The efficacy of probiotics in modulating *Clostridium difficile* spore germination, growth and toxin production in an in vitro human gut model

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Abstract
Background and aims

Clostridium difficile infection (CDI) remains a healthcare burden and recurrent CDI (rCDI) still affects 20-30% of patients. Probiotics are live microorganisms that confer a host health benefit, but evidence of their efficacy in CDI prevention/treatment is controversial. Non-toxigenic C. difficile (NTCD) have been used successfully used in animals/humans to reduce CDI. The present study aimed to assess efficacy of two probiotics, Lactobacillus casei Shirota (LcS, Yakult) and NTCD, in preventing simulated CDI in an in vitro human gut model.

Methods

C. difficile-negative pooled faeces from healthy volunteers (>65 yrs) was used to inoculate the gut model. Two probiotics, LcS (6.2 x 10^9 cfu) and NTCD (1 x 10^8 spores), were dosed into separate gut models prior to C. difficile (CD) ribotype 027 spores (RT027, 1 x 10^8). LcS was chosen given its commercial availability, in addition to studies that report the prevention of antibiotic-associated diarrhoea by LcS, and its role in preventing CDI. Similarly, NTCD was chosen given reports of its antagonism against toxigenic C. difficile. Probiotic dosing was for 28d; LcS once-daily and NTCD spores once-weekly. The rationale for dosing is consistent with literature and the ease of obtaining the probiotic. For example LcS is commercially available whereas NTCD had to be prepared using a rigorous process over a duration of 7 – 10 days. Various antimicrobial groups such as lincosamides, aminopenicillins, fluoroquinolones, and third generation cephalosporins was used to disrupt the gut microflora in separate experiments. Gut model contents were assayed for microflora composition using viable counting techniques and CD cytotoxin production using a Vero cell cytotoxicity assay. Probiotic dosing ceased 14 days before the end of the experiment.

Results

LcS dosing resulted in marked increases in lactobacilli and bifidobacterial viable counts. However, during clindamycin (DA) dosing these viable counts declined by 4-log_{10}cfu/mL. RT027 spore germination and cell proliferation was observed during/after antimicrobial instillation. Interestingly, another cycle of growth/cytotoxin was observed after LcS dosing ceased. NTCD did not colonise the gut model prior to DA instillation; spores were quiescent and washed out. NTCD spores germinated and vegetative cells multiplied, whereas, RT027 spores did not germinate and no cytotoxin was produced. NTCD remained vegetative until the end of the experiment and isolated cells retained their non-toxigenic phenotype.

Conclusions

Instillation of NTCD prevented primary CDI in a human gut model, whereas dosing with LcS did not. LcS, if beneficial in the antagonism of CD, is unlikely to be due to nutrient/adhesion competition or
production of antimicrobial substances. NTCD may be beneficial not only in treating rCDI but also in the prevention of primary infection. Further work is needed to better understand the protection that NTCD might confer.
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Abbreviations

AAD   Antibiotic- associated diarrhoea
AMP   Ampicillin
ATCC  American Type Culture Collection
BaiCD Bile acid - inducible C.difficile
BBE   Bacteroides Bile Aesculin
BHI   Brain Heart Infusion
BSAC  British Society for Antimicrobial Chemotherapy
CA-CDI Community associated Clostridium difficile infection
CBA   Columbia blood agar
CCEY  Cefoxitin/Cycloserine Egg Yolk
CCNA  Cell culture cytotoxicity neutralisation assay
CcpA  Carbon catabolite control/repressor protein
CDAD  Clostridium difficile associated disease
CDC   Centre of Disease Control
CDI   Clostridium difficile infection
CDRN  Clostridium difficile ribotyping network for England and Northern Ireland
CDT   Clostridium difficile binary toxin
Cfr   Chloramphenicol florfenicol resistance
CFU   Colony forming unit
ClosER Clostriduim difficile European Resistance
CLSI  Clinical & Laboratory Standards Institute
Cwp    Cell wall protein
DA     Clindamycin
DMEM   Dulbecco Modified Eagle’s medium
CFX    Cefotaxime
Cipro  Ciprofloxacin
DNA    Deoxyribonucleic acid
ECDC   European centre of disease prevention and control
EIA    Enzyme Immunoassays
EMEM   Eagle’s Minimum Essential Medium
Erm    Erythromycin ribosomal methylase
ESCMID European Society for Clinical Microbiology and Infectious Diseases
ESGCD  European Study Group on Clostridium difficile
EUCAST European Committee on Antimicrobial Susceptibility Testing
FAA    Fastidious Anaerobe Agar
FAO    Food and Agriculture Organisation
FDA    Food and Drug Administration
FMT    Faecal microbiota transplantation
G+C    guanine-cytosine
GDH    glutamate dehydrogenase
Gyr    Gyrase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>HAI</td>
<td>Health Care- Associated Infections</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>ICDS</td>
<td>International C. difficile Symposium</td>
</tr>
<tr>
<td>ITS</td>
<td>Intergenic spacer region</td>
</tr>
<tr>
<td>KAA</td>
<td>Kanamycin Aesculin Azide</td>
</tr>
<tr>
<td>LAMVAB</td>
<td><em>Lactobacillus</em> Anaerobic de Man, Rogosa and Sharpe with Vancomycin and Bromocresolgreen</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>LeS</td>
<td><em>Lactobacillus casei</em> Shirota</td>
</tr>
<tr>
<td>LCT</td>
<td>Large clostridial toxins</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<tr>
<td>ML</td>
<td>Millilitres</td>
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<tr>
<td>MLSb</td>
<td>Macrolide, lincosamide and streptogramin B</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi-Locus Sequence Typing</td>
</tr>
<tr>
<td>MLVA</td>
<td>Multi- Locus variable number tandem repeat analysis</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant Staphylococcus aureus</td>
</tr>
<tr>
<td>NAAT</td>
<td>Nucleic acid amplification test</td>
</tr>
<tr>
<td>NAP1</td>
<td>North American pulse- field type 1</td>
</tr>
<tr>
<td>NI</td>
<td>No Intervention</td>
</tr>
<tr>
<td>NTCD</td>
<td>Non-toxigenic C. difficile</td>
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</table>
PaLoc Pathogenicity locus

PBP Penicillin-binding protein

PBS Phosphate Buffer Saline

PCR Polymerase chain reaction

P-cresol Para-cresol

PFGE Pulse- field gel electrophoresis

pH Potential of Hydrogen

PHE Public Health England

PMC Pseudomembranous colitis

RCDI Recurrent *Clostridium difficile* infection

RNA Ribonucleic acid

Rpo RNA polymerase

RT Ribotype

RU Relative Unit

SAB Schaedler's Anaerobe Broth

SDW Sterile Distilled Water

SNP Single Nucleotide Polymorphism

SP Spore

SPP Species

TC Toxigenic Culture

TcdA Toxin A
TcdB   Toxin B
UN    United Nations
VNTR  Variable number tandem repeat
WBC   White blood cell count
WCA   Wilkins Chalgrens Agar
WGS   Whole genome Sequencing
WHO   World Health Organisation
CHAPTER ONE

1.0 Introduction

1.1 The triple-stage gut model

The triple-staged continuous culture system was validated by MacFarlane et al., (1998) to assess the effect of retention time on the ecology and metabolism of bacteria in the human colon. Unlike single stage fermenters that inaccurately depicts the entire colon as having the same pH and nutrient availability, the triple-stage system was designed to mirror the nutritional, physical, and chemical properties of the proximal, transverse, and distal colon. The gut model was made up of three glass fermentation vessels operating in a weir-cascade system, flowing into a waste unit. The uppermost vessel has a higher substrate availability, promoting increased bacterial growth, and is operated at an acidic pH mimicking the environment of the proximal colon. In contrast, the lowest vessel is consistent with the neutral pH, low substrate availability, and slow bacterial growth, which is obtainable in the distal regions of the colon. Growth medium is supplied to the gut model with the aid of a peristaltic pump. This model is widely used to study antimicrobial induction of simulated CDI, in addition to studying the efficacy of CDI treatments (Baines et al., 2008; 2009; Freeman et al., 2005; 2007, Crowther et al., 2013; 2014; 2016; Chilton et al., 2014; 2015; Moura et al., 2019). This is in part because the system allows for the growth and retention of bacterial groups for a prolonged duration (Freeman et al., 2007). Although pH is controlled in the system, it is still a useful model to study probiotics that act by altering the pH of the environment. This is because the pH control is slow and gradual, thereby affording the probiotic strains time to outcompete its pathogenic counterparts. Besides, the different vessels are set to different pHs. Nevertheless, a major limitation of the gut model is its inability to mimic immunological events. Additionally, the gut model design makes it very difficult to investigate bacterial populations in biofilms but allows the retrieval of bacterial populations in planktonic state with relative ease. Furthermore, the gut model is also limited in terms of bacteria-host interactions. Nevertheless, reports suggest that data from the gut model closely reflect in vivo observations (Freeman et al., 2003; Moura et al., 2019).
1.2 Historical background of *Clostridium difficile*

*C. difficile* is a spore-forming, Gram-positive obligately anaerobic bacterium. The organism was first identified by Hall and O’Toole in 1935 as part of the microflora of healthy infants. The authors described this bacterium as an ‘’actively motile, heavy-bodied rod with an elongated sub terminal or nearly terminal spores’’ (Hall & O’Toole, 1935). The bacterium was named *Bacillus difficilis* at that time to mirror the difficulty experienced in isolating and culturing it. Although the bacterium was present as a commensal organism in infants, the early researchers observed that it could cause disease in animals, possibly due to the production of a toxin (Hall & O’Toole, 1935). Subsequent research showed that the main disease-causing (virulence) factors were the high molecular weight clostridial toxins: toxin A and toxin B (Cebeci & Gürakan, 2003). It was in the 1970s that the bacterium then called *Clostridium difficile* was shown to be involved in human disease (Tedesco et al., 1974). This disease became widely known as *C. difficile*-associated diarrhoea/disease (CDAD) and then *C. difficile* infection (CDI). In more recent times Lawson and Rainey (2015) proposed that the genus *Clostridium* be restricted to *Clostridium butyricum* and related species. The proposal has implications for *C. difficile* because it has been reported to be phylogenetically distant from the rRNA clostridial cluster I (Collins et al., 1994). The ‘rRNA cluster I’ is referred to species of the genus *C. butyricum* which can be called *Clostridium sensu stricto* (Lawson & Rainey, 2015). Accordingly, ‘’Clostridioides difficile’’ was proposed as a reclassification of the bacterium. Since both names can be used, and have been validly reported in accordance with the requirements of the Prokaryotic Code (Oren & Garrity, 2016; Oren & Rupnik, 2018), the name ‘’Clostridium difficile’’ shall be used throughout this work.

Yutin and Galperin (2013) had previously attempted to change the taxonomy of *Clostridium* based on phylogenetic relationships. They proposed the genus ‘’Peptoclostridium’’ to cover *C. difficile* and some other *Clostridium* species, but no validation request was sent to the International Journal of Systemic and Evolutionary Microbiology (IJSEM) List Editors (Oren & Rupnik, 2018). This is seen as positive event, as the acceptance of ’’Peptoclostridium’’ might have meant abbreviations such as CDI, CDAD and *C. difficile* would not have been retained, and this would have brought even bigger confusion especially in clinical settings. This would have been in addition to the potential global economic waste
that would have accompanied an update of product packaging, trademarks, brand names, web pages, and educational materials for healthcare staff, patients, and customers.

1.2.1 Epidemiology of *C. difficile*

In the early 2000s, increasing rates of CDI mortality and incidence in North America, and afterwards in a high number of European countries were documented. These were linked with PCR ribotype 027 (Kuijper *et al.*, 2006; He *et al.*, 2013) and to a lesser extent, PCR ribotype 078 (Freeman *et al.*, 2010). Similar increases have also been documented in Asia, Australia, and Central America (Van Gessel, 2008; Collins *et al.*, 2013; ). It is noteworthy that CDI and its associated epidemics are not restricted to these ribotypes. Accounts of outbreaks for strains of other ribotypes such as 017 (Kuijper *et al.*, 2006), 018 (Pépin *et al.*, 2005), 106 (Cebeci & Gürakan, 2003), 176 (Brazier *et al.*, 2001), and 244 (Purdell *et al.*, 2011) have been observed. In addition, ribotypes 001, 002, and 014/020 often give rise to CDI clusters in Europe and the US. (Tickler *et al.*, 2014; Bauer *et al.*, 2011). The European CDI study (ECDIS) undertaken by the European Centre for Disease Prevention and Control (ECDC) showed that PCR ribotypes 015 and 056 could also cause severe CDI (Kuijper *et al.*, 2006).

Following a survey of hospital-acquired infection by the ECDC in 2011/2012, *C. difficile* was identified as the most documented pathogen linked with gastrointestinal disease of healthcare origin in European hospitals; accounting for over 47% of all gastrointestinal diseases (ECDC, 2013a). It was estimated based on this data that about 153,000 new CDI cases occur in Europe every year with an incidence of 3 cases in every 10,000 population. Furthermore, Cassini *et al.*, (2016) reported that CDI was associated with up to 8382 deaths every year in Europe. Additionally, CDI is increasingly occurring in people in the community with no traditional risk factors such as recent hospitalisation and antimicrobial use (Wilcox *et al.*, 2008). Epidemiological data on *C. difficile* in the Middle East and Africa are scanty (Freeman *et al.*, 2010).
1.2.2 The emergence of hypervirulent PCR ribotype 027

The epidemic of CDI about 15 years ago owing to the emergence and predominance of a hypervirulent strain that caused several outbreaks and increased disease severity (Loo et al., 2005; Pepin et al., 2005). This strain was characterised as polymerase chain reaction ribotype 027, restriction endonuclease analysis group BI, and pulse-field gel electrophoresis type NAP1, designated BI/NAP1/027 (McDonald et al., 2005; Kilgore et al., 2008). This strain has since been isolated from patients in several countries around the world including the UK, US, Canada, and Australia (Kato et al., 2007; Hensgens et al., 2009; Riley et al., 2009). Several studies have associated this strain with increased toxin production, high sporulation rates and fluoroquinolone resistance (Akerlund et al., 2008; McDonald et al., 2005; Warny et al., 2005; He et al., 2013). This is in addition to higher resistance to tetracycline, aminoglycosides, and erythromycin (Knetsch et al., 2018).

Prior studies reported that the elevated toxin production by BI/NAP1/027 strains was due to a Δ117 frameshift mutation and an 18bp deletion in the tcdC gene (Warny et al., 2005; MacCannell et al., 2006). In contrast Merrigan et al., (2010) demonstrated that no significant difference exists in the amounts of toxin produced by hypervirulent and non-hypervirulent strains in the exponential phase, suggesting the tcdC did not play a repressive role. These findings were supported by Cartmen et al., (2012) who reported no association between tcdC status and toxin production in both hypervirulent strain R20291 and wild type C. difficile 630.

Furthermore, the use of trehalose as a food additive possibly contributed to the dissemination of RT027 strains. This ribotype is thought to have acquired unique mechanisms to utilise low trehalose concentrations. Ribotype 027 strains exhibit a single point mutation in the trehalose repressor which results in a more than 500-fold increase of sensitivity to trehalose and also increased the virulence of RT027 strains in mouse models of CDI (Collins et al., 2018). The authors suggested that the incorporation of trehalose as a food additive into the human diet, shortly prior to the emergence of the epidemic lineage, helped select for RT027 emergence and contributed to hypervirulence (Collins et al., 2018). The role of trehalose metabolism on CDI severity may be due to the increased toxin production.
that accompanies trehalose utilisation by RT027 strains (Collins et al., 2018). However, Eyre et al., (2019) did not find any correlation between trehalose metabolism variants and severe disease outcomes.

1.2.3 Molecular typing of *C. difficile*

Due to their capacity to type a strain precisely, discriminatory power, and high reproducibility, genotyping methods are employed for *C. difficile* typing. PCR ribotyping, pulsed-field gel electrophoresis (PFGE), and restriction enzyme analysis (REA) are commonly used molecular typing methods. In the past, PCR ribotyping which is based on rRNA-based phylogenetic analyses has been the preferred method in Europe, whereas PFGE and REA were mainly used in North America. Given the 16S rRNA gene is the most conserved among the rRNA genes, 16S rRNA gene sequencing has been employed as the gold standard for the taxonomic classification and identification of bacterial species (Mishra & Swain, 2020). PCR ribotyping employs primers directed at conserved regions of the 16S rRNA and 23S rRNA ribosomal genes to amplify the intergenic spacer region between these sequences (Tanis et al., 2015). Given distinct *C. difficile* strains may possess varied complements and configurations of rRNA operon, amplification of the intergenic spacer region culminates in a reproducible and highly discriminatory banding pattern that could be used for *C. difficile* outbreak investigations and surveillance purposes (Tanis et al., 2015).

The use of different approaches by different regions have led to a situation in which epidemic strains are usually known by multiple typing names (Tenover et al., 2011). For example, PCR ribotype 027 are also known as PFGE type NAP1 and REA group BI. In a similar manner, PCR ribotype 078 strains are classified as PFGE type Nap7/NAP8 and REA group BK. Owing to the need for global surveillance, efforts are geared to reconcile the different typing schemes (Smits et al., 2016). Sequence-based methods such as whole genome sequencing (e.g. single nucleotide polymorphism (SNP)), and multilocus sequence typing have drawn more attention, especially in phylogenic studies (Knetsch et al., 2013). Multilocus variable number tandem repeat analysis (MLVA) is often employed to study the relationship between strains in an outbreak (Knetsch et al., 2013). Data from a European survey on *C.
difficile showed that the most applied method for C. difficile characterisation for the purpose of surveillance was PCR ribotyping (used in 25 countries), followed by MLVA (13 countries), whereas whole genome sequencing was used only in five countries (Krutova et al., 2018). In the UK, the C. difficile reference laboratory is situated within the Public Health England and C. difficile Ribotyping Network in Leeds, and new ribotypes are assigned from the reference laboratory. Currently there are over 950 distinct C. difficile PCR ribotypes (Dr Warren Fawley – personal communication).

1.2.4 Life cycle

C. difficile is transmitted through the faecal-oral route. Due to the fact that metabolically active cells are unlikely to survive the acidic conditions of the stomach or in the oxygenated conditions outside the host, it is thought that spores are the infectious form of the organism. Spores are resistant to environmental conditions (Debast et al., 2014; Paredes-Sabja et al., 2014), in addition to a high number of antimicrobials (Baines et al., 2009; Paredes-Sabja et al., 2012) and some disinfectants. Structurally, spores are made of several layers (Henriques & Moran, 2007). The core of the endospore contains ribosomes, RNA, DNA, and a large quantity of dipicolinic acid, chelated with calcium which bestows heat resistance (Paredes-Sabja et al., 2014; Gil et al., 2017). An inner membrane made of phospholipids surrounds the spore core, followed by a layer of germ-cell wall that subsequently becomes the cell wall of the vegetative cell during outgrowth (Shrestha & Sorg, 2019). A specialised thick layer of peptidoglycan cortex protects the spore core against osmolyis by surrounding the germ-cell wall. The cortex is surrounded by an outer membrane derived from the parent cell, spore coat proteins, and an exosporial layer (Paredes-Sabja et al., 2014; Zhu et al., 2018; Henriques & Moran, 2007). Upon favourable environmental conditions, spores germinate, and outgrow to the vegetative forms of the bacterium (Zhu et al., 2018; Burns et al., 2011).

Germination of spores is thought to occur when the germinant receptor CspC senses primary bile acids in the gut such as taurocholate (produced by the liver). Conversely, C. difficile spore germination is inhibited by secondary bile acids in the large intestine (Paredes-Sabja et al., 2014; Theriot & Young,
Moreover, glycine is capable of acting as a germinant via an unknown mechanism (Sorg & Sonenshein, 2008). A proteolytic cascade results in the breakdown of the spore peptidoglycan, calcium dipicolinate release, and the rehydration of spore, eventually leading to an outgrowth of the vegetative cell (Paredes-Sabja et al., 2014). More recent research indicates that a minor mutation in the region of cspBA that codes for cspA culminated in spore germination in response to taurocholic acid alone, with no role for an amino acid, strongly suggesting that the amino acid germinant receptor is the CspA protein (Shrestha & Sorg, 2018).

*C. difficile* spores have the ability to adhere to colon cells prior to germination into vegetative cells (Paredes-Sabja & Sarker, 2012). In addition, the shift between the motile phase of *C. difficile* and the sessile phase is closely regulated by cyclic di-GMP, a secondary messenger (Bordeleau & Burrus, 2015). Furthermore, *C. difficile* is also able to, (at least in vitro) form biofilms and the spore form of *C. difficile* has been isolated from multi-species biofilms and also pure culture biofilms, which may facilitate survival of the organism in adverse environmental conditions (Crowther et al., 2014). Also the ability to form spores has been proposed to contribute to the difference in virulence between RT027 and other *C. difficile* strains (Burns et al., 2010; Lanis et al., 2010).

1.2.5 Risk factors

Age (above 65 years), previous hospitalization, and chiefly, the use of antibiotics are known risk factors for development of CDI. All classes of antimicrobials have been implicated in CDI, but clindamycin, third generation cephalosporins, and fluoroquinolones are more often associated with CDI (Freeman et al., 2015). Microbial imbalance of the protective intestinal microbiota caused by antibiotics is usually the underlying cause of *C. difficile* outgrowth and toxin production (Venugopal & Johnson, 2011). The alteration of the intestinal microbiota can persist for more than 3 months after antibiotic treatment, therefore patients might remain susceptible to CDI development even after the conclusion of treatment (Leeds et al., 2013). McDonald et al., (2015) reported a close association between proton pump inhibitors and CDI. Biologically, there is a plausibility for the suppression of gastric acid as a CDI risk
factor because a loss of acidity might distort the normal microbial diversity of the gastrointestinal tract (Seto et al., 2014) and even prolong the survival of C. difficile spores (Jump et al., 2007). Nonetheless, the specific role proton pump inhibitors play in CDI remains elusive (Lewis et al., 2009). About 20 – 30% antibiotic-associated diarrhoea is caused by C. difficile. Non-beneficial bacteria such as Enterococci increase in number in older individuals at the expense of beneficial short chain fatty acid-producing bifidobacteria (Goldstein et al., 2011), this might in part explain why elderly individuals are more prone to CDI.

An immunocompromised state and medical comorbidities are also known CDI risk factors in the elderly (McDonald et al., 2017). However, CDI risk factors in children are not well defined. In addition, current understanding of CDI in children is even made more difficult owing to the fact that about 70% of infants less than a month to over 1 year of age may be asymptotically colonised with C. difficile, as a result do not experience clinical illness until 1-2 years of age (McFarland et al., 2000). This may in part because children are thought to lack the toxin receptors (Eglow et al., 1992; Nicholson et al., 2014). Accordingly, a closer assessment of available evidence is required to better grasp the implications and significance of potential CDI risk factors for children.

1.2.6 Toxin A and B

The cytotoxic effect of C. difficile was initially associated to one toxin molecule (Lyerly et al., 1982). However, the exact molecular weight of the resultant toxin showed dissimilarities between researchers. Taylor & Bartlett, (1979) estimated the toxin to be 240 kDa, which was inconsistent to the 530 kDa reported by Rolfe & Finegold, (1979). This inconsistency was resolved when amongst others, Banno et al., (1984) reported that C. difficile produced two antigenically different molecules named toxins A and B, with molecular weights of 308 kDa and 270 kDa respectively.

Toxin A has been historically seen as a more potent enterotoxin, as introduction of purified TcdA to the intestines of rodents and rabbits triggered tissue necrosis (Lyerly et al., 1985). In contrast, high levels
of TcdB in similar experiments did not bring about these effects (Smits et al., 2016). It is pertinent to note that these results are a representation of only the response of the small intestine.

The significance of TcdA in CDI became questionable due to the existence pathogenic *C. difficile* isolates that were shown to produce TcdB but not TcdA (Drudy et al., 2007). These isolates gave rise to the full clinical spectrum of CDI with some researchers even suggesting that these strains give rise to more serious CDI than strains that produced both Toxins A and B (Drudy et al., 2007). Unlike the studies which employed purified toxins, these variant strains showed that TcdA was not necessary for CDI onset and that strains producing only TcdB were as virulent as strains producing both toxins in patients. Consequently, these works revealed that TcdB carried out a more pivotal role in disease than reported from earlier studies. This argument gained more support as the use of purified TcdB was shown to elicit an acute inflammatory response and resulted in more severe damage than TcdA to the intestinal epithelium of mice (Smits et al., 2016). Furthermore, it is now well established that both toxins are crucial for inducing the death of colonocytes and colitis, as well as mounting evidence suggesting their role in CDI extra-intestinal effects (Bella et al., 2016).

A high number of functional and structural studies have elucidated the mechanism of action of TcdA and TcdB. Upon secretion, these toxins enter and bind the colonic epithelium, and brings about the production of inflammatory chemokine and cytokine, neutrophil influx, fluid secretion, tight junction disruption, and ultimately epithelial cell death (Shen, 2012).

### 1.2.7 Clindamycin

Clindamycin is a broad-spectrum antimicrobial that mainly targets anaerobic bacteria, as well as Gram-positive aerobic bacteria (Dhawan & Thadepalli, 1982). The antimicrobial is primarily excreted in bile and is able to reach very high concentrations in the intestinal lumen, culminating in considerable imbalances in the gut microbiota (Sullivan et al., 2001; Jernberg et al. 2010). Following a culture-based study of the faecal microbiota of healthy individuals, the administration of clindamycin prompted a significant decline in anaerobic bacterial populations (*Bacteroides*, bifidobacteria, clostridia, and
lactobacilli) and an increase in Enterococci and Enterobacteria (Citrobacter, Enterobacter, and Klebsiella), which are thought to be intrinsically resistant to clindamycin (Orrhage et al., 1994). Another culture-based study evaluating the impact of clindamycin prophylaxis in surgical patients reported a transient decline in Enterococci and Streptococci during antimicrobial administration (Kager et al., 1981). A substantial decline in anaerobic bacterial populations also occurred following clindamycin treatment, however the bacterial populations returned to their initial concentrations within two weeks.

1.2.8 Symptoms of CDI

The symptoms of CDI vary from self-limiting, mild diarrhoea to fulminant pseudomembranous colitis, sepsis, toxic megacolon, multiple organ failure, and death. Mild cases are characterised by slight fever, abdominal pains, and loose stools (Welfare et al., 2011). C. difficile colitis might start with non-specific signs such as erythema and oedema on endoscopy. The left colon predominantly becomes inflamed. The condition can advance to severe colitis with characteristic adherent pseudo-membranes. Severe CDI is associated with fever, hypoalbuminemia (Joint, 2001), and leucocytosis, which usually precedes signs of colitis (Ohashi & Ushida, 2009). Despite diarrhoea being the symptomatic hallmark of CDI, it might be absent initially, owing to a possible colonic dysmotility from the disease process or previous conditions (Jaber et al., 2008). This is particularly crucial in surgical patients that might have an accompanying ileus. Therefore, it is important to have a high suspicion index in surgical patients for CDI development (Sartelli et al., 2019).

To evaluate the prognostic value of leucocytosis, renal failure, and fever, Bauer et al., (2012) examined the data from two randomised controlled trials (RCTs), that contained data on 1105 CDI patients. The authors reported that both renal failure and leucocytosis were important predictors of severe CDI. Miller et al., (2013) reported an analysis of the two same clinical trials to validate a system of categorisation to classify patients of CDI into mild, moderate, or severe. A blend of five commonly available and simple laboratory and clinical variables determined during CDI diagnosis were used accurately to predict treatment response upon CDI therapy. The criteria were: age, treatment with antibiotics,
leucocyte count, serum albumin, and serum creatinine levels (ATLAS). The ATLAS predictors of severe CDI included: WBC > 15 x 10^9/L, Temperature > 38.5 °C, Albumin < 2.5 g/dL, and increase in serum creatinine level ≥ 133 µM/L.

Recently, di Masi et al., (2018) showed that human serum albumin is able to bind toxins A and B, therefore impeding their internalisation to host cells; this may offer some explanation to the heightened CDI severity that hypoalbuminemic patients suffer.

1.3 *C. difficile* pathogenicity locus

There are several virulence factors that contribute to the retention of *C. difficile* within the gastrointestinal tract GIT (van Nood et al., 2013), however, the exotoxins TcdA and TcdB are the main virulence factors produced by *C. difficile* (Sokol et al., 2016). Clinical strains that do not produce at least one of these toxins are avirulent in animal models (Youngster et al., 2014). The genes that encode both toxins are situated within the pathogenicity locus or PaLoc, which is a 19.6 kb region of the chromosome (Forssten et al., 2015). In addition to the toxin genes, *tcdA* and *tcdB*, the pathogenicity locus also encodes TcdR, TcdC, and TcdE, proteins that are thought to function in the regulation of toxin production (Pirker et al., 2013). TcdR is a member of the extracytoplasmic function (ECF) family of alternative sigma factors and is crucial for up-regulating the expression of PaLoc genes (McFarland, 2006).

Experimental data indicates that TcdC is an anti-sigma factor that regulates the expression of *tcdA* and *tcdB* by preventing the association of the core RNA polymerase with TcdR (Pirker et al., 2013). Epidemic ribotype 027 strains have a nonsense mutation within the *tcdC* gene, fuelling the hypothesis that de-repression of the toxin genes by inactivation of TcdC may be partly responsible for the reported elevated toxin expression in these strains and their elevated virulence (Wong et al., 2014). The role of TcdC remains controversial since conflicting data have been documented about the exact function of TcdC in toxin production and virulence (Smits, 2013).
TcdE is likely to be a holin family protein which might play a role in the export of TcdA and TcdB from the cell (Hundsberger et al., 1997; Tan et al., 2001). Nonetheless, the specific role of this protein is also enmeshed in controversy, as different studies reported conflicting data on the ability of TcdE to effect the export of TcdA and TcdB from C. difficile (Govind & Dupuy, 2012; Olling et al., 2012).

TcdA and TcdB share a high degree of similarity at the amino acid level, an indication that a gene replication event might have given rise to the separate toxins (von Eichel-Streiber et al., 1992). Multiple conserved domains shared by the toxins include a catalytic glucosyltransferase domain situated at the N-terminus, a cysteine protease domain that is essential for auto-processing and transport of the catalytic domain to the intoxicated cell. In addition, there is a hydrophobic translocation domain situated in the centre of the toxins, which is involved in pore formation and toxin insertion into the endosomal membrane before they are released into the cytosol. The fourth is a C-terminal receptor binding domain that consists of a repeating series of oligopeptides; that has been reported to bind to unknown receptors on target cells (Smits et al., 2016).

Furthermore, a direct relationship between a quorum-sensing system and C. difficile toxin production and pathogenesis is thought to exist (Darkoh et al., 2015). The authors demonstrated that C. difficile toxins are regulated via an accessory gene regulator (Agr) quorum-sensing system. The purified quorum signal from the culture supernatant and faecal samples of CDI patients brought about toxin synthesis in non-hypervirulent and hypervirulent C. difficile strains. Strikingly, the sequenced genomes of all the C. difficile strains encode a cysteine-containing AgrDI autoinducer prepeptide. Hydroxylamine treatment which is known to disrupt thioester bonds (List et al., 2012) led to a loss of activity in the toxin-inducing (TI) signal. A suggestion that a thioester bond is present in the TI signal and necessary for its activity. Following a series of experiments, Darkoh et al., (2015) further suggested that the TI signal is a novel thiolactone. They postulated that following a disruption of the colonic microbiota by antimicrobial therapy which allows C. difficile to proliferate, the rapidly increasing bacterial population synthesises and releases the TI signal, which accumulates in the extracellular milieu. Consequently, the local concentration of the TI reaches a threshold level at high cell density and activates an Agr system leading to transcriptional activation of the toxin genes. The detection of the TI signal in faecal samples from
CDI patients is an indication that this process is active in human patients and contributes significantly to CDI.

1.3.1 Binary toxin CDT.

*C. difficile* transferase (CDT) or binary toxin is the third toxin produced by some strains of *C. difficile*; the epidemic PCR ribotypes 027 and 078 in particular (Smits *et al.*, 2016). There has been increased interest in CDT due to its increasing prevalence in human and animal *C. difficile* isolates (Gerding *et al.*, 2014). The *cdtA* and *cdtB* genes are situated in an operon on the binary toxin locus encodes CDT (Goncalves *et al.*, 2004). The binary toxin locus (CdtLoc) has an approximately 2kb deletion in CDT-negative stains (Stare *et al.*, 2007). The role of CDT in the pathogenesis of CDI remain elusive, however, it is a member of the binary ADP-ribosylating toxin family and is made up of two components: one enzymatic component (CDTα) and a binding component (CDTβ) that enhances the passage of the enzymatic component to the cell cytosol (Gerding *et al.*, 2014). Because of its ADP ribosyltransferase activity, CDTα once in the cytosol initiates a total destruction of the actin cytoskeleton, and eventually cell death (Popoff *et al.*, 1988).

There are contradictory reports of the role of CDT in disease. Very rare cases of *C. difficile*-dependent enterocolitis have been traced back to CDT in the absence of toxins A and B in animal models. Following a study that investigated the virulence of isogenic toxin mutants in a hamster CDI model, researchers showed that CDT increased the virulence of *C. difficile* when toxins A and B were present (Kuehne *et al.*, 2014). One possible mechanism of action proposed by the authors was that CDT might increase the adherence of *C. difficile* owing to the formation of a network of microtubule-based protrusions. However, CDI was observed following infection with a *C. difficile* strain that was toxin A- and B-negative but CDT positive (Androga *et al.*, 2015).
1.4 Biofilm formation

Biofilms are multicellular communities of microorganisms that grow on abiotic and biotic surfaces, and are enmeshed in a matrix of extracellular polymeric substances (EPS) (Heydorn et al., 2000), conferring on the microbe an effective protection from antimicrobials and disinfectants (Peng et al., 2002), and an increased possibility of survival in nutrient-deficient conditions (Koch et al., 2001).

Biofilm formation has been thought to promote the persistence and pathogenesis of C. difficile (Vuotto et al., 2018). The growth of biofilms is seen as a potent threat due to the associated increase in bacterial resistance which render antimicrobials often ineffective. In spite of the likely contribution of biofilms to the pathogenesis of CDI, the precise mechanism governing C. difficile biofilm formation, in addition to the role of biofilms in CDI have not been clearly elucidated.

Recently, Soavelomandroso et al., (2017) reported that C. difficile formed biofilms in the caecum and colon of a mono-associated mouse model. Although the mono-associated mouse model may not present an accurate representation of the human colon given its lack of microbial diversity, it allows for the study of C. difficile behaviour in vivo. Additionally, C. difficile is capable of coexisting in multicellular communities, as well as in human periodontal-disease-associated biofilms (Semenyuk et al., 2015; Colombo et al., 2016). In a similar manner, C. difficile has been shown to form thick biofilms in vitro in the presence of Finegoldia magna (Donelli et al., 2012). Such in vitro biofilms confer protection of C. difficile against antimicrobials such as metronidazole and vancomycin (Dapa & Unnikrishnan, 2013). In contrast, antimicrobials such as fidaxomicin and surotomycin were shown to have a capacity to distort the biofilm structure (James et al., 2018).

Structurally, the matrix of C. difficile biofilms is made up of extracellular DNA (eDNA), proteins, and polysaccharides (such as polysaccharide II) (Dawson et al., 2012; Dapa & Unnikrishnan, 2013). Toxins were reported to be found in the biofilm matrix and might contribute to CDI upon a rupture of such biofilms. C. difficile has been also shown to form spores in biofilms (Semenyuk et al., 2014). Additionally, Crowther et al., (2014) reported that C. difficile spores were preferentially found in biofilm structures in a triple stage human gut model of complex multi-species biofilms.
Researchers have sought to ascertain if the formation of biofilms was related to the PCR-ribotype and sequence type. Pantaleon, et al., (2018) demonstrated that biofilm formation is not uniform among *C. difficile* strains, for example ribotypes 020 and 005 formed high and medium biofilms; sequence type 19 strains formed high, medium, and low biofilms, an indication that there is no relationship between the ability of a *C. difficile* strain to form biofilms and clinical symptoms during infection.

1.5 Diagnosis

There should be a suspicion of *C. difficile* infection in patients with more than three loose stools in a 24 hour period without another clear possible explanation such as the use of laxatives, especially in a setting with known risk factors such as: advanced age, antibiotic use, and hospitalisation (Sartelli et al., 2019). A precise and quick diagnosis is central to effective CDI management. Such early diagnosis enables early treatment and may likely improve outcomes. Similarly, a quick isolation of patients with CDI infection is crucial in mitigating *C. difficile* transmission (Barbut et al., 2014).

Clinical signs and symptoms in addition to laboratory tests should form the basis for CDI diagnosis (Sartelli et al., 2019). The diagnostic tests for *C. difficile* can test for metabolic products of *C. difficile* such as glutamate dehydrogenate (GDH), aromatic fatty acids, TcdA and/or TcdB. The detection of toxin-producing *C. difficile* can be achieved by culture methods, whereas tests for the amplification of nucleic acid can be carried out for *C. difficile* genes to detect 16S rRNA, the GDH-encoding gene, or toxin genes (Van Tassell & Miller, 2011). The choice of test is important to differentiate between asymptomatic carriers and CDI patients. Tests that detect the bacterium might suggest colonisation and not necessarily the disease, in contrast those that detect toxins are distinct to CDI (Wong et al., 2015).

Due to huge differences in sensitivity and specificity of the different diagnostic tests, a two-step algorithm, which includes a test for *C. difficile* presence and the other to detect free toxins in the faecal sample has been recommended by the European Society of Clinical Microbiology and Infectious Diseases (Debast et al., 2014). Another diagnostic algorithm employs a coupled enzyme immuno-assay which detects TcdA or TcdB and GDH simultaneously (Ota & McGowan, 2012). For the reason that
the sensitivity of the toxin element of the test is unreliable, GDH-positive samples but toxin-negative, are made to undergo further evaluation to find out if a toxigenic *C. difficile* is present using a nucleic acid amplification test (Smits et al., 2016)

Dependence on molecular tests for diagnosing CDI without testing for toxins will lead to over-diagnosis and inappropriate treatment (Polage et al., 2015). Due to the fact that toxins can be found even after successful treatment (Louie et al., 2012), it is not advised to monitor patients regularly using toxin tests. Patients might be colonized asymptotically by *C. difficile* and diarrhoea caused by other factors or patients may in fact be suffering from CDI but toxin levels are under the limit of detection of the chosen assay. For such patients, a clinical examination is useful to ascertain if treatment for CDI is required.

The use of stool specimen limits the ability of clinicians to diagnose suspected severe CDI in patients complicated by ileus. Therefore, following a study by Kundrapu et al., (2012), evidence was presented that suggested PCR testing of perirectal swabs was an acceptable substitute to stool sample analysis for patients with ileus who might not be able to pass stool specimens.

The presence of a consistent clinical picture and context such as recent antimicrobial use and hospitalisation are important in deciding patients that should be tested for CDI. Abdominal pain, fever, leucocytosis, together with other laboratory investigations (e.g. serum lactate and creatinine) are important in ascertaining the severity of infection. Additionally, any of the following may be predictors of severe CDI a white blood cell count of $>15 \times 10^9$/L, temperature of $>38.5$ °C, serum creatinine level of $\geq 133$ μM/L or $\geq 1.5$ times premorbid level, or Albumin $<2.5$ g/dL (Sartelli et al., 2019).

There is still no consensus on a single stool test that can be referred to as the reference standard for CDI diagnosis. Historically, toxigenic culture (TC) was used by many microbiologists as the preferred method for CDI diagnosis. The TC procedure involves stool culture for *C. difficile* in a selective medium and an assay to determine the capacity of colonies to produce toxins. Despite TC previously considered as the gold standard technique, the prolonged turnaround time, and its incapacity to discover toxins presence in stool are marked issues with the method. This might also result in false positive results
because about 7% of asymptomatic hospitalised patients might be colonised with toxigenic strains of
*C. difficile* (Kyne *et al*., 2003).

The use of Nucleic Acid Amplification Tests (NAATs) for the identification of *C. difficile* in stool samples from diarrhoeal patients was reported about three decades ago (Lyerly *et al*., 1991). NAATs possess low complexity, fast turnaround time, excellent specificity and sensitivity, simplified reporting, and a decreased requirement for repeat testing (Chen *et al*., 2017; Gerding *et al*., 2014; Smits *et al*., 2016). Despite the superior specificity and sensitivity of NAATs, this assay is not routinely performed in many laboratories (Polage *et al*., 2012). Besides some setbacks have been linked with NAATs (Schmidt & Gilligan, 2009). An obvious limitation of NAATs is its inability to accurately differentiate between *C. difficile* colonisation and disease, which might culminate in over-diagnosis and overtreatment. There is still an ongoing debate if a positive stool sample from a molecular assay requires a confirmatory free toxin assay (Planche *et al*., 2013), because it is able to detect toxigenic *C. difficile* in asymptomatic patients. This reinforces the importance of testing only symptomatic patients.

In some clinical settings, supplementary methods of testing such as radiological diagnostic imaging might be employed for diagnosing CDI. Computerised tomography (CT) imaging may provide useful information for early diagnosis, and may assist in ascertaining disease severity in CDI patients (Napolitano & Edmiston, 2017). CT has been widely studied as an imaging method for diagnosing *C. difficile* colitis (CDC) (Ros *et al*., 1996; Merine *et al*., 1987; Fishman *et al*., 1991; Boland *et al*., 1994). The most common CT finding of CDI is a thickened colonic wall. Although this is not specific and may be found in other colitis forms, it is more prominent in CDI (Sartelli *et al*., 2019).
1.6 Treatment

1.6.1 Antimicrobials used to treat CDI

1.6.2 Metronidazole

Metronidazole has been the primary CDI treatment choice for over 3 decades. Nevertheless, reduced efficacy of metronidazole in treating CDI was documented by Musher et al. (2005). Metronidazole exerts its antimicrobial action by damaging bacterial DNA directly after reduction of its nitro group as soon as enters a bacterium (Baines & Wilcox, 2015). Reduced initial response to metronidazole and elevated recurrence of CDI following metronidazole first-line therapy have also been reported by Pépin et al. (2005), in addition to studies that have reported reduced susceptibility to metronidazole (Freeman et al., 2008; Brazier et al., 2001). Furthermore, metronidazole-resistant/reduced susceptible *C. difficile* have been reported in several studies (Brazier et al., 2001; Lynch et al., 2013; Orden et al., 2017). Notwithstanding the reports of reduced susceptibility and resistance to metronidazole, failure in CDI treatment has not been associated with antimicrobial resistance in *C. difficile* (Purdell et al., 2011).

In *Helicobacter pylori* and *Bacteroides fragilis*, metronidazole resistance is often facilitated by nitroimidazole (*nim*) genes (Gal & Brazier, 2004), yet these genes have not been discovered in *C. difficile* (Moura et al., 2014). Despite gaps in knowledge, data from studies on strains ribotype (RT) 010 and RT 027 indicate that metronidazole resistance in *C. difficile* is multifactorial with roles for altered metabolic pathways, such as nitro-reductase activity, DNA repair, and iron uptake (Moura et al., 2014; Chong et al., 2014). Additionally, the formation of biofilms has been suggested to contribute to metronidazole resistance in *C. difficile* (Vuotto et al., 2016). Although the exact role of biofilm growth in *C. difficile* metronidazole resistance remains unclear, Spigaglia et al., (2018) hypothesized that the biofilm matrix may act as a protective barrier, causing a change in the physiological state of the bacteria in the biofilm that brings about an increased resistance to antibiotics.

Patients with moderate CDI may be treated with oral metronidazole, while on the contrary, for complicated or severe infections, oral vancomycin is recommended (Debast et al., 2014). Repeat courses of metronidazole are discouraged owing to the risk of cumulative and very likely irreversible
neurotoxicity (Kuriyama et al., 2011). Indeed, recent guidelines from the Infectious Disease Society of America (IDSA) suggested that metronidazole be administered only to patients with an initial non-severe CDI episode in settings with limited access to vancomycin or fidaxomicin (McDonald et al., 2018).

Findings from different randomized controlled trials showed vancomycin was superior to metronidazole based on clinical cure rates in CDI patients (clinical cure rates 81.1% versus 72.7%) (Johnson et al., 2014). Nonetheless, CDI patients with toxic megacolon or paralysed bowel, such that oral antibiotics are unable to reach the site of disease, can be treated with rectally delivered vancomycin plus intravenously delivered metronidazole (Smits et al., 2016). Furthermore, after the successful resolution of an initial CDI episode, the possibility of a recurrence in a period of 8 weeks is up to 25%, even worse for a patient with one or two prior recurrences, the risk of further recurrences is as high as 65% (Kelly, 2012).

1.6.3 Vancomycin

In a similar manner to metronidazole, vancomycin has been a first-line treatment option for moderate to severe CDI for over 30 years (Jarrad et al., 2015); its concentrations usually go beyond 1000 mg/L in faeces following oral administration (Baines & Wilcox, 2015) due to its poor absorption in the gastrointestinal tract (Keith et al., 2000). Vancomycin continues to have the desired activity against C. difficile, even against epidemic strains, and strains with reduced metronidazole susceptibility (Baines & Wilcox, 2015). Vancomycin brings about its antibacterial action by binding to polypeptide intermediates terminating in -D-Ala-D-Ala effectively inhibiting the biosynthesis of peptidoglycan, an important component of bacterial cell wall.

There are sporadic reports of reduced susceptibility to vancomycin over in recent years, this is in addition to the emergence of single and multiple isolates from 6 European countries, resistant to vancomycin (MIC ≥ 8 mg/L) (Baines & Wilcox, 2015). The vancomycin resistance/reduced susceptibility mechanism(s) in C. difficile remain unclear. Despite Tn1549-like elements been identified
in several *C. difficile* strains (Brouwer *et al*., 2011; 2012), these elements, unlike the original Tn1549 element reported in *E. faecalis*, have no functional *vanB* operon. Strikingly, a *vanG*-like gene cluster similar to that reported in *E. faecalis* have been also identified in *C. difficile* but it appears incapable of promoting resistance to vancomycin (Ammam *et al*., 2012; 2013). Interestingly, vancomycin-resistant mutants, with an amino acid change Pro108Leu in MurG were obtained *in vitro* recently (Leeds *et al*., 2014). Given that MurG plays a role in the membrane-bound phase of peptidoglycan assembly, this amino acid substitution might affect vancomycin activity. Additionally, biofilm formation has been previously reported to contribute to vancomycin resistance. Indeed *C. difficile* within biofilms showed more resistance to high vancomycin concentrations (20 mg/L), and inhibitory and sub-inhibitory concentrations of vancomycin appeared to enhance biofilm production (Dapa *et al*., 2013).

A systematic review and meta-analysis in 2015 that compared the safety and efficacy of vancomycin monotherapy and metronidazole monotherapy in CDI patients showed that there was no statistically significant difference in the rate of clinical cure between vancomycin and metronidazole for mild CDI. However, the rate for clinical cure was higher for vancomycin than metronidazole for severe CDI (Li *et al*., 2015). More recently, in 2017, a Cochrane review was published that suggested vancomycin was superior to metronidazole in all CDI cases (Nelson *et al*., 2017). This might be due to the superior pharmacokinetics of vancomycin, resulting in substantially higher intra-luminal gut concentrations compared to metronidazole.

### 1.6.4 Fidaxomicin

Fidaxomicin is a narrow spectrum, macrocyclic, bactericidal antimicrobial drug, which is absorbed poorly following oral administration, and inhibits bacterial RNA polymerase at a site different from the rifamycins (Venugopal & Johnson, 2011). Fidaxomicin is the foremost new drug licensed for CDI treatment in 3 decades; and is now recommended for treatment in recurrent bouts of CDI (Debast *et al*., 2014). Fidaxomicin has a high potency against *C. difficile*, with MICs typically 0.02-0.025 mg/L (Freeman *et al*., 2015). Although there are very few reports of reduced susceptibility (MIC 2-4 mg/L)
(Leeds et al., 2013) and resistance (MIC 16 mg/L) (Goldstein et al., 2011), even these higher MICs are about two orders of magnitude lower than fidaxomicin concentrations attainable in the gut lumen (Baines & Wilcox, 2015).

The antibiotic agent (orally 200 mg twice a day for 10 days) shows cure rates superior to vancomycin and a considerably lower CDI recurrence rate (Louie et al., 2011). Similarly, two prospective trials showed that fidaxomicin was superior to vancomycin for initial CDI cure (Fekety et al., 1984; Barlett et al., 1980). Furthermore, its deleterious effect on the composition of the indigenous microbiota, especially on Bacteroides species is minimal (Tannock et al., 2012). Accordingly, fidaxomicin may be considered for treating patients at increased risk of recurrence.

Mutations in CD22120 (or rpoB), which encodes for a MarR (multidrug resistance-associated transcriptional regulator) homologue have been identified in C. difficile mutants showing resistance to fidaxomicin obtained in vitro (Leeds et al., 2014). Given the mutations that cause fidaxomicin resistance appear in rpoB gene at a different loci compared to those that cause resistance to rifamycins (Spigaglia et al., 2018), fidaxomicin does not lose activity against rifamycin-resistant strains (Anti-Infective Drugs Advisory Committee Briefing Document, Optimer Pharmaceuticals, Inc. 2018).

The use of other antimicrobials such as teicoplanin, fusidic acid, rifamixin (Cornely et al., 2012), nitazoxanide (Musher et al., 2007) and tigecycline (El-Herte et al., 2012; Herpers et al., 2009) for CDI treatment has been reported, however, they are not recommended currently for general use.

1.7 Recurrent CDI

A diagnosis of recurrent CDI (rCDI) is made if CDI recurs less than 8 weeks following the resolution of an initial episode, as long as the symptoms from the earlier episode resolved upon completing the first treatment and other causes are excluded (Sartelli et al., 2019). Symptomatic rCDI occurs in about one in five of CDI patients (Eyre et al., 2012). Accordingly, experienced clinicians should direct the treatment of rCDI patients. Oral vancomycin (125 mg four times daily for 14 days) may be used for the
treatment of first CDI recurrence (especially if the first episode was treated with metronidazole) or fidaxomicin (200 mg twice a day for 10 days).

In rCDI, vancomycin is usually administered by a prolonged tapered regimen which is thought to be more efficient than the standard 10 to 14 days course, even though no RCTs have been published in second or later CDI recurrences (Cornely et al., 2012). Fidaxomicin is associated with a lower propensity of CDI recurrence after an initial recurrence (Louie et al., 2011; Cornely et al. 2012; O’Horo et al. 2014). However, there is still an absence of prospective RCTs investigating the potency of fidaxomicin in patients with multiple RCDI. Nonetheless, faecal microbiota transplant have been reported to resolve rCDI (Gough et al., 2011).

1.8 Non-antimicrobial treatments

1.8.1 Prebiotics

Changes to the microbiota that result in reduced strength of the mucosal barrier can result in CDI. Accordingly, a plan to boost mucosal barrier function will confer health benefits in a healthy individual and may play a role in disease prevention.

Prebiotics are non-digestible (by the host) oligosaccharides that selectively enhance the activity and/or growth of a limited number of beneficial bacterial species in the gut which benefits host health (Gibson & Roberfroid, 1995). Therefore, adding prebiotics to the diet might increase the activity and biomass of lactic acid-producing bacteria (lactobacilli and bifidobacteria), that boosts the host’s mechanism of defence. Some compounds that have been documented to show prebiotic activity are fructo-oligosaccharides, lactulose, galacto-oligosaccharides (Gibson et al., 2004), and inulin (Joshi et al., 2018). Some of these prebiotic compounds for example inulin are present naturally in foods such as bananas, onions, leeks, garlic, asparagus, and wheat (van Loo et al., 1995). Prebiotic compounds can also be extracted from tubers and vegetables, for example Jerusalem artichoke and chicory root. Pectins and xylans are examples of polysaccharides in plant cell walls with a growing potential to be prebiotics.
(Scott et al., 2019). The growing popularity of such prebiotic candidates is largely due to their indigestibility within the upper GIT and their selective fermentation by microbiota in the colon.

Current prebiotics are mainly carbohydrate-based, however, other compounds like polyunsaturated fatty acids and polyphenols might have prebiotic activity (Gibson et al., 2017). It is important to highlight that increased ingestion of prebiotic foods might not lead to an expression of a profound prebiotic effect given the dose of prebiotic required for such beneficial health effect. Although the optimum prebiotic dose is unclear, studies have reported different prebiotic doses ranging from 5-8g/d (Bosscher et al., 2006; Ramnani et al., 2010) 12 g/d (Lewis et al., 2005; Van Loo et al., 2005), 15 g/d (Den Hond et al., 2000; Jain et al., 2004), 25 g/d (Welters et al., 2002), to 32 g/d (Anderson et al., 2004) that recorded improved clinical outcomes such as anti-carcinogenic effect, improved nutritional status, as well as a reduced incidence of hypercholesterolemia. Accordingly, frequently consumed foods may be fortified with prebiotics.

More recent knowledge of the gut microbiota composition has revealed that lactobacilli and bifidobacteria which were the original targets of prebiotics constitute only a small component of the gut microbiota which is predominantly dominated by Firmicutes and Bacteroides, including Ruminococcaceae and Lachnospiraceae. Specifically increasing concentrations of butyrate, the metabolic outcome of some prebiotics can be improved (Riviere et al., 2016). Research has now shown that inulin-type fructans (ITFs) enhance the growth of bifidobacteria, as well as increase the populations of Faecalibacterium prausnitzii (Ramirez-Farias et al., 2008). Similarly, Neyrinck et al., (2011) reported that Roseburia species numbers are enhanced by arabinoyxans. Roseburia spp. and F. prausnitzii constitute the most prevalent producers of butyrate in the human gut (Louis et al., 2010).

There is evidence that various bacterial genera are capable of utilising prebiotics as growth substrates in vitro (Scott et al., 2014). Observed effects in vitro may not guarantee similar effects in vivo because other individual factors may affect the capacity of prebiotics to bring about beneficial effects. For example, different individuals have a tendency to be colonised by distinct particular bifidobacterial species, and in the Bifidobacterium genus there exists a marked difference in the capacity of different species to utilise differing chain lengths of inulin-type fructans (Selak et al., 2016). Therefore, if the
indigenous microbiota lacks the particular bacterium that is able to utilise the added prebiotic, the effects may not be seen. The structure of most prebiotics often consists of sugars with $\beta(2\rightarrow1)$, $\beta(1\rightarrow6)$, $\beta(1\rightarrow3)$, or $\beta(1\rightarrow4)$ linkages. This linkage bonds in FOS and GOS are readily degraded by $\beta$-fructanosidase and $\beta$-galactosidase enzymes respectively, which are abundant in particular bifidobacterial species (Gibson et al., 2017). Additionally, Scott et al., (2014) demonstrated that the chain length of some prebiotics for example fructans is an important criterion in ascertaining which bacteria can ferment them.

It is equally important to highlight that gut bacteria coexist in a mixed, competitive ecosystem. This implies that results from pure culture experiments do not necessarily mean a particular strain can utilise the same substrate in a competitive condition. Some bacteria depend on metabolites released by other bacteria for growth. This was shown in a co-culture experiment where *Eubacterium hallii* was unable to grow on FOS substrate, whereas *Bifidobacterium adolescentis* grew well on FOS, subsequently releasing acetate and lactate. Interestingly, in the mixed culture, q-PCR quantification confirmed the growth of both bacteria, and no lactate, only butyrate was detected in the growth medium (Belenguer et al., 2006; Moens et al., 2017).

Batch-culture experiments using isolated bacteria *in vitro* showed that bifidobacteria (Gibson & Wang, 1994), in addition to some lactobacilli, such as *Lactobacillus plantarum* (Cebeci & Gürakan, 2003) grew in culture medium with oligofructose or inulin as the single source of carbon, as well as a corresponding decrease in faecal pathogens, clostridia, coliforms, and *Bacteroides* spp. This is consistent with a report of the bifidogenic effects of oligofructose and inulin in human subjects that were fed oligofructose 15g/d with a supplementation of the diet with inulin 15g/d for 2 weeks (Bosscher et al., 2006). The numbers of fusobacteria, clostridia, and *Bacteroides* spp. decreased considerably ($P < 0.01$). Consequently, prebiotics have become an attractive intervention to potentially prevent CDI. Indeed, Piotrowski et al., (2019) reported a concentration-dependent anti-adhesive effect of mannose and fructooligosaccharides against *C. difficile* by significantly reducing the adhesion of *C. difficile* strains to various human epithelial cell lines.
1.8.2 Probiotics

The gut microbiota is a very diverse and complex community of microbes which has co-evolved in a commensal manner with humans (Donaldson et al., 2016). This community of microorganisms in a healthy state confers protection to the host by preventing colonisation by pathogens; so-called colonisation resistance. However, the intestinal microbiota can be disturbed by exposure to antimicrobial agents, resulting in a decreased species diversity and microbial abundance. This in turn can compromise the gut barrier and can culminate in antibiotic-associated diarrhoea (Valdes-Varela et al., 2018). In some individuals (ca. 20% of antibiotic associated diarrhoea), the dysbiosis upon antibiotic treatment permits an overgrowth of *C. difficile* owing to the fact that the perturbed intestinal environment has a high abundance of carbohydrates and primary bile acids, a decreased concentration of short chain fatty acids, and an immunocompromised host with no microbial competitors in the gut (Lawley & Walker, 2013).

The standard treatment for CDI is metronidazole, vancomycin, or fidaxomicin, but reduced susceptibility and resistance to these agents, as well as the recurrence rate of CDI and a perpetration of dysbiosis has prompted scientists to seek alternative therapies which have been reviewed by different researchers (Martin & Wilcox 2016; McFarland 2016; Padua & Pothoulakis, 2016). Consequently, probiotics have been suggested as a potential therapy for the prevention of microbiota dysbiosis caused by antibiotic administration, and restoration of the microbiota following antibiotics use (Reid et al., 2011).

Probiotics are live organisms that present beneficial effects to the health of host when administered in sufficient quantity (Joint, 2001). To an increasing extent, they are readily available as dairy-based products and capsules, and can be purchased in supermarkets. Inasmuch as there exist a high number of commercially available probiotics, their beneficial effects (if any) and the specific organisms that might be most potent in a specific patient group remains debatable (Hempel et al., 2012). Commonly employed microorganisms in probiotic preparations include *Bifidobacterium* spp., *Bacillus* spp., *Lactobacillus* spp., *Saccharomyces* spp., and *Escherichia* spp. (Ohashi & Ushida, 2009).
Many strain- and species-specific factors dictate what benefit a probiotic might confer. A probiotic must be able to colonise the GIT by adhesion to the gastrointestinal mucosa (Van Tassell & Miller, 2011) in order to bring about its beneficial effects. Even though it still remains unclear, present evidence indicates that the adhesive characteristics of probiotics might be as a result of distinctions in the expression of large surface proteins and their interactions with mucus-binding proteins (Van Tassell & Miller, 2011).

Lactobacilli and bifidobacteria are thought to possess immune-modulating abilities. In mouse experiments (Pouwels et al., 1996), in addition to human studies (Kaila et al., 1992), the oral administration of lactobacilli has been demonstrated to lead to an increased production of gamma-interferon and marked increased IgA secretory responses against Salmonella, and elevated stimulation of lymphocytes, macrophages, and natural killer cells. Similarly, there is some evidence that Saccharomyces boulardii is capable of reducing the incidence of AAD (McFarland, 2010), notwithstanding, there is evidence of the probiotic properties of Lactobacillus rhamnosus GG (McFarland, 2006), L. acidophilus La-5 and Bifidobacterium Bb-12 (Wenus et al., 2008), and Streptococcus thermophilus (Hickson et al., 2007). A meta-analysis carried out by Hempel et al., (2012) included 82 randomised controlled trials, out of which 17 involved Lactobacillus interventions. The meta-analysis showed that probiotic administration was linked to reduced risk of AAD, although, there were differences in study designs, probiotic strain type, length of treatment, dosage, and study population, probably providing an explanation to previous discrepancies (McFarland, 2006).

The use probiotics in preventing AAD and CDI in patients in spinal injuries centres was reported by Wong et al., (2015), this is in tandem with the findings of Lewis et al., (2009) that suggested probiotic fermented milk products containing probiotics reduced the risk of AAD and CDI by a number of mechanisms such as: competition for colonization sites and nutrients, SCFA production which reduces the pH of the gut, immune stimulation, and production of antimicrobials such as bacteriocins that directly antagonize pathogenic bacteria. Bifidobacterium have been reported to produce proteinaceous compounds that impact on the growth of a vast array of both Gram-positive and Gram-negative bacteria.
(Gibson & Wang, 1994). Fukuda et al., (2011) demonstrated that the acetic acid produced by bifidobacteria has a key role in the protection against invading microbial pathogens.

The capacity of probiotics to inhibit *C. difficile* growth has been documented by employing various experimental techniques (Auclair et al., 2015; Forssten et al., 2015; Valdes-Varela et al., 2016b; Fredua-Agyeman et al., 2017). In spite of the large number of studies undertaken *in vitro* for the identification of probiotics with activity against *C. difficile* and the selection of probiotic strains for the treatment or prevention of CDI, strong evidence from human trials on a CDI-specific benefit of probiotic therapy is still limited. Various probiotic strains have been associated with an increased colonisation resistance against *C. difficile* (Hopkins & Macfarlane 2003; Auclair et al. 2015; Forssten et al. 2015). Some lactobacilli and bifidobacterial strains have been reported to decrease the adhesion to intestinal mucus or intestinal epithelial cells by *C. difficile* (Collado et al., 2005; Banerjee et al., 2009) or shown an ability to prevent *C. difficile* growth (Lee Chung & Seo 2013; Valdes-Varela et al. 2016b). Additionally, animal studies appear to reaffirm a likely benefit of probiotics in increasing colonisation resistance against *C. difficile* (Mansour et al., 2017). Nonetheless, a majority of the clinical studies have been centred on prevention, and there is an absence of data on the possible use of probiotics for CDI treatment.

Despite the large number of probiotic strains studied *in vitro*, almost all the evidence from clinical trials were obtained from a handful of strains. Among the screened strains, the ability of the yeast *Saccharomyces boulardii* (Kotowska et al., 2005; Besirbelloglu et al., 2006) or *Lactobacillus rhamnosus* strain GG (Vanderhoof et al., 1999) in preventing AAD has been widely reported.

Despite limitations due to inter-product and inter-strain variability, meta-analysis studies of probiotics for AAD prevention have continuously presented evidence for a beneficial role (Cremonini et al., 2002; Sazawal et al., 2006; Goldenberg et al., 2015). Moreover, some studies have paid specific attention to confirmed CDI and such studies have also reported positive results for primary CDI prevention (Gao et al., 2010; Allen et al., 2013; Dietrich et al., 2014; Maziade et al., 2015). For example, a Canadian hospital, the “Pierre-Le Gardeur” administered a probiotic mix simultaneously with any antibiotic after a *C. difficile* outbreak. This culminated in a marked reduction in the number of CDI cases (Maziade et
al., 2015). In general, the data from systematic reviews and meta-analysis support a beneficial role of probiotics on the primary prevention of CDI. Although, the heterogeneity that exists between clinical studies makes it more challenging to define the most suitable probiotic to use, the dose, and its administration regime.

Even though no cases of translocation of microorganisms have been reported in clinical trials with probiotics for antibiotic-associated diarrhoea or CDI, probiotics should be used with caution. Several studies of invasive disease have been reported, resulting from the use of probiotics such as *Saccharomyces boulardii* in debilitated or immunocompromised patients (Enache-Angoulvant, & Hennequin, 2005; Munoz et al., 2005). Besides, probiotics were linked with increased mortality, partly due to non-occlusive mesenteric ischaemia, in a randomized controlled trial in acute pancreatitis (Besselink et al., 2008).

The available data on the use of probiotics in preventing CDI recurrence is more limited than that of primary CDI prevention. Some intervention studies have been carried out with varied outcomes (McFarland et al., 1994; Surawicz et al., 2000), with meta-analysis and reviews suggesting there exists only limited evidence on the benefits of probiotics in secondary CDI prevention (Allen et al. 2013; O’Horo et al., 2014; McFarland 2015). The absence of sufficient evidence on secondary CDI prevention reinforces the need for more intervention studies in this regard.

Until recently, cost-effectiveness analyses of probiotics in CDI prevention have not been undertaken, with different results (Leal 2019; Starn et al., 2016), necessitating further research to be conducted in different healthcare systems.

### 1.8.3 Faecal microbiota transplantation

Evidence of the effectiveness of faecal microbial transplantation (FMT) continues to expand, yet it is still a non-regulated product. FMT entails implanting beneficial intestinal bacteria from a healthy donor to the gut of an individual with a dysfunctional microbiome. FMT is a very efficient rescue treatment and should be contemplated in patients who have had more than two recurrences, as antibiotics efficacy
in these patients is not more than 30% (Smits et al., 2016). The rationale behind FMT is that intestinal dysbiosis might be corrected by reintroducing normal flora through healthy donor faeces, and normal bowel functioning restored (Bakken et al., 2011).

Van Nood et al., (2013) reported that FMT was 81% effective in treating multiple recurrent CDI in a randomised controlled trial, although only 16 patients participated in the study. It is better to set aside FMT for patients with several recurrences of CDI who do not respond to other options of treatment (Bakken et al., 2011; Johnson et al., 2012; Cammarota et al., 2014; Goldenberg et al., 2017). Nonetheless, FMT lacks public acceptance and it is not a standard procedure, and the long-term effects of changing the gut microbiota of a patient are not known. These acceptability and safety concerns have meant FMT is yet to be adopted widely as a therapy. Therefore, a number of national guidelines have been devised to standardize FMT. Such guidelines include donor screening and selection (Sokol et al., 2016).

Data from a preliminary study of patients with a degenerative CDI showed that FMT using encapsulated inoculum (frozen) from different donors culminated in an improved outcome (Youngster et al., 2014). Such encapsulated preparations are simpler to administer and are less invasive, which might improve cost-effectiveness (Hirsch et al., 2015). Despite the high success rates of FMT (Orenstein et al., 2016), the “'handling’” of faeces and the enteral instillation techniques are not only unattractive for patients and physicians but it is also laborious. There is an increasing likelihood that future research will result in the production of cocktails of important microorganisms, combined according to the roles of the microorganisms in the microbiota against CDI, in place of FMT. This might improve the social acceptance and feasibility of microbiota transplantation. Petrof et al., (2014) reported that a mixture of 33 bacteria was effective against CDI in two patients. Similarly, bacteriotherapy using a cocktail of twelve bacteria retrieved from healthy donors resolved CDI and prevented CDI recurrence inside 30 days in 64% of patients (Tvede et al., 2015). In a murine study, Buffie et al., (2015) demonstrated that a mixture of four bacterial species protected mice from CDI, suggesting an activity via indirect effect on metabolism of bile acid.
Following a systemic review in 2011, Gough et al. reported that out of 317 CDI patients treated across 27 case series and reports, FMT was effective, recording CDI resolution in 92% of cases. Furthermore, 35% of patients in those studies administered FMT through enema, with a response of 95%, 19% of patients were administered through colonoscopy with a response rate of 89%, and 23% via naso-jejunal tube by gastroscope, with 76% response rate. FMT effectiveness differed by volume of FMT received, route of administration, treatment prior to instillation, and relationship to the donor (Gough et al., 2011).

Another systemic review by Cammarota et al., (2014) that included 20 full-text case series, 15 case reports, and 1 RCT, in which the majority of participating patients that were treated with FMT had suffered recurrent CDI episodes with diarrhoea in spite of standard antibiotic therapy. Out of 536 patients that received FMT, 87% (467) had their diarrhoea resolved. The rates of diarrhoeal resolution varied depending on infusion site: 93% in the ascending colon/caecum, 86% in the jejunum/duodenum, 84% in the distal colon, and 81% in the stomach. Severe adverse events due to the procedure were not reported.

More recently, Quraishi et al., (2017) published a review that evaluated the efficacy of FMT in the treatment of recurrent CDI; thirty case series and 7 RCTs were included. The authors concluded that FMT was more efficient than vancomycin in resolving recurrent CDI. Across all studies, clinical resolution of CDI was 92%. A marked difference was recorded between upper GI (88%) and lower GI delivery (95%) \( (p=0.02) \). No difference was observed between frozen and fresh FMT \( (p=0.84) \). Instilling consecutive FMT courses upon failure of initial FMT led to an incremental effect. There was a consistent donor screening, although there were differences in FMT volume and recipient preparation. Severe adverse events were not common.

Nevertheless, serious side effects such as disease flares have been reported following FMT (Cui et al., 2015; Costello et al., 2017). A case of aspiration pneumonia was reported in a patient that received FMT via the nasogastric route (Vermeire et al., 2016), in addition to mortality due to sepsis and toxic megacolon (Grewal et al., 2016).
Successful FMT practice requires long-time safety data, owing to the far-reaching consequences of the gut microbiota in health especially how a new gut microbiota may affect immune responses and brain function.

1.8.4 Non-toxigenic *C. difficile*

Non-toxigenic strains of *C. difficile* (NTCD) are avirulent due to the absence of the major virulence factors of Toxins A and B within the *C. difficile* genome. In theory, it might be attainable for NTCD to displace their toxigenic counterparts in infected or colonised individuals by competing for germinants and essential growth factors. A phase 2 double-blind, randomised, dose-ranging placebo-controlled trial assessed the ability of a NTCD strain in preventing recurrent CDI (RCDI) in patients with primary or recurrent infection who had completed treatment with vancomycin, metronidazole, or both antimicrobials (Gerding et al., 2015). The NTCD-M3 strain successfully colonised 69% of recipients. In the NTCD-colonised subjects, CDI recurrence rates were 2%, in comparison to 31% of recipients who were not colonised (p < 0.001), which was similar to placebo. This is an indication of the relationship between NTCD-colonisation and clinical efficacy. Strikingly, subjects that were colonised with NTCD at week 6 were no longer colonised at week 26. However, it has been shown experimentally that NTCD strains can be converted by genetic recombination and horizontal gene transfer into toxin producers *in vitro* (Brouwer et al. 2013). It is worrisome that distinct types of the pathogenicity locus could be acquired and transferred seemingly by any strain as this makes all the NTCD strains candidates for becoming toxin producers. Whether this acquisition of a toxigenic PaLoc can occur *in vivo* however, remained to be determined. The potential acquisition of the pathogenicity locus by NTCD strains that already show high resistance to antimicrobials widely employed in CDI treatment is a very troubling concern and therefore needs to be fully evaluated in any NTCD being considered as probiotics. For example, RT 010 strains are highly resistant to metronidazole (Moura et al., 2013). These findings about the PaLoc by Brouwer *et al.* might have far-reaching consequences on the probiotic prospect of NTCD, besides these events may be relevant to other pathogenic and commensal bacteria as well. A possible commercial development of NTCD strains remains to be seen. Unfortunately, it is very challenging to
develop new treatment/management for CDI, which will require well-designed studies with clearly defined populations.

1.8.5 Monoclonal antibodies

Owing to the fact that an expression of toxins A and/or B is essential for CDI development, the invention of monoclonal antibodies that work towards the prevention of the cytotoxic effect of the toxins holds promising potentials for disease prevention. The Food and Drug Administration (FDA) in 2016 approved bezlotoxumab, a human monoclonal antibody to decrease CDI recurrence in adults receiving antibiotic treatment for CDI and are at a heightened CDI recurrence risk. Bezlotoxumab (MK-6072) reduces RCDI by interfering with the binding of toxin B to cells of the host, hence mitigating epithelial damage and enhancing microbiota recovery (Barlett, 2017). Actoxumab is another human monoclonal antibody developed recently to neutralise *C. difficile* toxin.

Data from two randomised, double blind, placebo-controlled phase 3 trials, MODIFY I and II, which involved 2655 patients receiving antibiotics for primary or RCDI showed that bezlotoxumab successfully brought about a significant benefit compared to placebo in treating RCDI (Wilcox et al., 2017). Patients were administered bezlotoxumab 10 mg/kg of body weight, bezlotoxumab plus actoxumab 10 mg/kg of body weight each, or placebo. Actoxumab alone was administered in MODIFY I.

In both studies, the RCDI rate was considerably higher with placebo than with bezlotoxumab alone. MODIFY I: 28% vs. 17% respectively, p < 0.001; MODIFY II: 26% vs 16% respectively, p < 0.001 (Lubbert & Nitschmann, 2017). Actoxumab when given alone was not effective and showed no additional benefit when co-administered with bezlotoxumab (Wilcox et al., 2017).
1.8.6 Bacteriophage therapy

Phages are viruses that specifically infect bacteria. Their use for the treatment of diseases has been widely discussed, and phages have been reported to remove or reduce specific bacteria that accounts for a number of infections in animals and humans (Loc-Carrillo & Abedon 2011; Hargreaves & Clokie 2014; Parison et al., 2014). There have been recommendations that phage therapy be used as an alternative to antibiotics intervention in CDI (Zucca et al., 2013; Hargreaves & Clokie 2014; Sangster et al., 2014). Moreover C. difficile phages with capacity to access the lytic life cycle have been isolated (Goh et al., 2005; Meader et al., 2010), although it is useful to point out that the isolation of phages for C. difficile is extremely challenging. Prior reports have shown C. difficile-specific phages form clinical and environmental samples did not follow a strictly lytic lifestyle, in spite of their lytic activity, as their genomes encode integrases (Goh et al., 2005; Govind et al., 2006; Sekulovic et al., 2014).

Bacteriophage therapy is another promising treatment approach for multidrug resistant bacteria although phage therapy also suffers from resistance development against phages, and possibly phage-mediated transfer of bacterial virulence and antibiotic resistance genes. Nonetheless, it is an attractive option for CDI treatment given its non-disruption of the microbiota and targeted mechanism of action.

Studies in this regard are limited in part due to concerns about employing temperate phages which may possibly incorporate viral nucleic acid into DNA of the host and a limited availability of recognised lytic phages that are specific for C. difficile. Following a study that evaluated the capacity of 7 distinct phages, alone and when combined, to decrease the bacterial load of eighty different C. difficile strains covering 21 different ribotypes, Nale and colleagues (2016) reported that the most effective phage combination was strain-specific. However, multiple four or three phage combinations completely lysed C. difficile cultures in vitro within 2 to 5 hours. Additionally, combination phage therapy-treated hamster showed reduced C. difficile colonisation at 36 hours post-infection, a 33-hour delay in the onset of symptoms compared to untreated animals, and a 4-log decrease in C. difficile spore and bacterial counts in the colon. This suggests a likely therapeutic value for phage combinations in CDI, however, it is unlikely that such phage combinations would be useful in CDI where there are such diverse an array of virulent C. difficile strains causing infections in the clinical setting.
More recently, Nale et al., (2018) demonstrated that an optimised 4-phage cocktail was able to clear clinical ribotype 014 / 020 cultures in fermentation vessels spiked with faecal slurry form healthy volunteers. *C. difficile* counts declined by about 6-log after 5 hours and a total eradication after 24 hours upon the prophylactic regimen. Similarly, the effectiveness of FMT in CDI is thought to be associated with the abundance of bacteriophage in the donor (Park et al., 2019). Nevertheless, developing a phage with no integrase activity to minimise the risk of transmitting mobile genetic elements, as well as safety concerns continue to be huge challenges with phage therapy.

1.9 Non-human *C. difficile* sources and reservoirs.

*C. difficile* can be found in humans, animals, and the environment. The sizeable overlap between ribotypes from all three reservoirs is an indication of extensive transmission.

1.9.1 *C. difficile* in animals

The presence of *C. difficile* in animals (horses and rabbits) and in the environment (sand, river mud, and hay) in Europe has been described in the literature over four decades ago (Hafiz, 1974). Subsequently, other researchers in different European countries have also reported the identification of *C. difficile* and infection in pigs (UK) Lysons et al., (1980), hares (France) (Dabard et al., 1979), goats (UK) (Hunter et al., 1981), Chickens, ducks, and geese (UK) (Borriello et al., 1983). The first mention of *C. difficile* in cattle in Europe in which *C. difficile* toxins were observed in samples from calves was published by Pirs et al., (2008).

The pathogenic potential of *C. difficile* in farm animals has been a subject of research over the past two decades (Pirs et al. 2008; Koene et al., 2012; Spigaglia et al., 2015). Although the interest in *C. difficile* as an infectious agent in livestock and the likely accompanying economic losses are driving forces, the principal aim of many of the studies globally has been focused on demonstrating the presence of an
animal reservoir and to find explanations on the association between CDI in humans and potential
reservoirs.

As interest about a potential zoonotic \textit{C. difficile} transmission increases, factors such as age, species,
seasonality, microbiota, and breeding effect have been correlated with colonisation in farm animals
(Rodriguez \textit{et al.}, 2016). Age is the most studied factor concerning \textit{C. difficile} colonisation in farm
animals. Studies indicate high colonisation rates in newly born animals and are significantly reduced or
eradicated in adult animals (Alvarez-Perez \textit{et al.}, 2009; Schneeberg \textit{et al.}, 2013a). This widely reported
reduction in both colonisation and infection prevalence with age means foodborne transmission risk
from contaminated animals and animal products during harvest is largely reduced. Another
consequence is that CDI in adult animals is sparse, accordingly, \textit{C. difficile} is not currently regarded as
a common health issue in adult farm animals.

\textbf{1.9.2 \textit{C. difficile} in the environment and food}

Food borne zoonotic pathogens are often transmitted by the consumption of contaminated food and
water. Since the initial report of the possible foodborne transmission of \textit{C. difficile} in Europe in 1983
(Borriello, 1983), the significance of the bacteria as a zoonotic disease remained uncertain. \textit{C. difficile}
colonises the GIT of animals, then the bacterial spores are excreted in the faeces. In this manner, animals
can act as a source of environmental contamination. Healthy animals may carry \textit{C. difficile} spores into
the slaughter phase and establish a potential risk of meat contamination in the course of processing.
Indeed, a number of studies have reported retail meat contamination with \textit{C. difficile} (Songer \textit{et al.},
2009; Bouttier \textit{et al.}, 2010; Weese \textit{et al.}, 2010)

Irrigation with contaminated water or manure spread would contaminate vegetables. Although even
without fertilizing, root vegetables can carry \textit{C. difficile} spores present in soil. Furthermore, the
bacterium (RT 009 and 066) has been isolated from zooplankton samples from the Gulf of Naples Italy
(Pasquale \textit{et al.}, 2011). The work of Pasquale \textit{et al.} showed for the first time that \textit{C. difficile} can be
adapted to aquatic marine populations, an indication that C. difficile can be transmitted by ingesting undercooked or raw seafood.

A proportion of C. difficile strains is probably transmitted constantly between humans, environment, and animals as partial overlap of PCR ribotypes isolated in the same country over a period of 3 years from humans, environment, and animals showed that 11 out of 90 PCR ribotypes were shared between all three reservoirs (Janezic et al., 2012). Even strains within a particular ribotype still represent a very heterogeneous group and WGS level is required for identity confirmation. So far, this has been undertaken in only two studies, one on ribotype 078 strains in Netherlands (Knight et al., 2016) and the other on ribotype 014 strains in Australia (Knetsch et al., 2014). Although the proportion of the shared strains in both studies was low, identity between human and pig strains was demonstrated.

Overall aim: To investigate a probiotic intervention than can modulate C. difficile spore germination, growth, and toxin production in an in vitro human gut model.

Hypothesis: The introduction of probiotic strains to an antimicrobial-depleted microbiota in an in vitro human gut model may modulate spore germination, growth, and toxin production by C. difficile due to an increased competition for nutrient and space, in addition to the production of substances by the probiotic strains.
CHAPTER TWO.

2.0 General materials and methods

This study was approved by the University of Hertfordshire ethics committee for studies involving human participants (LMS/SF/UH/00103).

2.1 Isolation and culture of *C. difficile*

Agar and liquid-based cultures were incubated and maintained in an anaerobic cabinet (MG500, Don Whitley Scientific UK) in conditions of 80%, 10%, and 10% nitrogen, carbon dioxide, and hydrogen respectively. Antibiotic preparations were filter-sterilized using a 0.22µm syringe filters (SLGP033RS Millipore, Carrigtwohill Ireland).

2.1.1 *C. difficile* strains

The *C. difficile* strains used in the gut model experiments are PCR ribotypes 027 and 010. RT 027 used in this study was isolated in 2005 from Portland Medical Centre, USA, and provided by Prof. Mark Wilcox, University of Leeds and Leeds Teaching Hospital NHS Trust. This strain was used because *C. difficile* PCR ribotype 027 has been responsible for the epidemic spread of CDI and produces higher levels of toxins (Debast, 2014). Moreover, RT 027 was responsible for the epidemics of CDI in North America and Canada between 2004 and 2005 (Loo *et al*., 2005; Warny *et al*., 2005) and England in 2005 (Smith, 2005). RT 010 strain was also provided by Prof. Mark Wilcox. RT 010 was the first metronidazole reduced susceptibility *C. difficile* strain reported in the UK (Brazier *et al*., 2001). The RT 010 strain has a metronidazole MIC of 16 mg/mL (by E-test), it is *nim* negative and non-toxigenic (Brazier *et al*., 2001).
2.1.2 Culture of *C. difficile* on solid medium

Brazier’s agar (LAB160, Lab M limited, Lancashire, UK) was supplemented with 2 vials/L cycloserine (250 mg/L) cefoxitin (8 mg/L) supplement (X093, Lab M limited, Lancashire, UK), 20mL/L lysed horse blood (SR0050C, Oxoid, Basingstoke UK) was used for the isolation of *C. difficile*. Agar plates were pre-reduced for 24 hours at 37°C in the anaerobic cabinet before inoculation. *C. difficile* colonies were observed as grey opaque, about 4mm in diameter, and with a phenolic or horse manure odour (Delmee, 2001).

2.1.3 Culture of *C. difficile* in liquid medium

Brain Heart infusion (BHI) broth (LAB049, Lab M limited Lancashire UK)) was prepared according to the manufacturer’s instruction. Schaedler’s anaerobic broth (CM496, Oxoid UK) was prepared according to the manufacturer’s instructions. In a similar manner to the solid medium, all liquid medium was pre-reduced for 24 hours at 37°C in the anaerobic cabinet before inoculation.

2.1.4 Preparation of *C. difficile* spores

*C. difficile* was cultured onto supplemented-Brazier’s agar plates and anaerobically incubated for 48 hours at 37°C. Colonies were sub-cultured onto 50 Columbia blood agar (CBA) (LAB001, Lab M limited, Lancashire, UK) (CBA) plates and incubated in anaerobic conditions for one week at 37°C. All growth was harvested from the CBA using a sterile swab (300230 Deltalab, Rubi Spain) and transferred into a 5mL of 50% ethanol (10048291 Fisher scientific UK). The spore suspension was stored at 4°C until needed.
2.2 Triple-stage gut model

The triple-staged continuous culture system was validated by MacFarlane et al., (1998) to assess the effect of retention time on the ecology and metabolism of bacteria in the human colon. The fermentation system was designed to mimic the spatial, temporal, nutritional, physical, and chemical properties of the proximal, transverse, and distal colon. The gut model equipment is shown in Figure 2.1. The gut model was made up of three glass fermentation vessels (Vessel 1 (V1), Vessel 2 (V2), and Vessel 3 (V3)) (Soham Scientific, UK) operating in a weir-cascade system, flowing into a waste unit. The fermentation vessels were made of an outer jacket through which heated deionised water at 37°C flowed from a circulating water bath (TC120 Grant, Royston UK) to maintain the contents of the inner reaction vessel at 37°C. V1 had an operating volume of 280 mL, while V2 and V3 had a working volume of 300 mL. V1 had a higher substrate availability, promoting increased bacterial growth, and was operated at a pH mimicking the environment of the proximal colon. Contrastingly, V3 was similar to the neutral pH, low substrate availability, and slow bacterial growth, which is obtainable in the distal regions of the colon. The pH in Vessels 1, 2, and 3 were detected using pH probes (238764 Hamilton Bonaduz, Switzerland) and maintained at pH 5.5, 6.2, and 6.8 respectively (to mirror the increasing alkalinity of the human colon from proximal to distal) employing pH controller units (Anglicon Solo 2, Brighton systems, Newhaven, UK). Fermentation vessels were magnetically stirred (SB-161-3, Stuart, Staffordshire UK) and sparged with oxygen-free nitrogen (Parker, Domnick Hunter, Gateshead UK) via a 0.3 µm air inlet through a filter (6723-5000 Whatman, Buckinghamshire UK) to maintain anaerobiosis. The tubing for attachments to the gut model was Marprene™ autoclavable tubing (PHY-695-061E 1.6mm wall, 6.4mm inner diameter, Watson-Marlow limited, Cornwall UK), whereas growth medium tubing was Tygon™ autoclavable tubing (1.6mm wall, 3.2mm inner diameter Fisher Scientific, Leicestershire UK). The constituents of the growth medium are listed in Table 2.1. Growth medium was supplied to the gut model at 13.2mL/h (Retention time 66.7h, D=0.015 h⁻¹) using a peristaltic pump (120S Watson-Marlow, Cornwall UK). Addition of 10% (v/v) polyethylene glycol (P3015, Sigma Aldrich Dorset UK) was added to vessels to prevent foaming ad libitum. Controller units automatically dispensed 1M NaoH (S/4880/60, Fisher UK) or 1M HCL (H/1200/PB17, Fisher UK) to maintain pH (±0.1 pH units) within the gut model vessels.
2.2.1 Preparation of gut model growth medium

Gut model growth medium was prepared in 2L volumes in conical flasks (Pyrex, England). Sterilization was carried out by autoclaving at 121°C for 15 minutes. Thereafter, resazurin (R7017-5G, Sigma USA) and glucose (G-5250 Sigma Aldrich, UK) was filter-sterilised into the medium to obtain final concentrations of 0.005g/L and 0.4g/L respectively.
Table 2.1 Gut model growth medium constituents (Macfarlane et al., 1998). Chemicals were obtained from Sigma-Aldrich, UK unless otherwise stated.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Catalogue number</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone water</td>
<td>CM0009</td>
<td>2.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>LP0021</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>S/3105/63/63</td>
<td>0.1</td>
</tr>
<tr>
<td>di-Potassium hydrogen phosphate (K$_2$HPO$_4$)</td>
<td>P/5240/53</td>
<td>0.04</td>
</tr>
<tr>
<td>Potassium di-hydrogen phosphate (KH$_2$PO$_4$)</td>
<td>P/4800/53</td>
<td>0.04</td>
</tr>
<tr>
<td>Magnesium sulphate (MgSO$_4$)</td>
<td>M-7506</td>
<td>0.01</td>
</tr>
<tr>
<td>Calcium chloride (CaCl$_2$)</td>
<td>C-7902</td>
<td>0.01</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate (NaHCO$_3$)</td>
<td>S/4240/60</td>
<td>2.0</td>
</tr>
<tr>
<td>Tween 80</td>
<td>P1754</td>
<td>2.0mL/L</td>
</tr>
<tr>
<td>Haemin</td>
<td>51280-5G</td>
<td>0.005</td>
</tr>
<tr>
<td>Cysteine HCL</td>
<td>111785000 Acros organics Loughborough, UK</td>
<td>0.5</td>
</tr>
<tr>
<td>Bile salts</td>
<td>B8756</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>G-5250</td>
<td>0.4</td>
</tr>
<tr>
<td>Arabinogalactan</td>
<td>10830</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin K$_1$</td>
<td>V3501</td>
<td>10µl/L</td>
</tr>
<tr>
<td>Pectin</td>
<td>416860025 Acros organics Loughborough, UK</td>
<td>2.0</td>
</tr>
</tbody>
</table>
2.2.2 Collection of human faecal samples

Sample donors were sourced by Dr. Simon Baines. Human faecal samples used for the inoculation of the continuous culture (gut model) were collected from five healthy elderly (over 65 years) donors that have not undergone antibiotic treatment 3 months before the collection date. Samples were conveyed in sealed bags containing an anaerobic gas generation system (TSMX5335D Oxoid, Basingstoke UK) and kept in the anaerobic cabinet within 6 hours of collection. The anaerobic gas generation system contains, activated carbon and ascorbic acid and upon contact with air, oxygen is absorbed, and a rapid production of carbon dioxide ensues, creating an ideal transport condition for anaerobes.

2.2.2.1 Screening for *C. difficile* in faecal samples

Faecal samples were streaked on to pre-reduced supplemented Brazier’s agar and incubated at 37°C for 48 hours in the anaerobic cabinet. *C. difficile*-containing faecal samples were not used for the gut model studies. *C. difficile* was identified based on growth on selective agar, colony morphology, smell, antimicrobial susceptibility, and UV fluorescence with reference to the Wadsworth anaerobic bacteriology manual (Jousimies-Somer et al., 2002).

2.2.2.2 Faecal sample preparation for inoculation of the continuous culture

*C. difficile*-negative samples (N=5) were pooled to make a faecal slurry used to inoculate the gut model in each experiment. This entailed suspending 50g of pooled samples in 500mL pre-reduced sterile phosphate buffered saline (PBS) (BR53 Oxoid UK) to yield a ca. 10% (w/v) faecal slurry. The PBS-faeces mixture was initially mixed in a stomacher (400 circulator, Seward, West Sussex UK) in 200mL sterile pre-reduced PBS to achieve a smooth suspension, then coarse-filtered using sterile muslin to get
rid of larger particles, following which further PBS was added to yield the required 500mL of faecal emulsion.

2.2.3 Preparation of *L. casei* Shirota probiotic from Yakult to inoculate the model

Yakult yoghurt drinks were purchased from Asda stores Hatfield. The batch number of 65mL bottles was K22B. In two 50mL sterile conical polypropylene centrifuge tubes (ThermoFisher Scientific, UK), 32.5mL of live bacteria-containing Yakult yoghurt was dispensed into and centrifuged (Hettich EBA 21, Germany) at 6000 rpm for 35 minutes. The clear supernatant was decanted into waste, then 4mL of sterile PBS was added to the pellet to re-suspend bacterial cells. These procedures were performed aseptically. The PBS-bacteria suspension was dosed into the gut model, which comprised the viable bacteria contained within one Yakult bottle per dose.

2.2.3.1 Enumeration of *L. casei* Shirota.

Five hundred microliters of Yakult was added to 4.5ml of peptone water to make a 1:10 dilution. This was serially diluted up to $10^{-8}$. Twenty microliters of each diluent was plated and spread on pre-reduced LAMVAB agar (Hartemink *et al.*, 1997) plates and incubated in an anaerobic chamber for 48 hours. Experiments were performed in triplicate. The above procedure was repeated for the PBS-bacteria suspension used to inoculate the models to ensure it contained similar amounts of LcS to the original yoghurt. Again, these procedures were performed aseptically. Total viable counts were determined by counting between 30-300 colony forming units and accounting for the dilution factor; and concentrations of bacteria were expressed as cfu/mL.
2.2.4 Solid medium to distinguish \textit{L. casei} Shirota from other \textit{Lactobacillus} spp.

In a one litre bottle, 52 grams of MRS broth (CM0361, Oxoid, UK) and 0.5 grams cysteine hydrochloride was added to 500 mL water (bottle A). In a different 500 mL bottle was added 20 g of agar technical number 2 (LP0011 Oxoid UK) to 500mL water (bottle B). Both bottles were autoclaved separately. Bottle B was added to bottle A, and mixed gently. Using filter sterilisation, 0.03g/L of 2, 3, 5-triphenyl-2H-tetrazolium chloride (A10870 Alfa-Aesar, Heysham, England) was added. The pH was adjusted to 5.0 and 20mg/L vancomycin was added (Sakai \textit{et al}., 2010). The modified rhamnose-2, 3, 5-triphenyl-2H-tetrazolium chloride-LBS-vancomycin (M-RLTV) agar was previously reported to distinguish between lactobacilli strains (Sakai \textit{et al}., 2010). Whereas \textit{L. casei} formed red colonies on the M-RLTV agar, other lactobacilli formed pink colonies or white colonies with a red spot.

2.2.5 Inoculation of the gut model

The smooth 10\% faecal slurry was used to inoculate Vessel 2 and Vessel 3 to approximately 130mL. The remaining slurry was added to V1, then the peristaltic pump, magnetic stirrer units, nitrogen generator, pH control units, and water baths were turned on. Vessels V2 and V3 were allowed to be fed from V1. The gut model was allowed to equilibrate in terms of bacterial populations for 14 days (period A) with no intervention. Bacterial populations were enumerated every other day during period A.

2.2.6 Enumeration and identification of faecal bacteria

Selective and non-selective agar-based culture medium was used for the isolation of gut-bacterial population from the gut model. A selective medium contains particular ingredients that inhibit the growth of certain microbes but support the growth of the organism of interest. However, a differential medium supports the growth of a wider range of microbes but distinguishes them based on how they metabolize or change the medium. Blood agar is an example of a differential medium as it distinguishes microbes based on their ability to lyse red blood cells. The modified \textit{Lactobacillus} anaerobic de Man, Rogosa and Sharpe with vancomycin and bromocresolgreen (LAMVAB) agar is an example of a
selective medium. The growth medium, supplements and instructions for their preparation are highlighted in Table 2.2. From each vessel 0.5mL of sample was taken and was serially diluted 10-fold to $10^8$ in 4.5mL pre-reduced peptone water (CM0009, Oxoid UK) in an anaerobic cabinet (Freeman et al., 2003). Twenty microliters of four suitable dilutions were used to inoculate quarter plates of the different culture medium in triplicate. Agar plates were incubated in an anaerobic cabinet for 48 hours. However, Inoculation and incubation of agar for facultative anaerobes were carried out aerobically. Following incubation, colonies were counted and identified on the basis of colony morphology, growth on selective growth medium, Gram reaction, microscopic appearance, biochemical reaction, and antimicrobial susceptibility. Viable counts (cfu/mL) was calculated as:

**Mean cfu per 20µL x 50 x 10^x**

**(X = dilution factor)**

**Viable count expressed as log_{10} (cfu/mL)**

Furthermore, all enumerated bacterial colonies were also identified with MALDI-TOF as previously described by Seng et al., (2009) and Carbonnelle et al., 2011). Briefly, a single bacterial colony was spotted onto the MALDI-TOF sample target plate with 1 µL of matrix solution containing 1.5 mg of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid and allowed to air dry at room temperature. Then, the plate was inserted into the mass spectrometer. The dried matrix-sample mixture was bombarded with a laser to create gas phase ions that are then pulsed into a flight tube. The species of interest were identified by their mass/charge ratio, the mass/charge value is obtained from the centroid of the peak. Peptidic spectra were compared with the Saramis database, version 4.12, and the identification score was recorded. Measurements were performed with an Axima Confidence mass spectrometer (Shimadzu, Japan) equipped with a 337-nm nitrogen laser and a 50Hz variable repetition rate N2 laser. Data were acquired automatically employing Flex control 3.0 (Bruker Daltonics GmbH, Bremen, Germany).
Table 2.2. Constituents of different aerobic and anaerobic agar used for viable counting and their target species.

<table>
<thead>
<tr>
<th>Growth Medium</th>
<th>Preparation</th>
<th>Target bacterial group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazier’s CCEY</td>
<td>Prepared according to manufacturer’s instruction + 2 vials of cycloserine-cefoxitin supplement + 20mL/L lysed blood after autoclaving. For the 027 plates, 2mg/L of moxifloxacin was added.</td>
<td>C. difficile.</td>
</tr>
<tr>
<td>Nutrient Agar</td>
<td>Prepared according to manufacturer’s instruction</td>
<td>Total facultative anaerobes.</td>
</tr>
<tr>
<td>Bacteroides Bile Aesculin Agar (BBE)</td>
<td>Prepared according to manufacturer’s instruction + 5mg/L haemin + 20 µL/L Vitamin K₁ prior to autoclaving, and 75 mg/L kanamycin + 7.5 mg/L vancomycin + 1 mg/L penicillin G. after autoclaving.</td>
<td>Bacteroides fragilis group.</td>
</tr>
<tr>
<td>MacConkey Agar</td>
<td>Prepared according to manufacturer’s instruction</td>
<td>Lactose fermenting Enterobacteriaceae</td>
</tr>
<tr>
<td>Fastidious Anaerobe Agar (FAA)</td>
<td>Prepared according to manufacturer’s instruction +</td>
<td>Total anaerobes (facultative + obligate).</td>
</tr>
<tr>
<td>Medium</td>
<td>Formula and Preparation</td>
<td>Bacteria</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>--------------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Beeren’s Agar</td>
<td>42.5 g/L Columbia agar base + 5 g/L agar technical + 5 g/L glucose + 0.5 g/L cysteine hydrochloride. Agar is steamed for 1 hour until completely molten. 5 ml/L propionic acid is added and pH is adjusted to 5.0</td>
<td>Bifidobacterium spp.</td>
</tr>
<tr>
<td>Modified <em>Lactobacillus</em> anaerobic de Man, Rogosa and Sharpe with vancomycin and bromocresolgreen (LAMVAB) agar.</td>
<td>In a 1L bottle, 52 MRS broth + 0.5g cysteine hydrochloride was added to 500 mL water (bottle A) in a different 500 mL bottle was added 20g agar technical to 500mL water (bottle B). Both bottles were autoclaved separately. Bottle B was added to bottle A, and mixed gently. The pH was adjusted 5.0. 20mg/L vancomycin was added.</td>
<td><em>Lactobacillus</em> spp.</td>
</tr>
<tr>
<td>Kanamycin Aesculin Azide Agar (KAA)</td>
<td>Prepared according to manufacturer’s instruction + 5g/L agar technical. After</td>
<td><em>Enterococcus</em> spp.</td>
</tr>
</tbody>
</table>
2.3 Enumeration of *C. difficile* spores

From each vessel 0.5mL of sample was taken and added to 0.5mL 99% ethanol (10048291 Fisher scientific UK) and allowed to stand for one hour at room temperature. Alcohol-shocked samples were serially diluted in pre-reduced peptone water up to $10^7$. Twenty microliters of each dilution was used to plate onto Braziers agar supplemented with cycloserine cefoxitin supplement, and 20mL/L lysed horse blood. Plates were incubated in an anaerobic cabinet for 48 hours. Upon incubation, single colonies were counted. The above procedure was used to count *C. difficile* on Braziers agar versus moxifloxacin supplemented Braziers agar to confirm counts were not reduced on moxifloxacin supplemented agar. Moxifloxacin incorporation was used when enumerating *C. difficile* ribotype 027 due to its high-level resistance to the antimicrobial agent; it was not incorporated into NTCD Brazier’s agar plates.

2.4 Passaging of Vero cells

African green monkey kidney cells (Vero cells, ECACC 84113001) were grown in 75cm² cell culture flasks containing supplemented 20mL of Dulbecco’s modified eagles medium (DMEM) (D6429, Sigma UK). Five hundred millilitres of DMEM was supplemented with 50mL newborn calf serum (N4637 Sigma UK), 5mL antimycotic solution (100U/mL penicillin, 100mg/L streptomycin, 0.25mg/L amphotericin, Sigma), and 5mL L-glutamine (G7513, Sigma). Cultures were incubated at 37°C in 5% CO₂ (MCO-18AIC (uv) Japan) until Vero cell monolayers became confluent following examination under an inverted microscope (CKX41 Olympus, Japan).
2.4.1 Cell culture cytotoxicity assay

Supplemented DMEM in the flask containing a confluent Vero cell monolayer was discarded. Cells were treated with 0.5mL of Hanks balanced salt solution (HBSS) (H9394, Sigma) that contained 0.25g/L trypsin-EDTA (T4174, Sigma). Upon a gentle swirling, excess HBSS-trypsin-EDTA solution was decanted out, and a further 3mL of HBSS-trypsin-EDTA solution was added. Vero cell monolayers were incubated in 5% CO₂ for 10 minutes at 37°C until monolayers were mobile. Trypsinised Vero cells were diluted 1 in 10 using fresh supplemented DMEM and 180µL dispensed into the wells of a flat-bottomed 96-well microtitre tray (3585 Corning NY USA) but for the wells due to receive 20µL C. sordellii antitoxin (Pro-lab diagnostics, UK) to which 160µL of trypsinised were added. Trays were incubated for 48 hours in 5% CO₂.

Five hundred microliters of each gut model/culture sample was centrifuged at 13300g for 15 minutes (4°C) and the supernatants removed. The supernatants from the gut models were serially diluted in 10-fold with 0.01M sterile phosphate buffered saline (pH 7.4) to 10⁻⁶. Twenty microliters of the appropriate dilution was added to each test wells containing Vero cell monolayer, in addition to a control well containing a further 20µL of C. sordellii antitoxin and undiluted culture (PL6508, Pro-lab diagnostics, UK) (diluted 1 in 10 in PBS).

Following incubation in 5% CO₂ for 24 and 48 hours, monolayers were examined under an inverted microscope. Cell rounding was an indication of a positive result with a side by side neutralisation effect by C. sordellii antitoxin. Cytotoxin titres were expressed as relative units (RU), and reported in the highest dilution with more than 70% cell rounding, that is 10⁰ = 1 RU, 10⁻¹ = 2RU, 10⁻² = 3RU, 10⁻³ = 4RU etc.
2.5 Determination of minimum inhibitory concentration

2.5.1 Antibiotic agar incorporation plates preparation

The susceptibility of *C. difficile* RT 027, RT 010, and *L. casei* Shirota to clindamycin was determined using agar incorporation as described by Freeman and Wilcox, (2001) and Chilton *et al.*, (2014). The same method was used to determine the minimum inhibitory concentrations (MICs) of cefotaxime, ciprofloxacin, ampicillin and tetracycline against RT 027 and RT 010. Briefly, antibiotic solutions were prepared in sterile distilled water. Antimicrobials dilutions were made to arrive at a final concentration range of 0.03 – 128 mg/L when 4mL was added to 36mL of molten Wilkins Chalgren anaerobe agar (CM0619, Oxoid UK). Each antibiotic-incorporated molten agar was mixed gently but thoroughly and poured into two sterile Petri dishes. Upon solidification, antibiotic agars were placed for 30 minutes in a 37°C incubator to remove excess moisture.

2.5.2 Preparation of bacterial inoculum

Bacterial isolates were grown anaerobically in pre-reduced Schaedler’s anaerobe broth (CM0479, Oxoid, UK) overnight (ca. 10⁷ cfu/mL). Approximately 400µL of each inoculum was added to the wells of the multipoint inoculator block (Mast group, Bootle, UK). The inoculator dispensed about 1µL per spot (10⁴ cfu) unto the pre-reduced antibiotic-incorporated agar plates. The multipoint inoculator pins were flamed in alcohol between each set of antibiotics. Agar plates with no antibiotic were inoculated at the beginning and end of the procedure and incubated aerobically and anaerobically at 37°C for 48 hours. MICs were read as the lowest dilution with no growth or a significant alteration in growth had occurred. Reference strains (ATCC 700057) were also included for MIC determinations.

Although the enumeration of faecal bacteria was undertaken in triplicates, each gut model experiment was carried out once, alongside the controls. This was due to the labour-intensive nature of the experiments, cost of resources, and the relatively long turnaround of the experiments.
CHAPTER THREE

3.0 Assessment of the effects of *L. casei* Shirota (LcS) (probiotic) intervention on *C. difficile* growth, spore germination, and toxin production in a human gut model of clindamycin-induced CDI.

3.1 Background

In the last decade, *C. difficile* has been identified as the main cause of nosocomial diarrhoea in Europe (ECDC, 2013) and most frequent healthcare linked pathogen in the USA (Magill *et al*., 2014). Besides strains that have been locally identified as the major cause of serious disease, the emergence of hypervirulent strains, PCR-ribotype 027 and 078 have been linked with the observed increase in morbidity and mortality of CDI (Cartman *et al*., 2010). Data from a recent European study (Davies *et al*., 2014) indicated that 23% of CDI go undetected and undiagnosed in Europe every year due to substandard laboratory methods and/or an absence of clinical suspicion. This underscores the need for a prophylactic measure against CDI.

Lactic acid bacteria (LAB) are often found in milk products and synthesise lactic acid as a principal end product of metabolism following carbohydrate fermentation. Antimicrobial substances are also produced by a considerable number of LAB strains which further antagonises pathogenic and spoilage microbes (Sonomoto & Yakota, 2011). Indeed by using co-culture experiments, Gibson and Wang (1994) demonstrated that bifidobacteria excrete an anti-microbial substance that was not an acid, with a wide range of activity against bacterial species belonging to the genera *Shigella, Listeria, Salmonella,* and *Campylobacter.* This demonstrates that bifidobacteria are capable of inhibiting other colonic bacteria by mechanisms other than the fermentative production of acids such as lactate and acetate. Similarly, there are suggestions that the high bifidobacterial amounts in breast-fed infants may be a contributing factor to the heightened competitive exclusion of pathogens observed in this group compared with formula-fed infants (Gibson *et al*., 1997)

Furthermore, studies have reported the role of prebiotics in stimulating endogenous lactic acid bacteria. Gibson *et al*., (1995) reported that the consumption of 15 g of oligofructose for 15 days led to an increase
in bifidobacterial counts in the stool samples of volunteers, whereas populations of clostridia and fusobacterial declined. This reflects the observations of Wang and Gibson (1993) who observed that the health-beneficial bifidobacterial populations were preferentially stimulated by inulin and oligofructose in vitro, while maintaining populations of potentially pathogenic *Escherichia coli* and *Clostridium* at low levels. They demonstrated through batch culture experiments that the growth of *Bifidobacterium infantis* inhibited *Clostridium perfringens* and *E. coli*.

The human microbiota presents a defensive barrier against pathogens in a phenomenon referred to as colonisation resistance. Disruption of the microbiota and the resultant decrease in the degree of colonisation resistance due to antibiotic use is thought to be the main cause of *C. difficile* infection (CDI). This disruption of the indigenous colonic microbiota often leads to the germination of exogenous or endogenous *C. difficile* spores in the host gut, and consequently the colonization of the colon. Thereafter, vegetative *C. difficile* cells that are able to escape the host innate immune response, multiply, and produce toxins A and B; the main virulence factors. Freeman *et al.*, (2005) reported that clindamycin was able to induce CDI in both *in vivo* and *in vitro* models. These findings are consistent with the reports of Crowther *et al.*, (2014) that CDI was induced by administering 33.9 mg/L (equivalent to human bile concentrations) clindamycin, four times a day, for 7 days in a triple-stage *in vitro* human gut model. Other studies have also reported the capacity of clindamycin to induce CDI in a human gut model (Baines *et al.*, 2008; 2011).

Clindamycin is mainly prescribed for infections due to anaerobic bacteria. It is effective against anaerobic Gram-negative rod-shaped bacteria such as *Fusobacterium*, some *Bacteroides*, and *Prevotella*, as well as Gram-positive aerobic cocci such as some members of *Streptococcus* and *Staphylococcus* genera. It is not active against Enterococci and resistance in *B. fragilis* is increasing (Brook *et al.*, 2005), mainly due to MLS-B mechanism mediated by *ermB*, *ermF*, and *ermG* genes) (Smith *et al.*, 1992). Clindamycin primarily exerts a bacteriostatic effect, but may be bactericidal at higher concentrations (Spižek & Rezanka, 2004). The drug acts through inhibiting protein synthesis by blocking ribosomal translocation by binding reversibly to the 50S rRNA of the large ribosome subunit of bacteria (Wilson, 2014).
Public Health England documented that the incidence of CDI in the UK in 2013 stood at 26.7 cases per 100,000, and the recurrence rate can be up to 35% of cases (Barbut et al., 2000). The cost of treating one individual with a recurrent episode of CDI was estimated to be approximately £11,000, owing to requirement for extended hospital stay (Ghantoji et al., 2010). Lee et al., (2010) reported that rehospitalisation for diarrhoea within 12 weeks was lower in patients treated with either metronidazole or vancomycin together with a probiotic, L. casei Shirota. They also observed a significant decrease in the incidence of recurrent C. difficile infection (p= 0.007).

Lactic acid bacteria have been utilised in the fermentation of food for over 4000 years (Rotar et al., 2007). In the course of the last fifteen years, human probiotics use has received increasing attention. This is due to an increased level of awareness with regards to gut health and the idea of preventive therapy. Lactic acid bacteria, particularly Lactobacillus spp., are the most frequently employed microorganisms as probiotics because they are ‘Generally Recognised As Safe’ (Shokryazdan et al., 2014). Species of LAB that are generally thought to possess probiotic properties include Lactobacillus acidophilus, L. fermentum, L. rhamnosus, L. gasseri, L. (para)casei, L. plantarum, L. helveticus, L. reuteri, and L. johnsonii (Castro et al., 2015; Linares et al., 2016b).

A recent in vitro study demonstrated the probiotic capabilities of nine selected Lactobacillus strains isolated from infant faeces, fermented dates, grapes, and human milk. All the nine isolates showed antimicrobial activity against 12 human pathogenic bacteria (Shokryazdan et al., 2014). In the same study, neutralised supernatants (pH 6.5) of all Lactobacillus strains, did not inhibit the activity against the indicator strain, suggesting the inhibitory effects of the Lactobacillus strains were because of their organic acid production and not bacteriocin or hydrogen peroxide production. The health gains of probiotic bacteria may be directly via interactions with the host or indirectly through metabolites synthesized in the course of fermentation (Gobbetti et al., 2010). Similarly, following a placebo-controlled, double blind, randomised trial undertaken in Germany, seventy chronically constipated individuals were given about 6.5 x 10⁹ LcS or placebo for 28 days. A marked improvement in some constipation symptoms were recorded from the second week for individuals in the probiotic group (P < 0.001) (Koebnick et al., 2003).
Recent literature suggests probiotics are able to re-balance intestinal microbiota, improve intestinal function, and also prevent/treat gastrointestinal disorders such as irritable bowel syndrome (Barrett et al., 2008), inflammatory bowel disease (Mitsuyama 2008), and antibiotic-associated diarrhoea (AAD) (Pirker et al., 2012). Other reported health benefits of probiotics include immunomodulatory effect against a breast tumor in a mouse model, and serum cholesterol reduction (Jones et al., 2012; Linares et al., 2014). One probable mechanism of lowering cholesterol by bile salt hydrolase (BSH)-active probiotics reported by Jones et al. (2012) is that increased intra-luminal BSH activity might result in increased excretion of deconjugated bile acids and a subsequent excretion of serum cholesterol by the liver, replacing bile acids lost from the enterohepatic recirculation. Jones et al., further reported that the metabolism of cholesterol to bile acids in the liver and their subsequent secretion and stool excretion is the principal route for excess cholesterol elimination.

There is an agreement between studies that in order to be effective, probiotic strains must show an ability to survive the gastric acidic conditions of the stomach during transit, in addition to coping with exposure to bile in the upper small intestine to bring about their beneficial effects in the colon. This is in spite of the fact that there exists compelling data on beneficial probiotic effects from dead cells (Mottet & Michetti, 2005; Ghadimi et al., 2008; Lopez et al., 2008). It stands to reason that the maximum probiotic effects of probiotics might be achieved if the probiotic strains colonise (at least temporarily) the surface mucus layer of the intestine given they can displace enteric pathogenic microbes, positively stimulate the host immune system, and possibly exert other effects via cell signalling (Ljungh & Wadstrom, 2009).

Probiotic interventions are thought to reconstitute a depleted gut microbiota and support competitive antagonism of pathogenic bacteria such as C. difficile. Probiotics prevent C. difficile proliferation through a wide range of mechanisms (Sekirov et al., 2010). Different probiotics have been shown to decrease the incidence of AAD (Lactobacillus rhamnosus GG, Saccharomyces boulardii), (McFarland et al., 2006). There are reports of the probiotic yeast strain S. boulardii preventing CDI recurrence, the potency of this probiotic was shown in two randomized controlled trials to be a successful treatment,
although it requires an extended treatment period of 4 weeks following the initial episode of CDI (McFarland, 1994). Nonetheless, a conclusive evidence of probiotics treatment of CDI remains elusive.

3.2 Aims

These series of experiments sought to ascertain the impact of LcS within the gut model before, during, and after clindamycin dosing. This is in addition to observing the population of *C. difficile* in the indigenous microbiota without antimicrobial disruption, and also to ascertain the propensity of clindamycin to induce simulated CDI in a human gut model. Furthermore, the ability of *L. casei* Shirota to prevent simulated CDI in a human gut model shall also be evaluated.

Clindamycin was used in the present study to bring about germination, growth, and cytotoxin production of *C. difficile* because of the high level of reproducibility recorded in previous gut model experiments (Freeman *et al.*, 2007; Baines *et al.*, 2008; Chilton *et al.*, 2015).
3.3 Materials and Methods

Materials and methods were earlier described in 2.0. Selective and non-selective agars used for enumeration of bacterial populations of the gut models included: Nutrient agar, MacConkey agar, KAA, FAA, BBE, Beerens agar, LAMVAB, and Brazier’s agar. (See Table 2.2).

3.3.1 Probiotic strain

*L. casei* Shirota, belonging to Yakult was isolated in Japan by Dr. Minoru Shirota in 1930. Yakult yoghurt drinks were purchased from Asda stores Hatfield. The batch number of 65mL bottles was K22B. Viability of LcS strains was confirmed prior to inoculation into the gut model. Details of preparation of *L. casei* Shirota probiotic from Yakult to inoculate the model, as well as viable counting of LcS prior to gut model inoculation are described in section 2.2.3

3.2.2 Solid media to distinguish *L. casei* Shirota from other *Lactobacillus* spp.

The components and mode of preparation of selective agar were earlier described in 2.2.4

3.4 Antimicrobial activity

The minimum inhibitory concentration (MIC) of clindamycin against *Lactobacillus* spp. *Bifidobacterium* spp., Enterococci, and Enterobacteriaceae were determined using agar incorporation. These bacterial groups were chosen so that the sharp increase or decrease of these bacterial groups during clindamycin dosing can be explained.

Antimicrobial solutions were prepared in sterile distilled water. Antimicrobials dilutions were made to arrive at a final concentration range of 0.03 – 128 mg/L when 4mL was added to 36mL of molten Wilkins Chalgren anaerobe agar (CM0619, Oxoid UK). Each antibiotic-incorporated molten agar was mixed gently but thoroughly and poured into two sterile Petri dishes. Upon solidification, antibiotic
agars were placed for 30 minutes in a 37°C incubator to remove excess moisture. Bacterial isolates were grown anaerobically in pre-reduced Schaedler’s anaerobe broth (CM0479, Oxoid, UK) overnight (ca. 10^7 cfu/mL). Approximately 400µL of each inoculum was added to the wells of the multipoint inoculator block (Mast group, Bootle, UK). The inoculator dispensed about 1µL per spot (10^4 cfu) unto the pre-reduced antibiotic-incorporated agar plates. The multipoint inoculator pins were flamed in alcohol between each set of bacterial group. Agar plates with no antibiotic were inoculated in the beginning and end of the procedure and incubated aerobically and anaerobically at 37°C for 48 hours. MICs were read as the lowest dilution with no growth or a significant alteration in growth had occurred. Reference strains (ATCC 700057) were also included for MIC determinations.

3.4.1 Antimicrobial preparation for gut model

Clindamycin hydrochloride (C5269, Sigma-Aldrich, UK) solution was prepared in sterile distilled water to obtain a final concentration of 33.9 mg/L upon instillation into the gut model (280 mL). This is the Clindamycin concentration attained in human bile upon one 600mg intravenous dosage (Brown et al., 1976) The antimicrobial solution was filter-sterilized using a 0.22µm syringe filters (SLGP033RS Millipore, Carrigtwohill Ireland) and aliquoted into 1 mL volumes into sterile micro-centrifuge tubes (ThermoFisher scientific, UK). One millilitre volume of antimicrobial was dosed into the gut model. Antimicrobial-containing tubes were stored at -20 °C until needed.

3.5 Experimental design

Time periods for these experiments are highlighted in tables 3.1 and 3.2. Upon inoculation of the gut model with a 10% faecal slurry, the model was allowed to equilibrate in terms of bacterial populations for 14 days (period A). Bacterial counts were monitored during this period. At steady state (when bacterial counts are constant for a number of days), the model was dosed with 6.2 x 10^9 CFUs LcS (Pirker et al., 2013) (the content of one 65mL bottle) daily for 7 days (period B). This was followed by an administration of C. difficile ribotype 027 (strain 210) spores (1 x 10^8 spores) (Baines et al., 2005;
2008), after which another single dose of *C. difficile* spores and 33.9mg/L clindamycin was administered 4 times daily for a further 7 days to achieve the faecal/biliary levels observed upon a single 600 mg dose (Brown *et al.*, 1976) (period C). LcS-dosing continued daily during clindamycin administration. The model was continuously dosed with LcS for a further 14 days (period D), upon which the model was allowed without any further intervention for another 14 days (period E). Bacterial populations and *C. difficile* cytotoxin titres were monitored throughout the period of the experiment (period A to E). Sampling was carried out at about the same time on each day.

Experimental time periods for the *L. casei* Shirota and clindamycin (DA) are shown in Table 3.1 whereas the experimental design of the *C. difficile, L. casei* shirota and clindamycin experimental are described in Table 3.2

<table>
<thead>
<tr>
<th>Day</th>
<th>0-14</th>
<th>15-22</th>
<th>23-29</th>
<th>30-45</th>
<th>45-54</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time period</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
</tr>
<tr>
<td>Steady state</td>
<td>6.2 x 10^9 CFUs LcS</td>
<td>33.9 mg/L clindamycin every 6 hours + LcS</td>
<td>LcS</td>
<td>Rest</td>
<td></td>
</tr>
<tr>
<td>Sampling frequency</td>
<td>2-daily</td>
<td>Daily</td>
<td>Daily</td>
<td>Daily</td>
<td>Daily</td>
</tr>
</tbody>
</table>
Table 3.2. Time periods in gut model experiments with *C. difficile* ribotype 027, *L. casei* Shirota and clindamycin to ascertain the ability of *L. casei* Shirota to prevent simulated CDI.

<table>
<thead>
<tr>
<th>Day</th>
<th>0-14</th>
<th>15-21</th>
<th>22-28</th>
<th>29-35</th>
<th>36-49</th>
<th>50-62</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time period</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>F</td>
</tr>
<tr>
<td>027 Control</td>
<td>Steady state</td>
<td>No intervention</td>
<td><em>C. difficile</em> spore instillation (1 x 10^8 spores)</td>
<td><em>C. difficile spore</em> + 33.9 mg/L clindamycin every 6 hours</td>
<td>No intervention</td>
<td>Rest</td>
</tr>
<tr>
<td>LcS-dosed</td>
<td>Steady state</td>
<td>6.2 x 10^9 CFUs LcS</td>
<td><em>C. difficile</em> spore instillation + LcS</td>
<td><em>C. difficile spore</em> + 33.9 mg/L clindamycin every 6 hours + LcS</td>
<td>LcS</td>
<td>Recovery</td>
</tr>
<tr>
<td>Sampling frequency</td>
<td>2-daily</td>
<td>2-daily</td>
<td>Daily</td>
<td>Daily</td>
<td>Daily</td>
<td>Daily</td>
</tr>
</tbody>
</table>
3.6 Results

3.6.1 Gut bacterial populations for the LcS and clindamycin control experiment.

Changes in bacterial populations were very similar in all three vessels of the gut model, hence, only those observed in vessel 3 are shown in Figures 3.1 and 3.2, except for the Bacteroides spp. where there are marked differences between the vessels. Populations of all bacterial groups remained steady but for minor fluctuations in all of the three vessels during period A. Viable counts of lactose fermenters, Enterococcus spp., lactobacilli and bifidobacteria are shown in Figure 3.1. Similarly, viable counts of facultative anaerobes, total anaerobes, and Bacteroides (in vessels 1, 2, and 3) are shown in Figure 3.2. Bacteroides counts were unstable in vessel 1 almost throughout the experiment. Bacteroides numbers in vessels 2 and 3 were however stable but for a minor decline towards the end of period C. Obligate anaerobes outnumbered facultative anaerobes during steady state (period A).

![Graph showing bacterial populations](image_url)

Figure 3.1. Mean (+/−SE) viable counts (log_{10}cfu/mL) of total culturable lactose fermenters, Enterococcus spp., Lactobacillus spp., and Bifidobacterium spp., in the LcS control gut model. Vertical lines indicate the final day of each experimental period.
MICs were carried out so that the high numbers of Enterococci and lactose fermenters, as well as the low numbers of bifidobacteria and lactobacilli during clindamycin dosing can be explained. The MICs of clindamycin against bacterial groups isolated from the gut model are shown in table 3.3

<table>
<thead>
<tr>
<th>Bacterial genera</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LcS (isolated from Yakult)</td>
<td>1</td>
</tr>
<tr>
<td><em>Lactobacillus</em> spp. (isolated from the gut model)</td>
<td>2</td>
</tr>
<tr>
<td><em>Bifidobacterium</em> spp.</td>
<td>0.06</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp.</td>
<td>64</td>
</tr>
<tr>
<td>Lactose fermenters</td>
<td>128</td>
</tr>
</tbody>
</table>

3.6.2 Effect of LcS dosing on the gut microbiota

Following LcS dosing in both control and experimental models, there was a decline in the lactose-fermenting Enterobacteriaceae and Enterococci populations by approx. $2 \log_{10} \text{cfu/mL}$ in all three vessels. This was accompanied by a $2 \log_{10} \text{cfu/mL}$ increase in bifidobacterial viable counts. *Lactobacillus* spp. viable counts also increased by $3 \log_{10} \text{cfu/mL}$, however, lactobacilli counts declined upon the cessation of LcS dosing, this was also the case for bifidobacterial populations. In contrast, following the cessation of LcS dosing lactose-fermenting Enterobacteriaceae and Enterococci populations increased during period E (Figure 3.1). *Bacteroides fragilis* group populations showed fluctuations upon LcS dosage. LcS and endogenous lactobacilli from the steady state period failed to show a distinguishable difference in terms of colony morphology on the modified-2, 3, 5-triphenyl-2H-
tetrazolium chloride-LBS-vancomycin (M-RTLV) agar which was previously reported to distinguish between lactobacilli strains (Sakai et al., 2010)

3.6.3 Effect of clindamycin instillation on the gut microbiota

Clindamycin instillation elicited marked declines in bifidobacterial populations (approx. 6 log\(_{10}\) cfu/mL), *Lactobacillus* spp. viable counts (approx. 4 log\(_{10}\) cfu/mL), and a less prominent decline in *Bacteroides fragilis* group in both experimental and control models. The observed decline in bifidobacterial populations due to clindamycin was more pronounced in vessel 1 (data not shown). However, enterococcal and total anaerobe viable counts increased by approx. 1 log\(_{10}\) cfu/mL and 2 log\(_{10}\) cfu/mL respectively. Additionally, lactose-fermenting Enterobacteriaceae viable counts increased by approx. 3 log\(_{10}\) cfu/mL. Upon cessation of clindamycin administration, lactobacilli and bifidobacteria groups recovered to at least steady state levels. However increased Enterobacteriaceae and enterococcal viable counts declined following cessation of clindamycin dosing (Figure 3.1)

![Figure 3.2](image.png)

Figure 3.2. Mean (+/-SE) viable counts (log\(_{10}\)cfu/mL) of total culturable facultative anaerobes, total anaerobes, and *Bacteroides fragilis* group in the **LcS control** gut model. Vertical lines indicate the final
day of each experimental period. SS is steady state, DA is clindamycin, LcS is *L. casei* Shirota, V1, V2 and V3 are Vessel 1, vessel 2, and vessel 3 respectively.

3.6.4 Gut bacterial population changes observed in the CD027 control and the CD027 vs LcS experiment.

The total bacterial viable counts remained stable but for minor fluctuations during period A (Figures 3.3 and 3.4) in all vessels of the gut model. Lactobacilli viable counts determined in the two experiments, revealed a difference of $1.3 \times 10^7$ cfu/mL between the LcS-dosed model and the 027 control model upon LcS dosage. The LcS-dosed model showed higher counts (Figure 3.3a). Lactobacilli viable counts declined in both models by $1.5 \log_{10}$ cfu/mL following clindamycin instillation (period D). Total anaerobes and *Bacteroides fragilis* group viable counts declined by $2.5 \log_{10}$ cfu/mL and $2 \log_{10}$ cfu/mL upon addition of clindamycin in the 027 control and LcS-dosed model respectively. Bifidobacterial populations increased by $1.6 \log_{10}$ cfu/mL in both models during period C but showed a pronounced decrease of $3.8 \log_{10}$ cfu/mL and $4.7$ in the 027 control and LcS-dosed model respectively upon clindamycin instillation (Figures 3.3 and 3.5). In contrast, although periods C and D were marked with fluctuations of Enterobacteriaceae counts, the counts increased upon instillation of clindamycin. However, LcS dosing led to a general decline in the Enterobacteriaceae viable counts throughout the experiment. Enterobacteriaceae populations in the LcS-dosed model were fewer than the 027 control model in all experimental periods (Figures 3.3 and 3.5). Furthermore, the addition of *C. difficile* spores coincided with a remarkable increase in enterococcal counts. Similarly, clindamycin instillation resulted in a $3 \log_{10}$ cfu/mL increase in total viable counts of *Enterococcus* spp. in both models. All bacterial groups recovered following the cessation of clindamycin instillation. The biofilm formation in both models are shown in Figure 3.7.
Figure 3.3a. Mean (+/-SE) viable counts (log_{10} cfu/mL) of total cultivable, lactobacilli in vessels 3 of LcS vs CD 027 model and 027 control model. Vertical lines indicate the final day of each experimental period. SS is steady state, DA is clindamycin, LcS is *L. casei* Shirota, and CD is *C. difficile* RT027. Lactobacilli 027 control is without LcS; it is CD 027 control in the LcS vs CD experiment.

Figure 3.3b Mean (+/-SE) viable counts (log_{10} cfu/mL) of total culturable bifidobacteria, lactobacilli, Enterococci, and lactose fermenting Enterobacteriaceae in vessel 3 of the LcS vs CD027 gut model. Vertical lines indicate the final day of each experimental period. SS is steady state, DA is clindamycin, LcS is *L. casei* Shirota, and CD is *C. difficile*.
Figure 3.4 Mean (+/-SE) viable counts (log$_{10}$ cfu/mL) of total culturable *Bacteroides fragilis* group, facultative anaerobes, and total anaerobes in vessel 3 of the LcS vs CD027 gut model. Vertical lines indicate the final day of each experimental period. SS is steady state, DA is clindamycin, LcS is *L. casei* Shirota, and CD is *C. difficile*.

Figure 3.5 Mean (+/-SE) viable counts (log$_{10}$ cfu/mL) of total culturable bifidobacteria, Lactobacilli, *Enterococcus*, and Enterobacteriaceae in vessel 3 the 027 control gut model. Vertical lines indicate the final day of each experimental period. SS is steady state, NI is no intervention, DA is clindamycin, and CD is *C. difficile*. 
Figure 3.6 Mean (+/-SE) viable counts (log10cfu/mL) of total culturable *Bacteroides*, Facultative anaerobes, and Total anaerobes in vessel 3 of the 027 control gut model. Vertical lines indicate the final day of each experimental period. SS is steady state, NI is no intervention, DA is clindamycin, and CD is *C. difficile*.

Figure 3.7. Biofilm formation in (a) 027 control (b) LcS-dosed gut model vessels. Arrow pointing at biofilm.
3.6.5 *C. difficile* growth, spore counts, and cytotoxin production

*C. difficile* was not recovered from the LcS-dosed model and the 027 control model before the inoculation of the models with *C. difficile* spores and no cytotoxin production was detected using the Vero cell cytotoxicity assay. Upon instillation with *C. difficile* spores (approx. $10^8$ spores) into vessel 1 of both models, *C. difficile* was detected in vessels 1 and 2 ($4\log_{10}$ units) of both models, total viable counts were equivalent to spore counts; thus suggesting spore forms predominantly dominated the populations. *C. difficile* was detected in vessels 3 of both models on the next day in similar numbers and mainly as spores. During experimental period C, *C. difficile* numbers in all 3 vessels of both models declined steadily (Figures 3.8 and 3.9). Upon further inoculation of vessels 1 of both models with *C. difficile* spores (approx. $10^8$ spores), a similar pattern of declining populations was observed in period D (CD+DA) (Figures 3.8 and 3.9)

A cytotoxin titre of 1 relative unit (RU) was detected in vessel 3 of the 027 control model five days after cessation of clindamycin instillation. Toxin titres increased to 2RU on the 7th day after clindamycin instillation ceased, before climaxing at 4RUs on the eleventh day after cessation of clindamycin instillation. However, it took one more day following cessation of clindamycin instillation before cytotoxin titres were detected in vessel 2 of the same 027 control model. Although toxin production profiles between vessels two and three were similar, 3RUs was the maximum titre units recorded from vessel 2. Similarly, cytotoxin production was detected in vessels 2 and 3 of the LcS-dosed model on the fifth day following the cessation of clindamycin instillation. It took a further two days for toxin titres to reach 4 RUs in vessel 3 and four days to attain 3RUs in vessel 2. Cytotoxin was detected in both models until the end of the experiment.
Figure 3.8. *C. difficile* total viable counts, spore counts ($\log_{10}\text{cfu/mL}$) and cytotoxin titres in vessels 3 of LcS-dosed gut model. Vertical lines indicate the final day of each experimental period. Unit on vertical axis is $\log_{10}\text{cfu/mL} \pm SE$ and relative $\log_{10}$ units, RU.
Figure 3.9. *C. difficile* total viable counts, spore counts (log_{10}cfu/mL) and cytotoxin titres in vessels 3 of 027 control gut model. Vertical lines indicate the final day of each experimental period. Unit on vertical axis is log_{10}cfu/mL +/-SE and relative log_{10} units, RU
3.7 Discussion

Vancomycin, metronidazole, and fidaxomicin are the preferred treatments for CDI (Debast et al., 2014) and have been previously studied in the triple stage in vitro human gut model (Baines et al., 2008; 2009; Freeman et al., 2005; 2007; Crowther et al., 2013; 2014; 2016; Chilton et al., 2014; 2015; Moura et al., 2020). In order not to perpetrate a cycle of further dysbiosis, and given antibiotics are chiefly responsible for CDI, there is an increasing interest to look away from antibiotics for treatment of CDI. Probiotics are thought to address one of the primary instigating factors in the development of CDI, gut dysbiosis, consequently there is a substantial research in this area of study, especially to evaluate if the risk of CDI can be reduced by consuming a probiotic before, during, or after antibiotics intake. A high number of studies have evaluated the potency of L. casei Shirota (in a probiotic milk drink) in this regard. Prior studies have mostly relied on human volunteers (Wong et al., 2013; Lee et al., 2013; Pirker et al., 2013) and this brings to fore a string of ethical issues and inaccessibility of the colon. Besides there may be concerns of inadequate compliance by some volunteers in studies that may require participants to take a specific dosage of a novel drug. The use of experimental models have some advantages over studies involving human participants. In animal models, such advantages include the ability to carry out invasive tests, the availability of study animals, and the possibility to test new treatment strategies. However, the use of animals in research still result in a number of ethical concerns. This is in addition to the fact that animals are not truly reflective of the human colon, as well as difficulty in handling animals. In contrast, the triple stage in vitro human gut model presents a human gut-reflective, accessible, and easily controllable system, although its incapacity to mirror immunological events of the human colon is limitation of the current system design (Macfarlane et al., 1998).

The present study is unique in that it is the first time that the behaviour of a commercial probiotic is studied in the presence of an antimicrobial with a high propensity to cause antibiotic associated diarrhoea in a model that closely mimics the human colon. The findings of this study contribute to the previous knowledge acquired during probiotic studies. Spanhaak et al., (1998) reported the effect of LeS on the intestinal microbiota and immune parameters in humans following batch culture experiments. However, following an experiment to ascertain factors that govern protease production in
the colon by *Clostridium perfringens* in both batch and continuous cultures, Allison & Macfarlane
(1989) suggested that batch culture or single stage continuous culture were not able to effectively mirror
the gut conditions.

Subsequently, Macfarlane *et al.*, (1998) developed and validated the triple-stage *in vitro* human gut
model, engineered to permit the study of intestinal bacterial populations in the carbohydrate-rich, more
acidic environment typical of the proximal colon, and the more neutral, carbohydrate-low conditions
characteristic of the distal colon. Physicochemical measurements were validated against gut contents of
sudden death victims (Macfarlane *et al.*, 1998). Baines and other researchers used the triple stage *in vitro*
human gut model in a study to compare oritavancin and vancomycin as treatments for
clindamycin-induced CDI (Baines *et al.*, 2008). Interestingly, observations from previous gut model
experiments mainly mirrored clinical observations; *C. difficile* exposure to antibiotics with CDI-
inducing tendency *in vivo* (e.g. ceftriaxone, clindamycin, and cefotaxime) caused germination, growth,
and toxin production in the gut model (Freeman *et al.*, 2003; 2005). However, the gut model is unable
to mirror immunological responses and its design makes it very challenging to obtain bacterial
populations in biofilms for investigation but allows the retrieval of bacterial populations in planktonic
state with more ease. This is in addition its limitation in terms of not being able to mirror bacteria-host
interactions.

The populations of major bacterial groups within the vessels in the LcS control experiment are
consistent with the literature in that obligate anaerobes such as *Bacteroides* spp. and *Bifidobacterium*
spp. were predominant over facultative anaerobes such as *E. coli* (Hopkins *et al.*, 2001), in both models
at steady state. Upon the addition of LcS to the model in the LcS control experiment, populations of
Enterococci and lactose-fermenting Enterobacteriaceae in the affected model declined. Similar
observations have been reported previously by Shokryazdan *et al.*, (2014) in which *L. casei* strains
showed antagonistic activity (inhibition zones of more than 20 mm) against lactose-fermenting
Enterobacteriaceae and *Staphylococcus epidermidis*. LcS is thought to exert its antimicrobial effect by
the production of antimicrobial substances such as ethanol, hydrogen peroxide, carbon dioxide, acetoin,
diacetyl, reuterin, bacteriocins, and organic acids (Suskovic *et al.*, 2010). This is in addition to the
mechanism of competition, where probiotic strains outcompete pathogens for attachment surfaces and available nutrients, effectively preventing pathogens from colonising the intestine (Saulnier et al., 2009). In the present study, the antimicrobial activity of LcS strains against Enterococci and Enterobacteriaceae may be due to their organic acid production profile. This reflects the observations of Jin, (1996), who reported that the antagonistic effects of 12 Lactobacillus strains on E. coli and Salmonella were because of their organic acids production. Although the study did not identify the specific organic acid, the inhibitory zones remained unchanged when the culture supernatants were treated with catalase and pronase, an indication that the inhibition was not due to the action of hydrogen peroxide and bacteriocin respectively. However, when Lactobacillus culture supernatants were adjusted to pH 6.5, no inhibitory zones were observed, suggesting the antimicrobial action of the Lactobacillus isolate was via the production of organic acids (Jin, 1996). This is also consistent with data that the antimicrobial effects of L. helveticus, L. acidophilus, L. gallinarum, and L. crispatus against Campylobacter jejuni strains were because of their lactic acid production, but not bacteriocin production (Neal-McKinney et al., 2012). Furthermore, Zalan et al., (2010) documented that the antifungal effect of lactobacilli against Fusarium spp. increased with increasing concentrations of organic acids (acetic and lactic acid) produced by the probiotic strains. They however noted that organic acid production differed between species and strains. Nonetheless, the production of lactic, succinic, and acetic acid in L. casei Shirota has been demonstrated (Zalan et al., 2010; Shokryazdan et al., 2014). In the present study, no considerable difference between the decline in the potentially pathogenic enterococcal viable counts between period E (two weeks of LcS-dosage) and period B (one week of LcS-dosage) was observed (Figures 3.3 and 3.5), suggesting patients may not benefit from an elongated consumption of LcS. Moreover, the decline in enterococcal populations in the non-LcS-dosed 027-control experiment were similar to the LcS-dosed model, indicating the microbiota might be recovering from the effects of clindamycin independent of LcS. Prior C. difficile studies on the human faecal microbiota using the triple-stage in vitro human gut model that employed clindamycin as the CDI-inducing antimicrobial have reported similar observations upon the cessation of clindamycin instillation (Freeman et al., 2005; Baines et al., 2008). However, following the cessation of LcS dosing, enterococcal viable counts increased (Figure 3.3), suggesting populations of Enterococcus spp. might have been kept in check by
LcS-administration. Furthermore, human volunteer studies of the effect of L. casei Shirota consumption on the gut microflora have reported a general increase in populations of Lactobacillus spp. and Bifidobacterium spp., as well as a decrease in Enterobacteriaceae populations in the faecal samples of volunteers (Sur et al., 2010; Nagata et al., 2011). Sur et al., (2010) reported a reduced occurrence of acute diarrhoea in children following a daily consumption of L. casei Shirota drink for 12 weeks.

The observations in this study mirror with the reports of Macfarlane et al., in that the triple stage in vitro human gut model was capable of maintaining a vast array of bacteria genera over a remarkable time duration (Macfarlane et al., 1998). In the LcS control experiment, populations of obligate anaerobes were slightly more than the facultative anaerobes (Figures 3.1 and 3.2). This is in slight contrast to common reports that obligate anaerobes are highly dominant to facultative anaerobes in numerical terms in the human gut (Pirker et al., 2013; Backhed et al., 2005). This discrepancy may just be due to the differences in individual gut bacterial populations. Bacteroides spp. counts in the present study were lower in vessel 1 in relation to vessels 2 and 3 (Figure 3.2). This was observed in a previous study in which viable counts of B. fragilis group in vessel 1 were slightly lower than vessel 2 and 3 of the gut model (Macfarlane et al., 1998). This may in part be due to the more acidic and unfavorable pH of vessel 1. An analysis of amplified 16S rRNA sequences showed a pH-dependence in populations of human fecal bacterial community in a continuous flow fermenter. Bacteroides spp. constituted 27% of 16S rRNA sequences detected at pH 5.5, in contrast, at pH 6.7 they accounted for 86% of sequences (Duncan et al., 2009). Similar observations have been made in prior gut model studies (Freeman et al., 2007).

Clindamycin administration in vessel 1 was at 33.9mg/L, which is the concentration attained in human bile upon one 600mg intravenous dosage (Brown et al., 1976). Changes in the gut model bacterial populations due to clindamycin instillation mainly reflected data from previous studies (Baines et al., 2008; Jernberg et al., 2010). Marked declines were observed in populations of lactobacilli (MIC 2mg/L) and even more remarkable declines in bifidobacterial populations in all three vessels during the antimicrobial instillation. These declines were expected owing to the known activity of clindamycin against these genera (Hopkins & Macfarlane, 2002; Hoque et al., 2010). However, Enterobacteriaceae,
Enterococci, and to a lesser extent *Bacteroides* spp. were either not severely affected, or even increased in viable counts, following clindamycin administration in the present study. The observations on *Bacteroides* spp. were consistent with the findings of a previous researcher that a clear correlation does not exist between reduced viable counts of *B. fragilis* and clindamycin concentrations above the MIC (Baines et al., 2006). Clindamycin was used in the present study to bring about germination, growth, and cytotoxin production of *C. difficile* because of the high level of reproducibility recorded in previous gut model experiments. Prior studies reported similar changes in the indigenous microbiota upon clindamycin instillation as seen in the present studies, such as decreased obligate anaerobes, stable or increased facultative anaerobes. *C. difficile* spores remained quiet before, during, and after clindamycin instillation (Baines et al., 2008). Although the antimicrobial concentrations in the gut model vessels were not determined in the present study, germination, growth and toxin production of *C. difficile* only occurred when concentrations of clindamycin had likely declined below the MIC (2mg/L) for *C. difficile* PCR ribotype 027. This is in tandem with the observations of Baines et al., (2008).

While bacterial spores are metabolically dormant, they retain the capacity to sense environmental conditions and germinate when conditions become favourable (Setlow, 2003). The onset of *C. difficile* germination and cytotoxin detection in the present study occurred on the same day, 5 days after the cessation of clindamycin administration in both the LcS-dosed model and the 027 control, an indication that LcS administration does not delay or prevent the germination of *C. difficile* ribotype 027 spores and its subsequent toxin production. Indeed, toxin production climaxed at 4RU (maximum titre in this study) 3 days earlier in the LcS-dosed model than the 027 control (3 versus 6 days after initial toxin detection). Baines et al., (2008) suggested that PCR ribotype 027 releases toxins (A and B) earlier than other *C. difficile* strains in the growth cycle. Warny et al., (2005) reported that *C. difficile* PCR ribotype 027 produced more substantial amounts of toxin A and B than comparator clinical strains. Baines et al., (2008) argued that Warny and his colleagues had failed to recognise the “statistically significant greater cell density” in the *C. difficile* PCR ribotype 027 cultures in comparison to *C. difficile* toxinotype 0, and that might have accounted for the increase observed by Warny and his colleagues. Owing to the fact that a very high adherence to the intestine is required to bring about some probiotic effects, the
failure of LcS to prevent germination, proliferation, and toxin production might be due to the absence of colon epithelial cells (that may be required for attachment) in the gut model, although reports of beneficial probiotic effects from dead cells (Ghadimi et al., 2008; Lopez et al., 2008) contradicts this reasoning. The present study favours the model where live probiotic strains are required to exert probiotic effects.

In a study using Caco-2 cell line, a human intestinal cell line frequently employed to evaluate bacterial attachment due to its close similarities with human enterocytes, the different adherence capabilities of nine Lactobacillus strains were assessed. Shokryazdan et al., (2014) showed that the adherence capacity differed among the strains, suggesting adherence is strain-specific. Similar observations have been documented by Jacobsen et al., (1999). Furthermore LcS might not have exerted its probiotic effects observed by Pirker et al., (2013); Hickson et al., (2007); and Lee et al., (2013) in the present study due to the possibility of a dilution or washout of a nutritional molecule (required for organic acid production) from the gut model by the gut model growth medium. Alternatively, it may initially seem like the gut model growth media does not have a sufficient energy source for the production of organic acids such as acetate and butyrate by lactobacilli that may potentially antagonise C. difficile (Rolfe, 1984; Wheeldon et al., 2008). Indeed several studies have reported that the growth of lactobacilli, and their production of antimicrobial substances rely on the constituents of the growth media in which they are cultivated (Tomás et al., 2002; Avonts et al., 2004; Zalán et al., 2005). The primary reason for such differences is the presence or absence of nutritional substances in the different growth media. Although the impact of growth conditions such as temperature, time, and pH cannot be neglected (Gourama & Bullerman, 1997). Using the gut model growth media, Macfarlane and co-workers reported substantially higher amounts of organic acids such as butyrate and acetate in vessel 1 of the gut model and suggested it was due to the fermentation of larger amounts of carbohydrates present in vessel 1 compared to vessels 2 and 3 (Macfarlane et al., 1998). Although organic acid concentrations in the gut models were not determined in the present study, it may be possible that the high quantities of organic acids produced in vessel 1 of the gut model were diluted into vessels 2 and 3 such that the levels of organic acids in vessels 2 and 3 (where C. difficile growth occurred) were below a concentration that
may exert an antimicrobial effect on *C. difficile*. A concentration-dependent inhibitory effect of acetic and lactic acids produced by lactobacilli have been demonstrated against *Fusarium* (Zalan *et al*., 2010) and *Bacillus subtilis* (Corsetti *et al*., 1996).

Egervarn *et al*., (2006) suggested that an increase in the inoculum size of bacteria gave rise to increased MICs (reduced antimicrobial activity). Consequently, the high initial numbers of *Bacteroides* spp. (7-log₁₀-units) in Vessel 2 and Vessel 3 of the LcS control experiment (Figure 3.2) might be a plausible explanation for the comparatively reduced activity of clindamycin against this bacterial group in the gut models. Clindamycin resistance in *Bacteroides* spp. is increasingly being reported (Brook *et al*., 2005; Roh *et al*., 2009; Eitel *et al*., 2013), although no evidence of this was observed in the *Bacteroides* spp. isolates from the gut model in the present study. Interestingly, both lactobacilli and bifidobacterial populations had higher initial numbers of over 10¹⁰ cfu/mL (Figure 3.1) and were still more susceptible to clindamycin than *Bacteroides* spp. in the present study. The difference in clindamycin susceptibility of bacterial groups in this study is more likely to be species or strain-specific and not due to the initial inoculum size as suggested by Egervarn *et al*., (2006).

Instillation of clindamycin in a previous similar study led to a drop in bifidobacterial populations (approx. 6 log₁₀ cfu/mL), below the limits of detection (2 log₁₀ cfu/mL) (Baines *et al*., 2008). This was not the case in the LcS control experiment in the present study. Although populations of bifidobacteria dropped by 6 log₁₀ cfu/mL, viable counts were still above the limit of detection. Antimicrobial susceptibility testing in the present studies using agar incorporation showed clindamycin to have an MIC of 0.06 mg/L to bifidobacteria (Table 3.3). A variance in the bifidobacterial strains present in different faecal samples used for the two studies might in part provide some explanation for this discrepancy, as well as the initial number of bifidobacterial populations in the gut model which was higher in the present study. Nonetheless, lactose fermenters and to a lesser degree enterococcal populations were not severely affected following clindamycin instillation. These observations reflect the findings of previous studies (Baines *et al*., 2008; Chilton *et al*., 2014). These findings were again not surprising as clindamycin showed a minimum inhibitory concentration of 128mg/L and 64mg/L (up to 2 folds higher than the clindamycin dosage of 33.9mg/L) to Enterobacteriaceae and *Enterococcus*
respectively, isolated from the gut model in the present studies (Table 3.3). Indeed, Enterobacteriaceae and enterococcal populations increased when antimicrobial concentrations may have dropped below their MICs (Figures 3.3 and 3.5). Clindamycin concentrations determined by prior gut model studies indicate that the peak antimicrobial concentration achieved was 62.3 mg/L in vessel 3 (Chilton et al., 2014; Baines et al., 2015), which is still below the MICs for Enterobacteriaceae and Enterococcus isolates from the gut model in the present study. This is coupled with an absence of optimum competition from depleted bacterial groups. In addition, the resistance of these genera to clindamycin is well documented (Singh et al., 2002; Engberg et al., 2007).

The steep decline in B. fragilis group populations observed in period D due to clindamycin instillation in the 027 control model was less severe in the LcS-dosed model Figures (3.4 and 3.6), suggesting LcS might be exerting its effects by playing a protective role in minimising the reduction in viable counts of this group, which are thought to have a possible role in colonisation resistance to CDI in the gut (Freeman et al., 2003). Although clindamycin resistant B. fragilis has been reported by different researchers (Brook 2007; Mandell et al., 2004), the isolates from the gut model after clindamycin dosing did not show clindamycin resistance. The possibility of a protective role for LcS is further given credence as B. fragilis group populations were seen to decline following cessation of LcS administration. Nevertheless, B. fragilis group viable counts in the 027 control recovered during the rest period and did not decline until the end of the experiment without LcS. The relationship between Bacteroides and LcS remains to be elucidated.

Bifidobacterial populations between the LcS-dosed model and the 027 control did not show a marked difference following LcS administration for 1 week. These observations do not reflect the author’s observations in the LcS control experiment, and it is also inconsistent with the findings of Nagata et al., (2011) who reported an increasing abundance of bifidobacteria upon LcS administration. They argued that since the abundance of bifidobacteria represents a crucial short chain fatty acid-producing population, and these SCFAs enhance the colonic blood flow, in addition to electrolyte and fluid uptake, the reduced SCFA production could lead to mechanisms responsible for diarrhoea. Indeed, populations of bifidobacterial in the LcS-dosed model suffered a bigger decline following clindamycin
administration, contradicting the findings of Pirker et al., (2013) that LcS prevents the decline in bifidobacteria populations caused by antibiotic agents. This might in part be due to suggestions that the beneficial characteristics of probiotics are not distributed in all probiotic strains (Ohashi & Ushida, 2009), and such effects might differ from one individual to another (Wong et al., 2014). Nonetheless, the validity of these reports relies on the reproducibility of their observations.

The LcS dose used in the present study was 6.2 x 10^9 CFUs, which is the total viable count determined from a commercially available 65 mL bottle in the present study, as against 6.5 x 10^9 documented by the manufacturers. The discrepancy in LcS total viable counts from the 65 mL bottle may be due to methodological differences. This dose was used given the effective dose of probiotics that bring about beneficial effects on human health is thought to be about 10^6 – 10^9 live microbial cells/day, even though it is strain-dependent (Karimi et al. 2012; Watson & Preedy, 2015). Lactobacilli viable counts increased by 1.3 x 10^7 cfu/mL in the LcS-dosed model compared to the 027 control following LcS dosage. The rather limited increase on the total lactobacilli count might be due to the pre-existing high indigenous lactobacilli numbers (8 log_{10} cfu/mL). This is an indication that the consumption of LcS in high amounts increases the total lactobacilli counts but does not substitute the indigenous lactobacilli in the microbiota. An effort to select for LcS in the present study using selective agar plates was unsuccessful. Spanhaak et al., (1998) demonstrated that the administration of LcS resulted in an observed decrease in β-glucosidase and β-glucuronidase activities. These effects are seen as beneficial as there are reports that link these enzymes to chemical carcinogenesis (Goldin & Gorbach, 1984). Furthermore, there are suggestions that LcS administration could lead to a reduced intestinal transit time (Spanhaak et al., 1998), and a shortened intestinal transit time has been thought to impede constipation and reduce the risk of colon cancer by facilitating the excretion of toxic compounds (Cummings et al., 1992).

In an effort to mirror the likely manner of acquiring C. difficile by patients in a hospital setting, spores of C. difficile was inoculated into vessels 1 of the gut models. This presented an internal test period to evaluate the ability of the unperturbed microbiota to inhibit C. difficile and prevent spore germination, outgrowth, and cytotoxin production. The dynamics of C. difficile in an undisturbed microbiota have been previously studied (Freeman et al., 2007; Baines et al., 2008). C. difficile was unable to colonise
the vessels of the gut model without antimicrobial disturbance and was consequently washed out from
the vessels of the gut model. Freeman et al. (2003) suggested these observations were similar to events
in vivo. Observations from the present study mirror the reports of Freeman et al., (2007) and Baines et
al., (2008), as C. difficile remained mainly as spores and was washed out (Figures 3.8 and 3.9) at the
rate of the flow rate of the vessels. Similar studies in animal models showed that C. difficile- spore-
treated-hamsters without antimicrobial administration showed no symptom of disease (Larson &
Borriello, 1990). Moreover, Borriello & Barclay, (1986) suggested that colonisation resistance to
disease (CDI) necessitated the presence of viable microbes instead of metabolites production. This was
not the case in the present study as C. difficile proliferation continued even after populations of
indigenous members of the microbiota recovered from the effects of clindamycin, suggesting a role for
a key metabolite(s). Reports of animal and in vitro studies aimed at ascertaining the role of organic acid
in CDI have presented varied results (Forssten et al., 2015).

Following cessation of clindamycin administration, decreased populations of lactobacilli and
bifidobacteria recovered to at least steady state levels during period D. This is suggestive of a fall in the
antimicrobial concentrations in the vessels of the gut model beneath the MICs of the affected bacterial
groups (Freeman et al., 2003; 2005; 2007). Surprisingly, lactobacilli populations were observed to
increase within 1 day of clindamycin dosing cessation. Inasmuch as antimicrobial concentrations might
be rapidly declining, it is very unlikely that clindamycin levels will drop to less than 2 mg/L within one
day. Perhaps some biofilm detached and was counted with planktonic phase bacteria. Alternatively it
can be speculated that the lactobacilli recovered from the models one day following cessation of
clindamycin instillation is LcS (from Yakult) introduced daily to vessel 1 of the model and not the
indigenous pre-Yakult lactobacilli. Selective agar prepared in the present study in an effort to
distinguish LcS (from Yakult) and lactobacilli recovered from faecal samples was not successful, in
part because they have very close MIC profiles. The genetic sequences of the species can be used to
differentiate them. However, the practicality and cost of such molecular techniques limited their
application in the present study.
Similarly bifidobacterial populations increased immediately upon cessation of clindamycin instillation, against previous gut model experiments where bifidobacteria was not detected for 7 days following cessation of clindamycin instillation (Baines et al., 2008). It is noteworthy that probiotics (LcS) were not administered in the gut model experiment by Baines and others. Matsumoto et al., (2010) reported that drinking a fermented beverage drink that contained $4 \times 10^9$ LcS CFUs for 4 weeks led to an increase in the intrinsic bifidobacterial populations of healthy volunteers. This is consistent with the observations of Spanhaak et al., (1998). In addition, the swift bifidobacterial recovery observed just 1 day following cessation of clindamycin instillation might in fact be a possible disruption of a biofilm within the vessel, releasing bifidobacterial colonies into the planktonic population. This is reflective of the observations of Crowther et al., (2014). They showed that whilst the planktonic *Bifidobacterium* spp. declined below the limit of detection upon administration of 33.9 mg/L clindamycin, four times daily, for 7 days in a triple stage *in vitro* human gut model, the sessile *Bifidobacterium* spp. populations from biofilms in the same vessel were detected at approximately $3.9 \log_{10}$ cfu/g. The decreased susceptibility of biofilm-associated microorganisms to antimicrobials is well-documented (Stewart & Costerton, 2001).

During the last 30 years, many studies have reported that consumption of the probiotic lactobacilli might reduce the length of diarrhoea due to infection, or antibiotic intervention (Sheen et al., 1995; Kaila et al., 1992). In addition, lactobacilli have been reported to influence certain aspects of the immune system (Pouwels et al., 1996). However, the effect of lactobacilli consumption on the gut of healthy individuals and during antibiotic therapy has not be sufficiently elucidated. In the present study, LcS dosage and subsequent increase in lactobacilli counts led to a decline in Enterococci and Enterobacteriaceae populations as shown in Figure 3.1. This is in tandem with the observations of Shokryazdan et al., (2014) who demonstrated the antagonistic activity of lactobacilli against 12 pathogenic bacteria. The antimicrobial activity of lactobacilli and other probiotic strains has been frequently linked to the production of organic acids (such as butyric, acetic, and propionic acid), bacteriocins, and the probiotic strains’ ability to outcompete pathogens for attachment sites and nutrients, effectively preventing pathogens from colonising the gut (Saulnier et al., 2009). The decline observed in the Enterococci and Enterobacteriaceae populations in the present study might be due to an increased production of organic
acids due to an increased lactobacilli population because a further increase in the lactobacilli populations was accompanied by a commensurate decrease in Enterococci and Enterobacteriaceae populations.

Zalan et al., (2010) showed that the antagonistic effect of organic acids against Fusarium increased with increasing organic acid concentration. Unfortunately, due to a machine breakdown, gut model samples collected to assay for organic acids by high performance liquid chromatography (HPLC) was stored for over three months at 4°C. Following HPLC, no useful data was derived (data not shown); a pointer to the difficulty associated with storing organic acids. Although the present study is unable to provide conclusive evidence on the mechanism of action of LcS against Enterococci and Enterobacteriaceae populations, the probiotic’s antagonism of Enterococci and Enterobacteriaceae is evident. One explanation might be that the organic acids produced by LcS rapidly diffused and resulted in a local decreased pH which is unfavourable to Enterococci and Enterobacteriaceae populations (Ouwehand & Vesterlund, 2004). The capacity of lactobacilli to survive at low pH conditions is well known (García-Ruiz et al., 2014). Inhibition of bacterial growth by lactic acid is thought to be by an enhanced leakage of hydrogen ions across the bacterial cell membrane culminating in an acidification of the cytoplasm and a dissipation of pH gradient (Blom & Mortvedt, 1991). Indeed Neal-McKinney et al., (2003) demonstrated that lactobacilli produce organic acids. The acidity due to LAB is well known to be effective in disrupting colonisation of surfaces with potentially infectious bacteria. The vagina is a good example of this. Vaginal epithelial cells colonisation with Lactobacillus prevents a subsequent colonisation of the cell surface with pathogenic bacteria, hence, decreasing the incidence of chronic vaginal yeast infections (Boris et al., 1989). Taken together, one cannot rule out a role for organic acids in the antagonism of the bacterial groups by LcS in the present study.

Nonetheless, it is noteworthy that lactobacilli populations declined following cessation of LcS-dosing, with an accompanying and proportional loss of the apparent effects of LcS. Such effects included, observed increase in lactobacilli and bifidobacterial populations and marked declines in Enterococci and Enterobacteriaceae populations. This is an indication of poor colonization by LcS within the gut model vessels. Similar observations have been made in prior research. Spanhaak et al., (1998) reported
that *L. casei* Shirota was unable to colonize the gut permanently following a study that involved twenty male volunteers. This mirrors the findings of several other studies that *L. rhamnosus* was unable to colonize the gut (*Saxelin et al.*, 1995; *Goldin et al.*, 1992). The continued interaction of LAB such as LeS with the mucosal epithelial cells of the GIT and lymphoid cells of the gut promote an immune response against pathogenic bacteria (*Bourlioux et al.*, 2003; *Mazahreh & Ershidat*, 2009). Since the triple stage *in vitro* human gut model used in the present study is limited in terms of immunological responses, it is very likely that the probiotic effects observed herein were not due to immunomodulation, but a likely prevention of colonisation by the affected bacterial groups, prevention of bacterial invasion, inhibition of adhesion to epithelial cells, and outcompeting the affected bacterial groups for nutrients (*Matsumoto et al.*, 2010). Lactobacilli have been previously reported to outcompete *Salmonella cholerasuis* for epithelial binding sites in a study that used Human Caco-2 epithelial cells (*Lin et al.*, 2008). It is also very unlikely that immunomodulation may confer a more prolonged probiotic effect given studies involving human subjects (*Saxelin et al.*, 1993; *Alander et al.*, 1999) have also reported the short term colonisation and temporary probiotic benefits of *Lactobacillus* species. More studies with an extended follow up period are needed to shed more light on this area.

Interestingly cytotoxin was detected on day 58 in the 027 control model when total counts of *C. difficile* was below the limit of detection suggesting an accumulation of earlier produced toxins in the vessels. This might be the case *in vivo* as CDI patients may still suffer the consequences of *C. difficile* toxins even when vegetative cells of the bacteria have been killed by an antimicrobial intervention. Alternatively, it might be that such toxins were released from biofilms following biofilm detachment. Toxin in biofilms has been demonstrated in a prior gut model study after it has declined below the detection limit in the main vessel liquid contents (*Crowther et al.*, 2014). Data from the present study provides evidence that toxin production by *C. difficile* PCR ribotype 027 mirrors its growth cycle (Figures 3.8 and 3.9). Toxin production in both LeS-dosed and 027 control models occurred for 20 days until the end of the experiment, and the sigmoidal growth patterns of *C. difficile* seen in Figure 3.8 is suggestive of a biphasic growth cycle of the experimental strain, and consequently bestow on *C. difficile* PCR ribotype 027 an advantage of perpetrating prolonged illness (*Freeman et al.*, 2007). Nevertheless,
the increased toxin production and higher vegetative growth observed in the LcS-dosed model is difficult to explain. Although it appears LcS encouraged the germination of CD spores in the present study, there is insufficient efficient to make this claim. Besides, to the best of the author’s knowledge, no study have reported the role of LcS in \textit{C. difficile} spore germination. It may be that LcS interfered with the delicate balance of the gut microbiota required for promoting colonisation resistance to \textit{C. difficile}. Furthermore, the gut model does not mirror \textit{in vivo} secretory or immunological responses. Indeed, a considerable number of studies have attributed the probiotic ability of LcS to its immunomodulatory effects (Nagao \textit{et al.}, 2000; Reale \textit{et al.}, 2011; Dong \textit{et al.}, 2013) which cannot be replicated in the \textit{in vitro} human gut model. It is therefore plausible to suggest that immunomodulatory events might determine LcS’ overall response to CDI.

In summary, the results of the present study confirm the capacity of the triple stage \textit{in vitro} human gut model to maintain different genera of bacteria over a prolonged period of time. This makes it a valuable resource in investigating the relationship between pathogens, antimicrobial agents, and the colonic microbiota. Clindamycin administration led to a dysbiosis of the indigenous gut microbiota and simulated CDI in the gut models although bacterial populations recovered following cessation of clindamycin dosage. \textit{L. casei} Shirota dosage impacted positively on the gut microbial community confirming earlier studies, however, long term colonization of the gut by LcS was absent. \textit{C. difficile} spores did not germinate, outgrow, or produce cytotoxins in an undisturbed colonic microbiota, on the contrary, upon dysbiosis precipitated by antimicrobial agents, \textit{C. difficile} germinated, grew, and produced its cytotoxins. Daily LcS administration of a high probiotic dose failed to prevent \textit{C. difficile} spore germination, outgrowth, and cytotoxin production although a decline in Enterobacteriaceae and Enterococci populations were observed.

It is important to interpret \textit{in vitro} research data alongside actual observations and treatment of disease in patients. Sadly, research on the roles of individual microbial genera to human physiological wellbeing is constrained to the study of a few number of culturable and previously isolated organisms. Nevertheless, an improved understanding of microbial metabolism derived from culture-independent research and improvement to presently used culture techniques are crucial to future success in this field.
Metagenomics provides a well detailed view of all the genes present within a community, providing detailed insight to function and composition alike in one experiment (Sekirov et al., 2010). These results could be meaningful. Further large sample size and double-blind design studies are required to demonstrate the wider significance of these observations in terms of the capacity of LcS to prevent CDI and to promote health in general.
4.0 Evaluation of the capacity of nontoxigenic *C. difficile* to prevent antimicrobial-induced *C. difficile* infection in a triple-stage *in vitro* gut model.

4.1 Background

Antibiotic therapy is the principal predisposing factor for *C. difficile* infection (CDI). Consequently, the indigenous gut microbiota is disturbed quantitatively and/or qualitatively, to bring about a colonisation of the gut by *C. difficile* (Borriello & Barclay 1986; Borriello 1998). Hence, the disruption of colonisation resistance is often the initial step in the pathogenesis of CDI. Upon ingestion of *C. difficile* spores, germination into vegetative cells is required for colonisation in the gut, with a successive toxin production culminating in clinical manifestations (Vedantam et al., 2012). Gut anaerobic bacteria are understood to play an important role in colonisation resistance although the exact components involved are yet to be well defined. Early studies employed *in situ* hybridization with 16S rRNA probes and bacterial culture to compare the components of faecal microbiota in children, young adults, healthy elderly individuals, and elderly patients diagnosed with CDI (Hopkins et al., 2001; 2002; Hopkins & Macfarlane, 2002). The faecal microbiota of CDI patients showed low bacterial quantity and reduced species diversity. CDI patients had a sharp decline in bifidobacteria, *Prevotella*, and *Bacteroides* and an increase in Enterobacteria, lactobacilli, and clostridia compared to other subject groups. Although the authors acknowledged that some of the changes might have been due to metronidazole administration to CDI patients before stool collection. Similarly, Chang et al., (2008) employed 16S rRNA gene clone libraries to compare the faecal bacterial communities of patients with an initial episode of CDI, patients with recurrent CDI, and healthy controls. In the patient group with an initial CDI episode and in the control subjects, a greater number of sequences matched with organisms in the Bacteroidetes and Firmicutes divisions, which are the two principal bacterial phyla in the gut of healthy individuals. On the contrary, the members of the faecal microbiota in patients with recurrent CDI were variable, and showed a considerable decline in bacterial diversity and quantity. In two of the patients
with recurrent CDI, the microbiota communities are dominated by Verrucomicrobia or Proteobacteria, which are usually minor components in a healthy gut microbiota.

Any factor that impacts on colonisation resistance may potentially affect the risk of CDI associated with individual antibiotics (Freeman & Wilcox, 1999). Strikingly, *C. difficile* is part of the normal gut microbiota in up to 80% of infants but often does not promote CDI in spite of reports that a considerable amount of the *C. difficile* strains isolated from infants are toxigenic (Adlerberth et al., 2014). Treatment with broad-spectrum antimicrobials such as lincosamides, aminopenicillins, fluoroquinolones, and third generation cephalosporins are the chief risk factors for CDI (Oldfield et al., 2014), with *C. difficile* colonisation rates up to 73% in elderly inpatients (Deneve et al., 2009). Antibiotic treatment with vancomycin and metronidazole is the standard therapy for CDI. However, the efficacy of these antimicrobials are limited, with 20-40% of patients suffering a recurrent episode of CDI (Zucca et al., 2013). In fact, the crucial role of an unperturbed microbiota which enables colonisation resistance is mirrored in the high rates of success of faecal microbiota transplantation which are up to about 92% in severe recurrent CDI cases (Gough et al., 2011).

Given the complex network of co-dependence that exists among components of the indigenous gut microbiota, the microorganisms that suffer the deleterious effect of antimicrobials are not necessarily confined to those directly targeted by such antimicrobial (Willing et al., 2011). Furthermore, the spread of antimicrobial resistance genes form commensal bacteria to their pathogenic counterparts in the gut by horizontal gene transfer remains a concern (Jernberg et al., 2010). The degree of antimicrobial-mediated intestinal microbiota disturbance depends on several factors: the antimicrobial used (mechanism of action, range of activity, extent of absorption, pharmacokinetic and pharmacodynamics properties, and *in vivo* drug activation), the dose and duration of therapy, the route of antibiotic administration and elimination, in addition to the extent of resistance of the microbial population (Jernberg et al., 2010; Britton & Young 2014). A number of molecular- and culture-based studies have investigated the long- and short-term impacts of antimicrobials on the intestinal microbiota of healthy volunteers and patients receiving clinical treatment (Sullivan et al., 2001; Rafii et al., 2008; Jernberg et al., 2010).
4.1.1 Clindamycin

Jernberg et al., (2007) employed terminal restriction fragment length polymorphism of the 16S rRNA gene to assess the long-term effects of a one-week clindamycin course on the faecal microbiota structure in four healthy volunteers. A substantial shift in the components of the total bacterial community was seen immediately after clindamycin administration, although the community restored its original state within 12 weeks after treatment ceased. In a sharp contrast, Bacteroides populations never returned to their pre-clindamycin levels and this imbalance remained up to two years after clindamycin treatment ceased. This is important as Bacteroides are thought to play a major role in C. difficile colonisation resistance (Freeman et al., 2003; 2007). Dramatic and prolonged increases in antibiotic resistance genes (erm) levels were also recorded in the faecal DNA samples following clindamycin exposure. The erm determinants were due to Bacteroides species. A number of CDI studies using the gut model have used clindamycin to induce simulated-CDI large due to its reproducibility (Baines et al., 2008; 2009; 2014; Chilton et al. 2014).

4.1.2 Cephalosporins

Given the range of activity of cephalosporins is wider than antimicrobial groups such as penicillins, a bigger disruption of the intestinal microbiota is expected following dosing in patients, especially with antimicrobials such as ceftriaxone that are eliminated through the biliary duct (Sullivan et al., 2001). Following culture-based studies, cephalosporin exposure was reported to reduce the abundance of Enterobacteria and elevate the concentrations of aerobic Gram-positive cocci, e.g. Intrinsically resistant Enterococci. Cephalosporins are also linked with the emergence of resistance in Enterobacteria (Sullivan et al., 2001; Rafii et al., 2008). Perez-Cobas et al., (2013) used a multi-omics approach to examine the intestinal microbiome of a patient receiving cefazolin intervention for 2 weeks. The patient’s faecal samples were collected before and during cefazolin treatment, as well as 40 days after cessation of the treatment. Before the antimicrobial treatment and during the early days of treatment, populations of the Firmicutes were predominant in the faecal microbiota. On the eleventh day, a
substantial reduction in microbial diversity was observed. There was a displacement of firmicutes and a marked increase in Bacteroidetes (Bacteroides and Parabacteroides genera) and betaproteobacteria. Metabolomic and proteomic analyses indicated the intestinal microbiota responded swiftly (on day 6) to antimicrobial administration by activating mechanisms such as multidrug efflux pumps or expression of β-lactamases to detoxify the drug. Forty days following the cessation of antimicrobial treatment, the components of the intestinal microbiota were very similar to the pre-antimicrobial state, indicating a recovery of the initial bacterial populations. Cefotaxime and ceftriaxone have been previously shown to induce simulated-CDI in the triple-stage in vitro human gut model (Freeman et al., 2003; Baines et al., 2013).

4.1.3 Fluoroquinolones

Fluoroquinolones are able to reach very high amounts in faeces given they are rapidly absorbed following oral administration (Sullivan et al., 2001). Enterobacteriaceae are susceptible to fluoroquinolones and consequently, they suffer a deleterious effect during exposure. Additionally some fluoroquinolones have been shown to reduce populations of Gram-positive cocci (Rafii et al., 2008). Dethlefsen and colleagues tracked disturbances to the distal gut bacterial populations of three healthy individuals prior to and after a five-day ciprofloxacin treatment (Dethlefsen et al., 2008; Dethlefsen & Relman 2011). Although the intestinal microbiota of each individual reacted in a unique manner to the antimicrobial, some common trends were observed. The impact of ciprofloxacin on the gut microbiota was swift and profound, bringing about a marked decrease in diversity, depleting up to 50% of the bacterial community, and causing major shifts in microbiota composition (with declines in Faecalibacterium and Ruminococcaceae) within 3 – 4 days of antimicrobial treatment initiation. Even though the microbiota composition was very similar to the pre-treatment state within 1 – 4 weeks following cessation of treatment, some bacterial taxa such as Bilophila and Clostridiales did not recover even after 6 months of ciprofloxacin treatment. The propensity of fluoroquinolones to induce CDI was reinforced following gut model experiments (Saxton et al. 2009).
4.1.4 Aminopenicillins

A number of studies have investigated the effect of amoxicillin, alone or in combination with the β-lactamase inhibitor clavulanate, on the human gut microbiota. Upon culture-based experiments, amoxicillin treatment with or without clavulanate was linked to resistant Enterobacterial species overgrowth (Rafii et al. 2008). Barc et al., (2004) employed a human faecal microbiota-associated mouse model to investigate the impact of a seven-day amoxicillin-clavulanate course on the intestinal microbiota. The dominant bacterial groups were quantified by flow cytometry with 16S rRNA oligonucleotide probes and fluorescence in situ hybridisation. The total anaerobic bacteria was stable all through the experiment, but there was a considerable decline in the amounts of Clostridium coccoides-Eubacterium rectale group coupled with an increase in the levels of Enterobacteriaceae and Bacteroides-Prevotella-Prevotella groups during antimicrobial treatment. Upon antimicrobial discontinuation, all bacterial groups returned to pre-treatment levels within one week. Young and Schmidt (2004) monitored transient changes in faecal microbiota diversity in a patient that developed antibiotic-associated diarrhoea during a 10-day course of amoxicillin-clavulanate. Faecal samples obtained on days 0, 4 and 24 were analysed using 16S rRNA gene clone libraries. Before antimicrobial exposure, the faecal microbiota was chiefly made up of Bifidobacterium, Bacteroides, and members of Clostridium cluster IV and XIVa (butyrate-producing bacteria). After four days of amoxicillin-clavulanate exposure, a considerable increase in Enterobacteriaceae populations were observed, but no sequences matching Clostridium cluster XIVa and Bifidobacterium were detected. The majority of these changes resolved after two weeks of antimicrobial cessation, except Bifidobacterium spp. which did not recover. Co-amoxiclav, a combination of amoxicillin and clavulanate have been previously reported to induce growth and toxin production in C. difficile RT027 in a human gut model (Chilton et al., 2012).

A significant number of studies have investigated potential CDI preventive strategies such as probiotics, vaccines, and monoclonal antibodies (Songer et al., 2007; Mizrahi et al., 2014; Arruda et al., 2016; Kociolek & Gerding, 2016). Among strategies that seek to prevent CDI, the administration of non-toxigenic C. difficile (NTCD) is emerging as a promising approach to prevent intestinal colonisation of toxigenic C. difficile strains (Songer et al., 2007; Gerding et al., 2015). Recently a NTCD, Z31 isolated
from a healthy dog was investigated for its potential use as a probiotic to prevent CDI in swine. Z31 has since been characterised by whole genome sequencing (WGS), which showed the presence of important genes associated with sporulation and colonisation (Pereira et al., 2016). Additionally, the capacity of Z31 to prevent CDI was shown in a hamster model (Oliveira Junior et al., 2016). NTCD strains do not have genes for toxin production and are often found in hospital environments and colonised patients, even though such patients are asymptomatic (Shim et al., 1998). NTCD strains have been shown to safely colonise volunteer patients aged over 60 years when administered at doses of $10^4$ – $10^8$ spores per day for 2 weeks upon vancomycin dosing to deplete the normal microbiota and mimic CDI treatment (Villano et al., 2012).

4.2 Aim

To ascertain the capacity of NTCD (RT010) to prevent antimicrobial-induced simulated-CDI in a triple stage *in vitro* human gut model.

4.2.1 Objectives

- To instil NTCD into a triple-stage human gut model prior to instillation of virulent *C. difficile* (RT027) and subsequent antimicrobial disruption with a range of recognised CDI-inciting agents (fluoroquinolones, aminopenicillins, lincosamides, and cephalosporins) in separate experiments.

- To evaluate the effects of a range of recognised CDI-inciting agents (fluoroquinolones, aminopenicillins, lincosamides, and cephalosporins) on spore germination, outgrowth and proliferation of NTCD (RT010) in a triple stage *in vitro* human gut model in separate experiments.
4.3 Materials and Methods

Materials and Methods were earlier described in 2.0. Selective and non-selective agars used for enumeration of gut model bacterial populations, in addition to methods of preparation and supplements are highlighted in Table 2.2. The minimum inhibitory concentrations (MICs) and the concentrations of antimicrobials used in the selective agars are shown in table 4.2.

4.3.1 *C. difficile* strains

The *C. difficile* strains used in the gut model experiments are described in 2.1.1

The NTCD used in the present studies contains the chromosomal *erm*(B) gene (Dr. César Rodríguez Sánchez - Personal Communication).

4.3.2 Determination of cytotoxicity of *C. difficile* strains

Brain Heart infusion (BHI) broth (LAB049, Lab M limited Lancashire UK) was prepared according to the manufacturer’s instruction and pre-reduced for 24 hours at 37°C in the anaerobic cabinet before inoculation with about 4 – 8 colony forming units of *C. difficile* strains (isolated from the gut models at the start and end of every experiment) grown on Brazier’s agar plate. The *C. difficile*-inoculated BHI broth was incubated for 72 hours at 37°C in the anaerobic cabinet. The bacterial liquid culture was centrifuged at 13300g for 15 minutes (4°C) and the supernatants used to test for cytotoxicity as described in section 2.4.1

4.3.2 Antimicrobial susceptibility testing

Antimicrobial susceptibilities were determined using agar incorporation as described in 2.4
4.3.3 Experimental design.

Experimental time periods are shown in Table 4.1. Following inoculation of the gut model with 10% faecal slurry, the gut model was allowed to equilibrate in terms of bacterial populations for 14 days (period A). Bacterial counts were monitored during this period. At steady state, the model was dosed with ~10⁸ NTCD spores, following which ~10⁸ NTCD spores and ~10⁸ RT027 spores were dosed into the experimental model. Thereafter, NTCD + 027 spores were added again with the appropriate dose of the experimental antimicrobial (clindamycin 33.9 mg/L, ciprofloxacin 139 mg/L, cefotaxime 20mg/L, and ampicillin 8mg/L) to achieve reported faecal/biliary levels (Ayliffe & Davies, 1965; Brown et al., 1976; Novick, 1982; Lambert-Zechovsky et al., 1985; Brismar et al., 1990). Clindamycin was instilled every 6 h for 7 days, ciprofloxacin and cefotaxime were instilled every 12 h for 7 days, ampicillin was instilled every 8 h for 7 days. The models were then allowed to a rest period of 14 days. The duration of each experiment was 9 weeks, with each antibiotic and C. difficile strain growth behaviour in completion and alone (control) evaluated in separate experiments.

Table 4.1. Time periods in gut model experiments with NTCD (RT010) vs RT027 using different antimicrobials

<table>
<thead>
<tr>
<th>Day (Time period)</th>
<th>0-14 (A)</th>
<th>15-21 (B)</th>
<th>22-28 (C)</th>
<th>29-35 (D)</th>
<th>36-62 (E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>027 Control</td>
<td>Steady state</td>
<td>No intervention</td>
<td>027 spores</td>
<td>027 spores + antimicrobial</td>
<td>Rest</td>
</tr>
<tr>
<td>027 vs 010 (experimental)</td>
<td>Steady state</td>
<td>~10⁸ 010 spores</td>
<td>010 + 027 spores</td>
<td>010 + 027 spores + antimicrobial</td>
<td>Rest</td>
</tr>
<tr>
<td>010 Control</td>
<td>Steady state</td>
<td>No intervention</td>
<td>010 spores</td>
<td>010 spores + antimicrobial</td>
<td>Rest</td>
</tr>
<tr>
<td>Sampling frequency</td>
<td>2-daily</td>
<td>Daily</td>
<td>Daily</td>
<td>Daily</td>
<td>Daily</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
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</tr>
</tbody>
</table>


4.4 Results

The NTCD used in the present study was not cytotoxic.

The antimicrobial concentrations used in the selective agar are shown in table 4.2. The difference in MICs between strains were exploited to select for strains in the agar plates. The MICs of all antimicrobials used in the gut model experiments were determined to ascertain if the MIC of the antimicrobials conferred an advantage to one of the strains in the gut model. This is in addition to determining the concentration of antimicrobial that can be used in agar for selection.

Table 4.2. Antimicrobial concentrations used in selective agar.

<table>
<thead>
<tr>
<th>Drug concentration in agar</th>
<th>Tetracycline MIC (mg/L)</th>
<th>Clindamycin MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3. Antimicrobial MICs for *C. difficile* strains used in the gut model experiments

<table>
<thead>
<tr>
<th>CD strain</th>
<th>Ciprofloxacin (mg/L)</th>
<th>Cefotaxime (mg/L)</th>
<th>Ampicillin (mg/L)</th>
<th>Clindamycin (mg/L)</th>
<th>Tetracycline MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTCD</td>
<td>2</td>
<td>128</td>
<td>4</td>
<td>&gt;128</td>
<td>0.06</td>
</tr>
<tr>
<td>RT010</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT027</td>
<td>8</td>
<td>64</td>
<td>4</td>
<td>2</td>
<td>16</td>
</tr>
</tbody>
</table>

Given the high consistency between the effects of the antimicrobials on the microbiota in all the models, only those observed in vessel 3 of the competition model are presented and shown in appropriate figures.
4.4.1 Gut bacterial populations in the clindamycin-dosed models

The bacterial populations in different experimental periods in the clindamycin-dosed model are shown in figures 4.1a and 4.1b. Obligate anaerobes outnumbered facultative anaerobes during steady state. The instillation of NTCD spores showed no impact on the bacterial groups. A re-administration of NTCD spores in addition to instillation of 027 spores coincided with a $2 \log_{10} \text{cfu/mL}$ decline in total anaerobes viable count, but showed no effect on the other bacterial groups. Upon instillation of clindamycin, bifidobacteria, lactobacilli, total anaerobes, and *Bacteroides fragilis* group were the most depleted bacterial groups with a $1.5 - 3.5 \log_{10} \text{cfu/mL}$ decline. In contrast, Lactose-fermenting Enterobacteriaceae, Enterococci, and total anaerobes counts increased by approx. $1 - 2.5 \log_{10} \text{cfu/mL}$. The bacterial populations generally recovered to or exceeded pre-antimicrobial levels within 7 days of administration of the final dose of clindamycin. Populations of Enterococci, facultative anaerobes, and lactose fermenting Enterobacteriaceae declined by approx. $0.5 - 1 \log_{10} \text{cfu/mL}$ during the rest period.

Figure 4.1a. Mean (+/-SE) viable counts ($\log_{10} \text{cfu/mL}$) of total culturable total anaerobes, lactose fermenting Enterobacteriaceae, *Enterococcus* spp., and *Bifidobacterium* spp, in the clindamycin (DA) 027 vs 010 competition gut model. Vertical lines indicate the final day of each experimental period. SS is steady state. 010 is *C. difficile* RT010, 027 is *C. difficile* RT027.
4.4.2 Gut bacterial populations in the cefotaxime-dosed models

The bacterial populations in different experimental periods in the cefotaxime-dosed model are shown in figures 4.2a and 4.2b. Obligate anaerobes outnumbered facultative anaerobes during steady state. The instillation of NTCD spores showed no impact on the bacterial groups. Similarly, a re-administration of NTCD spores in addition to instillation of 027 spores showed no effect on the bacterial groups but for a minor decline in the total anaerobes viable counts. Following cefotaxime instillation, an approximately 0.5 log_{10} cfu/mL decline was observed in the total anaerobe population. Lactose fermenters, bifidobacteria, and Bacteroides spp. populations declined by approximately 1 – 2.5 log_{10} cfu/mL. There was no observable effect on the counts of facultative anaerobes and Enterococci. In contrast, lactobacilli populations rose by 1 log_{10} cfu/mL upon cefotaxime instillation. Upon the cessation
of cefotaxime instillation, the total anaerobe, *Bacteroides* spp., bifidobacteria, and lactose fermenters recovered to at least steady state levels, whereas lactobacilli populations rose beyond the levels observed during cefotaxime instillation. No further change was observed during the rest period, but for a very minor decline in *Enterococcus* spp.

![Graph](image)

**Figure 4.2a.** Mean (+/-SE) viable counts (log\(^{10}\) cfu/mL) of total anaerobes, *Bacteroides fragilis* group, *Bifidobacterium* spp. and *Lactobacillus* spp. in the cefotaxime (CFX) competition gut model. Vertical lines indicate the final day of each experimental period. SS is steady state. 010 is *C. difficile* RT010, 027 is *C. difficile* RT027.
Figure 4.2b. Mean (+/-SE) viable counts (log_{10}cfu/mL) of total culturable Enterococci, lactose fermenting Enterobacteriaceae, and facultative anaerobes in the cefotaxime (CFX) competition gut model. Vertical lines indicate the final day of each experimental period. SS is steady. 010 is *C. difficile* RT010, 027 is *C. difficile* RT027.

4.4.3 Gut bacterial populations in the ciprofloxacin-dosed models.

The bacterial populations in different experimental periods in the ciprofloxacin-dosed model are shown in figures 4.3a and 4.3b. Bacterial populations showed minor fluctuations during steady state. Obligate anaerobes such as bifidobacteria outnumbered the facultative anaerobes during steady state. The instillation of NTCD showed no considerable effect on the bacterial populations. The instillation of NTCD spores together with 027 spores coincided with a slight increase and decrease of *Bacteroides fragilis* group and Enterococci respectively, but showed no impact on other bacterial groups. Upon ciprofloxacin instillation, the counts of *Bacteroides* spp. decreased by approx. 1 log_{10}cfu/mL. Similarly, bifidobacteria, lactobacilli, and Enterococci counts decreased by 1 – 2 log_{10} cfu/mL. Additionally, lactose fermenters showed about 5 log_{10}cfu/mL decline below the limit of detection. However, the total anaerobe and facultative anaerobe populations were largely unaffected. Within 7 – 9 days of ciprofloxacin instillation cessation, the bacterial counts generally recovered to steady state levels,
except the lactose fermenters, which required 16 days to achieve steady state levels. No further change was observed during the rest period.

Figure 4.3a. Mean (+/-SE) viable counts (log$_{10}$ cfu/mL) of total culturable *B. fragilis* group, *Bifidobacterium* spp., *Lactobacillus* spp., and *Enterococcus* spp. in the ciprofloxacin (Cipro) competition gut model. Vertical lines indicate the final day of each experimental period. SS is steady state. 010 is *C. difficile* RT010, 027 is *C. difficile* RT027.
Figure 4.3b. Mean (+/-SE) viable counts (log$_{10}$cfu/mL) of total culturable lactose fermenting Enterobacteriaceae, total anaerobes, and facultative anaerobes in the ciprofloxacin (Cipro) competition gut model. Vertical lines indicate the final day of each experimental period. SS is steady state. 010 is *C. difficile* RT010, 027 is *C. difficile* RT027.

4.4.4 Gut bacterial populations in the ampicillin-dosed models.

The bacterial populations in different experimental periods in the ampicillin-dosed model are shown in figures 4.4a and 4.4b. Obligate anaerobes such as bifidobacteria and *Bacteroides fragilis* group outnumbered the facultative anaerobes during steady state. The instillation of NTCD showed no considerable effect on the bacterial populations. Similarly, the instillation of NTCD spores together with 027 spores did not show any considerable effect on the bacterial populations. Following administration of ampicillin, populations of *Bacteroides* spp., and bifidobacteria declined by approx. 1.5 – 2.5 log$_{10}$ cfu/mL, whereas populations of facultative anaerobes, and lactose fermenters, and total anerobes increased by approx. 1.0 – 3.0 log$_{10}$ cfu/mL. Bacterial populations recovered to steady state levels 8 days after the cessation of ampicillin instillation. No further changes were observed during the rest period.
Figure 4.4a. Mean (+/-SE) viable counts (log_{10} cfu/mL) of total culturable *Bacteroides fragilis* group, *Bifidobacterium spp.*, facultative anaerobes, and lactose fermenting Enterobacteriaceae in the ampicillin (Amp) competition gut model. Vertical lines indicate the final day of each experimental period. SS is steady state. 010 is *C. difficile* RT010, 027 is *C. difficile* RT027.
Figure 4.4b. Mean (+/-SE) viable counts (log$_{10}$cfu/mL) of total culturable Enterococci, Lactobacilli, and total anaerobes in the ampicillin (Amp) competition gut model. Vertical lines indicate the final day of each experimental period. SS is steady state. 010 is *C. difficile* RT010, 027 is *C. difficile* RT027.

4.4.5 *C. difficile* populations and cytotoxin production upon clindamycin administration

Prior to clindamycin administration, *C. difficile* remained as spores in all three models (027 control, NTCD control, and 027 vs NTCD experimental model) and the spores were washed out of the models with no evidence of cytotoxin production. Four days into clindamycin administration the NTCD germinated in the NTCD control model. This was seen as a considerable divergence of the *C. difficile* total viable count and spore count. Ribotype 027 germinated 10 days after clindamycin instillation cessation in the RT027 control model followed by a high toxin production (4 RUs) on the next day. In the NTCD/RT027 competition model, NTCD germinated 4 days into clindamycin administration, whereas RT027 remained quiescent as spores, with no evidence of germination/outgrowth/proliferation or cytotoxin production throughout the experimental period. The total count of NTCD remained considerably above the spore count for the duration of the experiments, while in the control experiment with *C. difficile* PCR ribotype 027, the total count and cytotoxin titres began to decline, with the cytotoxin titre being 1 RU at the end of the experiment. *C. difficile* total viable counts, spore counts,
and cytotoxin titres in the NTCD vs 027 competition model, 027 control model, and the NTCD control model are shown in figures 4.5 a, b, and c respectively.

Figure 4.5a. *C. difficile* total viable counts, spore counts (log_{10} cfu/mL ±SE) and cytotoxin titres (relative log_{10} units, RU) in V3 of RT010 vs RT027 clindamycin (DA) competition model. Vertical lines indicate the final day of each experimental period. LLOD is lower limit of detection.
Figure 4.5b. *C. difficile* total viable counts, spore counts (log_{10}cfu/mL ±SE) and cytotoxin titres (relative log_{10} units, RU) in V3 of RT027 control clindamycin (DA) model. Vertical lines indicate the final day of each experimental period. LLOD is lower limit of detection.

Figure 4.5c. *C. difficile* total viable counts, spore counts (log_{10}cfu/mL ±SE) and cytotoxin titres (relative log_{10} units, RU) in V3 of RT010 control clindamycin (DA) model. Vertical lines indicate the final day of each experimental period. LLOD is lower limit of detection.
4.4.6 *C. difficile* populations and cytotoxin production upon cefotaxime administration

*C. difficile* remained quiescent in all the models prior to cefotaxime administration, until the fifth day of cefotaxime administration when NTCD germination (an increase in TVC and a decrease in spores count) was observed in both the competition and NTCD control models. No toxin was detected in both models throughout the experimental period. There was no evidence of ribotype 027 germination/outgrowth/proliferation or cytotoxin production in the competition model throughout the experiment. In contrast, in the 027 control model, ribotype 027 germinated on the sixth day of cefotaxime administration with a toxin production of 3RUs. After 10 days of initial toxin detection, toxin production declined to 1RU until the end of the experiment. *C. difficile* total viable counts, spore counts, and cytotoxin titres in the NTCD vs 027 competition model, 027 control model, and the NTCD control model are shown in figures 4.6 a, b, and c respectively.

![Figure 4.6a](image.png)

Figure 4.6a. *C. difficile* total viable counts, spore counts (log_{10}cfu/mL ±SE) and cytotoxin titres (relative log_{10} units, RU) in V3 of RT010 vs RT027 competition cefotaxime (CFX) model. Vertical lines indicate the final day of each experimental period. LLOD is lower limit of detection.
Figure 4.6b. *C. difficile* total viable counts, spore counts (log_{10} cfu/mL ±SE) and cytotoxin titres (relative log_{10} units, RU) in V3 of RT027 control cefotaxime (CFX) model. Vertical lines indicate the final day of each experimental period. LLOD is lower limit of detection.

Figure 4.6c. *C. difficile* total viable counts, spore counts (log_{10} cfu/mL ±SE) and cytotoxin titres (relative log_{10} units, RU) in V3 of RT010 control cefotaxime (CFX) model. Vertical lines indicate the final day of each experimental period. LLOD is lower limit of detection.
4.4.7 *C. difficile* populations and cytotoxin production upon ciprofloxacin administration

*C. difficile* remained as spores in all the models prior to ciprofloxacin administration. On the fourth and fifth day after ciprofloxacin administration, germination of NTCD occurred in the NTCD vs 027 competition model and NTCD control model respectively. No toxins were detected in both models throughout the experimental period. There was no evidence of ribotype 027 germination/outgrowth/proliferation or cytotoxin production in the competition model. However, in the 027 control model, *C. difficile* PCR ribotype 027 germinated 7 days after ciprofloxacin administration, with an accompanying toxin production of 5 RU 2 days after. *C. difficile* total viable counts began to decline towards the end of the experiment in all of the models and cytotoxin titres also declined towards the end of the experiment in the 027 control model, with the cytotoxin titre being 1 RU at the end of the experiment. *C. difficile* total viable counts, spore counts, and cytotoxin titres in the NTCD vs 027 competition model, 027 control model, and the NTCD control model are shown in figures 4.7 a, b, and c respectively.

![Graph](image)

Figure 4.7a. *C. difficile* total viable counts, spore counts (log_{10}cfu/mL ±SE) and cytotoxin titres (relative log_{10} units, RU) in V3 of RT010 vs RT027 competition ciprofloxacin (Cipro) model. Vertical lines indicate the final day of each experimental period. LLOD is lower limit of detection.
Figure 4.7b. *C. difficile* total viable counts, spore counts ($\log_{10}\text{cfu/mL} \pm \text{SE}$) and cytotoxin titres (relative $\log_{10}$ units, RU) in V3 of RT027 control ciprofloxacin (Cipro) model. Vertical lines indicate the final day of each experimental period. LLOD is lower limit of detection.

Figure 4.7c. *C. difficile* total viable counts, spore counts ($\log_{10}\text{cfu/mL} \pm \text{SE}$) and cytotoxin titres (relative $\log_{10}$ units, RU) in V3 of RT010 control ciprofloxacin (Cipro) model. Vertical lines indicate the final day of each experimental period. LLOD is lower limit of detection.
4.4.8 *C. difficile* populations and cytotoxin production upon ampicillin administration

*C. difficile* remained as spores in all the models prior to ampicillin administration. On the third day of ampicillin administration NTCD germinated in the NTCD control experiment. This was followed by the germination of ribotype 027 in the 027 control model on the next day. NTCD germinated in the competition model on day 5 of ampicillin instillation. There was no evidence of ribotype 027 germination/outgrowth/proliferation or cytotoxin production in the competition model throughout the experiment. Similarly, no toxins were detected in the NTCD control model throughout the experimental period. However, a maximum cytotoxin titre of 3 RUs was observed in the 027 control model following germination and growth of ribotype 027. Cytotoxin titres declined to 2RUs in the 027 control model towards the end of the experiment. *C. difficile* total viable counts, spore counts, and cytotoxin titres in the NTCD vs 027 competition model, 027 control model, and the NTCD control model are shown in figures 4.8 a, b, and c respectively.

![Graph showing log10 cfu/mL ±SE and relative log10 units, RU of *C. difficile* total viable counts, spore counts (log10 cfu/mL ±SE) and cytotoxin titres (relative log10 units, RU) in V3 of RT010 vs RT027 experimental ampicillin (Amp) model. Vertical lines indicate the final day of each experimental period.](image)

Figure 4.8a. *C. difficile* total viable counts, spore counts (log10 cfu/mL ±SE) and cytotoxin titres (relative log10 units, RU) in V3 of RT010 vs RT027 experimental ampicillin (Amp) model. Vertical lines indicate the final day of each experimental period.
Figure 4.8b. *C. difficile* total viable counts, spore counts (log$_{10}$ cfu/mL ±SE) and cytotoxin titres (relative log$_{10}$ units, RU) in V3 of RT010 control ampicillin (Amp) model. Vertical lines indicate the final day of each experimental period.

Figure 4.8c. *C. difficile* total viable counts, spore counts (log$_{10}$ cfu/mL ±SE) and cytotoxin titres (relative log$_{10}$ units, RU) in V3 of RT027 control ampicillin (Amp) model. Vertical lines indicate the final day of each experimental period.
4.5 Discussion

*C. difficile* PCR ribotype 027 have been often revealed to be overrepresented in nosocomial outbreaks and have been associated with increased mortality and morbidity. Even though the mechanism through which it perpetrates hyper-virulence continues to be foment controversy, the capacity of RT027 strains to spread across the world may be an indication of an acquisition of a heightened capacity to cause disease. In the present study, it was hypothesized that the differed physiology could confer a competitive advantage on a non-toxigenic *C. difficile* (NTCD) strain (RT010) over a virulent strain and contribute to the body of published evidence suggesting a benefit of NTCD in interrupting the cycle of CDI. The triple-stage *in vitro* human gut model was employed for these studies since this model system has been shown to accurately reflect *in vivo* observations with respect to CDI induction and treatment (Freeman *et al.*, 2003; Baines *et al.*, 2008; Chilton *et al.*, 2012; Crowther *et al.*, 2015; Moura *et al.*, 2020). The gut model allows for the study of the capability of NTCD to interfere with a cycle of simulated CDI (RT027) following perturbation of the gut microbiota by a range of antimicrobial agents. This model is superior to studies of *C. difficile* in hamsters (Ebright *et al.*, 1981; Larson & Welch 1993; Alcantara *et al.*, 2001; Sambol *et al.*, 2001; Razaq *et al.*, 2007) and studies in faecal emulsions (Borriello & Barclay, 1986; Wilson & Perini, 1988; Larson & Welch, 1993b) in that animals are not truly reflective of the human colon and faecal antibiotic concentrations and *C. difficile* exposure in studies using faecal emulsions are not well controlled. Even though antimicrobial concentrations within each vessel were not measured in the present studies, the instillation of antimicrobials at regular intervals was intended to mirror the fluctuation of antimicrobial concentrations in the colon. In this manner, the present study instilled antimicrobial agents to achieve published faecal concentrations of the antimicrobials and prior studies have demonstrated that the gut model dosing in this fashion achieves clinically relevant antimicrobial concentrations of fluoroquinolones, clindamycin, cephalosporins, and aminopenicillins (Freeman *et al.*, 2003; Baines *et al.*, 2008; Saxton *et al.*, 2009; Chilton *et al.*, 2012).

Different antimicrobials (clindamycin, cefotaxime, ciprofloxacin, and ampicillin) were used in the present studies to ascertain if the type of antimicrobial impacted on the competition of the toxigenic and non-toxigenic strains in the models. Additionally the minimum inhibitory concentration (MIC) of the
inducing antimicrobial on the competition was also evaluated. This is because *C. difficile* outgrowth occurs when antimicrobial levels are below the MIC (Baines *et al.*, 2008; 2013; Crowther *et al.*, 2015). Both strains in the present studies appeared to respond to antimicrobial exposure; entering a cycle of spore germination, outgrowth, and proliferation when in monoculture in the gut model (control experiments).

Studies on antimicrobial-enhancement of *C. difficile* toxin production have reported conflicting results (Nakamura *et al.*, 1982; Barc *et al.*, 1992; Drummond *et al.*, 2003; Pultz & Donskey, 2005). The present studies showed *C. difficile* inactivity in the absence of antimicrobial disturbance in the gut models. Freeman *et al.*, (2003) suggested that growth and toxin production by *C. difficile* occurs upon an effect of antimicrobials on members of the microbiota, instead of a direct effect on *C. difficile*. This they believe accounts for why CDI does not occur within a short time frame of of antimicrobial instillation, but days or even weeks later. However, Drummond *et al.*, (2003) reported that the effect of antimicrobials on *C. difficile* growth and toxin production varied with strain and the type of antimicrobial, suggesting a more direct and complex relationship. They demonstrated that sub-inhibitory concentrations of clindamycin potentiated higher levels of toxin production and in a shorter time when compared with antibiotic-free controls. Antibiotics are expected to cause stress in bacteria (including *C. difficile*). The bacteria under stress ‘‘switch on’’ a number of genes, which the toxin promoters might be affected by (Drummond *et al.*, 2003). Indeed, the physiological stress of increased temperature was shown to lead to higher toxin production in *C. difficile* (Onderdonk *et al.*, 1979). The same authors showed an increased toxin production upon sub-inhibitory penicillin and vancomycin concentrations. Hennequin *et al.* (2001) demonstrated that *C. difficile*, when cultured with antimicrobials, produces higher amounts of GroEL, a 58 kDa surface adhesin that might assist *C. difficile* to colonise recently vacated binding sites left due to the depletion of the microbiota. Similarly, Deneve *et al.*, (2008) demonstrated that both non-toxigenic and toxigenic *C. difficile* strains overexpressed the three adhesins Fbp68, Cwp66, and the S-layer protein P47 (although to different degrees) following exposure to sub-inhibitory antimicrobial concentrations in the growth medium. Ampicillin and clindamycin exposure led to higher levels of expression of the adhesins. Strikingly,
exposure to kanamycin led to a 1.3 – 2.3-fold increase in the adhesins for a toxigenic strain (79-685) and a 3.2 to 5-fold increase for a non-toxigenic strain (ATCC 43603). Upregulation of the genes encoding the adhesins was not associated with the resistance level of the strains (Deneve et al., 2008). Furthermore, Deneve et al., (2008) showed that the adherence of strain ATCC 43603 to Caco-2/TC7 cells was increased 1.8-fold by clindamycin and 2.2-fold by ampicillin, consistent with the increased adhesin gene expression. Given the roles of S-layer protein, Fbp68, and Cwp66 in C. difficile adherence (Waligora et al., 2001; Calabi et al., 2002; Cerquetti et al., 2002), coupled with observations from the present studies where no strain of C. difficile germinated/grew/proliferated or produced cytotoxin prior to antimicrobial instillation, in addition to the likelihood that both C. difficile strains in the present studies proliferated when antimicrobial levels declined below their MICs for the strains in the control models, as well as the inability of ribotype 027 to germinate/outgrow/proliferate or produce cytotoxin in the presence of NTCD, it might be that the antimicrobials used in the present study (clindamycin, cefotaxime, ciprofloxacin, and ampicillin) increased colonisation factor gene expression in NTCD more than in RT027, leading a better adherence by NTCD and in turn a better utilisation of the new niche created by the depletion of bacterial groups by antimicrobials in the gut models. Such improved adherence might have conferred advantage on NTCD. Nonetheless, it is pertinent to state the inherent difficulties associated with comparing pure cultures and complex microbiota data.

Upon addition of C. difficile (toxigenic and non-toxigenic) spores to the gut models, and in the absence of antimicrobial perturbation in the present studies, no spore germination, growth, or toxin production were observed. This work demonstrates for the first time that ribotype NTCD (RT010) strains can directly outcompete ribotype 027 strains in a complex faecal microbiota. Given all the major antimicrobial groups were represented in the present studies, it is very unlikely that that the observed increased fitness of NTCD was due to a direct advantage by an antimicrobial. Similar amounts of both strains ($10^8$) were added to the gut models but only NTCD was able to germinate and grow, fuelling the suspicion that these particular strains cannot coexist. This is despite data from studies that showed two different C. difficile strains simultaneously proliferated in a complex in vitro model upon antimicrobial instillation, suggesting a possible in vivo coinfection by multiple virulent strains (Baines et al., 2013;
Crowther et al., 2015). Although the physiological differences present in ribotype 010 strains that enabled them to outcompete 027 is currently unknown, it is unlikely that the competitive advantage of RT010 over RT027 in the present studies was because of a simple advantage in growth rate since there was no evidence that RT027 spores germinated, unlike prior gut model studies where these antimicrobials have yielded growth and cytotoxin production for ribotype 027 (Freeman et al., 2003; Baines et al., 2008; Saxton et al., 2009; Chilton et al., 2012). Robinson et al., (2014) showed using in vitro batch culture experiments in several different laboratory media that no significant distinctions exists between the growth rate of RT027 strains and other ribotypes. Even though it is plausible to suggest that RT010 strains are able to directly antagonise RT027 strains, it is more likely that RT010 strains possess a heightened cellular fitness that enables them to compete better for limiting nutrients, hence indirectly leading to the elimination of RT027 strains from the gut model vessels. Nonetheless, it is plausible to suggest that RT010 might have produced substance(s) that may hinder the growth of RT027 in the present studies. Given RT010 and RT027 were not grown in a pure culture together, it can only be speculated that such a phenomenon happened with the strains used herein. Even though the present studies did not determine and characterise the presence of such inhibitory substances, it cannot be totally ruled out. The characterisation of a potential inhibitory substance may be undertaken by treating the culture supernatants with catalase, pronase, or adjusting the culture supernatant to pH 6.5 to ascertain if such inhibitory substance is hydrogen peroxide, bacteriocin, or an organic acid respectively. In theory, if RT010 produces substances that decrease the growth rate of RT027 even when RT027 is provided with a nutritionally rich medium. The effect will be a decrease in the maximum growth rate achievable by RT027. Such decrease in the maximum growth rate of RT027 would directly decrease its competitive capacity in a continuous culture environment such the triple-stage in vitro human gut model (Monod, 1950; Fredrickson, 1977). Some bacteria produce antibacterial compounds that can prevent/treat CDI. Bacteriocin molecules produced by Bacillus thuringiensis DPC 6431 (Mills et al., 2018) and reuterin produced by Lactobacillus reuteri have been reported to reduce the growth of C. difficile in vitro (Spinler et al., 2017).
Competition for nutrients may be another likely mechanism through which NTCD in the present studies prevented RT027 spores from germinating and growing. Chemostat theory holds that if different bacterial populations are growth-limited by a common substrate in a continuous culture, they will compete for that substrate. The population capable of depleting the common substrate to the lowest concentration while dividing at the dilution rate of the continuous culture will displace the other populations (Freter et al., 1983). Antimicrobials can impact on the availability of microbial resources in the colon in several ways. As an effect of decreasing bacterial biomass, antimicrobials reduce competition for nutrients and also establish previously unavailable ecological niches. Additionally, bacteria lysis due to antimicrobials give off carbon sources that may be utilised by the remaining members of the microbial community (Britton & Young, 2014).

Over 30 years ago, Wilson and Perini demonstrated that components of mucin, N-acetylneuraminic acid (a sialic acid) and N-acetylglucosamine promoted *in vitro* growth of *C. difficile*. Moreover, the introduction of these monosaccharides to a culture of mouse caecal microbiota completely restored the capacity of the microbial community to prevent *C. difficile* proliferation upon a transfer to germ-free mice, an indication that these monosaccharides enhance the expansion of specific intestinal bacteria that can utilise them efficiently, thus outcompeting *C. difficile* (Wilson & Perini, 1988). More recently Ng et al., (2013) showed that under normal conditions, the indigenous intestinal microbiota competes for the utilisation of sialic acids which are given off from the colon’s mucus lining. Nevertheless, upon a disturbance of the resident microbiota by antimicrobial agents, sialic acids are left unutilised, thereby providing a window of opportunity for *C. difficile* to utilise these carbohydrates and proliferate. It is therefore plausible to suggest that in the present studies ribotype 010 have more affinity for these sialic acids or may have an improved utilisation profile in comparison to ribotype 027. This may explain why ribotype 027 did not proliferate in the gut models in the presence of ribotype 010 despite antimicrobial disturbance of the microbiota. This thinking is also reinforced by the inability of ribotype 010 to gain a foothold in the gut models prior to antimicrobial instillation, and the germination and growth of RT027 in the absence of RT010. Future studies to investigate the substrate affinity and sialic acid utilisation profiles of toxigenic and NTCD may shed more light on this area.
An aspect of bacterial physiology that might likely affect competition outcome is differences in sporulation rates between strains. Despite reports that RT027 strains sporulate more efficiently than other ribotypes (Akerlund et al., 2008; Merrigan et al., 2010), bigger studies that compared many isolates of distinct ribotypes concluded that no significant association exists between ribotype and sporulation efficiency (Burns et al., 2011; Carlson et al., 2013). Given *C. difficile* was dosed into the gut models as spores in the present studies, the strains’ ability and speed to germinate will likely be more crucial for competitive advantage rather than their reduced sporulation efficiency. Besides, the present study does not have data to ascertain if the RT027 strains used in the present studies sporulate more efficiently than RT010 strains. *C. difficile* sporulation dynamics in the context of growing in a complex faecal microbiota should be an area of future research. Although it is crucial to consider the effect of sporulation dynamics on the outcome of bacterial competition, it is very unlikely that sporulation rates is responsible for ribotype 010 strains outcompeting ribotype 027 in the present studies.

Differences in germination is an aspect of bacterial physiology that might have a role in competitive fitness in the simulated CDI model. Differential germination might have played a role in the competitive advantage of RT010 strains in the gut models given competition in the present studies was initiated by an administration of spores. Francis et al., (2013) reported that muricholic acids (murine bile acids) inhibit the germination of *C. difficile* spores, and that a strain variability exists in this inhibition. Additionally, there is evidence to suggest a significant difference in germination rates and substances that act as germinants (at least *in vitro*) among *C. difficile* strains of different ribotypes (Heeg et al., 2012). Cholate and chenodeoxycholate are the primary bile acids produced by the liver in humans. They are often conjugated to glycine or taurine (for example taurocholate). The portion of produced bile acids (less than 5%) that is not reabsorbed in the small intestine (distal ileum) is passed into the colon, where they are subjected to bacterial metabolism (Gerard, 2014). A small fraction of intestinal bacteria can metabolise primary bile acids into secondary bile acids such as deoxycholate and lithocholate (Jones et al., 2008). *In vitro* research have demonstrated that deconjugated and conjugated forms of cholate act together with the amino acid glycine to induce *C. difficile* spore germination, whereas
chenodeoxycholate strongly inhibits germination of *C. difficile* spores (Sorg & Sonenshein, 2008; 2009). Lithocholate (a secondary bile acid) inhibits spore germination, whereas deoxycholate enhances spore germination, but because of its toxicity to *C. difficile* vegetative cells, it prevents it growth (Sorg & Sonenshein, 2008; 2010). Under normal physiological functioning chenodeoxycholate competitively inhibits taurocholate-promoted spore germination in the colon, in addition to preventing *C. difficile* outgrowth similar to deoxycholate (Sorg & Sonenshein, 2008; 2009; 2010).

Nevertheless, when the intestinal microbiota is disturbed by antimicrobials, primary bile acids are not metabolised into secondary bile acids, and this may ultimately enhance the germination and growth of *C. difficile* (Sorg & Sonenshein, 2009; 2010). In fact patients with severe CDI were shown to have very low or undetectable faecal secondary bile acid levels (Weingarden *et al.*, 2014). Buffie *et al.*, (2015) recently reported that *Clostridium scindens*, a bile acid 7α-hydroxylation bacterium, showed inhibition against *C. difficile* in the microbiota of antimicrobial-treated humans and mice in a bile acid-dependent manner. The 7α-hydroxylation activity is thought to involve the metabolism of primary bile acids into secondary bile acids, and a limited number of intestinal bacterial species among the *Eubacterium* and *Clostridium* genera possess this activity (Gerard, 2013). *C. difficile* spores are necessary for infection transmission, but to cause disease spores must return to vegetative cell growth via germination. Indeed the germination response to bile salts differs among spores of different isolates (Heeg *et al.*, 2012; Weingarden *et al.*, 2016). This same group of researchers reported that despite its ability to inhibit spore germination, chenodeoxycholate did not show inhibition to germination or vegetative cell outgrowth in some isolates. Similarly, there was a considerable variation in the extent and rate of germination among different *C. difficile* isolates in response to taurocholate. Taken together, and given that ribotype 027 strains did not germinate like ribotype 010 in the present studies, it can only be speculated that bile acids may have inhibited the germination of RT027 spores but not RT010 given the strain variability, or perhaps RT010 spores were able to utilise a nutrient or chemical (amino acid or bile salt) as a germinant while RT027 were unable to utilise same. It may also be that the germination or inhibition of *C. difficile* by bile acids may be in a concentration-dependent manner, such that RT010 may require very minimal amounts of bile acids for germination when compared to RT027. Ribotype 010 may also
require higher amounts of secondary bile acids to inhibit its germination. In fact Theriot et al., (2015) demonstrated that secondary bile acids that significantly interfered with taurocholate-mediated spore germination in *C. difficile* do so in a concentration-dependent manner. They demonstrated that *C. difficile* VPI 10463 spores germination was inhibited only at the appropriate concentration of secondary bile acids (α-muricholate 0.004% and lithocholate 0.01%), in the presence of known germinants in murine caecum. Gut model studies may be designed with an instillation of different concentrations of bile acids to elucidate optimum bile acid concentrations that may enhance germination of NTCD or an inhibition of toxigenic *C. difficile* spores in the presence of a complex human intestinal microbiota.

Exploiting the capacity of NTCD spores to germinate before its toxigenic counterparts is particularly promising in terms of preventing recurrent CDI (rCDI), which is a relapse of CDI symptoms within 2 – 8 weeks of successful treatment of the initial episode (McDonald et al., 2018). This is because unlike commercially available standard probiotics such as *L. casei* Shirota and *Saccharomyces boulardi*, NTCD strains are able to sporulate. Therefore if the conditions in the colon permitted *C. difficile* spore germination after an initial successfully treated episode of CDI, it is the NTCD spores that may germinate and prevent toxigenic counterparts from germinating and growing, thus holding a huge potential to prevent rCDI. Colonisation with a NTCD in a randomised clinical trial was correlated with reduced rCDI (Gerding et al., 2015). The researchers reported a 31% recurrence of CDI in patients who were not colonised with NTCD as against 2% in patients who were colonised. These findings provide more impetus for research on targeted microbiome-based therapy to treat and prevent CDI.

Owing to the reported inhibitory effect of chenodeoxycholate on *C. difficile* spore germination, it has been proposed as potential prophylactic to prevent CDI (Sorg & Sonenshein, 2010). However, Heeg et al., (2012) demonstrated that chenodeoxycholate does not inhibit the spore germination of every *C. difficile* strain. Accordingly, a chenodeoxycholate-based drug might be insufficient to tackle CDI, given its inhibitory effects on spore germination is not likely to apply to all *C. difficile* strains. To fully exploit the potential of germination-based interventions, more research on *C. difficile* spore germination mechanisms are required. Even though the presence of germinant receptors is supported by evidence (Sorg & Sonenshein, 2008), there is no evidence to suggest if germinants (for example taurocholate)
bind to a receptor or just move though the spore by diffusion and initiate the germination cascade. Therefore, it is difficult for the present study to speculate on what the molecular basis for the difference in germination characteristics of RT010 and RT027 in the present study. Investigating the germination differences between a potential probiotic candidate such as the NTCD RT010 strain and its toxigenic counterparts in more detail is a clear area for future research.

The capacity to adapt effectively to environmental changes is a global trait in the bacterial world. Colonisation resistance by the host microbiota is crucial in preventing CDI and C. difficile must overcome it to proliferate in the colon (Wilson & Freter, 1986). Competition for nutrients is one way in which the indigenous microbiota mediate colonisation resistance. The production of TcdA and TcdB (large enterotoxins) thought to be the principal virulence factors (Lyra et al., 2009; Kuehne et al., 2010), by C. difficile responds to the availability of specific nutrients (at least in vitro) (Yamakawa et al., 1996), an indication of the close link between virulence and metabolism. Colonisation of the colon by NTCD is frequently observed in hospitalised patients, possibly due to an ingestion of spores from the hands of clinicians or the environment (Shim et al., 1998). Studies in patients and hamster suggest a prevention of CDI by a colonisation with NTCD (Wilson & Sheagren, 1983; Borriello & Barclay 1985; Seal et al., 1987). Subsequent studies in hamster showed long-term protection against toxigenic C. difficile (Sambol et al., 2002; Merrigan et al., 2003).

Although the precise mechanism by which NTCD prevents CDI is unknown, the protection requires the presence of viable NTCD strains. No protection against toxigenic C. difficile was observed when study animals were given a heat-killed suspension of NTCD strains (Borriello et al., 1984). Similarly, in the same study, when the protective NTCD dose was decreased to undetectable amounts by vancomycin treatment prior to challenge with toxigenic C. difficile, no protection was observed. This is an indication that there was no memory for protection and that viable NTCD strains were necessary for such protection. This data suggests that a likely immune protective response is not the main factor involved in a protective role NTCD. Indeed, administration of NTCD to hamsters did not induce anti-toxin production in faecal pellets (Borriello et al., 1984). Given NTCD strains were unable to degrade C. difficile toxins (Borriello et al., 1984), in vivo degradation of the toxin as a mechanism of protection is
very unlikely. The protection by NTCD strains observed following the experiments of Borriello and co-workers (1984) is very specific in that other clostridial species did not protect the animals against CDI. Similarly, a prior colonisation of the study animals with NTCD strains did not protect the animals from disease and death upon exposure to a distinct clostridial pathogen, *C. spiroforme*.

The specific nature of protection coupled with the requirement for the presence of viable NTCD strains suggests that the means of protection against RT027 in the present studies might be competition for specific ecological niches (for example those at mucosal receptor sites) or limited nutrients. It is likely that RT010 occupies the same adherence or metabolic niche in the colon as does RT027, and once established, is capable of outcompeting newly ingested or resident RT027 strains. Mucosal association is a crucial pathogenic means for a number of bacteria. Studies have previously shown the intimate association of toxigenic *C. difficile* with the rectal mucosa of humans (Borriello, 1979), in addition to the mucosal lining of the caecum in hamsters (Borriello, 1984). Furthermore, Borriello et al., (1984) showed that a NTCD associated closely with the mucosal lining of the caecum, and that the colonisation was prolonged. Taken together, there is a likelihood that the non-proliferation of RT027 strains in the present studies might be due to the competition from RT010 for the binding niches within the biofilms in the gut models which are likely to be similar in their structure to *in vivo* biofilms which may be necessary for establishment of the toxigenic strain.

The mechanisms through which *C. difficile* responds to Butyrl CoA is of huge potential interest. The role of butyrate in inducing toxin synthesis has been reported (Karlsson et al., 2000; Dubois et al., 2016). Butyrl CoA is the precursor of both butanol and butyrate. Strikingly, butanol is an inhibitor of *C. difficile* toxin synthesis (Karlsson et al., 2000). Given NTCD strains’ lack the pathogenicity locus (PaLoc) and do not produce toxins, it is tempting to speculate that these strains convert more Butyrl CoA to butanol and less butyrate for biological efficiency, since they do not need to induce toxin synthesis. High amounts of butanol produced by the NTCD strains may then proceed to inhibit toxin synthesis in toxigenic strains, assuming ribotype 027 strains outgrew at low levels below the limits to detect it with microbial culture used in the present studies. In fact revealing how toxigenic *C. difficile* strains ‘decide’ whether to convert butyrl CoA to butyrate or butanol and the ratios will discover a
crucial decision point for the bacterium and may potentially provide new approaches of decreasing virulence. Further work at the level of gene expression may provide more robust conclusions.

The likelihood that NTCD strains could obtain toxin genes form toxigenic *C. difficile in vivo* remains a concern. An *in vitro* transfer for the pathogenicity locus (which contains toxins A and B) to NTCD has been successful in the laboratory (Brouwer *et al.*, 2013). A toxigenic strain, 630Δerm, was used as a donor strain to transfer the pathogenicity locus to 3 NTCD strains at a frequency of about $7.5 \times 10^{-9}$ transconjugants per donor. Different large DNA fragments that contained the PaLoc were transferred, and toxin B production by one of the transconjugants was demonstrated (Brouwer *et al.*, 2013). Although these *in vitro* PaLoc transfer observations raise concern that transfers might happen *in vivo*, it is important to state that *in vivo* PaLoc transfer has not been demonstrated. Nevertheless, they, reemphasise the need to eliminate toxigenic *C. difficile* with treatment to reduce the likelihood of *in vivo* conjugation and PaLoc transfer. Although this is not presented as an evidence of the lack of PaLoc transfer in the present study, it is worth mentioning that the NTCD strain isolated from the gut models after the experiments using antimicrobial-embedded selective agar was grown in brain heart infusion broth (BHIB) and tested using Vero cell cytotoxicity assay. No toxin production by the NTCD was observed. Given the low PaLoc transfer frequency of about $7.5 \times 10^{-9}$ reported by Brouwer *et al.*, (2013), the few CFUs of NTCD (about 5 – 10) picked up in the present studies for the cytotoxicity assay are insufficient representatives of about $10^9$ bacterial cells. Additionally, Brouwer *et al.*, (2013) also reported that the transfer of PaLoc often happens with co-transfer of conjugative transposons that encode antibiotic resistance genes. They demonstrated this by the transfer of the PaLoc and a conjugative transposon that encodes tetracycline resistance Tn5397 on the same DNA fragment from 630Δerm to CD37. While the present study acknowledges the low frequency of such transfer events, the NTCD strain (RT010) used in the present study retained its antimicrobial susceptibility profiles (at least MICs of tetracycline, clindamycin, ciprofloxacin, cefotaxime, and ampicillin) after the study, suggesting a non-receipt of antimicrobial resistance genes from toxigenic *C. difficile* or components of the faecal microbiota. Antimicrobial agar plates were quality controlled daily by inoculating both strains
on the two different selective agar plates. Studies in and out of the triple-stage *in vitro* human gut model are required with RT010 and RT027 to fully elucidate this phenomenon.

New antimicrobial intervention upon a loss of NTCD colonisation could very likely place patients at CDI risk again if they ingest a toxigenic *C. difficile*. Injectable CDI antitoxin vaccines currently in clinical development would confer more prolonged protection, but need weeks to months to mount an antibody response, particularly in aged individuals (Foglia *et al.*, 2012). An administration of NTCD to patients at high risk of CDI or rCDI might confer transient protection while waiting for a more durable vaccine to become effective. Additionally, given the NTCD prevented proliferation and cytotoxin production of RT027 in the gut models in the present studies, it might decrease the risk of toxigenic *C. difficile* (for example RT027) transmission in high-risk settings such as nursing homes and hospitals, an effect that is not likely with current monoclonal antibodies and antitoxin vaccines which do not affect colonisation. Although the NTCD strains were detected in the gut models until the end of each experiment in the present studies, it is believed that the colonisation of the gastrointestinal tract by NTCD is not long-lived (Borriello & Barclay 1985; Villano *et al.*, 2012; Gerding *et al.*, 2015). This is not likely to be a problem as the loss of NTCD colonisation probably happens due to a restoration of the indigenous microbiota, which is capable of protection against subsequent CDI.

Given the design of the experiments, the present studies are unable to provide evidence whether the NTCD strains can displace established toxigenic strains of *C. difficile* as a treatment for existing CDI. The extent to which this sort of prophylactic approach employed in the present studies may be therapeutically useful in managing already developed CDI remains to be known. Patients with a history of recurrent CDI may benefit from being colonised with NTCD strains against recolonization by a toxigenic strain. Borriello and Honour (1983) simultaneously isolated both non-toxigenic and toxigenic strains of *C. difficile* from the faeces of a patient with pseudomembranous colitis and cytotoxin detected. Whether their findings suggest an absence of antagonism by the NTCD strains is difficult to evaluate in the context of the results from the present studies, particularly as the sequence of colonisation of the patient was unknown. The lack of antagonism by the NTCD reported by Borriello and Honour (1983) may just be due to strain variability.

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In the present studies, ribotype 027 germinated, grew, and produced toxins in all of the RT027 control experiments. The timing of germination and toxin production showed no correlation with the extent of antimicrobial disturbance on the bacterial community (at least for the bacterial groups isolated in the present study). For example the cefotaxime and ampicillin-dosed models showed a comparatively reduced microbiota depletion when compared with the ciprofloxacin-dosed model. This is similar to the observations of Chilton et al., (2012) and Freeman et al., (2003), but the less disrupted models (cefotaxime- and ampicillin-dosed) recorded the earliest *C. difficile* germination and growth (Figures 4.2a & 4.4a). It may be that cefotaxime and ampicillin disrupts the microbiota just enough for *C. difficile* proliferation or that these antimicrobials may promote *C. difficile* colonisation in a manner other than disruption of the intestinal microbiota as described earlier.

Interestingly, in all of the control experiments in the present studies, RT027 seemed to produce toxin in V2 and V3 and not V1. This is despite an observation of *C. difficile* germination and growth in V1. Similar observations have been reported by Freeman et al., (2003) following a study using an *in vitro* gut model. The authors suggested that either the toxins produced were quickly degraded or toxin production was inhibited at low pH. Reduced levels of toxin production in *C. difficile* have been linked to low pH by prior studies (Onderdonk et al., 1979; Borriello & Barclay, 1986), similarly Qa’Dan, et al., (2000) reported a pH-induced conformational changes in *C. difficile* toxin B. Furthermore inhibition of spores at acidic pHs have been demonstrated in *C. sporogenes* (Valero et al., 2020) and *C. botulinum* (Blocher & Busta, 1985; Wong et al., 1988). Whereas the present study takes into consideration the effect of pH on *C. difficile* germination/growth, the absence of toxins in V1 in the present studies may be due to an abundance of metabolised carbon sources and nutrients such as glucose or amino acids for example cysteine that interfere with *C. difficile* toxin synthesis (Dupuy & Sonenschein, 1998; Karlsson et al., 2000; 2008). It is very likely that following instillation of antimicrobials into V1, essential germinants or nutrients such as sialic acid which are left unused due to the depletion of the microbiota flow into V2 and V3 where they are utilised by *C. difficile* in its preferred more alkaline pH (May et al., 1994; Engevik et al., 2014; Wetzel & McBride, 2020) obtainable in V2 and V3. Events *in vivo* may be similar to these observations.
Unlike the NTCD-M3 strain that was shown to colonise the colon and significantly reduce CDI recurrence following a phase 2 randomised controlled trial (Gerding et al., 2015), the non-toxigenic C. difficile strain used in the present study is clindamycin resistant (MIC >128 mg/L). The MIC of clindamycin to NTCD-M3 is 0.5 mg/L (Merrigan et al., 2003). Although the effectiveness of NTCD in preventing CDI have been demonstrated in the present and prior studies (Borriello & Barclay 1985; Villano et al., 2012; Gerding et al., 2015), there are important factors in protection and timing of colonisation which are associated with antimicrobial susceptibility of the strains (probiotic and challenge strain). Many toxigenic epidemic C. difficile strains are clindamycin resistant (Johnson et al., 1999; Sambol et al., 2001). Consequently, they are more likely to infect and cause CDI during and after clindamycin treatment. This in turn elongates the period which patients are at risk of infection, as against clindamycin susceptible toxigenic C. difficile strains which are not likely to cause disease during clindamycin treatment. Sambol et al., (2002) demonstrated that colonisation by a clindamycin resistant NTCD during clindamycin treatment prevented CDI in hamsters when challenged with a toxigenic C. difficile. In contrast, the clindamycin susceptible NTCD-M3 failed to colonise during a multiple dose course of clindamycin, and prevented CDI in hamsters only if the hamsters were challenged ≥2 days after the last dose of clindamycin treatment (Sambol et al., 2002). This is an indication that the NTCD-M3 strain is inadequate for effective prevention of CDI during antimicrobial administration. Therefore, resistance to an antimicrobial by NTCD such as RT010 used in the present studies may be useful in establishing colonisation and prevention of CDI during administration of such antimicrobial. Such potential benefit due to antimicrobial resistance was highlighted by the results of the control experiments in which the two strains responded differently to clindamycin, i.e. earlier germination of NTCD than ribotype 027. Furthermore, the NTCD used in the present studies contains the chromosomal erm(B) gene (Dr. César Rodríguez Sánchez - Personal Communication) that encodes a 23s rRNA methylase which confers resistance to macrolides, lincosamides, and streptogramines (MLS). Accordingly, an MLS mode of resistance to clindamycin is likely. Erm(B)-containing strains differ as to the presence of Tn5398 (mobilizable element) which was shown to transfer resistance (Farrow et al., 2001; Spigaglia & Mastrantonio 2002), although erm(B) could still be transferred even in the absence of Tn5398 (Merrigan et al., 2003). Accordingly it is crucial to ascertain the transfer frequency of erm(B).
to toxigenic *C. difficile* and other bacteria resident in the colon. Nonetheless, the most ideal probiotic *C. difficile* strain will be a NTCD strain that is both antimicrobial resistant and potent in preventing CDI but does not carry the risk of resistance transfer. Prior to consideration for human use, the clindamycin-resistant NTCD strain employed in the present studies, should undergo rigorous genetic analysis and gene transfer experiments to ascertain the risk of resistance transfer. Such experiments may include but not limited to mating experiments used by Brouwer *et al.*, (2013) to identify non-toxigenic *C. difficile* strains with the least likelihood of receiving the pathogenicity locus. Furthermore, the genome of non-toxigenic *C. difficile* strains should be examined to ascertain the presence of mobile genetic elements that are in the form of conjugative transposons. These mobile elements are thought to be responsible for the acquisition of a wide range of genes involved in antimicrobial resistance, host interaction, and virulence (Sebaihia *et al.*, 2006).

In conclusion, the present studies provide evidence that NTCD RT010 has an ecological advantage over RT027 in a complex faecal microbiota. Similarly, the present studies favours the model in which RT010 is able to outcompete RT027 in the triple stage *in vitro* human gut model due to its capacity to more efficiently exploit the limited resources in the bacterial community by demonstrating a fitness advantage despite the type of antimicrobial used to deplete the microbiota. These observations may mirror events in the human intestinal environment *in vivo*. NTCD behaves like its toxigenic counterpart in the absence of antimicrobials, i.e. it is unable to proliferate in an unperturbed microbiota. The present studies further expand on aspects of *C. difficile* physiology, which could be exploited by bacterial therapies to prevent/treat CDI, as well as demonstrating that the physiology of *C. difficile* is a crucial factor in its success or failure as a pathogen. A clear understanding of the conditions that favour PaLoc transfer to NTCD is crucial for its success as a probiotic, in addition to evaluating the propensity of mobile genetic element transfer of antimicrobial resistance genes to other members of the gut microbiota.
4.6 General discussion and conclusions

The role of antibiotics in shaping the landscape of modern medicine and the ability of humans to survive numerous bacterial diseases are significant. However, the effects of antimicrobials on the micro-ecology of the gut microbiome is becoming increasingly relevant. CDI is one of such negative consequences of antimicrobial use. FMT has been successfully used to treat rCDI, with resolution rates of about 90%. Notwithstanding, the composition and behaviour of the gut microbiota, especially in ill-defined microbiota replacement intervention such as FMT remains to be fully elucidated.

The triple-stage in vitro human gut model was validated against the intestinal contents of sudden-death victims and is a reliable tool to depict or mirror bacterial activities and microbial content of the human colon (Macfarlane et al., 1998). This model has been previously employed to evaluate the tendency of various antimicrobials to stimulate CDI, as well as the potency of treatments (Baines et al., 2009; 2013; 2015; Freeman et al., 2003; Chilton et al., 2012; 2014; Moura et al., 2019; 2020). Data obtained from the gut model have been reported to be consistent with phase III clinical trials (Freeman et al., 2003; Moura et al., 2019), an indication of the clinical importance of this tool.

*Bacteroides* spp., *Lactobacillus* spp., *Bifidobacterium* spp. and *Clostridium* spp., constitute highly abundant bacterial genera in the human colon that are frequently depleted by antimicrobial intervention, as demonstrated by in vitro and ex vivo studies (Ben-Amor et al., 2005; Louie et al., 2012; Baines et al., 2013; Rajilic-Stojanovic & de Vos, 2014; Kelly et al., 2016). The depletion of bacterial populations brings about a potential new niche for *C. difficile* spore germination, cell growth, and toxin production, culminating in CDI (Freeman et al., 2003). Given the high degree of consistency and accuracy of gut model studies which show a depletion of bacterial populations upon antimicrobial intervention, it seems plausible to suggest that such bacterial populations provide an insight into a healthy microbiota (Rajilic-Stojanovic & de Vos, 2014).

Following the present studies, the administration of various antimicrobial groups such as lincosamides, aminopenicillins, fluoroquinolones, and third generation cephalosporins into the gut models culminated in simulated CDI, reflecting prior in vivo clinical observation (Freeman et al., 2003). Furthermore, *L. casei* Shirota dosage impacted positively on the gut microbial community confirming earlier studies.
however, long term colonization of the gut by LcS was absent. Similarly, a daily administration of a high dose of LcS failed to prevent *C. difficile* germination, outgrowth, and cytotoxin production in the gut model, although a decline in potentially pathogenic Enterobacteriaceae and Enterococci populations were observed. Although the gut model is limited by an absence of immunological responses, it is unlikely that immunomodulation may confer a more prolonged probiotic effect given studies involving human subjects (Saxelin et al., 1993; Alander et al. 1999) have also reported the short term colonisation and temporary probiotic benefits of *Lactobacillus* species. Nevertheless, the role of host immune system in preventing CDI is widely reported, especially in recurrent CDI and the inability of the gut model to mirror secretory and immunological events is acknowledged here.

The failure of LcS to prevent *C. difficile* germination, outgrowth, and cytotoxin production in the gut model was initially thought to be due to the gut model growth media, as the production of substances such as acetic and butyric acid that are potentially inhibitory to *C. difficile* is largely dependent on constituents of the growth media in which they are cultivated (Tomás et al., 2002; Avonts et al., 2004; Zalán et al., 2005). However, using the gut model growth media, Macfarlane and colleagues demonstrated substantially high amounts of organic acid production in vessel 1 of the gut model, thereby fuelling speculations that such inhibitory effect of organic acids produced by lactobacilli may be concentration-dependent, such that the resultant concentrations of the organic acids in the vessels 2 and 3 in the present study were below a concentration that may exert an antimicrobial effect on *C. difficile*. Large double-blind design studies are required to demonstrate the wider significance of these observations in terms of the capacity of LcS to prevent CDI.

In the present series of experiments, *C. difficile* remained quiescent as spores prior to antimicrobial instillation. This internal control period of spore dormancy is an indication that the unperturbed gut microbiota may have a role in preventing *C. difficile* germination, growth, and toxin production. Although declines in the members of the microbiota due to antimicrobials did not correlate with the timing and extent of *C. difficile* germination and toxin production. Cefotaxime and ampicillin exerted less deleterious effects than ciprofloxacin and clindamycin upon the gut microbiota enumerated, but induced *C. difficile* germination and toxin production in a shorter time. Similarly, ciprofloxacin and
clindamycin had very profound deleterious effects on the microbiota enumerated, but differed in the maximum cytotoxin titres produced by *C. difficile* following their administration. Furthermore, in the control experiments, the absence or presence of a particular bacterial group showed no relationship with *C. difficile* germination, growth, and toxin production. This reinforces suggestions that the relationship between *C. difficile* and the indigenous microbiota may be more complex than simple colonisation resistance, and that other factors may contribute. Although it could just be that the particular members of the indigenous microbiota responsible for colonisation resistance were not cultured and enumerated in the present studies.

Antimicrobials were dosed into the gut models to mirror *in vivo* faecal/biliary levels, as well as the fluctuation of antimicrobial concentrations in the colon. An evaluation of *C. difficile* germination profiles of individual antimicrobial experiments in the present studies in conjunction with previous gut model data pertaining to antimicrobial induction of CDI (Freeman *et al.*, 2003; Baines *et al.*, 2008; 2013; Chilton *et al.*, 2014; Moura *et al.*, 2019) indicated that *C. difficile* germination only occurred when antimicrobial levels declined to below the MIC for the *C. difficile* strain. This was particularly evident upon clindamycin administration, when germination of the NTCD with a MIC of >128 mg/L occurred on the fourth day of clindamycin instillation in the control model, whereas RT027 with a MIC of 2 mg/L required additional 13 days to germinate in the control model. Similar patterns were observed albeit less pronounced with the other antimicrobials. This might present further evidence to support the role of sub-MIC antimicrobial concentrations in promoting *C. difficile* spore germination and subsequent cytoxin production, in addition to the importance of *C. difficile* susceptibility or resistance to the antimicrobial, rather than the susceptibility of the microbiota. It may also explain why some antimicrobials carry more risk to induce CDI than others. This might be due to the MIC of the treatment antimicrobial to the infecting strain. If the concentration of antimicrobial does not decrease below the MIC before spores are eliminated (washed out), then symptomatic disease cannot occur. It was earlier suggested that the decline in antimicrobial concentrations may be a removal of an inhibitory barrier to germination rather than a direct stimulus for germination (Freeman *et al.*, 2005). The present study favours the model in which sub-MIC antimicrobial concentrations induce stress in *C. difficile*, leading
to an improved adherence and utilisation of the new niche created by the depletion of bacterial groups by antimicrobials in the gut models. It is likely that NTCD produced higher amounts of such surface adhesins in response to the sub-MIC concentrations of antimicrobials used in the present studies leading to a competitive advantage.

The present studies consistently instilled approximately $10^8$ *C. difficile* spores into the gut models. This is likely higher than the number of spores patients are exposed to per time in the environment. The precise infective dose of *C. difficile* in humans remains to be ascertained. This is crucial as the inoculum size may influence the progression of colonisation to CDI. A smaller inoculum is more likely to be washed out of the gut by peristalsis before antimicrobial concentrations decrease to below inhibitory levels. Similarly, the antimicrobial concentrations achieved in the gut may also have a role in symptomatic disease. Antimicrobials present in lower gut concentrations might decrease to sub-inhibitory levels before *C. difficile* spores are washed out of the gut by peristalsis. Further gut model studies using varying inoculum sizes are required.

Given the concentrations of a particular antimicrobial in a population of individuals might differ substantially (Wilcox *et al.*, 2001), and the results from the present studies reinforce evidence that antimicrobial levels are crucial in dictating *C. difficile* germination and cytotoxin production. It therefore appears that such individual factors may impact on the capacity of *C. difficile* to germinate and produce cytotoxin *in vivo*. This may in part explain why some individuals do not experience symptomatic CDI despite treatment with a high predisposing antimicrobial.

This work is limited because variations in microbial populations were investigated using bacterial culture. This limits the investigation to only culturable bacterial groups, as bacterial populations such as *Clostridium leptum* and *Prevotella* spp. that do not have a selective agar cannot be studied using bacterial culture despite their importance in a healthy microbiome (Ben-Amor *et al.*, 2005; Hayashi *et al.*, 2005; Louie *et al.*, 2012). It is also a huge burden on personnel time as anaerobic bacterial culture plates require up to 48 h of incubation for colony growth, which may delay interventions. Molecular techniques such as real-time PCR can be employed to study gut microbiota variations quantitatively and in a lesser turnaround time (Bartosch *et al.*, 2004; Louie *et al.*, 2012; Thorpe *et al.*, 2018). However
it is pertinent to state that toxin production and spore counts cannot be quantified by molecular methods such as qPCR. For example the presence of toxin gene(s) may be determined by PCR, but such a molecular method may not provide adequate information on the actual amounts of toxins produced per time. Similarly current understanding of gut microbiota depletion by antimicrobials can be improved by employing 16S sequencing (Hayashi et al., 2005; Kelly et al., 2016). However, given bacterial groups differ in the number of 16S gene copies encoded on their genomes, such variations determined by 16S sequencing are not quantitative but qualitative. Besides by detecting DNA, molecular methods such as qPCR cannot differentiate between non-viable and viable cells. In spite of the longer turnaround time associated with culture-based methods, it represents cell viability and thus provides information on the most abundant viable bacterial population at a given time in the gut model. This permits for a better understanding of active metabolic pathways and their likely relevance in disease. Besides, following a study that compared methods for the direct enumeration of bacterial species using the in vitro human gut model, Moura et al., (2020) observed a > 85% correlation between direct culture and qPCR for a majority of the bacterial populations investigated. Nonetheless, by employing bacterial culture, the present series of experiments were able to monitor the different phases of C. difficile life cycle by distinguishing vegetative cells and spores which cannot be achieved by molecular techniques such as qPCR.
4.7 Future work

This study reinforced the gut model as a useful tool in which the interplay between the gut microbiota, antimicrobials, and C. difficile can be effectively studied. Even though minor differences in the impact of antimicrobials on C. difficile and the gut microbiota was observed between repeat experiments, the general reproducibility of events within the gut model was obvious. Given the gut model appeared reflective of in vivo observations with regards to the impact of antimicrobials on the gut microbiota and C. difficile, as well as the capability of NTCD to interfere with a cycle of CDI, it may be employed in future studies to examine the potency of novel interventions to prevent and/or treat CDI.

Despite results from the present study as well as data from animal models and phase two clinical trials (Zhang et al., 2015; Gerding et al., 2015) indicating that NTCD is able to colonise the colon and significantly reduce CDI without adverse effects in the human trials, the likelihood that NTCD strains could obtain toxin genes from toxigenic C. difficile in vivo remains a concern given an in vitro transfer of the pathogenicity locus to NTCD has been successful in the laboratory (Brouwer et al., 2013). NTCD strain isolated from the gut models after the experiments using antimicrobial-embedded selective agar was grown in brain heart infusion broth (BHIB) and tested using Vero cell cytotoxicity assay. No toxin production by the NTCD was observed. Similarly, although the present study acknowledges the low frequency of PaLoc transfer events (Brouwer et al., 2013), the NTCD strain (RT010) used in the present study retained its antimicrobial susceptibility profiles after the study, suggesting a non-receipt of antimicrobial resistance genes from toxigenic C. difficile or components of the faecal microbiota.

Nonetheless, Riedel et al (2017) reported that functional binary toxin-associated genes were encoded by a C. difficile bacteriophage genome. In a like manner, Ramirez-Vargas et al., (2018) demonstrated that C. difficile strains carried tcdB toxins on extrachromosomal elements, suggesting the transfer of toxin genes from toxigenic C. difficile to NTCD is more likely than previously thought. This raises questions about the distribution of C. difficile toxin loci and the pathogenicity mechanisms of this pathogen, in addition to reinforcing the notion that the epidemiology of this bacterium is not completely understood. Furthermore, a C. difficile isolate HMX-149 was associated with lower hamster mortality figures and a lower cell cytotoxicity than a NAP1 strain despite considerably producing more TcdB in
vitro (Ramirez-Vargas et al., 2018). This is in contrast to the widely propagated notion that hypervirulence is linked with tcdB overproduction (Warny et al., 2005), although in vivo toxin measurements are needed to confirm it. This controversy raises concerns that necessitate a continued surveillance on C. difficile using comprehensive genomic approaches.

Nonetheless, gut model studies in addition to rigorous genetic analysis are required to investigate whether NTCD (RT010) can be converted into a toxigenic strain by receiving a C. difficile pathogenicity locus. This is in addition to studies to evaluate the capacity of NTCD to interfere with already established CDI. Furthermore, optimising NTCD spore dose to prevent development of CDI in the gut models will contribute to strategies aimed at preventing CDI.
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