Effects of Prebiotics on Gut Bacterial Communities and Healing of Induced Colitis in Mice

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EFFECTS OF PREBIOTICS ON GUT BACTERIAL COMMUNITIES AND
HEALING OF INDUCED COLITIS IN MICE

by

Krystyn Elizabeth Davis

A Thesis
Submitted to the Graduate School
and the Department of Biological Sciences
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Science

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ABSTRACT
EFFECTS OF PREBIOTICS ON GUT BACTERIAL COMMUNITIES AND HEALING OF INDUCED COLITIS IN MICE
by Krystyn Elizabeth Davis
August 2016
Inflammatory Bowel Diseases (IBD) cause chronic inflammation of the gastrointestinal tract and debilitating symptoms in those suffering from the diseases. After inducing colitis in a mouse model using Dextran Sulfate Sodium (DSS), prebiotics inulin and oligofructose enriched inulin (OEI) were used as treatments to determine their effects on the gut microbial community, physiological healing process, and immune response in the mice after initial inflammation and before subsequent inflammation, or relapse. The treatment with inulin led to an increase in regulatory T cell number, but this increase was not as significant as the increase induced by the OEI. Inulin increased the inflammation in the mouse colon, whereas inflammation was decreased in the colons of the mice treated with OEI. A three percent increase in butyrate producing bacteria, Clostridium cluster XIVa spp., was observed in mice treated with OEI before the relapse period when compared to untreated mice with colitis. The proposed mechanism for how the OEI led to decreased inflammation in the colons of the treated mice was that the introduction of the prebiotic allowed for an increase in butyrate producing Clostridium cluster XIVa spp., which led to a direct increase in butyrate production in the colon. In turn, this butyrate production led to an
increase in differentiation of regulatory T cells and an overall reduction of the immune response and inflammation in the mice treated with OEI. This reduction of immune response and inflammation allowed the mice that were treated with OEI to be more resistant to induced relapse.
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CHAPTER I - INTRODUCTION

Inflammatory Bowel Diseases (IBD) cause chronic inflammation of the gastrointestinal tract. The two major forms of IBD are Crohn’s disease and ulcerative colitis. Crohn’s disease may affect any part of the gastrointestinal tract including every layer of the intestine. Ulcerative colitis, however, only takes place in the colon. The diseases may consist of remission periods when one experiences no symptoms, followed by “flare-ups” where debilitating symptoms such as weight loss, severe abdominal pain, rectal bleeding, vomiting, and diarrhea may be experienced. These random “flare-ups” are what make the diseases so frustrating and hard to study and treat. Although these diseases are rarely fatal, they can severely affect a person’s quality of life in a negative way. As of the year 2014, IBD was shown to account for more than 700,000 physician visits, 100,000 hospitalizations, and disability in 199,000 patients yearly in the United States alone, and had an overall health care cost of over $1.7 billion (Centers for Disease Control and Prevention [CDC], 2014).

During IBD, the immune system mistakes normal intestinal components for foreign or pathogenic substances. This causes an immunological response that leads to chronic inflammation. The cause of this occurrence is largely unknown. However, several factors have been found to contribute to the development of the disease. One major factor that has gained increasing interest is the host’s gut microbial community. A number of studies suggest that gut microbiota must play a role in the cause and development of IBD. According to Swidsinski et al. (2002), colon inflammation tends to occur in segments with...
the highest concentrations of bacteria associated with the mucosa. Bibiloni, Mangold, Madsen, Fedorak, and Tannock (2006) found that patients with IBD contained atypical intestinal microbial composition. According to D’Haens et al. (1998), diversion of the fecal stream treats some forms of IBD, and intestinal inflammation returns upon the reintroduction of the fecal stream. Also, Sartor (2006) mentions that luminal commensal bacteria are required for chronic inflammation in most rodent models of IBD. In summary, multiple studies have provided evidence that gut microbiota may play a major role in the onset of the disease. However, it is still largely unknown as to why or how this occurs.

There are several theories on why gut microbes are involved in the pathogenesis of IBD. One theory is that microbial pathogens cause intestinal inflammation. These pathogens may include traditional pathogens such as *Mycobacterium avium* subspecies *paratuberculosis*, or they may include commensal bacteria such as *Escherichia coli* or *Staphylococcus aureus* that have been altered functionally (Sartor, 2008). The second is the idea that host immunoregulation does not function properly during the disease (Sartor, 2008). In other words, the host has lost the ability to distinguish between beneficial and pathogenic bacteria and has begun attacking all gut bacteria, which causes an overaggressive immune response and subsequent inflammation. A third theory is that the dysbiosis of gut microbiota causes the disease (Sartor, 2008).

Dysbiosis, or microbial imbalance, is being observed and studied in the intestines of patients with IBD. A decreased ratio of protective commensal species compared to aggressive species was observed in patients with IBD
(Frank et al., 2007). An increase in microbial number and a decrease in microbial diversity have also been seen in patients with IBD (Sartor & Mazmanian, 2012). However, it is not known whether the disease causes the dysbiosis or the dysbiosis causes the disease. Although it is not known whether commensal microbiota dysbiosis is a cause or effect of IBD, it can be inferred that microbial dysbiosis can cause negative effects in patients by shifting the balance of the patient’s flora in favor of harmful or proinflammatory microbial species. Therefore, finding ways to correct this dysbiosis could be beneficial in the treatment, healing process, and/or prevention of relapse of the disease.

In addition to microbial dysbiosis, short chain fatty acid (SCFA) levels are lower than normal within the colon of a person with IBD (Huda-Faujan et al., 2010; Kumari, Ahuja, & Paul, 2013). SCFAs are beneficial to the host and are formed when dietary fiber is fermented in the colon. One particular SCFA, butyrate, has been shown to be very beneficial to the health of the colon. It is an important energy source to colonocytes and has been shown to inhibit carcinogenesis and inflammation (McIntyre, Gibson, & Young, 1993; Andoh, Bamba, & Sasaki, 1999). Butyrate has also been shown to enhance the colonic defense barrier by stimulating increased mucin production (Finnie, Dwarakanath, Taylor, & Rhodes, 1995). It also induces the differentiation of colonic regulatory T cells, which could in turn contribute to the suppression of an inflammatory response in the host (Furusawa et al., 2013). Therefore, increasing levels of butyrate would seem to benefit a person with IBD, and this could be achieved directly or indirectly by introducing butyrate producing bacteria or altering the
colonic environment in favor of the growth of butyrate producing bacteria by giving the patient prebiotics.

Currently, there is no cure for IBD, and the only treatments for the disease are anti-inflammatory and immunosuppressant drugs. These drugs can be detrimental to human health and can lead to harmful side effects such as severe toxicity, decrease in immune function, and increased risk of contracting opportunistic infections. Therefore, other forms of treatment are urgently needed. The uses of antibiotics, probiotics, and prebiotics have been studied as possible treatments for IBD. Probiotics and prebiotics are more natural forms of treatment that lack negative side effects, hence their appeal.

Broad spectrum antibiotics have been shown, in some cases, to benefit patients with Crohn’s disease (Lal & Steinhart, 2006). However, they are not known to be able to keep the disease under control and do not appear to be effective against ulcerative colitis (Sartor, 2004). In addition, it is well known that prolonged use of antibiotics can contribute to the development of resistant bacteria, and the antibiotics can kill some of the beneficial microbiota in the intestines. Probiotics, on the other hand have been shown to prevent recurrence of intestinal inflammation and treat IBD with no dangerous side effects in a limited number of studies (Dieleman et al., 2003; Gionchetti et al., 2000). A study by Venturi et al. (1999) on patients with ulcerative colitis showed that probiotics allowed 75% of the treated patients to remain in remission. Kruis et al. (2004) also reported maintenance of remission in patients with IBD by using the probiotic *Escherichia coli* Nissle 1917. Although probiotics can maintain
remission of IBD, a mechanism of action of the probiotics has not been found. Suggested mechanisms include preventing invasion by harmful bacteria, improving epithelial and/or mucosal barrier, and altering the control of the immune response (Sartor, 2004).

Another form of possible therapy for IBD, which has only recently begun to be studied, is prebiotics. Prebiotics are carbohydrates incorporated into the diet but are indigestible by the host. They cannot be absorbed until they reach the colon, where they are fermented by certain commensal bacteria to produce short chain fatty acids (SCFAs) and lactate (Scaldaferri et al., 2013). Short chain fatty acid concentrations are reduced in the fecal contents of patients with IBD (Packey & Sartor, 2008). Therefore, the supplementation of prebiotics into the diet of a patient with IBD can be beneficial in helping to restore the SCFA concentration to that of a normal person. Prebiotics also stimulate the growth of beneficial and protective commensal bacteria that can help lower the number of harmful bacteria by decreasing the luminal pH, preventing epithelial attachment, and secreting bactericidal substances (Sartor, 2004). Although it can be seen that prebiotics have tremendous potential as a treatment for the disease, they “have not been adequately tested yet in IBD” (Sartor, 2004).

The idea of synbiotics, which combines the use of probiotics and prebiotics, has become very appealing recently. This approach could speed up recovery or decrease the duration or frequency of probiotic administration due to synergy between probiotic and prebiotic treatments. This would lead to a decrease in costs and an increase in patient readiness to accept it as a form of
treatment. Neither probiotics nor prebiotics have been approved as a reputable form of treatment for IBD due to a lack of consistent evidence supporting the idea that they work. Although several studies have been done to show the positive effect of probiotics and prebiotics in the treatment of IBD, there is still much more to be done before they can be considered as serious forms of treatment. Specifically, the role of the prebiotic inulin has not been studied in preventing relapse and maintaining remission in IBD (Kelly, 2009).

The objective of this research is to determine whether prebiotics improve healing and enhance resistance to recurrence of IBD in mice, and if so, to describe possible mechanisms for this. The hypothesis I propose to test is that prebiotics promote the proliferation of butyrate producing gut bacteria in the colon and that the increased production of butyrate stimulates an anti-inflammatory immune response by increasing the differentiation of regulatory T cells. If this beneficial environment remains within the colon, the immune system of the mouse should prevent the reoccurrence of or, at least, mitigate the disease.

In order to test my hypothesis I planned to use a chemically induced mouse model of colitis. After inducing inflammation, the mice would be treated with prebiotics then allowed an extended period of recovery or healing before reintroducing them to the chemical (DSS) that induces colitis. The physiological and immunological response would be examined in the mice by looking at body weight change, colon length, histology of the colon, and regulatory T cell counts in the spleen. The fecal bacterial community would also be sequenced and analyzed for each mouse, and the community would be searched for changes in
abundance of specific known butyrate producing bacteria. In support of my hypothesis, it would be expected that with an increase in butyrate producing bacteria, an increase in regulatory T cells should be observed. This increase in regulatory T cells should then correlate with an increase in body weight and colon length when compared to DSS control groups, as well as a decrease in damage to the colon.
CHAPTER II - METHODS

Mice and Experimental Treatment

All animal procedures were approved by the University of Southern Mississippi IACUC Committee (Protocol Numbers 13121204 and 15120901). Female mice (BALB/c) (six-eight weeks old) from Jackson Laboratory were housed under standard conditions in shoebox cages with filter tops, with a twelve hour photoperiod, and received a standard mouse diet (Teklad Global Soy Protein-Free Extruded Rodent Diet (Sterilizable)) and unrestricted access to water. Fourteen groups of mice were made (n=3 for each group), and mice were weighed before the implementation of DSS (Dextran Sulfate Sodium) treatment. On the first day of experimentation, ten of the fourteen groups of mice were given 3% DSS (MW~ 36,000-50,000) (MP Biomedicals, Cat. No. 160110) \textit{ad libitum}, in autoclaved H\textsubscript{2}O to chemically induce colitis in the mice. The 3% DSS solution was sterilized by filtration (0.22 \textmu m) before use. This supplementation into the drinking water continued for four days total. Mice were weighed and observed once daily for water/food consumption, stool consistency, and blood in the feces after the start of DSS treatment. The remaining four groups of mice (control groups) were given autoclaved water only during this time. After the four day supplementation of DSS, mice were given autoclaved drinking water for three days, then two groups of mice were euthanized by standard CO\textsubscript{2} inhalation euthanasia, including one control group and one DSS treated group.

On Day 7 of the study, three groups of DSS treated mice received a daily supplement of the prebiotic inulin (NOW Certified Organic Inulin, Code 2944B)
(3% w/v) suspended in their autoclaved drinking water ad libitum. Another three groups received the prebiotic Oligofructose Enriched Inulin (OEI) (Prebiotin™) (3% w/v) suspended in their autoclaved drinking water ad libitum. Both solutions were filter sterilized through a 0.22 um filter before use. The remaining six groups were given autoclaved drinking water during this time. This supplementation lasted for two weeks. After the two week period was over, four groups of mice, including one control group, one DSS group, one inulin treated group, and one OEI treated group were euthanized. The remaining eight groups of mice were given autoclaved drinking water for four more weeks. When the four week period was over, four groups of mice, including one control group, one DSS group, one inulin treated group, and one OEI treated group were euthanized. The remaining four groups of mice were given a low concentration (1.5%) of DSS in autoclaved drinking water ad libitum for four days to attempt to reactivate the colitis. They were given autoclaved drinking water for three more days then euthanized.

This entire experiment was then repeated once more exactly, except that inulin supplementation was no longer used.

Assessment of Disease State

Mouse body weight was measured every day during DSS treatment, and weight loss was used to help determine disease severity. Fecal occult blood tests were carried out by using a ColoScreen-ES test kit (Helena Laboratories, Cat. No. 5086). Presence of blood in the feces indicated that the DSS had caused intestinal inflammation, leading to bleeding. After euthanasia, the colon of each mouse was harvested, and their lengths were measured. Differences in length of
the colons indicated damage done by the DSS. The shorter the colon was, the more damage was assumed (Kim, Shajib, Manocha, & Khan, 2012).

Flow Cytometry

Whole spleens were collected from mice on the day of euthanasia and placed in five ml of RPMI-1640 + 10% fetal bovine serum (FBS) on ice. Each spleen was crushed between two microscope slides and the released cells suspended in the same medium. Cells were spun down at 500 X g for five minutes, and supernatant was removed. Red blood cells were lysed by re-suspending the cells in one ml of 1X Red blood cell lysis buffer (Sigma, Cat. No. R7757) for five minutes. After stopping cell lysis by adding nine ml of 1X phosphate-buffered saline (PBS) + 2% FBS, the cells were pelleted again by centrifugation. The supernatant was again removed and the cells, resuspended in 1X PBS + 2% FBS, were filtered through a 70 μm cell strainer to remove cellular or tissue debris. The cells were pelleted one more time and then resuspended in five ml of 1X PBS + 2% FBS for counting using a hemocytometer and light microscope.

Once counted, cells were diluted with 1X PBS + 2% FBS to reach approximately one million total cells per one ml. An Fc block was performed by adding 0.5 μl of Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc block) (BD Biosciences, Cat. No. 553141) for 10 minutes at 4°C, to prevent the staining of cells other than T lymphocytes, such as B cells, monocyte/macrophages, natural killer cells, and neutrophils. Cells were washed with 500 μl of PBS + 2% FBS and spun down at 500 X g for five minutes. After discarding the supernatant, cells
were resuspended in 20 μl of 1X PBS + 2% FBS, 2.5 ul of FITC (Anti-Mouse CD3e) (eBioscience, Ref. No. 11-0031-85) and 0.75 μl of APC-eFluor 780 (Anti-Mouse CD4) (eBioscience, Ref No. 47-0041-82). Cells were allowed to incubate for one hour at 4°C in the dark. After incubation, cells were washed two times with 1X PBS + 2% FBS, and supernatant was discarded. The pellet was then resuspended in 500 μl of 1X Fix/Permeabilization working solution from Foxp3 staining buffer set (eBioscience, Ref. No. 00-8333-56). This was incubated for 40 minutes at 4°C in the dark. Cells were spun down and then washed twice with 500 μl of 1X permeabilization buffer from Foxp3 staining buffer set. Cells were resuspended in 20 μl of 1X permeabilization buffer and 2.5 μl of APC (Anti-Mouse/Rat Foxp3) (eBioscience, Ref. No. 17-5773-82) and allowed to incubate for one hour at 4°C in the dark. Cells were washed twice with 1X permeabilization buffer, resuspended in 1X permeabilization buffer, stored overnight at 4°C in the dark, and observed the next day. Using a BD LSRFortessa flow cytometer, 10,000 events were recorded. Live cells were gated off using the Forward-scattered light (FSC) and Side-scattered light (SSC) plots, and only live populations were observed and used for counting. The percentage of Foxp3+ cells recorded was the percentage of live cells that were CD3+, CD4+, and Foxp3+.

Feces Collection and DNA Extractions

One fecal pellet was collected from each mouse individually immediately before euthanasia. Fecal pellets were stored individually in 1.5 ml sterile microcentrifuge tubes at -20°C until DNA extraction. Fecal DNA extraction was
performed using a PowerSoil DNA extraction kit (MO BIO Laboratories, Cat. No. 12888-100) and the manufacturer's protocol. DNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer and the DNA stored at -20°C until needed.

Fixing, Embedding, and Staining the Colon

After harvesting the colons, they were rolled into a Swiss roll formation, placed in 10% neutral buffered formalin, and allowed to fix for 48 hours at 4°C. The tissues were then dehydrated sequentially starting in 50% ethanol for two hours, 70% ethanol overnight, and 95% ethanol for two hours, followed by three changes of 100% ethanol for one hour each. To prepare samples for wax infiltration and sectioning, ethanol was replaced with xylene by first placing samples in a 1:1 solution of ethanol:xylene for one hour and then two changes of 100% xylene for one hour each. Samples were then moved to an oven set at 35°C, and paraffin wax chips were added slowly every two hours until the wax stopped melting. The samples were then transferred to an oven set at 60°C, where half of the paraffin/xylene mixture was poured out and replaced with already melted paraffin from the oven. This addition of fresh paraffin happened every two hours and was allowed to sit overnight after a complete change of paraffin to allow complete infiltration.

Samples were then imbedded in molds in Paraplast plus paraffin for sectioning at 10 um. The sections were then mounted on glass slides and warmed at 30°C until they underwent hematoxylin and eosin staining. To begin the staining process, slides were immersed in two changes of xylene for five
minutes each to dewax the slides. Samples were then rehydrated by two changes of 100% ethanol and two changes of 95% ethanol at three minutes each. Slides were rinsed with distilled water for three minutes, then stained with Richard-Allan Scientific Hematoxylin (Thermo scientific, Ref. No. 7211) for one minute and 40 seconds. Slides were later immersed in clarifier (VWR differentiation RTU, Cat. No. 95057-856) for one minute and 30 seconds followed by a rinse step in distilled water for one minute. The slides were then placed in bluing solution (VWR bluing reagent RTU, Cat. No. 95057-852) for 30 seconds and rinsed again with distilled water for one minute followed by staining in Eosin (VWR Phloxie-eosine, Cat. No. 95057-846) for 20 seconds. Samples were then dehydrated again by passing through 95% ethanol for one minute, two changes of 100% ethanol for two minutes each, and two changes of xylene for two minutes each. Coverslips were then applied onto each slide using mounting medium Permount, and the slides cured on a hot plate overnight at 60°C.

Assessment of Histological Damage

An unbiased observer scored each histological slide by using a previously published scoring system, including scores for the following: crypt architecture (normal, 0 - severe crypt distortion with loss of entire crypts, 3), degree of inflammatory cell infiltration (normal, 0 – dense inflammatory infiltrate, 3), muscle thickening (base of crypt sits on the muscularis mucosae, 0 – marked muscle thickening present, 3), goblet cell depletion (absent, 0 – present, 1) and crypt abscess (absent, 0 – present, 1) (Cooper, Murthy, Shah, & Sedergran, 1993; Kim et al., 2012). The final histological damage score was the sum of each individual
score. The number of lymphoid aggregates was also counted for each sample. Representative images were taken for each histological sample using a Leica MC170 HD camera and a Leica DM IL light microscope. Examples of histological images that display muscle thickening and crypt loss or distortion after induction of colitis can be seen in Figure 2.

16S rRNA Illumina Sequencing

The gut microbial community of each mouse was assessed by sequencing the V3-V4 region of the bacterial 16S rRNA gene extracted from fecal samples from the first experiment. Sequencing was performed by the core sequencing facility at the University of Mississippi Medical Center, Jackson MS in both directions using Illumina MiSeq. The following forward and reverse primers were used to amplify the V3-V4 region:

16S-F:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

16S-R:
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGA\nCTACHVGGGTATCTA\nATCC

Only full length, quality filtered, and overlapped reads were used in the sequencing data analysis.

Sequence Analysis

The FASTX-Toolkit (version 0.0.14) was used to trim sequences of their primers. In USEARCH (version 8.1.1825), a similarity threshold of 97% was used in assigning operational taxonomic units (OTUs) as well as for removing
chimeras. Sequences were classified at the genus level using the Ribosomal Database Project (RDP) (version 11.4) (Wang, Garrity, Tiedje, & Cole, 2007) with default parameters. Classified sequences with their respective counts were exported into Microsoft Excel 2013 and used to construct bar graphs of relative abundances of 16s rRNA genes. R software package (version 3.2.3) was used to determine significant indicator OTUs from each treatment group, and the indicator OTUs were classified at the lowest level of classification possible using RDP (version 11.4) (Wang et al., 2007) with default parameters.

Data Analysis

Graph Pad Prism (Version 6) was used to analyze and make figures from all data from flow cytometry and histological scoring methods. Significance (*P<0.05, **P<0.01, and ***P<0.001) was determined by unpaired parametric t tests with Welch’s correction and a 95% confidence level.
CHAPTER III - RESULTS

Body Weight Change

The body weight of mice with induced colitis decreased abruptly on Day 5 of treatment (Figure 1a). After the reintroduction of 1.5% DSS to cause relapse of colitis in the mice, the DSS and inulin treatment groups showed a gradual loss of body weight (Figure 1b), while the control groups and OEI treated groups showed relatively stable body weight throughout treatment and a sharp increase in body weight on Day 7 (Figure 1b).
**Figure 1.** Effect of Induced Colitis and Prebiotic Treatments on Mouse Body Weight

Mean body weight change (%) in mice from (a) after 3% DSS treatment and (b) after reintroduction of 1.5% DSS. Error bars indicate standard error of the mean (SEM).
Histological Images

*Figure 2. Effect of Induced Colitis on Microscopic Anatomy of the Colon*

Representative images of colon sections from control mouse (a) and mouse after induced colitis (b). The arrow points out areas of crypt damage or loss, while the star points out muscle thickening.

**Colon Length**

Colon length was significantly shortened in mice with induced colitis (Figures 3a and b). Treatment with OEI led to longer colon length than those treated with DSS alone (Figure 3b). After the reintroduction of 1.5% DSS to stimulate relapse in the mice, the inulin treated group had significantly shorter colon length than both the control and DSS treated groups (Figure 3d). In contrast, the colon length in the OEI treated group was significantly longer than that of the DSS treated group (Figure 3d).
Effect of Induced Colitis and Prebiotic Treatments on Colon Length

Colon length measurements (mm) from (a) after 3% DSS treatment (b) after inulin and/or OEI prebiotic treatments (c) after four week recovery period and (d) after reintroduction of 1.5% DSS. Error bars indicate SEM (n=6 for all groups except DSS+Inulin, in which n=3). Significance (*P<0.05, and ***P<0.001) was determined by unpaired parametric t tests with Welch’s correction and a 95% confidence level.

Histological Scores

DSS treated groups had higher histological scores than control groups (Figures 4a, b, c, and d). However, this increase was not found to be significant. Although the change was not significant, when compared to the DSS treated groups, the OEI treated groups showed less histological damage overall, specifically directly after prebiotic treatment (Figure 4b). In contrast, the inulin treatment group showed a significantly higher histological score after the four week recovery period (Figure 4c) and higher scores than the control, DSS, and OEI treated groups after the reintroduction of 1.5% DSS, though this difference was not significant (Figures 4b and d).

Figure 4. Effect of Induced Colitis and Prebiotic Treatments on Histological Scores of the Colon

Histological scoring of colon sections (a) after 3% DSS treatment (b) after inulin and OEI prebiotic treatments (c) after four week recovery period and (d) after reintroduction of 1.5% DSS. Error bars indicate SEM (n=6 for all groups except
DSS+Inulin, in which n=3). Significance (*P<0.05) was determined by unpaired parametric t tests with Welch's correction and a 95% confidence level.

**Lymphoid Aggregate Count**

The number of lymphoid aggregates was higher in mice treated with DSS initially, though this was not significant (Figure 5a). However, the number was lower in DSS treated mice after relapse when compared to healthy controls (Figure 5d). The count was higher in the prebiotic treated groups than control and DSS treatment groups after the four week recovery period and the reintroduction of DSS (Figures 5c and 5d). Furthermore, the count in the OEI treated group after the four week recovery period was significantly higher than the control group (Figure 5c).

![Graphs showing lymphoid aggregate count](image)

**Figure 5.** Effect of Induced Colitis and Prebiotic Treatments on Lymphoid Aggregate Counts in the Colon

Lymphoid aggregate count in histological sections from (a) after 3% DSS treatment (b) after inulin and OEI prebiotic treatments (c) after four week recovery period and (d) after reintroduction of 1.5% DSS. Error bars indicate SEM (n=6 for all groups except DSS+Inulin, in which n=3). Significance (*P<0.05) was determined by unpaired parametric t tests with Welch's correction and a 95% confidence level.
Regulatory T cell Quantification

Induced colitis led to a significant increase in the number of Foxp3+ Treg cells (Figure 6a). After prebiotic treatment, the number of Foxp3+ Treg cells was higher in the DSS treatment group than in the control group and increased even more in the OEI treatment group (Figure 6b). The number of Foxp3+ Treg cells was significantly lower in the inulin treatment group when compared to the control, DSS, and OEI treatment groups (Figure 6b). Treg cell number was significantly lower in DSS treated and OEI treated groups after the four week recovery period when compared to control mice (Figure 6c). However, after the reintroduction of 1.5% DSS to induce relapse, the number of Foxp3+ Treg cells was significantly increased in the inulin and OEI treatment groups when compared to the control group and DSS treatment group (Figure 6d). This significance was greater in the OEI treatment group than the inulin treatment group (Figure 6d).

Figure 6. Effect of Induced Colitis and Prebiotic Treatments on Regulatory T cell counts in Mouse Spleen

Percentage of FoxP3+ cells from total live cell population from (a) after 3% DSS treatment (b) after inulin and OEI prebiotic treatments (c) after four week recovery period and (d) after reintroduction of 1.5% DSS. Error bars indicate SEM.
(n=6 for all groups except DSS+Inulin, in which n=3). Significance (*P<0.05, **P<0.01, and ***P<0.001) was determined by unpaired parametric t tests with Welch’s correction and a 95% confidence level.

Note: Results from the first experiment four week healing period are not shown due to unreliable measurements (n=3 for figure 6c).

**Fecal Bacterial Community Composition**

Overall, there was a relative increase in the number of *Barnesiella* spp. from 37% to 51.62% in the DSS treated group and decrease in *Anaeroplasma* spp. from 22.97% to 0.72% when compared to control mice (Figure 7a). The proportion of *Clostridium cluster XIVa* spp. and *Clostridium cluster XVIII* spp. was increased from 4.52% to 8.81% and from 0.06% to 3.3% respectively in the DSS treated group when compared to controls (Figure 7a).

After prebiotic treatment, there was a relative increase in *Barnesiella* spp. in the inulin (70.3%) and OEI (63.75%) treatment groups when compared to the control (45.07%) and DSS (43.04%) treatment groups (Figure 7b). Relative abundance of *Clostridium cluster XIVa* spp. was increased from 4.7% in the control group to 10.29% in DSS, 6.97% in inulin, and 9.14% in OEI treatment groups, while the abundance of bacteria belonging to *Lactobacillus* spp. were decreased overall in the three groups (Figure 7b). An increase in abundance of *Lachnospiracea_incertae_sedis* spp., *Flavonifractor* spp., *Oscillibacter* spp., and *Ruminococcus* spp. was observed in the DSS treated group, while these groups were closer to levels seen in the control group or lower in number in the inulin and OEI treated groups (Figure 7b).

After the four week recovery period, relative abundance of *Anaeroplasma* spp., *Clostridium sensu stricto* spp., *Lachnospiracea_incertae_sedis* spp., and
Oscillibacter spp. was increased in the DSS treatment group while abundance of these groups was decreased or closer to control levels in the inulin and OEI treated groups (Figure 7c). Ruminococcus spp. were completely absent in DSS, inulin, and OEI treated groups while Turicibacter spp. were only found in DSS and OEI treated groups (Figure 7c). The relative proportion of Flavonifractor spp. was increased in DSS and OEI treated groups (Figure 7c). Barnesiella spp. numbers were decreased in DSS when compared to the control group and increased in the inulin and OEI treatment groups (Figure 7c). Abundance of Lactobacillus spp. was decreased in DSS and inulin treatment groups, while its abundance was closer to control levels in the OEI treatment group (Figure 7c). Finally, the proportion of Clostridium cluster XIVa spp. was decreased from 16.74% in the control group to 9.41% in the DSS group and 1.06% in the inulin treated group. However, the proportion was closer to that of the control group in the OEI treated group, being 12.48% (Figure 7c).

Following the reintroduction of 1.5% DSS, proportions of Barnesiella spp., Clostridium cluster XVIII spp., Enterococcus spp., and Escherichia/Shigella were increased in DSS, inulin, and OEI treatment groups (Figure 7d). Numbers of Clostridium cluster XIVa spp. and Oscillibacter spp. were lower in DSS, inulin, and OEI treatment groups than in the control group (Figure 7d). The relative abundance of Flavonifractor spp. was seen to be decreased in the DSS group but restored to abundance similar to that of the control group in the inulin and OEI treatment groups (Figure 7d). Lachnospiracea_incertae_sedis spp. abundance was decreased in DSS and inulin treated groups and restored to
abundance similar to the control group in the OEI treatment group (Figure 7d). *Lactobacillus* spp. were seen in higher numbers in DSS and inulin treated groups, but were observed in lower numbers than the control group in OEI treated mice (Figure 7d). *Ruminococcus* spp. numbers were decreased in DSS treated mice but were increased in inulin and OEI treated mice (Figure 7d).
Figure 7. Effect of Induced Colitis and Prebiotic Treatments on the Fecal Bacteria Composition at the Genus Level

Composition of the V3-V4 region of 16s rRNA counts at the genus level. (a) after 3% DSS treatment (b) after inulin and OEI prebiotic treatments (c) after four week recovery period and (d) after reintroduction of 1.5% DSS. Each bar represents the average composition of the three samples within each treatment group (n=3). No error bars are shown. Unclassified bacteria are not shown.
CHAPTER IV - DISCUSSION

Studies have shown that numbers of butyrate producing bacteria are decreased in patients with IBD (Frank et al., 2007; Machiels et al., 2013). This lack of potential butyrate production could be the cause of the reoccurrence of inflammation. Butyrate has been shown to increase protein production in colonocytes (Frankel et al., 1994). It has been shown to reduce the translocation of bacteria across epithelium that was metabolically stressed (Lewis et al., 2010). Butyrate has also been shown to stimulate mucin production in the colon (Finnie et al., 1995) and induce the differentiation of colonic regulatory T cells, which are critical in maintaining homeostasis in the intestine (Furusawa et al., 2013). Therefore, the number of butyrate producing bacteria in an organism could potentially directly correlate with the number of regulatory T cells in the organism. By attempting to increase the abundance of butyrate producing bacteria in the gut through the introduction of prebiotics, we attempt to increase the number of regulatory T cells in the organism as well. The beneficial effects of butyrate mentioned above, as well as an increase in regulatory T cell number, should allow for an enhanced healing process in the diseased mice as well as a reduced chance of relapse.

When measuring percent body weight change in the mice after the initial introduction of 3% DSS (Figure 1a) and the reintroduction of 1.5% DSS (Figure 1b), the mice treated with 3% DSS began to lose weight around Day 5 of DSS treatment, which was to be expected due to the inflammation in the guts of the mice. When observing the body weight changes after the prebiotic treatments
and reintroduction of DSS, the DSS and inulin treated groups (Figure 1b) had a more significant decrease in body weight during the 1.5% DSS treatment than the control or OEI treated groups had. The OEI treated group was more resistant to the decrease in body weight, as it maintained levels similar to the control group.

After induction of colitis, the colon length of the DSS treated mice was shorter than the control mice (Figure 3a) and the histological score was higher in the DSS treated mice (Figure 4a). This shortening of the colon and higher histological score indicated the presence of inflammation in the colon (Kim et al., 2012). The shortening of the colon and high histological scoring was even more severe in the inulin treated mice (Figures 3d, 4c, and 4d). Overall, the OEI treated groups exhibited less severe inflammation than the DSS or inulin treated groups (Figures 3b, 3d, and 4b). The histological scores of the OEI treated group were lowest directly after prebiotic treatment (Figure 4b) indicating a possible enhanced healing process in these prebiotic treated mice.

A correlation has been shown between an increase in the number of lymphoid aggregates and severity of colonic inflammation (Nascimbeni et al., 2005). However, the role of these lymphoid aggregates in an inflamed colon may actually be beneficial. Lymphoid aggregates have been shown to facilitate the healing of intestinal injury by increasing cell proliferation in the intestinal epithelium (Saxena, Thompson, & Sharp, 1997). Mice treated with DSS had higher counts of lymphoid aggregates than the control mice during the first three time points (Figures 5a-c). However, after relapse, the lymphoid aggregate
counts were lower in DSS treated mice than in control mice (Figure 5d). Mice treated with the prebiotic OEI had higher lymphoid aggregate counts than DSS treated mice during two time points (Figures 5c and d). The increase in lymphoid aggregates in the OEI treated mice could have caused the lowering in histological score for this group of mice by allowing increased cell proliferation and a faster healing response. A combination of an increase in the number of lymphoid aggregates and an increase in the number of regulatory T cells could further improve the healing process.

The number of Foxp3+ regulatory T cells was initially increased in the spleens of DSS treated mice (Figures 6a and b) when compared to the control group. This initial slight increase in regulatory T cells could be due to the immune system of the mouse trying to prevent excessive inflammation in the gut. Since the method of inducing colitis in the mice was chemically induced, the mice had fully functioning immune systems. Therefore, it is not surprising that their immune systems initially increased the production and/or differentiation of regulatory T cells in order to attempt to attenuate the inflammation. However, the number of regulatory T cells in the DSS treated mice after healing (Figure 6c) was significantly lower than in the control group. This signifies that the regulatory T cell response was not sustained in these animals or that it was down regulated at some point. In contrast, the number of regulatory T cells in the OEI treatment group after the reintroduction of DSS was significantly higher than in the control or DSS treated groups (Figure 6d). This significant increase in regulatory T cells
in the OEI treatment group could have contributed to the decrease in inflammation in this group when compared to DSS treated controls.

Sequencing data was analyzed and classified at the genus level in order to attempt to identify possible butyrate producing genera of bacteria. When analyzing the gut bacterial community directly after introduction of 3% DSS, an increase in potentially beneficial bacteria, such as *Lactobacillus* spp., and possible butyrate producing bacteria, such as *Clostridium cluster XIVa* spp, was observed. This contradicts earlier studies by Wang et al. (2014), Sokol et al. (2009), and Takaishi et al. (2008) that showed an increase in *Lactobacillus* spp, but a decrease in the *Clostridium cluster XIVa* group in human patients with active IBD. The reason for the increase in potential butyrate producing bacteria such as *Clostridium cluster XIVa* in mice treated with DSS is unknown. However, it may be due to the mouse’s immune system trying to compensate for the damage in the colon by increasing the likelihood of survival for beneficial bacteria capable of producing butyrate, which can be used by colonocytes as an energy source for tissue repair. The idea of altering gut microbial diversity through immune regulation has been shown in a study by Kawamoto et al. (2014). In this study, regulatory T cells were shown to regulate the secretion of immunoglobulin A, which, in turn, regulated the diversity of species of bacteria within the intestine (Kawamoto et al., 2014). The regulatory T cells in their study specifically facilitated the selection and diversification of the *Firmicutes* phyla, and more specifically, bacteria belonging to *Clostridium clusters IV and XIVa* (Kawamoto et al., 2014).
After the four week healing period and the reintroduction of DSS, the abundance of this potentially beneficial *Clostridium cluster XIVa* group was lower in DSS treated mice than in the control group (Figures 7c and d). Therefore, the higher level of butyrate producing bacteria could not be maintained in the mouse gut, probably due to prolonged inflammation and an altered environment within the gut. *Clostridium cluster XIVa* spp. were nearly absent after the four week healing period in the inulin treated mice. However this group was found to be higher in abundance in the OEI treatment group than the DSS treatment group and was nearly the same as the level found in the control group of mice at this time point. The OEI treatment must have altered the environment within the colon in favor of the conservation of this *Clostridium* group, either by directly feeding the bacteria or by feeding other beneficial bacteria that produce side products, such as lactic acid, that the *Clostridium* species have been shown to utilize in their production of butyrate (Louis & Flint, 2009). The OEI prebiotic stimulated an increase in *Clostridium cluster XIVa* bacteria from the time in which it was administered to the time right before the reintroduction of DSS. The maintenance of this potential butyrate producing group of bacteria could have led to the increase in the expansion of regulatory T cells that was seen after the reintroduction of DSS (Figure 6d). The increase in regulatory T cell number could in turn allow the *Clostridium* group to be proliferated and diversified in favor of the production of even more butyrate.

After reintroduction of DSS (Figure 7d) an outgrowth of *Escherichia/Shigella* occurred in one mouse from each of the following groups:
DSS, DSS+Inulin, and DSS+OEI. This signifies that recurrent use of DSS in a mouse model may induce an environment that favors the overgrowth of certain species of bacteria that can be adherent or invasive. However, the age of the mice could have caused this outgrowth as well. Langille et al., (2014) showed that the gut community is constantly changing in an aging mouse, so this should be taken into consideration when observing the gut community of older mice. Also, it should be noted that neither the body weight of the mice nor the feces looked abnormal or different than the other mice that underwent the same 1.5% DSS treatment. There were no signs of infection in the mice.

Significant indicator organisms were identified for each treatment group. These OTUs were individually classified at the lowest level of classification possible. A majority of the significant OTUs in each treatment group were only identified down to the family level. Therefore, without having classified these bacteria, we have no real way of knowing what role they may be playing in the gut during these time points. Even with the abundant amount of sequencing data we have, a majority of the bacterial species within the gut are still unclassified. Some of the OTUs found could possibly be important in the healing process and could potentially be used as probiotics if properly studied. In addition, some of the indicator OTUs found in the DSS treated mice may be key in triggering or maintaining inflammation in the gut, and if we could identify them, we could possibly learn how to control them.

Most sequencing analyses done on mice treated with DSS have only identified bacteria at the phylum level with some being identified down to the
family level. However this level of identification is not deep enough to distinguish between beneficial and potentially pathogenic bacteria, nor to identify potential butyrate producing bacteria in a community. The studying of changes at the genus level mainly occurs in human studies and have not been thoroughly examined in mice. This study attempts to classify bacteria down to the genus level in order to identify possible butyrate producing groups in the mice.

Future directions may include sampling the mucosal associated bacterial community rather than the fecal community. The mucosal associated community has been shown to be significantly different from the fecal community. Van den Abbeele et al. (2013) found that Bacteroidetes and Proteobacteria dominated the luminal content of an in vitro gut model, while Firmicutes, specifically bacteria from Clostridium cluster XIVa, were more abundant in the mucin layer of the gut. Therefore, the butyrate producing bacteria may actually be more abundant in the mucosal associated tissue in order to enhance butyrate production near the tissue and site of inflammation itself to enhance its repair and regeneration (Van den Abbeele et al., 2013). More drastic or significant changes in the bacterial community of the gut may be seen if the mucosal community is studied rather than the fecal community.

Another study that could be done is to measure the number of regulatory T cells in the colon during the different time points and see if the number of cells is increased there as well after prebiotic treatment. Knowing that there is an increase in regulatory T cells in the blood and spleen after prebiotic treatment is a good start, but we also need to determine if these cells are migrating to the
colon where the inflammation is occurring and where they need to be active. One last future direction may be to determine how much butyrate is actually being taken up and utilized by the colonocytes. Ahmad et al. (2000) showed that butyrate oxidation by colonocytes is impaired in mice with DSS induced colitis. Therefore, increasing levels of butyrate in the gut through the use of prebiotics is a good start to treating IBD. However, we need to figure out how to repair the damaged colonocytes more quickly and efficiently so they can utilize the excess butyrate available to them. A combination of corticosteroids and prebiotics could possibly be used. Corticosteroids could decrease the inflammation enough to help put the patient in a state of remission and help colonocytes be able to function properly and utilize butyrate. The dampening of inflammation could also allow for the microbial community to return to a somewhat normal state, and the prebiotic supplementation would ensure that the butyrate producing community is present to provide energy to the colonocytes. A consistent use of prebiotics could possibly help the patient remain in remission.

In conclusion, I propose that when inflammation occurs in the gut, an environmental change takes place within the gut that allows for beneficial butyrate producing bacteria to be outcompeted. When this happens, the colonocytes cannot receive proper amounts of butyrate for energy to repair and replace themselves, and further inflammation may occur. If the proper microbial balance within the gut is not restored, by the use of prebiotics for example, this inflammation can becoming a recurring event and lead to inflammatory bowel disease. Through the use of prebiotics, we can ensure that the butyrate
producing bacteria of the gut have a food source from which they can produce butyrate, which in turn will give colonocytes the energy to allow them to reproduce more quickly and allow more rapid healing of the inflamed gut. OEI is a particular prebiotic that shows promising results in this study by inducing the proliferation of butyrate-producing bacteria which led to the increase in regulatory T cell production and down regulation of inflammation in diseased mice. Therefore, this prebiotic should be studied in more detail in the future.
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

NOTICE OF COMMITTEE ACTION

The proposal amendment noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: 13121204
PROJECT TITLE: The effects of probiotics and prebiotics on gut microbiota and colitis in mice
PROPOSED PROJECT DATES: 02/2015 - 09/2017
PROJECT TYPE: Modification
PRINCIPAL INVESTIGATOR(S): Xiaowu Wang
DEPARTMENT: Biological Sciences
FUNDING AGENCY/SPONSOR: N/A
IACUC COMMITTEE ACTION: Full Committee Approval
PROTOCOL EXPIRATION DATE: September 30, 2017

[Signature]
Frank Moore, PhD
IACUC Chair

Date: 02/13/2015
NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: 15120901 (Replaces 13121204)
PROJECT TITLE: The Effects of Probiotics and Prebiotics on Gut Microbiota and Colitis in Mice
PROPOSED PROJECT DATES: 12/2015 - 09/2018
PROJECT TYPE: New
PRINCIPAL INVESTIGATOR(S): Shiao Wang
DEPARTMENT: Biological Sciences
FUNDING AGENCY/SPONSOR: N/A
IACUC COMMITTEE ACTION: Full Committee Approval
PROTOCOL EXPIRATION DATE: September 30, 2018

[Signature]
Date

Frank Moore, Ph.D.
IACUC Chair
REFERENCES


