Examining the Effects of Defined Microbial Ecosystems on *Clostridioides difficile* Growth and Virulence

by

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ABSTRACT

EXAMINING THE EFFECTS OF DEFINED MICROBIAL ECOSYSTEMS ON CLOSTRIDOIDES DIFFICILE GROWTH AND VIRULENCE

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Many cases of Clostridioides (formerly, Clostridium) difficile infection (CDI) are unresponsive to current antibiotic treatment strategies, and often patients suffer from recurrent infections characterized by severe diarrhea and colonic inflammation. We have developed a defined and standardized stool-derived microbial ecosystem therapeutic (MET-1), which was used to cure two patients of recurrent CDI (rCDI) in a proof-of-principle trial. To investigate the mechanisms behind the ability of the healthy human gut microbiota to protect against C. difficile in vitro, we used MET-1 and other defined microbial ecosystems to model health and disease states. Using a single-stage chemostat distal gut model to support the growth of bacterial communities, we characterized the compositional and metabonomic profiles of two defined microbial ecosystems derived from the microbiota of a healthy donor (MET-1 and DEC58), and two ecosystems representative of a dysbiotic state. Dysbiotic ecosystems were individually created through both the omission of Lachnospiraceae from DEC58, and treatment of chemostat-cultured DEC58 with ciprofloxacin, a broad-spectrum fluoroquinolone antibiotic. Both perturbed ecosystems were shown to have altered, but distinct taxonomic and metabonomic compositions compared to DEC58. We then examined the effects of defined microbial ecosystem-associated metabolites on the vegetative cell growth, sporulation, germination, spore outgrowth, toxin gene
expression and secretion of two clinically important *C. difficile* ribotype strains, 027 and 078. Additionally, the cytotoxicity and metabonomic profiles of *C. difficile* were assessed in response to treatment with each defined microbial ecosystem. Although there was large heterogeneity in the growth and virulence determinants of *C. difficile* strains in response to defined microbial ecosystems, the results from this study suggest that defined microbial ecosystem-associated metabolites may influence *C. difficile* virulence by decreasing secreted TcdA and TcdB levels *in vitro* and protecting against TcdB-mediated cytotoxicity. The identification of these antagonistic properties complements our existing knowledge of gut microbiota-specific anti-virulence mechanisms against *C. difficile*, and will guide the development and optimization of novel defined microbial ecosystem formulations for the effective treatment of rCDI.
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AUTHOR’S DECLARATION OF WORK PERFORMED

I, Christian Carlucci, declare that all of the work presented in this thesis was completed by me and carried out during my PhD research with the exception of the following:

The MET-1 and DEC58 communities were isolated and identified by Michelle Daigneault and Eric Brown. Chris Ambrose, Erin Bolte, Ian Brown, Michelle Daigneault, Carys Jones, Kaitlyn Oliphant, Dr. Rafael Peixoto, and Dr. Kathleen Schroeter assisted with periodic chemostat vessel set-up, maintenance, and inoculation helping to ensure proper operation of the system. Sandi Yen and Kaitlyn Oliphant performed the 1D $^1$H NMR spectroscopy scans. Illumina MiSeq beyond PCR purification and Agilent Bioanalyzer to determine RNA quality, were conducted by the Advanced Analysis Centre at the University of Guelph. Kaitlyn Oliphant provided invaluable assistance with bioinformatics data analysis. Carys Jones assisted in the experimental setup and data analysis of *C. difficile* viability, toxin gene expression, and toxin secretion assays and Avery Robinson performed the cytotoxicity assays and cell enumeration.
Scanning electron microscope (SEM) image of human gut microbiota grown in a continuous-culture bioreactor
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LIST OF ABBREVIATIONS

°C – degree Celsius
× g – times gravity
1D – one-dimensional
2D – two-dimensional
AUC – area under the curve
BH – Benjamini-Hochberg
BHI – brain heart infusion
BHIS – brain heart infusion supplemented
BMI – body mass index
BSCFA – branched short chain fatty acid
CaCl₂ – calcium chloride
CDI – Clostridioides difficile infection
cDNA – complementary DNA
CdtLoc – binary toxin locus
Cq – quantification cycle
DEC58 – Defined experimental community 58
DMEM – Dulbecco’s Modified Eagle’s Medium
DNA – deoxyribonucleic acid
DSS – 4,4-dimethyl-4-silapentane-1-sulfonic acid
ELISA – enzyme-linked immunosorbent assay
ESBL-Kp – extended spectrum beta-lactamase producing Klebsiella pneumoniae
F.S. – filter sterilized
FAA – fastidious anaerobe agar
FBS – fetal bovine serum
FDA – Federal Drug Administration
FDR – false discovery rate
FMT – fecal microbiota transplantation
gDNA – genomic deoxyribonucleic acid
GI – gastrointestinal
HCl – hydrochloric acid
IBD – inflammatory bowel disease
MET-1 – Microbial ecosystem therapeutic-1
NAD+/NADH – nicotinamide adenine dinucleotide
NIH 3T3 – mouse embryonic fibroblast cell line
NMDS – non-metric multidimensional scaling
NMR – nuclear magnetic resonance
NOESY – nuclear Overhauser effect spectroscopy
OTU – operational taxonomic unit
PaLoc – pathogenicity locus
PBS – phosphate buffered saline
PC – principal component
PCR – polymerase chain reaction
PLS-DA – partial least squares-discriminant analysis
qPCR – quantitative polymerase chain reaction
rCDI – recurrent Clostridioides difficile infection
RCT – randomized controlled clinical trial
RNA – ribonucleic acid
rRNA – ribosomal ribonucleic acid
SCFA – short chain fatty acid
ST80 – PBS containing 0.1% Tween 80
UC – ulcerative colitis
V4 – variable region of the 16S rRNA gene
VRE – vancomycin-resistant enterococci
Chapter 1: Introduction

1.1 The human gut microbiome

The human gut microbiome is a complex ecosystem of fundamental importance to human health. The human gut microbiome can be defined as the collection of microorganisms, their gene products and corresponding physiological functions found in the human gastrointestinal (GI) tract (Gill et al., 2006). Our gut microbiome influences many aspects of our physiology including the production of vitamins and nutrients, regulation of metabolism, exclusion of pathogens, and maintenance of the immune system (Lozupone et al., 2012). Much of our current understanding of the human gut microbiome has arisen from ‘omics-based’ initiatives such as the Human Microbiome Project Consortium (Human Microbiome Project, 2012). These studies have provided great insight into the composition and diversity of gut microbial life.

1.1.1 How is a ‘healthy’ microbiome defined?

The average healthy adult colon contains a dense and diverse community of bacterial species (Qin et al., 2010). Phylogenomic studies of stool samples, primarily from Western populations, have shown that although gut microbial composition varies greatly between individuals there are general trends among healthy adults. Bacteroidetes and Firmicutes are the most prevalent bacterial phyla, while Actinobacteria and Proteobacteria are also prominent members (Human Microbiome Project, 2012). The large interindividual variation in the ratios of these representative phyla can be attributed to numerous influences; a recent study found many factors that significantly correlated with overall microbiota compositional variation including medication/drug use, health-status, age, sex, lifestyle, host genetics, diet, and animal exposure (Falony et al., 2016).
1.1.2 Functional profiles and short-chain fatty acid production in the human gut microbiome

The functional profile of the gut microbiome, which can be defined as the metabolic capacity of bacterial ecosystems, is more conserved than the taxonomic composition (Qin et al., 2010). Multiple studies have shown that the overall metabonomic (complex system-associated metabolomic profile) (Marchesi and Ravel, 2015) and genetic capabilities of gut microbial ecosystems appear to be somewhat consistent across healthy adults (Human Microbiome Project, 2012; Qin et al., 2010; Turnbaugh et al., 2009). For example, short-chain fatty acids (SCFAs) including acetate, propionate and butyrate, are produced by many gut microbial species through carbohydrate fermentation and are the most abundant of the SCFAs in the colon (Marchesi et al., 2015; Ríos-Covián et al., 2016; Wong et al., 2006). SCFAs provide 10% of our daily energy requirements, regulate host energy demands, intestinal epithelial cell homeostasis, and also support the growth of microbiota itself (Ríos-Covián et al., 2016; Wong et al., 2006). In humans, the majority of propionate and acetate is taken up by the liver, while the remainder is sent to peripheral tissues; butyrate is primarily metabolized by colonic epithelial cells (den Besten et al., 2013b). Acetate, propionate, and butyrate are present in an approximate molar ratio of 60:20:20 in the colon and stool, and these compounds can reach a total concentration of 20 to 70 mM in the distal colon (den Besten et al., 2013b).

In addition, butyrate and propionate have been shown to indirectly transcriptionally regulate immune effectors exerting anti-inflammatory properties, thus implicating SCFAs in the regulation of immune function (Furusawa et al., 2013; Lukovac et al., 2014; Marchesi et al., 2015). Acetate is the most abundant SCFA present in the gut, and is primarily produced by gut bacterial fermentation of pyruvate via acetyl-CoA (Miller and Wolin, 1996) (Figure 1.1). Butyrate is produced by anaerobic bacteria within the Firmicutes phyla, and is primarily
Figure 1.1 Microbial metabolic pathways responsible for the production of SCFAs and other major metabolites from carbohydrate fermentation and bacterial cross-feeding in the human colon. Acetate can be produced by many enteric bacteria through the conversion of pyruvate to acetyl-CoA and by acetogenic bacteria via the Wood-Ljungdahl pathway. The primary pathway for butyrate synthesis is the CoA-transferase route. The succinate pathway is the major pathway for propionate production among gut bacteria, while two other pathways of propionate production; the acrylate and propanediol pathways are found in other gut bacteria. Species shown in purple are able to consume lactate to form butyrate, while species shown in blue can use lactate to form propionate. Additionally, specialist succinate users (shown in green) can convert succinate into propionate. Dominant pathways are shown in red. PEP, phosphoenolpyruvate; DHAP, dihydroxyacetonephosphate. (Adapted from Flint et al., 2015 and Louis et al., 2014).
synthesized by the actions of acetyl-CoA and butyryl-coenzyme A (CoA):acetate CoA-transferase (Louis et al., 2004; Macfarlane and Macfarlane, 2003). Gut microbiota can generate propionate via three independent pathways. The succinate pathway, which is utilized by Bacteroidetes and some Firmicutes, is responsible for the greatest abundance of propionate generated from hexose and pentose carbohydrates in the gut (Reichardt et al., 2014). The propanediol pathway uses deoxyhexose sugars to generate propionate and is found in some Lachnospiraceae including Blautia and Roseburia species and at least one known Proteobacteria, Salmonella enterica. Lastly, propionate can also be produced by the lactate pathway by a limited number of gut bacteria restricted to Firmicutes, and for the most part, Lachnospiraceae (Louis et al., 2014; Reichardt et al., 2014). Amino acids valine, leucine, and isoleucine obtained from proteolytic breakdown can form branched-chain SCFA (BSCFA) including isobutyrate, isovalerate, and 2-methyl butyrate, which marginally contribute to total SCFA production (~5%) (Ríos-Covián et al., 2016).

Mutualistic cross-feeding mechanisms that occur between bacteria are ubiquitous in the gut and significantly influence colonic SCFA levels (Flint et al., 2015; Heinken and Thiele, 2015). In vitro studies have shown that two mechanisms of cross-feeding occur between microorganisms; consumption of metabolic end products such as lactate and acetate, and partial breakdown products from complex carbohydrate substrates (Belenguer et al., 2006). For example, lactate is normally present in low amounts in the human colon; however, lactate (and acetate) produced by Bifidobacterium and Lactobacillus can be used by Eubacterium hallii and Anaerostipes spp. to produce butyrate (Duncan et al., 2004; Flint et al., 2015; Munoz-Tamayo et al., 2011) (Figure 1.1). In vitro single-stage continuous bioreactors seeded with human fecal samples have indeed shown that lactate generated through cross-feeding mechanisms contributes
to ~20% of total butyrate quantities (Belenguer et al., 2011). A recent study has also shown that acetate fermentation to butyrate is a mainstay of bacterial cross-feeding, though fermentations of butyrate to propionate and propionate to acetate do also occur to a lesser degree (den Besten et al., 2013a). These mechanisms highlight the complex nature of bacterial metabolic interactions and augment the difficulty in predicting metabonomic outcomes in multi-species bacterial ecosystems. Overall, defining the taxonomic and metabonomic interactions exhibited by gut microbial ecosystems will be necessary to understand how these complex systems operate to maintain gut homeostasis.

1.2 Gut microbial dysbiosis

Gut dysbiosis can be defined as an imbalance in a microbial ecosystem characterized by a shift in the composition or function of microbes, which can lead to conditions conducive to pathogenesis (Figure 1.2). Antibiotics, toxic compounds, poor diet, medical interventions, and disease can cause disturbances to, and potentially loss of diversity within the gut microbiota ecosystem (Hell et al., 2013). Diet is a major modulator of the gut microbiota (David et al., 2014); in the absence of dietary complex carbohydrates (the main substrates for SCFA production), microbial metabolism will shift towards proteolytic fermentation, which results in the generation of potentially toxic, pro-inflammatory and pro-carcinogenic compounds such as amines, ammonia, phenols, sulfides, branched-chain fatty acids and p-cresol (Louis et al., 2014; Vipperla and O'Keefe, 2016). Although not yet clearly elucidated, such compounds have been implicated in gut dysbiosis and the development of colorectal cancer and inflammatory bowel disease (IBD) (Vipperla and O'Keefe, 2016). Defining gut microbial dysbiosis is a persistent challenge because of the compositional variability of the microbial ecosystem across different individuals. Although currently there is no biomarker for dysbiosis, recent work suggests that
Figure 1.2 Alternative stable states (ball and cup model) of human gut microbial ecosystems. Here we conceptualize the ecological stability of the human gut microbiota as inferred through its species richness. The gut microbial ecosystem is represented as a ball that exists within an equilibrium represented as a cup. The depth of each cup is symbolic of the resilience of a given ecosystem and is related to species richness, where lower diversity leads to lower resilience and the greater the likelihood that the “ball” will roll out of the “cup” into a new state of equilibrium that may be less stable than the first. Perturbational stresses caused by numerous factors such as poor diet, disease state, drug use (including antimicrobials), immunosenesence as examples can impact bacterial diversity and force the ecosystem into a less-stable equilibrium state. Repeated stresses can cause a situation where function may be reduced to the point that dysbiosis and the development of disease ensue. (Adapted from Relman, 2012 and Folke et al., 2004).
certain correlations may become diagnostically useful. For example, low fecal chromogranin A concentrations (a neuroendocrine peptide secreted in high levels in IBD and IBS), alongside higher microbial diversity and functional richness has been found to associate with higher high-density lipoprotein (HDL) concentrations (Zhernakova et al., 2016).

1.2.1 Antibiotics and gut microbiota dysbiosis

Antibiotics are life-saving medications essential in basic health-care and their use has become omnipresent worldwide (WHO, 2015). However, antimicrobial use is a growing public health concern, due to the increased emergence of antibiotic resistant bacterial strains around the globe (Ferrer et al., 2016; WHO, 2015). Furthermore, it is becoming increasingly evident that antibiotic use has major deleterious consequences for the human gut microbiome.

i. The impact of antibiotics on microbiota composition

Recent studies have shown that broad-spectrum antibiotic use disrupts host-microbiota homeostasis by decreasing gut microbiota abundance, diversity, and community structure, and altering metabonomic functional profiles (Dethlefsen et al., 2008; Perez-Cobas et al., 2013a; Perez-Cobas et al., 2013b; Theriot et al., 2016; Theriot et al., 2014). Ciprofloxacin, a broad-spectrum fluoroquinolone antibiotic used to treat a variety of mixed aerobic and anaerobic infections (Stein and Goldstein, 2006), has been shown to affect human gut microbiota community composition. In a study by Dethlefsen et al., 2008, three patients were exposed to a 5-day pulse of ciprofloxacin, and immediately following treatment there were significant reductions in bacterial diversity and abundance, specifically in Bacteroidetes and species of Lachnospiraceae and Ruminococcaceae families within the Firmicutes phylum. Two weeks post-ciprofloxacin exposure, the majority of taxa had recovered to a similar state that existed before
treatment (Dethlefsen et al., 2008). Such resilience has been displayed by gut microbial ecosystems in other studies (Antonopoulos et al., 2009; Young and Schmidt, 2004); however, with repeated exposure and long-term ciprofloxacin therapy, extensive damage resulted in an altered community where specific species present in the pre-treatment communities did not recover (Dethlefsen et al., 2008). Specifically, ciprofloxacin treatment in human subjects has been shown to affect the abundance of a wide variety of gut bacterial genera including: Alistipes, Anaerostipes, Bacteroides, Bifidobacterium, Blautia, Clostridium, Coprococcus, Dialister, Escherichia, Eubacterium, Faecalibacterium, Roseburia, Ruminococcus, and Veillonella (Ferrer et al., 2016). Because of the high variation in human gut microbiota among individuals, it is unlikely that ciprofloxacin and other broad-spectrum antibiotics would disturb taxonomic composition uniformly across different individuals.

ii. The impact of antibiotics on microbiota function

Although many studies have investigated taxonomic compositional changes in antibiotic-induced dysbiotic gut environments, functional changes to the gut microbiota have been studied to a much lesser extent. Analysis of the collective microbial components/contributions (e.g. genes, proteins, small molecules) is arguably more indicative of alterations within the gut environment than taxonomic composition, and provides detailed insight into the metabolic activity of microbial communities at a given time.

A study by Antunes et al., 2011 examined stool from streptomycin-treated mice and found that antibiotic treatment altered intestinal homeostasis and the levels of many intestinal fecal metabolites. In particular, bile acid metabolism and hormone synthesis were the main metabolic pathways affected (Antunes et al., 2011). Bile acids play an important role in the intestinal absorption of lipids and fat-soluble vitamins but also have antibacterial properties
Accordingly, select members of the gut microbiota modify primary bile acids through bile salt hydrolysis and bile acid \(7\alpha\)-dehydroxylation producing secondary bile acids (Begley et al., 2005; Boesjes and Brufau, 2014; Ridlon et al., 2014; Theriot et al., 2016). Although there is a complex relationship between gut microbiota and bile acid homeostasis, alterations in bile acid metabolism have been associated with many diseases (Baars et al., 2015; Bernstein et al., 2005; Charach et al., 2011; Duboc et al., 2013; Ma and Patti, 2014). Antibiotics have also been shown to alter bile acid metabolism in human subjects and murine models (Antunes et al., 2011; Perez-Cobas et al., 2013b; Theriot et al., 2016; Theriot et al., 2014; Vrieze et al., 2014). Specifically, decreases in secondary bile acids and increases in primary bile acids have been observed after antibiotic treatments (Theriot et al., 2016; Theriot et al., 2014; Vrieze et al., 2014), consistent with decreased bacterial conversion of bile acids. Another study, assessed the comprehensive changes of fecal microbiota from one individual after two weeks of beta-lactam therapy and found that fundamental bacterial processes including glycolysis, vitamin production, protein expression, bile acid metabolism and cholesterol synthesis were altered, affecting the amount of amino acids, sugars, SCFAs, and other essential molecules produced in the gut (Perez-Cobas et al., 2013b).

In a human gut in vitro batch fermentation model supporting fecal microbial communities, SCFA production was monitored after treatment with physiological concentrations of ampicillin or gentamicin (Johnson et al., 2015). Ampicillin was shown to slightly decrease acetate, butyrate and propionate levels in a similar manner while gentamicin had the opposite effect (Johnson et al., 2015). Of note, SCFA concentrations varied substantially in this model, likely due to disparity in inoculation materials, as different fecal specimens were used to seed each replicate vessel (Johnson et al., 2015). Post-antibiotic fecal metabonomic profiles have been investigated
in murine models using nuclear magnetic resonance (NMR) spectroscopy (Romick-Rosendale et al., 2009; Zhao et al., 2013). Gentamicin and/or ceftriaxone treatment decreased SCFAs, monosaccharides (glucose and fructose, xylose and galactose), amino acids and primary bile acids (cholic acid, taurocholic acid) while oligosaccharides (sucrose, cellobiose, raffinose, stachyose), d-pinitol, choline and secondary bile acids (deoxycholic acid) were increased (Zhao et al., 2013). Romick-Rosendale et al., 2009 profiled the fecal extracts from enrofloxacin-treated mice, and found significant decreases in acetate, butyrate, propionate, alanine, isoleucine, leucine, threonine, and valine, while urea was the only compound increased (Romick-Rosendale et al., 2009). Overall, these studies suggest that bacterial fermentation and protein degradation were suppressed by the actions of these antimicrobials.

Multiple studies have associated decreases in SCFA-producing bacteria and SCFA levels to different GI conditions where antimicrobials are utilized in disease management. For example, Young and Schmidt, 2004 found a 25% reduction in Lachnospiraceae and an 18% reduction in Faecalibacterium in antibiotic-associated diarrhea (AAD) cases treated with amoxicillin-clavulanic acid (Young and Schmidt, 2004). Ulcerative colitis (UC) has also been associated with decreases in species within the Lachnospiraceae family and Faecalibacterium prausnitzii (Frank et al., 2007; Lepage et al., 2011; Rossen et al., 2015). As known butyrate producers, the reduction of these species may account for the depletion of this SCFA observed in some UC patients (Lepage et al., 2011). Additionally, Akkermansia muciniphila, a known propionate and acetate producer (through the degradation of mucin) and commensal gut microbe that contributes to the homeostatic balance of the intestinal mucus layer, was found to be reduced in UC patients (Png et al., 2010). Antibiotics have also been implicated in gut microbial dysbiosis and the development of Clostridioides difficile infection (CDI).
1.3 *C. difficile* infection

*C. difficile* is a Gram-positive, anaerobic, sporulating, bacterial pathogen that is the etiological agent of CDI (Abt et al., 2016; Bartlett et al., 1978; Hall and O'Toole, 1935). Recently, the nomenclature of this bacterial pathogen has been reclassified from “*Clostridium difficile*” to “*Clostridioides difficile*” based on its phylogenetic diversity from rRNA cluster I clostridial species (Collins et al., 1994; Lawson et al., 2016; Lawson and Rainey, 2015). CDI is the most commonly diagnosed cause of healthcare-associated and AAD and is associated with high patient morbidity, disease outbreaks and increased health-care costs in industrialized countries. In 2011, there were 453,000 CDI cases in the USA, resulting in 29,300 deaths, and since this time, *C. difficile* has been listed as an urgent drug-resistant threat according to the Centers for Disease Control and Prevention (CDC, 2013; Lessa et al., 2015). CDI is associated with a range of signs and symptoms from mild diarrhea to pseudomembranous colitis, diarrhea, and more life-threatening conditions including bowel perforation, toxic megacolon and sepsis (Gerding et al., 1995).

1.3.1 Gut dysbiosis and *C. difficile* infection

Under normal circumstances, it is thought that the resident gut microbiota facilitates colonization resistance against *C. difficile* suppressing its pathogenic activity in the colon (Theriot et al., 2014). However, broad-spectrum antibiotic use disrupts host-microbiota homeostasis by decreasing gut microbiota abundance, diversity, community structure, and altering metabonomic functional profiles (Dethlefsen et al., 2008; Perez-Cobas et al., 2013a; Perez-Cobas et al., 2013b; Theriot et al., 2016; Theriot et al., 2014) (Figure 1.3 A). Broad-spectrum antibiotic use is the main risk factor for CDI, with clindamycin, cephalosporins, penicillins and fluoroquinolones presenting the greatest risk (Owens et al., 2008).
Figure 1.3 Microbiome-based therapeutics aim to restore the gut dysbiosis observed in CDI. Under normal circumstances, the resident gut microbiota facilitate colonization resistance against *C. difficile*, suppressing its pathogenic activity in the colon. However, broad-spectrum antibiotic use disrupts host-microbiota homeostasis by decreasing gut microbiota abundance, diversity, community structure, and altering metabonomic functional profiles. In these conditions, *C. difficile* is able to establish and maintain itself in the gut, where the production of toxins A and B primarily mediate CDI symptoms (A). Therapeutic interventions including probiotics, FMT, and stool-substitute therapies have direct antagonistic mechanisms against *C. difficile* growth/virulence and are thought to reestablish colonization resistance against *C. difficile* through restoration of the healthy gut microbiome (B).
Many studies have described microbial taxonomic alterations in CDI patients (Table 1.1). In general, the gut microbiota profiles of CDI patients display reduced overall bacterial diversity, characterized by increases in Proteobacteria with decreases in Firmicutes and Bacteroidetes phyla in comparison to healthy subjects (Antharam et al., 2013; Chang et al., 2008; Fuentes et al., 2014; Rea et al., 2012; Schubert et al., 2014; Shahinas et al., 2012). However, taxonomic changes do not appear to be consistent between distinct patient cohorts; this is likely because of the large variation in human gut microbiota among individuals (Girotra et al., 2016; Schubert et al., 2014).

Metabonomic alterations as a result of antibiotic use are important in the context of CDI (Theriot et al., 2014). Butyrate-producing bacteria, mainly those from Ruminococcaceae and Lachnospiraceae families, are depleted in CDI patients (Antharam et al., 2013; Fuentes et al., 2014). *Enterococcus, Lactobacillus,* and *Veillonella* genera are often more abundant in CDI patient stool compared to healthy individuals, as are members of the Proteobacteria phylum, including *Klebsiella pneumoniae, Enterobacter aerogenes,* and *Sutterella* spp. (Fuentes et al., 2014). Through network analysis, these signatures were found to be negatively correlated to the abundance of common butyrate-producing species such as *Eubacterium rectale* and *Roseburia intestinalis* (Fuentes et al., 2014). Given the important role of butyrate to gut homeostasis, preservation of these bacteria is thought to be critical for the maintenance of gut health.

Bile acids have been shown to mediate colonization resistance to the GI pathogen *C. difficile,* thus disruptions to the balance of bile acid levels can negatively impact gut homeostasis (Buffie et al., 2015; Perez-Cobas et al., 2013b; Theriot et al., 2016; Theriot et al., 2014; Weingarden et al., 2014). As an example, primary and secondary bile acids have been shown to exert markedly different effects on *C. difficile;* primary bile acids promote growth of this
Table 1.1 Microbial taxa (phylum level) positively correlated to CDI in human subjects

<table>
<thead>
<tr>
<th>Study size/details:</th>
<th>Changes associated with condition*:</th>
<th>Reference:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-FMT and post-FMT fecal samples from n=3 elderly CDI patients</td>
<td>↑ Proteobacteria, ↑ Firmicutes, ↓ overall microbial diversity</td>
<td>Hamilton et al., 2013</td>
</tr>
<tr>
<td>Fecal samples from n=7 elderly CDI patients (n=4 initial episode of CDI, n=3 recurrent CDI cases), and n=3 elderly control subjects</td>
<td>Changes from the normal ratio of Bacteroidetes to Firmicutes, ↑ Proteobacteria, ↑ Verrucomicrobia, ↓ overall microbial diversity</td>
<td>Chang et al., 2008</td>
</tr>
<tr>
<td>Fecal samples from n=14 recurrent CDI patients (4 within this group given FMT)</td>
<td>↑ Proteobacteria, ↓ Bacteroidetes, ↓ Firmicutes</td>
<td>Weingarden et al., 2015</td>
</tr>
<tr>
<td>Fecal samples from n=94 CDI, and n=89 non-CDI inpatients</td>
<td>↑ Proteobacteria, ↓ Firmicutes (except Enterococcus and Erysipelotrichia and some Lachnospiraceae)</td>
<td>Schubert et al., 2014</td>
</tr>
<tr>
<td>Fecal samples from n=24 CDI patients, and n=48 healthy age/sex matched controls</td>
<td>↑ Small subset of Bacteroidetes and Firmicutes, no change in bacterial diversity</td>
<td>Manges et al., 2010</td>
</tr>
<tr>
<td>Fecal samples from n=9 recurrent CDI patients given FMT in van Nood et al., 2013 study</td>
<td>↑ Proteobacteria, ↑ Bacilli, ↓ Bacteroidetes, ↓ Firmicutes, ↓ overall microbial diversity compared to post-FMT and healthy donor samples</td>
<td>Fuentes et al., 2014</td>
</tr>
<tr>
<td>Fecal samples from n=22 C. difficile culture-positive elderly subjects, and n=252 healthy elderly subjects</td>
<td>↑ Proteobacteria, Firmicutes (↓ Enterococcaceae and ↑ Lactobacillaceae), ↓ overall microbial diversity</td>
<td>Rea et al., 2012</td>
</tr>
<tr>
<td>Fecal samples from n=39 CDI patients, n=36 C. difficile-negative nosocomial diarrhea patients, and n=40 healthy control subjects</td>
<td>↓ Firmicutes, ↑ Proteobacteria, ↓ Microbial diversity in both diarrheal groups compared to healthy controls</td>
<td>Antharam et al., 2013</td>
</tr>
<tr>
<td>Stool samples from n=6 CDI patients given FMT</td>
<td>↑ Proteobacteria, ↓ Bacteroidetes, ↓ Firmicutes (specifically Lachnospiraceae), ↓ overall microbial diversity</td>
<td>Shahinas et al., 2012</td>
</tr>
<tr>
<td>Fecal samples from n=5 elderly patients with rCDI given FMT compared with non-FMT matched rCDI patients.</td>
<td>No change in Firmicutes, ↑ Proteobacteria,</td>
<td>Girotra et al., 2016</td>
</tr>
</tbody>
</table>

*Phylum level taxonomic changes are listed unless otherwise described.
pathogen, while secondary bile acids are inhibitory. Thus, an antibiotic-induced perturbation that reduces secondary bile acid production can promote CDI (Theriot et al., 2016). A study investigating the metabonomic changes across diarrheal patients found that *C. difficile* colonized patients that were toxin positive (p/p) had increased levels of primary bile acids compared to both *C. difficile* colonized toxin negative (p/n) and non-*C. difficile* colonized control (n/n) patients (Rojo et al., 2015). Interestingly, primary bile acid levels were also increased in the p/n group compared to the n/n group regardless of the presence or absence of previous antibiotic treatment history, suggesting that antibiotic treatment may not be the sole factor in the observed differences of bile acids (Rojo et al., 2015).

Broad-spectrum antibiotic treatment prior to the development of CDI alters other aspects of the colonic metabonome providing a favourable environment for *C. difficile*. Commensal gut microbes that encode sialidases can cleave mucosal carbohydrate moieties on the surface of host epithelial cell glycoproteins, releasing free sialic acid into the lumen (Ng et al., 2013; Sonnenburg et al., 2005) where a diverse range of bacterial species, predominantly from the Firmicutes and Proteobacteria phyla, can consume this host-derived sugar (McDonald et al., 2016; Vimr et al., 2004). As mentioned previously, primary fermenters such as Bacteroidetes can break down complex carbohydrates into succinate, which is normally rapidly consumed by commensal bacteria in the gut (den Besten et al., 2013b; Ferreyra et al., 2014; Fischbach and Sonnenburg, 2011). However, following antibiotic perturbation, free sialic acid and succinate levels in the gut accumulate, and these carbohydrates are not readily consumed because commensal microbes are depleted. In gnotobiotic murine models, *C. difficile* can capitalize on these metabonomic alterations as it can utilize excess sialic acid and succinate to proliferate (Ferreyra et al., 2014; Ng et al., 2013). Rojo et al., 2015 also completed metabonomic analysis.
investigating changes to succinate and sialic acid levels and found increasing levels of both compounds in the p/n, p/p, and n/n groups respectively. These results also suggest that increased levels of succinate and sialic acid are actively consumed by *C. difficile* (even more so by strains actively producing toxin), and that these bacterial-metabolic interactions may be occurring in humans (Rojo et al., 2015).

### 1.4 *C. difficile* growth and virulence factors

It is important to consider the life cycle and virulence factors of *C. difficile* to understand disease pathogenesis within the context of the host GI physiology and the gut microbiome. Sporulation and toxin production are considered the major virulence factors of *C. difficile* and are expanded upon in detail below.

#### 1.4.1 Sporulation

As an obligate anaerobic bacterium, *C. difficile* cannot survive outside the anoxic condition of the gut lumen in the vegetative form. The production of metabolically dormant endospores (spores) allows *C. difficile* to persist and spread in unfavourable environments (Fawley et al., 2007; Paredes-Sabja et al., 2014; Sorg and Sonenshein, 2008). *C. difficile* spores have been shown to resist oxygen, extreme temperatures, desiccation, and chemical disinfectants aiding in their transmission (Dawson et al., 2011b; Fawley et al., 2007; Rodriguez-Palacios and Lejeune, 2011). Spores are therefore the primary infective form. Sporulation is important in the disease pathogenesis of CDI, as spores transmitted via the fecal-oral route can resist the acidity of the stomach, persist in the colon and withstand antibiotic treatments (Baines et al., 2009; Deakin et al., 2012; Howerton et al., 2013; Nerandzic et al., 2009) (Figure 1.4). Once in the small intestine, exposure to primary bile acids and glycine stimulate the germination of spores
Figure 1.4 Pathophysiology of CDI in humans. Spores are primarily transmitted via the fecal-to-oral route in the hospital setting. Ingested spores are able to resist the acidic environment of the stomach where they travel to the small intestine. In a healthy host, an intact microbiota metabolizes primary bile acids into secondary bile acids in the small intestine. Secondary bile acids (e.g. deoxycholate) inhibit the growth of *C. difficile* vegetative cells. However, upon antibiotic treatment, bile acid metabolism is significantly impaired due to the loss of commensal bacterial species. A reduction of secondary bile acids and increase of primary bile acids in the small intestine promotes an environment where *C. difficile* can germinate and colonize the host. Once in the colon, *C. difficile* can produce toxins damaging the intestinal epithelium causing diarrhea and other symptoms commonly associated with CDI. Furthermore, sporulation of *C. difficile* in the colon allows for its persistence in the gut environment and contributes to disease recurrence (Adapted from Sorg and Sonenshein, 2008).
into vegetative cells. If the infecting agent is a toxigenic strain of *C. difficile*, vegetative cells are then able to grow and secrete toxins when secondary bile acid levels are low, as they often are in the antibiotic-perturbed gut (Sorg and Sonenshein, 2008).

Much of what is known about sporulation in *C. difficile* has been derived from mechanistic studies in the spore-forming bacterium *Bacillus subtilis*, and several mechanisms are conserved between these two species (de Hoon et al., 2010; Fimlaid et al., 2013). In axenic culture, sporulation initiation begins when nutrients are limited during stationary phase (Edwards and McBride, 2014) (Figure 1.5). Spo0A, the master transcriptional factor for sporulation, initiates sporulation during nutrient limitation by integrating different environmental signals (Edwards and McBride, 2014). Sensor histidine kinases and SigH (σ^H^) have been shown to positively regulate Spo0A expression, which activate four sporulation-specific sigma factors (σ^F^, σ^E^, σ^G^, σ^K^) coordinating the physiological changes necessary for forespore formation in the mother cell (Fimlaid et al., 2013; Saujet et al., 2011). Spo0A is critical for the regulation of the complex series of events in sporulation – *C. difficile* spo0A mutants are asporogenic, and defective in persistent infection and host-to-host transmission in mice (Deakin et al., 2012). In the presence of nutrients, spo0A gene expression is also repressed by transcription factors involved in metabolic sensing including CodY and CcpA (Antunes et al., 2012; Dineen et al., 2010; Dineen et al., 2007; Nawrocki et al., 2016). As the forespore matures, the mother cell is lysed releasing the metabolically inactive spore into the environment (Fimlaid et al., 2013). Further details of these global metabolic regulators are discussed in section 1.4.3 ii., below, as these proteins are also involved in the regulation of toxin production.
Figure 1.5 Simplified schematic of the sporulation and germination pathways in *C. difficile*. During nutrient limiting conditions, the transcription factor Spo0A is phosphorylation and activated by five sensor histidine kinases. Phosphorylated Spo0A directs the complex genetic regulation and morphological changes necessary for sporulation. Briefly, the activation of $\sigma^H$ creates a positive feedback loop with Spo0A, which activates four sporulation-specific sigma factors ($\sigma^F$, $\sigma^G$, $\sigma^E$, $\sigma^K$) that coordinate asymmetric cell division and engulfment of the forespore by the mother cell. Once the spore has been lysed from the mother cell it can persist in the environment for extended periods of time. During favourable environmental conditions, germination of *C. difficile* spores into vegetative cells can occur. Germination is initiated by taurocholate and glycine. Although no receptor has been identified for glycine, taurocholate is recognized by the CspC receptor encoded by the *cspBAC* locus. Although this process is highly complex and involves hundreds of genes (Dembek et al., 2013), CspC mediates the activation of CspB, which results in cortex degradation by SleC and the subsequent outgrowth of a new vegetative cell. (Adapted from Abt et al., 2016).
1.4.2 Germination

During favourable environmental conditions, germination of *C. difficile* spores into vegetative cells can occur (Figure 1.5). The *cspBAC* gene locus orchestrates germination in *C. difficile* (Cartman and Minton, 2010). Briefly, germination in *C. difficile* is co-stimulated by bile acids such as taurocholate and the amino acid glycine (discussed in detail above) (Sorg and Sonenshein, 2008). The bile acid germinant receptor has been identified (CspC), but the glycine-sensing receptor remains elusive (Francis et al., 2013). By an unknown mechanism, stimulation of CspC activates CspB, which is necessary for downstream activation of the lytic enzyme SleC to mediate the degradation of the spore cortex during germination (Adams et al., 2013; Burns et al., 2010b; Gutelius et al., 2014; Kevorkian et al., 2016).

1.4.3 Toxin production

i. TcdA and TcdB

*CDI* is a bacterial exotoxin-mediated disease. Most virulent *C. difficile* isolates produce and secrete two large exotoxins: toxin A (TcdA) and toxin B (TcdB), which are responsible for many symptoms in CDI (Abt et al., 2016). Toxins A and B are among the largest bacterial toxins known (308 kDa and 270 kDa respectively), and are encoded within a 19.6-kb pathogenicity locus (PaLoc) on the *C. difficile* bacterial chromosome (Dingle et al., 2014; Hammond and Johnson, 1995; von Eichel-Streiber et al., 1996) (Fig 1.6 A). Both TcdA and B are glucosyltransferases that share 47% amino acid homology and contain four structurally homologous domains (Chumbler et al., 2016). Toxins are internalized into colonic epithelial cells through the recognition of different receptors; TcdA binds to carbohydrate moieties while TcdB binds to poliovirus receptor-like 3 (PRL3) and Wnt receptor frizzled family (FZDs) on the apical
surface (LaFrance et al., 2015; Tao et al., 2016). Once inside the epithelial cell, acidification of the endosome promotes a series of events whereby the cysteine protease domain (CPD) and the glucosyl transferase domain (GTD) are translocated into the cytosol. Once in the cytoplasm of target cells, both toxins glycosylate small guanosine triphosphate hydrolysing proteins (GTPases) including Rho, Rac and Cdc42 proteins (Just et al., 1995a; Just et al., 1995b; Voth and Ballard, 2005). Inactivation of these GTPases causes a cascade of cellular events leading to major cytoskeletal changes including the loss of epithelial cell integrity and tight junction function, resulting in cell death, inflammation and diarrheal symptoms common to CDI patients (Meyer et al., 2007; Voth and Ballard, 2005).

Despite similarities in the mechanisms of action of TcdA and B, these toxins exhibit different potencies suggesting that they may play disproportionate roles in CDI pathogenesis (Meyer et al., 2007). Studies aiming at understanding which toxin is more essential for C. difficile virulence in hamsters have given rise to conflicting results (Kuehne et al., 2014; Lyras et al., 2009); however, the current consensus is that both toxins, as well as a third toxin known as C. difficile binary toxin (CDT) are all important in the pathogenesis of CDI (Kuehne et al., 2014).

ii. Regulation of tcdA and tcdB expression in C. difficile

The PaLoc is crucial to the regulation of C. difficile toxins. Variations in this region determine the various toxinotypes of this pathogen (Rupnik, 2008). For example, non-toxigenic (TcdA’B’) isolates have a highly conserved non-coding sequence in place of the PaLoc region (Monot et al., 2015). In toxigenic C. difficile isolates, TcdA and TcdB production is regulated by three proteins encoded on this locus: TcdR, TcdC, and TcdE (see Figure 1.6 A). During late log/early stationary phase of C. difficile in brain heart infusion growth medium, TcdR (an alternative RNA polymerase sigma factor) activates tcdA and tcdB transcription (Hundsberger et
Figure 1.6 Simplified schematic of the loci responsible for toxin production in *C. difficile*. The pathogenicity locus (PaLoc) (A) and binary toxin locus (CdtLoc) (B) are shown. Green arrows indicate positive regulation, whereas red arrows indicate inhibition.
TcdR can also bind its own promoter ($tcdR$) creating a positive feedback loop inducing constitutive toxin production (Mani et al., 2002). It is thought that TcdC represses toxin expression by interfering with TcdR activity (Matamouros et al., 2007), while TcdE forms a holin-like protein allowing for toxin secretion into the extracellular environment (Govind and Dupuy, 2012). Production of TcdR, and thus TcdA and TcdB is dependent on environmental cues including nutrient limitation, temperature and subinhibitory antimicrobial levels. For example, an environmental temperature of 37ºC (physiological temperature), and presence of fluoroquinolone antibiotics or butyrate have each been shown to induce toxin production in vitro (Adams et al., 2007; Karlsson et al., 2003; Karlsson et al., 2000; Saxton et al., 2009) (Figure 1.7). Conversely, increased concentrations of glucose, cysteine, proline, butanol or biotin are known to reduce toxin production (Karlsson et al., 1999; Karlsson et al., 2000; Yamakawa et al., 1996).

Four known global metabolic regulators (Rex, CcpA, PrdR and CodY) and two proteins that are also involved in spore formation (SigH and Spo0A) co-regulate toxin gene expression in C. difficile (Bouillaut et al., 2015; Martin-Verstraete et al., 2016) (Figure 1.7). PrdR is a central metabolic regulatory protein that mediates proline-dependent repression of $tcdA$ during growth in vitro (Bouillaut et al., 2013). When proline is in excess, the expression of proline reductase by PrdR stimulates the reduction of proline coupled to the oxidation of NADH (Bouillaut et al., 2013). Likewise, Rex (redox-dependent transcriptional repressor) is a transcription factor that actively represses transcription of genes required to synthesize butyrate from succinate and acetyl-CoA during low intracellular NADH/NAD$^+$ ratio conditions (Bouillaut et al., 2015). The mechanism by which butyrate triggers toxin production in C. difficile is not understood, and it is counterintuitive, given that a healthy gut microbiota is often associated with butyrate production. Butyrate is, however, produced by vegetative C. difficile cells, and it is possible that the
Figure 1.7 Schematic of *C. difficile* toxin regulation. Various metabolites and regulatory proteins influence toxin A and B synthesis in *C. difficile*. Question marks represent unknown mechanisms of inhibition or activation (Adapted from Bouillaut et al., 2015).
bacterium utilizes a butyrate concentration gradient in addition to other environmental triggers to sense its environment before committing to toxin production, an energetically expensive process.

During exponential phase, CodY (a global transcriptional regulator of metabolism) represses all PaLoc genes mediated through the binding to the promoter of tcdR (Dineen et al., 2007). CodY is highly active when cells can sense increased amounts of branched-chain amino acids in the immediate environment, including isoleucine, leucine, valine and GTP (Dineen et al., 2007). However, in stationary phase, when nutrient limitation occurs, reduction of intracellular levels of amino acids and GTP decrease the ability of CodY to bind the tcdR promoter and, accordingly, tcdR expression is increased (Dineen et al., 2007) (Figure 1.7). This explains why a mixture of nine amino acids was used in experiments to suppress total C. difficile toxin yield (Karlsson et al., 1999). A codY mutant has been shown to have impairments in PaLoc gene repression, and displays overexpression of genes associated with amino acid biosynthesis (leucine, histidine, isoleucine, valine, arginine, glycine), sporulation, protein degradation and fatty acid membrane synthesis in C. difficile 630 (Dineen et al., 2010).

CcpA (a regulator of global transcriptional responses to rapidly catabolizable carbohydrates) mediates glucose-dependent repression of toxin production via interactions with the PaLoc (Antunes et al., 2012). CodY, CcpA and Rex also repress transcription of genes required to synthesize butyrate from succinate and acetyl-CoA, thus repressing butyrate and toxin production (Antunes et al., 2012; Dineen et al., 2010) (Figure 1.7). As mentioned previously, production of CodY and CcpA also represses sporulation (Antunes et al., 2012; Dineen et al., 2010; Nawrocki et al., 2016). All endospore-producing bacteria possess Spo0A, another global transcriptional regulator, which is also involved in initiation of sporulation, virulence, colonization, and metabolism regulation in C. difficile (Pettit et al., 2014). Spo0A is
important for *C. difficile* persistent infection, recurrence, and transmission in murine models (Deakin et al., 2012). Spo0A also represses toxin gene expression in two ribotype 027 isolates but exerts negligible effects on toxin gene expression in ribotype 078 and 012 strains (Mackin et al., 2013). Furthermore, Spo0A is thought to regulate genes involved in bacterial chemotaxis, adherence, and antimicrobial resistance and has been shown to positively regulate glucose fermentation pathways and butyrate production in *C. difficile* (specifically, a *spo0A* mutant produced less butyrate than its parent 630 strain) (Pettit et al., 2014). Spo0A has a reciprocal regulatory role with another sporulation regulatory sigma factor called SigH. SigH not only positively regulates sporulation, but is also implicated in PaLoc gene expression and other *C. difficile* virulence factors including surface adhesins, S-layer protein precursors and flagellar components. For brevity, these factors will not be discussed in detail here since they were not studied as part of this work (Saujet et al., 2011).

iii. *C. difficile* toxin (CDT)

*C. difficile* strains causing increased disease severity and recurrence, including the BI/NAP1/027 isolate, frequently contain CDT (Gerding et al., 2014; Marsh et al., 2012). CDT is part of the family of ADP-ribosylating binary toxins most closely related to *Clostridium perfringens* iota toxins (Gerding et al., 2014) and consist of two separate domains: the active or enzymatic portion (CDTa) and the binding portion (CDTb). Both domains are encoded by *cdtA* and *cdtB* respectively, and these genes reside outside of the PaLoc on a 6.2-kb region known as the CdtLoc (Carter et al., 2007) (Figure 1.6 B).

CDTb is a 98.8 kDa protein that is involved in the binding of CDT to colonic epithelial cells. Once cleaved/activated by serine-type proteases, the large CDTb portion is thought to form heptamers which partly facilitate CDT binding to the lipolysis-stimulated lipoprotein receptor
(LSR) on the host cell surface (Gerding et al., 2014; Gulke et al., 2001; Papatheodorou et al., 2011). After the toxin-receptor complex is internalized by endocytosis, the acidification of the endosome promotes CDTb to initiate membrane insertion and pore formation, translocating the CDTa subunit (~53 kDa) into the cytosol (Barth et al., 2004). Once in the cytosol, the activity of CDTa inhibits actin polymerization through its ADP-ribosylating activity, causing destruction of the actin cytoskeleton (Aktories et al., 2011). Such structural changes promote microtubule protrusions near the cell membrane, which enhance adherence to the epithelial surface promoting colonization of the pathogen in this niche (Schwan et al., 2009).

Interestingly, supernatants derived from TcdA’B’CDT+ C. difficile isolates were shown to cause an inflammatory immune response in rabbit ileal loop models. However, when antibiotic-treated hamsters were colonized with these strains, they were unable to cause diarrhea or death (Geric et al., 2006). These results suggested that CDT might not be sufficient to cause disease symptoms. More recently, it was demonstrated that purified CDT can cause death in both mice and hamsters (Wang et al., 2016) and strains expressing CDT may augment virulence in C. difficile by the suppression of host colonic eosinophils (Cowardin et al., 2016).

iv. Regulation of cdtA and cdtB toxin gene expression in C. difficile

Upstream of the cdtA and B genes is the putative response regulator gene, cdtR (see Figure 1.6 B). Through complementation studies, CdtR was shown to be required for optimal expression of the binary toxin and a deletion within the cdtR gene resulted in severely reduced CDT levels (Carter et al., 2007). In a more recent and comprehensive study, Metcalf et al. sequenced the entire CdtLoc of ten environmental C. difficile isolates and assessed the expression of cdtA and cdtR genes in these strains during growth. Interestingly, truncation mutations were identified in cdtR of 078 ribotype isolates, but cdtA was still expressed in 078
ribotype strains, suggesting that a truncated CdtR is still functional, or that additional proteins exist to regulate CDT activity (Metcalf and Weese, 2011). Furthermore, CdtR may not be restricted to CDT regulation; a recent study demonstrated that CdtR upregulates not only CDT, but also TcdA and TcdB in two 027 ribotype *C. difficile* strains (Lyon et al., 2016). Although the role of CDT in CDI virulence and pathogenesis is incomplete, these studies have provided valuable insight into the genetic and evolutionary differences among *C. difficile* ribotypes, highlighting the importance of assessing multiple and diverse clinical isolates.

In summary, numerous metabolic transcriptional regulatory proteins can integrate and respond to complex environmental signals to tightly control *C. difficile* sporulation and toxin production. As the primary source of energy in *C. difficile* comes from carbohydrates and amino acids, strict regulation of toxin production and sporulation in *C. difficile* provides a potential mechanism of dispersal to inhabit new nutrient-rich environments that may be more beneficial to the overall fitness of this bacterium.

### 1.5 Emergence of *C. difficile* strains associated with severe disease and hospital outbreaks

A ribotype 027 *C. difficile* strain was responsible for the largest reported CDI epidemic to date, which occurred in Quebec, Canada in 2005 (Yakob et al., 2015). *C. difficile* strains, particularly those of the BI/NAP1/027 designation, have been frequently associated with increased antibiotic resistance, severe disease, recurrence, and mortality compared to endemic strains (Barbut et al., 2007; Goorhuis et al., 2008). Although not fully understood, many studies have investigated strain-specific attributes to understand this ‘hypervirulence’ phenomenon.

Specific strains of *C. difficile* have been reported to be more virulent and infectious than their endemic counterparts, but to date there are conflicting results in different studies. Increased
toxin A and B production in ribotype 027 isolates compared to non-027 ribotype strains has been described (Warny et al., 2005). Authors of this and other studies (Carter et al., 2011; Dupuy et al., 2008), have attributed an 18-bp deletion in \textit{tcdC}, to be responsible for the increased toxin production; however, \textit{in vitro} work has suggested TcdC has no impact on toxin expression (Bakker et al., 2012; Cartman et al., 2012), and that these mutations are not solely responsible for the augmentation of virulence traits in these strains (Goldenberg and French, 2011; Stabler et al., 2009). Merrigan et al., 2010 found that toxin production did not differ between tested isolated identified as ‘hypervirulent’, but increased sporulation (rate and total spores) was observed, implicating this virulence determinant as of importance to hypervirulence (Merrigan et al., 2010). Some studies have ascribed increases in both toxin and sporulation levels to hypervirulent strains (Akerlund et al., 2008; Carlson et al., 2013; Vohra and Poxton, 2011). However, comprehensive \textit{in vitro} studies have provided conflicting results regarding sporulation proficiencies in epidemic strains, and have challenged the methodological reliability in many of the above studies (Burns et al., 2010a; Burns and Minton, 2011; Carlson et al., 2013). Interestingly, sequence variation has been described in TcdB isolated from hypervirulent vs. non-hypervirulent strains, which is thought to impact toxin structure, antigenicity, potency and lethality contributing to the virulence properties of these isolates (Lanis et al., 2010; Lanis et al., 2013; Stabler et al., 2009).

Using a human fecal-based bioreactor model and a humanized murine model of infection, 027 strains were shown to have an increased ecological advantage compared to non-hypervirulent clinically relevant ribotypes of \textit{C. difficile} (Robinson et al., 2014). Production of \textit{p}-cresol by \textit{C. difficile} has also been shown to differ in 027 and 012 ribotype strains (Dawson et al., 2011a). As \textit{C. difficile} can tolerate this bacteriostatic compound, increased levels produced
by hypervirulent 027 isolates may also provide increased ecological fitness in the gut environment (Dawson et al., 2011a). However, a recent study using a simulated epidemiological model found that the ability for hypervirulent *C. difficile* strains to outcompete endemic strains present in the host gut is not a primary factor contributing to the observed global spread and clonal dominance of ribotype 027, compared to other properties such as infectivity and rates of severe disease (Yakob et al., 2015).

Recently, ribotype 078 isolates causing CDI have increased in prevalence and have been categorized as hypervirulent. Compared to ribotype 027, the emergence of hypervirulent ribotype 078 has been associated with younger patient populations and community-acquired disease, though overall disease severity was comparable (Goorhuis et al., 2008). The frequent isolation of 078 from livestock (particularly cattle and swine) and other food sources coupled with the high genetic relatedness of 078 isolates between human and animal reservoirs provide evidence to support the community-acquired nature of infection (Costa et al., 2012; Keel et al., 2007; Metcalf et al., 2010b; Weese, 2010). However, a recent study showed that many ribotypes other than 078 are shared between human and animal hosts, suggesting zoonotic transmission is likely not exclusive to this ribotype (Janezic et al., 2012).

Overall, various *C. difficile* ribotypes have been responsible for severe disease and hospital outbreaks around the world (Barbanti and Spigaglia, 2016; Burke and Lamont, 2014), and as it stands, there is no current molecular definition for ‘hypervirulence.’ Thus, the emergence of *C. difficile* strains associated with high disease severity and infection is not ribotype specific. However, given the high genome plasticity of *C. difficile* and large heterogeneity of this species, the study of multiple isolates is important in the context of disease...
management and population-based infection control (Dingle et al., 2014; Monot et al., 2015; Sebaihia et al., 2006; Stabler et al., 2006).

1.6 Treatments for \textit{C. difficile} infection

1.6.1 Standard treatment practices

After an individual is diagnosed with primary CDI, implementation of infection control policies and cessation of the inciting antibiotic is important to reduce diarrheal symptoms and decrease risk of recurrence of disease (Cohen et al., 2010; Hu et al., 2009; Mullane et al., 2011). First-line antibiotic monotherapy consists of oral metronidazole or vancomycin, which kill vegetative \textit{C. difficile}, providing symptomatic relief and allowing for restoration of colonization resistance (Cohen et al., 2010; Debast et al., 2014; Surawicz et al., 2013). Clinical trials have shown comparable efficacy using both metronidazole and vancomycin to treat mild-to-moderate CDI (see Table 1.2 for severity index and treatment recommendations for CDI) (Surawicz et al., 2013; Wenisch et al., 1996; Zar et al., 2007). Traditionally, although metronidazole has been favoured as first-line therapy due to its low cost, vancomycin has been shown to be superior in the prevention of recurrence and for use in severe CDI cases (Johnson et al., 2014).

For treatment of severe CDI, oral vancomycin is currently recommended (Cohen et al., 2010). In approximately 25\% of cases, persistent \textit{C. difficile} (as endospores) or introduction of a new strain can lead to disease recurrence, which is more likely when there is incomplete recovery of the gut microbiota (Cohen et al., 2010). Upon a first relapse, repetition of the initial treatment or treatment with a new antibiotic, for instance, fidaxomicin, is recommended (Debast et al., 2014). Fidaxomicin is a narrow-spectrum bactericidal macrocyclic antibiotic that was approved for CDI by the Food and Drug Administration (FDA) in 2011 (Venugopal and Johnson,
<table>
<thead>
<tr>
<th>Disease severity:</th>
<th>Diagnostic criteria:</th>
<th>Treatment:</th>
<th>Comment:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild-to-moderate</td>
<td>Diarrhea plus symptoms that do not meet the criteria of severe or complicated disease (described below)</td>
<td>Metronidazole (500 mg orally) three times a day for 10-14 days. If unable to take metronidazole, give vancomycin (125 mg orally) four times a day for 10-14 days</td>
<td>If no improvement in 5–7 days, consider change to vancomycin (125 mg orally) four times a day for 10-14 days</td>
</tr>
<tr>
<td>Severe</td>
<td>Serum albumin &lt; 3 g/dL plus either: WBC ≥ 15,000 cells/mm³ or abdominal tenderness</td>
<td>Vancomycin (125 mg orally) four times a day for 10-14 days</td>
<td>If no clinical improvement, increase vancomycin dose to 500 mg</td>
</tr>
</tbody>
</table>
| Severe and complicated | Any of the following:  
- Admission to ICU for CDI  
- Hypotension  
- Fever ≥ 38.5°C  
- Ileus or significant abdominal distention  
- Mental status alterations  
- WBC ≥ 35,000 or < 2,000 cells/mm³  
- Serum lactate levels > 2.2 mmol/L  
- End organ failure (e.g. mechanical ventilation, renal failure, etc.) | Vancomycin (500 mg orally) four times a day and metronidazole (500 mg IV every 8 h, and vancomycin per rectum (500 mg in 500 mL saline as enema) four times a day for 10-14 days | Surgical consultation recommended. Intracolonic vancomycin should be restricted to patients with severe ileus who are non-responsive to oral antibiotic therapy |
| Recurrent CDI | Recurrent CDI within 8 weeks of completion of therapy | Repeat metronidazole or vancomycin pulse treatment regimen or use fidaxomicin (200 mg orally) twice a day for 10 days | Can begin pulse-dosing after second recurrence episode with or without probiotics. Consider FMT after three recurrence episodes |

(Adapted from Surawicz et al., 2013)

Abbreviations: C. difficile infection (CDI), fecal microbiota transplantation (FMT), intensive care unit (ICU), intravenous (IV), and white blood cells (WBC).
In comparison to vancomycin, fidaxomicin has been shown to decrease CDI recurrence, have a greater sustained clinical response and provide extended reduction of *C. difficile* toxin levels (Cornely et al., 2014; Eyre et al., 2014; Lee et al., 2016; Louie et al., 2011; Thabit et al., 2016). Interestingly, lower recurrence rates were not observed after fidaxomicin treatment for BI/NAP1/027 strains (Louie et al., 2011). A recent study has also shown that, in comparison to fidaxomicin, vancomycin causes greater disruption to the gut microbiota (reduction of Firmicutes and increase in Proteobacteria abundances), leading to increased susceptibility of patients to vancomycin-resistant enterococci (VRE) and extended-spectrum-β-lactamase-producing *Klebsiella pneumoniae* (ESBL-Kp) infections (Deshpande et al., 2016).

In summary, management of recurrent CDI (rCDI) can be a major and persistent clinical challenge; patients that are non-responsive to antimicrobial therapy require alternative treatment options (Lapointe-Shaw et al., 2016).

### 1.6.2 Probiotics

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (WHO, 2002). As an alternative treatment for CDI, it is proposed that probiotics may act through one or more mechanisms: direct antagonism against *C. difficile*; protection of the epithelial barrier; ability to reinstate microbial diversity, and regulation of innate and adaptive immune responses within the gut (Ollech et al., 2016).

Although probiotics are generally regarded as safe and are normally well tolerated, the study of their usefulness as treatment for CDI is plagued by the lack of mechanistic data, standardization of administration, and agreement between randomized clinical trials (RCTs)
Thus, probiotics are not currently recommended for the treatment of CDI (Cohen et al., 2010; Debast et al., 2014).

*Lactobacillus rhamnosus, Saccharomyces boulardii, and select multistrain preparations containing lactobacilli and bifidobacteria have each been tested in clinical trials for use in the prevention and treatment of antibiotic-associated CDI (Allen et al., 2013; Ouwehand et al., 2014; Pochapin, 2000; Surawicz et al., 2000; Thomas et al., 2001). However, these studies have largely reported conflicting results in terms of the levels of success and there is limited evidence of the direct benefits of probiotics for rCDI cases (Evans and Johnson, 2015). The yeast probiotic Saccharomyces boulardii has been the most extensively studied for rCDI prevention (McFarland et al., 1994; Surawicz et al., 2000), though no decrease in recurrence resulted after administration of this probiotic alone. Conversely, S. boulardii is thought to be beneficial in adjunct therapy with vancomycin for rCDI (Surawicz et al., 2000) possibly through its ability to interfere with or degrade C. difficile toxins (Figure 1.3 B), transiently colonize the antibiotic-cleared niche (Castagliuolo et al., 1999; Pothoulakis et al., 1993) and restore intestinal SCFA levels (More and Swidsinski, 2015). Although adverse event reporting in probiotic trials is poorly documented, reports of fungemia after S. boulardii use has been described, cautioning its use (Cohen et al., 2010).

In 1987, Seal and colleagues cured two recurrent CDI patients using a defined non-toxigenic strain of C. difficile (Seal et al., 1987). More recently, a non-toxigenic C. difficile spore-based probiotic was shown to prevent CDI recurrence in a phase II RCT (Gerding et al., 2015). The underlying mechanism by which this non-toxigenic strain reduces rCDI is unknown; however, it may potentially inhabit the same physical or metabolic space that toxigenic C. difficile would normally occupy, thereby hindering pathogen establishment (Gerding et al., 2015).
As for most probiotics, the ability of non-toxigenic *C. difficile* to colonize the gut of rCDI patients is transient, although long enough to inhibit *C. difficile* expansion and toxin production, providing a window where the normal microbiota can recover and presumably reinstate colonization resistance (Gerding et al., 2015).

Given the unique pathogenesis of *C. difficile*, it has been postulated that only a multistrain probiotic mixture emulating a healthy gut microbiota may confer protection against recurrence and provide clinical resolution of CDI (Hell et al., 2013). The success of multistrain probiotic formulations and stool-based therapeutics for rCDI support this hypothesis.

1.6.3 Modulation of the gut microbiome using stool-based therapeutics

In Table 1.3, we list representative examples of live microbial preparations and microbial-based products that are currently being developed with therapeutic manipulation of the gut microbiome or its associated host interactions in mind. Fecal-microbiota derived products, probiotic, prebiotic, and targeted molecule therapeutics have been developed for a variety of indications. In the following section, we specifically discuss the use of complex stool-based therapeutics for rCDI.

1.6.4 Fecal microbiota transplantation (FMT)

FMT aims to restore gut microbiota diversity by transferring feces from a healthy donor to a sick patient. The therapy has been extensively used in the treatment of rCDI with good success, likely because the donated gut microbial ecosystem can replace the microbiota lost through antibiotic use and thus suppress *C. difficile* overgrowth, promoting patient recovery (Seekatz et al., 2014) (see Figure 1.3 B). Recent studies have demonstrated effectiveness of FMT for clinical cure of rCDI around 90% (Agrawal et al., 2016; Kelly et al., 2016; van Nood et al.,
<table>
<thead>
<tr>
<th>Active agent</th>
<th>Presumed mechanism of action</th>
<th>Indication</th>
<th>Research status</th>
<th>Company/innovator</th>
<th>Reference/clinical trial identifier*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stool</td>
<td>Restoration of colonization resistance through FMT</td>
<td>CDI</td>
<td>Non-profit stool bank, screening, and FMT product development</td>
<td>OpenBiome</td>
<td>Kazerouni et al., 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stool bank and screening</td>
<td>Advancing Bio</td>
<td>AdvancingBio, 2016</td>
</tr>
<tr>
<td>RBX2660</td>
<td>FMT-derived bacteria to restore gut microbiota</td>
<td>CDI, IBD</td>
<td>Phase II RCT complete for CDI</td>
<td>Rebiotix Inc.</td>
<td>NCT01925417</td>
</tr>
<tr>
<td>MET-1</td>
<td>33 strain defined bacterial ecosystem that restores colonization resistance</td>
<td>Recurrent CDI</td>
<td>Proof-of-principle clinical study</td>
<td>NuBiyota</td>
<td>Petrof et al., 2013</td>
</tr>
<tr>
<td>SER-109</td>
<td>Purified bacterial spore-based ecological formulation to restore gut microbiota after antibiotic treatment</td>
<td>Recurrent CDI</td>
<td>Missed primary endpoint in phase II RCT</td>
<td>Seres Therapeutics Inc.</td>
<td>Khanna et al., 2016; NCT02437487</td>
</tr>
<tr>
<td>SER-287</td>
<td>Mild-to-moderate UC</td>
<td></td>
<td>Phase I RCT</td>
<td></td>
<td>NCT02618187</td>
</tr>
<tr>
<td>VE-202</td>
<td>Oral formulation of live Clostridial bacteria that restore immune system homeostasis (increase regulatory T cells)</td>
<td>IBD</td>
<td>Pre-clinical</td>
<td>Vedanta</td>
<td>Atarashi et al., 2013; Furusawa et al., 2013</td>
</tr>
<tr>
<td>CBM588</td>
<td><em>Clostridium butyricum</em> bacterial probiotic</td>
<td>Pediatric AAD, UC with pouchitis</td>
<td>Phase II RCT</td>
<td></td>
<td>Seki et al., 2003</td>
</tr>
<tr>
<td>Lactin-V</td>
<td><em>Lactobacillus crispatus</em> bacterial probiotic</td>
<td>Bacterial vaginosis, Recurrent UTIs</td>
<td>Phase II RCT complete</td>
<td>Osel Inc.</td>
<td>NCT00635622; Hemmerling et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CVD and obesity</td>
<td>Phase II RCT complete</td>
<td>OptiBiotix Health PLC</td>
<td>NCT00305227; Stapleton et al., 2011</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>Probiotic to to modulate the gut microbiome</td>
<td></td>
<td>Phase II RCT complete</td>
<td></td>
<td>Patent #: WO2015067948A1</td>
</tr>
</tbody>
</table>
## Table 1.3 Select gut microbiome-based therapies in clinical or pre-clinical testing

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Description</th>
<th>Disease(s)</th>
<th>Stage</th>
<th>Sponsor/Institution</th>
<th>Registry Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNDO-201</td>
<td><em>Trichuris suis</em> ova, nonpathogenic helminth probiotic to modulate immune system</td>
<td>Crohn’s disease</td>
<td>Missed primary endpoints in two phase II RCTs</td>
<td>Fortress Biotech</td>
<td>NCT01576471; NCT01279577</td>
</tr>
<tr>
<td>Blautix</td>
<td>Proprietary live bacterial therapeutic</td>
<td>IBS</td>
<td>Phase I RCT complete</td>
<td>4D Pharma Research Ltd.</td>
<td>NCT02704728; Eudract 2014-005666-29</td>
</tr>
<tr>
<td>Thetanix</td>
<td>Anti-inflammatory protein derived from <em>Bacteroides thetaiotaomicron</em></td>
<td>Pediatrics Crohn’s disease</td>
<td>Phase I RCT</td>
<td>4D Pharma Research Ltd.</td>
<td>NCT02704728; Eudract 2014-005666-29</td>
</tr>
<tr>
<td>SYN-004</td>
<td>Targeted molecule that degrades IV ß-lactam antibiotics specifically in gut to protect microbiome</td>
<td>CDI, antibiotic-resistant infections and AAD</td>
<td>Phase II RCT</td>
<td>Synthetic Biologics Inc.</td>
<td>NCT02563106</td>
</tr>
<tr>
<td>Avidocins</td>
<td>Genetically modified R-type bacteriocins that have specific antibacterial activity</td>
<td>CDI, foodborne pathogens</td>
<td>Pre-clinical</td>
<td>AvidBiotics</td>
<td>Gebhart et al., 2015; Scholl et al., 2012, 2009</td>
</tr>
<tr>
<td>AG014</td>
<td>Genetically modified <em>Lactococcus lactis</em> probiotic strain that secretes anti-inflammatory factors</td>
<td>IBD</td>
<td>Phase I RCT complete</td>
<td>Intrexon</td>
<td>Vandenbroucke et al., 2010</td>
</tr>
<tr>
<td>SHP-01</td>
<td>Narrow-spectrum lysin (antimicrobial enzyme) specifically targeting C. difficile</td>
<td>Acute and recurrent CDI</td>
<td>Pre-clinical</td>
<td>Symbiotic Health</td>
<td>Hirsch et al., 2015; Wang et al., 2015</td>
</tr>
<tr>
<td>VT301</td>
<td>Genetically engineered lactic acid bacterial probiotic that produce elafin to heal gut epithelial lining</td>
<td>IBD</td>
<td>Pre-clinical</td>
<td>ViThera Pharmaceuticals</td>
<td>Motta et al., 2011</td>
</tr>
<tr>
<td>SGM-1019</td>
<td>Small molecule inhibitor that modulates host-microbiota interactions</td>
<td>IBD</td>
<td>Phase I RCT complete</td>
<td>Second Genome</td>
<td>Ratner, 2015</td>
</tr>
<tr>
<td>NM504/505</td>
<td>Modulating the gut microbiome using prebiotics to improve glucose tolerance and other metabolic parameters</td>
<td>Metformin-intolerant type 2 diabetes</td>
<td>Phase 0 RCT</td>
<td>MicroBiome Therapeutics</td>
<td>NCT01866462; Burton et al., 2015</td>
</tr>
</tbody>
</table>

*Applicable clinical trial registry numbers or primary literature references are included where publicly available.

**Abbreviations:**
Antibiotic-associated diarrhea (AAD), *Clostridium difficile* infection (CDI), cardiovascular disease (CVD), fecal microbiota transplant (FMT), inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), randomized controlled clinical trial (RCT), ulcerative colitis (UC), urinary tract infection (UTI).
2013; Youngster et al., 2016; Youngster et al., 2014) and FMT is now recommended for multiple recurrent CDI nonresponsive to conventional antibiotic therapy (HQO, 2016; Lapointe-Shaw et al., 2016; Merlo et al., 2016). Although the failure rate for this treatment modality is low, failure of a first FMT is strongly associated with previous history of CDI-related hospitalization events, pre-existing IBD, being an inpatient while receiving FMT, or severe/complicated rCDI status (Fischer et al., 2016; Khoruts et al., 2016).

Large compositional changes in gut microbiota occur after FMT. Specifically, decreases in Proteobacteria, and increases in Firmicutes and Bacteroidetes have been widely observed in CDI patients following FMT (Fuentes et al., 2014; Hamilton et al., 2013; Kelly et al., 2016; Khoruts et al., 2010; Li et al., 2016; Seekatz et al., 2014; Shahinas et al., 2012; van Nood et al., 2013; Weingarden et al., 2015) (see Table 1.1). Post-FMT fecal samples have also been shown to contain increased numbers of Lachnospiraceae compared to pre-FMT samples, and decreased levels of potentially pathogenic bacteria including *Proteobacteria (Enterobacter aerogenes and Escherichia coli)*, *Enterococcus*, *Lactobacillus* and *Veillonella* species (Fuentes et al., 2014; Hamilton et al., 2013; Khoruts et al., 2010; Shahinas et al., 2012).

It has been reported that post-FMT microbial ecosystem compositions are highly similar to donor samples following FMT (Hamilton et al., 2013; Weingarden et al., 2015), and donor strains were shown to durably colonize and establish themselves alongside or in place of the pre-existing microbiota in FMT recipients after 3 months (Li et al., 2016). Furthermore, this study demonstrated that strain-specific dominance in FMT recipients is influenced in part by the source of donor strains and by pre-existing recipient community, suggesting that similar to allogenic organ transplantation, gut microbiome compatibility between FMT donor and recipient may be important for a successful clinical response (Li et al., 2016).
Given the high compositional variability in donor material, but widespread success of FMT for CDI, it has been proposed that restoration of functional imbalances rather than the replacement of specific taxa *per se* may determine treatment success. Butyrate-producing bacteria, mainly from Ruminococcaceae and Lachnospiraceae families, are depleted in CDI (Antharam et al., 2013), and the observation that FMT can restore SCFA short-chain fatty acid production by the gut microbiota (Fuentes et al., 2014) has encouraged the investigation of metabonomic changes in the gut microbiome post-FMT. In one study, post-FMT patient metabolome profiles (derived from fecal samples) shifted from their pre-treatment state to closely resemble their donor metabolome counterparts (Weingarden et al., 2014). Increased amounts of secondary bile acids were found to be the main FMT-induced metabolic change, suggesting that restoring bile acid composition with FMT may play an important role in treating rCDI (Weingarden et al., 2014).

Although largely promising, the use of stool as medicine, albeit from healthy donors, is not without problems. Pathogen transmission, and/or unintended alterations in metabolic function may occur as a result of FMT. For example, a normal-weight rCDI patient developed obesity (BMI > 30) after receiving FMT from a healthy but slightly overweight (BMI = 26.4) fecal donor (Alang and Kelly, 2015). Furthermore, the transfer of stool from monozygotic or dizygotic twins discordant for obesity into ‘humanized’ germ-free mice resulted in increased adiposity and the transmission of obesity-associated metabolic phenotypes, corroborating these findings (Ridaura et al., 2013). Less pressing, but equally challenging, is the regulation of stool-based therapies. Because fecal material is extremely complex, containing living microbes, microbial metabolites, host cells, and food particles; it is not inert and thus cannot be standardized as a drug in terms of pharmacokinetics, pharmacodynamics, or consistency of
chemical composition. Accordingly, the FMT regulatory landscape poses one of the biggest obstacles to efficient delivery of FMT to rCDI patients, as well as to the exploration of potential uses of FMT for other diseases. While there are some companies that currently fulfill the needs of physicians by providing pathogen-screened stool products from selected, healthy individuals (see Table 1.3), such products cannot be standardized and do not fulfill the criteria for acceptance as a biologic drug. There is thus a pressing need for the development of an effective, microbiota-based, defined ‘drug’ to ultimately replace FMT to minimize risk to patients and facilitate rapid treatment options.

1.6.5 Stool-substitute therapies

Stool-substitute therapies aim to combine the high-effectiveness of FMT with the excellent safety-profile of probiotics to mitigate many of the concerns of each respective therapeutic option. To date, this approach has been used only for the treatment of rCDI. Stool-substitute therapies use defined, standardized preparations of stool-derived products, while retaining the compositional, metabolic and transcriptomic properties of fecal communities. Tvede and Rask-Madsen were among the first to use a stool-derived microbial consortium of 10 bacterial strains to cure six patients with rCDI (Tvede and Rask-Madsen, 1989). However, at the time of the study, rCDI was considered an uncommon complication of antibiotic use and no real progress was made in the field for almost three decades.

More recently, gut microbiota constituents have been used to develop defined microbial ecosystem formulations for use in murine CDI models. In one study, a six-strain murine bacterial community was shown to influence microbial community structure, promote the resolution of CDI, and decrease \textit{C. difficile} transmission in this animal infection model (Lawley et al., 2012). A second study used a more targeted approach by screening human and murine intestinal
bacteria for predicted inhibitory activity against *C. difficile*. Buffie et al., 2014 used a four-strain formulation consisting of *Clostridium scindens*, *Barnesiella intestihominis*, *Pseudoflavonifractor capillosus*, and *Blautia hansenii*, as well as a single-species formulation of *C. scindens* alone, to investigate whether engraftment of these bacteria could resolve CDI in mice. The authors found that both formulations could impact resistance to *C. difficile* through antagonistic mechanisms mediated by the production of secondary bile acids (Buffie et al., 2015).

In a small proof-of-principle trial our group demonstrated a successful cure of two rCDI patients, using a stool-substitute preparation called RePOOPulate or microbial ecosystem therapeutic-1 (MET-1) (Petrof et al., 2013). This treatment was composed of 33 purified bacterial strains, which had been derived from a single, healthy donor (Petrof et al., 2013). As for FMT, the microbiota profiles of the treated patients indicated incorporation of the MET-1 composition strains after a single administration of the therapeutic. Signatures identifying with MET-1 strains were present in the patients 6 months post-treatment, suggesting that a subset was able to colonize the recipients; this sets MET-1 apart from conventional probiotics assessed to date, and highlights the therapeutic potential representative of a multi-species microbial ecosystem (Petrof et al., 2013). A company called Seres Therapeutics Inc. has also developed stool-substitute therapies for rCDI with a varied approach by using a spore-based microbial ecosystem formulation (Khanna et al., 2016). Results from their first RCT with product SER-109 appeared promising; however, interim results from their phase II RCT have been largely disappointing (Seres Therapeutics, 2016). In general, as with FMT, the utility of stool-substitute therapies for treatment of rCDI requires further research to understand, and capitalize upon, their mechanisms of action.
1.7 Research rationale

Many cases of CDI are non-responsive to traditional antibiotic treatments, and often patients suffer from recurrent infections characterized by severe diarrhea and colonic inflammation. As CDI has been associated with recent epidemiological outbreaks, high patient morbidity and increased healthcare costs, novel therapeutics for this condition are desperately needed. Fecal bacteriotherapy is as an alternative, highly effective treatment for rCDI; however, it is not without risk, and is typically reserved for severe cases. To circumvent safety issues associated with administration of undefined microbes from donor stool, the Allen-Vercoe laboratory has developed a defined microbial ecosystem therapeutic (MET-1) composed of a defined and standardized stool-derived microbial community. MET-1 has been shown to cure two patients with rCDI in a proof-of-principle trial and mitigate many of the issues surrounding FMT. However, the precise mechanisms through which health is restored in rCDI patients after MET-1 treatment are largely unknown. Recently, MET-1 has been shown to provide protection against \textit{C. difficile} in a murine model of colitis, and the effects mediated through the inhibition of \textit{C. difficile} TcdA (Martz et al., 2016). However, whether MET-1 can affect the production of TcdB and CDT in \textit{C. difficile} is unknown. Furthermore, the effects of MET-1 on \textit{C. difficile} sporulation and spore outgrowth have yet to be explored.

Chemostats inoculated with pooled fecal communities have previously been used to assess the impacts of healthy and antibiotic-disturbed microbial communities on \textit{C. difficile} germination, growth, toxin production and sporulation (Baines et al., 2008; Freeman et al., 2005; Freeman et al., 2012). Since MET-1 could cure rCDI, we wanted to investigate the influence of this defined microbial ecosystem on \textit{C. difficile} in a similar manner. Defined microbial ecosystems have been previously used to represent simplified versions of more complex fecal
communities (Natividad et al., 2015; Yen et al., 2015). Although the metabonomic profiles (functionality) are dissimilar between defined and fecal microbial ecosystems within the chemostat model, defined microbial ecosystems are useful surrogates to explore various ecological principles, especially in the context of host GI disease states (Yen et al., 2015). To our knowledge, the effects of various perturbations on the taxonomic composition and metabonomic profiles of healthy defined microbial ecosystems have yet to be examined in the context of CDI. Furthermore, the effects of metabolites from healthy and perturbed defined microbial ecosystems supported by chemostat vessels have not been explored on *C. difficile* growth and virulence.

As the mechanisms behind the effectiveness of stool-derived therapies for rCDI are unravelled, many findings emphasize the importance of a robust gut microbiome to host health. Overall, this thesis presents work that investigated the mechanisms behind the success of microbial ecosystem therapeutics, including MET-1, for the treatment of CDI. Specifically, the compositional and mechanistic properties of defined gut microbial ecosystems and their in vitro effects on *C. difficile* growth and virulence were examined. Results from this study should impact our understanding of the gut microbiome in the context of CDI and provide directions for the continued development and optimization of stool-substitute therapies for this indication.

**Chapter 2** covers the methodology for the work performed throughout this thesis, including the development of healthy and perturbed defined microbial ecosystems. Assays used to investigate the effects of cell-free defined microbial ecosystem supernatants on *C. difficile* growth and virulence were described.

In **Chapter 3**, the taxonomic composition of two representative healthy and perturbed defined microbial ecosystems supported by an *ex vivo* human gut model were investigated, and a
preliminarily assessment of ecosystem functional profiles were achieved using a targeted metabonomic approach.

In chapter 4 the representative healthy and perturbed defined microbial ecosystems characterized in the previous chapter were investigated for their ability to modulate the growth and virulence of *C. difficile*.

In chapter 5 a summary of the work presented in this thesis was provided and several methodological limitations were discussed. Lastly, continued research expanding on this work was suggested and the development and optimization of future stool-substitute therapeutics are considered.
Chapter 2: Materials and Methods

2.1 Defined microbial ecosystem development and preparation

2.1.1 Development of defined microbial ecosystems

A detailed explanation of the protocol for culturing microorganisms from donor stool has been previously described (Petrof et al., 2013). Briefly, stool from one healthy 41-year old female donor was extensively cultured. The selected donor had a healthy lifestyle, normal BMI, and experienced very few antibiotic exposures in childhood and within the last ten years. Strains isolated from donor stool were purified by repeated plate subculture under strict anaerobic conditions at 37ºC and subsequently stored at -80ºC. Genomic DNA was extracted from all isolates using the Maxwell® 16 DNA purification kit (Promega, Madison, Wisconsin, USA) and identified by 16S rRNA gene sequencing using V3kl and V6r primers (Table 2.1) under the following cycling conditions: 94ºC for 2 min, 94ºC for 30 s, 60ºC for 30 s, and 72ºC for 30 s repeated for 30 cycles, followed by a final elongation at 72ºC for 5 min.

2.1.2 Preparation of defined microbial ecosystems

Purified strains were individually cultured on fastidious anaerobe agar (Lab M Ltd. Heywood, Lancashire, UK) with or without 5% defibrinated sheep’s blood (Hemostat Laboratories, Dixon, California) under anaerobic conditions at 37ºC for 48 h. Bacterial isolates specific to each respective community (Tables 2.2 and 2.3) were combined and resuspended in 50 mL of pre-reduced 0.9% saline before inoculation into a single-staged chemostat vessel (Infors, Switzerland). MET-1 and DEC58 communities were constructed from a subset of strains isolated from donor stool. Briefly, strains within the MET-1 ecosystem were highly and reproducibly culturable and selected to represent a balanced ecosystem from healthy individuals.
<table>
<thead>
<tr>
<th>Name:</th>
<th>Amplicon size (bp):</th>
<th>Primer concentration (nM):</th>
<th>Primer Sequence (5’-3’):</th>
<th>Reference:</th>
</tr>
</thead>
<tbody>
<tr>
<td>V3kl/V6r 16S rRNA</td>
<td>~735</td>
<td>500</td>
<td>F’-TACGG[AG]AGGCAGCAG R’-AC[AG]ACACGAGCTGACGAC</td>
<td>Gloor et al., 2010</td>
</tr>
<tr>
<td>V4 region 16S rRNA</td>
<td>~253</td>
<td>500</td>
<td>F’-GTGCCAGCMGCCGCGGTAA R’-GGACTACHVGGGTWTCTAAT</td>
<td>Kozich et al., 2013</td>
</tr>
<tr>
<td>Ribotyping</td>
<td>N/A (variable patterns)</td>
<td>500</td>
<td>F’-GTGCGGCTGGATCACCTCCT R’-CCCTGCACCCCTTAATAACCTTGACC</td>
<td>Bidet et al., 1999</td>
</tr>
<tr>
<td>Toxinotyping B1</td>
<td>~3100</td>
<td>150</td>
<td>F’-AGAAAAATTTTATGAGTTTAGTTAATAGAAA R’-CAGATAATGTAGGAAAGTAGCTATAG</td>
<td>Rupnik et al., 1997</td>
</tr>
<tr>
<td>Toxinotyping A3</td>
<td>~3100</td>
<td>150</td>
<td>F’-GGAGGTTTTATGTCTTTAATATCTAAAGA R’-CCCTCTGTATTGTAGGTAGCATTTA</td>
<td>Rupnik et al., 1997</td>
</tr>
</tbody>
</table>
Furthermore, all isolates included in MET-1 displayed favourable antimicrobial resistance profiles, and were shown to be effective in a proof-of-principal human trial for rCDI.

To make a community depleted of Lachnospiraceae, DEC58 isolates identified as part of the Lachnospiraceae bacterial family according to the All Species Living Tree (Release LTPs123, September 2015, www.arb-silva.de/projects/living-tree) (Munoz et al., 2011; Yarza et al., 2008) were excluded from this defined microbial ecosystem inoculum (Table 2.2). The ciprofloxacin-treated DEC58 community was created by culturing the ecosystem in chemostat vessels and pulse-dosing with 137.5 mg of ciprofloxacin (dissolved in a 0.1 N HCl solution, 5.5 mL of 25 mg/mL) every 12 h starting at day 7 post-inoculation. The concentration of ciprofloxacin delivered to the vessel after each dose was 0.344 mg/mL. Samples for subsequent 16S rRNA analysis (see section 2.2.2) were directly taken from the vessel immediately following the administration of each ciprofloxacin dose.

The development and operation of bioreactor (chemostat) vessels and culture medium to model communities derived from human fecal ecosystems have been described elsewhere (McDonald et al., 2013). Briefly, a continuous-culture bioreactor system was used to support the growth of each microbial ecosystem. Culture vessels were maintained under anaerobic conditions through the continuous sparging of N2 gas, and held at 37°C and a pH of 6.9-7.0 to mimic the conditions of the human distal gut. The culture medium was added at a rate of 400 mL/day to give a retention time of 24 h, and contents were gently stirred to ensure even nutrient dispersion. Chemostat vessel contents were sampled daily and harvested 9 days post-inoculation. Immediately following the chemostat run, vessel contents were ultracentrifuged at 92,387 \times g for 2 h at 4°C and the clarified supernatants were filtered through a 0.22 μm sterile filter. The resulting, cell-free supernatants from these defined microbial ecosystems were stored at -20°C.
Table 2.2 List of cultured bacterial isolates used to generate the DEC58 and DEC58-Lachnospiraceae communities used in this study

<table>
<thead>
<tr>
<th>Internal strain ID:</th>
<th>% identity:</th>
<th>Phylum:</th>
<th>Family:</th>
<th>*Closest species match:</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 FMU</td>
<td>99</td>
<td>Verrucomicrobia</td>
<td>Akkermansiaceae</td>
<td>Akkermansia muciniphila</td>
</tr>
<tr>
<td>32-6-S 28 TSA</td>
<td>100</td>
<td>Firmicutes</td>
<td>Acidaminococcaceae</td>
<td>Acidaminococcus intestini</td>
</tr>
<tr>
<td>16-6-I 18 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Agathobacter rectalis</td>
</tr>
<tr>
<td>16-6-I 1 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Agathobacter rectalis</td>
</tr>
<tr>
<td>16-6-I 29 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Agathobacter rectalis</td>
</tr>
<tr>
<td>16-6-I 6 FM</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Agathobacter rectalis</td>
</tr>
<tr>
<td>32-6-S 17 FAA</td>
<td>100</td>
<td>Bacteroidetes</td>
<td>Rikenellaceae</td>
<td>Alistipes finegoldii</td>
</tr>
<tr>
<td>32-6-I 29 FAA</td>
<td>100</td>
<td>Bacteroidetes</td>
<td>Rikenellaceae</td>
<td>Alistipes putredinis</td>
</tr>
<tr>
<td>32-6-I 10 FAA NB</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Anaerostipes hadrus</td>
</tr>
<tr>
<td>32-6-I 11 MRS</td>
<td>99</td>
<td>Actinobacteria</td>
<td>Atopobiaceae</td>
<td>Atopobium minutum</td>
</tr>
<tr>
<td>32-6-S 1 D5 FAA AER</td>
<td>100</td>
<td>Firmicutes</td>
<td>Bacillaceae</td>
<td>Bacillus muralis</td>
</tr>
<tr>
<td>32-6-I 19 FAA NB</td>
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<td>Bacteroidaceae</td>
<td>Bacteroides caccae</td>
</tr>
<tr>
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<td>Bacteroidetes</td>
<td>Bacteroidaceae</td>
<td>Bacteroides fragilis</td>
</tr>
<tr>
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<td>Bacteroidaceae</td>
<td>Bacteroides fragilis (dorei)</td>
</tr>
<tr>
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<td>Bacteroidaceae</td>
<td>Bacteroides ovatus</td>
</tr>
<tr>
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<td>Bacteroidetes</td>
<td>Bacteroidaceae</td>
<td>Bacteroides vulgatus</td>
</tr>
<tr>
<td>32-6-I 1 TSA</td>
<td>99</td>
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<td>Bifidobacteriaceae</td>
<td>Bifidobacterium adolescentis</td>
</tr>
<tr>
<td>32-6-S 24 FAA</td>
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<td>Actinobacteria</td>
<td>Bifidobacteriaceae</td>
<td>Bifidobacterium longum</td>
</tr>
<tr>
<td>32-6-I 11 D6 FAA</td>
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<td>Actinobacteria</td>
<td>Bifidobacteriaceae</td>
<td>Bifidobacterium pseudocatenulatum</td>
</tr>
<tr>
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<td>Lachnospiraceae</td>
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<td>Lachnospiraceae</td>
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<tr>
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<td>Lachnospiraceae</td>
<td>Blautia stercoris</td>
</tr>
<tr>
<td>16-6-I 11 FM</td>
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<td>Lachnospiraceae</td>
<td>Blautia wexlerae</td>
</tr>
<tr>
<td>32-6-I 2 FAA NB</td>
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<td>Firmicutes</td>
<td>Clostridiaceae</td>
<td>ButyrivibrioH pullicaecorum</td>
</tr>
<tr>
<td>32-6-I 24 FAA NB</td>
<td>99</td>
<td>Firmicutes</td>
<td>Veillonellaceae</td>
<td>Candidatus ‘Dielma fastidiosa’</td>
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<tr>
<td>16-6-S 15 LS</td>
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<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Clostridium aldenense</td>
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<tr>
<td>16-6-S 4 LS</td>
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<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Clostridium citroniae</td>
</tr>
<tr>
<td>32-6-S 28 D6 FAA</td>
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<td>Firmicutes</td>
<td>**Lachnospiraceae</td>
<td>Clostridium lactatifementans</td>
</tr>
<tr>
<td>32-6-I 30 D6 FAA</td>
<td>97</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Clostridium oroticum</td>
</tr>
<tr>
<td>32-6-S 4 CNA</td>
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<td>Lachnospiraceae</td>
<td>Clostridium scindens</td>
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<tr>
<td>32-6-I 7 MET</td>
<td>94</td>
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<td>Erysipelotrichaceae</td>
<td>Clostridium sp.</td>
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<tr>
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<td>100</td>
<td>Actinobacteria</td>
<td>Coriobacteriaceae</td>
<td>Collinsella aerofaciens</td>
</tr>
<tr>
<td>32-6-I 16 NA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Coprococcus comes</td>
</tr>
</tbody>
</table>
Table 2.2 List of cultured bacterial isolates used to generate the DEC58 and DEC58-Lachnospiraceae communities used in this study

<table>
<thead>
<tr>
<th>Internal strain ID:</th>
<th>% identity</th>
<th>Phylum:</th>
<th>Family:</th>
<th>*Closest species match:</th>
</tr>
</thead>
<tbody>
<tr>
<td>32-6-I 1 BHI AER</td>
<td>99</td>
<td>Actinobacteria</td>
<td>Corynebacteriaceae</td>
<td>Corynebacterium aurimucosum</td>
</tr>
<tr>
<td>32-6-I 16 TSA</td>
<td>98</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Dorea formicigeners</td>
</tr>
<tr>
<td>32-6-I 1 NA</td>
<td>98</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Dorea longicatena</td>
</tr>
<tr>
<td>32-6-I 6 NA</td>
<td>96</td>
<td>Firmicutes</td>
<td>Eggerthellaceae</td>
<td>Eggerthella lenta</td>
</tr>
<tr>
<td>16-6-S BF 7</td>
<td>99</td>
<td>Proteobacteria</td>
<td>Enterobacteriaceae</td>
<td>Enterobacter aerogenes</td>
</tr>
<tr>
<td>32-6-S 14 BHI</td>
<td>100</td>
<td>Proteobacteria</td>
<td>Enterobacteriaceae</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>16-6-I F1 FAA</td>
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<td>Lachnospiraceae</td>
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<td>Eubacterium fissicatena</td>
</tr>
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<td>Lachnospiraceae</td>
<td>Eubacterium hallii</td>
</tr>
<tr>
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<td>Eubacteriaceae</td>
<td>Eubacterium limosum</td>
</tr>
<tr>
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<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Eubacterium ventriosum</td>
</tr>
<tr>
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<td>Firmicutes</td>
<td>Ruminococcaceae</td>
<td>Faecalibacterium prausnitzii</td>
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<tr>
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<td>unclassified_Clostridiales</td>
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</tr>
<tr>
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<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Hungatella hathewayi</td>
</tr>
<tr>
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<td>Lachnospira pectinoschiza</td>
</tr>
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<td>Lactobacillaceae</td>
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<tr>
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<td>Lachnospiraceae</td>
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<td>Porphyromonadaceae</td>
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<tr>
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<td>Bacillaceae</td>
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<td>Lachnospiraceae</td>
<td>Roseburia faecis</td>
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<tr>
<td>16-6-I 31 FAA</td>
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<td>Lachnospiraceae</td>
<td>Roseburia intestinalis</td>
</tr>
<tr>
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<td>Staphylococcaceae</td>
<td>Staphylococcus capitis</td>
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<tr>
<td>32-6-S 3 D6 FAA</td>
<td>95</td>
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<td>Staphylococcaceae</td>
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<tr>
<td>32-6-S 2MET</td>
<td>97</td>
<td>Proteobacteria</td>
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<td>99</td>
<td>Firmicutes</td>
<td>Veillonellaceae</td>
<td>Veillonella dispar</td>
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</table>

Strains highlighted in orange are the Lachnospiraceae that were excluded for the DEC58-Lachno community inoculum. *Closest species match was inferred by alignment of 16S rRNA gene sequences (V3kl and V6r) to NCBI BLAST. Percent identity corresponds to the closest species match. **Clostridium lactatifeormentans was not excluded from DEC58-Lachno community as it was not identified as Lachnospiraceae on the all species living tree.
Table 2.3 List of cultured bacterial isolates used to generate the MET-1 community used in this study

<table>
<thead>
<tr>
<th>Internal strain ID:</th>
<th>% identity:</th>
<th>Phylum:</th>
<th>Family:</th>
<th>*Closest species match:</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-6-S 14 LG</td>
<td>99</td>
<td>Firmicutes</td>
<td>Acidaminococcaceae</td>
<td>Acidaminococcus intestini</td>
</tr>
<tr>
<td>16-6-I 29 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Agathobacter rectalis</td>
</tr>
<tr>
<td>16-6-I 6 FM</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Agathobacter rectalis</td>
</tr>
<tr>
<td>16-6-I 18 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Agathobacter rectalis</td>
</tr>
<tr>
<td>16-6-I 5 MM</td>
<td>99</td>
<td>Bacteroidetes</td>
<td>Bacteroidaceae</td>
<td>Bacteroides ovatus</td>
</tr>
<tr>
<td>16-6-I 11 FAA</td>
<td>99</td>
<td>Actinobacteria</td>
<td>Bifidobacteriaceae</td>
<td>Bifidobacterium adolescentis</td>
</tr>
<tr>
<td>16-6-I 20 MRS</td>
<td>99</td>
<td>Actinobacteria</td>
<td>Bifidobacteriaceae</td>
<td>Bifidobacterium adolescentis</td>
</tr>
<tr>
<td>16-6-I 4 FM</td>
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<td>Actinobacteria</td>
<td>Bifidobacteriaceae</td>
<td>Bifidobacterium longum</td>
</tr>
<tr>
<td>16-6-I 2 FAA</td>
<td>99</td>
<td>Actinobacteria</td>
<td>Bifidobacteriaceae</td>
<td>Bifidobacterium longum</td>
</tr>
<tr>
<td>16-6-I 2 MRS</td>
<td>95</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Blautia luti</td>
</tr>
<tr>
<td>16-6-I 27 FM</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Blautia stercoris</td>
</tr>
<tr>
<td>16-6-I 11 FM</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Blautia wexlerae</td>
</tr>
<tr>
<td>16-6-I 48 FAA</td>
<td>95</td>
<td>Firmicutes</td>
<td>Clostridiaceae</td>
<td>Butyricoccus pullicaecorum</td>
</tr>
<tr>
<td>16-6-I 21 FAA</td>
<td>92</td>
<td>Firmicutes</td>
<td>Erysipelotrichaceae</td>
<td>Clostridium sp.</td>
</tr>
<tr>
<td>16-6-I 3 FM</td>
<td>99</td>
<td>Firmicutes</td>
<td>Coriobacteriaceae</td>
<td>Collinsella aerofaciens</td>
</tr>
<tr>
<td>16-6-I 42 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Dorea longicatena</td>
</tr>
<tr>
<td>16-6-I 10 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Dorea longicatena</td>
</tr>
<tr>
<td>16-6-S BF 7</td>
<td>100</td>
<td>Proteobacteria</td>
<td>Enterobacteriaceae</td>
<td>Enterobacter aerogenes</td>
</tr>
<tr>
<td>16-6-S 3 FM 4i</td>
<td>100</td>
<td>Proteobacteria</td>
<td>Enterobacteriaceae</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>16-6-I F1 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Eubacteriaceae</td>
<td>Eubacterium eligens</td>
</tr>
<tr>
<td>16-6-S 13 LG</td>
<td>97</td>
<td>Firmicutes</td>
<td>Eubacteriaceae</td>
<td>Eubacterium limosum</td>
</tr>
<tr>
<td>16-6-I 47 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Eubacteriaceae</td>
<td>Eubacterium ventriosum</td>
</tr>
<tr>
<td>16-6-I 40 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Ruminococcaceae</td>
<td>Faecalibacterium prausnitzii</td>
</tr>
<tr>
<td>16-6-I 34 FAA</td>
<td>95</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Lachnospira pectinoshiza</td>
</tr>
<tr>
<td>16-6-I 6 MRS</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lactobacillaceae</td>
<td>Lactobacillus casei</td>
</tr>
<tr>
<td>16-6-I 25 MRS</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lactobacillaceae</td>
<td>Lactobacillus paracasei</td>
</tr>
<tr>
<td>16-6-I 5 FM</td>
<td>99</td>
<td>Bacteroidetes</td>
<td>Porphyromonadaceae</td>
<td>Parabacteroides distasonis</td>
</tr>
<tr>
<td>16-6-I 39 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Roseburia faecalis</td>
</tr>
<tr>
<td>16-6-I 31 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Lachnospiraceae</td>
<td>Roseburia intestinalis</td>
</tr>
<tr>
<td>16-6-I 9 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Ruminococcaceae</td>
<td>Ruminococcus faecis</td>
</tr>
<tr>
<td>16-6-I 30 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Ruminococcaceae</td>
<td>Ruminococcus faecis</td>
</tr>
<tr>
<td>16-6-S 50 FAA</td>
<td>99</td>
<td>Firmicutes</td>
<td>Streptococcaceae</td>
<td>Streptococcus dentisani</td>
</tr>
</tbody>
</table>

*Closest species match was inferred by alignment of 16S rRNA gene sequences (V3kl and V6r) to NCBI BLAST. Percent identity corresponds to the closest species match.
until use in further experiments. Preparation of defined microbial communities and chemostat runs were completed in biological duplicate \((n = 2)\).

2.2. Characterizing the taxonomic composition of defined microbial ecosystems

2.2.1 Genomic DNA (gDNA) extraction

Total cellular gDNA was extracted from defined microbial ecosystem samples from the chemostat vessels as previously described with some modifications. Briefly, 200 µL of defined microbial ecosystem sample was added to a screw-cap microfuge tube containing 10 µL of 20 mg/mL proteinase K (in 0.1 mM CaCl2), 300 µL of SLX buffer (E.Z.N.A. Kit Omega Bio-Tek, Norcross, Georgia, USA), and 200 mg of 0.1 mm zirconium beads. Samples were then homogenized using a bead-beater, Digital Disrupter Genie (Scientific Industries Inc., Bohemia, New York, USA) at maximum speed for 3 min. Tubes were then incubated at 70°C for 10 min and at 90°C for 2 min before the addition of 100 µL of Buffer P2 (E.Z.N.A. Kit). After vortexing each sample for 30 s, tubes were incubated on wet ice for 5 min and subsequently centrifuged at 14,000 \(\times\) g. The resulting supernatant was transferred to a new microfuge tube containing 200 µL HTR reagent (E.Z.N.A. Kit), mixed and incubated at room temperature for 2 min. After spinning at 14,000 \(\times\) g for 2 min, gDNA was extracted from the resulting supernatant using the Maxwell® 16 DNA Purification Kit (Promega), according to the manufacturer’s instructions. gDNA sample concentration and quality was evaluated using the NanoDrop 8000 spectrophotometer (ThermoScientific), and stored at -20°C.

2.2.2 Illumina MiSeq 16S rRNA gene sequencing

The V4 region of the bacterial 16S rRNA gene was PCR amplified (see primer Table 2.1) from bacterial gDNA templates in 20 µL reaction volumes containing 1-3 µL gDNA, using
GoTaq® DNA Polymerase (Promega). Cycling conditions were as follows: 95°C for 3 min, 98°C for 20 s, 65°C for 20 s, 72°C for 60 s, and repeated for 25 cycles followed by a 72°C elongation for 10 min. Amplification was carried out in triplicate and successfully replicated amplicons were pooled, purified using the EZ-10 Spin Column PCR Products Purification kit (BioBasic, Ontario, Canada) and quantified using the NanoDrop 8000 spectrophotometer (ThermoScientific) before being sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) at the Advanced Analysis Centre (University of Guelph) according to manufacturer’s recommended protocols.

Raw data files from Illumina MiSeq were prepared and analyzed according to the established protocol by (Kozich et al., 2013). Briefly, Mothur software was used to assemble and process contigs. Sequences were aligned to the SILVA bacterial 16S rRNA reference sequences trimmed to the V4 region (Quast et al., 2013). Misaligned sequences, sequences above 275 base pairs, homopolymers greater than 8 base pairs and chimeras were removed. Sequences were classified against a custom taxonomy database generated from the 16S rRNA gene sequences of the bacterial isolates used in this study, using an 80% alignment cut-off. Operational taxonomic units (OTUs) were assigned using the phylotype method and were included in the analysis if they were represented by at least 0.1% of all sequences. To do this, briefly, the number of sequences in each community was subsampled, and rarefied using the sample containing the lowest sequence count (sample coverages were above 99.9%). Statistical analyses were completed on sequencing data derived from two biological replicates from each chemostat run using the Metastats, non-metric multidimensional scaling (NMDS), and analysis of molecular variance (AMOVA) commands within the Mothur software package (White et al., 2009). The false discovery rate (FDR) method of Benjamini and Hochberg (BH) was used to adjust for multiple
comparisons on Metastats-generated \( p \)-values for each taxonomic rank using RStudio version 0.99.896 (www.rstudio.com/).

To decompose closely related microbial taxa to the species level we used oligotyping (Eren et al., 2014). The oligotyping pipeline as described by Eren et al., 2013, was used to decompose the highly similar V4 region of Bacteroides, Bifidobacterium, Blautia, and Roseburia OTUs as determined by 16S rRNA gene sequencing. Shannon entropy analysis was conducted on mothur-generated FASTA files of aligned sequences for each of the above genera to reveal positions of highest sequence variation. Two entropy components, and a minimum substantive abundance parameter of 50 were used to resolve the oligotypes within the corresponding aligned sequences. Oligotype identification was confirmed using NCBI nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/) and cross-referenced to each defined microbial ecosystem community. The matrix-percent file was then used to determine the percent contribution of each oligotype to the total genus-level abundance. To determine statistical significance between mean abundance values, a one-way ANOVA with the FDR method of Benjamini and Hochberg was used and FDR adjusted \( p \)-values were reported. All statistical analyses were completed using GraphPad Prism 7.0 (GraphPad Software, La Jolla California USA, www.graphpad.com).

2.3 Characterizing the metabonomic profiles of defined microbial ecosystems

2.3.1 Sample preparation and 1D \(^1\)H NMR scanning

Samples (0.22 \( \mu \)m filtered cell-free supernatants from defined microbial ecosystems, section 2.1) were diluted to 10\% (v/v) and combined with the internal standard, 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), to a final concentration of 0.5 mM. Samples were then transferred to Wilmad 5 mm glass NMR tubes (Sigma), and stored at 4\(^{\circ}\)C overnight before
scanning using a Bruker Avance 600 MHz spectrometer. All NMR spectra were obtained using the first increment of a 1D NOESY pulse sequence with a t mix of 100 ms, 4 s acquisition time, 1 s relaxation delay, and a spectral width of 12 ppm.

2.3.2 1D ¹H NMR spectra pre-processing

Spectra files were pre-processed in their entirety using Chenomx NMR Suite 7. (Chenomx Inc., Edmonton, Canada). Briefly, all spectra were batch phase corrected using the Chenomx auto-phase step, and manually adjusted if the automated procedure resulted in a spectrum that remained out of phase. Manual baseline spline correction was then completed for each spectrum by monitoring and adjusting changes to the internal standard DSS, as well as various metabolite clusters across the entire spectrum. Finally, shim and chemical shape correction were automatically applied in reference to the DSS peak, and respective pH (± 0.5) values were entered into each spectral file prior to metabolite profiling.

2.3.3 1D ¹H NMR spectra metabolite profiling

Targeted profiling was used to identify and quantify compounds in the pre-processed NMR spectra. Briefly, a library of 36 compounds was generated from the internal Chenomx/Bruker’s 600 MHz compound library (Table 2.4). Compounds were profiled based on a set of criteria previously described (Yen et al., 2015). Briefly, compounds were fit to spectra based on their signatures and properties obtained from the Chenomx software database, The Human Metabolome Database (Wishart et al., 2013) and the Kyoto Encyclopedia of Genes and Genomes (Kanehisa et al., 2016). Metabolite concentrations were generated from the area of the projected signal after it was fit to the peak centers during identification. Errors in identification and quantification were monitored throughout the targeted profiling of each spectrum using a
Table 2.4 List of compounds identified in targeted 1D $^1$H NMR spectroscopy analysis

<table>
<thead>
<tr>
<th>Compound:</th>
<th>Reason for choice of compound:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>Product of bacterial fermentation</td>
</tr>
<tr>
<td>Acetone</td>
<td>Product of bacterial fermentation</td>
</tr>
<tr>
<td>Alanine</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Betaine</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Butyrate</td>
<td>Product of bacterial fermentation</td>
</tr>
<tr>
<td>Choline</td>
<td>Product of bacterial catabolism</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Product of bacterial fermentation</td>
</tr>
<tr>
<td>Formate</td>
<td>Product of carbohydrate metabolism</td>
</tr>
<tr>
<td>Fructose</td>
<td>Substrate for carbohydrate metabolism</td>
</tr>
<tr>
<td>Fumarate</td>
<td>Product of carbohydrate metabolism</td>
</tr>
<tr>
<td>Galactose</td>
<td>Substrate for carbohydrate metabolism</td>
</tr>
<tr>
<td>Glucose</td>
<td>Substrate for carbohydrate metabolism</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Glycine</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>Product of bacterial fermentation</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>Product of bacterial fermentation</td>
</tr>
<tr>
<td>Lactate</td>
<td>Product of carbohydrate metabolism</td>
</tr>
<tr>
<td>Leucine</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Lysine</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Methanol</td>
<td>Product of bacterial fermentation</td>
</tr>
<tr>
<td>Methionine</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Proline</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Propionate</td>
<td>Product of bacterial fermentation</td>
</tr>
<tr>
<td>Pyroglutamate</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>Product of bacterial fermentation</td>
</tr>
<tr>
<td>Succinate</td>
<td>Product of bacterial fermentation</td>
</tr>
<tr>
<td>Threonine</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Thymine</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Product of protein catabolism</td>
</tr>
<tr>
<td>Valine</td>
<td>Product of protein catabolism</td>
</tr>
</tbody>
</table>
confidence ranking of compounds based on the number of clusters in the compound’s signal, the amount of signal convolution with other compound’s signals, and the signal-to-noise ratio. Compounds without high confidence rankings were omitted from downstream metabonomic analysis. Raw compound concentration data were represented in heat maps generated using Graph Pad Prism 7.0 (GraphPad Software, La Jolla California USA, www.graphpad.com).

2.4 Examining the effects of defined microbial ecosystems on C. difficile growth and virulence

2.4.1 Characterization of C. difficile isolates used in this work

The C. difficile isolates used in this work can be found in Table 2.5. All C. difficile strains were obtained from Dr. Scott Weese, University of Guelph, Ontario, Canada except for 630Δerm and P4D3A1-1 (Petrof et al., 2013). Ribotyping and toxinotyping were performed on P4D3A1-1 as previously described (Bidet et al., 1999; Rupnik et al., 1998) (Figure A 2.1).

C. difficile strains were spread onto BHIS agar supplemented with 0.1% sodium taurocholate (Sigma), D-cycloserine (Sigma), and cefoxitin (Sigma) from -80°C freezer stocks and incubated anaerobically at 37°C for 72 h. To measure growth kinetics, individual C. difficile strains were inoculated into 5 mL BHI (F.S) liquid broth and grown for 3 h. Cultures were diluted 1/100 and transferred to a 96-well plate, incubated anaerobically at 37°C for 48 h, and the OD₆₀₀ was recorded every 60 min using the Victor³ plate reader (Perkin Elmer, USA). All growth measurements were completed in eight technical replicates.

2.4.2 Experimental design

To assess the in vitro effects of defined microbial ecosystems on C. difficile growth and virulence, sterile cell-free supernatants from defined microbial ecosystems were inoculated in an
<table>
<thead>
<tr>
<th>Strain:</th>
<th>Ribotype</th>
<th>Toxinotype</th>
<th>Toxins encoded</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>630Δerm</td>
<td>012</td>
<td>I</td>
<td>A+B+CDT-</td>
<td>Hussain et al., 2005</td>
</tr>
<tr>
<td>CD186</td>
<td>027</td>
<td>III</td>
<td>A+B+CDT+</td>
<td>Quebec, 2003 (outbreak)</td>
</tr>
<tr>
<td>P4D3A1-1</td>
<td>027</td>
<td>III</td>
<td>A+B+CDT+</td>
<td>Petrof et al., 2013</td>
</tr>
<tr>
<td>MOH978</td>
<td>027</td>
<td>III</td>
<td>A+B+CDT+</td>
<td>Martin et al., 2008 (non-outbreak)</td>
</tr>
<tr>
<td>CD973</td>
<td>078</td>
<td>V</td>
<td>A+B+CDT+</td>
<td>Ontario, 2008 (non-outbreak)</td>
</tr>
<tr>
<td>CD247</td>
<td>078</td>
<td>V</td>
<td>A+B+CDT+</td>
<td>Ontario, 2004 (non-outbreak)</td>
</tr>
<tr>
<td>MOH744</td>
<td>078</td>
<td>V</td>
<td>A+B+CDT+</td>
<td>Martin et al., 2008 (non-outbreak)</td>
</tr>
<tr>
<td>MOH16</td>
<td>078</td>
<td>V</td>
<td>A+B+CDT+</td>
<td>Martin et al., 2008 (non-outbreak)</td>
</tr>
</tbody>
</table>
equal volume with a ribotype 027 (CD186) or 078 (CD973) strain (Table 2.5) and cultures were grown to $\text{OD}_{600} = 0.1-0.2$ in BHI broth. Samples were incubated at anaerobically at 37°C for 12, 24, and 48 h (see experimental overview, Figure 2.1).

2.4.3 Enumeration of *C. difficile* vegetative cells and spores

Total *C. difficile* cell counts (cells/mL) were generated by serially diluting and plating 0.1 mL of each treated culture on BHIS agar + 0.1% sodium taurocholate. Spore counts were generated by treating 0.5 mL of the above treatment/control cultures with an equal volume of 100% ethanol for 1 h at room temperature. Samples were then pelleted by centrifugation at $14,000 \times g$ for 5 min, and the supernatant was removed. Spore-containing pellets were then resuspended in 0.5 mL of PBS, serially diluted and plated on BHIS agar + 0.1% sodium taurocholate. Plates were incubated anaerobically at 37°C and enumerated after 24 h. Vegetative cell counts (cells/mL) were derived from subtracting the spore count from matched total cell count observations. All experiments were carried out in biological triplicate. Mean vegetative cell and spore counts were determined from three technical replicate spread-plating experiments and statistical significance was determined using a one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test in Graph Pad Prism 7.0 (GraphPad Software, La Jolla California USA, www.graphpad.com).

2.5 Investigating the effects of defined microbial ecosystems on *C. difficile* toxin gene expression (RT-qPCR assays)

2.5.1 Sample collection and RNA stabilization

Approximately 10 mL of treatment/control samples were pelleted by centrifugation at $4,686 \times g$ for 10 min at 4°C. Each pellet was resuspended in 1 mL of RNAProtect reagent
Figure 2.1 Experimental methods used to assess the effects of defined microbial ecosystems on *C. difficile* growth effects *in vitro*.
(Qiagen) and incubated for 5 min at room temperature. Samples were then centrifuged again as above, the supernatants decanted and discarded, and the pellets frozen at -80°C until RNA extraction.

2.5.2 RNA extraction and assessment

Total bacterial RNA was isolated and purified using the RNeasy Mini kit (Qiagen) following manufacturer’s instructions with some modifications. Briefly, frozen RNAprotect-treated pellets were thawed on wet-ice and resuspended in 1 mL of buffer RLT (Qiagen) + 0.01% (v/v) β-mercaptoethanol (lysis buffer). Resuspended pellets were transferred into 1.5 mL screw-cap tube containing 200 mg (± 10 mg) of 0.1 mm zirconium beads. Samples were lysed using a bead-beater homogenizer (Scientific Industries) at maximum speed for 2 x 60 s, placing on ice for 2 min between each homogenization step. Beads were then pelleted by centrifugation at 21,000 × g for 1 min and the supernatants were transferred to a separate sterile/RNase-free tube, and then mixed with 650 µL of 100% ethanol. The resulting solution from each sample was then added to Qiagen RNeasy Mini columns in 700 µL aliquots. Protocol 7 in the Qiagen RNeasy Mini Kit Handbook was followed with some modifications: All centrifugations were carried out at 15,500 × g, except for the second spin after the second Buffer RPE wash, which was carried out at 21,000 × g for 1 min. Samples were then eluted in 50 µL of RNase-free H₂O, and the concentration and purity of each RNA sample was quantified spectrophotometrically using a NanoDrop 8000 instrument (ThermoScientific). All samples were immediately stored at -80°C.
2.5.3 Removing gDNA from RNA samples

Contaminating gDNA was removed from the total RNA isolations in 50 µL reaction volumes using the RapidOut DNA Removal Kit (Thermo Scientific) according to manufacturer’s instructions. Generally, multiple DNase treatments were required to purify RNA samples. A reverse transcriptase-minus qPCR was completed using 1 µL of DNase-treated RNA sample as template. Samples with a C\textsubscript{q} > 26 when amplified by \textit{rrs} (Table 2.6) were considered free of contaminating gDNA.

2.5.4 Confirmation of RNA quality

Following DNase treatment of all RNA samples, RNA integrity was assessed using the Agilent Bioanalyzer system (Agilent Technologies) in the Genomics Facility of the Advanced Analysis Centre (University of Guelph, Guelph, ON, Canada) with visual inspection of a 23S and 16S rRNA gene banding pattern on a 1% agarose gel by loading 500 ng of RNA into each well. Samples with a RNA integrity number (RIN) value ≥ 5 and/or clearly distinct rRNA banding patterns with little degradation were considered of appropriate quality for reverse transcription.

2.5.5 cDNA preparation

cDNA was generated from 500 ng of each RNA sample using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random hexamers. Briefly, up to 10 µg of total RNA was added in 20 µL total reaction volumes and reverse transcription was completed according to manufacturer’s instructions under the following conditions: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, hold at 4°C. All cDNA samples were immediately stored at -20°C. Prior to qPCR, cDNA samples were diluted 1:6 for target genes of interest (\textit{tcdA}, \textit{tcdB}, \textit{cdtA}, \textit{cdtB}).
Table 2.6 Properties of primers used in RT-qPCR to assess the effects of defined microbial ecosystems on *C. difficile* toxin gene expression

<table>
<thead>
<tr>
<th>Gene:</th>
<th>Protein encoded:</th>
<th>Amplicon size (bp):</th>
<th>Primer Efficiency:</th>
<th>R² value:</th>
<th>Melting Temp. (˚C):</th>
<th>Primer Sequence (5’-3’):</th>
<th>Reference:</th>
</tr>
</thead>
<tbody>
<tr>
<td>tcdA</td>
<td>Toxin A (enterotoxin)</td>
<td>56</td>
<td>1.84</td>
<td>0.994</td>
<td>75.9</td>
<td>F'-TGTCAGAAGCTCGCTCCACA R'-AGCTGACGCATAAGCTCCTGGAC</td>
<td>This study</td>
</tr>
<tr>
<td>tcdB</td>
<td>Toxin B (cytotoxin)</td>
<td>167</td>
<td>1.78</td>
<td>0.998</td>
<td>74.2</td>
<td>F'-CCCTGGAGATGGTGAAATAC R'-GCTGCTTCTATTTCTGTGG</td>
<td>(Metcalf, 2012)</td>
</tr>
<tr>
<td>cdtA</td>
<td>Binary toxin enzymatic</td>
<td>81</td>
<td>1.79</td>
<td>0.998</td>
<td>72.5</td>
<td>F'-TGCAATAACTACTTAAAGCTCTTATAGA R'-TCTTTCCCATTCTTTAGCCTTTTC</td>
<td>(Carter et al., 2007)</td>
</tr>
<tr>
<td>cdtB</td>
<td>Binary toxin binding</td>
<td>158</td>
<td>1.84</td>
<td>0.998</td>
<td>76.0</td>
<td>F'-CCGTTAGTTGCAGCATATCCA R'-CCTACATTAACAGACACACACCAGC</td>
<td>This study</td>
</tr>
<tr>
<td>rrs</td>
<td>16S ribosomal RNA</td>
<td>120</td>
<td>1.83</td>
<td>0.999</td>
<td>80.8</td>
<td>F'-GGGAGACTTGAGCGAGGAG R'-GTGCCTCGAGCGTACGTAGT</td>
<td>(Denève et al., 2008)</td>
</tr>
</tbody>
</table>
2.5.6 qPCR

qPCR was carried out in 15 µL reaction volumes containing 5 µL of diluted cDNA, 7.5 µL of PerfeCTa SYBR® Green SuperMix with ROX (Quantbio) and 500 nM of each forward and reverse primer (Table 2.6) using the StepOnePlus Real-Time PCR System (Life Technologies). Thermocycling conditions were as follows: 95ºC for 33 s and 60ºC for 30 s, repeated for 40 cycles. PCR product specificity was determined by melt curve analysis generated by completing a stepwise gradient 60ºC to 95ºC at a rate of 0.3ºC per second at the end of the qPCR run.

2.5.7 Validation of qPCR

To assess the efficiency of all primers used in this study, serial ten-fold dilutions of gDNA extracted from a ribotype 078 human isolate of C. difficile were used as template in the qPCR under the same conditions as above. A standard curve was generated from three technical replicates of at least five of the serial dilution points for each primer set using the StepOnePlus Real-Time software (Life Technologies).

2.5.8 Gene expression analysis and statistics

A threshold of 0.5 was used to determine the C<sub>q</sub> value for each amplicon using the StepOnePlus Real-Time software (Life Technologies). Gene expression was normalized to both the C. difficile reference gene rrs, and respective control samples using the ΔΔC<sub>q</sub> method. Error bars represent the standard deviation of three biological replicate experiments run in technical triplicate. Normality of ΔC<sub>q</sub> values were assessed at 12, 24, or 48 h time points for each C. difficile ribotype strain using the D’Agostino & Pearson normality test. To determine the
significance between treatments and corresponding control samples of normally distributed data, a one-way ANOVA followed by Dunnett’s multiple comparisons test was performed on $\Delta C_q$ values for each data set. To determine the significance between treatments and corresponding control samples of non-normally distributed data, a Kruskal-Wallis test followed by Dunn’s multiple comparisons test was performed on $\Delta C_q$ values for each data set. All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla California USA, www.graphpad.com).

2.6 Investigating the effects of defined microbial ecosystems on *C. difficile* TcdA and TcdB expression

2.6.1 Preparation of cell-free supernatants for quantification of *C. difficile* toxins TcdA and TcdB

Approximately 10 mL of treatment/control samples were spun down at 4,686 $\times$ g for 10 min. For making filter-sterilized samples, supernatants were sterile-filtered directly through a 0.22 μm disposable filter (GE-Whatman) as above. Supernatants were transferred to a fresh tube and subsequently frozen at -20°C until the ELISAs were performed.

2.6.2 Quantification of *C. difficile* toxins TcdA and TcdB

Sterile-filtered supernatants were thawed to room temperature and then subsamples were either left undiluted or diluted 1:2 prior to addition to ELISA wells. The separate quantification of TcdA and TcdB *C. difficile* toxins was completed using the TGC E002-1 toxin ELISA kit (tgcBIOMICS, Bingen, Germany) following manufacture’s instructions. Two-fold serial dilutions of TcdA and TcdB standard recombinant toxins were included on each ELISA plate to generate a toxin calibration curve for accurate quantification. Toxin quantification levels were normalized to matched vegetative cell count data for each replicate. To determine statistical
significance, pairwise comparisons were made between all groups, using a one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test in Graph Pad Prism 7.0 (GraphPad Software, La Jolla California USA, www.graphpad.com).

2.7 Examining the effects of defined microbial ecosystems on *C. difficile* toxin biological activity

2.7.1 Maintenance of NIH 3T3 fibroblasts for *C. difficile* cytotoxic assays

NIH 3T3 fibroblast cells were maintained as previously described (ATCC, 2016). Briefly, cells were grown at 37°C in 5% CO₂ in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin solution (Gibco) and 0.2% plasmocin (InvivoGen) in a 24-well plate to 80% confluency. Cells from passages 2-6 were used for all experiments. Filter-sterilized supernatants from defined microbial ecosystems were added to each well at a 1:10 dilution and incubated at 37°C under the above conditions for 4 h followed by aspiration and washing of wells with PBS. One-millilitre of supplemented DMEM containing 400 pg/mL of TcdA (List Biological Laboratories Inc. Campbell, California, USA) or 2 pg/mL of TcdB (List Biological Laboratories Inc. Campbell, California, USA) isolated from *C. difficile* 630 (ribotype 012) was then added to each well and plates were incubated for a further 2 h at 37°C. Each treatment or control treated-sample well was prepared in biological triplicate. Images of fibroblast cells were taken from 3-5 different representative fields of view using an inverted microscope (DM IL LED, Leica Microsystems GmbH, Wetzlar, Germany) at 100x total magnification equipped with a Jenoptik ProgRes CT3 digital microscope camera. A blinded experimenter conducted manual cell counts on printed microscopy images. Rounded cells were defined by their circular morphology and lack of elongated processes (fibroblast extensions). Percent rounding was determined by dividing the
number of rounded cells by the total number of cells counted per field of view from each image. To determine significance, pairwise comparisons were made between the percent rounded cells from each treatment or control group, using a one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test in GraphPad Prism 7.0 (GraphPad Software, La Jolla California USA, www.graphpad.com).

2.8 Assessing the germination and spore outgrowth properties of \textit{C. difficile} in response to defined microbial ecosystems

2.8.1 \textit{C. difficile} spore purification

Spores were purified from \textit{C. difficile} isolates using a combination of previously described methods (Edwards and McBride, 2016; Hasan et al., 2011) with modifications. Briefly, \textit{C. difficile} isolates were spread onto BHIS agar plates and incubated anaerobically at 37°C for 7 days. On the second day, plates were wrapped in parafilm to prevent drying. After 7 days, plates were removed from the anaerobic chamber and flooded with 5 mL of ice-cold ST80. Culture collected from each plate was pooled and centrifuged at 14,000 × g for 5 min at 4°C. Culture was resuspended in an equal volume of sterile ddH$_2$O and heat-treated at 65°C to kill any vegetative cells. After heat treatment, the spore preparations were centrifuged again at 14,000 g for 5 min, the supernatant was removed and the resulting pellets were resuspended in 20% Nycodenz (w/v). Bacterial-Nycodenz suspensions were then layered onto two volumes of 50% Nycodenz (w/v) and centrifuged at 14,000 × g for 15 min at 4°C. The vegetative cell debris at the gradient interface was removed from each tube, and the spore pellet was washed three times (14,000 × g, 5 min) in 1 mL ice-cold ST80, and finally resuspended in 1 mL of ice-cold sterile ddH$_2$O. All spore suspensions were then visualized under phase-contrast microscopy to assess spore purity.
2.8.2 Monitoring changes to *C. difficile* spore loss of OD$_{600}$ and spore outgrowth in response to ethyl acetate-extracted metabolites from defined microbial ecosystems

Chemostat vessel contents were collected on day 9, and immediately frozen at -20°C. Small molecules were extracted from these samples by adding an equal volume of ethyl acetate (99.8%, Sigma-Aldrich), shaking vigorously and allowing to settle and then repeating the procedure. The aqueous organic solvent phase was removed using glass-pipettes and transferred to separate glass bottles. Silicone tubing connected to an air-line was used to accelerate the rate of evaporation of the solvent phase, and the remaining dried extracts were stored at -20°C. Dried extracts were resuspended in equal volumes of brain-heart infusion (BHI) broth, filter-sterilized through a 0.22 µm using a vacuum, and stored at 4°C.

Purified spore suspensions were diluted 1:5 in each liquid extract in a 96-well plate, and germination was induced with 2 mM of sodium taurocholate (Sigma). Plates were incubated anaerobically at 37°C in a Victor$^3$ plate reader (Perkin Elmer, USA) for 22 h and growth measurements (OD$_{600}$) were automatically recorded. The area under the curve (AUC) was calculated from spore loss of OD$_{600}$ curves run in biological triplicate from $x = 0$-20.25 using a baseline of $y = 0$. To determine statistical significance, pairwise comparisons were made between treatment and control groups using a one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test in Graph Pad Prism 7.0 (GraphPad Software, La Jolla California USA, www.graphpad.com).
2.9 Assessing the metabonomic profiles of mixed defined microbial ecosystem supernatants and *C. difficile* culture

NMR pre-processing and profiling of defined microbial ecosystems were carried out using identical methods to those detailed in Section 2.3. Experiments were done in triplicate.

2.9.1 Metabonomic quantification and analysis of 1D $^1$H NMR profiled spectra

Profiled NMR spectra data were batch exported into concentration tables (.csv format) that were uploaded to the MetaboAnalyst 3.0 online server (Xia and Wishart, 2011) for subsequent analysis. For both CD186 and CD973 groups, all samples (rows) were normalized to the pooled average samples from the filter sterilized chemostat medium control treatment and compounds (columns) were normalized by the auto-scaling function. For multivariable statistical analyses, 2D PLS-DA score and loading plots were generated using MetaboAnalyst 3.0. To determine statistical significance between normalized concentration values, a one-way ANOVA was used to compare the means between each treatment group. The FDR method of Benjamini and Hochberg was used to correct for multiple comparisons when evaluating normalized metabolite concentration data, and FDR adjusted $p$-values were reported. All statistical analyses were completed using GraphPad Prism 7.0 (GraphPad Software, La Jolla California USA, www.graphpad.com). Heat maps were also generated from the mean normalized concentration data using GraphPad Prism.
Chapter 3: Characterization of defined gut microbial and therapeutic ecosystems

3.1 Introduction

The human gut microbiota is critical for the maintenance and development of many fundamental physiological processes, including the production of certain vitamins and nutrients, regulation of metabolism, protection from pathogens, and maintenance of the immune system. Notably, disturbances to our indigenous gut microbes can alter intestinal homeostasis leading to the development and progression of both GI and non-GI related indications. Given this relationship, examining the compositional and functional properties of healthy and dysbiotic gut microbial ecosystems is a pertinent research initiative.

An understanding of the compositional and functional changes of gut microbial communities exposed to various perturbations can be accomplished using chemostats supporting human gut microbial ecosystems. Knowledge of the metabonomic properties of gut ecosystems can be used as an important diagnostic tool and exploited for use in human medicine. As a simple example, if butyrate production is significantly reduced through antibiotic insult, the targeted replacement of the damaged microbiota with butyrate-producing bacterial species may help return the community to a stable equilibrium, preventing gut dysbiosis.

Comprehensive analysis of the effects of antibiotics and targeted taxonomic depletion on the composition and metabolic function of defined microbial ecosystem preparations supported by an ex vivo human gut model has yet to be reported.
1. I hypothesize that disturbed/perturbed defined microbial ecosystems supported by our chemostat model system will have atypical taxonomic compositions compared to those of healthy defined microbial ecosystems.

   a. Furthermore, I predict that chemostats supporting healthy defined microbial ecosystems will resemble the taxonomic compositions of healthy fecal communities previously described in the literature, while perturbed ecosystems will have profiles commonly associated with gut microbial dysbiosis.

3.1.1 Research objectives

AIM 1: Determine the differences in taxonomic composition between healthy and perturbed defined microbial ecosystems

AIM 2: Determine the differences in metabonomic profiles between healthy and perturbed defined microbial ecosystems

3.2 Results

To establish defined gut microbial ecosystems to model health and disease states, a total of four communities were developed from bacterial isolates derived from the stool of a single, healthy fecal donor (previously described, Petrof et al., 2013). The first two formulations were representative of a healthy state of human gut microbiota (HS) and included: 1) a defined experimental community of 58 bacterial isolates (DEC58); and 2) a subset community of DEC58 species comprising 33 bacterial isolates, (microbial ecosystem therapeutic-1 (MET-1), representative of an ecosystem that has previously been utilized as a clinical intervention for the treatment of CDI (Petrof et al., 2013) (Tables 2.2 and 2.3, respectively). Other preparations were
developed to represent dysbiotic gut microbial ecosystems (DS), and were formulated either 1) through deliberate omission of Lachnospiraceae family microbes from DEC58 (DEC58-Lachno) (Table 2.2), or 2) through application of ciprofloxacin, a clinically relevant broad-spectrum antibiotic associated with CDI, to chemostat-cultured DEC58. All bacterial communities were seeded into a continuous-culture bioreactor system (chemostat) and supported for 9 consecutive days of growth. Experiments were carried out in biological duplicate. The use of a chemostat system to model the distal gut has been previously described, and represents a highly suitable model for the study of microbial-ecosystem dynamics in a reproducible manner (MacDonald et al., 2013).

Using 16S rRNA gene sequencing, taxonomic profiles were generated for all defined microbial gut ecosystems and resolved to the phylum, family, and species level (Figure 3.1 A-C). We then applied NMDS ordination on the 16S rRNA gene sequencing data to visualize whether we could discern clustering disparities in the community structure of the four different defined microbial ecosystems examined in this study (Figure 3.1 D). Pairwise AMOVA between each defined microbial ecosystem group showed significant independent clustering (MET-1 vs. DEC58, \( p = 0.027 \); DEC58 vs. DEC58-Lachno, \( p = 0.029 \); DEC58 vs. DEC58+cipro, \( p = 0.016 \)), indicating that the community structures of each ecosystem were distinct. By visualization of the NMDS ordination plot, DEC58+cipro displayed the greatest separation from DEC58 compared to that of MET-1 and DEC58-Lachno (Figure 3.1 D).
Figure 3.1 Taxonomic characterization of chemostat-supported defined gut microbial ecosystems used to model health and disease states. Abundance plots of the phylum (A), family (B), and species (C) level taxonomic compositions are shown for MET-1, DEC58, DEC58-Lachno, and DEC58+cipro defined microbial ecosystems. Mean abundance values were generated for four replicate samples derived from two biological replicate chemostat vessels. For clarity, error bars representing the standard error of the means are not shown. NMDS ordination plot was used to plot the Yue & Clayton dissimilarity measure of 16S rRNA gene sequencing data from all defined microbial ecosystems at the species level (D). Pairwise AMOVA comparisons between each defined microbial ecosystem group are as follows: MET-1 vs. DEC58, \( p = 0.027 \); DEC58 vs. DEC58-Lachno, \( p = 0.029 \); DEC58 vs. DEC58+cipro, \( p = 0.016 \). Plots were generated from data obtained using the Metastats and NMDS commands within the Mothur software package. OTUs that required further species-level resolution were determined using the Oligotyping method.
3.2.1 Health-associated defined microbial ecosystems derived from the same source have similar taxonomic profiles

Within the healthy defined microbial communities, MET-1 displayed an increased relative abundance in Proteobacteria \( (p = 0.04703) \) and Actinobacteria \( (p = 0) \) compared to DEC58, but no significant change in the remaining representative bacterial phyla (Figure 3.1 A and Figure 3.2). Of the bacterial families common to both health-associated defined microbial ecosystems, Porphyromonodeceae \( (p = 0.0378) \), Enterobacteriaceae \( (p = 0.0475) \), Coriobacteriaceae \( (p = 0.01705) \), and Bifidobacteriaceae \( (p = 0.04287) \) were decreased, while Ruminococcaceae \( (p = 0.01705) \) was the only bacterial family significantly increased in DEC58 compared to MET-1 (Figure 3.3). Of the species common to both MET-1 and DEC58 ecosystems, MET-1 was enriched in \textit{Bacteroides ovatus} \( (p = 0.001625) \), \textit{Roseburia rectale} \( (p = 0.001625) \), \textit{Parabacteroides distasonis} \( (p = 0.04092) \), \textit{Escherichia coli} \( (p = 0.03058) \), \textit{Eubacterium eligens} \( (p = 0.03962) \), \textit{Collinsella aerofaciens} \( (p = 0.03962) \), \textit{Bifidobacterium longum} \( (p = 0.007772) \), \textit{Eubacterium ventriosum} \( (p = 0.03122) \), and \textit{Faecalibacterium prausnitzii} \( (p = 0.04835) \), while DEC58 was enriched in \textit{Clostridium glycyrrhizinilysiticum} \( (p = 0.04835) \) (Figure 3.1 C and Figure 3.4).

3.2.2. Alteration of a defined microbial ecosystem modeling a healthy gut community (DEC58) by the removal of species within the Lachnospiraceae bacterial family

The Lachnospiraceae-depleted DEC58 community was enriched in Bacteroidetes \( (p = 0.04073) \), Actinobacteria \( (p = 0.01943) \), and Proteobacteria \( (p = 0.034) \) compared to DEC58 (Figure 3.1 A and Figure 3.2). As expected, the relative abundance of Lachnospiraceae in this ecosystem was decreased compared to DEC58; 16.8% to 1% \( (p = 0) \) (Figure 3.1 B and Figure
Figure 3.2 Significant phylum level changes in defined microbial ecosystems supported by an ex vivo human gut model. Error bars represent the standard error of the means for four replicate samples. BH FDR-adjusted $p$-values are shown. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Only significant trends are shown for clarity.
Figure 3.3 Significant family level changes in defined microbial ecosystems supported by an ex vivo human gut model. Samples containing bacterial families represented at a relative abundance greater than 0.1% are shown in Panel A and those with relative abundances less than 0.1% are shown in Panel B. Error bars represent the standard error of the means for four replicate samples. BH FDR-adjusted p-values are shown. *, \( p \leq 0.05 \); **, \( p \leq 0.01 \); ***, \( p \leq 0.001 \); ****, \( p \leq 0.0001 \). Only significant trends are shown for clarity.
Figure 3.4 Significant species level changes in defined microbial ecosystems supported by an *ex vivo* human gut model. Samples containing bacterial species represented at a relative abundance greater than 0.1% are shown in Panel A and those with relative abundances less than 0.1% are shown in Panel B. Error bars represent the standard error of the means for four replicate samples. BH FDR-adjusted *p*-values are shown. *, *p* ≤ 0.05; **, *p* ≤ 0.01; ***, *p* ≤ 0.001; ****, *p* ≤ 0.0001. Only significant trends are shown for clarity.
3.3). Non-Lachnospiraceae species to be significantly decreased in this ecosystem compared to DEC58 were *Bacteroides fragilis* (*p* = 0.001175) and *Veillonella dispar* (*p* = 0.04335) (Figure 3.1 C and Figure 3.4). Additionally, the relative abundances of multiple bacterial species were increased in the DEC58-Lachno chemostat samples compared to DEC58, including *Bacteroides caccae* (*p* = 0.001175), *Bacteroides ovatus* (*p* = 0.001175), *Enterobacter aerogenes* (*p* = 0.04906), *Parabacteroides distasonis* (*p* = 0.03525), *Escherichia coli* (*p* = 0.03525), *Collinsella aerofaciens* (*p* = 0.03525), and *Atopobium minutum* (*p* = 0.04573) (Figure 3.1 C and Figure 3.4).

3.2.3 Ciprofloxacin treatment alters the taxonomic composition of DEC58

Ciprofloxacin antibiotic treatment induced changes in the taxonomic composition of the DEC58 community at all phylogenetic levels investigated (Figure 3.1 A-C). At the phylum level, there was a significant reduction in Bacteroidetes (*p* = 0.002288), and a ~18% increase in Proteobacteria (*p* = 0.0184) compared to the DEC58 ecosystem (Figure 3.1 A). The relative abundances of three bacterial families, Bacteroidaceae (*p* = 0), Acidaminococcaceae (*p* = 0.0266), and Ruminococcaceae (*p* = 0.02454) were significantly decreased with ciprofloxacin treatment, (Figure 3.1 B and Figure 3.3) and this was found to be due to a reduction in *Bacteroides caccae* (*p* = 0.002167), *Bacteroides fragilis* (*p* = 0.002167), and *Bacteroides ovatus* species (*p* = 0.0001093), *Acidaminococcus intestini* (*p* = 0.02413) and *Flavonifractor plautii* (*p* = 0.0237) (Figure 3.4). *Escherichia coli* was the only species significantly enriched in the ciprofloxacin-treated DEC58 community in comparison to DEC58 (*p* = 0.0237).
3.2.4 Differences in the taxonomic composition between defined microbial ecosystem inocula and chemostat-supported communities

i. MET-1

Although 69.7% of the MET-1 inoculum consisted of bacteria within the Firmicutes phylum (see Table 2.3), an abundance of only 42.3% of Firmicutes were detected in the MET-1 ecosystem after 9 days of growth using 16S rRNA gene sequencing (see Figure 3.2). Conversely, there were only 6.1% Bacteroidetes present in the MET-1 inoculum, but the MET-1 ecosystem was largely enriched in Bacteroidetes (50.8%) when grown in the chemostat vessels for 9 days. Additionally, approximately 1/3 of Lachnospiraceae were represented in the MET-1 inoculum; however, only 21.3% of this bacterial family was detected in the MET-1 bacterial ecosystem by 16S rRNA gene sequencing analysis (Figure 3.3). Although only one Acidaminococcus strain was included in the MET-1 community (Acidaminococcus intestini), there was a high mean abundance corresponding to this bacterial isolate at the species level (17.4%) (Figure 3.4). Sequencing data revealed that all of the bacterial families included in the MET-1 inoculum were detected in samples taken from the chemostat vessels, except for Lactobacillaceae and Streptococcaceae (Table 3.1).

ii. DEC58

Similar to MET-1, although 70% of the DEC58 inoculum consisted of bacteria within the Firmicutes phylum (see Table 2.2), only 48.4% Firmicutes was detected in the DEC58 ecosystem from samples derived from duplicate chemostat runs (see Figure 3.2). Additionally, although there was only 13.3% Bacteroidetes present in the DEC58 inoculum, the DEC58 ecosystem abundance of Bacteroidetes was increased (45.2%) when grown in the chemostat vessels. Similar
to the community composition of MET-1, Acidaminococcus intestini was the only *Acidaminococcus* species included in the DEC58 community, and a high abundance of this taxon was also observed at the species level (15.1%) in chemostat vessels supporting this ecosystem (Figure 3.4). Likewise, the only species within the Verrucomicrobia phylum included in DEC58 was Akkermansia muciniphila, which contributed to 4.4% of the total abundance of all bacterial phyla within this ecosystem. In contrast to MET-1, Bacillaceae, Bifidobacteriaceae, Coriobacteriaceae, Corynebacteriaceae, Erysipelotrichaceae, Lactobacillaceae, Micrococcaceae, Ruminococcaceae, Staphylococcaceae and Sutterellaceae bacterial families included in the DEC58 inoculum were not detected above 0.1% as determined by 16S rRNA gene sequencing of chemostat samples supporting this ecosystem (see Table 3.1).

**iii. DEC58-Lachno**

Twenty-five strains in the DEC58 community identified as Lachnospiraceae were excluded from the DEC58-Lachno chemostat inoculum. Similar to MET-1 and DEC58, the proportion of Firmicutes in the DEC58-Lachno inoculum was greater than what was detected through 16S rRNA gene sequencing of chemostat-derived samples (45.7% inoculum vs. 27.6%, chemostat-derived) (see Table 2.2 and Figure 3.2). Furthermore, there was 22.9% Bacteroidetes present in the DEC58-Lachno inoculum, but Bacteroidetes was detected in greater proportions (59.3%) when this community was actively supported by the chemostat vessels. Comparable proportions of Proteobacteria were present in both the DEC58-Lachno inoculum (8.6%) and vessel contents of this ecosystem (6.9%). As reported in section 3.2.2, although Lachnospiraceae were largely removed from the DEC58-Lachno inoculum, 1% of this bacterial family was present in the DEC58-Lachno chemostat vessel samples (see Figure 3.2). Although only one isolate within the Acidaminococcaceae bacterial family was included in the DEC58-Lachno
community inoculum, there was a high mean abundance of *Acidaminococcus intestini* (10%). In chemostat samples supporting DEC58-Lachno, Bacillaceae, Clostridiaceae, Corynebacteriaceae, Eggerthellaceae, Micrococcaceae, Rikenellaceae, Staphylococcaceae and Sutterellaceae bacterial families were not detected by 16S rRNA gene sequencing, although these families were represented in the inoculum of this ecosystem (see Table 3.1).

### 3.2.5 SCFA concentrations in healthy vs. disturbed defined microbial ecosystems

Using 1D $^1$H NMR spectroscopy, we were able to reveal targeted metabolite profiling of both HS and DS defined microbial ecosystems after 9 days of growth in a continuous-culture bioreactor system used to model the human distal gut (Figure 3.5). Both MET-1 and the unaltered DEC58 ecosystem displayed similar quantities of propionate (29.76 and 26.87 mM, respectively), formate (0.047 and 0.031 mM, respectively), and isovalerate (0.528 vs. 0.0566 mM, respectively), though amounts of acetate and butyrate were increased ~1.7-fold in DEC58 compared to MET-1 (Figure 3.5 A).

Production of butyrate was marginally reduced in DEC58-Lachno compared to DEC58 (24.47 vs. 28.6 mM, respectively), but increased compared to MET-1 (16.34 mM) (Figure 3.5 A). Propionate levels were consistent when comparing DEC58-Lachno and MET-1 or the native DEC58 ecosystem. In the DEC58-Lachno community, acetate levels were highly comparable to those of MET-1 (53.08 vs. 52.41 mM, respectively), but reduced by approximately 50% in comparison to DEC58. DEC58-Lachno had increased levels of proline but most other examined compounds were present in similar abundances to those measured in the MET-1 and DEC58 ecosystems (Figure 3.5 A).
Figure 3.5 Targeted metabonomic profiles of defined, chemostat-supported gut microbial ecosystems. All compound concentrations (mM) were determined using 1D $^1$H NMR spectroscopy. SCFA concentrations are shown in (A). Additional compounds were selected for targeted analysis based on their involvement in carbohydrate or proteolytic fermentation, or importance to general gut microbial metabolism (B). Uninoculated sterile chemostat medium was also profiled as a control.
Ciprofloxacin also impacted the SCFA composition of DEC58 in a major way, i.e., acetate, butyrate, isovalerate and propionate levels were all decreased following this treatment in comparison to the untreated DEC58 community (acetate, 96.58 to 15.58 mM; butyrate, 28.6 to 3.36 mM; isovalerate, 0.566 to 0 mM; propionate 26.87 to 3.09 mM), and were similar to levels measured in uninoculated chemostat medium controls (Figure 3.5 A). Formate was the only SCFA increased in DEC58+cipro compared to all other microbial ecosystems examined in this study. Other compounds including lactate, isoleucine, valine, glutamate and pyroglutamate were present in chemostat medium controls but found to be increased in DEC58+cipro, while galactose and glucose were increased in DEC58+cipro compared to chemostat medium control and all other defined microbial ecosystems (Figure 3.5 A). Of note, isobutyrate was not present in the chemostat medium control or any of the defined microbial ecosystems examined. Increased succinate levels in MET-1 and DEC58+cipro were found compared to all other defined microbial ecosystems.

3.2.6 Concentrations of other compounds in healthy and disturbed defined microbial ecosystems

Galactose and glucose were increased in DEC58+cipro compared to chemostat medium control and all other defined microbial ecosystems (Figure 3.5 B). Compounds including lactate, isoleucine, valine, glutamate and pyroglutamate were present in chemostat medium controls but found to be increased in DEC58+cipro. DEC58-Lachno had increased levels of proline but most other examined compounds were present in similar abundances to those measured in the MET-1 and DEC58 ecosystems. Increased succinate levels in MET-1 and DEC58+cipro were found compared to all other defined microbial ecosystems.
3.3 Discussion

Given the importance of our gut microbiota to human health, examining the properties of gut microbial ecosystems in health and disease states is an active research initiative. To develop bacterial ecosystems representative of human gut microbiota, our group has extensively cultured bacterial isolates from a healthy stool donor. We used these isolates to develop two defined microbial ecosystems, one composed of 58 bacterial isolates, DEC58, and a second representing a 33-species subset of this community, MET-1. The MET-1 ecosystem (RePOOPulate) was selected for use in this study, as this consortium of 33 bacterial strains was shown to be effective for two patients with recurrent CDI (rCDI) in a proof-of-principle trial (Petrof et al., 2013). DEC58 was used to represent a more ‘robust’ ecosystem of bacterial species isolated from the same donor as MET-1, as this ecosystem contains increased species diversity (see lists of defined microbial ecosystems in Tables 2.2 and 2.3). This allowed us to sufficiently study alterations in taxonomic and metabolic composition under controlled perturbational stresses (e.g. through specific taxa removal and antibiotic treatment).

3.3.1 MET-1 vs. DEC58

Using 16S rRNA gene sequencing and 1D $^1$H NMR spectroscopy, we completed taxonomic and targeted metabolite profiling of defined microbial ecosystems. Both MET-1 and DEC58 were enriched in Bacteroidetes as determined by 16S rRNA gene sequencing, despite the low abundance of bacteria corresponding to this phylum in the inocula of these communities. In contrast, after 9 days of continuous growth in chemostat vessels, the proportion of Firmicutes within both MET-1 and DEC58 was decreased compared to the proportion of this phylum in the inocula of these communities. These results indicate that the taxonomic composition of defined microbial ecosystems changes when examined in the context of an in vitro chemostat model of
the distal gut. Interestingly, although only one strain of *Acidaminococcus* was included in MET-1 and DEC58, OTUs corresponding to this species were represented in high abundance within these bacterial ecosystems. Similarly, one isolate of *Akkermansia muciniphila* was included in the DEC58 inoculum, and this sole member of the Verrucomicrobia phylum was highly represented in the DEC58 ecosystem when supported by the chemostat vessels. The dominance of the above species within defined microbial ecosystems is supported by previous reports, which have described both *Acidaminococcus intestini* and *Akkermansia muciniphila* as abundant commensals of the human gut (D'Auria et al., 2011; Derrien et al., 2004).

Within the defined microbial ecosystems representative of a healthy community, there was a 5.1% decrease in Actinobacteria and Proteobacteria in DEC58 compared to MET-1, but no significant difference in other representative bacterial phyla including Firmicutes, Bacteroidetes, or Verrucomicrobia. *Collinsella aerofaciens* was significantly enriched in the MET-1 ecosystem in comparison to DEC58, and overrepresentation of the Actinobacteria phylum in MET-1 is likely due to increased abundance of this bacterium. However, MET-1 had an increased relative abundance of Ruminococcaceae, which perhaps resulted from the addition of two *Ruminococcus faecis* isolates, not included in the DEC58 inoculum.

Based on the preliminary metabonomic data presented here, DEC58 produced greater quantities of acetate and butyrate compared to MET-1 under our culture conditions. Because of the increased species diversity in DEC58, increases in these SCFAs may be directly related to this ecosystem’s capacity for increased bacterial metabolic activity. As acetate is a product of general bacterial metabolism in a wide variety of species, this could be indicative of a highly diverse community. Both MET-1 and DEC58 contain multiple butyrate-producing bacterial genera (Louis et al., 2014). As determined from 16S rRNA gene sequencing results, there was an
increased abundance of key butyrate-producing species such as *Faecalibacterium prausnitzii* and *Roseburia rectale* in MET-1 compared to DEC58. However, additional butyrate-producing species in DEC58 such as *Eubacterium hallii, Anaerostipes hadrus* and *Coprococcus comes* were not included in the MET-1 inoculum, and these bacteria may be contributing to the increases in butyrate observed.

### 3.3.2 DEC58-Lachno vs. DEC58

By removing strains in DEC58 from the Lachnospiraceae bacterial family prior to inoculation, we aimed to diminish butyrate production, creating a dysbiotic ecosystem. We observed slightly decreased butyrate levels in DEC58-Lachno compared to DEC58; however, butyrate levels were still 8.13 mM higher than in MET-1. Non-Lachnospiraceae butyrate producers present in the healthy DEC58 ecosystem should include *Faecalibacterium prausnitzii, Eubacterium limosum* and *Flavonifractor plautii* (Carlier et al., 2010; Louis et al., 2014; Vital et al., 2014). Such species may be responsible for consistency of butyrate quantities and such activity in the context of complex defined microbial ecosystems warrants further investigation.

Although we aimed to fully remove Lachnospiraceae bacterial taxa from the inoculum, there was 1% relative abundance of Lachnospiraceae present in DEC58-Lachno chemostat samples. Ambiguities in the classification of species within this community resulted in the unintended inclusion of this bacterial family in the chemostat inoculum. Our custom taxonomy database was constructed using bacterial classifications from NCBI GenBank, where *Clostridium lactatifermentans* is currently classified as a Lachnospiraceae. However, when we ran the DEC58-Lachno chemostat vessel, *Clostridium lactatifermentans* was not classified as a Lachnospiraceae using the All Species Living Tree (Release LTPs123, September 2015,
Based on this observation, we included this strain in the DEC58-Lachno inoculum. The presence of even a restricted range of Lachnospiraceae within this microbial ecosystem may have flourished to fill this vacant niche and accordingly contributed to the butyrate production observed.

Interestingly, the depletion of Lachnospiraceae from the DEC58 ecosystem, did not significantly impact the community diversity compared to DEC58 ($p = 0.9137$), as assessed through the inverse Simpson index (Figure A 3.1). However, the exclusion of Lachnospiraceae from DEC58 resulted in the detection of Bifidobacteriaceae, Coriobacteriaceae, and Erysipelotrichaceae, which were undetected in DEC58. For example, Lachnospiraceae may be occupying the same niche as the above bacterial families, suppressing their growth. Although this was not directly examined in this work, the microbial interactions responsible for the altered taxonomic composition of DEC58 in response to the removal of Lachnospiraceae are relevant in the context of gut microbial health, and should be further investigated upon.

Elevated levels of Collinsella aerofaciens and Parabacteroides distasonis in the DEC58-Lachno ecosystem compared to DEC58 were responsible for the associated increases in Coriobacteriaceae and Porphyromonadaceae, as these are the only two isolates that provide coverage for these families in these defined microbial ecosystems. Additionally, reductions in Bacteroides fragilis and Veillonella dispar were noted in DEC58-Lachno. Veillonella dispar is a known fermenter of lactate, producing propionate and acetate as a result of this metabolism. Reductions in the abundance of this species may have contributed to the reduced acetate levels in comparison to DEC58. Although many bacterial species contribute to acetate production, because neither DEC58-Lachno nor MET-1 contain Veillonella dispar and had comparable acetate levels, this suggests that Veillonella dispar may be a major acetogen within these defined
microbial ecosystems. Propionate levels were consistent between the DEC58 and DEC58-Lachno microbial ecosystems even though key Lachnospiraceae had been removed and *Bacteroides fragilis* and *Veillonella dispar* levels were reduced. Maintenance of propionate levels in this ecosystem may have been facilitated by other species within the Bacteroidetes phyla, through the succinate pathway (see overview of gut microbial metabolism pathways in Figure 1.1). Given that the succinate pathway is thought to be the main pathway for propionate production in the gut (Salonen et al., 2014), the high abundance of Bacteroidetes in DEC58-Lachno, may maintain propionate levels predominantly through this route.

3.3.3. DEC58+cipro vs. DEC58

Treatment of DEC58 with a physiologically relevant dose of ciprofloxacin was used as a strategy to study the impact of common broad-spectrum antimicrobial treatments on healthy defined bacterial communities. The quantity of ciprofloxacin used to dose the chemostat vessels (137.5 mg) was derived from the percent fecal-excretion (27.5%) of a 500 mg oral dose of ciprofloxacin in human subjects (FDA, 2004). Given that there is no absorption of antibiotic within the chemostat vessel, the measure of fecal-excretion is suitable to estimate the physiologically relevant doses of ciprofloxacin in this model. Interestingly, ciprofloxacin treatment, did not significantly impact the community diversity of DEC58 (*p* > 0.9999), as assessed through the inverse Simpson index (Figure A 3.1). This is contrary to previous reports where ciprofloxacin was shown to diminish taxonomic richness, diversity and evenness of gut microbiota in stool samples from human subjects (Dethlefsen et al., 2008; Dethlefsen and Relman, 2011). Although markedly different gut microbial communities were assessed in the study by Dethlefsen et al., 2008, high interindividual variation in gut microbial community composition following ciprofloxacin was described, which we similarly observed here (see
At the phylum level, DEC58+cipro had decreased abundance of Bacteroidetes compared to DEC58, which corroborates findings in the literature from studies that have measured this phylum in CDI patient stool samples (see phylum-level taxonomic changes in CDI patients Table 1.1). Ciprofloxacin treatment also caused a drastic decrease in acetate, butyrate, propionate and isovalerate to levels similar to that of sterile chemostat medium controls. The uninoculated chemostat medium did not contain any bacterial species, thus in the absence of bacterial metabolism, quantities of these SCFAs should be negligible. The decreased SCFA levels as a result of broad-spectrum antibiotic use are indicative of a loss of bacterial fermentation. Other studies have associated antibiotic use to the decreased production SCFAs in the gut. Numerous studies have shown decreased fecal SCFAs in patients with antibiotic-associated diarrhea, infectious diarrhea and/or the lack of dietary prebiotic substrates (Clausen et al., 1991; Gustafsson et al., 1998; Hove et al., 1996; Hoverstad et al., 1986; Siigur et al., 2011). Many metabonomic studies have shown that antibiotic treatment dramatically alters SCFA levels in human and murine models (Antunes et al., 2011; Ferreyra et al., 2014; Perez-Cobas et al., 2013b; Theriot et al., 2014; Zhao et al., 2013). In agreement with our findings, the study by (Johnson et al., 2015) showed that levels of acetate, butyrate and propionate were lower in samples obtained from ampicillin-treated fecal communities supported by in vitro batch culture fermentation vessels compared to controls.

DEC58+cipro vessels were found to have increased levels of formate compared to DEC58. In the gut, formate is a major product of mixed acid anaerobic fermentation, primarily produced by Enterobacteriaceae (Leonhartsberger et al., 2002) and is important to anaerobic metabolism in methanogen and sulfate-reducing microorganisms (Leonhartsberger et al., 2002;
Rey et al., 2010) (see bacterial metabolic pathways in Figure 1.1). In the DEC58+cipro community, significant increases in *Escherichia coli* ($p = 0.0237$) in comparison to DEC58 could be responsible for the elevated formate levels observed.

The expansion of this species in DEC58+cipro highlights the importance of studying specific gut microbes in the context of whole bacterial ecosystems rather than in isolation. Given that ciprofloxacin is normally effective against Gram-negative bacteria such as *E. coli*, it is possible that *E. coli* acquired ciprofloxacin resistance within the chemostat vessel (Ferrer et al., 2016). Although we did not examine this possibility, resistance rates of *E. coli* to fluoroquinolones have been increasingly reported and mutations in several genes including *gyrA* (DNA gyrase A) have been commonly found in clinical isolates of this species (Fasugba et al., 2015; Yoshida et al., 1990). An important future direction would be to sequence *gyrA* alleles to determine the amount of ciprofloxacin resistance that was acquired by bacterial populations in DEC58+cipro compared to the native DEC58 community. Further studies examining additional mechanisms utilized by this species to persist in this perturbed gut environment representative of dysbiosis are warranted.

Relative abundance levels of *Bacteroides caccae, Bacteroides fragilis, Bacteroides ovatus, Acidaminococcus intestini* and *Flavonifractor plautii* were significantly reduced in DEC58+cipro. *Flavonifractor plautii* is a known producer of acetate and butyrate (Carlier et al., 2010), while *Bacteroides* species are known acetate and propionate producers (den Besten et al., 2013b); reductions in these species may contribute to the decreases in these SCFAs in this dysbiotic ecosystem. In contrast, ciprofloxacin may precipitate metabonomic changes through modulation of gut bacterial gene expression (Maurice et al., 2013). In a recent study, a course of ciprofloxacin was administered to 10 healthy patients, and after one-month, predictive
metagenomics analysis on collected fecal samples suggested that pyruvate metabolism was significantly reduced compared to the placebo group. As the pyruvate pathway is utilized in the synthesis of butyrate from complex carbohydrates (polysaccharides), impacts on these metabolic processes may play a role in decreasing butyrate concentrations (Zaura et al., 2015). Future in vitro experiments utilizing RT-qPCR to assess SCFA-related gene expression levels of entire defined microbial ecosystems and individual species, will demonstrate how and to what extent these metabonomic changes occur.

Impairment of hexose and pentose carbohydrate metabolism (e.g. glycolysis) in the DEC58+cipro sample may have resulted in the increased levels of galactose and glucose compared to all other defined microbial ecosystems and the control. Given that ciprofloxacin treatment decreased total bacterial cell density in DEC58+cipro (Figure A 3.2), the total metabolic output of this ecosystem in turn might have been affected. Similarly, deficiencies in DEC58+cipro involved in one-arm of the biosynthesis of propionate might explain the decreased quantities of propionate and corresponding increase of lactate. As lactate has been shown to be further metabolized to acetate, propionate and butyrate via cross-feeding bacteria (Belenguer et al., 2006; Morrison and Preston, 2016), it would be erroneous to attribute the variation in metabolite concentrations within these communities to one bacterial species given the extensive cross-feeding mechanisms that exist in gut microbial ecosystems (Flint et al., 2015; Morrison and Preston, 2016; Ríos-Covián et al., 2016).

Compounds including glutamate, pyroglutamate, isoleucine, valine, and lactate were increased in DEC58+cipro compared to DEC58, but present in comparable levels to chemostat medium controls. The reduction of the branch-chained amino acids (BCAAs) isoleucine and valine is indicative of increased protein fermentation. Microbial metabolism dominated by
proteolytic fermentation can result in the generation of amines, ammonia, phenols and sulfides, increased levels of which are associated with gut dysbiosis and are thought to have detrimental effects on the host (Russell et al., 2011; Vipperla and O'Keefe, 2016; Windey et al., 2012). Antibiotics have been shown to alter glutamate metabolism, which could result in the increased levels of glutamate we observed in the DEC58+cipro ecosystem (Perez-Cobas et al., 2013b). Increased levels of pyroglutamate are associated with high abundance of Lactobacilli and have also been identified in IBS phenotypes (Ponnusamy et al., 2011). Marginal increases in the relative abundance of Lactobacilli in the DEC58+cipro ecosystem may have contributed to this metabonomic alteration. However, since ciprofloxacin decreased total bacterial cell load, and levels of glutamate, lactate, pyroglutamate, isoleucine, and valine are comparable to that of chemostat medium control, these metabolite signatures may be more reflective of the metabolite make-up of the chemostat medium itself.

Though ciprofloxacin induced major changes in both biological replicate vessels, a large variance in taxonomic composition resulted (see Table 3.1, Figure 3.1, and Figure A 3.1). Whether this large difference is due to the small number of biological replicates or biological variation in the way ciprofloxacin affects defined bacterial communities has yet to be determined, though ciprofloxacin has been shown to have contrasting effects on species diversity, as determined by 16S rRNA gene pyrosequencing on human stool samples (Dethlefsen et al., 2008). Further experiments including additional replicates of DEC58+cipro chemostat vessels will help elucidate whether the effect of ciprofloxacin affects the DEC58 community in a consistent manner.
Table 3.1 Differences in the taxonomic composition between defined microbial ecosystem inocula and chemostat-supported communities. Bacterial families included in the inocula of defined microbial ecosystems are listed in each column. Families that were detected at over 0.1% relative abundance of 16S rRNA V4 gene sequences are highlighted in bold.

<table>
<thead>
<tr>
<th>MET-1</th>
<th>DEC58</th>
<th>DEC58-Lachno</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidaminococcaceae</td>
<td>Acidaminococcaceae</td>
<td>Acidaminococcaceae</td>
</tr>
<tr>
<td>Bacteroidaceae</td>
<td>Atopobacteriaceae</td>
<td>Atopobacteriaceae</td>
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<tr>
<td>Bifidobacteriaceae</td>
<td>Bacillaceae</td>
<td>Bacteroidaceae</td>
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<tr>
<td>Clostridiaceae</td>
<td>Bacteroidaceae</td>
<td>Clostridiaceae</td>
</tr>
<tr>
<td>Coriobacteriaceae</td>
<td>Bifidobacteriaceae</td>
<td>Clostridiaceae</td>
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<tr>
<td>Enterobacteriaceae</td>
<td>Clostridiaceae</td>
<td>Eubacteriaceae</td>
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<tr>
<td>Erysipelotrichaceae</td>
<td>Coriobacteriaceae</td>
<td>Erysipelotrichaceae</td>
</tr>
<tr>
<td>Eubacteriaceae</td>
<td>Corynebacteriaceae</td>
<td>Eubacteriaceae</td>
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<tr>
<td>Lachnospiraceae</td>
<td>Eggerthellaceae</td>
<td>Eubacteriaceae</td>
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<tr>
<td>Lactobacillaceae</td>
<td>Enterobacteriaceae</td>
<td>Enterobacteriaceae</td>
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<tr>
<td>Porphyromonadaceae</td>
<td>Erysipelotrichaceae</td>
<td>Erysipelotrichaceae</td>
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<tr>
<td>Ruminococcaceae</td>
<td>Eubacteriaceae</td>
<td>Fumaricutes_unclassified</td>
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<tr>
<td>Streptococcaceae</td>
<td>Lachnospiraceae</td>
<td>Lachnospiraceae</td>
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<td></td>
<td>Lactobacillaceae</td>
<td>Lactobacillaceae</td>
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<td>Micrococcaceae</td>
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<td></td>
<td>Oscillospiraceae</td>
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<td>Porphyromonadaceae</td>
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<td>Porphyromonadaceae</td>
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<td>Rikenellaceae</td>
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<td>Staphylococcaceae</td>
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<td>Sutterellaceae</td>
<td>Sutterellaceae</td>
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<td>Veillonellaceae</td>
<td>Veillonellaceae</td>
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<tr>
<td>Verrucomicrobiaceae</td>
<td>Verrucomicrobiaceae</td>
<td>Verrucomicrobiaceae</td>
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</tbody>
</table>
3.4 Conclusions and future directions

Together, the taxonomic and functional data obtained from the various defined microbial communities examined in this study have provided an increased understanding of ecosystem behaviour in a simplified fermentation system. Here we show the effects of specific taxa removal (DEC58-Lachno) and antibiotic treatment (DEC58+cipro) on the composition and function of defined microbial ecosystems compared to their native ecosystem (DEC58). While removing Lachnospiraceae from DEC58 changed the ecosystem taxonomic composition, the metabonomic profiles of butyrate and propionate were not considerably altered. Ciprofloxacin treatment, however, induced drastic taxonomic and metabonomic changes to DEC58, specifically notable reductions in all three major SCFAs, demonstrating the deleterious consequences of antibiotic use on the gut microbiota.

We selected ciprofloxacin to disturb the DEC58 community as this fluoroquinolone antibiotic has been shown to affect a wide-variety of bacterial species (Ferrer et al., 2016; Dethlefsen et al., 2008), including species within the *Bacteroides* genera, which are predominant commensal anaerobes contributing to homeostasis in the human gut microbiota (Wexler, 2007). Furthermore, ciprofloxacin is a known risk factor for *C. difficile* infection (Owens et al., 2008). Compared to newer generation fluoroquinolones, ciprofloxacin has less coverage of Gram-positive and anaerobic bacterial organisms, but has extended activity against Gram-negative aerobes such as *Pseudomonas aeruginosa* (Oliphant, 2002). Given its low cost and decreased toxicity compared to newer generation fluoroquinolones, ciprofloxacin is still often prescribed for a variety of clinical indications including skin, bone, joint, lower respiratory, and urinary tract infections (FDA, 2004).
In this study, only a select group of compounds produced by defined microbial ecosystems were investigated (see Table 2.4). We elected to highlight the SCFAs, especially acetate, butyrate, and propionate, as these bacterial fermentation products are general indicators of microbial metabolism and have known benefits to both host cells and the gut microbiota (Ríos-Covián et al., 2016; Wong et al., 2006). However, bile acids have also been shown to be important gut microbial ecosystem modulators (Begley et al., 2005; Ridlon et al., 2014), and alterations to the levels of secondary and primary bile acids in the gut have been observed after antibiotic treatments (Theriot et al., 2016; Theriot et al., 2014; Vrieze et al., 2014). In the present study, we did not investigate bile acid homeostasis in the defined microbial ecosystems characterized, but additional NMR experiments to elucidate the quantities of bile acids is an important future direction of this work.

Limitations of this study include the small number of biological replicates and lack of temporal analysis when investigating taxonomic and metabonomic changes in the defined microbial ecosystems grown in the chemostat vessel. Chemostat vessel preparation and maintenance are extensive procedures and because both singular and duplicate biological replicates have been published in previous work, we limited our analysis to two replicate vessels per ecosystem (Baines et al., 2005; Carman and Woodburn, 2001; Crowther et al., 2014; Freeman et al., 2005; Freeman et al., 2012; Hopkins and Macfarlane, 2003; Santiago-Rodriguez et al., 2015). Increasing the number of biological replicate vessels would allow for increased statistical power and the ability to discern taxonomic changes between these defined microbial ecosystems with greater confidence. Although defined microbial communities have been shown to reach a steady-state composition much earlier than whole fecal communities (McDonald et al., 2013; Schroeter, 2014), it would be valuable to extend the duration of the DEC58+cipro vessels
to examine the long-term ecological implications (e.g. stability and resilience) that may result from this specific antibiotic exposure.

Additionally, this study did not take into consideration the host-derived influence on gut bacterial ecosystems. Although mucin was added as a substrate to the chemostat medium for bacterial growth, host diet, genetics, immunological responses, epithelial cell homeostasis, and other variables were not investigated, but are important in the context of gut microbial diversity (Falony et al., 2016; Nuding et al., 2013; Wang et al., 2016). Conversely, variations in ecosystem dynamics precipitated by host-related effects are bypassed using this gut model, allowing for more robust and precise analyses of microbe-microbe interactions (Fujisaka et al., 2016). Because bacterial taxonomic inclusion was pre-determined, a situation unlike that employed in large cohort human population studies, interindividual (chemostat) variation is inherently reduced compared to that of fecal samples.

Furthermore, only one segment of the gut, the distal colon, was modeled using our system. Spatial location of the gut microbiota in the human GI tract has been shown to influence gut microbiota development and maintenance, which is applicable to human health and perturbational states (Donaldson et al., 2016; Li et al., 2015). For example, antibiotic or diet-induced perturbations may affect the luminal-associated gut microbiota to a greater extent than a community that exists in colonic microenvironments, the latter would include the biofilms, mucosal layers, and crypts (Donaldson et al., 2016). However, unlike sequencing or metabonomic analysis on fecal samples, our model does take into consideration the biogeography of the gut microbiota, although this is so far limited to the distal colon. This qualification should be considered when interpreting results or generating future hypotheses from this work.
Modulations to gut microbial ecosystems in response to the perturbations induced here can be applied broadly to the therapeutic setting. For example, as MET-1 has been used to successfully treat two patients with rCDI, examination of this therapeutic ecosystem’s ability to influence *C. difficile* in comparison with other defined microbial ecosystems studied will help determine the importance of gut microbial composition and function in this disease-state. Ultimately, these basic mechanistic studies will guide the development of robust and effective gut microbial ecosystems for CDI and other conditions where the gut microbiota is implicated.
Chapter 4: Examining the effects of defined microbial ecosystems on *C. difficile*

4.1 Introduction

FMT has been shown to be beneficial as a treatment for rCDI that works through modulation of the gut microbiome (see Table 1.1) and is currently recommended for patients with rCDI who are repeatedly non-responsive to antimicrobial therapy (HQO, 2016; Lapointe-Shaw et al., 2016; Merlo et al., 2016). Although FMT is highly effective for rCDI, the use of stool as a therapeutic presents many logistical, regulatory, and safety challenges (Carlucci et al., 2016). Consequently, stool-substitute therapies have been developed for rCDI to retain the beneficial therapeutic properties of FMT, while addressing many of the concerns surrounding the use of stool as a therapeutic agent (see gut microbiome-based therapies summary Table 1.3). Our group has developed a microbial ecosystem therapeutic (MET-1) formulation, which has been shown to cure rCDI in a recent proof-of-principle trial (Petrof et al., 2013). The MET-1 formulation aimed to improve upon FMT by using defined, standardized preparations of stool-based products, while preserving the compositional, metabolic, and transcriptomic properties of fecal communities. Currently, the mechanisms through which health is restored in rCDI patients after MET-1 treatment are largely unknown. Recently, MET-1 was shown to provide protection against *C. difficile* in a murine model of colitis through the inhibition of *C. difficile* TcdA. To understand the success of stool-substitute therapies for rCDI, we aimed to characterize the effects of gut microbial ecosystem-associated metabolites on *C. difficile* growth and virulence *in vitro*. Specifically, the effects of representative healthy and perturbed defined microbial ecosystem metabolites on vegetative cell growth, sporulation, toxin gene expression, toxin production and germination for *C. difficile* ribotype strains 027 and 078 were assessed. Additionally, the impact
of *C. difficile* metabolism on the targeted metabonomic profiles of defined microbial ecosystems was examined.

1. I hypothesize that the sterile cell-free supernatants derived from defined microbial ecosystems representative of a healthy state (MET-1 and DEC58) will have a greater antagonistic impact on *C. difficile* growth and virulence than the cell-free supernatants derived from perturbed defined microbial ecosystems (DEC58-Lachno and DEC58+cipro) – i.e., perturbed ecosystem components will either augment or have negligible effects on *C. difficile* growth and virulence *in vitro* compared to the behaviour of representative healthy ecosystems.

2. I hypothesize that alterations in the metabolic profiles of representative dysbiotic ecosystems may help to elucidate mechanisms of impaired antagonism of *C. difficile* growth and virulence compared to their native ecosystem counterparts.

3. Lastly, given the genetic and physiological heterogeneity observed among *C. difficile* isolates, I speculate that the *C. difficile* ribotype strains 027 and 078 examined in this study will exhibit distinct growth and virulence characteristics in response to the metabolites derived defined microbial ecosystem preparations.

4.1.1 Research objectives

To test the hypotheses listed above, we carried out several experiments under the following broad aims:

AIM 1: Determine changes in *C. difficile* vegetative cell growth and sporulation in response to cell-free supernatants derived from defined microbial ecosystems
AIM 2: Assess *C. difficile* germination and spore outgrowth in response to small molecules extracted from defined microbial ecosystems

AIM 3: Determine the effects of defined microbial ecosystems on *C. difficile* toxins

AIM 4: Identify metabolite signatures of cell-free defined-microbial ecosystem treated *C. difficile* culture supernatants

4.2 Results

4.2.1 AIM 1: Effects of defined microbial ecosystems on *C. difficile* vegetative cell growth and sporulation

To determine changes in *C. difficile* viability in response to cell-free supernatants derived from defined microbial ecosystems, the growth characteristics of each *C. difficile* ribotype isolate was measured in response to the metabolites of defined microbial ecosystems. Specifically, sterile cell-free supernatants from the four defined microbial ecosystems previously developed and characterized (see Chapter 3), were separately co-incubated for 24 or 48 h with a clinical isolate of *C. difficile* representative of ribotypes 027 or 078 (CD186 and CD973, respectively). Cell viability assays were completed in biological triplicate to determine alterations in vegetative cell growth and sporulation. To determine the vegetative cell count for each replicate experiment, the number of colony forming units (CFU) per mL recovered on solid medium after ethanol shock (spore count) was subtracted from matched observations of total cell counts spread-plated on the same medium.

MET-1, DEC58 and DEC58-Lachno defined microbial ecosystem supernatants increased *C. difficile* CD186 vegetative cell growth after 24 h *in vitro* in comparison to uninoculated medium control ($p \leq 0.0001$), while DEC58+cipro did not induce a change in vegetative cell
growth under these conditions ($p > 0.05$) (Figure 4.1 A). Conversely, after 48 h of incubation there were no significant differences in vegetative cell growth of this strain in response to any of the defined microbial ecosystems tested compared to control ($p > 0.05$) (Figure 4.1 A). For CD973, vegetative growth did not differ after 24 h in response to any of the defined microbial ecosystems ($p > 0.05$), except for DEC58 ($p \leq 0.01$). After 48 h, CD973 vegetative cell growth was significantly increased with treatment of DEC58-Lachno and DEC58+cipro ($p \leq 0.001$ and $p \leq 0.01$, respectively) (Figure 4.1 B).

*C. difficile* spore counts were determined via enumeration of CFU from ethanol-shocked liquid cultures plated on BHIS agar supplemented with 0.1% sodium taurocholate, a known *C. difficile* spore germinant. Treatment with the cell-free supernatants from DEC58, DEC58-Lachno and DEC58+cipro decreased CD186 spore recovery on this medium ($p \leq 0.05$), while the cell-free supernatant from MET-1 increased sporulation of this 027-ribotype strain after 24 h ($p \leq 0.001$) (Figure 4.1 C). After 48 h, a significant decrease in spore recovery remained for the cell-free supernatants of DEC58, DEC58-Lachno, and DEC58+cipro ($p \leq 0.01$) compared to control, though spore recovery for CD186 treated with the cell-free supernatants of MET-1 was not significantly different ($p > 0.05$) (Figure 4.1 C). Treatment with MET-1 cell-free supernatants resulted in increased spore recovery of CD973 at both 24 and 48 h ($p \leq 0.05$ and $p \leq 0.0001$ respectively) (Figure 4.1 D). In contrast, there was no significant difference in spore recovery at 24 h ($p > 0.05$) and decreased spores were observed at 48 h in response to the cell-free supernatants of DEC58, DEC58-Lachno, and DEC58+cipro ($p \leq 0.01$, $p \leq 0.001$, and $p \leq 0.01$ respectively).
Figure 4.1 *C. difficile* vegetative cell growth and sporulation after treatment with filter-sterilized defined microbial ecosystems for 24 and 48 h. Vegetative cell counts for CD186 (A) and CD973 (B) were determined by subtracting the total number of spores produced by CD186 (C) and CD973 (D) from the total cell count, respectively. Colony forming units represent cells that have completed sporulation, resisted ethanol treatment, completed germination and returned to vegetative cell growth. Control bars indicate treatment with the cell-free supernatant from sterile uninoculated chemostat medium. Error bars represent the standard error of the means for three replicate experiments. *P*-values were determined using a one-way ANOVA followed by Dunnett’s multiple comparisons test. NS, *p > 0.05*; *, *p ≤ 0.05*; **, *p ≤ 0.01*; ***, *p ≤ 0.001*; ****, *p ≤ 0.0001*. 

---

**A**
- Control
- MET-1
- DEC58
- DEC58-Lachno
- DEC58+clpro

**B**
- Control
- MET-1
- DEC58
- DEC58-Lachno
- DEC58+clpro

**C**
- Control
- MET-1
- DEC58
- DEC58-Lachno
- DEC58+clpro

**D**
- Control
- MET-1
- DEC58
- DEC58-Lachno
- DEC58+clpro
4.2.2 AIM 2: Determination of *C. difficile* germination and spore outgrowth in response to defined microbial ecosystems

To determine the germination rates of *C. difficile* isolates in response to metabolites of defined microbial ecosystems, purified spore suspensions were resuspended in BHI containing ethyl acetate extracted metabolites from each defined microbial ecosystem. After the addition of metabolites from defined microbial ecosystems, uninoculated chemostat medium or BHI control, 2 mM sodium taurocholate was added to each well to stimulate the germination of *C. difficile* spores. The transition of spores into vegetative cells was then measured anaerobically at 37°C over two time points in the spore loss of OD₆₀₀ experiment (Figure 4.2). Between samples, a reduced OD₆₀₀ value at $t = 0.25$ in comparison to $t = 0$ would indicate increased germination.

Interestingly, germination did not significantly differ when comparing any defined microbial treatment group to other groups or to chemostat medium control for either CD186 or CD973 ($p > 0.05$ for all ecosystem metabolite groups tested).

To investigate whether *C. difficile* spore outgrowth differed in response to ethyl acetate-extracted metabolites from defined microbial ecosystems, we measured the return of *C. difficile* vegetative cell growth from germinated spores by OD₆₀₀ over approximately 22 h. Upon entering stationary phase, maximum OD₆₀₀ values were measured for the ethyl acetate-extracted defined microbial ecosystem and control samples described above.

By area under the curve (AUC) analysis, spore outgrowth levels were assessed in response to ethyl acetate-extracted metabolites from defined microbial ecosystems and controls. Ethyl acetate-extracted metabolites from MET-1 significantly decreased the mean AUC for CD186 compared to DEC58+cipro ($p < 0.0001$) and DEC58-Lachno ($p < 0.0001$) metabolite extracts (Figure 4.2 A). In contrast, spore outgrowth did not differ between MET-1 and sterile...
Figure 4.2 *C. difficile* spore loss of OD$_{600}$ in response to defined microbial ecosystem metabolites. Purified spores from CD186 (A) and CD973 (B) were resuspended in ethyl acetate extracted metabolite formulations derived from defined microbial ecosystems and incubated anaerobically at 37°C for 24 h. Error bars represent the standard error of the mean OD$_{600}$ value obtained from three replicate experiments. Area under the curve (AUC) analysis was completed for spore outgrowth of *C. difficile* ribotype 027 (CD186) and 078 (CD973) in response to ethyl acetate-extracted defined microbial ecosystems (C). Error bars represent the standard error of three replicate experiments. Pairwise comparisons were made between all groups, using a one-way ANOVA followed by Tukey's multiple comparisons test in Graph Pad Prism 7.0. NS, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. 
chemostat control metabolite extracts in CD186 \( (p = 0.3041) \). Ethyl acetate-extracted metabolites from DEC58 significantly decreased the mean AUC for CD186 spore outgrowth compared to DEC58+cipro \( (p < 0.0001) \) but not DEC58-Lachno \( (p = 0.1608) \). Strikingly, CD186 reached highest cell density as measured by OD\(_{600}\) values in cultures treated with DEC58+cipro extracted metabolites. In CD973, mean OD\(_{600}\) values were lower for all treatments/controls compared to CD186, and this was also confirmed using AUC analysis (Figure 4.2). In contrast to CD186, spore outgrowth for CD973 was consistent in response to metabolite extracts of MET-1 compared to DEC58+cipro \( (p = 0.4874) \) and DEC58-Lachno \( (p = 0.9994) \) (Figure 4.2 B). However, in comparison to the DEC58, the metabolite extracts from MET-1 \( (p < 0.0001) \), DEC58-Lachno \( (p < 0.0001) \), DEC58+cipro \( (p = 0.0001) \), and chemostat medium control \( (p < 0.0001) \) all increased CD973 spore outgrowth.

4.2.3 AIM 3: Effects of defined microbial ecosystems on *C. difficile* toxins

A. Effects of defined microbial ecosystems on *C. difficile* toxin gene expression

To understand whether *C. difficile* toxin gene expression was influenced by components of our tested microbial ecosystems, RT-qPCR assays were carried out on *C. difficile* grown in BHI and mixed in equal volumes with sterile cell-free supernatants of defined microbial ecosystems or chemostat medium control. Briefly, changes in the expression of \( tcdA, tcdB, cdtA, \) and \( cdtB \) of *C. difficile* were investigated (see Table 2.6 for properties of RT-qPCR primers used in this study).

At 12 h, the metabolite supernatants from MET-1 increased the expression of all toxin genes examined, except for \( cdtA \) CD973 \( (p = 0.4014) \) and \( cdtB \) CD186 \( (p = 0.2716) \) (Figure 4.3). Additionally, the metabolite supernatants from all other defined microbial ecosystems
Figure 4.3 *C. difficile* toxin gene expression after incubation with cell-free defined microbial ecosystem supernatants for 12 h. Negative values represent a repression in gene transcription whereas positive values represent an increase in transcription compared to control, *C. difficile* incubated with the cell-free supernatant of chemostat medium. Error bars represent the standard error of the means for at least three technical and biological replicate experiments. *P*-values were determined using a one-way ANOVA followed by Dunnett’s multiple comparisons test. For non-normally distributed ΔCq data (CD186 *cdtB*, and CD973 *cdtA* and *cdtB*), a Kruskal-Wallis test followed by Dunn’s multiple comparisons test was performed. NS, *p* > 0.05; *, *p* ≤ 0.05; **, *p* ≤ 0.01; ***, *p* ≤ 0.001; ****, *p* ≤ 0.0001.
significantly increased \( cdtA \) expression in CD186; however, only DEC58 and DEC58+cipro increased the expression of this toxin gene in CD973 \((p = 0.0423\) and \(p = 0.0056\), respectively). None of the culture supernatants from defined microbial ecosystems significantly repressed toxin gene expression in either of the ribotype isolates tested at this time point.

Metabolites from MET-1 supernatants significantly repressed the expression of all toxin genes investigated in CD186 after 24 h \((tcdA, p \leq 0.0001; \ tcdB, p = 0.0056; \ cdtA, p \leq 0.0001; \ cdtB, p \leq 0.0001)\) (Figure 4.4). Additionally, at the 24 h time point, DEC58, DEC58-Lachno, and DEC58+cipro metabolite supernatants repressed \( tcdA \) and \( cdtB \) expression, but not \( tcdB \) or \( cdtA \) \((p > 0.05)\). Conversely, none of the defined microbial ecosystem metabolite supernatants influenced toxin gene expression \((p > 0.05)\) in CD973, except for MET-1, which was shown to repress only \( cdtB \) at 24 h \((p \leq 0.05)\).

At 48 h the changes to toxin gene expression in response to defined microbial ecosystems were less consistent. Similar to the 24 h time point, repression of \( tcdA \) was observed in CD186 for treatment with DEC58 and DEC58-Lachno metabolite supernatants; however, \( tcdA \) expression was not altered by DEC58+cipro at 48 h (Figure 4.5). In contrast to the toxin gene expression results obtained after 24 h, MET-1 significantly increased the expression of all CD186 toxin genes examined at 48 h. Additionally, the metabolite supernatants of DEC58 and DEC58+cipro, but not DEC58-Lachno significantly induced expression of both CDT genes at 48 h. In CD973, all defined microbial ecosystem metabolite supernatants increased expression of \( tcdA, cdtA \) and \( cdtB \), except for MET-1, which did not affect levels of \( cdtA \) and \( cdtB \) after 48 h.

Of note, expression of \( tcdB \) in CD973 could not be determined at the 12, 24 or 48 h time points as the \( C_q \) values were below the detection limit of qPCR (Figures 4.3-4.5).
Figure 4.4 *C. difficile* toxin gene expression after incubation with cell-free defined microbial ecosystem supernatants for 24 h. Negative values represent a repression in gene transcription whereas positive values represent an increase in transcription compared to control, *C. difficile* incubated with the cell-free supernatant of chemostat medium. Error bars represent the standard error of the means for three replicate experiments. *P*-values were determined using a one-way ANOVA followed by Dunnett’s multiple comparisons test. For non-normally distributed ∆Cq data (CD186 tcdB), a Kruskal-Wallis test followed by Dunn’s multiple comparisons test was performed. NS, *p* > 0.05; *, *p* ≤ 0.05; **, *p* ≤ 0.01; ***, *p* ≤ 0.001; ****, *p* ≤ 0.0001.
Figure 4.5 *C. difficile* toxin gene expression after incubation with cell-free defined microbial ecosystem supernatants for 48 h. Negative values represent a repression in gene transcription whereas positive values represent an increase in transcription compared to control, *C. difficile* incubated with the cell-free supernatant of chemostat medium. Error bars represent the standard error of the means for three technical and biological replicate experiments. *P*-values were determined using a one-way ANOVA followed by Dunnett’s multiple comparisons test. NS, *p > 0.05*; *, *p ≤ 0.05*; **, *p ≤ 0.01*; ***, *p ≤ 0.001*; ****, *p ≤ 0.0001.*
B. Effects of defined microbial ecosystems on *C. difficile* secreted TcdA and TcdB levels

i. Toxin secretion in CD186

To corroborate toxin gene expression findings, the effects of defined microbial ecosystem metabolite supernatants on secreted toxin production in *C. difficile* were also examined. Because decreases were specifically observed in the 24 and 48 h toxin gene expression data, we further investigated toxin (protein) production at these time points. In agreement with *tcdA* and *tcdB* gene expression results, at the 24 h time point, there were significant decreases in the levels of TcdA and TcdB secreted by CD186 exposed to the cell-free supernatants derived from MET-1 (*p* ≤ 0.0001), DEC58 (*p* < 0.0001), DEC58-Lachno (*p* < 0.0001) and DEC58+cipro (*p* ≤ 0.0066) compared to chemostat medium control. Similarly, at the 48 h time point there were significant decreases in TcdA and TcdB production by CD186 exposed to the cell-free supernatants derived from MET-1 (*p* ≤ 0.0119), DEC58 (*p* ≤ 0.0195), DEC58-Lachno (*p* ≤ 0.0079) and DEC58+cipro (*p* ≤ 0.0066) compared to chemostat medium control, with the exception of DEC58+cipro, where a significant decrease in TcdB was observed (*p* ≤ 0.0116), but not TcdA (*p* = 0.5255) (Figure 4.6 and 4.7).

However, at 24 h, there were no differences in the toxin levels secreted by CD186 exposed to metabolite profiles derived from MET-1 (*p* ≥ 0.9294), DEC58 (*p* ≥ 0.8338) and DEC58-Lachno (*p* ≥ 0.8338). Similarly, at the 48 h time point there were no differences in toxin levels secreted by CD186 exposed to metabolites derived from MET-1 (*p* ≥ 0.9596), DEC58 (*p* ≥ 0.8126) and DEC58-Lachno (*p* ≥ 0.8126). CD186 toxin A and B production was increased in DEC58+cipro compared to both DEC58 (*p* ≤ 0.0091) and DEC58-Lachno at 24 h (*p* ≤ 0.0043), but not at 48 h (*p* ≥ 0.3480, *p* ≥ 0.1608 for DEC58 and DEC58-Lachno respectively).
Figure 4.6 *C. difficile* secreted toxin A and B levels are modulated by cell-free defined microbial ecosystem supernatants after 24 h co-incubation. TcdA and B levels of CD186 (A) and CD973 (B) were quantified using the TGC biomics-E002 ELISA kit. Quantities shown are relative to the mean of matched vegetative cell counts for each sample. All ecosystem treatments for CD186 (A) were significantly decreased compared to chemostat medium control at 24 and 48 h. Vegetative cell counts were calculated by subtracting the spore recovery count from the total cell count obtained by spread-plating culture. Error bars represent the standard error of the means for three replicate experiments. *P*-values were determined using a one-way ANOVA followed by Tukey’s pairwise comparisons test. *, *p* ≤ 0.05; **, *p* ≤ 0.01; ***, *p* ≤ 0.001; ****, *p* ≤ 0.0001. Non-significant trends were omitted for clarity.
Figure 4.7 *C. difficile* secreted toxin A and B levels are modulated by cell-free defined microbial ecosystem supernatants after 48 h co-incubation. TcdA and B levels of CD186 (A) and CD973 (B) were quantified using the TGC biomics-E002 ELISA kit. Quantities shown are relative to the mean of matched vegetative cell counts for each sample. All ecosystem treatments for CD186 (A) were significantly decreased compared to chemostat media control at 24 and 48 h. Vegetative cell counts were calculated by subtracting the spore recovery count from the total cell count obtained by spread-plating culture. Error bars represent the standard error of the means for three replicate experiments. *P*-values were determined using a one-way ANOVA followed by Tukey's pairwise comparisons test. *, *p* ≤ 0.05; **, *p* ≤ 0.01; ***, *p* ≤ 0.001; ****, *p* ≤ 0.0001. Non-significant trends were omitted for clarity.
ii. Toxin secretion in CD973

Toxin levels secreted by CD973 in response to all defined microbial ecosystem metabolites were similar to CD186 at 48 h, but not at 24 h (Figure 4.6 and 4.7). In comparison to chemostat medium control, the metabolites from MET-1, DEC58 and DEC58+cipro defined microbial ecosystems decreased TcdA secretion at the 24 h ($p \leq 0.0068$, $p \leq 0.0031$, $p \leq 0.0068$ respectively) and 48 h ($p \leq 0.0021$, $p \leq 0.0033$, $p \leq 0.0020$ respectively) time points.

Interestingly, CD973 toxin production did not vary in response to the defined microbial ecosystem metabolites of DEC58-Lachno at 24 h ($p = 0.7233$), but decreased at 48 h ($p = 0.0079$) in comparison to control. TcdB levels were differentially expressed at the 24 and 48 h time points. While there was no significant change in TcdB quantity after 24 h treatment with metabolite supernatants from MET-1 ($p = 0.9996$), DEC58 ($p = 0.1184$), DEC58-Lachno ($p = 0.4153$), DEC58+cipro ($p = 0.1263$) compared to control, TcdB was decreased in response to the metabolite supernatants derived from MET-1 ($p \leq 0.0051$), DEC58 ($p \leq 0.0008$), DEC58-Lachno ($p = 0.0155$), DEC58+cipro ($p = 0.0007$) after 48 h. Between representative healthy ecosystem metabolites of MET-1 and DEC58 in CD973, there were no significant differences in toxin production detected at 24 h (TcdA, $p = 0.9795$; TcdB, $p = 0.0878$) or 48 h (TcdA, $p = 0.9972$; TcdB, $p = 0.6719$). Similarly, there were no significant changes in CD973 toxin production in the defined microbial ecosystem metabolites of DEC58+cipro vs. DEC58 at the 24 h (TcdA, $p = 0.9801$; TcdB, $p > 0.9999$) and 48 h (TcdA, $p = 0.9956$; TcdB, $p > 0.9999$) time points investigated. However, the metabolite supernatants derived from DEC58-Lachno increased TcdA quantities in CD973 at 24 h compared to treatment with the metabolite supernatants of DEC58 ($p = 0.0006$) and DEC58+cipro ($p = 0.0012$).
C. Effects of defined microbial ecosystems on *C. difficile* toxin activity in NIH 3T3 fibroblast cells

To determine if the cell-free supernatants from defined microbial ecosystems could diminish the cytopathic effects of TcdA or TcdB, we used an established cell-rounding assay with NIH 3T3 fibroblast cells. Because NIH 3T3 cells are morphologically filamentous, TcdA and TcdB-induced rounding can be identified microscopically and used to assess the cytopathic effects to cells under different experimental conditions. Pre-treatment of NIH 3T3 cells with the cell-free supernatants from any defined microbial ecosystem did not decrease cell rounding compared to chemostat medium controls in response to the addition of TcdA (Figure 4.8 A). However, in comparison to chemostat medium control, increased cell rounding was observed in TcdA-treated NIH 3T3 cells after pre-treatment with DEC58-Lachno \((p = 0.0335)\) and DEC58+cipro \((p = 0.0125)\) cell-free defined microbial ecosystem supernatants.

Interestingly, pre-treatment of NIH 3T3 cells with the cell-free supernatants of DEC58 and DEC58-Lachno significantly decreased TcdB-induced cell rounding compared to chemostat medium control \((p < 0.0001\) and \(p = 0.0009\) respectively) (Figure 4.8 B). Conversely, this effect was not observed in response to MET-1 \((p = 0.1179)\) or DEC58+cipro \(0.1488)\) metabolite supernatants. Though cell-free MET-1 supernatants did not decrease TcdB-induced cell rounding in comparison to chemostat media, cell rounding did not significantly differ to that observed in negative control treated NIH 3T3 cells in this group \((p = 0.0510)\) (Figure 4.8 C). Similarly, the cell rounding effects in response to DEC58 and DEC58-Lachno metabolite supernatants after TcdB treatment was not significantly different than that observed in negative control treated NIH 3T3 cells \(p = 0.7644, p = 0.2411\) respectively).
Figure 4.8 Cell-free metabolite supernatants of DEC58 and DEC58-Lachno confer protection against TcdB cytotoxicity. NIH 3T3 fibroblast cells were seeded in a 24-well plate and grown to 80% confluency in high glucose DMEM supplemented with 10% FBS, 1% penicillin/streptomycin solution (Thermo Fisher Scientific) and 0.2% Plasmocin (InvivoGen) in an incubator at 37ºC supplied with 5% CO₂. Cells were pretreated with cell-free defined microbial ecosystem supernatants and incubated for 4 h. Following incubation, 400 pg/mL of TcdA (A) and 2 pg/mL of TcdB (B) from C. difficile 630 dissolved in new media was added to PBS washed wells and plates were incubated for a further 2 h. The effects of cell-free defined microbial ecosystem supernatants without the addition of toxins (C) are shown in comparison to TcdA and TcdB treated samples. Images were taken with a DM IL LED inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a Jenoptik ProgRes CT3 digital microscope camera. Errors bars represent the standard error of the means for at least three replicate experiments. p-values were determined using a one-way ANOVA followed by Dunnett’s multiple comparisons test in GraphPad Prism 7.0. NS, p > 0.05; *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001.
4.2.4 AIM 4: Targeted metabolite profiling of samples using 1D $^1$H NMR spectroscopy

To determine metabolite signatures of cell-free defined microbial ecosystem treated *C. difficile* culture supernatants, a targeted approach was used to analyze 1D $^1$H NMR-derived spectra for three replicate experiments. A visualization of the 2D PLS-DA scores plots (produced in MetaboAnalyst) reveals distinct clusters of each treatment group based on normalized compound concentrations for both CD186 (Figure 4.9 A) and CD973 (Figure 4.9 B). The quality and robustness of each model was validated using $Q^2$ assessment and permutation testing; these measures prevent data over-fitting and ensure the statistical model is built correctly. For the CD186 data set, using four components gave the best performance based on $Q^2$ assessment ($Q^2 = 0.98194$; see Figure A 4.2). For the CD973 data set, using five components gave the best performance based on $Q^2$ assessment ($Q^2 = 0.97873$; see Figure A 4.3). Principal components (PC) that explained the largest amount of variance were included in the 2D plots. For the targeted metabolite profiles of defined microbial ecosystem treated CD186, PC 1 and 2 accounted for 84.9% of the total variance, whereas PC 1 and 3 accounted for 59.8% of the total variance in the CD973 group of samples.

A. Multivariable analysis of metabonomic data from cell-free defined microbial ecosystem treated CD186 culture supernatants

In the CD186 2D PLS-DA plot, MET-1, DEC58 and DEC58-Lachno treatment groups visually cluster close together, while control and DEC58+cipro are distinctly separated (Figure 4.9 A). Compounds contributing to the increased separation of each treatment group are illustrated in the corresponding 2D PLS-DA loading plot (Figure 4.9 A). Alanine, methionine, tyrosine, galactose, glucose, lactate, isovalerate, formate, and fumarate are the prominent compounds contributing to the separation of DEC58+cipro. Compounds including acetate,
Figure 4.9 2D PLS-DA score and loading plots of normalized targeted metabolites from *C. difficile* treated with defined microbial ecosystems. Score and loading plots from CD186 (A) and CD973 (B) were constructed using normalized metabolomic data generated in turn from 1D $^1$H NMR spectroscopy concentration data. Normalization of concentration data of both CD186 and CD973 data sets was completed and scores plots, cross-validation and permutations of both PLS-DA models were performed and generated using Metaboanalyst 3.0.
butyrate, propionate, lysine, isoleucine, glutamate, glycine, \( p \)-cresol and choline are responsible for the separation observed in MET-1, DEC58 and DEC58-Lachno treatment groups. Additionally, mean normalized compound concentration values for the CD186 data set were used to generate heat-maps for both SCFAs and the remaining compounds within the treatment groups (Figure 4.10). For the data presented in CD186 heat maps, the mean normalized compound concentration standard deviations ranged from 0-1.26 units (raw data not shown).

B. SCFA levels in cell-free defined microbial ecosystem treated CD186 culture supernatants

The relative mean normalized concentrations of SCFA compounds present in the supernatants of each defined microbial ecosystem treated CD186 culture group are illustrated in Figure 4.10 A. Below we detail the observed differences in the production of SCFAs among select defined microbial ecosystem treatment groups.

i. MET-1 vs. DEC58

Greater levels of acetate (\( p < 0.0001 \)) and butyrate (\( p < 0.0001 \)) were observed in DEC58 compared to MET-1 microbial ecosystem treated CD186 supernatants (Figure 4.10 A). Formate and isovalerate concentrations did not vary amongst MET-1 and DEC58 treatment groups (\( p = 0.9293 \) and \( p > 0.9999 \)), while propionate was found at greater levels in MET-1 compared to DEC58 (\( p = 0.002 \)).

ii. DEC58-Lachno vs. DEC58

Acetate, butyrate, and propionate levels were significantly decreased in DEC58-Lachno microbial ecosystem treated CD186 supernatants compared to DEC58 (\( p < 0.0001 \), \( p < 0.0001 \), and \( p = 0.0166 \) respectively), while formate, isobutyrate, and isovalerate levels in DEC58-Lachno did not significantly differ in comparison to the DEC58 treatment group (\( p = 0.9293 \), \( p = 0.8904 \), and \( p > 0.9999 \) respectively).
Figure 4.10 Targeted metabolite profiling of *C. difficile* treated with cell-free defined microbial ecosystem supernatants. Mean normalized concentrations of CD186 (A) and CD973 (B) treated with cell-free defined microbial ecosystem supernatants are shown. All compounds were determined using 1D $^1$H NMR spectroscopy. *C. difficile* treated with uninoculated sterile chemostat medium was also profiled as a control. Normalized compound concentrations were generated using Metaboanalyst 3.0 and the heat-map was constructed using GraphPad Prism 7.0.
0.5294, and $p > 0.9999$) (Figure 4.10 A).

iii. DEC58+cipro vs. DEC58

Acetate, butyrate, isobutyrate, and propionate levels were significantly decreased in DEC58+cipro microbial ecosystem treated CD186 supernatants compared to DEC58 ($p < 0.0001$) (Figure 4.10 A). Interestingly, levels of acetate and propionate were decreased to levels that were not significantly different than those observed in the chemostat medium control group ($p = 0.1021$ and $p = 0.9937$ respectively). Formate and isovalerate were both increased in DEC58+cipro microbial ecosystem treated CD186 supernatants comparison to DEC58 ($p < 0.0001$). Although significantly decreased from control, formate levels in DEC58+cipro were significantly higher compared to MET-1, DEC58 and DEC58-Lachno treatment groups ($p < 0.0001$). Of note, isovalerate was the only SCFA to be increased in DEC58+cipro microbial ecosystem treated CD186 supernatants compared to all other experimental groups ($p < 0.0001$).

iv. Other compounds of interest

In CD186, lower levels of choline were observed in MET-1 ($p = 0.0009$), DEC58 ($p = 0.0099$) and DEC58-Lachno ($p = 0.0001$) microbial ecosystem treated CD186 supernatants compared to DEC58+cipro. Also, isoleucine was present in higher quantities in MET-1 ($p = 0.0023$), DEC58 ($p < 0.0001$) and DEC58-Lachno ($p < 0.0001$) microbial ecosystem treated CD186 supernatants compared to the DEC58+cipro treatment group (Figure 4.10 A). Glycine was another metabolite detected at greater levels in the cell-free supernatants of CD186 treated with MET-1 ($p < 0.0001$) and DEC58-Lachno ($p = 0.0002$) compared to DEC58. A significant increase in both glucose and lactate was also observed in DEC58+cipro microbial ecosystem treated CD186 supernatants compared to all other ecosystem treatment groups examined ($p <$
C. Multivariable analysis of metabonomic data from cell-free defined microbial ecosystem treated CD973 culture supernatants

Similar to the CD186 multivariable analysis metabonomic results (see above), control and DEC58+cipro cell-free defined microbial ecosystem treated CD973 culture metabolite groups are distinctly separated from MET-1 and DEC58 groups as illustrated in the 2D PLS-DA plot (Figure 4.9 B). Compounds contributing to the increased separation of each treatment group are shown in the corresponding PLS-DA loading plot (Figure 4.9 B). Glycine, isoleucine, methionine, valine, galactose, and glucose are main compounds contributing to the separation of the DEC58+cipro treatment group. Compounds including acetate, butyrate, propionate, succinate, lysine, glutamate, betaine, p-cresol, and choline are responsible for the separation observed of MET-1, DEC58 and DEC58-Lachno treatment groups in the 2D PLS-DA plot. Additionally, mean normalized compound concentration values for the CD973 data set were used to generate heat-maps for both SCFAs and other targeted compounds within the treatment groups (Figure 4.10 B). For the data presented in CD973 heat maps, the mean normalized compound concentration standard deviations ranged from 0-2.23 units (raw data not shown).

D. SCFA levels in cell-free defined microbial ecosystem treated CD973 culture supernatants

i. MET-1 vs. DEC58

Similar to the levels of SCFA observed in cell-free defined microbial ecosystem treated CD186 culture supernatants, none of the SCFAs investigated were present in higher quantities in the cell-free components of MET-1 compared to DEC58 treated CD973 culture supernatants (Figure 4.10 B). Increased levels of acetate \((p < 0.0001)\), butyrate \((p < 0.0001)\) and formate \((p < 0.0001)\) for both glucose and lactate).
0.0001) were observed CD973 treated with DEC58 microbial ecosystem supernatants, compared to MET-1, while there was no change in isobutyrate and propionate concentrations under the same conditions ($p = 0.9946$ and $p = 0.9649$ respectively).

ii. DEC58-Lachno vs. DEC58

In contrast to the SCFA metabolite profiles of cell-free defined microbial ecosystem treated CD186 culture supernatants, acetate ($p < 0.0001$), butyrate ($p < 0.0001$), formate ($p < 0.0001$) and propionate ($p < 0.0001$) were all significantly lower in DEC58-Lachno CD973 culture supernatants compared to DEC58. Of note, there were no detected levels of isobutyrate or isovalerate in either of these ecosystems (Figure 4.10 B).

iii. DEC58+cipro vs. DEC58

Acetate ($p < 0.0001$), butyrate ($p < 0.0001$), formate ($p < 0.0001$) and propionate ($p < 0.0001$) levels were significantly lower in cell-free DEC58+cipro treated CD973 culture supernatants compared to DEC58 (Figure 4.10 B). Interestingly, the levels of acetate, butyrate and propionate in DEC58+cipro treated CD973 culture supernatants were similar to those detected in the chemostat medium control group ($p = 0.9423$, $p = 0.2899$ and $p = 0.999$ respectively). Isobutyrate was the only SCFA investigated that was greater in DEC58+cipro treated CD973 culture supernatants compared to DEC58 ($p < 0.0001$). In the CD973 group, formate levels in cell-free DEC58+cipro treated CD973 culture supernatants were significantly lower compared to DEC58 ($p < 0.0001$) and chemostat medium control ($p < 0.0001$), but significantly higher compared to the DEC58-Lachno ($p < 0.0001$) treatment group.
v. Other compounds of interest

The level of lactate was lower in the cell-free supernatant DEC58-Lachno treated CD973 group compared to that of DEC58+cipro \( (p = 0.0004) \) (Figure 4.10 B). Choline levels were consistent between cell-free DEC58-Lachno and DEC58+cipro treated CD973 supernatants \( (p = 0.14) \), but lower in both these groups compared to the cell-free supernatant DEC58-treated group \( (\text{DEC58-Lachno}, p = 0.0005; \text{DEC58+cipro}, p = 0.0048) \). A significant increase in glucose was also observed in DEC58+cipro treated CD973 supernatants compared to the remaining ecosystem groups examined \( (p < 0.0001 \text{ for MET-1, DEC58, and DEC58-Lachno treatment group}) \). Levels of isoleucine were greater in the DEC58+cipro treated CD973 supernatants than the DEC58-Lachno group \( (p = 0.0003) \), and both dysbiotic ecosystem treated CD973 supernatant groups contained higher quantities of this compound than MET-1 \( (p < 0.0001) \), DEC58 \( (p < 0.0001) \) and chemostat medium control \( (p < 0.0001) \) groups.

4.3 Discussion

To determine the potential mechanisms that may be responsible for the positive clinical outcome of use of MET-1 to treat rCDI, we aimed to investigate the effects of metabolites derived from this community as well as other defined microbial ecosystems (as detailed in Chapter 3) on \textit{C. difficile} growth, virulence, toxins, and metabolism \textit{in vitro}.

Based on compositional and functional studies of the human gut microbiota, we hypothesized that the representative healthy ecosystems (MET-1 and DEC58) would have a greater antagonistic impact on \textit{C. difficile} growth compared to representative perturbed ecosystems (DEC58-Lachno and DEC58+cipro). However, in contrast to our expectations we found that vegetative cell growth of strain CD186 was increased compared to control in response
to exposure to supernatants of all microbial ecosystems tested, except for DEC58+cipro after 24 h. To determine if residual ciprofloxacin influenced *C. difficile* vegetative cell growth, we tested the antibiotic resistance profile of this CD186 isolate (Table A 4.1). Similar to other 027 isolates examined (Drudy et al., 2007), CD186 was resistant to ciprofloxacin (as determined through disc diffusion antibiotic sensitivity testing with 5 µg ciprofloxacin), indicating that this antibiotic did not influence vegetative cell growth of this strain at the 24 or 48 h time points.

Although it is uncertain why DEC58+cipro supernatants did not influence CD186 vegetative cell growth compared to MET-1, DEC58 and DEC58-Lachno treatment groups at 24 h, metabonomic changes detected in the profiles of the latter ecosystems may potentially be responsible for these growth-altering effects (Figure 4.10 A). For example, the amino acids glycine, methionine and threonine are required for optimal growth of *C. difficile* in defined media (Bouillaut et al., 2015; Karasawa et al., 1995). The high levels of glycine in supernatants derived from chemostat cultures of either MET-1 or DEC58-Lachno groups may have supported the increased growth of CD186 in these samples. Additionally, we found increased abundance of acetate, butyrate, isobutyrate and propionate in MET-1, DEC58, and DEC58-Lachno cell-free supernatants compared to those of DEC58+cipro and chemostat medium control. SCFAs are known to support the growth of various gut microbial species including *C. difficile*, except butyrate, which is thought to have no effect on *C. difficile* vegetative growth as it produces this SCFA itself (Karlsson et al., 2003; Karlsson et al., 2000; Pettit et al., 2014).

Although vegetative cell growth in control-treated samples did not significantly vary between the different *C. difficile* strains at 24 h (see Figure 4.1 A and B and Figure A 4.1), CD973 vegetative growth was not increased in the cell-free supernatant MET-1 and DEC58-Lachno groups as they were in CD186 (Figure 4.1 B). Additionally, the cell-free supernatants
from both perturbed ecosystems increased vegetative cell growth of *C. difficile* CD973 at 48 h compared to control medium. These results underline the heterogeneous response of different *C. difficile* ribotypes to defined microbial ecosystem-derived metabolites. Investigation into the direct inhibitory effect of live MET-1 constituents (species) against *C. difficile* was also conducted for CD186 and CD973. Growth of CD186 and CD973 vegetative cells and spore recovery were not decreased by live MET-1 cultures compared to levels of control medium (Figure A 4.4), suggesting that neither direct microbial species interactions nor their associated metabolic products can inhibit the growth or sporulation of these *C. difficile* isolates *in vitro*.

In addition to the increased vegetative cell production, there was increased sporulation in response to cell-free metabolites from MET-1 treated CD186 and CD973 culture supernatants compared to all other defined microbial ecosystems and controls tested at 24 h (Figure 4.1 C and D). Furthermore, DEC58, DEC58-Lachno and DEC58+cipro cell-free defined microbial ecosystem supernatants decreased sporulation in CD186 at 24 and 48 h, and CD973 at 48 h compared to control. The environmental stimulus for *C. difficile* sporulation is complex and not fully understood, but is thought to begin under nutrient limiting conditions (Bouillaut et al., 2015). The apparent ability for MET-1 to increase sporulation is inconsistent with the abundance of nutrients present in the cell-free supernatants of this defined microbial ecosystem. Interestingly, methanol was significantly diminished in both CD186 and CD973 MET-1 metabonomic profiles compared to all other groups (Figures 4.10). As this compound has been previously shown to inhibit sporulation in *Bacillus subtilis* (Rigomier et al., 1980) future studies examining this link may elucidate whether this metabonomic change contributes to increased sporulation in this ecosystem. Clinically, increased sporulation of *C. difficile* in response to MET-1 components may provide relief for the host from *C. difficile* toxin production in the gut.
environment, presumably allowing the restoration of colonization resistance and subsequent inhibition of germination. Conversely, as spores are essential to the infectivity and transmissibility of *C. difficile*, increased sporulation may be unfavourable to recurrence and management of CDI.

Interestingly, ethyl acetate-extracted metabolite supernatants derived from MET-1 decreased stationary-phase spore outgrowth of CD186 but not CD973 when compared to similar extracts from DEC58+cipro and DEC58-Lachno (Figure 4.2). Nonetheless, CD186 spore outgrowth did not differ between the extracted metabolites from MET-1 and sterile chemostat controls. Strikingly, the highest mean OD<sub>600</sub> values detected were those in CD186 cultures treated with DEC58+cipro extracted metabolites. These results indicate that a component specific to the chemostat medium and MET-1 (thus one that is being metabolized by DEC58, DEC58-Lachno and DEC58+cipro) may be responsible for this growth limitation effect. As the ethyl acetate-extracted metabolites were not profiled by NMR spectroscopy (as undertaken for the *C. difficile*-treated defined microbial ecosystem samples), no speculations to the identity of such compound(s) could be made. Furthermore germination rates were not significantly different between the extracted metabolites from any defined microbial treatment group or chemostat medium control in the CD186 or CD973 loss of spore OD<sub>600</sub> assays. Taken together, these results suggest that the reduction of *C. difficile* viability is not the main mechanism by which microbial ecosystem therapeutics may be beneficial for CDI.

All defined microbial ecosystem cell-free supernatants decreased secreted levels of *C. difficile* TcdA and B by 48 h, with the exception of the cell-free supernatants of DEC58+cipro in CD186, where no decrease in TcdA was observed at the 48 h time point. This did not support our hypothesis whereby perturbed ecosystems were predicted to be impaired in their ability to
antagonise *C. difficile* toxin production. However, previous studies have described increases in TcdA and TcdB secreted levels by ribotype 027, toxinotypes III *C. difficile* isolates in response to subinhibitory ciprofloxacin exposure (Aldape et al., 2013), which may contribute to the results observed here. Collectively, this suggest that factors shared among all ecosystems may be responsible for inhibiting TcdA and B secretion or alternatively, the effect may be non-specific and a result of additional factors present in all defined microbial ecosystem that are not present in the filter-sterilised chemostat medium control. To assess these possibilities, we conducted 1D $^1$H NMR spectroscopy to analyze a targeted set of metabolites (see Chapter 3) within the 24 h defined microbial ecosystem and control-treated *C. difficile* cultures.

Although TcdA and B levels were lower in all cell-free defined microbial ecosystem-treated CD186 culture supernatants compared to control, secreted toxins in MET-1, DEC58, and DEC58-Lachno groups were all significantly decreased compared to DEC58+cipro at 24 h. Acetate, butyrate and propionate greatly contributed to the separation of MET-1, DEC58, and DEC58-Lachno metabolic profiles compared to DEC58+cipro (Figure 4.9 A) and these SCFAs were all significantly lower in cell-free DEC58+cipro treated CD186 culture supernatants. Thus, DEC58+cipro may influence CD186 toxin production in part by the combined SCFA alterations described here.

Interestingly, isoleucine was present in greater amounts in CD186 treated with the cell free supernatants of MET-1, DEC58, and DEC58-Lachno compared to DEC58+cipro. This amino acid has been previously associated with decreased toxin expression in ribotype 087 *C. difficile* isolates (Karlsson et al., 1999), and further work investigating its role in TcdA and B production with the specific *C. difficile* isolates in this study would be of interest. Likewise, isobutyrate was undetectable within all groups of uninoculated chemostat vessel content cell-free
supernatants (see Figure 3.5), but was increased in cell-free MET-1, DEC58, and DEC58-Lachno treated CD186 culture supernatants compared to DEC58+cipro (see Figure 4.10 A). Isobutyrate is a branched-short chain fatty acid (BSCFA) and known protein fermentation product of C. difficile (De Vos et al., 2009), but the effects of isobutyrate on toxin production have not been described in the literature. Unlike the results described for CD186, increased quantities of isobutyrate were detected in cell-free DEC58+cipro treated supernatants of CD973 compared to all other ecosystem supernatants. As components in DEC58+cipro supernatants did not significantly influence toxin production in comparison to the supernatants of MET-1 or DEC58 in CD973, it is likely that synergistic metabonomic interactions mediate toxin regulation and/or the effects of individual compounds such as isobutyrate act against C. difficile in a strain-specific manner. Increased levels of choline were present in representative healthy cell-free defined microbial ecosystem supernatants (MET-1 and DEC58) and in DEC58-Lachno for CD186, but only in representative healthy cell-free defined microbial ecosystem supernatants for CD973. Rojo et al., 2015 found an inverse relationship in choline production with toxin production in CDI patients (Rojo et al., 2015). Our results support this association; there are low levels of choline in control samples where toxin production is high and increased levels of choline present in the above ecosystems that repress secreted toxin levels in both C. difficile isolates tested (Figures 4.10 and Figure 4.6). However, in CD973 there was increased toxin levels in the cell-free DEC58-Lachno treated supernatants compared to all other ecosystems, but no significant difference in choline abundance was discerned between DEC58+cipro and DEC58-Lachno groups. Consequently, we postulate that choline may be another compound involved in potential synergistic metabonomic regulation of toxin production.
Another important finding was that the overall secreted levels of TcdA and TcdB in CD973 were lower compared to CD186. This is in agreement with previous data wherein ribotype 027 strains produced higher levels of TcdA and TcdB compared to strains of other ribotypes (Merrigan et al., 2010; Vohra and Poxton, 2011; Warny et al., 2005). Additionally, TcdA was determined to be less potent than TcdB to NIH 3T3 fibroblast cells when grown in supplemented DMEM controls, given that 200 times more toxin was required to achieve similar levels of cell rounding (see Figure 4.8 A and B). Similar results have been previously reported in a variety of different cell lines, including mouse adrenal tumour cells (Y1), rat hepatoma (MHC), chinese hamster ovary (CHO), human cervical epithelium (HeLa), and human carcinoma T84 cells (Chaves-Olarte et al., 1997; Donta et al., 1982).

As discussed above, the general trend at 24 and 48 h for both ribotypes examined was a repression of secreted TcdA and TcdB in response to cell-free defined microbial ecosystem supernatants, except for in CD973 where no significant differences in the levels of TcdB were detected. Accordingly, it is possible that defined microbial ecosystem metabolites may have distinct effects on these two effector toxins. Recent work using live bacterial MET-1 preparations has supported this hypothesis – MET-1 has been shown to provide protection in a \textit{C. difficile} colitis mouse model, and these effects were mediated through the specific inhibition of TcdA (Martz et al., 2016). In accordance with our study, MET-1 did not reduce the viability of \textit{C. difficile} vegetative cells, suggesting that this mechanism contributes little to the positive effects observed.

To determine if the cell-free supernatants from defined microbial ecosystems could influence the cytopathic effects of TcdA or TcdB, we monitored cell rounding in NIH 3T3 fibroblast cells. Because of technical issues in the individual purification of TcdA and TcdB
from CD186 and CD973 isolates used throughout this study, both TcdA and TcdB isolated from *C. difficile* 630 (ribotype 012) were obtained from a commercial source. Both toxins were able to significantly induce cell rounding in the NIH 3T3 cells as previously described (Olling et al., 2011). Pre-treatment of NIH 3T3 cells with the cell-free supernatants of DEC58 or DEC58-Lachno prevented TcdB-mediated cytopathic effects. Interestingly, this effect was not observed for TcdA-treated NIH 3T3 fibroblast cells. Given that the cell-free supernatants from DEC58, but not MET-1 were protective against TcdB induced cytotoxicity, these results suggest that a component associated with DEC58 and may mediate these protective effects. Despite the depletion of Lachnospiraceae, the DEC58-Lachno ecosystem supernatant was also able to protect against TcdB induced cytotoxicity. Given these results, we hypothesize that a component derived from a non-Lachnospiraceae species may be a likely candidate in this regard.

In contrast, Martz et al., 2016 demonstrated that live formulations of MET-1 could protect against TcdA-mediated cytotoxicity (Martz et al., 2016). As we did not detect decreased TcdA-induced cell rounding in response to the cell-free supernatants from MET-1, this suggests that factors associated with live MET-1 bacterial species may be required to induce protective effects against this toxin. Examining whether live MET-1 and the other defined microbial ecosystem formulations can influence TcdB-mediated cytotoxicity will further support the above hypothesis. Together, these results suggest that the biological activity of TcdB is diminished in response to the presence or absence of a metabolite specific to DEC58 and DEC58-Lachno microbial ecosystems. Further *in vivo* studies investigating the effects of all defined microbial ecosystems and their metabolites in *C. difficile* colitis rodent models will indicate whether these defined microbial ecosystems can also exert similar protective effects to MET-1.
To corroborate the toxin secretion results, we aimed to determine whether the decreases in TcdA and B levels in response to cell-free defined microbial ecosystem supernatants were influenced at the transcriptional level. To do this, we investigated the effects of tcdA and tcdB gene expression in response to the cell-free metabolites derived from MET-1, DEC58, DEC58-Lachno, and DEC58+cipro (see Figures 4.3-4.5). With the exception of the 12 h gene expression results, CD186 and CD973 displayed diverse responses to the four cell-free defined microbial ecosystem supernatants, highlighting the heterogeneity between these two C. difficile strains.

Interestingly, all variations of DEC58 ecosystem components decreased tcdA and tcdB expression in CD186 after 24 and 48 h, except for CD186 tcdB at 24 h, indicating that the decrease in toxin secretion by ecosystem metabolites may be in part facilitated at the transcriptional level. Similarly, cell-free MET-1 metabolite supernatants repressed toxin A and B gene transcription at 24 h. However, toxin gene expression was increased at 12 and 48 h, but the increases were not reflected in the secreted toxin levels. This may indicate a delay between these transcriptional modifications and protein output. Indeed, similar temporal disparities between gene and protein expression of TcdA and TcdB have been described previously (Aldape et al., 2013).

In contrast to the discrepancy between gene and protein expression, MET-1 components may influence tcdE expression at the 12 and 48 h time points. As tcdE is known to encode a holin-like protein important for the secretion of TcdA and TcdB (Govind and Dupuy, 2012; Govind et al., 2015), repression of this gene may ultimately impede the release of C. difficile toxins into the extracellular environment (culture supernatant). Alternatively, MET-1 components may post-transcriptionally or post-translationally regulate tcdA and tcdB transcripts. For example, post-translational mechanisms such as the proteolytic degradation of TcdA and/or
TcdB may occur via proteases present within the MET-1 metabolite supernatants. Indeed, the yeast probiotic, *Saccharomyces boulardii* (Hansen CBS 5926) is thought to be useful in adjunctive therapies for CDI through the proteolytic inhibition of TcdA (Castagliuolo et al., 1999). Accordingly, future investigation of the potential proteases in these defined microbial ecosystem cell-free supernatants is warranted.

We additionally investigated CDT transcript levels in this work because of the association of this toxin with high virulence of *C. difficile* (Cowardin et al., 2016; Gerding et al., 2014; Kuehne et al., 2014). The cell-free metabolites from the supernatants of MET-1 significantly repressed expression of *cdtA* and *cdtB* compared to control in CD186 at 24 h. Although both CDT genes are under the control of the same promoter (Carter et al., 2007) (see Figure 1.6 B), MET-1 supernatants significantly repressed expression of only *cdtB* in CD973 at this time point. Expression of *cdtA* was decreased by MET-1 supernatants compared to control at 24 h, but not significant likely because of the large standard error within the biological replicates of this treatment. Similar discrepancies were also detected in the remaining defined microbial ecosystem treated CD186 groups at this time point.

At the 12 h time point, MET-1 supernatants significantly increased the expression of *cdtA* in CD186 and *cdtB* in CD973. However, at 48 h, the expression of *cdtA* and *cdtB* was increased in only CD186 under these conditions. Because metabolomic profiling was not completed at these additional time points, and protein levels of CDT were not investigated, explanations behind this temporal dissimilarity remain elusive. Nevertheless, the decrease of CDT gene expression at 24 h may provide insight into the therapeutic value of MET-1, especially in regards to disease recurrence.
4.3.1 Limitations to this study, conclusions and future directions

In this study, we inoculated *C. difficile* cultures with the cell-free supernatants from defined microbial ecosystems rather than the live bacterial samples taken directly from chemostat vessels. As our chemostat vessels are not physically contained in biological safety cabinets, the inoculation of *C. difficile* spores or vegetative cells into vessels under positive pressure would be a biohazard risk. Also, toxin gene expression assays could not be completed on *C. difficile* treated with live defined microbial ecosystem preparations because of consistent non-specific amplification in samples when using multiple reference and target genes. Consequently, we specifically investigated the effects of secreted products derived from gut microbial communities, without considering bacterial and metabolic interactions that would undoubtedly occur in culture with *C. difficile*. For instance, because *C. difficile* was the only bacterium present, no competition for nutrients with other gut microbial residents would impact its metabolism or growth.

As such, metabolite quantities in this artificial system were not expected to be consistent with those found in the human gut *in vivo*. For example, SCFAs produced by bacteria through carbohydrate fermentation are primarily absorbed in gut – 95% of SCFAs are absorbed by colonocytes and the residual 5% of SCFAs are secreted in the feces (den Besten et al., 2013b). When these cell-free defined microbial ecosystems were mixed with *C. difficile* in non-continuous culture, we expected the accumulation of SCFAs to decrease the sample pH (den Besten et al., 2013b). Although these samples were not pH controlled, the only treatment group to significantly decrease pH (7.0 to 5.5) was DEC58+cipro for CD186. A recent study showed that growth of *C. difficile* at pH 7 and 5.5 are similar, and thus we speculate that the final pH of our samples did not impact growth measurements (Scaria et al., 2015).
Another major limitation to this study is that our model only mimics the luminal environment of the gut and host-associated interactions are not accounted for. For example, immunological and secretory events in the gut may significantly impact the effects of defined microbial ecosystems on \textit{C. difficile}. Moreover, any effects that result from nutrient limitation, which would not occur in the host, cannot be distinguished from that of the defined microbial ecosystems. Additionally, various metabolites in each defined microbial ecosystem may provide readily available forms of energy for \textit{C. difficile}. The corresponding increases in vegetative cell growth and repression of TcdA and B levels may be reflective of this, and is an unavoidable limitation in our study design.

In regards to the gene expression assays completed in this work, the only suitable reference gene demonstrating stable and constitutive expression across all treatment groups was \textit{rrs}, even though numerous reference genes were validated in this study (Table A 2.1). Using more than one reference gene for RT-qPCR studies is highly recommended (Bustin et al., 2009; Vandesompele et al., 2002); however, because of low reference gene stability in \textit{C. difficile}, often only one reference gene is used (Aldape et al., 2013; Deneve et al., 2009; Govind et al., 2009). Indeed, there are studies that have not used and/or reported inappropriate reference genes for gene expression normalization (Dembek et al., 2013; Gerber et al., 2008).

In this study, NIH 3T3 cells were pre-treated with defined microbial ecosystem cell-free supernatants prior to the addition of purified \textit{C. difficile} TcdA and TcdB toxins and cytotoxicity was subsequently assessed. There are many drawbacks to this cytotoxicity assay, including the source of tissue culture cells (mouse), and the use of non-live defined microbial ecosystem components and \textit{C. difficile}. To address these concerns, future experiments utilizing live microbial ecosystems in co-culture with human intestinal epithelial cells under conditions more
representative of the intestinal-bacteria interface, will be more biologically relevant and result in an increased understanding of the molecular interactions occurring. A previously described microfluidics-based \textit{in vitro} model of the gut-bacterial interface, called HuMiX, has been used to recapitulate various \textit{in vivo} responses in human intestinal epithelial cells in response to co-culture with bacteria (Shah et al., 2016). Briefly, this model has the capacity to support the interaction and growth of both defined microbial ecosystem live compositions (anaerobic conditions) and human intestinal epithelial cells (aerobic conditions), whereby any protective effects against \textit{C. difficile} or its toxins may be examined.

In conclusion, the results from this study suggest that defined microbial ecosystems may influence \textit{C. difficile} virulence through decreased secretion of toxin A and B \textit{in vitro} and protection against TcdB-mediated cytotoxicity in NIH 3T3 cells. The work described here supplements the findings of Martz et al., 2016, who described the protective effects of the defined microbial ecosystem MET-1 against \textit{C. difficile} TcdA. Future studies examining the components of defined microbial ecosystems responsible for the antagonistic mechanisms described here will complement our existing knowledge on gut microbiota-specific anti-virulence mechanisms and contribute to the optimization of novel defined microbial ecosystem formulations for the effective treatment of rCDI.
Chapter 5: Summary, conclusions and future directions

As standard treatment practices are not effective in all rCDI cases, there has been a surge in the development of microbiome-based therapeutics for this indication. Among novel therapeutics, FMT is a highly effective intervention for rCDI, and is thought to restore microbial ecosystem imbalances and have direct antagonistic mechanisms against *C. difficile* (Figure 1.3). However, the use of stool as medicine is not without risks and logistic challenges. To address the safety and reproducibility issues associated with FMT, we have developed a defined microbial ecosystem therapeutic (MET-1), which has been used to cure two patients of recurrent CDI in a proof-of-principle trial (Petrof et al., 2013). Understanding the mechanisms by which defined microbial ecosystems are beneficial for rCDI is crucial to the development of fecal-microbiota based therapeutics for CDI.

Although the precise mechanisms through which health is restored in rCDI patients after MET-1 treatment are not fully understood, preliminary evidence of host-associated protective effects and direct antagonistic mechanisms against *C. difficile* have been described (Martz et al., 2016; Munoz et al., 2016). This study aimed to investigate the specific *in vitro* antagonistic mechanisms of defined microbial ecosystem components against two clinically important ribotypes of *C. difficile*.

To determine the influence of defined microbial ecosystems on *C. difficile* growth and virulence, we first developed and characterized 4 defined microbial ecosystems (detailed in chapter 3). The growth of two bacterial ecosystems developed from a healthy fecal community (MET-1 and DEC58), and two perturbed ecosystems (DEC58-Lachno and DEC58+cipro) were supported by an *ex vivo* human gut model previously described (McDonald et al., 2013). The taxonomic composition and metabonomic profiles of all ecosystems were determined using 16S
rRNA gene sequencing and 1D $^1$H NMR spectroscopy on chemostat samples derived from the vessels supporting these bacterial communities. Using the same techniques, we additionally resolved the taxonomic composition and targeted metabonomic profiles of cell-free defined microbial ecosystem supernatant-treated *C. difficile* cultures. Finally, using a variety of *in vitro* assays, we investigated the effects of cell-free defined microbial ecosystem supernatants on *C. difficile* growth and virulence (detailed in chapter 4).

The results from this study suggest that defined microbial ecosystem supernatants may influence *C. difficile* virulence by decreasing secreted toxin A and B levels. The identification of these antagonistic properties against *C. difficile* complements our existing knowledge of gut microbiota-specific anti-virulence mechanisms. For example, Martz et al., 2016 described antagonistic properties of MET-1 against *C. difficile* TcdA *in vitro* and in a mouse model of CDI. As we determined that the cell-free supernatants of DEC58 and DEC58-Lachno are associated with protective effects against *C. difficile* TcdB, further studies investigating the effects of these microbial ecosystems and their associated metabolite supernatants on TcdB *in vivo* is warranted. Although MET-1 has been shown to modulate TcdA in a *C. difficile* colitis mouse model, these effects were mediated by live bacterial formulations. Thus it would be useful to examine how live formulations and secreted bacterial products of all 4 defined microbial ecosystems differ in their ability to influence toxin production in *C. difficile* colitis rodent models.

Although the mechanisms behind *C. difficile* metabolism and virulence are complex, they are tightly integrated (Bouillaut et al., 2015). A more holistic understanding of toxin regulation in response to defined microbial ecosystem components could be achieved by examining the expression of other factors directly involved in toxin production, including CdtR, TcdC, TcdR, and TcdE regulation under these experimental conditions.
By using a targeted metabonomic approach we aimed to identify specific compounds and associate them to growth and virulence alterations observed in defined microbial ecosystem treated *C. difficile* cultures. Although only a small subset of compounds was explored, an untargeted approach may help identify metabolite signatures of gut microbial dysbiosis in disturbed defined microbial ecosystems, such as DEC58+cipro. By integrating the metabonomic and toxin production results, it is likely that defined microbial ecosystem metabolites synergistically influence toxin production. Determining whether toxin inhibition can be mediated by the individual components from single species within defined microbial communities will support this hypothesis and is an important future direction of this work.

In this study, we used two *C. difficile* isolates of different ribotypes that are important to human nosocomial and community-acquired infections and the changing epidemiology of CDI (Burke and Lamont, 2014). Throughout this study, these two strains displayed heterogeneous virulence and metabonomic profiles, confirming that extrapolating the behaviour of one ribotype to another is erroneous. We also examined germination properties of a variety of *C. difficile* 027 and 078 ribotype strains of clinical importance, and found significantly altered germination rates within ribotype groups (Figure A 5.1). Thus, extrapolating the properties of the specific 027 and 078 isolates used in this study to other *C. difficile* strains of the same ribotype is inappropriate and would require the testing of those strains under the same treatment conditions. The ability of defined microbial ecosystems to antagonize growth and virulence in many different *C. difficile* strains is underscored by the emergence of novel ribotypes and heterogeneous epidemiology in terms of strain diversity worldwide. For example, the incidence of BI/NAP1/027 isolates has steadily decreased in the United Kingdom and other areas of Western Europe since the late 2000s (Martin et al., 2016). The diverse distribution of *C. difficile* strains worldwide coupled
with the large interindividual variation in human gut microbiota will undoubtedly complicate the development of novel microbiota-based therapeutics.

Nevertheless, the work described here suggests that gut microbial ecosystems represent a valuable target for therapeutic intervention in the context of CDI and provides mechanistic insight into how defined gut microbial ecosystems influence *C. difficile* growth and virulence *in vitro*. Further investigation of antagonistic mechanisms against *C. difficile* will contribute to the development and optimization of defined microbial ecosystem formulations and novel stool-based therapeutics for the effective treatment of rCDI.
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291. Theriot, C.M., Bowman, A.A., and Young, V.B. (2016). Antibiotic-induced alterations of the gut microbiota alter secondary bile acid production and allow for *Clostridium difficile* spore germination and outgrowth in the large intestine. mSphere 1.


Appendices:
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein encoded:</th>
<th>Amplicon size (bp)</th>
<th>Primer Efficiency</th>
<th>Melting Temperature (°C)</th>
<th>Primer Sequences (5’-3’):</th>
<th>Reference</th>
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<tr>
<td>adk</td>
<td>adenylate kinase</td>
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<td>1.99</td>
<td>78</td>
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<td>2.00</td>
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<td>1.96</td>
<td>75</td>
<td>F’-AAGAAGCTAAGGCTACAAA  R’-CATAATATTGGGCTATCTAC</td>
<td>(Lemee et al., 2004)</td>
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Table A 4.1 Resistance profiles of CD186 and CD973 to common antimicrobials used in the healthcare setting

<table>
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<th>Antibiotic class:</th>
<th>CD186 zone diameter (mm):</th>
<th>CD973 zone diameter (mm):</th>
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<tbody>
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<td>β-lactam</td>
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<td>36</td>
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<td>Ceftazidime (30 µg)</td>
<td>3rd generation cephalosporin</td>
<td>12</td>
<td>0</td>
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<tr>
<td>Ceftriaxone (30 µg)</td>
<td>3rd generation cephalosporin</td>
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<td>20</td>
</tr>
<tr>
<td>Ciprofloxacin (5 µg)</td>
<td>2nd generation fluoroquinolone</td>
<td>0</td>
<td>14</td>
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<td>Lincosamide</td>
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<td>Vancomycin (30 µg)</td>
<td>Glycopeptide</td>
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Figure A 2.1 An 027 ribotype, toxinotype III strain was isolated from the stool of a patient with severe recurrent CDI. Ribotyping (A) and toxinotyping (B) were performed on the unknown isolate.
Figure A 3.1 Comparison of the community diversity between defined gut microbial ecosystems used in this study. Inverse Simpson diversity estimate analysis was completed in the Mothur software package using the OTU abundance. One-way ANOVA followed by Tukey’s multiple comparisons were performed between each defined microbial ecosystem group using GraphPad Prism 7.0. All pair-wise comparisons were not significant (NS), $p > 0.05$. 
Figure A 3.2 Qualitative differences in bacterial cell density between DEC58+cipro (right) and DEC58 culture (left). DEC58+cipro has less bacterial density compared to DEC58, and resembles the opacity of sterile chemostat media. E.g. For the first replicate vessels run, gDNA isolated from 200 µL of MET-1, DEC58 and DEC58-Lachno day 9 chemostat samples resulted in concentrations of 36.49, 36.17 and 39.49 ng/µL respectively, compared to 2.31 ng/µL for the same volume of DEC58+cipro day 9 chemostat sample.
Figure A 4.1 Growth curves of select *C. difficile* isolates used in this study. *C. difficile* cultures were grown to exponential phase (3-4 h) in BHI, diluted to an OD600 < 0.100, and transferred to a 96-well plate. Plates were incubated anaerobically at 37ºC and growth measurements were recorded every 1 h for a total of 24 h in a Victor³ plate reader. Error bars represent the variation 8 technical replicates.
Figure A 4.2 Data normalization and model validation for CD186 metabonomic analysis. Compound (A) and sample (B) normalization of CD186 concentration data was performed by normalization to pooled sample from control group and auto-scaling. PLS-DA multivariate statistical analysis was performed and validated as an appropriate model by a permutation test (C) and cross-validation (D) using Metaboanalyst 3.0 software.
Figure A 4.3 Data normalization and model validation for CD973 metabonomic analysis. Compound (A) and sample (B) normalization of CD973 concentration data was performed by normalization to pooled sample from control group and auto-scaling. PLS-DA multivariate statistical analysis was performed and validated as an appropriate model by a permutation test (C) and cross-validation (D) using Metaboanalyst 3.0 software.
Figure A 4.4 Vegetative cell and spore recovery counts of *C. difficile* treated with live MET-1 bacterial preparations. CD186 and CD973 vegetative cell counts (A) and spore recovery (B) after treatment with live MET-1 chemostat contents compared to control chemostat media for three replicate experiments. Student's *t*-tests were performed between treatment and control for each *C. difficile* isolate using GraphPad Prism 7.0. NS, *p* > 0.05; *, *p* ≤ 0.05; **, *p* ≤ 0.01; ***, *p* ≤ 0.001; ****, *p* ≤ 0.0001.
Figure A 5.1 The rate and extent of *C. difficile* germination varies among different clinical isolates. Purified spores from a variety of 027 and 078 isolates were resuspended in a sodium phosphate buffer (100 mM) supplemented with 0.1% sodium taurocholate and 0.2% glycine. Purified spores were incubated aerobically at 37°C for 140 min and the OD$_{600}$ was recorded at ~2.5 min intervals.