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World J Gastroenterol 2005;11(33):5185-5192
World Journal of Gastroenterology ISSN 1007-9327

• *BRIEF REPORTS* •

Preventative effects of a probiotic, *Lactobacillus salivarius ssp. salivarius*, in the TNBS model of rat colitis

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Supported by the Spanish Ministry of Science and Technology, No. SAF2002-02592 and by Instituto de Salud 'Carlos III', No. PI021732, with Funds from the European Union, and by Junta de Andalucia (CTS 164). Monica Comalada is a recipient of Juan de la Cierva Program from Spanish Ministry of Science and Technology. Laura Peran is a Recipient From Puleva Foundation Spain
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Received: 2004-11-23 Accepted: 2005-02-28

Abstract

AIM: To investigate the intestinal anti-inflammatory effect and mechanism of a probiotic *Lactobacillus salivarius ssp. salivarius* CECT5713 in the TNBS model of rat colitis.

METHODS: Female Wistar rats (180-200 g) were used in this study. A group of rats were administered orally the probiotic *L. salivarius ssp. salivarius* (5×10^8 CFU suspended in 0.5 mL of skimmed milk) daily for 3 wk. Two additional groups were used for reference, a non-colitic and a control colitic without probiotic treatment, which received orally the vehicle used to administer the probiotic. Two weeks after starting the experiment, the rats were rendered colitic by intracolonic administration of 10 mg of TNBS dissolved in 0.25 mL of 500 mL/L ethanol. One week after colitis induction, all animals were killed and colonic damage was evaluated both histologically and biochemically. The biochemical studies performed in colonic homogenates include determination of myeloperoxidase (MPO) activity, glutathione (GSH) content, leukotriene B_4 (LTB_4) and tumor necrosis factor α (TNF-α) levels, as well as inducible nitric oxide synthase (iNOS) expression. In addition, the luminal contents obtained from colonic samples were used for microbiological studies, in order to determine Lactobacilli and Bifidobacteria counts.

RESULTS: Treatment of colitic rats with *L. salivarius ssp. salivarius* resulted in amelioration of the inflammatory response in colitic rats, when compared with the corresponding control group without probiotic treatment. This anti-inflammatory effect was evidenced macroscopically by a significant reduction in the extent of colonic necrosis and/or inflammation induced by the administration of TNBS/ethanol (2.3±0.4 cm *vs* 3.4±0.3 cm in control group, $P<0.01$) and histologically by improvement of the colonic architecture associated with a reduction in the neutrophil infiltrate in comparison with non-treated colitic rats. The latter was confirmed biochemically by a significant reduction of colonic MPO activity (105.3±26.0 U/g *vs* 180.6±21.9 U/g, $P<0.05$), a marker of neutrophil infiltration. The beneficial effect was associated with an increase of the colonic GSH content (1 252±42 nmol/g *vs* 1 087±51 nmol/g, $P<0.05$), which is depleted in colitic rats, as a consequence of the oxidative stress induced by the inflammatory process. In addition, the treatment of colitic rats with *L. salivarius* resulted in a significant reduction of colonic TNF-α levels (509.4±68.2 pg/g *vs* 782.9±60.1 pg/g, $P<0.01$) and in a lower colonic iNOS expression, when compared to TNBS control animals without probiotic administration. Finally, treated colitic rats showed higher counts of Lactobacilli species in colonic contents than control colitic rats, whereas no differences were observed in Bifidobacteria counts.

CONCLUSION: Administration of the probiotic *L. salivarius ssp. salivarius* CECT5713 facilitates the recovery of the inflamed tissue in the TNBS model of rat colitis, an effect associated with amelioration of the production of some of the mediators involved in the inflammatory response in the intestine, such as cytokines, including TNF-α and NO. This beneficial effect could be ascribed to its effect on the altered immune response that occurs in this inflammatory condition.

Key words: *Lactobacillus salivarius ssp. salivarius*; TNBS rat colitis; Probiotic; Tumor necrosis factor α; Nitric oxide

Peran L, Camuesco D, Comalada M, Nieto A, Concha A, Diaz-Ropero MP, Olivares M, Xaus J, Zarzuelo A, Galvez J. Preventative effects of a probiotic, *Lactobacillus salivarius ssp. salivarius*, in the TNBS model of rat colitis. *World J Gastroenterol* 2005; 11(33): 5185-5192
http://www.wjgnet.com/1007-9327/11/5185.asp

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disease of

the digestive tract, and usually refers to two related conditions, namely ulcerative colitis and Crohn's disease, characterized by chronic and spontaneously relapsing inflammation. Although the etiology of IBD remains unknown, there is increasing experimental evidence to support a role for luminal bacteria in the initiation and progression of these intestinal conditions; probably related to an imbalance in the intestinal microflora, relative predominance of aggressive bacteria and insufficient amount of protective species[1,2]. This could justify the remission achieved in intestinal inflammation, after treatment with antibiotics such as metronidazole or ciprofloxacin[3], or the fact that germ-free animals may fail to develop experimental intestinal inflammation[4]. In consequence, a possible therapeutic approach in IBD therapy is the administration to these patients of probiotic microorganisms, defined as viable nutritional agents conferring benefits to the health of the human host. In fact, it has been reported that administration of a mixture of *Bifidobacterium* and *Lactobacillus*[5] or of non-pathogenic viable *Escherichia coli*[6] prolongs remission in ulcerative colitis. Moreover, there are reports on successful induction and maintenance of remission of chronic pouchitis after oral bacteriotherapy[7,8]. However, treatments of Crohn's disease with probiotic preparations reported conflicting results[9-12].

Different mechanisms have been proposed to participate in the therapeutic effects exerted by probiotic microorganisms. First, probiotic microorganisms may exert their action through a modulation of the intestinal bowel flora, which may result from competitive metabolic interactions with potential pathogens, production of anti-microbial peptides, or inhibition of epithelial adherence and translocation by pathogens[5,13]; second, probiotics have been proposed to modulate the host defenses by influencing the intestinal immune system[14,15]; and third, these microorganisms have been reported to positively affect the intestinal barrier function[16,17]. However, the detailed mechanisms by which these bacteria mediate their effects are not fully understood.

Although the results obtained after probiotic treatment in both human IBD and experimental colitis are promising, new studies are required in order to further understand this new concept for the therapy of IBD, even if we consider the fact that many studies have shown that not all bacterial species have equal activities in reducing intestinal inflammation[18,19]. Hence, the selection of new probiotic strains for the treatment of IBD can be based on their ability to regulate the immune response of the intestinal mucosa. This can be the case of *Lactobacilli* strains, which were able to downregulate the production of tumor necrosis factor α (TNF-α). In fact, previous *ex vivo* experiments have reported the ability of *L. casei* and of *L. bulgaricus* to downregulate TNF-α production in colonic explants from patients with Crohn's disease[20], thus supporting their future development for IBD therapy. This may be of special relevance, since several studies have attributed a key role in the pathogenesis of IBD to this pro-inflammatory cytokine, as evidenced by the increased production of TNF-α in the intestinal mucosa from IBD patients[21,22] as well as by a number of clinical studies using anti-TNF-α mAb therapy that have clearly shown a beneficial effect in these patients[23].

The aim of the present study was to test the preventative effects of a *L. salivarius ssp. salivarius* strain in the trinitrobenzenesulfonic acid (TNBS) model of rat colitis, a well-established model of intestinal inflammation with some resemblance to human IBD[24]. The selection of this lactobacilli strain was based on previous *in vitro* studies that showed its ability to adhere to human intestinal cells, to inhibit pathogenic bacterial growth (unpublished results) and to reduce the production of inflammatory cytokines by immune cells. Special attention was paid to its effects on the production of some of the mediators involved in the inflammatory response, such as TNFα, leukotriene B_4 (LTB_4) and nitric oxide (NO). In addition, the correlation among the intestinal anti-inflammatory effect of *L. salivarius ssp. salivarius* and modifications on colonic flora induced by this probiotic was also studied.

MATERIALS AND METHODS

This study was carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the National Institute of Health.

Reagents

All chemicals were obtained from Sigma Chemical (Madrid, Spain), unless otherwise stated. Glutathione (GSH) reductase was provided by Boehringer Mannheim (Barcelona, Spain).

In vitro modulation of cytokine production by bacteria

Puleva Biotech's lactic acid bacteria collection was screened for *Lactobacilli* bacteria with the ability to reduce the production of inflammatory cytokines by activated macrophages. For this purpose, rodent bone marrow-derived macrophages, obtained as previously described[25], were stimulated with 100 ng/mL LPS, in the presence or absence of 10^6 CFU/mL of each bacteria for 2 h. Then, cells were washed with culture media to eliminate non-attached bacteria, and cultured with new media for 12 h. TNF-α, IL-12, and IL-10 production was evaluated by ELISA in cell supernatants (CytoSets™, Biosource International, Nivelles, Belgium) following manufacturer's instructions.

Preparation and administration of the probiotic

L. salivarius ssp. salivarius CECT5713 was provided by Puleva Biotech (Granada, Spain) and it was normally grown in MRS media at 37 ℃ in anaerobic conditions using the AnaeroGen system (Oxoid, Basingstoke, UK). For probiotic treatment, bacteria was suspended in skimmed milk (10^9 CFU/mL) and stored at -80 ℃ until usage.

Experimental design

Female Wistar rats (180-200 g) were obtained from the Laboratory Animal Service of the University of Granada (Granada, Spain) and maintained in standard conditions. The rats were randomly assigned to three groups (n = 10); two of them (non-colitic and control groups) received no probiotic treatment and the other (treated group) received orally the probiotic (5×10^8 CFU suspended in 0.5 mL of skimmed milk) daily for 3 wk. Both non-colitic and control groups received orally the vehicle used to administer the probiotic (0.5 mL daily). Two weeks after starting the

experiment, the rats were fasted overnight and those from the control and treated groups were rendered colitic by the method originally described by Morris *et al*.[26]. Briefly, they were anesthetized with halothane and given 10 mg of TNBS dissolved in 0.25 mL of 500 mL/L ethanol by means of a Teflon cannula inserted 8 cm through the anus. Rats from the non-colitic group were administered intracolonically 0.25 mL of PBS instead of TNBS. All rats were killed with an overdose of halothane, 1 wk after induction of colitis.

Assessment of colonic damage

The body weight, water and food intake were recorded daily throughout the experiment. Once the rats were killed, the colon was removed aseptically and placed on an ice-cold plate, longitudinally opened and luminal contents were collected for the microbiological studies (see below). Afterwards, the colonic segment was cleaned of fat and mesentery, blotted on filter paper; each specimen was weighed and its length measured under a constant load (2 g). The colon was scored for macroscopically visible damage on a 0-10 scale by two observers unaware of the treatment, according to the criteria described by Bell *et al*.[27] (Table 1), which takes into account the extent as well as the severity of colonic damage. Representative whole gut specimens were taken from a region of the inflamed colon corresponding to the adjacent segment to the gross macroscopic damage and were fixed in 4% buffered formaldehyde. Cross-sections were selected and embedded in paraffin. Equivalent colonic segments were also obtained from the non-colitic group. Full-thickness sections of 5 μm were obtained at different levels and stained with hematoxylin and eosin. The histological damage was evaluated by two pathologist observers (AN and AC), who were blinded to the experimental groups, according to the criteria described previously by Stucchi *et al*.[28] (Table 2). The colon was subsequently divided into four segments for biochemical determinations. Two fragments were frozen at -80 ℃ for myeloperoxidase (MPO) activity and inducible nitric oxide synthase (iNOS) expression, and another sample was weighed and frozen in 1 mL of 50 g/L trichloroacetic acid for total GSH content determinations. The remaining sample was immediately processed for the measurement of TNF-α and LTB_4 levels. All biochemical measurements were completed within 1 wk from the time of sample collection and were performed in duplicate.

MPO activity was measured according to the technique described by Krawisz *et al*.[29]; the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 μmol hydrogen peroxide/min at 25 ℃. Total GSH content was quantified with the recycling assay described by Anderson[30], and the results were expressed as nanomole per gram of wet tissue. Colonic samples for TNF-α and LTB_4 determinations were immediately weighed, minced on an ice-cold plate and suspended in a tube with 10 mmol/L sodium phosphate buffer (pH 7.4) (1∶5 w/v). The tubes were placed in a shaking water bath (37 ℃) for 20 min and centrifuged at 9 000 r/min for 30 s at 4 ℃; the supernatants were frozen at -80 ℃ until assay. TNF-α was quantified by ELISA (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the results were expressed as picogram per gram of wet tissue. LTB_4 was determined by enzyme immunoassay (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the results expressed as nanogram per gram of wet tissue.

iNOS expression was analyzed by Western blotting as previously described[31]. Control of protein loading and transfer was conducted by detection of the β-actin levels.

Table 1 Criteria for assessment of macroscopic colonic damage

Score	Criteria
0	No damage
1	Hyperemia, no ulcers
2	Linear ulcer with no significant inflammation
3	Linear ulcer with inflammation at one site
4	Two or more sites of ulceration/inflammation
5	Two or more major sites of ulceration and inflammation or one site of ulceration/inflammation, extending >1 cm along the length of the colon
6-10	If damage covers >2 cm along the length of the colon, the score is increased by one, for each additional centimeter of involvement

Table 2 Criteria for assessment of microscopic colonic damage

Mucosal epithelium	
	Ulceration: none (0); mild - surface (1); moderate (2); extensive-full thickness (3)
Crypts	
	Mitotic activity: lower third (0); mild mid-third (1); moderate mid-third (2); upper third (3)
	Mucus depletion: none (0); mild (1); moderate (2); severe (3)
Lamina propria	
	Mononuclear infiltrate: none (0); mild (1); moderate (2); severe (3)
	Granulocyte infiltrate: none (0); mild (1); moderate (2); severe (3)
	Vascularity: none (0); mild (1); moderate (2); severe (3)
Submucosal	
	Mononuclear infiltrate: none (0); mild (1); moderate (2); severe (3)
	Granulocyte infiltrate: none (0); mild (1); moderate (2); severe (3)
	Edema: none (0); mild (1); moderate (2); severe (3)

Maximum score: 27. Modified from Stucchi *et al*.[28].

Microbiological studies

Luminal content samples were weighed, homogenized, and serially diluted in sterile peptone water. Serial 10-fold dilutions of homogenates were plated on specific media for *Lactobacillus* (MRS media, Oxoid) or *Bifidobacterium* (MRS media supplemented with 0.5 mg/L dicloxacillin, 1 g/L LiCl and 0.5 g/L L-cysteine hydrochloride) and incubated under anaerobic conditions in an anaerobic chamber for 24-48 h at 37 ℃. Coliforms and enterobacteria were also determined by using specific Count Plates Petrifilm (3M, St. Paul, MN). After incubation, the final count of colonies was reported as $\log_{10}$ colony forming units per gram of material.

Statistical analysis

All results are expressed as mean±SE. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and post hoc least significance tests. Non-parametric data (score) are expressed as the median (range) and were analyzed using the Mann-Whitney *U*-test. Differences between proportions were analyzed with the χ^2 test. All statistical analyses were carried

out with the Statgraphics 5.0 software package (STSC, MD), with statistical significance set at $P<0.05$.

RESULTS

More than 30 lactic acid bacterial strains with the ability to adhere to human intestinal cell lines and to inhibit pathogenic bacterial growth *in vitro* belonging to the own Puleva Biotech collection were screened for their ability to modulate the production of inflammatory cytokines in LPS-stimulated macrophages. The results obtained were highly diverse, including bacteria with the ability to enhance or to reduce inflammatory cytokine production (TNF-α and IL-12) modifying or not the expression of the anti-inflammatory cytokine IL-10 (data not shown).

Among all the screened bacteria, *L. salivarius ssp. salivarius* (CECT5713) showed the best TNF-α/IL-10 and IL-12/IL-10 ratio (Figure 1), since it was able not only to reduce the LPS-induced TNF-α and IL-12 production, but also to increase the levels of IL-10. For these reasons, we decided to use this strain to test its ability to prevent the inflammatory response in the *in vivo* assay of experimental colitis.

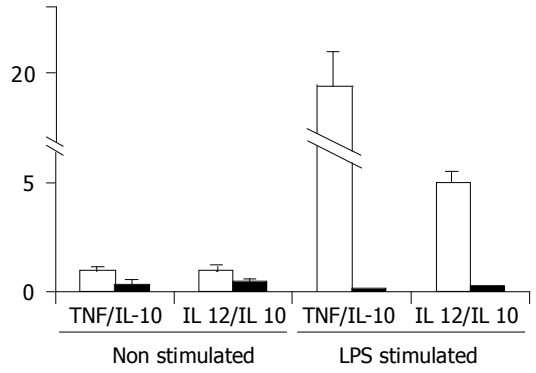

Figure 1 Production of inflammatory cytokines by bone marrow-derived macrophages (BMDM). TNF-α, IL-12, and IL-10 production was analyzed by ELISA in the supernatants of BMDM stimulated or not with LPS (100 ng/mL) and incubated with 10^6 CFU/mL of *L. salivarius ssp. salivarius* (CECT5713) (black bars) or in absence of bacteria (gray bars). The results are the mean of three assays±SE of the ratio between the pro-inflammatory cytokines (TNF-α and IL-12) and IL-10.

L. salivarius ssp. salivarius administration for 2 wk did not induce any symptoms of diarrhea or affected weight evolution. However, once the colitis was induced, the probiotic-treated rats showed an overall lower impact of TNBS-induced colonic damage compared to the TNBS control group. The anti-inflammatory effect was evidenced macroscopically by a significantly lower colonic damage score than that of control rats ($P<0.05$), with a significant reduction in the extent of colonic necrosis and/or inflammation induced by the administration of TNBS/ethanol (Table 3). This anti-inflammatory effect was also associated with a significant reduction in the colonic weight/length ratio between both colitic groups, an index of colonic edema, which increased significantly as a consequence of the inflammatory process (Table 3). The histological studies confirmed the intestinal anti-inflammatory effect exerted by *L. salivarius* (Figure 2). Histological assessment of colonic samples from the TNBS control group revealed severe transmural disruption of the normal architecture of the colon, extensive ulceration and inflammation involving all the intestinal layers of the colon, giving a score value of 18.9±1.1 (mean±SE). Colonic samples were characterized by severe edema, interstitial micro-hemorrhages and diffuse leukocyte infiltration, mainly composed of neutrophils in the mucosa layer and, to a lesser extent, lymphocytes in the submucosa. Most of the rats showed epithelial ulceration of the mucosa affecting over 75% of the surface. The inflammatory process was associated with crypt hyperplasia and dilation, and moderate goblet cell depletion. However, histological analysis of the colonic specimens from rats treated with the probiotic revealed a more pronounced recovery in the intestinal architecture than controls, with a score of 11.2±2.4 (mean±SE) ($P<0.01$ *vs* TNBS control group). Thus, most of the samples (7 of 10) showed almost complete restoration of the epithelial cell layer, in contrast to the extensive ulceration observed in non-treated animals; in fact, the zones with ulceration were surrounded by tissue in process of re-epithelization. Moreover, the transmural involvement of the lesions was reduced. The goblet cell depletion was less severe and thus they appeared replenished with their mucin content, and no dilated crypts were observed. The improvement in colonic histology was accompanied by a reduction in the inflammatory infiltrate, which was

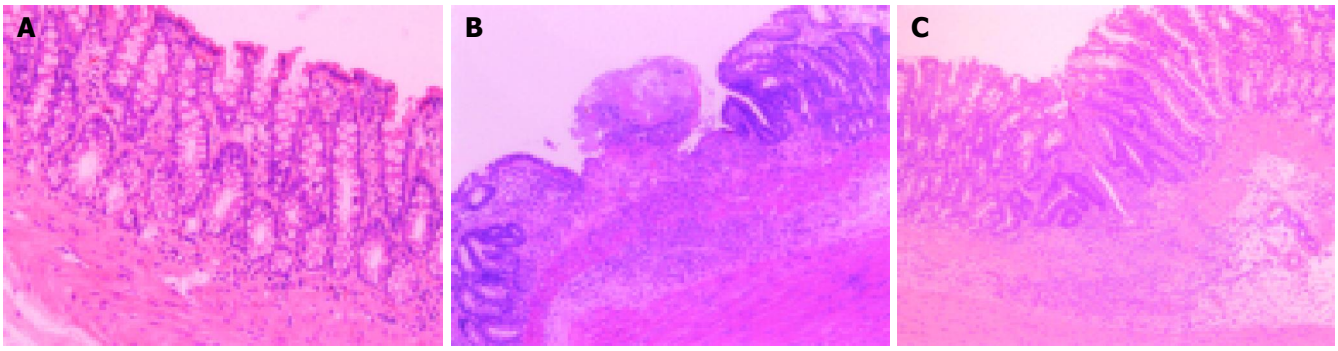

Figure 2 Histological sections of colonic mucosa from colitic rats 1 wk after TNBS instillation stained with hematoxylin and eosin. **A**: Non-colitic group showing the normal histology of the rat colon (original magnification ×20); **B**: TNBS control group showing complete destruction of the mucosa, which has been substituted by inflammatory granulation tissue. There is evident edema and intense diffuse transmural inflammatory infiltrate (original magnification ×100); **C**: *L. salivarius ssp. salivarius* treated group showing amelioration of the inflammatory process and 'restoration' of the mucosal tissue with presence of mucin replenished goblet cells (original magnification ×100).

Table 3 Effects of *L. salivarius ssp. salivarius* (5×10^8 CFU/rat·d) treatment on macroscopic damage score, extent of the inflammatory lesion along the colon and changes in colon weight in TNBS experimental colitis in rats

Group (n = 10)	Damage score (0-10)	Extent of damage (cm)	Colon weight (mg/cm)
Non-colitic	0	0	63.3±2.5
TNBS control	6.5 (5-8)	3.4±0.3	209.7±17.0
TNBS probiotic	5 (3-7)[a]	2.3±0.4[b]	143.3±11.8[b]

Damage score for each rat was assigned according to the criteria described in Table 1 and data are expressed as median (range). Extent of damage and colon weight data are expressed as mean±SE. [a]$P<0.05$, [b]$P<0.01$ *vs* TNBS control. All colitic groups differ significantly from non-colitic group.

slight to moderate with a patchy distribution, although neutrophils were the predominant cell type.

The lower leukocyte infiltration was also assessed biochemically by the reduction in colonic MPO activity, a marker of neutrophil infiltration that was enhanced in the TNBS control group (Table 4). In addition, probiotic-treated colitic rats showed a significant increase in colonic GSH content, which is depleted in colitic rats as a consequence of the colonic oxidative stress induced by the inflammatory process, as previously reported in this model of experimental colitis[32] (Table 4). Finally, the colonic inflammation induced by TNBS was characterized by increased levels of colonic TNF-α and LTB_4 (Table 4) as well as by higher colonic iNOS expression (Figure 3) in comparison with non-colitic animals. Treatment of colitic rats with *L. salivarius* resulted in a significant reduction of colonic TNF-α levels (Table 4), that did not show any statistical differences with normal rats. No significant modification was observed on colonic LTB_4 levels. Finally, lower colonic iNOS expression was also seen in colitic animals that received the bacterial suspension, when compared to TNBS control animals (Figure 3).

Effects of L. salivarius administration on colonic bacterial profile

TNBS colitis resulted in a significant reduction in fecal lactobacilli count in comparison with normal rats ($P<0.05$). Probiotic-treated colitic rats showed higher counts of Lactobacilli species in colonic contents than control colitic rats, without showing statistical differences with both non-colitic and colitic control groups (Figure 4). No statistical differences were observed in Bifidobacteria counts among three groups ($P>0.1$, Figure 4) nor in the amount of other fecal potential pathogenic bacteria such as enterobacteria or coliform bacteria (data not shown).

DISCUSSION

The results obtained in the present study reveal the efficacy of probiotic therapy with a *L. salivarius ssp. salivarius* strain in intestinal inflammation, incorporating a new microorganism to the probiotics that have been reported to attenuate the development of colonic injury in experimental and human IBD[33]. Thus, oral administration of the probiotic facilitated recovery from TNBS-induced colonic damage, as it was evidenced histologically, with a significant reduction in the extent and severity of inflamed tissue. This beneficial effect was also stated biochemically by a decrease in colonic MPO activity, a marker of neutrophil infiltration that has been previously described to be upregulated in experimental colitis[29], and is widely used to detect and follow intestinal inflammatory processes. In consequence, a reduction in the activity of this enzyme can be interpreted as a manifestation of the anti-inflammatory activity of a given compound[34]. The ability of the probiotic to reduce granulocyte infiltration, showed by MPO activity reduction, was confirmed histologically, since the level of leukocyte infiltrate in the colonic mucosa was lower in treated colitic animals than in

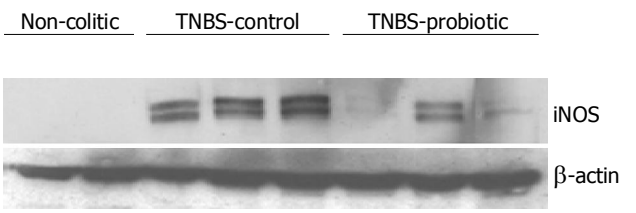

Figure 3 Effects of *L. salivarius ssp. salivarius* treatment (5×10^8 CFU/rat·d) on colonic nitric oxide synthase (NOS) expression in TNBS experimental colitis in rats.

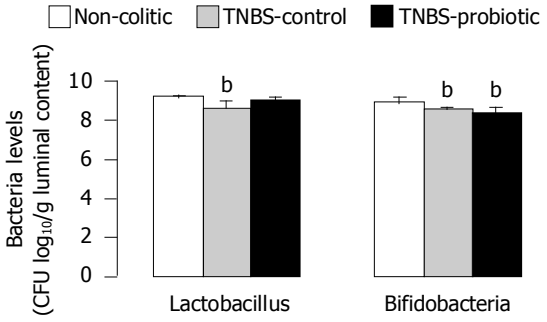

Figure 4 Effects of *L. salivarius ssp. salivarius* (5×10^8 CFU/rat·day) treatment on bacteria levels (Lactobacillus and Bifidobacteria) in TNBS experimental colitis in rats. [b]$P<0.01$ *vs* non-colitic group.

Table 4 Myeloperoxidase (MPO) activity, total GSH content, TNF-α and LTB_4 levels in colon specimens from non-colitic rats, TNBS control colitic rats and TNBS colitic rats treated with *L. salivarius ssp. salivarius* (5×10^8 CFU/rat·d)

Group (n = 10)	MPO activity (units MPO/g)	GSH (nmol/g)	LTB_4 (ng/g)	TNF-α (pg/g)
Non-colitic	23.4±7.2	1 540±41	2.9±0.4	441.5±39.1
TNBS control	180.6±21.9[d]	1 087±51[d]	6.5±0.9[d]	782.9±60.1[d]
TNBS probiotic	105.3±26.0[a,d]	1 252±42[a,d]	6.9±0.8[d]	509.4±68.2[b]

Data are expressed as mean±SE. [a]$P<0.05$, [b]$P<0.01$ *vs* TNBS control group; [d]$P<0.01$ *vs* non-colitic group.

the corresponding TNBS control groups. The inhibitory effect on the infiltration of inflammatory cells into the colonic mucosa might account for the beneficial effect of this probiotic against tissue injury, because margination and extravasation of circulating granulocytes contribute markedly to the colonic injury in this model of IBD[35]. These results are in agreement with other studies, that describe the attenuation exerted by several probiotics in leukocyte-endothelial cell adhesion in this experimental model of rat colitis[36]. This effect can justify the inhibition of the synthesis and/or release of different mediators that participate in the inflammatory process, such as NO, since probiotic treatment of colitic rats was associated with a reduction in colonic iNOS expression. Moreover, this can also explain the improvement in the colonic oxidative stress in colitic rats after probiotic treatment, as evidenced by a partial restoration of the GSH depletion that took place as a consequence of the TNBS colonic damage.

During the last decade, it has become increasingly evident that chronic colonic inflammation, both in human IBD and in experimental colitis, is associated with enhanced NO production, mainly via iNOS activity[37-39], as well as with increased release of reactive oxygen metabolites, including superoxide[40-42]. The simultaneous overproduction of NO and superoxide can yield the highly toxic radical, peroxynitrite in the inflamed intestine[43], which have been demonstrated to produce widespread colonic injury[44]. It is important to note that neutrophils are thought to be important source of both NO[45,46] and reactive oxygen metabolites[47]. Considering the above, the effect exerted by *L. salivarius ssp. salivarius* in decreasing the neutrophil infiltration that occurs in response to TNBS, may preserve the colonic mucosa from oxidative insult. In fact, beneficial effects have previously been reported either after NOS inhibition[37,38] or by antioxidant therapy[31,42] in different experimental models of intestinal inflammation.

Probiotic treatment could attenuate neutrophil infiltration via inhibition of different mediators with chemotactic activity. The results obtained in the present study revealed that probiotic treatment did not significantly modify colonic LTB_4 levels, an eicosanoid with chemotactic activity involved in the pathogenesis of IBD[1]. In consequence, the inhibitory effect of leukocyte infiltration exerted by the probiotic should be related to the downregulation of other pro-inflammatory mediators, given the ability of this lactobacilli strain to modulate the immune response as demonstrated by the *in vitro* studies. In fact, the intestinal anti-inflammatory activity exerted by *L. salivarius ssp. salivarius* was also characterized by downregulation of colonic TNF-α. This may be relevant since TNF-α acts as a potent chemoattractant, thus contributing to the recruitment of neutrophil in the inflamed colonic mucosa and initiating the inflammatory pathogenic cascade that definitively perpetuates colonic inflammation[48]. The important role attributed to TNF-α in intestinal inflammation is strongly supported by the fact that different drugs capable of interfering with the activity of this mediator are being developed for IBD therapy[23]. The ability of probiotic bacteria to downregulate TNF-α production has been reported previously for other lactobacilli strains such as *L. casei* and of *L. bulgaricus*, when they were cultured with inflamed mucosa from patients with Crohn's disease[20]. This effect was attributed to the existence of a cross talk between bacteria and mucosal cells, being able to downregulate the degree of activation of intestinal immune cells[20]. This has also been demonstrated in the present study for *L. salivarius ssp. salivarius* since it was able to modify the cytokine profile in macrophages, reducing the amount of inflammatory cytokines (TNF-α and IL-12), while increasing the amount of the anti-inflammatory cytokine IL-10. The high diversity of immuno-modulatory action of probiotics observed in the screening of the bacteria are in concordance with previous works showing both the ability of some lactic bacteria to promote TNF-α production[49] while others such as *L. rhamnosus* GG (LGG) reduced it[50]. LGG, a probiotic that also reduces the ratio TNF-α/IL-10, has been reported to exert intestinal anti-inflammatory effects both in human[12] and in experimental intestinal inflammation[51]. This effect of some probiotics on the immune response may be of special relevance because it would promote a possible shift from a T_H1-mediated immune response toward a T_H2/T_H3 profile, similarly to that proposed to occur with Lactobacillus GG[15]. It is important to note that replacing the bacteria responsible for the constant antigenic drive leading to T_H1 cellular activation with probiotic species that preferentially induce protective immune responses may alter the normal course of these relapsing intestinal conditions. In addition, probiotics like *Bifidobacterium longum* or *L. bulgaricus* have been shown to inhibit the IL-8 secretion in intestinal epithelia, when stimulated by the pro-inflammatory cytokine TNF-α, thus reducing the activity of other pro-inflammatory cytokines with chemotactic activity[52].

However, the participation of the modification in the immune response in the intestinal anti-inflammatory effect exerted by this probiotic does not exclude mechanisms proposed for other probiotics, mainly due to a role in preventing the imbalance in the intestinal microflora, given the relative predominance of aggressive bacteria and insufficient amount of protective species that has been reported in these intestinal conditions[1,2]. Previous studies have suggested that in TNBS-induced colitis, specific strains from colonic microflora invades the colonic wall after disruption of the epithelium and the presence of bacteria within the wall participates in the transmural inflammation[53]. In fact, the present study reveals that the colonic damage induced by TNBS was associated with a significant reduction of lactobacilli count in the colonic lumen, which was counteracted after the probiotic treatment, since probiotic-treated rats showed no statistical differences from non-colitic rats in the lactobacilli content.

In conclusion, administration of the probiotic *L. salivarius ssp. salivarius* CECT5713 facilitates the recovery of the inflamed tissue in the TNBS model of rat colitis, an effect associated with amelioration of the production of some of the mediators involved in the inflammatory response of the intestine, such as cytokines, including TNF-α, and NO. This beneficial effect could be ascribed to its effect on the altered immune response characteristic of this inflammatory condition, which would attenuate the exacerbated immune response evoked by the colonic instillation of the hapten TNBS in the rats.

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