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Prevalence and control of *Clostridium difficile* in patients with cystic fibrosis

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

By

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Declaration

This Thesis has not been previously submitted, in part or in whole, to this or any other university for any degree and is, unless otherwise stated, the original work of the author

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Author Contribution

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

Chapter 2

Daniel Burke and Dr Mike Harrison are joint first authors of this chapter. All experimental work for this chapter was carried out by Daniel Burke. Statistical analysis for this chapter was performed by Dr Mike Harrison

PCR-ribotyping for this study was performed at the *Clostridium difficile* Ribotyping Network for England (CDRNE) at Leeds University Hospital, Leeds, UK.

Chapter 3

Dr Orla O'Sullivan conducted part of the bioinformatics analysis of pyrosequencing data.

Chapter 4

Dr Mary Rea performed pulsed field gel electrophoresis (PFGE) on *Clostridium difficile* strains and dendographic analysis of the resulting PFGE profiles

Dr Caitriona Guinane performed dendograpgic analysis of *Clostridium difficile* sequence types.

Chapter 5

Dr Jakki C. Cooney and Dr Todd F. Kagawa generated and analysed the bacteriophage ϕCD6356 endolysin model.

_____

Daniel Burke

Abstract

The overall aim of this thesis was to investigate the cause of high prevalence of *Clostridium difficile* in patients with cystic fibrosis (CF), to monitor it and ultimately to control its spread.

Initially the carriage rate of *C. difficile* and characterisation of the strains present in patients with CF was investigated (Chapter 2). *C. difficile* was found in 50% (30/60) of CF patient stool, compared to just 2% (2/99) of healthy controls. *C. difficile* toxin was detected in the stool of 31.66% (19/60) of CF patients, all of which contained *C. difficile* strains capable of producing toxin. Toxin was not detected in any stool samples containing toxin negative *C. difficile* strains. *C. difficile* strains detected were typed using PCR-ribotyping. This revealed sixteen distinct ribotypes in CF patients. The most prevalent of which was ribotype 140 which did not produce toxin. However, the second most prevalent was ribotype 046 which is closely related to the hyper-virulent ribotype 078 which was also reported in this study. All strains detected were susceptible to commonly prescribed anti-*C difficile* drugs including, metronidazole, vancomycin, fusidic acid and rifampicin, while they were highly resistant to drugs commonly prescribed to treat Pseudomonal lung infections. No correlation was observed between the carriage of *C. difficile* and any recorded patient clinical parameters or treatment received.

The aim of Chapter 3 was to investigate the effect of CF and its treatment on the composition of the intestinal microbiota of patients with CF by comparing it to that of a healthy volunteer group. Overall, the CF associated microbiome had reduced microbial diversity relative to the healthy volunteer group. This was accompanied by a shift towards an increased Firmicutes to Bacteroidetes ratio. The most profound differences in composition were observed between the CF associated microbiota and that of healthy controls, with eight phyla, twenty-seven families, and forty-nine genera significantly increased or decreased between these two groups. However, compositional differences were also reported between CF patients grouped by patient clinical

parameters and treatments received. The CF associated microbiome is therefore altered from that of healthy controls by a combination of the disease and its treatment.

In Chapter 4, multilocus sequence typing (MLST) was assessed as an alternative to PCR-ribotyping (PCR-RT) for typing *C. difficile* in patients at high risk of *C. difficile* infection. Comparison of the discriminatory power of MLST and PCR-RT was performed and compared to that of pulsed field gel electrophoresis (PFGE) for typing *C. difficile* strains. The index of discrimination (ID) of MLST (0.93) compared favourably with that of the more commonly used PCR-RT (0.95). However, both were found to be less discriminatory than the lesser used PFGE method. While MLST yielded easily interpretable and transferable results allowing for rapid identification and typing of *C. difficile* in high risk patient groups, its current cost may prove prohibitive to its wide-scale adoption.

In chapter 5, the *C. difficile* bacteriophage ΦCD6356 and its biologically active endolysin were assessed as novel antimicrobials for the treatment of *C. difficile* infection. The bacteriophage was effective at reducing viable *C. difficile* by 1.75 logs over 24h in an *in vivo* model of the human distal colon. However, following initial infection all surviving *C. difficile* were immune to infection due to the uptake of the prophage (super infection). The endolysin encoded by ΦCD6356, an n- acetylmuramoyl -l-alanine amidase named LysCD was cloned and expressed in *Escherichia coli*. The recombinant endolysin was biologically active as evident bylysis of *C. difficile* cells in a zymogram. Treatment of mid log phase *C. difficile* cells with a crude preparation of the endolysin resulted in roughly a 3 log reduction in viable *C. difficile* after 3h. The endolysin was modelled on the previously determined structure of a similar endolysin, CD27l. This revealed the presence of zinc coordination at the active site which was experimentally determined to be required for lytic activity. Due to its lysogenic nature, the *C. difficile* bacteriophage ΦCD6356 is not suitable for the treatment of *C. difficile* infection. However, its endolysin shows potential as a novel antimicrobial for treating *C. difficile* infection.

Publications

D. G. Burke, P. D. Cotter1, R. P. Ross, and C. Hill

Ch 15. Microbial production of bacteriocins for use in foods. *Microbial Production of Food Ingredients, Enzymes and Nutraceuticals.* Mar 2013; ISBN: 978-0-85709-343-1

Abbreviations

AAD Antibiotic associated diarrhoea

ATCC American type culture collection

BHI Brain heat infusion

BMI Body mass index

CCEY Cycloserine cefoxitin egg yolk agar

CDRNE Clostridium difficile Ribotyping Network for England

CDT *Clostridium difficile* toxin

CF Cystic fibrosis

CFTR Cystic fibrosis transmembrane conductance regulator

CFU Colony forming units

DGGE Denaturing gradient gel electrophoresis

DIOS Distal intestinal obstruction syndrome

DNA Deoxyribonucleic acid

DPC Dairy production centre

EDTA Ethylenediaminetetraacetic acid

FAA Fastidious anaerobic agar

FEV_1% Predicted Forced expiratory volume in one second expressed as a percentage of the average for persons of the similar age, sex, and body composition.

GI Gastrointestinal

GIT Gastrointestinal tract

IBD Inflammatory bowel disease

IBS Irritable bowel syndrome

ID Index of discrimination

Ig Immunoglobulin

Il Interleukin

IPTG Isopropyl β-D-1-thiogalactopyranoside

IV Intravenous

IVAB Intravenous antibiotics

kDa kilo Dalton

LGG *Lactobacillus rhamnosus* GG

MI Meconium ileus

MIC Minimum inhibitory concentration

MIC_{50} Minimum inhibitory concentration required to inhibit 50% of strains tested

MIC_{90} Minimum inhibitory concentration required to inhibit 90% of strains tested

MIP-2 Macrophage inflammatory protein - 2

MLST Multilocus sequence typing

MOI Multiplicity of infection

MRD Maximum recovery diluent

OD Optical density

OR Odds ratio

PCR Polymerase chain reaction

PFGE Pulsed field gel electrophoresis

PFU Plaque forming units

PGE Prostaglandin E

$PGF_{2\alpha}$ Prostaglandin F2 alpha

PPI Proton pump inhibitor

PT Pulse type

RCM Reinforced clostridial medium

RT Ribotype

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SFI-CSET Science foundation Ireland centre for science, engineering and technology

SIBO Small intestinal bacterial overgrowth

ST Sequence type

TNFα Tumour necrosis factor alpha

WT Wild type

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Chapter 1

A review of the effects of cystic fibrosis and its treatment on the intestinal microbiota

1.1 Abstract:

Cystic Fibrosis (CF) is an autosomal recessive disease resulting from a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Progressive pulmonary failure resulting from chronic infection of the CF lung is the number one cause of death in people with CF. The lung microbiome of CF has extensively been studied, however, the effect of CF associated gastrointestinal disorders, and intensive therapies prescribed to treat pulmonary infection on the composition of the CF gut microbiota have received comparatively little attention. The disease itself and the antibiotics used to treat it undoubtedly cause a constant state of flux in the gut microbiota which will in itself impact on patient health. In this review, we discuss the impact of CF and its treatment on the gut microbiota, and suggest strategies to minimize this impact to improve patient care.

1.2 Introduction:

Cystic Fibrosis (CF) is the most common fatal autosomal recessive disease among people of European decent, affecting one in every 2,500 live births (Welsh MJ, 1995). The disease affects multiple organs and manifests itself in the pancreas, liver, gastrointestinal tract, and most notably, the lungs. Cystic fibrosis results from a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which codes for a chloride ion transport protein expressed in exocrine epithelial cells (Bear et al., 1992). Dysfunction of this transport protein causes abnormal mucus production resulting in airway surface liquid depletion and adhesion of thick viscous mucous to pulmonary epithelia. This reduces mucilliary clearance in the lungs, facilitating bacterial colonization and consequently the development of chronic pulmonary infection (Matsui et al., 1998). Progressive lung disease resulting from chronic pulmonary

infection is the most common cause of morbidity and mortality patients with CF and, therefore is the focus of the majority of CF therapeutic strategies (Ramsey, 1996).

The term "cystic fibrosis" was first used to describe plugging of glandular ducts in the pancreas of malnourished infants. The disease was characterized by malabsorption, failure to thrive, and pulmonary infection believed to arise from damage to the pancreas resulting in malnutrition. In 1938, the life expectancy of a child diagnosed with CF was only 6 months (Andersen, 1938). Now thanks to improvements in disease management, life expectancy has improved dramatically. Analysis of US, England and Wales death registry data has revealed that CF life expectancy has increased by 0.543 life years per year between 1972 and 2009 (Hurley et al., 2014). However, with the CF patient population aging, new challenges are emerging.

Development of the human gut microbiota begins at birth. Vaginally-delivered babies are predominantly colonized by *Lactobacillus* and *Prevotella* species derived from their mother's vaginal canal during delivery (Dominguez-Bello et al., 2010). *Bifidobacterium* then become the dominant genus in the infant gut microbiota (Aires et al., 2011). Subsequently, metabolic niches in the GI tract are successively filled until the GI microbiota reaches an "adult-like" state (Koenig et al., 2010). The adult human gut microbiota is temporally stable and dominated by the phyla, Firmicutes, Bacteriodes, Proteobacteria, and Actinobacteria (Bäckhed et al., 2005). Composition of the gut microbiota is influenced by both extrinsic and intrinsic factors including, diet, age, environment, and host genetics (Benson et al., 2010, Turnbaugh et al., 2009). These environmental and genetic factors may account for large inter-subject variability frequently reported in the gut microbiota (Dethlefsen et al., 2008, Duytschaever et al., 2011). However, a "core" microbiota, consisting of greater than fifty taxa has been reported in nearly half of human faecal samples (Qin et al., 2010).

In recent years, the reduced cost of metagenomic sequencing has facilitated the study of the gut microbiota and its effects on human health. The gut microbiota has been described as having the metabolic capacity equal to that of a virtual organ (Bocci, 1992). In a healthy individual, the gut microbiota plays a number of important roles in digestion and health; therefore as with all organs, its malfunctioning can have a profound effect on health. A number of studies have observed alterations in the gut microbiota in disease situations such as obesity (Turnbaugh et al., 2006), inflammatory bowel disease (Kassinen et al., 2007), and type II diabetes (Larsen et al., 2010) when compared to that of healthy individuals. To date no such study has been performed on adult patients with CF. We propose that the changes in the intestinal physiology resulting from mutation in the CFTR gene, as well as the chemotherapeutic drugs prescribed to patients with CF significantly alter the gut microbiota resulting in additional negative effects on the patient. In this review, we discuss the GI manifestations of CF, its treatment, and their possible effect on the gut microbiota.

1.3 Physiology of CF GI Tract:

Mutation of the CFTR gene results in poor chloride transport by gut epithelia. Consequently, defective chloride regulation in the intestinal lumen results in highly viscous mucus secretions which have been implicated in slowed gastric transit and intestinal blockage reported in patients with CF (De Lisle, 2007, Hedsund et al., 2012). Gastrointestinal (GI) transit has been studied in patients with CF as well as CF animal models. In one study of GI transit in CF mice, it was reported that gastric emptying was slightly reduced, while transit in the small intestine was significantly increased compared to wild type controls (De Lisle, 2007). Antibiotic treatment was also found to increase intestinal transit time in WT mice, but had no effect on intestinal transit in CF mice. Therefore it was hypothesised that the gut microbiota may play a role in the

regulation of GI transit (De Lisle, 2007). This hypothesis was supported De Lisle et al., 2010 (De Lisle et al., 2010) who found a reduction in major prostaglandin (PG) degenerative gene expression in CF mice as well as in WT mice treated with antibiotics. This resulted in a significant increase in PGE2 and PGF2α which impaired intestinal circular muscle activity. Resolution of bacterial overgrowth by oral laxative was found to improve CF muscle activity by increasing the expression of PG degenerative genes, thereby decreasing PGE2 levels (De Lisle et al., 2010). While, Hedsund et al found oro-cecal transit time to be significantly increased in patients with CF (Hedsund et al., 2012), other studies have found inter-digestive mobility to be comparable to that of healthy controls (Hallberg et al., 2001, Hedsund et al., 2012). Despite these findings, constipation and intestinal obstruction are frequently reported in patients with CF (Doef et al., 2011) – both of which likely affect the microbial composition of the gut.

Meconium ileus (MI) is a disorder uniquely described in CF, occurring in 13 to 25 % of CF neonates (Doef et al., 2011). Meconium ileus occurs in the neonatal period and is characterized by complete intestinal blockage resulting from accumulation of viscid meconium. Distal intestinal obstruction syndrome (DIOS) is a similar condition which occurs in adults with CF (Doef et al., 2011). Blockage occurs after accumulation of viscid faecal material in the terminal ileum and proximal colon. DIOS occurs in 14 to16 % of patients with CF throughout their lifetime, with prevalence increasing with age (Andersen et al., 1990, Dray et al., 2004). As stated above, constipation is another GI manifestation of the disease which is frequently reported (Doef et al., 2011). Constipation is distinct from DIOS, resulting from gradual accumulation of faecal material and reduction in the frequency of bowel movements; while DIOS is defined by acute onset (Houwen et al., 2010). Between 26 and 47% of patients with CF report problems with constipation. Previous incidence of MI has been cited as a risk factor for developing constipation (Doef et al., 2011, Rubinstein et al., 1986). Typically, constipation and DIOS are successfully treated by administration of laxatives, however, some cases of DIOS and MI that do

not respond to this may require surgical intervention (Doef et al., 2011).Gastrointestinal disorders resulting in delayed gastric transit and intestinal blockage have been suggested to predispose patients to bacterial overgrowth (Dukowicz et al., 2007).

The efficiency of the host innate immune defence in the CF intestine is reduced due to limited dissolution and accumulation of paneth cell granules within intestinal crypts (Clarke et al., 2004). This impedes the transport of antimicrobial peptides to the intestinal lumen, potentially facilitating overgrowth by pathogenic bacteria (Clarke et al., 2004). Small intestinal bacterial overgrowth is an overgrowth of bacteria in the small intestine in excess of 106 CFU/ ml, typically with colonic-type microbes (Gasbarrini et al., 2007, Gregg, 2002). Small intestinal bacterial overgrowth has been reported to occur in 30 – 50 % of patients with CF, mainly determined by the breath test to determine bacterial fermentation resulting in hydrogen or methane gas formation (Fridge et al., 2007, Lewindon et al., 1998, O'Brien et al., 1993). Small intestinal bacterial overgrowth affects the normal function of the small intestine which can result in diarrhoea, steatorrea, and macrolytic anaemia (Hoffmann and Zeitz, 2002). Orocecal transit may also be delayed as a result of small intestinal bacterial overgrowth (Virally-Monod et al., 1998). Resolution of small intestinal bacterial overgrowth by antibiotic therapy in diabetic patients has been shown to significantly improve orocecal transit. A study of bacterial overgrowth in CFTR null mice, revealed a forty fold increase in bacterial load in the CF mouse small intestine compared to controls (Norkina et al., 2004). It was also demonstrated that oral antibiotic therapy with ciprofloxacin and metronidazole not only reduced bacterial load by over 400-fold, but also reduced the expression of inflammatory markers in the small intestine (Norkina et al., 2004). Therefore, it is important to treat small intestinal bacterial overgrowth as it can have multiple detrimental effects on the CF gut, including but not limited to gastrointestinal inflammation.

Pulmonary as well as gastrointestinal inflammation is a typical feature of CF pathogenesis. This is evident by the observation of increased expression of inflammatory markers, both in patients with CF, and in CF animal models (Bruzzese et al., 2004, Koller et al., 1996, Norkina et al., 2004, Raia et al., 2000, Smyth et al., 2000). Smyth et al observed that paediatric CF patients have increased output of albumin, IgG, IgM, eosinophil, cationic protein, neutrophil elastase, IL-1β, and Il-8 in the gastrointestinal tract, indicating immune activation in the gastrointestinal tract (Smyth et al., 2000). Kahn et al, reported increased neutrophil and IL-8 in the lungs of paediatric CF patients prior to colonization with known pathogens using culture-based techniques (Khan et al., 1995). At the time of this study, highly sensitive molecular based techniques were not available and thus un-culturable pathogens may not have been detected. In addition, Smyth et al made their conclusion without assessing the bacterial population in the small intestine and thus cannot be certain inflammation was not due to the presence of pathogenic bacteria.

Resolution of bacterial overgrowth in the small intestine by antibiotic administration has been shown to reduce inflammatory markers in CF mice (Norkina et al., 2004). Further to this, supplementation with the probiotic, *Lactobacillus GG* has been shown to reduce the intestinal inflammatory biomarkers, faecal calpronectin, and nitric acid (Bruzzese et al., 2004). In inflammatory bowel disease (IBD) patients, significant decreases in microbial diversity as well as significant alteration of their microbial composition have been described relative to that of healthy controls (Frank et al., 2007). It is likely that, as in some IBD patients, alterations in the CF gut microbiota may be responsible for observed intestinal inflammation.

With an aging CF population, the risk of cancer undoubtedly increases. In a retrospective cohort study, patients with CF were found to be at increased risk of developing digestive tract cancer; however risk of developing all other cancers were comparable (Neglia et al., 1995). Similarly, IBD patients have been shown to have an increased risk of developing certain

digestive tract cancers (Bernstein et al., 2001). Intestinal inflammation resulting in oxidative stress has been proposed for the increased risk of colorectal cancer observed in IBD patients (Itzkowitz and Yio, 2004). Therefore the CF gut microbiota might offer a target to reduce the risk of digestive tract cancer in patients with CF by reducing inflammation.

1.4 CF intestinal microbiota

In a study of the development gut and lung microbiota of CF infants Bacteriodetes, Bifidobacterium and Veillonella were reported as the most abundant genera in the CF gut, accounting for 40% of sequencing reads (Madan et al., 2012). The gut microbiota of CF infants increases in diversity over time responding to changes in diet (Dominguez-Bello et al., 2011, Madan et al., 2012). In another study by Duytschaever et al, 2011, denaturing gradient gel electrophoresis (DGGE) analysis of the gut microbiota of paediatric patients with CF found them to have comparable species richness to that of their healthy siblings. However, the gut microbiota of patients with CF was found to be more variable in its compositional complexity and have more pronounced fluctuations over time than that of their healthy siblings (Duytschaever et al., 2011). As part of the same study, selected bacterial groups were enumerated by culture based techniques which revealed that levels of Bacteriodes/Prevotella were found to be 1.5 mean log CFU g-1 lower in paediatric patients with CF than in their siblings with borderline significance ($P = 0.07$). While counts of *Enterobacteriaceae* (0.5 mean log CFU g-1 difference) and *Clostridia* (0.2 mean log CFU g-1 difference) were higher in CF patients (Duytschaever et al., 2011). Longtitudal analysis of the gut microbiota of these CF patients over 2 years revealed higher counts for all bacterial groups in siblings, except for Enterobacteriaceae which was again found to be higher in CF patients (Duytschaever et al.,

2011). Temporal fluctuations in the composition of CF gut microbiota and decreases in bacterial numbers are undoubtedly as a result of frequent antibiotic disturbances.

1.5 Effect of CF treatment on gut microbiota:

Proton pump inhibitors

Pancreatic insufficiency is a common feature of CF, affecting 80 – 85% of patients with CF (Werlin et al., 2010). This is typically corrected by treatment with pancreatic enzyme supplementation. However, steatorrea may persist due to failure of pancreatic enzymes to be released in an acidic environment (Proesmans and De Boeck, 2003). Duodenal pH has been shown to drop post prandial due to impairment of bicarbonate secretion in the CF duodenum. Treatment with proton pump inhibitors increases duodenal pH, thereby improving fat absorption (Proesmans and De Boeck, 2003). Proton pump inhibitors are widely prescribed and are considered very safe (Vanderhoff and Tahboub, 2002). However, gastric acid is a significant barrier to bacterial colonization and its neutralization can increase the risk of enteric infection. In fact, in a systematic review to evaluate the association between acid suppression and enteric infection, Leonard et al., 2007 found an increased risk (adjusted odds ratio [OR] 3.33, 95% Confidence Interval [CI] 1.84–6.02) of enteric infection in those taking proton pump inhibitors (Leonard et al., 2007). In one study investigating the risk of small intestinal bacterial overgrowth in non-CF patients receiving proton pump inhibitors , small intestinal bacterial overgrowth was detected by glucose hydrogen breath test in 50% of patients, compared to just 6% of controls. The authors also noted an increase in prevalence of small intestinal bacterial overgrowth after one year of proton pump inhibitor use (Lombardo et al., 2010). This is of particular interest as patients with CF with pancreatic insufficiency who do not respond to pancreatic enzymes alone will require proton pump inhibitor therapy for their entire life, greatly increasing their risk of

small intestinal bacterial overgrowth. A correlation between the risk of *Clostridium difficile* associated diarrhoea and proton pump inhibitor use has also been reported by a number of authors (Kim et al., 2010). This may, in part, explain the high prevalence of *C. difficile* reported in patients with CF (Yahav et al., 2006).

Antibiotic treatment

Chronic pulmonary infection resulting in obstructive pulmonary disease is the number one cause of morbidity and mortality in patients with CF, accounting for 90% of deaths (Abman et al., 1991). Colonization of the lungs with *Pseudomonas aeruginosa* can occur at a very young age (Burns et al., 2001) and has been shown to correlate with a more rapid reduction in lung function than in those free from *P. aeruginosa* infection (Govan and Nelson, 1992). Therefore, in their life long battle with chronic pulmonary infection patients with CF are likely to undergo countless courses of aggressive antibiotic therapy to control this infection. Due to the polymicrobial nature of CF pulmonary infection combinations of broad spectrum antibiotics may be required. Early treatment with a combination of oral ciprofloxacin and nebulized colistin has been shown to eradicate *P. aeruginosa* in up to 80% of patients with CF (Hansen et al., 2008). Long term maintenance therapy with oral azithromycin improves lung function, reduces need for oral antibiotics, and results in fewer pulmonary exacerbations (Wolter et al., 2002).

But what are the long term implications of prolonged antibiotic treatment for pulmonary infections on the gut microbiota? With the advent of next-generation sequencing platforms, such as the Roche 454 and Illumina sequencers, it has been possible to make detailed observations of compositional shifts in the GI microbiota in response to antibiotic treatment (Southern et al., 2012). Extensive reviews on the effect of antimicrobial treatment on the gut microbiota are available by Rafii, 2008 (Rafii et al., 2008) and Cotter et al, 2012 (Cotter et al., 2012), however,

in this section we will focus on antibiotics typically prescribed to treat pulmonary infection in CF.

Ciprofloxacin is an effective anti-pseudomonal antibiotic frequently prescribed to patients with CF (Church et al., 1997). Dethlesfsen et al 2008 (Dethlefsen and Relman, 2011) examined the effect of 5 days of twice daily treatment with 500 mg ciprofloxacin in humans and reported an initial reduction in species richness, diversity, and evenness in the gut microbiota. However, after four weeks, the gut microbiota was found to mostly recover to pre-treatment composition. After six months a number of taxa failed to return to pre-treatment numbers, including *Clostridiales* and *Bilophia* (Dethlefsen et al., 2008). Similar observations were made in another study following repeated courses of ciprofloxacin. However, alterations in community composition were found to be stable over the final two months of a ten month study (Dethlefsen and Relman, 2011). Therefore antibiotics prescribed to treat *P. aeruginosa* in the CF lung are likely to have a profound and lasting effect on the CF gut microbiota.

Oral clindamycin can be used to treat Staphylococcal pulmonary infection in patients with CF (Shapera et al., 1981). However, clindamycin use has been shown to select for clindamycin resistant *Bacteriodetes* clones, which dominate the community after treatment (Jernberg et al., 2007). While these resistant clones were not detected in the gut, it is likely they would also be present due to the swallowing of sputum. Antibiotic therapy can reduce clonal diversity and select for antibiotic resistant bacteria which can persist in an environment even after removal of antibiotic selective pressure (Jernberg et al., 2007). In one study examining the effect of short-term antibiotic treatment on the gut microbiota, high levels of the macrolide resistance gene erm (B) were detected up to four years post- treatment, despite a lack of selective pressure (Jakobsson et al., 2010). Antibiotic resistance in anaerobic faecal microbiota has also been shown to increase relative to the duration of antibiotic therapy as well as hospital stay (Stark et al., 1993).

The healthy gut microbiota can act as a barrier to colonization, preventing overgrowth by potential pathogens. Disruption of the indigenous gut microbiota can result in a reduction in colonization resistance allowing for colonization by opportunistic pathogens, such as *Clostridium difficile* (Croswell et al., 2009, Pépin et al., 2005). Fluoroquinolones, including ciprofloxacin, have emerged as a major risk factor for the acquisition of *C. difficile* (Pépin et al., 2005). The risk of *C. difficile*-associated diseases is also increased in respect to duration of antibiotic treatment and use of multiple antibiotics in combination (Owens et al., 2008). Administration of two antibiotics by intravenous injection for 14-21 days has been suggested as appropriate treatment for pulmonary exacerbations in patients with CF (Turpin SV, 1993). Therefore antibiotic treatment coupled with the above mentioned proton pump inhibitors are likely contributes to the high carriage of *C. difficile* in patients with CF (Yahav et al., 2006).

1.6 Minimising the effect of antibiotic therapy on the gut microbiota:

While it is not possible to cease the use of antibiotics in patients with CF, it may be possible to reduce their effect of the gut microbiota. Oral and intravenous (IV) antibiotics prescribed to treat pulmonary infection can modify the gut microbiota either directly by transit through the GI tract or by systemic dissemination, reaching the gut through GI secretions (Edlund and Nord, 1999). Aerosolized antibiotic therapy offers a valuable alternative to oral and IV antibiotics for the treatment of pulmonary infection. The advantages of aerosolized antibiotics are that the drug quickly reaches high concentrations at the site of infection while undergoing minimal systemic penetration (Zarogoulidis et al., 2013). This minimizes the impact of the antibiotic on the gut microbiota while still providing an effect treatment (Geller et al., 2002).

As stated previously, maintenance therapy with azithromycin can result in the development of macrolide resistant bacteria (Hansen et al., 2008, Phaff et al., 2006, Tramper-

Stranders et al., 2007). However, short term (< 6months), treatment with azithromycin has been shown to improve lung function (Tramper-Stranders et al., 2007), reduce the frequency of pulmonary exacerbations (Southern et al., 2012), and reduce the prevalence of *Staphylococcus aureus*, *Streptococcus pneumonia* and *Haemophilus influenza* (Hansen et al., 2009). Despite an increase in macrolide resistance among *S. aureus* strains following long term low doses of Azithromycin, no resistance was observed in *S. pneumonia* or *H. influenza.* Strict control of *S. aureus* and reduction in its prevalence were proposed to negate the emergence of macrolide resistance on treatment outcome (Hansen et al., 2009). Reduction in the frequency of pulmonary exacerbation greatly reduces the burden of antibiotic treatment on patients with CF due to the length and intensity of treatment required to treat exacerbations (Turpin SV, 1993).

There is increasing evidence to suggest host immune modulation occurs in response to probiotic administration, both in IBD patients, as well as in patients with CF (Bruzzese et al., 2004, Meijer and Dieleman, 2011). As well as this, some studies have reported probiotics as having a protective role against respiratory infection (Alvarez et al., 2001, Bruzzese et al., 2007, Weiss et al., 2010). For instance, *Lactobacillus casei* has been shown to enhance the clearing of *P. aeruginosa* from the lungs of young non-CF mice (Alvarez et al., 2001). In another study, treatment with the commercial probiotic *Lactobacillus rhamnosus GG* (LGG) was found to significantly lower the risk of upper respiratory tract infection in children (Hojsak et al., 2010). Interestingly, similar protective effects were observed in two separate pilot studies in patients with CF with LGG and, Bio-plus (*Lactobacillus bulgaricus, Bifidobacterium bifidum*, and *Streptococcus thermophiles*). In both studies, probiotic therapy was shown to significantly reduce the frequency of pulmonary exacerbation. (Bruzzese et al., 2007, Weiss et al., 2010). Probiotic therapy may therefore be useful in reducing pulmonary exacerbations and thus, the burden of antibiotics on patients with CF. However, much larger scale trials are required due to the low numbers involved in these studies (36 and 10 respectively).

Therapeutic regimes for patients with CF, while effective may potentially be harmful to the GI microbiota. Patients with CF are likely to be at greater risk of enteric pathogens, including *C. difficile,* due to the action of proton-pump inhibitors and antibiotics. Frequent and prolonged antibiotic treatment is also likely to select for and maintain antibiotic resistance. Therefore patients with CF may act as a reservoir for *C. difficile*, as well as antibiotic resistant pathogens.

1.7 Conclusions:

From observations made in patients with CF as well as in other disease states, it is clear that the CF gut microbiota is likely to differ significantly from that of healthy individuals, undoubtedly undergoing frequent fluctuations due to the disease and its treatment, resulting in lower temporal stability and diversity. However, due to a lack a study incorporating high-throughput sequencing to examine the composition of CF gut microbiota, the extent to which it differs will largely remain unknown. Careful consideration should be taken by clinicians in the treatment of CF so as to reduce antibiotic induced alteration of the gut microbiota as well as to prevent the spread of *C. difficile* and antibiotic resistant pathogens. As understanding of the CF gut microbiota improves, it may become a target for treatment of gastrointestinal and pulmonary disease in patients CF by way of probiotic administration and targeted nutrition.

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Table 1.1 Gastrointestinal disorders typically associated cystic fibrosis, their treatment, and prevalence.

GI disorder	Description	Treatment	Prevalence
Meconium ileus	Complete intestinal blockage resulting from accumulation of viscid mucous in the neonatal period.	Laxatives and/or surgical intervention	13 – 25% (neonates)
Distal intestinal obstructive syndrome	Complete blockage of the terminal ileum and proximal colon due to accumulation of viscid mucus.	Laxatives and/or surgical intervention	14 – 16% (adult)
Small intestinal bacterial overgrowth	Overgrowth of bacteria in the small intestine exceeding 10^6 bacteria /ml of luminal fluid. Colonic-type species are also typically reported.	Antibiotics	30 – 50%
Constipation	Infrequent or difficult bowel movements.	Laxatives	26 – 47%
Steatorrea	Poor fat absorption resulting in excess faecal lipid content.	Pancreatic enzyme supplementation and proton pump inhibitors	80 – 85%

Chapter 2

Cystic fibrosis suffers have a high incidence (50%) of *Clostridium difficile* carriage including hypervirulent strains.

2.1 Abstract:

Clostridium difficile carriage is frequently reported in patients with cystic fibrosis (CF). Despite high carriage rates and the presence of *C. difficile* toxin in stool, patients with CF rarely appear to develop *C. difficile* infection (CDI). In this study, we examined the carriage and ribotype distribution of *C. difficile* in a cohort of 60 adult patients with CF who were pre-transplant. *C. difficile* was detected in 50% (30/60) of patients with CF by culturing for the bacteria. *C. difficile* toxin was detected in 63% (19/30) of *C. difficile* positive stool samples. All toxin positive stool samples contained toxigenic *C. difficile* strains harbouring toxin genes, tcdA and tcdB. Despite the presence *of C. difficile* and its toxin in patient stool, no acute gastrointestinal symptoms suggesting CDI were reported. Ribotyping of *C. difficile* strains revealed 16 distinct ribotypes (RT), including the hypervirulent RT078. All strains were susceptible to vancomycin and metronidazole as well as fusidic acid and rifampicin. As expected, all strains were resistant to antibiotics prescribed to treat pseudomonal and other gram negative infections in the lungs. No correlation was observed between carriage of *C. difficile* and any recorded patient clinical parameters or treatment received.

2.2 Introduction:

Progressive pulmonary disease resulting in respiratory failure is the leading cause of morbidity and mortality in patients with CF. However, median predicted survival continues to increase, rising from approximately 28 years in 1990 to 41 years in 2012 using US data (Marshall, 2013) Analysis of European data suggests a predicted median survival of 50 yrs for the birth cohort of the year 2000 (Dodge et al., 2007). This increasing survival is likely due to a number of factors including: the co-ordination of care through specialised CF centres using a multi-disciplinary approach, improved nutritional support, and the development of new therapeutic agents to treat

CF, including inhaled anti-pseudomonal antibiotics, inhaled mucolytics, maintenance macrolide therapy, inhaled hypertonic saline, and the intense use of intravenous antibiotics (Plant et al., 2013, Ramsey et al., 1999, Fuchs et al., 1994, Elkins et al., 2006, Saiman et al., 2003) However, as people with CF survive longer, new focus has been brought on extra-pulmonary disease in CF(Yahav et al., 2006a, Theunissen et al., 2008, Smyth et al., 2000, Duytschaever et al., 2011b), and its potential role in improving health outcomes and survival. CFTR-dysfunction in the gut has long been associated with reduced intestinal motility due to accumulation of mucus in the gut (Hedsund et al., 2012), distal intestinal obstruction (Dray et al., 2004), small intestinal bowel overgrowth (Fridge et al., 2007), and based on recent data in a murine model, altered composition of the gut microbiota (Lynch et al., 2013). Emerging data suggest a role of the gut and, specifically, the gut microbiota, in influencing health outcomes for people with CF (Munck, Duytschaever et al., 2011a, Duytschaever et al., 2012, Bruzzese et al., 2007, Weiss et al., 2010). With increasing survival and increasing cumulative antibiotic exposure (Rabin et al., 2004), manipulation of the "gut-lung axis" in the health of people with CF may provide novel therapeutic strategies to improve health outcomes. Interestingly, the role of the gut microbiota may extend beyond influencing gastrointestinal health in CF. A recent study reported that colonization of the respiratory tract by microbes is presaged by colonization of the gut and demonstrated a role of nutrition in development of the respiratory microbiota (Madan et al., 2012).The use of broad-spectrum antibiotics has been shown to influence the gut microbiota in non-CF populations causing significant alterations in the gut microbiota, in particular the overgrowth of *Clostridium difficile* resulting in CDI and pseudomembranous colitis (Gorbach, 1999, Bartlett et al., 1978). Limited data exist regarding the effect of repeated courses of broad-spectrum antibiotics on alterations in the CF gut microbiota, including the incidence of *C. difficile* (Duytschaever et al., 2011a, Duytschaever et al., 2012, Wu et al., 1983b, Welkon et al., 1985b, Peach et al., 1986a, Pokorny et al., 1992, Yahav et al., 2006a). Asymptomatic carriage of

C. difficile has been reported in up to 46.6% of CF patients (Yahav et al., 2006a), compared to 1.1% - 15% in healthy adults (Nakamura et al., 1981, Aronsson et al., 1985, Clayton et al., 2012). Despite high carriage rates and the presence of toxin in stool, CDI in people with CF is rare (Nakamura et al., 1981, Viscidi et al., 1981, Yahav et al., 2006a, Wu et al., 1983b, Welkon et al., 1985b, Peach et al., 1986a). However, the risk of CDI increases 3-fold in CF patients undergoing lung transplantation (Theunissen et al., 2008). This is particularly worrying, considering the atypical presentation and high mortality rate (50%) associated with CDI reported in post-transplant CF patients (Dallal et al., 2002, Yates et al., 2009).

To better understand carriage of *C. difficile* in people with CF and its influence on health outcomes, patients attending the Cork Adult Cystic Fibrosis Centre at Cork University Hospital were assessed for carriage of *C. difficile* and presence of its toxin in their faeces. *C. difficile* isolates were also characterised by ribotype and antibiotic susceptibility. Finally, we examined the relationship between *C. difficile* carriage, CF patient clinical parameters, and a same-site healthy volunteer group.

2.3 Materials and methods:

Subject recruitment and sample collection.

Consecutive adult patients with CF who were pre-lung transplant were recruited prospectively on attendance at routine adult outpatient clinics and at presentation to the Cystic Fibrosis Ambulatory Care facility at Cork University Hospital over an 18-month period beginning in January 2012. All participants donated a fresh 5 gram stool sample at study entry. Post-transplant patients with CF were excluded from this analysis. Participant clinical characteristics were recorded at time of study entry including age, gender, genotype, pancreatic sufficiency

status, clinical status at study entry (i.e. clinically stable patients or patients experiencing a pulmonary exacerbation), best FEV1% predicted and body mass index (BMI) over the 12 months prior to study entry, along with the number of days of intravenous antibiotics and the number of inpatient days in the 3 years prior to study entry. Use of macrolides, proton pump inhibitors, and H2-antagonist in the 6-months prior to study entry was also recorded. A control group consisting of 99 healthy volunteers was recruited from the university. These volunteers were aged between 18 and 65 years (61 female) and had not received antibiotics or reported gastrointestinal symptoms in the previous 6 months. Informed written consent was obtained from all participants in accordance with local research ethics committee guidelines. The study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals.

Isolation of Clostridium difficile.

Samples were collected from subjects at the Cork Adult Cystic Fibrosis centre and frozen at -20˚C within 24h of sampling. *C. difficile* were isolated from stool by ethanol shocking and plating on cycloserine cefoxitin egg yolk agar (Lab M, Bury, United Kingdom), as previously described (Clayton et al., 2009). Isolates displaying characteristic *C. difficile* morphology and "horse-stable" odour were further assessed microscopically and biochemically. Those found to be Gram-positive anaerobic spore-forming rod-shaped bacteria that were non-haemolytic, L-proline aminopeptidase positive and who gave a positive result using the Oxoid *Clostridium difficile* test kit (Oxoid Basinstoke, UK) were determined to be *C. difficile*. Strains were stocked at -80˚C on Microbank beads (Pro-Lab Diagnostics, Ontario, Canada). For routine use, strains were sub-cultured onto Fastidious Anaerobic Agar (FAA) (Lab M, Heywod, Lancs UK) containing 7% (w/v) defibrinated horse blood and grown anaerobically in a Don Whitley anaerobic chamber at 37˚C.

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 respectively for the presence of tcdA and tcdB toxin genes. *C. difficile* ATCC 700057 was used as a control strain for antibiotic susceptibility testing.

PCR ribotyping.

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

Enzyme immunoassay for C. difficile toxin and detection of toxin genes, tcdA and tcdB by PCR.

In vivo toxin production was assessed from stool samples using the commercial kit, Toxin A+/B+: ProSpecT II (Oxoid). Assays were performed according to manufacturers' instructions. DNA was extracted from isolates according to Rea et al (Rea et al., 2011). Briefly, *C. difficile* isolates were streaked onto horse blood agar and 5-6 colonies were picked and suspended in 5% w/v Chelex-100 resin. Colonies were then heated to 56ºC for 30 minutes, followed by 100ºC for 8 minutes. The cell suspension was then centrifuged in a desktop centrifuge for 3 minutes at 16,000 x g to remove cell debris. This was then used as DNA template for amplification of the toxin genes tcdA and tcdB using the primers described by Terhes et al, 2004.(Terhes et al., 2004). Amplification of each gene was performed in separate reaction mixtures (25 µl) containing 12.5 µl Biomix Red (Bioline, London, UK), 0.5µl of each forward and reverse primer (tcdA or tcdB) 2 µl template DNA and 9.5 µl H2O. Gene amplification consisted of denaturation

at 95°C for 20 s, annealing at 62°C for 45 s, and extension at 72°C for 45 s. PCR products were visualized following electrophoresis in 1% agarose gel with ethidium bromide (0.5 μg/ml) in Tris-Acetate EDTA buffer (40mM Tris, 20mM acetic acid, and 1mM EDTA).

Antibiotic susceptibility.

The E-test system (BioMérieux, Hamshire, United Kingdom) was used to screen isolates for antimicrobial resistance against a range of commonly prescribed antibiotics for treating pulmonary exacerbations in patients with CF, as well as vancomycin, metronidazole, rifampicin and fusidic acid. The E-test was performed and interpreted as per the manufacturer's instructions; however Reinforced Clostridial Agar (Merck, Darmstadt, Germany) was substituted for the recommended media. *C. difficile* ATCC 700057 was included as an internal control. Minimum inhibitory concentration50 (MIC50) and MIC90, defined as the minimum inhibitory concentration observed to impede the growth of 50% and 90% of isolates tested respectively were calculated as described by Drummond et al (Drummond et al., 2003). Strains were deemed susceptible or resistant to the test antibiotic, according to documented pharmacological breakpoint values defined by the Swedish Reference Group for Antibiotics (SRGA) (http;//www.srga.org) where available.

Statistical analysis.

All statistical analyses were carried out using IBM SPSS Statistics 21. Chi-square testing was used to examine for a relationship between the carriage of *C. difficile* and gender, genotype, pancreatic sufficiency status and macrolide, PPI and H2-antagonist use. Mann-Whitney testing

was used to investigate the relationship between carriage of *C. difficile*, lung function, BMI and exacerbation rate.

2.4 Results

Sixty adult participants with CF were recruited. Table 1 describes the characteristics of the study cohort. None of the study participants reported new gastroenterological symptoms that were clinically suspicious for CDI at the time of enrolment.

Characterization of C. difficile isolates.

Standard culturing techniques were used to determine the rate of carriage of *C. difficile*. C. difficile was isolated from 50% (30) of patients with CF (see Table 2) while *C. difficile* was only detected in 2% (2) of healthy volunteers.

Detection of C. difficile toxin (stool antigen ELISA) and toxin genes (tcdA/tcdB)

C. difficile toxin was detected in 31.7% of faecal samples from patients with CF by ELISA. All *C. difficile* strains isolated from toxin positive faecal samples carried toxin genes, tcdA and tcdB as revealed by PCR amplification. *C. difficile* strains harbouring toxin genes were not found in any toxin-negative faecal samples.

PCR ribotyping and virulence.

PCR ribotyping of the *C. difficile* isolates revealed 16 distinct ribotypes (see Table 2). Eleven toxigenic ribotypes were detected, including the emerging hypervirulent ribotype 078. The most

commonly isolated toxigenic ribotype was 046, which, although not commonly disease-causing locally, this strain has been identified as disease-causing in a geographic cluster in Sweden (Akerlund et al., 2011). Four non-toxigenic ribotypes were also revealed, namely 009, 010, 039, and 140. One of the detected ribotypes was a novel ribotype not present in the *Clostridium difficile* Ribotyping Network database for England and Northern Ireland. Two distinct ribotypes were identified in the healthy control group; the rare toxigenic ribotype 062 and a non-toxigenic ribotype 026, neither of which were present in the CF cohort.

Antibiotic susceptibility.

The range of activity of an array of antibiotics against the *C. difficile* strains isolated is summarized in Table 3. Antibiotics that are commonly used in clinical practice, both in treating CF lung disease and in treating CDI were selected. MIC50 and MIC90 values for each determined. According to the pharmacological breakpoints recommended by the Swedish Reference Group for Antibiotics (SRGA) (http;//www.srga.org), a MIC90 of ≤ 2mg/L indicates susceptibility to both metronidazole and vancomycin. All *C. difficile* isolates tested in our study were susceptible to both metronidazole (MIC90 of 0.38μg/ml) and vancomycin (MIC90 of 0.75μg/ml).While there are no published clinical breakpoints for rifampicin, fusidic acid, meropenem or linezolid against *C. difficile*, these drugs performed favourably against the isolates recording MIC90 values of <0.002 μg/ml, 0.19μg/ml, 0.75μg/ml and 1.5μg/ml, respectively. High levels of resistance to azithromycin (>256 μg/ml), tobramycin (>256 μg/ml), aztreonam (>256 μg/ml), ceftazidime (>256 μg/ml), and ciprofloxacin (>256 μg/ml) were ubiquitous among isolates.

Relationship between Clostridium difficile and clinical parameters.

No significant associations were found between the presence of *C. difficile* and best FEV1 % predicted in the previous 12 months (p=0.173), age (p=0.07), PPI use(p=0.796), pancreatic insufficiency (p=0.122), BMI (p=0.07), maintenance macrolide therapy (p=0.953), gender (p=0.752), Class 1-3 mutation (p=0.07), clinical stability at time of sample donation (p=0.538), number of courses of intravenous antibiotics (p=0.440) or number of inpatient days in the 3 years prior to study entry (p=0.541). Furthermore, there were no significant associations between either the presence of toxigenic *C. difficile* or the presence of a *C. difficile* isolate of a known disease-causing ribotype (i.e. virulent vs non-virulent) and any recorded clinical parameters.

2.5 Discussion

We report a *C. difficile* carriage rate of 50% in adult patients with CF who are pre-transplant. Earlier studies show a trending increase in *C. difficile* carriage in patients with CF with reported carriage rates in studies since the 1980s of 22% -50% (Wu et al., 1983b, Welkon et al., 1985b, Peach et al., 1986a, Yahav et al., 2006b, Pokorny et al., 1992). However, these earlier studies reported carriage rates in cohorts that may not be representative of current-day patients with CF. Wu et al from 1983 reported a carriage rate of 31% (n=11/35), with the carriage rate increasing to 50% (n=11/22) in patients with CF who were receiving continuous oral or intravenous antibiotics for at least 2 months prior to the time of culturing. Peach et al, 1986 (Peach et al., 1986b) reported a 32 % carriage rate of *C. difficile* in patients with CF, and 43% in adults with CF who were receiving antibiotics. Yahav et al, 2006 (Yahav et al., 2006a) reported a carriage rate of 46% (n=14/30) with a carriage rate of 74% (n=11/19) in the subgroup who were receiving continuous antibiotics at time of sampling. Wu et al, 1983 (Wu et al., 1983a) and Peach et al, did not isolate *C. difficile* from any patients not receiving antibiotics, while Yahav et

al, isolated *C. difficile* in 27% (n=3/11) of patients with CF who were not taking continuous antibiotics (Yahav et al., 2006b, Wu et al., 1983b). In our study, the carriage rate of *C. difficile* was 46% (n=6/13) in patients with CF receiving antibiotics. All of these patients were receiving short courses (≤ 2 weeks) of antibiotics for treatment of an infective pulmonary exacerbation. Most modern CF programs, including ours, do not routinely prescribe continuous oral antibiotics. While in earlier studies the carriage rate of *C. difficile* increased in patients receiving long-term or continuous anti-pseudomonal antibiotics, we did not see the same effect in the small number of patients in our study who were taking short courses of anti-pseudomonal antibiotics. This increased *C. difficile* carriage rate in all patients with CF without an increase in carriage rates seen in patients receiving either short-term or continuous antibiotic therapy in our study suggests that novel factors other than antibiotic usage that are as yet undetermined may contribute. Despite the relatively high carriage rate and presence of *C. difficile* toxin in faeces, no participants reported new GI symptoms that suggested occult CDI. Asymptomatic carriage of *C. difficile* despite the presence of toxin has been reported previously (Peach et al., 1986b, Welkon et al., 1985a). This may be due in part to CF patients (without a history of CDI) having higher circulating serum anti-toxin A and B antibodies than patients with CDI (Monaghan et al., 2013).

Cystic fibrosis intestinal dysbiosis has been demonstrated to cause alterations in the composition of the gut microbiota (Duytschaever et al., 2011a), which may allow a unique commensal microbiota profile that protects the host by preventing potentially pathogenic *C. difficile* from overcoming colonization resistance, proliferating within the colon, and producing toxins A and B. Cystic fibrosis patients often present with atypical symptoms and the diagnosis of *C. difficile* colitis can be missed (Binkovitz et al., 1999, Barker et al., 2008, Schmitt-Grohé et al., 2002). Furthermore, systemic symptoms of CDI are derived from toxin-induced inflammatory mediators (IL-1, IL-6, IL-8, TNF-α, MIP-2,) (Castagliuolo et al., 1998, Flegel et

al., 1991) released locally in the colon. Altered innate immune responses in CF (del Fresno et al., 2008, del Fresno et al., 2009, O'Connell O.J. et al., 2014) may result in a reduced ability or an inability to produce an inflammatory response to *C. difficile* toxin.

We report the detection of toxigenic *C. difficile* in 32% (n=19) of our total patient cohort based on the detection of toxins A/B in faeces and on the presence of toxin-producing genes tcdA and tcdB in genomic DNA of these isolates, which corresponds to the detection of toxigenic *C. difficile* in 63% (n=19/30) of all *C. difficile* isolates detected. The prevalence of toxigenic *C. difficile* in people with CF varies greatly between previous studies. Peach et al reports 75% (n=9) of isolates produced toxin B (cytotoxin) in vivo however, toxin B was only directly detected in the stool of 3 of these samples; detection of toxin A (enterotoxin) was not performed (Peach et al., 1986a). Welkon et al described 22% toxigenic *C. difficile* carriage based on the detection of cytotoxin (Welkon et al., 1985b). Wu et al found that only 27% of isolates were positive for cytotoxin while detection of enterotoxin was not performed (Wu et al., 1983b). In a more recent study (2006), toxin was detected in 14 of 30 samples tested, with toxin A detected in 3 of 14 and toxin A or B detected in 11 of 14 samples tested, while culture detection of *C. difficile* was not reported (Yahav et al., 2006b). The predominance of toxigenic *C. difficile* in these patients is in contrast to a recent study which reported high carriage of non-toxigenic strains in CF patients (Bauer et al., 2014).

The thirty *C. difficile* strains isolated in this study were distributed across 16 distinct ribotype (RT) groups. Four of the ribotypes reported in this study have appeared in the top five most prevalent disease-causing ribotypes reported in Ireland in the last four years (RT002, RT014, RT015, RT078), with RT014 and RT078 consistently the most prevalent ribotypes across the last 4 years of surveillance data (Executive, 2013). Ribotype 078 is associated with increased mortality, and has been responsible for an increasing number of CDI outbreaks in recent years, including an outbreak in an Irish population resulting in a mortality rate of at least

33% directly attributable to CDI (Burns et al., 2010). Ribotype 046 was the most prevalent pathogenic ribotype reported in this study accounting for 13.33% of *C. difficile* strains isolated. While, to date, ribotype 046 has not been associated with significant morbidity or mortality in an Irish population, it has been associated with CDI outbreaks in other countries (Bauer et al., 2011, Pituch et al., 2006, Akerlund et al., 2011). Both RT078 and RT046 have been found in pigs and could suggest zoonotic acquisition (Debast et al., 2009, Norén et al., 2013). The diverse nature of the PCR ribotypes reported in this study and the relative under-representation of commonly reported Irish ribotypes may support the theory of community acquisition as proposed by Clayton et al. (Clayton et al., 2009).

Subjects in this study received an average of 1.6 (SD ± 2.2) intravenous antibiotic (IVAB) courses spread over an average of 63.8 (SD ± 82.2) days in the 12 months prior to donation of their stool sample. The majority of study participants (79.4%) were receiving maintenance macrolide antibiotics in the previous 12 months. Antibiotic administration is frequently cited as a risk factor for the development of CDI in non-CF cohorts (Bignardi, 1998, Owens et al., 2008), with antibiotics commonly prescribed to treat pulmonary infection in people with CF (third-generation cephalosporins, fluoroquinolones and macrolides) associated with an increased risk of CDI (Bauer et al., Bignardi, 1998, Pépin et al., 2005, Gerding, 2004). Despite this, our study revealed no association between the presence of *C. difficile* and the use of short-term anti-pseudomonal antibiotics or continuous macrolide therapy over the previous 12 months.

Reassuringly, all isolates tested were susceptible to both metronidazole and vancomycin according to the breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (Testing, 2013). While breakpoints are not available for the other antibiotics tested, all detected isolates were sensitive to fucidic acid, linezolid, meropenem and rifampicin. As expected *C. difficile* isolates were resistant to antibiotics prescribed to treat

Pseudomonal and other gram-negative lung infections that have poor activity against Gram positive organisms (azithromycin, aztreonam, ciprofloxacin, ceftazadime and tobramycin).

2.6 Conclusion

C. difficile carriage in people with CF is common and appears to be increasing, with half of the adults in this study carrying *C. difficile* in their gut. The advent of ribotyping analysis allows us to comprehensively characterise the strains detected in people with CF, with many toxigenic and hypervirulent strains detected in patients who did not manifest symptoms of CDI.

C. difficile carriage in an asymptomatic patient with CF is an especially challenging clinical scenario given the potential consequences of CDI in the post-transplant period. A randomised controlled trial examining the effects of eradication of *C. difficile* on health outcomes in both pre- and post-transplant patients with CF is required to clearly establish the optimum strategy for management of *C. difficile* carriage in this cohort.

2. 7 Limitations

This study is a single-centre study and examines 60 patients with CF from a single geographical area, which may not represent the patterns of *C. difficile* carriage seen in other geographical areas. However the high carriage rate and significant rate of detection of virulent *C. difficile* amongst patients with CF are concerning and supports the role of a larger multi-centre study.

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Table 2.1: Characteristics of CF patient study cohort.

Variable	**n=60**
Age in yrs (median, interquartile range [IQR])	27 (24-37)
Male Gender	37 (62%)
FEV_1 % predicted (median, [IQR])	65 (46-83)
PPI use	31 (52%)
Macrolide use	46 (77%)
Pancreatic insufficiency	49 (82%)
Class 1-3 mutation	45 (75%)
Exacerbating at time of sample donation	14 (23%)
On IVAB at time of sample donation	11 (18%)
On po AB at time of sample donation	2 (3%)
Not on antibiotic therapy at time of sample donation	1 (2%)
BMI (median)	22.4 (23%)

Table 2.2: Toxin gene detection, direct stool toxin, ribotype and virulence of *Clostridium difficile* strains detected.

Patient (n=30)	*tcd-A	§tcd-B	Stool toxin (EIA)	Ribotype	Virulent
CF3	+	+	+	014	Yes
CF5.	+	+	+	002	Yes
CF7	-	-	-	039	No
CF8	+	+	+	126	Yes
CF9	+	+	+	001	Yes
CF10	+	+	+	001	Yes
CF13	-	-	-	140	No
CF15	+	+	+	078	Yes
CF18	-	-	-	140	No
CF21	-	-	-	140	No
CF22	-	-	-	009	No
CF24	-	-	-	010	No
CF26	+	+	+	001	Yes
CF27	+	+	+	046	Yes
CF29	+	+	+	014	Yes
CF34	+	+	+	046	Yes
CF41	+	+	+	045	Yes
CF44	-	-	-	Unknown	No
CF46	-	-	-	039	No
CF47	+	+	+	046	Yes
CF48	+	+	+	046	Yes
CF51	+	+	+	078	Yes
CF52	+	+	+	126	Yes
CF53	-	-	-	140	No
CF61	+	+	+	011	Yes
CF64	+	+	+	092	Yes
CF65	-	-	-	010	No
CF66	-	-	-	140	No
CF71	+	+	+	087	Yes
CF73	+	+	+	356	Yes

Table 2.3: Susceptibility of *Clostridium difficile* isolates to commonly used antibiotics

Antibiotic	Range of activity (mg/l)	*Antibiotic break point (mg/L)
Metronidazole	0.016 – 0.5	2
Vancomycin	0.19 – 0.5	2
Fusidic acid	0.016 – 0.75	n/a
Rifampicin	< 0.002	n/a
Meropenem	0.125 – 1	n/a
Linezolid	0.38 – 4	n/a
Ciprofloxacin	> 32	n/a
Ceftazadime	>256	n/a
Tobramycin	> 256	n/a
Aztreonam	> 256	n/a
Azithromycin	> 256	n/a

*Antibiotic break point represents the defined EUCAST clinical break point for *C. difficile*

Chapter 3

Cystic fibrosis and its treatment affect the composition of the intestinal microbiota.

3.1 Abstract

Cystic fibrosis (CF) is an autosomal recessive disease that affects the function of a number of organs, including the lungs and gastrointestinal (GI) tract of patients. The manifestations of CF in the GI tract, as well as frequent antibiotic treatment for pulmonary infection, undoubtedly disrupt the composition of the intestinal microbiota composition. To analyse the intestinal microbiota of CF patients, we compared the composition of 68 CF patients to that of 69 healthy non-CF controls through 16S rRNA amplicon sequencing. The impact of the clinical manifestation/progression and treatment of the disease were also assessed. Overall, the CF associated microbiome had reduced microbial diversity. This was accompanied by a shift towards an increased Firmicutes to Bacteriodetes ratio. While the greatest numbers of differences in taxonomic abundances of the intestinal microbiota were observed between CF patients and healthy controls, compositional differences were also reported between CF patients grouped by disease manifestation/progression or treatments received.

3.2 Introduction

The healthy human gut is populated with in excess of 1011 microbes per millilitre of luminal content (Savage, 1977). The composition of this gut microbial community is generally stable over time in healthy adults, however, inter-individual variation can be high, which is thought to be associated with extrinsic factors including age, diet, environment and host genetics (Costello et al., 2009, Malinen et al., 2005). This microbial community plays an essential role in healthy intestinal development, maturation of host immune response, and provision of metabolic capabilities otherwise unavailable to the host (Hooper, 2004, Hooper et al., 2002, Mazmanian et al., 2005, Round and Mazmanian, 2010). Alteration of the normal gut microbiota has been linked to a number of diseases including, diabetes, obesity, irritable bowel syndrome, and

irritable bowel disease (Kassinen et al., 2007, Larsen et al., 2010, Malinen et al., 2005, Scanlan et al., 2006, Turnbaugh et al., 2006).

Cystic Fibrosis (CF) is the most common life shortening autosomal recessive disease, affecting one in 2,000 Caucasians (Hodson., 1984). It is caused by a mutation in the Cystic fibrosis transmembrane conductance regulator (CFTR) gene, which can result in expression of a dysfunctional Cl- ion transport protein, or complete non-expression of this protein in epithelial cells. This prevents the adequate hydration in the lumen of tubular organs, resulting in viscous mucus accumulating along a variety of epithelial surfaces including the lungs and gastrointestinal (GI) tract (Greger, 2000). Patients with CF suffer from a number of gastrointestinal complications including, pancreatic insufficiency, slowed gastric transit, mal-absorption, and obstruction (Andersen et al., 1990, Bruzzese et al., 2004, De Lisle, 2007, De Lisle et al., 2010, Gasbarrini et al., 2007, Rubinstein et al., 1986). Progressive pulmonary disease due to chronic infection is the number one cause of death in CF patients. Therefore CF patients undergo frequent courses of broad spectrum antibiotics (Ramsey, 1996, Szaff et al., 1983). Previous studies in non-CF patients have shown antibiotic therapy to reduce the diversity of the intestinal microbiota as well as altering the relative abundances of susceptible bacterial species (Jakobsson et al., 2010, Jernberg et al., 2010).

Typically, the intestinal microbiota returns to a normal pre-treatment state within weeks of cessation of antibiotic therapy. However, it has been noted that some taxonomic changes can persist for long periods after antibiotic treatment (Dethlefsen et al., 2008, Dethlefsen and Relman, 2011, Jernberg et al., 2007, Jernberg et al., 2010). Frequent courses of antibiotics are also likely to increase the risk of developing antibiotic resistant strains, as well as reducing the hosts colonization resistance, allowing for colonization and proliferation of opportunistic pathogens such as *C. difficile* (Hirschhorn et al., 1994, Safdar and Maki, 2002). Previous investigations revealed that paediatric CF patients had lower counts of all taxa examined by

culture except for Enterobacteria and lower temporal stability in their gut microbiota relative to sibling controls (Duytschaever et al., 2011). Frequent antibiotic therapy to treat chronic pulmonary infection as well as the inherent effect of CF on the gastrointestinal tract have been proposed as possible causes of this dysbiosis in CF patients gut microbiota (Duytschaever et al., 2011). This theory is supported by studies conducted in CF mice which have demonstrated decreases in the richness, evenness, and diversity of the small intestinal microbiota relative to non-CF mice (Lynch et al., 2013). A study into the development of the gut and lung microbiome in paediatric CF patients revealed both microbial communities develop simultaneously and share a number of colonising species(Madan et al., 2012). It was also revealed that the appearance of some species in the gut can foretell of their appearance in the lungs, suggesting the gut microbiota may help shape the development of the lung microbiota (Madan et al., 2012). This coupled with the success of probiotic trials at reducing GI inflammation and exacerbation frequency in CF patients (Bruzzese et al., 2004, Bruzzese et al., 2007, Weiss et al., 2010) highlights the importance of understanding the CF gut microbiota and the effect of disease manifestation and its treatment on the gut microbiota.

Previous studies investigating the CF gut microbiota have varied in approach adopting both culture-dependent and culture-independent approaches in either paediatric CF patients (Duytschaever et al., 2011, Madan et al., 2012) or CF animal models (Lynch et al., 2013). In this study the effect of CF and its treatment on the gut microbiota of 68 adult patients with CF was investigated by high-throughput pyrosequencing. This study demonstrates that the gut microbiota of patients with CF is significantly different to that of a healthy control group. A shift towards an increased Firmicutes to Bacteriodetes ratio was also observed in patients with CF. Microbial diversity and composition were also found to be affected by the disease and its treatment.

3.3 Materials and methods

Study Participants.

A total of seventy three individuals with CF were recruited from the Cork Adult Cystic Fibrosis Centre, Cork University Hospital for this study. None of these patients were suffering any form of gastrointestinal illness at the time of sampling. Informed written consent was obtained from all subjects in accordance with local research ethics committee guidelines. We were unable to produce Amplicons from three patients stool samples, while a further two patients samples did not meet the minimum quality scores for sequencing and thus were not included in this study. Therefore sixty eight CF patients were entered into the study of which 61.4% were male and they ranged in age from 17 – 77 (median age 29)

A total of 69 non-CF volunteers were recruited from the greater Cork community as a control group for comparison of their gut microbiota. Non-CF volunteers were eligible for inclusion provided they were between 20 and 80, reported no gastrointestinal illness at the time of sampling, and had not received antibiotics within the previous 6 months.

The present study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals.

Pyrosequencing and bioinformatics

Genomic DNA was extracted from faecal samples as per the manufacturer's instructions using the QIAamp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK) with the addition of an initial bead-beating step. Samples were prepared for compositional sequencing by taq-based

PCR amplification of the V4 region of the 16s rRNA gene using universal 16S primers as described by Ribosomal Database Projects (RDP) Pyrosequencing pipeline (http://pyro.cme.msu.edu/pyro/help.jsp). The resulting amplicons were sequenced using a Roche 454 GSFLX by using Titanium chemistry at the Teagasc 454 sequencing platform. Raw sequences were quality trimmed using the Qiime Suite of programmes, any reads not meeting the quality criteria of a minimum quality score of 25 and sequence length shorter than 150bps for 16S amplicon reads were discarded. Trimmed FASTA sequences were BLASTed against the SILVA 16S rRNA (version 106) database. The resulting BLAST outputs were parsed using MEGAN (Huson et al., 2007). MEGAN assigns reads to NCBI taxonomies using the lowest common ancestor algorithm. Cut off bit scores were from within MEGAN to filter the results prior to tree construction an summarization; a cut-off bit score of 86 was used (Urich et al., 2008). Phylum, family, and genus counts for each individual were extracted from MEGAN. Operational taxonomic unit (OTU) assignment, chimera checking, clustering and α and β diversities of reads were implemented with QIIME (Caporaso et al., 2010). The resulting principal coordinate analysis was visualised within KiNG (http://kinemage.biochem.duke.edu).

Statistical analysis

The non-parametric Kruskal-Wallis test, applied using Minitab Release 15.1.1.0 (Minitab Inc., 2007), was used to estimate the relations between groups. Statistical significances were accepted at $P < 0.05$.

3.4 Results

Compositional differences in gut microbiota of people with CF compared to healthy controls.

The microbial composition of CF patients gut microbiota of was evaluated through high-throughput 16S amplicon sequencing derived from faecal samples. A total of 2,099,804 reads were sequenced, corresponding to an average 23,331 reads/ sample.

Principle coordinate analysis plots generated using weighted and un-weighted Unifrac distance matrix showed that CF patients clustered separately to healthy controls (fig. 3.1). However, CF patients did not cluster based on carriage of *Clostridium difficile* (previously reported Burke et al, 2014), phenotype (class 1-3 mutation, FEV1% <68%, pancreatic insufficiency) or treatments received (proton pump inhibitors, no. of home IVAB days, inpatient days, macrolide antibiotics or lung transplantation (figure 3.2). This suggests CF has a greater effect on the diversity of the gut microbiota than any individual treatment.

The gut microbiota of CF patients was found to be significantly less diverse healthy controls based on five diversity metrics (Chao1, Simpson, Shannon, and phylogenetic diversity and species richness) as shown in figure 3.3. Assigned sequence reads were used to assess differences in taxonomic abundances between CF patients and healthy controls at phylum, family, and genus levels. At the phylum level there was a significant ($P < 0.05$) decrease in the populations of Actinobacteria, Proteobacteria, Cyanobacteria, Verrucomicrobia, RF3, Tenericutes, and Lentisphaerae in patients with CF relative to healthy controls (figure 3.4 (a)). Notably there was a significant ($P < 0.05$) increase in Firmicutes of 8.18% in CF patients relative to healthy controls. At the family level, a total of 27 families were significantly ($P < 0.05$) different in CF patients compared to healthy controls (11 increased and 16 decreased). The largest difference was seen in *Lachnospiraceae* which was increased in CF patients by and 5.17% respectively. Conversely, decreases of 7.03%, 9.22% and 4.36% in *Rikenellaceae*,

Ruminococcaceae and *Prevotellaceae* respectively were found in CF patients relative to healthy controls. At the genus level 19 genera were increased and 30 were decreased in patients with CF. This included a 5.94% increase in *Lachnospiraceae Incertae Sedis* and decreases of 4.81%, 4.04%, and 4.09% in *Alistipes, Prevotella, and Faecalibacterium* respectively in patients with CF.

The effect of macrolide antibiotics on the gut microbiota of people with cystic fibrosis.

The effect of macrolide antibiotics in previous six months was investigated for its impact on the composition of the gut microbiota in CF patients. Macrolide antibiotics had been given to 58 of the 68 CF patients in this study in the previous 6 months. The composition of their gut microbiota was compared to that of the patients who had not received the antibiotic (n= 10). Patients receiving macrolide antibiotics had no significant differences in the diversity or species richness of their gut microbiota relative to CF patients who had not received macrolide antibiotics. At the phylum level, those receiving macrolide antibiotics showed significant ($P < 0.05$) decreases in Verrucomicrobia and Eukaryota. At the family level *Bacteroidaceae* were significantly ($P < 0.05$) increased however, *Bifidobacteriaceae Family XIII Incertae Sedis*, *Gammaproteobacteria, Akkermansiaceae, Pasteurellaceae,* and *Desulfovibrionaceae* were all significantly ($P < 0.05$) reduced. These changes were also evident at the genus level, with a significant ($P < 0.05$) increase *Bacteroides* and reductions in *Pseudobutyrivibrio, Bifidobacterium, Akkermansia, Acinetobacter,* and *Enterococcaceae* evident in those receiving macrolide antibiotics as can be seen in the supplementary material.

The effect of proton pump inhibitors on the gut microbiota of people with cystic fibrosis.

Proton pump inhibitors (PPI) are commonly prescribed to patients with CF to aid in pancreatic enzyme absorption in those that suffer malabsorption. The effect of PPIs on the gut microbiota of patients with CF was examined by comparing the composition of the gut microbiota of patients on PPIs (n = 37) to those not on PPI therapy (n = 31). No significant ($P > 0.05$) differences were observed in the diversity or species richness of CF patients receiving PPI therapy relative to those not receiving PPIs. At the phylum level, CF patients on PPIs had a significant ($P < 0.05$) decrease in *Fusobacteria* relative to those not receiving PPI. Not surprisingly, *Fusobacteriaceae* was significantly decreased at the family level, in CF patients receiving PPI therapy. At the genus level a significant ($P < 0.05$) increase in *Veillonella* was observed as well as significant ($P < 0.05$) decreases in the proportions of *Dialister*, *Subdoligranulum*, *Clostridiaceae*, and *Fusobacterium* in CF patients receiving PPI therapy.

The effect of duration of intravenous antibiotic treatment on gut microbiota.

To examine the effect of duration of intravenous antibiotic (IVAB) treatment on the gut microbiota, CF patients were divided into a high IVAB treatment days group (> 55days) and low IVAB treatment days group (< 55 days) depending on the number of days spent receiving IVAB in the previous year. The gut microbiota of the high IVAB treatment days group (n = 23) was compared to the low IVAB treatment days group (n = 45). The high IVAB treatment day's group had significantly ($P < 0.05$) less diverse gut microbiota and lower species richness relative to the low IVAB treatment days group. No significant differences were observed at the phylum level between CF patients in the high IVAB treatment days group and those in the low group. Four families were significantly different in CF patients in the high IVAB treatment days group. *Enterococcaceae* were significantly ($P < 0.05$) increased in this group while *Alcaligenaceae*,

Veillonellaceae, and *Prevotellaceae* were significantly ($P < 0.05$) decreased relative to CF patients in the low IVAB treatment days group.

The effect of duration of hospital stays on the gut microbiota.

To examine the effect of duration of hospital stay on the gut microbiota, CF patients who spent greater than 18 days as a hospital inpatient ($n = 13$) in the previous year were compared to those who had spent less than 18 days as an inpatient ($n = 55$). According to two of the five measurements used (Simpson and Shannon) CF patients with a high number of inpatient days had a significantly less diverse gut microbiota than those with lower than 18 inpatient days. No significant ($P > 0.05$) differences were observed in patients with greater than 18 days as a hospital inpatient at the phylum level. At the family level, increased duration of time spent as an inpatient was significantly ($P < 0.05$) associated with reductions in Lachnospiraceae, Veillonellaceae, and Clostridiaceae. These CF patients also had significantly ($P < 0.05$) higher proportions of *Enterococcus*, *Clostridium* (*Clostridiaceae*), *Coriobacteriaceae*, and *Bifidobacteriaceae* at the genus level. While *Dorea*, *Pseudobutyrivibrio*, and *Blautia* were all significantly ($P < 0.05$) reduced in these patients.

The impact of lung transplantation on the gut microbiota.

To examine the impact of lung transplantation on the gut microbiota of CF patients, the gut microbiota of post lung transplantation CF patients ($n = 11$) was compared to CF patients who have not undergone lung transplant ($n = 57$). No significant ($P > 0.05$) differences in the diversity or species richness were observed between these two groups. At the phylum level a shift towards an increased Firmicutes to Bacteriodetes ratio was observed in post-transplant CF

patients relative to non-transplant CF patients. Firmicutes were significantly ($P < 0.05$) increased by 20.76% while Bacteriodetes were significantly ($P < 0.05$) reduced by 21.21% in this group. A significant ($P < 0.05$) increase in Eukaryota was also observed in this group. At the family level post lung transplant patients had a significant ($P < 0.05$) reduction in *Bacteroidaceae*, while *Streptococcaceae*, *Lachnospiraceae*, *Lactobacillaceae*, and *Betaproteobacteria* were all significantly ($P < 0.05$) increased in this group. At the genus level post-transplantation CF patients had significant ($P < 0.05$) increases in *Streptococcus, Lachnospiraceae Incertae Sedis, Blautia, Lactobacillus, Veillonella, Weissella, Acinetobacter, and Allisonella*. Post-transplant CF patients also had a significant ($P < 0.05$) decrease in their proportion of *Bacteroides* and *Alistipes* relative to non-transplant CF patients.

Changes in gut microbiota during pulmonary exacerbation.

To determine if there is an association between pulmonary exacerbation and the composition of the gut microbiota in CF patients the gut microbiota of CF patients suffering pulmonary exacerbation at the time of sampling ($n = 12$) were compared to stable CF patients ($n = 56$). Exacerbating CF patients had significantly ($P < 0.05$) less diverse gut microbiota and species richness than non-exacerbating CF patients according to four of five measures used (Simpson, $P = 0.062$). No significant differences were observed at the phylum level in CF patients suffering exacerbations. However, at the family level, proportions of *Lactobacillaceae, Veillonellaceae,* and *Clostridiaceae* were significantly ($P < 0.05$) reduced while *Bacilli* was significantly ($P < 0.05$) increased. At the genus level patients experiencing pulmonary exacerbations had significant ($P < 0.05$) reductions in their proportions of *Streptococcus, Pseudobutyrivibrio, Blautia, Clostridium (Clostridiaceae),* and *Lactobacillus*. Pulmonary exacerbation was also

associated with a significant ($P < 0.05$) increase in proportions of *Erysipelotrichaceae Incertae Sedis* relative to CF patients not experiencing pulmonary exacerbations.

Alteration of gut microbiota with decreasing FEV1%.

Forced expiratory volume in 1 second (FEV1) is a measure of lung function used to measure the progression of pulmonary disease in CF patients. To assess whether the gut microbiota changes with progression of lung disease progression in CF patients, we compared the gut microbiota of CF patients with FEV1 <68% to CF patients with an FEV1 >68%. CF patients with depressed lung function had significantly ($P < 0.05$) less diversity and species richness in their gut microbiota relative to those with an FEV1 of > 68%. At the phylum level CF patients with depressed lung function had significantly ($P < 0.05$) increased proportions of *Proteobacteria.* While at the family level, these patients had higher proportions of *Bacteroidaceae* and *Streptococcaceae*. CF patients with depressed lung function also had significantly ($P < 0.05$) lower proportions of *Lachnospiraceae* and *Prevotellaceae* at the family level.

At the genus level, CF patients with depressed lung function had significantly ($P < 0.05$) decreased proportions of *Pseudobutyrivibrio, Blautia, Clostridium, and Lactobacillus*. These patients also had increased proportions of *Streptococcus* and *Clostridiaceae* relative to CF patients with an FEV1 > 68%.

Changes in the gut microbiota of pancreatic insufficient CF patients.

Pancreatic insufficiency is a common manifestation of CF. To examine the effect of pancreatic insufficiency on the composition of the gut microbiota in CF patients we compared the gut microbiota of CF patients with pancreatic insufficiency (n = 59) to those who were pancreatic

sufficient (n = 9). No significant differences in diversity or species richness were observed between pancreatic insufficient and pancreatic sufficient CF patients. Pancreatic insufficiency had no significant impact on the gut microbiota at a phylum level in the CF patients tested. At the family level *Lachnospiraceae* was significantly ($P < 0.05$) increased while Ruminococcaceae was significantly ($P < 0.05$) decreased in pancreatic insufficient CF patients relative to pancreatic sufficient CF patients. At the genus level pancreatic insufficient CF patients had a significant ($P < 0.05$) increase in *Lachnospiraceae Incertae Sedis*, while *Ruminococcus, Subdoligranulum, Bifidobacteriaceae, Anaerotruncus, Allobaculum, Clostridiaceae, Parvimonas*, and *Arcobacter* were all significantly ($P < 0.05$) decreased relative to pancreatic sufficient CF patients.

Cystic fibrosis is caused by a mutation in the CFTR gene which codes for a chloride ion transport protein expressed at epithelial cell surfaces. Mutations in the CFTR gene are classified into 6 classes according to the mechanism by which they disrupt the action of the CFTR protein. Class 1 – 3 mutations are considered the most severe as they tend to result in a complete loss of function. To assess the impact of class 1-3 mutations on the composition of the gut microbiota, the gut microbiota of CF patients with class 1 – 3 mutations was compared to CF patients with less severe mutations. No significant difference in species richness of microbial diversity were observed between the CF patients with class 1 – 3 mutations and CF patients with other mutations. CF patients with class 1 – 3 mutations had no significant differences in the composition of their gut microbiota at phylum level. At the family level *Enterococcaceae* was significantly ($P < 0.05$) increased while *Ruminococcaceae* was significantly ($P < 0.05$) decreased relative to those with less severe mutations. At the genus level, *Alistipes, Ruminococcus, Subdoligranulum, Faecalibacterium, Paraprevotella, Anaerotruncus*, and *Barnesiella* were all significantly ($P < 0.05$) decreased in CF patients with severe mutations (class 1-3).

Alterations in gut microbiota of CF patients carrying of Clostridium difficile in their stool.

To assess whether alterations in the gut microbiota may predispose CF patients to colonisation with *C. difficile*, we compared the gut microbiota of CF patients who were asymptomatic carriers of *C. difficile* to CF patients negative for carriage of *C. difficile*.

A significant ($P < 0.05$) reduction in microbial diversity was observed in CF patients carrying *C. difficile*. Patients carrying *C. difficile* had significant ($P < 0.05$) decrease in proportions of Actinobacteria, Proteobacteria, and Fusobacteria at the phylum level relative to *C. difficile* free CF patients. While at the family level significant ($P < 0.05$) increases in *Anaerococcus and Erysipelotrichaceae Incertae Sedis* were observed in CF patients carrying *C. difficile* as well as a significant ($P < 0.05$) decrease in *Fusobacteriaceae*. At the genus level this group of patients had significant ($P < 0.05$) reductions in *Parasutterella, Paraprevotella, Barnesiella, Fusobacterium, Weissella,* and *Peptostreptococcaceae* (Clostridiales) relative to *C. difficile* free CF patients. A significant ($P < 0.05$) increase *in Erysipelotrichaceae Incertae Sedis* was also observed in CF patients carrying *C. difficile*.

3.5 Discussion

In this study high throughput compositional pyrosequencing of the faecal microbiota was used to investigate the impact of cystic fibrosis on the composition of the gut microbiota of patients with CF. In addition, patient medical histories were used to establish whether disease manifestation/progression or treatment of CF had an impact on the gut microbiota of CF patients. The intestinal microbiota of CF patients was shown to be altered to that of healthy controls as evident by their separate clustering by principle coordinate analysis. The gut microbiota of CF patients was significantly ($P < 0.05$) less diverse than that of healthy controls

and alterations in taxonomic abundances were observed between the two groups at the phylum, family and genus level. The disease and its treatment were also found to contribute to the dysbiosis of the CF patient gut microbiota with differences in diversity and taxonomic abundances observed among CF patient groups.

A shift toward an increased Firmicutes to Bacteriodetes ratio was observed in CF patients relative to the non-CF control group which may partially be explained by diet. Similar observations have been made in studies of obesity (Bervoets et al., 2013, Turnbaugh et al., 2006) with diet proposed as a major factor in the development of the gut microbiota (Moschen et al., 2012). Under nutrition in CF patients is associated with poor clinical outcome, therefore CF patients are recommended to consume a diet high in fat and protein (Borowitz et al., 2002). Diets high in fat have been shown to increase the Firmicutes to Bacteriodetes ratio in mice independent of obesity (Hildebrandt et al., 2009). However, dietary information was not collected as part of this study, and therefore no definite conclusion can be made.

With the survival rate of CF patients increasing, the risk of patients developing cancer also increases. The risk of cancer in CF patients is similar to that of the healthy population, however CF patients have a higher ratio of observed to expected digestive cancers (Neglia et al., 1995, Maisonneuve et al., 2003, Maisonneuve et al., 2013). In this study, *Fusobacterium*, a bacterium which is seen as a risk factor for developing colorectal cancer (CRC) (Flanagan et al., 2014) was detected in the intestinal microbiota of four CF patients, while it was notably absent from healthy volunteers. *Fusobacterium nucleatum* has been show to induce the growth of CRC by inducing oncogenic and inflammatory responses (Rubinstein et al., 2013). *Fusobacterium* is more abundant in the stool of CRC patients (Flanagan et al., 2014) and may play a role in the development of digestive tract cancers in CF patients.

The gut microbiota of CF patients was found to be significantly less diverse and had lower species richness than that of a healthy control group. CF patient's intestinal microbiota also displayed an increased Firmicutes to Bacteriodetes ratio relative to the control group. Intestinal dysbiosis observed in CF patients was influenced by a combination of the disease and its treatment. The results presented here further develop the understanding of the intestinal microbiota of CF patients. In the future, this may be exploited as a target to improve the gastrointestinal, as well as pulmonary health of CF patients. However, longtitudinal studies are required to improve our understanding of the influence of CF and its treatment on the gut microbiota over time.

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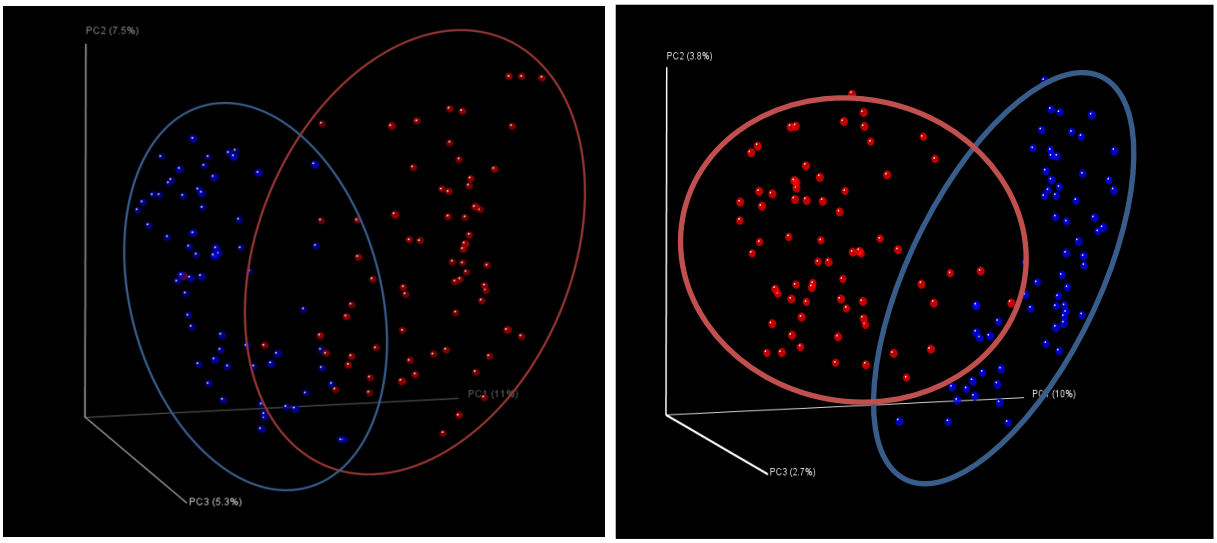
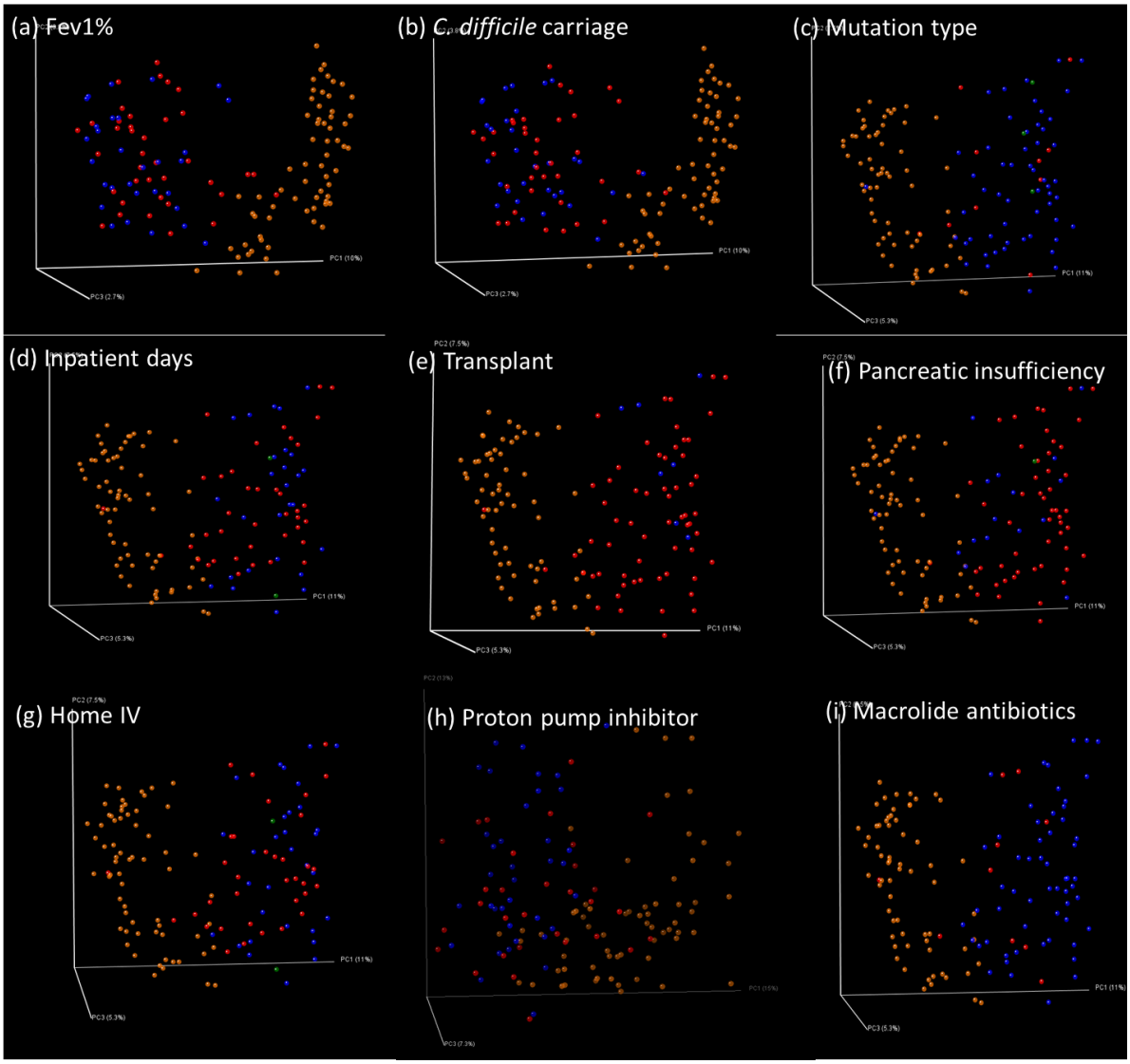
Figure 3.1 Principle coordinate analysis plots of weighted (a) and unweighted (b) unifrac distance matricies of CF patient compared to healthy controls.

(a) (b)



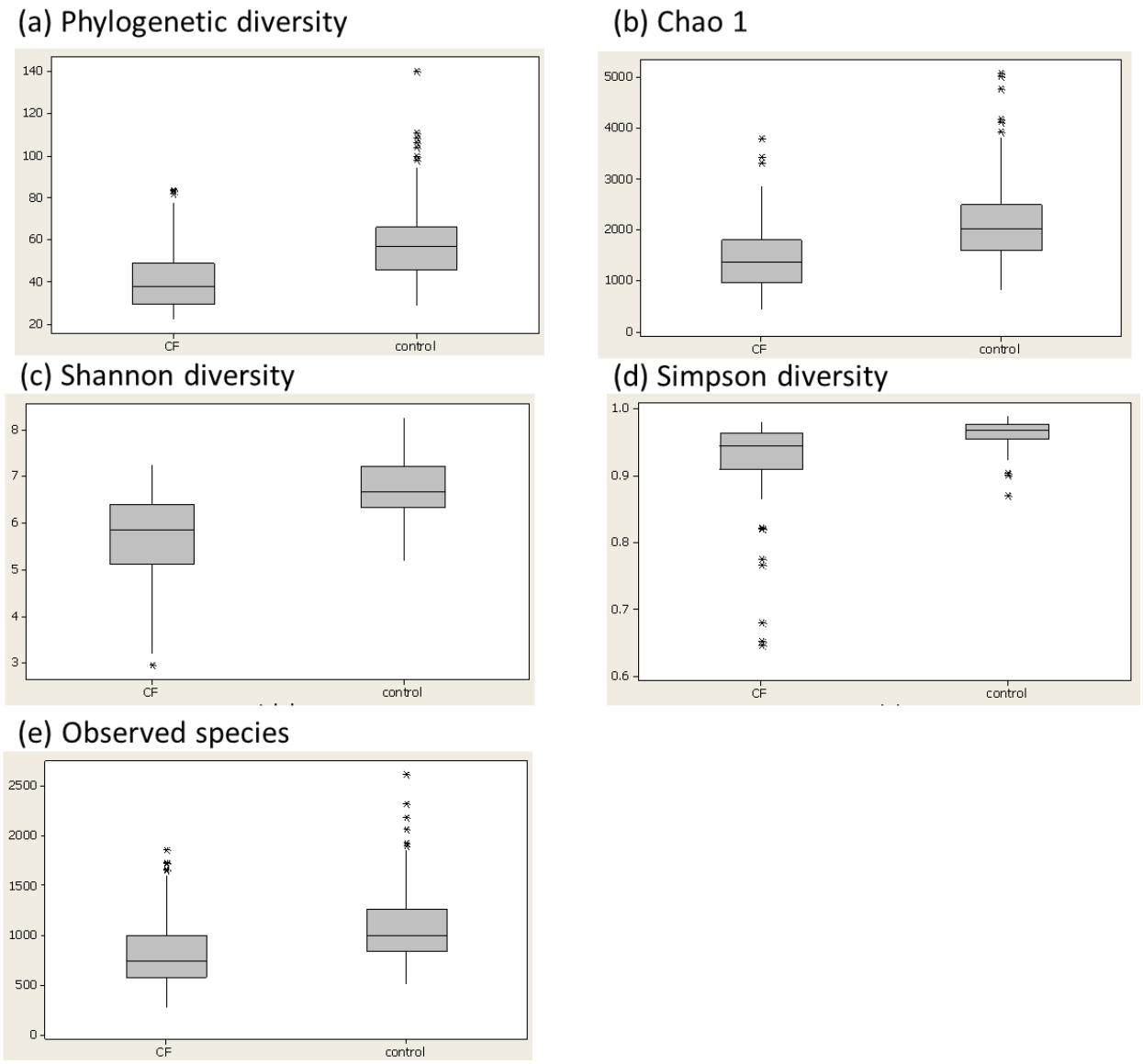
Beta diversities of reads were implemented with QIIME. The resulting principal coordinate analysis was visualised within KiNG (http://kinemage.biochem.duke.edu). Cystic fibrosis patients are represented by blue dots, while healthy controls are represented by blue dots. Red and blue ovals indicate separate clustering of these groups.

Figure 3.2: Principle coordinate analysis plots of weighted unifrac distance matrices of CF patients based on clinical parameters and treatments received as well as healthy controls to healthy controls.



Healthy controls are represented by yellow dots in all graphs. In graph (a), patients with an FEV_1 of > 68% are represented in red and those with < 68% in blue. In graph (b), *C. difficile* negative CF patients are represented by red and *C. difficile* positive in blue. In graph (c) patients with class 1 - 3 mutations are represented in blue and non 1 – 3 mutations in red. In graph (d), patients with a high number of inpatient days are represented in blue and those with lower inpatient days in red. In graph (e), non-lung transplant patients are coloured in red and transplant patients in blue. In graph (f), pancreatic sufficient patients are coloured in red and pancreatic insufficient patients in blue. In graph (g), patients with a high number of home IV antibiotic days are represented in blue and those with a low number in red. In graph (h), those receiving proton pump inhibitors are coloured in blue and those not receiving PPIs in red. In graph (i), patients receiving macrolide antibiotics are coloured in blue and those not receiving the treatment in red.

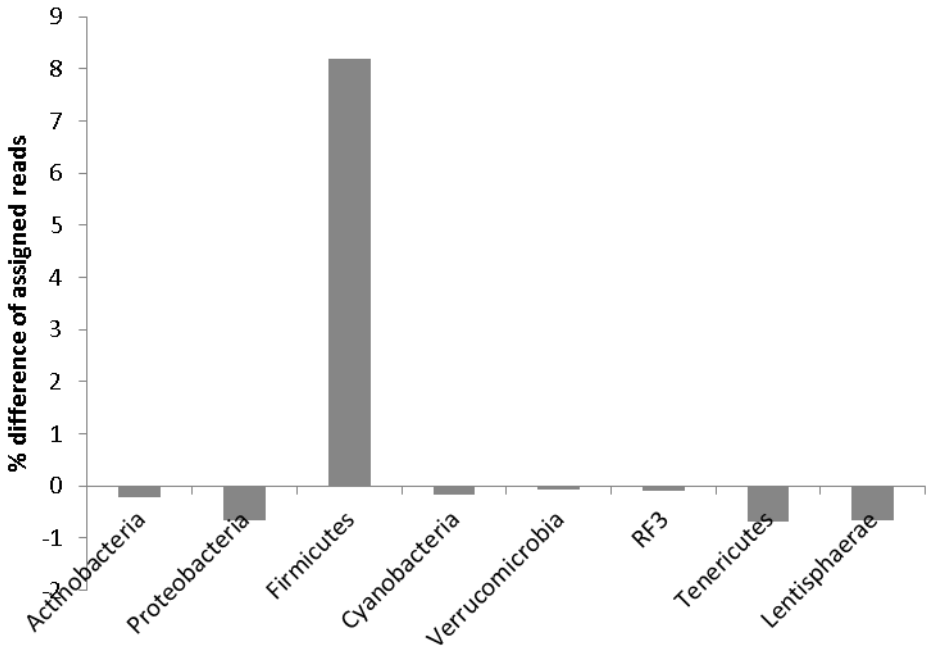
Figure 3.3 Box plots comparing the diversity of CF intestinal microbiota compared to healthy controls using the following diversity metrics; Phylogenetic diversity (a), Chao 1 (b), Shannon (c), Simpson (d), and species richness (e).



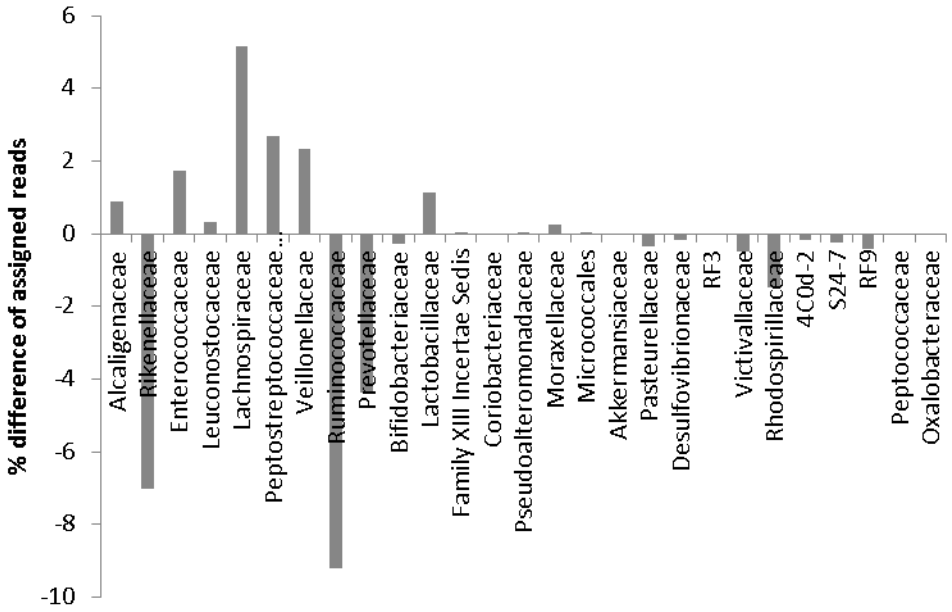
Alpha diversities of reads were implemented with QIIME and box plots created using Minitab 15.

Figure 3.4 Significant differences in microbial abundances observed between CF patient and phylum (a), family (b) and genus (c) level.

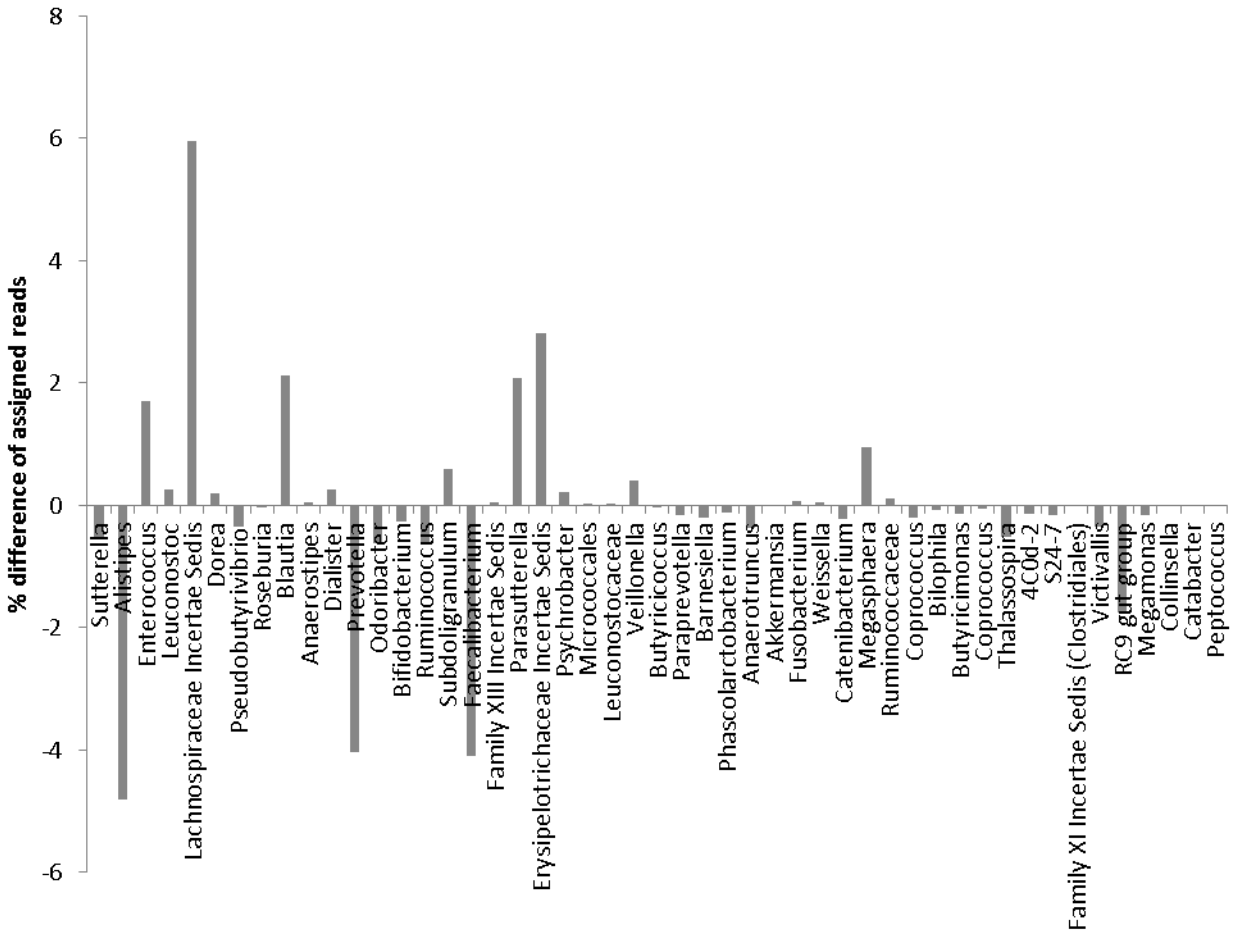
(a) Phylum level differences in taxonomic abundance between CF and healthy controls.



(b) Family level differences in taxonomic abundance between CF and healthy controls.

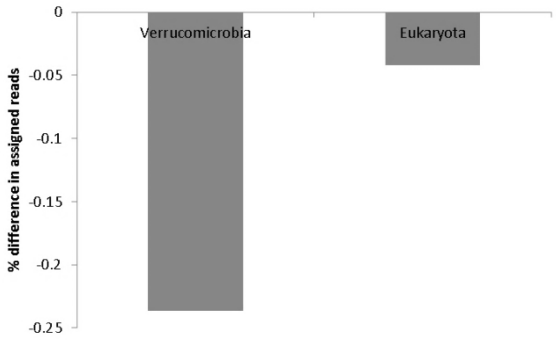


(c) Genus level differences in taxonomic abundance between CF and healthy controls.

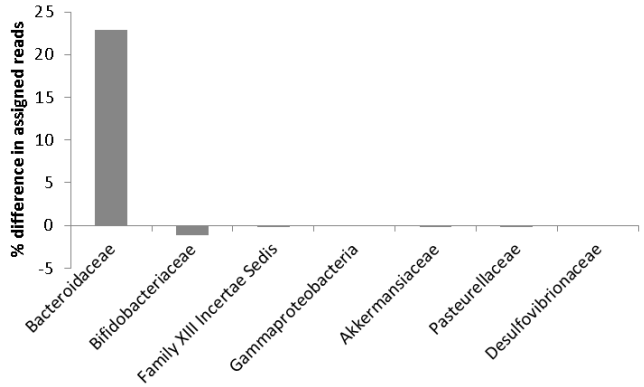


Supplementary figure 3.1: Significant changes in taxonomic abundances at the phylum (a), family (b), and genus (c) level in CF patients receiving macrolide antibiotics compared to CF patients not receiving the treatment based on assigned reads. The non- parametric Kruskal–Wallis was test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$.

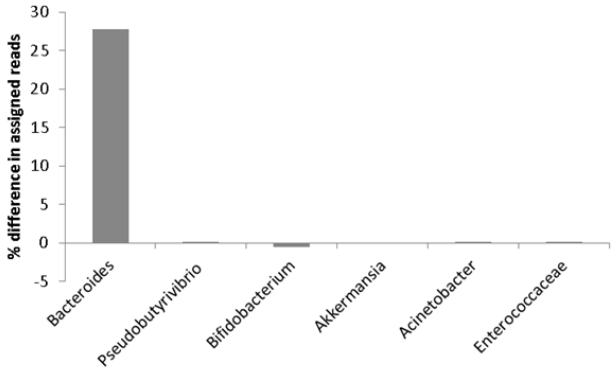
(a)



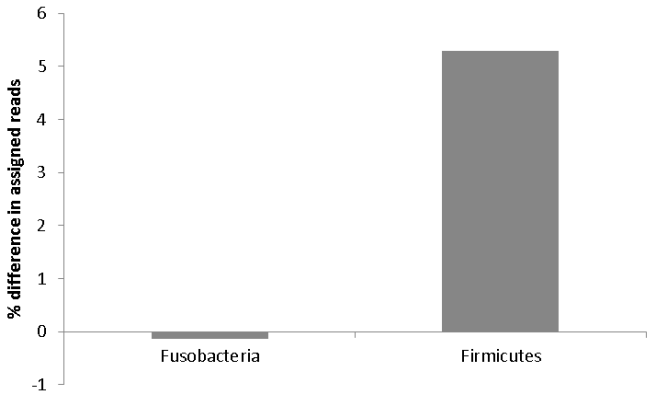
(b)



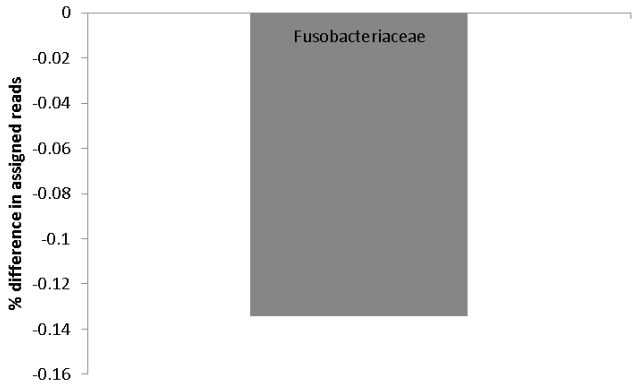
(c)



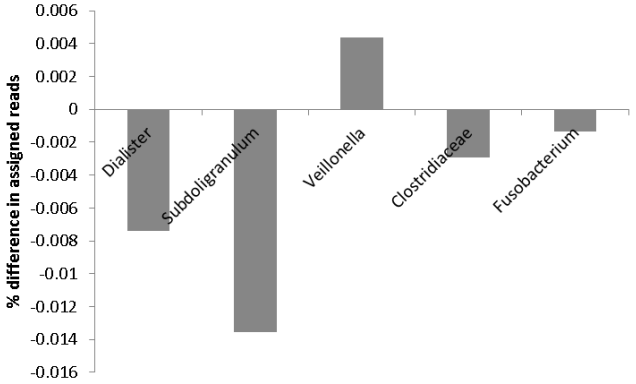
Supplementary figure 3.2: Significant changes in taxonomic abundances at the phylum (a), family (b) and genus (c) level in CF patients receiving proton pump inhibitors compared to CF patients not receiving the treatment based on assigned reads. The non- parametric Kruskal–Wallis was test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$.



(a)

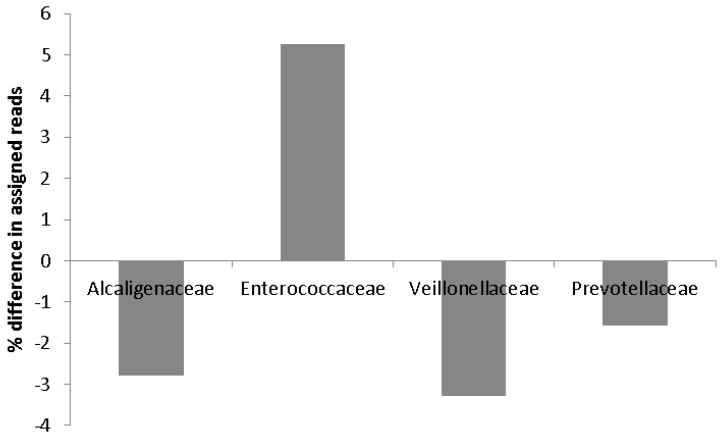


(b)

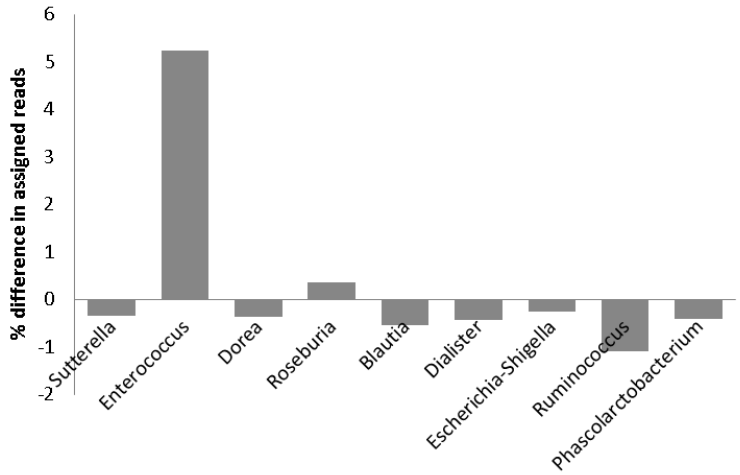


(c)

Supplementary figure 3.3: Significant changes in taxonomic abundances at the family (a), and genus (b) level in CF patients receiving IVAB for >55 days compared to CF patients who received the treatment over a shorter duration (< 55 days) based on assigned reads. The non-parametric Kruskal–Wallis was test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$.

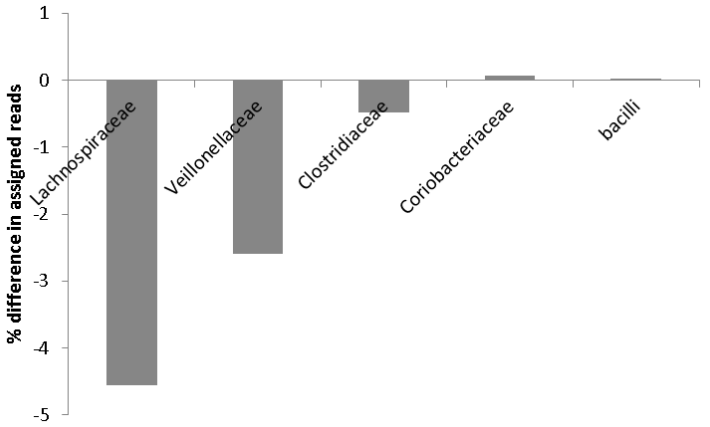


(a)

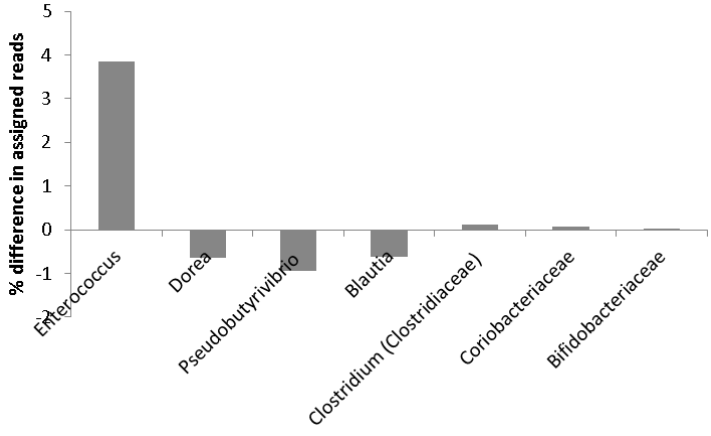


(b)

Supplementary figure 3.4: Significant changes in taxonomic abundances at the family (a) and genus (b) level in CF patients who spent >18 days as a hospital inpatient to CF patients who spent < 18 days as a hospital inpatient based on assigned reads. The non- parametric Kruskal–Wallis was test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$.

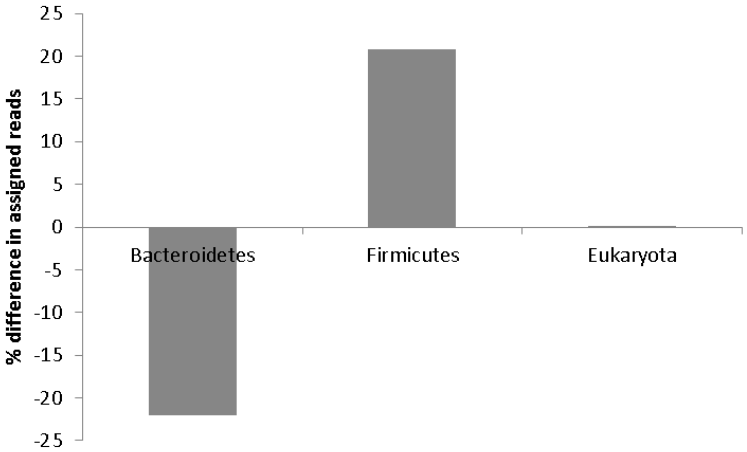


(a)

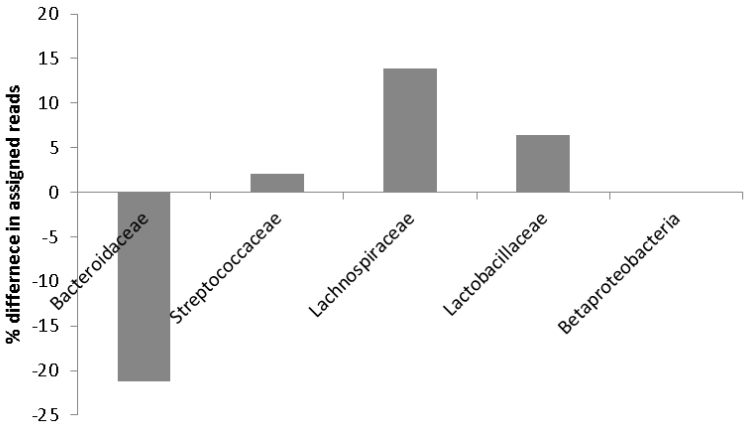


(b)

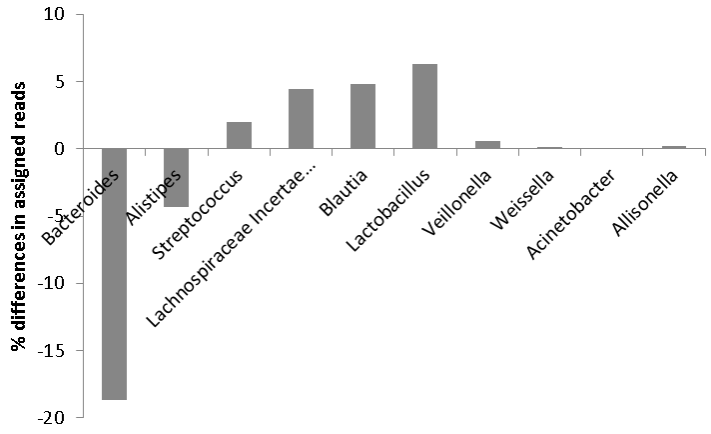
Supplementary figure 3.5: Significant changes in taxonomic abundances at the phylum (a), family (b) and genus (c) level in post-lung transplant CF compared to non-transplant patients based on assigned reads. The non- parametric Kruskal–Wallis was test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$.



(a)

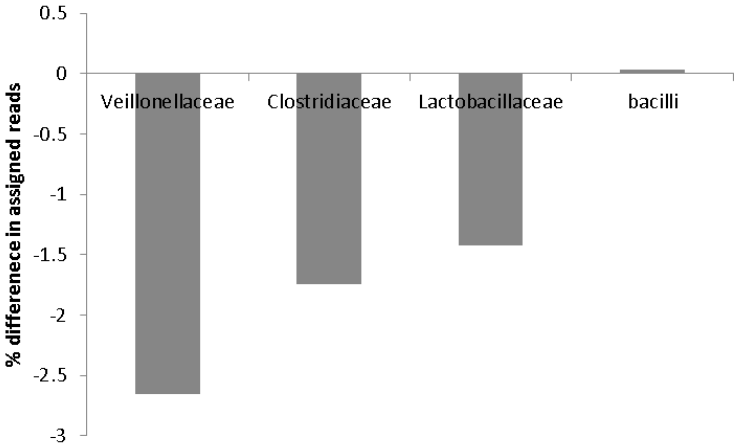


(b)

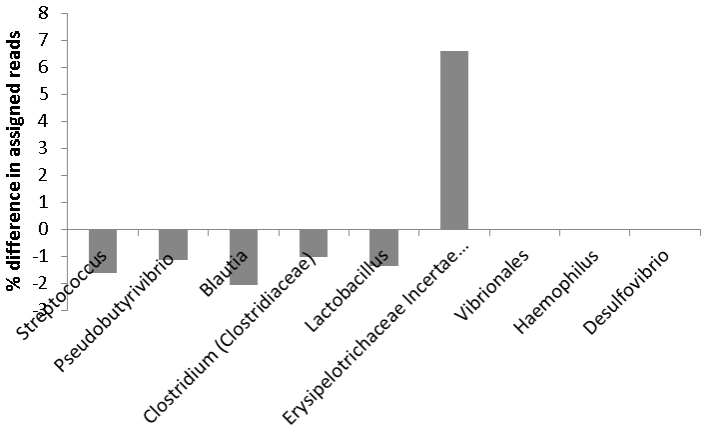


(C)

Supplementary figure 3.6: Significant changes in taxonomic abundances at the family (a) and genus (b) level in CF patients suffering pulmonary exacerbation compared to non-exacerbating CF patients based on assigned reads. The non- parametric Kruskal–Wallis was test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$.

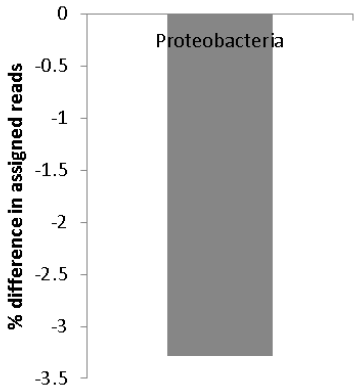


(a)

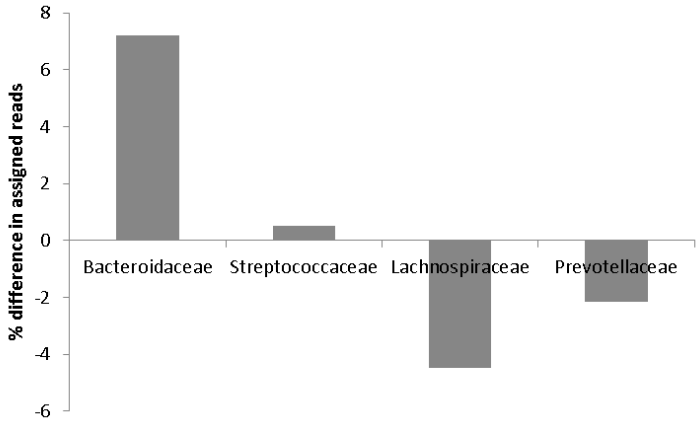


(b)

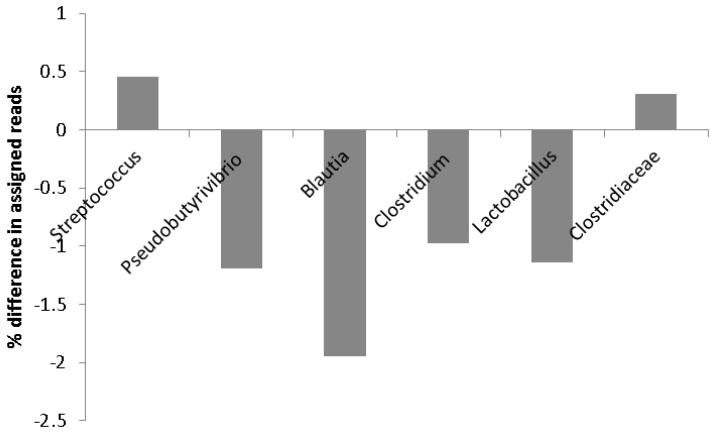
Supplementary figure 3.7: Significant changes in taxonomic abundances at the phylum (a), family (b) and genus (c) level in CF patients with an FEV_1 of less than 68% compared to CF patients with an FEV_1 of greater than 68% based on assigned reads. The non- parametric Kruskal–Wallis was test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$.



(a)

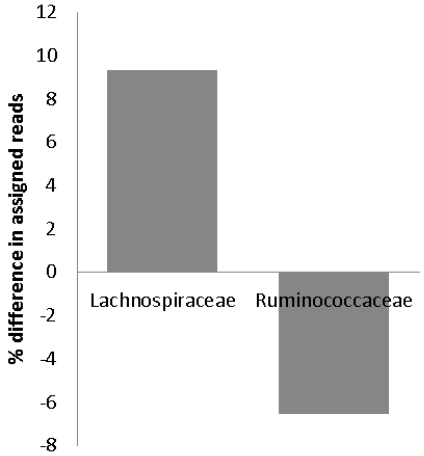


(b)

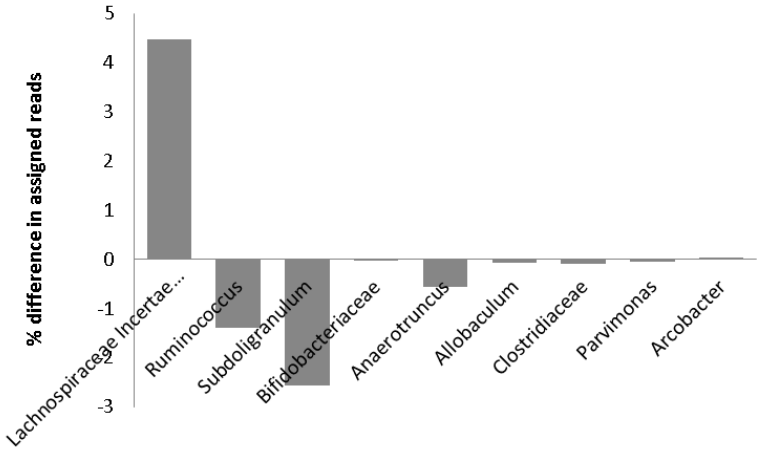


(c)

Supplementary figure 3.8: Significant changes in taxonomic abundances at the family (a) and genus (b) level in pancreatic insufficient CF patients compared to pancreatic sufficent CF patients based on assigned reads. The non- parametric Kruskal–Wallis was test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$.

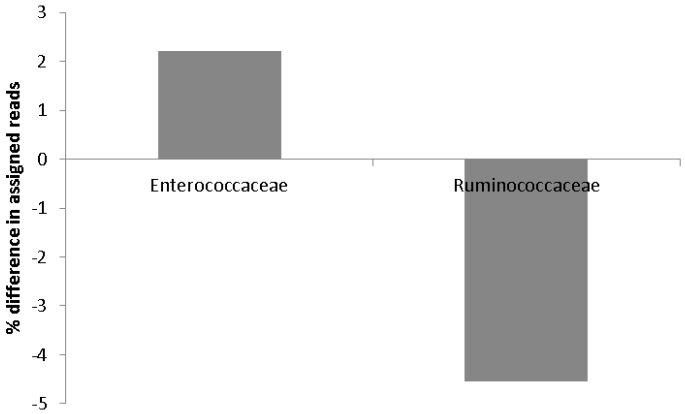


(a)

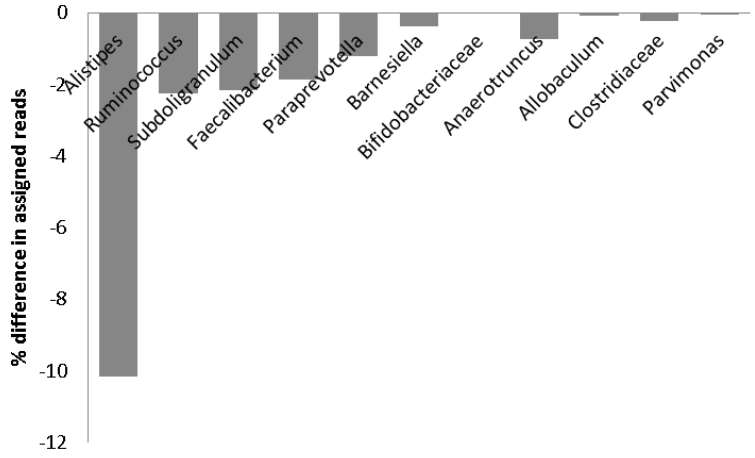


(b)

Supplementary figure 3.9: Significant changes in taxonomic abundances at the family (a) and genus (b) level in CF patients with severe class 1 – 3 mutations compared to CF patients with less severe mutations based on assigned reads. The non- parametric Kruskal–Wallis was test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$.

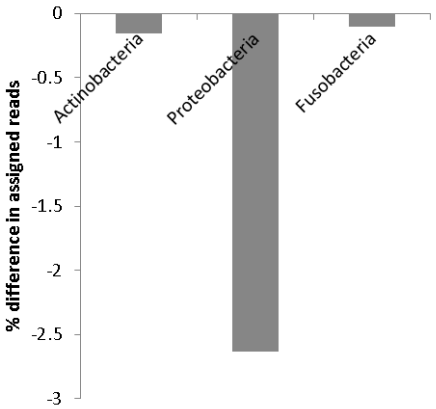


(a)

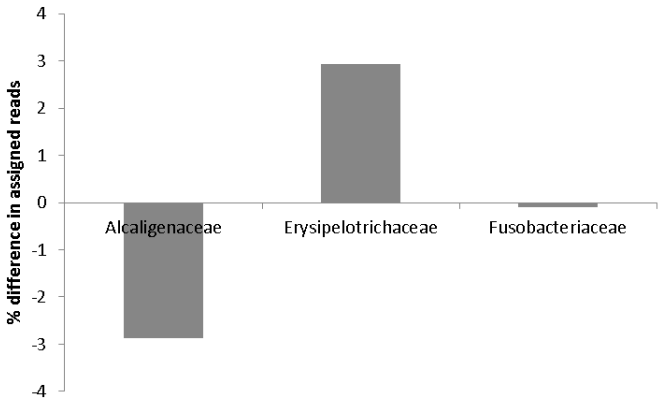


(b)

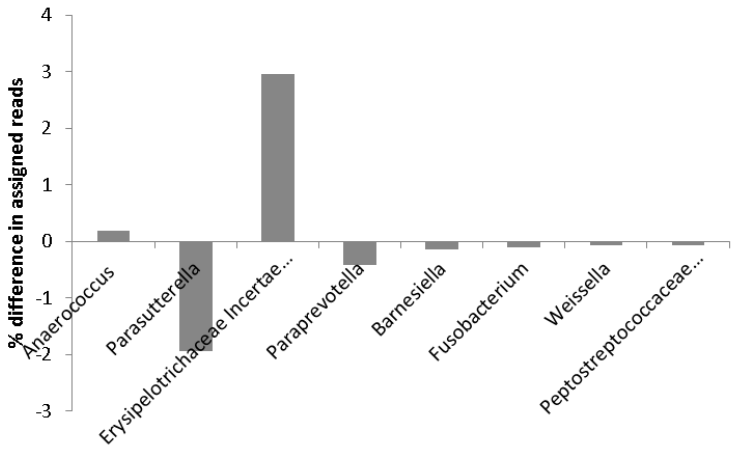
Supplementary figure 3.10: Significant changes in taxonomic abundances at the phylum (a), family (b), and genus (c) level in *C. difficile* positive CF patients compared to CF patients not carrying *C. difficile* based on assigned reads. The non- parametric Kruskal–Wallis was test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$.



(a)



(b)



(c)

Chapter 4

Multilocus sequence typing as an alternative to PCR-ribotyping for typing *Clostridium difficile* in patients with high risk of infection.

4.1 Abstract:

A collection *Clostridium difficile* strains isolated from a number of patient cohorts with increased risk of *C. difficile* infection (CDI) were typed using multilocus sequence typing (MLST). The discriminatory power of MLST for typing *C. difficile* was compared to that of PCR-ribotyping and pulsed-field gel electrophoresis (PFGE). In total, 28 MLST sequence types (ST) and 33 ribotypes (RT) were described in 112 *C. difficile* strains isolated from these patient cohorts. A subset of 28 strains was also typed using pulsed field gel electrophoresis (PFGE). Pulsed field gel electrophoresis typing of this subset resulted in 18 pulse types (PT), 12 sequence types, and 12 ribotypes. Multilocus sequence typing compared favourably with PCR-ribotyping for its discriminatory power, having an index of discrimination (ID) of 0.93 compared to 0.95 for PCR-ribotyping. However, PFGE was the most discriminatory with an ID of 0.96. Despite this, there was a high level of concordance between the three typing techniques. The sequence type, ST3, which corresponded to RT001 and RT072 was the most frequently isolated sequence type reported in this study accounting for 19.4% of isolates tested. All RT072 strains were isolated from elderly patients; however, these strains were not identical according to PFGE analysis. The second most frequently reported (11.6%) strain, ST46/RT087 was predominantly found in elderly patients with colon cancer. Multilocus sequence typing of *C. difficile* strains isolated from patient cohorts with increased risk of *C. difficile* infection was comparably discriminatory to the widely used PCR-ribotyping scheme. Multilocus sequence typing yielded easily interpretable and transferable results, allowing for rapid identification and typing of *C. difficile* in high risk patient groups.

4.2 Introduction:

Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacillus, first described as the aetiological agent of pseudomembranous colitis in 1978 (Larson et al., 1978). With the widespread use of broad-spectrum antibiotics, *C. difficile* has increased in both prevalence, and severity worldwide, and is now recognized as causing virtually all cases of pseudomembranous colitis (Owens, 2007). *C. difficile* infection (CDI) is mediated by the production of two toxins - an endotoxin, toxin A and a cytotoxin, toxin B (Kelly et al., 1994) which cause a range of diseases from mild diarrhoea, to life-threatening pseudomembranous colitis. As an opportunistic pathogen, CDI typically occurs following disruption of the normal gut microbiota following antibiotic treatment (McFarland et al., 1989). *C. difficile* is also recognised as a significant nosocomial pathogen, responsible for the majority of cases hospital acquired diarrhoea (Aslam et al., 2005). In addition to antibiotic use and prolonged hospital stay, advanced age, chemotherapy, and gastric acid suppression medication have all been shown to increase the risk of CDI (Bignardi, 1998). *C. difficile* spores are highly resistant and can persist in the environment for long periods. Spores are spread by a faecal oral route, by person to person contact or through contact with contaminated surfaces (Weber et al., 2010). Patients with increased risk of acquiring *C. difficile* due to their comorbidity, or the treatment they receive may act as a reservoir for dissemination of *C. difficile* in hospitals or long stay care facilities.

There has been a rise in the incidence and severity of CDI globally since 2003, partially attributed to the emergence of a number of highly virulent strains, including ribotype 027 (RT027). The highly virulent strain, RT027 was first identified in North America in 2003 during an outbreak of severe CDI (Pepin et al., 2004, Pépin et al., 2005). The severity of disease associated with this ribotype is speculated to be as a result of production of binary toxin coupled with production of a hypertoxic and antigenically variable version of toxin B (Warny et al., Lanis et al., 2013). It has subsequently become a major epidemic strain, frequently isolated in

hospitals across North America and Europe (O'Connor et al., 2009, Warny et al.). More recently, a second ribotype, RT078 has also been associated with more virulent cases of CDI and it is notable in that in can cause disease in patients with no recent history of antibiotic treatment or hospitalization (Goorhuis et al., 2008). Community acquisition of *C. difficile* from asymptomatic carriers has also been proposed after an association was observed between the presence of strains in a hospital with those found in the surrounding community (Angulo, 2007, Curry et al., 2013, Thompson, 2008). Cases of CDI are increasing in Europe and therefore active surveillance and reporting is important to control its spread and understand its changing epidemiology (Bauer et al., Benson et al., 2007, DePestel and Aronoff, 2013). Therefore there is a need for improved monitoring of *C. difficile* to better track and control CDI.

Multilocus sequence typing (MLST) has been proposed as a general genotyping procedure suitable for tracking the epidemiology of bacterial species, while providing accurate data which can easily be shared between laboratories (Maiden et al., 1998). The PCR based nature of MLST allows for it to be performed directly on DNA isolated from stool, thereby removing the need to culture, thus increasing the speed of strain identification (Griffiths et al., 2010). Multilocus sequence typing is performed by amplifying and sequencing several (7-10) housekeeping gene loci. Alleles at each locus are assigned an allele number, unique combinations of which designate a strain's sequence type (ST). A number of clinically relevant bacterial species have been typed using MLST, including *C. difficile* (Griffiths et al., 2010, Lemee et al., 2004, Maiden et al., 1998). Based on MLST analysis, it has been established that *C. difficile* is distributed across five evolutionary clades, with sequence types from each clade being associated with CDI (Dingle et al., 2011, Griffiths et al., 2010, Mamoon A. Aldeyab et al., 2011, Patterson et al., 2012, England, 2014). An extensive study into the relationship between *C. difficile* strains, host biomarkers, and mortality rates in CDI using MLST was recently carried out by Walker et al, 2013 (Walker et al., 2013). In the study described here MLST was

compared to PCR-ribotyping and pulsed-field gel electrophoresis (PFGE) for its suitability as a typing scheme to track the epidemiology of *C. difficile* strains isolated from high risk patient groups in addition to identifying the distribution of *C. difficile* genotypes within these cohorts. To this end, a collection of 112 *C. difficile* strains isolated from a number of patient groups including people with inflammatory bowel disease, irritable bowel syndrome, cystic fibrosis, colon cancer, patients with CDI, as well as elderly persons (>65 y) and healthy volunteers were typed and compared using MLST and PCR-ribotyping, while a subset of 28 isolates were also typed using PFGE.

4.3 Materials and methods

Study populations and Bacterial strains

A total of 112 *C. difficile* isolates were obtained from the Teagasc Food Research Centre (DPC) culture collection, the APC culture collection, Alimentary Pharmabiotic Centre, University College Cork, and the Eldermet culture collection, University College Cork. Isolates were routinely cultured on Fastidious Anaerobic agar (FAA) (Lab M, Lancashire, UK) supplemented with 7% defibrinated horse blood, at 37 ºC under anaerobic conditions. All strains were stocked on microbank beads (Pro-lab diagnostics, Cheshire, UK) and stored at -80ºC.

PCR ribotyping (PCR-RT)

Ribotyping was performed by the *C. difficile* Ribotyping Network for England (CDRNE), at the Microbiology Reference Laboratory, Leeds General Infirmary, United Kingdom. Strains were

analysed by capillary gel electrophoresis and compared to over 500 ribotypes housed in the CDRNE ribotype reference library.

Pulsed field gel electrophoresis (PFGE)

Isolates were sub-cultured from -80ºC stocks onto FAA Blood agar and grown for 48h anaerobically at 37ºC. Approximately 5 colonies were inoculated into 10ml of BHI broth which had previously been boiled and cooled under anaerobic conditions. Following overnight incubation 200µl was sub-cultured into a fresh 10ml of BHI broth and grown for ~5h at 37ºC. Genomic DNA was prepared in agarose plugs, lysed and subsequently digested with Sma1 as described previously (Janezic and Rupnik, 2010). Electrophoresis was performed using the Biorad Chef-DR II instrument and DNA macro-restriction profiles visualised using the Alpha Imaging system. DNA macro-restriction patterns were stored as TIFF files and imported into Bionumerics software (Version 3 Applied Maths, Kortrijk, Belgium) for dendrographic analysis. Salmonella Braenderup H9812 restricted with Xba1 at 37°C for 2h was used as a molecular weight marker for analysis using the Bionumerics software. Strains were assigned to the same pulse types (PT) if they had banding patterns of 95% similarity or greater.

Multilocus sequence typing (MLST)

Genomic DNA was extracted from isolates according to Rea et al (Rea et al., 2012). Briefly, five to six colonies from an overnight culture of C. difficile grown on fastidious anaerobic agar containing 7% defibrinated horse blood were resuspended in 200µl of 5% w/v Chelex-100 (Sigma). Cell suspension was heated for 30min at 56°C, followed by 100°C for 8 min. Cell debris was removed by centrifugation at 16,000 x g for 3 min and supernatant containing DNA

was stored at -20˚C. Seven housekeeping genes (adk, atpA, dxr, glyA, recA, sodA and tpi) were amplified for MLST analysis using primers designed by Griffiths et al, 2010 (Griffiths et al., 2010). PCR reaction mixtures (15µl) consisted of 5.5 µl molecular biology grade water (Millipore), 0.5 µl (10 pM) of each forward and reverse primer, 7.5 µl KAPA HiFi HotStart readymix (2x) and 1 µl of genomic DNA. As per manufacturer's instructions, thermoycycler conditions outlined by Griffiths et al (Griffiths et al., 2010) were modified to accommodate the use of KAPA HiFi HotStart DNA polymerase, as follows; 95˚C for 5 min, followed by 35 cycles of 98˚C for 20 s, 57˚C for 30 s and 72˚C for 40 s, with a final extension of 72˚C for 5 min and storage at 10˚C. Amplicons were purified by enzymatic treatment with Exonuclease I (20units/µl) and Antarctic Phosphatase (5units/µl) (NEB,). Briefly, a (2x) reaction mixture of Exonuclease I, Antarctic Phosphatase, 10x Antarctic Phosphatase buffer and dH2O in a 1:1:1:17 ratio respectively was added to an equal volume of PCR products, then incubated at 37˚C for 15 min, followed by 80˚C for 15 min to deactivate the enzyme. Consensus sequencing of both DNA strands was performed using the amplication primers by Beckman Coulter Genomics (Hope End, Takeley Essex CM22 6TA, UK). Obtained housekeeping gene sequences for each strain were queried against the *C. difficile* MLST reference database (http://pubmlst.org/cdifficile/) and allele numbers assigned. Sequence types were assigned based on the combination of alleles corresponding to each strain. Reconstruction of evolutionary relationships was performed using the MEGA 5 package (Tamura et al. 2011). Concatenated MLST sequence data obtained from the MLST online database (http://pubmlst.org/cdifficile/) was used to construct a consensus neighbour joining tree from 500 bootstrapping replicates (Tamura et al., 2011) (Fig 4.2).

Detection of C. difficile toxin genes tcdA and tcdB.

Pathogenicity potential of *C. difficile* isolates was screened for by PCR amplication of the toxin genes tcdA and tcdB using the primers designed by Terhes et al.(Terhes et al., 2004). Briefly, amplication of each gene was performed in a separate reaction mixture (25 µl) containing, 12.5 µl Biomix Red (Bioline, London, UK), 0.5µl of each forward and reverse primer (tcdA or tcdB), 2 µl template DNA and 9.5 µl H2O. Toxin genes were amplified by 30 cycles of 95°C for 20 s, 62°C for 45 s, and 72°C for 45 s, followed by a final elongation of 72°C for 5 min.

4.4 Results

MLST, PCR-RT and PFGE typing of *C. difficile* isolated from different patient groups.

Multilocus sequence typing of *C. difficile* has previously been shown to deliver rapidly generated, easily interoperable results without then need for an in-house strain library (Griffiths et al., 2010). This makes MLST a more attractive typing scheme for high risk patient groups in a healthcare setting. Therefore to compare the type-ability of this typing scheme to established methods, MLST and PCR-ribotyping were applied to a collection of 112 human *C. difficile* isolates, while PFGE was applied to a subset of 28 isolates from elderly patients. These strains were previously isolated during a number of studies investigating the carriage of *C. difficile* in patient groups at increased risk of CDI or those with CDI (Clayton et al., 2009, Clayton et al., 2012, Rea et al., 2012). Patient cohorts included those with inflammatory bowel disease, irritable bowel syndrome, cystic fibrosis, colon cancer, CDI patients, as well as elderly (>65 y) persons, and healthy volunteers. Two *C. difficile* type strains; *C. difficile* 630 and *C. difficile* VPI 10463 were also included in this study. Genotyping resulted in a total of 28 sequence types and 33 ribotypes. Pulsed field gel electrophoresis typing of a subset of 28 strains using yielded 18 pulse

types compared to 12 sequence types and 12 PCR-ribotypes, which can be seen in fig. 4.1 Pulsed field gel electrophoresis was more discriminatory than either PCR-RT or MLST revealing subtypes within sequence types and ribotypes. The 12 RT072/ST3 types included in the 28 strains typed by PFGE were subtyped into 4 separate PTs. However, these strains clustered together at 90% similarity indicating their relatedness (fig. 4.1). Similar observations were made for RT027/ST1 and RT216/ST33 strains which were subtyped into separate PTs (RT027/ST1 into PT1 and PT2 and RT216/ST33 into PT12 and PT13) while maintaining >90% similarity. Both RT308/ST37 strains typed by PFGE were 100% identical.

Toxin production

All strains included in this study were screened for toxin production capability by PCR amplification of the two toxin genes tcdA and tcdB. Toxigenic *C. difficile* harbouring both toxin genes accounted for 88.4% (n = 112) of strains tested. The remainder did not harbour either toxin gene and thus were considered non-pathogenic. No tcdA- / tcdB+ toxin variants were identified in this study, while strains were not screened for binary toxin. Toxin negative strains were typed as; ST15 (RT010), ST7 (RT026), ST26 (RT039, RT140) and ST31 (Unknown RT). Pathogenic and non-pathogenic *C. difficile* strains were distinct and did not share either sequence type or ribotype, however, they were not confined to a single MLST clade.

Comparison of typing schemes for epidemiologic study.

Thirty three distinct RTs, including one novel, previously un-described RT, belonging to ST 31, were included in this study. MLST compared quite favourably to PCR-RT for discrimination of *C. difficile* strains (index of diversity [ID] of 0.93 and 0.95 respectively) according to the

formula described by Hunter and Gaston (Hunter and Gaston, 1988). However, for a subset of 28 strains PFGE was found to be more discriminatory than either typing scheme (ID of 0.96 compared to 0.80 for MLST and PCR-RT).

Distribution of STs among study cohorts.

The distribution of the most prevalent sequence types and their corresponding ribotype are detailed in table 4.2. The most prevalent sequence type reported was ST3, accounting for 19.6% of the strains tested. The twenty two ST3 strains consisted of one RT009, three RT001/072, seven RT001, and eleven RT072. All RT072/ST3 and RT001/072/ST3 strains were isolated from elderly persons while the seven RT001/ST3 and one RT009/ST3 strains were isolated from multiple sources. The eleven 072 strains from elderly patients were found to consist of multiple subtypes based on PFGE fingerprinting. This suggests the prevalence of this ribotype in elderly patients is not due to horizontal transfer of a single strain. The second most prevalent sequence type was ST46 accounting for 11.6% of strains tested. All ST46 strains were isolated from colon cancer patients. However likely, we cannot be certain these strains were acquired from a single source due to the lower discriminatory power afforded by MLST and PCR-ribotyping compared to PFGE. Likewise all seven toxin negative ST26 were solely found in CF patients but could not for certain be attributed to single source acquisition.

4.5 Discussion

This study was undertaken to assess the suitability of MLST as a typing scheme to track the epidemiology of *C. difficile* strains isolated from high risk patient groups in addition to identifying the distribution of *C. difficile* genotypes within these cohorts. Overall, MLST was

found to be comparable to PCR-ribotyping for discriminating *C. difficile* isolates yielding 28 sequence types versus 33 PCR-ribotypes (ID of 0.93 and 0.95 respectively) from 112 isolates. This compares favourably to the discriminatory ability of MLST previously reported by Griffiths et al (ID of 0.90) (Griffiths et al., 2010). Contrary to a previous study by Kilgore et al, all three techniques could be interpreted with confidence (ID > 0.9) (Hunter and Gaston, 1988, Killgore et al., 2008). Pulsed field gel electrophoresis was found to be more discriminatory (ID = 0.96) than either PCR-ribotyping or MLST and could identify subtypes within primary types. Multilocus sequence typing and PCR-ribotyping on the other hand could only identify primary types as has been reported previously (Killgore et al., 2008). There was good concordance between the three techniques for typing this subset of strains. Subtypes of MLST and PCR-ribotype primary types identified by PFGE all shared >90% similarity indicating they were closely related, as can be seen in fig. 4.1.

The most common sequence type reported in this study was ST3 which consisted of two ribotypes, RT001 and RT072. *C. difficile* RT001 has previously been associated with CDI in Southern Germany and has been reported as one of the most frequently isolated ribotypes in the Netherlands (Borgmann et al., 2008, Hensgens et al., 2009). However, neither RT001 nor RT072 have been reported in the top five ribotypes reported in Ireland between 2010 and the beginning of 2013 (Centre, 2013). Despite the fact that all RT072/ST3 strains were isolated from elderly patients (>65 years), it is unlikely they were all acquired from a single source due to the presence of multiple pulse types based on PFGE analysis. The second most prevalent sequence type was ST46 (RT087) which was predominantly found in patients with colon cancer. This sequence type also included the *C. difficile* type strain VPI 10463 (ATCC 43255) which produces large amounts of toxin and is typically used as a positive control in tests for toxin production (Åkerlund et al., 2008). The emerging hyper-virulent strain, RT078 (Goorhuis et al., 2008) was typed as ST11, the third most prevalent ST reported in this study. As well as RT078,

RT045 and RT126 also belong to the ST11 sequence type. While RT078 was reported in over 10% of cases of CDI annually in Ireland between 2010 and 2013 (Centre, 2013), RT045 and RT126 are not frequently reported in humans, however, all three have frequently been isolated from animal sources (Burns et al., 2010, Indra et al., 2009, Schneeberg et al., 2013, Zidaric et al., 2012). Therefore ST11 likely represents an emerging zoonotic *C. difficile* ST that evolved separately from the majority of human isolates, as evident from its outlying position in the MLST dendogram (fig. 4.2). All epidemic RT027 were typed as ST1 as previously described (Griffiths et al., 2010). Of the three RT027/ST1 strains typed by PFGE, two were identical (PT1) while the third (PT2) shared 94% similarity. With the exception of RT014 which was typed as both ST2 and ST14, all other ribotypes described in this study were associated with a single sequence type.

The distribution of *C. difficile* strains can vary both by region as well as time (Wilcox et al., 2012). All patients from which *C. difficile* strains were isolated were recruited from Cork hospitals and thus may not be indicative of the entire country. *C. difficile* strains were also isolated between 2006 and 2013. This may partially explain the difference in prevalence of *C. difficile* strains reported in this study to those reported in Ireland between 2010 and 2013 (Centre, 2013). The majority of *C. difficile* strains included in this study were isolated from patients who were asymptomatic for CDI in contrast to strains reported in the Enhanced Surveillance of *Clostridium difficile* reports are likely from patients with active CDI. This may lead to a biased view of *C. difficile* strains in the community as only those resulting in CDI are likely to be reported. Asymptomatic carriers of *C. difficile* have been proposed as potential disease reservoirs as they have higher rates of skin and environmental contamination compared to non-carriers (Riggs et al., 2007, Kim et al., 1981, McFarland et al., 1989). However, the exact contribution of asymptomatic carriers to the transmission of *C. difficile* is unknown (Eyre et al., 2013).

PCR-ribotyping in an established typing method widely used to tract the epidemiology of *C. difficile* in Europe. Its application coupled with mandatory case reporting was successful in stemming the increase in cases of CDI following years of steady rise in the UK. Previous issues surrounding pattern interpretation and reproducibility with PCR-ribotyping have been solved with the adaptation of high resolution capillary gel electrophoreses PCR-ribotyping (Indra et al., 2008). However, identification of ribotypes still requires access to a collection of reference strains. The Braziers collection of 20 of the most common *C. difficile* ribotypes is available to reference laboratories in Europe, while the CDRNE collection at Leeds houses over 500 ribotypes. In contrast, sequence data for MLST is freely available online from the *C. difficile* MLST database (http://pubmlst.org/cdifficile/), which is constantly updated. Contrary to PCR-RT data, MLST sequence data is readily transferable, highly reproducible, and is easily interpreted (Knetsch et al., 2013). MLST may also be performed directly on DNA isolated from patient stool, removing the need to culture for *C. difficile*, greatly reducing diagnosis and identification time (Griffiths et al., 2010). While PFGE was the most discriminatory typing scheme reported in this study, its laborious nature and sometimes difficult to interpret data reduce its applicability in high risk patient groups. Despite the number of advantages MLST holds over PCR-ribotyping and PFGE, the high cost of sequencing multiple gene targets is still prohibitive to its wide scale adoption for typing *C. difficile*. However, with the rapid advances in sequencing technologies these costs are reducing annually.

In conclusion, MLST was found to be as effective as PCR-RT for typing *C. difficile* in patients with a high risk of CDI. Although both techniques would need to be combined with a more discriminatory typing technique such as PFGE or multilocus variable-tandem repeat analysis to study clonal distribution in an outbreak setting.

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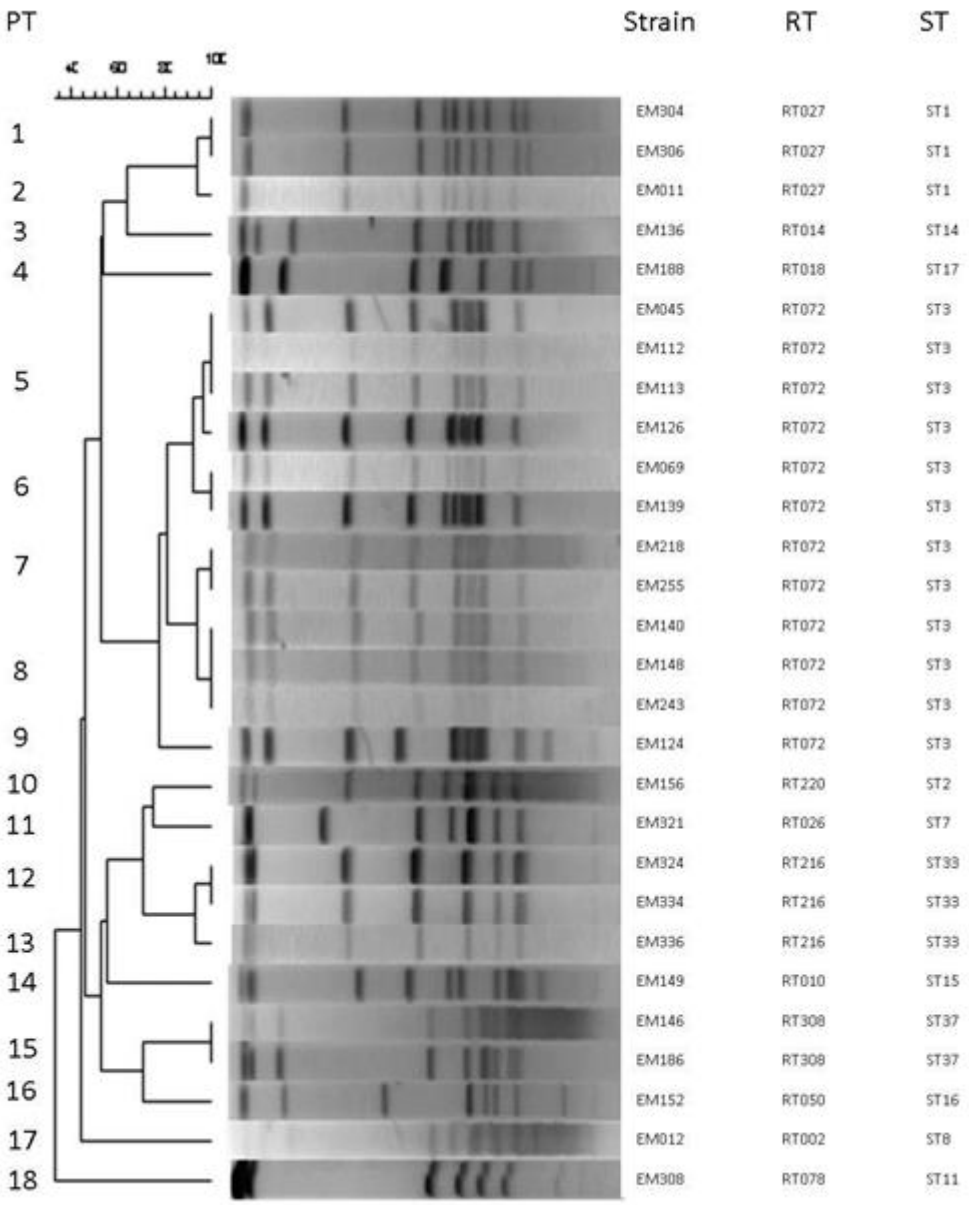
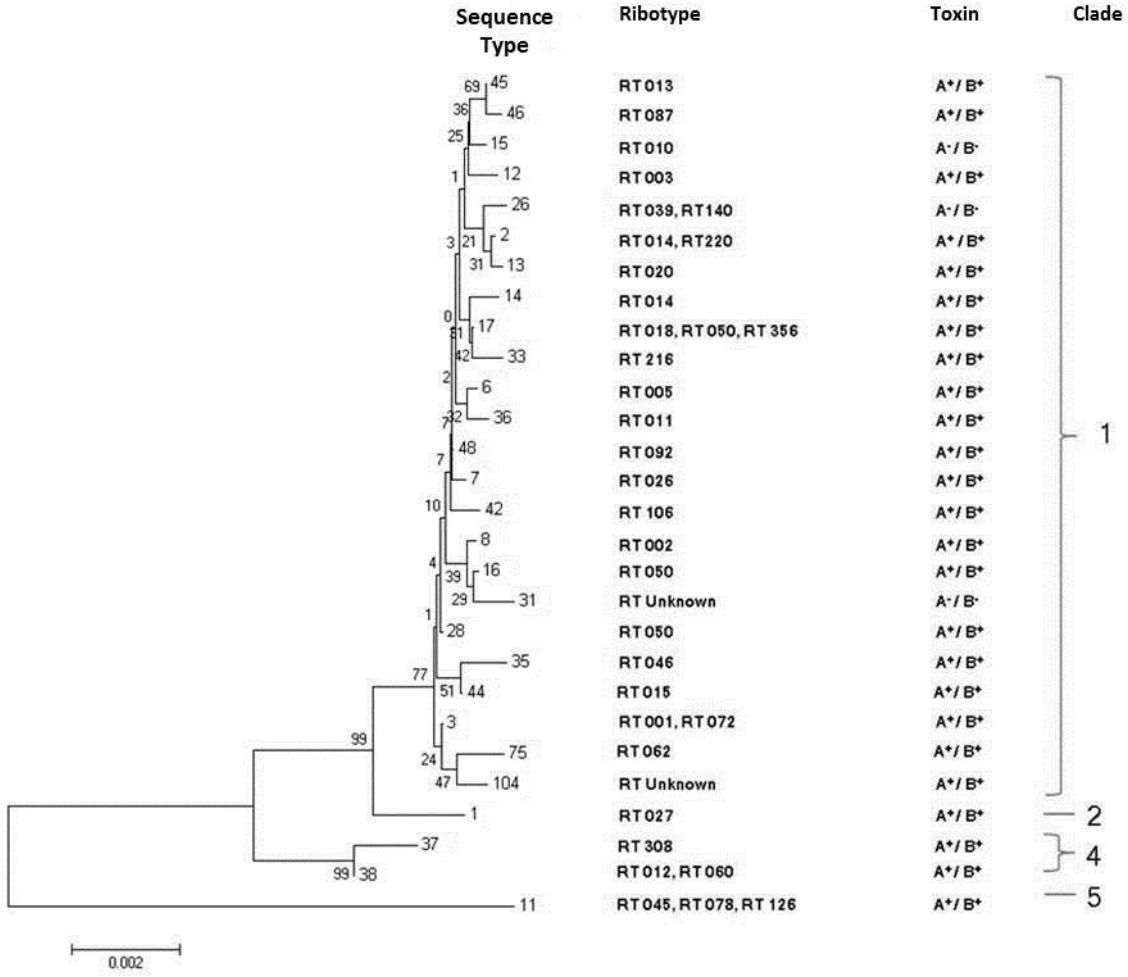
Figure 4.1 Dendrographic analysis of pulsed-field gel electrophoresis Sma 1 restriction profiles of *C. difficile* isolates with their corresponding PCR-ribotype and multilocus sequence type.



Pulsed field gel electrophoresis was performed using the Biorad Chef-DR II instrument and DNA macro-restriction profiles visualised using the Alpha Imaging system. DNA macro-restriction patterns were stored as TIFF files and imported into Bionumerics software (Version 3 Applied Maths, Kortrijk, Belgium) for dendrographic analysis. *Salmonella Braenderup* H9812 restricted with *Xba*1 at 37°C for 2h was used as a molecular weight marker for analysis using the Bionumerics software. Strains were assigned to the same pulse types (PT) if they had banding patterns of 95% similarity or greater.

Figure 4.2 Dendrographic analysis of *C. difficile* sequence type along with their corresponding PCR-ribotype and presence of toxin genes.



Sequence types were assigned based on the combination of alleles corresponding to each strain. Reconstruction of evolutionary relationships was performed using the MEGA 5 package (Tamura et al. 2011). Concatenated MLST sequence data obtained from the MLST online database (http://pubmlst.org/cdifficile/) was used to construct a consensus neighbour joining tree from 500 bootstrapping replicates. Information to the right of the tree, details corresponding ribotype, toxin genes and the clade for each sequence type.

Table 4.1: Table of *C. difficile* strains, their source of isolation, ribotype and sequence type.

Strain	Source	Ribotype	Sequence type
APC 1413	Cystic Fibrosis patient	014	ST 2
APC 1402	Cystic Fibrosis patient	001	ST 3
APC 1403	Cystic Fibrosis patient	001	ST 3
APC 1408	Cystic Fibrosis patient	009	ST 3
APC 1411	Cystic Fibrosis patient	001	ST 3
APC 1426	Cystic Fibrosis patient	001	ST 3
APC 1425	Cystic Fibrosis patient	005	ST 6
APC 1399	Cystic Fibrosis patient	002	ST 8
APC 1401	Cystic Fibrosis patient	126	ST 11
APC 1405	Cystic Fibrosis patient	078	ST 11
APC 1416	Cystic Fibrosis patient	045	ST 11
APC 1421	Cystic Fibrosis patient	078	ST 11
APC 1422	Cystic Fibrosis patient	126	ST 11
APC 1398	Cystic Fibrosis patient	014	ST 13
APC 1409	Cystic Fibrosis patient	010	ST 15
APC 1428	Cystic Fibrosis patient	010	ST 15
APC 1432	Cystic Fibrosis patient	356	ST 17
APC 1400	Cystic Fibrosis patient	039	ST 26
APC 1404	Cystic Fibrosis patient	140	ST 26
APC 1406	Cystic Fibrosis patient	140	ST 26
APC 1407	Cystic Fibrosis patient	140	ST 26
APC 1418	Cystic Fibrosis patient	039	ST 26
APC 1423	Cystic Fibrosis patient	140	ST 26
APC 1429	Cystic Fibrosis patient	140	ST 26
APC 1417	Cystic Fibrosis patient	Unknown	ST 31
APC 1412	Cystic Fibrosis patient	046	ST 35
APC 1414	Cystic Fibrosis patient	046	ST 35
APC 1419	Cystic Fibrosis patient	046	ST 35
APC 1420	Cystic Fibrosis patient	046	ST 35
APC 1424	Cystic Fibrosis patient	011	ST 36
APC 1430	Cystic Fibrosis patient	011	ST 36
APC 1410	Cystic Fibrosis patient	015	ST 44
APC 1431	Cystic Fibrosis patient	087	ST 46

APC 1415	Cystic Fibrosis patient	092	ST 48
APC 1427	Cystic Fibrosis patient	092	ST 48
EM 304	Elderly person Rehab	027	ST 1
EM 306	Elderly person Rehab	027	ST 1
EM 011	Elderly person Day	027	ST 1
EM 156	Elderly person Long stay	220	ST 2
EM 069	Elderly person Rehab	072	ST 3
EM 112	Elderly person Long stay	072	ST 3
EM 113	Elderly person Rehab	072	ST 3
EM 124	Elderly person Long stay	072	ST 3
EM 126	Elderly person Long stay	072	ST 3
EM 139	Elderly person Long stay	072	ST 3
EM 140	Elderly person Long stay	072	ST 3
EM 148	Elderly person Rehab	072	ST 3
EM 218	Elderly person Long stay	072	ST 3
EM 243	Elderly person Long stay	072	ST 3
EM 255	Elderly person Long stay	072	ST 3
EM 286	Elderly person	001/072	ST 3
EM 359	Elderly person	001/072	ST 3
EM 420	Elderly person	001/072	ST 3
EM 045	Elderly person Day	072	ST 3
EM 321	Elderly person Rehab	026	ST 7
EM 012	Elderly person Day	002	ST 8
EM 308	Elderly person Rehab	078	ST 11
EM 366	Elderly person	078	ST 11
EM 136	Elderly person Long stay	014	ST 14
EM 149	Elderly person Community	010	ST 15
EM 152	Elderly person Rehab	050	ST 16
EM 188	Elderly person Long stay	018	ST 17
EM 324	Elderly person Rehab	216	ST 33
EM 334	Elderly person Rehab	216	ST 33
EM 336	Elderly person Community	216	ST 33
EM 146	Elderly person Long stay	308	ST 37
EM 186	Elderly person Long stay	308	ST 37
EM 397	Elderly person	106	ST 42
EM 426	Elderly person	013	ST 045
EM 436	Elderly person	087	ST 46
EM 447	Elderly person	087	ST 46
EM 501	Elderly Colon cancer patient	087	ST 46
EM 503	Elderly Colon cancer patient	087	ST 46
EM 504	Elderly Colon cancer patient	087	ST 46

EM 505	Elderly Colon cancer patient	087	ST 46
EM 506	Elderly Colon cancer patient	087	ST 46
EM 510	Elderly Colon cancer patient	087	ST 46
EM 524	Elderly Colon cancer patient	087	ST 46
EM 525	Elderly Colon cancer patient	087	ST 46
EM 527	Elderly Colon cancer patient	087	ST 46
APC 11	Healthy volunteer	026	ST 7
APC 32	Healthy volunteer	018	ST 17
APC 14	Healthy volunteer	062	ST 75
APC 40	CDI patient	001	ST 3
APC 24	CDI patient	001	ST 3
APC 25	CDI patient	018	ST 17
APC 26	CDI patient	018	ST 17
APC 27	CDI patient	106	ST 42
APC 28	CDI patient	106	ST 42
APC 41	CDI patient	308	ST 37
APC 34	Irritable bowel syndrome patient	005	ST 6
APC 35	Irritable bowel syndrome patient	050	ST 16
APC 36	Irritable bowel syndrome patient	050	ST 17
APC 37	Irritable bowel syndrome patient	060	ST 28
APC 38	Irritable bowel syndrome patient	005	ST 38
APC 39	Irritable bowel syndrome patient	050	ST 104
APC 16	Inflammatory bowel disease patient	027	ST 1
APC 17	Inflammatory bowel disease patient	001	ST 3
APC 10	Inflammatory bowel disease patient	005	ST 6
APC 9	Inflammatory bowel disease patient	005	ST 6
APC 29	Inflammatory bowel disease patient	078	ST 11
APC 3	Inflammatory bowel disease patient	003	ST 12
APC 6	Inflammatory bowel disease patient	020	ST 13
APC 4	Inflammatory bowel disease patient	010	ST 15
APC 29	Inflammatory bowel disease patient	050	ST 42
APC 5	Inflammatory bowel disease patient	015	ST 44
APC 7	Inflammatory bowel disease patient	015	ST 44
APC 1	Inflammatory bowel disease patient	015	ST 44
APC 2	Inflammatory bowel disease patient	015	ST 44
APC 8	Inflammatory bowel disease patient	062	ST 75
ATCC BAA-1382 , 630	Clinical isolate	012	ST 38
ATCC 43255, VPI 10463	Abdominal wound	087	ST 46

Table 4.2: Table of most prevalent sequence types, their correspondine ribotypes, their source of isolaton and precentage of total strains.

(a)

Sequence type	Ribotype	% of strains	Source
ST3	RT001, RT009, RT072	19.6	Elderly (14), CDI (2), CF(5), IBD (1)
ST46	RT087	11.6	Colon cancer (11), CF (1), VPI10463 (1)
ST11	RT045, RT078, RT126	7.1	CF (5), Elderly (2), IBS (1)
ST26	RT039, RT140	6.3	CF (7)
ST17	RT018, RT050, RT356	5.4	CDI (2), CF(1), Elderly (1), HV(1), IBS (1)
ST1	RT027	4.5	EM (4), IBD (1)
ST44	RT015	4.5	CF (1), IBD (4)

EL = Elderly person, CF = Cystic fibrosis patient, CDI = *C. difficile* infection,

IBS = Irritable bowel syndrome, IBD = Inflammatory bowel disease, HV = Healthy volunteer, CC = colon cancer.

Chapter 5

Assessment of the bacteriophage ΦCD6356 and its biologically-active endolysin as novel antimicrobials targeting *Clostridium difficile*

5.1 Abstract

Clostridium difficile infection is typically associated with the use of broad-spectrum antibiotics. Consequently, there is a need for the development of specific antimicrobials which target *C. difficile* but do not result in collateral effects on the microbiota. The *C. difficile* bacteriophage ΦCD6356 can proliferate in an ex vivo model of the human distal colon resulting in a 1.75 log reduction in viable *C. difficile* over 24 hours. However, due to its lysogenic nature, 100% of *C. difficile* surviving in the presence of ΦCD6356 were found to harbour prophage. The endolysin encoded by ΦCD6356 was cloned and expressed in *Escherichia coli*. This recombinant endolysin, LysCD, has lytic activity against heat-killed and live *C. difficile* cells. Crude LysCD was capable of reducing *C. difficile* by ~ 3 log units within 2 hours. The theoretical 3D structure of the endolysin was determined based on amino acid sequence homology to experimentally determined endolysins revealing zinc coordination at the endolysins active site. Endolysin lytic activity was eliminated by treatment with EDTA but activity was restored when zinc and other divalent metal ions were reintroduced. The endolysin also retained activity after heating to 90ºC for 5 min.

5.2 Introduction:

Clostridium difficile is an opportunistic enteric pathogen which causes a wide range of disease, from mild diarrhoea to pseudomembranous colitis (Knoop et al., 1993). *C. difficile* infection (CDI) commonly occurs following antibiotic therapy and as such is thought to be the result of altering the innate protection afforded by the intestinal microbiota (Bibbò et al., 2014). Indeed, *C. difficile* is responsible for causing between 15 and 25% of cases of antibiotic associated diarrhoea (AAD) worldwide (Bartlett, 2002). The organism can form extremely resistant spores that can persist for long periods in the environment. This, coupled with its resistance to heat and

antiseptic cleaners has allowed *C. difficile* to become particularly problematic in hospitals and rest homes, where it has emerged as a major cause of nosocomial diarrhoea (Weber et al., 2010). Outbreaks of CDI can be extremely costly for healthcare providers with the average case costing between $9179 and $11 456 (McGlone et al., 2012). *Clostridium difficile* infection is typically treated with either metronidazole or vancomycin; however, treatment failure is common with a failure rate of 22.4% and 14.2% associated with these two antibiotics respectively (Vardakas et al., 2012). Relapse after treatment is also common and is likely due to failure of the enteric microbiota to recover following treatment with these broad spectrum antibiotics (Vardakas et al., 2012). This highlights the need for highly effective therapeutics with a narrow spectrum of inhibition to target *C. difficile* while leaving the majority of the intestinal microbiota undisturbed. Bacteriophage (bacterial viruses) and the endolysins they produce may offer such a solution.

While there are two types of bacteriophage replication cycle, lytic and lysogenic, *C. difficile* bacteriophage are typically lysogenic. Lytic bacteriophage replicate in the host then lyse the host cell wall releasing new progeny. While lysogenic bacteriophage can do likewise, they can also insert into the host genome and replicate with the host as a prophage (Adams, 1959). Bacteriophage therapy was first trialled in 1919, however, was mostly abandoned after the introduction of antibiotics in the 1930's (Wittebole et al., 2013). There has been a resurgence of interest in bacteriophage therapy in recent times with the emergence of antibiotic resistance. The effectiveness of bacteriophage for treating *C. difficile* induced ileocecitis was demonstrated by Ramesh et al, 1999 (Ramesh et al., 1999) in a hamster disease model. The authors reported recovery of all but one animal after bacteriophage treatment while all animals in the control group died within 96 hours (Ramesh et al., 1999). *C. difficile* bacteriophage have also demonstrated their effectiveness in preventing growth and toxin production by *C. difficile* in a batch fermentation model of the human distal colon. Prophylactic treatment with bacteriophage

at a multiplicity of infection (MOI) of 10 eliminated all *C. difficile* after 48 hours. At an MOI of 7 the authors reported prevention of growth and reduction in toxin production (Meader et al., 2010).

Recombinant bacteriophage endolysins have also been demonstrated as effective narrow spectrum antimicrobials against a number of human pathogens (Daniel et al., 2010, Fenton et al., 2010, Lim et al., 2012, Mayer et al., 2008, Son et al., 2012). An endolysin is a bacteriophage encoded cell wall hydrolase required to cleave the host cell wall, allowing for release of bacteriophage progeny (Young, 1992). Recombinant endolysins have also been assessed as potential treatment for CDI. Mayer and colleagues achieved rapid lysis of C. difficile in vitro using the recombinant endolysin CD27L (Mayer et al., 2008).

The study reported here was undertaken to assess the efficacy of the bacteriophage ΦCD6356 and its recombinant endolysin, LysCD as potential treatments for CDI.

5.3 Materials and Methods

Bacterial and bacteriophage culture

All *C. difficile* strains used in this study were obtained from the DPC (Dairy Production Centre, Teagasc Food Research Centre, Moorepark, Cork, Ireland) culture collection including DPC 6356 from which the bacteriophage ΦCD6356 was induced (Horgan et al., 2010). *C. difficile* strains were routinely sub-cultured onto Fastidious Anaerobic Agar (FAA) (Lab M Lancashire, UK) containing 7% defibrinated horse blood and grown anaerobically in a Don Whitley anaerobic chamber at 37˚C. Bacteriophage ΦCD6356 was propagated in early log phase cultures and concentrated to a high titre as described previously (Alemayehu et al., 2009, Horgan et al.,

2010, Moineau et al., 1994). Phage numbers were routinely estimated by plaque assay using 0.7% Brain Heat Infusion (BHI) agar seeded with 2% overnight culture of *C. difficile* overlaid onto a 1.5% BHI agar base. The spectrum of infection of the bacteriophages was assessed by plaque assay on 40 human *C. difficile* isolates housed in the DPC collection here at Teagasc Food Research Centre, Moorepark. *Escherichia coli* XL1-Blue was grown in Luria broth (Merck, Darmstadt Germany) shaking (160 rpm) at 37˚C.

DNA isolation and PCR analysis for prophage

Genomic DNA was isolated from *C. difficile* strains recovered from fermentation vessels as described by Rea et al (Rea et al., 2012). *C. difficile* isolates were suspended in 5% w/v chelex-100 resin and heated to 56 ºC for 30 min followed by 100 ºC for 8 min. The cell suspension was then centrifuged at 16,000 x g for 3 min to remove cell debris. The resulting supernatant was used as the template DNA for subsequent PCR reactions. Phage DNA was isolated by phenol-chloroform extraction as described by Moineau, et al (Moineau et al., 1994). Prophage was detected by PCR amplication with primers designed to amplify the three structural genes, namely the putative major capsid protein A (ORF 5), the putative phage tail protein (ORF 16), and the N-acetylmuramoyl-L-alanine amidase endolysin (ORF 28). Primers, product size, and annealing temperature are outline in Table 5.1. Isolates were deemed to harbour the prophage if all three structural genes were present. All *C. difficile* strains (40) held in the DPC culture collection were also screened for prophage carriage by PCR.

Efficacy of the bacteriophage ΦCD6356 in treating C. difficile infection

Batch fermentations modelling the human distal colon were performed as described by Rea et al (Rea et al., 2011). Briefly, the fermentation medium was prepared according to Fooks and Gibson, 2003 (Fooks and Gibson, 2003). Fermentation vessels from the Multifors fermentation system (Infror UK), containing fermentation media (160ml) were autoclaved at 121˚C for 15 min. The pH of the media was adjusted to 6.8 and the pH maintained throughout the experiment by addition of HCl (1M) and NaOH (1M). Nitrogen was sparged through the fermentation media for at least 2h prior to inoculation to remove oxygen and create an anaerobic environment. The system was run under anaerobic conditions by sparging continuously with N2. A composite faecal sample was prepared by combining fresh faecal samples obtained from two healthy donors aged between 21 and 45 years old who had not received antibiotics in the previous three months and who tested negative for carriage of *C. difficile*. Composite faecal samples were diluted in pre-reduced 50 mM phosphate buffer (pH 7.2) and homogenised by stomaching for 1 min at high speed to achieve a 20% w/v suspension. The resulting faecal slurry (35ml) was inoculated into each fermentation vessel along with 2ml of an overnight culture of *C. difficile* DPC 6219 (ribotype 001) (1x 106 CFU/ ml final concentration) as well as 1ml of CaCl2 (1M). Two ml bacteriophage (1x 108 PFU/ml) suspended in 0.1M ammonium acetate was added to the experimental vessels, while 2 ml of ammonium acetate (0.1M) was added to the control vessels. Batch fermentations were run for 24h and maintained at 37 ºC pH 6.8. Samples were taken at 2, 4, 6, 8, and 24 h time to estimate the numbers of viable *C. difficile* and bacteriophage. Samples were serially diluted 10-fold in pre-reduced maximum recovery diluent (MRD) and plated on Cefoxitin-Cycloserine Egg-Yolk (CCEY) agar for counting *C. difficile* or filter sterilized and used for plaque assay as described above. Plates were incubated anaerobically at 37˚C for 48h, after which cell and plaque counts were made. To determine if bacteriophage entered the lysogenic phase during infection, 5 colonies of *C. difficile* from the highest countable dilution

were selected from CCEY plates from each vessel prior to inoculation with bacteriophage at 0h and again at hour 24h. DNA was extracted and screened for prophage structural genes by PCR amplification.

Sub-cloning of bacteriophage endolysin gene into E. coli

The endolysin gene (ORF 28) of bacteriophage ΦCD6356 described by Horgan et al (Horgan et al., 2010), here after referred toas lyscd, was amplified from genomic DNA using primers designed to introduce an NcoI restriction site at the 5' end (5'-ATATCCATGGAGGTTGTACTAACAGCAG -3' where underlining indicates restriction site) and a BglII site downstream of the coding site (5'-CCCAGATCTTTTCTTAATAAAATCTAATACT -3'). Amplicons were restriction digested with NcoI and BglII, then ligated into the expression vector pQE-60 (Qiagen) using T4 DNA ligase (NEB). The resulting construct, pQE-60lyscd, was transformed into chemically competent *E. coli* XL1-Blue and selected for by ampicillin (100μg/ml). Endolysin gene was identified by restriction analysis of the vector using NcoI and BglII and PCR amplication with lyscd primers.

Protein expression

The endolysin was induced by addition of 1mM isopropyl β-D-thiogalactopyranoside (IPTG) to a culture of *E. coli* XL1-Blue.(pQE-60lyscd) at an optical density (600nm) of 0.3, followed by incubation at 26ºC for 14h to avoid inclusion bodies as previously described (Horgan et al., 2009). Bacterial cells were suspended in TN buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl) and disrupted by four rounds of bead beating with 0.1mm acid washed beads (Sigma) with 1min on ice between 1min bursts. Cell debris was removed by centrifugation at 16,000 x g x 20 min

followed by filter sterilization using a 0.45 μM pore filter (Merck Millipore, Darmstadt, Germany). His-tagged endolysin was partially purified under native conditions using the HisTrap FF system (GE healthcare). Protein was eluted in 20mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4. Protein lysate and partially purified endolysin were stored at -20ºC until required.

SDS-PAGE and Zymogram analysis

Lytic activity of the crude endolysin preparation was visualized by zymogram analysis. Zymograms were prepared by incorporating a 20-fold concentration of heat killed (100ºC for 10 min) mid log phase *C. difficile* DPC 6219 cells into a 12% SDS-PAGE as described by Donovan et al (Donovan et al., 2006). Briefly, zymograms were run in parallel with a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, with the same gel mix used for both. A mid–log phase culture of *C. difficile* in 100ml of BHI broth was centrifuged at 16,000 x G for 5 min to remove cells. The resulting pellet was washed once in sterile distilled water and harvested by centrifuging again before being heated to 100 ˚C for 10 min to kill the target cells. Cells were resuspended in 5 ml of the zymogram gel mix prior to the addition of TEMED (N,N,N',N"-tetramethylethylenediamine). Gels were run at 100 V for 2h in a Bio-Rad Mini-PROTEAN 3 gel apparatus. Zymograms were washed in distilled water and incubated overnight in 2.5% Triton X-100 solution. SDS-PAGE gels were stained with comassie blue.

Generation and analysis of a model for ϕCD6356 lysin.

A model for ϕCD6356 lysin, LysCD, was built using MODELLER (Sali and Blundell, 1993) with the amidase domain of the endolysin CD271 (3QAY) as the template structure (Mayer et al., 2011).

Effect of EDTA and requirement of divalent ions on endolyisn activity.

The effect of divalent ions on endolysin activity was determined as previously described (Schmelcher et al., 2012b). Endolysin was incubated with 50 mM ethylenediaminetetraacetic acid (EDTA), a chelating agent, for 15mins at room temperature to remove metal ions. EDTA was removed by passing the sample through an Amicon® Ultra-15 3K filter (Millipore). The resultant protein extract was then re-suspended in TN buffer (10 mM Tris-HCl pH 7.6, 10 mM NaCl). The EDTA treated endolysin was tested for lytic activity by zymogram with and without the addition of metal ions (ZnCl2, MgCl2, MnCl2 or CaCl2, at concentrations of 1mM or10 mM). The addition of 10 mM Zn2+ resulted in precipitation and therefore was not tested.

Activity of endolysin against live C. difficile

C. difficile were grown to mid-log phase (OD 600nm 0.6), then harvested and re-suspended in pre-reduced MRD to an OD 600 of ~1.2.Washed *C. difficile* cells (200µl) were incubated with protein extract (300 µg final concentration) in TN buffer (100µl) from induced or uninduced *E. coli* for 2 h anaerobically at 37 ºC. Following incubation *C. difficile* were 10-fold serially diluted and plated on pre reduced BHI agar to enumerate surviving cells.

5.4 Results

The association of *C. difficile* with antibiotic associated diarrhoea (Bartlett, 2002) as well as the emergence of metronidazole resistant and vancomycin insensitive strains (Peláez et al., 2002) has led to increased interest in narrow spectrum antimicrobials with activity against *C. difficile.* Therefore we investigated the *C. difficile* bacteriophage ΦCD6356 and its associated endolysin for their ability to kill *C. difficile*.

Bacteriophage spectrum of inhibition and prophage carriage

The spectrum of infection of ΦCD6356 was examined by plaque assay against 40 human *C. difficile* isolates housed in the DPC culture collection (Table 5.2). The bacteriophage was capable of forming plaques on 11 of 40 strains, including ribotype 078 (1) and ribotype 001(3) which are typically associated with CDI (Goorhuis et al., 2008b, Borgmann et al., 2008). *C. difficile* isolates were also examined for carriage of prophage by PCR amplication of phage structural genes. Prophage was detected in 6 of 40 isolates tested, including DPC6356 (ribotype 005) from which the phage was originally induced. Not surprisingly, lysogenic strains were not susceptible to infection by the bacteriophage. Prophage was not detected in *C. difficile* DPC6219 which was used in the ex vivo model of the distal colon. Isolates harbouring prophage were identified as ribotype 106(1), 005 (2), 050 (3).

Efficacy of the bacteriophage ΦCD6356 in treating C. difficile infection

A model of the human distal colon was used to assess the efficacy of using the bacteriophage ΦCD6356 to treat *C. difficile* infection. Bacteriophage was added to the system at an MOI of 1 (1x 106 PFU/ml) resulting in a highly significant (P= <0.001) nearly 2-fold reduction in the

recovery of *C. difficile* relative to control vessels at 24 h. A 1.75 log reduction in *C. difficile* was observed over 24 h following a single dose of bacteriophage at an MOI of 1. Rapid proliferation of bacteriophage was observed between hour 2 and 4 for the experiment 6 (fig. 5.1B), which preceded a sharp decline in *C. difficile* after 4 hours (fig. 5.1A). Bacteriophage increased rapidly during active growth of *C. difficile*, however, this seemed to cease as *C. difficile* reached its stationary phase at hour 6 (fig. 5.1B).

To assess the frequency to which bacteriophage may enter the lysogenic life cycle during treatment; five isolates from each vessel were examined for prophage by PCR. Prophage carriage was defined by PCR amplication of three phage structural genes from the genomic DNA of *C. difficile* isolates. Prophage was not detected in any *C. difficile* isolated before the addition of bacteriophage or from control vessels. However, prophage was detected in 100% of *C. difficile* isolated from bacteriophage treated vessels at 24 h following bacteriophage treatment.

Cloning and expression of the biologically active endolysin in E. coli

The gene encoding the ΦCD6356 bacteriophage endolysin lyscd was successfully cloned and expressed with a six–histidine tag in *E. coli* under IPTG induction. The ~31 kDa endolysin was visible in protein lysate of induced *E. coli* XL1-Blue(pQE-60lyscd) on SDS-PAGE, but not in uninduced lysate. The identity of the endolysin was confirmed by zymogram run in parallel with an SDS-PAGE (fig 5.3). Zones of clearing in the zymogram corresponded with the size of the endolysin. Partial purification of the endolysin was achieved using the HisTrap FF system however; endolysin activity was highly unstable in partially purified fractions. For this reason all further experiments were carried out using crude protein extracts.

Generation and analysis of a model for ϕCD6356 lysin

A model for ϕCD6356 lysin, LysCD, was built using MODELLER (Sali and Blundell, 1993) with the amidase domain of the endolysin CD27l (3QAY) as the template structure (Mayer et al., 2011) (fig. 5.4). Sequence comparisons of the amidase domains from ϕCD6356 and CD271 indicated there were 69 identities between the two sequences and these were distributed along the length of the domain(51% identity overall). Critically, this analysis showed conservation of key residues in ϕCD6356 which had previously been implicated in the activity of CD27l (Mayer et al., 2011).

The model of the ϕCD6356 amidase domain shows a typical αβ amidase fold with a central six stranded mixed β-sheet flanked by five α-helices (Fig. 5.4). The RMSD Cα for superposition of the ϕCD6356 amidase on the CD27l amidase is 0.975 Å. Two loops in the ϕCD6356 amidase domain are truncated (labelled Loop I and Loop II in Fig. 5.4) with respect to the CD27l template. This is a result of deletion of 2 and 5 residues in the ϕCD6356 amidase domain sequence respectively. The active site of the amidase domain found in many bacteriophage endolysins is composed of two conserved His residues (His9 and His 84 in CD271) and a conserved Glu residue (Glu26 in CD271) which are involved in coordination of the catalytic Zn atom (Mayer et al., 2011). The three catalytic residues are augmented by a Glu (Glu144 in CD271) which is proposed to act as a proton acceptor during catalysis (Mayer et al., 2011, Korndorfer et al., 2006). All four residues are conserved in the ϕCD6356 amidase domain (His9, His77, Glu24, and Glu 137 respectively) (Fig. 5.6). The solvent exposed nature of the active-site cleft in the ϕCD6356 model is consistent with the conformation found in other endolysin amidase domains (Korndorfer et al., 2006, Mayer et al., 2011, Yang et al., 2012, R.Zhang, 2008, T.Yamane, 2003). The rim of the active site cleft has electropositive character

while the region immediately proximal to the catalytic residues has electronegative character which is similar to the structure of CD27l (Fig. 5.5). As previously described this distribution in charge character was seen in the CD27l amidase domain (Mayer et al., 2011) and has been proposed to serve a role in allowing the enzyme's active site to access its buried substrate.

Two other key features of the solved CD27l amidase are preserved in the ϕCD6356 amidase. There is a series of conserved residues (Asn 86, Glu 96 and Arg 122 in CD271) which has been mooted as a proton relay system to the proton accepting Glu (Glu 144 in CD271) in the amidase active site (Korndorfer et al., 2006, Mayer et al., 2011, T.Yamane, 2003). These residues are present and conserved in the ϕCD6356 model (Asn 79, Glu 91 and Arg 117) (Fig. 5.5), and in line with the conservation between this model and the CD271 template residue Ile93 in the ϕCD6356 model corresponds to residue Leu98 of CD27l. This residue is of significance as it is part of a spatial cluster of three residues in CD27l proposed to interact with the substrate. The additional residues are Leu130/Tyr131 in CD27l, and Leu126/Tyr127 in the ϕCD6356 model (Fig. 5.5). Studies on the CD27l enzyme have shown that mutation of the Leu98 residue to a Trp, analogous to the PlyPSA enzyme, enhanced the activity of the mutant enzyme towards cells targeted by the PlyPSA enzyme, i.e. enhanced lysis of Listeria monocytogenes (Mayer et al., 2011).

Effect of EDTA and requirement of divalent ions on endolysin activity

To determine whether zinc was required for lytic activity, EDTA treated and untreated endolysin with reintroduced divalent ions were tested for activity using a zymogram. A short treatment of 15 min with the chelating agent EDTA (50mM) was sufficient to completely remove lytic activity. Lytic activity was restored by addition of either 1mM or 10mM of each divalent ion (MgCl2, MnCl2 or CaCl2), in the case of ZnCl2 only 1mM was tested.

Endolysin kills live C. difficile

To assess whether the endolysin was capable of lysing live cells, crude LysCD was assayed against mid-log phase *C. difficile* at 37ºC, under anaerobic conditions. Protein extract from uninduced *E. coli* was used as a control. Crude LysCD reduced *C. difficile* by ~3 logs in 2 hours compared to uninduced protein extract (Fig 5.2).

5.5 Discussion

In this study, we evaluated the bacteriophage ΦCD6356 and its associated recombinant endolysin, LysCD for their potential as therapeutics for CDI. The bacteriophage ΦCD6356 was examined using an ex vivo model of the human distal colon as previously described (Meader et al., 2010, Rea et al., 2011). We demonstrated that a single dose of the bacteriophage at an MOI of 1 was sufficient to significantly reduce the number of viable *C. difficile* by 1.75 log units within 24 hours. Previous studies have also demonstrated the effectiveness of a single dose of bacteriophage in treating infection (Ramesh et al., 1999, Smith and Huggins, 1982). In fact, Ramesh and colleagues found a single dose of bacteriophage (108 PFU) to be sufficient to treat hamsters with *C. difficile* induced ileocecitis. Following 96h, the majority of bacteriophage treated hamsters had survived while all control hamsters had died (Ramesh et al., 1999). This is likely due to the rapid proliferation of bacteriophage in situ (Smith and Huggins, 1982) as was observed in our experiment (fig. 5.1 B). Bacteriophage increased by 1 log preceding the rapid reduction in *C. difficile* as can be seen in graphs 5.1 A and 5.1 B. In a similar experiment, Meader et al found that not only did bacteriophage treatment reduce the number of viable *C. difficile* but also prevented toxin production (Meader et al., 2010). As *C. difficile* have been reported to be excreted at between 107 and 109 CFU/g of faeces (Mutters et al., 2009), a reduction of 1.75 log units coupled with prevention of toxin production may be sufficient to

alleviate symptoms of CDI. In the study by Meader et al they found that by increasing the MOI from 7 to 10 resulted in complete elimination of *C. difficile* after 48 hours (Meader et al., 2010).

Typically, the use of lysogenic bacteriophages is not efficacious for bacteriophage therapy due to their potential to relysogenise, and thus conferring immunity to the host against further infection (called "super infection"). A PCR screen for prophage structural genes identified the prophage in all *C. difficile* recovered from bacteriophage treated vessels but none were found in those from control vessels. A similar observation was made by Meader et al after treatment with the bacteriophage ΦCD27. Following the 48 hour treatment, bacteriophage was present in 100% of surviving *C. difficile* isolates as evident by induction of bacteriophage using mitomycin C (Meader et al., 2010). As well as conferring immunity on the host, prophage have also been associated with horizontal gene transfer between bacteria (Casjens, 2003). Prophage have also been linked to increased toxin production in hyper-virulent *C. difficile* ribotype 027 strains (Sekulovic et al., 2011). Prophage carriage has also been cited as a possible reason for the narrow host range of most *C. difficile* bacteriophages (Ramesh et al., 1999, Raya et al., 2006).While ΦCD6356 was only lytic against 11 of 40 strains tested; this included two ribotypes frequently associated with CDI, 001 and 078 (Borgmann et al., 2008, Goorhuis et al., 2008a). This is encouraging as ribotype 001isolates with reduced susceptibility to metronidazole have been reported (Baines et al., 2008) and 078 is emerging as a new hyper-virulent strain (Goorhuis et al., 2008a). However, due to the lysogenic nature of ΦCD6356 it would not make a suitable treatment for CDI. Therefore, the focus of this research shifted towards the bacteriophages endolysin (probable N-acetylmuramoyl-l-alanine amidase ORF 28), previously described by Horgan et al (Horgan et al., 2010).

The endolysin, designated LysCD was cloned into *E. coli* XL1 Blue and expressed by induction with IPTG. N-acetylmuramoyl-l-alanine amidase endolysins have previously been reported in *C. difficile* bacteriophages ΦCD27 and ΦCD119 (Govind et al., 2006, Mayer et al., 2008), however

these only share 51% and 41% identity to LysCD, respectively. This is likely due to ΦCD27 and ΦCD119 belonging to the myoviridae family and ΦCD6356 belonging to the siphoviridae family of viruses (Govind et al., 2006, Horgan et al., 2010, Mayer et al., 2008). Crude protein extracts containing recombinant endolysin displayed lytic activity against live and heat killed *C. difficile* DPC6219 in vivo. Unfortunately, lytic activity was quickly lost after partial purification, and therefore further work needs to be done to stabilize endolysin activity.

A model for ΦCD6356 lysin, LysCD, was built using MODELLER (Sali and Blundell, 1993) with the amidase domain of the endolysin CD271 (3QAY) as the template (Mayer et al., 2011). This revealed the presence of zinc coordination at the enzymes catalytic domain. Removal of zinc by treatment with EDTA eliminated the lytic activity of the endolysin. Reintroduction of zinc as well as other divalent metal ions (Mn2+, Mg2+and Ca2+) to EDTA treated endolysin restored lytic activity. This flexibility in divalent ions has been reported previously in other bacteriophage endolysins (Schmelcher et al., 2012b, Son et al., 2012). In fact, Schmelcher et al reported a 2.4 fold increase in activity relative to control after addition of 1 mM Mn2+, and thus this could be exploited to increase lytic activity of LysCD (Schmelcher et al., 2012b).

Endolysins have been reported to have similar if not broader host range than that of parent bacteriophage while still specifically targeting bacteria within the species or genera (Horgan et al., 2009, Mayer et al., 2008, O'Flaherty et al., 2005, Schmelcher et al., 2012a). Endolysins have also been shown to have rapid action at relatively low concentrations. To date resistance against endolysins has not yet been reported (Schmelcher et al., 2012a).

Therefore, while the bacteriophage ΦCD6356 is not suitable for bacteriophage therapy due to its lysogenic nature, its cloned, biologically active endolysin, LysCD has demonstrated potential as a novel narrow spectrum antimicrobial for the treatment of CDI.

5.6 Acknowledgements

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Table 5.1: PCR primers, annealing temperatures and product size

Gene	Primer	Annealing temperature (ºC)	Product size (bp)
Endolysin *LysCD* (ORF 28)	F 5'- ATATCCATGGAGGTTGTACTAACAGCAG R 5'- CCCAGATCTTTTCTTAATAAAATCTAATACT	56	804
Major capsid protein A (ORF 5)	F 5'- GCTGACAGGGCGATACTTGA R 5'- CCAGCTGACCCAATTCCCAT	57	809
Phage tail protein (ORF 16)	F 5'- TTGCTTTGTGGGTTGGTGAC R 5'- ACACCCCCAAATAAAGCGGAT	57	435

Table 5.2: *C. difficile* strains examined for prophage carriage and susceptibility to infection with bacteriophage ΦCD6356.

Strain	*Source*	*Ribotype*	*Prophage*	*Infection*
DPC6219	CDI patient	001	-	+
DPC6538	Inflammatory bowel disease patient	001	-	+
DPC6534	Irritable bowel syndrome patient	001	-	+
DPC6510	Irritable bowel syndrome patient	005	-	-
DPC6514	Irritable bowel syndrome patient	005	-	-
DPC6353	Ulcerative colitis patient	005	-	-
DPC6355	Ulcerative colitis patient	005	+	-
DPC6356	Ulcerative colitis patient	005	+	-
DPC6507	Crohns disease patient	010	-	-
ATCC BAA-1382 , 630	ATCC, Clinical isolate	012	-	-
DPC6360	Crohns disease patient	015	-	-
DPC6362	Crohns disease patient	015	-	-
DPC6357	Ulcerative colitis patient	015	-	-
DPC6358	Ulcerative colitis patient	015	-	-
DPC6220	CDI patient	018	-	+
DPC6221	CDI patient	018	-	+
DPC6366	Healthy adult	18	-	+
DPC6363	Crohns disease patient	020	-	-
DPC6505	Healthy adult	026	-	-
DPC6511	Irritable bowel syndrome patient	050	+	-
DPC6512	Irritable bowel syndrome patient	050	+	-
DPC6515	Irritable bowel syndrome patient	050	+	-
DPC6513	Irritable bowel syndrome patient	060	-	-
DPC6361	Crohns disease patient	062	-	-
DPC6506	Healthy adult	062	-	+
DPC6359	Ulcerative colitis patient	078	-	+
ATCC 43255, VPI 10463	ATCC, Abdominal wound	087	-	-
DPC6350	CDI patient	106	-	-
DPC6351	CDI patient	106	-	-
DPC6539	Inflammatory bowel disease patient	106	+	-
DPC6535	CDI patient	308	-	-
ATCC 43600	ATCC, antibiotic-associated colitis	N/A	-	+
ATCC 43593	ATCC, Human faeces	N/A	-	-
DPC6535	CDI patient	N/A	-	-
DPC6365	Irritable bowel syndrome patient	N/A	-	-
DPC6365	Irritable bowel syndrome patient	N/A	-	+
DPC6508	Ulcerative colitis patient	N/A	-	+

Figure 5.1 (a): *C. difficile* counts in control and bacteriophage treated fermentation vessels over 24h.

A.

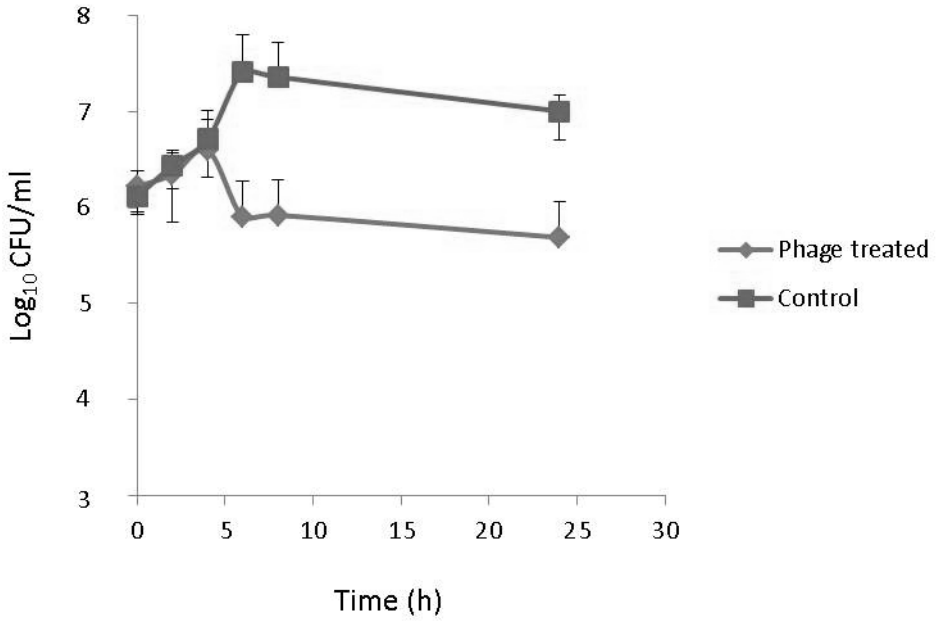


Figure 5.1 (b): Bacteriophage ΦCD6356 counts in bacteriophage treated vessels over 24hours.

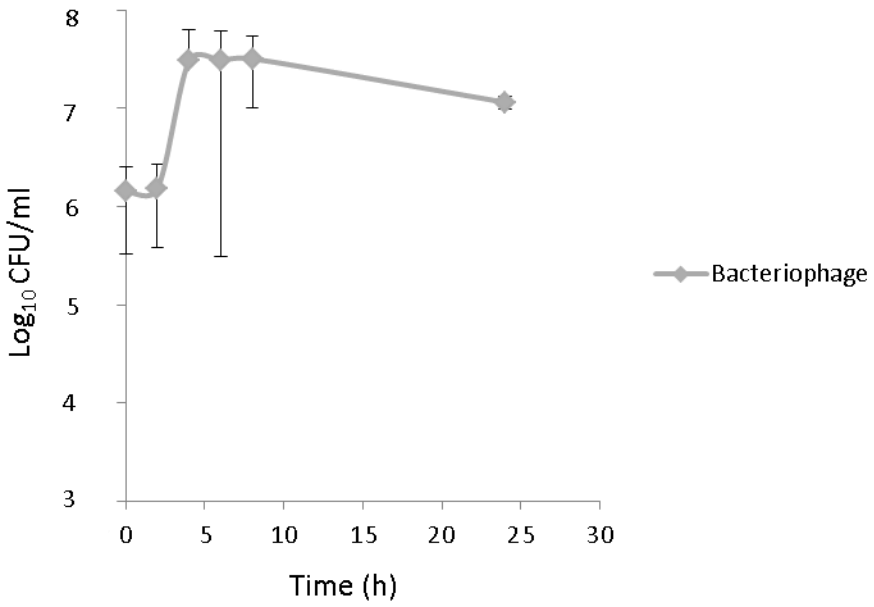
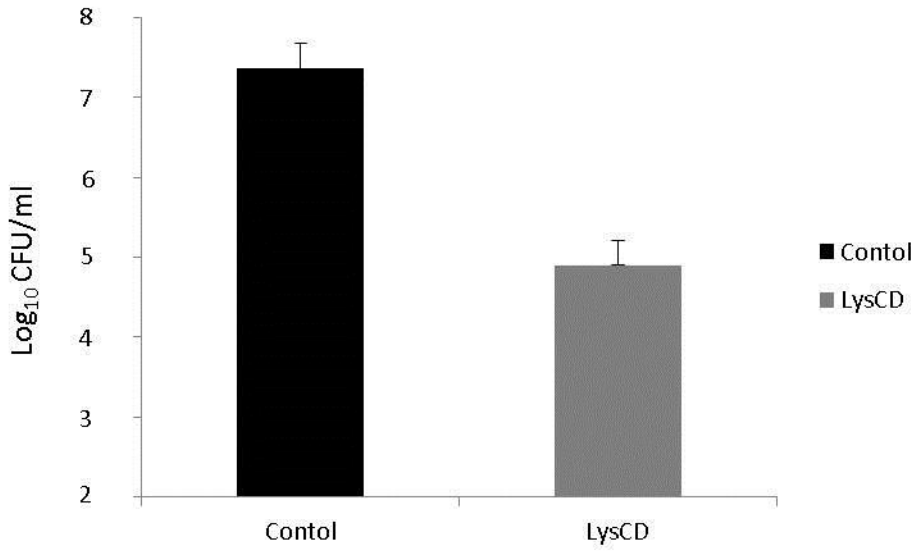
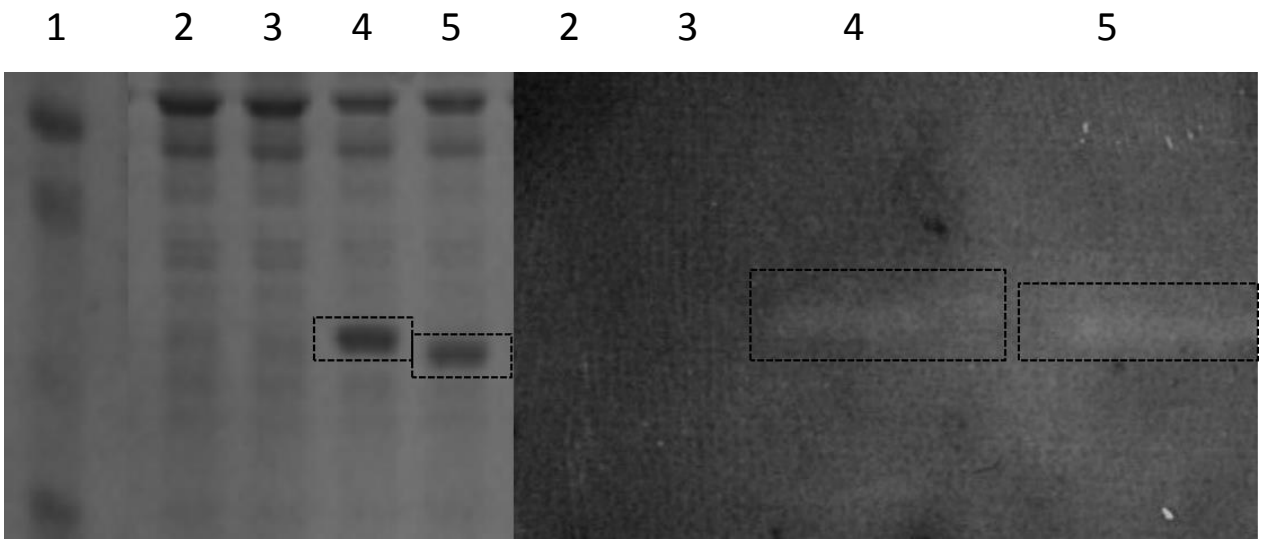


Figure 5.2: Graph of the reduction in *C. difficile* numbers after 2hours incubation with the endolysin, LysCD.



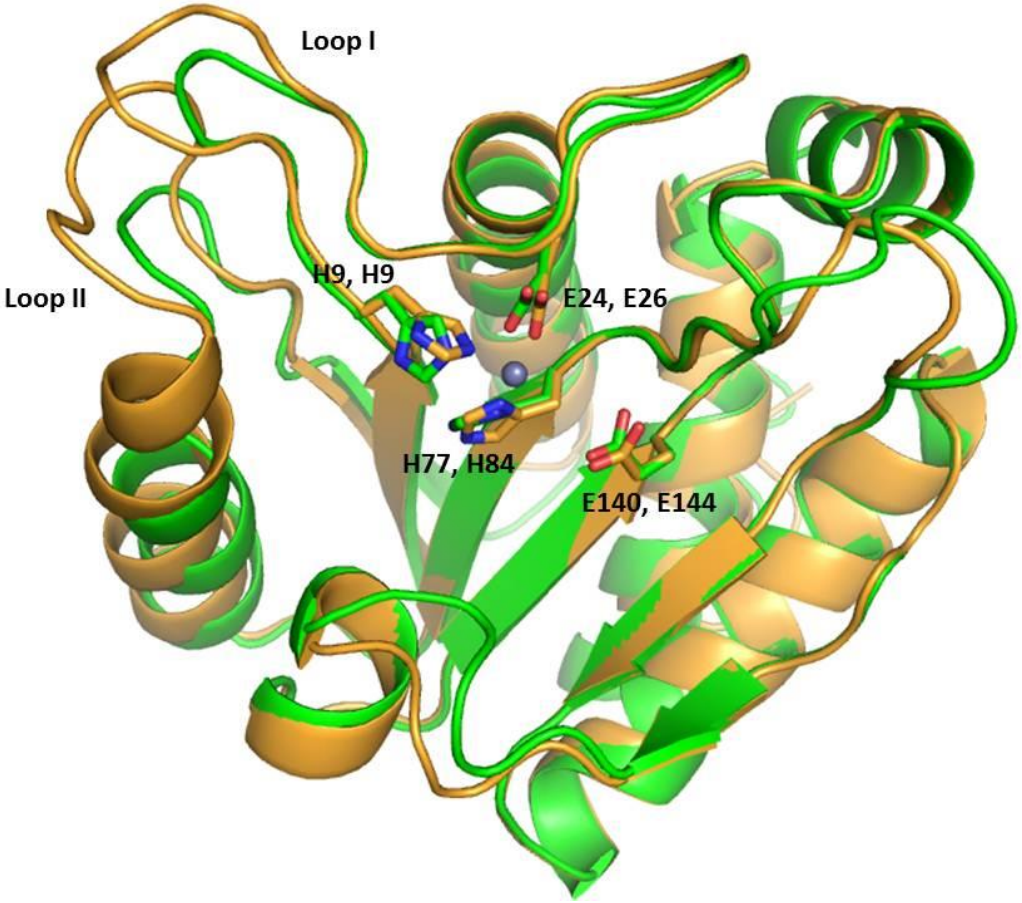
Mid-log phase *C. difficile* cells were incubated at 37ºC, under anaerobic conditions with crude protein extract from uninduced (control) and induced *E. coli* XL1-Blue (pQE-60*lyscd*) for 2h. Viable *C. difficile* were estimated by plate count on BHI agar.This experiment was performed in triplicate on two separate days.

Figure 5.3: SDS-PAGE of endolysin LysCD and LysCD His6x and corresponding zones of clearing in a zymogram gel containing heat killed *C. difficile* DPC 6219.



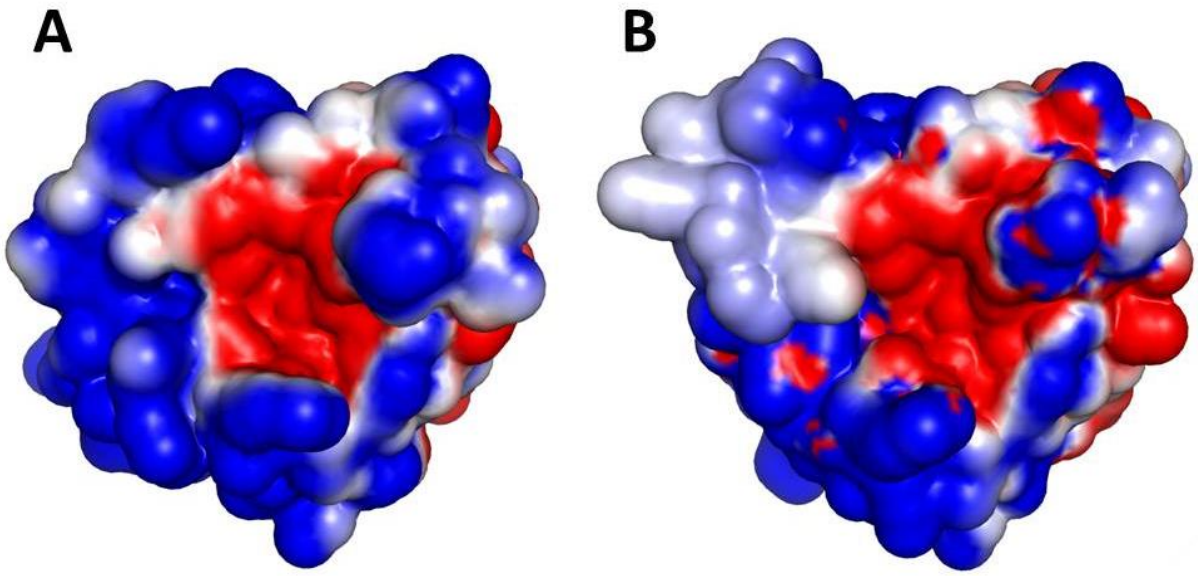
SDS-PAGE lanes from left to right: Lane 1, molecular weight marker; lane 2, uninduced *E. coli* protein extract of histidine tagged endolysin clone; lane 3, uninduced *E. coli* protein extract of untagged endolysin clone; lane 4, induced *E. coli* protein extract of histidine tagged endolysin clone; Lane 5, induced *E. coli* protein extract of untagged endolysin clone. Zymogram was run in parallel with SDS-PAGE and lanes correspond to their equivalent numers in the SDS-PAGE gel. Square boxes indicate the endolysin bands in both SDS-PAGE and zymogram gels.

Figure 5.4: Conservation of active site residues in the ϕCD6356 amidase domain.



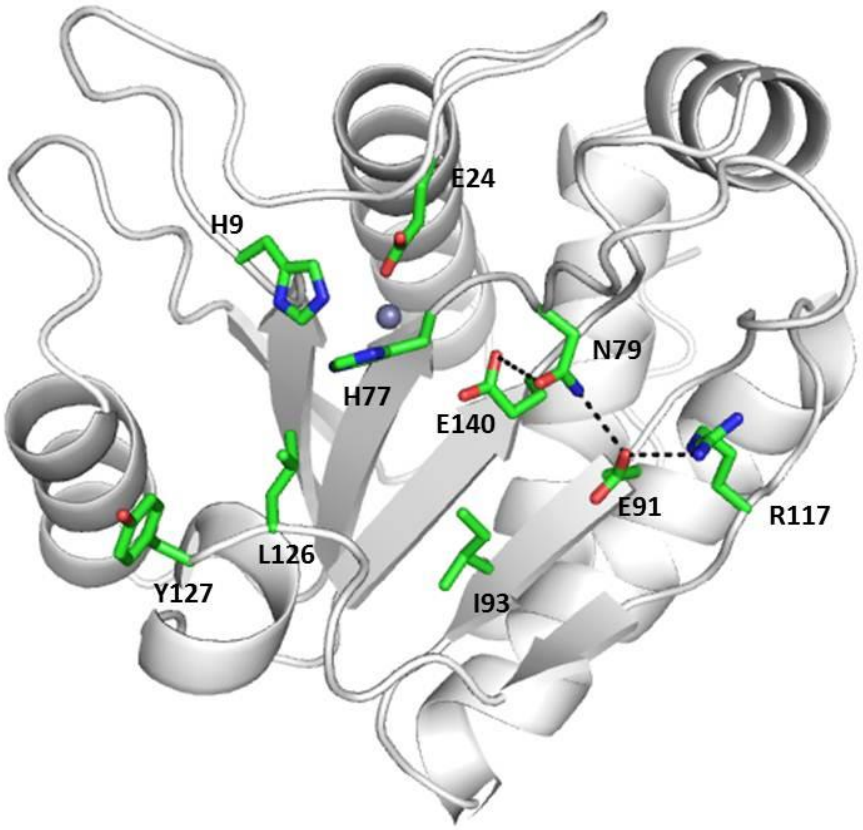
The model of the the ϕCD6356 amidase domain (green) is superimposed on the structure of the CD271 (3QAY) amidase domain (orange). Catalytic His and Glu residues are rendered in stick and coloured by atom. The catalytic Zn atom is rendered as a purple sphere. Loop structure in the ϕCD6356 amidase domain with sequence deletions when compared to CD271 are labelled Loop I and Loop II.

Figure 5.5: Electrostatic surfaces of the ϕCD6356 and CD271 amidase domain.



The electrostatic surface was calculated using the Adaptive Poisson-Boltzmann Solver (APBS) for the ϕCD6356 amidase domain (Panel A) and the CD271 amidase domain (Panel B). Electropositive surface is rendered in blue and electronegative surface is rendered in red.

Figure 5.6: The proton relay and substrate binding residues in the ϕCD6356 amidase model.



For clarity the fold of the ϕCD6356 amidase model is rendered in white cartoon. Catalytic His and Glu residues are rendered in green stick and coloured by atom. The catalytic Zn atom is rendered as a purple sphere. Residues Asn79, Glu91, and Arg117 are suggested to be part of a proton relay system delivery protons to Glu140 in the active site. The path of the transfer is indicated by a black dashed line. Residues Ile93, Leu126 and Tyr127 are part of the proposed substrate recognition interface.

Chapter 6

General discussion

This thesis concerned the study of the gut microbiota in patients with CF and how various antimicrobial treatments affected this bacterial community. Prior to these studies it was known that asymptomatic carriage of *Clostridium difficile* is common in patients with CF. Indeed, it has been reported to be as high as 46% (Yahav et al., 2006). Despite high carriage rates and detection of C. difficile toxin in stool, patients with CF rarely appear to develop CDI (Peach et al., 1986, Welkon et al., 1985, Wu et al., 1983, Yahav et al., 2006). Documented cases of CDI in patients with CF report atypical disease presentation which potentially leads to delayed diagnosis and increased risk of severe complications (Barker et al., 2008, Egressy et al., 2013, Yates et al., 2007).

In this thesis bacterial culturing revealed that 50% of patients with CF tested were colonised with *C. difficile* compared to just 2% of healthy volunteers. The majority of *C. difficile* strains cultured (19/30) were capable of producing both toxins A and B. Indeed, toxins A/B were detected in 32% of stool samples from patients with CF - all of which were confirmed to contain *C. difficile* strains capable of producing toxin by culturing. This is in direct contrast to a recent study carried out in Rotterdam (Bauer et al., 2014a). The authors reported a similar carriage rate (47%), however the majority of the *C. difficile* isolates (77%) were non-toxigenic (Bauer et al., 2014a). Carriage of non-toxigenic *C. difficile* has been proposed to protect the host from colonization with toxigenic strains (Wilson and Sheagren, 1983) and was proposed by the authors as an explanation for the low incidence of CDI in CF patients (Bauer et al., 2014a). Despite detection of toxin in the stool of our CF patients, none reported any gastrointestinal symptoms indicative of CDI. Peach et al 1986 reported similar findings, although they failed to detect toxin A (Peach et al., 1986). A commercial *C. difficile* toxin A/B kit was used in this study and therefore it is not possible to determine if one or both toxins were present. However, PCR analysis of *C. difficile* strains revealed the presence of both toxin genes in all strains cultured from toxin positive stool. Increased circulating antibody and memory B-cell responses to *C.*

difficile toxin A and B have been observed in patients with CF (Monaghan et al., 2013) which may explain the absence of CDI despite the presence of *C. difficile* toxins in the stool. The intestinal microbiota of patients with CF may also help protect against CDI. The stool of patients with CF in a previous study was found to contain several bacteria, including *Lactobacillu*s species with known inhibitory effects on *C. difficile* and was proposed to impart protection against CDI (Welkon et al., 1985). Interestingly, in this thesis, a significant increase (1.1%) in the family *Lactobacillaceae* was observed in CF patients relative to healthy controls in chapter 3. Misdiagnosis of mild cases of CDI due to CF patients displaying symptoms of constipation rather than diarrhoea may also contribute to the low incidence of CDI in CF patients (Binkovitz et al., 1999a).

In chapter 2 *C. difficile* was isolated from 30 patients, comprising 16 distinct ribotypes, while only two were detected in the control group; interestingly, neither of the ribotypes found in the control group were found detected in our CF patient group. Of the 16 ribotypes detected, the non-toxin producing ribotype RT140 was the most prevalent (5/30). In Chapter 4, RT140 was found to share a sequence type with the likewise non-toxin producing RT039 (ST26). All seven ST26 strains were isolated from CF patients comprising 6.3% of the total isolates tested. The most prevalent toxin producing ribotype in CF patients was RT046 (4/30), which was unique to this group as seen in chapter 4. This ribotype has previously been associated with out-breaks of CDI (Bauer et al., Pituch et al., 2006, Viscidi et al., 1981), however it is not among the most frequently reported ribotypes in Ireland (Executive, 2013). Surprisingly, only three of the sixteen ribotypes reported in chapter 2 (RT002, RT014 and RT078) have featured in the top five ribotypes reported in Ireland each year from 2010-2013 (Executive, 2013). As well as this, only the ribotypes RT078 and RT010 were found in the other patient groups included in chapter 4. Intriguingly, six *C. difficile* ribotypes (RT009, RT010, RT039 RT046, RT078, and RT140) were reported in both our CF patients and those in the study by Bauer et al, 2013 (Bauer et al., 2014b).

The emergence of a number of shared ribotypes between geographically distinct CF patients groups (this study and that of Bauer et al, 2013) coupled with the infrequency with which some of these strains are reported could indicate a core of strains associated with CF patients. Further research into *C. difficile* carriage using a longitudinal study from a young age to monitor acquisition and maintenance of *C. difficile* strains in CF patients may reveal a core of strains associated with CF patients or this group's potential role in the spread of *C. difficile.*

Reassuringly, all *C. difficile* strains isolated from CF patients were susceptible to vancomycin and metronidazole. Fusidic acid, meropenem, linezolid and rifampicin were also effective against these strains. Not surprisingly, these strains were highly resistant to antibiotics typically prescribed to treat pulmonary infections including, ciprofloxacin, ceftazadime, tobramycin, aztreonam, and azithromycin. Further research into *C. difficile* carriage using a longitudinal study from a young age to monitor acquisition and maintenance of *C. difficile* strains in CF patients may reveal a core of strains associated with CF patients or their potential role in the spread of *C. difficile.*

Asymptomatic carriers of *C. difficile* have higher percentage of skin and environmental contamination with *C. difficile* and have been proposed as a significant contributor to the spread of *C. difficile* (Riggs et al., 2007). The high asymptomatic carriage of *C. difficile* among CF patients reported in this thesis is worrying as these patients may act as a reservoir for the spread of *C. difficile.* Therefore monitoring of *C. difficile* in this group is vitally important for controlling *C. difficile* spread in the hospital environment. In chapter 4, MLST was found to be comparable to PCR-ribotyping for typing a collection of 112 *C. difficile* strains isolated from high risk patients (Index of Discrimination [ID] of 0.93 and 0.95 respectively). In total, 28 sequence types and 33 PCR-ribotypes were reported in the *C. difficile* collection examined. While PCR-ribotyping is the most frequently used typing scheme in the United Kingdom and Europe, this method relies on comparison of data to a ribotyping reference library, such as the

one housed at the Reference Laboratory, Leeds General Infirmary, UK by the *Clostridium difficile* Ribotyping Network for England (England, 2014). Currently there is no dedicated *C. difficile* ribotyping reference laboratory in Ireland. Multilocus sequence typing has been shown to be an effective typing scheme generating easily accessible and interpretable results with an ever expanding freely accessible online database (http://pubmlst.org/cdifficile/). Therefore MLST could be employed to monitor *C. difficile* acquisition and carriage in CF patients without the need to send strains to England for typing.

The risk of acquiring *C. difficile* is higher for patients with CF due to frequent hospital admission and proton pump inhibiter therapy (Bignardi, 1998, Clabots et al., 1992, Dial et al., 2004, Pépin et al., 2005, Safdar and Maki, 2002). Patients with CF are also frequently exposed to broad spectrum antibiotics to treat pulmonary infection (Ramsey, 1996), and this likely contributes to the elevated carriage rates observed in this group (Yahav et al., 2006). In fact, ciprofloxacin and azithromycin have both been proposed to increase the risk of CDI (McCusker et al., 2003, Gorenek et al., 1999). However, no correlation could be made between any recorded patient clinical parameters and the carriage of *C. difficile* in chapter 2. Although, these factors combined may contribute to the high carriage rate (50%) observed. Lower microbial diversity has been reported in non-CF individuals suffering *C. difficile* associated disease (Rea et al., 2012, Chang et al., 2008). High throughput compositional pyrosequencing of the faecal microbiota revealed lower species richness and diversity in CF patients relative to healthy controls. This is contrary to a report by Duytschaever et al, 2011 (Duytschaever et al., 2011) who found species richness to be comparable between patients with CF and healthy siblings; However, lower temporal stability was reported (Duytschaever et al., 2011). Further investigation revealed that duration of intravenous antibiotic treatment, length of hospital stay and decreased lung function were all associated with lower species richness and microbial diversity in CF patients. Lower diversity observed in CF patients with decreased lung function is

likely due chronic pulmonary infection requiring more frequent antibiotic therapy. Most interesting was the observation that patients carrying *C. difficile* had decreased microbial diversity compared to *C. difficile* free CF patients. This suggests that the acquisition of *C. difficile* in patients with CF is likely due to alteration of the intestinal microbiota by a combination of the disease and its treatment. Minimising exposure to antibiotics by increasing the use of inhaled antibiotics (Geller et al., 2002) and probiotic supplementation to reduce exacerbation frequency (Bruzzese et al., 2007, Weiss et al., 2010) may allow the intestinal microbiota to recover and reduce the rate of *C. difficile* carriage.

While CDI is rare among patients with CF, documented cases can be difficult to diagnose due to atypical presentation and can be quite severe (Barker et al., 2008, Binkovitz et al., 1999b, Egressy et al., 2013, Yates et al., 2007). As well as this patients with CF may also act as a potential reservoir for *C. difficile* and therefore there exists a need for rapidly acting narrow spectrum antimicrobials to treat CDI in high risk patient groups such as patients with CF or to eradicate it from asymptomatic carriers. In chapter 5 of this thesis, the *C. difficile* bacteriophage ΦCD6356 and its associated endolysin were investigated for their effectiveness at killing *C. difficile*. Bacteriophage have previously been shown to be effective at treating CDI. Ramesh and colleagues (Ramesh et al., 1999) found a single dose of bacteriophage to be sufficient to treat hamsters with *C. difficile*-induced ileocecitis. Following 96h, the majority of bacteriophage-treated hamsters had survived, while all the control hamsters had died (Ramesh et al., 1999). In this thesis, we report that a single dose of the bacteriophage ΦCD6356 was sufficient to reduce viable *C. difficile* by 1.75 logs over 24h in an ex vivo model of the human distal colon. However, PCR analysis revealed 100% of surviving *C. difficile* carried the ΦCD6356 prophage by detection of bacteriophage structural genes in their genome. These structural genes were not detected in *C. difficile* recovered from the untreated control vessels. A similar observation was made by Meader et al, 2008, who induced prophage from surviving *C. difficile* using mitomycin

C, following treatment with the *C. difficile* bacteriophage ΦCD27 (Meader et al., 2010). To date, no lytic C. difficile bacteriophage have been reported (Hargreaves and Clokie, 2014). The lysogenic nature of discovered *C. difficile* bacteriophage, including ΦCD6356 renders them unsuitable for bacteriophage therapy due to the immunity acquired via lysogeny (called "superinfection immunity"). As well as conferring immunity on the host, prophage have also been associated with horizontal gene transfer between bacteria (Casjens, 2003). Prophage have also been linked to increased toxin production in hyper-virulent *C. difficile* ribotype 027 strains (Sekulovic et al., 2011). Therefore *C. difficile* bacteriophage are not a viable option for the eradication of *C. difficile* from asymptomatic CF carriers.

Bacteriophage endolysins have promise as potential alternatives to antibiotics for the treatment of a number of infections (Schmelcher et al., 2012). The endolysin gene encoded by ΦCD6356 was previously identified as a probable N-acetylmuramoyl-l-alanine amidase, located at ORF 28 (Horgan et al., 2010). This gene, lysCD, was cloned into *E. coli* and expressed under IPTG induction. A model for ΦCD6356 lysin, LysCD, was built using MODELLER (Sali and Blundell, 1993) with the amidase domain of the endolysin CD27l (3QAY) as the template structure (Mayer et al., 2011). This revealed the presence of zinc coordination at the enzymes catalytic domain. Removal of zinc by treatment with EDTA eliminated the lytic activity of the endolysin. Reintroduction of zinc as well as other divalent metal ions (Mn2+, Mg2+and Ca2+) to EDTA treated endolysin restored lytic activity. This flexibility in divalent ions has been reported previously in other bacteriophage endolysins (Son et al., 2012, Schmelcher et al., 2012). In fact Crude protein extract containing the endolysin was active against live *C. difficile* in vivo resulting in a nearly 3 log reduction in viable *C. difficile* after 2h. However, enzymatic activity was unstable after partial purification. This work proves promising for the development of LysCD as a novel *C. difficile* antimicrobial with narrow spectrum of activity. However, for this

to be realised further work on the purification and characterising of the endolysin would need optimization and scaling-up.

In conclusion, the data presented in this thesis reveals an alarmingly high burden of *C. difficile* carriage in patients with CF. Among the strains recorded were a number of virulent strains typically associated with CDI outbreaks. This highlights that good tracking of *C. difficile* and recording of epidemiological data using techniques such as MLST are required to minimise the spread of *C. difficile* in this group and to the community as a whole. The composition of the CF intestinal microbiota is significantly altered from that of healthy individuals through a combination of the disease and its treatment, potentially creating a niche for colonization by *C. difficile*. *C. difficile infection* in CF patients, while rare can be potentially life threatening, especially in post lung transplant patients (Egressy et al., 2013). Cystic fibrosis patients may also act as potential reservoirs for dissemination of a great variety of unrelated *C. difficile* strains. Therefore antimicrobials with minimal effect on the intestinal microbiota are required for the treatment and eradication of *C. difficile* in people with CF to prevent reinfection and spreading of *C. difficile*. Narrow spectrum antimicrobials such as bacteriophage endolysins could therefore be of great benefit in patients with CF and thus warrant greater investigation. Further studies into longitudinal development of the CF intestinal microbiota in paediatric patients and *C. difficile* carriage in post-lung transplant patients can further inform the treatment and gastrointestinal health of patients with CF.

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Appendix

Microbial production of bacteriocins for use in foods

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Abstract:

Bacteriocins are ribosomally synthesised, antimicrobial peptides produced by bacteria. Many bacteriocins produced by food-grade lactic acid bacteria inhibit food-borne spoilage and pathogenic bacteria in food. Here we reviews the means via which these bacteriocins can/are employed by the food industry i.e. through the production of bacteriocins by bacteriocinogenic bacteria from within the food or through the addition of bacteriocin to the food in the form of an ingredient or preservative.

Keywords: Bacteriocin, Lactic Acid Bacteria, bacterial fermentation, biopreservatives, bacteriocin purification

1. Introduction

Bacteriocins are ribosomally synthesised, small heat stable, antimicrobial peptides produced by bacteria. They are typically active against closely related species but can also have a broad spectrum of activity across genera. Bacteriocinogenic bacteria are protected from the bacteriocins which they produce as a consequence of the production of dedicated immunity (self-protective) proteins (Cotter *et al.*, 2005, Rea *et al.*, 2011). Gram positive bacteriocins can be divided into two classes i.e. Class I, the post-translationally modified bacteriocins and Class II, the unmodified bacteriocins (Cotter et al., 2005, Rea et al., 2011). It is estimated that between 30-99% of bacteria produce at least one bacteriocin (Klaenhammer, 1988, Riley, 1998). Indeed, the frequency with which bacteriocin encoding gene clusters occur has been borne out by genome sequencing studies (Begley *et al.*, 2009, Marsh *et al.*, 2010). This fact indicates that there continues to be great opportunities for the discovery and development of new bacteriocins for commercial applications. To date such commercial applications have most frequently involved the use of bacteriocins to control spoilage or pathogenic bacteria in food. The lactic

acid bacteria (LAB) are the most important bacteriocin producers with regard to such applications. LAB have been used for millennia for the preservation and microbial safety of fermented foods by inhibiting the growth of pathogenic and spoilage bacteria (Caplice & Fitzgerald, 1999). Food preservation is mediated by the production of a number of end products of LAB fermentation such as organic acids, ethanol, hydrogen peroxide, and, of course, bacteriocins (Jack *et al.*, 1995).

Many bacteria isolated from food fermentations have been found to produce bacteriocins. Examples include nisin and lacticin 3147, which are Class I bacteriocins (or lantibiotics). These bacteriocins are produced by members of the genus *Lactococcus* and the benefits of using producers of such bacteriocins in cheese manufacture have been highlighted (Roberts *et al.*, 1992, Ryan *et al.*, 1996). Similarly the Class II pediocin PA-1 producing *Pediococcus acidilactici* have been used in the fermentation of dry fermented sausage (Foegeding *et al.*, 1992) and several bacteriocin producing *lactobacilli* have been employed in the fermentation of sausages and olives (Dicks *et al.*, 2004, Messens *et al.*, 2003, Ruiz-Barba *et al.*, 1994). As a consequence of their long history of safe use, LAB have attained a generally regarded as safe (GRAS) status. Despite this, nisin remains the only bacteriocin approved for use as a preservative in foods. There are however, products such as the pediocin PA-1-containing Alta 2341® (Kerry Bioscience, Carrigaline, Co. Cork, Ireland) and other fermentates which are employed by the food industry. As a consequence of its approved status, nisin has been the focus of much attention. It was first discovered in 1928 (Rogers & Whittier, 1928) and was first marketed in England in 1953. In 1969, nisin was assessed as safe for use in food by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) and was later added to the European food additive list and given the number E234 by the EEC (EEC, 1983). It has also been approved for use in food by the U.S. Food and Drug Administration. While nisin is

widely available as Nisaplin™ (Danisco, Copenhagen, Denmark), other commercial preparations are also available.

There are numerous benefits to the use of bacteriocins to preserve and increase the microbial safety of food. Firstly, as bacteriocins are metabolites of bacteria they are seen as "natural" products that can be used in place of chemical preservatives in foods, therefore gaining wider acceptance by consumers. Bacteriocins can also be applied in combination with other treatments such as high pressure or temperature to improve food preservation. This is exemplified by lacticin 3147 which, when combined with hydrostatic pressure, has an increased killing effect on *Staphylococcus aureus* and *Listeria innocua* than was observed with either treatment alone (Morgan *et al.*, 1999). Similarly, a combination of the *Enterococcus*-produced enterocin AS-48 with a mild heat treatment (80-95˚C for 5 mins) caused a considerable reduction in the viability of *Bacillus coagulans* CECT 12 endospores when compared to the heat treatment alone, which did not impact significantly on the endospores (Lucas *et al.*, 2006). Thus the effective use of bacteriocins in hurdle technology can result in increased food safety and nutritional quality through the improved killing of contaminating bacteria and reducing the need for harsh processing procedures, respectively.

This chapter will focus on the use of bacteriocins for the preservation of food through their production by microbes *in-situ* in foods and their incorporation as preservatives/fermentates, and will review the different approaches which have been taken to optimise their yield.

2. *In-situ* production of bacteriocins in food

As previously stated, LAB have been used for millennia in food fermentations. However, defined starter cultures have replaced traditional undefined mixed culture starters in modern commercial fermentations. Use of defined starter culture systems allows for improved control

over fermentations as well as the selection of strains that possess specific beneficial traits, such as bacteriocin production. In the last few decades bacteriocinogenic strains have been studied extensively with regard to their potential as starter cultures, starter adjuncts and protective cultures to control the growth of pathogenic and food spoilage bacteria. This section shall explore the topic of the *in-situ* bacteriocin production for food preservation.

2.1 Bacteriocinogenic starter cultures.

Unbeknownst to us, mankind may have been reaping the benefits of in-*situ* bacteriocin production in fermented foods since the practice of fermentation first began. Ancient fermentations were the result of the outgrowth of microflora naturally present on the raw material from which the fermented product was made. Backslopping i.e. the practice of inoculating a new fermentation with a small quantity of fermented product from a successful fermentation was later used as a means of replicating successful fermentations. In effect this process lead to the selection of the best starters, although the underlying science was not understood at the time (Leroy & De Vuyst, 2004). Modern industrial fermentations utilise well characterized defined starter systems with properties which are beneficial from the perspective of the end product. Such properties include rapid acid production, phage resistance, and the formation of aromatic compounds and, of course, the production of bacteriocins. Bacteriocin production by starter cultures can contribute to food safety and preservation thereby limiting the need for chemicals preservatives.

Lactococci are commonly used as starter cultures in the dairy industry, most notably as starters in the manufacture of cheese. As previously stated, nisin is produced by a number of *L. lactis* strains, some of which have been investigated with a view to their use as starter cultures for the

control of pathogenic and spoilage bacteria in various food systems. Rodríguez *et al* (1998) used a nisin producing *L. lactis* strain, ESI 515, in the production of Manchego style cheese made from raw ewe's milk. The strain displayed desirable properties in terms of making this cheese but, importantly, also produced sufficient nisin to reduce counts of *Listeria innocua* by 4.08 log units relative to a control cheese produced with a non-bacteriocinogenic starter culture. *L. lactis* DPC 3147 and a transconjugant, *L. lactis* DPC 4275, both producing the broad spectrum two-component bacteriocin lacticin 3147 have also been successfully used to manufacture Cheddar cheese. Levels of the bacteriocin remained constant over a six month ripening period (see fig. 1) and were sufficient to control non-starter LAB (Ryan et al., 1996), which can lead to inconsistencies and off-flavours. This strain was also used as a starter for cottage cheese manufacture, where it produced 2560 activity units (AU) ml^{-1} of lacticin 3147. There was a 99.9% reduction in *L. monocytogenes* Scott A numbers in cheese made with the lacticin 3147 producing starter after five days whereas no change in pathogen levels was seen in the control cheese (McAuliffe *et al.*, 1999).

In addition to inhibiting pathogens, bacteriocins can be of great use with respect to controlling spoilage bacteria. Bacterial spores surviving milk pasteurisation is a significant problem as they can contaminate cheese and sporulate during ripening causing the formation of off odours and a late blowing defect due to butyric acid formation. The latter phenomenon is caused by the out-growth of clostridial spores and is a major cause of spoilage in hard and semi-hard cheeses (McSweeney *et al.*, 2004). Garde *et al* (2011) successfully prevented the out-growth of *Clostridium beijerinckii* spores in ovine milk cheese by using a bacteriocinogenic *L. lactis* starter culture. Production of nisin and another Class I bacteriocin, lacticin 481, by the starter culture during fermentation prevented outgrowth of the spores and hence late blowing. After 120 days ripening this defect occurred in the control cheese but not in cheese made with the

bacteriocinogenic starter. As well as preventing spoilage, the starter also generated the desired sensory characteristics for this type of cheese and thus was deemed a suitable replacement starter. The use of bacteriocinogenic cultures in the production of cheese to prevent late blowing offers a natural alternative to lysozyme, which is commonly added to cheese to prevent this defect (Crawford, 1987). This method of prevention is not only more cost effective, but also lysozyme has become an increasingly less attractive preservative in recent years as a consequence of the fact that it is purified from eggs and thus there are fears associated with its potential allergenicity (Carmen Martínez-Cuesta et al., 2010).

Moving away from the topic of dairy products temporarily, the production of dry fermented sausages is also worthy of discussion. This practice traditionally involves fermentation followed by drying without a heat treatment. This minimal processing can potentially lead to contamination and the proliferation of spoilage and pathogenic bacteria, such as *L. monocytogenes*. Pediococci are commonly used as starters in the fermentation of sausages, and many *Pediococcus*-produced bacteriocins (also known as pediocins), are active against important pathogenic bacteria, such as those of *Listeria* and *Clostridium* sp. (Christensen & Hutkins, 1992, Luchansky *et al.*, 1992, Nieto-Lozano *et al.*, 2010). In one such study a pediocin PA-1 producer, *Pediococcus pentosaceus* BCC 3772 was selected as a consequence of its anti-listerial activity and was evaluated as a starter culture for the fermentation of Nham (Thai traditional pork sausage). The strain performed agreeably when used as a starter culture in the fermentation of Nham in that it did not significantly alter the sensory characteristics of the sausage. *In-situ* production of PA-1 was sufficient to reduce spiked *L. monocytogenes* numbers by 3.2 logs compared to initial counts, within 18-24 h (Kingcha *et al.*, 2011). *Pediococcus acidilactici* MCH14, a starter culture commonly used in the Spanish meat industry also produces pediocin PA-1 and was tested to assess its ability to control the growth of *L. monocytogenes* in

Spanish dry-fermented sausage. Sausages were made using either the bacteriocinogenic starter, or a non-bacteriocin producing *P. acidilactici* as a control, and were spiked with *L. monocytogenes* (10^5 CFU g^{-1}) before being vacuum stuffed and stored for four weeks. After ripening, the numbers of *L. monocytogenes* were reduced by 2 logs (2×10^1 CFU g^{-1}) in the MCH14-containing sausage compared to the control (7×10^3 CFU g^{-1}) but, importantly, no significant difference was observed between the two starters with regard to A_w and lactic acid production (Nieto-Lozano et al., 2010). Members of the genus *Lactobacillus* are also commonly used as starter cultures for the fermentation of meat (Hammes *et al.*, 1990). Bacteriocin production appears to provide such *lactobacilli* with a competitive advantage in such environments, as a number of bacteriocinogenic *lactobacilli* have been isolated from fermented sausages (Aymerich *et al.*, 2000, Schillinger & Lucke, 1989, Vignolo *et al.*, 1993, Vogel *et al.*, 1993). For example, *Lactobacillus sake* CTC 494, which was isolated from dry fermented sausage, has been found to have excellent starter capabilities, as well as the ability to produce the *Listeria*-active bacteriocin sakacin K (Hugas *et al.*, 1996), under conditions (pH and temperature) similar to that found during the fermentation of dry fermented sausage (pH 5.0 - 5.5, 20 - 25˚C) (Leroy & De Vuyst, 1999b). This strain was tested for its ability to control the growth of *L. monocytogenes* in three types of fermented sausage; Belgin-type, Cacciatore-type and Italian salami. Notably, *L. monocytogenes* numbers were reduced by between 0.6 and 1.0 log CFU g^{-1} when compared to bacteriocinogenic starter free controls after the completion of sausage production (Ravyts *et al.*, 2008). Another example relates to *Lactobacillus pentosus* 31-1. This strain was isolated from a Chinese meat product (Xuanwei ham) and produces the bacteriocin pentocin 31-1. This strain was evaluated as a starter culture for the production of fermented sausage. The strain performed very well, producing a product with desirable organoleptic properties. During challenge tests with *L. innocua* and *Staphylococcus aureus*, pathogen cell numbers in the *Lb. pentosus* 31-1 sausage were reduced by between 4.4 and 5.1

log units compared to the control. Antimicrobial activity was detected up to 7 days post production in homogenized sausages whereas, as expected, no activity was observed in the controls. Activity was lost during the ripening period suggesting that the bacteriocin was inactivated over time (Liu *et al.*, 2008a).

2.2 Bacteriocinogenic starter culture adjuncts.

Bacteriocinogenic strains are not always suitable as starter cultures as they may not be capable of the requisite acidification rates or lack the proteolytic activity required. Such problems have previously been associated with some nisin producing strains (Lipinska, 1973, Lipinska, 1977). This problem can be solved by introducing the bacteriocin encoding genes into a suitable starter by conjugation or genetic manipulation or, more simply, by combining the bacteriocinogenic culture with a suitable starter culture that is resistant to the bacteriocin being produced.

A number of authors have described the use of bacteriocinogenic enterococci as starter-culture adjuncts (Arantxa *et al.*, 2004, Giraffa *et al.*, 1995, Nascimento *et al.*, 2008, Oumer *et al.*, 2001). However due to the ongoing debate with respect to the safety of enterococci in food, these shall not be discussed in this section.

The combination of bacteriocinogenic adjunct cultures with resistant/insensitive commercial starter cultures is an economical alternative to the application of either chemical preservatives or purified bacteriocin preparations for controlling spoilage and pathogenic bacteria. Rilla *et al* (2003) successfully controlled the growth of *Clostridium tyrobutyricum* CECT4011, which has been associated with the previously described butyric acid formation/late blowing defect in cheese, through the use of a mesophilic mixed starter, IPLA-001, in combination with the nisin Z producing strain, *L. lactis* subsp. lactis IPLA 729. Nisin Z is a variant of nisin (nisin A) which differs by one amino acid. Nisin Z levels reached 1600 AU/ml in this cheese after day 1, this level of activity was retained for 15 days. The experimental cheese, along with a control and a

commercial cheese made with the anti-blowing agent potassium nitrate, were spiked with the CECT4011 strain. During ripening, *Cl. tyrobutyricum* numbers were reduced from 1.2 x 10^5 CFU g^{-1} to 1.3x 10^3 CFU g^{-1} in the nisin Z cheese, but increased to 1.99 x 10^9 CFU g^{-1} and 3.5 x 10^7 CFU g^{-1} in the control and commercial cheeses, respectively. The nisin Z producing starter *L. lactis* subsp. lactis biovar diacetylactis UL719 was also successfully used as a starter adjunct by Bouksaim *et al* (2000) in the production of Gouda cheese. Nisin Z levels reached a maximum of 512 AU g^{-1} after 6 weeks ripening but decreased to 128 and 32 AU g^{-1} after 27 and 45 weeks, respectively. The level of nisin present is significant as it has been shown that 40 Au g^{-1} is sufficient to prevent butyric acid formation (Hugenholtz & De Veer, 1991).

As previously stated, bacteriocins can potentially be used in combination with other treatments (or hurdles) to maximise food quality and safety. By utilising two bacteriocinogenic cultures producing different bacteriocins, with differing mechanisms of action but with coinciding spectrums of activity, it is possible to affect a synergistic killing of a target bacterium. This approach can be particularly useful in preventing the emergence of resistance (Vignolo *et al.*, 2000). *Lb. plantarum* LMG P-26358, produces an anti-listerial class II bacteriocin with 100% homology to plantaricin 423 was used as a starter adjunct in combination with a nisin-producer, *L. lactis* CSK65 in laboratory scale cheese production. In challenge trials, cheese produced with the *Lb. plantarum* LMG P-26358 starter adjunct had a greater killing effect against *L. innocua* than observed in cheese produced with the nisin producer alone. It was also noted that when used in combination, no viable *L. innocua* were recovered after 28 days ripening. Mass spectrometry also revealed that both bacteriocins were present after 18 weeks ripening (Mills *et al.*, 2011). Despite the success of this approach, it is important to ensure the bacteriocins produced are not overly antagonistic against the other bacteriocinogenic culture(s) present. Mills *et al* (2011) did observe the inhibition of *Lb. plantarum* LMG P-26358 by nisin, which slowed

its growth during the first eight hours of fermentation. However, despite this, the *Lb. plantarum* strain did reach the optimal cell density required for bacteriocin production (i.e.10^8 CFU ml^{-1}) on day one of cheese production in this case.

In addition to inhibiting spoilage and pathogenic microbes, bacteriocins can also be exploited to partially lyse starter and/or adjunct cultures during cheese production which is enhances cheese maturation. This is due to the release of intracellular enzymes, such as proteinases, peptidases, amino acid catabolic enzymes and esterases, all of which impact upon flavour formation (Lortal & Chapot-Chartier, 2005).A number of authors have described the use of bacteriocinogenic adjunct starter cultures to increase starter cell lysis (Ávila *et al.*, 2005, Garde *et al.*, 2006, Garde *et al.*, 2005, Garde *et al.*, 2002, Lortal & Chapot-Chartier, 2005, Morgan *et al.*, 1997, O'Sullivan *et al.*, 2002, O'Sullivan *et al.*, 2003).

2.3 Bacteriocinogenic protective cultures.

In situ bacteriocin production for the protection of foods from pathogenic and spoilage bacteria is not an option which is exclusively reserved for fermented foods. Bacteriocinogenic bacteria can also be applied to the surface of non-fermented foods, allowing for in *situ* bacteriocin production. A suitable bacteriocinogenic protective culture must not be capable of causing spoilage, must grow at the intended storage temperature (usually refrigeration temperatures), must not affect the organoleptic properties of the food during storage and must produce sufficient bacteriocin to have a protective role under these conditions.

Two Lactobacilli producing bacteriocin-like inhibitory substances were assayed for their ability to enhance the preservation of vacuum-packed sliced beef meat stored at 4˚C over 28 days by Katikou, *et al* (2005). The two lactobacilli, *Lb. sakei* CECT 4808 and *Lb. curvatus* CECT 904^T,

were applied to the sliced beef meat either individually and in-combination. Counts of Enterobacteriaceae, *Pseudomonas* spp., LAB, *Brochothrix thermosphacta*, yeasts and moulds as well as the organoleptic properties of the beef were assayed over the 28 days. Beef inoculated with either the *Lb. sakei* or the *Lb. sakei* in combination with *Lb. curvatus* had significantly ($P < 0.05$) lower counts of spoilage microbes than beef inoculated with *Lb. curvatus* alone or controls. The use of *Lb. sakei* in isolation was more effective in controlling spoilage microbes and the associated product attained greater organoleptic scores than was achieved by the other treatments. The sakacin P producer *Lb. curvatus* CWBI-B28 has also been shown to be effective as a protective culture, in this instance when employed on the surface of cold-smoked salmon under vacuum-packed and refrigerated conditions. Counts of *L. monocytogenes* on this product were reduced to below the detectable limit (0.7 CFU/cm^2) within the first week of storage after the application of *Lb. curvatus* as a protective culture. However a 1.3-log increase in colony numbers was observed after 14 days (Ghalfi *et al.*, 2006). *Lb. curvatus* CWBI-B28 has also been shown to be effective with respect to controlling *L. monocytogenes* in raw beef and in raw chicken meat when combined with the sakacin G producing *Lb. sakei* CWBI-B1365 (Dortu *et al.*, 2008).

When considering commercial applications for bacteriocinogenic protective cultures storage, the retention of antimicrobial activity may become an issue. Spray-drying offers an inexpensive method for industrial-scale production of cultures containing high levels of viable bacteriocinogenic bacteria suitable for storage and transport. Silva *et al* (2002) assessed the antimicrobial activity of three bacteriocinogenic protective-cultures for their antimicrobial activity before and after spray-drying. All three strains, *Lb. sakei*, *Lb. salivarius* and *Carnobacterium divergens*, exhibited antimicrobial activity against the target strains, i.e. *Staph. aureus*, *L. innocua* and *L. monocytogenes*. After spray-drying, the cultures were stored at either

4˚C or 18˚C and at a relative humidity of 0.3%. Although *Carn. divergens* lost its antimicrobial activity against *Staph. aureus* immediately after spray drying, it retained activity against *L. innocua* and *L. monocytogenes*. The two lactobacilli retained antimicrobial activity against all target bacteria after spray-drying. Storage temperature had a significant impact on survival of the bacteriocinogenic spray-dried cultures. Numbers of *Carn. divergens* and *Lb. sakei* decreased during storage at 4˚C and18˚C, with a greater decrease being seen at 18˚C. Numbers of *Lb. salivarius* didn't significantly decrease until the 3rd month of storage. Rodgers *et al* (2002) also demonstrated the retention of nisin and pediocin A production by the protective cultures, *L. lactis* CSCC 146 and *P. pentosaceus* ATCC 43200 respectively, after freeze-drying. On the basis of these results spray- and freeze-drying could become an important process in the production of bacteriocinogenic cultures for use in food production.

2.4 *In-situ* bacteriocin production – Conclusion

The use of physical processes (heating, hydrostatic-pressure, gamma radiation etc.) to kill undesirable microbes in foods can be expensive and can negatively impact on the sensory characteristics of certain foods and therefore are not always suitable. The addition of chemical preservatives is highly regulated and is negatively perceived by consumers. For these reasons bacteriocin production *in-situ* is a very attractive alternative for maintaining food safety and quality. Also, because the bacteriocin is produced *in-situ* by GRAS cultures this provides an alternative to the use of concentrated bacteriocin preparation as food preservatives and associated regulatory restrictions. It is important to note, however, that the bacterial strains to be used as starter, adjunct or protective cultures for in-*situ* bacteriocin production need to be extensively tested within the food environment as both their growth rate and levels of bacteriocin

production can be affected by environmental factors that prevail within the food environment (Leroy & de Vuyst, 1999a, Neysens *et al.*, 2003, Sarantinopoulos *et al.*, 2002).

3. Ex-*situ* production of bacteriocins:

Bacteriocins for use as preservatives and food additives can be produced as either purified preparations or as fermentates (see Table1). Like many industrial fermentations, maximal product formation is only achieved through tight control of fermentation conditions. For this reason there have been many studies into the effects of medium composition, temperature, pH, and fermentation design on bacteriocin production.

3.1 Media Composition.

Production of bacteriocins on an industrial scale is an expensive process. This is due in part to the high cost of commercial media required for the cultivation of the bacteriocin producing LAB, examples include; deMan, Rogosa, Sharpe media (MRS), Tryptone, Glucose, Yeast extract media(TGY) and All Purpose Tween media(APT) (Daba *et al.*, 1993, De Vuyst, 1995, Jensen & Hammer, 1993). While these general media may be ideal for the growth of LAB in the laboratory, they may not be optimal for bacteriocin production.

MRS is commonly used for the production of sakacin A (180 $AUml^{-1}$) from *Lb. sakei*. Trinetta *et al* (2008) used a 'one variable at a time' approach to develop an alternative culture medium for the production of sakacin A. Using this approach a medium, optimal for sakacin A production and was developed which cost 50% less. In this alternative medium consisting of, bactopeptone, meat peptone, milk whey, yeast autolysate, glucose and calcium carbonate, sakacin A production was increased to 480 AU ml^{-1}. Juárez Tomás *et al* (2010) designed a medium optimal for growth and production of the Class II bacteriocin, salivaricin CRL 1328 by *Lb. salivarius* CRL 1328.

Growth and bacteriocin production were assessed under a range of culture conditions. Using a desirability function, an optimal medium composition was predicted which cost between 25-40% less than commercial alternatives i.e. MRS.

Cultivation of bacteriocinogenic bacteria in medium based on industrial waste products has also been assessed in a bid to reduce the cost of bacteriocin production. LAB require diverse peptic sources due to their fastidious nature. Peptones from fish, while uncommon, are a quite good alternative to peptone sources commonly used such as bactopeptone, tryptone, yeast extract and meat extract (Vázquez *et al.*, 2004). Octopus peptone (OP) media made from octopus tissue (as peptone source) was compared to MRS and a medium (medium B) made with commercial peptones (bactopeptone), for its ability to support growth and the production of nisin and pediocin by strains of *L. lactis* and *P. acidilactici*, respectively. Increased nisin and biomass production was observed in OP relative to that achieved in either MRS or medium B. OP outperformed medium B for biomass and pediocin production but not MRS (Vázquez et al., 2004). A similar study was performed using peptones derived from fish visceral and muscle residues. Nisin and pediocin PA-1 production was assessed in medium prepared with fish peptones (FP) or bactopeptone (D medium) as well as in MRS. FP compared favourably to D medium and MRS for biomass and nisin production by *L. lactis.* Pediocin and biomass production in *P. acidilactici* improved 500% with FP compared to medium D or MRS (Vázquez *et al.*, 2006). Snow crab hepatopancreas (a by-product of crustacean processing) medium has also been shown to be an effective substrate for the production of bacteriocins. A maximum activity of 3.7×10^4 AU ml^{-1} of divergicin M35 was produced by *Carnobacterium divergens* M35 in snow crab hepatopancreas medium after 10h (Tahiri *et al.*, 2009).

Cereal based by-products from various industrial processes have also been investigated for their application as low-cost medium for large scale bacteriocin production. Condensed corn soluble (CSS) is a by-product of fuel ethanol production. CSS was found to be considerably cheaper

than the commercial media Laurel-Tryptose broth (LT broth) for the production of nisin when buffered with $NaHCO_3$. Costs were reduced from $600/Kg nisin (LT broth) to $35-40/Kg nisin (Wolf-Hall *et al.*, 2009). Malt-sprout extract (MSE) medium is another low cost cereal-based medium derived from food industry by-products. MSE medium supplemented with glucose (G) and yeast extract (YE) was investigated as an alternative to MRS for the growth and production of antimicrobial activity by *Lb. plantarum* VTT E-79098. MSE(GYE) compared favourably with MRS for biomass production and interestingly, was superior for the production of antimicrobial activity. The cost of MSE medium was estimated to be 20% that of MRS (Laitila *et al.*, 2004).

A number of studies have demonstrated that milk and by-products of the dairy industry (whey) are cost effective food grade substrates for commercial bacteriocin production (Arakawa *et al.*, 2008, Daba et al., 1993, Dimov, 2007, Goulhen *et al.*, 1999, Guerra & Pastrana, 2001, Liu *et al.*, 2005, Morgan et al., 1999). Ananou *et al* (2008) found production of the bacteriocin AS-48 to be significantly cheaper in a commercial whey (supplemented with 1% glucose) when compared to the cost of production in BHI (€1.53/Kg Vs €140.00/Kg). As well as being cost-effective food grade substrates, milk and whey have also been used to produce bacteriocin containing bioactive powders by lyophilising the fermentate of bacteriocinogenic cultures, i.e. Nisaplin™ and Alta 2341®. Due to the fastidious nature of LAB, the low concentration or absence of certain growth factors (vitamins and amino acids) in whey may be limiting for bacteriocin production (Guerra & Pastrana, 2001). The addition of a complex nitrogen source can facilitate increased bacteriocin production through the availability of free amino acids, short peptides as well as additional growth factors (Aasen *et al.*, 2000, Cheigh *et al.*, 2002). This is exemplified by addition of yeast extract to whey, which has been shown to increase bacteriocin production in a number of studies (Anthony *et al.*, 2009, Avonts *et al.*, 2004, Cladera-Olivera *et al.*, 2004, Enan & Al Amri, 2006, Liao *et al.*, 1993, Pérez Guerra *et al.*, 2005). The availability of carbon can also impact upon

bacteriocin production. At low concentrations, carbon availability can limit biomass production and hence final bacteriocin titres (De Vuyst & Vandamme, 1992). However, it has been shown that increasing the initial carbon concentration does not result in a proportional increase in bacteriocin titre, and above a certain concentration can result in a reduction in biomass and bacteriocin formation (Parente *et al.*, 1997). Maximum bacteriocin titre is usually reached at the end of the log phase or early stationary phase, after which time a decrease in bacteriocin activity is commonly seen (Callewaert *et al.*, 2000, Leroy & De Vuyst, 2002, Mataragas *et al.*, 2003). The depletion of the energy source has been proposed for this decline in bacteriocin activity (Callewaert et al., 2000, De Vuyst *et al.*, 1996). Parente *et al*, (1997) observed that at low glucose concentrations (<25g L^{-1}) bacteriocin activity dropped after it had reached its peak. At higher glucose concentrations, no decline in activity was observed.

3.2 Environmental Conditions

Bacteriocin production has frequently been reported as being greatest during the active growth phase, displaying primary metabolite kinetics (De Vuyst et al., 1996). While bacteriocin production is growth associated, conditions required for maximum growth rate may not be optimal for maximum bacteriocin production (Nel *et al.*, 2001). A number of studies have shown that temperatures favouring bacteriocin production are lower than those required for optimum growth (Bizani & Brandelli, 2004, Cheigh et al., 2002, Delgado *et al.*, 2007, Delgado *et al.*, 2005, Mataragas et al., 2003, Messens et al., 2003). Aasen *et al* (2000) observed that sakacin P production ceased earlier when grown at 30˚C (optimum for growth) rather than at 20˚C, and that lower quantities of glucose were consumed at 30˚C than was observed when grown at 20˚C. The authors proposed that the greater bacteriocin activity observed at 20˚C was due to rate limiting reactions dependant on temperature, resulting in more efficient carbohydrate utilization

at lower growth rates. It has also been proposed that a decrease in bacteriocin activity at higher temperatures could be due to the action of proteases (Bizani & Brandelli, 2004, Messens et al., 2003).

The pH of the growth medium during fermentation can have a profound effect on the yield of bacteriocin. As with temperature, suboptimal pH has frequently been reported as being required for optimal bacteriocin production (Drosinos *et al.*, 2006, Herranz *et al.*, 2001, Mataragas et al., 2003, Matsusaki *et al.*, 1996, Parente & Ricciardi, 1994). Once an optimal pH for bacteriocin production has been established, pH controlled fermentations can be used to maintain maximum bacteriocin production throughout the fermentation (Abriouel *et al.*, 2003, Liu et al., 2010, Mataragas et al., 2003, Naghmouchi *et al.*, 2008, Wolf-Hall et al., 2009). In contrast to this, the natural fall in pH resulting from the production of lactic acid by LAB has also been shown to be beneficial in increasing bacteriocin yields. Guerra and Pastrana (2003) investigated the effect of pH drop on nisin and pediocin PA-1 production in whey. They found that nisin and pediocin production rates were higher in un-buffered fermentations than in those buffered with different concentrations of potassium hydrogen phthalate-NaOH. Specific bacteriocin production rates increased with decreasing pH, until a final pH inhibitory to the producer was reached. Similar results were reported by Cabo *et al* (2001) and Yang & Ray (1994). The adsorption of bacteriocins to producer cells at high pH has also frequently been described (Bhunia *et al.*, 1991, D'Angelis *et al.*, 2009, Klaenhammer, 1988, Ray, 1992, Wu *et al.*, 2008, Zhang *et al.*, 2009), and occurs once growth associated bacteriocin production has ceased (De Vuyst et al., 1996). In such situations, a reduction of the culture pH to around 2.0 once maximum bacteriocin titre has been reached can be employed to prevent adsorption to producer cells and release any bacteriocin already bound to the producers cells (Yang *et al.*, 1992).

3.3 Effect of fermentation processes on bacteriocin production.

Batch fermentation is what is described as a "closed system", whereby the substrate and producing microorganism are added to the system at time zero and are not removed until the fermentation is complete. This represents the simplest and most commonly employed method of fermentation for the production of bacteriocins. Optimization of bacteriocin production in batch fermentation is achieved by the manipulation of growth conditions (pH, temperature etc.) and medium composition. Batch fermentation is ideal for studying bacteriocin production in the lab or in small scale trials, but is not economically viable on a commercial scale (De Vuyst & Vandamme, 1991, Liao et al., 1993). Fed-batch fermentation is a modified form of batch fermentation whereby growth limiting substrates are fed into the fermenter at a controlled rate. This allows for a tight control over growth rate and can alleviate problems such as catabolite repression. By controlling the sucrose feeding rate in a fed-batch system, Lv, Zhang & Chong (2005) increased the maximum nisin titre from 2658 IU ml^{-1} in batch fermentation to 4185 IU ml^{-1}. Controlled carbohydrate feeding facilitated a maximum growth rate without substrate inhibition impacting upon the rate of bacteriocin production. A number of studies have demonstrated that bacteriocin production can be improved through the use of fed-batch, rather than batch fermentations (Ekinci & Barefoot, 2006, Guerra *et al.*, 2005, Lv *et al.*, 2005, Paik & Glatz, 1997). While promising, these experiments were performed on a small scale and thus, it is difficult to determine how scaling up of the process would impact upon bacteriocin yields. In one study, bacteriocin production by *Propionibacterium thoenii* in small and large scale fed-batch fermentations has been assessed. Paik & Galtz (1997) found that scaling up of the process led to a reduction in bacteriocin activity. Even with the reduced activity, the authors concluded that fed-batch fermentations have the potential to facilitate the production of high concentrations of bacteriocins by propionibacteria.

While fed-batch fermentation is an improvement over batch fermentation, it has been proposed that continuous culture techniques are superior to both. Continuous fermentation is preferred to the batch process due to its high productivity, reduced product inhibition and no batch to batch variation and ultimately reduced production costs (Tejayadi & Cheryan, 1995). Continuous fermentation is an "open system" whereby sterile substrate is added to the system at a specific dilution rate (*D*, volume of vessel h^{-1}), while an equal volume of converted substrate containing the product along with the producer is simultaneously removed. The continuous flow through the system maintains fermentation conditions at the optimum required for bacteriocin production. This results in the producer being maintained at the growth rate/phase which results in the specific bacteriocin production rate being optimal (Bhugaloo-Vial *et al.*, 1997). The dilution rate (*D*, vessel volume h^{-1}) in a continuous fermentation can greatly affect the productivity of the process. The maximum dilution rate in a continuous fermentation must not exceed the specific growth rate of the bacterial strain being used to prevent cells being washed out of the system. Kaiser & Montville (1993) compared the production of the Class II bacteriocin Bavaricin MN by *Lb. bavaricus* MN in batch and continuous culture. In a pH and temperature controlled batch fermentation a maximum titer of 3200 AU ml^{-1} was reached. However this fell to 800 AU ml^{-1} after 76h. In a continuous culture at the same temperature and pH conditions a maximum titer of 6400 AU ml^{-1} was obtained, which was subsequently maintained for 345h. The effect of carbon sources as well as dilution rates on the production of the Class I bacteriocin, Plantaricin C, by *Lb. plantarum* LL441 was investigated in a continuous culture (Bárcena *et al.*, 1998). The carbon sources, glucose, sucrose and fructose were tested at different dilution rates to determine the optimum conditions for bacteriocin production. Plantaricin C was only detected at low *D* (0.05 h^{-1}) when glucose was used as the carbon source. From this observation it was postulated that glucose may mediate catabolite repression, and therefore the use of sucrose and fructose as

carbon sources became a priority. With these carbon sources plantaricin C production was detected at *D* (0.1-0.12 h^{-1}) i.e. double that of glucose. Under optimum conditions, similar bacteriocin titres (~3200 AU ml^{-1}) in culture supernatants were achieved for all three carbon sources, however bacteriocin yield was doubled in media containing sucrose and fructose due to the higher dilution rates.

It has also been noted that immobilisation of bacteriocin producing cells onto a solid matrix facilitates the application of dilution rates far in excess of the maximum specific growth rate, without cell washout (Lamboley *et al.*, 1997), and can also provide increased cell density (Dervakos & Webb, 1991) and plasmid stability (Huang *et al.*, 1996). In one instance nisin Z production by *L. lactis* UL179 immobilised in κ-carrageenan/locust bean gum gel (IC) and in free cell culture (FC) was compared in a continuous fermentation. It was noted that nisin Z production increased with increasing *D* in IC fermentations relative to FC fermentations. Sonomoto *et al* (2000) compared nisin Z production by FC and IC adsorbed to ENTG-3800 in continuous fermentation. FC displayed good nisin Z production at a dilution rate of 0.1 h^{-1} but, at a dilution rate of 0.2 h^{-1}, cell wash out resulted in reduced production. In contrast an increase in productivity at higher dilution rates was observed in IC fermentations. This phenomenon was also reported by Liu *et al* (2005) during the production of nisin by *L. lactis* subsp. *lactis* ATCC 11454 i.e. the authors observed that nisin activity increased with increased dilution rates up to a *D* of 0.31 h^{-1}, although further increase in *D* resulted in a decrease in nisin activity.

The immobilisation of bacteriocinogenic bacteria in calcium alginate gel beads has also been frequently reported (Bhugaloo-Vial et al., 1997, Ivanova *et al.*, 2002, Naghmouchi et al., 2008, Scannell *et al.*, 2000, Wan *et al.*, 1995). Calcium alginate is an appropriate scaffold for cell immobilisation due to its low cost, food grade status and relative ease in encapsulation of cells (Bhugaloo-Vial et al., 1997). The production of nisin and lacticin 3147 by two *L. lactis* strains

either immobilized in calcium alginate beads (IC) or in free culture (FC) was assessed in continuous fermentation (Scannell et al., 2000). The beads were found to be quite stable over 180h, although some cell leakage from the beads was reported. While both nisin and lacticin 3147 were detected earlier in FC bioreactors, a sharp decline in production occurred mid way through the fermentation. Bacteriocin production took longer to reach its maximum in IC bioreactors but, once achieved, maximum production was maintained for the remainder of the fermentation. It should be noted, however, that Mg^{2+}, $MgSO_4$, acetate, citrate and phosphate ions can cause instability in calcium alginate beads (Vignolo *et al.*, 1995, Yang & Ray, 1994) and that the stability is also affected by the strain that is being encapsulated (Scannell et al., 2000).

The natural attachment of cells to fibrous surfaces in packed bed bioreactors has also been employed when immobilising of bacteriocinogenic bacteria (Cho *et al.*, 1996, Liu et al., 2005). Using this design, Liu *et al* (2005) immobilised the nisin producer, *L. lactis* subsp. *lactis* ATCC 11454, in a packed bed bioreactor. This bioreactor was run continuously for six months without encountering any problems. Under optimal conditions a maximum nisin titre of 5.1×10^4 AU ml^{-1} was achieved.

As well as being used in continuous fermentations immobilised cells have also been investigated in repeated-cycle batch (RCB) fermentations. In RCB fermentation, immobilised bacteriocinogenic bacteria are used to perform consecutive batch fermentations. After each fermentation cycle the fermentate is removed and replaced with fresh media and the process is repeated. During 1-h cycle RCB cultures the nisin Z producer *L. lactis* UL719, when immobilised in calcium alginate beads, was capable producing up to 8200 IU ml^{-1} of the bacteriocin. This corresponds to a volumetric productivity of 5730 IU ml^{-1}. An aerated continuous IC culture with the same strain run at a D of 2.0 h^{-1}, resulted in a maximum

volumetric productivity of only 1760 IU ml^{-1} (Bertrand *et al*, 2001). RCB fermentations have also been assessed with respect to pediocin PA-1 production by *P. acidilactici* UL5 immobilised in κ-carrageenan/locust bean gum gel. Maximum pediocin PA-1 activity, 4096 AU ml^{-1}, was obtained after 0.45 and 2 h of incubation in MRS and supplemented whey permeate respectively. This corresponded to volumetric productivities of 5461 and 2048 AU ml^{-1} h^{-1}. In contrast, pediocin activity in pH-controlled batch fermentations with free cells yielded only 4096 AU ml^{-1} after a12 h incubation, resulting in a much lower volumetric productivity of just 342 ml^{-1} h^{-1} (Naghmouchi et al., 2008).

3.4 Purification of bacteriocins.

The purification of bacteriocins from culture media is necessary to study their composition and mode of action. It is also required for the production of pure peptides for application as natural biopreservatives in food. The purification of bacteriocins from the culture media is a notoriously difficult process and so many methods have been developed (Saavedra & Sesma, 2011). This section will give a brief overview of the methods employed for the purification of bacteriocins. For a more detailed review, please refer to the review by Saavedra & Sesma (2011).

When purifying bacteriocins the first step is to concentrate the peptides. This process is compounded by the presence of a substantial quantity of small peptides of similar size to that of most bacteriocins (3,000 - 6,000 Da) in the complex media required for the cultivation of bacteriocinogenic LAB (Parente & Ricciardi, 1999). Therefore, it is not possible to simply concentrate the peptides by water removal. The two most widely reported methods for the concentration of bacteriocins are acid extraction (Yang *et al.*, 1992) and salt precipitation (Muriana & Klaenhammer, 1991). Acid extraction relies on the adsorption and desorption of a

bacteriocin to its producing cells at different pHs. Yang *et al* (1992) studied this phenomenon with the bacteriocins nisin, pediocin PA-1, sakacin A and leuconocin Lcm1. It was discovered that by adjusting the pH to ~ 6.0 (bacteriocin specific), it was possible to adsorb between 93 - 100% of bacteriocin molecules to the surface of the bacteriocin producing cells. Cells were removed from the culture supernatant by centrifuging and released from the cells by adjusting the pH to ~ 2.0 (bacteriocin specific) in the presence of 100mM NaCl. Over 90% recovery was reported for nisin, pediocin PA-1 and leuconocin Lcm1. However, sakacin A recovery was lower i.e. 44%. This is a relatively simple method that provides good yields of bacteriocin and so could be suitable for the concentration of bacteriocin peptides on an industrial scale. The other most common method for concentrating bacteriocins from the culture media is salt precipitation with ammonium sulphate. Ammonium sulphate is added to the culture supernatant (CS) to the point of saturation. The saturated CS is then gently mixed overnight and the peptides are allowed to precipitate out of solution. The precipitate is subsequently removed from the culture by centrifuging and is washed in buffer (Muriana & Klaenhammer, 1991). While this is the more commonly applied method for peptide bacteriocin concentration the percentage recovery can vary greatly, from below 10 to nearly 100% (Bayoub *et al.*, 2011, Kamoun *et al.*, 2005, Kumar *et al.*, 2010, Liu *et al.*, 2008a).

After concentration, the bacteriocin peptides still need to be separated from the contaminants present in the media. Generally this is achieved by applying concentrated peptides to a cation exchange column (Callewaert & De Vuyst, 1999, Uteng *et al.*, 2002). Anionic compounds can pass freely through the column, while bacteriocins, which are frequently cationic, are retained in the columns matrix. Bacteriocins are subsequently eluted from the column using a NaCl solution (Uteng et al., 2002). Active fractions containing bacteriocin peptides are then applied to a reverse-phase high-performance liquid chromatography (RP-HPLC) column for final

concentration. Bacteriocins are eluted from the column using a gradient of water miscible organic solvents (Saavedra & Sesma, 2011).

3.5 Partially purified bioactive powders.

Purification of highly purified bacteriocins on an industrial scale is not viable due to the long processing time and low percentage recovery. For these reasons, bacteriocins are more commonly produced as partially purified bioactive powders such as Nisaplin™ and Alta 2341™ (Danisco, Kerry bioscience). The long term stability and ease of transport associated with powdered preparations also make them a more attractive option (Gardiner *et al.*, 2000). Spray dried bioactive powders have also been described in the literature (Ananou *et al.*, 2008, Morgan et al., 1999). A lacticin 3147 containing powder has been produced by Morgan *et al* (1999). Briefly, *L. lactis* DPC 3147 was inoculated into reconstituted demineralised whey (10%) and fermented for 24 h under constant pH. The resulting fermentate was pasteurized (72˚C for 15s) and concentrated by evaporation to 40% solids. The concentrate was then spray-dried to produce a bioactive lacticin 3147 powder. The addition of 10% total product weight of lacticin 3147 powder was required to sufficiently reduce numbers of *Bacillus cereus* and *L. monocytogenes* in a range of food trials (Morgan *et al.*, 2001). While this exhibits the potential of a lacticin 3147 based bioactive powder, the addition of 10% total product weight of such a powder is neither feasible nor economic.

A spray dried bioactive powder containing the enterocin AS-48 has also been produced. *E. faecalis* A-48-32 was cultivated in the whey derived substrate Esprion-300 (E-300), at 28˚C for 18-20h under controlled a pH of 6.5. After fermentation AS-48 was recovered from the E-300 by cation exchange chromatography on a carboxymethyl Sephadex CM-25. The recovered fractions

were either heat treated (80˚C, 20 mins) or UV light irradiated (5mins) to inactivate the producer and subsequently spray dried (Ananou *et al.*, 2010). However, yet again, 5% and 10% wt/vol was still required to control the growth of *L. monocytogenes* and *S. aureus* in skim milk respectively (Ananou et al., 2010). Also while both the lacticin 3147 and the AS-48 powders retain full activity for at least 4 months at refrigeration temperatures (4-5˚C), however both undergo a 50% reduction in activity after 9 months at room temperature (Ananou et al., 2010, Morgan et al., 2001). The stability of these powders clearly does not match that of the commercial nisin preparation Nisaplin™ which is stable between 4˚C and 25˚C over 2 years from the date of manufacture (Morgan et al., 2001). For such powders to reach their true commercial potential, the issues of stability and total activity will have to be addressed.

4. **Improvement of bacteriocinogenic bacteria.**

Genetic manipulation of bacteriocinogenic bacteria can be exploited to address many of the problems associated with bacteriocin production for food applications, such as low production, production in suitable bacteria and spectrum of activity. A number of authors have demonstrated the effectiveness of genetic manipulation in improving bacteriocin production (Cheigh *et al.*, 2005, Cotter *et al.*, 2006, Heinzmann *et al.*, 2006). While the genetically modified nature of the strains in question usually precludes their use in food, such studies have often provided valuable insights and may be of relevance to the food industry in the future.

Cotter *et al* (2006) investigated the provision of additional copies of the lacticin 3147 genes on the production of lacticin 3147 in *L. lactis* MG1363 (pMRC01), a strain expressing the parental lacticin 3147 encoding plasmid (pMRC01). A high copy number plasmid containing the entire lacticin 3147 encoding region (pOM02) was introduced into MG1363 by electroporation and the

resulting strain, MG1363(pMRC01, pOM02) was found to produce 4-fold more lacticin 3147 than strains containing either plasmid alone. Further investigation revealed that additional copies of the two lacticin 3147 structural genes (*ltnA1A2*) was not necessary, as addition of a plasmid containing all other lacticin 3147 encoding genes resulted in a 3.5 fold increase in production relative to the strain carrying pMRC01 alone. In another instance, an increase in the production of nisin Z by *L. lactis* A163 was achieved by introducing a multiple copy plasmid harbouring the nisin regulatory genes, *nisR* and *nisK*. This resulted in an increase in production from 16,000 AU ml^{-1}, observed in the control to 25,000 AU ml^{-1} in the strain over-expressing *nisRK*. This was as a consequence of the increased transcription of the *nisZ* gene (Cheigh et al., 2005).

As enterocin producing *Enterococci* can potentially carry virulence genes and therefore are not considered GRAS organisms (Eaton & Gasson, 2001, Franz *et al.*, 2001, Shankar *et al.*, 2002), there has been a growing interest in producing enterocins in GRAS hosts. An example of this is the heterologous expression of the Class II bacteriocin enterocin P (EntP) from *E. faecium* P13 by a strain of *L. lactis*. The EntP structural gene, *entP*, and its immunity gene, *entiP* were cloned into *L. lactis* NZ9000 under a nisin inducible expression system. The resulting strain, *L. lactis* NZ9000(pJR199), exhibited a higher specific activity than was produced by any other *L. lactis* hosts or the parent strain *E. faecium* P13 (Gutiérrez *et al.*, 2006). Liu *et al* (2008b) heterologously expressed the Class II bacteriocin, enterocin A (EntA) in *L. lactis* MG1614(pLP712). The EntA encoding plasmid pEnt02 was introduced into *L. lactis* MG1614(pLP712) by electroporation and although the resulting strain, *L. lactis*$_{Ent+}$ produced 4-fold less EntA than the parent strain *E. faecium* DPC 1146, however this was deemed acceptable due to the strong anti-listerial properties of EntA.

The natural transfer of bacteriocin-encoding plasmids by conjugation has also been used to confer a bacteriocin producing phenotype on strains that are better adapted to specific food

environments. Over 30 food-grade starter cultures producing lacticin 3147 have been constructed by exploiting the conjugal nature of the lacticin 3147 encoding plasmid pMRC01 (Coakley *et al.*, 1997, Trotter *et al.*, 2004), some of which have also been used as protective cultures in food fermentations (Coffey *et al.*, 1998, McAuliffe *et al.*, 1999). One such transconjugant, *L. lactis* DPC 4275 was found to produce variable titres of lacticin 3147. In this strain it was found that an 80kb cointegrate plasmid, pMRC02 had formed from the incorporation of the lacticin 3147 genes into the resident plasmid pMT60. It was revealed that when pMRC02 was present at a high copy number lacticin 3147 titre was roughly double that of a low copy variant (Trotter et al., 2004).

The limited antimicrobial potency and spectrum of inhibition of some bacteriocins restrict their value with respect to food related applications. One solution to this problem is the creation of multi-bacteriocinogenic bacteria producing two or more bacteriocins to enhance or broaden their activity. By combining unrelated bacteriocins with different modes of action, it is also possible to prevent the emergence of resistance to either bacteriocin (Horn *et al.*, 1999). Gutiérres *et al* (2006) described the heterologous production of enterocin P in the nisin producing strain *L. lactis* DPC 5598. The resulting transformant was capable of simultaneous production of both bacteriocins, although it should be noted that the levels of EntP activity of this strain were lower than that observed among other *L. lactis* host strains. Lower bacteriocin production relative to the parent strain was also observed when enterocin A and pediocin PA-1 were heterologously co-produced in *L. lactis* IL1403, although this could be attributed to an inefficient host (Martínez *et al.*, 2000).

Genetic manipulation has also been employed to modify bacteriocins with a view to improving their spectrum of inhibition, antimicrobial activity and the solubility and stability of the

bacteriocins for applications in food environments. This is generally achieved either by mutagenesis of the bacteriocin encoding genes or by fusing genes from different species to create chimeric bacteriocins (Gillor *et al.*, 2005). Yuan *et al* (2004) created a number of mutants producing derivatives of nisin Z by site directed mutagenesis at the hinge region of the nisin Z gene, *nisZ*. The resulting mutants had decreased activity versus *Micrococcus flavus* NCIB8166 and *Streptococcus thermophilus*. However, peptides with enhanced activity against Gram negative, i.e. *Shigella*, *Salmonella* and *Pseudomonas* species, or with increased solubility and stability compared to that of nisin Z were identified. Random mutagenesis has also been employed to create the largest bank of randomly mutated nisin derivates (Field *et al.*, 2008). Use of this approach resulted in the identification of derivatives with enhanced activity against *L. monocytogenes*, *S. aureus Streptococcus agalactiae* (Field et al., 2008) and various mycobacteria (Carroll *et al.*, 2010) and others with apparently enhanced ability to diffuse through complex matrices (Rouse *et al.*, 2012).

Notably, although the initial producers of these variants were genetically modified microorganisms (GMM), strategies exist which can facilitate the generation of corresponding strains through self-cloning, meaning that such producers would fall outside the scope of directives regarding the contained use of GMMs. This fact may ultimately facilitate the application of these nisin derivatives for food applications.

An alternative strategy, i.e. DNA shuffling, was employed to develop an 'improved' derivative of pediocin PA-1. A DNA shuffling library was created by shuffling four specific regions of the N-terminal half of pediocin PA-1 with 10 other class IIa bacteriocins. A library of 280 shuffled DNA mutants was created, 63 of which displayed antimicrobial activity. Shuffled mutants displayed increased activity against various species of *Lactobacillus, Pediococcus,* and *Carnobacterium*. One of the mutants identified was also active against *L. lactis*, which was immune to the parent pediocin PA-1 (Tominaga & Hatakeyama, 2007).

5. Conclusions:

The *in-situ* production of bacteriocins by bacteriocinogenic starter, adjunct or protective cultures has been demonstrated to be an effective delivery system with respect to the incorporation of bacteriocins into the food environment. As a consequence of the diverse array of bacteriocinogenic LAB that are available, and the fact that they can be produced as lyophilised bacteriocinogenic starter, adjunct or protective cultures, there is great potential for the use of cultures as biopreservatives in food. As there are no regulatory issues that limit the use of bacteriocinogenic LAB in food, this approach may be an economical alternative to the application of chemical preservatives or purified bacteriocin preparations for controlling spoilage and pathogenic bacteria.

The optimisation of fermentation processes, as well as the development of food grade media from industrial waste products, has greatly reduced the cost of producing bacteriocins by large scale fermentation. However, the production of purified bacteriocin peptides can still be a difficult and expensive process and peptides other than nisin have not been approved for use as food preservatives. Therefore, the production of bioactive fermentates containing bacteriocins is preferred. Such bioactive fermentates can be added to foods as food ingredients rather than as preservatives and thus are not subject to the same regulatory scrutiny as a purified peptide preservative. While the production of bacteriocins as bioactive fermentates is relatively cheaper and less complex than the production of purified bacteriocin peptides, in some instances the antibacterial activity and stability of such preparations would need to first be improved before commercial applications could be considered.

Finally, many strategies have been employed to improve bacteriocinogenic bacteria and the bacteriocins they produce, as current regulations prohibit the use of GMMs in foods and consumer resistance to GMMs also hinder their application in food production, for the short to

medium term there will continue to be an emphasis on the use of food grade strategies to generate new and improved bacteriocinogenic strains.

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