Evaluating the effectiveness of select bacteria against *Clostridium difficile*

by

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The student author and the program of study committee are solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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NOMENCLATURE

CDI	*Clostridium difficile* Infection
CDAD	*Clostridium difficile*-Associated Diarrhea
AAD	Antibiotic-Associated Diarrhea
CDC	Center for Disease Control and Prevention
SCFA	Short-Chain Fatty Acids
FMT	Fecal Microbiota Transplants
GRAS	General Recognized As Safe
FDA	Food and Drug Administration (America)

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ABSTRACT

Clostridium difficile infection (CDI) causes roughly 30,000 deaths each year in the United States and the number has been increasing since the early 2000s. Traditionally, CDI is treated with antibiotics (metronidazole or vancomycin). However, this method is only effective in 50% of the patients since it only affects the vegetative *C. difficile* cells and not the spores. Since the early 2000s fecal microbiota transplants (FMTs) have been used as a treatment method, leading to over 80% success rate. However, FMTs are still considered an investigational therapy since the mechanism of how these FMTs treat CDI are unknown. This has led to research into probiotics as a possible treatment method. In this study, the objectives were to identify major bacteria species common across 20 FMTs, and investigate if some of these bacteria could be potential probiotics. Our secondary focus was to evaluate microorganisms found in kefir, for effectiveness against *C. difficile*. *Bacteroides* and *Ruminococcaceae* were found to be the most prevalent bacteria family across the 20 FMTs. Potentially probiotic *Lactobacillus* and *Bifidobacterium* only made up roughly 0.2% of the bacteria population. The kefir and the different kefir components (cell-free supernatant, cell lysate, fat) inhibition capabilities were tested against *C. difficile*. It was found that neither the different components nor the entire kefir matrix showed any inhibition against *C. difficile*. However, a *Lactobacillus paracasei* isolate was found to not only grow alongside *C. difficile*, but to have resistance against the antibiotic vancomycin, making it a potential candidate for competitive inhibition against *C. difficile*. *L. paracasei* showed little inhibition when inoculated at the same population concentration as the *C. difficile* and also did not significantly inhibit *C. difficile* growth when used at a higher population. With such studies, we will gain a better understanding of the mechanisms of action and potential treatment for CDI.

CHAPTER 1: GENERAL INTRODUCTION

Clostridium difficile infection (CDI) causes roughly 30,000 deaths each year in the United States and the number has been increasing since the early 2000s (Center for Disease Control and Prevention; Lessa et al. 2015). This is partially due to the ability of *C. difficile* to form heat-resistant and antibiotic-resistant spores (Mullany and Roberts 2010), but also because of the increase in hyper-virulent strains that have recently started to emerge (Butler et al. 2011). Traditionally, CDI affects mostly elderly patients in hospitals, but there has been an increase of CDI cases in younger individuals (Bakken et al. 2011); cases have increased to almost 3 CDI cases per 100 hospitalizations in patients under 18 years of age (Lessa et al. 2012). Along with this, about 20% of the cases of CDI are from community-acquired *C. difficile* and 35% having CDI symptoms without prior exposure to antibiotics (Auclair et al. 2015).

C. difficile is transmitted though ingestion of the spores. These spores remain metabolically dormant until a favorable environment become present through a disruption in the natural gut microbiota (Mullany and Roberts 2010; Vedantam et al. 2012). When there is a disruption of the native bacteria, *C. difficile* is able to start germinating and producing toxins. These toxins lead to the formation of toxin-mediated diarrhea and pseudomembranous colitis, which is known as *C. difficile*-associated diarrhea (CDAD) (Plummer et al. 2004; Mullany and Roberts 2010). Because *C. difficile* spores are resistant to several antibiotics, they remain in the gut even after treatment; this leads to 15-30% of CDI patients having a relapse of CDI, which leads to an increase in mortality and a more chronic CDI (Leffler and Lamont 2015).

Currently, there are only two main treatment methods used to treat CDI: antibiotics and fecal microbiota transplants (FMTs). Most times, the antibiotics metronidazole or vancomycin are used as the first treatment method for patients with CDI. However, this method is only effective in 50% of the patients since it only affects the vegetative *C. difficile* cells and not the spores (Leffler and Lamont 2015). Along with this, antibiotics further disrupt the native microbiota and decrease the overall bacterial diversity, allowing the *C. difficile* spores to germinate after the treatment (Bakken 2014). With the low success rate associated with antibiotics, a new method of treatment for CDI was needed. Fecal microbiota transplants (FMTs) are fecal microbiota obtained from healthy human donors. They are thought to help "re-inoculate" the patient's infected colon with the beneficial bacteria (Shankar et al. 2014; Leffler and Lamont 2015). Studies have shown that FMTs can be successful in treating CDI in roughly 90% of patients. However, since FMTs are still considered an experimental procedure, it can be difficult as well as expensive, for patients to undergo this treatment method (Bakken 2014).

Recently, more studies have started to investigate the use of probiotics as a treatment method. Probiotics are known to provide a benefit to the host in several different mechanisms, including competitive inhibition, lowing the pH and the production of organic acids (Jardine 2009). Both *Lactobacillus* and *Bifidobacterium* have been used in clinical trials with CDI patients. Several studies have shown a decrease in CDAD in patients when given a probiotics supplement with antibiotics, with some showing an 80% recovery rate without a relapse in CDI (Hickson et al. 2007; Gao et al. 2010; Bakken 2014). However, most of these studies have not investigated the mechanism of the probiotics and overall there is not always consistency across the studies (Johnston et al. 2012), thus, leading to an

increased need for research into probiotics that specifically help treat CDI and prevent *C. difficile* growth.

This thesis focuses on FMTs and their microbiota population to determine which species could potentially contributing to the 80% success in treating CDI patients. A second focus of the thesis investigates a selection of microorganisms found in kefir, to determine if they possess the ability to inhibit C. difficile growth. The review of literature in the following chapter introduces the issues associated with CDI, the current research into FMTs and the lack of understanding their mechanism, and the use of probiotics as a treatment method for patients with CDI.

The objective of this research project was to determine the major bacteria species that are common across several FMTs that have demonstrated success in treating patients with CDI, and investigate if some of these bacteria could be potential probiotics (*Lactobacillus* & *Bifidobacterium* species). Along with the FMTs, probiotics found in kefir may also have the ability to inhibit *C. difficile* growth either from production of antimicrobial compounds or competitive inhibition. The following hypotheses were tested:

1) There are bacteria commonalities across FMTs; among them, potential probiotics include *Lactobacillus* and *Bifidobacterium* bacteria.
2) The probiotic bacteria present in kefir produce compounds that inhibit *Clostridium difficile* growth.

CHAPTER 2: LITERATURE REVIEW

Clostridium difficile Infection

Clostridium difficile are toxin-producing, heat-resistant, spore-forming, anaerobic bacteria. *C. difficile* are transmitted through the fecal oral route, from ingesting the spores, but it is estimated that 1-3% humans have *C. difficile* already in their microbiome. The spores that form are metabolically dormant, resistant to both heat and acid, and are highly resistant to most standard disinfection methods. In favorable environments, typically in susceptible hosts, *C. difficile* spores can start to germinate and cause what is known as *C. difficile* infection (CDI) (Vedantam et al. 2012). In an individual with a healthy gut microbiota, the risk of CDI developing is low (Butler et al. 2011). However, this risk increases when a patient becomes susceptible due to a disturbance to their gut microbiota from antimicrobials or other illnesses (Mullany and Roberts 2010). Other factors also

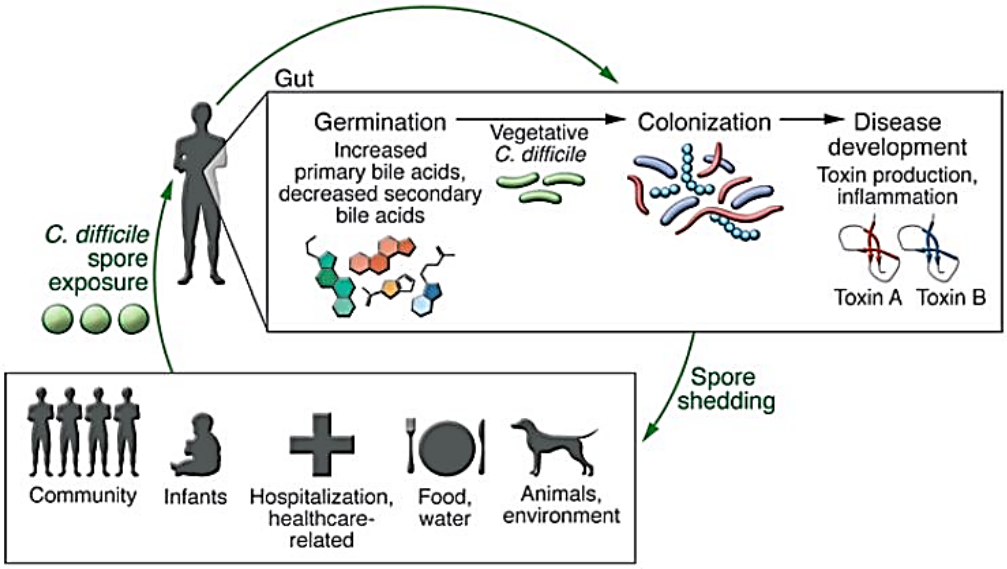

Figure 2.1. CDI pathogenesis in humans. (Adapted from Seekatz and Young 2014)

increase the risk of CDI development, such as the host immunity, gender, age or gastrointestinal procedures (Butler et al. 2011).

In healthy patients, normal microbiota produce short-chain fatty acids and lactic acid due to breaking down the non-absorbable carbohydrates; this leads to a decrease in pH, which can prevent pathogens from germinating. A healthy microbiota also prevents pathogenic bacteria from growing due to colonization resistance, which includes competitive exclusion. With a disruption of these microbiota in the colon, they can no longer break down the present carbohydrates and the microbiota population decreases, allowing for a pathogen such as *C. difficile* to germinate. Germination of *C. difficile* also requires the bile salt, taurocholate, and glycine, is which are naturally present; these two components must be present to trigger the spore to start germinating. When *C. difficile* starts to colonize the colon, it starts to produce two exotoxins: TcdA, the primary enterotoxin and TcdB, a cytotoxin (Plummer et al. 2004). These two toxins causes inflammation in the colon, which leads to tissue damage and further disrupts the normal microflora (Vedantam et al. 2012). Overall, they lead to the symptoms known as toxin-mediated diarrhea and pseudomembranous colitis (Mullany and Roberts 2010). Together, this is classified as *C. difficile*-associated diarrhea (CDAD) (Johnston et al. 2012) in patients with *C. difficile*.

The biggest threat for patients with CDI, is when it becomes recurrent. Recurrent CDI happens in 15-30% of patients after the initial bout of CDI. When CDI is recurring, it can become more chronic and unresponsive to treatments, which can lead to an increase death risk (Bakken et al. 2011). Recurring CDI happens due to spores that are still present in the gut even after antibiotic treatment, an impaired immune response to the infection and a weakened barrier function of the colonic microbiota (Leffler and Lamont 2015).

According to the Centers for Disease Control and Prevention (CDC) roughly 30,000 individuals died in the United States due to CDI in 2011 (Center for Disease Control and Prevention; Lessa et al. 2015). Unfortunately, this number of deaths has increased over the last few years due to emergence of hyper-virulent and antibiotic-resistant strains of *C. difficile*. Traditionally, *C. difficile* affects older individuals in a hospital or those exposed to antibiotic treatments. About 90% of the deaths due to *C. difficile* are in patients 65 years or older, making it the 18th leading cause of death of this age division (Lessa et al. 2012; Evans and Safdar 2015). However, there has been an increase of CDI in young, healthy individuals who acquire *C. difficile* from the environment (Bakken et al. 2011). In a study by Lessa et al. (2012), it was found that there has been a substantial increase in CDI in individuals under the age of 18; cases have increased from 0.7 per 1000 hospitalizations in 1997 to 1.28 per 1000

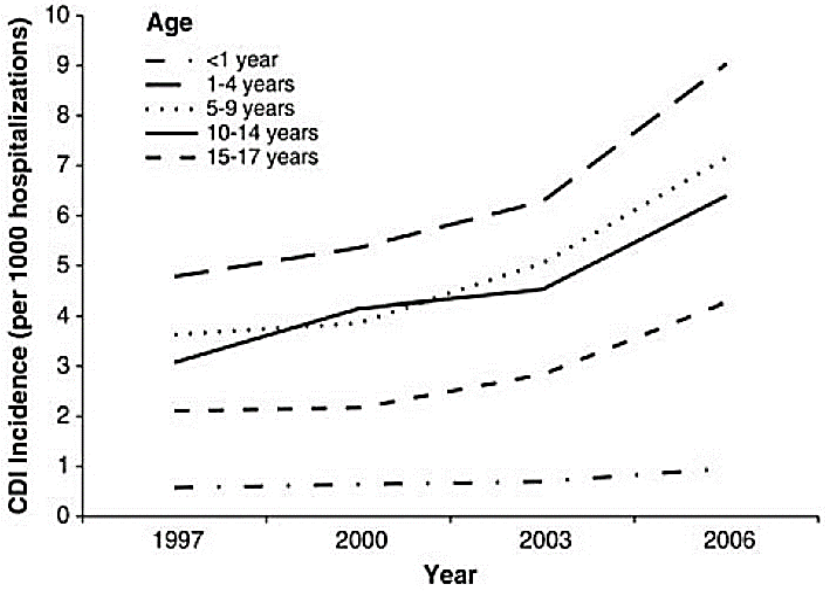

Figure 2.2. Incidences of *C. difficile* infection per 1000 hospitalizations by age. Abbreviation: CDI, C. difficile infection (Adapted from Lessa et al. 2012)

hospitalizations in 2006 (Figure 2.2). This is believed to be due to the increase in community acquired C. difficile and the emergence of the hyper-virulent strains. About 20-28% of all the CDI cases are from community acquired *C. difficile* and around 35% of these patients form CDI without exposure to an antibiotic (Auclair et al. 2015). The hyper-virulent strains that have been emerging since the early 2000s have an increase in toxin production, including a new binary toxin, have increased sporulation, which increases its ability to spread, and high levels of resistance to some antibiotics (Butler et al. 2011). It was shown in a study by (Lessa et al. 2012), that the amount of C. difficile infections in the US from 2000 to 2014 increased in several age groups; with it more severely in the older age groups (shown in Figure 2.3). Our understanding of C. difficile and CDI is continuing to improve, but with the emerging new strains and an increase in community acquired C. difficile, CDI has

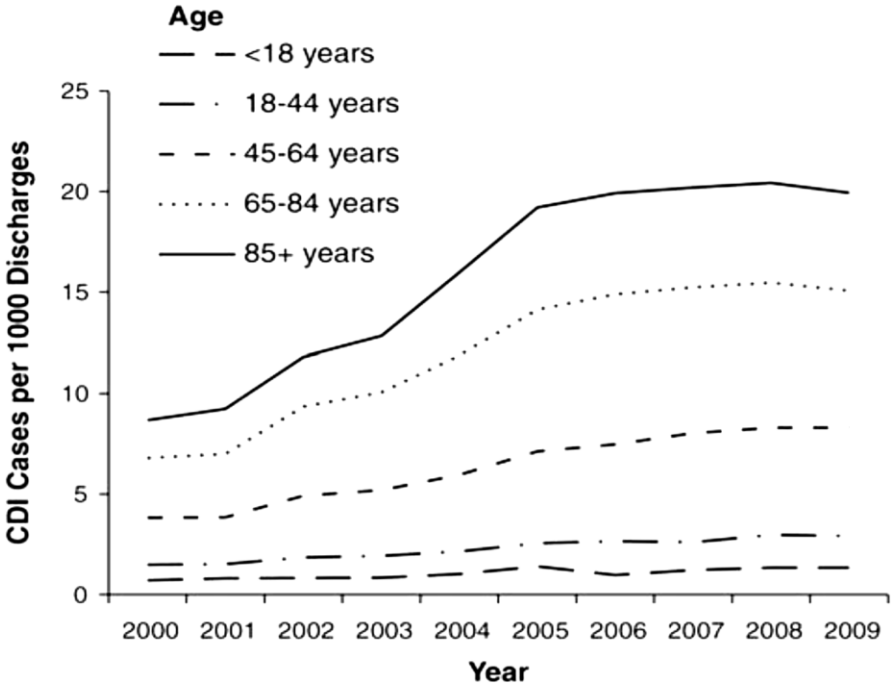

Figure 2.3 Discharge rate for *Clostridium difficile* infection from US short-stay hospitals by age (Lessa et al. 2012)

become one of the more important health-care associated pathogens. Thus, leading to the need for increased research into current and alternative treatment methods.

Current Treatments for *Clostridium difficile* Infection: Antibiotics and Fecal Microbiota Transplants

Currently, there are two main methods used to treat CDI: antibiotics and fecal microbiota transplants (FMTs). Traditionally, antibiotics are used as the first treatment method for patients with CDI. The primary antibiotics used are metronidazole and vancomycin. However, it is found that antibiotics are only successful in 50% of patients with recurring CDI (Leffler and Lamont 2015). Antibiotics are used to treat patients with CDI because they can potentially reduce the germinated *C. difficile* strains, however, vancomycin and metronidazole further disrupt the microbiota in the intestinal tract, which then allows the *C. difficile* spores, which are resistant to the antibiotics, to germinate; thus, leading to recurring CDI (Bakken 2014; Leffler and Lamont 2015). The interaction of antibiotics for CDI patients have been described as a three phase system by Louie et al. (2015). The first phase is the introduction of the antibiotics to the intestinal microbiota, which impairs the native microbiota and leads to an overgrowth of *C. difficile*. The second phase is the production of toxins and inflammation, which further disrupts the native microbiota, leading to the onset of diarrhea. Lastly, there is the struggle between the native microbiota trying to reestablish themselves in both diversity and quantity, but must try to outgrow the *C. difficile* that have established themselves. With increased use of antibiotics has been the emergence and spread of *C. difficile* strains that are resistant to both metronidazole and vancomycin. This resistance is caused when increased use provides antimicrobial selection pressure through either mutation of new resistance genes or alteration of the bacterial ecology (i.e.

transfer of naturally occurring resistances from one strain to another) (Heywood and Netts 1993). Since the recent emergence of some antibiotics-resistant *C. difficile* strains, and due to antibiotics' low success rate, FMTs have started to become an alternative treatment method for CDI.

As stated before, the colonic microbiota, which provide colonization resistance against pathogens, are thought to be the main way to prevent CDI (Leffler and Lamont 2015). When a patient has CDI, she or he also has a decrease in the normal gut microbiota, which leads to the infection and potential recurring CDI. Fecal Microbiota Transplants (FMTs) are fecal microbiota obtained from a healthy human donor that are utilized to treat patients who have recurrent CDI. Each fecal donation is put through a rigorous screening process to look for several pathogens or viruses (Bakken et al. 2011; Shankar et al. 2014). It is thought that through the FMT, the patient's infected colon gets "re-inoculated" with beneficial microbiota. This then leads to an increase in their growth and reestablishing the colonization resistance against *C. difficile*. FMTs have been successful in treating roughly 90% of patients with CDI (Leffler and Lamont 2015). In a study done by Patel et al. (2013), 31 patients were administered FMTs to investigate the effectiveness for treating CDI as well as identifying any side-effects from the procedure. Prior to the FMT, all the patients suffered

Table 2.1 Fecal Microbiota Transplantation Primary Outcomes (Adapted from Patel et al. 2013)

	Diarrhea		Abdominal Pain		Fatigue	
Outcome	Patients, No.	Mean d (range)	Patients, No	Mean d (range)	Patients, No	Mean d (range)
Not improved	1	3 (1-18)	6	3 (1-30)	13	6 (1-90)
Improved	7		5		8	
Resolved	22		12		8	

from CDAD, 81% had abdominal pain, and 90% suffered from fatigue. After the treatment, patients were evaluated on their improvement or resolution of these symptoms. In Table 2.1, it shows the improvement or resolution of each symptom after FMT procedure. Overall, the majority of the patients had some type of resolution or improvement of diarrhea and abdominal pain. Only 3 out of the 31 patients had a recurring CDI (81% recovery rate). In a similar study from van Nood et al. (2013), shown in Figure 2.4, 13 out of 16 patients (81%) were cured after the first FMT infusion, and 2 out of 16 after the second infusion; resulting in a 94% cure rate through FMTs. With antibiotics alone, only 31% (4 out of 13) of the patients were cured using only vancomycin and 23% (3 out of 13) cured using vancomycin and a bowel lavage. These findings support the statement that FMTs have a significant higher success rate compared to antibiotics.

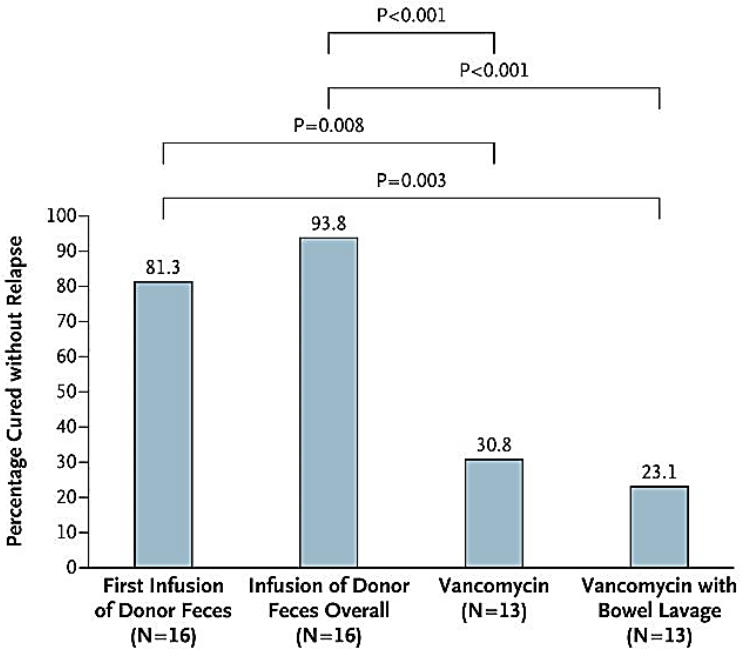

Figure 2.4. Rates of Cure without Relapse for Recurrent *Clostridium difficile* Infection. (Adapted from van Nood et al. 2013)

As stated before, the success rate from FMTs are thought to be related to increasing the patients' native gut microbiota diversity. During CDI, the patients have a shift in their gut microbiota. In one study by van Nood et al. (2013), they found that patients with CDI had low overall gut diversity compared to a healthy individual. This study also investigated how the diversity changed from before to after the FMT infusion. Figure 2.5 shows how patients' gut microbiota diversity changed before and after the infusion of the FMTs; it shows that after the FMT, there was an increase in the diversity so that it starts to resemble that of the healthy donors'. It is well documented that in a healthy human gut, Bacteroidetes and Firmicutes are the two most dominate bacterial divisions (Khoruts et al. 2010) and that both of these decrease in patients with CDI (Khoruts et al. 2010; Louie et al. 2015). Similarly, in a study by Shankar et al. (2014), low diversity of intestinal microbiota was found in CDI patients compared to the healthy donors and after the FMT infusion, the CDI patients had both an increase in diversity but also had similar microbiota population of the donor. This

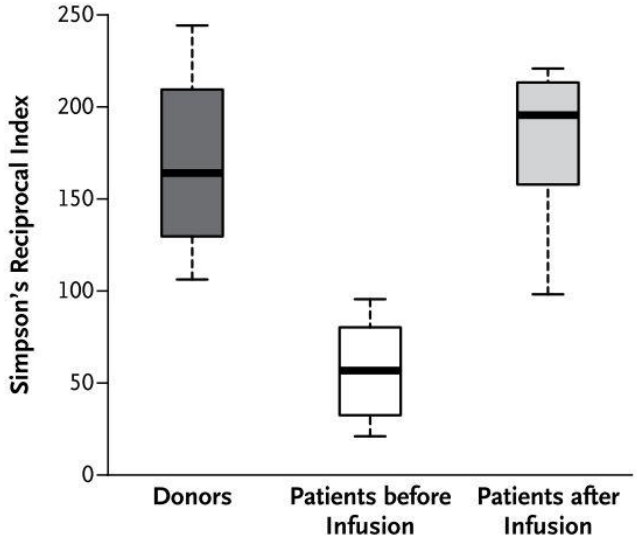

Figure 2.5. Microbiota Diversity in Patients before and after Infusion of Donor Feces, as Compared with Diversity in Healthy Donors (Adapted from van Nood et al. 2013)

microbiota community of the CDI patients' was monitored over a four month period and it was found that the CDI patients continued to have high microbiota diversity of that of the donors' FMT (Shankar et al. 2014), supporting that the FMTs are possibly "re-inoculating" the patients' intestinal tracts. However, the key mechanism, or bacteria from the FMTs that the patients benefited the most from, are still unknown (Schenck et al. 2015).

Even though FMTs have been shown to be very successful, there are still some downsides to this procedure. FMTs are considered to be an investigational therapy or used as a "last resort" therapy; therefore some third party payers and medical services have been unwilling to pay for the procedure. Along with this, there is a lack of access to health care centers that can perform FTMs. This leads to patients having to pay more out of pocket or finding alternative treatment methods, hence other treatment methods for CDI are needed (Patel et al. 2013; Bakken 2014).

Probiotics as a Treatment Method for *Clostridium difficile* Infection

In the digestive tract in humans there are several main microbiota genera; these are *Bacteroides, Enterococci, Lactobacillus and Bifidobacterium* (Jardine 2009). Many factors, such as illness, age, and use of antibiotics, can affect the diversity and genera of bacteria present. As stated before, patients with CDI have a change in their gut microbiota; the Bacteroides and Firmicutes start to decrease, along with an overall decrease in the diversity of bacteria species. Through FMTs it is thought to help reintroduce the native gut bacteria, but FMTs are not always an option, thus, additional treatment methods are need (Patel et al. 2013; Bakken 2014). Probiotics as a treatment for patients with CDI, is one area that is starting to be investigated. Probiotics are defined as "live microorganisms that when ingested by in adequate amounts, confer a health benefit on the host" (World Health Organization and

Food and Agriculture Organization of the United Nations 2002). There have been several genera of bacteria that have been identified as probiotic bacteria; the primary genera being *Lactobacillus* and *Bifidobacterium.* Each specific potential probiotic bacteria strain must go through a complex process, using genetic and phenotypic techniques, to establish the identity, type and classification of the probiotic. In addition to this, the bacteria must undergo a functional aspect and safety assessment to determine its probiotic functional mechanisms as well as determine if the bacteria has "General Recognized As Safe" (GRAS) status, which is set by the American Food and Drug Association (FDA). Each probiotic strain must have a documented health effect and be clinically validated (Lee & Salaminien

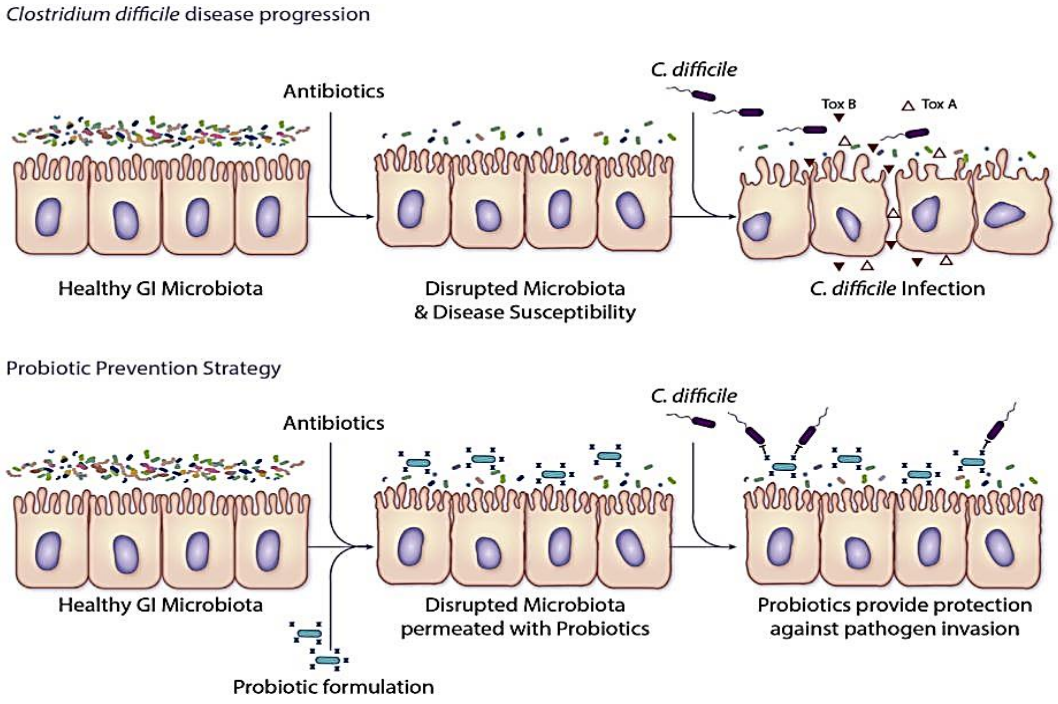

Figure 2.6. Probiotic Prevention Strategy against CDI. (Adapted from Spinler et al. 2016). Disruption of a healthy GI microbiota by antibiotics increases CDI susceptibility. Adjunctive probiotic therapy provides protection against CDI by stabilizing the GI microbiota protecting the host by various mechanisms and preventing *C. difficile* invasion.

2009; Kneifel et al. 2010). There are many different probiotic functional mechanisms needed to be considered a probiotic, some of which provide health benefits and these different mechanisms are important to know, since the help establish a potential bacteria as a probiotic. To start, probiotics need to be able to withstand the passage from consumption to intestine and then must be able to then survive in the human GI track. Therefore, the bacteria must be resistant to gastric conditions (i.e. bile and acids) as well as adherence to mucus and human epithelial cells (Lee & Salaminien 2009). Some known probiotics also have functions to help decrease or prevent the growth of pathogenic bacteria; they do this through several different mechanisms. One is by the production of different organic acids and gut protective metabolites (short-chain fatty acids, acetate, lactic acid, diacetyl, hydrogen peroxide), which help in lowering the pH of the gut environment and strengthens the gut barrier function, to create an environment that pathogenic bacteria cannot survive in (Jardine 2009; Lee & Salaminien 2009; Spinler et al. 2016). Probiotic bacteria can also produce different antimicrobials such as bacteriocins. These bacteriocins are protein groups that can interfere with enzymatic reactions, bacterial membranes, and effect the transcription, translation and replication of many pathogenic bacteria (Fijan 2016; Tenea and Yépez 2016). The other mechanism for preventing pathogenic growth is through competitive inhibition (competitive exclusion). Competitive inhibition can be driven by competing for bacterial adhesion sites in the intestinal epithelial surface or competing for a similar nutrient source. Many probiotics are able to out compete pathogens just by having a higher population number, using coaggregation mechanisms (bacteria to bacteria interactions) and by regulation the intestinal motility and mucus secretion. In addition, probiotics may also utilizing some of the other probiotic functional mechanisms to increase their competitive inhibition advantage over

intruding pathogens (Liong 2011; Ötleş 2014). Some other mechanisms are stimulating the immune system, and binding and metabolizing toxic substances (Jardine 2009; Lee & Salaminien 2009; Fijan 2016; Tenea and Yépez 2016; Spinler et al. 2016).

Two main probiotic genera present in healthy humans are *Lactobacillus* and *Bifidobacterium.* In some studies, it has been shown that with illness and age, the Bifidobacterium start to decrease in the gut (Jardine 2009). However, in a study done by Louie et al. (2015), many of the *Bifidobacterium* and *Lactobacillus* species remained prevalent in the gut even after treatment with both vancomycin and fidaxomicin. This suggests that, potentially, these types of probiotics could be utilized in CDI patients since they seem to be able to survive and have some resistance to the antibiotics used. Plummer et al. (2004) suggests that probiotics therapy (Biotherapy) acts similarly to FMTs in that their role is to restore the colonization resistance of the gut microbiota, , through several of the probiotic functional aspects, that was disrupted during antibiotic treatment to help prevent recurrent CDI or even the initial onset of CDI. In this study, Plummer found that there was a 4% decrease in *C. difficile* in the fecal sample of the patients in the probiotic group compared to the placebo as well, as a decrease in the toxins from *C. difficile* (Plummer et al. 2004). This suggests that the probiotics may have an impact in both prevention of *C. difficile* spread but also with keeping the present *C. difficile* in an asymptomatic carrier state, meaning in spore form.

Similarly, two clinical studies focused on treating CDI patients with probiotics to prevent recurrent CDI along with an antibiotic treatment. The study done by Gao et al. (2010) used a probiotic capsule containing *Lactobacillus acidophilus* and *Lactobacillus casei*. In this study, there were three groups: placebo, probiotic group 1 (given 1 capsules)

and probiotic group 2 (given 2 capsules). Gao and colleagues found that there was a decrease in CDAD in both the probiotic groups compared to the control; 28% of the placebo group tested positive for CDAD while only 9% in probiotic group 1 and 1% in probiotic group 2. In the second study, Hickson et al. (2007) looked at a probiotic yogurt drink, Actimel, that contained *Lactobacillus casei*, *L. bulgaricus* and *Streptococcus thermophilus*. The Actimel was found to decrease CDAD compared to those in the control by 17% and no *C. difficile* toxins were found in the probiotic group compared to 52% of the control group testing positive for toxins.

Another study, done by Bakken (2014), took a slightly different approach; they looked at using kefir, a fermented milk beverage, as the probiotic supplement with a staggered and tapered antibiotic withdraw (STAW). STAW simply means that the dose of antibiotics is decreased over time. Kefir was chosen as a probiotic supplement due to its diverse collection of probiotics strains (Bakken 2014). This method was used because it is postulated that the STAW allows the *C. difficile* spores time to start germinating, between the drug-free periods so that the majority of *C. difficile* (vegetative cells and spores) are removed from the patient and the kefir would help enrich and increase diversity of the native microbiota that are affected by the antibiotics. In this study, Bakken focused on recurrent CDI, having each patient act as their own control, since they had several rounds (between 2-9 relapses) of CDI that were not cured with antibiotics alone. The kefir supplement along with the STAW displayed an 84% (21 out of 25 patients) effectiveness of treating CDI. Even after a 9-month follow up, only 4 of the 25 patients had a relapse of CDI. Of the 21 patients with a successful treatment, all had reestablished normal bowel functions and 20 remained symptom-free after 12 months. This study shows that the use of probiotics could be as

successful in treating CDI as FMTs, however, the authors did not investigate which of the probiotics in the kefir were contributing to the 84% success.

In a meta-analyses from Johnston et al. (2012), of several clinical studies (including the from Gao et al & Hickson et al.), probiotics tended to be favored over the control when used to treat CDAD. However, most of these studies focused on CDAD and if the probiotics supplement decrease CDAD. They did not look into specifically which of the probiotic strains were providing the increased treatment success. Johnston et al. (2012) also showed that even though there is a positive trend for probiotics, the results are not always consistent across the studies. This just goes to show that more research is needed in this area as well as more investigation into the specific probiotic strains that contribute to the decrease of CDI and the prevention of *C. difficile* growth.

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CHAPTER 3: IDENTIFYING COMMONALITIES OF BACTERIA FOUND IN FECAL MICROBIOTA TRANSPLANTS

ABSTRACT

In this study, the objective was to identify major bacteria species common across 20 fecal microbiota transplants (FMTs) that are used to treat *Clostridium difficile* infection (CDI), and investigate if some of these bacteria could be potential probiotics. CDI is traditionally treated with antibiotics (metronidazole or vancomycin). However, this method is only effective in 50% of the patients since it only affects the vegetative *C. difficile* cells and not the spores. Since the early 2000s fecal microbiota transplants (FMTs) have become a popular treatment method for CDI patients, leading to over 80% success rate. However, FMTs are still considered an investigational therapy since the mechanism of how these FMTs treat CDI are unknown. It was hypothesis that there are bacteria commonalities across FMTs; among them, potential probiotics include *Lactobacillus* and *Bifidobacterium* bacteria. *Bacteroides* and *Ruminococcaceae* were found to be the most prevalent bacteria family across the 20 FMTs. *Faecalibacterium prausnitzii* was one the most prevalent species present across all the FMTs, making about 15% of the gut microbiota. The probiotic *Lactobacillus* and *Bifidobacterium* only made up roughly 0.2% of the bacteria population.

INTRODUCTION

A diverse commensal microbiota of humans has been well documented as being crucial in maintaining health. Several disease have been associated with a dysbiosis in the gut microbiota; one of these is *Clostridium difficile* infection (CDI) (Ranjan et al. 2016). CDI is typically associated with hospitals and older adults (65 years and older) caused from

the spread of *C. difficile* spores. CDI has caused roughly 30,000 deaths in the US and has been increasing due to hyper-virulent and antibiotic *C. difficile* strains (Center for Disease Control and Prevention; Lessa et al. 2012; Evans and Safdar 2015). The biggest risk for CDI patients is when the infection becomes recurrent; this happens in 15-30% of CDI patients. When CDI become recurrent, it becomes more difficult to treat and can lead to an increase risk for death. Recurrent CDI happens due to spores, which are resistant to the antibiotics, that remain in the gut and can start germinating after treatment causing several bouts of infection (Bakken et al. 2011; Leffler and Lamont 2015).

Currently the two main treatment methods for CDI are through antibiotics or fecal microbiota transplants (FMTs). However, antibiotics have a low success rate, roughly 50% compared to FMTs, which have around 80% success (Leffler and Lamont 2015). It is postulated that FMTs work in treating CDI by re-inoculating the intestinal tract to re-establish normal bowel function (Bakken et al. 2011). But even with FMTs' high success with treating CDI, there is still a sense of reluctance to fully utilize this procedure. FMTs are still considered an investigational therapy since the mechanism of how FMTs work to treat CDI are unknown, along with many of the risks involved (Patel et al. 2013). Because of this, many patients are forced to pay for FMTs out of pocket or seek out other treatment methods. This increases the need for further research into the type of microbiota present in FMTs as well as establishing what bacteria population make up a healthy microbiota.

Metagenomic sequencing, such as whole genome shotgun sequencing (WGS) and 16s rRNA, is a valuable tool in determining and analyzing complex microbial communities. It is through these sequencing techniques that many of the human gut microbiota influences, such as on health and physiology, have been established (Qin et al. 2010). WGS and 16s rRNA are

essential for identifying and enumerating the bacterial species that are represented in the gut along with identifying some of their complex interactions between bacteria species and bacteria and the host. Sequencing has enabled determining the major bacteria phyla found in the gut, such as the Bacteroidetes and Firmicutes, which are known to represent the majority of human gut microbiota. Compared to the more modern sequencing, 16s rRNA, WGS can more clearly define the taxa present, down to the species level (Ranjan et al. 2016). One of the biggest challenges with FMTs is that the bacteria community and diversity varies from donor to donor, so to identify the major commonalities across several FMTs may start to help identify the crucial microbiota needed to establish a healthy microbiota.

The purpose of this study was to start identifying the bacterial commonalities across several FMTs that have been used successfully to treat CDI as well as determine if some of these common bacteria are *Lactobacillus* and *Bifidobacterium* species that are common to dairy products.

MATERIALS AND METHODS

Sample acquisition

Although stool was acquired from healthy donors, and stored at OpenBiome (Somerville, MA) stool bank, human subjects' research paperwork was filed with the ISU Institutional Review Board prior to acquisition of any stool samples. Twenty different fecal samples, from 20 different healthy individuals who were approved donors, were acquired from OpenBiome's frozen storage.

DNA extraction, amplicon preparation, pyrosequencing, and data analysis

Genomic DNA from bacteria in stool was purified using the The PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc. Carlsbad, CA). DNA preparations were quantified

using Quan-iT™ PicoGreenH dsDNA Kit (Invitrogen, Carlsbad, CA, USA) and a Nanodrop 3300 Fluorospectrometer (Thermo Fisher Scientific, Wilmington, DE). Each genomic DNA sample was subjected to whole genome DNA amplification (Illustra™ GenomiPhi™ V2 DNA Amplification Kit, GE Health Sciences, Piscataway, NJ) following the manufacturer's standard protocol. The resulting DNA was purified using ethanol-precipitation and resuspended in Qiagen AE elution buffer. These were quantified using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The resulting products were used as templates for both Shut-gun Whole Genome (Ranjan et al. 2016) and 16s rRNA libraries, conducted at Argonne National Laboratory (Chicago, IL). The sizes of all PCR products were confirmed by agarose gel electrophoresis on 1% SB buffer gels (Faster Better Media LLC, Hunt Valley, MD). The quality of the 16S rDNA amplicon libraries were tested by running them, under the supervision of collaborator Phillips, on a 2100 Agilent bioanalyzer on a DNA High Sensitivity chip (Iowa State University DNA Facility, Ames, IA).

Characterization of bacteria

Sequence handling and analysis for determination of operational taxonomic units (OTUs) was determined using MG-RAST pipeline (Glass et al. 2010) for the WGS and the QIIME program (Caporaso et al. 2010) for the 16s rRNA sequences, following the standard procedure methods. The WGS sequences were compared with the GenBank database analysis program through MG-RAST using the analysis program on MG-RAST. The frequency of bacteria in each phylum, class, order, family and genus were determined and classified. Due to backlogs with the MG-RAST pipeline, only 19 of the 20 FMTs WGS were analyzed through the MG-RAST pipeline.

RESULTS AND DISCUSSION

Through both 16s RNA and WGS, it was determined that the main bacteria phyla across the FMTs were Bacteroidetes and Firmicutes, making up 37% and 55% of the gut microbiota, respectively (Figure 3.1 & Figure 3.2). This was expected, since these two phyla have been well established as the main human gut microbiota (Eckburg et al. 2005; Heinken et al. 2014). Of the Bacteroidetes, 34% were made up of the genus *Bacteroides* (family: *Bacteriodacea*), which was the largest population in the fecal samples (Figure 3.3). However, no one *Bacteroides* species could be identified as a major commonality across the FMTs; meaning there was very high diversity of *Bacteroides* in the gut microbiota. Twenty different *Bacteroides* species were found to be common across the FMTs, *Bacteroides vulgatus* (5% of bacteria in FMTs) being the most prevalent (Table 3.1). This is similar to the findings by Bakken et al. (2011) and Khoruts et al. (2010), with *B. vulgatus* being the primary Bacteriodacea in the FMTs as well as in the CDI patients after an FMT infusion.

Unfortunately, many of the *Bacteroides* species, including *B. vulgatus,* do not have defined host interactions and it is still unknown if they provide any beneficial capabilities for the human host. Some of these could potentially be probiotics but would have to go through the process, mentioned in Chapter 2, to be established as probiotic. However, studies show mixed reviews on whether *Bacteroides* species provide any benefits; Cuív and others (2011) state that many studies show *B. vulgatus* both capable of promoting and protecting against colitis and can be considered an opportunistic pathogen. One hypothesis is, that in the correct concentration, *B. vulgatus* could be beneficial, but with too high of a population, could cause complications. Identifying its mechanism in the gut environment would be crucial in establishing if it is beneficial with treating CDI. Nevertheless, knowing that these 20 species

make up most of the human *Bacteroides* may provide better guidelines for selection of potential beneficial bacteria in treating CDI.

Over 22% of the population of the FMTs was composed of the family *Ruminococcaceae* (Phylum: Firmicutes). Unlike the *Bacteroides* species, one major species was identified across each FMT; *Faecalibacterium prausnitzii* made up 15% of the microbiota bacteria and 25% of the overall Firmicutes species. It is generally accepted that *F. prausnitzii* makes up 5-15% of healthy human microbiota (Hold et al. 2003; Miquel et al. 2013). Unlike many of the *Bacteroides* species, *F. prausnitzii* is known to provide some benefits to the host. In studies by both Hold et al. (2003) and Khan et al. (2014), they found that *F. prausnitzii* is a major butyrate-producer and also produces anti-inflammatory compounds. Butyrate is one of several short-chain fatty acids (SCFA) that are known to play an important role in providing protection against cancer and ulcerative colitis. Several studies have shown that with disease, there is a shift in the gut microbiota (Clemente et al. 2012). Miquel et al. (2013) determined that with many diseases, such as inflammatory bowel syndrome (IBS) or ulcerative colitis (UC), there is a decrease in *F. prausnitzii*, and *F. prausnitzii* can potential help treat IBS patients. However, it has not been well documented if *F. prausnitzii* decreases in CDI patients. Although *F. prausnitzii* is not fully established as a probiotic, due to its high oxygen sensitivity (Miquel et al. 2013), it could be an important beneficial bacteria for treating CDI patients.

Originally, the hope was that there would be a large concentration of lactic acid-producing bacteria (*Lactobacillus*) and other known probiotic bacteria (*Bifidobacterium)* in the FMTs since these were the probiotic bacteria used in other clinical studies (Hickson et al. 2007; Gao et al. 2010; Bakken 2014). Many species of these bacteria are considered

probiotic because they have established benefits for human health and have documented inhibition capabilities for other pathogenic bacteria (Jardine 2009; Lee & Salaminien 2009). However, it was found from the WGS data that *Lactobacillus* and *Bifidobacterium* only made up an average of 0.19% and 0.17% of the bacterial population across the 20 fecal samples, ranging in each FMT from 0.01% to 1% (Figure 3.4), respectively. Even though these two probiotic genera are only present in small concentration, their benefits are well established, as mentioned in Chapter 1, and many are already being investigated as potential probiotics to use to treat patients with CDI. Therefore, knowing which species are most common across the FMTs could be a valuable tool in creating a probiotic supplement to treat CDI patients.

The WGS identified a large diversity of *Lactobacillus* species. There were 34 different *Lactobacillus* species present across the 19 FMTs (Table 3.3), with around 17 of them being present in all 19 in larger percentages (greater than 0.003% of total FMT microbiota and greater than 1% of *Lactobacillus* total population). *Lactobacillus delbrueckii* (*L. delbrueckii* subsp *bulgaricus*) was found to be the most prevalent, making up 12% of the *Lactobacillus* species. *L. delbrueckii* subsp *bulgaricus* is one of the two bacteria required for yogurt to be called yogurt. A study by Hickson et al. (2007) utilized *L. delbrueckii* with several other probiotics in a probiotic yogurt drink, Actimel, and found that it significantly decreased *C. difficile*-associated diarrhea (CDAD). Along with *L. delbrueckii, L. salivarius, L. paracasei, L. plantarum,* and *L. reuteri* were also common across the FMT samples, making up 10%, 8%, 8% and 7% of the *Lactobacillus* population, respectively. Similar to *L. delbrueckii*, these species are found in fermented dairy products, and have also been utilized in studies to treat CDI patients and are known to help treat antibiotic-associated diarrhea (AAD) or acute pediatric diarrhea (McFarland 2015). In a study by Ambalam et al. (2015),

L. paracasei was found to inhibit *C. difficile* in competitive inhibition tests. *L. casei* and *L. acidophilus* are two other main probiotics that have been utilized in CDI treatments since they are known to help improve normal microbiota and also help with AAD. They were found to make up only 3% of the *Lactobacillus* species in the present study.

Unlike the *Lactobacillus* species, the *Bifidobacterium* species had less diversity. The WGS identified 13 different *Bifidobacterium* species across the 19 FMTs (Table 3.4), with 11 being present in all 19 samples (in populations greater than 0.003% of total FMT bacteria, greater than 1% of *Bifidobacterium* total population). *Bifidobacterium longum* made up an average of 50% of the total *Bifidobacterium* population across the 19 FMTs. *B. animalis* (*Bifidobacterium animalis* subsp. *lactis*) and *B. bifidum* made up 14% and 11% of the *Bifidobacterium* population. These species, much like many of the *Lactobacillus* ones, are commonly found in fermented dairy products and have also be used in CDI studies. In a study by (Kondepudi et al. 2012), they found that *B. longum* exhibited high antimicrobial activity against *C. difficile*, making it possibly one important probiotic needed to treat CDI. *B. bifidum* is known to help displace pathogenic bacteria and has been utilized with other probiotics in CDI clinical trials (Johnston et al. 2012; McFarland 2015). *B. animalis* is known to improve normal microbiota as well as showed the ability to inhibit *C. difficile* in a study by Schoster et al. (2013). Much like with *Lactobacillus*, knowing the major *Bifidobacterium* present could help with narrowing in on the crucial probiotics to consider for food-based treatment or prevention of CDI.

Both *Lactobacillus* and *Bifidobacterium* species were identified in the WGS, and the major bacteria species for each genera were also identified. Figure 3.5 displays which *Lactobacillus* species could potentially make up a food-based cocktail, based on the results

from the WGS of the 19 FMTs. Knowing the major commonalities of probiotics naturally found in healthy human microbiota donors may help with identifying the "ideal" probiotic culture for treating or preventing CDI.

CONCLUSION

Overall, Bacteroidetes and Firmicutes phyla made up the largest population across all 19 FMTs, suggesting that these bacteria may play an important role in inhibition, prevention or treatment of *C. difficile.* However, many of these species are not fully understood regarding how they help benefit gut microbiota. *Faecalibacterium prausnitzii* was one the most prevalent species present across all the FMTs and is known to provide the host with butyrate and anti-inflammatory compounds; it has been used to treat IBS. Even though the *Lactobacillus* and *Bifidobacterium* species only made up a small percentage of the FMTs, knowing the main species and their population that are present in the gut could lead to creating a probiotic cocktail that more specifically mimics that of healthy human adults. Knowing how the *Lactobacillus* and *Bifidobacterium* species work together may help establish a better understanding of how to utilize them in treating CDI patients. As technologies improve in the area of culturing and genomic sequencing, more understanding of how the gut microbiota function, may be further understood.

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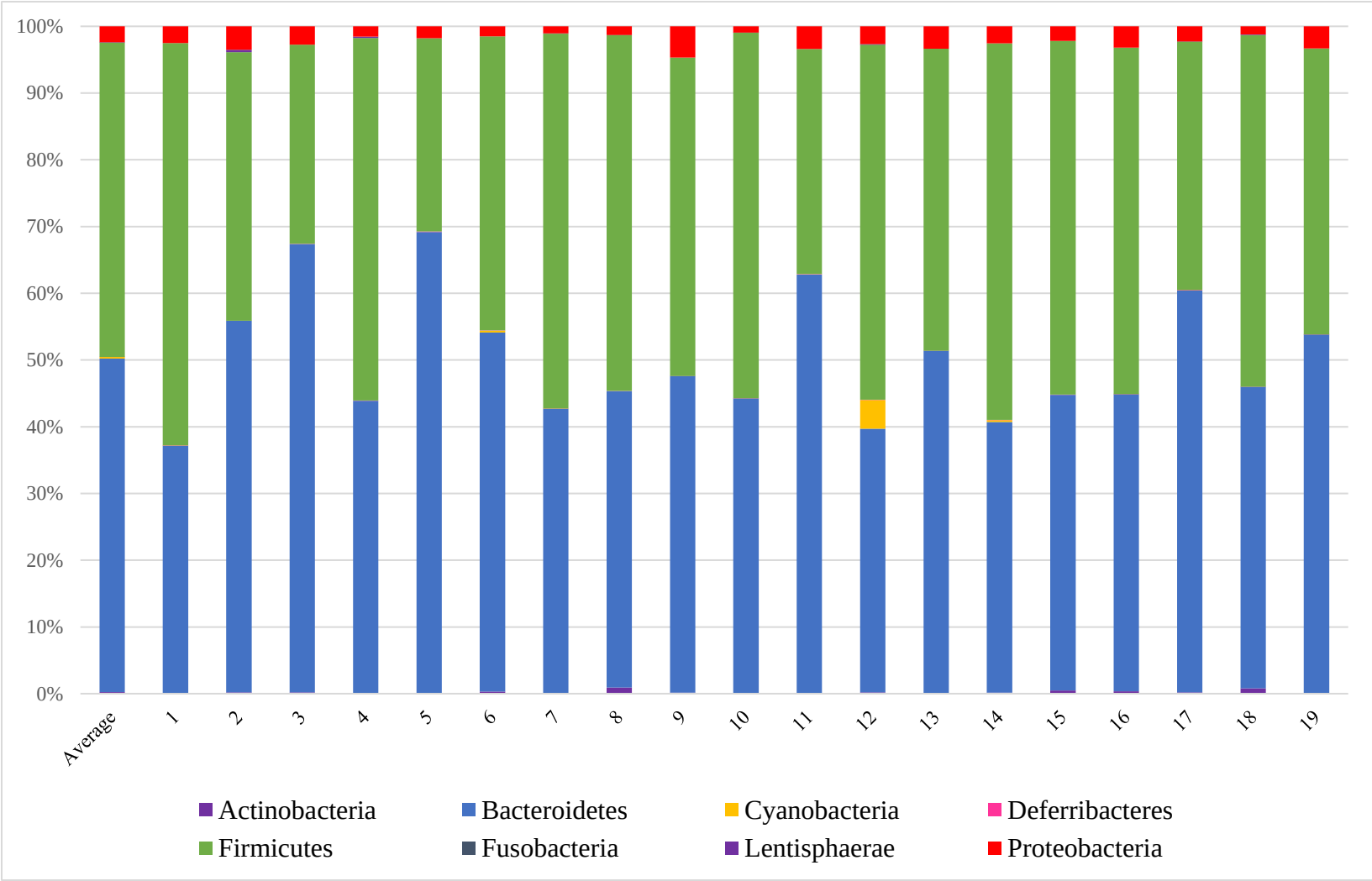

Figure 3.1. Main bacteria phylum identified through 16s rRNA sequencing across 20 fecal microbiota transplants. 1-19 represent the different FMTs

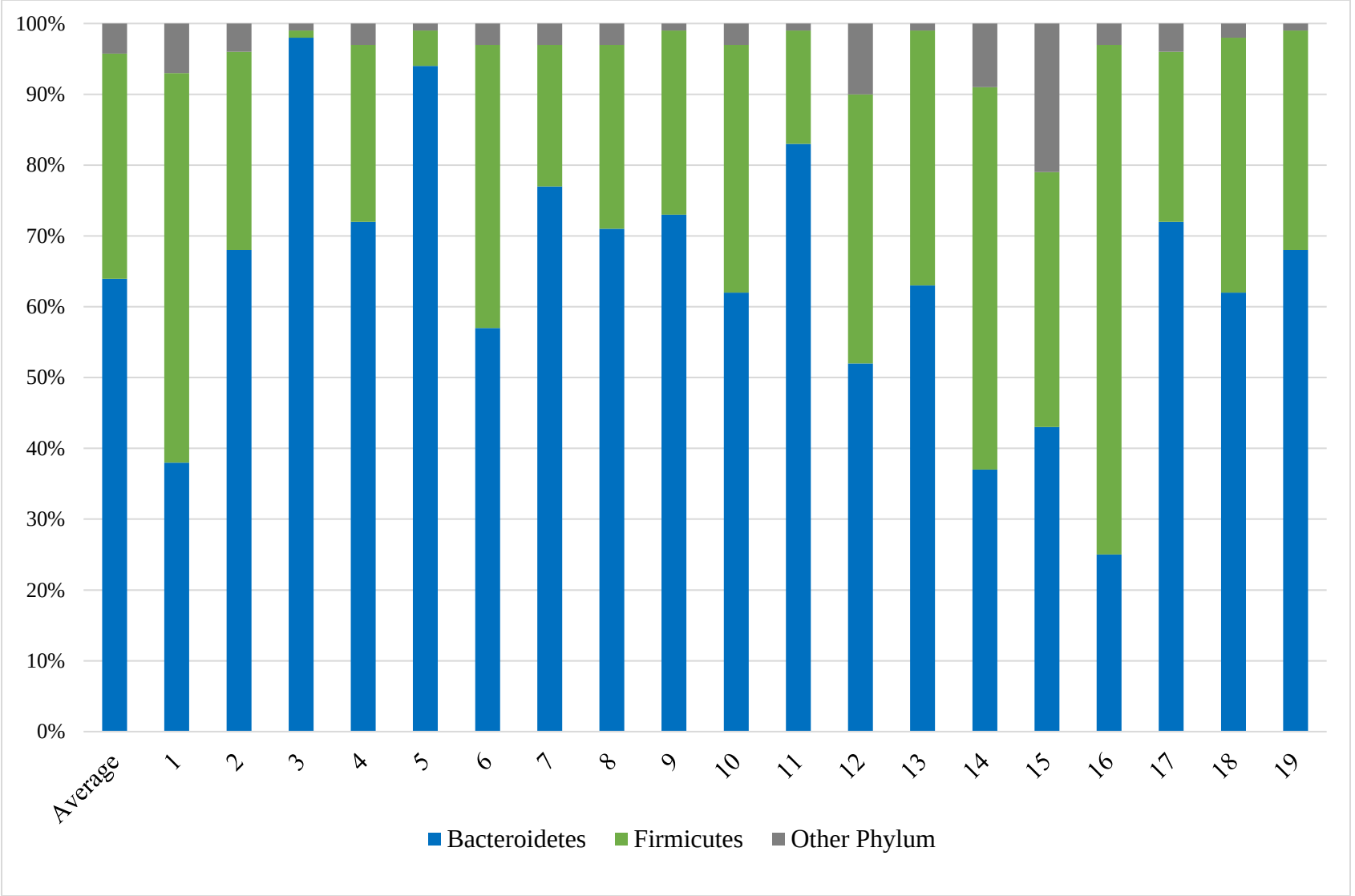

Figure 3.2. Main bacteria phylum identified through whole genome shotgun sequencing across 19 fecal microbiota transplants. 1-19 representing the different FMTs

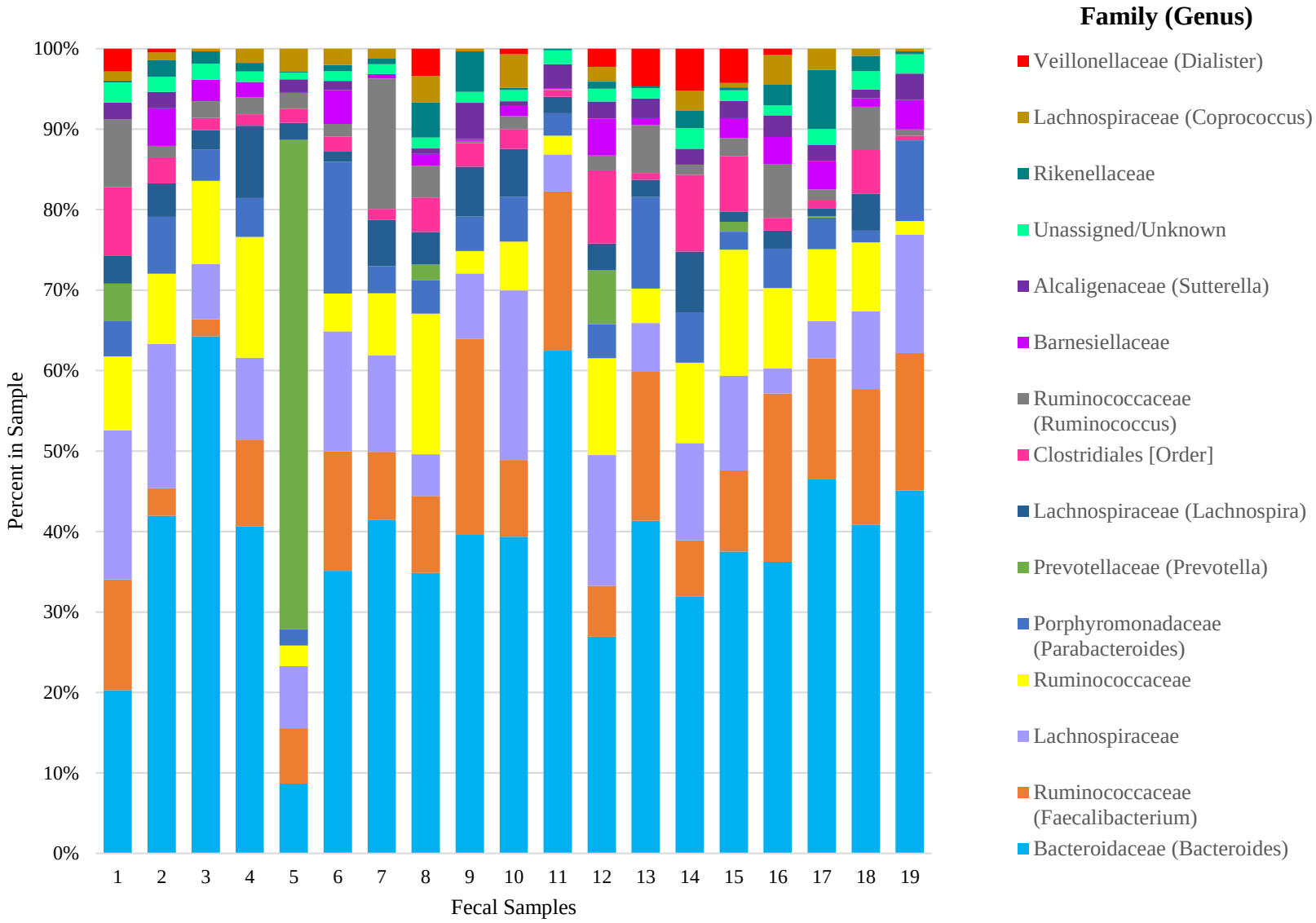

Figure 3.3. Main bacteria family groups identified through 16s rRNA across 19 fecal microbiota transplants. 1-19 representing the different FMTs

Table 3.1. Average frequency of most prevalent *Bacteroides* species found in 19 fecal microbiota transplants

FAMILY	SPECIES	AVERAGE PERCENT IN FMTS
BACTERIODACEA	*Bacteroides vulgatus*	5.06%
	Bacteroides eggerthii	4.30%
	Bacteroides dorei	3.91%
	Bacteroides stercoris	3.13%
	Bacteroides uniformis	3.1%
	Bacteroides helcogenes	2.96%
	Bacteroides ovatus	1.71%
	Bacteroides cellulosilyticus	1.19%
	Bacteroides intestinalis	1.15%
	Bacteroides thetaiotaomicron	0.98%
	Bacteroides fragilis	0.86%
	Bacteroides finegoldii	0.76%
	Bacteroides xylanisolvens	0.62%
	Bacteroides pectinophilus	0.60%
	Bacteroides caccae	0.40%
	Bacteroides coprocola	0.39%
	Bacteroides capillosus	0.36%
	Bacteroides plebeius	0.34%
	Bacteroides coprophilus	0.34%

Table 3.2. Average frequency of *Ruminococcaceae* species found across 19 fecal microbiota transplants

FAMILY	SPECIES	AVERAGE PERCENT IN FMTS
RUMINOCOCCACEAE	*Faecalibacterium prausnitzii*	14.99%
	Subdoligranulum variabile	0.57%
	Anaerotruncus colihominis	0.56%
	Ruminococcus gnavus	0.28%
	Ruminococcus obeum	0.23%
	Ruminococcus albus	0.12%
	Ruminococcus torques	0.10%
	Ethanoligenens harbinense	0.06%
	Ruminococcus lactaris	0.06%
	Acetivibrio cellulolyticus	0.03%

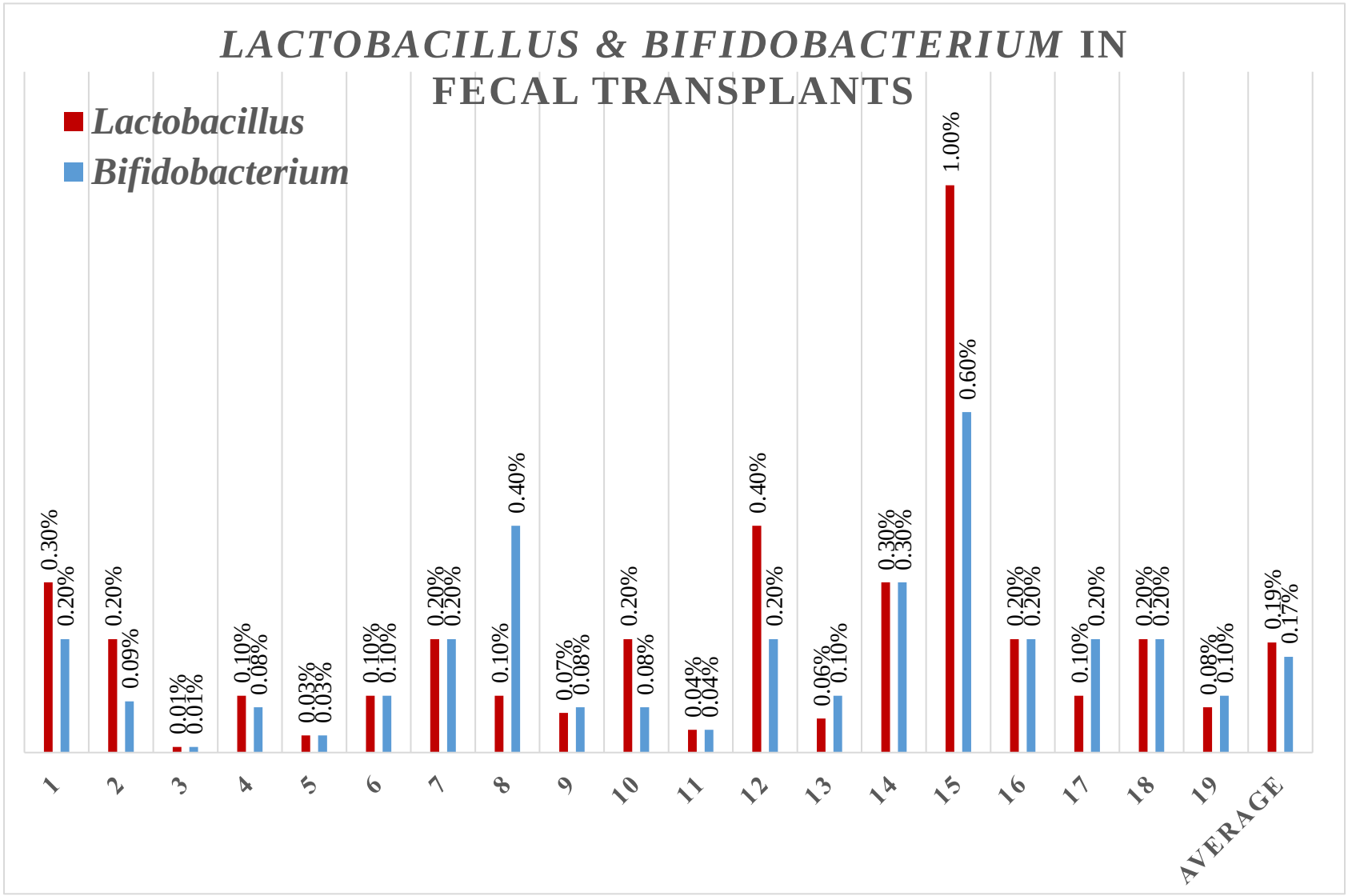

Figure 3.4. Percent of *Lactobacillus* & *Bifidobacterium* species found across 19 fecal transplant materials. 1-19 representing the different FMTs.

Table 3.3 Average frequency of each *Lactobacillus* species out of the total *Lactobacillus* genus found in the 19 fecal microbiota transplants.

SPECIES	PERCENT OF SPECIES IN *LACTOBACILLUS* GROUP
L. delbrueckii	12.16%
L. salivarius	9.84%
L. paracasei	7.89%
L. plantarum	7.89%
L. reuteri	6.85%
L. brevis	6.79%
L. gasseri	6.05%
L. ruminis	6.0%
L. helveticus	4.15%
L. johnsonii	4.03%
L. iners	3.36%
L. rhamnosus	3.33%
L. acidophilus	3.25%
L. casei	3.09%
L. fermentum	3.01%
L. amylovorus	2.52%
L. jensenii	1.67%
L crispatus	1.39%
L. ultunensis	1.08%
L. coleohominis	1.06%
L. vaginalis	0.99%
L. antri	0.81%
L. amylolyticus	0.70%
L. buchneri	0.52%
L. hilgardii	0.46%
L. oris	0.45%
L. sanfranciscensis	0.05%
L. sakei	0.05%
L. agilis	0.02%
L. pentosus	0.004%
L. dextrinicus	0.003%
L. taiwanensis	0.003%
L. paraplantarum	0.0005%

Table 3.4 Average frequency of each *Bifidobacterium* species out of the total *Bifidobacterium* genus found in the 19 fecal microbiota transplants.

SPECIES	PERCENT OF SPECIES IN *BIFIDOBACTERIUM* GROUP
B. longum	50.05%
B. animalis	14.52%
B. bifidum	10.74%
B. dentium	8.32%
B. adolescentis	5.44%
B. breve	2.67%
B. catenulatum	2.22%
B. pseudocatenulatum	2.08%
B. gallicum	1.68%
B. angulatum	1.20%
Bifidobacterium sp. 12_1_47bfaa	1.02%
B. ruminantium	0.02%
B. asteroides	0.004%

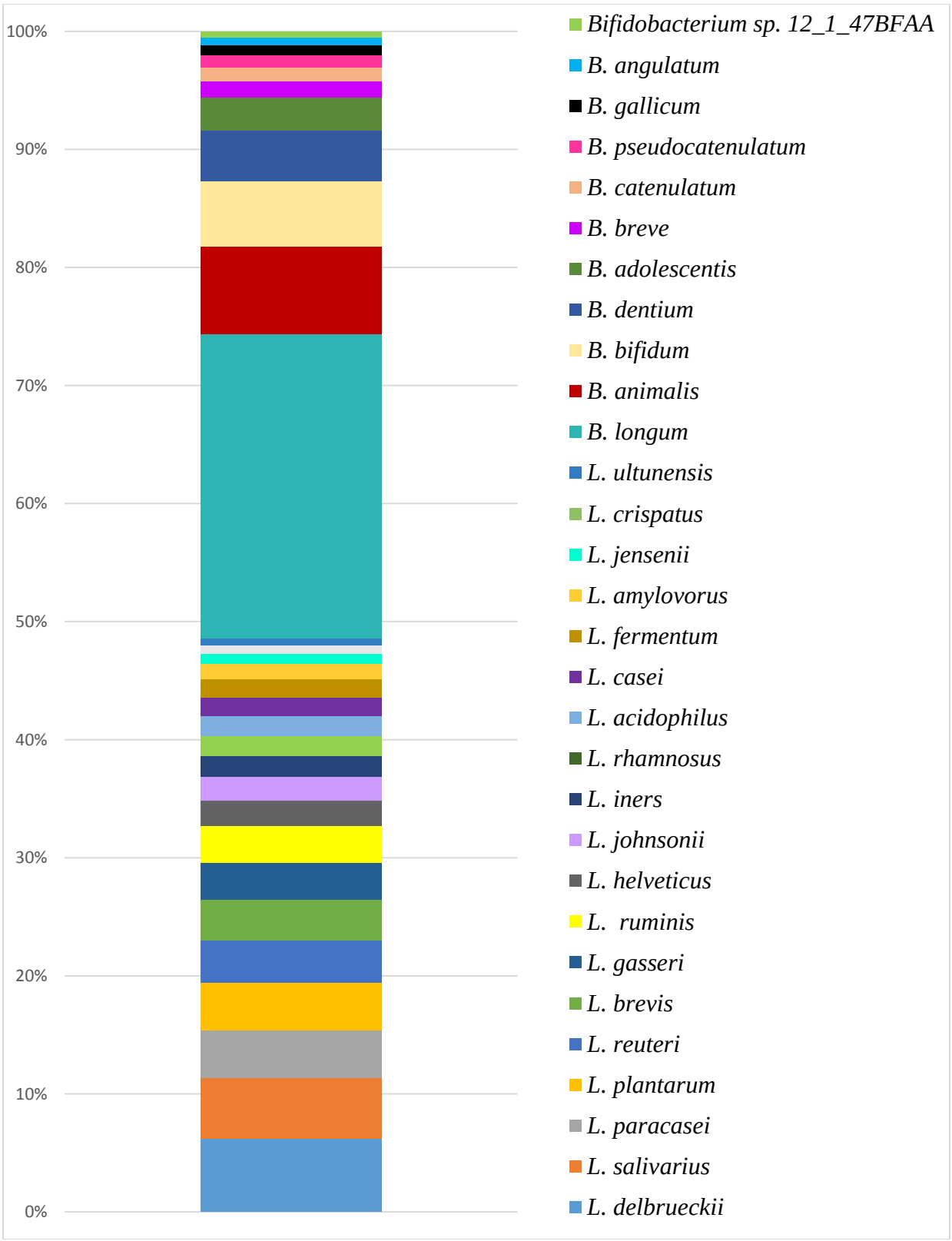

Figure 3.5. Frequency of *Lactobacillus* and *Bifidobacterium* species from 19 fecal microbiota transplants.

CHAPTER 4: EVALUATION OF *CLOSTRIDIUM DIFFICILE* INHIBITION BY KEFIR AND SELECT ISOLATE FROM KEFIR, *LACTOBACILLUS PARACASEI*

ABSTRACT

With *Clostridium difficile* infection (CDI) causing 30,000 deaths in the United States each year, better treatment methods (other than antibiotics) are needed. Fecal microbiota transplants have been one method to emerge in the early 2000s, however it is considered an investigational therapy. This has led to research into the area of probiotics as a potential treatment method for CDI. The purpose of this study was to evaluate microorganisms found in kefir, for effectiveness against *C. difficile*. The kefir and the different kefir components (cell-free supernatant, cell lysate, fat) inhibition capabilities were tested against *C. difficile*. It was found that neither the different components nor the entire kefir matrix showed any inhibition against *C. difficile*. However, a *Lactobacillus paracasei* isolate was found to not only grow alongside *C. difficile*, but to have resistance against the antibiotic vancomycin, making it a potential candidate for competitive inhibition against *C. difficile*. *L. paracasei* showed little inhibition when inoculated at the same population concentration as the *C. difficile* and also did not significantly inhibit *C. difficile* growth when a one log population (CFU/ml) difference was used.

INTRODUCTION

With the increase in *Clostridium difficile* infections (CDI), new methods of treatment are needed. Fecal microbiota transplants (FMTs) are starting to replace the traditional

antibiotic treatments. FMTs are fecal microbiota from healthy human donors that are used to treat patients with CDI. Each fecal donations is highly screened for unwanted pathogens and viruses and are thought to help "re-inoculate" the CDI patient intestinal tract, with beneficial bacteria (Bakken et al. 2011; Shankar et al. 2014). However, since FMTs are still investigational and not always an option for CDI patients, much research has turned to probiotics as a potential treatment method for CDI (Patel et al. 2013; Bakken 2014). A disruption in the native gut microbiota allow for spores of *C. difficile* to start germinating in the gut; it is postulated that probiotics can help re-populate the gut and protect the patient through their several known inhibitory mechanisms (Plummer et al. 2004).

Two main probiotics genera, *Lactobacillus* and *Bifidobacterium*, have been utilized in several CDI clinical studies. Both Gao et al. (2010) and Hickson et al. (2007) found a decrease in *C. difficile*-associated diarrhea (CDAD) when patients were given a probiotic supplement along with antibiotics. Bakken (2014) utilized a Lifeway® kefir regimen with a staggered and tapered antibiotic withdraw (STAW) treatment. A STAW treatment simply means that the dose of antibiotics is decreased over time as well as given less times per day. In this study, Bakken found a significant improvement in CDI symptoms along with a decreased risk of recurrent CDI. Kefir, a fermented milk beverage, contains a diverse collection of potentially probiotic bacteria. Due to the high bacterial diversity in kefir, it is hypothesis that the bacteria are able to "re-inoculate" the gut, similar to FMTs, and through either production of antimicrobials (bacteriocins or short-chain fatty acids) or competitive exclusion, inhibit *C. difficile* germination and growth (Lee & Salaminien 2009; Bakken 2014). In another study done by Ambalam et al. (2015), four probiotics strains, which are also found in Lifeway® kefir (*L. paracasei, L. plantarum, B. breve* and *B. animalis*), were

used in *in vitro* competitive inhibition tests against *C. difficile*. In this study, these probiotics were found to inhibit *C. difficile* growth. This suggests that some (or all) of these probiotics may be potentially beneficial in treating CDI.

Although several meta-analysis studies of clinical studies have suggested that probiotics tend to be favored over the control when used to treat CDI, the studies are limited and contain mixed results (Johnston et al. 2012). Limited research has been done to identify the mechanisms probiotics use to enable treatment of CDI patients with success.

Many probiotics are known to produce different types of organic acids and antimicrobials, such as butyrate, lactic acid, acetate and bacteriocins (Lee & Salaminien 2009; Fijan 2016; Spinler et al. 2016). These compounds would be present in the cell-free supernatant of the kefir product. The objective of this research was to investigate the ability of kefir, its cell-free components, and individual kefir bacteria to inhibit *C. difficile* growth either from production of antimicrobial compounds or competitive inhibition.

MATERIALS AND METHODS

Kefir Cell-Free Supernatant Extraction.

Commercial Lifeway® kefir, purchased from local grocery stores, was centrifuged at 15,000 x g at 4 °C for 15 minutes in 50 ml sterile centrifuge tubes. The supernatant was decanted after centrifugation and was filtered through 0.8 µm Millipore filters. Kefir Supernatant, cell lysate and fat separation were used in zone of inhibition tests. The fat portion was separated by adding 5% polyethylene glycol (FisherChemicals, NJ) to the fat and cell lysate sections and centrifuging again at 10,000 x g for 15 minutes. The cell lysate was

decanted after centrifugation, leaving the fat section. All kefir components were stored at -80 C until use.

Isolation of and identification of *Lactobacillus paracasei* from Kefir

Isolation of *Lactobacillus paracasei* was done using cycloserine-cefoxitin-fructose agar with sodium taurocholate (TCCFA) (*Clostridium difficile* agar, BioWorld, Ohio; *C. difficile supplements*, Thermo Scientific; Bovine Blood Defibrinated, Lampire® Bilogical Lab, PA). 500ul of kefir was added to 10ml of Brain-Heart Infused (BHI) broth (FisherScientific) and vortex. This solution was streaked on TCCFA plates to obtain an isolated colony and incubated in an anaerobic chamber for 48 hours. One colony from streak-plate was added to new BHI broth and incubated in anaerobic chamber for 48 ours. After incubation, 500ul was spread onto TCCFA plates and taken to the Iowa State VetMed Diagnostic lab (Ames, IA) for Matrix-assisted laser desorption/ionization identification. 100ul of *L. paracasei* broth culture was added to 500ul of glycerol to make glycerol-stock and stored at -80 C.

Zone of inhibition studies

Pure *C. difficile* glycerol stock cultures (isolated at ISU VetMed) were used. Each strain was grown up to 10^8 CFU/ml by first swabbing the pure culture onto TCCFA plates. Isolated colonies were added to 10ml Brain-Heart Infused (BHI) broth (FisherScientific) and incubated in anaerobic chamber for 12 hours. The disk diffusion agar overlay method, as described in Xia et al. (2012) was used with modifications. In brief, overnight pure cultures (10^8 CFU/ml) of *C. difficile* strains were used to create lawns on solid agar plates, grown in BHI broth. Individual *C. difficile* strains were used to evaluate various levels of virulence.

Strains included Ribotype 027/Toxino Type III/NAP 1 (hyper-virulent), Ribotype 020, Ribotype 012, Ribotype 002, Ribotype 001, Ribotype 056, Ribotype 126 and Ribotype 010 (negative toxin production). Pour plates were made with cycloserine-cefoxitin-fructose agar with sodium taurocholate (TCCFA) (*Clostridium difficile* agar, BioWorld, Ohio; *C. difficile supplements*, Thermo Scientific; Bovine Blood Defibrinated, Lampire® Bilogical Lab, PA) and allowed to cool/harden. A 200ul culture suspension of *C. difficile* was spread onto to each respective plate.

For kefir component (cell-free supernatant, cell lysate, fat) or full kefir (no separation), drops (10-100ul) of each sample along with vancomycin and BHI were added on top the seeded agar overlay, in four separate quadrants of the plate. The plates were inverted and incubated anaerobically in an anaerobic chamber overnight at 37°C. Vancomycin was used as positive control, and BHI was used as negative control. Growth was observed after 48 hours and each separation and strain was done in duplicate.

***In vitro* competitive growth assay**

Competitive inhibition properties of *Lactobacillus paracasei* against *C. difficile* was measured through *in vitro* competitive growth assay following the method of Cornick et al 2017, with some modifications. In short, three strains of *C. difficile* (Ribotype 027/Toxino Type III/NAP 1 (hyper-virulent), Ribotype 002, and Ribotype 020) and the *L. paracasei* strain (isolated from kefir), were grow separately overnight in BHI broth in an anaerobic chamber to create the stock culture of vegetative cells. BHI broth was used to obtain vegetative *C. difficile* cells and promote their growth to prevent sporulation. *L. paracasei* (1000ul) was added to each competitive inhibition BHI broth and its control. Individually 10ul of each *C. difficile* strain (in duplicate) was added to the competitive inhibition broth

(Table 4.3) and its control BHI broth. Serial dilutions were done of each stock culture to determine initial populations. After 48 hour incubation, plate counts were done to determine colony-forming units per milliliter (CFU/mL). Competitive inhibitions and controls were checked after 24 hours. Serial dilutions were performed again, along with plate counts, after 48 hours. A t-test was used to determine if there was any significant difference between the *C. difficile* grown with *L. paracasei* and on its own, using JMP (JMP®, Version *Pro 12*. SAS Institute Inc., Cary, NC, 1989-2007).

RESULTS AND DISCUSSION

Lifeway® kefir has been shown to have a possible benefit to patients with *Clostridium difficile* infection (CDI) (Bakken 2014), but no mechanism has been confirmed. Because of this, Lifeway® kefir and kefir components (cell-free supernatant, fat, and cell lysate) were used in zone of inhibition tests against different *C. difficile* strains. Each component (cell-free supernatant, fat, and cell lysate) was used in the zone of inhibition test against the *C. difficile* strain, Ribotype 027/Toxino Type III/NAP 1 human hyper-virulent strain. This strain was used because there are several hundred different pathogenic human *C. difficile* strains, each which produces different amounts of the toxins (toxins A & toxin B). This hyper-virulent strain produces higher amounts of both toxins and tends to be hard to treat in patients, thus it was used as the first step in investigating kefir's potential inhibition capabilities.

For all components of the kefir, no inhibition zones were found (Table 4.1), thus demonstrating that cell-free supernatant, cell lysate and fat from commercial Lifeway® kefir may not contain inhibitory compounds that prevent growth of *C. difficile* Ribotype 027.

Another hypothesis is that in the Lifeway® kefir, the antimicrobials and organic acids were not present in high enough concentrations to inhibit *C. difficile* Ribotype 027. Further investigations to identify if bacteriocins and short-chain fatty acids are present in Lifeway® kefir, and to test at which concentrations such compounds could inhibit *C. difficile*, would help answer this question.

Since no inhibition was found from the separated components, the entire kefir matrix (no separation) was investigated with the zone of inhibition test. Since there are several hundred different human *C. difficile* strains with different toxin production and resistances, a selection of nine different strains (Table 4.2) were utilized in zone of inhibition tests to evaluate differences between *C. difficile* strains, in kefir's ability to inhibit its growth. Unfortunately, the kefir did not show any inhibitory effect on the nine different *C. difficile* strains. This was not expected since kefir was shown to be an effective supplement for CDI patients in the study by Bakken (2014). Again, this result may have been because of low concentrations of the potentially beneficial microorganisms or inhibitory compounds in the kefir. In addition to this, the kefir microorganisms may inhibit *C. difficile* through competitive exclusion, which was not tested through the zone of inhibition test. To test this, a competitive inhibition test using just the isolated probiotics strains from kefir, would be needed; this is what was done next.

Through the zone of inhibition study, an unknown bacteria from the kefir was found to grow alongside *C. difficile.* The fact that it grew alongside *C. difficile* means that it was resistant to the antibiotic, Vancomycin. After isolation and Matrix-assisted laser desorption/ionization (MALDI) identification test from the ISU VetMed Diagnostic lab, the microorganism was identified as *Lactobacillus paracasei*. The concentration of *L. paracasei*,

determined in two lots of Lifeway® kefir, was 10^9 CFU/mL. Because *L. paracasei* was found to grow on the same selective *C. difficile* agar with *C. difficile*, it was hypothesized that *L. paracasei* could potentially act as a competitive inhibitor, through competitive exclusion, against *C. difficile*. Competitive exclusion, a form of competitive inhibition, were the probiotic bacteria outcompetes the pathogenic bacteria for bacterial adhesion site (in intestine or on media) or outcompetes for similar nutrients. Because *L.* paracasei was found to grow on a similar selective media agar as *C. difficile*, it was hypothesized that it could inhibit *C. difficile* through competitive exclusion. Along with this, *L. paracasei* in combination with other probiotics, was found to inhibit *C. difficile* in a similar study by Ambalam et al. (2015). In this study, the probiotics *L. paracasei, L. plantarum, B. breve* and *B. animalis*, were evaluated as a group and if they used cross-feeding in the co-cultures to enhance their inhibition against *C. difficile*. Cross-feeding is common amongst several probiotic species; it is the growth enhancement of probiotics by the byproducts of other probiotic bacteria. Ambalam et al (2015) found that *L. paracasei, L. plantarum, B. breve* and *B. animalis* used together did show inhibition *C. difficile* growth.

To investigate further, *L. paracasei* was used in an *in vitro* competitive growth assay. For this study, a greater population concentration of the *L. paracasei* was used against a smaller population concentration of *C. difficile* to test if having an increase in the probiotics would prevent *C. difficile* growth. However, as shown in Table 4.3, *L. paracasei* did not significantly inhibit the growth of any of the three *C. difficile* strains used (Ribotype 027/Toxino Type III/NAP 1 (hyper-virulent), Ribotype 002, and Ribotype 020) through competitive exclusion. One hypothesis for this is that not one probiotic is needed to out-compete *C. difficile*, but many. It has been well established that probiotics can stimulated

other the growth of other potentially probiotic bacteria, through cross-feeding, by producing by-products that are usable as energy; this concept was established with *L. paracasei* and *B. breve* in Ambalam et al. (2015).

This hypothesis that a cocktail of potential beneficial bacteria may be appropriate for treating CDI is supported by the results found in Chapter 3. The human gut is made up of a diverse amount of beneficial probiotic bacteria and many *Lactobacillus* and *Bifidobacterium* species were found to be common across 19 healthy donors. Thus, it could be more beneficial to utilize a treatment of a large diversity (one similar to a healthy FMT donor) as a treatment for CDI, not just a few select ones.

CONCLUSIONS

Using Lifeway® kefir (and its components) in zone of inhibition test was unsuccessful at inhibiting *C. difficile*. *Lactobacillus paracasei*, a probiotic found in kefir, was identified as a potential competitive exclusion bacteria since it could grow on similar selective nutrient media and was resistant to one of the main antibiotics used to treat CDI, vancomycin. But in the competitive inhibition study, *L. paracasei* did not significantly decrease *C. difficile* growth, even when present in a higher cell concentration. Further research is warranted to investigate if *L. paracasei*, when combined with additional select bacteria, could inhibit *C. difficile*.

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Table 4.1. Zone of inhibition test of kefir components against *C. difficile* Ribotype 027/Toxino type III/Nap 1 human hyper-virulent strain

Kefir Component		Vancomycin (10ul)	BHI (10ul)
Fat (100ul)	No inhibition	inhibition	No inhibition
Cell Lysate (10ul)	No inhibition	inhibition	No inhibition
Combine (Fat & Cell-Lysate) (100ul)	No inhibition	inhibition	No inhibition

Inhibition is defined as any clearing, or zone of inhibition of *C. difficile* growth

Table 4.2. Summary of zone of inhibition tests of kefir against 9 human virulent *C. difficile* strains.

***C. difficile* Strain**	**Kefir**	**BHI (negative control)**	**Vancomycin (positive control)**
Ribotype 027/Toxino Type III/NAP 1 hyper-virulent strain[2]	No inhibition	No inhibition	Inhibition
Ribotype 030/double positive[1,2]	No inhibition	No inhibition	Inhibition
Ribotype 012/double positive[1,2]	No inhibition	No inhibition	Inhibition
Ribotype 002[2,3]	No inhibition	No inhibition	Inhibition
Ribotype 020/double positive[1,2]	No inhibition	No inhibition	Inhibition
Ribotype 001[2,3]	No inhibition	No inhibition	Inhibition
Ribotype 056/double positive[1,2]	No inhibition	No inhibition	Inhibition
Ribotype 126/double positive[1,2]	No inhibition	No inhibition	Inhibition
Ribotype 010/Double Negative[3,4]	No inhibition	No inhibition	Inhibition

[1] double positive means produces both toxin A & B
[2] All *C. difficile* strains are human virulent strains
[3] toxin production is unknown
[4] double negative means no toxins (A & B) are produced
Each strain done in 4 replications
Inhibition is in regards to *C. difficile* growth

Table 4.3. Competitive inhibition of *Lactobacillus paracasei* against *C. difficile* strains. Competitive means grown with *L. paracasei*, control is without. No inhibition was found to be significant between strains competitive group and control.

C. difficile strain	*Competitive (Average log CFU/ml)*	*Control (Average log CFU/ml)*	*P-value*
Ribotype 002[2,3]	7.9	7.3	$p > 0.05$
Ribotype 020/double positive[1,2]	8.1	7.9	$p > 0.05$
Ribotype 027/Toxino Type III/NAP 1 hyper-virulent strain[2]	7.7	7.7	$p > 0.05$

[1] double positive means produces both toxin A & B
[2] human virulent strains
[3] toxin production is unknown

CHAPTER 5: GENERAL CONCLUSION

Through this study, it was established that fecal microbiota transplant material from 19 healthy humans were primarily composed of Bacteroidetes and Firmicutes bacteria. *Faecalibacterium prausnitzii* made up one of the largest species populations present across the 19 FMT donors; the microorganism has been established to produce high amounts of butyrate and anti-inflammatory compounds. However, very few studies have investigated if this microorganism is effective against *C. difficile* and if it could potential help treat CDI patients.

The WGS of the 19 FMTs also displayed that a healthy microbiota is made up of several different *Lactobacillus* and *Bifidobacterium* species. Many of these potentially probiotic bacteria species are also present in kefir products. However, Lifeway® kefir, which was effective in helping CDI patients recover one previous study, was found to not inhibit several different *C. difficile* strains.

Lactobacillus paracasei, which was isolated from Lifeway® kefir, showed some potential to act as a competitive inhibitor through competitive exclusion, since it was resistant to vancomycin and utilized similar nutrients to *C. difficile.* Yet it was found to not significantly decrease the growth of three strains of *C. difficile* in the competitive inhibition studies.

Overall, through the WGS of FMTs and kefir inhibition tests, the present research suggests that many different potentially probiotic species need to be investigated, as cocktails for their ability to inhibit *C. difficile* growth. With such studies, we will gain a better understanding of the mechanisms of action and potential treatment for CDI.

CHAPTER 6: FUTURE RESEARCH

Probiotics have shown some success in treating CDI patient in clinical trials, however more research is needed. WGS has displayed that several different probiotic bacteria species are present cross many healthy donors, suggesting that it may take more than one or two to help treat CDI. To test this hypothesis, future research in the area of probiotics as a treatment method for CDI, needs to focus on several probiotic bacteria species, such as *Lactobacillus* and *Bifidobacterium*, they are commonly present in a healthy human GI tract instead of just looking at one or two. Understanding how these gut probiotic bacteria function could lead to better understanding into the type of probiotics need to treat CDI patients.

Research has shown that dairy products such as kefir, can be beneficial in treating CDI; however more research is needed in investigating the types of antimicrobial or short-chain fatty acids produced by the probiotics as well as establishing the concentration that would be need to potential inhibit *C. difficile.* In addition, more research focusing on *Lactobacillus paracasei*, the probiotic isolated from kefir, could provide more insight into if it can be beneficial in treating CDI. In other studies, it was found to have an increased growth when grown with other *Lactobacillus* and *Bifidobacterium* species; further suggesting that a cocktail of several different probiotics could increase inhibition of *C. difficile.*

Along with this, *Faecalibacterium prausnitzii*, which a major gut microbiota (making up 15% of gut microbiota), has been established as a bacteria helpful in treating IBS patients and produces butyrate and anti-inflammatories. Research is very limited if *F. prausnitzii* is present in CDI patients and if it could be utilized to treat CDI. As technologies improve in the area of genomic sequencing and culturing, our understanding of how the gut microbiota

works and promotes human health, with only increase our ability to treat intestinal disease and infections, such as CDI.