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Lactobacillus fermentum, a probiotic capable to release glutathione, prevents colonic inflammation in the TNBS model of rat colitis

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Abstract *Background and aims:* Inflammatory bowel disease is associated with intestinal oxidative stress. In the present study we test the preventative effect of *Lactobacillus fermentum*, a probiotic that produces per se glutathione, in the trinitrobenzenesulphonic acid (TNBS) model of rat colitis. *Methods:* Colitis was induced in rats by intracolonic administration of 10 mg of TNBS dissolved in 0.25 ml of 50% ethanol. *L. fermentum* was administered orally (5×10^8 CFU suspended in 0.5 ml of skim milk) to a group of rats for 3 weeks, starting 2 weeks before colitis induction. Colonic damage was evaluated both histologically and biochemically, and the colonic luminal contents were used for bacterial studies as well as for short chain fatty acid (SCFA) production. *Results:* *L. fermentum* treatment resulted in an amelioration of the inflammatory response in colitic rats as evidenced histologically and by a significant reduction of colonic MPO activity ($P<0.05$). The probiotic partially counteracted the colonic glutathione depletion induced by the inflammatory process. In addition, probiotic-treated colitic rats showed significant lower colonic tumour necrosis factor (TNF)α levels ($P<0.01$) and inducible nitric oxide synthase (iNOS) expression when compared to non-treated rats. Finally, the probiotic induced growth of *Lactobacilli* species and production of SCFA in colonic contents in comparison with control colitic rats. *Conclusion:* Administration of the probiotic *L. fermentum* facilitates the recovery of the inflamed tissue in the TNBS model of rat colitis, an effect associated with increased levels of glutathione as well as with amelioration of the production of some of the mediators involved in the inflammatory response of the intestine, such as TNFα and NO.

Keywords *Lactobacillus fermentum* · TNBS experimental rat colitis · Glutathione · Oxidative stress

Introduction

Inflammatory bowel disease (IBD) is a chronic disease of the digestive tract, and usually refers to two related conditions, ulcerative colitis and Crohn's disease, which are characterised by chhronic and spontaneously relapsing inflammation. Although the aetiology 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]. A possible therapeutic approach in IBD therapy is the administration of probiotic microorganisms, defined as viable nutritional agents conferring benefits to the health of 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 of successful induction and maintenance of remission of chronic pouchitis after oral bacteriotherapy [7, 8].

Different mechanisms have been proposed to participate in the therapeutic effects exerted by probiotic microorganisms. Firstly, these microorganisms could 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, 9]; secondly, probiotics have been proposed to modulate the host defenses by influencing the intestinal immune system [10, 11]; and thirdly, these microorganisms have been reported to positively affect the intestinal barrier function [12, 13]. Moreover, an interesting approach in IBD treatment is the administration of probiotics capable of delivering in the intestinal lumen compounds that have been reported to exert beneficial effects in these intestinal conditions. Thus, the use of genetically modified *Lactococcus lactis* able to promote the delivery of either the anti-inflammatory cytokine mIL-10 [14] or trefoil factors [15] in the intestine cures or prevents experimental enterocolitis in mice. In addition, nitric oxide released by *Lactobacillus farciminis* improves experimental colitis in rats [16].

It is well reported that IBD is characterised by an unbalanced formation of reactive oxygen species and antioxidant micronutrients, and this may be important in the pathogenesis and/or perpetuation of the tissue injury in IBD [17, 18], which may provide a rationale for therapeutic modulation of these intestinal conditions with antioxidants. Thus, antioxidant therapy has been shown to be beneficial in experimental models of colitis [19–21], having been proposed that the beneficial effects exerted by 5-aminsalycilic derivates in human IBD are derived from their antioxidant properties [22]. In addition, glutathione, the major component of the endogenous nonprotein sulfhydryl pool, is an endogenous antioxidant that is essential in maintaining mucosal integrity; and some experimental data confirm this important role. Firstly, the inflammatory status in experimental colitis is associated with its depletion [17, 18]; secondly, when the sulfhydryl blocker iodoacetamide is administered intracolonically to rats, they develop colonic inflammation [23]; and thirdly, glutathione supplementation improves colonic damage in experimental colitis [24, 25]. Considering all of the above, a probiotic strain able to directly produce or promote the intestinal release of glutathione could have potential use in the treatment of IBD.

The aim of the present study was to test the preventative effects of a *Lactobacillus fermentum* strain in the trinitrobenzenesulphonic acid (TNBS) model of rat colitis, a well-established model of intestinal inflammation with some resemblance to human IBD [26]. The selection of this lactobacilli strain was based on its capacity to produce glutathione, an uncommon feature amongst lactobacilli strains. Special attention was paid to its effects after oral administration to colitic rats on the colonic glutathione levels and on the production of some of the mediators involved in the inflammatory response, such as tumour necrosis factor α (TNFα), leukotriene B_4 (LTB_4) and nitric oxide (NO). In addition, the correlation between the intestinal anti-inflammatory effect of *L. fermentum* and the modifications induced on colonic flora and on SCFA production in the luminal contents 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, and was approved by the Animal Research and Ethic Committee of the University of Granada (Spain).

Reagents

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

Glutathione production in bacteria

Puleva Biotech lactic acid bacterial collection was screened for Lactobacilli bacteria with the ability to produce glu-

tathione. Cultures were grown in MRS medium at 37°C for 24 h and used to inoculate 50-ml Falcon tubes containing MRS medium. Cells were incubated for 24 h and 1-ml samples were taken to analyse glutathione content. Cells were washed with distilled water, suspended in 300 µl of TCA 7.5% (w/v) and disrupted by stirring. The mixture was centrifuged (at 10,500 *g* for 2 min) and 100 µl from the supernatant was transferred to a new tube containing 300 µl of MilliQ water. A portion (20 µl) from this solution was mixed with 340 µl of 0.6 M phosphate buffer (pH 7.8) and 340 µl of 1.25 mM Tris (carboxyethyl) phosphine HCl (TCEP) in 20 mM HCl. The sample was placed in the dark for 15 min, and then 800 µl of 12 mM *ortho*-pthalaldehyde in 50 mM sodium acetate was added and samples were placed at 4°C for 15 min. Samples were analysed by HPLC using a Spherisorb S3 ODS column at 0.8 ml/min in isocratic mode using 50 mM sodium acetate (pH 7.7)/acetonitrile (96:4) as mobile phase.

Preparation and administration of the probiotic

L. fermentum 5716, a human breast milk derived strain [27], was obtained from Puleva Biotech (Granada, Spain) and was normally growth in MRS media at 37°C under anaerobic conditions using the Anaerogen system (Oxoid, Basingstoke, UK). For probiotic treatment, bacteria was suspended in skim milk (10^9 CFU/ml) and stored at −80°C 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 under 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 the probiotic orally (5×10^8 CFU suspended in 0.5 ml of skim milk), daily for 3 weeks. Both non-colitic and control groups were given daily administration of the vehicle used to administer the probiotic (0.5 ml of skim milk). Two weeks after the treatment was started, 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. [28]. Briefly, they were anaesthetised with halothane and given 10 mg of TNBS dissolved in 0.25 ml of 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm through the anus. Rats from the non-colitic group were administered intracolonically with 0.25 ml of phosphate-buffered saline instead of TNBS. All rats were killed with an overdose of halothane 1 week after induction of colitis.

Assessment of colonic damage

Body weight, water and food intake were recorded daily throughout the experiment. After the rats were sacrificed, the colon was removed aseptically and placed on an ice-cold plate, and longitudinally opened; then the luminal contents were collected for microbiological studies and for SCFA quantification (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 who were unaware of the treatment, according to the criteria described by Bell et al. [29] and Camuesco et al. [30], which take 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 taken at different levels and stained with haematoxylin and eosin. The histological damage was evaluated on a 0–27 scale by two pathologist observers (A.N. and A.C.), who were blinded to the experimental groups, according to the criteria described previously [30]. The colon was subsequently divided into four segments for biochemical determinations. Two fragments were frozen at −80°C 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 glutathione content determination. The remaining sample was immediately processed for the measurement of TNFα and leukotriene B_4 (LTB_4) levels. All biochemical measurements were performed in duplicate and completed within 1 week of sample collection.

MPO activity was measured according to the technique described by Krawisz et al. [31]; the results were expressed as MPO units per gram of wet tissue; 1 unit of MPO activity was defined as that degrading 1 µmol hydrogen peroxide/min at 25°C. Total glutathione content was quantified with the recycling assay described by Anderson [32], and the results were expressed as nmol/g wet tissue. Colonic samples for TNFα and LTB_4 determinations were immediately weighed, minced on an ice-cold plate and suspended in a tube with 10 mM sodium phosphate buffer (pH 7.4) (1:5 w/v). The tubes were placed in a shaking water bath (37°C) for 20 min and centrifuged at 9,000 *g* for 30 s at 4°C; the supernatants were frozen at −80°C until assay. TNFα was quantified by enzyme-linked immunoabsorbent assay (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the results were expressed as pg/g wet tissue. LTB_4 was determined by enzyme immunoassay

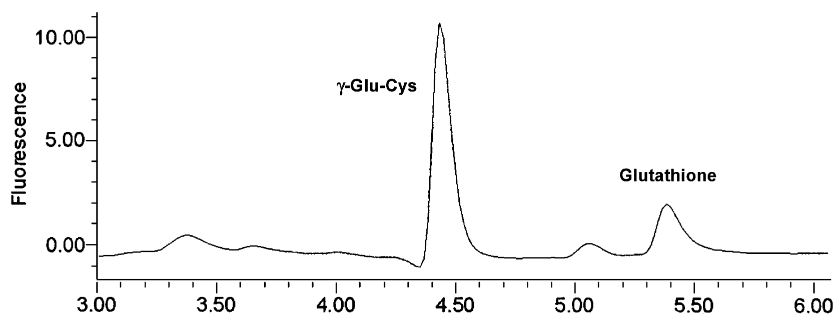

Fig. 1 HPLC analysis of glutathione and γ-Glu-Cys dipeptide production by *L. fermentum* 5716

(Amersham Pharmacia Biotech) and the results expressed as ng/g wet tissue. iNOS expression was analysed by Western blotting as previously described [20], and control of protein loading and transfer was conducted by detection of the β-actin levels.

Microbiological studies

Luminal content samples were homogenised in peptone physiological saline (100 mg faeces/ml). Tenfold serial dilutions were made in the same medium and aliquots of 0.1 ml of the appropriate dilution were spread onto the following agar media: MRS agar for lactobacilli, MRS agar supplemented with 0.5 mg/l dicloxacillin, 1 g/l LiCl and 0.5 g/l L-cysteine hydrochloride for *Bifidobacterium*; reinforced clostridial containing 20 μg/ml de polymyxin for Clostridium. All media were obtained from Oxoid (Basingstoke, UK), whereas antibiotics and other supplements were obtained from Sigma (St. Louis, MO). Culture plates were incubated in absence of oxygen at 37°C for 24–48 h. Similarly, 1 ml of suitable dilution was spread onto specific Count Plates Petrifilm (3M, St. Paul, MN) for coliforms, for total aerobes and for Enterobacteriaceae. Plates were incubated at 37°C for 24 h. After the incubation, the specific colonies grown on the selective culture media were counted and the number of viable microorganism per gram faecal (CFU/g) was calculated. The mean and standard error per group were calculated from the log values of the CFU/g.

SCFA quantification in colonic contents

To quantify the SCFA concentration in the colonic luminal contents, samples were homogenised with 150 mM $NaHCO_3$ (pH 7.8) (1:5, wt/v) in an argon atmosphere. Samples were incubated for 24 h at 37°C and stored at −80°C until the extraction. To extract the SCFAs, 50 μl of the internal standard 2-methylvaleric acid (100 mM), 10 μl of sulphuric acid and 0.3 ml of ethyl acetate were added to 1 ml of the homogenate and then centrifuged at 10,000 *g* for 5 min at 4°C. The supernatants were dehydrated with sodium sulphate anhydrous and centrifuged 10,000 *g* for 5 min at 4°C. Later, 0.5 ml of the sample was splitless inoculated into a gas chromatograph (Varian CP-3800) equipped with an ID (CPWAX 52CB 60 m×0.25 mm), and connected to a FID detector (Varian, Lake Forest, CA). Helium was used as the carrier and the make-up gas, with a flow rate of 1.5 ml/min. Injection temperature was 250°C. Acetate, propionate and butyrate concentrations were automatically calculated from the areas of peaks using the Star Chromatography WorkStation program (version 5.5), which was on-line connected to the FID detector.

Statistics

All results are expressed as the mean±SEM. 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 analysed using the Mann–Whitney *U*-test. Differences between propor-

Table 1 Effects of *L. fermentum* (5×10^8 CFU/rat·day) 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	60.4±2.8
TNBS control	7 (6–9)	4.0±0.3	226.5±17.6
TNBS probiotic	5.5 (2–7)*	2.6±0.5**	149.7±13.0**

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±SEM

*$P<0.05$, **$P<0.01$ vs TNBS control. All colitic groups differ significantly from non-colitic group ($P<0.01$, not shown)

tions were analysed with the chi-square test. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Maryland), with statistical significance set at $P<0.05$.

Results

Intestinal anti-inflammatory activity of *L. fermentum* administration in rats with TNBS-induced colitis

More than 50 strains of lactic acid bacteria belonging to Puleva Biotech's collection were screened for their ability to produce glutathione. The results confirmed that the ability to produce glutathione is not a common feature in the lactobacilli group, although it has been reported in other prokaryotic microorganism [33, 34]. In fact, the production of glutathione was detectable in the strain *L. fermentum* 5716, which, in addition to its ability to produce glutathione (1.4±0.3 mM in culture media), was also able to generate the antioxidant dipeptide γ-Glu-Cys (2.3±0.2 mM in culture media) (Fig. 1). For this reason, we decided to use this strain to test its ability to prevent the inflammatory response in the in vivo assay of experimental colitis.

L. fermentum 5716 administration for 2 weeks failed to induce any symptoms of diarrhoea or effect in the weight evolution (data not shown). However, once 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 of the extent of colonic necrosis and/or inflammation (Table 1). This anti-inflammatory effect was also associated with a significant reduction of the colonic weight/length ratio between both colitic groups, an index of colonic oedema that is increased significantly as a consequence of the inflammatory process (Table 1). The histological studies confirmed the intestinal anti-inflammatory effect exerted by *L. fermentum* (Fig. 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 21.6±2.3 (mean±SEM). Colonic samples were characterised by severe oedema, interstitial microhaemorrhages and diffuse leucocyte 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

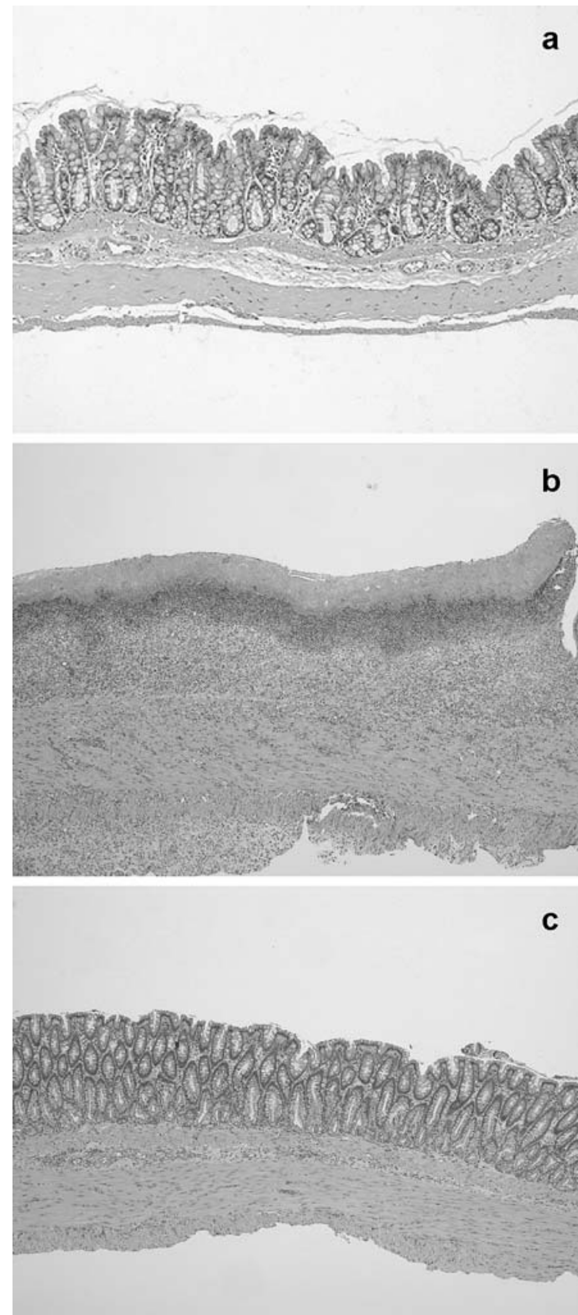

Fig. 2 Histological sections of colonic mucosa from colitic rats 1 week after TNBS instillation stained with haematoxylin and eosin. **a** Non-colitic group showing the normal histology of the rat colon (original magnification ×20). **b** TNBS control group showing complete destruction of the mucosa, which has been substituted by inflammatory granulation tissue. There is evident edema and intense diffuse transmural inflammatory infiltrate (original magnification ×100). **c** *L. fermentum*-treated group showing amelioration in the inflammatory process and 'restoration' of the mucosal tissue with the presence of mucin-replenished goblet cells (original magnification ×100)

Table 2 Myeloperoxidase (MPO) activity, total glutathione (GSH) content, TNFα and LTB_4 levels in colon specimens from non-colitic rats, TNBS control colitic rats and TNBS colitic rats treated with *L. fermentum* (5×10^8 CFU/rat·day)

Group	MPO activity (units MPO/g)	GSH (nmol/g)	LTB_4 (ng/g)	TNFα (pg/g)
Non-colitic (*n*=10)	8.3±1.8	1,937±37	3.7±0.4	331.6±25.7
TNBS control (*n*=10)	313.0±6.1$^{\#\#}$	1,331±62$^{\#\#}$	14.6±1.7$^{\#\#}$	680.9±52.8$^{\#}$
TNBS probiotic (*n*=10)	193.7±25.9$^{\#\#,**}$	1,614±85$^{\#,*}$	13.2±1.6$^{\#\#}$	469.5±67.6$^{*,\#}$

Data are expressed as mean±SEM
**P*<0.05, ***P*<0.01 vs TNBS control group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 vs non-colitic group

moderate to severe goblet cell depletion. However, histological analysis of the colonic specimens from rats treated with the probiotic revealed a more pronounced recovery of the intestinal architecture than controls, with a score of 9.4±1.9 (mean±SEM) (*P*<0.01 vs TNBS control group). Thus, most of the samples (nine of ten) 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-epithelisation. 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 slight to moderate with a patchy distribution, although neutrophils were the predominant cell type.

The lower leucocyte infiltration was also assessed biochemically by the reduction of colonic MPO activity, a marker of neutrophil infiltration that was enhanced in the TNBS control group (Table 2). In addition, probiotic-treated colitic rats showed a significant increase of colonic glutathione 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 [35] (Table 2). Finally, the colonic inflammation induced by TNBS was characterised by increased levels of colonic TNFα and LTB_4 (Table 2) as well as by a greater colonic iNOS expression (Fig. 3) in comparison with non-colitic animals. Treatment of colitic rats with *L. fermentum* resulted in a significant reduction of colonic TNFα levels (Table 2), but no significant modification of colonic LTB_4 levels was obtained between both colitic groups (Table 2). Finally, a lower colonic iNOS expression was also observed in colitic animals that received the bacteria suspension when compared to TNBS control animals (Fig. 3).

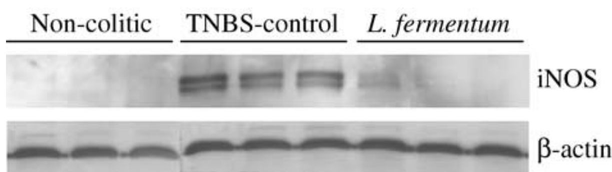

Fig. 3 Effects of *L. fermentum* treatment (5×10^8 CFU/rat·day) on colonic nitric oxide synthase (*NOS*) expression in TNBS experimental colitis in rats

Effects of *L. fermentum* administration on colonic bacterial profile

TNBS colitis resulted in a significant reduction of faecal lactobacilli count in comparison with normal rats (*P*=0.003). Probiotic-treated colitic rats showed significantly higher counts of *Lactobacilli* species in colonic contents in comparison with control colitic rats (*P*=0.039), without showing statistical differences with non-colitic control group (Fig. 4). No statistical differences were observed in Bifidobacteria counts amongst three groups (*P*>0.1; Fig. 4) or in the amount of other faecal potential pathogenic bacteria such as entherobacteria or coliforms (data not shown).

Effects of *L. fermentum* administration on SCFA production

When the colonic contents from TNBS control rats were incubated for 24 h, a reduction of the levels of SCFA was

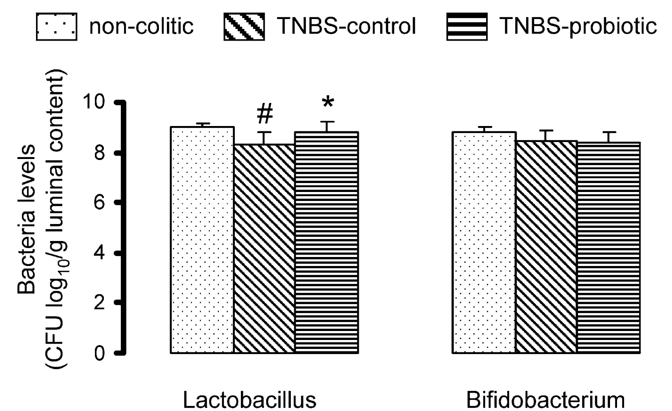

Fig. 4 Effects of *L. fermentum* (5×10^8 CFU/rat·day) treatment on bacteria levels (Lactobacillus and Bifidobacteria) in TNBS experimental colitis in rats. **P*<0.05 vs TNBS control; $^{\#}P$<0.01 vs non-colitic group

Table 3 Effects of *L. fermentum* (5×10^8 CFU/rat·day) treatment on short chain fatty acid production in colonic contents in TNBS experimental colitis in rats

Group	Acetate (mg/g faeces)	Propionate (mg/g faeces)	Butyrate (mg/g faeces)
Non-colitic (*n*=8)	25.3±2.9	11.5±1.0	6.3±1.1
TNBS control (*n*=9)	6.2±1.6	2.7±0.6	0.8±0.2
TNBS probiotic (*n*=10)	13.3±1.9*	5.3±0.7**	2.6±0.7**

Data are expressed as mean±SEM

*$P<0.05$, **$P<0.01$ vs TNBS control. All colitic groups differ significantly from non-colitic group ($P<0.01$, not shown)

observed compared to non-colitic rats ($P<0.01$, Table 3), similar to that described previously in this model of experimental colitis [36]. However, the intestinal contents obtained from the colitic treated rats showed greater acetate, butyrate and propionate production than those from TNBS control rats without probiotic treatment ($P<0.01$, Table 3).

Discussion

IBD is characterised by the abnormal production of free radicals with resultant oxidant-induced tissue injury and reduced antioxidant defenses [17, 18]. For this reason, antioxidant therapy can constitute an interesting approach in the downregulation of this inflammatory condition. In fact, the beneficial effects exerted by the 5-aminosalicylic derivatives in the treatment of IBD have been partially attributed to their antioxidant and free radical scavenger properties [22]. Moreover, several antioxidant compounds, such as flavonoids or vitamin E, have been reported to exert anti-inflammatory activity in experimental models of rat colitis [19–21], which was associated with restoration of glutathione colonic mucosal levels. Glutathione is a sulfhydryl-derived compound that actively participates in the antioxidant mechanisms of the intestinal mucosa, preserving it from oxidant-induced tissue damage. Different studies have reported diminished glutathione content in these intestinal conditions, both in humans [37] and in experimental models of rat colitis [19–21], and that glutathione supplementation results in beneficial effects in experimental colitis [24, 25]. All these facts prompted us to evaluate the intestinal anti-inflammatory effect of the probiotic *L. fermentum*, a microorganism that has been demonstrated in vitro to produce antioxidant compounds, such as glutathione and its precursor the dipeptide γ-Glu-Cys, in the TNBS model of rat colitis.

The results obtained in the present study reveal the efficacy of *L. fermentum* in this experimental model of colitis, thus incorporating a new microorganism to the probiotics that have been reported to attenuate the development of colonic injury in experimental and human IBD [38]. This beneficial effect was histologically evidenced with a significant reduction of the extent and severity of inflamed tissue achieved after probiotic treatment in comparison with non-treated colitic rats. The anti-inflammatory effect was also stated biochemically, since its administration to colitic rats resulted in a significant inhibition of colonic MPO activity, a marker of neutrophil infiltration previously described to be upregulated in experimental colitis [31], and widely used to detect and follow intestinal inflammatory processes. In consequence, a reduction of the activity of this enzyme can be interpreted as a manifestation of the anti-inflammatory activity of a given compound [39]. The ability of the probiotic to reduce granulocyte infiltration was confirmed histologically since the level of leucocyte infiltrate in the colonic mucosa was lower in treated colitic animals than in the corresponding TNBS control groups. This may account for the beneficial effect showed by this probiotic because margination and extravasation of circulating granulocytes contributes markedly to the colonic injury in this model of IBD [40]. These results are in agreement with other studies that describe the attenuation exerted by several probiotics in leucocyte–endothelial cell adhesion in this experimental model of rat colitis [16]. The inhibitory effect on leucocyte infiltration may be the consequence of the preventative effect exerted by the probiotic against the free radical derived oxidative injury that takes place after TNBS instillation in the colonic tissue [25, 41], since the intestinal anti-inflammatory effect was associated with a restoration of the colonic glutathione levels in comparison with non-treated colitic rats. The production of γ-Glu-Cys, precursor of glutathione, by this Lactobacilli strain may play a key role, since it has been described to be more efficiently uptaken than glutathione in the intestine. Although γ-Glu-Cys can be also substrate for other enzymes, like gamma-glutamylcyclotransferase, glutathione synthesis is increased in animal cells because of its higher affinity for the enzyme glutathione synthetase [42]. The free radical scavenger properties attributed to both compounds, γ-Glu-Cys and glutathione, produced by this probiotic seem to be crucial in its anti-inflammatory effect. In fact, it has been proposed that free radical generation in the inflamed tissue constitutes an early signal that promotes the infiltration of neutrophils into colonic tissue, which in turn produce a large amount of free radicals that actively participate in the perpetuation of the inflammatory response [43]. For this reason, the rapid neutralisation of these reactive oxygen species would result in the inhibition of neutrophil infiltration, as observed in the present

study. The inhibitory effect of the probiotic on the production and/or release of others mediators with chemotactic properties, like LTB_4, can be ruled out because the probiotic treatment was not associated with a significant modification of the colonic levels of this eicosanoid in comparison with non-treated colitic rats.

Moreover, this inhibitory effect on neutrophil infiltration attributed to the probiotic may also justify the inhibition of the synthesis and/or release of NO, another mediator that participates in the inflammatory process, since probiotic treatment of colitic rats was associated with a reduction in colonic iNOS expression. During the last decade, it has become increasingly clear that chronic colonic inflammation, both in human IBD and in experimental colitis, is associated with enhanced NO production, mainly via iNOS activity [44–46]. The simultaneous overproduction of NO and reactive oxygen metabolites, like superoxide anion, can yield the highly toxic radical peroxynitrite in the inflamed intestine [17]. Since neutrophils have been also considered as an important source of NO [47, 48], the effect exerted by *L. fermentum* in decreasing the neutrophil infiltration may in turn contribute to preserve colonic mucosa from peroxynitrite insult.

The present study also reveals that probiotic treatment promotes the downregulation of TNFα, a pro-inflammatory mediator that has been proposed to play a key role in colonic inflammation [49]; in fact, different drugs capable of interfering with the activity of this mediator are being developed for IBD therapy [50]. The ability of probiotic bacteria to downregulate TNFα 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 [51]. 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 [51]. The results obtained in the present study show that, in the case of *L. fermentum*, this relationship between bacteria and mucosal cells may be driven by SCFA, mainly butyrate. In fact, when colonic contents were incubated for 24 h, SCFA production was increased in probiotic-treated colitic rats in comparison with the corresponding control rats without probiotic treatment. Thus, the inhibitory effect of probiotic administration on cytokine production may be related to the ability of SCFA to interfere with transcription factors. Nuclear factor-kappa B (NF-κB) is a transcription factor that, in combination with others, plays a central role in regulating the expression of genes encoding numerous cytokines in immune and inflammatory responses [52]. Thus, it has been previously reported that butyrate decreases TNFα production by intestinal biopsies and by isolated lamina propria mononuclear cells via inhibition of NF-κB activation and IκBα degradation [53]. The inhibitory effect of butyrate on NF-κB activation in HT-29 cells, probably derived from its ability to inhibit deacetylases, has also been reported [54]. However, the amelioration of the colonic oxidative stress observed after probiotic treatment to colitic rats may also account for its effect on cytokine production, since NF-κB is a redox-sensitive transcription factor activated by oxidant stress in the inflamed intestinal mucosa [55].

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 by other probiotics, mainly a role in prevention in the imbalance in the intestinal microflora, given the relative predominance of aggressive bacteria and insufficient amount of protective species that has been reported to occur 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 contributes to the transmural inflammation [56]. 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 probiotic treatment without showing statistical differences with non-colitic rats.

In conclusion, administration of the probiotic *L. fermentum* 5716 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, including TNFα and NO. This beneficial effect could be ascribed to its ability to prevent oxidative stress that occurs in this inflammatory condition, through the increased production of glutathione, which might attenuate the exacerbated immune response evoked by the colonic instillation of the hapten TNBS in the rats.

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