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Genetically Engineered Immunomodulatory *Streptococcus thermophilus* Strains Producing Antioxidant Enzymes Exhibit Enhanced Anti-Inflammatory Activities

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The aims of this study were to develop strains of lactic acid bacteria (LAB) having both immunomodulatory and antioxidant properties and to evaluate their anti-inflammatory effects both *in vitro*, in different cellular models, and *in vivo*, in a mouse model of colitis. Different *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains were cocultured with primary cultures of mononuclear cells. Analysis of the pro- and anti-inflammatory cytokines secreted by these cells after coincubation with candidate bacteria revealed that *L. delbrueckii* subsp. *bulgaricus* CRL 864 and *S. thermophilus* CRL 807 display the highest anti-inflammatory profiles *in vitro*. Moreover, these results were confirmed *in vivo* by the determination of the cytokine profiles in large intestine samples of mice fed with these strains. *S. thermophilus* CRL 807 was then transformed with two different plasmids harboring the genes encoding catalase (CAT) or superoxide dismutase (SOD) antioxidant enzymes, and the anti-inflammatory effects of recombinant streptococci were evaluated in a mouse model of colitis induced by trinitrobenzenesulfonic acid (TNBS). Our results showed a decrease in weight loss, lower liver microbial translocation, lower macroscopic and microscopic damage scores, and modulation of the cytokine production in the large intestines of mice treated with either CAT- or SOD-producing streptococci compared to those in mice treated with the wild-type strain or control mice without any treatment. Furthermore, the greatest anti-inflammatory activity was observed in mice receiving a mixture of both CAT- and SOD-producing streptococci. The addition of *L. delbrueckii* subsp. *bulgaricus* CRL 864 to this mixture did not improve their beneficial effects. These findings show that genetically engineering a candidate bacterium (e.g., *S. thermophilus* CRL 807) with intrinsic immunomodulatory properties by introducing a gene expressing an antioxidant enzyme enhances its anti-inflammatory activities.

Inflammatory bowel disease (IBD) is a group of chronic inflammatory disorders of the gut with a substantial socioeconomic impact worldwide (1). The two major forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by an uncontrolled inflammatory response to luminal content and which differ mostly by the region of the gut where inflammation progresses and the depth of inflammatory damage. Conventional IBD therapy with antibiotics and corticosteroids is often associated with negative side effects. The difficulty in developing new therapeutic options for the treatment of IBD is thus a major challenge, from a medical and patient care point of view and in regard to economic and social aspects as well.

Currently, numerous studies have suggested possible health benefits associated with the consumption of probiotics ("live microorganisms which when administered in adequate amounts confer a health benefit on the host" [2]). In particular, probiotics represent a potential alternative for IBD treatment due to their active role in enhancing the intestinal barrier function, by either modifying the intestinal microbiota or modulating the host immune response (3, 4). Notably, it has been previously shown that a yogurt prepared with a pool of candidate probiotic strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* from the Culture Collection of the Centro de Referencia para Lactobacilos (CERELA) (San Miguel de Tucumán, Argentina) diminished the severity of inflammation in a mouse model of chemically induced colitis by modulating the immune response of the host, mainly due to an increase in the number of interleukin-10 (IL-10)-producing cells at the intestinal level with a significant decrease of IL-17 and IL-12 production (5, 6). More recently, it has been described that genetically engineered lactic acid bacteria (LAB) producing antioxidant enzymes such as catalase (CAT) or superoxide dismutase (SOD) were able to reduce the inflammation in different murine models of chemically induced colitis through a different mechanism, which was a reduction of reactive oxygen species (ROS) levels in the gut (7–10). From this perspective, we hypothesized that the expression of such antioxidant enzymes in LAB strains with proven intrinsic immunomodulatory properties could enhance their anti-inflammatory activities. Therefore, the aims of this study were to construct a novel anti-inflammatory LAB strain with immunomodulating as well as antioxidant properties and to evaluate its anti-inflammatory activity *in vitro* and *in vivo*.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. delbrueckii* subsp. *bulgaricus* (CRL 861, 863, 864, 866, 869, 871, 872 and 887) and *S. thermophilus* (CRL 806 and 807) strains from the CERELA Culture Collection (Tucumán, Argentina) were grown for 16 h at 37°C without agitation in MRS (Britania, Buenos Aires, Argentina) and LAPTg (1% glucose, 1.5% peptone, 1% tryptone, 1% yeast extract, and 0.1% Tween 80) medium, respectively.

For fermented milk preparation, bacterial strains were grown overnight in 5 ml reconstituted sterile (autoclaved at 110 to 115°C for 10 min) nonfat milk (Milkaut, Argentina). These cultures were used to inoculate the milk with either *L. delbrueckii* subsp. *bulgaricus* or *S. thermophilus* strains at a concentration of 1% (vol/vol) and incubated without agitation for 16 h at 37°C. Yogurt was prepared freshly by inoculating milk with the pools of lactobacilli and streptococci (grown in milk) at a concentration of 0.5% (vol/vol) each and incubated without agitation for 16 h at 37°C.

In vitro **assays using different cellular models.** Commercial peripheral blood mononuclear cells (PBMC) from healthy donors were obtained from StemCell Technologies (Grenoble, France) and grown in RPMI 1640 medium (Sigma, St. Louis, MO, USA) with 1% L-glutamine and 10% heat-inactivated fetal bovine serum (FBS) in a humidified 10% CO_2 atmosphere at 37°C (32). Isolation of Peyer's patch mononuclear cells (PPMC) from healthy mice was performed as previously described (11).

For PBMC, cells were spread in 24-well culture plates at 1×10^6 cells/well. The LAB strains were grown in MRS overnight (16 h), and then the cultures were diluted to an optical density (OD) at 600 nm of 0.3, washed with 0.01 M phosphate-buffered saline (PBS) (pH 7.4), resuspended in PBS, and added at a multiplicity of infection (MOI) of $1{:}10^2$ (cells/bacteria). Plates were coincubated for 24 h at 37°C in 10% CO_2 and samples collected for IL-10 and IL-12 determination by enzyme-linked immunosorbent assay (ELISA) (MABTECH AB, Sweden). PBS without bacteria was used as negative control.

For PPMC isolated from mice, cells were spread in 6-well culture plates at 2×10^6 cells/well. Bacteria were grown in milk because the *in vivo* assay would be performed with fermented milk. Bacterial counts were determined in MRS agar plates, and PPMC were cocultured in RPMI 1640 for 24 h at 37°C with 20 μl of each individual strain grown in milk for 16 h (1×10^9 CFU/ml; MOI of 1:10 [cells/bacteria]) or with noninoculated milk or yogurt as negative and positive controls, respectively. IL-10 and gamma interferon (IFN-γ) concentrations from PPMC supernatants were measured using BD OptEIA cytokine ELISA sets (BD Bioscience, San Diego, CA, USA).

In vivo **evaluation of the intestinal cytokine profiles induced by the administration of selected LAB to healthy mice.** BALB/c mice (female, 5 weeks old, weighing 20 to 25 g) were obtained from the inbred animal facilities at CERELA. All animal protocols were approved by the Animal Protection Committee of CERELA (CRL-BIOT-LT-2010/1A), and all experiments comply with the current laws of Argentina.

Groups of mice ($n = 5$) were fed for 10 days *ad libitum* with either unfermented milk or milk fermented by *L. delbrueckii* subsp. *bulgaricus* CRL 864 or *S. thermophilus* CRL 807. The mice were then sacrificed, and the intestinal contents were collected from their large intestines with 1 ml PBS and immediately centrifuged at $5{,}000 \times g$ for 15 min at 4°C. The supernatants were recovered and stored at −20°C until determination of IFN-γ and IL-10 concentrations using BD OptEIA cytokine ELISA sets (BD Bioscience).

The large intestines and ceca were removed and prepared for histological evaluation using standard methods. Cytokine-positive cells were detected by indirect immunofluorescence on large intestine tissue slides, using a previously described technique (12). Rabbit anti-mouse IL-10 (ProSci Inc., Poway, CA, USA) or goat anti-mouse IL-17 (BD Bioscience, San Diego, CA, USA) polyclonal antibodies were used as primary antibodies, and goat anti-rabbit or rabbit anti-goat antibodies conjugated with fluorescein isothiocyanate (FITC) (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) were used as secondary antibodies. The number of fluorescent cells was counted by two scientists (two individual blinded counts per sample), and the results were expressed as the number of positive cells for each cytokine in 10 fields of vision as seen at a magnification of ×1,000 using a fluorescence light microscope (Carl Zeiss, Germany).

DNA manipulations. The plasmids used in this study (pIL253-*sodA* and pIL253-*mnkat*) were isolated from the *Lactobacillus casei* BL23 strain harboring the vector of interest (BL23:SOD and BL23:CAT, respectively) (13). Briefly, lysis was performed by incubating bacterial pellets with 10 mg/ml lysozyme for 1 h at 37°C. After this step, the Accuprep Plasmid Mini Extraction kit (Bioneer, Daejeon, South Korea) was used according to manufacturer's instructions. *S. thermophilus* strain CRL 807 was transformed with either pIL253-*sodA* or pIL253-*mnkat* plasmid using a modified electroporation protocol (14). Briefly, in order to obtain competent cells, this strain was grown in 5 ml of M17 medium (Difco, Detroit, MI, USA) containing 1% glucose (GM17) for 16 h at 37°C. This culture was diluted in 200 ml of GM17 supplemented with 1% glycine and grown at 37°C to an optical density (OD) at 600 nm of 0.4 to 0.6, and the cells were harvested by centrifugation at $5{,}000 \times g$ (4°C). After 4 washes with ice-cold 0.5 M sucrose containing 10% glycerol, the cells were finally resuspended in 1 ml of cold 30% polyethylene glycol (PEG) 3000–10% glycerol and stored in aliquots (100 μl) at −80°C until further use. For electroporation, 100 μl was thawed on ice, mixed with 100 to 200 ng of plasmid, and then transferred to an ice-cooled electroporation cuvette. The sample was subjected to a 2.5-kV, 200-Ω, 25-μF electric pulse in a 0.2-cm cuvette by using a Gene Pulser and a Pulse Controller apparatus (Bio-Rad, Richmond, CA, USA). Transformants were selected based on the antibiotic resistance on GM17 agar plates containing 2.5 μg/ml erythromycin as described previously (15) and then transferred to fresh broth GM17 medium with 5 μg/ml erythromycin in order to confirm the acquired resistance in the recombinant strains. Plasmid DNA was extracted as described above and characterized by enzymatic digestion. Recombinant *S. thermophilus* CRL 807 strains harboring either pIL253-*mnkat* or pIL253-*sodA* plasmid were named *S. thermophilus* CRL 807:CAT and CRL 807:SOD, respectively. Plasmid stability was evaluated by growing the transformants without a pressure selection (i.e., use of antibiotics) for 200 generations, and we confirmed the presence of *kat* and *sod* genes by PCR. We also confirmed their identity by restriction enzyme analysis. The same protocol was used to detect the presence of plasmids after milk fermentation.

Characterization of recombinant LAB strains. Transformant candidates were confirmed by PCR using previously described specific primers for the *mnkat* (16) or *sodA* (10) gene and by detection of CAT and SOD activities. Cell pellets resuspended in MilliQ water were used as templates for amplification. PCR was performed using *Taq* DNA polymerase (Invitrogen Life Technologies, São Paulo, Brazil) according to the manufacturer's instructions.

In order to detect enzyme production, cultures of recombinant LAB strains grown overnight in medium or in milk were inoculated at a 1:50 dilution in 10 ml fresh medium supplemented with erythromycin. At an OD at 600 nm of 0.4 to 0.6, cultures were centrifuged at $5{,}000 \times g$ for 5 min and bacterial pellets resuspended in 1 ml of cold 50 mM potassium monobasic phosphate buffer (pH 7.0) and homogenized in a Bead Beater apparatus (BiospecProducts Inc., Bartlesville, OK, USA) with 0.1 mm zirconia-silica beads (catalogue number 110791012; Biospec Products Inc.). After 4 cycles of disruption (2 min) alternating with 2 min of incubation on ice, samples were centrifuged ($8{,}000 \times g$, 10 min, 4°C), and the supernatants were immediately used to determine enzymatic activities. The protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) based on method of Bradford (17), using bovine serum albumin as a standard. CAT and SOD activities were determined as previously described (18).

Preparation of bacterial suspensions and fermented milk for mouse administration. Wild-type (WT) and recombinant streptococci (i.e., *S. thermophilus* CRL 807:CAT and CRL 807:SOD) were grown in 10 ml of LAPTg medium, washed, and resuspended in 1 ml of saline solution in order to obtain a final concentration of 1×10^{10} CFU/ml. Coadministra-

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tion of *S. thermophilus* CRL 807:CAT and CRL 807:SOD (here called *S. thermophilus* CRL 807:CAT/SOD) was performed by mixing each strain suspension in a 1:1 ratio.

Before being used in the preparation of the fermented milk or administered to the mice, recombinant streptococcal cultures were washed twice with 5 ml of saline solution (0.9% NaCl) in order to eliminate any remaining traces of the antibiotic. The oral administration of 100 μl of each strain, strain mixture suspension, or saline solution was performed daily using a catheter attached to a micropipette.

For fermented milk administration, reconstituted sterile nonfat milk (Milkaut, Argentina) was inoculated with the WT, CAT, or SOD-producing streptococci strains described above at a concentration of 1% (vol/vol) or with the *S. thermophilus* CRL 807:CAT/SOD mixture (0.5% each strain). Milk was incubated statically for 16 h at 37°C.

The fermented milk prepared under these conditions contained an average number of live bacteria of 1×10^{10} CFU/ml. Mice received the fermented milk products or unfermented milk *ad libitum*. Fermented or unfermented milk was prepared and changed daily (with checking that the quality of the product was maintained), and the intake per mouse was followed. Each animal in this trial drank approximately 3 ml of milk per day.

Colitis induction and administration of bacteria. Induction of trinitrobenzenesulfonic acid (TNBS) colitis was achieved essentially as previously described (5). Briefly, BALB/c mice (female, 5 weeks old) were fully anesthetized with an intraperitoneal injection of ketamine hydrochloride (Holliday-Scott S.A., Buenos Aires, Argentina) (100 μg/g body weight) mixed with xylazine hydrochloride (Rompun; Bayer, Division Sanidad Animal, Buenos Aires, Argentina) (5 μg/g body weight). Colitis was then induced by intrarectal inoculation of TNBS solution (Sigma, St. Louis, MO, USA) (2 mg/mouse) dissolved in 0.01 M phosphate-buffered saline (PBS) (pH 7.4) and mixed with an equal volume of ethanol (50% ethanol), using a 4-cm-length catheter. Control mice (mock group) received only PBS mixed with ethanol (without TNBS) using the same technique.

TNBS-treated mice were subdivided into 5 groups ($n = 6$): (i) inflammation control group (or TNBS group), (ii) mice receiving the *S. thermophilus* CRL 807 WT strain (WT group), (iii) mice receiving *S. thermophilus* strain CRL 807 producing CAT (*S. thermophilus* CRL 807:CAT group), (iv) mice receiving *S. thermophilus* strain CRL 807 producing SOD (*S. thermophilus* CRL 807:SOD group), and (v) mice receiving coadministration of both recombinant *S. thermophilus* CRL 807 strains producing CAT and SOD (*S. thermophilus* CRL 807:CAT/SOD group). LAB were administered to mice one day before TNBS injection and for 3 consecutive days after colitis induction as a suspension in a volume of 100 μl of saline solution using a gavage syringe or in the fermented milk *ad libitum*. TNBS and mock groups received either 100 μl of saline solution by gavage or unfermented milk *ad libitum*.

All groups were fed *ad libitum* with balanced rodent diet and maintained in a room with a 12-h light/dark cycle at 18 ± 2°C. Body weight and animal mortality rates were controlled daily.

Assessment of colonic inflammation. Four days after TNBS injection, 3 mice per group were sacrificed. Large intestines and ceca were removed, visually inspected for macroscopic evaluation, and prepared for histological analysis using standard methods. Serial paraffin sections of 4 μm were made and stained with hematoxylin-eosin (HE) for light microscopy examination. Macroscopic lesions and extent of colonic damage and inflammation were assessed using previously described grading systems (13). The analyses were performed by two different scientists. High macroscopic or histological damage scores indicate increased damage in the intestines. Microbial translocation to liver was determined essentially as previously described (19). Briefly, the liver was aseptically removed, weighed, and homogenized in 5.0 ml sterile 0.1% (wt/vol) peptone solution. Serial dilutions of the homogenate were plated in triplicate in MRS, MacConkey, and LAPTg media to detect a wide range of microorganisms. Bacterial growth was evaluated after incubation of the plates at 37°C for 48 to 72 h.

Enzyme activity in the intestinal contents was determined. Large intestines were washed with 500 μl of cold 50 mM potassium monobasic phosphate buffer (pH 7.0). The samples were centrifuged at 3,000 × *g* and 4°C for 15 min, the supernatants were collected, and the pellet was resuspended in 500 μl of cold 50 mM potassium monobasic phosphate buffer and homogenized in a Bead Beater apparatus with 0.1-mm zirconia-silica beads, as described above. CAT and SOD activities and protein concentration in both the supernatant and pellet were determined as previously described (18). Results were expressed as specific units for the enzymatic activity from the addition of the results obtained for the supernatant and the respective pellet.

Cytokine producer cells in the intestinal tissue samples were also analyzed. IL-10- and IL-17-positive cells were detected by indirect immunofluorescence following the technique described above.

Statistical analyses. The *in vitro* analyses were performed in triplicate. For animal experiments, 3 mice of each group (from a group of 6 animals [see above]) were sacrificed and samples collected. The experimental protocol was performed three times (no interactions between these 3 independent trials were observed), and the results (from the 3 trials) were analyzed together ($n = 9$). Statistical analyses were performed using MINITAB 15 software. A factorial design (replicates-treatment) was used for the experiments. Comparisons were done with an analysis of variance (ANOVA) general linear model followed by Tukey's *post hoc* test, and unless otherwise specified, a P value of <0.05 was considered significant.

RESULTS

Cytokine profiles induced by preselected strains of lactobacilli and streptococci *in vitro*. As shown in Fig. 1A, cocultures of PBMC with *L. delbrueckii* subsp. *bulgaricus* CRL 887, *S. thermophilus* CRL 806, and *S. thermophilus* CRL 807 resulted in the highest anti-inflammatory cytokine/proinflammatory cytokine (IL-10/IL-12) ratio (Fig. 1A). The cytokine profile was also studied using murine intestinal PPMC, which are more closely related to the *in vivo* site of action of the putative anti-inflammatory strains in the model used further in this study, mice. Fermented milk with each LAB was evaluated, and IFN-γ was selected as the proinflammatory cytokine because it would also be evaluated in the intestinal fluids of mice. The highest IL-10/IFN-γ ratios were observed in the supernatants obtained from cocultures with milk fermented by *L. delbrueckii* subsp. *bulgaricus* CRL 864 or *S. thermophilus* CRL 807 (Fig. 1B). These ratios were even higher that the ones obtained with yogurt (our anti-inflammatory control). We thus decided to test *in vivo* the immunomodulatory effects of milk fermented by these two strains.

***In vivo* effects of the potentially anti-inflammatory selected strains.** As shown in Fig. 2A, samples of large intestine fluids from mice that received milk fermented by either *L. delbrueckii* subsp. *bulgaricus* CRL 864 or *S. thermophilus* CRL 807 for 10 days showed a significant increase ($P < 0.05$) of the IL-10/IFN-γ ratio. IL-17 was evaluated as another inflammatory cytokine because of its relation with intestinal inflammation. For this study, samples of intestinal tissues were analyzed and showed a significant increase ($P < 0.05$) in the ratio of IL-10$^+$ cells to IL-17$^+$ cells in the mice given fermented milk compared to control mice (Fig. 2B). No significant difference was observed in the body weights of animals fed with the fermented milk in comparison with control animals that received unfermented milk (data not shown).

Determination of CAT and SOD in recombinant streptococci. The results obtained by PCR confirmed a single amplicon of approximately 600 bp in *S. thermophilus* CRL 807 transformed with the pIL253-*mnkat* plasmid (*S. thermophilus* CRL 807:CAT), as was also observed in the positive-control *L. casei* BL23 harbor-

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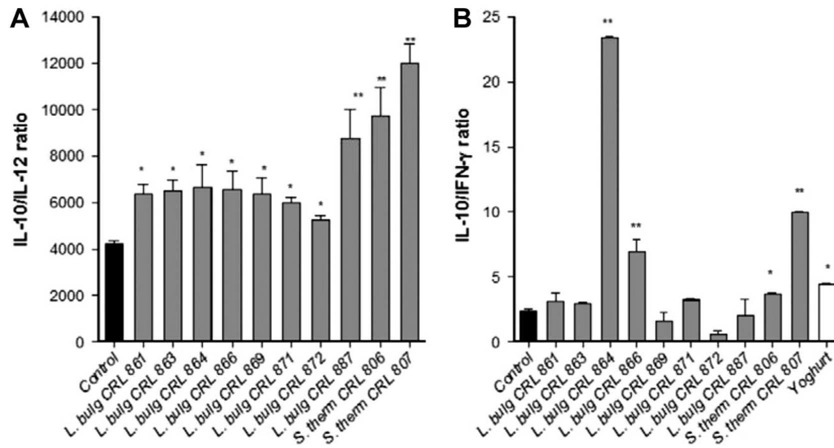

FIG 1 Cytokine profile after coincubation of LAB with primary cultures of mononuclear cells. (A) IL-10 and IL-12 cytokines were quantified by ELISA after coincubation of the different LAB with PBMC for 24 h, and IL-10/IL-12 ratios were determined; PBS was used a negative control. (B) IL-10 and IFN-γ cytokines were quantified by ELISA after coincubation of each individual strain grown in milk, yogurt (positive control), or unfermented milk (negative control) with Peyer's patch mononuclear cells for 24 h, and IL-10/IFN-γ ratios were determined. Results are expressed as means ± standard deviation (SD). Means with asterisks differ significantly from control values (*, $P < 0.05$; **, $P < 0.01$).

ing the same plasmid. Similarly, an amplicon of approximately 1,200 bp was observed in *S. thermophilus* CRL 807 transformed with the pIL253-*sodA* plasmid (*S. thermophilus* CRL 807:SOD), as was observed in the positive-control *L. casei* BL23 harboring the same plasmid. No amplification was observed for WT *S. thermophilus* CRL 807 (Fig. 3C). Furthermore, CAT (Fig. 3A) and SOD (Fig. 3B) activities were detected in the *S. thermophilus* CRL 807: CAT and CRL 807:SOD strains, respectively, confirming biological activity of the antioxidant enzymes in recombinant streptococci.

Genetically engineered *S. thermophilus* CRL 807 producing antioxidant enzymes reduces the severity of TNBS-induced chronic colitis. As shown in Fig. 4, mice receiving *S. thermophilus* CRL 807:CAT, *S. thermophilus* CRL 807:SOD, or a mixture of these strains (*S. thermophilus* CRL 807:CAT/SOD) orally using a gavage syringe showed a significant improvement in weight loss (Fig. 4A), significantly lower macroscopic and histological damage scores (Fig. 4C), and a significantly lower liver microbial translocation (Fig. 4E) than TNBS control mice ($P < 0.05$). Similar results were observed when animals were fed *ad libitum* with milk fermented by *S. thermophilus* CRL 807:CAT, CRL 807:SOD, or both strains (*S. thermophilus* CRL 807:CAT/SOD) (Fig. 4B, D, and F). Animals receiving the WT strain also showed significantly ($P <$ 0.05) lower macroscopic and microscopic damage scores (Fig. 4C and D) and a lower liver microbial translocation than the TNBS group (Fig. 4E).

Strikingly, animals that received coadministration of CAT- and SOD-producing strains showed the lowest mortality rates (data not shown). Considering the three independent trials together, until day 4 no deaths were observed when the strains were coadministered as a bacterial suspension, and only 7% mortality was observed in the group given the strains in fermented milk, compared to mortalities of between 16% and 33% in the other test groups and 50% in the TNBS group (data not shown). The mix-

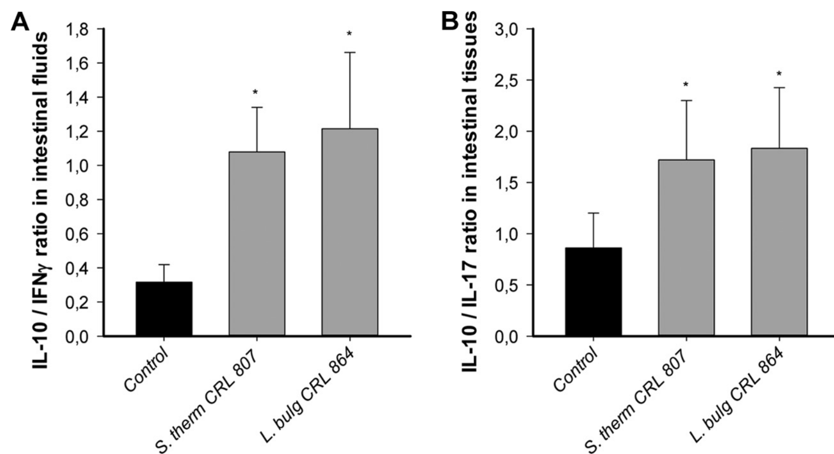

FIG 2 Evaluation of anti-inflammatory potential effects of selected lactobacilli and streptococci *in vivo*. The ratio of anti-inflammatory cytokines to proinflammatory cytokines induced in large intestine fluids (A) or cells in the intestinal tissues (B) of mice fed with unfermented milk (negative control) or with milk fermented by *S. thermophilus* CRL 807 or *L. delbrueckii* subsp. *bulgaricus* CRL 864 were determined. Results are expressed as means of ± SD ($n = 9$). Means with asterisks differ significantly from control values (*, $P < 0.01$).

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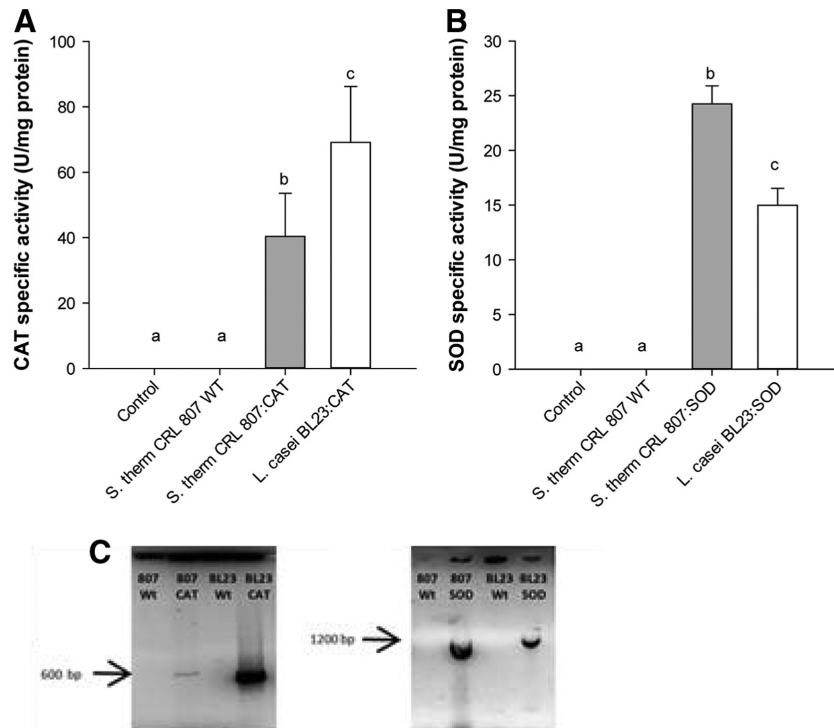

FIG 3 Detection of enzymatic activity and confirmation of recombinant streptococci. (A and B) Catalase (A) or superoxide dismutase (B) specific to WT *S. thermophilus* CRL 807, CRL807:CAT (*S. thermophilus* CRL 807 transformed with the pIL253-*mnkat* plasmid) or *S. thermophilus* CRL 807:SOD (*S. thermophilus* CRL 807 transformed with the pIL253-*sodA* plasmid) grown in LAPTg medium is expressed as enzymatic units (U) per mg of proteins and compared to the enzymatic activity of *L. casei* BL23 harboring the same plasmid (*L. casei* BL23:CAT and BL23:SOD). The control is the culture medium. Results are expressed as means ± SD. Means without a common letter differ significantly ($P < 0.05$). (C) PCR confirming single bands of approximately 600 and 1,200 bp that correspond to the CAT and SOD enzymes, respectively, in the recombinant *S. thermophilus* CRL 807 (807:CAT and 807:SOD) and in the *L. casei* BL23 positive controls harboring the same plasmid (BL23:CAT and BL23:SOD). No amplification was observed in the WT strains.

ture of recombinant streptococci was also administered with *L. delbrueckii* subsp. *bulgaricus* CRL 864 (the other strain selected on the basis of anti-inflammatory potential) and analyzed in the IBD model; however, no additional benefits were observed compared to those with the mixture of antioxidant enzyme-producing streptococci (data not shown).

The increase in the antioxidant enzyme activities was related to the improved anti-inflammatory effect exerted by the genetically engineered *S. thermophilus* CRL 807 in the colitis model. Mice that received the *S. thermophilus* CRL 807:CAT strain as a suspension orally administered using a gavage syringe or *ad libitum* in milk fermented by this strain had significantly increased catalase activity in their intestinal samples (Fig. 5A). The same was observed in mice that received the *S. thermophilus* CRL 807:SOD strain (in both feeding protocols), which significantly increased SOD activity in the large intestine samples, compared to those in the mock and TNBS groups (Fig. 5B). Animals that received coadministration with CAT- and SOD-producing strains also showed significant increases in both CAT and SOD activities in their intestines compared to the other groups (Fig. 5A and B).

A mucosal decrease in IL-17-producing cells correlates with the protective effects of recombinant *S. thermophilus* CRL 807 producing antioxidant enzymes. Mice that received the CRL 807 WT strain or the antioxidant enzyme-expressing strains had significantly decreased ($P < 0.05$) numbers of IL-17-producing cells in the large intestines compared to those in the mice from TNBS group (Fig. 6A), showing values similar to those in the mock group. IL-10-producing cells increased significantly in all the mice inoculated with TNBS, compared to the mock group (Fig. 6B); thus, the ratio of IL-10$^+$ cells to IL-17$^+$ cells increased significantly in mice given any bacterial supplementation, compared to the inflammation control (TNBS group) (Fig. 6C). Remarkably, this ratio was significantly increased in some groups of mice that received the antioxidant-expressing strains compared to the mock mice.

DISCUSSION

In this study, two strains (*S. thermophilus* CRL 807 and *L. delbrueckii* subsp. *bulgaricus* CRL 864) from a pool of LAB from the CERELA Culture Collection present in a yogurt, with proved anti-inflammatory properties in an IBD model in mice (5), were selected for their immunomodulatory potentials. The *in vitro* assays demonstrated that these strains showed elevated IL-10/IL-12 and IL-10/IFN-γ ratios in coculture with human PBMC and mouse mononuclear cells isolated from Peyer's patches (PPMC), respectively. In this sense, previous *in vitro* trials using human PBMC have allowed a preliminary classification of candidate beneficial strains according to their immunomodulatory capacities (20). Cytokine responses were also evaluated using cocultures of LAB with PPMC of healthy mice because they may have a higher correlation with the immunomodulatory effects expected *in vivo* in the IBD mouse model, since it is known that PPs are of great importance in the immune response at the mucosal level (21). In this assay, the strains were grown in milk because the selected fermented milk

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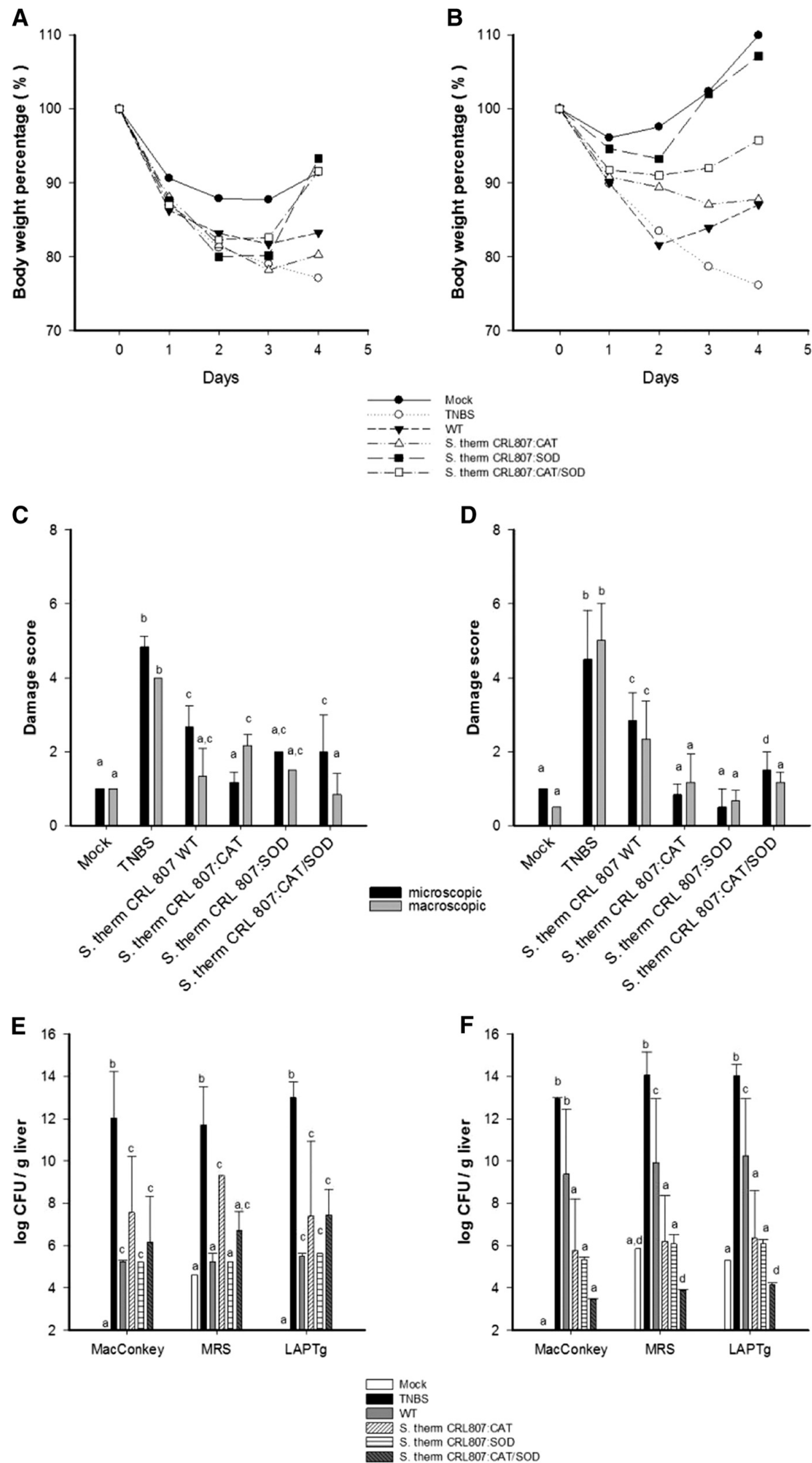

FIG 4 Effects of recombinant streptococci expressing antioxidant enzymes on TNBS-induced inflammation in mice. The body weight percentage (A and B), macro- and microscopic damage scores (C and D), and liver microbial translocation (E and F) were evaluated in mice from the mock group, mice from the TNBS group, and mice that received the WT (*S. thermophilus* CRL 807 WT), *S. thermophilus* CRL 807:CAT (*S. thermophilus* CRL807 transformed with the pIL253-*mnkat* plasmid), *S. thermophilus* CRL 807:SOD (*S. thermophilus* CRL 807 transformed with the pIL253-*sodA* plasmid), or *S. thermophilus* CRL 807:CAT/SOD (coadministration of both recombinant streptococci) in suspension (A, C, and D) or in fermented milk (B, D, and F). Body weight was measured from the day of TNBS inoculation up to 4 days after TNBS inoculation and they is represented as a percentage of the initial mouse body weight. Microscopic (black bars) and macroscopic (gray bars) damage scores correspond to samples taken 4 days after TNBS inoculation. Each value represents the mean ± SD ($n = 9$). Microbial growth in MacConkey, MRS, or LAPTg of liver samples obtained from different groups was evaluated. Results are expressed as means ± SD of the log CFU/g liver. Means for each value without a common letter differ significantly ($P < 0.05$).

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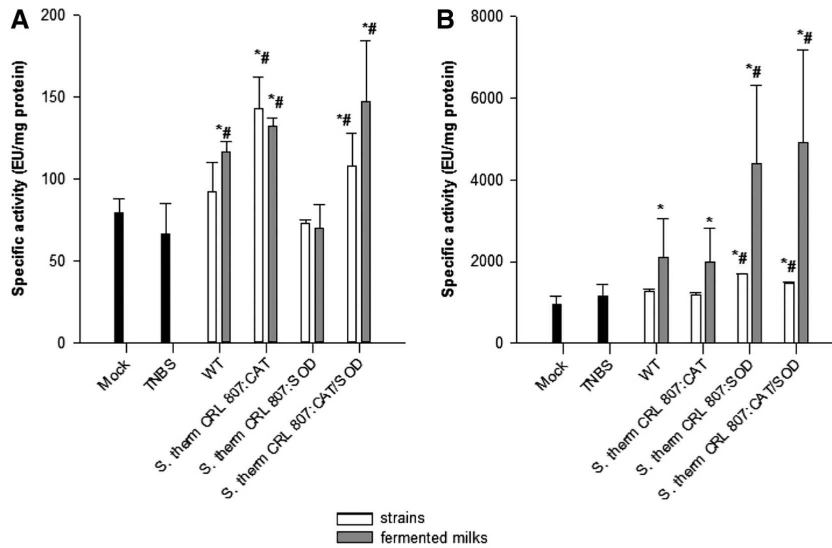

FIG 5 Enzymatic activity in the large intestine contents. CAT (A) and SOD (B) specific activities were determined in the intestinal contents of mice from the mock group, mice from the TNBS group, and mice that received the WT (*S. thermophilus* CRL 807 WT), *S. thermophilus* CRL 807:CAT (*S. thermophilus* CRL 807transformed with the pIL253-*mnkat* plasmid), *S. thermophilus* CRL 807:SOD (*S. thermophilus* CRL 807 transformed with the pIL253-*sodA* plasmid), or *S. thermophilus* CRL 807:CAT/SOD (coadministration of both recombinant streptococci) in suspension (white bars) or in fermented milk (gray bars). The results are expressed as the means of the enzymatic units (EU) per mg of protein. * and #, significantly different from the mock or TNBS group, respectively ($P < 0.01$).

would afterwards be evaluated *in vivo*. It is known that IL-12 plays a major role in IBD by inducing lymphocytes to produce IFN-γ, so IFN-γ in the supernatant from PPMC was evaluated because of the predominance of lymphocytes in these isolated cells. According to these results, and considering the complexity of the intestinal environment, the selected *S. thermophilus* CRL 807 and *L. delbrueckii* subsp. *bulgaricus* CRL 864 were also analyzed *in vivo* by evaluating the cytokine pattern in the intestinal fluids and tissues of healthy mice fed with milk fermented by these LAB, demonstrating a good correlation between *in vitro* and *in vivo* results (Fig. 1 and 2).

Several studies have confirmed the role of ROS in inflammation and the importance of antioxidant enzymes as a new treatment approach for IBD (22–24). Carroll et al. reported that treatment with SOD-producing *Lactobacillus gasseri* reduced inflammation in IL-10 knockout mice (8). Han et al. showed that

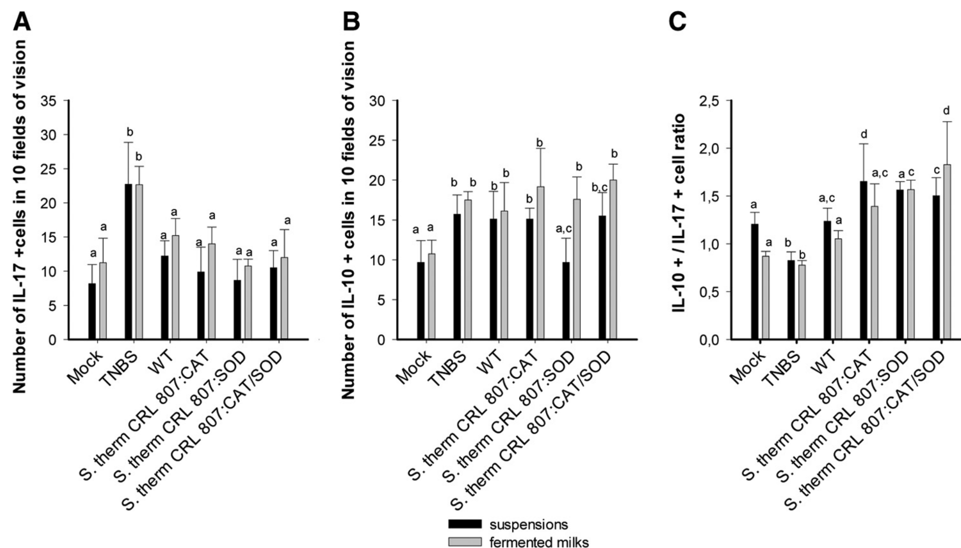

FIG 6 IL-17- and IL-10-producing cells in the large intestine tissues. (A and B) IL-17 (A)- and IL-10 (B)-positive cells were evaluated by immunofluorescence in mice from the mock group, mice from the TNBS group, and mice that received the WT (*S. thermophilus* CRL 807 WT), *S. thermophilus* CRL807:CAT (*S. thermophilus* CRL807 transformed with the pIL253-*mnkat* plasmid), *S. thermophilus* CRL 807:SOD (*S. thermophilus* CRL 807 transformed with the pIL253-*sodA* plasmid), or *S. thermophilus* CRL 807:CAT/SOD (coadministration of both recombinant streptococci) in suspension (black bars) or in fermented milk (gray bars). The results are expressed as the means of the total number of positive cells counted in 10 fields at a magnification of ×1,000. (C) Ratio between the number of IL-10^+ cells and the number of IL-17^+ cells for each group. Data correspond to the means ± SD ($n = 9$). Means for each value without a common letter differ significantly ($P < 0.01$).

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administration of SOD-producing *Lactobacillus lactis* and *Lactobacillus plantarum* diminished inflammation in a rat TNBS-induced colitis model (9).

Recently, our group also reported that oral administration of CAT- and SOD-producing *L. casei* BL23 was capable of reducing colon damage scores in DSS-induced (10, 25) as well as TNBS-induced (18) colitis mouse models. However, the most remarkable effect of these strains was through their antioxidant mechanisms and not through an immunomodulating mechanism (18).

Considering these previous results, the LAB selected for their anti-inflammatory potential in this study were subjected to different transformation protocols to introduce the antioxidant enzyme genes into them. After many attempts, transformation of *L. delbrueckii* subsp. *bulgaricus* CRL 864 was not possible, and we continued with the transformation of the selected streptococcal strain. This difficulty has previously been encountered with other *L. delbrueckii* subsp. *bulgaricus* strains and has been explained by the presence of different restriction modification systems in their genomes (26).

It is important to note that *mnkat* and *sodA* genes used in the present study were cloned from *L. plantarum* ATCC 14432 and *Lactococcus lactis* MG1363, respectively, which are also generally recognized as safe (GRAS) microorganisms. Even though native strains of *S. thermophilus* have also been reported to possess the *sodA* gene (27), the *S. thermophilus* CRL 807 strain did not possess SOD activity or the presence of this gene (Fig. 3), but since the complete genome of this strain has not been determined, we cannot discard the possibility that a *sod* gene might be present in this strain and that the PCR parameters were not appropriate to show it (10, 16). Due to the fact that *S. thermophilus* lacks the enzymes to catalyze the reaction that detoxifies H_2O_2 into H_2O and O_2, such as catalase, Fu et al. (33) have also recently developed an *S. thermophilus* strain that produces heterologous catalase (encoded by *katE*) from *L. brevis* ATCC 367. Interestingly, in that work it was shown that KatE expression in *S. thermophilus* ST5 was beneficial to *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 cocultured in milk, which confirms the functionality of this enzyme on this particular food matrix. In contrast to that previous report, our work did not show any additional benefits with the mixture of CAT- and/or SOD-producing *S. thermophilus* CRL 807 with *L. delbrueckii* subsp. *bulgaricus* CRL 864 (WT strain) compared to the mixture of antioxidant enzyme-producing streptococci.

Finally, the anti-inflammatory potentials of the recombinant streptococci were analyzed *in vivo*. Both protocols for administration (as a bacterial suspension by gavage or as fermented milk *ad libitum*) of the antioxidant enzyme-producing *S. thermophilus* strains used in this study decreased the severity of the inflammation, with less body weight loss, lower macroscopic and histological damage scores in the large intestine, and a decrease of bacterial translocation to liver, compared to the inflammation control (Fig. 4). These beneficial modifications were even increased compared to those in the mice that received the WT strain. The remarkable decrease in the mortality observed in mice receiving coadministration of CAT- and SOD-producing streptococci is of particular interest. We hypothesize that the increased survival of these animals might be related to a more efficient reduction of ROS in the presence of both enzymes (CAT and SOD), conferring a higher antioxidant protection. Moreover, in a recent study it has been shown that an anti-inflammatory strain of *L. rhamnosus* CNCMI-3690 protected against oxidative stress and increased the life span in *Caenorhabditis elegans* through a differential expression of the DAF-16/insulin-like pathway (28). It is also important to note that in both protocols of administration, WT *S. thermophilus* CRL 807 also decreased the severity of the inflammation compared to that in the TNBS control group, corroborating the innate anti-inflammatory potential of this strain selected by *in vitro* and *in vivo* assays. In fact, a significant increase in the number of IL-10-producing cells was observed in the large intestines of mice receiving WT *S. thermophilus* (Fig. 5A); however, the elevated anti-inflammatory/proinflammatory cytokine-producing cell ratio was maintained or even increased in those animals that received the transforming strains, confirming an additive effect (their antioxidant activities) in these mice (Fig. 5C).

To our knowledge this is the first time that an enhancement of the anti-inflammatory activity has been achieved for an immunomodulatory strain of *S. thermophilus* by conferring on it the capacity to produce antioxidant enzymes. Our findings suggest that LAB strains that are able to modulate immune responses and that also express antioxidant enzymes could be useful in the development of novel therapeutic products that prevent IBD. Although genetically engineered strains are normally used to demonstrate a "proof of concept," some human clinical trials using modified lactic acid bacteria have already been performed without any significant negative side effects on the host: a phase 1 trial that evaluated the safety of a strain of *L. lactis* expressing human IL-10 for the treatment of Crohn's disease (29) and, more recently, a phase 1b study that evaluated a recombinant *L. lactis* strain secreting the mucosal protectant human trefoil factor 1 (30). These studies set the precedent for the clinical use of engineered probiotic microorganisms as potential treatment options.

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