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Investigation of diabetes-induced changes in the lung microbiome

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Thesis submitted for the Degree of Doctor of Philosophy

2022

Declaration

I, Stanislavs Vasiljevs, declare that all the work presented in the thesis is my own. Any contributions from collaborators, or where information has been derived from other sources, is explicitly referenced in the text.

SVasiljevs

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Abstract

Through the development of next-generation sequencing, it was demonstrated that the lungs are inhabited by resident bacteria and possess a unique microbiome. The airway epithelium and the airway surface liquid (ASL) play a vital role in maintaining a healthy microbiome and suppressing the growth of pathogenic species.

Glucose concentration in ASL is much lower than that of blood. It was proposed that low glucose concentration contributes to innate protection against the growth of pathogenic organisms, which can utilise glucose for growth. Studies demonstrated that increased glucose concentration in ASL *in vitro* and in animals with diabetes led to increase in the growth of pathogenic bacteria. However, there is limited data on the effects of diabetes on the lung microbiome in people or animals.

This thesis investigated the following: 1) The use of diabetic mouse models to study the effects of diabetes on the lung microbiome. 2) The use of cystic fibrosis (CF) sputum to study the effects of diabetes on the lung microbiome. 3) The use of artificial sputum medium to study the effects of hyperglycaemia on the common lung pathogens *in vitro*. 4) The use of oral washes as a non-invasive proxy to study the lung microbiome.

Results demonstrated that 1) Diabetes changes the lung microbiome in mice and enhances the growth of specific pathogens. 2) Diabetes modifies the human sputum microbiome in CF. 3) Hyperglycaemia did not affect the growth of either *Staphylococcus aureus* or *Pseudomonas aeruginosa* when grown in co-culture in the artificial sputum medium. 4) Changes in the sputum microbiome are not reflected in the oral wash samples.

This thesis highlights how diabetic hyperglycaemia changes the lung microbiome and can affect the abundance of pathogens in the lungs.

Table of Contents

Declaration.....	ii
Acknowledgements	iii
Abstract.....	iv
Table of Contents.....	v
Abbreviations	xiv
1 Literature review	1
1.1 Lung anatomy.....	2
1.2 ASL and lung defence.....	4
1.3 Presence of glucose in the ASL.....	5
1.4 Effects of inflammation on the glucose concentration in the ASL.....	6
1.5 Effects of the diabetes on the lung health	7
1.6 Assessing lung microbiome	9
1.7 Culture-independent approach of studying microbiome	11
1.8 MiSeq	14
1.9 16S rRNA sequencing	17
1.10 Effects of diabetes on the microbiome in various body systems	21
1.10.1 Effects of diabetes on the gut microbiome.....	22
1.10.2 Effects of diabetes on the oral microbiome.....	24
1.10.3 Effects of diabetes on the skin microbiome	25
1.10.4 Effects of diabetes on the lung microbiome.....	26
1.11 Effects of diabetes on the lung microbiome in individuals with CF	28

1.12 Effects of diabetes on the lung microbiome in individuals with COPD	31
2 Investigating diabetes-induced changes in the lung microbiome of diabetic mouse models.....	33
2.1 Introduction.....	35
2.1.1 Studying lung microbiome in animals	35
2.1.2 Non-rodent animal models of diabetes	36
2.1.3 Rodent models of diabetes	37
2.1.4 Mouse models of diabetes	38
2.1.4.1 Db/db mice	39
2.1.4.2 GK ^{+/-} mice	39
2.1.4.3 Streptozotocin mice.....	39
2.1.4.4 Ob/ob mice	40
2.1.5 Comparison of mouse models of diabetes	40
2.2 Aims and objectives	43
2.3 Materials and methods.....	44
2.3.1 Db/db mice	44
2.3.2 GK ^{+/-} mice	44
2.3.3 BAL and blood sampling	45
2.3.4 DNA extraction, quantification and 16S sequencing	46
2.3.5 Data analysis	48
2.3.6 Statistical analysis	49
2.4 Results.....	50
2.4.1 Mouse BAL sample collection and DNA extraction	50
2.4.2 Weight and blood glucose in db/db and wildtype mice.....	50
2.4.3 Total OTU amount in BAL samples from db/db and wildtype mice.....	52

2.4.4 Alpha diversity of db/db and wildtype mice	52
2.4.5 Beta diversity of the lung microbiome between db/db and wildtype mice	55
2.4.6 Bacterial abundance at genus level between db/db and wildtype mice	56
2.4.7 Bacterial abundance at genus level compared between different diabetic mouse models	58
2.4.8 Bacterial abundance at phyla level compared between different diabetic mouse models	61
2.5 Discussion	63
2.5.1 Stability of the lung microbiome across various mice models housed in different environments	63
2.5.2 Effects of diabetes on the lung microbiome	65
2.5.3 Comparison of lung microbiome between mice and humans	68
2.6 Summary	70
2.7 Limitations and future work	71
3 Investigating diabetes-induced changes in the lung microbiome in individuals with cystic fibrosis	72
3.1 Introduction	73
3.1.1 Revisiting cystic fibrosis and cystic fibrosis-related diabetes	73
3.1.2 Designing the study	73
3.1.3 Clinical markers of lung health in CF individuals	75
3.1.3.1 Spirometry tests	75
3.1.3.2 HbA1c	76
3.1.4 Using sputum to analyse lung microbiome	78
3.2 Aims and hypothesis	80

3.3 Materials and methods.....	81
3.3.1 Study design and sample acquisition.....	81
3.3.2 Targeted amplicon sequencing.....	81
3.3.2.1 DNA extraction.....	81
3.3.2.2 Amplicon library preparation and sequencing	82
3.3.3 Data analysis	83
3.3.4 Statistical analysis	83
3.4 Results.....	84
3.4.1 Blood HbA1c and glucose concentrations in CF and CFRD individuals.....	84
3.4.2 Spirometry measurements in CF and CFRD individuals.....	86
3.4.3 Total OTU count in the sputum samples of CF and CFRD individuals	88
3.4.4 Alpha diversity of the microbiome in the sputum of CF and CFRD individuals.....	89
3.4.5 Beta diversity of microbiome in the sputum of CF and CFRD individuals.....	92
3.4.6 Phylum-level analysis of sputum microbiome in CF and CFRD individuals	94
3.4.7 Family-level analysis of sputum microbiome in CF and CFRD individuals.....	95
3.4.8 Genus-level analysis of sputum microbiome in CF and CFRD individuals.....	96
3.4.8.1 Genus-level classification.....	96
3.4.8.2 Analysis of twelve most abundant genera in the sputum of CF and CFRD individuals.....	96
3.4.8.3 Genera detected in the sputum of CF and CFRD individuals modified by diabetes	97
3.4.9 Abundance of Staphylococcus and Pseudomonas in the sputum of CF and CFRD individuals	101
3.4.10 Understanding the effects of high Pseudomonadaceae abundance in the sputum of CFRD individuals.....	103
3.4.11 Age of participants as a confounding variable in the lung microbiome study	108
3.5 Discussion	112

3.5.1 Use of sputum to investigate lung microbiome	112
3.5.2 Effects of diabetes on the lung microbiome.....	113
3.5.3 Diabetes-induced changes in the abundance of specific genera detected in the sputum of CF and CFRD individuals.....	115
3.5.4 Importance of <i>Staphylococcus</i> and <i>Pseudomonas</i> in the sputum of CF and CFRD individuals	118
3.5.5 Effect of <i>Pseudomonas</i> infection on the lung microbiome of CFRD individuals..	120
3.5.6 Understanding the effects of age of participants on the lung microbiome	121
3.5.7 Understanding the effects of antibiotics on the lung microbiome	122
3.6 Summary	124
3.7 Limitations and future work	125
4 Use of the Artificial Sputum Medium for the in vitro analysis of interactions between common lung pathogens <i>Staphylococcus aureus</i> and <i>Pseudomonas aeruginosa</i> in the hyperglycaemic environment.....	126
4.1 Introduction.....	128
4.1.1 <i>Staphylococcus aureus</i>	128
4.1.2 <i>Pseudomonas aeruginosa</i>	129
4.1.3 Relationship between <i>S. aureus</i> and <i>P. aeruginosa</i> in the human lungs.....	131
4.1.4 Effects of glucose on the growth of <i>S. aureus</i> and <i>P. aeruginosa</i> in CF lungs....	132
4.1.5 Use of Artificial Sputum Media to study effects of glucose on the growth of <i>S. aureus</i> and <i>P. aeruginosa</i>	133
4.2 Hypothesis and Aims	135
4.3 Materials and methods.....	136
4.3.1 Bacterial strains	136

4.3.2 Bacterial stocks	136
4.3.3 Culturing bacteria from stocks.....	136
4.3.4 Bacterial media and agar plates	137
4.3.5 Single colony bacterial plates.....	137
4.3.6 ASM bacterial preparation.....	138
4.3.7 Quantification of bacteria	138
4.3.8 Preparation of ASM media and variants	139
4.3.9 Silver stain analysis.....	140
4.3.10 Glucose measurement	140
4.3.11 Lactate measurement.....	140
4.3.12 Statistical analysis	141
4.4 Results.....	142
4.4.1 The physical appearance of <i>S. aureus</i> and <i>P. aeruginosa</i> on agar plates	142
4.4.2 Quantification of bacteria	142
4.4.2.1 Quantification using OD600 measurement.....	142
4.4.2.2 Quantification of <i>S. aureus</i> and <i>P. aeruginosa</i>	144
4.4.3 Bacterial growth in the ASM compared to MH.....	145
4.4.4 Bacterial growth in the ASM and MH in the presence of glucose	147
4.4.5 Bacterial growth and glucose depletion in the ASM	150
4.4.6 Bacterial lactate production and depletion in the ASM	152
4.4.7 <i>S. aureus</i> growth in the ASM, supernatant, and boiled supernatant in presence and absence of glucose.....	153
4.4.8 <i>P. aeruginosa</i> growth in the ASM, supernatant, and boiled supernatant in presence and absence of glucose	156

4.4.9 Co-culture of <i>S. aureus</i> and <i>P. aeruginosa</i> in the ASM and derived media	159
4.4.10 Effects of glucose on the establishment of co-culture of <i>S. aureus</i> and <i>P. aeruginosa</i> in the ASM	162
4.4.11 Establishing stable co-culture of <i>S. aureus</i> and <i>P. aeruginosa</i> in a 50:50 mix of ASM and supernatant.....	165
4.4.12 Silver staining of supernatant.....	166
4.5 Discussion	169
4.5.1 Quantification of live bacteria	169
4.5.2 Evolution of the ASM model.....	172
4.5.3 The role of glucose in the ASM model	174
4.6 Summary	176
4.7 Future work and limitations	177
5 Using oral washes as an alternative to invasive lung microbiome sampling	178
5.1 Introduction.....	179
5.1.1 Non-invasive sampling of the lung microbiome	179
5.1.2 Oral wash sampling as a proxy of the lung microbiome	180
5.1.3 Using sputum samples derived from COPD individuals to study lung microbiome	181
5.2 Aims and hypothesis	183
5.3 Materials and methods.....	184
5.3.1 Study design	184
5.3.2 Study documentation.....	185
5.3.3 Sample collection	185
5.3.4 Targeted amplicon sequencing	186

5.3.5 Data and statistical analysis.....	186
5.4 Results.....	187
5.4.1 Recruiting individuals for the study.	187
5.4.2 Bacterial OTUs observed in oral wash and sputum samples.....	188
5.4.3 Alpha diversity in the oral wash and sputum samples.....	190
5.4.4 Correlation of lung function decline with the alpha diversity of oral wash and sputum samples.....	192
5.4.5 Beta diversity of the microbiome in the oral wash and sputum samples	193
5.4.6 Analysis of genera detected in the oral wash and sputum samples	195
5.4.7 Common bacterial lung pathogens in the sputum and oral wash samples from individuals with COPD	203
5.5 Discussion	204
5.5.1 Patient recruitment process and study design improvements.....	204
5.5.2 Lung microbiome comparison between CF and COPD individuals	205
5.5.3 Future uses for oral wash samples	206
5.6 Summary	208
5.7 Covid-19 impact statement	209
5.8 Limitations and future work	210
6 Final summary.....	211
7 References.....	213
8 Attachments	257
8.1 CF sample information	257
8.2 COPD sputum and oral wash study documentation	261

8.2.1 Participant information sheet.....	261
8.2.2 Consent form	265
8.2.3 Case report form.....	267
8.2.4 Original project proposal	275

Abbreviations

AMOVA	analysis of molecular variance
ASL	airway surface liquid
ASM	artificial sputum medium
BAL	bronchoalveolar lavage
CF	cystic fibrosis
CFRD	cystic fibrosis-related diabetes
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony forming units
COPD	chronic obstructive pulmonary disease
EMT	epithelial-to-mesenchymal transition
FMT	faecal microbiota transplantation
FVC	forced vital capacity
GDM	gestational diabetes mellitus
HbA1c	haemoglobin A1c
HMP	Human Microbiome Project
HOMOVA	homogeneity of molecular variance
i.p.	intraperitoneal
IL	interleukin
kD	kilodalton
LB	lysogeny broth
MH	Mueller-Hinton
mM	millimolar
MMP	matrix metalloproteinase
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
FEV ₁	forced expiratory volume
NGS	next-generation sequencing

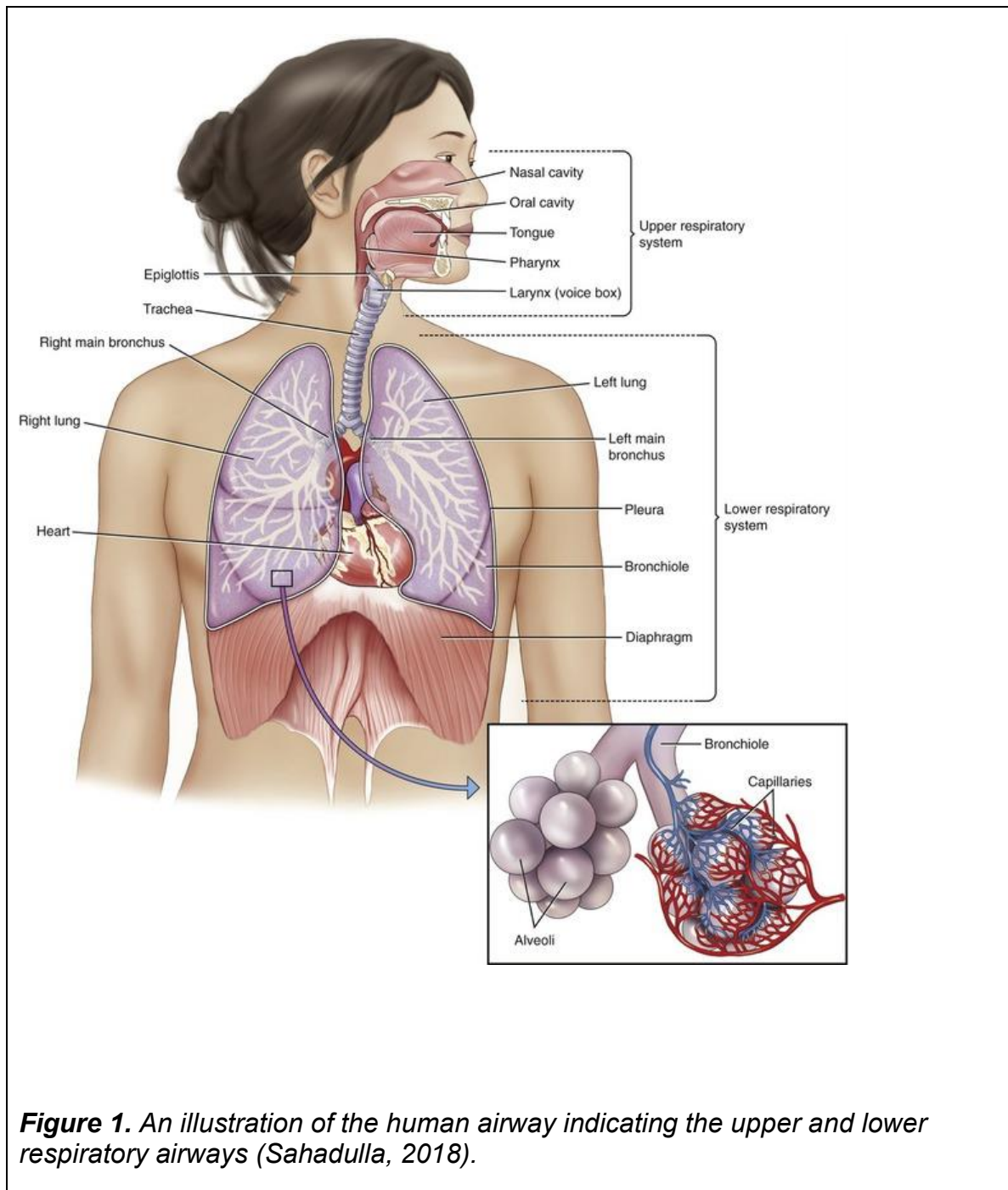
OD	optical density
OGTT	oral glucose tolerance test
OTU	operational taxonomic unit
OW	oral wash
PBS	phosphate-buffered saline
PCoA	Principal Coordinates Analysis
PCR	polymerase chain reaction
PLC	periciliary liquid
SGLT	sodium-glucose linked transporter
TNF	tumour necrosis factor
STZ	Streptozotocin
TSB	tryptic soy broth
WT	wild type
μm	micromolar

Chapter 1

Literature review

1.1 Lung anatomy

The lungs are the primary organs of the respiratory system, whose vital function is to enable gas exchange between the environment and the bloodstream. Oxygen gets transported through the alveoli into the capillary network. The gas exchange takes place, ultimately allowing tissue perfusion (Chaudhry and Bordoni, 2019). The respiratory system is composed primarily of the upper respiratory tract and lower respiratory tract. The upper respiratory tract is comprised of the nasal cavity, pharynx, larynx and trachea. The lower respiratory tract is comprised of the trachea, left and right primary bronchi and bronchioles (Figure 1).



Like skin, lungs are constantly exposed to diverse pathogens and foreign materials (Martin and Frevert, 2005). On average, humans perform 11-17 breaths per minute, with each breath averaging 450-600ml of air, meaning that humans inhale approximately 7 litres of air each minute (Schlesinger, 1988). It was shown that the number of bacteria in the air could range anywhere between 10^2 and 10^6 CFU/m³ (Jo and Seo, 2005; Bowers *et al.*, 2009; Andualem *et al.*, 2019). After a simple calculation, we can conclude that at any moment, our average human

could be inhaling between 1 and 7000 bacterial CFU every minute. Human lungs have evolved and adapted to this constant stress into a complicated system where changes in human physiology could be reflected in the health of the lung.

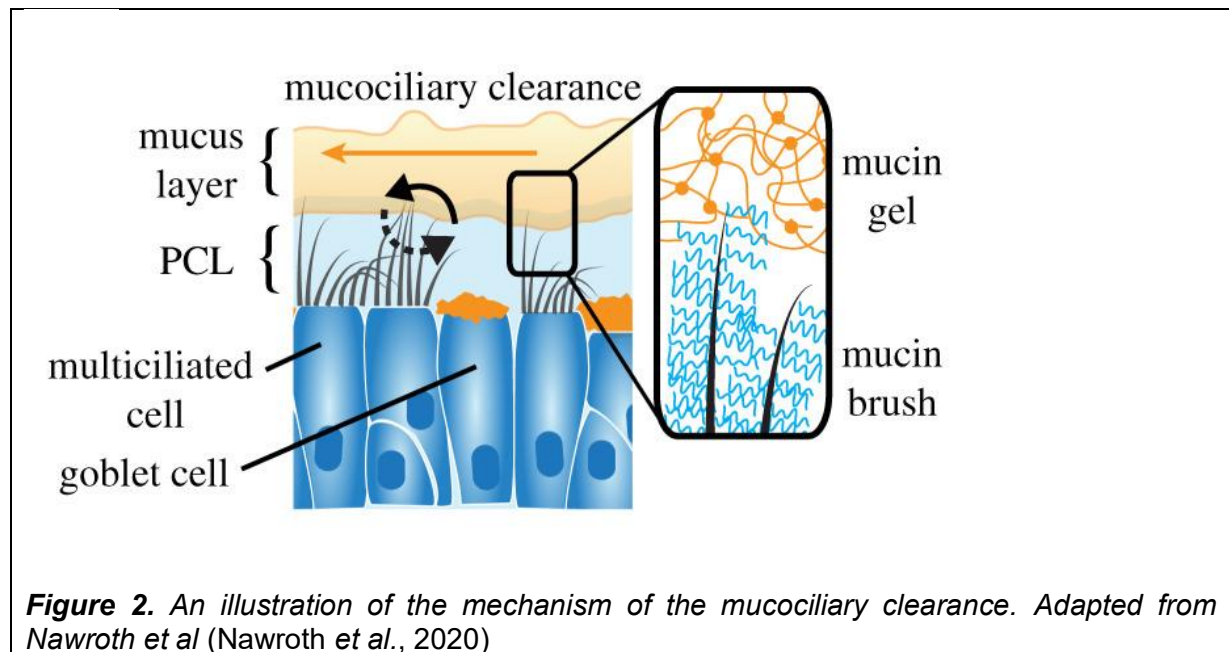
Throughout this chapter, the complex physiology and microbiology of the airways will be explored; initially outlining the structure of the airway surface liquid and the role of glucose in the airways, later exploring glucose homeostasis in the airways and the effects of sustained blood hyperglycaemia on the glucose concentration in the lung, followed up by the exploration of the human microbiome and its role in various body systems, especially lungs, and finally outlining what effects diseases have on both the physiology and microbiology of the lung.

1.2 ASL and lung defence

The airway epithelium plays a vital role in the defence against the invasion of foreign pathogens, as it is the largest surface of the body that has direct contact with the environment. Because of this, the lungs have developed a complex system of defence.

Respiratory epithelial cells create multiple barriers mediated by their secretory products, surface glycocalyxes, membranes, and intercellular junctional proteins (Patke *et al.*, 2016). The luminal surface of the airways is lined with a thin layer of fluid called airway surface liquid (ASL) (Wanner *et al.*, 1996). The ASL is comprised of two layers, a mucus component and a periciliary liquid layer (PLC). The PLC layer allows the separation of mucus from the underlying epithelial cell layer. In healthy individuals, the PLC has a depth of 5µm - 10µm, covering the cilia, allowing them to beat (Figure 2) (Button *et al.*, 2012). The mucus layer on top of the PLC has a varying depth, from 7µm to 70µm. Mucus plays a primary role in innate immune defence by trapping invading pathogens and foreign particles (Wine, 2004; Fahy and Dickey, 2010). Foreign particles are neutralised by the antibacterial agents, such as β -defensins (Singh *et al.*, 1998; Boucher, 1999), and broad-spectrum antimicrobial peptide products produced in epithelia, abundant in the ASL (Tarran, 2004). Cilia, located on the airway epithelial cells, beat

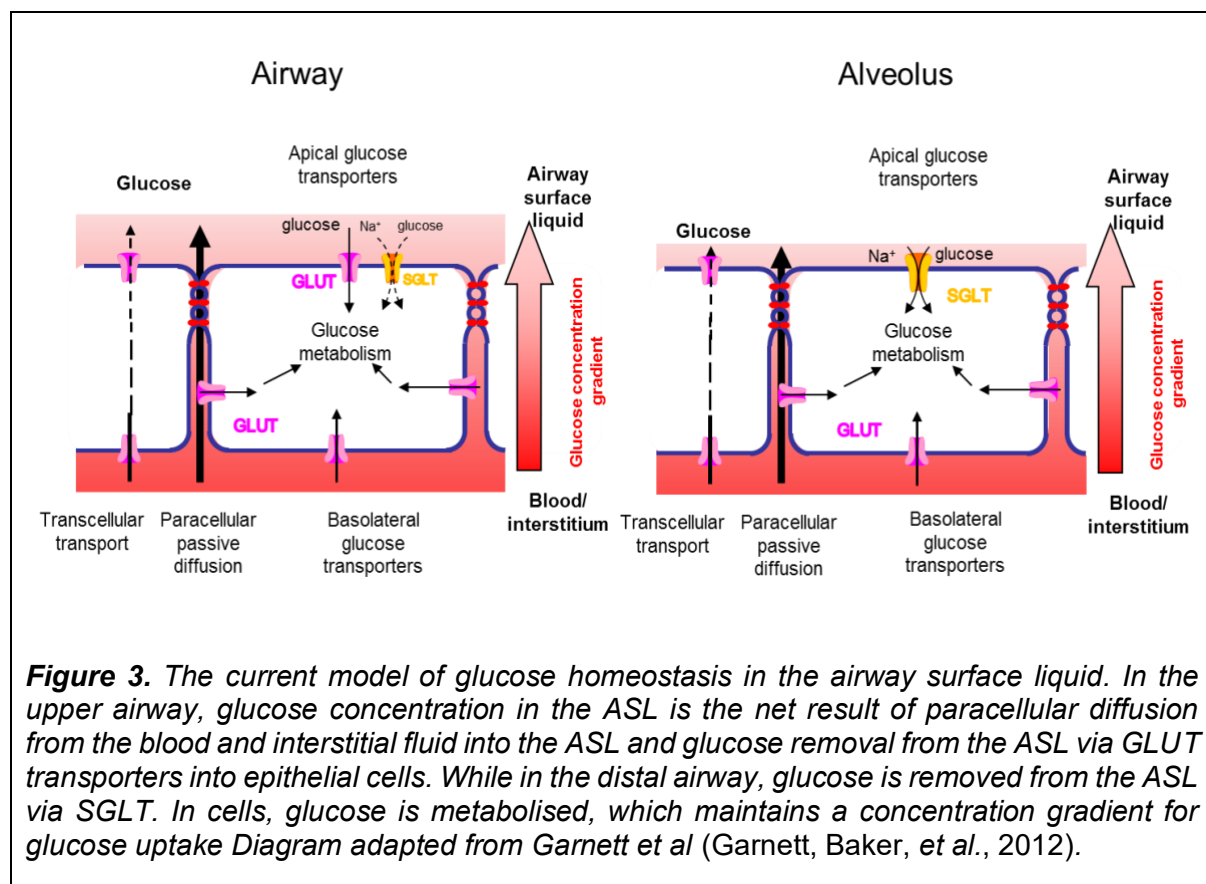
in a wave pattern to move the mucus into the nasopharynx, where bacteria and foreign objects trapped in the mucus layer are swallowed and destroyed by stomach acids (Wanner *et al.*, 1996; Mitchison and Mitchison, 2010).



1.3 Presence of glucose in the ASL

Like any other cells, lung epithelial cells use glucose metabolism to generate the energy required for cellular processes. Epithelial cells receive glucose from the blood (interstitium); however, some glucose can potentially be taken up from the ASL. In previous research by our group, intracellular glucose sensor was used to measure glucose concentrations in airway epithelial cells. Inhibition of facilitative glucose transporters (GLUTs) with cytochalasin B reduced intracellular glucose concentration. It was also demonstrated that glucose in the cells is processed by hexokinase-dependent and hexokinase-independent metabolic pathways to maintain low intracellular glucose concentrations (Bearham *et al.*, 2019). Glucose moves into ASL from the interstitium paracellularly down the concentration gradient, and the rate of movement is determined by epithelial permeability to glucose (Saumon and Loiseau, 1996; Kalsi *et al.*, 2009). Glucose concentration in the ASL is much lower than that of blood. It was

shown that the glucose concentration in healthy ASL is about 0.4 mM, approximately 12.5 times lower than that of blood (~5 mM) (Baker *et al.*, 2007). Glucose in the ASL is maintained at this constant low concentration via facilitative glucose transporters (GLUT) and less by sodium-glucose linked transporter (SGLT) (Figure 3). The gradient for removing glucose from the ASL is generated by cellular metabolism and glucose uptake (Saumon and Loiseau, 1996; Kalsi *et al.*, 2009). It is proposed that low glucose concentration contributes to innate protection against the growth of pathogenic organisms. Common pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* can utilise glucose for growth (Garnett *et al.*, 2013; Garnett *et al.*, 2013).



1.4 Effects of inflammation on the glucose concentration in the ASL

It was demonstrated that the glucose concentration in the ASL is increased in respiratory diseases (Philips *et al.*, 2005). One of the proposed mechanisms responsible for the increase in ASL glucose concentration is respiratory inflammation. Inflammation is generally

characterised by the increased activity of the neutrophils in the ASL. Activated neutrophils release molecules such as human neutrophil elastase and myeloperoxidase, which lead to bronchial inflammation, fibrosis and emphysema (Epstein and Weiss, 1989; Borregaard *et al.*, 2007; Ilumets *et al.*, 2008). Several mediators trigger neutrophil recruitment, such as tumour necrosis factor (TNF)- α , interleukin (IL)-8 and IL-1 β . These mediators were shown to have a profound ability to disrupt tight junction integrity (Coyne *et al.*, 2002). Tight junction disruption, in turn, leads to a decrease in epithelial resistance, allowing increased non-specific flux of molecules such as glucose across the epithelium. These inflammatory mediators were associated with the severity of airway damage in COPD and CF (Caramori *et al.*, 2014; Courtney *et al.*, 2004; A.-X. Huang *et al.*, 2016).

1.5 Effects of the diabetes on the lung health

Diabetes mellitus is a chronic condition in which the body either cannot produce a sufficient amount of insulin or cannot utilise insulin to control blood glucose concentration within tight limits of 5-6 mM (American Diabetes Association, 2004). Type 1 diabetes mellitus is caused by the autoimmune destruction of β -cells found in the pancreas (Srikanta *et al.*, 1983; Schranz and Lernmark, 1998; Seissler *et al.*, 1998). Damage to the pancreas results in the inability of the pancreas to produce insulin in concentrations required to control blood glucose levels. Type 2 diabetes mellitus results from developing insulin resistance (Wiegand *et al.*, 2004). The pancreas secretes large amounts of insulin in response to increased blood glucose concentration. High exposure to insulin causes cells to become less responsive to the effect of the hormone, resulting in insulin resistance and the inability to control blood glucose (Oh *et al.*, 2018). In both scenarios, blood glucose increases beyond the normal range (random plasma glucose concentration ≥ 11.1 mmol/l, fasting plasma glucose concentration ≥ 7.0 mmol/l, or glycated haemoglobin (HbA1c) level of 47.5 mmol/mol or higher) leading to sustained hyperglycaemia.

A sustained increase in blood glucose concentration leads to increased glucose concentration in the ASL in both human lungs and animal lung models (Baker et al., 2007; Garnett, Gray, et al., 2013; Åstrand et al., 2017). This similarity between humans and animals allows the use of diabetic animals to investigate the effects of sustained blood hyperglycaemia on the microbiome of the lung.

A study utilising hyperglycaemic ob/ob and db/db mice showed a clear link between an increase in blood glucose, ASL glucose concentration and increased growth of *P. aeruginosa* inoculated into the lungs. These bacteria utilise glucose as a growth substrate. Increasing glucose concentration in the ASL had no effect on a mutant strain of *P. aeruginosa* (edd⁻ mutant) (edd gene encodes for 6-phosphogluconate dehydratase, which is one of the critical enzymes for glucose utilisation), which cannot utilise glucose as a growth substrate (Pezzulo et al., 2011). Decreasing blood and bronchoalveolar lavage (BAL) glucose concentrations in db/db mice with the antidiabetic drug dapagliflozin decreased *P. aeruginosa* growth to the levels seen in wildtype mice (Åstrand et al., 2017).

Measuring ASL glucose concentration in live individuals remains a significant challenge. Various approaches have been developed to aid the exploration of diabetes-induced changes to the respiratory microbiota. In one study, breath condensate was collected and analysed by chromatography with pulsed amperometric detection to estimate glucose concentrations. Data showed that increased glucose observed in the samples was associated with increased pulmonary infections, specifically methicillin-resistant *Staphylococcus aureus* (MRSA) (Baker et al., 2007). Another study utilised bronchial aspirates from intubated individuals and showed a link between increased glucose concentration in the aspirate and acquisition of MRSA (Philips et al., 2005). It was observed that poor glycaemic control in diabetic individuals increases the likelihood of acquiring pneumococcal pneumonia (Rueda et al., 2010).

Using a database of over a million individuals, a longitudinal study was conducted to investigate the association between a diagnosis of diabetes and pulmonary diseases. The

study reported that incidents of asthma, COPD, fibrosis and pneumonia were higher in individuals with diabetes (Ehrlich *et al.*, 2010). Several studies have reported that individuals with diabetes mellitus experience a significant decrease in lung functions (measured in terms of Forced Expiratory Volume (FEV₁) and Forced Vital Capacity (FVC)) over time and independent of smoking (Abdel-Azeem *et al.*, 2013; Irfan *et al.*, 2011; Kinney *et al.*, 2014; Shah *et al.*, 2013). One of the potential causes for the deterioration of lung function in diabetic individuals could be increased inflammation and fibrotic changes in the lungs, as demonstrated in multiple studies (Ban & Twigg, 2008; Hu *et al.*, 2014; Talakatta *et al.*, 2018; J. Yang *et al.*, 2011). Fibrosis was shown to be induced via epithelial-to-mesenchymal transition (EMT) signalling pathways as mesenchymal cells are involved in the tissue fibrosis process (Kalluri and Weinberg, 2009). Glucose restriction therapy decreased inflammation and promoted mesenchymal cells reversal into epithelial cells, suggesting that hyperglycaemia could be the primary cause of lung fibrosis (Talakatta *et al.*, 2018).

1.6 Assessing lung microbiome

The human microbiota refers to all the microbial species found on and within the human body. In contrast, the human microbiome refers to the genetic content of these bacteria (Ursell *et al.*, 2012).

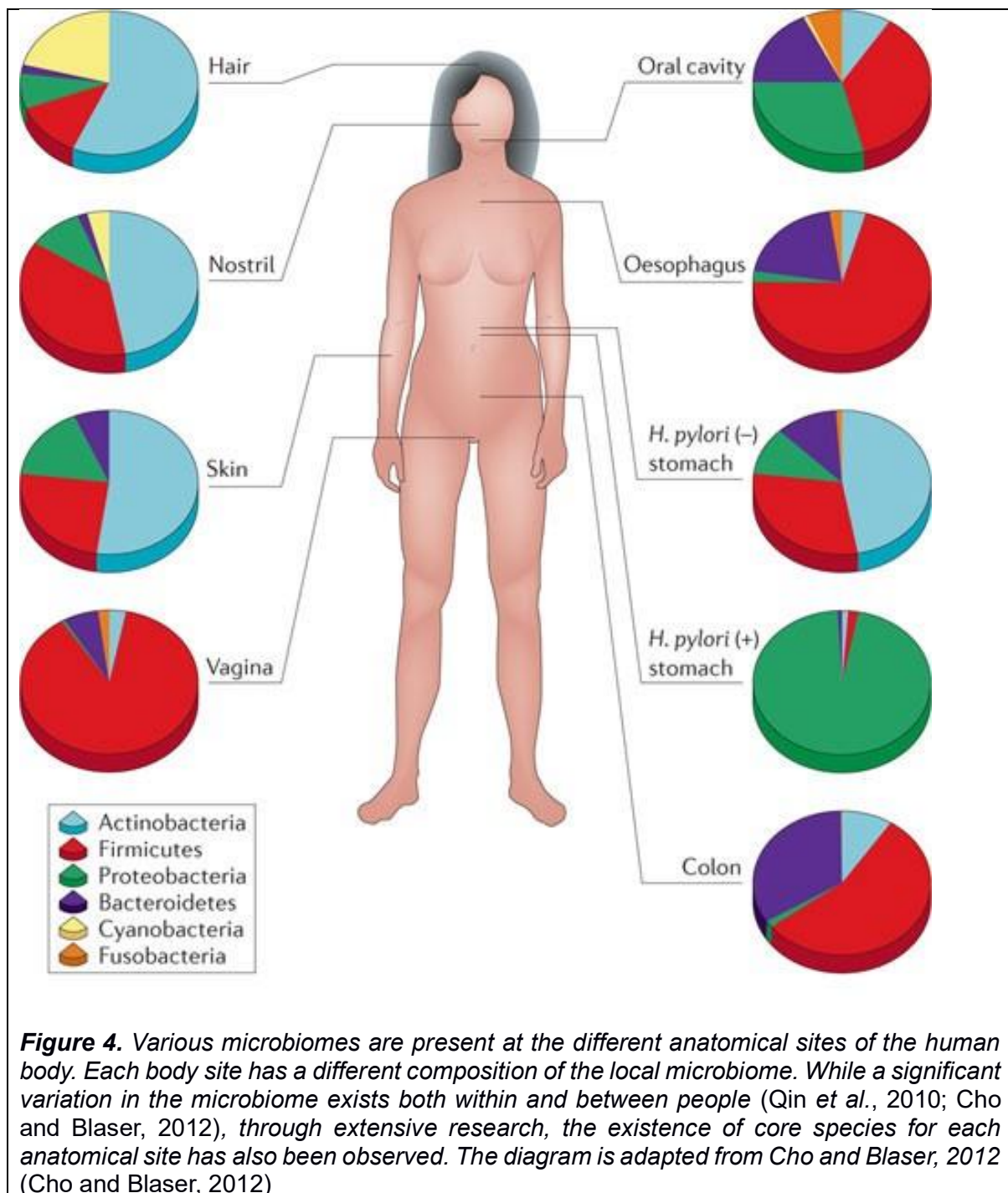
Bacteria are found in the most hostile environments on Earth: arctic ice, acidic conditions and deep in Earth's core (Rampelotto, 2013). Animals have evolved to coexist with bacteria in mutually beneficial relationships. They are now known to play a vital role in the healthy life of humans. Bacteria were shown to be involved in digestion, the function of the immune system and even mental health (Turnbaugh *et al.*, 2006; Heijtz *et al.*, 2011; K. M. Neufeld *et al.*, 2011; Cani, 2013).

Culture-independent, genomic approaches have transformed our understanding of the role of the human microbiome in healthy and diseased states. Culture-dependent techniques have

been used for decades to assess the microbiota. Such techniques were considered to give an accurate estimate of such microbiota. However, new genomic approaches have challenged the established culture-dependent findings. A study in 2012 estimated that less than 30% of gut microbiota detected using sequencing approaches was identified using culture-based techniques (Fraher *et al.*, 2012). Moreover, the lungs were considered sterile because of the very low numbers of bacteria detected using traditional culturing techniques. However, new culture-dependent and culture-independent approaches have identified complex lung microbiota. Culture-enriched molecular profiling uses information acquired through bacterial genomic sequencing to improve and optimise culture conditions and target hard-to-culture bacteria. The majority of lung and gut microbiota detected using sequencing technology by various research groups can now be cultured using such techniques (Sibley *et al.*, 2011; Browne *et al.*, 2016; Lau *et al.*, 2016; Whelan *et al.*, 2018). Some groups claim that culture-enriched molecular profiling, using 16S rRNA gene sequencing information, permits 95% of bacteria present in the gut microbiota to be cultured (Lau *et al.*, 2016). This approach can now target previously unculturable bacteria to allow phenotypic analysis and to explore mechanisms of microbiota-host interactions.

The human microbiome has been extensively studied to uncover the importance of bacteria in the human body. Large scale studies, such as the Human Microbiome Project (HMP) by the National Institutes of Health in USA and Metahit, the European Union Project on Metagenomics of the Human Intestinal Tract, as well as smaller individual studies over the years, have generated terabytes of data from hundreds of individuals from all the possible human body sites. Through the efforts of thousands of scientists, it is now known that specific anatomical sites possess their own unique microbiomes, which play unique roles in health and sickness (Figure 4). The exact role bacteria play in each body site and the importance of dysbiosis is still debated due to the complexity of microbiome studies and great inter- and intra-human variability. This project will focus on uncovering the role of the microbiome in the lungs and the changes it undergoes under the effect of sustained hyperglycaemia. This project

will also investigate the effects of sustained hyperglycaemia on the lung microbiome in individuals with chronic lung diseases.



1.7 Culture-independent approach of studying microbiome

Sequencing technologies have undergone unprecedented development in the past decades, allowing quicker, more reliable sequencing for ever-decreasing prices. The roots of all the

sequencing technologies stem back to 1953 when James Watson and Francis Crick uncovered the double helix DNA structure (Watson and Crick, 1953). However, it was not until 1977 that Frederick Sanger and Walter Gilbert independently developed the first methods for long DNA sequencing (F. Sanger *et al.*, 1977; Maxam and Gilbert, 1977). Almost a decade later, Sanger's DNA sequencing with chain-terminating inhibitor (F Sanger *et al.*, 1977) was partially automated by a group based in California (Smith *et al.*, 1986). Later, in 1986, it was fully automated by Applied Bioscience (Giani *et al.*, 2020) which became pivotal for the research in the coming years. Research involving sequencing gained much traction in the scientific community, with The International Project on Human Genome being announced in 1990 that would be predicted to last 15 years. The project would ultimately be completed in 2003, two years before the deadline, and would become a historical mark in the history of science. Between the creation of Sanger sequencing and the completion of the Human Genome Project, Sanger sequencing has undergone multiple improvements, which allowed it to be used for large-scale projects and increased its popularity and use in the scientific community. Ultimately, however, Sanger sequencing still bore a high cost, required specialised equipment and highly trained professionals to perform the sequencing and then analyse it (McCombie *et al.*, 2019). The success of Sanger sequencing led to the development of a new generation of sequencing technologies. These new technologies collectively became known as Next-generation Sequencing (NGS) platforms. Massively Parallel Signature Sequencing (MPSS), developed in 2000 by Lynx Therapeutics (Brenner *et al.*, 2000), was the first NGS released into the world. However, NGS is not a brand-new technology and is, instead, a successor to Sanger sequencing. Unlike Sanger sequencing, NGS allows one to perform both the enzymology and data acquisition in a controlled step-by-step process, generating thousands to billions of templates simultaneously (McCombie *et al.*, 2019).

The development of NGS was rapid, with multiple companies competing for market dominance. This competition has reduced cost (Figure 5) and time and increased sequencing accuracy, enabling many samples to be examined at greater depth. Next-generation

sequencing (NGS) followed by computational analysis has become routine practice for phylogenetic analysis of bacterial communities.

Several different NGS sequencing platforms are now available, such as HiSeq, MiSeq and NextSeq by Illumina and Ion Torrent PGM and Proton by Life Technologies. Many early microbiome studies were performed on the GS FLX 454 platform, generating reads of ~700bp long. Still, the phasing out of this platform has meant that the Illumina MiSeq, with its 2x250bp and 2x300bp chemistry, has become the platform of choice for 16S studies. Illumina holds dominance over the sequencing market with a 70% share of the sequencing market. Between 2007 and 2016, Illumina platforms generated 90% of the worlds sequencing data (Zhang, 2016). This research project will use the Illumina MiSeq platform for microbiome analysis.

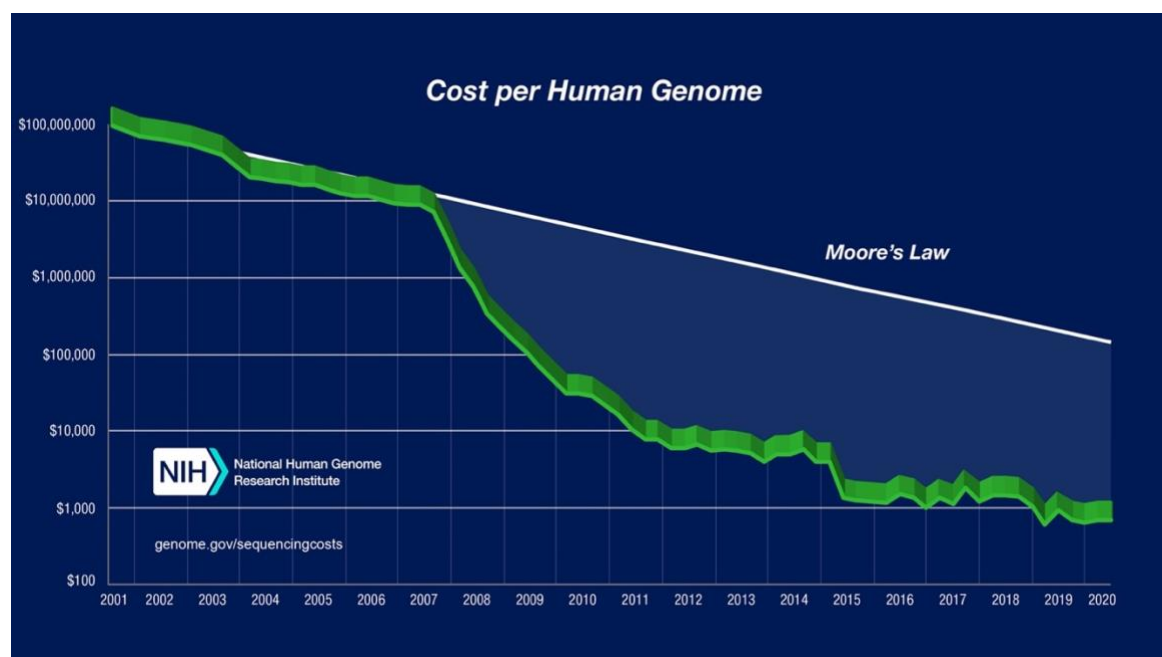


Figure 5. Sequencing cost per Megabase 2001-2020.

The cost of DNA sequencing has decreased dramatically over the past two decades, with the most significant price drop observed in 2007. (National Human Genome Research Institute (NHGRI), 2017)

1.8 MiSeq

For this research, it was decided that all of the sequencing would be performed using the Illumina MiSeq platform. As was previously discussed, Illumina platforms hold an absolute dominance over the sequencing data generated worldwide. With more and more microbiome studies making their raw sequencing databases publicly available, it would allow us to cross-compare data generated in this study with other lung microbiome studies, compensating for potentially small sample sizes and a high variability within the microbiome that is often observed. Datasets, generated using the MiSeq platform, from studies investigating lung microbiome in healthy people and people with CF, COPD, and other chronic lung diseases, are readily available in various online databases such as MG-RAST (Meyer *et al.*, 2008).

MiSeq platform, unlike SOLiD and 454, utilises sequencing by synthesis technology with bridge amplification. The general workflow for the Illumina platform is described in Figure 6. Original DNA is fragmented prior to the library preparation. Sequencing adapters are then ligated to each end of the DNA fragments to create fragment libraries. These libraries are then amplified by PCR using primers designed for the adapters. Fragments are added onto the flow cell, coated with oligonucleotides that complement the adaptors, where DNA fragments are hybridised to the surface of the flow cell. Unlike other platforms, Illumina systems allow PCR amplification to be performed directly on the flow cell. The attached DNA fragments are amplified by bridge amplification (Figure 7). Once amplification is complete, DNA molecules are linearised, and clusters with an identical sequence are formed, covering the flow cell. Fragments are then ready to be sequenced. Illumina platforms utilise a method that is in its core similar to Sanger sequencing. All four nucleotides and DNA polymerase are simultaneously added to the flow cell. Modified dNTPs are used, which contain a terminator. The presence of the terminator ensures that the polymerase enzyme adds only a single base to each DNA copy strand, and the reaction is immediately stopped. Each nucleotide has a unique fluorescent label attached to it. Once nucleotides are incorporated, the cell is imaged

using four different wavelengths corresponding to each nucleotide. Residual nucleotides are then washed, and a new cycle begins. Each cycle adds a single nucleotide to each copy strand, hence the name – sequencing by synthesis. An algorithm then assigns sequences with quality values to each read. The quality values are then evaluated with low-quality sequences removed. (Bentley *et al.*, 2008; Mardis, 2008; Tucker *et al.*, 2009; Berglund *et al.*, 2011)

As discussed previously, Illumina platforms utilise bridge amplification for cluster generation. Once DNA libraries are hybridised onto the flow cell, a complementary strand is synthesised by the polymerase, followed by denaturation of the said double strand. The original template is then washed off, and the newly synthesised strand flips over to adjacent complementary primer creating a single-stranded bridge. This strand is extended by a polymerase, forming a double-stranded bridge. The bridge is then denatured, resulting in two single-stranded templates. The single-stranded template can now form a new bridge repeating the cycle. The repetition of this cycle leads to localised clusters of identical strands (Figure 7).

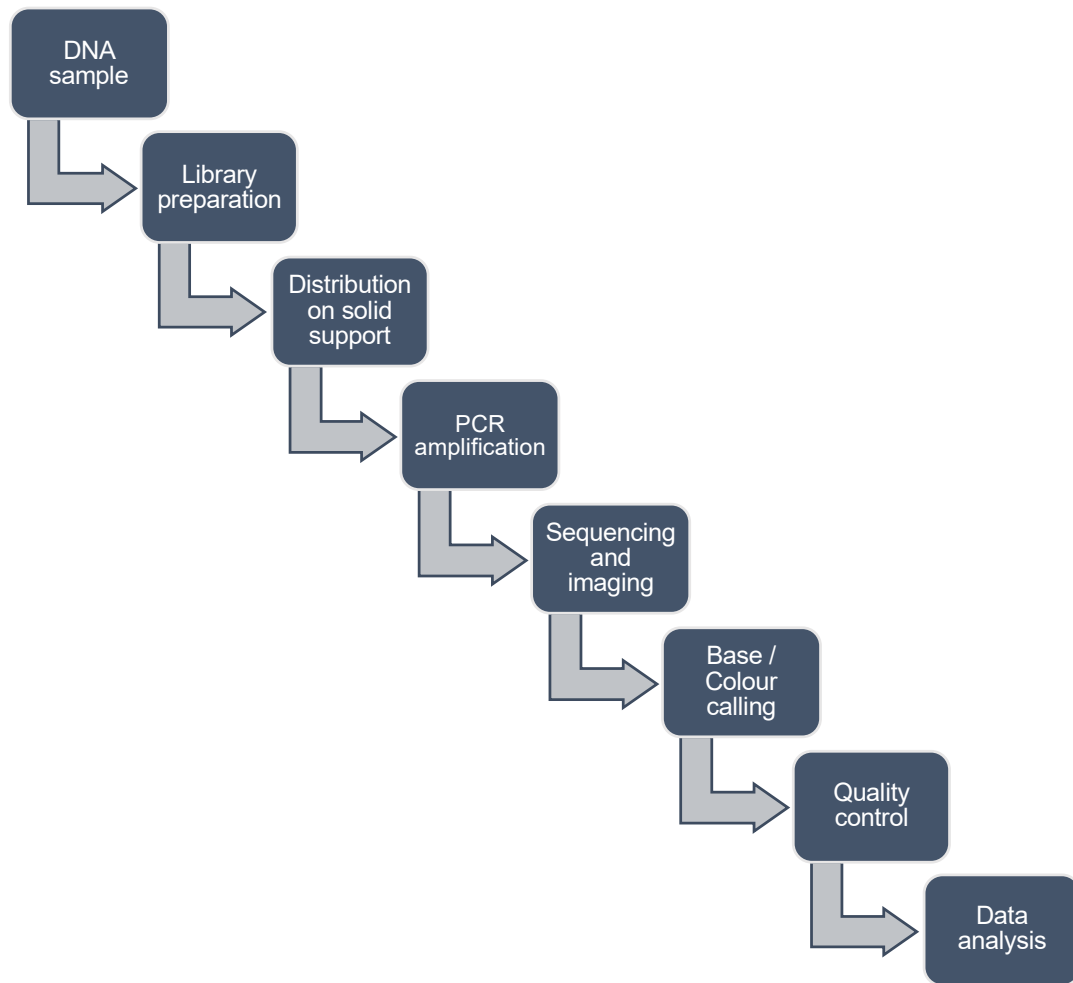
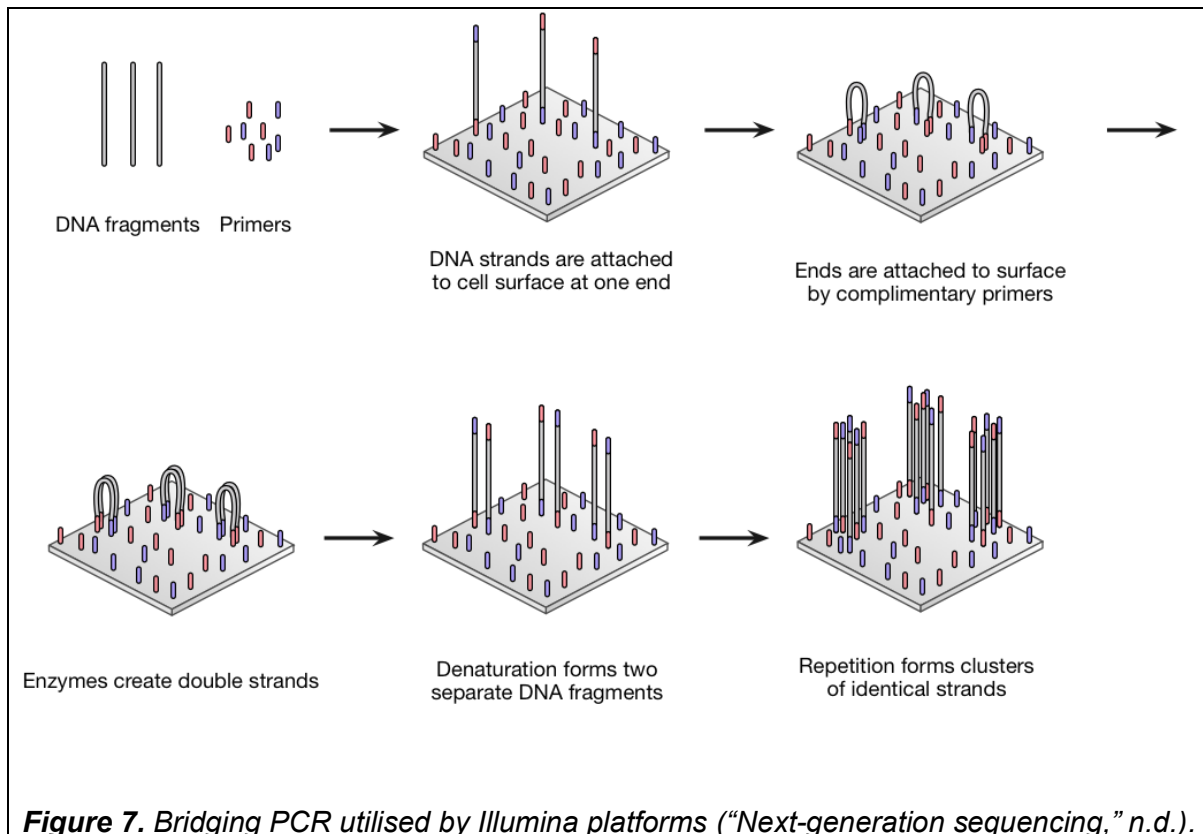


Figure 6. Illumina platform general workflow. Self-made diagram.



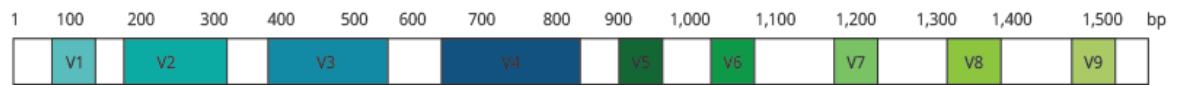
1.9 16S rRNA sequencing

Many of the modern-day high-throughput sequencing technologies for studying microbial communities are based on sequencing portions of the genome coding for the bacterial 16S ribosomal RNA subunit, which is used as a phylogenetic marker. This approach enables the prediction of the composition of bacterial communities in the samples. It allows differences in diversity and abundances of individual bacterial species between microbiota samples to be explored.

Ribosomes are essential for the survival of the cell. Because of this, ribosome complexes are highly conserved and are found in all living creatures. The ribosomal complex is made up of subunits. In prokaryotic cells, 70S ribosomes consist of small 30S and large 50S subunits. These subunits are composed of 16S RNA, 5S RNA and 23S RNA subunits (Noller *et al.*, 2005; Tu *et al.*, 2009). The 16S rRNA was established as a marker for phylogenetic studies in 1991 due to its structural relationship driven by evolutionary constraints (Weisburg *et al.*,

1991). The bacterial 16S rRNA subunit is made up of highly conserved regions that are flanked by nine hypervariable regions (Figure 8). Universal primers can target the conserved regions, thus allowing the hypervariable regions to undergo amplification. These amplified regions can then be sequenced and used for phylogenetic analysis. Target amplified regions are aligned to a set of reference sequences with pre-assigned taxonomy. Sequences are grouped according to their similarity (usually 97-99% homology) into operational taxonomic units (OTUs). OTUs allow approximation of genus and species-level phylogeny. As a result, it is possible to approximate the abundance of each species in a sample (Dickson *et al.*, 2016). These sequence databases are available as public taxonomic databases, such as EzBioCloud (Yoon *et al.*, 2017), Ribosomal Database Project (Cole *et al.*, 2014), SILVA (Mitrović *et al.*, 2011) and GreenGenes (DeSantis *et al.*, 2006). These are regularly updated, and all of the sequences in the database are validated.

A.



B.

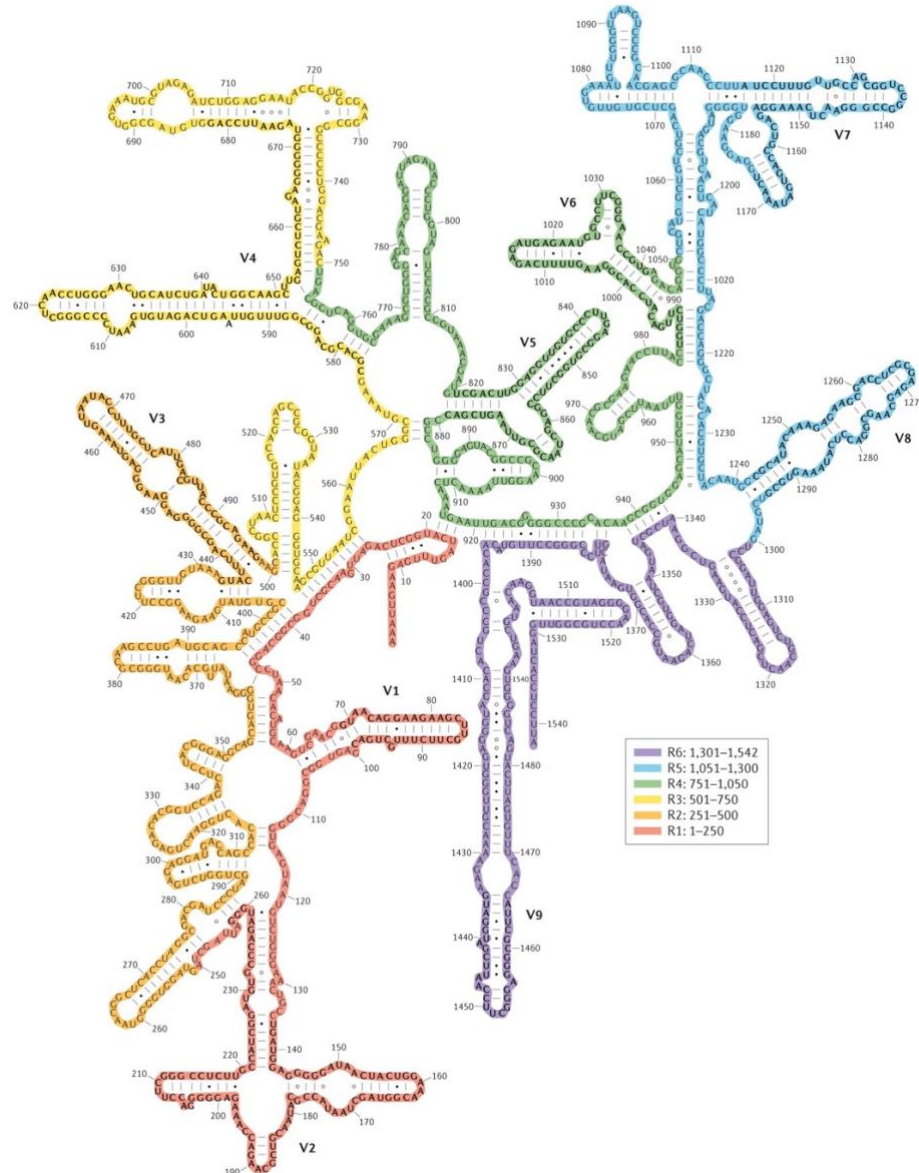


Figure 8 A. Conceptual representation of the 16S rRNA gene sequence with the conserved region in white and hypervariable regions (V1 – V9) in blue-green **B.** Secondary-structure model of the 16S DNA where double lines indicate variable regions, black lines indicate conserved regions and hypervariable regions are marked V1-9 (Yarza et al., 2014).

Any of the nine hypervariable regions could be targeted for amplification. The choice of the target variable region and primers for the 16S sequencing largely depends on the choice of the target organism. The capabilities of the sequencing platform determine the length of the target fragment. For example, MiSeq is capable of sequencing 2x300 base pairs of DNA, while Roche 454 - 700 base pairs of DNA. Henceforth to use these platforms, primers would need to produce amplicons within these read lengths. Several studies have been performed to evaluate the use of primers targeting particular hypervariable regions either by using *in silico* methods or using mock communities of known bacteria (Barb et al., 2016; Chakravorty et al., 2007; Hiergeist et al., 2016; Klindworth et al., 2013b; Thijs et al., 2017; B. Yang et al., 2016). The results varied, with some claiming the V4-V6 regions to be the most consistent, while V2 and V8 regions being the least consistent (Yang *et al.*, 2016). Some showed that V2, V4 and V6-7 as most reliable regions for identifying communities in the mock sample, showing the least divergence from known mock samples, with V4 regions being the most reliable and V9 being the least reliable (Barb *et al.*, 2016). Some studies showed that particular hypervariable regions are helpful for targeting known pathogens. The V1 region was most efficient at differentiating *Staphylococcus aureus* and coagulase-negative *Staphylococcus sp.*; the V2 region was best for distinguishing among *Mycobacterial* species and the V3 region for distinguishing among *Haemophilus* species (Chakravorty *et al.*, 2007). There currently is not an agreed single set of perfect primers used. Each primer set needs to be evaluated in line with the experimental setup, targeted species and sample origin.

There are, however, some limitations to be aware of when working with 16S rRNA sequencing. First, the total number of 16S rRNA gene copies varies between bacterial species. This variation could lead to bacterial species with a higher number of 16S gene copies being over-amplified and their abundance to be inflated in the resulting sequence (Lee *et al.*, 2018). Secondly, generated sequencing data is aligned with one of the reviewed databases to identify bacterial species. Errors could occur if the sequenced data is not perfectly aligned with the database. These errors would limit the precision at which sequences would be matched and

taxonomy assigned. These errors, however, primarily affect the taxonomy above the family/genus level (Lee *et al.*, 2018).

1.10 Effects of diabetes on the microbiome in various body systems

Several studies have demonstrated a link between the gut microbiome and depression/anxiety disorders (Clarke *et al.*, 2013; Desbonnet *et al.*, 2010; Heijtz *et al.*, 2011; K.-A. M. Neufeld *et al.*, 2011). It was shown that the gut microbiome might even affect autistic spectrum disorders (Mayer *et al.*, 2014). The gut microbiome has also been shown to be essential for the regulation of fat storage, and changes in the gut microbiota have led to the development of obesity in mice. Studies utilising germ-free mice have shown that normal or obese gut microbiota transfer severely alters the animal's ability to harvest and store energy (Backhed, 2004; Turnbaugh *et al.*, 2006). In more recent discoveries, it was shown that the efficiency of certain cancer drugs differs based on the presence of specific gut microbiota in humans (Geller *et al.*, 2017; Guthrie *et al.*, 2017).

It is well known that chronic low-level inflammation and obesity are closely linked to the development of Type 2 diabetes. Inflammatory mediators, such as TNF- α and IL-6, play an essential role in the regulation of the chronic inflammation observed in type 2 diabetes (Dandona *et al.*, 2004; Calle and Fernandez, 2012). In recent years, however, more research has focused on understanding the role of the human microbiome in diabetes. The interaction between diabetes and microbes living within the body, specifically those in the gut, has become a point of interest because of the emerging role of these resident microbes in the regulation of host energy metabolism, lipid accumulation, immunity and inflammation. Moreover, diabetes is associated with increased infections where pathogenic strains dominate the microbiome (Bäckhed *et al.*, 2007; Vogensen *et al.*, 2010; Jie, Han, Yang, Cai, Dai, J. J. Wang *et al.*, 2012; Tremaroli *et al.*, 2013; Zhang *et al.*, 2013; Kootte *et al.*, 2017). Therefore, how diabetes affects the balance of bacterial communities and the consequence of human health is critical to understand.

1.10.1 Effects of diabetes on the gut microbiome

The theory of the interaction between the gut microbiome composition and diabetes has evolved from the early studies looking at gut microbiome role in the metabolism using germ-free mice. In a critical study from 2004, it was observed that germ-free mice had lower body fat content than conventionally raised animals, despite having a higher caloric intake. These germ-free mice were then populated with gut microbiota collected from conventionally raised animals. Within 14 days, these mice produced a 60% increase in body fat content and had higher circulating insulin and glucose levels while having overall lower caloric intake (Bäckhed *et al.*, 2004). Another study utilising germ-free mice showed that these animals are protected from developing obesity when fed a Western-style diet (Bäckhed *et al.*, 2007). These mice gained significantly less weight and did not develop insulin resistance as conventionally raised animals would when fed the same diet (Bäckhed *et al.*, 2007). These observations indicated a potential link between the gut microbiome and the development of type 2 diabetes mellitus.

The effects of gut microbiota on the body's metabolic state were further investigated in a series of experiments where Jie and colleagues performed faecal microbiota transplant (FMT) procedures in obese patients with metabolic syndrome. Patients were randomly split into two groups given either autologous (from the same individual) or allogeneic (from a lean donor) FMT. Patients were controlled for diet and hormonal treatments. During the first study in 2012, it was observed that after six weeks of allogenic FMT, there was a significant improvement in peripheral insulin sensitivity as well as increased microbiome diversity. Interestingly, the total number of bacteria from faecal samples did not differ between patients receiving allogeneic or autologous FMT (Jie, Han, Yang, Cai, Dai, J. Wang, *et al.*, 2012). The follow-up study investigated the viability of FMT treatment as a long-term treatment for metabolic syndrome. In the same setup as the previous study, it was shown that FMT from lean donors had a short-term beneficial effect (at six weeks) on peripheral insulin sensitivity (from 25.8 to 28.8 $\mu\text{mol kg}^{-1} \text{min}^{-1}$, $p < 0.05$) as well as a slight decrease in HbA1c levels (from 39.5 to 38, mmol/mol, $p < 0.01$) However, by week 18 post-treatment gut microbiome reverted to the baseline and

there was no significant improvement in insulin sensitivity. Similar results were observed for single and repeated allogenic FMT treatments (Kootte *et al.*, 2017). These results demonstrate the potential of targeting the gut microbiome as a part of the treatment for poorly controlled diabetes; however, more research needs to be performed to assert the viability of such a strategy.

With overwhelming evidence generated from animal and human studies, it is now apparent that the gut microbiome plays a vital role in developing metabolic conditions by alterations in insulin resistance, weight gain and low-grade chronic inflammation. However, the role of the gut microbiome in diabetes progression remains to be determined.

Studies investigating the association between the human microbiome and type 2 diabetes have revealed substantial evidence of a correlation between diabetic status and gut microbiome (Vogensen *et al.*, 2010; Jie, Han, Yang, Cai, Dai, J. Wang, *et al.*, 2012; Tremaroli *et al.*, 2013; Zhang *et al.*, 2013). Several studies have looked into the effects of antidiabetic drugs on the gut microbiome. It was shown that treatment with metformin (a common drug for diabetes) produces a specific shift in the gut microbiome that could account for some of the metformin antidiabetic properties (Forslund *et al.*, 2015; Wu *et al.*, 2017).

Following the evidence of gut microbiome involvement in metabolic syndrome, diabetes and insulin insensitivity, some research groups investigated the gut microbiome in women with gestational diabetes mellitus (GDM). Gestational diabetes is a condition in which pregnant women with no previous history of diabetes develop abnormal blood glucose levels due to the hormones released in pregnancy causing insulin resistance (Mack and Tomich, 2017). Conclusions derived from these studies, however, were variable and conflicting. Some studies claimed that the gut microbiome in women with GDM was significantly different from the microbiome in normoglycaemic women. Gut microbiome composition in these women was similar to type 2 diabetic patients (Franke *et al.*, 2018; Wang *et al.*, 2018). Another study demonstrated that the intestinal and vaginal microbiome were significantly different between

women with GDM and normoglycaemic women, with the microbiome in women with GDM showing decreased diversity (Cortez *et al.*, 2018). However, the oral microbiome did not differ between the groups. Lastly, a study of 128 women (60 GDM-positive and 68 non-GDM control) demonstrated no significant differences between the gut microbiome of the two groups (Hasan *et al.*, 2017). Although the gut microbiome could potentially play a role in GDM development, these studies demonstrate that more work needs to be performed before conclusions can be drawn.

1.10.2 Effects of diabetes on the oral microbiome

The evidence that chronic inflammation associated with diabetes exacerbates other chronic inflammatory diseases has been described for periodontitis. Periodontal disease is an inflammatory condition of the gums surrounding the teeth. The oral cavity is rich in bacteria that generally do not cause infections. However, the accumulation of bacteria could trigger an inflammatory response, leading to the loss of collagen tissues that maintain teeth attached to the jawbone. The loss of collagen tissues eventually leads to the loss of teeth if left untreated (Loesche and Grossman, 2001).

Over a decade ago, a meta-analysis study showed a clear relationship between people with diabetes who were at higher risk of developing periodontitis and that diabetes was a risk factor for oral inflammatory diseases (Tsai *et al.*, 2002; Salvi *et al.*, 2008). It was also observed that periodontitis in diabetic individuals adversely affects glycaemic control and could contribute to diabetic complications (Khader *et al.*, 2006). It was shown that non-surgical periodontal therapy with antibiotics significantly reduced HbA1c levels in individuals with diabetes mellitus compared to the individuals who received only surgical treatment (Munjal *et al.*, 2019).

More researchers have shifted their focus towards changes to the oral microbiome in periodontal diseases in individuals with diabetes in recent years. Models using hyperglycaemic mice demonstrated that pathogen-induced periodontitis elicits an immune response that causes changes in the oral microbiome and increases insulin resistance

(Blasco-Baque *et al.*, 2016). It was demonstrated that in diabetic individuals, there was an apparent reduction of diversity in the oral microbiome when compared to normoglycaemic people (Saeb *et al.*, 2019). Moreover, it was shown that oral microbiota contained more pathogenic species in individuals with a combination of diabetes and periodontitis when compared to individuals with a single pathology (Babaev *et al.*, 2017). These observations were also confirmed by transferring oral microbiota into germ-free mice. Germ-free mice inoculated with the oral microbiome from diabetic donor mice showed a greater inflammatory response when compared to the mice that received non-diabetic microbiome (Xiao *et al.*, 2017).

The relationship between periodontal diseases and glycaemic control in diabetic individuals remains a topic of debate and research. Ohtsu and colleagues in 2019 orally administered suspension of *P. gingivalis* (a pathogenic bacteria involved in the development of periodontitis) into mice with streptozotocin-induced (STZ mice) diabetes to investigate links between oral and gut microbiome. It was observed that *P. gingivalis* altered the gut microbiota and worsened glycaemic control, increasing fasting glucose levels in these mice compared to control STZ mice (Ohtsu *et al.*, 2019). This data demonstrates the interplay between the oral and gut microbiome and how both could affect the status of diabetic animals and humans.

1.10.3 Effects of diabetes on the skin microbiome

It is widely known that diabetic individuals suffer a wide range of skin conditions with persistent bacterial and fungal infections and slow-healing wounds that lead to secondary infections. The American Diabetes Association even warns that skin complications are usually the first indicators that a patient has diabetes (Gardiner *et al.*, 2017). Human skin presents a rather unwelcoming environment for microorganisms – it is nutrient-poor and acidic. This, however, does not prevent bacteria from colonising and it is well known that skin possesses its unique microbiome (Byrd *et al.*, 2018). Due to the high burden of skin conditions in individuals with diabetes, much research has focused on understanding the underlying causes of these

conditions and the relationship to changes to the skin microbiome in diabetes. Two studies demonstrated increased bacterial diversity in diabetic foot ulcers and that the diversity of the microbiome was directly proportional to the depth and severity of the ulcer (Gardner *et al.*, 2013; Redel *et al.*, 2013). Similarly, increased bacterial diversity was found on the intact skin of diabetic individuals with foot ulcers (Gontcharova, 2010; Gardiner *et al.*, 2017). All of these studies agreed that chronic wounds in diabetic individuals were dominated by *Staphylococcus*, with one study specifying that superficial ulcers had a higher abundance of *Staphylococcus* than deep ulcers (Gardner *et al.*, 2013). The skin microbiome was so significantly different between diabetic and non-diabetic individuals that the diabetic status of the individual could be predicted using the foot skin microbiome (Gardiner *et al.*, 2017). Although there is a lack of evidence to derive conclusions about the effect of diabetes on the skin microbiome and chronic skin diseases progression, it is notable that there is a higher prevalence of opportunistic wound pathogens in diabetic individuals with ongoing diabetes-related skin conditions.

1.10.4 Effects of diabetes on the lung microbiome

Despite rapid advancements, the lung microbiome has avoided the spotlight. Lungs were not included in the original Human Microbiome Project since they were considered sterile. The first studies utilising NGS to delve deeper into the lung microbiome focused on individuals with CF as it was predicted their lungs would not be sterile. For the first time, it was shown that the lung microbiome was much more diverse than expected (Rogers *et al.*, 2003, 2004). Following this, research studies have looked at the healthy human lung microbiome as well as other diseases. The lung microbiome was shown to change with diseases, such as COPD, asthma, bronchiectasis and CF. Moreover, it was found that changes in the microbiome composition are associated with the severity of lung disease (Byun *et al.*, 2017; Hilty *et al.*, 2010; Sethi *et al.*, 2006). The lungs and airways contain diverse communities of microbes in both healthy and diseased states (Dickson *et al.*, 2013).

The composition of the lung microbiome, like any other system, is determined by the elimination of bacteria, immigration of bacteria and relative reproduction of bacteria within the communities (Dickson, Erb-Downward, *et al.*, 2014; Dickson, Martinez, *et al.*, 2014). Any changes within the lung's ecosystem would be a direct consequence of changes to these three factors. A healthy lung has a low-density microbiota, and maintenance of a small number of bacteria is crucial for the health of the lung (Mathieu *et al.*, 2018). In a healthy lung, the balance of microbiota is regulated primarily by immigration and elimination of the species. It is when the standard pulmonary defence mechanisms are impaired, such as in chronic lung disease, that the local growth of bacteria becomes overwhelming and overcomes the net effects of elimination and immigration on the lung microbiota composition (Dickson, Martinez, *et al.*, 2014).

Bacteria could be introduced into the lungs by various measures, such as inhalation of air (which contains between 10^2 and 10^6 CFU/m³, as previously discussed), direct mucosal dispersion and microaspiration (Dickson, Martinez, *et al.*, 2014). Microaspiration during sleep was shown to happen to both diseased and healthy individuals (Gleeson *et al.*, 1997; Cvejic *et al.*, 2011). Elimination of bacteria from the airways is driven primarily by the mucociliary clearance, immune defence mechanisms and physical dispersal driven by cough.

There has been much controversy in the claims of the origin of lung microbiota; however, many studies have now demonstrated that microbial immigration from the oral cavity is a major source of lung microbiota in healthy individuals (Bassis *et al.*, 2015; Charlson *et al.*, 2011; Erb-Downward *et al.*, 2011; Morris *et al.*, 2013; Wu and Segal, 2017). It has also been noted that the bacterial biomass decreases significantly towards the distal part of the lung when compared to the proximal lung, where bacterial biomass in the upper airways was 2-4 logs higher than that of the lung (Charlson *et al.*, 2011). While the lung microbiome shared many similarities with that of the mouth, it was shown that the lung exhibited significant selective elimination of bacteria derived from the oral cavity (Morris *et al.*, 2013; Bassis *et al.*, 2015). This shows that the homeostasis of the lung microbiome is not a passive balance between

immigration and elimination but that the lung microbiome is an actively maintained environment.

Multiple studies have demonstrated that, while each human's lung microbiome fingerprint is unique, there exists a core of communities found in human lungs. Lung microbiota is often defined by Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria phyla, and by *Streptococcus*, *Haemophilus*, *Veilonella*, *Pseudomonas* and *Prevotella* genera (Cameron et al., 2016; Einarsson et al., 2016; Erb-Downward et al., 2011; Hosgood et al., 2014; Lee et al., 2016; Liu et al., 2018; Morris et al., 2013; Park et al., 2014; Pragman et al., 2018; Wang et al., 2016; Yan et al., 2015; Yu et al., 2016).

There is currently no published research that explores diabetes-induced changes to the lung microbiome using NGS in individuals without other chronic lung diseases. All the research that looks at the diabetes-induced changes to the lung microbiome includes individuals with CF, asthma, COPD and emphysema. Below we will explore the effects of sustained hyperglycaemia on the lung microbiome in individuals with two of the most prominent chronic lung diseases – CF and COPD.

1.11 Effects of diabetes on the lung microbiome in individuals with CF

Cystic fibrosis is an autosomal recessive genetic disease. Mutations associated with the disease alter the function of the cystic fibrosis transmembrane regulator (CFTR) gene. This gene codes for an anion channel crucial for maintaining healthy flowing secretions from organs, including the lungs, pancreas, and intestines. Defective anion transport results in water being unable to enter the ASL, causing mucus to thicken and the ASL to acidify (Haq et al., 2016). As water leaves the periciliary layer of the ASL, the cilia of the airway become inoperative, preventing the removal of mucus and trapped particles. This environment

encourages chronic infections within the airways that eventually can lead to lung failure (Button *et al.*, 2012).

Another effect of the CFTR protein mutation is the blockage of pancreatic ducts with thick mucus, causing an inability of the pancreas to secrete insulin, thus impairing the ability to maintain healthy glucose levels in the blood. Enzymes trapped by the thickened mucus contribute to the damage to the pancreas, decreasing the number of β -cells over time (Brennan & Beynon, 2015; Kayani *et al.*, 2018). Damage to the pancreas and inability to sustain adequate insulin levels leads to the condition known as cystic fibrosis-related diabetes (CFRD). CFRD shares similar pathophysiology with Type 1 diabetes mellitus and has the same effect on the ASL glucose concentration as described above (Moran *et al.*, 2010; O'Shea and O'Connell, 2014).

CF is one of the more extensively studied lung diseases. The microbiome of spontaneously expectorated sputum (Rogers *et al.*, 2003), BAL and, protected specimen brushing (PSB) (Maughan *et al.*, 2012; Harris *et al.*, 2007; Hogan *et al.*, 2016; Muhlebach *et al.*, 2018) and tissue biopsies (Rudkjøbing *et al.*, 2012; Maughan *et al.*, 2012; Willner *et al.*, 2012) have all been studied.

Currently, the primary method of monitoring lung function in CF individuals is spirometry, specifically looking at FEV₁ and FVC. To assess the degree of disease progression FEV₁ is divided by FVC, and the resulting ratio is expressed as FEV₁%. It is the best available predictor for the survival rate of CF individuals (Taylor-Robinson *et al.*, 2012; Kerem *et al.*, 2014). The complex polymicrobial community is present in the lungs of CF individuals. *P. aeruginosa* and *S. aureus* are the most common bacterial pathogens isolated from the respiratory tract of CF individuals. They cause chronic pulmonary infections and are associated with a decline in lung functions (Ahmed & Mukherjee, 2018; Emerson *et al.*, 2002; Parkins & Floto, 2015). However, the increased use of antibiotic therapies targeting *S. aureus* and *P. aeruginosa* has led to the emergence of new pathogens in the respiratory system of CF individuals, such as

Stenotrophomonas maltophilia and *Achromobacter* spp. (Parkins and Floto, 2015). The clinical relevance of these pathogens is not yet established. Few studies have attempted to correlate the CF microbiome with future clinical outcomes using established markers of disease progression such as FEV₁ and comparing microbiota from the sputum samples from individuals in the stable and exacerbated state. Changes in the bacterial community diversity in the airways of CF individuals were associated with the decline of lung function (Paganin *et al.*, 2015; Acosta *et al.*, 2018; Carmody *et al.*, 2018). A significant increase in anaerobic bacteria was also noted (Carmody *et al.*, 2018). However, the bacterial overgrowth was not shown to be causative of exacerbations (Jones *et al.*, 2017). These observations create an exciting potential to use changes in the lung microbiota composition as a biological biomarker of long-term CF progression. Microbial community analysis could potentially identify CF individuals at high risk of respiratory decline. Individuals with CF who suffer from CFRD have been shown to have accelerated pulmonary decline compared to CF individuals with normal glucose tolerance. Studies by Baker *et al.* and Brannan *et al.* have demonstrated increased glucose concentration in the nasal secretions and breath condensates in individuals with CF who suffer from CFRD (Baker *et al.*, 2007; Brennan *et al.*, 2007). An increase in glucose concentration could lead to the proliferation of glucose consuming pathogens in the lungs, accounting for the rapid decline of pulmonary functions. To support this theory, a study from 2016 demonstrated that individuals with CFRD, and particularly those with poor glycaemic control, were more likely to be co-infected with *S. aureus* and *P. aeruginosa*, which in turn was correlated to a decrease in lung functions and increase in the frequency of pulmonary exacerbations (Limoli *et al.*, 2016). All of the studies investigating the effects of CFRD on the lung microbiome have focussed on specific pathogens of interest, and there has not been a study that has explored the effects of CFRD on the whole lung microbiome.

1.12 Effects of diabetes on the lung microbiome in individuals with COPD

COPD is characterised by chronic airway inflammation that leads to an impairment of airflow. One of the clinical presentations is pathogenic precipitation (pathogen deposition in lungs) due to impaired airflow (inability to exhale at normal speed and normal volume). Bacterial, viral and fungal infections all contribute to disease progression. Deposited pathogens induce the release of a cocktail of inflammatory mediators impairing the innate and adaptive immune system (Hassett et al., 2014). The primary cause of COPD is cigarette smoking, air pollution and smoke inhalation. It is known that cigarette smoke increases the number of macrophages and macrophage subtypes residing in alveolar tissues responsible for non-specific pathogen clearance (Wallace et al., 1992). Lung macrophages are split into two distinct phenotypes – M1 and M2. M1 macrophages produce pro-inflammatory cytokines, these are bactericidal macrophages activated by Lipopolysaccharide and IFN- γ . M1 macrophages secrete pro-inflammatory cytokines such as IL-1 β , TNF, and IL-12. In contrast, M2 macrophages are characterised by allergic responses and protection against parasites. These macrophages are involved in tissue remodelling and secrete anti-inflammatory cytokines, including IL-10, TGF- β and others. In COPD, macrophages generate a more pro-inflammatory response (M1 phenotype) that damages tissue via pro-inflammatory mediators and proteases, which contributes to COPD progression. Macrophages indirectly contribute to the COPD progression through defective clearance of bacterial pathogens and apoptotic cells (Yamasaki and Van Eeden, 2018; Hiemstra, 2019). Despite an increase in macrophage numbers, smoke inhalation impairs activation patterns of anti-inflammatory macrophages, causing defective phagocytosis and apoptosis, resulting in pathogenic colonisation of lungs (Woodruff *et al.*, 2005). Smoke exposure has also been shown to result in increased release of several enzymes (cysteine and matrix metalloproteinases (MMP)) that lead to the physical destruction of lung elastin fibres, further impairing the ability of lungs to sustain healthy airflow (Russell *et al.*, 2002). As discussed above, both inflammation and tight junction disruption could

contribute to the increased leak of glucose into the ASL, further contributing to the enhanced pathogenic growth.

There has been a lot of epidemiological data showing an increased prevalence of diabetes in individuals with COPD compared to the general population. More importantly, diabetes in COPD individuals is associated with more frequent exacerbations and worse outcomes in the events of hospitalisation. This result persisted after adjusting cohorts for age, sex and weight. (European Respiratory Society. *et al.*, 2013; Meteran *et al.*, 2015; Kozhevnikova *et al.*, 2016; Stojkovicj *et al.*, 2016; Ho *et al.*, 2017; Gayle *et al.*, 2019; Rambaran *et al.*, 2019). Despite the epidemiological evidence, the mechanisms underlying the high prevalence of type II diabetes in COPD individuals remain unclear and a point of debate. We have discussed above that both COPD and type 2 diabetes mellitus are linked with low-grade chronic inflammation and thus share common pathophysiological pathways. This could, in part, explain the comorbidity of COPD and type 2 diabetes mellitus. It should also be noted that reduction in lung functions and development of type 2 diabetes mellitus could indicate the overall declining health of the individual, which is often correlated with obesity, a common confounding factor for both COPD and type 2 diabetes mellitus.

Several studies utilising bacterial 16S rRNA genomic sequencing have associated the alterations in the composition of the lung microbiome with worsening of lung function, adverse clinical outcomes and exacerbation status in individuals with COPD (Cameron *et al.*, 2016; Diao *et al.*, 2018; Erb-Downward *et al.*, 2011; Jubinville *et al.*, 2018; Mayhew *et al.*, 2018; Muhlebach *et al.*, 2018; Sze *et al.*, 2015, 2012). Management of the symptoms is vital for the treatment of COPD individuals. However, the link between changes in the lung microbiome and symptoms is not currently well established. Using culture-dependent techniques, many pathogens, such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*, have been shown to play a vital role in triggering exacerbations in individuals with COPD (Lieberman *et al.*, 2001; Rosell *et al.*, 2005; Papi *et al.*, 2006). Notably, the same pathogens were observed to be associated with exacerbations in individuals with COPD using

16S rRNA sequencing (Huang *et al.*, 2014; Wang *et al.*, 2016; Jubinville *et al.*, 2018; Mayhew *et al.*, 2018).

While there has been much research looking at the effects of type 2 diabetes mellitus in individuals with COPD and the changes in the lung microbiome of COPD individuals; there has not been a study that has investigated changes in the lung microbiome of COPD individuals with type 2 diabetes mellitus. Such a study could offer an invaluable opportunity to breach the gap in understanding the underlying changes in the lungs of COPD individuals with type 2 diabetes mellitus and, perhaps, help explain the rapid decline in lung functions of these individuals.

Chapter 2

Investigating diabetes-induced changes in the lung microbiome in diabetic mouse models

2.1 Introduction

2.1.1 Studying lung microbiome in animals

Studying the lung microbiome in humans is a complex endeavour: recruiting volunteers for a clinical study is complicated and has a low success rate, and sample acquisition is an invasive procedure that requires trained clinical staff. During collection, the lung microbiome could be affected by a variety of factors. It was previously demonstrated that the environment is a major factor affecting the composition of the lung microbiome, mainly via inhalation of airborne bacteria, viruses, and fungi, as well as particle deposition in the lung (Cui *et al.*, 2014; Dickson and Huffnagle, 2015; Man *et al.*, 2017; Hosgood *et al.*, 2019). The use of anti-inflammatory medications and antibiotics can also influence the composition of the lung microbiome (Segal and Blaser, 2014; Langdon *et al.*, 2016; Vandeplassche *et al.*, 2020). While limited evidence exists that the lung microbiome is affected by diet, several studies have demonstrated the existence of a gut-lung microbiome axis (Marsland *et al.*, 2015; Dumas *et al.*, 2018; Enaud *et al.*, 2020). For example, evidence exists that modifying diet in children with CF alters composition of lung microbiome. Both nutritional factors and gut colonisation patterns are responsible for changes in the respiratory microbiome (Madan *et al.*, 2012). With diet having a strong influence on the composition of the gut microbiome, it could also potentially result in an altered lung microbiome via the gut-lung axis. These factors contribute to the variation of the microbiome between individuals, creating lung microbiomes unique to each individual. Such variation could potentially mask disease-specific changes to the lung microbiome. To account for these variables and observe disease-specific changes in the lung microbiome, large numbers of individuals need to be recruited for the studies. This in combination with difficulties in recruitment of individuals often results in underpowered studies.

Animal models of diabetes could offer preliminary insight into the effects of diabetes on the lung microbiome without the challenges of recruitment of human individuals. Animals used in research are generally sourced from licenced suppliers allowing the purchase of animals of standardised breeds for the experimental set-up. In cases where diabetes is chemically induced, littermates can be used as experimental controls, whereas, in models where a random mutation causes diabetes, littermates without the mutation can be used as controls. Animals purchased from the licenced supplier are screened for underlying health conditions (other than diabetes) that could affect the experimental results, such as lung infections. Animal housing and diet can also be controlled to account for the effect of the environment on the lung microbiome. Thus, using animal models has the potential to reduce variability of the microbiome in individual animals allowing specific factors (ie disease conditions) to be interrogated.

Over the years, various animal models for diabetes have been developed, ranging from human primates to zebrafish. Animals could be used to model both type 1 and type 2 diabetes mellitus; some animal models could be used to create hybrid models possessing properties of both type 1 and type 2 diabetes mellitus.

2.1.2 Non-rodent animal models of diabetes

Large animals serve a specific purpose in research in bridging the gap between rodent (and other small animals) models and human studies.

The use of primates as a model for diabetes is rare but not unheard of. In 1986 Hansel and Bodkin used 42 adult male rhesus monkeys in a longitudinal study to describe the step-by-step development of Type 2 diabetes from lean young adults. The study identified early changes such as increased fasting plasma insulin level and increased insulin secretion in response to a glucose stimulus, which preceded glucose intolerance by several years. This study helped to describe the changes to plasma insulin and blood glucose levels, amongst

other events that lead to the development of type 2 diabetes (Hansen and Bodkin, 1986). In 2011 a study utilising rhesus monkeys attempted to develop a type 2 diabetes mellitus model using a high-fructose diet. A high-fructose diet in rhesus monkeys produced insulin resistance and many features of the metabolic syndrome, including central obesity and inflammation within a brief period of time. Some rhesus monkeys developed type 2 diabetes (Bremer *et al.*, 2011). Primate models of diabetes are used to validate pharmacological intervention prior to human studies and are usually reserved for studies that have extensively demonstrated research in other animal models.

Pigs emerged as a large animal of choice for many studies, as organ systems of this species, as well as physiological and pathophysiological responses, resemble those of the human (Larsen and Rolin, 2004). The size and weight of pigs allow the use of standard diagnostic and surgical techniques that are utilised in humans. Together with the resemblance of the pig pancreatic duct system to the human system makes pigs an attractive model for diabetes research (Wolf *et al.*, 2014). Domestic pigs have similar fasting glucose to humans, plasma glucose and insulin increase with age in a similar manner, and glucose tolerance can be expected to deteriorate with age in both humans and pigs (Larsen and Rolin, 2004).

Using big animal models, however, comes with significant drawbacks. Cases of spontaneous diabetes or diabetic mutations in pigs and primates are extremely rare, so models of diabetes must be induced experimentally or surgically. Big animals require extensive veterinary care to provide diabetic control and manage adverse effects, as well as specialised facilities. Due to these drawbacks, big animal models are not often used for exploratory studies and are reserved for pre-clinical trial treatment validations instead.

2.1.3 Rodent models of diabetes

Rodents are commonly used in research to model diseases including diabetes. Rats are a common model of diabetes. In rats' diabetes could be chemically induced with drugs, such as

streptozotocin and alloxan (Szkudelski, 2001); rats could also be bred for genetic onset of diabetes with such models as BB rats (Mordes *et al.*, 2004) and Zucker diabetic fatty rats (Shiota and Printz, 2012). In this study, however, mouse models of diabetes were chosen over rat models of diabetes, due to the expertise in handling and housing of mice.

2.1.4 Mouse models of diabetes

Mouse models of diabetes are commonly used in exploratory studies as an alternative to large animal models. Despite having fewer similarities to human physiology, mice offer a model that is easier to control and easier to maintain.

In humans, an accepted and validated method of sampling the lung microbiome is bronchoalveolar lavage (BAL). BAL allows sampling of the large surface area of the distal lung and airways and produces high-volume samples rich in bacteria. A similar technique can be used in mice to sample the lung microbiome. In humans a saline solution is delivered into the lungs using bronchoscope, the liquid is then used to wash the airways and capture a fluid sample containing bacteria. Due to restricted size of mice, it is impossible to perform this procedure on a live animal. Instead, animal is terminated and the whole lung is lavaged. The sample volume generated in BAL procedures in mice rarely exceeds 1ml and requires termination of the animal (Carney *et al.*, 2020).

The mouse lung microbiome was shown to be easily influenced by a myriad of environmental factors (McGinniss and Collman, 2018). This demonstrates the importance of properly controlling the experiment: using genetically similar mice, preferably littermates, and eliminating any environmental variables by housing animals in the same environment in neighbouring cages, using the same food (chow), carrying out procedures on the same day. It was also reported that lung bacteria communities in mice cluster strongly by cage, shipment, and vendor, adding the necessity to purchase all experimental animals in batches from the same vendor (Dickson *et al.*, 2018).

2.1.4.1 Db/db mice

Mice homozygous for diabetes (*Lepr^{db/db}* or db/db respectively) mutation were among the earliest diabetic mouse models and remains a popular choice for diabetes research. Leptin is a hormone that regulates appetite, and mutations in the leptin receptor (*Lepr*) results in unregulated feeding, leading to obesity and diabetes. The homozygous nature of mutation allows using heterozygous littermates (*Lepr^{+/-db}*) as an experimental control. The db/db mouse model is currently the most widely used mouse model of type 2 diabetes mellitus (The Jackson Laboratory, no date b; Wang *et al.*, 2014). These mice exhibit elevated blood glucose levels at the age of 4 weeks and severe hyperglycaemia at 8 weeks of age. Early-onset of hyperglycaemia makes this model perfect for studies that do not require animals to have sustained hyperglycaemia until older age, as the health of these mice deteriorates quickly and causes severe discomfort in the later stages of life (The Jackson Laboratory, no date b). Db/db mice develop obesity without requiring a high-fat diet, which is beneficial for studies, as both diabetic and control mice could be fed the same diet. This removes the potential effect on the lung microbiome via gut-lung axis, if mice were fed different diet.

2.1.4.2 GK^{+/-} mice

Heterozygous glucokinase knockout mice (GK^{+/-}) are an uncommon diabetes model; however, were shown to respond well to drugs that lower blood glucose levels (such as metformin, glipizide, and others) and improve glycaemic control (Baker *et al.*, 2014). These mice develop robust hyperglycaemia when fed a high-fat diet and are a highly reproducible model, offering a translatable model to human type 2 diabetes mellitus (Baker *et al.*, 2014).

2.1.4.3 Streptozotocin mice

Streptozotocin-induced diabetic mice model (STZ) is one of the few chemically induced mice models that does not require genetic mutations to be present in mice. Streptozotocin is an antibiotic that causes pancreatic islet β -cell destruction leading to insulin deficiency. STZ mice

are the most common type 1 diabetes mellitus model (Furman, 2021). The loss of insulin causes hyperglycaemia; STZ mice lack the confounding effect of insulin resistance and high circulating fatty acids, which is present in other type 2 diabetes mellitus mice models such as db/db and ob/ob mice (Graham et al., 2011; Levant et al., 2013; Murray et al., 2005; Tang et al., 2017). As these mice do not develop insulin resistance, insulin therapy has a profound glycaemic control (Grossman *et al.*, 2010; Deeds *et al.*, 2011) and so this is a useful model to investigate insulin responsive factors and insulin therapies. There are however more ethical considerations to be taken into account with STZ mice. The administration of streptozotocin and consequent destruction of β -cells causes great amount of pain and discomfort to animal, requiring careful management of the animal welfare.

2.1.4.4 Ob/ob mice

Similar to db/db mice, homozygous for obese (*Lep^{ob}* or ob/ob respectively) mutation are one of the earliest and most popular mouse models for diabetic research (Wang *et al.*, 2014). Ob/ob mice have a mutation in the leptin gene (*Lep*), resulting in the decrease in leptin secretion, causing unregulated feeding and obesity. While both db/db and ob/ob mice models exhibit obesity and diabetes, ob/ob do not develop stable hyperglycaemia and are not a good model for studies focusing on the effects of hyperglycaemia. Ob/ob mice also require a high-fat diet to develop obesity leading to diabetes, which makes it harder to control accurately (The Jackson Laboratory, no date).

2.1.5 Comparison of mouse models of diabetes

In order to choose diabetic mice models for this study, different mouse models were evaluated. For this study we are looking at mice that develop severe hyperglycaemia, which should translate to elevated glucose concentration in the ASL, detected in BAL (Holmberg *et al.*, 2017). The ideal mouse model would develop hyperglycaemia in the early stage of life, allowing hyperglycaemia to affect lung microbiome for prolonged period. A model in which

diabetic and non-diabetic animals would consume standard rodent chow is preferable, as it eliminates the potential effect of the diet on the lung microbiome.

GK^{+/-} mice were previously used in a collaborative study (Holmberg et al., 2017), and it was demonstrated that glucose and lactate concentrations in the BAL sample were elevated in GK^{+/-} mice compared to WT mice. A direct correlation between blood glucose and BAL glucose concentrations was also established. Due to the availability of the animals, previous experience with this model and validated lung hyperglycaemia with normal insulin levels this model was previously chosen to investigate the effects of hyperglycaemia on the lung microbiome. This model, however, requires mice to be fed a high-fat diet in order to maintain stable hyperglycaemia (Gorman *et al.*, 2008), which could affect the composition of the mouse microbiome. Therefore, in this study, I decided to utilise a murine model that does not require a high-fat diet. One of the most used diabetic mice models that do not require a specialised diet is the db/db mouse model. These mice develop stable hyperglycaemia at the age of 6 weeks (Kobayashi *et al.*, 2000) and maintain hyperglycaemia throughout their lifespan. Db/db mice were previously used for research in our laboratory, where it was demonstrated that glucose concentration was significantly elevated in both blood and BAL samples of db/db mice compared to non-diabetic littermates (Åstrand *et al.*, 2017). More prolonged exposure to hyperglycaemia and elevated glucose in the lung lumen increasing the likelihood of driving changes to the lung microbiome.

STZ mice were initially considered for this research, however, were decided against. While db/db and GK^{+/-} are genetically modified, STZ mice require pharmacological destruction of b-cells. At the time of this research, facilities for the generation, housing, and maintenance of STZ mice on-site was restricted. Moreover, STZ mice require a high-fat diet, which, as discussed above, would potentially affect the mouse microbiome.

Overall, the db/db model was chosen as the prime diabetic animal model for this study. This allowed me to collect microbiome data under standardised conditions and procedures in a well-controlled environment.

2.2 Aims and objectives

Hypothesis: Hyperglycaemia associated with diabetes modifies the lung microbiome, decreasing bacterial diversity and increasing the presence of pathogenic bacterial species in diabetic mice models.

Aim 1: To compare the lung microbiome composition between diabetic and non-diabetic mice.

Aim 2: To investigate the difference in bacterial species abundance and diversity in the lung microbiome of db/db and GK^{+/-}.

Aim 3: To compare the lung microbiome composition between db/db mice acquired from the same source but processed at two independent laboratories.

2.3 Materials and methods

2.3.1 Db/db mice

Seven-week-old female db/db mice and non-diabetic littermate controls were purchased from Envigo (UK). A total of 30 animals were purchased: 15 db/db (BKS.Cg-+Leprdb/+Leprdb/OlaHsd) and 15 non-diabetic littermates (BKS.Cg-(Lean)/OlaHsd). Mice were housed in five-animal cages in a standard animal housing room. Mice were allowed to acclimatise for three weeks before lung microbiome collection. Animals were kept in a facility with 12h light/dark cycle; water and standard rodent chow were available *ad libitum*. Water consumption was monitored daily for each cage.

Prior to termination, the weight of each mouse was recorded. The animals were terminated with an overdose of pentobarbital (0.2ml of 100mg/ml i.p.). BAL and blood samples were collected as described below.

As part of a previous study, we received BAL samples from db/db mice housed in Sweden in AstraZeneca facilities (Gothenburg). These mice were kept in similar conditions, received the same food, and were terminated using the same methodology. A total of 6 BAL samples were received: 3 diabetic and 3 non-diabetic samples. These samples were processed alongside samples obtained from residential db/db mice to assess the similarities between lung microbiome of similar mice hosted in a different environment.

2.3.2 GK^{+/-} mice

As a part of a previous study carried out by a former student at St George's University of London, raw microbiome data was available from GK^{+/-} mice housed in AstraZeneca facilities in Sweden. These mice were kept in a facility with 12h light/dark cycle; water and standard rodent chow were available *ad libitum*. A total of four diabetic and four non-diabetic mice were used in the study. The resulting bacterial DNA from each animal was pooled into a single

sample to increase the amplification success rate. Library preparation and 16s sequencing were performed by Barts Genome Centre. Raw microbiome data generated in the project was reanalysed in this project alongside other diabetic mice samples to compare the lung microbiome.

2.3.3 BAL and blood sampling

Skin and muscles of the neck were dissected using sterile scissors, and the trachea was exposed. A small incision in the trachea was created using surgical scissors (501777, WPI). A sterile catheter tube (Jelco 4030, Smiths Medical) was inserted into the opening of the trachea and connected to a sterile 1ml syringe (BD572, Appleton Woods Ltd) (Figure 9). A catheter was secured by tightening a piece of sterile surgical thread around the intubated trachea to create an air-tight seal. 1 mL of sterile phosphate-buffered saline (PBS) was gently instilled into the lungs using a sterile syringe. While lungs filled with PBS, mouse chest was gently massaged for 30 seconds, after which liquid was collected. The procedure was repeated the second time using the same liquid, resulting in 1 mL of the final BAL sample. Sterile PBS was taken up into the syringe and expelled via a catheter into a new collection tube to act as environment control. BAL samples were snap-frozen and stored at -80°C . At termination, blood was collected from the heart. Blood glucose concentration was immediately measured using Accu-Chek Mobile (Roche).

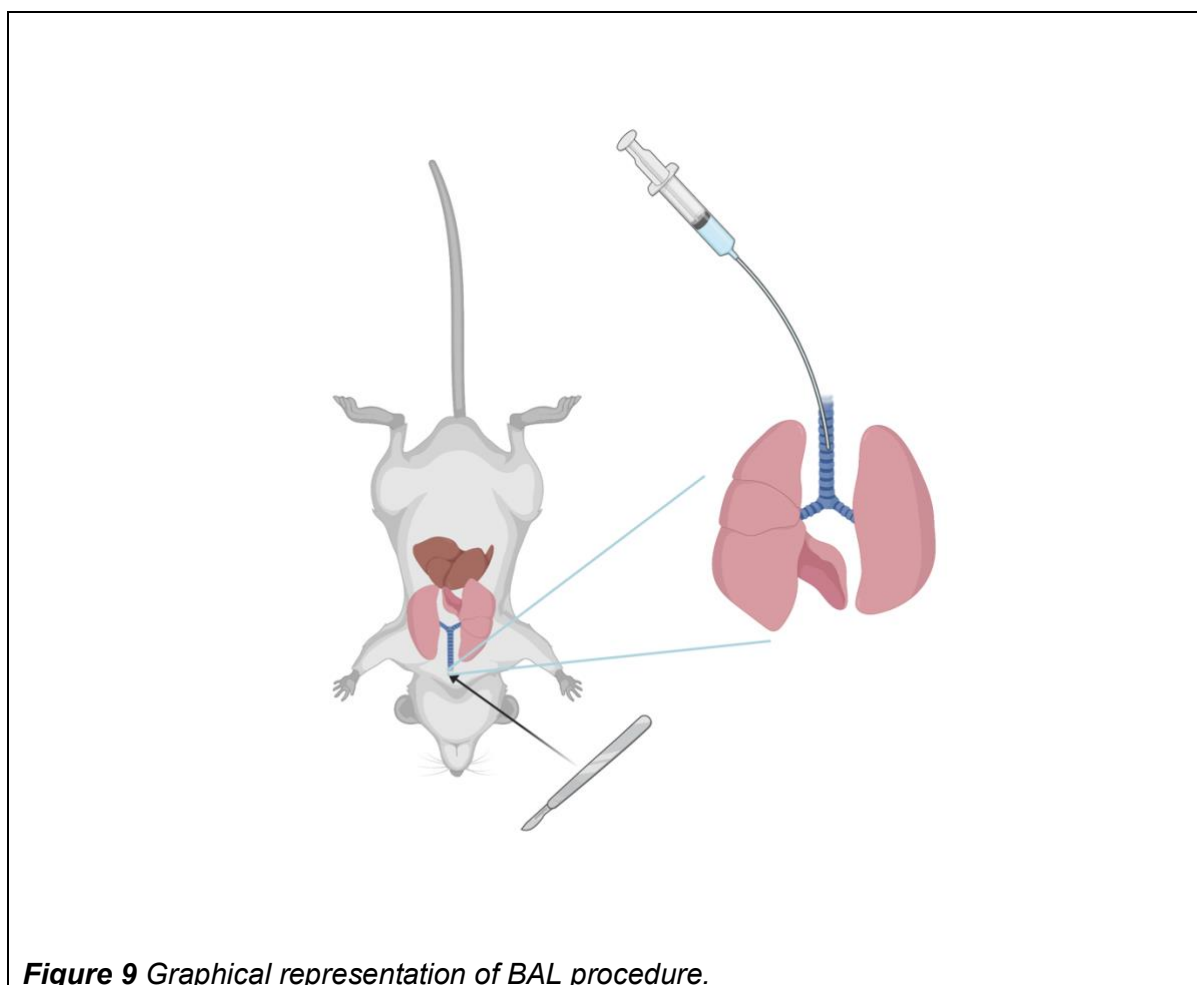


Figure 9 Graphical representation of BAL procedure.

2.3.4 DNA extraction, quantification and 16S sequencing

Bacterial DNA was extracted from BAL samples using QIAamp DNA Microbiome Kit (Qiagen). Extractions were performed as per manufacturers guidelines. This kit utilises an additional step of digesting host DNA to aid bacterial DNA purification. As the volume of BAL samples was 1 ml, no additional centrifugation was required to generate a pellet; the whole sample was used for extraction instead. Buffer AHL was added to the sample and incubated for 30 minutes at room temperature with an end-over-end rotation, which allowed protein denaturation to release DNA. The sample was then centrifuged, and supernatant removed. Buffer RDD and Benzonase were further added to the sample and incubated at 37 °C for 30 minutes. Benzonase is used to hydrolyse nucleic acid and reduce sample viscosity. Buffer RDD is used for on-column digestion of DNA. Following Proteinase K was added to the sample and

incubated further at 56 °C for 30 minutes for protein digestion and removal of contaminants. Buffer ATL (containing Reagent DX) was further added to the sample. Buffer ATL contains sodium dodecyl sulfate, which acts as a detergent and aids in cell lysis. Whole content of the tube was transferred to the Pathogen Lysis Tube L. FastPrep-24 (MPbio) was used to lyse bacteria with Pathogen Lysis Tube L. All samples were shaken twice at a velocity of 6.5 m/s for 45 s with a 5-minute interval with storage on ice. Pathogen Lysis Tube L was then centrifuged to remove the foam and the sample was transferred into a fresh microcentrifuge tube without glass beads. Additional Proteinase K was added to the sample and incubated at 56 °C for 30 minutes. Following incubation Buffer APL2 was added to the sample and mixed well, and the sample was incubated at 70 °C for 10 minutes. Buffer APL2 aids cell and virus lysis, as well as denatures RNase and DNase enzymes. Ethanol was added to the sample and thoroughly mixed by pulse vortexing. The mixture was then applied to the QIAamp UCP Mini Column and centrifuged. Flow-through was discarded and buffer AW1 was applied to the column. As before, flow-through was discarded and buffer AW2 was applied. Buffers AW1 and AW2 cause DNA to bind to the silica membrane preventing its elution during centrifugation. Following discarding of flow-through, column was centrifuged to dry the membrane. At the final step DNA was eluted in 50 µl of Buffer AVE which causes DNA to be released from the silicon membrane.

Extracted DNA was quantified using Qubit Fluorometric Quantification (ThermoFisher Scientific) using dsDNA High Sensitivity kit.

Nextera XT DNA Library Preparation Kit (Illumina) was utilised to prepare the library for sequencing. This study utilised primers targeting the V3-V4 region of the 16S gene using specific primers 341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3'. Each sample reaction was assembled to a total volume of 25 µl containing 12.5ng of template DNA, 5 µl of 1 µM Forward and Reverse Primers, 12.5 µl of 2 x KAPA HiFi HotStart ReadyMix. The PCR programme consisted of initial denaturation at 95 °C for 3 minutes; 25 cycles of denaturation at 95 °C for 30 seconds, annealing at 55°C

for 30 and extension at 72 °C for 30 seconds; completing with a final extension at 72 °C for 5 minutes. The PCR products were purified by AMPure XT beads (Beckman Coulter Genomics) using a magnetic stand and ethanol washes as per manufacturers guidelines. AMPure beads bind DNA allowing contaminants to be washed out. Purified products were quantified using Qubit (ThermoFisher Scientific). Dual index adapters for sequencing on the Illumina MiSeq platform were attached using the Nextera XT Index kit (Illumina). Each reaction was set up to contain 5 µl of Index Primer 1 and 2, 25 µl of 2x KAPA HiFi HotStart ReadyMix, 5 µl of product DNA and 10 µl of PCR grade water. PCR programme consisted of initial denaturation at 95 °C for 3 minutes; 8 cycles of denaturation at 95 °C for 30 seconds, annealing at 55°C for 30 and extension at 72 °C for 30 seconds; completing with a final extension at 72 °C for 5 minutes. Following PCR, final purification with AMPure XT beads (Beckman Coulter Genomics) was performed. Prepared libraries were sent to Glasgow Polyomics for the library quantification, normalisation and MiSeq sequencing. All sequencing was performed off-site. These libraries were sequenced in a 300bp paired-end run. Raw FASTA files were generated and received for further analysis on-site.

2.3.5 Data analysis

Bioinformatic analysis was performed using Mothur v1.39.5 as per the MiSeq SOP pipeline (Schloss *et al.*, 2009). For genus-level analysis, final alpha diversity, beta diversity, and PCoA loadings were used. SILVA v138 was utilised as a reference dataset for alignment purposes. OTUs were generated at a 97% similarity threshold. Analysis was performed on St George's University of London Stats3 remote server with 72 CPU and 300 GB RAM. After removing contaminant sequence reads, downstream statistical analyses were performed using R statistical software and GraphPad Prism version 9.

2.3.6 Statistical analysis

Beta diversity between diabetic and non-diabetic mice was compared using Analysis of Molecular Variance (AMOVA) within Mothur software based on the dissimilarity of matrix centerpoints between two groups. Homogeneity of Molecular Variance (HOMOVA) was performed within Mothur software. HOMOVA is a non-parametric test based on Bartlett's test for homogeneity of variance, which allows to assess whether genetic diversity within two populations is homogenous (Schloss, 2008). Differences in specific bacteria genus (and higher-level taxonomy) between diabetic and non-diabetic lung microbiome was assessed using Multiple t-tests using GraphPad Prism 9 software (GraphPad). Correlation between blood glucose concentration and bacterial species or α -diversity was analysed using a simple linear regression test using GraphPad Prism 9 software (GraphPad).

2.4 Results

2.4.1 Mouse BAL sample collection and DNA extraction

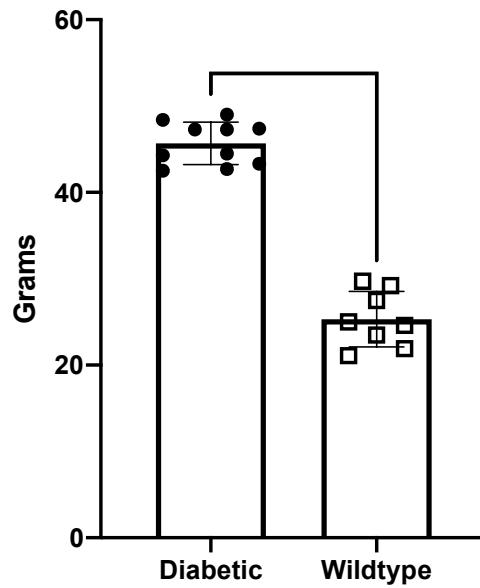
A total of 15 db/db and 15 db/wt mice were sampled at the age of 11 weeks. A total of 6 BAL extractions failed due to procedural error. As diabetic mice age, they become sicker, resulting in organ fragility, making it easier to pierce the lung during BAL procedure and making it harder to secure the catheter in the trachea with a suture. Another 6 BAL samples failed to produce enough DNA during the extraction process. Attempts were made to amplify these samples; however, not enough DNA was present to generate the library. Many studies pool mouse BAL samples to avoid having samples yield insufficient bacterial DNA. I did not pool samples to allow β -diversity analysis of the microbiome and further statistical analysis.

2.4.2 Weight and blood glucose in db/db and wildtype mice

The weight of the diabetic mice was significantly higher than that of non-diabetic mice (Figure 10a, $n=9$, $p<0.0001$). The mean weight of db/db mice was 45.67 ± 2.46 g, while that of wildtype was 25.31 ± 3.22 g.

Random blood glucose measurement was higher in db/db than wildtype mice (Figure 10b, $n=9$, $p<0.0001$). Mean blood glucose in diabetic mice was 25.06 ± 2.85 mM, while mean blood glucose in wildtype mice was 8.31 ± 0.93 mM. BAL glucose levels were not measured immediately upon collection. When BAL glucose was measured during DNA extraction, all the glucose appeared to be metabolised and no glucose was detected. This prevented measurement of BAL glucose directly.

A Weight of db/db and wildtype mice



B Blood glucose of db/db and wildtype mice

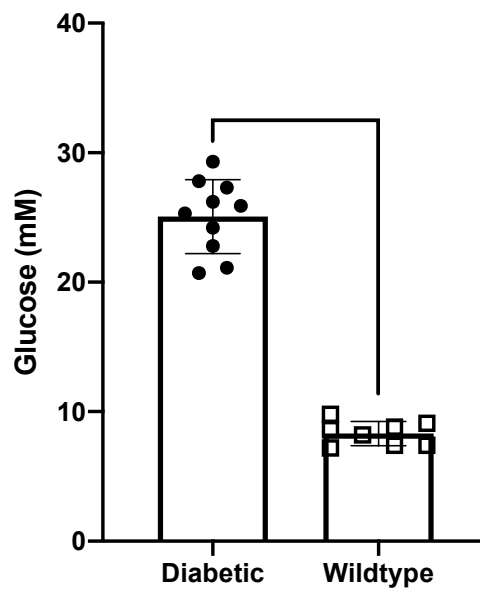
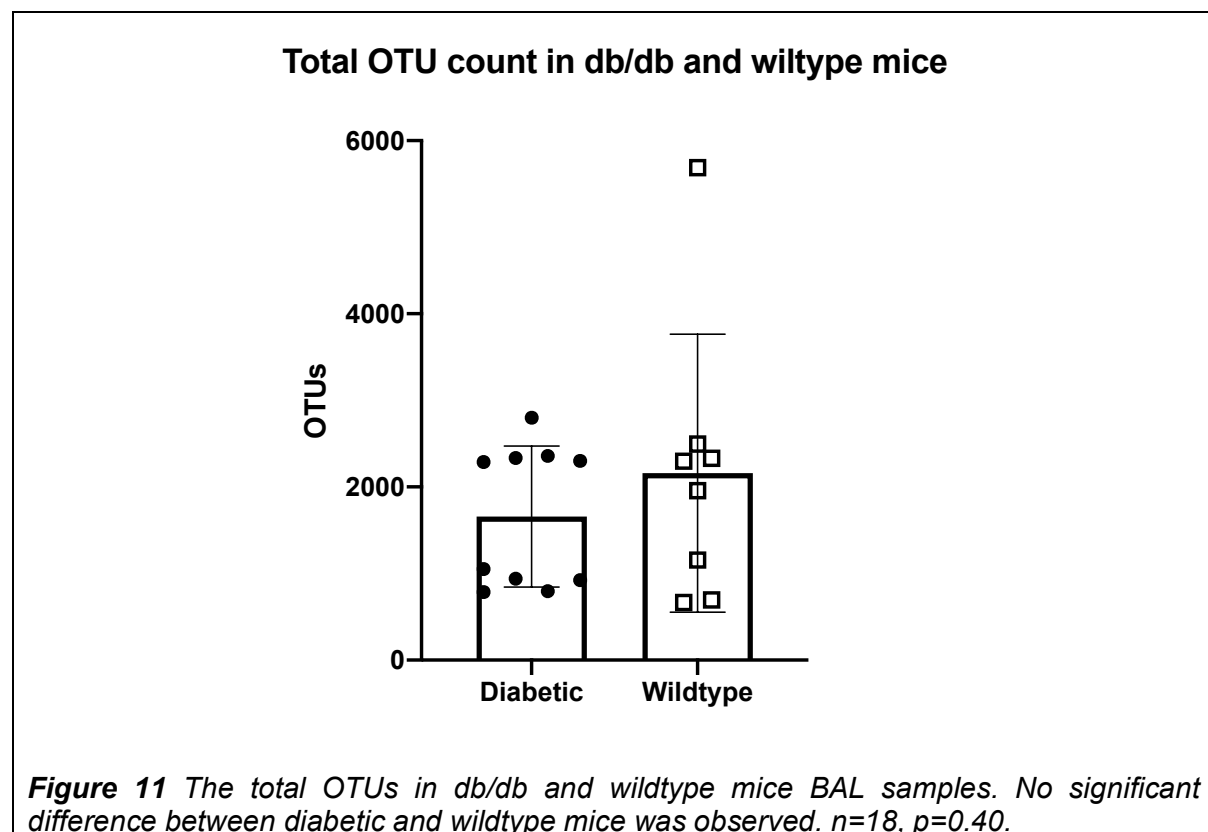


Figure 10 Blood glucose and weight are elevated in hyperglycaemic mice. (A) Weight shown as values in grams $\pm$ SD and plotted for individual db/db and wildtype and (B) Blood glucose concentration shown as values in mM $\pm$ SD and plotted for individual db/db and wildtype. $n=9$ for both (A) and (B). **** $p < 0.0001$ with unpaired t-test

2.4.3 Total OTU amount in BAL samples from db/db and wildtype mice

The total number of OTUs observed in the BAL samples from db/db, and wildtype mice showed no significant difference between the two groups (Figure 11, $n=9$, $p=0.40$). The mean amount of OTUs found in the BAL samples of db/db mice was 1659 ± 815 , while that of the wildtype mice was 2161 ± 1605 . This demonstrates that sustained blood hyperglycaemia does not result in a change to lung bacterial load.

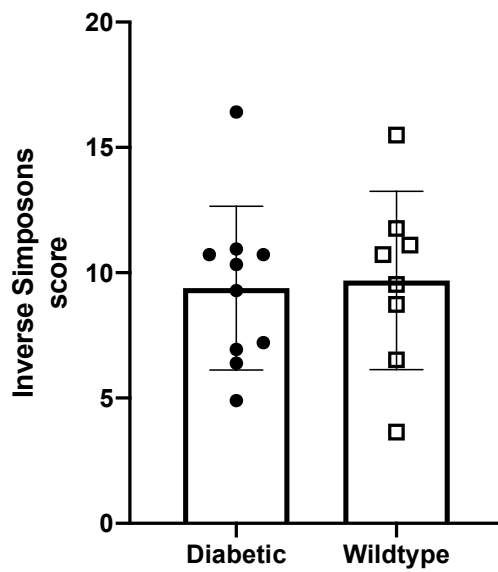


2.4.4 Alpha diversity of db/db and wildtype mice

Alpha diversity of the microbiome extracted from BAL samples from db/db and wildtype mice was measured using an inverse Simpson diversity index. This measurement of α -diversity is preferred for work with samples with uniform evenness and similar levels of diversity. Simpson-based metrics are also less affected by sampling efforts, such as sampling duration, depth, and intensity, which is hard to control in a BAL procedure.

There was no statistically significant difference in α -diversity in diabetic and wildtype mice (Figure 12a, $n=9$, $p=0.8536$). Simple linear regression showed no relationship between blood glucose concentration and α -diversity of lung microbiome (Figure 12b, $n=9$, $R^2=0.038$, $p=0.4372$) as the slope was not significantly different from zero. This demonstrates that sustained hyperglycaemia does not affect microbiome diversity within the lung of mice.

A Alpha diversity scores in db/db and wiltype mice



B Correlation of alpha diveristy changes with blood glucose concentration

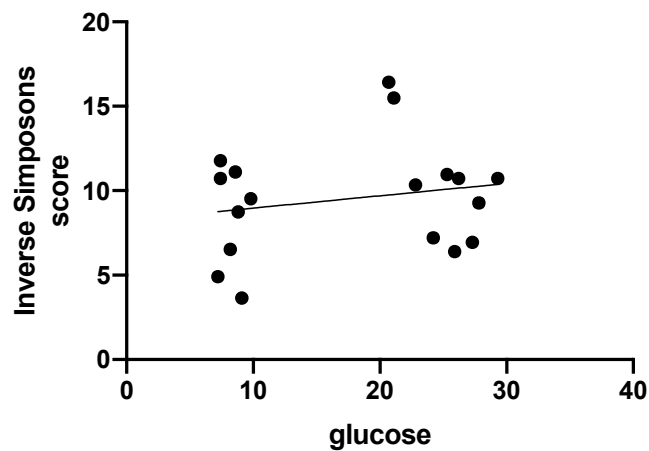


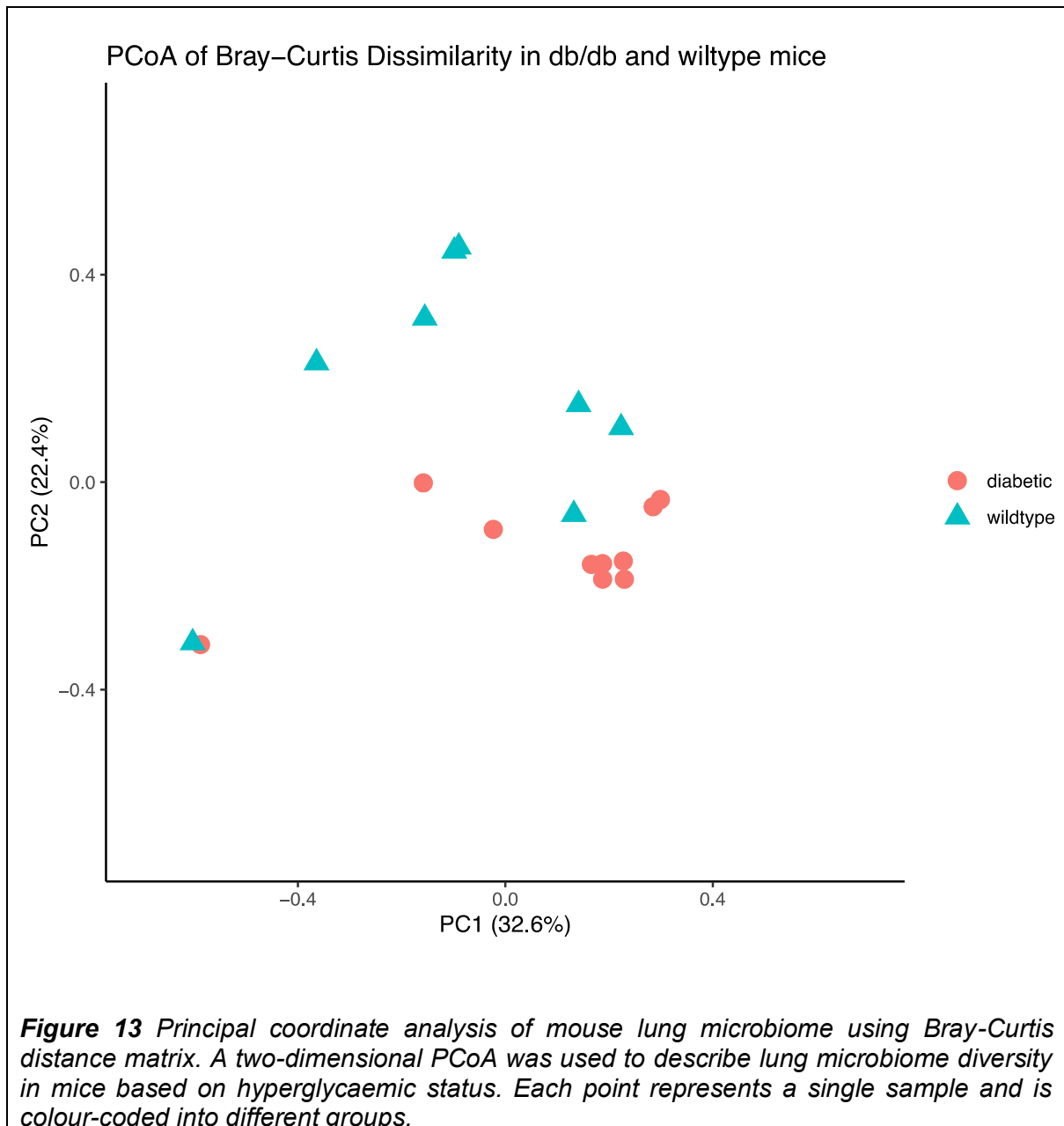
Figure 12 α -diversity in the lung microbiome of mice does not change with sustained hyperglycaemia. (A) Inverse Simpson scores are shown as values $\pm$ SD and plotted for individual db/db and wildtype mice (B) Inverse Simpson scores are plotted against blood glucose concentration for individual mice. $n=9$ for both (A) and (B)

2.4.5 Beta diversity of the lung microbiome between db/db and wildtype mice

Beta diversity is a measure of similarity or dissimilarity of two microbiomes from different environments. It is a helpful tool to assess if the environment that microbiome data is collected from affects the composition of the said microbiome. b-diversity in this study is calculated using Bray-Curtis dissimilarity scores and is presented as the Principal Coordinates Analysis (PCoA) graph for visualisation purposes (Figure 13).

Statistical analysis using AMOVA shows a significant difference in the centroids of the matrixes between the lung microbiome of diabetic and non-diabetic mice ($n=9$, $p=0.009$); thus, the separation between lung microbiome of diabetic and non-diabetic mice is statistically significant. This confirms that sustained hyperglycaemia affects b-diversity of the mouse lung microbiome and changes the composition of the microbiome.

Using HOMOVA, shows that variation in the lung microbiome in the lungs of diabetic mice is not significantly different from that variation in the lungs of non-diabetic mice ($n=9$, $p=0.59$). This shows that both diabetic and non-diabetic mouse lung microbiome is equally variable, suggesting the presence of the stable core microbiome that does not change under the effects of persistent hyperglycaemia.

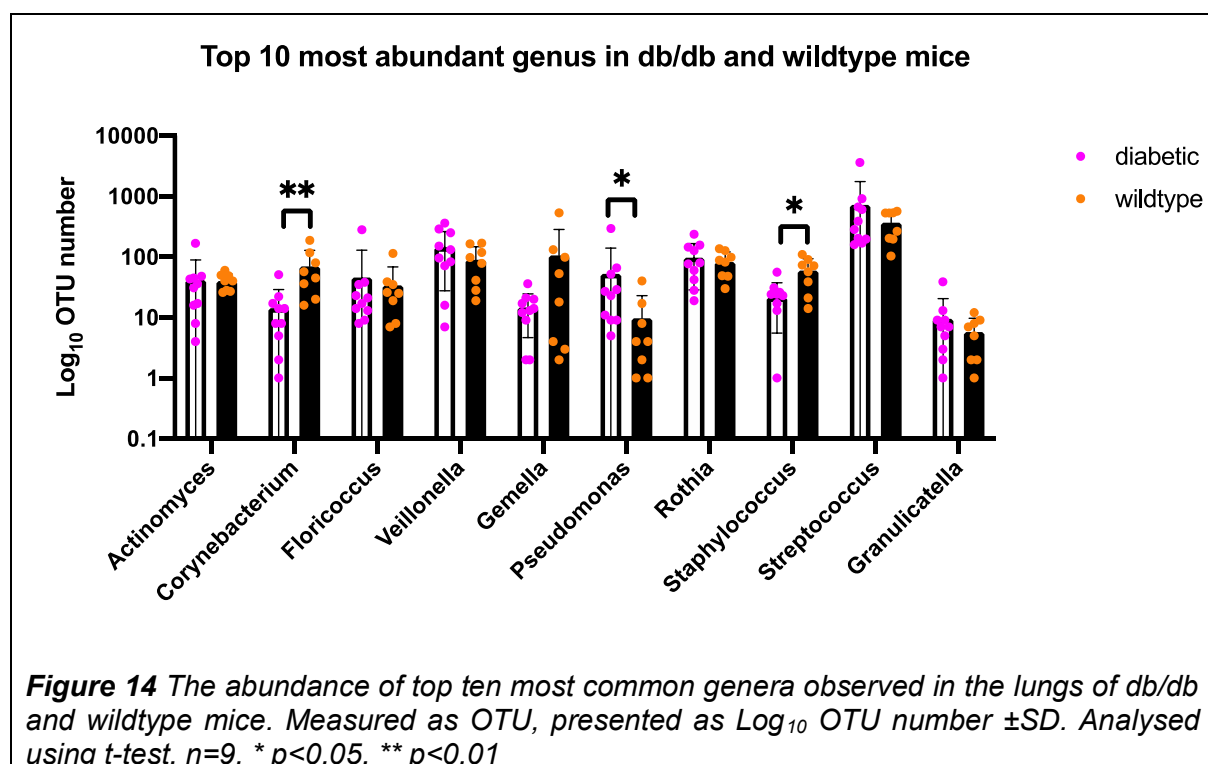


2.4.6 Bacterial abundance at genus level between db/db and wildtype mice

There were 1166 total unique genera of bacteria detected in the BAL samples from db/db and wildtype mice. However, a large proportion of genera were unclassified or poorly aligned, creating uncertain genus classifications. A total of 35% of all genera detected could not be identified. For the purpose of the analysis, ten top genera were picked to analyse the effects

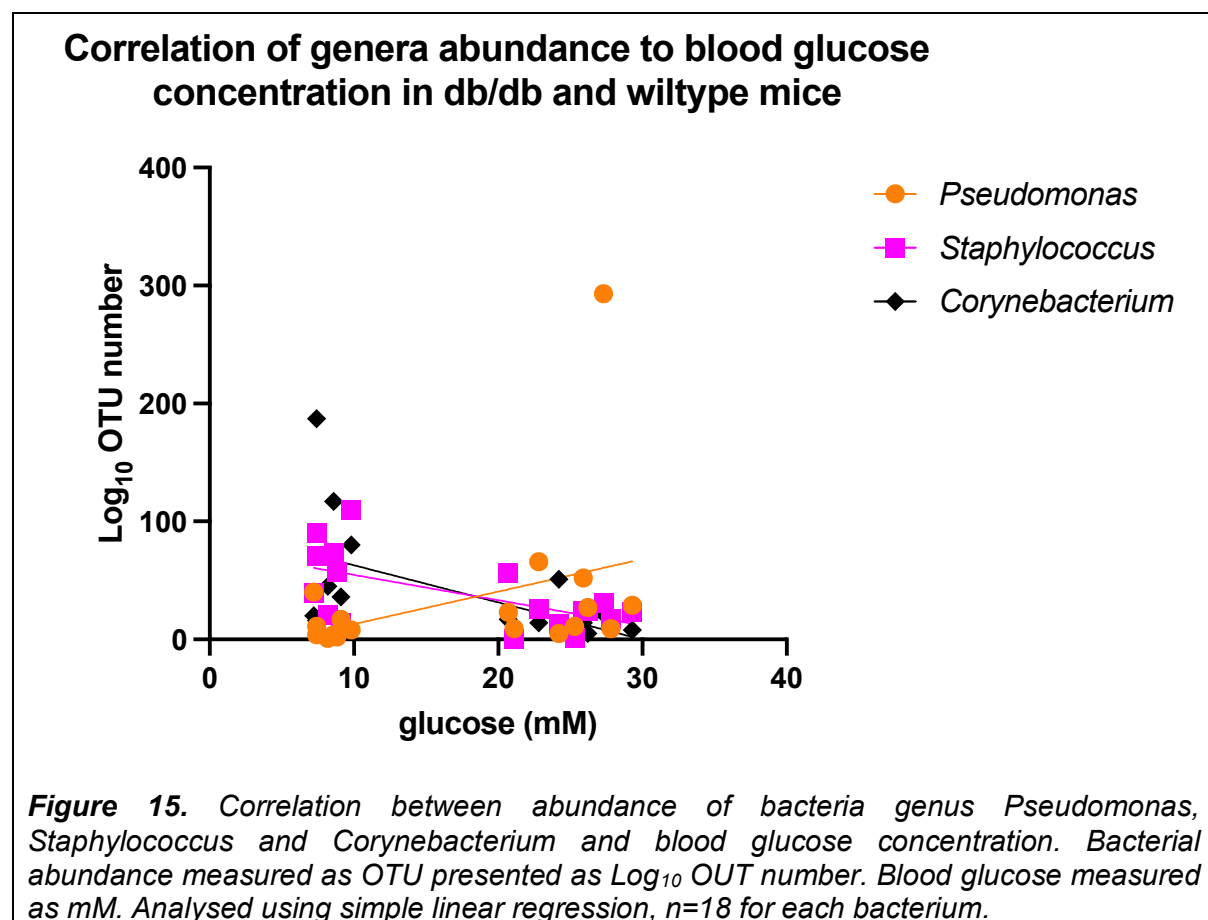
of sustained hyperglycaemia (Figure 14). These top genera account for 85.58% of all detected OTUs.

Within the top ten genera detected, three genera were statistically significantly different. It was shown that genus *Corynebacterium* (n=9, $p=0.0018$) and genus *Staphylococcus* (n=9, $p=0.0190$) are significantly lower in the lungs of diabetic mice than that of wildtype, while genus *Pseudomonas* is significantly more abundant in the lungs of diabetic mice than that of wildtype (n=9, $p=0.0106$). Moreover, genus *Streptococcus* accounted for 46% of all OTUs detected in the lungs of db/db and wildtype mice. This data demonstrates that while the overall lung microbiome of diabetic mice was different from the lung microbiome of non-diabetic mice, a small proportion of genera were different between the two.



Using simple linear regression, I also analysed whether an increase or decrease in specific pathogens numbers correlated with the blood glucose concentration. In Figure 15 I demonstrate that the abundance of genera *Corynebacterium* and *Staphylococcus* significantly correlate with blood glucose concentrations ($p=0.0115$ and $p=0.0074$ respectively, n=9 for

both) while the presence of genus *Pseudomonas* does not significantly correlate with blood glucose concentrations ($p=0.1397$, $n=9$). In combination with the data presented above, I can suggest that, in this study, changes in the abundance of *Corynebacterium* and *Staphylococcus* in the lungs of diabetic mice are primarily due to the changes in glucose concentration. In contrast, lung glucose concentration alone may not be responsible for the changes in the abundance of *Pseudomonas*.

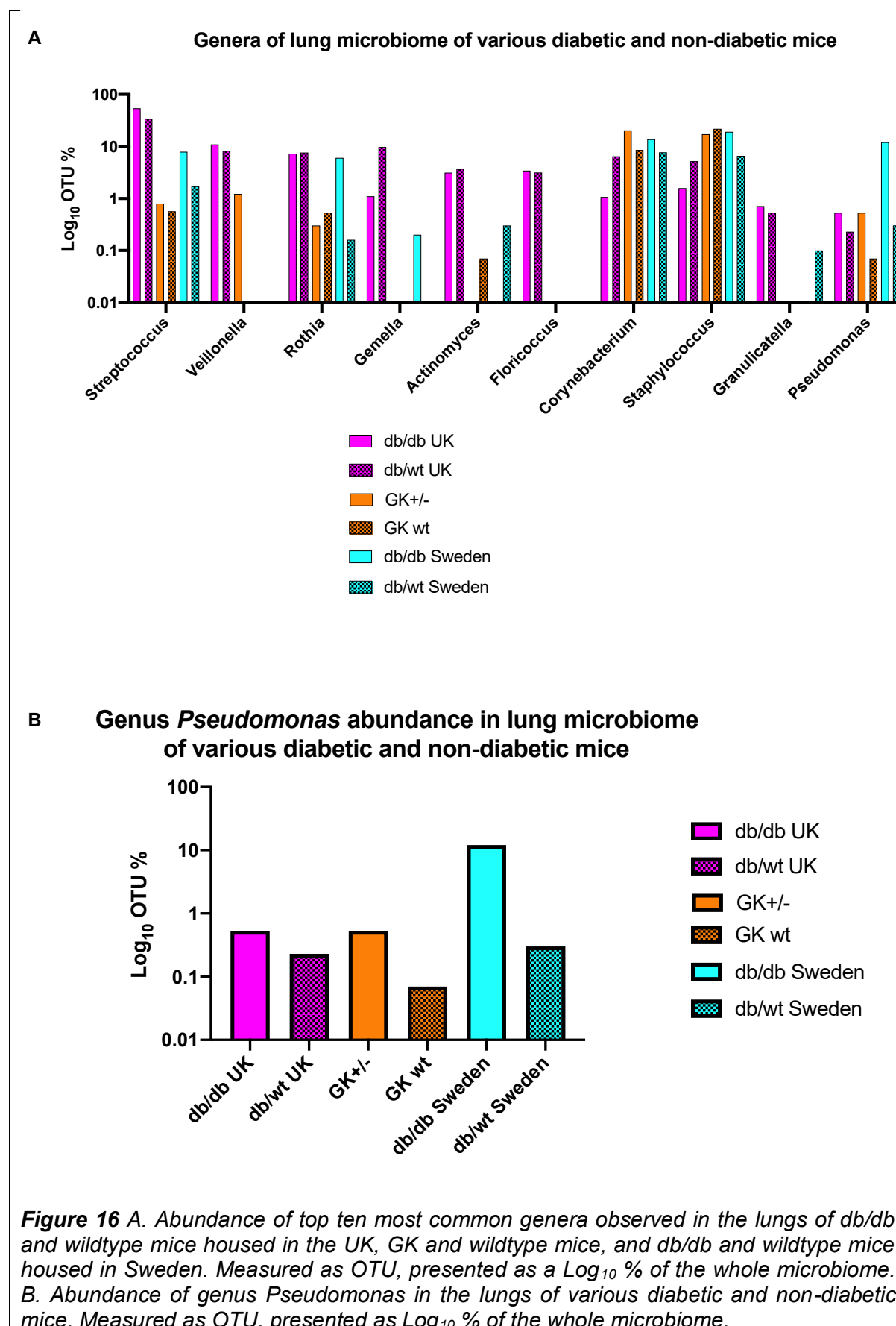


2.4.7 Bacterial abundance at genus level compared between different diabetic mouse models

In order to evaluate the similarities and differences of the lung microbiome in diabetic and non-diabetic mice housed in different environments and to mice of different genetic makeup, I used the top genera found in db/db and wildtype mice housed in the UK (Figure 16A) and compared

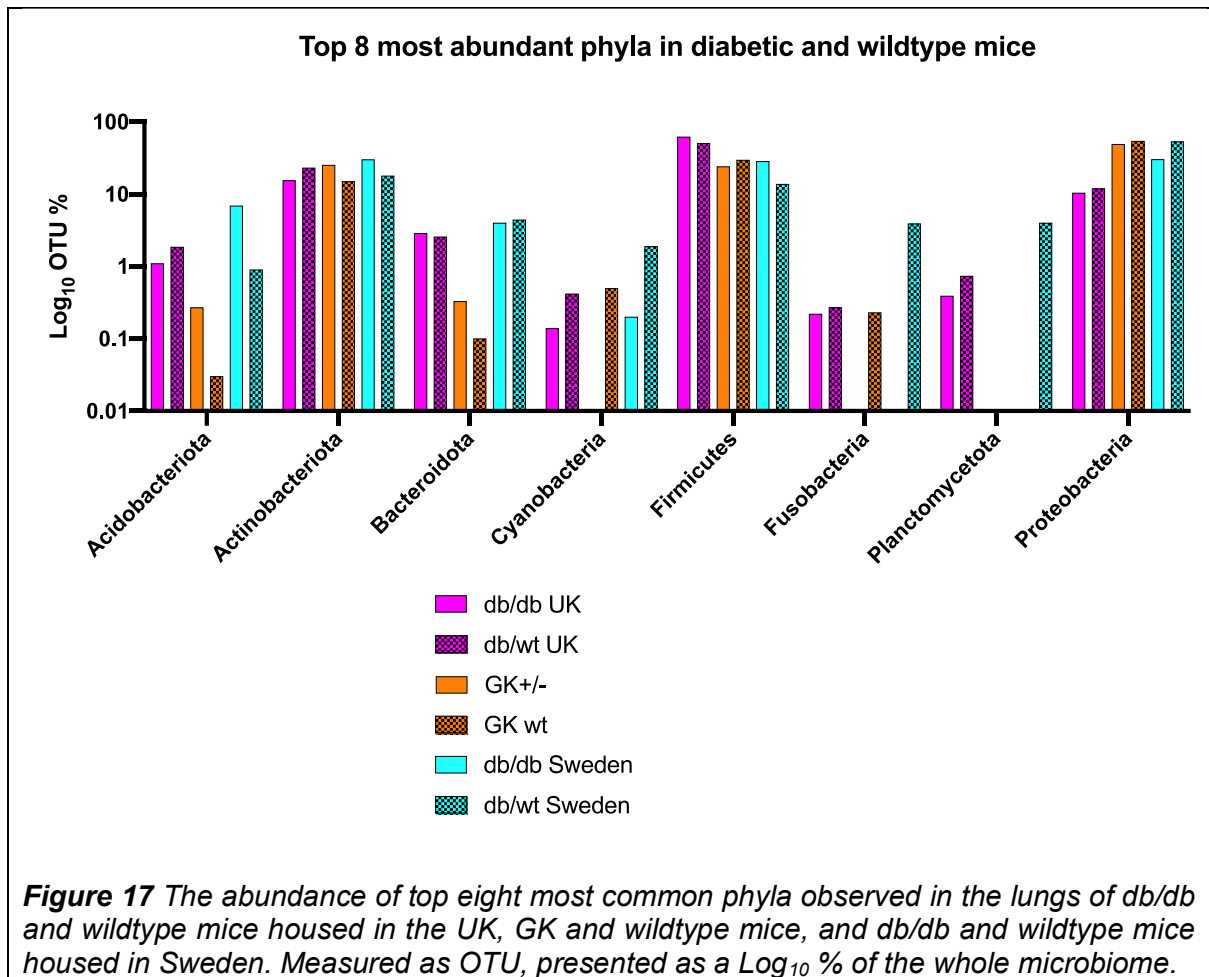
the abundance of these to other mice models. As some of these samples were pooled and the number of animals varied significantly, it was difficult to statistically analyse the differences. Because different BAL samples were not normalised prior to sequencing, using the absolute number of OTUs to compare the data was not possible. When comparing data from different microbiomes, values are expressed as % of the total microbiome.

The lung microbiome appeared to significantly differ between mice housed in different conditions and between different diabetic mouse models. Genera *Streptococcus*, *Rothia*, *Corynebacterium*, *Staphylococcus* and *Pseudomonas* were detected in all mouse lungs, diabetic and non-diabetic. Bacteria of genus *Floricoccus* were only detected in db/db and wildtype mice housed in the UK and not in the db/db or GK mice housed in Sweden. While the majority of the microbiome in db/db and wildtype mice housed in the UK was made up of genera *Streptococcus*, this was not the case in db/db mice housed in Sweden and GK mice housed in Sweden. The lung microbiome in db/db mice housed in Sweden and GK mice housed in Sweden was primarily made up of genera *Corynebacterium* and *Staphylococcus*. While performing statistical analysis on this data is complicated, a trend could be seen in genus *Pseudomonas* where the amount of the bacteria was consistently higher in all the diabetic mice than their respective non-diabetic counterparts across all diabetic mice models (Figure 16B). This data demonstrates that the lung microbiome of mice is highly heterogeneous and is affected by both the environment and the genetic background of the mice.



2.4.8 Bacterial abundance at phyla level compared between different diabetic mouse models

As was discussed in 2.4.7 lung microbiome of mice is very unstable, with different genera contributing differently to the microbiome. In order to evaluate the stability of the microbiome, I have analysed the phyla distribution in the lung microbiome in the different diabetic models. When assessing the lung microbiome composition on a phylum level, it appears to be more homogenous between different models of diabetic mice at different conditions (Figure 17). In both db/db and wildtype mice housed in UK and Sweden, as well as GK^{+/-} and wildtype mice housed in Sweden, the lung microbiome is primarily made up of phyla *Actinobacteriota*, *Firmicutes* and *Proteobacteria*. The top ten genera were found in almost all BAL samples, with a few notable exceptions. Bacteria of phylum *Fusobacteria* were not observed in GK^{+/-} mice housed in Sweden and db/db mice housed in Sweden. Bacteria of phylum *Planctomycetota* were only found in the lungs of db/db and wildtype mice housed in the UK and wildtype mice housed in Sweden. Bacteria from phylum *Cyanobacteria* were not observed in the lungs of GK^{+/-} mice housed in Sweden. This demonstrates a stable core microbiome observed in all diabetic mice and their wildtype littermates irrespective of housing environment and genetic makeup when analysing the microbiome at a higher taxonomic level.



2.5 Discussion

2.5.1 Stability of the lung microbiome across various mice models housed in different environments

In this study, I observed that predominant phyla were similar between db/db mice housed in UK and Sweden, GK mice housed in Sweden and their respective wildtype controls. I previously discussed that the lung microbiome is highly affected by the environment, animal origin, and diet. Several studies have demonstrated these patterns in mouse lungs. Dickson and colleagues in 2018 demonstrated that the similarity of lung microbiome across mice was greatest when comparing mice sharing a single cage and was increasingly distinct when compared across cages, shipments, and vendors. They used *Streptococcaceae* family as an example to show changes in the abundance in these different environments. The differences were so significant that in some mice, *Streptococcaceae* family made up a significant portion of the microbiome, while in others, it was barely detectable (9.5% of all sequences vs 0.2% of all sequences). Moreover, the diversity of the lung microbiome was also different between cages, shipments, and vendors (Dickson *et al.*, 2018). In this study I have observed similar variation in the abundance of *Streptococcus* in different mice groups. Genus *Streptococcus* was the most predominant genus in the lungs of db/db mice housed in UK, while in the lungs of db/db and GK mice housed in Sweden, this genus contributed to a much smaller proportion of the microbiome. In mice housed in Sweden majority of the lung microbiome was made up of genera *Corynebacterium* and *Staphylococcus* instead.

Dickson and colleagues also analysed the plasticity of the lung microbiome by obtaining healthy, genetically identical mice from two different vendors and co-housing them. Lung microbiome samples from these animals were acquired on days 0, 1 and 7 to demonstrate the changes in the lung microbiome over time. While initially lung microbiome in these mice was completely different and clustered by vendor, by day 7, they were completely indistinguishable

(Dickson *et al.*, 2018). This demonstrates how easily the environment can affect the lung microbiome and reinstates the importance of letting mice settle down after acquisition to allow the lung microbiome to stabilise. I made similar observations in this study, where bacteria of genus *Floricoccus* were only detected in mice housed in the UK and not in Sweden and bacteria of genus *Veilonella* were mostly present in the mice housed in UK and not in Sweden despite being acquired from the same breeder.

In my experiments, we allowed newly purchased mice to acclimatise for three weeks before performing BALs. Other groups have tracked microbiome development from an early age of mice into adulthood. It was demonstrated that a continuous significant increase in microbiome diversity and richness is observed with age (Singh *et al.*, 2017; Kostic *et al.*, 2018). An important observation made by several groups that in the lung microbiome, dynamic changes were often observed at the genus level; however, relative stability was observed at phyla level with dominant phyla often being *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Yun *et al.*, 2014; Segal *et al.*, 2016; Singh *et al.*, 2017).

In support of this, while the lung microbiome remained stable across different mouse models at phylum-level, a high variance was observed at genus level, with only a few genera found in all mice. High variation of the lung microbiome at genus level between genetically similar mice housed in the different environments makes it harder to understand the physiological effect of diseases on the lung microbiome. However, when similar patterns at the genus level are observed between different models of diabetic and non-diabetic mice housed in a different environment, it implies a strong confidence that the changes observed are true and warrant a more thorough investigation. For example, abundance of genus *Pseudomonas* was consistently higher in the lungs of diabetic mice than non-diabetic across different mouse models and housing locations.

2.5.2 Effects of diabetes on the lung microbiome

From the data above, the observation is that diabetes changes the overall microbiome of the lung in mice. However, only the abundance of several bacterial species was significantly affected. At the genus level, the abundance of 3 pathogens was shown to be different between diabetic and non-diabetic mice: *Corynebacterium*, *Pseudomonas* and *Staphylococcus*. Reduced abundance of two pathogens correlated with increased blood glucose concentration: *Corynebacterium* and *Staphylococcus*. Abundance of *Pseudomonas* was increased in the lungs of diabetic mice but was not directly correlated with blood glucose concentration. This could mean that other factors, potentially related to diabetes, could influence abundance of this pathogen. For example, the decrease in the abundance of *Staphylococcus* and *Corynebacterium* could promote the increase in the abundance of *Pseudomonas*. Alternatively, these results could be due to a small sample size.

Most studies looking at the effects of diabetes on the mouse microbiome focus on the gut and not the lung. With few comparable studies, this research offers novel insight into the potential effects of diabetes on the mouse lung microbiome.

While 16S sequencing doesn't offer species-level specification, genus-level information could be used to estimate the species observed in the microbiome. In this study I have detected high abundance of genera *Pseudomonas* and *Staphylococcus* in the lungs. Common lung pathogens of this genera include *Staphylococcus aureus* and *Pseudomonas aeruginosa*. I will, therefore, suggest that potentially these pathogens are observed in the mouse lung microbiome.

To date, no studies have analysed the lung microbiome of people with and without diabetes in the absence of any other lung conditions. Studies investigating lung microbiome of diabetic mice are also sparse. There are, however, studies in humans which could help me understand the importance of the changes in the lung microbiome that I recorded.

A subset of individuals in which *Staphylococcus aureus* and *Pseudomonas aeruginosa* are prevalent pathogens are individuals with CF. In a 2017 study by Lehoux Dubois *et al.*, while non-significant, a trend was observed where the prevalence of *P. aeruginosa* was increased in the lungs of individuals with CFRD when compared to non-diabetic CF individuals, while the prevalence of *S. aureus* was decreased (Lehoux Dubois *et al.*, 2017), which is similar to my observations in mice. CFRD is a major comorbidity of individuals with CF, associated with more rapidly progressing lung disease, lung function decline and early mortality (Moran *et al.*, 2009; Lewis *et al.*, 2015). Individuals with CFRD also appear to be at greater risk of infection with detrimental CF pathogens, such as *Burkholderia cepacia complex* and *Stenotrophomonas maltophilia*, than CF individuals without CFRD (Olesen *et al.*, 2020). These data correlate to the observation I made in this study, where a higher abundance of *Pseudomonas* and a lower abundance of *Staphylococcus* was seen in the lungs of diabetic mice. *In vitro* Garnett and colleagues demonstrated that increased glucose concentration promoted growth of *P. aeruginosa* on primary CF and non-CF human bronchial epithelial cell monolayers (Garnett *et al.*, 2013). This study demonstrates that increased glucose concentration could be explanatory to the increased abundance of *P. aeruginosa* observed in the lungs of CFRD patients.

Baker and colleagues have previously demonstrated that in intubated patients glucose detected in the bronchial aspirates was associated with acquisition of *Staphylococcus aureus* infection (Philips *et al.*, 2005). Increase in glucose concentration leading to increase in the growth of *S. aureus* was also demonstrated *in vitro* (Garnett *et al.*, 2013). This was further demonstrated by Garnett and colleagues in diabetic db/db mice, where diabetic mice infected with *S. aureus* exhibited higher bacterial load than non-diabetic control animals. Further treatment of these mice with metformin reduced abundance of *S. aureus* in the airways (Garnett *et al.*, 2013). In this study, however, I have observed a reduced abundance of *Staphylococcus* in the lungs of diabetic mice. It is possible that increase in the abundance of *Pseudomonas* caused it to outcompete *Staphylococcus*, leading to the decrease in its'

abundance. Similar patterns are often seen in CF individuals, where *S. aureus* is a predominant lung infection in children and young adults and *P. aeruginosa* becomes dominant pathogen with age, causing abundance of *S. aureus* to decrease (Cystic Fibrosis Foundation, 2021). The importance of glucose in modification of the relationship between these pathogens is not well studied.

Corynebacterium is a known member of the normal respiratory microbiome (Kanmani *et al.*, 2017). *Corynebacterium* species are considered beneficial due to their potential to hinder the spread and establishment of opportunistic pathogens. The abundance of *Corynebacterium* species was shown to correlate with a reduced abundance of *Streptococcus pneumoniae* in humans (Bomar *et al.*, 2016; Kelly *et al.*, 2017; Xu *et al.*, 2021). *Corynebacterium accolens* specifically were shown to release anti-pneumococcal free fatty acids *in vitro* (Bomar *et al.*, 2016). The abundance of *Corynebacterium* was shown to be associated with a more stable airway microbiome in adults and children (Bosch *et al.*, 2017; de Steenhuijsen Piter *et al.*, 2019; Kelly *et al.*, 2022). This suggests that *Corynebacterium* species may play an important role in protecting lungs from pathogenic species. These observations were also confirmed in the mouse lung model, where the colonisation of lungs with *Corynebacterium* reduced *Streptococcus pneumoniae* establishment, as well as reduced levels of pro-inflammatory mediators in the lungs (Horn *et al.*, 2022). *Corynebacterium pseudodiphtheriticum* species were shown to activate respiratory innate immunity. Nasal priming with viable bacteria triggers toll-like receptor 3 activation and activation of CD3⁺CD4⁺IFN- γ ⁺ and CD3⁺CD4⁺IL-10⁺ T cells in mice (Kanmani *et al.*, 2017) which offers protection against pathogens such as *Streptococcus pneumoniae*. Non-viable bacteria did not offer the same protection, suggesting live bacteria are required. With this and the data presented above, it is possible that *Corynebacterium* offers protection against the establishment of *Pseudomonas* in the lung of mice. I observed an increase in the abundance of *Pseudomonas* and a decrease in the abundance of *Corynebacterium* in the lungs of diabetic mice. This could potentially suggest

that hyperglycaemia driven reduction in the abundance of *Corynebacterium* promotes growth of *Pseudomonas* in the lungs of diabetic mice.

The evidence presented in this chapter demonstrates a shift in the lung microbiome of diabetic mice. It is possible that the shift in the lung microbiome promotes the growth of pathogenic bacteria. Where glucose itself provides a source of nutrition for pathogenic bacteria, the loss of commensal bacteria, which offer protection, could also allow pathogens to proliferate. In Chapter 1, I have discussed how studies demonstrate that diabetic individuals are at an increased risk of developing infections. In mice studies, it was demonstrated that diabetes impedes the lung bacterial clearance and causes an exaggerated but less effective inflammatory response to bacteria inoculation (Hunt *et al.*, 2014) in mice. I suggest that microbiome shift could contribute to the susceptibility of individuals with diabetes to lung infections.

2.5.3 Comparison of lung microbiome between mice and humans

The lung microbiome varies greatly between species and individuals, but the majority of bacteria compromising the lung microbiome in both humans and animals belong to four phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* (Yun *et al.*, 2014; Segal *et al.*, 2016; Dickson *et al.*, 2018). While the four main bacterial phyla found in lungs are identical in humans and mice, some differences are found between the two. *Bacteroidetes* and *Firmicutes* predominate in humans, whereas *Proteobacteria* and *Firmicutes* predominate in mice (Mathieu *et al.*, 2018). It is difficult to predict how well mice lung microbiome research would translate to human research. To date, there are no studies investigating the similarities between the human lung microbiome and the corresponding mouse model lung microbiome. However, evidence shows that lung function defects in mice follow similar patterns to human pathophysiology (Devos *et al.*, 2017). The majority of the research comparing human and mouse microbiomes focuses on the gut and shows great variation due to different anatomy, living conditions and disease models used. However, even with these differences using mouse

models in gut microbiome studies has brought more insights into the pathological mechanisms of several diseases (Nguyen *et al.*, 2015). For example, when the impact of the western diet on the gut microbiome was investigated, similar changes were observed in human and mouse gut microbiomes (Nguyen *et al.*, 2015).

In human lungs, several pathogens such as *S. aureus* and *P. aeruginosa* could lead to severe lung health deterioration. Mouse microbiome studies could be combined with existing human data to aid our understanding of the pathogenicity of these bacteria. The changes in the abundance of lung pathogens in mice observed in this study correlated with the changes seen in humans with respiratory diseases, such as CF and COPD, as discussed above in section 2.5.2.

2.6 Summary

In this chapter, I have demonstrated that the mouse lung microbiome is subject to high variation at the genus level under different environmental pressure; however, it maintains a strong core comprised of four main phyla: *Actinobacteriota*, *Bacteroidota*, *Firmicutes* and *Proteobacteria*. I have also demonstrated that diabetes inherently does not affect the amount of bacteria found in the mouse lungs and does not affect the α -diversity of the lung microbiome. However, I have demonstrated that diabetes does change the composition of the lung microbiome in diabetic mice when compared to non-diabetic littermates. When analysing microbiome data at the genus level, I have observed a decrease in the amount of *Staphylococcus* and *Corynebacterium*, and an increase in the amount of *Pseudomonas* observed in the lungs of diabetic mice. I have also demonstrated that the decrease in the amount of *Staphylococcus* and *Corynebacterium* found in lungs was associated with an increase in glucose concentration, while an increase in the abundance of *Pseudomonas* was not correlated with blood glucose concentration.

2.7 Limitations and future work

Next-generation sequencing offers a unique opportunity to analyse the lung microbiome. This technology, however, comes with its drawbacks. Currently, the limitations of 16S sequencing mean that we can only identify bacteria down to genus level and not species level. While I can make an assumption that bacteria of the genus *Staphylococcus* and *Pseudomonas* identified in this study correspond to *Staphylococcus aureus* and *Pseudomonas aeruginosa* based on previous research and literature, I cannot claim it with certainty. In order to identify bacteria down to species level in the future, I would need to perform a species-specific qPCR or a whole-genome sequencing, such as Nanopore.

In order to further understand the effects of diabetes on the lung microbiome, we could introduce glycaemic control drugs for diabetic mice. We would observe whether the lung microbiome would revert to that of non-diabetic mice upon reaching normoglycaemic blood glucose concentration. We would also observe whether the abundance of bacteria of the genus *Staphylococcus* and *Corynebacteria* would increase in the lungs of diabetic mice with better glycaemic control and whether that would lead to the decrease in the abundance of bacteria of the genus *Pseudomonas*. These results would become a first step for designing a larger human study, in which we could investigate whether the effects of glycaemic control on the lung microbiome could be reproduced in human individuals. We could also explore effects of lifestyle on the bacterial growth, quorum sensing, and biofilm formation.

Chapter 3

Investigating diabetes-induced changes in the lung microbiome of individuals with cystic fibrosis

3.1 Introduction

3.1.1 Revisiting cystic fibrosis and cystic fibrosis-related diabetes

As previously discussed in Chapter 1 section 1.11, CF is a disease of genetic origin with a high worldwide prevalence and is one of the best-studied lung diseases. Disease progression in individuals with CF is characterised by increasing colonisation with pathogenic bacteria and decreasing resistance to lung infections due to a decrease in pulmonary health. Microbiome changes in the CF lung have been extensively studied, and some studies have demonstrated that changes in lung microbiome profile are associated with a decrease in lung function in individuals with CF (Layeghifard *et al.*, 2019; Cuthbertson *et al.*, 2020). It was also previously discussed that a common CF comorbidity is Cystic Fibrosis Related Diabetes (CFRD), a unique type of diabetes stemming from CF-related pancreatic damage associated with pulmonary decline.

3.1.2 Designing the study

Cystic fibrosis affects around 100,000 people worldwide, of which 10,600 live in the UK, according to Cystic Fibrosis Trust (Cystic Fibrosis Trust, 2022). Of these individuals, approximately 7,000 are over the age of 16 (Keogh *et al.*, 2020). These are the individuals that are most often recruited from a hospital for potential studies. For this research, I was particularly interested in individuals with CFRD. Of all adult individuals with CF, approximately 40-50% develop CFRD, with this number increasing with age (lafusco *et al.*, 2021). This creates a pool of 2,800 to 3,500 individuals I could potentially recruit in England.

According to Cystic Fibrosis Trust, there are 21 Cystic Fibrosis clinics in England (*Adult specialist CF centres in the UK*, no date). Studies working in the CF field based in England

would normally recruit individuals directly from these clinics. However, this process requires a direct clinical contact at the CF clinic who would be responsible for participant recruitment, sample acquisition and transportation. Studies that do not have direct access to the CF clinics often rely on NHS tissue banks that would either hold samples in frozen form or recruit individuals on behalf of the researcher. Use of these facilities usually incurs a fee per sample.

As was discussed in Chapter 2, the lung microbiome is influenced by the environment in which individuals live. In the lung, where the bacterial load is low, small changes in bacterial abundance can significantly affect the profile of the microbiome and the variation between individuals. This makes it challenging to understand the relationships between the lung microbiome and the general health of the human lung. Frequently, studies exploring the CF lung microbiome have used hundreds of participants and samples in order to power the studies appropriately to accommodate such variation and derive statistically significant conclusions (Paganin *et al.*, 2015; Zemanick *et al.*, 2017; Acosta *et al.*, 2018; Carmody *et al.*, 2018; Cuthbertson *et al.*, 2020). The importance of small-scale studies, however, should not be under-appreciated. These studies often show the potential for further investigation and/or highlight specific microbiome changes to pursue. Large scale clinical studies are not always possible due to the complexity behind the recruitment of individuals with CF. Data generated in small studies then underpin and justify the cost of a large scale study (Rogers *et al.*, 2003; Hahn *et al.*, 2016, 2020; Muhlebach, Zorn, *et al.*, 2018; Ahmed *et al.*, 2019).

Which samples to include in a CF lung microbiome study requires careful consideration of inclusion and exclusion criteria. Ideally, individuals age should be matched between experimental and control groups as the lung microbiome and prevalence of lung pathogens change with age (Mac Aogáin *et al.*, 2019). All individuals with CF, due to their condition, would be on antibiotic therapy. Antibiotics are known to affect the composition of the microbiome (Elvers *et al.*, 2020; Ramirez *et al.*, 2020) so, ideally, individuals should be taking the same antibiotics in all groups. Lung function is one of the major drivers of lung microbiome composition in CF individuals (Cuthbertson *et al.*, 2020) and ideally individuals need to be

matched by their lung functions. Evidence exists to show that lung microbiome in CF individuals is affected by geographical location (Cuthbertson *et al.*, 2020). Effects of geographical location on the lung microbiome was also shown in mice, as described in Chapter 2.

3.1.3 Clinical markers of lung health in CF individuals

3.1.3.1 Spirometry tests

In order to relate microbiome changes to the lung health of individuals with CF, several lung function measurements are often utilised.

Spirometry is the most used test to measure lung function in individuals with CF. Forced expiratory volume in one second (FEV₁) measures the volume of air expelled at the end of the first second of a forced expiration. The use of FEV₁ is to assess the health of large airways, and it is considered the most valuable spirometric measure. FEV₁ is used to indicate the degree of lung obstruction (eg. caused by inflammation, mucous plugging) preventing forced exhalations. FEV₁ scores are often expressed as a percentage of the predicted value, which is calculated for each individual (David and Edwards, 2020). FEV₁ measurements are often used in conjunction with forced vital capacity (FVC) measurements. FVC is the maximum amount of air an individual can forcibly exhale after a full inhalation. The ratio of FEV₁ to FVC (FEV₁/FVC, also known as FEV₁%) can help distinguish obstructive and restrictive lung diseases. In obstructive diseases FEV₁ would be reduced, while FVC would remain unaffected, reducing FEV₁%. In restrictive diseases however, FVC would be reduced while maintaining normal FEV₁, resulting in a higher FEV₁%.

The importance of FVC and FEV₁ measurements was underlined by the national CF registry, which for many years required FVC and FEV₁ measurements to be provided for each individual. Meanwhile, the Cystic Fibrosis Clinical Practice Guidelines recommend that decline in pulmonary function is measured using FEV₁ (Yankaskas *et al.*, 2004; König *et al.*, 2018).

FEV₁ measurements for individuals with CF dates back to 1992, when a pivotal study aimed to predict the risk of death due to respiratory failure using pulmonary function measurements. The study examined 673 records of individuals from 1977 to 1989. This study demonstrated the value of using FEV₁ measurements to reliably predict a decline in pulmonary function. A decrease in FEV₁ was shown to be the most significant predictor of mortality in individuals with CF and suggested lung transplantation for individuals with values below 30% of the predicted value (Kerem *et al.*, 1992; Breuer *et al.*, 2018).

Since the original study in 1992, numerous studies have evaluated the risk factors predicting and/or contributing to the decline of FEV₁ values in individuals with CF. In 2016 Harun SN *et al.* published a systematic review attempting to correlate the effects of known risk factors in CF to the FEV₁% decline. This review evaluated the effects of both non-modifiable (sex, genotype, age of diagnosis, meconium ileus, pancreatic insufficiency, baseline FEV₁ values) and modifiable (chronic *P. aeruginosa* infection, infection with other pathogens, nutritional status, CFRD) risk factors on the pulmonary decline in individuals with CF. An initial search of 2307 studies were filtered to 39 studies included in the review. This review demonstrated that infection with *P. aeruginosa* and pancreatic insufficiency was associated with lower baseline FEV₁ and quicker decline of FEV₁ than predicted. While the study notes that more data are warranted to correlate other risk factors with FEV₁ values, it demonstrates that spirometry alone is not always enough to assess pulmonary health (Harun *et al.*, 2016).

Despite the evidence that FEV₁ measurement alone is not sufficient to predict a decline in lung function in individuals with CF, it remains a staple measurement to record when working with CF individuals until new methods of assessing lung function decline are established.

3.1.3.2 HbA1c

In people with CFRD, poor glycaemic control is associated with a decline in pulmonary function and is an important metric to monitor (Milla *et al.*, 2000; Hameed *et al.*, 2010). However, the best screening methods for CFRD in CF individuals are debated. One of the most popular

tests utilised to screen individuals with CF is the annual oral glucose tolerance test (OGTT). An individual is asked to fast for 8-12 hours prior to this test. A blood sample is collected in the hospital to determine baseline blood glucose levels. The individual is then given a liquid containing glucose to drink. Another blood test is performed 2 hours post liquid consumption to measure new blood glucose levels. Depending on the new blood glucose concentration, it is determined if the individual is at risk of developing CFRD, has CFRD, or has healthy glucose control. Individuals whose fasting blood glucose concentration is under 6 mmol/L, and at the two-hour mark is under 7.8 mmol/L are not considered at risk of diabetes. Individuals whose fasting blood glucose concentration is between 6 and 7 mmol/L, and after two hours reaches between 7.9 and 11 mmol/L are considered to have impaired glucose tolerance. These individuals are advised of lifestyle changes and glucose-lowering medications to prevent the development of diabetes. Individuals whose fasting blood glucose concentration is over 7 mmol/L, and at the two-hour mark reaches over 11 mmol/L are considered to have diabetes and will be prescribed glucose-lowering medications (*Glucose Tolerance Test*, 2022). However, glycaemic control in individuals with CF often fluctuates due to insulin resistance and inflammation, which could lead to misdiagnosis if the 2 hour measurement period falls within a period of strong fluctuation (Sterescu *et al.*, 2010; Leclercq *et al.*, 2014).

Unlike OGTT, measuring haemoglobin A1c (HbA1c) shows glycaemic control over a more extended period. HbA1c is glycated haemoglobin, and it originates from haemoglobin reacting with glucose in the blood. Levels of HbA1c are directly proportional to the blood glucose concentration. As the lifespan of haemoglobin on average is around four months, measurements of HbA1c can be used to reflect average blood glucose levels over that duration of time (WHO, 2011; *Guide to HbA1c*, 2022). The HbA1c value does not rely on taking measurements at a specific time or under specific conditions, such as fasting, making it an ideal tool to measure long-term glycaemic control in individuals during a routine health check. While HbA1c is not recommended for the screening of individuals with CF for the development of CFRD (Gillett *et al.*, 2012; Choudhury *et al.*, 2019), it is a good indicator of long term

glycaemic control. In this project, samples from individuals with CF diagnosed with CFRD will be examined for the effect of hyperglycaemia on the lung microbiome. Measuring HbA1c is preferable to OGTT in this study, as individuals will not be required to fast prior to attending a hospital appointment and will not be required to stay in the hospital for a prolonged period of time to acquire results. As spontaneously expectorated sputum will be acquired from individuals with CF during hospital visits, measuring HbA1c will account for glycaemic control fluctuations.

3.1.4 Using sputum to analyse lung microbiome

In CF, the gut and lung microbiome are of the most interest to researchers – offering a new perspective on the health of the individual and the pathophysiology of the disease (Héry-Arnaud *et al.*, 2019). Stool samples are most commonly used to assess the microbiome in gut microbiome studies. Stool samples are easy to collect, non-invasive, and have a high bacterial load with 10^{11} colony forming units per gram of stool (Françoise and Héry-Arnaud, 2020). Studying the lung microbiome, however, relies on sampling the respiratory tract, which is harder to access and has a lower bacterial load (Françoise and Héry-Arnaud, 2020). Historically bronchoalveolar lavage (BAL) and protected specimen brushings (PSB) samples have been used to sample the lung microbiome. BAL is a procedure in which a bronchoscope is used to deliver a small amount of sterile saline solution into the airways, “washing” the surface of the airways and is afterwards collected for future analysis. PSB is a procedure in which a bronchoscope is inserted into the airways with a protected specimen brush. The brush is then extended from the bronchoscope, swabbing the surface of the airways to acquire a bacterial sample. These techniques, however, are invasive, uncomfortable for the individual and require a trained specialist.

Sputum collected from individuals with CF offers a non-invasive alternative to studying the lung microbiome, as it is often spontaneously expectorated in these individuals and does not require additional procedures. In people who cannot passively produce sputum, production of

sputum can instead be induced, which requires trained personnel, but is far less invasive than BAL and PSB. Induced sputum has been shown to be a viable alternative to invasive BAL (Ronchetti *et al.*, 2018) and was shown not to be contaminated by the oral microbiome (Rogers *et al.*, 2006).

3.2 Aims and hypothesis

Hypothesis: Cystic Fibrosis-related diabetes has a significant effect on the lung microbiome of people with cystic fibrosis, lowering its diversity and increasing the abundance of pathogenic species

Aim 1: To investigate the difference in the bacterial species abundance and diversity in sputum samples from people with CF and CFRD.

Aim 2: To correlate HbA1c with lung function and microbiome diversity in the lungs

Aim 3: To evaluate whether changes in the composition of the lung microbiome can be further investigated as an indicator of chronic lung disease progression in humans.

Aim 4: To evaluate the relationship between *Pseudomonas* and *Staphylococcus* in individuals with CF and CFRD.

3.3 Materials and methods

3.3.1 Study design and sample acquisition

A total of 30 spontaneously expectorated sputum samples were purchased from Manchester Allergy, Respiratory and Thoracic Surgery (ManARTS) Biobank. 14 sputum samples were acquired from individuals with CF, and 14 were acquired from CF individuals with CFRD. 2 samples were acquired from individuals with pre-diabetes. CFRD individuals were defined as having HbA1c >48 mmol/mol (>7.8mmol/L) as per WHO recommended guidelines (WHO, 2011). Spirometry data was provided for each individual. Consent was taken prior to sample collection by ManARTS biobank staff. Samples were collected voluntarily under a biobank licence (IRAS ID 180280, REC reference 15/NW/0409) in North West Lung Research Centre at the University Hospital of South Manchester NHS Foundation Trust (UHSM). Table containing sample information could be found in Attachment 1.

Spontaneously expectorated sputum was collected into clinic sputum sample pots with a target volume of at least 1 gram. After labelling, samples were immediately transported to the lab. Collected samples were frozen within 120 minutes.

Samples were transported to the St George's University of London on dry ice using a courier service and stored at -80°C until processing.

3.3.2 Targeted amplicon sequencing

3.3.2.1 DNA extraction

All work with raw sputum was performed in a microbiological laminar flow cabinet (BSC0010, Walker Safety Cabinets). Sputum samples were thawed on ice and washed with sterile phosphate-buffered saline to remove saliva. Sputum was liquified in Remel™ Sputasol (Thermofisher). Bacterial DNA from liquified sputum samples was extracted using a QIAamp DNA Microbiome Kit (Qiagen) according to the manufacturer's guidelines as described in

Chapter 2, section 3.4. QIAamp kits utilise a pre-lysis step that digests extracellular DNA, preventing potential bias in the final analysis. Bacteria were lysed using a FastPrep-24™ Classic bead beating grinder and a lysis system (MPbio) at a velocity of 6.5m/s for 45 s with a 5-minute interval to ensure lysis of gram-positive bacteria whose tough cell walls are resistant to chemical lysis. The total DNA was eluted in 50µl of Elution buffer and stored at -80°C until further use.

3.3.2.2 Amplicon library preparation and sequencing

This study utilised primers targeting the V3-V4 region of the 16S gene (341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3'). The 5' ends of the primers were tagged with specific barcodes unique for each sample. The PCR reaction was prepared using Q5® High-Fidelity DNA Polymerase (New England Biolabs). Each sample reaction was assembled to a total volume of 25 µl containing 25ng of template DNA, 5 µl 5X Q5 Reaction Buffer, 0.5 µl 10mM dNTPs, 1.25 µl of 10 µM Forward and Reverse Primers, 0.25 µl of Q5 High-Fidelity DNA Polymerase and 5 µl of 5X Q5 High GC Enhancer. PCR-grade water was used to adjust the volume of each reaction to 25 µl. Touchdown PCR was used for the product amplification. The PCR programme consisted of initial denaturation at 98 °C for 30 seconds; 10 cycles of denaturation at 98 °C for 10 seconds, annealing at 65°C for 30 seconds, with each consecutive step reducing annealing temperature by 1°C, and extension at 72 °C for 45 seconds; this was followed by 25 cycles of denaturation at 98 °C for 10 seconds, annealing at 54°C for 30 seconds, and extension at 72 °C for 45 seconds; completing with a final extension at 72 °C for 10 minutes. The PCR products were purified by AMPure XT beads (Beckman Coulter Genomics) and quantified by Qubit (Invitrogen). Sample quality control and sequencing were performed by LCScience (USA). Amplicon pools were prepared for sequencing, and the size and quantity of the amplicon library were assessed on Agilent 2100 Bioanalyzer (Agilent) and with the Library Quantification Kit for Illumina (Kapa Biosciences), respectively. The libraries were sequenced on the NovaSeq PE250 platform. Raw FASTA files were generated for further analysis.

3.3.3 Data analysis

Bioinformatic analysis was performed using Mothur v1.39.5 as per the MiSeq SOP pipeline (Schloss et al., 2009). Analysis was performed as described in Chapter 2, section 3.5.

3.3.4 Statistical analysis

Statistical analysis was performed as described in Chapter 2, section 3.6. α - and β -diversity were calculated using Mothur integrated AMOVA and HOMOVA analysis. GraphPad Prism version 9 was used to perform linear regression analysis, multiple t-test analysis and calculate Pearson correlation coefficients.

3.4 Results

In the results section, normoglycaemic individuals with CF will be denoted as CF, while individuals with CF with cystic-fibrosis related diabetes will be denoted as CFRD.

In this study sputum samples were used to investigate lung microbiome. While sputum samples may not represent distal lung microbiome as well as BAL and PSB samples, they offer a non-invasive approach to study lung microbiome. Further in this chapter lung microbiome would be used instead of sputum microbiome to denote changes observed in the sputum microbiome of CF individuals.

3.4.1 Blood HbA1c and glucose concentrations in CF and CFRD individuals

Blood HbA1c concentration was higher in individuals with CFRD than in individuals with CF (Figure 18, $p < 0.0001$). Mean blood HbA1c concentration in individuals with CFRD was 58.00 ± 6.05 mmol/mol, while mean blood HbA1c concentration in individuals with CF was 35.36 ± 3.77 mmol/mol.

HbA1c concentration in normoglycaemic and diabetic CF patients

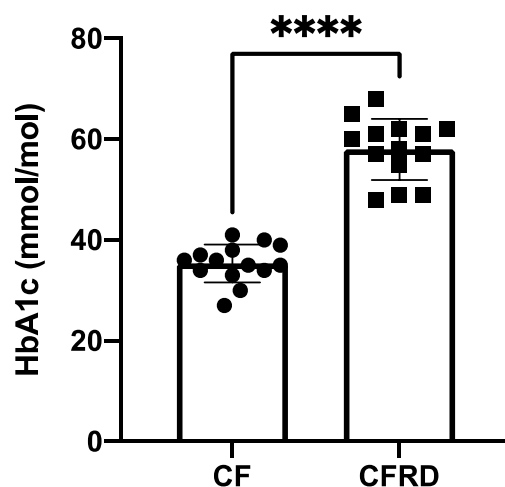
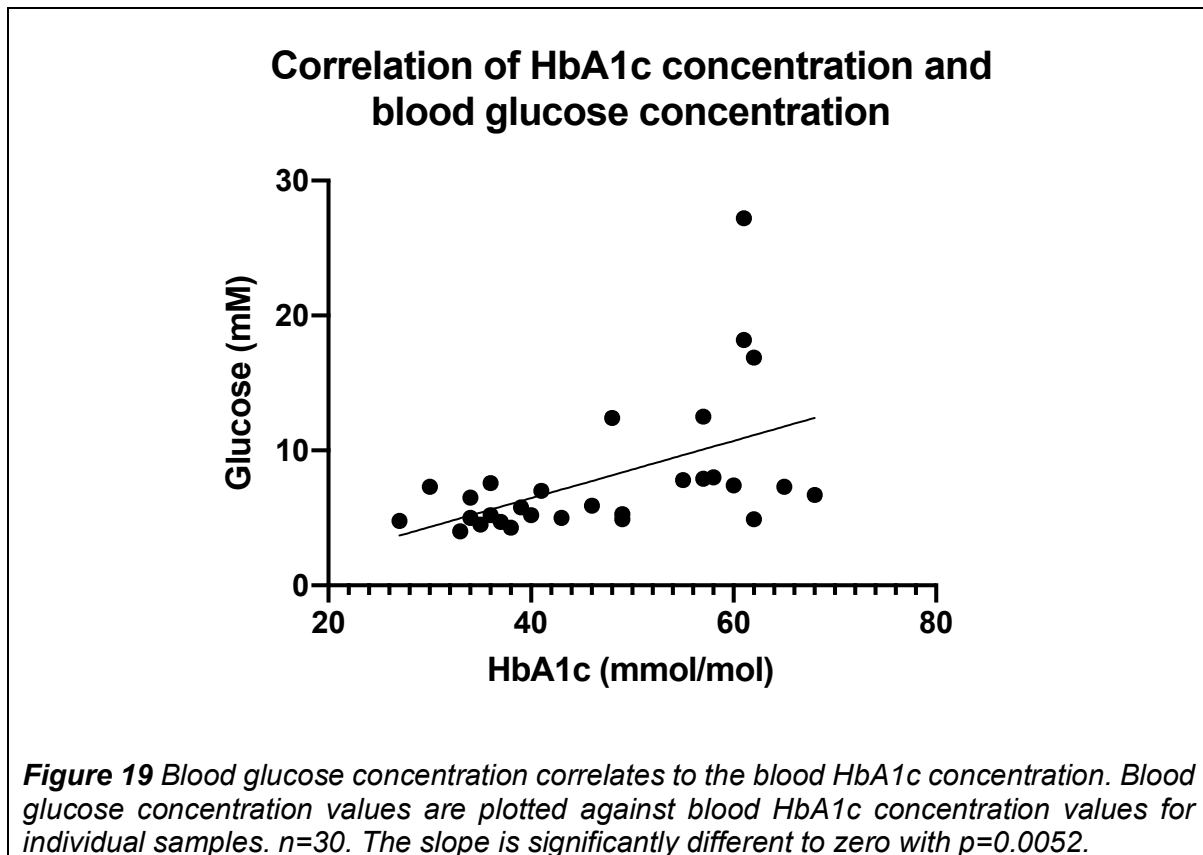


Figure 18 Blood HbA1c concentration was elevated in individuals with CFRD. HbA1c concentration shows as values in mmol/mol $\pm$ SD and plotted for individual CF and CFRD samples. $n=28$, **** $p < 0.0001$ with unpaired t-test

The majority (29) of the individual had given blood to measure random blood glucose concentration. Blood glucose concentration in humans varies throughout the day and depends on food intake. individuals were not asked to fast prior to blood collection for glucose measurement. Using Pearson correlation calculations, there was an increase in random blood glucose which correlated with an increase in HbA1c concentration (Figure 19, $p=0.0052$, $R^2=0.2553$, $n=30$). The spread in blood glucose values, however, was a lot higher than that of HbA1c, which demonstrates why using HbA1c concentration is more reliable to indicate chronic elevation of blood glucose than random blood glucose concentration or could indicate poor long-term glycaemic control in CFRD individuals.



3.4.2 Spirometry measurements in CF and CFRD individuals

Lung function in individuals with CF was measured as FEV_1/FVC . The values were then divided by the predicted value to calculate % of the predicted value. Using a t-test, lung function is significantly lower in individuals with CFRD than those without associated diabetes (Figure 20, $p=0.0018$, $n=28$).

Spirometry measurements in normoglycaemic and hyperglycaemic CF patients

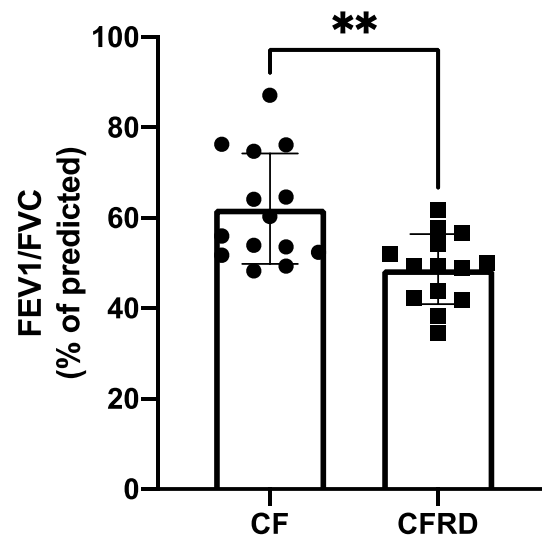
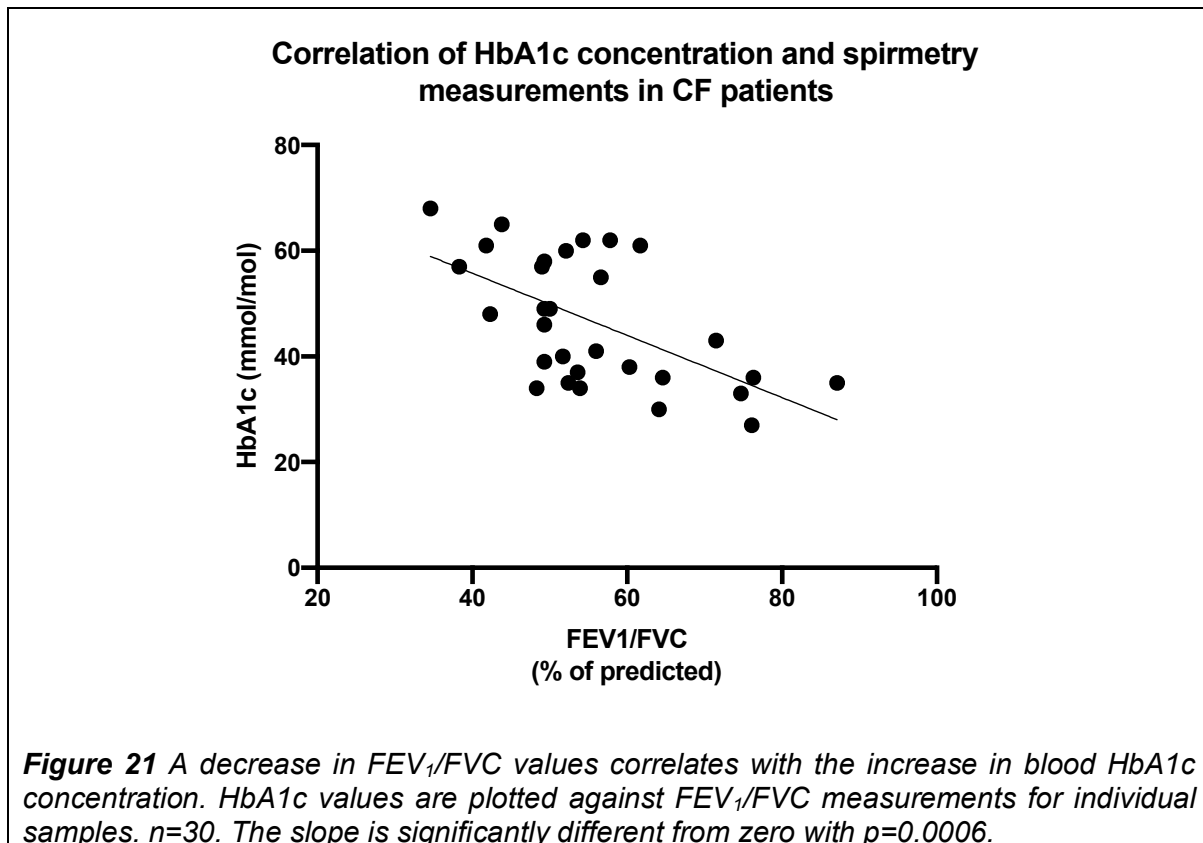


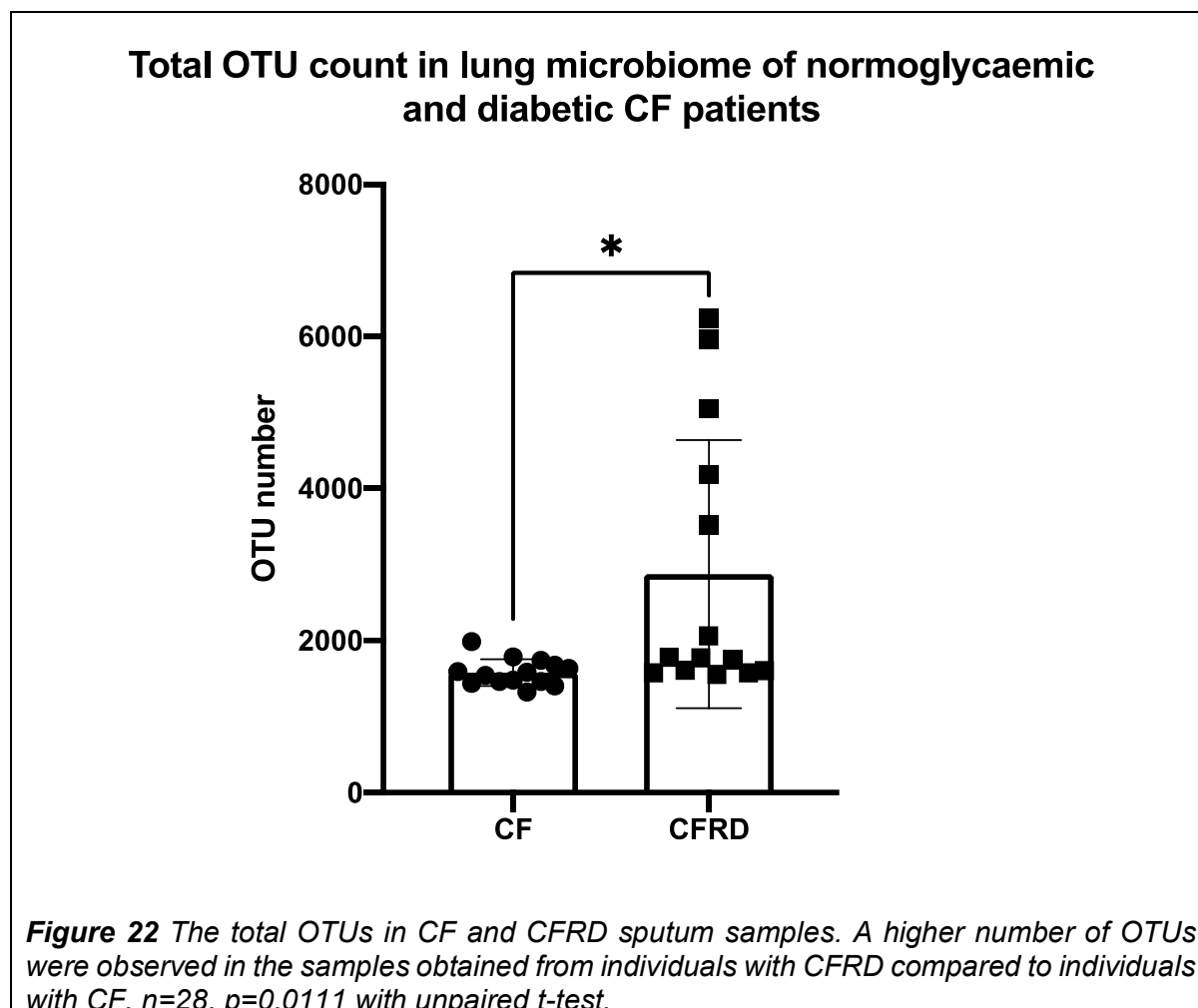
Figure 20 FEV_1/FVC values are lower CFRD compared to CF. FEV_1/FVC values are shown as % of the predicted $\pm$ SD and plotted for individual CF and CFRD samples. $n=28$, ** $p < 0.01$ with unpaired t -test

The decline of lung function correlated with the increase in blood HbA1c concentration (Figure 21, $p=0.0006$, $n=30$). This demonstrates that individuals with CFRD have lower lung function, which correlated with the severity of the sustained hyperglycaemia.



3.4.3 Total OTU count in the sputum samples of CF and CFRD individuals

The total number of OTUs observed in the sputum samples showed a significantly higher number of OTUs in individuals with CFRD compared to CF. (Figure 22, $n=28$, $p=0.0111$). The mean OTUs found in the sputum samples of individuals with CF was 1579 ± 175 , while that of the individuals with CFRD was 2873 ± 1763 . There were five individuals with CFRD who had a significantly higher number of OTUs in their sputum which contributed to the higher mean in this group.



3.4.4 Alpha diversity of the microbiome in the sputum of CF and CFRD individuals

There was a statistically significant difference in the α -diversity of the lung microbiome in individuals with CF and CFRD when measured using Shannon's diversity index (Figure 23, $p=0.0155$, $n=28$). This demonstrates that CFRD increases microbiome diversity within the sputum of individuals with CF.

Alpha diversity in the lung microbiome of normoglycaemic and diabetic CF patients

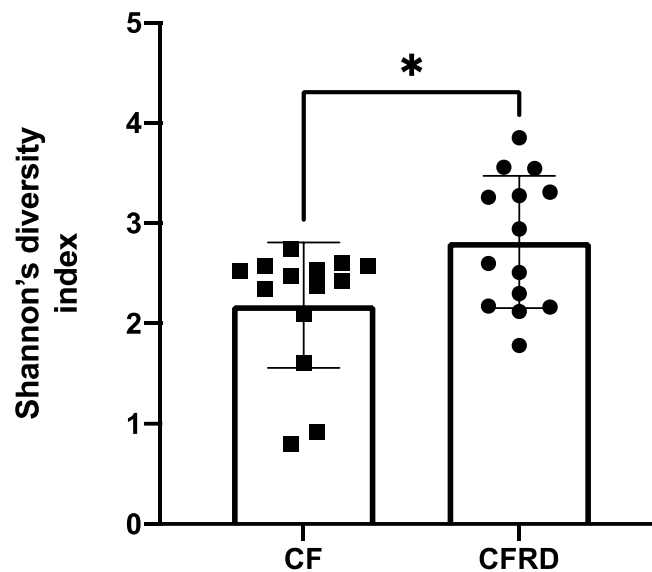
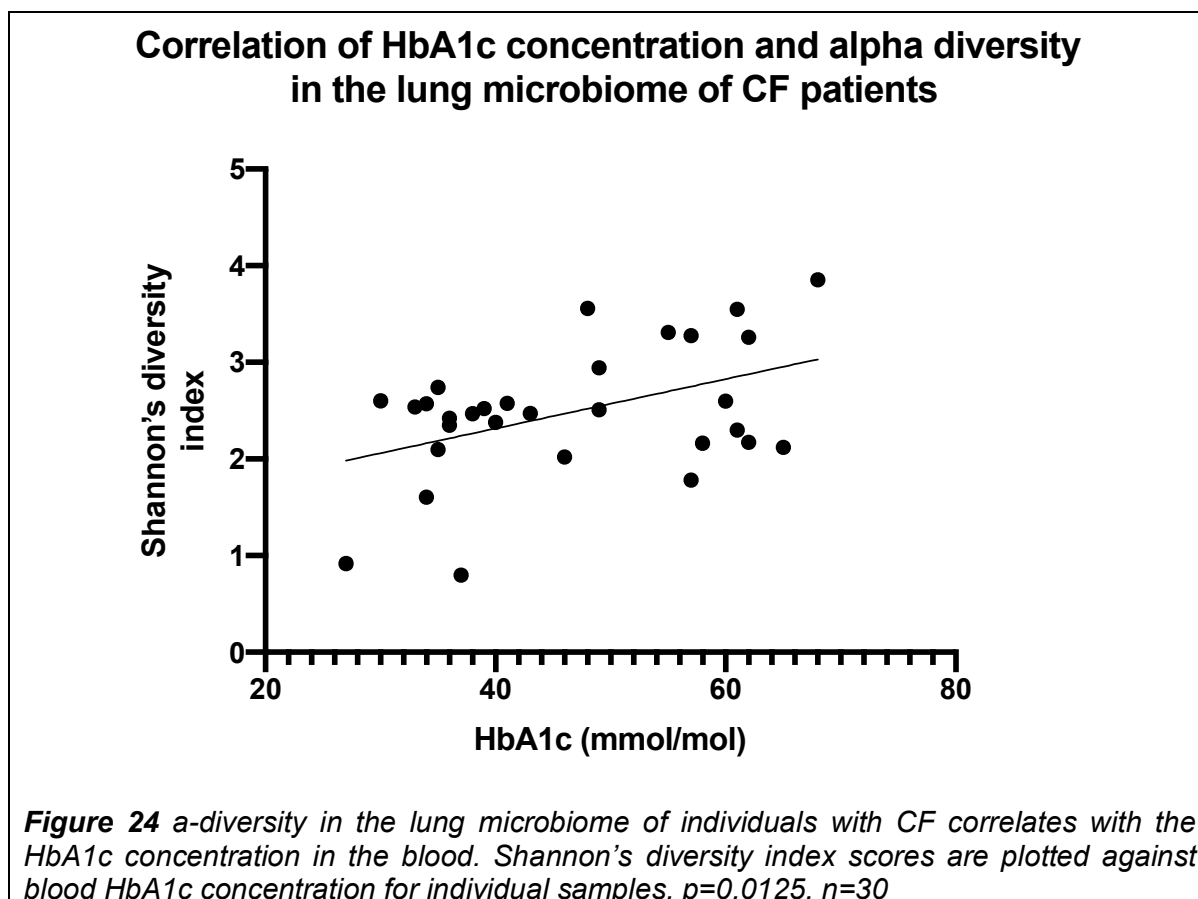
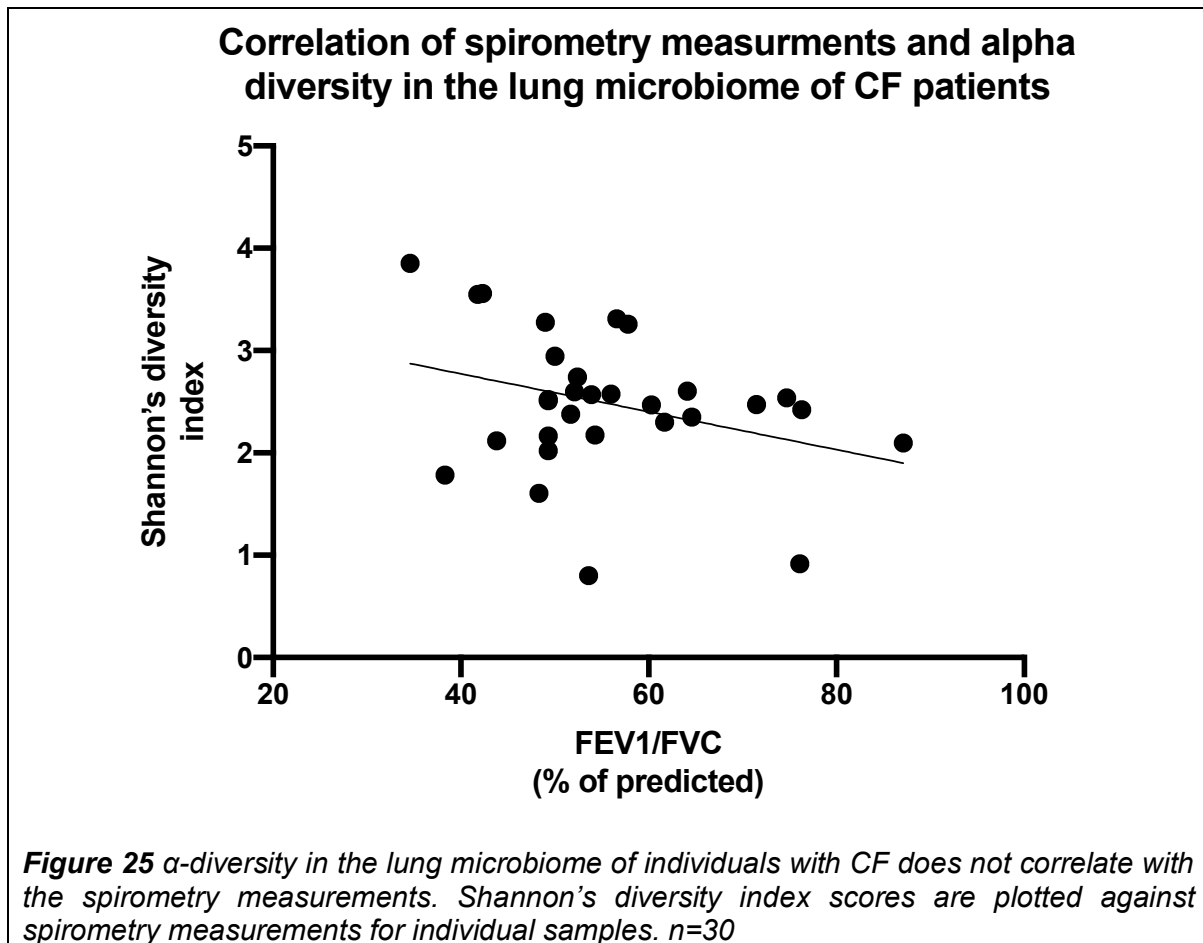


Figure 23 α -diversity in the lung microbiome of individuals with CF, changes with sustained hyperglycaemia. Shannon's diversity index scores are shown as values $\pm$ SD and plotted for individual CF and CFRD samples. $p=0.0155$, $n=28$.

Calculating the Pearson correlation coefficient showed a significant association between blood HbA1c concentration and the α -diversity of the lung microbiome in individuals with CF and CFRD (Figure 24, $p=0.0125$, $R^2=0.2029$, $n=30$). Simple linear regression analysis showed that the slope is significantly different from zero. This demonstrates that microbiome α -diversity within the sputum of CF individuals positively correlates with the changes in the blood HbA1c concentrations.



Pearson's correlation coefficient showed no significant correlation between blood spirometry measurements and the α -diversity of the lung microbiome in individuals with CF and CFRD (Figure 25, $p=0.0782$, $R^2=0.1066$, $n=30$). Simple linear regression analysis showed that the slope is not significantly different from zero. While there was no statistically significant association between lung functions and α -diversity in individuals with CF, a low p -value and a general trend of the data indicates that the results may be biologically significant.

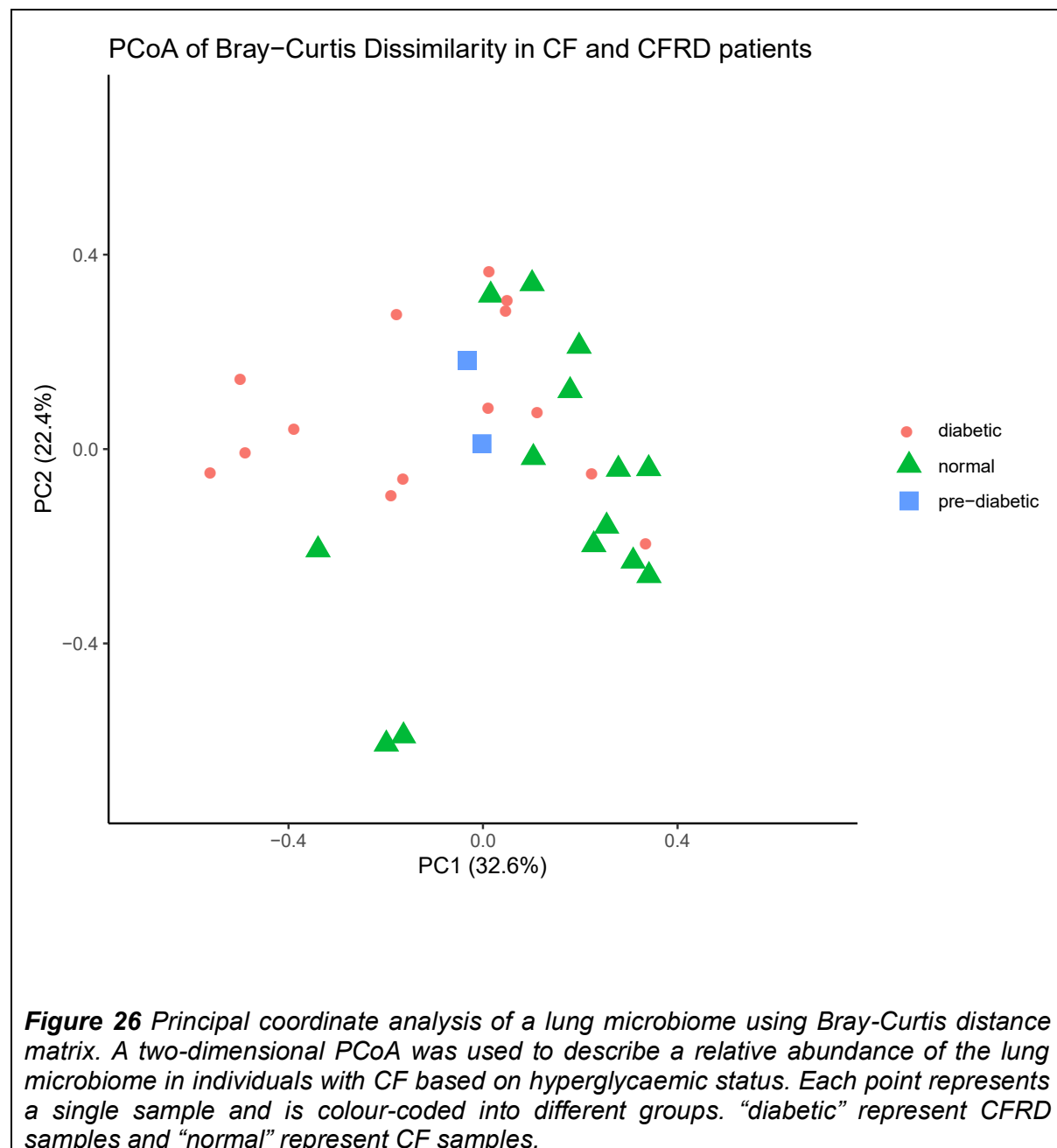


3.4.5 Beta diversity of microbiome in the sputum of CF and CFRD individuals

β -diversity in this study was calculated using Bray-Curtis dissimilarity scores and is presented as the Principal Coordinates Analysis (PCoA) graph for visualisation purposes (Figure 26). In Figure 26 I observe that CF and CFRD samples tend to cluster separately. Interestingly, the two samples acquired from pre-diabetic CF individuals appear to cluster between the samples acquired from CF individuals and those with CFRD. This would suggest a potential shift in the microbiome diversity with the progression of diabetes but more samples from pre-diabetic CF individuals are required to draw any conclusions.

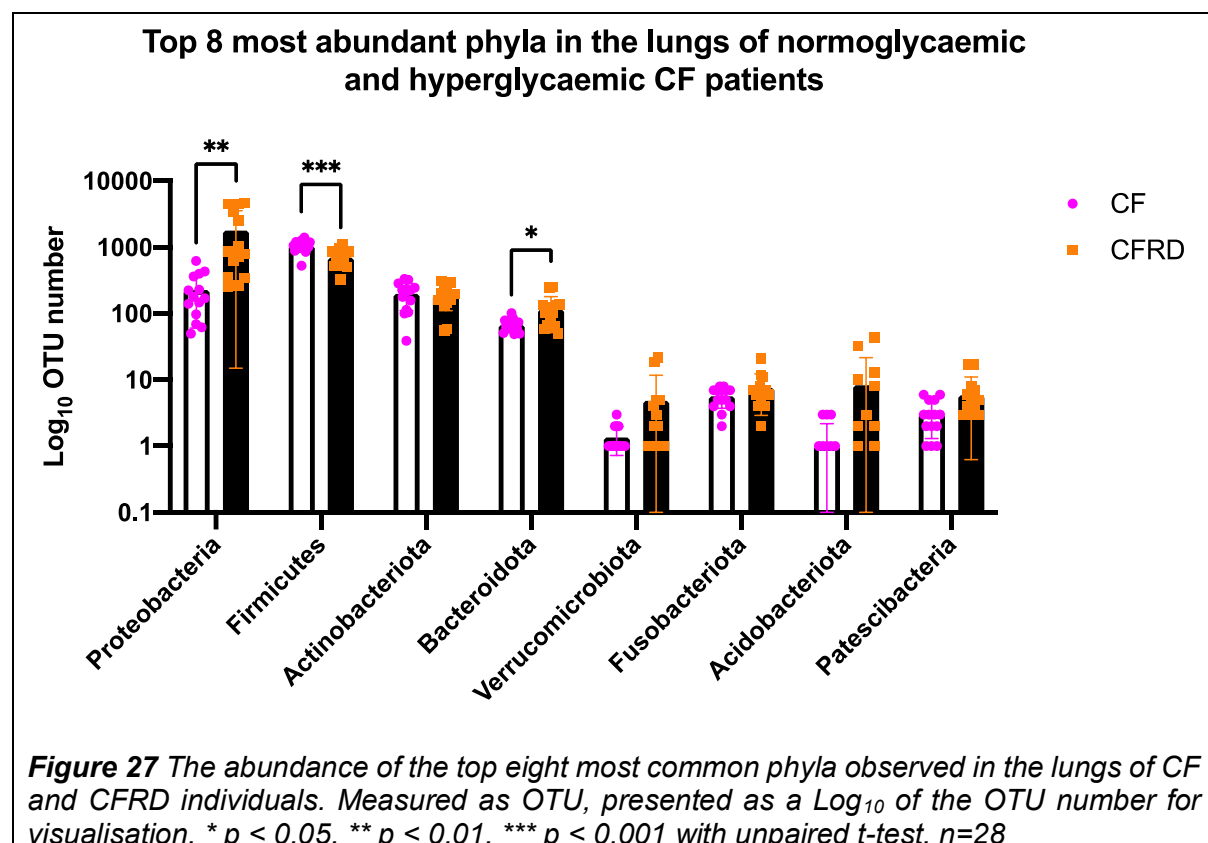
Statistical analysis using AMOVA shows a significant difference between the lung microbiome of individuals with CF and CFRD ($p=0.002$, $n=28$), confirming that the separation visualised in

Figure 26 between CF and CFRD samples is significant. This shows that diabetes was associated with changes in the lung microbiome of individuals with CF, resulting in dissimilar lung microbiomes in the lungs of people with CFRD and non-diabetic CF.



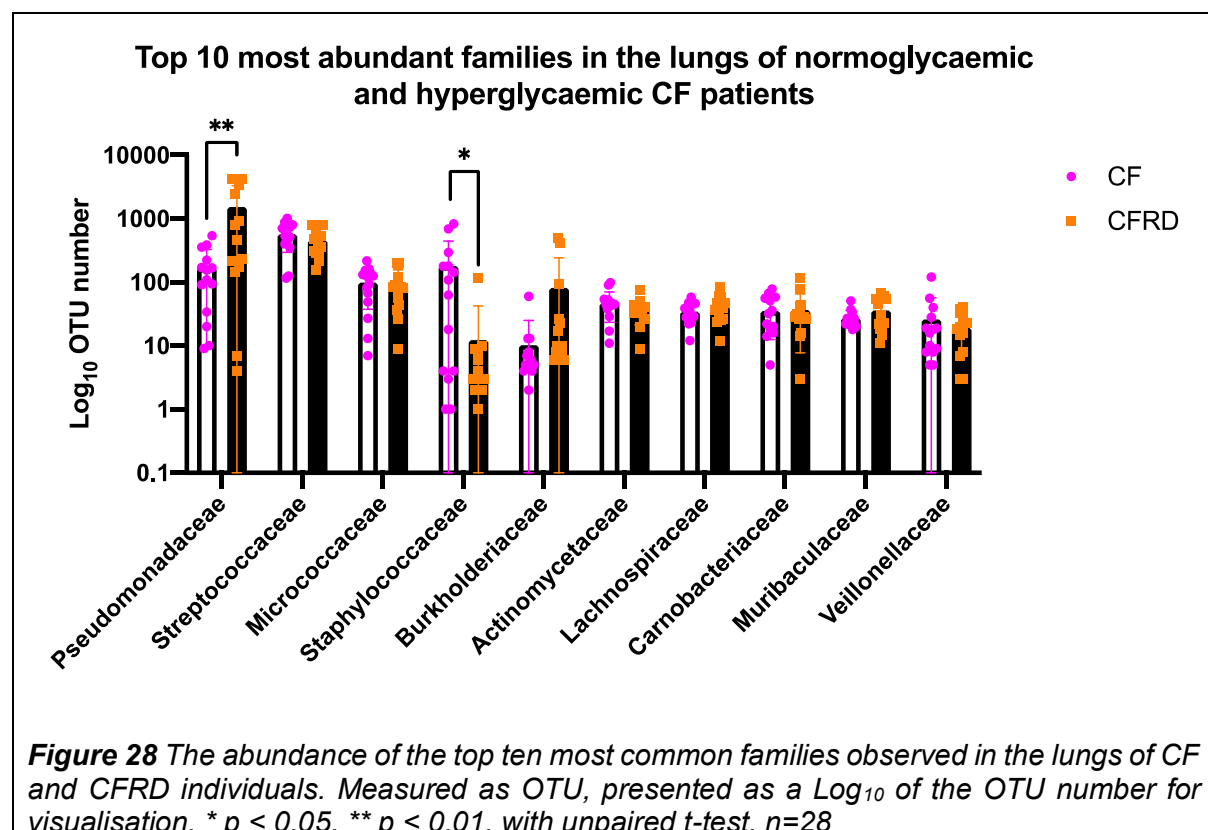
3.4.6 Phylum-level analysis of sputum microbiome in CF and CFRD individuals

At the phylum level, the top 8 most abundant phyla make up 98.39% of all the observed OTUs; of these, *Proteobacteria* make up 44% of the microbiome, while *Firmicutes* make up 40% of all the observed microbiome. The abundance of *Proteobacteria* was higher in the lungs of CFRD than in CF (Figure 27, $p=0.0029$, $n=28$), while the abundance of *Firmicutes* and *Bacteroidota* was higher in CF individuals (Figure 27, $p=0.00012$ and $p=0.011$ respectively, $n=28$). This demonstrates a diabetes-induced change in the abundance of the major phyla of bacteria in the lungs, with both *Proteobacteria* and *Firmicutes* OTU being affected by hyperglycaemia.



3.4.7 Family-level analysis of sputum microbiome in CF and CFRD individuals

A total of 441 families of bacteria were detected in the sputum of individuals with CF. The top ten most abundant families comprise 79% of all detected OTUs. While the number of bacterial families observed is significantly higher than that of phyla, there is not much variation in the abundance of various families between CF and CFRD individuals. Two bacterial families whose abundance in the lungs is predominantly different between CF and CFRD individuals are *Pseudomonadaceae* and *Staphylococcaceae*. I observed a significant increase in the mean abundance of *Pseudomonadaceae* in the lungs of hyperglycaemic individuals (Figure 28, $p=0.0075$, $n=28$), while the abundance of *Staphylococcaceae* was significantly decreased in hyperglycaemic individuals (Figure 28, $p=0.0256$, $n=28$).



3.4.8 Genus-level analysis of sputum microbiome in CF and CFRD individuals

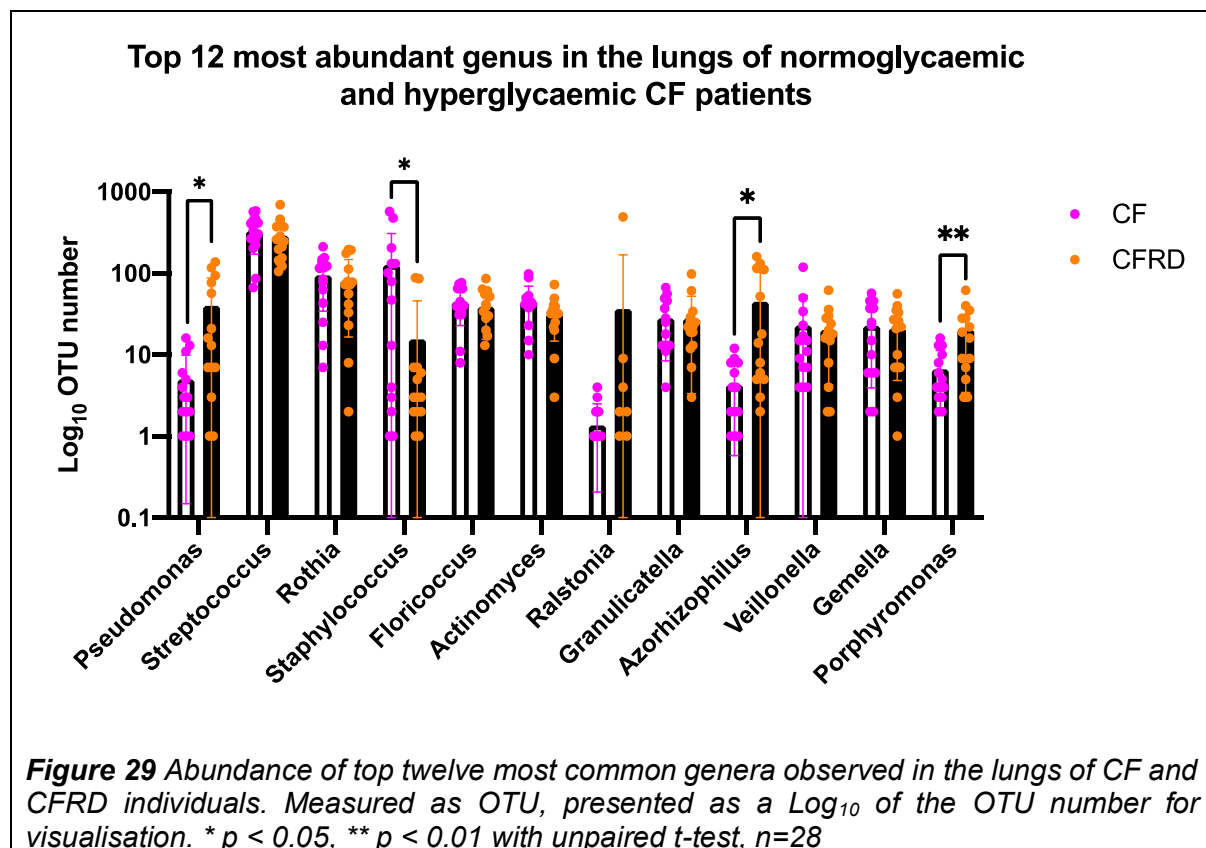
3.4.8.1 Genus-level classification

There were 1011 total unique genera of bacteria detected in the sputum samples from individuals with CF. Poor alignment at the genus level caused many bacteria to be unclassified past the family level. The majority of the *Pseudomonadaceae* family did not generate the required alignment and were classified as “unidentified”, with a total of 22603 OTUs being attributed to genus “unidentified” and only 636 OTUs being attributed to genus *Pseudomonas*. A similar observation was made for the family *Streptococcaceae* where 5397 OTUs were not identified, and 8751 OTUs were identified as belonging to the genus *Streptococcus*. While we can assume the attribution of unidentified genera to identified ones, making such an assumption may affect the distribution of the microbiome. Approximately 56% of the OTUs could not be assigned to a specific genus, with *Pseudomonadaceae_unclassified* making up 61% of the unidentified OTUs. This is likely due to a combination of poor sequencing data and the use of specific primers that were not specific enough for the particular bacteria. While a large proportion of bacteria could not be identified at the genus-level, further analysis would only be using identified OTUs. As the decrease in OTU count was proportional across all samples, the overall changes induced by diabetes were not affected by the drop in OTU number at the genus level.

3.4.8.2 Analysis of twelve most abundant genera in the sputum of CF and CFRD individuals

For the analysis in this section, abundances of the top 12 most abundant classified genera were selected and presented in Figure 29. At the genus level, I have observed a significantly lower abundance of genus *Staphylococcus* in the sputum of CF individuals ($p=0.032$, $n=28$). The abundance of genera *Pseudomonas*, *Azorhizophilus* and *Porphyromonas* were

significantly higher in the lungs of diabetic CF individuals ($p=0.011$, $p=0.014$ and $p=0.0069$ respectively, $n=28$). The top twelve genera comprised 71% of the total identified genera.



3.4.8.3 Genera detected in the sputum of CF and CFRD individuals modified by diabetes

The difference in the abundance of all genera in the lungs of CF and CFRD individuals was analysed using a t-test. The abundance of 27 identified genera was found to be different in the lungs of CF and CFRD individuals.

The most abundant significantly different genus observed in the lung microbiome was *Staphylococcus*, which was found in a higher abundance in the lungs of CF and was less abundant in the sputum from individuals with CFRD (Figure 30B, $p=0.024$, $n=28$). Genus *Pseudomonas* was shown to be more abundant in the sputum of individuals with CFRD when compared to CF (Figure 30A, $p=0.011$, $n=28$). Similarly, genera *Azorhizophilus* and

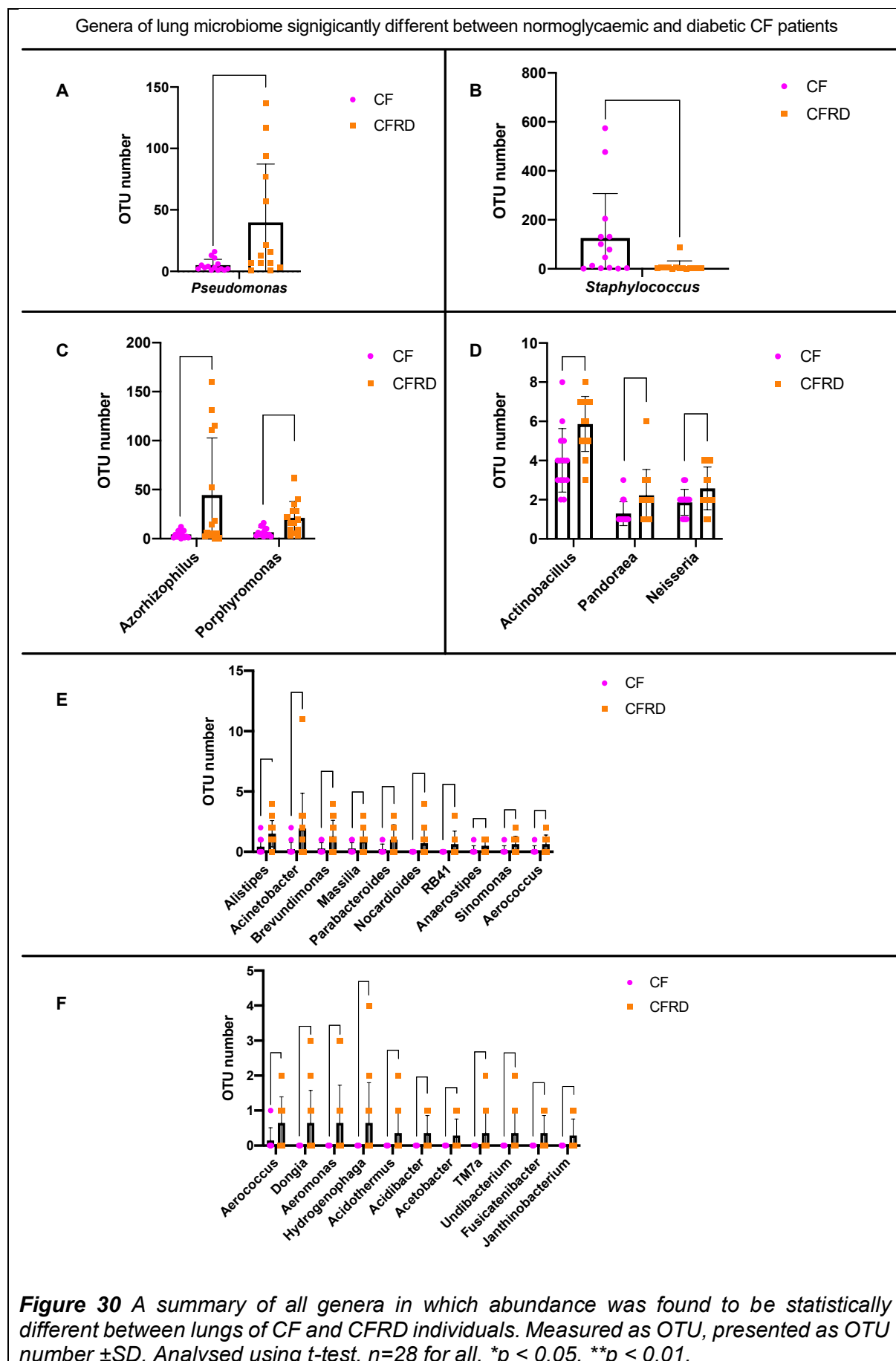
Porphyromonas were found in a higher quantity in the lungs of CFRD compared to individuals with CF (Figure 30C, $p=0.016$ and $p=0.004$ respectively, $n=28$). While not as abundant as the genera previously discussed, genera *Actinobacillus*, *Pandoraea* and *Neisseria* were observed in all sputum samples collected from individuals with CF and were seen in higher abundance in the lungs of CFRD individuals (Figure 30D, $p=0.003$, $p=0.023$, and $p=0.045$ respectively, $n=28$).

Ten other genera were found to be more abundant in the lungs of CFRD individuals (Figure 30E), however, were not seen in all the sputum samples. Found in small amounts in the majority of the samples, the mean abundance was elevated by a few samples rich in these genera. Genera *Alistipes* ($p=0.0039$, $n=28$), *Acinetobacter* ($p=0.040$, $n=28$), *Brevundimonas* ($p=0.013$, $n=28$), *Massilia* ($p=0.027$, $n=28$), *Parabacteroides* ($p=0.033$, $n=28$), *Nocardioides* ($p=0.026$, $n=28$), *RB41* ($p=0.035$, $n=28$), *Anaerostipes* ($p=0.044$, $n=28$), *Sinomonas* ($p=0.016$, $n=28$) and *Aerococcus* ($p=0.032$, $n=28$).

Lastly, eleven other genera were found to be more abundant in the samples from CFRD when compared to CF (Figure 30F). Interestingly all of these bacteria were absent from the samples derived from CF individuals (with the exception of two samples containing genus *Aerococcus*). These bacteria were present in the sputa of CFRD individuals, however, in extremely small amounts. Genera *Aerococcus* ($p=0.032$, $n=28$), *Dongia* ($p=0.015$, $n=28$), *Aeromonas* ($p=0.035$, $n=28$), *Hydrogenophaga* ($p=0.046$, $n=28$), *Acidothrmus* ($p=0.044$, $n=28$), *Acidibacter* ($p=0.012$, $n=28$), *Acetobacter* ($p=0.031$, $n=28$), *TM7a* ($p=0.044$, $n=28$), *Undibacterium* ($p=0.044$, $n=28$), *Fusicatenibacter* ($p=0.012$, $n=28$) and *Janthinobacterium* ($p=0.031$, $n=28$) were all found to be more abundant in the lungs of CFRD individuals.

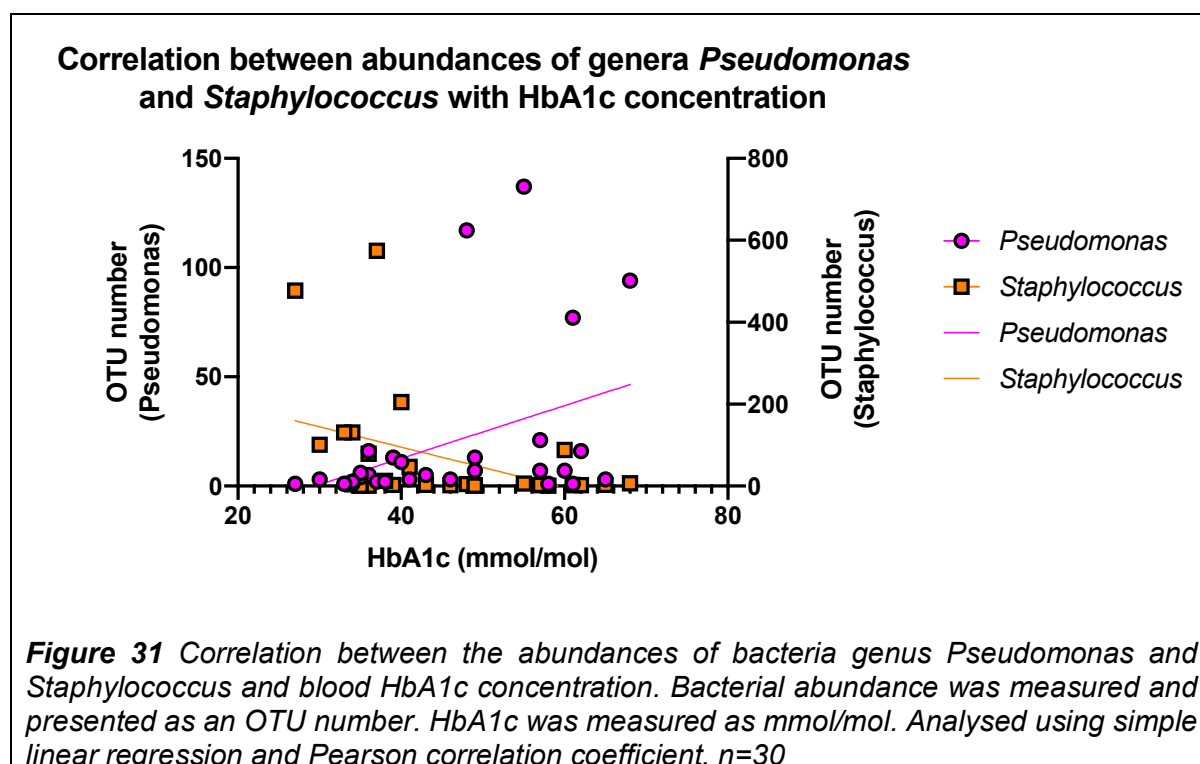
It is important to note that while the abundance of genera presented in Figure 30E and Figure 30F was found to be statistically significantly different between the sputa from CF and CFRD individuals, this difference is unlikely to be biologically significant. These bacteria were found in extremely small amounts in the lungs compared to the whole microbiome, and the results

were often skewed by several individuals containing a higher number of these bacteria than the rest of the cohort.

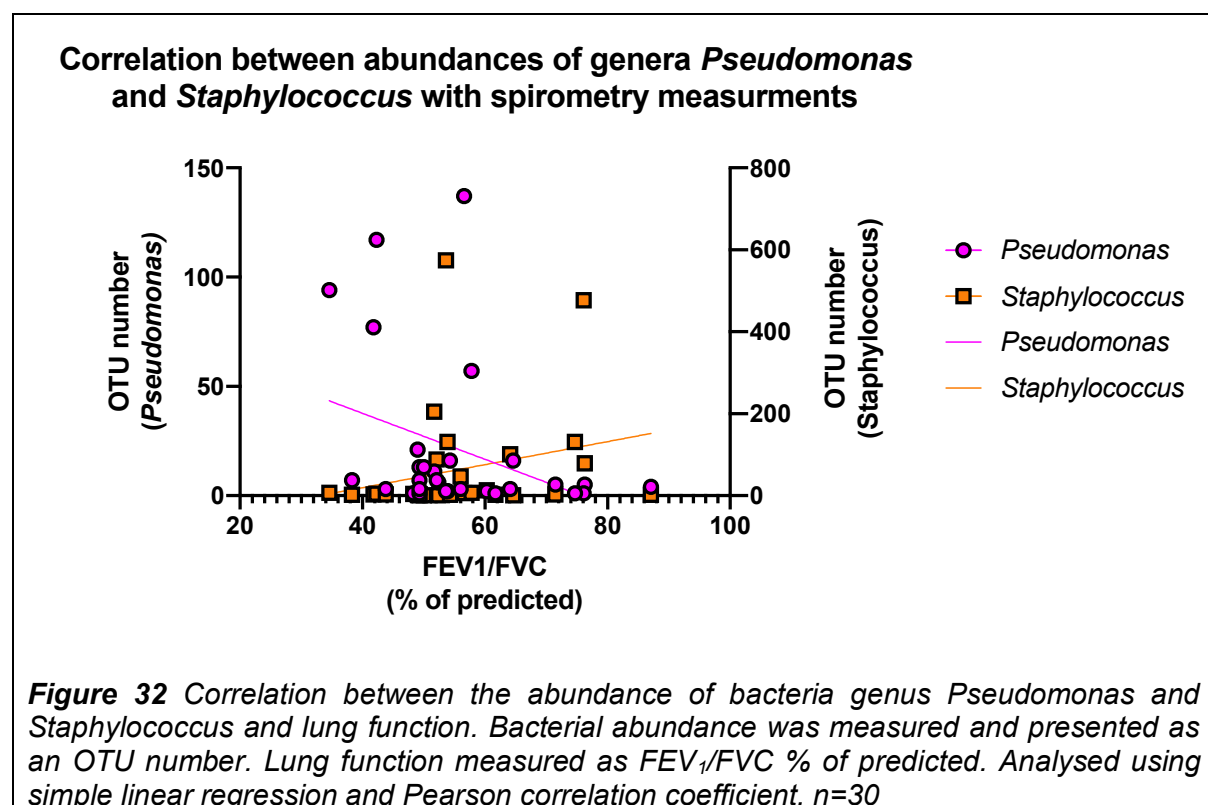


3.4.9 Abundance of Staphylococcus and Pseudomonas in the sputum of CF and CFRD individuals

To further understand the difference in the abundance of genus *Pseudomonas* and genus *Staphylococcus* in the sputum from individuals with CF, I used a simple linear regression and calculated Pearson's correlation coefficient to see whether the increase or decrease in specific pathogen number correlated with the blood HbA1c concentration. In Figure 31 I demonstrate that the abundances of both *Staphylococcus* and *Pseudomonas* correlate significantly with the blood HbA1c concentration ($p=0.020$ and $p=0.034$, $n=30$). I have observed that the abundance of *Pseudomonas* in the lungs of individuals with CF increases with the increase in blood HbA1c concentration, while the abundance of *Staphylococcus* decreases with the increase in blood HbA1c concentration.



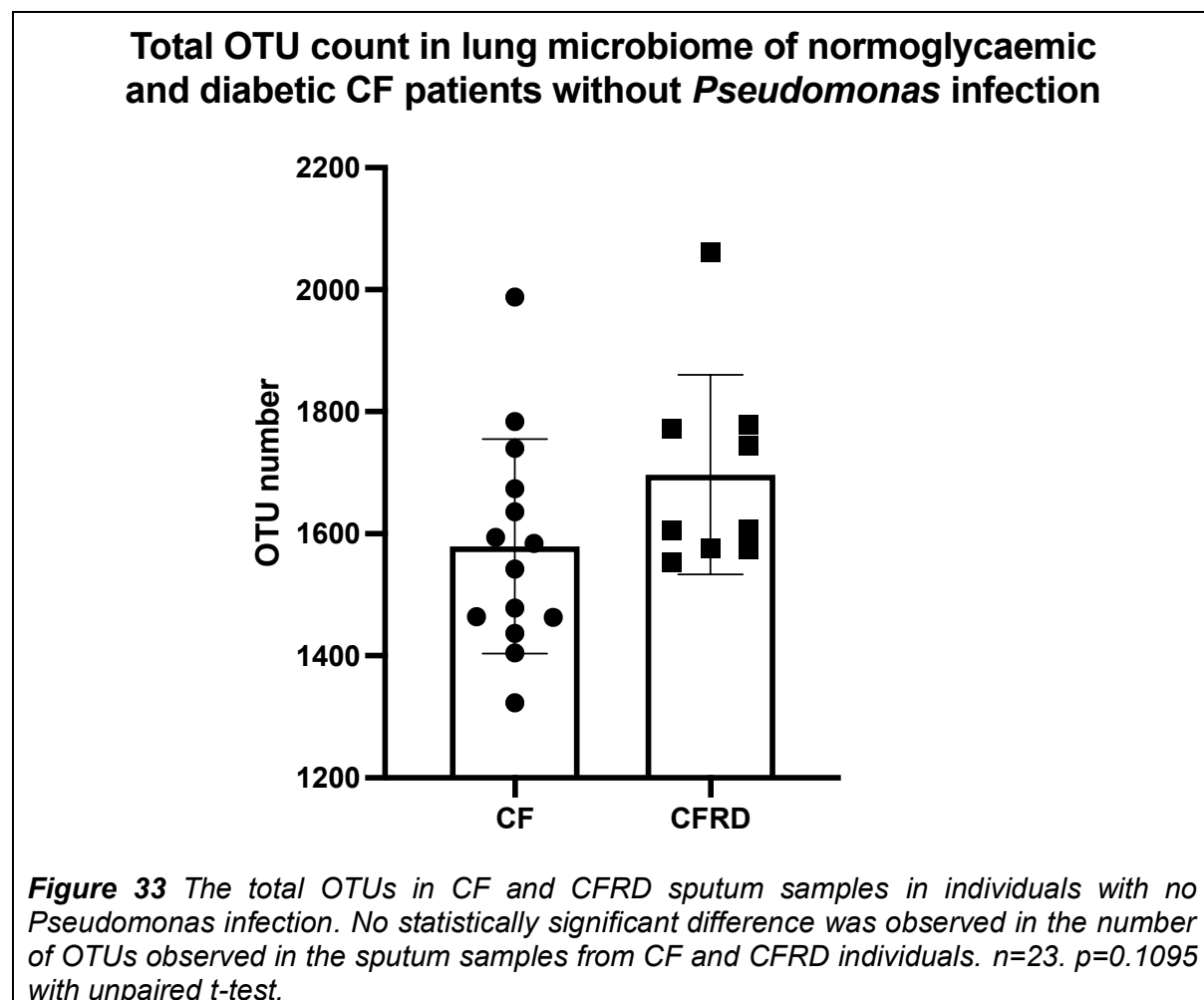
Using simple linear regression and calculating Pearson's correlation coefficient, I also analysed whether an increase or decrease in specific pathogen numbers correlates with the lung function of individuals with CF. Presence of neither *Staphylococcus* nor *Pseudomonas* significantly correlated with the lung function of individuals with CF ($p=0.183$ and $p=0.0586$, $n=30$). However, it is important to note that the p -value for the genus *Pseudomonas* was 0.0586 and that, in combination with a poor genus-level identification of the microbiome data discussed in 3.8.1, it warrants a more specific investigation of the effects of *Pseudomonas* on the lungs and lung function. It is plausible that increasing the study sample size and focusing on the abundance of *Pseudomonas* specifically may affect the final data.



This data demonstrates that while changes in the blood HbA1c levels affect the abundance of both *Pseudomonas* and *Staphylococcus*, these two pathogens are unlikely to be solely responsible for the decrease in the lung function observed in CFRD individuals.

3.4.10 Understanding the effects of high *Pseudomonadaceae* abundance in the sputum of CFRD individuals

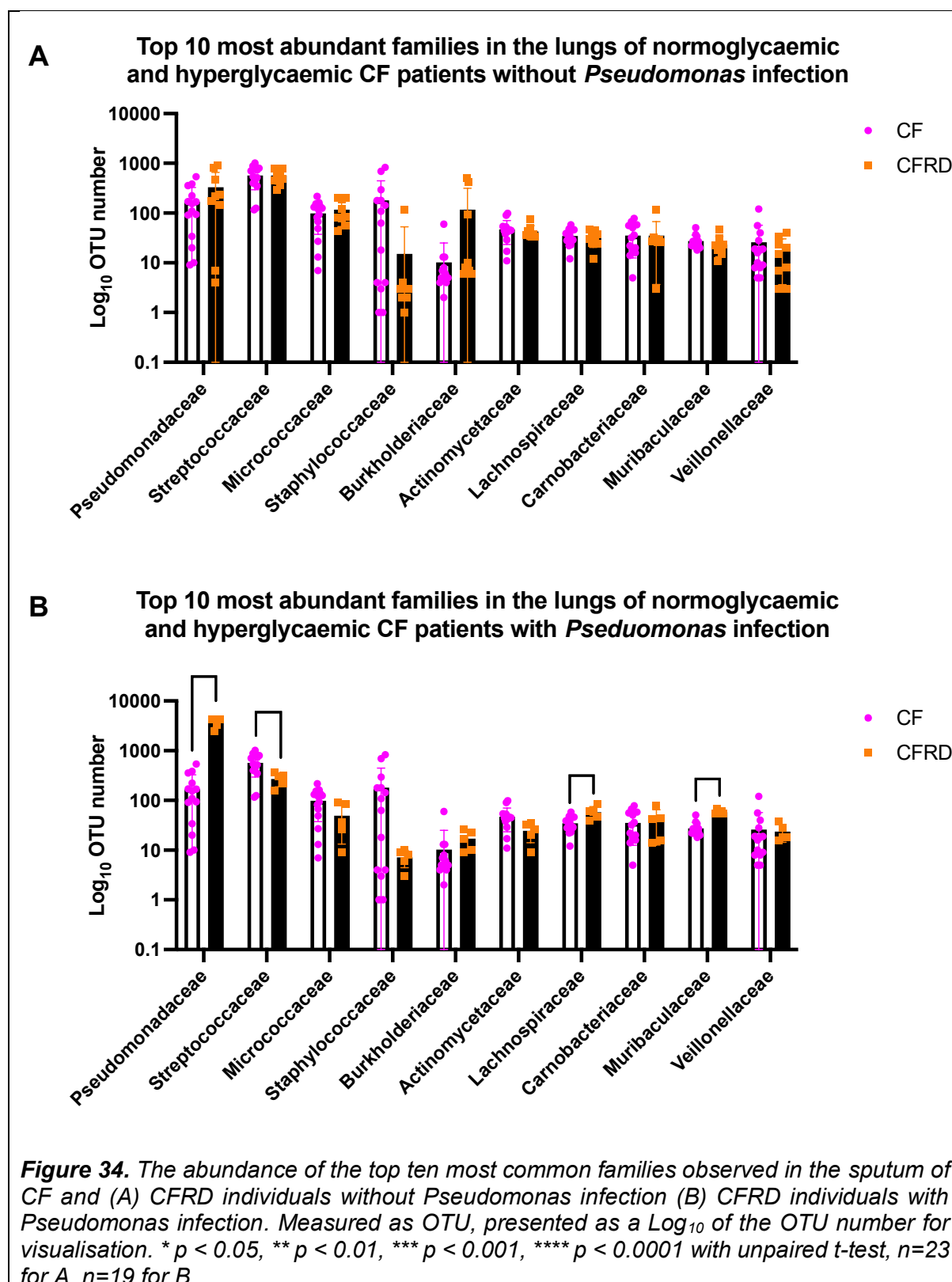
Once five patients with high abundance of *Pseudomonadaceae* in the sputum of CFRD individuals are removed from the study the amount of OTU detected in the sputum of individuals with CF and CFRD is not statistically significantly different ($p=0.8648$, $n=23$).



Re-evaluating the family- and genus-level analysis from section 3.4.7 and 3.4.8, I could separate two groups of CFRD individuals: one with a high abundance of *Pseudomonadaceae* in the sputum and one with a normal abundance of *Pseudomonadaceae* in the sputum.

Removing five individuals with high *Pseudomonadaceae* abundance in the sputum resulted in no statistically significant difference observed in the abundance of top ten family-level bacteria found in the sputum of CF individuals (Figure 34A). However, while not statistically significant, amount of *Pseudomonadaceae* appeared to be higher in CFRD individuals ($p=0.1321$), amount of *Staphylococcaceae* in CF individuals appeared to be higher than in CFRD individuals ($p=0.078$) and abundance of *Burkholderiaceae* appeared to be higher in CFRD individuals than CF individuals ($p=0.0526$).

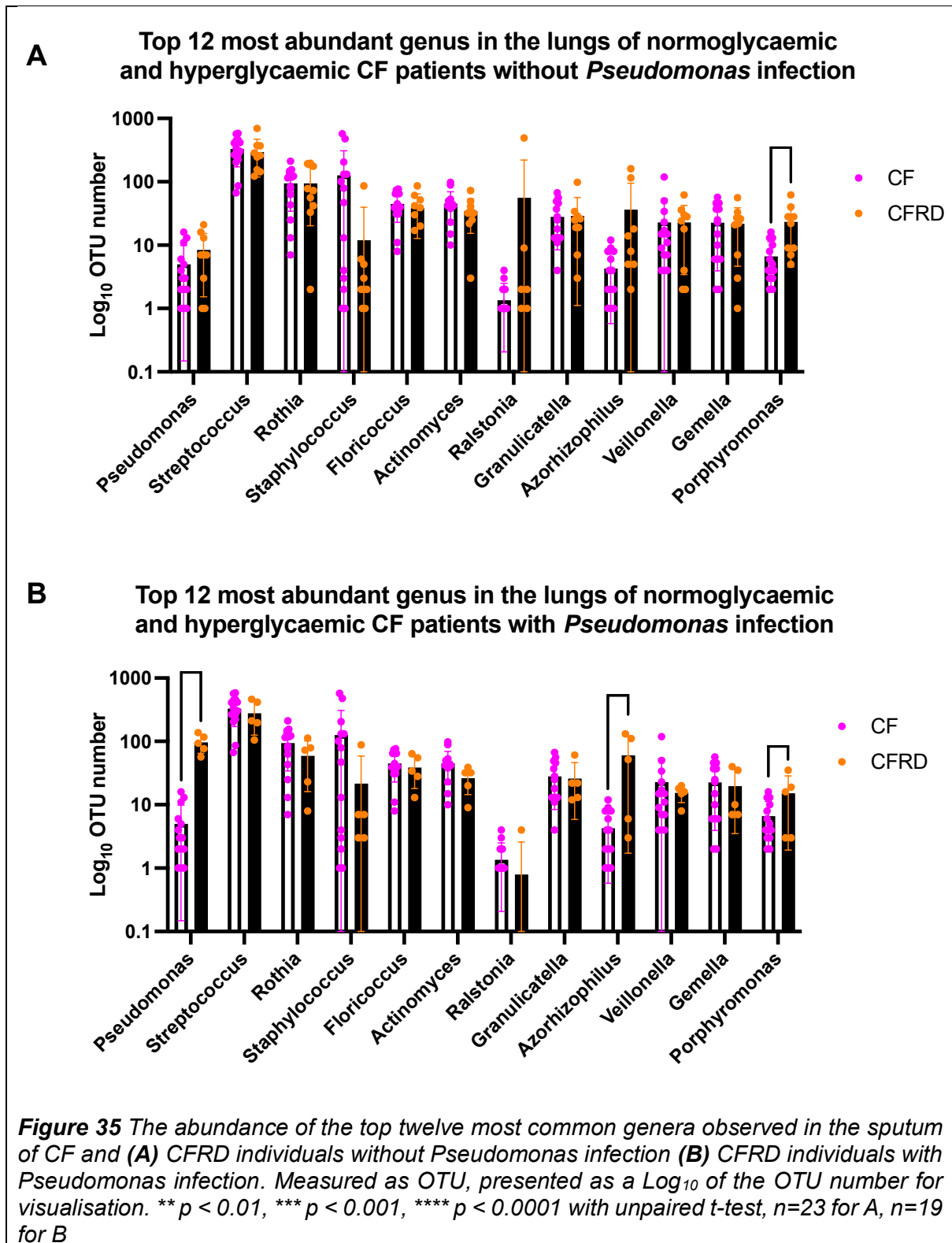
When analysing the difference in the abundance of bacteria observed in the sputum of CF individuals and individuals with CFRD and high *Pseudomonadaceae* abundance a different trend could be observed (Figure 34B). Abundance of *Pseudomonadaceae* appeared to be significantly higher in sputum of CFRD individuals than CF individuals ($p>0.0001$), abundance of *Streptococcaceae* was significantly higher in CF than CFRD individuals ($p=0.0131$), abundance of *Muribaculaceae* was higher in CFRD than CF sputum ($p<0.0001$) and abundance of *Lachnospiraceae* was higher in the sputum of CFRD individuals than CF individuals ($p=0.0041$). While not statistically significantly different, a trend could be observed where abundance of *Staphylococcaceae* was higher in sputum of CF individuals than CFRD individuals ($p=0.1679$), abundance of *Micrococcaceae* was higher In CF than CFRD individuals ($p=0.1103$), and abundance of *Actinomycetaceae* was higher In CF than CFRD individuals ($p=0.0599$).



At the genus level, removing five individuals with a higher *Pseudomonadaceae* abundance (Figure 35A) result in only one genus being statistically significantly different between CF and CFRD patients – *Porphyromonas* ($p=0.0042$). While not statistically significant different, a

trend is observed where abundance of *Pseudomonas* is higher in CFRD individuals ($p=0.1736$), abundance of *Staphylococcus* is higher in individuals with CF than CFRD ($p=0.0769$) and abundance of *Azorhizophilus* is higher in CFRD individuals ($p=0.0517$).

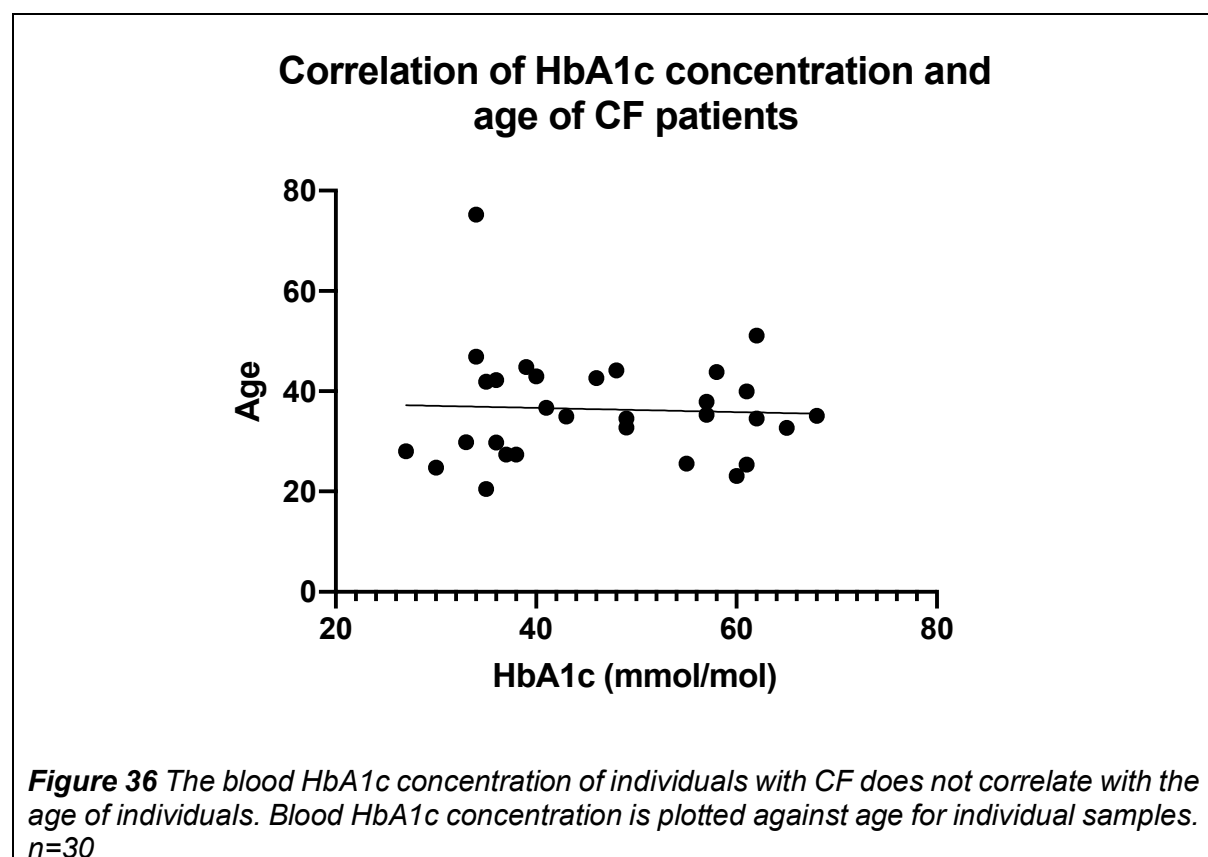
At genus level, focusing on the five CFRD individuals with high *Pseudomonadaceae* abundance a different trend is observed (Figure 35B). Abundance of *Pseudomonas* is significantly higher in individuals with CFRD ($p<0.0001$), other genera abundance of which is higher in individuals with CFRD include *Azorhizophilus* ($p=0.0016$) and *Porphyromonas* ($p=0.0451$). Not statistically different, but abundance of *Actinomyces* ($p=0.1151$) and *Staphylococcus* ($p=0.2253$) appears to be higher in individuals with CF than CFRD.



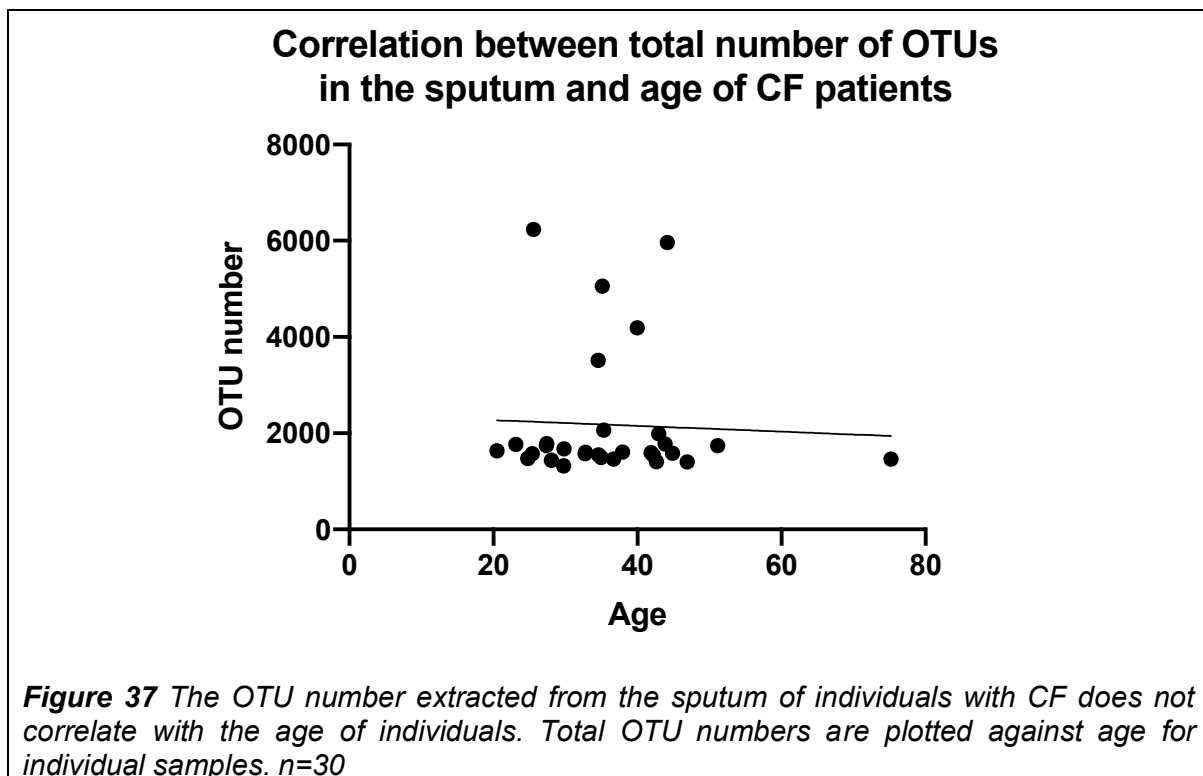
3.4.11 Age of participants as a confounding variable in the lung microbiome study

In this study, we did not control for the age of the participants; however, the lung health of individuals with CF deteriorates with age. In order to understand the potential confounding effect of age on the changes in the lung microbiome, I have analysed the correlation of key observations with the age of participants.

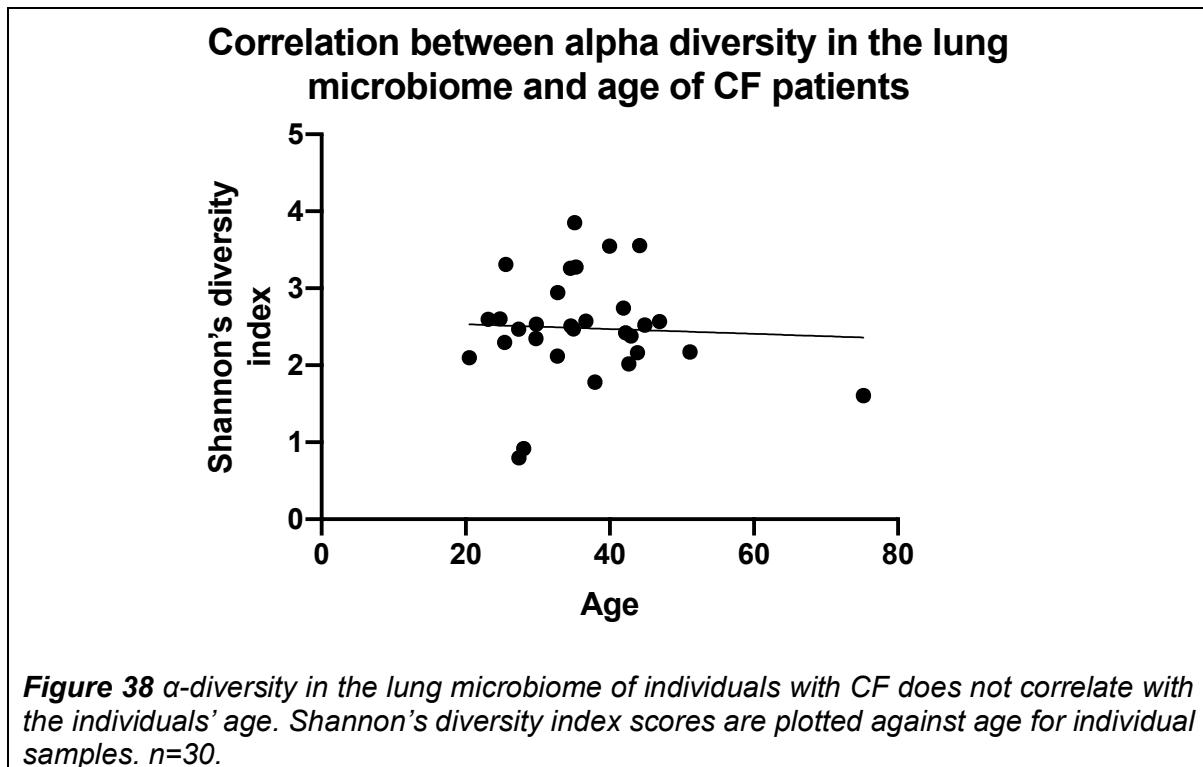
I used a simple linear regression and calculated the Pearson correlation coefficient to see whether the concentration of blood HbA1c correlates with the age of individuals with CF (Figure 36). There was no correlation between the age of participants and the blood HbA1c concentration ($p=0.8082$, $n=30$).



There appears to be no correlation between the age of participants and the total number of bacteria extracted from the sputum samples of individuals with CF (Figure 37, $p=0.8051$, $n=30$).



Changes in the α -diversity did not correlate with the age of participants (Figure 38, $p=0.7991$, $n=30$). OTU and α -diversity data combined demonstrate that age's effect on the overall lung microbiome is minimal.



Finally, to ensure that the age of participants does not affect the specific bacteria found in the lungs, I have correlated the abundance of genera *Pseudomonas* and *Staphylococcus* with the age of participants (Figure 39). The abundance of *Pseudomonas* and *Staphylococcus* did not correlate with the age of participants ($p=0.8481$ and $p=0.2515$, $n=30$). The combination of OTU and α -diversity data presented above demonstrates that the effect of age on the specific bacteria within the lung microbiome of individuals with CF is minimal. These data show that the age of participants does not need to be controlled for in the studies analysing lung microbiome in CF individuals.

Correlation between abundances of genera *Pseudomonas* and *Staphylococcus* with age

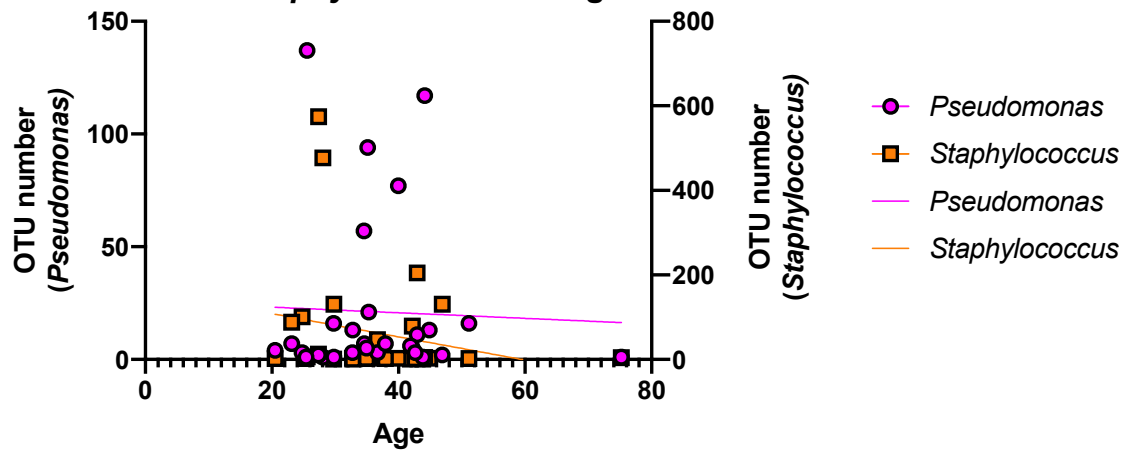


Figure 39 Correlation between the abundance of bacteria genus *Pseudomonas* and *Staphylococcus* and age of individuals. Bacterial abundance was measured and presented as an OTU number. Analysed using simple linear regression and Pearson correlation coefficient. $n=30$

3.5 Discussion

3.5.1 Use of sputum to investigate lung microbiome

In this study I have used sputum samples to investigate lung microbiome. As discussed in section 1.4 sputum offers a non-invasive proxy to study lung microbiome. Sputum is the most common way to clinically evaluate the lung microbiome in CF individuals and is commonly used in studies to investigate lung microbiome (Hogan *et al.*, 2016). A study investigated the similarities of the lung microbiome observed in sputum, BAL and PSB of individual with CF. It was demonstrated that microbiome observed in BAL, PSB and sputum differs, however bacteria with the highest abundance and most abundant pathogens were similar between all three samples. The most difference observed between the samples was derived from genera with low abundance (<2%). Importantly, the study notes that bronchoscopy did not detect any new bacteria beyond those identified in sputum samples (Hogan *et al.*, 2016). This would suggest that sputum does not necessarily accurately represents bacterial composition of the lung, however, allows to investigate major lower airway pathogens.

In this study it was not possible to obtain BAL and PSB samples, as these are not routinely collected from individuals with CF due to invasive nature of sampling. This limitation means that any diabetes-induced changes in the sputum microbiome observed in this study may not extrapolate to the lower-lung microbiome. However, evidence presented above suggests that changes in the major genera or main CF pathogens observed in this study should translate to the whole lung microbiome.

Sputum samples were previously used in major CF studies with hundreds of participants (samples) to analyse lung microbiome (Coburn *et al.*, 2015; Cuthbertson *et al.*, 2020; Garcia-Núñez *et al.*, 2020; Heirali *et al.*, 2020; Raghuvarshi *et al.*, 2020; Weiser *et al.*, 2022). Using sputum samples in this study allows direct comparison of the microbiome data to generated in other studies, offsetting one of the major limitations of this study – low sample size.

3.5.2 Effects of diabetes on the lung microbiome

CFRD is a known comorbidity in individuals with CF and is associated with a decrease in lung function, independent of nutritional status (Moran *et al.*, 2009; Leclercq *et al.*, 2014; Lewis *et al.*, 2015). It was shown that even prior to the onset of diabetes, abnormal glucose tolerance in individuals with CF is associated with a decline of FEV₁% (Elidottir *et al.*, 2021). Glucose intolerance was also shown to be a strong determinant of future lung function decline in individuals with CF (Milla *et al.*, 2000). Early intervention and glycaemic control are known to be associated with a reduction in pulmonary exacerbations and improved clinical prognosis in individuals with CF (Gojsina *et al.*, 2021). While the connection between CFRD and lung function is often noted in clinical settings, published literature often focuses on specific pathogenic species seen in CF lungs, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia complex*, and *Stenotrophomonas maltophilia*. There appears to be a lack of studies in which the lung microbiome in full is compared between CF individuals without diabetes and those with CFRD.

In this study, I observed a higher number of bacterial OTUs detected in the sputum of individuals with CFRD. These results were predominantly associated with 5 individuals who had a significantly higher amount of bacteria family *Pseudomonadaceae* detected in the lungs compared to other CF and CFRD individuals. Such high levels of *Pseudomonadaceae* were only seen in the CFRD group. While genus-level analysis failed to assign a genus to the majority of the *Pseudomonadaceae* family, it is not uncommon for the genus *Pseudomonas* to account for 30-50% of the total microbial community of the lungs (Fodor *et al.*, 2012; Scialo *et al.*, 2021; Zhao *et al.*, 2021). Neither is it uncommon for CF individuals to develop chronic infection with multi-drug resistant *Pseudomonas aeruginosa* over the course of their life. A recent study showed that 25% of CF patients (56 total samples) were chronically infected with multi-drug resistant *Pseudomonas aeruginosa* (Berghea, 2022). I previously discussed that individuals with CFRD are at a higher risk of developing chronic infections with *Pseudomonas*

aeruginosa. This would increase the chance of CFRD individuals developing multi-drug resistant infection, preventing treatments from fully clearing out the infection. It is possible that five individuals with a high abundance of *Pseudomonadaceae* have a chronic infection with *Pseudomonas aeruginosa* which is not cleared despite the antibiotic treatments.

In this study, there was no significant correlation between lung function and the α -diversity of the sputum microbiome. If anything, the data trend indicated an increase in α -diversity in individuals with worse lung function. This contrasted with other studies of the lung microbiome in individuals with CF which have reported a decrease in α -diversity correlated to the decrease in lung function (Cox *et al.*, 2010; Fodor *et al.*, 2012; Carmody *et al.*, 2013; Boutin *et al.*, 2017; Cuthbertson *et al.*, 2020). This study is unique as it includes the microbiome of CF individuals with CFRD and there was an increase in the α -diversity of the lung microbiome in individuals with CFRD. Increased α -diversity also correlated with an increase in blood HbA1c concentrations. Thus, these data indicate that sustained hyperglycaemia, associated with diabetes, correlates with increased microbiome diversity, potentially changing the relationship between microbiome diversity and lung function.

Diabetes appears to significantly modify the lung microbiome as demonstrated by the calculation of β -diversity (Bray-Curtis dissimilarity matrix). Statistical analysis showed that the lung microbiome of CF individuals was different from those with CFRD (AMOVA). There are currently no other studies investigating the difference in the lung microbiome of CF and CFRD; however, the presence of diabetes is associated with changes in the gut microbiome in people, where changes in the abundance of specific bacteria and decrease in α -diversity was observed in diabetic individuals (Doumatey *et al.*, 2020; Chen *et al.*, 2021).

3.5.3 Diabetes-induced changes in the abundance of specific genera detected in the sputum of CF and CFRD individuals

To understand the importance of these changes, a deeper analysis of individual phyla, families, and genera of bacteria was undertaken. I observed that the majority of lung microbiome in individuals with CF was composed of two phyla – *Proteobacteria* and *Firmicutes*, which make up 84% of the entire microbiome. Other phyla that greatly contributed to the lung microbiome were *Actinobacteriota* and *Bacteroidota*, which make up 9.09%, and 4.17% of the lung microbiome. These data correlate strongly with another major study, which analysed samples from over 275 CF adults over the course of 16 years. In this study, Acosta and colleagues demonstrated that across the sample population, the majority of the OTUs were classified as *Proteobacteria* (47.7%), followed by *Firmicutes* (41%), *Actinobacteria* (4.6%), and *Bacteroidota* (4.4%) (Acosta *et al.*, 2017). This demonstrates a robust core microbiome that dominates the lungs of individuals with CF. It is a common observation that phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria* constitute over 99% of CF lung microbiome (Françoise and Héry-Arnaud, 2020). I observed that phyla *Proteobacteria* and *Bacteroidota* were less abundant in the sputa of CF when compared to CFRD individuals; however, phylum *Firmicutes* was found in a higher abundance in the lungs of CF individuals. Currently, it is hard to predict the effect of these changes in the lungs of individuals with CF, whereas in the gut microbiome, *Firmicutes/Bacteroidota* ratio is a known marker of disease. Increased or decreased *Firmicutes/Bacteroidota* ratio in the gut is often regarded as dysbiosis (Stojanov *et al.*, 2020). In the gut microbiome of individuals with type 1 diabetes mellitus, a decline in the abundance of *Firmicutes* and an increase in the abundance of *Bacteroidota* was observed compared to non-diabetic individuals (Murri *et al.*, 2013; Doumatey *et al.*, 2020; Zaky *et al.*, 2021). In this study I observed a *Firmicutes/Bacteroidota* ratio of 15.5 in CF individuals and ratio of 7.9 in CFRD individuals. The ratio in diabetic individuals was almost two times lower than in normoglycaemic individuals similarly to the decrease in the ratio in the gut discussed above. Changes in the abundance of *Firmicutes* and

Bacteroidota in the lungs of people with diabetes may be governed by the same physiological mechanisms that change the abundance of these bacteria in the gut. Currently, many researchers are trying to understand the gut-lung axis and its importance in microbiome maintenance. Further understanding of the gut-lung axis may help us to explain why similar changes are observed in the gut and the lung microbiomes of diabetic individuals.

The CF lung microbiome is a well-studied field, where a core microbiome across different countries and continents was identified. The genera most observed in CF lungs are *Streptococcus*, *Pseudomonas*, *Prevotella*, *Rothia*, *Veillonella*, *Actinomyces*, *Porphyromonas*, *Ralstonia*, and *Gamella* (Van Der Gast *et al.*, 2011; Fodor *et al.*, 2012; Coburn *et al.*, 2015; Acosta *et al.*, 2017; de Almeida *et al.*, 2020). Amongst the top genera observed in our samples, most of the findings were consistent with the literature. Unexpected genera observed in the sputum of individuals with CF included *Floricoccus*, *Granulicatella*, and *Azorhizophilus*. These, however, made up a small proportion of the lung microbiome: 1.97%, 1.27%, and 1.04% respectively. *Granulicatella* was previously observed in the expectorated sputum of individual with CF during exacerbations (Zemanick *et al.*, 2013). *Azorhizophilus* was previously isolated from the lungs of non-cystic fibrosis bronchiectasis (Woo *et al.*, 2019). There is no literature on the presence of *Floricoccus* in the lungs of individuals with CF. The abundance of these three genera is not affected in diabetes.

The majority of the genera significantly affected by CFRD were found in small amounts in the lungs. For example, genera *Acidibacter* and *Aeromonas* were not detected in the lungs of CF individuals at all but were present in the lungs of 5 and 7 CFRD individuals respectively in small amounts. For most of the genera where the abundance was affected by CFRD, the total OTU count appeared to be less than 100 in all of the samples combined (out of 65255 total OTU). Such low abundance indicates that these bacteria are unlikely to prove biologically significant or be used as biomarkers of disease progression. Genera of note, where the abundance was affected by diabetes, were *Actinobacillus*, *Porphyromonas*, *Azorhizophilus*, *Staphylococcus* and *Pseudomonas*. These bacteria were detected in all the collected

samples. *Staphylococcus* and *Pseudomonas* are the most studied CF pathogens and will be discussed separately. It is interesting to note that of 27 genera, the abundances of all but one bacterium were increased in the lungs of diabetic CF individuals. The only genus which was more abundant in the lungs of normoglycaemic lungs is *Staphylococcus*.

Genus *Actinobacillus* is commonly found in the lungs of individuals with CF (Rogers *et al.*, 2004; Beaume *et al.*, 2017; Acosta *et al.*, 2021) and is considered to be a traditional CF pathogen associated with respiratory secretions (Bevivino *et al.*, 2019). One study observed *Actinobacillus* replacing *Pseudomonas* as a dominant genus after lung transplantation in individuals with CF (Beaume *et al.*, 2017). There is currently not enough evidence to suggest whether the increase in the abundance of *Actinobacillus* in diabetic lungs is associated with lung function decline or contributes to disease progression.

Porphyromonas are strict anaerobes commonly detected in CF lungs (similar to *Rothia*, *Veillonella* and *Prevotella*) (Muhlebach, Hatch, *et al.*, 2018; Cuthbertson *et al.*, 2020). In this study, I observed an increase in the abundance of this bacteria in the lungs of diabetic CF individuals. However, genus *Porphyromonas* (and the specie *Porphyromonas gingivalis*) is most known as an oral pathogen causing chronic periodontitis. In Chapter 1, I discussed how diabetic individuals are at higher risk of developing chronic oral periodontitis, which is primarily driven by *P. gingivalis*. I also discussed how *P. gingivalis* altered the gut microbiome in the STZ mice and worsened glycaemic control in these animals. It is, therefore, possible that the same mechanisms increasing the abundance of *P. gingivalis* in the oral cavity increase its abundance in the lungs of CFRD individuals. The implications of *P. gingivalis* worsening glycaemic control and the existence of the gut-lung axis should be further explored to understand whether this pathogen could be responsible for the lung disease progression of CF individuals. The importance of *Porphyromonas* in the lungs of individuals with CF was first brought to light by Keravec *et al.*, who observed an increase in the abundance of *Porphyromonas* in individuals unaffected by *Pseudomonas* and a subsequent decrease in the abundance of *Porphyromonas* following *Pseudomonas* infection (Keravec *et al.*, 2019).

However, no direct antimicrobial activity of *Porphyromonas* against *Pseudomonas* was observed (Guilloux *et al.*, 2021). A most recent study by Webb and colleagues demonstrated that the presence of *Porphyromonas* in the lungs of individuals with CF was associated with a more significant annual decline in lung function (Webb *et al.*, 2022). I also observed a decline in lung function and an increase in the abundance of *Porphyromonas* in the lungs of CFRD individuals. I therefore suggest that *Porphyromonas* could contribute to the decline of lung function via impairment of glycaemic control in CFRD individuals.

Azorhizophilus is a genus from the family of *Pseudomonadaceae*, with one known species (*Azorhizophilus paspali*), and is not a well-studied bacteria. There is currently no literature on its presence or role in CF. Two studies identified this bacterium in the lungs of individuals with non-cystic fibrosis bronchiectasis (Woo *et al.*, 2019; Wang *et al.*, 2022). This genus is unlikely to be important in the lung function decline in CFRD individuals or be a key member of the lung microbiome.

3.5.4 Importance of *Staphylococcus* and *Pseudomonas* in the sputum of CF and CFRD individuals

I observed an increase in the abundance of *Pseudomonas* and a decrease in the abundance of *Staphylococcus* in the lungs of people with CFRD compared to those with CF. The changes in the abundance of these pathogens correlated with blood HbA1c levels and lung function. Consistent with the presence of these genera, two of the most common and well-studied CF pathogens are *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Respiratory infections with *Staphylococcus aureus* and *Pseudomonas aeruginosa* are known to increase the risk of disease progression in individuals with CF (Dasenbrook *et al.*, 2010; Sly *et al.*, 2013; Caudri *et al.*, 2018). *Staphylococcus aureus* infections mainly affect young children and are detected in approximately 75% of individuals aged 2 to 18, while *Pseudomonas aeruginosa* becomes a dominant pathogen in adults and is found in 55% of individuals aged 25 and over, often

coinciding with the development of CFRD (Cystic Fibrosis Foundation, 2021). Thus, it is not uncommon for both pathogens to co-infect the lungs of individuals with CF.

In individuals with CF, it is well-documented that CFRD is associated with higher odds of being chronically infected with *Pseudomonas aeruginosa* (Olesen *et al.*, 2020). Hyperglycaemia was shown to be associated with an increase in the growth of both *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Brennan *et al.*, 2007). Pre-CFRD, early glucose abnormalities were shown to be associated with higher rates of *Pseudomonas aeruginosa* infection (Prentice *et al.*, 2021). *In vitro* increase in glucose concentration was shown to promote the growth of *Pseudomonas aeruginosa* (Garnett *et al.*, 2013). The use of anti-diabetic drugs reduced growth of the *Pseudomonas aeruginosa* (Patkee *et al.*, 2016) and *Staphylococcus aureus* (Garnett *et al.*, 2013) *in vitro*. Thus, the increase in abundance of *Pseudomonas* in CFRD is well supported in the literature. However, evidence to explain the decrease in the abundance of *Staphylococcus* seen in CFRD individuals *in vitro* is lacking. Clinical data exists, however, that demonstrates a decrease in the abundance of *Staphylococcus* and increase in the abundance of *Pseudomonas* in CF individuals with age. The data presented in the CF repository does not mention prevalence of CFRD, however, the onset of CFRD in CF individuals increases with age, reaching 40-50% by adulthood (Moran *et al.*, 2009). It is possible that CFRD plays an important role in the prevalence of these pathogens. The results presented in this study are supported by the mouse data presented in Chapter 2, where I demonstrated that diabetes in mice was associated with higher abundance of *Pseudomonas* and lower abundance of *Staphylococcus*.

Studies have demonstrated that early-colonising isolates of *Pseudomonas aeruginosa* in CF lungs show strong antagonism towards *Staphylococcus aureus* (Baldan *et al.*, 2014; Michelsen *et al.*, 2014). *Pseudomonas aeruginosa* in the lungs of chronically infected CF individuals lacks the antagonistic properties (mostly secretion of anti-staphylococcal compounds), and so *Pseudomonas aeruginosa* and *Staphylococcus aureus* could co-exist (Limoli *et al.*, 2016; Michelsen *et al.*, 2016; Tognon *et al.*, 2017). It is possible that in CFRD,

Pseudomonas begins producing anti-staphylococcal compounds (Hotterbeekx *et al.*, 2017), decreasing the abundance of *Staphylococcus*.

Thus, I propose that diabetes promotes the growth of *Pseudomonas* which impacts the growth of *Staphylococcus*.

3.5.5 Effect of *Pseudomonas* infection on the lung microbiome of CFRD individuals

Earlier in this chapter, I demonstrated that in the CFRD cohort, five individuals presented with a high abundance of *Pseudomonadaceae*, which skewed the total amount of bacterial OTUs detected in the sputum of individuals with CF. Removing these patients from the analysis showed no statistically significant difference in the number of OTUs observed in CF and CFRD individuals. These data demonstrate the importance of a large dataset when working with clinical samples, as a small number of samples can significantly skew the results.

It is possible that these five individuals had an ongoing infection with *Pseudomonas aeruginosa* at the time of the sputum collection. Infection with a lung pathogen such as *Pseudomonas aeruginosa* could potentially disrupt the microbiome masking the effects of diabetes on the lung microbiome. To understand the significance of the high abundance of *Pseudomonadaceae* on the lung microbiome, I further stratified individuals with CFRD into two groups: individuals with a normal abundance of *Pseudomonadaceae* (10) and individuals with a high abundance of *Pseudomonadaceae* (5).

Removing a large portion of samples severely decreases study power, resulting in many observations being not statistically significant, despite the trends observed (p -values 0.05-0.2).

Stratifying CFRD samples into two groups changed the sputum microbiome composition at the family level. In the CFRD group with low levels of *Pseudomonadaceae*, a higher abundance of *Burkholderiaceae* was observed in individuals with CFRD. Populations of *Burkholderia* were previously shown to invade populations of *Pseudomonas* and inhibit biofilm formation in *Pseudomonas* (Schwab *et al.*, 2014). However, in another study, *Pseudomonas* was shown to inhibit growth of *Burkholderia* (Costello *et al.*, 2014). This would suggest that the outcome of competition between *Pseudomonas* and *Burkholderia* could be dependent on the environment.

In the CFRD group with high abundance of *Pseudomonadaceae*, a higher abundance of *Muribaculaceae*, *Lachnospiraceae* and *Streptococcaceae* was observed. Interestingly, in both groups of CFRD individuals, an abundance of *Pseudomonadaceae* was higher than in CF individuals, and the abundance of *Staphylococcaceae* was lower than that of CF individuals.

At the genus level, stratifying CFRD samples into two groups did not majorly affect the abundance of bacteria in the sputum. Whether samples were derived from CFRD individuals with a high or low abundance of *Pseudomonadaceae*, abundances of *Porphyromonas*, *Pseudomonas* and *Azorhizophilus* were generally higher in CFRD individuals than CF individuals, while the abundance of *Staphylococcus* was higher in CF individuals.

This demonstrates that despite potential lung infection at the time of the sample collection, effects of diabetes on the lung microbiome at the genus level could still be observed.

3.5.6 Understanding the effects of age of participants on the lung microbiome

Bacterial communities residing in the lungs of people with CF are influenced by a multitude of external and internal factors. Several studies have focused on the longitudinal analysis of the CF lung microbiome to understand the differences in younger versus older CF airways. Understanding the effects of a single factor on the lung microbiome in a complex disease such

as CF is challenging. Studies striving to understand the effects of ageing on the CF lung microbiome have primarily focused on the presence of pathogenic species, the presence of antibiotic resistance genes and generic species diversity. Cox and colleagues demonstrated that in a cohort of clinically stable individuals, the microbiome of older individuals with decreased lung function tends to be more uneven and cluster separately than the microbiome of younger people (Cox *et al.*, 2010). They have also demonstrated a progressive loss of diversity with age (Cox *et al.*, 2010). Lee and colleagues, however, showed that α -diversity was comparable between young and elderly groups in non-CF healthy adults. Instead, α -diversity correlated to lung function decline (Lee *et al.*, 2019), which has also been observed in individuals with CF in other studies (Coburn *et al.*, 2015; Cuthbertson *et al.*, 2020). Age appears to influence the abundance of core CF lung bacteria, such as *Streptococcus*, *Prevotella*, *Rothia*, *Veillonella*, *Actinomyces*, *Pseudomonas* and *Stenotrophomonas* (Coburn *et al.*, 2015; Zemanick *et al.*, 2017; Lee *et al.*, 2019). In individuals with CF, with age, lung function declines and incidents of acquisition of lung pathogens increase. These changes could be responsible for the observed changes in the lung microbiome independent of the participant's age. In this study, I did not observe changes in the α -diversity or total OTU count that correlated with the age of participants. This study, however, introduced comorbidity in the form of CFRD, which was not present in other studies. Prevalence of CFRD normally increases with age and affects 40-50% of the adults with CF (Moran *et al.*, 2009). In this study, however, blood HbA1c concentration did not correlate with age. Therefore, I believe age on its own was not a confounding variable in this study.

3.5.7 Understanding the effects of antibiotics on the lung microbiome

All the recruited individuals in this study were being actively treated with several antibiotics. Antibiotics disrupt the microbiome (Langdon *et al.*, 2016; Ramirez *et al.*, 2020) and could introduce variation in the results that would not persist through different studies. Most of the

knowledge on the effects of antibiotics on the microbiome is derived from gut research. Many people with CF take antibiotics daily to prevent new lung infections, exacerbations or reduce the strength of a long-term infection, so recruiting individuals with CF for the study with no recent use of antibiotics would be challenging. A study in 2012 by Fodor and colleagues investigated the effects of antibiotics on the lung microbiome of individuals with CF. It was demonstrated that microbial community composition did not substantively change after treatment with broad-spectrum antibiotics. Initially, a slight decrease in richness was observed following treatment; however, the effect was limited to taxa with low initial relative abundance. The most abundant taxa, such as *Prevotella* and *Streptococcus*, showed little change in response to antibiotics. The authors suggest that persistent use of antibiotics encourages the establishment of a stably antibiotic-resistant lung microbiome (Fodor *et al.*, 2012). In conjunction with the complexities of recruiting individuals with CF with no recent use of antibiotics, these data suggest that studies should not exclude individuals based on antibiotic use and instead recruit these individuals to increase the study sample size.

3.6 Summary

In this chapter, I have provided preliminary evidence on how CFRD influences the composition of the lung microbiome in CF disease. I have demonstrated an increase in the α -diversity of the lung microbiome in CFRD compared to CF that was associated with hyperglycaemia, despite the decrease in the lung function of these individuals. The core composition of the lung microbiome does not change in diabetes; however, diabetes-induced changes in the abundance of *Staphylococcus*, *Pseudomonas*, *Azorhizophilus*, and *Porphyromonas* were observed. Interestingly, I expected to see an increase in the abundance of both *Staphylococcus* and *Pseudomonas* in individuals with CFRD but observed a decrease in the abundance of *Staphylococcus*. I suggest that the relationship between *Staphylococcus* and *Pseudomonas* in diabetic lungs should be investigated further. I also demonstrated that age does not appear to affect the lung microbiome of people with CF in this study and that antibiotics might not significantly affect the lung microbiome in these individuals either.

3.7 Limitations and future work

As was previously discussed in Chapter 2, section 7, the current 16S sequencing employed in this study is limited to the genus-level identification and could not be used to confirm the species of detected bacteria. Due to the small amount of extracted sputum provided by the biobank, all the material was used to extract bacterial DNA and subsequent 16S analysis. In future work, a small amount of material should be preserved and used in qPCR with species-specific primers to identify bacteria of interest up to species level.

Microbiome analysis was limited to the molecular methods and no bacteria from the sputum were cultured in this experiment. Due to this limitation no information on the biofilm formation could be obtained. Biofilm formation is known to affect relationship between *S. aureus* and *P. aeruginosa*. It is possible that presence of glucose affects biofilm formation, which could explain some of the results we are observing in CF individuals. In the future studies, bacteria from sputum should be cultured to observe biofilm formation in presence of glucose.

Individuals with CF are prone to cross-infection during hospital visits, and social interactions (Farrell *et al.*, 1997; Bhagirath *et al.*, 2016), and individuals visiting the same clinics tend to acquire the same pathogens. This could affect the lung microbiome and cause it to cluster similarly. A larger dataset should be used to investigate the lung microbiome with samples acquired from individuals with CF across different clinics and locations.

In this study use of antibiotic medications was noted, however, no information on anti-reflux medications was collected. CF individuals are commonly prescribed anti-reflux medications to help deal with acid secretions in the stomach. Evidence exists that reflux disorders affect oral microbiome, which could potentially affect lung microbiome. In the future studies use of anti-reflux medications should be noted, as it could help explain some of the bacterial changes we observe.

In this study, blood glucose concentration was calculated to understand the effects of hyperglycaemia on the lung microbiome. However, diabetes is a complicated disease which affects multiple body systems, such as the immune system, which affects the body's ability to hinder pathogen spread. In this study, I have not measured inflammatory markers and other markers of diabetic damage. While I make an assumption that hyperglycaemia is responsible for the changes in the lung microbiome observed, the potential effects of diabetes could not be ignored.

In this study, alongside bacterial DNA extraction, peptides were extracted from the sputum of CF and CFRD individuals. We planned to analyse extracted peptides using gas chromatography mass spectrometry analysis. However, performing this analysis in the scope of this study did not appear to be feasible. Samples are currently frozen in a stable form and will be analysed in the future. Understanding the hyperglycaemia-induced changes in peptidome would potentially help us understand how bacteria interaction changes in diabetes and would help us understand how changes in the abundances of certain bacteria may affect the abundance of others.

Chapter 4

**Use of the Artificial Sputum Medium
for the in vitro analysis of
interactions between common lung
pathogens *Staphylococcus
aureus* and *Pseudomonas
aeruginosa* in the hyperglycaemic
environment.**

4.1 Introduction

4.1.1 *Staphylococcus aureus*

Staphylococcus aureus is gram-positive bacteria that evolved to be both a commensal bacterium in the human body and a major human pathogen. It was estimated that approximately 28% of the UK general population (Gamblin *et al.*, 2013) and approximately 27% of the German population (Holtfreter *et al.*, 2016) are carriers of *S. aureus*. Humans primarily carry *S. aureus* inside the nose and on their skin: armpits and groin being the main sites (Gamblin *et al.*, 2013). *S. aureus* can cause several infections in humans, such as endovascular infections, pneumonia, septic arthritis, endocarditis, and various skin and soft-tissue infections (David and Daum, 2010). The transformation of *S. aureus* from a commensal organism to a pathogen is poorly understood. Using a murine lung model, it was demonstrated that the transcriptome of *S. aureus* is altered when transferred from bacterial culture to the *in vivo* (mouse lung) environment and was further altered during the initial stages of infection development. It was shown that the expression of genes responsible for central metabolic pathways, amino acid synthesis and RNA translation was significantly altered upon introduction into the lung environment. Virulence factors were initially downregulated but later significantly upregulated within the first few hours of the transfer (Chaffin *et al.*, 2012).

S. aureus is an opportunistic pathogen that does not commonly cause infections in healthy individuals. However, it is more often found in individuals with chronic lung diseases and can persist in the lungs of these individuals for several years, despite antibiotic treatment, by utilising genomic, proteomic and metabolic changes (Tan *et al.*, 2018). *S. aureus* is found in 70% of Cystic Fibrosis individuals' lungs in the 10-20-year-old age group, decreasing steadily to approximately 25% in 60-year-olds (Cystic Fibrosis Foundation, 2018). *S. aureus* is commonly found in the lungs of COPD individuals; however, there is no centralised database for the prevalence of pathogens in the lungs of individuals with COPD. Various studies utilising sputum samples, bronchoalveolar lavage (BAL) and protective specimen brushings (PSB)

from COPD individuals demonstrated the occurrence of *S. aureus* in 1-40% of all COPD individuals (Sethi and Murphy, 2001; Curran *et al.*, 2007; Bafadhel *et al.*, 2015; Wang *et al.*, 2015). *S. aureus* was shown to be associated with severe worsening of asthma leading to exacerbations and hospitalisations and is often detected in the sputum of individuals with asthma (Zhang *et al.*, 2012).

S. aureus manipulates mucosal immunology via its proteins (such as superantigens, protein A and serine-protease-like proteins) by causing the release of inflammatory substances such as Interleukin-33 and type 2 cytokine (Bachert *et al.*, 2020). Currently, despite the apparent involvement of *S. aureus* in many chronic lung conditions and high prevalence, its growth patterns in the lung are still not well understood. Understanding what drives the pathogenicity of *S. aureus* and its relationship with other lung pathogens would benefit the treatment of individuals with chronic lung diseases.

4.1.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a common gram-negative opportunistic pathogen known to cause severe respiratory infections in humans. *P. aeruginosa* has been extensively studied in CF individuals. It is not surprising, as *P. aeruginosa* is found in approximately 70% of CF individuals by the time they reach 30 years of age (Cystic Fibrosis Foundation, 2018). *P. aeruginosa* is one of the most critical lung pathogens and the predominant cause of morbidity and mortality in individuals with CF. During the colonisation of CF lung, *P. aeruginosa* adapts to the lung environment. To survive and persist in the lung, it undergoes both genotypic and phenotypic changes such as increased antibiotic resistance, decreased metabolism, and loss of motility (Bhagirath *et al.*, 2016). Once established, *P. aeruginosa* becomes almost impossible to eradicate from CF lungs. CF individuals undergo frequent rounds of antibiotic therapy, and as was shown in a 2019 study, *P. aeruginosa* can quickly respond to antibiotic therapy and develop resistance. Moreover, these changes are frequently patient-specific, making it harder to use established broad treatments protocols (Tueffers *et al.*, 2019). Like

with *S. aureus*, individuals with COPD are at constant risk of infection with *P. aeruginosa*. Studies have reported the prevalence of *P. aeruginosa* in COPD individuals from 4% to 34%. Despite the considerable variation, these studies showed an association between *P. aeruginosa* isolation and the severity of the condition, frequency of readmissions and frequency of exacerbations (Lieberman and Lieberman, 2003; Murphy *et al.*, 2008; Gallego *et al.*, 2014; Rodrigo-Troyano *et al.*, 2018).

In non-CF bronchiectasis presence of *P. aeruginosa* is associated with increased disease severity, greater airflow obstruction and poorer quality of life. However, whether *P. aeruginosa* infection is directly responsible for the decline in the lung health of these individuals is not conclusive. Multiple studies have shown that *P. aeruginosa* tends to infect people with worse pulmonary functions and does not seem to affect the rate of pulmonary decline (Evans *et al.*, 1996; Miskiel *et al.*, 1997; Davies *et al.*, 2006). This indicates that *P. aeruginosa* serves the role of a marker of diseases severity, as people with more severe cases of bronchiectasis are more likely to be infected with *P. aeruginosa*. A study in 2018 investigated the strain stability in individuals with non-CF bronchiectasis over six months. It was demonstrated that in 67% of the individuals positive for *P. aeruginosa*, a single strain persisted for six months, with 33% of individuals acquiring a new strain of *P. aeruginosa* either via strain displacement, temporary disruption or chaotic airway infection (Woo *et al.*, 2018). These studies demonstrate the ability of *P. aeruginosa* to adapt, survive and persist in the human lungs despite the changes in the environment or treatment. With the frequency of *P. aeruginosa* colonisation of lungs of individuals with chronic lung diseases and its ability to persist through the challenges, it is becoming increasingly important to understand the mechanisms of its survival and adaptation within the lung, its relationship with other common lung pathogens and its effects on the lung physiology.

4.1.3 Relationship between *S. aureus* and *P. aeruginosa* in the human lungs

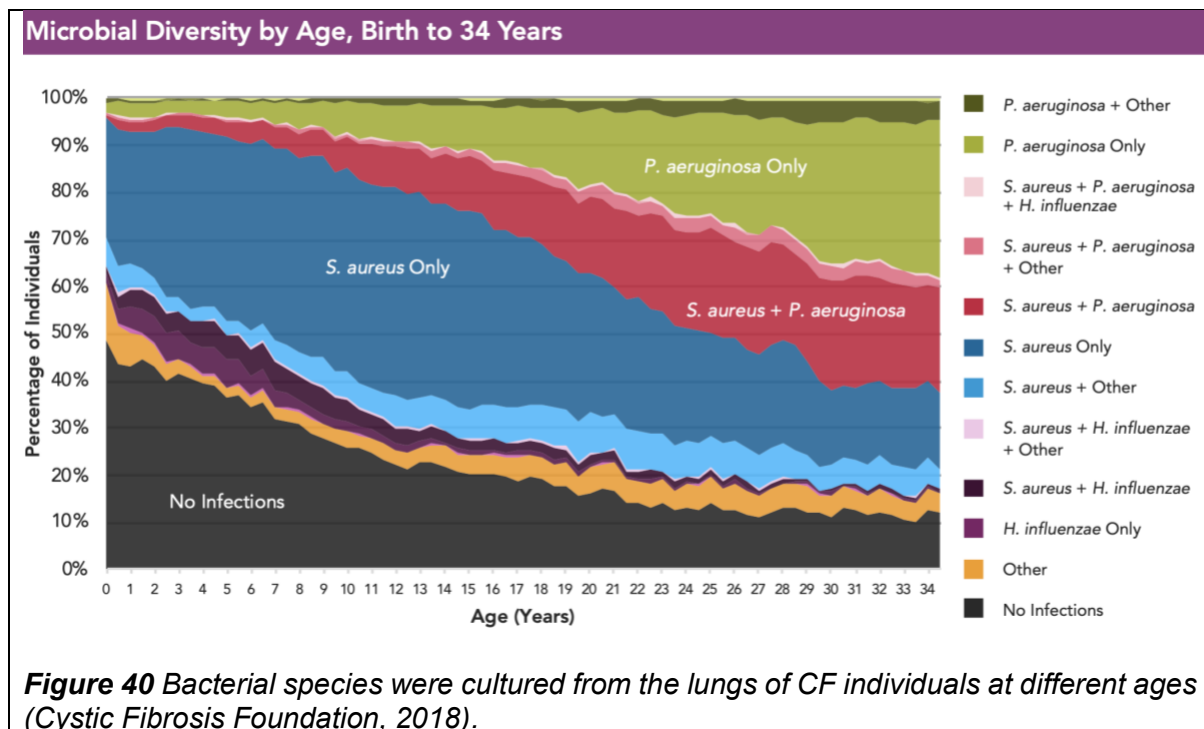
Both *S. aureus* and *P. aeruginosa* are highly versatile organisms that quickly adapt to a wide variety of environments. As discussed above, both microorganisms are readily abundant in the lungs, infect the same categories of chronically ill individuals and can have devastating effects on the lungs. It is no surprise that both pathogens have been extensively studied and their relationship explored. However, the interactions between *S. aureus* and *P. aeruginosa* remain a topic of discussion.

Looking at the 2018 Annual Data Report from Cystic Fibrosis Foundation Patient Registry (Cystic Fibrosis Foundation, 2018), a clear trend emerges. The lungs of individuals with CF are predominantly infected with *S. aureus* at a younger age, with *P. aeruginosa* appearing at the age of 10 and onwards. From data presented in Figure 40 it could be suggested that after the introduction of *P. aeruginosa*, either both pathogens learn to co-exist in the lungs, or *P. aeruginosa* outcompetes *S. aureus*. In individuals where *P. aeruginosa* and *S. aureus* have been isolated from lungs, both pathogens seem to contribute additively to the disease severity in CF individuals (Sagel *et al.*, 2009; Hotterbeekx *et al.*, 2017). Numerous studies have looked at the relationship between these pathogens to better understand their pathogenicity in the lungs. In theory, there is an antagonistic relationship between *P. aeruginosa* and *S. aureus*. *P. aeruginosa* produces various molecules inhibiting the growth of *S. aureus*, often outcompeting and eliminating it from the lungs (Hotterbeekx *et al.*, 2017). However, through the literature review, I can see the evidence of these two pathogens forming a dual-species biofilm. While two pathogens compete extensively *in vitro*, evidence shows that the presence of environmental pressure, like the presence of antibiotics or effects of the immune system, stimulates synergistic relationship and biofilm formation between *S. aureus* and *P. aeruginosa* (DeLeon *et al.*, 2014; Kumar and Ting, 2015; Beaudoin *et al.*, 2017; Orazi and O'Toole, 2017). It was shown that in the presence of *S. aureus* exoproducts, *P. aeruginosa* forms different

biofilm architecture (Beaudoin *et al.*, 2017). More importantly, there is evidence that dual-species biofilm enhances *S. aureus* resistance to multiple antibiotics, such as vancomycin (Orazi and O'Toole, 2017; Kayumov *et al.*, 2018), ampicillin (Kayumov *et al.*, 2018), gentamicin, ciprofloxacin and tetracycline (DeLeon *et al.*, 2014). In contrast, studies have also demonstrated that while resistance to certain antibiotics is heightened, *S. aureus* becomes more sensitive to antibiotics such as ciprofloxacin and aminoglycosides (Kayumov *et al.*, 2018) and chloroxylonol (Orazi *et al.*, 2019). Confusingly, increased sensitivity and resistance appear mediated via small molecule 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO) produced by *P. aeruginosa*. Understanding how two pathogens interact in human lungs would help design better design treatment regimens for individuals with multi-pathogen infections.

4.1.4 Effects of glucose on the growth of *S. aureus* and *P. aeruginosa* in CF lungs

As was previously discussed, both pathogens can utilise glucose for growth, which is important in individuals with chronically high glucose levels in the blood, resulting in increased glucose in the ASL. These individuals include people with CFRD – a subset of CF individuals who developed diabetes due to cystic fibrosis. Lungs of these individuals, like the majority of individuals with CF, may get colonised by *P. aeruginosa* and *S. aureus*; however, these individuals may also have high glucose in the ASL, further enhancing bacterial growth. The prevalence of CFRD increases with age, with the median onset being 20 years. However, only a small proportion of young people develop early-onset CFRD, with 40% of CF individuals developing it by the age of 40. The lack of a centralised database of CFRD individuals makes it harder to determine the effect of CFRD on the prevalence and relationship of *S. aureus* and *P. aeruginosa* in the lungs; as evidence shows that by the age of twenty, almost 70% of all cultured bacterial species in the lungs of CF individuals are either *S. aureus*, *P. aeruginosa* or a combination of two (Figure 40).



4.1.5 Use of Artificial Sputum Media to study effects of glucose on the growth of *S. aureus* and *P. aeruginosa*

Some studies looking at the interactions between *S. aureus* and *P. aeruginosa* utilise a skin model of colonisation, which may not be an appropriate model for colonisation of the lung. Other studies have investigated the relationship between these pathogens *in vitro* using popular bacterial media such as tryptic soy broth (TSB) and lysogeny broth (LB). While these media ensure the good growth of bacteria, they are designed as a highly nutrient-rich environment and do not represent the lung environment, which is nutrient-poor. Glucose in these models would not have the same effect on bacterial growth as it would in the lungs. I have identified this as an opportunity to develop *in vitro* model that could be used to investigate lung pathogens in a controlled environment that is more relevant to the lung.

Artificial sputum medium (ASM) is a culture medium designed to mimic sputum from CF individuals. It contains components of CF sputum, such as amino acids, mucin and DNA (Sriramulu *et al.*, 2005). It was initially designed to test antibiotic efficiency against *P.*

aeruginosa. It was shown that *P. aeruginosa* growth in the ASM is similar to the growth in the lungs of CF individuals (Garbe *et al.*, 2010; Naughton *et al.*, 2011), supporting its validity as an *in vitro* model to mimic the *in vivo* environment. The use of ASM, a nutrient-poor media, would allow us to investigate the impact of glucose on the growth of *P. aeruginosa* and *S. aureus* while controlling nutritional content and conditions of the growth.

In this chapter, I hypothesise that the ASM model could be adapted to study the interaction between *S. aureus* and *P. aeruginosa* in a controlled environment that is more relevant to the *in vivo* environment of CF lungs.

4.2 Hypothesis and Aims

Hypothesis: Presence of high glucose concentration in the Artificial Sputum Medium affects growth of *S. aureus* and *P. aeruginosa* compared to normoglycaemic medium.

Aim 1: To investigate the effects of glucose on the growth patterns of *S. aureus* and *P. aeruginosa* in the ASM

Aim 2: To establish a stable co-culture of *S. aureus* and *P. aeruginosa* in the ASM

Aim 3: To investigate the effect of glucose on the relationship between *S. aureus* and *P. aeruginosa* when grown in a co-culture scenario

4.3 Materials and methods

4.3.1 Bacterial strains

Staphylococcus aureus (ATCC29213) and *Pseudomonas aeruginosa* (H174) were used for experimental design. H174 strain of *P. aeruginosa* is a modified PAO1 strain with inserted *lux* genes. With these genes correctly inserted, bacteria will continuously produce light. Measurement of produced light allows precise real-time quantification of viable bacteria in the media.

4.3.2 Bacterial stocks

To generate frozen bacteria stocks, 1ml of the overnight culture was added to 0.5ml of 100% sterile glycerol (BDH) in a cryogenic vial (Nalgene). Glycerol stocks could then be stored long-term at -80°C.

4.3.3 Culturing bacteria from stocks

To culture bacteria from frozen stocks, a frozen sample was scraped using an inoculation loop, added to MH broth, and incubated at 37°C, shaking 225rpm overnight.

4.3.4 Bacterial media and agar plates

All the culture media were sterilised by autoclaving at 15 lbs p.s.i (121°C) for 15 min.

Table 1. *Composition of Mueller-Hinton broth and Mannitol salt agar*

Mueller-Hinton broth		
Amount	Reagent	Source
2 g/L	Beef infusion solids	Sigma-Aldrich
17.5 g/L	Casein hydrolysate	Sigma-Aldrich
1.5 g/L	Starch	Sigma-Aldrich
Mannitol salt agar		
Amount	Reagent	Source
5 g/L	Casein peptone	Sigma-Aldrich
10 g/L	D-mannitol	Sigma-Aldrich
1 g/L	Meat extract	Sigma-Aldrich
5 g/L	Meat peptone	Sigma-Aldrich
0.025 g/L	Phenol red	Sigma-Aldrich
75 g/L	Sodium chloride	Sigma-Aldrich
15 g/L	Agar	Sigma-Aldrich

4.3.5 Single colony bacterial plates

Bacteria cultured overnight were streak-plated using sterile inoculation loops onto MH agar plates and allowed to grow overnight at 37°C in a benchtop incubator (211DS, Labnet International). This plate acted as a stock plate, allowing for single colony selection for future overnight culture preparations. Plated bacteria were stored in a fridge at 4°C to slow down

further growth. Plated bacteria were kept for three weeks, they were then discarded, and new plates were prepared.

4.3.6 ASM bacterial preparation

Overnight cultures were prepared under sterile conditions from single colonies isolated from MH agar plates. Bacteria were inoculated into Mueller-Hinton agar (MH) and grown overnight at 37°C 225rpm in a Labnet 211DS shaking incubator. The following day bacteria were quantified using Optical Density (OD) measurement. For the experimental setup, approximately 3×10^5 CFU/ml bacteria were added to 15ml of ASM in a 50ml tube. Bacteria were then incubated at 37°C, 225rpm in an Excella E25 shaking floor incubator. Where appropriate, cultures were supplemented with glucose.

4.3.7 Quantification of bacteria

P. aeruginosa was quantified by measuring luminescence using a Tecan microplate reader (Life Sciences). 200ml of pure culture was added into a white 96 well plate (Thermofisher). Background luminescence was subtracted by measuring no-bacteria culture. Colony Forming Units (CFU) per millilitre were calculated using the constructed standard curve. Bacterial culture was serially diluted in sterile PBS (Sigma-Aldrich) and plated onto the Mannitol salt agar plates. Plated bacteria were incubated at 37°C in a benchtop incubator for 24 hours and, formed colonies were then counted. Final CFU/ml was calculated using equation $\text{CFU/ml} = (\text{colony count} * \text{dilution factor}) / \text{volume of culture plated}$.

Both *P. aeruginosa* and *S. aureus* colonies were confirmed using MALDI-TOF Mass Spectrometry to ensure culture purity due to the absence of selective antibiotics in the media and plates.

4.3.8 Preparation of ASM media and variants

Table 2. *Composition of Artificial Sputum Medium*

Amount	Reagent	Source
5 g/L	Pig stomach mucin (MUC2)	Sigma-Aldrich
4 g/L	low molecular-weight salmon sperm DNA	Sigma-Aldrich
5 g/L	Sodium chloride	Sigma-Aldrich
2.2 g/L	Potassium chloride	Sigma-Aldrich
1.81 g/L	Tris base	Sigma-Aldrich
5.9 mg/L	diethylene triamine pentaacetic acid (DTPA)	Sigma-Aldrich
5 g/L	Casamino acids	ThermoFisher Scientific
5 ml/L	Egg yolk emulsion	Sigma-Aldrich

For 1L of ASM mucin, DNA, DTPA, NaCl, KCl, and Tris base and amino acids were dissolved in 800ml of distilled water using a magnetic stirrer. Once fully dissolved, pH was balanced to 7.0 using Tris base and volume was adjusted to 1L. ASM was then sterilised at 121 °C for 15 minutes. After sterilisation, ASM was thoroughly cooled, and egg yolk emulsion was added under sterile conditions. ASM was then aliquoted into 50ml tubes and kept in the fridge for up to 4 weeks.

To collect bacterial supernatant, ASM with bacteria was spun at 5200 RCF for 10 minutes at 4°C to halt growth. The supernatant was then moved into a new sterile 50ml tube without disturbing the pellet, and the pellet was discarded. If further sterilisation was required, the supernatant was autoclaved at 121 °C for 15 minutes.

4.3.9 Silver stain analysis

Protein changes in the ASM were analysed using Silver Staining. The total protein amount was quantified using Pierce™ BCA Protein Assay Kit (ThermoFisher). Staining was performed using the Pierce Silver Stain Kit (ThermoFisher) using standard manufacturers protocol. Stained proteins were visualised using NuPAGE™ 4 to 12% Bis-Tris gels (ThermoFisher).

The gel was washed twice in ultrapure water for 5 minutes each time. The gel was then fixed in 30% ethanol:10% acetic acid solution for 30 minutes, changing the solution once in between. The gel was then washed twice with 10% ethanol for 5 minutes and twice with ultrapure water for 5 min each. The gel was then sensitised with a sensitizer working solution for 1 minute. Following sensitisation, the gel was washed twice with ultrapure water for 1 min. The gel was then stained for 30 min using a stain working solution. Before developing, the gel was washed twice with ultrapure water for 20 s. The gel was then developed using a developer working solution for 2-3 minutes until the bands were clearly visible. The development was stopped by washing gel in 5% acetic acid for 10 minutes

4.3.10 Glucose measurement

Glucose utilisation by bacteria in the ASM was quantified by the glucose oxidase method using the Analox Glucose Analyzer GM9D (Analox Instruments) according to the manufacturer's guidelines. For the glucose measurement, bacterial supernatant was used, rather than a pure culture, to avoid machine contamination.

4.3.11 Lactate measurement

Lactate production in the ASM was measured using Lactate Assay Kit MAK064 (Sigma-Aldrich), following the manufacturer's guidelines. ASM supernatant was used for the lactate measurement. A total of 10 µl of 100 nmol/µl lactate standard was diluted with 990 µl lactate assay buffer to generate a 1 nmol/µl standard solution. The volumes of the 1 nmol/µl lactate

standard solution used to generate the standard curve were 0, 2, 4, 6, 8, and 10 µl. Lactate concentration in the sample was measured by reading absorbance at 570 nm on a transparent microplate reader. The final concentration was calculated using the standard curve.

4.3.12 Statistical analysis

The effect of glucose on bacterial growth was analysed using Two-Way ANOVA with factors including time and presence of glucose independently. Data are shown as individual points and/or mean $\pm$ standard deviation. Pearson correlation coefficient was calculated between bacteria growth and glucose depletion, the coefficient of determination (R^2) is presented. Significant differences are indicated with *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. GraphPad Prism version 9 (GraphPad Software) was used for statistical analysis and illustrations.

4.4 Results

4.4.1 The physical appearance of *S. aureus* and *P. aeruginosa* on agar plates

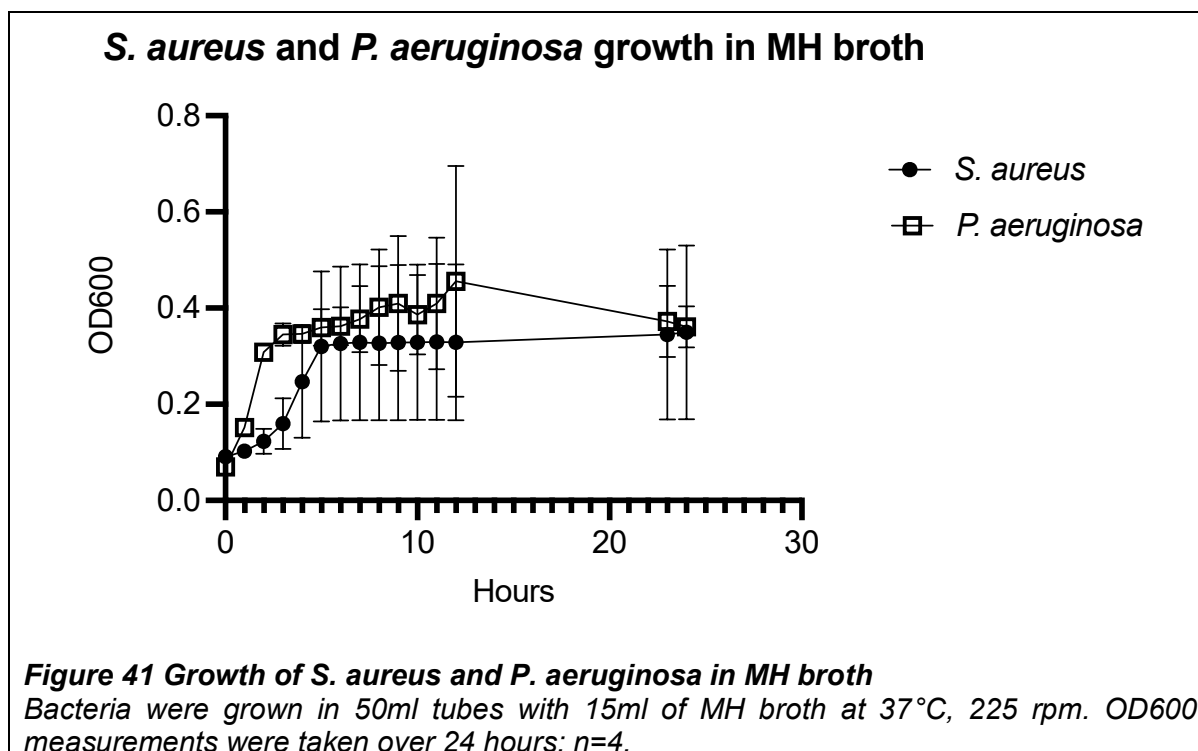
Staphylococcus aureus was grown in MH broth and, when cultured on mannitol salt agar plates, produced uniform yellow colonies. Colonies were distinctly separated, were visible and could be counted after 24-hour incubation at 37 °C. *S. aureus* grown in the ASM and cultured on mannitol salt agar plates produced distinctive uniform yellow colonies similar to that of *S. aureus* grown in MH broth. Colonies were visible and could be counted after 36-hour incubation at 37 °C, indicating that growth in ASM was slower.

Pseudomonas aeruginosa, grown in MH broth and cultured on MH agar plates, produced green colonies varying in size. Colonies were clustered together; however, single colonies could still be identified. Colonies were visible and could be counted after 16-hour incubation at 37 °C. *P. aeruginosa*, grown in the ASM and cultured on MH agar plates, also produced green colonies varying in size. However, colonies were frequently merged, making it difficult to identify single colonies and accurately calculate CFU. Colonies were visible after 16-hour incubation at 37 °C. Merging of colonies could be due to the increased biofilm and mucus production.

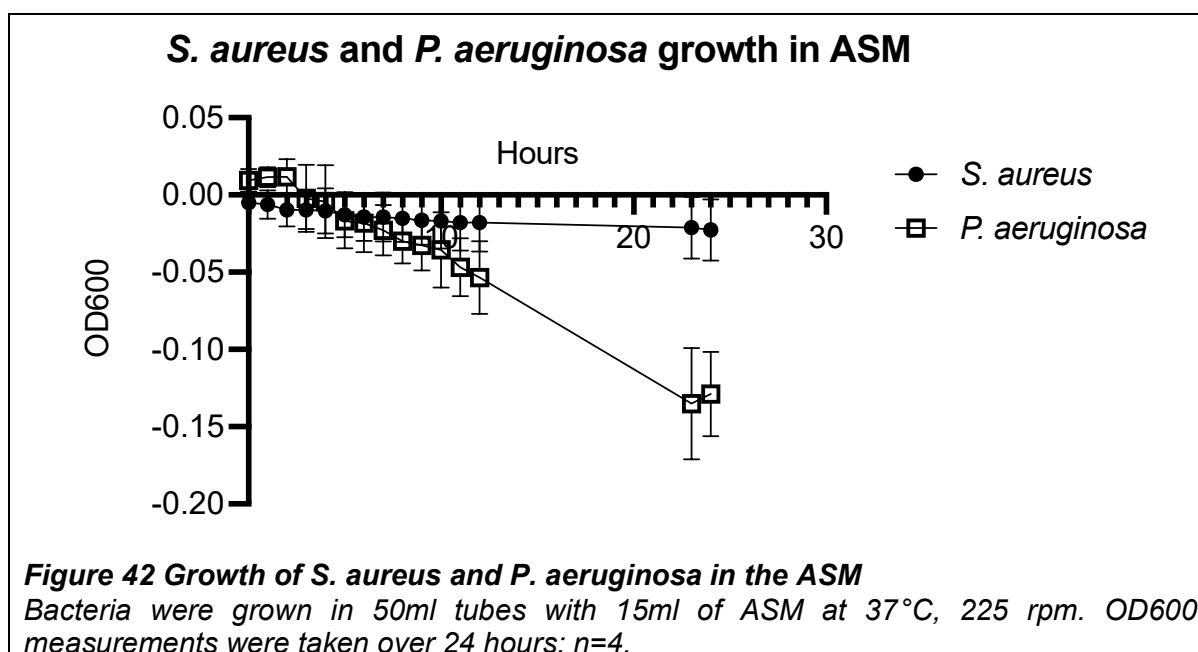
4.4.2 Quantification of bacteria

4.4.2.1 Quantification using OD600 measurement

Estimating bacterial growth by spectrophotometric measurements at 600 nm (OD600) is a standardised procedure widely used by microbiologists worldwide. It offers a cheap and quick method to estimate bacterial growth based on the increased turbidity due to bacterial growth. Growth of both *S. aureus* and *P. aeruginosa* in MH broth could easily be measured using this technique and offered no challenges (Figure 41).



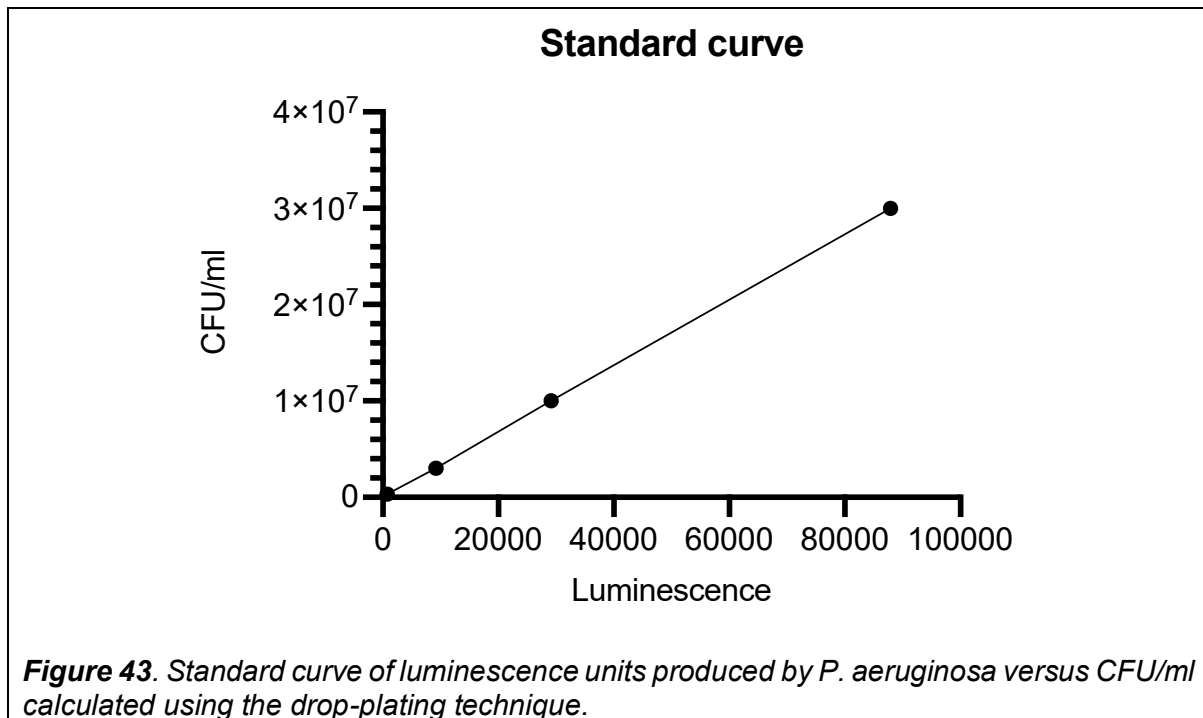
However, when OD was measured for *S. aureus* and *P. aeruginosa* over 24 hours, absorbance has steadily decreased for both pathogens indicating bacterial lysis (Figure 42). In order to confirm these observations, media was plated at various time points, and CFU was calculated. CFU calculations indicated strong bacterial growth throughout a 24-hour period, conflicting with spectrophotometry data. A decrease in OD could have been caused by bacteria-mediated changes in the ASM. Due to conflicting spectrophotometry results, it was decided not to employ spectrophotometry readings further in this study.



4.4.2.2 Quantification of *S. aureus* and *P. aeruginosa*

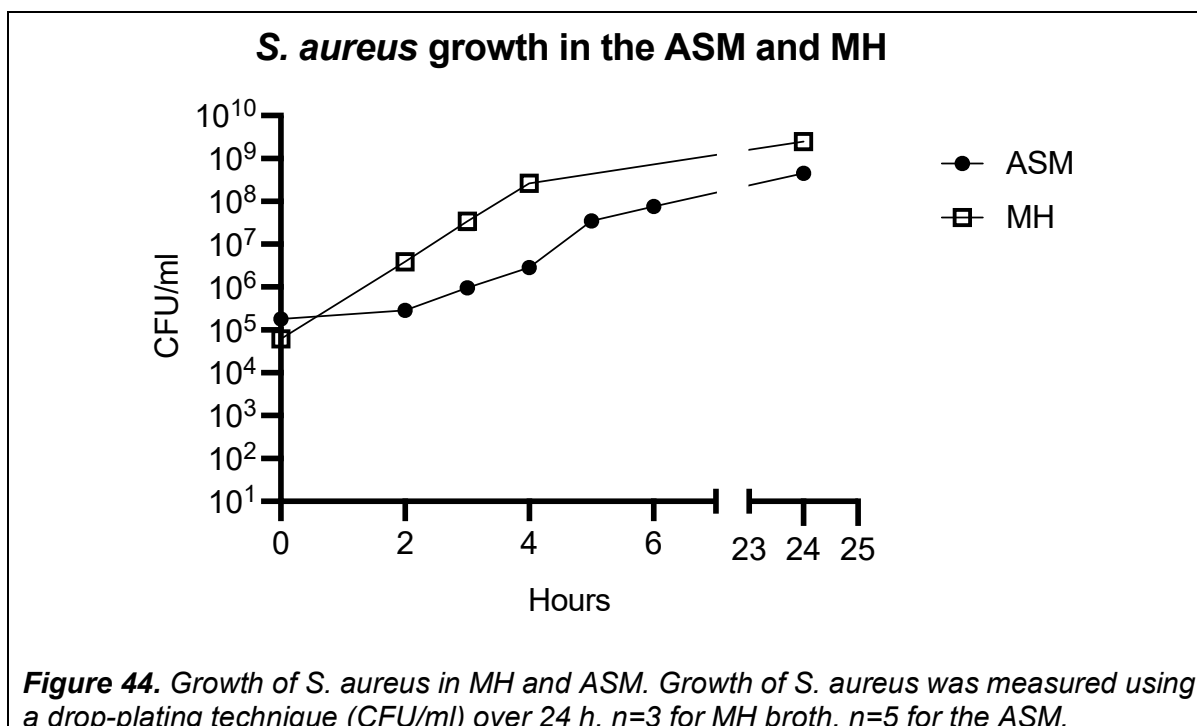
Growth of *S. aureus* and viable CFU were calculated by serially diluting the culture and plating bacteria on the mannitol salt agar. This technique allowed accurate quantification of the growth. By using Mannitol salt agar instead of the standard MH agar, it was possible to selectively grow *S. aureus* from the culture containing both *S. aureus* and *P. aeruginosa*.

As discussed in 4.4.1, *P. aeruginosa* grown in ASM, when plated, produced colonies that were hard to distinguish and quantify. Bioluminescent *P. aeruginosa* strain was utilised instead to observe growth directly. The growth of *P. aeruginosa* was quantified by measuring luminescence. Using a standard curve, luminescence units could be translated into the CFU/ml. By comparing live CFU bacterial count quantified by serial dilution to fluorescence output, the standard curve was constructed with a good correlation between bacterial growth and observed fluorescence (Figure 43). The ease of performing luminescence analysis allowed high throughput of experiments and allowed to balance out labour-intensive colony counting required for *S. aureus*.



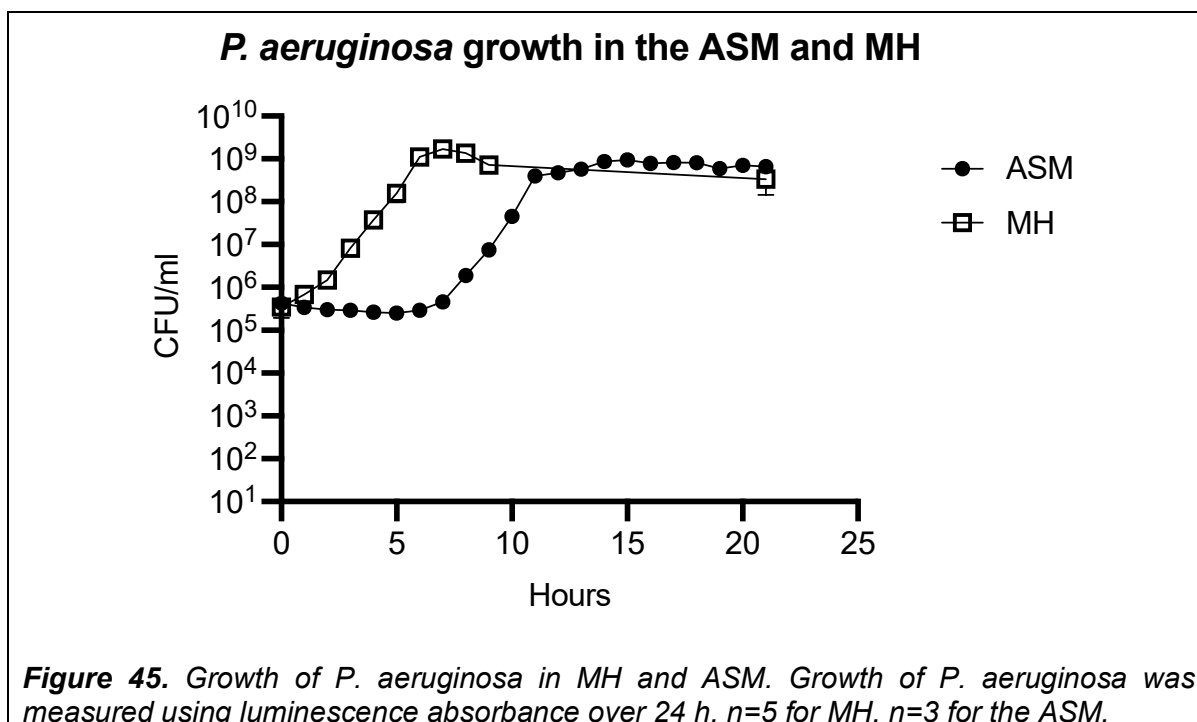
4.4.3 Bacterial growth in the ASM compared to MH

When added into the MH broth, *S. aureus* exhibited rapid growth, reaching 5×10^8 CFU/ml in 4 hours from a starting concentration of 9×10^4 CFU/ml. After 24 hours of incubation, the total amount of CFU in the culture reached 2×10^9 (n=3). *S. aureus* that was added into the ASM had a slower growth rate for the first 4 hours, reaching 7×10^7 CFU/ml from the initial 2×10^5 CFU/ml after 6 hours (n=5). After 24 hours of incubation, the final concentration of *S. aureus* in the ASM was 4×10^8 , almost ten times less than that of the MH broth.



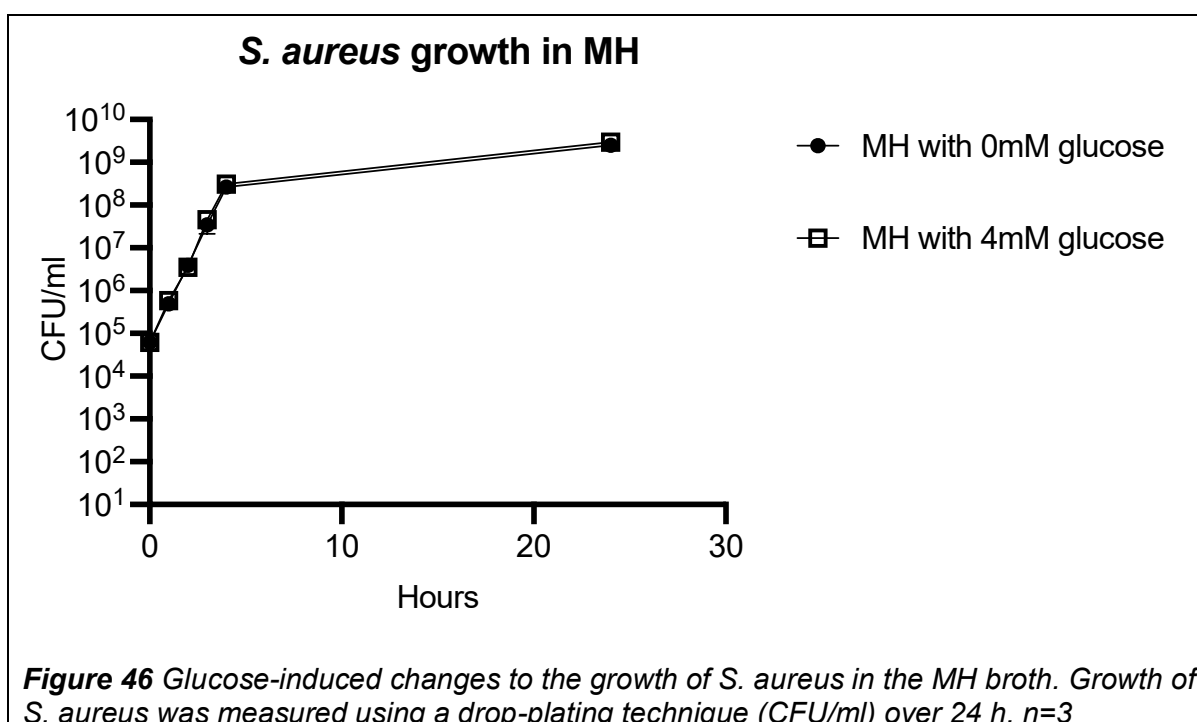
Like *S. aureus*, *P. aeruginosa* exhibited rapid growth in MH, reaching 1×10^9 CFU/ml in 6 hours, at which point growth seized, and CFU/ml plateaued ($n=5$). However, *P. aeruginosa* that was added to the ASM underwent a more prolonged lag phase of 7 hours ($n=3$) (Figure 45).

P. aeruginosa reached similar final CFU/ml concentrations in ASM to that of MH at 20-24 hours. These data indicate that *P. aeruginosa* requires several hours to begin active growth in nutrient-poor ASM media and that *S. aureus* adapted quicker to this growth medium.

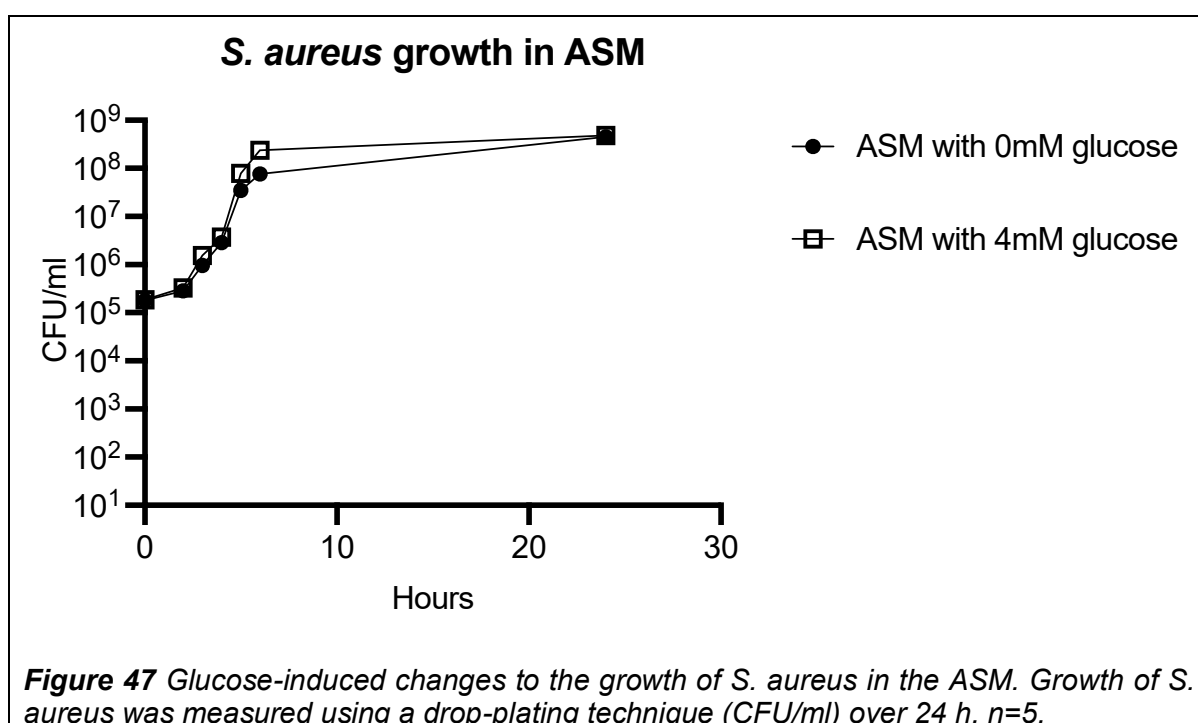


4.4.4 Bacterial growth in the ASM and MH in the presence of glucose

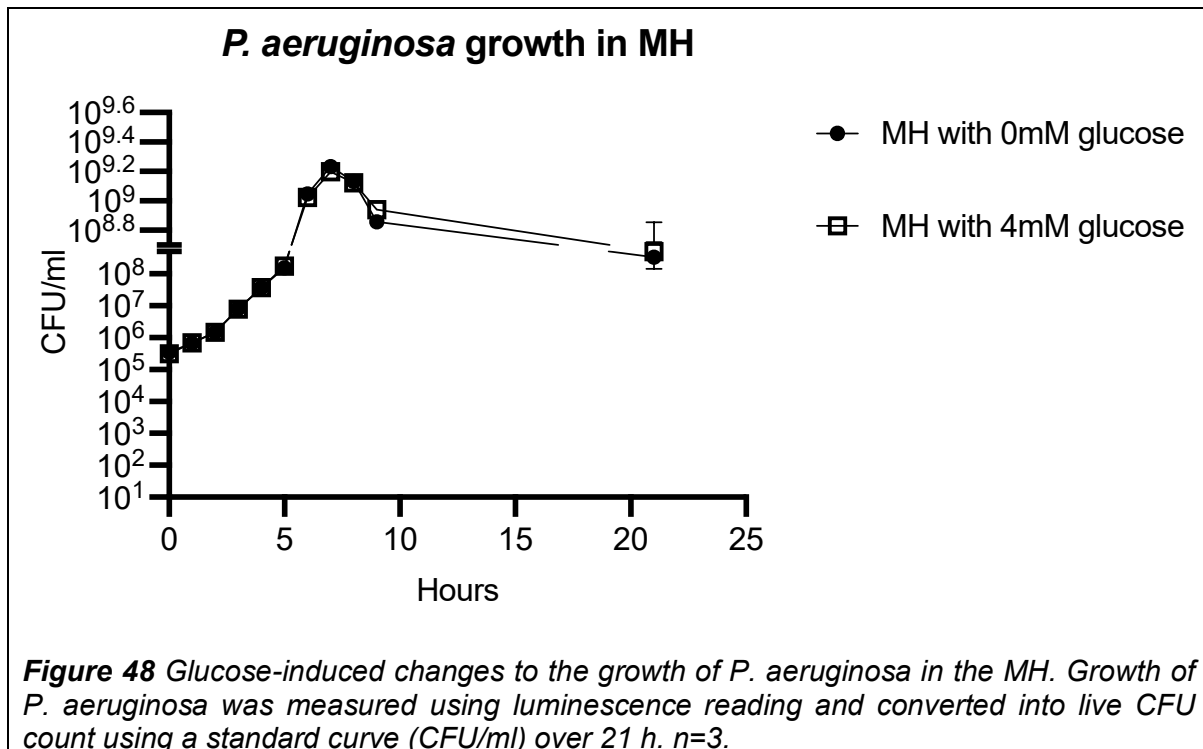
The addition of 4 mM glucose to the *S. aureus* culture in the MH broth did not affect the growth of the bacteria. The growth remained consistent in both normoglycaemic and hyperglycaemic conditions, and both cultures reached similar CFU/ml values after 24 hours of incubation.



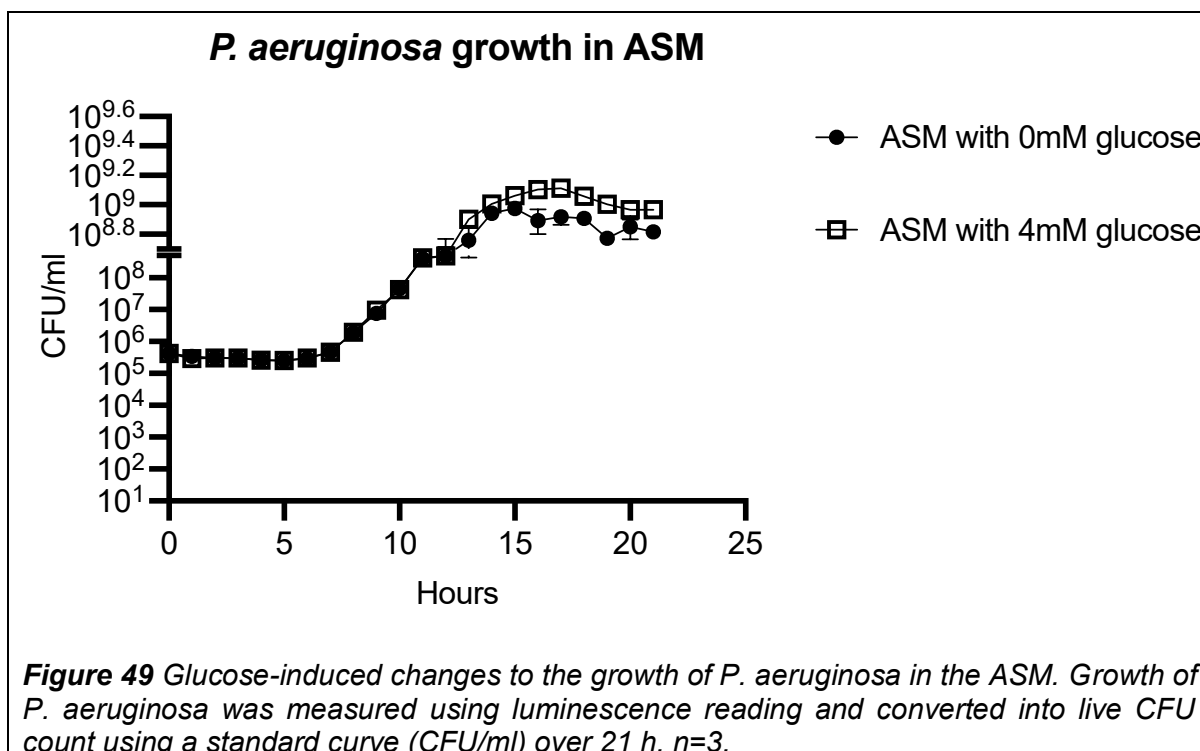
The addition of 4mM of glucose to the *S. aureus* cultures in the ASM stimulated bacterial growth. Two-way ANOVA showed that growth was dependent on both time and glucose ($p<0.0001$); and that glucose ($p=0.0037$) and time ($p<0.0001$) independently affected bacterial growth. However, the addition of glucose did not affect the final concentration of bacilli in the culture, reaching 4.5×10^8 in 0mM glucose and 4.8×10^8 CFU/ml in 4mM glucose ($n=5$). This demonstrates that the effects of glucose on bacterial growth can be observed in the low-nutrient environment of ASM and not in the high-nutrient environment such as MH.



Similar to *S. aureus*, the addition of glucose to MH broth did not affect the growth of *P. aeruginosa* ($n=5$). In both normoglycaemic and hyperglycaemic cultures, *P. aeruginosa* followed the same growth pattern as shown in Figure 48.

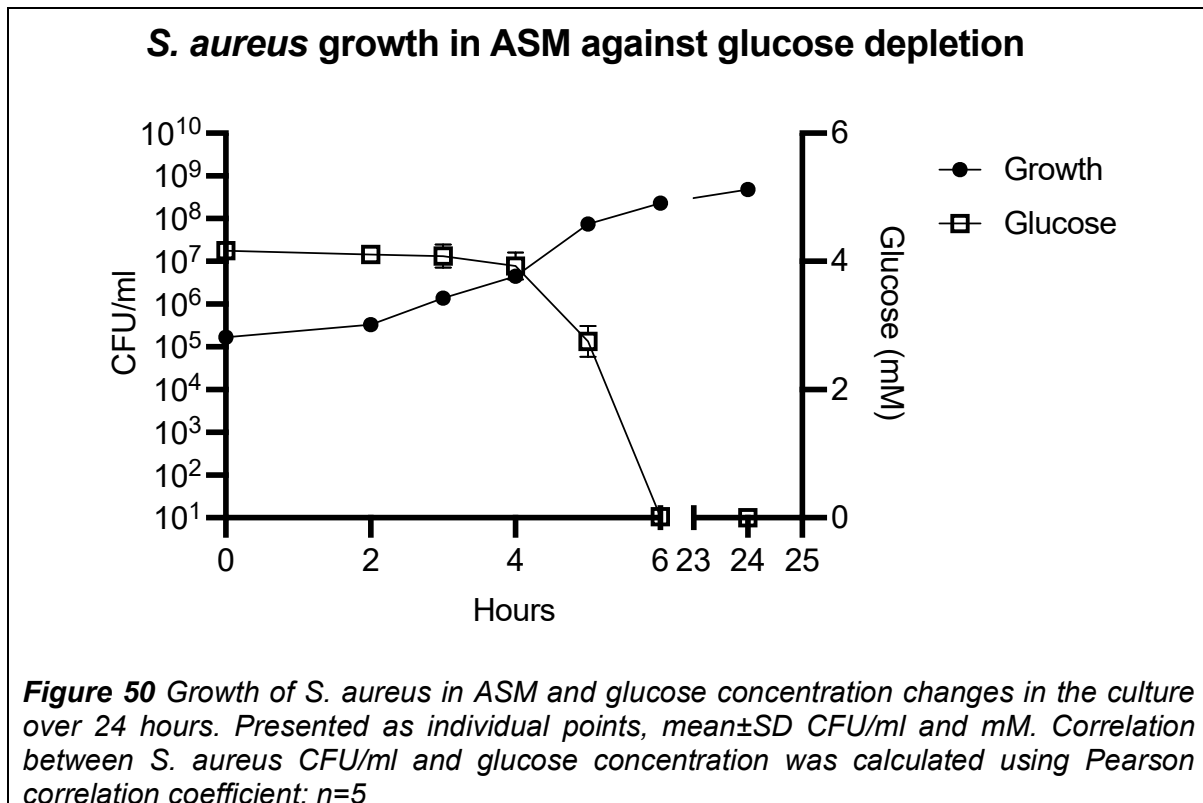


The addition of 4mM of glucose to the *P. aeruginosa* cultured in ASM affected the growth ($p=0.0006$, $n=3$) independently of time ($p<0.0001$). The addition of glucose did not affect the prolonged lag phase ($n=3$). However, at 12 hours, there was more *P. aeruginosa* in the hyperglycaemic ASM than the normoglycaemic ASM, and this difference was maintained for the rest of the experiment. After 16 hours of incubation in normoglycaemic ASM, there were approximately 7×10^8 CFU/ml, while in the hyperglycaemic ASM, there were approximately 1×10^9 CFU/ml of *P. aeruginosa* ($p=0.0022$, $n=3$).

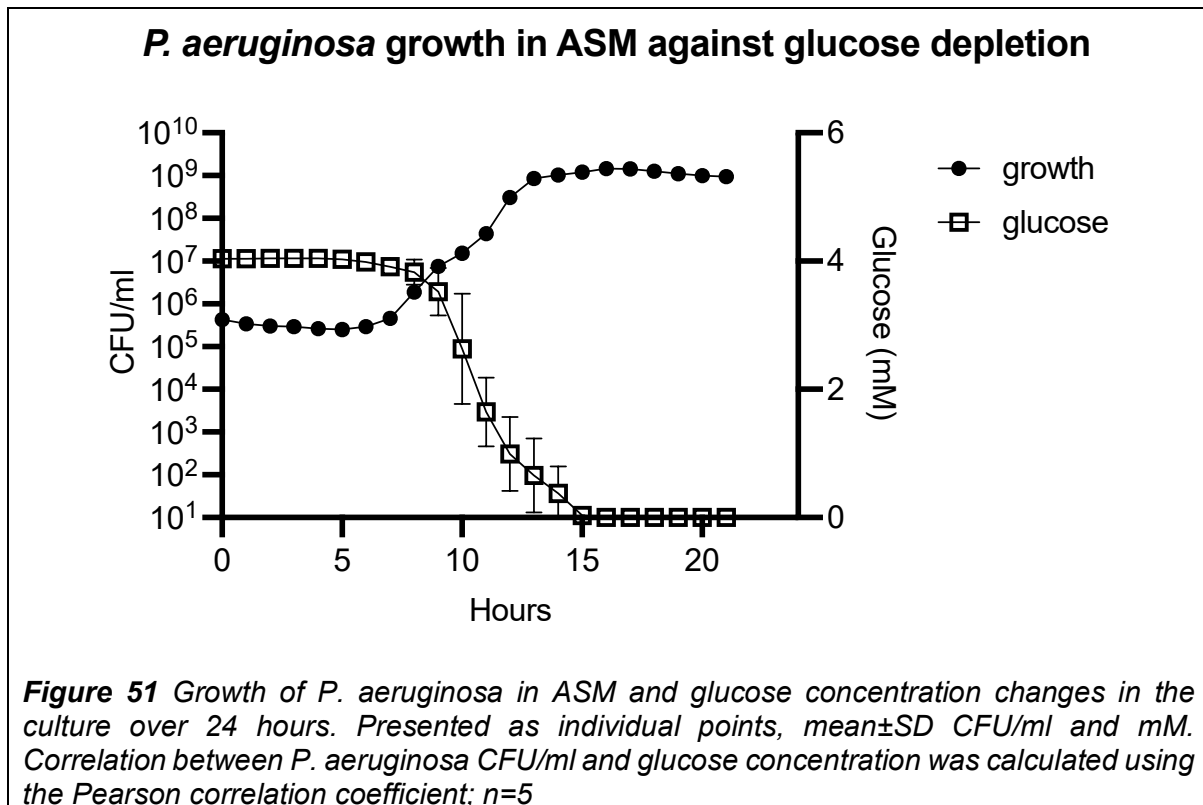


4.4.5 Bacterial growth and glucose depletion in the ASM

In ASM containing 4mM glucose, the glucose concentration did not significantly decrease in the first 4 hours after the addition of *S. aureus* into the media, while *S. aureus* increased from 1.5×10^5 to 4×10^6 CFU/ml. During the accelerated period of growth between 4 to 6 hours, all of the glucose in the media was depleted, while CFU/ml increased from 4×10^6 to 2.2×10^8 (Figure 50). From this point onwards, bacterial growth plateaued until the end of the experiment at the 24-hour mark. Pearson correlation coefficient showed that bacterial growth correlated with glucose depletion ($R=0.8343$, $p=0.004$, $n=5$).



During the lag phase of *P. aeruginosa* in the ASM, which lasted the first 7 hours, no bacterial growth was observed, and minimal glucose depletion occurred. *P. aeruginosa* underwent a rapid growth between 7 -13 hours, increasing in numbers from 4×10^5 to 8×10^8 CFU/ml, and glucose concentration in the ASM decreased from 4mM to ~ 0.65 mM and was undetectable in the following 2 hours. After 13 hours, *P. aeruginosa* plateaued at approximately 1×10^9 CFU/ml until the end of the experiment (Figure 51). Pearson correlation coefficient showed that *P. aeruginosa* growth correlated with glucose depletion ($R=0.8528$, $p<0.0001$, $n=5$)



4.4.6 Bacterial lactate production and depletion in the ASM

As was previously demonstrated, *S. aureus* growth correlated with depletion of glucose. I have speculated that an increase in lactate concentration proportional to the decrease in glucose concentration would be observed. While lactate concentration increased in the first 2 hours, it was quickly depleted in the subsequent 2 hours and reached 0 by hour 5. At the 6-hour mark, both the glucose and lactate concentration in the media were at 0 and remained constant until the experiment's end at 24 hours. There was no statistically significant relationship between glucose and lactate concentrations.

Depletion of glucose and lactate by *S. aureus* in the ASM

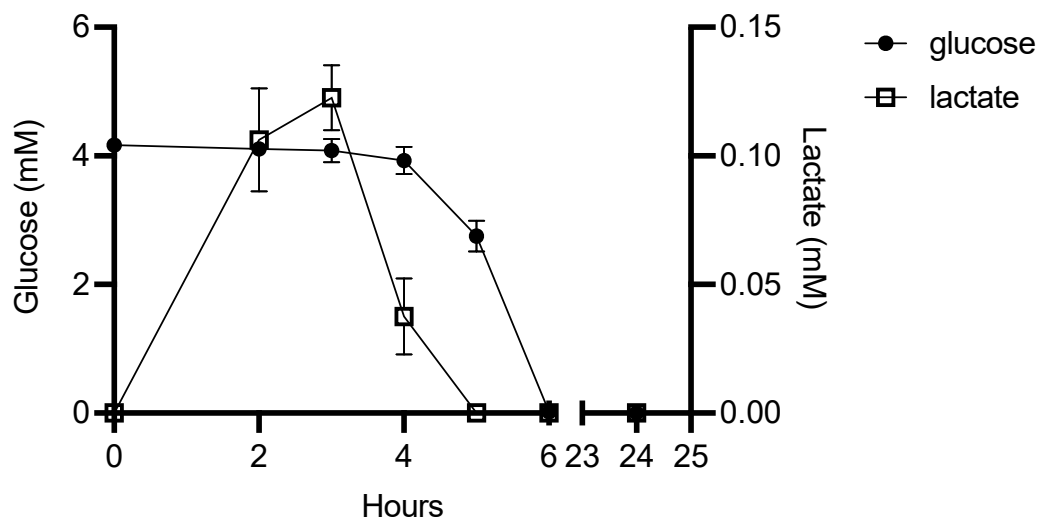
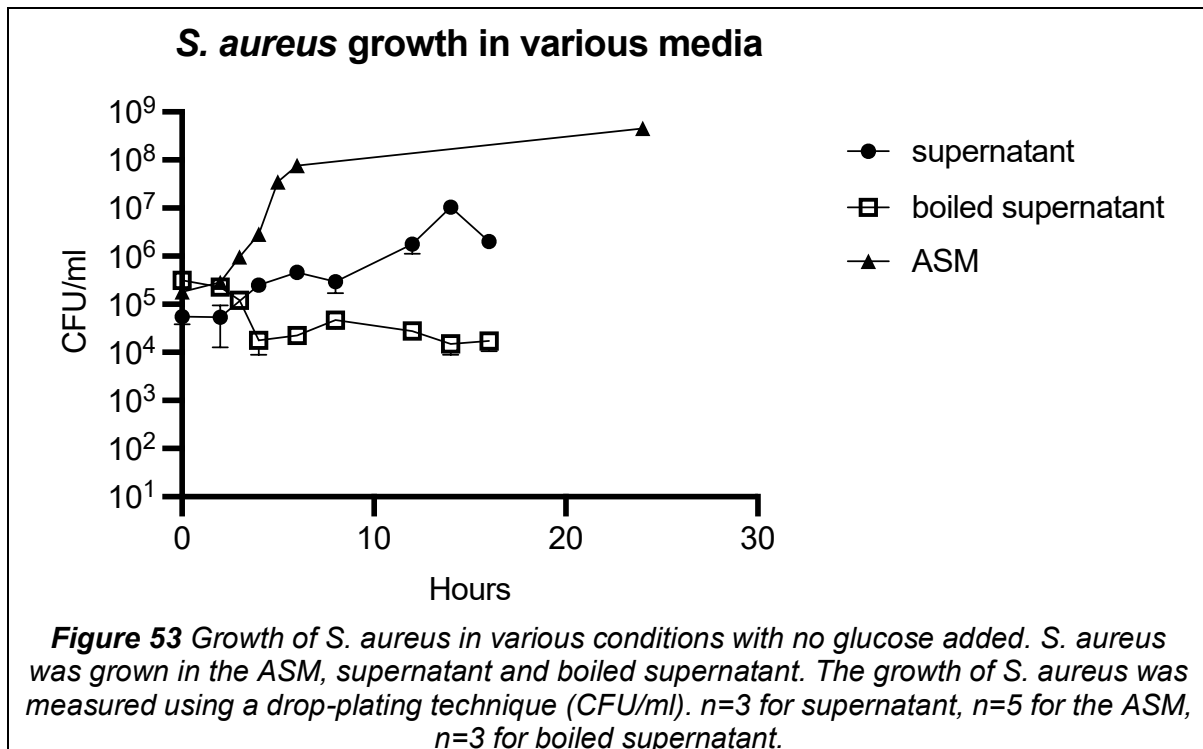


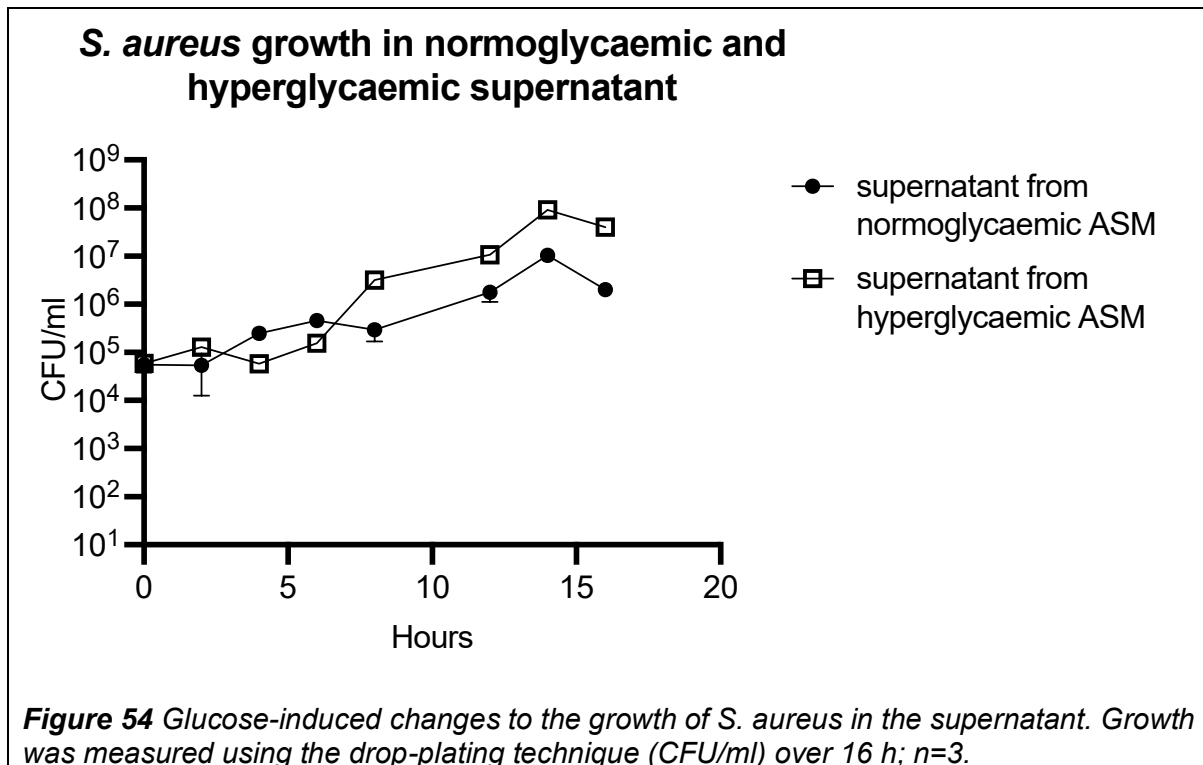
Figure 52 Lactate and glucose concentration changes in *S. aureus* culture in ASM were observed over 24 hours. Presented as individual points, mean $\pm$ SD mM. Correlation between lactate and glucose concentration was calculated using Pearson correlation coefficient; $n=5$

4.4.7 *S. aureus* growth in the ASM, supernatant, and boiled supernatant in presence and absence of glucose

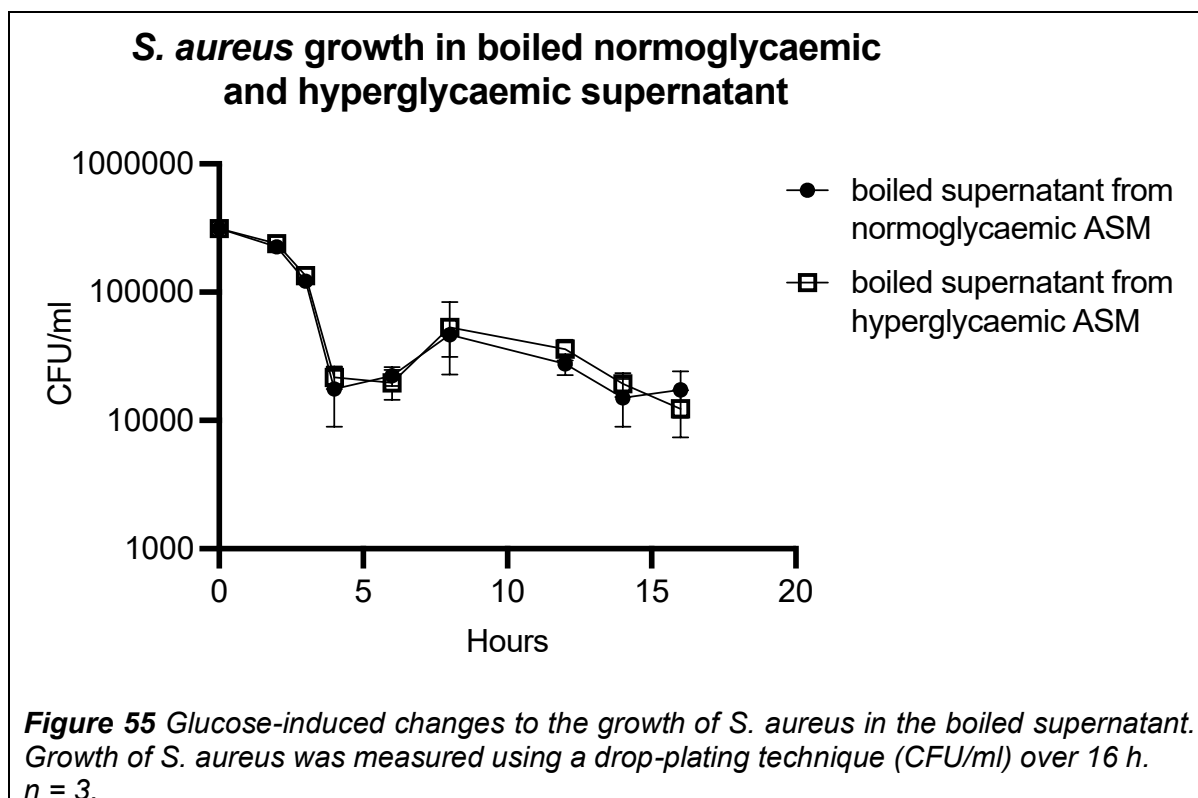
S. aureus showed consistent growth in the ASM, reaching 7×10^7 CFU/ml in 6 hours, with a peak of 4×10^8 after 24 hours of growth. In the supernatant, *S. aureus* did not exhibit the same growth patterns as in ASM. The number of live bacteria in the culture peaked after 14 hours at 1×10^7 CFU/ml. In the following hours live population of bacteria declined. The growth rate was much slower than what was observed in the pure ASM culture. In the boiled supernatant, *S. aureus* did not exhibit any growth. Within the first 3 hours, the bacterial population declined from 3×10^5 to 3×10^4 . CFU/ml remained relatively stable at 3×10^4 through 16 hours of incubation.



The presence of glucose in the ASM at the supernatant generation stage was shown to significantly affect the subsequent bacterial growth in said supernatant (Figure 54, $p=0.0003$, $n=3$). While both supernatants were glucose-free at the time of the experiment, *S. aureus* grown in the supernatant derived from hyperglycaemic ASM had peak live bacteria count 19-fold higher than that of the *S. aureus* grown in the supernatant derived from normoglycaemic ASM. This demonstrates that the presence of glucose plays an important role at the supernatant generation stage, and the effects of glucose on bacterial growth extend past the simple concept of a nutrient supplement.



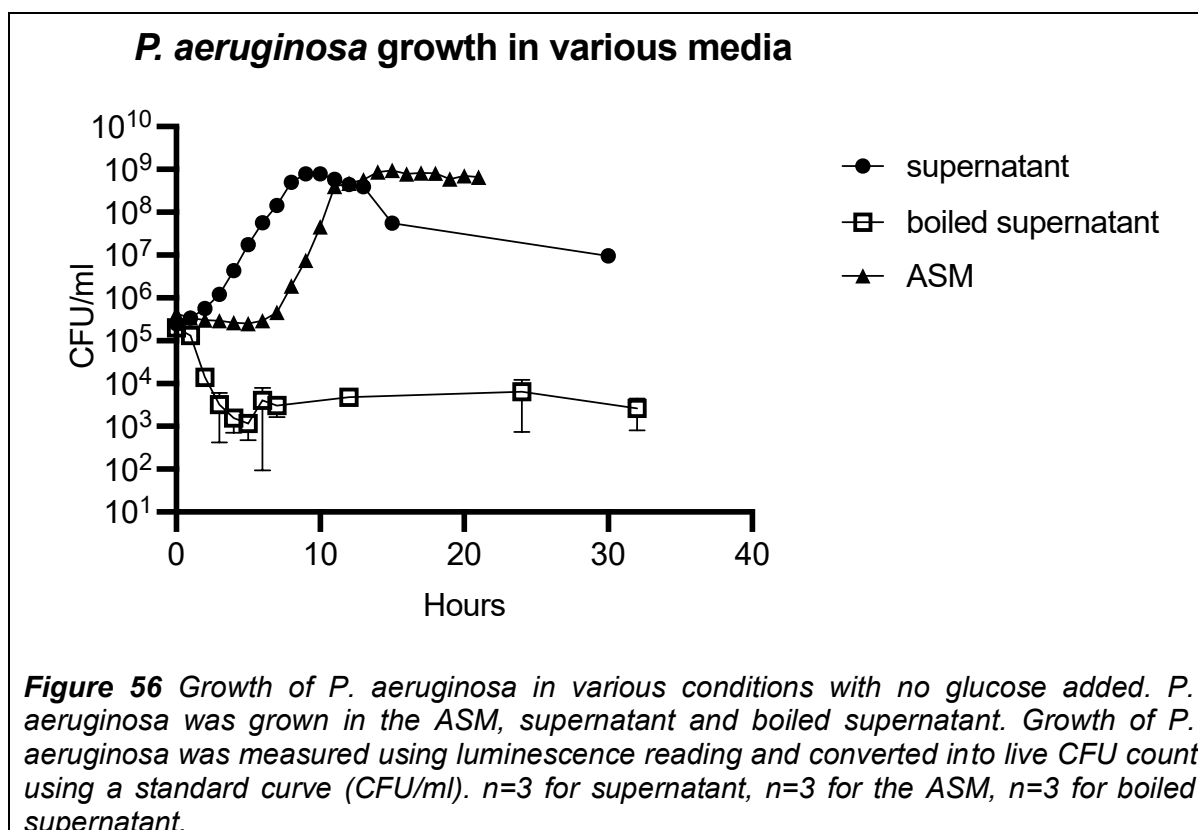
As previously discussed, *S. aureus* did not exhibit growth in the boiled supernatant, and the number of live bacteria remained stable for the 16 hour-incubation. Whether media was derived from the hyperglycaemic or normoglycaemic ASM prior to boiling produced no significant effect on the bacterial growth rate. *S. aureus* exhibited similar growth patterns in both media.



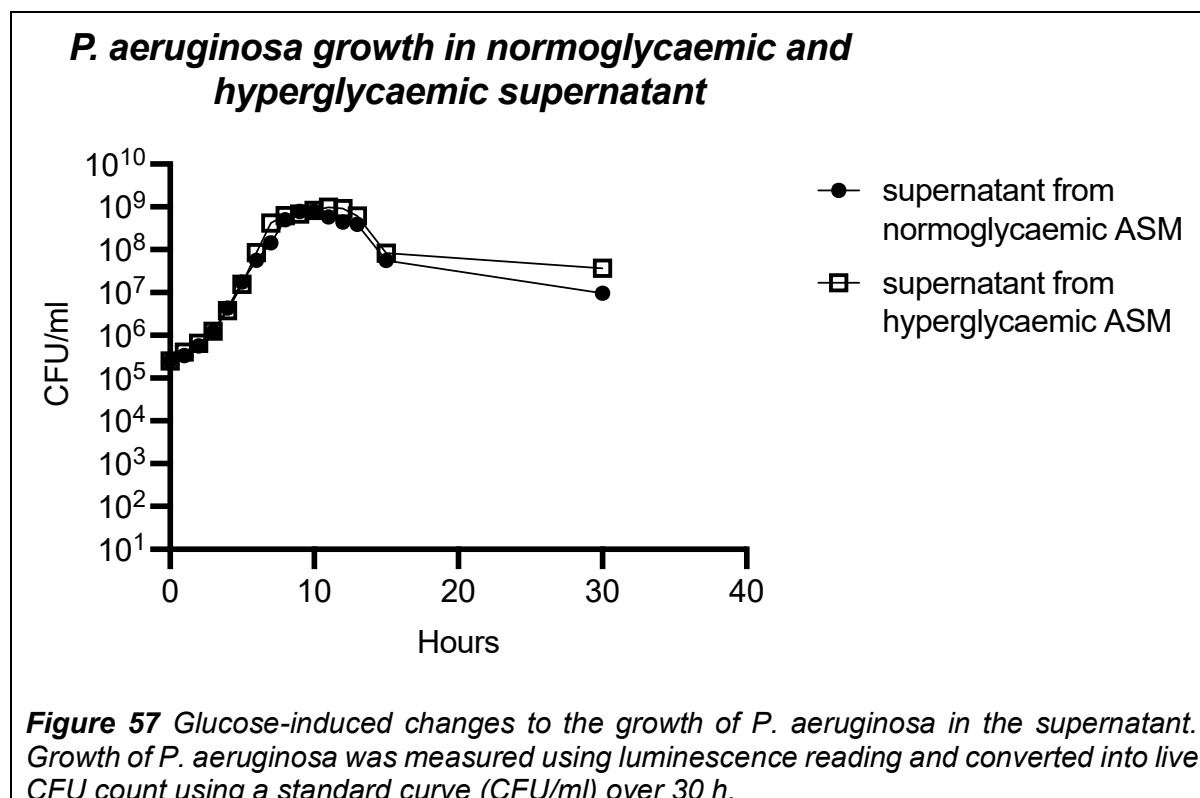
4.4.8 *P. aeruginosa* growth in the ASM, supernatant, and boiled supernatant in presence and absence of glucose

The growth of *P. aeruginosa* was severely altered depending on the media it was grown in. When added into the pure ASM, *P. aeruginosa* did not show any growth in the first 7 hours, where the number of live bacteria remained stable. After 7 hours, *P. aeruginosa* began rapid growth, where the concentration of live bacteria in the media increased from 4×10^5 CFU/ml to 4×10^8 CFU/ml. Growth has plateaued after 12 hours and remained stable at 7×10^8 CFU/ml until the end of the experiment. When *P. aeruginosa* was added into the supernatant, which was derived from *S. aureus* culture, it did not show the initial lag phase seen in the pure ASM. Instead, *P. aeruginosa* began rapid growth and reached the peak CFU/ml after 9 hours of incubation. The peak live bacteria amount in the supernatant was similar to that of the ASM, with a plateau at 8×10^8 CFU/ml. When *P. aeruginosa* was added into the boiled supernatant, a rapid drop in the live bacteria was observed, with the number of live bacteria decreasing

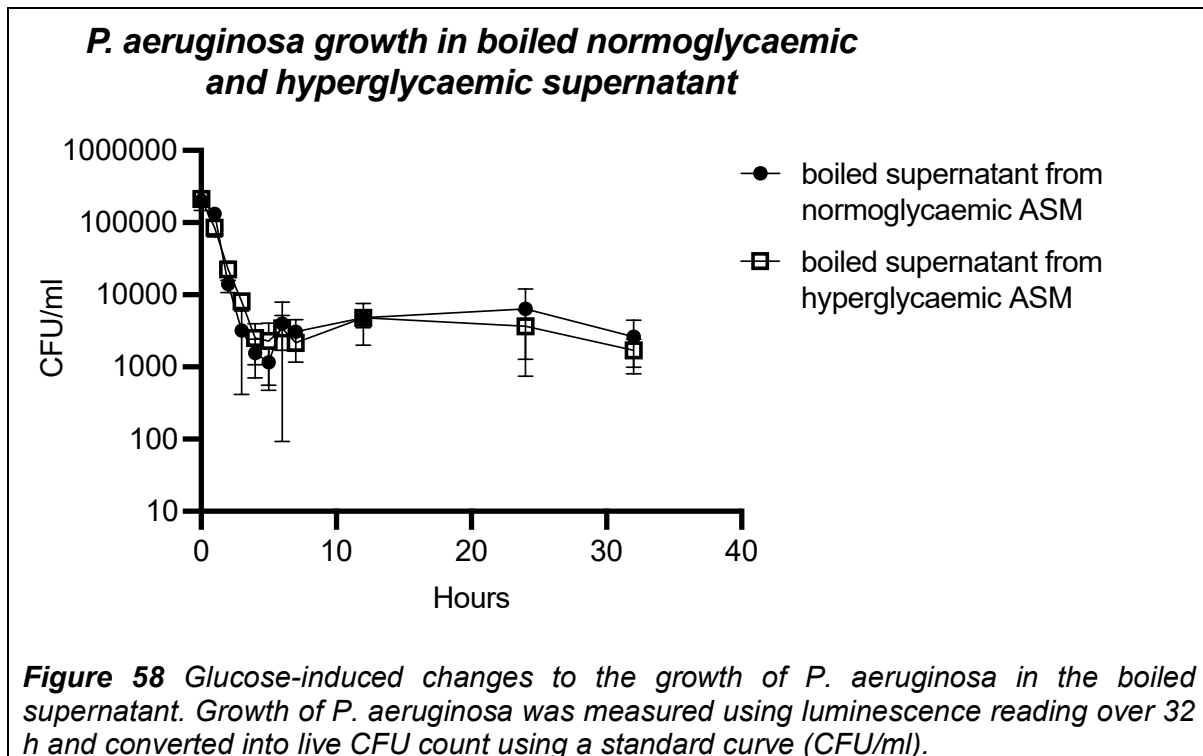
from the initial 2×10^5 CFU/ml to 5×10^3 CFU/ml over the first 3 hours. After the initial decrease, the amount of live bacteria remained stable at 4×10^3 CFU/ml for the next 30 hours.



Whether supernatant was derived from the normoglycaemic or hyperglycaemic ASM did not affect the growth of *P. aeruginosa*. In both supernatants, *P. aeruginosa* began rapid growth upon introduction to the media and reached a plateau simultaneously (Figure 57, $n=3$).

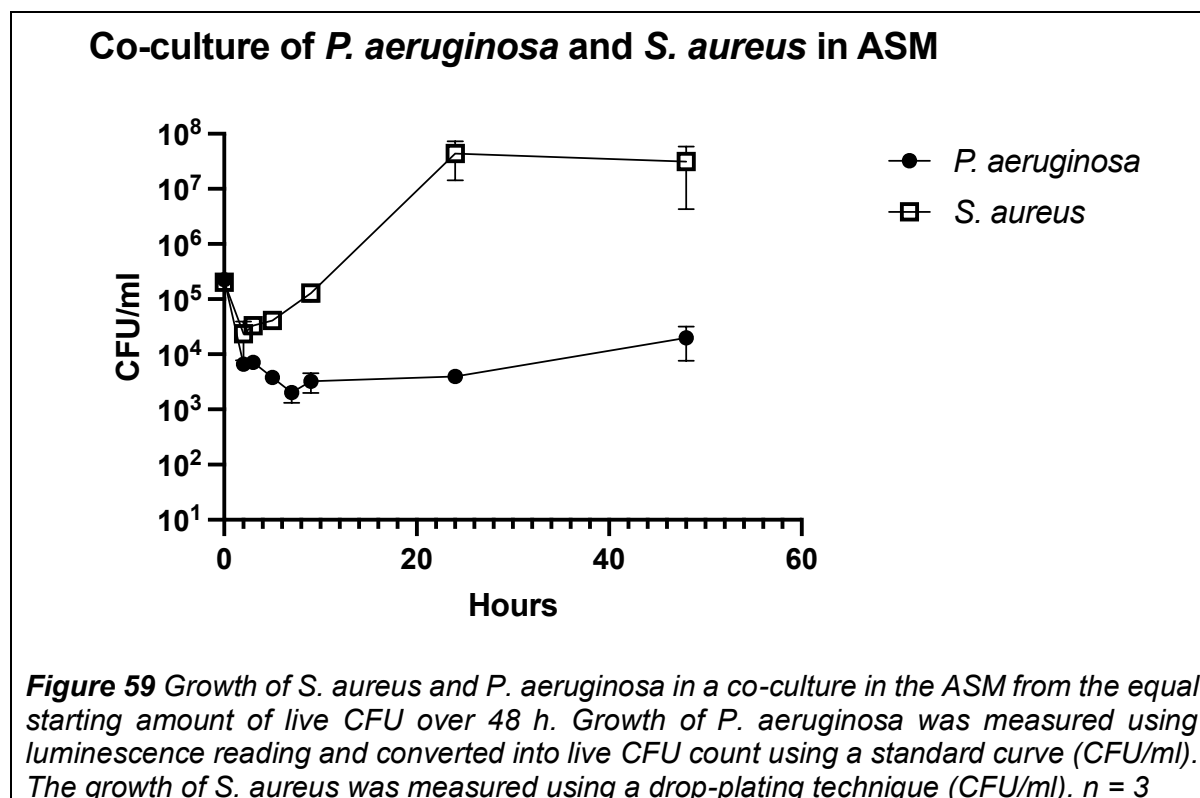


Similar to the growth in the supernatant, whether boiled supernatant was derived from the normoglycaemic or hyperglycaemic ASM did not affect the growth of *P. aeruginosa*. No growth was observed in either media.



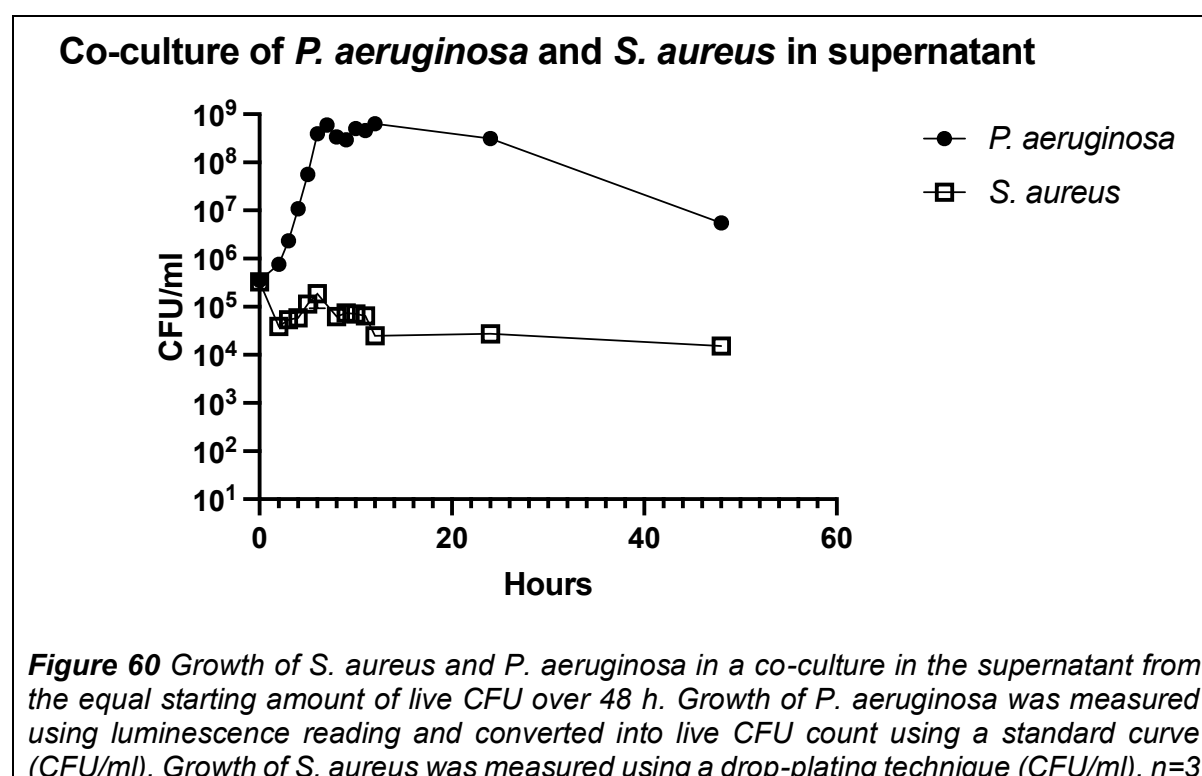
4.4.9 Co-culture of *S. aureus* and *P. aeruginosa* in the ASM and derived media

To assess the growth of *S. aureus* and *P. aeruginosa* in a scenario of co-infection, I attempted to create a stable co-culture by simultaneously adding both bacteria in equal amounts. This simulated scenario where a patient is infected with both pathogens simultaneously without prior history of infections. When the same amount of *S. aureus* and *P. aeruginosa* were added into the ASM, the amount of both bacteria decreased in the first two hours. *S. aureus* later recovered and in 22 hours increased from 5×10^4 to 9×10^7 CFU/ml, where it plateaued. *P. aeruginosa*, on the other hand, did not recover in the same manner and continued to decrease until it reached 2×10^3 CFU/ml after 7 hours of incubation. The amount of *P. aeruginosa* remained stable in the culture, increasing slightly from 2×10^3 CFU/ml at 7 hours to 2×10^4 CFU/ml at 48 hours (Figure 59).



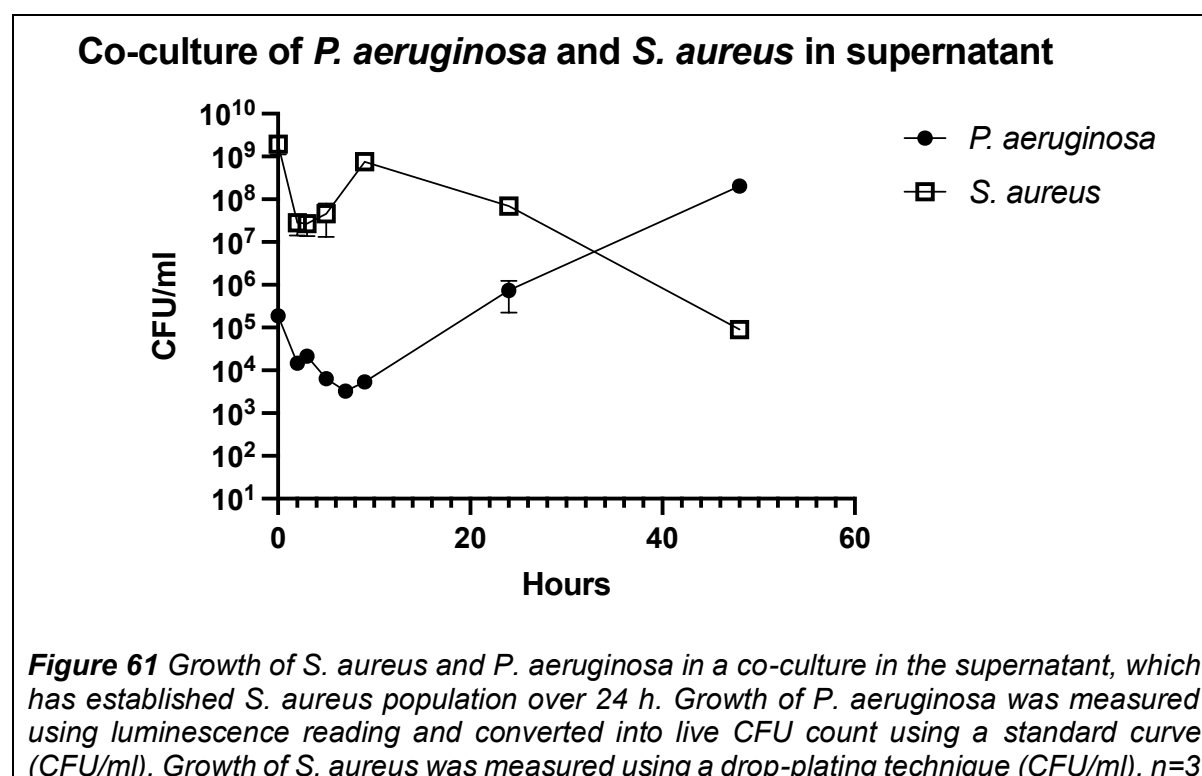
It is, however, unusual for both pathogens to simultaneously infect a host who has not previously been exposed to either of these pathogens. As previously discussed, in individuals with CF, I observe initial infection with *S. aureus*, which later evolves into co-infection of *S. aureus* and *P. aeruginosa* or becomes a mono-infection of *P. aeruginosa*. To simulate a scenario where both pathogens are present in a patient with a previous *S. aureus* infection, I added both pathogens in equal numbers into the supernatant derived from 24-hour *S. aureus* culture. When the same amount of *S. aureus* and *P. aeruginosa* were added into the supernatant media, I observed a different growth pattern than the one seen in Figure 59. Unlike with the ASM, *P. aeruginosa* did not exhibit a decrease of live CFU in the first hours and instead immediately began rapid growth. *P. aeruginosa* numbers increased from 3×10^5 CFU/ml to 4×10^8 in just 6 hours. For the next 18 hours amount of live CFU of *P. aeruginosa* in the culture remained stable around 5×10^8 , decreasing slowly between 24 hours and 48 hours. Unlike *P. aeruginosa*, *S. aureus* initial numbers decreased 10 times in the first two hours. The amount of live *S. aureus* in the culture remained low for the experiment duration, fluctuating

between 1×10^4 and 9×10^4 CFU/ml (Figure 60). Co-culture of *P. aeruginosa* and *S. aureus* in the supernatant derived from *S. aureus* culture demonstrated completely inverted growth compared to the ASM co-culture, with *P. aeruginosa* exhibiting rapid growth while *S. aureus* numbers remained low. This demonstrates that priming media with *S. aureus* affects subsequent growth of *P. aeruginosa* in co-culture, enabling rapid growth upon introduction into new media.



To better mimic *in vivo* model where *P. aeruginosa* infects patients with a stable infection of *S. aureus*, I allowed *S. aureus* to grow in the ASM for 24 hours. At this time-point, 2×10^5 CFU/ml of *P. aeruginosa* was added into the media. After 24 hours of incubation, *S. aureus* had plateaued at 2×10^9 and remained in the stationary phase. Immediately after the addition of *P. aeruginosa*, both pathogens experienced a rapid drop in the live CFU numbers. In the first two hours, *P. aeruginosa* amount decreased from 2×10^5 to 1×10^4 CFU/ml, while *S. aureus* amount decreased from 2×10^9 to 3×10^7 CFU/ml. It took 9 hours for *S. aureus* to recover to initial values, while the amount of live CFU of *P. aeruginosa* continued to decrease for 9 hours. Between 9 hours and 24 hours of incubation, however, the amount of *P. aeruginosa* rapidly

increased from 5×10^3 to 1×10^6 CFU/ml, while *S. aureus* decreased 10 to 7×10^7 . Between 24- and 48-hours, *P. aeruginosa* continued to grow while the amount of *S. aureus* decreased. After 48 hours in the culture, there was 2×10^8 CFU/ml of *P. aeruginosa* and 9×10^4 CFU/ml of *S. aureus* (Figure 61). This demonstrated the ability of *P. aeruginosa* to outgrow *S. aureus* in the established *S. aureus* media.



4.4.10 Effects of glucose on the establishment of co-culture of *S. aureus* and *P. aeruginosa* in the ASM

The addition of glucose into the ASM had no significant effects on the growth of either *P. aeruginosa* or *S. aureus* when analysed using two-way ANOVA. Both bacteria followed similar growth patterns as in the normoglycaemic media.

Co-culture of *P. aeruginosa* and *S. aureus* in ASM

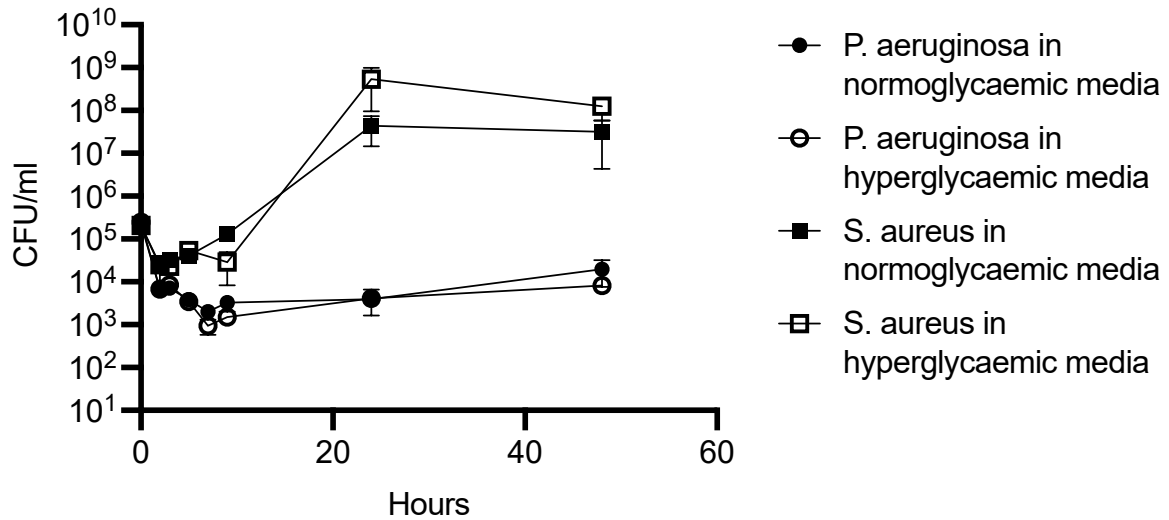


Figure 62 Glucose-induced changes to the growth of *S. aureus* and *P. aeruginosa* in a co-culture in the ASM from the equal starting amount of live CFU over 48 h. Growth of *P. aeruginosa* was measured using luminescence reading and converted into live CFU count using a standard curve (CFU/ml). Growth of *S. aureus* was measured using a drop-plating technique (CFU/ml). $n=3$

Whether supernatant was derived from the hyperglycaemic or normoglycaemic ASM significantly affected the growth of both *S. aureus* ($p=0.0027$, $n=3$) and *P. aeruginosa* ($p=0.0089$, $n=3$) when analysed using two-way ANOVA (Figure 63). The presence of glucose in the source ASM, however, did not change the overall behavioural patterns of the two pathogens. *P. aeruginosa* dominated the culture from the initial culture set up a step until the end of the experiment 48 hours later, even though *S. aureus* grew noticeably better in the supernatant derived from the hyperglycaemic ASM. This shows that while the presence of glucose at the supernatant generation stage plays an important role for both pathogens, it does not, however, dramatically alter the competition between two pathogens.

Co-culture of *P. aeruginosa* and *S. aureus* in supernatant

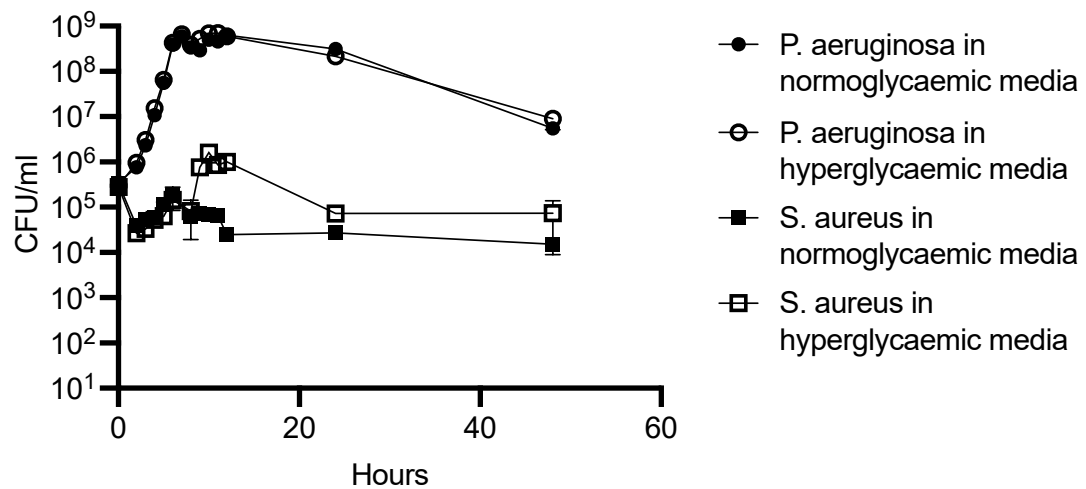


Figure 63 Glucose-induced changes to the growth of *S. aureus* and *P. aeruginosa* in a co-culture in the supernatant from the equal starting amount of live CFU over 48 h. Growth of *P. aeruginosa* was measured using luminescence reading and converted into live CFU count using a standard curve (CFU/ml). Growth of *S. aureus* was measured using a drop-plating technique (CFU/ml). $n=3$.

Whether *S. aureus* grew for 24 hours in normoglycaemic or hyperglycaemic ASM prior to the addition of *P. aeruginosa* did not change the overall growth patterns of the two pathogens. Media was initially dominated by *S. aureus* and was later overtaken by rapidly growing *P. aeruginosa*. The presence of glucose significantly affected the growth of *S. aureus* ($p=0.0003$, $n=3$) and did not significantly alter the growth of *P. aeruginosa* when analysed using two-way ANOVA.

Co-culture of *P. aeruginosa* and *S. aureus* in supernatant

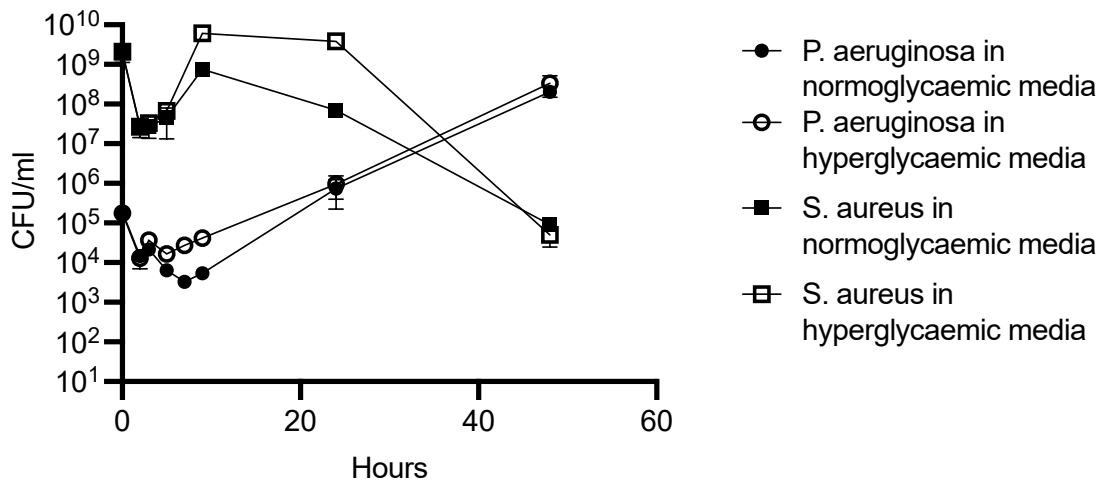


Figure 64 Glucose-induced changes to the growth of *S. aureus* and *P. aeruginosa* in a co-culture in the ASM which has established *S. aureus* population over 48 h. Growth of *P. aeruginosa* was measured using luminescence reading and converted into live CFU count using a standard curve (CFU/ml). Growth of *S. aureus* was measured using a drop-plating technique (CFU/ml). $n=3$.

4.4.11 Establishing stable co-culture of *S. aureus* and *P. aeruginosa* in a 50:50 mix of ASM and supernatant

To establish an *S. aureus* and *P. aeruginosa* co-culture model where both respiratory pathogens exist in balance, ASM was mixed 50:50 with supernatant and 4mM of glucose was added to the hyperglycaemic media mix. Similar amounts of *S. aureus* and *P. aeruginosa* were added simultaneously to the media; both pathogens exhibited rapid growth, with *S. aureus* reaching 1×10^9 CFU/ml after 24 hours of incubation, while *P. aeruginosa* reaching 1×10^8 CFU/ml after 24 hours of incubation. While *P. aeruginosa* remained stable in the culture at 5×10^8 CFU/ml, *S. aureus* decreased to 2×10^6 CFU/ml but remained at this level from 48-72 hours (Figure 65). The presence of glucose in the co-culture did not significantly affect the growth of either *S. aureus* or *P. aeruginosa*. Thus, I have demonstrated the possibility of establishing a stable co-culture of *S. aureus* and *P. aeruginosa* over 72 hours in a controlled environment.

Co-culture of *P. aeruginosa* and *S. aureus* in the 50:50 ASM-supernatant

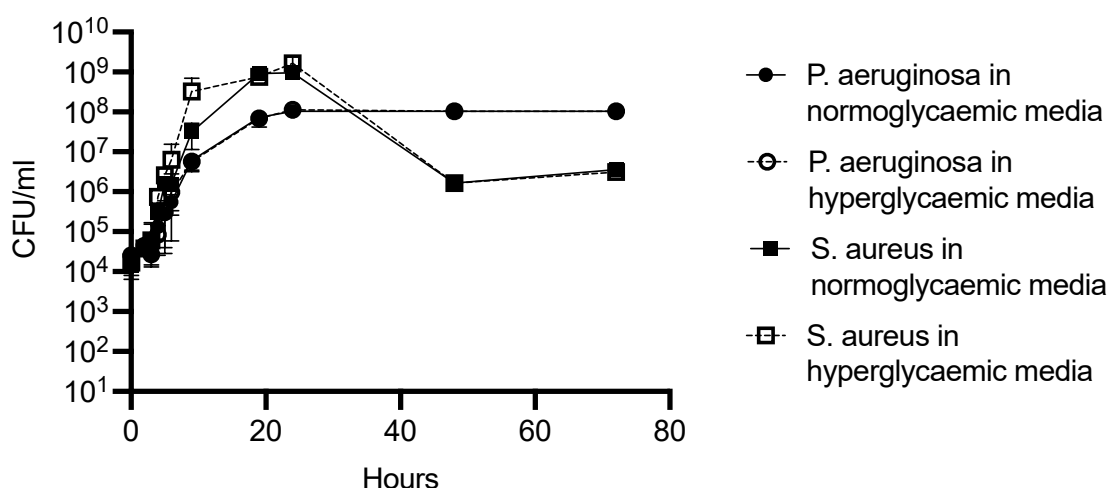


Figure 65 Co-culture of *P. aeruginosa* and *S. aureus* in 50:50 ASM-supernatant mix. Equal amounts of *S. aureus* and *P. aeruginosa* were added to the culture. Glucose was initially added into the culture at the supernatant generation stage. Additional glucose was then added at the stage of inoculation. The growth of pathogens was observed over 72 hours. Presented as individual points, mean±SD CFU/ml. The effect of glucose was compared using two-way ANOVA.

4.4.12 Silver staining of supernatant

As observed in 4.7 and 4.8, both *S. aureus* and *P. aeruginosa* have different growth patterns in the supernatant compared to the ASM and do not grow in the supernatant that was boiled. I theorise that boiling denatures proteins and peptides used by pathogens for growth. It was also observed that the presence of glucose at the supernatant generation stage affected the growth of *S. aureus* in the supernatant. While glucose did not have the same effect on *P. aeruginosa*, whether it was grown in ASM or supernatant changed the growth pattern, removing the prolonged lag phase in the supernatant compared to the ASM. In order to investigate the changes that ASM undergoes in the presence of *S. aureus* and glucose, I looked at the protein profiles of ASM and both supernatants derived from hyperglycaemic and normoglycaemic ASM using silver staining (Figure 66).

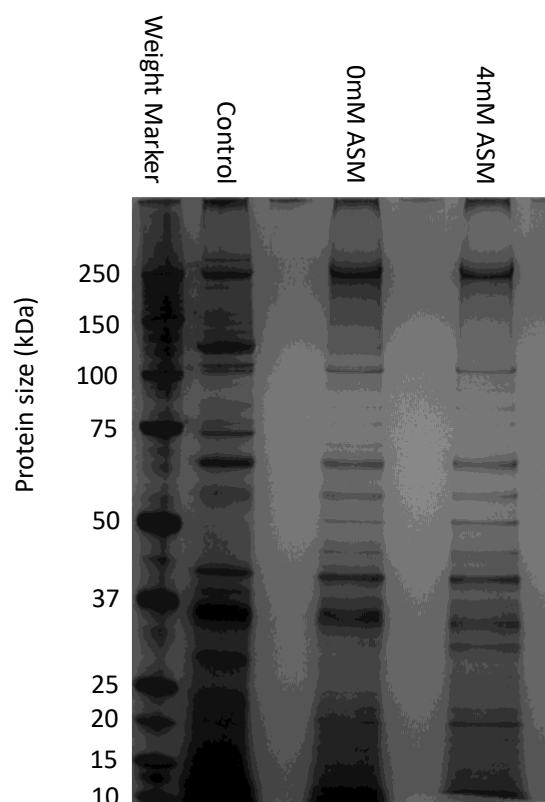
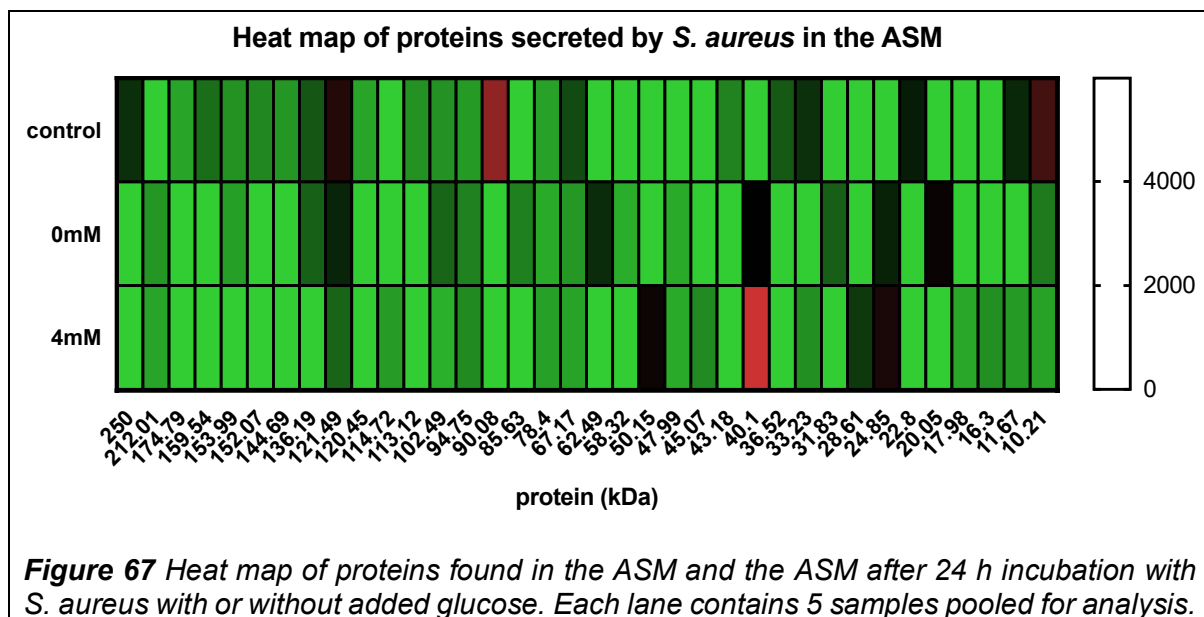


Figure 66 Silver staining of proteins found in the ASM (control) and the ASM after 24 h incubation with *S. aureus* with or without added glucose. Each band represents a protein detected

Using silver staining, the intensity of the protein bands was recorded, and a heatmap was constructed (Figure 67) for ASM and both supernatants. It was observed that following the 24-hour growth of *S. aureus* in the ASM, the protein profile of the media shifted: the amount of large proteins found in the ASM decreased and the amount of smaller proteins in the supernatant increased. Notably, proteins with a molecular weight of 250 kD, 90.8 kD and 67.17 kD found in abundance in ASM were not observed in the supernatant, while the abundance of protein with a molecular weight of 121.49 kD appeared to be reduced in the supernatant. Some smaller proteins were also reduced or absent in normoglycaemic supernatant, such as 33.23 kD, 20.8 kD, 11.67 kD and 10.21 kD. Several proteins were more abundant in the supernatant, which was not observed in the ASM, such as 62.49 kD, 40.1 kD, 24.85 kD and 20.5 kD. To assess the effect that the presence of glucose has at the ASM generation step, I could compare protein profiles of the supernatant derived from hyperglycaemic and

normoglycaemic ASM. Protein with a molecular weight of 40.1 kD was observed in both normoglycaemic and hyperglycaemic supernatants but increased in the latter. Proteins with a molecular weight of 50.15 kD and 28.61 kD were present in the hyperglycaemic supernatant and not in the normoglycaemic one. Whereas proteins with the molecular weight of 62.49 kD and 20.05 kD were found in the normoglycaemic supernatant and not hyperglycaemic ones. Glucose appears to change the resulting supernatant protein profile at the generation stage, which could in part be responsible for the change in behaviour of *P. aeruginosa*.



4.5 Discussion

In this chapter, I have demonstrated the potential to use ASM to better study the effects of glucose on bacterial growth in the airway. I propose that ASM better represents the nutrient status of ASL. In this environment, the data support previous findings that changes in glucose concentration promote the growth of *S. aureus* and *P. aeruginosa* (Åstrand et al., 2017; Garnett et al., 2012; Garnett et al., 2013; Garnett et al., 2013; Gill et al., 2016). I believe that the model for the stable co-culture of *S. aureus* and *P. aeruginosa* both resemble *in vivo* CF sputum and allows precise control of the environment with high reproducibility.

4.5.1 Quantification of live bacteria

Measurement of optical density (OD) at a wavelength of 600nm (OD600) is the most commonly used approach for the quantification of bacteria in a liquid suspension (Myers *et al.*, 2013). OD measurements are popular among scientists due to being a fast, inexpensive way to estimate bacterial count without interfering with the experiments. Moreover, it could easily be automated for prolonged experiments. While other techniques, such as microscopy, flow cytometry and drop-plating, offer superiority in quantifying live bacteria, they often lack the unique selling points of OD. Despite widespread use, OD measurement has significant downsides. A study in 2016 demonstrated that OD measurements are proportional to the cell number only in a limited range of conditions and are usually not linearly related (Stevenson *et al.*, 2016). Previous studies have also demonstrated that to relate OD measurements to cell count or compare measurements between instruments and experiments; it is necessary to calibrate the machine using reference material (Beal *et al.*, 2020). Such practice is not widespread, so OD measurement could not be considered a valid method of live bacteria quantification.

For this study, OD measurement was initially chosen due to the potential to automate the process of estimation of bacterial count and instant feedback on bacterial growth.

Quantification using OD600, however, generated unusual results (as can be seen in 4.4.2.1). Both *S. aureus* and *P. aeruginosa* grew as expected in MH broth, and growth was observed using OD measurement. In ASM, however, negative OD600 values were observed throughout the whole 24 hours of the experiment. Despite negative OD600 values, the growth of the pathogens was confirmed using CFU calculation using the drop-plating technique. A potential explanation of the negative values observed could be due to the composition of the media. While MH broth starts out clear and becomes turbid with bacterial growth, ASM starts out turbid due to egg yolk emulsion. I have observed that media became clearer with bacterial growth when compared to the original turbid solution, which explains OD600 values being negative. A possible explanation for the clarification of media would be that bacteria utilise media components such as egg yolk emulsion, leading to the clearing of the media. Due to the complexity of relating OD600 values to bacterial growth, it was decided not to utilise OD600 readings for the bacterial growth quantification.

Following attempts to quantify bacteria using OD600, I decided to quantify bacteria using plating of serial dilutions and subsequent quantification of colony-forming units. The drop plate method is economical, does not require specialised equipment, and is used in microbiology research laboratories worldwide (Naghili *et al.*, 2013). This approach is, however, labour intensive. The one obvious downside to using drop plating is the lack of standardised procedural protocols. The size of drops, the number of replications or the number of dilutions used per plate are decided by the investigator and could lead to variations on repeats (Chen *et al.*, 2003). The initial preparation for the experiment involved extensive trials on the number of dilutions per agar plate and the optimum volume of drops required for the accurate quantification of bacteria.

When grown in the MH broth and plated on agar plates, both *S. aureus* and *P. aeruginosa* formed well-defined colonies that were easy to quantify. Likewise, *S. aureus* grown in the ASM also formed well-defined colonies after overnight incubation and could be reliably quantified. However, *P. aeruginosa* grown in the ASM did not follow the same pattern and instead formed

colonies that lacked definition and could not be reliably quantified due to the merged borders of colonies. Comparing the growth of *P. aeruginosa* in MH broth and ASM, I observed an increased amount of biofilm formation by *P. aeruginosa* grown in the ASM. I hypothesise that the lack of distinct colonies on the agar plates is due to the excessive production of the biofilm, which creates the appearance of conjoint colonies. My hypothesis is supported by the observations that *P. aeruginosa* is known to form biofilms in chronic infections in individuals with CF (Moreau-Marquis *et al.*, 2008; Starkey *et al.*, 2009; Høiby *et al.*, 2010; Kovach *et al.*, 2017). ASM is designed to mimic the sputum of individuals with CF, so *P. aeruginosa* may present a similar phenotype in the ASM that is observed in CF sputum.

As previously mentioned, one of the downsides of using drop plating is the lengthy manual procedure of setting up serial dilutions and plating bacteria. Due to the experimental design, it was impossible to prepare plates for both *S. aureus* and *P. aeruginosa* between readings. In addition to the inability to reliably quantify *P. aeruginosa* grown in the ASM, it was decided to find an alternative method to quantify live *P. aeruginosa*. From collaborators, I acquired *P. aeruginosa* strain H174, a luminescent strain created by Kai Hilpert at St Georges University of London (Hilpert and Hancock, 2007). This modified strain was built based on PAO1 and had a luminescent gene cassette (*luxCDABE*) inserted into it. This modification allows accurate, rapid quantification of live bacteria by measuring emitted light. Direct measurement of emitted light allowed me to bypass the necessity to quantify *P. aeruginosa* using the drop plate technique, speed up quantification of co-culture and have an instant measurement of viable bacteria in the culture. Removing the need to plate *P. aeruginosa* allowed me to use the drop plating technique to quantify *S. aureus*, increase the frequency of measurements and acquire more precise growth curves.

4.5.2 Evolution of the ASM model

P. aeruginosa thrived in the environment pre-conditioned by *S. aureus*. Ample evidence demonstrates that *S. aureus* and *P. aeruginosa* can modify each other's behaviour, pathogenicity and metabolism (Mashburn *et al.*, 2005; Hoffman *et al.*, 2006; Korgaonkar and Whiteley, 2011; Billings *et al.*, 2013; Pastar *et al.*, 2013). The majority of the studies, however, focus on the effects of *P. aeruginosa* on *S. aureus* (Billings *et al.*, 2013; Filkins *et al.*, 2015; Hoffman *et al.*, 2006; Korgaonkar and Whiteley, 2011; Mashburn *et al.*, 2005; Pernet *et al.*, 2014). Relatively little is known about the effects of *S. aureus* on *P. aeruginosa*. In a human skin model of co-infection, *S. aureus* acts as a pioneer, priming the environment and promoting the attachment of *P. aeruginosa* to the keratinocytes (Alves *et al.*, 2018).

The initial design of the model involved the simultaneous addition of *S. aureus* and *P. aeruginosa* into the ASM. In this model, *S. aureus* outcompeted *P. aeruginosa* over 48 hours, preventing any meaningful growth of *P. aeruginosa*, resulting in the amount of live *S. aureus* being 1000-fold higher than that of *P. aeruginosa*. These results were not surprising as a prolonged lag phase was observed in the monoculture of *P. aeruginosa* in the ASM, which was not present in *S. aureus*, which gives *S. aureus* ample time to establish in the media. This growth pattern, combined with literature evidence showing that *P. aeruginosa* prefers an environment that was pre-conditioned by *S. aureus*, led to the model's design based on *S. aureus* pre-conditioned media (supernatant). Upon adding *P. aeruginosa* into the ASM that was conditioned by *S. aureus*, the lag phase was not observed, and *P. aeruginosa* immediately began logarithmic growth. Based on these results, the next stage of model development was to add both *S. aureus* and *P. aeruginosa* simultaneously into the supernatant in equal amounts. In this scenario, *P. aeruginosa* began rapid growth, suppressing any growth of *S. aureus*. This showed a complete mirror image to that of the ASM, confirming our belief that conditioning of the environment by *S. aureus* is crucial for the growth of *P. aeruginosa* in the ASM.

In vivo, *P. aeruginosa* and *S. aureus* would not populate lungs simultaneously. As previously demonstrated in individuals with CF, *P. aeruginosa* infection follows initial infection by *S. aureus*. In order to mimic this scenario *in vitro*, I added *P. aeruginosa* into the media with an established *S. aureus* population. Initially, both pathogens decreased in their numbers. After initial 6 hours, however, *P. aeruginosa* began growth, while the population of *S. aureus* began to decline. After 48 hours, *P. aeruginosa* amount in the culture surpassed that of *S. aureus*.

In vivo, however, sputum is continuously replaced and consists of a combination of new and conditioned sputum. I modified the model to take this into account by mixing 50:50 of the ASM and conditioned supernatant. Simultaneous addition of both pathogens into the mixed media saw both pathogens begin growth, with *S. aureus* initially outgrowing *P. aeruginosa*. However, after 48 hours of incubation, the amount of *S. aureus* in the media dropped below that of *P. aeruginosa*. After an initial 48 hours amount of live *S. aureus* and *P. aeruginosa* remained stable for the next 24 hours. This version of media is the first instance where *S. aureus* and *P. aeruginosa* co-existed without eliminating each other with a stable continuous CFU count.

Previous *in vitro* models of co-culture of *S. aureus* and *P. aeruginosa* were either stable for less than 24 hours (Michelsen *et al.*, 2014; Filkins *et al.*, 2015; Tognon *et al.*, 2019; Wijesinghe *et al.*, 2019) or were performed on solid support media (plastic/agar plates) (Antonic *et al.*, 2013; Fugère *et al.*, 2014; Michelsen *et al.*, 2014). Our model offers the ability to observe bacterial behaviour over a prolonged period and ease of modification.

Our data indicate that the *S. aureus* associated factor(s) in ASM that promoted *P. aeruginosa* were heat-labile. It is, currently, impossible to speculate on the nature of these changes using existing literature, as previous transcriptomic and proteomic analysis demonstrated extreme metabolic plasticity of *P. aeruginosa* and the ability to shift metabolism to adjust to the varying carbon sources depending on the environment (Dolan *et al.*, 2020). A more thorough proteomic study is required to understand the changes introduced to the media by *S. aureus*, which allow more rapid growth of *P. aeruginosa*.

4.5.3 The role of glucose in the ASM model

While the addition of glucose did not affect the growth of *S. aureus* or *P. aeruginosa* in MH, it promoted growth when added to ASM (Section 1.4). It is a common practice to use high nutrient media to explore the growth and interaction of these pathogens (Palmer *et al.*, 2005; Ma *et al.*, 2006; Briaud *et al.*, 2019; Tognon *et al.*, 2019; Wijesinghe *et al.*, 2019). Our data indicated that the strain of *S. aureus* I used better adapted to the low nutrient ASM than *P. aeruginosa* as it did not have the same lag in growth when inoculated into ASM. Both *P. aeruginosa* and *S. aureus* utilised glucose for growth in monoculture in the ASM.

It was also reported that *P. aeruginosa* drives *S. aureus* from aerobic respiration towards the fermentation pathways in order to utilise produced lactate and that when *S. aureus* ferments glucose into lactate *P. aeruginosa* increases the expression of lactate transporters, which allows it to utilise lactate as a preferential source of carbon (Filkins *et al.*, 2015; Tognon *et al.*, 2019). However, whilst our data indicate that in ASM with glucose, *S. aureus* did produce lactate, this was rapidly depleted and was absent when *P. aeruginosa* growth was initiated in co-culture in ASM. Thus, our data indicate that factors other than glucose and lactate promoted the growth of *P. aeruginosa* in *S. aureus* conditioned medium. Nevertheless, I recognise that there are limitations to these experiments when compared to the *in vivo* scenario (Filkins *et al.*, 2015). ASM contained a finite supply of glucose, limiting lactate production. *In vivo*, with respiratory disease and hyperglycaemia, there is the potential for continuous glucose leak into the ASL for bacterial fermentation, and lactate could enter the ASL from other cellular sources (Garnett *et al.*, 2016).

The role of glucose was less evident in co-culture, where in some cases glucose promoted growth on either *S. aureus* or/and *P. aeruginosa*; however, it often had no significant effect on the growth of either. Studies have shown that in the presence of *S. aureus*, *P. aeruginosa* downregulates genes responsible for sugar and carbohydrate metabolism (Tognon *et al.*, 2019). This change in gene expression could explain why glucose did not induce changes to

the growth of *P. aeruginosa* in co-culture. The model should be further developed to allow continuous replenishing of glucose to simulate realistic *in vivo* conditions. Until the continuous flow of glucose is established *in vitro*, it is hard to speculate on the true role of glucose in the relationship of *S. aureus* and *P. aeruginosa* in co-culture. The main challenge in establishing realistic glucose conditions is continuously measuring glucose concentration in the media without affecting the volume, temperature, and conditions of the media. The data acquired in this research does, however, demonstrate that the role of glucose in the co-culture is not simply a source of nutrients and plays a vital role in the establishment of the environment to maximise bacterial growth.

4.6 Summary

Mixing ASM with supernatant 1:1 allowed me to establish a relatively stable co-culture of *S. aureus* and *P. aeruginosa*. This model now permits further exploration of the interaction between these organisms and others (e.g. *Haemophilus influenzae*), factors that change in the ASL, and responses to antimicrobial agents (Dolan *et al.*, 2020). I have demonstrated that the role of glucose in altering the relationship between *S. aureus* and *P. aeruginosa* is limited and that other factors must play determining factors that allow these two pathogens to co-infect CF individuals' lungs.

4.7 Future work and limitations

The ASM model designed in this study offers a unique approach to investigate the activity of microbial pathogens in the low-nutrient environment mimicking that of the lung. However, to understand the effects of glucose on the growth of pathogens, a modification to the model may be required to increase the robustness of the study. In vivo, a continuous flux of glucose is present, creating stable hyperglycaemic conditions. I did not create a stable hyperglycaemic condition as pathogens depleted glucose. The rate of glucose depletion could be measured in the future, and the addition of a peristaltic pump set to the rate of replenishing could be utilised to maintain glucose concentration. This would allow us to understand further the role of glucose in the low-nutrient environment and the modifications bacterial pathogens undergo to adapt to constant hyperglycaemia.

In section 4.4.12, I have demonstrated that *S. aureus* modifies ASM, changing its protein profile and that presence of glucose changes proteins present in the media. Silver staining analysis is a good first step to visualise potential changes; however, it cannot be used to identify proteins. In the future, proteins and peptides from the supernatant and the ASM could be separated and analysed using mass spectrometry to help us identify the proteins responsible for the changes in the growth of the *P. aeruginosa*. If identified, it would be possible to synthesise these peptides to selectively add them into the media to confirm the effects on bacterial growth.

Chapter 5

Using oral washes as an alternative to invasive lung microbiome sampling

5.1 Introduction

5.1.1 Non-invasive sampling of the lung microbiome

I previously discussed in Chapter 3 that current methodologies for collecting lung microbiome samples rely on invasive techniques, such as protected specimen brushing and bronchoalveolar lavage. Due to the invasive nature of these procedures, only medically trained professionals could collect samples. These techniques are usually reserved for use in individuals suffering from severe respiratory infections or in those with chronic lung diseases. An alternative for sampling the lung microbiome without the use of invasive techniques is collecting sputum, which is produced in excess by some individuals. The leading causes of excess sputum production are smoking, asthma, CF, COPD, and respiratory tract infections. The inability to collect sputum samples from healthy individuals limits its potential use as an exploratory technique for sample collection in healthy individuals.

In this thesis, I discussed the potential to use lung microbiome data to investigate changes in the microbiome that could be associated with diabetes and pre-emptively predict those most at risk of developing chronic respiratory infections. Individuals for whom this might be most useful may not produce excess sputum. As these individuals will not yet be suffering from severe respiratory infections, they are unlikely to agree to an invasive procedure such as BAL and PSB to collect lung microbiome data. Thus, an alternative sampling method, which would allow the analysis of lung microbiome data would be useful for these individuals but also others.

Several studies have investigated the origin of the lung microbiome and how it relates to the other linked sites, such as the oral, nasal and gut microbiome. These studies used sputum, nasal brushes, oral washes, PSB and BAL to compare microbiomes in various locations. It

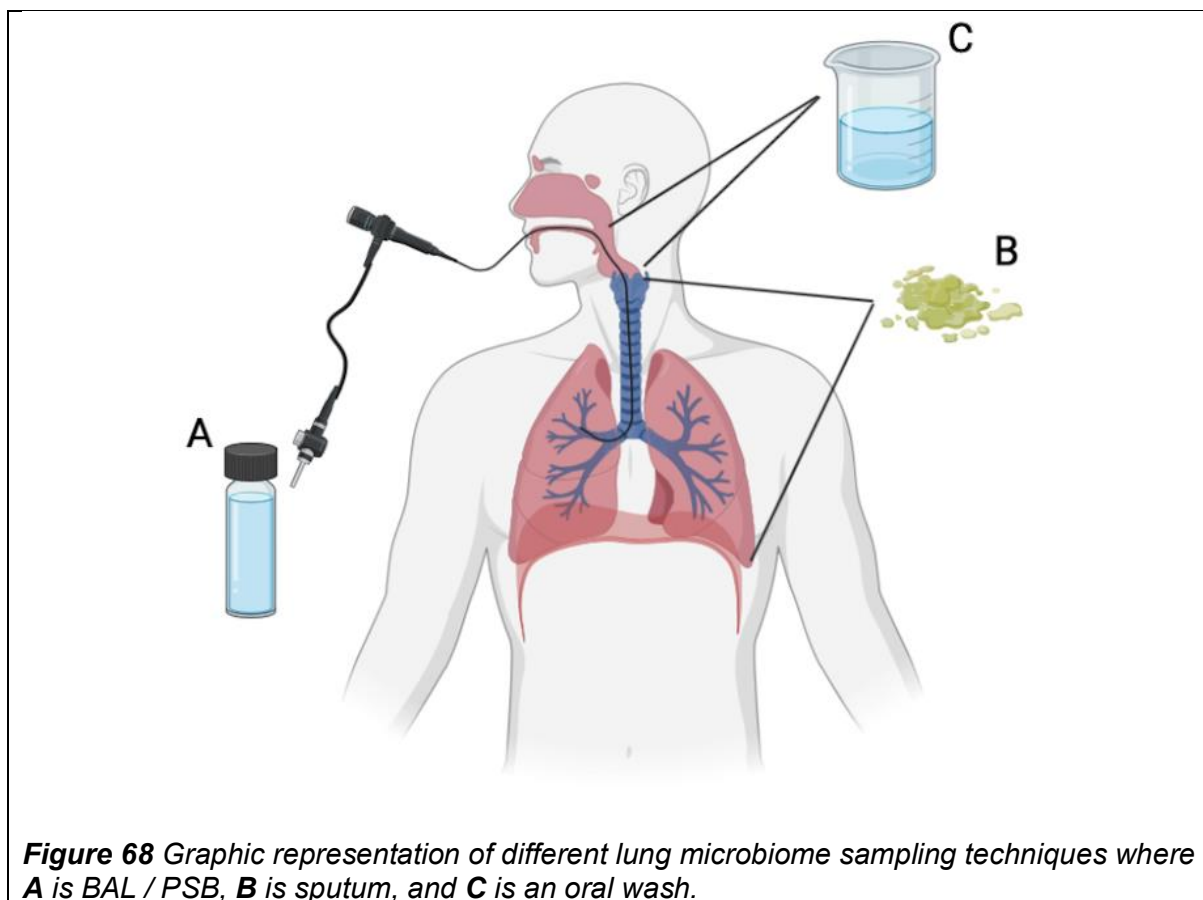
was shown that the abundance of genera detected in the PSB samples correlated with those detected in oral washes but not with those in nasal brush samples (Durack *et al.*, 2018). Unlike PSB and BAL, sputum samples contain a mixture of upper and lower airway microbiomes. Sputum samples were shown to have great similarities to the microbiome obtained by oral washes (Durack *et al.*, 2018; Sulaiman *et al.*, 2018). However, the similarities between sputum and samples obtained biomicroscopically are more apparent in individuals with lung diseases (Hogan *et al.*, 2016; Durack *et al.*, 2018). Another study has demonstrated that bacterial communities found in healthy lungs were similar to that of the mouth; however, the relative abundance of bacterial OTUs was different in the lungs and mouth (Bassis *et al.*, 2015). This demonstrates that, while oral wash samples cannot be used to study the lung microbiome, they may provide an adequate estimation of bacteria found in the lungs and help predict changes in their populations (Charlson *et al.*, 2011; Morris, James M Beck, *et al.*, 2013; Bassis *et al.*, 2015; Samuelson *et al.*, 2018).

Above I discussed the importance of developing an alternative non-invasive method for sampling the lung microbiome in people who do not naturally produce sputum. Currently, there are no efficient ways to non-invasively sample lung microbiome in healthy individuals. However, throughout this thesis, I have discussed the importance of several pathogens in the lungs of diabetic individuals and how early detection of these pathogens may play an important role in preventing the establishment of chronic lung infections. I therefore suggest that oral wash samples may be used as a non-invasive approach to investigate the presence of pathogenic bacteria in the lung microbiome. Using oral washes as a first step could aid clinical decisions regarding administration of bronchoscopic sampling for individuals.

5.1.2 Oral wash sampling as a proxy of the lung microbiome

Oral sampling has previously been used to detect dozens of human pathogens, including pathogens whose primary sites of infection are outside of the oral cavity, such as the respiratory pathogens *Mycobacterium tuberculosis* and SARS-CoV-2 (Valinetz and Cangelosi,

2021). In this study, I will be using oral wash samples, sometimes described as oral rinses or gargle lavages, by different research groups. This sampling technique involves gargling sterile saline solution before spitting it into a sterile container. The resulting sample contains a mixture of saliva and oropharyngeal secretions (Larsen *et al.*, 2004; Goterris *et al.*, 2019; Mittal *et al.*, 2020). Figure 68 demonstrates how different sampling techniques could be used to acquire lung microbiome samples from various locations and how oral wash samples compare to the BAL, PSB and sputum samples.



5.1.3 Using sputum samples derived from COPD individuals to study lung microbiome

In previous chapters, I used sputum samples from individuals with CF to look at the lung microbiome. Due to the low prevalence of CF in the UK and the complications these individuals suffer, they generally attend specialised clinics. This makes it hard to recruit these individuals

for studies outside specialised clinics. Purchasing sputum samples from the biobank has a significant cost attached to it and is not always possible for a small exploratory study. Because of these reasons, I decided to instead focus on individuals with COPD.

It is estimated that there are around 1.2 million people (2% of the population) suffering from COPD in the UK (Snell *et al.*, 2016). This is significantly higher than that of CF, of which there are around 10 600 in the UK (Cystic Fibrosis Trust, 2022). Individuals with COPD regularly attend hospital appointments for health check-ups where they could be recruited for a simple study like the one I designed. Individuals with COPD are known to suffer from excess sputum production (Burgel, 2012; Maestrelli *et al.*, 2001; Miravittles, 2011; Ramos *et al.*, 2014), which could be collected during a routine procedure. Importantly for us, diabetes mellitus is a common comorbidity in individuals with COPD. It was reported that individuals with COPD have a higher prevalence of diabetes, adjusting for age, sex, race, smoking, body mass index and education (Mannino *et al.*, 2008). While individuals with COPD were shown to be substantially more likely to have pre-existing diabetes (Feary *et al.*, 2010), it was also shown that a decrease in spirometry measurements (such as the ones seen in individuals with COPD) is an independent predictor of the development of type 2 diabetes mellitus (Yeh *et al.*, 2005). The frequency of diabetes diagnosis in individuals with COPD was essential in the decision to base our study on individuals with COPD. We predicted that we would be able to recruit both diabetic and non-diabetic COPD individuals without the need to target diabetic individuals specifically.

5.2 Aims and hypothesis

Hypothesis: Oral wash samples can be used to predict the abundance of respiratory pathogens in sputum.

Aim 1: To investigate the difference in the bacterial species abundance and diversity in sputum and oral wash samples in individuals with COPD

Aim 2: To evaluate whether changes in the composition of the lung microbiome observed in sputum samples could also be observed in oral wash samples

Aim 3: To evaluate the effects of diabetes on the lung microbiome in individuals with COPD

5.3 Materials and methods

5.3.1 Study design

This study was approved by the NHS Health Research Authority (IRAS project ID: 256124, REC reference: 19/NE/0122) to be conducted at St Georges Hospital in London, United Kingdom. The named chief investigator for the study was confirmed to be Stanislavs Vasiljevs, who received both Good Clinical Practice and Consent Taking training and certificates.

The cohort included individuals with COPD attending their regular appointment in the chest clinic. The attending consultant screened the patient file against inclusion and exclusion criteria prior to approaching the patient. If a patient matched the criteria, during their appointment the attending consultant briefly explained the study and offered to arrange for the patient to talk to the named chief investigator. If a patient was willing to participate, then the patient was enrolled in the study. All documents were completed prior to sample collection; both oral wash and sputum samples were collected during a single visit, and no follow-up visits were required.

The study aimed to collect a total of 30 patient samples from 15 diabetic and 15 non-diabetic individuals. CFRD individuals were defined as having HbA1c >48 mmol/mol (>7.8 mmol/L) as per WHO recommended guidelines (WHO, 2011). Inclusion criteria consisted of: a patient with a confirmed COPD case, a patient with an FEV₁/FVC ratio below 0.7, and a patient being an active sputum producer. Exclusion criteria for the study consisted of: the use of antibiotics in the last 30 days prior to the sample collection, a patient having any lung infections in the last 30 days, and individuals having other chronic lung diseases except for COPD. Relevant clinical information, such as blood glucose levels, HbA1c levels and spirometry data, was collected from the patient's file with the patient's permission. Upon entering the study, the patient was assigned a randomised number, and no further identifying information was featured in the study.

5.3.2 Study documentation

The study was designed in full by Stanislavs Vasiljevs to be conducted in St George's Hospital. All of the study documentation is available in full in Attachment 2. In short, a patient was presented with a Participant Information Sheet which, in detail, described the study, explained the patient's rights, procedure, the samples to be collected, what would happen to the samples and how it would be disposed of, and which data would be generated during the study. If satisfied, the patient then signed an informed consent form. Consent was taken by chief investigator Stanislavs Vasiljevs. Copies of both informed consent and participant information sheets were given to the patient.

Prior to sample collection, patient information was entered into the case report form. No identifying information was noted in the case report form, and only an anonymised personal number was used. First of all, inclusion and exclusion criteria were reconfirmed to ensure individuals' eligibility for the study. The patient's date of birth, gender and smoking history were noted. Spirometry data, relevant medical history (diabetes, gastroesophageal reflux and dental problems) and medical history were collected and noted. The patient was also asked about the dates of previous chest infections and the frequency of chest infections in a typical year. Where available, blood glucose and HbA1c levels were checked with the patient's medical notes.

5.3.3 Sample collection

Following completion of the notes, oral wash and sputum samples were collected. Importantly, an oral wash sample was collected prior to the sputum collection, as coughing sputum could contaminate the oral cavity and affect oral wash results. Study participants were asked to gargle 10ml of sterile saline solution for 20 seconds and to spit the solution into a sterile 50ml falcon tube (StarLab). Study participants were then asked to bring up and expectorate sputum

into a 50ml falcon tube (StarLab). Both tubes were immediately placed on ice and transported to the laboratory, where they were frozen in a -80° C freezer. Sample collection was performed over a single visit, and study participants were not asked to return for further sample acquisition.

5.3.4 Targeted amplicon sequencing

Sputum and oral wash samples were stored at -80° C until the collection of samples was completed. All work with raw samples was performed in a microbiological laminar flow cabinet (BSC0010, Walker Safety Cabinets). Samples were thawed on ice prior to DNA extraction. Sputum samples were washed with sterile phosphate-buffered saline to remove saliva and liquified with Remel™ Sputasol (Thermofisher). Oral wash samples were centrifuged at 8000 RPM, the supernatant was removed, and the pellet was liquified in Remel™ Sputasol (Thermofisher). As described in Chapter 2 section 2.2.1, bacterial DNA was extracted using Qiagen QIAamp Microbiome Kit. Extracted DNA was quantified using Qubit (Invitrogen). Primers targeting the V3-V4 region of the 16S gene (341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3') were used to amplify the extracted DNA. DNA was then purified using AMPure XT beads (Beckman Coulter Genomics) and quantified by Qubit (Invitrogen). DNA was then sent to LCScience (USA) for amplicon library preparation and sequencing as described in Chapter 2, section 2.2.2. Raw FASTA files were generated for further analysis.

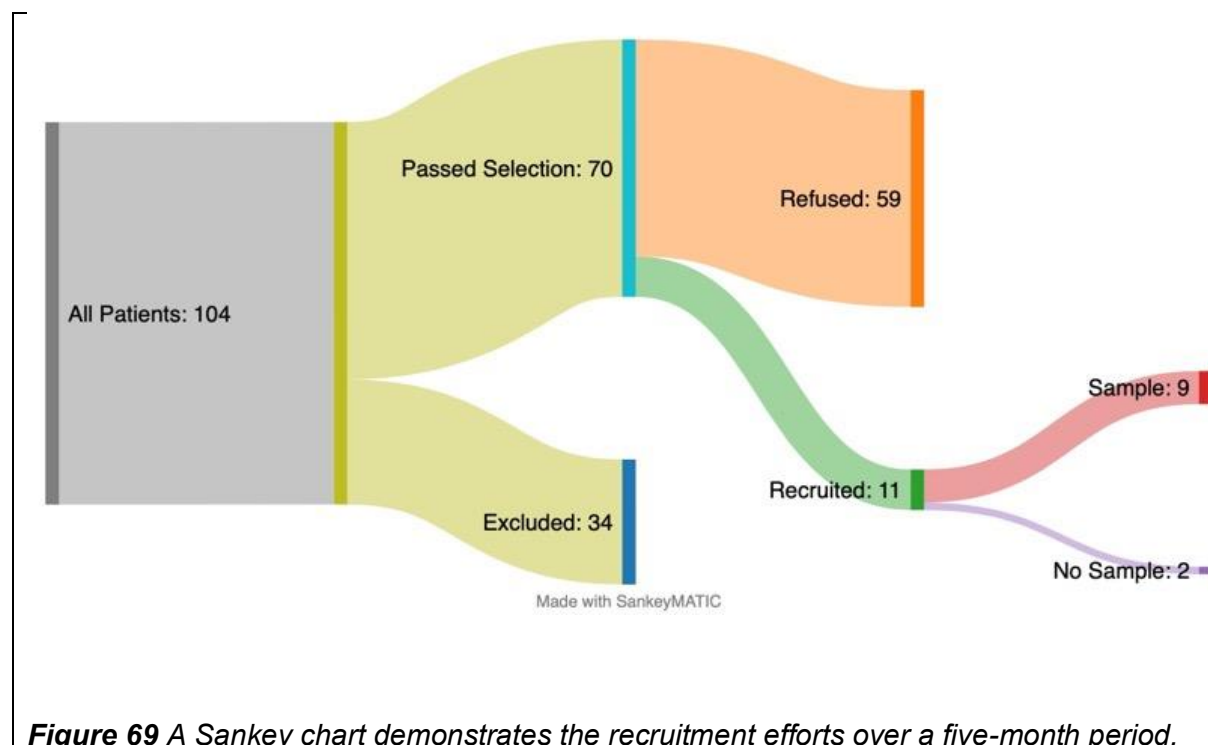
5.3.5 Data and statistical analysis

Bioinformatic analysis was performed using Mothur v1.39.5 as described in Chapter 2, section 3.5. Statistical analysis was performed as described in chapter 2 section 3.6. α - and β -diversity were calculated using Mothur integrated commands. GraphPad Prism version 9 was used to perform a statistical analysis of the data, and to compare sputum and oral wash samples.

5.4 Results

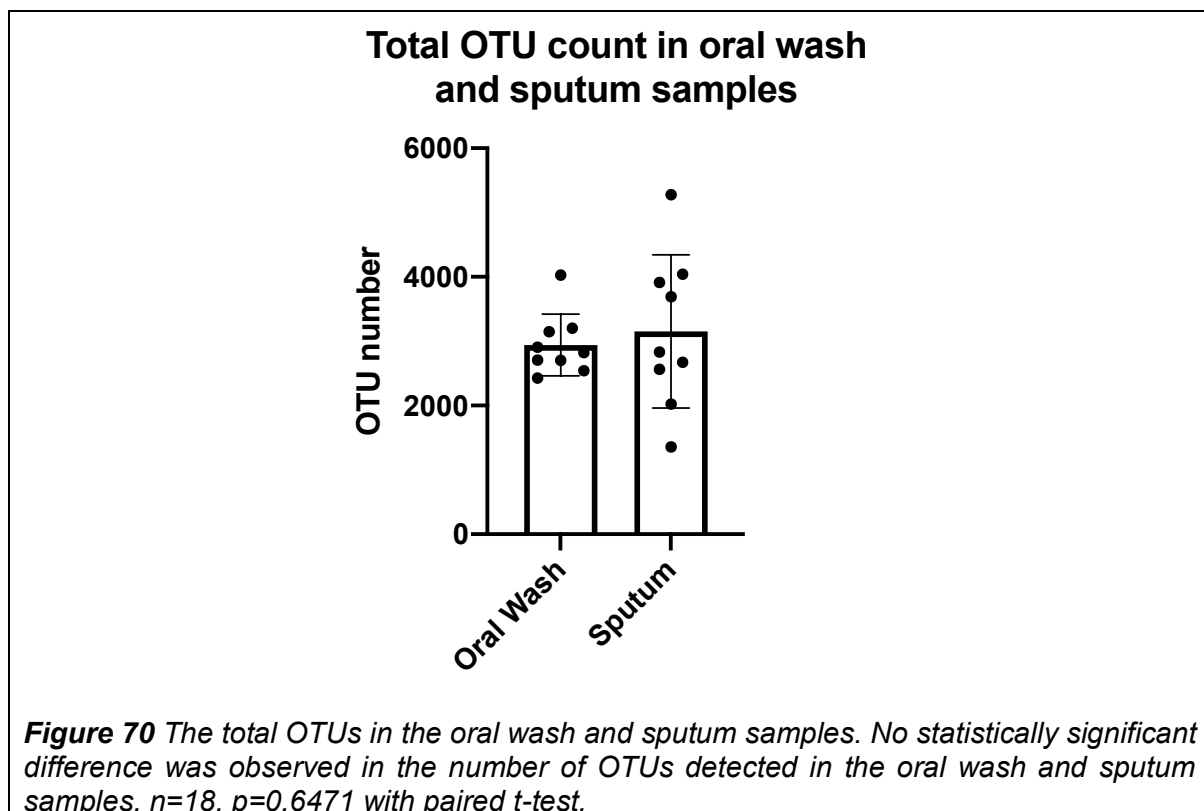
5.4.1 Recruiting individuals for the study.

In this study, I aimed to recruit 30 individuals with COPD – 15 with diabetes and 15 without diabetes. Over five months, 104 individuals passed through the clinic in which recruitment was taking place. Of those individuals, 70 passed the inclusion and exclusion criteria and were approached to recruit for the study. A total of 11 individuals agreed to participate in the study. Two individuals, however, failed to expectorate sputum during the appointment. Therefore, the recruitment rate for the clinic was 11% of all individuals and 15% of eligible individuals. During the recruitment process, I did not see individuals with diabetes that would qualify for the study. All diabetic individuals who attended the COPD clinic during recruitment suffered from ongoing lung infections and were being treated with antibiotics.

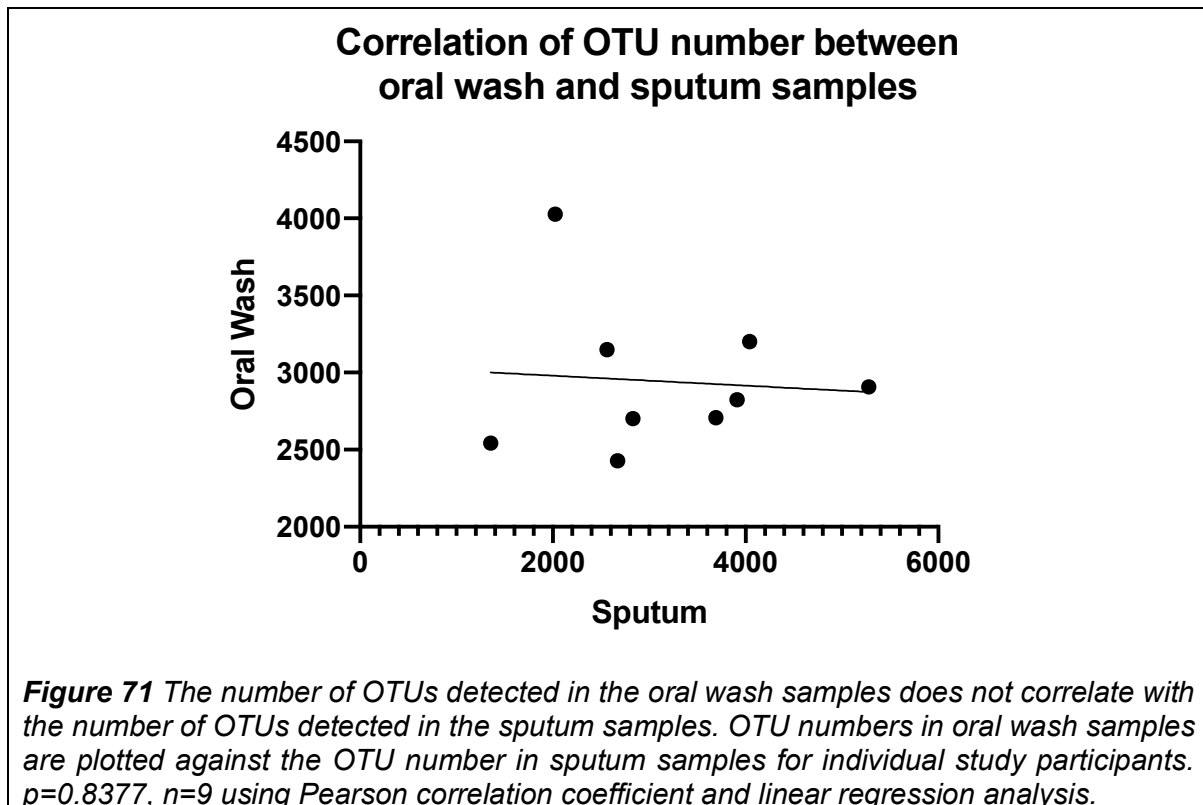


5.4.2 Bacterial OTUs observed in oral wash and sputum samples

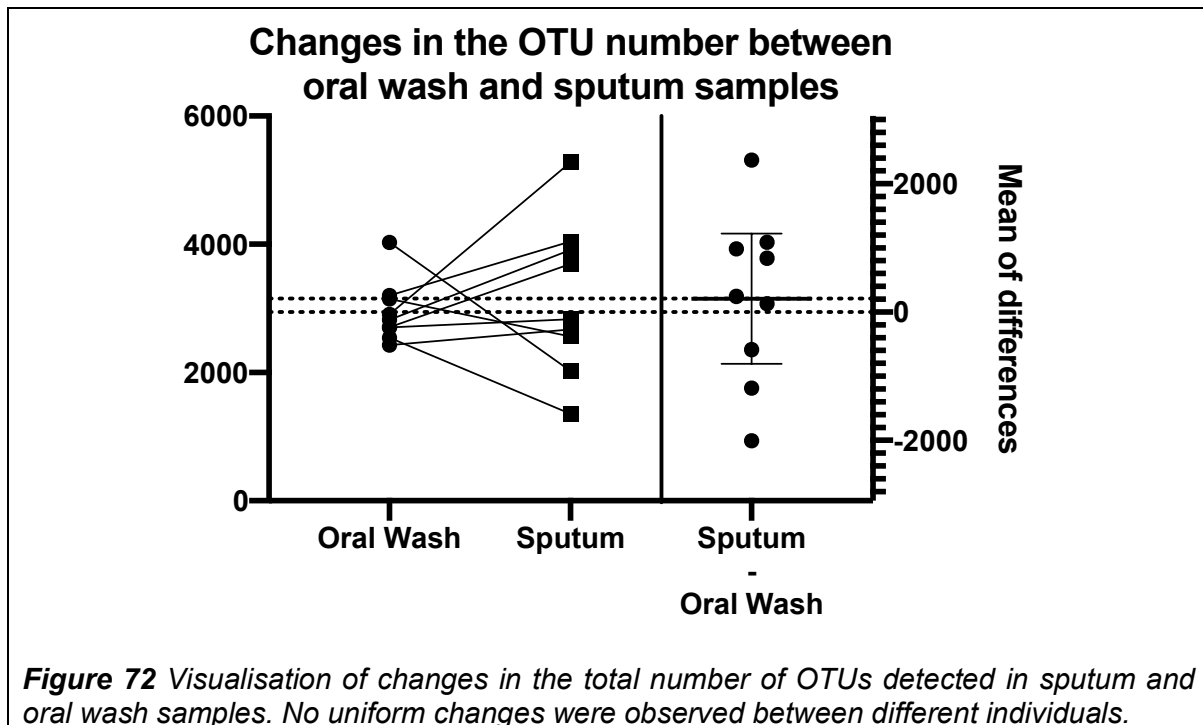
The total number of OTUs observed in the sputum and oral wash samples were not statistically significantly different (Figure 70, $p=0.6474$, $n=9$). The mean amount of OTUs found in the sputum samples was 3152 ± 1191 , while that of the oral wash samples was 2942 ± 479 .



To understand whether the number of OTUs found in each patient's sputum and oral washes correlates, I calculated Pearson correlation coefficients. The number of OTUs detected in sputum, and oral wash samples did not appear to correlate (Figure 71., $p=0.8377$, $n=9$).



In some individuals the number of OTUs observed in sputum samples was higher than that of oral washes, while in other individuals, the opposite was observed (Figure 72). Based on these data, there is no evidence to suggest that oral wash samples could be used to approximate the amount of OTU that would be detected in the sputum samples.



5.4.3 Alpha diversity in the oral wash and sputum samples

Using Shannon's diversity index, I have demonstrated that there is no statistically significant difference in the α -diversity between sputum and oral wash samples (Figure 73, $p=0.6720$, $n=9$).

Alpha diversity scores in oral wash and sputum samples

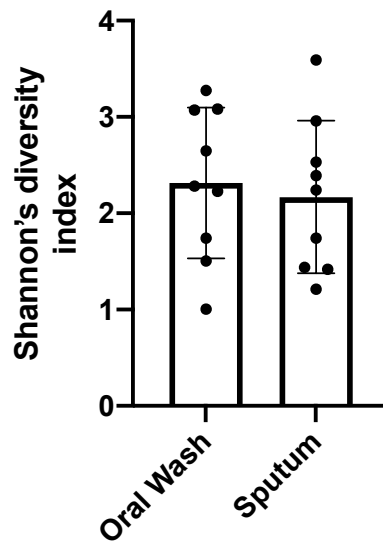
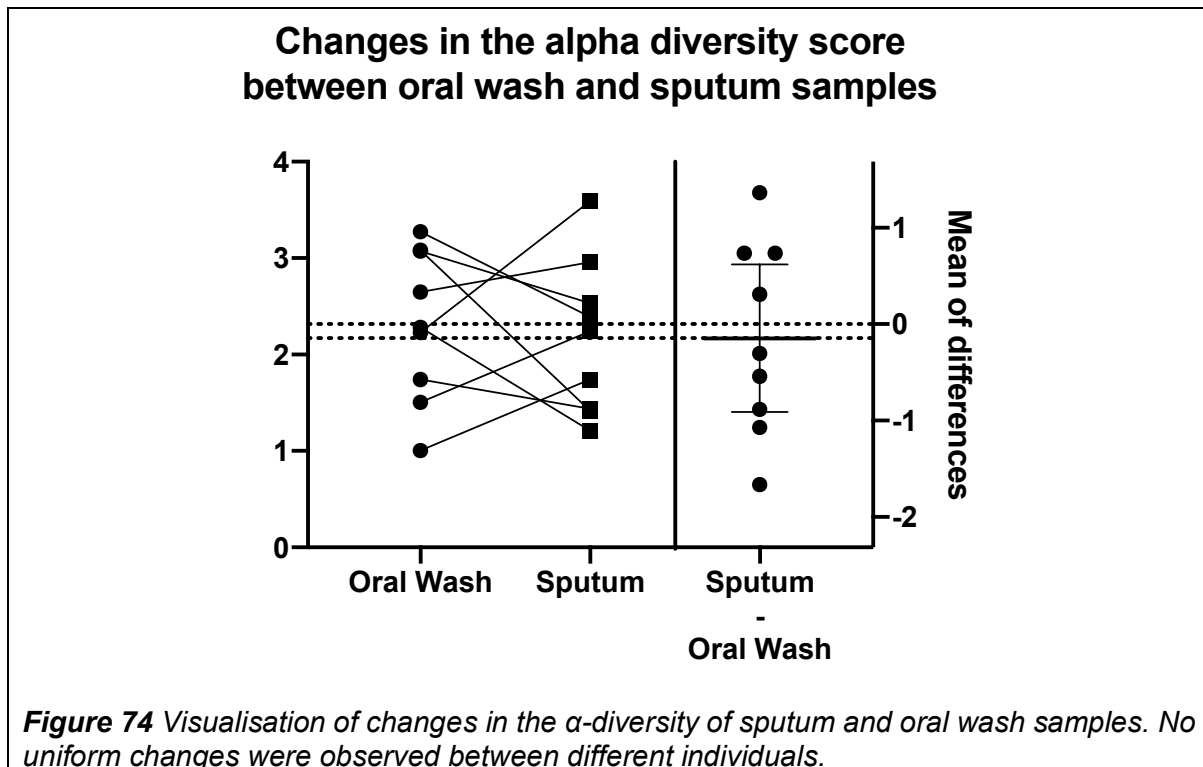


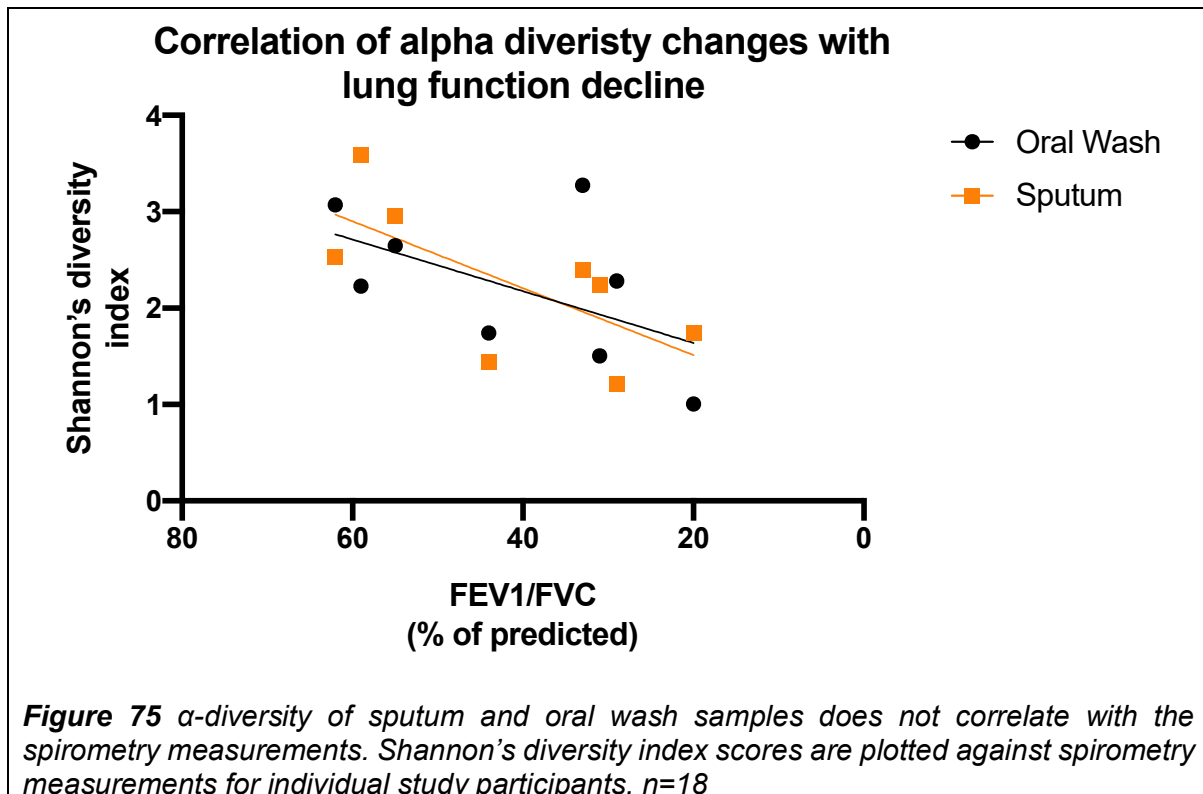
Figure 73 α -diversity in the oral wash and sputum microbiome of individuals with COPD is not statistically significantly different. Shannon's diversity index scores are shown as values $\pm$ SD and plotted for individual samples. $n=18$

Differences in the α -diversity of sputum and oral wash samples appeared to be sporadic, whereas in some individuals α -diversity in the sputum appeared higher, while in others, the opposite was observed. Similar to the OTU count, this data suggests that oral wash samples cannot be used to approximate α -diversity in the sputum samples.



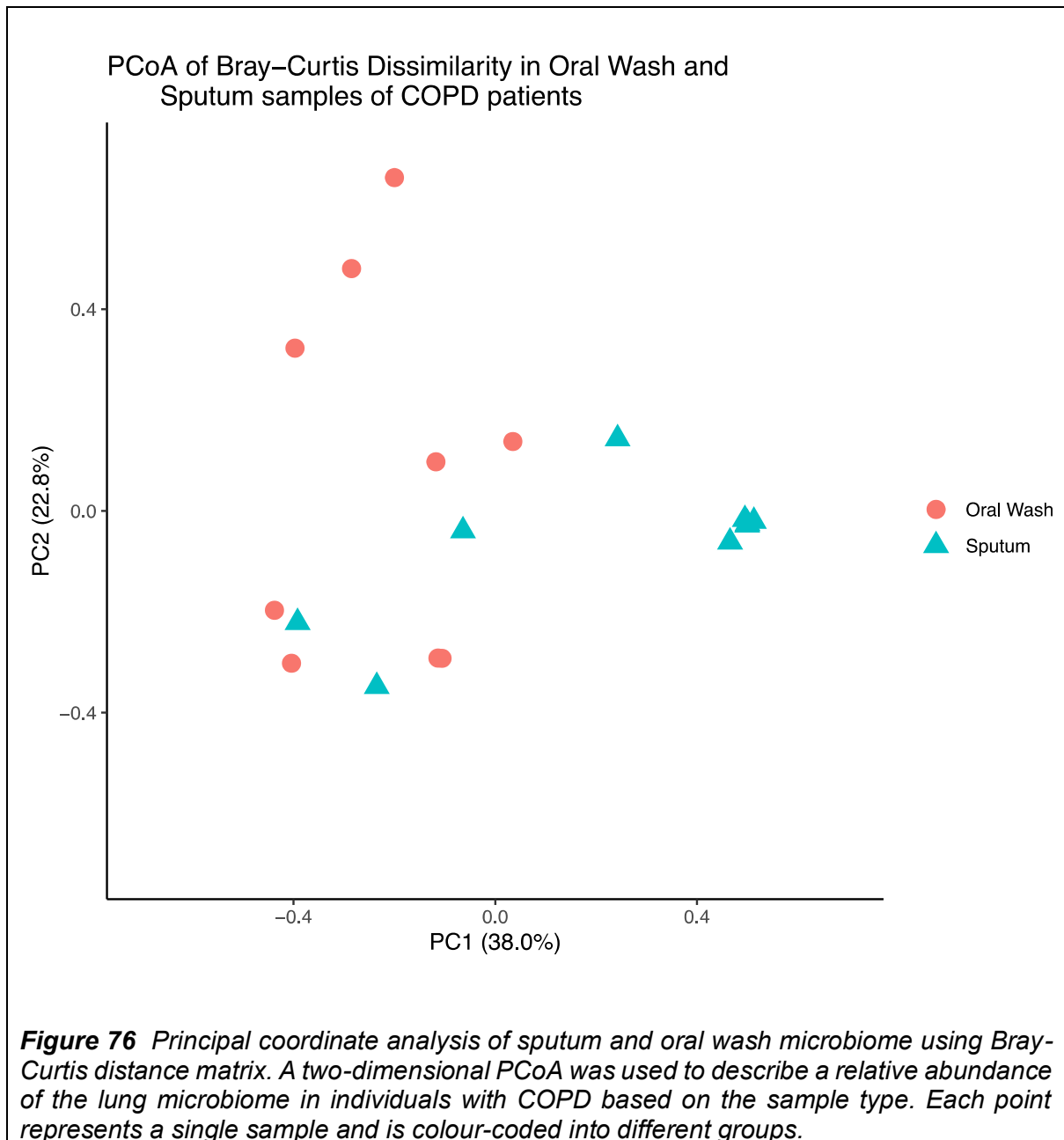
5.4.4 Correlation of lung function decline with the alpha diversity of oral wash and sputum samples

Pearson's correlation coefficient showed no significant correlation between lung function and the α -diversity of the sputum and oral wash samples (Figure 75, $p=0.0602$, $p=0.1673$, $n=9$). Simple linear regression analysis showed that the slope is not significantly different from zero. While there was no statistically significant association between lung function and α -diversity of the sputum and oral wash samples, a low p -value and a general trend of the data indicate that the results may be biologically significant.

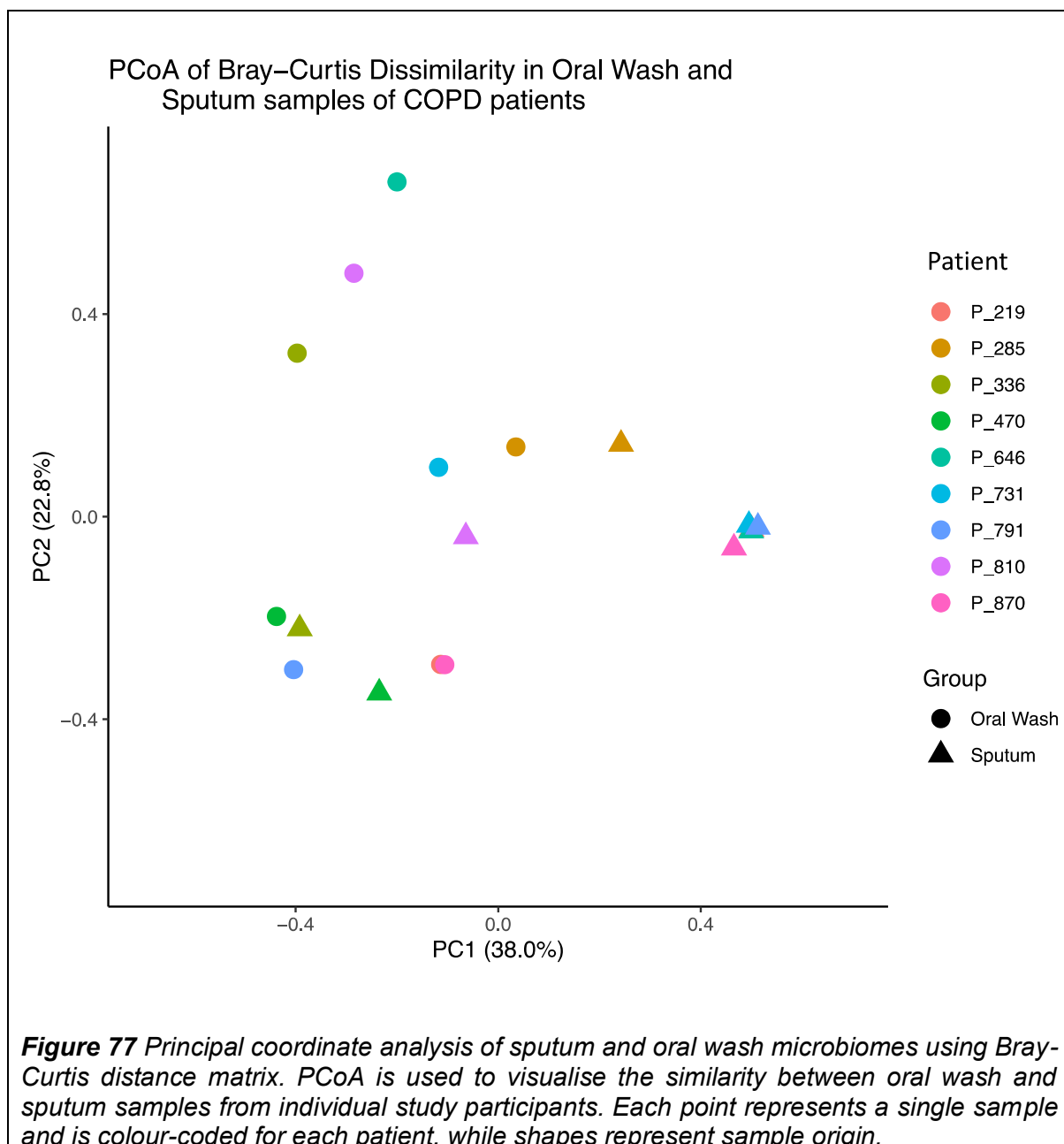


5.4.5 Beta diversity of the microbiome in the oral wash and sputum samples

Using the Bray-Curtis dissimilarity score, β -diversity of sputum and oral wash samples was visualised using a PCoA plot (Figure 76). I observed that oral wash and sputum samples did not cluster together. While some sputum samples are clustered together, most samples appear to be scattered. Statistical analysis using AMOVA shows a significant difference between the oral wash and sputum samples ($p=0.006$, $n=9$), confirming that the separation visualised in Figure 76 is significant.



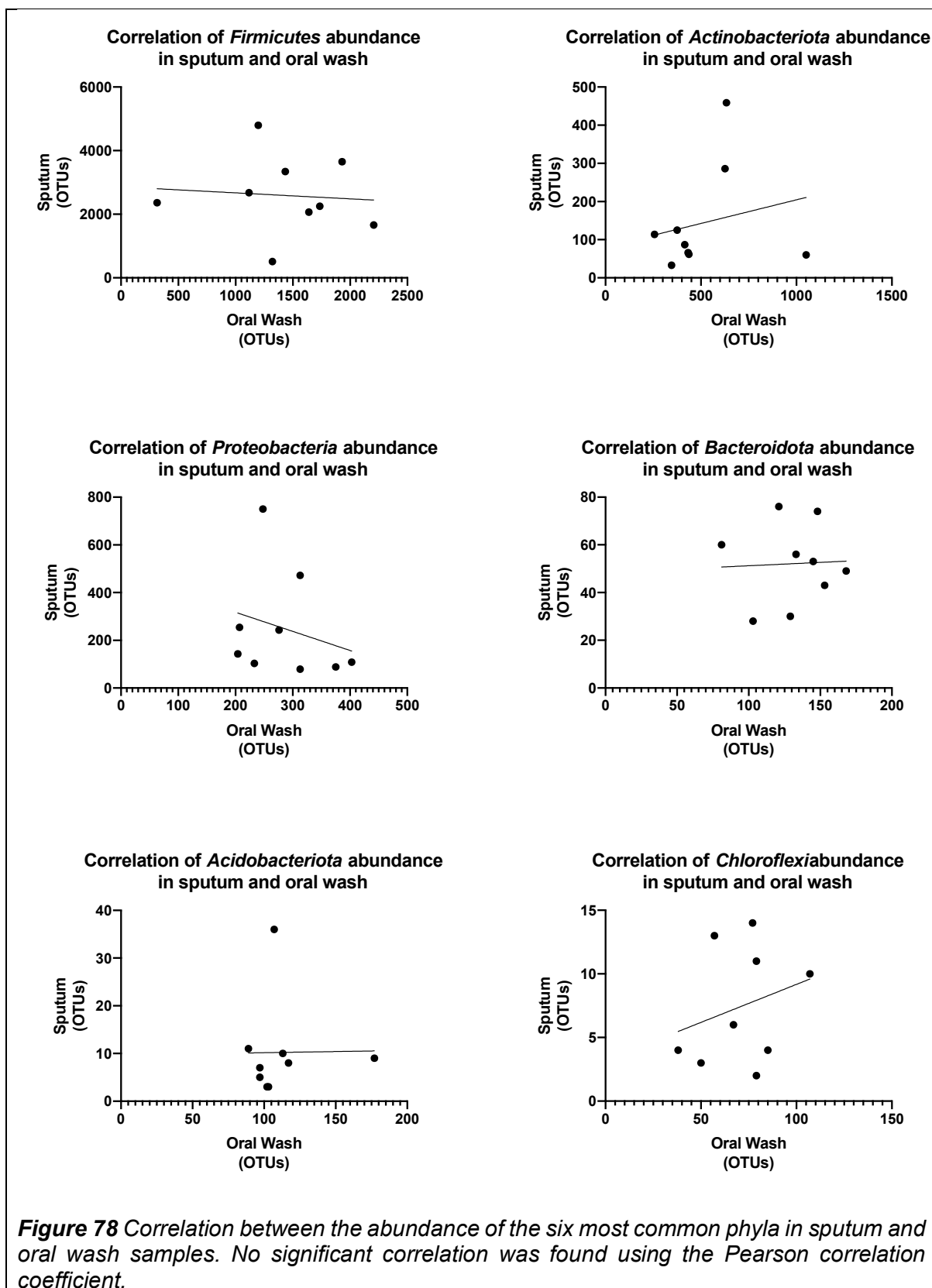
Sputum and oral wash samples from the same individuals do not appear to cluster together (Figure 77). Only two out of nine sets of samples appear in proximity on the PCoA plot: the samples derived from individuals 285 and 470. This indicates that while, in some cases, the microbiome of the sputum and oral wash samples from the same patient appear to be similar, in the majority of the cases, the two sites have their own distinct composition.



5.4.6 Analysis of genera detected in the oral wash and sputum samples

In order to understand whether oral wash samples could be used to predict the abundance of specific bacteria in the sputum, I have analysed the abundance of all phyla- and genus-level bacteria in both types of samples.

The microbiome of both sputum and oral washes was made up of phyla commonly observed in respiratory samples. Phyla *Firmicutes*, *Actinobacteriota* and *Proteobacteria* made up much of the microbiome. Using linear regression and Pearson's correlation coefficients, I have analysed whether an abundance of specific phyla correlates between oral wash and sputum samples. There were no statistically significant correlations in any of the detected bacterial phyla. Figure 78 shows an example of linear regression analysis of the six most abundant phyla in the oral wash and sputum samples. These data indicate that it would not be possible to predict the abundance of specific phyla in the sputum using oral wash samples in individuals with COPD.

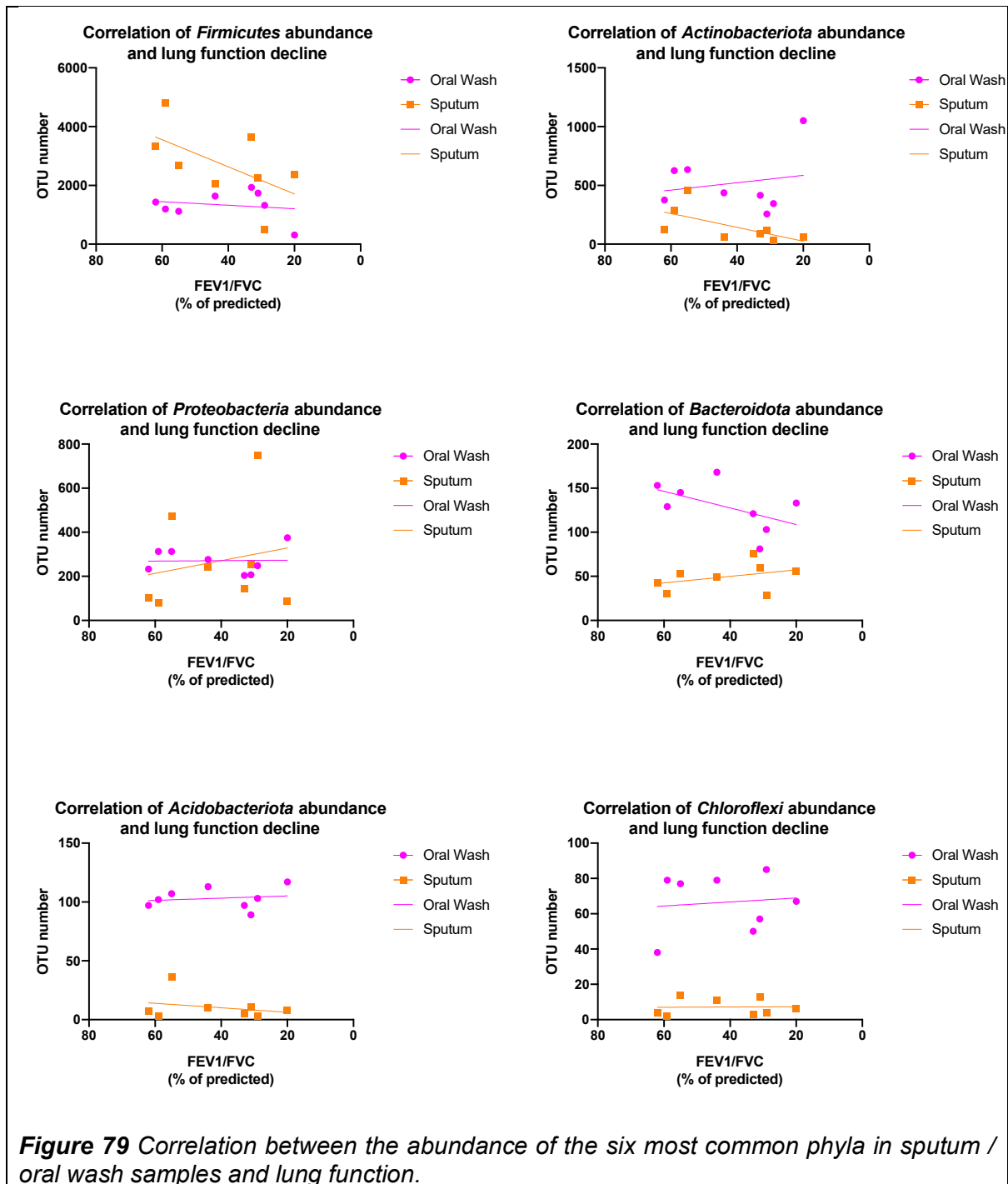


While oral wash samples could not be used to predict the abundance of bacterial phyla in the sputum, I used the generated data to understand whether changes in the abundance of

bacteria in the sputum would be reflected in the oral wash samples. Initially, I intended to evaluate whether changes in the bacterial abundance in the oral wash and sputum samples correlate to the difference in blood glucose levels between diabetic and non-diabetic individuals. In this study, however, we did not recruit any diabetic individuals. Instead, I evaluated changes in the microbiome against a decline in lung function in individuals with COPD.

I used a linear regression analysis to evaluate whether lung function decline causes similar changes in the abundance of bacteria in both sputum and oral washes. Simple linear regression analysis can be used to assess whether slopes and intercepts are significantly different. A p -value lower than 0.05 would indicate that slopes and intercepts are significantly different. However, in this study, I am evaluating whether similar trends are observed in the sputum and oral washes. I can, therefore, use an inverse analysis, where a p -value higher than 0.95 would indicate that slopes and intercepts between two samples are significantly similar. In this analysis, a p -value of 1 would indicate that both the slopes and the intercepts are precisely the same.

Changes in the abundance at the phylum level appeared to be sporadic across different samples (Figure 79). For example, the abundance of *Actinobacteriota* appears to increase in oral wash samples and decrease in sputum samples as the lung function of individuals with COPD declines. The abundance of bacteria of phylum *Acidobacteriota* remained constant in both sputum and oral wash samples, irrespective of lung function. There were no bacteria that changed in a similar manner and correlated with lung function levels.



Similar to the analysis above, I investigated whether an abundance of the individual genus of bacteria in the sputum could be predicted using oral wash samples. Linear regression and Pearson's correlation coefficient revealed that there was no bacterial genera abundance that correlated between oral wash and sputum samples. An example of linear regression analysis could be seen in Figure 80, demonstrating graphs corresponding to the top six most abundant genera.

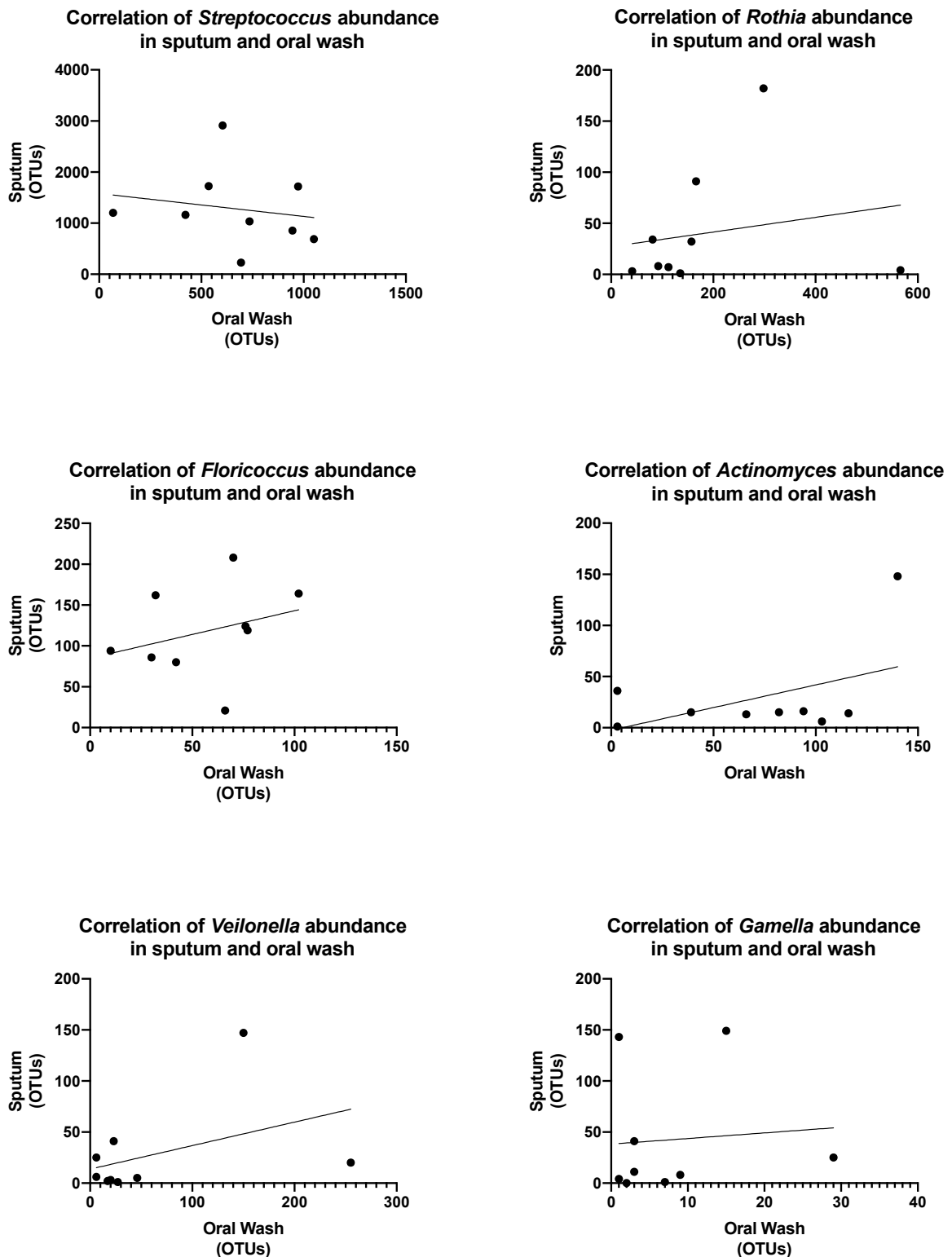
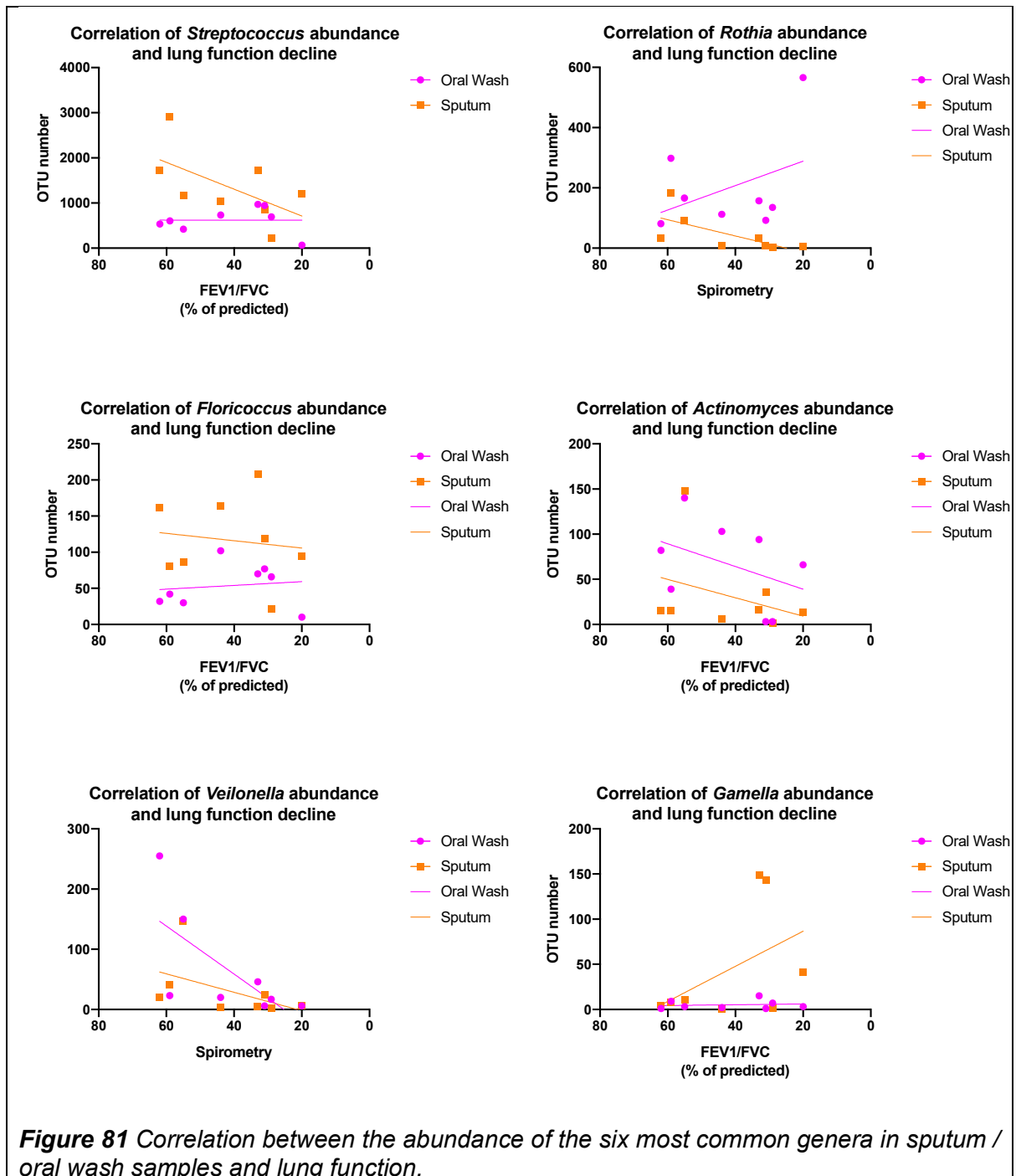


Figure 80 Correlation between the abundance of the six most common genera in sputum and oral wash samples. No significant correlation using the Pearson correlation coefficient.

Similar to phylum-level analysis, I have analysed whether bacterial abundance at genus level changes in sputum and oral wash samples in a similar manner in individuals with various levels of lung function. Bacterial abundance changed differently in the oral wash and sputum samples, where, for example, the abundance of *Streptococcus* decreased in the sputum samples with the decline of lung function while remaining consistent in the oral wash samples. The only notable exception is the abundance of *Actinomyces*. The abundance of *Actinomyces* dropped in both sputum and oral wash samples as lung function declined. More importantly, the decline was consistent in both sputum and oral wash samples, with a slope of 1.258 in oral wash samples and 1.017 in sputum samples. Simple linear regression returned a *p*-value of 0.8874 for the slopes and intercepts being equal. While not statistically significant, it does demonstrate that there are potentially bacteria where changes in the abundance could be similar between sputum and oral wash samples.



The data above demonstrate that it is unlikely that oral wash samples could be used to estimate the abundance of bacteria in the sputum or to predict the changes in the abundance in the sputum.

5.4.7 Common bacterial lung pathogens in the sputum and oral wash samples from individuals with COPD

In the sputum and oral wash samples of individuals with COPD, I observed a low abundance of common lung pathogens such as *Pseudomonas* and *Staphylococcus*. Genus *Staphylococcus* was present in nearly all samples; however, only 1-3 OTUs belonging to *Staphylococcus* were detected in each sputum and oral wash sample. Genus *Pseudomonas* was found in oral wash samples (2 OTUs total) but was absent from all sputum samples. Genus *Moraxella* was present in 2 of the sputum and 2 of the oral samples in extremely low quantities (1 OTU in each). Genus *Haemophilus* was one of the most common genera detected in the oral wash and sputum samples, with 270 OTUs between all samples. This, however, is skewed by two individuals whose sputum accounted for the majority of OTUs (178 and 48 OTUs), while other samples contained a low number of OTUs (0-5).

While COPD individuals' sputum and oral wash microbiome contained common respiratory bacteria and presented a typical respiratory microbiome profile, our interest in this study lies in tracking bacterial respiratory pathogens. These data would suggest that healthy individuals with COPD may not be an ideal cohort for studies focusing on respiratory pathogens.

5.5 Discussion

5.5.1 Patient recruitment process and study design improvements

One of the reasons we failed to recruit enough individuals for this study is the general unwillingness of individuals to participate in the study. The most common reason for not participating in the study was individuals feeling tired after having multiple appointments in a single day. A few individuals could not bring up sputum to expectorate and informed me that generally, they can only spontaneously expectorate sputum in the morning after they wake up.

A few modifications could be added to the recruitment protocol to aid recruitment chances. Several individuals brought up that peak sputum production is in the morning, after awakening, and they struggle to expectorate sputum later in the day. Individuals could potentially be given sterile tubes to expectorate sputum in the morning and bring it with them to the clinical appointment. The delay before freezing would potentially affect microbiome composition (Cuthbertson *et al.*, 2014); however, if the delay would only be a few hours, the effect on the microbiome could be minimal. The protocol would need to be redesigned, and a safety assessment would be required to assess the risks of individuals carrying contaminated sputum. If implemented, it would increase the number of potential study participants.

Individuals unwilling to participate in the study due to exhaustion after hospital appointments could be offered a reimbursement for transportation costs, such as a taxi home. The prospect of staying longer at the hospital for an appointment in exchange for the comfort of travelling home could sway individuals to participate in the study.

The initial study design suggested recruiting individuals with no antibiotic use in the last 30 days. However, during the study, it became apparent that recruiting diabetic COPD individuals with no recent antibiotic use may be problematic, as these individuals are at higher risk of respiratory infections (Mirrakhimov, 2012). In order to recruit 15 qualifying diabetic COPD

individuals, a more prolonged study would be required. Alternatively, a larger research team spanning multiple hospitals would greatly increase recruitment success.

5.5.2 Lung microbiome comparison between CF and COPD individuals

In this study, I decided to recruit individuals with COPD instead of individuals with CF. COPD is a much more prevalent disease in the population than CF. Individuals with COPD often attend chest clinics at their local hospital, allowing recruitment during scheduled hospital visits. Recruiting individuals also removes the cost of sample acquisition, allowing us to gather more samples.

At the phylum level, the composition of the sputum microbiome was distinctively similar between individuals with CF and COPD. Both microbiomes primarily comprised *Proteobacteria*, *Firmicutes* and *Actinobacteriota*. In individuals with CF, however, *Proteobacteria* were most abundant, while in individuals with COPD, *Firmicutes* were most abundant. The difference is primarily driven by *Pseudomonas* (phylum *Proteobacteria*), which is the most abundant bacteria in the lungs of individuals with CF and is nearly wholly absent from the lungs of individuals with COPD.

At the genus level, the microbiome comprised similar bacteria in CF and COPD sputum. Genera *Streptococcus*, *Rothia*, *Floricoccus*, *Actinomyces*, *Veilonella* and *Gamella* were some of the most abundant bacteria in both samples. The difference between CF and COPD microbiomes was primarily driven by pathogenic species such as *Staphylococcus*, *Pseudomonas* and *Ralstonia*. Sputum samples acquired from individuals with CF contained a high abundance of pathogenic bacteria, while sputum samples from COPD individuals lacked these pathogenic bacteria. This difference is primarily driven by different use of antibiotics in two cohorts. Individuals with CF are regularly treated with antibiotics to maintain lung function; this leads to the establishment of antibiotic-resistant pathogens in the lungs, as discussed in

Chapter 3 section 4.4. On the other hand, individuals with COPD do not regularly use antibiotics as long-term prophylaxis with antibiotics was not shown to reduce exacerbations or prevent pulmonary infections in these individuals (MacNee and Calverley, 2003). In COPD, antibiotics are used to treat occasional lung infections. This doesn't lead to the establishment of antibiotic-resistant lung pathogens as it does in CF and so common lung pathogens are not normally found in COPD individuals (Devine, 2008). On occasion that pathogens colonise the lungs of individual with COPD they are cleared rapidly cleared with antibiotics. This could potentially explain the lack of bacterial pathogens detected in the sputum of individuals with COPD.

In this study, I also observed that COPD microbiomes follow the same pattern observed in individuals CF, where α -diversity decreases as lung function of the patient declines (Cox *et al.*, 2010; Fodor *et al.*, 2012; Carmody *et al.*, 2013; Boutin *et al.*, 2017; Cuthbertson *et al.*, 2020). With the information presented above, I suggest that individuals with COPD are an excellent cohort to recruit for studies focusing on the lung microbiome and assessing the impacts of treatments, conditions, and the environment on the lung microbiome. Individuals with COPD present a typical lung microbiome profile with the usual core taxa present. However, individuals with COPD may not be the ideal cohort for studies focusing on investigating the bacterial pathogens in the lungs as in this study, individuals with COPD appear to lack the common respiratory pathogens in sputum samples.

5.5.3 Future uses for oral wash samples

This study was designed to assess whether oral wash samples could be used to aid lung microbiome studies in individuals that do not expectorate sputum and do not want to undergo invasive procedures. I have established that oral wash and sputum samples share the majority of species and are predominantly made up of the same bacteria. However, at this stage, with limited sample size, it was not possible to predict the OTU count or α -diversity of the sputum from oral wash samples in this study. Changes in the abundance of specific bacteria in the

sputum were not reflected in the oral wash samples. I have observed one potential genus where changes in the abundance in the sputum appeared to correlate with changes in the oral wash. However, the results were not statistically significant. Changes in the sputum microbiome did not appear to translate to the oral wash microbiome, limiting its potential uses. A niche use for oral wash samples could be to confirm the presence or absence of a particular pathogen, which is how other research groups have previously used oral wash samples to detect respiratory pathogens such as *Mycobacterium tuberculosis* and SARS-CoV-2 (Valinetz and Cangelosi, 2021). While the abundance of bacteria did not correlate between sputum and oral wash samples, bacteria present in the sputum were also present in the oral wash samples.

5.6 Summary

In this chapter, I have explored the potential to use oral wash samples as a non-invasive alternative to detect and predict the lung microbiome. I have described a study that I designed and received approval to conduct in a clinical setting, where I recruited a cohort of individuals with COPD. In this study, I collected oral wash and sputum samples from individuals, extracted bacterial DNA, and compared the microbiome data. I observed remarkable similarities between oral wash and sputum microbiomes and noted a possibility of using oral wash samples to confirm the presence of specific bacteria, as previously described in the literature. I have, though, observed no correlation between the abundance of bacteria in the oral wash and sputum samples. It was not possible to predict the abundance of specific bacteria in the sputum using oral wash samples.

Moreover, changes in the abundance of bacteria with a decline in lung function did not correlate between oral wash and sputum samples. The cohort of recruited individuals lacked common lung bacterial pathogens, preventing me from investigating whether pathogen abundance could correlate between oral wash and sputum samples. At present, that the data do not support that oral wash samples could be used to study lung microbiome in individuals with no ongoing lung infections.

5.7 Covid-19 impact statement

This study was approved for the duration of 1 year as we estimated this to be enough time to recruit a full cohort of individuals. Individuals with COPD tend to attend a chest clinic every 4-6 months for a regular check-up. It was decided that if a patient is willing to participate in the study, but is currently being treated with antibiotics, then a follow-up appointment would be set, to collect samples from the patient once they match the criteria. This would have aided the recruitment of individuals who are more likely to have respiratory infections, such as diabetic individuals.

Due to unforeseen circumstances, five months into the recruitment process the UK entered a national lockdown to curb the spread of Covid-19. Recruitment for this study had to be curtailed, as the work was not classed as essential. Individuals with COPD are extremely vulnerable to respiratory pathogens, so any hospital visits in person were cancelled, and virtual appointments were held instead. This prevented the recruitment of any additional individuals after nine samples were collected. Any follow up appointments were cancelled, resulting in no diabetic sample acquisition. Unfortunately, the IRAS study approval expired over the course of the lockdown. When individuals returned for in-person appointments, consultants did not feel that exposing individuals to more personnel would be beneficial to the individuals' health, so withdrew their support for the study.

Though this study was not completed due to reasons beyond our control, samples collected prior to the lockdown were processed and analysed after the Chief Investigator was allowed to return to site. This was possible due to the clause in the Patient Information Sheet, which stated that in the event the study was not completed, all collected samples would be processed and analysed as outlined in the study design.

5.8 Limitations and future work

Initially this study was designed to determine whether specific lung pathogens are detected in the oral wash samples of diabetic individuals with COPD. However, I was unable to recruit any individuals with diabetes for this study. If this study is to be repeated in the future, an alternative approach should be utilised to collect samples from diabetic individuals with COPD. The study timeframe should be prolonged to allow diabetic individuals with ongoing lung infections to recover, and alternative appointments could be set to collect samples. Alternatively, exclusion criteria could be adjusted to include individuals with antibiotic use, greatly increasing study intake and netting a bigger cohort. The study design could also be modified to allow contacting diabetic individuals with COPD via hospital letters inviting them to participate in the study during their next hospital visit. This would require approval of the IRAS, hospital ethics board, and the attending consultant; however, it would aid recruitment of diabetic individuals with COPD.

In this study, I have focused on individuals with COPD with no recent use of antibiotics. This resulted in a lung microbiome containing small amounts of classic bacterial lung pathogens. The absence of lung pathogens prevented me from completing one of the initial goals of the study – to investigate whether the abundance of bacterial pathogens correlates between oral wash and sputum samples. If this study were to be repeated, recruiting individuals with CF instead of COPD could potentially allow the tracking of common lung pathogens. However, the logistics of recruiting individuals with CF are a lot more complicated than recruiting individuals with COPD.

6 Final summary

In this thesis, I have explored the effects of diabetes on the lung microbiome using diabetic mice, human sputum samples, and *in vitro* artificial sputum medium. I demonstrated that diabetes modifies the lung microbiome in both mouse and human samples. However, understanding the importance of changes in the microbiome composition is challenging due to the complexity of the microbiome and the effects of diabetes on the body, which are not limited to hyperglycaemia.

In humans, I have demonstrated how diabetes modifies the lung microbiome in a non-conventional way and links it to other body systems. For example, diversity of the lung microbiome increased in individuals with CFRD despite being associated with a decrease in lung functions. I have shown that the abundance of *Firmicutes* and *Bacteroidota* in the sputum changed in diabetic individuals in the same manner as it was previously observed to change in the gut by other research teams. I have highlighted that the abundance of *Porphyromonas* increased in the lungs of CFRD individuals. While this bacterium is not well studied in the lungs, it is a leading cause of chronic periodontitis, frequency of which is increased in diabetic individuals. Though this study did not offer any explanatory mechanisms behind the changes observed, it highlighted the scope of the changes that diabetes could be imposing on the lung microbiome, which should be further researched in the future.

In mouse samples, I observed an increase in the abundance of *Pseudomonas* and a decrease in the abundance of *Staphylococcus* in diabetic animals. These changes were also observed in the human CF sputum samples, where an increase in the abundance of *Pseudomonas* and a decrease in abundance of *Staphylococcus* were seen in individuals with CFRD. To understand the effects of diabetes on the relationship between these two pathogens, I set up an artificial sputum medium *in vitro* model. In this model, I observed a gradual increase in the abundance of *Pseudomonas* and a decrease in the abundance of *Staphylococcus* until growth

of both bacteria plateaued. However, in my model, although the presence of glucose promoted the growth of both species in the separate cultures, the presence of added glucose did not modify the relationship between the two pathogens in the final model. This model offers a novel way to establish a stable co-culture of several bacteria and could be further modified to explore the effects of glucose (eg with constant glucose influx, as would be found *in vivo*.) on bacterial growth.

Work presented in this thesis highlights diabetes-induced changes in the lung microbiome that should be explored further to aid our understanding of the importance of the microbiome in the maintenance of the lung health.

7 References

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8 Attachments

8.1 CF sample information

Individuals with CF did not receive all of the stated drugs at the same time. However, exact date of administration of each drug is not available.

Sample ID	Age	Sex	FVC	FEV ₁	FEV1/FVC (%)	HbA1c	Glucose	Status	Drugs
1	44.85	F	3.5	1.725	49.3	39	5.8	normal	Azithromycin
2	32.76	M	2.95	1.475	50.0	49	4.9	diabetic	Tobramycin Temocillin
3	44.14	M	2.6	1.1	42.3	48	12.4	diabetic	Azithromycin Clindomycin
4	32.72	M	3.95	1.73	43.8	65	7.3	diabetic	Azithromycin Promixin
5	42.65	M	3.75	1.85	49.3	46	5.9	pre-diabetic	Azithromycin
6	41.91	F	3.55	1.86	52.4	35	4.5	normal	Azithromycin Colistin
7	46.90	F	2.41	1.3	53.9	34	5	normal	Azithromycin Meropenem
8	27.39	M	3.99	2.14	53.6	37	4.7	normal	Azithromycin Colistin
9	29.80	M	4.95	3.7	74.7	33	4	normal	Azithromycin Promixin

									Salbutamol
10	42.2 1	F	3.75	2.86	76.3	36	5.2	normal	Promixin Salbutamol
11	42.9 7	M	3.54	1.83	51.7	40	5.2	normal	Tobramycin Flucloxacillin Colisitin
12	24.7 6	M	4.59	2.94	64.1	30	7.3	normal	Azithromycin Flucloxacillin TIP Colobreathe
13	29.7 5	M	4.46	2.88	64.6	36	7.6	normal	Azithromycin Promixin
14	20.5 1	M	3.88	3.38	87.1	35	-	normal	Azithromycin Colistin Co-amoxiclav
15	25.3 9	F	2.69	1.66	61.7	61	18.2	diabetic	Azithromycin Aztreonam Colobreathe
16	43.8 3	F	2.25	1.11	49.3	58	8	diabetic	Azithromycin
17	27.3 6	F	2.24	1.35	60.3	38	4.3	normal	Colistin Ipra Flucloxacillin
18	34.9 4	M	4.04	2.89	71.5	43	5	pre- diabetic	Azithromycin Colistin Salbutamol

19	34.5 8	M	2.25	1.11	49.3	49	5.3	diabetic	Azithromycin Promixin Tobramycin Ciprofloxacin Septrin
20	51.1 3	M	3.61	1.96	54.3	62	4.9	diabetic	Azithromycin Colistin Aztreonam Salbutamol
21	36.7 0	F	1.75	0.98	56.0	41	7	normal	Azithromycin Colobreathe Aztreonam
22	75.2 2	M	2.07	1	48.3	34	6.5	normal	x
23	28.0 6	M	3.31	2.52	76.1	27	4.8	normal	Azithromycin Colistin Tobramycin
24	25.5 6	M	5.3	3	56.6	55	7.8	diabetic	Azithromycin Colistin
25	35.1 3	M	3.38	1.17	34.6	68	6.7	diabetic	x
26	39.9 6	M	2.37	0.99	41.8	61	27.2	diabetic	Aztreonam Colistin Tobramycin Salbutamol
27	23.1 2	F	2.82	1.47	52.1	60	7.4	diabetic	Azithromycin

28	37.9 3	M	3.84	1.47	38.3	57	12.5	diabetic	Azithromycin Rifaximin Tobramycin
29	35.3 3	F	2.43	1.19	49.0	57	7.9	diabetic	Azithromycin Aztreonam Meropenem
30	34.5 6	F	2.3	1.33	57.8		16.9	diabetic	Azithromycin Promixin Meropenem

8.2 COPD sputum and oral wash study documentation

8.2.1 Participant information sheet

Participant Information Sheet (PIS)

Title of Study: Investigation of diabetes-induced changes to the lung microbiome

IRAS Ref: 256124

Participants will be given a copy of this information sheet

Invitation Paragraph

I am a PhD student at the St George's University of London. I would like to invite you to participate in this research project which forms part of my PhD research. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask me if there is anything that is not clear or if you would like more information.

What is the purpose of the study?

When we breathe in, germs (bacteria and viruses) enter our lungs. As the lungs have a strong immune system for protection, most of the time this doesn't cause a chest infection. One part of the lung immune system is a mechanism to keep lung secretions 'sugar-free'. This means that there is no sugar for the bacteria to eat in lung secretions, making it harder for them to grow. Lung sugar concentrations rise in people with high blood sugar (diabetes) and in people with lung disease (sugar leaks out of the blood into lung secretions). This increases the risk of infection.

In this study, we look at what bacteria live in lungs and how these bacteria change between people with normal and high blood sugar. Usually studying bacteria in the lungs is hard and requires unpleasant procedures. This study will look into alternative ways to sample lung bacteria by analysing oral washes and sputum samples.

We will take all of the samples into the laboratory where bacteria DNA from your sample will be extracted and sequenced to find out what bacteria are present in the sample. The rest of the sample will be destroyed. No human material will be used.

Why have I been invited to take part?

You have been invited to take part because you have a chronic lung condition and are able to cough up phlegm and provide a sample.

Do I have to take part?

No, participation is voluntary. You do not have to take part. You should read this information sheet and if you have any questions you should ask the research team.

What will happen to me if I take part?

The study will take up to 45 minutes of your time.

We will ask you a few questions, ask you to gargle some clean salt water for 30 seconds and take a sample of your phlegm (sputum).

Questions. We will ask you a few questions about diet and medical history and take a list of your medicines. With your permission, we will be able to get some of this information from your medical notes.

Oral wash sample. We will ask you to gargle 10ml of saline solution for 30 seconds and spit out solution into the container. This could take around 2 minutes.

Sputum sample. We will ask you to perform some simple breathing and coughing manoeuvres to bring up a fresh sample of phlegm from your lungs. A physiotherapist may help with this, particularly if you are already being treated by one. This could take around 5 minutes.

Your GP would be notified of your participation in this study as a standard procedure.

What are the possible benefits and risks of taking part?

There are no specific benefits for you in taking part in this study. In the future, we hope it will generate new knowledge to help us develop new ways of preventing lung infections and analysing lung bacteria.

The blood test can be moderately uncomfortable. You will feel squeezing, then a sharp prick on your arm. Sometimes your arm can feel slightly bruised after the test has been done.

If your blood sugar levels are unexpectedly high, we will tell your hospital doctors or GP with your permission so that it can be checked in more detail. Note that high blood sugar levels are not necessarily indicative of diabetes, however, we would advise to consult with GP for further investigation.

How is the project being funded?

The project is funded by using funding available to Stanislavs Vasiljevs, as a part of his PhD research project.

This study has been given a favourable ethical opinion by the Ethics Committee.

What if something goes wrong?

If you wish to make a complaint about the conduct of the study you can contact student, supervisor, course director or independent researcher using the details below for further advice and information:

████████████████████
████████████████████
████████████████████
████████████████████
████████████████████

[REDACTED]

[REDACTED]

[REDACTED]

The **University** has in force the relevant insurance policies which apply to this study. If you wish to complain or have any concerns about any aspect of the way you have been treated during the course of this study then you should follow the instructions given above.

Who should I contact for further information?

If you have any questions or require more information about this study, please contact me using the following contact details:

[REDACTED]

Who is Handling My Data?

St George's University of London, as the sponsor, will act as the 'Data Controller' for this study. We will process your personal data on behalf of the controller and are responsible for looking after your information and using it properly. This information will include your NHS number, medication regime and health information, which is regarded as a 'special category personal data'. We will use this information as explained in the 'What is the purpose of the study' section above.

What will happen to my data?

As a publicly-funded organisation, we have to ensure when we use identifiable personal information from people who have agreed to take part in research, this data is processed fairly and lawfully and is done so on the basis of **public interest**. This means that when you agree to take part in this research study, we will use your data in the ways needed to conduct and analyse the research study.

All project data will be held for at least **10** years, your personal data will be held and processed in the strictest confidence, and in accordance with current data protection regulations.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you decide to withdraw your data from the study, we may not be able to do so. We will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information <https://www.sgul.ac.uk/privacy>

Or contact our university Data Protection Officer at:

Email: dataprotection@sgul.ac.uk

Tel: 020 8725 0668

What will happen to the results of the study?

I will produce a final report summarising the main findings. I also plan to disseminate the research findings through publication and conferences.

Pseudonymised data will be deposited or submitted to an open source online research data repository at the end of the study. This data may be used for future research.

Will my data be used for future research?

When you agree to take part in a research study, the information we collect may be provided to researchers running other research studies in this organisation and in other organisations. These organisations may be universities, NHS organisations or companies involved in research in this country or abroad. Your information will only be used by organisations and researchers to conduct research and processed on the basis of public interest.

This information **will not identify you** and **will not be combined** with other information in a way that could identify you. The information will only be used for the purpose of research and cannot be used to contact you or to affect you. It will not be used to make decisions about future services available to you, such as insurance.

What if I want to complain about the way data is handled?

If you wish to raise a complaint on how we have handled your personal data, you can contact our Data Protection Officer who will investigate the matter. If you are not satisfied with our response or believe we are processing your personal data in a way that is not lawful you can complain to the Information Commissioner's Office (ICO) (<https://ico.org.uk/>).

Thank you for reading this information sheet and for considering taking part in this research.

8.2.2 Consent form

CONSENT FORM FOR PARTICIPANTS IN RESEARCH STUDIES

Please complete this form after you have read the Information Sheet and listened to an explanation about the research.

Title of Study: Investigation of diabetes-induced changes to the lung microbiome

IRAS Ref: 256124

Thank you for considering taking part in this research. The person organising the research must explain the project to you before you agree to take part. If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

By ticking/initialling each box you are consenting to this element of the study. It will be assumed that un-ticked/un-initialled boxes mean that you DO NOT consent to that part of the study and you may be deemed ineligible for the study.

	Please tick
1. I confirm that I have read and understood the information sheet dated 14/05/2019 Version 1.2 for the above study. I have had the opportunity to consider the information and asked questions which have been answered satisfactorily.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time during the study without giving any reason and without being disadvantaged in any way.	☐
3. I consent to the processing of my personal information for the purposes explained to me. I understand that such information will be handled in accordance with current data protection regulations.	☐
4. I understand that my information may be subject to review by responsible individuals from the St George's University of London and/or regulators for monitoring and audit purposes.	☐
5. I understand that confidentiality and anonymity will be maintained, and the researcher will not identify me in any research output.	☐
6. I agree that the research team may access my medical records for the purposes of this research project.	☐
7. I agree that the research team may use my data derived from this research for future research and understand that any use of identifiable data would be reviewed and approved by a research ethics committee. (In such cases, as with this project, data would not be identifiable in any report).	☐

8. I agree that my GP may be contacted if any unexpected results are found in relation to my health. I will be informed when this happens ☐
9. I agree for my samples to be collected and used for research and understand that any use of identifiable data would be reviewed and approved by a research ethics committee. (In such cases, as with this project, data would not be identifiable in any report). ☐

Name of Participant

Date

Signature

Name of Researcher

Date

Signature

8.2.3 Case report form

CASE REPORT FORM (CRF)

Study title: Investigation of diabetes-induced changes in lung microbiome using next-generation sequencing.

Investigator Signature: _____

Patient initials:

Screening number:

Subject number:

IRAS Project ID: 256124

Section 1- SCREENING

Informed consent:	
Consent received	Yes ☐ No ☐
Date volunteer signed written consent form: __ __ / __ __ / __ __ (DD) / (MMM) / (YYYY)	

Inclusion criteria:			
Able and willing to give Informed consent?	Yes	☐	No ☐
Have more than 10 pack-years of smoking?	Yes	☐	No ☐
Is active sputum producer?	Yes	☐	No ☐

Exclusion criteria:			
Main chronic lung condition is not COPD If yes please specify.	Yes	☐	No ☐

Use of antibiotics in the previous 30 days? If yes please specify.	Yes	☐	No ☐

Section 2- RANDOMISATION

Participant study Randomisation Number allocated:	_____
Date of Randomisation:	____/____/_____ (DD / MMM / YYYY)

Section 3 - PATIENT INFORMATION

Demographic Data	
Date of Birth	/ / (DD) / (MMM) / (YYYY)
Gender	Female ☐ Male ☐

Smoking History
☐ Current smoker Started smoking (MMM/YYYY): ____ / ____ Number of cigarettes : ____ /day Pack year history: ____ Other: _____ Type of smoking: ☐ Tobacco ☐ E-cigarettes ☐ Other
☐ Ex-Smoker Pack year history: ____ Smoked Till: Days: ____ Weeks: ____ Months: ____ Years: ____ Other: _____

Spirometry data	
FEV1	_____ / _____ % of predicted
FVC	_____ / _____ % of predicted
FEV1:FVC	_____

Other relevant medical conditions			
Condition	Present? Yes/No	Start date (DD/MMM/YYYY)	End date (DD/MMM/YYYY) Or tick if ongoing.
Diabetes			☐
Gastroesophageal reflux			☐
Dental problems / false teeth			☐

Is the volunteer on any <u>relevant</u> medication?					Yes	No
Medication	Dose and units	Daily frequency	Route of administration	Start date (DD/MMM/YYYY)	End date (DD/MMM/YYYY) Or tick if ongoing.	
						☐
						☐
						☐
						☐

Last chest infection (with dates): _____

Number of chest infections in last year: _____

Section 4 – Blood glucose

Blood glucose

	Value
HbA1C	
Blood glucose	
CRP	
WCC	

Section 4 – Oral Wash

Oral Wash collected? If no, complete below.	Yes ☐	No ☐

Why not collected:

Short term storage (fridge/freezer/etc):

	Value
Weight of the sample	

Section 4 – Sputum collection

Sputum collected? If no, complete below.	Yes	☐	No	☐

Why not collected:

Sputum collected

Short term storage (fridge/freezer/etc):

	Value
Weight of the sample	

8.2.4 Original project proposal

Study synopsis

Brief title:	Comparison of approaches for lung microbiome analysis between patients with or without diabetes
Official title:	Investigation of diabetes-induced changes in lung microbiome using next-generation sequencing
Study design	Prospective observational cross-sectional pilot (exploratory)
Study Population/disease condition	COPD patients
Eligibility criteria:	Inclusion criteria: - More than 10 pack-years of smoking - FEV/FVC less than 0.7 - Be active sputum producer - Have confirmed COPD
Study design	Exclusion criteria: - Use of antibiotics in the previous 30 days - Have other chronic lung diseases except for COPD - Unable to give consent
The target number of participants	30
Criteria for evaluation	- Primary outcome measure(s) This study will compare operational taxonomic units (OTUs) between participants with hyperglycaemia and normoglycaemia. OTUs are the most commonly used units of microbial diversity when performing the 16S analysis.
	- Secondary outcome measure(s) Paired analysis of OTUs, as well as β-diversity, will be compared between gargle and sputum samples in all participants groups, including normoglycaemic, hyperglycaemic and participants with impaired glucose tolerance.
Sources of funding Anticipated start date: Anticipated primary completion date:	PhD project funding
Sources of funding Anticipated start date: Anticipated primary completion date:	27/05/2019
Sponsor/Co-Sponsor	27/11/2019
Key Contact names	

Background

Contrary to the popular belief that the lungs are sterile, it was recently discovered that the lung is inhabited by resident bacteria and that it possesses its own unique microbiome. Several chronic lung conditions, such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), have been shown to induce changes to the resident lung microbiota.

High blood glucose (hyperglycaemia) has been linked to increased glucose concentration in the airway surface liquid (ASL). This has been shown to be associated with an increased risk of developing a pulmonary infection in patients in the intensive care unit and in patients who have diabetes and chronic lung disease.

Next-generation sequencing utilising 16S ribosomal RNA amplification approach has become the standard for identifying bacterial species in the microbiota. 16S gene is present in all bacteria and allows precise species identification from a small amount of starting material, making it a strong choice for analysis of the samples with low bacterial yield, such as samples of lung microbiome.

Currently, sampling lung microbiome requires invasive methods to acquire representative samples such as lung biopsy, bronchoalveolar lavage or induced sputum.

Study Rationale

Previous studies have shown that gargle sample allows adequate estimation of the lung microbiome. (Charlson et al., 2011; Morris, James M Beck, et al., 2013; Bassis et al., 2015; Samuelson et al., 2018). However, no such work was performed in patients with diabetes or/and chronic lung disorders.

Study objectives

This study will investigate whether less invasive gargling approach can provide a true estimate of lung microbiome to replace more invasive methods.

Primary objective

To determine whether lung microbiome of people with chronic lung conditions differs between those with hyperglycaemia and normoglycaemia.

Secondary objectives

To determine whether gargle samples can be used as an alternative for sputum samples to study lung microbiome.

Trial design

Overall design

Prospective observational cross-sectional pilot (exploratory)

This will be a prospective cross-sectional study.

People with primary COPD and hyperglycaemia, as well as people with COPD and normoglycaemia, will be recruited from secondary care clinics in their order of attendance. They will be seen at a single time point when blood glucose and HBA1C will be measured and gargle and sputum samples will be collected.

Consent for all of the procedures and sequencing of the data will be obtained prior to the sampling.

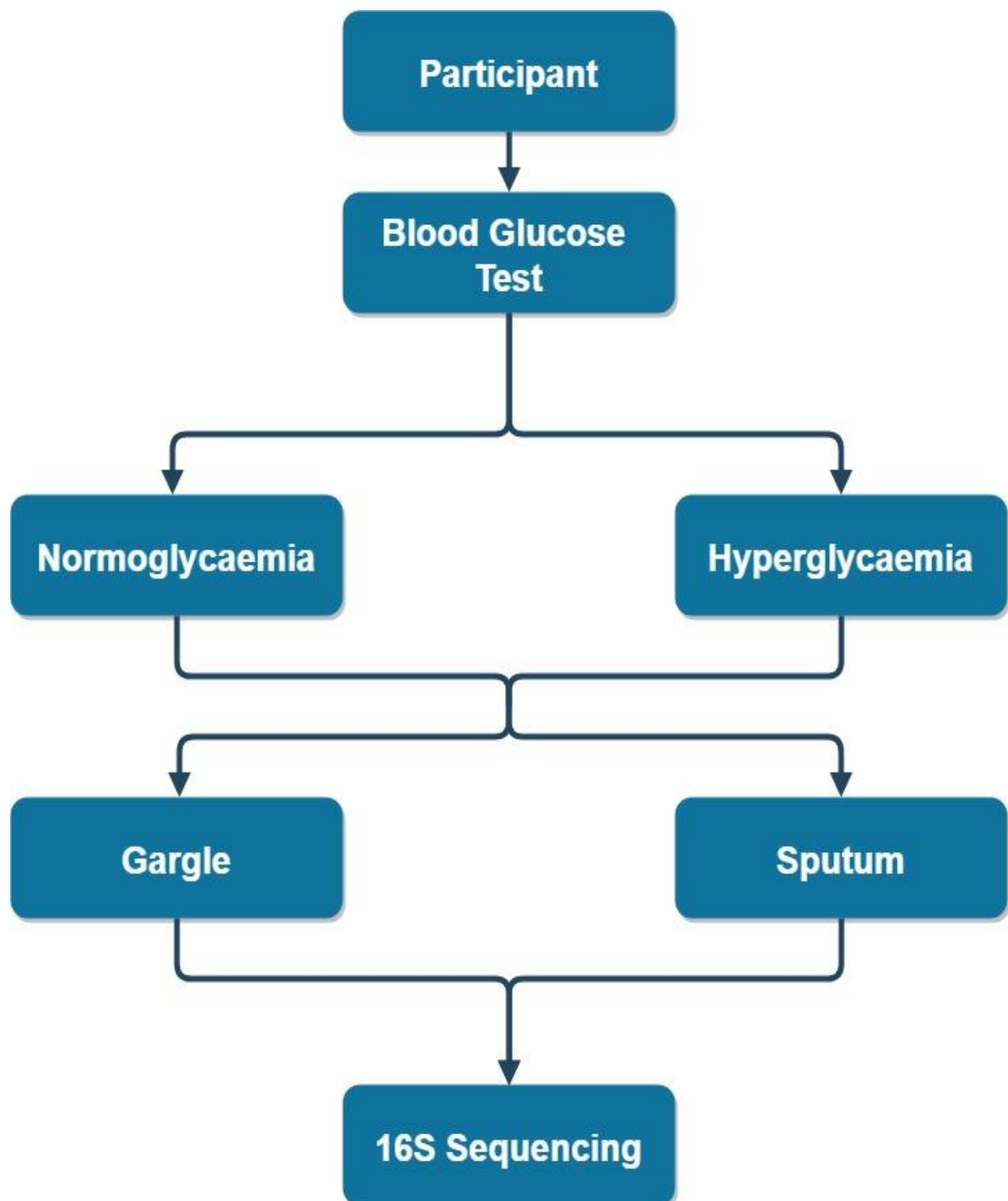
Treatment/intervention plan and rationale

There is no treatment or intervention plan.

Additional treatment/ interventions

No additional treatment or intervention will be performed.

Schematic of Study design



Participation selection criteria

There will be no exceptions (waivers) to eligibility criteria prior to participant inclusion into the study. Any questions raised about eligibility should be addressed prior to entering the participant.

The eligibility criteria have been carefully considered and are standards used to ensure the trial results can be appropriately used to make future treatment decisions for other people with similar disease or medical condition. It is, therefore, vital exceptions are not made to the following detailed selection criteria.

Participants will be considered eligible for enrolment into this trial if they fulfil all the inclusion criteria and none of the exclusion criteria as defined below.

Participants with COPD will be screened against inclusion and exclusion criteria. Participants will be stratified into groups by HBA1C. Participants with HBA1C of <42mm will be considered normoglycaemic. Participants with HBA1C of >48mm will be considered hyperglycaemic. Participants who fall between 42mm and 48mm will be considered to have impaired glucose tolerance. Samples from these participants will not be used for answering the primary objective. However, their samples will still be analysed to compare gargle and sputum microbiome to answer the second objective.

Inclusion criteria

People seen at St George's University Hospitals NHS Foundation Trust with the chronic obstructive pulmonary disorder (COPD) who are able and willing to give informed consent will be recruited to the study.

Participants will need to fill out all of the following criteria:

- More than 10 pack-years of smoking
- FEV/FVC less than 0.7
- Be active sputum producer
- Age over 18 years
- Have confirmed COPD

Exclusion criteria

Participants will be excluded from the study if they meet at least one of the following criteria:

- Use of antibiotics in the previous 30 days
- Have other chronic lung diseases except for COPD
- Unable to give consent

Participant Recruitment process

Patient recruitment at a site will only commence once evidence of the following approval/essential documents is in place:

1. REC approval, if applicable HRA approval
2. Final sponsorship and host site permissions, (confirmation of capacity and capability)

All subjects who wish to enter the study will be fully screened and consented by the Chief Investigator, or an appropriate delegate. As detailed below Participants will be recruited during their visits at the St George's University Hospitals NHS Foundation Trust.

Study procedures

Informed consent

Consent will be taken by CI of this trial Stanislavs Vasiljevs. Prior to the beginning of the study, Stanislavs will undergo Good Clinical Practice (GCP) training.

Training for Stanislavs has been arranged for the week commencing 13th of May 2019 and will be completed prior to the beginning of the study. Training will take place at St George's University of London. Stanislavs would also shadow Professor Emma Baker in the clinic to gain experience and would be assisted in the beginning of the study until enough experience is obtained to work independently.

Participants will be approached to discuss the study. They will be given a patient information sheet and have as long as they see fit to consider whether or not to take part in the study. If they agree to take part consent will be taken at the study visits prior to any procedures.

The Investigator or designee will explain that the patients are under no obligation to enter the trial and that they can withdraw at any time during the trial, without having to give a reason.

A copy of the signed Informed Consent Form (ICF) along with a copy of the most recent approved Patient Information Sheet (PIS) will be given to the study participant. An original signed & dated consent form will be retained in the ISF and a copy will be placed in the medical notes

If the new information results in significant changes to the risk-benefit assessment, the consent form will be reviewed and updated if necessary. All participants, including those already being treated, will be informed of the new information, given a copy of the revised consent form and asked for re-consent if they choose to continue in the study.

Discontinuation/withdrawal of participants and stopping rules

As participation in the trial is entirely voluntary, the participant may choose to discontinue participation at any time without penalties or loss of benefits to which they may be entitled. Although not obliged to give a reason for discontinuing their participation a reasonable effort should be made to establish this reason, whilst remaining fully respectful of the participant's rights. Participants who discontinue study participation, for any of the above reasons, should remain in the study for the purpose of follow up and data analysis.

Participants may withdraw at any stage. Participants will not be followed up. *If the participant confirms they do not wish to participate in the scheduled follow up data collection visits then data that has already been collected should be kept and analysed according to the ITT principle for all participants who stop follow up early.*

Definition of the End of Trial

End of the trial is defined as the Last Patient Last Visit (LPLV).

Study Procedures

Participants will only be seen once. All of the procedures will be performed consecutively.

Screening assessments

Patients will be screened for suitability for the study against inclusion and exclusion criteria using medical records.

Baseline assessments

Participants' blood levels of Hemoglobin A1C (HBA1C) will be measured with a blood test. Clinical data including age, nature of underlying lung disease, comorbidities and inflammatory markers (where available) will be collected from patient notes and electronic records.

Participants will be asked to gargle 10ml of sterile saline and sample will be collected. Once gargle is collected participants will be asked to give a sample of non-induced sputum. This may be obtained with the help of the respiratory physiotherapists. Both sputum and gargle samples will be collected into chilled pre-weighed containers, placed on ice and transported into a laboratory where they will be snap frozen in liquid nitrogen and stored at -80 °C until future work.

Subsequent assessments

No, follow up will be performed in this study.

Summary flow chart of study assessments

Study procedure	Screening	First visit
Informed consent		X
Inclusion/exclusion criteria	X	
Medical history	X	
Demographics	X	
Baseline		X
Screening		X
Blood test		X
Sputum sample		X
Gargle samples		X
Questionnaire		X

Methods

Laboratory procedures

Once all of the required samples are collected, they will be thawed on ice and processed to extract bacterial DNA. Bacterial DNA will then be sequenced using next-generation sequencing in order to determine bacterial species present in the samples.

Safety Events

Confidentiality

All data will be handled in accordance with the Data Protection Act 2018.

Data collection tool

Case Report Forms will be designed by the CI. All data will be entered legibly in black ink with a ball-point pen. If the Investigator makes an error, it will be crossed through with a single line in such a way to ensure that the original entry can still be read. The correct entry will then be clearly inserted. The amendment will be initialled and dated by the person making the correction immediately. Overwriting or use of correction fluid will not be permitted.

Participants' clinical data will be taken from the medical notes. Blood glucose HBA1C levels will be stored in the electronic patient record. Gargle and

sputum samples will be used for research only. Data will be recorded from medical records onto the CRF.

Incidental Findings

If participants have an unexpectedly elevated blood HBA1C levels this will be reported to their hospital doctor or GP using the study reporting letter.

Data handling and analysis

Data will be entered into a Microsoft Excel spreadsheet.

Statistical design

Statistical input in the trial design

Samples will be analysed individually. Microbiome data will be analysed against blood glucose concentration, to assess the effect of increased blood glucose on the lung microbiome. Microbiome data from gargle and sputum samples will be compared to assess the similarity between the samples.

Endpoints

Primary endpoints

This study will compare operational taxonomic units (OTUs) between participants with hyperglycaemia and normoglycaemia. OTUs are the most commonly used units of microbial diversity when performing the 16S analysis.

Secondary endpoints

Paired analysis of OTUs, as well as β -diversity, will be compared between gargle and sputum samples in all participants groups, including normoglycaemic, hyperglycaemic and participants with impaired glucose tolerance.

Sample size and recruitment

Sample size calculation

This is a forefront scientific hypothesis of no similar precedent. Therefore, there is no preliminary input such as effect size to test for to perform a power calculation and estimate the number of participants required for this study. Previous studies looking at the lung microbiome with gargle sampling recruited between 6 and 78 participants, making it extremely hard to estimate the required number of participants. We propose this study as a

feasibility study and expecting to recruit 30 patients for both hyperglycaemic and non-hyperglycaemic groups.

Planned recruitment rate

We plan on recruiting every eligible patient attending COPD clinic as there are no invasive procedures involved and procedures are not time-consuming. We expect the recruitment rate to be at least 2 participants a week for 12 weeks.

Statistical analysis plan

Summary of baseline data and the flow of patients

Microbiome data will be grouped and measured for changes in alpha diversity (species diversity in each individual sample), bacterial richness, the total number of unique OTUs, phylogenetic diversity (the relative amount of diverse phylogenetic lineages), and species evenness in each sample. The difference will be measured between gargle and sputum samples as well as correlated to the blood glucose concentration.

Data will be a group using QIIME software. Statistical tests will be performed in Prism 7 (GraphPad software). Patients will be grouped to minimise the effect of multiple variables. Where possible, Wilcoxon t-test will be performed on the mean relative abundance of OTUs between the gargle and sputum samples to determine the statistical significance, using the MASS package in R. AMOVA analysis of variance will be performed to assess dissimilarity between samples from normoglycaemic and hyperglycaemic patients.

Primary endpoint analysis

All the variables in the data will be explored using graphs and summary statistics to understand their nature and distributional assumptions. Continuous data will be summarized using means, standard deviations, medians, interquartile values and ranges and binary/categorical data as proportions.

Each individual will be assessed for the burden and abundance of microbes. Each participant will be assigned a microbial spectrum and its individual distribution. Detailed microbial distributions will be derived for each patient. For the purpose of statistical analysis, i.e. to understand potential differences between groups average distributions, it is likely to disentangle 5 categories of main classes of microbes present throughout - one category possibly deemed as “others” to include those in very small proportions. This will define a multinomial statistical outcome and investigated with a multinomial logistic regression. The differences between groups will be adjusted for demographics and other collected clinical variables. Predictions following models’ fit to the data would be used for patterns in the average distribution of the microbiome across groups defined by glucose concentration and other

significant demographic or clinical variables. Results would provide solid statistical input to estimate the sample size for a larger study.

Committees involved in the trial

As this is not a trial study, it will be overseen by a clinician.

Direct access to source data

The Investigator(s)/institution(s) will permit trial-related monitoring, audits, REC review, and regulatory inspection(s), providing direct access to source data/documents. Trial participants are informed of this during the informed consent discussion. Participants will consent to provide access to their medical notes.

Finance

This study is being conducted as a project which forms part of a PhD research. Consumables will be paid for by student bench fees. There is no other funding required for this study.

Insurance and indemnity

The St George's University of London holds insurance to cover participants for injury caused by their participation in the clinical trial. Participants may be able to claim compensation if they can prove that St George's has been negligent. This includes negligence in the writing of the protocol or selection of trial resources.

Where the Trial is conducted in a hospital, the hospital has a duty of care to participants. The St George's University of London will not accept liability for any breach in the hospital's duty of care or any negligence on the part of hospital employees. Hospitals selected to participate in this clinical trial shall provide clinical negligence insurance cover for harm caused by their employees and a copy of the relevant insurance policy or summary shall be provided to the St George's University of London, upon request.

Participants may be able to claim compensation for injury caused by participation in this Trial without the need to prove negligence on the part of the St George's University of London or another party.

If a participant indicates that they wish to make a claim for compensation, this needs to be brought to the attention of the St George's University of London immediately.

Failure to alert the St George's University of London without delay and to comply with requests for information by the sponsor or any designated Agents may lead to a lack of insurance coverage for the incident.

IP and development policy

Unless otherwise specified in agreements, the following guidelines shall apply: All Intellectual Property Rights and Know-How (IP) related to the Protocol and the Trial are and shall remain the property of the Sponsor excluding

- 1) Pre-existing IP related to clinical procedures of any Hospital.
- 2) Pre-existing IP related to analytical procedures of any external laboratory.

All contributors

Shall assign their rights in relation to all Intellectual Property Rights and in all Know How, not excluded above to the Sponsor and at the request and expense of the Sponsor, shall execute all such documents and do all such other acts as the Sponsor may reasonably require in order to vest fully and effectively all such Intellectual Property Rights and Know-How in the Sponsor or its nominee.

Shall promptly disclose to the Sponsor any Know-How generated pursuant to this Protocol and not excluded above and undertake to treat such Know-How as confidential information jointly owned between it and the Sponsor

Nothing in this section shall be construed so as to prevent or hinder and medical professional from using Know How gained during the performance of the Trial in the furtherance of its normal business activities, to the extent such use does not result in the disclosure or misuse of Confidential Information or the infringement of any Intellectual Property Right of the Sponsor.

Publication policy

Publication: "Any activity that discloses, outside of the circle of trial investigators, any final or interim data or results of the Trial, or any details of the Trial methodology that have not been made public by the Sponsor including, for example, presentations at symposia, national or regional professional meetings, publications in journals, theses or dissertations."

All scientific contributors to the Trial have a responsibility to ensure that results of scientific interest arising from Trial are appropriately published and disseminated. The Sponsor has a firm commitment to publish the results of the Trial in a transparent and unbiased manner without consideration for commercial objectives.

To maximise the impact and scientific validity of the Trial, data shall be consolidated over the duration of the trial, reviewed internally among all investigators and not be submitted for publication prematurely. Lead in any publications arising from the Trial shall lie with the Sponsor in the first instance.

Before the official completion of the Trial

All publications during this period are subject to permission by the Sponsor. If an investigator wishes to publish a subset of data without permission by the Sponsor during this period, **the Funder** shall have the final say.

Exempt from this requirement are student theses that can be submitted for confidential evaluation but are subject to embargo for a period not shorter than the anticipated remaining duration of the trial.

Up to 180 days after the official completion of the Trial

During this period the Chief Investigator shall liaise with all investigators and strive to consolidate data and results and submit a manuscript for peer-review with a view to publication in a reputable academic journal or similar outlet as the Main Publication.

- The Chief Investigator shall be the senior and corresponding author of the Main Publication.
- Insofar as compatible with the policies of the publication outlet and good academic practice, the other Investigators shall be listed in alphabetic order.
- Providers of analytical or technical services shall be acknowledged, but will only be listed as co-authors if their services were provided in a non-routine manner as part of a scientific collaboration.
- Members of the Steering Group shall only be acknowledged as co-authors if they contributed in other capacities as well.
- If there are disagreements about the substance, content, style, conclusions, or author list of the Main Publication, the Chief Investigator shall ask the Steering Group to arbitrate.

Beyond 180 days after the official completion of the Trial

After the Main Publication or after 180 days from Trial end date any Investigator or group of investigators may prepare further publications. In order to ensure that the Sponsor will be able to make comments and suggestions where pertinent, material for public dissemination will be submitted to the Sponsor for review at least sixty (60) days prior to submission for publication, public dissemination, or review by a publication committee. Sponsor's reasonable comments shall be reflected. All publications related to the Trial shall credit the Chief and Co-Investigators as

co-authors where this would be in accordance with the normal academic practice and shall acknowledge the Sponsor and the Funders.

Statement of Compliance

The trial will be conducted in compliance with the protocol, Sponsor's Standard Operating Procedures (SOPs), GCP and the applicable regulatory requirement(s).

The study conduct shall comply with all relevant laws of the EU if directly applicable or of direct effect and all relevant laws and statutes of the UK country in which the study site is located including but not limited to, the Human Rights Act 1998, the Data Protection Act 2018, the Human Medicines Regulations 2012, ICH GCP, the World Medical Association Declaration of Helsinki entitled 'Ethical Principles for Medical Research Involving Human Subjects' (2008 Version), the UK Policy Framework for Health and Social Care Research (Version 3.2, October 2017).

This study will be conducted in compliance with the protocol approved by the REC and according to GCP standards. No deviation from the protocol will be implemented without the prior review and approval of the Sponsor and REC except where it may be necessary to eliminate an immediate hazard to a research subject. In such a case, the deviation will be reported to the Sponsor and REC as soon as possible.