5-9-2015

Effect of Probiotics in the Mitigation of Clostridium difficile Associated Disease

Oluwaseyi Adekunle

EFFECT OF PROBIOTICS IN THE MITIGATION OF *CLOSTRIDIUM DIFFICILE* ASSOCIATED DISEASE

By

OLUWASEYI ADEKUNLE

Under the Direction of ANDREW GEWIRTZ PhD

ABSTRACT

Clostridium difficile is one of the most important and widespread causes of acute gastroenteritis. Due to the limitations of current therapeutic options there is a need for the development for new treatment methods. Probiotics are an alternative treatment option that have gained prominence in the last few years, and the ability of various probiotics to mitigate *C. difficile* infection was tested. The probiotics *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii* demonstrated the greatest mitigative effect, showing signification protection against *C. difficile* induced death, bioactive toxin and shedding of pathogen within feces in the germ free mouse model. The probiotics also demonstrated protective effects by delaying the onset of *C. difficile* induced mortality as well in the conventional mouse model. Thus, some probiotics could potentially be used to help mitigate symptoms of *C. difficile* associated disease.

INDEX WORDS: Probiotics, *Clostridium difficile*, *Lactobacillus rhamnosus GG*, *Saccharomyces boulardii*, Fecal Microbiota Transplant, *Lactobacillus acidophilus*

EFFECT OF PROBIOTICS IN THE MITIGATION OF CLOSTRIDIUM DIFFICILE ASSOCIATED DISEASE

by

OLUWASEYI ADEKUNLE

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2015

EFFECT OF PROBIOTICS IN THE MITIGATION OF CLOSTRIDIUM DIFFICILE ASSOCIATED DISEASE

by

OLUWASEYI ADEKUNLE

Committee Chair: Andrew Gewirtz

Committee: Didier Merlin

Parjit Kaur

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

May 2015

DEDICATION

To my parents Kayode and Mosun Adekunle, who never stopped believing in me; and to my grandmother Musilimat; who was always there when I needed someone to talk to, and give advice when I needed to hear it.

ACKNOWLEDGEMENTS

The first of my acknowledgements must go to my mentor Dr. Andrew Gewirtz. I will always be grateful that you took a chance and allowed me to join your laboratory. Your door was always open whenever I needed advice, guidance, or just to humor me with the random questions that popped into my head. Your guidance was instrumental, not only in helping to guide the direction of this project and keeping it on tract but also in helping to ensure I had the skills to be successful in my research career. You are an amazing mentor and I am extremely glad that I got to experience research in your lab.

I would also like to thank my committee members Dr. Parjit Kaur and Dr. Didier Merlin, who were always there to give me advice when I needed it.

I cannot, of course, forget Dr. Lucie Etienne-Mesmin. While Dr. Gewirtz helped lay out the general plan it was you who was with me in the trenches, helping me learn how to perform techniques, and analyzing the data. Through this project I learned that sometimes, even great ideas don't always pan out, and you were there giving me perspective in hard times. Without your tireless efforts this project would cease to be. Thank you for all that you have done, you are an excellent teacher and I owe a great deal of my newfound knowledge and confidence in research to you. I am very grateful for your assistance.

I would also like to thank the rest of my lab members. Dr. Benyue Zhang for taking the time out of your day to teach me to handle animals. Dr. Benoit Chassaing who was always there whenever I needed to bounce experimental designs off of. Dr. Robin Uchiyama who gave me sound advice and feedback when this project was nothing but a faint idea in the back of my mind. Zhang Zhang and Zhenda Shi who were always there to answer my numerous procedural questions. Hasrat Shah who's efforts helped ensure the timely arrival of all of our lab supplies.

Hao Tran who provided a sounding board as another comrade in arms going through a research project. Also Jennifer Miles and Aneseh who together with the rest of the lab made coming to work something I looked forward too. I thank all of you sincerely!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

1.1 Clostridium difficile

Acute gastroenteritis, characterized by inflammation of the gastrointestinal tract, can lead to symptoms such as fever, diarrhea, vomiting, and general malaise. The root cause of gastroenteritis can be linked to a variety of sources such as bacteria or viral infections, certain toxins, parasites and other factors. The incidence of this disease has been steadily increasing over the past few years with around 170 million cases occurring annually in just the U.S. alone (Scallan et al., 2011). One of the more notable causes of gastroenteritis which has gained notoriety in the last few years is *Clostridium difficile* (*C. difficile*).

C. difficile is a gram positive, spore forming, obligate anaerobic bacterium (Aslam et al., 2005). While not a normal part of the human microbiota it can still be found in around 3% of healthy asymptomatic carriers. This number increases dramatically, however, when looking at hospital patients where the number of infected individuals can reach as high as 35% (Aslam et al., 2005). In fact, *C. difficile* is one of the main causes of nosocomial diarrhea in the US and in parts of Europe (Gorbach et al., 1999). The high infection rate of hospital patients compared to the normal population is directly correlated with the use of antibiotics in hospital environments (Hall et al., 2012). While it is known that antibiotics are extremely useful in controlling, preventing, and curing a wide variety of bacterial infections it is also documented that antibiotics, particularly those of the broad-spectrum variety, do not discriminate between the normal commensal microbiota and pathogenic bacteria (Lessa et al., 2012). The normal microbiota is one of the key features that helps to protect the body from pathogenic organisms through methods such as outcompeting, limiting areas where pathogens can establish themselves, and by limiting growth of pathogenic

microorganisms (Gareau et al., 2010). These protective effects are especially important within the gastrointestinal tract because it faces daily exposer to various foreign microorganisms.

C. difficile, setting aside the antibiotic resistant strains, is usually not extremely resistant to antibiotic treatment. However, *C. difficile* is able to rebound from antibiotic treatment faster when compared to the normal microbiota. This leads to large proliferation of *C. difficile* and thus massive secretion of its two toxins; toxin A, encoded by the *tcdA* gene, and toxin B, encoded by *tcdB* gene, which work together to cause severe colitis. These toxins act by damaging the intestinal epithelial cells as well as acting as attracting leukocytes, primarily neutrophils (Sunenshine et al., 2006). This recruitment of neutrophils causes severe diarrhea, abdominal pain, malaises and, in severe cases, can lead to characteristic pseudomembranous colitis, toxic megacolon, intestinal perforation, and eventually death.

The treatment for this disease is cessation of antibiotic treatment and, if necessary, administration of metronidazole or vancomycin, two antibiotics used to treat *C. difficile* infection (Aslam et al., 2005). This treatment poses a few problems, notably that the antibiotic administration was likely important for treatment of a prior illness, while metronidazole and vancomycin use is discouraged to prevent the formation of antibiotic resistant strains. This antibiotic treatment is also not extremely effective, resulting in relapse rates of around 26% (Aslam et al., 2005). Those that relapse also have a higher chance of undergoing further episodes of relapse, especially if they take anymore antibiotics (Aslam et al., 2005).

The diarrhea of those infected with *C. difficile* contains its spores which can then be spread throughout the hospital. These spores can be ingested by other patients leading to their colonization and infection. Since these patients are likely to be on antibiotics, when colonized by *C. difficile*

they quickly become infected themselves and become a new source of spore sheading. This cycle can lead to outbreaks of *C. difficile* occurring within the hospital environment.

One of the most effective treatments for *C. difficile* is the administration of a Fecal Microbiota Transplant (FMT), which consist of taking fecal samples from healthy, *C. difficile* free patients and giving them to infected individuals (Rohlke F et al., 2012). This approach has a high success rate, however, some limitations include the fact that administration of FMT has not been approved by the FDA and thus to undergo the treatment one must first attempt rounds of traditional treatment with metronidazole and vancomycin, as well as other requirements which vary from hospital to hospital, must be met to undergo the procedure. Locating and screening healthy *C. difficile* free donors for fecal transplants also adds to the time and cost of this treatment. The idea of taking in fecal material from another person can be a concept that some people might find to be undesirable. FMTs can also have unintended side effects. For instance, there was a case where a patient who underwent FMT with feces from an obese donor became obese themselves (Alang et al., 2015).

Therefore there is a search for low cost, less invasive, and more effective ways in which to treat *C. difficile* associated disease (CDAD).

1.2 Probiotics

One option which has gained some prominence in the past few years are probiotics. Probiotics are defined as, “Live microorganisms that, when administered in adequate amounts, confer a health benefit to the host” (Guarner et al., 1998). These organism have been purported to promote healthy gut function in ways such as helping to reduce effects of lactose intolerance, reducing constipation, helping ameliorate gut pain caused by Crohn’s disease or Ulcerative colitis, and elimination of pathogenic microbes such as *H. pylori* (Sanders et al., 2013). The means of

probiotic action can vary from between the various probiotics but some purported mechanisms include acting as a physical barrier, increasing mucosal production, improving tight junctions, releasing antimicrobial peptides, and regulation of the immune response (Gareau et al., 2010).

Probiotics come in a wide variety of various types including both prokaryotic and eukaryotic organisms. Various studies have demonstrated that probiotics can have an effect against many kinds of pathogens, including some studies on *C. difficile*. For instance, the probiotic *Lactobacillus rhamnosus GG* (LGG), has been demonstrated to enhance the efficacy of a vaccine against rotavirus within the neonatal pig model of infection (Vlasova et al, 2013). LGG has also, in conjunction with the probiotic *Bifidobacterium breve* (BB12), been shown to be effective in reducing the duration of diarrhea induced by mild gastroenteritis in a trial using children attending day care (Rosenfeldt et al., 2002). LGG, among other lactic acid producing bacteria such as *Lactococcus lactis*, *Bifidobacterium lactis*, and BB12 have been shown to inhibit the growth of *C. difficile* after coculturing *in vitro* (Lee et al., 2013).

The probiotic *Saccharomyces boulardii* has demonstrated the ability to secrete a protease that interferes with the effects of one of *C. difficile* toxins, toxin A, within the rat ileum (Castagliuolo et al., 1996). It has also been shown in various clinical trials to exhibit protective effects against *C. difficile* infection (Tung et al., 2009).

Gaining a better understanding of which probiotics can be effective against *C. difficile* in vivo and then studying their mechanism of action could lead to better use of probiotics in disease treatment. Thus, this study seeks to determine the efficacy and mechanism by which certain probiotics can ameliorate *C. difficile* infection.

2 METHODS

2.1 Animal Experiments

Mice were housed at Georgia State University's animal facility. WT C57BL/6 mice were obtained from Jackson Laboratories. Mice were transferred to autoclaved cages, food, and water.

2.1.1 Germ free experiments

Germ free mice of WT C57BL/6 or Swiss Webster genetic background which were raised in GSU germ free facility were transferred into autoclaved changes containing both autoclaved food and water. The mice were split into groups and each group was given 200 ul of 10^7 CFU/mL via oral gavage of one out of the four different probiotics (*Lactobacillus rhamnosus GG*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, or *Saccharomyces boulardii* while the control group received 200 ul of PBS. After 24 hours the mice were then challenged with *C. difficile* (VPI 10463 reference strain ATCC 43255, tcdA +, tcdB+) with each mouse receiving 200 ul of 5 x 10^3 CFU/mL for the C57BL/6 mice or 5 x 10^4 for the Swiss Webster background.

2.1.2 Conventional Experiments

C57BL/6 mice were ordered from Jackson Laboratory where upon arrival were transferred into autoclaved cages containing both autoclaved food and water. All mice were put on an antibiotic cocktail containing the following antibiotics (metronidazole (0.215 mg/ml), vancomycin (0.045 mg/ml), gentamicin (0.035 mg/ml), colistin (850 U/ml), kanamycin (0.4 mg/ml)) within their drinking water for three days. After the 3rd day mice were switched to regular autoclaved water. 2 days after switching to regular water mice received of clindamycin via intraperitoneal injection. One day post clindamycin injection

mice were challenged with *C. difficile* (VPI 10463) with each mouse receive 200 ul of 1 x 10^5 CFU/ml.

2.2 *In vitro* Experiments

2.2.1 qPCR of Clostridium difficile from fecal samples

PBS+Tween 0.1% was added to fecal samples to obtain a concentration of 100mg/ml. Samples were shaken using a bead beater for 3 minutes and 250 ul of the fecal suspension was removed and 50 ul of SDS 10% was added to each sample. Samples were incubated at 64ºC for 5 minutes and then 300 ul of Tris-Phenol was added and incubated again at 64ºC for 5 minutes. 300 mg of glass beads were added and samples were shaken through a bead beater apparatus, heated, and centrifuged at 12,000 rpm 5 minutes. Supernatant was removed and added to 250 ul of Tris HCL and 400 ul of Tris phenol. Samples were vortexed and centrifuged at 12,000 rpm for 5 minutes. Supernatant was removed and added to 750ul of chloroform and centrifuged at 12,000 rpm for 5 minutes. Supernatant was removed and added to 900 ul of ethanol and incubated at -20 ºC overnight. Samples were then centrifuged 12,000 rpm for 30 minutes before removal of supernatant. 300 ul of 75% ethanol was added to pellet and samples centrifuged again for 12,000 rpm 5. Supernatant was discarded, pellet was dry and finally resuspeneded in 100ul of molecular grade water, diluted one 1:5, and stored in -20 until used for qPCR.

Cell Preparation: Vero cell (ATCC CCL-81) were grown in Dulbecco's Modified Eagle Medium (altered with 10% heat inactivated fetal bovine serum, 1% glutamine, 1% Penicillin/Streptomycin) at 37ºC at 5% CO2. On day of infection cells were washed with PBS and then resuspended in DMEM at a concentration of 1 x 10^6 CFU/ml. 50 ul of suspension was added to 96 well plate.

Sample Preparation: PBS+Tween 0.1% was added to fecal samples to obtain a concentration of 100mg/ml. Samples were shaken for 2 minutes before being centrifuged at 12,000 rpm 10 minutes. The supernatant was collected and passed through a 0.22 um filter. Samples were then diluted at a 1/10 serial fold dilution in DMEM. 50 ul of samples were added to the Vero cells. The cells were incubated for 24 hours at 37ºC at 5% CO2. Supernatant was then removed and cells were stained with crystal violet. Presence of toxin was determined by visual destruction of vero cell monolayer.

C. difficile spores were cultured in Dr. Shonna McBride's laboratory at Emory University. Spores were kept at room temperature until use, before infection cells were heated at 55ºC for 20 min in order to eliminate vegetative cells.

2.2.2 Probiotic Culture

20 ul of *Lactobacillus rhamnosus GG* stock, ATCC 53103, was added to 10 mL of MRS broth (Difco Lactobacilli MRS Broth 288130) in aerobic conditions overnight at 37ºC. The culture was centrifuged (10 minute 2000 rpm), the supernatant was removed and the pellet was resuspended in 10 mL PBS. The culture was then diluted in PBS to desired concentration (1×10^7 CFU/mL).

20 ul of *Bifidobacterium breve*, Chr-Hansen DSM15954, was added to 10 mL of MRS broth (Difco Lactobacilli MRS Broth 288130) in anaerobic conditions, anaerobic Oxoid Jar, overnight at 37ºC. The culture was vortexed and centrifuged (10 minutes 2000 rpm). The supernatant was removed and the pellet was resuspended in 10 mL PBS. Culture was then diluted in PBS to desired concentration (1×10^7 CFU/mL).

20 ul of *Lactobacillus acidophilus*, Chr-Hansen DSM13241, was added to 10 mL of MRS broth (Difco Lactobacilli MRS Broth 288130) in aerobic conditions overnight at

37ºC. Culture was vortexed and centrifuged (10 minutes 2000 rpm). The supernatant was removed and pellet resuspended in 10 mL PBS. Culture was then diluted in PBS to desired concentration (1 x 10^7 CFU/mL).

250 mg of *Saccharomyces boulardii*, FLORASTOR CAPSULE 7.5 x 10^7 CFU/mL, was dissolved in 5 mL PBS, vortexted and centrifuged (10 minutes at 2000 rpm). The supernatant was discarded and the pellet was resuspended in 5 mL PBS. Yeast then diluted in PBS to desired concentration (1 x 10^7 CFU/mL).

2.2.3 Probiotic Plating

Probiotic colony forming units (CFU) was determined by growing culture on plates. LGG and La5 were grown on Lactobacilli MRS Agar and cultured overnight at 37ºC in aerobic conditions. BB12 was grown on Lactobacilli MRS Agar overnight at 37ºC in anaerobic conditions. *S. boulardii* was grown on Difco Sabouraud Dextrose Agar for 2 days at 30ºC. Serial dilutions of probiotics were done in triplicate. O.D. of initial culture prior to dilution was taken (O.D. 620)

3 RESULTS

3.1 Fecal Microbiota Transplant and *C. difficile*

3.1.1 Clostridium difficile and Fecal Microbiota Transplant

FMT transplants are well documented as an effective method in treating *C. difficile* infection, however, the efficacy in treating certain strains is unknown. For the experiments in this project the *C. difficile* strain VPI 10463 was chosen because infection causes death in susceptible mice beginning two days after infection. This allows for easy determination to see if the treatment is or is not effective in mitigating the acute phase of *C. difficile* infection. It is assumed that the microbiota contained within the FMT is the key mechanism in providing protection against *C. difficile* infection. To test this hypothesis, three groups of C57BL/6 mice were placed on antibiotics and given two doses of either PBS, frozen

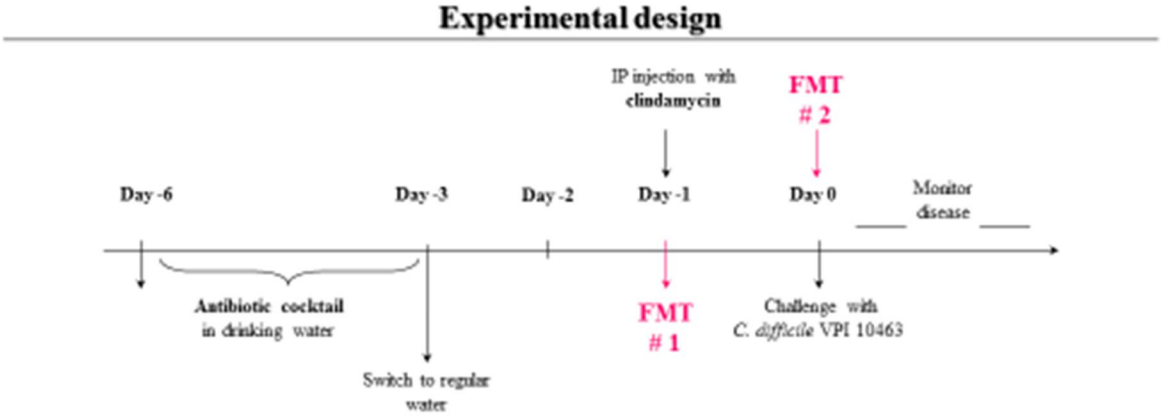

Figure 3.1 Mouse Germ Free Fecal Microbiota Transplant Experimental Design

feces from germ free mice, or frozen feces from healthy C57BL/6 mice (Figure 3.1).

The results of the experiment show that both of the FMT treatments were able to provide some protection against *C. difficile* infection in a variety of aspects such as reduced

weight loss, death, and reducing the amount of *C. difficile* shed in feces. The FMT of both conventional and germ free also resulted in lower levels of bioactive toxin (Figure 3.2).

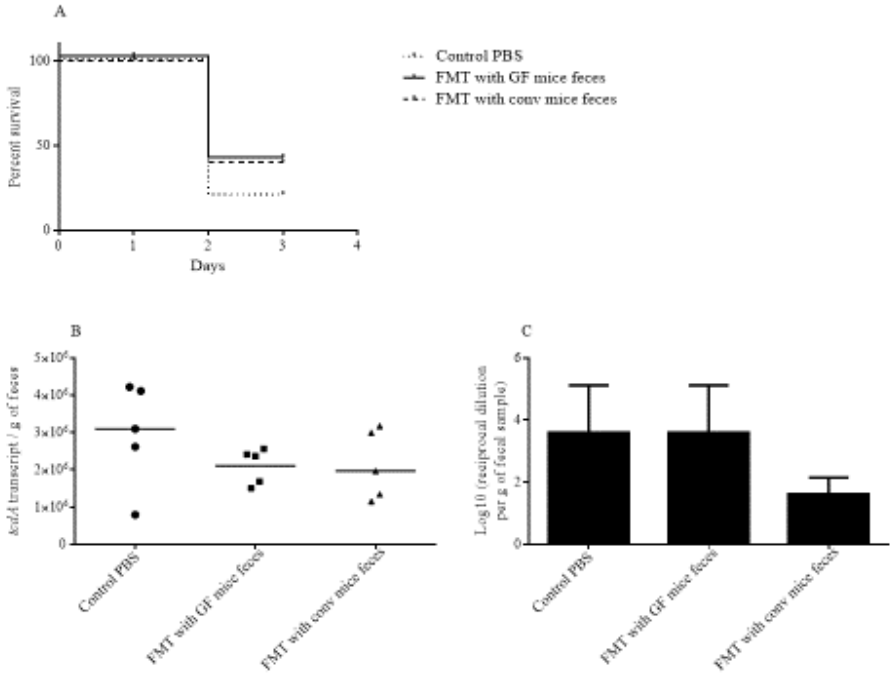

Figure 3.2 Fecal Microbiota Transplant Results: A) Mouse survival, B) *tcdA* transcript Day 1 Post Infection C) Vero cell Toxicity Assay Day 1 Post Infection

However, the most impressive result was clearly the FMT delivered from conventional feces. This result gives credence to the idea that it is the microbes that provide the protection. This experiment also used frozen fecal samples, however, which likely reduced the amount of active microbes that were successfully transferred. The experiment was repeated, but this time comparing between fresh and frozen feces from conventional C57BL/6 mice. This experiment showed a dramatic increase in protection in the fresh fecal group (Figure 3.3). In this experiment the extensive delay in *C. difficile* induced mortality was particularly notable. There were also decreases in the amount of *C. difficile* and lower levels of bioactive toxin of the Fresh group when compared with the PBS and Frozen fecal

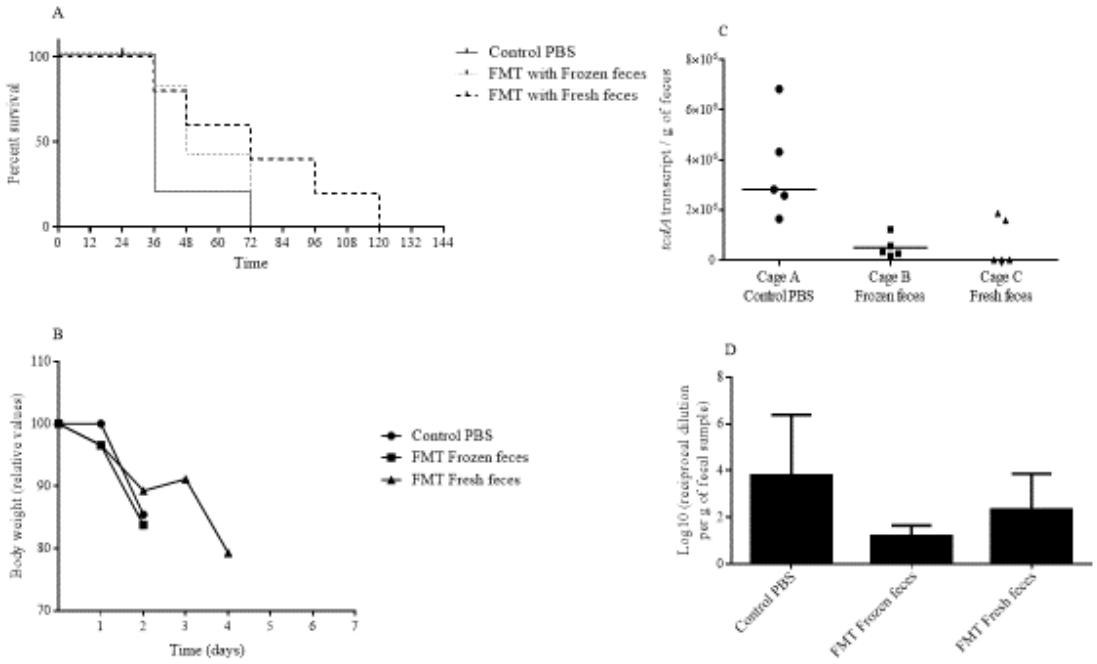

Figure 3.3 Fecal Microbiota Transplant Fresh Samples Results: A) Mice Survival B) Body Weight Change C) *tcdA* Transcript Day 1 Post Infection D) Vero Cell Assay Day 1 Post Infection

group. These results help strengthen the idea that the main factor in FMT induced protection is the microbiota. The focus now turned to see if this protective effect could be mimicked using probiotics.

3.2 *Clostridium difficile* and Probiotics in Germ Free Mouse Model

3.2.1.1 Determination of Probiotic efficacy in germ free model

Four different probiotics were used to test their efficacy in mitigating *C. difficile* infection using a germ free mouse model. Five groups of germ free Swiss Webster mice

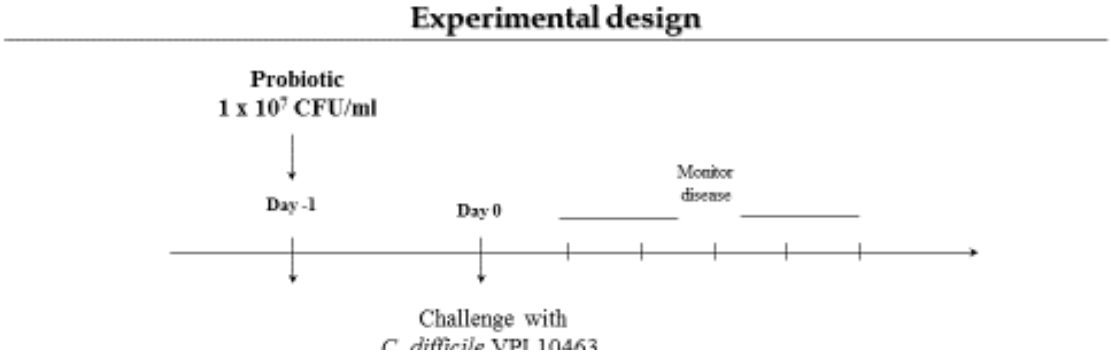

Figure 3.4 Germ Free Swiss Webster Mouse Experiment Probiotic Design

were each given either PBS, *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus acidophilus* (La-5), *Bifidobacterium breve* (BB12), or *Saccharomyces boulardii* one day prior to *C. difficile* challenge as described in the experimental design (Figure 3.4).

The probiotics all showed various levels of protection in different areas depending on the probiotic used. Of the four, LGG displayed the most effectiveness with 100% of the mice surviving *C. difficile* infection versus the PBS control group. The LGG treated group also showed lower levels of *C. difficile* shed in feces when compared to PBS group. The other probiotics showed smaller protective effects versus *C. difficile* infection with *S. boulardii* being the second most prominent probiotic in terms of protective effect.

Lactobacillus acidophilus and *Bifidobacterium breve* showed slight protection by delaying the onset of *C. difficile* induced mortality and severity of mortality respectively (Figure 3.5).

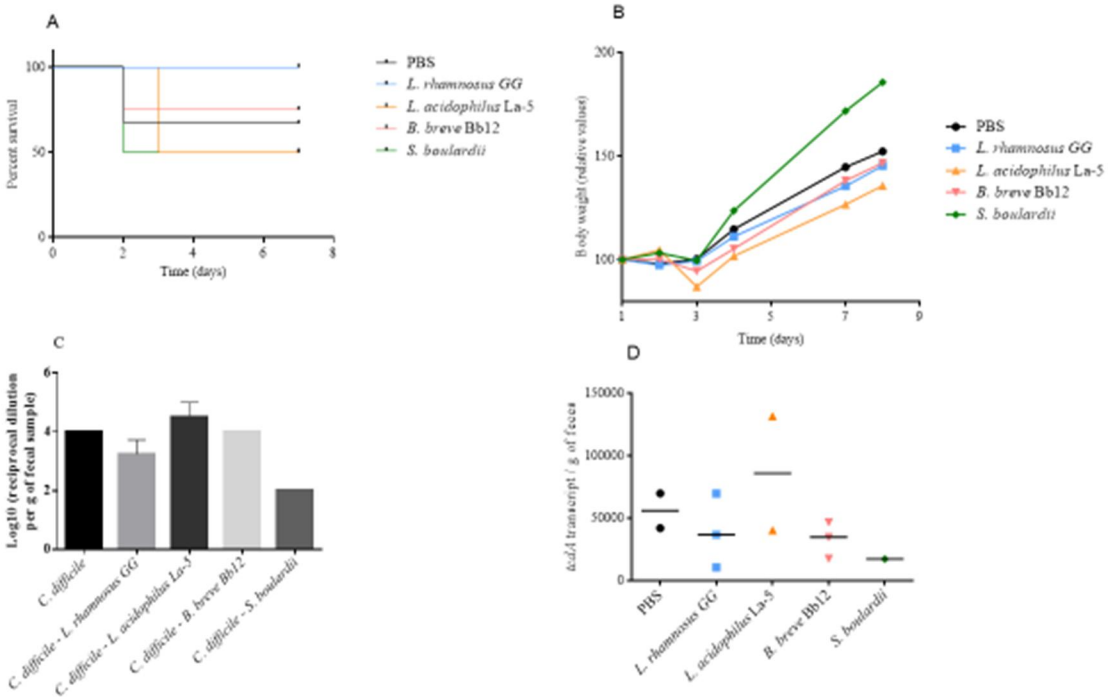

Figure 3.5 Probiotic Results: A) Mice Survival B) Weight Change C) Vero Cell Toxicity Assay Day 2 Post Infection D) *tcdA* Transcript Day 2 Post Infection

Based on these results the focus turned to study the ability of LGG and *S. boulardii* to protect against *C. difficile* associated disease.

3.2.2 Lactobacillus rhamnosus GG and Clostridium difficile in a Germ Free Mouse Model

Further experiments were undertaken to determine the efficacy of LGG in protecting against *C. difficile* associated disease. The above experiment was repeated, this time utilizing C57BL/6 germ free mice in lieu of Swiss Webster. Based on prior observations Swiss Webster mice seem to be more resistant to certain pathogenic infections when compared to mice of the C57BL/6 background. The experiment was the same save for the mouse strain and also that the mice received 1 log lower dose of *C. difficile*. The LGG treated mice again showed 100% survival against *C. difficile* induced death as well as a

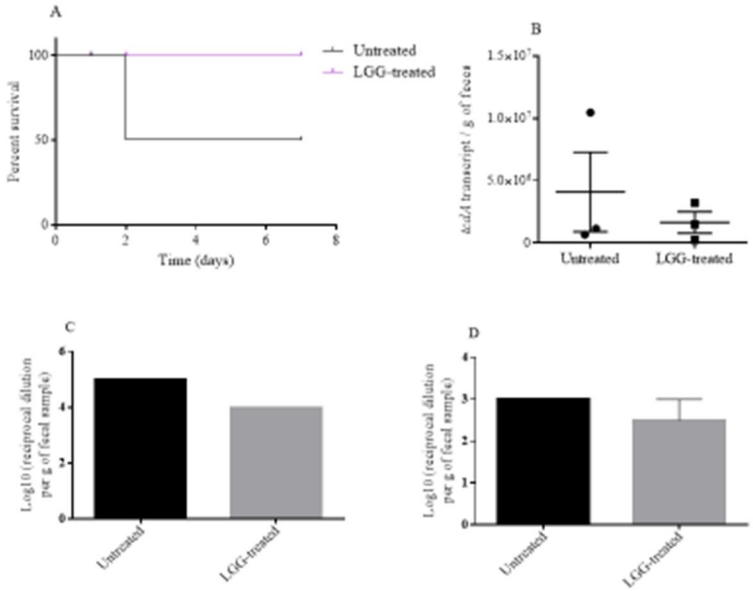

Figure 3.6 *Lactobacillus rhamnosus* GG and *C. difficile* GF C57BL/6 Mice A) Mice Survival B) *tcdA* transcript Day 1 Post Infection C) Vero Cell Toxicity Assay Day 1 Post Infection D) Vero Cell Toxicity Assay Day 2 Post Infection

lower amount of *C. difficile* and bioactive toxin that was shed in the feces. These results indicate that LGG is able to provide protection of at least two different strains of mice against *C. difficile* infection in germ free conditions (Figure 3.6).

3.2.3 S. boulardii and Clostridium difficile in a Germ Free Mouse Model

The probiotic *S. boulardii* was also utilized to determine its protective effects of *C. difficile* in a germ free mouse model. *S. boulardii* was given twice, once 24 hours prior to *C. difficile* infection and once 8 hours prior to *C. difficile* infection. In these experiments the yeast demonstrated its ability to protect against *C. difficile* Associated Disease. It was able to prevent death and showed strong protection against body weight loss. Also, the amounts of bioactive toxin in the feces were significantly lower in the yeast treated group compared to the control group on day 1 post infection (Figure 3.7).

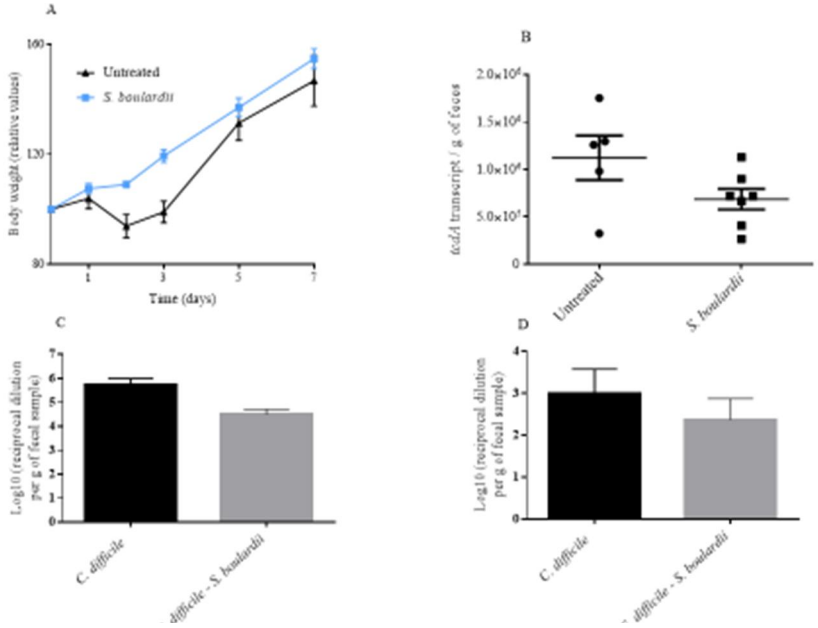

Figure 3.7 *S. boulardii* and *C. difficile* Germ Free Mice: A) Weight Change B) *tcdA* transcript Day 1 Post infection C) Vero Cell Assay Day 1 Post infection D) Vero Cell Assay Day 2 Post infection

3.3 *Clostridium difficile* and Probiotics in a Conventional Mouse Model

3.3.1 Clostridium difficile and LGG in a Conventional Mouse Model

Probiotics showed great efficacy in protecting against *C. difficile* when given just one dose for LGG or two doses for *S. boulardii* 24 hours prior to *C. difficile* infection in the germ free model of mice. One of the key the limitations within the germ free model, however, includes the fact that the probiotics do not have to interact with the normal microbiota which could either assist or hinder the probiotics ability to protect against infection. To determine the effect that the microbiota has on the ability of probiotics to mitigate *C. difficile* infection, C57BL/6 mice were placed on an antibiotic cocktail for 3 days before being switched to regular water. One day prior to challenge the mice received

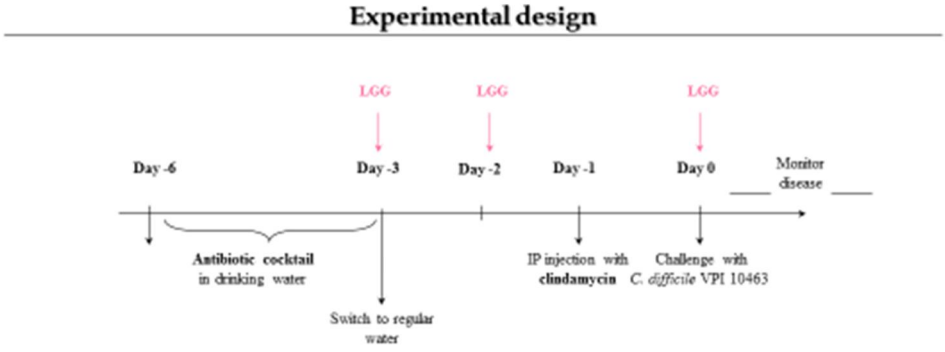

Figure 3.8 *Lactobacillus rhamnosus GG* + *C. difficile* Conventional Mice Experiment

an intra-peritoneal injection of clindamycin. The mice were given 3 doses of probiotics prior to *C. difficile* challenged (Figure 3.8).

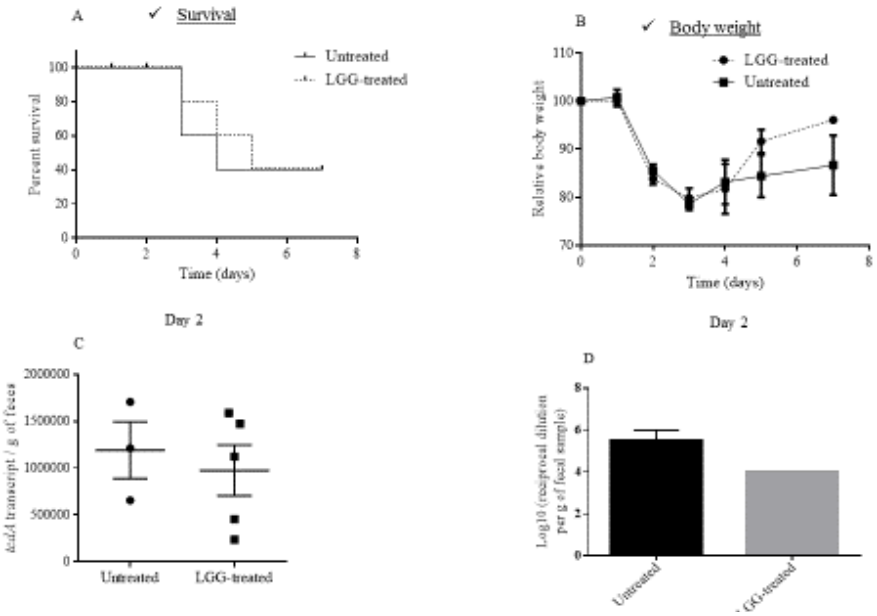

Figure 3.9 *Lactobacillus rhamnosus GG* and *C. difficile* Conventional Mice A) Mice Survival B) Weight Change C) *tcdA* Transcript Day 2 PI D) Vero Cell Toxicity Assay Day 2 PI

The protective effect of LGG was lower in the conventional experiments when compared to the protective effects observed with germ free experiments, however, LGG was still able to delay the onset of *C. difficile* induced death. The probiotic was also able to provide a significant reduction in the amount of bioactive toxin as well as increasing the weight gain after acute phase of infection had passed (Figure 3.9).

3.3.2 C. difficile and Probiotic Combination in a Conventional Mouse Model

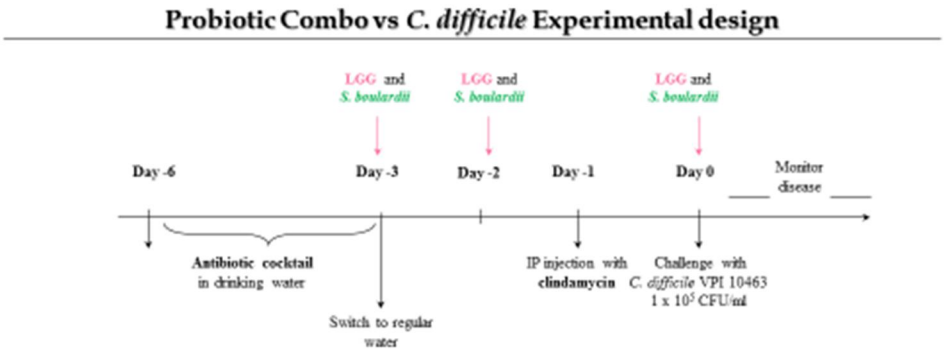

Figure 3.10 Probiotic Combination Experimental Design

Based on these results, it was thought that using a combination of probiotics would be even more effective in protecting against *C. difficile*. The two probiotics which showed the highest effectiveness against *C. difficile*, *S. boulardii* and LGG, were selected. Both probiotics were administered at the same time (Figure 3.10). The probiotic combination showed slight protection in *C. difficile* induced death. The majority of the probiotic combination protective effect in resisting C. difficile induced death seemed to be concentrated within the first 48 to 72 hours post infection. The combination did provide a significant reduction in the amount of C. difficile shed in feces as well as lowering the amount of bioactive toxin found in the feces when compared to the *C. difficile* only group (Figure 3.11).

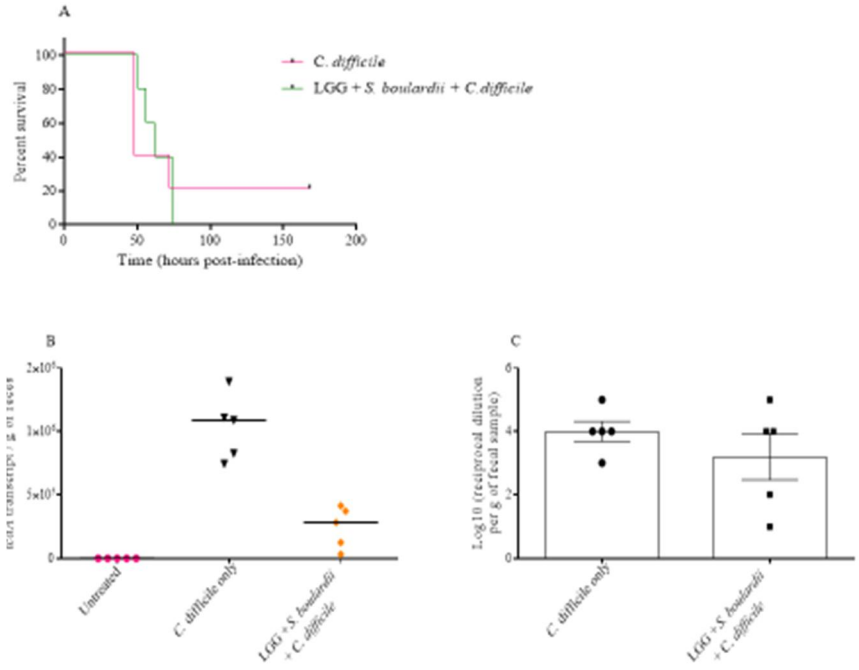

Figure 3.11 Probiotics and *C. difficile*: A) Mice Survival B) *tcdA* transcript C) Vero Cell Toxicity Assay

4 CONCLUSIONS

4.1 Discussion

The gut microbiota and its association with the host results in a very complex system with numerous different species of microorganisms interacting with each other as well as with the host. This delicate balance however, as anyone who has been infected with a food borne pathogen such as *E. coli* can asset to, can be altered by the addition of a single species resulting in discord. If the introduction of one or two species of microorganisms can tilt this balance to disorder, then the addition of one or two species of other microorganisms might be able to shift this balance back to its orderly state.

This study sought to discover if the use probiotics could be utilized to prevent the effects of *C. difficile* infection. Four different probiotics were used and each demonstrated various effects in protecting against *C. difficile* infection. Both LGG and Bb12 displayed protection against

C. difficile induced mortality, while La5 demonstrated the ability to delay the onset of death. *S. boulardii* treatment showed remarkable protection versus weight loss as well as lowering the levels of *C. difficile* 1 day post infection while LGG and BB12 showed lower levels on day 2 post infection. These differences in the effects demonstrate a key fact that all probiotics are not equal on their effect against *C. difficile* infection. Some, such as LGG, and *S. boulardii*, stand out from the pack but their effect is not uniform. The difference in their effects leads credence to the idea that these probiotics are each working in different ways.

One must now ask how are the probiotics providing protection? One of the key correlations between mice that survive and mice that do not are the lower levels of bioactive toxin. This reduction in toxin is also associated with lower levels of *C. difficile* in the feces. Based on this information it is likely that the introduction of probiotics leads to the reduction in the amount of *C. difficile* in the gut. This, in turn, leads to the reduction in the amount of bioactive toxin. This reduced toxin amount allows the mice remain more active, letting them consume more food and notably water, which prevents them from dying of dehydration.

How are the probiotics accomplishing the initial reduction in *C. difficile*? Based on the performance of the germ free mouse experiments compared to the conventional ones, the bacterial probiotics such as LGG could be acting by outcompeting and limiting the ecological niches that is normally available for *C. difficile* to colonize. It is also possible that LGG and BB12 could be releasing antimicrobial factors that can directly target and limit *C. difficile* growth directly (Lee et al., 2013).

Interestingly, *S. boulardii* also lowered the amount of *C. difficile* present in the feces. Of the four probiotics tested, *S. boulardii* showed the lowest amount of colonization potential, indicating that the yeast presence in the gut is very transit. This reduces the likelihood that

S. boulardii mechanism of action against *C. difficile* is operating by outcompeting or taking up colonization space. Studies have shown that *S. boulardii* has a protease that can target *C. difficile* toxins (Castagliuolo et al., 1996). This antitoxin activity could explain the reduction in bioactive toxin found in *S. boulardii* experiments, however, this activity would not account for the reduction in the amount of *C. difficile* shed in the feces. *S. boulardii* modulation of the host could be a possible explanation for this difference. The yeast could be priming the host immune system, making it response better to the *C. difficile* presence. The yeast could also be altering local environmental conditions in the gut, such as altering bile acids.

Some studies have shown that *C. difficile* show inhibited growth in the presence of secondary bile acids (Sorg et al., 2008). Probiotics could potentially modulate these host factors to reduce the growth of *C. difficile*, which would result in lower amounts of toxin. An experiment to determine the effect of probiotics on secondary bile acid production could be performed to test this theory. Searching for other probiotics which also have the capability of increasing secondary bile acids and testing to see if they can demonstrate protective effects against CDAD could also provide useful information (Buffie et al., 2014).

The location of *C. difficile* infection also seems to be important (Koenigsknecht et al., 2015). The *C. difficile* spores start to germinate in the small intestine, and there is even *C. difficile* present in the small intestine during infection, but this does not seem to correlate with disease. Rather, *C. difficile* presence in the large intestine, particularly the cecum, is an important site for both sporulation and toxin production. Selecting probiotics which can colonize these areas with high efficiency could be one method in which to block *C. difficile* from establishing itself.

The fact that the probiotics showed a smaller protective effect within the conventional model of *C. difficile* infection demonstrated some of the limitations in the experimental approach.

The mice receiving probiotics in germ free mouse experiments showed significant protection versus the conventional experiments. The large difference of effectiveness in the conventional mouse model compared with the germ free mouse model most likely lies in two major factors; the presence of the antibiotics as well as the presence of the microbiota. LGG was sensitive to the presence of gentamycin and clindamycin while displaying resistance to the other antibiotics used in the cocktail (Table 1).

Table 1 *Lactobacillus rhamnosus GG* Antibiotic Sensitivity

Kanamycin	Resistant
Gentamicin	Sensitive
Metronidazole	Resistant
Vancoymicin	Resistant
Colistin	Resistant
Clindamycin	Sensitive

This sensitivity, particularly towards clindamycin, is important to note. In this model it is not known for how long that the antibiotics linger within the mouse after inoculation. Lingering amounts of antibiotics from the cocktail as well as the clindamycin injection could potentially have adverse effects on the ability of LGG to maintain a presence within the gut. Also, the 12 hour time window between clindamycin administration and the final dose of LGG may not allow for clearance of *C. difficile* from the mouse intestine. The amount of antibiotics lingering within the mouse could reduce the probiotics ability to remain active within the mice, leading to a reduced action of the probiotic.

Some ways in which to alter the design and get a better perspective on the probiotic effect within the conventional model would be to delay the challenge of *C. difficile* by one day as well

as adding another dose of probiotic. This window should reduce the amount of active antibiotics that could be interfering with the probiotic effect. Neutrophil infiltration is also associated with CDAD, and measuring serum KC levels could be used to see if probiotic administration is altering the inflammatory response to *C. difficile*, either lowering it to reduce the amount of inflammatory damage or increasing it to provide better clearance of the pathogen. The bleeding required would likely provide undue stress for the mouse, so another approach would be preferable. Another marker that can be used to determine probiotic effectiveness would be to measure Lcn-2 within the feces (Chassaing B et al., 2012). This approach would look for general inflammatory response rather than neutrophil specific, however, it does provide the advantage of not adding undue stress for the mice, allowing one to see if the long term effects of treatment without the risk of mice death due to blood collection.

Based on these experiments probiotics do have the ability to provide some modest protection and mitigate some symptoms of *C. difficile* infection. The question that remains then is how effective the probiotic treatment is when compared to the current gold standard of FMTs? The probiotics used demonstrated less protection when compared to the FMT treatment. The cause of this difference could lie in many areas, but the likeliest resides in the difference in microbial content. This result is not too surprising, the FMT contain a complex mixture of a wide variety of microorganisms compared to the probiotics used in this study which included only of a single strain of probiotic used in each treatment, or two in the case of the probiotic combination approach.

Is there a role for probiotics in the future of gastroenterology? Yes, the effects of probiotics are well documented. Could there be a role for probiotics in the treatment of *C. difficile* in lieu of FMT? Perhaps, to discover this effect the experiments could be repeated with a less virulent strain of *C. difficile*, or restructured to allow more time in-between antibiotic and probiotic

administration. Selecting different probiotics, such as those with higher colonization efficiency, resistance to certain antibiotics, or those that alter bile acids could result in better protection against CDAD, but, with the results demonstrated here FMT still remains the most effective method of treating *C. difficile* associated disease.

REFERENCES

Aslam S, Hamill RJ, Musher DM. Treatment of *Clostridium difficile*-associated disease: old therapies and new strategies. The Lancet Infections Diseases. 2005 Sept; 5(9): 549-557

Alang N, Kelly RC. Weight Gain After Fecal Microbiota Transplantation. Open Forum Infectious Diseases. 2015 February

Scallan E, Griffin PM, Angulo FJ, Tauxe RV, Hoekstra RM. Foodborne Illness Acquired in the United States-Unspecified Agents. Emerging Infectious Diseases. 2011Jan; 17(1)

Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, No D, Liu H, Kinnebrew M, Viale A, Littmann E, van den Brink MR, Jenq RR, Taur Y, Sander C, Cross J, Toussaint NC, Xavier JB, Pamer EG. Precision microbiome reconstitution restores bile acid mediated resistance to *Clostridium difficile*. Nature. 2014 October; 517, 205-208

Castagliuolo I, LaMont JT, Nikulasson ST, Pothoulakis C. *Saccharomyces boulardii* protease inhibits *Clostridium difficile* toxin A effects in the rat ileum. Infection and Immunity. 1996 Dec; 64(12): 5225-5232

Chassaing B, Srinivassan G, Delgado MA, Young AN, Gewirtz AT, Kumar MJ. Fecal lipocalin-2, a sensitive and broadly dynamic non-invasive biomarker for intestinal inflammation. PLoS One. 2012 Sept; 7(9);e44328.

Collins LE, Lynch M, Marszalowska I, Kristek M, Rochfort K, O'Connell M, Windle H, Kelleher D, Loscher CE. Surface layer proteins isolated from *Clostridium difficile* induce

clearance responses in macrophages. Elsevier Microbes and Infection. 2014 May; 16(5): 391-400.

Gareau MG, Sherman PM, Walker WA. Probiotics and the gut microbiota in intestinal health and disease. Nature Review Gastroenterology and Hepatology. 2010 July; 7, 503-514

Gorbach SL. Antibiotics and *Clostridium difficile*. New England Journal of Medicine. 1999 Nov; 341: 1690-1691

Gray J. Rotavirus vaccines: safety, efficacy and public impact. Journal of Internal Medicine. 2011 Sep; 270(3): 206-214

Guarner F, Schaafsma GJ. Probiotics. International Journal of Food Microbiology. 1998 Feb; 39(3): 237-238

Hall AJ, Curns AT, McDonald LC, Parashar UD, Lopman BA. The Roles of *Clostridium difficile* and Norovirus Among Gastroenteritis-Associated Deaths in the United States, 1999-2007. Clin Infect Dis. 2012 Jul;55(2):216-23

Kochanek KD, Xu J, Murphy SL, Minio AM, Kung HC. Deaths: Preliminary Data for 2009. National Vital Statistics Report.

Koenigsknecht MJ, Theriot CM, Bergin IL, Schumacher CA, Schloss PD, Young VB. Dynamics and Establishment of *Clostridium difficile* Infection in the Murine Gastrointestinal Tract. Infection and Immunity. March 2015; 83(3) 934-941

Lee JS, Chung MJ, Seo JG. In Vitro Evaluation of Antimicrobial Activity of Lactic Acid Bacteria against *Clostridium difficile*. Toxicological Research. 2013 Jun; 29(2): 99-106

Lessa FC, Mu, Y, Choen J, Dumyati G, Farley MM, Winston L, Kast K, Holzbauer S, Meek J, Beldays S, McDonald LC, Fridkin SK. Presented at the IDWeek 2012, Annual Meeting of the infectious disease Society of America, Society for Healthcare Epidemiology,

Pediatric Infectious Disease Society, and HIV Medical Association; San Diego, Ocboter 2012

Parashar UD, Hummelman EG, Bresee JS, Miller MA, Glass RI. Global Illness and Death Caused by Rotavirus Disease in Children. Emerging Infectious Diseases. 2003 May; 9(5): 565-572

Rohlke F, Stollman N. Fecal microbiota transplantation in relapsing *Clostridium difficile* infection. Therapeutic Advances in Gastroenterology. 2012 March; 5(6):403-420

Rosenfeldt V, Michaelsen KF, Jakobsen M, Larsen CN, Moller PL, Tvede M, Weyrehter H, Valerius NH, Paerregaard A. Effect of probiotic Lactobacillus strains on acute diarrhea in a cohort of nonhospitalized children attending day-care centers. Pediatric Infectious Disease Journal. 2002 May; 21(5): 417-419

Sanders ME, Guarner F, Guerrant R, Holt PR, Quigley EM, Sartor RB, Sherman PM, Mayer EA. An Update on the Use and Investigation of Probiotics in Health and Disease. *Gut.* May 2013; 62.5, 787–796.

Sorg, Joseph A., and Abraham L. Sonenshein. Bile Salts and Glycine as Cogerminants for *Clostridium difficile* Spores. Journal of Bacteriology. 2008 April; 190.7 2505–2512

Sunenshine RH, McDonald LC. *Clostridium difficile*-associated disease: New challenges from an established pathogen. Cleveland Journal of Medicine. 2006 February; 73(2), 187-197

Tung, Jennifer M, Lisa R Dolovich, and Christine H Lee. Prevention of*Clostridium Difficile* Infection with *Saccharomyces Boulardii*: A Systematic Review. *Canadian Journal of Gastroenterology.* 2009 December; 23.12: 817–821.

Vlasova AN, Chattha KS, Kandasamy S, Liu Z, Esseili M, Shao L, Rajashekara G, Saif LJ. *Lactobacilli* and *Bifidobacteria* Promote Immune Homeostasis by Modulating Innate

Immune Reponses to Human Rotavirus in Neonatal Gnotobiotic Pigs. PLoS One. 2012 Oct; 8(10)