensis	5.778	48.41	0	0	20
Staphylococcus succinus	4.636	53.05	0	4.35	11.2
Streptococcus pseudopneumoniae	4.3	57.35	8	0.786	2.6
Burkholderia multivorans	**2.844**	**60.19**	**3.72**	**3.84**	**0**

SIMPER analysis showing the first 10 taxa. Bold text denotes species of interest regarding NTM and CF pathogens. Also given is within category mean percent abundance for taxa, Percentage contribution of each taxon to the average dissimilarity between the two groups being compared. Taxa with higher values in this column contribute more to the overall dissimilarity between the groups. Percentage cumulative contribution of each taxon to the average dissimilarity between the two groups. The taxa are typically listed in descending order of their contribution. The list of species is not exhaustive, so cumulative percent does not sum to 100%. Given the length of the ribosomal sequences analysed, species identities should be considered putative. (Full analysis Supplementary materials 11.10)

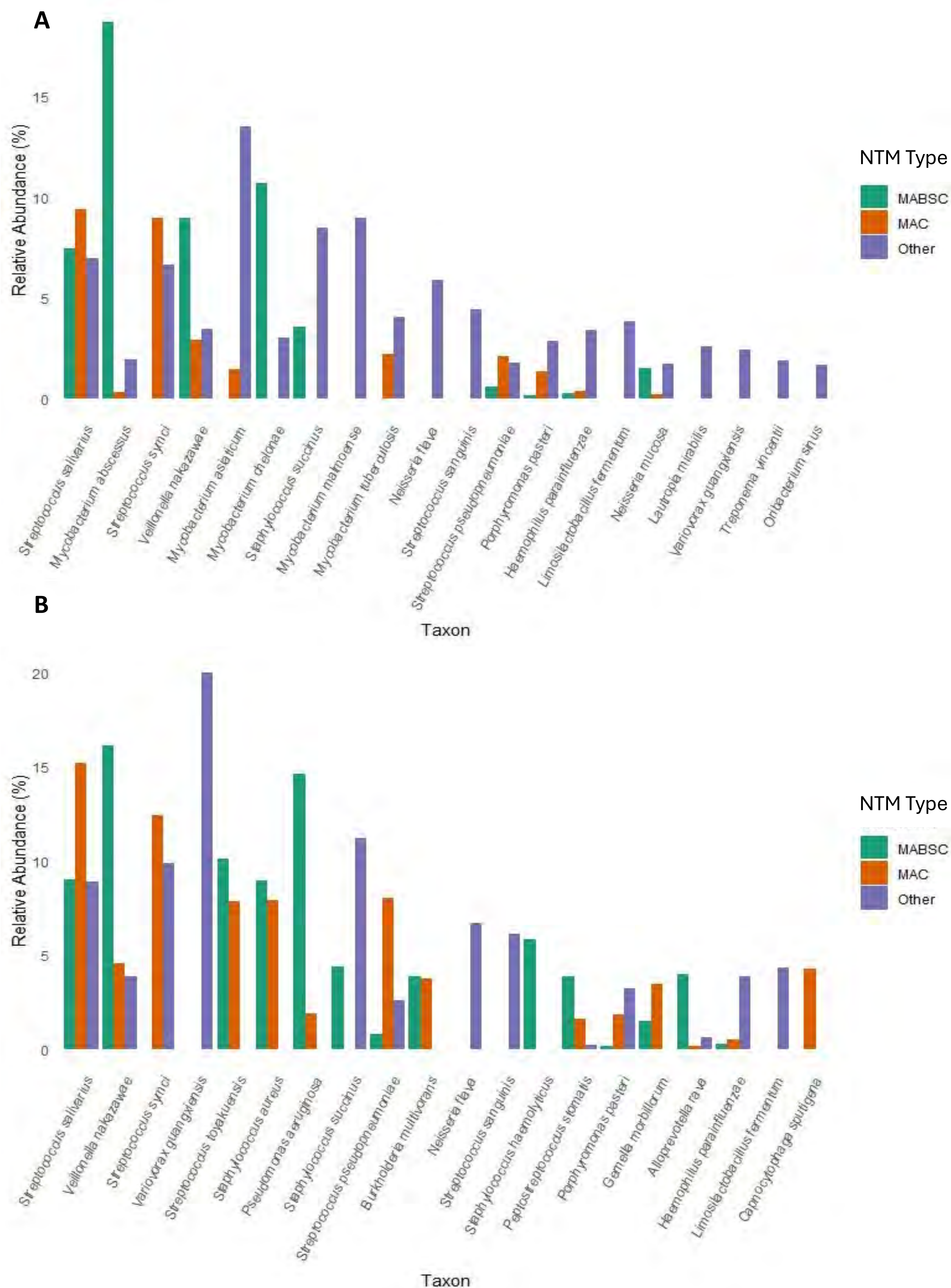


Figure 5.13 The relative abundance as calculated by the SIMPER analysis (Table 5.6/5.7) Shows the mean relative abundance for each species detected within the NTM type group (**A**) The mean relative abundance of the 16S rRNA sequencing analysis with NTM taxa included. (**B**) The mean relative abundance of the 16S rRNA sequencing without the NTM taxa included. Given the length of the ribosomal sequences analysed, species identities should be considered putative.

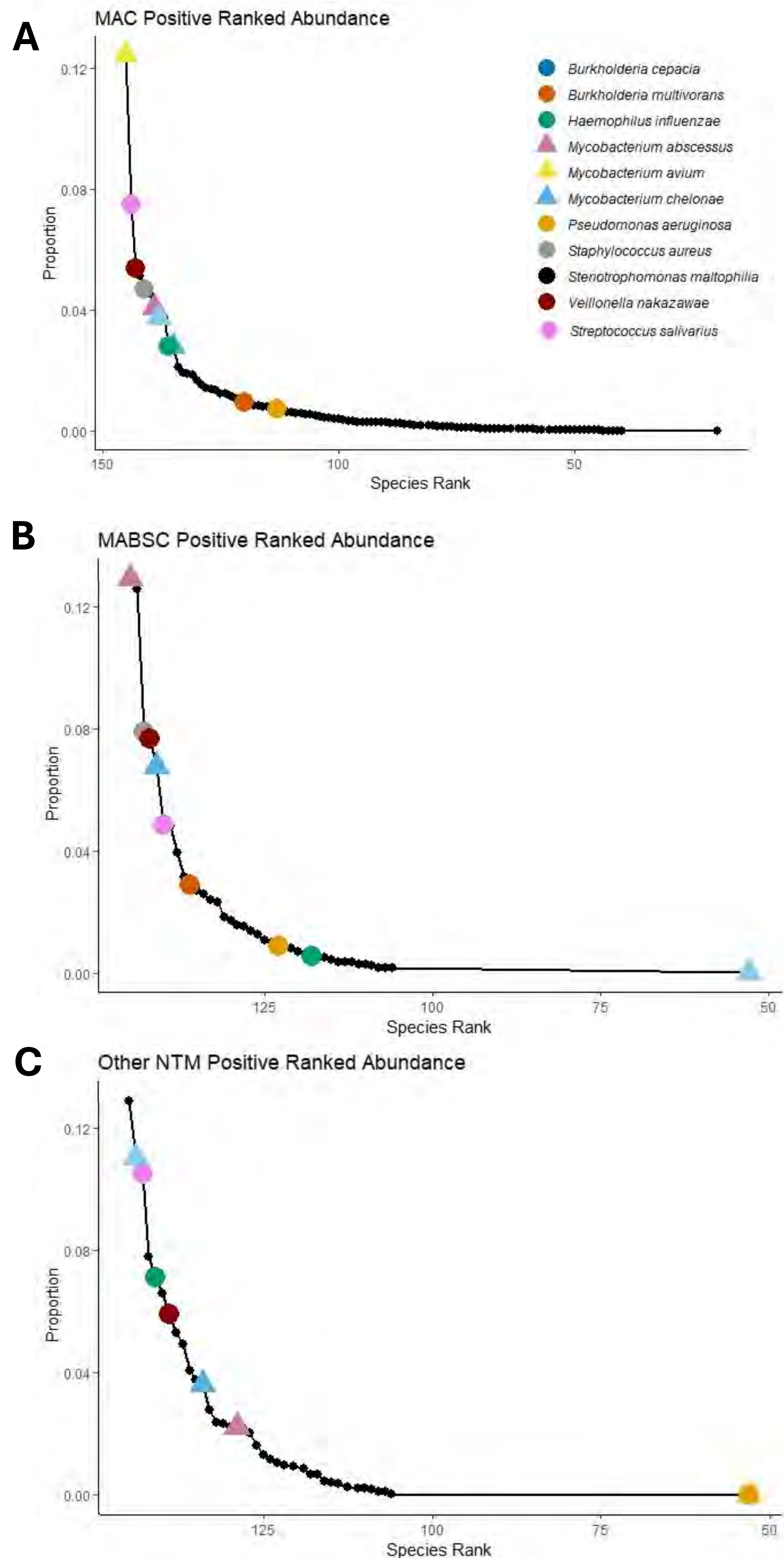


Figure 5.14 Rank-abundance curves. Only taxa and NTM canonical CF pathogens are highlighted by coloured shape which are plotted in terms of hierarchy abundance within the NTM type group. MAC (**A**) shows *M. avium* second followed by *S. salivarus* as the highest ranked in abundance. MABSC (**B**) Shows *M. abscessus, S. aureus* as the highest ranked. Other NTM (**C**) shows *M. chelonae and S. salivarius* are the most abundant.

5.5.4 NTM Complexes

5.5.4.1 Sequencing Analysis

The *rpo*B gene sequencing used in this chapter and chapters 4 and 6, yielded a total of 3998441 mycobacterial raw sequences. The internal filtering and quality control steps performed by DADA2 (Callahan et al., 2016) found 0.63% chimeric sequences and successfully merged 49% of sequences.

In this chapter 45 of the samples (Table 5.1/5.2), were used (mean = 698.91±3290 SD, sequences per sample) (Figure 5.15), were assigned to a taxonomic classification. Reads were distributed unevenly across the samples (minimum of 117 and a maximum of 52945 reads). In total 19 mycobacterial OTUs (total sequences; 371823) were assigned after manual removal of chloroplasts, mitochondrial, environmental contaminants, and any sequences unassigned to a genus.

The rarefaction analysis (Figure 5.16) (n=45) showed that samples had a range of sequencing depth and species abundance. The curves saturate at different points which is indicative of unique species distribution per sample. The sharp rise of the curve that plateaus rapidly suggests a high initial species diversity that drops, implying that a few species dominate and reside with less common species. The shorter the curve length the less sequencing depth of the sample.

The NTM complex richness, diversity, and community composition for samples positive for MAC, MABSC and other NTM acquired from patients on modulator therapy was assessed for each sample by *rpo*B gene sequencing targeting only *Mycobacteria* species and subspecies. Some of the negative samples were sequence positive for NTM and were and grouped with their corresponding complex. MAC group (n = 14) The MABSC group (n = 8) NTM Other group (n= 6) these samples were included in alpha and beta diversity analysis .

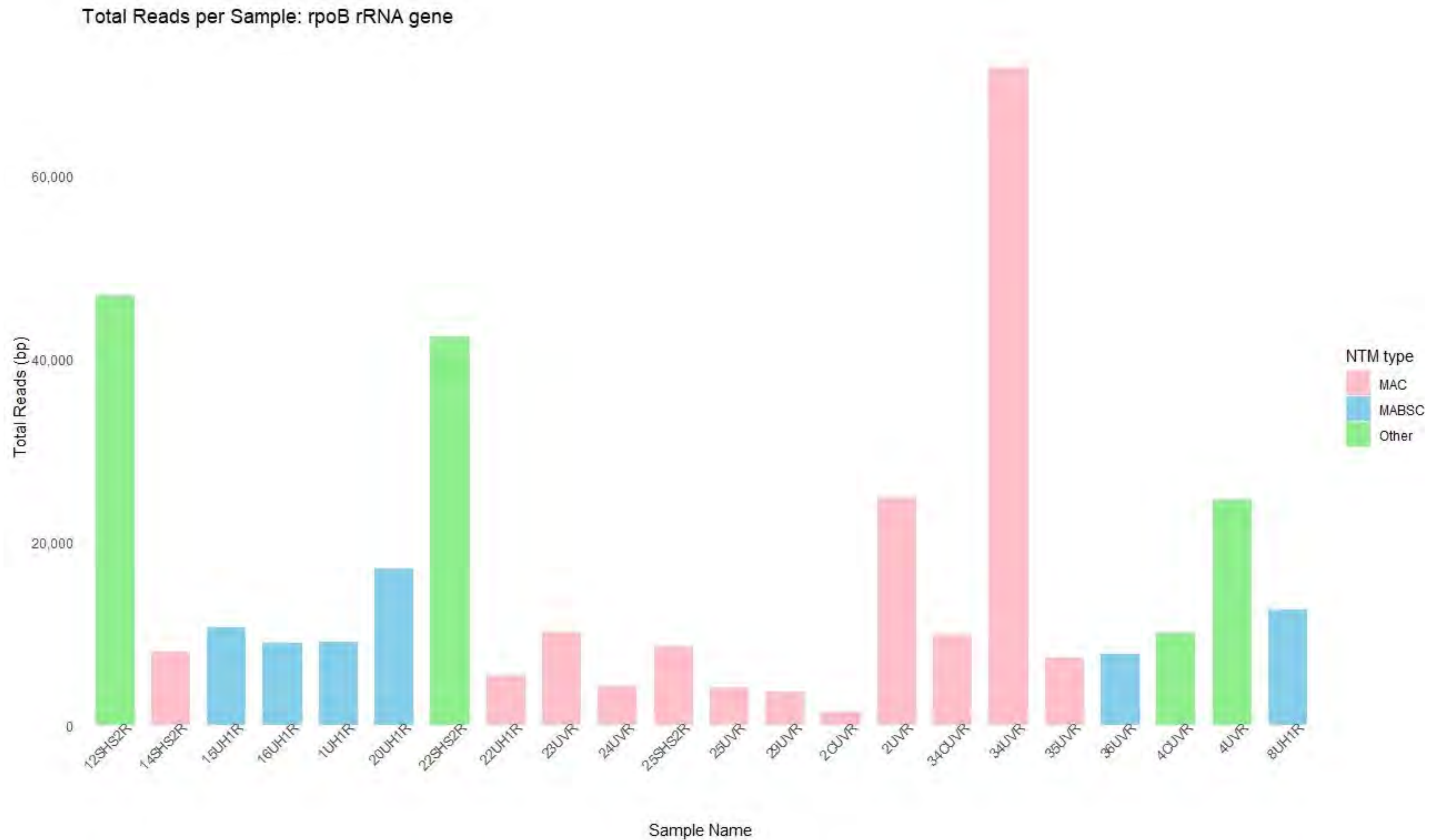


Figure 5.15: *rpo*B gene target total reads used per-sample after built-in DADA2 filtering. Split into NTM types Showed an uneven distribution of reads across the samples in both groups (mean = 698.91±3290, Range; min =117, max=52945) . Sample names on the x- axis are the sample names assigned by the clinic.

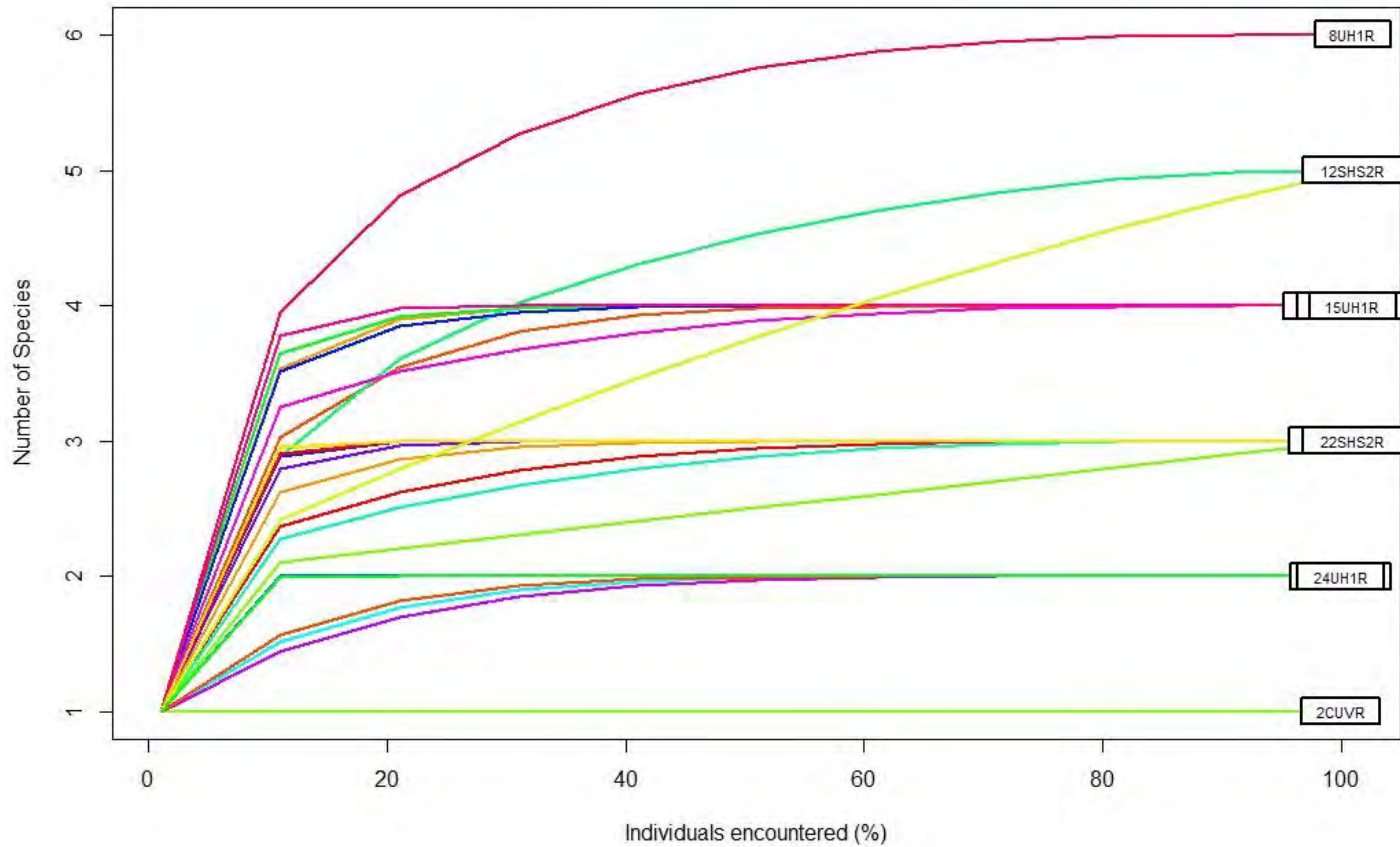


Figure 5.16 : Rarefaction curve of the *rpo*B gene sequencing reads after filtering, quality control and removal of unwanted sequences. Each curve represents a patient sample with the sample ID. The curve shows the percentage of individual species detected corresponding to the number of species in each sample.

5.5.4.2 NTM Diversity and Composition

The MABSC group (*n* =8) had a higher taxa (OTU) richness (mean=3.37±1.3 SD) than the MAC group (*n* = 14, mean= 3.07±1.2 SD) and the Other NTM group (*n*= 6, mean=3.0±1.0 SD). The Fishers' alpha diversity (Figure 5.17B) complemented these results showing that the MABSC group (mean= 0.33±0.13 SD) had a higher range of diversity than the MAC group (mean=0.308±0.11 SD) and the Other NTM group (mean=0.26±0.08 SD). The Berger-Parker index of dominance (Figure 5.17C) showed that the MAC group (mean=0.60±0.18 SD), MABSC (mean=0.59±0.22 SD) and the NTM other groups (mean=0.55±0.10 SD) all have dominant taxa. But all three tests of alpha diversity showed no statistical significance (Kruskal-Wallis test: H=0.37, p =0.82), (H= 1.38, p= 0.50), (H= 0.23, p =0.88) respectively)).

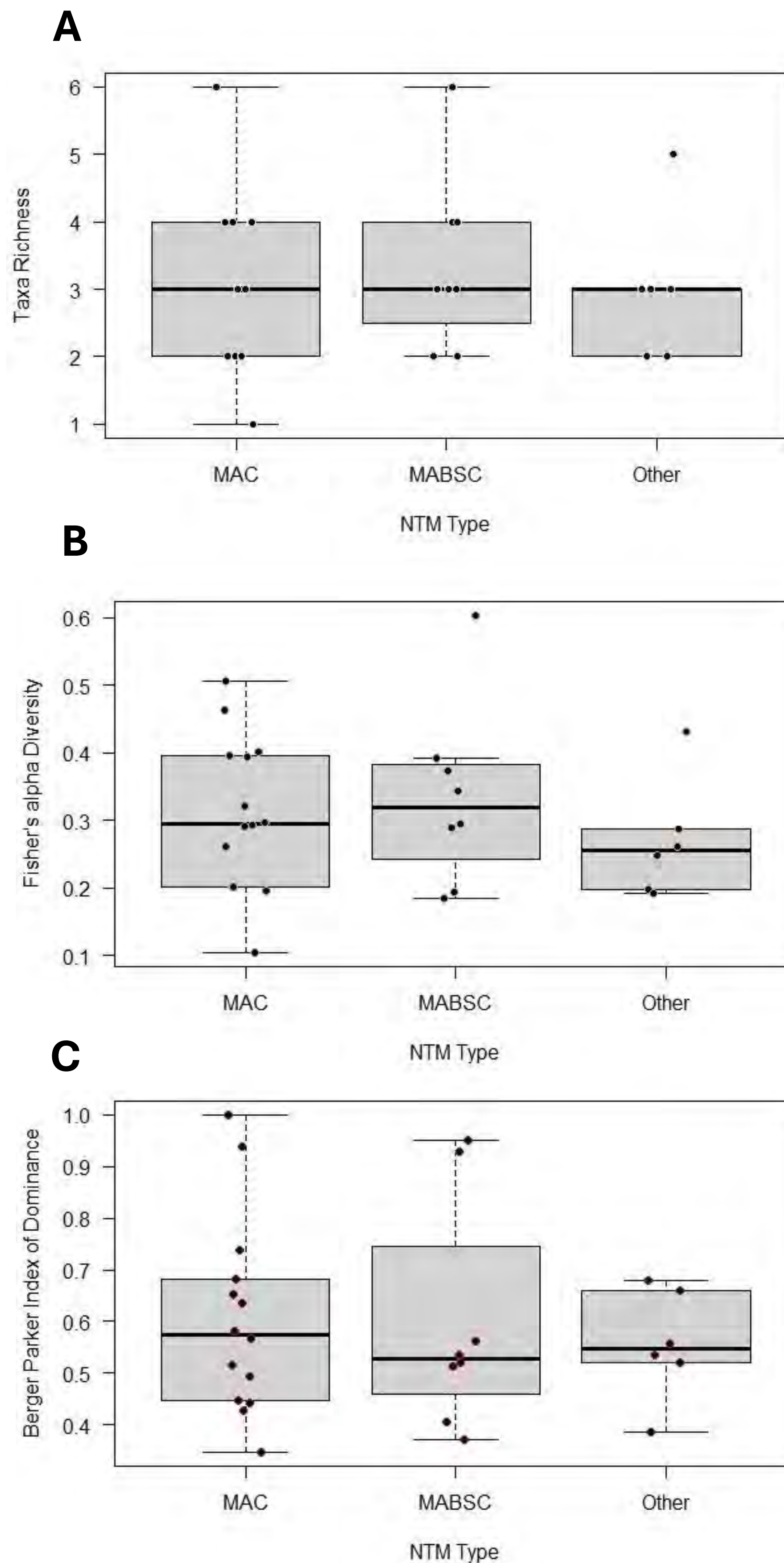


Figure 5.17 NTM complex alpha diversity and composition of NTM types for patients on modulator therapy and culture positive for NTM. MAC group (*n* = 14) The MABSC group (*n* = 8) NTM Other group (*n*= 6). Taxa richness (**A**), Fishers' Alpha diversity (**B**). Berger-Parker index of dominance (**C**). All three tests showed no statistical significance (p =≥0.05)

5.5.4.3 NTM Complex Similarity

To determine the change between species/sub-species in the NTM complexes (MAC, MABSC and Other NTM) the Bray-Curtis (abundance) Sørensen (absence/presence) indices of similarity were used (Figure 5.18/Table 5.8) and statistical significance was measured using Bray-Curtis based ANOSIM with Bonferroni correction.

The Bray- Curtis (Figure 5.18A) and Sørensen (Figure 15.8B) both show that whilst there was some very small level of similarity between each group, the NTM complexes are exclusive from eachother. These data showed significance (Bray-Curtis based ANOSIM: R = 0.8671, p=0.0001) which remained after applying a Bonferroni correction for multiple comparisons (Table 5.8). These results indicate that the type of NTM complex present is associated with unique members.

Table 5. 8 Statistical Analysis of the Similarity Indices for the NTM Complexes

NTM type	Bray-Curtis mean	Sørensen mean	Bonferroni p value
MAC	0.45±0.23	0.66±0.25	
MAC vs MABSC	0.003±0.01	0.01±0.06	0.0006
MAC vs other	0.11±0.19	0.14±0.2	0.0006
MABSC	0.37±0.2	0.64±0.15	
MABSC vs other	0.002±0.006	0.04±0.09	0.0012
Other	0.22±0.25	0.42±0.23	

The statistical analysis for the similarity indices of the NTM complexes. .Mean, ± 1 standard deviation throughout. [a] NTM type- Samples culture positive for MAC (*n* = 12), MABSC (*n* = 6), Other NTM type (*n* = 4) Statistical significance was measured using Bray-Curtis based ANOSIM with Bonferroni correction. A similarity of 1 indicates groups are identical whereas, a similarity of 0 signifies no matching species.

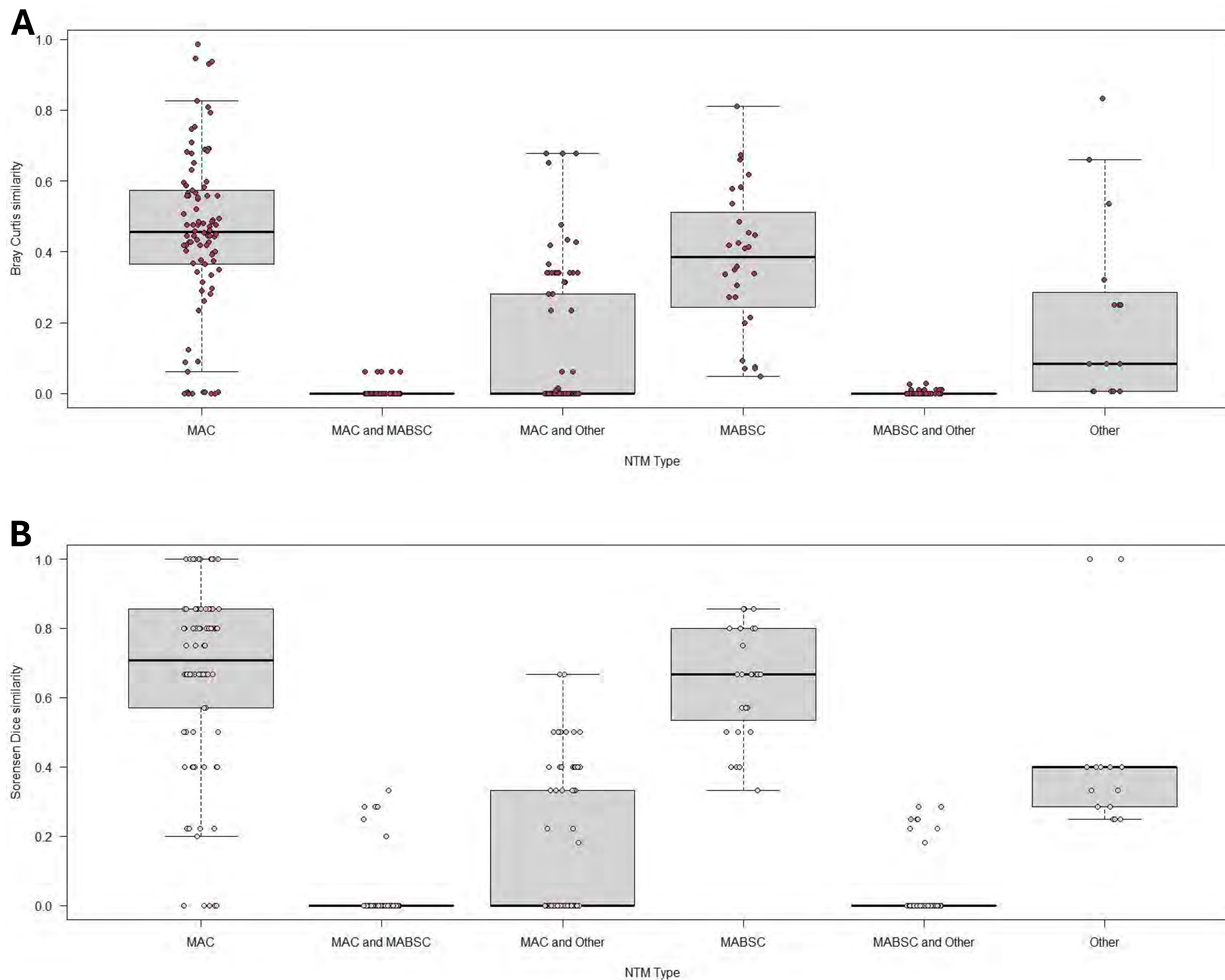


Figure 5.18 Similarity index of the NTM Complexes. (**A**) Using Bray-Curtis's measure of similarity (**B**) Sørensen indices of similarity. The plot demonstrates the similarity between the NTM type groups, with 0 denoting completely unique species and 1 identical species. The statistical analysis is displayed in table 5.8.

5.5.4.4 Metric Multidimensional Scaling

To illustrate the patterns of similarities between the groups an MDS plot was created (Figure 5.19). The plot including the NTM taxa (Figure 5.19), does show some similarities between the NTM type group, like the similarity analysis (Section 5.5.4.3). But most of the NTM complexes are distinct from each other. However, the samples in each group share some large similarities further reinforcing that the NTM complexes are associated with unique members. The statistical significance was assessed using PERMANOVA. The test showed statistical significance between the groups ($p=0.001$, $R^2= 0.6667$, $Df= 18.0$).

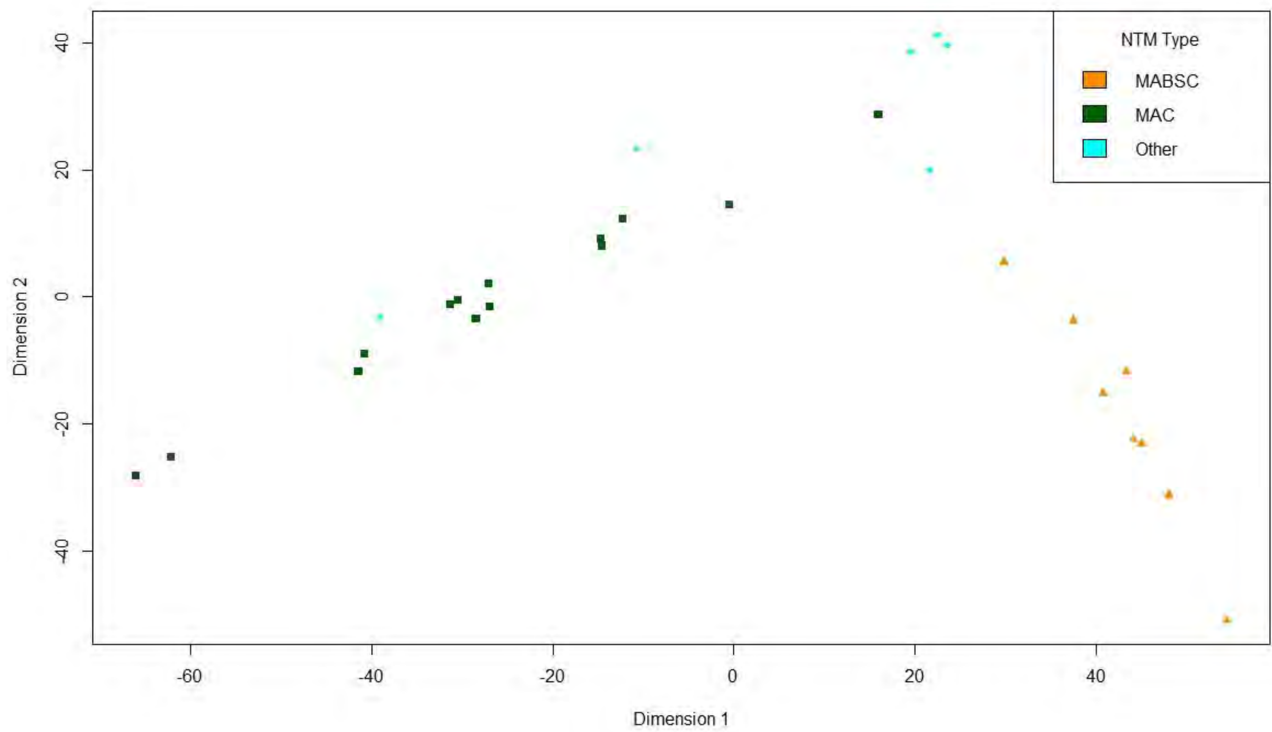


Figure 5.19 MDS plot showing how the groups are distributed by similarity. MDS of the rpo*B* sequencing. The coloured shapes represent the NTM complex groups, the closer the points are the more similarities shared.

5.5.4.5 NTM Complex SIMPER and Rank-Abundance

A Similarity Percentage (SIMPER) analysis (Table 5.9/Figure 5.20) was conducted to determine the main dissimilarities between the groups. It shows the main drivers of dissimilarity between the groups are *M. avium (*19.48%), *M.* a*bscessus subsp. abscessus* (10.98%) and *M. avium subsp. hominissuis* (9.73%) with a total dissimilarity of 95.83% showing the NTM complexes are indeed unique.

The relative abundance (Table 5.6) results within each NTM group the showed that the MAC group was predominantly made up of known members of the *M. avium* complex*; M. avium* (44.9%)*, M. avium subsp. hominissuis* (23.2%) *M. avium subsp. Paratuberculosis* (15.8%) and *M. chimera* (13%). The rank-abundance for the MAC group (Figure 5.9A) is also as expected, dominated by *M. avium* complex members but, with M. chimera as the highest ranked followed by; *M. avium* , *M. avium subsp. hominissuis* , then *M. avium subsp. Paratuberculosis.*

The MABSC group followed a similar trend where in both the relative abundance (Table 5.9) and the rank-abundance (Figure 5.21B) where largely comprised of known *M. abscessus* complex species.

The Other NTM group (Figure 5.21C) however, showed no trend with the highest taxa ranked and relative abundance as *M. asiaticum*, followed by *M. chelonae* and *M. phlei*.

Table 5. 9 Similarity Percentage Analysis (SIMPER) *rpo*B gene Sequencing of NTM Complexes

Taxon	Contrib. %	Cumulative %	Mean Relative abundance		
			MAC	MABSC	Other
Mycobacterium avium	19.48	19.48	44.9	0	17
Mycobacterium abscessus subsp. abscessus	10.98	30.46	0	32.1	0
Mycobacterium avium subsp. hominissuis	9.738	40.2	23.2	0	0
Mycobacterium asiaticum	8.725	48.92	0	0	30.9
Mycobacterium abscessus	8.041	56.96	0.432	23.4	0.177
Mycobacterium avium subsp. Paratuberculosis	6.604	63.57	15.8	0	0
Mycobacterium abscessus subsp. massiliense	6.458	70.02	0	18.9	0
Mycobacterium abscessus subsp. bolletii	6.32	76.34	0	18.5	0
Mycobacterium chimera	5.455	81.8	13	0	0
Mycobacterium immunogenum	4.268	86.07	0	0	15.1
Mycobacterium malmoense	3.573	89.64	0	0	12.7
Mycobacterium chelonae	2.66	92.3	0.112	0	9.28
Mycobacterium phlei	2.352	94.65	0	0.68	7.59
Mycobacterium xenopi	2.229	96.88	0	6.51	0
Mycobacterium simiae	1.906	98.79	0	0	6.75
Mycobacterium intracellulare	0.6034	99.39	1.44	0	0
Mycobacterium kansasii	0.4397	99.83	0.957	0	0.151
Mycobacterium chelonae subsp. gwanakae	0.1052	99.93	0	0	0.373
Mycobacterium terrae	0.06605	100	0.158	0	0

Given is within category mean percent abundance for taxa, Percentage contribution of each taxon to the average dissimilarity between the groups being compared. Taxa with higher values in this column contribute more to the overall dissimilarity between the groups. Percentage cumulative contribution of each taxon to the average dissimilarity between the two groups. The taxa are typically listed in descending order of their contribution.

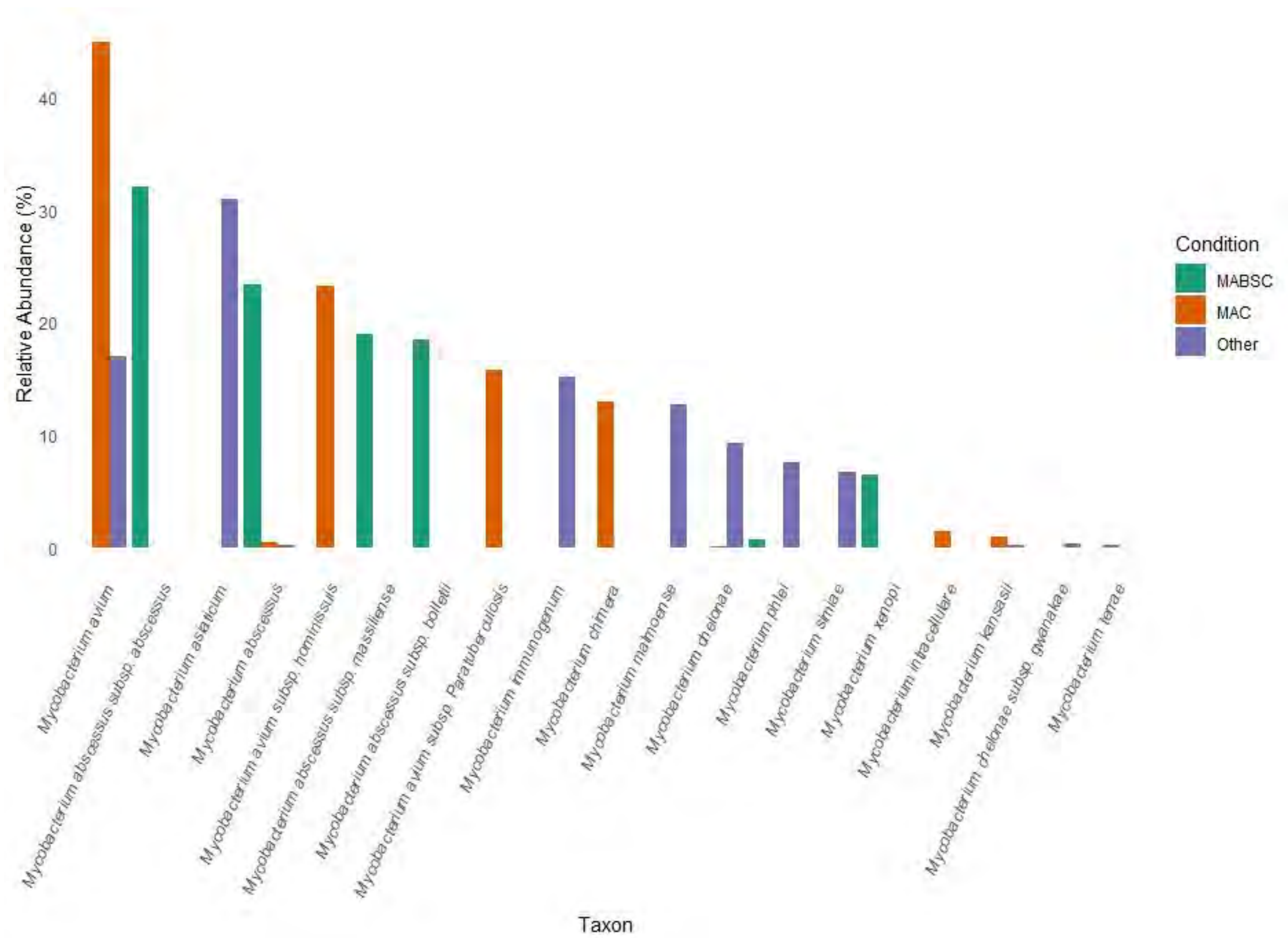


Figure 5.20 The relative abundance as calculated by the SIMPER analysis (Table 5.9). Shows the mean relative abundance for each species detected by *rpo*B gene sequencing within the NTM type complexes.

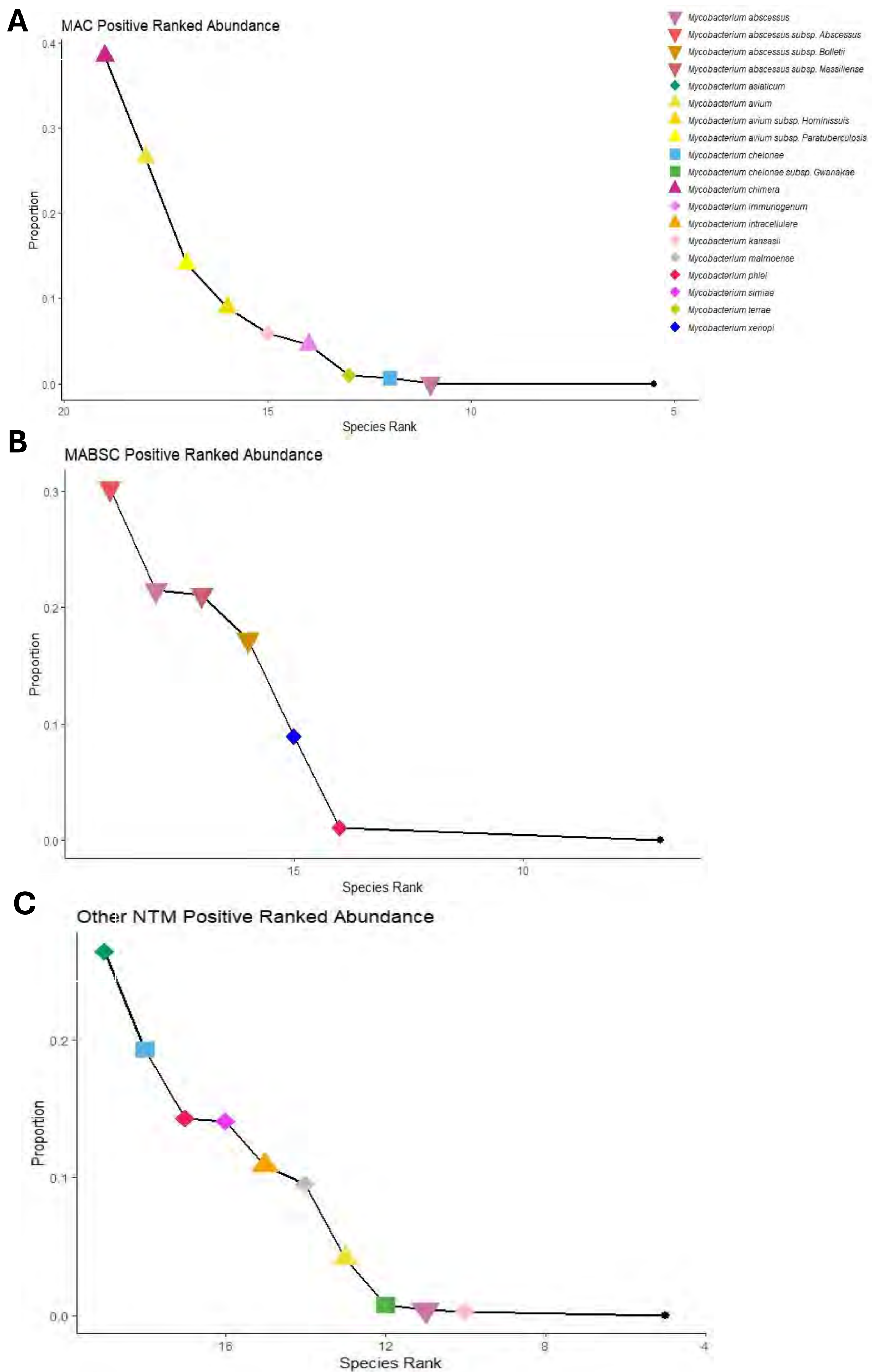


Figure 5.21 Rank-abundance curves. The coloured shapes are the NTM species and sub species which are plotted in terms of hierarchy abundance within the NTM complex group. MAC (**A**) MABSC (**B**) Other NTM (**C**). Each complex is dominated by known NTM complex members.

5.5.5 Taxa comparison

To provide a comparison of the microbiome between patients taking CFTR modulators (Chapter 5) and those who are not (Chapter 4) the relative abundance of the NTM status was examined (Figure 5.22). This showed the species, and their distribution was arranged differently between the two studies. Chapter 4 showed more opportunistic pathogens in higher ranking positions than the current chapter, which had higher abundance of oral taxa.

In Chapter 4 (Figure 5.22A), *B. multivorans* was ranked the highest in relative abundance in the positive group, followed by *S. aureus*, then *M. avium*. In Chapter 5 (Figure 5.22B), *M. avium*, *S. salivarius*, and *M. abscessus*, the taxa ranked the highest in the positive group. The negative groups showed a different distribution of relative abundance in both the studies in Chapter 4 (Figure 5.22A). *P. aeruginosa* is ranked much higher than other species, and *B. multivorans* and *S. aureus* follow it. Chapter 5 (Figure 5.22B) had higher levels of *S. aureus* then *S. salivarius* and *P. aeruginosa.*

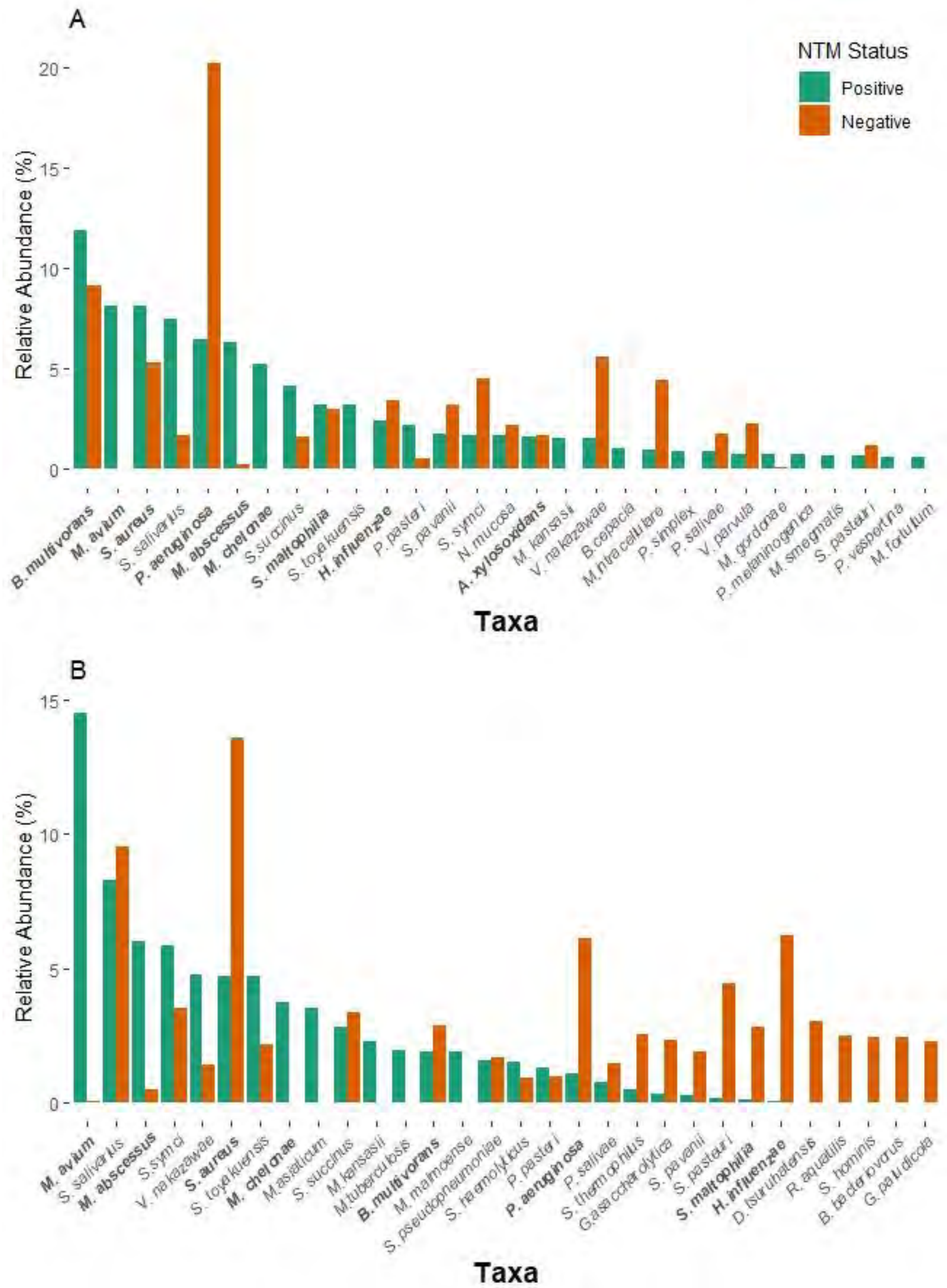


Figure 5.22 Relative Abundance (%) of the positive/negative 16S rRNA data generated in chapter 4 (**A**) and chapter 5 (**B**). Taxa in bold indicates taxa of interest including canonical CF pathogens and *Mycobacteria.*

5.6 Discussion

The introduction of CFTR modulators has significantly changed the treatment scheme for PWCF whom fit the genetic eligibility criteria (e.g., homozygous for ΔF508 or with single copies of residual function mutations (Van Goor et al., 2009; Vertex Pharmaceuticals, 2012)). These therapies have the potential to improve airway clearance, reduce antibiotic requirements and directly impact the airway microbiology of CF patients, leading to clinical benefits and improved quality of life (Ramsey et al., 2011; Rogers et al., 2020; National Jewish Health, 2023).

Previous research focusing on the effect of modulators on the microbiota, whilst limited, have shown some interesting trends. For example, one review posited how understanding the role of CF airway microbiota changes in response to modulator therapy plays an important part in informing treatment strategies for infection management (Yi et al., 2021). While another review discussed the potential for CFTR modulators altering the airway microbiota resulting in a change to traditional CF airway microbiology models (Rogers et al., 2020). Regarding the effects of CFTR modulators and NTM in CF, one of the few studies performed so far found reduced NTM culture positivity in patients using modulator therapy (Ricotta et al., 2022). The current study examined a cohort on modulator therapy, and were selected due to NTM culture positivity and compared against a group with no history of NTM infection or culture positivity. Therefore, one cannot draw a direct comparison between studies.

This chapter investigated the differences in the CF lung microbiota composition in patients currently on CFTR modulator therapy in terms of positive or negative NTM infection. The chapter also aimed to explore the differences in microbiota composition in patients' culture positive for MAC, MABSC and other NTM species undergoing modulator therapy, this was achieved through the application of 16S rRNA gene sequencing. It also further aimed to analyse the NTM specific complex composition by examining NTM specific sequencing data obtained by *rpo*B gene sequencing in the same group of patients.

5.6.1 Sequencing Analysis

The 16S rRNA and *rpo*B samples for this chapter were sequenced along with the samples for chapter 4 and 6. The results of the internal quality control by the MiSeq is discussed in Chapter 4, Section 4.6.1. Here a brief overview will be given as to how these metrics affected the downstream analysis.

The initial quality control metrics were good and analysis and the DADA2 algorithm (Callahan et al., 2016) was used to further quality control and align the sequences. The data analysed by DADA2 suffered large losses. The fastQ files used in this chapter had considerably less reads assigned per sample (Figure 5.1) then the files used in chapter 4 (Figure 4.1), which also suffered loss of data which was most likely due to multiple factors: the introduction of PCR artefacts leading to chimeric sequences, poor sample starting quality, possible DNase contamination and sequencing errors. The losses seen here were much larger and whilst some of the issues mentioned most likely contributed the loss of data is most likely due to the large number of samples that were collected via cough swab rather than through sputum (Table 5.1). Another factor which likely affected the data recovery is that some of the samples were transported via air from the United States to the United Kingdom and would have undergone several security screening measures. Airport security screening uses radiation which can damage DNA, this method of sample transportation should be avoided if possible (Gloor et al., 2006).

Recovery of microbial DNA from the respiratory system for sequencing studies has always been challenging as most non-invasive methods: spontaneous sputum or cough swabs, are always highly contaminated with oral microbiota and host DNA which, results in low bacterial biomass recovery which, then results in low sequencing depth and poor-quality data (Thornton et al., 2022). The use of cough swabs in particular are found to provide less sensitivity and an inaccurate view of the microbiome (Fenn et al., 2022). Therefore, data collected from them should be viewed as putative. However, in patients on CFTR modulators sampling the lung microbiome is difficult due to the lung returning to normal function (Ramsey et al., 2011), and resulting in lower sputum production (Rogers et al., 2020). Leaving only the use of cough, nasopharyngeal swabs or the invasive bronchial lavage (BAL) as viable collection methods (Thornton et al., 2022).

With this in mind, the analysis was carried out on the samples and the variability of sequencing depth was further highlighted in both the *rpo*B (Figure 5.17) and 16S rRNA

gene targets (Figure 5.2), by the rarefaction curves, and the inability to analyse the data through NMDS plots, which lead to the analysis being performed in the less computationally rigorous MDS plot instead.

5.6.2 The Presence of NTM in the Microbiota During Modulator Therapy

When considering the microbiota diversity and composition (Section 5.5.2.1), the NTM positive and negative groups showed no significance in any of the univariate tests. However, both measures of richness (Figure 5.3A/B) showed more taxa identified in the NTM positive group than the NTM negative group and the Berger-Parker index of dominance (Figure 5.3C) showed both groups have dominant species.

The multivariate tests (Section 5.5.2.2) showed significance between the groups, but showed a different distribution of taxa when compared to the same analysis in chapter 4 (Figure 5.22A).Here *M. avium* is highest in rank-abundance (Table 5.2/Figure 5.7), followed by *S. aureus* then mostly by taxa known to be recovered from the oral cavity (Kaci et al., 2014; Mashima et al., 2021; Qi et al., 2021) and other pathogens such as *P. aeruginosa* and *B. multivorans* being ranked lower in abundance. The rise in the detection of oral microbiota is likely due to the patients limited ability to spontaneously produce sputum (Tewkesbury et al., 2021) and the lower ranking pathogens are possibly because of an undesirable environment created by modulator therapy (Héry-Arnaud et al., 2019). The similarity and distribution of the groups with the NTM taxa removed and like chapter 4 (Section 4.5.2.2) found that there whilst the removal of the taxa resulted in a slight shift in the order of the abundance the overall result remained the same. Conversely, the data found in this chapter remained significant after the removal of the NTM taxa whereas in chapter 4 the significance was lost. These results indicate that the presence of the NTM were a main factor in the distribution of the taxa.

The shift seen here where common CF pathogens are in lower abundance in patients taking modulator therapy in comparison to patients not on modulator therapy (Chapter 4) could be due to the change in environment created by the modulators as suggested by (Héry-Arnaud et al., 2019) making it a less desirable habitat for those pathogens. Another factor to consider when evaluating the differences in microbiota composition is the reduced ability of patients to spontaneously produce sputum when on modulator therapy (Tewkesbury et al., 2021), this accounts for the increased recovery of “oral flora” such as

S. salivarius (Kaci et al., 2014), *V. nakazawae* (Mashima et al., 2021) and *S. symci* (Qi et al., 2021) seen in the SIMPER and Rank-abundance results (Section 5.5.2.4).

However, to fully evaluate any changes in microbiome composition during NTM infection and modulator therapy the type of NTM present must be considered as treatment regimens are variable and dependant on NTM type culture positivity.

5.6.3 The NTM Type and the Microbiota in Modulator Therapy

The alpha diversity analysis (Section 5.5.3.1) revealed the MAC group had a higher richness (Figure 5.9A) and diversity (Figure 5.9B) than the MABSC and Other NTM groups These findings suggest that while there may be some differences in richness, diversity, and dominance between the three groups they are not distinct enough to be considered statistically significant. However, with a larger sample size this may not have been the case. Therefore, to gain a true understanding of the alpha diversity in patients positive for certain NTM complexes further study should be done with larger groups per NTM complex.

However, the similarity indices (Section 5.5.3.2/Figure 5.10) indicated that any microbiota compositions between the MAC, MABSC, and Other NTM groups were highly dissimilar and significantly different, showing a unique composition dependant on which NTM was present.

The SIMPER (Table 5.5) and rank-abundance curves (Figure 5.14) show similarities in the presence and absence of species. All groups had some species in common but in different levels of abundance, this was notably the detection of anaerobes from the *Prevotella* and *Veillonella* genus some anaerobes have been linked to a possible role in the pathogenesis of NTM (Yamasaki et al., 2015). There was overlap in the presence of some NTM species, with all three groups showing the presence of *M. abscessus* however, in terms of abundance the presence of *M. abscessus* in the MAC group was minimal and *M. avium* was the dominant species (Figure 5.13A). These results are reinforced by the Berger-Parker index of dominance (Figure 5.9C) showing that all groups have dominant taxa. When the analysis was repeated with the NTM taxa omitted the significance was lost in all comparisons. The lower means reported in (Table 5.5), showed less similarity within each group which likely indicates the presence of NTM was driving the similarity.

These comparisons show that NTMs are the main driver of compositional changes in these patients and there was distinct differences in the microbiota found in this chapter and the microbiota found in chapter 4 where there were higher levels of pathogens present during NTM infection. Which could indicate that some pathogens are inhibited by the presence of modulators whilst others are able to thrive. However, the groups examined were small for the comparison and therefore may not give an accurate view of the factors influencing the microbiome at this time.

5.6.4 NTM Complexes and the Microbiota in Modulator Therapy

The alpha diversity tests (Section 5.5.4.1) showed no significance at this level, again suggesting there are some small differences, with the MABSC group having higher taxa richness (Figure 5.17A) and diversity (Figure 5.17B). The Berger-Parker index of dominance (Figure 5.17C) showed that there were dominant species within each group, this was further supported by the multivariate tests which shows significance and very small similarities between the groups.

The SIMPER (Table 5.9) analysis shows that only *M. abscessus* was detected in all three groups but, in the groups other than MABSC, was in very low abundance (Figure 5.21A/C). The NTM other group did share some taxa with the other two groups mainly *M. phlei*, a possible environmental contaminant found in aerosols and soils (Johnson, 2013) which is considered non-pathogenic except in very rare cases (Tanaka et al., 2019). It also shared *M. kansasii* with the MAC group, this supports the univariate tests for similarity showing the groups share some species in low proportion (Figure 5.9). The ranked abundance also supports the Berger-Parker index of dominance showing that within the NTM complex groups there is a species present in a higher proportion even within NTM complexes (*M. avium* in the MAC complex and *M. abscessus* in the MABSC).

Whilst the work done here evaluating the differences in the microbiome in terms of the presence or absence of NTM in patients taking modulator therapies may need substantial future work, the results highlight there is still potential for pathogens to endure. So, understanding what members of the microbiota are still present (pathogens or not) is extremely important in informing treatment strategies for infection management (Yi et al., 2021).

It is worth noting that the cohort for this chapter was very small and therefore is most likely influencing the analysis done here. A larger sample group would provide a more

robust and detailed analysis of all aspects studied in this chapter. That the data obtained from the sequencing suffered large losses therefore the viability of the results is questionable and should be repeated with a larger sample group. It would also be interesting to see the differences in the microbiota composition depending on the modulator therapy the patient is taking. Unfortunately, whilst this study did have some participants on different modulators the groups were too small to gain a meaningful analysis.

5.7 Conclusion

This study provides novel insight into the microbiota composition in relationship to NTM and CFTR modulator status. While small differences in richness, diversity, and dominance were observed between NTM positive and negative groups and between different NTM types (MAC, MABSC, and Other NTM), these differences were not statistically significant. This suggests that other factors may play a more important role in distinguishing between these groups such: as type of modulator, other members of the microbiota, genotype, the co-existence of infection, age, or gender. However, significant differences in microbiota composition were found between the groups, with some overlap in the presence of certain species but different levels of abundance. This indicates that the type of NTM has an influence on the microbiota and it is not just the presence of NTM that makes a difference. Despite the effects of CFTR modulators altering the environment in which the microbiota resides, opportunistic pathogens such as NTMs are still finding a niche, which could have serious long-term implications for patient outcomes.

Further research is needed to examine the influence of factors such as age and lung function on the composition of the CF lung microbiota and NTM infection. This could provide valuable insights into how these factors may affect the microbiota and its interactions with NTM, as well as the potential impact of CFTR modulators on these relationships. Understanding the mechanisms behind these associations could help inform the development of more effective treatments for NTM infection in individuals with CF. This highlights the importance of continued research in this area to improve our understanding and management of NTM infection in the context of CFTR modulator therapy.

Chapter 6: The Characteristics of Host and NTM Infection and Their Impact on Lung function

6 Introduction

Cystic fibrosis (CF) associated airway disease is the leading cause of morbidity and mortality in CF patients (The Cystic Fibrosis Trust, 2009; Turcios, 2020). It was reported that between 2017 and 2019, 70.2% of recorded deaths in the CF population was due to respiratory/cardiorespiratory disease and the overall mean predicted survival age was 49.1 years (The Cystic Fibrosis Trust, 2019; 2020; Sokhi et al., 2021). Inflammation, impaired mucociliary clearance and chronic infection lead to respiratory decline which results in pulmonary exacerbations and eventual respiratory collapse (Flume et al., 2009). Therefore, it is crucial to relate any changes in the structure or composition of the microbiota to lung function measurements to assess their clinical significance.

6.1 Monitoring of Pulmonary Status in CF

Lung function is monitored in CF patients during clinical visits to determine the cause of possible pulmonary decline, and to evaluate the best treatment strategies if needed it is monitored by the following methods (National Guideline Alliance (UK), 2017):

- Pulmonary function tests (PFT): spirometry, cardiopulmonary exercise testing and lung clearance index.
- Imaging techniques: X-ray and CT scan
- Non-invasive microbiological investigation: Induced sputum samples, nasopharyngeal aspiration, nose, and throat swabs.
- Invasive microbiological investigation: bronchial lavage (BAL) (National Guideline Alliance (UK), 2017).

PFT are often used to help classify patients based on their percentage predicted forced expiratory volume in 1 second (%FEV_1) into 3 lung disease categories: mild (≥70%), moderate (40-69%) and severe (<40%). A deterioration in %FEV_1 could be indicative of pulmonary decline (Abbott et al., 2013).

However, to accurately interpret changes in %FEV_1 in CF patients and make informed decisions about their management and prognosis, it is important to understand the inherent variability of %FEV_1 measurements within individuals (Taylor-Robinson et al., 2012). This helps determine whether a change in %FEV_1 is clinically significant or simply due to measurement error or recoverable fluctuations in lung function (Szczesniak et al.,

2017). Understanding this variability is also crucial for making valid inferences about the association between covariates and %FEV_1 in observational studies (Taylor-Robinson et al., 2012; Szczesniak et al., 2017; Kakavas et al., 2021).

6.1.1 Cystic Fibrosis Pulmonary Decline

Associations with the covariates age, gender and %FEV_1 need to be considered. Those diagnosed in childhood were found to have more severe disease than those diagnosed as adults (Nick et al., 2010), with 74.1% of patients being diagnosed via newborn screening (The Cystic Fibrosis Trust, 2020). At the ages of 18 to 22 patients experience an acceleration in lung function decline in comparison to younger patients (Vandenbranden et al., 2012). CF patients experience %FEV_1 decline by an average of 1-3% a year (Hatziagorou et al., 2023), and microbial diversity was higher in younger patients than in older patients (Cox et al., 2010; Zemanick et al., 2017).

Gender was also found to be a risk factor with females showing worse clinical outcomes than males (Harness-Brumley et al., 2014). Research has shown that females are more likely to acquire chronic *Pseudomonas aeruginosa* infection 1.7 years sooner than their male counterparts and suffered worse %FEV_1 readings (Demko et al., 1995; Konstan et al., 2012; Harness-Brumley et al., 2014), and suffer from more exacerbation events (Montemayor et al., 2021).

Airway inflammation is another factor that can have a major effect on the development of CF lung disease. It is characterised by neutrophils arriving in the lungs and releasing reactive oxygen species (ROS) and proteolytic enzymes damaging the surrounding tissues. The activation of neutrophils is possibly due to increased presence of CF pathogens (De Rose, 2002). C-reactive protein (CRP) is also produced in response to inflammation, and elevated levels of CRP is associated with increased inflammation in CF patients (Sharma et al., 2017).

6.1.2 The Microbiome and Pulmonary Decline

Studies have shown that when the lung microbiota is dominated by a small group of pathogens patients showed poorer clinical outcomes in relation to lung function (LiPuma, 2012). Research has shown that patients with lower microbial diversity are also at risk of more frequent pulmonary exacerbations, which in turn leads to faster pulmonary decline (Carmody et al., 2013; Lee et al., 2019). When examining the lung microbiota in association with covariates such as age. Studies found older people with chronic lung

disease had an over representation of *Proteobacteria* and less representation of *Firmicutes*, which is indicative of dysbiosis associated with disease when compared to healthy subjects (Chotirmall and Burke, 2015; Lee et al., 2019). These overrepresentations were shown to be indicative of decline in %FEV_1 in older patients (O'Dwyer et al., 2016; Budden et al., 2019; Lee et al., 2019).

The association of gender, the microbiota and decline in %FEV_1 are covariates that have been studied in pairs, but few studies look at all three possible associations. We have already established in previous sections that microbiota diversity is essential for healthy %FEV_1 (Carmody et al., 2013; Lee et al., 2019; Cuthbertson et al., 2020), and that gender has an influence on %FEV_1 (Demko et al., 1995; Konstan et al., 2012; Harness-Brumley et al., 2014). A study exmaining the lung microbiome in cancer pathogensis highlighted how females are at higher risk of chronic pulmonary disease then their male counterparts (Wong et al., 2020). Murine models examining microbiome diffrences in gender found that the pulmonary microbiome is influenced by gender (Barfod et al., 2015; Roggenbuck et al., 2016). The Human Microbiome Project also highlighted diffrencs in microbial communites in the upper airways (Turnbaugh et al., 2007; Beauruelle et al., 2021).

6.1.3 Non-tuberculosis mycobacteria and Pulmonary Decline

Infection with opportunistic pathogens such as non-*tuberculosis mycobacteria* (NTM) have been associated with lung function decline and worse clinical outcomes (Park et al., 2016). There have been few studies focusing on change in %FEV_1 in patients with NTM pulmonary disease (NTM-PD) of certain genders. Generally, more than 50% of adults diagnosed with NTM-PD are female (Roux et al., 2009; Floto et al., 2016). In some instances, females with distinct features (Caucasian, non-smokers, approximately 59 years old, tall, and slim) have be found to be pre-disposed to higher rates of *Mycobacterium avium* infection (Kim et al., 2008); this predisposition is known as Lady Windermere syndrome (Reich and Johnson, 1992). A retrospective study found that NTM-PD is associated with greater %FEV_1 decline in young males, with bronchiectasis and with high radiographic scores (Lee et al., 2013). These contrasting results highlight that risk factors for NTM-PD are likely to be multifaceted.

Age has also been highlighted as a risk factor for increased risk of NTM positivity especially in CF patients (Reynaud et al., 2020). Incidence increased from 10% in children aged ten years to over 30% of adults aged 40 and above (Roux et al., 2009; Floto et al.,

2016). A study found NTM treatment success decreased as age increased, and that people over the age of 80 are at higher risk of NTM infection (Kim et al., 2022). Another study added further to this by concluding that the increase of NTM-PD prevalence is partly due to an aging population (Al-Houqani et al., 2012). Whilst there is evidence that these factors influence %FEV_1 as does NTM positivity there is little research addressing the effects of NTM positivity in combination with host factors and the effects on %FEV_1.

In terms of the NTM species causing the pulmonary disease much of the literature is indicative that this is mostly down to geographical location. NTM-PD accounts for 80-90% of all NTM-associated diseases (Prevots and Marras, 2015; Chindam et al., 2021; Saptawati et al., 2022; Mejia-Chew et al., 2023). *Mycobacterium avium* complex (MAC) and *Mycobacterium abscessus* complex (MABSC) are associated with around 90% of the total reported cases of NTM-PD (Olivier et al., 2012; Schiff et al., 2019; Lipman et al., 2020). MAC is more commonly identified in patients residing in North America (Floto et al., 2016), and MABSC is more commonly isolated in the UK (The Cystic Fibrosis Trust, 2009). Adults aged 25 and above were found to have MAC isolated more routinely from sputum cultures, whereas MABSC was common across all age groups, particularly in adolescents aged 11-15 (Roux et al., 2009; Floto et al., 2016). There has been a noticeable increase in NTM prevalence in respiratory samples from CF patients, making NTM a rising concern for patients living with CF (Caverly et al., 2016; Floto et al., 2016).

The themes explored earlier in this thesis (Chapters 4 & 5) looked exclusively at NTM culture status and how its presence influence the composition of the wider lung microbiome. However, it has yet to address the combination of age, gender, CFTR modulator status and NTM culture status on %FEV_1 in the patients samples.

6.2 Chapter Aims and Objectives

It is essential to relate any alterations in the structure or composition of the microbiota to lung function measurements to assess their clinical significance. This chapter aimed to examine if age, gender, culture positivity for NTM, CFTR modulator status and alpha diversity measures of the lung microbiome have any influence on %$FEV_{1.}$ This was achieved by:

1. Examination of patient demographic data and its relationship to %FEV_1 using a minimum adequate model of statistics.
2. Investigation of culture NTM status and its effects on %FEV_1 using a minimum adequate model of statistics.
3. To study microbial community data acquired from 16S rRNA and *rpo*B gene sequencing and association its lung function, using a minimum adequate model of statistics and correlation analysis.

6.3 Methods

Participants were recruited as part of a longitudinal and cross-sectional study of adults as described in Chapter 2. Patients who were culture positive at the time of sampling were denoted as “NTM positive”, those who have no history of positive NTM culture were “NTM negative”. Patients are sub-grouped according to their culture status designated by the hospital, including their “NTM type” status which is the species of NTM they were culture positive at the time (*M. avium* complex (MAC), *M. abscessus* complex (MABSC) and Other). Culture of patient samples was done and confirmed by University Hospital Southampton (UHS) and The University of Vermont (UVM) microbiology departments. The %FEV_1 reading was performed using via spirometry during clinic visits, those participants using the postal pack (Chapter 2) provided the most recent spirometry reading available from UHS and UVM CF clinics.

6.3.1 Sample Preparation and DNA Extraction

Gene sequencing methods are outlined in Chapter 2 “Core Methods” and detailed in Chapter 3 “Optimisation of Amplicon Sequencing for the Detection of NTM in Microbiota.”

Prior to DNA extraction, patient sputum samples were prepared in a class II cabinet. The samples were centrifuged for 10 minutes at room temperature with a speed of 1107xg, the supernatant was discarded, and the pellet was resuspended in 900µl of phosphate buffered saline (PBS) and centrifuged again under the same conditions for 5 minutes, supernatant was discarded, and the pellet resuspended in 500µl of PBS (Rogers et al., 2006). Samples were then, transferred into a 1.5ml amber micro-centrifuge tube (Sigma Aldrich, UK) and 1.25µl of PMA (Biotium, USA) was added for DNA cross-linking. The samples were then lysed via bead-beating . Nucleic acid extraction was performed following manufacturer’s instructions using Quick-DNA/RNA miniprep kit (Zymo-research, USA). DNA was then stored at -20˚C for future use and RNA was stored at -80˚C.

6.3.2 DNA Sequencing

The microbiome of the samples was assessed by 2 step amplicon-based sequencing using the Illumina MiSeq system.

First step amplicon PCR was done in two batches the first batch targeted the V4-V5 Regions of the 16S rRNA gene. Each PCR 25µl reaction consisted of 12.5µl 2x Q5 Master Mix (New England Biolabs, Hitchin, UK), 2µl (0.2µM) of phased primer pool (Invitrogen,

Paisley, UK) (further detailed in Chapter 3) and 8.5µl of ultra-pure, molecular grade water (Cytiva, Fisher Scientific UK Ltd.).

The following parameters were used; 95°C for 3 minutes proceeded by 30 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds ending with one cycle at 72°C for 5 minutes.

The second batch of amplicon PCR targeted the *rpo*B gene and the reaction consisted of 12.5µl 2x Q5 Master Mix (New England Biolabs, Hitchin, UK), 1µl (0.1µM) of phased primer pool (Invitrogen, Paisley, UK) 12% molecular grade dimethyl sulfoxide (Thermo Fisher, UK) and 8.5µl of ultra-pure, molecular grade water (Cytiva, Fisher Scientific UK Ltd).

The following parameters were used; 94°C for 3 minutes proceeded by 45 cycles at 94°C for 60 seconds, 60°C for 60 seconds and 72°C for 60 seconds ending with one cycle at 72°C for 10 minutes (Gebert et al., 2018) Both PCR reactions were confirmed by gel electrophoresis (Section 2.5.1.1).

On completion of the PCR, a 2% agarose (Thermo Fisher, UK) gel was prepared with 1x TAE buffer (tris base/ acetic acid/ Ethylenediaminetetraacetic Acid (EDTA)) with the addition of SYBR safe DNA Gel stain (Invitrogen, Paisley, UK). Electrophoresis was carried out at 100V for 60 minutes then visualised using the Bio-Rad ChemiDoc Transilluminator (Bio-Rad Laboratories, Inc. UK). Following successful amplicon amplification AMpure XP PCR purification beads (Beckman Coulter Life Sciences, USA) were used to remove any PCR fragments of 350bp and below, following manufacturer's instructions.

6.3.2.1 Index PCR

Each 25µl PCR reaction consisted of 12.5µl, 2x Q5 Master Mix (New England Biolabs, Hitchin, UK), 1.25mM of each primer, 5µl cleaned PCR product and 2.5µl of ultra-pure molecular grade water (Cytiva, Fisher Scientific UK Ltd).

The following parameters were used; 95°C for 3 minutes followed by 20 cycles at 95°C for 30 second, 55°C for 30 seconds and 72°C for 30 seconds ending with one cycle at 72°C for 5 minutes. The fragment was confirmed using gel electrophoresis and then purified as described in section 2.5.2. and reconfirmed using the Agilent Bioanalyzer (Agilent, USA) (Illumina, 2013).

6.3.2.2 Normalisation and Sequencing

Purified PCR products were quantified then normalised to a 1nM library and processed as stated in the MiSeq Denature and Dilute guide (Illumina, 2013; 2019), 5µl of the library was denatured with 0.2N NaOH for 5 minutes at room temperature and transferred to a heat block at 95°C for 5 minutes. 990µl of pre-chilled HT1 buffer was added to the denatured library, and this created the 10pM denatured library which was stored on ice. The final library consisted of the denatured library of 7pM and a 30% PhiX spike. The samples were sequenced on the Illumina MiSeq platform using the Illumina MiSeq V3 600 cycle reagent kit (Illumina Cambridge Ltd, Cambridge, UK).

6.3.2.3 Sequencing Analysis

FastQ files were downloaded from Illumina base-space. The analysis of raw sequence data was then performed through the DADA2 pipeline (Callahan et al., 2016), Using R version 4.2.2 (R Core Team, 2023). Forward and reverse reads were truncated at 200bp and 175bp, respectively. This truncation was based on the quality scores provided by the DADA2 pipeline and aimed to remove low quality bases and retain the required overlap of the paired ends reads for merging downstream (Callahan et al., 2016). The Silva Database version 138.1 release (Quast et al., 2013) was used to assign taxonomy to the sequences. Any unassigned amplicon sequence variants (ASV) where manually assigned using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) nucleotide database (Sayers et al., 2022) and matched with sequences based on a minimum of 95% query coverage, with the lowest possible e-value and a minimum of 95% identity cutoff. Multiple sequences assigned to the same ASV were condensed into OTU for statistical analysis. Any chloroplasts, mitochondria or environmental contaminants were manually removed following sequence assignment. Given the length of the sequences analysed, these identities should be considered putative.

6.3.3 Statistical Analysis

Statistical analysis and calculations was performed in R version 4.2.2 (R Core Team, 2023). Data were analysed by ANOVA using model simplification to generate the minimum adequate model (Crawley, 2013). Alpha diversity was analysed using Fisher's alpha index of diversity and Berger-Parker index of dominance and correlation was assessed via Spearman's rank correlation which is a non-parametric test that is unaffected by sample distribution (Gautheir, 2001).

6.3.3.1 Analysis of Variance

The analysis of variance (ANOVA) method is used to determine significance between the means of three or more groups by examining variance within the data. It works by splitting the data by systematic factors and random factors. Were the systemic factors have a statistical influence on the data set and the random factors have no influence on the data (St and Wold, 1989; De Iorio et al., 2004).

The relationship between the covariates (age, gender, CFTR modulator status, pulmonary status), NTM status and $\%FEV_1$ was measured for statistical significance using a minimum adequate model with an ANOVA applied.

The model initially includes all the variables and interactions in the study, this is the "saturated model" (West et al., 2012). An ANOVA is then applied to the model to measure the total variability within it. The non-significant values are then removed, and the ANOVA is conducted again, this is repeated until the minimum adequate model is found. The minimum adequate model is the simplest model that explains the which factors carry the most significance (West et al., 2012; Crawley, 2013). This was performed on R version 4.2.2 (R Core Team, 2023).

Table 6. 1 Participant Demographics used in this Chapter.

Origin	Sample ID	Collection method	Age	Gender	Type of Sample	Genotype	%FEV$_1$	Exacerbation	NTM	NTM Type	Modulator
UHS	107AS	Clinic	27	Female	Sputum	Homozygous	49	Exacerbation	Positive	Other	
UHS	107BBS	Clinic	26	Male	Sputum	Heterozygous	68	Stable	Positive	Other	
UHS	107BS	Clinic	20	Female	Sputum	Homozygous	88	Stable	Positive	Other	
UHS	107CS	Clinic	23	Male	Sputum	Homozygous	50	Exacerbation	Positive	Other	
UHS	10UH1s	Clinic	30	Female	Sputum	Homozygous	40	Exacerbation	Positive	MABSC	
UHS	11SHS2S	Postal	39	Female	Sputum	Heterozygous	73	Stable	Negative	Negative	Symkevi
UHS	11UH1S	Clinic	37	Female	Sputum	Heterozygous	68	Stable	Positive	MABSC	
UVM	11UVS	Postal	33	Female	Swab	Homozygous	50	Stable	Negative	Negative	Kaftrio
UHS	12SHS2S	Postal	32	Female	Sputum	DF508	45	Stable	Positive	Other	Kaftrio
UHS	12UH1	Clinic	31	Female	Sputum	Homozygous	57	Exacerbation	Positive	MAC	
UVM	12UVS	Postal	28	Male	Swab	Homozygous	97	Stable	Negative	Negative	Kaftrio
UHS	13UH1s	Clinic	33	Male	Sputum	Heterozygous	36	Stable	Positive	MABSC	Ivacaftor
UVM	13UVS	Postal	33	Female	Swab	Homozygous	59	Stable	Negative	Negative	Kaftrio
UHS	148BS	Clinic	25	Male	Sputum	Heterozygous	49	Exacerbation	Positive	MAC	
UVM	14BUVS	Postal	36	Male	Sputum	Heterozygous	51	Exacerbation	Negative	Negative	Kaftrio
UHS	14SHS2S	Postal	22	Female	Swab	Heterozygous	75	Stable	Positive	MAC	Kaftrio
UHS	14UHS1	Clinic	21	Female	Sputum	Heterozygous	95	Stable	Positive	MABSC	
UVM	14UVS	Postal	37	Male	Sputum	Heterozygous	52	Exacerbation	Negative	Negative	Kaftrio
UHS	153S	Clinic	25	Male	Sputum	Homozygous	86	Exacerbation	Negative	Negative	

UHS	15UH1s	Clinic	29	Female	Sputum	Homozygous	22	Exacerbation	Positive	MABSC	Symkevi
UVM	15UVS	Postal	30	Female	Swab	Homozygous	44	Stable	Negative	Negative	Kaftrio
UHS	164AS	Clinic	56	Female	Sputum	Heterozygous	51	Stable	Negative	Negative	
UHS	164BS	Clinic	22	Male	Sputum	Homozygous	82	Exacerbation	Negative	Negative	
UVM	16BUVS	Postal	35	Female	Swab	Homozygous	71	Exacerbation	Negative	Negative	Kaftrio
UHS	16UH1s	Clinic	28	Male	Sputum	Heterozygous	86	Exacerbation	Positive	MABSC	Ivacaftor
UVM	16UVS	Postal	36	Female	Swab	Homozygous	72	Exacerbation	Negative	Negative	Kaftrio
UHS	179S	Clinic	27	Female	Sputum	Homozygous	63	Exacerbation	Negative	Negative	
UHS	17UH1s	Clinic	30	Female	Sputum	Homozygous	56	Exacerbation	Negative	Negative	
UVM	17UVS	Postal	35	Female	Swab	Homozygous	101	Stable	Negative	Negative	Kaftrio
UHS	186S	Clinic	28	Male	Sputum	Heterozygous	104	Exacerbation	Positive	MAC	
UHS	187AS	Clinic	23	Female	Sputum	Homozygous	94	Exacerbation	Positive	MAC	
UHS	187CS	Clinic	28	Male	Sputum	Heterozygous	45	Stable	Positive	MAC	
UHS	188AS	Clinic	26	Male	Sputum	Heterozygous	45	Stable	Positive	MAC	
UHS	188BS	Clinic	22	Female	Sputum	Heterozygous	45	Exacerbation	Positive	MAC	
UHS	18UH1s	Clinic	31	Female	Sputum	Homozygous	52	Stable	Positive	MABSC	
UVM	18UVS	Postal	23	Female	Swab	Heterozygous	83	Exacerbation	Negative	Negative	Kaftrio
UHS	190CCS	Clinic	25	Female	Sputum	Heterozygous	52	Exacerbation	Positive	MAC	
UHS	190CS	Clinic	19	Female	Sputum	Heterozygous	49	Stable	Positive	MAC	
UHS	197S	Clinic	25	Female	Sputum	Heterozygous	106	Stable	Negative	Negative	
UHS	199S	Clinic	56	Male	Sputum	Homozygous	88	Stable	Negative	Negative	
UHS	19UH1s	Clinic	20	Female	Sputum	Heterozygous	51	Exacerbation	Positive	MAC	
UVM	19UVS	Postal	23	Male	Swab	Homozygous	76	Stable	Negative	Negative	Kaftrio
UHS	1SHS2S	Postal	39	Female	Sputum	Heterozygous	73	Exacerbation	Positive	MAC	Ivacaftor

UHS	1UH1s	Clinic	28	Female	Sputum	Heterozygous	27	Exacerbation	Positive	MABSC	Ivacaftor
UVM	1UVS	Postal	31	Female	Sputum	Heterozygous	37	Stable	Negative	Negative	
UHS	203AS	Clinic	23	Female	Swab	Heterozygous	40	Stable	Positive	MAC	
UHS	203BBS	Clinic	20	Female	Sputum	Heterozygous	40	Exacerbation	Positive	MAC	
UHS	203BS	Clinic	20	Male	Sputum	Homozygous	95	Exacerbation	Positive	MAC	
UHS	204AS	Clinic	20	Female	Sputum	Homozygous	63	Exacerbation	Negative	Negative	
UHS	204BS	Clinic	19	Female	Sputum	Homozygous	32	Exacerbation	Negative	Negative	
UHS	204CS	Clinic	20	Female	Sputum	Heterozygous	112	Stable	Negative	Negative	
UHS	205AS	Clinic	21	Female	Sputum	Heterozygous	88	Stable	Positive	MAC	
UHS	20AS	Clinic	29	Female	Sputum	Heterozygous	52	Exacerbation	Negative	Negative	
UHS	211S	Clinic	19	Male	Sputum	Heterozygous	35	Stable	Negative	Negative	
UHS	213S	Clinic	18	Male	Sputum	Heterozygous	52	Exacerbation	Positive	MAC	
UHS	21UH1s	Clinic	19	Male	Sputum	Homozygous	97	Exacerbation	Negative	Negative	
UVM	21UVS	Postal	32	Female	Sputum	Homozygous	95	Exacerbation	Negative	Negative	Kaftrio
UVM	22SHS2S	Postal	28	Female	Swab	Homozygous	99	Stable	Negative	Other	Kaftrio
UVM	22UH1S	Postal	44	Female	Sputum	Homozygous	28	Exacerbation	Positive	MAC	Symkevi
UVM	22UVS	Postal	28	Female	Swab	Homozygous	99	Stable	Negative	Negative	Kaftrio
UVM	23CUVS	Postal	27	Male	Swab	Homozygous	25	Stable	Positive	MAC	Kaftrio
UHS	23UH1S	Clinic	30	Female	Sputum	Homozygous	85	Exacerbation	Positive	MABSC	Symkevi
UVM	23UVS	Postal	27	Male	Swab	Homozygous	25	Stable	Positive	MAC	Kaftrio
UVM	24CUVS	Postal	24	Female	Swab	Homozygous	74	Stable	Positive	MAC	Symkevi
UHS	24SHS2S	Postal	39	Female	Sputum	Heterozygous	73	Exacerbation	Positive	Other	Symkevi
UHS	24UH1S	Clinic	37	Female	Sputum	Heterozygous	71	Stable	Positive	Other	Symkevi
UVM	24UVS	Postal	24	Female	Swab	Homozygous	74	Stable	Positive	MAC	Symkevi

UHS	25SHS2S	Postal	28	Female	Sputum	Heterozygous	72	Stable	Positive	MAC	Ivacaftor
UHS	25UH1s	Clinic	27	Female	Sputum	Homozygous	66	Exacerbation	Negative	Negative	
UVM	25UVS	Postal	47	Female	Swab	Heterozygous	93	Stable	Positive	MAC	Kaftrio
UVM	26UVS	Postal	25	Female	Swab	Heterozygous	94	Stable	Negative	Negative	Kaftrio
UVM	27UVS	Postal	31	Male	Swab	Homozygous	25	Stable	Negative	Negative	Kaftrio
UVM	28UVS	Postal	38	Female	Swab	Homozygous	100	Stable	Negative	Negative	Kaftrio
UHS	2CUVS	Clinic	29	Female	Sputum	Homozygous	22	Exacerbation	Positive	MAC	Kaftrio
UHS	2UH1s	Clinic	33	Female	Sputum	Heterozygous	47	Stable	Positive	MABSC	
UHS	2UVS	Clinic	29	Female	Sputum	Homozygous	22	Exacerbation	Positive	MAC	Kaftrio
UVM	30UVS	Postal	50	Male	Swab	Homozygous	56	Stable	Negative	Negative	
UVM	33UVS	Postal	29	Male	Swab	Homozygous	64	Stable	Negative	Negative	Kaftrio
UVM	34UVS	Postal	41	Female	Swab	Homozygous	80	Stable	Positive	MAC	Kaftrio
UVM	36UVS	Postal	53	Male	Swab	Heterozygous	104	Stable	Negative	MABSC	Ivacaftor
UHS	3UH1s	Clinic	30	Female	Sputum	Homozygous	84	Stable	Positive	MABSC	
UVM	3UVS	Postal	23	Male	Sputum	Homozygous	80	Stable	Negative	Negative	
UHS	46S	Clinic	29	Female	Sputum	Homozygous	59	Exacerbation	Negative	Negative	
UHS	4SHS2S	Postal	23	Female	Sputum	Heterozygous	45	Stable	Positive	MABSC	
UVM	4UVS	Postal	34	Male	Sputum	Homozygous	75	Stable	Positive	Other	Kaftrio
UHS	54AS	Clinic	22	Female	Sputum	Homozygous	45	Stable	Negative	Negative	
UHS	54BS	Clinic	28	Male	Sputum	Homozygous	91	Exacerbation	Negative	Negative	
UHS	54CS	Clinic	24	Female	Sputum	Homozygous	78	Stable	Negative	Negative	
UHS	55S	Clinic	29	Female	Sputum	Heterozygous	23	Stable	Negative	Negative	
UHS	5UH1s	Clinic	21	Female	Sputum	Heterozygous	99	Stable	Positive	MABSC	

UVM	5UVS	Postal	31	Male	Sputum	Homozygous	41	Stable	Negative	Negative	Kaftrio
UHS	6AS	Clinic	30	Female	Sputum	Heterozygous	41	Exacerbation	Positive	MAC	
UHS	6CS	Clinic	27	Female	Sputum	Homozygous	91	Stable	Positive	MAC	
UVM	6UVS	Postal	26	Male	Swab	Homozygous	80	Stable	Negative	Negative	Kaftrio
UHS	73BS	Clinic	25	Male	Sputum	Heterozygous	55	Stable	Positive	MAC	
UHS	81S	Clinic	26	Female	Sputum	Heterozygous	59	Exacerbation	Positive	MAC	
UHS	8UH1s	Clinic	28	Female	Sputum	Homozygous	27	Exacerbation	Positive	MABSC	Ivacaftor
UHS	9BUH1s	Clinic	33	Female	Sputum	Heterozygous	39	Stable	Positive	MABSC	
UHS	9SHS2S	Postal	33	Female	Sputum	Homozygous	55	Stable	Positive	MABSC	
UHS	9UH1S	Clinic	28	Female	Sputum	Homozygous	27	Exacerbation	Positive	MABSC	
UVM	9UVS	Postal	30	Male	S Swab	Homozygous	145	Stable	Negative	Negative	Kaftrio

Participant demographics for the samples used in this chapter.
Origin of the sample UHS: University Hospital Southampton, UVM: University of Vermont.
[b] Collection method: Clinic: if the sample was collected in the clinic, pre-COVID 19 pandemic or via the postal-pack method which is denoted as Postal.
[C] Type of sample provided Sputum or Cough Swab.
[D] CFTR genotype- CF transmembrane conductance regulator genotype. Homozygous ΔF508, two copies of the ΔF508 gene mutation, ΔF508 heterozygous, single copy of ΔF508 gene mutation and another mutation.
[e] Percent predicted forced expiratory volume in 1 second (%FEV1).
[f] NTM type- Samples culture positive for *M. avium* complex (MAC), *M. abscessus* complex (MABSC), Other NTM.

Table 6. 2 Participant Demographic Summary used in this Chapter

Demographics	NTM Positive	NTM Negative
Number of Patients (*n*=101)	52	49
Age (Years)		
Mean (±SD)	30.4 ± 10.14	31± 10.72
Median	28	28
Range (Min-Max)	19-70	18-63
Gender		
Male	15	18
Female	41	26
CFTR Genotype [a]		
ΔF508 homozygous	29	33
ΔF508 heterozygous	27	11
CFTR Modulator		
None	36	24
Ivacaftor®	6	1
Symkevi®	5	1
Kaftrio®	10	21
NTM Type [b]		
MAC	32	-
MABSC	16	-
Other	8	-
Sequencing Status		
Positive	48	11
Negative	4	38
Exacerbation status		
Stable/ recovering	20	34
Exacerbation	32	15
Mean predicted %FEV1 (±1SD)	59.92 ± 25.07	71.4 ± 26.56

SD denotes standard deviation of the mean. [a] CFTR genotype- CF transmembrane conductance regulator genotype. Homozygous ΔF508, two copies of the ΔF508 gene mutation, ΔF508 heterozygous, single copy of ΔF508 gene mutation and another mutation. [b] NTM type- Samples culture positive for *M. avium* (MAC), *M. abscessus* (MABS), Other NTM.

6.4 Results

6.4.1 Lung Function and Patient Factors

To establish the relationships between %FEV_1, NTM status and patient factors, clinical data was evaluated for significance using ANOVA.

The results indicated (Figure 6.1A) the influence between gender and %FEV_1 was non-significant ($F(1,92) = 1.23$, $p = 0.280$), males (*n*=36) on average had higher %FEV_1 (mean= 67.7±27% SD) than females (*n*=73, mean= 59.9±26% SD).

Similarly, the influence mutation has on %FEV_1 (Figure 6.1B) was also found to be non-significant ($F(2,92) = 0.49$, $p = 0.610$) with those homozygous for ΔF508 (*n*=47) having the highest %FEV_1 on average (mean= 66.7±24.5% SD) when compared to those heterozygous for ΔF508 (*n*=52, mean= 59.7±27.1% SD) or with other/unknown mutations (*n*=12, mean= 57.18±30% SD).

This trend of non-significance continued with the %FEV_1 and CFTR modulator status (Figure 6.1C) ($F(3,78) = 1.23$, $p = 0.304$), but it did show that with patients taking Kaftrio having a higher average %FEV_1 (*n*=33, mean= 67.25±30.6% SD) than those on Symkevi (*n=10,* mean= 55.7±29%SD), Ivacaftor (*n*=5, mean= 51.8±31% SD) and none (*n*=60, mean= 61.5±22.8% SD).

The effect of pulmonary exacerbation on %FEV_1 (Figure 6.1D) was also found to be non-significant ($F(1,78) = 0.98$ $p = 0.324$) although, the patients who were stable (*n*=63, mean= 67.5±26.1% SD) had a higher average %FEV_1 than the exacerbation group (*n*=42, mean= 60±23%S D). The influence of age on %FEV_1 (Figure 6.2) was also found to be non-significant ($F(1,60) = 0.96$ $p = 0.330$).

There were significant interactions occurring between gender, exacerbation status and %FEV_1 ($F(1,33) = 5.87$, $p = 0.021$) and with gender, modulator status, exacerbation status and %FEV_1 ($F(1,33) = 4.68$, $p = 0.039$), indicating that these relationships are multifactorial and that these factors may play a role in the development of exacerbation.

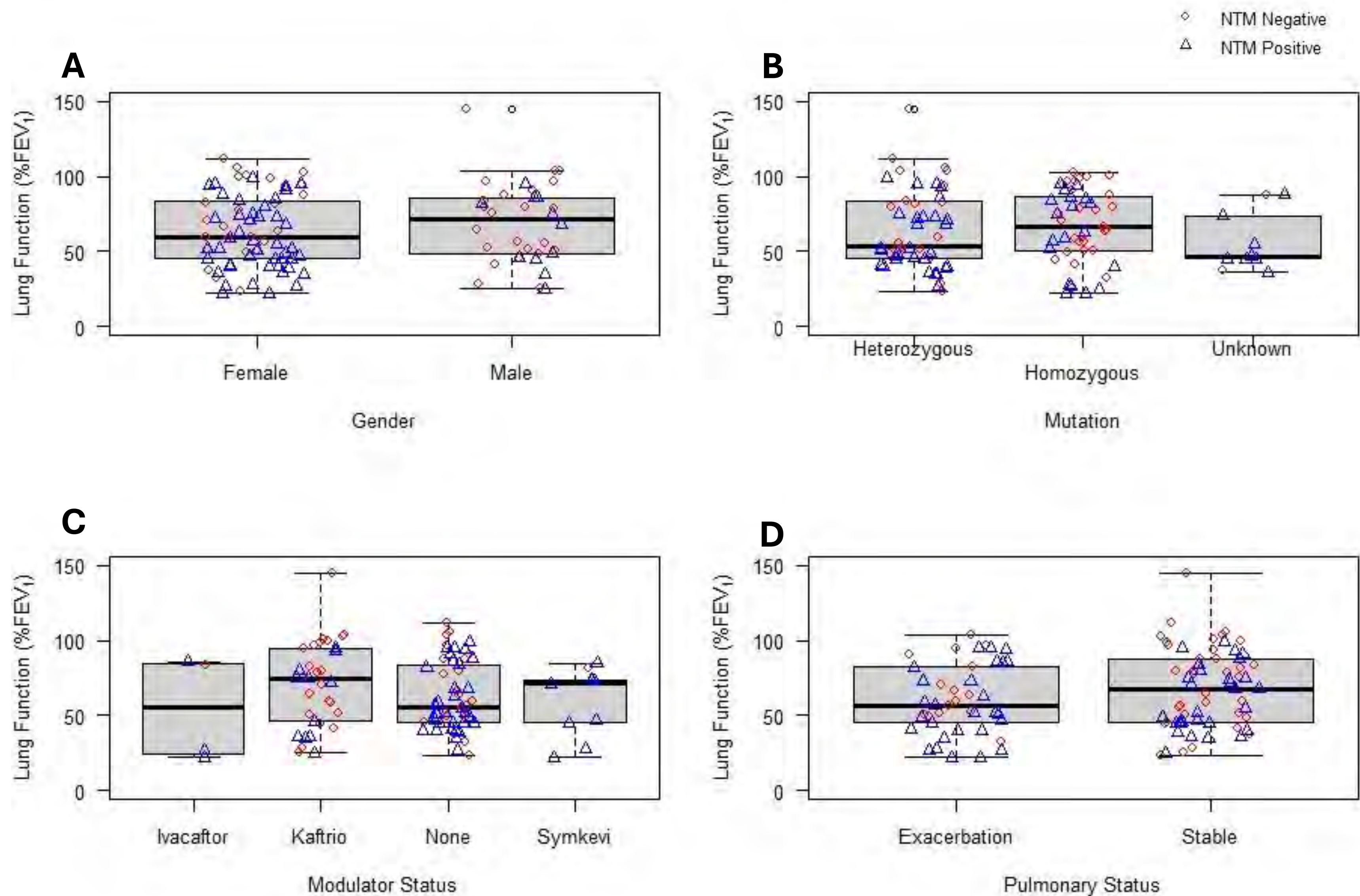


Figure 6. 1 Patient Factors influencing %FEV_1, the coloured shapes represent the NTM culture status, red squares NTM culture negative and blue triangles NTM culture positive. (**A**) %FEV_1 and gender **(B)** %FEV_1 and Mutation, Patients who are heterozygous, homozygous for the ΔF508 mutation and unknown mutation. (**C**) %FEV_1 and CFTR modulator status shows data for patients on the different types of modulators. (**D**) %FEV_1 and exacerbation status; this shows patients experiencing exacerbation events at the time of sampling.

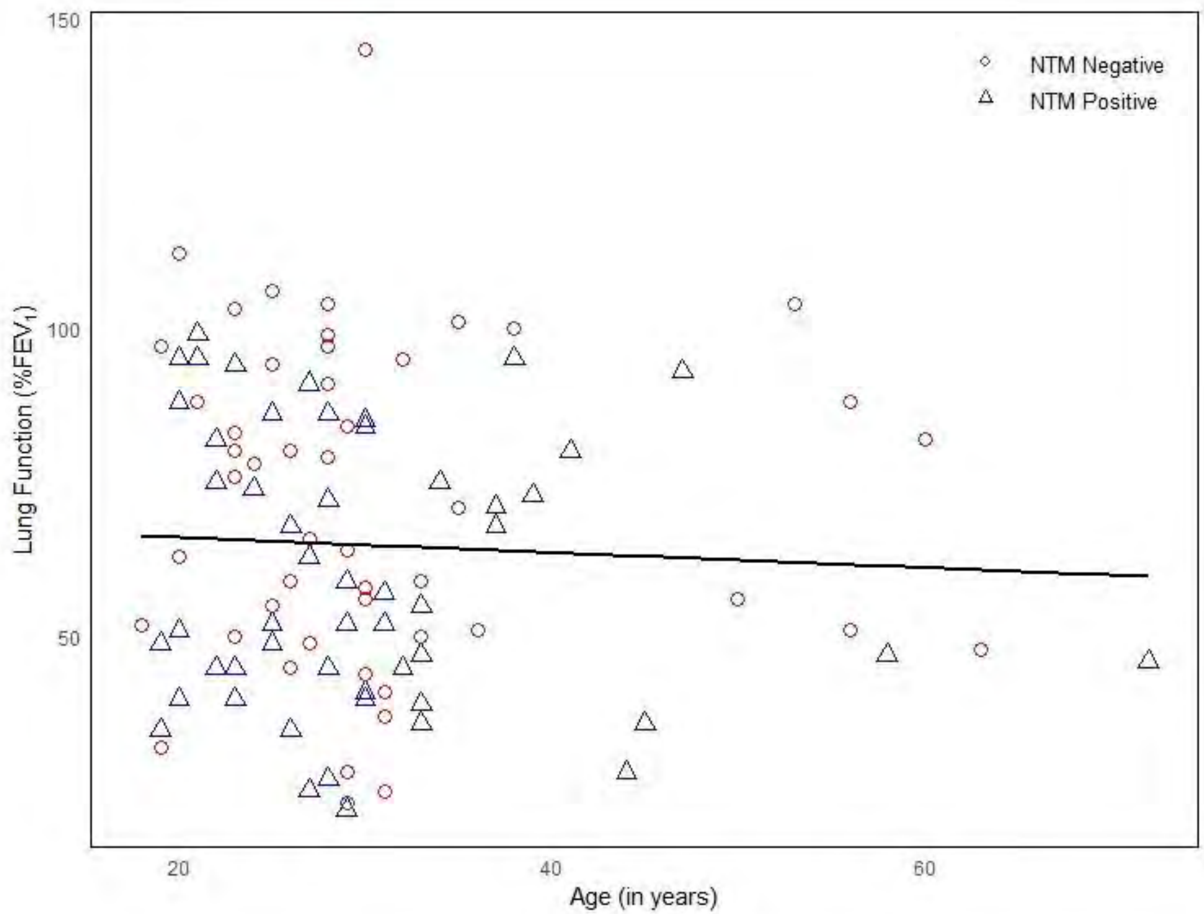


Figure 6. 2 Age group and its relationship to %FEV_1, the red circles show the NTM negative group, and the blue triangles show the NTM positive group. The black regression line shows a negative relationship. r^2= -0.006, F=0.46, p=0.49

6.4.2 NTM Status and %FEV_1

The presence of NTM infection and its impact on %FEV_1 was evaluated. This was investigated using culture diagnosis as the primary measure of NTM status and compared to the sequencing results. NTM culture status (Figure 6.3A) had a significant ($F (1,80) = 3.91$, $p = 0.050$) influence on %FEV_1 and showed that patients who were culture negative for NTM (*n* =39) have an overall higher mean %FEV_1 (71.15 ± 25.5%SD), than the NTM culture positive group (*n*=45, 60.3 ± 24.2%SD). Conversely results for the NTM sequencing status (Figure 6.3B) showed non-significance ($F (1,80) = 2.15$ $p = 0.146$) when assessed against %FEV_1 but, it showed similar results with the sequencing negative group having higher average %FEV_1 (*n*= 33, 70.15±26.3%SD) than the sequencing positive group (*n*= 51, 62.29±24.35%SD). The NTM type and its association with %FEV_1 (Figure 6.3C) was also found to be non-significant ($F (3,80) = 1.19$, $p = 0.315$) but showed a similar trend in means with the negative group having the highest average %FEV_1 (*n*=38, 70.81±25.20%SD) when compared to the NTM type groups (MAC *n*=12, 63.08±31.79%, MABSC *n*= 29, 61.13±23.27%SD, Other *n*=5, 64.2±16.33%SD). The evaluation of NTM sequencing status its relationship to NTM type and %FEV_1 (Figure 6.3D) was again non-significant ($F (1,76) = 0.21$, $p = 0.885$).

A significant relationship was found between the type of NTM and exacerbation status in relation to %FEV_1 ($F (2,40) = 4.25$, $p = 0.021$). Additionally, a significant association was observed between NTM type culture, MABSC and MAC positive sequencing data ($F (1,40) = 4.58$, $p = 0.038$). Furthermore, a significant correlation was identified between NTM type culture, exacerbation status, and NTM positive sequencing data ($F (2,40) = 4.40$, $p = 0.047$).

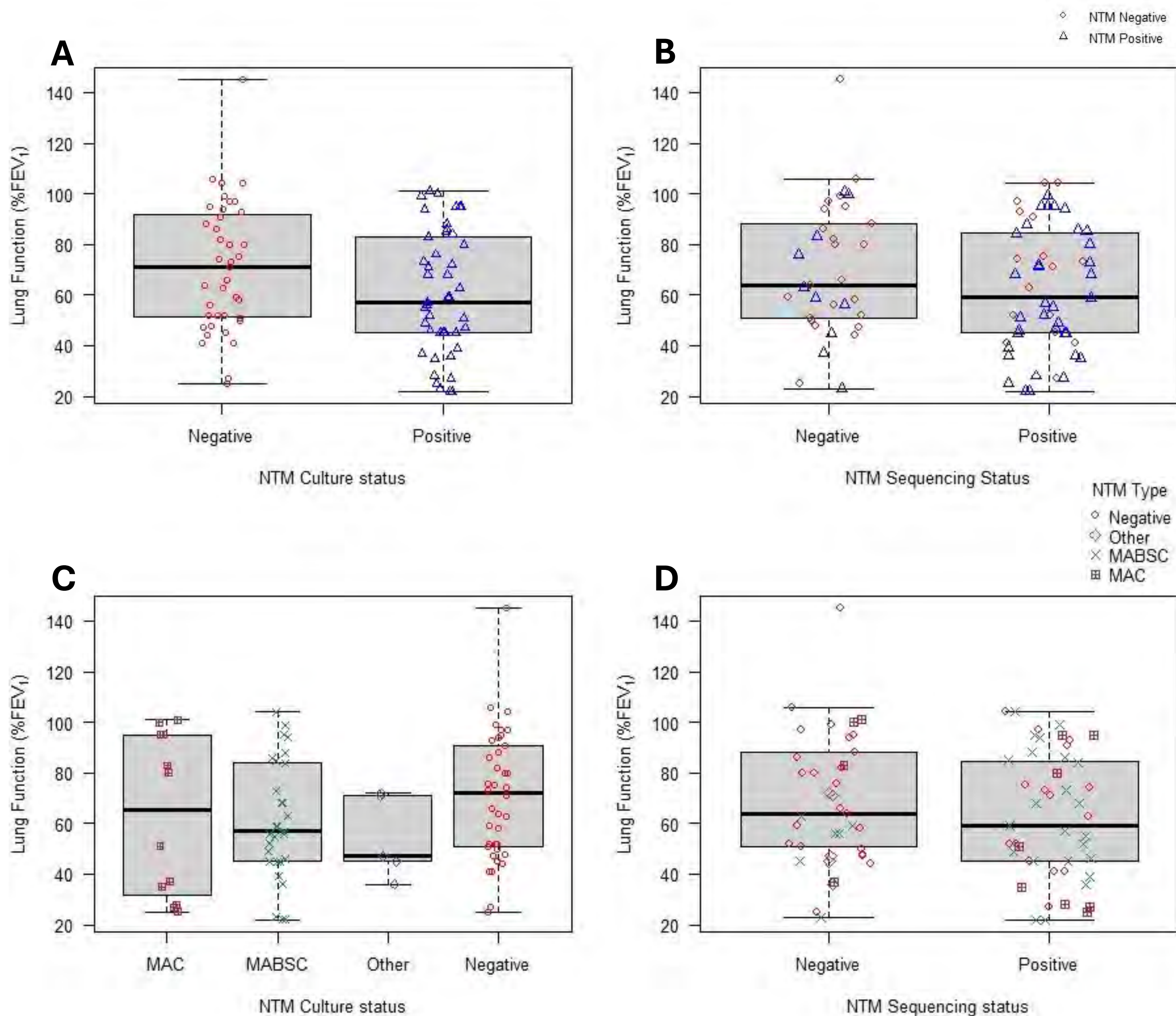


Figure 6. 3 NTM status and its influence on %FEV_1 (**A)** %FEV_1 and NTM positive or negative culture status (**B**) %FEV_1 and NTM sequencing status, where red circle represents culture negative samples and blue triangles NTM culture positive samples. (**C**) NTM culture type and %FEV_1 (**D**) NTM sequencing status with the NTM type overlayed and its relationship to %FEV_1. The different shapes represent the culture positive NTM types.

6.4.3 NTM Alpha Diversity and its Association with %FEV_1

Data obtained through NTM *rpo*B gene sequencing on NTM culture positive samples was used to evaluate the alpha diversity in relationship to %FEV_1 (Figure 6.4). The relationship was evaluated using Spearman's rank correlation. Species richness and evenness was measured using Fishers alpha index diversity (Figure 6.4A) and was found to be slightly negative but, non-significant in its relationship with %FEV_1 (Spearman's rank correlation: Rho=-0.12, p= 0.440). Species dominance as measured by Berger Parker index of dominance was also found to be non-significant and slightly positive Spearman's rank correlation: Rho=-0.05 p= 0.745).

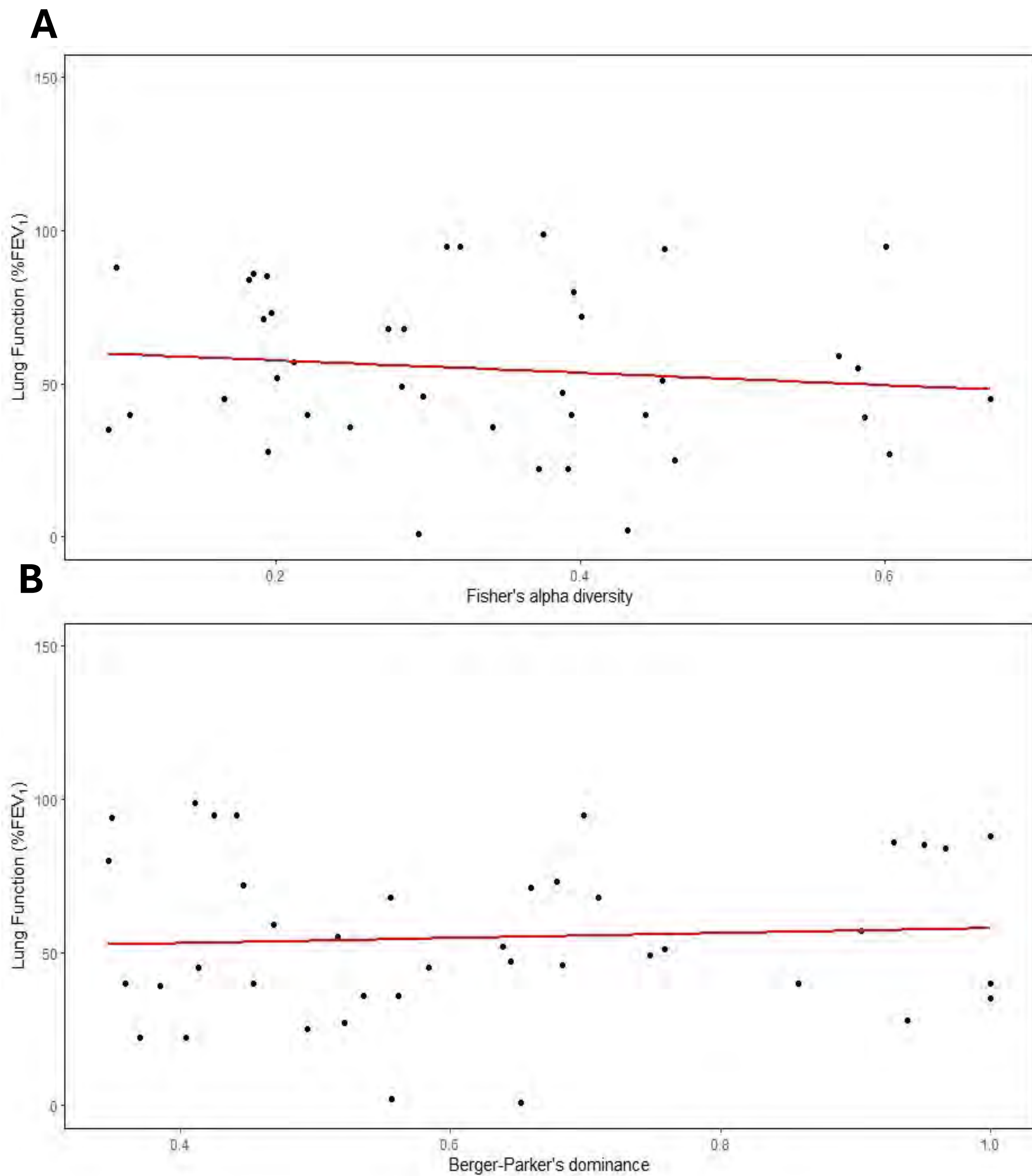


Figure 6. 4 The alpha diversity measures in relationship to $\%FEV_1$ using *rpo*B sequencing data from NTM culture positive patients and a regression line in red. (**A**) Fishers' alpha diversity against $\%FEV_1$ with R^2=0.01,($p>0.05$). (**B)** Berger Parker dominance and $\%FEV_1$, R^2=0.004, ($p>0.05$).

6.4.4 16S Alpha Diversity and its Effect on %FEV_1

Data obtained through 16S rRNA gene sequencing on all samples was used to assess the alpha diversity in association to %FEV_1 (Figure 6.5). The relationship was evaluated using Spearman's rank correlation and species richness and evenness was measured using Fishers alpha index diversity (Figure 6.5A). This slight positive relationship was found to be non-significant (Spearman's rank correlation: Rho=-0.02, p= 0.849). As was species dominance as measured by Berger Parker index of dominance (Spearman's rank correlation: Rho=-0.01, p= 0.900).

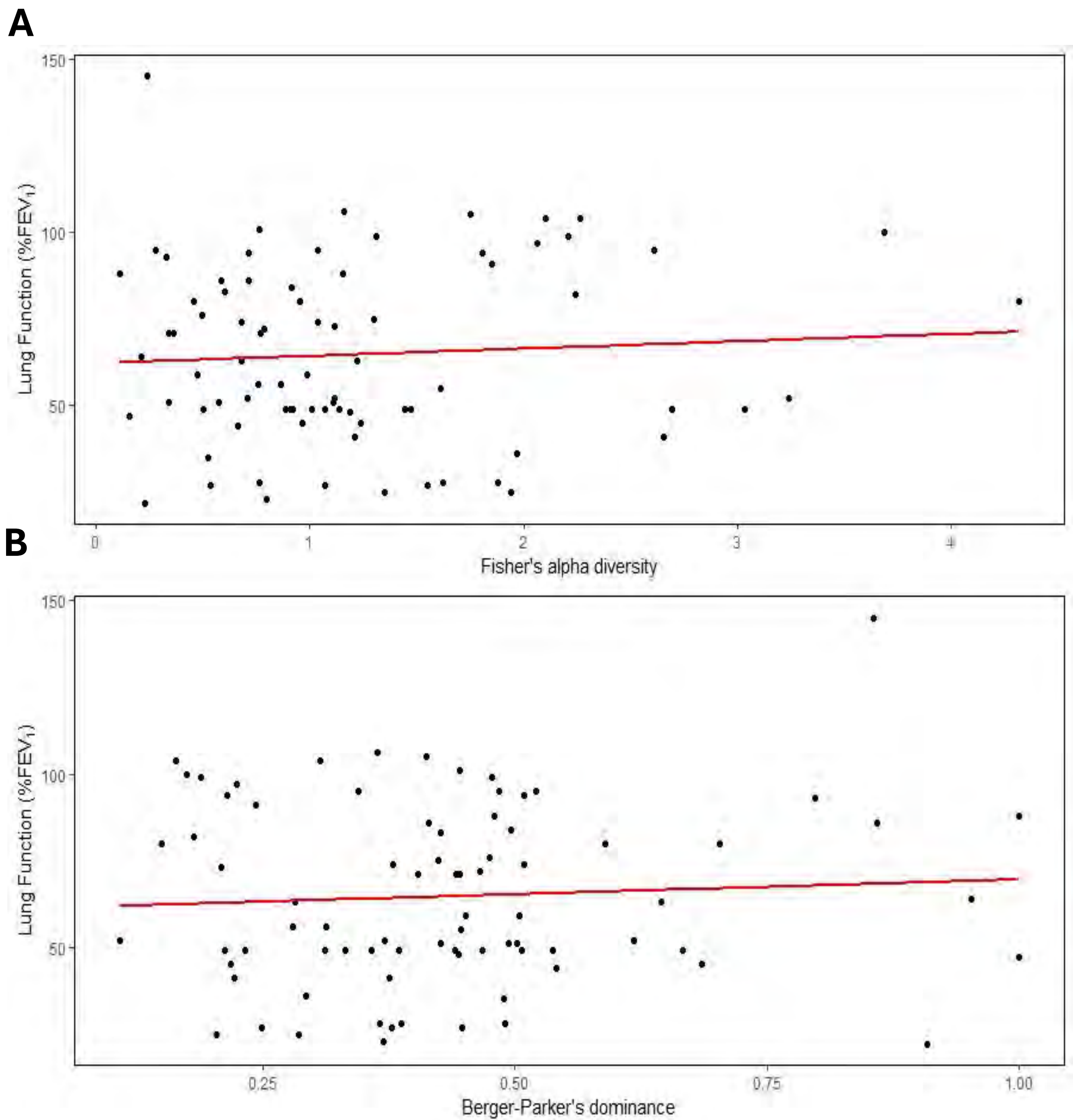


Figure 6. 5 The alpha diversity measures in relationship to %FEV_1 using 16S rRNA sequencing data from all patients and a regression line in red. (**A)** Fishers' alpha diversity against %FEV_1 R^2=0.004, (p>0.05) **(B)** Berger Parker dominance and %FEV_1, R^2=0.004, showing a non-significant positive correlation (p>0.05).

6.5 Discussion

Chapters 4 and 5 examined NTM culture status and how its presence influences the composition of the wider lung microbiome. But, neglected to address how covariates and NTM culture status affects %FEV_1 in the patients samples.

The aim of this chapter was to evaluate the association of age, gender, CFTR modulator status and NTM culture status on %FEV_1 in this cohort.

6.5.1 Lung Function and Patient Factors

Assessment of %FEV_1 in patients with CF is a standard practice to evaluate the progression of lung disease and is used in clinical settings (Taylor-Robinson et al., 2012). Several risk factors have been identified that influence decline in %FEV_1 and include CFTR genotype (Rueda-Nieto et al., 2022), age (Konstan et al., 2012), gender (Gee et al., 2003; Kim et al., 2018) and colonisation with bacterial pathogens (Cuthbertson et al., 2020), however, this study found that single factors such as age, gender and genotype have no significant (Figure 6.1/6.2) influence on %FEV_1 within this cohort. Previous research suggests that a combination of factors influence %FEV_1 decline (Demko et al., 1995; Amadori et al., 2009; Konstan et al., 2012). In relationship to gender and %FEV_1 (Figure 6.1A), previous studies found that females were more likely to develop chronic infection 1.7 years sooner than males, leading to worse lung function (Demko et al., 1995). Another study showed that oestrogen has been linked to reduce mucociliary clearance contributing to worse pulmonary outcomes in females but, the study also suggests that the relationship is likely to be complex and other factors could be contributors (Lam et al., 2021).

The NTM positive group in the current study was dominated by females which could lead to a gender bias in the results. This unevenness could be due to females being at higher risk of chronic pulmonary disease then males (Wong et al., 2020; Beauruelle et al., 2021). Previously, when studied *in vivo* diffrences in gender was found to influence the microbiome (Barfod et al., 2015; Roggenbuck et al., 2016); The Human Microbiome Project also highlighted diffrencs in microbial communites in the upper airways between genders (Turnbaugh et al., 2007; Beauruelle et al., 2021). This corresponds to the significant relationships found in this study where the combination of gender and exacerbation status effects %FEV_1 and gender, modulator status and exacerbation status has effects on %FEV_1, demonstrating multifactorial influences on %$FEV_{1.}$

When evaluating the relationship between age and %FEV_1 (Figure 6.2) this study found a non-significant relationship. Other studies found the rate of lung function decline was higher in young adults then in adolescence but were associated with a range of factors such as *P. aeruginosa* infection, BMI, and nutritional status (Vandenbranden et al., 2012). Other work also found that annual decline in %FEV_1 is extremely variable depending on population and individual factors (Liou et al., 2010). Studies examining the composition of the pulmonary microbiota in ageing patients found that older people with chronic lung disease had an over representation of Proteobacteria and less representation of Firmicutes, which is indicative of disease when compared to healthy subjects (Chotirmall and Burke, 2015; Lee et al., 2019). These irregularities were shown to be indicative of decline in %FEV_1 in older patients (O'Dwyer et al., 2016; Budden et al., 2019; Lee et al., 2019). Highlighting that age alone is not responsible for decline in %FEV_1 and the decline is also influenced by microbiota composition.

6.5.2 NTM Status and %FEV_1

NTM culture status (Figure 6.3A) in this study was found to be significant in association with %FEV_1, and patients culture negative had higher mean %FEV_1 than those who are culture positive. These results are like other studies which found that patients who were infected with *M. abscessus* had an increased rate of %FEV_1 decline (Esther et al., 2010; Qvist et al., 2016; Skolnik et al., 2016). Other studies determined that infection with NTM in general is likely to have detrimental effects on %FEV_1 and that severity is likely due to the NTM species causing the infection (Floto et al., 2016; Skolnik et al., 2016; Haworth et al., 2017). On the contrary, the data acquired from the *rpo*B gene sequencing of NTM culture positive patients (Figure 6.3B) found no significance in its association to %FEV_1. The non-significance is most likely due to the detection of NTM species within both groups or other external factors such as modulator and exacerbation status. The study failed to find any significance between %FEV_1 and NTM culture type (Figure 6.3C), which was unexpected as patients infected with rapid growing mycobacteria (RGM) are more likely to suffer from more aggressive disease than those infected with slow growing mycobacteria (SGM) (Esther et al., 2010; Qvist et al., 2016; Skolnik et al., 2016). This result could be again due to the detection of NTM species within the negative group (Figure 6.3D) and low sample numbers of leading inaccuracies in the analysis.

It was found that the relationships between NTM culture type and exacerbation status had significant influence on %FEV_1. The data showed that patient's culture positive for *M.*

abscessus had higher %FEV_1 then those positive for MAC or other NTMs. When the NTM culture type was paired with sequencing data this was found to have a significant relationship to %$FEV_{1.}$ As was the relationship between NTM culture type, exacerbation status and NTM sequencing data. Previous studies have shown that patients positive for MABSC have worse clinical outcomes than those culture positive for MAC (Esther et al., 2010; Qvist et al., 2016; Skolnik et al., 2016). Patients positive with MAC have little or no decline in %FEV_1 (Skolnik et al., 2016).

The findings of the study are important in understanding the how the type of NTM influences the lung function during exacerbation and indicates that NTMs are a significant factor in patient exacerbation which leads to longer periods of inflammation which is associated with decline in %FEV_1 (Chmiel et al., 2002; Waters et al., 2012).

6.5.3 Alpha Diversity and its Effect on %FEV_1

The alpha diversity tests (Figure 6.4/6.5) using data generated from *rpo*B and 16S rRNA gene sequencing, showed both the NTM specific *rpo*B and 16S rRNA also failed to reveal any significance regarding species diversity, dominance, and their effects on %$FEV_{1.}$ Indicating that the alpha diversity has no influence on %$FEV_{1.}$ When examining NTM specific alpha diversity in CF research in the area is extremely limited with no comparable studies. However, there is extensive research examining 16S rRNA alpha diversity and its effects on %FEV_1 with other studies showing increases in dominant species and reduced diversity results in decrease in %FEV_1 (Cuthbertson et al., 2020; Metzger et al., 2021; Frey et al., 2022). However, these results could have been influenced by low sample numbers, leading to inaccuracies in the analysis.

6.6 Conclusions and Future work

Although most of the results in this are statistically non-significant, they do show that factors influencing %FEV_1 in CF populations is multifactorial. The main findings from this study is that NTM culture status does influence %FEV_1 which has implications for treatment and NTM type significantly affects exacerbation status which leads to prolonged periods of inflammation, potentially causing faster decline in %FEV_1.

Further work examining the multifactorial importance of NTM on %FEV_1 decline, would be beneficial to gain a wider understanding of all the potential influencing factors. The inclusion of a wider range of CFTR modulators, NTM culture types and additional factors such as BMI, antibiotic treatments, different genotypes, and positivity for other pathogens would provide a much better understanding of how %FEV_1 decline is influenced.

Future work examining the regulation of NTM virulence genes during exacerbation states via RT-qPCR. This would enable the determination of some of the mechanisms NTMs use to evade, survive, or attack host cells.

Chapter 7: Interactions Between Host Immune Response and NTM During Respiratory Infection

7 Introduction

7.1 Non-*tuberculosis* mycobacteria

Non-*tuberculosis* mycobacteria (NTM) are mycobacteria that do not cause tuberculosis (TB) or leprosy. Both *Mycobacterium avium* complex (MAC) and *Mycobacterium abscessus* complex (MABSC) have been found to cause lung infection in immunocompromised groups such as those suffering with chronic obstructive pulmonary disease (COPD), bronchiectasis and cystic fibrosis (CF) (Cullen et al., 2000; Coutinho et al., 2008).

There are currently 197 species and 14 sub-species of NTM that have been identified and can be sub-divided by growth rate (Forbes, 2017; Parrish, 2019). Rapid growing mycobacteria (RGM), based on growth in 7 days or less, include the major taxa *Mycobacterium fortuitum* group and the *M. abscessus* complex (MABSC) comprising of three subspecies: *M. abscessus subsp. abscessus*, *M. massiliense* and *M. bolletii*. The lesser groups are the *M. smegmatis, M. mucogenicum* and the *M. magertitense/ wolinskyi* (Brown-Elliott and Philley, 2017).

The slow growing mycobacteria (SGM), which take seven days or more to grow, can be further subdivided by pigmentation; Group I: Pigmentation when exposed to light (photochromogenic), Group II: always pigmentated (scotochromogenic), Group III: No/weak/late pigmenting regardless of light exposure (non-photochromogenic) (Turenne, 2019). The major taxa of this group are: *M. avium* complex (MAC) which consists of the main species *M. avium, M. chimaera and M. intracellulare* (Hwang et al., 2017) *M. kansasii, M. malmoense* and *M. simiae* (Falkinham, 2009).

The prevalence of NTM infections is increasing globally (Brode et al., 2014; Jarchow-MacDonald et al., 2023). Pulmonary disease caused by NTM (NTM-PD) is the most common type of infection. MAC and MABSC are among the most identified cause of infection (Griffith et al., 2007; Floto and Haworth, 2015).

7.1.1.1 *Mycobacterium avium*

MAC has a complex and diverse genetic makeup, with multiple subspecies and genotypes (Shin and Shin, 2021). This complex includes *M. avium*, *M. intracellulare* and *M. chimera* (Moravkova et al., 2008; To et al., 2020). Among all NTM, MAC is the most common cause

of pulmonary disease in humans, particularly in individuals with underlying lung diseases such as bronchiectasis, CF, or COPD. NTM-PD caused by MAC are often less aggressive than infections caused by RGMs (Griffith et al., 2007; Diel et al., 2018).

MAC can attach to bronchial and intestinal epithelial cells via the fibronectin-attachment protein (FAP) (Honda et al., 2015), or the hemagglutinin binding protein (Menozzi et al., 1998) found on its surface. The FAP interacts with the host fibronectin and creates a connection binding to integrin receptors located on the surface of mucosal cells. Once entry into the cell has occurred, the *Mycobacterium* undergoes a phenotypic change, increasing its ability to invade macrophages (McGarvey and Bermudez, 2002; Honda et al., 2015). This invasion does not trigger an inflammatory response and there has been an association with the suppression of chemokines reducing the host response to the infection (Sangari et al., 2000; To et al., 2020).

7.1.1.2 *Mycobacterium abscessus*

The RGM MABSC can exist in two different morphotypes: a smooth (S) variant and a rough (R) variant. It can transition between the morphotypes to ensure the best chance of survival (Jönsson et al., 2007; Rüger et al., 2014). This transition is mediated by the presence/absence of surface-associated glycopeptidolipids (GPL), which this existence or non-existence considerably influences the physiological and physio-pathological abilities of MABSC. The GPL have various functions, including biofilm formation, host-cell interaction and intramacrophage trafficking (Howard et al., 2006; Medjahed et al., 2010; Gutiérrez et al., 2018).

MABSC is known to modulate host immune responses, triggering a proinflammatory response that contributes to chronic inflammation and tissue damage (Kim et al., 2020). It is also able to acquire resistance to antibiotics through various mechanisms, including intrinsic resistance, efflux pumps, and acquired resistance genes (Luthra et al., 2018)

7.1.2 NTM and CF Exacerbation

CF patients often experience periods of pulmonary exacerbation, these periods are associated with a decrease in lung function which can eventually lead to lower survival rates (Stanford et al., 2021). These exacerbation events are usually caused by the bacteria *Pseudomonas aeruginosa (Mayer-Hamblett et al., 2012), Staphylococcus aureus* (Cogen et al., 2015), although some NTM or other pathogenic bacteria can cause worsening symptoms (Floto et al., 2016). These periods are associated with lower airway

inflammation which are often neutrophil mediated, there is often an increased expression of inflammatory markers like C-reactive protein (CRP), white cell counts, interlukin-8 (IL-8), and others (McGrath et al., 1999; Bhatt, 2013).

7.1.2.1 The role of Neutrophils in CF Exacerbation

During CF exacerbation phases, neutrophils play a crucial role in the host immune response (Downey et al., 2008; Malech et al., 2014). Neutrophils are the first white blood cells to be recruited to sites of acute inflammation, in response to chemotactic prompts like IL-8 which is produced by stressed cells and resident macrophages (De Oliveira et al., 2016). It has been detected that during exacerbation patients showed an increase of CD11b expression in neutrophils when compared to stable CF patients, indicating a circulating activation state due to an infection (Martin et al., 2022). CD11b is a protein subunit that forms the heterodimeric integrin alpha-M beta-2 (αMβ2) molecule, also known as macrophage-1 antigen (Mac-1) or complement receptor 3 (CR3). This molecule plays an important role in cell migration, adhesion, and transmigration across blood vessels (Springer, 1990). A similar study showed neutrophils migrated into the CF airway *in vitro* and showed reduced phagocytic receptor expression and bacterial killing, but heightened granule release, immunoregulatory function and metabolic activities, including glycolysis, and oxidant production (Forrest et al., 2018). It was also found that neutrophils from CF patients spontaneously generated higher levels of superoxide after exacerbation phases have ended, suggesting that the immune system in CF patients remains in a heightened state after infection has been resolved (Brockbank et al., 2005).

Neutrophil counts are often monitored in CF patients as it can provide valuable information about the status of the immune system and its ability to respond to infection (Goss and Burns, 2007). Research has shown that increased neutrophil count correlates with increased disease severity (Khan et al., 2019; Martin et al., 2022).

7.1.2.2 The role of C-reactive protein in CF exacerbation.

CRP is a biomarker of inflammation in CF and is often used as a marker of systemic inflammatory response to infection and useful in monitoring exacerbation phases (VanDevanter et al., 2022). During inflammatory events CRP concentration increases and is deposited at sites of tissue damage (Braig et al., 2017). It has been shown to have a protective role against bacterial pathogens through the activation of the complement

(Sproston and Ashworth, 2018) and it causes agglutination and lysis of bacterial pathogens (Kindmark, 1972; Ngwa and Agrawal, 2019)

However, in terms of use as a clinical marker some research has shown CRP concentration during exacerbation states was a poor predictor of treatment response and that CRP measurement may be an ineffective measure of recovery (Sharma et al., 2017; VanDevanter et al., 2022).

7.1.2.3 Treatment for Exacerbation

Exacerbation treatment is often complex and can involve long term antibiotics, physiotherapy, and inhaled medications (Bhatt, 2013). Treatment regimens are often individualised and can be dependent on the specific needs of the patient and the cause of the exacerbation (Bhatt, 2013; Stanford et al., 2021). In some instances, prophylaxis is prescribed to help manage and reduce the risk of exacerbations, this is usually targeted against common CF pathogens (Flume et al., 2009; Bhatt, 2013; Smyth et al., 2014).

7.1.3 *Mycobacterium* Infection and Pathogenesis

Many studies in this area have focused on understanding the molecular and physiological basis of virulence in mycobacteria. Liu et al. (2017) found that several well-established virulence factors in mycobacteria include the surface-exposed lipids in the mycobacteria outer membrane, as well as the ESX family proteins and the Pro-Glu (PE)/ Pro-Pro-Glu (PPE) family proteins, which are believed to be responsible for host cell interaction and immune response regulation (Qian et al., 2020). Another study showed *Mycobacterium tuberculosis* (MTB) uses its range of virulence factors to invade through the airway, undermine host immunity, establish survival and escape whilst setting in active disease (Rahlwes et al., 2023). It has also been found that *M. abscessus* can cope with intracellular stress through gene regulation and can also switch to a slower growth phenotype to enable its survival in macrophages (Dubois et al., 2019; Ferrell et al., 2022). Some research has also focused on the opportunistic pathogenicity of NTM in different environments (Pereira et al., 2020) and others have examined the coordination of metabolism and gene expression (Gouzy et al., 2014; Rieck et al., 2017; Gago et al., 2018).

Mycobacteria gain entry to the host respiratory system through bacilli contained in aerosolised droplets in the air (Frieden et al., 2003; Abukhalid et al., 2021). It starts to induce infection by attacking alveolar macrophages (Cohen et al., 2018), via attachment to pathogen-associated molecular patterns (PAMPs) (Jo, 2008; Mortaz et al., 2015),

identification by the host macrophage pattern recognition receptors (PRRs) (Kleinnijenhuis et al., 2011) and the stimulation and activation of intracellular signalling pathways leading to phagocytosis and infection of the macrophages (Kleinnijenhuis et al., 2011; Cohen et al., 2018). This is an attack feature shared by mycobacteria in both the RGM (Byrd and Lyons, 1999) and SGM groups (Appelberg and Orme, 1993; Early et al., 2011).

Once engulfed by the host macrophage the *Mycobacterium* resides in the phagosome (Ferrari et al., 1999), usually a phagosome maturation cycle occurs, which involves the fusion of the phagosome with lysosomes forming a mature phagolysosome (Vieira et al., 2002; Nguyen and Yates, 2021). The phagolysosome has degrative and bactericidal capabilities which eliminates the phagosome contents (Nathan and Hibbs Jr, 1991; Vieira et al., 2002). However, *Mycobacterium* can survive elimination by inhibiting phagosome-lysosome fusion (Frehel et al., 1986; Carranza and Chavez-Galan, 2019), and evade the destructive environment of the phagolysosome (Forrellad et al., 2013). The host contains the infection through the formation of granulomas, with recruitment of immune cells including neutrophils, which stops the infection spreading throughout the host (Volkman et al., 2004; Ehlers and Schaible, 2013). MTB uses the granuloma to remain dormant (latent tuberculosis) and later cause necrosis to the surrounding neutrophils and proliferate (Ehlers and Schaible, 2013; Kapoor et al., 2013). It is undetermined if tissue necrosis starts with the central narcotisation of the granuloma (Ehlers and Schaible, 2013). The necrotic tissue is characterised by a caseating manifestation which is associated with apoptosis of surrounding cells (Fayyazi et al., 2000). Though, MAC and *M. kansasii* are also known to create necrotising granulomas neither of them has been found to cause latent infection and show less virulence (Inderlied et al., 1993; Griffith et al., 2007). There are some differences in NTM and MTB pathogenesis. But they share some of same mechanisms have been found to be important in both the control and understanding of NTM and TB.

7.1.4 Virulence gene expression

During infection the *Mycobacterium* will express several virulence genes to activate the processes involved in its survival. This is complex and several gene pathways are working in unison (Echeverria-Valencia et al., 2018). This study examines some of the genes known to be expressed in MTB virulence to see if NTMs express them during exacerbation phases.

The leprosy serum reactive clone 2 (*Lsr2*) gene is a pleiotropic, master transcriptional regulator and has critical roles in virulence, cell wall lipid biosynthesis and host-induced stress response (Liu and Gordon, 2012). It is found in several mycobacteria *species:* MTB (Gordon et al., 2010), *M. leprae* (Laal et al., 1991), *M. smegmatis (Chen et al., 2006)* and *M. abscessus* (Le Moigne et al., 2019). It is able to protect the *Mycobacterium* against reactive oxygen intermediates (ROI) during macrophage infection by regulating oxygen levels (Bartek et al., 2014). *Lsr*2 has been shown to facilitate DNA structural conditions and is often upregulated during stress conditions enhancing its survivability (Kriel et al., 2018), therefore could be actively being regulated during latent periods. However, the role of *lsr2* varies between *Mycobacterium* species. For instance, in *M. smegmatis* it has roles in biofilm formation, adaptation to hypoxia and antibiotics (Chen et al., 2006). In MABSC, it has roles in morphotype switching, ROS protection, antibiotic resistance, and persistent infection (Gerges et al.), which could be expressed to ensure the survival of the *Mycobacterium.*

The superoxide dismutase (*sodA)* gene encodes for the enzyme super oxide dismutase which converts free radicals generated by host macrophages into hydrogen peroxide (H_2O_2), enabling higher survivability once engulfed (Allen et al., 2008). It also has an active role in allowing MTB to grow within macrophages once engulfed, it works alongside the *kat*G gene to achieve this (Ehrt and Schnappinger, 2009). It is found to be expressed in higher quantities in pathogenic *Mycobacterium* than in non-pathogens (Echeverria-Valencia et al., 2018).

NTMs can also regulate genes in response to antibiotic treatment to further ensure their survival (Poole, 2012). Another role of *lsr*2 is enabling the *Mycobacterium* actively to adapt to environmental stresses to ensure its survival, one of these stressors is antimicrobials (Kołodziej et al., 2021). Another gene found to regulated during antimicrobial treatment is the erythromycin ribosomal methylase 41 (*erm41)* gene. This gene confers inducible resistance to macrolides in *M. abscessus* and is considered the primary mechanism of intrinsic resistance to macrolides in (Nash et al., 2009), this resistance is associated with the function of efflux pumps (Remm et al., 2021). Efflux pumps transport toxins out of the cell (Webber and Piddock, 2003). MABSC can acquire this resistance through post transcriptional methylation of the 23S rRNA gene which inhibits drug attachment (Bastian et al., 2011). *M. massiliense* has a short *erm*41 gene and

has shown intrinsic sensitivity to clarithromycin (Kim et al., 2010). Active regulation of *erm*41 has been found during macrolide treatment in MABSC (Richard et al., 2020). There is, however, no mention of this resistance in MAC.

Macrolides are often used in the treatment of MAC (Floto et al., 2016; Alexander et al., 2020) and in the general management for CF lung infection (Binder et al., 2013). Macrolides function by inhibiting protein synthesis stopping cell growth (Coolen et al., 2015).

Whilst there has been studies looking at the function and expression of *Mycobacterium* repair (*lsr*2), protection *(sodA),* and efflux (*erm41*), there have not been any studies to my knowledge looking at the regulation of these genes during CF exacerbation in patients positive for NTM-PD.

7.2 Aims and Objectives

This chapter aims to undertake an initial investigation into the interplay between the immune response, gene regulation and the presence of NTMs in CF sputum samples from patients. This was achieved by completing the following objectives:

1. Monitor changes in gene expression of repair (*lsr*2), protection *(sodA),* and efflux (*erm41*), utilizing quantitative reverse transcription polymerase chain reaction (RT-qPCR)
2. Correlate changes in gene expression with neutrophil, and CRP values due to the regulation of the virulence genes.
3. Determine the role of CRP, neutrophils and NTM virulence on the lung function of patients.

7.3 Methods

7.3.1 Patient Recruitment and Sample Collection

Participants were recruited as part of a longitudinal and cross-sectional study of adults as described in Chapter 2. Patients who were culture positive at the time of sampling were denoted as "NTM positive", those who have no history of positive NTM culture were "NTM negative". Patients are sub-grouped according to their culture status designated by the hospital, including their "NTM type" status which is the species of NTM they were culture positive at the time (*M. avium* complex (MAC), *M. abscessus* complex (MABSC) and Other). Culture of patient samples was done and confirmed by the University Hospital Southampton (UHS) microbiology department. The %$FEV_{1,}$ neutrophil and CRP measurements were taken during clinic visits.

Spontaneous expectorated sputum samples were collected from CF patients in clinic during routine appointments by the regular CF team. Collected specimens were stored at -80°C. Then transported via special packaging on dry ice to the laboratory at Manchester Metropolitan University (MMU), the samples were then stored at -80°C until processing.

7.3.2 Mycobacteria Standards

Mycobacteria strains were used to optimise the RT-qPCR protocols and used as standards during the RT- qPCR analysis. The strains used were *M. abscessus* for the rapid growing mycobacteria group (RGM) *and M. avium* for the slow growing mycobacteria group (SGM) standards (Section 2.1.2, Table 2.).

7.3.3 Sample Preparation

Prior to DNA extraction, patient sputum samples were prepared in a class II cabinet. The samples were centrifuged for 10 minutes at room temperature with a speed of 1107xg, the supernatant was discarded, and the pellet was resuspended in 900µl of phosphate buffered saline (PBS) and centrifuged again under the same conditions for 5 minutes, supernatant was discarded, and the pellet resuspended in 500µl of PBS (Rogers et al., 2006).

500µl of washed sputum was transferred into a 1.5ml amber micro-centrifuge tube (Sigma Aldrich, UK) and 1.25µl of Propidium monoazide (PMA) (Biotium, USA) was added to each tube, then incubated for 15 minutes at room temperature. The mixture was then

transferred into a clear micro-centrifuge tube then added to an LED lightbox for a further 15 minutes, as previously described by Rogers et al. (2008); Cuthbertson (2014).

7.3.3.1 Bead-beating and DNA/RNA Extraction

500µl of sputum was added to a capped 1.5ml microcentrifuge tube which were previously prepared with one tungsten carbide bead and glass beads (Merck, Dorset, UK) filled up to the 0.5ml mark. Then 400µl of DNA/RNA lysis buffer (Zymo research, USA) was added. The microcentrifuge tubes were inserted into a FastPrep-24 homogeniser (MP Biomedicals, Loughborough, UK) and processed for 30 seconds in two bursts.

Nucleic acid extraction was performed following manufacturer's instructions using Quick-DNA/RNA miniprep kit (Zymo-research, USA). DNA was then stored at -20˚C for future use and RNA was stored at -80˚C.

7.3.3.2 Reverse Transcription

Extracted ribonucleic acid (RNA) from patient samples were converted into copy DNA (cDNA) via reverse transcription by using the Applied Biosystems High-Capacity RNA-to-cDNA™ Kit (ThermoFisher Scientific, UK). The cDNA was then stored at -20°C until needed.

Table 7. 1 Participant Demographics used in this Chapter

Sample ID	Age	Gender	Genotype	%FEV1	Pulmonary Status	NTM Type	CFTR Modulator	C-Reactive Protein (mg/L)	Neutrophil (x 10^9/L)
1UHS1s	28	F	Heterozygous	37	Exacerbation	MABSC	Ivacaftor	16	15.2
3UHS1s	30	F	Homozygous	84	Stable	MABSC	None	30	7.3
4UHS1s	22	F	Other	45	Stable	MABSC	None	89	5.1
5UHS1s	31	F	Homozygous	52	Stable	MAC	None	2	4.9
7UHS1s	22	F	Other	45	Exacerbation	MABSC	None	18	6.4
8UHS1s	28	F	Homozygous	27	Exacerbation	MABSC	Ivacaftor	25	14.6
10UHS1s	30	F	Homozygous	73	Exacerbation	MABSC	None	11	6.3
12UHS1s	31	F	Homozygous	57	Exacerbation	MABSC	None	2	4.9
15UHS1s	29	F	Homozygous	22	Exacerbation	MABSC	Symkevi/Ivacaftor	91	7.4
16UHS1s	28	M	Heterozygous	86	Exacerbation	MABSC	Ivacaftor	1	4.2
17UHS1s	30	F	Homozygous	56	Exacerbation	Negative	None	1	5.6
19UHS1s	20	F	Heterozygous	51	Exacerbation	MAC	None	8	8.8
20UHS1s	29	F	Homozygous	22	Exacerbation	MABSC	Symkevi	131	6.8
22UHS1s	44	F	Homozygous	28	Exacerbation	MAC	Symkevi	24	18.3
24UHS1s	37	F	Heterozygous	71	Stable	Other	Symkevi	7	4.1
25UHS1s	27	F	Homozygous	66	Exacerbation	Negative	None	9	6.2

Participant demographics for the samples used in this chapter.
All samples and data within this table originated from University Hospital Southampton.
All samples where sputum. No cough swabs were used in this part of the study.
[D] CFTR genotype- CF transmembrane conductance regulator genotype. Homozygous ΔF508, two copies of the ΔF508 gene mutation, ΔF508 heterozygous, single copy of ΔF508 gene mutation and another mutation.
[e] Percent predicted forced expiratory volume in 1 second (%FEV1).
[f] NTM type- Samples culture positive for *M. avium* complex (MAC), *M. abscessus* complex (MABSC), Other NTM.

Table 7. 2 Participant Demographic Summary Used in this Chapter

Demographics	
Number of Patients	16
Age (Years)	
Mean	29 ± 5.69
Median	19
Range (Min/Max)	20/44
Male	1
Female	15
CFTR Genotype [a]	
ΔF508 homozygous	10
ΔF508 heterozygous	4
Other	2
CFTR Modulator	
None	9
Ivacaftor®	4
Symkevi®	3
NTM Type [b]	
MAC	3
MABSC	10
Other	1
Negative	2
Exacerbation status	
Stable/ recovering	4
Exacerbation	12
Mean predicted %FEV_1	51.3 ± 20.87

SD denotes standard deviation of the mean. [a] CFTR genotype- CF transmembrane conductance regulator genotype. Homozygous ΔF508, two copies of the ΔF508 gene mutation, ΔF508 heterozygous, single copy of ΔF508 gene mutation and another mutation. Other, Other mutation no ΔF508 mutation. [b] NTM type- Samples culture positive for *M. avium* (MAC), *M. abscessus* (MABS), Other NTM.

7.3.3.3 Primer optimisation

To validate the primers the cDNA of the *mycobacteria* strains underwent PCR. A 12.5 µl master mix was prepared for each primer pair using BioMix™ Red *Taq* DNA polymerase (Scientific Laboratory Supplies Limited, Nottingham, UK), 0.5 µM (200nM concentration) of each primer, 1ng/µl of cDNA and 4.75 µl of ultra-pure molecular grade water (Cytiva, Fisher Scientific UK Ltd). This was amplified on a thermocycler (PCRmax, UK) using the following parameters: 95°C for 3 minutes followed by 30 cycles at 95°C for 30 seconds, 59°C for 30 seconds and 72°C for 30 seconds ending with one cycle at 72°C for 5 minutes.

The PCR product of the primer validation was cleaned up using AMpure XP PCR purification beads (Beckman Coulter Life Sciences, USA) as directed by the manufacturer. The purified PCR product was then quantified using the Qubit fluorometer (Thermo Fisher, UK) and diluted in ultra-pure molecular grade water (Cytiva, Fisher Scientific UK Ltd) to 0.01 ng/µl in triplicate and a 1:10 serial dilution was performed as described by (Elston and Deatherage, 2019).

A 10µl master mix was prepared for each primer set using 5µl SYBR Green PCR Master Mix (Applied Biosystems, Paisley, UK), 2µl ultra-pure molecular grade water (Cytiva, Fisher Scientific UK Ltd), 1µl (200nM concentration) of each primer and 1µl of serial diluted template. This was then amplified via RT-qPCR on the Bio-Rad CFX connect (Bio-Rad Laboratories, Ltd, Watford, UK) machine with the following parameters 95°C for 10 minutes proceeded by 40 cycles at 95°C for 10 seconds, 59°C for 30 seconds and 72°C for 30 seconds followed by a melt curve.

Table 7. 3 Primers Used In this Chapter

Target	Primer Sequence (5' to 3')	Function	Reference
hsp65 Tb11/Tb12	**Tb11** ACCAACGATGGTGTGTCCAT **Tb12** CTTGTCGAACCGCATACCCT	**Reference gene** 439bp	(Telenti et al., 1993)
***sod*A** SODLGF/SODLGR	**SODLGF** GAAGGAATCTCGTGGCTGAATAC **SODLGR** AGTCGGCCTTGACGTTCTTGTAC	**Gene of interest** 540bp	(Blauwendraat et al., 2012)
Lsr2 Lsr2QPCRF/Lsr2QPCRR	**Lsr2-QPCR-F** GAGACCGTTGAATTCGGTG **Lsr2-QPCR-R** GCTGATTACGCAGCTTCTCC	**Gene of interest** 82bp	*(Le Moigne et al., 2019)*
Erm41 86F/65R	**erm-86F** GACCGGGGCCTTCTTCGTGAT **erm64R** GACTTCCCCGCACCGATTCC	**Gene of interest** 396bp	(Mase et al., 2019)

qRT-PCR primer sequence table. States the target gene and the primer name. The primer sequence used to amplify the target region and the primer name. The function which states whether the target gene functions as a reference gene or a gene of interest. This column also includes the size of the target in base pairs (bp). The reference column where the primer sequence originated.

7.3.3.4 Optimal cDNA Concentration

cDNA created from the mycobacteria strains was serially diluted and underwent optimisation to determine the ideal starting concentration for cDNA. The RT-qPCR was prepared the serially diluted cDNA was used as the template.

A 10µl master mix was prepared for each primer and cDNA concentration set using 5µl SYBR Green PCR Master Mix (Applied Biosystems, Paisley, UK), 2µl ultra-pure molecular grade water (Cytiva, Fisher Scientific UK Ltd), 1µl (200nM concentration) of each primer and 1µl of serial diluted template. This was then amplified via RT-qPCR on the Bio-Rad CFX connect (Bio-Rad Laboratories, Ltd, Watford, UK) machine with the following parameters 95°C for 10 minutes proceeded by 40 cycles at 95°C for 10 seconds, 59°C for 30 seconds and 72°C for 30 seconds followed by a melt curve.

7.3.3.5 Reference Gene Standards

Standards were created using *M. abscessus* and *M. avium* targeting the *hsp*65 gene. Standards are used as positive controls during the experimental stage of the gene expression assay and are also used as a reference to measure the relative change in gene expression. The reference gene allows for accurate and reliable measurement of gene expression levels. They help establish a calibration curve and contribute to quality control (Bustin et al., 2009).

7.3.4 Experimental Gene Expression Assays

The RT-qPCR gene expression assays were executed in triplicate with both *M. avium* and *M. abscessus* standards as positive controls. A 10µl master mix was prepared for each primer set using 5µl SYBR Green PCR Master Mix (Applied Biosystems, Paisley, UK), 2µl ultra-pure molecular grade water (Cytiva, Fisher Scientific UK Ltd), 1µl (200nM concentration) of each primer and 1µl of serial diluted template. This was then amplified via RT-qPCR on the Bio-Rad CFX connect (Bio-Rad Laboratories, Ltd, Watford, UK) machine with the following parameters 95°C for 10 minutes proceeded by 40cycles at 95°C for 10 seconds, 59°C for 30 seconds and 72°C for 30 seconds followed by a melt curve.

7.3.4.1 Quality Control

The data acquired was quality controlled to assess its reliability and reproducibility as described by the MIQE guidelines (Bustin et al., 2009). This assessment was done with the production of a standard curve which enables the calculation of primer efficiency (Ruijter et al., 2009)(Equation 7.1), which should be within the range of (90-110%) (Bustin et al., 2009) .

Equation 7.1 Primer Efficiency

$$Effciency = 10^{-1/slope} - 1$$

The coefficient of determination (R^2) was calculated using R version 4.2.2 (R Core Team, 2023) to determine how well the data fits into the linear regression line on the standard curve and should be ≥99 (Bustin et al., 2009).

The coefficient of variance was assessed on the intra-assay replicates (Equation 7.2). This method examines the variability of results between results on a single plate or experiment (Boda et al., 2009). No inter-assay of variation was performed on this data as all corresponding replicates were performed on a single assay.

Equation 7.2 Coefficient of Variance

$$CV(\%) = \frac{Standard\ deviaton\ of\ Ct\ values}{Mean\ of\ Ct\ values} \times 100$$

7.3.5 Statistical Analysis

Statistical analysis and calculations was performed in R version 4.2.2 (R Core Team, 2023). Relative gene expression ratios where calculated (Pfaffl, 2001). Statistics were analysed by Analysis of Variance (ANOVA) (Armstrong, 2014) to generate the minimum adequate model (Crawley, 2013) on patient demographic data (Table 7.1/7.2) and RT-qPCR results. Relationship correlation was determined using Spearman’s correlation (Gautheir, 2001).

7.3.5.1 Relative Gene Expression Ratio

The relative gene expression ratios were calculated using the Pfaffl method (Equation 7.3). This method calculates relative gene expression, whilst accounting for differences in primer efficiencies, increasing experimental reproducibility (Pfaffl, 2001).

Equation 7.3 Relative Gene Expression Ration

$$Gene\ Expression\ Ratio = \left(\frac{E_{target}}{E_{ref}}\right)\frac{\Delta Ct\ target}{\Delta Ct\ ref}$$

Where:

^{E}*target* and ^{E}*ref* are the efficiencies of the target and reference genes, respectively. Δ*Ct target* and Δ*Ct ref* are the differences in Ct values between the control and sample for the target and reference genes, respectively (Pfaffl, 2001).

7.3.5.2 Minium adequate model

The relationships between the %$FEV_{1,}$ CRP, neutrophil count and exacerbation status were measured. As well as the associations between gene expression, %$FEV_{1,}$ CRP, neutrophil count and exacerbation were measured. Using a minimum adequate model with an ANOVA applied.

The model initially includes all the variables and interactions in the study, this is the “saturated model” (West et al., 2012). An ANOVA is then applied to the model to measure the total variability within it. The non-significant values are then removed, and the ANOVA is conducted again, this is repeated until the minimum adequate model is found. The minimum adequate model is the simplest model that explains the which factors carry the most significance (West et al., 2012; Crawley, 2013). This was performed on R version 4.2.2 (R Core Team, 2023).

7.4 Results

7.4.1 Quality Control

The primer efficiency (83.81%), R^2 (0.998) and CV (6.627%) values, for the reference gene *hsp65* was determined using a standard curve (Figure 7.1). The curve showed that the R^2 was within the threshold of ≥0.99, the primer efficiency was just below the range of 90-110%, and the CV was within the recommended ranges (≤10%) as set out by the MIQE guidelines (Bustin et al., 2009).

The quality control (Figure 7.2) shows the primer efficiencies, R^2 and CV for the genes of interest did not meet all the criteria set out in the MIQE guidelines (Bustin et al., 2009). The *lsr*2 target (Figure 7.2A) had the lowest primer efficiency (66.54%), and its CV value (80.121%) was poor, both figures are not within the recommended ranges . Neither did the R^2 (0.9072) value. The standard curve for the *erm*41 gene (Figure 7.2B) showed excellent efficiency (126.51%), but poor R^2 (0.5923) and CV (52.72%). The quality control for the *sodA* gene (Figure 7.2C) showed excellent efficiency (116.25%), R^2 (0.9825) and poor CV (24.11.%) This suggests that the previously published primer sets may have some issues regarding the consistency of the data, the results presented here should be considered putative (Bustin et al., 2009).

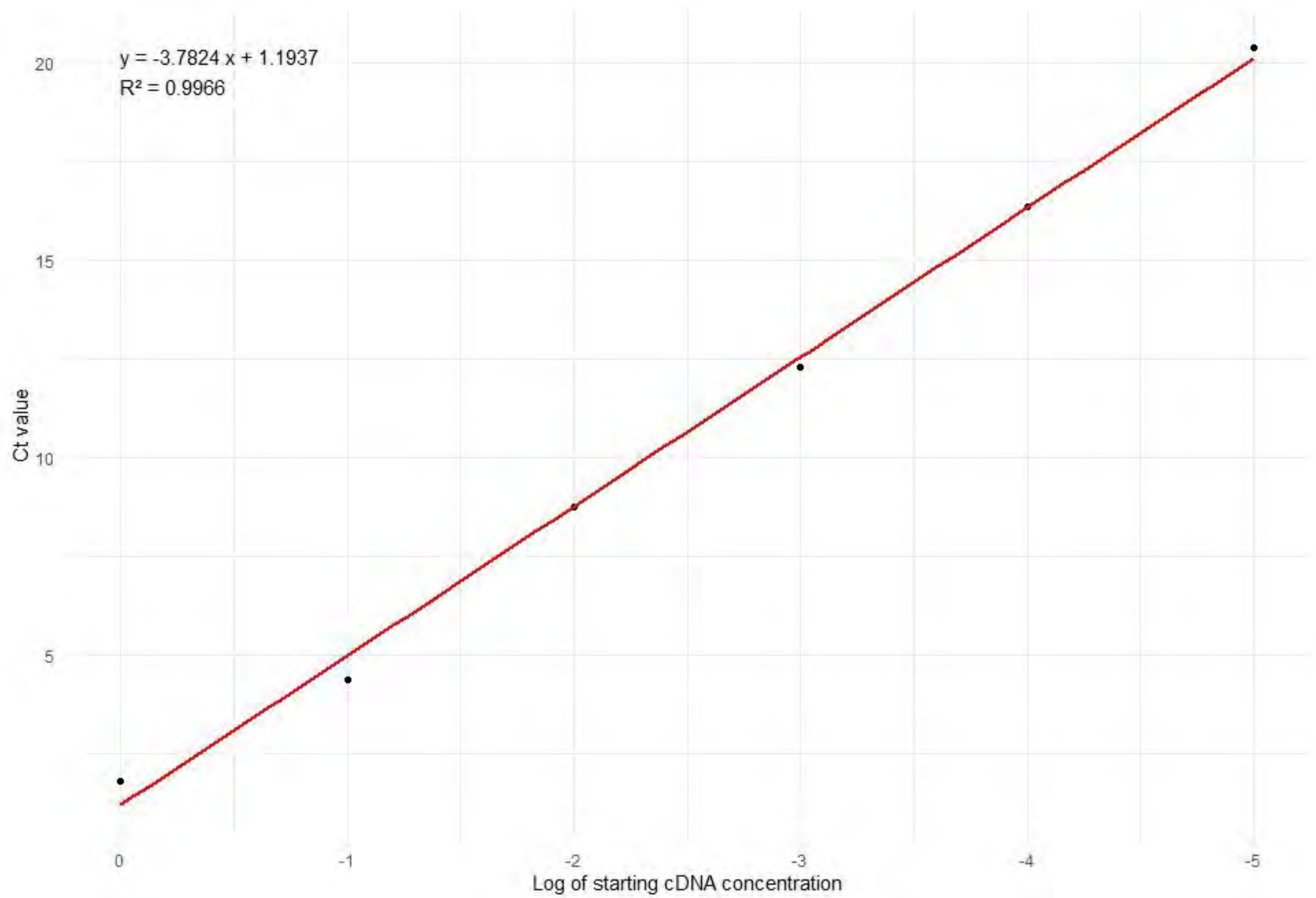


Figure 7. 1 Standard curve of the *hsp*65 reference gene. The y axis shows the average cycle threshold (Ct) value. The x axis shows the log of the starting cDNA concentration. The curve is annotated with the y-intercept equation which is used to calculate the primer efficiency. The R^2 value is also displayed, this value represents how well the data fits into the linear regression line.

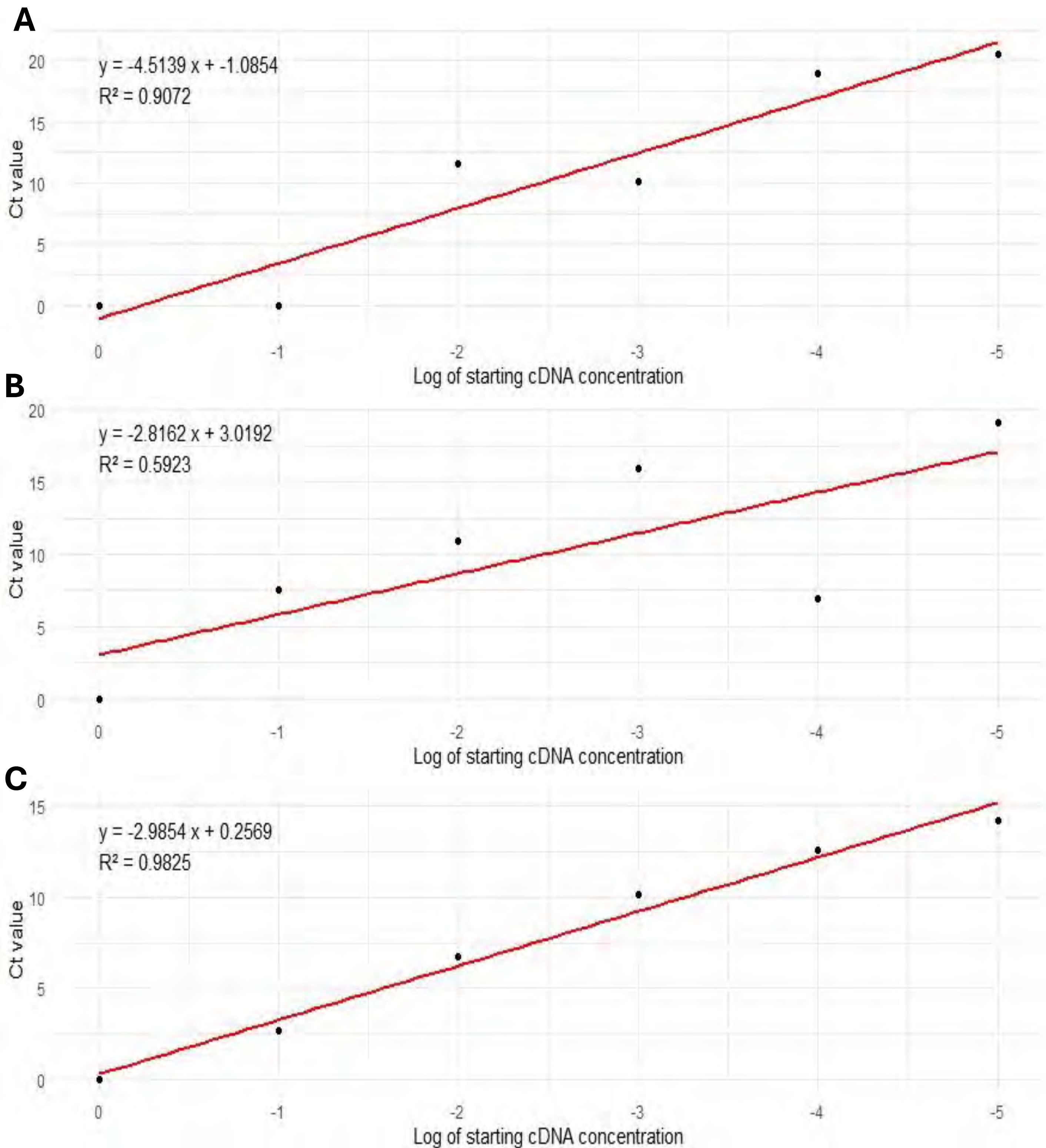


Figure 7. 2 Standard curve of the genes of interest. **(A)** *lsr*2 **(B)** *erm*41 **(C)** *sod*A. The y axis shows the average cycle threshold (Ct) value. The x axis shows the log of the starting cDNA concentration. The curve is annotated with the y-intercept equation which is used to calculate the primer efficiency. The R^2 value is also displayed, this value represents how well the data fits into the linear regression line.

7.4.2 The Effects of Pulmonary Status on Clinically Derived Metrics

Patient lung function (%FEV_1) measures of inflammation, C-reactive protein (CRP) levels, and neutrophil concentration were assessed against pulmonary status as the determining factor (Figure 7.3). The analysis found that the %FEV_1 of the stable group was not significantly ($F (1,14) = 1.734$, $p = 0.209$) higher mean %FEV_1 (*n*=4, mean= 63.0 ± 17.7% SD) than the exacerbation group (*n*=12, mean= 47.5±21% SD) (Figure 7.3A). Having shown that lung function has no difference in exacerbating patients, the analysis turned to measures of inflammation. Here, CRP in stable patients (mean= 32 ± 39.9 SD mg/L) was assessed against patients in exacerbation (mean= 28±40.4 SD mg/L). However, this was non-significant ($F (1,14) = 0.028$, $p=0.868$). Staying on trend with the other two tests, the neutrophil concentration (Figure 7.3C) was found to be non-significant ($F (1,14) = 1.97$, $p= 0.181$), in the exacerbation group (mean= 8.7 ± 4.6x10^9/L SD) and the stable group (mean= 5.3 ± 1.3 x10^9/L SD).

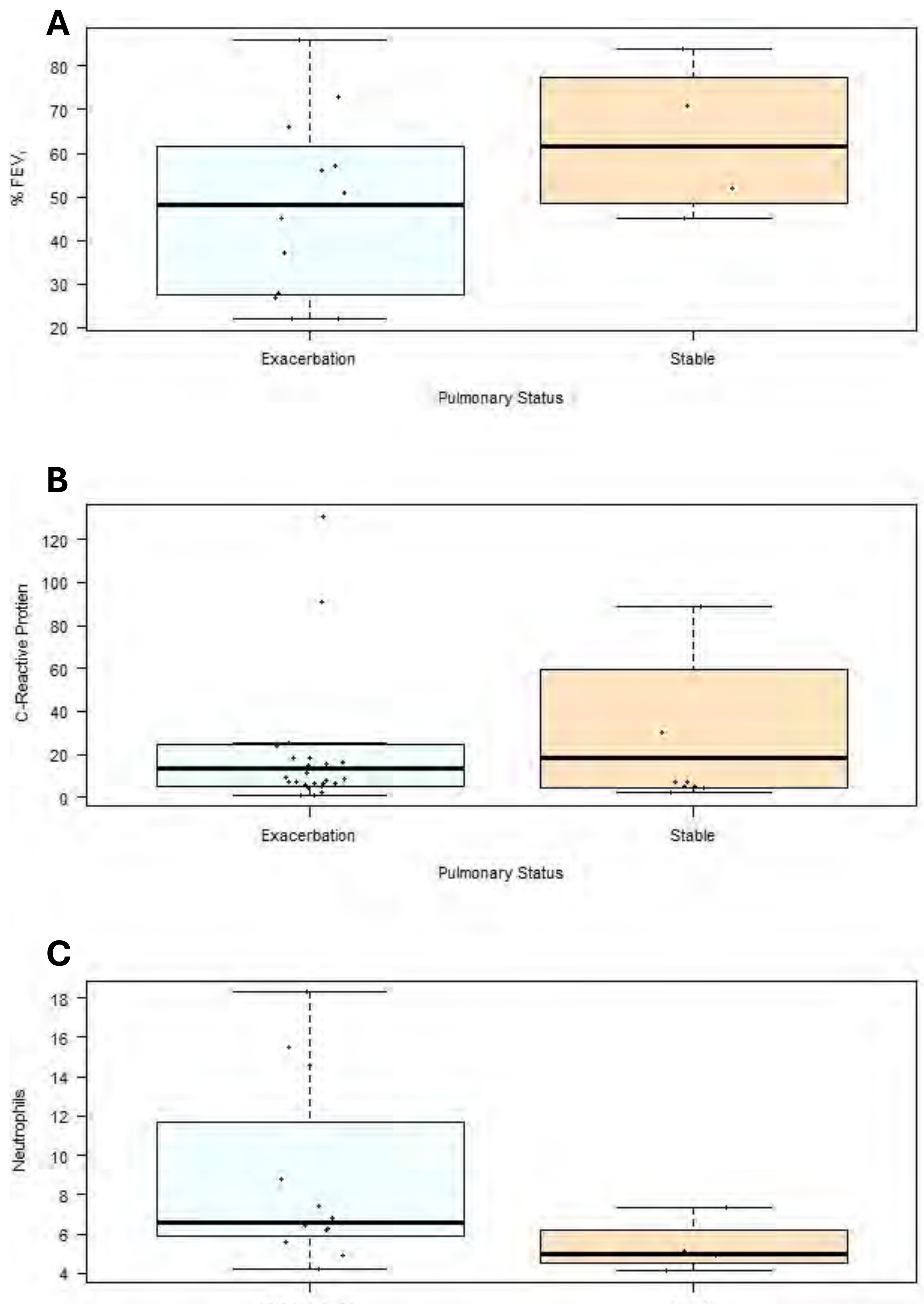


Figure 7. 3 The influence of %FEV_1 (**A**) C-reactive Protein count (mg/L) (**B**) and Neutrophil count (x10^9/L) (**C**) on pulmonary status, exacerbation (*n*=12) and stable (*n*=4).

7.4.3 Lung Function

Neutrophil, CRP count and exacerbation status were assessed against %FEV_1 using generalised minimum models and Spearman's rank correlation (Figure 7.4). The relationship between %FEV_1 and exacerbation status was significant ($F (1,8) = 5.9$, $p = 0.040$), as were the relationships between %FEV_1, neutrophils ($F (1,8) = 11.5$ $p = 0.009$), and CRP ($F (1,8) = 18.9$, $p = 0.002$). The relationship between %FEV_1, exacerbation status and neutrophil count was also found to be significant ($F (1,8) = 5.3$, $p = 0.0502$). Both Neutrophil count (Spearman's rank correlation: Rho=-0.621 $p < 0.010$) (Figure 7.4 A) and CRP (Spearman's rank correlation: Rho -0.63, $p < 0.008$) (Figure 7.4B) showed a similar trend of a significant negative relationships with lung function.

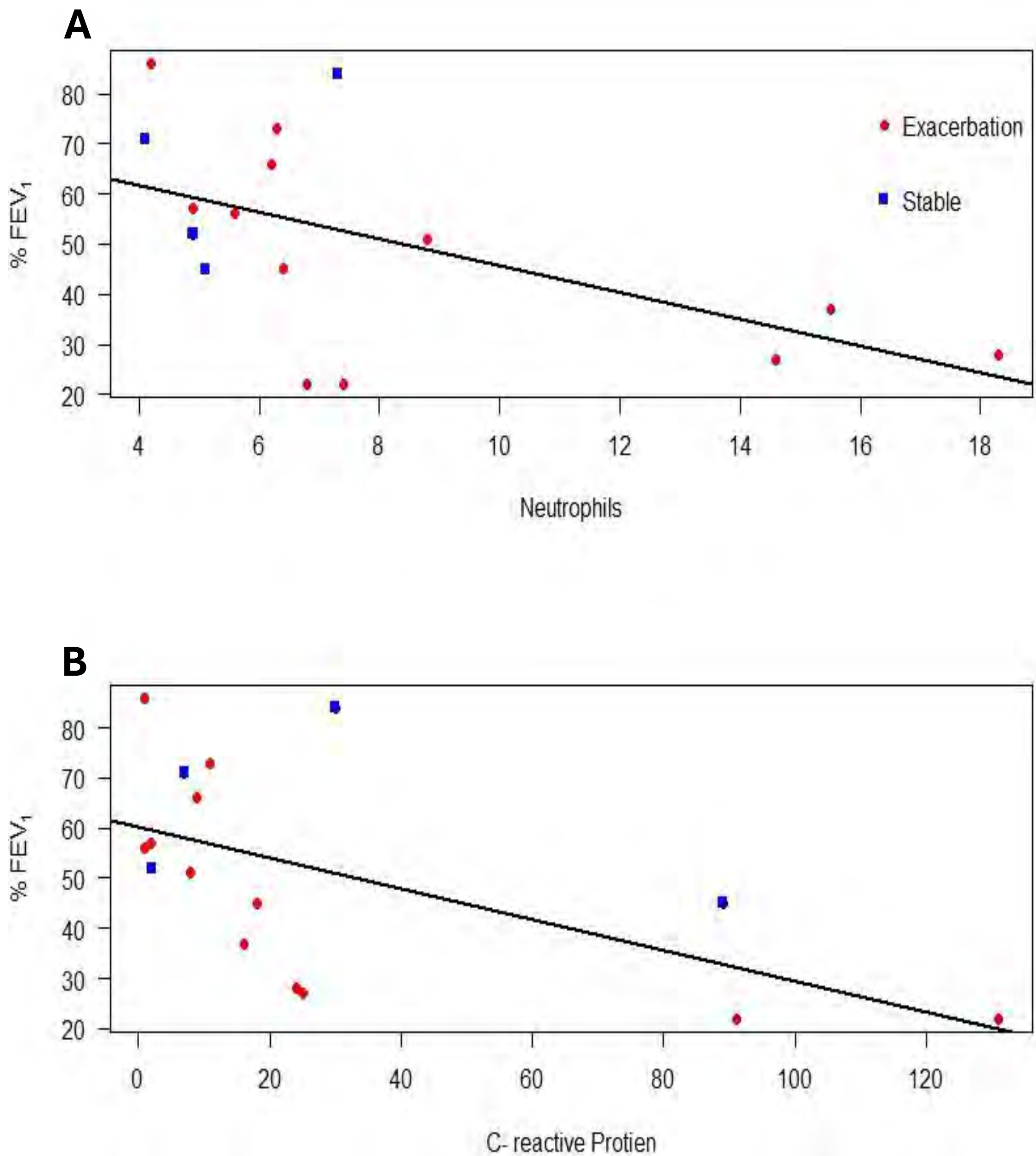


Figure 7. 4 Lung function (%FEV1) and its relationship to Neutrophil count (x10^9/L) $r^2$0.30 (**A**) and C-reactive Protein count (mg/L) r^2=0.33 (**B**).

7.4.4 Gene Regulation

Having established that there was a relationship between lung function, inflammation, and pulmonary status in these patients, the interplay between the host and infecting *Mycobacterium* was assessed. Here, three pathways, integral to bacterial survival in the host, were investigated (repair of cell walls, protection from reactive oxygen species, and efflux pumps) were tested against lung function and inflammation. The results show the regulation of repair (*lsr2* gene) has a non-significant negative relationship with neutrophil count (Figure 7.5A) (Spearman's rank correlation: Rho = -0.27, p = 0.296). The correlation to CRP (Figure 7.5B), also showed a non-significant negative relationship (Spearman's rank correlation: Rho = -0.48, p = 0.059). On the contrary the relationship between regulation of repair and %FEV_1 result showed a positive correlation but was still non-significant (Spearman's rank correlation: Rho = 0.42, p = 0.102).

The regulation of protection (sodA) exhibited non-significant association with neutrophil count (Figure 7.6A) (Spearman's rank correlation: Rho = -0.35, p = 0.181). The correlation to CRP (Figure 7.6B) again showed a non-significant relationship (Spearman's rank correlation: Rho = -0.104, p = 0.699), and the connection between regulation of repair and %FEV_1 result was non-significant (Spearman's rank correlation: Rho = 0.17, p = 0.512)

The results show the regulation of efflux (*erm41*) again had non-significant association with neutrophil count (Figure 7.7A) (Spearman's rank correlation: Rho = 0.06, p = 0.815), CRP (Figure 7.7B) (Spearman's rank correlation: Rho = 0.10, p = 0.691), or %FEV_1 (Figure 7.5C) (Spearman's rank correlation: Rho = -0.03, p= 0.896).

When the regulation of each gene was compared to each other (Figure 7.8) the results showed a significant negative correlation between the regulation of efflux and repair (Figure 7.6A) (Spearman's rank correlation: Rho = -0.73, p= 0.001) but non-significant relationship between efflux and protection (Figure 7.8B) (Spearman's rank correlation: Rho = 0.16, p= 0.534) or between repair and protection (Figure 7.8C) (Spearman's rank correlation: Rho = 0.05, p= 0.854).

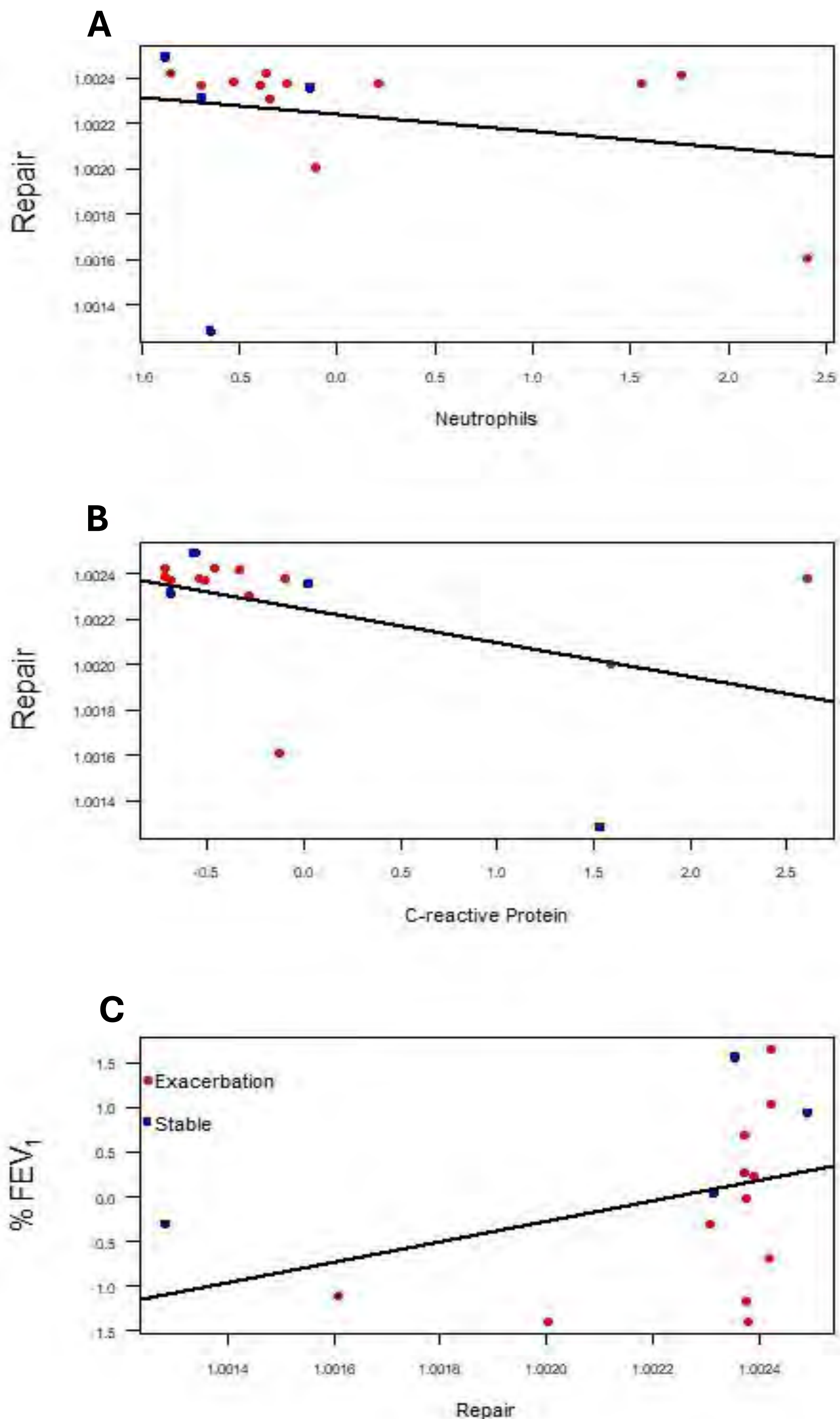


Figure 7. 5 The regulation of repair, showing the relative gene expression of the *lsr2* repair gene with the exacerbation (red circle) and stable (blue square) groups highlighted. Plotted against Neutrophil count ($x10^9/L$) r^2=0.2 (**A**), C-reactive protein count(mg/L) $r^2$0.14 = (**B**), %FEV_1(%) r^2=0.31 (**C**).

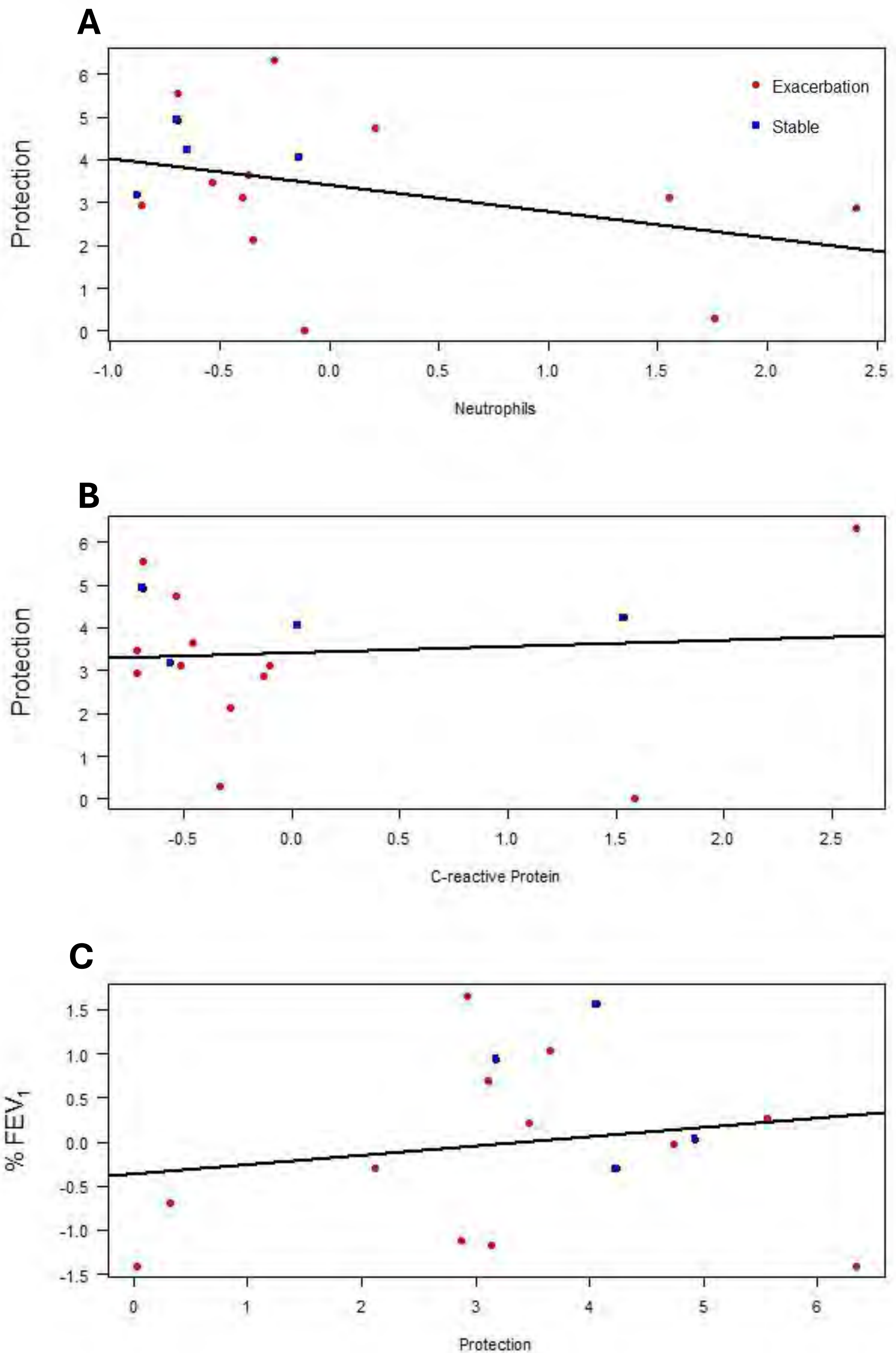


Figure 7. 6 regulation of protection, showing the relative gene expression of the *sodA* gene with the exacerbation (red circle) and stable (blue square) groups highlighted. Plotted against Neutrophil count (x10^9/L) r^2= 0.1(**A**), C-reactive protein count (mg/L) r^2=0.008 (**B**), %FEV_1 (%) $r^2$0.03 (**C**).

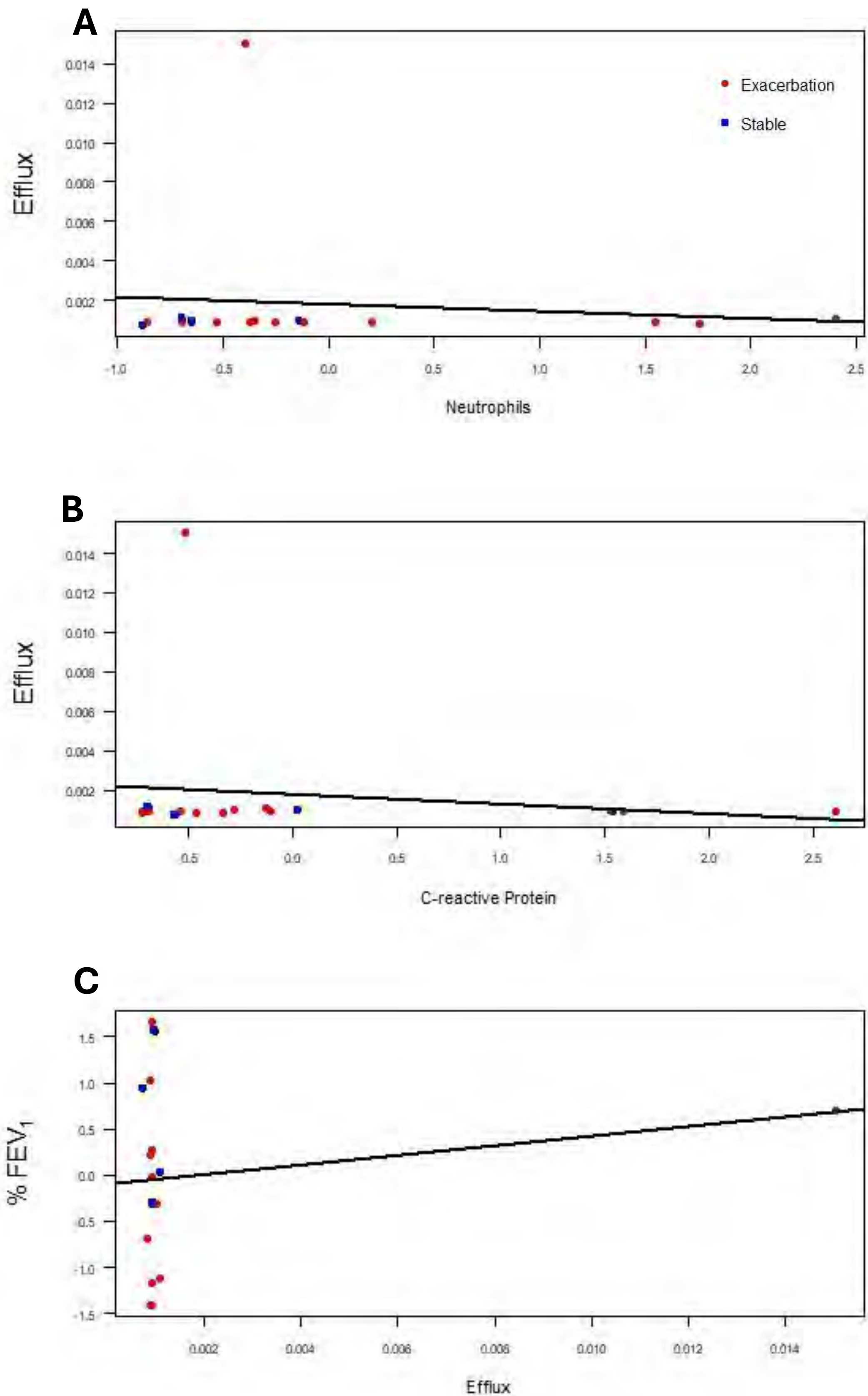


Figure 7. 7 The regulation of efflux showing the relative gene expression of the *erm41* efflux gene with the exacerbation (red circle) and stable (blue square) groups highlighted. Plotted against Neutrophil count (x10^9/L) r^2= 0.01(**A**), C-reactive protein count(mg/L) r^2= 0.01 (**B**), %FEV_1 (%) r^2=0.3 (**C**).

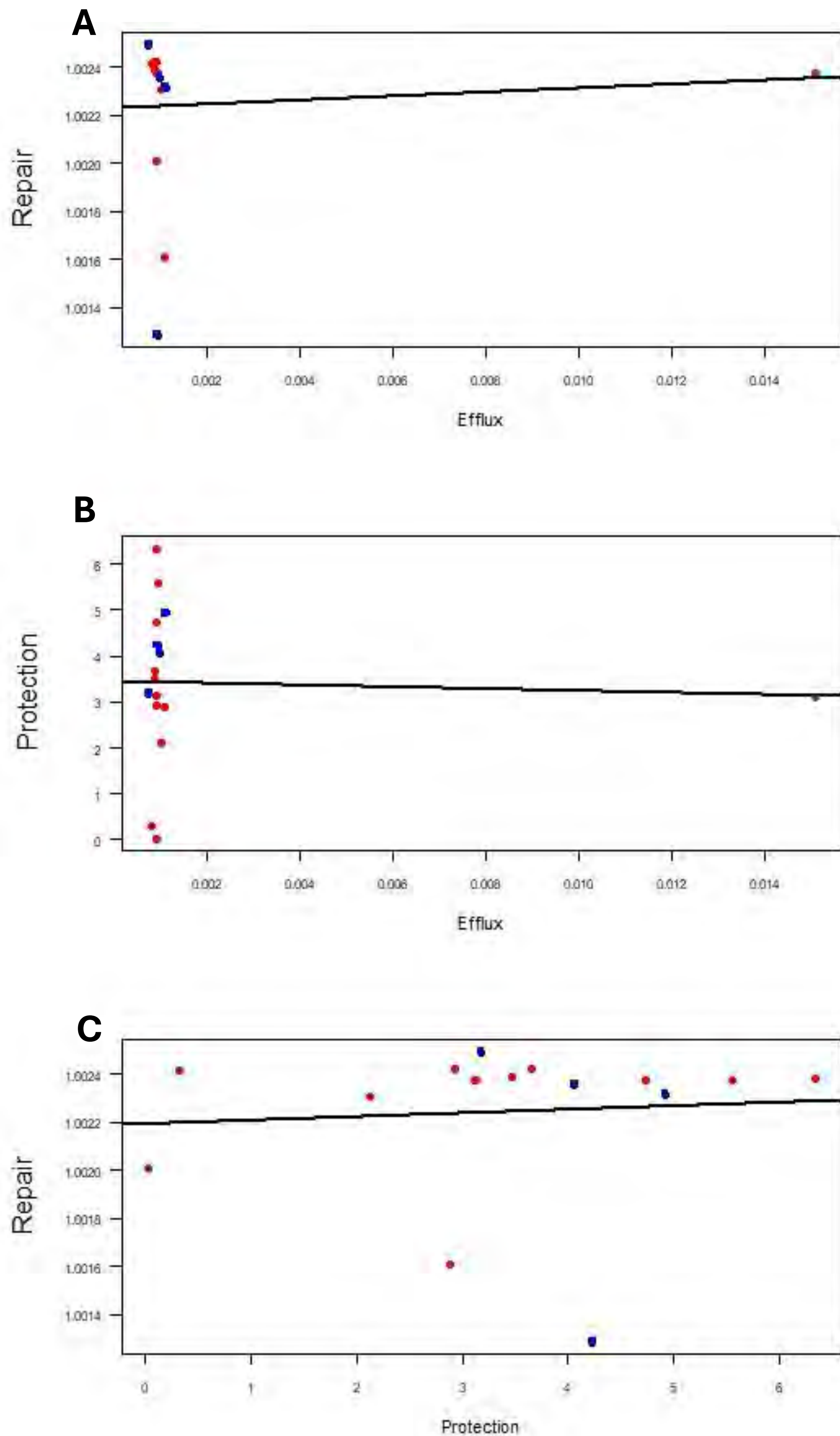


Figure 7. 8 The regulation of all genes of interest, showing the relative gene expression of the repair *(lsr2)*, protection *(sodA)*, and efflux *(erm41)* genes with the exacerbation (red circle) and stable (blue square) groups highlighted. Plotted repair and efflux r^2= 0.1 (**A**), protection and efflux r^2 =0.008 (**B**), repair and protection r^2= 0.03(**C**).

7.5 Discussion

Research focusing on mycobacteria regulation of virulence genes is an active area of investigation with studies aiming to comprehend the physiological and molecular mechanisms of virulence in mycobacteria (Smith, 2003; Pereira et al., 2020; Ferrell et al., 2022). Some studies have investigated the opportunistic pathogenicity of mycobacteria in various environments while others have explored the interplay between metabolism and gene expression (Gouzy et al., 2014; Rieck et al., 2017; Gago et al., 2018). *Mycobacterium* can gain a foothold in infection by using multiple strategies to effectively navigate the host immune defence system; evasion, survivability, and capture (Abe et al., 2020; Chai et al., 2020; Habtamu et al., 2022).

Mycobacterium gene expression to facilitate infection and survival during CF exacerbation is complex and will need a range of studies to fully understand the mechanisms occurring. By examining some of the genes that could be regulated during these events contributes to this understanding. Whilst there has been studies looking at the function and expression of *Mycobacterium* repair (*lsr*2), protection *(sodA),* and efflux (*erm41*), there have not been any studies to my knowledge looking at the regulation of these genes during CF exacerbation in patients positive for NTM-PD.

This chapter was designed as a small preliminary study to examine the mycobacteria expression of repair (*lsr2*), protection (*sodA*) and efflux pump (*erm41*) genes in CF sputum samples to evaluate any significant relationships between the regulation of these genes of interest to lung function (%FEV_1), and neutrophil and CRP count.

7.5.1 Lung Function

The effects of %FEV_1, CRP and neutrophils was assessed against pulmonary status (stable or exacerbation) to establish if a relationship was present (Figure 7.3). This study found that the patients who were not experiencing exacerbation (stable group) had a higher %FEV_1 than those who were suffering exacerbation. This result was also shown in Chapter 6 and is expected from previous research (Amadori et al., 2009; Bouzek et al., 2022) because reduction in lung function is one of the key clinical markers that herald an exacerbation (Waters et al., 2012). When assessing the effects of pulmonary status against neutrophil count this work showed a non-significant relationship but, when examined against %FEV_1, found a significant negative correlation (Figure 7.4A). However,

other studies have found a significant increase in the density of neutrophils during exacerbation states and an increase of CD11b expression (Martin et al., 2022) which is part of the phagocytic response (Agramonte-Hevia et al., 2002). This could be due to differences in study design. For example, Martin et al. (2022) examined blood samples using flow cytometry. Their cohort consisted of non-CF patients as the control group whereas, this study used RT-qPCR based methods and focused on the expression levels of genes between stable and exacerbating CF patients. The use of a non-CF control group in the Martin et al. (2022) study whilst beneficial in establishing a normal baseline, CF patients have higher baseline levels of neutrophils than non-CF groups (Dittrich et al., 2018; Bezzerri et al., 2019; Khan et al., 2019). Therefore, when comparing the two groups the CF group will always have a higher level of neutrophils. The use of a non-exacerbation CF group would have provided a more robust baseline in this instance.

The current study would have benefited with the inclusion of flow cytometry to add another dimension to the data. RT-qPCR is effective at measuring changes in gene expression levels, but it misses cellular changes (Adams, 2020). The inclusion of flow cytometry would have enabled the study to examine the changes in neutrophil or macrophage density in relationship to exacerbation status (Martin et al., 2022). Using the stable group to establish a baseline.

Other research examining changes in CRP counts found that the counts are highly unpredictable between individuals but there was an increase in levels during exacerbation in comparison to a recorded baseline, which suggests that the use of CRP as an marker for exacerbation may be unreliable due to this variability (Jung et al., 2021). When comparing increases in CRP and its relationship to %FEV_1 this study found a significant negative correlation (Figure 7.4B) which is synonymous with other studies. Shaaban et al. (2006) found a negative relationship between CRP and %FEV_1 in smokers during longitudinal study. As did a study focusing on CF patients, which also found in addition to a negative relationship it showed a more rapid decline in %FEV_1 in patients with high CRP levels (Matouk et al., 2016).

7.5.2 Gene regulation

This study then focused on the metabolic behaviour of the NTMs present in the infection. Regulation of repair (*lsr2* gene) (Figure 7.5), protection (*sodA* gene) (Figure 7.6) and efflux (*erm41* gene) (Figure 7.7), were all measured against %FEV_1, neutrophil and CRP count to

find any potential relationships between the host and infective agent. The results found that the repair was significantly upregulated with increasing neutrophil count, but non-significant correlations with %FEV_1 and CRP count. Whilst there are no other studies looking specifically at the regulation of *lsr2* and exacerbation states this does enable the hypothesis that when CF patients are undergoing exacerbation the *Mycobacterium* is actively regulating repair mechanisms to aid its survival. This could provide new insights into how NTM survives and proliferates during CF exacerbation. If repair is being prioritised, then *lsr*2 could be used as a potential drug target. As disruption of this gene would stop several vital survival and regulation processes (Chen et al., 2006; Gordon et al., 2010; Bartek et al., 2014; Le Moigne et al., 2019; Kołodziej et al., 2021). In MTB targeting *lsr*2 has been discussed in the past as disrupting, or incapacitating, its function would stop important cellular processes (Liu and Gordon, 2012). However, in NTMs this has yet to be explored.

The regulation of protective genes (*sodA*) and efflux (*erm41*) showed no significance to any of the factors tested. But, when efflux and repair (Figure 7.8) were compared the study found that there was a significant negative relationship indicating that the *Mycobacterium* is prioritising the exportation of moieties from the cell over repair, this allocation of resources could be potentially exploited for therapeutic benefit.

7.6 Conclusion and Next steps

This small study has demonstrated that there are some significant relationships between gene regulation and patient factors, it helps to inform future research about the regulation of neutrophils and membrane repair.

This study has numerous caveats such as small control groups, limited variation in patient demographics and small panel of target genes. The results have shown an under studied area in terms of mycobacteria gene expression and future work could include a panel of genes focusing on specific areas such as membrane repair or protection and incorporation of longitudinal analysis to examine patient gene expression levels at different stages of exacerbation. Highlighting these areas could help to inform drug target studies and enable a further understanding of the mycobacteria regulation of gene expression during CF exacerbation.

Chapter 8: Discussion

8 Introduction

The field of medical microbiology research has been transformed since the advent of next generation sequencing (NGS). This technology has allowed researchers to examine the complex relationships of the microbiome independent of traditional culture techniques (Hodkinson and Grice, 2015; Feigelman et al., 2017; Wensel et al., 2022). Microbiome research has highlighted that lung infection in diseases such as cystic fibrosis (CF) are polymicrobial in nature and often driven by a range of pathogens (Rogers et al., 2003; Dickson et al., 2013). Whilst this research is constantly evolving there are still research areas which need to be addressed.

One area that is of particular interest, in terms of CF lung disease progression, is the involvement of the emerging opportunistic pathogens known as non-tuberculosis mycobacteria (NTM), which have been increasingly detected during lung infection (Floto et al., 2016; Adjemian et al., 2018; Sokhi et al., 2021). In clinical settings detection of NTM is highly dependent on culture and requires specialist referral; this process is often lengthy and increases patient treatment times and could have detrimental long-term consequences on patient outcomes (Griffith et al., 2007; Daley et al., 2020). Certain NTM's are inherently resistant to some antimicrobials making them extremely hard to treat (Lambert, 2002; Skolnik et al., 2016), therefore, rapid species identification is extremely important (Tortoli, 2012; Daley et al., 2020).

The understanding of NTM complexes and their effects on the composition of the lung microbiota are research areas that need addressing. Studies in this area are sparse and the few available have focused on the prevalence (Floto and Haworth, 2015; Lipman et al., 2020; Abidin et al., 2021) or the clinical impact of NTMs on CF patients (Olivier et al., 2003; Bryant et al., 2013) rather than the changes in microbiota composition in the presence/absence of NTM complexes. With the introduction of CF transmembrane conductance regulator (CFTR) modulator therapy (Vertex Pharmaceuticals Incorporated, 2012), a drug that potentially changes the lung environment (Donaldson et al., 2018; Mok et al., 2023), this raises more questions regarding changes in microbiota community composition (Héry-Arnaud et al., 2019) and its effects on the dynamics of NTM lung infection in positive patients. The assessment of the changes in the lung microbiota in relationship to NTM infection and modulator therapy can help to further understand the long-term effects of this condition.

This thesis aimed to investigate the differences in microbiome communities in the CF lung in patients with positive or negative NTM lung infection. To better understand how NTM positivity influences the composition of the CF microbiome. The aims were achieved by optimisation of next-generation sequencing (NGS) techniques to enable the detection of species in low diversity samples such as metalworking fluids (MWF)(Chapter 3) and CF sputum. This optimised method was then utilised to understand the community composition of the microbiome in patients positive for NTMs, how the different types of NTMs influence the surrounding microbiome and how NTM complexes are structured in CF lung infection (Chapters 4 and 5). The thesis also investigated the relationships between NTM positivity and patient factors: percentage predicted forced expiratory volume in 1 second (%FEV1), gender, CF transmembrane regulator (CFTR) modulator status, exacerbation status and CFTR genotype (Chapter 6). To begin to understand the role of NTMs in disease, a small preliminary study was done using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) examining the regulation of NTM gene expression during exacerbation states and its relationships to the same patient factors measured in the previous chapter (Chapter 7).

8.1 Optimisation of NGS for Low Diversity Samples.

Chapter 3 focused on optimising multiplex NGS gene sequencing on the Illumina MiSeq to sequence the 16S total microbiome and a target specific to NTM complexes using phased primers. In order to mitigate known issues with cluster identification in low diversity sample sets (Krueger et al., 2011), like the MWF used in this chapter as experimental samples or CF sputum (used in subsequent chapters 4-6) a multiplexed and phased primer (Figure 3.1) approach was used. Introducing artificial base diversity as described previously by Wu et al. (2015) and Naik et al. (2020) (Section 3.4.4).

Parts of this chapter focused on the optimisation and comparison of two known sequencing NTM targets; *hsp*65 (Telenti et al., 1993) and *rpo*B (Manjeese et al., 2017). When examining other studies using NGS sequencing protocols for detection of NTMs some exclusively used one gene target such as the 16S rRNA v4 region (Caverly et al., 2016), *hsp*65 target (Cowman et al., 2018), or *rpo*B target (Manjeese et al., 2017; Nasiri et al., 2017). Others sequenced multiple targets separately such as 16S rRNA V4 and hsp65 (Gebert et al., 2018), 16S rRNA and *rpo*B (Shen et al., 2022), *hsp*65 and *rpo*B (Clarke et al., 2022). In terms of multiplexing 16S rRNA and an NTM specific target using phased primer sets there have been no other studies to my knowledge using this methodology.

There is also a group of studies focused on determining what gene target is more superior in terms of NTM detection. Most studies found the 16S rRNA gene not efficient in NTM identification due to its inability to differentiate between extremely high similarity sequences (Slany et al., 2010; Van Ingen et al., 2010; Kim and Shin, 2018; Morais et al., 2022). This is synonymous with the work done here which showed that the 16S rRNA gene sequencing is adequate in detection of NTM genera in low diversity samples. But it failed to detect specific NTM species within the mock community (Figure 3.14), only detecting 30% of NTM strains. However, in terms of giving a clear overview of the total microbiota the 16S rRNA gene target is sufficient as it detected the widest range of bacterial species in the mock community, though some species detected were not expected. The unexpected species were most likely due to the primers inability to distinguish between closely related species (Kim et al., 2011), external contaminants (Sheik et al., 2018) or incorrect allocation of taxa during sequence analysis (Schloss and Westcott, 2011).

Through robust optimisation and testing it was found that the *rpo*B gene successfully detected a wider range of slow growing (SGM) and rapid growing (RGM) NTM taxa in both the mock community, (~90% expected taxa, (Figure 3.16)) and in the experimental samples (17 total NTM species and subspecies (Figure 3.17)), which was higher than the *hsp*65 gene (mock community ~40% expected taxa, 2 additional (Figure 3.15) experimental 4 total NTM species and subspecies (Figure 3.17)). While identification was limited the *hsp*65 primers did detect *M. avium, M. avium paratuberculosis, M. bovis, M. kansasii* and *M. austroafricanum* all of which are SGM (<10 days incubation time). It also detected members of the *M. abscessus* complex (MABSC) and *M. chelone* both considered RGM (>7-day incubation time). Though the *hsp*65 gene detected less NTM species and subspecies compared to *rpo*B target (Figure 3.15/3.16), the NTMs that were detected were predominantly of the SGM group. This result is similar to research conducted previously by Kim and Shin (2018) and Ong et al. (2010) which showed *hsp*65 detecting mostly (SGM) NTM species. This result could indicate that target gene selection is dependent on what mycobacteria is being examined/expected. When testing polymicrobial samples *rpo*B would be a better target due to its wider capture of species as shown here and therefore was selected as the NTM sequencing target for the studies in chapters 4-6.

The method optimised here was adopted for the NTM and the wider CF lung microbiome studies in this thesis (Chapters 4-6), however, the method performed worse in chapters 4 and 5 then during the optimisation stages. This is likely due to a range of factors: different sample types (sputum was used in subsequent chapters), transportation of samples and sample handling. Another factor to consider when evaluating differences in the performance is that MWF will be more selective to certain types of bacteria and mycobacteria and samples originating from CF sputum/swabs will be selective for a different range of microorganisms, with some species may be detectable in both mediums (Bonnet et al., 2020).

This method has a lot of potential in the field of NTM research because it has enabled the investigation of the composition of specific NTM complexes, which can be applied to NTM complexes in other pulmonary disorders such as bronchiectasis, asthma, and COPD. It can also be applied to NTM complexes in extrapulmonary disorders and environmental systems, enabling a greater understanding of how NTM complexes are composed in relationship to their host, associated diseases, and surroundings.

8.2 The Presence of NTM in CF Lung Disease

Previous research has shown that patients who have a microbiota with low diversity and high species dominance tend to have poorer clinical outcomes in relation to lung function (LiPuma, 2012; Cuthbertson et al., 2020; Metzger et al., 2021). These patients are also at higher risk of increased frequency of pulmonary exacerbation which, leads to a faster progression of lung disease (Carmody et al., 2013; Metzger et al., 2021). Infection with opportunistic pathogens such as NTMs has also been associated with poor clinical outcomes and decline in pulmonary function (Floto and Haworth, 2015; Park et al., 2016). Earlier research analysing the community composition in CF lung microbiota and its relationship to NTM positivity is extremely limited, therefore highlighting a research gap that needs to be addressed. This study adopted a layered approach to assessing the overall microbiota, the type of NTM present in the microbiota and the composition of the NTM complexes.

8.2.1 The Wider Lung Microbiota and NTM Positivity

This study found differences between the composition of the CF lung microbiota in NTM positive and negative patients (Chapter 4) and those on modulator therapy (Chapter 5); using the data generated by 16S rRNA gene sequencing obtained from methods developed in Chapter 3. The sample cohorts were measured for alpha diversity and composition similarity. In both studies the alpha diversity analysis was non-significant (Section 4.5.2/Figure 4.4;Section 5.41/Figure 5.5.2).

The evaluation of the composition did find clear and significant differences in the composition of the NTM positive/negative cohort (Figure 4.8)(Section 4.5.2.4) with those patients positive for NTM having lower levels of *Pseudomonas aeruginosa* and higher levels of *Staphylococcus aureus* than the NTM negative group. This is in line with previous work which found NTM positive patients had lower levels of *P. aeruginosa* (Kamata et al., 2017) and higher levels of *S. aureus* (Olivier et al., 2003; Wickremasinghe, 2005; Aksamit et al., 2017). As to why the presence of NTM shows lower levels of *P. aeruginosa* and higher levels of *S. aureus* is unknown. This could be due to treatment for NTM using aminoglycosides which some patients may have been taking at the time (Raaijmakers et al., 2021). Aminoglycosides have activity against Gram-negative bacteria like *P. aeruginosa* which in turn enables the proliferation of Gram-positive bacteria such as *S. aureus.* This study did find that the NTM positive cohort had elevated levels of *Burkholderia multivorans (*Table 4.3/4.4, Figure 4.8/4.9) and remained high when

examining the rank abundance of the NTM type (MAC, MABSC, Other NTM) (Table 4.5/4.6, Figure 4.14). This fits into the hypothesis that NTM treatment creates a niche for other bacteria to thrive like *B. multivorans* which is intrinsically resistant to some aminoglycosides (Moore et al., 2001).

Another theory to consider is the possible competitive effects of NTMs on other usually dominant species. Whilst this has not been an area of direct research, we are able to postulate that with the unique physiology of NTMs (Section1.4.5) this is a possibility. For example, some NTMs are able to survive within several host immune cells such as macrophages (Sousa et al., 2019), this is likely due to the mycolic-acid, lipid-rich membrane (Figure 1.2), which grants the intrinsic resistance to extreme stress and resistance to antibiotics (Hoffmann et al., 2008; Zuber et al., 2008; Pereira et al., 2020). Competitive effects of pathogenic bacteria have been examined in a wide range of other studies. One study found that *Burkholderia dolosa* can displace other bacteria in the CF lung causing *cepacia* syndrome via growth inhibition mechanisms (Perault and Cotter, 2018). Other studies showed that some strains of *Achromobacter xylosoxidans* was able to inhibit the growth and pigmentation of *P. aeruginosa* (Menetrey et al., 2020). *P. aeruginosa* is able to outcompete other species via production of antimicrobial chemicals (Tashiro et al., 2013). Furthermore, the high levels of *B. multivorans* detected in this study is of great concern clinically as a member of the *Burkholderia cepacia* complex (BCC) (Vandamme and Dawyndt, 2011). BCC is known to cause *cepacia* syndrome which causes fatal necrotizing pneumonia and occasionally bacteraemia (Silva et al., 2016; Scoffone et al., 2017).

Chapter 5 examined the microbiome of CF patients on CFTR modulator therapy who were positive or negative for NTMs. The multivariate tests (Section 5.5.2.2) showed significance between the NTM positive and negative groups but showed a different distribution of taxa when compared to the same analysis in chapter 4 (Figure 5.22B). The taxa recovered from chapter five showed higher levels of taxa which are known to be recovered from the oral cavity (Kaci et al., 2014; Mashima et al., 2021; Qi et al., 2021) and other pathogens such as *P. aeruginosa* and *B. multivorans* being ranked lower in abundance. The rise in the detection of oral microbiota is likely due to the patients limited ability to spontaneously produce sputum (Tewkesbury et al., 2021) and the lower ranking pathogens are possibly because of an undesirable environment created by modulator therapy (Héry-Arnaud et al., 2019).

8.2.2 NTM Complexes

The NTM complexes detected by the sequencing of the *rpo*B gene found significance in the Fisher's alpha diversity measure in chapter 4 (Figure 4.18) this is indicative of differences in diversity within the groups (MAC, MABSC and Other NTM). However, the study found non-significance in all the other alpha diversity measures in both chapter 4 and 5. This is likely due to inadequate sample sizes used for the analysis. If there were larger groups for comparison the results may have been significant.

However, the measures of similarity show large dissimilarity between the NTM positive and Negative groups in both chapter 4 (Section 4.5.4.3) and Chapter 5 (Section 5.5.4.2), the dissimilarity is due to the presence/ absence of certain NTM taxa from each group. The results produced were expected when examining different complexes in terms of the examination of NTM complexes and their composition in CF lung infection but has not been evaluated before. Studying this area is vital to understand the structure of NTM complexes in CF lung infection, which could provide future drug targets and enhance our understanding of the survivability of these organisms.

8.2.3 Host Characteristics and Its Relationships to Lung Function

When evaluating the findings in Chapters 4 and 5 and relating them to NTM status and percentage predicted forced expiratory volume in 1 second (%FEV_1)(Section 6.3.4), this study found that NTM culture positivity had a significant association with %FEV_1 and on average patients negative for NTM had a higher average %FEV_1) than those culture positive as expected from previous work which showed patients infected with *M. abscessus* showed increased decline in %FEV_1 (Esther et al., 2010; Qvist et al., 2016; Skolnik et al., 2016). NTM positive patients have higher levels of pathogens than negative patients (Table 4.3), this reduction in average %FEV_1 is to be expected as previous research has shown that microbiomes with dominant pathogens and less diversity have faster rates of declining %FEV_1 (Cuthbertson et al., 2020; Frey et al., 2022). However, when evaluating the association of modulator therapy and %FEV_1 (Section 6.4.1) this study showed non-significance. This was unexpected as previous studies have reported an improvement in %FEV_1 when patients start modulator therapy (Ramsey et al., 2011; Burgener and Moss, 2018; Muilwijk et al., 2022). The work done in chapter 5 showed less recovery of pathogens (Table 5.3/5.4) and patients in this cohort (Table 5.1/5.2) taking Kaftrio had a higher average %FEV_1 than those on Symkevi, Ivacaftor and no modulator.

Chapter 6 also evaluated patient demographic data (Table 6.1/6.2) against %FEV_1. Its main finding was that examining single factors such as age, gender, CF genotype and CFTR modulator status against %FEV_1 showed non-significance. Previous research suggests that influence on %FEV_1 is multifactorial (Demko et al., 1995; Amadori et al., 2009; Konstan et al., 2012) and this study found that when %FEV_1 was examined against multiple factors; gender and exacerbation status, gender, modulator status and exacerbation status some of the relationships were significant, aligning to trends in studies mentioned previously (Demko et al., 1995; Amadori et al., 2009; Konstan et al., 2012).

Chapter 6 showed the importance of considering a wide range of factors when evaluating patient lung function. It also showed that NTM culture status is a factor in lung function decline but, it should be considered alongside other influences like, gender, modulator therapy and exacerbation status. This information plays an important part in individual treatments and further adds to the growing area of research involving NTMs and CF patient outcomes.

The examinations carried out in chapters 4, 5 and 5 are impactful to the NTM field and because the results have increased understanding of how NTM positivity could cause a difference in composition in comparison to NTM negative samples. With differences in the distribution of CF pathogens. It also showed that with the dawn of modulator therapy and the change in the environment created by this treatment infection can still endure. It also added to the understanding of how NTM positivity and the type of NTM present can affect the patient through evaluation of %FEV_1 correlation and that other important factors, age, gender, and genotype must be considered.

The detection of the NTM complexes and their arrangements are also important to the field, this study has shown that the methods implemented are affective at detecting NTMs down to sub-species levels, though further investigation is needed, this study provides evidence that slight differences do exist in the arrangement of the NTM complexes. This information can be used to further understand how the complexes are arranged, how this arrangement could affect NTM pathogenicity and create further studies for potential drug targets.

8.2.4 Interactions Between Host Immune Response and NTM During Respiratory Infection

Chapter 7 consisted of a preliminary study aiming to evaluate the interplay between host immune response; C-reactive protein (CRP) and neutrophil counts and the regulation of repair, protection, and efflux by NTMs during exacerbation phases. It found similarly to Chapter 6 and other studies that patients experiencing exacerbation (Figure 7.4A) had a higher %FEV_1 than those who were stable (Amadori et al., 2009; Bouzek et al., 2022). This study also showed a significant negative correlation in the relationship between CRP and %FEV_1 (Figure 7.4B) which is in line with a previous study that found a negative relationship between the same two variables and postulated that low-grade inflammation complements reduction in pulmonary function (Aronson et al., 2006; Shaaban et al., 2006; Matouk et al., 2016). The use of CRP as a marker for exacerbation has also been debated in the literature. Jung et al. (2021) found that CRP levels vary between patients therefore using it as a measure of pulmonary exacerbation is problematic. This was also confirmed by several other studies with one concluding that the use of CRP was unable to detect treatment associated differences in response to exacerbation (VanDevanter et al., 2022), and CRP levels are not associated with exacerbation severity but, are influenced by other factors such as pathogen colonisation and antibiotic treatment (Girón-Moreno et al., 2014). A negative relationship was also found when examining neutrophil count and %FEV_1 (Figure 7.4B). Some studies have shown that heightened levels of circulating neutrophils are associated with increased decline in lung function by causing airway injury during chronic inflammation (Jasper et al., 2019; Backman et al., 2020; Voynow and Shinbashi, 2021).

The study shifted its focus to the associations between the mechanisms for the regulation of repair (Figure 7.4), protection (Figure 7.5), and efflux (Figure 7.6) against %FEV1, neutrophil and CRP count, with no significant relationships observed. Conversely, when the regulation of efflux and repair (Figure 7.7A) expressions were measured against each other, a significant negative correlation was found. This could indicate that the NTM prioritises exporting poisons out of its cell rather than focusing on cell growth or repair.

This was a very small preliminary study, though it did find that some associations are of interest it gives future research a starting point and further adds to the area of NTM gene expression work whilst highlighting an under researched area.

8.3 Study limitations

The study had several limitations throughout.

The *hsp65* primers had to undergo a large amount of optimisation, this could have affected its downstream capabilities resulting in a less optimal primer. In some instances, optimisation by altering the annealing temperature (Ta) can change the primers specificity, if the Ta is too high then the primer could produce low yields and poor-quality sequences. If the Ta is too low, then non-specific amplification can occur (Rychlik et al., 1990). Both examples can lead to downstream issues in sequencing library preparation including the formation of chimeras, loss of sequencing data and low-quality data.

This study was limited by non-standardised databases for the analysis of *rpo*B and *hsp*65 sequences. The database used was NCBI BLAST, which while useful for 16S rRNA sequencing proved difficult when accurately assigning taxa via the *rpo*B or h*sp6*5 data. To alleviate this issue only sequences with <99% similarity were used. The limitations of using a targeted amplicon such as *rpo*B or *hsp*65 is the partial coverage within the genus, as this study has shown both primer sets succeeded in detecting NTMs within both the mock community and the experimental samples but differed in the taxa identified.

Chapters 4, 5 and 6 had similar limitations. The main limitation was the use of the postal pack sample collection method (Section 2.1.1.1). Even though this method allowed the continuation of sample collection during the pandemic the metadata obtained was collected by the participants which could contain inaccuracies regarding NTM positivity, treatment, or lung function. The main reasons for the inaccuracies would largely be due to the participants lacking access to up-to-date medical records and in some cases would have been providing information from memory. Therefore, to ensure clinical metadata is accurate and up to date liaison with clinical personnel is essential as discrepancies could create inaccurate results.

Chapter 5 suffered from a drop in data quality when compared to chapter 4. There are a few possible reasons for this; most samples collected were via cough swab. The recovery of microbial DNA from cough swabs have been found to be contaminated, provide less sensitivity, and produce an inaccurate view of the lung microbiome (Fenn et al., 2022; Thornton et al., 2022). Additionally, the samples collected from the University of Vermont (UVM) in the United States were transported back to the United Kingdom via commercial airline. The samples were extracted onsite at UVM and transported as DNA extractions.

As part of routine airport security measures, the samples would have gone through a cabinet x-ray system which produces radiation that can degrade double-stranded DNA (Gloor et al., 2006). Therefore, transportation via airline should be avoided where possible.

The NTM positive group was dominated by females which could lead to a gender bias. Previous research has stated that some females with certain morphological features have been pre-disposed to MAC infection regardless of an underlying health condition (Reich and Johnson, 1992; Kim et al., 2008) which could be creating a bias toward female disposition in this study.

The analysis regarding modulator therapy would have benefited from a more comprehensive selection of modulators used in the study. The NTM positive and negative group participants mostly used Kaftrio, with few taking other types. This meant that, unfortunately, this study was unable to examine the differences in NTM complexes and lung function in each type of modulator therapy, as the sample group would have been too small.

Chapter 7 was a very small preliminary study which had numerous limitations. Firstly, the sample set was small which limits the accurate determination of the results. The study demographics were also limited due to this small sample size with small variations in CFTR modulator status, NTM type and gender which leads to a poor representation of the target population. The study design was limited by a small panel of genes being tested which is not representative of all virulence expression occurring at the time.

8.4 Future work

The optimisation and multiplex method could be used to test a range of sequencing targets but needs further streamlining to enable maximum data capture. This would lead to the testing of other target specific amplicons in pathogenic complexes such as *Burkholderia cepacia* complex or *Mycobacterium tuberculosis* complex. The method is also transferrable between NTM complexes in other chronic pulmonary and extrapulmonary disease.

The adaption of these methods to target larger amplicons of the target genes using different sequencing technologies such as the Nanopore GridION™ (ONT Ltd.), would further elevate this and future studies. Platforms used in third generation sequencing

produce longer reads than its predecessor (NGS) which can increase the coverage and resolution of the target. There is also less chance of PCR artefacts being introduced during library preparation, providing higher quality data (Athanasopoulou et al., 2021).

Chapter 4 highlighted an increased level of *B. multivorans* within the NTM positive group, further investigation of this and the potential niches created by NTMs would be beneficial for the understanding of the effects of treatment on the host microbiome and for informing future research. This investigation could be carried out *in vitro* through examination of NTM complexes and *B. multivorans* co-habitation and their combined response to antimicrobial therapy to see if this does create an opening for the *B. multivorans* to proliferate. This type of model could be applied to several other co-habitation scenarios; NTM and MTB, NTM and *P. aeruginosa* or NTM and *A. fumigatus,* even MAC and MABSC co-habitation could be tested this way. Investigation of the possibility of inhibitory peptides could also be examined in these scenarios to see if one organism has a competitive edge over others.

Testing NTM complexes in modulator therapy in patients taking a range of modulators would be beneficial to examine if the type of modulator has any effect on NTM culture positivity or detection of NTM from sequencing data.

Longitudinal examination on NTM complex composition and changes in patients pre and during modulator therapy would increase understanding on how NTM complexes are adapting to modulators and if treatment length shows any reduction in NTM positivity.

The preliminary study conducted in Chapter 7 did highlight some significant relationships occurring between the regulation of NTM survival and patient factors and highlighted an area for future work. The examination of a larger panel of genes possibly through RNA sequencing which would provide information about the gene expression pathway rather than focusing on single genes, would give a comprehensive overview of the mechanisms occurring during patient exacerbation. A further study with a wider patient demographics and larger cohort would be beneficial to increase the knowledge in this area and further understand how NTMs work during exacerbation stages.

9 Conclusion

This research investigated differences in the lung microbiota in patients' culture positive or negative for non-tuberculosis *mycobacteria* and in those on CFTR modulator therapy. This was achieved by the optimisation of multiplex next generation sequencing methods using 16S rRNA and *rpo*B targeted amplicon phased primer sets.

The following conclusions can be drawn from this research:

- The multiplex amplicon sequencing of 16S rRNA and *rpo*B using phased primers is an innovative method for detecting non-tuberculous mycobacteria (NTM) species and the background microbiome concurrently. This approach holds the potential to significantly reduce the time and cost required for NTM detection in clinical and experimental samples.
- The evaluation of NTM and the lung microbiota showed the detection of NTM in CF sputum samples is more complex than just a positive or negative diagnosis. The presence of these microorganisms influences the possibility for other pathogens to gain an advantage such as members of BCC which can have detrimental outcomes for patients.
- Novel insight was gained through the evaluation of the differences in the composition of CF lung microbiota in relationship to NTM and CFTR modulator status. It showed small differences in richness, diversity, and dominance but these differences were not statistically significant. However, significant differences in microbiota composition were found between the groups when evaluated using multivariate methods.
- Factors influencing $\%FEV_1$ in CF populations is multifactorial and that NTM culture status does influence $\%FEV_1$ which has implications for treatment.
- There are some significant relationships between gene regulation and lower $\%FEV_1$. This information helps to inform future research about the regulation of neutrophils and membrane repair, which could potentially be used as drug targets for *Mycobacterium* treatment.

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11 Supplementary Materials

11.1 Patient Questionnaire

When providing each sputum sample, please complete this questionnaire.

To assist accuracy, please write in CAPITALS using a black or blue pen

Patient ID ________________________ (*Provided by CF clinic*)

Sample Number___________________

Sputum sample Date (*DD/MM/YY*)**:** _____/_____/_____

Is this your First /Second / Third (*Please circle*) **sample provided for this study?**

Age (*years*):_________________ **Sex:** Male/Female/Prefer not to say (Please circle)

Genotype 1:____________________ **Genotype 2:**______________________ (if known)

Have you started Kaftrio treatment: Yes / No (*Please circle*)

If yes, date Kaftrio Treatment started (*DD/MM/YY*)**:** ______/_____/_____

Have you previously received Orkambi, Symkevi, Kaftrio, or similar (please state which and approximate dates of treatment, otherwise state N/A):

____

____

____

Do you have CF related diabetes: Yes / No (*Please circle*)

Do you take Creon with your meals: Yes / No (*Please circle*)

Most recent FEV1 % result: ________________Date of result: ____/_____/_____

Was this recorded on home spirometry or hospital equipment? Home/hospital

How would you describe your current health status: Stable / Treating new infection/recovering/declining (*Please circle*)

When was your first NTM infection identified: (date) _____/_____/_____

Are you currently being treated for NTM infection? Yes/No

Please turn over....

What antibiotics are you currently taking?	What antibiotics have you taken in last 3 months?	What anti-fungal treatment are you currently taking?
Please circle all that apply		
Augmentin Azithromycin Aztreonam (Cayston) Cefuroxime Ceftazidime Ciprofloxacin Clarithromycin Colistimethate/Colistin/Promixin Doxycycline Erythromycin Etracycline Flucloxacillin Imipenem Levofloxacin Meripenem Minocycline Tigecycline Tobramycin	Augmentin Azithromycin Aztreonam (Cayston) Cefuroxime Ceftazidime Ciprofloxacin Clarithromycin Colistimethate/Colistin/Promixin Doxycycline Erythromycin Etracycline Flucloxacillin Imipenem Levofloxacin Meripenem Minocycline Tigecycline Tobramycin	Itraconazole Voriconazole

Vancomycin	Vancomycin	
Other: please name	Other: please name	Other: please name

Any additional information you would like to add?

Thank you for participating in our study.

11.2 Postal Pack

User Guide

Respiratory Sample Collection Through the Postal Service.

Contents

Kit Description 3

Kit Contents 4

Important Please Read! 5

Getting Started 6

How to use the postal kit if you are producing sputum. 8

How to use the postal kit if you are not producing sputum. 11

Returning the Pack 15

Kit Description

This single use , pre-prepared kit contains:

- 1 x 15ml Sterile Falcon tube with a red lid Tube A
- 1 x 5ml Sterile Bijou tube Tube B
- 1x Cough swab in a 15ml transport tube Tube C
- 1x Sealable polythene bag

Tube A - is empty and is for collection of sputum.

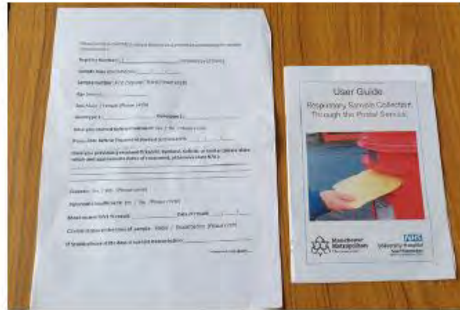
Tube B ***(please see safety sheet on page 5)*** - contains DNA/RNA Shield™, which preserves and protects the quality of the sample in transit to the lab (Zymo Research).

Tube C - is a cough swab in a clear transport tube.

Also included - Participant questionnaire, sealable polythene bag

Please do not discard the envelope the kit comes in as this is pre-paid and needed to return the sample.

Kit Contents



Kit guide and patient questionnaire

Self addressed pre-paid envelope

Tube A, 15ml Sterile Falcon tube with a red lid

Tube B, 5ml Sterile Bijou tube, contains DNA/RNA Shield™.

Tube C, Cough swab in a 15ml transport tube

1x Sealable polythene bag

Important Please Read!

Please read this information before using the kit.

Please keep this leaflet. You may need to refer to it again.

If you have any questions, or if there is anything you do not understand please ask the clinic.

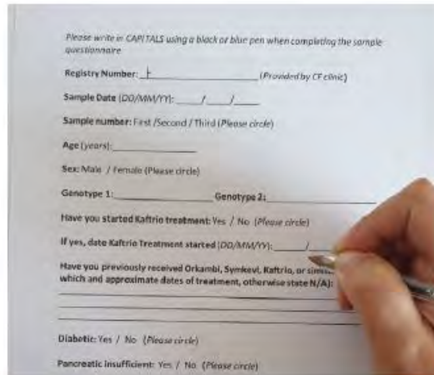
DNA/RNA ShieldTM (Zymo Research) Tube B safety information. Warnings and precautions:

- ***FOR EXTERNAL USE ONLY.***
- ***DO NOT*** drink
- Avoid contact with skin.
- The DNA/RNA Shield™ can be ***HARMFUL IF SWALLOWED*** and may cause irritation if exposed to skin and eyes.
- If DNA/RNA ShieldTM comes into contact with your skin, immediately wash the affected area with soap and water.
- If the DNA/RNA ShieldTM comes into contact with your eyes, immediately flush eyes with water.
- Notify your doctor if irritation develops.
- ***DO NOT*** use if tube is damaged.
- ***DO NOT MIX*** with bleach or other household cleansers.
- ***STORAGE AND STABILITY:*** This product should be transported and stored in its original container until use.
- For further safety information contact Zymo Research Corporation directly.

H302: Harmful if swallowed
H315: Causes skin irritation
H319: Causes eye irritation

Getting Started

Step 1: Carefully read the safety information included in this guide on page 5.



Please write in CAPITALS using a black or blue pen when completing the sample questionnaire

Registry Number: ______ *(Provided by CF clinic)*

Sample Date (DD/MM/YY): ___/___/___

Sample number: First /Second / Third (*Please circle*)

Age (years): ______

Sex: Male / Female (Please circle)

Genotype 1: ______ **Genotype 2:** ______

Have you started Kaftrio treatment: Yes / No (*Please circle*)

If yes, date Kaftrio Treatment started (DD/MM/YY): ___/___

Have you previously received Orkambi, Symkevi, Kaftrio, or simi... which and approximate dates of treatment, otherwise state N/A):

Diabetic: Yes / No (*Please circle*)

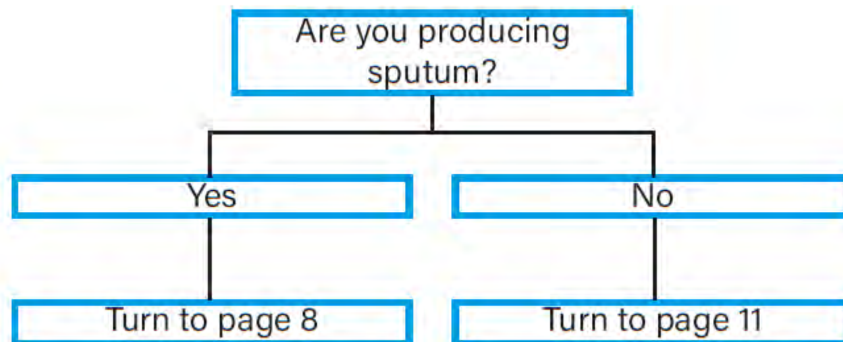
Pancreatic insufficient: Yes / No (*Please circle*)

Step 2: Complete the participant questionnaire.

Step 3: Clean the area you wish to use before starting sample collection.

Step 4: Wash your hands with warm water and soap for atleast 20 seconds.

Step 5: Proceed with sample collection using flow chart below:



How to use the postal kit if you are producing sputum.

Step 1.

Fill out the label attached to the side of Tube A.

Step 2.

Collect sputum into Tube A.

Step 3.

Pour the contents of Tube B (DNA/RNA Shield™) into Tube A.

Step 4.

Ensure lid is securely on Tube A.

Step 5.

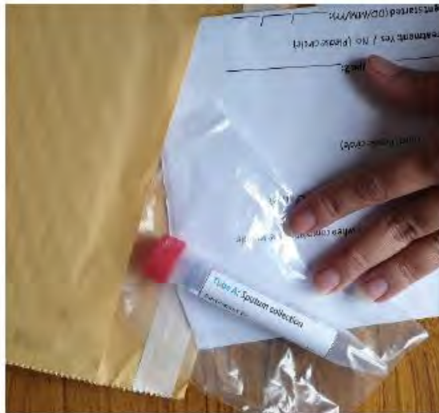
Gently invert Tube A to mix ***(do not shake).***

Step 6.

Put Tube A into the polythene bag provided and seal.

Step 7.

Dispose of Tube B and any unused tubes in general waste. ***Wash hands thoroughly.***



Step 8.

Put Tube A in the sealed polythene bag and the completed questionnaire in the pre-paid and addressed packaging provided. Seal the envelope. Follow the instructions for returning the pack on page 15.

How to use the postal kit if you are not producing sputum.

Step 1.

Fill out the label on the side of Tube C

Step 2.

Open swab Tube C. Avoid touching the cotton end once open.

Step 3.

Place swab on a clean surface using the lid as a stand.

!!! MAKE SURE YOU HAVE REMOVED THE SWAB FROM TUBE C. !!!

Step 4.

Pour the contents of Tube B (DNA/RNA shield™). Into the ***empty*** Tube C.

!!! MAKE SURE YOU HAVE REMOVED THE SWAB FROM* TUBE C *!!!

Step 5.

Hold swab inside the mouth as far to back as comfortable. Cough on the swab three times.

Step 6.

Put the swab back in Tube C and detach the white lid from the swab by gently pulling it away allowing it to drop into the tube.

Step 7.

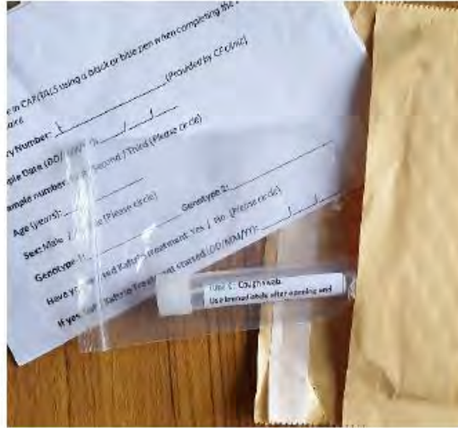
Replace the lid for Tube c, ensure it is secure.

Step 8.

Put Tube C into the polythene bag provided and seal.

Step 9.

Dispose of Tube B and any unused tubes in general waste. ***Wash hands thoroughly.***



Step 10.

Put Tube C in the sealed polythene bag and the completed questionnaire in the pre-paid and addressed packaging provided. Seal the envelope. Follow the instructions for returning the pack on page 15.

Returning the Pack

Once all the sample collection steps are complete follow the checklist below to make sure you have included every thing.

Pack Return Checklist	Please tick:
Completed patient questionnaire and put in envelope	
If you ***are*** producing sputum: ***Tube A*** with completed label in sealable bag.	
If you ***are not*** producing sputum: ***Tube C*** with completed label in sealable bag.	
Required tubes A or C in polythene bag and self addressed envelope	
Seal envelope	
Dispose of any unused tubes in normal household waste.	

All done?

Please put the sealed envelope in any post-box.

Thank you for your time and participation in our study!

11.3 SIMPER analysis Background microbiota and NTM positive vs NTM With NTM (Chapter 4)

Taxon	**Contrib. %**	**Cumulative %**	**Mean**	
			Positive	Negative
Burkholderia multivorans	**9.73**	**21.86**	**11.9**	**9.15**
Mycobacterium avium	**4.416**	**37.12**	**8.13**	**0**
Staphylococcus aureus	6.327	28.19	8.08	**5.31**
Streptococcus salivarius	**4.52**	**32.71**	**7.45**	1.66
Pseudomonas aeruginosa	**12.13**	**12.13**	**6.47**	**20.2**
Mycobacteroides abscessus	**3.482**	**40.61**	**6.3**	**0.245**
Mycobacteroides chelonae	2.844	55.61	5.24	0
Staphylococcus succinus	**2.855**	**52.77**	**4.14**	1.57
Stenotrophomonas maltophilia	3.154	47.03	3.19	**2.99**
Streptococcus toyakuensis	1.72	67.33	3.17	0
Haemophilus influenzae	**2.821**	**58.43**	**2.35**	**3.41**
Porphyromonas pasteri	**1.391**	**73.41**	**2.18**	0.498
Stenotrophomonas pavanii	2.541	63.76	1.73	3.2
Streptococcus symci	2.882	49.91	1.66	**4.51**
Neisseria mucosa	1.847	65.61	1.64	2.17
Achromobacter xylosoxidans	1.626	68.95	1.56	1.67
Mycobacterium kansasii	0.8399	80.79	1.55	0
Veillonella nakazawae	**3.27**	**43.88**	**1.51**	5.6
Burkholderia cepacia	**0.535**	**87.16**	**0.985**	**0**
Mycobacterium intracellulare	2.784	61.22	0.918	**4.39**
Peribacillus simplex	**0.464**	**88.58**	**0.854**	0

Prevotella salivae	1.262	76	0.829	1.74
Veillonella parvula	**1.539**	**70.49**	**0.71**	2.27
Mycobacterium malmoense	0.4102	88.99	0.696	0.067
Mycobacterium gordonae	0.3999	89.39	0.692	0.0471
Prevotella melaninogenica	0.3698	90.53	0.681	0
Mycolicibacterium smegmatis	0.3558	91.6	0.655	**0**
Staphylococcus pasteuri	**0.9047**	**79.06**	**0.614**	1.17
Prevotella vespertina	0.3007	92.23	0.554	0
Mycolicibacterium fortuitum	0.2944	92.52	0.542	**0**
Moraxella osloensis	0.3926	89.78	0.541	**0.193**
Acinetobacter baumannii	0.268	93.06	0.493	**0**
Staphylococcus hominis	**0.2627**	**93.33**	**0.484**	0
Streptococcus sanguinis	0.2557	93.58	0.471	0
Mycobacterium paraintracellulare	**0.7722**	**83.17**	**0.451**	1.02
Prauserella isguenensis	0.2498	94.08	0.423	0.0517
Limosilactobacillus vaginalis	0.8035	82.4	0.395	1.12
Mycobacteroides immunogenum	0.3247	91.92	0.394	0.216
Mycobacterium malmoense	0.9879	78.16	0.338	1.54
Lachnoanaerobaculum saburreum	0.2757	92.8	0.337	0.187
Lancefieldella parvula	0.3621	90.89	0.333	0.392
Proteus mirabilis	0.1682	95.49	0.31	0
Porphyromonas catoniae	0.1671	95.66	0.308	0
Alloprevotella rava	0.253	93.84	0.287	0.197
Veillonella massiliensis	0.1407	95.96	0.259	0

Gemella morbillorum	0.7065	85.37	0.257	1.09
Enterococcus faecalis	0.1529	95.81	0.25	0.0328
Prevotella oris	0.1331	96.09	0.245	0
Fusobacterium nucleatum	0.124	96.47	0.228	0
Streptococcus pseudopneumoniae	**0.8892**	**79.95**	**0.227**	**1.52**
Mogibacterium diversum	0.1172	96.59	0.216	0
Schaalia odontolytica	0.7303	84.67	0.209	**1.21**
Mycobacterium haemophilum	0.6267	86	0.202	0.975
Prevotella nanceiensis	0.1065	97.02	0.196	0
Capnocytophaga granulosa	0.2346	94.32	0.193	0.253
Epilithonimonas bovis	0.1013	97.12	0.186	0
Megasphaera micronuciformis	0.1912	95.16	0.177	0.192
Fusobacterium periodonticum	0.13	96.35	0.177	0.0665
Prevotella shahii	0.09551	97.32	0.176	0
Prevotella histicola	0.4835	87.64	0.17	0.754
Oligella urethralis	0.09716	97.22	0.155	0.0272
Gemella asaccharolytica	0.08359	97.4	0.154	0
Streptococcus gordonii	0.08184	97.65	0.151	0
Murinocardiopsis flavida	0.07851	97.73	0.145	0
Lactobacillus paragasseri	0.07566	97.88	0.139	0
Lactobacillus iners	0.07533	98.03	0.139	0
Streptococcus thermophilus	0.1104	96.7	0.138	0.0787
Prevotella pallens	0.06978	98.18	0.128	0
Lentilactobacillus kefiri	0.06602	98.31	0.122	0

Haemophilus parainfluenzae	0.1699	95.33	0.118	0.207
Veillonella dispar	0.08316	97.48	0.117	0.0436
Prevotella jejuni	0.06345	98.37	0.117	0
Parvimonas micra	1.168	77.17	0.11	**2.06**
Burkholderia cenocepacia	**0.05653**	**98.61**	**0.104**	0
Pseudoleptotrichia goodfellowii	0.05585	98.67	0.103	0
Prevotella intermedia	0.0538	98.77	0.0991	0
Tannerella serpentiformis	0.05325	98.88	0.098	0
Anaerococcus obesiensis	0.05103	98.93	0.094	0
Prevotella timonensis	**0.8044**	**81.6**	**0.0924**	**1.4**
Alteribacillus alkaliphilus	0.04983	98.98	0.0917	0
Rothia mucilaginosa	0.0463	99.03	0.0852	0
Chryseobacterium koreense	0.04577	99.07	0.0843	0
Brevundimonas vesicularis	0.04506	99.21	0.083	0
Cutibacterium acnes	0.08281	97.57	0.0803	0.0813
Limosilactobacillus fermentum	0.05391	98.72	0.0747	0.0269
Neisseria flavescens	0.07545	97.96	0.0709	0.0751
Prevotella scopos	0.03841	99.33	0.0707	0
Anaerostipes caccae	0.03372	99.4	0.0621	0
Brevibacterium otitidis	0.03235	99.44	0.0596	0
Granulicatella elegans	0.03115	99.53	0.0573	0
Limosilactobacillus reuteri	**0.03094**	**99.56**	**0.057**	0
Selenomonas felix	0.04564	99.12	0.0565	0.0292
Mycobacterium lacus	0.03019	99.59	0.0556	0

Streptococcus vulneris	0.02955	99.62	0.0544	0
Prevotella aurantiaca	0.356	91.24	0.0537	**0.611**
Porphyromonas endodontalis	0.07406	98.11	0.0514	0.0901
Mycobacterium tuberculosis	0.3776	90.16	0.047	**0.654**
Massilia frigida	0.02525	99.67	0.0465	0
Staphylococcus argenteus	0.02269	99.75	0.0418	0
Finegoldia magna	0.02248	99.77	0.0414	0
Staphylococcus epidermidis	0.02248	99.79	0.0414	0
Prevotella pleuritidis	0.02165	99.81	0.0399	0
Mycobacterium attenuatum	0.01895	99.83	0.0349	0
Actinomyces vulturis	0.0159	99.85	0.0293	0
Pseudomonas mangiferae	0.01504	99.86	0.0277	0
Paracoccus angustae	0.01459	99.88	0.0269	0
Peptostreptococcus stomatis	0.01342	99.89	0.0247	0
Actinomyces bouchesdurhonensis	0.01209	99.9	0.0223	0
Actinomyces graevenitzii	0.01112	99.91	0.0205	0
Mycobacterium aemonae	0.01092	99.92	0.0201	0
Brevibacterium ravenspurgense	0.009634	99.93	0.0177	0
Butyrivibrio fibrisolvens	0.008117	99.94	0.0149	0
Stomatobaculum longum	0.2203	94.54	0.0146	0.394
Comamonas jiangduensis	0.007598	99.95	0.014	0
Enterococcus faecium	0.007194	99.96	0.0132	0
Atopobium parvulum	0.006595	99.97	0.0121	0
Capnocytophaga gingivalis	0.006229	99.98	0.0115	0

Burkholderia glumae	0.005995	99.98	0.011	0
Mycobacterium asiaticum	**1.326**	**74.74**	**0.00955**	2.43
Prauserella marina	0.003957	99.99	0.00729	0
Morococcus sicca	0.003383	99.99	0.00623	0
Neisseria subflava	0.003177	99.99	0.00585	0
Burkholderia pyrrocinia	0.002578	100	0.00475	0
Cryptobacterium curtum	0.002029	100	0.00374	0
Mycobacterium heidelbergense	0.04535	99.16	0.00155	0.0821
Corynebacterium aurimucosum	0.000659	100	0.00121	0
Escherichia coli	1.532	72.02	0	2.82
Moraxella catarrhalis	0.766	83.94	0	1.41
Paraburkholderia fungorum	0.6255	86.63	0	1.15
Streptococcus agalactiae	**0.4672**	**88.11**	**0**	**0.86**
Fusobacterium pseudoperiodonticum	0.217	94.76	0	0.4
Pseudomonas mendocina	0.2085	94.97	0	0.384
Haemophilus parahaemolyticus	0.1303	96.22	0	0.24
Paucibacter toxinivorans	0.1081	96.81	0	0.199
Oribacterium asaccharolyticum	0.1077	96.92	0	0.198
Ralstonia pickettii	0.07844	97.8	0	0.144
Veillonella atypica	0.06676	98.24	0	0.123
Veillonella rogosae	0.06177	98.43	0	0.114
Capnocytophaga bilenii	0.06041	98.49	0	0.111
Rothia dentocariosa	0.05953	98.55	0	0.11
Deinococcus xinjiangensis	0.05367	98.83	0	0.0988

Prevotella loescheii	0.04375	99.25	0	0.0806
Lautropia mirabilis	0.04077	99.29	0	0.0751
Paraburkholderia phenazinium	0.03755	99.37	0	0.0691
Facklamia hominis	0.03181	99.47	0	0.0586
Peptoniphilus grossensis	0.03129	99.5	0	0.0576
Lachnoanaerobaculum gingivalis	0.02718	99.65	0	0.05
Capnocytophaga leadbetteri	0.02467	99.7	0	0.0454
Alloscardovia omnicolens	0.02466	99.72	0	0.0454
Mycobacterium xenopi	0.007387	99.96	0	0.0136
Mogibacterium timidum	0.001762	100	0	0.00324
Actinomyces lingnae	0	100	0	0
Sphingomonas pseudosanguinis	0	100	0	0
Ligilactobacillus salivarius	0	100	0	0
Streptococcus anginosus	0	100	0	0
Catonella massiliensis	0	100	0	0
Abiotrophia defectiva	0	100	0	0
Bdellovibrio bacteriovorus	0	100	0	0
Prevotella oulorum	0	100	0	0
Lysobacter xinjiangensis	0	100	0	0
Fusobacterium animalis	0	100	0	0
Lipingzhangella halophila	0	100	0	0
Mycolicibacter terrae	0	100	0	0
Methylobacterium radiotolerans	0	100	0	0
Burkholderia contaminans	0	100	0	0

Nonomuraea roseoviolacea	0	100	0	0
Bifidobacterium longum	0	100	0	0
Neisseria perflava	0	100	0	0
Paenibacillus tyrfis	0	100	0	0
Actinomyces naeslundii	0	100	0	0
Corynebacterium durum	0	100	0	0
Mycolicibacterium insubricum	0	100	0	0
Mycolicibacterium monacense	0	100	0	0
Proteus vulgaris	0	100	0	0
Streptococcus australis	0	100	0	0
Leptotrichia hongkongensis	0	100	0	0
Delftia tsuruhatensis	0	100	0	0
Actinomyces pyogenes	0	100	0	0
Dialister invisus	0	100	0	0
Achromobacter marplatensis	0	100	0	0
Staphylococcus ureilyticus	0	100	0	0
Dialister pneumosintes	0	100	0	0
Selenomonas sputigena	0	100	0	0
Prevotella saccharolytica	0	100	0	0
Pedobacter nutrimenti	0	100	0	0
Capnocytophaga sputigena	0	100	0	0
Prevotella oralis	0	100	0	0
Streptococcus rubneri	0	100	0	0
Selenomonas artemidis	0	100	0	0

Mycobacterium kansasii	0	100	0	0
Actinomyces oris	0	100	0	0
Neisseria oralis	0	100	0	0
Oribacterium sinus	0	100	0	0
Cupriavidus gilardii	0	100	0	0
Peptostreptococcus anaerobius	0	100	0	0
Lactobacillus kalixensis	0	100	0	0
Treponema maltophilum	0	100	0	0
Granulicella paludicola	0	100	0	0
Gemella sanguinis	0	100	0	0
Alloprevotella tannerae	0	100	0	0
Tannerella forsythia	0	100	0	0
Reyranella aquatilis	0	100	0	0
Mycolicibacter engbaekii	0	100	0	0
Cutibacterium porci	0	100	0	0
Porphyromonas gingivalis	0	100	0	0
Cardiobacterium valvarum	0	100	0	0
Phreatobacter cathodiphilus	0	100	0	0
Corynebacterium segmentosum	0	100	0	0
Campylobacter gracilis	0	100	0	0
Enhydrobacter aerosaccus	0	100	0	0
Mycobacterium simiae	0	100	0	0
Treponema vincentii	0	100	0	0
Morococcus cerebrosus	0	100	0	0

Treponema lecithinolyticum	0	100	0	0
Streptococcus timonensis	0	100	0	0
Mycobacterium doricum	0	100	0	0
Streptococcus shenyangsis	0	100	0	0
Lancefieldella rimae	0	100	0	0
Capnocytophaga ochracea	0	100	0	0
Variovorax guangxiensis	0	100	0	0
Bacteroides heparinolyticus	0	100	0	0
Arachidicoccus terrestris	0	100	0	0
Prevotella denticola	0	100	0	0
Staphylococcus haemolyticus	0	100	0	0
Stenotrophomonas geniculata	0	100	0	0
Campylobacter concisus	0	100	0	0
Lactobacillus gasseri	0	100	0	0
Prevotella koreensis	0	100	0	0
Selenomonas flueggei	0	100	0	0
Phenylobacterium muchangponense	0	100	0	0

11.4 Full SIMPER Analysis Background microbiota and NTM positive vs NTM Without NTM (Chapter 4)

Taxon	Contrib. %	Cumulative %	Mean relative abundance	
			Positive	Negative
Burkholderia multivorans	10.58	24.32	13.8	8.73
Streptococcus salivarius	6.538	39.51	11.1	1.59
Pseudomonas aeruginosa	13.74	13.74	9.61	20.8
Staphylococcus aureus	8.648	32.97	7.58	10.6
Staphylococcus succinus	3.734	47.46	5.78	1.5
Neisseria mucosa	3.433	54.36	4.63	2.06
Streptococcus toyakuensis	2.323	71.95	4.21	0
Achromobacter xylosoxidans	2.995	67.02	4.1	1.59
Haemophilus influenzae	3.466	50.93	3.69	3.25
Stenotrophomonas maltophilia	3.304	61	3.51	2.85
Porphyromonas pasteri	1.653	73.6	2.64	0.477
Veillonella nakazawae	4.22	43.73	2.29	6.84
Streptococcus symci	3.336	57.7	2.11	4.92
Stenotrophomonas pavanii	2.6	69.62	1.91	3.04
Prevotella salivae	1.631	75.23	1.39	1.9
Burkholderia cepacia	0.7294	84.85	1.32	0
Prevotella melaninogenica	0.6751	86.24	1.22	0
Prevotella vespertina	0.6304	86.87	1.14	0
Peribacillus simplex	0.548	88.63	0.993	0
Veillonella parvula	3.029	64.03	0.838	4.81
Prauserella isguenensis	0.4083	91.35	0.705	0.0492
Staphylococcus pasteuri	1.511	76.74	0.652	2.25
Moraxella osloensis	0.4494	90.5	0.641	0.184
Acinetobacter baumannii	0.3489	91.7	0.632	0
Streptococcus sanguinis	0.337	92.04	0.61	0
Staphylococcus hominis	0.32	92.7	0.58	0
Alloprevotella rava	0.4413	90.94	0.548	0.274
Limosilactobacillus vaginalis	1.15	81.75	0.483	1.65
Lancefieldella parvula	0.4631	89.6	0.457	0.463
Lachnoanaerobaculum saburreum	0.3362	92.38	0.449	0.182
Proteus mirabilis	0.235	93.19	0.426	0
Fusobacterium nucleatum	0.2255	93.42	0.408	0
Porphyromonas catoniae	0.2248	93.64	0.407	0
Veillonella massiliensis	0.1893	94.9	0.343	0
Prevotella oris	0.1872	95.08	0.339	0
Streptococcus pseudopneumoniae	1.176	79.43	0.335	1.94
Schaalia odontolytica	0.833	82.58	0.269	1.34
Enterococcus faecalis	0.1634	95.25	0.266	0.0313
Murinocardiopsis flavida	0.1437	95.85	0.26	0
Gemella morbillorum	0.7185	85.57	0.254	1.1
Oligella urethralis	0.1494	95.71	0.248	0.0261
Capnocytophaga granulosa	0.2622	92.96	0.243	0.246
Megasphaera micronuciformis	0.2218	93.86	0.239	0.183

Mogibacterium diversum	0.1287	96.26	0.233	0
Epilithonimonas bovis	0.1219	96.63	0.221	0
Massilia frigida	0.1163	96.87	0.211	0
Prevotella shahii	0.1164	96.75	0.211	0
Neisseria flavescens	0.1514	95.56	0.21	0.0715
Prevotella histicola	0.6078	87.48	0.201	0.937
Haemophilus parainfluenzae	0.2149	94.08	0.2	0.201
Prevotella pallens	0.1102	96.98	0.2	0
Streptococcus gordonii	0.1101	97.09	0.199	0
Lentilactobacillus kefiri	0.1087	97.2	0.197	0
Prevotella intermedia	0.1059	97.3	0.192	0
Fusobacterium periodonticum	0.1385	96.13	0.191	0.0634
Lactobacillus paragasseri	0.1004	97.71	0.182	0
Prevotella nanceiensis	0.1259	96.51	0.177	0.0545
Lactobacillus iners	0.09069	97.81	0.164	0
Parvimonas micra	1.175	80.6	0.16	1.99
Streptococcus thermophilus	0.161	95.41	0.158	0.152
Veillonella dispar	0.1039	97.61	0.154	0.0415
Brevibacterium otitidis	0.08449	98.07	0.153	0
Prevotella jejuni	0.08026	98.15	0.145	0
Burkholderia cenocepacia	0.07707	98.3	0.14	0
Pseudoleptotrichia goodfellowii	0.07514	98.45	0.136	0
Prevotella scopos	0.07	98.52	0.127	0
Tannerella serpentiformis	0.06778	98.59	0.123	0
Limosilactobacillus fermentum	0.0777	98.23	0.118	0.0256
Prevotella timonensis	0.7932	83.38	0.117	1.33
Alteribacillus alkaliphilus	0.06385	98.79	0.116	0
Anaerococcus obesiensis	0.06143	98.85	0.111	0
Rothia mucilaginosa	0.05904	99.03	0.107	0
Anaerostipes caccae	0.05838	99.08	0.106	0
Chryseobacterium koreense	0.05509	99.2	0.0998	0
Cutibacterium acnes	0.08875	97.98	0.0921	0.0778
Brevundimonas vesicularis	0.04954	99.35	0.0897	0
Porphyromonas endodontalis	0.08954	97.9	0.0818	0.0875
Granulicatella elegans	0.0419	99.39	0.0759	0
Limosilactobacillus reuteri	0.04106	99.43	0.0744	0
Streptococcus vulneris	0.04029	99.47	0.073	0
Prevotella pleuritidis	0.03749	99.55	0.0679	0
Selenomonas felix	0.05145	99.3	0.067	0.0278
Prevotella aurantiaca	0.5063	89.14	0.0637	0.863
Staphylococcus epidermidis	0.02706	99.7	0.049	0
Finegoldia magna	0.02706	99.67	0.049	0
Staphylococcus argenteus	0.02427	99.75	0.044	0
Peptostreptococcus stomatis	0.02323	99.82	0.0421	0
Pseudomonas mangiferae	0.02023	99.84	0.0366	0
Paracoccus angustae	0.01762	99.86	0.0319	0
Actinomyces vulturis	0.01748	99.88	0.0317	0
Actinomyces bouchesdurhonensis	0.01605	99.89	0.0291	0

Comamonas jiangduensis	0.0156	99.91	0.0283	0
Actinomyces graevenitzii	0.01223	99.92	0.0222	0
Brevibacterium ravenspurgense	0.01088	99.93	0.0197	0
Enterococcus faecium	0.009808	99.94	0.0178	0
Butyrivibrio fibrisolvens	0.009785	99.95	0.0177	0
Stomatobaculum longum	0.2146	94.29	0.0158	0.376
Capnocytophaga gingivalis	0.008381	99.96	0.0152	0
Burkholderia glumae	0.008173	99.97	0.0148	0
Atopobium parvulum	0.00795	99.98	0.0144	0
Morococcus sicca	0.005857	99.98	0.0106	0
Prauserella marina	0.004468	99.99	0.00809	0
Burkholderia pyrrocinia	0.003515	99.99	0.00637	0
Neisseria subflava	0.003495	99.99	0.00633	0
Cryptobacterium curtum	0.002446	100	0.00443	0
Corynebacterium aurimucosum	0.000899	100	0.00163	0
Escherichia coli	1.508	78.25	0	2.73
Moraxella catarrhalis	0.7416	84.12	0	1.34
Paraburkholderia fungorum	0.6056	88.08	0	1.1
Streptococcus agalactiae	0.4523	90.05	0	0.819
Fusobacterium pseudoperiodonticum	0.2101	94.5	0	0.38
Pseudomonas mendocina	0.2018	94.71	0	0.366
Gemella asaccharolytica	0.1416	95.99	0	0.257
Haemophilus parahaemolyticus	0.1262	96.39	0	0.229
Oribacterium asaccharolyticum	0.1043	97.51	0	0.189
Paucibacter toxinivorans	0.1046	97.41	0	0.189
Ralstonia pickettii	0.07593	98.38	0	0.138
Prevotella loescheii	0.06513	98.66	0	0.118
Veillonella atypica	0.06463	98.72	0	0.117
Capnocytophaga bilenii	0.05961	98.97	0	0.108
Veillonella rogosae	0.0598	98.91	0	0.108
Rothia dentocariosa	0.05763	99.14	0	0.104
Deinococcus xinjiangensis	0.05195	99.25	0	0.0941
Lautropia mirabilis	0.03947	99.51	0	0.0715
Paraburkholderia phenazinium	0.03635	99.59	0	0.0658
Facklamia hominis	0.03099	99.62	0	0.0561
Peptoniphilus grossensis	0.03029	99.65	0	0.0549
Lachnoanaerobaculum gingivalis	0.02631	99.73	0	0.0477
Capnocytophaga leadbetteri	0.02388	99.78	0	0.0433
Alloscardovia omnicolens	0.02388	99.8	0	0.0432
Mogibacterium timidum	0.001706	100	0	0.00309
Corynebacterium segmentosum	0	100	0	0
Treponema lecithinolyticum	0	100	0	0
Treponema vincentii	0	100	0	0
Capnocytophaga ochracea	0	100	0	0
Streptococcus timonensis	0	100	0	0
Variovorax guangxiensis	0	100	0	0
Morococcus cerebrosus	0	100	0	0
Bacteroides heparinolyticus	0	100	0	0

Arachidicoccus terrestris	0	100	0	0
Prevotella denticola	0	100	0	0
Stenotrophomonas geniculata	0	100	0	0
Lactobacillus gasseri	0	100	0	0
Campylobacter concisus	0	100	0	0
Prevotella koreensis	0	100	0	0
Selenomonas flueggei	0	100	0	0
Phenylobacterium muchangponense	0	100	0	0
Ligilactobacillus salivarius	0	100	0	0
Staphylococcus haemolyticus	0	100	0	0
Actinomyces lingnae	0	100	0	0
Sphingomonas pseudosanguinis	0	100	0	0
Streptococcus anginosus	0	100	0	0
Catonella massiliensis	0	100	0	0
Lysobacter xinjiangensis	0	100	0	0
Abiotrophia defectiva	0	100	0	0
Fusobacterium animalis	0	100	0	0
Lipingzhangella halophila	0	100	0	0
Methylobacterium radiotolerans	0	100	0	0
Prevotella oulorum	0	100	0	0
Lancefieldella rimae	0	100	0	0
Streptococcus shenyangsis	0	100	0	0
Bdellovibrio bacteriovorus	0	100	0	0
Nonomuraea roseoviolacea	0	100	0	0
Bifidobacterium longum	0	100	0	0
Paenibacillus tyrfis	0	100	0	0
Enhydrobacter aerosaccus	0	100	0	0
Campylobacter gracilis	0	100	0	0
Proteus vulgaris	0	100	0	0
Streptococcus australis	0	100	0	0
Burkholderia contaminans	0	100	0	0
Neisseria perflava	0	100	0	0
Leptotrichia hongkongensis	0	100	0	0
Actinomyces pyogenes	0	100	0	0
Dialister invisus	0	100	0	0
Actinomyces naeslundii	0	100	0	0
Achromobacter marplatensis	0	100	0	0
Staphylococcus ureilyticus	0	100	0	0
Dialister pneumosintes	0	100	0	0
Delftia tsuruhatensis	0	100	0	0
Prevotella saccharolytica	0	100	0	0
Selenomonas sputigena	0	100	0	0
Corynebacterium durum	0	100	0	0
Prevotella oralis	0	100	0	0
Pedobacter nutrimenti	0	100	0	0
Capnocytophaga sputigena	0	100	0	0
Streptococcus rubneri	0	100	0	0
Selenomonas artemidis	0	100	0	0

Actinomyces oris	0	100	0	0
Neisseria oralis	0	100	0	0
Oribacterium sinus	0	100	0	0
Peptostreptococcus anaerobius	0	100	0	0
Lactobacillus kalixensis	0	100	0	0
Treponema maltophilum	0	100	0	0
Granulicella paludicola	0	100	0	0
Gemella sanguinis	0	100	0	0
Alloprevotella tannerae	0	100	0	0
Cupriavidus gilardii	0	100	0	0
Tannerella forsythia	0	100	0	0
Reyranella aquatilis	0	100	0	0
Cutibacterium porci	0	100	0	0
Porphyromonas gingivalis	0	100	0	0
Phreatobacter cathodiphilus	0	100	0	0
Cardiobacterium valvarum	0	100	0	0

11.5 SIMPER analysis Background microbiota and NTM Type with NTM(Chapter 4)

Taxon	Contrib. %	Cumulative %	Mean Relative abundance		
			MAC	MABSC	Other
Burkholderia multivorans	11.36	11.36	12.2	8.92	24.8
Mycobacteroides abscessus	8.659	20.02	0.302	15	16.3
Streptococcus salivarius	8.446	28.46	2.82	18.6	0
Mycobacteroides chelonae	7.243	35.71	0.0238	16.6	0
Mycobacterium avium	6.813	42.52	14	0	1.25
Staphylococcus aureus	5.904	48.42	4.68	4.51	17.9
Pseudomonas aeruginosa	5.47	53.89	10.7	0.376	2.93
Streptococcus toyakuensis	3.727	57.62	1.49	7.38	0
Staphylococcus succinus	3.661	61.28	5.95	1.36	3.68
Haemophilus influenzae	3.23	64.51	0.924	1.67	15.1
Stenotrophomonas maltophilia	2.696	67.21	5.59	0	0
Porphyromonas pasteri	1.839	69.05	3.81	0	0
Achromobacter xylosoxidans	1.669	70.71	1.3	2.6	0
Streptococcus symci	1.488	72.2	1.94	1.74	0
Stenotrophomonas pavanii	1.459	73.66	3.02	0	0
Neisseria mucosa	1.434	75.09	2.32	1.01	0
Veillonella nakazawae	1.358	76.45	1.48	2.11	0
Mycobacterium kansasii	1.306	77.76	2.71	0	0
Mycobacterium malmoense	0.9652	78.72	0	2.21	0
Mycobacterium gordonae	0.9597	79.68	0	2.2	0

Mycobacterium intracellulare	0.9169	80.6	1.24	0	2.44
Burkholderia cepacia	0.8316	81.43	1.72	0	0
Prevotella salivae	0.7876	82.22	0.954	0.902	0
Staphylococcus pasteuri	0.7777	83	0.107	0.214	4.09
Moraxella osloensis	0.7509	83.75	0	1.72	0
Prevotella vespertina	0.7445	84.49	0.0621	1.65	0
Peribacillus simplex	0.7213	85.21	1.5	0	0
Staphylococcus hominis	0.7125	85.93	0.0964	0.628	2.7
Mycobacterium malmoense	0.632	86.56	0	0	3.94
Prevotella melaninogenica	0.6298	87.19	0.928	0.48	0
Veillonella parvula	0.6133	87.8	1.04	0.366	0
Mycolicibacterium smegmatis	0.6129	88.41	1.03	0	0.772
Acinetobacter baumannii	0.5631	88.98	0.32	0.988	0
Mycolicibacterium fortuitum	0.5093	89.49	0.835	0	0.757
Streptococcus sanguinis	0.3975	89.88	0.824	0	0
Mycobacterium paraintracellulare	0.3808	90.27	0.789	0	0
Mogibacterium diversum	0.375	90.64	0.0434	0	2.23
Prauserella isguenensis	0.374	91.01	0.619	0.169	0.193
Alloprevotella rava	0.3551	91.37	0.114	0.707	0
Limosilactobacillus vaginalis	0.3337	91.7	0.692	0	0
Mycobacteroides immunogenum	0.333	92.04	0.69	0	0
Lachnoanaerobaculum saburreum	0.3073	92.34	0.493	0.175	0
Proteus mirabilis	0.2917	92.63	0.416	0.229	0
Prevotella oris	0.287	92.92	0.14	0.526	0

Lancefieldella parvula	0.2839	93.21	0.525	0.104	0
Mycobacterium haemophilum	0.2808	93.49	0	0.644	0
Porphyromonas catoniae	0.2598	93.75	0.538	0	0
Epilithonimonas bovis	0.2586	94.01	0	0.593	0
Oligella urethralis	0.2278	94.23	0.00859	0.336	0.524
Veillonella massiliensis	0.2187	94.45	0.453	0	0
Enterococcus faecalis	0.2115	94.66	0.438	0	0
Lactobacillus paragasseri	0.1932	94.86	0	0.443	0
Fusobacterium nucleatum	0.1927	95.05	0.399	0	0
Lactobacillus iners	0.1924	95.24	0	0.441	0
Streptococcus pseudopneumoniae	0.1915	95.43	0.397	0	0
Gemella morbillorum	0.1866	95.62	0.387	0	0
Schaalia odontolytica	0.1761	95.8	0.365	0	0
Capnocytophaga granulosa	0.163	95.96	0.338	0	0
Megasphaera micronuciformis	0.1493	96.11	0.309	0	0
Fusobacterium periodonticum	0.1492	96.26	0.309	0	0
Prevotella shahii	0.1485	96.41	0.308	0	0
Prevotella histicola	0.1433	96.55	0.297	0	0
Prevotella nanceiensis	0.1379	96.69	0.286	0	0
Prevotella jejuni	0.1361	96.82	0.0684	0.247	0
Anaerococcus obesiensis	0.1303	96.95	0	0.299	0
Streptococcus gordonii	0.1272	97.08	0.264	0	0
Prevotella pallens	0.1229	97.2	0.153	0.131	0
Murinocardiopsis flavida	0.122	97.33	0.253	0	0

Chryseobacterium koreense	0.1169	97.44	0	0.268	0
Cutibacterium acnes	0.1114	97.55	0	0.255	0
Lentilactobacillus kefiri	0.1026	97.66	0.213	0	0
Parvimonas micra	0.1018	97.76	0.144	0.0903	0
Streptococcus thermophilus	0.1005	97.86	0.0699	0.164	0
Haemophilus parainfluenzae	0.09922	97.96	0.206	0	0
Veillonella dispar	0.09856	98.06	0.204	0	0
Rothia mucilaginosa	0.0928	98.15	0.0671	0.149	0
Burkholderia cenocepacia	0.08788	98.24	0.182	0	0
Pseudoleptotrichia goodfellowii	0.08682	98.32	0.18	0	0
Prevotella intermedia	0.08363	98.41	0.173	0	0
Tannerella serpentiformis	0.08278	98.49	0.172	0	0
Brevibacterium otitidis	0.08262	98.57	0	0.19	0
Limosilactobacillus reuteri	0.07901	98.65	0	0.181	0
Prevotella timonensis	0.07803	98.73	0.162	0	0
Alteribacillus alkaliphilus	0.07746	98.81	0.161	0	0
Brevundimonas vesicularis	0.07004	98.88	0.145	0	0
Limosilactobacillus fermentum	0.06309	98.94	0.131	0	0
Neisseria flavescens	0.05986	99	0.124	0	0
Prevotella scopos	0.0597	99.06	0.124	0	0
Finegoldia magna	0.0574	99.12	0	0.132	0
Staphylococcus epidermidis	0.0574	99.17	0	0.132	0
Anaerostipes caccae	0.05242	99.23	0.109	0	0
Paracoccus angustae	0.05022	99.28	0	0	0.313

Granulicatella elegans	0.04842	99.33	0.1	0	0
Selenomonas felix	0.04771	99.37	0.0989	0	0
Mycobacterium lacus	0.04693	99.42	0.0973	0	0
Streptococcus vulneris	0.04594	99.47	0.0952	0	0
Prevotella aurantiaca	0.04534	99.51	0.094	0	0
Porphyromonas endodontalis	0.04338	99.55	0.0899	0	0
Mycobacterium tuberculosis	0.03969	99.59	0.0823	0	0
Massilia frigida	0.03924	99.63	0.0813	0	0
Staphylococcus argenteus	0.03527	99.67	0.0731	0	0
Prevotella pleuritidis	0.03366	99.7	0.0698	0	0
Actinomyces bouchesdurhonensis	0.03089	99.73	0	0.0709	0
Mycobacterium attenuatum	0.02946	99.76	0.0611	0	0
Actinomyces vulturis	0.02472	99.79	0.0512	0	0
Pseudomonas mangiferae	0.02337	99.81	0.0484	0	0
Peptostreptococcus stomatis	0.02086	99.83	0.0432	0	0
Comamonas jiangduensis	0.0194	99.85	0	0.0445	0
Actinomyces graevenitzii	0.01728	99.87	0.0358	0	0
Mycobacterium aemonae	0.01698	99.89	0.0352	0	0
Brevibacterium ravenspurgense	0.01498	99.9	0.031	0	0
Butyrivibrio fibrisolvens	0.01262	99.91	0.0262	0	0
Stomatobaculum longum	0.01234	99.93	0.0256	0	0
Enterococcus faecium	0.01118	99.94	0.0232	0	0
Atopobium parvulum	0.01025	99.95	0.0212	0	0
Capnocytophaga gingivalis	0.009683	99.96	0.0201	0	0

Burkholderia glumae	0.009319	99.97	0.0193	0	0
Mycobacterium asiaticum	0.008064	99.97	0.0167	0	0
Prauserella marina	0.006151	99.98	0.0127	0	0
Morococcus sicca	0.005259	99.99	0.0109	0	0
Neisseria subflava	0.004938	99.99	0.0102	0	0
Burkholderia pyrrocinia	0.004007	99.99	0.00831	0	0
Cryptobacterium curtum	0.003154	100	0.00654	0	0
Mycobacterium heidelbergense	0.001305	100	0.0027	0	0
Corynebacterium aurimucosum	0.001025	100	0.00212	0	0

11.6 Full SIMPER Analysis Background microbiota and NTM type without NTM

Taxon	**Contrib. %**	**Cumulative %**	**Mean Relative abundance**		
			MAC	MABSC	Other
Burkholderia multivorans	12.84	26.25	12.7	13.1	21.3
Staphylococcus aureus	8.215	34.47	5.05	7.61	20.8
Haemophilus influenzae	5.388	53.16	1.04	2.45	20.4
Achromobacter xylosoxidans	5.134	58.29	3.24	0	17.8
Pseudomonas aeruginosa	7.736	42.2	14.2	0.468	6.12
Staphylococcus pasteuri	0.9977	83.38	0.161	0.512	3.54
Staphylococcus succinus	4.815	63.11	7.79	2.24	3.19
Staphylococcus hominis	0.7147	86.6	0.447	0.109	2.34
Mogibacterium diversum	0.3858	90.91	0.0515	0	1.71

Brevibacterium otitidis	0.2774	92.91	0	0	1.3
Oligella urethralis	0.3907	90.52	0.00883	0.472	1
Paracoccus angustae	0.05785	99.44	0	0	0.271
Prauserella isguenensis	0.5849	87.89	1.01	0.232	0.167
Streptococcus salivarius	13.41	13.41	4.09	32.5	0
Neisseria mucosa	5.567	47.77	2.71	11.2	0
Streptococcus toyakuensis	3.575	66.68	5.7	2.59	0
Stenotrophomonas maltophilia	2.76	69.44	5.69	0	0
Veillonella nakazawae	2.238	71.68	1.87	4.29	0
Porphyromonas pasteri	2.072	73.75	4.27	0	0
Streptococcus symci	1.89	75.64	2.32	2.57	0
Prevotella vespertina	1.689	77.33	0.0682	4.15	0
Stenotrophomonas pavanii	1.504	78.84	3.1	0	0
Prevotella salivae	1.432	80.27	1.28	2.27	0

Prevotella melaninogenica	1.074	81.34	1.64	0.805	0
Burkholderia cepacia	1.038	82.38	2.14	0	0
Moraxella osloensis	0.9683	84.35	0	2.42	0
Peribacillus simplex	0.7795	85.13	1.61	0	0
Alloprevotella rava	0.7632	85.89	0.125	1.78	0
Veillonella parvula	0.7031	87.31	1.11	0.567	0
Acinetobacter baumannii	0.4963	88.39	1.02	0	0
Streptococcus sanguinis	0.4794	88.87	0.988	0	0
Prevotella oris	0.4225	89.29	0.171	0.881	0
Proteus mirabilis	0.422	89.71	0.454	0.549	0
Lachnoanaerobaculum saburreum	0.4216	90.13	0.541	0.433	0
Limosilactobacillus vaginalis	0.3794	91.29	0.782	0	0
Lancefieldella parvula	0.3744	91.66	0.649	0.211	0
Epilithonimonas bovis	0.3334	92	0	0.834	0
Fusobacterium nucleatum	0.3208	92.32	0.661	0	0
Porphyromonas catoniae	0.3198	92.64	0.659	0	0
Lactobacillus paragasseri	0.2747	93.19	0	0.687	0
Veillonella massiliensis	0.2692	93.46	0.555	0	0
Streptococcus pseudopneumoniae	0.2634	93.72	0.543	0	0
Lactobacillus iners	0.2481	93.97	0	0.621	0
Schaalia odontolytica	0.2115	94.18	0.436	0	0
Enterococcus faecalis	0.2093	94.39	0.431	0	0
Prevotella pallens	0.2068	94.6	0.181	0.331	0
Murinocardiopsis flavida	0.2043	94.8	0.421	0	0

Gemella morbillorum	0.1991	95	0.411	0	0
Streptococcus thermophilus	0.193	95.19	0.0868	0.394	0
Capnocytophaga granulosa	0.1911	95.39	0.394	0	0
Megasphaera micronuciformis	0.1876	95.57	0.387	0	0
Prevotella jejuni	0.1804	95.75	0.075	0.374	0
Anaerococcus obesiensis	0.168	95.92	0	0.42	0
Prevotella shahii	0.1655	96.09	0.341	0	0
Massilia frigida	0.1654	96.25	0.341	0	0
Neisseria flavescens	0.1652	96.42	0.34	0	0
Prevotella histicola	0.158	96.58	0.326	0	0
Haemophilus parainfluenzae	0.1572	96.73	0.324	0	0
Streptococcus gordonii	0.1566	96.89	0.323	0	0
Lentilactobacillus kefiri	0.1547	97.04	0.319	0	0
Chryseobacterium koreense	0.1507	97.19	0	0.377	0
Prevotella intermedia	0.1507	97.35	0.311	0	0
Fusobacterium periodonticum	0.1502	97.5	0.31	0	0
Parvimonas micra	0.1429	97.64	0.2	0.137	0
Cutibacterium acnes	0.1391	97.78	0	0.348	0
Prevotella nanceiensis	0.1388	97.92	0.286	0	0
Rothia mucilaginosa	0.1216	98.04	0.0764	0.226	0
Veillonella dispar	0.121	98.16	0.25	0	0
Limosilactobacillus reuteri	0.1123	98.27	0	0.281	0
Burkholderia cenocepacia	0.1096	98.38	0.226	0	0

Pseudoleptotrichia goodfellowii	0.1069	98.49	0.22	0	0
Prevotella scopos	0.09957	98.59	0.205	0	0
Tannerella serpentiformis	0.09641	98.68	0.199	0	0
Limosilactobacillus fermentum	0.09232	98.78	0.19	0	0
Prevotella timonensis	0.0915	98.87	0.189	0	0
Alteribacillus alkaliphilus	0.09083	98.96	0.187	0	0
Anaerostipes caccae	0.08304	99.04	0.171	0	0
Finegoldia magna	0.07402	99.12	0	0.185	0
Staphylococcus epidermidis	0.07402	99.19	0	0.185	0
Brevundimonas vesicularis	0.07046	99.26	0.145	0	0
Porphyromonas endodontalis	0.06428	99.32	0.133	0	0
Granulicatella elegans	0.0596	99.38	0.123	0	0
Streptococcus vulneris	0.05732	99.5	0.118	0	0
Prevotella pleuritidis	0.05332	99.55	0.11	0	0
Selenomonas felix	0.05263	99.6	0.109	0	0
Prevotella aurantiaca	0.05002	99.65	0.103	0	0
Actinomyces bouchesdurhonensis	0.04391	99.7	0	0.11	0
Comamonas jiangduensis	0.04267	99.74	0	0.107	0
Staphylococcus argenteus	0.03453	99.78	0.0712	0	0
Peptostreptococcus stomatis	0.03305	99.81	0.0681	0	0
Pseudomonas mangiferae	0.02877	99.84	0.0593	0	0
Actinomyces vulturis	0.02487	99.86	0.0513	0	0
Actinomyces graevenitzii	0.0174	99.88	0.0359	0	0

Brevibacterium ravenspurgense	0.01547	99.9	0.0319	0	0
Enterococcus faecium	0.01395	99.91	0.0288	0	0
Butyrivibrio fibrisolvens	0.01392	99.92	0.0287	0	0
Stomatobaculum longum	0.01243	99.94	0.0256	0	0
Capnocytophaga gingivalis	0.01192	99.95	0.0246	0	0
Burkholderia glumae	0.01163	99.96	0.024	0	0
Atopobium parvulum	0.01131	99.97	0.0233	0	0
Morococcus sicca	0.008331	99.98	0.0172	0	0
Prauserella marina	0.006355	99.99	0.0131	0	0
Burkholderia pyrrocinia	0.004999	99.99	0.0103	0	0
Neisseria subflava	0.004971	100	0.0102	0	0
Cryptobacterium curtum	0.00348	100	0.00717	0	0
Corynebacterium aurimucosum	0.001279	100	0.00264	0	0
Corynebacterium segmentosum	0	100	0	0	0
Treponema lecithinolyticum	0	100	0	0	0
Treponema vincentii	0	100	0	0	0
Streptococcus timonensis	0	100	0	0	0
Variovorax guangxiensis	0	100	0	0	0
Moraxella catarrhalis	0	100	0	0	0
Morococcus cerebrosus	0	100	0	0	0
Bacteroides heparinolyticus	0	100	0	0	0
Arachidicoccus terrestris	0	100	0	0	0
Escherichia coli	0	100	0	0	0

Prevotella denticola	0	100	0	0	0
Prevotella loescheii	0	100	0	0	0
Stenotrophomonas geniculata	0	100	0	0	0
Lactobacillus gasseri	0	100	0	0	0
Capnocytophaga ochracea	0	100	0	0	0
Paucibacter toxinivorans	0	100	0	0	0
Campylobacter concisus	0	100	0	0	0
Prevotella koreensis	0	100	0	0	0
Selenomonas flueggei	0	100	0	0	0
Phenylobacterium muchangponense	0	100	0	0	0
Ligilactobacillus salivarius	0	100	0	0	0
Staphylococcus haemolyticus	0	100	0	0	0
Actinomyces lingnae	0	100	0	0	0
Paraburkholderia fungorum	0	100	0	0	0
Sphingomonas pseudosanguinis	0	100	0	0	0
Streptococcus anginosus	0	100	0	0	0
Catonella massiliensis	0	100	0	0	0
Capnocytophaga leadbetteri	0	100	0	0	0
Lysobacter xinjiangensis	0	100	0	0	0
Abiotrophia defectiva	0	100	0	0	0
Lachnoanaerobaculum gingivalis	0	100	0	0	0

Oribacterium asaccharolyticum	0	100	0	0	0
Lipingzhangella halophila	0	100	0	0	0
Methylobacterium radiotolerans	0	100	0	0	0
Veillonella atypica	0	100	0	0	0
Prevotella oulorum	0	100	0	0	0
Lancefieldella rimae	0	100	0	0	0
Streptococcus shenyangsis	0	100	0	0	0
Bdellovibrio bacteriovorus	0	100	0	0	0
Bifidobacterium longum	0	100	0	0	0
Pseudomonas mendocina	0	100	0	0	0
Paenibacillus tyrfis	0	100	0	0	0
Enhydrobacter aerosaccus	0	100	0	0	0
Campylobacter gracilis	0	100	0	0	0
Proteus vulgaris	0	100	0	0	0
Burkholderia contaminans	0	100	0	0	0
Lautropia mirabilis	0	100	0	0	0
Neisseria perflava	0	100	0	0	0
Peptoniphilus grossensis	0	100	0	0	0
Leptotrichia hongkongensis	0	100	0	0	0
Actinomyces pyogenes	0	100	0	0	0
Dialister invisus	0	100	0	0	0
Actinomyces naeslundii	0	100	0	0	0
Staphylococcus ureilyticus	0	100	0	0	0
Dialister pneumosintes	0	100	0	0	0

Paraburkholderia phenazinium	0	100	0	0	0
Delftia tsuruhatensis	0	100	0	0	0
Streptococcus agalactiae	0	100	0	0	0
Prevotella saccharolytica	0	100	0	0	0
Alloscardovia omnicolens	0	100	0	0	0
Rothia dentocariosa	0	100	0	0	0
Selenomonas sputigena	0	100	0	0	0
Prevotella oralis	0	100	0	0	0
Achromobacter marplatensis	0	100	0	0	0
Pedobacter nutrimenti	0	100	0	0	0
Capnocytophaga bilenii	0	100	0	0	0
Capnocytophaga sputigena	0	100	0	0	0
Streptococcus rubneri	0	100	0	0	0
Fusobacterium pseudoperiodonticum	0	100	0	0	0
Selenomonas artemidis	0	100	0	0	0
Gemella asaccharolytica	0	100	0	0	0
Actinomyces oris	0	100	0	0	0
Facklamia hominis	0	100	0	0	0
Streptococcus australis	0	100	0	0	0
Neisseria oralis	0	100	0	0	0
Corynebacterium durum	0	100	0	0	0
Oribacterium sinus	0	100	0	0	0
Veillonella rogosae	0	100	0	0	0

Peptostreptococcus anaerobius	0	100	0	0	0
Lactobacillus kalixensis	0	100	0	0	0
Treponema maltophilum	0	100	0	0	0
Deinococcus xinjiangensis	0	100	0	0	0
Granulicella paludicola	0	100	0	0	0
Nonomuraea roseoviolacea	0	100	0	0	0
Gemella sanguinis	0	100	0	0	0
Ralstonia pickettii	0	100	0	0	0
Alloprevotella tannerae	0	100	0	0	0
Cupriavidus gilardii	0	100	0	0	0
Tannerella forsythia	0	100	0	0	0
Reyranella aquatilis	0	100	0	0	0
Haemophilus parahaemolyticus	0	100	0	0	0
Fusobacterium animalis	0	100	0	0	0
Cutibacterium porci	0	100	0	0	0
Porphyromonas gingivalis	0	100	0	0	0
Phreatobacter cathodiphilus	0	100	0	0	0
Mogibacterium timidum	0	100	0	0	0
Cardiobacterium valvarum	0	100	0	0	0

11.7 SIMPER: Background Microbiota and NTM Status with NTM (Chapter 5)

Taxon	Contrib. %	Cumulative %	Positive	Negative
Staphylococcus aureus	8.444	8.444	4.72	13.6
Mycobacterium avium	7.746	16.19	14.5	0.0379
Streptococcus salivarius	7.059	23.25	8.31	9.53
Streptococcus symci	4.367	27.62	5.85	3.52
Pseudomonas aeruginosa	3.504	31.12	1.07	6.09
Streptococcus toyakuensis	3.374	34.49	4.69	2.13
Mycobacteroides abscessus	3.343	37.84	5.99	0.49
Haemophilus influenzae	3.338	41.17	0.0568	6.2
Staphylococcus succinus	3.056	44.23	2.8	3.36
Veillonella nakazawae	2.873	47.1	4.78	1.42
Staphylococcus pasteuri	2.399	49.5	0.136	4.41
Burkholderia multivorans	2.392	51.89	1.87	2.86
Mycobacteroides chelonae	1.998	53.89	3.74	0
Mycobacterium asiaticum	1.886	55.78	3.53	0
Delftia tsuruhatensis	1.61	57.39	0	3.01
Streptococcus thermophilus	1.56	58.95	0.474	2.51
Streptococcus pseudopneumoniae	1.544	60.49	1.57	1.68
Stenotrophomonas maltophilia	1.524	62.02	0.0682	2.79
Gemella asaccharolytica	1.384	63.4	0.335	2.3
Reyranella aquatilis	1.328	64.73	0	2.48
Staphylococcus hominis	1.291	66.02	0	2.41
Bdellovibrio bacteriovorus	1.29	67.31	0	2.41

Staphylococcus haemolyticus	1.243	68.55	1.48	0.919
Granulicella paludicola	1.219	69.77	0	2.28
Mycobacterium kansasii	1.199	70.97	2.24	0
Porphyromonas pasteri	1.125	72.09	1.31	0.949
Stenotrophomonas pavanii	1.124	73.22	0.241	1.91
Prevotella salivae	1.086	74.3	0.726	1.44
Mycobacterium tuberculosis	1.034	75.34	1.93	0
Mycobacterium malmoense	0.9993	76.34	1.87	0
Limosilactobacillus fermentum	0.9146	77.25	0.799	0.989
Peptostreptococcus stomatis	0.8782	78.13	1.61	0.069
Oligella urethralis	0.8702	79	0.0677	1.58
Streptococcus sanguinis	0.8614	79.86	0.922	0.822
Schaalia odontolytica	0.7622	80.62	0.0319	1.4
Achromobacter xylosoxidans	0.7552	81.38	0	1.41
Neisseria flava	0.7208	82.1	1.22	0.14
Capnocytophaga sputigena	0.7107	82.81	1.33	0
Mycobacterium intracellulare	0.6757	83.49	1.26	0
Lachnoanaerobaculum saburreum	0.6735	84.16	0.806	0.552
Gemella morbillorum	0.6342	84.79	1.19	0
Prevotella histicola	0.5707	85.36	0.34	0.791
Paenibacillus tyrfis	0.559	85.92	0	1.05
Prauserella isguenensis	0.5444	86.47	0	1.02
Fusobacterium periodonticum	0.5415	87.01	0	1.01
Haemophilus parainfluenzae	0.5221	87.53	0.976	0

Veillonella parvula	0.5196	88.05	0.474	0.561
Neisseria mucosa	0.4717	88.52	0.882	0
Streptococcus gordonii	0.4582	88.98	0	0.857
Alloprevotella rava	0.4184	89.4	0.782	0
Prevotella melaninogenica	0.4097	89.81	0.57	0.246
Lautropia mirabilis	0.3512	90.16	0.539	0.141
Prevotella nanceiensis	0.3505	90.51	0.655	0
Stomatobaculum longum	0.3467	90.86	0.648	0
Pedobacter nutrimenti	0.336	91.19	0.628	0
Ligilactobacillus salivarius	0.3085	91.5	0	0.577
Veillonella rogosae	0.2899	91.79	0.145	0.411
Veillonella dispar	0.2887	92.08	0.506	0.0577
Tannerella serpentiformis	0.2849	92.36	0.533	0
Lactobacillus gasseri	0.279	92.64	0	0.522
Lancefieldella parvula	0.2751	92.92	0.487	0.0411
Megasphaera micronuciformis	0.2726	93.19	0.51	0
Variovorax guangxiensis	0.2662	93.46	0.498	0
Streptococcus timonensis	0.2584	93.72	0	0.483
Mycolicibacterium fortuitum	0.23	93.95	0.43	0
Phenylobacterium muchangponense	0.22	94.17	0.412	0
Parvimonas micra	0.2151	94.38	0.0536	0.357
Lactobacillus kalixensis	0.2094	94.59	0	0.392
Treponema vincentii	0.207	94.8	0.387	0
Prevotella pallens	0.2023	95	0.36	0.0198

Fusobacterium nucleatum	0.1994	95.2	0.277	0.119
Paraburkholderia fungorum	0.1976	95.4	0.369	0
Oribacterium sinus	0.1884	95.59	0.342	0.0116
Actinomyces lingnae	0.1772	95.76	0.331	0
Streptococcus rubneri	0.172	95.93	0	0.322
Corynebacterium segmentosum	0.1561	96.09	0	0.292
Capnocytophaga granulosa	0.144	96.23	0.0781	0.199
Veillonella massiliensis	0.1415	96.38	0.265	0
Acinetobacter baumannii	0.1411	96.52	0	0.264
Capnocytophaga leadbetteri	0.1369	96.65	0.18	0.0828
Haemophilus parahaemolyticus	0.1309	96.79	0	0.245
Nonomuraea roseoviolacea	0.1288	96.91	0	0.241
Porphyromonas endodontalis	0.1209	97.04	0.226	0
Mycobacterium paraintracellulare	0.1206	97.16	0.226	0
Streptococcus anginosus	0.12	97.28	0	0.224
Actinomyces naeslundii	0.1139	97.39	0	0.213
Prevotella intermedia	0.1113	97.5	0.0853	0.131
Streptococcus australis	0.1113	97.61	0.208	0
Neisseria oralis	0.1108	97.72	0.207	0
Prevotella vespertina	0.09915	97.82	0.143	0.046
Mycolicibacterium smegmatis	0.09637	97.92	0.18	0
Actinomyces oris	0.09298	98.01	0	0.174
Mycobacterium simiae	0.08971	98.1	0.168	0
Proteus mirabilis	0.08501	98.19	0.159	0

Streptococcus vulneris	0.08468	98.27	0.158	0
Staphylococcus epidermidis	0.08043	98.35	0	0.15
Prevotella oulorum	0.07775	98.43	0	0.145
Burkholderia cenocepacia	0.07048	98.5	0.132	0
Mycobacterium gordonae	0.06779	98.57	0.127	0
Neisseria perflava	0.06712	98.63	0.0918	0.0367
Prevotella denticola	0.061	98.7	0	0.114
Mycobacterium malmoense	0.06008	98.76	0.112	0
Selenomonas felix	0.05898	98.81	0.103	0.00776
Mycolicibacter terrae	0.05792	98.87	0.108	0
Capnocytophaga gingivalis	0.05674	98.93	0.106	0
Streptococcus shenyangsis	0.05325	98.98	0	0.0996
Staphylococcus ureilyticus	0.05141	99.03	0.0961	0
Treponema lecithinolyticum	0.0503	99.08	0.0941	0
Prevotella oris	0.05008	99.13	0	0.0937
Mycobacterium haemophilum	0.04855	99.18	0.0746	0.0177
Leptotrichia hongkongensis	0.04827	99.23	0.0903	0
Abiotrophia defectiva	0.04468	99.28	0.0836	0
Campylobacter concisus	0.04441	99.32	0.0696	0.0161
Actinomyces vulturis	0.03925	99.36	0.0734	0
Cutibacterium acnes	0.03561	99.4	0	0.0666
Methylobacterium radiotolerans	0.03528	99.43	0	0.066
Treponema maltophilum	0.03473	99.47	0.065	0
Prevotella koreensis	0.03287	99.5	0.0615	0

Gemella sanguinis	0.02858	99.53	0	0.0534
Sphingomonas pseudosanguinis	0.02794	99.55	0	0.0522
Bacteroides heparinolyticus	0.02756	99.58	0.0515	0
Lipingzhangella halophila	0.02725	99.61	0.051	0
Prevotella jejuni	0.02618	99.64	0.049	0
Capnocytophaga ochracea	0.02588	99.66	0.0484	0
Oribacterium asaccharolyticum	0.02566	99.69	0.048	0
Achromobacter marplatensis	0.02487	99.71	0	0.0465
Peptostreptococcus anaerobius	0.02382	99.74	0	0.0445
Prevotella oralis	0.02288	99.76	0	0.0428
Selenomonas sputigena	0.02131	99.78	0.0399	0
Porphyromonas gingivalis	0.02116	99.8	0.0396	0
Alloprevotella tannerae	0.02105	99.84	0.0254	0.0152
Prevotella loescheii	0.02105	99.82	0.0394	0
Brevibacterium otitidis	0.01905	99.86	0.0356	0
Actinomyces graevenitzii	0.01805	99.88	0.0338	0
Cupriavidus gilardii	0.01757	99.9	0.0329	0
Actinomyces pyogenes	0.01561	99.91	0	0.0292
Lysobacter xinjiangensis	0.01329	99.93	0.0249	0
Selenomonas artemidis	0.01309	99.94	0.0245	0
Lancefieldella rimae	0.01167	99.95	0	0.0218
Dialister invisus	0.01142	99.96	0.0214	0
Campylobacter gracilis	0.009686	99.97	0	0.0181
Prevotella saccharolytica	0.009448	99.98	0.0177	0

Dialister pneumosintes	0.007683	99.99	0	0.0144
Prauserella marina	0.005839	100	0	0.0109
Enhydrobacter aerosaccus	0.004429	100	0.00828	0

11.8 SIMPER: Background Microbiota and NTM Status without NTM (Chapter 5)

Taxon	Contrib. %	Cumulative %	Mean Postive	Mean Negative
Staphylococcus aureus	9.295	9.295	6.56	13.6
Streptococcus salivarius	8.868	18.16	12.1	9.77
Streptococcus symci	5.631	23.79	8.28	3.53
Pseudomonas aeruginosa	5.574	29.37	5.2	6.1
Streptococcus toyakuensis	4.522	33.89	6.86	2.13
Veillonella nakazawae	4.469	38.36	7.76	1.42
Staphylococcus succinus	3.509	41.87	3.61	3.36
Haemophilus influenzae	3.388	45.26	0.0717	6.2
Streptococcus pseudopneumoniae	3.327	48.58	4.77	1.79
Burkholderia multivorans	2.969	51.55	2.98	2.87
Staphylococcus pasteuri	2.461	54.01	0.229	4.41
Variovorax guangxiensis	2.257	56.27	4.17	0
Streptococcus thermophilus	1.806	58.08	0.934	2.51
Delftia tsuruhatensis	1.632	59.71	0	3.01
Stenotrophomonas maltophilia	1.552	61.26	0.0861	2.79
Gemella asaccharolytica	1.445	62.7	0.424	2.3
Staphylococcus haemolyticus	1.377	64.08	1.7	0.92
Staphylococcus hominis	1.346	65.43	0	2.49
Reyranella aquatilis	1.345	66.77	0	2.48
Bdellovibrio bacteriovorus	1.312	68.08	0	2.42
Porphyromonas pasteri	1.297	69.38	1.63	0.949
Granulicella paludicola	1.235	70.62	0	2.28
Prevotella salivae	1.233	71.85	1.03	1.44
Stenotrophomonas pavanii	1.189	73.04	0.342	1.91
Gemella morbillorum	1.173	74.21	2.17	0
Capnocytophaga sputigena	1.159	75.37	2.14	0
Peptostreptococcus stomatis	1.084	76.45	1.97	0.069
Streptococcus sanguinis	1.059	77.51	1.27	0.825
Limosilactobacillus fermentum	0.9795	78.49	0.902	0.989
Oligella urethralis	0.8897	79.38	0.0887	1.58
Lachnoanaerobaculum saburreum	0.8331	80.22	1.09	0.553
Neisseria flava	0.8219	81.04	1.39	0.141
Schaalia odontolytica	0.8064	81.84	0.036	1.46
Achromobacter xylosoxidans	0.7651	82.61	0	1.41
Alloprevotella rava	0.728	83.34	1.34	0
Haemophilus parainfluenzae	0.6141	83.95	1.13	0
Prevotella histicola	0.6121	84.56	0.414	0.791

Veillonella parvula	0.6112	85.17	0.642	0.564
Paenibacillus tyrfis	0.5663	85.74	0	1.05
Neisseria mucosa	0.5651	86.31	1.04	0
Prauserella isguenensis	0.5515	86.86	0	1.02
Prevotella melaninogenica	0.5496	87.41	0.833	0.246
Fusobacterium periodonticum	0.5486	87.96	0	1.01
Stomatobaculum longum	0.5466	88.5	1.01	0
Lancefieldella parvula	0.5315	89.03	0.954	0.0411
Prevotella nanceiensis	0.4946	89.53	0.913	0
Streptococcus gordonii	0.4642	89.99	0	0.857
Megasphaera micronuciformis	0.4511	90.44	0.833	0
Tannerella serpentiformis	0.4508	90.89	0.832	0
Pedobacter nutrimenti	0.4163	91.31	0.769	0
Lautropia mirabilis	0.3953	91.71	0.612	0.141
Veillonella dispar	0.3793	92.09	0.666	0.058
Veillonella rogosae	0.3714	92.46	0.303	0.411
Prevotella pallens	0.3357	92.79	0.602	0.0198
Fusobacterium nucleatum	0.3164	93.11	0.494	0.119
Ligilactobacillus salivarius	0.3125	93.42	0	0.577
Lactobacillus gasseri	0.2827	93.7	0	0.522
Phenylobacterium muchangponense	0.2726	93.98	0.503	0
Paraburkholderia fungorum	0.2635	94.24	0.486	0
Streptococcus timonensis	0.2618	94.5	0	0.483
Actinomyces lingnae	0.2543	94.76	0.47	0
Oribacterium sinus	0.2511	95.01	0.453	0.0116
Treponema vincentii	0.2387	95.25	0.441	0
Parvimonas micra	0.2201	95.47	0.0581	0.357
Lactobacillus kalixensis	0.2122	95.68	0	0.392
Streptococcus rubneri	0.1743	95.85	0	0.322
Proteus mirabilis	0.1711	96.02	0.316	0
Capnocytophaga granulosa	0.1674	96.19	0.122	0.199
Corynebacterium segmentosum	0.1588	96.35	0	0.293
Veillonella massiliensis	0.1554	96.51	0.287	0
Porphyromonas endodontalis	0.1522	96.66	0.281	0
Streptococcus australis	0.1494	96.81	0.276	0
Acinetobacter baumannii	0.143	96.95	0	0.264
Capnocytophaga leadbetteri	0.1387	97.09	0.18	0.0828
Haemophilus parahaemolyticus	0.1326	97.22	0	0.245
Prevotella intermedia	0.1326	97.35	0.126	0.131
Nonomuraea roseoviolacea	0.1305	97.48	0	0.241
Neisseria oralis	0.1278	97.61	0.236	0
Streptococcus anginosus	0.1226	97.74	0	0.226
Streptococcus vulneris	0.1215	97.86	0.224	0
Actinomyces naeslundii	0.1161	97.97	0	0.214
Prevotella vespertina	0.107	98.08	0.155	0.046
Selenomonas felix	0.0972	98.18	0.172	0.00776
Actinomyces oris	0.0942	98.27	0	0.174
Capnocytophaga gingivalis	0.08608	98.36	0.159	0

Burkholderia cenocepacia	0.08321	98.44	0.154	0
Neisseria perflava	0.08243	98.52	0.118	0.037
Staphylococcus epidermidis	0.08148	98.6	0	0.15
Prevotella oulorum	0.07877	98.68	0	0.145
Campylobacter concisus	0.06649	98.75	0.109	0.0161
Staphylococcus ureilyticus	0.06369	98.81	0.118	0
Prevotella denticola	0.0618	98.87	0	0.114
Leptotrichia hongkongensis	0.05981	98.93	0.11	0
Actinomyces vulturis	0.05972	98.99	0.11	0
Abiotrophia defectiva	0.05826	99.05	0.108	0
Treponema lecithinolyticum	0.05801	99.11	0.107	0
Streptococcus shenyangsis	0.05394	99.16	0	0.0996
Prevotella oris	0.05073	99.22	0	0.0937
Prevotella koreensis	0.04286	99.26	0.0791	0
Oribacterium asaccharolyticum	0.0406	99.3	0.0749	0
Treponema maltophilum	0.04005	99.34	0.0739	0
Actinomyces graevenitzii	0.03829	99.38	0.0707	0
Cutibacterium acnes	0.03637	99.41	0	0.0671
Bacteroides heparinolyticus	0.03593	99.45	0.0663	0
Methylobacterium radiotolerans	0.03574	99.49	0	0.066
Prevotella jejuni	0.03413	99.52	0.063	0
Capnocytophaga ochracea	0.03375	99.55	0.0623	0
Selenomonas sputigena	0.03372	99.59	0.0622	0
Gemella sanguinis	0.02895	99.62	0	0.0534
Sphingomonas pseudosanguinis	0.02853	99.64	0	0.0527
Lipingzhangella halophila	0.02761	99.67	0.051	0
Porphyromonas gingivalis	0.02759	99.7	0.0509	0
Cupriavidus gilardii	0.02654	99.73	0.049	0
Achromobacter marplatensis	0.0254	99.75	0	0.0469
Alloprevotella tannerae	0.02527	99.78	0.0327	0.0152
Brevibacterium otitidis	0.02435	99.8	0.045	0
Dialister invisus	0.02423	99.83	0.0447	0
Peptostreptococcus anaerobius	0.02413	99.85	0	0.0445
Prevotella oralis	0.02317	99.87	0	0.0428
Prevotella loescheii	0.02313	99.9	0.0427	0
Lysobacter xinjiangensis	0.01784	99.91	0.0329	0
Selenomonas artemidis	0.01707	99.93	0.0315	0
Actinomyces pyogenes	0.01581	99.95	0	0.0292
Prevotella saccharolytica	0.01232	99.96	0.0227	0
Lancefieldella rimae	0.01191	99.97	0	0.022
Campylobacter gracilis	0.009813	99.98	0	0.0181
Dialister pneumosintes	0.007783	99.99	0	0.0144
Prauserella marina	0.005915	99.99	0	0.0109
Enhydrobacter aerosaccus	0.005775	100	0.0107	0

11.9 SIMPER analysis: NTM Type and the Microbiome Chapter 5

Taxon	Contrib. %	Cumulative %	MAC	MABSC	Other
Mycobacterium avium	8.995	8.995	29	0	0
Staphylococcus aureus	7.37	16.37	5.09	7.45	0
Streptococcus salivarius	6.985	23.35	9.38	7.42	6.98
Streptococcus symci	4.593	27.94	8.95	0	6.63
Mycobacteroides abscessus	4.288	32.23	0.303	18.7	1.9
Streptococcus toyakuensis	3.599	35.83	4.93	7.62	0
Veillonella nakazawae	3.236	39.07	2.91	8.94	3.43
Staphylococcus succinus	3.183	42.25	0	3.55	8.49
Pseudomonas aeruginosa	2.85	45.1	1.23	1.58	0
Mycobacteroides chelonae	2.626	47.73	0	10.7	2.99
Mycobacterium asiaticum	2.498	50.22	1.43	0	13.5
Haemophilus influenzae	2.48	52.7	0.114	0	0
Burkholderia multivorans	2.238	54.94	1.91	3.13	0
Staphylococcus pasteuri	1.803	56.74	0.271	0	0
Streptococcus pseudopneumoniae	1.514	58.26	2.09	0.555	1.74
Mycobacterium malmoense	1.407	59.67	0	0	8.97
Staphylococcus haemolyticus	1.393	61.06	0	5.08	0
Mycobacterium kansasii	1.386	62.44	3.76	1.24	0
Mycobacterium tuberculosis	1.276	63.72	2.18	0	4.04
Streptococcus thermophilus	1.26	64.98	0.948	0	0
Delftia tsuruhatensis	1.19	66.17	0	0	0
Porphyromonas pasteri	1.179	67.35	1.34	0.142	2.87

Stenotrophomonas maltophilia	1.142	68.49	0.136	0	0
Gemella asaccharolytica	1.122	69.61	0.114	0.953	0
Peptostreptococcus stomatis	1.057	70.67	1.27	3.19	0.183
Prevotella salivae	0.9998	71.67	0.539	1.57	0
Reyranella aquatilis	0.9813	72.65	0	0	0
Neisseria flava	0.9689	73.62	0	0	5.85
Streptococcus sanguinis	0.9664	74.59	0	0	4.43
Limosilactobacillus fermentum	0.9617	75.55	0	0	3.83
Staphylococcus hominis	0.9542	76.5	0	0	0
Bdellovibrio bacteriovorus	0.9536	77.46	0	0	0
Stenotrophomonas pavanii	0.9083	78.37	0	0.828	0
Granulicella paludicola	0.9008	79.27	0	0	0
Capnocytophaga sputigena	0.8254	80.09	2.66	0	0
Mycobacterium intracellulare	0.7928	80.88	1.73	1.37	0
Gemella morbillorum	0.7438	81.63	1.66	1.21	0
Haemophilus parainfluenzae	0.6853	82.31	0.376	0.268	3.41
Lachnoanaerobaculum saburreum	0.6825	83	1.31	0.509	0
Oligella urethralis	0.6674	83.66	0	0	0.325
Neisseria mucosa	0.6095	84.27	0.178	1.5	1.71
Schaalia odontolytica	0.5747	84.85	0	0	0.153
Achromobacter xylosoxidans	0.5582	85.41	0	0	0

Alloprevotella rava	0.5461	85.95	0.095	2.19	0.462
Prevotella histicola	0.5267	86.48	0.118	0.208	1.06
Veillonella parvula	0.5057	86.98	0.638	0	0.745
Lautropia mirabilis	0.4523	87.44	0	0	2.59
Pedobacter nutrimenti	0.4494	87.89	0	2.15	0
Prevotella melaninogenica	0.4316	88.32	1.14	0	0
Prevotella nanceiensis	0.4197	88.74	0.915	0	0.95
Paenibacillus tyrfis	0.4131	89.15	0	0	0
Stomatobaculum longum	0.4026	89.55	1.3	0	0
Prauserella isguenensis	0.4023	89.95	0	0	0
Fusobacterium periodonticum	0.4002	90.36	0	0	0
Variovorax guangxiensis	0.3748	90.73	0	0	2.39
Streptococcus gordonii	0.3387	91.07	0	0	0
Veillonella dispar	0.3362	91.4	0.49	0.347	0.769
Tannerella serpentiformis	0.3309	91.74	1.07	0	0
Megasphaera micronuciformis	0.3167	92.05	1.02	0	0
Lancefieldella parvula	0.3133	92.37	0.973	0	0
Mycolicibacterium fortuitum	0.3076	92.67	0	1.47	0
Phenylobacterium muchangponense	0.2943	92.97	0	1.41	0
Treponema vincentii	0.2914	93.26	0	0	1.86
Oribacterium sinus	0.2615	93.52	0	0	1.64

Veillonella rogosae	0.247	93.77	0.29	0	0
Actinomyces lingnae	0.237	94	0	1.14	0
Prevotella pallens	0.2309	94.24	0.72	0	0
Paraburkholderia fungorum	0.2295	94.46	0.739	0	0
Ligilactobacillus salivarius	0.228	94.69	0	0	0
Fusobacterium nucleatum	0.2099	94.9	0.554	0	0
Lactobacillus gasseri	0.2062	95.11	0	0	0
Streptococcus timonensis	0.191	95.3	0	0	0
Veillonella massiliensis	0.1892	95.49	0	0.907	0
Parvimonas micra	0.1761	95.67	0	0.184	0
Streptococcus australis	0.1567	95.82	0	0	0.999
Neisseria oralis	0.156	95.98	0	0	0.994
Lactobacillus kalixensis	0.1548	96.13	0	0	0
Porphyromonas endodontalis	0.143	96.28	0.361	0	0.219
Capnocytophaga leadbetteri	0.1419	96.42	0.36	0	0
Mycobacterium paraintracellulare	0.1401	96.56	0.451	0	0
Mycolicibacterium smegmatis	0.1357	96.69	0	0	0.865
Streptococcus rubneri	0.1271	96.82	0	0	0
Mycobacterium simiae	0.1263	96.95	0	0	0.805
Capnocytophaga granulosa	0.1241	97.07	0.156	0	0

Prevotella vespertina	0.1191	97.19	0	0.491	0
Corynebacterium segmentosum	0.1154	97.31	0	0	0
Streptococcus vulneris	0.1133	97.42	0	0.543	0
Acinetobacter baumannii	0.1043	97.52	0	0	0
Prevotella intermedia	0.1016	97.62	0.171	0	0
Proteus mirabilis	0.09873	97.72	0.318	0	0
Haemophilus parahaemolyticus	0.09676	97.82	0	0	0
Burkholderia cenocepacia	0.09604	97.92	0	0.145	0.43
Nonomuraea roseoviolacea	0.09517	98.01	0	0	0
Streptococcus anginosus	0.08872	98.1	0	0	0
Mycobacterium malmoense	0.08459	98.18	0	0	0.539
Actinomyces naeslundii	0.08417	98.27	0	0	0
Mycobacterium gordonae	0.08151	98.35	0.17	0	0.201
Mycolicibacter terrae	0.07746	98.43	0	0.371	0
Treponema lecithinolyticum	0.07082	98.5	0	0	0.452
Neisseria perflava	0.07035	98.57	0.184	0	0
Staphylococcus ureilyticus	0.06875	98.64	0	0.33	0
Actinomyces oris	0.06872	98.71	0	0	0
Selenomonas felix	0.06689	98.77	0.206	0	0
Capnocytophaga gingivalis	0.0659	98.84	0.212	0	0
Leptotrichia hongkongensis	0.06456	98.9	0	0.31	0
Staphylococcus epidermidis	0.05945	98.96	0	0	0

Prevotella oulorum	0.05746	99.02	0	0	0
Mycobacterium haemophilum	0.05273	99.07	0.149	0	0
Abiotrophia defectiva	0.0519	99.13	0.167	0	0
Campylobacter concisus	0.05069	99.18	0.067	0.124	0
Treponema maltophilum	0.0489	99.22	0	0	0.312
Actinomyces vulturis	0.04729	99.27	0.0862	0.104	0
Prevotella denticola	0.04509	99.32	0	0	0
Streptococcus shenyangsis	0.03936	99.36	0	0	0
Prevotella koreensis	0.03818	99.39	0.123	0	0
Prevotella oris	0.03701	99.43	0	0	0
Bacteroides heparinolyticus	0.03201	99.46	0.103	0	0
Lipingzhangella halophila	0.03165	99.5	0.102	0	0
Prevotella jejuni	0.03041	99.53	0.0979	0	0
Capnocytophaga ochracea	0.03006	99.56	0.0968	0	0
Oribacterium asaccharolyticum	0.0298	99.59	0.096	0	0
Prevotella loescheii	0.02816	99.61	0	0.135	0
Cutibacterium acnes	0.02632	99.64	0	0	0
Methylobacterium radiotolerans	0.02608	99.67	0	0	0
Selenomonas sputigena	0.02475	99.69	0.0797	0	0
Cupriavidus gilardii	0.02473	99.72	0	0	0.158

Porphyromonas gingivalis	0.02458	99.74	0.0791	0	0
Brevibacterium otitidis	0.02213	99.76	0.0713	0	0
Alloprevotella tannerae	0.02129	99.78	0.0508	0	0
Gemella sanguinis	0.02112	99.8	0	0	0
Actinomyces graevenitzii	0.02097	99.83	0.0675	0	0
Sphingomonas pseudosanguinis	0.02065	99.85	0	0	0
Lysobacter xinjiangensis	0.01871	99.87	0	0	0.119
Achromobacter marplatensis	0.01838	99.88	0	0	0
Peptostreptococcus anaerobius	0.0176	99.9	0	0	0
Prevotella oralis	0.01691	99.92	0	0	0
Selenomonas artemidis	0.0152	99.93	0.049	0	0
Dialister invisus	0.01327	99.95	0.0427	0	0
Actinomyces pyogenes	0.01153	99.96	0	0	0
Prevotella saccharolytica	0.01097	99.97	0.0353	0	0
Lancefieldella rimae	0.008623	99.98	0	0	0
Campylobacter gracilis	0.007159	99.98	0	0	0
Dialister pneumosintes	0.005678	99.99	0	0	0
Enhydrobacter aerosaccus	0.005144	100	0.0166	0	0

Prauserella marina	0.004316	100	0	0	0

11.10 SIMPER analysis: NTM Type and the Microbiome Chapter 5 without NTM

Taxon	Contrib. %	Cumulative %	Mean MAC	Mean MABSC	Mean Other
Variovorax guangxiensis	5.778	48.41	0	0	20
Staphylococcus succinus	4.636	53.05	0	4.35	11.2
Streptococcus symci	7.466	17.19	12.4	0	9.88
Streptococcus salivarius	9.726	9.726	15.2	9.01	8.9
Neisseria flava	1.925	64.22	0	0	6.66
Streptococcus sanguinis	1.759	71.64	0	0	6.09
Limosilactobacillus fermentum	1.251	77.46	0	0	4.33
Veillonella nakazawae	7.325	24.52	4.52	16.1	3.88
Haemophilus parainfluenzae	1.337	76.21	0.484	0.29	3.87
Porphyromonas pasteri	1.632	73.27	1.82	0.154	3.24
Lautropia mirabilis	0.8486	84.13	0	0	2.94
Streptococcus pseudopneumoniae	4.3	57.35	8	0.786	2.6
Oribacterium sinus	0.6281	89.25	0	0	2.17
Treponema vincentii	0.611	90.48	0	0	2.12
Neisseria mucosa	1.166	78.63	0.3	1.67	1.95
Streptococcus australis	0.3825	94.82	0	0	1.32
Prevotella histicola	0.4804	93.15	0.194	0.226	1.21
Neisseria oralis	0.327	95.5	0	0	1.13
Veillonella parvula	0.6466	88.62	0.821	0	1.11
Prevotella nanceiensis	0.8504	83.28	1.38	0	1.07
Veillonella dispar	0.6	91.08	0.751	0.377	0.868
Alloprevotella rava	1.6	74.87	0.122	3.96	0.613
Treponema lecithinolyticum	0.1485	97.63	0	0	0.514
Burkholderia cenocepacia	0.1979	96.98	0	0.177	0.489
Oligella urethralis	0.123	98.17	0	0	0.426
Treponema maltophilum	0.1025	98.81	0	0	0.355
Porphyromonas endodontalis	0.2575	96.58	0.458	0	0.249
Cupriavidus gilardii	0.06793	99.19	0	0	0.235
Peptostreptococcus stomatis	1.913	66.13	1.61	3.83	0.208
Schaalia odontolytica	0.04999	99.7	0	0	0.173
Lysobacter xinjiangensis	0.04568	99.75	0	0	0.158
Pseudomonas aeruginosa	5.947	42.63	1.88	14.6	0
Streptococcus toyakuensis	6.153	30.67	7.86	10.1	0
Staphylococcus aureus	6.013	36.68	7.9	8.96	0
Staphylococcus haemolyticus	2.108	62.3	0	5.82	0
Burkholderia multivorans	2.844	60.19	3.72	3.84	0
Pedobacter nutrimenti	0.9536	81.55	0	2.63	0
Phenylobacterium muchangponense	0.6244	89.87	0	1.73	0

Prevotella salivae	1.004	79.63	1.07	1.7	0
Actinomyces lingnae	0.5825	91.66	0	1.61	0
Gemella morbillorum	1.874	68.01	3.46	1.5	0
Gemella asaccharolytica	0.4834	92.67	0.169	1.17	0
Stenotrophomonas pavanii	0.4244	94.44	0	1.17	0
Veillonella massiliensis	0.356	95.18	0	0.984	0
Streptococcus vulneris	0.2783	95.78	0	0.769	0
Lachnoanaerobaculum saburreum	0.9644	80.59	1.82	0.603	0
Prevotella vespertina	0.1928	97.17	0	0.533	0
Staphylococcus ureilyticus	0.1459	97.77	0	0.403	0
Leptotrichia hongkongensis	0.137	98.05	0	0.379	0
Parvimonas micra	0.07207	99.05	0	0.199	0
Actinomyces vulturis	0.1059	98.5	0.135	0.147	0
Prevotella loescheii	0.05297	99.65	0	0.146	0
Campylobacter concisus	0.1043	98.6	0.14	0.134	0
Capnocytophaga sputigena	1.873	69.88	4.28	0	0
Stomatobaculum longum	0.8837	82.43	2.02	0	0
Lancefieldella parvula	0.8353	84.96	1.91	0	0
Streptococcus thermophilus	0.8177	85.78	1.87	0	0
Prevotella melaninogenica	0.7299	86.51	1.67	0	0
Megasphaera micronuciformis	0.7292	87.24	1.67	0	0
Tannerella serpentiformis	0.7287	87.97	1.66	0	0
Prevotella pallens	0.5268	92.19	1.2	0	0
Fusobacterium nucleatum	0.4326	93.59	0.988	0	0
Paraburkholderia fungorum	0.4259	94.01	0.973	0	0
Proteus mirabilis	0.2766	96.06	0.632	0	0
Veillonella rogosae	0.2655	96.32	0.606	0	0
Staphylococcus pasteuri	0.2002	96.78	0.457	0	0
Capnocytophaga leadbetteri	0.1578	97.33	0.36	0	0
Selenomonas felix	0.1509	97.48	0.345	0	0
Capnocytophaga gingivalis	0.1392	97.91	0.318	0	0
Prevotella intermedia	0.1101	98.28	0.251	0	0
Capnocytophaga granulosa	0.1068	98.39	0.244	0	0
Neisseria perflava	0.1035	98.7	0.236	0	0
Abiotrophia defectiva	0.09419	98.9	0.215	0	0
Stenotrophomonas maltophilia	0.0754	98.98	0.172	0	0
Prevotella koreensis	0.06929	99.12	0.158	0	0
Oribacterium asaccharolyticum	0.06564	99.25	0.15	0	0
Haemophilus influenzae	0.06283	99.31	0.143	0	0
Actinomyces graevenitzii	0.06191	99.38	0.141	0	0
Bacteroides heparinolyticus	0.05809	99.43	0.133	0	0
Prevotella jejuni	0.05519	99.49	0.126	0	0
Capnocytophaga ochracea	0.05456	99.54	0.125	0	0
Selenomonas sputigena	0.05452	99.6	0.124	0	0
Lipingzhangella halophila	0.04464	99.79	0.102	0	0

Porphyromonas gingivalis	0.0446	99.84	0.102	0	0
Brevibacterium otitidis	0.03937	99.88	0.0899	0	0
Dialister invisus	0.03918	99.91	0.0895	0	0
Alloprevotella tannerae	0.02863	99.94	0.0654	0	0
Selenomonas artemidis	0.02759	99.97	0.063	0	0
Prevotella saccharolytica	0.01992	99.99	0.0455	0	0
Enhydrobacter aerosaccus	0.009336	100	0.0213	0	0
Paenibacillus tyrfis	0	100	0	0	0
Dialister pneumosintes	0	100	0	0	0
Streptococcus timonensis	0	100	0	0	0
Nonomuraea roseoviolacea	0	100	0	0	0
Streptococcus shenyangsis	0	100	0	0	0
Delftia tsuruhatensis	0	100	0	0	0
Bdellovibrio bacteriovorus	0	100	0	0	0
Actinomyces naeslundii	0	100	0	0	0
Streptococcus rubneri	0	100	0	0	0
Streptococcus gordonii	0	100	0	0	0
Cutibacterium acnes	0	100	0	0	0
Streptococcus anginosus	0	100	0	0	0
Methylobacterium radiotolerans	0	100	0	0	0
Staphylococcus hominis	0	100	0	0	0
Corynebacterium segmentosum	0	100	0	0	0
Staphylococcus epidermidis	0	100	0	0	0
Sphingomonas pseudosanguinis	0	100	0	0	0
Ligilactobacillus salivarius	0	100	0	0	0
Achromobacter xylosoxidans	0	100	0	0	0
Reyranella aquatilis	0	100	0	0	0
Lancefieldella rimae	0	100	0	0	0
Lactobacillus kalixensis	0	100	0	0	0
Prevotella oulorum	0	100	0	0	0
Lactobacillus gasseri	0	100	0	0	0
Prevotella oris	0	100	0	0	0
Prevotella oralis	0	100	0	0	0
Haemophilus parahaemolyticus	0	100	0	0	0
Campylobacter gracilis	0	100	0	0	0
Prevotella denticola	0	100	0	0	0
Granulicella paludicola	0	100	0	0	0
Prauserella marina	0	100	0	0	0
Prauserella isguenensis	0	100	0	0	0
Gemella sanguinis	0	100	0	0	0
Actinomyces pyogenes	0	100	0	0	0
Peptostreptococcus anaerobius	0	100	0	0	0
Fusobacterium periodonticum	0	100	0	0	0
Actinomyces oris	0	100	0	0	0

Acinetobacter baumannii	0	100	0	0	0
Achromobacter marplatensis	0	100	0	0	0