UNIVERISTY OF CALIFORNIA, SAN DIEGO

Administration of Probiotics Normalizes Deficits in the Microbiota-Gut-Brain Axis Induced by DSS-Colitis

A thesis submitted in partial satisfaction of the requirements for the degree of

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by

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2015

The thesis of Kevin Huynh is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2015

DEDICATION

I dedicate this thesis to my family.
For Anh, Eron, Jamie, Ken and Alice.

EPIGRAPH

In the beginner's mind there are many possibilities, but in the expert's there are few.

Shunryu Suzuki

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ABSTRACT OF THE THESIS

Administration of Probiotics Normalizes Deficits in the Microbiota-Gut-Brain Axis Induced by DSS-Colitis

by

Kevin Huynh

Master of Science in Biology

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Professor Melanie G. Gareau, Chair

Professor Andrew Chisholm, Co-Chair

Altered behavior and mood disorders, including anxiety, depression and cognitive dysfunction, are increasingly found to occur in the context of intestinal diseases such as inflammatory bowel disease (IBD) and negatively affect

patient quality of life. The aim was to determine whether colonic inflammation precipitates behavioral deficits and whether these changes can be ameliorated by administration of probiotic organisms. Dextran sodium sulfate (DSS; 3% w/v) was administered to 6-7 week old C57BL/6 mice via drinking water for 5 days followed by either 3 days (8 d post-DSS; height of disease) or 9 days (14 d post-DSS; resolution of disease) of normal drinking water. A subset of mice was given probiotics (*Lactobacillus rhamnosus* [R0011] and *L. helveticus* [R0052]; 10^9 CFU/ml orally) starting 7 days prior to DSS and continuing until the end of the experiment. Changes in weight, colon length, behavior and microbiota were assessed. At 8 d post-DSS, weight loss and colonic shortening (both $p<0.01$) were observed, indicating colonic disease, and dysbiosis was also present. DSS mice (*vs.* controls) demonstrated impairments in recognition memory ($p<0.01$) and the presence of anxiety-like behavior ($p<0.05$), which were both resolved by 14 d post-DSS. Administration of probiotics ameliorated colonic disease ($p<0.05$), deficits in behavior ($p<0.05$ for cognition and anxiety-like behavior), and normalized levels of c-fos expression ($p<0.05$) in the CA-1 region as well as partially restored the composition of the microbiota. Taken together, these findings indicate the presence of dysregulation of the microbiota-gut-brain axis in the setting of DSS-induced colitis that can be prevented by treatment with probiotics.

INTRODUCTION

Microbiota-gut-brain (MGB) axis

Communication between the gut and the brain has long been recognized to play an important role in maintaining homeostatic processes such as satiety and hunger. The bi-directional communication between these two major organs, referred to as the gut-brain axis, is becoming an area of major interest in the context of gastrointestinal and cognitive processes(1). It is becoming increasingly clear that the microbiota, or flora, of the gut plays a major role in modulating the gut-brain axis. The microbiota-gut-brain axis has been coined to describe the role for the microbiota in maintaining communication between the brain and the gut via the bacteria that inhabit the intestinal lumen (1, 2).

The microbiota consists of trillions of micro-organisms including bacteria, archaea, yeast, and fungi that colonize our skin, gut, and numerous other sites starting at birth. The microbiota plays a major role in maintaining proper metabolic and homeostatic functions in the gut, however it is increasingly being shown also to modulate processes in the brain, including the regulation of behavior. For example, the microbiota can modulate normal stress responses via regulation of the hypothalamus-pituitary-adrenal (HPA) axis (3). In a germ free (GF) setting, where there is a complete lack of a microbiota, mice display an exaggerated stress response due to a hyperactive HPA-axis as determined by elevated serum corticosterone levels (4). Interestingly, adult GF mice also have a more permeable blood brain barrier (BBB) compared to specific pathogen free (SPF) mice (5). The BBB permeability defect observed in this study was ameliorated when the mice became colonized by short chain fatty

acid (SCFA)-producing bacteria or when SCFA was introduced to their gastrointestinal tract via oral gavage (5). This suggests that the gut microbiota, and its secreted by-products, can potentially influence the brain. Studies that investigate the impact of the microbiota on the gut-brain axis rely heavily on animal models to understand the consequences of gastrointestinal events on the brain by measuring behavior. These validated behavioral tests utilized to assess these changes represent powerful readout methods to assess behavioral and cognitive changes associated with modulation of the microbiome.

Inflammatory bowel disease (IBD)

IBDs are characterized by chronic, relapsing and remitting inflammation of the gastrointestinal tract, which can severely decrease quality of life in patients. The close proximity of trillions of gut microbes adjacent to an area of high cell-turnover, the intestinal epithelium, requires a delicate balance between pro-inflammatory and anti-inflammatory responses to maintain health. Although the exact etiology remains unknown, an imbalance in the microbiota, in a genetically susceptible host, following exposure to unknown environmental triggers are thought to lead to development of IBD (6). IBDs consists of two subsets of disease: ulcerative colitis (UC) and Crohn's disease (CD). Both diseases are characterized by acute or chronic episodes of inflammation in the gut. Patients with UC experience continuous, mucosal inflammation of the colon

(7). In contrast, patients with CD experience discontinuous, transmural inflammation throughout the entire length of the gut, often in patches or segmentally (7). Although UC and CD are very different in terms of site and extent of inflammation, both are believed to be heavily influenced by the gut microbiota.

The gut microbiota interacts with and can modulate the host immune system. The intestinal microbiota is crucial to maintaining gut homeostasis, as it plays an important role in the development of local and systemic immunity (8). The importance of the gut microbiota in immune function has largely been established through studies of GF mice. The immune system of GF mice is impaired and less able to resist infection from bacterial pathogens (9, 10) In the absence of a gut microbiome, GF mice have altered intestinal architecture, impaired lymphoid tissues, and reduced Peyer's patches (both size and numbers) (11). In patients with IBD, the gut microbiota is shifted significantly from healthy subjects (12). This dysbiosis, or alterations to the composition of the microbiome, identified in patients with IBD suggest that the microbiota may play a large role in disease pathogenesis (13). In addition to inflammation, patients with IBD are also characterized as having increased barrier permeability (14). This "leaky gut" is widely believed to allow unrestricted passage of bacterial antigens, including lipopolysaccharides (LPS), and other endotoxins from the microbiota across the intestinal barrier and potentially contributing to the strong inflammatory response seen in patients with IBD.

In IBD, an overt immune response is seen in the gut, resulting in significant inflammation and damage. Genetic knockout mice for regulatory cytokines like IL-2 and IL-10 have been shown to develop spontaneous colitis compared to wild type controls (15). In patients with IBD, the pro-inflammatory cytokine IL-6 is often significantly increased compared with healthy controls (16). Current treatment options for patients with IBD center primarily on modulation of this pro-inflammatory state and commonly involve treatment with anti-inflammatories like prednisone or newer recombinant antibodies against pro-inflammatory cytokines; although in clinical settings these have often yielded mixed results (15). This suggests that there exists a network of cytokines that regulate mucosal inflammation and that the effects of various cytokines can be pleiotropic.

Mouse models of colitis

To study disease, as seen in humans, numerous mouse and rat models of IBD have been established and validated, with each displaying certain features of disease, but none completely recapitulating this complex clinical phenotype. Given the unknown etiology of IBD and the likely multifactorial nature of the disease, there has not been a successful *in-vitro* model developed to date (17). Dextran-sodium sulfate (DSS) is a widely used and accepted acute model of colitis in mice (17). DSS-induced colitis is readily accessible, easily reproducible, and can be manipulated to induce acute or chronic colitis

depending on the dosing regimen. The ability for mice to recover from disease following removal of the DSS also allows the opportunity to study resolution of disease, which is not possible in the genetic susceptibility models where severity increases with time. Mice administered DSS daily in their drinking water display pathologies similar to UC such as bloody stools, diarrhea, weight loss, and ulcers (18). In terms of therapeutic responses, mice with DSS-induced colitis have shown similarities to IBD observed in humans (17). In these particular studies, the DSS-colitis model was chosen for the ease of use and low intrusive nature of administration of DSS, via the drinking water, in contrast to more invasive models involving colonic instillation of compounds, such as dinitrobenzene sulfonic acid (DNBS) or trinitrobenzene sulfonic acid (TNBS) for example (19).

Probiotics

Within the gastrointestinal microbiota resides a large portion of beneficial, or probiotic, bacteria that help maintain health and well-being. Probiotics are defined as live microorganisms that confer health benefits to the host, beyond their inherent nutrition, when ingested in adequate quantities (20) Potential probiotic organisms must possess several characteristics and abilities to improve overall heath in order to be classified as a probiotic. Primarily, any beneficial (21) organism must be able to survive transit through the various parts of the digestive system, be able to proliferate in the intestinal environment, and lastly, promote

changes in the composition of the microbiota to confer its benefits (21, 22). Probiotics typically come from fermented milk products, such as yogurt, but are increasingly being supplemented in numerous food products, including cheese and dark chocolate. Many current industrial probiotics consist of lactic acid bacteria that include genera like *Lactobacillus, Streptococcus,* and *Bifidobacteria* as well as the probiotic yeast *Saccharomyces boulaardi* (23, 24). In the literature, probiotics have been reported to have protective effects and ameliorate defects in intestinal physiology and dysbiosis (25–27). Beyond the clinical applications of probiotic use in health and disease, they may also serve as useful tools to further study the MGB axis and understand the mechanisms of communication between the gut and the brain.

Probiotics and IBD

Historically, treatment of IBD has focused on reducing inflammation in the gut through administration of various pharmacological or biological therapeutics. NSAIDs, steroids, and other anti-inflammatory medications, including antibodies to cytokines or cytokine receptors, represent the primary clinical options to treat patients with IBD (28). These biologic strategies using antibodies and recombinant cytokines represent the more recent advances to treat IBD, including anti-TNFα monoclonal antibodies or introducing recombinant IL-10 into the systemic circulation, with both strategies having some clinical success (29, 30). Due to the chronic and remitting nature of IBD, patients must often undergo

long-term therapies which may be accompanied by significant side-effects from the drugs used in treatment (31, 32).

Probiotics have been suggested as a potential alternative therapeutic option to treat patients with IBD, by targeting the dysbiosis seen in patients as a means to decrease gut inflammation (33). Numerous animal studies have been conducted that support the therapeutic potential of probiotics in treating IBD. For example, in an acetic acid injury model of colitis in rats, administration of *L. reuteri* was shown to be effective in preventing colitis (34). In another study, *L. lactis* that was genetically engineered to secrete active IL-10 prevented onset of colitis in IL10 deficient (-/-) mice (35). In a murine model of infectious colitis induced by *Citrobacter rodentium*, a probiotic cocktail consisting of *L. helveticus* and *L. rhamnosus* was effective at preventing disease (36). These preclinical studies, and numerous others, suggest that probiotics can have protective effects against colitis, although clinical studies performed to date have been far less promising.

IBD and MGB axis: inflammation and depression

Numerous patient studies support a strong association between mood disorders, such as depression and anxiety, with an increased risk of IBD (37–39). Moreover, patients with IBD that also experience comorbid depression and anxiety have a greater risk of disease relapse, hospitalization, and reduced quality of life (39–41). IBD patients that experience mood disorders also have a 28% increased risk of surgery, suggesting that depression and anxiety

correspond to a more severe disease state (42). Taken together, these significant comorbidities between IBD and mood disorders implicate a potential role of the MGB axis in this association (43, 44).

MRI studies of the brain of CD patients showed that there was decreased gray matter volume in the frontal cortex and anterior mid-cingulate cortex, compared to healthy controls, which is involved in cognitive processes (45). Moreover, the number of white matter lesions was significantly higher in IBD patients relative to controls (46). This suggests that chronic gastrointestinal inflammation may correlate to physical changes in the brain.

There is also increasing evidence that link the presence of elevated pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α with the onset of depression (47, 48). Interestingly, mice without an adaptive immune system such as those with severe combined immune deficiency (SCID) are also significantly impaired in cognitive processes such as learning and memory (49, 50). This finding was supported by a recent paper published from our group that demonstrated that $Rag1^{-/-}$ mice, deficient in both B and T cells, are innately anxious and exhibit cognitive deficits, which are accompanied by alterations in both colonic physiology and composition of the microbiota (25). These deficits could be restored by administration of Lactobacillus-containing probiotics starting at weaning (25). Together, these studies support a role for the adaptive immune system in regulating the MGB axis.

The high comorbidities observed between IBD and mood disorders, as well as numerous studies linking inflammation to changes in cognition, suggest

that IBD may result in changes in the brain through the MGB axis. However, it remains unclear whether IBD can precipitate cognitive deficits. Therefore, the aim of this study was to assess whether IBD can precipitate cognitive deficits in a murine model and whether administration of probiotics could serve as a potential therapeutic option in treating cognitive deficits associated with colitis.

MATERIALS AND METHODS

Animals

Male and female C57BL/6 mice (6-8 weeks of age) were used for all experimental groups. Mice were housed in cages lined with chip bedding with free access to food and water in a 12:12 light/dark cycle. Animals were kept at a UC San Diego animal care facility and all behavioral testing was performed in a biosafety cabinet. All procedures and protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at UC San Diego.

DSS-induced colitis

DSS (Affymetrix®) solution was prepared to a concentration of 3% w/v in deionized water, which was given to mice as drinking water for 5 days. After 5 days, DSS-supplemented water was replaced with regular drinking water for either 3 or 9 days.

Probiotics

Probiotic solutions were prepared using Lacidofil (Kindly provided by Dr. Thomas Tompkins, Lallemand Health Solutions®) lyophilized powder, which contains two bacterial species: *L. helveticus* (R0052) and *L. rhamnosus* (R0011). Probiotic solution was prepared daily (10^9CFU/100ul) by addition of probiotic powder to sterile Luria broth. Placebo solution consisted of a similar (w/v) solution of maltodextrin dissolved in sterile Luria broth. Probiotics were administered daily starting one week prior to induction of DSS colitis and continued until the end of the experiment at day 8 post-DSS.

Study design

8-day and 14-day DSS

C57BL/6 mice were given DSS administered in their drinking water. After 5 days, the DSS drinking solution was removed and replaced with normal drinking water for the rest of the study. Control mice were given normal drinking water during the entirety of the study and the weight of all mice was collected daily. For the day 8 experiments, a subset of mice underwent behavioral testing and were euthanized, corresponding to the height of acute inflammation. Another subset of mice were continued on the water regimen until day 14 post-DSS, when they underwent behavioral testing and were euthanized following resolution of colonic inflammation and weight loss.

Probiotics and DSS

C57BL/6 mice were orally administered a probiotics solution, or placebo, for seven consecutive days, after which the mice began the DSS regimen. As described previously, after 5 days of DSS, normal drinking water was resumed for 3 days and weight measured daily. At 8 days post-DSS the mice underwent behavioral testing and were subsequently sacrificed for sample collection. The placebo- and probiotics-only groups consisted of oral administration of placebo or probiotics throughout the same time period with normal drinking water.

Behavioral testing

On test day, mice were transferred in their original cages to a biosafety cabinet and allowed to acclimatize (1hr) to this new environment. Following

acclimatization, mice underwent the light/dark box test. Immediately after completion of light/dark box testing, mice were placed in clean individual cages and allowed to habituate (1hr). After habituation, mice underwent NOR testing. Once behavior testing was completed, the mice were sacrificed by CO_2 inhalation followed by cervical dislocation. At the time of sacrifice, colons and brains were isolated and fixed in formalin. Fecal samples were collected for DNA isolation.

NOR test

The NOR test was performed to assess recognition memory (51). Each mouse was placed into a clean cage and was allowed to habituate to the new environment (1hr). Following habituation, mice underwent a training phase in which they were exposed to two objects, objects 1 and 2, for five minutes. After the training phase, the objects were removed and the mice were given a 15 minute rest period, which was immediately followed by the testing phase. During the testing phase, each mouse wass introduced to two objects where one object was from the training phase (object 2B) and a novel object (object 3). During both the training and testing phases, behavior was recorded by video camera and analyzed for the number of times that the mouse investigated each object. From this, an exploration ratio ((exploration ratio = freq smell #3/(freq smell #3 + freq smell #2B) x 100) was generated. The exploration ratio quantifies the frequency of exploring the novel object relative to the number of times the

mouse investigated both objects. A high ratio indicates a high level of recognition memory.

Light/dark box

The light/dark box was used to evaluate anxiety-like behavior of the mice in each test group(52). The box was designed so that one-third of the box is dark while the other two-thirds is kept lit. A divider containing a small opening separated the two areas, allowing the mice to traverse between the dark and well lit areas freely. Mice have an inherent curiosity to explore a new environment, however, they are more comfortable in the dark. Mice that spend more time in the dark and less in the lit environment are considered to be more anxious. In addition, the number of transitions between the light and dark compartments of the box are indicative of overall activity. The total amount of time the mouse spent in the lit portion and the number of transitions that mice undergoes from dark to lit portion of the box were counted.

Microbiota Analysis

Fecal samples were collected from the colon immediately following sacrifice and stored at -80C until further use. DNA was extracted from fecal pellets using a QIAmp DNA Stool Mini Kit (Qiagen™). DNA samples were then analyzed by qPCR using the SYBR green system (Applied Biosystems™). Primers for: *Lactobacillus, Eubacterium rectale, Bacillus*, *Bacteroides, Enterobacteriacease,*

Firmicutes, and segmented filamentous bacteria (SFB) were used to assess relative levels of abundance of each bacteria as compared to the total bacteria using *Eubacteria* universal primers (**Table 1**).

Histology

Colonic tissue was collected during sacrifice, fixed in 10% formalin, processed, and embedded in paraffin. Sections of the colon were then cut and subsequently stained using a hematoxylin and eosin (H&E) protocol. Stained slides were then observed by light microscopy under blinded conditions to minimize observer bias.

Immunohistochemistry (c-fos)

Fixed and processed brains were sectioned and stained for c-fos. Slides were de-paraffinized with xylene and rehydrated using a gradient of isopropyl alcohol. Following rehydration, brain sections underwent antigen retrieval using boiling citrate buffer. Following antigen retrieval, the slides were blocked with Vector Bloxall™ Blocking Solution (Vector Labs) for 10 minutes followed by a bovine serum albumin (BSA) blocking solution with 5% (w/v) normal goat sera (1hr). Following blocking, the slides were then exposed to the primary antibody at 1:200 dilution (rabbit derived c-Fos antibody, Abcam®) overnight. The following day, the primary antibody was washed off and immediately exposed to secondary antibody using the Vectastain Elite ABC Kit (Anti-rabbit; 1hr). Secondary antibody was then washed off and, per instructions from the Vectastain kit, the ABC solution was prepared and added to slides for 30

minutes. The ABC solution was then washed off the slides and ImmPACT DAB Peroxidase (Vector Laboratories) substrate was added while observing under a microscope. Once the cells in the hippocampus became sufficiently brown (2-10 minutes), the DAB solution was rinsed off to stop peroxidase activity. The slides were then counterstained in hematoxylin and mounted with coverslips using non-aqueous mounting media.

Table 1: Primer list for fecal microbiota qPCR. List of qPCR primers used to assess microbiota content (25).

TARGET	FORWARD (5'-3')	REVERSE (5'-3')
Eubacteria	ACTCCTACGGGAGGCAGCAGT	ATTACCGCGGCTGCTGGC
Bacillus	GCGGCGTGCCTAATACATGC	CTTCATCACTCACGCGGCGT
Bacteroides	GAGAGGAAGGTCCCCCAC	CGCTACTTGGCTGGTTCAG
Enterobacteriaceae	GTGCCAGCMGCCGCGGTAA	GCCTCAAGGGCACAACCTCCAAG
Lactobacillus/ Lactococcus	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG
Segmented filamentous bacteria	GACGCTGAGGCATGAGAGCAT	GACGGCACGGATTGTTATTCA
Eubacterium rectale	ACTCCTACGGGAGGCAGC	GCTTCTTAGTCAGGTACCGTCAT

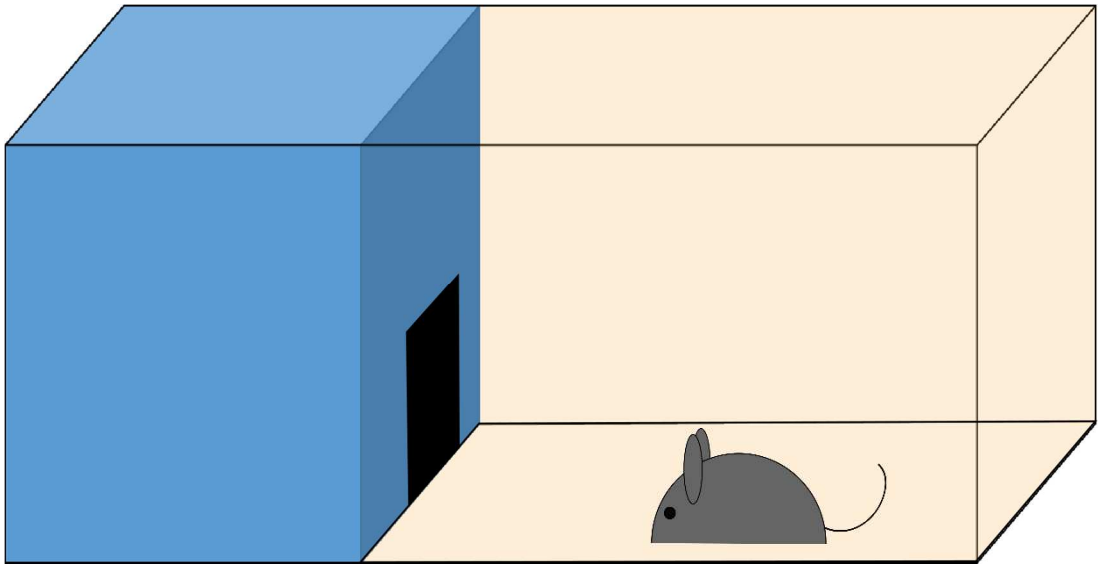
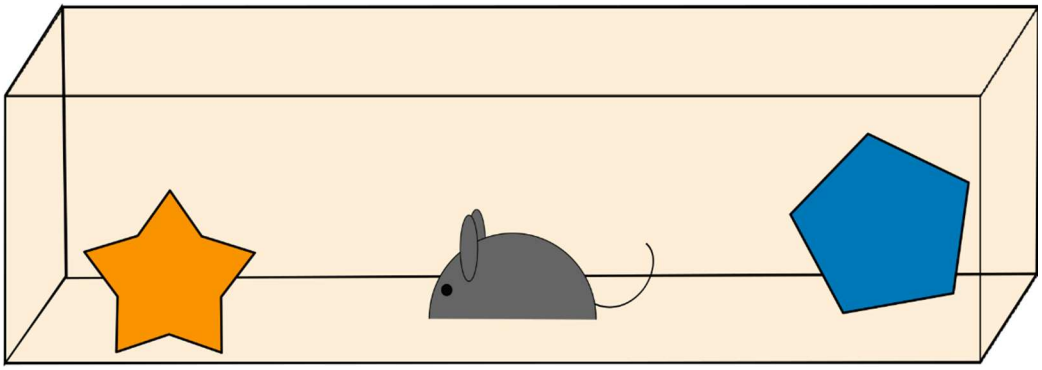


Illustration 1: Light-dark box test. Each mouse is placed into a cage in which one-third of the box is dark and two-third of the box is lit. The mouse is allowed to traverse between the two regions via an opening. The amount of time the mouse spends in the lit portion is quantified and the number of transitions from dark to lit portions of the box is tallied.

A



B

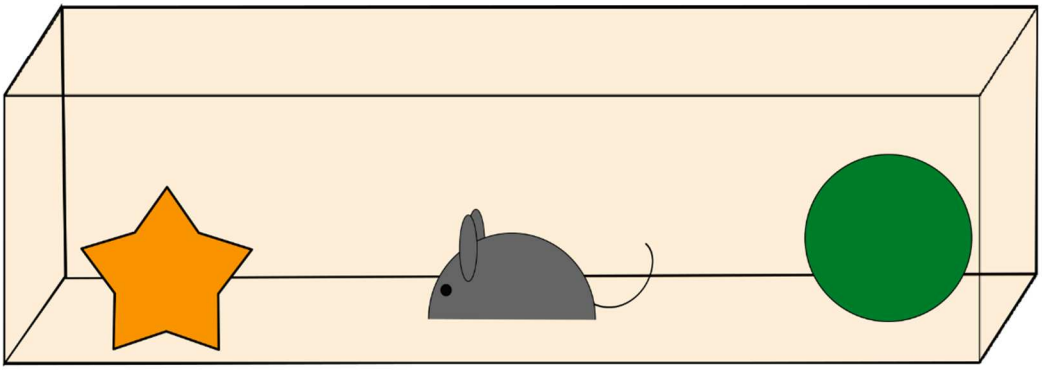


Illustration 2: Novel Object Recognition (NOR) Test. The mouse first undergoes training phase where the mouse is given two objects to explore for a period of 5 minutes **(A)**. During the testing phase, the mouse is exposed to a familiar object and a novel object **(B)**. The number of times the mouse investigates each object is tallied and quantified as the exploration ratio.

RESULTS

DSS induces colitis-like disease in mice

We first wanted to determine the optimal dose of DSS for inducing a reproducible colonic disease in our colony, as disease induction (3-5% w/v) can vary between vivaria. Administration of a 3% (w/v) DSS solution to adult C57BL/6 mice in the drinking water for 5 days caused a robust, colitis-like pathology including weight loss, bloody stool, and colonic inflammation at 8 days post-DSS. This corresponded with the peak of weight loss and the height of acute inflammation **(Figure 1)**. Restoration of weight began starting at 9 days post-DSS and was normalized by 14 days post-DSS **(Figure 1)**. Colon length measured at 8 days post-DSS was significantly reduced compared to control colons, with length significantly improved by 14 days post-DSS **(Figure 2)**. Colons collected on day 8 and day 14 were processed for histological analysis by staining with hematoxylin and eosin (H&E) and were analyzed for signs of damage and inflammation **(Figure 3)**. Extent of the DSS-induced damage was quantified by a histological damage score which evaluated loss of tissue architecture, inflammatory infiltrate, presence of crypt abscesses, and several other pertinent criteria. This was done under conditions where the observer was blind to the treatment group and performed by myself and another researcher in the laboratory in order to minimize bias. As expected, mice at day 8 post-DSS had a higher histological damage score relative to water controls and by day 14, recovery of colonic damage was observed **(Figure 3),** although not completely normalized.

Mice administered DSS display anxiety-like behavior

Using the light/dark box test, anxiety-like behavior was assessed in mice at 8 and 14 days post-DSS. Mice studied at the peak of DSS-induced inflammation displayed anxiety-like behavior compared to water controls (**FIGURE 4**). Anxiety was assessed by measuring the amount of time spent in the lit portion of the box, as mice have an innate preference for dark versus light but are also inherently exploratory. Mice subjected to DSS-induced colitis spent significantly less time in the light relative to controls. **(Figure 4A)**. The anxiety-like behavior observed at peak of inflammation (day 8) and was normalized by 14 days post-DSS **(Figure 4A)**. The number of transitions from the dark portion of the box to the lit portion was quantified and used as an indicator of general health and behavior. The frequency of transitions was found to be similar between groups, indicating that overall activity of the mice was not affected by colitis and suggesting that presence of sickness behavior wasn't affecting general behavior, potentially confounding the anxiety-like behavior we observed.

DSS administration is associated with memory deficits

Recognition memory was assessed following exposure to DSS using the novel object recognition (NOR) test. The NOR test evaluates the mouse's ability to recall a familiar object that it was previously exposed to when presented with a novel object. An exploration ratio was calculated, which quantifies the number

of times mice explore a new object relative to the number of times it explores both objects. Thus, a higher exploration ratio signifies that the mouse recalled the familiar object. Mice administered DSS displayed deficits in recognition memory as indicated by a decrease in exploration ratio compared to controls **(Figure 5)**. Impairments observed at day 8 post-DSS were normalized by day 14 post-DSS as demonstrated by similar exploration ratios compared to the control group **(FIGURE 5)**.

DSS administration results in shifts of the composition of the gut microbiota

The composition of the microbiota was assessed by qPCR of the 16S rRNA genes of several bacterial targets. Relative abundance was quantified based on normalization to total bacteria by measuring *Eubacteria*, a universal bacterial primer. Relative to controls, the proportion of *E. rectale*, *Bacillus, Lactobacillus*, and segmented filamentous bacteria (SFB) decreased at 8 days post-DSS compared to the control group. By 14 days post-DSS, levels of *E. rectale*, *Bacillus,* and *Lactobacillus* were normalized while a partial recovery of SFB was observed. In contrast, *Bacteroides, Enterobacteriaceae,* and *Firmicutes* were not affected by DSS administration **(Figure 6)**.

Administration of probiotics reduces colonic damage following DSS-induced colitis

Given their beneficial effect in other models of colitis and intestinal inflammation, probiotics (*L. rhamnosus* and *L. helveticus*) were administered daily by oral swabbing starting one week prior to commencing DSS-induced colitis and continued for the duration of the experiment. DSS mice that received the probiotic regimen showed a reduction in weight loss relative to DSS treated mice administered a placebo **(Figure 7)**. This difference was particularly striking at 7 to 8 days post-DSS, which corresponds to the height of disease. Consistent with our observations in weight changes, colonic shortening was also improved in DSS-induced mice treated with probiotics compared to mice administered a placebo **(Figure 8)**. Significant colonic shortening was still observed in the DSS + probiotics group relative to probiotics- and placebo-alone control groups. Similarly, no difference in colon length was observed between placebo- and probiotic-alone treated groups. In terms of colonic damage, the DSS + placebo group had a significantly higher histological damage score compared to DSS + probiotics group indicating greater extent of disease **(Figure 9)**. As expected, both DSS + placebo and DSS + probiotic groups had substantially higher damage scores than both placebo- or probiotics-alone groups.

Probiotics administration reduced anxiety-like behavior and cognitive deficits seen in DSS-induced colitis

Anxiety-like behavior, assessed as previously described using the light-dark box test, showed that anxiety-like behavior in DSS-induced colitis was ameliorated following administration of probiotics compared to placebo **(Figure 10A)**. However, placebo administered mice exhibited greater anxiety-like behavior than probiotics-treated groups. The number of transitions between all four groups were not significantly altered, indicating that all mice displayed similar levels of overall activity **(Figure 10B)**.

The NOR test was again used to determine whether administration of probiotics could rescue cognitive deficits observed in mice subjected to DSS-induced colitis. **(Figure 11)**. Administration of probiotics starting one week prior to DSS was able to normalize cognitive deficits associated with DSS-induced colitis, compared to placebo treated controls. No significant difference was observed between placebo-alone, probiotics-alone, and DSS + probiotics treated groups.

Probiotic administration normalized c-fos expression in the CA-1 region of hippocampus

Expression of the immediate early gene, c-fos, serves as a surrogate marker of neuronal activity; in the CA-1 region of the hippocampus, this is correlated with spatial and recognition memory(53). Previous work has demonstrated that probiotics have the potential to increase c-fos expression in the brain(25). Greater c-fos expression correlates to greater neuronal activity in

the region of interest. Using immunohistochemistry staining techniques on formalin-fixed, paraffin embedded, coronal brain sections, the number of c-fos positive cells was quantified as a percent of the total number of cells in the field. Mice administered DSS + placebo had a decrease in c-fos expression relative to the placebo-only and probiotics-only groups **(Figure 12)**. In contrast, administration of probiotics to DSS mice was able to normalize c-fos expression to levels similar to those seen in the placebo-only group.

Probiotic administration results in partial normalization of the microbiota

The microbiota was assessed in all placebo- and probiotic-treated groups with administration of *L. rhamnosus* and *L. helveticus* able to partially restored the microbiota. Relative to placebo only treated groups, levels of *E. rectale, Lactobacillus,* and *Bacillus* were found to be decreased in DSS + placebo treated groups **(Figure 13)**. Levels of *E. rectale, Lactobacillus,* and *Bacillus* were increased in DSS + probiotics relative to DSS + placebo group. In all groups, levels of *Enterobacteriaceae, Firmcutes,* and *Bacteroides* were found to be not significantly different between treated groups **(Figure 13)**. SFB was observed to significantly increase in probiotic treated alone but not in the DSS + probiotic group **(Figure 13)**. Overall, administration of probiotics shifted the composition of the microbiota relative to placebo administered groups.

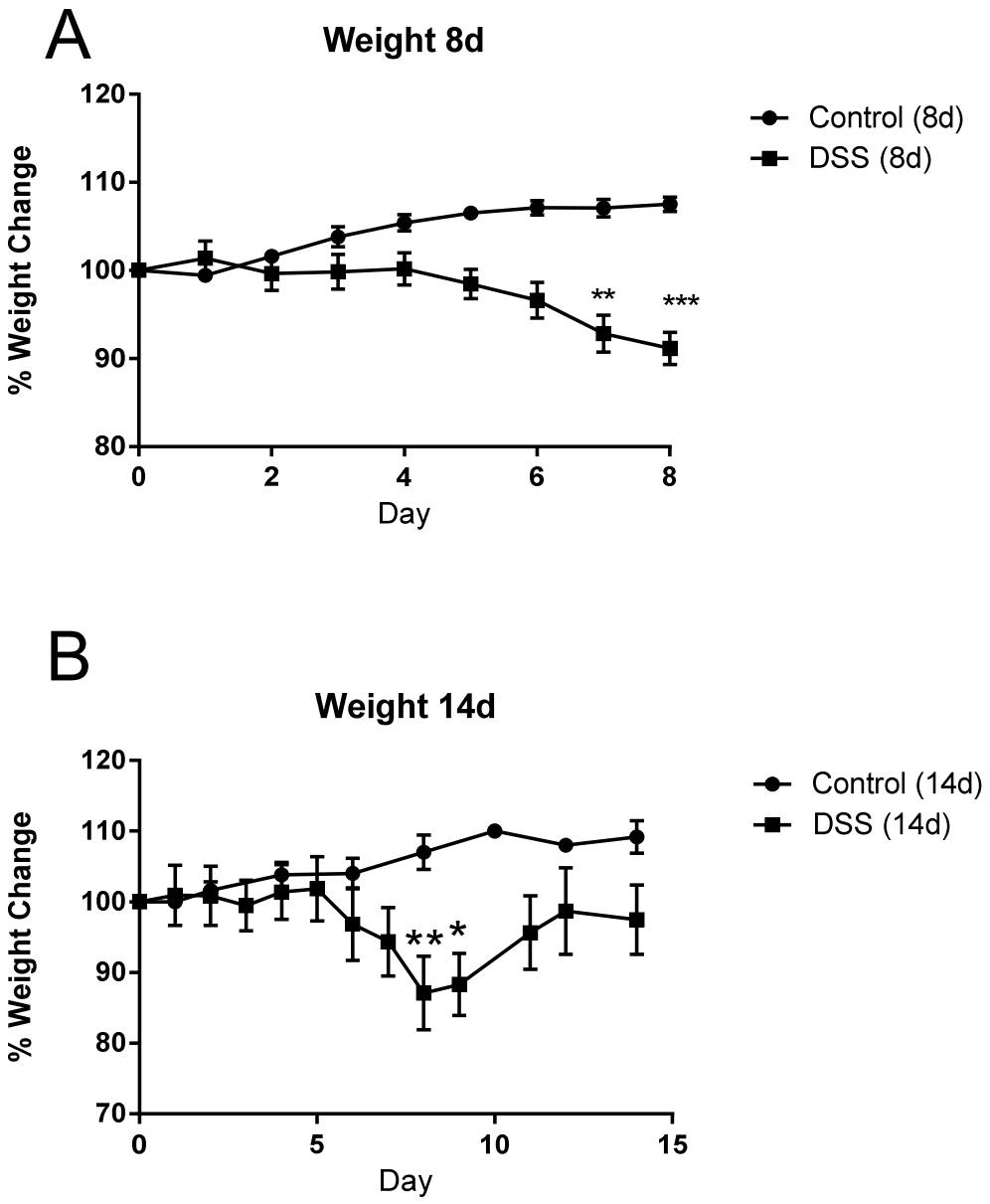


Figure 1: Percent weight change in the murine DSS colitis model. Administration of DSS (3% w/v) in the drinking water resulted in severe weight loss. (**A**). Peak of weight loss was seen at 8 days post-DSS and recovery began at 9 days post-DSS, continuing until 14 days post-DSS (**B**). (Error = ± SEM, **p< 0.01, *p<0.05 via Student's t-test. N=14-16.)

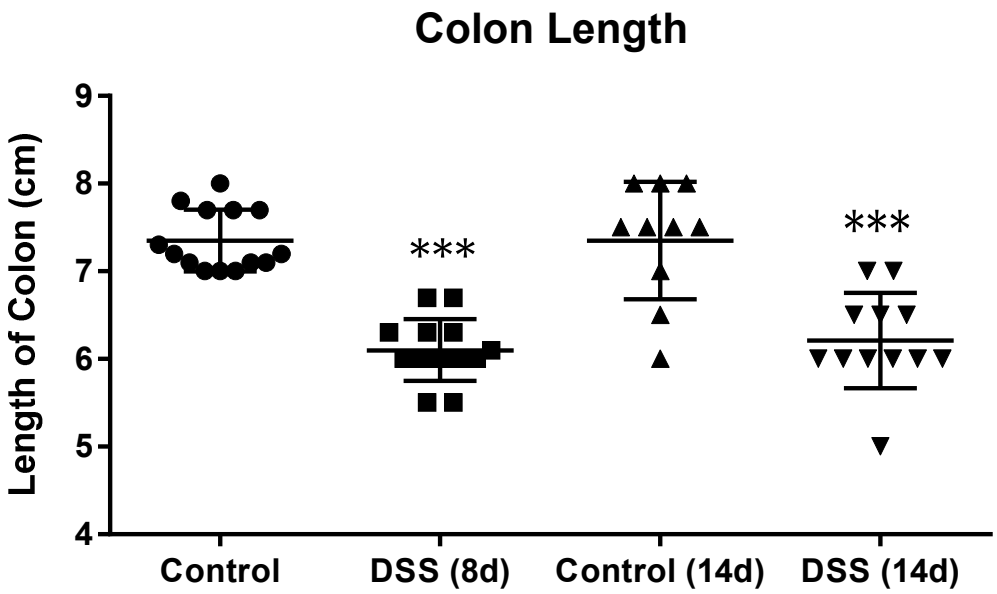


Figure 2: DSS-induced colonic shortening in mice. Administration of DSS (3% w/v) in the drinking water for 5 days resulted in colonic shortening by 8 days, which was not resolved by 14 days post-DSS. (***$p<0.001$ by one-way ANOVA compared to respective controls. N = 8-14.)

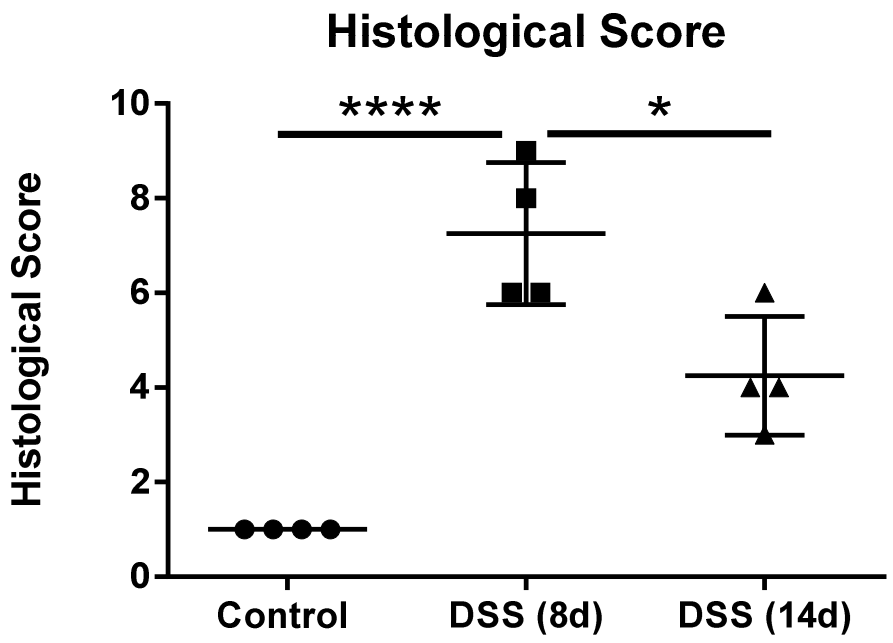


Figure 3: DSS-induced colonic damage in mice. Administration of DSS (3% w/v in the drinking water for 5 days) resulted in severe colonic damage which was quantified as an increase in histological damage score. (*$p<0.05$, ****$p<0.0001$ by ANOVA. N=4.)

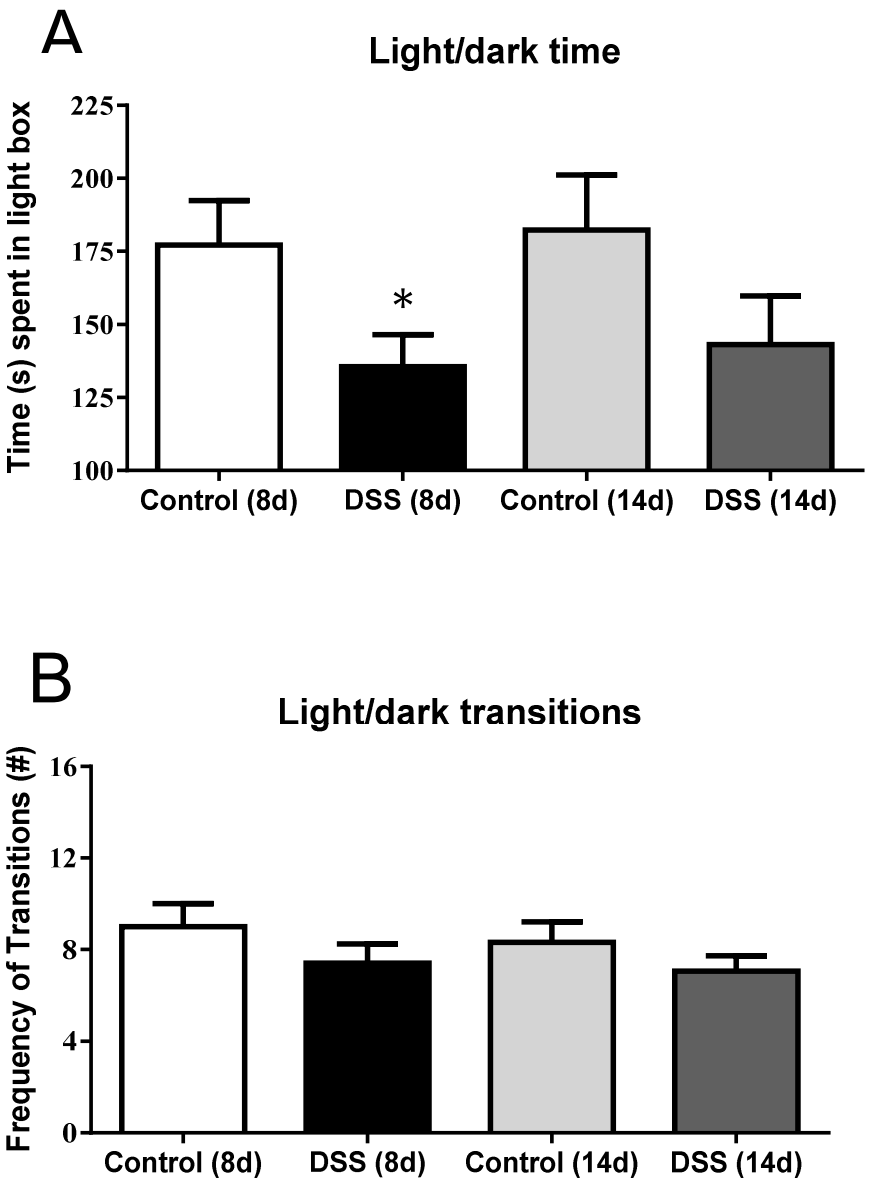


Figure 4: Anxiety-like behavior in mice administered DSS. Administration of DSS (3% w/v in drinking water for 5 days) resulted in increased anxiety-like behavior at 8 days post-DSS which was normalized by 14 days (**A**). Similar number of transitions were seen between all groups (**B**). (*p<0.05 ANOVA compared to control. N=10-12.)

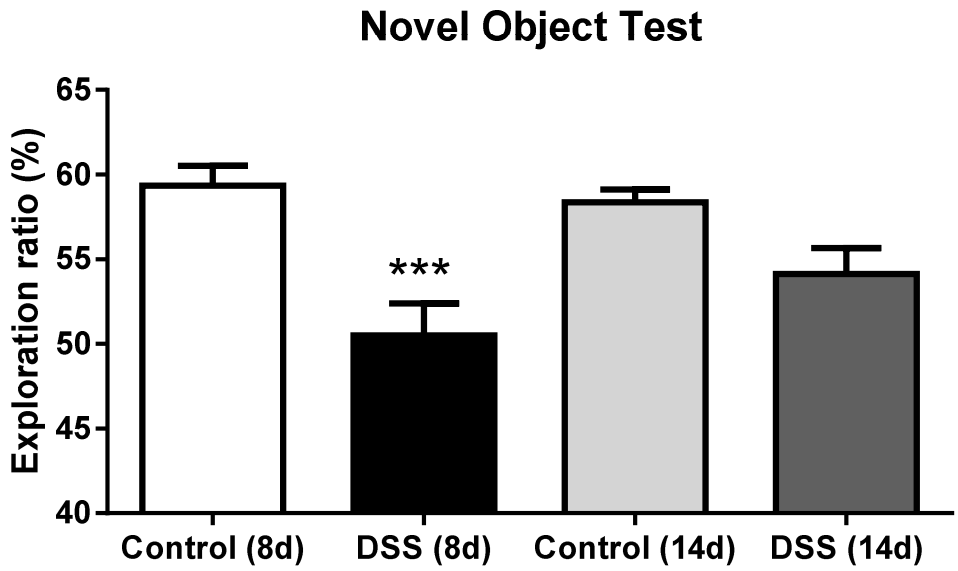


Figure 5: Recognition memory dysfunction in mice administered DSS. Memory impairment was observed at 8 days post-DSS as seen through a significantly lower exploration ratio. The exploration ratio quantifies the number of times a mouse investigates the novel object relative to the familiar object. Memory improved by 14 days post-DSS. (***$p<0.001$ via one-way ANOVA compared to respective controls. N =10-12)

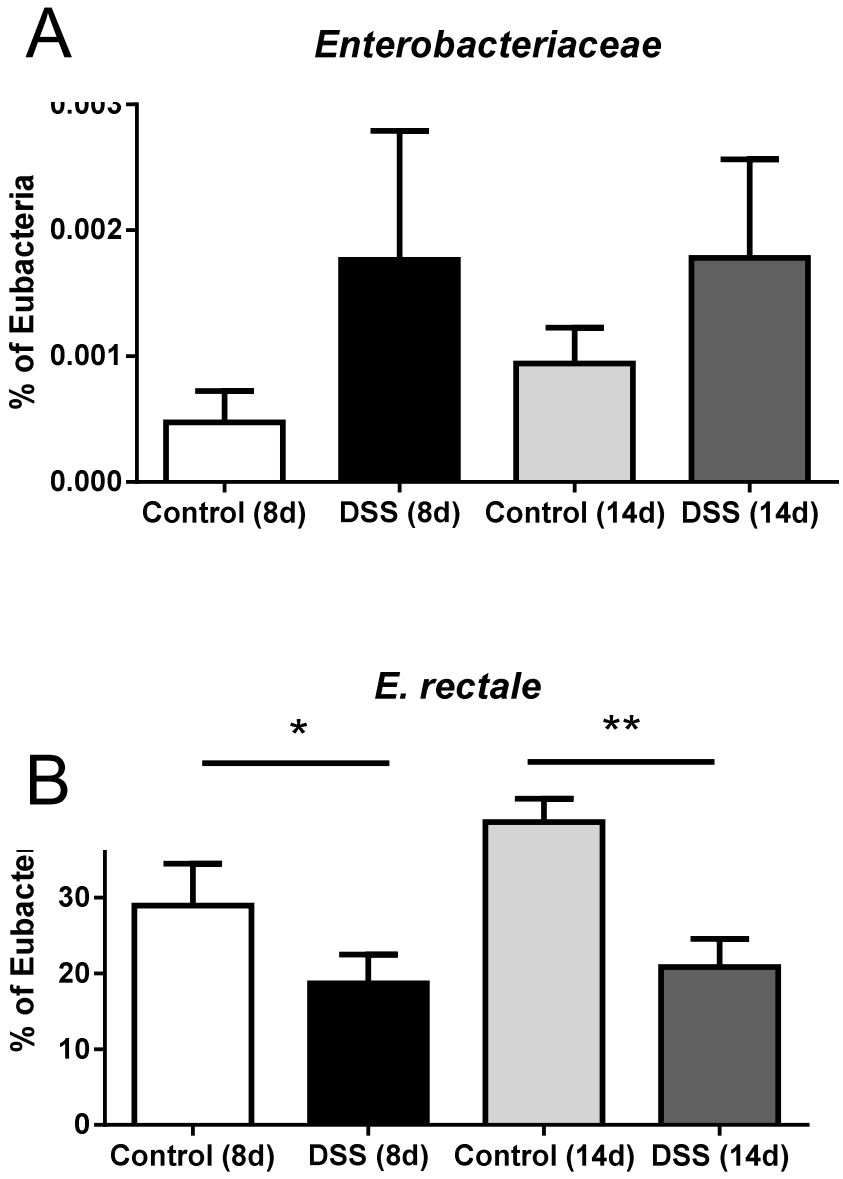
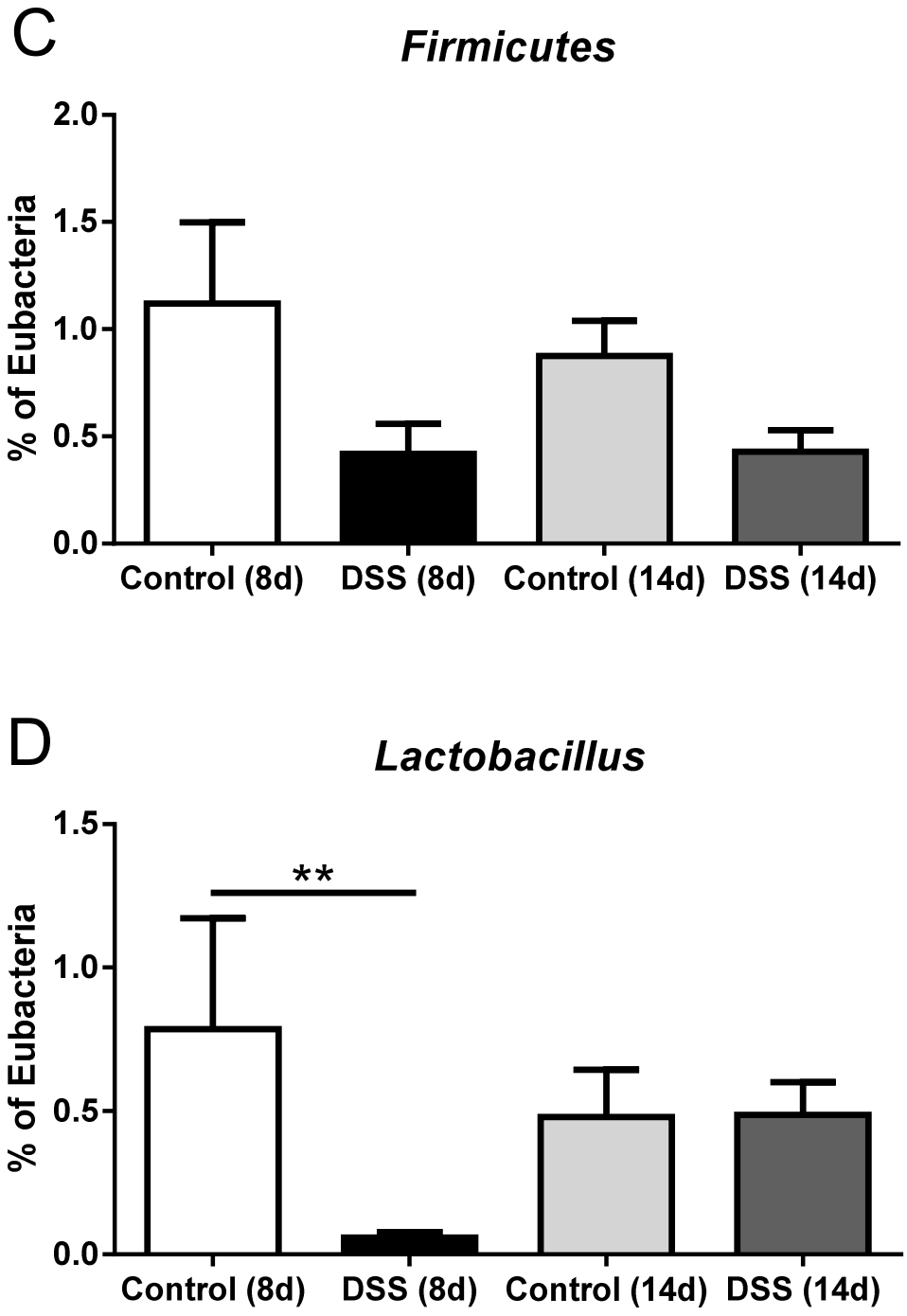


Figure 6: Alterations in the composition of the gut microbiota in mice following administration of DSS. Levels of *E. rectale* (**B**), *Lactobacillus* (**D**), *Bacillus* (**E**), and SFB (**F**), significantly decreased at 8 post-DSS. The changes in levels of *E. rectale* (**B**), *Lactobacillus* (**D**), *Bacillus* (**E**), and SFB (**F**) were normalized by 14 days post-DSS. Levels of Enterobacteriaceae (**A**) Firmicutes (C), and *Bacteroides* (**G**) levels were not impacted by DSS administration. (*p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA. N= 4-8.)



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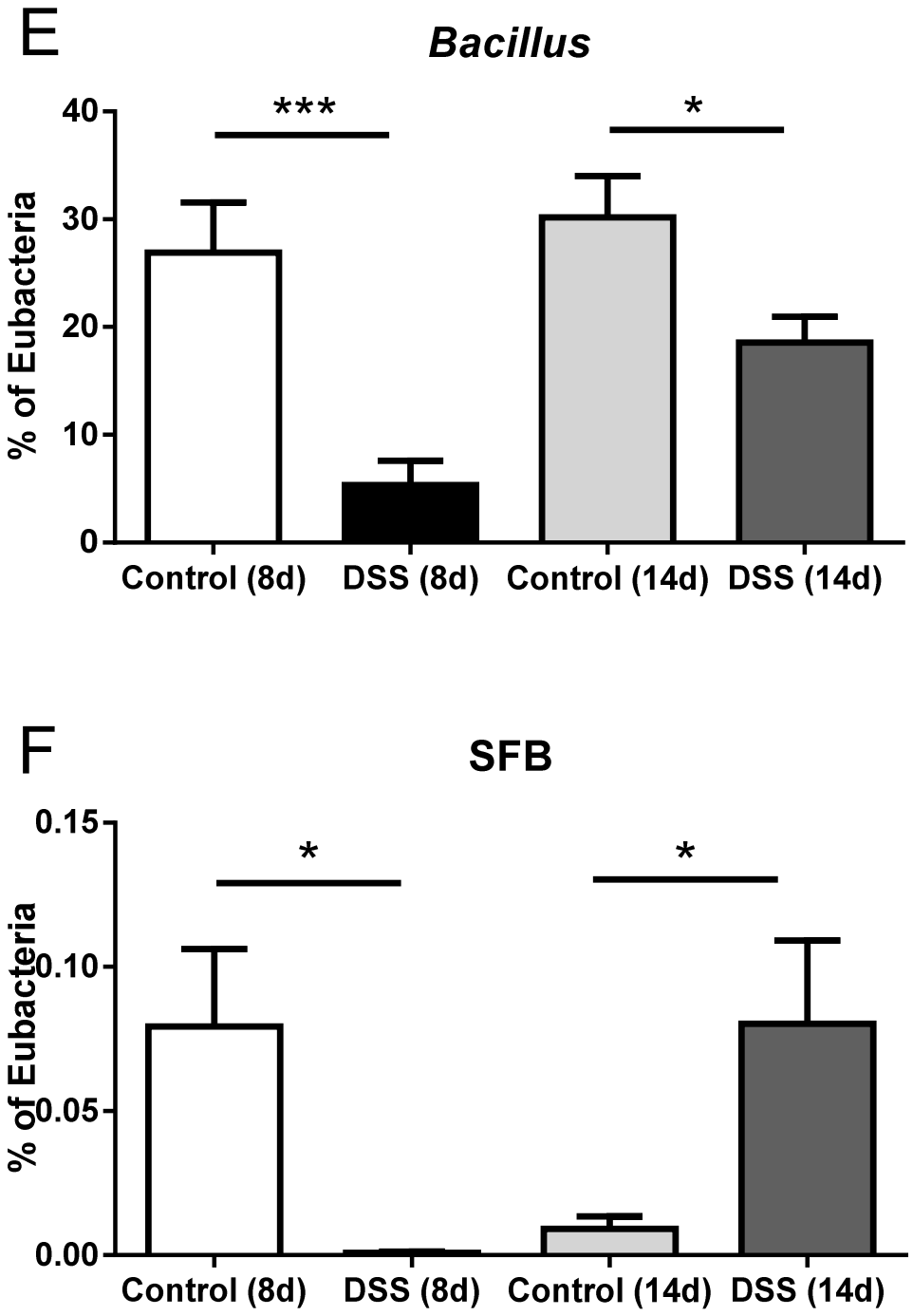

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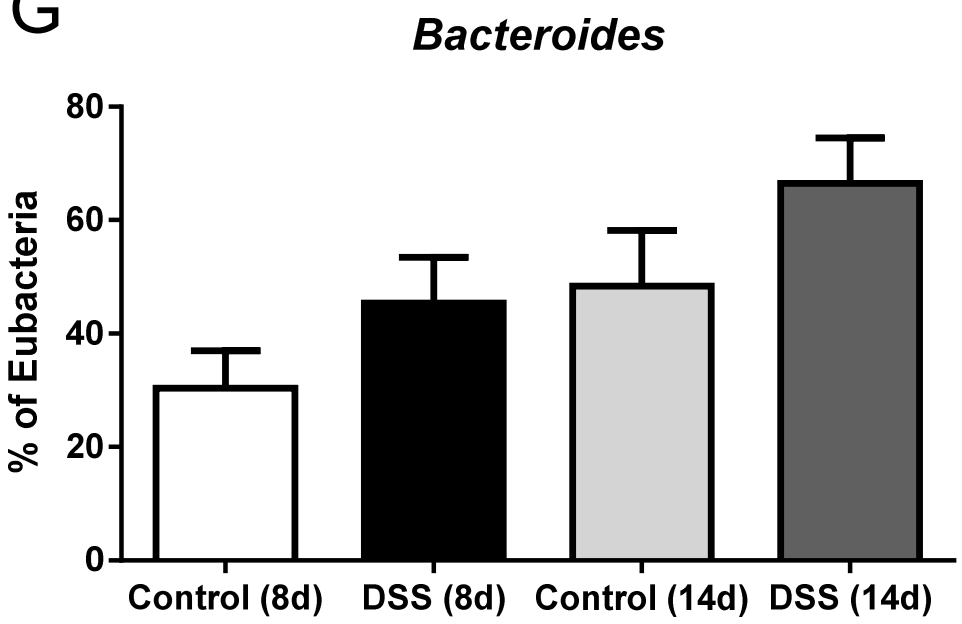

Bacteroides
% of Eubacteria
80
60
40
20
0
Control (8d)
DSS (8d)
Control (14d)
DSS (14d)

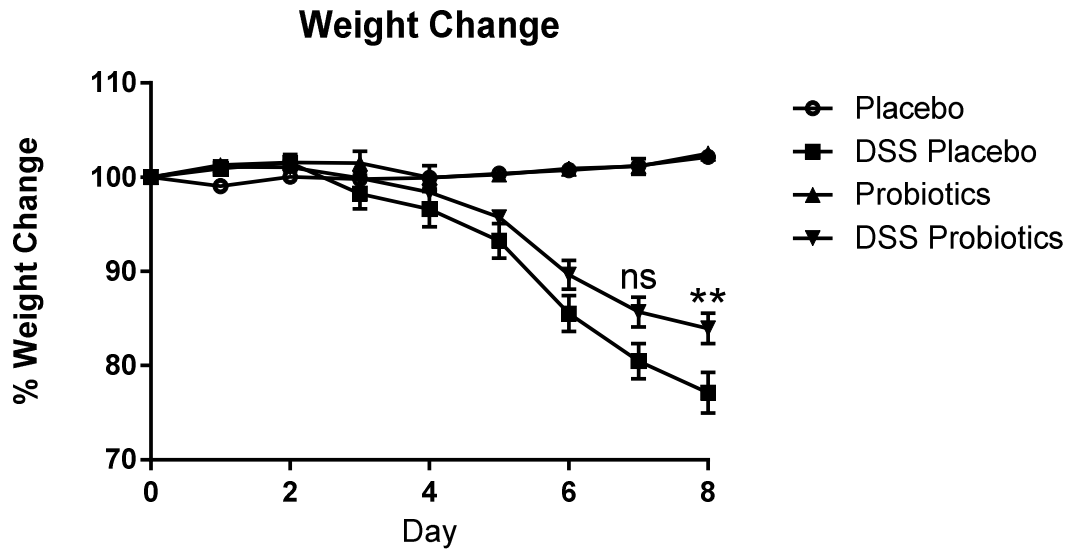


Figure 7: Percent weight change following probiotic administration to mice subjected to DSS colitis. Administration of probiotics attenuates weight loss in DSS (3% w/v in drinking water for 5 days). Day 0 correlates to start of DSS regimen. Mice administered DSS + probiotics experienced significantly less weight loss compared to DSS + placebo group. No difference in weight observed between groups given only placebo and probiotics. (**$p<0.01$ compared to DSS + placebo via one-way ANOVA, N=4-8.)

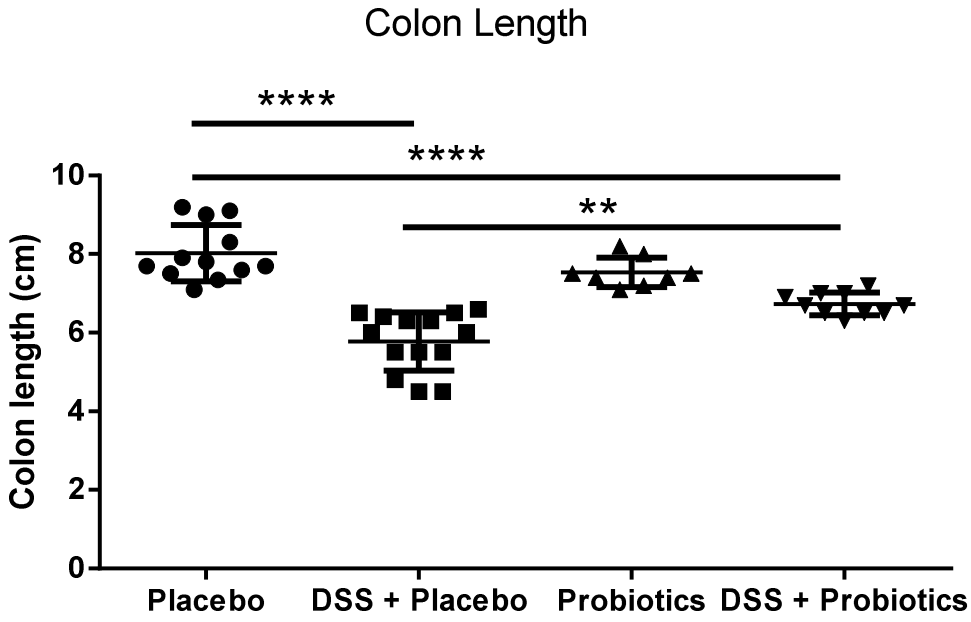


Figure 8: Colonic shortening was attenuated by administration of of probiotics to mice subjected to DSS colitis. Administration of probiotics decreases colonic shortening associated with administration of DSS at day 8 (3% w/v in drinking water for 5 days) (****p<0.0001, **p<0.01 via ordinary one-way ANOVA. N= 8-14.)

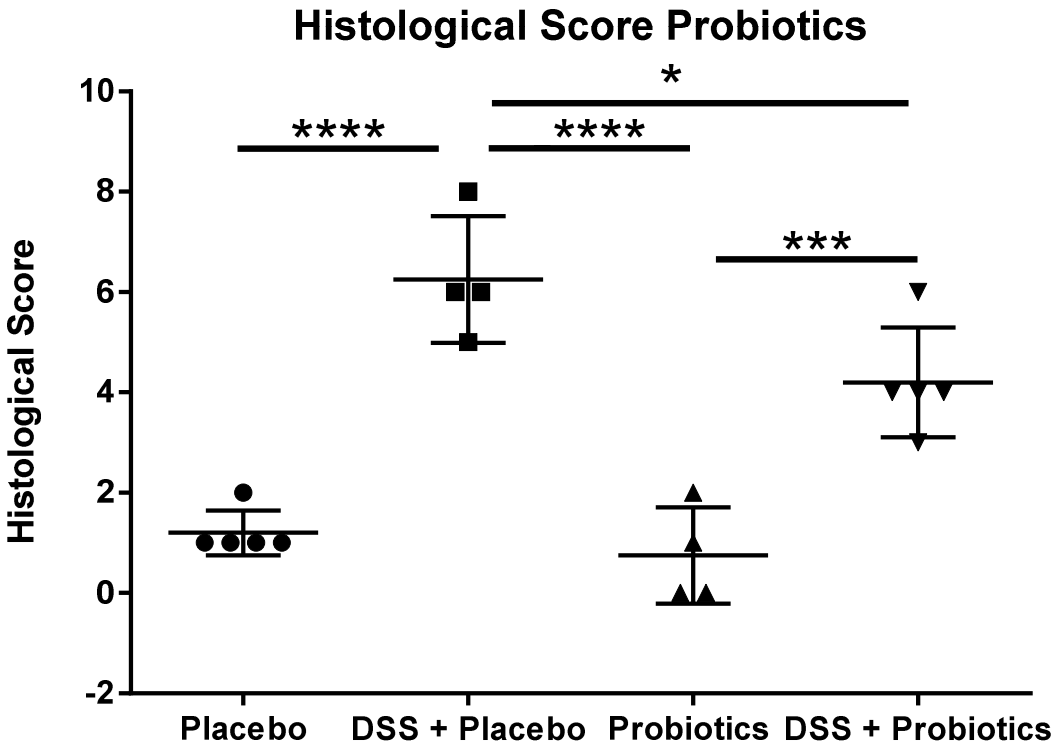


Figure 9: Administration of probiotics reduces colonic damage in mice subjected to DSS colitis. Administration of probiotics decreases colonic damage associated with administration of DSS at day 8 (3% w/v in drinking water for 5 days)(****p<0.0001, ***p<0.001, *p<0.05 via one-way ANOVA. N = 4-5.)

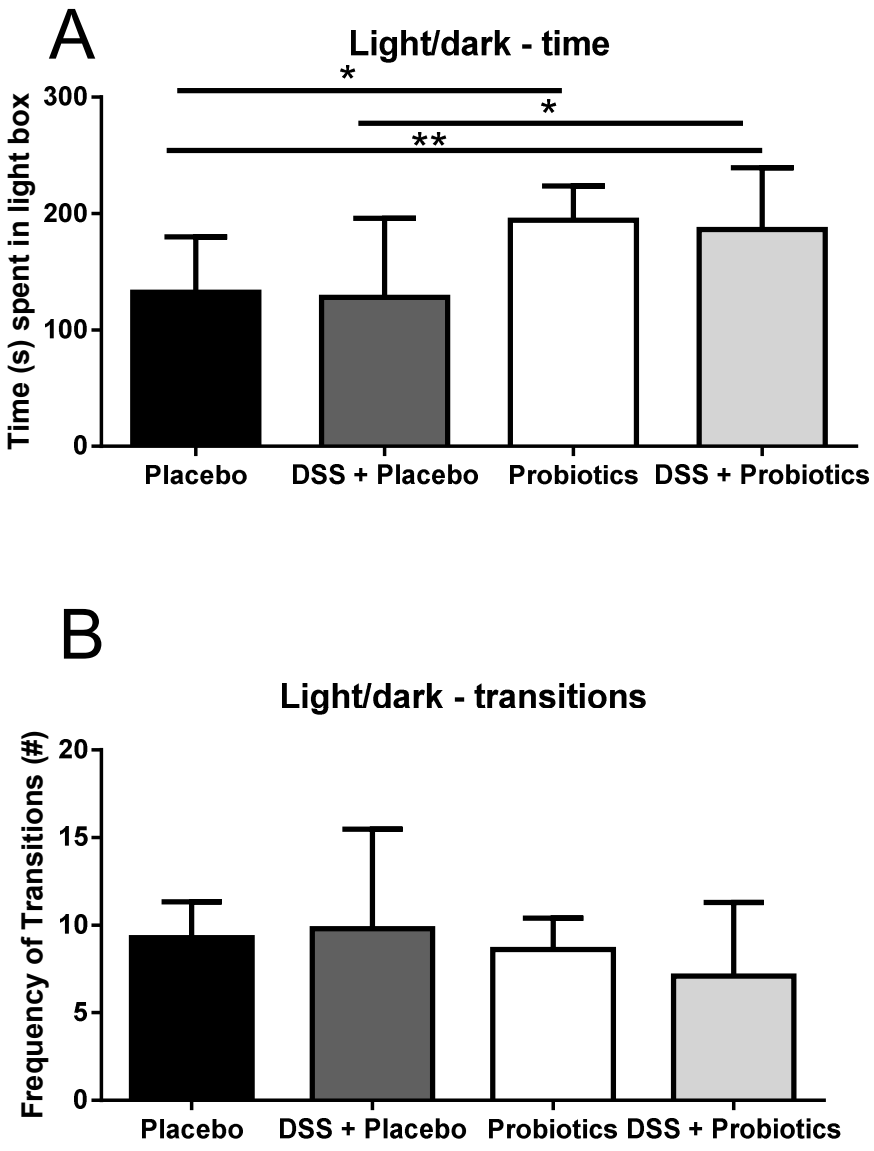


Figure 10: Probiotics decrease anxiety-like behavior in mice subjected to DSS colitis. Administration of probiotics decreases anxiety-like behavior in DSS (3% w/v in drinking water for 5 days) treated mice (**A**). Total activity of mice was not affected by DSS exposure or probiotic administration (**B**). (**p<0.01, *p<0.05 via one-way ANOVA. N=4-8.)

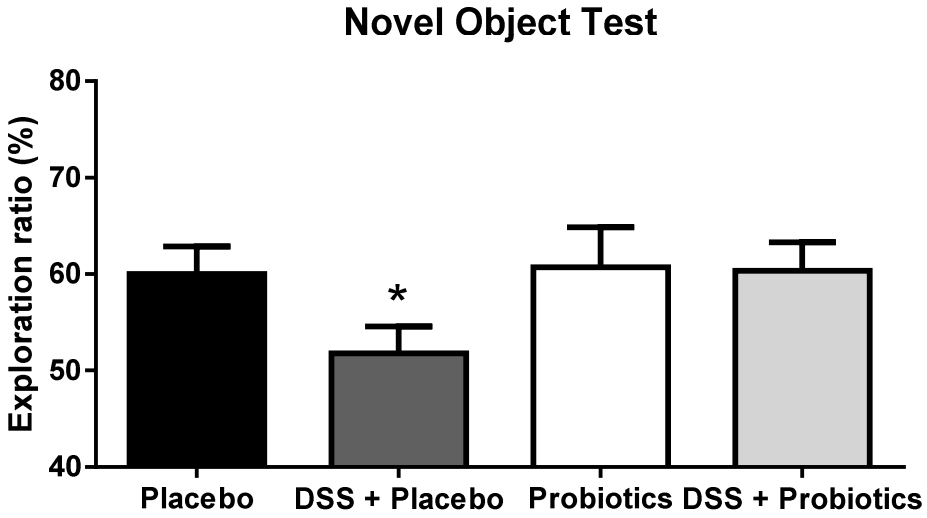


Figure 11: Probiotics improve recognition memory in DSS-treated mice. Administration of probiotics normalized the memory deficit associated with DSS-colitis (3% w/v in drinking water for 5 days). (*$p<0.05$ via one-way ANOVA. N= 7-10.)

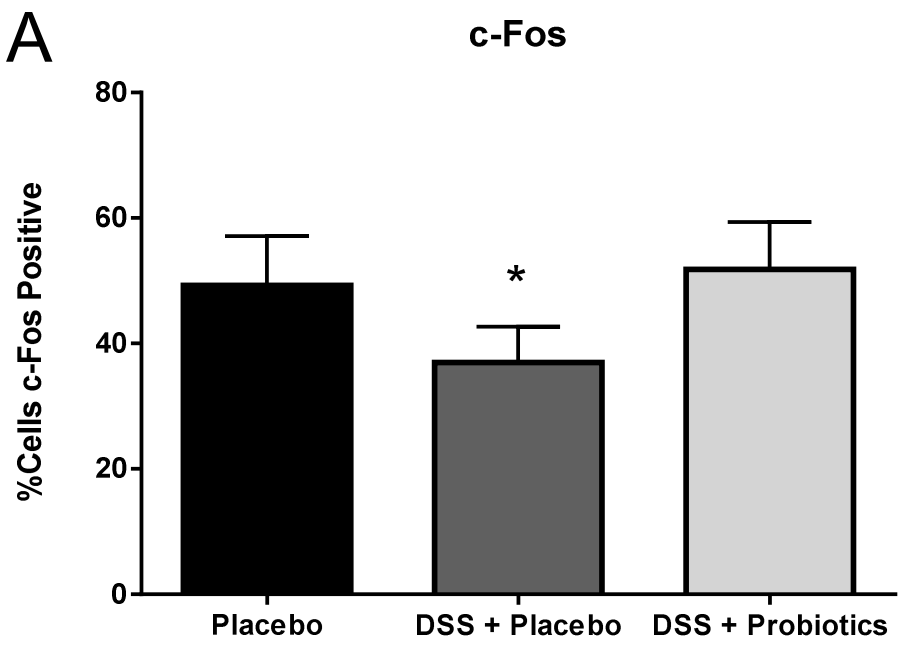
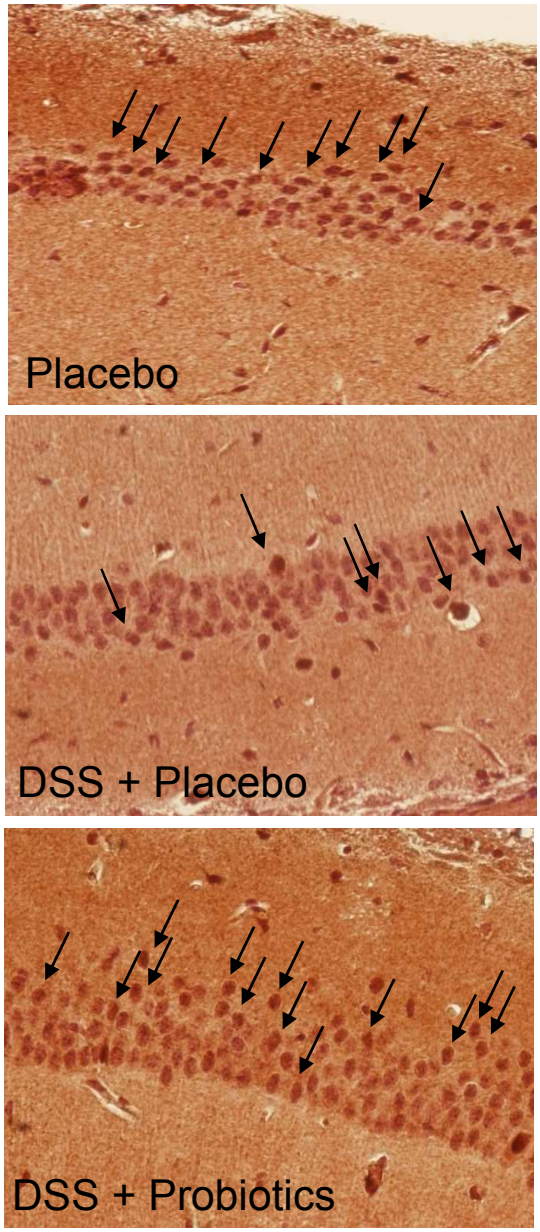


Figure 12: c-Fos expression in the CA-1 region of the hippocampus in mice subjected to DSS colitis. Administration of probiotics normalizes c-Fos expression in DSS (3% w/v) treated mice. Quantification of number of c-fos positive cells per total cells in view (**A)**. Representative images with c-fos positive cells indicated (**B**). (*$p<0.05$ via one-way ANOVA. N=4-5.)

B



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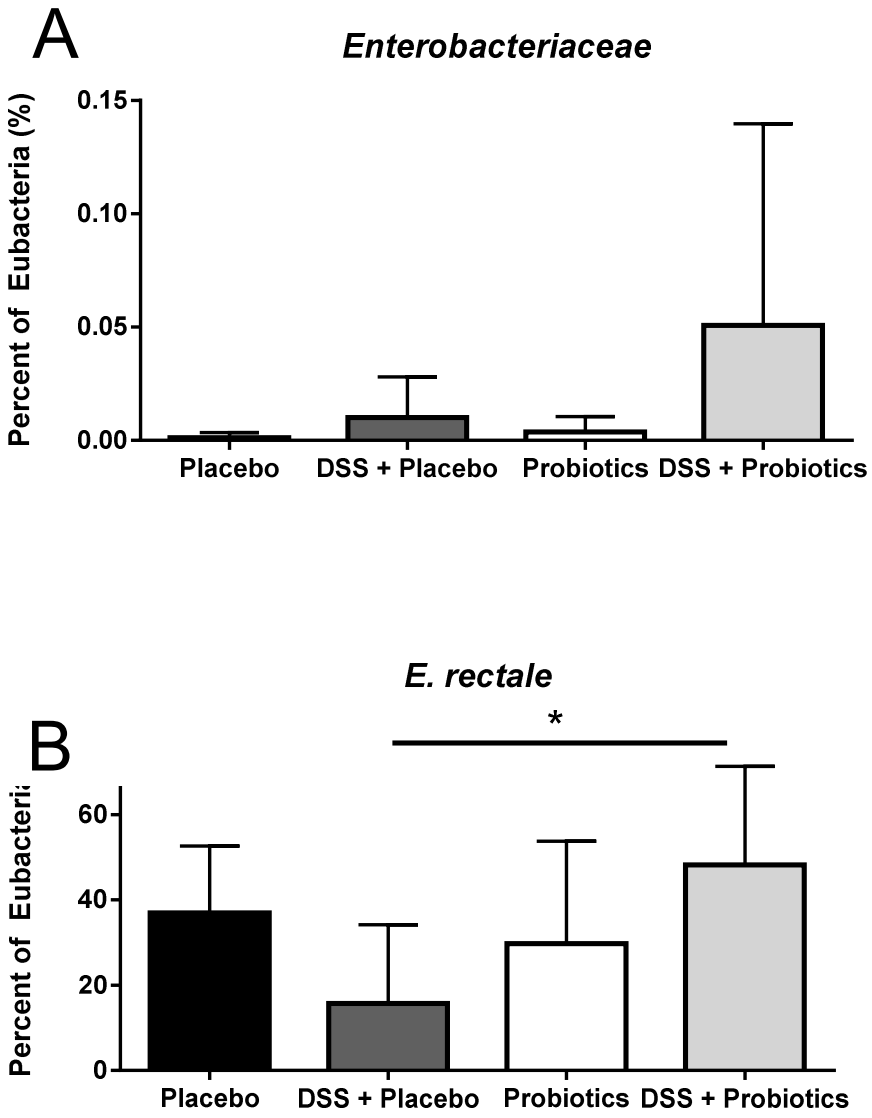
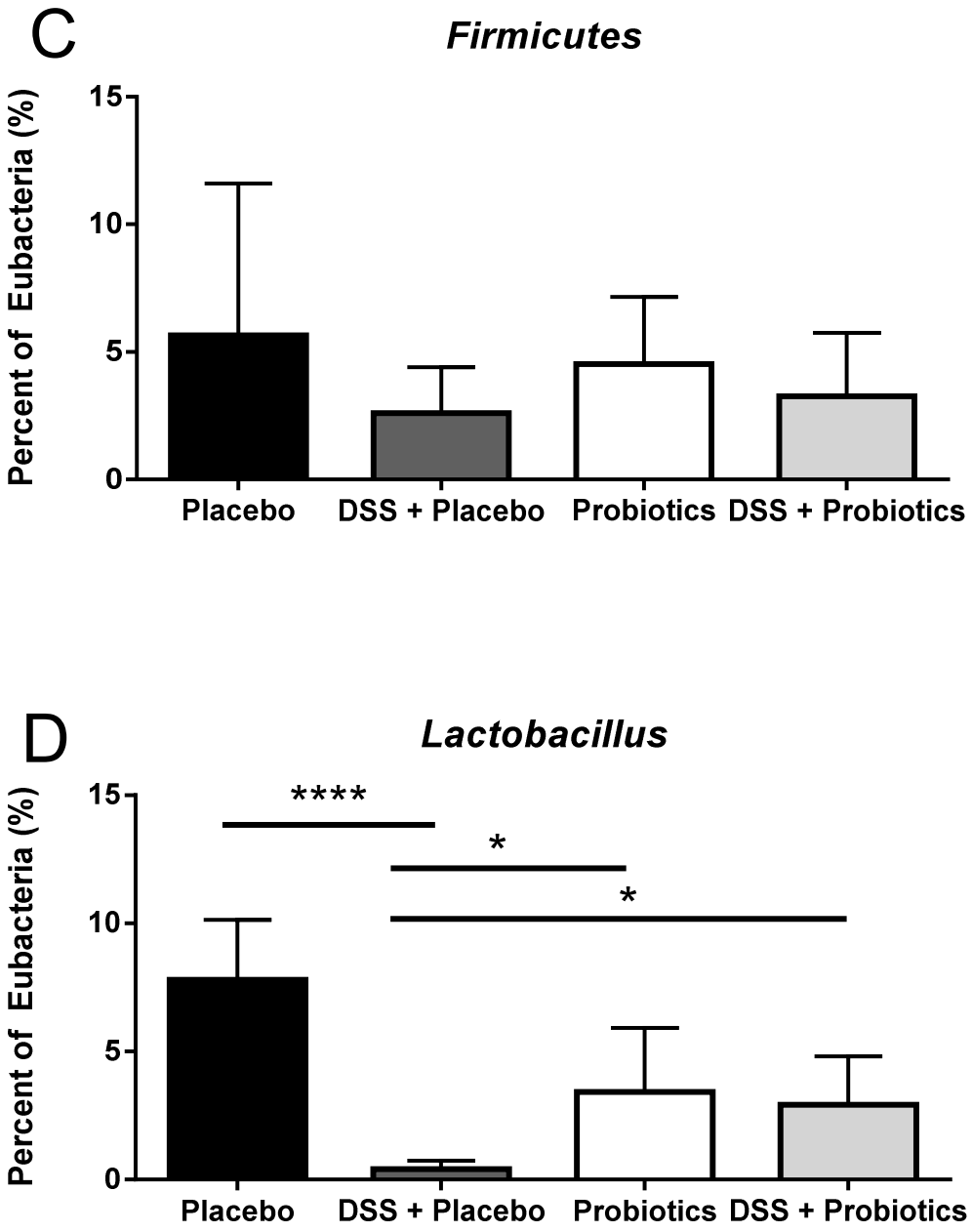


Figure 13: Administration of probiotics alters the gut microbiota composition in mice subjected to DSS colitis. Levels of *E. rectale* (**B**), *Lactobacillus* (**D**), *Bacillus* (**E**) and SFB (**F**) significantly decreased in the DSS + placebo group (3% DSS w/v in drinking water for 5 days). Levels of *E. rectale* and *Lactobacillus* (**D**) was normalized by administration of probiotics. Decreased levels of *Bacillus* in the DSS administered group were restored by administration of probiotics. The proportion of *Enterobacteriaceae* (**A**), *Firmicutes* (**C**), *Bacteroides* (**G**), was not impacted by administration of DSS or DSS+probiotics. (*p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA. N= 4-8.)



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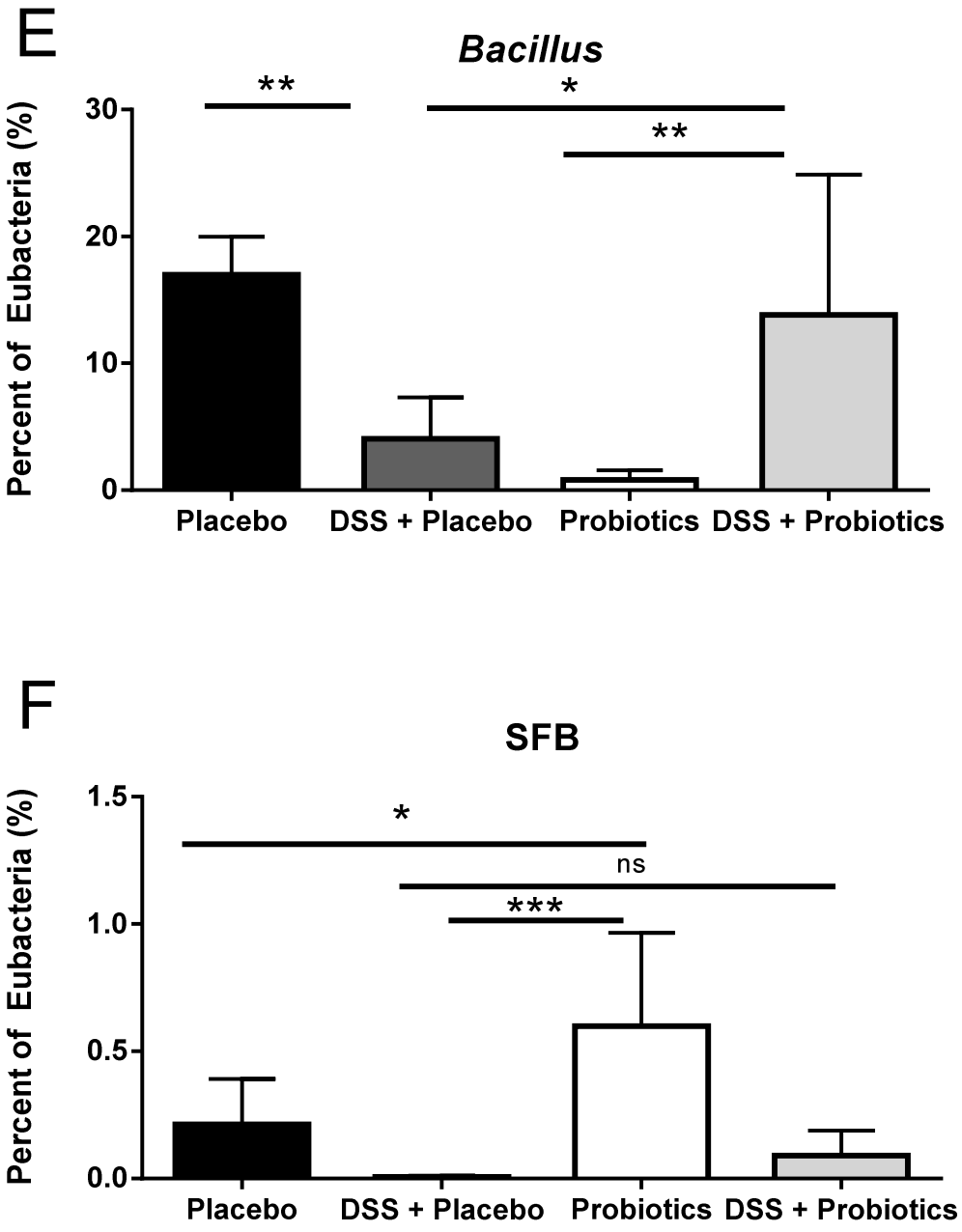

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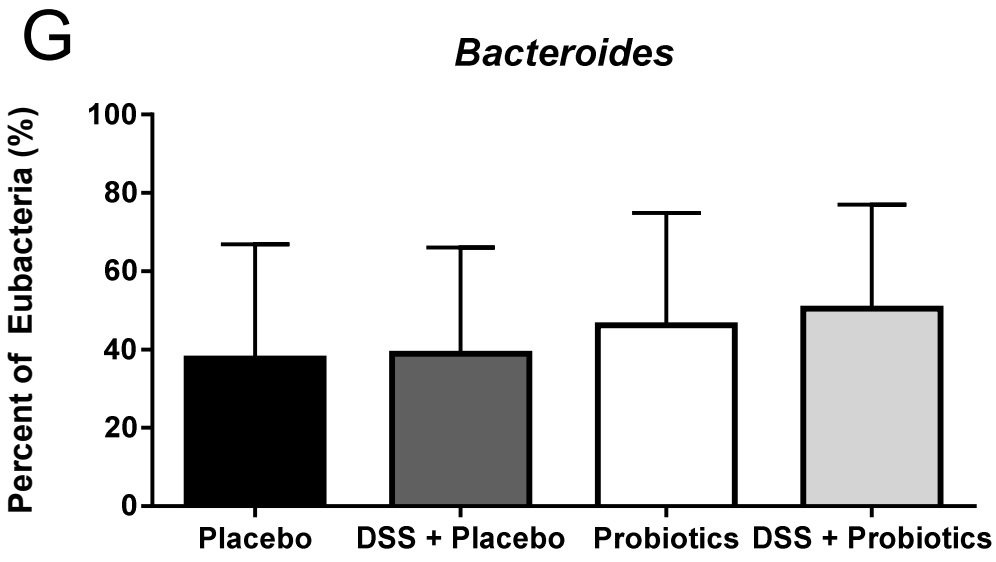

Figure 13. continued

I would like acknowledge Jacob Emge who obtained preliminary data as well as Kim Barrett and Melanie Gareau who oversaw this project.

DISCUSSION

Using the DSS-induced mouse model of acute colitis we have demonstrated the presence of cognitive deficits as well as anxiety-like behavior during colonic inflammation, compared to control mice. These changes in behavior were reversed, along with resolution of disease, by 14 days post-DSS. Administration of a Lactobacillus-containing probiotic cocktail, starting one week prior to induction of DSS and continued thereafter, was able to correct behavioral deficits as well as confer protection against DSS-induced colitis. Taken together, these studies suggest that acute colitis leads to transient behavioral deficits that correlate with the presence of colonic disease.

An increase in anxiety is often found in patients with IBD compared to both healthy controls and patients with non-gastrointestinal diseases (54). In mice, the light dark box test can be used as a means to assess anxiety-like behavior by quantifying the amount of time each mouse spent in the lit portion of the box, with decreased time a reflection of increased anxiety (52). Mice administered DSS exhibited anxiety-like behavior when compared to controls, spending less time in the light box. This anxiety-like behavior was normalized at day 14 corresponding with resolution of disease. Previous studies using different gastrointestinal injury models have demonstrated similar results. In a study using an infectious model, the *Trichuris muris* parasite, mice were found to exhibit anxiety-like behavior (55). Similarly, in a study using a chronic model of IBD, consisting of 3 cycles of DSS, mice were also shown to exhibit increased anxiety-like behavior (56). In addition to calculating time, the number of transitions between the light/dark compartments was also quantified in the

test as an indicator of overall activity. Similar frequencies of transitions were observed between the DSS administered groups and controls groups, suggesting that administration of DSS did not impair overall activity levels of the animals, leading to sickness behavior and explaining in the behavior deficits observed. Similar associations between inflammation and mood disorders are also being observed in a clinical setting (43). Patients who had lifelong anxiety disorder reported higher rates of medical illnesses compared to those without anxiety (57). Patients with chronic inflammatory illnesses, such as multiple sclerosis, are associated with increased anxiety levels (58). Thus the immune system is postulated to play a role in modulating mood and anxiety in a disease setting.

In addition to behavioral changes, cognitive deficits are also known to occur in settings of chronic disease. Consequently, an increasing interest exists in characterizing cognitive function in pre-clinical disease models. The NOR test is a well-established behavioral test that evaluates recognition memory in mice (51). Acute administration of DSS in mice was associated with deficits in recognition memory, as quantified through a decrease in exploration ratio compared to controls. Impairments to memory were normalized by day 14, concurrent with resolution of colonic disease. This suggests that colonic inflammation may lead to short-term cognitive deficits, in the setting of colitis, and a more thorough evaluation of cognitive function is therefore warranted.

The gut microbiota is increasingly being found to play a role in physiology and disease outside of the local gut environment, including

modulating behavior and memory. Previous work has linked the microbiota GF mice are a useful tool to study physiology in the absence of a microbiota, in a sterile environment. Consequently, mice that are raised GF exhibit reduced anxiety-like behavior compared to SPF mice (59). This suggests that the commensal microbiota are important in regulating processes that govern anxiety. GF mice also have a reduced mucosal immune system in the absence of a microbiota, which has been associated with alterations in behavior (60). Previous work studying gut inflammation and the microbiota, using IL-10$^{-/-}$ mice that spontaneously develop IBD, demonstrated that a pro-inflammatory environment can cause shifts to the composition of the microbiota (61). In the same IL10$^{-/-}$ mice, these shifts in the microbiota were associated with an impairment in memory as measured using a Barnes maze (62).

Behavioral defects observed in the setting of IBD are likely due to a combination of dysbiosis and concurrent inflammation. The presence of inflammation in the gastrointestinal tract increases barrier permeability, allowing passage of bacterial effectors such as LPS, which can elicit a strong pro-inflammatory response, perpetuating this deficit. The translocation of LPS into systemic circulation contributes to systemic-wide inflammation and is associated behavioral changes (63). These changes have been observed in studies in which LPS, when administered systemically, enhanced sickness behaviors and increased depression-like behavior (64). Moreover, studies analyzing serum concentrations of IgM and IgA against LPS have shown that patients with major depression have significantly higher concentrations of

antibodies to LPS suggesting greater translocation of LPS into systemic circulation (65). Whether the presence of LPS or the host immune response to the LPS is involved in the mechanisms seen following behavioral changes in DSS is still uncertain. In this context, our study demonstrates that DSS induced colitis can precipitate behavioral changes which is likely mediated by the microbiota and inflammation.

In the current studies, the microbiota was assessed through qPCR analysis of 16S rRNA extracted from fecal samples. Previous work from our group demonstrated that bacterial infection with the enteric pathogen *C. rodentium* induced changes in the composition of the microbiota of mice, which also demonstrated behavioral deficits following exposure to acute stress (36). Employing the same technique for analysis of fecal samples from DSS-treated mice, we observed significant changes in the composition of the microbiota compared to controls. In particular, SFB and *Lactobacillus* were identified to be significantly decreased following DSS administration in mice and were both recovered by disease resolution, similar to the changes seen in behavior.

Decreased levels of *Lactobacillus* were also observed at height of DSS-induced colitis compared to control mice. Many species of *Lactobacillus* are commonly used in the production of fermented products such as yogurts and kefir and many have probiotic, or beneficial bacterial, properties. This genus consists of rod shaped, gram-positive, and non-sporulating bacteria that are characterized by their ability to ferment lactose, producing lactic acid(66). In this study, *Lactobacillus* decreased significantly at the peak of inflammation but was

restored by day 14 post-DSS. *Lactobacillus* has previously been shown to affect cognition and protect against IBD. Administration of *L. helveticus (R0052)* helped to improve memory and decreased anxiety-like behavior relative to controls using the Barnes maze test, and decreased severity of disease in IL-10-/- mice (62). Ingestion of *Lactobacillus* has been associated with increased GABA expression in the central nervous system that was only observed in non-vagotomized mice (67). This suggests that *Lactobacillus* may exert an influence on the MGB axis through the vagus nerve. It is possible that the decrease in levels of *Lactobacillus* may play a role in the manifestation of behavioral deficits observed in 8 days post-DSS and that this signaling occurs via the vagus nerve. One possibility to investigate the underlying mechanism of our probiotic cocktail would be to perform vagotomies on mice treated with probiotics and/or DSS. Previous studies have demonstrated that heat killed probiotic cocktails can still provide protection against DSS-induced colitis (68). Thus, future studies may also involve administering heat-killed probiotics to evaluate whether these effects are mediated by a product produced by the probiotics or whether it is mediated by live microbial interactions between the probiotics and the gut microbiota.

The increasing establishment of the microbiota as a major influence in physiology has made it an attractive target for the development of potential therapeutics for a range of diseases. One popular therapeutic option that has been around for centuries, but has more recently been investigated, are probiotic organisms. Particularly, species of *Lactobacillus or bifidobacteria* are

increasingly being shown to have potential for a range of therapeutic applications. In this study, we utilized a probiotic cocktail containing two species of *Lactobacillus.* Previous studies from our group demonstrated that administration of *L. rhamnosus* and *L. helveticus* was able to correct behavioral deficits observed in Rag1-/- immunodeficient mice (25). In the current study, *L. rhamnosus* and *L. helveticus* were again tested as a potential therapeutic to treat colitis-like pathologies and behavioral deficits induced by DSS. Treatment with *L. rhamnosus* and *L. helveticus* conferred protection against DSS-induced colitis, both locally within the gut and systemically with changes in behavior. Administration of probiotics, beginning seven days prior to DSS administration, ameliorated weight loss relative to mice that were exposed to DSS plus placebo. Colonic shortening as well as histological damage were also attenuated by administration of probiotics, demonstrating that pre-treatment of probiotics was effective at reducing disease indices associated with colitis. These findings are similar to previous studies that demonstrate therapeutic abilities of probiotics. For example, in a recent study, suspensions of heated killed *L. bulgaricus* (OLL1181) were shown to inhibit DSS-induced colitis when given orally to mice(68). Similarly, oral administration of the probiotic *Bifidobacterium longum* was demonstrated to significantly reduce inflammation in a murine DSS-induced model of IBD (69). These studies provide promising evidence for a role of probiotics in ameliorating colonic disease, however, precise mechanisms of action remain to be elucidated.

Administration of *L. rhamnosus* and *L. helveticus* not only reduced severity of disease but also corrected defects in recognition memory and anxiety-like behavior. Probiotics administered both prior to and during exposure to the DSS regimen were shown to correct deficits in recognition memory. Mice on a DSS + probiotic regimen exhibited similar exploration ratios to mice given either probiotics or placebo alone. Anxiety-like behavior was decreased in DSS + probiotics and probiotics-only groups. Surprisingly, mice given only placebo exhibited behavior similar to the DSS + placebo group. A recent study has shown that chronic restraint stress can induce anxiety-like behaviors(70). It is possible that daily administration of placebo, which involves scruffing of the mouse, may have caused mild restraint stress over time. This may contribute to the increased anxiety-like behavior which was observed in the placebo group. Despite this, administration of probiotics was shown to decrease anxiety-like behavior in both the probiotic alone and DSS + probiotic treated groups, suggesting the probiotic cocktail could overcome anxiety-like behavior associated with any potential exposure to chronic restraint stress.

Our study demonstrates that administration of this *Lactobacillus* cocktail can normalize behavior deficits associated with DSS colitis. Similar studies have demonstrated that probiotics can influence cognitive function and depression-like behaviors in various murine models. In IL10-/- mice, administration of *L. helveticus* (R0052) not only attenuated colonic damage but also improved memory and decreased anxiety-like behavior relative to controls (62). In a model of infection and inflammation utilizing *C. rodentium,* mice were

shown to demonstrate exhibit memory dysfunction in the context of stress(60). However, a cocktail of probiotics containing various species of *Lactobacillus* was able to prevent memory dysfunction (60). To investigate cellular changes associated with memory, I performed immunohistochemistry staining for c-fos. I was able to identify a significant decrease in c-fos expression in the CA-1 region. The CA-1 region of the hippocampus plays a major role in memory and high levels of c-fos expression has been associated with higher cognitive strength (25, 53). These results suggest that administration of probiotics can influence physical processes in the brain. However, the precise mechanism via which these probiotic organisms lead to changes in behavior and cognition remain to be determined and studies to elucidate these are currently ongoing in the laboratory.

In addition to an observed effect on behavior, probiotic administration also partially corrected the observed shifts in the microbiota associated with DSS-induced colitis. In particular, levels of *Lactobacillus* were observed to be restored in both probiotics-only and DSS + probiotics treatment groups, as would be expected with supplementation of probiotics. Interestingly, levels of SFB significantly increased in the group administered probiotics-alone compared to the DSS + placebo group. SFB has been shown to induce intestinal Th17 cells (71) which has been shown to mediate epithelial homeostasis and host defenses (72). SFB was found to be slightly increased in the DSS + probiotics group but not to statistical significance. SFB are gram-positive, spore forming bacteria that are known to colonize mice and humans

(73). They adhere tightly to the intestinal mucosa and play an important role in maintaining homestasis (74). In a recent study, SFB colonization was shown to be sufficient for significant induction of T helper 17 (Th17) cells (71). Th17 cells mediate responses to inflammation through secretion of various cytokines such as IL-17 and IL-22 (75). Secretion of these cytokines allows Th17 cells to attract macrophages and neutrophils via chemotaxis (76). Th17 cells have shown to SFB have been shown to have protective effects by inhibiting colonization of potentially pathogenic bacteria likely through mediating induction of Th17 cells (74). It remains possible that the probiotic cocktail administered helped to increase SFB levels which in turn provided immune stimulation. Currently, SFB remains unculturable and challenging to study to possible mechanistic properties. Based on this study, experiments utilizing flow cytometry may be useful to characterize levels of Th17. Other shifts in the microbiota associated with levels of *Firmicutes* and *Bacteroides* were not associated with behavioral changes. Changes in levels of *Bacillus* were also observed. In general, higher levels of *Bacillus* were associated with improved cognitive performance. Although qPCR evaluation of the microbiota has associated various changes to levels, it is limited in the sense that it is difficult to access for the numerous species and strains of various genera. A high-through put metagenomics approach may yield additional information about changes in the microbiota associated with administration of DSS and probiotics.

Various mechanisms by which probiotics likely influence the gut and the brain have been proposed. A recent study using *B. longum* showed the ability of

probiotics to normalize anxiety-like behavior in a chronic DSS-colitis murine model (56). In contrast, this normalization was not observed in vagotomized mice, suggesting a potential role of the vagus nerve in mediating MGB-axis interactions. Likewise, increases in various pro-inflammatory cytokines, such as IL-6 and TNF-α, have been implicated in psychiatric mood disorders (41, 42). This suggests that there may be multiple routes of communication between the brain and the gut, including both neural and humoral.

It is unlikely that all probiotics confer their health benefits through the same mechanism. Many mechanism are likely in play, which can complicate investigations. In our DSS model of acute colitis, changes in behavior were associated with inflammation. These behavioral changes were rescued following administration of probiotics, which implicates a role of the microbiota. Comorbidities seen in psychiatric and gastrointestinal illness also implicate the MGB axis as a major mediator in disease pathogenesis. Thus, further studies into the MGB-axis may yield further strategies not only in treating IBD but also lead to insights into psychiatric illnesses.

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