Gut microbiota interactions with *Clostridioides difficile* and the antimicrobials used to treat *C. difficile* infection

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Submitted in accordance with the requirements for the degree of

Master of Philosophy

The University of Leeds

Leeds Institute of Medical Research

Faculty of Medicine

November 2022

The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Acknowledgements

Having 8 years ago excitedly embarked on a PhD to then making the difficult decision to transfer to an MPhil, for the sake of my health, has been a rollercoaster ride, to say the least. I am indebted to so many wonderful people for helping me through the highs and lows this experience has brought.

First and foremost, I would like to express my deepest gratitude to my supervisory team. Dr. Caroline Chilton, your unwavering patience, support and guidance through this unconventional academic trajectory has been invaluable. Your passion and enthusiasm for the subject was inspiring and integral to the development of the work presented in this thesis. Dr. Jane Freeman, your encouragement and sage advice helped me through some of the most difficult points in this journey, along with Dr. Chilton, I may not have got to the finish line without you. Prof. Mark Wilcox, your guidance and honest feedback were critical to my academic development and thesis production. It has been a pleasure working with you all.

Dr. Charmaine Normington and Duncan Ewin, thank you for coming to my rescue with laboratory assistance when my body was working against me! I must extend my deepest thanks again to Dr. Normington for always making time for tea to listen to my caffeine-fueled ramblings, and for supporting me through the thesis writing process. Special thanks to the HCAI team for accommodating my special needs in the lab and to Dr. Kerrie Davies for your advice in negotiating the ethics process.

My wonderful friends Dr. Thomas Mathis, Dr. Tina Davies and Dr. Lucy Prodgers; thank you all for your advice and for supporting me through a very hard academic decision.

Lastly, none of this would have been possible without my amazing husband, Ash, who has kept this ship afloat at every stage of the journey.

Abstract

Prior antibiotic use is the highest risk factor for acquiring *Clostridioides difficile* infection (CDI), a leading cause of infective diarrhoea, due to its deleterious effect on resident gut microbiota and consequential loss of colonisation resistance. Antimicrobial CDI treatment is paradoxical since this can further impair the microbiota, increasing risk of recurrent infection. This thesis explores interactions between the gut microbiota and *C. difficile,* and the underexplored interactions between the faecal microbiota and anti-*C. difficile* agents.

In Chapter 1, a batch-culture colonisation resistance assay was refashioned comparing *C. difficile* growth, germination and sporulation in raw and heat-sterilised *in vitro* gut model samples. Colonisation resistance was achieved in raw samples seeded with vegetative *C. difficile* (TVCs reduced by 6 $\log_{10}$CFU/mL after 48 hours) and lost in sterilised (no reduction of TVCs), as expected. Unexpectedly, colonisation resistance remained in sterilised samples seeded with *C. difficile* spores.

In Chapter 2, *C. difficile* interactions with potentially inhibitory gut microbiota species were investigated when incubated in co-culture. Of the select organisms only *Bifidobacterium* and *Lactobacillus* species reduced the growth of *C. difficile* by 1.4 and 2.2 $\log_{10}$CFU/mL after 48 hours, respectively. However, the assay was limited in its range of microbiota-*C. difficile* interactions, as key species known to inhibit *C. difficile in vivo,* had no effect in co-culture.

In Chapter 3, the effect of faeces on antimicrobial bioactivity was measured by antimicrobial bioassay. Considerable decreases of fidaxomicin (74.7%), vancomycin (60.5%) and metronidazole (99.9%) bioactivity after 24 hours in raw faeces were observed. No decrease was seen in metronidazole in sterilised faeces, suggesting microbiota involvement, which may partly account for the poor clinical efficacy of this antimicrobial in CDI treatment.

This thesis highlights the utility of simple *in vitro* methods to investigate gut microbiota and *C. difficile* interactions and explore faecal microbiota effects on antimicrobial concentrations.

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Abbreviations

Alc	Alcohol
ANOVA	Analysis of Variance
BHI	Brain Heart Infusion
CA-CDI	Community Aquired *C. difficile* Infection
CBA	Columbia Blood Agar
CCEYL	Cycloerine Cefoxitine Egg Yolk Lysozme
CDI	*C. difficile* Infection
CDT	Biniary Toxin
CFU	Colony Forming Unit
CHO	Carbohydrate
CRACs	Colonisation Resistance Assay for *C. difficile* in Stool
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EPEC	Enteropathogenic *E. coli*
ESBL	Extended-Spectrum Beta_Lactamase
F	Fidaxomicin
FAPO	Food and Agriculture Organization of the United Nations
FMT	Faecal Microbiota Transplantation
GIT	Gastrointestinal Tract
GM	Geometric Mean
GMM	Gut Model Growth Media
GMS	Gut Model Samples
GTPases	Guanosine Triphosphatases
HBSS	Hanks Balanced Salt Solution
HIO	Human Intestinal Organoids
HPLC	High Performance Liquid Chromatography
IDSA	Infectious Diseases Society of America
Kcal	Kilocalorie
LLOD	Lower Limit of Detection
M	Metronidazole
MAbs	Monoclonal Antibodies
MALDI-TOF	Matrix-Assisted Laser Desorption Ionisation Time of Flight
MIC	Minimum Inhibitory Concentration
mRNA	Messenger Ribonucleic Acid

NAP1	North American Pulsed-field type 1
NHS	National Health Service
NSP	Non-Starch Polysaccaride
PaLoc	Pathogenicity Locus
PBS	Phosphate Buffered Solution
PCR	Polymerase Chain Reaction
PMC	Pseudomembranous Colitis
PPI	Proton Pump Inhibitor
Prot	Protein
rCDI	Recurrent *C. difficile* Infection
RCT	Randomised Clinical Trial
REA	Restriction Endonuclease Analysis
rRNA	Ribosomal RNA
RU	Relative Units
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SCC	*C. difficile* Supernatant Containing Cytotoxin
SHEA	Society for Healthcare Epidemiology of America
Spp.	Species
STEC	Shiga-Toxin-Producing *E. coli*
tdcA	Gene responsible for toxin A production
tdcB	Gene responsible for toxin B production
TVC	Total Viable Count
V	Vancomycin
WHO	World Health Organisation

Introduction

1.1 Clostridioides difficile

1.1.1 History and Clinical Manifestations

Clostridioides difficile is a Gram positive, strictly anaerobic, spore forming bacillus. It was first isolated in 1935 from the colonic microbiota of a breast-fed neonate and originally named *Bacillus difficilis* in recognition of the difficulty in isolating and studying the bacteria [1]. Further investigation revealed different *B. difficilis* strains, although morphologically and culturally similar, had varying pathogenicity when cultures or filtrates were injected subcutaneously into animal models [2]. However, no pathogenic effects were seen when the cultures or filtrate were ingested by healthy animals. In 1938, *B. difficilis* was reclassified to the genus *Clostridium* and *C. difficile* was the adopted nomenclature until 2016 when, based on phenotypic, chemotaxonomic and phylogenetic profiles separate from other *Clostridium* spp., it was transferred to the novel genus *Clostridioides* [3].

C. difficile was not identified as an aetiological agent of antibiotic-associated diarrhoea until the 1970s, following the discovery of faecal cytotoxin presence in cases of antibiotic-induced pseudomembranous colitis (PMC) [4, 5]. During the 80s and 90s, the development of enzyme immunoassays for cytotoxins enabled early diagnosis [6-8], and *C. difficile* infection (CDI) was regarded as a treatable complication of broad-spectrum antibiotic use within hospitals; usually affecting the elderly [9, 10]. *C. difficile* is now considered the most common cause of infectious diarrhoea in the nosocomial setting, and since the beginning of the 21st century, worldwide incidence and severity have increased, as have the number of deaths attributed to CDI [11]. Previously thought low-risk groups - such as younger adults without antibiotic history - have also been affected, community-acquired CDI (CA-CDI) is more prevalent and recurrent infection has become increasingly difficult to treat [12].

CDI is defined by the occurrence of symptoms or colonoscopic evidence of colonic inflammation in combination with detection of stool toxigenic *C. difficile* or toxin [13]. It can result in a wide spectrum of health states from mild self-limited diarrhoea to pseudomembranous colitis (PMC) leading to life-threatening toxic megacolon [14]. The latter is characterised by pain, abdominal distention, pyrexia, marked leucocytosis and raised C-reactive protein, and can require a colectomy to save the life of the patient [15]. Stool *C. difficile* or toxin detection in the absence of diarrhoea, colonoscopic or histopathogenic findings is generally classed as asymptomatic carriage [16].

CDI continues to place a major clinical and economic burden on healthcare facilities, with the most recent figures suggesting around 23 cases per 100 000 per year [17], and treatment costs per patient estimated at £12,710 for a first episode, and £31,121 for recurrent CDI (rCDI) over 28 days [18].

1.1.2 Epidemiology

In the UK the incidence of CDI has been reported voluntarily since 1990 when it was regarded primarily as a nosocomial pathogen. In 2004 mandatory surveillance of > 65-year-old patients was introduced for acute trusts in England in response to the emergence of a new, more virulent strain, known as North American pulsed-field type 1 (NAP1), restriction endonuclease analysis (REA) type BI or PCR ribotype 027 strain (NAP1/B1/027) [19]. This strain had previously caused a rapid rise in incidence and deaths attributable to *C. difficile* in North America in the early 2000s [20]. The first known outbreak of 027 in the UK occurred in 2003/2004 at the Stoke-Mandeville hospital which resulted in 174 cases and 19 deaths [21]. Outbreaks followed in the Netherlands and Belgium in 2005, and France and southern Europe thereafter [19]. As well as increased incidence, an increase in severity was also associated with the 027 strain [22]. In 2007 mandatory surveillance in the UK was extended to all inpatients >2 years, allowing for more comprehensive monitoring and exploration of

patient risk factors, as well as tracking the patterns of strains involved in infection. The advent of increased surveillance corresponded with a dramatic decline of 75.9% in total reported CDI cases per year between 2007/08 (55,498 cases) and 2013/14 (13,361 cases) [17]. Antibiotic stewardship, with particular emphasis on the restriction of fluoroquinolone prescribing in the UK, coincided with a reduction of CDIs attributable to 027 [23], and 001 [24], since 2008. However, since 2014 CDI has continued to burden the NHS with no significant decline in yearly incidence, and 12,503 cases were reported in the UK between April 2020 and March 2021 [17]. The current pattern of strains causing CDI are more complicated compared to 15 years ago when 027 dominated, with a more heterogeneous distribution of ribotypes [24]. New identification techniques available have shown that a significant number of cases are not linked, suggesting that patient-to-patient spread in healthcare facilities is not the only mode of transmission [24]. Although there is increasing awareness of CDI not associated with hospital admission, it has been suggested that half of CA-CDI cases may go undetected, especially in demographics previously considered low-risk, likely due to a lack of clinical suspicion in patients <65 years [25].

1.1.3 *C. difficile* Reservoirs

CDI patients can shed spores for 6 weeks after antibiotic treatment and resolution of diarrhoea, facilitating community transmission and the chance of recurrent infection [26]. *C. difficile* spores have also been isolated from domesticated pets [27], livestock [28], meat products for human consumption [29-31], and soil and vegetables [32, 33]. Although *C. difficile* has been isolated from these sources, the relevance of these reservoirs to CA-CDI in humans is still unknown.

1.1.4 Pathogenesis

CDI results from a disruption of the normal colonic microbiota, which allows colonisation with opportunistic *C. difficile*, and the subsequent release of toxins that cause inflammation and damage. Transmission occurs via the faecal-oral route with

the ingestion of highly resistant spores able to bypass gastric acid [34]. *C. difficile* spores are ubiquitous in the environment and are highly resistant to extremes of temperature and some disinfectants [35, 36]. The shedding of spores from CDI patients, and contamination of hospital surfaces [37], mediates the transmission of *C. difficile* spores attributing to the high nosocomial dissemination [38-40].

The development of CDI is then dependent on *C. difficile* spores encountering an intestinal environment conducive to germination and colonisation. Spore germination mainly occurs in the ileum, when primary bile acid germinants are present at high levels and the pH has increased sufficiently [41]. After germination, vegetative *C. difficile* colonisation and outgrowth can occur in the colon when there has been a disturbance in the resident microbiota [42], most often due to antibiotic-induced dysbiosis [43]. Colonic colonisation is facilitated by various virulence factors such as fibronectin-binding proteins, surface layer and cell wall proteins, and flagella [44, 45].

CDI symptoms are the result of the ability of certain strains to produce toxins. The most widely researched *C. difficile* toxins are toxin A, and B. Both toxins are cytotoxic, eliciting the destruction of the actin cytoskeleton through the glucosylation of guanosine triphosphatases (GTPases). This results in increased epithelial permeability, fluid secretion and the release of pro-inflammatory cytokines, promoting an influx of inflammatory cells, and adding to the intestinal inflammation [46]. The genes responsible for toxin production, *tcdA* and *tcdB*, are located on the *C. difficile* pathogenicity locus, PaLoc. Variations in PaLoc are used to differentiate *C. difficile* variant strains into ‘toxinotypes’ [47]. Non-toxigenic *C. difficile* either do not contain the PaLoc genes, present a modified PaLoc unable to produce toxin A or B, or produce such a low amount of toxin their cytotoxic activity is undetectable [48].

The relative importance of these toxins in the pathogenesis of *C. difficile* has been debated. Earlier papers suggested virulence was attributed to toxin A, as administration of purified toxin A alone to murine and leporine models elicited a more

toxic response than toxin B [49, 50]. However, increasing numbers of *tcdA*$^{-}$ *tcdB*$^{+}$ strains have been isolated from patients with severe CDI [51], and further animal studies have shown hamsters infected with either knockout *tcdA*/*tcdB* gene mutants succumbed to CDI with either toxin alone [46, 52]. Highlighting that both toxins should be considered in the virulence of *C. difficile* and that the natural infection process is important to elucidate the role of toxins in pathogenesis. PaLoc also contains genes that regulate toxin expression, *tcdR* (positive regulatory), *tcdC* (negative regulator), and the gene encoding a membrane lysis protein, *tcdE* [53]. The importance of these genes in the virulence of *C. difficile* strains is still under review.

Some *C. difficile* strains, notably 027 and 078, also produce a binary toxin (CDT) [54]. CDT is an actin-specific ADP-ribosultransferase, encoded by two genes, *cdtA* and *cdtB*, located outside the PaLoc, which transcribe an enzymatic portion (CDTa) and a component responsible for membrane binding (CDTb) [55]. Although the role of CDT in the pathogenesis of *C. difficile* is still not fully understood, its presence in clinical isolates is associated with increased disease recurrence and mortality [56].

The strict anaerobic nature of *C. difficile* renders it incapable of surviving in aerobic environments in its vegetative form, therefore the pathogenesis of CDI is reliant on its metabolically dormant, aero-tolerant spore morphotype as the main vehicle of infection, persistence, transmission and recurrence [57]. *C. difficile* spores consist of a partially dehydrated core containing supercoiled DNA, encapsulated by an inner membrane, a germ cell wall, and the spore cortex, encased in a thick spore coat [58, 59]. These features make the spore resistant to antibiotics [60], disinfectants [61], alcohol [62], host immune system [63], as well as heat and radiation [64]. Some strains possess an outer exosporium layer, thought to aid adhesion [65]. The exact signals required to initiate the sporulation process in vegetative *C. difficile* cells are still unknown, however, studies of spore-forming *Bacillus* and *Clostridium* species have demonstrated low nutrient availability and high cell density are essential for sporulation [66, 67]. Sporulation-deficient *C. difficile* strains are incapable of

persisting in the environment or the gastrointestinal tract (GIT), highlighting the integral role of the spore CDI [68].

1.1.5 Risk Factors

Prior antibiotic exposure is the main risk factor for an initial CDI episode [69-71]. Different classes of antibiotics have differential risks for CDI, most likely due to the varying levels of disturbance to the host's gut microbiota [72] combined with *C. difficile* strain variation in antibiotic susceptibility [73]. Antibiotics associated with a higher predisposition to CDI include ampicillin, amoxicillin, cephalosporins [74], clindamycin and fluoroquinolones [75-77]. Clinical guidelines now recommend considering the restriction of fluoroquinolones, clindamycin and cephalosporins [13]. In contrast, a meta-analysis concluded tetracyclines to be lower risk, [78] possibly due to a shorter period of disruption to the gut microflora [79], alongside low *C. difficile* resistance rates [80]. Cumulative antibiotic exposure also appears to be associated with an increased risk of CDI [81]

Advanced age is considered another significant risk factor for CDI [82-84]. Decreases in microbiota diversity alongside an inadequate immune response with increasing age [85] may account for this observation, however, other factors such as frailty and comorbidities resulting in increased exposure to antibiotics and longer-term stays in healthcare environments may also increase the risk of CDI in this age group [86]

Other risk factors include gastrointestinal (GI) surgery, inflammatory bowel disease, proton pump inhibitor (PPI) use, immunosuppression and immunodeficiency [13, 83, 87-90].

1.1.6 Recurrent CDI

Recurrent CDI (rCDI) is most commonly described as a resurgence of CDI occurring within 2 to 8 weeks of the previous episode [13]. However, the validity of this definition is still under review as it may be too short a window to expose the true extent of recurrent infections [91]. Approximately 25% of patients who initially

respond to antimicrobial treatment encounter rCDI [92, 93] with the risk of further recurrence increasing with each subsequent episode (Fig 1.) [94, 95], placing a major burden on patients and healthcare services. Many factors have been implicated in the incidence of rCDI, including prolonged changes in the indigenous gut microbiota populations caused by antimicrobial therapy [96], an ineffective host humoral immune response with insufficient antibody production against *C. difficile* toxins [97-99], as well as the presence of particular *C. difficile* ribotypes associated with recurrence [100, 101]. The latter has been documented for the ribotype 027 and it has been hypothesised that this could be due to earlier and more extensive sporulation in this isolate, thus increasing the potential number of spores with which to cause a relapse [102].

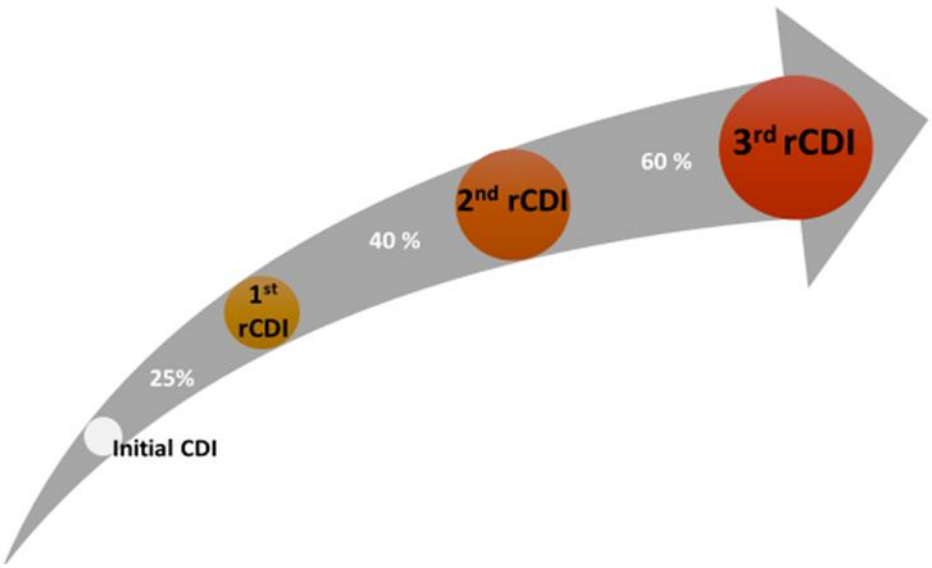

Fig 1. The CDI recurrence escalator. The risk of recurrence increases with each subsequent episode of rCDI.

rCDI can be subdivided into two contexts: relapse of the initial infection from spores persisting in the gut, or reinfection with spores from the environment. Distinguishing between the two is challenging as PCR ribotyping may not be able to discriminate between reinfection with a genotypically similar isolate [103], a factor further complicated by some patients hosting multiple distinct *C. difficile* genotypes [104].

1.2 Treatment of CDI

1.2.1 Antimicrobials

Antibiotics continue to be the standard treatment for CDI. Historically, metronidazole was recommended as first-line therapy for mild CDI, with vancomycin reserved for severe cases and those that did not respond to metronidazole [105]. However, clinical guidelines now recommend vancomycin or fidaxomicin over metronidazole for first-line therapy since evidence revealed metronidazole is inferior to vancomycin on an intent-to-treat basis [106, 107], and fidaxomicin is similar to vancomycin for initial clinical cure and superior in preventing recurrent disease [108]. Although metronidazole is no longer recommended as a first-line treatment [13, 109], it is still commonly prescribed [110], possibly due to its low cost, availability and lack of prescriber awareness, most likely in primary care for CA-CDI. Fidaxomicin, the most recently licenced treatment for CDI, was initially found to be more active against *C. difficile in vitro* than vancomycin [111, 112]. Clinically, fidaxomicin has demonstrated non-inferiority to vancomycin but has the advantage of reducing recurrent disease incidence [108]. Fidaxomicin is a macrolytic narrow-spectrum antibiotic, which has shown reduced microbiota disturbances compared to vancomycin. It is thought that this sparing of gut microbiota populations, particularly of the *Bacteroides* and *Prevotella* genera, may be responsible for the decreased rates of recurrence [113]. The high cost of fidaxomicin compared to the alternatives has historically limited its use as a first-line treatment [114], however, despite the higher acquisition costs, reduced recurrence incidence may result in lower incremental costs [115, 116], especially for subgroups of patients at higher risk of severe or complicated CDI [117]. Previous guidelines recommended the prescription of the same antimicrobial agent for rCDI episodes [118]. Updated guidelines now suggest a tapered/pulsed vancomycin regimen for rCDI [13] based on fairly weak evidence [95], or standard fidaxomicin therapy [93]. One clinical study has also shown an extended-pulsed fidaxomicin regime to be superior to vancomycin for rCDI [119]. Other antimicrobial

agents demonstrating an antagonistic effect on *C. difficile* and undergoing clinical evaluation for CDI treatment include teicoplanin [120], rifaximin [121], ridinilazole [122], nitazoxanide [123], ramoplanin and rifampin [124]. Although some of these agents have shown promise, a lack of large clinical trials and the potential for antimicrobial resistance currently hinders their use as first-line therapy.

The treatment of CDI with antimicrobials is somewhat paradoxical, as antibiotic exposure is the main risk factor for CDI [69, 70], and an altered colonic microbiota caused by antibiotics as an important pathophysiological factor for rCDI has been hypothesised [96]. The success of alternative therapies that restore gut microbiota diversity, and thus colonisation-resistance, such as faecal microbiota transplantation (FMT) have been the mainstay of this hypothesis [125-128].

1.2.2 Antimicrobial Alternatives and Adjuvants

1.2.2.1 Faecal Microbiota Transplantation (FMT)

FMT involves the transplantation of minimally processed faeces from a healthy donor to a patient with the intention of restoring the gut microbial populations required to prevent the re-establishment of CDI after antibiotic therapy. FMT was first documented in the 1950s as a treatment for pseudomembranous colitis [129] and since the 1980s has been perceived as a promising treatment for rCDI as indicated by retrospective case reports [130]. The first randomised clinical trial (RCT) assessing FMT efficacy compared to standard treatments was carried out in 2013 [126]. The FECAL study randomly assigned 42 patients to either; FMT via nasogastric (NG) tube with bowel lavage, a standard vancomycin regimen, or a standard vancomycin regimen with bowel lavage. The primary outcome measure was cure without relapse within 10 weeks of the administration of therapy. Resolution occurred in 81% of the FMT patients compared to 31% receiving vancomycin alone, and 23% vancomycin with bowel lavage [126]. The study was terminated after an interim analysis due to a high number of control group patients relapsing. These patients were offered FMT off

protocol. Although this was a landmark paper with encouraging results, it was open-label, had a relatively low sample number and did not disclose details of the randomisation process. Furthermore the study may not have run for long enough to see the full picture of relapse, and did not include a tapered vancomycin arm with which to compare. Another RCT in 2016, a double-blind trial, compared FMT using a donor stool (n=22) with autologous stool (n=24) administered via colonoscopy [131]. Interestingly, resolution occurred in 91% of the donor FMT patients and 63% of those treated with their own stool. The reasons why treatment with autologous stool could be curative for rCDI are unclear. However, as rCDI cure rates with autologous FMT in this study were similar to treatment with vancomycin [95, 132], and faecal microbiota analyses from the study showed a greater abundance of *Clostridia* before autologous FMT in many of the successfully treated patients [131], this raises the possibility that those successfully treated were already hosting a gut microbial community inhibitory to further CDI.Conversely, another RCT comparing FMT via a single enema to a tapered vancomycin regimen reported FMT to be inferior to antibiotic therapy in treating rCDI [133].

These studies highlight that different modes of FMT delivery, control treatment, and treatment used for the initial CDI may affect the efficacy outcome of FMT. Several systematic reviews have indicated the clinical effectiveness of FMT to vary with the instillation method [130, 134, 135], with the most comprehensive review to date concluding lower GI endoscopy is superior to all other delivery methods [134].

The Infectious Diseases Society of America/Society for Healthcare Epidemiology of America (IDSA/SHEA) guidelines now recommend FMT for multiple rCDI [136] as FMT is associated with increased microbial restoration of inhibitory interactions of commensal gut bacteria on *C. difficile* and lower incidence of rCDI [126, 134, 137-139]. However, there are safety concerns with transferring a minimally processed stool from donor to patient. Serious adverse events have been reported, including death, after the transfer of extended-spectrum beta-lactamase-producing (ESBL)

Escherichia coli [140]. Further reports of patients infected with enteropathic *E. coli* (EPEC) and Shiga-toxin-producing *E.coli* (STEC) following FMT have also been declared [141-143]. Although mandatory FMT screening guidelines could potentially reduce adverse events, they would not address the issue of emerging infectious agents, such as SARS-CoV-2, which some studies suggest can be shed in stools for days [144, 145]. Current screening guidelines also cannot identify agents with the potential to cause long-term side effects or diseases possibly linked with the gut microbiome, such as Parkinson's disease [146], obesity [147], and various autoimmune diseases [148-150], which are poorly understood and yet to be established.

1.2.2.2 Probiotics

The term 'probiotic', is historically described by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organisation (WHO) as "*live microorganisms which when administered in adequate amounts confer a health benefit on the host*" [151] is somewhat controversial and often misused, especially in reference to consumer products [152]. The exploitation of the term in the food industry led to a review of the definition of what constitutes a probiotic by an international panel in 2014 [153], who added that "*evidence of health benefit is required for a probiotic.*"

Human studies investigating the impact of single, or select multiple probiotic species on the prevention of primary CDI in at-risk patients have had limited success or inconsistencies in CDI diagnostic measures [154-156]. The largest randomised, double-blind, placebo-controlled study to date investigating a mixed probiotic of *Bifidobacterium* and *Lactobacillus* spp. in the prevention of primary CDI in elderly patients was unclear as too low a number of CDI cases occurred in both the control and test cohorts [157]. Clinical studies assessing the treatment of primary or rCDI after antimicrobial therapy with single or select multiple strain probiotics are scarce, owing to limited knowledge and consensus on the beneficial underlying mechanisms

of particular species and strains. One study assessing a probiotic formulation of 12 species, including *Lactobacillus, Bifidobacterium and Saccharomyces* spp. in a kefir drink alongside a tapered antibiotic regime reported resolution of symptoms in 84% of patients in the treatment group following a diagnosis of rCDI [158]. However, the study was carried out on a small, somewhat homogenous cohort, and the results have not yet been reproduced in a larger multi-site study. Furthermore, murine models of CDI treated with the same formulation did not demonstrate the same level of efficacy [159].

Although FMT after antibiotic therapy has demonstrated that restoration of the gut microbiota is associated with increased clinical response [126] it remains an invasive option for the prevention and/or treatment of rCDI and carries the potential risk of transmission of other opportunistic pathogens [140, 141]. More recently, an oral microbiome therapeutic composed of approximately 50 species of purified Firmicutes spores from screened healthy stool samples, SER 109, has been developed and was shown to be superior to placebo in reducing the risk of rCDI up to 8 weeks after antibiotic treatment in a phase 3 trial [160]. Spore-forming Firmicutes are ideal candidates for an oral probiotic mix as they can resist residual antibiotics that disrupt other gut microbiota species and remain viable after passage through the stomach. They may also compete with *C. difficile* for nutrients and/or metabolise key *C. difficile* germinants [161], as well as potentially possessing a range of host-protective and microbiota modulating effects, such as those mentioned previously. Although an open-label trial of SER 109 is underway, additional investigations at species level will also be required to elucidate the functional pathways and interactions these, and other gut bacteria species, have with each other as there may be wider-reaching effects beyond the treatment of CDI.

1.2.2.3 Monoclonal Antibodies

Direct neutralisation of toxins A and B with human monoclonal antibodies (MAbs) is a different approach for the potential treatment of rCDI. Actoxumab and bezlotoxumab

are two such MAbs with the ability to bind and neutralise toxins A and B, respectively [162]. Phase III trials have shown only bezlotoxumab is associated with significantly lower rates of rCDI alongside antimicrobial therapy compared to placebo [163] and is now an approved adjunctive therapy for patients receiving antimicrobial treatment for CDI, at higher risk of recurrent infection [13].

1.3 Gut microbiota

1.3.1 Overview

The gut microbiota is the collective term for the commensal, symbiotic and pathogenic bacteria, fungi, viruses and parasites helminths indigenous to the intestinal tract [164]. The dominant phyla of the human intestinal microbiota are Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Tenericutes [165]. Although specific gut microbiota composition is highly variable between individuals and can fluctuate over time within individuals, studies have indicated the existence of core genes and key functions, which persist long-term in the absence of perturbation. A critical aspect of conferring this functional stability is a highly diverse microbiota. Microbial diversity results in increased functional redundancy, therefore functionality of the microbiota may be more important than the exact composition [166, 167]. A rich and diverse gut microbiota confers a range of physiological functions to the host such as strengthening gut integrity [168], nutrient metabolism [169, 170], drug metabolism [171], immunomodulation [172] and providing colonisation resistance against pathogens invasion.

1.3.2 Influences on Microbiota Composition

The composition and functionality of the gut microbiota varies considerably between locations in the GIT, between hosts, and within individuals throughout their life. The composition and function of the gut microbiota are shaped by many factors, including - but not limited to – age [173, 174], mode of birth [175], genetics [176, 177] gender

[178], health status and medication [179], geographical origin and diet [180], and antibiotics.

Host age has a significant impact on gut microbiota composition and function. The neonatal microbiota is simple and hypervariable and has shown to be highly dependent on external factors such as the route of delivery and modality of feeding [175, 181]. Taxonomic diversity increases into childhood and adolescence [182-185], but may still be compositionally and functionally distinct from an adult microbiota [185]. The elderly microbiota has been characterised by a decline in diversity compared to younger adults [186]. This may be due to a combination of lifestyle and physiological changes such as reduced dietary variety [187], decreased gastrointestinal motility [188], and increased gastric atrophy [189].

Genetics have been shown to have a limited role in shaping an individual's gut microbiota and environmental factors such as diet are thought to have a stronger influence [190]. Studies have demonstrated the manipulation of long- and short-term diet can alter colonic microbial profiles [191-193]. Although dramatic short-term dietary changes can alter microbial composition quickly they are transient and have not shown to persist over time [193]. Habitual long-term diet patterns have been shown to correlate with microbiota composition [191], with balanced diets that are high in plant-based foods associated with increased microbial diversity [194, 195].

Antibiotic therapy induces the most drastic perturbation to gut microbiota composition, density and diversity. The degree of impact is dependent on the target spectrum of the antibiotic, route of administration, dose and duration, as well as on the original resident microbial populations [196, 197]. Faecal microbiota data has shown that even very short courses of antibiotics can cause significant disruption [197], however, the disturbances in response to antibiotic therapy are individual-specific, with pre-antibiotic composition significantly determining the impact [198, 199].

Loss of colonisation resistance and increased susceptibility to pathogens such as *C. difficile* is the most significant threat of gut microbiota alteration after antibiotic treatment yet the understanding of what changes are clinically meaningful is still in its infancy.

1.3.3 Colonisation resistance

Colonisation resistance was first described in the 1970s following observations that antibiotic disruption to the indigenous microbiota of mice coincided with colonisation with orally introduced *Escherichia coli.* Colonisation resistance was re-established when antibiotic treatment ceased [200]. A rich and diverse gut microbiota is thought to confer colonisation resistance, protecting the host from endogenous opportunistic and exogenous pathogens, including *C. difficile*. However, the underlying mechanisms by which colonisation resistance is conferred are complex and not fully understood. Multiple interrelated functions have been suggested including but not limited to, direct inhibition, competition for nutrients and space, and the regulation and stimulation of host immunity.

Studies have demonstrated *C. scindens* can directly inhibit *C. difficile* infection by converting primary bile acids required for *C. difficile* spore germination into secondary bile acids [43, 161]. Direct inhibition can also occur via the production of antimicrobial peptides such as bacteriocins, which can selectively inhibit growth and kill competing bacteria [201, 202]. More general studies have found that resident gut microbial communities under homeostatic conditions compete for nutrients and space, with exogenous bacteria, such as *C. difficile* unlikely to find unchallenged niches and obtainable substrates [203, 204]. The gut microbiota has also been found to regulate the immune response by promoting differentiation of T cells [205] and stimulating innate immune receptors promoting the expression of bactericidal lectins by the intestinal epithelial cells [206].

Studies on the individual roles of microbiota constituents in colonisation resistance are complicated by the practical constraints that hinder the isolation and characterisation of all bacterial species. The multiple mechanisms of microbiota-mediated colonisation resistance are diverse and can function simultaneously, which further complicates the evaluation of the relative importance of individual pathways in this environment.

1.3.4 Microbiota dysbiosis

Gut microbial imbalances characterised by an inability to function optimally are often referred to as ‘dysbiosis’ [148, 207, 208]. Microbiota dysbiosis is a nebulous term as there is significant variability between individuals at both the genus and species level. Bacterial communities in the gut are unique to the host and dependent on multiple factors described previously and there is currently no consensus on which imbalances constitute a ‘dysbiotic’ microbiota. This can be problematic for clarity in CDI-microbiota research.

1.4 Models for Studying the Gut Microbiota, CDI, and CDI antimicrobials

1.4.1 *In vivo* models

As previously described, animal models have an important role in facilitating the study of host-pathogen interactions, host-drug interactions as well as host-microbiome interactions. In contrast to human clinical studies where patients are often receiving a cocktail of therapies and other confounding issues making it difficult to delineate direct associations, animal studies provide an opportunity to standardise host and treatment variables. Animals used for studies are typically from inbred stock, thus reducing genetic variability, and can be microbially manipulated to harbour microbiome profiles of interest. Several animal species have been used to study CDI but the most common species to feature in studies are mice and hamsters [4, 209-

211]. Murine and hamster models have been used to demonstrate colonisation resistance of the gut microbiota - and the loss of under antibiotic challenge - to CDI [212-214]. Gnotobiotic mice have allowed for a range of standardised microbiota profiles to study, from germ-free and monoexenic animals to highly diverse profiles using a donor [215-217], however the underdeveloped immune system in gnotobiotic mice is not reflective of normal conditions of humans or animals and results must be interpreted with caution [218, 219]. The main limitations of animal models are the distinct pathophysiological differences compared to humans. For example, in hamsters the site of infection is within the caecum [220]. CDI in hamsters is also rapidly fatal [221], which is inconsistent with human disease and limits the duration of experiments, which prevents them from being used for rCDI studies. Cross-contamination arising from conditions the animals live in, or behavioural traits, such as coprophagia, can also compromise study validity and reproducibility.

There are ethical and moral considerations to be made for animal models, especially since interspecies differences can make extrapolating results relative to humans difficult.

1.4.2 *In vitro* models

1.4.2.1 Batch Culture

Batch culture models are inexpensive and require minimal specialised equipment. They offer considerable flexibility over experimental parameters, allow for a greater number of biological replicates over other methods and are relatively quick to perform. Borriello and Barclay [222] described an *in vitro* batch culture colonisation resistance model that determined *C. difficile* growth and toxin production was inhibited in the presence of faeces from healthy volunteers but not faeces from patients receiving antibiotics. They also demonstrated the inhibitory effect was lost when faeces were sterilised by heat and filtration, thus attributing colonisation resistance to a ‘healthy’ microbiome. This batch culture colonisation resistance model

[222] was later assessed for its predictive value by comparing an *in vivo* antibiotic-challenged hamster model with an *in vitro* batch culture model using the cecal contents of antibiotic-treated hamsters [213, 223]. The *in vivo* and *in vitro* results were closely correlated. Batch culture models have also been used to assess the effect *C. difficile* has on 'healthy' and 'dysbiotic' faecal microbiotas [224], interactions with selective strains of bacteria with *C. difficile* [225], and the activity of novel *C. difficile* therapeutics [226].

Although batch culture methods are limited by reliance on stool donations, they cannot reflect the behaviour of bacterial species *in vivo,* and are subject to short experimental duration due to the depletion of nutrients and accumulation of waste products, they are a useful tool to perform high-throughput initial assays focusing on specific C*. difficile* – microbiota – therapeutic interactions. The results of which can be used to base more sophisticated research protocols.

1.4.2.2 Continuous Culture Systems

Continuous culture systems offer *in vitro* assay growth dynamics that are more reflective of the gut than batch culture and enable controlled interventions and monitoring over a longer period. This has allowed for longer-term evaluation of factors involved in CDI development, including the exploration of faecal microbiota components that may be responsible for colonisation resistance [227, 228], expanding on those in animal and batch culture studies [212-214, 229]. Wilson *et al.* [230] demonstrated the degree of *C. difficile* suppression by hamster caecal microbiota seeded in a single-stage continuous culture was similar to the same cecal microbiota seeded into germ-free mice. Further investigation using the same model protocol was used to investigate nutrient competition between the microbiota and *C. difficile*. However, the use of hamster-derived microbiota limited its relevance to human microbiotas [228]. Another study using a single-stage system addressing this limitation reported *C. difficile* was more strongly inhibited by models seeded with *C. difficile* negative human infant faeces than *C. difficile* positive faeces [227]. The same

study noted there was a greater diversity of species in the *C. difficile* negative faeces compared to positive, and inhibition of *C. difficile* may be due to competition for amino acids [227]. However, the single-stage system used was not pH controlled and the decrease in *C. difficile* growth seen may have been confounded by the pH decrease.

Although single-stage continuous models are suitable for longer-term studies than batch culture, they are still limited in reflecting the full spectrum of environmental conditions in the distinct anatomical regions of the colon. Two-stage continuous systems have been used to examine colonisation resistance, with an emphasis on changing microbial populations and function by stimulating bifidobacterial growth with non-digestible oligosaccharides [231]. Two-stage models have the advantage over single-stage by offering seeded bacteria conditions of nutrient excess, such as those in the proximal colon, and nutrient limitation, such as those in the distal colon.

The triple-stage human gut model first described by MacFarlane [232], consisting of three vessels connected in a weir cascade top fed with growth media at a set flow rate, was designed to replicate the spatial, temporal, nutritional and physiochemical environment of the proximal, transverse and distal colon. It has been validated by the colonic contents of sudden death victims [232] when seeded with a pooled faecal slurry from healthy donors and allowed to equilibrate over 14 days.

The microbiota within the triple-stage gut model has been shown to exhibit colonisation resistance against CDI during its steady state cycle [233] and has been used extensively to evaluate microbial populations and *C. difficile* response under various simulated gut conditions and treatment regimes [234-238]. Antimicobials can be instilled into the model reflective of clinical dosing regimes, and depending on experimental aims, the model can be run for months, facilitating daily measurements of microbial populations and *C. difficile* in response to interventions [234, 239, 240].

Although the human gut model cannot consider host factors such as immunological functions, intestinal secretions or absorption of metabolites, it does provide a useful

and validated tool for investigating the gut microbiota and it's interactions with *C. difficile* and antimicrobials. Standard protocols for equipment, methodology and monitoring of continuous models make them more reproducible than animal models. However, they are more labour-intensive and expensive than batch culture and require specialist equipment and technical experience. These factors reduce the number of biological replicates per investigation.

1.4.2.3 Organoids

Human intestinal organoids (HIO) are 3D spheroids of human epithelium grown *in vitro* from either adult multipotent or induced pluripotent stem cells [241, 242] cultured in stromal replacement scaffolding resembling *in vivo* structures, which provide a modifiable structurally and functionally complex intestinal model system [243]. They have shown an ability to perform *in vivo* functions such as producing mucus, and secretory and absorptive processes [244], therefore offering the opportunity for host tissue function and therapeutic targets to be studied simultaneously, as well as host-pathogen interactions. Although HIOs cannot be co-cultured with obligate anaerobic microorganisms, microinjections of *C. difficile* directly into the lumen of an organoid have been found to persist for at least 12 hours [245], suggesting that they may be useful in the study of CDI. They have been used to study human inflammatory responses such as the effect of *C. difficile* on host mRNA expression [246] and to define a role for inflammasomes in CDI pathogenesis [247]. Pharmacological studies using HIO models of CDI have also been considered [248]. Whilst HIOs offer a distinct physiological advantage over other models to study specific host tissue-*C. difficile* interactions in isolation, co-cultures of complex microbial communities and organoid relationships are still technically challenging due to the divergent requirements regarding oxygen levels and nutrients.

1.4.3 Human Faeces

In vitro models, both batch culture and continous culture, are most often seeded with human faeces to represent gut microbiota populations. Considering faecal matter is an abundant resource, can be used as a reserve of disease biomarkers [249] and has a potential application as a therapeutic substance [131, 134, 250, 251], the overall composition of human faeces, beyond the microbial component, has not been extensively studied; but is known to vary widely between individuals and is strongly influenced by host diet [252-255] and health status [254].

Studies have demonstrated there are differences between faecal and colonic mucosal microbiota composition [256, 257]. Obtaining mucosal microbiota samples is laborious, invasive and requires technical skill, which limits accessibility for high numbers of biological replicates for research. Fortunately, although the faecal and mucosal microbiota may be structurally distinct microbial niches, they are highly correlated [257, 258] and therefore faeces serve as a pragmatic proxy for investigating gut microbiota interactions. Faeces can be easily and naturally collected without aid and do not require invasive procedures to procure them. In practice, however, poo taboo is very real and many producers of this valuable resource may be unwilling to participate.

1.5 Study Aims

This study aims to assess gut microbiota interactions with *C. difficile* and associated antimicrobials using simple batch culture methods.

1.5.1 Primary objectives

- Determine if samples taken from a triple-stage human gut model can be used in place of fresh faeces to replicate a batch culture colonisation resistance model by seeding raw and autoclaved gut model samples with *C. difficile.*
- Determine if batch co-culture of single gut microbiota species with *C. difficile* is suitable to assess the inhibitive potential of such species by measuring growth of gut microbiota species with and without *C. difficile*
- Determine whether faecal slurry affects the bioactivity of antimicrobials used to treat *C. difficile* in batch culture as measured by large plate antimicrobial bioassay

Chapter 1 Validation of a colonisation resistance model using samples from a triple-stage gut model

1.1 Background and Rationale

The colonisation of the gut by *C. difficile* is strongly associated with a depleted microbiota [72]. Investigating the importance of a disrupted gut microbiota and loss of colonisation resistance in the development of CDI via clinical trials, *in vitro* continuous culture models, and animal models are labour intensive, expensive, logistically challenging and have ethical implications [163, 239, 259, 260]. The concept of colonisation resistance has been demonstrated using simple *in vitro* batch culture assays observing a lack in vegetative *C. difficile* growth in 'healthy' faeces compared to heat sterilised faeces or faeces from individuals with a depleted microbiota [222, 224]. More recently a colonisation resistance assay for *C. difficile* in stool (CRACS) was optimised using *C. difficile* spores to investigate the changes in colonisation resistance following antibiotic exposure in healthy volunteers [261]. Germination of spores is an essential first step in pathogenesis prior to *C. difficile* outgrowth, colonisation and toxin production [262], and is, therefore, important to consider alongside vegetative *C. difficile* when designing colonisation resistance assays.

Although human stool samples serve as a useful proxy for investigating gut microbiota–*C. difficile* interactions, acquiring samples from volunteers can be difficult due to 'poo taboo'. Obtaining sequential samples, even from a willing single donor, at precise time points is logistically very difficult, and inflicting different disease states upon humans to investigate faecal samples is ethically and logistically problematic. The validated and clinically reflective continuous chemostat triple-stage human gut model primed with pooled human faeces offers an opportunity to overcome the limitations of procuring sequential faecal samples at different points and disease states without inconveniencing a patient [232]. Samples from the gut model are easy

to obtain at the exact time points of interest for immediate testing and the model can be subjected to interventions that disrupt the microbiota that mirror *in vivo* colonic conditions [263, 264].

Here, a colonisation resistance model was refashioned using fresh (raw) and autoclaved samples from multiple separate gut models to compare *C. difficile* growth, germination, sporulation, and cytotoxin production in;

- raw and heat-sterilised Gut Model Samples (GMS)
- raw GMS from different time points in the gut model cycle
- non-GMS controls consisting of a *C. difficile* growth media and gut model media

It is hypothesised that colonisation resistance assays using gut model samples will replicate those using faeces directly from the human source with colonisation resistance prevailing in undisrupted gut model samples and *C. difficile* proliferating in sterile or depleted gut model samples.

1.2 Materials and Methods

For full constituents for all agars used in this study please see Appendix A.1.1.

1.2.1 Vegetative *C. difficile* Preparation

Toxigenic *C. difficile* ribotypes 027and 001and non-toxigenic ribotype 010, were individually maintained on modified Brazier's Cycloerine Cefoxitine Egg Yolk Lysozyme (CCEYL - containing 5mg/L lysozyme and 2% lysed horse blood) agar (Oxoid, UK) (full constituents in Appendix A.1.1) at 37^0C anaerobically (Don Whitley A95 anaerobic workstation). All *C. difficile* strains used were clinical isolates obtained from the *C. difficile* ribotyping network (CDRN). Single colonies were inoculated into 5ml pre-reduced Brain Heart Infusion (BHI) broth (Oxoid, UK) and incubated anaerobically overnight at 37^0C. Overnight cultures were adjusted to an OD_{600} of 0.5±0.3 (Genesys 20 spectrophotometer, Thermo Scientific), in an attempt to standardise inoculum. Serial dilutions and plating of viable counts of this standardised inoculum at this density was shown to be equivelent to a ~7 $\log_{10}$CFU/mL concentration (data not shown). $\log_{10}$CFU/mL concentration (data not shown).

1.2.2 *C. difficile* Spore Preparation

C. difficile ribotypes 027, 001 and 010 were sub-cultured onto pre-reduced Columbia Blood Agar (CBA) (full constituents in Appendix A.1.1) plates and incubated anaerobically at 37^0C for 10 days. Growth was removed from the CBA plates with a sterile swab and emulsified in a 20mL sterile saline and ethanol solution (50% v/v). The suspension was incubated at ambient temperature for 1 hour before centrifugation at 16000g for 10 minutes. The supernatant was discarded and the pellet was re-suspended in sterile saline. Viable counts of the spore preparation were measured and then adjusted to a concentration of ~7 $\log_{10}$CFU/mL by diluting in saline.The spore preparation was further diluted when required to create various spore inoculum concentrations (as detailed throughout the methods and results).

1.2.3 Cytotoxicity/Neutralisation Assay Preparation

Vero cells (African Green Monkey Kidney Cells, ECACC 84113001) were cultured in 20mL Dulbecco's Modified Eagles Medium (DMEM) (Sigma) supplemented with newborn calf serum (50mL) (Gibco, Paisley, UK), antibiotic/antimycotic solution (5mL) (Sigma) and L-glutamine (5mL) (Sigma) in a flat bottom T75 tissue culture flask and incubated at 37°C in 5% CO_2 (Sanyo, Watford, UK). When confluent monolayers of Vero cells formed – confirmed by inverted light microscopy (Olympus UK Ltd, Middlesex, UK) – they were harvested by removal of DMEM and rinsing with 1ml of Hanks Balanced Salt Solution (HBSS) (Sigma) containing trypsin-EDTA (0.25g/ L) (Sigma). HBSS-EDTA (6mL) was added to the flask and incubated for 10 minutes at 37°C in 5% CO_2. Once the cells detached from the flask, further passage was achieved by diluting the HBSS-EDTA cell mixture (1:20) in DMEM in a 96F microtiter tray (Nunc). Vero cells were harvested (160 µl) and inoculated into wells to which antitoxin would later be added. To other wells trypsinised Vero cells (180 µL) were added. Trays were incubated for 2 days at 37°C at 5% CO_2 prior to assay and growth confluence examined microscopically before samples were added.

1.2.4 Control Test Medias

BHI broth was made with sterile water according to the manufacturer's instructions.

Gut Model Media (GMM) contained (g/L): peptone water (2.0), yeast extract (2.0), NaCl (0.1), K_2HPO_4 (0.04), KH_2PO_4 (0.04), $MgSO_47H_2O$ (0.01), $CaCl_22H_2O$ (0.01), $NaHCO_3$ (2.0), haemin (0.005), cysteine HCl (0.5), bile salts (0.5), arabinogalactan (1), pectin (2), starch (3). Vitamin K 10 µL/L and Tween 80 0.2% (v/v). Additions after autoclaving (g/L): glucose (0.4), resazurin anaerobic indicator (0.005).

1.2.5 *In vitro* gut model

The *in vitro* gut model is a triple-stage chemostat model consisting of three glass fermentation vessels (Soham Scientific, Ely, UK) connected in a weir cascade system (Fig. 1- 1) based on that of MacFarlane *et al.* [232]. Vessels 1 (280mL), 2 (300mL),

and 3 (300mL) were kept at 37˚C by a circulated heated water bath (Grant Instruments, Cambridgeshire, UK) connected to a jacketed system, and anaerobic by a continuous source of oxygen-free nitrogen (Parker Domnick Hunter, VWR, Leicestershire, UK). The pH level of each vessel was maintained at levels reflecting the increasing alkalinity of the gut; 5.5 (± 0.2), 6.2 (± 0.2), 6.8 (± 0.2) for vessels 1, 2 and 3 respectively. The pH was monitored by probes (P200 chemotrode, Hamilton, USA) and maintained using pH controllers (Brighton Systems, Sussex, UK) by the addition of 1 M solutions of NaOH and HCl (Fisher Scientific, Loughborough, UK), administered by a controller unit (Biosolo, Brighton Systems, UK). Excessive foaming and loss of vessel volume was prevented with the addition of 0.5mL of 10% (w/v) polyethylene glycol (Sigma). A peristaltic pump was used to feed gut model growth media (GMM) (as described above) through the Weir cascade at a flow rate of 13.2ml/h. Multiple gut models were used throughout these investigations to provide samples to generate biological replicates. The gut models were set-up and maintained by research technicians within the Healthcare Associated Infection Research Group (HCAI).

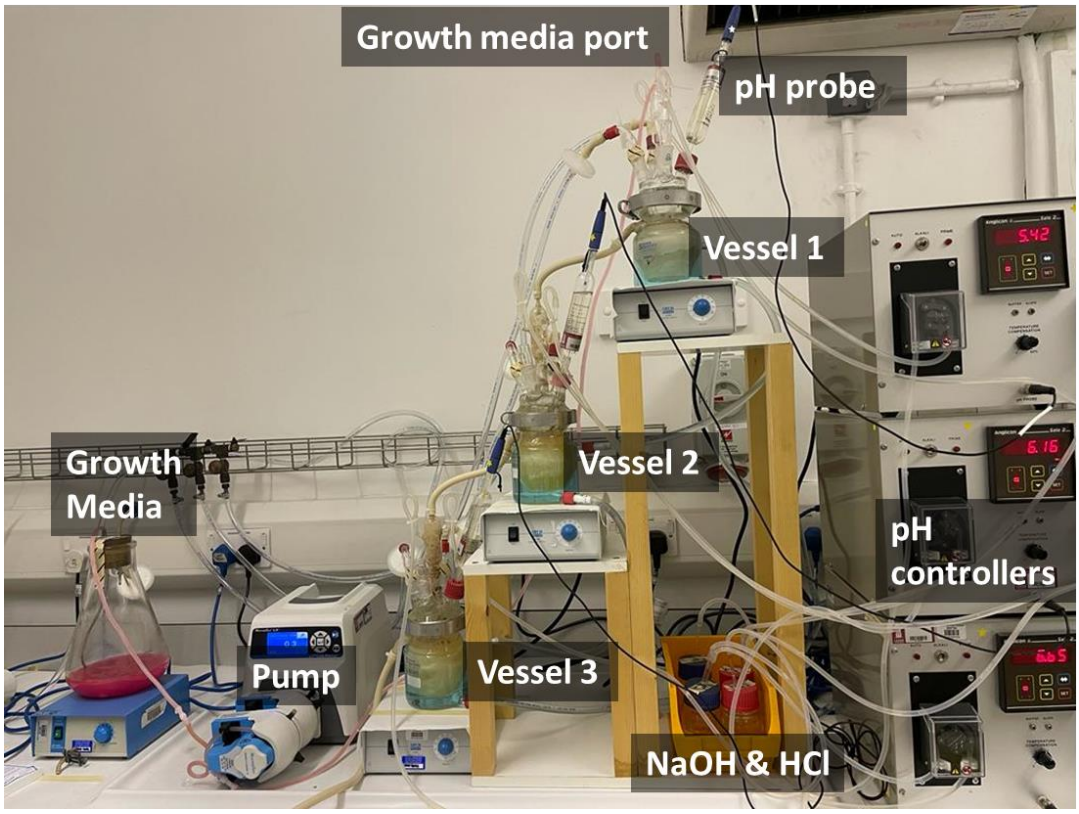

Fig. 1- 1 *In vitro* gut model. Three vessels are connected in a weir cascade, top-fed via a peristaltic pump with a complex growth media. A pH probe in each vessel is connected to a pH controller that automatically adjusts levels using NaOH and HCl.

1.2.6 Gut Model and Gut Model Sample (GMS) Preparation

Each gut model providing GMS for the batch culture assays was seeded with pooled faeces obtained from 3 healthy adult donors (no antibiotic history within 3 months of donation). Faeces were collected by the donors and transported in a sealable plastic pouch with an AneroGen™ Compact sachet (Oxoid, UK) within 12 hours for storage in an anaerobic cabinet. Each faecal donation was screened for *C. difficile* by plating on CCEYL in duplicate and anaerobically incubating for 48 hours. *C. difficile* positive faeces were discarded. Viable faeces were emulsified in pre-reduced phosphate buffer solution PBS (BioVision, USA) 10% w/v. The faecal slurry was homogenised in a stomacher (Stomacher Lab-Blender 400, Borolabs, Aldermaston, UK), and course-filtered through sterile muslin (Bigger Trading Limited, Watford, UK).

Faecal slurry (150mL) was added to each gut model vessel. GMM (prepared as described in section 1.2.4) was then added to vessel 1 to create a total volume of 280mL, after which GMM was pumped through the weir cascade system. The gut model was maintained in biologically reflective conditions as described above and in Freeman *et al.* (2003) [233].

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Fig. 1- 2 Gut model cycle. Samples were extracted at point A = 4 days steady state (GMS A); point B = 10 days steady state prior to inoculation of *C. difficile* spores (GMS B); and point C = 7 days post clindamycin treatment (GMS C); (Qds (four times daily))

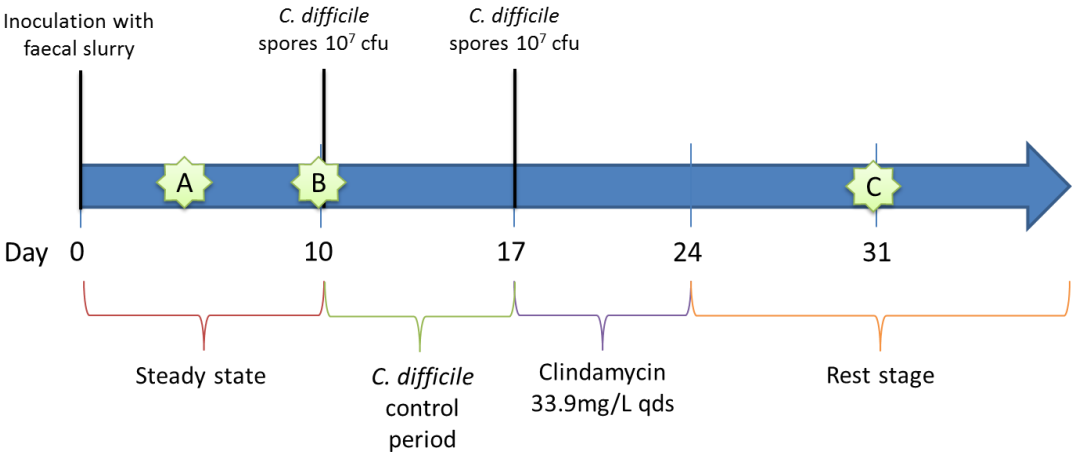

Fig. 1- 2 Gut model cycle. Samples were extracted at point A = 4 days steady state (GMS A); point B = 10 days steady state prior to inoculation of *C. difficile* spores (GMS B); and point C = 7 days post clindamycin treatment (GMS C); (Qds (four times daily))

The gut model cycle timeline and specific time points GMS was extracted are outlined in Fig. 1- 2. GMS was extracted from the third vessel of each gut model at: 4 days steady state (GMS A), 10 days steady state (GMS B) when full microbiota populations are intact and colonisation resistance would be expected (prior to the introduction of any *C. difficile* spores), and 31 days during the rest stage (GMS C). GMS taken at each point were divided into two portions; one raw sample placed directly into a Don Whitley A95 anaerobic workstation and another aliquoted 5ml in glass Wasserman's to be heat sterilised in an autoclave at 123ºC for 26 minutes and

immediately placed in anaerobic conditions. Autoclaved GMS was tested for sterility by plating onto CBA plates and incubating anaerobically and aerobically for 48 hours

1.2.7 Preliminary Experiment 1: C*. difficile* survival, sporulation and toxin production in different media types

BHI, GMM and raw and autoclaved GMS extracted from 2 separate gut models at steady state points A and B (Fig. 1- 2) (4.5ml) were inoculated individually with 0.5ml *C. difficile* preparations containing ~5 x 6 $\log_{10}$CFU 027, 001 or 010 vegetative cells or spores (prepared as outlined in sections 1.2.1 and 1.2.2), and incubated anaerobically at 37°C for 48 hours. GMS was taken from 2 separate gut models running simultaneously as biological duplicates.

Fig. 1- 2 Gut model cycle. Samples were extracted at point A = 4 days steady state (GMS A); point B = 10 days steady state prior to inoculation of *C. difficile* spores (GMS B); and point C = 7 days post clindamycin treatment (GMS C); (Qds (four times daily))

Samples were taken at 24 and 48 hours under anaerobic conditions and diluted 10-fold to 10^{-6} in pre-reduced peptone water. Each dilution was plated onto pre-reduced modified Brazier's CCEYL agar, in triplicate, and incubated at 37˚C, anaerobically for 48 hours before total viable counts (TVCs) were enumerated by eye. Spores were measured by treating a portion of each sample with an equal volume of 97% ethanol and incubating at room temperature for 1 hour. The alcohol shocked sample was serially diluted and plated as above. Plates were incubated anaerobically at 37°C for 48 hours prior to enumeration by eye.

Mean *C. difficile* TVC and spore counts in BHI, GMM and GMS (raw and autoclaved) were amalgamated from the individual 027, 001 and 010 as technical triplicates to increase heterogeneity. Mean GMS data was amalgamated from samples taken from two different gut models, at 2 separation extraction time points (BHI and GMM n=3, GMS n=12 (Fig. 1- 3)). Error bars represent the standard error (±SE) of the mean. Statistical analysis was performed on Microsoft Excel version 2202. A two-sample t-

test assuming unequal variances was employed to determine the significance of differences in log_{10}CFU/mL of *C. difficile* TVCs and spore counts between the various media. A statistical significance level of <0.05 was adopted, <0.01 very significant, and <0.001 highly significant.

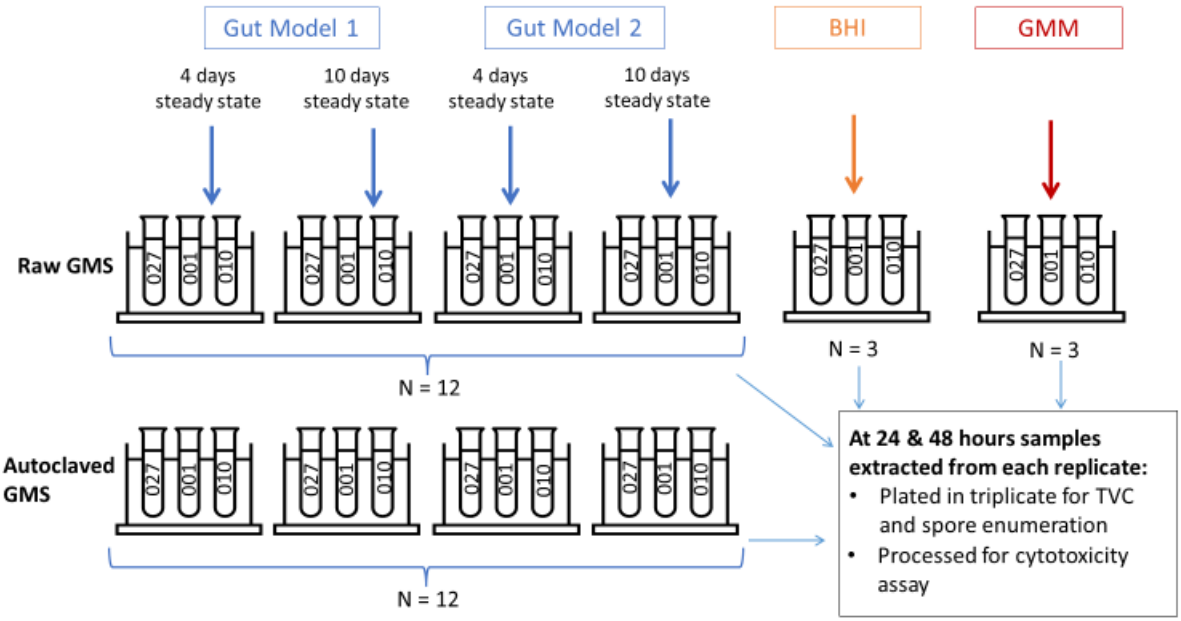

Fig. 1- 3 Preliminary Experiment 1 schematic overview of technical (*C. difficile* ribotypes 027, 001, 010) and biological replicates (GMS taken from 2 separate gut models at 2 different time points). Process carried out individually for vegetative *C. difficile* and *C. difficile* spore inoculation.

Cytotoxin titres were measured by the Vero cell cytotoxicity assay: samples taken at 24 and 48 hours were centrifuged at 16000g, the supernatant removed, and filtered through a 0.22-m filter (Millipore Corporation, Billerica, MA, USA). The supernatant was serially diluted 10-fold to 10^{-7} in PBS. Each dilution (20µL) was added in triplicate at an initial 1:10 dilution to a 96-well microtitre tray containing Vero cell culture monolayers and serially diluted to 10^{-6} (prepared as described in section 1.2.3 and shown visually in Fig. 1- 4). *Clostridium sordellii* antitoxin (Prolab Diagnostics, Bromborough, UK) diluted 1 in 10 in PBS (inoculated into the tray 1:10 with a final concentration of 1:100 v/v) was used to neutralise any cytotoxic effect caused by *C. difficile* toxins. A positive control using the supernatant of *C. difficile* 027

210 grown in BHI broth for 48 hours anaerobically at 37°C was also applied to each tray. Cell growth and appearance were assessed using inverted microscopy at 24 and 48 hours. Trays were then incubated at 37°C in 5% CO_2. Assays were examined after 24 and 48 hours under an inverted microscope. Positive reactions were indicated by ≥80 % cell rounding and confirmed by parallel neutralisation with *sordellii* antitoxin to ensure cell rounding was not caused by non-specific toxins found in the gut model fluid. Toxin titres are expressed in relative units (RU); a positive reaction in the neat dilution was assigned 1 RU, 10^{-1} dilution = 2 RU, 10^{-2} dilution = 3 RU, and so on.

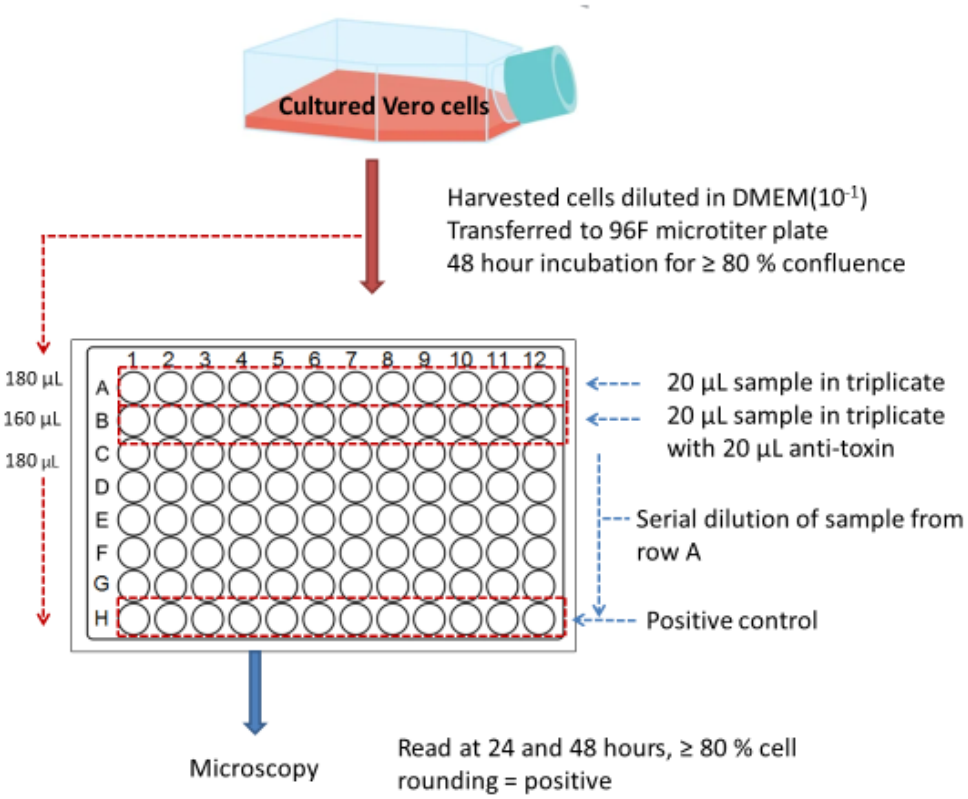

Fig. 1- 4 Cytotoxicity/Neutralisation assay preparation graphical representation

1.2.8 Preliminary Experiment 2: *C. difficile* survival, sporulation and toxin production in GMS from different stages of the gut model cycle

GMS (4.5ml) extracted at points A, B and C (Fig. 1- 2) from 2 separate gut models (biological duplicates) were seeded with 0.5ml *C. difficile* preparation containing ~5 x

6 log_{10}CFU ribotypes 001, 010 or 027 (technical triplicates) vegetative cells (prepared as outlined in section 1.2.1) and incubated at 37˚C anaerobically for 48 hours. As *C. difficile* had been added to the gut models between points B and C a control sample of GMS C was tested for residual *C. difficile* TVCs and spores (quantities detailed in results). TVCs, spore counts and toxin titres were established as described in section 1.2.7 after 24 and 48 hours. Mean *C. difficile* TVC and spore counts in raw GMS extracted from different stages of the gut model cycle were amalgamated from 3 individual vegetative *C. difficile* ribotypes (technical triplicates) and two different gut models (GMS A n=6, GMS B n=6, GMS C n=6 (Fig. 1- 5)).

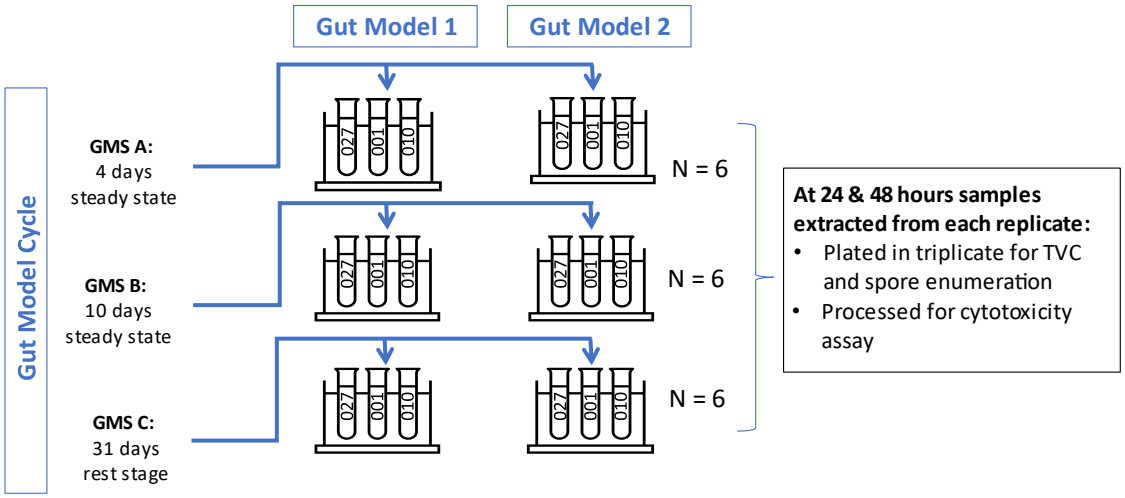

Fig. 1- 5 Preliminary Experiment 2 schematic overview of technical (*C. difficile* ribotypes 027, 001, 010) and biological replicates (2 gut models) at each extraction time point in the gut model cycle.

1.2.9 Colonisation Resistance Assay 1 – Seeded with 027 Vegetative and Spore Preparations

Raw and autoclaved GMS (4.5ml) extracted from the gut model at point B (10 days steady state) from 3 individual gut models (biological replicates) were separately inoculated with 0.5ml *C. difficile* preparations containing ~5 x 7 log_{10}CFU ribotype 027 spores or vegetative cells (prepared as described previously) and incubated

anaerobically at 37°C for 48 hours. TVCs and spore counts were enumerated at 0 and 48 hours as described above (Raw and autoclaved GMS at both time points n=3).

1.2.10 Colonisation Resistance Assay 2 – Seeded with 027 Spore Preparations of Varying Concentrations

Raw and autoclaved GMS B from 3 individual gut models (biological replicates) were separately inoculated with *C. difficile* 027 spore preparations of varying concentrations: 6, 5, 4 and 3 $\log_{10}$CFU/mL (prepared as described above). TVCs and spore counts were enumerated at 0 and 48 hours as described previously (Raw and autoclaved GMS at both time points n=3).

1.2.11 Statistical Analysis for Colonisation Resistance Assays 1 and 2

Data are presented as the calculated mean TVCs and spore counts from *C. difficile* incubation in GMS B from 3 individual gut models. Error bars represent the standard error (±SE) of the mean. Statistical analysis was performed on Microsoft Excel version 2202. As the sample size was small apaired t-test was employed to determine the significance of differences in $\log_{10}$CFU/mL of *C. difficile* TVCs and spore counts in raw and autoclaved GMS between 0 and 48 hours. A statistical significance level of <0.05 was adopted, <0.01 very significant, and <0.001 highly significant.

Results

1.2.12 Preliminary Experiment 1: Comparison *C. difficile* Survival and Toxin Production in Different Media Types

Each sample was inoculated with ~5 x 6 log_{10}CFU spores or vegetative cells to give expected TVCs and spore counts of approximately 6 log_{10}CFU/mL at 0 hours. Cytotoxin titres were averaged from the two toxigenic strains, exclusively.

The highest number of *C. difficile* TVCs was observed in BHI compared to other media inoculated with vegetative cells (~9 log_{10}CFU/mL after 24 hours). After 48 hours TVCs in BHI were still ~4 log_{10}CFU/mL higher than both the raw and sterilised GMS ($p<0.001$) (Fig. 1- 6). Similar TVCs and spore counts were seen from BHI seeded with *C. difficile* spores (Fig. 1- 7) to that inoculated with vegetative cells, which may indicate germination of spore cells. However, without a confirmatory 0 hour reading the extent of true germination is unknown. The highest cytotoxin RUs were observed in BHI seeded with both vegetative and spore ribotypes 001 and 027 (Fig. 1- 6 and Fig. 1- 7), with RUs increasing by 1 between 24 and 48 hours.

GMM supported less initial survival than BHI inoculated with vegetative *C. difficile* with TVCs attaining ~7 log_{10}CFU/mL after 24 hours. After 48 hours TVCs remained stable and ~4 log_{10}CFU/mL higher than both raw and sterilised GMS ($p<0.001$) (Fig. 1- 6). Cytotoxin titres from GMM seeded with vegetative *C. difficile* were lower than BHI but still increased by 0.5RU between 24 and 48 hours. GMM supported more germination and growth when seeded with *C. difficile* spores than any other media, with TVCs reaching 9 log_{10}CFU/mL and spore counts of ~3 log_{10}CFU/mL after 24 hours (Fig. 1- 7). However, after 48 hours TVCs decreased by ~3 log_{10}CFU/mL, whereas spores counts and cytotoxin titres remained stable, suggesting considerable cell death.

In raw GMS inoculated with vegetative *C. difficile,* cell death and sporulation occurred after 24 hours as indicated by almost equal TVCs and spore counts at ~4

log_{10}CFU/mL. Vegetative *C .difficle* TVCs in autoclaved GMS were similar to raw at 24 hours, however, spore counts were almost 1 log_{10}CFU/mL lower. Raw and autoclaved GMS inoculated with spore cells seemingly remained as spores with TVCs and spore numbers fairly similar at 24 and 48 hours; ~5 – 5.5 log_{10}CFU/mL in raw, and ~ 6.5 log_{10}CFU/mL in autoclaved. GMS inoculated with spores were slightly higher than that inoculated with vegetative cells, with approximately ~2 log_{10}CFU/mL difference. Toxin titres from all GMS states were negative reflecting the lack of growth or germination.

Minimal differences in growth and germination were observed between the 3 ribotypes used for this preliminary study (Appendix A.1.2). Mean toxin results were calculated from ribotypes 001 and 027 only as 010 is a non-toxigenic strain.

The error bars suggest a lot of variation, which could be indicative of sampling from the gut model at various time points and/or multiple ribotypes used for this preliminary data. The missing time point of 0 hours also limits the ability to fully see the extent of growth, germination and sporulation and thus true vegatitive inocula for assays seeded with vegetative cells. These limitations were accounted for in colonisation resistance assays 1 and 2.

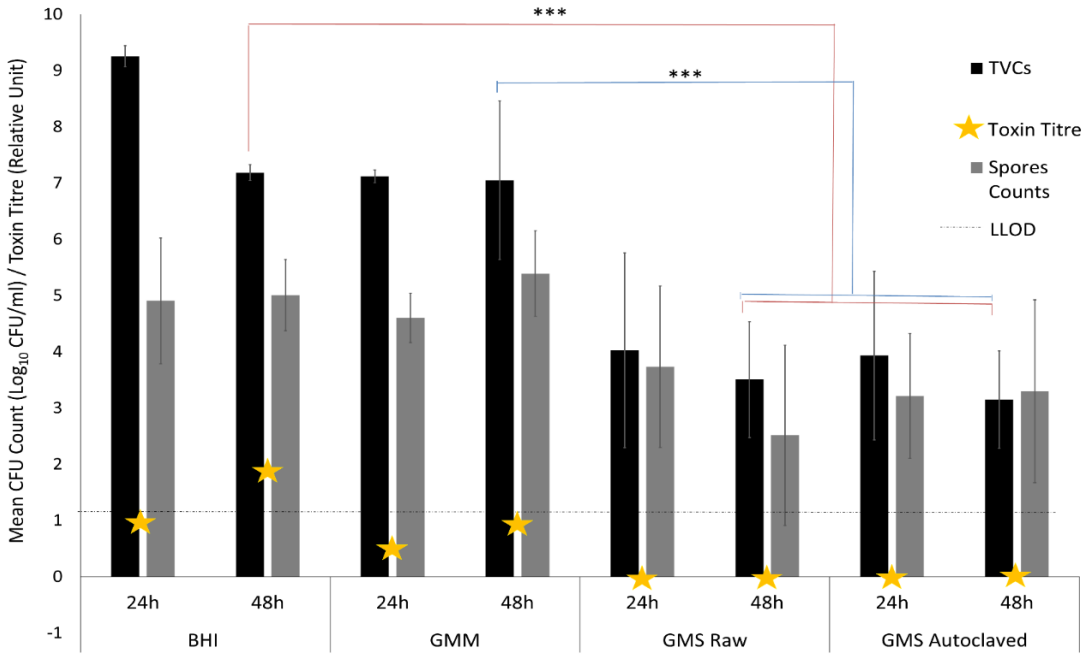

Fig. 1- 6 Preliminary Experiment 1: Mean (±SE) TVCs and spore counts of vegetative *C. difficile* seeded in BHI, GMM, raw and autoclaved GMS after 24 and 48 hour anaerobic incubation. BHI and GMM mean of vegetative *C. difficile* ribotypes 001, 010, 027 as technical triplicates (n=3). GMS mean of technical triplicates and biological duplicates at 4 and 10 day steady state extractions (GMS A and B) (n=12). Highly significant results are highlighted by * (p<0.001). Starting inoculum ~6 log_{10}CFU/ml at 0 hours. Mean toxin titre RUs from ribotypes 001 and 027 only. LLOD = Lower Limit of Detection of TVCs and spores.**

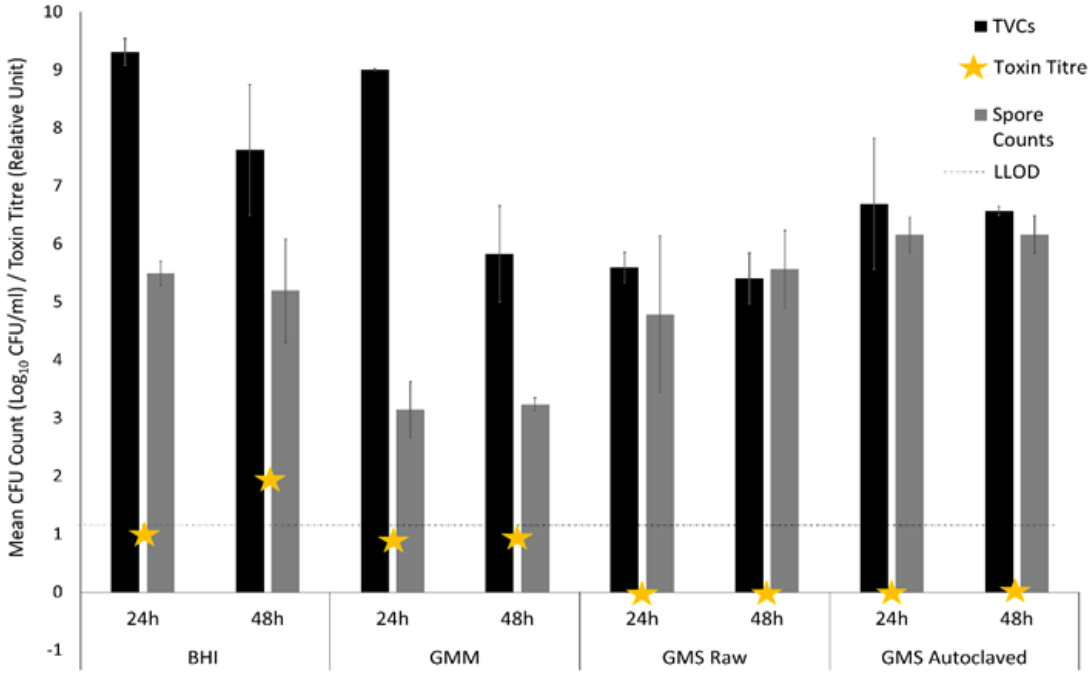

Fig. 1- 7 Preliminary Experiment 1: Mean (±SE) TVCs and spore counts of *C. difficile* spores seeded in BHI, GMM, GMS A and B (4 and 10 days steady state) raw and autoclaved after 24 and 48 hours anaerobic incubation. BHI and GMM mean of *C. difficile* spore ribotypes 001, 010, 027 as technical triplicates (n=3). GMS mean of biological duplicates at both steady state points (GMS A and B) and technical triplicates (n=12). Starting inoculum approximately 6log_{10}CFU/ml at 0 hours. Mean toxin titre RUs from ribotypes 001 and 027 only. LLOD = Lower Limit of Detection of TVCs and spores

1.2.13 Preliminary Experiment 2: Comparison of *C. difficile* survival and Toxin Production at Different Points in the Gut Model Cycle

Although *C. difficile* TVCs were highest in GMS A (4 days steady state) and lowest in GMS B (10 days steady state), growth of *C. difficile* was not observed in any of the samples. With TVCs of at least 6 $\log_{10}$CFU/mL expected to be achieved at 0 hours, GMS A did not support the proliferation of *C. difficile* growth after 24 hours as TVCs only achieved ~5.5 $\log_{10}$CFU/mL and decreased by a further ~1.5 $\log_{10}$CFU/mL after 48 hours, whilst spores increased by ~0.5 $\log_{10}$CFU/mL (Fig. 1- 8)

Spurious counts were observed from GMS B, with spores outnumbering TVCs by 2 $\log_{10}$CFU/mL at 24 hours with large error bars. As TVCs equal the total number of viable organisms (vegetative cells plus spores) this is most likely indicative of experimental error. Spores can clump together and without vigorous agitation, unequal numbers of spores can be plated. At 48 hours spores were still higher than TVCs but closer in range (Fig. 1- 8).

At GMS C TVCs and spores ~3.5 $\log_{10}$CFU/mL at both time points (Fig. 1- 8). As *C. difficile* was added to the gut models before extraction point C a control sample was taken to account for residual *C. difficile.* The GMS C control indicated TVCs of 2.5 $\log_{10}$CFU/mL and spore counts of 2.4 $\log_{10}$CFU/mL (data not shown).

Unfortunately, GMS seeded with spore cells were not available at each GMS time point to compare with vegetative inoculation.

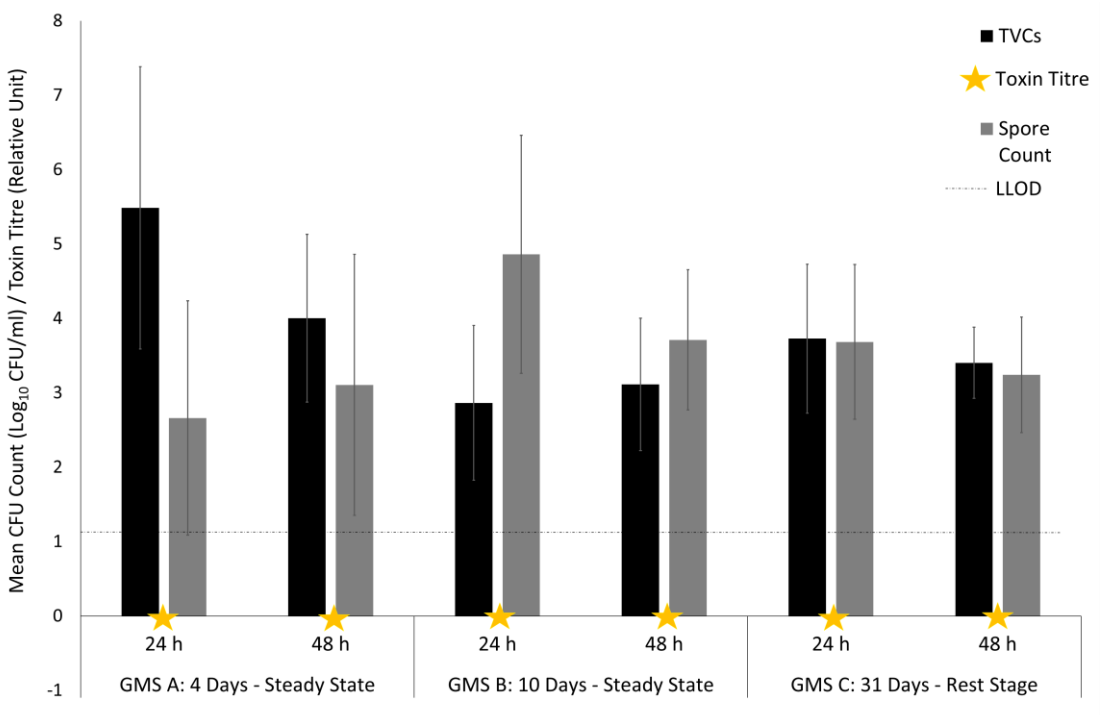

Fig. 1- 8 Preliminary Experiment 2: Mean (±SE) TVCs, spore counts and cytotoxin titres at 24 and 48 hours incubation of vegetative *C. difficile* seeded in raw GMS taken from various stages in the gut model cycle. Mean calculated from biological duplicates and technical triplicates made up of 3 *C. difficile* ribotypes (n=6). Starting inoculum approximately 6log10CFU/ml at 0 hours. Mean toxin titre RUs from ribotypes 001 and 027 only. LLOD = Lower Limit of Detection of TVCs and spores.

1.2.14 Colonisation Resistance Assay 1

C. difficile 027 vegetative cell death occurred in raw GMS B with a significant decrease of 6.1 log_{10}CFU/mL ($p=0.007$) in TVCs. Both TVCs and spores fell below the limit of detection after 48 hours. Vegetative cell numbers were maintained in autoclaved GMS from 0 hours to 48 hours, with TVCs remaining ~6 log_{10}CFU/mL and spores ~2 log_{10}CFU/mL with no significant differences at each time point (Fig. 1- 9).

Interestingly, no significant difference in TVCs or spore counts was observed between 0 and 48 hours in raw or autoclaved GMS B inoculated with *C. difficile* spores (Fig. 1- **10**). The lack of germination in autoclaved GMS inoculated with spores was unexpected as it was hypothesised that once the gut bacteria was destroyed colonisation resistance mechanisms would have also diminished. As the starting inoculum of *C. difficile* spores was relatively high in this assay the second assay examined smaller inoculum numbers.

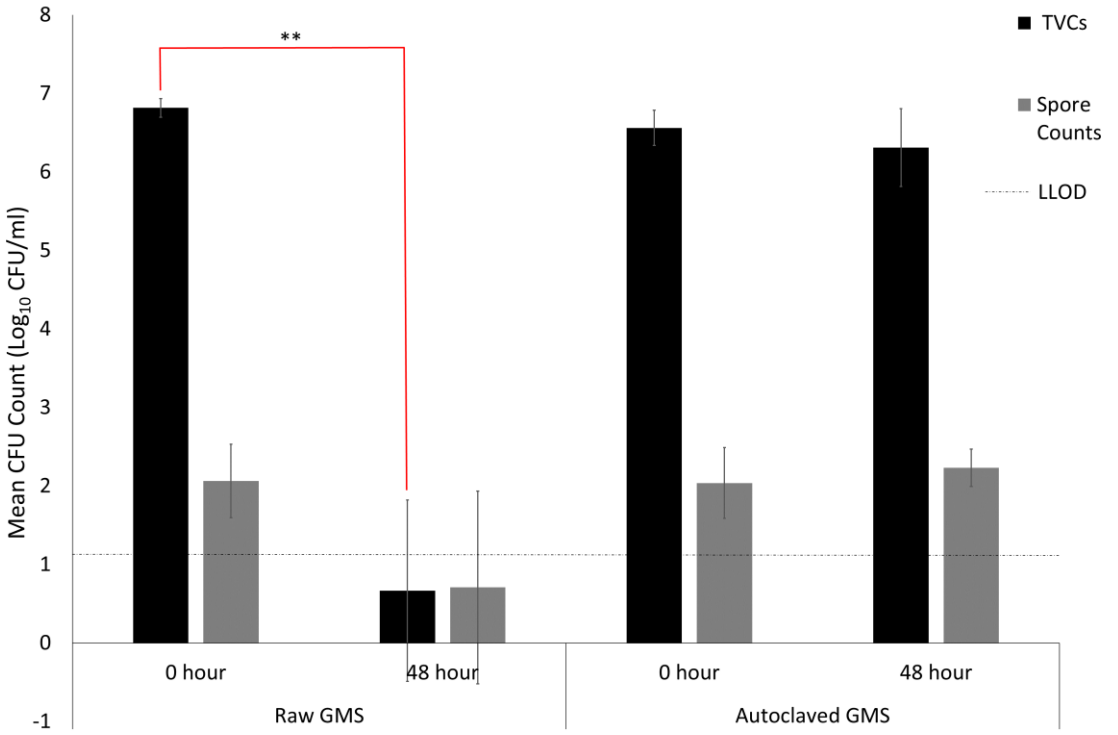

Fig. 1- 9 Mean (±SE) TVCs and spore counts from vegetative *C. difficile* 027 seeded in GMS B taken from 3 gut models at 10 days steady state (3 biological replicates (n=3)). Very significant results are highlighted with ** (p<0.01). LLOD = Lower Limit of Detection for TVC and Spore Counts

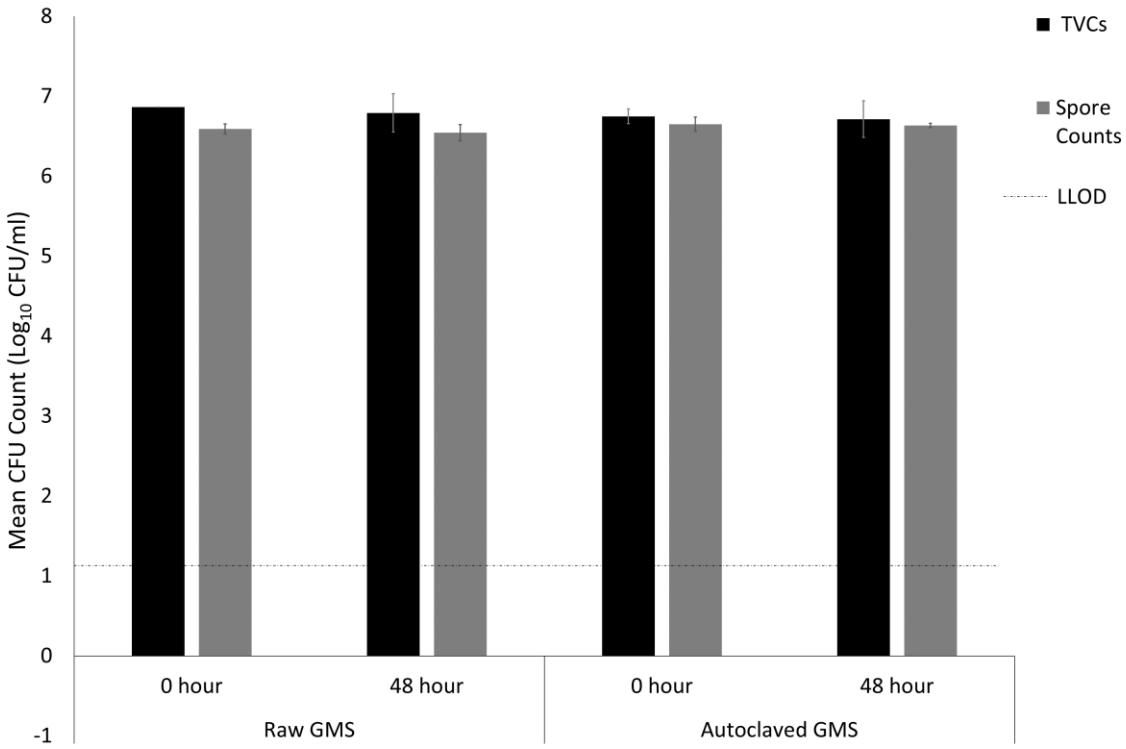

Fig. 1- 10 **Mean (±SE) TVCs and spore counts from GMS seeded with *C. difficile* 027 spores. GMS B taken from 3 gut models at 10 days steady state (3 biological replicates (n=3)). No differences were seen between recovery at 24 hours and 48 hours (data shown in Appendix A. 1.3). LLOD = Lower Limit of Detection for TVC and spore counts**

1.2.15 Colonisation Resistance Assay 2

No significant differences in TVCs or spore counts were observed between 0 and 48 hours for any inoculum number of *C. difficile* spores in either raw or autoclaved GMS (Fig. 1- 11).

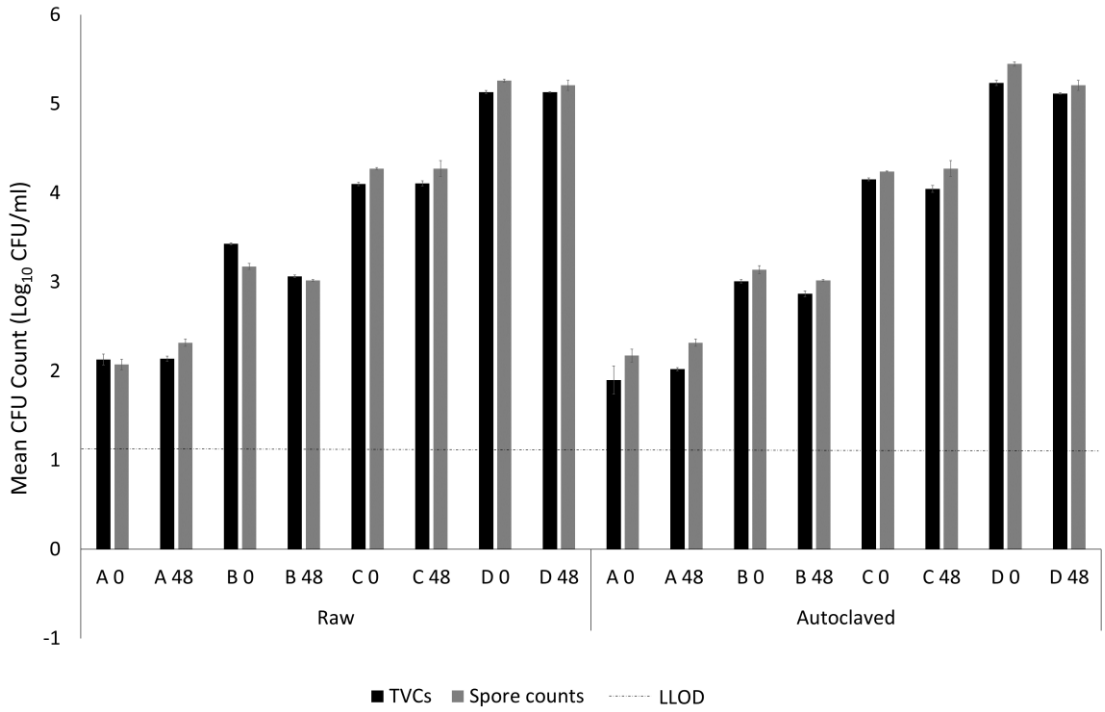

Fig. 1- 11 Mean (±SE) TVCs and spore counts from raw and autoclaved GMS Bseeded with various inoculum quantities of *C. difficile* 027 spores. Inoculum quantities: A = 2 log_{10}CFU/mL, B = 3 log_{10}CFU/mL, C = 4 log_{10}CFU/mL, D = 5 log_{10}CFU/mL. GMS taken from 3 individual gut models at 10 days steady state (3 biological replicates (n=3)). LLOD = Lower Limit of Detection

1.3 Discussion

Both growth and cytotoxin production from vegetative *C. difficile* seeded in raw GMS was inhibited compared to that seeded in autoclaved GMS, BHI or GMM. As expected, germination, growth and cytotoxin production occurred in BHI and GMM seeded with *C. difficile* spores and inhibited in raw GMS, but unexpectedly, was also inhibited in autoclaved GMS.

The preliminary data demonstrated that BHI was superior in supporting the survival and cytotoxin production, with consistently high numbers of viable cells from both inoculation with *C. difficile* spores and with vegetative cells. BHI is routinely used for growth, germination and cytotoxin experiments on *C. difficile* [262, 265-267] and comes as no surprise that this was the superior media. GMM also supported survival and allowed for toxin production but not as reliably as BHI. BHI has established itself as an ideal medium for future experiments concerning the growth and survival of *C. difficile* in batch culture.

In the preliminary experiments, *C. difficile* growth, germination and cytotoxin production was not observed in either raw or autoclaved GMS. As the raw GMS contained a full mix of gut microorganisms it was correctly predicted that the colonisation resistance mechanisms seen in previous studies using faeces [222, 224, 261] would prevail. However, TVCs and spore counts from both vegetative and spore *C. difficile* seeded in the autoclaved GMS, in which sterility was confirmed, were surprisingly similar to the results attained in the raw GMS. Since the gut microorganisms were assumed to have been destroyed in the autoclaved GMS, this was puzzling as previous experiments had shown a loss of colonisation resistance against *C. difficile* in heat or filter-sterilised faecal samples [222, 224, 261]. Vegetative *C. difficile* inoculated in raw GMS taken at various points in the gut model cycle showed some variation in growth, with higher TVCs seen in GMS taken at 4 days steady state compared to 10 days. This is more in keeping with the colonisation resistance model as the seeded faecal bacteria is still in the process of establishing

itself at 4 days in the gut model cycle compared to at 10 days when bacterial populations have reached a steady state and represent a rich and diverse microbiota.

In the preliminary studies, three *C. difficile* ribotypes were used as replicates to increase the heterogeneity of the results, helping to ensure that the observed results are not strain-specific, as it has been reported that different *C. difficile* strains can display variable growth, sporulation and toxin production patterns [268]. Since the different ribotypes demonstrated little variation between growth and germination in different media (Appendix A.1.2) other than cytotoxin production (with 010 a non-toxigenic strain), only ribotype 027 was used for further experimentation due to its clinical relevance and ability to produce cytotoxin. GMS from both points A (4 days) and B (10 days) in the cycle were also amalgamated to increase the number of biological replicates. Spurious spore counts were also encountered in the preliminary studies, with spore numbers outnumbering TVCs. As TVCs equal the total number of viable organisms (vegetative cells plus spores) this is most likely indicative of experimental error. The large error bars show that there was a lot of variation in the raw data, which may indicate that the spore samples were not mixed thoroughly enough before plating. Unpublished experience from the HCAI research group suggests that without vigorous agitation to ensure spores are well mixed an unequal number of spores can be plated.

The results from the colonisation resistance assay comparing *C. difficile* death, growth, germination and sporulation in raw and autoclaved GMS are in line with previous observations inoculating fresh and sterile faeces with vegetative cells [222, 224]. Significant cell death was observed between the point of inoculation and 48 hours incubation in raw GMS and maintenance of *C. difficile* turnover in autoclaved GMS – in which the gut bacteria were destroyed – indicating a loss of colonisation resistance against vegetative *C. difficile* in autoclaved samples. However, the same loss of colonisation resistance was not seen in autoclaved GMS seeded with *C. difficile* spores, which was unexpected especially given the virulence of the 027

ribotype [269]. Resporulation after 48 hours is unlikely as TVC and spore counts recovered at 24 hours were similar to 48 hours (Appendix A.1.3). Harris *et al.* [261] observed germination of *C. difficile* ribotype 027 spores in heat and filter sterilised faeces using a lower inoculum, therefore the current experiment was repeated by inoculating raw and autoclaved GMS with lower spore inoculums. No germination was observed with any inoculum quantity in the raw or autoclaved GMS.

This could indicate that components of GMS, even once heat sterilised and the gut bacteria destroyed, are not conducive to *C. difficile* spore germination. It is unlikely that the media alone is hostile to *C. difficile* germination as *C. difficile* spores readily germinated in GMM (gut model media before it was fed through the gut model), even with a high inoculum number, in the preliminary investigations. Other factors that could be considered include, but are not limited to, other methodological issues such as the viability of the spore preparation used. Due to time constraints, and lack of laboratory assistance, spores were not inoculated into a BHI control in the latter colonisation resistance assays, and therefore any issues with the spores themselves cannot be ruled out. However, the spores readily germinated on CCEYL agar for enumeration and so other factors in the GMS should still be considered. Inhibitory secretions by commensal gut bacteria after heat exposure such as those affecting pH may be worth investigating further since it has been demonstrated previously that germination and outgrowth of *C. difficile* 027 spores is prevented at pH <6.19, where proliferation of vegetative cell suspensions has still occurred at pH >5.67 [270]. Consumption of germinants in GMM by microbiota before heat sterilisation should also be considered. A useful addition to this study would have been to assess the microbiota populations from the gut model on the day of extraction for a more in-depth analysis of populations involved in colonisation resistance. This was not possible due to a lack of appropriate laboratory support.

The underlying mechanisms as to why a loss of colonisation resistance was not seen in heat sterilised GMS seeded with *C. difficile* spores is beyond the scope of this

experiment. To investigate why the results of the spore seeded colonisation resistance assay did not concur with that using faeces [261] it may be prudent to elucidate differences in quantities of potential germinants, such as primary bile acids [160], and pH between GMS and faecal slurry. As well as investigating various inoculum numbers, further work may include investigating other ribotypes as well as filter sterilised GMS, alongside autoclaved GMS.

This study was useful in confirming GMS is a valuable media with which to carry out microbiota – *C. difficile* interaction studies using ribotype 027 vegetative cells but currently not for germination studies using 027 spores. It was also useful in reaffirming that BHI is a suitable growth medium for further analysis of *C. difficile* growth, germination, sporulation and toxin production.

Chapter 2 Interactions between intestinal bacteria and *C.difficile* in batch co-culture

2.1 Background and Rationale

A healthy and diverse gut microbiota confers colonisation resistance against *C. difficile*; with key commensal microbe-derived metabolites, such as secondary bile acids, inhibiting *C. difficile* spore germination and proliferation [161, 271]. CDI can occur when there is a disturbance or depletion in the microbiota, and conditions in the gut become favourable for *C. difficile* germination [43, 229, 272-274].

Although the greatest risk factor for microbiota depletion and loss of colonisation resistance against CDI is the use of antibiotics, standard CDI treatments also involve antimicrobial regimes. With an estimated ~25% of patients encountering rCDI [108, 275] and a further 40 – 60% chance of recurrence after the second infection [125]. Collateral damage from antimicrobials used to treat CDI on the recovering host microbiome may reduce the necessary microbial composition and metabolic processes that confer colonisation resistance to prevent further CDIs [276, 277].

Although FMT has proven to be a useful therapy for multiple rCDI [13, 134], it is still a relatively rudimentary treatment protocol and questions have been raised regarding its safety, especially concerning screening for emerging infectious pathogens [144, 145]. A selection of key organisms with roles in providing colonisation resistance could be safer than a full FMT to prevent rCDI after antimicrobial therapy to treat the initial infection, however, at the time of designing this study the species potentially involved were only just beginning to be explored. Identifying bacterial species which can inhibit the proliferation of *C. difficile* could help towards creating a mix of target species to be used as a treatment or adjunct treatment to reduce the collateral damage on the microbiota without the safety concerns of using FMT. *In vitro*, batch co-culture methods are an ideal starter to screen for interactions of key gut bacteria species with *C. difficile*. They are simple and cheap to run and the results of which

can be used to focus on probiotic permutations to be considered for more technical studies. These co-culture experiments are also useful to concurrently assess any antimicrobial interactions *C. difficile* may have upon microbiota species. As well as toxins, *C. difficile* has also been documented to secrete antibacterial compounds such as proline-based cyclic dipeptides that may inhibit commensal gut bacteria [271] and therefore the effect of a *C. difficile* cell-free supernatant upon these selected species is a worthy addition to the study.

Here, bacterial species previously isolated from the gut model and identified, are co-cultured in a liquid suspension with *C. difficile* to determine any inhibitive effects. A range of species from the major culturable gut organisms were selected for investigation.

The primary aim of this study was to evaluate the *in vitro* potential of a selection of gut microbiota species to directly inhibit *C. difficile* growth and toxin production in a BHI broth co-culture assay. It is hypothesised that many of the microbiota species selected for this study will have an inhibitive effect on *C. difficile* growth and/or toxin production in a co-culture.

The secondary aim of this study was to assess the effect of cell-free *C. difficile* supernatant containing cytotoxin (SCC) on the proliferation of the same selected gut microbiota species.

2.2 Materials and Methods

2.2.1 Preparation *of C. difficile* and *C. difficile* Supernatant Containing Cytotoxin (SCC)

C. difficile ribotype 027 was maintained on modified Brazier's CCEYL agar incubated at 37^0C anaerobically. A single colony of *C. difficile* was inoculated in pre-reduced BHI broth and incubated overnight at 37^0C anaerobically.

To produce the SCC, a single *C. difficile* colony was inoculated into pre-reduced BHI broth and incubated at 37°C anaerobically. After 48 hours the culture was centrifuged at 16000g for 10 minutes, and the supernatant was filtered through a 0.22µm filter (Millex-GP Merck Millipore LTD) to remove contaminants. SCC was confirmed by a cytotoxicity/neutralisation assay as described in Chapter 1 (section 1.2.3). SCC was stored at 5 – 8^0C.

2.2.2 Preparation of Test Bacteria Species

Each bacterial test species (detailed inTable 2-1), excluding *C. Scindens*, was isolated from the gut model, purified, identification verified by MALDI-TOF, and stored at -80°C. *C. scindens* was kindly donated by Nottingham University and maintained as a spore preparation in saline. Test species were streaked on CBA and incubated for 48 hours. A single colony of each species was inoculated into pre-reduced BHI broth and incubated overnight at 37°C, anaerobically.

Table 2-1 Individual species isolated from the gut model and identified via MALDI-TOF

Organism	Source
Lactobacillus delbrueckii	Gut Model
Lactobacillus paracasei	Gut Model
Lactobacillus rhamnosus	Gut Model
Lactobacillus gasseri	Gut Model
Lactobacillus plantarum	Gut Model
Clostridium butyricum	Gut Model
Clostridium cadaveris	Gut Model
Clostridium baratii	Gut Model
Clostridium tertium	Gut Model
Clostridium glycolicum	Gut Model
Clostridium scindens	Nottingham University
Bacteroides fragilis	Gut Model
Bacteroides thetaiotamicron	Gut Model
Bacteroides ovatus	Gut Model
Bifidobacterium longum	Gut Model
Bifidobacterium bifidum	Gut Model
Bifidobacterium dentium	Gut Model
Bifidobacterium breve	Gut Model
Bifidobacterium catenulatum	Gut Model
Clostridioides difficile	The PCR ribotype 027 strain used in this experiment (027 210) was isolated during an outbreak of CDI at the Maine Medical Centre (Portland, ME, USA) and was kindly supplied by Dr Rob Owens

2.2.3 SCC and Test Species Co-Culture

Equal measures (0.5mL) of overnight cultures from individual test species (Table 2-1) and SCC were added to 4ml pre-reduced BHI and incubated anaerobically at 37ºC. Controls consisted of 0.5mL individual test species culture or SCC in 4.5mL. Samples taken at 24 and 48 hours were serially diluted 10-fold to 10^{-6} in pre-reduced peptone water. Each dilution (20µl) was plated onto ¼ pre-reduced CBA plates, in triplicate, and incubated at 37˚C, anaerobically for 48 hours before CFUs were enumerated by eye.

2.2.4 *C. difficile* and Test Species Co-Culture Initial Screen

Equal measures (0.5mL) of overnight cultures from individual test species (Table 2-1and *C. difficile* were added to 4ml pre-reduced BHI. Controls consisted of 0.5mL overnight cultures added to 4.5ml pre-reduced BHI. Co-cultures and controls were incubated anaerobically at 37°C. Samples taken at 24 and 48 hours were enumerated as previously described.

At 24 hours only 1ml aliquots of the co-culture and *C. difficile* controls were processed for the cytotoxicity/neutralisation assay. Cytotoxin titres are expressed in relative units (RU).

2.2.5 *C. difficile* in Co-culture with Selected Test Species of Interest

C. scindens, B. dentium, B. breve, L. rhamnosus, L. delbrueckii and *C. difficile* were grown on selective and non-selective agars (Table 2-2) and incubated at 37º anaerobically for 48-hours. A single colony of each organism was inoculated into pre-reduced BHI broth and incubated overnight at 37°C anaerobically. Monocultures of test species and *C. difficile* were quantified spectrophotometrically and adjusted to a OD_{600} of 0.5± 0.03. Equal measures (0.5mL) test species and *C. difficile* preparations (containing approximately7 $\log_{10}$CFU/mL as verified by a quality control TVC count) were added to 4ml pre-reduced BHI and incubated anaerobically for 72 hours, in triplicate (n=3). Samples were taken at 24, 48 and 72 hours, plated onto

selective and non-selective agars (Table 2-2) and enumerated as described previously. Cytotoxin was not measured.

2.2.5.1 Statistical Analysis for C. difficile growth in Co-culture with Selected Species

Data are presented as the calculated mean of a minimum of three independent experiments. Error bars represent the standard error of the mean. Statistical analysis was performed on Microsoft Excel version 2202. As the sample size was small a two-sample t-test assuming unequal variances was employed to determine the significance of differences in $\log_{10}$CFU/L between *C. difficile* in co-culture with selected test species of interest and the *C. difficile* mono-culture control. A statistical significance level of <0.05 was adopted, <0.01 very significant, and <0.001 highly significant.

Table 2-2 Selective agars for test species of interest (full agar constituents in Appendix A.1.1)

Target Organism	Agar	Supplements	Growth Conditions
B. breve	Beerens agar - 42.5 g/L Columbia agar, 5 g/L agar technical	0.5 g/L cysteine HCl, 5 g/L glucose and 5 ml propionic acid, adjusted to pH 5	48-hour at 37˚C Anaerobically
B. dentium	Beerens agar - 42.5 g/L Columbia agar, 5 g/L agar technical	0.5 g/L cysteine HCl, 5 g/L glucose and 5 ml propionic acid, adjusted to pH 5	48-hour at 37˚C Anaerobically
C. difficile	Brazier's CCEYL with 2% lysed horse blood	5 mg/L lysozyme, 250 mg/L D-cycloserine, 8 mg/L cefoxitin	48-hour at 37˚C Anaerobically
C. scindens	Fastidious anaerobe agar	5% horse blood	48-hour at 37˚C Anaerobically
L. delbrueckii	Fastidious anaerobe agar (this *Lactobacillus* spp. is sensitive to vancomycin and does not grow on LAMVAB)	5% horse blood	48-hour at 37˚C Anaerobically
L. rhamnosus	LAMVAB agar - 52.5 mg/L MRS broth and 20 mg/L agar technical	0.5 g/L cysteine HCl, 20mg/L vancomycin	48-hour at 37˚C Anaerobically

	with 0.5 g/L L-cysteine and 20 mg/L vancomycin

2.3 Results

2.3.1 Initial Screen of *C. difficile* Growth in Co-Culture with Test Species

Each *Bifidobacterium* spp. investigated had an inhibitory effect on *C. difficile* growth in co-culture (Fig. 2- 1). The most substantial decrease in *C. difficile* TVCs was seen in co-culture with *B. dentium, B. catenulatum* and *B. breve* with a 3.6-, 5.1- and 6.1-$\log_{10}$CFU/mL difference compared to the *C. difficile* control after 48 hours, respectively. Cytotoxin titres were negative from *C. difficile* in individual co-cultures with these three species after 24 hours. Cytotoxin recovered from co-cultures with *B. longum* and *B. bifidum* was 1RU lower than that from the *C. difficile* control.

All the *Lactobacillus* spp. tested had a slight inhibitory effect on *C. difficile* in co-culture compared to the *C*. *difficile* control (Fig. 2- 2). *L. delbrueckii* had the greatest effect with a decrease of *C. difficile* of 2.2$\log_{10}$CFU/mL seen after 48 hours compared to the *C. difficile* control. *C. difficile* in co-culture with *L delbrueckii*, *L. paracasai* and *L. rhamnosus* produced negative cytotoxin titres after 24 hours. *L. plantarum* and *L. gasseri* co-cultures yielded titres 1RU lower than the *C. difficile* control at 24 hours.

Of the three *Bacteroides* spp. tested, only co-culture with *B. ovatus* reduced *C. difficile* TVCs, with a decrease of 1.6 $\log_{10}$CFU/mL counts after 48 hours (Fig. 2- 3). *C. difficile* in co-culture with *B. thetaiotaomicron* had a small decrease of 0.01 $\log_{10}$CFU/mL, and with *B. fragilis* a slight increase of 0.22 $\log_{10}$CFU/m L. Cytotoxin titres were equal to the control at 1RU for co-cultures with each *Bacteroides* spp..

Co-culture with *Clostridium* spp. (Fig. 2- 4) slightly increased the growth of *C. difficile*. *C. cadaveris* and *C. glycolicum* increased TVCs of *C. difficile* by ~2 $\log_{10}$CFU/mL compared to the control after 48 hours. *C. baratii*, *C. tertium* and *C. scindens* co-

cultures had marginally higher *C. difficile* TVCs, <0.5 log_{10}CFU/mL compared to the control. Only *C. difficile* in co-culture with *C. butyricum* reduced *C. difficile* growth, but again this was a small 0.07 log_{10}CFU/mL decrease. Four of the five available cytotoxicity titres for co-culture with the *Clostridium* spp. were equal to the control (2RU), whereas co-culture with *C. butyricum* was negative. Cytotoxicity assay results were unavailable for *C. scindens* co-cultures.

Full results including test species TVCs in mono- and co-cultures are included in Appendix A.2

2.3.2 Initial Screen of Test Species Growth when Incubated with SCC

No discernible effect on the growth of 18 test species in the presence of SCC at a concentration of 2RU was seen after 24 or 48 hours (Fig. 2- 5) except for *L. delbrueckii*, which had an increase in growth ~1 log_{10}CFU/mL in the presence of SCC compared to the monoculture control between 24 and 48 hours.

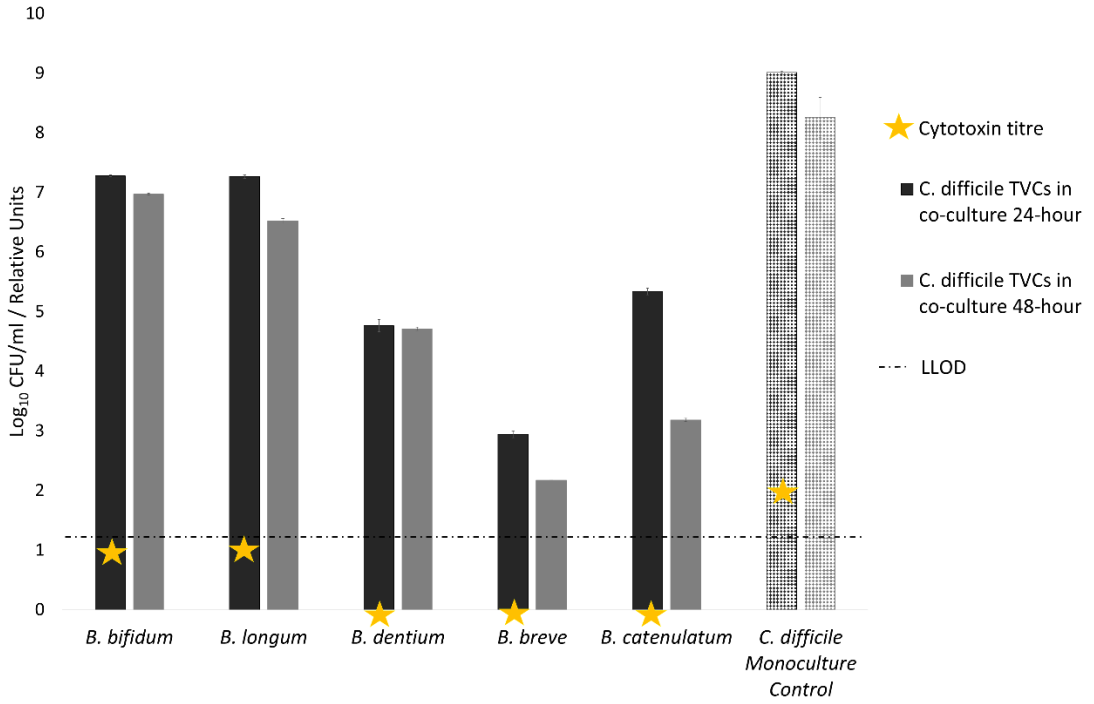

Fig. 2- 1 *C. difficile* TVCs in co-culture with *Bifidobacterium* spp. and in a monoculture control at 24 and 48 hours. Cytotoxin titre (RUs) at 24 hours. LLOD = Lower Limit of Detection of TVCs. Associated test *Bifidobacterium* spp. TVCs recovered from co-culture in appendix A.2.

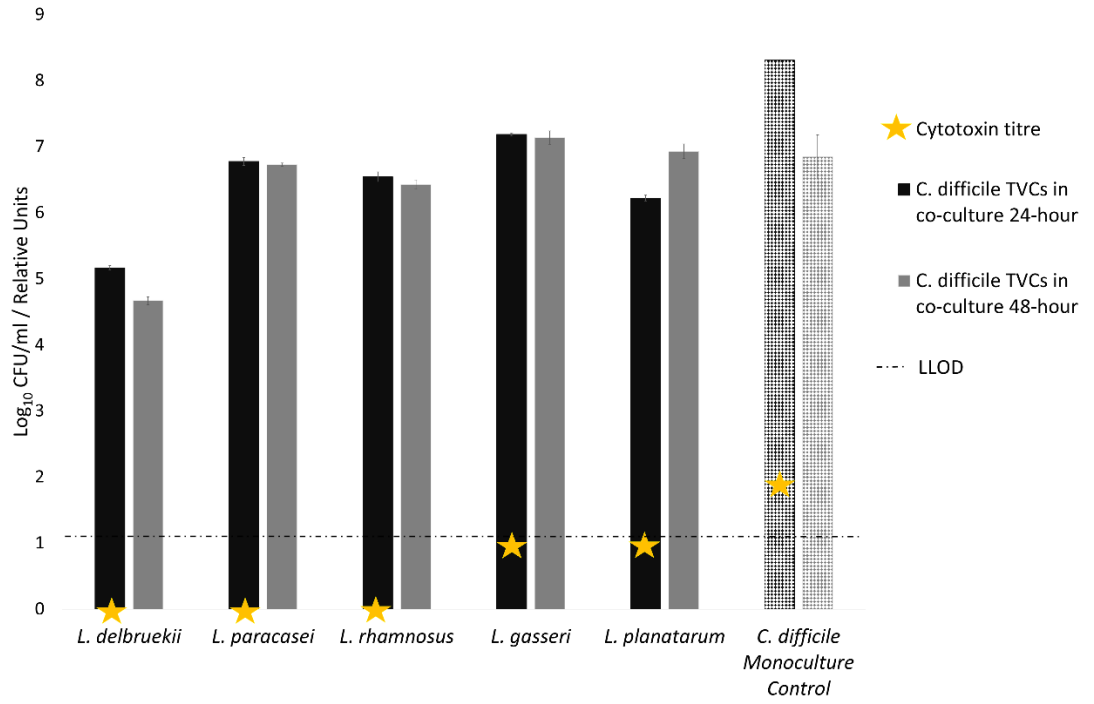

Fig. 2- 2 *C. difficile* TVCs in co-culture with *Lactobacillus* spp. and in a monoculture control at 24 and 48 hours. Cytotoxin titre (RUs) at 24 hours. LLOD = Lower Limit of Detection of TVCs. Associated test *Lactobacillus* spp. TVCs recovered from this co-culture in appendix A.2.

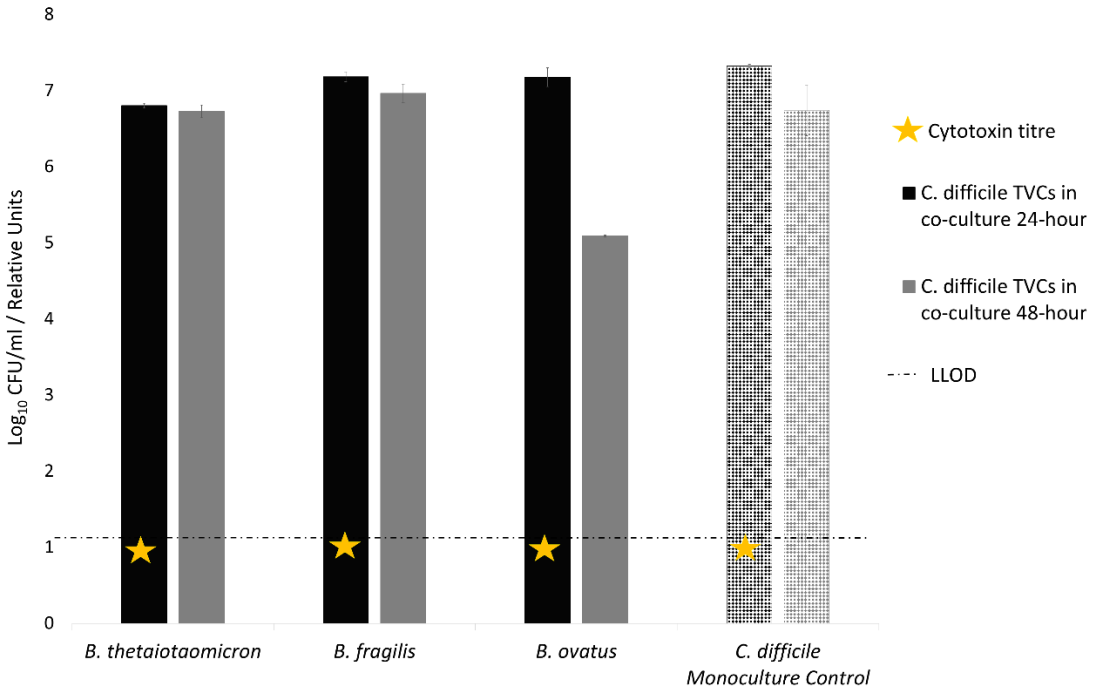

Fig. 2- 3 *C. difficile* TVCs in co-culture with *Bacteroides* spp. and in a monoculture control at 24 and 48 hours. Cytotoxin titre (RUs) at 24 hours. LLOD = Lower Limit of Detection of TVCs. Associated test *Bacteroides* spp. TVCs recovered from this co-culture in appendix A.2.

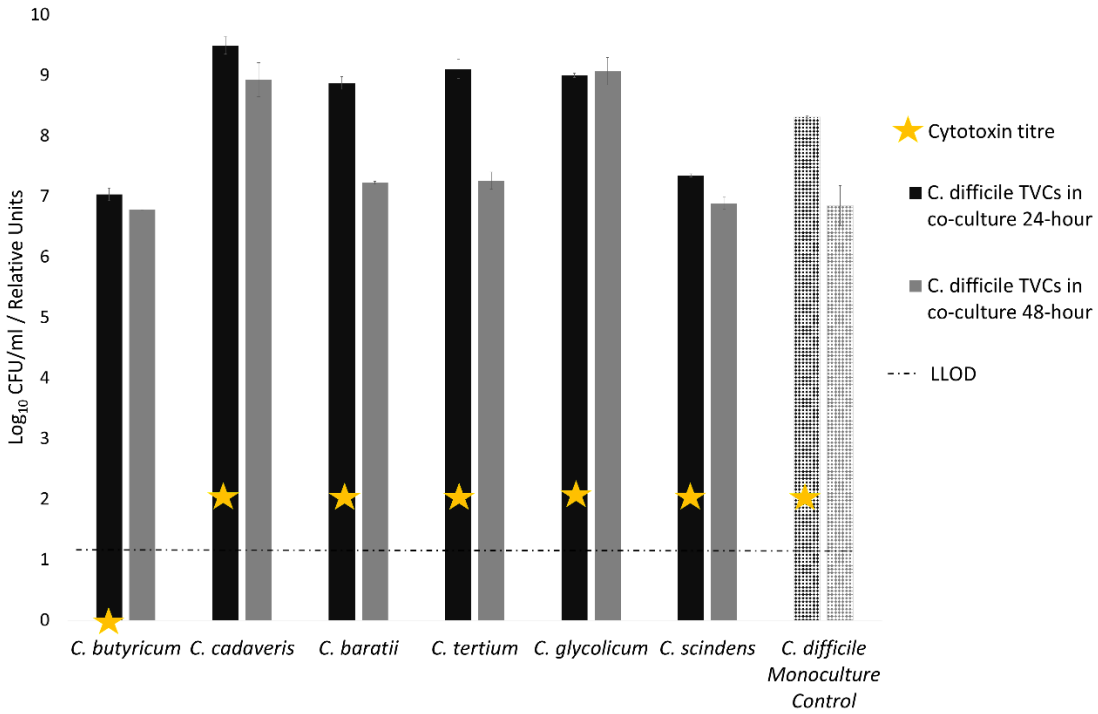

Fig. 2- 4 *C. difficile* TVCs in co-culture with *Clostridium* spp. and in a monoculture control at 24 and 48 hours. Cytotoxin titre (RUs) at 24 hours. LLOD = Lower Limit of Detection of TVCs. Associated test *Clostridium* spp. TVCs recovered from this co-culture in appendix A.2.

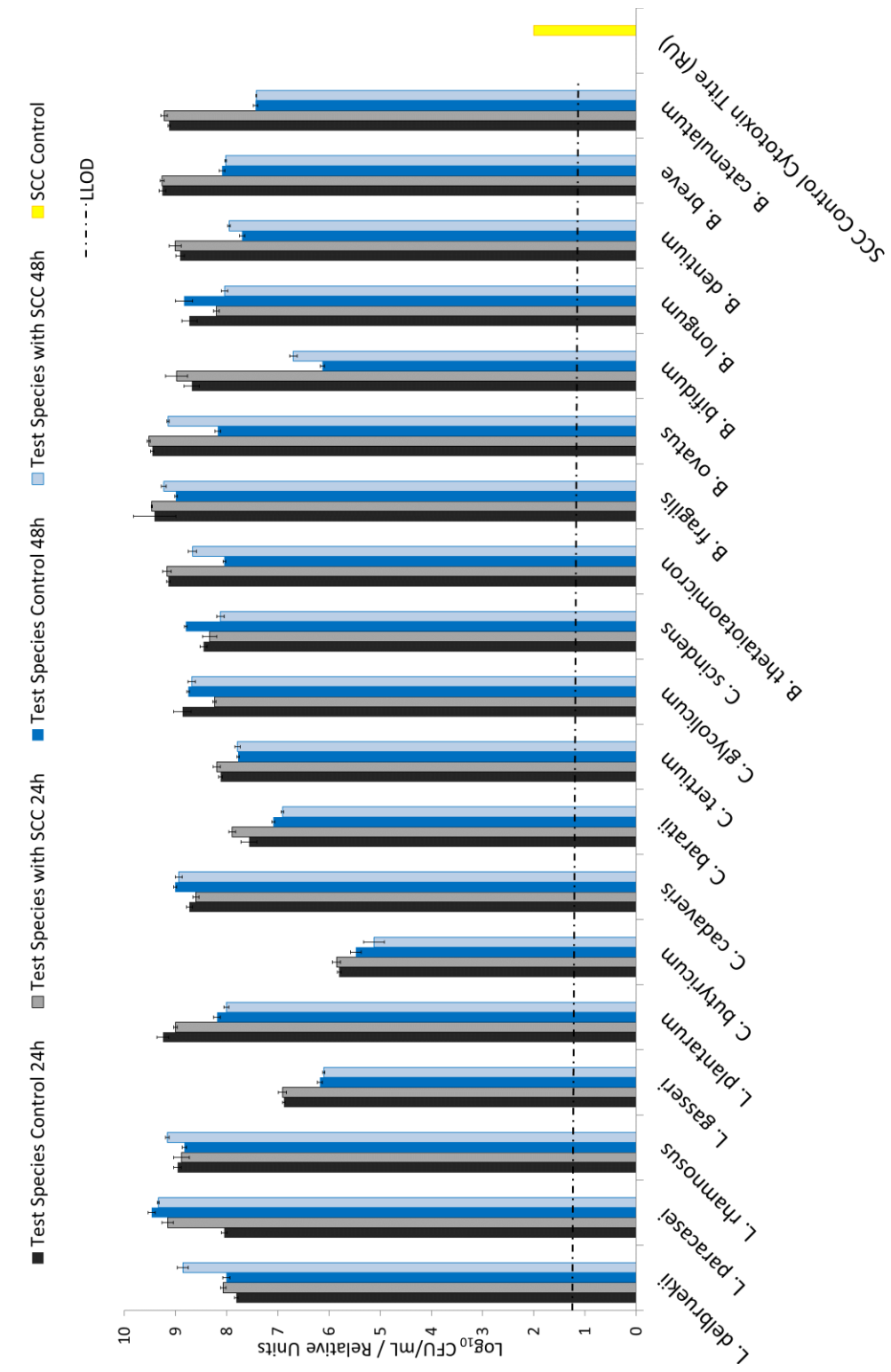

Fig. 2- 5 Test species TVCs recovered from culture with SCC compared to test species TVCs recovered from monoculture controls at 24 and 48 hours. Cytotoxin titre recorded as Relative Units after 24 hours incubation. LLOD = Lower Limit of Detection

2.3.3 *C. difficile* Growth in Co-Cultures with Selected Test Species

L. rhamnosus, *B. dentium, B. breve* and *L. delbrueckii* were selected for further examination as they had shown varying degrees of inhibition on *C. difficile* growth in the initial co-culture screens. *C. scindens* was also included as this is a clinically important species concerning *C. difficile* germination and disease progression [161].

Co-culture with *L. rhamnosus* showed a similar degree of inhibition to the initial screen with small significant decreases on *C. difficile* growth compared to the control of 1.4 $\log_{10}$CFU/mL ($p=0.025$), 2.2 $\log_{10}$CFU/mL ($p=0.028$) and 1.6 $\log_{10}$CFU/mL ($p=0.015$) at 24, 48 and 72 hours, respectively (Fig. 2- 6)

Co-culture with *B. dentium, B. breve* and *L. delbrueckii* showed smaller decreases than previously seen compared to the *C. difficile* control 48 hours: 1.4 $\log_{10}$CFU/mL ($p=0.027$) (Fig. 2- 7), 1.1 $\log_{10}$CFU/mL ($p<0.001$) (Fig. 2- 8), and 0.7 $\log_{10}$CFU/mL ($p=0.02$) (Fig. 2- 9), respectively.

C. difficile in co-culture with *C. scindens* showed non-significant ($p>0.05$) decreases of 0.5, 0.5 and 0.4 $\log_{10}$CFU/mL compared to the control, at 24, 48 and 72 hours (Fig. 2- 10), respectively

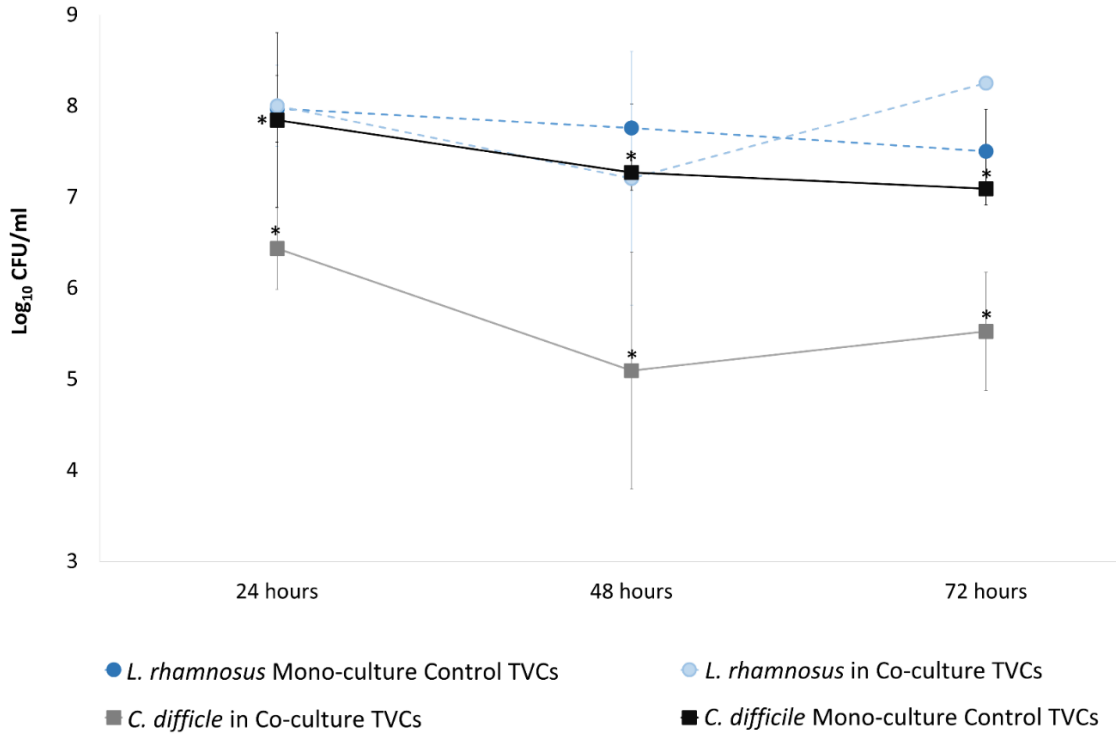

Fig. 2- 6 Mean (±SD) *L. rhamnosus* and *C. difficile* TVCs from mono- and co-cultures in BHI at 24, 48 and 72 hours. Expected 0 hour TVCs ~6 log_{10}CFU/mL of both species in each mono- and co-culture. Significant results highlighted with * (p<0.05), very significant ** (p<0.001), or highly significant with * (p<0.001), where available.**

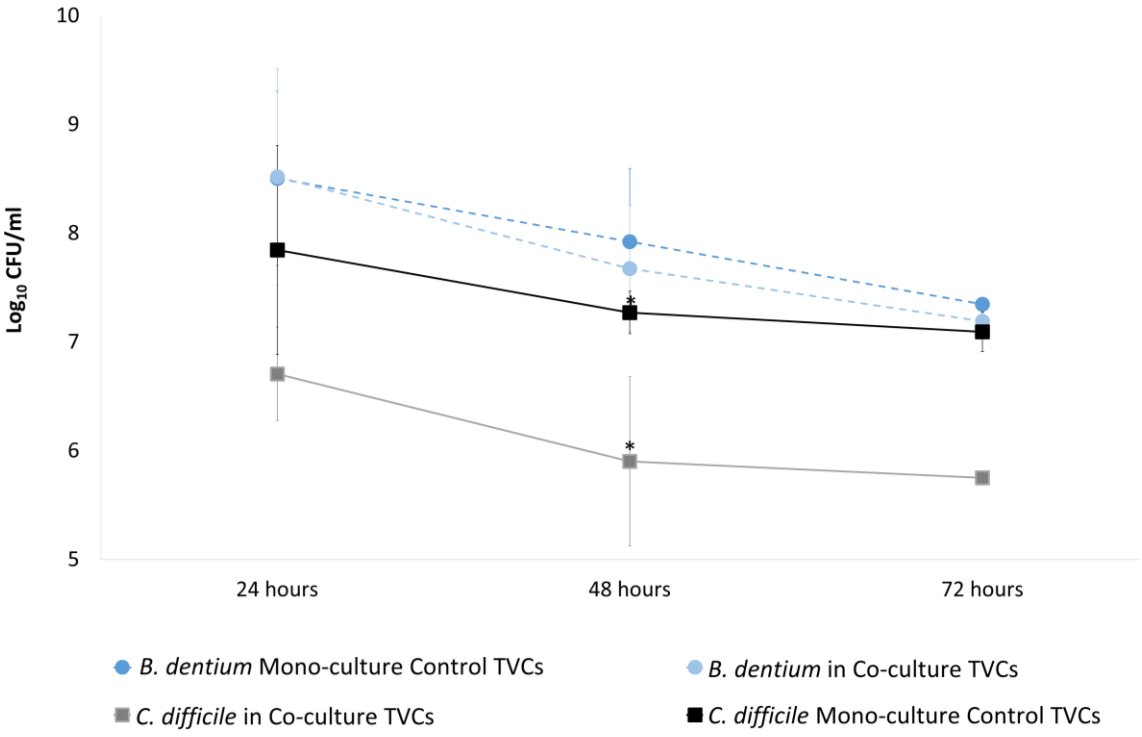

Fig. 2- 7 Mean (±SD) *B. dentium* and *C. difficile* TVCs from mono- and co-cultures in BHI at 24, 48 and 72 hours. Expected 0 hour TVCs ~6 log_{10}CFU/mL of both species in each mono- and co-culture. Significant results highlighted with * (p<0.05), very significant ** (p<0.001), and highly significant with * (p<0.001), where available.**

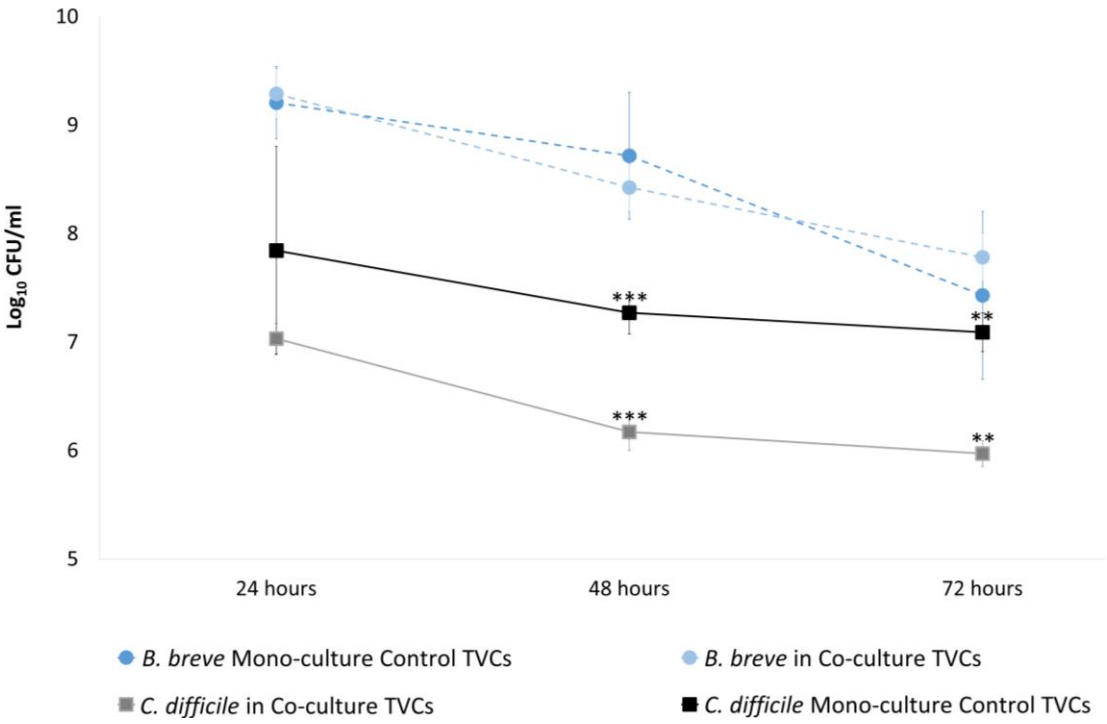

Fig. 2- 8 Mean (±SD) *B. breve* and *C. difficile* TVCs from mono- and co-cultures in BHI at 24, 48 and 72-hours. Expected 0 hour TVCs ~6 log_{10}CFU/mL of both species in each mono- and co-culture. Significant results highlighted with * (p<0.05), very significant ** (p<0.001), and highly significant with * (p<0.001), where available.**

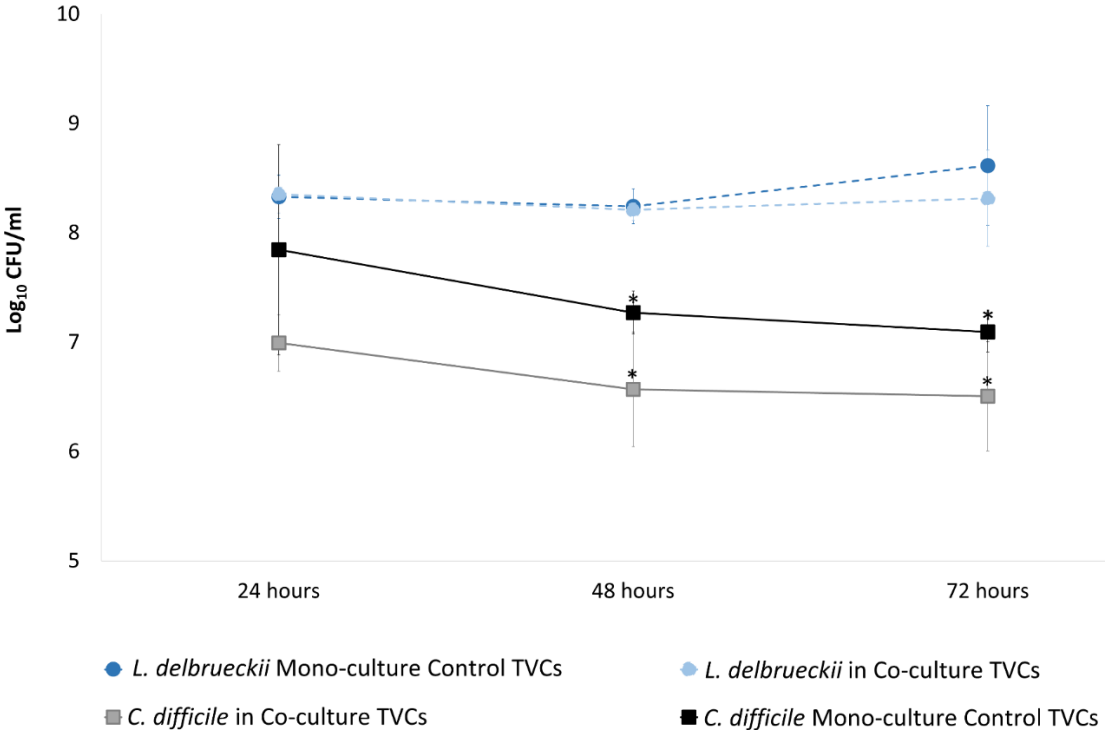

Fig. 2- 9 Mean (±SD) *L. delbrueckii* and *C. difficile* TVCs from mono- and co-cultures in BHI at 24, 48 and 72-hours. Expected 0 hour TVCs ~6 log_{10}CFU/mL of both species in each mono- and co-culture. Significant results highlighted with * (p<0.05), very significant ** (p<0.001), and highly significant with * (p<0.001), where available.**

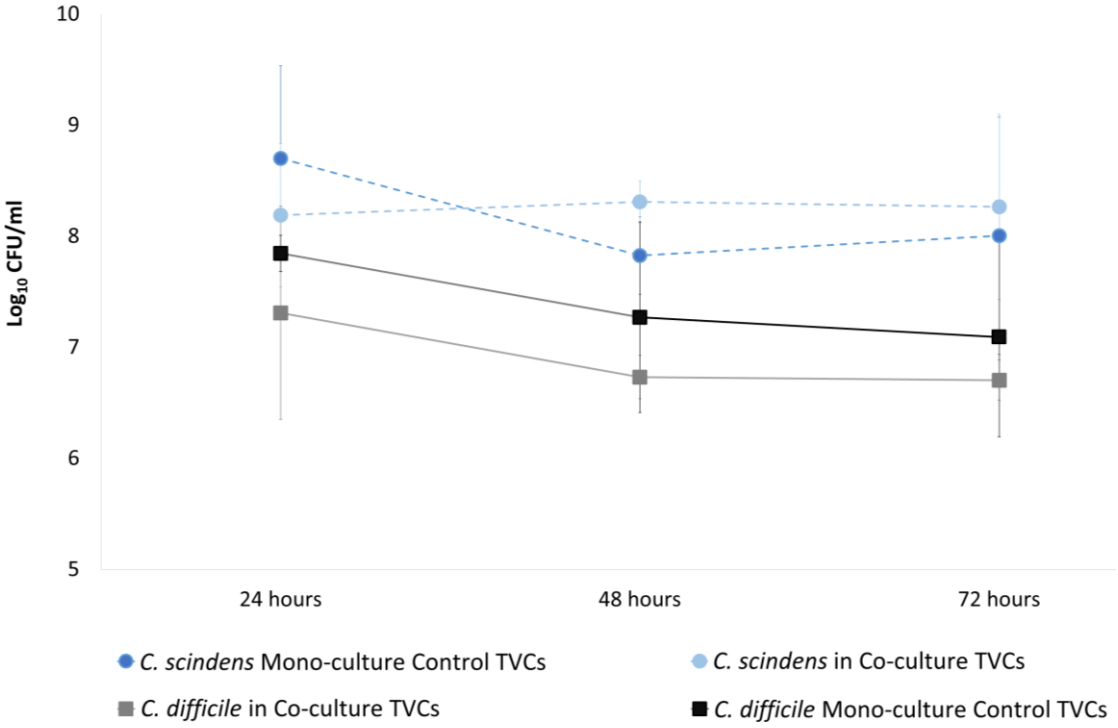

Fig. 2- 10 Mean (±SD) *C. scindens* and *C. difficile* TVCs from mono- and co-cultures in BHI at 24, 48 and 72-hours. Expected 0 hour TVCs ~6 log_{10}CFU/mL of both species in each mono- and co-culture. All differences between the *C. difficile* mono- and co-cultures were insignificant. No significant differences to highlight.

2.4 Discussion

C. difficile growth and toxin production varied considerably when incubated in co-culture with a range of gut microbiota species. The *Bifidobacterium* and *Lactobacillus* spp. were the most antagonistic on *C. difficile* growth and toxin production of all the species tested, whilst generally maintaining the same growth behaviour as seen in the mono-cultures (Appendix A.2). This observation is consistent with other *in vitro* studies using various methodologies with single *Bifidobacterium* and *Lactobacillus* spp. in co-culture with *C. difficile*, with fluctuating levels of inhibitive effect between studies [278-283]. Differing magnitudes of inhibitory effect on *C. difficile* between studies investigating the same *Bifidobacterium* and *Lactobacillus* spp. is perhaps due to the variation in their methodologies; the inhibitory mechanisms utilised by these particular species may be enhanced or impeded by different growth mediums and assay type [284] compared to the BHI broth co-culture used in the current study. As well as possible strain-dependent mechanisms underlying the antibacterial activity as seen in these species against *C. difficile* and other gut pathogens [278, 285]. *B. breve, B. dentium, L. delbrueckii* and *L. rhamnosus* were selected for more rigorous testing based on the results from the initial screens and previous reports of inhibition or displacement of *C. difficile* using a variety of experimental methods [279, 283, 286-288]. These species all showed some slight differences in inhibitive effect on *C. difficile* growth between the initial screen results and secondary testing. *B. breve* had a smaller inhibitive effect compared to the initial screen, whereas *L.rhamnosus,* which showed very little inhibitive effect in the initial screen, exhibited the most inhibition on *C. difficile* growth in the secondary tests. This may be due to differences in inoculum quantity between the two assays as the overnight cultures of bacteria were only quantified and adjusted to a standard amount in the second test. The results from the second assay are in keeping with Chapman *et al*. [279] who observed *L. delbrueckii* and *L. rhamnosus* exhibited a greater inhibitory effect on *C. difficile* compared to *B. breve*, using an agar spot test. The underlying mechanisms as to how these

particular *Bifidobacterium* and *Lactobacillus* spp. inhibited the growth of *C. difficile* is beyond the scope of the study as only TVCs counted from the co-culture of vegetative cells were investigated. Other studies have suggested species more antagonistic to *C. difficile* growth produced higher levels of H_2O_2, lactic - and other organic - acids, altering the pH of the growth medium [279, 289, 290]. The production of bacteriocins has also been considered [291], but in a batch culture such as that used for this study competition for nutrients cannot be discounted. Future similar studies could easily measure the pH of the medium to discount this as an implicatory factor in the inhibition of *C. difficile* growth.

Cytotoxin titres were reduced in all the *C. difficile* co-cultures with *Bifidobacterium* and *Lactobacillus* spp. tested compared to the *C. difficile* mono-culture control. This may be due to the overall reduction on *C. difficile* proliferation; however, studies have indicated a neutralising effect also occurs with cytotoxin in a cell-free supernatant incubated with particular strains of these two genera [283, 287, 290]. More intricate studies have reported various *Lactobacillus* spp. can produce bioactive molecules in their culture medium that can down-regulate *C. difficile* virulence genes, and interfere with quorum sensing, thus reducing toxin production and adhesion to colonic cell lines [292-294]. Unfortunately, the cytotoxicity assays performed on SCC in co-culture with these species in this study had inconsistent control data and confidence in observations was too low to confirm any neutralising effect.

Little has been documented on *in vitro* batch co-culture of *C. difficile* with *Clostridium* spp. Clinical studies have reported that a reduction in host-microbiota Clostridiales is associated with an increased risk of nosocomial CDI [295], and patients with an abundance of Clostridiaceae were more likely to respond to CDI treatment first time [296]. Although the particular *Clostridium* species investigated here are only a tiny fraction of this order contained in the gut microbiota, interestingly, no inhibition of growth was observed. *C. scindens*, which had little inhibitory effect on *C. difficile* growth in co-culture, has been shown to enhance resistance to CDI *in vivo* [161].

Buffie *et al.* [161] demonstrated that *C. scindens* reduces the proliferation of *C. difficile* by metabolising bile acids necessary for spore germination. It is therefore unsurprising that little effect was seen, as the current study only examined the growth of vegetative cells in the absence of bile acids. Out of the six *Clostridium* spp. screened only *C. difficile* in co-culture with *C. butyricum* attained negative cytotoxin titres. Cytotoxin suppression effect without reducing *C. difficile* counts has been reported previously [297, 298]. Woo *et al.* (2011), indicated the probiotic strain *C. butyricum* MIYAIRI 588 reduced *C. difficile* cytotoxicity *in vitro* with cell-to-cell contact, without reducing *C. difficile* growth [297]; and Oka *et al.* (2018) observed orally administered *C. butyricum* MIYAIRI 588 decreased diarrhoea and cytotoxin titres, but not faecal *C. difficile* numbers, in a novel rat CDI model [298]. However, 10x concentrated cell-free supernatants of the *C. butyricum* T58A strain have been shown to inhibit *C. difficile* growth *in vitro* [299]. These differences in effect may be strain- (for either *C. butyricum* or *C. difficile*) or concentration-dependent. The *C. butyricum* strain used throughout the current study was isolated from the gut model and is therefore different from the various strains used in other studies. *C. butyricum* has also been shown to support the growth of endemic gut *Bifidobacterium* and *Lactobacillus* spp. [300, 301], modulate gut microbiota composition and enhance colonisation resistance against CDI in murine models [302]. This range of potential host protective effects by *Clostridium* spp. were not elucidated during the current study as time constraints and lack of laboratory support thwarted onward progression from the initial screens.

Although only a small number of *Bacteroides* spp. were examined in this study, it was interesting they did not affect *C. difficile* counts, as high numbers of this genus in CDI patient faeces have also been associated with treatment success and lower chance of recurrence [296]. In particular, *B. fragilis* abundance has been reported to have an inverse association with CDI [303]. This may indicate that batch co-culture experiments such as this cannot expose the inhibitory mechanisms *Bacteroides* spp.

exert on *C. difficile*, or that they have a wider role in colonisation resistance such as a promoting effect on inhibitory species or working synergistically with anti-CDI agents. The *B. fragilis* strain ZY-312 has been observed to inhibit *C. difficile* adherence to colonic HT-29 cells, maintain colonic epithelial integrity upon *C. difficile* toxin exposure *in vitro*, and prophylactically increase the survival rate of CDI mice [304], which suggests promotion of host protective mechanisms are involved.

The presence of SCC did not affect the growth of any of the gut microbiota species included in the initial screen. Although a cytotoxin concentration of 2RU may be relatively low compared to levels seen in simulated gut model CDI (~5RU) [305], the lack of an inhibitory effect on the species tested here would still be expected at higher concentrations since the cytotoxic action of *C. difficile* toxins is mammalian cell-specific [306]. As no inhibitory effect was observed further analysis of other components in SCC was not assessed.

As demonstrated in previous studies, and the results from Chapter 1, vegetative *C. difficile* proliferation and toxin production can be inhibited when co-cultured with a healthy intestinal microbiota [222]. Chapter 2 investigated this phenomenon at its simplest level, single species isolated from the gut model grown in a co-culture with *C. difficile.* Although a batch broth co-culture assay cannot reflect the intricate environment of the colon and the complex interactions between *C. difficile* and the gut microbiota, they are a valuable method to consider the individual relationships between single species of the microbiota and *C. difficile*, with which to drive further experiments. BHI was chosen as the growth medium for this study as it is one of the most widely utilised broth formulations for *C. difficile* enrichment and growth in research labs. Results from Chapter 1 also confirmed BHI was optimal for *C. difficile* growth, allowing for a full appreciation of any inhibition. All the test species extracted from the gut model used in this experiment grew well in a BHI monoculture. BHI was therefore deemed an ideal medium for the co-culture experiments. *C. difficile* ribotype 027 was used because of its clinical relevance and production of cytotoxin.

Unfortunately, co-cultures inoculated with *C. difficile* spores were not investigated due to limited lab support allocation. This would have helped produce a more thorough exploration of growth, germination and sporulation in co-cultures with single or multiple microbiota species.

It is unlikely that one species or strain alone would be sufficient to restore a depleted gut microbiota and confer lasting colonisation resistance. As these results and similar studies have shown, specific gut bacteria species have a wide range of potential CDI-inhibiting functions, which most likely work synergistically.

This study demonstrated a small range of gut microbiota species can individually inhibit the growth and toxin production of *C. difficile* in a liquid co-culture, in keeping with other similar studies. However, the assay did not lend itself to expressing the full range of mechanisms from species known to have specific inhibitive effects on *C. difficile*

Chapter 3 Effect of Faeces on the Bioactivity of Antimicrobials used to Treat *C. Difficile*

3.1 Background and Rationale

For metronidazole, vancomycin and fidaxomicin to effectively treat CDI, *C. difficile* inhibitory intracolonic levels need to be achieved. Faecal concentrations between these three antimicrobials vary widely [307-309]. Variations in faecal concentrations are also seen between patients receiving the same antimicrobial [310-312].

Vancomycin and fidaxomicin both have low bioavailability and therefore reach the colon at high concentrations - ~500mg/L [308] and >1000mg/L [310], respectively - whereas metronidazole is highly bioavailable (>90% absorption) [313], is metabolised in the liver and is mainly excreted via the kidneys (~77%) [314], with variable faecal excretion [309]. Although differing mechanisms of delivery of these antimicrobials to the colon can partially explain variations in faecal concentrations of different antibiotics, other factors are likely to contribute to the observed differences between individuals [307-309].

Previous studies have indicated that faecal matter may inhibit antibiotic activity [315-318] and that the inhibition can vary between individual stool samples [319]. However, these studies are few and did not explore antibiotics used in CDI therapy.

A pilot study evaluating the effect of faecal matter from three individuals on metronidazole, vancomycin and fidaxomicin showed substantial inhibition of bioactivity [320]. The level of inhibition varied between different faecal samples and appeared to correlate with the diversity of the faecal microflora. As the pilot study only included faecal samples from three individuals, it remains unclear how much variation was due to the direct action of microflora populations present or other components in faecal matter.

Many factors affect gut microbiome composition, including long- and short-term diet [191-193]. As well as modulating gut microbiota populations and diversity, diet also

influences the metabolites produced by specific microbial communities and host cells [321, 322]. Food consumption also directly contributes to the major organic components in faeces, such as undigested polysaccharides [255], proteins [252] and fats [323], as well as inorganic minerals [254]. There is no data to the authors knowledge on the effect of dietary composition on the bioactivity of antimicrobials in the lower digestive tract. Most data related to diet and the efficacy of antibiotics is rightly related to the bioavailability of antibiotics with particular foods in the upper GIT [324]. However, dietary macronutrients could potentially have an influence on antimicrobial bioactivity within the colon, especially for large molecule antibiotics that are not absorbed in the upper GIT.

The potential inhibitory effect of faecal matter on antimicrobials requires further exploration to elucidate the underlying mechanisms at play. Few safe alternative first-line therapies exist for CDI and as resistance to current antimicrobial treatments becoming a major issue, it is vital to have a treatment regime that is used optimally. Therefore, it is necessary to assess the impact of gut microflora diversity on antimicrobial activity and the effect diet may play to enhance our understanding.

The primary aim of this study is to investigate the effect of raw and sterilised faecal matter on the bioactivity of metronidazole, vancomycin and fidaxomicin. It is hypothesised that raw faeces will have more of an inhibitory effect on antibiotic bioactivity than sterile faeces or a non-faeces control.

The secondary aims of this study are to determine potential relationships between particular bacterial populations in the gut microbiota, at the genus level, or macronutrients from the diet with antimicrobial bioactivity.

3.2 Materials and Methods

3.2.1 Faecal Donors

Faecal samples were donated by informed and consenting volunteer donors, recruited from a range of people known to the author, including friends, family, students and staff of the University of Leeds, and their family and friends. Participant criteria included any healthy (no diagnosed health issues or issues under investigation), adults (≥18 years old) with no history of antimicrobial therapy in the previous 3 months. Participant metrics were not recorded as these were deemed unnecessarily intrusive to an already labour-intensive participant protocol.

Volunteers were asked to provide a faecal sample and to fill in an optional food diary for 3 days in the lead-up to providing the sample.

Each sample and corresponding food diary was assigned a study number to provide pseudonymity.

3.2.2 Ethical Considerations

Ethical approval for the study was granted by the University of Leeds Faculty of Medicine and Health Ethical Review Panel, reference MREC16-002 (Appendix A.3.1).

All potential volunteers were provided with a participant information sheet (Appendix A.3.2) with full details of the study and given a minimum of 24 hours to read it and ask questions. Willing volunteers were provided with a consent form (Appendix A.3.3) to confirm they understood the purpose and nature of the study, what they were being asked to do, and that they were under no obligation to take part.

3.2.3 Preparation of Faecal Samples

Eighteen faecal samples were donated. Faeces were collected by the donating volunteer, placed in a sterile plastic pot and were either immediately placed into a sealable plastic pouch with an AneroGen™ Compact sachet to reduce the oxygen

content to 1% within 30 minutes, or placed directly into a Don Whitley A95 anaerobic workstation for a maximum of 8 hours before being processed.

The 18 faecal samples were individually emulsified in pre-reduced phosphate buffer solution PBS (BioVision, USA) 20% w/v, mixed using a stomacher, and course-filtered through a sterile muslin to remove particulate and fibrous matter. The emulsion was then divided into three portions:

- Raw: no further intervention
- Filtered: Centrifuged 10000g for 10 minutes and the supernatant filtered through a 0.22m filter (Millex-GP Merck Millipore LTD)
- Autoclaved: Heat sterilised in 5ml aliquots in glass Wasserman tubes at 126°C for 15 minutes.

Each preparation was immediately pre-reduced once processed. Autoclaved and filtered faeces were tested for sterility by plating onto CBA plates and incubating anaerobically and aerobically for 48 hours.

3.2.4 Enumeration of Microbiota Populations from Raw Faecal Emulsion

For all target microbiota populations, excluding spores, the raw faeces in PBS 20% w/v emulsion was diluted 10-fold to a 10^{-7} concentration in pre-reduced peptone water. Spores were enumerated by adding an equal volume of 96% ethanol to the raw faeces-PBS emulsion, incubating at room temperature for 1 hour, and diluting 10-fold to a 10^{-3} concentration in peptone water. Each dilution (20µl) was plated onto pre-reduced selective and non-selective agars (constituents Appendix A.1.1), in triplicate, and incubated as indicated in Table 3-1.. Post-incubation CFU were counted by eye.

Table 3-1 Selective and non-selective agars used to enumerate target gut microbial populations and their growth conditions

Target populations	Agar	Supplements	Growth Conditions
Total anaerobes and total spores	Fastidious anaerobe agar	5% horse blood	48-hour at 37˚C Anaerobically
Bifidobacteria	Beerens agar - 42.5 g/L Columbia agar, 5 g/L agar technical	0.5 g/L cysteine HCl, 5 g/L glucose and 5 ml propionic acid, adjusted to pH 5	48-hour at 37˚C Anaerobically
***Bacteroides fragilis* group**	Bacteroides bile esculin agar	5mg/L haemin, 10µL/L vitamin K, 7.5 mg/L vancomycin, 1 mg/L penicillin, 75 mg/L kanamycin and 10 mg/L colistin	48-hour at 37˚C Anaerobically
Lactobacilli	LAMVAB agar – 20 g/L agar technical, 52.2 g/L MRS broth	0.5 g/L cysteine HCl, 20mg/L vancomycin	48-hour at 37˚C Anaerobically
Total facultative anaerobes	Nutrient agar		24-hours at 37˚C Aerobically
Lactose fermenting *Enterobacteriaceae*	MaConkey's agar		24-hours at 37˚C Aerobically
Enterococci	Kanamycin Aesculin Azide Agar	10 mg/L nalidixic acid, 10 mg/L aztrianoam and 20 mg/L kanamycin	48-hours at 37˚C Aerobically

3.2.5 Preparation of Antimicrobials

Metronidazole (Sigma-Aldrich) and vancomycin (Cayman Chemical Company) were prepared with sterile H_2O and fidaxomicin (APEXBIO) with Dimethyl Sulfoxide (DMSO) (Thermo Scientific), to make a 2000mg/L concentration stock solution. Fresh antibiotic stock solutions were made <30 minutes before adding them to the faecal samples. For each experimental condition, a separate H_2O and DMSO control were included.

3.2.6 Incubation of Antimicrobials in Faecal Slurry and PBS-Controls

Stock solutions of metronidazole, vancomycin and fidaxomicin were diluted 1:10 to give a 200mg/L concentration in each of the 18 raw, autoclaved and filtered faecal preparations, pre-reduced PBS control, and aerobically refrigerated PBS control (Fig. 3- 1).DMSO and H_2O controls (without fidaxomicin) were also carried out in each faecal slurry preparation and PBS. All 18 faecal preparations were incubated anaerobically at 37˚C and sampled after 24 hours. Of these 18 preparations, 8 were sampled at timed intervals (1, 2, 4 and 6 hours), and 3 of these 8 were also sampled at 0.5-hours. At each sample time, 1ml of each preparation was removed, centrifuged (10000g) and the supernatant was stored at -20˚C.

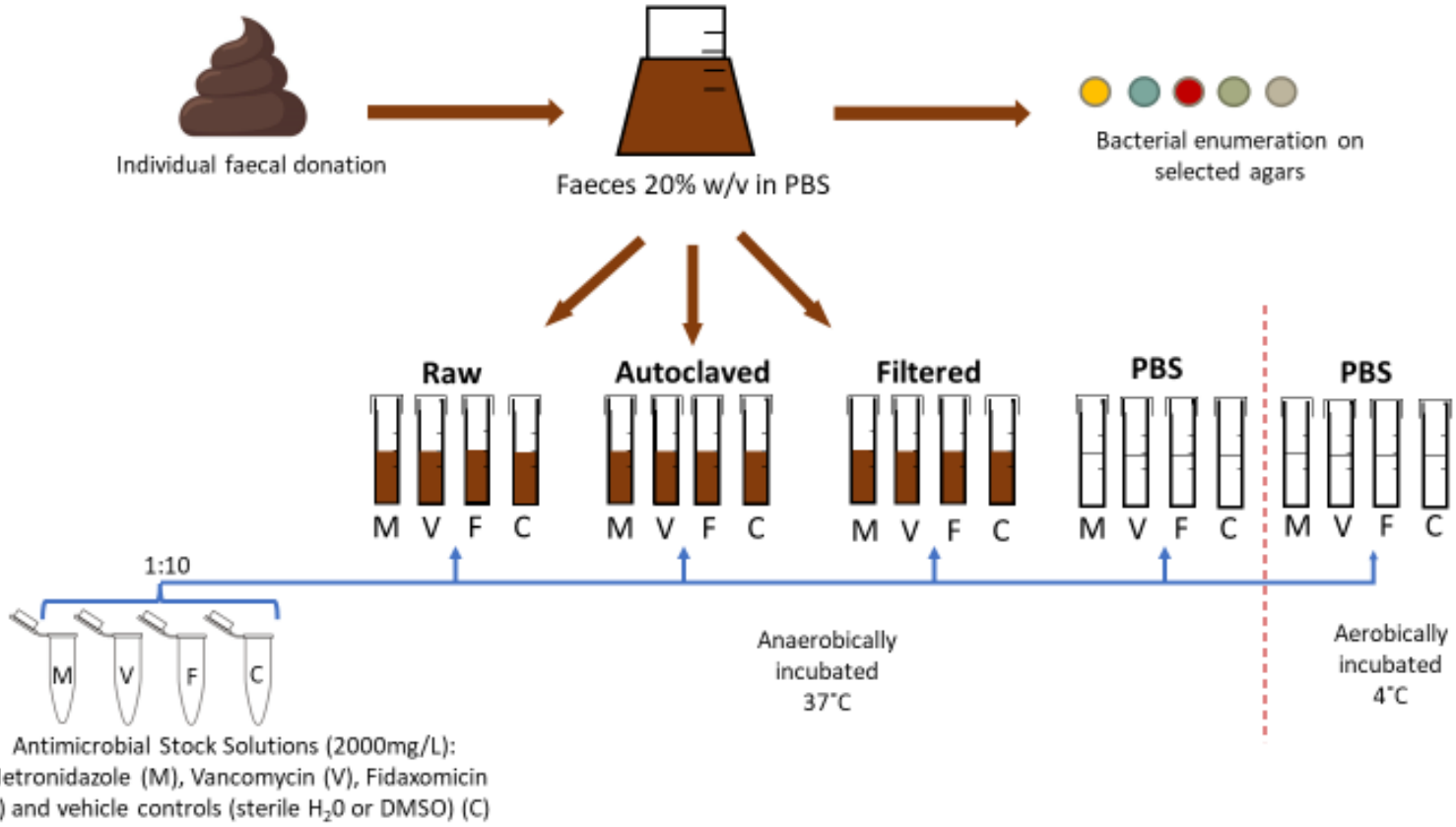

Fig. 3- 1 Overview of the methodology used to process each faecal donation and non-faeces controls. Each faecal donation was divided into 3 parts; raw, autoclaved and filtered. Three antimicrobials (Metronidazole (M), Vancomycin (V), Fidaxomicin (F)) and vehicle controls (C) (H_2O or DMSO) were administered to each faecal preparation and PBS-controls to a final concentration of 200mg/L. All 18 faecal donations were sampled to assay for antimicrobial concentration at 24 hours. 8 of the 18 faeces were also sampled for assay at 1, 2, 4, and 6 hours. Three of the 8 faeces were sampled at 30 minutes.

3.2.7 Determination of Antimicrobial Bioactivity by Bioassay

Bioactive antimicrobial concentrations following incubation of each of the individual faecal samples were determined by large plate bioassay using the following indicator organisms and media: metronidazole on Wilkins-Chalgren agar (Oxoid) with a laboratory strain *Clostridium sporogenes,* isolated in house from the gut model and identified with MALDI-TOF. *C. sporogenes* was incubated anaerobically overnight in BHI; vancomycin on Mueller-Hinton agar (Oxoid) with *Staphylococcus aureus* (ATCC 29213); fidaxomicin on Wilkins-Chalgren agar with *Kocuria rhizophila* (ATCC 9341). Bioassay agar (100ml) was sterilised by autoclave, cooled to 50˚C and inoculated with 1ml indicator organism (0.5 McFarland standard suspension measured by densitometer) and poured into 245mm^2 dishes. Once set and dried, 25 x 9mm wells were dug in the agar (no.5 cork borer). This process was carried out under anaerobic conditions for the *C. sporogenes* bioassay. A doubling dilution calibration series from 4 - 256mg/L was prepared for each antibiotic. Antibiotic calibrator or defrosted and centrifuged test samples were added to the wells (20µl), and incubated for 24 hours aerobically (vancomycin and fidaxomicin) or anaerobically (metronidazole). Zones of inhibition were measured using callipers accurate to 0.1mm. Calibration lines were plotted from zone diameters squared against calibration series concentrations Log2, and active concentrations of samples were determined by calculating the diameter of the sample against the calibration line. Each test sample was assayed between 3 and 6 times on separate bioassay plates.

3.2.8 Antimicrobial Activity in Faeces Statistical Analysis

Data distribution testing and statistical analysis were performed on SPSS version 22. Before statistical analyses, data were assessed for normality using Shapiro-Wilk and Kolmogorov-Smirnov tests. A one-way ANOVA with post hoc Tukey test was utilised to determine the significance of differences between the mean antimicrobial concentrations from the raw, autoclaved and filtered faecal preparations and the PBS control. A paired t-test was employed to determine the significance of differences

between mean antimicrobial concentrations in a particular faecal preparation over time. A statistical significance level of <0.05 was adopted, <0.01 very significant, and <0.001 highly significant.

N=the number of individual faecal samples before processing. Means of antimicrobial bioactivity from the raw, autoclaved and filtered faecal slurry samples, and PBS controls, at their respective time point (n at each time point is stated throughout results) are reported with standard error of the mean (±SE).

3.2.9 Microbiota Populations and Antimicrobial Activity Statistical Analysis

Genus level population data collected as log_{10}CFU/mL was compared to the bioactivity data in raw faeces for all three antibiotics correlated and the percentage of variation was calculated (R^2). For each antimicrobial, the time point with the greatest number of samples with detectable antimicrobial activity was analysed.

3.2.10 Participant Nutritional Data

A semi-weighed account of all foods, drinks and supplements consumed was recorded by 8 participants in a standard paper food diary (Appendix A.4.3) Participants were instructed to detail approximate portion sizes, or exact weights if known, as well as brand names and weights given on packages of processed foods, during the 3 days leading up to the production of the donated stool sample. Each 3-day food diary data was input into WinDiets Professional (Robert Gordon University), a suite of tools based on the UK and US nutrition databases that allow for extensive nutritional analysis. Diary data was inputted by the author, utilising specialist nutritional experience and knowledge to compensate for any gaps or discrepancies in the diet records, along with the application of appropriate UK population-specific resources [325, 326]. The 3-day food data was averaged for one day and presented as: percentage of energy intake (%E) from protein, carbohydrate and fat, Kcal/day for overall energy intake, and g/day for non-starch polysaccharides (NSP). Participants

were also asked to categorise their diet into a specific diet category as per Table 3-2 List of Diet Categoriesand to note any dietary supplements or nutritional complementary therapeutics, including pre or probiotics.

Table 3-2 List of Diet Categories

Diet	Description
Omnivorous 1	Eats red meat, poultry or fish at least once per day
Omnivorous 2	Eats red meat, poultry or fish at least three times per week
Semi-vegetarian 1	Eats poultry, fish, dairy or eggs regularly but rarely eats red meat
Semi-Vegetarian 2 (Pescatarian)	Eats fish, dairy or eggs regularly but rarely eats poultry or red meat
Vegetarian	Eats dairy or eggs
Vegan	Does not eat anything of animal origin

3.2.11 Nutritional Data and Antimicrobial Activity Analysis

The average 3-day percentage of total percentage energy (%E) from each macronutrient (protein, fat, carbohydrate and alcohol), Kcalories and NSP in grams from 8 participants were compared with the bioactivity data for each antibiotic at the 1-hour time interval, correlated and the percentage of variation calculated (R^2).

3.3 Results

3.3.1 Antimicrobial Activity in Raw Faeces Compared to Autoclaved and Filtered Faeces, and a PBS Control after 24-Hours Incubation

The bioactive concentrations of all three antimicrobials in raw faeces were significantly lower compared with the PBS control solutions at 24 hours (n=18): Observed decreases compared to the PBS control were: 74.7% decrease for fidaxomicin ($p<0.001$), 60.5% for vancomycin ($p<0.001$), and 99.9% for metronidazole ($p<0.001$) (Fig. 3- 2)

Metronidazole concentrations in raw faeces were also significantly lower than in autoclaved (99.8%) and filtered faeces (99.8%) ($p<0.001$). Whereas there was no significant difference between the fidaxomicin in raw and autoclaved faeces, and vancomycin bioactivity was 42.1% lower in autoclaved faeces compared to raw ($p=0.031$). Both the fidaxomicin and vancomycin bioactivity in raw and autoclaved faeces were significantly lower than in filtered and PBS controls ($p<0.001$) (Fig. 3- 2).

3.3.2 Antimicrobial Activity Over Time

Mean bioactivity in raw faecal samples was similar for all antimicrobials between the 0.5-hour (n=3) and 1 hour (n=8) assay times (Fig. 3- 3), therefore the 1 hour measurement was used for further analysis due to the larger sample size.

A significant decrease in bioactive concentrations was seen in raw faeces compared to the PBS control: at 1 hour metronidazole bioactivity decreased by 77.5% ($p<0.001$), and continued to decrease over time by 93.9% at 2 hours ($p<0.001$), 98.6% at 4 hours ($p<0.001$), and the concentration falling below the limit of detection at 6 hours (Fig. 3- 3a). A 39.2% decrease for vancomycin ($p<0.001$) (Fig. 3- 3b), and a 64.8% decrease for fidaxomicin were observed at 1 hour ($p<0.001$) (Fig. 3- 3c). Although vancomycin and fidaxomicin bioactivity in raw faeces appear to slightly

decrease further over time (Fig. 3- 3 b & c) this decrease was not statistically significant between 1 and 24 hours.

3.3.3 Control and Quality Control Data

No significant difference was seen between the pre-reduced, anaerobically incubated PBS control and the aerobically refrigerated PBS control (data not shown). DMSO incubated in faeces without fidaxomicin did not have any inhibitory effect on the bioassays (data not shown). All CBA plates testing for autoclaved and filtered faeces sterility were clean after 48 hours.

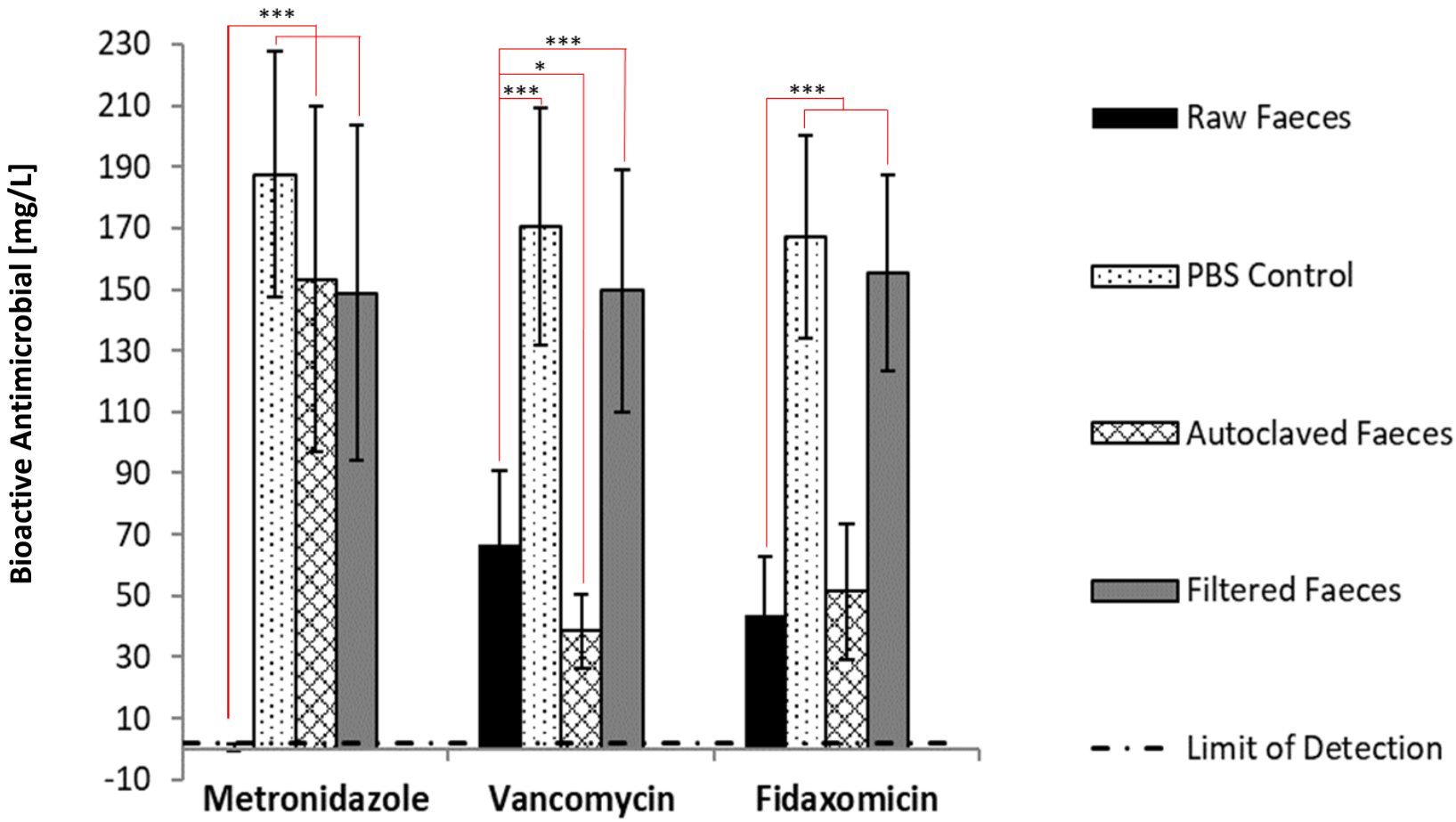

Fig. 3- 2 Mean (±SD) antimicrobial concentrations (mg/L) in raw (n=18), autoclaved (n=18) and filtered (n=18) faecal samples and a PBS control (n=14) after 24-hour incubation as measured by bioassay with a minimum of 3 technical repeats. Significant results highlight with * ($p<0.05$), very significant ** ($p<0.001$), and highly significant with * ($p<0.001$). Limit of detection = ~2mg/L**

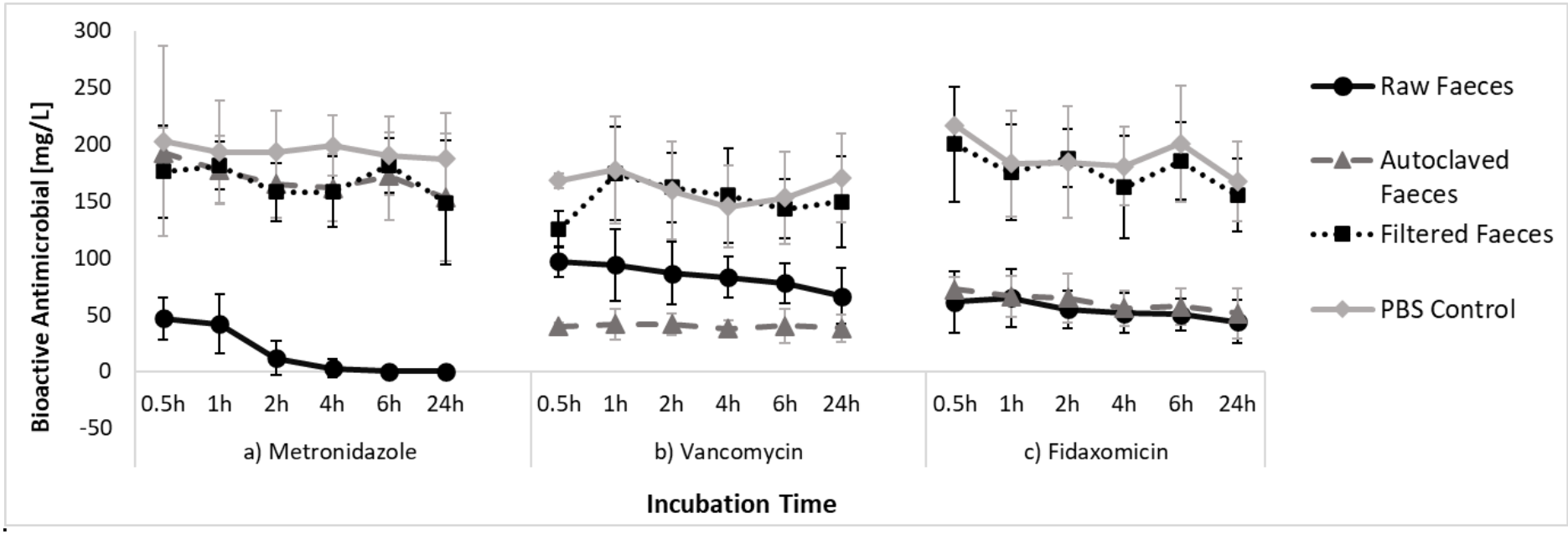

Fig. 3- 3 Mean bioassay concentrations (mg/L) at different time points of individual faecal samples incubated with a) metronidazole, b) vancomycin, c) fidaxomicin (0.5 hour: n=3 / 1,2,4,6 hour: n=8 / 24 hour: n=18). PBS Controls (0.5 hour: n=2 / 1,2,4,6 hour: n=6 / 24 hour: n=14). Limit of detection = ~2mg/L. Each faecal sample and control was inoculated with (1:10) 2000mg/L stock solution of antimicrobials to achieve a starting concentration of 200mg/L at 0 hour.

3.3.4 Variation in Bioactivity Between Individual Raw Faecal Samples

Variation in antimicrobial bioactivity was observed between individual raw faecal samples with decreases compared to the PBS control ranging from 27.5 – 86.6% for vancomycin, and 48.9 – 86.9% for fidaxomicin at 24 hours (n=18) (data not shown). Metronidazole variation is best demonstrated at the 1-hour measurement as there was bioactivity recorded in all 8 samples taken at that time point with decreases of 57.8 – 97.7% in raw faeces compared to the PBS control. The time point at which metronidazole became undetectable also differed between the 8 individual samples; 2 samples were undetectable at 2 hours, and all 8 samples by 6 hours (Fig. 3- 4). Only 1 of the 18 raw faecal samples measured at 24 hours had detectable metronidazole concentrations, with a 97.4% decrease from the PBS control (data not shown).

Although mean metronidazole concentrations were similar at 0.5-hour (46.6mg/L) (n=3) and 1 hour (41.7mg/L) (n=8) in raw faeces (Fig. 3- 3), Fig. 3- 4shows that the 3 faecal samples measured at 0.5 hour decreased by 61.9% (p=0.03) between 30 minutes and 1 hour.

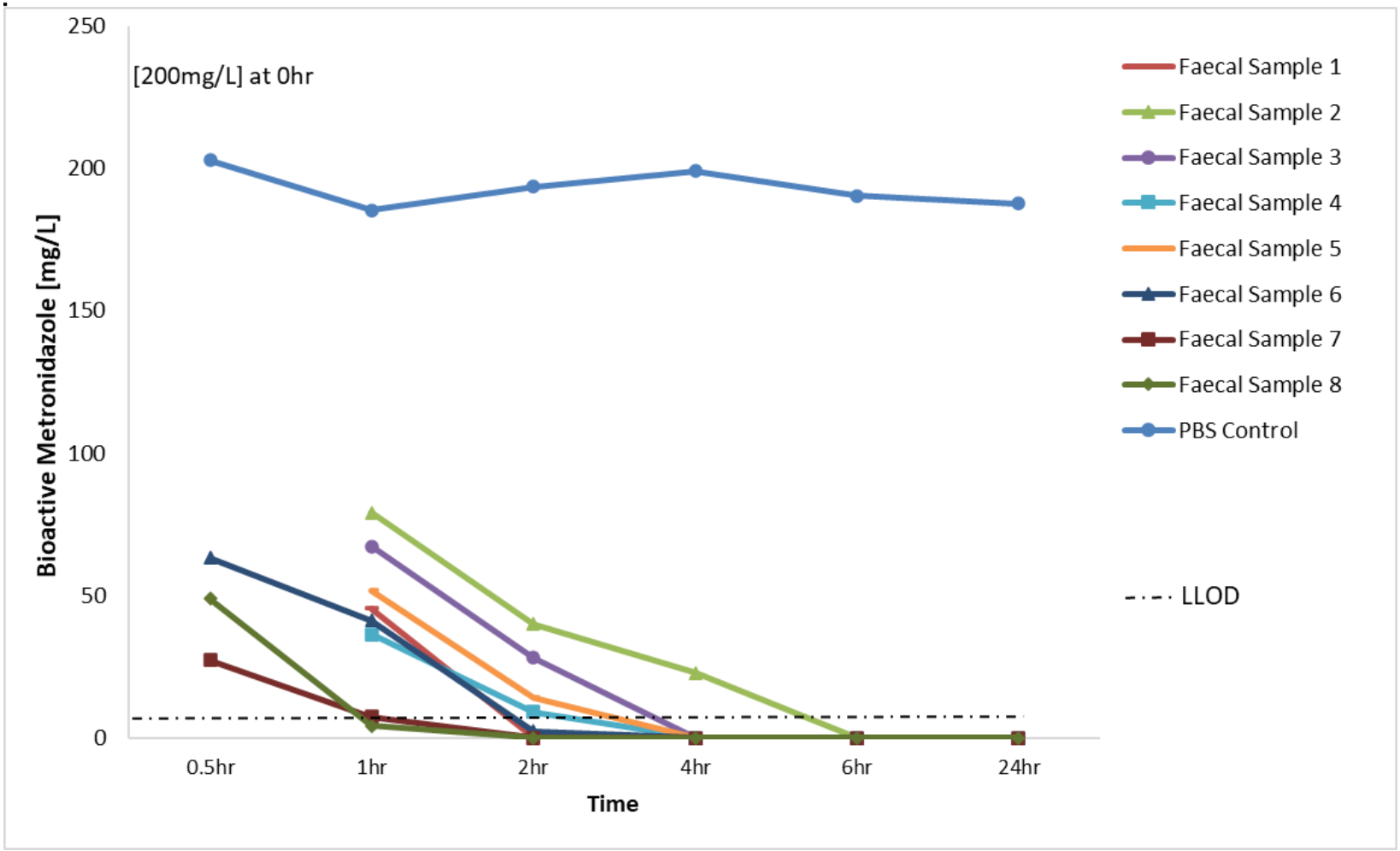

Fig. 3- 4 Metronidazole bioactive concentrations in 8 individual faecal samples over time. All samples were prepared with a 2000mg/L stock solution of metronidazole to achieve an initial concentration of 200mg/L at 0 hours. 3 of 8 samples also measured at 0.5-hours. LLOD = Lower limit of detection

3.3.5 Participant Microbiota Data

The percentage abundance of the bacterial groups recovered from bacterial culture on selective agars from each of the 18 participants are detailed in Fig. 3- 5. *Bacteriodes* data was unavailable for samples 5 and 11. Two samples (4 and 5) had undetectable *Lactobacilli.*

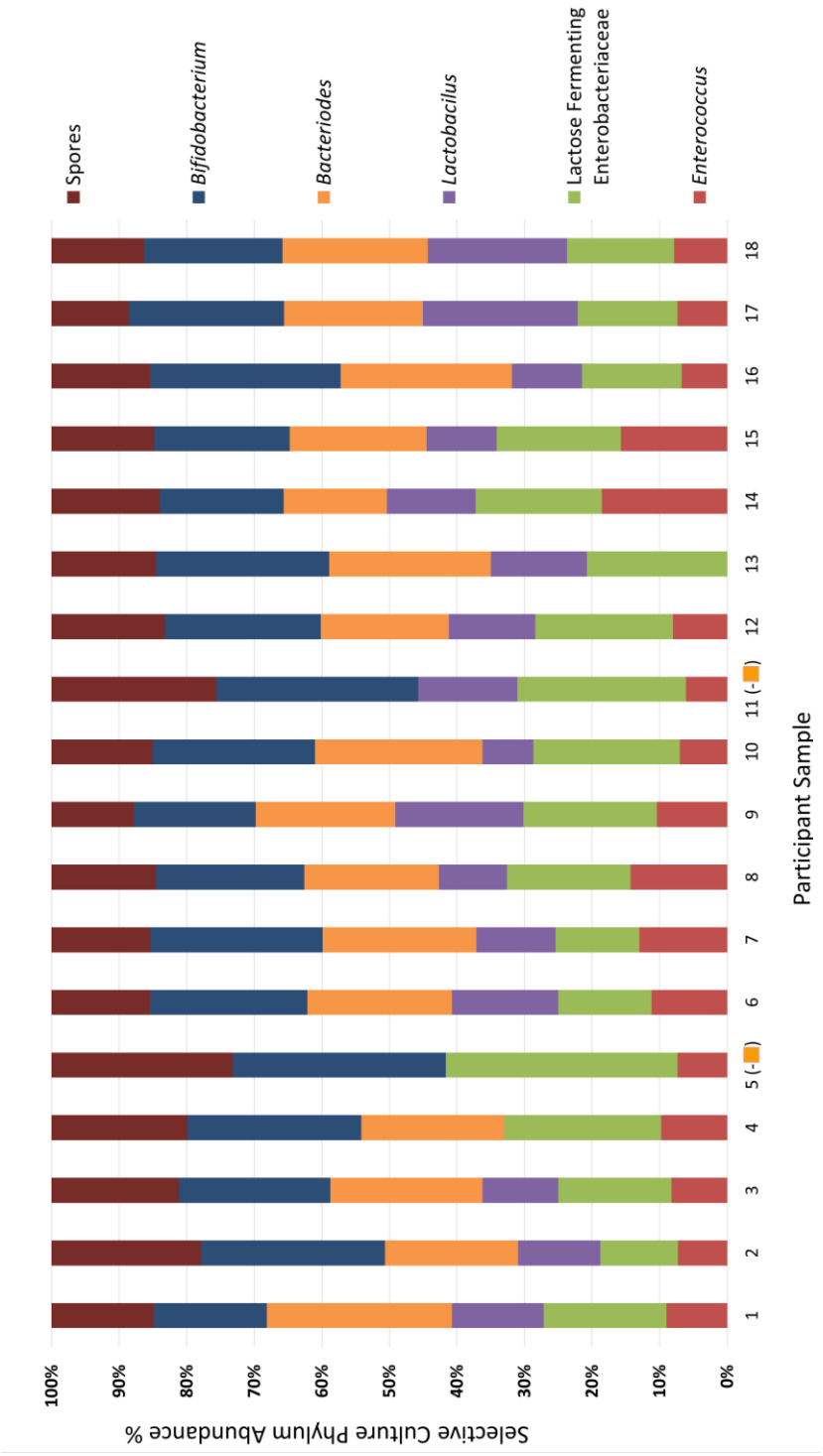

Fig. 3- 5 Bacterial taxonomic abundance of groups recovered from each participant faecal sample. Bacteria counted by eye from cultures grown on selective agars. Bacteriodes were unavailable for samples 4 and 5.

3.3.6 Metronidazole in Raw Faeces and Microbiota Populations

Metronidazole bioactivity at 1 hour in raw faeces from 8 individuals was compared to the cultured genus level microbiota population data. Of the genus groups observed, only lactobacilli were found to have a significant correlation of decreasing bioactive metronidazole concentrations with increasing CFU $\log_{10}$ lactobacilli (n=8) R^2=0.7 (p=0.01) (Fig. 3- 6). It is also worth noting that the only sample with recorded metronidazole bioactivity (4.9mg/L) at 24 hours was also two samples without detectable lactobacilli (Fig. 3- 5). Unfortunately, there was no incrementally timed data for these samples.

No other correlations were found between metronidazole and other genus level groups (data not shown).

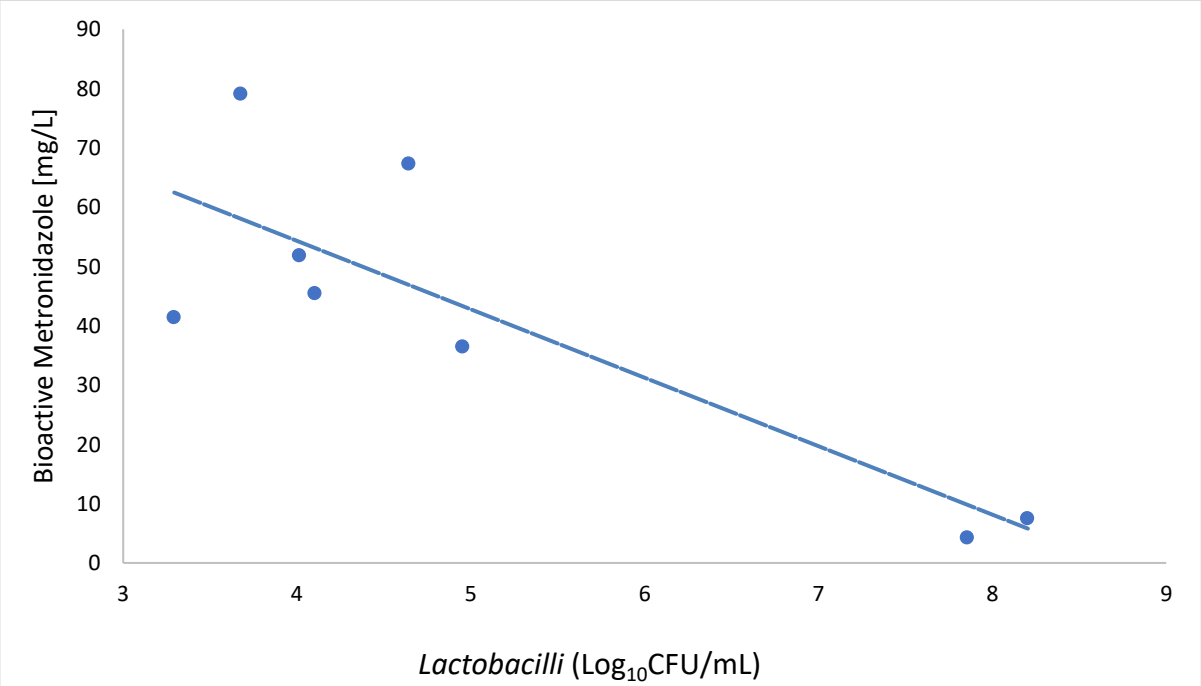

Fig. 3- 6 Individual metronidazole concentrations as measured by antimicrobial bioassayafter 1 hour incubation in 8 raw faecal samples compared to their respective lactobacilli TVCs recovered on LAMVAB agar.

3.3.7 Participant Nutritional Data

Table 3-3 Individual participant diet categories and mean 3-day macronutrients as a percentage of diet energy (%E), overall energy intake (Kcal), and non-soluble polysaccharides (NSP) (g) from semi-weighed food diary

		Mean of 3 days					
Participant	**Diet Type**	**Kcal**	**Fat %E**	**Prot %E**	**CHO %E**	**Alc %E**	**NSP (g)**
1	Omnivorous 1	2907	34%	20%	46%	0%	18.7g
2	Omnivorous 1	2975	36%	15%	48%	0%	9.8g
3	Omnivorous 2	2269	41%	12%	47%	0%	18.5g
4	Omnivorous 2	3050	38%	19%	39%	5%	14.8g
5	Omnivorous 1	2542	40%	16%	37%	7%	16.3g
6	Omnivorous 1	2242	37%	18%	37%	7%	14.5g
7	Vegan	3499	27%	13%	54%	7%	55.2g
8	Vegan	2496	29%	10%	61%	0%	29.5g

Of the small number of food diaries collected from participants four ate an omnivorous diet with meat every day (omnivorous 1), two ate an omnivorous diet with meat at least 3 times a week (omnivorous 2) and two ate a vegan diet (no meat or animal products). From this very small data set it appears the vegan diet had a higher content of carbohydrate and NSP than an omnivorous diet and lower protein and fat percentages, but these differences were not significant ($p>0.1$) (Fig. 3- 7).

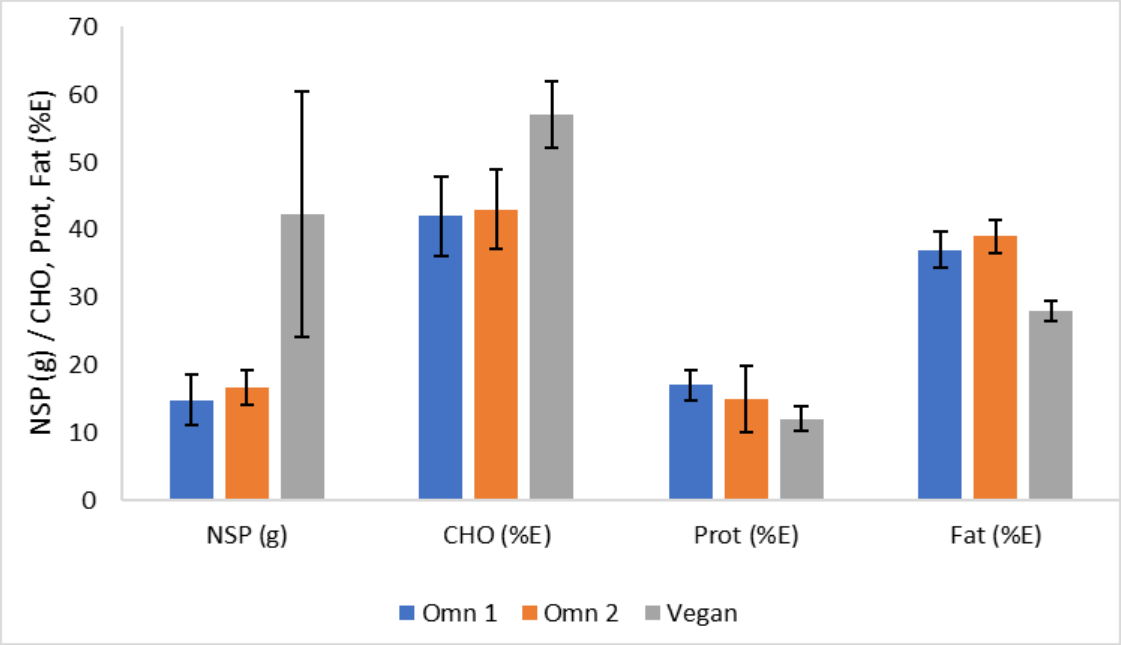

Fig. 3- 7 Mean non-soluble polysaccharide (NSP) (g), carbohydrate (CHO) (%E), protein (Prot) (%E) and fat (%E) from 3 day food diaries. Omnivorous 1: n=4; Omnivous 2: n=2; Vegan: n=2.

3.3.8 Nutritional Data and Antimicrobial Activity Analysis

Individual participant NSP, carbohydrate, protein, fat and Kcal were compared to their respective bioactive antibiotic concentrations at 1 hour. There were no significant correlations for the percentage energy of fat, protein, carbohydrate or grams of NSP with metronidazole in raw faeces, or vancomycin or fidaxomicin in raw or autoclaved faeces (data not shown).

3.4 Discussion

3.4.1 Antibiotic Bioactivity in the Presence of Faeces

Raw faeces have a significant inhibitory effect on the bioactivity of all three antimicrobials. The inhibition is most marked for metronidazole. A decrease occurs within 30 minutes of raw faeces exposure for all agents. While antimicrobial bioactivity is retained for vancomycin and fidaxomicin over time, it decreased to undetectable levels for metronidazole. The variation in bioactive concentrations between antimicrobials, and between the different faecal preparations, suggest inhibitory mechanisms specific to individual antibiotics. The significant decrease of metronidazole bioactivity in raw faeces compared with sterile filtered and autoclaved faeces implies live microorganism involvement. Conversely, the lack of a significant difference for fidaxomicin bioactivity between raw and autoclaved faeces, and lower vancomycin bioactivity in autoclaved compared with raw, may indicate antimicrobial binding to components in non-filtered faeces, >0.22µm, independent of live microorganisms, as indicated by significantly higher bioactivity in filtered faeces.

Other studies have reported inactivation of β-lactam antibiotics in the presence of faeces [315, 318], with the deactivation differing between individuals, as well as samples from the same individuals at different time points [319]. Nagy and Foldes [327] observed *in vitro* inactivation of metronidazole occurring in the presence of *Enterococci faecalis* and a decline in concentrations with cell-free *E. faecalis* supernatants, suggesting that specific species of the faecal microbiota might mediate the activity of antimicrobial agents. Interestingly, the data (not shown) from the current study did not see a correlation between numbers of *Enterococcus* spp. and metronidazole activity or any considerable metronidazole decreases in deactivated (autoclaved) or cell-free (filtered) faeces. However, a significant negative correlation between metronidazole bioactivity after 1 hour in raw faeces and *Lactobacillus* spp. recovery was observed. Although the correlation was drawn from a small sample size

(n=8), it should also be noted that the only sample showing metronidazole activity at 24 hours had no detectable *Lactobacillus* spp. (data not shown). This finding corresponds with Lee *et al* [328] who reported that non-target *Lactobacillus* spp. may sequester metronidazole and lower the amount available to target organisms using an ordinary differential equation model, validated by *in vitro* co-cultures and clinical cohorts of women with bacterial vaginosis.

Inhibition of metronidazole in the presence of a particular group of organisms is an important consideration, particularly for CDI patients – for whom metronidazole treatment continues to be used, despite guidelines recommending against it [110, 329] – as they are more likely to have considerable gut microbiota dysbiosis with disproportionately high levels of particular organisms such as *Enterococcus* spp. [273]. Single and multi-strain *Lactobacillus* and *Enterococcus* spp. are also readily available in many commercial probiotic products promoted for general well-being [330] and diseases of the digestive system [331-334], including mild cases of CDI [158]. Potential inhibition of metronidazole by *Lactobacillus* and *Enterococcus* spp. would not only have implications for the treatment of CDI but also for other disease states, such as bacterial vaginosis, for which probiotic *Lactobacillus* spp. are a consideration alongside first-line metronidazole [335, 336].

Faeces from CDI patients were not tested in this study due to potential confounding by the presence of other antimicrobials. Greater diversity of gut microbiota in healthy patients may be associated with more metronidazole inhibition, in which case bioactive levels may be higher in patients with a disrupted microbiota during CDI. However, dysbiosis in CDI patients, potential intake of indiscriminate probiotics, and promotion of particular groups of organisms may exacerbate the inactivation of metronidazole.

Metronidazole has a broad spectrum of activity [311]. It can be ‘uptaken’ by anaerobic and aerobic cells, via passive diffusion [337], however, it is only cytotoxic to obligate,

and some facultative, anaerobic organisms [338]. In susceptible organisms, the metronidazole nitro group is reduced producing a free radical, which causes DNA damage and thus cell death [339]. *In vitro* studies have shown that decreases in metronidazole concentrations correlate with bactericidal activity when incubated with susceptible organisms [327, 340, 341]. Additionally, it has been reported that metronidazole can be inactivated in the presence of some non-susceptible bacteria [340, 342]. Although the exact mechanisms by which non-susceptible organisms inactivate metronidazole are not confirmed, this sequestering effect by non-susceptible organisms along with consumption by susceptible organisms may be a possible reason for the decline in bioactive metronidazole concentrations in the presence of raw faeces.

Bolton and Culshaw [309] reported metronidazole achieves low faecal levels (~9mg/L) in loose stools from CDI patients at the start of treatment, which then decreases during recovery with considerably lower concentrations in formed stools. They postulated that the decrease may be due to metronidazole's pharmacokinetics, whereby it is absorbed in the upper gastrointestinal tract and secreted into the colon depending on the degree of local inflammation; the more inflammation and looser the stool, the higher secretion of metronidazole into the colon [309]. The results of the present study suggest low faecal levels of metronidazole may be exacerbated by deactivation upon "healthy" faecal exposure, therefore it is possible that even if inhibitory levels were achieved during initial CDI treatment, residual *C. difficile* in the gut could be exposed to sub-inhibitory concentrations during disease resolution as the stools firm, not only increasing the chance of recurrence but also potentially driving up MIC. Reduced susceptibility to metronidazole has been reported for the hyper-virulent *C. difficile* ribotype 027 (geometric mean (GM) MIC 1.24mg/L) and the emerging ribotype 198 (GM MIC 1.68mg/L) [73]. Furthermore, studies have shown that high concentrations of haem produced by colonic inflammation during CDI can be utilised by *C. difficile* to protect itself against oxidative stress from antimicrobials,

and a haem-dependant increase in metronidazole MIC has been seen across a range of clinically relevant ribotypes. In multivariate analysis, a metronidazole MIC ≥1mg/L was an independent predictor of initial clinical failure in patients receiving metronidazole treatment [347]. With increased metronidazole MICs for prevalent *C. difficile* strains [73], it is conceivable that the metronidazole inactivation upon exposure to colonic contents observed in the present study could affect clinical efficacy.

Despite the statistically significant reduction in bioactivity for fidaxomicin and vancomycin upon exposure to raw faeces, the clinical significance of this finding is less evident. The detected bioactive concentrations of 44mg/L and 66mg/L for fidaxomicin and vancomycin, respectively, in raw faeces even after 24 hours remain much higher than recently reported MICs of prevalent *C. difficile* ribotypes (MIC_{90} 0.125mg/L for fidaxomicin, and MIC_{90} 1mg/L for vancomycin [73]). Given that a starting 200mg/L concentration of antibiotic was examined, which is lower than recorded faecal levels during therapy (>1000mg/L for fidaxomicin [310] and ~500mg for vancomycin [308]), reductions in bioactivity of the magnitudes observed over time are unlikely to affect clinical efficacy.

Interestingly, concentrations of fidaxomicin and vancomycin also significantly decreased in autoclaved faeces compared to filtered faeces implying a lack of live microbial involvement in the mechanisms of inhibition. Although both antimicrobials retained bioactivity throughout the 24-hour sampling period, there was variation between individual faeces. Fidaxomicin and vancomycin in raw and autoclaved faeces from the same individuals followed a similar pattern of inhibition, supporting the notion that a component of the faeces - not removed or deactivated by heat sterilisation - may be inhibiting these antimicrobials. Where the presence of a live microbiota affecting faecal concentrations was not apparent, an exploration of other faecal components beyond the microbiota, such as diet,may be a useful future addition to understanding any potential limitations of these agents against CDI.

3.4.2 Diet and Antimicrobial Activity

No correlations were found between macronutrients or total energy intakes (as determined by dietary records) and antibiotic activity in faeces. However, the sample set was very small (n=8) and there was little variation in diet type and macronutrient content between individuals to draw out potential associations. To date, this is the first assessment of direct host diet and antibiotic bioactivity in faeces. Although no correlations were found in this small data set, the profound influence diet has on stool composition [252, 253, 255, 323, 348, 349] makes it a worthy add-on to experiments investigating the possible mechanisms underlying the inhibition of anti-CDI agents upon exposure to faeces. The mechanisms by which fidaxomicin and vancomycin concentrations decreased in raw and autoclaved faeces but not in filtered faeces is beyond the scope of this study, but it was hoped that a factor could be alluded to from the diet analysis results. Vancomycin is known to adhere to proteins in blood [350] and therefore, it is possible vancomycin could bind to proteins from undigested food residue, metabolites, or host cells affecting its concentrations in deactivated (autoclaved) faeces. Less is known regarding fidaxomicin's ability to bind to protein, however, other components in faeces that may be worthy of exploration concerning antibiotic bioactivity and diet are microplastics. There is an increasing body of evidence that microplastics have entered the human food system [351-356] and the presence of microplastics in human faeces has been identified [357-359]. Since fidaxomicin is a hydrophobic compound that may adsorb non-specifically to plastic surfaces [360] and vancomycin has also shown an ability to adsorb to plastics [361, 362] a regressive analysis of faecal antibiotic active levels with direct faecal microplastic concentrations may warrant further investigation given the extent of inhibition seen in raw and autoclaved faeces reported here.

Although no direct diet–antibiotic associations were found in this small study, diet-microbiome interactions have been found to drive antibiotic efficacy; with a synergistic effect of a high-fibre diet increasing the ability of antibiotics to reduce pathogenic

bacteria, and a high-fat diet associated with decreased antibiotic efficacy in mice [363], and therefore is worthy of exploration in humans.

3.4.3 Strengths, Limitations and Future Research

3.4.3.1 Donor Faeces

A range of healthy adult participants donated their stools to this study. Although a gut microflora lacking in diversity is integral to loss of colonisation resistance against - and the main risk factor for - CDI, obtaining faecal samples only from high-risk groups would have had an impact on sample number. Faeces from high-risk groups would have also risked confounding the results due to other variables such as residual antibiotic contamination and other drugs, given the frequent presence of co-morbidities in this cohort. Finding and accessing these particular participants would have also had ethical implications, as this would require obtaining sensitive data to identify them as a high risk of CDI. The sample numbers achieved in this study were adequate to show statistically significant differences between antimicrobial levels in faeces compared to controls but were not large enough to make confident inferences on the secondary objectives comparing microbiota populations and diet with antimicrobial concentrations.

3.4.3.2 Dietary Assessment

Although food transit time varies between individuals, and also within the same individual at different times and after consuming different food [364, 365] and substances consumed also do not move uniformly through the digestive system [366], for this preliminary study a short-term dietary assessment (3 days preceding stool production) was deemed an appropriate time scale to potentially reflect dietary components within the resulting faeces based on average transit times from multiple reports [252, 253, 255, 348, 349, 364, 365].

A detailed prospective semi-weighed account of all foods and drinks consumed was utilised to assess the participant's current direct diets. Although this will have incurred

a loss of precision compared with a weighed account, the semi-weighed approach is less labour-intensive and more practical for the respondent [367]. There were seemingly considerable differences in macronutrient density and NSP quantities in the vegan compared to the omnivorous diets, although these were not found to be significant from the small sample data in this study, the results were consistent with similar reports investigating omnivorous and vegan diets in Europe [368-372]. Diet categorisation, e.g. omnivore, vegan, etc. is a vague form of dietary assessment, however, as long-term diet can affect microbial communities it is a useful, low respondent and researcher burden method assessing long-term diet and can be combined with a prospective diet record, such as a semi-weighed account to validate nutrient densities and quantities, as seen from this study.

Future research in this area may want to consider new technologies in the study design to determine remnant nutrients in faeces, for example, faecal nutrimetabolomics, as an adjunct to traditional dietary assessment methods. However, nutrimetabolomics is a relatively new technology and although food intake biomarkers are assumed to provide a more objective reflection of intake, few biomarkers are sufficiently validated [373, 374], and should only be used as a long side traditional assessment methods.

3.4.3.3 Microbiota Populations

In this study bacterial populations in each stool sample were elucidated via culture on a series of solid selective agars. Although the original proposal for this study intended to use PCR 16S rRNA profiling to compare and confirm observed bacterial populations via culture methods and implement appropriate diversity indices with which to compare with antimicrobial data, this was not possible due to cost and time constraints.

3.4.3.4 Assessment of Antibiotic Activity

Some previous studies have measured faecal concentrations of antimicrobials and their metabolites via HPLC [309, 310]. Whilst HPLC is a sensitive, fast and precise method of quantifying the amount of antibiotic in a solution [375] it does not determine the bioactivity, and therefore their results may not reflect the overall potency of the antibiotic measured within the faecal sample. A bioassay, as utilised in the present study, can estimate both potency and bioactivity [376] and is arguably more clinically relevant, however, as with many non-automated assay techniques it can lack the precision afforded by automated quantitative techniques. Many factors can contribute to bioassay variability, including but not limited to agar thickness, inoculum concentration, incubation temperature, exposure-time duration and sample preparation. Samples were repeated on a minimum of 3 bioassay plates to increase confidence and multiples of the same sample were tested on the same bioassay plate to reduce the differential effects from growth, time and temperature between plates.

Due to the laborious nature of the assay and lack of laboratory assistance, not all of the 18 faecal samples were measured at timed intervals between 0 and 24 hours. The samples that were measured at smaller time intervals showed a significant loss of bioactivity in the raw faeces compared to controls in a short time frame. Further work could include even smaller time intervals after the addition of the antimicrobial to the faecal samples to assess the rate at which bioactivity declines.

Although faecal concentrations differ between the antimicrobials assessed in this study, a standard starting concentration (200mg/L) of each antimicrobial was tested, rather than reflective clinical concentrations, to compare each antimicrobial on an equal standing.

3.5 Conclusion

This study demonstrated raw faeces have a considerable inhibitory effect on the bioactive concentrations of fidaxomicin, vancomycin and metronidazole. To treat CDI effectively, antibiotics must achieve sufficient intra-colonic levels. The inhibition of fidaxomicin and vancomycin is unlikely to impact treatment efficacy due to the high faecal levels achieved in vivo. By contrast, the marked reduction in metronidazole bioactivity is particularly likely to result in sub-effective intra-colonic concentrations. It is conceivable that the inactivation documented in this study at least partly explains the relatively poor clinical efficacy of metronidazole.

Conclusions

Perturbations of the gut microbiota leading to a loss of colonisation resistance predispose individuals to CDI [42, 229], with antibiotic use the leading risk factor for acquiring infection [69, 70, 72, 75, 76, 79, 196]. The treatment of CDI is paradoxically reliant on antimicrobial therapy, which may cause further collateral damage to the gut microbiota [377, 378], increasing the risk of rCDI [379]. Treating CDI and reducing the risk of rCDI is therefore a delicate balance of achieving adequate bioactive antimicrobial concentrations in the colon to eradicate *C. difficile* whilst minimising disruption to the resident microbiota.

Colonisation resistance arises when a rich and diverse resident gut microbial community interacts and operates in concert to suppress *C. difficile* germination and proliferation. Although the role of some specific organisms in colonisation resistance against *C. difficile*, such as *C. scindens*, have been identified, there may be other mechanisms of microbiota-mediated colonisation resistance yet to be elucidated. Concurrent research investigating treatment failure and rCDI following antimicrobial therapy has rightly focused heavily on the effect of anti-CDI agents on the resident gut microbiota, as well as resistance mechanisms in *C. difficile*. However, the effects the resident gut microbiota have on therapeutic antimicrobial potency have been overlooked. Large-scale studies using molecular sequencing techniques to investigate complex microbial communities in the gut have the potential to identify key groups and species involved in colonisation resistance and assess microbiota-antibiotic associations. However, these are prohibitively expensive, labour intensive, and the recruitment of faecal donors can be problematic. Animal models are often used, which come with ethical considerations, especially since the extrapolation of results relative to humans is complicated due to interspecies differences. Simpler methods can be a useful initial step to identify microbiota-*C. difficile* and microbiota-antimicrobial interactions of interest and so to direct further research. This thesis

aimed to explore these interactions using simplistic batch culture methods. Firstly, the feasibility of using samples from the Leeds triple-stage chemostat gut model in place of fresh human faeces for a colonisation resistance model was assessed. Secondly, the inhibitive potential of select gut microbiota species isolated from the gut model was explored. Finally, the effect of the faecal microbiota on the bioactivity of antimicrobials used to treat CDI was investigated.

In chapter 1, samples from the Leeds gut model were used in place of faeces to assess the utility of such samples in a batch culture colonisation resistance assay akin to Borriello and Barclay's [222] original *in vitro* assay. Raw and sterilised GMS seeded with vegetative *C. difficile* replicated the original faecal colonisation resistance assay. Unexpectedly, *C. difficile* spores did not germinate when seeded in sterilised GMS.

Borriello and Barclay's [222] *in vitro* model of CDI colonisation resistance demonstrated growth of vegetative *C. difficile* in heat and filter sterilised human faecal samples but not in raw samples, indicating colonisation resistance was intact. This study attempted to replicate their assay using GMS as obtaining GMS are logistically easier than faecal donations from humans and can be taken from the same gut model at different time points undergoing simulated disease states. This would enable longitudinal studies on the colonisation resistance of the microbiota under different conditions. Unfortunately, the assay did not demonstrate a loss of colonisation resistance against *C. difficile* spores in heat sterilised GMS, which diminishes its application for these types of studies as sterilised samples are expected to act as a positive control. Further work is needed to explore why spores did not germinate in heat sterilised GMS, as well as exploring spore germination in filtered sterilised GMS.

In Chapter 2, a select range of gut microbiota species were singularly co-cultured with *C. difficile* to investigate potential inhibitive effects. Although colonisation resistance

of the gut microbiota occurs via the many interactions and functions conveyed by multiple different species, the assessment of single species in a co-culture is a cheap, quick and relatively easy method to assess direct inhibitive interactions, identify key functional species and direct further research. Of the species tested for *C. difficile* inhibitive capacities, only members of the *Bifidobacterium* and *Lactobacillus* spp. reduced the growth of *C. difficile* in batch culture. The inhibitive effects of *Bifidobacterium* and *Lactobacillus* spp. on *C. difficile* seen here corroborate other studies [279, 283, 286-288]. Other species known to enhance resistance to *C. difficile in vivo*, such as *C. scindens* [161], had little inhibitive effect in co-culture, highlighting the limitations of this model to investigate the full range of microbiota interactions with *C. difficile*.

In Chapter 3, the effect of the faecal microbiota on the bioactivity of antimicrobials was investigated. The paradoxical use of antimicrobials for the treatment of *C. difficile* has led to much research on the effect antimicrobials have on the gut microbiota, with little investigation of the antithesis to the author's knowledge. Here, we observed raw faeces had a considerable inhibitory effect on the bioactivity of fidaxomicin, vancomycin and metronidazole, the extent of which is unlikely to affect the treatment success of fidaxomicin and vancomycin as they achieve high colonic levels. However, the significant decrease in metronidazole bioactivity in the presence of raw faeces may partly account for the relatively poor clinical outcomes for this antimicrobial. Although this study tested stool samples from healthy donors, a significant negative correlation between metronidazole and numbers of *Lactobacilli* recovered from the faeces was observed, in keeping with another study that suggested *Lactobacillus* spp. may sequester metronidazole [328]. As CDI patients are more likely to have gut microbial perturbations resulting in disproportionately high levels of a particular species [273], further research is warranted on the effect a ‘dysbiotic’ faecal microbiota may have on metronidazole activity. Although correlations between diet and antibiotic activity were not observed in this study, this may be indicative of low

sample size and is still worthy of further exploration as diet-microbiome interactions have been found to drive antibiotic efficacy in animal studies [363].

In summary, the work presented in this thesis highlights the utility of simple *in vitro* methods to investigate interactions between the gut microbiota and *C. difficile,* and importantly explored the effects microbiota can have on faecal antimicrobial concentrations. The impact the latter may have on CDI treatment warrants further investigation.

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Appendix

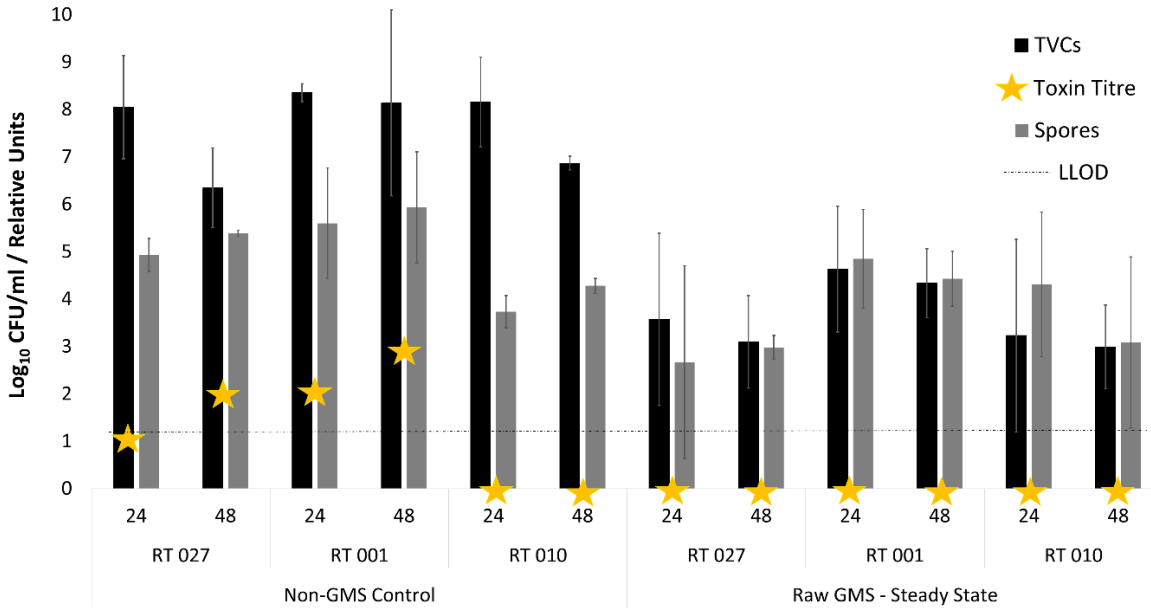
A.1.1 Constituents of the culture media used to enumerate microbial populations

Agar	Typical formula	Amount (g/ L)
Brazier's CCEY agar (LabM) – LAB160	Peptone Mix	23.0
	Sodium chloride	5.0
	Soluble starch	1.0
	Agar	12.0
	Sodium bicarbonate	0.4
	Glucose	1.0
	Sodium pyruvate	1.0
	Cysteine HCl	0.5
	Haemin	0.01
	Vitamin K	0.001
	L-arginine	1.0
	Soluble pyrophosphate	0.25
	Sodium succinate	0.5
	Cholic acid	1.0
	p-Hydroxyphenylacetic acid	1.0
Bile aesculin agar (Oxoid) – CM0888	Peptone	14.0
	Bile salts	15.0
	Ferric citrate	0.5
	Aesculin	1.0
	Agar	15.0
Columbia blood agar base (Oxoid) – CM0331	Special peptone	23.0
	Starch	1.0
	Sodium chloride	5.0
	Agar	10.0

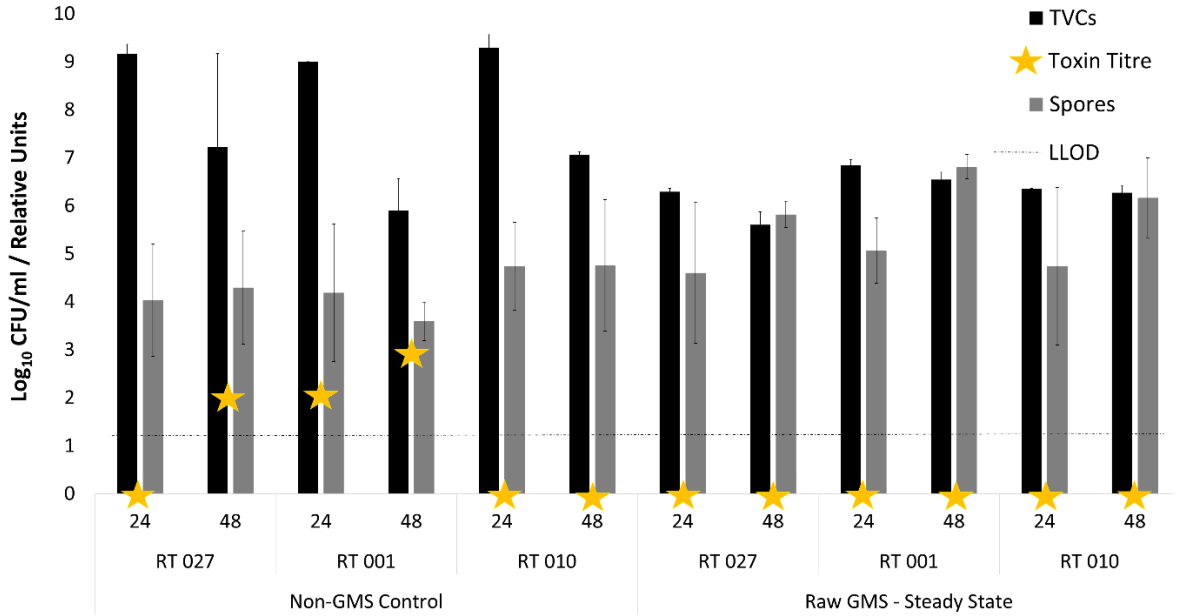
Fastidious anaerobe agar with horse blood (Oxoid) – PB0225A	Peptone Mix	23.0
	Sodium chloride	5.0
	Soluble Starch	1.0
	Agar No.2	12.0
	Sodium bicarbonate	0.4
	Glucose	1.0
	Sodium pyruvate	1.0
	Cysteine HCl monohydrate	0.5
	Haemin	0.01
	Vitamin K	0.001
	L-Arginine	1.0
	Soluble pyrophosphate	0.25
	Sodium succinate	0.5
	Defibrinated horse blood	50 mL
Kanamycin aesculin azide agar base (Oxoid) - CM0591	Tryptone	18.8
	Yeast extract	5.0
	Sodium chloride	5.0
	Sodium citrate	1.0
	Aesculin	1.0
	Ferric ammonium citrate	0.5
	Sodium azide	0.15
	Starch	0.6
	Mix for Streptococci	0.6
	Agar	10.0
MacConkey agar No.3 (Oxoid) – PO0495A	Peptone	20.0
	Lactose	10.0
	Bile salts No. 3	1.5
	Sodium chloride	5.0
	Neutral red	0.03

	Crystal violet	0.001
	Agar	15.0
MRS Broth (De Man, Rogosa, Sharpe, Oxoid) – CM0359	Peptone	10.0
	'Lab-Lemco' powder	8.0
	Yeast extract	4.0
	Glucose	20.0
	Sorbitan mono-oleate	1 mL
	Dipotassium hydrogen phosphate	2.0
	Sodium acetate $3H_2O$	5.0
	Triammonium citrate	2.0
	Magnesium sulphate $7H_2O$	0.2
	Manganese sulphate $4H_2O$	0.05
Nutrient agar (Oxoid) – PO0155A	Protease peptone	15.0
	Yeast extract	5.0
	Liver digest	2.5
	Sodium chloride	5.0
	Agar	12.0

A.1.2 Comparison of different *C. difficile* ribotypes growth in various media

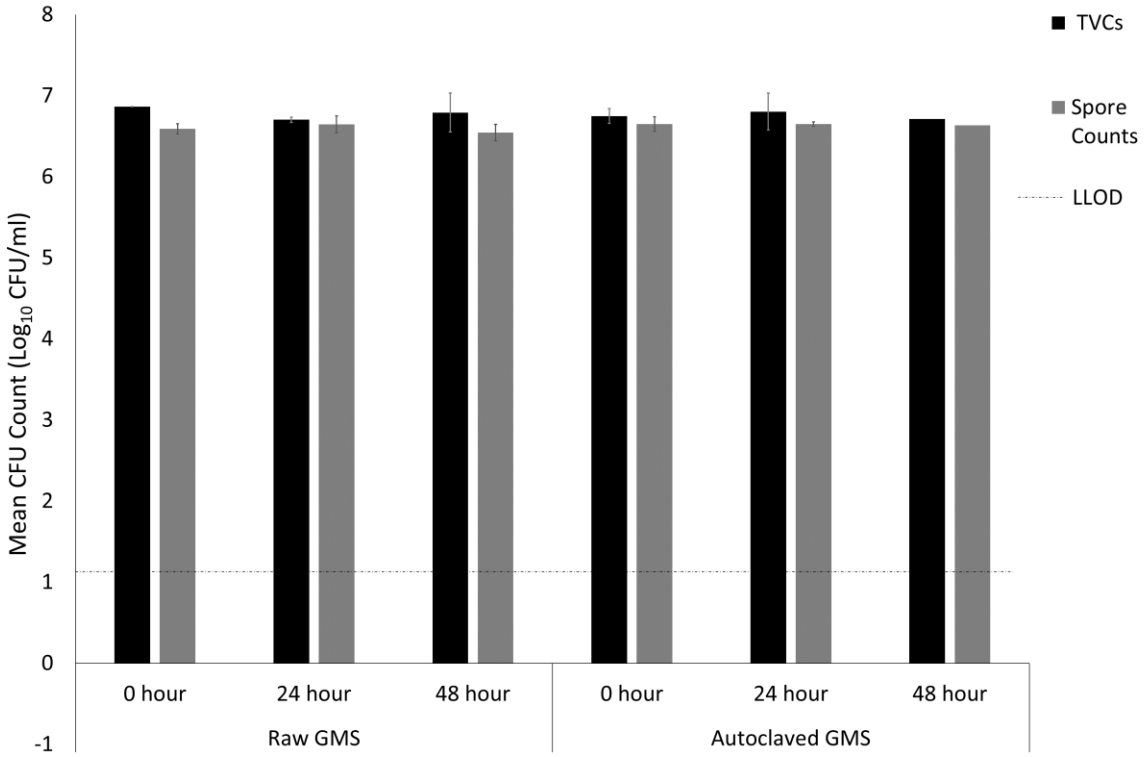


Mean (±SE) *C. difficile* TVCs and spores recovered from non-GMS controls (BHI and GMM amalgamated) (n=2) and raw GMS (n=4) seeded with vegetative *C. difficile* after 24 and 48 hours anaerobic incubation. Starting inoculum ~6 log_{10}CFU/mL at 0 hours. LLOD = Lower Limit of Detection for TVC and Spore Counts.



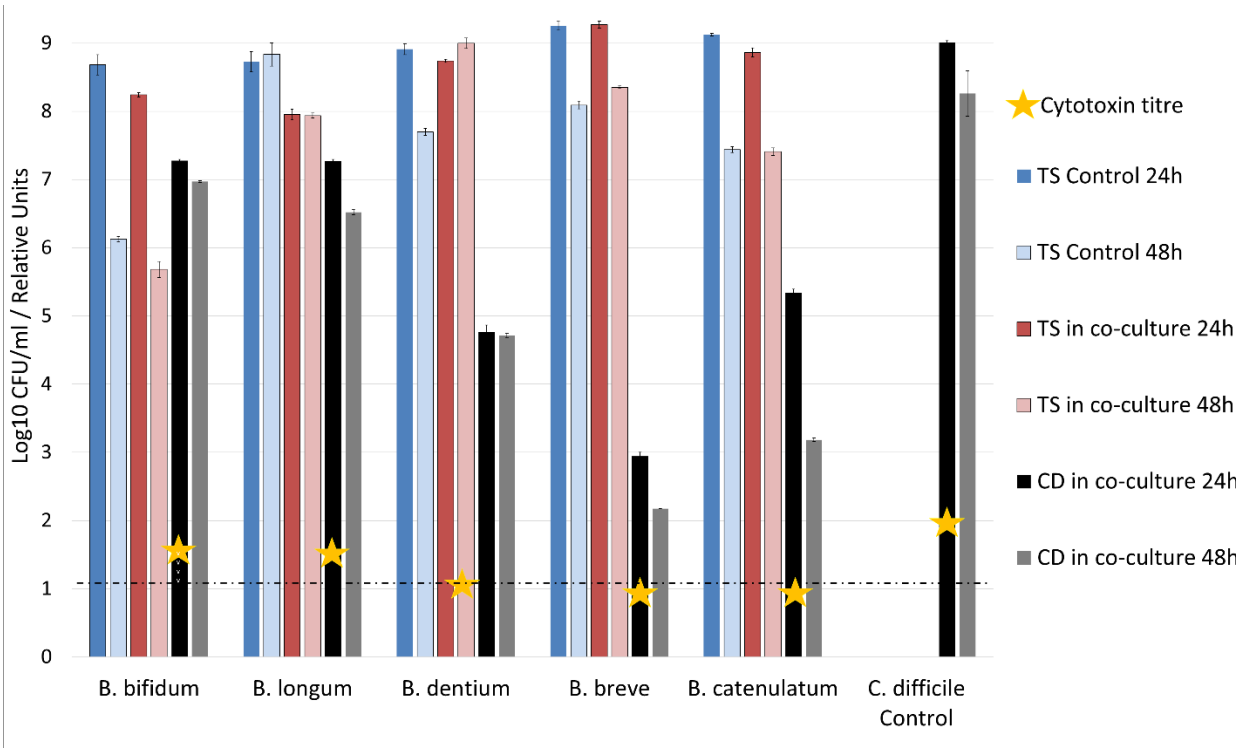
Mean (±SE) *C. difficile* TVCs and spores recovered from non-GMS controls (BHI and GMM amalgamated) (n=2) and raw GMS (n=4) seeded with *C. difficile* spores after 24 and 48 hours anaerobic incubation. Starting inoculum ~6 log_{10}CFU/mL at 0 hours. LLOD = Lower Limit of Detection for TVC and Spore Counts.

A.1.3 Recovered *C. difficile* TVCs and spore counts in GMS seeded with *C. difficile* spores at 24 and 48 hours

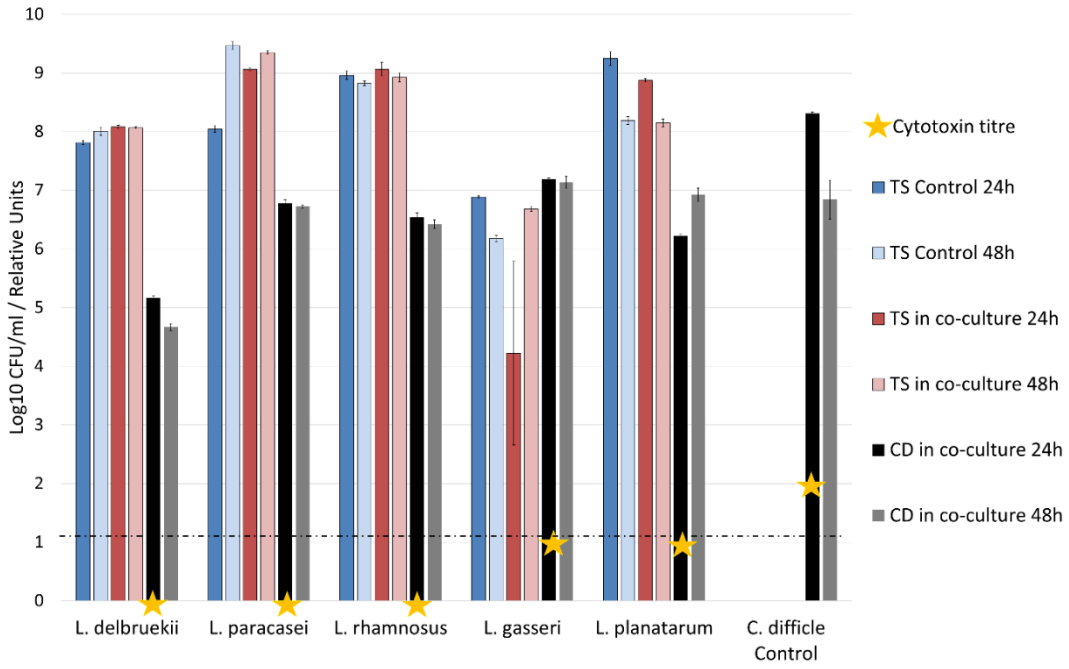


Mean (±SE) *C. difficile* TVCs and spores recovered from GMS (n=3) seeded with *C. difficile* spores after 24 and 48 hours anaerobic incubation. LLOD= Lower Limit of Detection for TVC and Spore Counts.

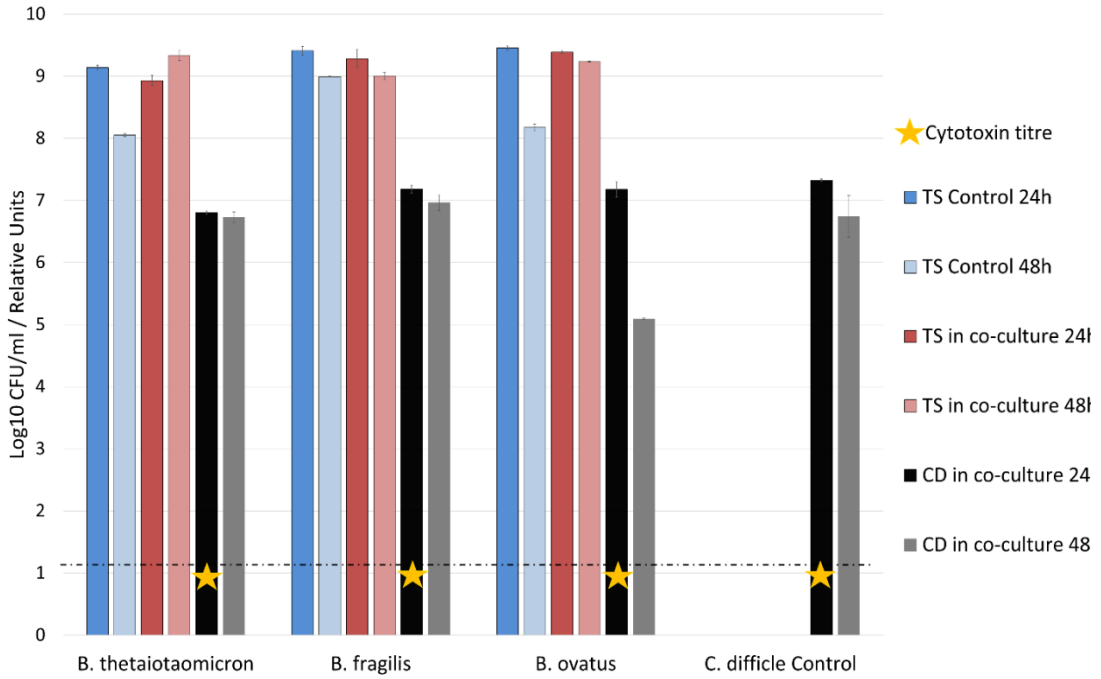
A.2 Recovered test species and *C. difficile* TVCs from co-cultures



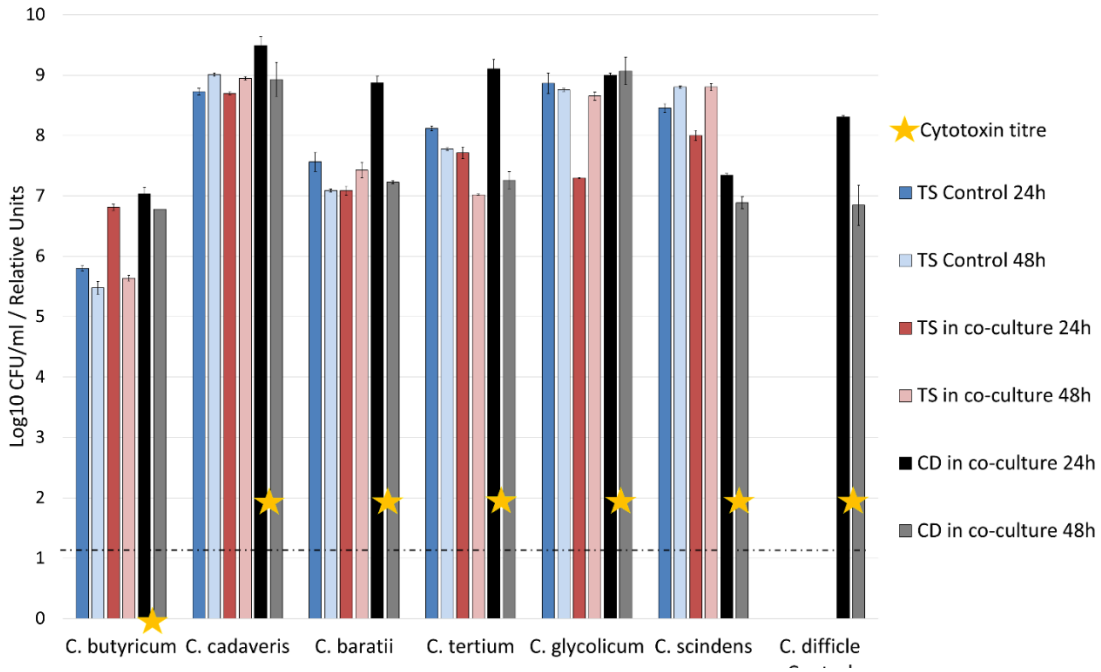
Test Bifidobacterium species (TS) and *C. difficile* TVCs recovered from co-cultures after 24 and 48 hours. Cytotoxin titre (RUs) at 24 hours. LLOD = Lower Limit of Detection.



Test Lactobacillus species (TS) and *C. difficile* TVCs recovered from co-cultures after 24 and 48 hours. Cytotoxin titre (RUs) at 24 hours. LLOD = Lower Limit of Detection.



Test Bacteroides species (TS) and *C. difficile* TVCs recovered from co-cultures after 24 and 48 hours. Cytotoxin titre (RUs) at 24 hours. LLOD = Lower Limit of Detection.



Test Clostridium species (TS) and *C. difficile* TVCs recovered from co-cultures after 24 and 48 hours. Cytotoxin titre (RUs) at 24 hours. LLOD = Lower Limit of Detection.

A.3.1 Ethical Aproval Letter

UNIVERSITY OF LEEDS

Faculty of Medicine and Health Research Office
School of Medicine Research Ethics Committee (SoMREC)

Room 9.29, level 9
Worsley Building
Clarendon Way
Leeds, LS2 9NL
United Kingdom

✆ +44 (0) 113 343 1642

05 January 2017

Ms Rebecca Sinclair
Leeds Institute of Biomedical & Clinical Sciences
Microbiology
University of Leeds
Old Medical School
Thoresby Place
LEEDS LS1 3EX

Dear Rebecca

Ref no: **MREC16-002**

Title: **Effect of faecal matter on antibiotics used to treat C difficile 1.0**

Your research application has been reviewed by the School of Medicine Ethics Committee (SoMREC) and we can confirm that ethics approval is granted based on the following documentation received from you.

Document	*Version*	*Date Submitted*
FullDatasetTrialForm V5.3.2	5.3.2 (2.0)	14/11/2016
COSHH Anaerogen	1.0	14/11/2016
Consent Form V2	2.0	14/11/2016
Participant Information Sheet V2	2.0	14/11/2016
Research lone working risk assessment CC	1.0	14/11/2016
Disposal of stool collection kit procedure	1.0	14/11/2016
3 Day Food Diary V2	1.0	14/11/2016
Sponsor opinion re HRA approval not required	1.0	11/08/2016
Research Proposal V7.1	7.1 (1.0)	11/08/2016

Please notify the committee if you intend to make any amendments to the original research ethics application or documentation. All changes must receive ethics approval prior to implementation. Please contact the Faculty Research Ethics Administrator for further information (fmhuniethics@leeds.ac.uk)

Ethics approval does not infer you have the right of access to any member of staff or student or documents and the premises of the University of Leeds. Nor does it imply any right of access to the premises of any other organisation, including clinical areas. The committee takes no responsibility for you gaining access to staff, students and/or premises prior to, during or following your research activities.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. You will be given a two week notice period if your project is to be audited.

It is our policy to remind everyone that it is your responsibility to comply with Health and Safety, Data Protection and any other legal and/or professional guidelines there may be.

We wish you every success with the project.

Yours sincerely

Dr Roger Parslow
Co-Chair, SoMREC, University of Leeds

(Approval granted by Dr Naomi Quinton on behalf of SoMREC Co-Chairs)

A.3.2 Participant Information Sheet

Rebecca Sinclair, Lead Researcher
PhD Student within Leeds Institute of Biomedical & Clinical Sciences
Email: fs11ras@leeds.ac.uk
V3 Project Number: MREC16-002

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Participant Information Sheet

Research Study: Effect of Faecal Matter on the Activity of Antibiotics

You have been invited to partake in a research study. Please read the following information carefully and discuss it with others if you wish. If you have any questions please do not hesitate to ask the lead researcher, Rebecca Sinclair (fs11ras@leeds.ac.uk), or PhD student supervisor within the Healthcare Associated Infection (HCAI) Research Group, Caroline Chilton (C.H.Chilton@leeds.ac.uk).

What is the purpose of the research?

This research is part of a PhD study exploring an important infection causing bacteria called *Clostridium Difficile*. This bacteria infects the gut, it can be life-threatening and is not easily treated with antibiotics; therefore further research is required to improve treatment options. For this research, we require stool (poo) samples from healthy volunteers to see how the gut bacteria (from within the stool sample) interacts with antibiotics which are used to treat the infection. We will also like to see what was eaten in the days leading up to the production of a stool sample to see if this has an effect on the gut bacteria.

Who can take part?

Any healthy adult, over the age of 18, who has not taken antibiotics in the past 3 months.

Do I have to take part?

Participation is entirely voluntary. If you decide to take part you will be given this information sheet to keep and a consent form to sign. Once you have signed the consent form you are still free to withdraw from the study, without giving a reason, until the collection of stool sample and food diary by Rebecca. Withdrawal after this time will not be possible as all samples collected will be given a number and it will not be possible to trace your particular sample or food diary information. If you wish, you may provide a stool sample without filling in the 3 day food diary.

Rebecca Sinclair, Lead Researcher
PhD Student within Leeds Institute of Biomedical & Clinical Sciences
Email: fs11ras@leeds.ac.uk
V3 Project Number: MREC16-002

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What am I being asked to do?

If you decide to take part you will be given a kit to enable you to provide a stool sample in the comfort of your own home. You will also be given a food diary to fill in for 3 days leading up the production of the stool sample. If filling a food diary is not possible, the donation of a stool sample alone is acceptable. Collection of the sample and diary will be at a prearranged time that is mutually convenient for you and Rebecca. It is important that the stool is collected within 24 hours of being produced. If you are not able to provide a stool in advance of the agreed collection date, and would still like to be included in the study, please contact Rebecca to rearrange. If you agree to being contacted on the consent form we may ask for another stool sample at a later date. However, even if you have initially agreed to this you can decline or withdraw at any time until the sample and diary are collected by Rebecca.

The stool collection kit consists of:

- Disposable gloves
- A disposable bowl
- Wooden scoop
- 2 x 60ml plastic pots
- 1 AnaroGen anaerobic pouch
- Small sealable bag
- Large plastic bag

Instructions:

1. Put on the gloves
2. Poo into a disposable bowl
3. Using the wooden scoop provided, scoop the poo into the 60ml pots – **half fill** each 60ml plastic pot and put any left over poo into the toilet
4. Firmly screw the lids back onto the 60ml pots
5. Put the 60ml pots into the small sealable bag, add the anaerobic pouch (tear open the foil sachet at the tear-nick indicated. Remove the AnaeroGen Compact paper

Rebecca Sinclair, Lead Researcher
PhD Student within Leeds Institute of Biomedical & Clinical Sciences
Email: fs11ras@leeds.ac.uk

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sachet from within and immediately place in the bag), remove as much air as possible and seal the bag so it is airtight

6. Put the gloves and any soiled items in the large plastic bag and dispose of with your general waste, just as you would a nappy
7. Wash your hands thoroughly with soap and warm water

If you are willing we will also provide a 3 day food diary to fill in; this will be accompanied by a guide how to complete.

Where can I access advice or support?

Please be assured that the HCAI Research Group deal with stool samples on a daily basis and will only be grateful for any contribution you can give to the study. Rebecca has plenty of experience in providing stool samples herself and can give advice if necessary. Within the research team we also have a research nurse, Peter Inns, who can be contacted via email at peter.inns@nhs.net, for any further support needs you may have.

What will happen to my sample and food diary?

The stool sample will be screened to see what bacteria it contains. The stool will then be processed and mixed with antibiotics to check how well the antibiotics work after they've been exposed to poo. Another portion will be used to test the effect of poo on *C. difficile* growth. This information will be compared to the bacterial screen to see if there are any connections. A portion of the stool sample will be frozen in a lockable freezer, for 3 years and will only be accessible to the research team. This is so a more thorough screen of the bacteria contained in the stool can be assessed at a later date and just in case the experiment needs to be repeated. After this time the stool sample will be destroyed.

The information in the food diary will be used to assess the proportions of key nutrients from your diet in the 3 days leading up to the stool sample. This information will be compared to the results from the stool experiments to see if there are any associations.

What are the possible benefits to me and others?

While there are no immediate benefits, your participation will contribute to the research and development of better treatment options for patients with this infection.

Rebecca Sinclair, Lead Researcher
PhD Student within Leeds Institute of Biomedical & Clinical Sciences
Email: fs11ras@leeds.ac.uk
V3 Project Number: MREC16-002

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How can I access the results of the study?

Once the study is completed, a summary sheet of its finding will be available on request to the lead researcher (contact details above). As it will not be possible to trace your individual information from the processed samples and food diaries, individual results cannot be given.

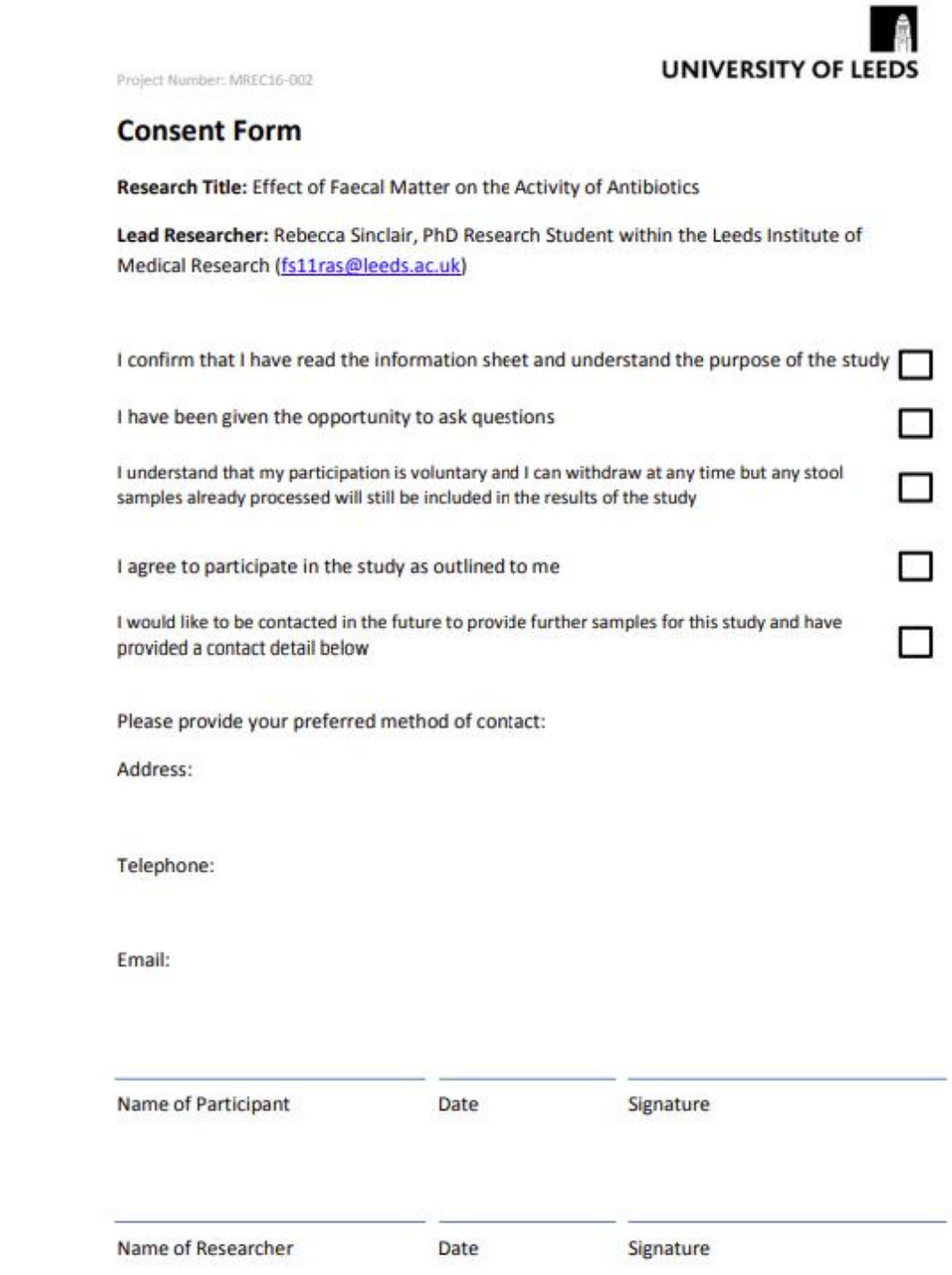
Confidentiality

Any information that is collected about you will be kept strictly confidential. Once collected, food diaries and samples will be assigned a study number and not be linked to any personal details that can identify you. If you have agreed to being contacted in the future, the link between your contact details and the study number of samples and food diaries will be kept on a password protected university computer. The information collected will not be used for any other purpose than that explained here.

Ethical approval has been sought from the School of Medicine Research Ethics Committee: project number MREC16-002.

Thank you for taking the time to read this information sheet and your interest in the study.

A.3.3. Consent Form



Project Number: MREC16-002

UNIVERSITY OF LEEDS

Consent Form

Research Title: Effect of Faecal Matter on the Activity of Antibiotics

Lead Researcher: Rebecca Sinclair, PhD Research Student within the Leeds Institute of Medical Research (fs11ras@leeds.ac.uk)

I confirm that I have read the information sheet and understand the purpose of the study ☐

I have been given the opportunity to ask questions ☐

I understand that my participation is voluntary and I can withdraw at any time but any stool samples already processed will still be included in the results of the study ☐

I agree to participate in the study as outlined to me ☐

I would like to be contacted in the future to provide further samples for this study and have provided a contact detail below ☐

Please provide your preferred method of contact:

Address:

Telephone:

Email:

Name of Participant	Date	Signature

Name of Researcher	Date	Signature

A.3.4. Food Diary

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Version 3.1
Project Number: MREC16-002

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3 Day Food Diary

Instructions:

- Write the day and time of each meal, snack or drink
- Record everything you eat. Keep the diary and a pen with you to record on the go. A square of chocolate or a cup of tea and a biscuit are still important to note.
- Be as specific as you can;
    - With type of food, e.g. granary bread or white bread; wholemeal or white pasta; semi-skimmed or whole milk; dark or white chicken meat, etc.
    - Include sauces, salad dressings, condiments and gravies
    - Give approximate portion sizes (or exact weights if known)
    - Give brand names and weights for processed foods such as ready meals, crisps, biscuits, etc.
    - Note all drinks including water and alcoholic beverages
- For homemade composite foods, e.g. lasagne, make a brief note of main ingredients (such as that in the example below)

An example Food Diary has been provided below to help guide your own notes.

Please answer the following:

1) Do you take any regular food supplements, for example, multi-vitamins, cod liver oil, glucosamine, probiotics, etc.? Please describe what you take (including brand name) and how often:

_____

_____

_____

2) Please select the option closest to your normal diet from the list:

Omnivorous 1 (eats red meat, poultry or fish at least once per day) ☐

Omnivorous 2 (eats red meat, poultry or fish at least 3 times per week) ☐

Semi-vegetarian (eats poultry, fish, dairy or eggs but **not** red meat) ☐

Semi-vegetarian or 'Pescetarian' (eats fish, dairy or eggs but **not** red meat or poultry) ☐

Vegetarian (eats dairy, eggs and honey) ☐

Vegan (does not eat any animal produce) ☐

Other (please describe):

_____

_____

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DAY: ***Example***

Time	Food	Drink
8am	*3 Weetabix with lots of semi-skimmed milk and a teaspoon of sugar* *2 slices granary toast with margarine and marmite*	*1 cup instant coffee with creamer*
10am		*1 cup green tea*
10.30am	*1 large banana*	*1 large glass water*
12pm	*1 large bowl tuna pasta salad (wholemeal pasta, red onion, tomato, cucumber with vinaigrette dressing)* *30g bag Macoy's Flame grilled crisps* *48g Snickers bar*	*330ml can regular Coca Cola*
3pm	*8 custard cream biscuits*	*1 tea with semi-skimmed milk and 1 sugar*
5pm		*1 small glass sugar-free orange squash*
7pm	*Large portion homemade lasagne (white pasta, tinned tomatoes, onion, garlic, mince beef, basil, béchamel sauce, cheddar cheese)* *5 broccoli florets* *garlic bread (small aprrox 6 inch white baguette)*	*2 large glasses red wine*
8.30pm	*125g pot Activia apricot yogurt*	
10pm		*1 cup hot chocolate made with semi-skimmed milk*

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Day:

Time	Food	Drink