DISEASES OF THE COLON & RECTUM

ORIGINAL CONTRIBUTION

Therapeutic Potential of Two Probiotics in Inflammatory Bowel Disease as observed in the Trinitrobenzene Sulfonic Acid Model of Colitis

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PURPOSE: The pathogenesis of inflammatory bowel disease is thought to be a multifactorial process. One of the leading hypotheses is that an imbalance in normal gut flora induces an excessive immune response and contributes to inflammation in the gastrointestinal tract. Administration of probiotic bacteria reduces symptoms in patients suffering from inflammatory bowel diseases, probably via both manipulation of the microflora and stimulation of the intestinal immune system. In the current study the therapeutic potential of two different probiotics–*Lactobacillus GG* and a mixture of *Streptococcus thermophilus, Lactobacillus acidophilus*, and *Bifidobacterium longum* (YO-MIX™ Y 109 FRO 1000)—in a rat model of colitis were evaluated.

METHODS: Male Wistar rats were administered probiotics for three days simultaneously with colitis induction. Colonic damage was evaluated histologically and biochemically and colonic tissues, as well as fecal samples, were used for bacterial studies using 16S rRNA gene primers.

RESULTS: Probiotics administration reduced the relative amounts of the pathogenic bacteria *Aeromonas* and *Escherichia coli* in the colonic tissue. However, whereas both probiotics affected colon morphology, only *Lactobacillus* GG administration reduced myeloperoxidase activity.

CONCLUSIONS: We report the therapeutic rather than preventive potential of two different probiotics in an animal model of colitis.

KEY WORDS: Inflammatory bowel disease; Experimental colitis; Probiotics; Microflora.

Inflammatory bowel disease (IBD) is a chronic and spontaneously relapsing disorder of the gastrointestinal (GI) tract that is characterized by inflammation and tissue damage. The mechanism as well as the exact etiology and pathogenesis of the development of this disease are not fully understood. Increasing experimental and clinical data suggest that the induction and pathogenesis of IBD is a multifactorial process that involves interactions among genetic, immune, enteric bacteria, and environmental factors.[1,2]

Several lines of evidence support the notion that an excessive immune response to an essentially normal population of gut microflora underlies the pathogenesis of IBD. Evidence that supports this hypothesis includes the observation that inflammation usually occurs in intestinal regions with the highest bacterial concentrations (ileum and colon),[3] that patients with IBD typically have greater numbers of adherent bacteria compared with healthy people,[4] and that antibiotic administration often is a viable treatment option.[5] Studies have shown that rats and mice remain healthy when housed in germ-free conditions but spontaneously develop colitis upon intestinal colonization by typical commensal bacteria.[6,7] These studies are consistent with the concept that normal enteric bacteria initiate and sustain inflammation in genetically susceptible rodents. In addition, in human IBD the inflammation is present in the parts of the gut that contain the highest bacterial concentrations. Because the intestinal microflora is essential for the development of IBD, administration of probiotic bacteria is one possible therapeutic approach. Lactobacillus GG is one of the most widely studied probiotic bacteria and has been shown to survive gastric and bile secretions, adhere to intestinal epithelial cells, and colonize the intestine.[8] It has been found to be beneficial and safe for the treatment of several GI conditions characterized by increased gut permeability.[9,10]

Lactobacillus species have been shown to prevent colitis in interleukin (IL)-10-deficient mice.[11] Gupta *et al.*[12] have suggested that Lactobacillus GG may improve gut barrier function and clinical status in pediatric Crohn's disease. In addition, *Bifidobacteria* are considered to be an important strain in the development of the neonatal intestinal microenvironment.[13] Menard *et al.*[14,15] have reported that *Streptococcus thermophilus* exerts an anti-TNF-α effect, stimulates the Th1 immune system, and reinforces the colonic epithelial barrier.

This study was designed to test the therapeutic potential of different strains of probiotic bacteria: *Lactobacillus* GG

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and a mixture of *Streptococcus thermophilus, Lactobacillus acidophilus*, and *Bifidobacterium longum* (YO-MIX™ Y 109 FRO 1000) in an animal model of trinitrobenzene sulfonate (TNBS)-induced colitis.

MATERIALS AND METHODS

Animals

Male Wistar rats ($n=20$; 300–450 g) were obtained from the Harlan Laboratory at The Weizmann Institute of Science (Rehovot, Israel). They were housed in metal cages in a room with controlled temperature (25±2°C), relative humidity (65±5 percent), and light (0800–2000 h). Ethics approval was obtained for the study. The procedures were conducted in full compliance with the strict guidelines of the *Hebrew University Policy on Animal Care and Use.*

Induction of Colitis and Probiotic Bacteria

Probiotic strain, *Lactobacillus* GG (Lgg), was provided by Valio (Helsinki, Finland) and YO-MIX™ Y 109 FRO 1000 probiotic bacterial mix (Y 109) was obtained from Danisco Cultures (Niebull, Germany). This probiotic formulation consists of three live bacterial strains: Streptococcus strain (thermophilus); Lactobacillus strains (acidophilus); and Bifidobacterium strains *(lactis)*. The two probiotics were administered in the drinking water, each in the amount of 1×10^8 colony forming units (CFU)/ml.

The rats were randomly assigned to four groups; two of them (noncolitic and control) received no probiotic treatment and the two remaining groups (treated groups) were induced colitis and received the probiotics daily for three days simultaneously with colitis induction. A modification of the procedure developed by Morris *et al.*[16] was used to induce colitis. Rats were lightly anesthetized with ether and a rubber catheter was inserted through the anal canal for a distance of 8 cm into the colon just proximal to the splenic flexure. Colitis was induced by administering 0.3 ml 2,4,6-trinitrobenzene sulfonic acid (TNBS; Sigma Chemical, St. Louis, MO; 100 g/l dissolved in 50 percent ethanol). Animals from all groups were killed 72 hours after colitis induction.

Assessment of Colonic Damage

Colitis severity was assessed in two ways: histologic evaluation, and quantification of granulocyte infiltration through measurement of myeloperoxidase (MPO) activity. Once the rats were killed, the colon was removed aseptically and divided into three segments. One fragment was fixed in 4 percent (v/v) buffered formaldehyde and the tissue was subsequently processed for histologic evaluation, and another sample was frozen at −80°C for MPO activity assay. The remaining sample was stored at −20°C for microbiologic studies. Equivalent colonic segments were obtained from the noncolitic group. Inflammation and crypt damage were assessed on hematoxylin and eosin-stained sections, using a modification of a validated scoring scheme described by Dieleman *et al.* (Table 1).[17] Each section was then scored for each feature separately and the total colonic histology scores are the sum of the five different subscores. Using this scoring system, the minimum score was 0 and the maximum score was 18.

MPO activity was assayed by determining the decomposition of hydrogen peroxide in the presence of *o*-dianisidine[18] 100 to 200 mg tissue was homogenized in hexadecyltrimethylammonium bromide (HTAB) buffer. The assay mixture, in a cuvette of 1 cm path length, contained 2.9 ml Dianizidine solution, 100 µl of the homogenate sample, and 10 µl H2O2. The homogenate was added last and the change in absorbance at 460 nm was followed for 90 seconds. All measurements were performed in duplicate. MPO activity was expressed in units per gram tissue.

Bacterial Genomic DNA Extraction

Bacterial genomic DNA was isolated from tissues by using a Wizard® Genomic DNA purification kit according to the manufacturer's protocol (Promega, Madison, WI).

Feces were treated as previously described by Zhu *et al.*[19] They were homogenized by vortexing with 4 mm diameter glass beads for three minutes. Debris was removed by centrifugation at 700 *g* for one minute, and the supernatant was collected and centrifuged at 12,000 *g* for five minutes. The pellet was washed twice with phosphate-buffered saline and stored at −20°C until DNA extraction.[19,20] For DNA purification, the pellet was resuspended

Table 1. Histologic scoring of colitis

Feature graded	*Score*	*Description*
Inflammation severity	0	None
	1	Mild
	2	Moderate
	3	Severe
Inflammation extent	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Regeneration	4	No tissue repair
	3	Surface epithelium not intact
	2	Regeneration with crypt depletion
	1	Almost complete regeneration
	0	Complete regeneration or normal tissue
Crypt damage	0	None
	1	Basal 1/3 damage
	2	Basal 2/3 damage
	3	Only surface epithelium intact
	4	Entire crypt and epithelium lost
Percent involvement	1	1–25
	2	26–50
	3	51–75
	4	76–100

in EDTA and treated with 10 g/l lysozyme (Sigma Aldrich, St. Louis, MO) for 45 minutes at 37°C. The bacterial genomic DNA was then isolated and its concentration and purity checked spectrophotometrically.

Polymerase Chain Reaction Amplification of Bacterial Genes Coding for 16S rRNA

Molecular techniques based on 16S rRNA gene sequences provide a reliable method for the detection and identification of bacterial species. 16S rRNA-gene-targeted group-specific primers were used to identify predominant bacteria in a semiquantitative polymerase chain reaction (PCR). *Lactobacillus* group-specific PCR was performed as described in Wang *et al.*[21] *Bifidobacterium* genus-specific PCR was performed by using 16S rRNA gene-targeted primers, as described by Langendijk *et al.*[22] *Escherichia coli* group-specific PCR was performed by using 16S rRNA gene-targeted primers according to Tsen *et al.*[23] *Streptococcus thermophilus* strain PCR was performed by using primers according to Furet *et al.*[24] *Aeromonas* strain-specific PCR was performed by using the 16S rRNA-targeted primers designed with the programs BLAST and RDPII (http://www.ncbi.nlm.nih.gov/BLAST, http://rdp.cme.msu.edu/html/). The target sites for the primers were identified as sequences that are invariant, or nearly so, in members of each bacterial group. The conserved 16S rRNA region, identifying all known bacteria, was amplified by using universal bacterial primers according to Amit-Romach *et al.*[20] The universal primer set was used to determine the total microflora population.[22,25] For PCR amplification of the bacterial targets from tissue, 100 ng of extracted DNA was amplified, and for the bacterial targets from feces, 50 ng of extracted DNA was amplified using GoTaq® Green Master Mix (Promega). DNA (5 μl) was added to 45 μl of PCR mixture containing 10 μl nuclease-free water, 5 μl of each primer, and 25 μl Taq polymerase. To determine the linear phase of the amplification, the PCR was run with different numbers of cycles (25, 30, 35, 40, 45, or 50) for each primer set. Amplification of the fragments was as follows: *Lactobacillus* spp.—a 286-bp 16S rRNA fragment, 35 cycles; *Bifidobacterium* spp.—a 510-bp 16S rRNA fragment, 42 cycles; *E. coli* strains—a 584-bp 16S rRNA fragment, 42 cycles; *Aeromonas* spp.—a 586-bp 16S rRNA fragment, 32 cycles; *Streptococcus thermophilus*—a 279-bp 16S rRNA fragment, 46 cycles; universal primers—a 611-bp 16S rRNA PCR product, 37 cycles.

Amplification conditions were: denaturation (94.5°C, 30 seconds), annealing (60°C, 1 minute), and extension (72°C, 50 seconds). PCR products were visualized by agarose (2 percent) gel electrophoresis stained with ethidium bromide, and quantified with a Gel-Pro Analyzer™ version 3.0 (Media Cybernetics, Inc., Bethesda, MD). The evaluation of the different PCR products was normalized to the density of the PCR product of the universal primers by densitometer scanning and was reported in arbitrary units as described previously.[20] The relative amount was defined as the amount of group-specific bacteria of the total amount of microflora population (universal primers). The primers used to amplify the bacterial 16S rRNA are shown in Table 2.

Statistical Analysis

Treatment-dependent changes were analyzed by using one-way analysis of variance (ANOVA). Statistical differences among means were considered significant at $P<0.05$. A posthoc test (Tukey-Kramer) was performed when the interaction between treatments was significant. Tests were performed within segments and not between them. JMP version 5.1 was used for all analyses. Values are presented as means ± standard error of the mean.

RESULTS

Effect of Probiotic Administration on Colonic Inflammation

Intrarectal administration of TNBS resulted in macroscopic colitis-associated damage, with diarrhea, shortening and thickening of the colon, and mucosal ulceration.

Table 2. PCR primers used to amplify bacterial 16S rRNA genes

Bacterial group	*Primers*	*Sequence(5′-3′)*	*Length (bp)*
Universal	Unibac- f	CGTGCCAGCCGCGGTAATACG	611
	Unibac- r	GGGTTGCGCTCGTTGCGGGACTTAACCCAACAT	
Lactobacillus	LAA-f	CATCCAGTGCAAACCTAAGAG	286
	LAA-r	GATCCGCTTGCCTTCGCA	
Escherichia coli strain	E.coli-f	GGGAGTAAAGTTAATACCTTTGCTC	584
	E.coli-r	TTCCCGAAGGCACATTCT	
Bifidobacterium	Bif164-f	GGGTGGTAATGCCGGATG	510
	Bif662-r	CCACCGTTACACCGGGAA	
Aeromonas	Aeromonas-f	CACCAAGGCGACGATCCCTAGCTGG	586
	Aeromonas r	AAGCCACGTCTCAAGGACACAGCCT	
Streptococcus thermophilus	St1	TTATTTGAAAGGGGCAATTGCT	279
	St2	GTGAACTTTCCACTCTCACAC	

Histologic assessment of colonic samples from the TNBS group revealed severe transmural disruption of the normal architecture of the colon, with extensive ulceration and inflammation involving all of its intestinal layers (score (mean ± standard error), 15.88±0.66). Colonic samples were characterized by neutrophil infiltration in the mucosal layer and submucosa. Most of the rats showed epithelial ulceration of the mucosa affecting >85 percent of the surface. The inflammatory process was associated with crypt loss. Histologic analysis of the colonic specimens from rats induced colitis and treated with probiotic revealed only a minor effect of Y 109 on colonic tissue, with a mean score that was not significantly different from TNBS group (13.67± 1.86). On the other hand, administration of Lgg improved colonic tissue architecture significantly compared with the TNBS group (mean score, 10.75±1.8). Tissue in the process of reepithelization surrounded the ulceration zone in a few of the Lgg-treated rats. Moreover, the transmural involvement of the lesions was reduced. In those rats, the improvement in colonic histology was accompanied by a reduction in inflammation infiltrate (Fig. 1).

Colonic damage was assessed biochemically by colonic MPO activity. MPO is an enzymatic marker of neutrophil infiltration into GI tissues. MPO activity was enhanced in the TNBS group compared with controls; however, it was only slightly reduced in some but not all of the Lgg-treated rats (Fig. 2).

Microbiologic Studies

The relative amounts of *Lactobacillus* spp. decreased slightly, but nonsignificantly, after TNBS induction. Administration of the Y 109 probiotic resulted in a small increase in the relative amounts of *Lactobacillus*. *Bifidobacteria* were almost undetectable in the colon tissues. TNBS induction did not change the relative amounts of *Bifidobacteria* relative to controls. Y 109 probiotic treatment significantly increased this bacterial population ($P<0.05$). The relative amount of *S. thermophilus* was the same in all groups (Fig. 3).

TNBS induction led to the appearance of *E. coli* strains and *Aeromonas* in the colon of two rats. These animals also had the highest colonic histology scores (i.e., their inflammation was more severe). Rats treated with Lgg tended to colonize less *E. coli* and *Aeromonas* than TNBS rats, whereas the Y 109 probiotic-treated rats showed variability in this parameter (Fig. 4).

Analysis of the colon-associated bacterial population revealed a large variation among individuals with respect to some bacterial species. The presence of different bacterial species in rat feces samples was analyzed as well.

The results indicated that the relative amounts of *Lactobacillus* spp. were slightly increased and the *Bifidobacteria* population in the feces was significantly reduced after TNBS induction. Administration of Lgg resulted in a small increase in the relative amounts of *Lactobacillus* compared with the TNBS group, whereas the Y 109 probiotic had no effect on relative *Lactobacillus* amounts but elevated the relative amount of *Bifidobacteria* spp. *S. thermophilus* was undetectable in feces samples from TNBS-induced rats. In the feces of the three other groups, it was detected in very small amounts (Fig. 5).

E. coli strains were detectable in only one fecal sample from each group, and there were no significant differences

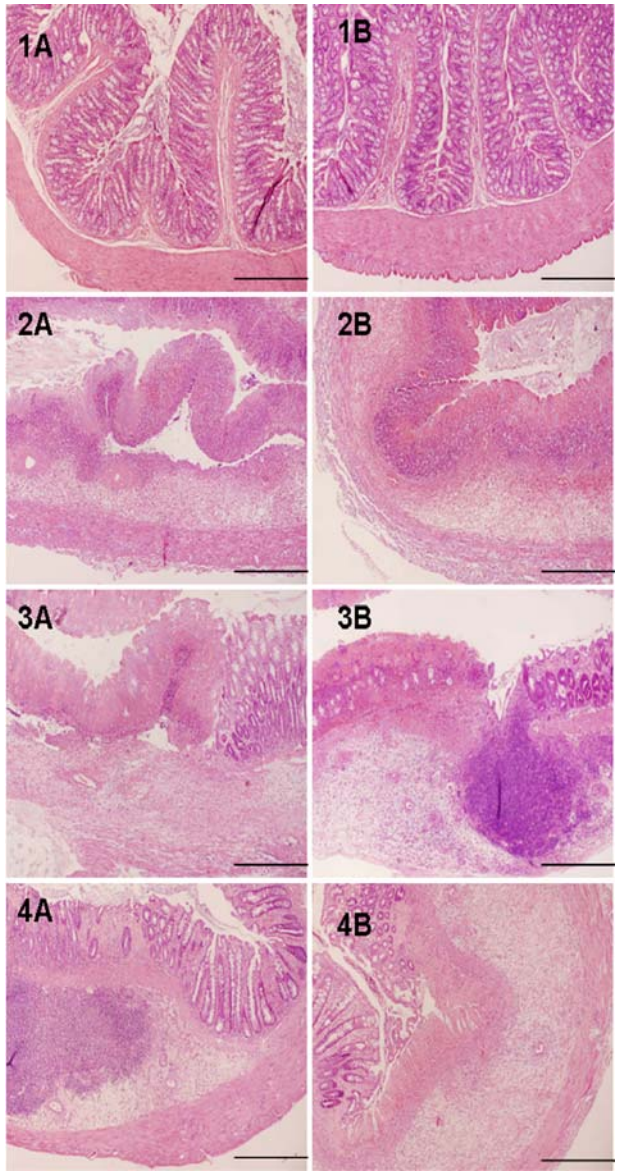

FIGURE 1. Histologic sections of colonic tissue stained with hematoxylin and eosin. Magnification x40; bar=500 μm. **1A-B**. Noncolitis group showing the normal histology of the rat colon. **2A-B**. Trinitrobenzene sulfonate colitis group showing complete destruction of the tissue. **3A-B**. Y 109-treated group showing a very slight improvement of the inflammatory process. **4A-B**. *Lactobacillus* GG-treated group showing improvement in both the inflammatory process and the colonic tissue architecture.

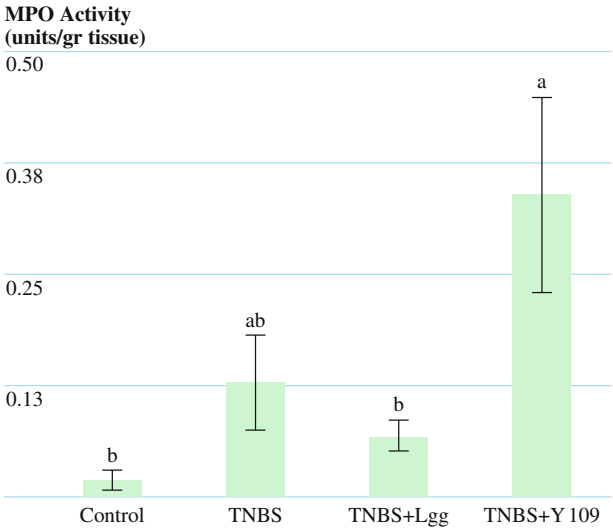

FIGURE 2. Effects of probiotic treatments on colonic myeloperoxidase (MPO) activity in rats with trinitrobenzene sulfonate (TNBS)-induced colitis. Values are mean ± standard error of the mean (n=5). The means of treated groups without a common letter differ ($P < 0.05$). Lgg = *Lactobacillus* GG.

between them. *Aeromonas* was identified in three fecal samples from the control group, one from the TNBS group, and two from the probiotics-treated group (Fig. 6).

DISCUSSION

This study reported the therapeutic potential of two probiotics—Lgg, and the Y 109 probiotics bacterial mix—on the course of inflammation in an animal model of colitis. The approach used included administration of probiotics with the drinking water for three days while simultaneously inducing colitis.

Previous studies have demonstrated the preventative effect of probiotics in the TNBS model.[26,27] In those studies, probiotics were given two weeks before induction of colitis, and all of the probiotics assayed were shown to have anti-inflammatory activity as evidenced histologically; however, each probiotic showed its own anti-inflammatory profile.

Although both probiotics helped to promote the recovery of colonic tissue after TNBS induction, the restoration with respect to crypt damage and to the extent and severity of the inflamed tissue was more evident in the rats that received Lgg than in those that received Y 109. This beneficial effect also was observed biochemically: Lgg decreased colonic MPO activity (Fig. 2). The observed inhibition of neutrophil infiltration may reflect one of the intestinal anti-inflammatory effects of the probiotics against tissue injury.

Previous studies have suggested that after TNBS induction, specific strains of luminal colonic microflora invade the disrupted colonic epithelial wall and the

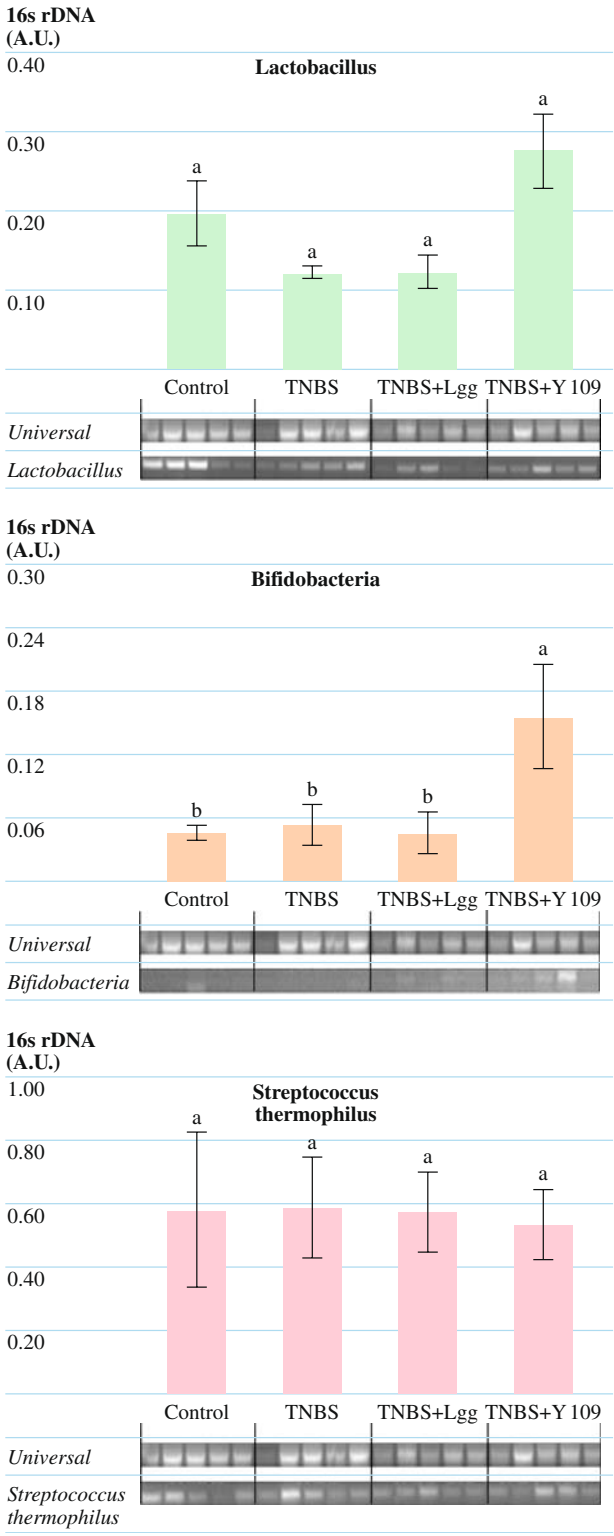

FIGURE 3. Effects of probiotic treatments on the population of beneficial bacteria in the colon of rats with trinitrobenzene sulfonate (TNBS)-induced colitis. A. Relative proportions of beneficial bacteria in the colon. B. Representative PCR products of 16S rDNA of the beneficial bacteria and invariant sequences of all known intestinal bacterial species (Universal) in the colon of all four groups. Values are mean ± standard error of the mean (n=5). The means of treated groups without a common letter differ ($P < 0.05$). Lgg = *Lactobacillus* GG.

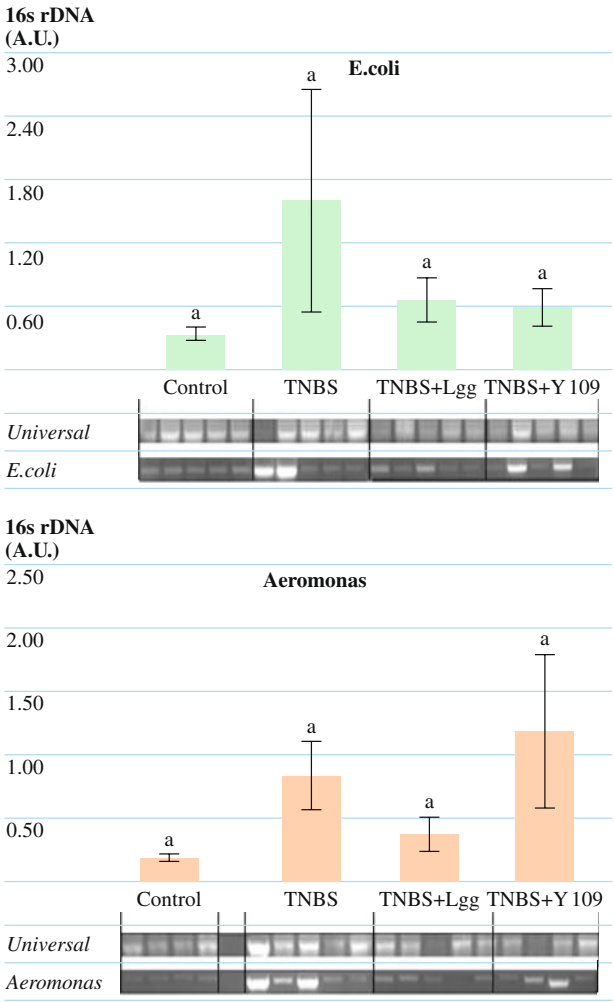

FIGURE 4. Effects of probiotic treatments on the pathogenic bacterial population in the colon of rats with trinitrobenzene sulfonate (TNBS)-induced colitis. A. Relative proportions of pathogenic bacterial in the colon. B. Representative polymerase chain reaction products of 16S rDNA of pathogenic bacterial and invariant sequences of all known intestinal bacterial species (Universal) in the colon of all four groups. Values are mean ± standard error of the mean (n=5). The means of treated groups without a common letter differ ($P<0.05$). Lgg = *Lactobacillus* GG.

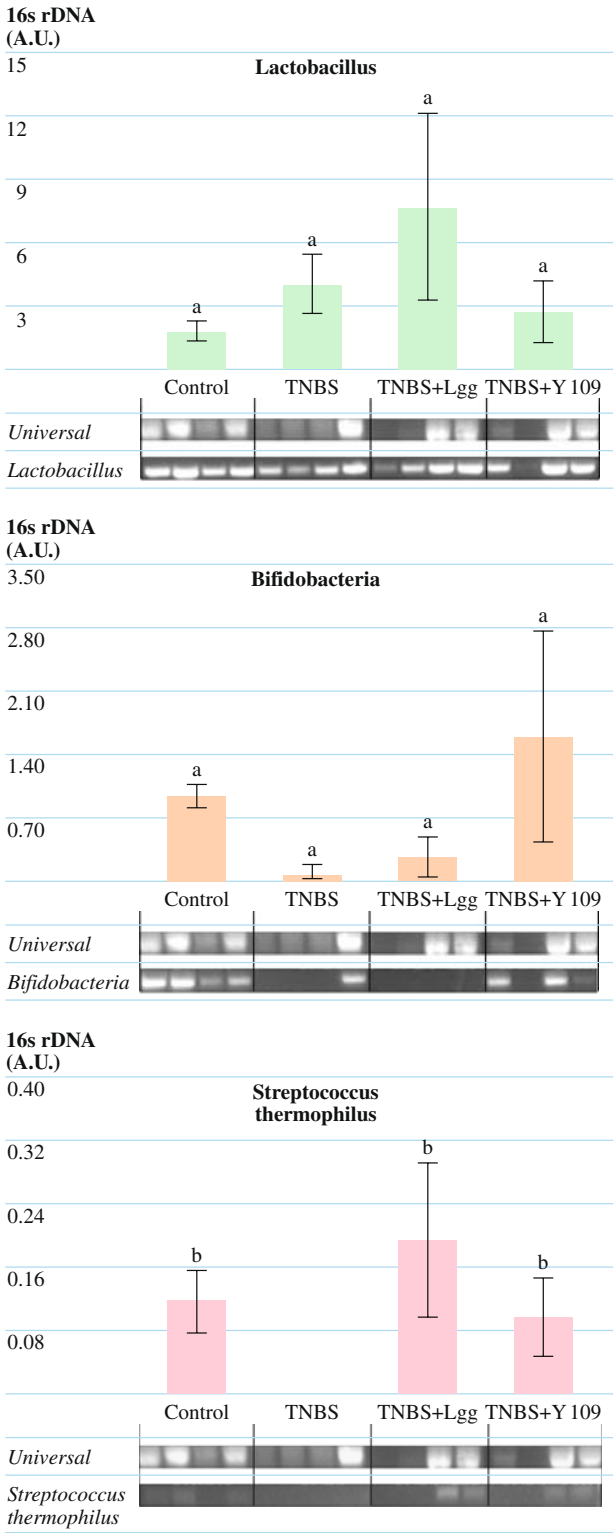

FIGURE 5. Effects of probiotic treatments on the beneficial bacterial population in feces of rats with TNBS-induced colitis. A. Relative proportions of beneficial bacteria in the colon. B. Representative polymerase chain reaction products of 16S rDNA of the beneficial bacteria and invariant sequences of all known intestinal bacterial species (Universal) in the colon of all four groups. Values are mean ± standard error of the mean (n=5). The means of treated groups without a common letter differ ($P<0.05$). Lgg = *Lactobacillus* GG.

existence of these bacteria within the wall contribute to the transmural inflammation.[28] Human and animal studies also have shown a significant decrease in the number of bacteria and *Lactobacillus* in IBD groups compared with healthy subjects.[27,29] It is possible that products of the commensal flora promote inflammation in the presence of an impaired mucosal barrier or injury to the mucosa.[30]

Because the microbial environment has been shown to play a role in the development of IBD, modulation of the microbiota presents an option for therapeutic intervention.[31] Administration of probiotics is one of the methods used to manipulate the intestinal microbiota in an attempt to reduce the inflammatory response. In fact, it has been

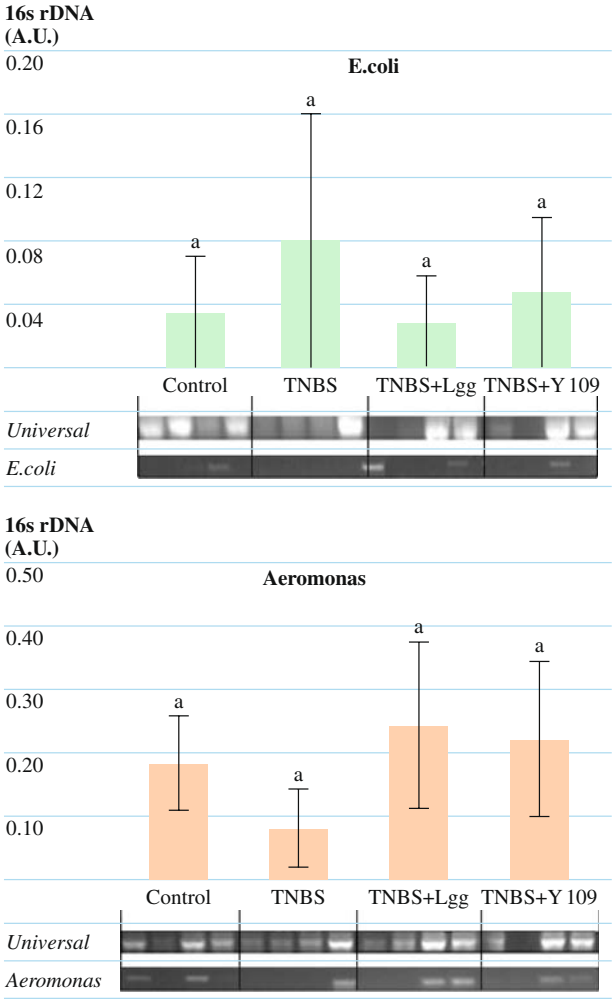

FIGURE 6. Effects of probiotic treatments on the pathogenic bacterial population in feces of rats with TNBS-induced colitis. A. Relative proportions of pathogenic bacterial in rats feces. B. Representative polymerase chain reaction products of 16S rDNA of pathogenic bacterial and invariant sequences of all known intestinal bacterial species (Universal) in rat feces. Values are mean ± standard error of the mean (n=5). The means of treated groups without a common letter differ ($P<0.05$). Lgg = *Lactobacillus* GG.

reported that administration of a mixture of *Bifidobacterium* and *Lactobacillus*[32] prolongs remission in ulcerative colitis.

In this study, attenuation of disease activity was associated with modulation of the gut microflora, as investigated by the culture-independent 16S rRNA gene target method. We demonstrated the ability of both probiotics to modify the colonic microflora, which had been altered as a consequence of the TNBS-induced colitis. TNBS induction resulted in the appearance of *E. coli* strains, mainly pathogenic ones, as reported by Tsen *et al.* who tested these specific primers.[23] *Aeromonas*, an extremely aggressive bacterium that leads to intestinal and nonintestinal diseases,[33] appeared as well. Probiotics treatments in our study reduced the relative amounts of both pathogenic bacteria in the colonic tissue.

Various probiotic bacteria have been found to possess different mechanisms of action. One common mechanism that is evident in a wide variety of probiotic strains is adherence to the intestinal mucosal surface, which prevents colonization of pathogenic bacteria.[34] This is a form of competition between the two species. Another common mode of action is via stimulation of the intestinal immune system. Several studies reported that lactobacilli are potent inducers of IL-12.[35,36]

Generally, luminal content and feces are used for microbial studies.[26,27] Here, the relative proportions and microbial profile of the colon-associated bacteria were found to differ from those of the feces-associated bacteria. These observations are in accordance with our previous work[37] and with that of Zoetendal *et al.*[38] These differences may be related to the fact that many pathogenic bacteria must bind to epithelial cells to affect the host: bacteria that fail to adhere to the intestinal epithelium are washed out with the luminal contents, leaving the body in the feces. Hence, the feces may contain mainly bacteria that could not adhere and, as such, it does not provide an accurate reflection of the colonic microbial ecology. Isolation of bacteria from the tissue and the feces may better reflect the actual colonized microbiota.

In this study, concurrent administrations of Lgg and Y 109 probiotics with induction of colitis were shown to possess anti-inflammatory activity in a rat model of TNBS-induced colitis. The different probiotic regimens differed in their efficacy as anti-inflammatory agents and did not share the same mechanisms of action. It is noteworthy that Lgg was found to be more effective than Y 109 as a probiotic. Nevertheless, both of these probiotics improved different parameters of inflammation, including colonic damage, and modulated the gut microflora. We suggest that the colonization of these probiotics in the colonic epithelium results in beneficial effects on GI inflammatory conditions, which are probably secondary to their immunomodulatory properties. It is possible that administration of probiotic bacteria downregulated colonic proinflammatory cytokines via the suppression of nuclear factor kappa B (NF-κB), in addition to increased production of mucosal IL-6. Suppression of cytokines production may improve restoration of intestinal barrier function and reduce proinflammatory mediators in intestinal lumen.[39]

In summary, the results obtained in this study emphasize the therapeutic potential of dietary probiotics in IBD in general and of Lgg in particular. Studies with human subjects are needed to substantiate these findings in patients with IBD.

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