Prevention of TNBS-Induced Colitis by Different *Lactobacillus* and *Bifidobacterium* Strains Is Associated with an Expansion of $\gamma\delta$T and Regulatory T Cells of Intestinal Intraepithelial Lymphocytes

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Background: Probiotics may protect against inflammatory bowel disease through regulation of lamina propria lymphocytes (LPLs) function. Data are lacking on possible involvement of intraepithelial lymphocytes (IELs). The aim of this study was to investigate whether different probiotic mixtures prevented gut inflammatory disease and the role of both IELs and LPLs.

Methods: BALB/c mice received 2 probiotic mixtures orally for 3 weeks, as Mix1 (*Lactobacillus acidophilus* and *Bifidobacterium longum*), or Mix2 (*Lactobacillus plantarum, Streptococcus thermophilus*, and *Bifidobacterium animalis* subsp. *lactis*). Colitis was induced by intrarectal administration of trinitrobenzene sulfonic acid (TNBS). Probiotics in stools were analyzed by real-time polymerase chain reaction (PCR). Colon subpopulations of IELs and LPLs were assayed by flow cytometry. Serum cytokines were measured by cytometric bead array (CBA).

Results: All probiotics colonized the intestine. The 2 mixtures prevented the TNBS-induced intestinal damage, and Mix1 was the most effective. The Mix1 protection was associated with a reduction in $CD4^+$ cells of IELs and LPLs, an increase in $\gamma\delta$T cells of IELs, and a decrease in $\gamma\delta$T cells of LPLs. An expansion of T regulatory (Treg) cells of IELs was induced by Mix1 and Mix2. Both probiotic mixtures inhibited tumor necrosis factor (TNF)-α and monocyte chemotactic protein (MCP)-1 production and upregulated interleukin (IL)-10. In addition, Mix1 prevented the TNBS-induced increase of IL-12 and interferon (IFN)-γ.

Conclusions: The 2 probiotic mixtures were able to prevent the TNBS-induced colitis; the *L. acidophilus* and *B. longum* mixture was the most effective. Other than an involvement of LPLs, our results report a novel importance of the IELs population in probiotic protection.

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Key Words: colitis, probiotic mixtures, intestinal epithelial cells, $\gamma\delta$T cells, regulatory T cells

Ulcerative colitis (UC) and Crohn's disease (CD) are 2 major healthcare problems of the digestive tract, commonly known as inflammatory bowel disease (IBD), characterized by chronic and spontaneous inflammation due to a complex interaction of genetic, microbial, and environmental factors, which results in continuous activation of the mucosal immune system.[1] Increasing evidence indicates that changes in gut microbiota, with an increase of pathogenic bacteria and a decrease of health-promoting symbionts, such as bifidobacteria and lactobacilli,[2,3] play an important role in promoting and maintaining intestinal inflammation in IBD.[4–7] Probiotics are microorganisms that confer health benefits in different ways, including modulation of immune response.[8,9] Probiotics have been proposed for IBD treatment and clinical studies have reported alleviation of symptoms and prevention of relapses in IBD.[10–12] The most widely used probiotics are bifidobacteria and lactobacilli, but other microorganisms, such as the nonpathogenic *E. coli* strain Nissle 1917 and the yeast *Saccharomyces boulardii*, have been successfully used in several clinical trials[13,14] and in animal models of experimentally induced colitis.[15–17] Despite the evidence that some probiotics can represent a valid therapeutic approach in IBD treatment, the mechanisms underlying the protection by probiotics in IBD is still largely unknown. In addition, not all probiotic strains are able to reduce intestinal inflammation.[18] Since probiotic activity is considered to be genera, species, and strain-specific, probiotic mixtures of strains belonging to different genera or species may be more effective than single probiotics by complementing each other's health effects.[19] For example, promising results were obtained after administration

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of the VSL#3, a mixture of 8 probiotic bacteria, to patients affected by UC.[20]

In recent years many murine models of mucosal inflammation mimicking human IBD have been described. One of these models, the hapten-induced colitis in mice caused by intrarectal injection of trinitrobenzene sulfonic acid (TNBS), is a Th1 T cell-mediated colitis with many features of CD.[21] By using the TNBS-induced murine colitis model, in this study we investigated the efficacy of mixtures of different *Lactobacillus, Bifidobacterium*, and *Streptococcus* strains in the prevention of the mucosal disease. We studied the role of lamina propria lymphocytes (LPLs) and intraepithelial lymphocytes (IELs) by analyzing the different lymphocyte subsets including the regulatory T cells (Treg). These cells are a subpopulation of $CD4^+CD25^+$ T cells expressing the Foxp3 transcription factor and play a role in preventing the development and activity of potentially autoreactive T cells.[22] An alteration in the frequency of Treg has been recently found in the peripheral blood and mucosa of patients with IBD.[23–25] Recent studies have shown an induction of Treg by some probiotic strains in IBD patients and mice with experimentally induced colitis.[26–28] LPLs constitute the major effector cells along gut mucosal surfaces, whereas IELs are interspersed with enterocytes, thus having direct contact with foreign antigens derived from the gut lumen, and are thought to play a key role in the immune responses toward these antigens and in the pathogenesis of a variety of diseases.[29,30] A role in the repair from experimental colitis in mice has been ascribed to IELs due to the activity of $\gamma\delta$T cells,[31–33] which represent a major T-cell subpopulation within IELs.[34] While some studies have indicated a regulation of LPLs function by probiotics in IBD,[35] up to now there are no data of a possible involvement of IELs in the protective activity of probiotics in colitis.

MATERIALS AND METHODS

Bacteria

Two different mixtures were prepared: Mix1, containing *Lactobacillus acidophilus* Bar 13 and *Bifidobacterium longum* Bar 33 (1:1); and Mix2, containing *L. plantarum* Bar 10, *Streptococcus thermophilus* Bar 20, and *B. animalis* subsp. *lactis* Bar 30 (1:1:1). The mixtures contained 1×10^9 colony-forming units (CFU) of each strain. The probiotics were supplied by Barilla G&R f.lli SPA (Parma, Italy). Strains belonging to these species are commonly used in several dairy fermented foods and have been demonstrated to exert immunomodulatory activity.[8,9] Among the strains used in this study, *L. acidophilus* Bar 13 and *B. longum* Bar 33 were shown to be the most effective in pathogen protection and cytokine regulation.[36] *S. thermophilus* has a bifidogenic effect,[37] and for this property it is often used in association with probiotic *Lactobacillus* and *Bifidobacterium* strains.[20,26] All these strains are potential candidates for the development of new functional foods due to their ability to survive in dry food matrices (P. Carnevali, pers. commun.).

Animals

Female 8-week-old BALB/c mice, obtained from Charles River Laboratories (Como, Italy), were kept at 23°C with a 12-hour light-dark cycle and fed ad libitum with standard laboratory diets. Three groups of animals received phosphate-buffered saline (PBS) orally (control: C group), or the probiotic mixtures Mix1 or Mix2 (Mix1 and Mix2 groups), daily for 3 weeks. This duration was chosen on the basis of preliminary results indicating the maximal effectiveness of probiotics in colitis prevention, in agreement with previous studies.[26] A subgroup of each group was treated with trinitrobenzene sulfonic acid (TNBS, Sigma, Milan, Italy), 48 hours before the end of the third week (C + TNBS, Mix1 + TNBS and Mix2 + TNBS groups). Mice were slightly anesthetized by inhalation of isoflurane (Isoba, Schering-Plough Animal Health, Uxbridge, UK), and TNBS (125 mg/kg body weight in 50% ethanol) was administered intrarectally using a 1.1 × 48 mm catheter (BD Insyte, Milan), inserted 4 cm proximal to anus, according to Foligné et al.[38] A group of control mice received 50% ethanol with the same technique. After the instillation, mice were kept vertical for 1 minute. Mice had free access to food and water. Body weight was recorded weekly and every day following TNBS administration. Feces of all groups were collected at the beginning of the experiments (time zero: T0), and at the end of each week (T1, T2, and T3) for microbiological analysis. At the end of the experimental periods, animals were anesthetized with an intraperitoneal injection of pentobarbital (10 mg/kg), blood was drawn via cardiac puncture, and colons were excised and placed in cold PBS. Animal studies were performed under conditions approved by the National Health Ministry, Department of Food, Nutrition, and Animal Health.

Histological Analysis

Small segments of colon were immersed in Bouin's fixative for 12 hours, washed in PBS for 24 hours, embedded in paraffin at 58°C, and sectioned to 7 μm. After Mallory's staining the intestinal sections were analyzed under a light microscope (Zeiss Axioscope, Zeiss, Thornwood, NY). Colon damage and inflammation were assessed blindly, and the scores were assigned according to previously described criteria[39] as follows: extent of destruction of normal mucosal architecture with ulcerations (0, normal; 1, mild; 2, moderate; and 3, extensive damage), presence and degree of cellular infiltration (0, normal; 1, mild; 2, moderate; and 3, extensive with infiltration of submucosa), presence or absence of crypt (0, absent; 1, present), and

presence or absence of goblet cell depletion (0, absent; 1, present).

Analysis of Fecal Bacteria

Extraction of Bacterial DNA from Mice Fecal Samples

Total bacterial DNA was extracted from 200 mg of fecal samples by using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except that lysis buffer was added in a volume of 1.6 mL and half tablet of InhibitEX was used.

Real-time Quantitative PCR

Quantitative polymerase chain reaction (PCR) was performed with a LightCycler instrument (Roche, Mannheim, Germany) and SYBR Green I fluorophore was used to correlate the amount of PCR product with the fluorescence signal. Total bacterial DNA extracted from fecal samples was amplified with species-specific primer sets targeted to the 16S rRNA (rrn) gene or 16S-23S rRNA spacer region. The primer sets and the amplification conditions used were previously described: BiLON1/BiLON2[40] for *B. longum*; Bflact2/Bflact5[41] for *B. animalis* subsp. *lactis*; Aci-ITS.L/Aci-ITS.R[42] for *L. acidophilus*; F_plan/IDL62R[43,44] for *L. plantarum*; and ThI/ThII[45] for *S. thermophilus*. They amplify regions of 827, 660, 199, 376, and 240 bp, respectively. Three subsamples of each DNA extract were amplified in a final volume of 20 μL containing 4 mM of $MgCl_2$, 0.5 μM of each primer, 2 μL of LightCycler-FastStart DNA Master SYBR Green I (Roche), and either 2 μL of template or water (no-template control). Quantification of rrn operons of each species was done using standard curves made from known concentrations of genomic DNA of the administered strains. The number of rrn operons in each genome were deduced from the sequenced genomes of *B. longum* NCC2705,[46] *L. acidophilus* NCFM,[47] *L. plantarum* WCFS1,[48] and *S. thermophilus* LMD-9.[49] Since a complete genomic sequence for the species *B. animalis* is not available, the rrn operon copy number for *B. animalis* subsp. *lactis* Bar 30 was evaluated using real-time PCR (50), and resulted in being equal to 3. Chromosomal DNA of the strains used as standards was extracted using the DNeasy Tissue Kit (Qiagen) and serially diluted from 10^5 to 10^1 molecules μL^{-1}. Results obtained by PCR were converted to the average estimate of total rrn operons from each species present in 1 μg of total DNA.

Lymphocyte Preparation

IELs

Colons were placed on ice in 10 mL RPMI-1640 medium, washed twice with cold PBS, longitudinally opened, and cut to small pieces. Intestinal pieces were washed in Hank's balanced salt solution Ca^{2+} and Mg^{2+} free (HBSS-CMF) and stirred twice for 45 minutes at 37°C in an orbital shaker in HBSS-CMF added with 100 g/L fetal calf serum (FCS, Euroclone, Milan), 1×10^5 U/L penicillin, 100 mg/L streptomycin, 1 mM EDTA, 5 mM Hepes, and 1 mM dithiotreithol. The solution was passed through 100 and 40 μm nylon cell strainers (BD Falcon, Oxnard, CA) and centrifuged at 650*g*. IELs were isolated from enterocytes by discontinuous 44/67% gradient Percoll (Percoll GE Healthcare, Milan) in RPMI-1640 medium (Sigma), centrifuged at 650*g* for 25 minutes.

LPLs

After separation from IELs, colon fractions were washed with HBSS and stirred for 1 hour at 37°C with 10 mL RPMI-1640 medium containing 100 g/L FCS, 5 mM Hepes, 1.2×10^5 U/L collagenase I, and 10 mg/L DNAse (both from Sigma). Cells were filtered and centrifuged as described for IELs.

Flow Cytometry Analysis

The following monoclonal antibodies (BD Pharmingen, San Diego, CA) were used: FITC anti-CD3 (clone 17.12), PE anti-CD4 (clone GK1.5), PE-Cy5 anti-CD8 (clone 53-67), PE-Cy5 anti-TCR $\alpha\beta$ (clone H57-597), PE anti-TCR $\gamma\delta$ (clone GL3), and anti-CD16/CD32 (clone 2.4G2). Each antibody was titrated to determine the optimal concentration for maximal staining. The IELs and LPLs (1×10^6 cells) were preincubated for 20 minutes with anti-CD16/CD32 to block Fc receptors, thus avoiding nonspecific binding. Cells were then washed and labeled with appropriate mixture of antibodies or isotype matched controls for 30 minutes, centrifuged at 650*g*, and resuspended in FacsFlow (BD Biosciences, San Jose, CA). Flow cytometry analysis was performed using a FACSCalibur flow cytometer (BD Biosciences). To exclude dead/dying cells and therefore nonspecific antibody-binding cells, lymphocytes were gated according to forward and side scatter. The percentages of $CD4^+$, $CD8^+$, $CD4^+$ $CD8^+$, $\alpha\beta$T, and $\gamma\delta$T cells subsets were calculated on $CD3^+$ gate. At least 10,000 events were acquired and analyzed. Data were analyzed using CellQuest software (BD Biosciences).

The analysis of $CD4^+CD25^+Foxp3^+$ cells was performed in IELs and LPLs with a specific kit (eBioscience, San Diego, CA) staining CD4 (FITC), CD25 (PE), and transcription factor Foxp3 (PE-Cy5) according to the manufacturer's instructions. Briefly, lymphocytes were first stained for surface markers CD4 and CD25 for 30 minutes at 4°C, then permeabilized and stained for intracellular Foxp3 for 30 minutes at 4°C. Cells were analyzed by flow cytometry and the percentage of $CD25^+Foxp3^+$ cells was calculated on $CD4^+$ gate.

Cytokine Analysis

The levels of serum cytokines were analyzed using a mouse cytometric bead array (CBA) inflammatory kit (BD Biosciences) for interleukin (IL)-12, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, and IL-10 detection according to the manufacturer's specifications. Briefly, microbeads with distinct fluorescence intensities, coated with capture antibody specific for each cytokine, were incubated with serum samples and PE-conjugated detection antibodies for 2 hours. The samples were then washed, resuspended in 300 μL wash buffer, and analyzed by flow cytometry using FCAP array software (BD Biosciences).

Statistical Analysis

The significance of the differences was evaluated by 1-way analysis of variance (ANOVA) followed by Fisher's test. Statistical evaluation of changes in quantitative PCR results was carried out by Mann–Whitney *U*-test. Differences with P values < 0.05 were considered significant. Statistical analysis was performed with the Statistica for Windows software package (Stat Soft, Tulsa, OK).

RESULTS

Different Protection by Mix1 and Mix2 Against TNBS-Induced Colitis

We first verified whether the administration of Mix1 and Mix2 induced changes in the body weight and damages to the colon, and the results indicated no difference between C, Mix1, and Mix2 mice (data not shown). We then evaluated the TNBS-induced damages and the potential protection by Mix1 and Mix2 by body weight measurement and both macroscopic and microscopic evaluation of colitis. TNBS administration induced a significant body weight reduction, but this reduction was lower in the probiotic-treated mice: in fact, body weight of both Mix1 + TNBS and Mix2 + TNBS mice after 24 hours of TNBS administration and body weight of Mix 1 + TNBS mice after 48 hours were higher than C + TNBS mice (Fig. 1A). The macroscopic analysis of the intestine indicated the presence of diarrhea in the C + TNBS mice, which was absent in the Mix1 + TNBS mice and notably reduced in the Mix2 + TNBS mice (Fig. 1B). The microscopic examination of the colon (Fig. 1C) showed that TNBS administration provoked loss of mucosal architecture to C + TNBS mice, with ulcerations, transmural immune cell infiltration through the mucosa and submucosa, edema, goblet cells depletion, and crypt necrosis. Treatment with Mix1 was able to prevent the morphologic alterations induced by TNBS. In the Mix2 + TNBS mice the intestinal damage was almost abrogated, with the exception of a modest inflammatory cell infiltration. The histological scores are reported in Table 1. Administration of ethanol in control mice did not cause any damage (data not shown).

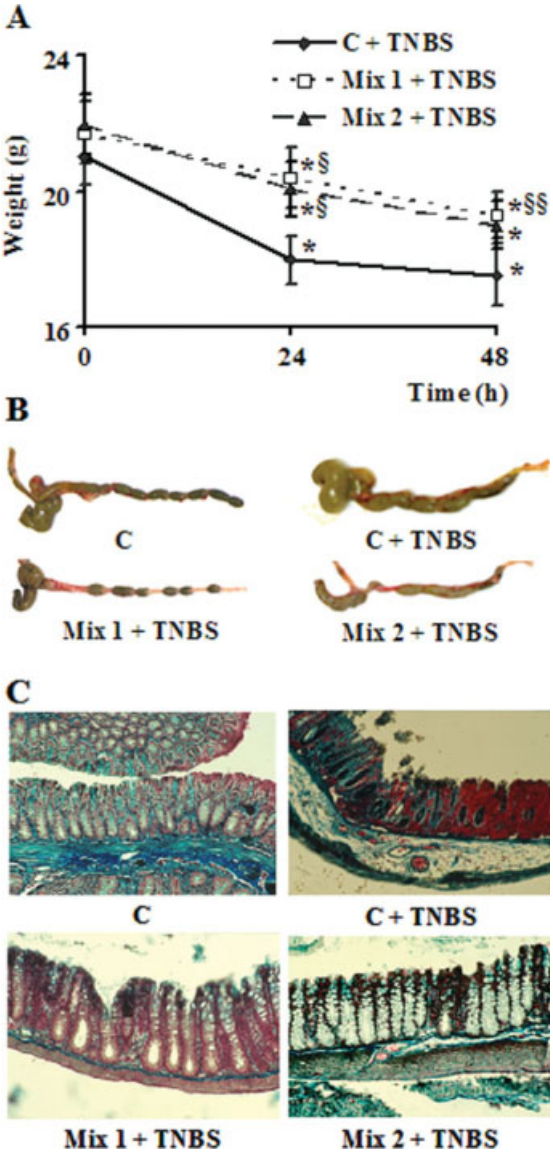

FIGURE 1. Protective effect of probiotic mixtures against the damage induced by TNBS. Mice received PBS orally (control, C), Mix1, containing *L. acidophilus* and *B. longum*, or Mix2, containing *L. plantarum, S. thermophilus*, and *B. animalis* subsp. *lactis* for 3 weeks. TNBS was administered rectally 48 hours before the end of the experimental period. (A) Body weight. (B) Representative macroscopic evaluation of colitis. (C) Representative microscopic evaluation of colitis, Mallory's staining was used. In panel A data represent the means ± SD of at least 10 mice. $^{*}P < 0.001$ versus time 0; $^{\S}P < 0.001$ versus C + TNBS; $^{\S\S}P < 0.05$ versus C + TNBS.

Colonic Bacterial Profile After Probiotic Administration

To verify whether the ingested bacteria were able to colonize the intestine and thus exert their protective

TABLE 1. Histological Scores

Damage Parameters	Mice			
	C	C + TNBS	Mix1 + TNBS	Mix2 + TNBS
Mucosal ulcerations	0.0 ± 0.0	2.9 ± 0.3*	0.0 ± 0.0	0.2 ± 0.4
Cellular infiltration	0.1 ± 0.3	2.9 ± 0.3*	0.2 ± 0.4	1.7 ± 0.5**
Crypt necrosis	0.0 ± 0.0	0.9 ± 0.3*	0.0 ± 0.0	0.0 ± 0.0
Goblet cells depletion	0.0 ± 0.0	0.9 ± 0.3*	0.0 ± 0.0	0.0 ± 0.0
Total score	0.1 ± 0.3	7.6 ± 1.2*	0.2 ± 0.4	1.9 ± 0.9**

Data are the means ± SD of at least 10 animals for each group. The score ranged from 0 to 3 as described in Materials and Methods.
*$P < 0.01$ versus all; **$P < 0.05$ versus all.

activity, we measured the amount of the probiotic strains in the feces at different times during the supplementation. The real-time PCR analysis indicated that at T0, that is, before Mix1 and Mix2 administration, all the probiotic species were present in the feces (Fig. 2A,B), with the exception of *L. plantarum*, which was completely absent (Fig. 2B). After 1 week of probiotic treatment (T1), the amount of *L. acidophilus* and *B. longum* (Fig. 2A), *L. plantarum, B. animalis* subsp. *lactis*, and *S. thermophilus* (Fig. 2B) was notably increased and remained high after another week of probiotic administration (T2). At T3, a decrease of the *B. lactis* population was observed in mice treated with TNBS and not in untreated mice, suggesting that this drug exerts a negative effect on the survival of *B. longum*. All the other probiotics were unaffected by TNBS administration and their concentration at T3 remained at values similar to those found at T2. No variation in microbiota composition was observed in the control mice group (data not shown).

Changes of T-cell Populations by Probiotics

To investigate whether the protection induced by the probiotics was exerted through modulation of intestinal T cells, we analyzed the $CD4^+$, $CD8^+$, $CD4^+CD8^+$, $TCR\alpha\beta^+$, and $TCR\gamma\delta^+$cell subsets in the gut. Besides the known involvement of LPLs in colitis, there is some evidence of a role of IELs in such disease. Thus, we analyzed the T-cell population of the 2 compartments of the intestine. In the intraepithelial compartment (Fig. 3), no differences were found between the C, Mix1, and Mix2 groups in the considered subpopulations. A significant decrease of the $CD4^+$ subpopulation was observed in the Mix1 + TNBS group as compared to the other groups. The Mix1 + TNBS mice were also characterized by a strong increase in the $TCR\gamma\delta^+$ subpopulation. Relating to the LPL subpopulations (Fig. 4), Mix1 and Mix2 did not induce significant changes in any of the subsets as compared to C mice. An increase in $CD4^+$ was induced by TNBS, which was prevented by Mix1 administration. In addition, a lower percentage of $\gamma\delta$T cells was found in the Mix1 + TNBS mice.

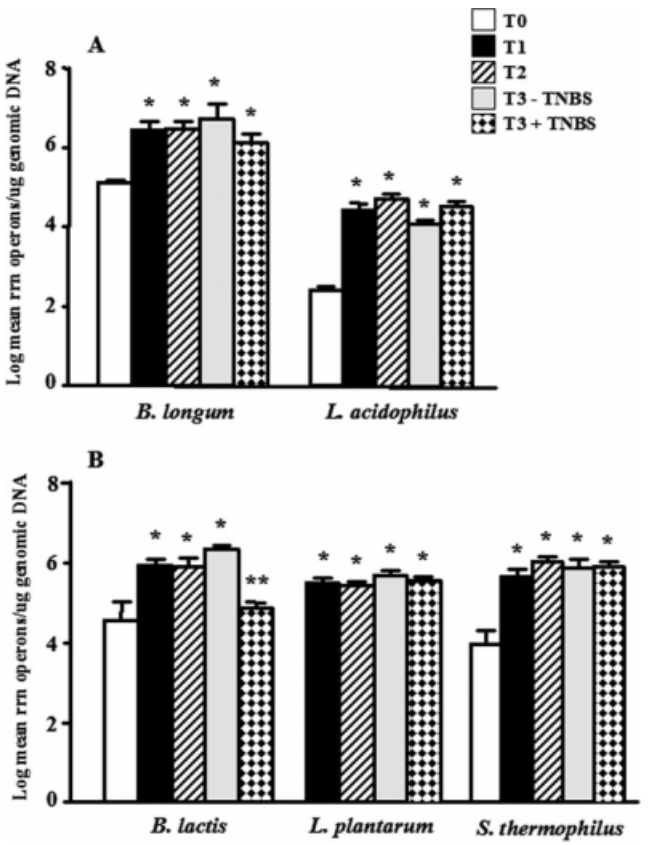

FIGURE 2. Real-time PCR evaluation of 16S rrn operons of probiotics present in the Mix1 (A) and Mix2 mice (B). Mice received orally Mix1, containing *L. acidophilus* and *B. longum*, or Mix2, containing *L. plantarum, S. thermophilus,* and *B. animalis* subsp. *lactis* for 3 weeks. TNBS was administered rectally 48 hours before the end of the third week. Stools were collected every week for 3 weeks, that is, at time (T) 0, 1, 2, 3. Data are expressed as log mean of rrn operons/μg of fecal genomic cDNA ± SD. *$P < 0.05$ versus T0; **$P < 0.05$ versus T3 - TNBS.

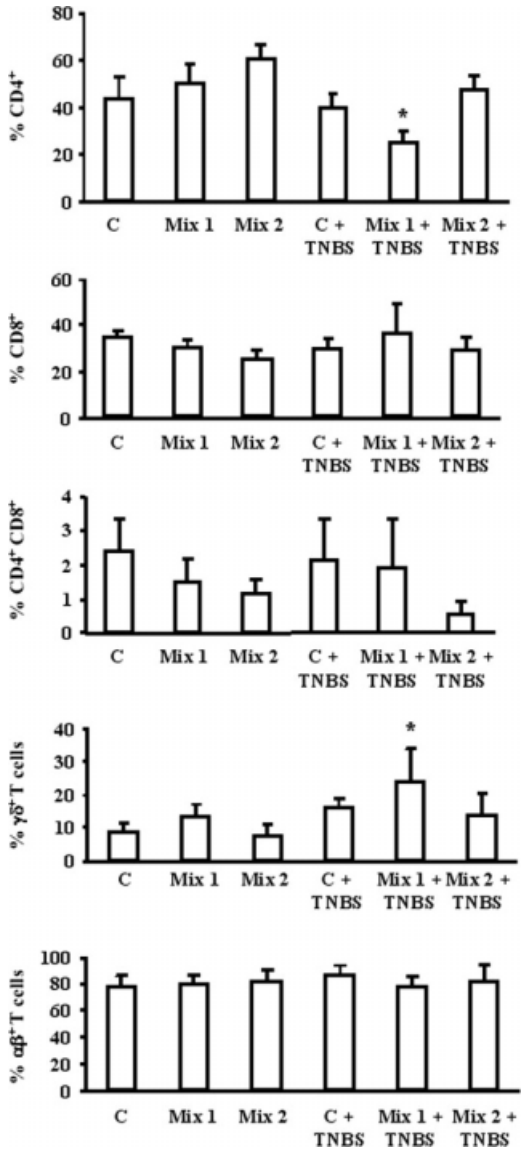

FIGURE 3. Effect of Mix1 and Mix2 on the frequency of intestinal intraepithelial lymphocytes subpopulations. Mice received PBS orally (control, C), Mix1, containing *L. acidophilus* and *B. longum*, or Mix2, containing *L. plantarum, S. thermophilus*, and *B. animalis* subsp. *lactis* for 3 weeks. TNBS was administered rectally 48 hours before the end of the experimental period. $CD4^+$, $CD8^+$, $CD4^+$, $CD8^+$, $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cell subsets were analyzed by flow cytometry. Data represent the means ± SD of at least 10 mice. $^*P < 0.05$ versus all.

Modulation of Intraepithelial Treg Cells by Probiotic Mixtures

Since an important role in colitis has been ascribed to Treg cells, we investigated whether the prevention of TNBS-induced damage was associated with a modulation of Treg population of both IELs and LPLs. In the intraepithelial compartment (Fig. 5A,C), although the frequency of $CD4^+CD25^+Foxp3^+$ cells was not changed in the C + TNBS mice, a strong increase in the frequency of these cells was observed in the Mix1 + TNBS and Mix2 +

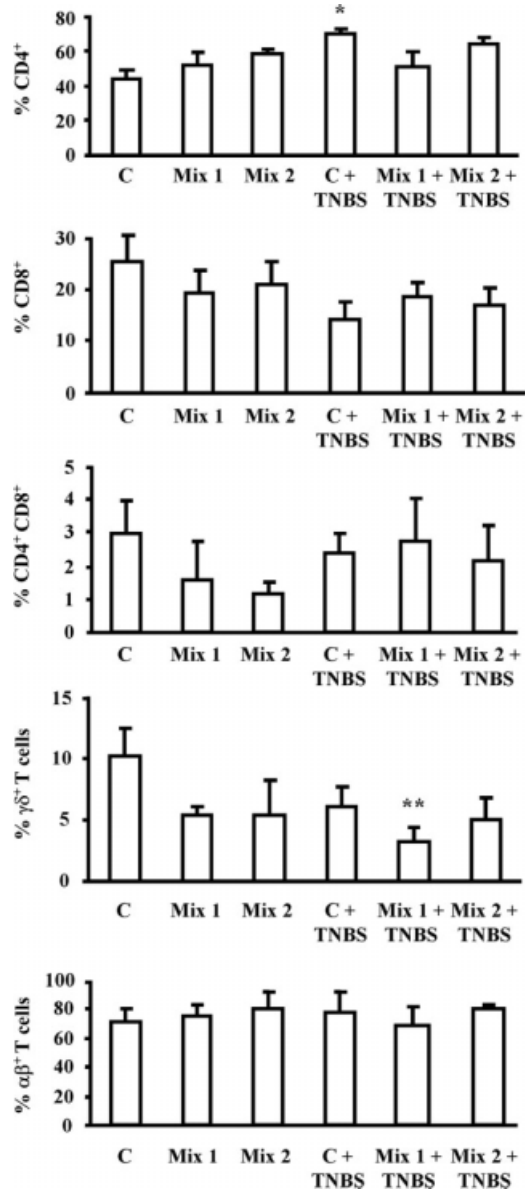

FIGURE 4. Effect of Mix1 and Mix2 on the frequency of intestinal lamina propria lymphocytes subpopulations. Mice received orally PBS (control, C), Mix1, containing *L. acidophilus* and *B. longum*, or Mix2, containing *L. plantarum, S. thermophilus*, and *B. animalis* subsp. *lactis* for 3 weeks. TNBS was administered rectally 48 hours before the end of third week. $CD4^+CD8^+$, $CD4^+CD8^+$, $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cell subsets were analyzed by flow cytometry. Data represent the means ± SD of at least 10 mice. $^*P < 0.05$ versus C and Mix1 + TNBS; $^{**}P < 0.05$ versus C and C + TNBS.

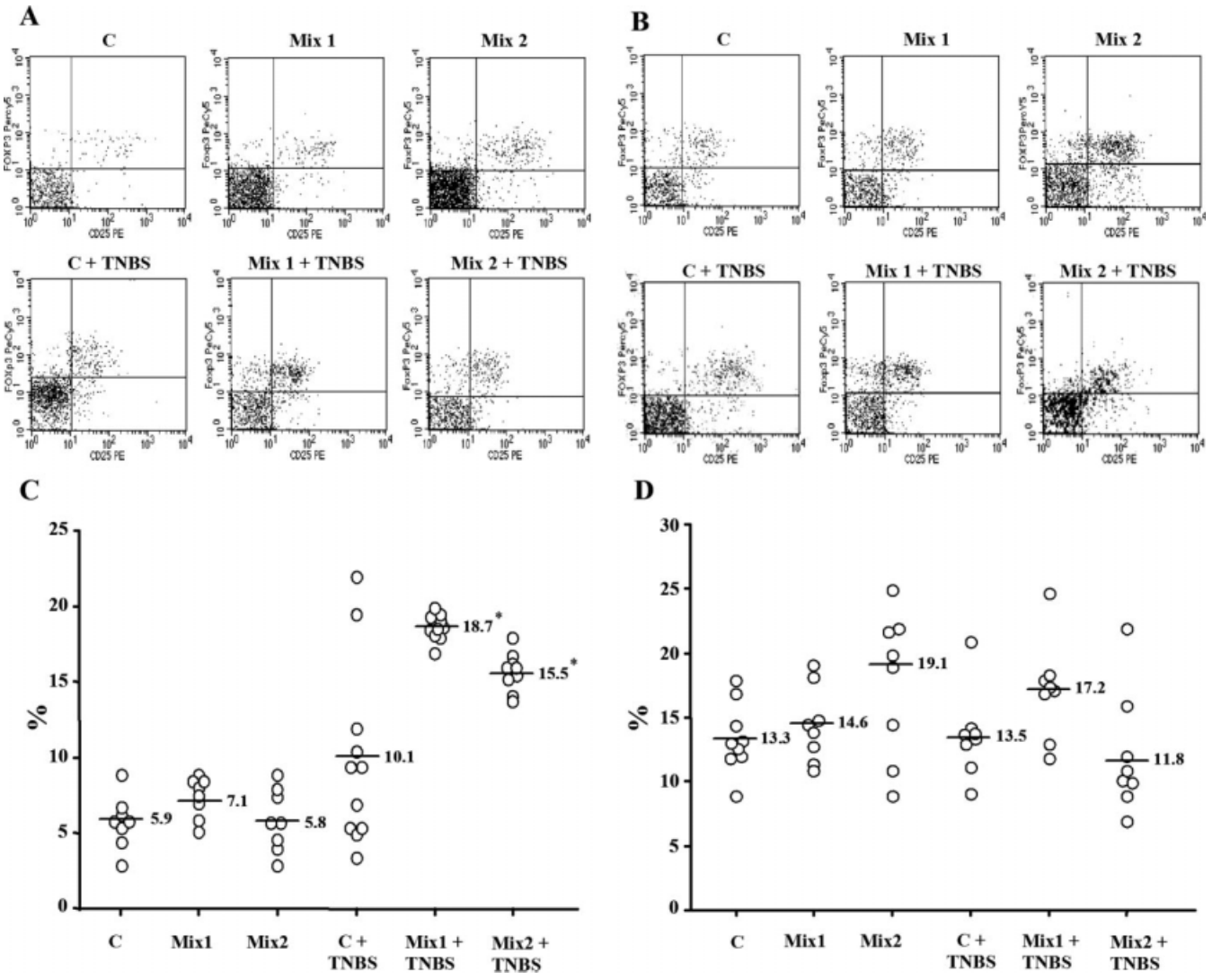

FIGURE 5. Modulation of Treg cells of intestinal intraepithelial lymphocytes (A,C) and lamina propria lymphocytes (B,D) by probiotic mixtures. Mice received PBS orally (control, C), Mix1, containing *L. acidophilus* and *B. longum*, or Mix2, containing *L. plantarum, S. thermophilus*, and *B. animalis* subsp. *lactis* for 3 weeks. TNBS was administered rectally 48 hours before the end of third week. (A,B) Representative dotplots of $CD4^+CD25^+Foxp3^+$ cells analyzed by flow cytometry. (C,D) Scatterplots of the percentage of $CD4^+CD25^+Foxp3^+$ cells. Numbers represent mean values. *$P < 0.01$ versus all.

TNBS groups that was significantly different from all the other groups. In the LPLs (Fig. 5B,D), no difference in the amount of Treg was found in any group of mice.

Modulation of Cytokine Production by Probiotic Mixtures

We then investigated whether the changes in lymphocyte populations were associated with modifications in pro- and antiinflammatory cytokine production (Fig. 6). A considerable increase of IL-12, IFN-γ TNF-α, and MCP-1 was induced by TNBS. When the mice received the probiotic mixtures before TNBS administration, a different cytokine profile was found. Indeed, Mix1 was able to prevent the increase of all these proinflammatory cytokines, while Mix2 was effective in inhibiting the production of TNF-α and MCP-1 but not IL-12 and IFN-γ. No difference in the IL-6 levels were observed. Both the Mix1 and Mix2 induced a significant increase in the antiinflammatory IL-10 production.

DISCUSSION

In this study we evaluated the antiinflammatory activity of 2 probiotic mixtures in a mouse model of

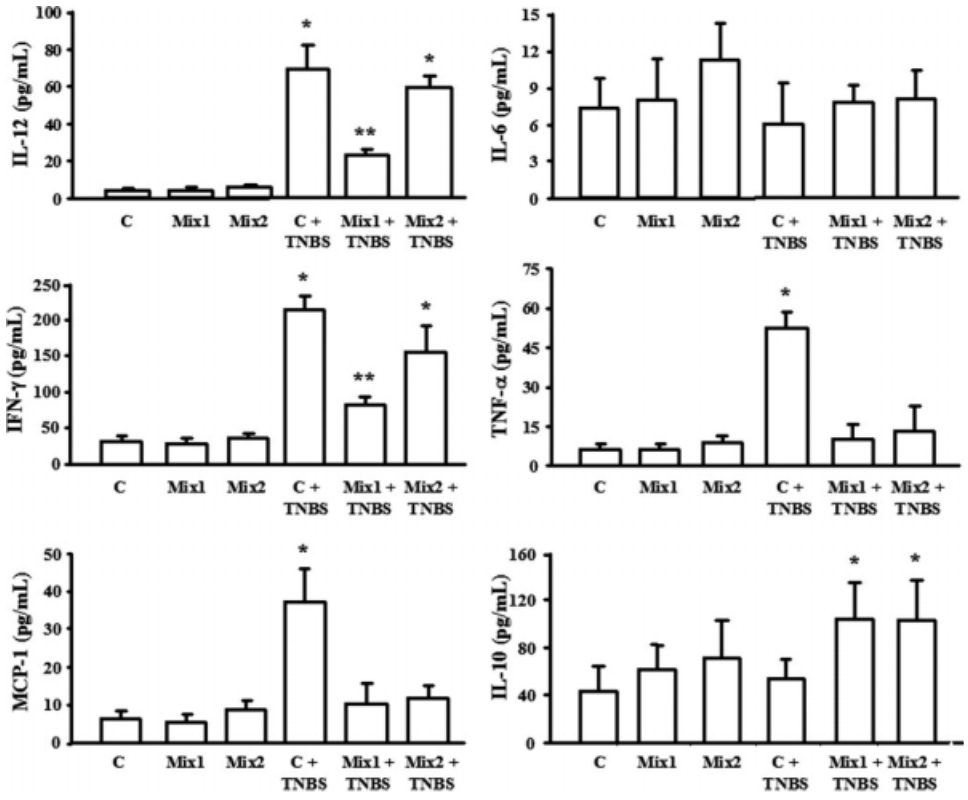

FIGURE 6. Modulation of cytokine production by probiotic mixtures. Mice received PBS orally (control, C), Mix1, containing *L. acidophilus* and *B. longum*, or Mix2, containing *L. plantarum, S. thermophilus*, and *B. animalis* subsp. *lactis* for 3 weeks. TNBS was administered rectally 48 hours before the end of third week. Cytokine levels were analyzed in serum by flow cytometry with a CBA inflammatory kit. Data represent the means ± SD of at least 10 mice. For IL-12, IFN-γ, TNF-α, and MCP-1: *$P < 0.001$, versus all; **$P < 0.05$, versus C, Mix1, Mix2, C + TNBS and Mix2 + TNBS. For IL-10: *$P < 0.05$, versus C and C + TNBS.

experimentally induced colitis mimicking human CD by performing for the first time, to our knowledge, an extensive analysis of the phenotypic heterogeneity and function of both IELs and LPLs of the colon. We report that the 2 probiotic mixtures were able to prevent the TNBS-induced intestinal inflammation and damage, and the most efficient was the mixture of *L. acidophilus* and *B. longum*. We provide evidence of a new ability of these probiotics to modulate the function of IELs, where a reduction of $CD4^+$ T cells and an expansion of $\gamma\delta$T cells as well of $CD4^+CD25^+Foxp3^+$ cells were induced. All the bacteria used in this study were able to colonize the intestine during the experimental period, indicating that the protective effects were effectively due to the increased number of probiotics.

Intestinal lymphocytes, including both IELs and LPLs, form highly specialized lymphoid compartments, and play a critical role in the mucosal immune system regulation by providing immune surveillance of the epithelium. IELs represent the first response to antigens and bacteria, as they establish the closest contact with the lumen, whereas LPLs, which reside in the underlying mucosa, act later. In particular, $\gamma\delta$ IELs are considered to be a T-cell population playing the first line of host defense against a variety of antigens and pathogens, contributing to the maintenance of intestinal homeostasis at epithelial sites.[51] These cells accumulate at inflammation sites, where they have been shown to reduce the inflammatory reaction and tissue damage in collagen-induced arthritis and insulin-dependent diabetes in mice.[52,53] Inagaki-Ohara et al[51] showed that $\gamma\delta$T cells are involved in colitis regulation and represent a significant protective T-cell subpopulation, since development of spontaneous colitis in $\gamma\delta$T-cell-deficient mice is suppressed by $\gamma\delta$ IELs transfer. In addition, several studies have indicated an increase in susceptibility to experimentally induced colitis in $\gamma\delta$T-cells-depleted mice.[32,51,54] In agreement with these findings, we report that the suppression of colitis was associated with an increase in $\gamma\delta$T cells of IELs induced by *L. acidophilus* and *B. longum*, suggesting a new function of these probiotics in the prevention of colitis. On the contrary, we found a small decrease in $\gamma\delta$T cells of LPLs after *L. acidophilus* and *B. longum* administration. Recently, an increase in $\gamma\delta$T cells was shown by some authors in lamina propria of $TCR\alpha^{-/-}$ mice that develop spontaneous colitis with several features of human UC.[55,56] The same authors have shown that $\gamma\delta$T cells exacerbated the colitis by inducing proinflammatory cytokines, neutrophils, and monocyte-chemoattractant chemokines in lamina propria.[56] These results suggest that $\gamma\delta$T cells of IELs and LPLs may have different and opposite roles in

colitis. Based on this consideration, the decrease of $\gamma\delta$T cells in LPLs induced by *L. acidophilus* and *B. longum* can be seen as a favorable change for the suppression of colitis.

Our results showed that the 2 probiotic mixtures induced an increase in the frequency of $CD4^+$ $CD25^+Foxp3^+$ cells of IELs and not of LPLs. Also in this case, the mixture of *L. acidophilus* and *B. longum* was more effective than the other probiotics mixture. The Treg cells have a fundamental role in maintaining gut immune homeostasis and may exert potent suppressive activity by inhibiting the proliferation of effector cells and production of Th1 and Th2 cytokines.[57] The importance of Treg in the prevention of colitis has been demonstrated by previous studies showing that transfer of naive $CD4^+$ T cells into immunodeficient mice in the absence of Treg cells resulted in colitis,[58,59] while transfer of Treg ameliorated or reversed pathology.[60,61] Furthermore, a reduced frequency of Treg was shown in peripheral blood in patients with IBD.[24,25] In contrast, an increased number of Treg cells was found in lamina propria, mesenteric lymph nodes, and intestinal inflamed mucosa of patients with either CD or UC,[23,24,62] although the increase in IBD lesions was lower compared with inflammatory controls.[24] These data suggest that Treg cells traffic to the site of inflammation in an attempt to halt the progression of the disease. Together, the results of these studies support the idea that expansion of the Treg cells may represent a therapeutic tool for the treatment of IBD. There is some evidence that probiotics supplementation is able to ameliorate the colitis by modulation of Treg cells. By using a mouse colitis model, some authors showed that the VSL#3 probiotic cocktail conferred protection against the chemically induced intestinal inflammation by the induction of $CD4^+$TGF-β-bearing Treg cells.[26] Furthermore, studies conducted in IBD patients demonstrated an antiinflammatory effect of *L. rhamnosus* and *L. reuteri* supplementation that paralleled the expansion of peripheral Treg cells.[27] Thus, our results of the Treg increase after probiotic administration are consistent with a role of probiotics in prevention of colitis through induction of Treg cells. The cytokine profiles found in this study are compatible with the ability to enlarge the Treg cells population and the antiinflammatory activity elicited by the probiotics. Indeed, we report that both probiotic mixtures inhibited the production of TNF-α and MCP-1, and the *L. acidophilus* and *B. longum* mixture was also able to reduce the production of IL-12 and IFN-γ. In addition, we found an increase of IL-10 induced by the 2 probiotic mixtures. Of these cytokines, TNF-α and IFN-γ are crucial mediators in the inflammatory cascade of IBD.[63,64] IL-12 is the primary cytokine in directing T-cell differentiation toward Th1 effector cells and thus is considered to be among the major cytokines in the pathogenesis of CD.[64,65] On the other hand, IL-10 is a critical factor for Treg function,[66] and is necessary to abrogate established intestinal inflammation, as demonstrated by the inability of Treg cells to cure colitis if the IL-10 signaling is blocked.[61] In addition, and in agreement with our results, an increase in IL-10 was found associated with colitis protection at the end of 3-week probiotic treatment before the establishment of TNBS colitis.[26]

In conclusion, we demonstrate that the 2 probiotic mixtures used in this study were able to protect against TNBS-induced colitis, and the *L. acidophilus* and *B. longum* mixture was the most effective by preventing all damages, suggesting a potential use of such bacteria to prevent gut inflammatory diseases such as CD. Other than an involvement of LPLs, our results report a novel importance of the IELs population in probiotic protection, where an expansion of $\gamma\delta$T cells and of $CD4^+CD25^+Foxp3^+$ cells was induced by probiotic administration.

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