PROBIOTIC PROTECTION AGAINST DYSREGULATED GUT MICROBIOTA AND INTESTINAL CELL FUNCTIONALITY INDUCED BY *CLOSTRIDIOIDES DIFFICILE*

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SUGGESTED SHORT TITLE

Effect of probiotics towards protection against *Clostridioides difficile* infection

ABSTRACT

Clostridioides (C.) *difficile* infection (CDI) is the leading cause of nosocomial infectious diarrhea that is of growing concern due to its rapid rise in recent years across industrialized countries. CDI is strongly associated with prior antibiotic therapy, manifesting as mild-severe diarrhea to life-threatening pseudomembranous colitis. Additionally, antibiotic therapy of CDI is associated with disrupted metabolic function and altered gut microbiota. The use of probiotics as an adjunct is being studied extensively due to their potential in maintaining intestinal homeostasis by modulation of gut microbiota, its function and host immune response. In the present work, normal and CDI-infected fecal samples were treated with several single strain and multispecies probiotics in a simulated gastrointestinal (GI) model. The use of an *in vitro* GI model in this regard has shown certain benefits of being capable to dynamically monitor changes to the gut microbiota and the colonic conditions under physiologically controlled conditions. Outcome measures included analyses of microbiota composition and its associated metabolic activity, antioxidant status of the colonic milieu, and, the inflammatory response of T84 human colon epithelial cells to GI-derived fecal water (FW). The findings from this dissertation showed that CDI fecal samples altered the antioxidant status of the colonic milieu, decreased microbial alpha diversity and dysregulated GI metabolic function by significantly ($p < 0.05$) decreasing short-chain fatty acids (SCFAs) and hydrogen sulfide (H_2S) production. Probiotic supplementation in CDI samples was associated with an improved antioxidant status which was attributed to increased copper chelation. Additionally, probiotic supplementation restored metabolic function in CDI samples via increased production of SCFAs and H_2S. Although probiotic strains were detected in treated normal and CDI fecal samples, no significant changes were observed in microbial composition. T84 intestinal cells exposed to CDI-FW exhibited increased cytotoxicity and proinflammatory cytokine production which was characterized by interleukin (IL)-8, CXCL5, MIF, TNFRSF8, and IL-32. These effects were diminished with exposure of T84 cells to CDI-FW treated with certain probiotics, such as the single-strain *Saccharomyces boulardii* CNCM I-1079 and *Lactobacillus rhamnosus* R0011. Overall, these findings indicate the potential of probiotics to

restore intestinal homeostasis in CDI as mediated via improvements in gut microbiota and host intestinal cell functionality.

RÉSUMÉ

L'infection par *Clostridioides* (C.) *difficile* (CDI) est la principale cause de diarrhée nosocomial infectieuse, ce qui est de plus en plus préoccupant parce qu'elle a augmenté rapidement pendant les dernières années dans des pays industrialisés. La CDI est fortement associée à des thérapies antibiotiques antérieures et ses symptômes vont d'une diarrhée légère ou sévère à une colite pseudomembraneuse qui peut mettre la vie en danger. De plus, la thérapie antibiotique pour CDI est associée à une fonction métabolique perturbée et un microbiote intestinal altéré. L'utilisation des probiotiques comme thérapie complémentaire est largement étudiée à cause de leur potentiel à maintenir l'homéostasie intestinale grâce à la modulation du microbiote intestinal, sa fonction et la réponse immunitaire de l'hôte. Dans ce travail, des échantillons fécaux normaux et infectés avec CDI ont été traités avec différents probiotiques d'une seule souche et multi-espèces dans un modèle gastro-intestinal (GI) simulé. L'utilisation d'un modèle GI *in vitro* à cet égard a montré certains avantages dont celui de pouvoir surveiller dynamiquement les changements du microbiote intestinal et les conditions du côlon dans des conditions physiologiquement contrôlées. Les mesures ont inclus des analyses de la composition du microbiote et son activité métabolique associée, l'état antioxydant du milieu côlonique, et la réponse inflammatoire des cellules humaines épithéliales du côlon T84 à l'eau fécale (FW) dérivée du GI. Les résultats de cette thèse montrent que les échantillons fécaux avec CDI altèrent l'état antioxydant du milieu côlonique, diminuent la diversité microbienne alpha et dérégulent la fonction métabolique du GI en diminuant significativement ($p < 0.05$) la production d'acides grass à chaîne courte (SCFA) et de sulfure d'hydrogène (H_2S). L'addition des probiotiques aux échantillons avec CDI a été associée à un état antioxydant amélioré, ce qui a été attribué à une augmentation de la chélation du cuivre. De plus, l'addition des probiotiques a restauré la fonction métabolique dans des échantillons CDI via une production accrue de SCFAs et H_2S. Malgré que des souches probiotiques ont été détectées dans les échantillons fécaux traités normaux et avec CDI, aucun changement significatif n'a été observé dans la composition microbienne. Les cellules intestinales T84 exposées à des CDI-FW ont montré une cytotoxicité et une production des cytokines pro-

inflammatoires accrues, caractérisées par l'interleukine (IL)-8, CXCL5, MIF, TNFRSF8, et IL-32. Ces effets ont diminué avec l'exposition des cellules T84 à des CDI-FW traités avec certains probiotiques, tels que ceux de souche unique *Saccharomyces boulardii* CNCM I-1079 et *Lactobacillus rhamnosus* R0011. Dans l'ensemble, les résultats de cette thèse montrent le potentiel des probiotiques pour restaurer l'homéostasie intestinale dans des cas de CDI via une amélioration du microbiote intestinal et de la fonctionnalité intestinale de l'hôte.

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PREFACE

This thesis is presented in a manuscript form and consists of six chapters. Chapter 1 presents a general introduction to the *Clostridioides difficile* infection, its relevance and the use of probiotics in its therapy. Chapter 2 presents a current and in-depth literature review of the topic covering different aspects of *Clostridioides difficile*, its pathophysiology, current challenges and how probiotics can be effective as adjuncts in the management of *Clostridioides difficile* infection. Chapter 3 through 6 are research-based on manuscripts that are bridged sequentially through connecting statements. Amongst these four manuscripts, Chapter 4 has been published in Nutrients, and Chapter 5 has been published in Microorganisms. Chapters 3 and 6 will be duly submitted for publication in Microorganisms and Frontiers in Microbiology, respectively. Finally, the last chapter presents an overall discussion and conclusion of the work presented throughout the thesis, along with contributions of knowledge and recommendations for future research. This dissertation is in accordance with guidelines for thesis preparation as published by the Faculty of Graduate Studies and Research of McGill University.

CONTRIBUTION OF AUTHORS

Mohd Baasir Gaisawat (Candidate) was responsible for the design of experiments and the experiment work, including developing experimental protocols, conducting the *in vitro* digestions and cull culture, performing all the associated assays (metabolic makers, metagenomic analyses, cell culture analyses and other wet-lab assays). The candidate also responsible for interpretation of data, performing statistical analyses and making the visualisations. Finally, the candidate wrote all the original drafts of the individual manuscripts and the entirety of this dissertation.

Dr. Stan Kubow (Candidate's supervisor) was responsible for conceptualisation of the research project, with critical input into study design, methodology and any ongoing guidance and feedback where required. Dr. Kubow supervised all aspects of the dissertation. Dr. Kubow also provided extensive feedback and input for all the manuscripts and the dissertation.

Dr. Michele Iskandar was involved in developing the methodology for cell culture experiments, along with critical feedback on aspects of methodology and data interpretation for each of the manuscripts. Dr. Iskandar also provided support in reviewing and editing the manuscripts presented in this thesis.

Dr. Luis Agellon, in conjunction with Dr. Stan Kubow**,** was responsible conceptualisation and development of the gastrointestinal model utilized in this dissertation. Dr. Agellon also gave critical feedback on Chapter 3.

Mr. Chad McPherson was involved in training of microbial analyses performed in the dissertations such as understanding and interpreting the 16S sequencing data and in the multiplex analysis for immune markers in Study 4. Moreover, Chad was also involved in critical feedback regarding methodology, data interpretation and manuscript revision.

Dr. Thomas A. Tompkins was responsible for critical input into study design, methodology and support in reviewing and editing the manuscripts.

Dr. Julien Tremblay was primarily responsible for running the bioinformatics for 16S rRNA sequencing utilized in Chapters 3 and 5, including running statistics, methodology and generating all visualisations from the analyses. Dr. Tremblay also supported manuscript review for those chapters.

Ms. Silvia Lopez helped perform the cell culture studies (presented in Chapter 6), including maintenance, treatment and performing the cell viability analysis.

Ms. Amanda Piano was involved in training, method development and in the performance of the rtPCR analysis presented in Chapter 5.

STATEMENT OF ORIGINALITY

A. Claims of Original Research

The overall aim of this doctoral thesis was to better understand the role of probiotic supplementation in the management of *Clostridioides (C.) difficile* infection outcomes using an *in vitro* gastrointestinal (GI) model to monitor changes occurring in the colonic environment.

In Study 1, the growth dynamics of the colonic microbiota and its associated metabolic functions were assessed over a two-week period in a recently developed small-scale, dynamic multi-compartmental GI model using human fecal matter inoculate. The findings from this study demonstrated the stabilization of the metabolic capacity in the different colonic reactors occurred within five days of fecal sample inoculation. Microbial composition analyses showed distinct community formation in the different colonic reactors within a 5-day period that remained stable up to day 9. Moreover, fecal inoculates from the same donor taken at different times produced similar microbial community results.

In Study 2, we assessed the efficacy of several probiotics to regulate changes in overall antioxidant status of fecal water (FW) mediated by *C. difficile* infection (CDI) in an *in vitro* gut model. The findings from this study showed that CDI in the model resulted in a dysregulated antioxidant status that was characterised by a lowered FRAP (ferric-reducing ability) and no inactivation of the DPPH radical. Supplementation with probiotics in the CDI model, resulted in an increased ferric-reducing ability that was associated with an increased ability to chelate copper.

In Study 3, the ability of probiotics to modulate the intestinal flora and function in an in vitro CDI model was assessed. Microbial composition analyses and metabolic function assays revealed that the in vitro CDI model had a lower microbial alpha diversity and an altered metabolic profile. Supplementation with probiotics was associated with a significantly increased ($p < 0.05$)

production of short-chain fatty acids and restoration of endogenous hydrogen sulfide production. Probiotics, however, did not result in significant changes to the simulated CDI microbiota.

Study 4 was conducted to assess for the potential role of probiotics in modulating CDI-mediated changes in T84 intestinal epithelial cells. The findings from this study showed that CDI-FW induced cytopathic effects on T84 cells, resulting in a significantly ($p < 0.05$) lower cell viability and a significantly ($p < 0.05$) higher cytotoxicity when compared to normal-FW treated T84 cells. Moreover, CDI-FW induced several inflammatory cytokines (IL-8, CXCL-5, MIF, TNFSRF8 and IL-32) in T84 cells. Notably, CDI-FW treatments containing probiotics were associated strain-specific effects in attenuation of its cytopathic effects on the T84 cells, including attenuation of cytokine production.

B. Research Publications in Peer-Reviewed Scientific Journals

- **Gaisawat, M. B.**, MacPherson, C. W., Tremblay, J., Piano, A., Iskandar, M. M., Tompkins, T. A., & Kubow, S. (2019). Probiotic Supplementation in a *Clostridium difficile*-Infected Gastrointestinal Model Is Associated with Restoring Metabolic Function of Microbiota. Microorganisms, 8(1), 60

- **Gaisawat, M. B.**, Iskandar, M. M., MacPherson, C. W., Tompkins, T. A., & Kubow, S. (2019). Probiotic Supplementation is Associated with Increased Antioxidant Capacity and Copper Chelation in *C. difficile*-Infected Fecal Water. Nutrients, 11(9), 2007

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ABBREVIATIONS

AC	Ascending colon
ASV	Amplicon sequence variant
BMI	Body mass index
CCL-21	Chemokine (C-C motif) ligand 21
CDI	*Clostridioides difficile* infection
CDI-FW	*Clostridioides difficile* infected fecal water
CNISP	Canadian Nosocomial Infections Surveillance Program
Cq	Quantification cycle
$CuSO_4.5H_2O$	Copper sulphate pentahydrate
CXCL5	C-X-C motif chemokine 5
DC	Descending colon
ddH_2O	Deionized distilled water
DMEM	Dulbecco's modified eagle medium
DNPH	2,4-Dinitrophenylhydrazine
DPD	N,N-Diethyl-p-phenylenediamine
DPPH	2',2'-Diphenyl-1-picrylhydrazyl
EDTA	Ethylene-diamine-tetraacetic acid
EIA	Enzyme immunoassay
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
FBS	Fetal bovine serum
FLASH	Fast length adjustment of short reads
FMT	Fecal microbiota transplant
FRAP	Ferric-reducing antioxidant power
FW	Fecal water

GC-FID	Gas chromatograph system equipped with a flame ionization detector
GI	Gastrointestinal
GDH	Glutamate dehydrogenase
GSH	Glutathione
GTP	Guanosine-5'-triphosphate
H_2S	Hydrogen sulfide
HCl	Hydrochloric acid
Ig	Immunoglobulin
IL	Interleukin
KNO_2	Potassium nitrite
KNO_3	Potassium nitrate
LAB	Lactic acid bacteria
LDH	Lactate dehydrogenase
LGG	*Lactobacillus rhamnosus* GG
MAP	Mitogen-activated protein
MIF	Macrophage inhibitory factor
mM	Millimolar
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide
NAAT	Nucleic acid amplification test
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NED	N-naphthyl-ethylenediamine
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NH_4	Ammonium
NO	Nitric oxide

OUT	Operational taxonomic unit
PCA	Principle component analysis
PCR	Polymerase chain reaction
PROTO	ProtecFlor™
R0011	*Lactobacillus rhamnosus* R0011
R0052	*Lactobacillus helveticus* R0052
R0175	*Bifidobacterium longum* R0175
R0343	*Lactobacillus rhamnosus* GG R0343
RCTs	Randomized controlled trials
RDP	Ribosomal database project
ROS	Reactive oxygen species
rtPCR	Reverse transcription polymerase chain reaction
SA	Sulfanilamide
SB	Saccharomyces boulardii CNCM I-1079
SCFA	Short-chain fatty acid
SEM	Standard error of mean
SHIME®	Simulator of the Human Intestinal Microbial Ecosystem
SOD	Superoxide dismutase
TC	Transverse colon
TcdA	*Clostridioides difficile* Toxin A
TcdB	*Clostridioides difficile* Toxin B
TEAC	Trolox-equivalent antioxidant capacity
TNFSRF8	Tumor necrosis factor receptor superfamily member 8
TNF-α	Tumor necrosis factor-α
TPC	Total phenolic content

CHAPTER 1: GENERAL INTRODUCTION

1.1 General Introduction

Clostridioides (C.) difficile is a gram-positive spore-forming bacillus that is the leading cause of nosocomial infectious diarrhea [1]. *C. difficile* infection usually manifests as mild-moderate diarrhea, and in more severe cases, pseudomembrane formation [2]. Pathogenesis of CDI is strongly associated with prior antibiotic therapy and an altered gut microbiota [3]. CDI patients exhibit an altered metabolic profile as a result of dysregulation in gut microbiota. This results in decreased production of secondary bile acids and short chain fatty acids, both of which play an important role in colonization resistance of the native microbiota to pathogens. Additionally, these metabolites possess important physiologic functions, as such as antimicrobial action, immune system function and maintenance of the intestinal epithelial barrier [4,5]. *C. difficile* toxins A and B are thought to be the largest contributing factor in CDI pathophysiology, with its envelope proteins playing a significant role in its colonization [6-8]. Toxins A & B induce apoptosis in intestinal cells through inactivation of small GTP-binding proteins and followed by an acute inflammatory response characterized by neutrophil recruitment and inflammasome activation [9-11]. In addition, the toxins also mediate several cell signalling pathways resulting in reactive oxidation species propagation and alteration in colonic redox status [9-11]. In this regard, probiotics have been considered as a viable adjunct therapy due to their ability to reduce symptoms of antibiotic-associated diarrhea and to beneficially modulate the gut microflora to restore colonization resistance [12,13].

Probiotics are defined as live microorganisms that impart beneficial effects on the host when given in adequate quantities [14]. Majority of the probiotics studied till date originate from *Lactobacilli* and *Bifidobacteria* spp. and yeast (*Saccharomyces*) groups. In the gastrointestinal tract, probiotics have shown strain-specific effects in their ability to improve metabolic function [15-18], to counteract infections through a variety of secreted molecules [19-21], regulate host immune response, and, decrease the severity of symptoms of several gastrointestinal (GI) disorders, including antibiotic-associated diarrhea [19,22-25]. However, in the context of CDI,

only a few probiotics have shown efficacy, of which *Saccharomyces (S.) boulardii*, *Lactobacillus (L.) rhamnosus* GG have shown the most promise in clinical studies [12,26]. The interaction of these strains and other probiotics in the different aspects of CDI pathogenesis in the colon is still poorly understood [27]. Given the ability of probiotics to exert a variety of effects in the gut lumen, it is important to be able to understand how they interact with CDI microbiota and its pathophysiology. This would also allow for better pre-clinical screening of different probiotic agents. Thus, in order to study these changes at the microbiota level, the use of *in vitro* GI models is seen to be beneficial due to their ability to simulate gut microbial complexity and its metabolic capacity as is observed in the *in vivo* gut lumen [28,29]. GI models have been utilized to study the effects of various substances on the composition and function of the gut microbiota, including changes in the diet, phytochemicals, drugs, probiotics, and, infectious pathogens [30-35]. In the context of CDI, these models have been utilized to study effects of various antibiotics on *C. difficile* growth patterns and commensal microbes [35].

1.2 Research Objectives

1. a) To establish an *in vitro* GI model capable of simulating a diverse gut microbial community and its associated metabolic capacity.
   b) To utilize this model to simulate *C. difficile* infection in the human colon.
2. To assess *C. difficile*-mediated changes in the gut microbiota through analysis of perturbations in antioxidant status of the colonic milieu, assessment of gut microbial composition using sequencing techniques, assessment of microbial metabolic function through metabolite quantification, and via assessment of cytotoxicity and immune marker production in the intestinal epithelial cells.
3. To evaluate the effects of several single-strain and multi-species combination probiotics on their ability to affect each of the previously described markers in both *C. difficile*-infected microbiota and in healthy microbiota.

CHAPTER 2: LITERATURE REVIEW

2.1 CLOSTRIDIOIDES DIFFICILE: AN OVERVIEW

Clostridioides (C.) *difficile* is an anerobic gram-positive spore-forming bacillus that is the leading cause of nosocomial infectious diarrhea [36]. *C. difficile* was first isolated from the feces of healthy infants in 1935 [37] where Hall and O'Toole showed that it had enterotoxin producing potential that was fatal in laboratory animals. However, as infants with virulent forms of *C. difficile* did not appear to show any clinical manifestations, this bacterium was largely out of the spotlight until late 1970s, where in 1978, Bartlett *et al.*, identified it as the causative agent of antibiotic-associated diarrhea and pseudomembranous colitis [38]. This was followed by development of a selective *C. difficile* growth medium by George *et al.* 1979 that allowed for numerous other groups to confirm the presence of *C. difficile* in patients with diarrhea and pseudomembranous colitis [39]. Since then, much has been learned about *C. difficile*, its virulence, toxicology and clinical manifestation.

2.1.1 Incidence

The incidence of CDI of has significantly changes since the 2000s with growth curves observed in USA, Canada, and Europe [40]. Quebec, in Canada was one of the first areas in North America to publish data in 2003 showing increased clinical severity and nearly a five-fold increase in number of cases and of within a decade, a rise from 35.6 per 100,000 population to 156.3. per 100,000 population [41,42]. Similar reports of large outbreaks were subsequently published from several regions in Europe, including the UK, Netherlands, Belgium and France [41]. In North America, there was a worrying trend amongst the elderly showing a nearly eight-fold increase in the early 2000s as compared to the 1990s [42]. Currently, the incidence in USA is estimated at a nearly 500,000 cases per year in hospitals and long-term care facilities, with mortality rates of approximately 5% [43,44]. These cases account for nearly 75% of the overall number of cases in USA, resulting in healthcare expenditures of USD $9,000 – $15,000 per patient resulting in an estimated USD $1.5 – 3.2 billion annually [45]. Following the large outbreaks in Canada, several

CDI surveillance programs including the Canadian Nosocomial Infections Surveillance Program (CNISP) network were founded with the objective to monitor and determine prevention methods for hospital-acquired CDI. In a report from 2019, it was seen that healthcare-associated-CDI showed a steady decline over time, ranging from 2.1 to 6.5 days per 10,000 in-patient days, conversely, community-associated CDI showed an increasing tread of incidence and proportions, accounting for nearly 35% of total CDI cases with estimates of 10-40 cases per 100,000 population in 2016 [46].

2.1.2 Transmission

C. difficile transmission is thought to occur by the fecal-oral route. The bacterium can be ingested in either the vegetative form or as spores through contact with symptomatic or asymptomatic patients, healthcare workers or contaminated surfaces [47]. Clabots *et al.* 1992, demonstrated the risk of newly admitted asymptomatic carriers of *C. difficile* as an important source of *C. difficile* transmission [48] however, infection control practices in healthcare settings were adapted towards symptomatic carriers due to the greater perceived risk of infecting surrounding environments and health care workers [47]. As a result, much of healthcare guidelines have focussed on *C. difficile* infection (CDI) prevention through identification and isolation of symptomatic patients. The current healthcare recommendations include the isolation of suspected CDI patients for period of up to 48 h along with certain recommended guidelines for the care of healthcare personnel. Briefly, this includes the required use of disposable gloves and gowns when caring for patients, frequenting hand-washing with soap after handling of equipment, reducing reliability of alcohol based sanitizers as they do not effectively kill the spores, and the use of chlorine based cleaning agents to clean equipment and surfaces [49]. These practices, along with a better understanding of CDI pathophysiology, including better and faster tools for diagnosis and detailed guidelines for patient care and treatment, have led to overall decline in healthcare associated outbreaks over the past few decades. However, the overall incidence is still on the rise, partly due to the prevalence of hypervirulent strains,

increased number of patients at risk undergoing antibiotic therapy, higher occurrence of recurrent CDI, and an overall rise in community-transmission of CDI as seen in Canadian provinces [46,50].

2.1.3 Clinical Manifestations

Clinical manifestations of CDI range can from asymptomatic carriers to those experiencing mild-moderate diarrhea, abdominal pain and systemic upset, to those with severe infections exhibiting life-threatening and sometimes fatal pseudomembranous colitis [51,52]. In rare cases, this may be accompanied by toxic megacolon, electrolyte imbalance and bowel perforation [53]. In most patients, symptoms only occur as mild diarrhea that disappears within a week of stoppage of antibiotic treatment [54]. However, diarrhea can also occur in the weeks after antibiotic withdrawal. In addition to diarrhea, other symptoms such as abdominal pain, nausea, fever and loss of appetite have also been reported [55]. In more severe cases, colonic lesions and patchy colitis, with or without the presence of pseudomembranes, can be found. In 1–3% of patients who exhibit fulminant colitis, the gut mucosal lining becomes necrotic with the formation of an exudative membrane containing leucocytes, epithelial cells and mucin [56]. In a colonic endoscopy, these pseudomembranes can appear as multiple white/yellow plaques of up to 2 cm in diameter [57]. The underlying intestinal submucosa can also show signs of necrosis and inflammation [55].

2.1.4 Risk Factors

Prior antibiotic therapy is seen to be a strong indicator of CDI occurrence, although several other factors increase vulnerability to CDI exposure such as being elderly, recent hospitalizations, previous CDI history and having a weakened immune/ gastrointestinal system [58]. Development of CDI in the 2-4 weeks following antibiotic therapy remains as the single largest risk factor for hospitalised patients [59]. Although all antibiotics have been associated with CDI, the antibiotics identified with the highest risk are clindamycin, broad-spectrum cephalosporins and

fluoroquinolones [59] presumably due to the development of antibiotic resistance by *C. difficile*, which, in addition to the disruption of the normal microflora by the antibiotics, allows for optimal germination of the spores [60]. Moreover, the risk for CDI increases with the dose, duration and previous history of antibiotic use [59].

Another well-defined risk factor for CDI is advanced age (> 65 years). Initial studies on CDI showed that despite infants showing a high incidence of *C. difficile* colonization that was frequently positive for toxigenic properties, they did not exhibit any symptoms. Although this might be due to infrequent use of antibiotics in their age group, it is hypothesized that this could also be the result of maternal transfer of protective antibodies, colonization resistance by the fecal microbiome, or even, a lack of *C. difficile* toxin receptors in their gut [61]. As the individual grows older and their microbiota matures, *C. difficile* detection in healthy populations decreases significantly, with only a small portion of individuals able to show asymptomatic colonization [62]. The risk of *C. difficile* colonization and subsequent clinical manifestation significantly increase with age, where it is particularly apparent in the elderly aged > 65 years who can show over 20-fold higher rates of incidence as compared to other age groups [63]. In a study by Ananthakrishnan (2011), it was seen that elderly patients with underlying illnesses are 73% likely to contract CDI during prolonged hospitalisations [64]. Other well-defined risk factors include co-morbidities such as inflammatory bowel disease and renal disorders, and individuals undergoing treatments with immunosuppressants and proton-pump inhibitors [60].

2.1.5 Recurrence

Recurrences of CDI are a serious concern with nearly 1 in 6 patients experiencing recurrent CDI in the 2 to 8 weeks following the first episode [65]. These recurrences increase the length and overall cost of hospitalization and can be difficult to manage in patients, particularly if they are elderly or have other comorbidities. In a study by McFarland *et al.* 2002, it was observed that patients who had at least one recurrence, were 45% more likely to have another episode of CDI [66]. In another study by Barbut *et al.* 2000, it was noted that 57/93 patients showed recurrent

CDI, with 25/93 showing a second recurrence and nearly 11/93 showing more than two subsequent CDI recurrences [65]. Interestingly, this study also noted that nearly 50% of these recurrences were due to re-infections rather than resurgence of *C. difficile* colonization. In this regard, recurrent CDI has been observed to be either due to a relapse where the same virulent strain persists in the GI tract of the host, or as a reinfection through either healthcare or community transmission. Most recurrences reported till date have been due to reinfections rather than a relapse, as was observed in the previously described study [65,67-69]. The risk factors behind this are thought to be a compromised immune response, altered gut microflora, continued use of antibiotics, use of antacids such as proton-pump inhibitors or those with advanced age [68]. In an investigation by Warny *et al.* 1994, it was demonstrated that patients with recurrent CDI had a significantly lower production of immunoglobulin (Ig)A and anti-toxin IgG [70]. Additionally, studies have shown that patients with continual use of antibiotics and those experiencing recurrent CDI have a severely impaired gut microflora, showing a decreased commensal microbial diversity [71].

2.1.6 Diagnosis

CDI diagnosis is usually considered by a combination of criteria; the presence of diarrhea (> 3 unformed stools the span of 24 h) within 8 weeks of antibiotic usage, and, a positive stool test for the presence of toxigenic *C. difficile* or its toxins along with a colonoscopy for lesions or pseudomembranous colitis [40,72-74]. Many different approaches in CDI diagnosis exist, most of which are based around the detection of toxins in the stool. The cytotoxin assay is the gold standard assay for CDI diagnosis, which involves identification of *C. difficile* toxin B in cell culture [75]. In this assay, patient stool is incubated with cultured Vero cells to look for signs of cytotoxicity exhibited by rounding and shrinkage of the cells. However, as this assay can take up to 72 h to perform and requires expensive equipment and skilled personnel, other rapid molecular-based assays are utilized instead [76]. The most commonly used detection method is the rapid enzyme immunoassay (EIA) which can be performed with relative ease and gives good

sensitivity (75-85%) and specificity (95-100%) [54]. Another frequently utilized rapid test is the *C. difficile* antigen test that detects glutamate dehydrogenase (GDH). Although this test is highly reliable in detecting GDH (~ 100% specificity), it does not have the capability to detect toxigenic strains [77,78]. More recently developed assays that are based on amplification of *C. difficile* toxin B encoding gene (nucleic acid amplification test, NAAT) using polymerase chain reaction or isothermal amplification result in a higher sensitivity (80-100%) and specificity (87-99%) in comparison to EIA tests [54]. Although, these results only show the presence of the toxin producing gene and may not accurately indicate the actual presence of toxins in the stool. Thus, in the case of NAAT test, a second test confirming toxin presence might be required for diagnosis. According to the guidelines published by European Society of Clinical Microbiology and Infectious Diseases (ESCMID), no test can be used as a stand-alone test to diagnose CDI [74]. The recommendation is to use two assay types in combination, one that can accurately predict negative results, such as NAAT, EIA or GDH, and a second test, such as toxin detection to accurately predict positive results. In this manner, if a toxin detection result shows as negative, but NAAT and/or GDH show positive, it can be inferred as an asymptomatic colonization [79].

2.1.7 Virulence Factors

C. difficile virulence can be broadly classified into a three-step process, beginning with the alteration of GI microbiota and bile acid metabolism through the use of antibiotics or other risk factors, which then allows for germination of ingested spores, leading to bacterial growth and colonization through adhesion onto the gut lumen, and finally, enterotoxin production [80].

The transmission of spores plays an important aspect in this process as vegetative cells are susceptible to the acidic conditions of the stomach. Thus, the passage of the spores and its subsequent germination in the small intestine by bile acids is an important factor for *C. difficile* colonization. The primary bile acids, cholate and chenodeoxycholate differently affect spore germination. Cholate along with the amino acid glycine promotes germination, whereas, chenodeoxycholate inhibits germination [81]. Additionally, the secondary bile acids deoxycholate

and lithocholate also have differential effects on the spores, where deoxycholate simulates germination and lithocholate inhibits it. Interestingly, although deoxycholate promotes germination, it is also seen to be inhibitory of vegetative cell growth in the colonic area [82]. During healthy conditions, in the small intestine, cholate activates germination of spores, but in the proximal colon, deoxycholate presence inhibits the growth of *C. difficile* cells, thereby providing colonization resistance. Alteration of the gut microbiota leads to a shift in this balance by reducing the conversion of primary bile acids into secondary bile acids, thus allowing for vegetative cells to survive in the absence of deoxycholate, resulting in colonization [83].

In order for *C. difficile* infection to result in diarrhea and colitis, a number of virulence factors are required. These include its ability to colonize the gut mucosal surface through a range of adherence and motility factors such as its flagella, fimbriae and S-layer proteins, and most importantly, its enterotoxin-producing ability. *C. difficile* produces toxins as a response to nutrient availability to target intestinal epithelial cells, promoting necrosis and thereby leading to a loss in epithelial barrier integrity and subsequent activation of an acute inflammatory response [83]. Although the surface proteins of *C. difficile* also contribute to intestinal inflammation, the primary mode of *C. difficile* pathogenesis is through the effects of its two toxins, Toxin A (TcdA) and Toxin B (TcdB).

TcdA and TcdB have been widely demonstrated to be responsible for the symptoms associated with CDI [84]. In animal models of hamsters and mice, administration of only the toxins was able to exhibit CDI symptoms of intestinal fluid accumulation, diarrhea, hemorrhage, and death [80]. In addition, hamsters infected with strains that did not produce either toxin were shown to be asymptomatic [85]. Interestingly, animal studies showed TcdA to be the potent toxin due to its ability to elicit CDI pathogenesis in the animal models, whereas TcdB did not appear to elicit any of symptoms [86]. Further investigation revealed that TcdB to be 500-1,000 times more cytotoxic than TcdA in *in vitro* cell culture studies [87]. Moreover, studies showing that TcdA-negative

TcdB-positive *C. difficile* strains could also cause significant disease [88], showing that both these toxins have a role in disease propagation.

TcdA and TcdB share nearly 66% of their amino acid structure which includes important domains that allow for their endocytosis, cleavage and subsequent activation [1]. The C-terminal receptor binding domain consists of a series of repeating oligopeptides that binds to, yet uncharacterised, cell surface receptors of epithelial cells [84]. This binding triggers endocytosis through a clathrin-dependant pathway [86]. Once inside the cells, a hydrophobic translocation domain inserts the toxins into the endosomal membrane through pore formation [1]. A cysteine protease domain, adjacent to the glucosyltransferase domain, then cleaves the toxin by utilizing host cell-derived molecule inositol hexakisphosphate into its biologically active form, a N-terminal glucosyltransferase molecule which is this then released into the cytosol [89]. Once in the cytosol, the N-terminal regions of TcdA and TcdB inactivates a number of cellular proteins belonging to the Rho family of small GTPases, affecting their interactions with regulatory molecules and interrupting their downstream signaling pathways [90].

This dysregulation leads to an impairment in the formation of the actin cytoskeleton, resulting in cell rounding and shrinking [84]. In addition, cellular apoptosis pathways are upregulated through the activation of caspase-3 and caspase-9 [91]. As a consequence, there is a disruption in the cellular tight junctions, leading to loss of epithelial barrier integrity and function, subsequently leading to an increased intestinal permeability and fluid accumulation which manifests as diarrhea, and, ultimately leads to neutrophil recruitment resulting in pseudomembranous formation in severe CDI colitis [1]. TcdA and TcdB have also been demonstrated to activate several pro-inflammatory cytokines and chemokines characterised by interleukin (IL)-8, IL-6, IL-1β, interferon-γ and tumor necrosis factor (TNF)-α amongst others. Moreover, the toxins have also been shown to affect the enteric nervous system to release several neuropeptides, such as neurotensin, that along with cytokines, form an acute inflammatory response in the colon [1,92].

C. difficile has also been shown to produce a third toxin in approximately 6 to 12.5% of strains, known as *C. difficile* transferase (CDT) [65]. Although its role in contributing to *C. difficile* disease pathology is poorly understood, studies have indicated that it may play a part in the adherence of the bacillus onto epithelia cells through the dysregulation of the cytoskeleton and formation of microtubules [93].

2.1.8 Treatment

The current recommended treatment of CDI is by a regimen of antibiotics for a period of 10-14 days which are prescribed based on clinical severity and patient history. The first step in treatment usually involves discontinuation of the intercurrent antibiotic followed by a treatment response based on clinical presentation, such as those set by The Infectious Diseases Society of America and the Society for Health Care Epidemiology of America [73]. Mild to moderate CDI is usually defined as diarrhea presentation (>3 unformed stools within 24 h) without any complications associated with severe CDI, whereas severe CDI is classified as the presentation of hypoalbuminemia (< 30 g/L) along with presentation of either abdominal tenderness or a high white-blood cell count (≥ 15,000 cells/mm^3) [53]. Metronidazole is the first-line antibiotic for mild-moderate cases, whereas vancomycin is usually the treatment for more severe cases [79]. In two multinational randomized controlled trials, Johnson *et al.* 2014, showed that vancomycin was more effective than metronidazole in treating mild-moderate CDI showing an efficacy of 81% in 259 patients versus 73% efficacy of metronidazole in 278 patients [94]. However, in a meta-analysis of various antibiotic treatments for CDI, Nelson *et al.* 2017, found only moderate evidence that vancomycin was more effective than metronidazole [95]. Each of these studies did not show any significant differences between vancomycin and metronidazole as the choice of antibiotic to treatment of severe CDI patients [94,95]. Treatment with metronidazole is still the most preferable primarily due its low costs in comparison to the other antibiotics [53]. Fidaxomycin, a novel macrocyclic narrow-spectrum antibiotic designed for gram-positive pathogens [96] has shown comparable or better efficacy in comparison to vancomycin [95,97]

and shows higher effectiveness in reducing CDI recurrence as compared to the other antibiotics [96]. In the study by Louie *et al.* 2011, CDI recurrence for fidaxomicin treated patients (n = 548) was significantly lower at 15.4% as compared to 25.3% in the vancomycin group [96].

Table 2.1 Antibiotics prescribed in *Clostridium difficile* infection treatment [53,54,73,98]

Antibiotic	Recommended Use	Dosage
Metronidazole	For mild-moderate cases.	500 mg x 3/d orally for 10-14 d (500 mg x 3/d via IV for severe cases along with vancomycin)
Vancomycin	For all levels of severity, but mostly used in severe and recurrent cases	125 mg x 4/d orally for 10-14 d (500mg every 6 h for severe cases)
Fidaxomycin	For moderate to severe; mostly used in cases where high chance of recurrence is present	200 mg x 2/d orally for 10 d

2.2 The Role of Gut Microbiota in *C. difficile* Infection

The gastrointestinal tract harbors the largest microbial population of up to 10^{14} microorganisms in the colonic area [99] of which bacteria compose the majority, followed by archaea, eukaryotes and viruses [100]. The role of gut microbiota in the maintenance of human physiology through metabolic regulation and immune system modulation has been widely established [101,102]. Furthermore, the role of the indigenous gut bacteria in protection against significant alterations in the gut lumen, such as that by pathogen invasion, has been previously established [103]. This phenomenon, known as colonization resistance, can occur through several different modes of action which primarily involve inhibition mechanisms to inhibit pathogen growth through

secretion of substances like bacteriocins [104], or depletion of essential nutrients like iron [105], or via prevention of colonization on the mucosa through competition of binding sites [106]. The microbiota also plays a significant role in the maintenance of epithelial barrier integrity and in the regulation of innate and adaptive immunity of the host through activation of T-cells and receptors such as Toll-like receptors, both of which also play a key role in maintaining intestinal homeostasis [83,107,108].

Pathogenic bacteria, such as *C. difficile*, can only cause infections after reaching sufficient population densities, which allow for expression of its enterotoxins that then interact with the intestinal epithelium to trigger its cytopathic and inflammatory pathways [55]. These alterations in the host immune system allow for further disruptions in the microbial balance, exacerbating the disease [109]. Colonization resistance can therefore play an important role in prevention of *C. difficile* growth and subsequent infection. In infants, where nearly 50-70% are considered to harbour toxigenic strains of *C. difficile*, yet rarely show any clinical manifestations [110]. In the adult population, many carriers of toxigenic strains are asymptomatic, presumably due to antibodies developed during their early years [111]. In addition, certain individuals show better resistance to severe and recurrent CDI, also thought to be associated with the properties of their microbiota [111]. Furthermore, re-establishment of the diversity and functionality of the native microbiota is associated with a decrease in clinical severity and pathogen clearance in cases antibiotic-associated diarrhea [112,113]. Thus, understanding the processes involved in conferring colonization resistance to CDI, therapeutics can be better designed/screened to modulate the CDI intestinal ecosystem in order to restore homeostasis.

2.2.1 Changes in Microbiota are Associated with Loss of Colonization Resistance

Numerous studies have been conducted to further understand the role of gut microbiota in colonization resistance against CDI and how the risk factors along with *C. difficile*-mediated effects can result in an altered gut microbiota [114,115]. Prior antibiotic therapy and the subsequent alterations in the native microflora is thought to be the primary risk factor in the

development of CDI [116]. The role of antibiotics in altering the indigenous microbiota was first noted in a study by Miller *et al.* 1957, where in a mice model of *Salmonella typhimurium* infection, the dosage required to infect the mice was reduced by 100,000-fold following antibiotic treatment [117]. Subsequently, several other studies have since used this methodology to first disrupt the native microbiota by antibiotics to create successful animal models of infection, including those of CDI [118-120].

Antibiotic therapy has been strongly associated with alterations of the native microbiota, leading to reduced diversity and dysregulated microbial metabolism [116]. Although these effects vary from one antibiotic type to another, several animal and human studies have demonstrated alterations in the microbiota may result in long-lasting effects, even after discontinuation of treatment [116,121]. Dethlefsen *et al.,* reported that although microbial community was re-established in the weeks following antibiotic treatment, the diversity did not revert back to its original state [121]. Theriot *et al*., demonstrated that in a mouse model, at the native state, mice exhibited colonization resistance to *C. difficile*, but when treated with the broad-spectrum antibiotic cefoperazone, the mice were completely susceptible to infection. Interestingly, after a 6-week recovery period, the mice again returned to state of attaining colonization resistance [122]. In a study by Reeves *et al.,* using a mice model of CDI pre-treated with antibiotics found that in the microbial composition, members from the *Lachnospiraceae* family showed a decrease accompanied with an increase in members from the *Enterobacteriaceae* family [123]. A follow-up study was conducted to understand their potential role in colonization resistance to *C. difficile*, where it was reported that mice vaccinated with *Lachnospiraceae* were able to suppress *C. difficile* colonization and toxin production, presumably attributed due to its butyrate producing abilities [124]. Similar shifts in microbial composition were observed in a study by Buffie *et al.,* which showed that mice pre-treated with the antibiotic clindamycin had reduced microbiota diversity for at least 28 days, characterized by decreased presence of *Bifidobacterium*, *Clostridium* and *Bacteroides*, and increased prevalence of *Enterobacteriaceae* [125]. Similar observations were noted in studies involving human subjects, such as the study conducted by

Antharam *et al.* 2013, who reported that in patients with antibiotic-associated diarrhea, butyrate producers *Ruminococcaceae*, *Lachnospiraceae* and *Clostridia* decreased, along with an increase in *Enterococci* and *Veillonellaceae* [4].

Microbial composition analysis can also provide certain insights into how an individual is able to confer colonization resistance, as in the case of asymptomatic carriers, or predict adverse outcomes in the case of CDI patients [126]. The studies have shown that the bacterial phyla, *Firmicutes* and *Bacteroidetes* were more diverse and higher in prevalence in asymptomatic carriers, who were associated with lower *Proteobacteria* in comparison to those who developed CDI [60]. In work done by Buffie *et al.* 2015, microbial community characterisation was done to identify potential bacterial groups that were significantly associated with resistance to CDI development. Their results showed that the preservation of *Clostridium scindens,* an important microbe involved dehydroxylation of primary bile acids, was associated with resistance to CDI [127]. Furthermore, in a study by Khana *et al.* 2016, statistical analysis of the microbial composition was able to predict patients who would respond to therapy and those with a higher chance of CDI recurrence. Patients who responded to treatment had higher levels of *Ruminococcaceae, Rikenellaceae, Clostridia, Bacteroides, Faecalibacterium,* and *Rothia* in comparison to the non-responders. Additionally, patients that experienced recurrent CDI had increased *Veillonellaceae, Enterobacteriaceae, Streptococci, Parabacteroides,* and *Lachnospiraceae* when compared to those without recurrent episodes [128].

Such alterations in the microbial community also significantly impact the metabolic activities of the gut microbiota, resulting in decreased presence of bacterial metabolites that might inhibit *C. difficile* growth [129]. Mouse studies have shown that antibiotic-mediated disruptions in the microbiota alters bile acid metabolism, resulting in decreased levels of secondary bile-acid cholate that plays an important role in the inhibition of *C. difficile* spores [116]. Changes in the microbial balance also results in an altered nutrient profile, that favors *C. difficile* growth.

Decrease in commensal bacteria leads to an accumulation of normally utilized substrates, such as sialic acid (component of the mucosal layer) and succinate (short-chain fatty acid), allowing *C. difficile* to catabolize these substrates for its own growth [130,131]. The short-chain fatty acid, butyrate has been demonstrated to inhibit *C. difficile in vitro* growth and modulate its subsequent inflammatory response [4]. Butyrate also plays several functional roles in the GI that maintain intestinal health and contribute to colonization resistance of the host [132]. Important butyrate producers such bacteria from *Lachnospiraceae* family or *Faecalibacterium prausnitzii* have been shown to be consistently decreased in patients undergoing antibiotic-treatment or those with CDI [4]. Furthermore, other antimicrobial secretion products such as bacteriocins can play a role in the inhibition of *C. difficile*, although this has not been well characterised. In a study by Rea *et al.* 2011, several bacteriocins showed capacity to suppress *C. difficile* growth, potentially showing another method of colonization resistance that could be lost from altered microbiota composition [133].

2.2.2 Restoration of Microbiota-mediated Colonization Resistance using Bacteriotherapy

Antibiotic treatment of CDI is seen to have overall success rates of 70-80% for symptomatic cure in mild-moderate CDI, although this decreases significantly in more severe cases and in recurrent CDI [134]. Furthermore, antibiotic treatment of CDI has been associated with several long-term effects on the GI tract, including alterations of the immune function [95], further exacerbating the loss of colonization resistance due to decreased microbial diversity, and in the development of antibiotic resistance in the host as well as in *C. difficile* strains [135]. Thus, alternative approaches to antibiotics alone are being widely explored. In this regard, bacteriotherapy, defined as the intentional use of bacteria or their products to treat any disorder, has shown potential to counteract some of these side-effects and aid in restoring colonization resistance [12,136,137]. The use of probiotics and fecal microbiota transplantation (FMT) are two such examples of bacteriotherapy of CDI.

FMT is the transplantation of the entire fecal population of organism from screened healthy individuals into CDI patients in order to directly change/restore their microbial composition, thereby leading to better health outcomes [138]. Mainstream use of fecal enemas for the treatment of CDI pseudomembranous colitis was first described in 1958 by Eiseman *et al.* 1958 [139] showing the potential of fecal microbes as therapeutic agents [140]. FMT can be administered through the nasogastric tube, upper tract endoscopy, colonoscopy, or retention enemas [141]. In CDI, FMT is currently recommended only for patients that have experienced > 3 recurrent CDI episodes [53]. Several case studies and a few randomised controlled trials have shown that FMT has the potential to successfully treat over 80-90% of recurrent CDI patients without any short-term adverse effects, however, its effectiveness in treating primary CDI could not be established [142]. Moreover, a systematic review also noted that the effectiveness of FMT greatly depended on the donor, the method of fecal inoculum preparation and route of administration [142]. Despite such promise of FMT in treating recurrent CDI, very few studies have assessed the long-term risks involved. Moreover, due to the complex nature of stool samples, its use as mainstream medicine cannot be defined.

In this regard probiotics are seen to be beneficial, even though they do not exhibit the same success rates of FMT. Probiotics have been generally regarded as safe to use in a wide variety of gastrointestinal disorders and can be pre-defined to address specific adverse outcomes in the gut lumen [143]. Moreover, several studies have shown beneficial effects of probiotic supplementation in the gastrointestinal tract such as improvements in fermentation abilities of the indigenous microbiota [15-18], preservation of colonization resistance of the host [19-21], and in regulating host immune function [19,22-24]. With regard to CDI, several probiotic strains have shown potential to reduce CDI-associated diarrhea, aid in prevention of primary CDI, and reduce episodes of recurrent CDI [12]. Of the several probiotics studied till date, *Lactobacillus (L.) rhamnosus* GG and *Saccharomyces (S.) boulardii* have shown the strongest clinical evidence [12,26].

2.3 USE OF PROBIOTICS IN CDI

The rationale for probiotic administration in CDI is largely due to their potential in restoring intestinal homeostasis through various mechanisms that restore microbial diversity and functional capacity [144]. Several clinical trials and *in vitro* studies have been conducted to assess the efficacy of probiotics in the management of *C. difficile* infection [12,27,145]. Although some *in vitro* evidence of the efficacy of probiotics have been demonstrated, such as the capability of *S. boulardii* to directly inhibit *C. difficile* growth and its toxins [146,147], this has not been observed in randomised controlled trials. In this regard, the use of probiotics has been particularly focussed on their ability to reduce the clinical manifestations and length of CDI-associated diarrhea. Moreover, there is promising evidence for probiotic supplementation in reducing recurrent CDI episodes when used as an adjunct with antibiotics [148].

2.3.1 *Saccharomyces boulardii* and CDI

S. boulardii is very closely related to *Saccharomyces cerevisiae*, which is commonly known as brewer's or baker's yeast. The role of *S. boulardii* as probiotic garnered interest through initial studies of its efficacy in reducing symptoms associated with *Vibrio cholerae* infection [149]. *S. boulardii* was shown to secrete proteases that inhibited cholera toxin action in the intestinal tract, thereby decreasing secretion of chloride and fluid in response to *Vibrio cholerae* infection [149]. Early studies of recurrent CDI in a hamster model also showed efficacy of *S. boulardii* to prevent *C. difficile* growth [150]. In CDI patients, two randomized controlled trials have examined the role of *S. boulardii* in the prevention of CDI and CDI-associated diarrhea [20,151]. The first trial, conducted by McFarland *et al.* 1994, supplemented 3×10^{10} cfu/d of *S. boulardii* for 28 d in 124 adult patients on varied doses of vancomycin or metronidazole, with a 4-week follow-up for assessment of recurrent CDI [20]. The findings from the study showed that the frequency of recurrent CDI in the probiotic supplemented groups (15/57) was significantly lower than those in control groups (30/67) [20]. Moreover, it was revealed that patients who already had a prior

episode of CDI had a more significant effect of *S. boulardii* supplementation (34.6 %) as compared to those who had their first episode of CDI (19.3%) [20]. In another randomised controlled trial by Surawicz *et al.* 2000, 2×10^{10} cfu / d of *S. boulardii* was supplemented for 28 d in 170 adult patients with recurrent CDI, in conjunction with high or low dose of vancomycin, or, metronidazole [151]. The findings showed that recurrence amongst patients in the *S. boulardii* (43.3%) was not significantly different from those in the placebo group, however, in the group that received high-dose vancomycin (2 g/d) and *S. boulardii* there was a significant reduction in recurrent CDI (3/18 vs. 7/14) [151]. Collectively, both these trials only indicate a potential role of *S. boulardii* in the prevention of recurrent CDI.

Despite limited evidence of *S. boulardii* in primary prevention of CDI as exhibited by the two RCTs, several pieces of evidence from animal models and *in vitro* systems show the potential of *S. boulardii* to manage *C. difficile* infection [149]. In a study by Pothoulakis *et al.*, *S. boulardii* exhibited inhibition of TcdA binding to the rat ileum and its subsequent enterotoxicity in [152]. Similarly, in a study by Tasteyre *et al.*, *S. boulardii* was shown to inhibit *C. difficile* binding onto Vero cells by exhibiting proteolytic action and stearic hindrance [153]. Furthermore, in the set of studies by Castagliuolo *et al.* 1996 & 1999, *S. boulardii* was shown to inhibit TcdA in rat ileum through protease secretion [146] and inhibit both TcdA and TcdB cytotoxicity by cleavage of the toxins through proteases in human colonic HT-29 cells [147]. *S. boulardii* has also shown efficacy in modulating host immune response to the inflammatory cascade activated by *C. difficile* and its toxins, through increasing intestinal anti-toxin IgA [154], regulating the activation of nuclear factor kappa-B and mitogen-activated protein kinase signaling pathways [155], and inhibiting production of pro-inflammatory cytokines such as IL-8 [156].

2.3.2 *Lactobacilli* and CDI

L. rhamnosus GG (LGG), a sub-strain of *L. rhamnosus*, was isolated from the intestinal tract by Gorbach and Goldin in 1983 [149]. LGG has been demonstrated to survive the acidic conditions of the digestive tract and colonize the gut lumen [157]. In the context of CDI, LGG has been

primarily studied for recurrent CDI and amelioration of CDI-associated diarrhea [158]. The first published study on LGG use in CDI patients was by Gorbach *et al*. 1987, who demonstrated that in LGG supplementation was successful in treating recurrent CDI in five adult patients [159]. Another case study by Biller *et al.* 1995, showed that supplementation of LGG alone (5 x 10^9 cfu) in four children for a period of two weeks was effective in preventing any further recurrent episodes [160]. In a follow-up study by the same group, Bennett *et al.* 1996, showed that LGG supplementation in 32 patients with prior CDI episodes (minimum 10^9 cfu/d for at least 10 days; treatment regimen differed based on patient condition) resulted in the successful treatment of recurrent CDI in 27/32 (84%) patients based on a 2-month follow-up period [148]. In a study by Pochapin (2000), LGG supplementation in 25 adults on antibiotic treatment for primary and recurrent CDI resulted in no significant difference between the probiotic and placebo group [161]. A more recent study by Lawrence *et al*. 2005, a small randomised controlled trial of 15 patients on antibiotic treatment, were supplemented with 8 x 10^{10} cfu/d during antibiotic usage and for 21 days thereafter[162]. Their findings showed that in the probiotic group 3/8 patients had recurrent CDI episodes whereas 1/7 in the control group relapsed, although this did not reach statistical significance [162]. Collectively, the clinical evidence of LGG is seen to be moderate in the prevention of recurrent CDI due to its small sample sizes and many uncontrolled-for studies.

LGG has shown beneficial effects in the prevention and treatment of the diarrhea of various aetiologies in children and in adults that could be mediated through a multitude of mechanisms indicated by *in vitro* and animal studies such as excretion of biosurfactants and organic acids [163], production of anti-microbial bacteriocins [164], and hydrogen peroxide propagation to inhibit pathogen colonization on epithelia [165].

Another bacterium in the *Lactobacilli* group, *L. plantarum* 229v has also been studied in a clinical trial for its efficacy on recurrent CDI. Wullt *et al.* 2003, conducted a multi-site randomised controlled trial with 20 adults with recurrent CDI (1-5 prior episodes), supplementing the probiotic group with 5 x 10^{10} cfu/d for 38 days with a 70-day follow-up period [166]. The findings

from the study showed no significant difference in the frequency of recurrent episodes in patients from the probiotic group (4/11, or 36%) when compared to the antibiotic-only group (6/9, or 67%) [166]. In a follow-up study, the group also showed that *L. plantarum* 299v was associated with an increase in total short-chain fatty acid (SCFA) and butyrate production of in the fecal samples from recurrent CDI patients [16].

2.3.3 Probiotic Mixtures and CDI

The use of probiotic mixtures in CDI has not been well established in current literature although, two recent randomized controlled trials indicate potential of using mixtures in prevention of CDI. Hickson *et al.*, studied the development of antibiotic-associated diarrhea in 135 inpatients on antibiotic treatment [167]. A secondary outcome of CDI-associated diarrhea through toxin presence in the stool was also analysed. Patients in the probiotic groups received a 97 mL drink per day containing *L. casei* DN-114 001, *S. thermophilus,* and *L. bulgaricus* until a week after antibiotic discontinuation. The control group received a sterile milkshake as a placebo. The findings from this study showed a significant reduction in antibiotic-associated diarrhea occurrence in the probiotic group as compared to control (12% vs 34%, $p < 0.01$). In the 4-week follow up, 9/53 patients in the control group developed CDI-associated diarrhea, whereas no occurrence was observed in the probiotic group [167]. However, due to extremely selective inclusion exclusion criteria and improper blinding of probiotic treatment, these results may not accurately portray its efficacy [149]. In a second randomised controlled trial, Gao *et al*. 2010 supplemented a mixture of *L. acidophilus* CL1285 and *L. casei* LBC80R in 255 adult inpatients (10 x 10^{10} and 5 x 10^{10} cfu/d) undergoing antibiotic therapy with a subsequent follow-up of three weeks [168]. The findings of the study showed that high dose probiotic group had a lower incidence of antibiotic-associated diarrhea (15.5% vs. 28.2%) when compared to the low-dose probiotic group. Each probiotic group had a significantly lower incidence of antibiotic-associated diarrhea in comparison to placebo group (44%). Moreover, the study showed reduction the length of adverse symptoms in the probiotic groups. Interestingly, nearly 25% of the placebo

group developed CDI-associated diarrhea in comparison to 1% in the high-dose probiotic group, and 9% in the low-dose probiotic group [168]. Despite such promising results, this study has drawn skepticism due to its unusually high incidence of CDI development in the placebo group, and a nearly complete prevention of primary CDI in the probiotic groups, both of which have not been observed in previous clinical studies [158].

2.3.4 Meta-analyses of Probiotic Supplementation in CDI

Several systematic reviews have been conducted to assess the efficacy and safety of probiotics in the prevention of CDI in adults and children. In a systematic review of probiotics for the prevention of AAD and CDI by McFarland (2006), six random controlled trials (RCTs) were included in the meta analyses, of which, five RCTs involved CDI patients being treated with a combination of antibiotic (either vancomycin or metronidazole) and probiotic [169]. The findings from the meta analyses revealed only one RCT had sufficient evidence to recommend using *S. boulardii* to prevent recurrent CDI, whereas the remaining RCTs did not show any significant differences between probiotic and placebo groups [169]. A systematic review by Pillai *et al.* 2008 assessed randomized, prospective studies for probiotic efficacy in resolution of CDI-associated diarrhea, and occurrences of recurrent CDI and adverse outcomes [170]. From the four RCTs that met the inclusion criteria, only one study showed a statistically significant benefit for the use of *S. boulardii* used in combination with antibiotics for secondary prevention of CDI. Neither of the other studies showed a significant benefit, thereby leading the authors to conclude there was insufficient evidence to support the use of probiotics as an adjunct to antibiotic therapy for either primary or recurrent CDI [170]. In a more recent systematic review assessing probiotic efficacy in preventing CDI-associated diarrhea in adults and children receiving antibiotics, Johnston *et al.* 2012, conducted a meta analyses of twenty trials involving probiotic intervention (with a total of 3818 patients) [171]. The outcome of the meta analyses found that probiotic supplementation significantly reduced the occurrence of CDI-associated diarrhea by 66% (RR 0.39; 95% CI 0.26 to 0.59) without an increase in adverse outcomes [171]. However, as there was missing data from

13/20 trials, the authors concluded that there was only moderate-quality evidence to suggest the use of probiotics in the treatment of CDI-associated diarrhea. Thus, although most systematic reviews have no found overall benefit in the use of probiotics in the primary prevention of CDI, there is moderate quality evidence showing their efficacy in reducing CDI-associated diarrhea, and in the case of *S. boulardii*, good quality evidence suggesting its use in recurrent CDI [149].

2.3.5 Safety of Probiotics in Clinical Intervention

The use of probiotics has been rarely associated with any adverse outcomes in the clinical trials of antibiotic-associated diarrhea and CDI [172]. Some mild-moderate adverse reactions have been reported, such as nausea, vomiting, abdominal cramps or pain and rash, these are infrequently reported and do not differ from the reactions reported in placebo groups [158]. Moreover, clinical trials testing probiotics have not reported any probiotic-mediated bacteremia, fungemia or instances of intestinal translocation [172]. In non-clinical trial settings, 12 cases of LGG-associated bacteremia, and 24 cases of *S. boulardii*-associated fungemia have been reported in pre-term neonates, severely debilitated or immuno-compromised patients, and in immunocompromised adults [158].

2.3.6 Advantages of Probiotic Therapy

The ability of probiotics to survive GI tract passage into the colonic region, allowing for its interaction with the intestinal lumen and gut flora through several modes of action confer certain advantages to its utilization in the management of antibiotic-associated diarrhea and CDI. Moreover, probiotic therapy has been demonstrated to be safe to use with no reported adverse clinical outcomes, or any known adverse interactions with antibiotics or in combination with probiotic agents [149].

An important advantage of probiotics is their ability to survive the gastric acidity and reach the intestinal lumen as viable microbes, and in some cases even result in colonization of the lumen

[173]. In both animal models and human subjects, supplemented probiotics have ben detected in the fecal content [174]. It has been proposed that detection of over 10^8 cfu probiotics/g feces can indicate successful survival through the GI tract [175]. Limited information exists on the survival rates of probiotics in the clinical studies assessing its efficacy on antibiotic-associated diarrhea and CDI. In one clinical study by Elmer *et al*. 1999, the authors noted that in the follow-up period after *S. boulardii* supplementation, patients with further episodes of recurrent CDI had a significantly lower count of S*. boulardii* detected in their stool as opposed to those who did not relapse (2×10^4 cfu/g feces vs. 1×10^6 cfu/g) [158,176].

Probiotics, once in the gut lumen, can then impart several putative mechanisms of action on the gut lumen. The modes of actions can be broadly classified into three main categories: interaction with gut microflora to modulate its diversity and metabolic abilities; competition for nutrients and production of antimicrobial secretions to inhibit infections; interaction with the gut lumen to maintain the mucosal layer and barrier integrity, and regulate host immunity [15-24]. Although not all probiotics have the capacity to exhibit each of these mechanisms, it has been demonstrated that each strain can affect the colonic milieu in a multitude of ways to protect against infection and restore intestinal homeostasis [177].

2.3.7 Limitations

Apart from the previously discussed limitations in the design and methodology of probiotic intervention studies, there are several drawbacks and unknowns in the use of probiotics for CDI. A major drawback in probiotic studies is the variation of doses utilized in animal models, *in vitro* systems or in human clinical trials. Currently, the range of doses utilized are between 10^7 to 10^{11} cfu / day [178]. In the meta analysis by McFarland (2006), it was reported that probiotic efficacy in antibiotic-associated diarrhea was significantly higher in trials that utilized $\geq 10^{10}$ cfu/d (8/12) as opposed to $< 10^{10}$ cfu/d (2/12) [178]. Despite these findings, the requirement of high-dose probiotics is still not considered a requirement, as many factors are poorly understood, including its pharmacokinetics in the GI tract, how certain physiological conditions and patient populations

could affect dosage, and importantly, strain specificity that might determine different dose requirements [158]. Another important consideration is the lack of quality control in commercial probiotic products. Although some are manufactured by established pharmaceutical companies with stringent manufacturing and quality control practices, not all commercial supplements comply to those standards [158]. Several studies assessing probiotic quality control in commercially available supplements have found presence of contaminants and discrepancies in stated dosage and number of strains present [179,180].

CONNECTING STATEMENT I

The literature review presented in Chapter 2 summarises the evidence on *C. difficile* pathophysiology and how probiotics might help in better management, recovery and possible prevention of recurrent *C. difficile* infection. To better understand the role of probiotics and their mechanism of action to counteract *C. difficile* infection-mediated changes, it is essential to be able to monitor these changes occurring in the gut lumen. This latter aspect can, however, be challenging to study in *C. difficile* patients as normal methods of testing stool samples only provide a limited amount of information regarding microbial or metabolic changes in the colon. Moreover, assessing any changes in colonic morphology or real-time sample collection can be invasive and pose several ethical considerations. In this regard, the use of *in vitro* gastrointestinal models is seen to be advantageous. These models simulate all components of the gastrointestinal tract, including enzymatic digestion, microbiota formation, metabolic activity and controlled environmental conditions. Moreover, these models have shown the capability to replicate *in vivo* colonic environments including the rich and diverse microbiota and its associated metabolic functions. Thus, such models can serve as reliable preclinical models to screen and test potential probiotics for their therapeutic ability in *C. difficile* infections. In Chapter 3 we assessed the ability of an *in vitro* gastrointestinal model developed in our lab to simulate the colonic environment.

CHAPTER 3: RESEARCH PAPER 1

Growth Dynamics of Human Fecal Microbiota Cultured in an *In Vitro* Model of the Human Digestive Tract

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3.1 ABSTRACT

In recent years, disruption to the delicate balance of gut microbiota has been implicated as a causal factor in a multitude of metabolic disorders making it crucial to understand the effects of various internal and external stressors on the gut microbiota. Given the complexity of studying dynamic changes in the gut lumen, *in vitro* gastrointestinal (GI) models, such as the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) have been used to simulate and monitor gut microbiota. However, not much is known about the growth dynamics of the microbiota and the associated metabolic functions in the colonic segments of such models. In the present study we assessed these dynamics over a two-week period in a recently developed small-scale, dynamic multi-compartmental GI model using human fecal matter inoculate. The findings from the study show that the colonic microbiota exhibited metabolic activities in accordance to previously reported values of the *in vivo* human colon. The rate of short chain fatty acid (SCFA) production was observed to stabilize within 5 days of fecal inoculation, whereas the production of ammonium and hydrogen sulphide remained stable across the two-week period. Microbial composition analyses showed distinct community formation within 5 days of inoculation in the different colonic reactors that remained stable up to day 9. In addition, the composition analysis revealed similar microbial community characteristics between fecal inoculates taken from the same donor at different times. These findings indicate the capability of this model to be utilized for future testing of various interventions with a close replication of metabolic function and a reproducible microbial community.

3.2 INTRODUCTION

There are over 100 trillion commensal microorganisms in humans, colonizing the gastrointestinal tract, genitourinary tract, oral cavity and the respiratory tract amongst others [1]. The gastrointestinal (GI) tract harbors the largest microbial population of up to 10^{14} microorganisms in the colonic area [1] of which bacteria compose the majority, followed by archaea, eukaryotes and viruses [2]. Advances in metagenomic sequencing and culturing techniques have helped characterize a number of species, although many remain to be characterized [3]. Amongst the characterized bacterial species, numerous studies have shown that the bacterial population is primarily composed of *Firmicutes*, *Bacteroidetes* and *Actinobacteria* phyla, followed by *Proteobacteria*, *Cyanobacteria*, *Fusobacteria*, and *Verrucomicrobia* [2, 4, 5]. Several factors such as age, sex, geographical origin, diet and antibiotic usage can differentially affect the composition of the gut microbiota [6-11].

The gut microbiota has demonstrated to play an essential role in the maintenance of human physiology through metabolic regulation and immune system modulation [12, 13]. They play a role in digestion of food through fermentation of dietary fibers producing several beneficial short-chain fatty acids (SCFAs) [14, 15] and through production of enzymes that biotransform phytochemicals, such as polyphenols, to facilitate their absorption [16]. Furthermore, commensal gut bacteria have been shown to protect against colonization of pathogenic bacteria by modulating epithelial barrier function, and through regulating the innate and adaptive immunity of the host [17-20]. Disruption or alteration of this microbial community, known as dysbiosis, has been widely associated with the development of physiological disorders such as metabolic and immune disorders [13, 21], and, in more recent research, development of mental health disorders [22-24]. Thus, numerous studies over the past couple of decades have focused on understanding the gut microbiota and its functionality, and how we can selectively modulate the composition and function of the microbiota to achieve beneficial health outcomes during GI disorders [25, 26]. Such studies often evaluate the gut microbiota and its related functionality

through *in vivo* animal studies or human clinical trials [27-29]. However, in doing so, several challenges arise in terms of limited accessibility, ethical complications, poor correlation to the human *in vivo* conditions and the inability to monitor the dynamic changes of the GI microbiota to certain stimuli. In this regard, i*n vitro* models that simulate GI gastrointestinal tract conditions are seen to be favourable as they have been demonstrated to simulate human GI conditions in the laboratory to a fairly good extent [30]. Various types of *in vitro* models have been developed by labs across the globe ranging from static-single reactor batch fermenter systems, to more complex dynamically controlled multicompartment systems such as the TNO *In Vitro* Model of the Colon (TIM-2), Computer-Controlled Multicompartmental Dynamic Model of the Gastrointestinal System (SIMGI), and the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) [30]. The SHIME® model first described by Molly *et al.* [36] is one of the most frequently used models due to its dynamic control of the microbiota and ability to customize the design based on potential applications. Moreover, the SHIME® model has been previously validated to simulate microbiota profiles in the colonic compartments and its associated metabolic functions, such as the production of ammonium (NH_4) and SCFAs [36, 37].

Although the ability of SHIME models to simulate gut microbiota has been previously demonstrated, there is limited knowledge on the growth dynamics of the microbiota and its metabolic functions during the widely accepted two-week period of fecal inoculum stabilization to colonic microbiota. In the work by Possemiers *et al.* 2004, bacterial and functional dynamics were monitored over a 3-week period using correlation analyses to indicate stability. The findings from the study showed that stability in the microbial composition took two weeks to achieve whereas metabolic function stability took nearly three weeks [38]. However, as noted in the study, these results may not necessarily be applicable to other SHIME-based model systems, as each model is usually adapted based on the goals and purposes of study objective. Moreover, the use of different fecal inoculates may factor in the stability periods of the community and metabolic output [38-40]. In our proposed study, the aim was to test a recently developed benchtop-scale GI model based on SHIME® to characterize the process of stabilization of the

colonic microbiota and its microbial function [41]. Metabolic functionality was assessed by quantification of microbial metabolic products such as SCFAs, hydrogen sulfide (H_2S) and ammonium (NH_4) followed by 16S rRNA gene amplicon sequencing to assess for microbial composition over a two-week period.

3.3 Materials and Methods

3.3.1 Design of Gastrointestinal Model

The GI model was designed based on the SHIME® model developed by Molly *et al.* [36]. Briefly, a series of five sequentially connected 250 mL autoclavable fermentation vessels housed in a bench-top Teflon and plexiglass structure. The caps for the fermentation vessels were custom designed to have two input ports and two output ports using barbed fitting units. The input ports were used for feed and acid/base input, and, for purging with oxygen-free nitrogen gas to maintain anerobic conditions in the vessel. The output ports were utilized for transfer of vessel contents with an in-line sampling port, and for gas output for pressure release. Each of the gas output ports were sequentially connected to the following vessel and finally into an exhaust pipe. Each of the vessel contents were continuously stirred at the lowest setting using magnetic stirrer plates and the inner temperature was maintained at 37°C using heated double-jacketed beakers (CG-1103-04, Chemglass, NJ, USA). All vessels were pH regulated through changes detected by a pH probe connected to an EZO™ pH circuit (Atlas Scientific, NY, USA) embedded in a Raspberry Pi microprocessor (ver. 1B). The pH changes outside ± 0.2 set pH level were buffered by addition of 0.5 M NaOH or 0.5 M HCl through micropumps (Kamoer, Shanghai, CN) that activate through relay circuits embedded in the Raspberry Pi. The movement of feed between two vessels was conducted using programmable peristaltic pumps (NE-9000B, New Era Pump Systems, NY, USA).

3.3.2 Simulations of Gastrointestinal Conditions

To simulate the human digestive tract, five vessels were connected sequentially to represent different parts of the GI tract, i.e., the stomach, the small intestine, ascending colon (AC), transverse colon (TC), and the descending colon (DC). The last colonic vessel was connected to the biohazard waste container as outlined in **Figure 3.1**.

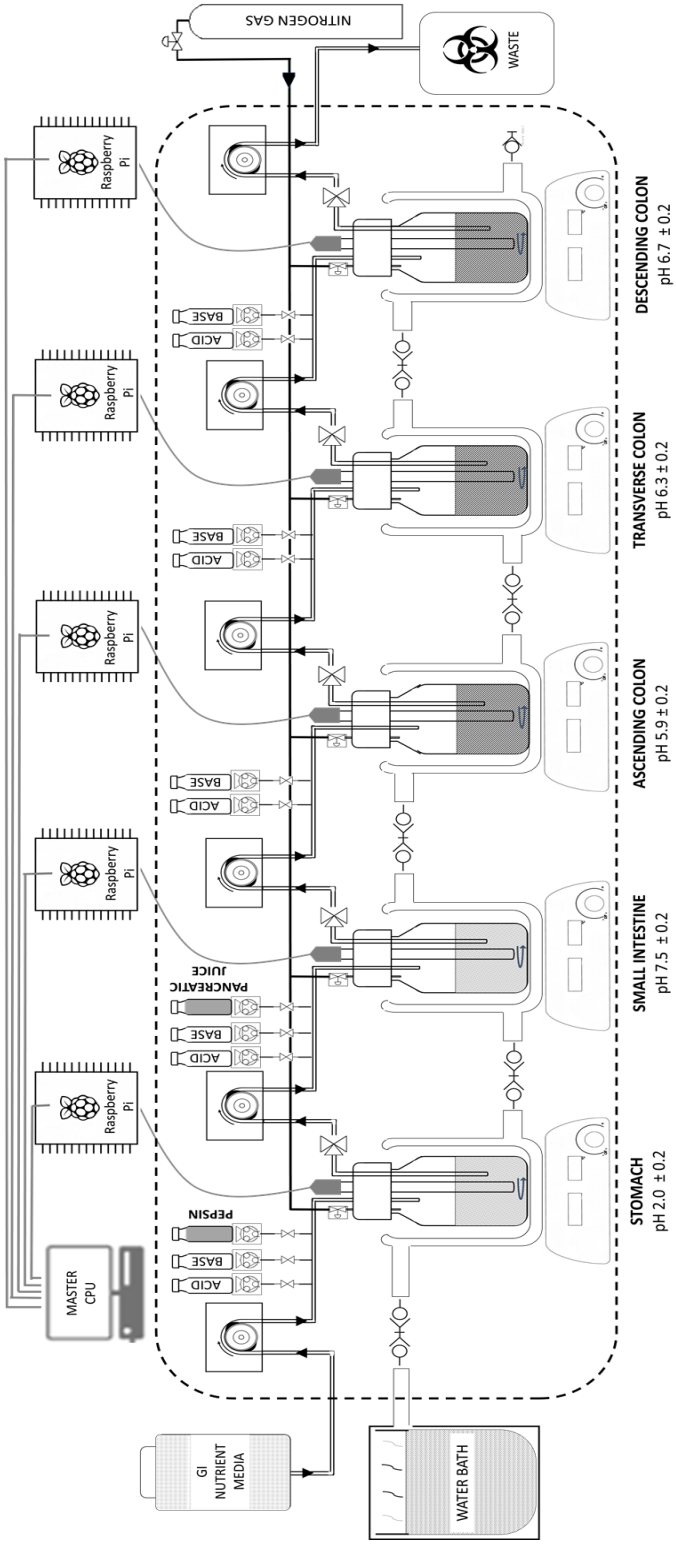

Figure 3.1. Schematic of the GI model

Table 3.1. GI model vessel parameters

Vessel	Volume	Transit Time	pH
Stomach	100 mL	2 h	2.0 ± 0.2
Small Intestine	150 mL	1.5 h	7.5 ± 0.2
Ascending Colon [AC]	175 mL	1.5 h	5.8 ± 0.2
Transverse Colon [TS]	175 mL	1.5 h	6.3 ± 0.2
Descending Colon [DC]	175 mL	1.5 h	6.7 ± 0.2

The parameters of each vessel are outlined in **Table 3.1**. Briefly, the stomach bioreactor was maintained at 2 ± 0.2 pH, the small intestine at 7.5 ± 0.2 pH, AC at 5.9 ± 0.2 pH, TC at 6.3 ± 0.2 pH, and, DC at 6.7 ± 0.2 pH level. The addition of feed into the stomach vessel and subsequent movement of digest from one vessel to the next was fully computer controlled and programmed into continuous 8 h cycles with specific transit times in each vessel as noted in **Table 3.1.** Addition of pepsin (1.5 g of pepsin in 1.5 mL of 1 M HCl; P7125, Sigma-Aldrich, Oakville, ON, Canada) into the stomach and pancreatic juice into the small intestine (20 mL of 12 g/L $NaHCO_3$, 6 g/L bile extract and 0.9 g/L pancreatin; P1750 Sigma-Aldrich, Oakville, ON, Canada) was done manually.

Fecal slurry for inoculation in the colonic vessels was prepared from fecal sample collected from a single healthy individual (male, aged 32 y and BMI of 23.4 kg/m^2) with no history of GI disorders or antibiotic use for a period of at least 6 mo prior. The two-week experimental run was performed two more times with the same fecal donor 6 mo apart to attain an experimental triplicate (n = 3).

Briefly, within 3 h of fecal sample collection, the sample was diluted at a ratio of 1:10 w/v in 0.9% saline and filtered to remove particulates (Whirl-Pak™ Sterile Filter Bags, Fisher Scientific, Ottawa, ON, Canada) prior to inoculation. Stabilization of the microbial populations in the fecal

inoculation was done as outlined in [42] and previously adapted by our group [43]. The model was run for a period of two weeks by supplementing the model with 50mL of filter-sterilized GI food, previously optimized by Molly *et al.*, [42], every 8 h cycle (composed of 1 g/L of arabinogalactan, 2 g/L of pectin, 1 g/L of xylan, 3 g/L of starch, 0.4 g/L of glucose, 3 g/L of yeast extract, 1 g/L of peptone, 4 g/L of mucin, 0.5 g/L of cysteine and 40 µL/L of minimum essential vitamin solution, was added to each vessel; Sigma-Aldrich, Oakville, ON, Canada). Samples were taken at 24 h intervals and were centrifuged at 2000 g for 10 min prior to storage. The supernatant (hereinafter referred to as fecal water, FW) was filtered using sterile 0.45 µm syringe filters and stored at -20°C, and, the fecal pellet was stored at -80°C. All vessels were purged with oxygen-free nitrogen gas for 15 min after taking samples to maintain anerobic conditions.

3.3.3 Assessment of Metabolic Activity

3.3.3.1 Short-Chain Fatty Acids (SCFA) Analysis

SCFA analysis was conducted by a gas chromatograph system equipped with a flame ionization detector (GC-FID) (6890A series, Agilent Technologies, Santa Clara, CA, USA) using the method outlined in [44]. The GC-FID equipped with a split/split-less inlet port and a fused capillary column (30m L x 250 µm ID x 0.25 µm film thickness; HP-INNOWAS, Agilent Technologies, Santa Clara, CA, USA) was set at 220°C at the inlet and 230°C at the detector. The separation method followed injection of 1 µL of pre-filtered (0.2 µm) fecal water samples into the GC-FID with the oven set at 100°C, held for 2 min, followed by an increase of 10°C/min until temperature reached 220°C where it was held for 1 min more. Helium was used as the carrier gas (1 mL/min). Characterization and quantification of SCFAs in samples was done by running a free-volatile fatty acid standard (46975-U, Sigma Aldrich, St. Louis, MO, USA). Individual SCFAs measured were acetate, propionate, butyrate, iso-butyrate, valeric acid, iso-valeric acid, caproic acid, iso-caproic acid, and, heptanoic acid. Samples were analyzed in duplicate from each fermentation experiment and values were reported as mM of SCFA.

3.3.3.2 Ammonium (NH_4) Determination Assay

Colorimetric determination of NH_4 present in the fecal water samples was done using an adapted method from [45] as outlined previously in [44]. Briefly, in a standard 96-well microplate 50 µL of sample is added to a solution of 25 µL of citrate reagent (0.2 M trisodium citrate in 0.5 M NaOH; 1110371000, Sigma Aldrich, St. Louis, MO, USA), 30 µL of salicylate-nitroprusside reagent (0.05 M sodium salicylate in 0.05 mM sodium nitroprusside; S3007 & 1614501, Sigma Aldrich, St. Louis, MO, USA), 25 µL of hypochlorite reagent (10:2:1 v/v/w of household bleach, NaOH and trisodium phosphate at pH 13.0), and, 145 µL of distilled water. The reaction was allowed to occur for 30 mins on a plate shaker and the absorbance was read at λ = 650 nm using a uQuant microplate reader (BioTek Instruments, Winooski, VT, USA). Oven-dried ammonium sulphate (A4418, Sigma Aldrich, St. Louis, MO, USA) was used as the analytical standard for quantification. All samples were analyzed in triplicate from each stabilization run.

3.3.3.3 Hydrogen Sulfide (H_2S) Determination Assay

Determination of H_2S in fecal samples was conducted by the zinc acetate precipitation method proposed by [46] and previously outlined in [44]. Briefly 0.7ml fecal water was added to 0.5 mL of alkaline zinc acetate. The mixture was centrifuged at 3000 g for 10 min to allow followed by a two-step washing of the pellet with 1.5 M sodium chloride (pH 8.0) and distilled water (pH 8.0). The pellet was then resuspended in 0.7 mL distilled water followed by the addition of 0.25 mL of acidified DPD reagent (0.1% w/v *N, N*-dimethyl-*p*-phenylenediamine monohydrochloride), and, 0.1 mL of 11.5 mM acidified ferric chloride. The reaction mixture was incubated at room temperature for 30 min for color development to occur. Absorbance was read at λ = 670 nm using a uQuant microplate reader (BioTek Instruments, Winooski, VT, USA). Sodium sulfide was used to generate the analytical standard curve and the values were reported in µM. All chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA. All samples were analyzed in triplicate from each stabilization run.

3.3.4 DNA Extraction and Analysis

3.3.4.1 DNA Extraction

Extraction of DNA from the collected fecal samples was done using the QIAamp® Fast DNA Stool Mini Kit (51604, Qiagen, Hilden, Germany) as per manufacturer's instructions with some modifications. Briefly, 300-500mg of pellet was pre-washed with 0.05 M phosphate buffer followed by addition of 1 mL InhibitEX, 300 mg of 0.1 mm zirconia beads (360991112, Thermo Fisher Scientific, USA) and agitation using a bead-beater (3 cycles of 4 m/s for 1 min; MP FastPrep®-24, MP Biomedicals, CA, USA). After centrifugation, DNA extraction of the samples followed the manufacturer's protocol. Concentration of extracted DNA was done by NanoDrop™ One (Thermo Fisher Scientific, USA) and DNA purity was assessed by 260/280 ratios. All DNA samples had ratios between 1.8–2.0, which were further processed for 16S rRNA gene amplicon sequencing.

3.3.4.2 16S rRNA Gene Amplicon Sequencing and Bioinformatics

16S rRNA gene amplicon sequencing was done on the extracted fecal DNA as previously described [46]. Briefly, a sequencing library (Illumina's "16 S Metagenomic Sequencing Library Preparation"; Part # 15044223 Rev. B) was constructed using extracted DNA with the following exceptions: Qiagen HotStar MasterMix for the amplicon PCR, and, half reagent volumes for the index PCR. The template specific primers (forward; 5'-CCTACGGGNGGCWGCAG-3' and reverse; 5'- GACTACHVGGGTATCTAATCC-3') were specific to bacterial organisms and targeted the V3-V4 hypervariable region [9]. Amplicon PCR was carried out for 25 cycles with annealing temperatures of 55 °C followed by a 500-cycle (paired-end sequencing configuration of 2 x 250 bp) on an Illumina MiSeq.

Sequencing data was analyzed using AmpliconTagger [48]. Briefly, raw reads were scanned for sequencing adapters and PhiX spike-in sequences and remaining reads were merged using their

common overlapping part with FLASH [49]. Primer sequences were removed from merged sequences and remaining sequences were filtered based on quality (Phred) score. Remaining sequences were clustered at 100% identity and then clustered/denoised at 99% identity (Vsearch v2.7.1, [50]). Clusters having abundances lower than 3 were discarded. Remaining clusters were scanned for chimeras with VSEARCH's version of UCHIME denovo and UCHIME reference [50,51] and clustered at 97% (VSEARCH) to form the final clusters/OTUs. A global read count summary is provided in Supplementary Table S1. OTUs were then assigned a taxonomic lineage with the RDP classifier [52] using an in-house training set containing the complete Silva release 128 database [53] supplemented with eukaryotic sequences from the Silva databases and a customized set of mitochondria, plastid and bacterial 16S sequences. The RDP classifier gave a score (0 to 1) to each taxonomic depth of each OTU. Each taxonomic depth having a score > 0.5 was kept to reconstruct the final lineage. Taxonomic lineages were combined with the cluster abundance matrix obtained above to generate a raw OTU table, from which bacterial organisms OTU table is generated. Five hundred 1,000 reads rarefactions were then performed on this latter OTU table and the average number of reads of each OTU of each sample is then computed to obtain a consensus rarefied OTU table (available in **Supplementary Table S3.2**). A multiple sequence alignment is then obtained by aligning OTU sequences on a Greengenes core reference alignment [54] using the PyNAST v1.2.2 aligner [54]. Alignments were filtered to keep only the hypervariable region of the alignment. A phylogenetic tree was then built from that alignment with FastTree v2.1.10 [56]. Alpha (observed species) and beta (weighted, unweighted UniFrac and Bray Curtis distances) diversity metrics and taxonomic summaries were then computed using the QIIME v1.9.1 software suite [55,57] using the consensus rarefied OTU table and phylogenetic tree (i.e. for UniFrac distance matrix generation).

3.3.4.3 Availability of Raw Data

Raw sequence reads of the 16S rRNA gene amplicon data were submitted to the NCBI sequence read archive under Bio Project PRJNA658988.

3.3.5 Statistical analysis

All data are reported as means ± standard error of mean (SEM). Data for SCFA, H_2S and NH_4 were analyzed using two-way ANOVA using Vessel (3 levels) and Time (15 levels) as factors. For multiple comparisons, Tukey's HSD post analysis was carried out to compare differences between vessels and time points. The means of all time points were jointly considered when no significant interactions in the two-way ANOVA were observed to compare significant differences between vessels. When significant interactions between time and vessel were observed, the means of each time point within a vessel were individually compared for significant differences within the vessel. Statistical significance was set at $p < 0.05$. All two-way ANOVA and post-hoc statistical analyses, and, visualisations for metabolite data were performed using JMP v14.4 (SAS Institute, Cary, NC, USA). Statistical tests for the sequencing data such as the two-way ANOVA analysis followed by matched pairs Student's t-test for Shannon index, and, PERMANOVA analyses for weighted UniFrac distance were done with R (v3.6.0) using the adonis2 function of the Vegan (v2.5-4) package. Taxonomic profiles, alpha- and beta-diversity plots were generated with R (ggplot2 v3.1.1).

3.4 RESULTS

In this study, metabolic functionality of the microbiota was assessed through changes in SCFA production and determination of H_2S and NH_4, each of which act as a relative measure of the metabolic health of the microbiota [58]. Furthermore, changes in antioxidant capacity was measured to indicate intestinal redox balance [59]. Changes in the microbial community was assessed by 16S rRNA gene amplicon sequencing of the bacterial genome in the fecal pellets obtained from the different colonic vessels at 24 h intervals.

3.4.1 SCFA Production Stabilised Within Five Days of Fecal Inoculation

The results of total SCFA determination in the colonic vessels using two-way ANOVA followed by Tukey's post-hoc analysis showed only a significant [$p < 0.05$] effect of time. No significant differences were found between the colonic vessels over the 14-day period. Total SCFA production levels appeared to stabilize following day 6 in the ascending colon, after day 3 in the transverse colon and after day 6 in the descending colonic vessel (**Figure 3.2**).

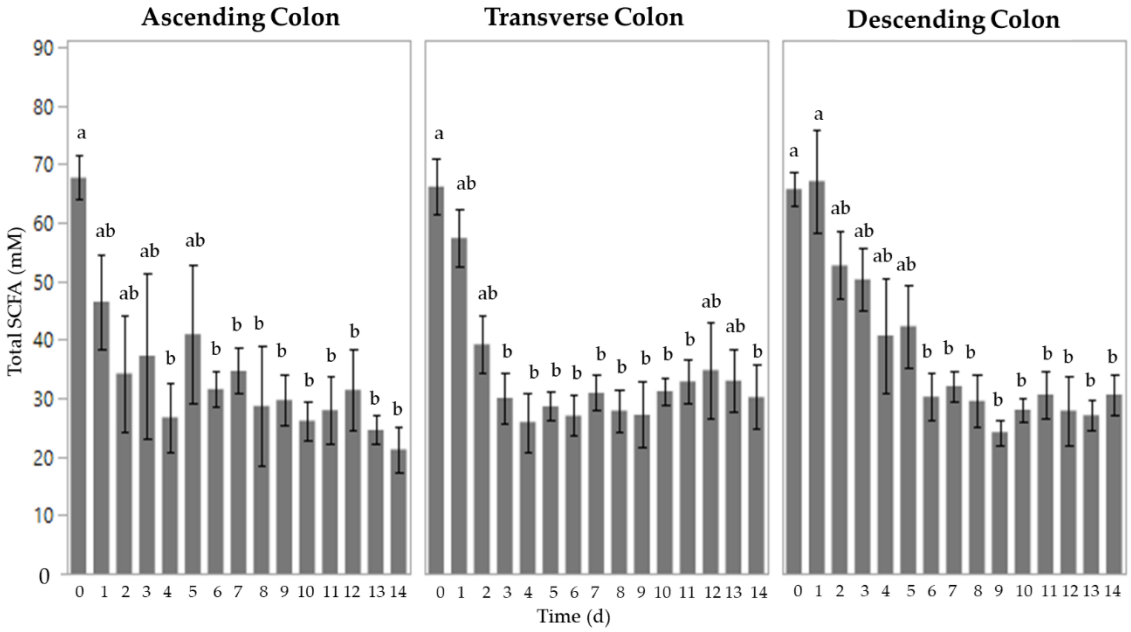

Figure 3.2. Total short-chain fatty acid (SCFA) production in the three different colonic vessels. Values are shown as mean ± SEM. Means at time points within colonic vessels without a common letter are significantly different ($p < 0.05$), n = 3.

Individual SCFAs such as acetate and propionate appeared to follow similar trends of the total SCFA production. Two-way ANOVA followed by Tukey's post-hoc analysis showed a significant ($p < 0.05$) main effect of time for acetate, propionate and butyrate. Acetate levels showed significant ($p < 0.05$) differences within vessels and appeared to stabilize day 3 onward in the AC

and TS vessels, and day 6 onward in the DC vessel. No such effect was observed with propionate and butyrate production, whereby levels did not statistically change across time within each vessel (**Figure 3.3**).

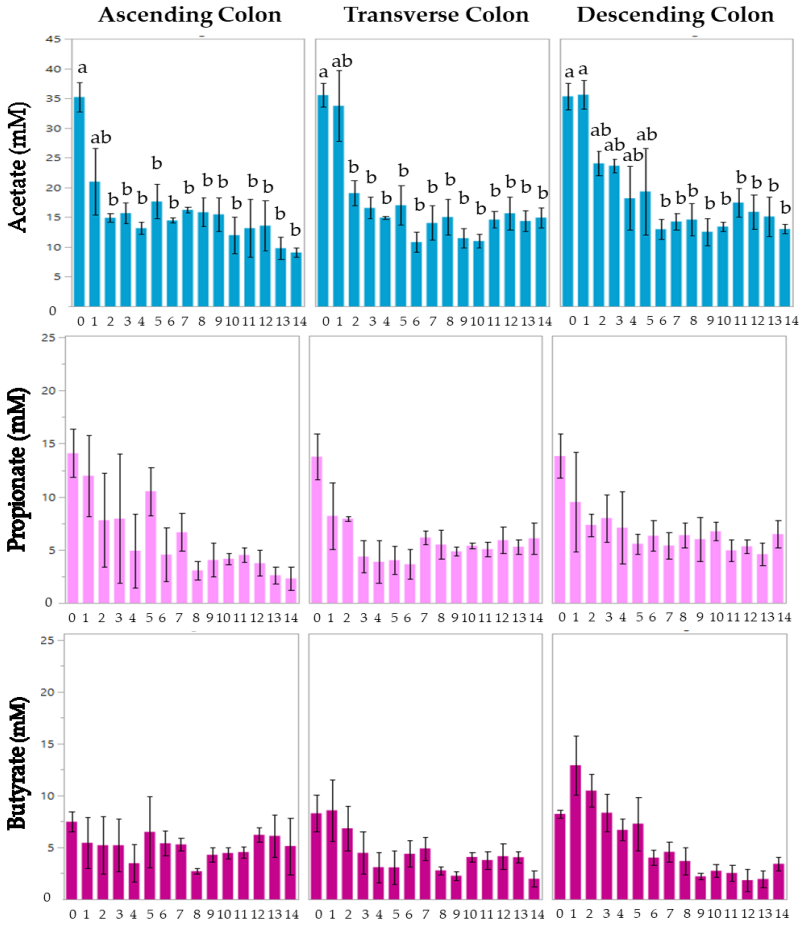

Figure 3.3. Acetate, propionate and butyrate production in the three different colonic vessels. Values are shown as mean ± SEM. Means at time points within colonic vessels without a common letter are significantly different ($p < 0.05$), n = 3.

3.4.2 NH_4 and H_2S Levels Remained Stable for the Two-Week Period

Two-way ANOVA results for NH_4 determination in FW showed no significant effects of time or colonic vessel. Ammonium levels appeared to stabilize in the DC vessel, although this did not reach statistical significance (**Figure 3.4**).

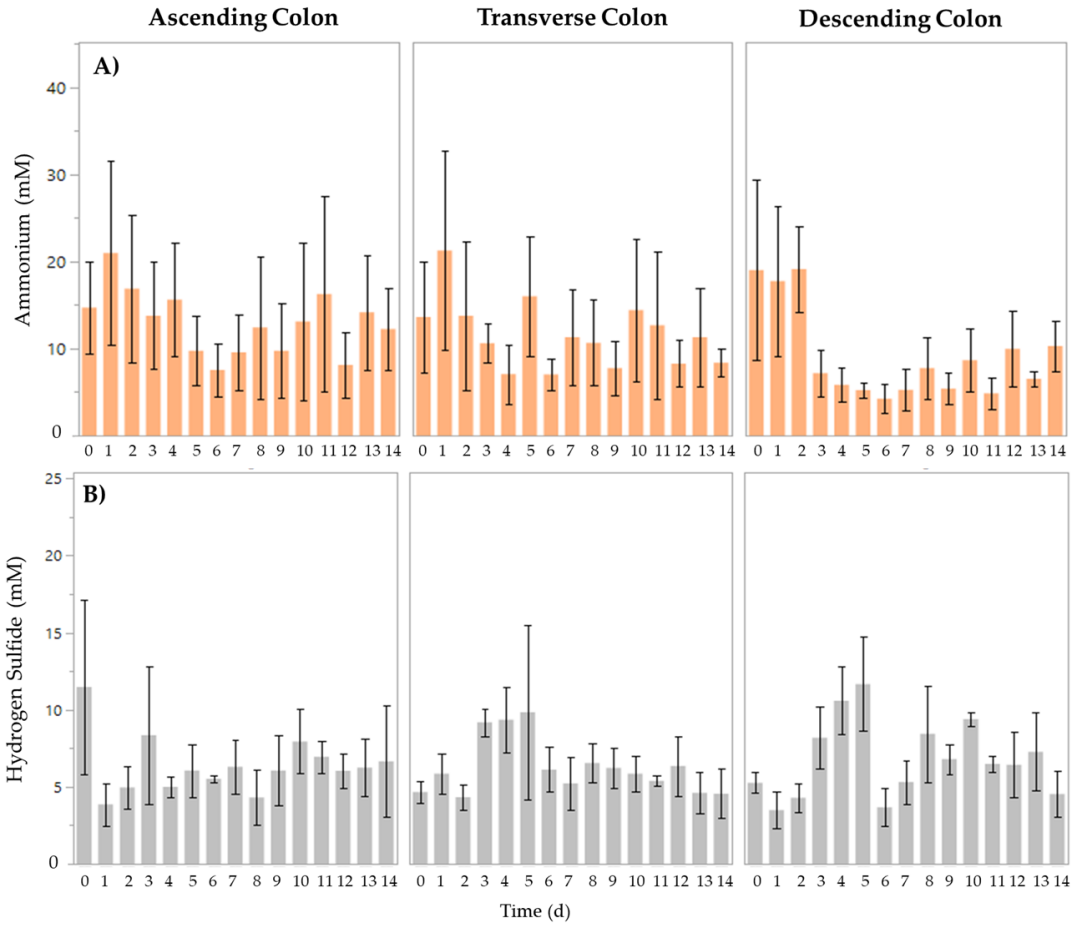

Figure 3.4. A) Ammonium production, and B) Hydrogen sulfide production across the three different colonic vessels. Values are shown as mean ± SEM, n = 3.

Similarly, two-way ANOVA results for H_2S production in FW showed no significant effects of time or colonic vessel. Levels appeared to stay stable across time and vessel across the 14-day gut model run period (**Figure 3.4**).

3.4.3 Antioxidant Capacity of Fecal Water Stabilized Within Three Days of Fecal Inoculation

The results of FRAP antioxidant capacity showed a significant effect of time using two-way ANOVA followed by Tukey's post-hoc analysis. There was no significant effect of colonic vessel or any interaction effects. The antioxidant capacity of FW showed stabilization following day 1 in the ascending colonic vessel, after day 3 in the transverse colonic vessel and day 4 in the descending colonic vessel (**Figure 3.5**).

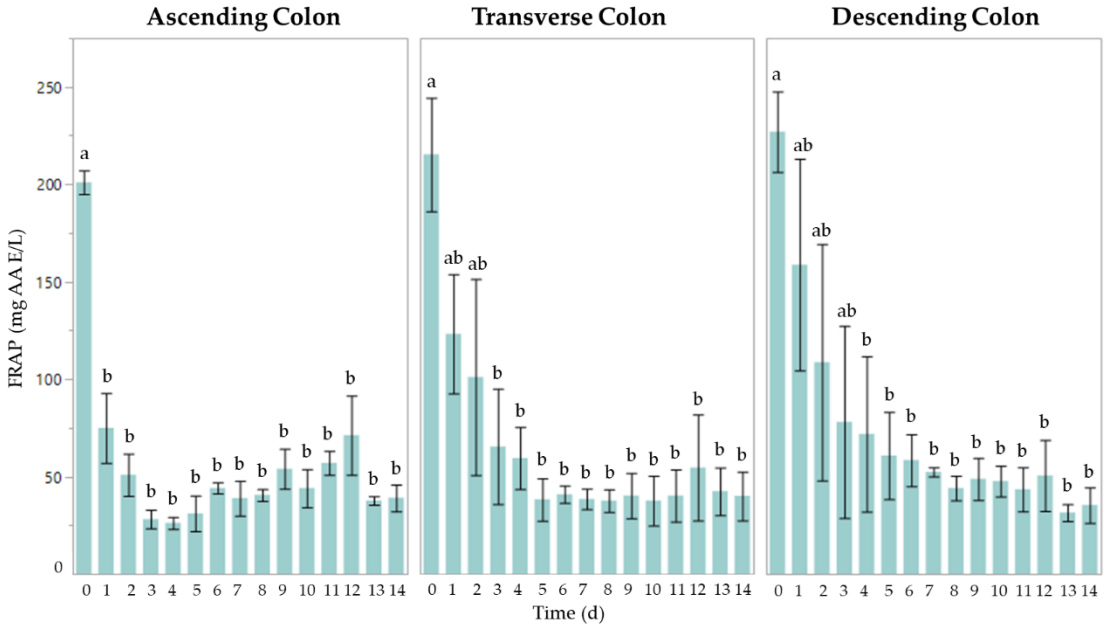

Figure 3.5. Antioxidant capacity as measured by ferric-reducing antioxidant power (FRAP) in the different colonic vessels. Values are shown as mean ± SEM. Means at time points within colonic vessels without a common letter are significantly different ($p < 0.05$), n = 3.

3.4.4 Microbial Community Profiling Exhibits A Shift in Community Dynamics Across Time

The 16S rRNA gene amplicon sequencing of the fecal samples was used to profile the microbiota community structure and composition (due to certain experimental limitations, only two repeats of the two-week study were analysed for their microbial composition by 16s sequencing). Metrics such as alpha diversity (Shannon index), beta diversity (Weighted UniFrac) and taxonomic relative abundance profiles of operational taxonomic units (OTUs) were used to assess changes to the fecal microbiota in the model until day 14 after inoculation.

To assess for changes in species richness, alpha diversity measured by Shannon index was analysed. Two-way ANOVA analysis followed by matched pairs Student's t-test on the Shannon index values showed that there was a significant decrease in microbial species diversity at day 7 and day 9 for the ascending colonic vessel when compared to day of inoculation, whereas, no significant changes were observed in the transverse and descending colonic vessels (**Figure 3.6**). Moreover, both the transverse and descending colonic microbiota appeared to have a higher microbial diversity when compared to the ascending colon vessel after day 9, although this did not reach statistical significance.

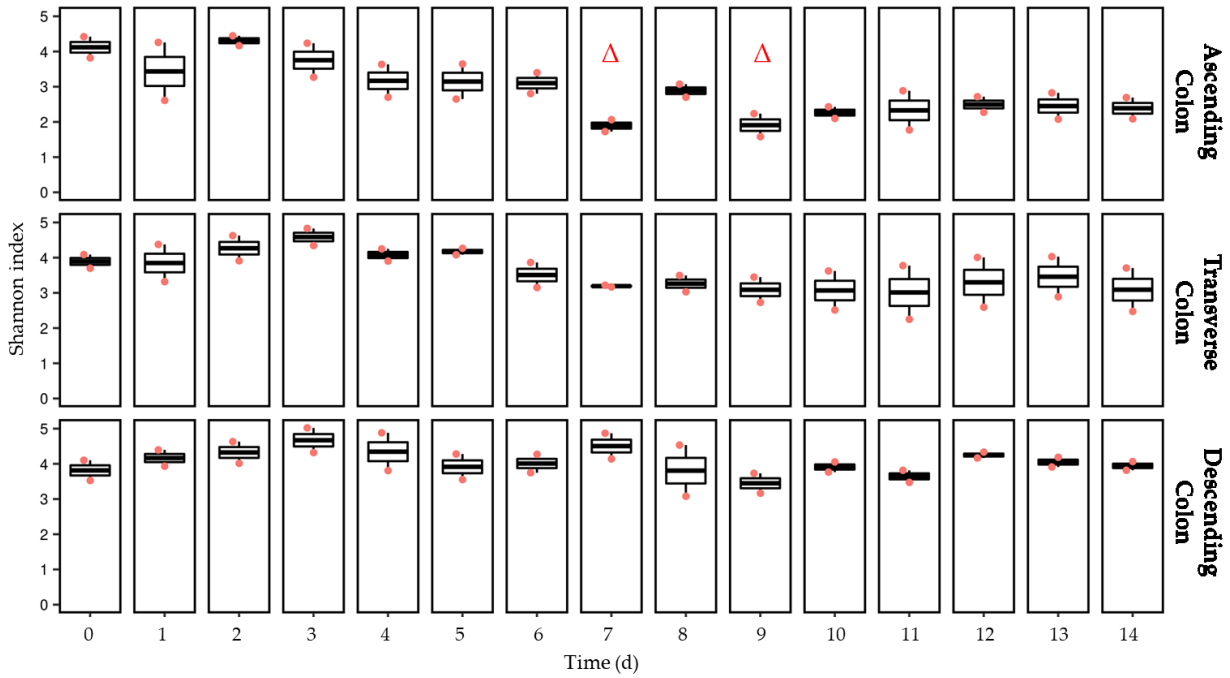

Figure 3.6. Microbial alpha diversity of fecal samples from the different colonic vessels over a period of 14 d as assessed by the Shannon index. The symbol Δ represents significant differences ($p < 0.05$) in alpha diversity between that day and day of inoculation (0 d) for that vessel.

PERMANOVA analysis of the beta diversity results on OTU abundance showed that there were significant differences ($p < 0.05$) within the microbial communities across time and colonic vessel. Fecal samples from the colonic vessels showed the formation of distinct microbial communities starting from the day of inoculation until day 9 after which, the microbial communities appear to show an increase in community similarity until day 14 (**Figure 3.7**). This observation is further confirmed by the beta diversity bi-plots that showed that the fecal samples clustered principally by time and colonic vessel indicating that these two variables are the main drivers in the formation of distinct communities (**Figure 3.7**). Similar clustering patterns were observed in OTU heatmaps, where clustering was based on vessel type and time (**Supplementary Figure S3.2**).

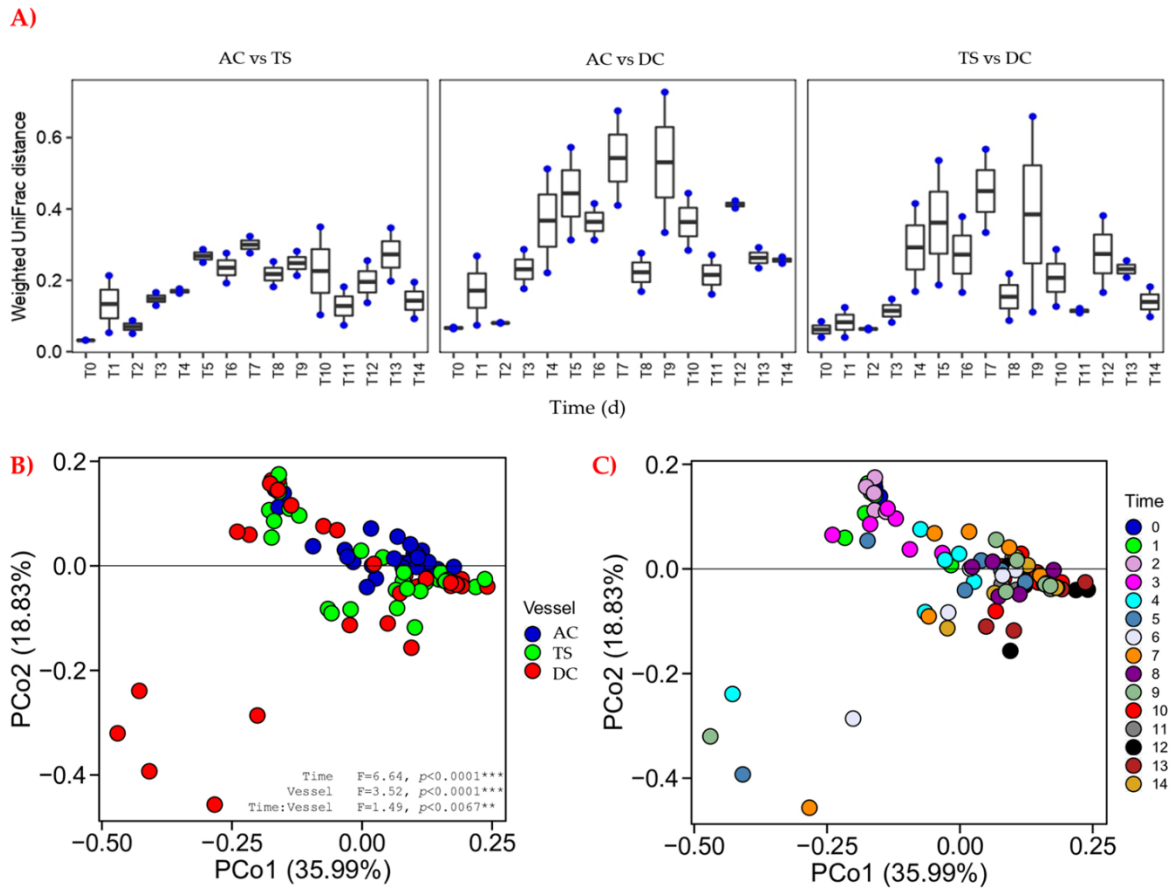

Figure 3.7. Weighted UniFrac beta diversity plots of fecal samples; A) distances between the reactor's compartments during time course, B) Principle coordinates analysis (PCoA) showing clustering by vessel, and, C) PCoA bi-plot showing clustering by time (d). AC = Ascending colon vessel; TS = Transverse colon vessel; DC = Descending colon vessel.

Analysis of the taxonomic abundance profiles was conducted to observe changes in the microbial communities and to assess for differences between the colonic vessels. **Table 3.2** shows the relative abundance of the taxonomic groups at the phylum and class level. The results show that three phyla *Actinobacteria*, *Bacteroidetes* and *Firmicutes* dominated the microbial communities at the time of inoculation with *Firmicutes* being much more abundant than *Bacteroidetes* and *Proteobacteria*. There were trends of higher proportions of *Actinobacteria* and *Bacteroidetes* in both the transverse and descending colon along with decreased proportions for *Actinobacteria*

in the ascending colon. The proportions of *Proteobacteria* increased in each of the vessels by day 8 and day 14. Moreover, the proportions of the phyla *Firmicutes* changed across time and vessel conditions.

Table 3.2. Relative abundance of higher taxonomic groups

Taxonomic group	Fecal inoculate [day 0]	% relative abundance					
		day 8			day 14		
		AC	TC	DC	AC	TC	DC
Actinobacteria	19.77	2.46	24.78	12.64	3.58	12.11	15.13
Bacteroidetes	0.43	1.51	13.23	11.03	3.65	14.62	15.65
Firmicutes, Bacilli	8.42	13.03	6.14	3.62	0.65	0.41	0.67
Firmicutes, Clostridia	35.11	0.26	2.27	20.80	0.12	0.29	7.04
Firmicutes, Others	35.01	48.61	30.86	18.49	67.52	50.07	33.73
Fusobacteria	< 0.01	< 0.01	7.29	< 0.01	< 0.01	0.45	3.91
Proteobacteria	0.35	32.76	11.12	9.975	23.55	17.63	17.41
Verrucomicrobia	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

Further analysis of the relative abundance of the lower phylogenetic groups revealed the differences in the microbial communities of the colonic vessels. The results show that the most prevalent taxa at the family level in the inoculum were *Erysipelotrichaceae*, *Lachnospiraceae* and *Bifidobacteriaceae*, followed by *Ruminococcaceae*, *Lactobacillaceae* and *Peptostreptococcaceae* (**Figure 3.8**). Bacteria that were characteristic for the ascending colon included *Coriobacteriaceae* and *Veillonellaceae*, followed by a rapid growth of *Veillonellaceae* and *Enterobacteriaceae*

towards the later half of the experiment. In the transverse colonic vessel, *Coriobacteriaceae*, *Veillonellaceae*, and *Bacteroidaceae* showed an increased presence after a few days of inoculation, after which there was also an increased presence of *Fusobacteria*, *Enterobacteriaceae* followed by other *Firmicutes*. The taxa *Veillonellaceae* also showed an increased rate of growth in the transverse colon during the latter stages, although this was less pronounced than in the ascending colonic vessel. Microbial composition in the descending colonic vessel continued to show the presence of the predominant families from the fecal inoculate until day 9, after which there was an increased presence of *Bacteroidaceae*, *Fusobacteria*, *Veillonellaceae*, *Enterobacteriaceae* and other species from the phyla *Firmicutes* (**Figure 3.8**).

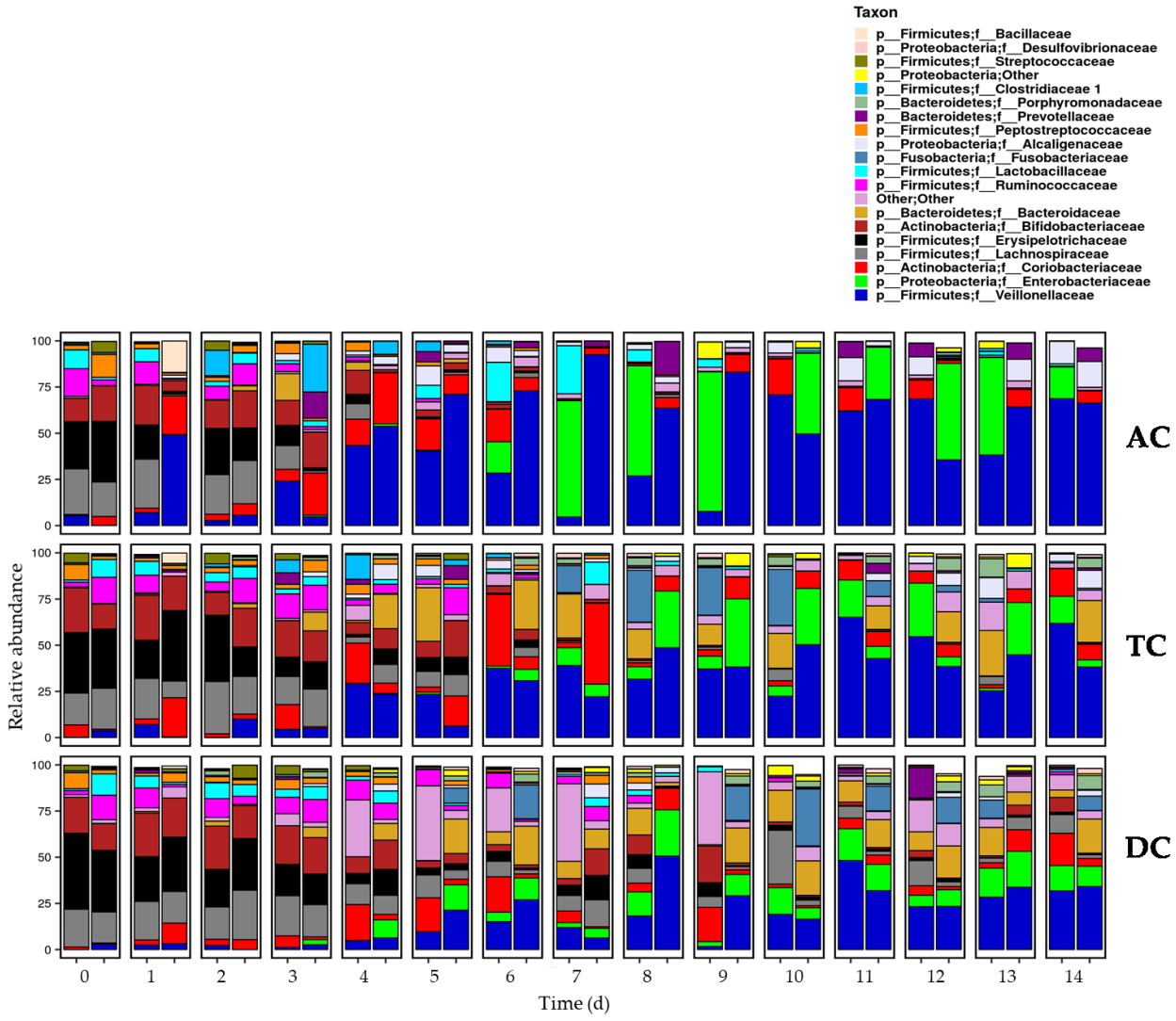

Figure 3.8. Microbial diversity (family level) of fecal samples from the different colonic vessels showing relative bacterial abundance over time. AC = Ascending colon vessel; TC = Transverse colon vessel; DC = Descending colon vessel.

3.5 DISCUSSION

The findings from the present work demonstrate stabilization of the metabolic capacity in the colonic reactors within a 5-day period following fecal sample inoculation. No significant differences were observed between the colonic reactors for any of the functional analytes assessed in this study. SCFA production showed an initial period of change due to the transit from *in vivo* fecal inoculum to *in vitro* conditions after which an apparent functional steady state was reached in each of the colonic vessels within 5 days. Although this latter phenomenon has not been observed in previous SHIME® studies, similarities with regard to total SCFA levels and its proportions of primary SCFA does exist [36,38,40]. In the study by Molly *et al.* 1994, total SCFA values of 77 ± 18 mg/L, 69 ± 16 mg/L, and 72 ± 18 mg/L, were obtained in the AC, TC and DC colonic reactors. In addition, molar ratios of the primary SCFAs (acetate:butyrate:propionate) of approximately 2.5:1:1 were obtained for each of the colonic reactors [36]. In comparison, the concentrations obtained in our study ranged between 20-60 mM in the two-week period with molar ratios of approx. 2:1:1 for each of the colonic vessels that remained stable across the two-week period (**Table S3.3**). These findings show that despite differences in total SCFA levels, owing to differences in reactor size, the molar ratios were comparable. Moreover, as noted in Molly *et al*., we also observed similar SCFA production across the three colonic segments [36]. In contrast, the study by Possemiers *et al.* 2004 showed very different SCFA profiles [38]. The AC reactor had over 98% acetate production, whereas in TC and DC vessels, acetate accounted for nearly 60-80% over the course of three weeks. Additionally, it was shown that SCFA levels took 3-weeks to stabilize in each of the colonic reactors [38]. Similarly, Van den Abeele *et al*. 2010 showed that the metabolic activities of the colonic reactors (NH_4, acetate, propionate, butyrate, and branched SCFAs) reached stabilisation after approximately three weeks [40]. However, SCFA molar ratios (3:1:1) were comparable to results from our study and those by Molly *et al.* [36,40]. In another SHIME® study by Sivieri *et al.* 2013, similar levels and molar ratios of SCFA were observed [39]. Comparison of molar ratios of primary SCFA has been shown to be a more relevant marker of metabolic function as it indicates the complex interactions between microbial communities

within the gut microbiota [60]. For example, the generation of butyrate and propionate by *Firmicutes* bacteria is though to occur through the utilization of acetate produced by *Bacteroidetes* and *Actinobacteria* [61].

Other functional markers, such the production of gaseous by-products NH_4 and H_2S, indicated no significant changes across the 2-week experimental run and reactor type. Additionally, antioxidant capacity of the fecal water showed a similar stabilization trend as SCFA, with antioxidant status stabilising within a few days after fecal inoculation (**Figure 3.5**). Ammonia is the only other functional marker that has been previously assessed in conjunction with SCFAs [36,38.40]. In the findings by Possemiers *et al.*, ammonia production increased over time in each of the colonic segments, with the highest production in the DC vessel. Additionally, it was noted that the levels of ammonia in the AC vessel did not reach stabilisation [38]. Results from Molly *et al.,* showed that ammonia reached 20-30 mM in each of the colonic segments with no significant differences between the vessels [36], further confirming similarities in fermentation potential between our models. To our knowledge, no other SHIME® study has assessed overall antioxidant capacity during or after stabilisation of the fecal inoculum.

In contrast to the metabolic abilities, the results from 16S rRNA sequencing of the simulated microbiota revealed shifts in microbial community dynamics for each of the colonic reactors over the two-week period after fecal inoculation. For the first 4-5 days following inoculation, the microbial community showed adaptation to the *in vitro* conditions followed by formation of distinct communities in the different colonic reactors by day 5 (**Figures 3.7 & 3.8**). This was characterised by the changes in microbial abundance of *Actinobacteria* (decreased in AC), *Bacteroidetes* (increased in TC and DC), *Clostridia* group (increased in DC) and in *Proteobacteria* (highest increase in AC, followed by TC and DC) across the vessels (**Table 3.2**). These distinct communities were further maintained for a period of 3 days in the AC reactor, and up to 6 more days in the TC and DC colonic reactors. In the latter stages of the experiment, however, growth of opportunistic *Veillonellaceae* and *Enterobacteriaceae* families were observed in the different

colonic reactors, showing a considerable presence in the AC reactor (**Figure 3.8**). Intriguingly, no significant ($p < 0.05$) differences in microbial alpha diversity (using Shannon Index) was observed in comparison to the initial fecal inoculum, except for a significant ($p < 0.05$) drop on day 7 and day 9 in the AC reactor (**Figure 3.6**).

Although similar growth patterns of opportunistic bacteria have not been reported in other large-scale GI models, it is presumed that this could be due to structural differences in the volume capacity which allows for considerably longer overall transit times of 72 h [36,38,40]. Additionally, recent evidence has suggested that the bacterial growth media (GI food) utilized lacks several key components such as mucin, SCFA, certain bile acids and inorganic salts, all of which play a key role in the growth of a diverse microbiota [62]. However, despite growth of the *Veillonellaceae* and *Enterobacteriaceae*, both alpha and beta diversity analyses show that for the most part (except for a drop in alpha diversity in the AC vessel at day 7 and 9) there were no significant perturbations in the community diversity or evenness. In this regard, microbiota evenness has been shown to be a good indicator of the resilience of the native microflora to external stressors [63]. Thus, these results suggest that the simulated microbiota in the GI model was able to maintain stability for the two-week in spite of the presence of opportunistic bacteria towards the latter part of the experiment.

In summary, the results from our study have shown that the metabolic functionality of fecal inoculate was stabilized within a 5-day period, along with distinct and stable community formation within the same time-frame, thus presenting a key advantage of such a small-scale GI model to run samples over shorter time frames as opposed to > 3 weeks in the larger SHIME® units. Moreover, the model shows consistency in microbial composition and metabolic function for the same donor over time, allowing for replicability of results rather than having to set-up multiple units of SHIME® in parallel. A potential limitation of this study is the use of a single donor fecal sample however it has been previously demonstrated that pooled samples and individual samples are comparable in terms of functionality and a majority of OTUs [64].

3.6 CONCLUSIONS

Overall, the present study demonstrates the potential of a small-scale GI model to stimulate gut lumen conditions as indicated by the various metabolic function analyses. SCFA production, and NH_4 and H_2S generation, were demonstrated to reach a steady state within 5 days of fecal inoculation. Importantly, the levels of each of these metabolic markers, including ratio amongst SCFA, were in accordance with previous literature [36, 59]. Microbial community analyses demonstrated distinct community formation in each of the colonic vessels and the maintenance of a stable and diverse microbiota. In conclusion, the results from this study demonstrate the ability of the GI model to accurately simulate human colonic functionality, and to an extent, its microbial composition, thereby showing the potential of this model to be utilized in the study of various stressors such as food components and probiotics on the gut microbiota and its metabolic activity.

3.7 ACKNOWLEDGMENTS

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3.9 SUPPLEMENTARY INFORMATION

Table S3.1. Reads count throughout key bioinformatics processing steps

http://doi.org/10.5281/zenodo.3997980

Table S3.2. Consensus rarefied OTU table

http://doi.org/10.5281/zenodo.3997980

Table S3. Proportions of the most prominent short-chain fatty acids (SCFAs) produced in the colonic vessels.

Colonic Vessel	Acetate (%)	Propionate (%)	Butyrate (%)
Ascending	48.48 ± 2.4	16.91 ± 2.4	16.03 ± 2.4
Transverse	50.09 ± 2.1	17.26 ± 2.4	12.40 ± 2.4
Descending	49.73 ± 1.7	18.42 ± 2.4	12.98 ± 2.4

*Values are shown as means ± SEM. (n = 3)

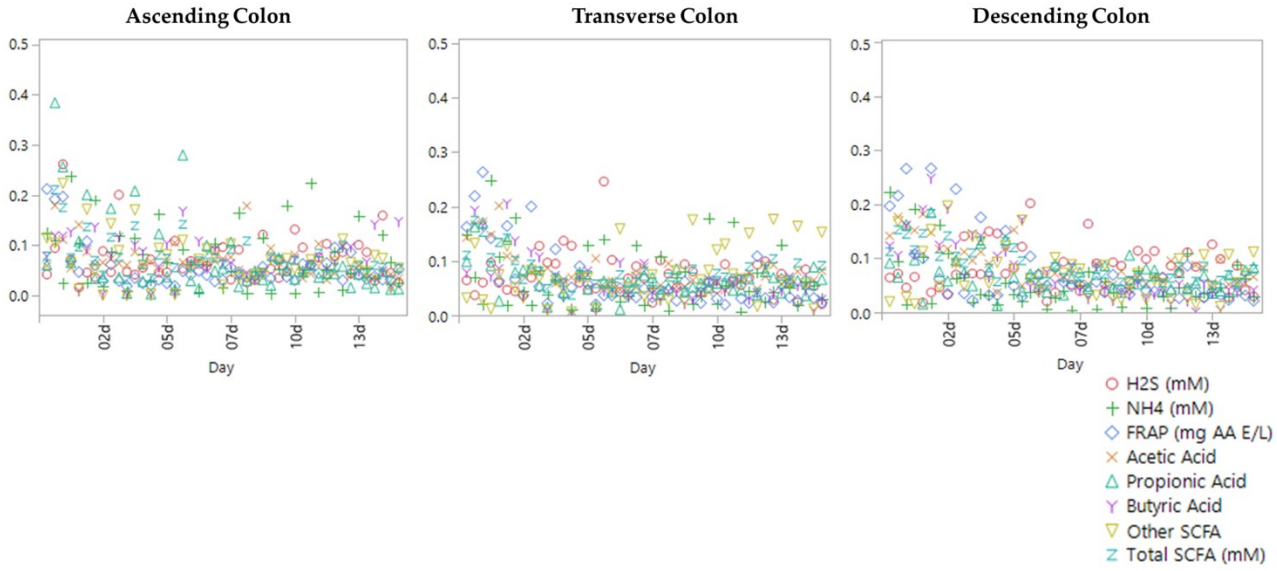

Figure S3.1. Cluster plot of normalised metabolite values over a two-week period. Metabolites indicated are; hydrogen sulfide (H_2S), ammonium (NH_4), ferric-reducing antioxidant power (FRAP), acetate, propionate, butyrate, other short-chain fatty acids (SCFAs), and, total SCFA.

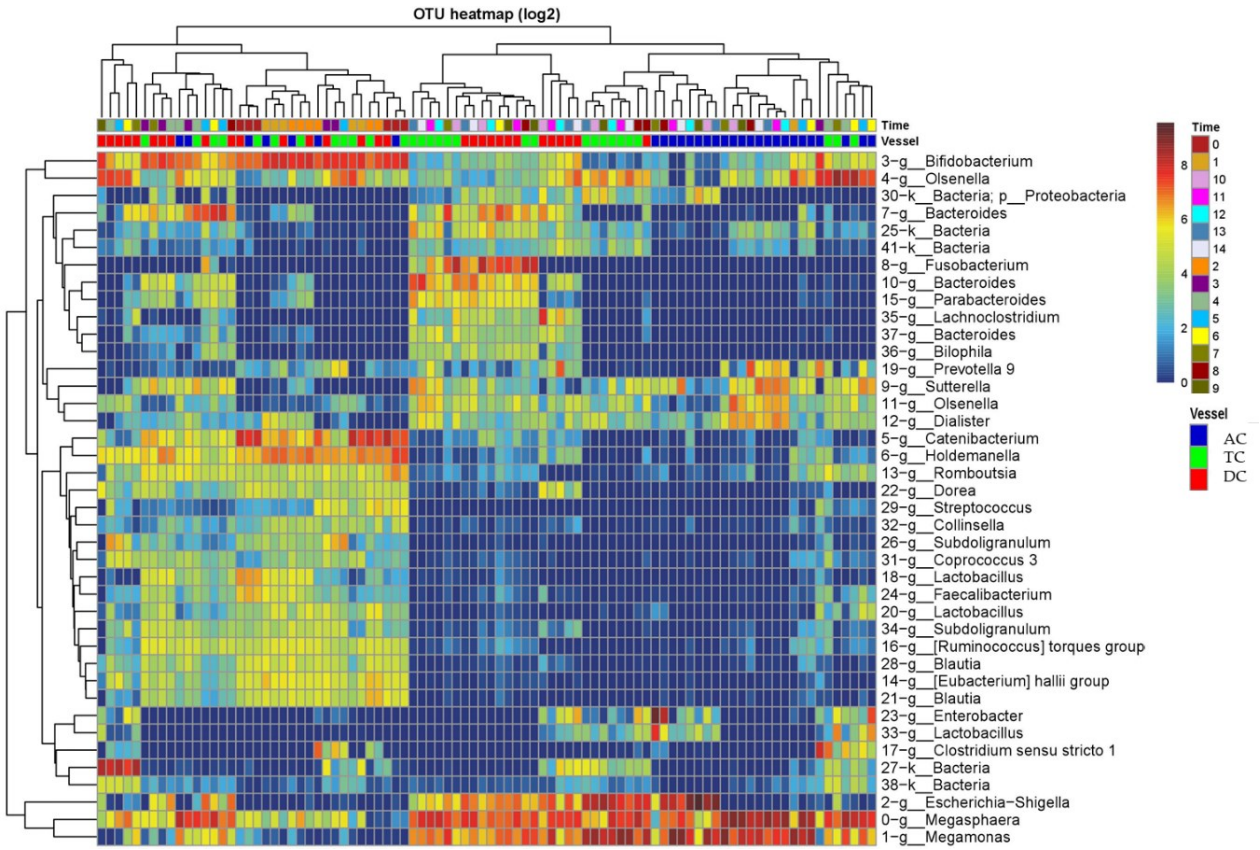

Figure S3.2. Heatmap of fecal samples over time for the different colonic vessels. Values are shown in operational taxonomic units (OTU). AC = Ascending colon vessel; TC = Transverse colon vessel; DC = Descending colon vessel.

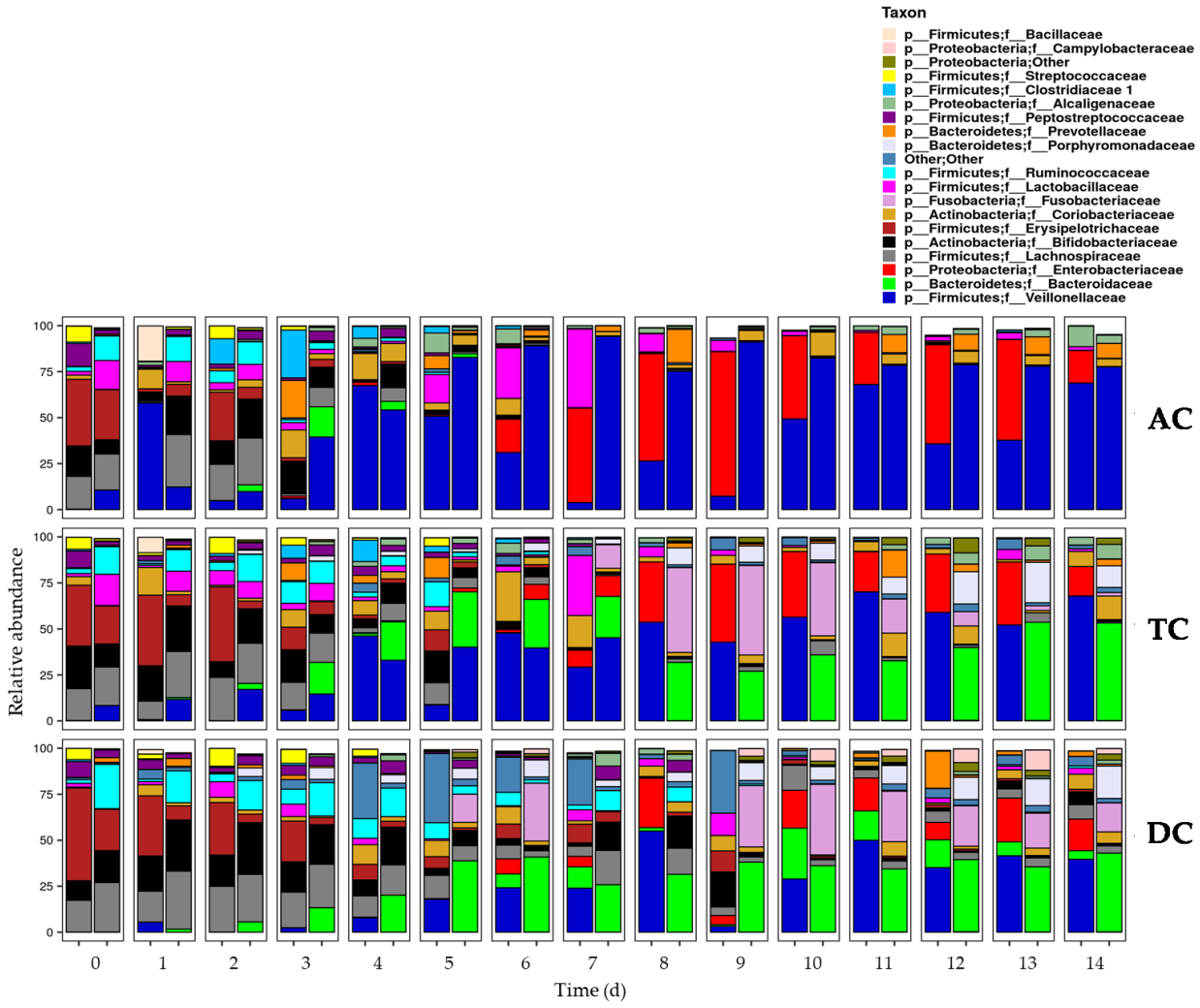

Figure S3.3. Taxonomic profiles (family level) of the colonic vessels showing relative abundance over time. Values are shown in amplicon sequence variants (ASVs). AC = Ascending colon vessel; TC = Transverse colon vessel; DC = Descending colon vessel.

CONNECTING STATEMENT II

The *in vitro* GI model developed by our lab in Chapter 3 showed promising results in simulating the colonic environment as assessed by metabolic functional makers and its ability to simulate a diverse and stable microflora. Thus, the use of this model system to study effects of different stressors on the native gut microflora and its functionality was further evaluated. In the subsequent studies, we utilized this model as a potential pre-clinical model to test and screen probiotics in *C. difficile* infection using batch culture fermentation. Batch culture fermentation involves the utilization of the model as individual, closed bioreactor vessels where enzymatic digestion, fecal inoculation and treatment addition are done sequentially in the same vessel. The benefit of using this adapted methodology as compared to the 2-week stabilization followed by a period of treatment is its ability to screen a large number of probiotics concurrently in a uniform and systematic manner, as each bioreactor had the same fecal inoculum and experimental conditions. In the following chapters, we assessed the ability of several single-strain and multi-strain probiotic combinations to causes changes in different aspects of *C. difficile* infection including changes in the colonic milieu, modulation of the native gut flora and its metabolic functionality, and, its effect on the intestinal epithelium. Chapter 4 aims to describe the effect of *C. difficile* infection on the overall antioxidant status of the colonic milieu and how probiotics might counteract these potential changes. The premise for assessing antioxidant status of the colonic milieu is its ability to indicate changes in gastrointestinal function and health through relatively simple measurement techniques. Chapter 4 has been published in Nutrients: Gaisawat M.B., Iskandar M.M., MacPherson C.W. Tompkins, T.A., Kubow S.; *Probiotic Supplementation is Associated with Increased Antioxidant Capacity and Copper Chelation in C. difficile-Infected Fecal Water.*

CHAPTER 4: RESEARCH PAPER 2

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Probiotic Supplementation is Associated with Increased Antioxidant Capacity and Copper Chelation in *C. difficile*-Infected Fecal Water

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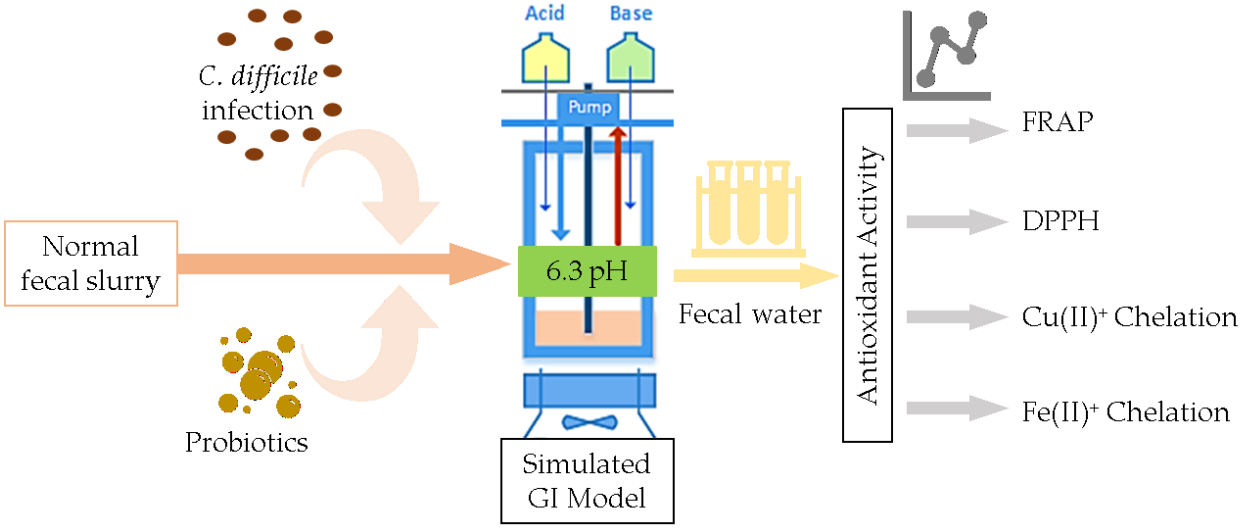
4.1 ABSTRACT

Probiotic supplementation plays a key role in maintaining intestinal homeostasis due to its ability to modulate gut microbiota. Although their potential as potent antioxidants have previously been explored, their ability to affect the redox status in the gut lumen of healthy subjects or those with gastrointestinal (GI) disorders remains unclear. In our study, we assessed the ability of single strain and multispecies probiotic supplementation to cause a change in the redox status of normal fecal water and in *Clostridium* (C.) *difficile*-infected fecal water using a simulated gastrointestinal model. Changes in redox status were assessed by ferric-reducing antioxidant power (FRAP), 2',2'-diphenyl-1-picrylhydrazyl (DPPH), and iron and copper chelation assays. The findings from our study showed that in normal fecal water, probiotic supplements, apart from Lactobacillus (L.) rhamnosus R0011, showed a significant increase in iron chelation ($p < 0.05$), which was associated with lower FRAP and copper chelation. In *C. difficile*-infected fecal water, all probiotic supplements showed a significant increase in FRAP ($p < 0.05$) and were associated with increased copper chelation. The DPPH assay showed no treatment effect in either fecal water. These findings suggest that *C. difficile* mediates dysregulation of redox status, which is counteracted by probiotics through ferric-reducing ability and copper chelation.

4.1.1 Graphical Abstract



4.2 INTRODUCTION

Probiotics have received extensive study as putative therapeutic agents for use toward the prevention and treatment of gastrointestinal (GI) disorders with altered gut microbiota profiles [1,2]. Probiotic supplementation has been well-established in various animal disease models to be associated with decreased intestinal cytotoxic damage, involving downregulation of inflammatory pathways [3,4]. Additionally, probiotics have been indicated to stabilize gut microbiota through different mechanisms such as iron chelation and anti-microbial metabolite production [2], which are associated with antioxidant action. In that regard, bacterial growth cultures have demonstrated that several probiotic strains possess significant *in vitro* antioxidant activity [5–7]. Enhanced plasma antioxidant capacity and decreased oxidative stress biomarkers have also been demonstrated in clinical trials involving probiotic supplementation [5–7].

The antioxidant potential of probiotic bacteria has been shown to be strain specific. For this reason, the antioxidant potential is not generalizable to the species level [8]. The strain-specific antioxidant properties of lactic acid bacteria (LAB) are well-documented, with a variety of LAB strains showing antioxidant capacity. However, the extent of their capabilities and the mechanisms by which they act can differ greatly [9]. The ability of probiotics to act as antioxidants is thought to be mediated via several potential mechanisms, i.e., producing antioxidant enzymes such as superoxide dismutase (SOD), glutathione S-transferase, and catalase, as well as by producing metabolites such as lactate [5,6]. In addition, probiotics work through indirect antioxidant mechanisms including reducing bioavailability of metals such as iron, which can also limit pathogen growth through regulating gut microbial profiles [10]. Fecal matter from healthy adults has been shown to possess antioxidant capacity and has been suggested as an important biomarker of gut microbiota homeostasis [11]. A variety of gastrointestinal disorders, such as inflammatory bowel disease and colon cancer, have been linked to an increase in oxidative stress in the gut lumen [12]. On the other hand, antioxidant action has been indicated to play a role in the therapeutic benefits of antioxidants used in the treatment of the gastrointestinal diseases,

such as salicylate, butylated hydroxyanisole, and vitamin E [11,13]. Several studies have associated improvements in gut health with the increase in gastrointestinal antioxidant capacity following intake of antioxidants such as polyphenols and tocopherols [14,15]. Moreover, a study by Bianchi et al. (2010) demonstrated that an increase in antioxidant content of feces is associated with an increase in stool bulk, indicating better gut function and health [16]. The potential of probiotic supplements in stabilizing the redox status of human fecal water from healthy subjects or those with GI disorders, however, is unclear.

The antioxidant potential of probiotic LAB could be particularly relevant to *Clostridium* (C.) *difficile*-mediated infection, as this involves enterotoxin-mediated intestinal toxicity, which is associated with increased free radical production and upregulation of inflammatory pathways [17–19]. As LAB strains possess multiple mechanisms of antioxidant action, combinations of these probiotics could increase the capability to regulate redox status changes caused by *C. difficile* infection. To date, *Saccharomyces* (S.) *boulardii* is the only reported probiotic to show an effective response to the enterotoxins produced by *C. difficile* [20], which could include indirect protective mechanisms involving reduction of free radical generation. There is limited literature on the direct antioxidant properties of *S. boulardii*, but Suryavanshi et al. [21] have shown that this probiotic can act, in growth cultures, as a potent free radical scavenger via production of antioxidant metabolites. Some studies have also shown a reduction in *C. difficile*-associated diarrhea when supplemented with *S. boulardii* and *Lactobacillus* (L.) *rhamnosus* GG [20].

Assessment of antioxidant activity of probiotics has been widely conducted using spectroscopic assays, such as ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), and Trolox-equivalent antioxidant capacity (TEAC) assays [22–26]. Each of these analyses are based on the principle of electron transfer between the oxidant and the antioxidant moieties, i.e., they measure the reduction potential of antioxidants present in a sample. The FRAP and TEAC assays have been shown to have similar redox potentials and are used interchangeably. The DPPH assay is used to determine radical scavenging potential of the sample using a stable synthetic

nitrogen-radical [27]. Metal chelation measurements have also been conducted, along with the traditional antioxidant assays, as a potential indication of their mechanism of action [9,28].

In the present study, commercially available strains of LAB and *S. boulardii*, as singular treatments and in combination, were used to assess the antioxidative potential of these strains when cultured in a simulated gut digestion model involving either *C. difficile*-infected fecal matter or healthy donor fecal matter. Fecal water (FW) digests were assessed for antioxidant capacity through the FRAP and DPPH assays and for the metal chelation ability of iron and copper.

4.3 Materials and Methods

4.3.1 Batch Culture Fermentation

Simulation of GI conditions was done using a dynamic computer-controlled model that utilizes several 250 mL independent fermentation vessels run in parallel. All vessels were maintained under anaerobic conditions by purging with oxygen-free nitrogen gas. Vessel contents were continuously stirred using magnetic stirrers and maintained at 37 °C using heated double-jacketed beakers. The pH monitoring and regulation of each vessel during enzymatic digestion and fermentation was done using python coding of a Raspberri Pi microprocessor (ver. 1B) with an embedded EZO™ pH circuit (Atlas Scientific, NY, USA). Addition of 0.5 M NaOH or 0.5 M HCl was carried out to maintain pH using computer-controlled peristaltic pumps.

One-hundred milliliters of filter-sterilized GI food, previously optimized by Molly et al., [29], composed of 1 g/L of arabinogalactan, 2 g/L of pectin, 1 g/L of xylan, 3 g/L of starch, 0.4 g/L of glucose, 3 g/L of yeast extract, 1 g/L of peptone, 4 g/L of mucin, 0.5 g/L of cysteine, and 40 µL/L of vitamin solution, were added to each vessel (Sigma Aldrich, St. Louis, MO, USA). For batch culture conditions, an adapted method from [30] was used. Oral enzymatic digestion was initiated by adjusting the media to a pH of 7.0 and adding 1.5 mL of ddH2O containing 1 g of α-amylase (A3176, Sigma-Aldrich, St. Louis, MO, USA). After 15 min of incubation, the pH of each

vessel was decreased to 2.0, followed by addition of 1.5 g of pepsin (P7125, Sigma-Aldrich, St. Louis, MO, USA) in 1.5 mL of 1 M HCl and incubated for 90 min. The vessels were then adjusted to a pH 8.0, followed by addition of 20 mL pancreatic juice (comprised of 12 g/L NaHCO3 (Sigma-Aldrich, St. Louis, MO, USA), 6 g/L bile extract (Sigma-Aldrich, St. Louis, MO, USA), and 0.9 g/L pancreatin (Sigma-Aldrich, St. Louis, MO, USA)). After 2 h of pancreatic digestion, 50 mL of fecal slurry were inoculated in each vessel (T = 0 h). Fermentation was carried out for a period of 24 h after inoculation with sampling after every 6 h. Samples taken at each time point were centrifuged at 2000 g for 10 min. The supernatant (hereinafter referred to as fecal water, FW) was filtered using sterile 0.45 µm syringe filters and stored at –20 °C for short-term use and –80 °C for long-term use. All experiments were performed in triplicate.

4.3.1.1 Fecal Slurry Preparation

Regular fecal samples were collected from a healthy adult male donor with no history of GI disorders or antibiotic use for a period of at least six months prior. Samples were collected at least three days apart to account for individual variability. Samples were processed within 3 h of collection and frozen at –80 °C in cryoprotectant solution (12.5% glycerol in 0.9% saline (v/v)) at a ratio of 1:3 w/v. Prior to inoculation in the gut model, the fecal sample was stabilized at 37 °C overnight under anaerobic conditions. *C. difficile* fecal samples were commercially sourced from BioIVT, USA (male adult; stool positive for toxins A & B). The fecal samples were processed to make a slurry in a similar manner. For each experiment, *C. difficile*-infected fecal samples were prepared by adding *C. difficile* fecal slurry into the regular fecal slurry at a ratio of 1:10 v/v (5 mL of slurry per vessel).

4.3.1.2 Probiotic Treatment Preparation

In this study, eight different probiotic treatments were utilized (five single-strain probiotics and three as combinations). All probiotics were acquired from Rosell® Institute for Microbiome and Probiotics (Montreal, QC, Canada) in powder format and stored at –20 °C until use. The

treatments, along with their codes, are as follows: *L. rhamnosus* R0011 (LR11), *L. helveticus* R0052 (LH52), *L. rhamnosus* GG (LGG), *S. boulardii* (SB), Bifidobacterium (B.) longum R0175 (BL175), ProtecFlorTM (commercially available combination of LRR, LHR, BLR, and SB) (PROTO), a combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175 (LR + LH + BLR), and a combination of LGG (10 billion cfu) and SB (5 billion cfu) (LGG + SB). Except where specified, all treatments were used at a dose of 1 billion cfu/vessel. All treatments were premixed at room temperature in 2 mL sterile 1× PBS before addition to any vessel (T = 0 h). Two milliliters of 1× PBS were used as negative control (blank).

4.3.1.3 Experimental Repeats

Each fermentation experiment of an individual treatment, including blank, was performed in triplicate. Samples of each repeat were tested in triplicate for all the assays performed.

4.3.2 Antioxidant Assays

4.3.2.1 Chemical Reagents

Chemicals, including 2,2-diphenyl-1-picrylhydrazyl radical (DPPH; S7670), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ; T1253), ferric chloride (236489), sodium acetate trihydrate (S7670), L-ascorbic acid (A7506), salicylic acid (247588), sodium hydroxide (221465), potassium nitrate (P6083), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 113784), 2,2',2'',2'''-(Ethane-1,2-diyldinitrilo)tetra acetic acid (EDTA) disodium salt dihydrate (ED2SS), L-cysteine (168149), sulphanilamide (S9251), and N-1-Naphthylethylenediamine dihydrochloride (N9125), were obtained from Sigma-Aldrich (St Louis, Mo). Cupric sulphate pentahydrate ($CuSO_4.5H_2O$; BP346-500), methanol, hydrochloric acid, sulfuric acid, and glacial acetic acid were obtained from Fisher Scientific (USA). Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; 218940050), ferrozine (3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5',5''-disulfonic acid disodium salt; 410570010), and pyrocatechol violet (3,3',4-trihydroxyfuchsone-2''-sulfonic acid;

146540050) were obtained from Acros Organics (Morris, NJ, USA). Water was purified with a MilliQ filtration system. All reagents were of analytical grade purity. Handling of chemicals and discarding of waste were done in accordance with safe lab procedures using a chemical hood and appropriate protective gear.

4.3.2.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was used to measure the total antioxidant capacity of FW based on a 96-well plate adapted method of Benzie et al. (1996) [31]. Briefly, in a 96-well plate, 30 µL dH_2O and 10 µL of sample or standard were added, followed by 200 µL of pre-incubated FRAP reagent at 37 °C (10:1:1 v/v/v of 300 mM sodium acetate at pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$). The contents of each well were mixed for 10 s and absorbance was read at λ = 593 nm after 8 min of reaction time using a uQuant microplate reader (BioTek Instruments, Winooski, VT, USA). Seven equally distributed serial dilutions of 1 mM ascorbic acid were used to generate the standard curve (R^2 = 0.999). Results were expressed as mg ascorbic acid equivalents per liter of FW (mg AA E/L).

4.3.2.3 2',2'-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The DPPH assay was used to assess the radical scavenging capacity of the FW toward the DPPH radical, leading to decolorization of the DPPH methanol solution. The method follows the procedure proposed by [32]. Undiluted aliquots from each experimental run were tested in triplicate. A standard curve was prepared with evenly spaced dilutions of 1 µM Trolox in methanol, with only methanol solvent as blank (R^2 = 0.998). In a 96-well plate, 190 µL of 0.15 mM DPPH-methanol stock reagent solution (pre-measured for a range of optical density value between 0.7–0.9 at 517 nm) was mixed with 10 µL of standard or sample and left to react for 30 min at 25°C. The decrease in absorbance was measured at λ = 517 nm using uQuant microplate reader (BioTek Instruments, Winooski, VT, USA). The antioxidant capacity (% of DPPH inhibition)

was calculated as [1 − (A Sample / A Blank)] × 100. Results were expressed as mg Trolox equivalents per liter (mg TE/L).

4.3.2.4 Cu (II)+ Chelation Assay

The ability of the FW to chelate Cu^{2+} was measured by the procedure outlined in [33]. Briefly, in a 96-well microplate, 30 µL of sample or standards were pipetted in triplicate, followed by the addition of 200 µL of 50 mM sodium acetate (at pH 6.0) and 30 µL of 100 mg/L $CuSO_4.5H_2O$. After 2 min of reaction time, 8.5 µL of 2 mM pyrocatechol was added. The microplate was placed on a shaker for 10 min, followed by 10 min of rest at 25 °C. Absorbance was read at λ = 632 nm and chelating ability was calculated as chelation (%) = [A Sample /A Control] × 100. A standard curve was prepared using serial dilutions of 100 mg/L disodium ethylenediamine tetraacetic acid ($EDTA.Na_2$) (R^2 = 0.99). Results were expressed as mg EDTA equivalents per liter (mg EDTA E/L).

4.3.2.5 Fe (II)+ Chelation Assay

The Fe^{2+} chelation capacity of the FW followed the methodology outlined in [33]. Briefly, in a 96-well microplate, 50 µL of sample or standards were pipetted in triplicate, followed by the addition of 160 µL H2O (at pH 6.0), 20 µL of 0.3 mM $FeSO_4$, and 30 µL of 0.8 mM ferrozine solution. Once mixed, the plates were incubated for 5 min at room temperature. In an additional well, ferrozine solution was replaced with dH_2O for each sample to account for background coloration. A volume of 50 µL of dH_2O alone along with all reagents was used as a control. Absorbance was read at λ = 562 nm. The Fe^{2+} chelating ability was calculated as chelation (%) = $[(A_{Sample} - A_{Solution\ w/o\ ferrozine})/A_{Control}] \times 100$. The standard curve was prepared using serial dilutions of 100 mg/L EDTA.Na2 (R^2 = 0.98). Results were expressed as mg EDTA E/L.

4.3.3 Statistical Analysis

All data are reported as means ± standard error of mean (SEM). Data for all assays were analyzed using two-way ANOVA using Probiotic Treatment (nine levels) and Time (five levels) as factors. For multiple comparisons, Tukey's HSD post hoc test was carried out to compare treatments to control. The means of all time points were jointly considered when no significant interactions in the two-way ANOVA were observed. When significant interactions between time and treatment were observed, the mean of each time point within a treatment was individually compared to its corresponding time point within the control. Correlation analysis was performed using non-parametric Spearman's rank-order correlation coefficient (ρ-value) to measure the degree of association between each pairwise variable. Statistical significance was set at $p < 0.05$. All statistical analyses and graphs were performed using JMP ® v14.2 (SAS Institute, Cary, NC, USA).

4.4 Results and Discussion

In this study, FRAP and DPPH assays were used to measure the redox status of the fecal water obtained from colonic reactors following simulated upper GI digestion involving probiotic treatments. Iron and copper chelation assays were conducted to assess the capability of the probiotics to affect the chelation ability of the fecal water.

4.4.1 FRAP and DPPH Antioxidant Capacity of Fecal Water

Two-way ANOVA results for FRAP showed significant ($p < 0.05$) main effects of treatment and time for both normal and *C. difficile*-infected FW. There was a significant ($p < 0.05$) interaction effect of time and treatment only in normal FW. Therefore, for normal FW, the mean FRAP for each time point within each treatment was compared to its corresponding time point within the blank. Singular probiotics LR11, LH52, and SB showed a significant ($p < 0.05$) increase in FRAP at time 24 h, time 12 h, and time 6 h, 12 h, and 24 h, respectively (**Figure 4.1**). No statistical significance was found for LGG and BL175 (*B. longum*-R175) at any given time point, which

indicated strain-dependent FRAP radical scavenging activity. Interestingly, a biphasic antioxidant response with time was seen with LR11 and LH52, which showed a significant decrease at 18 h. Although a biphasic response in antioxidant capacity has not been demonstrated with probiotics, it could be speculated that this could be related to binding and release of bacterial exopolysaccharides with gastrointestinal fecal matter at different time points of the digestion processes [34]. Neither of the probiotic combinations appeared to have a significant effect of treatment at a given time point, potentially due to antagonistic effects in the multispecies probiotic combinations. Ranadheera et al. (2014) [35] previously demonstrated that certain probiotic combinations could show a decreased ability to survive in gastrointestinal conditions or adhere to the mucosal layer when compared to their individual probiotics. Furthermore, multispecies combinations could show decreased viability due to competition for essential minerals or secretion of specific bacteriocins [36].

For the *C. difficile*-infected FW, as there was no significant interaction of time and treatment, all time points were jointly considered within each treatment. All probiotic treatments, including the probiotic combinations, showed a significantly ($p < 0.05$) higher FRAP as compared to the control. It is conceivable that the enhanced antioxidant capacity of the combination treatments in the *C. difficile*-infected FW could be related to its cytotoxic characteristics, which led to an upregulation of the reactive oxygen species (ROS) defense mechanisms of the probiotics. Multispecies probiotic combinations have been noted to work in a synergistic manner to maintain redox balance [8].

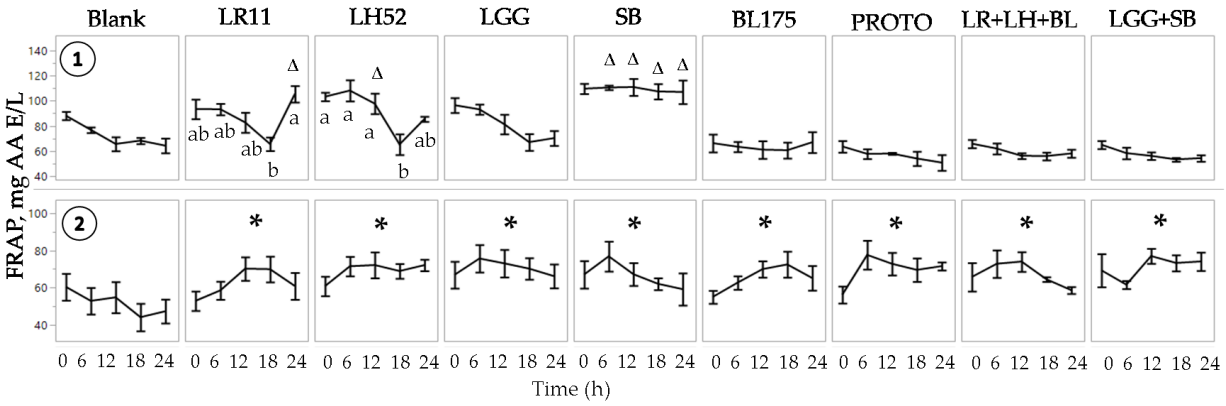


Figure 4.1. Antioxidant capacity of fecal water (FW) as measured by ferric reducing antioxidant power (FRAP). 1) Normal FW, 2) *C. difficile*-infected FW. Values are shown as mean ± SEM. The symbol Δ represents significant differences ($p < 0.05$) between treatment at a particular time point and blank at the corresponding time point. The symbol * represents significant differences between treatment and blank ($p < 0.05$) when the means of all time points are jointly considered. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). LR11 = *L. rhamnosus* R0011; LH52 = *L. helveticus* R0052; LGG = *L. rhamnosus* GG; SB = *S. boulardii*; BL175 = *B. longum* R0175; PROTO = ProtecFlorTM; LR+LH+BL = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; LGG+SB = combination of *L. rhamnosus* GG and *S. boulardii*.

The antioxidant potential of the specific strains used in this study have not been documented previously either in the context of fecal water or fermentation cultures. Similar strains of Lactobacilli, *B. longum*, and *S. boulardii*, however, have been associated with antioxidative properties [9,37]. LAB have been shown to produce high levels of glutathione and enzymes such as SOD, along with organic acids such as lactate, which contribute to their overall antioxidant properties [5]. Similarly, *S. boulardii* is thought to produce a range of phenolic metabolites in culture, such as vanillic acid, that contribute to their antioxidative properties [37]. It is thus possible that strains used in the present study possess similar antioxidant mechanisms to increase the reducing ability of FW in both the normal and *C. difficile* FW.

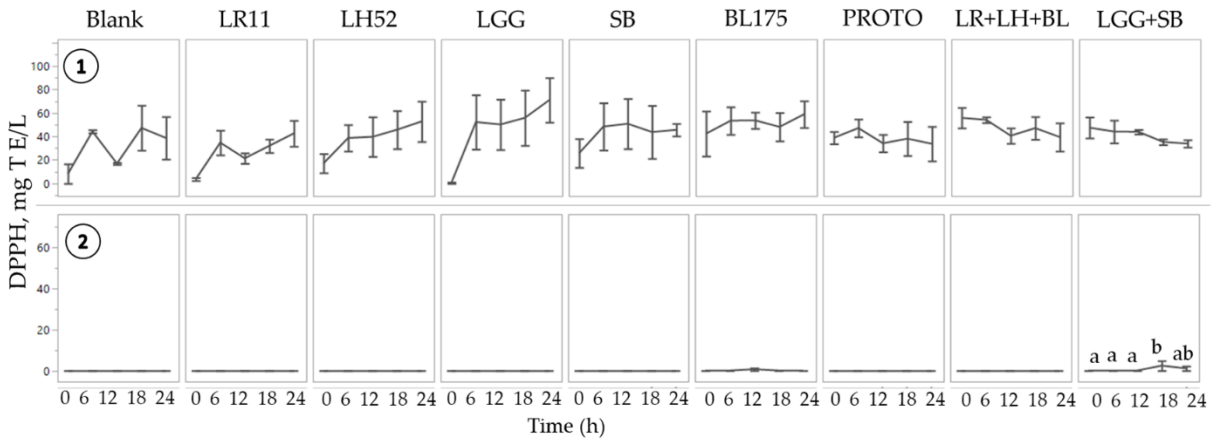


Figure 4.2. Antioxidant capacity of fecal water (FW) as measured by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). 1) Normal FW, 2) *C. difficile*-infected FW. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). LR11= *L. rhamnosus* R0011; LH52 = *L. helveticus* R0052; LGG = *L. rhamnosus* GG; SB = *S. boulardii*; BL175 = *B. longum* R0175; PROTO = ProtecFlorTM; LR+LH+BL = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; LGG+SB = combination of *L. rhamnosus* GG and *S. boulardii*.

The DPPH assay results showed the potential of normal FW to inhibit or scavenge the DPPH radical, whereas *C. difficile* FW showed no or minimal inhibition across samples (**Figure 4.2**). Supplementation with probiotics showed no significant change in DPPH radical scavenging potential in either normal or in *C. difficile*-infected FW. The inability of probiotics to show an effect in the DPPH radical scavenging capacity of either FW could be due to the nature of the DPPH radical and the assay itself. The extremely stable DPPH radical has been shown to have very slow reaction kinetics with some antioxidants as compared to reactions with highly reactive and unstable radicals generated *in vivo* [27]. Moreover, the assay was conducted using a methanol solvent that might interfere with the reactions that usually happen in aqueous media. From our study findings, it appears that the FRAP assay is a more sensitive indicator for assessing antioxidant reducing ability in FW. The differences in the capacity for inhibition of the DPPH

radical between normal FW and *C. difficile*-infected FW could be due to differences in the redox potential of the microbiota itself. In *C. difficile*-infected FW, the absence of DPPH radical inactivation could be due to the increase in ROS production, resulting in a pro-oxidant status [17].

4.4.2 Metal Chelation Capability of Fecal Water

Analysis of metal chelation was done to assess the ability of FW to decrease the bioavailability of bivalent metals such as copper and iron. An increase in metal chelation would reduce the available iron and copper used to catalyze free radical polymerization. Studies have previously demonstrated metal chelating potential of certain strains of LAB [9,28,38].

4.4.2.1 Determination of Copper Chelation

Two-way ANOVA results for the copper chelation assay showed significant ($p < 0.05$) main effects of treatment for both normal and *C. difficile*-infected FW and no interaction effects. In normal FW, there was a significantly ($p < 0.05$) higher chelation ability of two individual probiotic treatments, *S. boulardii* SB and *L. rhamnosus* LR11 (**Figure 4.3**), when compared to blank. Conversely, *B. longum* B175 showed significantly ($p < 0.05$) lower chelating capacity as compared to blank. The probiotic combination treatments (PROTO, LR+LH+BL, LGG+SB) showed no differences in copper chelation relative to the blank despite containing the LR11 and *S. boulardii* SB strains. FW samples with *C. difficile*-infected microbiota showed that only the treatments containing the yeast *S. boulardii* (SB, PROTO, and LLG+SB) showed a significantly ($p < 0.05$) higher chelation capability than blank.

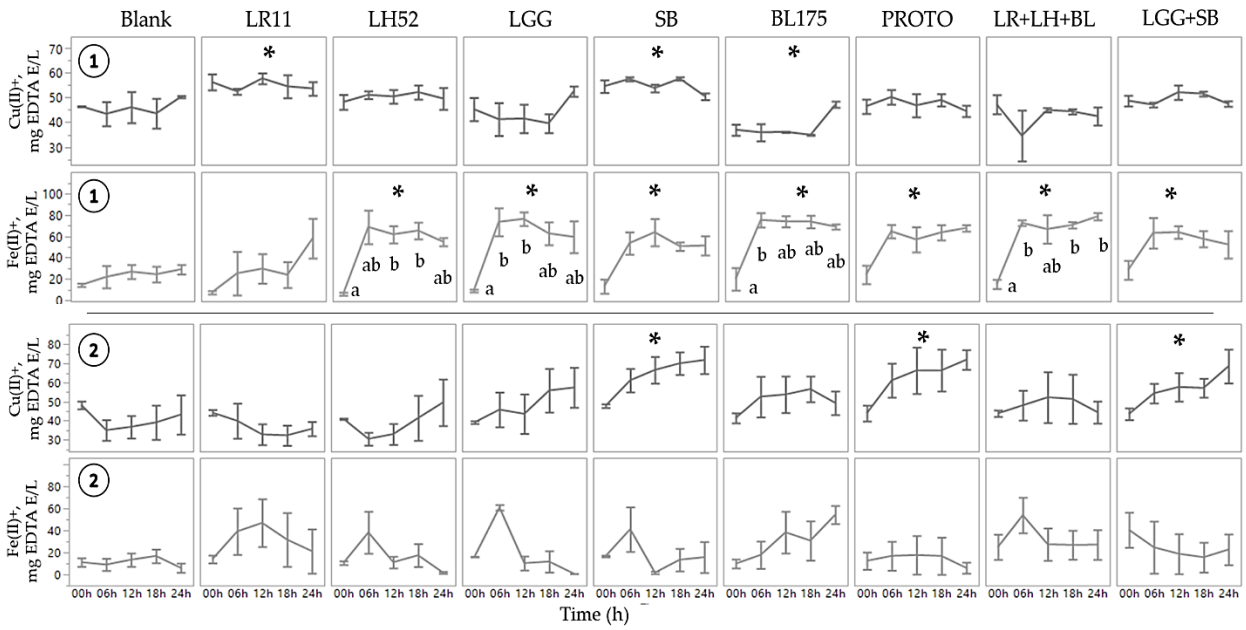


Figure 4.3. Copper and iron chelation ability of fecal water (FW). Normal fecal sample; *C. difficile*-infected fecal sample. The symbol * represents significant differences between treatment and blank ($p < 0.05$) when the means of all time points are jointly considered. (LR11 = *L. rhamnosus* R0011; LH52 = *L. helveticus* R0052; LGG = *L. rhamnosus* GG; SB = *S. boulardii*; BL175 = *B. longum* R0175; PROTO = ProtecFlorTM; LR+LH+BL = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; LGG+SB = combination of *L. rhamnosus* GG and *S. boulardii*).

4.4.2.2 Determination of Iron Chelation

Two-way ANOVA for iron chelation showed a significant effect on treatment only for normal fecal water. There were no interaction effects of time and treatment. Therefore, all of the time points were jointly considered when comparing treatments to blank. Iron chelation results showed a significant ($p < 0.05$) increase in the chelation capability of normal FW supplemented with probiotics, apart from *L. rhamnosus* LR11 when compared to blank. Conversely, the *C. difficile* FW showed no significant increase in the iron chelation capacity of any probiotic supplementation (**Figure 4.3**). The results of the probiotic-supplemented normal FW are in

concert with previous findings showing that LAB possess metal chelation ability. Lin and Yen et al. (1999) [9] showed that the strain Streptococcus thermophilus 821 showed the highest chelating ability for both iron and copper among 19 LAB strains. Six L. bulgaricus strains showed high copper chelation and two *B. longum* strains showed a high capacity to chelate both iron and copper. In another study, L. casei KCTC 3260 demonstrated the highest iron and copper chelation out of the four LAB strains [28]. Their results also showed that *L. rhamnosus* GG had chelating capacity for both iron and copper and was the only strain to possess significant SOD activity. In another study utilizing LAB, *L. helveticus* CD6 strain showed significant iron chelation ability [38].

The results from the present study show the capacity of *S. boulardii* to chelate copper in normal FW, and more importantly, in *C. difficile*-infected FW where it showed significant chelation. Toxins produced by *C. difficile* have been previously linked to enhanced ROS formation, which could lead to metals being used to catalyze oxidation through the Fenton reaction [39]. The ability of *S. boulardii* to reduce ROS formation, as shown by [21], could in part explain its continued capability to chelate copper. The LR11 probiotic, however, was shown in the present study to possess copper chelating ability only in normal FW. Interestingly, all probiotic treatments apart from LR11 showed the capability to chelate iron in normal FW but failed to show any chelation capacity in the presence of the induced *C. difficile* infection.

4.4.3 Determination of Nitrite, Nitrate, and, Protein Carbonyls

Toxins secreted by *C. difficile* have been associated with an upregulation of ROS production in a variety of cell types [17,40]. To assess for compounds generated from ROS reactions, assessments were done for nitric oxide (NO) derivatives and protein oxidation compounds (as outlined in **Supplementary Methods S4.1**).

Nitrite and nitrate quantification were performed in FW as an indirect assessment of NO production. NO, an essential immunomodulatory molecule, is implicated in several pathological conditions when upregulated [41]. *In vivo* NO is short-lived and rapidly converted to its more

stable oxidation end products, nitrite and nitrate. Nitrite quantification in *C. difficile*-infected FW showed low mean quantification values of ~ 0.5 µM in all samples (**Supplementary Figure S4.1**). No differences amongst the treatments were observed. Similarly, for nitrate quantification, for each treatment, considering all the time points, mean values of ~ 60 µM were observed. Many values were below the detection limit with a tendency for values to decrease with time for all treatments (**Supplementary Figure S4.2**).

Detection of protein carbonylation is another important method of detection of the extent of oxidative stress. Protein carbonyl groups are stable end-products of protein oxidation, particularly through the formation of aldehyde and ketone groups. These groups could also be formed via oxidative breakdown of the proteins or through lipid peroxidation [42]. In terms of detection of the protein carbonyl moieties in the *C. difficile* FW, only a handful of samples showed carbonyl formation with quantification values of less than 1 nmol/mg protein. The rest of the samples were below the limit of detection of 0.15 nmol/mg protein (**Supplementary Figure S4.3**). Low detection of these groups could be due to the formulation of the GI food in the fermentation units that have a low overall protein content (13.3 mg/mL) and a lack of a source of lipids, both of which lead to formation of carbonyl groups. Additionally, it has been stipulated that the critical step in the formation of hydrazones occurs at slightly acidic pH of 3–5 [42]. The pH of fecal water samples was closer to a neutral pH of 6–7, which could have contributed to a lower yield. Overall, the results from nitrite, nitrate, and protein carbonyl quantification showed no apparent increase in oxidation status for these measures in the *C. difficile* FW.

4.4.4 Correlation of Antioxidant Capacity Assays

The results of antioxidant and metal chelation assays were assessed for correlations to investigate predominant mechanisms of action of probiotics when supplemented *in vitro*. The data set of each assay (n = 15 for each treatment; n = 135 in total) was compared using pairwise correlation analysis to assess for any correlations between the antioxidant capacity assays, metal chelation assays and between each of the antioxidant capacity and metal chelation ability assays.

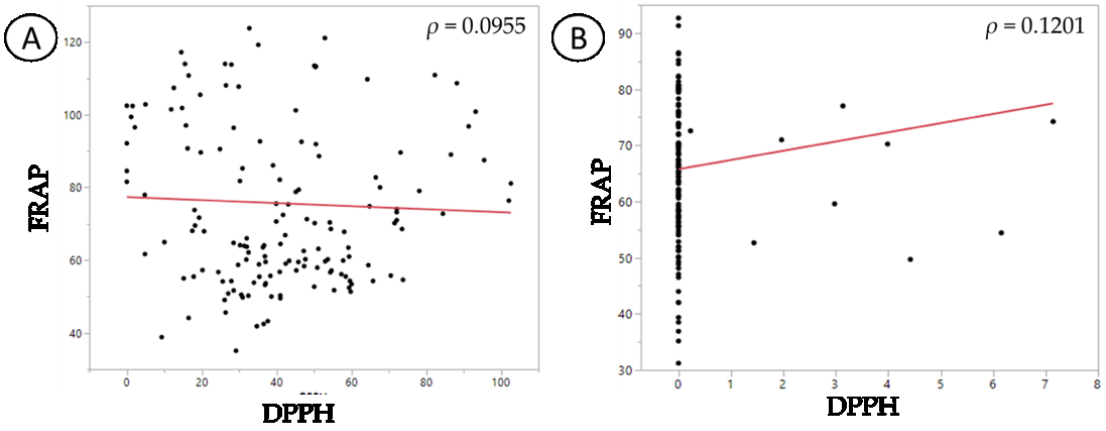


Figure 4.4. Spearman's correlation analysis between the antioxidant capacity assays, ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). A) Normal fecal sample; B) *C. difficile*-infected fecal sample.

The results from the correlation analysis between FRAP and DPPH for each fecal water showed no correlation or trend **(Figure 4.4**). The samples of *C. difficile*-infected FW were heavily clustered at the baseline due to the absence of DPPH radical inactivation.

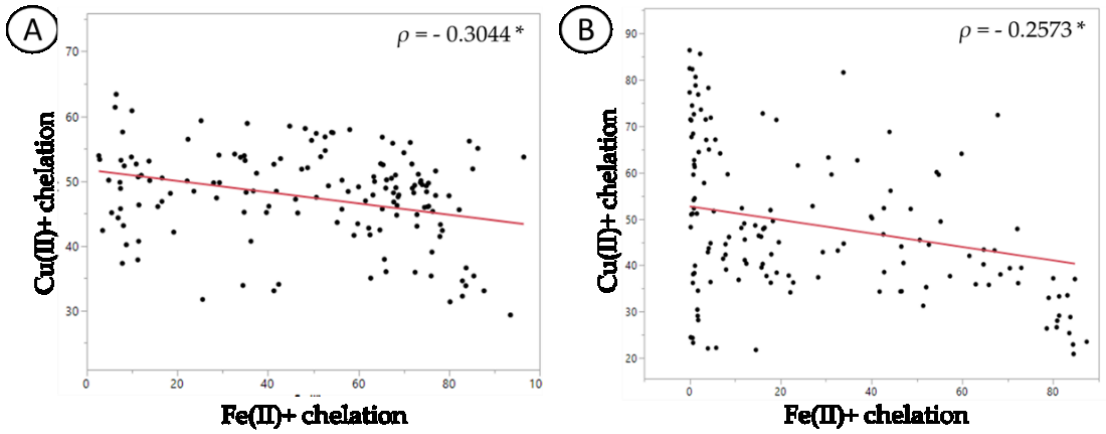


Figure 4.5. Spearman's correlation analysis between copper and iron chelation assays: A) Normal fecal sample; B) *C. difficile*-infected fecal sample. The symbol * represents significant correlation ($p < 0.05$).

Comparison of the copper and iron metal chelating ability showed that the copper chelating ability of probiotics was negatively correlated with iron chelation ability in both the normal and *C. difficile*-infected FW **(Figure 4.5**; **Tables 4.1 and 4.2**). These results indicate that an increase of copper chelation in FW was associated with lower iron chelation.

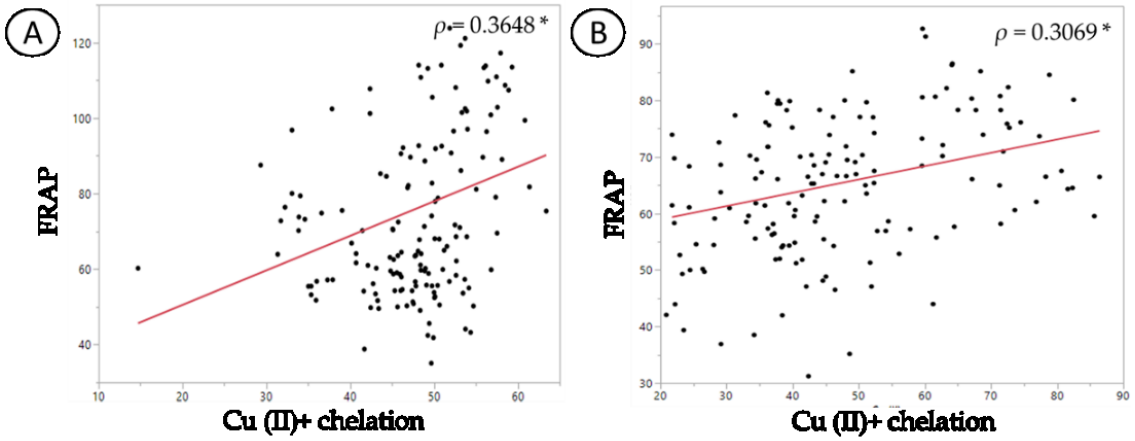


Figure 4.6. Spearman's correlation analysis between FRAP and copper chelation ability: A) Normal fecal sample; B) *C. difficile*-infected fecal sample. The symbol * represents significant correlation ($p < 0.05$).

The FRAP assay results showed a positive correlation with copper chelation data in each type of fecal water (ρ = 0.36 in normal FW and ρ = 0.31 in *C. difficile*-infected FW) (**Figure 4.6**). Iron chelation showed a strong positive correlation with the DPPH data in the normal fecal sample (ρ = 0.41), but no significant correlation was seen in the *C. difficile*-infected fecal sample (**Tables 4.1 and 4.2**). Likewise, the comparison between FRAP and iron chelation showed a negative correlation in the normal fecal sample (ρ = -0.23), whereas no such correlation was observed in the *C. difficile*-infected fecal sample (**Supplementary Figure S4.4**). Negative correlation in the normal FW for FRAP and iron chelation could stem from the difference in bacterial products such phenolic metabolites and exopolysaccharides, both of which have been previously demonstrated to possess specificity in transition metal binding [33,43]. Furthermore, no correlations were observed between DPPH and copper chelation in either type of FW (**Supplementary Figure S4.4**).

Table 4.1. Spearman's correlation (ρ) of normal fecal samples.

Variables	FRAP	DPPH	Cu (II)+ chelation	Fe (II)+ chelation
FRAP	1 *p = n/a*			
DPPH	- 0.0645 *p = 0.4572*	1 *p = n/a*		
Cu (II)+ chelation	0.3648 *p = 0.0001 **	- 0.1497 *p = 0.0831*	1 *p = n/a*	
Fe (II)+ chelation	- 0.2289 *p = 0.0076 **	0.4149 *p < 0.0001 **	- 0.3044 *p = 0.0116 (*)*	1 *p = n/a*

*$p < 0.05$

Table 4.2. Spearman's correlation (ρ) of *C. difficile*-infected fecal samples

Variables	FRAP	DPPH	Cu (II)+ chelation	Fe (II)+ chelation
FRAP	1 *p = n/a*			
DPPH	0.1201 *p = 0.1653*	1 *p = n/a*		
Cu (II)+ chelation	0.3069 *p = 0.0003 **	0.1187 *p = 0.1704*	1 *p = n/a*	
Fe (II)+ chelation	- 0.0162 *p = 0.8516*	0.0822 *p = 0.3429*	- 0.2573 *p = 0.0026 (*)*	1 *p = n/a*

*$p < 0.05$

Comparing pairwise correlations in each type of FW showed that FRAP and copper correlated positively ($\rho > 0.31$) and copper and iron chelation correlated negatively ($\rho < -0.22$) in both sets of samples, with both correlations showing statistical significance. The lack of other significant correlations in *C. difficile*-infected fecal samples could have been partly due to the low DPPH values, where almost no inactivation of the DPPH radical was observed. These latter results indicate that the probiotic supplements that show higher reducing ability by FRAP also possess a higher capability to chelate copper. Also, it appears that probiotic supplements that showed an increased copper chelating ability possessed decreased iron chelation ability. The mechanism of action of the apparent affinity for copper could stem from the different profiles of the bacterial secretion product, exopolysaccharides. Bacterial exopolysaccharides have been shown to possess a significant ability to adsorb transition metals such as copper, but they vary greatly in

function based on chemical structure and growth conditions, leading to strain specific interactions [43,44].

Similar correlations have only been previously performed in studies assessing antioxidant properties of fruit and vegetable extracts. Santos *et al.* (2017) compared the antioxidant properties of coffee extracts using FRAP, DPPH, total phenolic content (TPC), and iron and copper metal chelation assays [33]. Pairwise correlation analysis conducted on their results showed significant positive correlations between copper chelation and DPPH, FRAP, and TPC. The iron chelation assay showed poor correlations with any of the other assays. This is similar to the present study findings with C-difficile FW, as iron chelating capability showed poor correlations with any of the assays. Another study with vegetable juice that underwent simulated digestion showed strong correlations between antioxidant assays FRAP, 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and TPC, but poor correlations of those assays with DPPH [45]. Their results showed significant positive correlations of copper chelation with DPPH, FRAP, and TPC assays. These correlations, however, stem from highly abundant polyphenolic matrices that are absent in our fecal water samples where the antioxidant potential of probiotics was assessed (**Figure 4.7**).

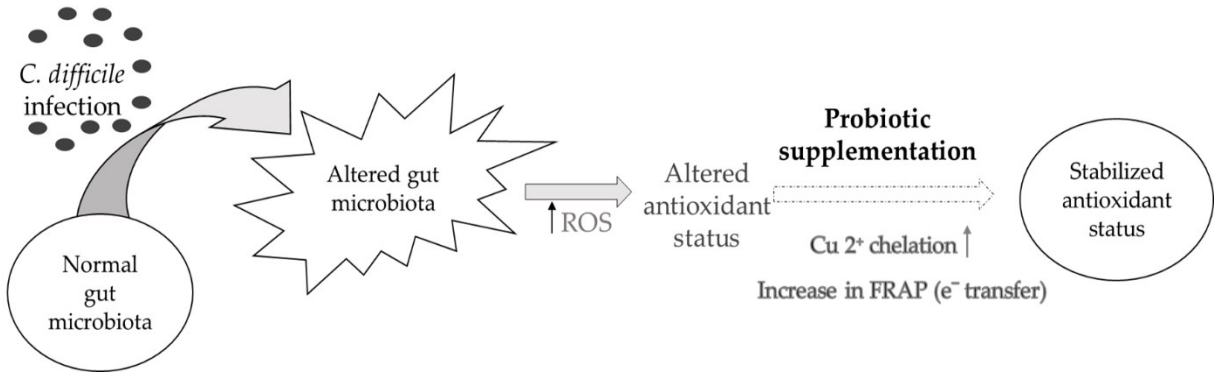


Figure 4.7. Proposed mechanism of probiotic regulation of redox potential in an altered gut microbiota.

4.5 CONCLUSIONS

In summary, the present findings have suggested dysregulation in the redox status of the *C. difficile*-infected FW. This phenomenon was indicated by the lack of radical inhibition in the DPPH assay and the reduced chelation capability seen with the iron chelating assay in those samples. Notably, probiotic treatment of the *C. difficile* FW was associated with higher FRAP reducing capacity, which was correlated with a higher capability to chelate copper. As reviewed by Wang et al. (2017), probiotics with antioxidant properties have been associated with protection against some GI diseases, which is linked to inhibition of the adverse effects caused with ROS generated within the GI milieu [6]. With respect to *C. difficile*, the present data supports the concept that probiotic strains with higher reducing and copper chelation abilities could provide an effective strategy to combat the altered redox status associated with *C. difficile* infection (**Figure 4.5**). Additionally, these probiotics might help stabilize the altered gut microbiota, an aspect that needs to be explored in more detail in future studies.

4.6 ACKNOWLEDGMENTS

The authors would like to thank Jocelyn Belvis for her extensive help in acquiring and culturing the probiotics. The authors would also like to acknowledge Dr. James Colee based at University of Florida for his input on the statistical analyses employed. Lastly, we would like to thank Ms. Xiaoci Hong for her support in performing antioxidant capacity assays.

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4.8 SUPPLEMENTARY INFORMATION

4.8.1 Supplementary Methods

4.8.1.1 Nitrite Determination

Nitrite was determined using the sequential-addition Griess reaction in which nitrite reacts with sulfanilamide (SA) in acidic conditions to form a diazonium salt, forming a stable pink azo compound after further reaction with N-naphthyl-ethylenediamine (NED) [1,2]. In this assay, 150 µL of FW was added to 150 µL of 1% (w/v) SA in 1 M HCl in a 1.5 mL microtube and agitated. One M HCl alone was used to correct for any background nitrite. The mixture is centrifuged at 10,000 g for 10 min at 4°C. An aliquot of 200 µL of the supernatant was transferred into a new microtube and 100 µL of 0.02% (w/v) NED in ultrapure water was added. After 15 min of reaction at room temperature, 200 µL was transferred into a standard 96-well assay plate and absorbance was measured at λ = 540 nm. A standard curve was prepared using equally spaced serial dilutions of 250 µM potassium nitrite (KNO_2) (R^2 = 0.99).

4.8.1.2 Nitrate Determination

Nitrate was measured using a microplate-adapted method of Cataldo *et al.* (1975) [3]. Briefly, salicylic acid undergoes nitration by dissolved nitrate under acidic conditions. The transient compound forms a yellow complex when further mixed with an alkaline solution. For the assay, in a 1.5 mL microtube, 10 µL of FW sample was added to 40 µL of 0.05% (w/v) salicylic acid in concentrated sulfuric acid and vortexed. Forty µL of sulfuric acid alone was used to determine the nonspecific background concentration. After 20 min of reaction at 25˚C, 1 mL of 8% (w/v) NaOH in ultrapure water was added to each tube. A 200 µL aliquot from each tube was added into a 96-well microplate and absorbance was read at λ = 410 nm. Stock nitrate standard solutions (8 mM) were prepared by dissolving 0.81 g oven-dried (100°C, 1 h) KNO_3 in 1 L ultrapure water. A standard curve was prepared using serial dilutions of 8 mM KNO_3 (R^2 = 0.99).

4.8.1.3 Protein Carbonyl Assay

Detection of protein carbonyl groups was done using the kit provided by Abcam (Cambridge, USA) with the catalogue no. ab126287. Procedures were followed as per manufacturer's instruction. This kit utilizes 2,4-dinitrophenylhydrazine (DNPH) to derivatize protein carbonyl groups, forming DNP hydrazones which are then quantified spectrophotometrically at λ = 375 nm. The results were expressed as nmol carbonyl/mg protein.

4.8.1.4 Supplementary Method References

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4.8.2 Supplementary Figures

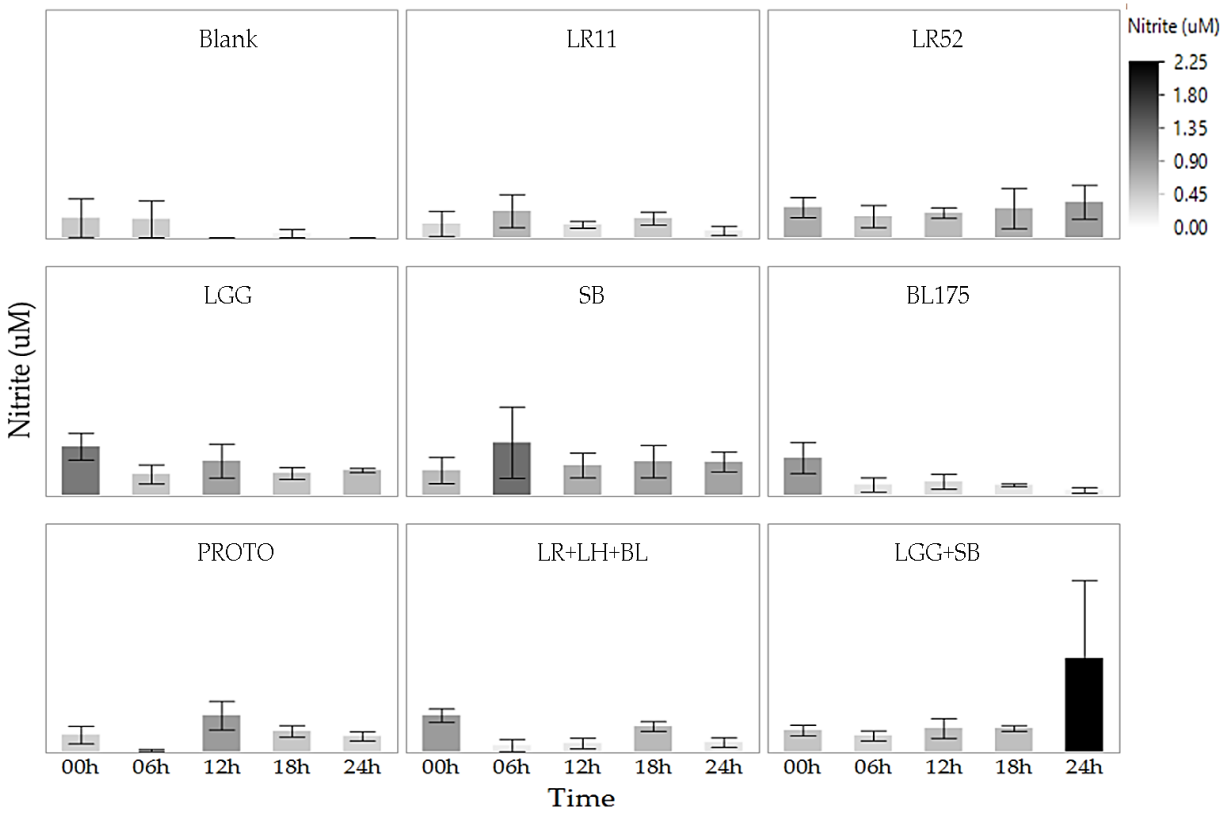

Figure S4.1. Nitrite determination in *C. difficile*-infected FW.

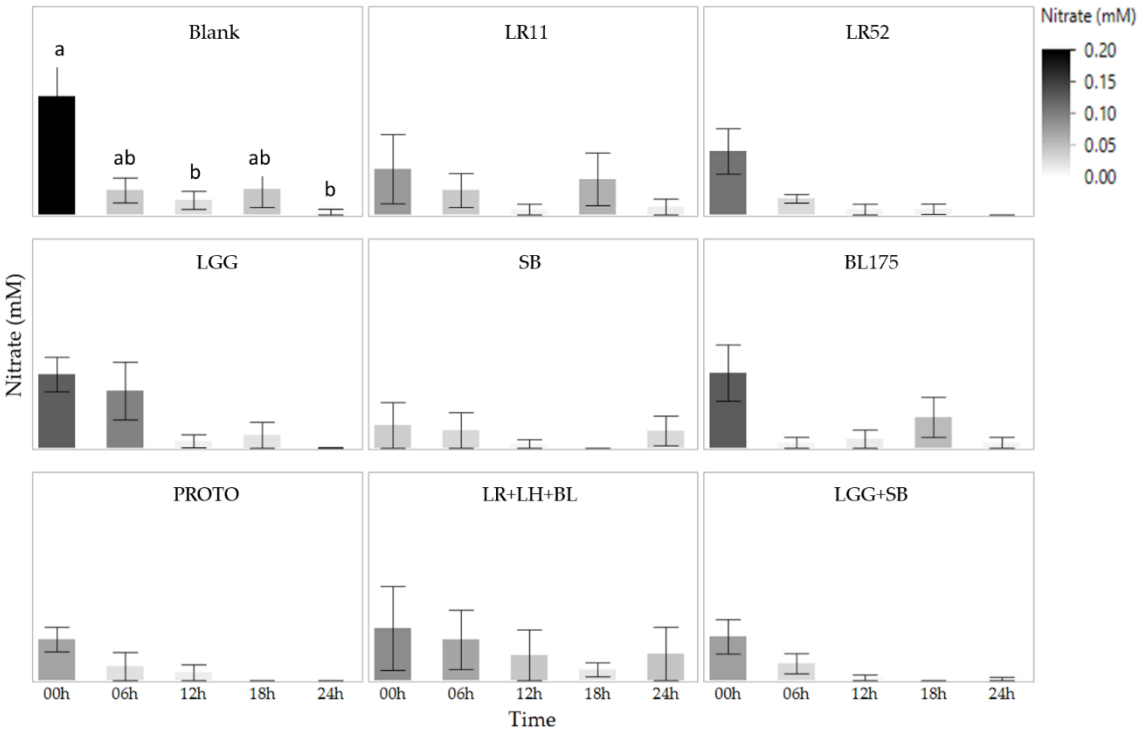

Figure S4.2. Nitrate determination in *C. difficile*-infected FW.

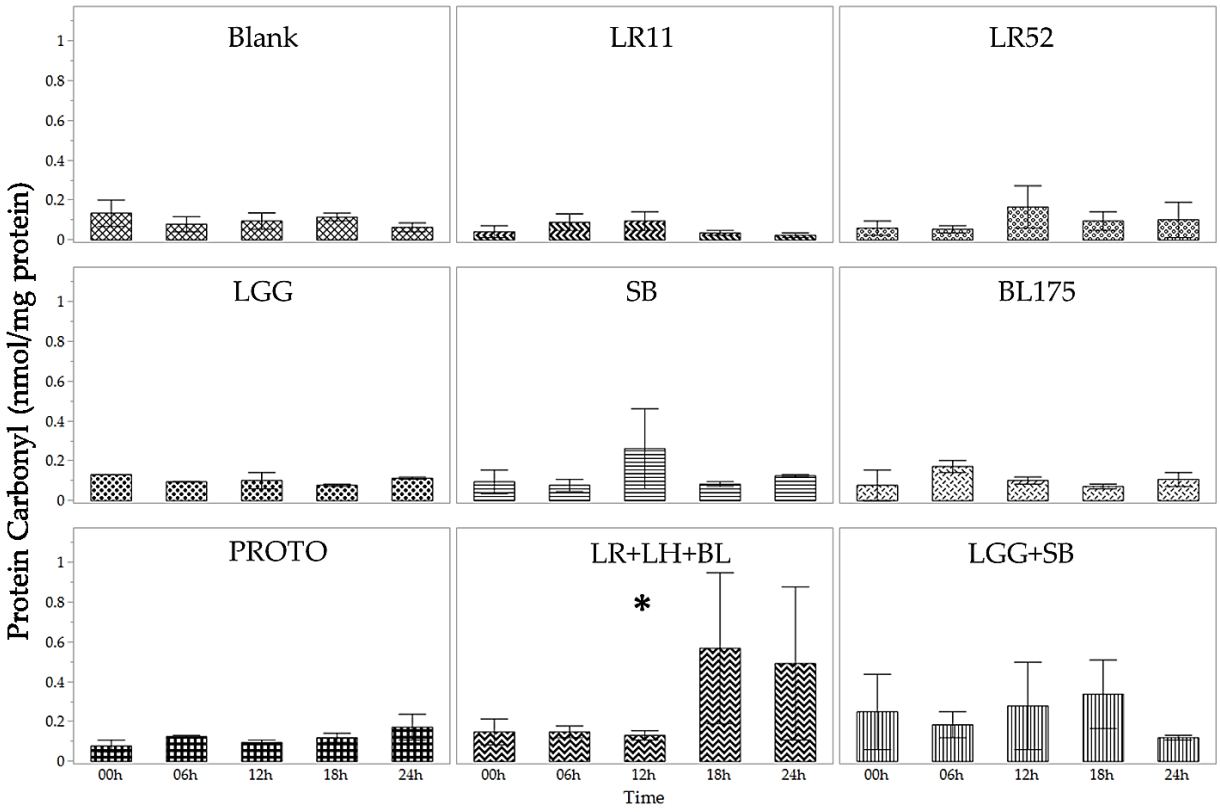

Figure S4.3. Protein carbonyl assessment of *C. difficile*-infected FW. The symbol * represents significant differences between treatments when compared to blank.

CONNECTING STATEMENT III

The findings from Chapter 4 showed dysregulation of the antioxidant status in fecal water mediated by *C. difficile* infection. This dysregulation was indicated by lower FRAP antioxidant activity and the absence of DPPH radical inhibition. Probiotic supplementation showed an ability to counteract this dysregulation by showing a significant ($p < 0.05$) increase in ferric-reducing ability, which was positively correlated with their ability to chelate copper. Further investigation into possible end-products of oxidative stress in the colonic milieu suggested that this dysregulation could be mediated through other metabolic markers, such as short-chain fatty acids, or through changes in the gut microflora, both of which play a key role in regulating colonic redox status and intestinal homeostasis. Thus, in Chapter 5, we assessed the ability of *C. difficile* infection to cause changes in the gut microflora and its metabolic function. Chapter 5 was accepted for publication in Microorganisms: Gaisawat M.B., MacPherson C.W., Julien T., Iskandar M.M., Tompkins, T.A., Kubow S.; *Probiotic Supplementation in a Clostridium difficile-Infected Gastrointestinal Model Is Associated with Restoring Metabolic Function of Microbiota*.

CHAPTER 5: RESEARCH PAPER 3

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Probiotic Supplementation in a *Clostridium difficile*-Infected Gastrointestinal Model Is Associated with Restoring Metabolic Function of Microbiota

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5.1 ABSTRACT

Clostridium (*C.*) *difficile* infection, a nosocomial gastrointestinal disorder, is of growing concern due to its rapid rise in recent years. Antibiotic therapy of CDI is associated with disrupted metabolic function and altered gut microbiota. The use of probiotics as an adjunct is being studied extensively due to their potential to modulate metabolic functions and the gut microbiota. In the present study, we assessed the ability of several single strain probiotics and a probiotic mixture to change the metabolic functions of normal and *C. difficile*-infected fecal samples. The production of short-chain fatty acids (SCFAs), hydrogen sulfide (H_2S), and ammonia was measured, and changes in microbial composition were assessed by 16S rRNA gene amplicon sequencing. The *C. difficile* infection in fecal samples resulted in a significant decrease ($p < 0.05$) in SCFA and H_2S production, with a lower microbial alpha diversity. All probiotic treatments were associated with significantly increased ($p < 0.05$) levels of SCFAs and restored H_2S levels. Probiotics showed no effect on microbial composition of either normal or *C. difficile*-infected fecal samples. These findings indicate that probiotics may be useful to improve the metabolic dysregulation associated with *C. difficile* infection.

5.2 INTRODUCTION

Clostridium (*C.*) *difficile* infection (CDI) is a toxin-mediated gastrointestinal (GI) disorder that is the leading cause of nosocomial infections [1]. CDI usually manifests as diarrhea, and in more severe cases, colonic inflammatory lesions and pseudomembrane formation [2]. An important factor in the pathogenesis of CDI is the presence of an altered gut microbial profile, which is strongly associated with antimicrobial therapy [3]. The GI concentrations of commensal microbes are decreased during CDI, which was shown to alter the colonic fermentative production of short chain fatty acids (SCFAs) [4,5]. Such metabolic disturbances could be important since SCFAs possess antimicrobial action, are critical in regulating immune function, and, in the case of butyrate, maintain intestinal cellular function and serve as a source of energy for colonic mucosal cells [4,5]. Furthermore, *C. difficile*-produced toxins A and B have demonstrated the potential to upregulate inflammatory pathways and induce cellular damage [6–8]. Probiotics have been considered as a therapeutic strategy to reduce the side effects of antibiotic therapy, counteract *C. difficile* growth, and reduce CDI-associated diarrhea [9,10].

Probiotics, defined as live microorganisms that impart beneficial effects on the host when given in adequate quantities [11], have shown beneficial effects in the GI tract such as improving metabolic function [12–15], counteracting infections [16–18], regulating immune function, decreasing GI disorder symptoms [16,19–21], and potentially lowering the risk of developing colon cancer [22]. Most of the probiotics utilized to date are usually from the *Lactobacilli*, *Bifidobacteria*, and yeast (*Saccharomyces*) groups. The efficacy and proposed mechanisms of action of these microbes in regulating intestinal microbiota functions are generally strain-specific. Some probiotic strains are thought to produce antimicrobial metabolites such as bacteriocins, to lower the pH by generating hydrogen peroxide and SCFAs, or to restrict pathogenic growth by competing for essential nutrients and adherence onto the gut mucosal barrier [18,23–27]. Several probiotics may reduce CDI-associated diarrhea and prevent primary CDI formation using some of the abovementioned mechanisms, but perhaps predominantly by

inhibiting the adhesion of *C. difficile* in the intestine [28,29]. In the case of *Saccharomyces (S.) boulardii*, the mechanism was shown to involve the proteolytic hydrolysis of the CD enterotoxins A and B [30]. Although the *Lactobacillus (L.) rhamnosus* GG strain and *S. boulardii* have been studied the most in the context of CDI-associated diarrhea [9,31], several other strains such as *Bifidobacterium (B.) longum* and *L. acidophilus* CL1285 have also shown efficacy against antibiotic-associated diarrhea [32–35]. Furthermore, strains such as *L. plantarum 299v* have been shown to enhance microbial function in CDI patients receiving antibiotic treatment by increasing butyrate and total SCFA production [13].

Despite the potential benefits of some probiotics in the management of CDI, much remains to be elucidated concerning their ability to combat *C. difficile* infection and its associated changes to the gut microbiota. In this study, we assessed the effects of several probiotic strains, individually or in combination, on CDI microbiota in an in vitro gut model system. In vitro GI models have been validated for the simulation of gut microbiota and its associated metabolic functions such as production of SCFAs and gaseous by-products such as ammonium (NH_4) and hydrogen sulfide (H_2S) [36,37]. Several studies have shown disruption in the metabolic capacity of gut microbiota within several gastrointestinal disorders and following exposure to certain medications such as antibiotics [38]. Moreover, microbial alterations can lead to proliferation of certain bacterial groups such as sulfate-reducing bacteria, leading to a dysregulation of the metabolic capacity and abnormal levels of NH_4 and H_2S. Altered production of these latter gases and SCFAs has been implicated in several gastrointestinal complications such as disrupted metabolism of intestinal cells [39], and disease states such as inflammatory bowel disorders and colorectal cancer [40]. In that regard, probiotic supplementation has shown the capacity to enhance production of SCFAs [12,41] and to help in restoring overall metabolic capacity through regulation of the microbiota [25,42]. Furthermore, such models have been previously utilized by our research group to study the effect of digestion on biotransformation of polyphenols and anthocyanins along with their effects on SCFA production and metabolite toxicity on intestinal cells [43,44]. In the context of CDI, GI models have been utilized to study the efficacy of various antibiotics and their effect on

C. difficile toxicity and commensal microbial communities [45]. The objective of the present study was to assess the changes in the metabolic function and microbial composition following *C. difficile* infection and determine whether probiotics could alleviate or minimize these changes. Individual strains *L. rhamnosus* R0011, *L. helveticus* R0052, *S. boulardii* CNCM I-1079, and *B. longum* R0175, along with a combination product, ProtecFlor^{TM} were studied for their efficacy. The study was conducted using an in vitro simulated GI model with *C. difficile*-infected fecal matter positive for both enterotoxins A and B. Metabolic function was assessed by quantification of microbial metabolites such as SCFAs, H_2S, and NH_4 [46]. Gut microbiota community structure of the fecal material from the fermentation experiments was assessed using 16S rRNA gene amplicon sequencing. Furthermore, detection of probiotic treatments was done using real-time polymerase chain reaction (qPCR) across time points to detect strain survivability.

5.3 Materials and Methods

5.3.1 Simulation of Gastrointestinal Conditions

This study utilized batch culture fermentation to simulate GI conditions as described previously [47]. Briefly, a dynamic computer-controlled model that houses 250 mL fermentation vessels was used to control for physiological colonic conditions such as pH, temperature, and an anaerobic environment. Each vessel was maintained at 37 °C using heated double-jacketed beakers and purged with oxygen-free nitrogen gas to maintain anerobic conditions. The pH was regulated continuously using an embedded EZO™ pH circuit (Atlas Scientific, Long Island City, NY, USA) controlled through a Raspberry Pi microprocessor (ver. 1B, Raspberry Pi Foundation, Cambridge, UK.

5.3.1.1 Fecal Slurry Preparation

Fecal slurry was prepared as previously described [47]. Briefly, normal samples were obtained from a healthy adult male donor with no previous history of GI disorders and no antibiotic use

within the past 6 months or more. Samples were diluted in 0.9% saline (1:3 *w*/*v*) and filtered using Whirl-Pak™ sterile filter bags (B01348WA, Thermo Fisher Scientific, Pittsburgh, PA, USA) followed by storage at −80 °C in a cryoprotectant solution (12.5% glycerol in 0.9% saline (*v*/*v*)) at a ratio of 1:3 *v*/*v*. *C. difficile* fecal samples were commercially sourced from BioIVT, Westbury, NY, USA (adult male; stool positive for enterotoxins A and B) and were processed in a similar manner. Regular fecal slurry was prepared by overnight stabilization of sample at 37 °C under anaerobic conditions. *C. difficile*-infected fecal slurry was prepared by adding *C. difficile* fecal slurry into the regular fecal slurry at a ratio of 1:10 *v*/*v* (5 mL of slurry per vessel).

5.3.1.2 Probiotic Treatment Preparation

Four commercial single strain probiotic treatments and one multi-strain probiotic treatment were tested in this study. The single strain probiotics used were *L. rhamnosus* R0011 (R0011), *L. helveticus* R0052 (R0052), *S. boulardii* CNCM I-1079 (SB), and *B. longum* R0175 (R0175). ProtecFlor™ (PROTO), a commercially available combination of R0011, R0052, R0175, and SB was used as the multi-strain probiotic. Probiotics were acquired from Lallemand Health Solutions Inc. (Montreal, QC, Canada) and stored at −20 °C until use. For inoculation in the fermentation vessel, each treatment was prepared by mixing the probiotic in sterile 1 x PBS and added at a dose of 1 billion cfu/vessel. Two treatment controls were used in this study: maltodextrin, the carrier base of the probiotic, was dissolved in 1 x PBS and used as vehicle control (hereinafter referred to as Vehicle), and 1 x PBS was used as the negative control (Blank).

5.3.1.3 Batch Culture Fermentation

For batch culture fermentation, a modified method of Tzounis et al. (2008) [48] was used. Briefly, 100 mL of GI food, previously optimized by Molly et al. (1994) [49] (composed of 1 g/L of arabinogalactan, 2 g/L of pectin, 1 g/L of xylan, 3 g/L of starch, 0.4 g/L of glucose, 3 g/L of yeast extract, 1 g/L of peptone, 4 g/L of mucin, 0.5 g/L of cysteine, and 40 µL/L of vitamin solution; Sigma Aldrich, St. Louis, MO, USA) was added to each vessel. This was followed by a sequential

enzymatic digestion in each vessel. Oral digestion was simulated by the addition of α-amylase (A3176, Sigma Aldrich, St. Louis, MO, USA) at pH 7.0 for 15 min, followed by stomach digestion by the addition of pepsin (P7125, Sigma Aldrich, St. Louis, MO, USA) at pH 2.0 for a period of 1.5 h, and pancreatic digestion by the addition of pancreatic juice (12 g/L $NaHCO_3$, 6 g/L bile extract, and 0.9 g/L pancreatin; Sigma Aldrich, St. Louis, MO, USA) at pH 8.0 for 2 h.

After completion of enzymatic digestion, each vessel was inoculated with 50 mL of prepared regular or *C. difficile* infected fecal slurry (T = 0 h). Premixed probiotic treatment or blank (1 x PBS) was added to each vessel and fermentation was carried out under anerobic conditions with pH regulated at 6.3 ± 0.3 for a 24 h period with sampling after every 6 h. Samples were centrifuged at 2000× *g* for 10 min. The supernatant was filtered using sterile 0.45 µm syringe filters into new sample vials for metabolite analysis (hereinafter referred to as fecal water, FW). The fecal pellet was used for 16S rRNA gene amplicon community profiling and was stored at −80 °C until extraction. Each treatment (*n* = 7) was run in triplicate for both regular fecal slurry and *C. difficile*-infected fecal slurry batch culture fermentations.

5.3.2 FW Metabolite Analysis

5.3.2.1 Short Chain Fatty Acids (SCFA) Analysis

SCFA analysis was conducted by a gas chromatograph system equipped with a flame ionization detector (GC-FID) (6890A series, Agilent Technologies, Santa Clara, CA, USA) using an adapted method outlined by Ekbatan et al. (2016) [43]. Briefly, 1 µL of 0.45 µm syringe filtered FW samples were directly injected into the GC-FID equipped with a fused capillary column (30 m × 250 µm ID × 0.25 µm film thickness; HP-INNOWAS, Agilent Technologies, Santa Clara, CA, USA). Helium was used as the carrier gas (1 mL/min). Inlet and detector temperatures were set at 220 and 230°C, respectively. For SCFA separation, the oven temperature was set at 100°C, held for 2 min followed by an increase of 10 °C/min until 220 °C where it was held for 1 min. Identification and quantification of individual SCFAs (acetate, propionate, butyrate, iso-butyrate, valeric acid, iso-

valeric acid, caproic acid, iso-caproic acid, and heptanoic acid) was done using a free-volatile fatty acid standard (46975-U, Sigma Aldrich, St. Louis, MO, USA) and values were reported in mM as total SCFA, and as a combination of acetate, propionate, butyrate, and the remainder SCFA. Samples were analyzed in duplicate from each fermentation experiment.

5.3.2.2 Hydrogen Sulfide (H_2S) Determination Assay

Colorimetric determination of dissolved H_2S in FW samples was conducted according to the zinc acetate precipitation method proposed by Gilboa-Garber (1971) [50]. Briefly, 0.5 mL of alkaline zinc acetate (2.6% *w*/*v* of zinc acetate and 6% *v*/*v* of sodium hydroxide mixed in a ratio of 5:1; Sigma-Aldrich, St. Louis, MO, USA) was added to 0.7 mL of FW sample. The mixture was centrifuged at 3000 *g* for 10 min to allow for precipitation of the zinc sulfide complex. After decanting the supernatant, the pellet was washed with 1.5 M sodium chloride (pH 8.0) and distilled water (pH 8.0). The pellet was then resuspended in 0.7 mL distilled water and vortexed, followed by the addition of 0.25 mL of *N, N*-dimethyl-*p*-phenylenediamine monohydrochloride (0.1% *w*/*v* in 5.5 N HCl; D5004, Sigma-Aldrich, St. Louis, MO, USA), and 0.1 mL of ferric chloride reagent (11.5 mM ferric chloride prepared with 0.6 N HCl; 157740, Sigma-Aldrich, St. Louis, MO, USA). The tubes were incubated at room temperature for 30 min for color formation to occur. An aliquot of 200 µL of solution was transferred into a 96-well microplate and absorbance was read at λ = 670 nm using a uQuant microplate reader (BioTek Instruments, Winooski, VT, USA). Seven equally distributed serial dilutions of 100 µM sodium sulfide were used to generate the analytical standard curve (R^2 = 0.99). All samples were analyzed in triplicate from each independent fermentation experiment.

5.3.2.3 Ammonium (NH_4) Determination Assay

Colorimetric determination of NH_4 was done using a microplate adapted method of the procedure outlined by Koroleff (1976) [51]. The procedure is based on the indophenol blue color formation when ammonium reacts with phenate in an alkaline solution in the presence of a

strong oxidizing agent such as hypochlorite, and a metal-containing catalyst such as sodium nitroferricyanide (nitroprusside). Briefly, in a 96-well plate, 50 µL of FW sample or standard was added. This was followed by 25 µL of citrate reagent (0.2 M trisodium citrate in 0.5 M sodium hydroxide; 1110371000, Sigma Aldrich, St. Louis, MO, USA), 30 µL of salicylate-nitroprusside reagent (0.05 M sodium salicylate in 0.05 mM sodium nitroprusside; S3007 and 1614501, Sigma Aldrich, St. Louis, MO, USA), and 25 µL of hypochlorite reagent (10:2:1 *v*/*v*/*w* of household bleach, sodium hydroxide, and trisodium phosphate at pH 13.0). An aliquot of 145 µL distilled water was finally added to a total volume of 275 µL per well. The microplate was incubated at room temperature on a plate shaker for 30 min for complete color development. Absorbance was read at λ = 650 nm. An analytical standard curve (R^2 = 0.99) was prepared using seven equally distributed serial dilutions of 36 mM ammonium sulphate (oven dried at 105 °C; A4418, Sigma Aldrich, St. Louis, MO, USA). Samples from each independent fermentation experiments were performed in triplicate.

5.3.3 DNA Extraction and Analysis

5.3.3.1 DNA Extraction

Extraction of fecal DNA was done using the QIAamp® Fast DNA Stool Mini Kit (51604, Qiagen, Hilden, Germany) as per the manufacturer's instructions. Prior to extraction, 300 to 500 mg of fecal pellet from each run were washed with 1 mL of 0.05 M phosphate buffer upon which InhibitEX (from the kit) and 0.1 mm zirconia beads (~300 mg/tube; 360991112, Thermo Fisher Scientific, Pittsburgh, PA, USA) were added. The sample tubes were then homogenized using a bead-beater (3 cycles of 4 m/s for 1 min; MP FastPrep®-24, MP Biomedicals, Irvine, CA, USA) followed by centrifugation at 13,000 rpm in a microcentrifuge for 3 min. DNA extraction was then carried out as per procedure outlined in the kit. Purity of extracted DNA was assessed by 260/280 ratios (absorbance at λ = 260 nm/280 nm) using NanoDrop™ One (Thermo Fisher Scientific, Pittsburgh, PA, USA). All DNA samples had ratios between 1.6–2.0. Samples were diluted in

molecular-grade water to attain final concentrations of 20 ng/µL and stored at −20 °C prior to 16S rRNA gene amplicon sequencing.

5.3.3.2 Detection of Probiotic Strains by Real-Time Polymerase Chain Reaction (qPCR)

Detection of individual probiotic strains, R0011, R0052, and R0175 was conducted in all extracted fecal DNA samples by real-time PCR (qPCR) once diluted five-fold in PCR-grade water. Strain-specific forward and reverse primers for R0011, R0052, and R0175 were obtained from Lallemand Health Solutions Inc. (Montréal, QC, Canada) and stored at −20 °C until use (Table 1). The qPCR assay specifications followed MIQE guidelines [52]. Each reaction consisted of 1X SYBR Select Master Mix (4472908, Applied Biosystems, Waltham, Massachusetts, USA), 300 nM of the respective forward and reverse primer, and 1 µL of template DNA. The 384-well qPCR assay plates were prepared by the epMotion 5075tc liquid handling robot (Eppendorf, Hamburg, Germany) by adding 9 µL of Mastermix and 1 µL of DNA to each well. Positive control DNA was extracted from pure overnight cultures of R0011, R0052, and R0175.

Table 5.1. Primer and target sequences for qPCR detection.

Bacterial Strain	Primer Name	Targeted Sequence	Gene Target	Amplicon size (bp)
B. longum R0175	R175_AP_HP10_F	GTC GCC ACA TTT CAT CGC AA	Hypothetical protein	99
	R175_AP_HP10_R	GAG AGC TTC GAT TGG CGA AC		
L. helveticus R0052	pIR52-1-orf5 F1	AGA ATC AAG CAG AGA CTG GCT ACG	An ORF in a plasmid specific to R0052	150
	pIR52-1-orf5 R1	GGA CCG GAT TTG AGT AGA GGT A		
L. rhamnosus R0011	113A29_293FL	ACT CCA AAG AGC ATT ACC TCC G	113A29 phage head protein	71
	113A29_321RU	TGA ATA TGC CGG ATC TAA GTC CA		

The following cycling conditions for each primer set (Table 1) were completed using the CFX384 Touch Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, United States): 50 °C for 2 min, followed by 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. A dissociation curve to ensure amplicon specificity was performed from 65 to 95 °C following the 40 cycles. The CFX Maestro™ software (version 1.1, Bio-Rad Laboratories, Hercules, CA, USA) was used to perform the data analysis.

5.3.3.3 16S rRNA Gene Amplicon Sequencing and Bioinformatics

Characterization of microbial communities was performed by 16S rRNA gene amplicon sequencing as previously described [53]. Briefly, extracted DNA was used to construct sequencing libraries according to Illumina's "16 S Metagenomic Sequencing Library Preparation" guide (Part

15044223 Rev. B), with the exception of using Qiagen HotStar MasterMix for the first PCR ("amplicon PCR") and halving reagent volumes for the second PCR ("index PCR"). The template specific primers were (without the overhang adapter sequence) the following: forward (5'-CCTACGGGNGGCWGCAG-3') and reverse (5'- GACTACHVGGGTATCTAATCC-3'), targeting the V3-V4 hypervariable region [54] specific to bacterial organisms and generating a fragment of around 460 bp. The first PCR ("amplicon PCR") was carried out for 25 cycles with annealing temperatures of 55 °C. Diluted pooled samples were loaded on an Illumina MiSeq system and sequenced using a 500-cycle (paired-end sequencing configuration of 2x250 bp) MiSeq Reagent Kit v3.

Sequencing data was analyzed using AmpliconTagger, the National Research Council of Canada's amplicon pipeline [55]. Briefly, raw reads were scanned for sequencing adapters and PhiX spike-in sequences and remaining reads were merged using their common overlapping part with FLASH [56]. Primer sequences were removed from merged sequences and remaining sequences were filtered based on quality (Phred) score. Remaining sequences were clustered at 100% identity and then clustered/denoised at 99% identity (Vsearch v2.7.1, [57]). Clusters having abundances lower than three were discarded. Remaining clusters were scanned for chimeras with VSEARCH's version of UCHIME denovo and UCHIME reference [57,58] and clustered at 97% (VSEARCH) to form the final clusters/operational taxonomic units (OTUs). A global read count summary is provided in Supplementary Table S1. OTUs were assigned a taxonomic lineage with the RDP classifier [59] using an in-house training set containing the complete Silva release 128 database [60] supplemented with eukaryotic sequences from the Silva database and a customized set of mitochondria, plasmid, and bacterial 16S sequences. The RDP classifier gave a score (0 to 1) to each taxonomic depth of each OTU. Each taxonomic depth having a score ≥0.5were kept to reconstruct the final lineage. Taxonomic lineages were combined with the cluster abundance matrix obtained above to generate a raw OTU table, from which a bacterial organisms OTU table was generated. Five hundred 1000 reads rarefactions were then performed on this latter OTU table and the average number of reads of each OTU of each sample was computed to obtain a consensus rarefied OTU table (available in Supplementary Table S2). A multiple sequence

alignment was obtained by aligning OTU sequences on a Greengenes core reference alignment [61] using the PyNAST v1.2.2 aligner [62]. Alignments were filtered to keep only the hypervariable region of the alignment. A phylogenetic tree was built from that alignment with FastTree v2.1.10 [63]. Alpha (Shannon index) and beta (weighted UniFrac distances) diversity metrics and taxonomic summaries were then computed using the QIIME v1.9.1 software suite [62,64] using the consensus rarefied OTU table and phylogenetic tree (i.e., for UniFrac distance matrix generation).

5.3.4 Statistical Analysis

All data are reported as means ± standard error of mean (SEM). Data for SCFA, H_2S, and NH_4 were analyzed using two-way ANOVA using probiotic treatment (7 levels) and time (5 levels) as factors. For multiple comparisons, Dunnett's post hoc test was carried out to compare treatments to control (blank). The means of all time points were jointly considered when no significant interactions in the two-way ANOVA were observed. When significant interactions between time and treatment were observed, the mean of each time point within a treatment was individually compared to its corresponding time point within the control along with Tukey's HSD post analysis to assess for significant differences within treatment. Statistical significance was set at $p < 0.05$. All two-way ANOVA and post-hoc statistical analyses, and visualizations for metabolite data were performed using JMP v14.2 (SAS Institute, Cary, NC, USA). PERMANOVA analyses were done with R (v3.6.0) using the adonis2 function of the Vegan (v2.5-4) package. Taxonomic profiles, alpha- and beta-diversity plots were generated with R (ggplot2 v3.1.1).

5.3.5 Availability of Data

Raw sequence reads of the 16S rRNA gene amplicon data were submitted to the sequence read archive under Bio Project PRJNA565012.

5.4 RESULTS

5.4.1 SCFA Determination in FW

Two-way ANOVA results for total SFCAs only showed a significant ($p < 0.05$) main effect of time for normal FW whereby time 0 h was significantly lower than time 12, 18, and 24 h. Supplementation with probiotics did not change the total SCFA levels in normal FW. The levels of acetate and butyrate, however, differed significantly ($p < 0.05$) amongst the probiotic treatments when compared to blank. The vehicle, R0052, and R0175 treatments showed significantly lower ($p < 0.05$) production of acetate whereas R0175 showed significantly higher ($p < 0.05$) production of butyrate (**Figure 5.1**).

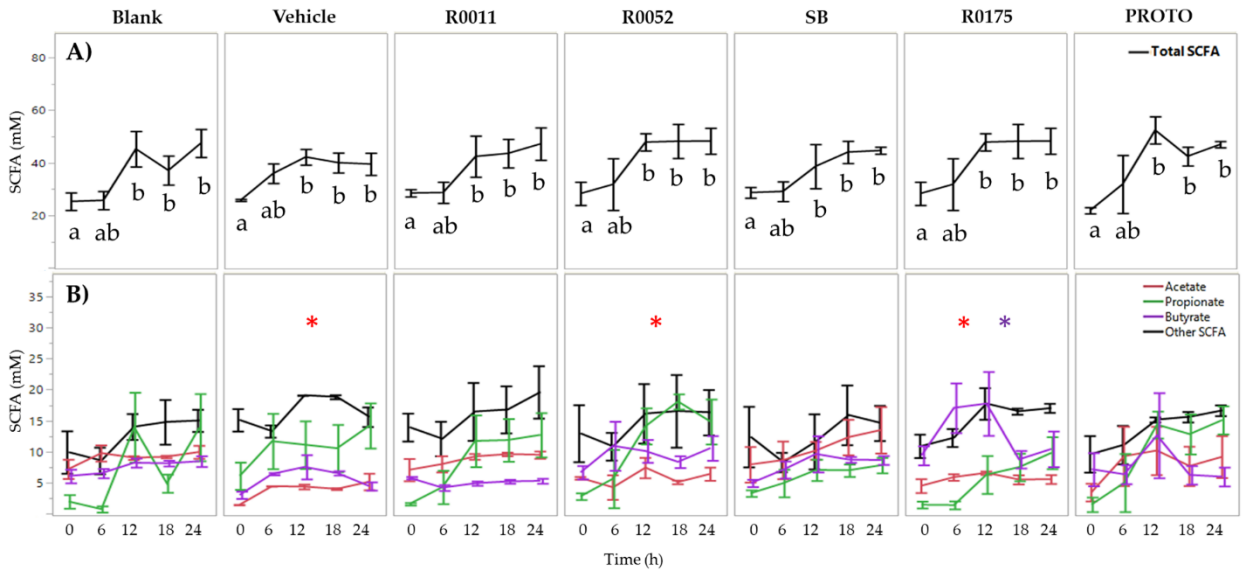

Figure 5.1. Short-chain fatty acid (SCFA) analysis of normal FW. (A) Total SCFA quantification and (B) individual SCFA quantification. Values are presented as the means ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * in red represents significant differences in acetate production between treatment and blank ($p < 0.05$) when the means of all time points are jointly considered. The symbol * in purple represents significant differences in butyrate production between treatment and blank ($p < 0.05$)

when the means of all time points are jointly considered. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor^{TM}.

In *C. difficile*-infected FW, two-way ANOVA results for total SCFA showed significant ($p < 0.05$) main effects of treatment, time, and an interaction effect of treatment and time. Therefore, the mean total SCFA for each time point within each treatment was compared to its corresponding time point of the blank. Each of the probiotic treatments showed a significant ($p < 0.05$) increase in total SCFAs. R0175 and PROTO showed a significant increase starting at time 6 h and at time 12 h. R0011 showed significantly ($p < 0.05$) higher total SCFAs at time 12 and 24 h whereas R0052 showed a significant ($p < 0.05$) increase at time 12, 18, and 24 h (**Figure 5.2**). The increase in total SCFA production for each of these probiotic treatments could be attributed to a significant ($p < 0.05$) increase in acetate production as compared to blank when the means of all time points were jointly considered. Furthermore, SB and R0175 showed a significantly ($p < 0.05$) higher butyrate production compared to the blank (**Figure 5.2**).

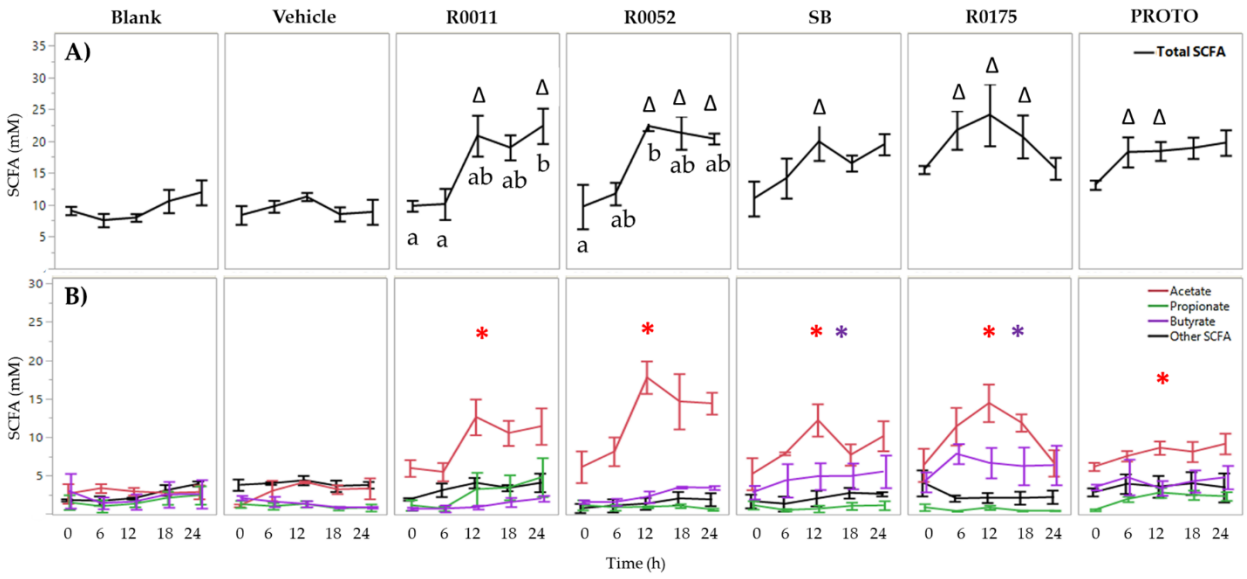

Figure 5.2. Short-chain fatty acid (SCFA) analysis of *Clostridium (C.) difficile*-infected normal fecal water. (A) Total SCFA quantification and (B) Individual SCFA quantification. Values are shown as mean ± SEM. The symbol Δ represents significant differences ($p < 0.05$) between treatment at a particular time point and blank at the corresponding time point. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * in red represents significant differences in acetate production between treatment and blank ($p < 0.05$) when the means of all time points are jointly considered. The symbol * in purple represents significant differences in butyrate production between treatment and blank ($p < 0.05$) when the means of all time points are jointly considered. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™.

In order to determine the ability of probiotic supplements to produce SCFAs in GI food, a batch culture experiment for a 24 h period was performed without the presence of fecal slurry. R0011, R0052, SB, and PROTO showed significantly ($p < 0.05$) higher total SCFA production when compared to Vehicle (**Figure S5.1**), whereas R0175 showed no significant effect. This observed increase in SCFAs was principally due to significantly ($p < 0.05$) higher acetate levels in R0011, SB,

and PROTO at 24 h. Moreover, significantly ($p < 0.05$) higher levels of butyrate were also observed in SB and PROTO cultures at 24 h (**Table S5.1**).

5.4.2 NH_4 and H_2S Determination in FW

Determination of NH_4 in FW showed no effect of probiotic supplementation in both normal FW and *C. difficile*-infected FW. Two-way ANOVA results for ammonium showed a significant ($p < 0.05$) main effect of time for both normal FW and *C. difficile*-infected FW. In normal FW, time 0 h was seen to be significantly ($p < 0.05$) lower than all the other time points (6 to 24 h) for Blank, R0011, R0052, and SB. Similarly, time 0 h was significantly ($p < 0.05$) lower than time 24 h in PROTO, and, lower than time 12 to 24 h in R0175. In *C. difficile*-infected FW, time 0 h was significantly ($p < 0.05$) lower than time 18 h and time 24 h in R0011, SB, R0175, and PROTO (**Figure 5.3**). However, despite the observed differences in ammonia production over time, no significant effect of treatment was observed in both normal FW and *C. difficile*-infected FW.

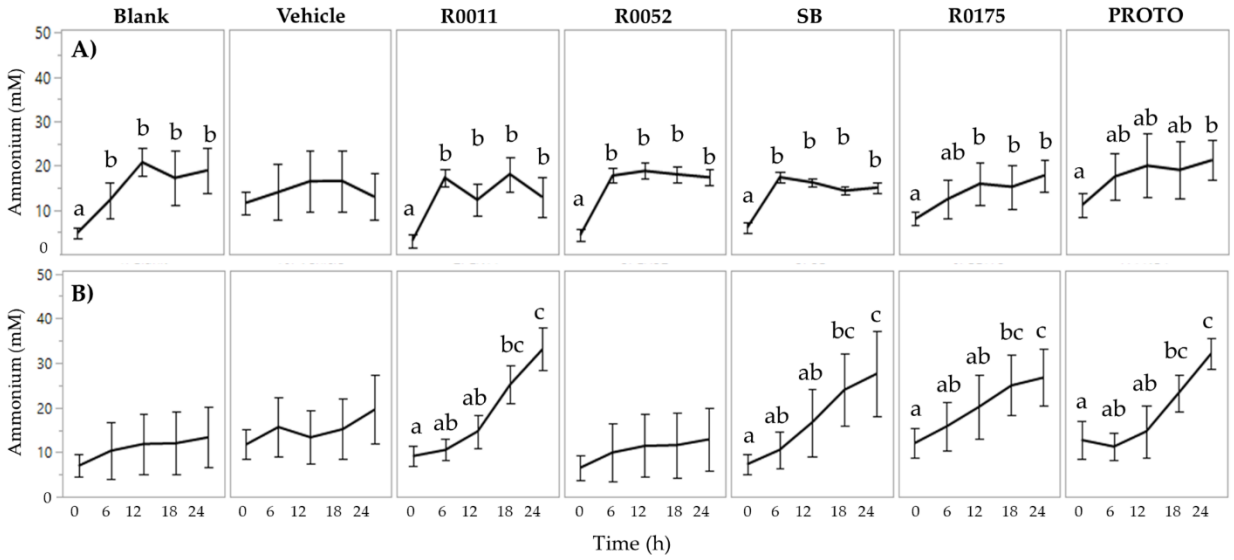

Figure 5.3. Determination of ammonium in fecal water (FW); (A) Normal FW and (B) *Clostridium* (*C.*) *difficile*-infected FW. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). R0011 = *L. rhamnosus*

R0011; R0052 = *L. helveticus* R0052; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™.

Hydrogen sulfide production in normal FW showed no significant main effects of treatment. In *C. difficile*-infected FW, however, two-way ANOVA results showed significant ($p < 0.05$) main effects for both time and treatment. Furthermore, H_2S levels were found to be lower in *C. difficile*-infected FW in comparison to normal FW, by 2.9-, 1.6-, 2.3-, 1.5-, and, 2.8-fold at times 0, 6, 12, 18, and 24 h, respectively. Supplementation with probiotics in *C. difficile*-infected FW resulted in a significant ($p < 0.05$) increase of H_2S production (**Figure 5.4**). Moreover, PROTO showed a significantly ($p < 0.05$) higher H_2S production at time 12 h compared to time 0 h in normal and *C. difficile*-infected FW.

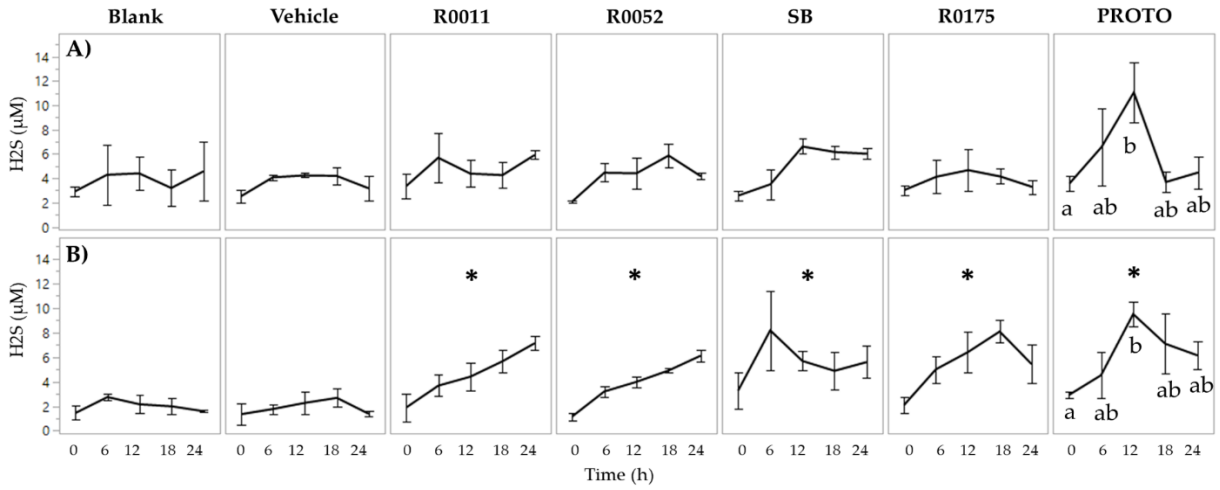

Figure 5.4. Determination of H_2S in fecal water (FW); (A) Normal FW and (B) *Clostridium* (*C.*) *difficile*-infected FW. Values are shown as mean ± SEM. The symbol * represents significant differences between treatment and blank ($p < 0.05$) when the means of all time points are jointly considered. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™.

5.4.3 Detection of Probiotic Strains by qPCR

Detection of the bacterial strains R0011, R0052, and R0175 was conducted across all the fecal samples. Positive detection was confirmed by comparing the amplicon melt curve to the positive control in samples with a threshold quantification cycle (Cq) value less than 30. The results from the qPCR detection show that the strains were positively detected in their respective samples in normal feces and *C. difficile*-infected feces across all time points of batch fermentation. As each of the individual bacterial strains are present in the PROTO probiotic mix, all the strains showed positive detection in samples from that treatment. Some false positive qPCR detections for R0052 at the 18 and 24 h time points were observed in one of the R0011 replicates, as well as for R0011 at the 0 h time point for one of the R0052 replicates. These false positive detections could be due to non-specific binding of the primers to similar sequences from other *Lactobacilli* in the microbiota (**Figure 5.5**), as reported previously when detecting *Bifidobacterium* strains [65].

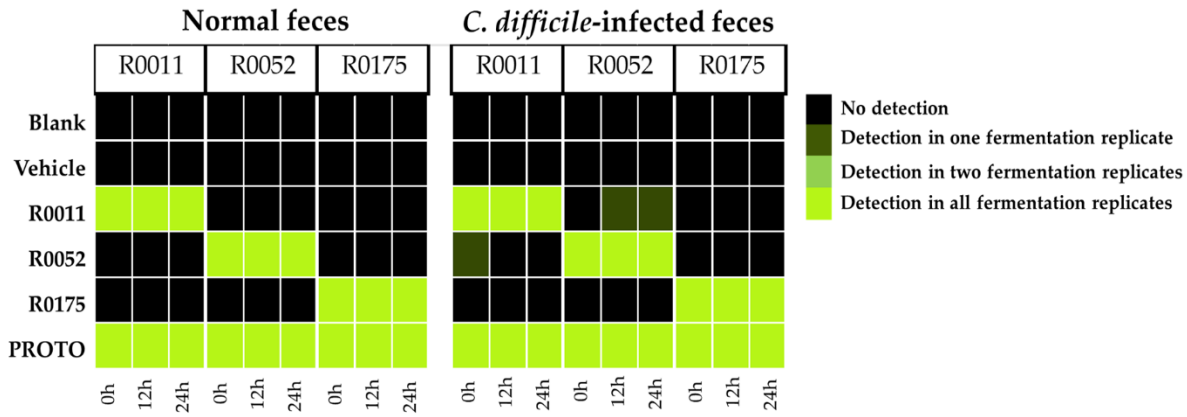

Figure 5.3. Detection of individual probiotic strains in extracted fecal DNA samples by real-time PCR (qPCR). (a) Detection of strain *B. longum* R0175; (b) Detection of strain *L. helveticus* R0052; (c) Detection of strain *L. rhamnosus* R0011. Each column of the corresponding time point represents an individual experiment along with corresponding quantification cycle (Cq) value. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0175 = *B. longum* R0175; PROTO = ProtecFlor™.

5.4.4. Microbial Community

16S rRNA gene amplicon sequencing was used to profile microbiota composition of fecal samples collected from batch culture fermentation at time 0, 12, and 24 h. Metrics such as alpha diversity (Shannon index), beta diversity (Weighted UniFrac) and relative abundance of observed species were used to characterize these microbial communities.

Two-way ANOVA analysis followed by matched pairs Student's t-test was conducted on Shannon index (alpha diversity) to assess for differences in microbial communities. To assess for differences between the fecal samples at time 0 h, pairwise comparisons showed that normal samples had an overall higher alpha diversity score as compared to *C. difficile*-infected samples, with all treatments except for PROTO showing a significant ($p < 0.05$) effect. The changes in microbial diversity within a given treatment was done by comparing the means of the time 12 and 24 h to the mean at time 0 h. The results for alpha diversity showed that in normal FW, there was a significant ($p < 0.05$) decrease in microbial species richness over time for all treatments. Blank, Vehicle, and R0175 showed a significant ($p < 0.05$) decrease starting at time 12 and 24 h, whereas R0011, R0052, PROTO, and SB showed a significant ($p < 0.05$) decrease only at time 24 h. In *C. difficile*-infected fecal samples, only PROTO showed a significant ($p < 0.05$) decrease at time 12 h (**Figure 5.6**). Differences in the microbial community richness (alpha diversity) between each treatment was done by comparing the values of a treatment at a particular time point to that of the blank at the corresponding time point. The results showed significant effects only in the *C. difficile*-infected fecal samples where Vehicle and R0011 showed significantly ($p < 0.05$) higher community richness at time 12 h, and PROTO showed a significantly ($p < 0.05$) higher diversity at time 12 and 24 h (**Figure 5.6**).

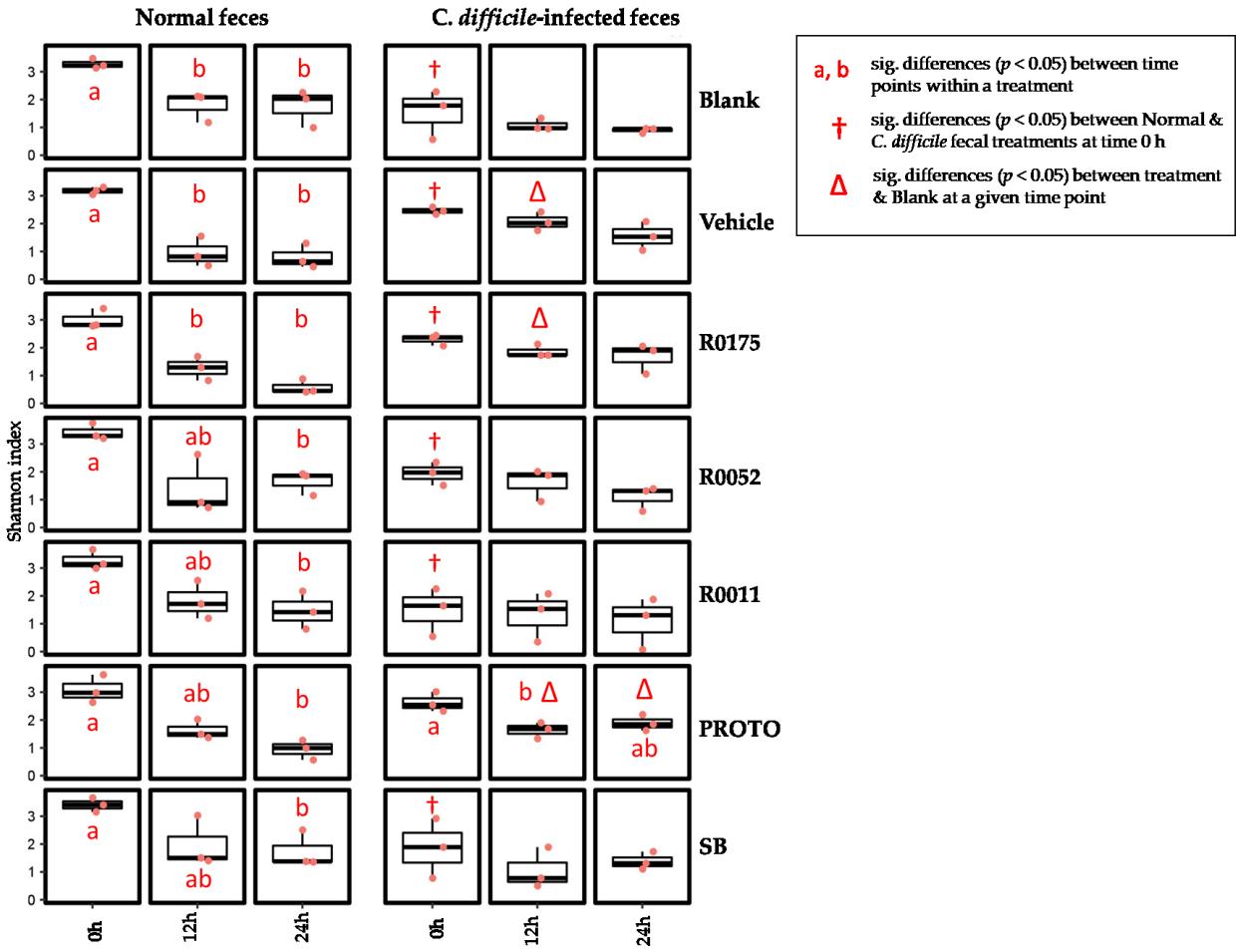

Figure 5.6. Microbial alpha diversity in normal and *Clostridium* (*C.*) *difficile*-infected feces assessed by the Shannon index. The symbol † represents significant (sig.) differences ($p < 0.05$) between feces for a particular treatment at time 0 h. The symbol Δ represents significant differences ($p < 0.05$) between treatment at a particular time point and blank at the corresponding time point. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™.

The results of the beta diversity showed that there were differences in the similarity of the microbial community structures over time. Samples of all treatments in both normal and *C. difficile*-infected feces showed an overall higher similarity of the microbiota at time 12 h when

compared to time 24 h, whereas the microbiota was relatively dissimilar at time 0 h when compared to time 24 h (**Figure 5.7**). This effect is less pronounced in samples of R0175, R0052, and R0011 when supplemented in *C. difficile*-infected feces, where there is less microbial community similarity when each of the time points were compared to each other.

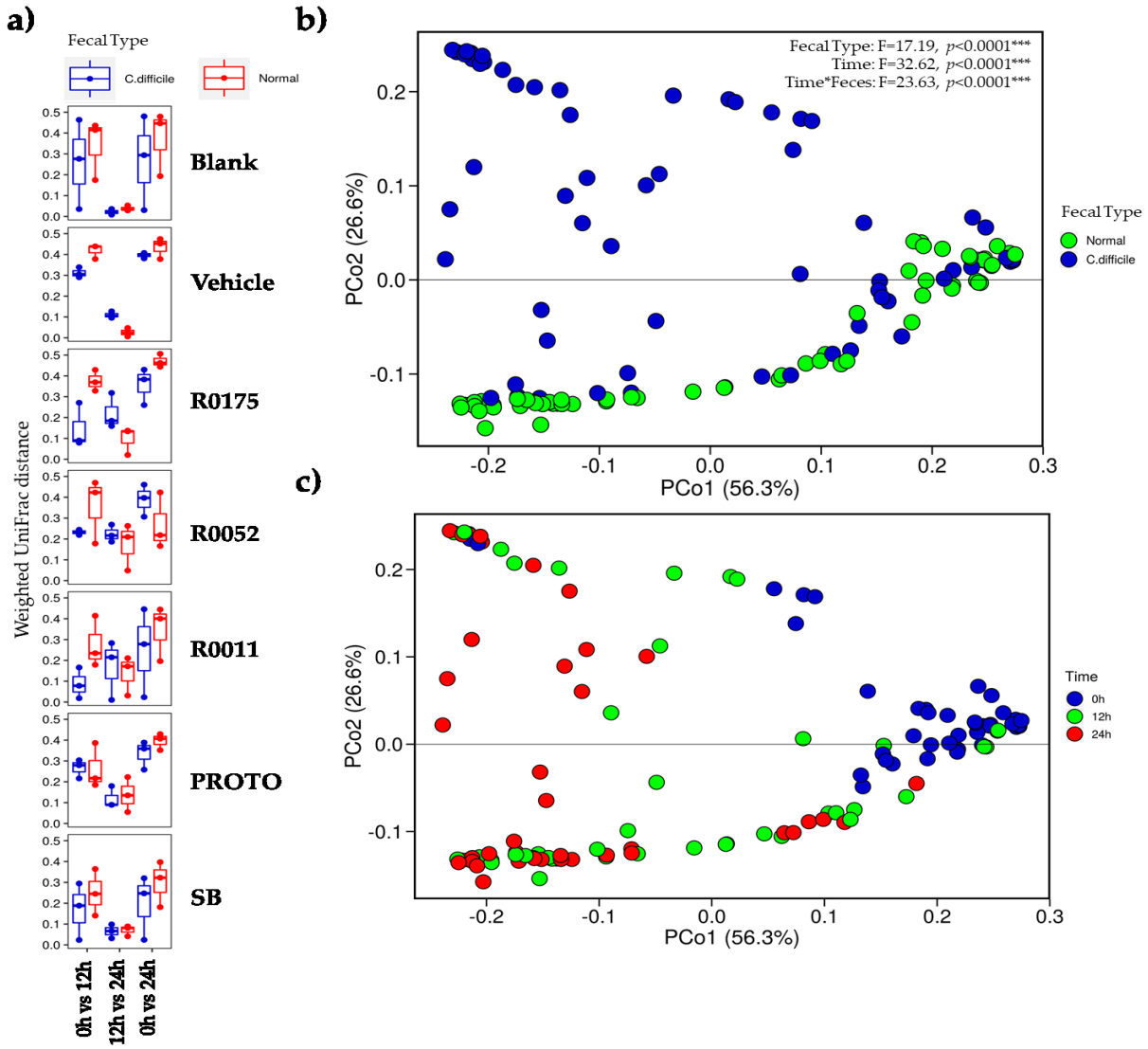

Figure 5.7. Beta diversity plots of normal fecal samples, and *Clostridium* (*C.*) *difficile*-infected fecal samples showing (**a**) Weighted UniFrac distance and PCA plots (**b**) clustered by type of feces, and (**c**) clustered by time. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™.

PERMANOVA analyses performed on weighted UniFrac distances (**Figure 5.7**) showed that samples cluster differed primarily by time and type of stool, indicating that these two variables are the main drivers in the formation of distinct communities. Interestingly, the normal fecal samples and *C. difficile*-infected fecal samples clustered together at time 0 h suggesting similar microbial community. The clustering of samples microbial communities was also observed in OTU heatmaps (**Supplementary Figures S5.2 and S5.3**), where the blanks of the respective fecal sample clustered at time 0 h.

Taxonomic profiles were generated to investigate microbial community structures across the experimental conditions. The relative abundance of the top 20 taxa down to the family level for both the fecal sample groups is shown in **Figure 5.8**. The results showed that the most prevalent taxa at the family level in normal fecal samples at time 0 h were of *Bifidobacteriaceae*, followed by *Lachnospiraceae* and *Coriobacteriaceae*. At time 12 and 24 h, however, the family *Veillonellaceae* becomes most abundant, followed by *Bifidobacteriaceae* for all treatments in normal fecal samples. In *C. difficile*-infected fecal samples at time 0 h, the taxa *Bifidobacteriaceae* and *Lactobacillaceae* were the most predominant, followed by *Peptostreptococcaceae* and *Coriobacteriaceae*. At time 12 and 24 h, *Lactobacillaceae* still show a high abundance, followed by an increase in abundance of *Veillonellaceae* and a decrease in abundance of *Bifidobacteriaceae*. Interestingly, the probiotic treatments were observed to have a higher proportion of *Bifidobacteriaceae* at time 12 and 24 h.

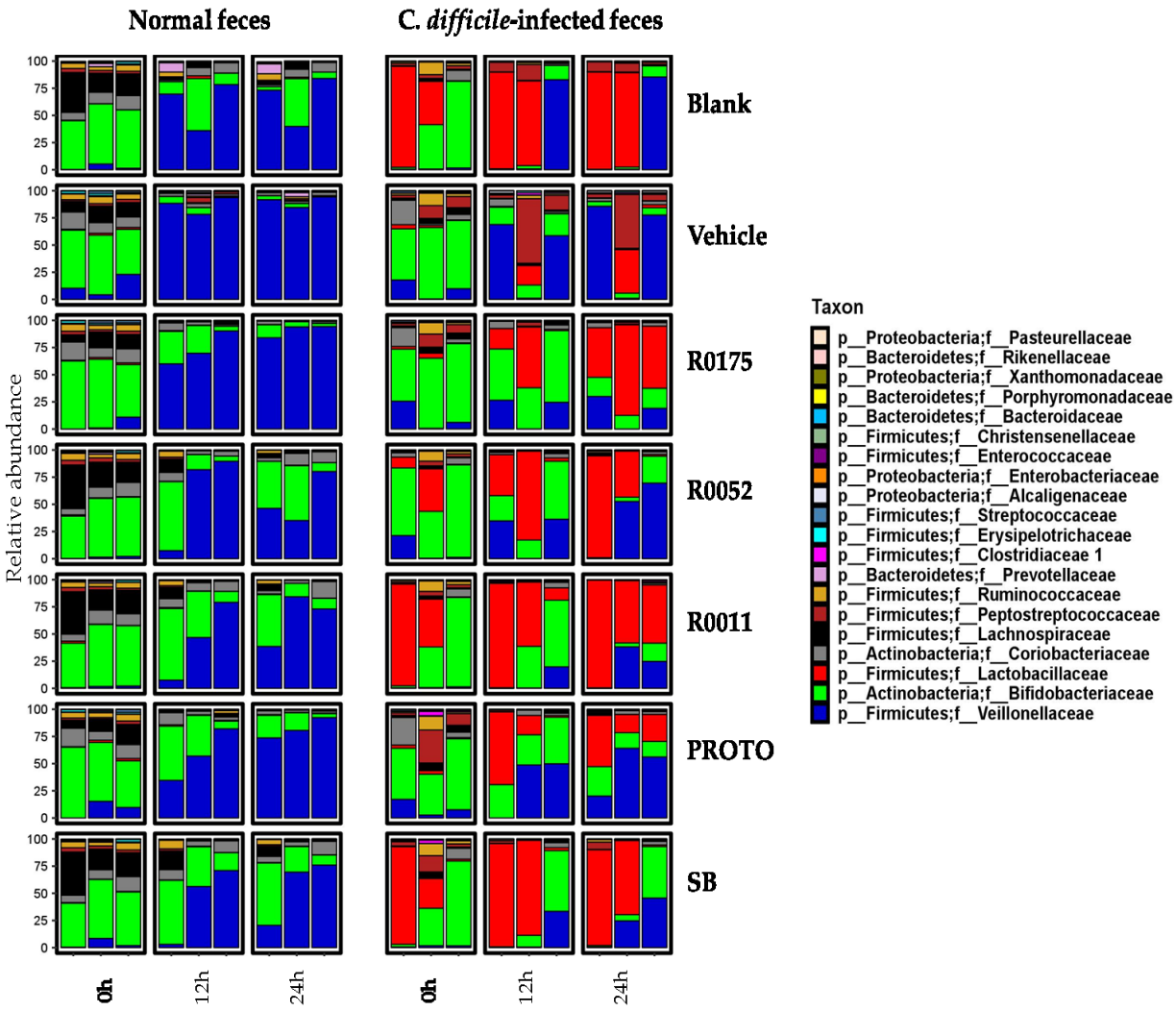

Figure 5.8. Taxonomic profiles (family level) of normal feces, and, *Clostridium* (*C.*) *difficile*-infected feces showing relative abundance over time. Values are shown in operational taxonomic units (OTUs). R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™.

One of the primary objectives for this study was to assess the effects of probiotic supplementation on the microbial composition of the fecal samples. Overall, no effect of probiotic treatments was noted across time for either of the fecal slurry preparations (**Figure 5.8**). On the other hand, notable differences were seen in alpha and beta diversity in the *C. difficile*-infected fecal samples with probiotic supplementation (**Figures 5.6 and 5.7**). In that

regard, PROTO showed increased alpha diversity at time 24 h compared with *C. difficile*-infected feces at the same time point, while the strains R0175, R0052, and R0011 appeared to show decreased changes in beta diversity in the *C. difficile*-infected feces. No major compositional changes were observed in the microbiota when comparing probiotic treatments to Blank or Vehicle (**Figure 5.8**). The above observations remained unchanged when the taxonomic profiles were assessed by amplicon sequence variants (ASVs) (**Figure S5.5**).

5.5 Discussion

The results from the present study showed that probiotic supplementation in *C. difficile*-infected fecal matter resulted in significant increases in the production of SCFAs and H_2S. In terms of the microbial communities, however, no overall effect of probiotics was observed with respect to changes in microbial composition in *C. difficile*-infected fecal matter when compared to the controls. Metabolite analyses showed that the levels of total SCFAs in normal fecal samples were similar to previously reported literature values of 20–70 mM for the transverse and proximal colonic regions [66]. Probiotic supplementation in normal FW was associated with no overall alteration in total SCFA production, although some changes were observed in individual SCFAs. Vehicle, R0052, and R00175 showed lower production of acetate, and R0175 treatment resulted in higher production of butyrate when compared to Blank. These latter differences in SCFA profiles, but not in total SCFA production, can be speculated to be due to differences in microbial interactions between the treatment groups. The increase in total SCFAs over time for the normal fecal samples could be attributed to the increased presence of the family *Veillonellaceae* (**Figure 5.8**), particularly that of *Megaspheara* spp. (**Supplementary Figure S5.1**). *Megaspheara* spp. have been shown to produce a range of SCFAs in the human gut through fermentation of lactate and glucose substrates [67,68]. More specifically, glucose utilization by *Megaspheara* spp. has been associated with the production of acetate, caproate, butyrate, and isovalerate, amongst other SCFAs [67].

In contrast to normal fecal samples, the total production of SCFAs in *C. difficile*-infected fecal samples was significantly ($p < 0.05$) reduced in both the controls, ranging between 5–15 mM total SCFAs. This observation is in line with reported literature whereby patients with *C. difficile* infection show hampered production of SCFAs [4,69]. Supplementation with each of the probiotic treatments resulted in significantly ($p < 0.05$) higher total SCFA production when compared to controls, reaching nearly 30 mM total SCFA at time 24 h. As seen in **Figure 5.2**, this latter increase in total SCFAs can be attributed to the significant ($p < 0.05$) increase in overall acetate production. The probiotics SB and R0175 also showed a significant ($p < 0.05$) increase in butyrate production. The ability of *Lactobacilli* spp. and *Bifidobacteria* spp. to regulate and increase acetate production in the human gut has been well documented [70,71]. In a study by Sivieri *et al.* (2013), supplementation with *L. plantarum* in a GI model resulted in higher levels of all the major SCFAs, with the highest increase seen in acetate production [72]. Moreover, *S. boulardii* has been previously associated with an increase in total SCFAs and individual SCFAs such as acetate, propionate, and butyrate [41,73]. The above findings are further supported by the results of SCFA production by the probiotic supplements in GI food culture in the absence of fecal microbiota. All supplements, except R0175, showed a significant ($p < 0.05$) increase in total SCFAs compared to Vehicle (**Figure S5.1**). Moreover, R0011 showed a significant ($p < 0.05$) increase in acetate, and SB and PROTO showed significant ($p < 0.05$) increases in acetate and butyrate (**Table S5.1**). The latter results provide further support for the potential of these supplements to contribute to the overall production of SCFA in the gut microbiota, particularly with respect to the increased acetate and butyrate levels observed in *C. difficile*-infected fecal samples. It is interesting to note that *B. longum* R0175 supplementation in the *C. difficile*-infected fecal slurry resulted in significantly ($p < 0.05$) higher levels of butyrate. *Bifidobacteria* fall under the category of acetogens, i.e., they have been shown to produce mainly acetate through carbohydrate fermentation pathways [71]. Although they have generally not be been seen as capable of producing butyrate through fermentation, many studies have speculated that due to symbiotic cross-feeding interactions between *Bifidobacteria* and butyrate-producing colonic bacteria, such as *Faecalibacterium prausnitzii*, *Eubacterium*, and *Roseburia* spp., supplementation with

Bifidobacteria could result in better survival of these bacteria and so lead to higher butyrate production [74,75].

Apart from products of carbohydrate fermentation, the protein fermentation products of NH_4 and H_2S were also measured to assess intestinal homeostasis. Each of these metabolites has been previously associated with changes in gut microbial composition and overall colonic health [39,76]. High levels of NH_4 have been linked to cytotoxic effects on the gut lumen, contributing to the formation of colorectal cancer [77,78]. Similarly, H_2S has been linked to a range of toxicity pathways [79,80]. The results from the present work show that NH_4 production in the GI model was within the normal range of NH_4 production in the human gut lumen [36,39]. Although the production of NH_4 significantly ($p < 0.05$) increased in all samples compared to time 0 h, which is indicative of the fermentation process, the levels of NH_4 thereafter remained stable with no statistical differences among timepoints. Furthermore, no effect of probiotics on NH_4 production was observed in either normal or *C. difficile*-infected fecal samples, and no significant differences were found between the two fecal types. It is possible that these latter results could be due to the limitation in the sensitivity of the assay, or that the level of protein in the GI food was not sufficient enough to see changes in NH_4 levels between the fecal types. With regards to H_2S production, normal fecal samples showed no overall differences between the treatments and the levels of H_2S were within the normal colonic range [40]. The *C. difficile*-infected fecal control samples, however, had lower levels of H_2S when compared to normal fecal samples. These levels appeared to be restored to the level found in the normal fecal samples when supplemented with each of the probiotic treatments (**Figure 5.4**). The depletion of H_2S observed in the *C. difficile*-infected fecal samples coincides with previous observations of inflammatory bowel conditions being associated with dysregulation of sulphate producing bacteria and disruption in some of the key functions of H_2S such as colonic mucus production and maintenance of microbiota biofilm [79,80]. The ability of probiotic supplementation to increase and restore H_2S levels in the *C. difficile*-infected fecal samples could be linked with the concurrent increased generation of acetate and butyrate. Production of acetate and butyrate by intestinal bacteria is thought to

occur via the glycolytic pathway, which converts carbohydrates to pyruvate and acetyl-CoA. This latter process generates H_2 as a by-product, which thereafter undergoes sulfate reduction in the gut to form H_2S [71].

16S rRNA gene amplicon sequencing was performed on all the samples to observe the changes occurring in the *C. difficile*-infected fecal samples and the possible shift in microbial communities during probiotic supplementation. These results showed that *C. difficile*-infected samples had a lower alpha diversity when compared to normal samples at time 0 h (**Figure 5.6**). Furthermore, relative abundance of the microbial communities showed that in both types of fecal samples, the richness in microbial diversity was not maintained across the time points, possibly arising from the batch culture conditions where poor microbiological control has been previously documented [37]. However, despite this limitation, normal samples had a more stable and richer microbiota when compared to *C. difficile*-infected samples at time 0 h (**Figure 5.8** and **Supplementary Figure S5.2**), as observed in previous literature [3]. Additionally, normal samples showed little variation between fermentation replicates whereas *C. difficile*-infected replicates failed to reproduce similar microbial relative abundances at time 0 h. The variation in the *C. difficile*-infected fecal slurry at time 0 h could be attributed to its lower initial diversity when compared to normal samples. Such lesser diversity could have resulted in different microbial interactions and growth rates leading to poorer microbial control. The results of beta diversity plots, however, showed that at time 0 h, normal and *C. difficile*-infected fecal samples clustered together, showing similarities in their microbial community structure. The reason for no major differences in initial microbial community structure could be due to the resilience of the normal fecal microbiota to compositional changes in the absence of antibiotic treatment [81]. Despite beta diversity plots showing community similarity at time 0 h, the patterns across time 12 and 24 h differed with time and type of fecal sample. Normal fecal samples were closely clustered at each corresponding time point, whereas *C. difficile* samples showed scattering across those time points (**Figure 5.6**), indicating variations in microbial communities. This above result was confirmed with the relative abundance data (**Figure 5.8**), which showed variations in microbial

groups such as *Lactobacillaceae*, *Veillonellaceae*, and *Bifidobacteriaceae* across treatments and time for the *C. difficile*-infected samples. Moreover, the strain R0175 and the probiotic mix PROTO seemed to show similar patterns of microbial communities (**Figure 5.8**) and were closely clustered in the heatmaps of each fecal type (**Supplementary Figure S5.3 and S5.4**), suggesting a possible dominant effect of R0175 in the mix. In the present study, however, no major shifts in microbial composition were observable when probiotics were supplemented in either fecal type. Similar observations were noted in previous studies; as shown by a study by Lahtinen *et al.* (2012) which demonstrated that *L. rhamnosus* HN001 and *L. acidophilus* NCFM were associated with changes to *Lactobacilli* and *C. difficile* but did not show any significant effects on major microbial groups [82]. Similarly, a study by Forssten *et al.* (2015) demonstrated that supplementation with *L. acidophilus* NCFM, and *L. paracasei* Lpc-37 did not show changes in the colonic microbiota in terms of reducing the *C. difficile* microbial population [83]. It has been suggested that this phenomenon could be due to slow growth rates of probiotics observed in the GI tract whist remaining metabolically active [25,84], thus explaining their inability to cause significant changes in the microbiota composition under batch culture conditions in the present study. Hence, inherent limitations of the batch culture design with respect to microbiological control could have masked the effects of the probiotics on the microbial communities in the fecal samples.

5.6 Conclusions

To summarize, the results of the metabolite assays of the *C. difficile*-infected fecal samples collectively showed a range of changes, which indicated impaired key metabolic functions. Probiotic supplementation (R0011, R0052, SB, R0175, and, PROTO) increased SCFA levels and restored depleted H_2S levels in *C. difficile*-infected fecal samples. In normal fecal samples, however, probiotics did not affect metabolic functions. Furthermore, 16S community profiling showed that normal fecal samples, across all treatments, had a closer similarity between its microbial communities at each time point, in contrast to *C. difficile*-infected fecal samples, which showed community similarity only at time 0 h signifying community disruption at time 12 and 24

h. Moreover, *C. difficile*-infected fecal samples displayed a lower diversity at time 0 h, in accordance with previous literature [3]. Despite the occurrence of strain-specific effects amongst the tested probiotics, such as the increase of microbial diversity by *B*. *longum* R0175 and ProtecFlor™ at certain time points, no drastic shifts in the microbiota composition were observed in the *C. difficile*-infected samples. Similarly, probiotic supplementation did not affect microbiota composition in normal fecal samples.

In conclusion, the present work has revealed that using an in vitro gastrointestinal model, metabolic functions changes induced by *C. difficile* infection (CDI) in a fecal sample were measurable, as well as the effect of probiotics on overall microbiota diversity and their metabolic output. Supplementation with single strain probiotics (*L. rhamnosus* R0011, *L. helveticus* R0052, *S. boulardii* CNCM I-1079, *B*. *longum* R0175) and a probiotic mixture (ProtecFlor™) restored microbial metabolic functions but was not associated with quantifiable changes in microbiota composition. Nevertheless, despite not having seen changes in *C. difficile*-infected microbiota in this model system, the metabolite analyses indicate the potential of probiotics to restore intestinal metabolic homeostasis, suggesting that they could be useful adjuncts to antibiotic therapy in CDI. Further research is warranted to establish the role of probiotics in restoring intestinal metabolic functionality in the context of CDI through the use of fecal samples from different population groups and from patients with different levels of CDI-pathophysiology.

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5.9 Supplementary Information

Table S5.1. Reads count throughout key bioinformatics processing steps

http://doi.org/10.5281/zenodo.3998006

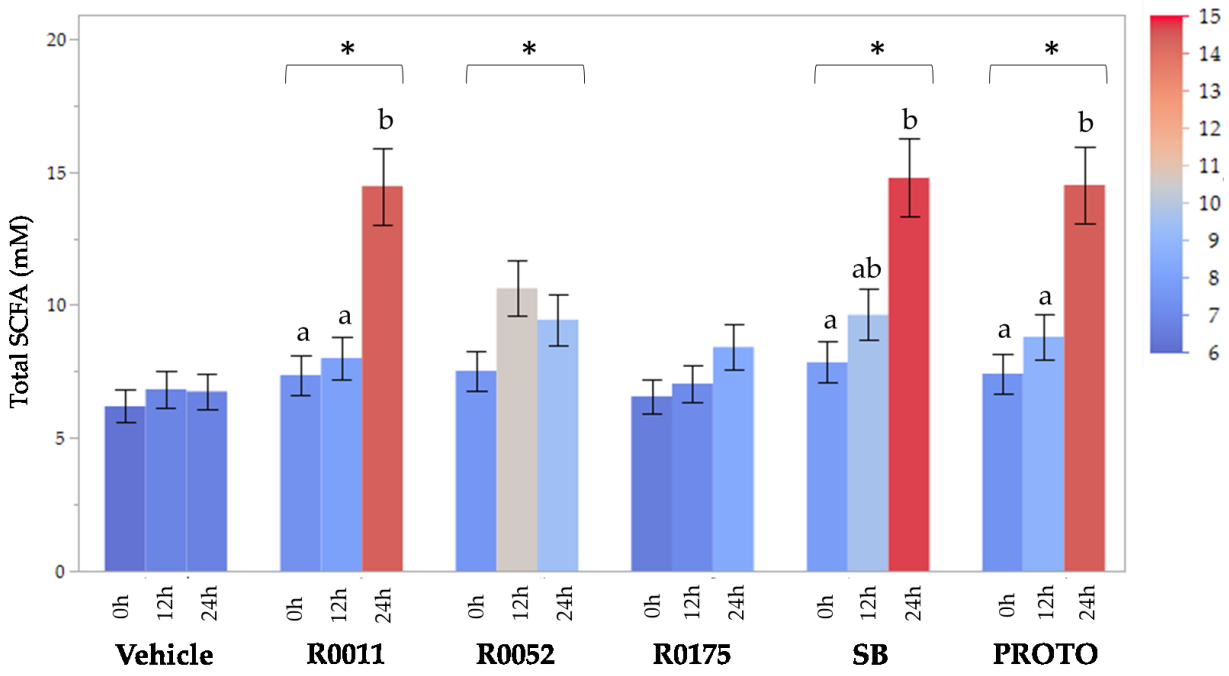
Table S5.2. Consensus rarefied OTU table

http://doi.org/10.5281/zenodo.3998006

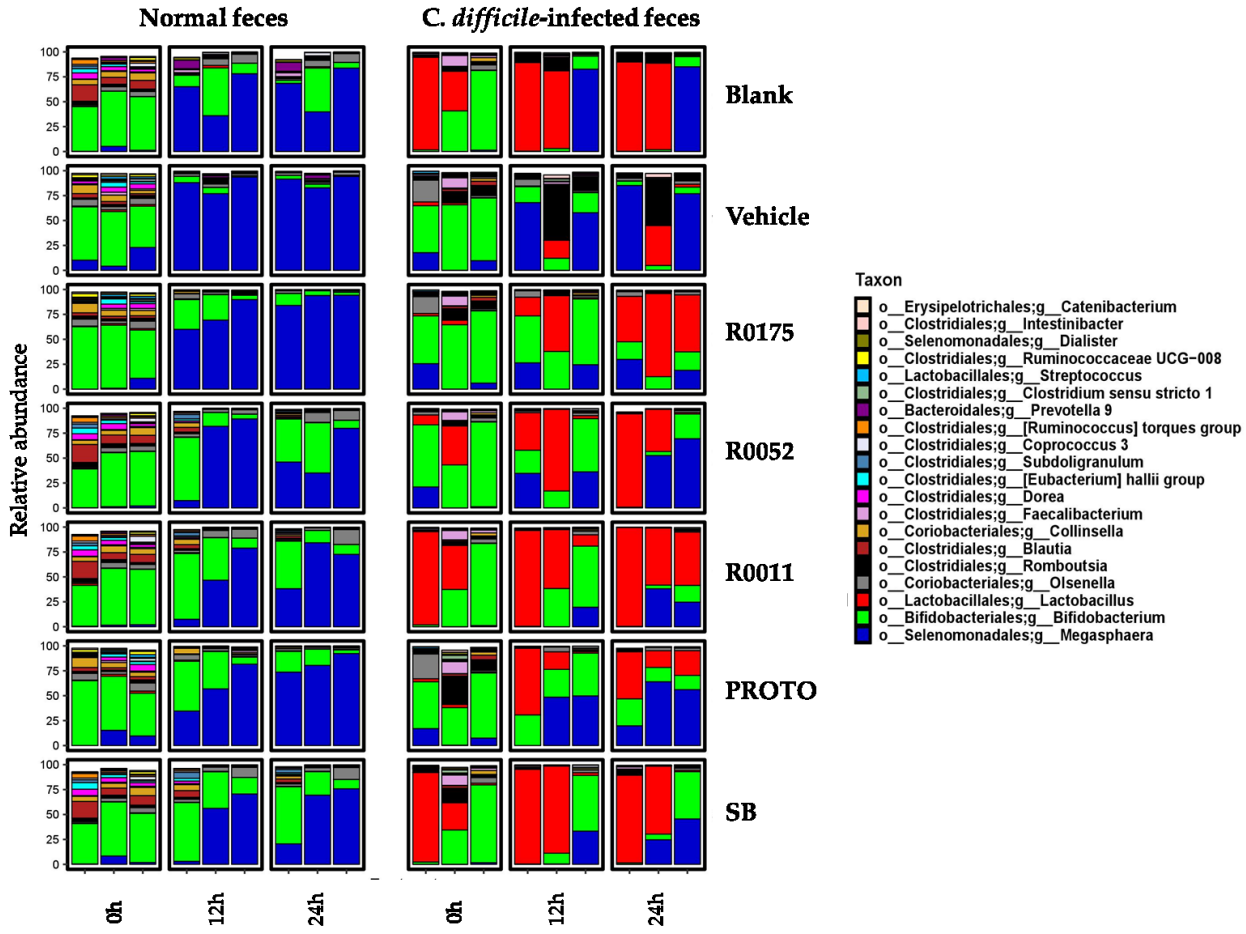
Table S5.3. Short-chain fatty acid (SCFA) concentrations in gastrointestinal (GI) food media supplemented with probiotics

Treatment	Time	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Remainder SCFA (mM)
Control (Vehicle)	0 h	5.79	0.15	0.16	0.19
	12 h	6.31	0.16	0.17	0.19
	24 h	5.07	0.59	0.20	0.89
L. rhamnosus R0011	0 h	6.94 [a]	0.18	0.10	0.15
	12 h	7.02 [a]	0.19	0.16	0.63
	24 h	13.22 [b]	0.76	0.10	0.38
L. helveticus R0052	0 h	6.96	0.22	0.17	0.17
	12 h	9.31	0.19	0.21	0.92
	24 h	8.62	0.20	0.17	0.45
B. longum R0175	0 h	6.25	0.12	0.09	0.10
	12 h	6.66	0.14	0.08	0.17
	24 h	7.98	0.18	0.14	0.12
S. boulardii	0 h	7.45 [a]	0.15	0.07 [a]	0.17
	12 h	9.11 [ab]	0.18	0.15 [a]	0.20
	24 h	11.94 [b]	0.18	2.43 [b]	0.22
Protecflor™	0 h	6.94 [a]	0.17	0.11 [a]	0.19
	12 h	8.25 [ab]	0.18	0.12 [a]	0.24
	24 h	12.46 [b]	0.10	1.76 [b]	0.18

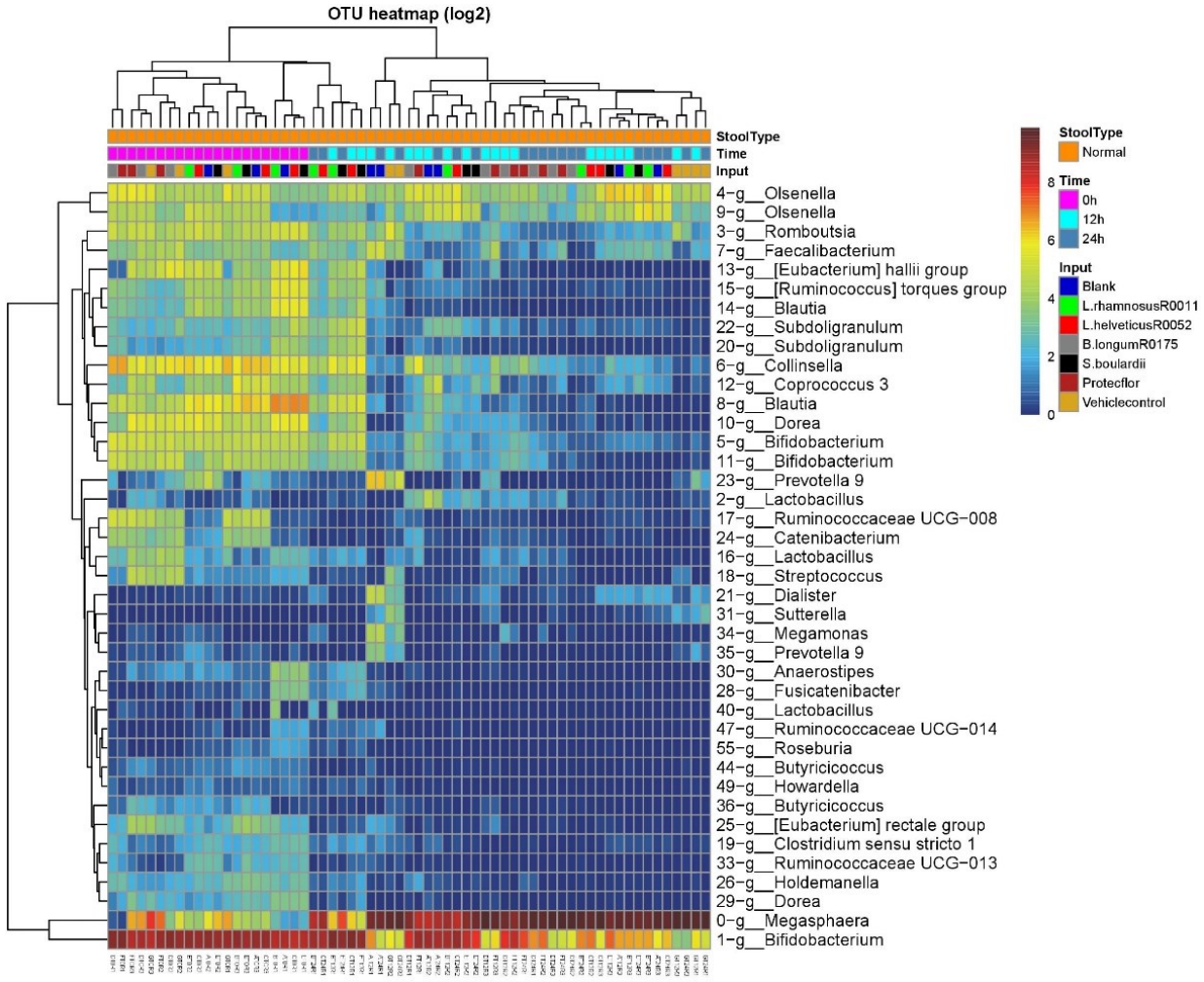
Remainder SCFA include: iso-butyrate, valeric acid, iso-valeric acid, caproic acid, iso-caproic acid, and, heptanoic acid. Values are shown as mean (n = 3).



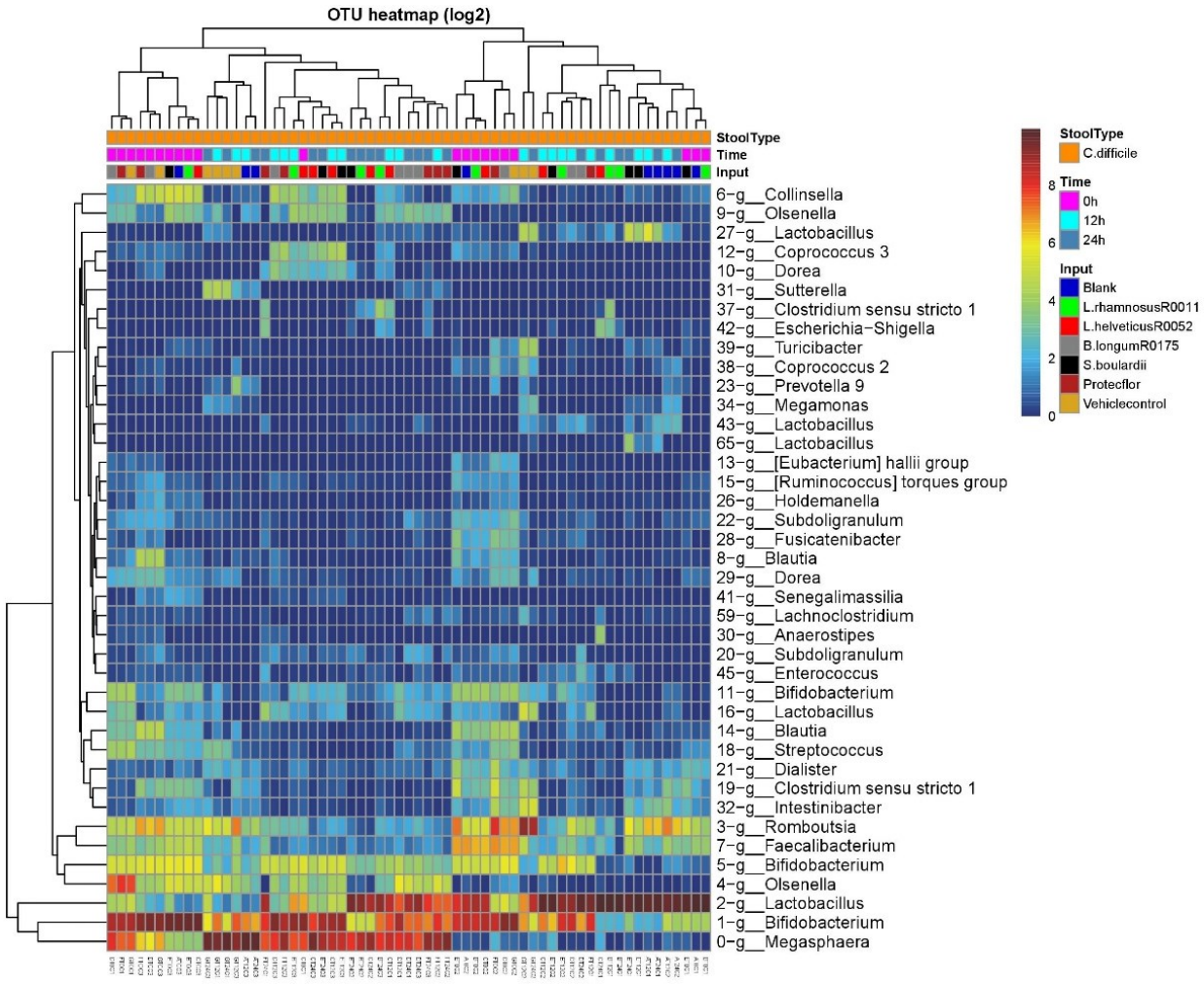
Supplementary Figure S5.1. Total short-chain fatty acid (SCFA) production by probiotic supplementation in gastrointestinal (GI) food media. Values are presented as means ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences in SCFA production between treatment and control ($p < 0.05$) when the means of all time points are jointly considered. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0175 = *B. longum* R0175; SB = *S. boulardii* CNCM I-1079; PROTO = ProtecFlor™.



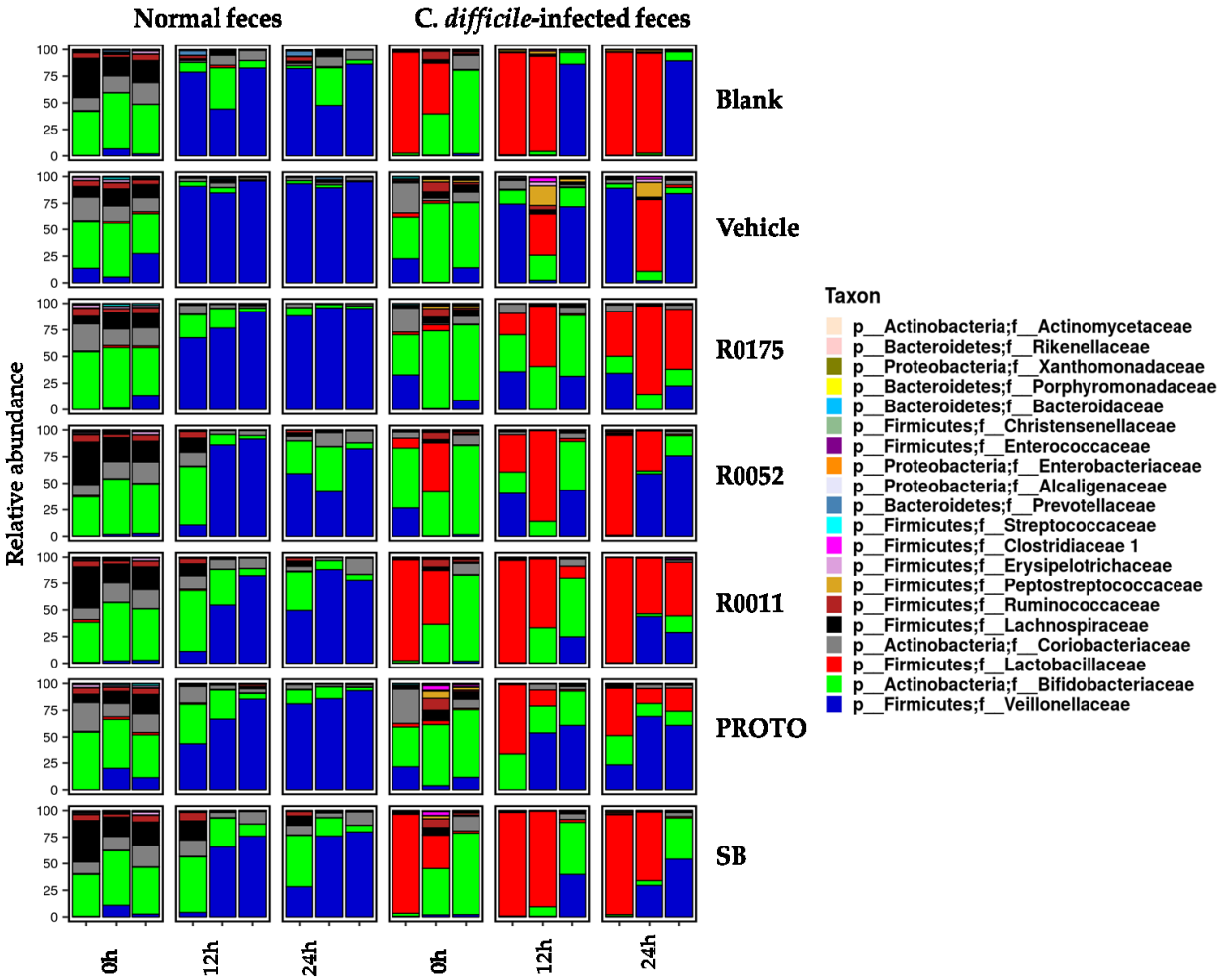
Supplementary Figure S5.2. Microbial diversity (genus level) of normal feces, and, *Clostridium* (C.) *difficile*-infected feces showing relative abundance over time. Values are shown in OTUs. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™.



Supplementary Figure S5.3. OTU heatmap of normal fecal samples showing clustering over time. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™.



Supplementary Figure S5.4. Heatmap of operational taxonomic units (OTUs) of *Clostridium* (C.) *difficile*-infected fecal samples showing clustering over time. Values are shown in OTUs. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™.



Supplementary Figure S5.5. Microbial diversity (family level) of normal feces, and, *Clostridium* (C.) *difficile*-infected feces showing relative abundance using amplicon sequence variants (ASVs). R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™.

CONNECTING STATEMENT IV

The findings from Chapter 4 and 5 clearly show the ability of *C. difficile* to mediate changes in the colonic environment. Chapter 4 showed its ability to mediate changes in the antioxidant status of colonic milieu, whereas, Chapter 5 showed *C. difficile* infection altered gut microbial community and decreased its diversity. Additionally, it dysregulated the gut metabolic function by significantly ($p < 0.05$) decreasing SCFA and H_2S production. In each of these studies, probiotic supplementation showed the ability to counteract these changes. In Chapter 4, probiotics showed an increase in antioxidant status through their ability to chelate copper. In Chapter 5, probiotics showed the ability to restore metabolic function of the gut flora by restoring H_2S levels and significantly ($p < 0.05$) increasing SCFA levels. Moreover, despite probiotic supplementation not being associated with major changes in *C. difficile*-infected microbiota, there was indication of their conservation of the commensal *Bifidobacteriaceae*. In order to comprehensively understand the changes mediated by *C. difficile* infection and probiotic action in the colon, it is imperative to understand the host response to these changes. Thus, Chapter 6 investigates *C. difficile*-mediated changes on the intestinal epithelium. In this study, fecal water digests from the GI model of *C. difficile*-infected microbiota were treated on cultured T84 cells to assess for changes in cell viability, cytotoxicity and inflammatory cytokine production.

CHAPTER 6: RESEARCH PAPER 4

Probiotic Supplementation in *Clostridioides difficile*-Infected Fecal Water Exhibits Strain-Specific Protective Effects Against Cytotoxic Damage and Inflammatory Marker Production in T84 Cells

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6.1 ABSTRACT

Clostridioides (C.) difficile infection (CDI) is frequently associated with intestinal injury and mucosal barrier dysfunction leading to an inflammatory response involving neutrophil localization and upregulation of pro-inflammatory cytokines. The extent of the immune response generated is associated with severity of clinical manifestations, regulation of which could lead to better management of the disorder. In this regard, probiotics have been extensively studied for their immunomodulatory ability in gastrointestinal disorders. In the present study, we assessed five single-strain and three multi-strain probiotics for their ability to modulate CDI fecal water (FW)-induced effects on T84 cells. The findings from our study showed that CDI-FW significantly ($p < 0.05$) decreased T84 cell viability. Moreover, CDI-FW exposed cells exhibited increased pro-inflammatory cytokine production, characterized by interleukin (IL)-8, C-X-C motif chemokine 5, macrophage inhibitory factor, IL-32 and tumor necrosis factor ligand superfamily member 8. Probiotics were associated with strain-specific attenuation of CDI-FW mediated effects, amongst which, *S. boulardii* CNCM I-1079 and *L. rhamnosus* R0011 were most effective in reducing pro-inflammatory cytokine production and in increasing T84 cell viability. ProtecFlor™, *L. helveticus* R0052 and *B. longum* R0175 showed moderate effectiveness, and *L. rhamnosus* GG R0343 along with the two other multi-strain combinations were the least effective. Overall, the findings support the concept that probiotic strains possess the capability to modulate CDI-mediated inflammatory response in the gut lumen.

6.2 INTRODUCTION

C*lostridioides (C.) difficile* (formerly *Clostridium difficile*) infection (CDI) is a toxin-mediated intestinal disease that is the most frequently identified cause of health care–associated infectious diarrhea [1]. Clinical manifestations of CDI range in severity from mild diarrhea to life-threatening pseudomembranous colitis [2]. Much of CDI pathophysiology is believed to be due to its production of enterotoxins A (TcdA) and B (TcdB), although the role of its S-layer proteins and flagellin are also thought to be significant contributing factors [3-5]. TcdA has demonstrated to induce apoptosis in intestinal cells through inactivation of small GTP-binding proteins and activation of pro-inflammatory cytokines characterized by neutrophil activation and recruitment followed by a subsequent acute inflammatory response, ultimately leading to intestinal epithelial damage [6]. TcdB is also thought to follow a similar mode of intracellular action but has been shown to differ greatly with respect to its enterotoxicity and cytotoxic potency. TcdB is suggested to be 500-1,000 times more cytotoxic than TcdA possibly owing to an increased enzymatic activity and density of toxin-specific receptors on the surface of intestinal cells [7]. Thus, it is suggested that the clinical manifestations of CDI can be attributed to the various *C. difficile* virulence factors in conjunction with host immune response [8]. In this regard, there is growing evidence to suggest that host immune response can be an important predictor of clinical severity and adverse outcomes in CDI patients [8-10]. A study by Feghaly RE *et al.* 2013, demonstrated that fecal C-X-C motif chemokine 5 (CXCL5) and interleukin (IL)-8, and not bacterial burden, were correlated with clinical severity in CDI patients [11]. Furthermore, a recent study by Dieterle et al. 2020 found that serum biomarkers of inflammation were good predictors of adverse outcomes in human and murine CDI [12]. In this regard, mitigation of CDI-mediated inflammation could play an important role in regulating host immune response leading to better management of CDI outcomes [8].

One of the most widely used therapeutic strategies to modulate host immune response in gastrointestinal disorders, including CDI, has been through probiotic supplementation [13].

Probiotics have been demonstrated to confer a wide variety of beneficial effects in the management of gastrointestinal (GI) disorders, including enhancement of mucosal barrier function [14,15], counteracting infections [16-18], modulating immune function [13] and attenuating clinical manifestations [16, 19-21]. Majority of the probiotics used commercially are from the *Lactobacilli*, *Bifidobacteria* and yeast (*Saccharomyces*) groups. Although research has demonstrated the potential of probiotics to act as immunomodulators, their effects are largely seen to be strain-specific and much is yet to be elucidated on their mechanisms of action. *S. boulardii* has been demonstrated to stimulate intestinal anti-toxin immunoglobulin A [22], inhibit IL-8 production, activate mitogen-activated protein (MAP) kinases [23], and produce soluble anti-inflammatory factors that inhibit nuclear factor (NF)-κB-mediated IL-8 gene expression [24]. Similarly, experimental evidence demonstrates the ability of several *Lactobacilli* spp. and *Bifidobacteria* spp. to modulate immune activity primarily through secretion of a variety of molecules that directly or indirectly promote the inactivation of NF-κB signaling pathways [25, 26] *Lacticaseibacillus (L.) rhamnosus* GG (previously known as *Lactobacillus rhamnosus*) was shown to prevent cytokine-induced apoptosis in several intestinal epithelial cell models [27], whereas, *L. rhamnosus* L34 and *L. casei* L39 were demonstrated to modulate CDI-mediated inflammation by decreasing IL-8 expression and inactivation of NF-κB [28]. Moreover, *L. rhamnosus* R0011 and *Lactobacillus helveticus* R0389 were shown to secrete bioactive molecules that were capable of downregulating IL-8 production in HT-29 epithelial cells [25,26].

Despite the promising role of probiotics to modulate host immune response to bacterial pathogens and stressors, much remains to be elucidated concerning their ability to mitigate CDI-associated inflammatory cascade. In the present study, we assessed the effect of fecal water (FW) from a gastrointestinal (GI) model of CDI on colonic adenocarcinoma derived T84 epithelial cell viability and immune marker production. Additionally, the effects of various probiotic supplemented CDI-FW were assessed for their efficacy in protection against potential CDI-mediated effects.

6.3 MATERIALS AND METHODS

6.3.1 Probiotic Treatments

In this study, eight different probiotic treatments were assessed for their ability to cause changes in *C. difficile*-infected microbiota. Five of the treatments were single strain probiotics, whereas, the rest three were various combinations of the single strain probiotics (**Table 6.1**).

Table 6.1. Probiotic treatments and dosage

Probiotic treatment	**Code**	**Dosage (cfu/vessel)**
L. rhamnosus R0011	R0011	1 x 10^9
L. helveticus R0052	R0052	1 x 10^9
L. rhamnosus GG R0343	R0343	1 x 10^9
S. boulardii CNCM I-1079	SB	1 x 10^9
B. longum R0175 (R0175)	R0175	1 x 10^9
ProtecFlor™ (combination of R0011, R0052, SB, R0175)	PROTO	1 x 10^9
Combination 2 (R0011, R0052 and R0175)	R0011+ R0052+ R0175	1 x 10^9 of each strain
Combination 3 (R0343 and SB)	R0343 + SB	10 x 10^9 of R0343 and 5 x 10^9 of SB

All probiotics were acquired from Lallemand Health Solutions Inc. (Montreal, QC, Canada) and stored at -20°C until use. For inoculation in batch culture fermentation, the probiotics were mixed in sterile 1 x PBS. 1 x PBS alone was used as the negative control (Blank).

6.3.2 Simulation of Gastrointestinal (GI) Conditions

Batch culture fermentation was performed to simulate the conditions of *C. difficile* infection using a computer-controlled GI model consisting of several independent anaerobic fermentation vessels run under physiological conditions as described previously in detail [29]. Briefly, 100 mL of filter-sterilized GI food, previously optimized by Molly *et al.* (1994) [30], consisting of arabinogalactan, pectin, xylan, potato starch, glucose, yeast extract, peptone, mucin and cysteine powders (Sigma Aldrich, St. Louis, MO, USA) were added to each vessel. This was followed by sequential enzymatic digestion by the addition of α-amylase (pH 7.0 for 15 min), followed by pepsin (pH 2.0 for 1.5 h), and finally, by pancreatic juice (12 g/L $NaHCO_3$, 6 g/L bile extract and 0.9 g/L pancreatin; pH 8.0 for 2 h). After completion of digestion, 50 mL of fecal slurry was inoculated (T = 0 h) to simulate the gut microbiota. Fecal sample obtained from a healthy male adult donor with no history of GI disorders and no antibiotic use in the past 6 mo was used to make normal fecal slurry, whereas, *C. difficile*-infected (CDI) fecal slurry was prepared using a 1:10 v/v fecal inoculation from a commercially obtained *C. difficile* fecal sample (male adult with stool positive for *C. difficile* toxins A & B; BioIVT, Westbury, NY, USA). Premixed probiotic treatments or blank were subsequently added to each vessel followed by anerobic fermentation at 6.3 ± 0.3 pH for 24 h with regular sampling at 6 h intervals. Samples taken at each timepoint were centrifuged at 2000 g for 10 min and stored at -80°C.

6.3.3 Sample Preparation for Cell Culture

The samples collected from the batch fermentation at T = 0 h and T= 24 h were further centrifuged at 13000 g for 20 min and the supernatant (hereinafter referred to as fecal water, FW) was collected, and filter sterilized with a 0.22 µm syringe filter (Fisher Scientific, Ottawa, ON, Canada). FW from each fermentation replicate was pooled before storage at −80 °C until treatment with the cells. Samples collected at T = 0 h from the probiotic blank vessels were considered as the controls for normal and CDI fecal matter respectively.

6.3.4 Cell Culture

Human colonic adenocarcinoma cell line, T84, were obtained from the American Type Culture Collection (ATCC, Burlington, ON, Canada) and cultured according to the company's procedures. Briefly, the T84 cells were cultured with Dulbecco's Modified Eagle Medium Nutrient Mixture (DMEM:F12) supplemented with 5% fetal bovine serum (FBS) in 75 cm^2 T-flasks until 80% confluency was reached. Cells were incubated at 37 °C with 5% CO_2 and 90% humidity and were monitored every second day with the appropriate sub cultivation ratio of 1:2 to 1:4 performed on a bi-weekly basis. Three separate cell passages (above passage number 15) were maintained concurrently for the treatment experiment.

For the experiment, T84 cells were subcultured at 80% confluence with 0.25% trypsin-EDTA solution for 5-10 min and subsequently seeded at a density of 2 x 10^6 cells/well onto 24 well plates (Costar® 24-well TC-treated Multiple Well Plates; Corning, NY, USA) and were grown overnight under the same incubation conditions mentioned earlier. Prior to starting the treatments, the confluency of monolayer formation was checked under the microscope. A dose-response experiment was previously carried out in order to determine the optimal dose of the FW on the T84 cells with minimum effect to their viability for an 8 h incubation period (> 90 % viability). As a result of this, 30 % (v/v) FW in cell culture medium was deemed appropriate for further use (data not shown). After monolayer formation in the plates, cell media was discarded followed by addition of 1% FBS supplemented fresh medium (1000 µL/well) along with filtered FW (500 µL/well). All treatments were added in triplicate for each cell passage number and subsequently incubated for a period of 8 h. After incubation, supernatant from each well was collected, centrifuged at 13000× g for 10 min, and, stored at −20 °C for further analyses. Cell viability was determined for the remaining cells in the 24-well plate.

6.3.5 Cell Viability Assays

6.3.5.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltretrazolium bromide (MTT) assay tetrazolium reduction assay was performed as a measure of cellular viability. The assay measures the ability of viable cells to covert the pale yellow MTT reagent to a purple-colored crystalline formazan through nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes [31]. Briefly, 500 µL of MTT solution (0.5 mg ml^{-1} MTT in phenol red-free DMEM:F12 media) was added to each well on a plate after the removal of supernatants from the experiment, followed by incubation for a period of 3 h. After incubation, the supernatant was discarded, 500 µL acidified isopropyl alcohol (0.4 N HCl) was added in each well and allowed to react for 5 min at room temperature. The contents of each well were transferred to a 96-well plate and was read spectrophotometrically at λ = 570 nm. The results were expressed as a percentage of untreated control cells.

6.3.5.2 Lactate dehydrogenase (LDH) Assay

Cellular damage was measured as a function of released lactate dehydrogenase (LDH) enzyme from T84 cells following exposure to the various treatments [32]. LDH determination was performed using the Cytotoxicity Detection Kit^{PLUS} (Roche Diagnostics GmbH, Mannheim, Germany) as per manufacturer's instructions. Supernatant from cells treated with only DMEM:F12 cell media were used to determine background control and those treated with lysis buffer were used for high control. The percentage of cytotoxicity was calculated as: cytotoxicity (%) = [(experimental value – background control)/(high control – background control)] × 100. The results were expressed as a percentage of untreated control cells.

6.3.6 Cytokine and Chemokine Determination

The detection of various cytokines and chemokines following the exposure of T84 cells to the treatments was determined by multiplex assays. Bio-Plex Pro™ Human Chemokine 40-plex Panel (cat. no. 171AK99MR2, Bio-Rad, Hercules, CA, USA) was used to detect chemokine expression, and, Bio-Plex Pro™ Human Inflammation 37-plex Panel (cat. no. 171AL001M, Bio-Rad, Hercules, CA, USA) was used to determine inflammatory cytokine expression. Each assay was performed according to the instructions of the manufacturer. Samples tested in these kits included the supernatants collected from T84 cell exposure to the normal FW Blank (T = 0 h and T = 24 h) and each of the *C. difficile*-infected FW treatments (Blank + 8 probiotic interventions; T = 0 h and T = 24 h). Each sample treatment was tested using three biological replicates. Standard curves for each cytokine and chemokine were generated in duplicate using serial dilutions of the premixed lyophilized standards provided in the kits. Data was acquired with the help of a Bio-Plex 200 instrument (Bio-Rad, Hercules, CA, USA) and analysed by the Bio-Plex Manager software (version 4.1, Bio-Rad, Hercules, CA, USA). Quality checks was done for each chemokine and cytokine using the respective working range and limit of detection data provided in the product lot sheets for each kit. Results for each marker was expressed as pg / mL.

6.3.7 Statistical analysis

All data are reported as means ± standard error of mean (SEM). Normality was assessed on original data sets with log transformations where necessary to align with statistical assumptions. Data for cell viability assays after treatment of cells with fecal water collected at 24 h were analyzed using one-way ANOVA for each fecal type using Treatment (9 levels) as a factor followed by Dunnett's post hoc analysis to compare with control. Data for cytokine analyses were assessed using two-way ANOVA for CDI-FW using Time (2 levels) and Treatment (9 levels) followed by Tukey's post hoc analysis. When significant interactions between time and treatment were observed, the means of each time point within a fecal type were individually compared for significant differences within the fecal type. Statistical significance was set at $p < 0.05$. All data

analyses and visualizations were performed using JMP v14.4 (SAS Institute, Cary, NC, USA), with the exception of the heatmap, which was generated with GraphPad Prism (v 7.04, GraphPad Software Inc., San Diego, CA, USA).

6.4 Results and Discussion

C. difficile toxins have been extensively studied in various intestinal cell cultures to elucidate their cytotoxic effects and its ability to induce inflammatory cytokines [3,4,10,11,33,34 The present investigation is the first to assess the cytotoxic and proinflammatory effects of *C. difficile*-infected fecal water such as obtained from a simulated human GI model. This approach allows for a more holistic approach to study CDI sample assessment involving human gut epithelial cells rather then *C. difficile* cultures or purified toxins [35,36]. In the present study, we assessed the ability of *C. difficile*-infected microbiota to cause changes to T84 cell viability and cytokine expression following exposure of these cells to FW collected from the batch fermentation at T = 0 h and T = 24 h.

6.4.1 Cell Viability

Two assays were performed to assess the effect of FW on T84 cell viability. The MTT assay determines cellular mitochondrial dehydrogenase activity from viable cells, and the LDH assay assesses cytosolic enzyme release from damaged cells. Each of these assays have been well documented to assess cell survival and growth [32,37].

In terms of the MTT assay, exposure to normal FW did not show a significant change in T84 cell viability. On the other hand, T84 exposure to CDI-FW resulted in 206% (2.1-fold) decrease in viability (**Figure 6.1**). In the cells treated with CDI-FW containing the probiotic treatments *L. rhamnosus* R0011 and *S. boulardii* CNCM I-1079, cell viability was significantly ($p < 0.05$) higher in comparison to the CDI-FW Blank sample (**Figure 6.1**). Interestingly, the cell viability of *L.*

rhamnosus R0011 and *S. boulardii* CNCM I-1079 CDI-FW treated cells was similar to untreated cells, indicating a protective effect of these two probiotic strains in CDI-FW.

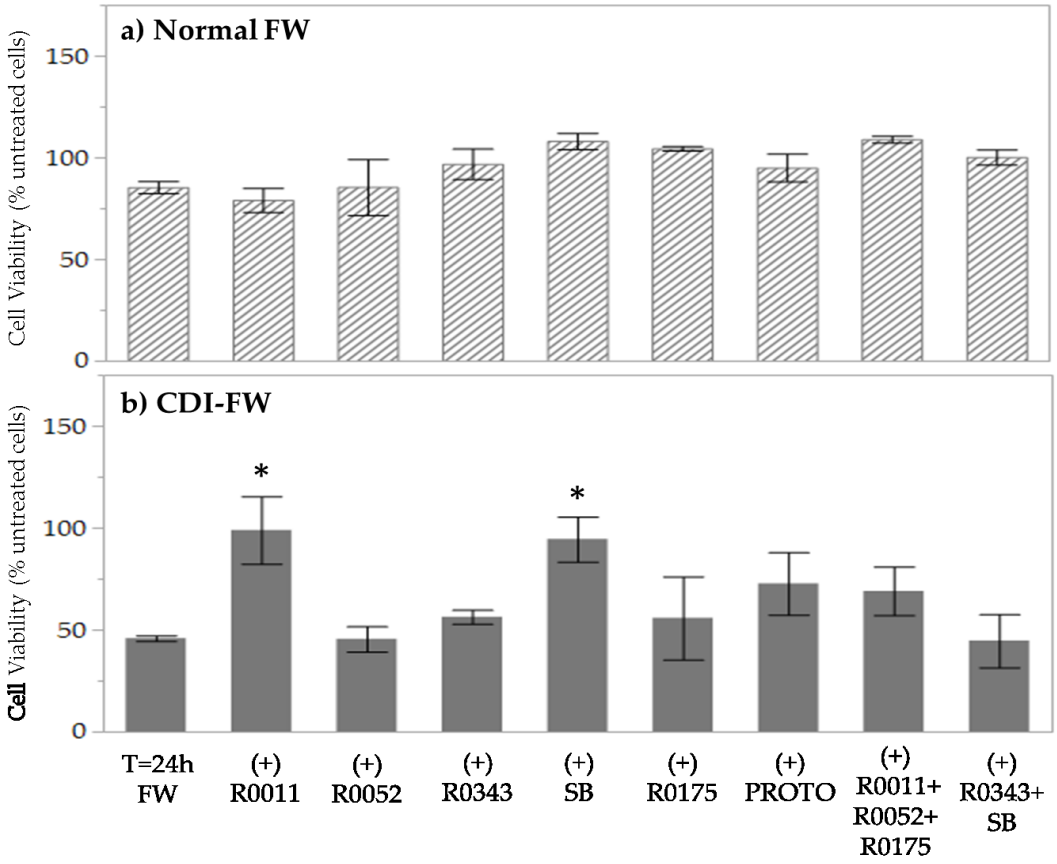

Figure 6.1. Cell viability following exposure of T84 cells to fecal water (FW) treatments as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A) Cells treated with Normal FW, and B) Cells treated with *Clostridioides difficile*-infected (CDI) FW. Values are shown as mean ± SEM. The symbol * represents significant differences between the means of treatments ($p < 0.05$). R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

In the results from the LDH assay, no significant effect was observed when cells were exposed to CDI-FW or normal FW collected at T = 24 h in comparison to untreated cells (**Supplementary Figure 6.1**). However, probiotic treated Normal FW and CDI-FW showed significant effects. In normal FW-exposed T84 cells, the probiotics *L. rhamnosus* R0011, *L. helveticus* R0052, *L. rhamnosus* GG R0343, *S. boulardii* CNCM I-1079, and, the combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079, showed a significant ($p < 0.05$) decrease in LDH production (**Supplementary Figure 6.1**). In contrast, the probiotic combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175 showed a significant ($p < 0.05$) increase. In the cells treated with probiotic supplemented CDI-FW, *L. rhamnosus* GG R0343, *S. boulardii* CNCM I-1079, *B. longum* R0175, and ProtecFlor^{TM} showed a significant ($p < 0.05$) decrease in LDH release (**Supplementary Figure 6.1**).

The results from the MTT assay are in concordance with previously documented studies showing cytotoxic potency of *C. difficile* and its toxins [3,6,34,38,39]. Notably, in our study this effect was observed by exposing cells to CDI-FW obtained from our simulated GI model of CDI microbiota, as opposed to cultured *C. difficile* strains or its purified toxins. Fecal water has been previously demonstrated shown to possess some cytotoxic activity [40,41], which in the case of CDI-FW, could have been further exacerbated by the presence of its enterotoxins, and secreted products such as proteolytic and hydrolytic enzymes [42]. This is further supported by previous studies by our group that have shown the potential of CDI-FW to indicate dysregulation in gut metabolic function and antioxidant status, potentially leading to a cytotoxic environment [29,43].

Probiotic supplemented CDI-FW showed, to an extent, the ability to counteract some of the cytotoxic effects of the CDI-FW. In particular, the probiotic supplements, R0011 and SB, demonstrated a significant ($p < 0.05$) increase in T84 cell viability, resulting in values similar to those observed in T84 cells exposed to normal FW (**Figure 6.1**). These results indicate that probiotics potentially act in a strain-specific manner to counteract *C. difficile* cytotoxicity, a phenomenon that has been previously demonstrated [44]. In CDI, *S. boulardii* is mainly thought

to act through immune system regulation [23,45] and the production of anti-toxin proteases, which could counteract CDI-mediated pathophysiology [46]. With regards to *Lactobacilli* spp. and *Bifidobacterium* spp., *in vitro* evidence is limited however, it is suggested that these probiotics might counteract CDI-mediated effects by preventing *C. difficile* adhesion [47,48], maintaining intestinal barrier integrity [49], and modulating host immune response [50].

6.4.2 Immune Response of T84 Cells Following FW Exposure

C. difficile exposure *in vitro* has been previously shown to be associated with intestinal tissue damage followed by a robust immune response that upregulates proinflammatory cytokines and recruits neutrophils, further leading to an acute inflammatory response [51]. Furthermore, it has been noted that monitoring the immune response in patients with CDI may be a more suitable marker for disease severity rather than bacterial burden [11].

In our study, we chose to assess a wide range of chemokines and cytokines as a tool to assess the host immune response of CDI-FW on T84 cells. These molecules were quantified using two multiplex assay kits comprising of a 40-plex chemokine panel and a 37-plex inflammatory cytokine panel (Bio-Rad, Hercules, CA, USA). The results from the multiplex assays, summarised in the heatmap (**Figure 6.2**), showed an increased production of a host of chemokines and inflammatory cytokines in the T84 cells following exposure to CDI-FW. These molecules primarily include the interleukins 8, 11, and 32, C-X-C motif chemokine 5 (CXCL5), tumor necrosis factor surface receptor 8 (TNFSR8), macrophage inhibitory factor (MIF) and C-C Motif Chemokine Ligand 21 (CCL21) amongst others. Notably, CDI-FW was shown to upregulate almost all of these chemokines and cytokines in comparison to normal FW, with the exception of IL-10. Most of these molecules have been previously associated with inflammatory pathways, chemotaxis, and in cytokine signalling of the tumor necrosis factor (TNF)-α and NF-κB pathways [52]. Importantly, the results from the multiplex assays showed anti-inflammatory effects of probiotic supplementation in CDI-FW at T = 24 h, whereby several probiotic treatments showed attenuation in the production of several chemokines and cytokines (**Figure 6.2**).

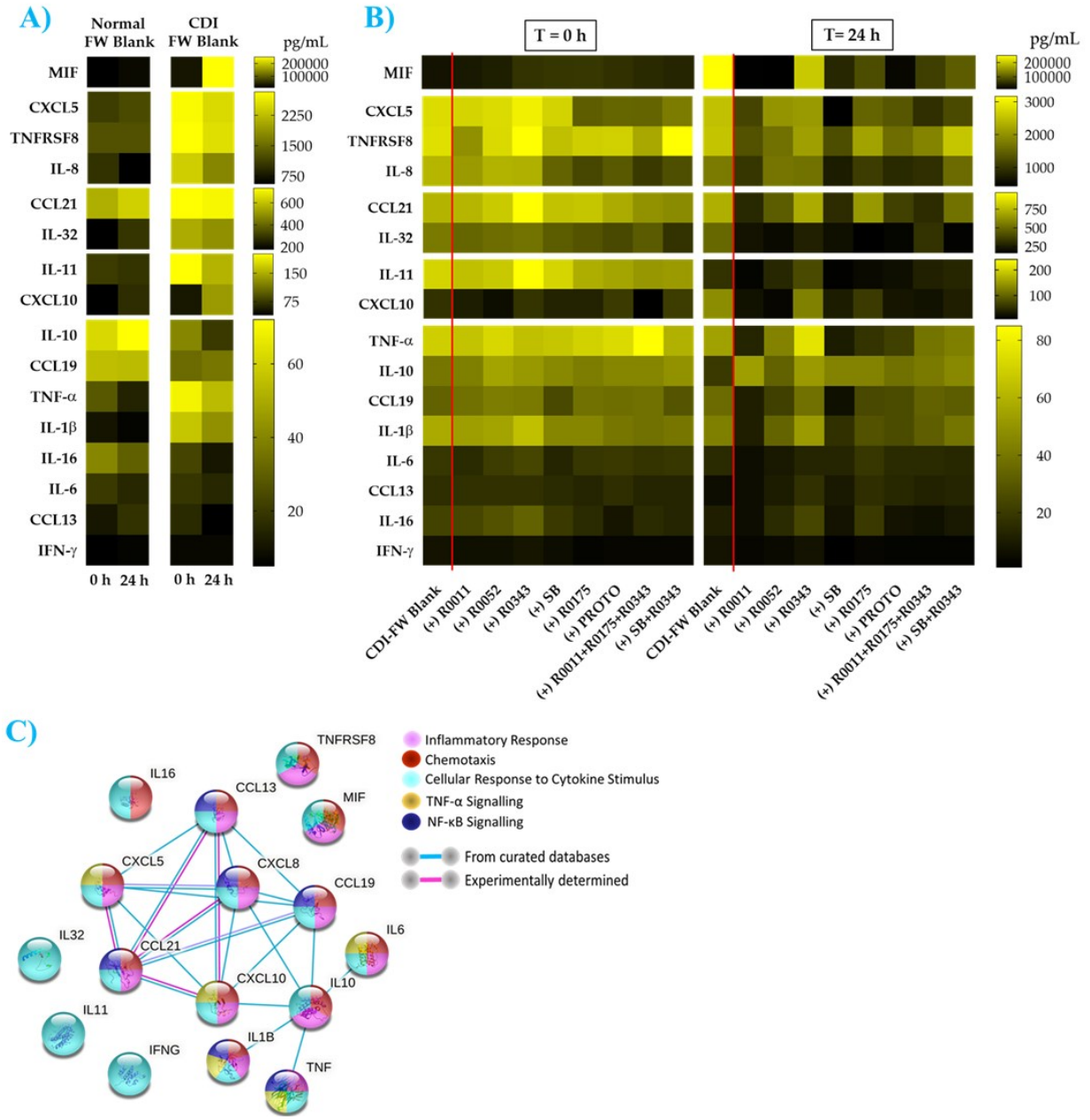

Figure 6.2. Cytokine and chemokine profiles from T84 cells exposed to the different fecal water (FW) treatments (collected at T = 0 h and T = 24 h from the batch fermentation) for a period of 8 h. **A)** Cytokine and chemokine profiles in Normal FW Blank and CDI-FW Blank; **B)** Cytokine and chemokine profiles in CDI-FW supplemented with probiotic treatments. Data shown are the mean cytokine/chemokine production (picograms per milliliter; *n* = 4). **C)** STRING v 11.0 analysis

showing functional links between each of the different cytokines/chemokines produced. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor^{TM}; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

6.4.2.1 Chemokine Expression

IL-8 and CXCL5 followed by CCL21 were the prominent chemokines expressed in the cultured T84 cells following exposure to CDI-FW. Both IL-8 (also known as CXCL8) and CXCL5 hail from the same family of CXC chemokines that are involved in activation of the CXCR2 receptor, ultimately leading to chemotaxis of neutrophils and setting of innate immunity [53]. CCL21, on the other hand, has shown to play a role in chemotaxis of leukocytes such as T cells [54].

The results of IL-8 production showed an increase of 212 % when T84 cells were exposed to CDI-FW when compared to normal FW at T = 24 h (**Supplementary Table S6.1**). In CDI-FW treatments, two-way ANOVA results showed significant ($p < 0.05$) effects of treatment, time and interaction, therefore, the mean IL-8 from cells exposed to CDI-FW collected at the two time points (T = 0 h and T = 24 h) were compared to its corresponding time point within the blank to assess for any differences (**Figure 6.3**). The probiotic, SB, was the only treatment that showed a significant ($p < 0.05$) decrease in IL-8 production when compared to control (T = 24 h). R0011 showed a significant ($p < 0.05$) decrease across time but did not show statistical significance when compared to control at the corresponding timepoint. No other probiotic supplemented CDI-FW showed a statistical difference when compared to the blank.

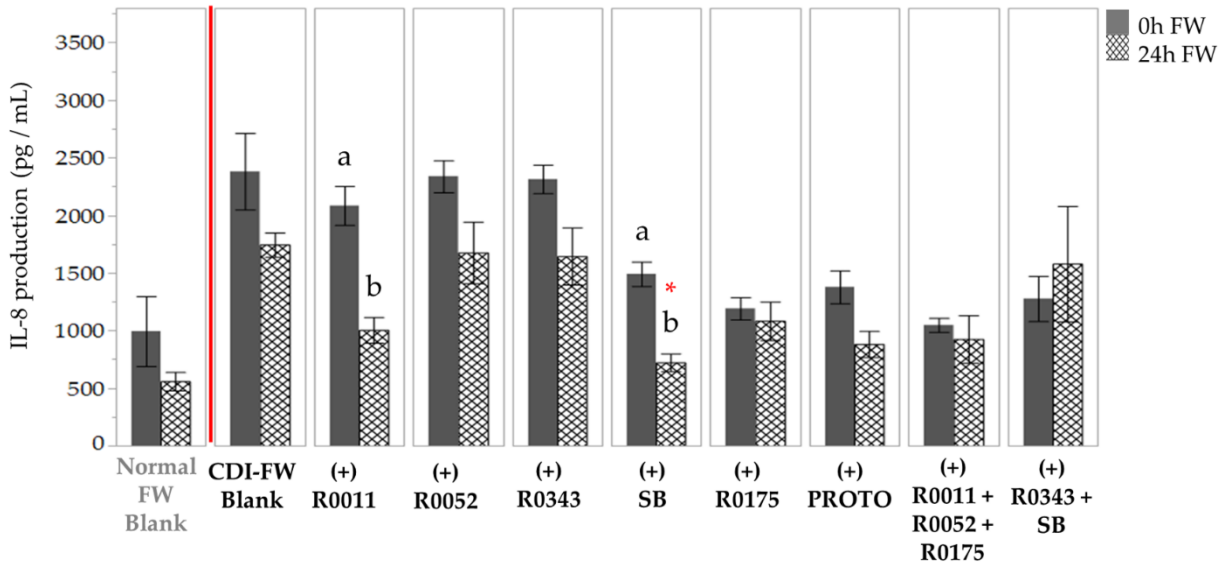

Figure 6.3. Detection of Interleukin-8 (IL-8) expression as measured by multiplex assay following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments. (■) cells treated with FW collected at T = 0 h, and (▩) cells treated FW collected at T = 24h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences ($p < 0.05$) between treatment and CDI-FW Blank at T = 24 h. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

CDI-FW also showed an upregulation in CXCL5 production in T84 cells at T = 24 h, showing a 105 % increase as compared to the normal FW treatment (**Supplementary Table S6.1**). Two-way ANOVA analysis of the data for cells exposed to CDI-FW treatments showed significant ($p < 0.05$) main effects of treatment, time and its interaction. CDI-FW supplemented with R0011 and SB were the only treatments that showed a significant ($p < 0.05$) decrease in CXCL5 production when compared to blank at T = 24 h (**Figure 6.4**).

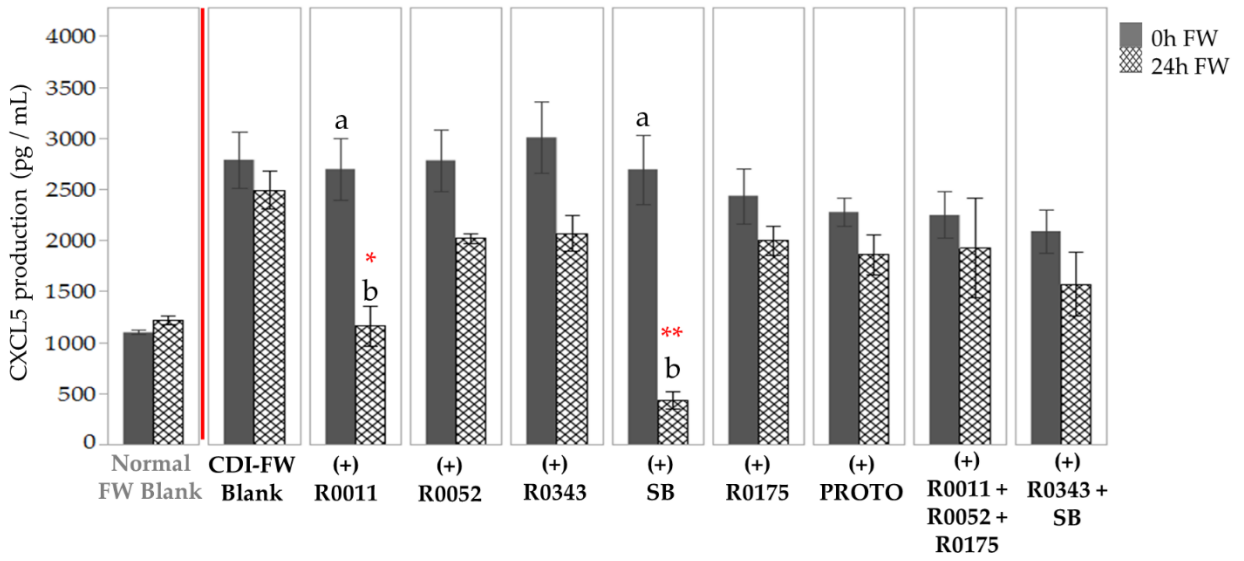

Figure 6.4. Detection of C-X-C motif chemokine 5 (CXCL5) production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (▢) cells treated with FW collected at T = 0 h, and (▨) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences ($p < 0.05$ = *; $p < 0.01$ = **) between treatment and CDI-FW Blank at T = 24 h . R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

In terms of CCL21, exposure of T84 cells to CDI-FW did not result in an increased production when compared to normal FW. The findings demonstrated, however, a significant ($p < 0.05$) decrease in CDI-FW treated with probiotics, indicating a potential role of R0011, R0052, SB, PROTO, and combination 2 (R0011 + R0052 + R0175) in modulating CCL21 production (**Supplementary Figure S6.2**).

6.4.2.2 Cytokine Expression

In addition to chemokine production, T84 cells challenged with CDI-FW were associated with increased production of several inflammatory cytokines such as MIF, TNFRSF8, and IL-32. MIF is an inflammatory cytokine that has been associated with host immune response to infectious pathogens such as CDI [55,56]. TNFRSF8, also referred to as CD30, has been previously shown to mediate signal transduction leading to the activation of NF-κB pathway [57]. Similarly, IL-32 has also been previously shown to induce cytokine signal pathway, that lead to the activation of NF-κB, TNF-α and IL-8 [58].

CDI-FW challenged T84 cells showed an initial 167 % difference in MIF production when compared to normal FW at T = 0 h. At T = 24 h, however, this difference in MIF production increased to 1111 % (**Figure 6.5**). The significant ($p < 0.05$) increase in MIF production showed attenuation when cells were exposed to probiotic-treated CDI-FW. The probiotic treatments, R0011, R0052, SB, and PROTO showed a significant ($p < 0.05$) decrease of MIF production at T = 24 h when compared to CDI-FW blank. A tendency for this attenuation was also observed in the probiotics R0175, combination 2, and combination 3, although this did not reach statistical significance. Interestingly, CDI-FW supplemented with R0343 was the only treatment that showed a significant ($p < 0.05$) effect of time, indicating no effect on MIF production in CDI-FW.

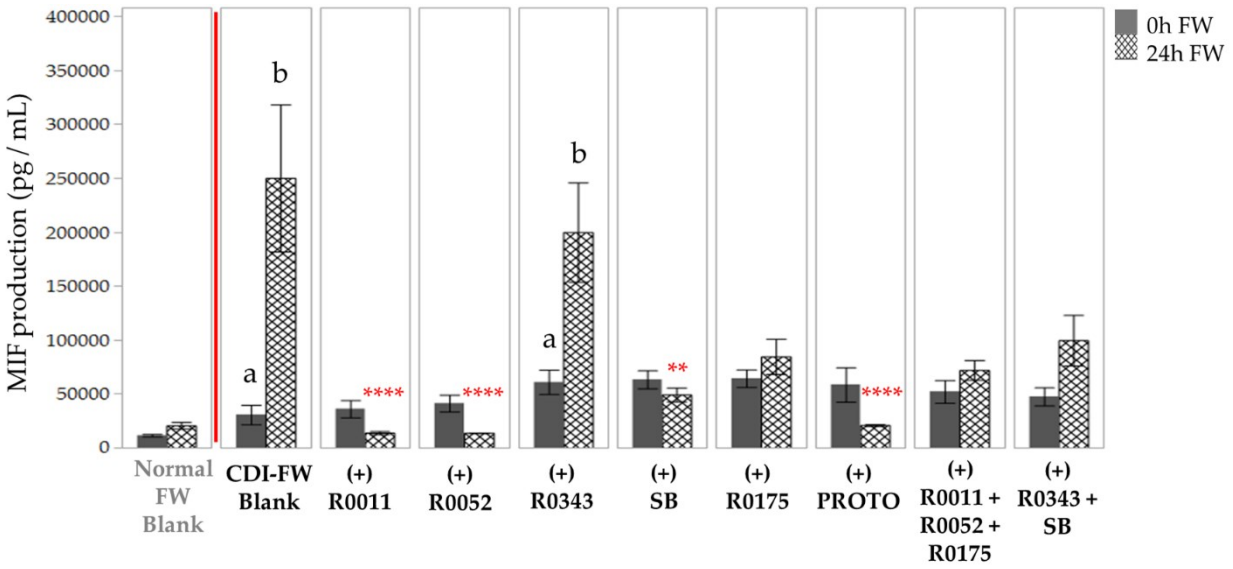

Figure 6.5. Detection of macrophage inhibitory factor (MIF) production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (▢) cells treated with FW collected at T = 0 h, and (▨) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different (p < 0.05). The symbol * represents significant differences (p < 0.05 = *; p < 0.01 = **; p < 0.001 = ***; p < 0.0001 = ****) between treatment and CDI-FW Blank at T = 24 h . R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

T84 cells exposed to CDI-FW also resulted in a 100 % increase of TNFRSF8 production in comparison to normal FW at T = 24 h (**Figure 6.6**). Each of the probiotic treatments in CDI-FW, except for combination 3 (R0343 + SB) showed a significant (p < 0.05) decrease in TNFRSF8 production at T = 24 h when compared to CDI-FW Blank.

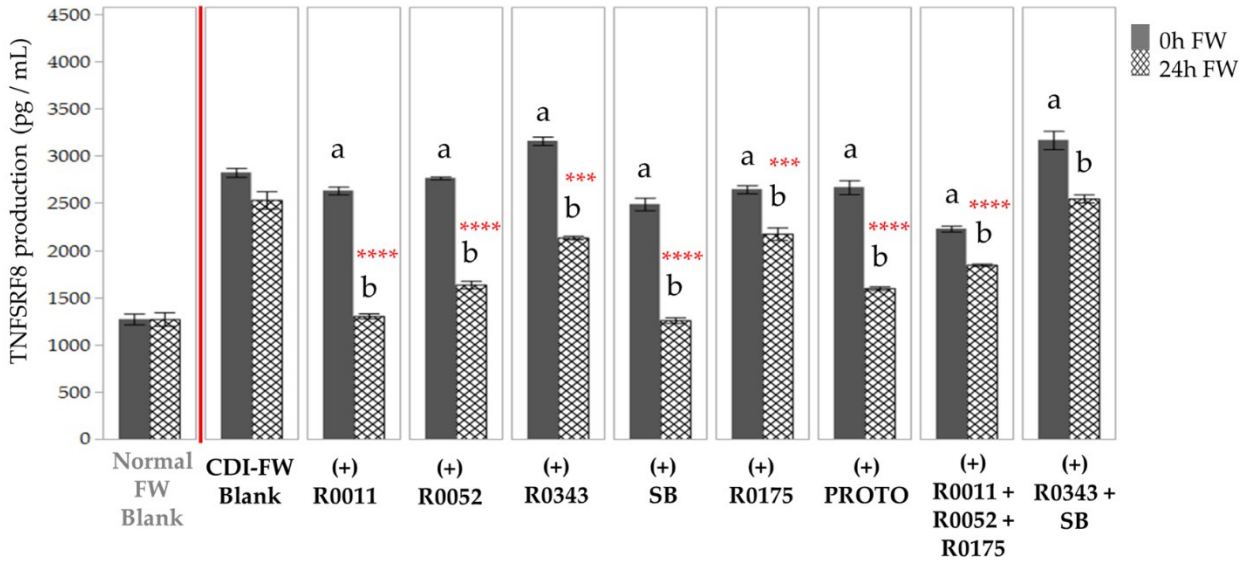

Figure 6.6. Detection of tumor necrosis factor receptor superfamily member 8 (TNFRSF8) production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. ( ) cells treated with FW collected at T = 0 h, and ( ) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences ($p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****) between treatment and CDI-FW Blank at T = 24 h . R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

The data of IL-32 followed a similar trend, showing a 69 % increase in the production of the cytokine when T84 cells were exposed to CDI-FW (T = 24 h) in comparison to normal FW. All probiotic-supplemented CDI-FW, with the exception of R0343 and combination 2 (R0011+ R0052 + R0175) showed a significant ($p < 0.05$) reduction of IL-32 production at T = 24 h (**Figure 6.7**).

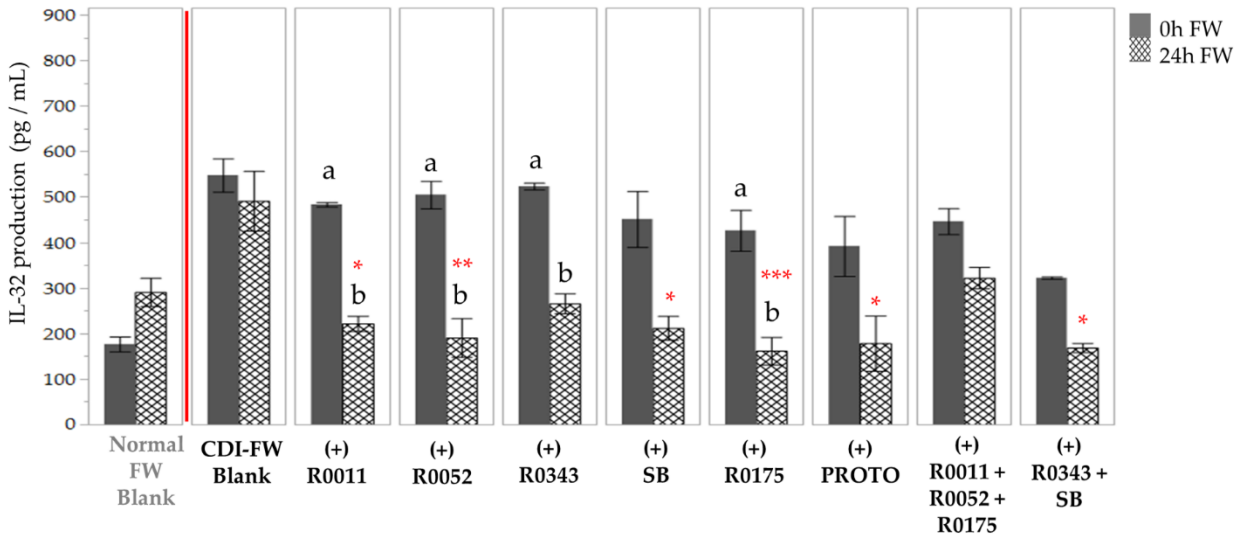

Figure 6.7. Detection of Interleukin-32 (IL-32) production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (▢) cells treated with FW collected at T = 0 h, and (▨) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences ($p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****) between treatment and CDI-FW Blank at T = 24 h . R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

Intriguingly, other commonly associated inflammatory cytokines in the context of CDI in *in vitro* studies, such as interferon (IFN)-γ, TNF-α, IL-6 or IL-1β [59], did not show significant activation in our study, showing detection levels below 100 pg / mL (**Supplementary Figures S6.4 - S6.12**). This could be presumably due to initial activation of IL-8 in T84 cells that is observed within a few hours of treatment exposure [60], whereas other cytokines such as TNF-α, have been shown to be produced in significant amounts only after 48 h of TcdA exposure [61]. Moreover, neutrophil activation and localization are seen to be the key hallmarks of CDI-mediated inflammation,

possibly explaining the prominent upregulation of both IL-8 and CXCL5 [8]. Despite the low concentrations observed, CDI-FW exposed cells showed an increased production in key cytokines, TNF-α and IL-1β (298 % and 593 % increase at T = 24 h respectively; **Supplementary Table S6.1**). No overall effect of CDI-FW was observed on IL-6 and IFN-γ concentrations. Interestingly, the anti-inflammatory cytokine, IL-10, was attenuated in CDI-FW exposed cells (72% decrease at T = 24 h; **Supplementary Table S6.1**). Additionally, IL-11, which plays a role in mediating an anti-inflammatory response through its interaction with the IL-6 signalling receptor [62], was also observed to be produced in association with both CDI-FW and normal FW. In this case, however, CDI-FW exposure did not result in a significant difference in its production when compared to normal FW (**Supplementary Figure S6.3**), suggesting that this cytokine did not play a contributing factor in CDI-mediated inflammatory response.

Overall, the findings from chemokine and cytokine analyses showed the ability of CDI-FW to induce production of inflammatory markers in T84 cells characterized by IL-8 and CXCL5 chemokines, and the cytokines MIF, TNFRSF8 and IL-32. In previous studies by Feghaly RE *et al.* 2013 and by Dieterle *et al.* 2020, increased levels of IL-8 and CXCL5 were characteristic of the immune profiles of CDI patients, and were key in predicting clinical severity in those patients [9,12]. Our findings further reiterate the association of IL-8 and CXCL5 with CDI-mediated effects in the gut mucosa. The results from our cytokine analyses, however, demonstrated the presence of cytokines such as TNFRSF8, IL-32 and MIF that have been sparsely documented with respect to CDI. The role of MIF in the intestinal lumen is thought to be multifaceted, where several *in vitro* studies have demonstrated its ability to maintain epithelial barrier function and integrity by modulating the epithelial tight-junction proteins [63]. Moreover, MIF has also been associated several other roles such as inhibition of cellular apoptosis by modulating mitogen-activated protein kinase signaling [62], eradication of gram-negative pathogens through macrophage action [64], and in regulation of the magnitude of inflammatory response via glucocorticoid modulation [65]. With regard to CDI, however, the only experimental evidence to date elaborating the role of MIF is a study by Jose *et al.* 2018, which showed that in a mouse model of

CDI, systemic MIF was significantly upregulated, neutralization of which, led to a decrease in tissue inflammation, reduction in diarrhea, and increased survival [55]. To our knowledge, the role of TNFSRF8 and IL-32 in CDI-associated inflammation has not been examined previously. Their role in CDI could presumably be linked to their subsequent activation of the NF-κB pathway, which leads to the activation of cytokines such as TNF-α, IL-6, IL-1β, and IL-8, all of which are more often tested for and associated with CDI [66].

Importantly, the results from the present study show the ability of several single strain and multi-strain probiotic supplements in protecting against CDI-FW mediated inflammatory marker production (**Figure 6.2**). The probiotic treatments showed varying effects on each of the cytokines detected in this study, supporting the concept that probiotics exert strain-specific effects on the intestinal epithelium to modulate its functionality and immune function [44]. Amongst all the probiotics, the single strain treatments, *S. boulardii* CNCM I-1079 (SB) and *L. rhamnosus* R0011 (R0011) were consistently associated with significant changes in inflammatory cytokine production at T = 24 h (in 12 out of a total 16 cytokines detected). R0011 was associated with a significant ($p < 0.05$) decrease in the levels of CXCL5, TNFRSF8, IL-32, MIF, CCL21, CXCL10, CCL19, TNF-α, IL-1β, IL-6 and IFN-γ, and a significant ($p < 0.05$) increase in anti-inflammatory IL-10. Similarly, *S. boulardii* CNCM I-1079 showed a significant ($p < 0.05$) decrease in the levels of IL-8, CXCL5, TNFRSF8, IL-32, MIF, CCL21, CXCL10, CCL19, TNF-α, IL-1β and IFN-γ, and a significant ($p < 0.05$) increase in anti-inflammatory IL-10. These findings indicate similar modes of immunomodulatory action for *S. boulardii* CNCM I-1079 and *L. rhamnosus* R0011 in the context of CDI. *S. boulardii* has been previously shown to inhibit IL-8 production induced by TcdA in human colonocyte NCM460 cells [23], reduce TNF-α expression in a hamster model of CDI [67], and exhibit immunomodulatory activity in the gut in clinical studies [68-70]. Although *L. rhamnosus* R0011 has not been previously examined in association to CDI, studies have demonstrated its ability to down-regulate IL-8 production in HT-29 epithelial cells via secretion of a range of bioactive molecules [25]. In a recent study by Jeffrey *et al.* 2020, the secretome of *L. rhamnosus* R0011 was shown to attenuate pro-inflammatory gene expression in HT-29 cells

challenged either with TNF-α or *Salmonella typhimurium* secretome [26]. In support of the present findings, *L. rhamnosus* R0011 secretome induced production of MIF, leading to a downregulation of NF-κB expression, indicating that MIF exhibits a context-dependent inflammatory response to bacterial challenges [28].

Amongst the rest of the probiotics, ProtecFlor™ was most effective (significant decrease observed at T = 24 h in 7/16 cytokines) followed by *L. helveticus* R0052 (6/16), *B. longum* R0175 (6/16), *L. rhamnosus* GG R0343 (3/16), combination of R0011+R0052+R0175 (3/16), and combination of R0343+SB (3/16). Interestingly, *L. rhamnosus GG* R0343, which has been demonstrated to prevent cytokine-induced apoptosis in several intestinal epithelial cell models [27] and to modulate serum cytokines in several clinical studies [71-73], did not appear to show any major effects on T84 cell viability or CDI-FW mediated inflammatory response. This could presumably be due to its differential mode of action that utilizes its pili to adhere onto the gut lumen followed by interaction with Toll-like receptor 2 and lipoteichoic acid to modulate IL-8 mRNA expression [74]. Thus, the absence of an intestinal lumen in the *in vitro* GI model used in this study could have potentially altered its immunomodulatory ability.

6.5 CONCLUSIONS

In summary, the results from our study demonstrated, for the first time, the ability of FW from CDI microbiota to adversely affect T84 cellular health and increase inflammatory marker production, including previously unreported cytokines. Specifically, CDI-FW exposure to T84 cells caused a significant ($p < 0.05$) decrease in cell viability (**Figure 6.1**). CDI-FW exposure also increased production of several pro-inflammatory markers, including the chemokines IL-8 and CXCL5, and the cytokines TNFSRF8, IL-32 and MIF, amongst others (**Figure 6.2**). Whilst the role of IL-8 and CXCL5 in CDI pathophysiology have been previously documented [11], this study shows a potential role of cytokines TNFSRF8, IL-32, and MIF in CDI-mediated inflammation. Notably, the present study shows the ability of several probiotics to protect against the CDI-FW mediated

inflammatory response. Probiotic supplementation in CDI-FW exhibited strain-specific modulation of cellular health and inflammatory marker production, amongst which, *S. boulardii* CNCM I-1079 and *L. rhamnosus R0011* were the most effective. In particular, these findings demonstrate that *L. rhamnosus* R0011 could play a role in modulating CDI-mediated inflammation, whilst further elucidating the potential modes of action of *S. boulardii* in this regard. Overall, the present data supports the concept that probiotic strains can modulate CDI-mediated changes in the lumen to impact upon the subsequent inflammatory response. This study also provides a novel systematic testing approach to assess probiotic efficacy in CDI involving cytokine production mediated by CDI fecal microbiota.

6.6 ACKNOWLEDGMENTS

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6.6 SUPPLEMENTARY INFORMATION

Supplementary Table S6.1. Percent change of cytokine production of CDI-FW Blank when compared to Normal FW Blank.

Production range (pg / mL)	Cytokine	% change at T = 0 h	% change at T = 24 h
0-50	**CCL19**	-40.37	-33.02
	IL-1β	452.85	593.33
	IFN-γ	52.63	17.39
	IL-6	-5.00	-1.69
	CCL13	45.45	-72.60
	IL-10	-35.22	-72.32
	IL-16	-42.41	-63.87
	TNF-α	149.85	298.15
51-200	**IL-11**	16.50	13.09
	CXCL10	37.61	106.52
201-3000	**CCL21**	31.65	13.60
	TNFRSF8	122.22	99.28
	IL-8	139.40	212.11
	IL-32	209.75	68.73
	CXCL5	154.21	104.76
10,000 +	**MIF**	167.48	**1111.29**

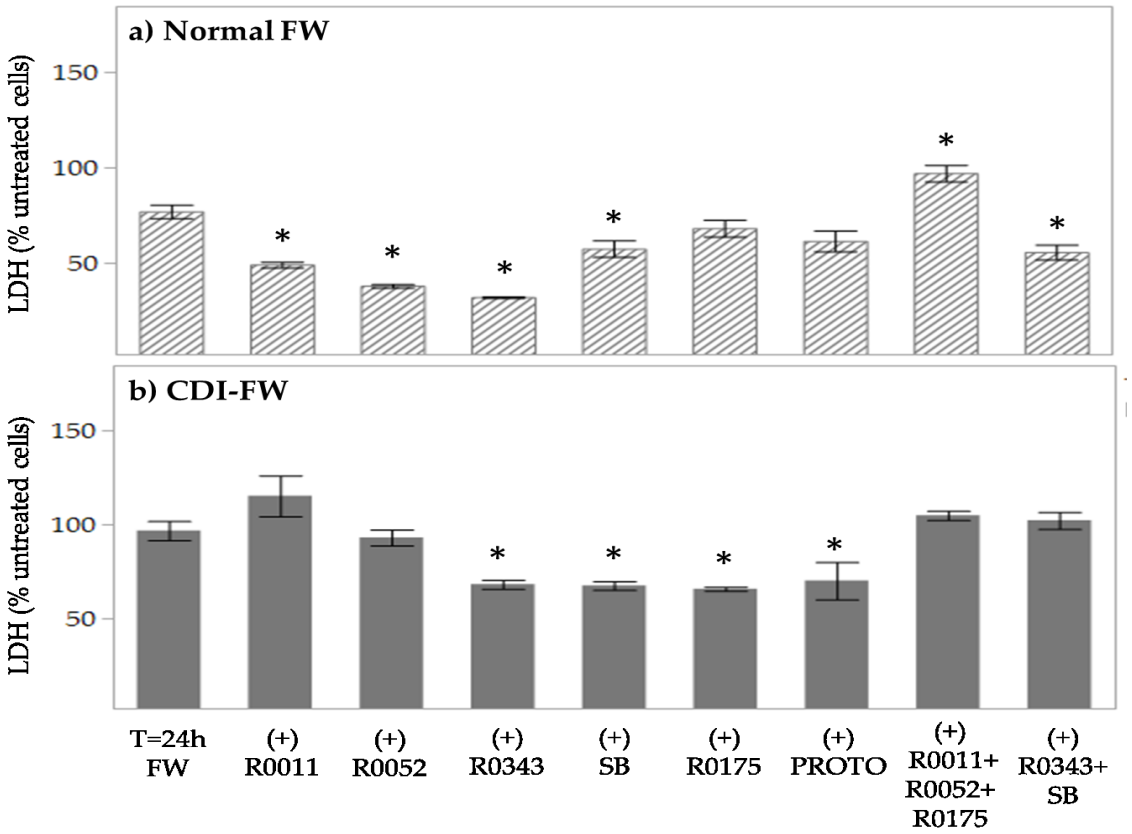

Figure S6.1. Determination of lactate dehydrogenase (LDH) following exposure of T84 cells to fecal water (FW) treatments. A) Cells treated with Normal FW, and, B) Cells treated with *Clostridioides difficile*-infected (CDI) FW. Values are shown as mean ± SEM. The symbol * represents significant differences between the means of treatments ($p < 0.05$). R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

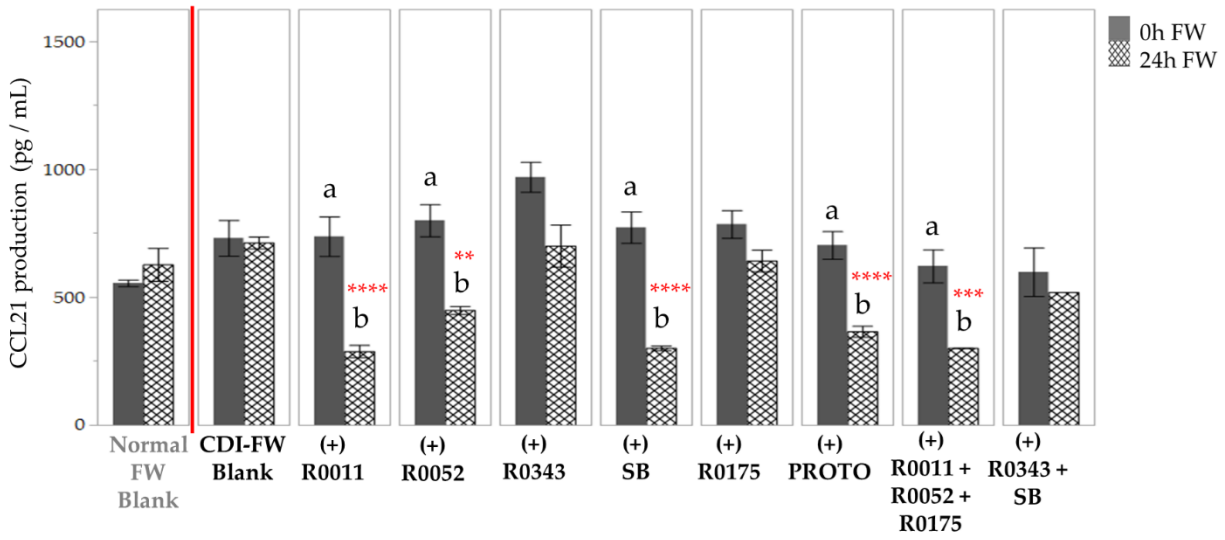

Figure S6.2. Detection of chemokine (C-C motif) ligand 21 (CCL21) production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (■) cells treated with FW collected at T = 0 h, and (▩) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences ($p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****) between treatment and CDI-FW Blank at T = 24 h. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

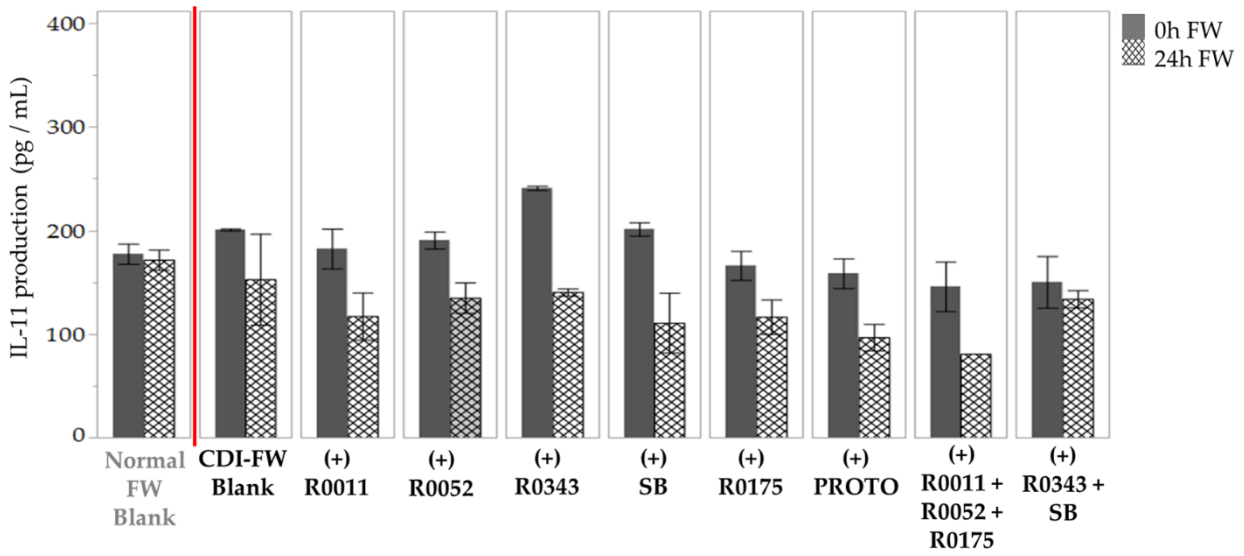

Figure S6.3. Detection of Interleukin-11 (IL-11) production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (■) cells treated with FW collected at T = 0 h, and (▩) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

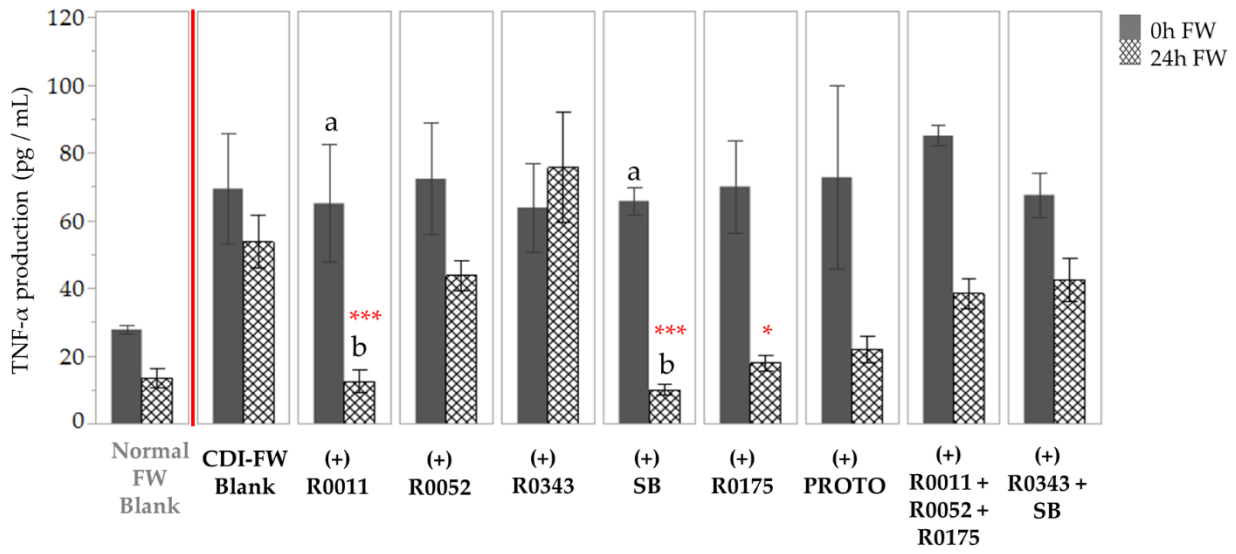

Figure S6.4. Detection of Tumor necrosis factor (TNF) - α production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (■) cells treated with FW collected at T = 0 h, and (▩) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences ($p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****) between treatment and CDI-FW Blank at T = 24 h. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

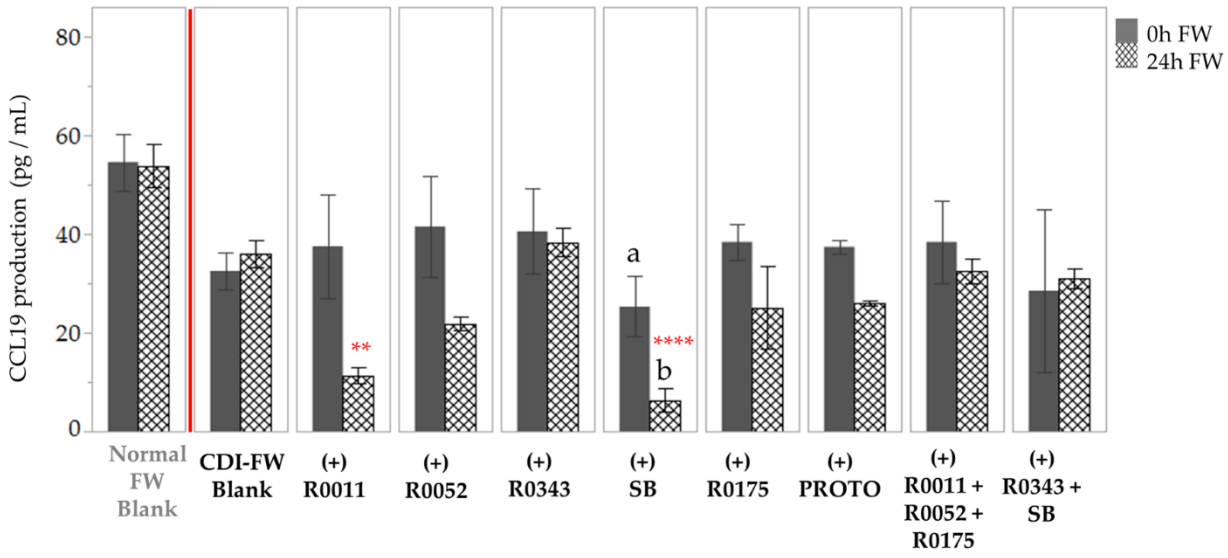

Figure S6.5. Chemokine (C-C motif) ligand 19 (CCL19) production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (■) cells treated with FW collected at T = 0 h, and (▩) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences ($p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****) between treatment and CDI-FW Blank at T = 24 h. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

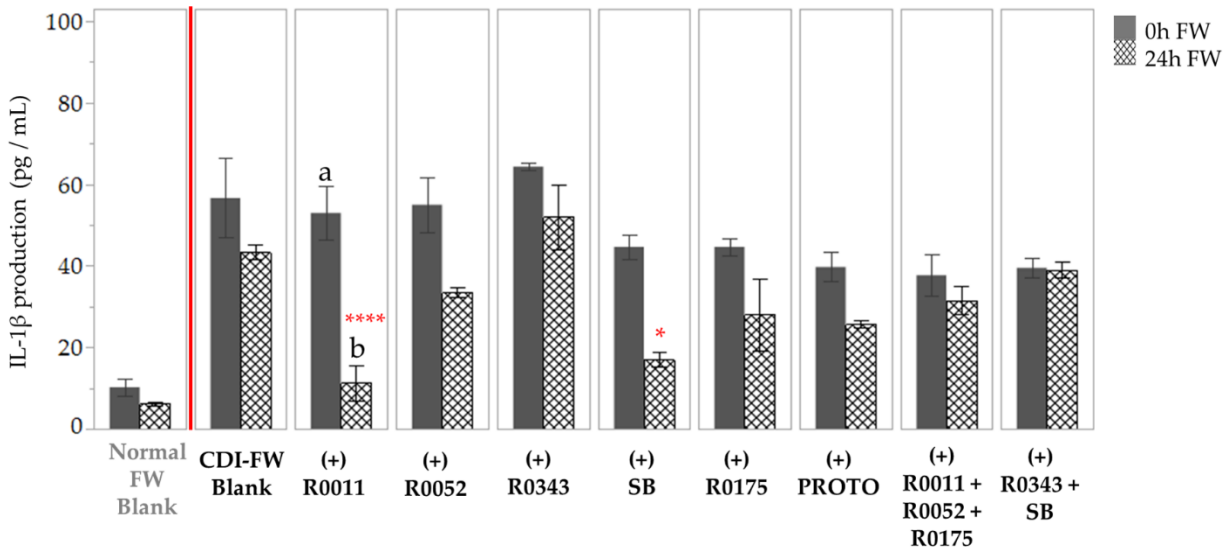

Figure S6.6. Interleukin (IL) -1β production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (■) cells treated with FW collected at T = 0 h, and (▩) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences ($p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****) between treatment and CDI-FW Blank at T = 24 h. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

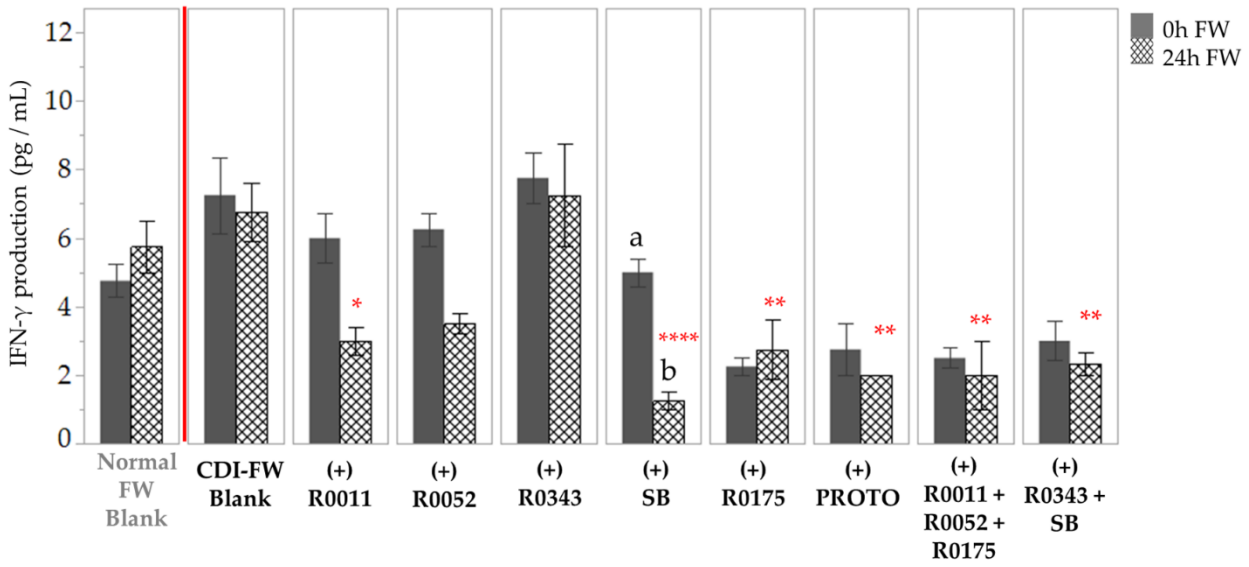

Figure S6.7. Interferon (IFN) -γ production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (■) cells treated with FW collected at T = 0 h, and (▩) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences ($p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****) between treatment and CDI-FW Blank at T = 24 h. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

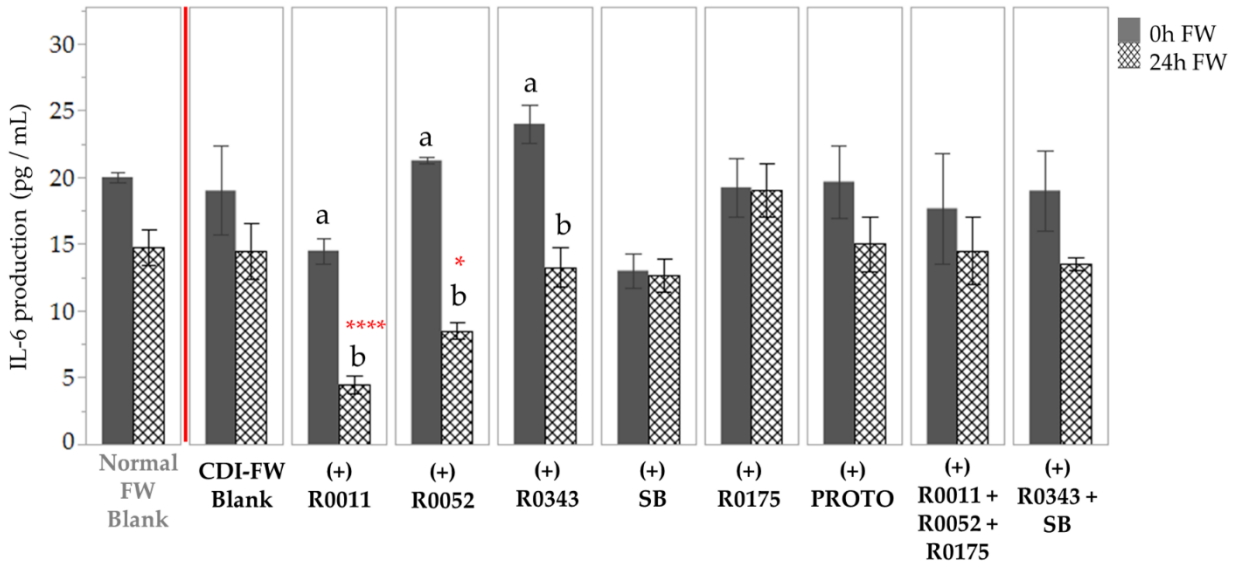

Figure S6.8. Interleukin (IL) -6 production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (■) cells treated with FW collected at T = 0 h, and (▩) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences ($p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****) between treatment and CDI-FW Blank at T = 24 h. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

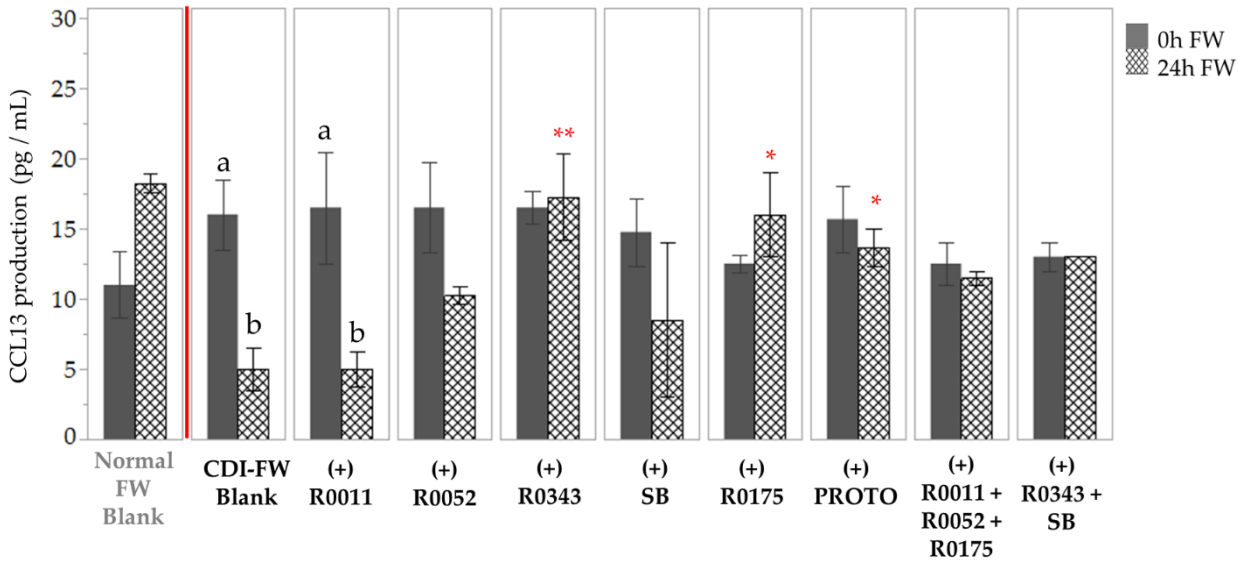

Figure S6.9. Chemokine (C-C motif) ligand 13 (CCL13) production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (■) cells treated with FW collected at T = 0 h, and (▩) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences ($p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****) between treatment and CDI-FW Blank at T = 24 h. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

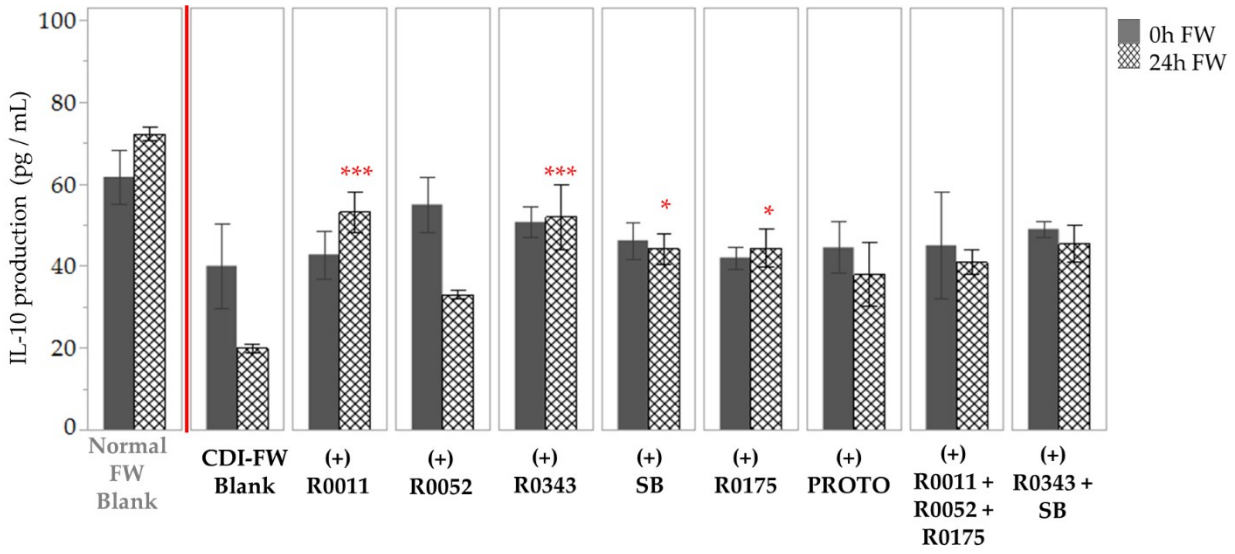

Figure S6.10. Interleukin (IL) -10 production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (■) cells treated with FW collected at T = 0 h, and (▩) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. The symbol * represents significant differences ($p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****) between treatment and CDI-FW Blank at T = 24 h. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

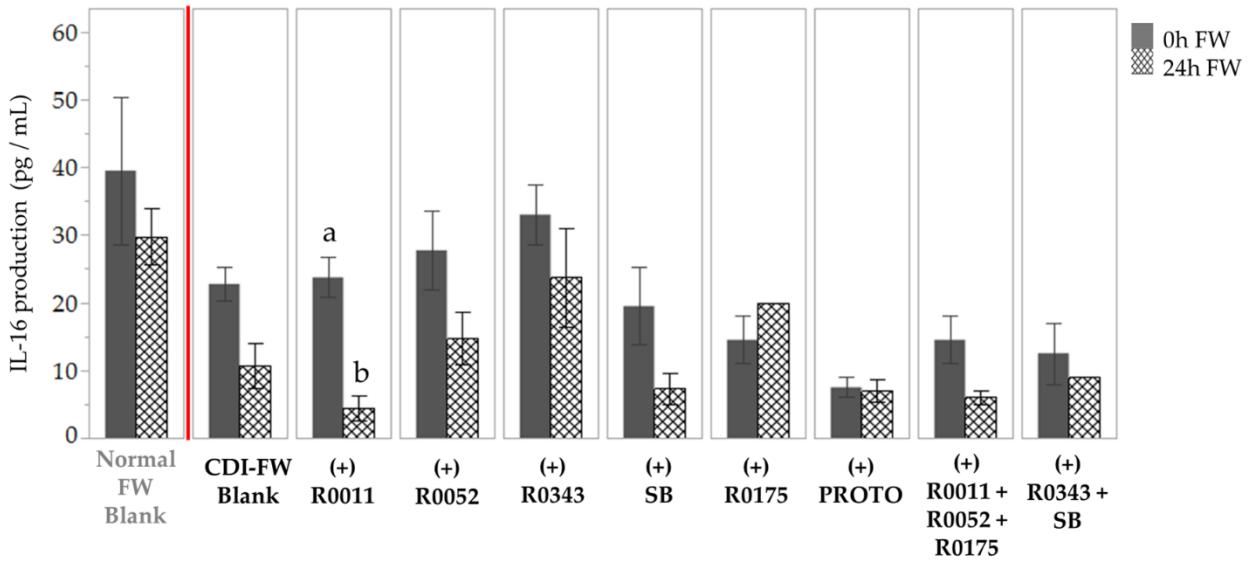

Figure S6.11. Interleukin (IL) -16 production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (■) cells treated with FW collected at T = 0 h, and (▩) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

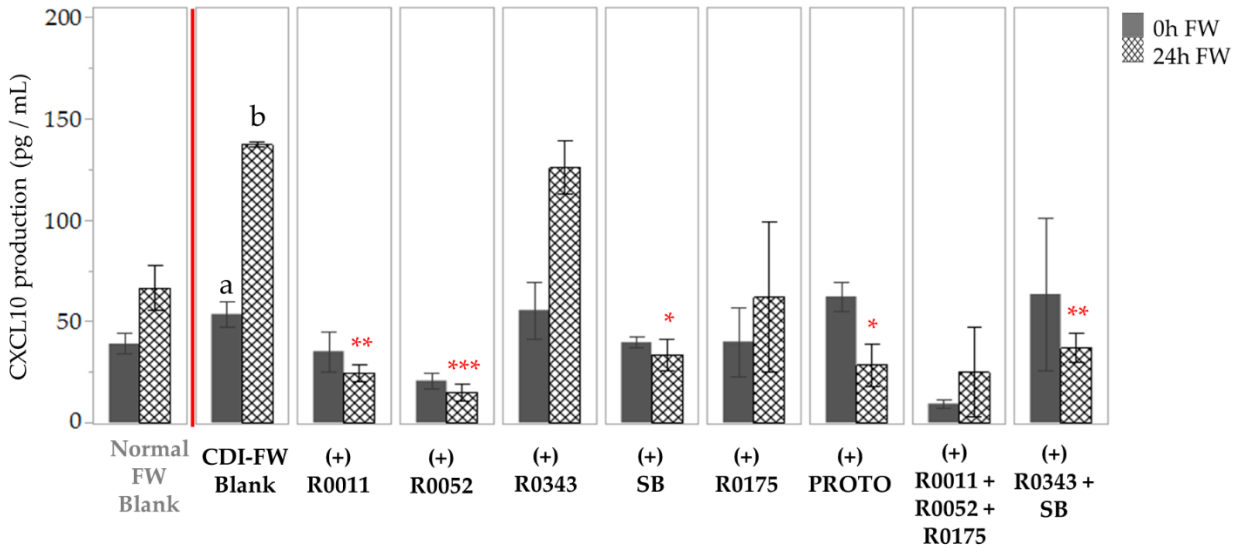

Figure S6.12. C-X-C motif chemokine ligand 10 (CXCL10) production following exposure of T84 cells with *Clostridioides difficile*-infected (CDI) fecal water (FW) treatments as measured by multiplex assay. (■) cells treated with FW collected at T = 0 h, and (▩) cells treated FW collected at T = 24 h. Values are shown as mean ± SEM. Means at time points within treatments without a common letter are significantly different ($p < 0.05$). The symbol * represents significant differences ($p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****) between treatment and CDI-FW Blank at T = 24 h. R0011 = *L. rhamnosus* R0011; R0052 = *L. helveticus* R0052; R0343 = *L. rhamnosus* GG R0343; SB = *S. boulardii* CNCM I-1079; R0175 = *B. longum* R0175; PROTO = ProtecFlor™; R0011+ R0052+R0175 = combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; R0343+SB = combination of *L. rhamnosus* GG R0343 and *S. boulardii* CNCM I-1079.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

This thesis describes the use of an *in vitro* GI model to simulate *C. difficile* infection conditions in the human gut lumen in order to study its effects on gut microbiota and its functionality, and, how probiotics might modulate some of those effects. Through a series of studies, the present work describes how *C. difficile* infection affects different aspects of GI health and function, and, how certain probiotics show the potential to protect against its associated toxicity.

7.1 Utilization of an *in vitro* Gastrointestinal Model to Study Changes in Gut Microbiota

Human colonic microbiota is a rich and highly diverse microbial community that is critical in the maintenance of human physiology through metabolic regulation and intestinal homeostasis [101,102]. Disruption or alteration of this microbial community, known as dysbiosis, has been widely associated with the development of physiological disorders such as metabolic, immune disorders and mental health disorders [102,181,182,183,184]. Thus, numerous studies have sought to further understand the role of the microbiota in various physiological processes such as food digestion, protection against pathogens and modulation of host immunity. Due to complexity in accessibility and ethical considerations of evaluating dynamic changes in the *in vivo* gut lumen to different stimuli, studies have instead sought to utilise *in vitro* fermentative models to simulate the gastrointestinal tract [185]. In Study 1, we assessed a modular *in vitro* computer-controlled dynamic gastrointestinal model (GI model) developed in our lab for its ability to simulate GI conditions, including the microbial composition and metabolic function. In our GI model, each of the physiological parameters were computer-controlled and closely monitored through the use of several pieces of custom hardware that monitored and regulated digestion, colonic pH, body temperature of 37˚C, and anaerobic conditions. The gut microbiota was simulated using fecal samples from a healthy donor with no history of GI disorders or antibiotic use in the last 6 months. Fecal microbial composition has been previously demonstrated to

represent the distal colonic microbiota [186]. These fecal samples are often used as inoculates in fermentation models to simulate the microbiota in the different colonic sections by adapting the fecal microbiota for a period of 2 weeks under controlled colonic pH and supplementation with microbial growth media, known as GI food [28]. This length of fecal microbiota adaptation is generally accepted across most GI models and was elaborated in a study by Possemiers *et al.* 2004 [187] where they observed that the *Bacteroides* phyla obtained stability within a 10-day period whereas less-abundant bacteria such as *Lactobacilli* needed 15 days of stabilization. Moreover, the study also noted stable metabolic functionality in terms of short chain fatty acid (SCFA) production required at least 15 days. The findings from our study after a two-week fecal inoculation showed the presence of a diverse intestinal microbiota for the length of the experiment. Moreover, metabolic functionality analysis showed a strong correlation of our findings to those from other in vitro models and reported in vivo data [28,188-190]. Interestingly, the data from our study showed that the microbial profile stabilized with distinct community formation in each colonic section within a week of fecal inoculation. This was further supported by the results of the microbial function analyses through the production of SCFA and gaseous by-products. These findings suggest that the use of small-scale bioreactors in the GI model might have a potential benefit in reducing the two-week stabilisation of fecal inoculates.

The methodology followed in Study 1 involved fecal microbiota stabilization for a period of 2 weeks, after which, usually a two to three-week period of treatment would follow [187]. However, in cases where numerous treatments need to be evaluated, this procedure can be extremely lengthy and laborious. Importantly, replicating the same fecal inoculum and its resulting microbial composition across different experimental repeats poses a considerable challenge. This has been previously noted in studies assessing variation between microbial communities between individuals and within the same donor over time [191]. In these instances, adopting batch culture fermentation is deemed more suitable. Batch culture fermentation involves the use of several bioreactors to independently simulate digestion rather than through a series of interconnected vessels, followed by fecal inoculation and subsequent addition of

treatments in each vessel. Most reported batch culture studies are run for a period of 12-48 h. This presents obvious drawbacks compared to the full methodology as it does not allow for enough time for fecal inoculum to stabilise to the colonic microbiota nor does it replicate the dynamic movement od digest from one section to another. However, despite these concerns, batch culture fermentation in GI models where environmental conditions are well controlled have shown evidence that they can effectively simulate metabolic function of the microbiota and maintains microbial stability for at least 24 h [192]. This allows for the potential to use this methodology as an effective screening tool in cases of multiple treatments, such as the studies described in this thesis. Thus, in our subsequent Studies 2-4, we utilized the batch fermentation methodology in our gut model to assess various probiotics for their effects in simulated *C. difficile* infected microbiota.

7.1.1 Assessment of Metabolic Function Through Biomarkers in Faecal Water

The major functions of the gut microbiota are broadly classified into its trophic action on intestinal epithelium, its ability to combat colonisation of pathogens in the gut lumen, and more importantly, its metabolic functions that are involved in a host of physiological functions. These metabolic activities include fermentation of substrates from the small intestine such as complex carbohydrates that generate SCFA or proteins that are degraded into phenolic and indolic metabolites along with various gaseous by-products such as ammonia and hydrogen sulfide [190]. Thus, changes in the diet or in the colonic milieu due to gastrointestinal disorders or other stimuli are usually reflected in the metabolites produced by the gut microbiota. Moreover, several studies have shown that assessment of metabolic markers might be more useful in assessing gastrointestinal health and disease as opposed to only assessing changes in microbial composition. This is presumably due to various factors such as the natural variations seen in the microbiota of an individual over time or that changes in the microbiota may not necessarily represent changes in metabolic capacity as seen by [191]. Furthermore, several studies, including those in presented in this thesis, have shown that various stimuli can cause changes in metabolic

function without affecting the microbiota [193,194]. In our Studies 1-4, metabolic function was measured through the analyses of SCFA and gaseous by-products (NH^+_4 and H_2S) generation. Moreover, the antioxidant capacity of the fecal water was assessed as an indirect maker for gastrointestinal health.

SCFAs, which are end products of fibre fermentation by the anerobic gut microbiota, have been well characterised for their numerous beneficial roles in human physiology, including maintenance of the gut barrier integrity, regulation of host immune response and modulating appetite [195]. Dysregulation of SCFA production is usually associated with changes in microbial composition induced through diet or stressors such as antibiotic usage or through inflammatory GI disorders that affect the mucosal epithelium such as colitis [196]. Monitoring colonic fermentation via SCFA production has proved to be a useful tool in assessing changes in the colonic milieu. In the results from Study 1, SCFA concentrations of 20-60 mM were found in each of the colonic sections, in consonance with previously reported values from in vitro GI models and in vivo gut lumen [28,188]. Although SCFA concentration in colon is largely determined by dietary intake of fibre, the proportions of the prominent SCFAs (acetate, propionate and butyrate) have been demonstrated to be indicative of the gut microbial composition. Complex associations between the three prominent bacterial phyla regulate SCFA production, where *Bacteroidetes* and *Actinobacteria* produce acetate and propionate as primary end products of fermentation, and *Firmicutes* utilize the acetate produced to generate butyrate [195]. The findings from Study 1 showed that the ratio of acetate:propionate:butyrate was approximately 60:20:20, which follows similar values reported by studies of the gut lumen [197,198]. SCFA results from Study 3 show a similar range of 25-60 mM total SCFA, however, the proportions of individual SCFA differ. The ratio of acetate:propionate:butyrate was found to be approximately 40:40:20, showing an increased presence of propionate in the normal fecal sample. In further analysis of the microbial composition, this was associated to an increased growth of fermenter bacteria, from the *Megaspheara* spp.

These symbiotic associations also regulate production of fermentation by-products such as gases (hydrogen, carbon dioxide, methane and ammonia) to avoid accumulation and promote excretion. For instance, saccharolytic bacteria from the *Bacteroidetes* phyla produce SCFA through the phosphoenolpyruvate pathway generating CO_2 and H_2 as by-products. These gasses are then utilized by Archaea to produce methane, and in the case of acetogens, produce acetate [199]. Thus, the monitoring of the SCFAs and gaseous by-products can be an important biomarker for assessing gut microbial community dynamics. Two such gaseous end-products assessed in our Studies (1 and 3) were ammonia and H_2S. H_2S plays an important role in the maintenance of the mucosal layer when production is regulated by the host and microbiota, but can be detrimental when accumulation occurs, leading to acute inflammation [200]. The production of H_2S occurs through degradation of sulfur-containing amino acids cysteine and methionine by bacteria such as *Enterococci*, *Enterobacteria,* and *Clostridia*, or through sulphates used in production of ATP by sulfate-reducing bacteria such as *Desulfovibrio* and *Desulfobulbus* [201]. Interestingly, H_2S generation involves the utilization of products from SCFA production such as molecular hydrogen and pyruvate as electron donors, showing the interplay of sulphate-reducing bacteria with SCFA producers. In Study 1, H_2S values of 5-15 mM were observed, whereas in the batch culture study, values between 3-10 mM were observed, both of which fall within reported healthy ranges of H_2S in human colon [202]. In Study 1, no significant changes were observed in H_2S production over the two-week period. Similarly, in the batch culture studies, H_2S levels stayed stable for the 24 h period in normal fecal samples.

The second gaseous by-product assessed in our studies was ammonia (NH^+_4 and NH_3). Ammonia in the colon is produced through deamination of dietary protein and through hydrolysis of endogenous urea [203]. Excess levels of ammonia produced by the microbiota have been associated with the development of hepatic coma [203]. Moreover, in a study by Cremin *et al.* 2003 excess ammonia was shown to inhibit oxidation of SCFA by colonocytes for energy utilisation [204]. Ammonia concentrations of 10-30 mM and 2-25 mM were reported in the two-week study and the batch culture fermentation methodologies respectively. In Study 1, no

significant changes were observed in ammonia production over the two-week period however, in the batch culture, study (Study 3), ammonia levels significantly increased over the 24h period. Although this increase was within healthy range of ammonia in the lumen, it was presumed that this stemmed from the closed conditions of the batch reactor, thereby leading to an increase in fermentation by-products.

7.1.2 Assessment of Gut Microbial Composition

The results of metabolic analyses of the faecal water from batch culture studies (Studies 2-4) and full-model fermentation studies (Study 1) show the ability of the model to simulate a healthy colonic milieu. Furthermore, they add to the literature showing that despite a considerable difference in model set-up and methodology, they both showed similarities in metabolic function as assessed by SCFA, H_2S and ammonia production. The results from bacterial sequencing, however, is where the difference in methodology showed an impact on the microbial composition. In Study 1, the results of microbial composition showed the ability of the model to simulate a diverse microbial community that stayed stable for the most part of the 2-week period, despite a growth of opportunistic bacteria from the *Veillonellaceae* family observed in the latter phases of the experiment. Relative abundance data showed the presence of the major phylogenetic groups *Actinobacteria*, *Bacteroidetes* and *Firmicutes* in concordance with reported phyla found in the gut lumen [187]. The reason for this shift in microbial community could stem from complex microbial interactions that compete for substrate utilization. A synthetic food composition previously optimised by Molly *et al.* 1993 [28] was utilized in the model to maintain a stable microbial growth however, recent studies have shown better growth and stability of the microbiota when the synthetic food is supplemented with prebiotics such as fructooligosaccharides [205]. However, despite this shift in microbial composition, both alpha and beta analysis of the 16S sequencing data showed that, for the most part, there were no significant perturbations in the microbial richness and community structure across the two-week period.

The results of metabolic assays from Study 1 and the batch culture studies in Studies 2-3 show the ability of the model to simulate metabolic function as is seen in *in vivo* gut lumen. Moreover, the results from Study 1 shows that a complex and stable gut microbial composition can be represented in the model as well. In the batch culture Studies 2-3, microbial composition shows good reproducibility, but lacks in the diversity and community structure as seen in the full model (2-week continuous vessel method) study.

7.2 Research Paper 2: Probiotic Supplementation is Associated with Increased Antioxidant Capacity and Copper Chelation in *C. difficile*-Infected Fecal Water

7.2.1 Contributions and Strengths

The findings from Study 2 revealed that *C. difficile* infection in our gut model was associated with dysregulation of antioxidant capacity of the fecal water. Probiotic supplementation in *C. difficile*-infected fecal water (CDI-FW) showed the potential to stabilise antioxidant status which was correlated with increased copper chelation. To our knowledge, this study is the first to provide novel insights into *C. difficile*-mediated changes in overall antioxidant status of the colonic milieu and subsequent mediation by probiotic supplementation. CDI-FW showed a significant reduction in antioxidant status when measured by FRAP and DPPH antioxidant assays. The ability of *C. difficile* infection to cause changes in the redox status of the gut lumen is poorly understood despite a well-characterised pathophysiology of CDI. In a study by Fiorentini *et al.* 1999, it was reported that *C. difficile* toxins caused oxidative imbalance in epithelial cells through depletion of cytosolic glutathione (GSH) [206]. This was further supported in a study by Sievers *et al.* 2018, which conducted thiol proteome profiling of *C. difficile* to indicate its ability to alter redox status [209]. Furthermore, a study by Macchioni *et al.* 2017, showed the involvement of TcdB in upregulating reactive oxidation species (ROS) pathways through NADPH oxidase without mitochondrial involvement in glial cells [208]. Although no glial cells were present in our study, it

can be assumed that CDI potentially mediated similar mechanisms of action in the intestinal microbes.

CDI-mediated dysregulation of the antioxidant status was counteracted by the supplementation of both single-strain and multi-species probiotics. This was particularly apparent in the FRAP antioxidant assay where all our probiotic treatments showed a significant ($p < 0.05$) increase in total antioxidant status. No such effect was observed in the DPPH assay, possibly owing to the different properties of the radical used in this assay [209]. Interestingly, the results from FRAP assay showed that probiotics can act in a bi-phasic manner to exert their antioxidant properties. Multispecies probiotics (ProtecFlor™; combination of *L. rhamnosus* R0011, *L. helveticus* R0052 and *B. longum* R0175; combination of *L. rhamnosus* GG and *S. boulardii*), which showed lowered FRAP status in normal FW, were observed to positively affect the antioxidant status in CDI-FW. This was presumed to be due to the presence of a pro-oxidant status in CDI-FW that potentially activated their antioxidant defense mechanisms [210].

In a study by Lin *et al.* 1999, 19 different strains of lactic acid bacteria (LAB) were assessed for their ability to chelate iron and copper as possible modes of antioxidant action. The study reported strain-specific effects, with *S. thermophilus* 821 showing the highest iron chelating ability and *B. longum* 15 708 highest copper chelating ability [211]. Similarly, a study by Lee *et al.* 2006, showed that *L. casei* KCTC 3260 showed resistance to induced ROS bacterial culture conditions through its ability to chelate iron and copper [212]. Thus, it was hypothesized that probiotics might attenuate the antioxidant status of CDI-FW using these chelating abilities. The data from iron and copper chelating ability of FW showed that probiotics increased copper chelation in CDI-FW largely driven by the presence of *S. boulardii* in the supplements. Correlation analyses performed on the data from both antioxidant assays and the metal chelation assays showed a significant positive correlation for FRAP and copper chelation, whereas copper and iron chelation were significantly negatively correlated. This indicates that the probiotics which showed an increase in FRAP antioxidant status were also likely to show an ability to chelate

copper. Surprisingly, none of the probiotics tested showed the ability to chelate iron in CDI-FW despite showing a significant ($p < 0.05$) increase in normal FW. This has been stipulated due to the regulation of Fur (ferric uptake regulator) by *C. difficile* to make iron less bioavailable during infections [213], thus accounting for the lack of iron chelation observed in our study. Overall, our findings suggest that probiotic strains with a higher reducing capacity and copper chelation ability can be considered as more suitable candidates for modulating CDI-mediated dysregulation of the redox status in the colonic milieu.

Study 2 shows several strengths over other studies assessing antioxidant potential of probiotic strains. A considerable portion of these studies measure the antioxidant potential of probiotics, including its ability to chelate iron and copper in simple bacterial cultures [214]. Very few studies detail probiotic ability to modulate pro-oxidant status in the colonic milieu, especially in the context of gastrointestinal infections such as that of *C. difficile*. Furthermore, the findings from our study suggest a novel mechanism of action of probiotics in attenuation of CDI-mediated disruption of the redox status in the colonic milieu through its reducing capability and chelation of copper.

7.2.2 Limitations and Future Considerations

While our study findings showed the ability of probiotics to counteract CDI-mediated dysregulation through changes in overall antioxidant status and metal chelation, the specifics of the mechanism involved in both CDI-mediated pro-oxidant status and the subsequent action of probiotics remain unclear. Although an effort was made to determine ROS species generated by CDI in the colonic milieu, as indicated by the nitrite, nitrate and protein carbonyl assays, this did not elucidate underlying mechanisms behind dysregulation of redox status. One possible explanation could be that in order to accurately detect ROS species production in the reactors, real-time flow-though analyses is required, an expertise we did not have access to. Moreover, the *in vitro* GI model used in this study lacks the presence of host epithelial or immune cells which possess the ability to interact with the gut microbiota to regulate oxidative stress. Although this

has been solved by the addition of an epithelial cell module by Marzorati and group [215] to a GI model, the integrated system has not been used in the presence of fecal samples and can be complex to work with when dealing with multiple treatments. The work-around used in these scenarios is to collect FW digests from the model and apply them as treatments on various cell lines. Regarding our study, it would be interesting to assess the effects of probiotics and CDI on cytosolic thiols or mitochondria-mediated oxidative stress pathways. Lastly, despite the role of probiotics to chelate copper in CDI-FW, it is possible that other mechanisms such as the probiotic production of GSH [214], antioxidant enzymes such as superoxide dismutase (SOD) and small peptides with radical scavenging activity [216] had an essential role in their modulation of antioxidant status. Thus, in future studies it would be interesting to assess the different modes of probiotic action in *C. difficile* infection, along with their effect on host cellular health and function.

7.3 Research Paper 3: Probiotic Supplementation in a *Clostridium difficile*-Infected Gastrointestinal Model Is Associated with Restoring Metabolic Function of Microbiota

7.3.1 Contributions and Strengths

In Study 3, we investigated the ability of probiotic supplementation to cause changes in the gut microbiota and the metabolic function in a *C. difficile* infected GI model. The results from our study showed that CDI mediated significant changes in the metabolic function and microbial diversity in the GI model. This is was supported by the decreased production of metabolites such as SCFA and H_2S, and through a decreased microbial community diversity when assessed by 16S rRNA sequencing. The findings from our study present the first piece of evidence that CDI-mediated changes in the metabolic activity of the gut microbiota were observed in an *in vitro* gastrointestinal model. Studies by Wilcox and group are the only other experimental pieces of evidence assessing *C. difficile* infection in the context of GI models, although their focus of

research is on assessing efficacy of potential antibiotics against *C. difficile* growth patterns and subsequent changes in the microbial composition [217-219]. In this regard, it was interesting to see that in their results, *C. difficile* spores did not result in major changes in the microbial composition, whereas in our model, clear differences were observed in normal and CDI microbiota. Microbial composition analyses from our findings showed a significant decrease in alpha diversity when compared to normal fecal samples and a shift in microbial community structure along with fermentation time as shown by beta diversity. Moreover, CDI samples were associated with higher levels of *Lactobacillaceae* and *Peptostreptococcaceae* in comparison to normal fecal samples, and lower abundance of *Bifidobacteriaceae* at T = 12 h and T = 24 h. These changes observed were in consonance with other pieces of literature that showed the microbiota of CDI patients were associated with lower microbial diversity and richness when compared to non-infected patients and exhibited an overrepresentation of bacterial families such as *Enterococcaceae*, *Lactobacillaceae* and *Clostridia* [220]. Thus, these results present evidence that our GI model was able to closely simulate the microbiota of CDI patients. Moreover, CDI-associated changes to the metabolic function were also observed in our study, supported by a significantly ($p < 0.05$) lower SCFA production and H_2S generation in comparison to normal FW.

Notably, the probiotic treatments employed in this study showed effectiveness in attenuating CDI-mediated changes in the metabolic function, although no major shifts in microbial composition were observed. *L. rhamnosus* R0011, *L. helveticus* R0052, *S. boulardii* CNCM I-1079, *B. longum* R0175, and the combination ProtecFlor^{TM} were all associated with a significant ($p < 0.05$) increase in total SCFA production and restoration of H_2S production to normal FW levels. The increase in SCFA was associated with increased acetate production by each of the supplements, and in the case of *B. longum* R0175 and *S. boulardii* CNCM I-1079, an increased butyrate production as well. The associations of several LAB strains and *S. boulardii* to increase SCFA in the human gut are well documented [221-223], although their ability to modulate metabolic function in the context of CDI has been seldom studied. Fascinatingly, the results from batch culture fermentation of these strains in GI food without the presence of any fecal inoculum,

showed that each strain was able to produce a range of SCFAs, but did not wholly account for the increased SCFAs observed in the CDI fecal samples. This suggests that these probiotic strains were able to modulate the metabolic function of the CDI microbiota to increase SCFA production, further supporting the clinical significance of this model to assess changes in the colonic environment. Although no quantifiable changes in the microbiota of probiotic-treated CDI samples were observed, the relative abundance data showed that all probiotics were associated with the protection of the *Bifidobacteriaceae* family, leading to a higher abundance at time 12 h and 24 h in comparison to the controls. This was interesting to note as it has been previously reported that the bacterium *B. longum* from the *Bifidobacteriaceae* family is associated with CDI-negative patients [224]. Moreover, the preservation of this bacterial family could be responsible for the observed changes in total SCFA production.

Overall, the findings from Study 3 have shown that the simulated CDI-model showed clinical resemblance to reported data on CDI patients, especially with regard to changes in the microbiota and altered metabolic function. Additionally, the study has shown that probiotics can be potent as an adjunct therapy in the management of CDI due to its ability to modulate the microbiota to restore metabolic function. Collectively these findings indicate that this methodology can be utilized for assess various probiotic strains for their effectiveness in CDI management as a pre-clinical screening tool.

7.3.2 Limitations and Future Considerations

The use of the batch culture fermentation method, as previously described, poses certain drawbacks over long term GI model methodologies, specifically with regard to the stability and diversity of the microbial community. Although this study showed similar changes with regard to certain bacterial families in comparison to CDI patient microbiota, the microbial community analyses showed the presence of only a handful bacterial families in CDI and normal FW samples that accounted for over 90 % of overall microbial composition. It also possible that due to the slow growth curves of the probiotics, no effect was observed on the microbial composition. Thus,

to better understand the role of CDI and subsequent changes by probiotic supplementation, it would be preferable to use a full-model method. In a similar manner to Study 2, another possible shortcoming is the lack of epithelial cells and a mucous layer. These additional physiological characteristics could give insight on the ability of *C. difficile* to colonize the gut lumen and subsequent alterations or competition by probiotics. In future studies, addition of mucin into the bioreactor vessels, as described by [225] could be considered.

7.4 Research Paper 4: Probiotic Supplementation in *Clostridioides difficile*-Infected Fecal Water Protects Against Cytotoxic Damage and Inflammatory Marker Production in T84 Cells

7.4.1 Contributions and Strengths

In Study 4 we investigated the ability of CDI-FW to cause changes in colonic adenocarcinoma-derived T84 epithelial cell lines. In addition, we assessed the effect of various probiotic-treated CDI-FW for their ability to modulate changes mediated by CDI-FW. The results from this study showed CDI-FW significantly ($p < 0.05$) decreased T84 cell viability and significantly increased ($p < 0.05$) cytotoxicity as measured by LDH release from the cells. Moreover, cells treated with CDI-FW showed an increased production of pro-inflammatory markers such as IL-8 and MIF amongst others. Each of these cytopathic effects were mediated in cells treated with probiotic-supplemented CDI-FW. Although strain-specific effects were observed, probiotic supplementation, overall, was associated with an increase in cell viability, decreased cytotoxicity and inflammatory cytokine production.

CDI-mediated cytopathic effects have been well documented through several studies of *C. difficile* toxins on epithelial and immune cell lines [226]. However, a majority of these studies assess the cytopathic effects of purified components of *C. difficile* such as their toxins A and B [227,228], or in the case of [229], the bacterium itself. In this regard, the use of fecal water

generated from CDI microbiota is seen to be more representative of the physiological conditions of the CDI colonic milieu. This hypothesis is further supported by studies that have shown the ability of *C. difficile* to induce inflammatory pathways through several means, such as its flagella or S-layer proteins, and even through a host of enzymes and metabolites [6]. Therefore, the effects observed through CDI-FW treatments of cells in Study 3, might be a more relevant indicator of CDI interaction with the gut lumen as opposed to toxin-challenged studies. In addition to testing to testing FW from CDI-microbiota, to our knowledge, this is the first study to assess the production of such a large range of chemokines and cytokines following *C. difficile* challenge to human epithelial cells. The results from our multiplex assays showed an upregulation of IL-8 and CXCL5 production in CDI-FW treated T84 cells which were in consonance with clinical studies showing the upregulation of these very chemokines in CDI patients and the essential role they play in predicting clinical severity [230,231]. Moreover, CDI-FW upregulated the production of TNFSRF8, IL-32 and MIF cytokines. Although *in vitro* cell culture studies have not associated TNFSRF8 and IL-32 upregulation with *C. difficile* or its toxins, their role in activation of the NF-κB pathway which further leads to the production of TNF-α, IL-6 and IL-1β, makes it in agreement with the studies showing regulation of the latter cytokines. One of significant findings of this study was the upregulation of MIF production observed in CDI-FW treated cells, especially at the end of the batch culture fermentation (T = 24 h CDI-FW). Until recently, the role of MIF in CDI pathophysiology was elusive. In the study by Jose *et al.* 2018, high systemic levels of MIF in CDI patients and CDI mice were observed. Furthermore, neutralization of MIF showed proved clinical outcomes in mice and showed potential in attenuating CDI-mediated colonic inflammation [232]. Our study further attests to the involvement of MIF in CDI-mediated cytopathy.

Notably, the cells treated with CDI-FW supplemented with probiotic strains, showed better cell viability, less cytotoxicity and markedly decreased pro-inflammatory cytokines. Of this these probiotic supplements, *L. rhamnosus* R0011 and *S. boulardii* CNCM I-1079 were consistently associated with significant effects in the prevention of the upregulation of cytokines and in

improving cellular health. The strain *L. rhamnosus* R0011 has previously shown potency to mediate intestinal cell chemokine and cytokine production following challenge with TNF-α through a variety of secreted molecules [233,234]. The role of *S. boulardii* in attenuation of pro-inflammatory cytokines, particularly in the context of TcdA and TcdB challenged cells and CDI animal models, has been previously noted [155,235]. The findings from this study also highlight the potential role of the other lesser associated probiotic strains (*L. helveticus* R0052, *B. longum* R0175, ProtecFlor™ and the combinations) with CDI in their modulation of proinflammatory cytokines, particularly that of MIF, TNFSRF8 and IL-32.

7.4.2 Limitations and Future Directions

An important consideration in study of CDI on host gut epithelium is the choice of cell lines that are employed. Numerous such cell lines have been utilized in studies based on the individual objectives of those studies. Of these, the most frequently utilized are the adenocarcinoma-derived Caco-2, HT-29 and T84 epithelial cell lines. These cell lines are frequently used to their reproducibility and ability to differentiate into cell representative of the gut epithelium. Our study chose to use T84 cells due to their close representation of colonocytes after differentiation in terms of morphology, presence of monocarboxylate transporter-1 and tight junction protein expression [236]. However, in this study, we did not utilize differentiated T84 cells on permeable supports due to the sheer complexity given the number of experimental treatments involved. Thus, in future studies, it might be helpful to assess pre-screened probiotic candidates on differentiated T84 cells for their effect on intestinal barrier function and tight junction protein expression. Another limitation of this study was the absence of toxin quantification data, as this would have allowed for better comparison of the magnitude of cytokine production between our fecal water study and other *in vitro* cell culture studies that have used purified toxins to challenge the cells. Moreover, in our study the sterile-filtered FW from the batch fermentation was stored at -20˚C until treatment on cells, which could have resulted in the degradation of the toxins as previously demonstrated, potentially resulting in inaccurate detection [237].

To better understand the mechanism of probiotics in protecting against CDI-mediated inflammation, further studies can be designed to assess their role in: i) modification of cytokine expression though mRNA analyses, ii) use of co-cultures of epithelial and immune cells to better assess inflammatory marker regulation, iii) compare the immune response in normal and primary human epithelial cell lines such as the HIEC-6, and, iii) identification of molecules secreted by probiotic strains responsible for modulation of inflammatory cytokines. These investigations could allow for pre-screening of probiotics for their effectiveness in CDI patients as adjunct therapies.

7.5 Overall Conclusions

In summary, this dissertation has presented a series of studies that have shown the potential role of commercial probiotic strains as therapeutic agents in the management of *C. difficile* pathogenesis. *C. difficile* infection in the *in vitro* gastrointestinal model resulted in the alteration of the gut microbiota and its function, accompanied by a dysregulation in intestinal homeostasis measured via antioxidant status, and, cytopathic effects on intestinal cells leading to a pro-inflammatory response. Probiotic supplementation in the *C. difficile*-infected fecal samples were associated with an increase in antioxidant capacity, improved metabolic function and an attenuation of intestinal cell cytotoxicity and pro-inflammatory marker production.

The findings from this work have demonstrated the ability of the GI model to simulate a colonic environment that is characteristic of *C. difficile* colonization in infected patients. Moreover, the results show for the first time the effect of probiotic supplementation on various aspects of CDI microbiota in an *in vitro* GI model. The results obtained from this work shows several novel insights into *C. difficile*-mediated dysregulation and probiotic action. CDI-mediated effects on antioxidant status and subsequent probiotic-mediated recovery through copper chelation were also reported for the first time. Moreover, the findings from the study on CDI-mediated effects on the gut microbial composition and metabolic capacity, showed that changes in metabolic function were achieved without major changes to the microbial composition, further adding to

the literature on the ability of probiotics to cause important metabolic changes in GI disorders. Lastly, the results from CDI-FW treated T84 intestinal cells showed the potential role of novel inflammatory cytokines previously not associated with CDI pathogenesis *in vitro*. Collectively, these results provide insight into possible pathways of probiotic action in *C. difficile* infection and set the basis for the use of *in vitro* GI models as a potential pre-clinical screening model for therapeutic agents.

7.6 General References

NOTE: *In accordance with the Guidelines for Thesis Preparation, each of the manuscript chapters (i.e. Chapters 3-6) contain their own reference list. Hence, the following reference list corresponds to the references included in the remaining chapters of the thesis (i.e. Chapters 1, 2, and 7).*

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