Original Article

The regulatory effects of *Bifidobacterium infantis* on the secretomotor activity of the enteric nervous system

Abstract

Background: *Bifidobacterium infantis* (BI) and other probiotics are non-pathogenic living organisms that have gained increased attention for their possible therapeutic implications on the health of the digestive tract. The mechanisms by which probiotics exert their effects are largely unknown.Aims: This study explored the protective and regulatory effect of oral BI on the enteric nervous system in the 2, 4, 6-trinitrobenzene sulfonic acid-induced colitis rats. **Materials and Methods:** Electrical field stimulation and chemical stimulation by 5 hydroxytryptamine or serotonin were used to elicit changes in short-circuit current response of the colonic rat tissue. **Results:** BI-fed colitis rats expressed trends of higher secretomotor activity and revealed signs of decreased macroscopic inflammatory damage when compared to sham-fed colitis rats, suggesting a protective and preventative role of oral BI. **Conclusion:** These findings may provide additional insights for understanding the prophylactic and therapeutic value of specific probiotics in intestinal inflammatory disorders, offering the possibility of a non-invasive alternative to toxic and immune-compromising drugs.

Key words:
Bowel disease, inflammation, probiotics, rat models

Introduction

Inflammatory bowel diseases

Digestive motility and secretion are regulated in order to maximize the digestion and absorption of ingested food. Maintaining fluid homeostasis is one of the many important roles of the gastrointestinal (GI) tract. Approximately, 9 L of fluid enters the small intestine on a daily basis, of which 80-85% is absorbed into the body. When homeostasis is challenged by pathogens or injury, inflammation occurs and the GI tract switches the balance from an absorptive state to a secretory state. This change results in a loss of electrolytes and water in the feces, leading to diarrhea.[1-4]

An inflammatory bowel disease (IBD) greatly disrupts an individual's quality of life and at this time has no cure. Crohn's disease and ulcerative colitis (UC) are the two most common IBDs. UC is a chronic inflammatory disease that acts on the lining of the colon and rectum. It is estimated that nearly 1.5 million Americans suffer from these IBDs,[5] which are responsible for 2.3 million physician visits,[6] 180,000 hospital visits,[7] and costs $6.3 billion annually.[8] IBDs are most commonly seen in Europe and North America. The primary symptoms of UC-diarrhea, abdominal pain, and urgency of defecation – are the result of non-immune cell dysfunction and superficial inflammation of the gut wall. These cells are typically smooth muscle cells, enteric neurons, and epithelial cells that regulate the motility and transport function of the colon.[9-11]

Microbial flora and the colon: A symbiotic relationship

It is estimated that the gut harbors 400-1000 different bacterial species.[12,13] These colonic micro-organisms are typically harmless and oftentimes beneficial. The microbial flora has the ability to out compete pathogenic microbes for nutrient and space, providing a degree of intestinal immunity and also help promote and regulate colonic motility and mucosal layer integrity.[3,14] The microbial flora and its interactions to maintain the health of the

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digestive system has been an interest of study in recent years. Colonization of the gut begins immediately at birth and is influenced by diet, hygiene, and medications. Studies have demonstrated different gut flora modulate different environments. Overall, it is thought that a healthy gut flora has the ability to maintain vascularity, digestive enzyme activity, and immune response.[14]

Probiotic therapy: *Bifidobacterium infantis*

Many diseases of the GI tract result from an imbalance in the normally occurring GI flora.[15] Probiotics are live micro-organism supplements that can colonize in the intestine to help provide microbial balance to the host.[16] This balance plays a pivotal part in preventing the over-growth of potentially pathogenic bacteria, which "helps maintain the integrity of the gut mucosal barrier."[17] Unlike traditional pharmacological therapies, probiotics are living organisms that are not metabolized or excreted from the body.[18] Probiotics protect themselves in acidic conditions and immune responses of the upper GI and make it through the digestive tract to colonize in the lower intestine.[19] No two probiotics are the same and direct comparisons from one strain to another should be avoided.[20] Previous research has focused on *Lactobacillus*, *Bifidobacterium*, and *Propionibacterium* species, yet additional investigation is needed to clarify the importance of individual strains.[10] BI is a specific probiotic that has been found to help in cases of digestive disorders.[21-24] BI has been linked to keeping a healthy colon and has been shown to colonize more efficiently in the human gut than other strains of bacteria, especially in the descending colon.[25,26] *Bifidobacterium* strains are thought to work in a number of ways to enhance the GI environment, (e.g., by influencing both the microbial and host physiology).[4,27]

Enteric nervous system

Digestive functions are controlled through complex interactions between the extrinsic and intrinsic neural innervations, as well as hormonal inputs and secretions. The enteric nervous system (ENS) is an extensive nerve network in the wall of the digestive tract that is independent of the rest of the nervous system. Links have been established between the ENS and the microbial flora that demonstrates probiotics work with the brain-gut system to help modulate GI function.[13,28] The ENS has nerve endings adjacent to the mucosal side of the absorptive epithelial cells. This location provides an ideal place for interaction and response to luminal bacteria, representing an essential connection between the microbes and the ENS in maintaining regular intestinal function.[28] Consequently, the parasympathetic vagal nerve and ENS have been increasingly recognized for their diverse GI signaling in helping regulate inflammatory responses.[29]

The chronic inflammation seen in UC leads to alterations in the neurotransmission, muscle contractility, and secretory functions.[30] Changing the secretomotor activity of the colon produces the symptoms seen in UC such as diarrhea, pain, and the potential for dehydration. It has been suggested that probiotics, such as BI, establish a "cross-talk" or "communication" with the host immune system.[31] This communication helps modulate immunity of the hosts system through several signal mediators but the exact mechanism is still unknown. Evidence suggests that the ENS may play a role, but the specific effects of probiotics on the ENS have not yet been studied.[25] Thus, the purpose of this investigation was to explore the protective and regulatory effect of oral BI on the ENS in the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis rats.

Objective

Recent studies have demonstrated that probiotics can decrease inflammation in the GI tract; but the mechanism through which this occurs is still unclear.[21,28] Therefore, the purpose of this study was to examine the effects of chemical and electrical stimuli on the intestinal secretormotor response by using 5 hydroxytryptamine or serotonin (5-HT) and electrical field stimulation (EFS). 5-HT is an instrumental neurotransmitter and paracrine signaling molecule for the bidirectional communication between the brain and the gut.[32] Approximately, 90% of 5-HT in the human body is produced within the GI tract where serotonin-containing enterochromaffin cells respond to chemical and mechanical stimuli by releasing 5-HT onto afferent nerve terminals that initiate GI reflexes.[32,33] The GI mucosa responds to the chemical stimuli differently between inflamed and non-inflamed tissues.[33] EFS allows for the recording of the short-circuit current (Isc), which portrays the equivalent to the algebraic sum of electrogenic ion movement by active ion transport.[34] These two techniques will help determine if a probiotic, like BI, can alter the ENS secretomotor response in the UC induced rat, contributing to the understanding of the complex interactions that occur between the gut wall, intestinal microbes, immune system, and endocrine system.

Materials and Methods

Experimental design

Ten Albino male Lewis rats of similar age (8-9 months) and mass (350-550 g) were randomized and separated into two populations. The rats were identified with tail markings and placed into the sham-fed colitis induced group or the BI fed colitis induced group. Laboratory living conditions were identical, and rats had free access to food and water. Animal protocol was approved by the institutional animal care and use committee.

Ten days prior to colitis induction probiotic was added to the BI fed colitis group's water supply, and weight, water/food consumption, and appearance documentation began for both groups. On day 3, 7 days prior to the onset of colitis, the rats were fed orally once a day through a curved oral

gavage.[35,36] After 10 days (3 days water supply and 7 days gavage), the rats were induced with UC that lasted for 7 days with continued oral feedings. At the conclusion of the 7 days, the rats were euthanized and tissues from the descending colon were harvested for Ussing chamber data collection.

Experimental protocol: Oral feedings and induction of colitis

The sham-fed colitis induced groups received a daily oral gavage feeding of 1.0 mL distilled water, whereas the BI fed group received 0.205 g of BI suspended in 1.0 mL distilled water. BabyLife, by Solaray, is a BI powder that has 3×10^9 colony forming units (CFU) of BI per 615 mg of powder. Oral feedings required a 1×10^9 CFU (54) dose, which contained 0.205 g of BabyLife BI in 1 mL of water for the feedings.

After 7 days of oral gavage feedings, the rats were fasted overnight, briefly anesthetized with IsoVet (Isoflurane, United States Patent) (Schering-Plough Animal Health Corp.), and given an enema of 1.0 mL 5% (w/v) TNBS (Sigma- Aldrich, USA), a chemical to induce colitis.[35-37] TNBS was delivered into the lumen of the colon through a polyethylene catheter inserted rectally 6 cm proximal to the anus.[35] TNBS administration was followed by 1.0 mL of air to ensure acute colitis induction. The rats were then kept in the Trendelenburg position for 5 min. The colitis conditions lasted 7 days.[36] Excess discomfort was continually evaluated, and data was collected for weight loss, blood loss, and stool activity[36] so that it could be evaluated on a disease activity index (DAI) that ranked the disease on a 4 point scale – 1 being normal and healthy and 4 being sickly.[38]

Experimental protocol: Tissue harvesting

After 7 days of UC the animals were euthanized with an overdose of IsoVet, followed by a thoracotomy. The descending colon of each rat was surgically removed, cleaned of fat and mesentery, and evaluated. Each colon was opened along the mesenteric border, and rinsed free of intestinal contents with ice-cold Krebs bicarbonate saline solution. Then, the colon samples were pinned down in a culture dish, so that the tissue could be stretched and prepared for mounting.[1,39] Every tissue was evaluated by two researchers and scored through a macroscopic scoring rubric for scoring UC severity. The criteria focused on ulceration size and appearance, adhesions, and diarrhea.[35,39]

Experimental protocol: Ussing flux chamber

The flat sheets of full-thickness colon tissue were mounted serosal side to the right in an oval CHM6 Ussing chamber. The Ussing chambers were bathed on either side with a volume of 10 mL solution containing (in mM): NaCl, 119; $CaCl_2$, 1.25; $MgCl_2$, 1; K_2HPO_4, 2.2; KH_2PO_4, 0.2; $NaHCO_3$, 21 and glucose, 10.[39] The solution was set at a pH of 7.4 and was bubbled with a gas mixture of 95% O_2-5% CO_2. A water bath maintained a temperature of 38°C. Ag-AgCl electrodes provided analysis and electrical current stimulation through the World Precision Instruments EVC-4000 Precision V/I Clamp. 150 mM KCl was used to create a 3% agar solution for the electrodes that recorded Isc. Information passed through the EVC-4000 to the iWorx 118 data acquisition system and presented through iWorx Systems, Inc., New Haven, USA. Tissue samples were allowed to stabilize for 30-40 min to create a consistent baseline Isc reading.[39] 5-HT was used to create a chemical stimulus.[1,39] Previous "in home" laboratory experiments found a concentration of 1×10^{-4} M to be effective. 5-HT was added to the serosal side of the mounted tissue and recorded for 3-5 min. EFS was given through the EVC-4000 at 0.1 A of continuous stimulation. Stimulations lasted for 20-30 s. Both chemical and electrical Isc recordings were calculated by taking the difference between the maximum current during stimulation by the initial basal levels.[1]

Statistical analysis

Data was expressed as means±SEM independent sample *t*-tests were used to test for the significance between two group means of the chemical and electrical tissue response, and analysis of variance (ANOVA) with *post-hoc* test, Sidak, were conducted to examine observational data of the BI-fed and sham-fed rats before and after the induction of UC. Categorical analysis was conducted with Chi-square tests. Results were considered significant at $P \leq 0.05$.

Results

Observational data: Weight, food intake, water intake, and DAI

Pre-TNBS percent body weight change between the BI-fed and sham-fed rats was statistically insignificant (P=0.3), but after the induction of TNBS the BI-fed rats presented with a significantly decreased percent body weight than the sham-fed rats (P=0.05) [Figures 1 and 2]. The weights correlated with the food intake, which found that after the first 6 days of TNBS the BI-fed rats ate significantly more food than the sham-fed rats (P=0.05) [Figure 3]. These findings propose a reduction of UC symptom with BI-feedings. This trend can be seen on the DAI scores taken throughout the pre-feedings and TNBS time period [Figure 4]. Water intake for the BI-fed rats was significantly more before and after TNBS (P<0.001) when compared to the sham-fed rats. Interestingly, the BI-fed rats drank significantly more water after TNBS than before (P<0.001), but the sham-fed rats displayed no significant difference between water consumption before and after TNBS induction (P=0.095) [Figure 5]. Observational results were compared using an ANOVA (with a weight of 5) to evaluate the BI-fed and sham-fed, pre- and post-TNBS data.

Macroscopic scoring of colonic inflammation and examination of other organs

Visual observation scoring by two researchers yielded significant morphological inflammatory changes in the

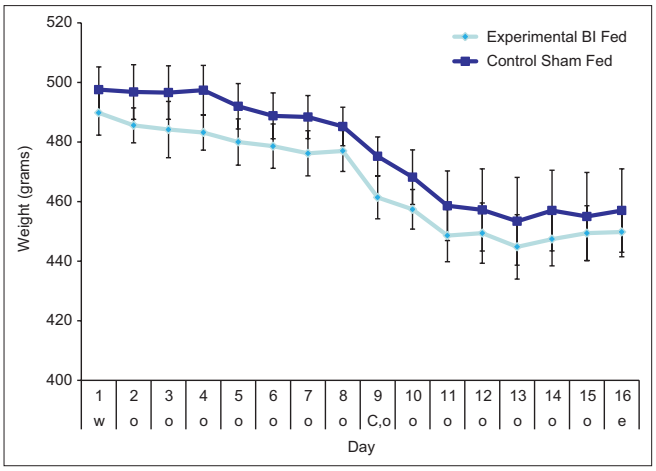

Figure 1: Average daily weight. Even before 2,4,6-trinitrobenzene sulfonic acid induction the rat weights from both groups declined, perhaps from the stress of the oral feedings. Weight loss between the groups was statistically insignificant prior to TNBS induction. A sharp decrease in weight is seen between day 8 and 9 because of the overnight fasting. Data expressed as daily group means±SEM. Please reference Figure 9b for post-TNBS induction weights (n=5). w – Water no feeding; o – Oral feeding; C – Colitis induction; e – Euthanized

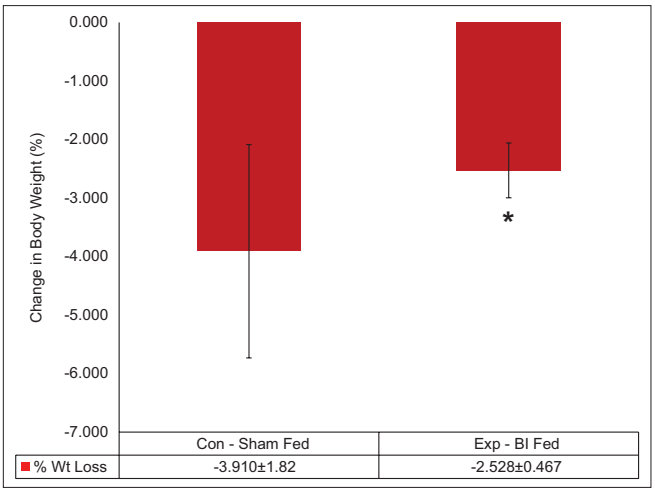

Figure 2: Change in % body weight between first and last day of ulcerative colitis. Data expressed as means±SEM. The percent weight loss was calculated between the first day and last day of colitis. The BI-fed rats lost significantly less weight over the ulcerative colitis time period (*P=0.05). Tested with an independent sample t-test (n=5)

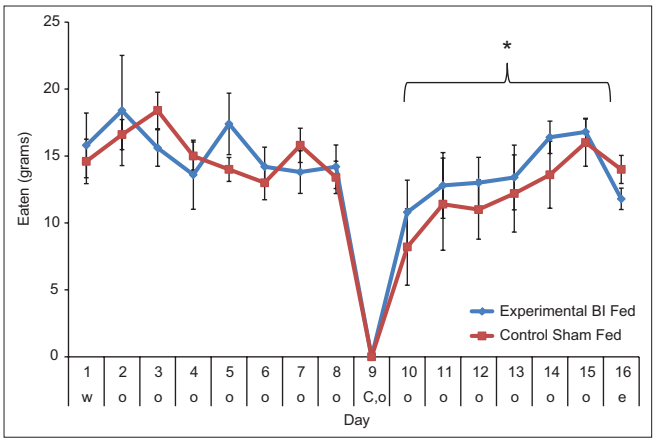

Figure 3: Average daily food intake. Food intake prior to TNBS induction was not significantly different. After the 1st 6 days of ulcerative colitis the BI-fed rats ate significantly more food (*P=0.05). Data expressed as means±SEM and analyzed with a weighted 5 ANOVA to account for small sample size (n=5). w – Water no feeding; o – Oral feeding; C – Colitis induction; e – Euthanized

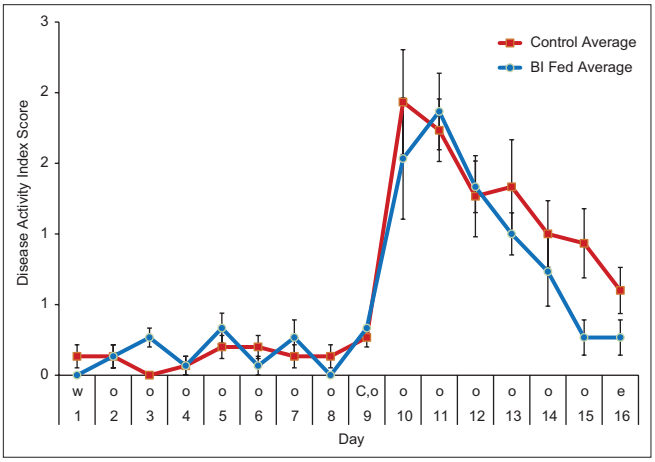

Figure 4: Disease activity index scores. At day 9, the 1st day of ulcerative colitis, a steep increase was seen in both groups. The score appears to decrease at a faster fate in the BI-fed rats, correlating with food, weight, and macroscopic colon damage data. Data expressed in means±SEM (n=5). w – Water no feeding; o – Oral feeding; C – Colitis induction; e – Euthanized

BI-fed versus sham-fed colitis rats (categorical Chi-square weighted 2, P=0.007; independent sample t-tests weighted 5, P=0.001) [Figure 6]. In addition, rough visual examination of the sham-fed rats' small intestine displayed more counted Peyer's patches per 1 inch section when compared to the BI-fed rats. Results from the macroscopic colonic inflammation and small intestine Peyer's patches suggest that BI decreases inflammation, which is evident from the decrease in the level of colonic ulceration, intestinal and peritoneal adhesions, wall thickness, and presence of Peyer's patches in the BI-fed rats.

Chemical and electrical response

5-HT chemical stimulation of the colonic tissue (n=5) displayed no significance between the BI-fed and sham-fed rats (P=0.922) [Figure 7]. A sample size of four per group was used for the EFS, because group 4 (rat 4 and rat 10) had a tissue sample that tore before the EFS could be applied. The EFS evoked an increase in Isc above the baseline in both the sham-fed (104.4±7.02 mV) and BI-fed (113.0±5.95 mV) tissues [Figure 8]. The response of the BI-fed rats tended

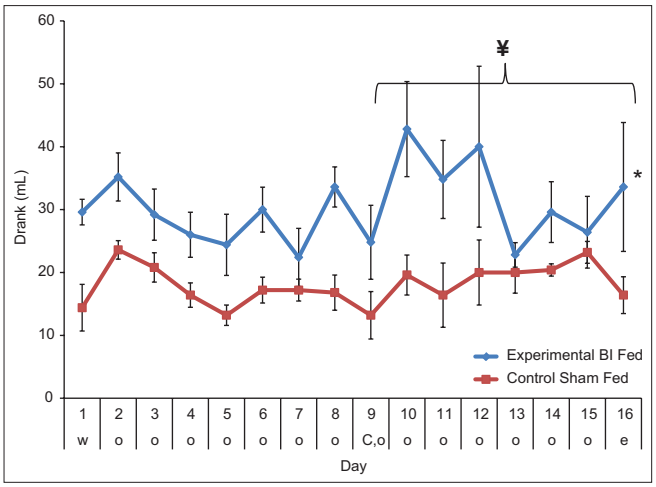

Figure 5: Daily average water intake. Water intake was significantly higher than the sham-fed rats before and after 2,4,6-trinitrobenzene sulfonic acid induction (*P<0.001). *Bifidobacterium infantis*-fed rats drank significantly more water post-TNBS than pre-TNBS (¥P<0.001). Data expressed as means±SEM and analyzed with a weighted 5 ANOVA to account for small sample size (n=5). w – Water no feeding; o – Oral feeding; C – Colitis induction; e – Euthanized

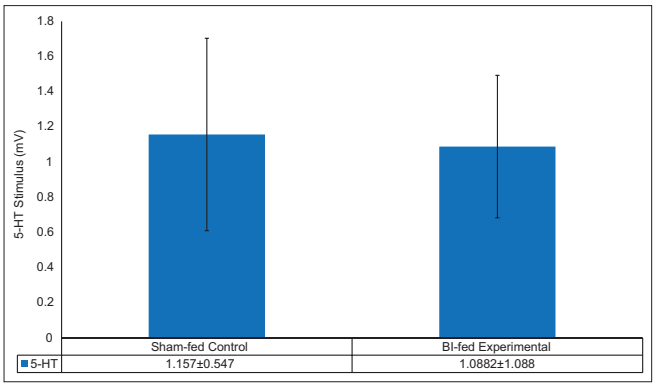

Figure 7: Chemical stimulation by 5 hydroxytryptamine or serotonin (5-HT). Data expressed in means±SEM. 5-HT chemical stimulation was not statistically different between the two groups. Independent sample t-test, P=0.922 (n=5)

to have greater EFS than the sham-fed [Figure 9], however, like the chemical stimulus, the EFS of the colonic tissue exhibited no significant difference between the BI-fed and sham-fed rats (P=0.387).

Discussion

A practical and effective treatment for IBD is still under investigation. Recently, significant attention has focused on the possibilities of probiotics.[4,38,40,41] However, no research to date has explained the mechanism for how these probiotics work.[42] Furthermore, BI has been found to provide protective effects without the presence of an intact vagus nerve, suggesting that probiotics may

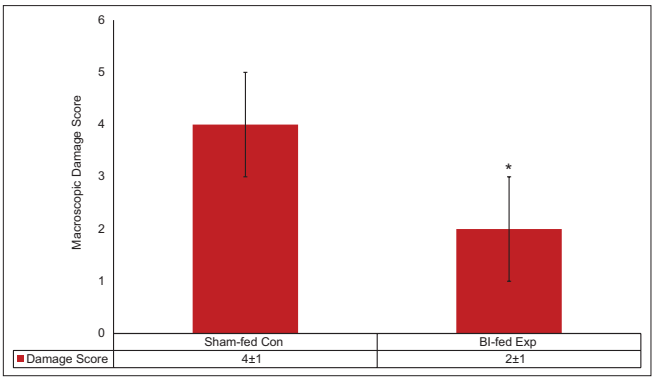

Figure 6: Macroscopic colonic tissue scores. The macroscopic damage seen in the dissected rat colon was significantly decreased in the *Bifidobacterium infantis*-fed rats (*P=0.007). Data expressed as means±SEM and analyzed with a weighted 5 t-test P=0.007 and weighted two Chi-square (P=0.001) to account for the small sample size (n=5)

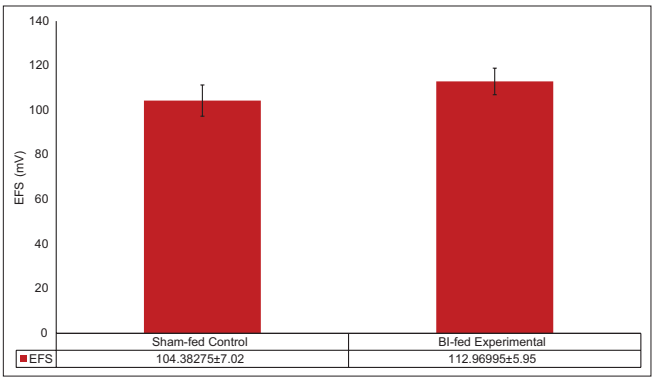

Figure 8: Average electric field stimulation. Data expressed in means±SEM. Electrical field stimulation was not statistically different between the two groups. Independent sample t-test, P=0.387 (n=4)

interact within a localized response such as the ENS.[28,36] The interaction between the ENS and probiotics remains a strong area of interest, and it has been suggested that the intestinal microflora may act with the ENS to regulate inflammation.[25,29,43]

The results obtained in this study found a significant decrease in food consumption and weight loss, supporting trends from previous studies that reflect the severity of UC.[35,42] Correlations with the DAI score[38] and macroscopic tissue damage score[35,44,45] caused by UC demonstrates a trend for decreased inflammation and symptoms within our BI-fed rats. This consistency with previous studies portrays the usefulness of the TNBS animal model as an instrumental tool for advancing our understanding of IBD.[11]

Trends seen with a high-low cross tabulation of EFS suggest BI may increase the secretomotor activity of the ENS [Table 1]. A possible increase in secretomotor activity

of the BI-fed tissue could be caused by several different events. It can be postulated that the increased secretions seen here were from a "healthy" flushing of the tissue. This may have been why the BI-fed rats had significantly higher water intake after TNBS induction then before. However, the increased water intake was most likely due to an innate difference in the osmolarity of the BI-water and the sham-fed distilled water. Keeping this in view, after the induction of colitis, the BI-fed rats were more capable of expelling the corrosive TNBS chemical than the sham-fed rats, producing less severe UC.

BI did not significantly decrease the secretomotor activity through chemical stimulation either. It has been shown that 5-HT is over stimulated in the presence of UC because secretions increase to expel the unwanted materials.[46] Perhaps, this over stimulation desensitized the tissue to the dosage of 5-HT that was found to stimulate healthy colon tissues in previous research within our laboratory. Future chemical stimulation could use varying concentration increments to determine a disparity.

Recent studies have suggested examining the effects of probiotics *in vivo*. *Bifidobacterium breve* was found to promote intestinal homeostasis by controlling chloride secretion. The Heuvelin *et al*.[47] study suggests that it may be through the decrease in the activity of serine/threonin kinases phosphorylating the inhibitor of NF-kappa-B alpha and p38 mitogen-activated PK that is responsible for the anti-inflammatory effects seen in the colon epithelial cells. The Heuvelin *et al*. study also found that the inhibitory effect on chloride secretion was obtained only when the entire bacteria complex was directly present on the tissue.[47] In this regard, *in vitro* tissue analysis may not be able to explain the full extent of the interaction, and future studies will need to exam probiotics effects *in vivo* to understand the full scope of the microbe-gut relationship.

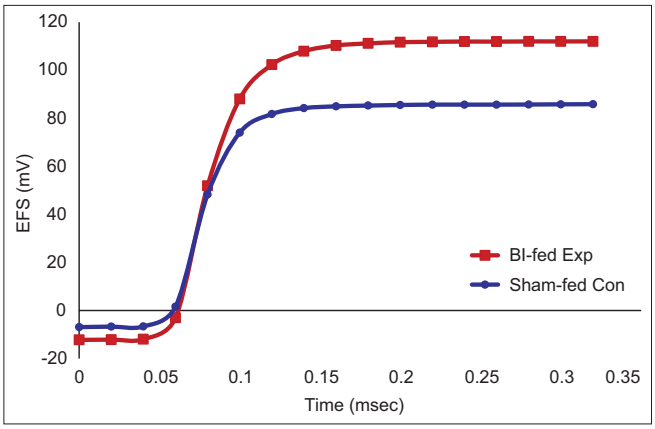

Figure 9: Electric field stimulation. Group 5 EFS shows a substantial difference between the *Bifidobacterium infantis*-fed and sham-fed rats (*n*=1)

Table 1: High-low cross tabulation of electrical field stimulation

N=4	Sham-fed Con (%)	BI-fed Exp (%)
High		
Greater than 100 μ/cm^2	50	100
Low		
Less than 100 μ/cm^2	50	0

High-Low Cross Tabulation

In conclusion, BI did not significantly decrease the secretomotor activity of the ENS as originally hypothesized. Instead, trends found that BI may increase secretions, perhaps offering protection and prevention to harm by expelling the TNBS chemical before it damages the colon. For example, in humans, long-term colitis and inflammation has caused damage to both the mucosa and submucosa layers. Probiotics may help shield these layers by increasing secretions, which could maintain the integrity of the protective system. These findings suggest that probiotics like BI may be more of a maintenance therapy to prevent relapse, and less of a treatment for inducing remission.

The findings of the study will have to be considered in light of limitations. First, the sample size for the two groups in the current study was too small. Furthermore, in the future a milk-based vehicle should be used with the feedings that supports probiotic growth. Afterwards, fecal samples could be collected to evaluate the presence and quantity of probiotic in the intestine. In addition, tissue samples were collected and preserved from each of the rats' colons and small intestines. These samples could be used to further the investigation of this study through histological and immunological analysis. Finally, in the future the muscularis layer should be dissected from the colon. This would allow the neuronal sub-mucosal receptors to be directly exposed to 5-HT, and therefore provide more accurate readings of the secretory response.

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