Cystic Fibrosis and the Gut Microbiota

A thesis presented to the National University of Ireland for the Degree of Doctor of Philosophy

By

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For my parents, Jim and Sandra, and my husband, Nathan.

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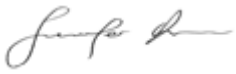
Declaration

I hereby certify that this thesis has not been previously submitted, in part or in whole, to this university or any other university for any degree and is, unless otherwise stated, the original work of the author.



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Author Contributions

All of the work herein was performed independently by the author, with the following exceptions:

Chapter 2: Dr. Orla O' Sullivan conducted part of the bioinformatic analysis of the sequencing data. Dr. Fiona Fouhy performed culture-independent related lab work and analyses. Dr. Fiona Fouhy and Jennifer Deane prepared the published manuscript.

Chapter 3: Ribotyping of *C. difficile* isolates was performed by *Clostridium difficile* Ribotyping Network for England (CDRNE).

Chapter 4: Analysis of whole genome sequence of *L. plantarum* 9 S_2 was performed by Dr. Damian Plichta (MS-Omics).

Chapter 5: Dr. Nicola Ronan conducted the clinical trial, collection of samples and clinical data. Dr. Orla O' Sullivan conducted part of the bioinformatic analysis of the sequencing data.

Chapter 6: Dr Gisli Einarsson conducted part of the bioinformatic analysis of the sequencing data. MS-Omics performed part of the analysis of the metabolomics data

Chapter 7: Dr Kiera Murphy performed part of the bioinformatics analysis of the sequencing data.

Publications

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Abbreviations

AAD Antibiotic Associated Diarrhoea

AMP Antimicrobial Peptide

AMR Antimicrobial Resistance

BHI Brain Heart Infusion Agar

BLAST Basic Local Alignment Search Tool

BMI Body Mass Index

BSH Bile Salt Hydrolase

CCEY Cycloserine Cefoxitin Egg Yolk Agar

CDAD *Clostridium difficile* Associated Diarrhoea

CDC Centre for Disease Control and Prevention

CDI *Clostridium difficile* Infection

CDRNE Clostridium difficile Ribotyping Network for England (CDRNE)

CF Cystic Fibrosis

CFQ-R Clinical Frequency Questionnaire

CFRD Cystic Fibrosis Related Diabetes

CFTR Cystic Fibrosis Transmembrane Conductance Regulator

CLA Conjugated Linoleic Acid

CRP C-reactive Protein

DIOS Distal Intestinal Obstruction syndrome

EAR Estimated Average Requirement

EDCD European Centre for Disease Control and Prevention

EDTA Ethylene-diamine-tetraacetic acid

EFSA European Food Safety Authority

EIA Enzyme Immunoassay

ELISA Enzyme-linked Immunosorbent assay

EPS Exopolysaccharide

ESBL Extended Spectrum Beta-Lactamase

FAA Fastidious Anaerobic Agar

FAO Food and Agriculture Organisation of the United Nations

FBS Fetal Bovine Serum

FDA Food and Drug Administration

FE-1 Faecal Elastase-1

FEV_1 Forced Expiratory Volume in 1 second

FLASH Fast Length Adjustment of Short Reads

FMT Faecal Microbial Transplant

GC-MS Gas chromatography- Mass spectrometry

GIT Gastrointestinal Tract

GRAS Generally Recognised as Safe

IBD Inflammatory Bowel Disease

IBS Irritable Bowel Syndrome

IV Intravenous

LAB Lactic Acid Bacteria

LBS Lactobacillus Selection Agar

MDR Multi Drug Resistance

MI Meconium Ileus

MIC Minimum Inhibitory Concentration

MRD Maximum recovery Diluent

mMRS Modified de Man, Rogosa and Sharpe media

MRSA Methicillin Resistant *Staphylococcus aureus*

OTU Operational Taxonomic Unit

PBS Phosphate-buffered saline

PB Phosphate Buffer

PCA Principle Components Analysis

PCR Polymerase Chain Reaction

PCoA Principle Co-ordinates Analysis

PFGE Pulse Field Gel Electrophoresis

PI Pancreatic Insufficiency

PO *per os*

PWCF Persons with Cystic Fibrosis

PyNAST Python nearest Alignment Space Termination

QIIME Quantitative Insights into Microbial Ecology

qPCR Quantitative Polymerase Chain Reaction

RBB Repeat Bead Beating

RNI Recommended Nutrient Intake

SCFA Short Chain Fatty Acid

TAE Tris Acetic EDTA buffer

TBE Tris Borate EDTA buffer

TCA Trichloroacetic acid

WCA Wilkens Chalgren Agar

WHO World Health Organisation

Abstract

The role of the gut microbiota in host health has been established in recent years and has been shown to be altered in numerous disease states, including Cystic Fibrosis (CF) cohorts. This thesis investigates the gut microbiota and microbiome in CF persons and how it may be manipulated through interventions with the potential to improve respiratory symptoms in CF persons. Firstly, in order to survey the CF gut microbiota composition, we performed a multicentre analysis of the prevalence of *C. difficile* in a CF cohort in three European sites (Ireland, UK and Belgium), reporting the highest carriage rate to date of 63-71% compared to 7% in age-matched healthy controls. *C. difficile* ribotypes were geographically distinct among the three sites, with a core of two ribotypes (046 and 078) shared among all sites. In order to identify probiotic species which would have potential to modulate the CF gut microbiota with respiratory benefits, we screened a biobank of lactobacilli and bifidobacterial isolates sourced from the CF gut microbiota for a putative probiotic with antibiotic tolerance, resulting in identification of *L. plantarum* 9_S2. In addition, the gut microbiota composition and function were examined following treatment with Cystic Fibrosis transmembrane conductance regulator (CFTR) modulation therapy, Ivacaftor. Despite clinical trials reporting improvements in lung function and reductions in pulmonary exacerbations, the effects on the gut microbiota composition, function and metabolome were subtle. Pancreatic function was not restored and intestinal inflammation was not reduced following commencement of Ivacaftor treatment. Finally, in order to overcome logistical issues of collecting sufficient amounts of fresh faecal samples on one day to perform distal colon models of the CF gut we investigated the viability of freezing faecal samples prior to preparation of faecal inoculum. Overall the results presented in this thesis demonstrate the differences in the gut microbiota between CF persons and healthy cohorts and demonstrate that interventions may result in subtle normalisations of the CF gut microbiota.

Chapter 1

Implications of long-term antibiotic use on gut health and microbiota in populations including patients with Cystic Fibrosis.

Published in full as a book chapter in ‘The Gut-Brain Axis- Dietary, Probiotic and Prebiotic Interventions on the Microbiota.’ Chapter 11, p. 223-260. Elsevier.

1.1 Abstract

Since the commercialisation of antibiotics in the 1940's, life expectancy has increased in parallel with their discovery. However, concerns regarding the collateral effects of antibiotic therapy on gut microbiota composition and functionality within the host have come to the fore. Antibiotic therapy disrupts the normal ecology of gut microbiome, resulting in altered composition, and in some cases function. Cystic Fibrosis (CF) is an ideal candidate group in which to examine antibiotic effects on the gut microbiota. Chronic antibiotic use, along with the typical high fat CF diet, CFTR malfunction itself, pancreatic enzyme supplementation and suppression of gastric acid, play a key role in defining the unique composition of the CF intestinal microbiota. Most available studies focus on the short term effects of an antibiotic course of limited duration (e.g. 7 days) and the pattern of recovery following such treatments. Here, we discuss the specific effects of different antibiotic families on the gut microbiota. We examine the multi-drug resistant (MDR), *C.difficile* and Antibiotic Associated Diarrhoea (AAD) risk and prevalence associated with chronic antibiotic use. We review the success, to date of probiotics to ameliorate CF symptomology. Finally, we explore alternative therapies for infection control currently under research.

1.2 Introduction

Since the discovery by Alexander Fleming of penicillin in 1929 and the subsequent journey of antibiotic research and discovery, antibiotics have revolutionised human health on a global scale. Antibiotic therapy can be acknowledged for an increase of life expectancy by 8 years between 1944 and 1972, a period of intensive and successful antibiotic research (Cotter *et al*. 2012). However, the negative effects of antibiotic therapy are well documented, most notably antimicrobial resistance which

is now accepted as a global issue spanning medical, economic and environmental fronts. WHO (World Health Organisation), CDC (Centre for Disease Control and Prevention) and ECDC (European Centre for Disease Prevention and Control) consider Multidrug Resistant (MDR) infections caused by antibiotic resistant bacteria as an emergent global disease and a major threat to public health (Roca *et al*., 2015). However, this review will focus on the more pervasive effects of antibiotic therapy on the gut microbiome and the far-reaching effects on the health of the host. The antibiotic-mediated perturbations of the microbiome and disruption of related functionality is an area of much research. In recent years the collateral damage caused by antibiotic use has come into focus (Blaser, 2011). Antibiotic therapy can be likened to a war of attrition on the gut microbiota as subsequent rounds of therapy result in loss of diversity, followed by incomplete recovery causing the bacterial population to shift from the initial assemblage. The control of infection through antibiotic use, while not diminishing its importance, has potentially devastating consequences downstream not only for the individual, as host-microbe interactions are disturbed, but also for society with the potential dissemination of multidrug resistance determinants. In this review we examine the literature regarding the effects of various families of antibiotics on the gut microbiota, discussing in particular issues with relevance to a Cystic Fibrosis (CF) cohort such as multidrug resistance as a result of chronic antibiotic use, *C.difficile* and disruption of host-microbiota mutualistic relationships.

Perturbations of the gut microbiota can occur via many external environmental factors, but perhaps the most dramatic modulatory effects are seen with antibiotic use. The immediate impact of short-term antibiotic therapy on the gut microbiota is extensively investigated. However longitudinal studies mapping the

recovery of the microbiota in the aftermath are limited, with even greater gaps in our knowledge regarding the long-term effects of chronic antibiotic use on the gut microbiota. Studies focussing on short term antibiotic use have demonstrated the profound and pervasive effects of the antibiotic, the resilience of the gut microbiota following treatment (Dethlefsen *et al*., 2008, Jernberg *et al*., 2007) and reduction of faecal bacterial load and diversity (Xu *et al*., 2014, Iapichino, 2008, Jakobsson *et al*., 2010). Others have demonstrated the differential effects of different classes of antibiotics on the gut microbiota and how the resultant assembly following antibiotic treatment may be influenced by mode of action (Perez-Cobas *et al*., 2013). It is widely reported that immediately following antibiotic treatment an increase in *Enterococcus* species, (Iapichino *et al*., 2008) and a decrease in *Bifidobacteria*, *Clostridia*, *Desulfovibrio*, *Faecalibacterium* and *Bacteroides* species (Adamsson *et al*., 1999, Bartosch *et al*., 2004, Jernberg *et al*., 2007, O' Sullivan *et al*., 2013) is expected. The majority of the microbiota recover after four weeks; however, a long-term persistence of expressed antibiotic resistance genes, particularly carried by *Enterococcus* species, following a short term antibiotic treatment was noted up to four years post treatment in some human studies (Jakobsson *et al*., 2010, Jernberg *et al*., 2007).

CF cohorts are ideal candidate groups to investigate the effects of antibiotic use on gut health and microbiota. CF is a monogenic disorder thereby reducing confounding factors and so facilitating inferences regarding antibiotic effects on the gut microbiota. CF is a potentially life threatening autosomal recessive disorder affecting 70,000 individuals worldwide with Ireland having the highest prevalence (approximately 1100 patients) and incidence rates (1 in 1353)(Farrell *et al*., 2002). It results from mutations in the Cystic Fibrosis Transmembrane Conductance

Regulator (CFTR) gene, located on the long arm of chromosome 7 (Collins FS, 1992), of which the most common is ΔF508 (a deletion of phenylalanine at codon 508). CFTR gene mutations result in defective chloride ion transport in the respiratory, hepatobiliary, gastrointestinal, reproductive tracts and the pancreas (Quinton, P.M, 1990). The natural progression of CF is characterised by chronic lung function decline punctuated by recurrent periods of temporary worsening of symptoms, known as pulmonary exacerbations (Amadori *et al*., 2009, Flume *et al*., 2009). Prophylactic antibiotic therapy plays a pivotal role in treating CF patients: combating frequent lung infections, slowing the rate of lung function decline and ultimately prolonging life. Indeed antibiotic discovery, amongst other therapeutic options, has significantly contributed to the increased life expectancy of CF sufferers meaning many survive into their thirties and beyond while in the 1950's few lived to attend primary school. The increasing life expectancy of CF individuals results further new therapeutic challenges (Plant *et al*., 2013). A global shifting pattern of antibiotic use is seen in antibiotic therapy for CF patients where prophylaxis, maintenance and chronic antibiotic therapy have been shown to improve clinical outcomes and survival (Moskowitz *et al*., 2008, Szaff *et al*., 1983).

While the gut microbiota of CF patients is poorly documented, it can be assumed that intensive and frequent courses of antibiotics alter its diversity and functioning as a symbiotic partner with the host. Seventy per cent of the immune system is harboured in the gastrointestinal tract, meaning perturbations in the microbiome at the very least will have implications for host immunity, but also for energy extraction efficiency from food, mental well-being and colonisation resistance against pathogenic microorganisms (Weiner, 2000, Faria and Weiner, 2005, Vighi *et al*., 2008). Defining and characterising the gut microbiota of a CF

population can provide information applicable to the wider medical community. A picture of the gut microbiota of CF patients as a unique entity that is a signature of the disease is emerging in recent research. The characteristic CF intestinal microbiome is thought to be as a result of a combination of factors including the CFTR malfunction itself, chronic antibiotic use, high fat diet, pancreatic enzyme supplementation and suppression of gastric acid (Bruzzese *et al*., 2014). The CF gut has been shown to have lower counts of lactic acid bacteria (LAB), *Clostridium* species, *Bifidobacterium* species, *Veillonella* species, and *Bacteroides-Prevotella* species, whereas Enterobacteriaceae counts were increased in the CF cohort (Duytchaever *et al*., 2011). As expected in a cohort with chronic antibiotic use, CF patients have been shown to have lower species richness, evenness, diversity and reduced temporal stability of intestinal microbiota (Scanlan *et al*., 2012). In particular, the reduction of diversity and abundance of *Bifidobacterium* species (*B.longum, B.catenulatum, B.pseudocatenulatum* and *B.adolescentis*), Clostridium cluster XIVa and Clostridium cluster IV (*R. bromii* and *F. prausnitzii*) has been described (Duytschaever *et al*., 2013). Bifidobacterium species have a greater antimicrobial susceptibility to many antibiotics used in the treatment of CF pulmonary exacerbations and reduced adhesion capacity to inflamed mucosa (Lee and O' Sullivan, 2010, Russell *et al*., 2011), perhaps explaining their reduction in a CF cohort. Evidence from Madan *et al*. (2012) revealed a core lung-gut microbiota dominated by *Veillonella* and *Streptococcus*, with similarities between population fluctuations between the two sites. Furthermore, they show select genera (*Roseburia, Dorea, Sporacetigenium, Coprococcus, Blautia, Enterococcus* and *Escherichia*) of the gut microbiota precede bacterial populations found in the lung. Allelic variations of the CFTR gene have also been shown to have effects on the gut microbiota, with

homozygous F508del and severe disease phenotype CF patients exhibiting greater dysbiotic faecal microbiota compared to heterozygous milder disease phenotype patients, a signature most likely reflective of reduced therapies (Schippa *et al*., 2013). It is hypothesised that imbalance of the intestinal microbiota of CF patients may be associated with, causes, or aids, the progression of the disease or may be a predictor of expected outcomes following antibiotic therapy (Duytschaever *et al*., 2011).

1.3 Antibiotics use among persons with Cystic Fibrosis (PWCF)

Antibiotic regimes for CF persons currently follow one of the following strategies: symptomatic regime which is treatment of pulmonary exacerbation upon notice of acute symptoms (Elphick *et al*., 2005); or elective regimes that may comprise of treatment of the chronic infection with IV, inhaled and/or oral antibiotics at regular intervals to delay or prevent long-term deterioration in the absence of recent clinical deterioration (Høiby 1993, Moskowitz *et al*., 2008). The latter elective regimes, constitutes chronic antibiotic usage or prophylaxis and have become routine practice to prevent or delay pulmonary exacerbations. CF patients, typically, undergo antibiotic treatment involving a combination of inhaled and oral therapies and potentially intravenous treatments also, from different mechanistic classes (e.g. ceftazidime and tobramycin) (Smyth *et al*., 2014, Flume *et al*., 2009). With the advent of efficient nebulisation devices in the 1990s, inhaled antibiotics became the mainstay in CF therapy maintenance, enabling eradication of initial *Pseudomonas* infection (Ramsey *et al*., 1999, Pamukcu *et al*., 1995, Wall *et al*., 1983, Moskowitz *et al*., 2008, Gibson *et al*., 2003). Inhaled antibiotics result in greater concentrations arriving directly to the respiratory system secretions, local to the infection (Moskowitz *et al*., 2008). Nebulised/inhaled tobramycin, aztreonam and colistin

form the main therapeutic options for eradication and/or suppression of *Pseudomonas aeruginosa,* in the lung (Doring *et al*., 2012). Nebulisation duration is associated with a significant treatment burden on the patient and may result in poorer clinical outcomes due to reduced compliance with the treatment regimen (Sawicki and Tiddens, 2012). Advances in addressing patient treatment burden are seen with the emergence of portable dry powder formulations of tobramycin (TOBI podhaler) and colistimethate sodium (Colobreathe) (Konstan *et al*., 2011, Schuster *et al*., 2013, Uttley *et al*., 2013). Rapid delivery systems for antibiotic therapies via portable inhalers significantly reduce treatment burden and increase adherence (Harrison *et al*., 2014). The success of a particular antibiotic treatment regime is assessed primarily by considering its efficacy at suppression or eradication of the infectious agents and subsequent improvement in lung function, measured as FEV_1. In addition to the need for hospitalisation; the length of time until the requirement for additional antibiotics; the time to reoccurrence of pulmonary exacerbation; the number and severity of adverse events experienced by patients and the convenience and patient satisfaction and hence ease of compliance for the patient are considered.

1.4 The modulatory effects of specific antibiotics on the gut microbiota (non CF hosts)

The human gut microbiota is individual specific, at the bacterial strain level (Turnbaugh and Gordon, 2009, Lozupone *et al*., 2012, Schloissnig *et al*., 2013). How antibiotic therapies interact and perturb the microbiota varies between individuals, with individuals on the same antibiotic, dose and time showing varying effects, albeit similar trends (Perez-Cobas *et al*., 2013). Dethlefsen *et al*., (2008) showed not only inter-individual variation in response to ciprofloxacin, but also different responses of taxa. Grouping of metagenomic data from several individuals may mask

individual perturbations. It is therefore preferable to measure distance from baseline in each individual to reveal the true effect of the antibiotic treatment (Engelbrektson *et al*., 2006). Diet, genetics and health status, as well as the dynamics of the microbiota itself, may contribute to stability within the intestinal microbiota, enabling some communities to have more resilience than others in response to antibiotic therapy (Perez-Cobas *et al*., 2013). This elaborate network fed by numerous variables present a huge challenge when analysing microbiota responses to antibiotic treatment. Countering the effect of the antibiotic is the resilience and stability of the microbiota. The balance of the microbiota to external pressures is mediated by host-microbe interactions, microbe-microbe interactions and physiochemical factors (Edlund *et al*., 2000).

The effects of the antibiotics on the gut microbiome, is unique to each antibiotic and depends on several factors: the target spectra; the dose and duration; method of administration and the pharma kinetic and pharma dynamic properties of the agent (incomplete absorption or secretion of intravenous antibiotics by the bile or intestinal mucosa may result in higher concentrations in the intestine with greater ecological effects) (Edlund *et al*., 2000, Jernberg *et al*., 2010, Adamsson *et al*., 1999). Disturbances may be assessed (1) qualitatively (emergence of novel bacteria types with resistance genes, plasmids and transposons) or (2) quantitatively (changes in microbiota composition due to antibiotic pressures) (Edlund *et al*., 2000). Broad-spectrum antibiotic treatments affect not only the aberrant pathogenic bacteria but also beneficial members of the gut community, reduce diversity and richness and disturb the ecology of the gut (Jernberg *et al*., 2007, Dethlefsen and Relman 2011, O' Sullivan *et al*., 2012). However, it remains a challenge to distinguish antibiotic effects on the gut microbiota from other confounding factors such as stress, diet and

host genetics (Jernberg *et al*., 2010). The route of administration has implications for antibiotic-mediated disturbances in the gut microbiota as oral, inhaled and intravenous routes result in different concentrations in different sites (Liu and Derendorf, 2003, Liu *et al*., 2002).

The gut microbiota has a degree of resilience to antibiotic treatment, the strength of which may vary depending on which microorganisms form a given individual's microbiota, some conferring more stability than others. Recovery of gut microbiota is often rapid, ranging from two days (Dethlefsen and Relman, 2011) to two weeks (Ubeda *et al*., 2010), however individual taxa have different recovery rates, some slower than others as seen with increased proportions of Lachnospiracaea and Clostridiacaea by Antonopoulos and colleagues (2009) six weeks post treatment with cefoperazone. For many recovery is often incomplete or partial (Dethlefsen and Relman, 2011). This resilience may be apparent in the first round of therapy and it is in subsequent, repetitive or prolonged rounds of therapy that shifts in the microbiota may be seen (Perez-Cobas *et al*., 2013). The magnitude and rate of the response is primarily dictated by the initial microbial community, with some communities displaying a prolonged directional shift and others more rapid response (Dethlefsen and Relman, 2011). Initial community composition could be an important indication of expected outcomes following antibiotic use such as likelihood of pathogen overgrowth or development of antibiotic associated diarrhoea (De La Cochetiere *et al* 2008, Ozaki *et al*., 2004). The gut microbiota while stable is never static. Its periodic variations within a normal range make it difficult to definitively attribute loss or gain of certain taxa to certain antibiotics. Dethlefsen and Relman (2011) explain that the long-term stability of the gut microbiota is maintained by restoring forces following external disturbances rather than rigidity or resistance to change.

However the restoring forces will not withstand repeated antibiotic courses, even in cases where the microbiota has complete recovery following an initial antibiotic treatment.

Antibiotics families vary in mode of action and target specificity and therefore in the fingerprint they leave on the gut microbiota. Perez-Cobas (2013) and colleagues showed the greatest influence on the microbiota is the antimicrobial effect and the mode of antibiotic action. At compositional level the mode of action exerts the greatest effect, while at the functional level the antimicrobial effect is the driving force (Perez-Cobas *et al*., 2013). The mode of action of the antimicrobial agent produces different effects on the gut community. O' Sullivan and colleagues (2013) noted a significantly greater reduction of *Bifidobacterium* species and significant differences in levels of *Anaerococcus*, *Denitrobacterium*, *Faecalibacterium*, *Lactonifactor* and *Proteus* species in subjects receiving only a nucleic acid inhibitor compared to subjects on antibiotics with cell envelope mode of action, while subjects receiving only cell envelope antibiotics had significantly increased levels (10-fold) of *Lactobacillus* species and significant differences in levels of *Ascomycota* and *Elusimicrobia*. They concluded that cell envelope inhibitors had less impact on the intestinal microbiota than nucleic acid synthesis inhibitors. The effects are taxa specific with some more susceptible than others, however signatures of antimicrobial classes can be seen in bacterial profiles following treatment, with individuals diverse microbiota converging to similar compositions when administered the same antibiotic (Perez-Cobas., 2013, Jernberg *et al*., 2010, Antonopoulos *et al*., 2009). The selection of antibiotic should be based not only on the expected success of the antibiotic treatment but also on the level of ecological disturbances (Edlund *et al*., 2000). There is a lack of information detailing the effects of chronic usage of

different antibiotic classes on the gut microbiota. Here, we discuss the signatures produced by short term use of different antibiotic classes as shown in human studies, unless stated otherwise.

Macrolides

Macrolides are so-called because of their macrocyclic ring which may be 14, 15 or 16-membered (Hamilton-Miller, 1992). They have a protein synthesis inhibitory mode of action. Macrolides have been shown to inhibit protein synthesis by causing a dissociation of the peptidyl-tRNA from the ribosome (Tenson *et al*., 2003). Azithromycin has been shown to significantly improve quality of life in CF patients, reduce C-reactive protein (CRP) levels, reduce the rate of lung function decline (FEV_1 measurements), reduce the number of respiratory exacerbations and significantly reduce systemic inflammation (Saiman *et al*., 2003, Wolter *et al*., 2002). Azithromycin is chosen for macrolide therapy over other macrolides, such as erythromycin, due to less adverse effects on the gastrointestinal tract. Azithromycin is also known to have negative effects on the bacterial motility of *P.aeruginosa* through suppression of flagellin expression at sub-inhibitory concentrations (Kawamura-Sato *et al*., 2000). In a study of 3 adults presenting with gastric and duodenal ulcers, Jakobsson *et al*. (2010) showed the negative impact of Clarithromycin on members of the Actinobacteria phyla, significant reductions of *E.coli*, suppression of *Bifidobacterium*, *Lactobacillus* and *Clostridium* species and an increase in *Enterococcus*, *Enterobacter*, *Citrobacter*, *Klebsiella* and *Pseudomonas* species. Clarithromycin has been shown to strongly suppress anaerobic populations in a study of healthy adults comparing clarithromycin to a fluoroquinolone, moxifloxacin based treatment regimen (Adamsson *et al*., 1999, Brismar *et al*., 1991, Edlund *et al*., 2000). This has important implications for total ecological

disturbances as the anaerobic population is mainly responsible for colonisation resistance (Waaij, 1989, Hertz *et al*., 2014). Adamsson *et al*., (1999) also showed a significant qualitative change with an increase of resistant *Bacteroides* from 2% to 76% in a study of thirty adults with *Helicobacter pylori* infection. This indicates an antibiotic class with the ability for ecological disruption and promotion of resistant strains. This contrasts with the findings of Morotomi *et al*. (2011) which show macrolide antibiotic treatments tended towards dominance of *Streptococcus* species and seemed to have less dramatic effects on the microbiota with samples remaining similar to healthy intestinal microbiota in a study of 29 healthy adults (Morotomi *et al*., 2011).

β-lactams

β-lactams, encompassing cephalosporins, carbapenems, penicillins, monobactams and cephamycins, are considered the most successful antibiotic class discovered (Lewis, 2013). *β-lactams* form an important part of treatment of pulmonary exacerbation for CF patients. Ceftazidime, meropenem, flucloxacillin, piperacillin/taxobactam and aztreonam are all *β-lactam* therapies commonly used by CF patients. *β-lactams* have a bactericidal mode of action which disrupts cell wall synthesis, specifically the final cross linking stage of the peptidoglycan layer (Page, 2012, Tipper, 1979, Tipper and Strominger, 1965). *β-lactams* demonstrate broad-range activity against both Gram-negative and Gram-positive bacteria (Holten *et al*., 2000). Discovered in 1928 by Alexander Fleming, in 1940 Penicillin was the first antibiotic to be produced in a large scale and is accredited with having saved millions wounded during World War II. However, prior to its discovery the first β-lactamase was identified in 1940 in *E. coli* (Abraham and Chain, 1940), which was countered by the first β-lactamase inhibitor (clavulanic acid) in 1976. Clavulanic

acid, along with amoxicillin, now forms part of Augmentin® (Foulstone and Reading, 1982). Following the first β-lactamase there have been many challenges to the success of *β-lactams.* Carbapenemases, classified as either molecular class B (metallo-β-lactamases), A or D (serine carbapenemases, also known as oxacillinases), form three of the four known classes of β-lactamases (Miriagou *et al*., 2010). The host of specific carbapenemases varies depending on class. Metallo- β-lactamases are disseminated mainly in *Pseudomonas aeruginosa*, but also *Acinetobacter baumanii*, *Enterobaceriaceae* and *Klebsiella pneumonia* (Queenan *et al*., 2007, Walsh *et al*., 2008). *Klebsiella pneumonia* the most common host of class A carbapenems (Queenan *et al*., 2007), although there have been reports of its occurrence in other species such as *Klebsiella oxytoca*, *Salmonella enterica*, *E. coli*, *Enterobacter* species and *Pseudomonas* species (Queenan *et al*., 2007, Deshpande *et al*., 2006, Navon-Venezia *et al*., 2006, Villegas *et al*., 2007, Bennett *et al*., 2009). Oxacillinases (class D) are common in *Acinetobacter baumanii*, (Queenan *et al*., 2007). Most recently, the emergence of New Delhi metallo-β-lactamase-1 in 2008, which has since spread worldwide, has transformed mildly pathogenic bacteria into lethal multidrug resistant bacteria (Rolain *et al*., 2010). The prevalence of metallo-β-lactamases in *P. aeruginosa* is of significant clinical concern, given the importance of *P. aeruginosa* in CF lung exacerbations. Metallo-β-lactamase producing *P. aeruginosa* have shown an increase in the last 10 years and have been implicated in septicaemia and pneumonia (Kouda *et al*., 2009, Gutiérrez *et al*., 2007, Pitout *et al*., 2007, Lagatolla *et al*., 2006). Extended spectrum β-lactamases (ESBLs) present another challenge to the treatment of Gram-negative infections and are the main source of hospital- and community- acquired infections (Pitout and Laupland, 2008). Mainly arising in Enterobacteriaceae such as *Escherichia coli* and *Klebsiella* species,

ESBLs have plasmid encoded capability of hydrolysing the *β-lactam* ring of the oxy-imino-cephalosporins (cefotaxime, ceftriaxone and ceftazidime) and monobactams (aztreonam) (Bush and Jacoby, 2010, Dortet *et al*., 2012). Cephamycins (cefoxitin) and carabapenems (imipenem and meropenem) retain their activity against ESBL-producing bacteria. Reassuringly, in some cases, β-lactamase inhibitors such as clavulanic acid and tazobactam still have the ability to inhibit ESBLs (Bush and Jacoby, 2010).

Bacteroides, *Blautia* and *Faecalibacterium* species have been shown to increase in a study of four adults following a single antibiotic treatment with *β-lactams* such as Ampicillin, Amoxicillin, Cefazolin and Sulbactam (Perez-Cobas *et al*., 2013, Monreal *et al*., 2005). This bacterial profile has been shown to be associated with bactericidal agents but not bacteriostatic antimicrobials. The increase seen in the *Bacteroides* and *Parabacteroides* species may be explained by the presence of a high rate of β-lactamase-producing strains observed in these genera. In addition, high levels of *cfiA* gene identified suggest these strains act as reservoirs for antibiotic resistance genes and, also, the common occurrence of *cepA* gene which is responsible for production of cephalosporinases and penicillinases (Nakano *et al*., 2011, Wybo *et al*., 2007). Morotomi *et al*., (2011) also reported a dominance of *Enterococcus* genus following a single *β-lactam* antibiotic treatment. Adamsson *et al*., (1999) show that compared with a macrolide treatment (clarithromycin) a *β-lactam* treatment showed reduced, although still significant, ecological impact on the anaerobic populations and less emergence of resistant strains following treatment. The reduced disruption to anaerobic populations was also noted by Stark *et al*., (1993). In addition, Perez-Cobas and colleagues (2013) have demonstrated the metabolic effects of *β-lactams* in the alteration of bile acids, cholesterol and

hormones by intestinal bacteria. They showed a higher expression of genes related to energy metabolism during *β-lactam* treatment. This supports the hypothesis that long term use of antibiotics undermines host-microbe mutualistic relationships.

Lincosamides

Clindamycin is a class of lincosamide antibiotics used to treat mainly anaerobic infections (Hedberg and Nord, 2002) with a bacteriostatic antimicrobial effect and a protein synthesis inhibitor mode of action. Resistance to clindamycin has increased significantly over the last two decades. Perez-Cobas and colleagues (2013) observed marked decreases in *Bacteroides* and *Blautia* genera immediately after administration, however, after three days a recovery of *Bacteroides* was observed suggesting acquisition of resistance genes by these bacteria. They also noted that clindamycin induced an increase in Enterobacteriaceae compensating for loss of other anaerobic bacteria. In comparison with other agents (flouroquinolone or β-lactams) clindamycin was shown to result in a more variable and stronger effect on microbial community structure. Clindamycin has been shown to result in an increase in abundance of resistance genes, relative to other antibiotics such as moxifloxacin, amoxicillin and cefazolin/ ampicillin/ sulbactam combination, mostly of the efflux pump class (Perez-cobas *et al*., 2013).

Fluoroquinolones

Fluoroquinolones inhibit bacterial cell proliferation by inhibiting DNA gyrase and topoisomerase IV, both essential enzymes in bacterial DNA transcription and replication (Hooper, 2001). In addition to inhibiting these two enzymes, fluoroquinolones sequester these enzymes into a drug/enzyme/DNA complex which holds double stranded DNA breaks together blocking replication (Drlica, 2014). Treatment with different antibiotics from the fluoroquinolone class presents a similar

initial response in the gut microbiota. Moxifloxacin showed a decrease in *Faecalibacterium* and *Bacteroides* with ciprofloxacin showing similar effects with additional decreases seen in Lachnospiraceae and Ruminococcaceae (Perez-Cobas *et al*., 2013, Dethlefsen and Relman 2011). Earlier fluoroquinolones such as ciprofloxacin, levofloxacin, norofloxacin and orofloxacin are target aerobic bacteria, mainly Gram-negatives but similarly to moxifloxacin, a methoxyquinolone, they result in reduction in numbers of Enterobacteriaceae. Moxifloxacin has a wider reaching activity than the others with activity against respiratory pathogens, including beta-lactamase-producing aerobic Gram-positive cocci, *Haemophilus* and *Moraxella*, aerobic Gram-negatives and intracellular and atypical microorganism such as *Legionella*, *Chlamydia* and *Mycobacterium*. Moxifloxacin also has activity against both Gram-positive and Gram-negative anaerobes. In a study looking at the effects of repeated courses of oral ciprofloxacin (500mg twice daily for five days at 2 month and 8 month time points in a 10 month study) and the disturbance to the gut microbiota in three subjects, Dethlefsen and Relman (2011) showed an almost complete return to the pre-ciprofloxacin state following the first treatment however following a second treatment the microbiota community composition was different to what it had been at the start of the study and appeared to be stable in its new state. Edlund *et al*., (2000) also showed significant decreases of enterococci, enterobacteria, bifidobacteria and clostridia during moxifloxacin treatment (400mg oral administration once daily for seven days).

An interesting feature of fluoroquinolones is that their ability to bind reversibly to bacteria and faecal material resulting in reduced amounts of the drug in the intestine, translating to less disturbance of the total microbial intestinal population by fluoroquinolones (Edlund *et al*., 2000, Lidbeck *et al*., 1988). Due to

this moxifloxacin has a more targeted effect on the gut microbiota with more limited ecological disturbances (Edlund *et al*., 2000).

Polmyxins

Colistin sulphomethate is cyclopeptide, belonging to the family of polymyxins, which is produced by *Bacillus polymyxa* var. *colistinus* (Jeong *et al*., 2009, Storm *et al*., 1977). Colistin is mainly active against Gram-negative organisms. Its mode of action involves inducing changes in the permeability of the cell wall by binding anionic lipopolysaccharide molecules and displacing calcium and magnesium thus causing cell leakage and death (Jeong *et al*., 2009). Orally administered colistin is known to be poorly absorbed in the gastrointestinal tract and studies show that when excreted it is bound to the faecal material (WHO, 2006). This may reduce the perturbation effect of the drug *in vivo*. Jeong *et al*., (2009) show that *E. coli* was the most susceptible to colistin. Colistin sulphomethate is one of the most frequently used antibiotics for treatment of exacerbations in CF patients. Early eradication of *Pseudomonas* infection with nebulised drug, aggressive treatment of acute exacerbations with intravenous therapy and long term suppressive maintenance nebulised therapy are the three primary modes of usage in CF management. Colistin forms part of each of these (Littlewood *et al*., 2000). *Pseudomonas aeruginosa* is known to frequently development resistance to *β-lactam* antibiotics and aminoglycosides. However, thus far *in vitro* resistance of *Pseudomonas* species to colistin is rare, cementing its place as a useful treatment for Pseudomonal infections for the foreseeable future. Wright *et al*., (2013) have shown using *in vitro* culture methods that antibiotics (ceftazadime, colistin, azithromycin and tobramycin) at sub-inhibitory concentrations result in the phenotypic population diversification of *P. aeruginosa*. Interestingly, they demonstrated that ceftazadime and colistin

contributed to more diversification than tobramycin and azithromycin. Diversification of the *P. aeruginosa* populations in the CF lung negatively affect antibiotic therapy success, leading to a chronic infection state and ultimately to progression of the disease. Whether this diversification pressure is also exerted on taxa in the gut has not been reported. Knowledge of differential diversification potential of antibiotics can contribute to more informed decisions for antibiotic selection in management and control of infection.

Nitrimidazoles

Metronidazole is the prototype of the nitroimidazole family and most widely used for treatment of anaerobic and protozoal infections (Lamp *et al*., 1999). Along with fidomoxacin and vancomycin, metronidazole is mainstay of *C.difficile* treatment (Surawicz *et al*., 2013, Cornely *et al*., 2014), estimated to have colonised up to 50% of CF patients (Binkovitz *et al*., 1999, Bauer *et al*., 2014). Metronidazole is known to have activity against both Gram-negative (*B.fragilis*) and Gram-positive (*C.difficile*) anaerobes (Lofmark *et al*., 2010). Metronidazole functions by entering the cell in its inactive or prodrug state, becoming activated in the bacterial cytoplasm through an electron transfer to the nitro group of the drug, or protozoa organelles, to a cytotoxic state with DNA-binding capabilities. Inhibition of DNA synthesis and DNA damage by oxidation leads to DNA degradation and cell death (Land *et al*., 1999, Diniz *et al*., 2000, Lofmark *et al*., 2010). Its activity is limited to anaerobic bacteria because aerobes lack the appropriate transfer proteins, conferring them with intrinsic resistance to metronidazole (Reysset *et al*., 1996). Mechanisms of resistance to metronidazole are primarily based on either decreased uptake of the prodrug or reduced electron transfer capabilities (Land *et al*., 1999). Nitroimidazole (*nim*) resistance genes encode an alternative reductase protein which converts

nitroimidazole to a non-toxic derivative, thus avoiding DNA damage (Reysset *et al*., 1996, Leiros *et al*., 2004, Lofmark *et al*., 2010). Metronidazole resistance is generally low, although there have been reports of resistance in *Sutterella* species (Jousimies-Somer *et al*., 2002). *C. difficile* maintains its susceptibility to metronidazole with no significant clinical resistance observed (Lofmark *et al*., 2010, Terhes *et al*., 2014). In a study using high through-put sequencing, Rea *et al*., (2011) demonstrated phylum (reduction of Firmicutes and Bacteriodetes and increase of Proteobacteria) and family (increasing Enterobacteriaceae) effects of administering metronidazole in a colonic model experiment. The concentration of metronidazole in faeces is low as a result of efficient absorption; therefore alterations to the normal flora following metronidazole treatment are minimal (Sullivan *et al*., 2001). Nagy and Foldes, (1991) propose a mechanism of inactivation of metronidazole by intestinal enterococci, thus reducing its effect on the gastrointestinal microbiota. The success of oral metronidazole in *C. difficile* infection treatment is a result of the elevated plasma serum levels which diffuses to through the damaged intestinal mucosa of infected individuals with greater efficiency (Sullivan *et al*., 2001).

Glyopeptides

Glycopeptide antibiotics have an inhibitory mode of action on late-stage peptidoglycan polymer synthesis. Glycopeptide antibiotics interfere with glycosylation of the assembled peptidoglycan polymer following transport to the cell membrane (Reynolds, 1989). Vancomycin, teicoplanin and, most recently, telvancin are the only FDA approved glycopeptide antibiotics (Bambeke *et al*., 2004, Damodaran *et al*., 2011). Glycopeptides are active against most Gram-positive organisms, but few Gram-negative organisms. Vancomycin remains the first line drug against MRSA, however, emergence of resistance in enterococci and

staphylococci has led to a restriction in the use of vancomycin, as well as teicoplanin (Van Bambeke *et al*., 2004, Damodaran *et al*., 2011). Yap and colleagues showed the disturbance effect of vancomycin on the gut microbiota in mice using 2 x 100mg/kg/day dose for 2 days. *C. leptum*, *C. coccoides*, *C. symbiosum* and *Photorhabdus luminescens* were lost immediately following treatment, with subsequent rapid recovery of *C .leptum*, *C. coccoides*. In an environmentally controlled 24 hour model distal colonic experiment Rea *et al*., (2011) showed a decrease in the major phyla, Firmicutes and Bacteroidetes, and an increase in Proteobacteria following addition of 90μM vancomycin at 8 hourly intervals. The rapid proliferation of Enterobacteriaceae at the expense of other Gram-negatives and Firmicutes during vancomycin treatment is reported (Rea *et al*., 2011, Yap *et al*., 2008). In a diet-induced obesity mouse model administering 2mg/day vancomycin for 20 weeks, Murphy and colleagues (2013) attributed the decrease in Firmicutes and Bacteroidetes at phylum level, to a decrease in Clostridaceae and Bacteroidaceae at family level, respectively, and increasing Proteobacteriaceae was attributed to increasing Enterobacteriaceae. At family level they also noted decreases in Porphyromonadaeae and increases in Streptococcaceae and Desulfovibrionaceae, while at genus level increases in *Lactococcus*, *Sutterella* and *Desulfovibrio* and decreases in *Clostridium* and *Odoribacter* were observed.

Table1. Summary of the effects of specific antibiotic classes on the intestinal microbiota

ANTIBIOTIC CLASS	**SPECIFIC ANTIBIOTIC EXAMPLES**	**MODE OF ACTION**	**GUT MICROBIOTA EFFECTS**	**REFERENCE**
AMINOGLYCOSIDES	Tobramycin	Bactericidal agents that inhibit protein	Little in literature. Bifidobacteria are	Bryan *et al*., 1979

		synthesis by binding the 30S prokaryotic subunit.	known to have innate resistance due to lack of cytochrome-mediated transport mechanism for aminoglycoside uptake.	
MACROLIDES	Azithromycin, erythromycin, Clarithromycin	Protein synthesis inhibitory mode of action by initiating a dissociation of the peptidyl-tRNA from the ribosome	Clarithromycin has been shown to reduce Actinobacteria phyla, *E.coli*, bifidobacteria, lactobacilli, clostridia and anaerobic populations and increase enterococci, *Enterobacter, Citrobacter, Klebsiella, Pseudomonas* and resistant *bacteroides*.	Jakobsson *et al*., 2010 Adamsson *et al*., 1999 Brismar *et al*., 1991 Edlund *et al*., 2000
B-LACTAMS	Penicillin, Ceftazidime, meropenem, flucloxacillin, co-amoxiclav, piperacillin/tazobactam, aztreonam	Bactericidal mode of action, disrupting cell wall synthesis, specifically in the final cross linking stage of the peptidoglycan layer.	Increase of *Bacteroides, Blautia, Faecalibacterium, Parabacteroides*- most probably due to high rate of resistance in these genera. Significant reduction of anaerobic population.	Perez-Cobas *et al*., 2013 Monreal *et al*., 2005 Morotomi *et al*., 2011 Adamsson *et al*., 1999
LINCOSAMIDES	Clindamycin	Bacteriostatic antimicrobial effect and protein synthesis mode of action	Decreases in *Bacteroides* and *Blautia*. Increase in Enterobacteriaceae.	Perez-Cobas *et al*., 2013
FLUOROQUINOLONES	Moxifloxacin, Ciprofloxacin	Inhibition of DNA gyrase and topoisomerase IV. Fluorquinlones sequester these	Decrease in *Faecalibacterium, Bacteroides,* enterococci, enterobacteria,	Perez-Cobas *et al*., 2013 Dethlefsen and Relman 2011 Edlund *et al*.,

		enzymes into a drug/enzyme/DNA complex which holds double stranded DNA breaks together blocking replication	Clostridia, bifidobacteria, Lachnospiraceae, Ruminococcaceae and Enterobacteriaceae,	2000
POLYMYXINS	Colistin sulphomethate	Induces changes to the permeability of the cell wall by binding anionic lipopolysaccharide molecules and displacing calcium and magnesium, leading to cell leakage and death.	Orally administered colistin is poorly absorbed in the gut, therefore it has little effect on the intestinal microbiota.	WHO, 2006
NITROIMIDAZOLES	Metronidazole	Entering cell in inactive state, becoming activated in the bacterial cytoplasm through electron transfer of the nitro group of the drug to a cytotoxic state with DNA binding capabilities.	Active against anaerobic bacteria. Decreases in Firmicutes and Bacteroidetes and increases in Enterobacteriaceae. Metronidazole concentration appears low in faeces; therefore intestinal microbiota effects are minimal.	Rea *et al*., 2010 Sullivan *et al*., 2001
GLYCOPEPTIDES	Vancomycin	Interferes with glycosylation of the assembled peptidoglycan polymer following transport to the cell membrane	Loss of *C.leptum, C.coccoides, C.symbiosum, Photorhabdus luminescens.* Decrease in Firmicutes, Bacteroidetes, Clostridiaceae,	Rea *et al*., 2011 Yap *et al*., 2008 Murphy *et al*., 2013

		Bacteroidaceae, Porphyromonadaceae, *Clostridium* and *Odoribacter*. Increase in Proteobacteria, Enterobacteriaceae, Streptococcaceae, *Lactococcus*, *Sutterella* and *Desulfovibrio*.

Effects of combination therapy on gut microbiota

Combination or multidrug therapy often confers advantages over single drug therapy in terms of a wider range of targets, synergistic effects, reduction of multidrug resistant organisms and reduction in total amount of antibiotic used, however it incurs high costs, complications for administration and drug related toxicity (Elphick *et al*., 2005, Perron *et al*., 2012, Gould and Van der Meer *et al*., 2007). Taylor (1993) reported that 80% of clinics had preference for combination therapy. Combinational antibiotic therapy poses challenges to identifying, quantifying and comparing the specific effects of antibiotic classes on the gut microbiota. At present, treatment of *P. aeruginosa* infection in patients with CF involves 2-3 antibiotics, demonstrating improved synergy (Zebouh *et al*., 2008, Hewer and Smyth, 2014). Generally this may include the use of inhaled antibiotics such as tobramycin and colistin (Littlewood, 1987, Ratjen, 2001), oral quinolones such as ciprofloxacin (Taccetti, 2005) and intravenous antibiotics usually involving a β-lactam antibiotic in combination with an aminoglycoside antibiotic (Doring, 2000, Hewer and Smyth, 2014).Combination therapies are a commonly used approach for biofilm management, with the rationale that multiple antibiotics, with a range of individual targets, can combine to help suppress or eradicate the varying antagonistic

phenotypes present in the biofilm (Barraud *et al*., 2013). Colistin is known to be active against bacterial cells with low metabolic activity, while tetracycline and ciprofloxacin are known to be active against metabolically active cells (Pamp *et al*., 2008, Herrman *et al*., 2010).

Conclusive results yielding specific guidelines for treatment of pulmonary exacerbations and delaying chronic *P. aeruginosa* infection in CF patients have yet to be reached on whether combinational or monotherapy is more effective due to a lack of studies testing combinational therapy options versus monotherapy in a head-to-head manner with control for confounding factors. Of the studies available it is difficult to unequivocally indicate which is the most effective since studies differ in length of administration of antibiotic, age, health status and history of patient cohort, differing times from detection to initiation of treatment, frequency of surveillance, markers of success and statistical analysis. However, the current physician consensus infers a preference to combinational therapy for patients with CF (Elpick *et al*., 2005).

1.5 Long-term issues of chronic antibiotic use

Multidrug resistance

Almost immediately after the introduction of the first antibiotics, a parallel emergence of resistance has inhibited their success (Davies and Davies, 2010). Most recently, a WHO report on antimicrobial resistance (AMR) (2014) warns of the dangers of entering a post-antibiotic era where minor infections could emerge as life threatening diseases in the face of an AMR pandemic. Multidrug resistance is a major public health issue facing the medical and wider community (Laxminarayan *et al*., 2013). In the EU AMR is estimated to responsible for 25,000 deaths each year, while health-care related costs are estimated to be €0.9 billion with a further €1.5

billion expenditure on productivity losses (Rodier, 2011, REPORT EEJT, 2009). Multidrug resistance is compromising our ability to manage infectious disease. Well documented multidrug resistant organisms include *P. aeruginosa* and *Burkholderia cepacia* complex, as well as emerging Methicillin-resistant *Stenotrophmonas maltophilia* and MRSA. Multidrug resistance was responsible for 23,000 deaths in the United States in 2013, of which MRSA and *Streptococcus pneumoniae* were responsible for 11,000 and 7,000 deaths, respectively (CDC, 2013, Atlanta, GA). Antibiotic overuse and misuse is the driving force of development of resistant organisms (Laxminarayan and Heymann, 2012). An empirical strategy of treatment based on expert consensus remains the mode of action on diagnosis of pulmonary exacerbation. This generalised treatment strategy inevitably contributes to the adverse effects of antibiotic overuse both in terms of dose and time. This is compounded by the increase in age of the CF demographic (Waters and Ratjen, 2006) globally (CFRI Annual report 2011, Davis, 2006, www.cff.org), with the ratio of adult CF patients to paediatrics increasing each year.

The human gastrointestinal tract is an environment where 10^{13}- 10^{14} microorganisms are in constant interaction providing an ideal situation for rapid dissemination of resistance genes between commensals and pathogens (Salyers *et al*., 2004, Davies, 1994). The human gut microbiota is now viewed as a reservoir where commensals and pathogens are in direct contact, thus facilitating the spread of resistance genes (Salyers *et al*., 2004, Sommer *et al*., 2009, Fouhy *et al*., 2014). Studies have established that the gut resistome is established in infancy, even in the absence of antibiotic therapy, growing and diversifying with age (Fouhy *et al*., 2014). In response to antibiotic therapy resistance genes are expressed and selected, with strains carrying resistance proliferating, amplifying the resistance reservoir (Lu

et al., 2014, Fouhy *et al*., 2014). In CF patients, or other groups on chronic antibiotic therapy the resistome is likely to have increased capabilities. Whole genome sequencing has shown that lateral gene transfer and mobilizable elements that incorporate into a variety of host bacteria are responsible for most resistance (Ochman *et al*., 2000). Transferable resistance genes may arise in commensals and through lateral gene transfer mechanisms spread to clinically relevant pathogenic strains. The gut microbiome, as an entity that may facilitate the acquisition of resistance by pathogenic strains, is postulated (Sommer *et al*., 2009). Sommer and colleagues (2009) used a functional metagenomic approach to characterise the antibiotic resistome of two healthy unrelated humans who had not been treated with antibiotics for a least one year. Phylogenetic profiling of aerobically cultured strains from the gut microbiome showed they were primarily members of the Proteobacteria phyla, and to a lesser extent Firmicutes and Actinobacteria. Resistance genes belonging to one of the following classes; tetracycline efflux pumps (TetA), two classes of aminoglycoside-modifying enzymes and three classes of beta-lactam-inactivating enzymes (TEM, AmpC, and CTX-M) were detected. Characterising the human gut resistome may increase understanding of the origin of the antibiotic resistance, ultimately aiding the address of the MDR issue.

The expression and subsequent persistence of resistance genes ensuing antibiotic treatment varies depending on antibiotic class, duration and dose strength of treatment. Persistence of resistance may be observed years after an antibiotic course. For instance, following a 5 day antibiotic treatment (500mg twice daily) with ciprofloxacin initial gut microbiota perturbations recover, however resistance to the particular antibiotic is enriched and may be observed for months or even years to follow (Dethlefsen *et al*., 2008). Similarly, Sjolund *et al*. (2003) showed that in 3 of

5 patients post clarithromycin treatment, erythromycin-resistance was present after one year and in one patient after three years. Jernberg *et al*., (2007) also showed the persistence of resistance genes (ermF, ermG and ermB) following clindamycin treatment up to two years following a seven day antibiotic treatment. Shoemaker *et al*., (2001) demonstrate that the presence of the *tet*Q gene has increased from 30% to 80% in all strains of *Bacteroides* as a result of conjugal gene transfer in the last three decades.

Some genera may contribute to multidrug resistance more than others by acting as a reservoir for mobilizable resistance determinants. *Bacteroides* and *Parabacteroides* species are mutualistic inhabitants of the gut preforming many beneficial functions for the host, such as carbohydrate metabolism (Xu *et al*., 2003). *Bacteroides* form 25% of the human gut microbiota (Salyers 1984) and have been implicated in many opportunistic infections, particularly *Bacteroides fragilis* (Wexler, 2007, Cao *et al*., 2014). They have an innate ability to produce β-lactamases such as cephalosporinases and penicillinases encoded by the *cepA* gene (Gutacker *et al*., 2000). Clindamycin resistance, encoded by the *ermF*, *ermB* or *ermG* genes or through efflux pumps, in *Bacteriodales* ranges from 10-42%, confers protection against macrolides, lincosamides and stretogramin B (Wybo *et al*., 2007, Gupta *et al*., 2003, Nakano *et al*., 2011, Shoemaker *et al*., 2001, Pumbwe *et al*., 2007). In a study by Nakano and colleagues (2011), 53.5% of tested *Bacteroides* and *Parabacteroides* strains were tetracycline resistant, primarily due to the acquisition of the *tetQ* ribosome protection protein. The rise of resistance genes in predominant and functionally vital genera of the human gastrointestinal tract provides pathogens with increased opportunity for acquisition of many resistance determinants.

Antimicrobial resistance rates are increasing. Studies such as the SMART (Study for Monitoring Antimicrobial Resistance Trends), PROTEKT and SENTRY seek to monitor and characterise antimicrobial resistance rates globally (Morrissey *et al*., 2013). The SMART study describes the increase in prevalence of ESBL's, conferring resistance to β-lactams, in Asia, Europe, Latin America, Middle East, North America and South Pacific. The study also reports the increasing resistance to fluoroquinolones; particularly in *P. aeruginosa* (increases from 22% to 33% were seen in North America). Macrolide resistance rates have also been shown to be increasing (Cresti *et al*., 2002). Recent studies have indicated this is due to the epidemic spread of *erm*B resistance genes among streptococcal populations and in particular *Streptococcus pyrogenes*, either by horizontal gene transfer (HGT) or clonal expansion of resistant isolates following selective pressures of antibiotic treatment (Jakobsson *et al*., 2010). Sjolund (2003) has reported the presence of macrolide resistant enterococci following selection by clarithromycin and metronidazole.

Development of opportunistic pathogens and Antibiotic Associated Diarrhoea (AAD)

Chronic antibiotic therapy results in significant alterations in the gut microbiota creating opportunities for opportunistic pathogens, primarily *C. difficile* to establish and proliferate. *C. difficile* is mostly a nosocomical infection of the elderly, but can also be community acquired, affecting younger populations and adults. The Centre for Disease Control (CDC) has linked *C. difficile* to 14,000 deaths in America (CDC, 2013). The European Centre for Disease Control (EDCD) estimates that *C. difficile* infection costs the European Union 3 billion per annum (EDCD, 2014). Broad-spectrum antibiotic therapy has been associated with increased incidence of *C. difficile* and is identified as the one of the most well recognised risk factors for *C.*

difficile and indeed recurrent and refractory CDI (*C. difficile* Infection) (Bartlett & Gerding, 2008, Diggs and Surawicz, 2009). Broad-spectrum antibiotics induce a dysbiotic gut microbiota, particularly amongst anaerobic populations, providing *C. difficile* with an opportunity to proliferate. Indeed, Owens and co-workers (2008) reported 94% of hospitalised patients with *C. difficile* infection had received antibiotics before or during their hospital stay. *C.difficile* is responsible for 20-25% of AAD cases and 90-100% of pseudomembraneous colitis (Rea *et al*., 2010, Cramer *et al*., 2008). Ironically, antibiotic treatment of *C. difficile* infection perpetuates its proliferation, increasing strains resistant to vancomycin, particularly enterococci (Chang *et al*., 2008), lowering diversity providing *C. difficile* with opportunity to proliferate. The association between ampicillin, cephalosporins, fluoroquinolones and *C. difficile* infection is well recognised in the literature (Gerding 2004, Sullivan *et al*., 2001). Interestingly, the emergence of particular antibiotics as risk factors for CDI has paralleled the frequency of that particular antibiotic class in general medical use. Initially the rise of CDI was associated with clindamycin, which is the preferential treatment for a variety of anaerobic, streptococcal and staphylococcus infections. In the past decade, the rise in the use of fluoroquinolones to treat a variety of infections has precipitated these antibiotics in becoming risk factors for CDI and have contributed to the emergence of virulent strains such as ribotype 027, which is characterised by fluoroquinlone resistance, binary toxin production and increased toxin production due to a mutation in the *tcdC* gene (Pepin *et al*., 2005, Kuijper *et al*., 2008). Recently, metronidazole and vancomycin have also been associated as risk factors for CDI. In cases where antibiotics are necessary to treat concomitant infection, alternative antibiotics to those identified as CDI development risk factors should be used where possible. As expected, increased duration antibiotic courses

and antibiotic combination therapy are associated with increased risk of CDI (Owens *et al*., 2008). Not all antibiotics for CDI infection are associated with increased risk. Piperacillin in combination with tazobactam has shown considerable success in treatment of CDI but with less associated risk of increased development of infection or relapse (Owen *et al*., 2008). Vancomycin and, more recently, Fidaxomicin (2011) are the only FDA-approved drugs for CDI treatment. Fidaxomicin is particularly effective in treating recurrent *C. difficile* infection as it has less impact on the faecal microbiota translating into a more resilient microbial assemblage following treatment. Fidaxomicin shows less risk of development of resistance than vancomycin in *in vitro* studies (Zar *et al*., 2007). Alternative therapies that may facilitate the reduction of antibiotics are necessary to treat CDI, such as Faecal Microbial Transplant (FMT) is an alternative therapy at conceptual stage with early pioneering case studies showing considerable success in patients with reoccurring symptomatic *C. difficile* (Petrof *et al*., 2013, Koenigsknecht and Young, 2013). Another alternative includes Thuricin CD, a novel antimicrobial peptide (AMP) or bacteriocin produced by *B. thuringiensis*, has been patented as a CDI therapy (Rea *et al*., 2010). The potency of thuricin CD has been shown to be greater than vancomycin and in some cases greater than metronidazole. In addition, thuricin CD shows no antimicrobial activity against a range of commercially available probiotics, demonstrating its narrow substrate range (Rea *et al*., 2011).

Asymptomatic carriage of *C. difficile* in CF is well recognised, however, the mechanisms of protection against infection, but not colonisation, are not fully understood. Carriage rates in healthy adults varies from 0-15% (Viscidi *et al*., 1981, Nakamura *et al*., 1981), compared to up to 50% in CF cohorts- a result of prophylaxis and frequent hospitalisations. However, unlike normal populations

where approximately 50% of colonised individuals develop CDI, or in more severe cases pseudomembranous colitis, development of response to *C. difficile* toxins is rare in CF patients (Kyne *et al*., 2000, Peach *et al*., 1986, Welkon *et al*., 1985). It is hypothesised that the unique microbial assemblage of CF patients with high levels of Enterbacteriaceae, lactobacilli, *Pseudomonas* and *Staphylococcus* confers protection and resistance against *C. difficile* growth, but not colonisation (Rolfe *et al*., 1981). Welkon and colleagues (1985) suggest an altered gut pH as a result of defective ion transport inhibits toxin production or degrades toxins, although there is no evidence to support this. Another possibility postulates that exposure to *C. difficile* at such an early age mediates an immune response which protects in later life (Welkon *et al*., 1985). The relatively low incidence of CDI despite high rates of toxigenic *C.difficile* colonisation and antibiotic usage is an area of much research.

Metagenomic studies in cohorts with *C. difficile*-associated diarrhoea (CDAD) that have investigated microbiome shifts noted increases in *C. difficile* and also Bacteroidetes, *C. coccoides*, *Eubacterium rectale*, *Ruminococcus gnavus* and *Clostridium nexile* (Chang *et al*., 2008). Interestingly the Bacteroidetes phylum seems to show a strong association with CDAD in a number of studies. Manges and colleagues (2010) show that low levels of Bacteroidetes are associated with CDAD. Tvede *et al*. (1989) demonstrate using bacteriotherapy experiments, the absence of *Bacteroides* species such as *Bacteroides ovatus*, *Bacteroides vulgaricus* and *Bacteroides thetaiotaomicron* may result in recurring *C. difficile* infection, while their abundance in the gut microbiota may afford protection against colonisation and proliferation against *C. difficile*. Low Firmicutes and Bacteroides ratios, along with increasing facultative anaerobes have been shown to associate with *C. difficile* colonisation in infants (Rousseau *et al*., 2011). Metagenomic studies of patients with

C. difficile infection noted a decrease in alpha diversity at the DNA level (Chang *et al*., 2008, Knecht *et al*., 2014). However Knecht *et al*., hypothesise that instead of a microbiota-wide compositional change, a loss of functionality of a single species may impair the resilience of the microbiota to *C. difficile* colonisation. These functions may include SCFA's or antimicrobial production which may protect against *C. difficile* proliferation. Recent research by Knecht and colleagues (2014) indicates that Lachnospiraceae may be one such species where low or high levels associate with protection against or colonisation to *C.difficile*. Antibiotic substitutions for other options with less risk of CDI development associated may be effective (Vonberg *et al*., 2008). Further research into drug development is warranted to provide options that cause less disruption to the microbiota, consequently affording *C. difficile* less opportunity to proliferate.

Interference of mutualistic host-microbe interactions through antibiotic induced alteration of gut microbiome.

The human intestinal microbiota develops a partnership with the host and is essential to health and functions such as nutrition, immunoregulation, metabolism, development and pathogen resistance. With approximately 100 times more genes than the human genome, the gut microbiome lends us functional features we have not had to evolve ourselves (Backhed *et al*., 2005). While the intestinal community does seem to have a level of taxonomic functional redundancy, antibiotic-mediated elimination or significant reduction of important members of the microbiota is likely to affect functionality and host physiology, particularly in the case of chronic antibiotic therapy (Perez-Cobas *et al*., 2013). Many key species of the human intestinal microbiota perform important functions; however, disruption of the core gut colonisers by antibiotic therapy may have more profound effect on the

functioning of the microbiota as a symbiotic partner of the host. Examples include *Bacteroides thetaiotaomicron*, a prominent mutualist in the intestinal microbiome, which has a genome encoded capacity for carbohydrate metabolism far greater than the carbohydrate metabolising capabilities of our human genome (Shipman *et al*., 2000, Backhed *et al*., 2005). The gut microbiota also functions in regulation of bile acid metabolism and choline metabolism. The gut microbiota ecosystem is comprised of a dynamic network of functional interactions. As in any ecosystem even targeted, or narrow spectrum, antibiotic effects will influence not only the target but also indirectly effect many interaction partners (Willing *et al*., 2011). Antibiotic therapy can induce host immunity effects through the loss of bacterial ligands, metabolites and specific bacterial signals (Willing *et al*., 2011). Receptors involved in host immunity, such as Toll-like receptors and NOD-like receptors, are activated by specific bacterial ligands such as lipopolysaccarhide, lipoteichoic acid, flagellin and peptidoglycan. The loss of specific bacterial ligands reduces immune signals (Wells *et al*., 2011). Metabolic profiles of antibiotic-treated mice show a reduction of Short Chain Fatty Acids (SCFAs), with downstream reduction in the anti-inflammatory effects of SCFAs as well as functions in regulation of cell proliferation, differentiation, growth, apoptosis, vasodilation and wound healing (Yap *et al*., 2008, Millard *et al*., 2002, Leung *et al*., 2009, Bergman, 1990). Populations of bacteria have specific signals associated with them, which may be lost following antibiotic treatment. In addition, many studies report reduced expression and secretion of AMPs, directly affecting first-line microbial defence (Meyer-Hoffert *et al*., 2008). Taken together these factors result in a more susceptible host to pathogen insult (Willing *et al*., 2011).

The diversity of the human gut microbiota is the result of natural selection at both the microbial level and the host level (Backhed *et al*., 2005). Diversity confers resilience to stress, such as antibiotics, by harbouring a diverse range of responses, known as the insurance hypothesis (Guarner *et al*., 2007). In general low diversity correlates to poor health status (Lozupone *et al*., 2012, Claesson *et al*., 2012). This is one of the primary caveats of gut health and is demonstrated in infants, adults and elderly populations. Many disease states have been associated with low diversity microbiota signatures in recent years. Antibiotics as agents that drive the microbiota to an altered assemblage through gradual drift or dramatic shift, which may lead to the onset of microbiota-related diseases associated with less diversity, such as obesity (Turnbaugh *et al*., 2006), diabetes (Larsen *et al*., 2010) and IBD (Dicksved *et al*., 2008). Turnbaugh *et al*., (2006) demonstrated colonisation of germ free mice with 'obese' microbiota resulted in development of obese phenotype and similarly in lean mice. Childhood antibiotic use and the resulting altered microbiota are, potentially, associated with asthma and atopic disease (Marra *et al*., 2006, Noverr and Huffnagle, 2005). Low gut microbiota diversity in infants has been associated with eczema (Abrahamsson *et al*., 2012), particularly of the phylum Bacteroidetes and its genus *Bacteroides*. Indeed, in a study of 123 CF children 59% presented with skin hypersensitivity allergy (Worner *et al*., 1976). Though not proposed by the authors at the time, low diversity gut microbiota may have contributed to the hypersensitivity observed. The reduction of gut microbiota diversity following exposure to antibiotics is well-documented. Antibiotic exposure may reduce overall diversity and/or diversity of select taxonomic groups, as seen by Jernberg *et al*. (2007) in a study of four patients exposed to 150mg clindamycin four times daily. They observed an overall reduction in gut microbiota diversity, but also a reduction

in diversity of the *Bacteroides* community and emergence of clindamycin resistant strains. In a study investigating the effects of ciprofloxacin (500mg, twice daily for five days) on the gut microbiota of adult patients, the abundance of a third of bacterial taxa were reduced (Dethlefsen *et al*., 2010). Similarly, Rea *et al.* (2010) showed reduction of diversity following exposure to metronidazole and vancomycin in a human colonic model, mostly accounted for by the proportional increase of Enterobacteriaceae. However despite the well acknowledged effect of the lack of diversity on the host health, the functional redundancy and resilience of the gut microbiota is also accepted. Indeed, Dethlefsen and colleagues (2008) have demonstrated that although the gut microbiota was disturbed following treatment with ciprofloxacin they observed a continuity of microbial functions. It remains to be investigated whether the low diversity microbiota disorders described above could be the result of perturbations brought about by antibiotics alone or a combination of factors where antibiotic therapy is perhaps the factor that tips the balance to a disease state.

The hygiene hypothesis is quoted frequently as an argument against sterilising our environment and indeed our bodies through antibiotic use (Wills-Karp *et al*., 2001,). Increasing incidence of autoimmune disorders such as asthma, eczema, coeliac disease and inflammatory bowel disease may be related to disruption of normal host-microbe interactions by antibiotics (Guarner, 2006). Guarner hypothesises that deficient exposure to mutualistic bacteria such as bifidobacteria and lactobacilli explains the rise of immunodysregulatory disorders in modern society. Guarner proposes the use of prebiotics and probiotics as more favourable method of infection control.

1.6 Modulation of the gut microbiota

Diet

Diet has rapid and profound modulatory effects on the gut microbiota composition and functionality. Various diet types, including high fat, high sugar, 'Western' and low fat, polysaccharide rich diets, have been associated with microbiota signatures differing at the both compositional and functional gene level. Particular bacterial species have genetically encoded specific metabolic capabilities. The gut microbiota adapts through selection by dietary intake to include bacterial populations with the genetic capabilities best suited for metabolism of substrates of a given diet (Scott *et al*., 2008, Power *et al*., 2014). The rapid response of the gut microbiota to changes in diet has been demonstrated by studies in germ-free mice. A change from a low fat/plant rich diet to a high fat and sugar/low plant polysaccharides 'Western diet' showed changes in the gut microbiota within a single day, with increases in Firmicutes and decreases in Bacteroidetes (Turnbaugh *et al*., 2009). Hildebrandt *et al*., (2009) showed a similar rapid response in the gut microbiota to a dietary change (high fat/low-fibre or low fat/high fibre) within 24 hours. Most pertinent to CF, are the effects of high fat diet on the gut microbiota composition. CF patient's nutritional requirements recommend 120-150% estimated average requirement (EAR) of energy and 200% reference nutrient intake (RNI) for protein (Littlewood and Macdonald., 1987, Pencharz and Durie., 1993). In addition, it is recommended that the CF total calorie intake should be comprised of 40% fat (White *et al*., 2004). The response of the gut microbiota to dietary fat is well documented. De Filippo (2010) established the profound difference between the gut microbiota of European children on a typical Western diet (high fat, sugar, animal protein and starch and low fibre content), and children in the African state of Burkina Faso who consumed a

predominantly vegetarian diet which was high in plant polysaccharides, starch and fibre and low in animal protein and sugar. The Burkina Faso children had a lower abundance of Firmicutes and a higher abundance of Bacteroidetes genera (Xylanibacter and Prevotella) involved cellulose and xylan hydrolysis, enabling efficient energy extraction from their plant rich diet. Indeed, a study by Wu *et al*., (2011) linked gut microbial enterotypes dominated by either Bacteroides or Prevotella with diets dominated by either protein and animal fat or carbohydrates along with low meat and dairy intake, respectively. A high fat diet has been shown to influence a less diverse microbiota and a higher proportion of Bacteroidetes, *Parabacteroides*, *Eubacterium*, *Anaerotruncus*, *Lactonifactor* and *Coprobacillus* in elderly subjects (Claesson *et al*., 2012). In the same study, community dwelling subjects having a predominantly low to moderate fat intake had higher abundances of *Prevotella*, *Coprococcus* and *Roseburia*. In addition to compositional microbial effects, a high fat diet has been shown to result in a low-grade intestinal inflammatory state which is a hallmark of CF (Arkan *et al*., 2005, Werlin *et al*., 2010).

Gastrointestinal manifestations of CF include exocrine pancreatic insufficiency, steatorrhoea, intestinal inflammation, Distal Intestinal Obstruction Syndrome (DIOS) and CF-related Diabetes (CFRD). Pancreatic insufficiency (PI) occurs in 90% of CF patients, resulting in malabsorption of fat, protein, fat soluble vitamins A, D, E and K and subsequent malnutrition (Fieker *et al*., 2011, Dodge and Turck, 2006). Undigested, unabsorbed dietary fat is excreted in stools, known as steahorrhoea (Somaraju *et al*., 2014) PI predisposes CF individuals to the onset of DIOS, an obstruction of faecal transit at the ileocaecal junction (Somaraju *et al*., 2014). DIOS occurs in 10-20% of patients due to increased water absorption as a

result of defective intestinal CFTR. CFRD occurs in approximately 2% of paediatrics, 19% of adolescents and 40-50% of adults presenting with CF. CFRD, distinct from Type I and Type II Diabetes found in the general population (Stecenko and Moran., 2010), is associated with poorer pulmonary function and nutritional status (Koch *et al*., 2001, Marshall *et al*., 2005). Although there is a paucity of information on how these gastrointestinal manifestations affect the intestinal microbiota, especially in the CF context, a microbiota response to such comorbidities is probable.

Positive modulation of the gut microbiota

Positive modulation of the gut microbiota and host health can be achieved using probiotics, most commonly *Lactobacillus* or *Bifidobacterium* species. The beneficial effects of probiotics are perhaps, more subtle than previously thought. Scientists are moving from a theory that probiotics alter total gut microbiome composition, to a concept of prebiotics conferring a direct health benefit on the host (Power *et al*., 2014, Ouwehand *et al*., 2002). Thus far, probiotics have been demonstrated to have therapeutic potential in treatment of Antibiotic Associated Diarrhoea (AAD) (Videlock and Cremonini, 2012, Hempel *et al*., 2012), CDI (Na and Kelly, 2011), IBD (Isaacs and Herfarth, 2008) and IBS (Andersen and Baumgart, 2006). There has been preliminary but promising studies trialling administration of *Lactobacillus rhamnosus* GG (LGG) as a probiotic in paediatric CF cohorts. Bruzzese *et al*. (2004 and 2014) showed the success of LGG in reducing intestinal inflammation as indicated by non-invasive inflammatory markers - nitric oxide and fecal calprotectin. The treated cohort also reported decreased abdominal pain. To further investigate the positive effects of LGG for the CF population Bruzzese *et al*., (2007) investigated the effects of LGG administration on the rate of pulmonary exacerbation. They

demonstrated a protective effect of LGG in children chronically infected with *Pseudomonas* and consequently a reduction in pulmonary exacerbations and increase in FEV_1. Weiss *et al*., (2010) showed a significant reduction in pulmonary exacerbations in children with CF infected with *P. aeruginosa* post administration of a probiotic cocktail containing *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Bifidobacterium bifidum* and *Streptococcus thermophilus*; 90% of patients were also receiving azithromycin treatment at the time. The offsite effects of *Lactobacillus* species on *Pseudomonas* in the lung suggests a direct effect between the former and the latter, supported by earlier studies in mice where LGG administration reduced Pseudomonas species and *Lactobacillus plantarum* showed inhibitory activity towards *Pseudomonas* species (Valdez *et al*., 2005). Bruzzese and colleagues (2014) showed partial recovery of gut microbiota in CF children on antibiotic treatments who were subsequently treated with LGG with significant increase of *Bacteroides* and a trend towards an increase in *Faecalibacterium prausnitzii*, a known anti-inflammatory.

Probiotic strains with antibiotic tolerance may have application following or during antibiotic therapy. However, antibiotic tolerance in probiotic bacteria is generally associated with safety concerns by food safety authorities such as EFSA (European Food Safety Authority) due to the risk of dissemination of resistance determinants from probiotics to commensal or pathogenic members of the gastrointestinal flora. Plasmid or mobile genetic element mediated resistance carried by many probiotic genera poses a serious safety issue (Sharma *et al*., 2014). In addition, there are issues for antibiotic-probiotic combination therapy and possible resulting antagonistic relationships that may exist. Increased bacterial load due to the presence of viable probiotics may reduce the potency of antibiotics. It has been

shown that the potency of vancomycin decreases depending on the numbers of bacteria exposed (Udekwu *et al*., 2009). Probiotic- antibiotic combinations must be selected that preserve both the potency of the antibiotic and the viability of the probiotic (Hammad and Shimamoto, 2010). Antibiotic tolerant probiotic therapies remain at the conceptual stage due to difficulty in determining safety. However, the potential for application in a cohort with chronic antibiotic usage warrants further research into this novel area.

Knowledge of which microbiota community composition resist change more efficiently may be important in probiotic development. It may be possible to direct microbial communities towards a more resilient assemblage in individuals for whom chronic antibiotic use is necessary. In future, probiotics may provide an adjunct therapy along with antibiotics for the treatment of CF symptoms. The possibility of reduction of antibiotics through use of alternative therapies, such as probiotics, is attractive to both patients and clinicians.

1.7 Future directions

Antimicrobial discovery has been in decline since the 1970's, while MDR is an increasingly serious threat to global public health. As an immediate response to MDR we can curb the inappropriate use and overuse of antibiotics, gain knowledge on antibiotic effects and administering at doses that target aberrant bacteria while beneficial bacteria are preserved can help preserve and maintain the effectiveness of antibiotics for the next generation. In this age of high-throughput, next generation sequencing technologies it is imperative that we use technologies available to us to tailor antibiotics currently available to the individual instead of the 'catch all' broad spectrum approach which has consequences on our antibiotic resources. CFMATTERS (Cystic Fibrosis Microbiome Derived Antimicrobial Therapy Trial in

Exacerbations Results Stratiified) is a EU funded multicenter study currently examing this question in Adult patients with CF (www.cfmatters.eu, https://clinicaltrials.gov/ct2/show/NCT02526004). Alternative additional strategies dealing with infection need to be brought from the bench to mainstream clinical practice. There have been a number of studies investigating the use of bacteriophage as a therapeutic to treat CF lung infection, particularly in treating Pseudmonas infection, but also *S. aureus* and *B. cepacia* (Brussow, 2012, Debarbieux *et al*., 2010, Alemayehu *et al*., 2012, Morello *et al*., 2011). Phage therapy is advantageous over antibiotic therapy because of its specificity to the target pathogen, its ability to replicate at the site of infection and its ability to evolve along with bacterial populations that may develop resistance. Alemayehu and colleagues (2012) have demonstrated the efficacy of a phage cocktail (a myovirus (ϕNH-4) and a podovirus (ϕMR299-2)) to clear murine Pseudomonas lung infection. Furthermore, it has been shown that phage not only cleared Pseudomonas infection but also prevented infection when administered 24 hours before infection, highlighting a potential role of phage in prophylaxis. (Debarbieux *et al*., 2010, Morello *et al*., 2011).

Inhibition of Pseudomonas virulence and biofilm formation through disabling quorum sensing networks is another potential therapeutic for infection control. O' Loughlin *et al*., (2013) have demonstrated the utility of a quorum sensing inhibitory molecule (meta-bromo-thiolactone) to block pathogenesis by interacting with the two major quorum sensing systems Lux I/R. In addition they demonstrated the potential for preventative as well as curative treatment using meta-bromo-thiolactone. Further work is needed in these areas.

1.8 Conclusion

The collateral effects of antibiotic therapy on the gut microbiota have been widely studied and it is accepted that chronic and/or longterm antibiotic use will have compositional and potentially functional effects on the gut microbiota. In a cohort with chronic antibiotic usage, such as CF, antibiotic resistance capabilities are expected to be high with consequences for subsequent antibiotic therapy. The long-term persistence of resistance genes in the gut microbiota in response to antibiotic therapy poses a concern for the clinical management of infection. Moving forward tailored individualised antibiotic strategies may prove critical along with additional options other than antibiotics. Phage therapy and targeting of quorum sensing networks may provide alternatives or adjunct therapies to antibiotic therapy in future. Probiotics may be used as restorative agents to reintroduce depleted populations and maintain the gut microbiota ecosystem. In the interim, the medical community and wider society must endeavour to preserve our antibiotic resources through strategic and rational administration.

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Chapter 2

The effects of freezing on faecal microbiota as determined using MiSeq sequencing and culture-based investigations

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2.1 Abstract

High-throughput sequencing has enabled detailed insights into complex microbial environments, including the human gut microbiota. The accuracy of the sequencing data however, is reliant upon appropriate storage of the samples prior to DNA extraction. The aim of this study was to conduct the first MiSeq sequencing investigation into the effects of faecal storage on the microbiota, compared to fresh samples. Culture-based analysis was also completed. Seven faecal samples were collected from healthy adults. Samples were separated into fresh (DNA extracted immediately), snap frozen on dry ice and frozen for 7 days at -80°C prior to DNA extraction or samples frozen at -80°C for 7 days before DNA extraction. Sequencing was completed on the Illumina MiSeq platform. Culturing of total aerobes, anaerobes and bifidobacteria was also completed. No significant differences at phylum or family levels between the treatment groups occurred. At genus level only *Faecalibacterium* and *Leuconostoc* were significantly different in the fresh samples compared to the snap and frozen groups ($p=0.0298$; $p=0.0330$ respectively). Diversity analysis indicated that samples clustered based on the individual donor, rather than by storage group. No significant differences occurred in the culture-based analysis between the fresh, snap or -80°C frozen samples. Using the MiSeq platform coupled with culture-based analysis, this study highlighted that limited significant changes in microbiota occur following rapid freezing of faecal samples prior to DNA extraction. Thus, rapid freezing of samples prior to DNA extraction and culturing, preserves the integrity of the microbiota.

2.2 Introduction

The human gut microbiota is a complex ecosystem, comprised of thousands of bacterial species which is increasingly being investigated for its role in health and

disease (Sekirov *et al*., 2010). Such complexity and diversity poses considerable challenges to researchers as to how best to investigate such an environment accurately and completely. In the past, studies relied heavily on culture-based approaches, known now to capture only 10-20% of the actual microbiota present (Eckburg *et al*., 2005). However, as molecular technologies advanced, studies increasingly applied molecular approaches such as PCR, denaturing gradient gel electrophoresis or temperature gradient gel electrophoresis to determine the gut microbiota with greater accuracy than culturing alone (Blaut *et al*., 2002). Over the past 2 decades, with our enhanced understanding of the human gut microbiota, researchers have investigated the contribution of gut microbes to diseases such as diabetes, obesity, inflammatory bowel disease and certain cancers (Cani, 2013, Kamada *et al*., 2013, Matsuki *et al*., 2014). These investigations have been enabled through the advent of next generation sequencing technologies including Roche 454-pyrosequencing, Illumina MiSeq and PacBio (Mardis, 2008).

With decreasing costs and increasing speed, high-throughput sequencing approaches are being applied more frequently to investigate human gut microbiota. The majority of sequencing studies sequence the hyper variable regions of the 16S ribosomal RNA (rRNA) bacterial gene. This enables the sequencing of all bacteria, without requiring prior knowledge of which populations are present, while also providing insights into populations present at low abundances that could be missed by culturing alone. The results from high-throughput sequencing can however be biased by numerous factors. Studies have shown that the DNA extraction procedure, primers and sequencing platform used, can impact on the accuracy of the results achieved (Cardona *et al*., 2012, Kennedy *et al*., 2014, Salter *et al*., 2014). In the majority of gut microbiota studies, faecal samples are used owing to the non-

invasive nature of collecting such samples. Recently, studies have highlighted that the storage conditions of the sample prior to DNA extraction can alter the microbiota detected, especially in cases where prolonged room temperature exposure occurs (Lauber *et al*., 2010). However, at present limited studies have addressed a critical comparison of microbiota composition in fresh faecal samples to snap frozen on dry ice, to samples frozen immediately at -80°C, using both culturing methods and sequencing on the MiSeq platform. In fact, there is a considerable paucity of studies investigating the ability to culture from frozen faecal samples. This is critical information as increasingly research collaborations between institutions are occurring, resulting in a necessity to collect, store and ship samples in an appropriate manner to enable accurate subsequent culture-based analysis. While studies using 454-pyrosequencing have been completed on this topic, to date we are unaware of any study using MiSeq sequencing to investigate the effects of such storage conditions on faecal microbiota. Given the increased sequencing depth achieved through MiSeq sequencing compared to 454-pyrosequencing, subtle changes in microbiota that may not be detected using 454-pyrosequencing, may be captured using the MiSeq sequencing platform. Thus, the aim of this study was to compare the faecal microbiota of 7 healthy adults using culturing and MiSeq sequencing approaches and to determine the effects of different storage conditions on the faecal microbiota detected. Such results will inform future studies using faecal samples and MiSeq sequencing on the most appropriate way to store samples prior to DNA extraction, when extraction from fresh samples is not appropriate.

2.3 Materials and Methods

Sample collection and experimental design

Fresh faecal samples were collected from 7 individuals (5 females). Participants were healthy adults, free from gastrointestinal conditions, with no antibiotic exposure in the 28 days prior to sample collection. Participants provided written informed consent. Ethical approval was received from the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Cork, Ireland. Fresh samples were collected and aliquoted within 4 hours of defecation. Each sample was homogenised and 250 mg aliquots from each faecal sample was added to 3 separate cryovials (Sarstedt, Wexford, Ireland) containing zirconia/silica bead mix (Stratech Scientific, UK). One aliquot per individual was immersed in dry ice for 4 minutes until completely frozen. These 'snap' frozen samples were then stored at -80°C for 7 days before DNA extractions and culturing were completed. The 'frozen' samples were immediately frozen at -80°C for 7 days following collection and aliquoting. The 'fresh' samples were processed within 4 hours of collection, and were stored at 4°C during this brief period between collection and DNA extraction. In the case of culture-based analysis, 1 g was taken from each homogenised faecal sample and stored under the 3 storage conditions detailed above, before being used for culturing.

Culture-based analysis

The effects of sample storage conditions on microbial populations were determined using culture-based approaches targeting total aerobes, total anaerobes and total bifidobacteria. One gram of fresh, snap or -80°C frozen faecal material per individual was taken and serially diluted in maximum recovery diluent (MRD) (Oxoid Ltd, Basingstoke, Hampshire, UK). Dilutions were then plated in triplicate on the respective media. Enumeration of total aerobes was performed by plating

samples from dilutions 10^{-4}-10^{-7} onto Brain Heart Infusion (BHI) agar (Merck, Darmstadt, Germany) supplemented with 50 units of Nystatin (Sigma Aldrich, Dublin, Ireland) and incubated for 5 days at 37˚C, aerobically. Enumeration of total anaerobes was determined by plating samples from dilutions 10^{-4}-10^{-7} on Wilkins Chalgren Agar (WCA) (Fluka analytical) supplemented with 50 units of Nystatin and 7.5% defibrinated horse blood (TCS Biosciences, Buckingham, UK) and incubated for five days at 37˚C anaerobically. Enumeration of bifidobacteria was assessed by plating samples from dilutions 10^{-1}- 10^{-5} for fresh samples, from 10^{-4}-10^{-7} for -80°C frozen and snap frozen samples onto de Man Rogosa Sharpe (MRS, Difco Laboratories, Detroit, MI, USA) agar supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (Sigma Aldrich), 100 µg/ml Mupirocin (Fluka) (Simpson *et al*., 2003) and 50 units of Nystatin. These plates were then incubated at 37˚C for 72 hours under anaerobic conditions (Anaerocult A gas packs; Merck). Following incubation, enumeration using plate counts was performed for all three groups.

DNA extraction

Total bacterial metagenomic DNA was extracted using a modified protocol which combined the repeat bead beating method (Yu and Morrison, 2004) with the QIAmp Fast DNA Stool Mini kit (Qiagen, UK). DNA from the fresh sample was extracted within 4 hours of collection, while DNA from the snap and -80°C frozen samples was extracted after 7 days at -80°C. Briefly, 1ml of lysis buffer (500mM NaCl, 50mM Tris-HCl pH8.0, 50mM EDTA and 4% sodium dodecyl sulphate) was added to the bead beating tubes containing the faecal sample. Samples were homogenised for 3 mins at max speed using the Mini Beadbeater (BioSpec). Samples were incubated at 70°C for 15 minutes to lyse the cells. Following centrifugation the supernatant was removed and the bead beating steps repeated. Following pooling of

the supernatant, samples were treated with 10M ammonium acetate (Sigma Aldrich, Ireland) and then the DNA was pelleted and washed with 70% ethanol. The DNA was then RNAse and proteinase K treated. Finally the DNA was washed using buffers AW1 and AW2 (QIAmp Fast DNA Stool Mini kit; Qiagen, UK) and eluted in 200 µl of ATE buffer. DNA was quantified using the Nanodrop 1000 (Thermo Scientific, Ireland).

16S rRNA amplification and MiSeq sequencing

The V3-V4 variable region of the 16S rRNA gene was amplified from 21 faecal DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). Two PCR reactions were completed on the template DNA. Initially the DNA was amplified with primers specific to the V3-V4 region of the 16S rRNA gene which also incorporates the Illumina overhang adaptor (Forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). Each PCR reaction contained DNA template (~10-12 ng), 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM), 12.5 µl 2X Kapa HiFi Hotstart ready mix (Anachem, Dublin, Ireland), PCR grade water to a final volume of 25 µl. PCR amplification was carried out as follows: heated lid 110°, 95°C x 3mins, 25 cycles of 95°C x 30s, 55°C x 30s, 72°C x 30s, then 72°C x 5mins and held at 4°C. PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5% agarose, 100V). Successful PCR products were cleaned using AMPure XP magnetic bead based purification (Labplan, Dublin, Ireland). A second PCR reaction was completed on the purified DNA to index each of the samples, allowing samples to be pooled for sequencing on the one flow cell and subsequently demultiplexed for analysis. Two

indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were used per sample. Each PCR reaction contained 5 µl index 1 primer (N7xx), 5 µl index 2 primer (S5xx), 25 µl 2x Kapa HiFi Hot Start Ready mix, 10 µl PCR grade water. PCRs were completed as described above, but only 8 amplification cycles were completed instead of 25. PCR products were visualised using gel electrophoresis and subsequently cleaned (as described above). Samples were quantified using the Qubit (Bio-Sciences, Dublin, Ireland), along with the broad range DNA quantification assay kit (BioSciences) and samples were then pooled in an equimolar fashion. The pooled sample was run on the Agilent Bioanalyser for quality analysis prior to sequencing. The sample pool (4 nM) was denatured with 0.2N NaOH, then diluted to 4 pM and combined with 10% (v/v) denatured 4 pM PhiX, prepared following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform in the Teagasc sequencing facility, using a 2 x 300 cycle V3 kit, following standard Illumina sequencing protocols.

Bioinformatics and statistical analysis

Three hundred base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME. Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit) (Edgar, 2014). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release 111. Alpha and beta diversities were

generated in QIIME (Caporaso *et al*., 2010) and calculated based on weighted and unweighted Unifrac distance matrices. Principal coordinate analysis (PCoA) plots were visualised using EMPeror v0.9.3-dev.

To determine if statistically significant differences occurred in microbial populations between the 3 storage groups, non-parametric Kruskall-Wallis analysis was completed using Minitab 15 statistical software package. Statistical significance was accepted as $p<0.05$, adjusted for ties. Microbiological enumeration data was log10 transformed prior to statistical analysis using GraphPad Prism 6 and RM one-way ANOVA testing with Geisser-Greenhouse correction.

2.4 Results

Culture-based analysis

To assess the effects of different storage conditions on the ability to culture anaerobes, aerobes and bifidobacteria, culture-based analysis was performed. Following incubation, enumeration using plate counts was performed for all three groups. Total aerobic populations ranged from 5.75–10.11 log colony forming units (CFU) g^{-1} (mean= 7.06 log CFU g^{-1}) from fresh samples, 6.02–8.47 log CFU g^{-1} (mean= 6.89 log CFU g^{-1}) for -80°C frozen samples and 5.98–8.37 log CFU g^{-1} (mean= 7.11 log CFU g^{-1}) for snap frozen samples. No significant differences between enumerated aerobic populations were found in the 3 different storage groups (p= 0.686; Figure 1A). Total anaerobic populations ranged from 5.88–9.60 log CFU g^{-1} (mean= 7.99 log CFU g^{-1}) for fresh faecal samples, 6.28–8.86 log CFU g^{-1} (mean= 7.78 log CFU g^{-1}) for -80°C frozen samples and 7.00–8.92 log CFU g^{-1} (mean= 7.89 log CFU g^{-1}) for samples plated from snap frozen faeces. No significant difference in numbers of total anaerobes occurred depending on storage conditions (p=0.350; Figure 1B).

Bifidobacteria counts ranged from 6.18–8.24 log CFU g^{-1} (mean= 7.56 log CFU g^{-1}) for fresh faeces, 6.27–8.12 log CFU g^{-1} (mean= 7.22 log CFU g^{-1}) for -80°C frozen samples and 4.82–8.02 log CFU g^{-1} (mean= 7.30 log CFU g^{-1}) for the snap frozen samples. No significant difference existed between bifidobacteria enumerated from the samples at different storage conditions (p=0.334; Figure 1C).

MiSeq analysis of faecal microbiota following different storage conditions

Following the extraction of DNA from the fresh, -80°C frozen and snap frozen faecal samples, MiSeq sequencing was used to determine the effects of storage on faecal microbiota. Samples were sequenced using the Illumina MiSeq platform, resulting in 13.7 million reads.

At phylum level the fresh, snap and -80°C frozen samples shared common phyla. The most prevalent phyla in all samples were the *Firmicutes* and *Bacteroidetes* with *Actinobacteria*, *Fusobacteria* and *Cyanobacteria* contributing smaller proportions of the sequencing reads. No significant differences between the levels of *Bacteroidetes* (p=0.428) or *Actinobacteria* (p=0.901) occurred (Figure 2). While there was an increase in the proportion of *Firmicutes* present in the -80°C frozen (78%) and snap frozen samples (75%) compared to the fresh samples (67%), this was not significant (p=0.205). The most prevalent families detected in all 3 treatment groups included Ruminococcaceae, Lachnospiraceae, and Bacteroidaceae. However, again storage conditions appeared to have no significant effects, as the proportions of any of the families present in the frozen and snap samples were not significantly different to the fresh samples (*Bifidobacteriaceae* p=0.809, Bacteroidaceae p=0.667, *Lactobacillaceae* p=0.912, Ruminococcaceae p=0.754 and Lachnospiraceae p=0.511). Additionally, populations that only contributed a minor proportion to the overall gut microbiota i.e. <1% of total reads e.g.

Verrucomicrobiaceae, Pseudomonadaceae, were also equally detected in all 3 groups (p=0.996 and p=0.880 respectively).

At genus level, again all 3 treatment groups were very similar in the genera that were detected (Figure 3). All genera detected in fresh samples were detected in the snap and -80°C frozen samples, and at very similar levels, with only 2 significant differences being noted between genera in the different treatment groups. The proportion of *Faecalibacterium* was significantly higher in snap frozen samples compared to fresh samples (p=0.029). No significant differences were found between proportions of *Faecalibacterium* in the fresh and the -80°C frozen samples (p=0.055) or between the -80°C frozen samples and snap samples (p=0.443). In contrast, the proportion of *Leuconostoc* was significantly higher in the fresh samples compared to the snap frozen samples (p=0.033), but no differences occurred between the fresh and -80°C frozen samples (p=0.054), or between the snap and -80°C frozen samples (p=1.000). No significant differences in any of the other genera including proportions of *Bifidobacterium* (p=0.591), *Bacteroides* (p=0.667), *Parabacteroides* (p=0.546), *Pseudomonas* (p=0.880) or *Clostridium* (p=0.932) were seen between the 3 groups.

Diversity analysis

To determine if alterations in microbial diversity occurred as a result of different faecal sample storage conditions, alpha and beta diversity were investigated. No significant difference in alpha diversity occurred between the fresh, snap or -80°C frozen samples, as determined using Chao1 (p=0.905), Simpson's diversity index (p=0.754) and Shannon index tests (p=0.662) (Table 1).

To determine if any significant differences in beta diversity occurred based on storage of the samples, PCoA plots were constructed based on weighted and unweighted UniFrac distance matrices. We sought to investigate if samples from the same individual would cluster together. As shown in Figure 4A, samples clustered according to the individual, with all 3 samples from the one individual clustering more closely together than to other samples from the same storage group (Figure 4B). The same was also true when the PCoA plots were constructed using weighted UniFrac distance matrices (data not shown).

2.5 Discussion

Despite high-throughput sequencing providing considerable insights into the microbiota of complex environments, including the human gut microbiota, many factors (Kennedy *et al*., 2004, Nechvatal *et al*., 2008), including sample storage prior to DNA extraction, are known to impact on the accuracy of the results achieved. While studies have investigated this, to date however, no study has used MiSeq sequencing to determine the effects of freezing on the faecal microbiota compared to the fresh sample. Aware of the increased sequencing depth and coverage achieved using MiSeq sequencing compared to 454-pyrosequencing, our aim was to determine if subtle changes occur following freezing of faecal samples that may not have been captured in previous studies using 454-pyrosequencing. Such studies are important, as researchers are increasingly using MiSeq sequencing instead of 454-pyrosequencing and thus our study aimed to clarify the most appropriate storage of faecal samples, prior to DNA extraction for MiSeq sequencing to limit biasing the results achieved. While DNA extraction from fresh faecal samples may be the ideal choice, in many situations this is not practical, especially given the growing number of large multicentre projects being undertaken. Thus, knowing the effects of different

storage conditions on faecal microbiota, as determined using MiSeq sequencing, is important for the design, analysis and interpretation of microbiota studies in the future.

Our study has further novelty as we investigated the ability to culture total aerobes, anaerobes and bifidobacteria from the snap and -80°C frozen samples compared to the fresh samples. We chose to culture and enumerate total aerobes and anaerobes as the best method of evaluating the global effect of storage condition on the culturable microbiota. Bifidobacteria were cultured and enumerated because of their low abundance in faecal microbiota and their assumed sensitivity to freezing. We rationalised that if bifidobacteria were recoverable following freezing at -80°C, accordingly other low abundance groups may be recoverable. However, the recovery rate of other such groups has yet to be investigated. Although microbiological culturing from faecal samples is for the most part performed on fresh samples (Gardiner *et al*., 2004, O' Sullivan *et al*., 2011), there is little information in the literature comparing the effects of storage conditions on the culturable bacterial populations present in faecal samples. Using culturing approaches, our results demonstrated that the levels of total aerobes, total anaerobes or bifidobacteria in the fresh samples were similar compared with either the snap frozen or -80°C frozen samples. The greatest differences were seen between samples from different individuals, rather than from different treatments. The results suggest that rapid freezing of samples following collection and the avoidance of prolonged periods at room temperature or the occurrence of freeze-thaw enables the recovery of comparable levels of aerobes, anaerobes and bifidobacteria from frozen samples, as from fresh samples. Snap freezing did not appear to afford any additional benefits over freezing the samples at -80°C. Importantly, we show that immediate freezing at

-80°C demonstrates no significant difference in enumerated total aerobes, total anaerobes and bifidobacteria from fresh or snap frozen, at global level without the addition of glycerol or other preservation media, such as RNA later. We acknowledge the limitations of this study, in that we can only comment on the global levels of the groups enumerated. In future studies, it may be interesting to examine the effects of storage on a wider range of bacterial groups and to investigate if the recovery of bacterial numbers at a global level translates to specific taxa.

When the microbiota of the fresh, frozen and snap frozen samples were compared using MiSeq sequencing, no significant differences occurred at phylum or family levels. The dominant phyla, families and genera were similar between the three groups. We did note non-significant higher levels of *Firmicutes* in the snap and -80°C frozen samples compared to the fresh samples. This supports previous research using qPCR, which demonstrated a significantly higher ratio of *Firmicutes* to *Bacteroidetes* in frozen samples compared to fresh (Bahl *et al*., 2012). This may be due to an increased extraction ability of DNA from Gram positive (*Firmicutes*) bacteria following frozen storage (Bahl *et al*., 2012). Our results of minimal and non-significant differences in microbiota following freezing of faecal samples are in agreement with previous research which used alternative analysis (Lauber *et al*., 2010, Carroll *et al*., 2012, Roesch *et al*., 2009). The increased depth of coverage achieved through MiSeq enabled us to identify genera present at very low levels e.g. *Bifidobacterium* at <0.001% of assignable phylum reads. However, even in these circumstances no significant difference occurred in samples stored under different storage conditions. Thus, using MiSeq we could confirm the suitability of sample freezing prior to DNA extraction, with results being comparable to fresh samples.

Though our research only investigated short-term (7 days) storage at -80°C, others who have investigated more prolonged storage at -80°C (up to 137 days) have also failed to find any significant effects on faecal microbiota (Lauber *et al*., 2010, Wu *et al*., 2010). However, we hope to further study these faecal samples after months and even years at -80°C, using both culture and MiSeq analysis. The research to date suggests that freezing, either snap or at -80°C, preserves the faecal microbiota, while long-term storage at 4°C, room temperature or samples which undergo freeze-thaw, results in altered microbiota compared to their fresh equivalent (Cardona *et al*., 2012, Roesch *et al*., 2009). Our fresh samples were processed within 4 hours of collection, during which time they were stored at 4°C. Such a short period of cold storage is unlikely to alter the results of these 'fresh' samples compared to the samples when defecated, with previous publications indicating minimal changes occur in faecal microbiota following short-term storage at room temperature or 4°C (Lauber *et al*., 2010, Carroll *et al*., 2012). In fact, recent studies on sputum samples from individuals with cystic fibrosis have suggested that 12 hours room temperature storage prior to -80°C storage, is unlikely to alter microbiota (Cuthbertson *et al*., 2014). Studies examining the effects of snap freezing are limited, but those which have been completed suggest freezing (either on dry ice or at -80°C) achieves the most accurate microbiota relative to the fresh sample (Ott *et al*., 2004). Though snap freezing results in samples reaching the same end-point temperature as samples placed at -80°C, the temperature drops at a greater rate, reducing ice crystal formation in the sample, thus retaining superior cell integrity. Though outside the scope of this study, it may be the case that snap freezing would be beneficial in cases where subsequent enzymatic or RNA-based analysis are to be completed (Flores *et al*., 2012). This study also investigated microbial diversity and found no significant

differences between the 3 treatment groups in terms of alpha or beta diversity. Previously, 454-pyrosequencing studies suggested that samples cluster based on the donor rather than the storage of the faecal sample (Wu *et al*., 2010). Our MiSeq results support these findings, with the greatest variation in microbiota being attributable to interpersonal differences rather than the storage treatment.

In conclusion, this is the first MiSeq-based study to examine the effects of different freezing methods on the faecal microbiota compared to fresh samples. Despite the increased sequencing depth achievable using MiSeq as compared to 454-pyrosequencing, our results support previous 454-pyrosequencing results that found the greatest differences between samples is due to the uniqueness of each individual's microbiota, rather than storage conditions prior to DNA extraction. Our results suggest that rapid freezing, either on dry ice or simply placed at -80°C prior to culturing or DNA extraction for sequencing analysis, will result in accurate microbiota results. Going forward, when comparing data sets from different studies, it will be important to consider how the samples were stored prior to extraction, with results from fresh or frozen samples being more reliable than samples stored at room temperature or 4°C for prolonged periods.

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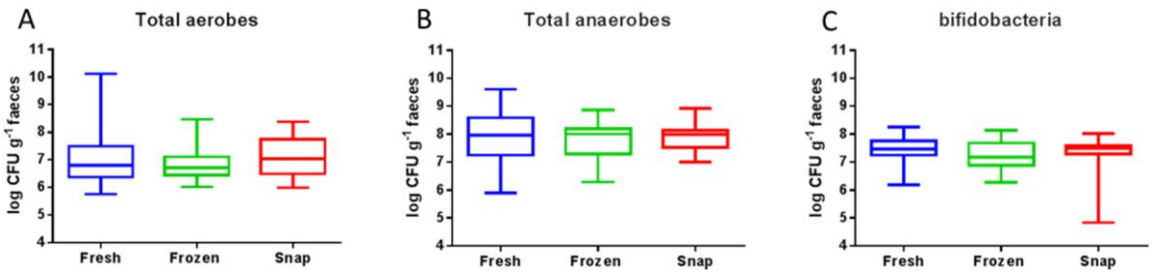

Figure 1**.** Microbiological enumeration of total aerobes (A), total anaerobes (B) and bifidobacteria (C) in log CFUg^{-1} faeces from fresh (blue), -80°C frozen (green) and snap samples (red). RM one-way ANOVA statistical testing showed no significant difference between storage conditions fresh, frozen at -80°C or snap frozen for the three groups tested.

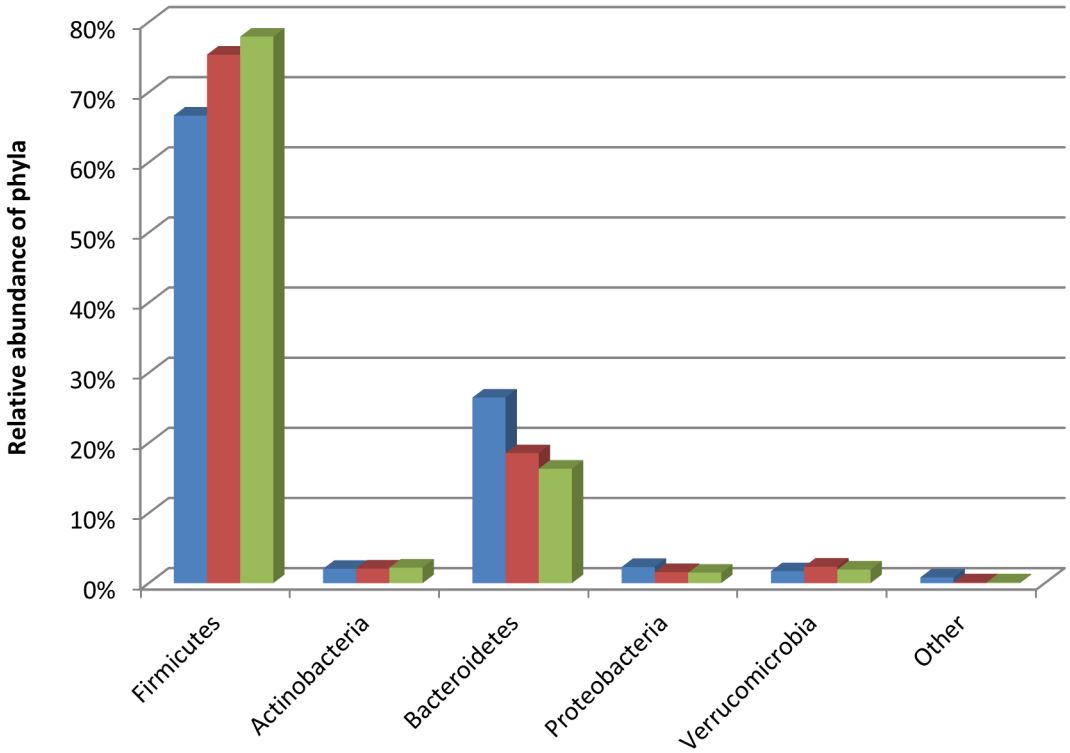

Figure 2. Relative abundances of bacterial phyla in the fresh (blue), snap (red) and -80°C frozen samples (green). Other contains phyla present < 1% of assignable sequences at phylum level

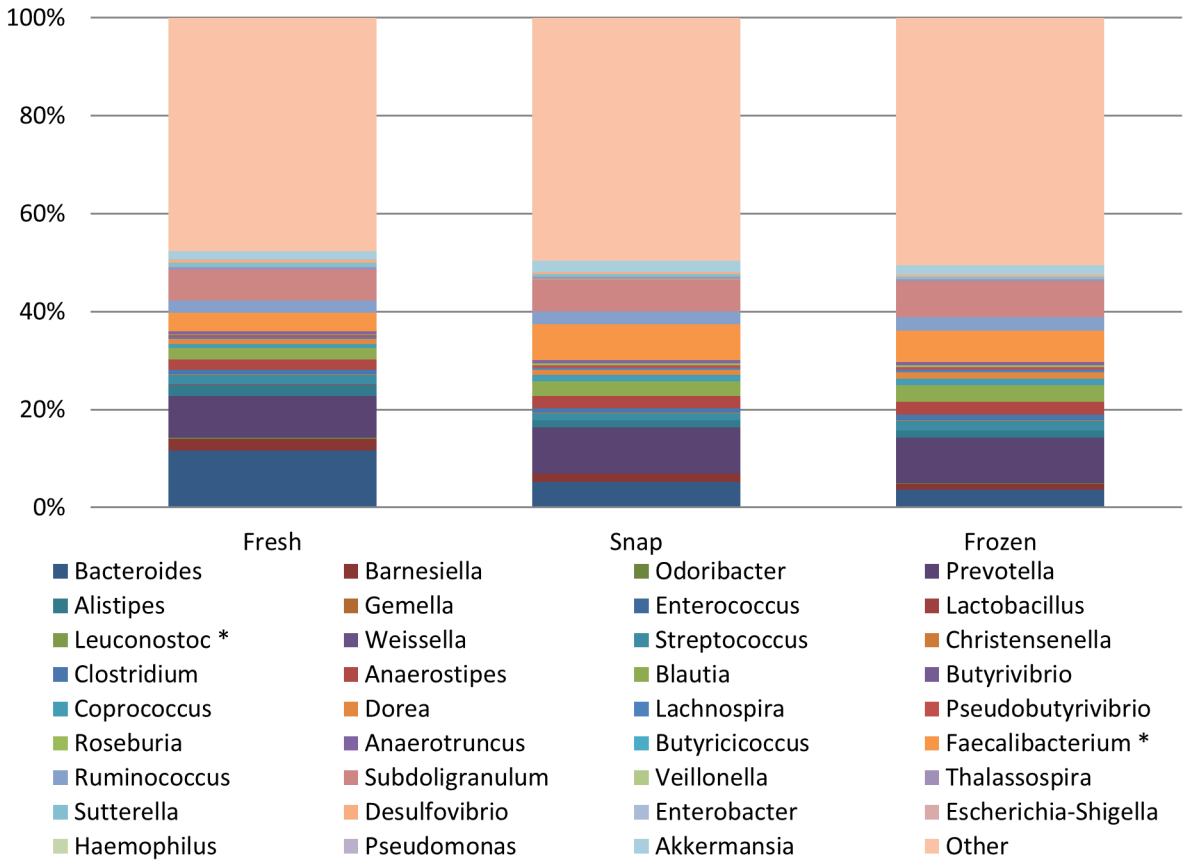

Figure 3. Relative abundances of bacterial genera in the fresh, snap and frozen samples. Statistically significant differences in genera are indicated with an asterisk (*) ($p<0.05$). The Other category contains all other genera present at <0.01% of assignable reads at genus level. No significant differences in any of these genera between the 3 groups was found using non-parametric Kruskall-Wallis analysis, where statistical significance was accepted as $p<0.05$, adjusted for ties.

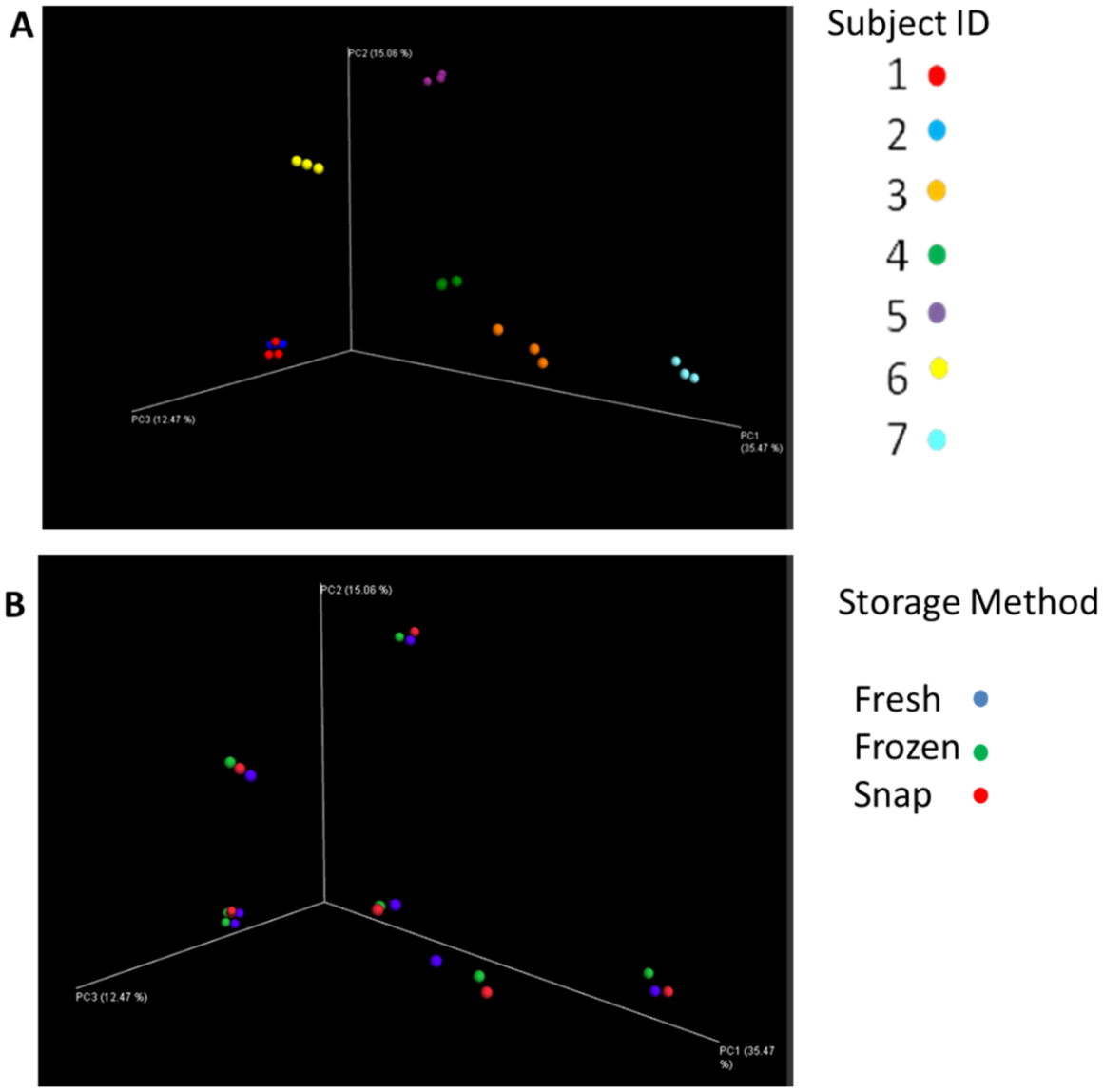

Figure 4: Visualisation of the PCoA analysis based on unweighted UniFrac distance matrices. Results indicate samples separate by subject ID rather than storage method. Section A: samples by subject ID. Section B: samples by storage method

Table 1: Estimates of alpha diversity for the fresh, -80°C frozen and snap frozen samples

Data set	Fresh	Frozen -80°C	Snap frozen	p-value
Chao1 richness estimate	846	964	878	0.905
Shannon index	5.75	5.97	5.87	0.662
Simpson's diversity index	0.95	0.96	0.95	0.754
Number of observed species	813	927	816	0.865

Chapter 3

A multicentre analysis of *C. difficile* in persons with Cystic Fibrosis demonstrates that carriage may be transient and highly variable with respect to strain and level.

3.1 Abstract

Clostridium difficile has been reported to occur in 50% of CF subjects, however, clinical *C. difficile* infection (CDI) is a rare occurrence in this cohort despite the presence of toxigenic ribotypes. Here, we present a multicentre (Ireland, UK, and Belgium) analysis of *C. difficile* prevalence among CF subjects from January 2015 to September 2016. Isolates were characterised in terms of ribotype, toxigenicity and antibiotic susceptibility to antibiotics routinely used in the treatment of CDI (metronidazole and vancomycin) and those implicated in induction of CDI (ciprofloxacin and moxifloxacin). Prevalence of *C. difficile* among CF subjects in the three sites was similar ranging from 47-50 %, while the healthy control cohort had a carriage rate of 7%. However, when we include subjects who were positive for *C. difficile* at any time point including baseline we observed an increased carriage rate of 71.4%, 66.7% and 63.2% in Ireland, UK, and Belgium, respectively. Ribotyping of 80 isolates from 45 CF persons, over multiple time points revealed 23 distinct ribotypes with a core of two ribotypes (046 and 078) which were shared by all centres. The rate of isolation of toxigenic isolates varied across the sites, ranging from 66% in Ireland to 53% in Belgium and 100% in UK. Antibiotic susceptibility rates to vancomycin, metronidazole, ciprofloxacin and moxifloxacin was 100%, 97.5%, 1.3% and 63.8%, respectively.

3.2 Introduction

C. difficile, a Gram-positive spore-forming anaerobe, is the causative agent of mostly nosocomical infections of the elderly, but is increasingly community acquired, affecting younger populations and adults. *C. difficile* is the most commonly isolated bacterium from antibiotic-associated diarrhoea (AAD) infections and the sole causative agent of pseudomembranous colitis (To and Napolitano, 2014, Rea *et al*.,

2010). *C. difficile* infection (CDI) has been linked to 14,000 deaths in America (CDC, 2013) and is estimated to cost the European Union ~€3 billion per annum (EDCD, 2014). Broad-spectrum antibiotic therapy, which disrupts the normal protective gut microbiota, has been associated with increased incidence of *C. difficile* and is identified as the major risk factor for CDI (Bartlett & Gerding, 2008, Diggs and Surawicz, 2009, Bakken *et al*., 2011).

Antibiotic resistance to *C. difficile* has been increasing in recent years contributing to the morbidity, mortality and increasing spread of this opportunistic pathogen. In the 1970's, clindamycin was a well-recognised risk factor for CDI (Bartlett *et al*., 1977). In the 2000's, fluoroquinolones became a risk factor for CDI, their use contributing to the rise in prevalence of virulent strains such as ribotype 027 (McDonald *et al*., 2005). In recent years, the epidemiology of *C. difficile* has changed with other ribotypes such as 001/072, 014/020 and 078 coming to the fore (Davies *et al*., 2016, Freeman *et al*., 2014). Increasingly, studies are reporting emergence of isolates resistant to metronidazole, a first line therapy for CDI, in addition to increasing resistance to fluoroquinolones and also rifampicin (Spigaglia, 2016).

Carriage rates of *C. difficile* in CF persons of up to 50% have been reported (Burke *et al*., 2016, Yahav *et al*., 2006), however, severe CDI is infrequently observed (Gelfond and Borowitz, 2013). CDI in CF subjects often presents with atypical symptoms, such as absence of diarrhoea, resulting in increased risk of misdiagnosis and delayed treatment (Piccolo *et al*., 2016). In CF lung post-transplant subjects, CDI is associated with increased mortality and morbidity (Yates *et al*., 2007). Asymptomatic carriage of *C. difficile* in CF is well recognised, however, the

mechanisms of protection against infection, but not colonisation, are not fully understood. Development of CDI, or in more severe cases, pseudomembranous colitis, in response to *C. difficile* toxins is rare in CF subjects (Kyne *et al*., 2000, Peach *et al*., 1986, Welkon *et al*., 1985). The reduced frequency of diarrhoea in CF persons carrying *C. difficile* may be due to insensitivity to *C. difficile* toxins as a result of CFTR dysfunction - similar to the protective effect of CFTR mutations against cholera toxin (Gelfond and Borowitz, 2013). Also hypothesised as reasons for high rates of asymptomatic carriage of *C. difficile* in CF persons are 1) an altered gut pH inhibiting toxin production, 2) early life exposure to *C. difficile* mediating an immune response that protects in later life (Welkon *et al*., 1985) and 3) a unique gut microbiota composition in CF subjects offering protection against *C. difficile* growth but not colonisation (Rolfe *et al*., 1981). The relatively low incidence of CDI despite high rates of toxigenic *C. difficile* carriage and antibiotic usage is an area currently being researched. In addition, there is little known about the clinical characteristics which may influence *C. difficile* carriage in a CF cohort. Recently, a study from our group (Burke *et al*., 2016) demonstrated trends towards correlation between BMI, age and mutation class and *C. difficile* carriage. Here, we expand the previous work by including CF subjects from three European centres at multiple time points including during pulmonary exacerbation.

3.3 Materials and Methods

Subject recruitment and sample collection

Faecal samples were collected from adult CF persons from three European centres; Cork University Hospital, Cork (Ireland), Papworth Hospital, Papworth (UK) and University Hospital Leuven, Leuven (Belgium) as part of the CFMATTERS clinical research trial (grant No.603038). Healthy control subjects were recruited from CF

centres (siblings) and the general population. Samples were collected at three monthly intervals following enrolment, at exacerbation and three months post exacerbation, where possible. Samples were collected within 24 hours of sampling and processed immediately from the Cork site. Samples from Papworth and Leuven were stored at -80^{o}C immediately following sampling and shipped to Cork on dry ice for subsequent study. Entry criteria for subjects to the CFMATTERS clinical research trial are as follows: 16 years or older, provision of written informed consent, proven CF diagnosis, confirmed pulmonary colonisation with *Pseudomonas* spp. at least twice over the previous year and a predicted FEV_1 of >25%. Subjects were excluded from the study if their life expectancy was less than 6 months, if they were post lung transplant (or other organ) or were being treated for non-tuberculous mycobacterium infection.

Isolation of C. difficile

C. difficile was isolated from faecal samples (fresh and frozen) by ethanol shocking in 50% ethanol followed by spread plating on cycloserine cefoxitin egg yolk agar (CCEY agar) (Lab M, Bury, UK), as previously described by Rea *et al.* (2012). Isolates displaying characteristic *C. difficile* morphology on CCEY agar, were Gram-positive, anaerobic, rod-shaped, with a distinctive 'horse-stable' odour and were positive using the Oxoid *Clostridium difficile* test kit (Oxoid, Basingstoke, UK) were stocked at -80^{o}C on Microbank beads (Pro-lab Diagnostics, Ontario, Canada). For routine testing, isolates were sub-cultured onto Fastidious Anaerobic Agar (Lab M, Heywood, Lancs, UK) supplemented with 7% defibrinated horse blood and incubated at 37^{o}C in a Don Whitley anaerobic chamber. As samples from Papworth and Leuven were frozen prior to culture of *C. difficile,* we investigated the impact of

freezing on the numbers of *C. difficile* recovered from samples. *C. difficile* was enumerated in 10 samples prior to freezing and following thawing.

Bacterial strains used

C. difficile VPI 10463 (ATCC 43255; A+/B+), *C. difficile* CUG 20309 (A-/B+) and *C. difficile* ATCC 43593 (A-/B-) were used as positive and negative controls for the detection of toxin genes. *C. difficile* ATCC 70057 was used as a control strain for antibiotic susceptibility testing.

PCR ribotyping

Ribotyping of *C. difficile* isolates for this study was performed by the *C. difficile* Ribotyping Network for England (CDRNE) based at the Microbiology Reference Laboratory, Leeds General Infirmary, United Kingdom. Isolates were analysed by capillary gel electrophoresis and compared to over 500 ribotypes contained in the CDRNE ribotype library.

In vivo *and* in vitro *enzyme immunoassay for* C. difficile *toxin and molecular detection of* tcdA *and* tcdB *toxin genes.*

In vivo and *in vitro* toxin production in faecal samples and *C. difficile* isolates, respectively, was assessed using a commercially available ELISA kit, Toxin A+/B+: ProSpecT II (Oxoid). The ELISA assays were performed as per the manufacturer's instructions. For the molecular detection of *tcdA* and *tcdB* toxin genes DNA was extracted from isolates as described by Rea *et al*., (2012). Summarily, isolates were sub-cultured on Fastidious Anaerobic Agar containing 7% defibrinated horse blood. Pure colonies (5-6) were picked and suspended in 5% Chelex-100 resin. Suspensions were then heated to 56^{o}C for 30 minutes, followed by 100^{o}C for 8 minutes and centrifuged at 16,000 X g for 3 minutes. The resulting DNA was used as a template

for *tcdA* and *tcdB* toxin genes as described by Kato *et al*., (1998). Each PCR amplification reaction (50µl) contained 25µl Biomix Red (Bioline, London, UK), 1 µl of NK2, NK3, NK9, NK11, NK104, NK105 (diluted to 20mmol concentration), 2µl template DNA and 17µl dsH_20. Amplification of toxin genes consisted of initial denaturation of 95ºC for 3 minutes followed by 35 cycles of: denaturation at 95ºC for 20 seconds; annealing at 62ºC for 120 seconds and extension at 72ºC for 120 seconds followed by a final extension step of 72ºC for 10 minutes. The resulting PCR products were visualised following electrophoresis in 1% agarose gel with ethidium bromide (0.5µg/ml) in 1X Tris-Acetate EDTA buffer (Sigma Aldrich, Darmstadt, Germany).

Antibiotic susceptibility testing of C. difficile isolates

Antibiotic susceptibility testing of *C. difficile* isolates was performed using the E-test system from BioMérieux (Hampshire, UK) as per manufacturer's instructions. The levels of antibiotic susceptibility to antibiotics commonly used to treat CDI and those implicated as risk factors for CDI (vancomycin, metronidazole ciprofloxacin and moxifloxacin) were tested. Strains were grown on Reinforced Clostridrial Agar (RCA, Merck, Darmstadt, Germany). *C. difficile* ATCC 70057 was used as a control strain for antibiotic susceptibility testing. Isolates were determined to be susceptible (S), intermediate (I) or resistant (R) to the test antibiotic according to pharmacological breakpoint values documented by the Swedish Reference Group for Antibiotics (SRGA) (http://www.srga.org/). The breakpoint values for *C. difficile* for the antibiotics used in this study are as follows: Metronidazole: susceptible <4mg/l, resistant >4mg/l; Ciprofloxacin: susceptible <2mg/l; intermediate 4mg/l; resistant > 8mg/l; Vancomycin: susceptible <4mg/l; resistant >4mg/l; Moxifloxacin: susceptible <2mg/l; intermediate 4mg/l; resistant > 8mg/l.

Statistical analysis

Statistical analysis of data and generation of graphs was performed using GraphPad Prism 5. Paired t-test was used to examine the difference between log CFU g^{-1} of *C. difficile* isolated from fresh and frozen faecal samples and the difference between *C. difficile* excretion rates during stability and enrolment.

3.4 Results

The effect of freezing on the microbiological enumeration of C. difficile in human faecal samples.

Due to the geographical spread of sampling sites (Cork, Ireland; Papworth, UK; Leuven, Belgium) it was necessary to freeze some samples prior to culturing. Thus the effect of freezing on *C. difficile* recovery from faecal samples was evaluated by enumerating the microorganism from samples (n=10) before freezing and after thawing. Results indicate that no significant differences existed between *C. difficile* numbers from both fresh and frozen samples ($p = 0.229$) (Figure 1).

Multicentre comparison of C. difficile prevalence

Faecal samples (161) from 66 persons (46.97% male, median age 29.4±11.6 years) with CF attending three European CF centres were screened for *C. difficile* carriage using culture methods. In addition, the *C. difficile* carriage rate in 14 age-matched healthy control subjects was also examined. Table 1 summarises the clinical characteristics of the subjects at baseline from the three centres. Subjects were screened on enrolment, at 3, 6, 9, 12, 15 months, during pulmonary exacerbation and three months post exacerbation, where faecal samples were available. The prevalence of *C. difficile* carriage in CF persons at time of enrolment in the three centres was as follows: Cork 48.57% (17/35 subjects), Papworth 50% (6/12 subjects) and Leuven 47.37% (9/19 subjects) (Table 1). Carriage rate in the healthy control cohort was 7.14%. If we expand our examination of *C. difficile* carriage rate to

include both subjects who carried *C. difficile* at baseline and at any other sampling point, carriage rates are as follows: Cork-71.4%, Papworth- 66.7% and Leuven-63.2%. Table 2 shows the number of *C. difficile* positive subjects among the three centres and at multiple time points for each subject. Most notably, no clear trend was seen in the prevalence of *C. difficile* between enrolment and exacerbation or 3 months post exacerbation among the three sites investigated. Figure 2A shows excretion rates of *C. difficile* at enrolment, 3, 6, 9, 12, and 15 monthly time points and exacerbation and 3 month post exacerbation (where samples were available) for all sites. We did not observe differences in the rate of excretion of *C. difficile* across time points, however we did not have sufficient data points for each subject to make statistical conclusions on this. We did observe intra-individual variability in *C. difficile* excretion rates sampled at different time points, which we examined in a subset of patients at enrolment and during exacerbation (Figure 2B). There was no significant difference in the rate of *C. difficile* excretion in subjects during periods of stability and exacerbation (paired t-test, p= 0.242)

PCR ribotyping and antibiotic susceptibility testing of C. difficile isolates from CF persons from three geographic locations.

Ribotyping of 80 isolates from three European centres (Cork (n=54), Papworth (n=9), Leuven (n=17)) revealed 23 distinct ribotypes. Only two ribotypes 046 and 078 were recovered from all centres with the most prevalent ribotypes in Cork, Papworth and Leuven being 078, 046 and 010, respectively (Figure 3 and 4). Interestingly, in this study we noted that some subjects sampled at multiple time points, including during exacerbation, revealed a change in ribotype from one time-point to the next (Table 3). Seventy-four percent (17/23) of ribotypes isolated were toxigenic, based on PCR detection of toxin genes *tcdA* and *tcdB* in combination with

EIA testing of stool. Sixty-seven per cent, 100% and 53% of isolates from the Cork, Papworth and Leuven sites were shown to be toxigenic *in vitro*. No toxin was detected from stool samples from 3 subjects (two from the Cork centre and one from Papworth) but the strains isolated from these samples carried the toxin genes and also produced toxin *in vitro* when grown in pure culture.

Eighty *C. difficile* isolates from 45 subjects sampled at multiple time points were tested for sensitivity to a range of antibiotics (Table 5). Susceptibility to metronidazole, ciprofloxacin, vancomycin and moxifloxacin was 97.5%, 1.3%, 100% and 63.8%, respectively. A comparison of the antibiotic susceptibility of *C. difficile* isolates between the three sites during stability, exacerbation and three months post exacerbation is shown in Table 5. No changes were observed in the levels of resistance to each of the antibiotics in any of the sites from enrolment to exacerbation and three months post exacerbation, although this is limited by the low number of samples received for exacerbation and 3 months post exacerbation.

With the exception of two isolates (ribotypes 001 and 039 isolated from the Cork centre), all isolates were susceptible to metronidazole (<4mg/L). All ribotypes recovered from all centres were resistant to ciprofloxacin (>8mg/L) with the exception of one toxigenic 001 isolate, also recovered from the Cork centre. No resistance to vancomycin was observed in any of the isolates. Among non-toxigenic ribotypes, three isolates (2 x 010 and 1 x 039) were resistant to moxifloxacin, both of which were isolated from subjects from the Leuven site. All 078 isolates were resistant to moxifloxacin, with the exception of three 078 strains (isolated from the Papworth and Cork sites). All 046 isolates were resistant to moxifloxacin with the exception of one isolate from the Cork site. One isolate (027) from the Leuven site

was also resistant to moxifloxacin. Table 4 shows the antibiotic susceptibility patterns of the isolates from the three centres in this study.

3.5 Discussion

This is the first multicentre longitudinal study, to our knowledge, that investigates the carriage of *C. difficile* in persons with CF. Here, we report a baseline carriage rate of 48.6%, 50% and 47.4% in Cork, Papworth and Leuven cohorts, respectively, which is consistent with carriage rates reported by a recent single centre study (Burke *et al*., 2016). However, when we include subjects who were positive for *C. difficile* at any time point including baseline we observed an increased carriage rate of 71.4%, 66.7% and 63.2% in Cork, Papworth and Leuven, respectively. Carriages rates reported here support the trend seen since the 1980's of increasing carriage rate of *C. difficile* in CF persons from 22% in a study by Welkon *et al*. (1985) to 47- 50% in recent years in studies by Bauer *et al*., (2014) and Burke *et al*. (2016) but are higher than any reported study to date. In addition, we observed that the carriage rate of healthy controls in this study (7%) is higher than earlier studies which reported rates of 1-2% (Clayton *et al*., 2012, Burke *et al*., 2016), however the increased carriage rate seen here may be indicative of the relatively small healthy control sample number.

In this study, we report that 67% (36/54), 100% (9/9) and 53% (9/17) of isolates in Cork, Papworth and Leuven, respectively were toxigenic, carrying both the *tcdA* and *tcdB* genes and produce the toxins *in vitro*. The overall incidence of toxigenic strains isolated during this study (67%) is comparable to a recent study in an Irish CF cohort (63%) (Burke *et al*., (2016). Other studies have shown varying levels of toxigenicity of *C. difficile* isolates from CF subjects and differing methodology makes direct comparison difficult but rates of 22%-77% have been

reported (Welkon, 1985, Wu, 1983, Peach *et al*., 1986, Yahav *et al*., 2006). The gold standard method for detection of toxin is a culture-cytotoxicity method whereby a human cell line is exposed to toxin B causing cell death; subsequently *C. difficile* is confirmed by reversal of cell damage by the addition of an antitoxin. However, testing for the presence of *C. difficile* by cell culture cytotoxicity is time consuming and not used for routine testing in most clinical laboratories with EIA testing the preferred method for routine diagnosis of *C. difficile* combined with PCR in a two-step algorithm. The EIA ProSpecT^{TM} has a sensitivity of 90.3% compared to cytotoxicity assays and specificity of 96.2%. Terhes *et al*., (2004) compared the molecular method used in this study with cytotoxicity assay and showed that both methods gave the same result for each subject tested. Therefore, for our study PCR is used as a reference, with results confirmed by EIA. In our study, three isolates (ribotypes 046, 017 and 012) from three different subjects were positive for toxin genes (confirmed by PCR and *in vitro* EIA), however negative for *in vivo* toxin production in stool (EIA test). Failure to detect toxin in the stool in these samples is likely attributable to the limit of detection and sensitivity of the EIA assay.

Ribotyping of strains revealed a high degree of heterogeneity between the three centres with relatively few ribotypes (046 and 078) shared between all three centres. This heterogeneity may reflect low levels of spread and cross-contamination among European countries. We observed some differences with ribotypes recovered from the three centres described here and a recent HSPC report which describes the top ranking riboptyes found in the EU, the UK and Ireland up to 2011 (Department of Health, Ireland, 2014), which probably reflects the changing epidemiology of *C. difficile* over time. The HSPC report shows a dominance of 027 in Ireland and the UK; while 014/020 dominate in the EU. In our study, we recovered 027 from one

subject in the Leuven site. Isolates from Cork, Papworth and Leuven were predominantly ribotypes 078, 046 and 010, respectively. A recent report on the epidemiology of *C. difficile* in Belgium (Neely *et al*., 2015) which details the prevalence of ribotypes up to and including 2015, shows a dominance of ribotype 014 followed by ribotypes 078, 020, 002, 005, 106 and 027 which was not reflected in the ribotypes isolated from Belgium in this study. Our Belgian cohort was dominated by 010 and also some ribotypes in the Neely *et al*., (2016) report are not recovered in our study (002, 020 and 106). The *C. difficile* Ribotyping Network (CDRN) for England and Northern Ireland biennial report which includes the first quarter of 2015 shows a dominance of ribotypes 015, 002, 078, 014 and 005. These results differ from the current study where ribotype 046 was most commonly isolated from the Papworth cohort. Ribotype 046 is a pathogenic *C. difficile* strain responsible for CDI outbreaks in Sweden and Poland (Noren *et al*.,2013, Obuch-Woszczatynski *et al*., 2013, Akerlund *et al*., 2010). Ribotype 078 was isolated from all three sites and its prevalence has increased in recent years (Burke *et al*., 2016, Bauer *et al*., 2014, Barbut *et al*., 2007). Both 046 and 078, isolated from all sites in this study, have been isolated from pigs, suggesting a zoonotic mode of transmission through farm workers to a hospital or community setting (Noren *et al*., 2013). In contrast, ribotype 027 was only found in the Leuven centre. This is a fluoroquinolone-resistant strain, responsible for major outbreaks in North America, Canada and Europe (Loo *et al*., 2005, Morfin-Otero *et al*., 2016), and over the past two decades is responsible for the increased mortality, morbidity and increased frequency of CDI. This decrease in prevalence of 027 most likely reflects a natural change in the epidemiology of *C. difficile* with other ribotypes such as 078 coming to the fore which has been reported in Pan-European and American studies (Bauer et

al., 2011, Kociolek *et al*., 2016). However, other European surveillance programs continue to report a dominance of 027, in contrast to results seen in our study (Davies *et al*., 2016, Freeman *et al*., 2017). The successful dissemination and enhanced pathogenicity of ribotypes 027 and 078 is probably a result of increased fluoroquinolone resistance, higher sporulation rates, rapid internalisation and pore formation by TcdB, increased cytotoxicity, increased toxin production relating to mutations in *tcdC* and increased production of binary toxin (Burnham *et al*., 2013).

In this study, we report the antibiotic susceptibility profiles of 80 *C. difficile* isolates from three geographical locations comprising of isolates from 45 subjects at multiple time points, where available. Vancomycin and metronidazole are the most commonly prescribed antibiotics to treat CDI, with oral metronidazole used to treat mild CDI and oral vancomycin for severe cases. While no resistance to vancomycin was detected in any of the isolates, two strains (ribotypes 001 and 039) were isolated which showed resistance to metronidazole (>4mg/L). In addition, 98.8% and 35% of the strains were resistant to ciprofloxacin and moxifloxacin, respectively. The ClosER study, a pan-European longitudinal study which examined antibiotic susceptibility profiles of *C. difficile* isolates from 41 sites in 28 countries, showed susceptibility rates of 97.9%, 98.6% and 63.1% for metronidazole, vancomycin and moxifloxacin, respectively, supporting results seen in our multi-centre study (Freeman *et al*., 2017). In a meta-analysis, comprising of 30 studies published from 2012-2015, Spigaglia (2016) reports high resistance rates to fluoroquinolones, including ciprofloxacin (99%) and moxifloxacin (34%), consistent with results seen here. In addition, they report resistance rates of 1.3% (0-18%, 28 studies) to metronidazole and 2.3% (0-47%, 25 studies) to vancomycin. Reassuringly, we see consistency between antibiotic susceptibility profiles in our CF cohort with results

from non-CF cohorts, despite chronic antibiotic therapy in the CF cohort, which would be considered a driver of antibiotic resistance.

While antibiotic usage has been identified as a risk factor for *C. difficile* carriage, this has not been shown in a CF cohort. Recently a case study by Piccolo *et al*. (2016) observed near fatal CDI in three persons with CF who had received IV antibiotics in the preceding 7-21 days. However, in our cohort, we did not observe links between IV antibiotics and *C. difficile* carriage. Indeed, in agreement with previous studies (Burke *et al*., 2014, Bauer *et al*., 2014), we did not observe any effect of IV, oral or nebulised antibiotics on *C. difficile* carriage. However, larger numbers are needed to perform a site-specific analysis and to make concert statistical correlations and conclusions regarding the influence of clinical parameters on *C. difficile* carriage.

There were no reports of CDI symptoms among CF subjects in this study. No subjects that were positive for *C. difficile* consistently reported presence of diarrhoea and only four subjects reported presence of abdominal pain frequently despite the detection of toxin in stool. Asymptomatic carriage of *C. difficile*, particularly among CF subjects, is well recognised but the underlying mechanisms are not fully understood. However, studies would suggest that it is host factors or the specific environment and gut microbiota assemblage in CF persons that determines whether or not colonisation leads to disease (McFarland *et al*., 1991). Recently, a study by Monaghan *et al*. (2013) established that CF subjects (without a history of CDI) have an enhanced immune response to *C. difficile* toxins A and B. This increase in antitoxin is likely due to increased exposure to *C. difficile* during colonisation and as a result of antibiotic-mediated disturbances of the gut microbiota.

This study shows that subjects may change in *C. difficile* status over multiple time points, in terms of carriage, excretion rate and ribotype, and this change is independent of pulmonary exacerbation. There was no difference in the carriage or excretion rate at baseline or exacerbation in the three centres suggesting that *C. difficile* carriage in the CF gut may be independent of overall health status of the persons with CF. In addition, the colonising ribotype was shown to change longitudinally in a number of subjects. These results suggest that *C. difficile* is a transient member of colonised individuals who acquire new ribotypes either from hospital or the environment.

Conclusion

This is the first longitudinal multicentre analysis, to our knowledge, which investigates *C. difficile* carriage in a CF population. *C. difficile* is an important pathogen in subjects with CF. Surveillance of *C. difficile* characteristics within this subject group is important to improve management of CDI within the CF group itself, but also for the wider hospital community. CF groups may act as an asymptomatic reservoir of virulent *C. difficile* isolates, putting other vulnerable subject groups at risk of CDI. Surveillance of the antibiotic susceptibility profiles of *C. difficile* is important to inform clinicians on the most effective therapy, to monitor the emergence of resistant ribotypes, identify antibiotics which may prove to be risk factors for CDI and also, particularly in the case of CDI to identify reoccurrence from treatment failure.

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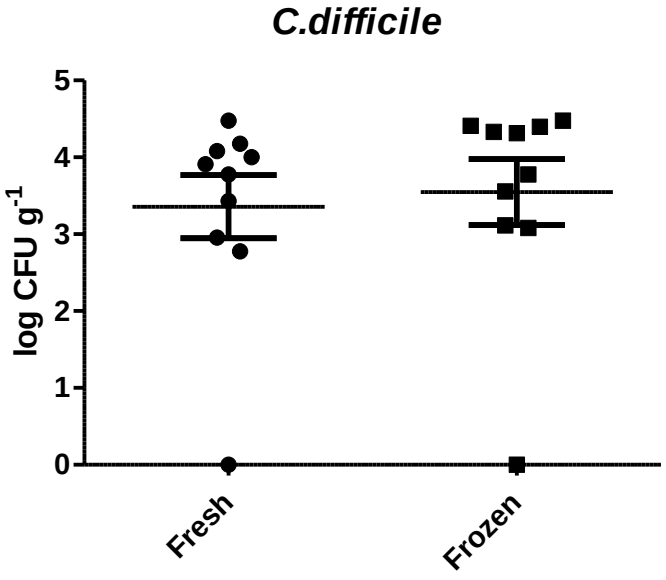

Figure 1 Comparison of *C. difficile* log CFU g^{-1} faeces recovered from a sample set of fresh and frozen faecal samples. Paired t-test was used to examine the difference between the log CFU g^{-1} of *C. difficile* isolated from fresh and frozen samples.

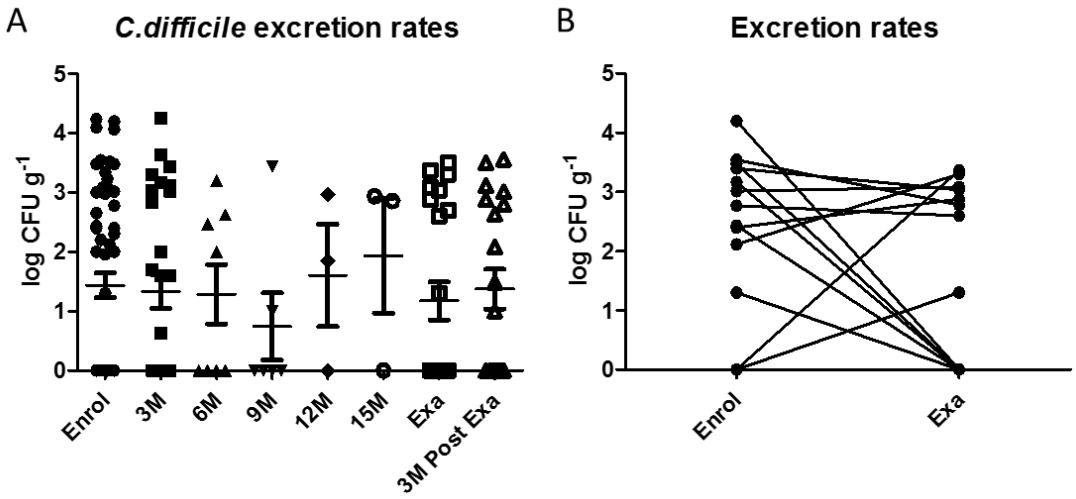

Figure 2. Excretion rates of *C.difficile* (log CFU g^{-1}) across all timepoints (A) and in a subset of subjects (n=21) at enrolement (Enrol) and during exacerbation (Exa) (B). Data from all three sites (Cork, Papworth and Leuven) is combined.

Table 1. Clinical characteristics of the CF cohort from three European sites (Cork, Ireland, Papworth, UK and Leuven, Belgium).

	Cork (n=35)	**Papworth (n=12)**	**Leuven (n=19)**
C. difficile culture positive %	48.57	50	47.37
Age in years (median, standard deviation)	28.16±12.72	28.27±12.65	32.4±6.88
Male gender %	51.43	50	36.84
FEV1 % predicted (median, standard deviation)	52.31±17.45	69.74±18.09	40±17.62
BMI (median, standard deviation)	21±2.80	23±1.85	20±2.68
Mutation class 1-3 %	100	100	100
Pancreatic insufficiency %	94.29	75	84.21
digestive score % (median, standard deviation)	91.67±12.87	79.17±16.08	83.33±9.07
PPI use %	31.43	75	47.37
Maintenance macrolide therapy %	91.43	50	84.21
IV antibiotics %	0	0	0
Oral antibiotics %	17.14	50	15.79
CFTR modulator therapy %	25.71	0	0
Nebulised/inhaled antibiotics			
Colistin %	45.71	66.67	68.42
Tobramycin %	62.86	58.33	31.58
Aztreonam %	62.86	33.33	63.16
Meropenem %	0	0	5.26

FEV_1 – forced expiratory volume in 1 second, BMI- Body Mass Index, PPI-Proton Pump inhibitor.

Table 2. Number of CF subjects positive for *C. difficile* carriage from Cork, Ireland, Papworth, UK and Leuven, Belgium at Enrolment (enrol), three (3m), six (6m), nine (9m), twelve (12m) and fifteen (15m) months, as well as at exacerbation (Exa) and at three months post exacerbation (3m post exa).

No. *C. difficile* positive (n)	**Enrol**	**3m**	**6m**	**9m**	**12m**	**15m**	**Exa**	**3m post exa**
Cork	17 (35)	9 (17)	4 (7)	2 (5)	2 (3)	2 (3)	5 (10)	9 (12)
Papworth	6 (12)	0 (1)	No sample	No sample	No sample	No sample	2 (6)	1 (3)
Leuven	9 (19)	4 (7)	1 (1)	No sample	No sample	No sample	2 (6)	1 (4)

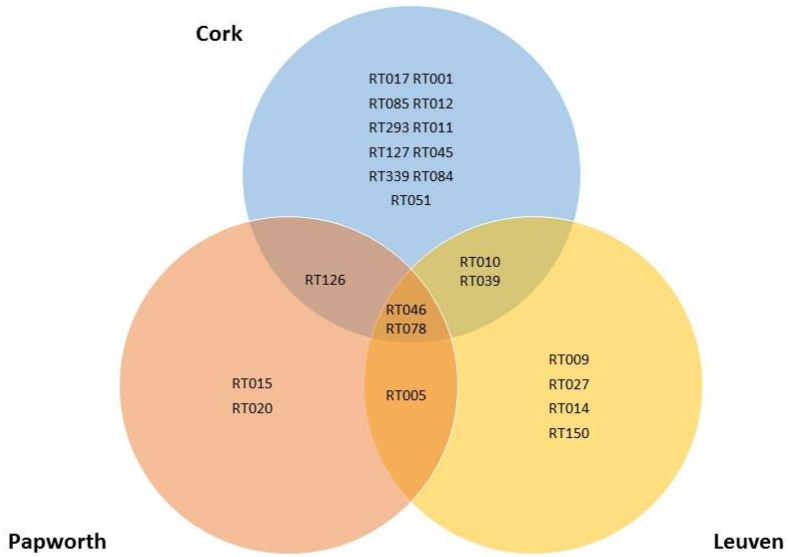

Figure 3. Venn diagram showing the spread of *C. difficile* ribotypes (RT) recovered from CF persons in the three centres: Cork, Papworth and Leuven.

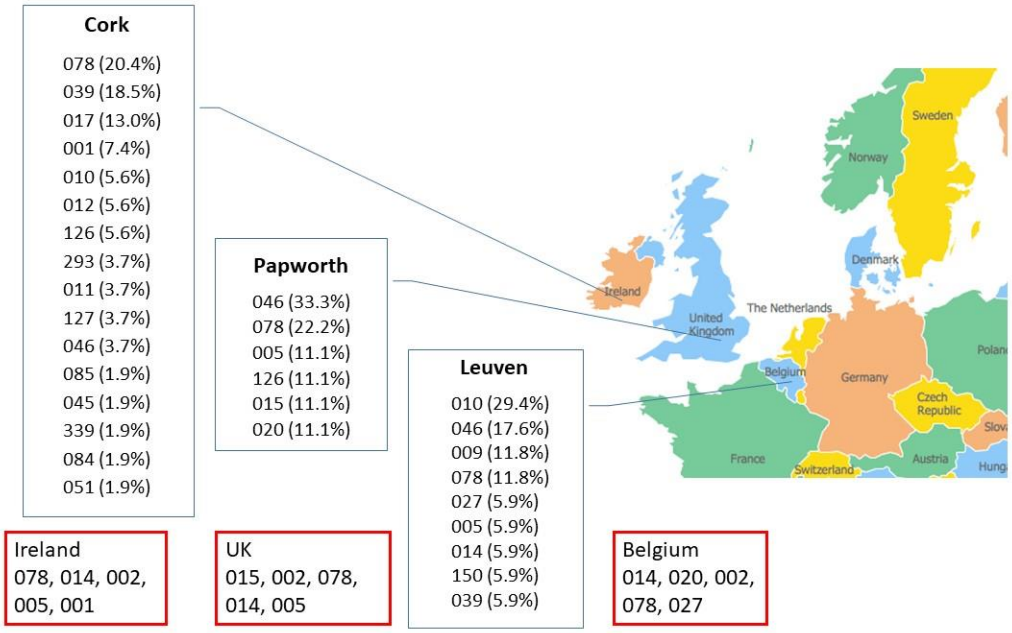

Figure 4. Geographical source and ribotypes, including the prevalence of each ribotype (in brackets following each ribotype) isolated from CF subjects at the three sites from this study. Most prevalent *C. difficile* ribotypes for Ireland, UK and Belgium for 2015-2016 period as reported by *C. difficile* national surveillance programs in each country (boxed in red).

Table 3. Ribotypes recovered from CF persons over time in the Cork, Papworth and Leuven cohorts. Subjects who provided faecal sample(s) but from which no *C. difficile* was isolated are marked CD-ve. Not included in the table are subjects who provided faecal samples at multiple time points but from which *C. difficile* was not isolated at any point. Blank boxes are where no sample was received at time point indicated. Highlighted in yellow are subjects from which different ribotypes were recovered over time.

Subject code				**Ribotype**					
	Location	**enrol**	**3m**	**6m**	**9m**	**12m**	**15m**	**exa**	**3m post exa**
CFA-5	Cork	039						039	
CFA-7	Cork	010	CD-ve					CD-ve	
CFA-9	Cork	078		127				127	
CFA-12	Cork	078	078					078	078
CFA-17	Cork	126	126	CD-ve		126		CD-ve	
CFA-19	Cork	012	293					012	293
CFA-20	Cork	CD-ve						045	011
CFA-25	Cork	CD-ve						CD-ve	039
CFB-1	Papworth	046						046	
CFB-2	Papworth	005	CD-ve					CD-ve	
CFB-5	Papworth	078						CD-ve	
CFB-7	Papworth							078	
CFC-3	Leuven	010						010	010
CFC-4	Leuven	078	078					CD-ve	
CFC-9	Leuven	CD-ve						009	CD-ve
CFC-11	Leuven	CD-ve	150					CD-ve	CD-ve
CFA-1	Cork	CD-ve	010	CD-ve	CD-ve	CD-ve	039		CD-ve
CFA-2	Cork	017	017	012	017	017	CD-ve		
CFA-3	Cork	001							CD-ve
CFA-4	Cork	001							

CFA-6	Cork	039						
CFA-8	Cork	085	CD-ve					
CFA-10	Cork	CD-ve	078					078
CFA-11	Cork	010						001
CFA-13	Cork	039						039
CFA-14	Cork	CD-ve	CD-ve	CD-ve	CD-ve		051	
CFA-15	Cork	CD-ve	078				078	
CFA-16	Cork	001						339
CFA-18	Cork	039	039	011	078	078		
CFA-21	Cork	CD-ve						046
CFA-22	Cork	017	017					
CFA-23	Cork	CD-ve	CD-ve	046				
CFA-24	Cork	084	039					
CFB-3	Papworth	126						
CFB-4	Papworth	CD-ve						015
CFB-6	Papworth	046						
CFB-8	Papworth	020						
CFC-1	Leuven	009						
CFC-2	Leuven	027						
CFC-5	Leuven	046	039					
CFC-6	Leuven	CD-ve	046	046				
CFC-7	Leuven	010	CD-ve					
CFC-8	Leuven	005						
CFC-10	Leuven	014						
CFC-12	Leuven	010						

A= Cork, Ireland, B= Papworth, UK, C= Leuven, Belgium.

Table. 4 Antibiotic resistance profiles of the *C. difficile* ribotypes isolated in this study.

Ribotype	% resistant			
	Metronidazole	Ciprofloxacin	Vancomycin	Moxifloxacin
017 (n=7)	0%	100%	0%	100%
001 (n=4)	25.00%	75.00%	0%	0%
039 (n=11)	9%	100%	0%	9% (10%i)
010 (n=8)	0%	100%	0%	25%
085 (n=1)	0%	100%	0%	0%
012 (n=3)	0%	100%	0%	0%
078 (n=15)	0%	100%	0%	20%
293 (n=2)	0%	100%	0%	0%
126 (n=4)	0%	100%	0%	25%
011 (n=2)	0%	100%	0%	50%
127 (n=2)	0%	100%	0%	100%
046 (n=8)	0%	100%	0%	87.5%
045 (n=1)	0%	100%	0%	100%
339 (n=1)	0%	100%	0%	0%
084 (n=1)	0%	100%	0%	0%
051 (n=1)	0%	100%	0%	0%
009 (n=2)	0%	100%	0%	0%
027 (n=1)	0%	100%	0%	100%
014 (n=1)	0%	100%	0%	100%
150 (n=1)	0%	100%	0%	0%
015 (n=1)	0%	100%	0%	0%
020 (n=1	0%	100%	0%	0%
005 (n=2)	0%	100%	0%	0%

Breakpoints for the antibiotics tested were as follows: Metronidazole: susceptible <4mg/l; resistant >4mg/l; Ciprofloxacin: susceptible <2mg/l; intermediate 4mg/l; resistant > 8mg/l ;Vancomycin: susceptible $\leq$4mg/l; resistant >4mg/l; Moxifloxacin :susceptible $\leq$2mg/l; intermediate 4mg/l; resistant $\geq$ 8mg/l. Number (n) of isolates detected for each ribotype in brackets after the ribotype.

Table 5. Comparison of the antibiotic susceptibility of *C. difficile* isolates between three geographical sites during stability (enrol), exacerbation (exa) and three months post exacerbation (3m post exa) for metronidazole, ciprofloxacin and moxifloxacin.

	Metronidazole			**Ciprofloxacin**			**Moxifloxacin**		
% resistant	**enrol**	**exa**	**3m post exa**	**enrol**	**exa**	**3m post exa**	**enrol**	**exa**	**3m post exa**
Cork	5.9% (1/17)	0% (0/5)	12.5% (1/8)	100% (17/17)	100% (5/5)	87.5% (7/8)	11.8% (2/17) (i 5.9%(1/17))	40% (2/5)	37.5% (2/8)
Papworth	0% (0/6)	0% (0/2)	0% (0/1)	100% (6/6)	100% (2/2)	100% (1/1)	50% (3/6)	50% (1/2)	0% (0/1)
Leuven	0% (0/9)	0% (0/1)	0% (0/1)	100% (9/9)	100% (1/1)	100% (1/1)	55.5% (5/9)	50% (0/1)	100% (1/1)

Vancomycin not included in the table as all isolates were susceptible to vancomycin.

Chapter 4

Screening of a biobank of *Lactobacillus* and *Bifidobacterium* strains for probiotic potential and safety assessment.

4.1 Abstract

Lactobacillus and *Bifidobacterium* strains were isolated from faecal samples from CF persons during periods of respiratory stability and screened for tolerance to antibiotics commonly used to treat CF respiratory infections. The stability of shortlisted strain *L. plantarum* 9_S2 under physiological stresses such as bile, heat and acid was investigated. *L. plantarum* 9_S2 did not show production of EPS (exopolysaccharide), CLA (Conjugated Linoleic Acid) activity or bacteriocin production. The strain had low levels of Bile Salt Hydrolyase activity against glycine conjugated bile salts. *L. plantarum* 9_S2 had improved capabilities to adhere to HT-29 cells compared to *L. rhamnosus* GG. Finally, the genome of *L.plantarum* 9_S2, a strain which had been shown to survive in the presence of vancomycin, metronidazole and ciprofloxacin, was sequenced and annotated.

4.2 Introduction

Cystic Fibrosis (CF) is an inherited disorder caused by a mutation in the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) gene which is characterised by reoccurring pulmonary exacerbations requiring chronic long-term antibiotic therapy (Amadori *et al*., 2009, Flume *et al*., 2009). The importance of the gut microbiota in the overall health of the host has been established in recent years (Guinane and Cotter, 2013) and the detrimental consequences of antibiotic therapy for gut microbiota composition are well documented (Dethlefsen *et al*., 2008, Jernberg *et al*., 2007, Cotter *et al*., 2012, Modi *et al*., 2014). Probiotics are defined as "live micro-organisms which when administered in adequate numbers confer a health benefit on the host" (FAO/WHO 2001). Probiotics function by competing with pathogenic species for nutrients, lowering the intestinal pH to exclude pathogens and inhibition of mucosal adherence of pathogens, stimulation of the

immune system or production of bacteriocins (Tytgat *et al*., 2016, Dobson *et al*., 2012, Ashraf and Shah, 2014, Walsham *et al*., 2016). Previously Bruzzese *et al*. (2004, 2007 and 2014) have shown beneficial respiratory effects of administration of *Lactobacillus rhamnosus* GG (LGG) in paediatric CF cohorts. They showed a reduction in intestinal inflammation, abdominal pain, rate of pulmonary exacerbation and an increase in FEV_1 (Forced Expiratory Volume in 1 second). They also demonstrated the protective effect of LGG against colonisation with *Pseudomonas* in children with CF. Weiss *et al*., (2010) have also shown a significant reduction in pulmonary exacerbations in children with CF infected with *P. aeruginosa* post administration of a probiotic cocktail containing *Lactobacillus acidophilus, Lactobacillus bulgaricus, Bifidobacterium bifidum* and *Streptococcus thermophilus*. In a chronic antibiotic-use cohort, probiotics could play an important role in the reduction of Antibiotic Associated Diarrhoea (AAD), which occurs in up 30% of subjects taking antibiotics (Elseviers *et al*., 2015). In meta-analysis of 82 randomised cross over trials, the majority of which used *Lactobacillus* species alone or in combination with other probiotic genera, Hempel *et al*., 2012 showed that probiotics correlated with a decreased incidence of AAD. In the present study, an antibiotic tolerant probiotic was isolated with potential as an adjunct therapy for treatment of patients on the antibiotic regimes common in CF treatment. It is hoped that this probiotic would have restorative effects on the gut microbiota following antibiotic therapy and protective effects during antibiotic therapy.

4.3 Materials and Methods

Sample Collection

Faecal samples were collected as part of the CFMATTERS (Grant agreement No. 603038) clinical trial from patients during periods of respiratory stability at the Cork Adult Cystic Fibrosis Centre, Cork University Hospital, Cork.

Lactobacillus and Bifidobacterium Strain Isolation

Faecal samples were stored at 4^{o}C and cultured within 24 hours of donation. One gram of fresh stool was serially diluted in maximum recovery diluent (MRD) (Oxoid Ltd, Basingstoke, Hampshire, UK) under anaerobic conditions and plated on *Lactobacillus* (LBS) and *Bifidobacterium* (mMRS) selective media. LBS (*Lactobacillus* Selective Agar) was prepared as per manufacturer's instructions and 50 U/ml Nystatin (Sigma Aldrich) added to inhibit yeast growth. Modified MRS was prepared according to manufacturer's instructions and 0.05% L-cysteine hydrochloride (Sigma Aldrich) and 50 U/ml Nystatin added. Colonies were purified on mMRS media and stored at -80 ^{o}C in 50% glycerol until characterisation.

Antibiotic susceptibility testing

Four hundred and eighty-one isolates (422 putative lactobacilli and 59 putative bifidobacterial strains) were tested for susceptibility to two antibiotics, chloramphenicol and ciprofloxacin using the Reference Agar Dilution Procedure (Wadsworth Method) with some modifications as described in the Clinical and Laboratory Standards Institute manual "Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard – Seventh edition (Vol.27 No.2). Isolates were inoculated into mMRS broth and incubated anaerobically until reaching exponential phase. The inocula were standardised to a turbidity equivalent to the 0.5 McFarland standard (Prolab Diagnostics, Merseyside, Liverpool, UK) and

one µl deposited onto previously prepared Isosensitest agar plates (90% Isosensitest agar (Oxoid Ltd, Basingstoke, Hampshire, UK), 10% mMRS) without antibiotics (controls) and onto plates containing a range of antibiotic concentrations. Plates were incubated anaerobically for 48 hours and subsequently checked for growth at 24 and 48 hours. Controls used were *Lactobacillus paracasei subsp. paracasei* LMG 12586, *Lactobacillus casei subsp. rhamnosus ATCC 334* and *Bifidobacterium longum* NCIMB 702259. Fifty-five strains that demonstrated high levels of antibiotic tolerance to ciprofloxacin but were susceptible to chloramphenicol were finger printed using Pulsed Field Gel Electrophoresis to identify genetically distinct strains.

Pulsed Field Gel Electrophoresis (PFGE)

Fifty-five isolates were finger printed by PFGE as described by Simpson *et al*. (2002). Electrophoresis was performed in 3 L 0.5× TBE buffer at 14°C for 16 h at 6 V/cm, with pulse times ramping linearly from 1 to 20s. Lambda Ladder PFG Marker (New England Biolabs) was used as molecular weight marker. After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/ml) for 1 h and washed with distilled water for 1 h. PFGE patterns showed 16 distinct patterns which then were further analysed for antibiotic susceptibility.

16S rRNA Sequencing

Genomic DNA was extracted from 16 distinct isolates, as established by PFGE results, using Sigma Aldrich DNA purification kit. 16S rRNA gene was PCR amplified using UNIF (5'- agagtttgatcctggctcagg -3') and UNIR (5'- acggcaaccttgttacgagtt -3') oligonucleotides. Purified PCR products from each strain were then sequenced using Sanger sequencing by Beckman Coulter genomics. Sequences were BLAST aligned using the NCBI database.

Minimum inhibitory concentration assays

Minimum inhibitory concentration (MIC) assays were performed on 17 distinct strains identified by PFGE (including control strains stated above) using VetMic assays (Lact-1 and Lact-2) obtained from the National Veterinary Institute, Sweden (National Veterinary Institute, Uppsala Sweden) as per the manufacture's protocol. Briefly, strains were grown for 48 hours and standardised to OD_{600} of 0.5, equating to $1x10^8$ CFU/ml. A final concentration of $5x10^5$ CFU/ml (100 µl) of each strain was added to MIC assay plates and the plates incubated anaerobically for 16 h at 37°C. MIC assays were performed in triplicate for all strains.

Characterisation of antibiotic tolerant strain.

Bacterocin production

Shortlisted strain, *L.plantarum* 9_S2 was screened for bacterocin production using the well diffusion assay with *Lactobacillus bulgaricus* as the target strain. 50 µl of cell-free supernatant of the test strain prepared from freshly grown inocula and pH adjusted to 6.5 with 1M NaOH was added to wells in an mMRS agar plate previously seeded with 0.5% *Lactobacillus bulgaricus*. Plates were incubated at 37^oC overnight and checked for zones of inhibition.

EPS production

EPS production of the shortlisted strain, *L.plantarum* 9_S2 was determined using the loop touch test. The strain was cultivated on mMRS agar containing 5% and 2% glucose, sucrose and lactose. Isolates were then grown for a further 48 hours and assessed for ropiness using the loop touch test. *Pedicoccus rhamnosus* was used as a positive control for EPS production.

Bile Salt hydrolyase activity

Bile salt hydrolase activity was assessed using the method previously described by Kumar *et al*., 2011. Briefly, the BSH assay was performed by measuring the release of amino acids from hydrolysis of glycine conjugated bile salts by the shortlisted strain analysed in this study. The test cultures were inoculated into MRS-thio broth and incubated for 16 hours at 37ºC. Exponentially grown cells were harvested by centrifugation at 5000 rpm, washed twice in peptone saline and resuspended in 0.1 M sodium acetate buffer (pH 5.0) to give an optical density of 2.5 at 600nm. The cell suspensions were then incubated at 37ºC with 10 mM (final concentration) of glycocholic acid for 30 minutes. The reaction was stopped by the addition of an equal volume of 15% TCA (w/v) followed by centrifugation at 18000g for 15 min. Subsequently, 200 µl of the supernatant was added to 1 ml distilled water and 1 ml ninhydrin reagent, vortexed, boiled for 14 minutes and cooled. Absorbance was determined at 570 nm with glycine or taurine as standards. One unit of BSH activity is defined as the amount of enzyme that liberated 1 nmol of amino acid from substrate per min per OD_{600}unit. *L. reuteri* APC 2587 (Cardioviva) was used as a positive control.

Conjugated Linoleic Acid Activity

The ability of the shortlisted strain to produce Conjugated Linoleic Acid (CLA) was assessed using a method developed by Barrett *et al*., (2007). Isolates were grown for 72 hours in mMRS broth containing 0.5 mg/ml free linoleic acid. Following incubation, 1 ml of culture was centrifuged for one minute at 16000xg. The was supernatant mixed with 2 ml isopropanol, allowed to stand for three minutes, before the addition of 1.5 ml hexane and allowed to stand for two minutes. Absorbance of the fat-soluble hexane layer was read at 233 nm. *Bifidobacterium breve* NCF2258

was used as a positive control and LBS media containing no strain was used as a negative control.

Screening for stress tolerance

Strains were incubated anaerobically for 16 hours at 37ºC or until the exponential growth phase was reached. Strains were standardised to OD_{600} of 1.0, harvested by centrifugation at 13,000 rpm x g for five minutes and washed twice with 20 mM sterile phosphate buffer, pH 7.0 (PB7). Strains were then resuspended in the following solutions for 30 minutes: 0.1% HCl pH 3.0 (acid stress tolerance assay), 0.1% (w/v) bile salts (bile stress tolerance assay) and 20 mM PB7 at 55°C (heat tolerance assay). Percentage survival was evaluated by spot plating, in triplicate, 5 µl of 10 fold serial dilutions of suspensions prior to suspension in stress tolerance solution (PB7 was used as a control solution), at 15 and 30 minute time points during the tolerance assays.

Assessment of ability of strain to adhere to HT-29 human colonic adenocarcinoma cell line.

The ability of *L. plantarum* 9_S2, isolated from faecal microbiota, to adhere to HT-29 human colonic adenocarcinoma cell line was assessed. The HT-29 cells were cultured in McCoy's 5A medium supplemented with 10% (v/v) FBS in 5% carbon dioxide at 37ºC, with media replaced on alternate days until 60% confluency was reached. Cells were then harvested by incubation with 3 ml of 0.25% trypsin-EDTA solution at 37ºC, resuspended in fresh media and transferred to 12-well tissue plates at a final cell count of 2×10^5 cells/ml. Prior to cell adherence assay 10% (v/v) FBS was replaced with 2% (v/v) FBS. 2% FBS was then removed and cells were washed with PBS three times. Strains to be tested were grown overnight in MRS broth and harvested at OD_{600}nm of 1.0. One ml of bacterial culture was added in triplicate to

the 12 well tissue culture plate and the plate was incubated for 2h at 37ºC under anaerobic conditions. Each well was then washed with PBS three times and lysed with PBS + 0.01% tritonX 100. Serial dilutions of the suspensions were plated on MRS plates and incubated for 24 h under anaerobic conditions to determine CFU/ml for adhered cells which was then compared to CFU/ml of starting inoculum to give % of cells adhered.

Antibiotic co-culture assays

Preliminary co-culture assays were preformed to investigate the survival of *L. plantarum* 9_S2 in the presence of antibiotic combinations and compare its survival to *L. rhamnosus* GG. Survival of *L. plantarum* 9_S2 and LGG was tested at two different concentrations of antibiotic combination as follows: 1x antibiotic dose (500 µg/ml vancomycin; 100 µg/ml metronidazole; 5µg/ml ciprofloxacin) and 2x antibiotic dose (1000 µg/ml vancomycin; 200 µg/ml metronidazole; 10 µg/ml ciprofloxacin). Both strains were grown in mMRS with either 1x or 2x antibiotic dose for 24 hours to assess survival.

Whole genome sequencing of strain L.plantarum 9_S2

Sample Preparation

L. plantarum 9_S2 was sequenced at the Clinical-Microbiomics facility in Hørsholm, Denmark using 2x100bp sequencing on Illumina's HiSeq 2500 platform. DNA was extracted as previously described using Sigma Aldrich DNA purification kit and prepared for sequencing using Illumina's Nextera sample preparation kit which uses an engineered transposon to fragment and tag DNA with unique adapter sequences. A limited cycle PCR was then used to amplify the insert DNA using the adaptors, simultaneously adding indices on both ends, allowing for dual index sequencing on the HiSeq platform.

Bioinformatic analysis

Quality trimming was performed with Trimmomatic and included trimming 15 leading bases and 20 trailing bases, a 6 bases sliding window trimming with minimum phred quality of 20 and retaining reads with minimum 60 bases (Bolger *et al*., 2014). De-novo genome assembly was performed using SPAdes with default settings (Bankevich *et al*., 2012). Quality of the assemblies was evaluated using Quast (Gurevich *et al*., 2013). Assembled genomes were submitted to RAST for gene prediction and functional annotation (Brettin *et al*., 2015, Overbeek *et al*., 2014). Predicted proteins were mapped to databases with virulence genes and resistance genes using global alignment (Usearch) (Edgar., 2010) and matches with minimum coverage of 80% and identity of 80% were retained (Chen *et al*., 2016, Zankari *et al*., 2012). Annotation for the possible bacteriocin gene clusters were performed using BAGEL3 (van Heel *et al*., 2013). A database of dnaK genes was built for Lactobacillus strains using Uniprot Swiss-Prot (Huang and Lee, 2011). Multiple-sequence alignments of the dnaK genes was produced using Clustal Omega (Sievers *et al*., 2011) and the approximate maximum-likelihood phylogenetic tree was produced using Fasttree (Price *et al*., 2010). Comparative analysis of *L. plantarum* 9_S2 and *L. plantarum* WCFS1 was performed using FIGfams (Meyer *et al*., 2009). Visualisation for the functional annotation and comparison was performed using EggNOG 4.5 database, based on the protein set eggnog4.proteins.core_periphery (Heurta-Cepas *et al*., 2016). The database was searched using global alignment with Usearch 8.1 and hits with at least 80% identity were accepted. The visualisation was made in BRIG (Alikhan *et al*., 2011).

4.4 Results

Biobank screen results

Four hundred and eighty-one isolates (422 lactobacilli and 59 bifidobacteria) were grown on 4 mg/l chloramphenicol (EFSA breakpoint for chloramphenicol) and 8mg /l ciprofloxacin. Fifty-five isolates that showed no growth on chloramphenicol (4 mg/l) and growth on 8 mg/l ciprofloxacin were carried forward for further analysis. These 55 strains were then genetically finger printed using PFGE resulting in 16 unique strains. 16S rRNA Sanger sequencing was used to speciate these isolates (Table 1). Table 1 shows 16S rRNA sequencing identified 6 *Lactobacillus mucosae* species, 5 *Lactobacillus amylovorous* species, 2 *Lactobacillus reuteri*, 1 *Lactobacillus plantarum* species, 1 *Lactobacillus gasseri* and 1 *Bifidobacterium bifidum* among the shortlisted strains.

Minimum inhibitory concentration assays

Minimum inhibitory concentration assays for a range of antibiotics were then performed on 16 shortlisted strains. The results for the MIC assays shown in tables 2 and 3 showed that two isolates *L. plantarum* and *B. bifidum* were compliant with EFSA breakpoints for food additives. *L. plantarum* and *B. bifidum* were then selected and tested for susceptibility to metronidazole. *L. plantarum* 9_S2 showed a higher tolerance to metronidazole (>256 µg/ml) compared to *B. bifidum* (2 µg/ml). *L. plantarum* 9_S2, therefore, was selected for further characterisation. As *L. rhamnsosus* GG is a commonly used probiotic and has been used in studies in CF subjects, the MIC breakpoints and tolerance to a range of antibiotics of LGG was compared to *L. plantarum* 9_S2. *L. plantarum* 9_S2 and *L. rhamnosus* GG were similar in their respective MIC profile for a range of antibiotics with the exception of

ciprofloxacin. *L. plantarum* 9_S2 and *L. rhamnosus* GG were shown to have MICs of 32 µg/ml and 2 µg/ml to ciprofloxacin, respectively (Table 4).

Stress tolerance assays

Stress tolerance assays of *L. plantarum* 9_S2 when incubated in 0.1% HCl pH 3.0 for 30 minutes showed no decease in viability after 15 or 30 minutes incubation. *L. plantarum* 9_S2 showed an approximately 2 log reduction following incubation at 55^{o}C and approximately 1.5 log reduction after 15 minutes incubation in 0.1% (w/v) bile salts. However, no further decrease in viability was observed after 30 min incubation in the presence of bile salts (Figure 1).

Evaluation of Technical properties

No bacteriocin, CLA or EPS production was observed in the *L. plantarum* 9_S2 candidate strain. The strain produced 8.4 $\pm$ 3.3U/min bile salt hydrolase activity which was lower than the positive control *Lactobacillus reuteri* APC 2587 (21.36$\pm$ 0.06U/min) (Cardioviva) (Figure 2).

Evaluation of adherence properties

The ability of *L.plantarum* 9_S2 and *L.rhamnosus* GG to adhere to HT-29 human colonic cell line was tested and the results shown in Figure 3. After 2 h incubation with HT-29 human cell *L.plantarum* 9_S2 showed a greater adherence ability (77.5%) to HT-29 human cell line than positive control LGG *rhamnosus* (64.7%).

Co-culture assays

L. plantarum and *L. rhamnosus* GG were co-cultured in an antibiotic combination treatment (vancomycin, ciprofloxacin and metronidazole) at concentrations which are relevant to a CF population in both single (1x) and double (2x) concentrations. Antibiotic concentrations for single dose was 500 µg/ml vancomycin, 100 µg/ml metronidazole and 5 µg/ml ciprofloxacin and for the double dose was 1000 µg/ml

vancomycin, 200 µg/ml metronidazole and 10 µg/ml ciprofloxacin. Co-culture of *L. plantarum* 9_S2 and *L. rhamnosus* GG at the two antibiotic doses relevant to a CF population showed that *L.plantarum* at both 1x (2 log) and 2x (4 log) antibiotic concentrations survived better than LGG (Figure 4). Increasing the antibiotic dose from 1x to 2x did not impact the survival of *L.plantarum* 9_S2, however *L. rhamnosus* GG showed approximately a 2.5 log reduction.

Whole genome sequencing of Candidate strain L.plantarum 9_S2

L. plantarum 9_S2 genome data assembled into 47 contiguous sequences amounting to 2, 680, 338 bp, with a 44.4% GC content and 3024 unique predicted genes. Blasting the *L. plantarum* 9_S2 genome against Bacterocin II database revealed a hits against Plantaricin_K and Plantaricin_N at orf_15 and orf_20, respectively. Mapping of predicted proteins to virulence and resistance gene databases showed no virulence or resistance genes in the *L. plantarum* 9_S2 genome. Despite *in vivo* assays showing tolerance to ciprofloxacin there were no matches to quinolone resistance genes even at 30% identity in nucleotide global alignment. Phylogenetic comparison to other *Lactobacillus* strains based on the dnaK marker gene showed a close clustering of the genome of *L. plantarum* 9_S2 to *L. plantarum* WCFS1 (Figure 5). EggNOG annotation showed 40% of genes found in *L. plantarum* 9_S2 are involved in metabolism, 20% information storage and processing and 17% cellular processes and signalling. The remainder of the genome is poorly characterised (Figure 6). Comparative analysis of *L. plantarum* 9_S2 and *L. plantarum* WCFS1, using FIGfams, showed that overall the two strains are very similar, but some distinct differences are noted. *L. plantarum* WCFS1 has 124 Figfams not present in *L. plantarum* 9_S2, while *L. plantarum* 9_S2 strain contains 67 Figfams not present in *L. plantarum* WCFS1. Also, when looking at protein

coding genes with no FIGfam annotation (phage proteins and unknown genes), they share 128 of such genes (out of a total of 190 and 171 unique genes in *L. plantarum* WCFS1 and *L. plantarum* 9_S2, respectively).

4.5 Discussion

In this study we screened the CF faecal microbiota for an antibiotic tolerant putative probiotic with potential to improve gut microbiota composition in a CF cohort, which resulted in identification of *L. plantarum* 9_S2 isolate. *L. plantarum* 9_S2 was then further characterised for probiotic properties such as bile tolerance, antimicrobial activity, BSH activity, CLA production, antibiotic susceptibility, cell adhesion properties and EPS production.

Lactobacillus species are Gram-positive non-pathogenic organisms with a Generally Recognised As Safe status (GRAS) which are desirable member of the gut microbiota community. Lactobacilli have been associated with health benefits including reduction of serum cholesterol levels (London *et al*. 2014, Anderson and Gilliand, 1999), enhancement of immune system (Perdigon *et al*., 2002) and contribution to colonisation resistance against infectious intestinal pathogens (Kim *et al*., 2008). *Lactobacillus plantarum* WCFS1 is one of the most well studied *Lactobacillus* species. Studies show a high survival rate of *L. plantarum* WCFS1 compared to other lactobacilli strains in the human intestinal tract increasing by 100-1000 fold in healthy volunteers (Van Bokhortst-van de Veen *et al*., 2012). However, despite high levels of adhesion to mucosa, *L. plantarum* WCFS1 is known to be a passenger of the gut microbiota rather than an effective intestinal coloniser which exerts its effect on transit through the intestine (Douillard and de Vos, 2014). *L.plantarum* has been associated with repair of gut barrier function in animal studies (Mao *et al*., 1996, White *et al*., 2006 and Mennigan *et al*., 2009). Indeed, Karczewski

and colleagues (2010) showed that introduction of *L. plantarum* WCFS1 into the duodenums of healthy subjects improved the organisation of epithelial tight junctions. Despite the many reports benefits associated with *L. plantarum* species we acknowledge that these benefits are strain specific, often differing even between different strains of the same genus and will need to be explored in the *L. plantarum* 9_S2 strain isolated in this study.

The lung-gut axis is increasingly being recognised along with the potential for manipulation of the gut microbiota being used as a therapeutic option for chronic lung infection diseases (Budden *et al*., 2016). Several studies have shown the beneficial respiratory effects of administration of probiotics, supporting the hypothesis of a lung gut axis. Yoda and colleagues (2012) showed that administration of *L. gasseri* in obese mice reduced inflammation in the lung. Alvarez *et al*., (2001) demonstrated increased clearance of *Pseudomonas aeruginosa* in mice that has been treated with *L. casei* prior to infection. By maintaining a healthy gut microbiota composition, we hypothesise that a healthier lung microbiota composition would be more probable, resulting in fewer exacerbations

Antibiotic therapy has well-documented negative effects on the gut microbiota diversity, composition and functionality (Blaser, 2011). In a cohort, such as a CF, who receive both maintenance and IV antibiotics on a regular basis, probiotics may be a useful adjunct therapy to aid maintenance of a healthy gut microbiota during therapy, restore the gut microbiota post antibiotic therapy and during periods of stability direct the gut microbiota to a healthier composition to reduce or delay pulmonary infections. To increase probability of survival we selected for strains with antibiotic tolerance to commonly used CF antibiotics, however this

antibiotic tolerance needs to fall within EFSA guidelines for food additives. EFSA (European Food Safety Authority) requires as part of its Qualified Presumption of Safety approach to assessment of bacteria deliberately introduced into the food chain that acquired resistance genes are absent. Intrinsic resistance poses a much lesser risk of lateral transfer of genes compared to acquired resistance. EFSA requires strains that are potentially for use as food additives to comply with defined microbiological cut-off values for the following antibiotics: ampicillin, kanamycin, vancomycin, gentamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol. *L. plantarum* 9_S2 shows compliance with EFSA requirements but also showed high levels of tolerance to ciprofloxacin (mic 32 µg/ml) and metronidazole (>256 µg/ml). Species of the lactobacilli genus tend to have intrinsic resistance to vancomycin and metronidazole (Hamilton-Miller and Shah, 1998, Danielson and Wind, 2003), affording them protection in a cohort treated with these antibiotics.

Bile salt hydrolases (BSH) have the ability to deconjugate bile salts, allowing bile acids to regulate functions such as cholesterol synthesis, there by being a desirable probiotic trait (Matsubara and Gonzalez, 2013). *L .plantarum* 9_S2 shows low levels of BSH activity towards glycine conjugated bile acids relative to established BSH producer *Lactobacillus reuteri* APC 2587. The majority of BSH active strains (including *L. reuteri* and *L. plantarum*) show a preference for glycine conjugated bile salts, which is readily available in the liver (Fang *et al*., 2009, Gaull *et al*., 1985). *L .plantarum* 9_S2 showed no EPS production or CLA activity, both which are considered desirable probiotic traits. Acid tolerance assay (0.1% HCl pH 3.0) showed no change in the log CFU ml^{-1} over 30 minutes, while both bile (0.1%

(w/v) bile salts) and heat tolerance (55^{o}C) assays showed a 2 log CFU ml^{-1} reduction over 30 minutes.

Finally, the whole genome sequence of *L. plantarum* 9_S2 was analysed. Whole genome analysis showed no antibiotic resistance or virulence genes present, supporting its suitability as a probiotic. Comparative analysis between *L. plantarum WCFS1* and *L. plantarum 9_S2* show that the strains have a high degree of similarity, however there are some distinct differences. *L.plantarum* WCFS1 has been shown to have bacteriocin activity (plantaricin A) which has a low minimum inhibitory concentration against *Enterococcus faecalis*. *L.plantarum* 9_S2 contains a plantaricin bacteriocin on orf 15 and 20, which may have similar activity to other plantaricins, though the effect has not been observed in vitro.

In conclusion, we present a study describing *L. plantarum* 9_S2 strain isolated from CF faeces, with antibiotic tolerance to ciprofloxacin, metronidazole and vancomycin and compliant with EFSA guidelines for food additives. In future studies this strain will be assessed *ex vivo* and *in vivo* assess its probiotic potential for as an adjunct therapy for CF.

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Table 1. Lactobacilli and Bifidobacterium species identified as part of Biobank screen for antibiotic tolerance.

Strain ID	Species	Query coverage %	E-value	Identity %
CF_1	*Lactobacillus mucosae* LM1, complete genome	93	0.00E+00	99
CF_6	*Lactobacillus mucosae* LM1, complete genome	92	0.00E+00	99
CF_9_S2	*Lactobacillus plantarum* strain K-46 16S ribosomal RNA gene, complete sequence	92	0.00E+00	100
CF_14	*Lactobacillus mucosae* LM1, complete genome	92	0.00E+00	99
CF_15	*Lactobacillus mucosae* LM1, complete genome	93	0.00E+00	99
CF_53	*Lactobacillus reuteri* strain I49, complete genome	92	0.00E+00	99
CF_7	*Lactobacillus mucosae* strain K129 16S ribosomal RNA gene, partial sequence	91	0.00E+00	99
CF_12	*Lactobacillus mucosae* strain K129 16S ribosomal RNA gene, partial sequence	90	0.00E+00	99
CF_50	*Lactobacillus amylovorus* gene for 16S rRNA, partial sequence, strain: JCM 8774	88	0.00E+00	100
CF_55	*Lactobacillus amylovorus* gene for 16S rRNA, partial sequence, strain: JCM 1126	92	0.00E+00	100
CF_53	*Lactobacillus reuteri* strain I49, complete genome	92	0.00E+00	100
CF_52	*Lactobacillus amylovorus* gene for 16S rRNA, partial sequence, strain: JCM 1032	91	0.00E+00	99
CF_54	*Lactobacillus amylovorus* gene for 16S rRNA, partial sequence, strain: JCM 1126	90	0.00E+00	100
CF_51	*Lactobacillus amylovorus* gene for 16S rRNA, partial	92	0.00E+00	100

	sequence, strain: JCM 1032			
CF_25	*Lactobacillus gasseri* strain SHZ67 16S ribosomal RNA gene, partial sequence	92	0.00E+00	99
CF_49	Lactobacillus amylovorus strain 30SC, complete genome	89	0.00E+00	99
	Bifidobacterium bifidum BGN4, complete genome	93	0.00E+00	97

Table 2. Antibiotic susceptibility testing of isolates for antibiotics with defined microbiological cut-offs required by EFSA for food additives. Microbiological cut-offs in mg/L. Gm: Gentmycin; Km: Kanamycin; Sm: Streptomycin; Tc: Tetracycline; Em: Erythromycin; Cl: Clindamycin; Cm: Chloramphenicol; Am: Ampicillin; Va: Vancomycin. MICs based on triplicate values.

Strain	**Gm**	**Km**	**Sm**	**Tc**	**Em**	**Cl**	**Cm**	**Am**	**Va**
EFSA cut-off (mg/L)	16[a]	64[b]	64[c]	8[d]	1	1[e]	4[f]	2[g]	n.r[h]
Lactobacillus mucosae CF_1	0.5	8	2	>64	>8	8	2	16	>128
Lactobacillus mucosae CF_6	0.5	8	2	>64	>8	8	2	>16	>128
Lactobacillus mucosae CF_14	0.5	4	1	>64	>8	>16	2	>16	>128
Lactobacillus mucosae CF_13	0.5	4	2	4	>8	16	4	2	>128
Lactobacillus mucosae CF_12	0.5	4	2	4	>8	>16	4	4	>128
Bifidobacteria bifidum CF_11	0.5	4	2	1	0.016	0.03	0.5	0.03	0.5
Lactobacillus mucosae CF_7	0.5	8	2	>64	>8	16	2	>16	>128
Lactobacillus gasseri CF_25	1	32	4	2	>8	1	2	0.24	1
Lactobacillus plantarum 9_S2	0.5	16	8	16	0.06	0.03	4	1	>128
Lactobacillus amylovorous CF_52	4	128	128	16	>8	>16	1	4	0.5
Lactobacillus amylovorous CF_54	2	32	64	64	0.016	0.5	2	8	0.5
Lactobacillus reuteri CF_53	0.5	4	2	>64	>8	4	0.5	0.5	>128
Lactobacillus amylovorous CF_49	1	8	16	64	>8	16	1	8	0.5
Lactobacillus amylovorous CF_51	2	32	64	64	>8	>16	2	2	0.5

Lactobacillus amylovorous									
CF_50	4	256	512	64	>8	>16	2	8	0.5
Lactobacillus amylovorous									
CF_55	2	32	2	64	0.016	0.06	2	8	0.5
Lactobacillus paracasei subsp.									
paracasei LMG 12586	0.5	8	4	1	0.12	0.03	2	1	>128
Lactobacillus casei subsp.									
rhamnosus ATCC 334	1	32	16	1	0.06	0.06	4	1	>128
Bifidobacterium									
longum NCIMB 702259	0.5	8	2	0.5	0.016	0.03	1	0.06	0.5

[a] exceptions *L .reuteri* EFSA cut-off 8mg/L, *Bifidobacterium* 64mg/L

[b] exceptions Bifidobacterium n.r, *L. amylovorous* and *L. gasseri* EFSA cut-off 16 mg/L, *L. mucosae* EFSA cut-off 32mg/L

[c] exceptions Bifidobacterium EFSA cut-off 128mg/L, *L. amylovorous* and *L. gasseri* EFSA cut-off 16 mg/L, *L. plantarum* n.r

[d] exceptions *L. amylovorous* and *L. gasseri* EFSA cut-off 4 mg/L, *L. plantarum* EFSA cut-off 32mg/L, *L. reuteri* EFSA cut-off 16mg/L

[e] exception *L. plantarum* EFSA cut-off 2mg/L

[f] exception *L. plantarum* EFSA cut-off 8mg/L

[g] exceptions *L. amylovorous* and *L. gasseri* EFSA cut-off 1 mg/L

[h] exceptions *L. amylovorous* and *L. gasseri* EFSA cut-off 2 mg/L, *Bifidobacterium* EFSA cut-off 2mg/L

Table 3. Antibiotic susceptibility testing of isolates. Microbiological cut-offs in mg/L. Nm: Neomycin; Pc: Penicillin; Quinupristin-dalfopristin; Lz; Linezolid; Tm; Trimethoprim; Ci: Ciprofloxacin. MICs based on triplicate values.

Strain	**Qda**	**Lz**	**Tm**	**Ci**	**Ri**	**Nm**	**Pc**
Lactobacillus mucosae CF_1	0.5	0.5	>64	>128	0.12	0.5	8
Lactobacillus mucosae CF_1	2	1	>64	>128	0.12	0.5	16
Lactobacillus mucosae CF_1	4	1	>64	>128	0.5	0.5	8
Lactobacillus mucosae CF_1	0.5	1	>64	32	0.24	0.5	1
Lactobacillus mucosae CF_6	2	2	8	>128	0.24	0.5	4
Lactobacillus mucosae CF_14	0.24	0.5	32	32	0.5	1	0.03
Lactobacillus mucosae CF_12	4	1	>64	>128	0.5	0.5	8
Bifidobacteria bifidum CF_11	0.24	1	0.12	16	0.24	4	0.06
Lactobacillus mucosae CF_7	0.5	2	4	16	0.5	0.5	1
Lactobacillus gasseri CF_25	1	1	8	16	2	16	0.5
Lactobacillus plantarum 9_S2	1	1	32	16	4	16	1
Lactobacillus amylovorous CF_52	0.24	0.24	4	16	0.12	0.5	2
Lactobacillus amylovorous CF_54	1	1	8	32	1	8	1
Lactobacillus reuteri CF_53	2	1	8	16	4	16	0.24
Lactobacillus amylovorous CF_49	1	1	16	8	2	16	1
Lactobacillus amylovorous CF_51	1	1	16	32	4	16	1
Lactobacillus paracasei subsp. paracasei LMG 12586	1	2	>64	2	0.5	0.5	0.5
Lactobacillus casei subsp. rhamnosus ATCC 334	2	2	>64	1	0.5	2	0.5
Bifidobacterium longum NCIMB 702259	0.12	0.24	32	8	0.5	1	0.06

Table 4. Comparison of MICS for *L. plantarum* 9_S2 strain and positive control strain LGG (L. *rhamnosus)*. MICS given in mg/L and based on triplicate values. Gm: Gentmycin; Km: Kanamycin; Sm: Streptomycin; Tc: Tetracycline; Em: Erythromycin; Cl: Clindamycin; Cm: Chloramphenicol; Am: Ampicillin; Va: Vancomycin; Ci: Ciprofloxacin; Met; Metronidazole.

	Gm	**Km**	**Sm**	**Tc**	**Em**	**Cl**	**Cm**	**Am**	**Va**	**Ci**	**Met**
EFSA cut-off	16	64	n.r,32	32,8	1	2,1	32,8	2,4	n.r	n/a	n/a
L.plantarum 9_S2	0.5	16	8	32	0.12	0.06	4	1	>128	32	<256
LGG L.rhamnosus	1	32	4	2	0.12	0.5	8	2	>128	2	<256

n.r not required, n/a not listed in EFSA cut-off requirements

In cases where EFSA cut-off column contains two values *L. plantarum* is first, *L. rhamnosus* is second.

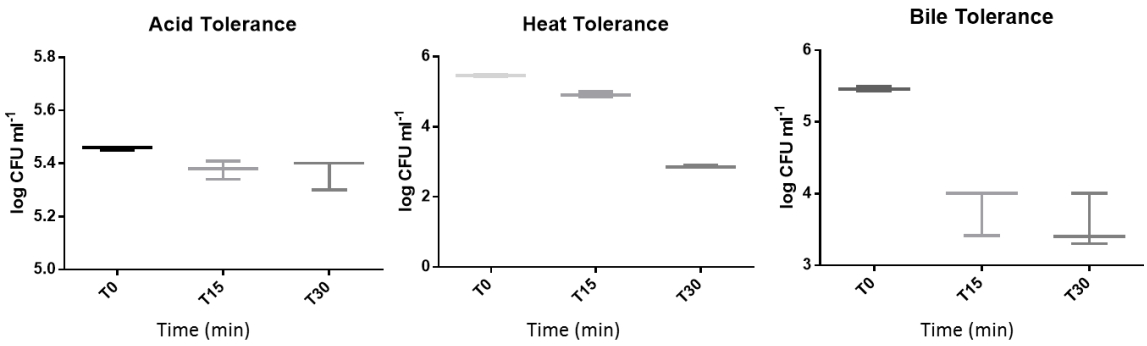

Figure 1. Tolerance of *L. plantarum* to acid, heat and bile physiological stresses. Strains were suspended 0.1% HCl pH 3.0 (acid stress tolerance assay), 0.1% (w/v) bile salts (bile stress tolerance assay) and 20 mM Phopshate Buffer pH7 at 55°C (heat tolerance assay) for 30 min and sampled at T0, T15 and T30. Results are the mean of 3 replicates and expressed as log CFU/ml.

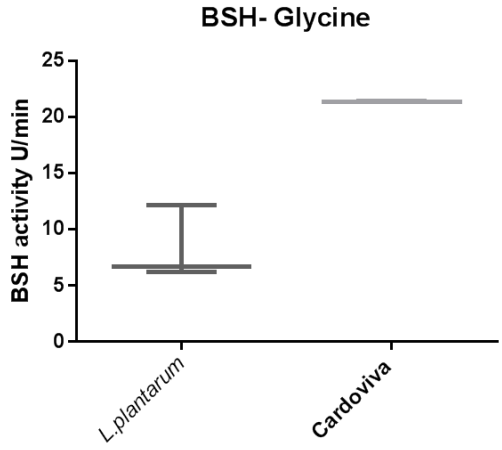

Figure 2. Comparison of Bile Salt hydrolyase activity of *L. plantarum* 9_S2 and *L. reuteri* (Cardioviva) using amino acid glycine as substrate. BSH activity (U/min) is defined as the amount of enzyme needed to liberate 1 nmol of amino acid from substrate per min per OD_{600}unit. Measurements are shown in triplicate.

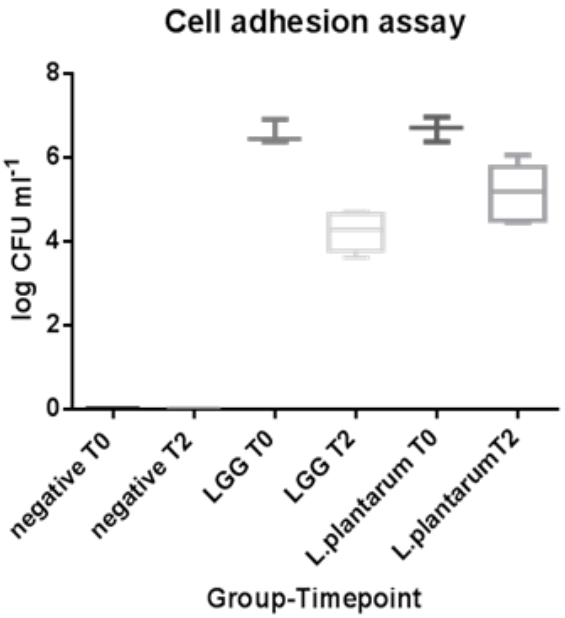

Figure 3. Adhesion of *L. plantarum* 9_S2 and *L. rhamnosus* LGG HT-29 cells after 2 h incubation. Results are expressed as the log CFU/ml of cells adhering to HT-29's after 2 h incubation compared to the log CFU/ml of the initial inoculum for each strain and the negative control.

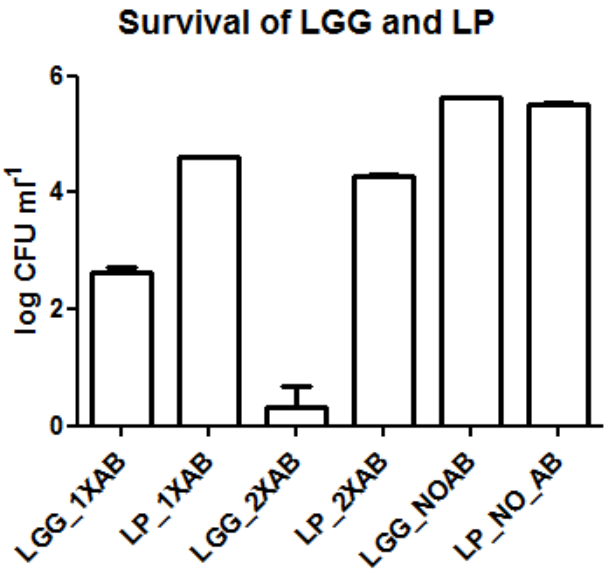

Figure 4. Survival of *L. plantarum* 9_S2 (LP) and *L. rhamnosus LGG* (LGG) in the presence of 1x (1XAB (500 µg/ml vancomycin; 100 µg/ml metronidazole; 5 µg/ml ciprofloxacin) and 2x (2XAB) (1000 µg/ml vancomycin; 200 µg/ml metronidazole; 10 µg/ml ciprofloxacin). Strains were incubated with both 1XAB and 2XAB and survival after 24 hours was evaluated by enumeration of viable colonies.

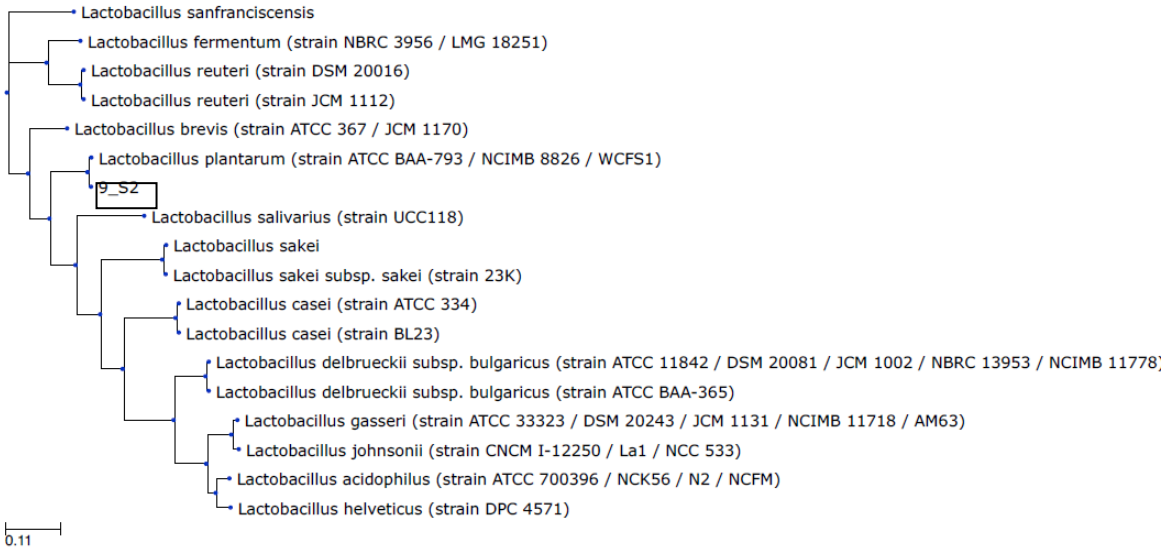

Figure 5. Phylogenetic tree showing relationship of *L. plantarum* 9_S2 candidate strain to other species within the lactobacillus genus based on dnaK marker gene.

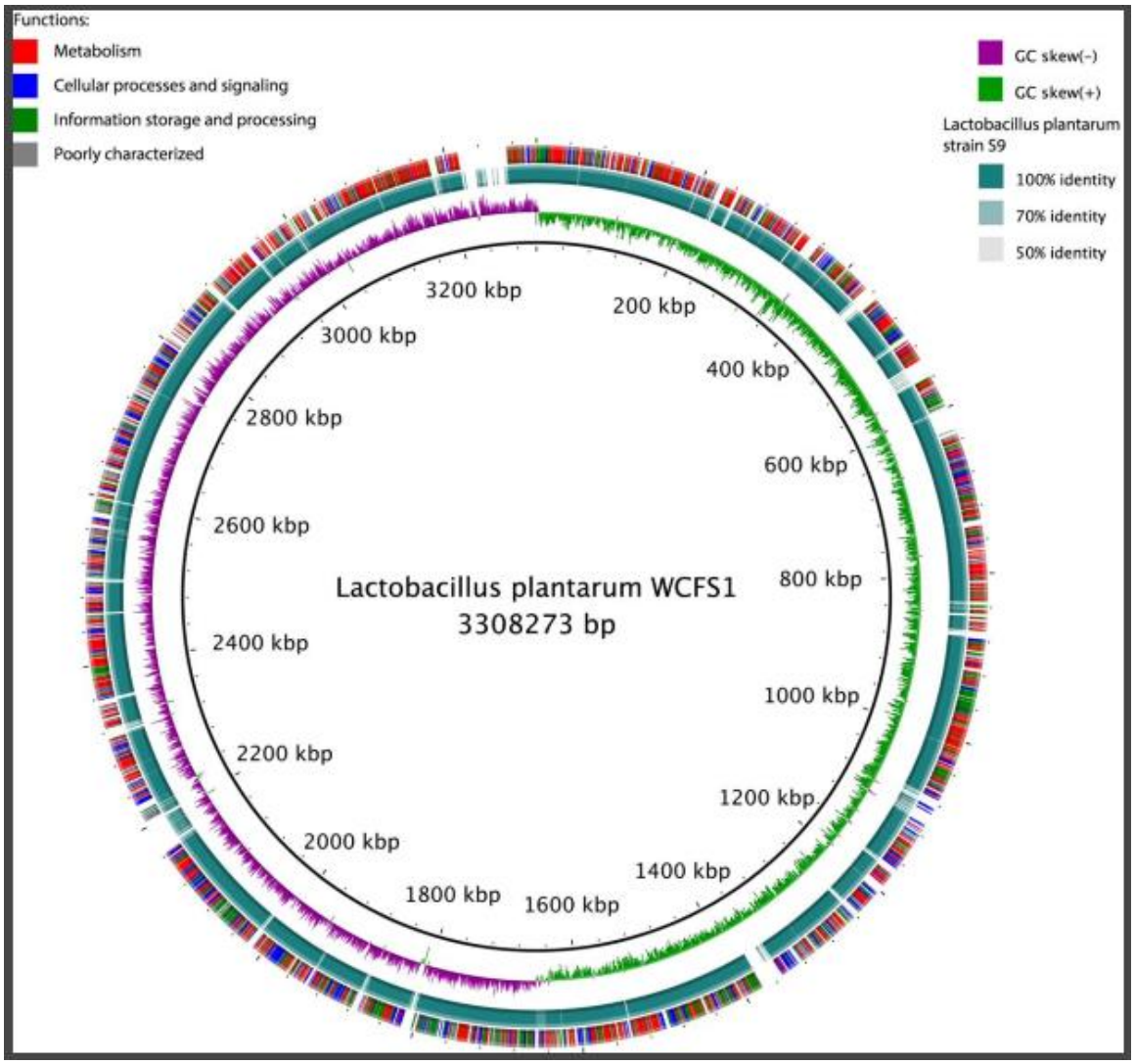

Figure 6. Comparative analysis of the genomes of *L. plantarum* WCFS1 (3,308,273 bp) and *L. plantarum* 9_S2 (2,680,338 bp). EggNOG annotation showed 40% of genes are involved in metabolism (red), 20% information storage and processing (green) and 17% cellular processes and signalling (blue). The remainder of the genome is poorly characterised (grey).

Chapter 5

Clinical Outcomes of Real World (Ivacaftor) Kalydeco™ (CORK)- Investigating the modulatory effects of CFTR potentiation on the intestinal microbiota, intestinal inflammation and pancreatic function.

5.1 Abstract

Ivacaftor, the first CFTR modulator to be licensed for treating patients with CF, is beneficial in treatment of persons with CF (PWCF) with the G551D gating mutation. We report data from the largest single-centre adult prospective study investigating the effects of Ivacaftor on the gut microbiome, pancreatic function, intestinal inflammation and clinical outcomes. The CORK study evaluated efficacy of Ivacaftor in a large (n = 16) adult single centre cohort for a median follow up period of 12 months after initiation of Ivacaftor treatment. Significant improvement in lung function, weight and reduction in sweat chloride were observed in this cohort in keeping with the findings of the clinical trials. A 75% reduction in pulmonary exacerbations requiring intravenous antibiotics was also observed. In the CORK cohort Ivacaftor does not influence gut microbial diversity, pancreatic function or intestinal inflammation. At the phylum, family and genus level we see subtle changes that indicate an altered gut microbiota profile in these patients. This study demonstrates real-world effectiveness of Ivacaftor in terms of improvement in lung function, BMI and reduction in pulmonary exacerbations. Our findings show that Ivacaftor, while life-changing clinically, has subtle effects on the gut microbiota which may reflect a reduction of antibiotic intake.

5.2 Introduction

Cystic Fibrosis (CF) is a multisystem autosomal recessive disorder due to mutation in the gene which codes for cystic fibrosis conductance transmembrane regulator (CFTR) protein (Riordan *et al.*, 1989). CFTR functions as an epithelial channel which plays a key role in regulating chloride transport in multiple organs including the lungs, gastrointestinal tract (GIT), pancreas, liver, reproductive tract and sweat glands. Over 1800 disease causing mutations have been identified which give rise to

a variety of phenotypes (Castellani *et al*., 2008). In the lung CFTR dysfunction results in dehydration of airway secretions, with resultant impairment of mucociliary clearance. Thus PWCF (persons with Cystic Fibrosis) cannot adequately clear their airways of thick secretions and suffer recurrent pulmonary exacerbations requiring both oral (PO) and intravenous (IV) antibiotics. Currently, respiratory compromise is the leading cause of death in PWCF. However, when first described by Dorothy Hansine Andersen in 1938 as cystic fibrosis of the pancreas, gastrointestinal manifestations predominated, and PWCF died of malnutrition and gastrointestinal complications in the first few months of life (Andersen, 1938).

Ivacaftor is the first medication which potentiates CFTR function in the setting of class III (gating) and class IV (conductance) CFTR mutations (Barry *et al*., 2015). Ivacaftor produces significant clinical benefit in patients with CF who carry the G551D mutation, with significant improvement in lung function (FEV_1), increase in BMI, improved respiratory symptoms, reduction in sweat chloride, and reduction in pulmonary exacerbations being reported in the clinical trials (Ramsey *et al*., 2011, Davies *et al*., 2013). Currently the G551D mutation is the most prevalent of the CFTR mutations amenable to treatment with Ivacaftor monotherapy - with a worldwide prevalence of 4-5% (Bobadilla *et al*., 2002). However, there is significant regional variation and prevalence of this mutation in Cork Adult Cystic Fibrosis Centre is 23% (De Boeck *et al*., 2014).

Pilcam studies have demonstrated increased inflammation in the CF gut compared to controls (Werlin *et al*., 2010). The histology of duodenal biopsies in CF patients presents a thickened mucus layer or mononuclear infiltration of the laminar propria (Werlin *et al*., 2010, Freye *et al*., 1964, Thomaidis *et al*., 1963, Eggermont, 1985, Raia *et al*., 2000). This is thought to be a result of increased intestinal

permeability due to almost constant exposure to broad spectrum antibiotics leading to dysregulation of innate immune mediators (Van Elburg *et al*., 1996, Claeys *et al*., 2005). Faecal calprotectin is present in high levels in individuals with intestinal inflammation and has been shown to be elevated in patients with CF compared to controls (Werlin *et al*., 2010, Bruzzese *et al*., 2004). Faecal lactoferrin is also regarded as a maker of gut inflammation and has previously been demonstrated in the CF gut (Kane *et al*., 2003, Lee *et al*., 2012). Previous work has demonstrated a reduction in sputum cytokines after Ivacaftor, thus we aimed to assess change in gut inflammatory markers.

In the pancreas CFTR dysfunction may result in disruption of pancreatic exocrine function giving rise to pancreatic insufficiency. In the pancreas reduced transport of pancreatic secretions containing pancreatic enzymes results in pancreatic auto digestion and resultant pancreatic insufficiency. Prevalence of pancreatic insufficiency is approximately 80-85% and increases with age (Kristidis *et al*., 1992). This may result in poor weight gain, malabsorption of fat and the fat soluble vitamins (Vitamins A, D, E and K), abdominal bloating, or flatulence (Stecenko and Moran, 2010). The KIWI study demonstrated improvements in exocrine pancreatic function (as reflected by faecal elastase) in very young children with CF aged 2-5 treated with Ivacaftor (Davies *et al*., 2016). Equally a recent case report highlighted dynamic changes in faecal elastase in an older child treated with Ivacaftor (Howlett *et al*., 2016). We aimed to assess the impact of Ivacaftor on faecal elastase in a cohort of adults with CF with the G551D mutation.

Emerging evidence, although limited by sample size, on the gut microbiota of patients with CF reveals a unique microbiome with lower species richness, evenness and diversity being reported (Bruzzese *et al*., 2014, Burke *et al*., 2017). There is

evidence to suggest that the gut microbiota may impact on the CF lung given than administration of probiotics to patients with CF has been associated with an improvement in FEV1, a decrease in pulmonary exacerbations, and a decrease in hospital administrations (Weiss *et al*., 2010, Bruzzese *et al*., 2007). This is thought to be as a result of a combination of factors including the CFTR dysfunction, chronic long-term antibiotic use, high fat diet, pancreatic enzyme supplementation and suppression of gastric acid. In addition, recent evidence from Madan *et al*. demonstrated a core lung-gut microbiota dominated by *Veillonella* and *Streptococcus*, with a high degree of concordance between population fluctuations between the two sites (Madan *et al*., 2012). Furthermore, they show certain genera (*Rosburia*, *Dorea*, *Sporacetigenium*, *Coprococcus*, *Blautia*, *Enterococcus* and *Escherichia*) appear in the gut microbiota bacterial populations before being observed in the lung. We aimed to evaluate the impact of Ivacaftor on the CF gut microbiome.

5.3 Materials and Methods

Subjects

Adult patients (age ≥16) attending Cork Adult Cystic Fibrosis centre commencing Ivacaftor were recruited from March 2013 and followed prospectively until October 2014. Sixteen adult Ivacaftor-naive persons with Cystic Fibrosis consented to participate in the current study. They were assessed on a three monthly basis in the routine CF clinic in line with local requirements for monitoring during the first year of treatment with Ivacaftor.

Patient Assessment

Participants attended the Cystic Fibrosis Day Centre when clinically stable and free from pulmonary exacerbation before commencing Ivacaftor, at which point written

informed consent to participate was obtained. Spirometry was performed according to ERS/ATS guidelines using CareFusion MicroLab™ spirometer which is calibrated on a regular basis. Additional clinical parameters including weight, height, sweat chloride, and Cystic Fibrosis Questionnaire Revised (CFQ-R is a CF specific quality of life questionnaire consisting of domains scored from 0 -100 with higher scores indicating that the patient is feeling better) (Quittner *et al*., 2005) were recorded at baseline. Sweat-testing was performed using a Macroduct™ system in accordance with manufacturer's guidelines. This clinical assessment was repeated after initiation of treatment for up to 12 months post-Ivacaftor. Numbers of antibiotic courses (Intravenous (IV) and oral (PO)) for pulmonary exacerbations were documented prospectively for 6 - 12 months after commencement of Ivacaftor and for the same matched period in the previous year depending on the period of patient follow-up. The patients consented to donate a stool sample before commencing Ivacaftor and 12 months post commencement of treatment, when possible. Stool samples were collected in sterile universal containers and frozen at -80°C for later DNA extraction, and analysis of faecal calprotectin, faecal lactoferrin and faecal elastase-1 measurement. Ethical approval was obtained from the Clinical Research Ethics Committee of the Cork Teaching Hospitals.

Evaluation of intestinal inflammation and pancreatic function

Faecal calprotectin, faecal lactoferrin and faecal elastase-1 were evaluated in 14 baseline samples and 14 matched follow up samples 12 months after Ivacaftor treatment. Faecal calprotectin was measured using the BÜHLMANN Calprotectin ELISA for faecal samples (BÜHLMANN, Switzerland). Briefly, the procedure for calprotectin analysis is as follows: faecal calprotectin extraction procedure is followed by a sandwich ELISA which specifically measures the calprotectin-antigen

using a monoclonal capture antibody (mAb) coated to the microtiter plate. Calibrators, controls and patient extracts were incubated for 30 minutes, washed and incubated with a detection antibody conjugated to Horse Radish Peroxidase (HRP). Following a further wash step and addition of tetramethylbenzidine (TMB), absorption was measured at 450nm using the Biotek Synergy 2 microplate reader using the Gen 5 data analysis software (Mason Technologies, Dublin, Ireland). Faecal lactoferrin was measured using the IBD-SCAN TECHLAB ELISA kit (Alere, Galway, Ireland). The microassay wells are coated with immobilised polyclonal antibody against lactoferrin. Lactoferrin in the samples and standards binds the immobilised antibody. Detection uses a polyclonal antibody conjugated to HRP. Absorbance is measured at 450nm. Faecal elastase-1 in stool was measured using the ScheBO Pancreatic Elastase 1 in Stool test kit (Schebo Biotech, Germany). Similarly, this kit employs a short extraction procedure prior to performing a sandwich ELISA. The ELISA plate is coated with a human pancreatic elastase 1 specific mAb which subsequently binds the samples and standards. A complex of monoclonal anti-Elastase 1-Biotin and Peroxidase (POD)-Strepavidin binds to E1 during the next incubation. The peroxidase oxidises ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). The absorbance is measured at 405nm using the Biotek microplate reader.

DNA extraction and high-throughput amplicon sequencing

Stool samples were stored at -80^{o}C for up to 9 months and then thawed on ice, prior to DNA extraction. DNA was purified from thawed faecal samples using the Repeated Bead Beating (RBB) DNA extraction method previously described by Yu and Morrison (2004). This involves a cell lysis step using 0.25g of faecal sample in 1 ml lysis buffer (500 mM NaCl, 50 mM Tris-HCl, Ph 8.0, 50 mM EDTA, and 4%

sodium dodecyl sulphate (SDS)) together with 0.4 g of zirconia beads, followed by homogenisation for 3 minutes using the Biospec Minibeadbeater. The homogenised samples were then heated to 70^{o}C for 15 minutes and centrifuged at 16,000xg for 5 minutes. The supernatant was then removed, fresh lysis buffer added and the bead beating, heating and centrifugation steps repeated. The nucleic acids were precipitated using 10M ammonium acetate, followed by addition of isopropanol. The nucleic acids were then pelleted, washed and resuspended in 1X TE buffer. Removal of RNA, protein and purification of the DNA was completed using components of the QIAGEN QIAamp DNA Stool Mini kit. DNA was subsequently stored at -20^{o}C.

16S r RNA amplicon MiSeq sequencing

The microbiota composition of the samples was established through amplicon sequencing of the commonly used 16S rRNA V3/V4 region according to Illumina's 16S metagenomic library prep guide. Each genomic DNA template was PCRed twice to complete complex library preparation: (1) 16S rRNA gene specific primers with integrated Illumina adaptor overhang nucleotide sequences (Forward Primer 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Reverse Primer = 5'

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) were used to initially isolate the 16S r RNA V3/V4 region, (2) Illumina Nextera XT (Illumina, Sweden) primer pairs were used to add dual indices and Illumina sequencing adaptors to PCR amplicons generated in step one. PCR's were performed using KAPA hifi hot start polymerase ready mix (KAPA Biosystems), purified using AMPure (Labplan, Ireland) and quantified using QUBIT dsDNA

broad range assay system (Invitrogen Life Technologies) prior to pooling the amplicons at a concentration of 4nM each. The pooled library was denatured by addition of 0.2M NaOH followed by heating, diluted to 4pM and combined with an internal control for low diversity libraries (10% PhiX). The prepared pools were then sequenced on the MiSeq platform using the 300bp V3 kit reagents (Illumina, Sweden).

Bioinformatics

Compositional metagenomic 16S rRNA data was obtained for 32 samples (16 samples at baseline and 16 post Ivacaftor treatment using the last available time point). Assembly of 300bp paired end reads was performed using FLASH (fast length adjustment of short reads to improve genome assemblies). Quality filtering, including removal of mismatched barcodes and sequences failing the length requirement, was achieved using QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso *et al*., 2010). USEARCH v7 (64-bit) (Edgar, 2010) was used for removal of background noise and chimeras, along with clustering of operational taxonomic units (OTUs). Alignment of OTUs was performed using PyNAST (Python Nearest Alignment Space Termination tool) (Caporaso *et al*., 2010), followed by taxonomy assignment using BLAST against the SILVA SSURef database release 111. Using phylogenetic trees created by PyNAST, alpha and beta diversities were calculated based on UNIFRAC distance matrices. Subsequent PCoA plots were visualised in EMPEROR v0.9.3-dev.

Quantitative PCR

Quantification of total faecal bacterial load of each subject was performed using qPCR. Samples and standards were analysed in triplicate using the Roche Lightcycler 480 platform and SYBR green reagent (Roche Diagnostics, UK). The

primers, previously described, were 5'-ACTCCTACGGGAGGCAGCAG-3' (Ahn *et al*., 2006) and 5'-ATTACCGCGGCTGCTGG-3' (Muyzer *et al*., 1993). A final concentration of 0.5 pmol of each primer was added to each reaction with a reaction cycle as follows: 95ºC for 5 minutes, 45 cycles of 95ºC for 10 seconds, 60ºC for 30 seconds and 72ºC for 30 seconds, and a final cooling step for 10 seconds. Mean concentrations were then expressed as $\log^{10}$ 16S rRNA copies per gram of faeces.

Statistical analysis

Clinical data was analysed using SPSS Version 22.0 and STATA. Paired Wilcoxon signed rank test was used to analyse change in clinical parameters, inflammatory markers and faecal elastase-1 levels. Change in categorical variables was analysed using Chi squared test. Non parametric Wilcoxon's signed rank test for paired samples was performed on microbiota data in R Studio Version 1.0.136 using R version 3.1.3 (R Core Team, 2016). P-values generated were corrected using Benjamini-Hochberg correction method for multiple testing. Significance was accepted at $p<0.05$. Graphpad Prism 6 was used for generation of graphs.

5.4 Results

Ivacaftor clinically improves the health status of CF patients with G551D mutation

Table 1 summarises participant's baseline characteristics. All patients were pancreatic insufficient and had a class I – III CFTR mutation as their second mutation. A larger percentage of patients were male (64.3%). Mean baseline FEV_1 was 65.5%, which is similar to baseline lung function in the phase III clinical trial. Mean baseline Sweat Chloride was high at 98 mmol/l. Significant improvements in FEV_1 % predicted ($p<0.01$), BMI ($p<0.01$), sweat chloride ($p<0.01$) were observed after Ivacaftor. Table 2 summarizes the changes in clinical parameters post-Ivacaftor. There was no statistically significant change in the CFQ-R eating domain

(p= 0.6), Body Image Domain (p=0.2) or Digestive Domain (p=0.72). A significant improvement in CFQ-R weight domain was observed after Ivacaftor (p=0.02). A significant 75% reduction in intravenous antibiotics requirements was observed post treatment. Table 3 details the number of exacerbations requiring intravenous antibiotics and the specific antibiotics used.

Evaluation of faecal calprotectin, lactoferrin and FE-1

There was no significant difference between the median faecal calprotectin quantified in pre-Ivacaftor (293.0 μg/g) and post-Ivacaftor (164.6 μg/g) treated patients (p=0.22, n=14) (Figure 1A). Corresponding with faecal calprotectin measurements, there was no significant difference between the median lactoferrin quantified in pre-Ivacaftor (49.6 μg/g) and post-Ivacaftor (33.1 μg/g) treated patients (n=14 p=0.27) (Figure 1B). FE-1 was measured in a CF cohort treated with Ivacaftor over a 12-month period, with a baseline faecal sample measured as a control. All subjects pre and post-Ivacaftor showed a faecal elastase concentration <100 μg FE-1/g of faeces, indicating severe pancreatic insufficiency. Statistical analysis showed no significant change (p=0.61) of FE-1 in response to treatment with Ivacaftor from baseline to post-Ivacaftor (n=14) (Figure 1C). This indicates no improvement of pancreatic function in response to Ivacaftor treatment after a median a median of 12 months.

The GIT microbial diversity in the CORK cohort is unaffected by Ivacaftor treatment

An average of 199,177 (± 75294) 16S rRNA reads were generated from faecal samples in this study. Rarefraction curves using Simpson, Chao1, Shannon and Phylogenetic whole-tree diversity measures established that extra sampling would have been unnecessary. β diversity, visualised using a PCoA of weighted UNIFRAC distances, demonstrated no clustering of treatment groups (Figure 2). Samples tended

to cluster by subject, irrespective of Ivacaftor treatment status. There was no significant difference in α diversity between pre and post Ivacaftor treated samples according to Chao1 (p=0.6603), Simpson (p=0.3655), Shannon (p=0.4534) parameters, phylogenetic diversity_whole tree (p=0.7761) or observed species (p=0.5872).

Proportions of several taxa are altered in the gut microbiota pre-Ivacaftor relative to post-Ivacaftor treatment

Reads corresponding to 12 phyla, 61 families and 154 genera were detected in samples both before and after commencement of Ivacaftor treatment. Following Ivacaftor treatment, phylum-level analyses showed no significant changes in any of the 12 phyla detected in the CORK samples. Figure 3 demonstrates that changes are occurring in the ratios of Firmicutes and Bacteroidetes at phylum level, however these changes are not significant. Firmicutes are the dominant phyla in samples before and after Ivacaftor, changing from 75% pre-Ivacaftor to 62% post-Ivacaftor. Bacteroidetes changed from 23% in pre to 34% in post Ivacaftor treated groups. Proteobacteria displayed stability, with approximately 1% in both groups. At the family level we note a non-significant increase in Bacteroidaceae (16%-27.5%) and parallel non-significant decreases in Enterococcaceae (3.2% -0.8%), Prevotellaceae (4.7%-2.1%) and Streptococcacae (7.3%-2.2%) post treatment (Figure 4). At genus level we saw a significant increase of Parabacteroides from 0.7%-2.1% (p=0.035). In addition, we observed non-significant increases in *Bacteroides* (16.5%-25%) and *Veillonella* (3%-6%) and parallel non significant decreases in *Prevotella* (4.7%-2.1%), *Enterococcus* (3%-0.8%), *Streptococcus* (7.2%-2.2%), *Blautia* (5%-3.3%) and *Escherichia* (3.3%-1.2%) (Figure 5). There was no significant difference in the

total bacterial load measured by qPCR, expressed as log^{10} per gram of faeces, between pre- and post-Ivacaftor treated groups (p= 0.7746).

5.4 Discussion

Ivacaftor is the first therapy to become available which modulates CFTR function. This is the largest investigation of the modulatory effects of Ivacaftor on the gut microbiome in an adult CF cohort. Given the relatively low prevalence of gating mutations worldwide, the high prevalence of the G551D mutation in the Cork Adult CF Centre, offers a unique opportunity to evaluate the impact of potentiating CFTR function on clinical parameters, gut microbiome, markers of GI inflammation and pancreatic exocrine function in a single centre cohort. The response of the gut microbiota and intestinal health to Ivacaftor administration has been unexplored to date. Intestinal health, as described by markers of intestinal inflammation (faecal calprotectin and faecal lactoferrin) and pancreatic function (faecal elastase-1), is not significantly impacted by Ivacaftor and does not exhibit restoration. In addition, findings suggest Ivacaftor has subtle effects on the gut microbiota despite its significant clinical benefit. Furthermore, the recovery of the gut microbiota in the event of antibiotic reduction following a sustained period of chronic antibiotic use is slow and subtle at phylum, family and genus levels.

This cohort experienced a significant improvement in lung function of a magnitude similar to that observed in the clinical trials, but larger than that reported in previous real world studies. Similarly mean increase in weight and BMI were of a similar magnitude to those observed in the phase III clinical trial, thus confirming the efficacy of Ivacaftor in a real world setting. Nearly half of this cohort had at least one course of Intravenous antibiotics in the year pre-Ivacaftor, thus they represent a cohort with chronic antibiotic exposure. A significant reduction (75%) in IV

antibiotics use was observed after 12 months of Ivacaftor. While no overall change in the gut microbiome was observed after 1 year of Ivacaftor treatment, it is possible that given the long duration of exposure to repeated courses of oral, intravenous and nebulised antibiotic usage a longer period of follow up may help to delineate the impact of modulating CFTR and resultant reduction in antibiotic usage on the CF gut microbiota.

Although faecal elastase-1 levels here are consistent with expectations of a CF cohort presenting with pancreatic insufficiency, they are significantly lower than what other groups have quoted. Walkowiak and colleagues (2001) found a median of 10μg FE-1/g faeces in a mostly paediatric cohort, while this study shows a median of 0.02 FE-1μg/g faeces. Recent case reports have raised the possibility of a restoration of pancreatic function in patients treated with Ivacaftor (Howlett *et al*., 2016). Similarly, the KIWI study which is investigating the use of Ivacaftor in children aged < 5 years suggest that Ivacaftor treatment is associated with improved pancreatic function as reflected by improvement in faecal elastase-1 levels (Davies *et al*., 2016). In contrast we observed no significant change in faecal elastase-1 in an adult cohort post Ivacaftor. This may reflect established pancreatic damage which is irreversible in an adult cohort, such as the CORK cohort (median age 27years, +/- 5.4 years) and would support a hypothesis that early Ivacaftor in children with the G551D mutation may maintain or enhance pancreatic function. Notwithstanding the lack of an enhancement of exocrine pancreatic function a significant weight gain was observed in this cohort. Thus it appears that other mechanisms underlie this weight gain.

In this study we looked at intestinal inflammation levels in a CF cohort prior to and during treatment with Ivacaftor, using faecal calprotectin and faecal

lactoferrin as biomarkers. Intestinal levels of both calprotectin and lactoferrin were consistent with expectations for a CF cohort. However, no significant change in intestinal inflammation was seen following treatment with Ivacaftor. Previous work in children with CF demonstrated a reduction in faecal calprotectin levels after treatment with a probiotic in the form of *Lactobacillus rhamnosus* (Bruzzese *et al*., 2014). It has been suggested that there may be a relationship between the intestinal microbiota composition and inflammation. The lack of a significant change in the gut microbiota in this study would be in keeping with this hypothesis in CF. A small study in ten patients treated with Ivacaftor demonstrated an improvement in gastrointestinal pH but no change in gastro-intestinal motility with restoration of CFTR function (Gelfond *et al*., 2017). However, despite the ability of Ivacaftor to improve gut pH no effect was noted on intestinal inflammation.

Gut microbiota diversity is an important indicator of the health of the host. Here, we saw individuals distinct from each other, with the individual microbiota dictating clustering above any other clinical features or treatments. Indeed, at phylum, family and genus levels the gut microbiota shows more similarity between individuals than between different persons in the same treatment group. A hallmark of individuals on antibiotic therapy (Jakobsson *et al*., 2010), and indeed CF patients (Duytschaever *et al*., 2013), is higher inter individual variation but low intra-individual diversity. Alpha diversity indices demonstrated that 12 months Ivacaftor therapy does not influence gut microbiota diversity. We hypothesise that an increase of gut microbiota diversity following years of chronic antibiotic therapy will be slow.

In depth compositional analysis using the MiSeq Illumina platform revealed a subtly altered gut microbiota following Ivacaftor treatment at the phylum, family and genus levels. At phylum level, a trend towards an increase in Bacteroidetes with a concurrent decrease of Firmicutes was seen (although both non-significant), most probably owing to the decrease in antibiotic intake. The decrease in Bacteroidetes following antibiotic therapy is well reported in the literature (Antonopoulos *et al*., 2009, Fouhy *et al*., 2012, Perez-Cobas *et al*., 2013). Here, we demonstrated the recovery of Bacteroidetes phyla in the event of a reduction in antibiotic intake. Previous work has demonstrated a reduction in the relative abundance of Bacteroidetes and increase in Firmicutes and in adults with CF compared to a control cohort at a phylum level (Burke *et al*., 2017). Interestingly patients with CF with the highest intravenous antibiotic requirements had the lowest Bacteroidetes and highest Firmicutes. We observed an increase in Bacteroidetes and reduction in Firmicutes after Ivacaftor treatment which was during a period in which IV antibiotic consumption decreased by 75% and thus this may represent the effects of reduced antibiotic usage.

At family and genus level increases in beneficial taxa, such as Bacteroidaceae, *Bacteroides* and *Parabacteroides* and decreases in more pathogenic taxa, such as Enterococcaceae, *Enterococcus*, Streptococcaceae, *Streptococcus*, *Blautia*, and *Escherichia* are observed, demonstrating a subtly improved gut microbiota. Taken together, these changes, while not significant, may be indicative of movement of the gut microbiota towards a healthier composition. However, subjects will need to be monitored prospectively for a longer period post-Ivacaftor in order to elucidate whether the gut microbiota composition will continue to improve. We also report a significant increase of Parabacteroides post-Ivacaftor. Interestingly,

decreases in intestinal *Parabacteroides* have been observed in paediatric CF cohorts prior to the onset of pulmonary exacerbation caused by *Pseudomonas* colonisation (Hoen *et al*., 2015). The increase in *Parabacteroides* post- Ivacaftor which parallels a decrease pulmonary exacerbations suggests that *Parabacteroides* may be intrinsically linked to respiratory health status.

The chemical structure of Ivacaftor contains a quinolone ring. Reznikov and colleagues (2014) have shown Ivacaftor to have antibacterial properties against *Staphylococcus aureus*, similar to vancomycin. In addition, they demonstrated the synergistic effects of Ivacaftor in combination with vancomycin against *S. aureus*. Ivacaftor has been identified at the highest levels of exposure in gastrointestinal tissue and the majority (88%) is eliminated through the faeces (Condren *et al*., 2013), where it is metabolised to M1 and M6. M1 has 1/6 of the potency of Ivacaftor and is considered pharmacologically active, while M6 has 1/50 of the activity of Ivacaftor, retaining none of its pharmacological activity (Wainwright *et al*., 2014). It is likely that the M1 metabolite exerts an antimicrobial effect, similar to quinolone antibiotics, on the gut microbiota. Quinolone antibiotics have shown activity against Ruminococcaceae, Lachnospiraceae, Enterobacteriaceae, *Haemophilus* and bifidobacteria (Dethlefsen *et al*., 2008, Perez-Cobas *et al*., 2013, Edlund *et al*., 2000) and are known to exert a limited ecological disturbance in the intestinal microbiota due to a more targeted effect (Lidbeck *et al*., 1988). In our cohort we saw reduction of Enterobacteriaceae, although not significant, from pre- to post-Ivacaftor. Although this is a signature of the quinolone group of antibiotics on the gut microbiota, we cannot say if the reductions we see here are a result specifically of the pharmacological activity of the M1 metabolite or a combination of a range of other factors.

Conclusions and study limitations

In conclusion, the response of the gut microbiota to Ivacaftor is subtle with changes seen at the phylum, family and genus level. This study also addresses the paucity of knowledge regarding long-term chronic antibiotic use and recovery of the gut microbiota following reduction of antibiotic intake, as observed in this cohort. There was no significant change in exocrine pancreatic function in an older cohort of patients with CF after commencement of Ivacaftor suggesting that it does not restore pancreatic function. This may reflect established long standing pancreatic destruction which is irreversible. There was no difference seen in the levels of intestinal inflammation post Ivacaftor suggesting chronic inflammation may be established in this cohort. A limitation of this study is the small sample size (n=16) and the lack of a control cohort. However, given that the global prevalence of the G551D mutation is 4-5% this represents a relatively large sample size.

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Table 1. Baseline clinical characteristics of the CF subjects.

Median Age (range)	29 (18-39)
Gender (% Male)	64.3%
Genotype	
G551D/F508del	78.58%
G551D/G551D	7.14%
G551D/R53X	7.14%
G551D/3028delA	7.14%
FEV_1 % predicted median (range)	65.5 (31-91)
BMI kg/m^2 median (range)	22.05 (18.2-29.5)

Table 2. Changes in clinical characteristics post-Ivacaftor. Values given are median and the range of each parameter is in brackets.

	Pre	**Post**	**P value**
FEV_1 % predicted	65.5 (31-91)	73.5 (44.5 – 106.5)	<0.01
Sweat Chloride (mmol/l)	98 (81-155)	43.8 (26.5 – 97)	<0.01
BMI (kg/m^2)	22.05 (18.2-29.5)	22.5 (19.9 – 30.3)	<0.01
CFQ-R			
Eating	100 (77.8-100)	100 (83.5-100)	0.6
Body Image	77.8 (11.1-100)	83.35 (11.1-100)	0.2
Digestive	88.9 (44.4-100)	90.35-100)	0.72
Weight	66.7 (0-100)	100 (33.3-100)	0.02

Table 3. Clinical characteristics and I.V (Intravenous) antibiotic intake details of subjects included in this study.

			No. Exacerbations requiring I.V antibiotic		I.V antibiotic	
Subject ID.	Gender	Age	1 year Pre	1 year Post	1 year Pre	1 year Post
108	M	39	0	0	0	0
148	M	29	0	0	0	0
288	F	30	1	0	Ceftazidime, Colomycin	0
299	F	30	2	1	Ceftazidime, Colomycin, Meropenem, Colomycin	Meropenem, Colomycin
307	F	n/a	0	0	0	0
319	m	25	1	0	Ceftazidime, colomycin	0
141	f	30	3	0	n/a	0
181	f	24	0	0	0	0
193	m	27	0	0	0	0
194	m	22	0	0	0	0
201	m	20	4	0	n/a	0
246	m	27	0	0	0	0
257	m	34	5	1	n/a	n/a
294	m	29	0	0	0	0
333	m	30	2	2	2 courses Meropenem, Tobramycin	2 courses Meropenem, Tobramycin
372	m	19	1	0	Ceftazadime, Tobramycin	0

n/a data not available.

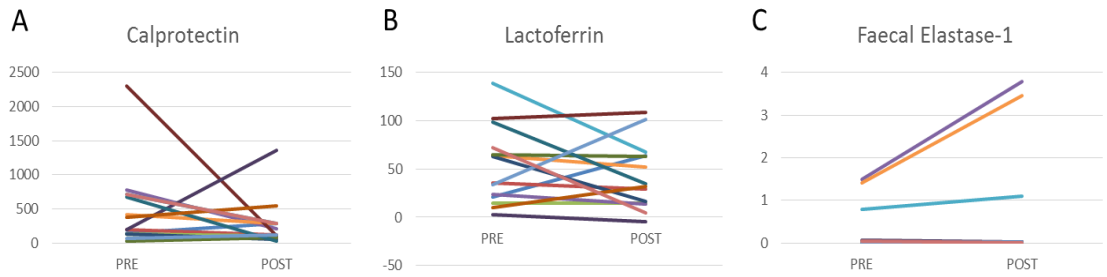


Figure 1. Calprotectin (A), Lactoferrin (B) and Faecal Elastase-1 (C) in pre and post-Ivacaftor treated subjects (n=14). Each line corresponds to data for each subject in pre and post-Ivacaftor.

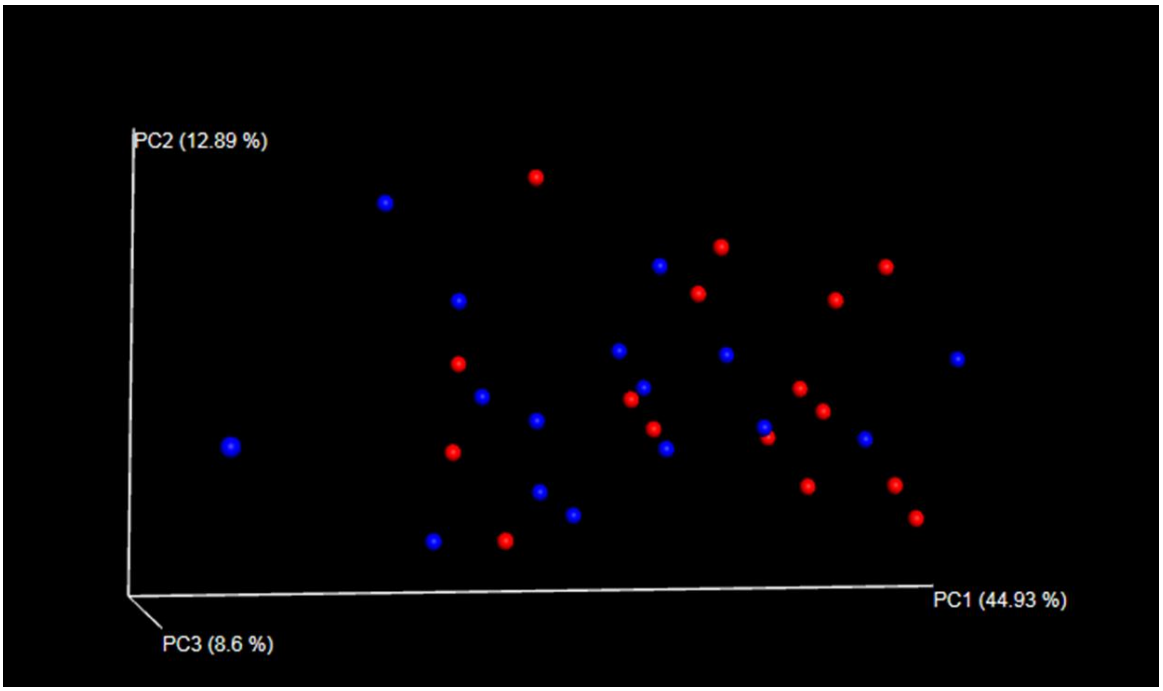
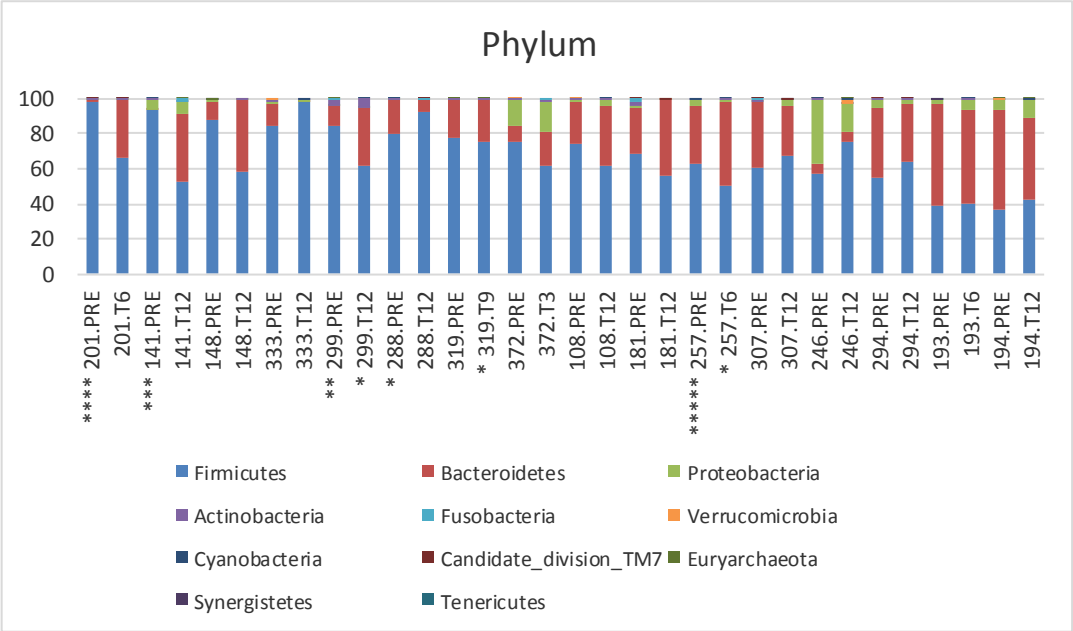
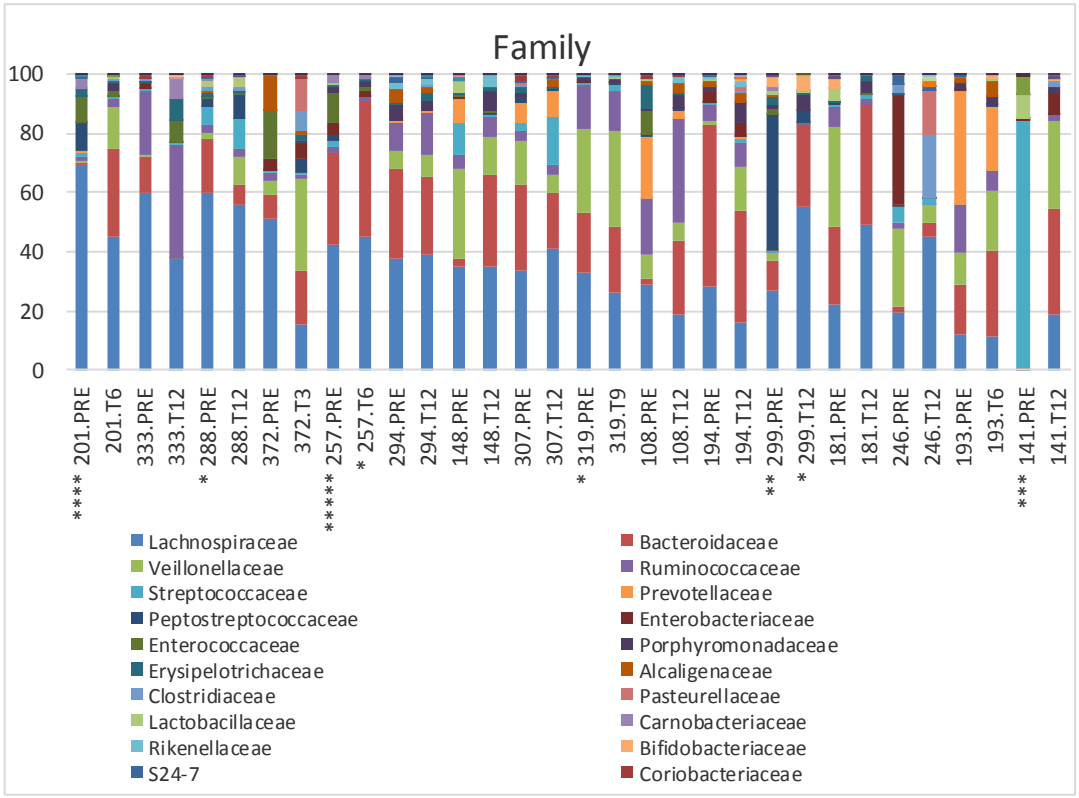


Figure 2. PCoA based on weighted UNIFRAC distances matrices of subjects pre and post-Ivacaftor treatment (Red=PRE, Blue= POST, n=16).



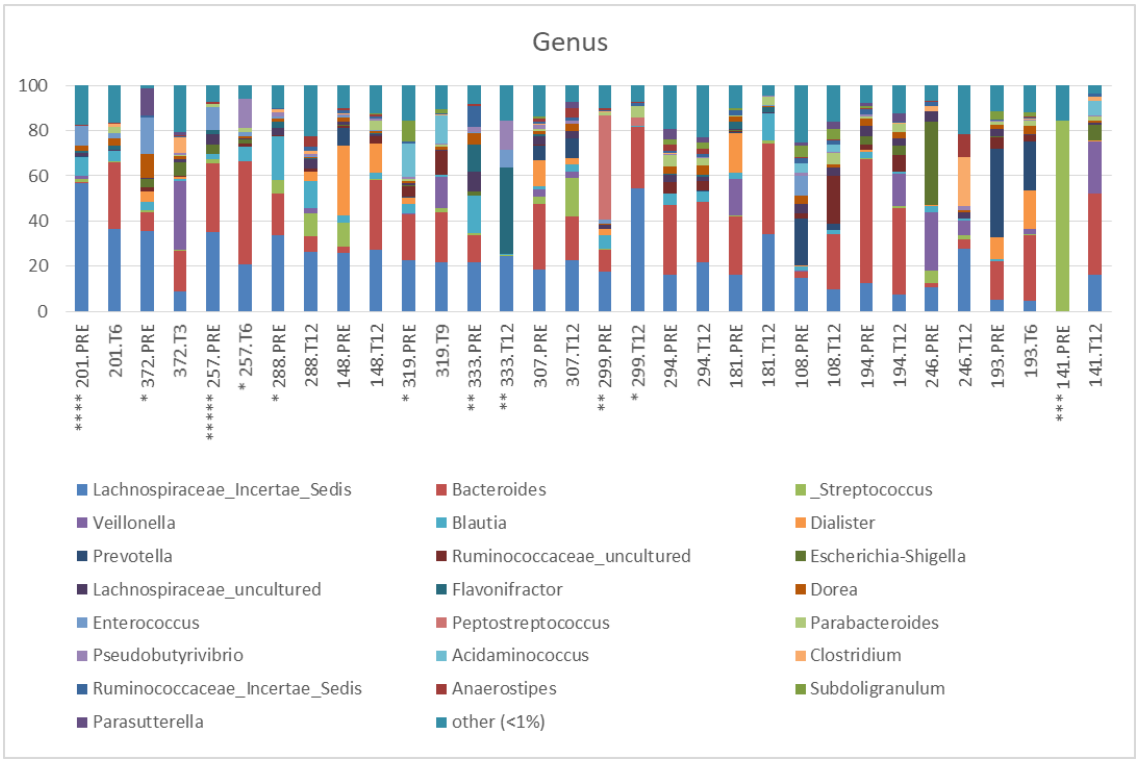
* Pulmonary exacerbation requiring IV antibiotic course

Figure 3. Relative abundance (%) of phylum taxa in pre and post-Ivacaftor treated subjects (n=16). PRE= pre-Ivacaftor, T3= 3 months post-Ivacaftor, T6= 6 months post-Ivacaftor, T9= 9 months post-Ivacaftor, T12= 12 months post-Ivacaftor.



* Pulmonary exacerbation requiring IV antibiotic course

Figure 4. Relative abundance (%) of the most dominant family level taxa in pre and post-Ivacaftor treated subjects. Taxa at <1% are presented together as 'other'. PRE= pre-Ivacaftor, T3= 3 months post-Ivacaftor, T6= 6 months post-Ivacaftor, T9= 9 months post-Ivacaftor, T12= 12 months post-Ivacaftor.



* Pulmonary exacerbation requiring IV antibiotic course

Figure 5. Relative abundance (%) of the most dominant genus level taxa in pre and post-Ivacaftor treated subjects (n=16). Taxa at <1% are presented together as 'other'. PRE= pre-Ivacaftor, T3= 3 months post-Ivacaftor, T6= 6 months post-Ivacaftor, T9= 9 months post-Ivacaftor, T12= 12 months post-Ivacaftor.

Chapter 6

Clinical Outcomes of Real-World Kalydeco (CORK) study: An analysis of the impact of CFTR potentiation on the functional metagenome and metabolome after 12 months

6.1 Abstract

In this study we investigated the impact of CFTR potentiation on the intestinal microbiome and metabolome 12 months post commencement of Ivacaftor therapy. Despite subtle differences in both the intestinal microbiome and metabolome following Ivacaftor treatment, high level functional categories remained unaffected. However, significant differences in the microbiome were observed in pathway abundances relating to protein metabolism, carbohydrate metabolism and vitamin synthesis. In addition, we investigated the effect of Ivacaftor treatment and the consequential reduction of antibiotic therapy on the antibiotic resistance capabilities of the gut microbiota. Although not significant, there was a reduction in the total numbers of antibiotic resistance genes post-Ivacaftor. We suggest that a larger cohort may need to be monitored for a longer period than 12 months for normalization of the intestinal metagenome and metabolome to be observed.

6.2 Introduction

Cystic Fibrosis (CF) is multisystem disorder resulting from mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene (Riordan *et al*., 1989). CF is characterized primarily by respiratory symptoms, most notably, reoccurring pulmonary exacerbations caused by poor mucociliary clearance. Gastrointestinal manifestations are the most prominent extra pulmonary complications of CF, with CF persons presenting with Distal Intestinal Obstruction Syndrome (DIOS) (10-47%) (Jensen, 1962, Rosenstein and Langbaum, 1983), pancreatic insufficiency (80-90%) (Kristidis *et al*., 1992), gastrointestinal inflammation (Werlin *et al*., 2010, Bruzzese *et al*., 2004) and CF-related Diabetes (CFRD) (prevalence may reach 30%) (Mackie *et al*., 2003). In addition, recent studies have shown that CF persons have an altered gut microbiota, both in

composition and functionality, compared to healthy controls (Duytschaever *et al*., 2011, Scanlan *et al*., 2012, Duytschaever *et al*., 2013, Debyser *et al*., 2015). Ivacaftor, a CFTR potentiator, has shown clinical benefits in CF persons with class III mutations, of which G551D is the most common, occurring at 4-5% internationally and 21% in the cohort from the Cork adult Cystic Fibrosis Centre (Bobadilla *et al*., 2004, De Boeck *et al*., 2014). Clinical trials have reported significant improvements in lung function (FEV_1), BMI, reduction in sweat chloride and in pulmonary exacerbations (Ramsey *et al*., 2011, Davies *et al*., 2013). Furthermore, the gastrointestinal specific effects of Ivacaftor therapy have been seen in the significant increase of intestinal pH to levels comparable with a non-CF cohort within one month of commencing treatment (Gelfond *et al*., 2017). Available in Ireland since March 2013, Ivacaftor potentiates the CFTR protein function by increasing the probability of the gating channel being in an open state.

While the gut microbiota composition has been extensively studied in many disease cohorts, explorations of the functional capabilities have to date been less common. Previously we have shown subtle changes in the gut microbiota in pre- and post-Ivacaftor treated persons (unpublished). In this study, we investigated the impact of CFTR potentiation on the gut microbiota functional metagenome and metabolome following 12 months of Ivacaftor therapy. Due to changes in the intake of antibiotics post-Ivacaftor treatment we were particularly interested in investigating if the antibiotic resistance capabilities of the gut microbiome had changed.

6.3 Materials and Methods

Subjects and sample collection

Adult persons (n=6) (age ≥16) attending Cork Adult Cystic Fibrosis centre commencing Ivacaftor were recruited from March 2013 until October 2014. Participants enrolled in the study were clinically stable and free from pulmonary exacerbation prior to commencing Ivacaftor, at which point written informed consent to participate was obtained. Persons were assessed pre and post Ivacaftor treatment and faecal samples were collected in sterile universal containers and stored at -80^{o}C for later DNA extraction and faecal water preparation.

DNA extraction and preparation of metagenomic libraries

Stool samples were stored at -80^{o}C for up to 9 months and then thawed on ice, prior to DNA extraction. DNA was purified from thawed faecal samples using the Repeated Bead Beating (RBB) DNA extraction method previously described (Yu and Morrison, 2004, Fouhy *et al*., 2015). This involves a cell lysis step using 0.25g of faecal sample in 1 ml lysis buffer (500 mM NaCl, 50 mM Tris-HCl, Ph 8.0, 50 mM EDTA, and 4% sodium dodecyl sulphate (SDS)) together with 0.4g of zirconia beads, followed by homogenisation for 3 minutes using the Biospec Minibeadbeater. The homogenised samples were then heated to 70^{o}C for 15 minutes followed by centrifugation at 16,000 x g for 5 minutes. The supernatant was removed, fresh lysis buffer was added and the beadbeating, heating and centrifugation steps were repeated. The nucleic acids were then precipitated using 10 M ammonium acetate, followed by addition of isopropanol. The nucleic acids were pelleted, washed and resuspended in 1X TE buffer. Removal of RNA, protein and purification of the DNA was completed using components of the QIAGEN QIAamp DNA Stool Mini kit. DNA was subsequently stored at -20^{o}C. Genomic DNA samples were prepared for

metagenomic sequencing using the Illumina Nextera DNA sample preparation kit as per protocol provided by Illumina (Illumina, San Diego, California, USA). Briefly, the Nextera kit uses an engineered transposome to fragment and tag input genomic DNA, adding adaptor sequences during the process. These adaptor sequences are then used to amplify the insert DNA during a limited cycle PCR. Indices were also added at both ends of the DNA fragments, allowing dual-indexed sequencing of pooled libraries on the Illumina Sequencing system. Following preparation library pools were then sequenced on Illumina's HiSeq 2500 (Beckman Coulter, New Jersey, USA) using v4 chemistry and 2 x 125bp read type.

Bioinformatic analysis of the shotgun metagenomic data

The quality of raw sequence data was checked using FastQC software version 0.11.4 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The ends of both forward and reverse reads were trimmed using the FASTX trimmer tool (http://hannonlab.cshl.edu/fastx_toolkit/). Following trimming of the reads, the forward and reverse reads were paired and PhiX internal control DNA was removed. Microbial composition profile was generated using BOWTIE2 version 2.2.6 (Langmead *et al*., 2012). Trimmed, assembled FastQ files containing contigs were uploaded to MG-RAST metagenomic analysis server to generate a functional and compositional microbial profile (Meyer *et al*., 2008). Functional profiles were generated based on both the COG and SEED databases. Additionally, HUMAnN2 (Abubucker *et al*., 2012) was used to assign function to reads based on UniRef (Suzek *et al*., 2007). The HUMAnN 2 gene abundance table was then regrouped into |BP|03|cellular amino acid metabolic processes, |BP|03|carbohydrate metabolic processes, |BP|03|lipid metabolic processes and |BP|03|xenobiotics metabolic processes based on GO terms. To assess the antibiotic resistance capabilities of the

gut microbiota pre- and post-Ivacaftor, trimmed FastQ files were compared to the Comprehensive Antibiotic Resistance Database (CARD) (Jia *et al*., 2017) using a local blastp tool. A 98% cut-off was considered acceptable for alignments. Procrustes and coinertia analyses was used to compare the metagenome and metabolome datasets. Procrustes was performed using 'vegan' package version 2.3-1 (Oksanen *et al*., 2007). Coinertia was performed using 'ade4' package version 1.7-2 (Dray and Dufour, 2007).

Sequence data availability

Sequences have been uploaded to the MG-RAST server under the following accession numbers: 4693382.3, 4683377.3, 4683379.3, 4683378.3, 4683375.3, 4683380.3, 4683376.3, 4683810.3, 4683381.3, 4684022.3, 4683371.3, 4683372.3, corresponding to 108 pre, 148 pre, 288 pre, 299 pre, 307 pre, 319 pre, 108 post, 148 post, 288 post, 299 post, 307 post and 319 post, respectively.

Quantitative PCR

The total faecal bacterial load of each subject was quantified in triplicate by qPCR using the Roche Lightcycler 480 instrument and SYBR green reagent (Roche Diagnostics, UK). Primers used were 5'-ACTCCTACGGGAGGCAGCAG-3' (Ahn *et al*., 2006) and 5'-ATTACCGCGGCTGCTGG-3' (Muyzer *et al*., 1993). A final concentration of 0.5 pmol of each primer was added to each reaction mix and qPCR was performed as follows: 95ºC x 5 minutes, 45 cycles x 95ºC 10 seconds, 60ºC 30 seconds, 72ºC 30 seconds, cooling for 10 seconds. Mean concentrations were then expressed as $\log^{10}$ 16S rDNA copies per gram of faeces.

Preparation of faecal water for metabolomic analysis

Faecal water was prepared from thawed stool samples that had been stored at -80ºC. Briefly, 200 mg of faecal sample was homogenised with 800 µl of sterile nuclease

free water (Qiagen, Dusseldorf, Germany) in a sterile 1.5 ml Eppendorf tube and centrifuged at 16000 x g for 30 minutes. The supernatant was removed and transferred to a new tube and centrifugation repeated twice. Finally, the supernatant was removed and added to a Corning Costar Spin-X tube with 0.22 µm filter. The filtrate was stored at -20^{o}C for subsequent metabolomics analysis. Metabolite analysis using Gas Chromatography-Mass Spectrometry (GC-MS) was carried out by MS-omics (Copenhagen, Denmark). Briefly, samples were initially derivatized using methyl chloroformate (MCF), which converts amino and non-amino organic acids into carbamates and esters. Samples, including a quality control sample, were then analysed by GC-MS. The GC-MS data was processed using the PARAFAC2 model, compounds extracted from the read-out and redundant peaks were removed.

Statistical analysis

Statistical analyses were carried out in R Studio Version 1.0.136 using R version 3.1.3 (R Core Team, 2016). Non parametric Wilcoxon's signed rank test for paired samples was performed on metabolome and microbiome data. P-values generated were corrected using Benjamini-Hochberg correction method for multiple testing. Significance was accepted at $p<0.05$. Graphpad Prism 5 was used for generation of graphs.

6.4 Results

Subject clinical characteristics

Six adult persons (median age 30 years, 1:1 Male: Female) were analyzed before and 12 months after Ivacaftor therapy commenced, or nearest available time point, as part of this sub-study; subject 319 provided a stool sample for analysis at 9 months post Ivacaftor commencement. The six subjects were selected to represent a range of responses to Ivacaftor from low responders to high responders, defined in terms of

FEV_1. The median FEV_1 pre-Ivacaftor was 73% (ranging from 31-86%). The median FEV_1 post-Ivacaftor was 74.5% (ranging from 45-96%). Median BMI ranged from 22.75- 22.85 pre- and post-Ivacaftor, respectively. Median numbers of pulmonary exacerbations requiring IV antibiotics ranged from 1-0 pre- and post-Ivacaftor commencement, respectively. Table 1 details the clinical characteristics of the subjects.

Impact of Ivacaftor on the intestinal microbiome

A total of 504,206,468 (504.2 million) amplicon reads were generated from the faecal samples of persons attending the Cork Adult Cystic Fibrosis Centre, with an average of 42,017,205 ($\pm$11,119,617) reads per sample. β-diversity, visualised using HUMAaN2 pathway abundances, showed clustering of samples based on individual rather than treatment status of subject with Ivacaftor when analysing the functional metagenome (Figure 1). Statistical analysis of 28 level 1 subsystem functional capabilities in MG-RAST, representing all the functional categories of the metagenomes showed no significant differences between pre- and post-Ivacaftor for any categories (Supplementary information Figure 1). Analysis of functional pathways using HUMAnN2 software showed significant reduction of gene pathway abundances post-Ivacaftor involved in valine biosynthesis (VALSYN-PWY, p=0.028), sulphate reduction (SO4ASSIM-PWY, p=0.025), sulphate assimilation and cysteine biosynthesis (SULFATE-CYS-PWY, p=0.056), aerobic respiration (PWY-7297, p=0.028), acetylene degradation (P161-PWY, p=0.026) and rhamnose biosynthesis (DTDPRHAMSYN, p=0.028). Significant increases post-Ivacaftor were observed in pathway abundance involved in phosphopantothenate biosynthesis (PANTO-PWY, p=0.041) (Table 2).

Biological processes involved in lipid, carbohydrate and protein metabolism and antibiotic catabolism were examined to identify changes post Ivacaftor. Gene Ontology terms involved in lipid (p=0.394), carbohydrate GO:0005975: [BP] (p=0.589) and xenobiotic GO:0017001: [BP] (p=0.937) metabolism showed no significant change post Ivacaftor (Figure 2). Gene ontology terms involved in protein metabolism were seen to increase post-Ivacaftor GO:0006520: [BP] (p=0.065). We noted subject 299 behaves differently to other subjects in terms of lipid, amino acid and carbohydrate metabolism, reflecting differences in the clinical characteristics in subject 299 from the other subjects.

Antibiotic resistance capabilities in the gut microbiome of pre- and post-Ivacaftor subjects

To mine further into the gut microbiome we looked at the antibiotic resistance capabilities of the microbiota in pre- and post-Ivacaftor treated subjects. Although not significant the mean total hits for resistance genes was lower post-Ivacaftor (pre-454.3$\pm$29.7, post 365.8. $\pm$77.4, p= 0.156). The number of antibiotic resistance gene hits found in each species were compared pre- and post-Ivacaftor, showing no statistical significant difference. *Escherichia coli* was found to carry the greatest burden of antibiotic resistance genes in pre-Ivacaftor while *Bacteroides fragilis* carried the most resistance genes post-Ivacaftor. *Bifidobacterium longum* was observed carrying high levels of antibiotic resistance genes in both pre- and post-Ivacaftor treated subjects. Together with *Escherichia coli* and *Bacteroides fragilis*, the following microorganisms, *Enterococcus faecalis*, *Bifidobacterium longum*, *Campylobacter jejuni* and *Enterococcus faecium* account for 264 and 209 antibiotic resistance gene hits pre and post Ivacaftor, respectively. Figure 3 shows the total antibiotic resistance hits found in pre compared to post Ivacaftor treated subjects and

the contributions of each species to the potential resistance within the gut. Antibiotic resistance in *Escherichia coli* is dominated by aminoglycoside resistance (APH(2")-IIa phosphorylation genes), (ACC(6')-lp acetylation genes), sulphonamide resistance genes (*sul*2) and *rpo*B rifampicin resistance genes. *Bacteroides fragilis* is dominated by tetracycline resistance genes *tet*Q, macrolide-lincosamide-streptogramin resistance protein ermF and tetracycline resistance gene *tet*X. *Enterococcus faecalis* is dominated by aminoglycoside bifunctional (acetylation and phosphorylation) resistance protein AAC(6')-APH(2'), followed by dihyrofolate reductase gene *dfr*G (trimethoprim resistance), *lsa*A (lincosamide and streptogramin resistance) and *lia*F (daptomycin resistance). *Bifidobacterium longum* carried only tetracycline resistance genes (*tet*W). *Campylobacter jejuni* was dominated by *tet*O tetracycline resistance genes. *Enterococcus faecium* was dominated by APH (2")-IIa aminoglycoside resistance protein.

An overall survey of the mechanisms of antibiotic resistance shows a dominance of tetracycline resistance followed by aminoglycoside, Macrolide/Lincosamide/Streptogramin beta-lactamase resistance genes and Multidrug resistance genes (Table 3). A reduction in resistance is observed in the major antibiotic classes from pre to post Ivacaftor including Tetracycline, Aminoglycoside, Macrolide/Stretogramin/Lincosamide, Multidrug, Trimethoprim and Fluoroquinolones, however this reduction is not significant.

Compositional analysis of microbiota pre and post-Ivacaftor

Alpha diversity analyses showed no significant difference in evenness or richness of the gut microbiota community composition between pre and post Ivacaftor subjects. The composition of the gut microbiota in pre and post Ivacaftor samples was analysed at phylum, genus and species levels. At phylum level we saw no significant

changes in the relative abundance of the dominant phyla pre compared to post-Ivacaftor. The gut microbiota in both pre and post Ivacaftor is dominated by Firmicutes (pre- 65.7% and post- 59.2%), followed by Bacteroidetes (pre- 27.3% and post-34.9%) (Figure 4). Although not significant, we noted a decrease in the Firmicutes and an increase in Bacteroidetes post-Ivacaftor. Proteobacteria and Actinobacteria remained constant in both pre- and post-Ivacaftor levels at 1.7% and 1.2%, respectively. Both pre- and post-Ivacaftor treated samples were dominated by *Bacteroides* (22% and 32%, respectively). Interestingly, we also note increases in the relative abundance, although not statistically significant, of *Parabacteroides* (133%) *Eubacterium* (63%), *Faecalibacterium* (53%), *Roseburia* (64%) and decreases of *Dialister* (51%), *Enterococcus* (75%), *Prevotella* (71%), *Peptostreptococcus* (91%) and *Acidaminococcus* (47%). Figure 5 shows the relative abundance of genera pre and post-Ivacaftor. At species level a dominance of *Bacteroides vulgatus* was observed in both pre- (9%) and post-Ivacaftor (10.2%) treated subjects. At species level, in both pre- and post-Ivacaftor samples, the gut microbiota is dominated by *Bacteroides s*pecies including *Bacteroides vulgatus, Bacteroides thetamicron, Bacteroides ovatus, Bacteroides dorei, Bacteroides fragilis, Bacteroides uniformis*. Although not statistically significant, we observed increases in *Eubacterium rectale* (57%), *Eubacterium eligens* (100%), *Bacteroides fragilis* (87%), *Bacteroides uniformis* (157%), *Bacteroides* spp. D20 (183%) and decreases in *Prevotella buccae* (78%), *Dialister invisus* (67%), *Prevotella copri* (92%) and *Streptococcus pneumoniae* (50%). Interestingly, we noted differences in subject 299 in the compositional data at phylum, and genus level, with increases in Bacteroidetes and *bacteroides* relative to other Ivacaftor treated subjects. In addition,

these differences were also seen in reduced alpha diversity in subject 299 post Ivacaftor relative to the post-Ivacaftor subjects.

Impact of Ivacaftor on the intestinal metabolome

A Principal Components Analysis (PCA) model (Figure 6) shows that the intestinal metabolome of persons does not cluster based on Ivacaftor treatment status. Intra-person changes in the levels of measured compounds were investigated. Intra-person changes revealed significant downregulation of fatty acid compound hexadecanoic acid ($p=0.026$) post Ivacaftor. Octadecenoic acid (=0.093) was also reduced post-Ivacaftor, however this was not significant.

The Procrustes model for comparing data sets was used to investigate similarities in the spread of data from the metagenomic and metabolomic datasets. Metagenomic data from both the COG and SEED databases were used. Procrustes analysis using metagenomics data from the SEED (Supplementary information Figure 2a) and COG (Supplementary Figure 2b) databases had r values of -0.224 and -0.1884 and significance of 0.919 and 0.94, respectively. Goodness of fit of the model (r^2 value) for the SEED, COG and metabolomics datasets was 0.998, 0.999 and 0.987, respectively. These p-values indicate that changes seen in the metagenomic dataset do not reflect changes seen in the metabolomics dataset.

Quantitative PCR

QPCR was performed to quantify total bacterial load in each subject to ensure differences observed were not due to changes to absolute bacterial load. Total bacterial load (log g^{-1} faeces) showed no significant difference between pre and post Ivacaftor samples (paired t-test, $p= 0.554$).

6.5 Discussion

Cystic fibrosis (CF) is a multisystem disorder caused by mutations in the CFTR gene, resulting in defective transport of chloride and bicarbonate across the CFTR epithelial transmembrane channel. Mutations in the CFTR gene results in viscous mucous at various exocrine sites mostly notably in the lung, but also affecting secretion of digestion enzymes from the pancreas and bile from the gallbladder (Davis, 2006). Ivacaftor is a CFTR potentiator which shows clinical benefit in CF persons with the G551D gating mutation. Clinical studies have shown a reduction in pulmonary exacerbations requiring IV antibiotics, improvement in FEV_1 and BMI and a reduction in sweat chloride levels (Ramsey *et al*., 2011, Davies *et al*., 2013). Previously we have shown subtle changes in the gut microbiota post Ivacaftor treatment, mainly in the Bacteroidetes phyla. In the current study we investigated the effects of Ivacaftor treatment and the resulting reduction of antibiotic therapy on the intestinal microbiome and metabolome. This is the first study, to our knowledge, that investigates the gut microbiome functionality in CF persons treated with Ivacaftor.

β-diversity, visualised using a PCoA plot of the HUMAnN2 pathway abundances, showed clustering of samples based on individual rather than Ivacaftor treatment. Analysis of high level functional capabilities using MG-RAST showed no significant differences between pre- and post-Ivacaftor treatment. To mine further into the specific functional capabilities of the gut microbiome we used the HUMAnN2 suite of analytical programs. This revealed statistically significant changes in a limited number of gene abundances involved in diverse functions such as cellular amino acid metabolism, xenobiotic metabolism, aerobic respiration, vitamin biosynthesis, fermentation and carbohydrate biosynthesis. VALSYN-PWY (valine biosynthesis) and SULFATE-CYS-PWY (cysteine biosynthesis) pathway

abundances were shown to be decreased post Ivacaftor. In addition, L-rhamnose biosynthesis (DTDPRHAMSYN) involved in carbohydrate biosynthesis decreased post Ivacaftor. Increased protein catabolism, resulting in excessive energy losses has been reported in CF persons (Ionescu *et al*., 2002, Moran *et al*., 2001). Increased protein metabolism in the gut microbiota of CF persons relative to healthy controls has been previously reported in our group, suggesting that the gut microbiota plays a role in contributing to increased protein metabolism (Fouhy *et al*., in press). Changes in specific pathways involved in protein and carbohydrate metabolising capabilities of the gut microbiota capabilities may be indicative of normalisation of the gut microbiota post Ivacaftor. However, these changes in specific pathways relating to carbohydrate and protein metabolism were not reflected in corresponding high level functional categories. We observed no change post Ivacaftor in the total carbohydrate metabolising capabilities using both MGRAST and HUMAaN2. We observed no change of total protein metabolising capabilities using MGRAST. However, we did see a nonsignificant trend towards increased protein metabolising capabilities using HUMAaN2. This is surprising given the results of previous studies and may be as a result of a small n number. Further studies will be needed to investigate if this trend is generalizable to larger cohorts post Ivacaftor and establish its clinical significance.

Given the high fat diet in persons with CF, the lipid metabolising functions of the gut microbiota and signatures of lipid metabolism in the metabolome are of particular interest. Increased fatty acid metabolism in the gut of CF individuals compared to healthy controls is well documented (Manor *et al*., 2016, Fouhy *et al*., in press) which results both from a high fat diet and malabsorption of fats due to exocrine pancreatic dysfunction. In addition, increased fatty acid metabolism has

been shown to correlate with increased intestinal inflammation, which we have previously shown to be unaffected by Ivacaftor treatment in this cohort. Analysis of the metabolome showed significant reduction of fatty acid compound hexadecanoic acid and a non-significant reduction of octadecanoic acid post Ivacaftor, which may be as a result of altered intakes of dietary fat post Ivacaftor. However, this reduction in compounds relating to lipid metabolism was not reflected in changes in pathway abundances relating to lipid metabolism. Therefore, while we see signals of change in the metabolome with respect to fatty acid metabolism we did not see corresponding changes in relative abundance of pathway abundances. In addition, we did not observe significant changes in high level functional categories relating to fatty acid metabolism.

The role of the gut microbiota in the metabolism of xenobiotic compounds has been established in healthy cohorts (Das *et al*., 2016, Saad *et al*., 2012). Due to chronic antibiotic therapy for treatment of reoccurring pulmonary exacerbations CF persons have been shown to have higher capabilities in their gut microbiota to metabolise xenobiotic compounds (Fouhy *et al*., in press). In the current study we observed pathway P161-PWY, which is involved in xenobiotic acetylene degradation, to be decreased post Ivacaftor. However, changes from pre to post Ivacaftor were not observed in antibiotic catabolising capabilities or aromatic compound metabolism. Phosphopantothenate (vitamin B5) biosynthesis pathway (PANTO-PWY) was shown to be increased post Ivacaftor, which may be indicative of improved vitamin biosynthesis capabilities post Ivacaftor. However, these changes were not reflected in level 1 vitamin synthesis functional capabilities using MGRAST. Collectively, the significant changes seen in a limited number of pathway abundances indicate changes in the functionality of the gut microbiota that

correspond to subtle signals of normalisation of the gut microbiota. However, it must be highlighted that these significant changes are at low abundances in the gut microbiota and no significant changes or trends were seen in the corresponding level 1 functional categories relating to these pathways. Such subtle differences in functionality between pre and post Ivacaftor suggests that these subjects may need to be treated with Ivacaftor for longer than the 12 month period of this study in order for significant changes to be observed.

Interestingly, subject 299 post-Ivacaftor was distinct from other persons in our compositional analysis of the microbiota, with high relative abundances of Bacteroidetes and *bacteroides*. In addition, a separation of this sample from other subjects was noted in the PCoA plot of the pathway abundance data reflecting clinical differences from the rest of the cohort. We also observed differences in the pattern of changes from pre to post Ivacaftor in subject 299 when looking at specific functional categories (lipid, carbohydrate and antibiotic metabolism capabilities) using HUMAaN2. Subject 299 was the only subject who required IV antibiotics (meropenem and colomycin) courses to treat pulmonary exacerbation in the year following commencement of Ivacaftor therapy, which explains the reduced diversity, seen both in measures of richness and evenness, in this subject. The length of time between sample collection for the 12-month post Ivacaftor time point and exacerbation is less than three months. Therefore, while the subject is returning to pre antibiotic treatment levels, diversity and microbiota composition has not fully recovered.

Furthermore, one of the findings of the study was the total antibiotic resistance gene hits were reduced post-treatment when compared to persons prior to commencing treatment with Ivacaftor, albeit this reduction was demonstrated to be

statistically non-significant. This is most likely as a result of a reduction in broad-spectrum antibiotic treatment post-Ivacaftor. It has been observed that the gut microbiota is a reservoir for antibiotic resistance, which increases with exposure to antibiotics (De Vries *et al*., 2011, Sommer *et al*., 2009). However, the effect of reducing antibiotics in a cohort chronically exposed to antibiotics from an early age has not been shown. Indeed, there are no studies, to our knowledge, examining the effect of chronic antibiotic therapy on the gut compared to short term antibiotic courses and investigations have mainly looked at the effect of short courses of antibiotic therapy on the gut. Persistence of antibiotic resistance following antibiotic therapy has been reported. Indeed, Dethlefsen *et al*., (2008) show that following cessation of antibiotic therapy the gut microbiota composition structure returns to pre-antibiotic organisation, however, antibiotic resistance capabilities appear to persist within their corresponding microbial communities. Furthermore, Slojund *et al*., (2003) observed clarithromycin resistance for up to three years post antibiotic therapy. Therefore, while we see a reduction in the total numbers of antibiotic resistant genes we may need to follow these subjects for longer than the 12-month period of study to see a significant reduction in antibiotic resistance capabilities.

There was no significant difference in the relative abundance of species which carried antibiotic resistance genes pre- compared to post-Ivacaftor treated persons. *Escherichia coli* was shown to carry the greatest burden of antibiotic resistant genes pre-Ivacaftor (15.8% pre Ivacaftor and 11.6% post Ivacaftor) and *Bacteroides fragilis* was shown to carry the greatest burden post- Ivacaftor (pre-Ivacaftor 12.4%, post-Ivacaftor 15%), although this change was not significant. Mean absolute numbers of gene hits mapped to *Escherichia coli*, *Bacteroides fragilis, Enterococcus faecalis, Bifidobacterium longum, Campylobacter jejuni*,

Enterococcus faecium, *Streptococcus suis* and *Clostridium difficile* were 303 and 242 in pre- and post-Ivacaftor treated subjects, respectively. *Escherichia coli*, *Enterococcus faecalis*, *Enterococcus faecium* have become recognised as nosomical multidrug resistant pathogens in recent years (Vincent, 2003, Rice, 2008) and are known to act as opportunistic pathogens in an immunocompromised individual, due to their ability to readily exchange plasmids that may confer antibiotic resistance during colonisation (Karami *et al*., 2007, Goren *et al*., 2010).

We detected genes conferring putative resistance to 17 antibiotic classes in both pre- and post-Ivacaftor samples. Fourteen of the 17 antibiotic classes show a reduction in the number of antibiotic resistance genes detected post-Ivacaftor, including most prevalent classes tetracycline, aminoglycoside, MLS and multidrug resistance, although none of these were shown to be significant. In total 154 types of antibiotic resistance genes were identified both pre- and post-Ivacaftor. Pre-Ivacaftor samples had 134 types of antibiotic resistance genes and post-Ivacaftor samples had 117 types of antibiotic resistance genes. However, this difference is not significantly different. In both pre- and post-Ivacaftor treated samples we see a dominance of tetracycline resistance genes (tetW, tetO and tetQ), followed by aminoglycoside resistance genes (APH(2")IIa and AAC(6')-APH(2") bifunctional resistance protein) and erythromycin resistance gene ermF. These results are in line with other studies that have seen a dominance *tetW*, followed by *tetO* and *tetQ* among tetracycline resistance genes. Shoemaker *et al*., (2001) showed *tetQ* to be the most abundant resistance gene, most commonly isolated from *Bacteroides* intestinal populations, increasing from 30% in the 1970s to 80% in the 2000s. Metagenomic studies (Forsland *et al*., 2013, Hu *et al*., 2013) have shown the omnipresence of tetracycline resistance genes and their dominance in the human gut resistome. Furthermore, they

showed the high prevalence of *ermF* among erythromycin resistance genes and its widespread nature among the members of the gut microbiota.

Study Limitations and conclusions

This study is limited by the small number of persons examined (n=6). A larger cohort may reveal significant changes in functionality or composition of the gut microbiota. In addition, persons were selected to give a range of change in FEV_1 values from pre- to post-Ivacaftor, which may mask changes which may be more prominent in a cohort with good response to Ivacaftor, in terms of FEV_1. This study underlines the functional stability and resilience of the human gut microbiota over time. Despite observing subtle significant changes in low abundance pathways which may correspond with normalisation of the functionality of the gut microbiota and non-significant changes in the antibiotic resistance capabilities of the gut microbiota, for the most part Ivacaftor treatment and its associating significant clinical improvements do not impact the high level functional capabilities of gut microbiota. The apparent stability of high level functionality and gut microbiota composition following intervention with Ivacaftor could be explained by the concept that stable equilibrium states exist for each individual's microbiota. The gut microbiota exhibits long-term stability that is maintained by the action of restoring forces that will try to maintain the community within a certain range even in the event of intervention, albeit with subtle differences (Dethlefsen *et al*., 2010, Lozupone *et al*., 2012, Beisner *et al*., 2003, Walker *et al*., 2004).

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Table 1. Clinical characteristics of the subjects included in this study.

			FEV1 %		BMI		No. Exacerbations requiring IV AB		IV AB	
Subject ID.	**Gender**	**Age**	**Pre**	**Post**	**Pre**	**Post**	**1 yr Pre**	**1 yr Post**	**1 year Pre**	**1 year Post**
108	M	39	77	78	23.7	24.7	0	0	nil	nil
148	M	29	86	94	24.8	26	0	0	nil	nil
288	F	30	37	45	22.6	19.7	1	0	Ceftazidime, colomycin	nil
299	F	30	31	49	19.1	20.4	2	1	Ceftazidime, colomycin Meropenem, colomycin	Meropenem, colomycin
307	F		69	71	20.2	21	0	0	nil	nil
319	M	25	77	96	22.9	25	1	0	Ceftazidime, colomycin	nil
	Median	30	73	74.5	22.75	22.85	1	0		

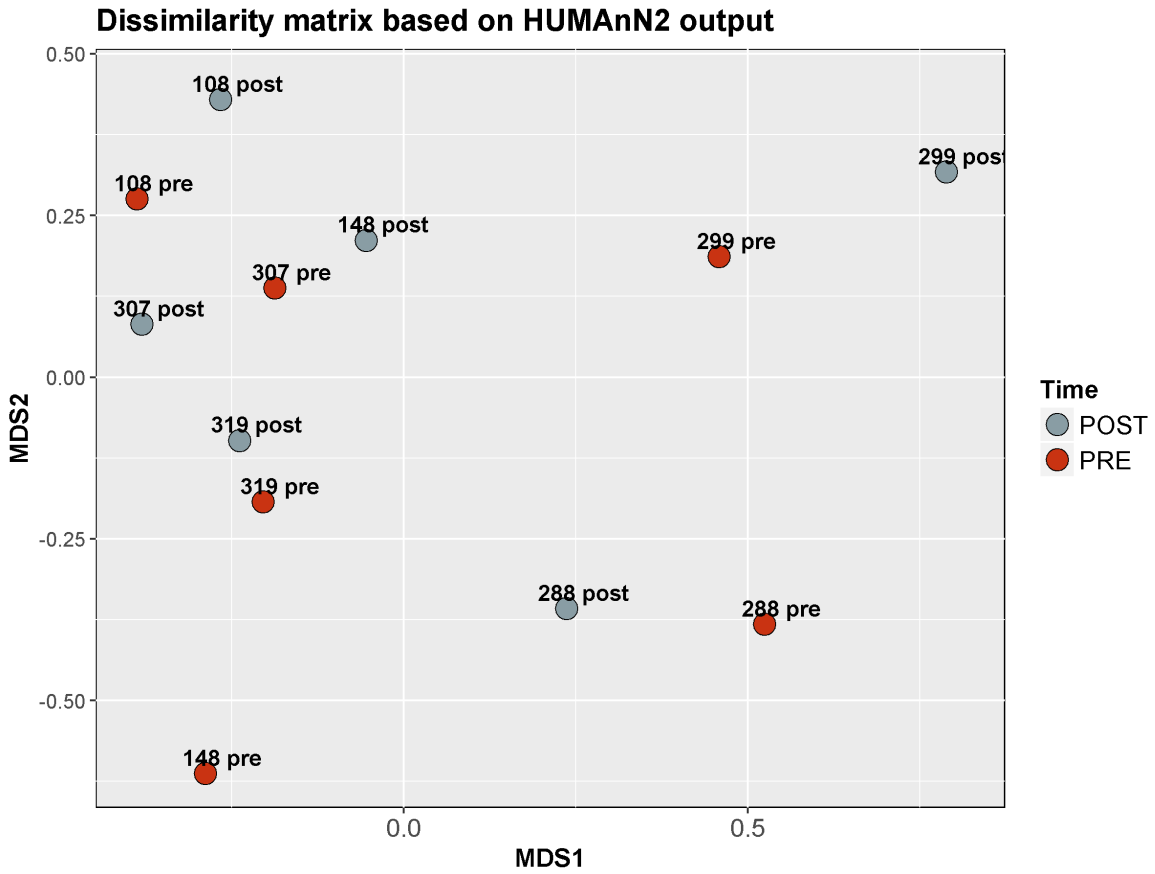


Figure 1. Dissimilarity matrix based on HUMAnN2 functional metagenome output of Pre Ivacaftor (red) and post Ivacaftor (green) treated subjects.

Table 2. HUMAnN2 functional profiling and pathway abundances significantly altered pre and post Ivacaftor

Superpathways	**Pathway**	**Pre**	**Post**	**% change**	**p-value (≤ 0.05)**
Cellular amino acid metabolic process	VALSYN-PWY: L-valine biosynthesis	2.22	0.00	-100.00	0.028
Inorganic nutrients metabolism	SO4ASSIM-PWY: sulfate reduction I (assimilatory)	2.77	0.78	-71.87	0.025
Cellular amino acid metabolic process	SULFATE-CYS-PWY: superpathway of sulfate assimilation and cysteine biosynthesis	5.72	1.71	-70.12	0.056
Aerobic respiration	PWY-7279: aerobic respiration II (cytochrome c) (yeast)	1.33	0.00	-100.00	0.028
Cofactors, Prosthetic Groups, Electron Carriers Biosynthesis	PANTO-PWY: phosphopantothenate biosynthesis I	307.94	437.95	42.22	0.041
Xenobiotic metabolism	P161-PWY: acetylene degradation	20.69	5.61	-72.89	0.026
Carbohydrate Biosynthesis	DTDPRHAMSYN-PWY: dTDP-L-rhamnose biosynthesis I	1.98	0.00	-100.00	0.028

Table 2 shows the relative abundances of gene pathways, including the superpathway they are involved in, which have been shown to be significantly different pre compared to post Ivacaftor.

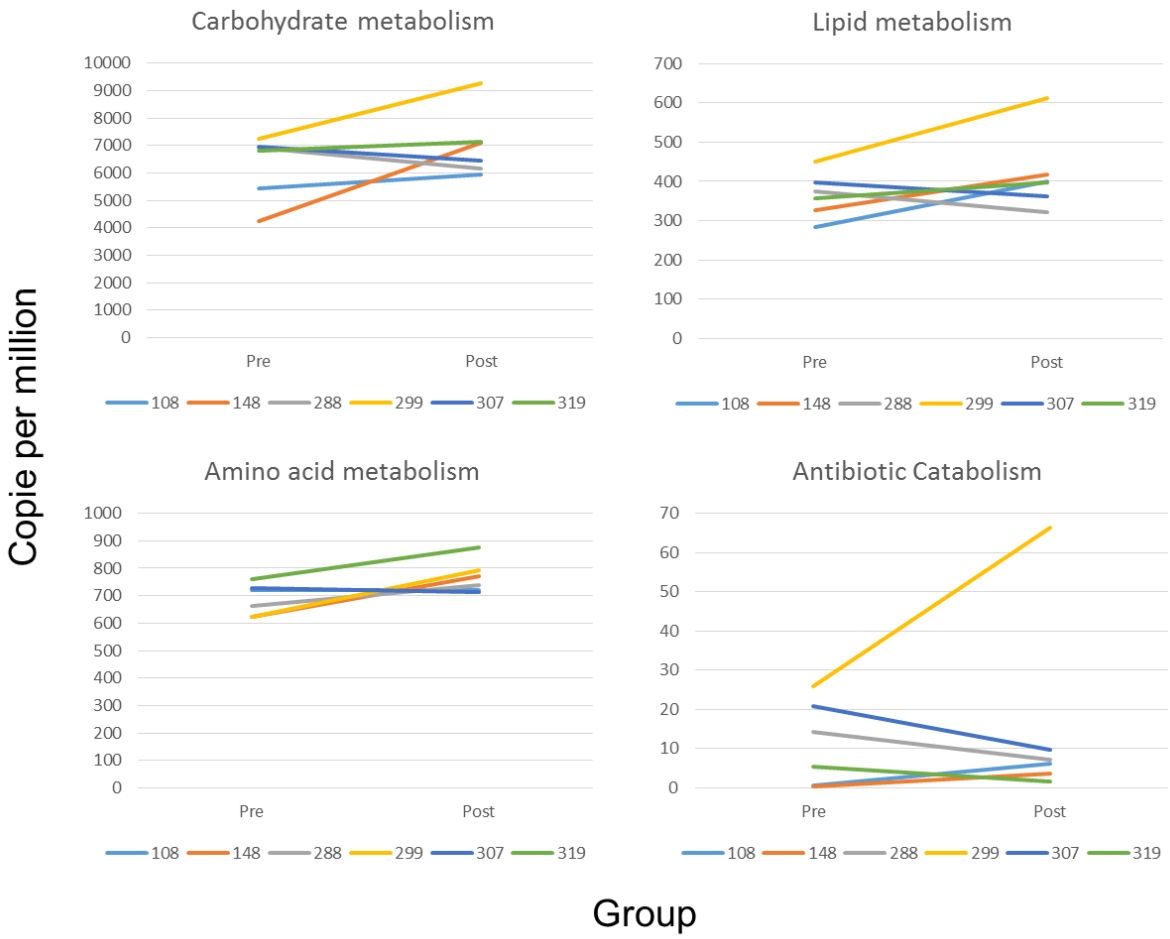


Figure 2. Abundance of Gene Ontology terms involved in lipid GO:000662: [BP], carbohydrate GO:0005975: [BP], amino acid GO:0006520: [BP] and antibiotic GO:0017001: [BP] metabolic processes pre and post- Ivacaftor. Values are given in copies per million.

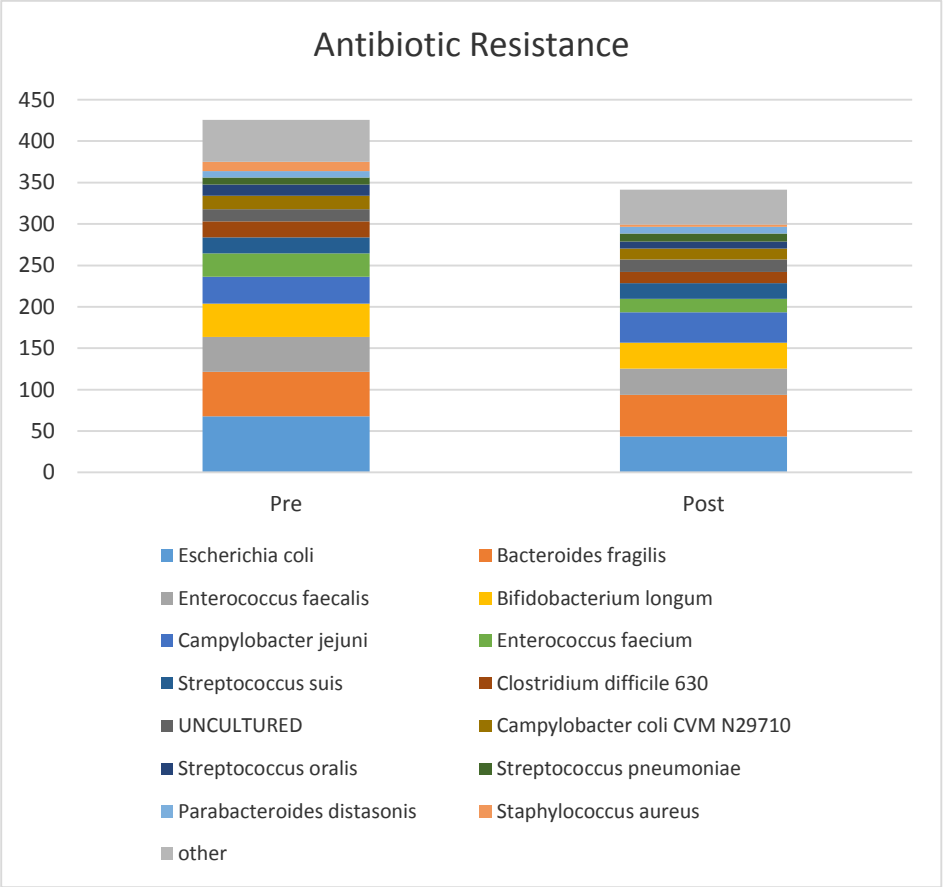


Figure 3. Total numbers of antibiotic resistance gene hits found in pre and post Ivacaftor treated subjects and the species which contribute to antibiotic resistance.

Table 3. Antibiotic classes (Mean and standard deviation) against which resistance genes were found pre and post Ivacaftor. Values given are the numbers of hits of resistance genes.

Antibiotic class	**Pre-Ivacaftor**		**Post-Ivacaftor**	
	Mean	**St. Dev**	**Mean**	**St.Dev**
Tetracycline	157.83	15.27	140.50	30.09
Aminoglycoside	78.83	14.19	63.50	17.13
Macrolide/Lincosamide/Streptogramin	54.83	13.62	47.17	18.39
Beta-lactamase	32.00	14.97	32.17	12.48
Multidrug	35.17	31.70	13.83	26.57
Trimethoprim	13.83	9.39	9.00	3.42
Fluoroquinolones	8.50	9.29	5.00	7.79
Lipopeptide	9.17	11.95	2.00	2.45
Vancomycin	2.33	3.94	8.17	10.98
Sulfonamide	5.33	6.62	5.17	5.84
Streptothricin	6.17	4.14	4.17	3.34
Rifampicin	5.67	5.25	3.50	5.19
Polymyxin	5.83	9.79	2.00	4.47
Elfamycin	5.67	4.11	1.83	1.57
Chloramphenicol	2.67	3.73	2.33	2.69
Penicillin	1.17	1.34	2.50	3.10
Bacitracin	1.00	1.41	0.33	0.75

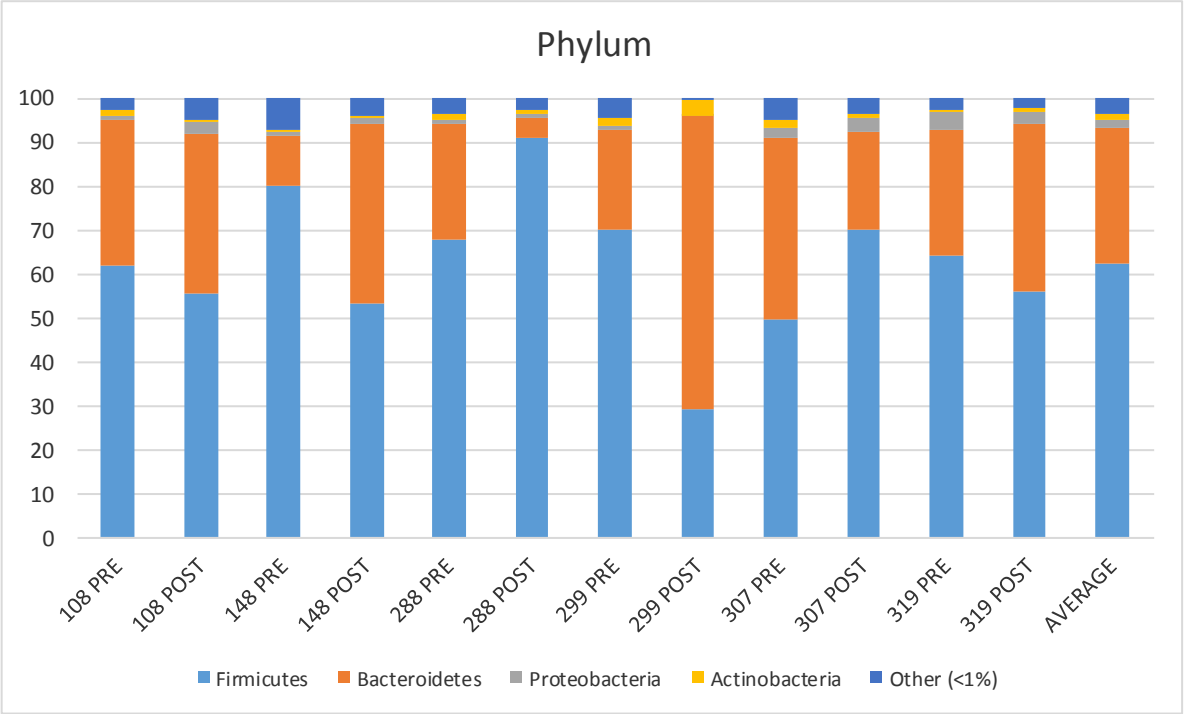


Figure 4. Percentage relative abundance of taxonomic groups at phylum level of individual subjects pre and post Ivacaftor treatment.

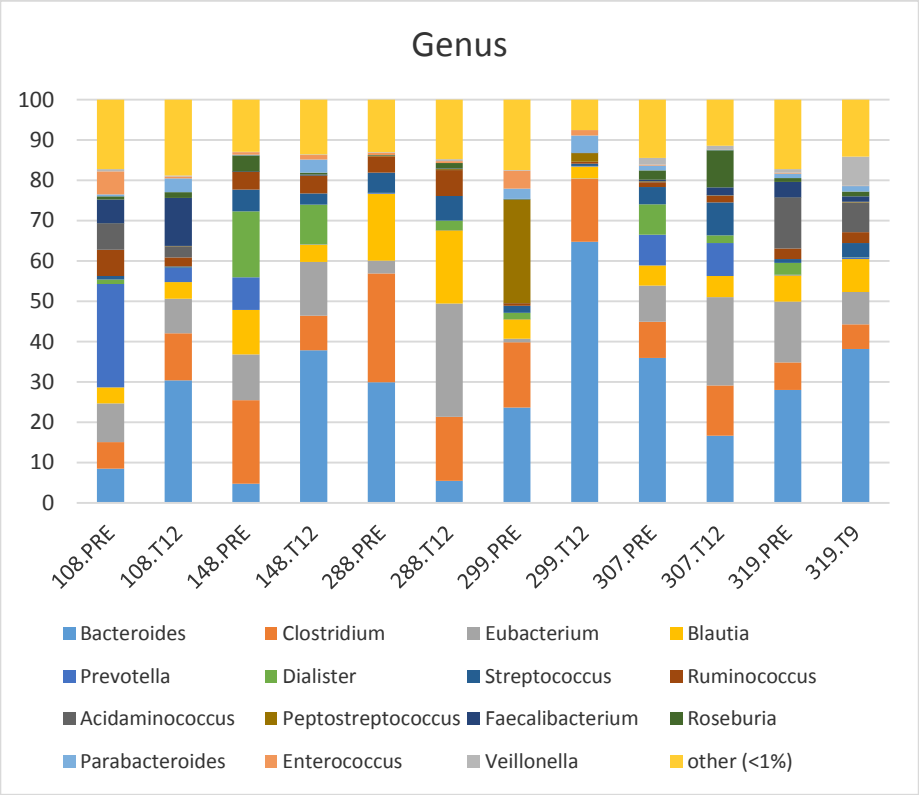


Figure 5. Percentage relative abundance of genera found in the gut microbiota at genus level in individual subject's pre and post-Ivacaftor. Genera that have a relative abundance greater than 1% are included in the figure. Genera at <0.1% abundance are grouped together as 'Other'.

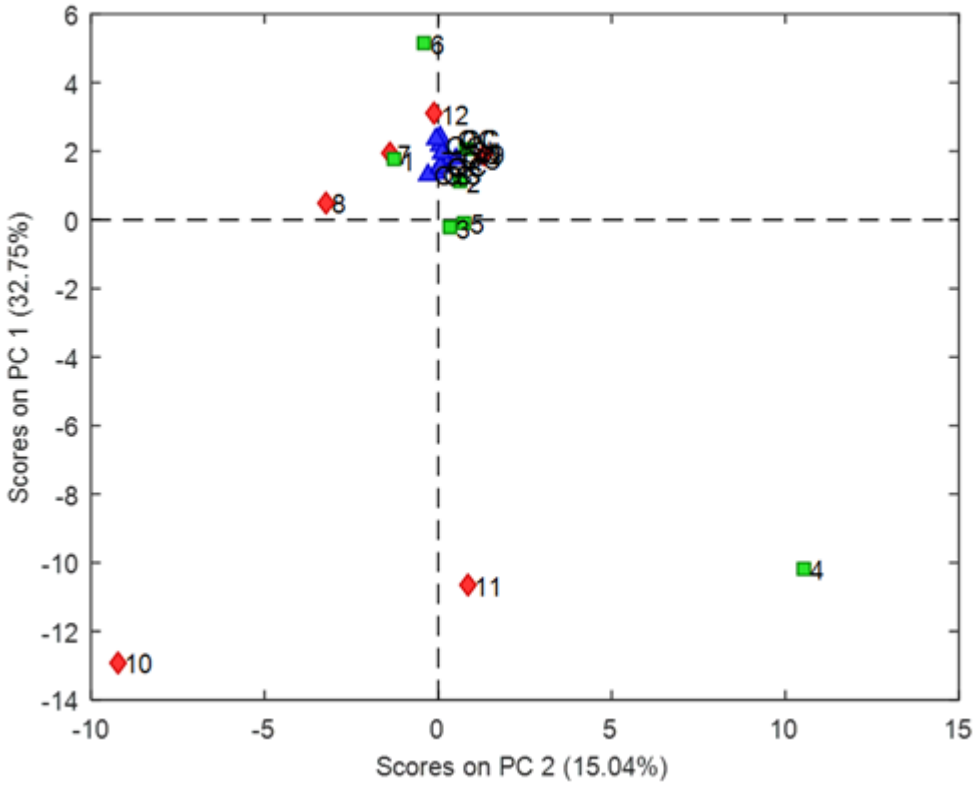
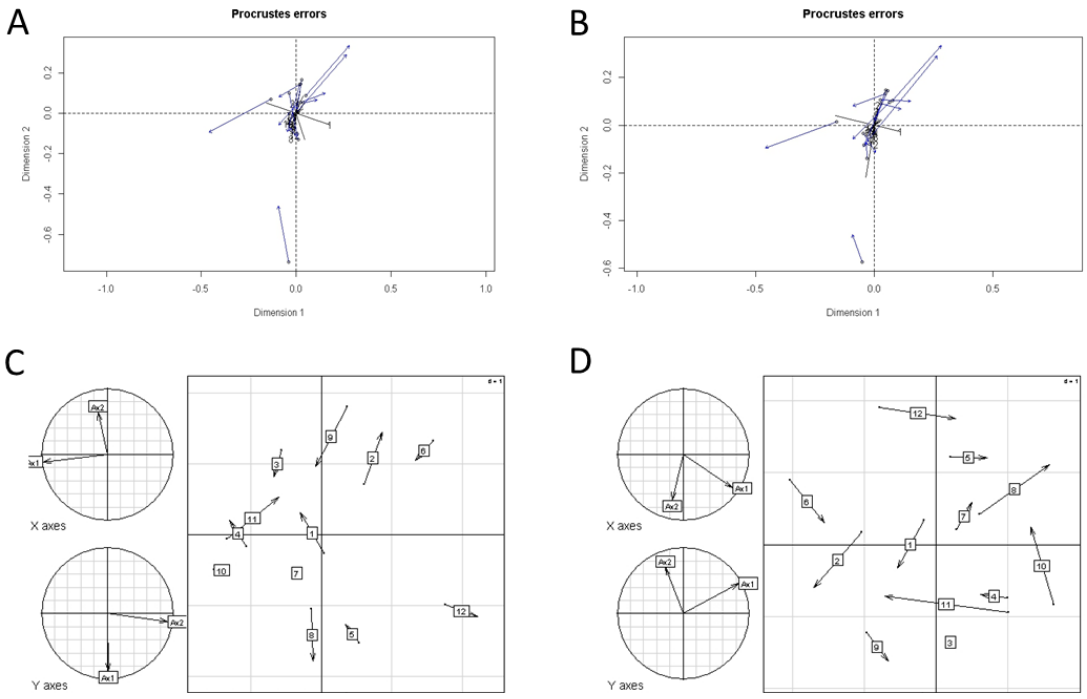


Figure 6. PCA plot of pre (green) and post (red) Ivacaftor treated samples based on the metabolite profile of each sample. Internal controls are shown in blue. Samples codes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 are 108 pre, 148 pre, 288 pre, 299 pre, 307 pre, 319 pre, 108 post, 148 post, 288 post, 299 post, 307 post and 319 post, respectively.

Supplementary Figure 1. Functional subsystems profiled using MGRAST metagenomics server.



Supplementary Figure 2. Procrustes and coinertia analysis of metagenomics and metabolomics based on SEED and COG databases. Procrustes analysis of metabolomics and metagenomics datasets using both SEED (A) and COG (B) shows no significant similarity in the spread of both datasets. Coinertia analysis of metabolomics and metagenomics datasets using both SEED (C) and COG (D) shows no significance in the axes of covariance of the eigenvalues of the metabolome and microbiome datasets.

Chapter 7

Validation of a method for preparation of faecal inoculum for faecal fermentation distal colon model studies

7.1 Abstract

Distal colon faecal fermentation models are valuable tools for assessment of the *ex vivo* effects of interventions on the human gut microbiota prior to progressing to costly human or animal model intervention trials. To conclude such experiments with valid results, a robust method for preparation of faecal inoculum is needed which accurately represents the human gut microbiota and is logistically feasible for the cohort being studied. Previously, a method has been developed for the preparation of faecal inoculum for distal colon models whereby fresh faecal samples from healthy adults were homogenised, washed in PBS and stocked in 10% glycerol until further use (O' Donnell *et al*., 2016). However, studies based on certain subject groups, such as CF cohorts may experience logistical difficulties in the collection of the required number of faecal samples on a single day. Here, we demonstrate that the preparation of faecal inoculum from frozen faecal samples is not a viable method for use in distal colon models compared to faecal inoculum preparation methods which use fresh faecal samples.

7.2 Introduction

The human gut microbiota is a complex ecosystem, inhabited by trillions of microbes, that influences the health of the host through its collective metabolic functions and cellular processes (Lozupone *et al*., 2012). The human gut microbiota is increasingly being shown to be linked to diverse disorders such as obesity (Ley *et al*., 2006, Turnbaugh *et al*., 2008), IBD (Dicksved *et al*., 2008, Frank *et al*., 2007) and cancer (Lupton, 2004). Furthermore, manipulations of the human gut microbiota, such as addition of probiotics or prebiotics, are proposed as therapies for various disorders, such as IBD (Mattila-Sandholm *et al*., 1999) and Antibiotic Associated Diarrhoea (AAD) (Hempel *et al*., 2012). Assessment of these interventions in an *ex*

vivo faecal fermentation model of the distal human colon is a useful tool to assess the value of such manipulations before proceeding to costly human and animal trials. *Ex vivo* batch faecal fermentation models of the distal human colon have been used to assess the effects of antibiotics (Rea *et al*., 2011), bacteriocins (Guinane *et al*., 2016), probiotics and prebiotics (Liu *et al*., 2016) on the gut microbiota. Faecal fermentation experiments require human faecal samples as the faecal inoculum, into which test components are added. Recently O' Donnell *et al*., (2016) developed a method to prepare a standardised faecal inoculum, prepared from fresh samples, aliquoted and stored at -80^{o}C until further use. This method provides a stable, standard inoculum with repeatable results which allows for comparability between experiments. However, this method depends on obtaining 4-6 fresh faecal samples on the day of preparation. For some study groups, such as a CF cohort obtaining the required number of faecal samples on a given day poses logistical issues. Here, we investigate the viability of a faecal inoculum prepared from frozen faecal samples, stocked in 15% mineral glycerol, and compare it to the standardisation method used by O'Donnell *et al*., (2016).

7.3 Methods

Sample collection

Faecal samples from four healthy adult donors providing signed consent, with no antibiotic usage in the last 6 months were collected and stored at 4^{o}C for 2-3 hours before faecal inoculum preparation either by method A or method B. Ethics for sample collection were provided by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (protocol no. APC022).

Inoculum preparation

Method A

Faecal samples were transferred into a Don Whitley (West Yorshire, UK) anaerobic chamber and processed as previously described by O' Donnell *et al*., (2016). Briefly, equal amounts of each faecal sample (25 g) were pooled and homogenised in a filtered stomacher bag (Sparks lab Supplies, Rathcoole, Co. Dublin) in a 1:1 w/v ratio (100 ml) with 50 mM sterile phosphate buffer (PB) with 0.05% L-cysteine hydrochloride (Sigma Aldrich, Ireland). The filtered slurry was then centrifuged at 4000 g for 30 minutes and the pellet resuspended in a final concentration of 10% glycerol (10 ml) made up to a final volume of 100 ml using 50mM PB. The inoculum was then aliquoted and frozen at -80^{o}C until further use. Prior to faecal fermentation experiments aliquots were thawed at 37^{o}C.

Method B

Faecal samples were stocked individually in a 1:1 ratio (w/v) in mineral glycerol (ingredients below) and frozen immediately at -80^{o}C. The final concentration of glycerol was 15%. After one week samples were thawed and homogenised as in method as previously described by O'Donnell *et al*., (2016) and refrozen until subsequent fermentation experiment.

Components in Mineral Glycerol used in method B.

A 3.8% salt solution 1 (0.6% $K_2 HPO_4$), 3.8% salt solution 2 (0.6% KH_2PO_4, 0.6% $(NH_4)_2SO_4$, 1.2% NaCl, 0.25% $MgSO_4 7H_2O$, 0.16% $CaCl_2 2H_2O$), 0.56% Sodium Hydrogen Carbonate, 0.1% Resazurin solution, 30% glycerol and 62% water were boiled together and allowed to cool while bubbling with CO_2. L-cysteine HCL (0.1%) was added after gasing. Liquid was aliquoted into Hungate tubes, autoclaved at 121^{o}C for 20 minutes and stored at -80^{o}C.

Distal colon model

Ex vivo distal colon fermentation experiments were carried out in the Multifors 4 vessel fermentation unit (Infors, UK) under anaerobic conditions. Growth media used in the fermentations was as described by (Rycroft *et al*., 2001, Olano-Martin *et al*., 2000). This medium contained (g l^{-1}): peptone water, 2 g; yeast extract, 2 g; NaCl, 0·1 g; K^2HPO^4, 0·04 g; KH^2PO^4, 0·04 g; $MgSO^4.7H^2O$, 0·01 g; $CaC^{l2}.6H^2O$, 0·01 g; $NaHCO^3$, 2 g; hemin (dissolved in a few drops of 1 mol l^{-1} NaOH), 0·05 g; cysteine.HCl, 0·5 g; bile salts, 0·5 g; Tween 80, 2 ml and 10 µl vitamin K1. The medium was adjusted to pH 7.0 and sparged for at least 2 hours before inoculation. Two hours before the fermentation experiment an aliquot of the frozen inoculum was thawed in the anaerobic chamber and added to the fermentation vessel to a final concentration of 14%. Fermentations were performed over 24 hours at 37ºC at pH 6.8 (which was maintained by addition of 2.5N NaOH or 1N HCL) and the system sparged with N_2 and stirred continuously at 100rpm. Six technical replicates were performed for each method of inoculum preparation. Samples were withdrawn from the vessels at T0 and T24 hours for subsequent analysis.

Culture-dependant analysis

The effects of the two methods of faecal inoculum preparation on the microbial populations were determined using culture based enumeration of total anaerobes and lactobacilli. One millilitre of faecal fermentation media at both T0 and T24 hours was removed and serially diluted in maximum recovery diluent (MRD) (Oxoid Ltd, Basingstoke, Hampshire, UK) and plated on the respective media. Total anaerobe numbers were evaluated by plating on Wilkins Chalgren Agar (WCA) (Fluka analytical) supplemented with 50 units of Nystatin and 7.5% defibrinated horse blood (TCS Biosciences, Buckingham, UK) and incubated for five days at 37˚C

anaerobically, as described by Fouhy *et al*., (2015). Total lactobacilli were enumerated by plating on Lactobacillus Selective agar (LBS) (DIFCO, New Jersey, USA) supplemented with 0.132% Glacial Acetic acid and 50 units of Nystatin and incubated for 3-4 days anaerobically.

Preparation of samples for 16S compositional sequencing and bioinformatics analysis of sequence data.

Two ml of liquid from the fermentation vessel was centrifuged at 16000xg for 5 minutes, the supernatant removed and the pellet frozen at -80^{o}C until further analysis. Samples were thawed and extracted by the Repeat Bead Beating method as previously described by Fouhy *et al*. (2015). Extracted DNA from 24 samples was then prepared for 16S rRNA compositional sequencing of the V3-V4 region using Illumina's MiSeq platform as previously described by Fouhy *et al*., (2015). Three hundred base pair paired end reads were then analysed using the bioinformatics pipeline as described by Fouhy *et al*., (2015)

Quantitative PCR

Total bacterial load and total lactobacillus numbers in samples at T0 and T24 for each faecal inoculum preparation method was measured using qPCR. Samples and standards were analysed in triplicate using the Roche Lightcycler 480 platform and SYBR green reagent (Roche Diagnostics, UK). For total bacterial load the primers: 5'-ACTCCTACGGGAGGCAGCAG-3' (Ahn *et al*., 2006) and 5'-ATTACCGCGGCTGCTGG-3' (Muyzer *et al*., 1993) were used. Quantification of lactobacilli was performed using the following primers: 5'-AGCAGTAGGGAATCTTCCA-3' and 5'-CGCCACTGGTGTTCYTCCATATA-3' (Furet *et al*., 2009). 0.5 pmol of each primer was added to each reaction and the following PCR cycle used: 95^{o}C x 5 minutes, 45 cycles x 95^{o}C 10 seconds, 60^{o}C 30

seconds, 72^{o}C 30 seconds, cooling for 10 seconds. Mean concentrations were then expressed as $\log^{10}$ 16S rRNA copies per gram of faeces.

7.4 Results

Culture dependant analysis

Culture dependant analysis, including microbiological counts of total anaerobic bacteria and total lactobacilli, showed an overall reduction of total anaerobes (WCA) of approximately 1.5 log in method B compared to method A at T0 and approximately 1 log reduction at T24 in method B compared to method A. Total lactobacilli counts showed a 1 log reduction in method B compared to method A at T0 and a 1.5 log reduction at T24 in method B compared to method A (Figure 1).

Culture independent analysis

Beta diversity visualised using a PCoA plot (Figure 2.) shows that samples from method A and B cluster together at the starting point (T0) of the fermentation, however after following 24 hours of fermentation in the distal colon model we see a distinct separation of method A and method B. Alpha diversity analysis (Figure 3) of the 16S rRNA compositional data shows a reduction in diversity across all metrics of diversity (Chao1, Simpson, Shannon and Observed_species) in method B at T24 relative to method A at T24. In addition the magnitude of change in diversity from T0 to T24 is greater for method B than method A in all measures of diversity, richness and evenness.

In method B_T24 at phylum level (Figure 4) a decrease was observed in Euryarchaeota, Bacteroides, Tenericutes, while Proteobacteria increased relative to A_T0, A_24 and B_T0. The relative abundance of Actinobacteria and Firmicutes at T24 are constant across both treatments. Proteobacteria show no change during

freezing AT0 v BT0, however this taxon increases from approximately 8% in AT24 to 13% in BT24.

In method B_24 relative to method A_T24 at family level (Figure 5) in taxa found at greater than 0.1% the relative abundance Bacteroidaceae, Porphyromonoadaceae, Prevotellaceae, Rikenellaceae, Christenellaceae, Defluviitalaceae, Lachnospiraceae, Ruminococcaeae, Alcaligenaceae, Desulfovibrionaceae and Verrucomicrobioaceae decreased and Clostridiaceae, Enterococcaceae, Peptostreptococcaceae, Acidaminococcaceae, Enterbacteriaceae increased. In method B_after 24h fermentation Clostridiaceae (20.9%), Enterococcaceae (9.15%), Peptostreptococcaceae (24.8%), Acidaminococcaceae (7.2%), Enterbacteriaceae (12.9%) together account for 75% of the microbial community compared to 12.7% in method A at the same time point.

At genus level (Figure 6) in taxa greater than 0.1% the relative abundance *Bifidobacterium*, *Bacteroides*, *Alistipes*, *Blautia*, *Lachonospiraceae insertae sedis*, *Roseburia*, *Faecalibacterium*, *Ruminococcus*, *Coprococcus*, *Dialister*, *Desulfvibrio* and *Akkermansia* decreased and *Enterococcus*, *Clostridiaceae*, *Acidaminococcus* and *Escherichia-shigella* increased in method B after 24 hours relative to method A at the same time point. *Enterococcus* (9.2%), *Clostridiaceae sensu stricto* (20%) *Peptostreptococcaceae insertae sedis* (24.5%), *Acidaminococcus* (3%) and *Escherichia-shigella* (12.3%) combined accounted for 69% of the relative abundance in method B after 24h fermentation (T24), compared to approximately 7% in method A at the same time point (T24).

Quantitative PCR

Using qPCR method as previously described the absolute abundance of total bacteria and lactobacilli were quantified. Total bacterial load increased from T0 to T24 in both treatment groups, however at T24 there is no difference between the two methods. Lactobacilli levels remained stable from T0 to T24 in both methods and there was no difference seen between the treatments at 24 hours, confirming 16S rRNA compositional analysis which showed no difference in lactobacilli and lactobacillaceae from A_T24 to B_T24 (Figure 7).

7.4 Discussion

The aim of the current study was to determine the viability of a standardised faecal inoculum prepared from faecal samples frozen in glycerol for *ex vivo* distal colon model experiments. Using culture-dependant methods (enumeration of total anaerobes and total lactobacilli), 16S rRNA compositional sequencing of the microbial communities and qPCR enumeration of total bacterial load and total lactobacilli, we established that faecal inoculum prepared from frozen samples had reduced alpha diversity due to non-viability of certain taxa following two freeze thaw cycles. Culture-dependant based analyses showed that after 24 hours method B had lower number of viable total anaerobes and total lactobacilli relative to method A, indicating that refreezing a frozen sample even when frozen in the presence of glycerol reduces the numbers of viable bacteria in the sample. In addition compositional analysis of microbial communities after 24 h fermentation showed distinct differences between method A and method B as visualised by PCoA plots of beta-diversity (diversity in microbial community between different subjects) at 24 hours (Figure 2). Over the course of 24 hours alpha diversity (number of species with a microbial environment), evenness and richness of the gut microbiota

community decreased in method B relative to method A. In addition, compositional changes were seen at phylum, family and genus levels. At phylum level there was a decrease in Bacteroidetes and an increase in Firmicutes in method B_T24 relative to method A_T24. This change in the ratio of Bacteroidetes to Firmicutes has been previously reported following freezing of faecal samples (Fouhy *et al*., 2015, Bahl *et al*., 2102). Decrease in alpha diversity measures of evenness and richness is explained by the bloom of Clostridaceae, Enterococcaceae, Peptostreptococcaceae, Acidaminococcaceae and Enterbacteriaceae at family level, and *Enterococcus*, *Clostridiaceae sensu stricto*, *Peptostreptococcaceae insertae sedis*, *Acidaminococcus* and *Escherichia-shigella* at genus level. These changes are likely due to an additional freeze thaw cycle of B_T0, resulting in loss of viability of taxa sensitive to freeze thaw, allowing taxa that are less sensitive to increase in relative abundance during the fermentation and thus filling the vacant niche. We also noted that while the samples are less diverse in method B prepared samples the total bacterial load as measured by qPCR does not change relative to method A prepared samples, suggesting that increases seen in taxa in B_T24 may be absolute changes, although has not been confirmed for individual taxa by qPCR. The extractability of DNA from certain taxa, especially Gram-positive groups (e.g *Enterococcus*), may improve following repeat freezing and thawing, thereby explaining their increase (Bahl *et al*., 2012). Interestingly, *bifidobacteria* and bifidobacteriaceae remained relatively resilient and displayed ability to resuscitate following one freeze thaw cycle (method A). However, despite similar levels of *bifidobacteria* and bifidobacteriaceae initially in method B at time 0 compared to method A at the same time point they were unable to grow to the same levels as in A T24. In line with these results, Fouhy *et al* (2015) have shown that bifidobacteria have the ability to

resuscitate and grow following one freeze thaw cycle. However this study shows that their ability to resuscitate following two freeze thaw cycles is greatly reduced. Various studies have established that the faecal microbiota is able to withstand one freeze thaw cycle (Fouhy *et al*., 2015, Lauber *et al*., 2010, O' Carroll *et al*., 2012, Roesch *et al*., 2009) but few have looked at the effect of multiple freeze thaw cycles on the ability of microbes to resuscitate and grow again. Indeed, frozen faeces have been successfully used in Faecal Microbiota Transplants (FMT) to treat CDI, demonstrating that the integrity of the gut microbiota is retained during one freeze cycle (Hamilton *et al*., 2013). Freeman and Wilcox (2003) showed that multiple cycles of refrigeration, freezing and thawing had little effect on the viability of *C.difficile*. This is in line with our results which show an increase in relative abundance of Clostridial species in B_24 relative to A_T24 at family and genus levels. However, Clostridia are spore forming bacteria and spores are more resistant to freezing than vegetative cells. While DNA based studies do not take into account the viability of a cell, faecal fermentation culture-based studies require cells to be viable and able to proliferate in order to sufficiently mimic the human gut.

A study by Fouhy *et al*., (2015) showed minimal effects of freezing on the faecal microbiota using both culture-dependant and sequencing methods and established that freezing of faecal samples was a viable method of storage prior to analysis. In addition, O' Donnell *et al*., (2016) demonstrated the suitability of using a standardised frozen faecal inoculum prepared from fresh samples for faecal fermentation experiments. In this study we compared the method of O' Donnell *et al* with a faecal inoculum prepared from samples which had been individually frozen in 15% glycerol and subsequently pooled and refrozen in glycerol before use. Pooling of faecal samples to create a standard has been established as an accepted method

and used by a number of groups (Rajilic-Stojanovic *et al*., 2010, Minekus *et al*., 1999, Van Neunen *et al*., 2003, Aguirre *et al*., 2014). Glycerol is a commonly used cryoprotectant for bacteria for many years, however protocols for glycerol concentration to be used are not well documented or standardised. Glycerol has been used at concentrations up to 50% (median 10%) for long-term storage of bacteria (Hubalek *et al*., 2003). In this study we used glycerol (final concentration of 15%) as a preservation media for faecal samples that were to be frozen at -80^{o}C prior to standardisation (Method B). We hoped that storage of faecal samples in 15% glycerol would have protected microbial communities found in stool through two freeze thaw cycles, allowing cells to successfully resuscitate and grow during faecal fermentation. However sequencing results show that many bacterial groups do not resuscitate following two freeze thaw cycles, despite the addition of glycerol and storage at -80^{o}C. High stresses are experienced by bacterial cells during freezing (formation of intracellular ice crystals) and thawing (osmosis) cycles. Furthermore, thawing at 37^{o}C as described in this protocol may be too fast and shock cells so they do not resuscitate. In addition glycerol may be used as a substrate for some organisms allowing their proliferation and outgrowth of organisms which may not prefer glycerol as a substrate. In a batch fermentation system clostridia and enterococcus bloom, competitively inhibiting lower abundance organisms which may have slower growth rates. In addition, adding of nutrients to the glycerol for storage may select for the growth of organisms which grow better in the presence of these nutrients.

In conclusion, faecal slurry prepared from fresh faecal samples immediately prior to fermentation has been the established methodology (Rea *et al*., 2011, Fooks and Gibson, 2003). Recently, O'Donnell *et al*., demonstrated that using a frozen

inoculum is an acceptable method for faecal fermentation experiments that is reproducible, stable allowing comparability of experiments run on different days. Here, due to logistical issues obtaining a sufficient number of faecal samples to prepare faecal standard on one day we investigated whether faecal samples could be frozen and thawed prior to the preparation of a pooled faecal inoculum which could subsequently be refrozen and thawed when required for faecal fermentation experiment. From the work reported here we conclude that subjecting stool to two freeze thaw cycles results in the loss in diversity, evenness and richness and results in a bloom of a small number of taxonomic groups such that the faecal inoculum is no longer an accurate representation of the composition of original stool samples.

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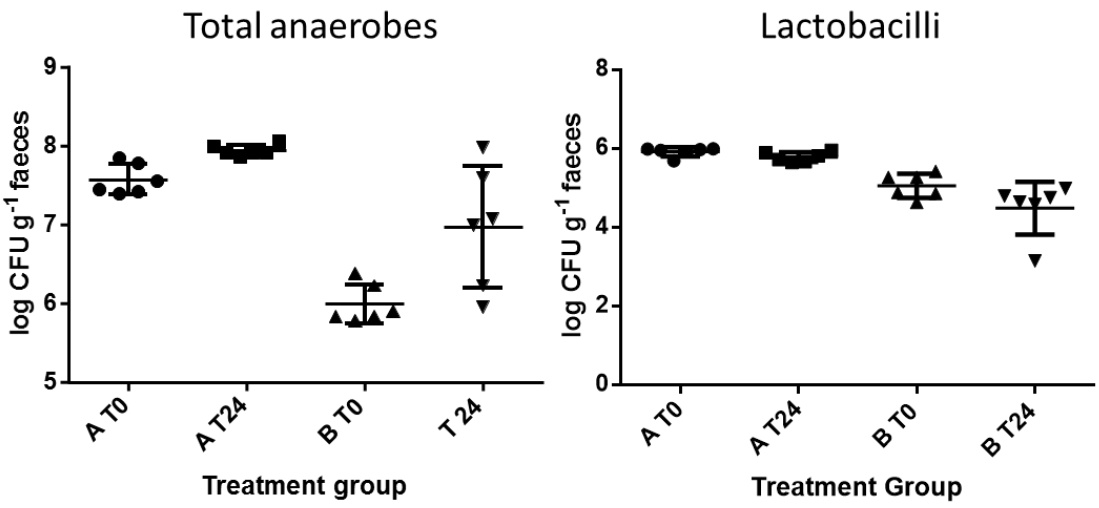

Figure 1. Viable counts (log CFU/g faeces) of total anaerobes and lactobacilli at time 0 (T0) and after 24h (T24) fermentation for faecal inoculum prepared by method A and method B.

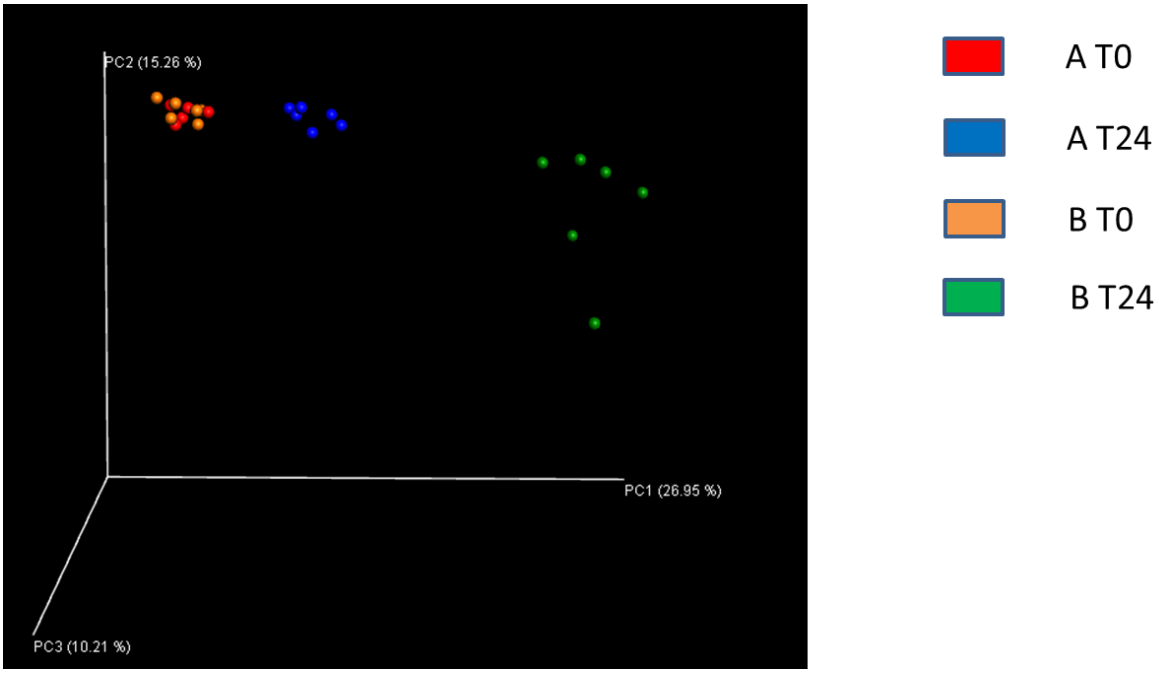

Figure 2. Principal Co-ordinate analysis (PCoA) using unweighted UNIFRAC distances visualising β-diversity of samples from method A T0 (red), method A T24 (blue), method B T0 (orange) and method B T24 (green).

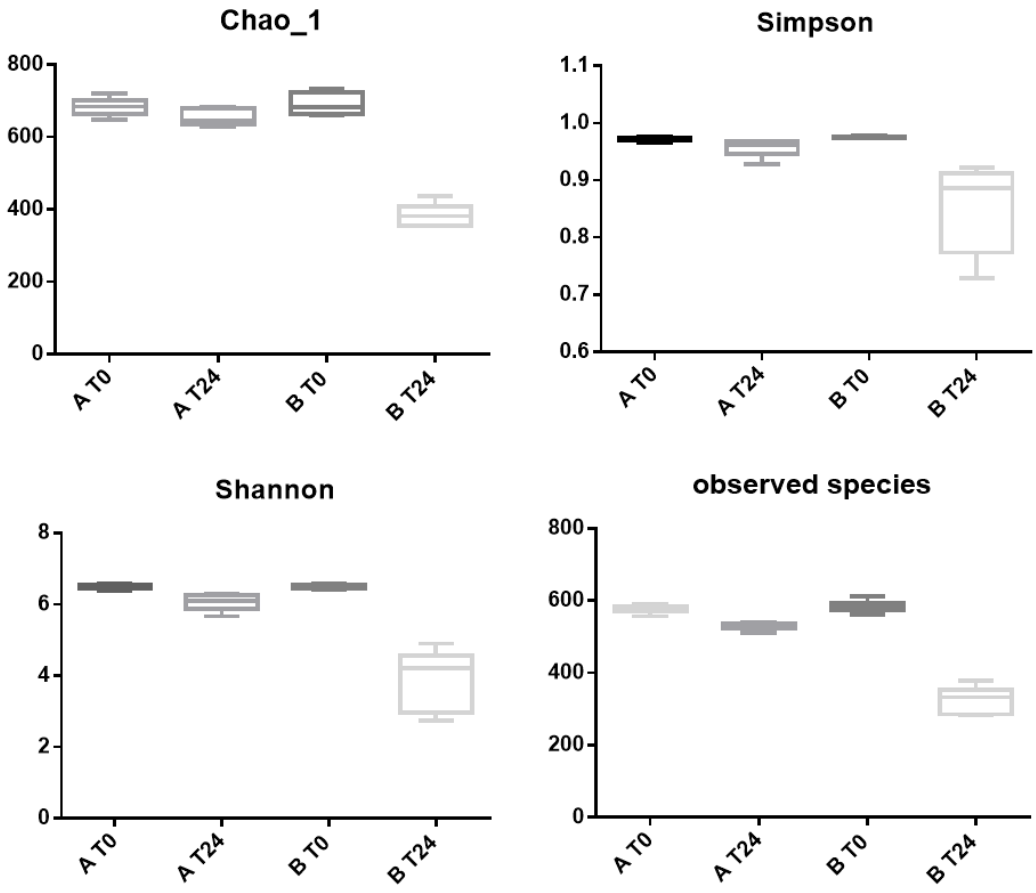

Figure 3. Alpha diversity metrics for Chao1, Simpson, Shannon and observed species for method A T0, method A T24, method B T0 and method B T24.

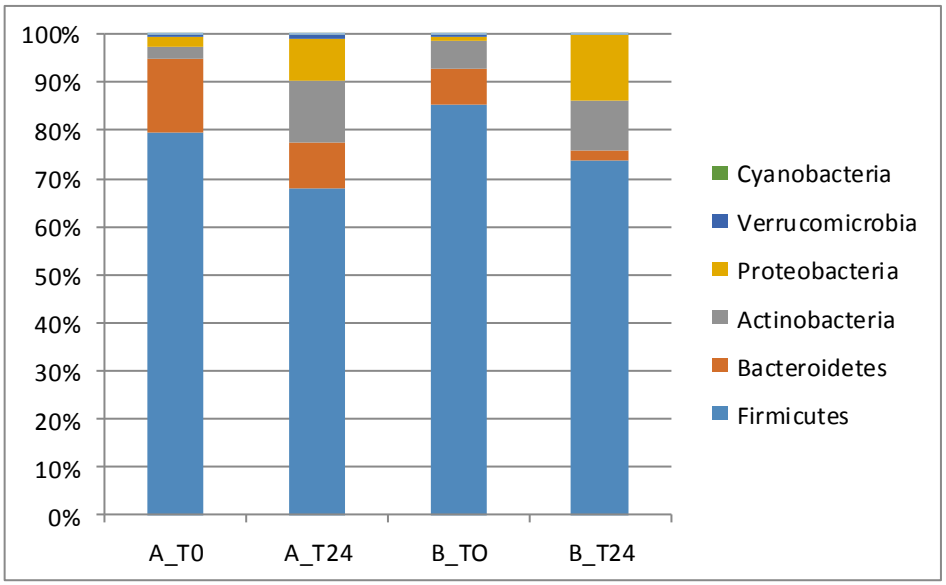

Figure 4. The relative abundance (%) of phyla (≥ 0.1%) for both methods of inoculum preparation A and B at beginning of fermentation (T0) and after 24h fermentation (T24).

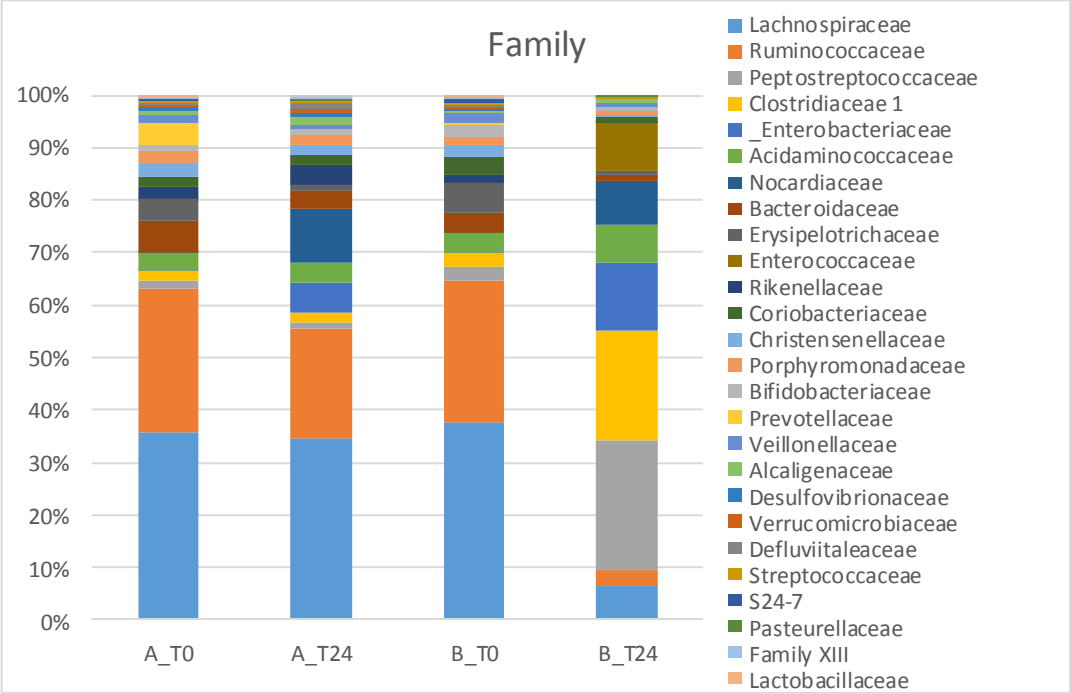

Figure 5. The relative abundance (%) of families (≥ 0.1%) for both methods of inoculum preparation A and B at T0 and after 24h fermentation (T24)

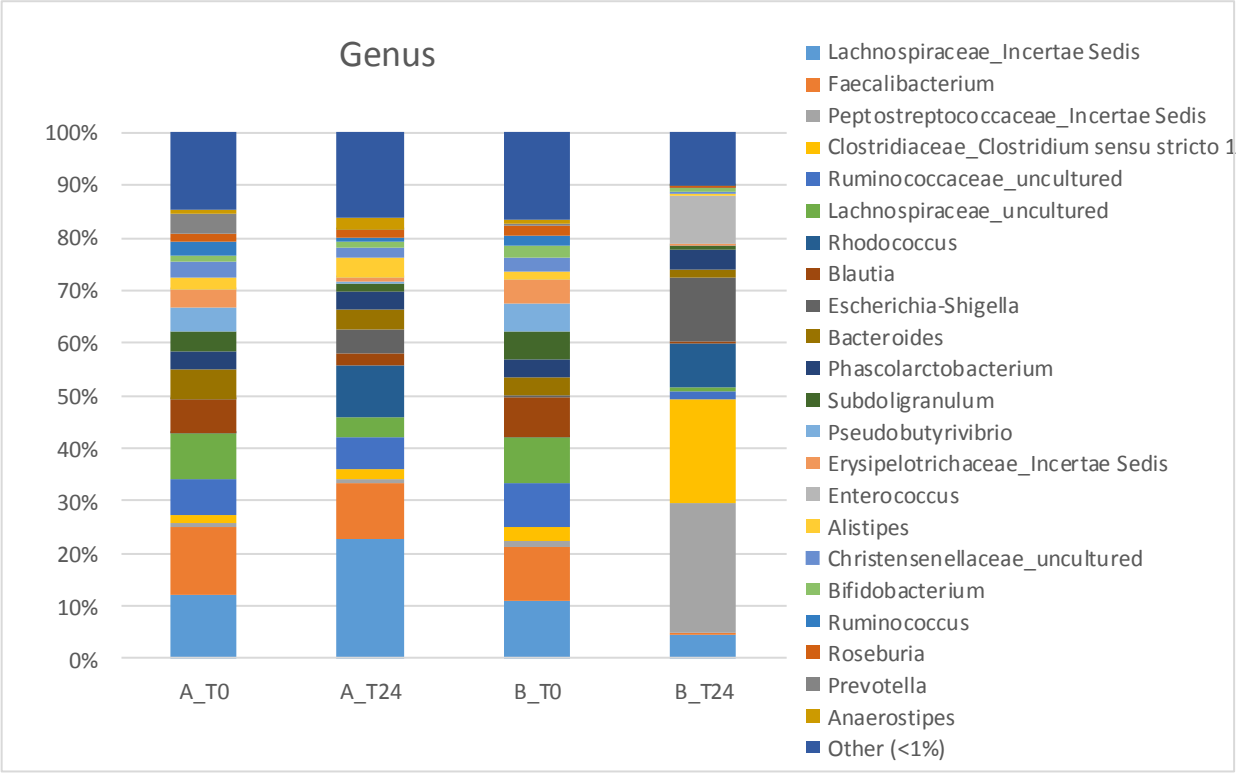

Figure 6. The relative abundance (%) of genera (≥ 0.1%) for both methods of inoculum preparation A and B at T0 and after 24h fermentation (T24).

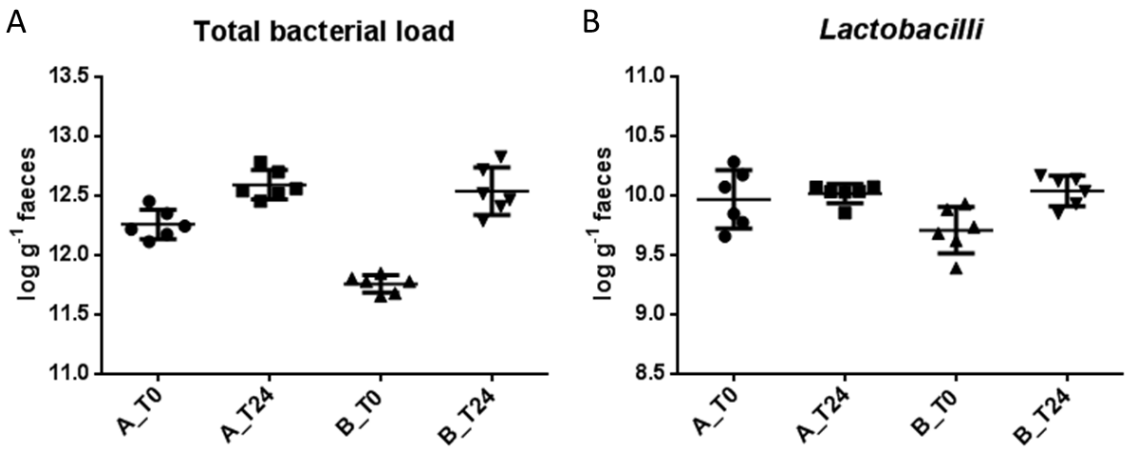

Figure 7. Quantification of total bacterial load (A) and lactobacilli (B) using quantitative PCR in method A and B prepared faecal inocula at time 0 (T0) hours and time 24 (T24) hours.

Chapter 8

General Discussion

The gut microbiota is a diverse and resilient ecosystem which responds to external influences such as diet (Lozupone *et al*., 2012, Flint *et al*., 2017, De Angelis *et al*., 2017) and antibiotic exposure (Langdon *et al*., 2016). Large scale studies, such as the European MetaHIT project (Metagenomics of the human intestinal tract) (Arumugam *et al*., 2011, Qin *et al*., 2010) and the US Microbiome Project (HMP) (HMP Consortium, 2012) have sought to establish what constitutes a healthy gut microbiota and how this differs in disease states. In recent years there has been increasing interest in understanding the gut microbiota in persons with Cystic Fibrosis. In an aging CF demographic, in which gastrointestinal manifestations are more frequently presented, a greater understanding of gastrointestinal complications and how the gut microbiota itself may influence the course of respiratory disease is necessary. Understanding the long-term effects of CF therapies, including chronic antibiotic therapy, on the gut microbiota and its functionality is important in order to gain knowledge on how intestinal-related comorbidities may be managed by manipulation of the microbiota to achieve a healthier composition with the ultimate goal of improving the overall health of the individual. The CF gut microbiota has been established to be distinct from healthy controls both in terms of composition and functionality (Burke *et al*., 2017, Fouhy *et al*., *under review*), most likely a result of the chronic antibiotic therapy, high fat diet and also the effects of the CFTR mutation itself. The gut microbiota of CF subjects has been shown to have reduced diversity from an early age through to adulthood in comparison with healthy controls and a positive correlation between lung function and gut microbiota diversity has been described (Nielsen *et al*., 2016, Burke et al., 2017, Scanlan *et al*., 2012). In particular, the reduction of diversity and abundance of *Bifidobacterium* species (*B.longum, B.catenulatum, B.pseudocatenulatum* and *B.adolescentis*), *Clostridium*

cluster XIVa and *Clostridium cluster* IV (*R. bromii* and *F. prausnitzii*) has been described (Duytschaever *et al*., 2013). In addition, the severity of CF disease and CFTR class has been shown to correlate with the magnitude of dysbiosis of gut microbiota (Schippa *et al*., 2013).

Validity of results and the value of research is rooted in robust experimental design. In order to reach reliable conclusions about the gut microbiota composition and evaluate manipulations of the gut microbiota the fundamental methodologies need to be validated. Logistical obstacles are often experienced by researchers in the collection of faecal samples for experiments, especially in the case of multicentre studies, such as CFMATTERS. In chapter 2 we established that the gut microbiota is stable following freezing, as surveyed by both next generation sequencing and culture dependant methods. This facilitated the undertaking of a multicentre analysis of the gut microbiota of CF persons, whereby samples could be frozen in dispersed sites and shipped to a central laboratory for analysis.

CF subjects have been shown to be frequently asymptomatically colonised with *C.difficile*. Since the 1980's, studies have shown *C. difficile* to be increasing in prevalence from 22% to 50% among CF cohorts (Welkon *et al*., 1985, Peach *et al*., 1986, Yahav *et al*., 2006, Burke *et al*., 2017). In this thesis we present the first multicentre analysis of *C. difficile* among CF persons, demonstrating the highest carriage rate reported to date of 63-71%, with 67% of isolates identified as toxigenic. Despite high carriage rates and detection of toxigenic *C.difficile* isolates, CF persons rarely present with CDI. CF persons presenting with CDI may have atypical symptoms resulting in delayed diagnosis and severe complications (Piccolo *et al*., 2017). The increasing prevalence of *C. difficile* among CF populations poses risks

for the general population, whereby CF populations may act as a silent reservoir of toxigenic *C.difficile* and also for the CF persons themselves due to presentation with atypical symptoms resulting in delayed diagnosis. Awareness of the increasing prevalence of *C. difficile* among CF populations may help to improve outcomes for both CF and non-CF populations. Promisingly, antibiotic susceptibility profiles for ciprofloxacin, vancomycin and moxifloxacin were similar to previous studies of antibiotic susceptibility of *C. difficile* isolate in CF cohorts. In addition, antibiotic susceptibility profiles of *C. difficile* isolates were similar to those reported in non CF populations, despite chronic antibiotic therapy in the CF population where it might be expected to increase antibiotic resistance in this gut pathogen. Reassuringly, all isolates tested were susceptible to vancomycin, the first line therapy for severe CDI. We report resistance towards metronidazole in two isolates (001 and 039), which was not reported in a previous study from our group (Burke *et al*., 2017). Reports of *C. difficile* resistance to metronidazole are found in the literature as (Pelaez *et al*., 2008, Kociolek *et al*., 2016). Furthermore, Lynch and colleagues (2013) reported a stable resistant isolate which was identified as ribotype 001. Emergence of resistance towards metronidazole is concerning and will need to be monitored in future studies to establish if this resistance is transient or reflective of a trend among clinical isolates. Ribotyping of isolates from three centres revealed distinct geographical clustering of ribotypes. A core of two ribotypes (046 and 078) were found to be common to all centres. Heterogeneity of ribotypes found among the three European centres surveyed in this study, may reflect low levels of spread between different geographical regions within Europe in the CF population, which is reassuring in the possible event of an epidemic involving virulent strains. Furthermore, the comparison of *C .difficile* ribotypes identified in this study to ribotypes identified in

similar geographic regions in recent years highlights the rapidly changing epidemiology of *C. difficile*. Finally, this study underlines the need for surveys of the gut microbiota to be longitudinal in nature, as sampling at single time points only gives a snapshot in time and may not reflect the true carriage rate. The dynamic fluctuations of the gut microbiota pose issues not only for studies endeavouring to define a 'healthy' gut microbiota composition, but also for studies such as ours which endeavour to survey for specific pathogens. In this study we established that *C.difficile* is a transient coloniser of the gut microbiota, the true prevalence of which may be missed in cross sectional studies.

The lung-gut axis is increasingly being recognised, along with the potential for using communication between the lung and the gut as a therapeutic tool, whereby manipulations of the gut microbiota may influence the lung microbiota (Budden *et al*., 2017, Skukla *et al*., 2017). The common co-occurrence of chronic lung diseases, such as asthma and COPD, along with chronic gastrointestinal diseases, such as IBD, highlights the importance of the lung-gut axis (Roussos *et al*., 2003, Rutten *et al*., 2014). CF itself, while primarily a disease of the respiratory system characterised by inflammation and infection, has multiple gastrointestinal co-morbidities. The role the gut microbiota plays in influencing health at distal sites and how it may be manipulated to direct host health is an area of much research. Further highlighting the role of the gut microbiota in respiratory health, studies (Hoen *et al*., 2015) have shown the reduction of *Parabacteroides* in the gut prior to chronic respiratory colonisation by *P. aeruginosa*. They also showed significant associations between gut microbial communities and early-life CF exacerbations, highlighting the crucial role of gut-lung communication. Madan *et al*., (2012) have shown that certain genera in the gut presage their appearance in the lung. Manipulation of the gut microbiota

with the aim of influencing a healthier lung microbiota composition is a potential tool that could be harnessed to improve respiratory health outcomes. Probiotics are a commonly used method for gut microbiota manipulation. Studies have shown the respiratory health benefits of probiotic therapy in CF cohorts (Bruzesse *et al*., 2004, 2014, 2007). In Chapter 4 we isolated a putative probiotic, *L. plantarum* 9_S2 with antibiotic tolerance to commonly used CF antibiotics (vancomycin, metronidazole and ciprofloxacin). *L. plantarum* 9-S2 has the potential to be used as an adjunct therapy along with antibiotics with the aim of maintaining a healthier gut microbiota composition, which in turn influences respiratory health. However, evaluation of the effect of *L. plantarum* 9_S2 on the gut microbiota composition and whether it may show respiratory benefits in a CF cohort will need to be further evaluated in future studies.

Ivacaftor, a CFTR potentiator, is the first CF therapy to treat the defective CFTR protein rather than the resultant symptoms. Ivacaftor has been shown by clinical trials to be effective in improving BMI, FEV_1, sweat chloride levels and rate of pulmonary exacerbations in CF subjects heterozygous for the G551D gating mutation (Ramsey et al., 2011 Davies et al., 2013). In addition, gastrointestinal specific effects were seen in the significant increase of intestinal pH to levels comparable with a non-CF cohort within one month of commencing treatment (Gelfond *et al*., 2017). G551D is prevalent at 4% internationally and at 23% in the Cork region, presenting a unique opportunity to access a relatively large cohort and examine the effects of Ivacaftor on the gut microbiota. In chapter 5 we examined the effects of Ivacaftor therapy on the gut microbiota composition, intestinal inflammation and pancreatic function. Subtle, non-significant changes in the gut microbiota were observed which were considered beneficial and may correspond

with a subtle normalisation of the gut microbiota. Increases in Bacteriodetes, Bacteroidaceae, *Bacteroides, Parabacteroides, Veillonella* and a parallel decreases in Firmicutes, Enterococcaceae, *Enterococcus,* Prevotellaceae, *Prevotella* and Streptococcaceae, *Streptococcus*, *Blautia* and *Escherichia* may be reflective of the gut moving towards a healthier composition at phylum, family and genus levels. Pancreatic insufficiency is observed in 80-90% of CF persons (Kristidis *et al*., 1992) and is managed through enzyme supplements, such as Creon, accompanying meals to aid nutrient absorption (Fieker *et al*., 2011). No significant difference in pancreatic function was observed post-Ivacaftor in the adult cohort studied here. In light of the results of the KIWI study (Davies *et al*., 2016) which showed the partial restoration of pancreatic function in children aged 2-5 years, the fact that no change in pancreatic function was seen over the course of the study suggests that pancreatic insufficiency is established in an adult cohort. Furthermore, a case study from our group (Howlett *et al*., 2016) concurred with results the KIWI study, demonstrating restoration of pancreatic function to normal levels 6 months post Ivacaftor and a reduction at 7 month to level consistent with moderate pancreatic insuffiency in a 6 year old subject. Our study addresses the paucity of information on the effect of Ivacaftor on pancreatic function in an adult cohort. However further longitudinal studies in adults beyond 12 months are needed to investigate if pancreatic function in this cohort is established or may be corrected following a longer period of intervention with Ivacaftor. Intestinal inflammation is a hallmark of CF, detected from early childhood, which is independent of pancreatic disease (Bruzesse *et al*., 2014, Werlin *et al*., 2010). We showed that intestinal inflammation, determined by measures of faecal calprotectin and lactoferrin, is not significantly reduced in an adult cohort following 12 months of Ivacaftor treatment. Bruzzese and colleagues

(2004) showed the reduction of intestinal inflammation in children (mean age 10.7 years) following a 4 week intervention with *L. rhamnosus* GG. In contrast, following intervention with Ivacaftor no significant change was seen in intestinal inflammation after one year in an adult cohort. This suggests that intestinal inflammation is established in an adult cohort and reduction of inflammation does not occur in 12 months. These subjects will need to be followed for longer than the 12 month period of this study to investigate if intestinal inflammation will reduce after a longer period of intervention with Ivacaftor.

In chapter 6 we investigated the effects of Ivacaftor on the intestinal microbiome capabilities and metabolome processes using a subset of subjects from chapter 5. We noted subtle alterations at low abundances in the microbiome and metabolome which may indicate normalisation of the gut microbiota. Significant increases in the microbiome post-Ivacaftor were observed in pathway abundances relating to protein metabolism, carbohydrate metabolism and vitamin synthesis, however no changes were observed in the high level functional categories relating to these pathways. Importantly, PCA plot of the microbiome shows subjects clustering by individual rather than by Ivacaftor treatment status, indicating that changes induced by Ivacaftor are not greater than inter-individual changes. Furthermore, chapter 6 investigates the gut microbiota antibiotic resistance capabilities post-Ivacaftor. Although not significant we observed a trend towards a decrease in antibiotic resistance gene hits post-Ivacaftor which corresponds to reductions in multidrug, tetracycline, aminoglycoside and Macrolide/lincosamide/Streptogramin (MLS) antibiotic resistance classes. Studies have shown that antibiotic resistance may persist for years following the antibiotic therapy (Jakobsson *et al*., 2010, Jernberg et al., 2007). The Ivacaftor studies add to knowledge on how the gut

microbiota behaves following chronic antibiotic therapy. Recovery of the gut microbiota composition and reduction of antibiotic resistance capabilities is slow and subtle.

In both Ivacaftor studies we noted subtle changes in the gut microbiota composition and functionality after 12 months, despite significant clinical changes reported in clinical trials. Intervention with Ivacaftor has not yet shifted their gut microbiota to a new or normal-like state. Subtle changes seen are consistent with 'nudges' towards normalisation however the gut microbiota composition or functionality cannot be considered to be normal following 12 months treatment with Ivacaftor. Further longitudinal monitoring of adult post-Ivacaftor subjects will be needed to establish if these subtle changes will continue to accumulate resulting in a complete shift of the gut microbiota environment. In summation, the Ivacaftor compositional and functional studies underline the resilience of the gut microbiota in the event of intervention. The gut microbiota will sustain a certain amount of perturbation before it will change to a different equilibrium state (Lozupone *et al*., 2012). This event has not yet occurred in the Ivacaftor subjects with the 12 month period studied.

Distal colon models are valuable tools for assessing the effectiveness of interventions on the gut microbiota prior to proceeding to costly animal trials. However, established methodologies require faecal samples to be collected on the day of experimentation, which is difficult to plan and reliant on collection of enough samples for experimentation to proceed. Previously, our group has established the validity of a method for preparation of a faecal inoculum, whereby faecal samples can be collected, pooled and frozen for use in subsequent distal colon model

experiments, which streamlines work for researchers (O' Donnell *et al*., 2016). However, for our study it was not possible to collect the required number of faecal samples in the recommended period of 4 hours from a CF cohort, an issue experienced by many who are working with specific disease groups. In chapter 7 we investigated the viability of a method for preparation of faecal inoculum for distal colon model experiments. We investigated whether samples could be collected, frozen individually in 15% glycerol, pooled when the required number of samples was reached and re-frozen for subsequent experiments. This was shown not to be a suitable method as two freeze thaw cycles resulted in an altered microbial community in terms of both diversity and composition compared to faecal inoculum prepared as described by O'Donnell *et al*. (2016). In future, alternative methods for preparation of faecal inoculum will need to be validated and established in order to perform robust distal colon models to evaluate interventions on the CF gut microbiota. Such methods are also transferable to other cohorts (e.g infants) that experience difficulties in obtaining sufficient volumes of stool in short collection period windows for colon model experiments.

To date a wealth of knowledge has been gathered on the gut microbiota, its functions and alterations in various disease states, including CF. However, moving forward from correlations to causal relationships and effective use of the gut microbiota to treat diseases such as CF requires future intervention studies manipulating the gut microbiota and evaluating its effect on the respiratory health of the host. In particular, further studies are required which establish how fluctuations in the lung and gut microbiome are related during periods of stability and exacerbation to give an improved understanding of how the gut microbiota may play a role in directing the lung microbiota towards a healthier composition.

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