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A comparative study of the preventative effects exerted by two probiotics, *Lactobacillus reuteri* and *Lactobacillus fermentum*, in the trinitrobenzenesulfonic acid model of rat colitis

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The intestinal anti-inflammatory effects of two probiotics isolated from breast milk, *Lactobacillus reuteri* and *L. fermentum*, were evaluated and compared in the trinitrobenzenesulfonic acid (TNBS) model of rat colitis. Colitis was induced in rats by intracolonic administration of 10 mg TNBS dissolved in 50 % ethanol (0·25 ml). Either *L. reuteri* or *L. fermentum* was daily administered orally (5×10^8 colony-forming units suspended in 0·5 ml skimmed milk) to each group of rats (n 10) for 3 weeks, starting 2 weeks before colitis induction. Colonic damage was evaluated histologically and biochemically, and the colonic luminal contents were used for bacterial studies and for SCFA production. Both probiotics showed intestinal anti-inflammatory effects in this model of experimental colitis, as evidenced histologically and by a significant reduction of colonic myeloperoxidase activity ($P<0·05$). *L. fermentum* significantly counteracted the colonic glutathione depletion induced by the inflammatory process. In addition, both probiotics lowered colonic TNFα levels ($P<0·01$) and inducible NO synthase expression when compared with non-treated rats; however, the decrease in colonic cyclo-oxygenase-2 expression was only achieved with *L. fermentum* administration. Finally, the two probiotics induced the growth of Lactobacilli species in comparison with control colitic rats, but the production of SCFA in colonic contents was only increased when *L. fermentum* was given. In conclusion, *L. fermentum* can exert beneficial immunomodulatory properties in inflammatory bowel disease, being more effective than *L. reuteri*, a probiotic with reputed efficacy in promoting beneficial effects on human health.

Probiotics: Inflammatory bowel diseases: Immunomodulation: Anti-inflammatory activity

Several studies have proposed that breast-feeding protects against many immune-mediated diseases, including those related to inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease (Klement *et al.* 2004). These observations confirm previous studies in which breast-milk feeding limited the development of colitis in IL-10 knock-out mice. This finding was explained by a change in the intestinal flora of the developing mice from pathogenic bacteria to non-adherent bacteria, promoted by milk oligosaccharides that stimulate *Bifidobacterium* and *Lactobacillus* growth (Kunz *et al.* 2000). In addition, the presence of lactic bacteria in breast milk could also account for its preventative effect against intestinal inflammation (Martin *et al.* 2003).

In fact, the administration of probiotic micro-organisms has been proposed to promote a balanced colonic microbial environment and thus probably help in both prevention and control of IBD. Previous studies have reported that the administration of a mixture of bifidobacteria and lactobacilli (Venturi *et al.* 1999) or *Escherichia coli* Nissle 1917 (Rembacken *et al.* 1999) prevents the relapse of ulcerative colitis, showing the latter to have an equivalent effect to mesalazine in maintaining remission. The studies performed both in human subjects and in animal models of intestinal inflammation have provided some clues about the different mechanisms involved in the therapeutic effects exerted by probiotic micro-organisms. First, probiotics could suppress the growth or epithelial binding and invasion of enteric pathogenic bacteria, maybe due to their ability to decrease luminal pH via production of SCFA (Sakata *et al.* 2003), promote the secretion of bactericidal proteins (Boris *et al.* 2001; Collado *et al.* 2005) and/or stimulate mucin production (Mack *et al.* 1999). Second, probiotics have been reported to exert immunoregulatory activities, either by inducing protective cytokines, such as IL-10 and transforming growth factor-β, or by suppressing pro-inflammatory cytokines, such as TNFα, in the intestinal mucosa (Borruel *et al.* 2002; Schultz *et al.*

Abbreviations: COX-2, cyclo-oxygenase-2; IBD, inflammatory bowel disease; iNOS, inducible NO synthase; LTB_4, leukotriene B4; MPO, myeloperoxidase; TNBS, trinitrobenzenesulfonic acid.

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2003; Pathmakanthan *et al.* 2004; Chen *et al.* 2005). And third, these micro-organisms positively affect the intestinal barrier function by decreasing mucosal permeability (Madsen *et al.* 2001). However, the detailed mechanisms by which these bacteria mediate their effects are not fully understood.

The aim of the present study was to compare the preventative effects of *Lactobacillus fermentum* CECT5716 and *L. reuteri* ATCC55730, two hetero-fermentative bacteria found in breast milk (Martin *et al.* 2005; BioGaia, 2006), in the trinitrobenzenesulfonic acid (TNBS) model of rat colitis. This is a well-established model of intestinal inflammation with some resemblance to human IBD (Jurjus *et al.* 2004). The selection of the probiotics was based on previous *in vitro* and *in vivo* studies that make them suitable candidates for the treatment of these intestinal conditions. In a previous study, we have reported that *L. fermentum* CECT5716 showed intestinal anti-inflammatory activity in the TNBS model of rat colitis (Peran *et al.* 2006). That effect was attributed, at least partially, to its ability to release glutathione and the antioxidant dipeptide γ-Glu-Cys, thus counteracting the damaging effects derived from the intestinal oxidative stress generated (Grisham *et al.* 1991), similarly to what occurs in human IBD (Grisham, 1994). This effect was also associated with a reduction in TNFα production and in inducible NO synthase (iNOS) expression in the inflamed tissue (Peran *et al.* 2006). On the other hand, different strains of *L. reuteri* have been described to show beneficial effects in several experimental models of colitis, both in mice (IL-10 and $CD4^{+}$T cell-induced colitis in the severe combined immunodeficient mouse) (Madsen *et al.* 1999; Moller *et al.* 2005), and in rats (acetic acid- and methothrexate-induced) (Mao *et al.* 1996; Holma *et al.* 2001). *In vitro* studies have shown that *L. reuteri* DSM12246 is able to down regulate the stimulated production of the pro-inflammatory cytokines IL-12 and TNFα in dendritic cells while inducing the anti-inflammatory cytokine IL-10 (Christensen *et al.* 2002). Similarly, another strain of *L. reuteri* inhibited mRNA up regulation, cellular accumulation and secretion of the chemokine IL-8 induced by TNFα in intestinal epithelial cells (Ma *et al.* 2004).

Materials and methods

The present 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 (Bethesda, MD, USA).

Reagents

All chemicals were obtained from Sigma Chemicals (Madrid, Spain), unless otherwise stated.

Preparation and administration of the probiotics

L. fermentum CECT5716 was provided by Puleva Biotech (Granada, Spain), *L. reuteri* ATCC55730 was obtained from a commercial dairy product licensed by BioGaia AB (Stockholm, Sweden). Lactobacilli strains were normally grown in De Man–Rogosa–Sharpe (MRS) media at 37°C in anaerobic conditions using the Anaerogen system (Oxoid Ltd, Basingstoke, Hants, UK). For probiotic treatment, bacteria were suspended in skimmed milk (10^9 colony-forming units/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), maintained in standard conditions and fed the Panlab A04 diet (Panlab, Barcelona, Spain) *ad libitum*. The composition of the diet was: 17·2 % protein, 2·7 % fat, 59·7 % carbohydrates, 3·9 % fibre (mainly cellulose), 4·4 % minerals and 12 % humidity. The rats were randomly assigned to four groups (n 10); two of them (non-colitic and control groups) did not receive probiotic treatment and the remaining groups (treated groups) received orally each probiotic (5×10^8 colony-forming units suspended in 0·5 ml skimmed milk) daily for 3 weeks. Both non-colitic and control groups received orally the vehicle used to administer the probiotic (0·5 ml daily). At 2 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.* (1989). Briefly, they were anaesthetised with halothane and given 10 mg TNBS dissolved in 0·25 ml ethanol (50 %, v/v) by means of a Teflon cannula inserted 8 cm through the anus. Rats from the non-colitic group were administered intracolonically 0·25 ml PBS instead of TNBS. All rats were killed with an overdose of halothane 1 week after induction of colitis. After killing, the following tissues were quickly removed and weighed: spleen, thymus, kidneys, liver and soleus muscle. Also the colon was obtained for the assessment of colonic damage.

Assessment of colonic damage

The body weight, water and food intake, as well as stool consistency, 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 the luminal contents were collected for the measurements of faecal moisture, pH and microbiological and SCFA production studies (see later). 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.* (1995), 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 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 (Camuesco *et al.* 2005). The colon was subsequently divided into four segments for biochemical determinations. Two

fragments were frozen at −80°C for myeloperoxidase (MPO) activity and iNOS and cyclo-oxygenase-2 (COX-2) expressions, and another sample was weighed and frozen in 1 ml TCA (50 g/l) for total glutathione content determinations. The remaining sample was immediately processed for the measurement of colonic TNFα, IL-1β, IL-10 and leukotriene B4 (LTB_4) levels. All biochemical measurements were completed within 1 week from the time of sample collection and were performed in duplicate.

MPO activity was measured according to the technique described by Krawisz *et al.* (1984). The results are expressed as MPO units per g wet tissue; one unit MPO activity was defined as that degrading 1 μmol H_2O_2/min at 25°C. Glutathione (reduced and oxidised) concentrations were assayed by HPLC with fluorimetric detection of oxidised and reduced glutathione, according to the method proposed by Martin &White (1991); the results are expressed as nmol glutathione/mg wet tissue. Colonic samples for cytokine 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 9000 ***g*** for 30 s at 4°C; the supernatant fractions were frozen at −80°C until assay. TNFα, IL-1β and IL-10 were quantified by ELISA (Amersham Pharmacia Biotech, Amersham, Bucks, UK) and the results were expressed as pg/mg protein; the detection limits were 31–2500 pg/ml for TNFα, 25·6–2500 pg/ml for IL-1β and 16–500 pg/ml for IL-10. LTB_4 was determined by enzyme immunoassay (Amersham Pharmacia Biotech) and the results expressed as pg/mg protein; the detection limits were 6·2–800 pg/ml.

The colonic expression of iNOS and COX-2 was analysed by Western blotting as previously described (Camuesco *et al.* 2004). The dilutions of each primary antibody were 1:2000 for iNOS (Transduction Laboratories, Becton Dickinson Biosciences, Madrid, Spain) and 1:1000 for COX-2 (Cayman Chemical Company, Montigny le Bretonneux, France), and incubated overnight at 4°C followed by peroxidase-conjugated anti-rabbit IgG antibody (1:3000) for 1 h. Control of protein loading and transfer was conducted by detection of the β-actin levels.

pH, moisture and short-chain fatty acid quantification in colonic contents

The pH values in the colonic contents were measured using a GLP21-21 pH-meter (Crison, Barcelona, Spain) after their suspension in water (1:5, w/v). The water content of the luminal stools was calculated by weight differences between fresh (immediately after collection) and dried (kept during 24 h at 65°C) samples.

To quantify the SCFA concentrations in the colonic luminal contents, the samples were homogenised with 150 mM-$NaHCO_3$ (pH 7·8) (1:5, w/v) in an Ar atmosphere. Samples were incubated for 24 h at 37°C and stored at −80°C until the extraction. To extract the SCFA, 50 μl of the internal standard 2-methylvaleric acid (100 mM), 10 μl sulfuric acid and 0·3 ml ethyl acetate were added to 1 ml of the homogenate and, then, centrifuged at 10 000 ***g*** for 5 min at 4°C. The supernatant fractions were dehydrated with sodium sulfate anhydrous and centrifuged at 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, USA). The carrier and the make-up gas was He, with a flow rate of 1·5 ml/min. The 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; Varian Inc., Palo Alto, CA, USA), which was on-line connected to the FID detector.

Microbiological studies

Luminal content samples were weighed, homogenised 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 dicloxacilin (0·5 mg/l), LiCl (1 g/l) and L-cysteine hydrochloride (0·5 g/l)) and incubated under anaerobic conditions in an anaerobic chamber for 24–48 h at 37°C. Coliforms and enterobacteria were also determined by using specific Count Plates Petrifilm (3M, St Paul, MN, Canada). After incubation, the final count of colonies was reported as $\log_{10}$ colony-forming units per g material.

Statistics

All results are expressed as means with their standard errors. Differences between means were tested for statistical significance using a one-way ANOVA and *post hoc* least significance tests. Non-parametric data (scores) are expressed as medians and ranges and were analysed using the Mann–Whitney *U* test. Differences between proportions were analysed with the χ^2 test. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Rockville, MD, USA), with statistical significance set at $P<0{\cdot}05$.

Results

Effects of probiotic administration on body and tissue weight in colitic rats

The administration of probiotics for 2 weeks before colitis induction did not affect rat weight gain compared with untreated rats (data not shown). The intracolonic administration of TNBS resulted in an intestinal inflammatory status in the rats characterised by anorexia, loss of weight and diarrhoea, which gradually increased. Thus, 1 week after colitis induction, body weight was reduced by 4·5 (SEM 1·9) % in the TNBS-treated rats, whereas in saline-treated rats it was increased by 4·8 (SEM 0·7) % ($P<0{\cdot}01$). Although none of the probiotics were able to inhibit the anorexia and the loss of weight in the acute phase of the inflammation (data not shown), both lactobacilli restored the animals' weight at the end of the study, since it was increased by 0·6 (SEM 2·5) and by 0·88 (SEM 2·6) % in the colitic rats that received *L. fermentum* or *L. reuteri*, respectively, without showing statistical differences with control groups.

The anorexia and the inflammatory response caused an important modification in the weight of some tissues such as muscle, thymus, spleen, while liver and kidneys did not

show any significant changes (Table 1). Soleus muscle weight was reduced in colitic rats in comparison with non-colitic rats, although the statistical differences were only obtained in the rats treated with *L. reuteri*. Moreover, the inflammatory process provoked a reduction in thymus weight and an increase in spleen weight. None of the probiotics were able to counteract the increase in spleen weight, and only *L. fermentum* was able to partially restore the thymus weight.

Effects of probiotic administration on colonic inflammation

L. fermentum administration showed an amelioration of the diarrhoeic process, resulting in a significantly lower incidence of diarrhoea (20 %) after 7 d when compared with untreated control rats (80 %; $P<0{\cdot}05$) (Table 2). The macroscopic evaluation of the colonic segments 1 week after colitis induction revealed the preventative effect exerted by probiotics. This was evidenced by a significant reduction of the colonic weight:length ratio ($P<0{\cdot}01$) in both cases (Table 2), as well as by a significantly lower colonic damage score in comparison with control colitic rats, derived from a decrease in the extent of colonic necrosis and the presence of intestinal adhesions induced by the administration of TNBS (Table 2). However, only the group of colitic rats treated with *L. fermentum* showed significant reduction in these inflammatory parameters in comparison with untreated colitic control rats; *L. reuteri* showed only a tendency to decrease them ($P=0{\cdot}07$; Table 2).

The histological studies revealed that *L. fermentum* was more efficient in promoting the recovery of colonic tissue than *L. reuteri*. Histological assessment of colonic samples from the TNBS control group showed 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 15·9 (SEM 2·5). The histological analysis of the colonic specimens from rats treated with *L. fermentum* revealed a more pronounced recovery of the intestinal architecture than controls, with a score of 9·4 (SEM 1·9) ($P<0{\cdot}05$ *v.* TNBS control group). Thus, most of the samples (eight out of ten) showed almost complete restoration of the epithelial cell layer, in contrast to the extensive ulceration observed in non-treated animals. 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 colonic specimens from colitic rats treated with *L. reuteri* also showed a higher recovery than the intestinal segments from control colitic rats, and they were assigned a score value of 10·8 (SEM 2·5), lower than in the control group, but without showing statistical differences ($P=0{\cdot}14$). Thus, four out of ten samples showed evident restoration of the epithelial cell layer, while in the rest of the samples the epithelial ulceration of the mucosa affected over 40–50 % of the surface, lower than in most of the specimens from control colitic rats. Similarly, the goblet cell depletion was also attenuated in this group, and the presence of mucin content was evident, together with an absence of dilated crypts. Finally, the inflammatory infiltrate was also attenuated, being moderate with a patchy distribution.

The biochemical analysis of the colonic specimens confirmed the intestinal anti-inflammatory effect exerted by the probiotics, although again some differences were observed in their effects on the different parameters assayed. Colonic MPO activity was reduced after treatment with *L. reuteri* or *L. fermentum* by approximately 40 % although only *L. fermentum* treatment reached significance (Table 3). Since colonic MPO activity is considered as a biochemical marker of neutrophil infiltration (Krawisz *et al.* 1984), these results confirm the lower leucocyte infiltration into the inflamed tissue after probiotic treatment observed in the histological studies. Furthermore, treatment of colitic rats with the probiotics showed an increase in colonic glutathione content (Table 3), depleted in colitic rats as a consequence of the colonic oxidative stress caused by the TNBS-induced inflammatory process (Galvez *et al.* 2003). However, although both probiotics restored the values observed in non-colitic rats, only the group of rats treated with *L. fermentum* showed statistical differences in comparison with control colitic rats ($P<0{\cdot}01$). The colonic inflammation induced by TNBS was also characterised by increased levels of colonic TNFα (Table 3), IL-1β (339·5 (SEM 43·9) *v.* 28·4 (SEM 3·4) pg/mg protein in the non-colitic group; $P<0{\cdot}01$) and LTB_4 (146·6 (SEM 33·1) *v.* 9·8 (SEM 2·5) pg/mg protein in the non-colitic group; $P<0{\cdot}01$), and a reduction in IL-10 production (5·1 (SEM 1·2) *v.* 18·3 (SEM 3·1) pg/mg protein in the non-colitic group; $P<0{\cdot}01$). Only TNFα production was significantly reduced after treatment with either *L. reuteri* or *L. fermentum* (Table 3). No statistical differences were observed in the other pro-inflammatory mediators assayed (data not shown).

Finally, the inflammatory process in the colonic tissue was also characterised by higher expression of both iNOS and COX-2 in comparison with non-colitic animals (data not

Table 1. Effects of probiotic treatment on tissue weights in trinitrobenzenesulfonic acid (TNBS) experimental colitis in rats (Mean values with their standard errors for ten rats per group)

	Muscle (mg/g rat)		Liver (mg/g rat)		Kidneys (mg/g rat)		Spleen (mg/g rat)		Thymus (mg/g rat)	
Group	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Non-colitic	6·6	0·1	31·3	1·3	5·9	0·1	2·2	0·2	1·9	0·1
TNBS control	6·3	0·2	31·9	1·1	6·2	0·1	3·0*	0·2	0·9*	0·1
Lactobacillus reuteri	6·0*	0·1	35·1	1·3	5·9	0·2	3·3*	0·4	0·9*	0·1
L. fermentum	6·5‡	0·1	34·9	1·1	6·1	0·2	2·9*	0·2	1·2*†‡	0·1

* Mean value was significantly different from that of the non-colitic group ($P<0{\cdot}05$).
† Mean value was significantly different from that of the TNBS control group ($P<0{\cdot}05$).
‡ Mean value was significantly different from that of the *L. reuteri* group ($P<0{\cdot}05$).

Table 2. Effects of probiotic treatment on diarrhoea, adhesions, damage score, extent of the inflammatory lesion along the colon and changes in colon weight in trinitrobenzenesulfonic acid (TNBS) experimental colitis in rats
(Percentages, medians and ranges, and mean values with their standard errors for ten rats per group)

			Damage score (0–10)§		Extent of damage (cm)		Weight/length (mg/cm)	
Group	Diarrhoea (%)	Adhesions (%)	Median	Range	Mean	SEM	Mean	SEM
Non-colitic	0	0	0	0	0	0	71·0	2·5
TNBS control	80*	80*	7*	6–8·5	3·6*	0·3	249·7*	22·1
Lactobacillus reuteri	50*	50*	6*	4–8	2·8*	0·3	175·9*	11·9
L. fermentum	20†	10*†‡	5·5*‡	4–6·5	2·4*†	0·3	145·7*†‡	7·6

* Percentage or mean value was significantly different from that of the non-colitic group ($P<0·05$).
† Percentage or mean value was significantly different from that of the TNBS control group ($P<0·05$).
‡ Percentage or mean value was significantly different from that of the *L. reuteri* group ($P<0·05$).
§ Damage score for each rat was assigned according to the criteria described previously by Bell *et al.* (1995).

shown). Treatment of colitic rats with *L. fermentum* resulted in a significant reduction of the expression of both inducible enzymes in eight out of ten rats, whereas *L. reuteri* was only able to significantly reduce iNOS expression, and this was achieved in seven out of ten rats.

Effects of probiotic administration on colonic short-chain fatty acid production and bacterial profile

No clear differences were observed in the pH values of the colonic contents among the different groups of rats (Table 4). Moreover, although a tendency to increase the faecal water content was observed in all the colitic rats, only those treated with *L. reuteri* showed a significant difference in the faecal moisture (Table 4).

When the colonic contents from colitic control rats were evaluated for SCFA production, no significant reduction in any of their levels was observed compared with non-colitic rats (Table 4). However, a significant reduction in all the analysed SCFA was observed in the *L. reuteri*-treated group in comparison with all the other experimental groups (colitic or not). In contrast, colitic rats treated with *L. fermentum* showed similar values to those observed in non-colitic rats (Table 4).

Table 3. Effects of probiotic treatment on colonic myeloperoxidase (MPO) activity, glutathione content and tumour necrosis factor α levels in trinitrobenzenesulfonic acid (TNBS) experimental colitis in rats
(Mean values with their standard errors for ten rats per group)

	MPO activity (units/g)§		Glutathione (nmol/g)		TNFα (pg/mg protein)	
Group	Mean	SEM	Mean	SEM	Mean	SEM
Non-colitic	80	12	1479	51	17·8	2·4
TNBS control	1325*	144	1093*	85	74·0*	9·6
Lactobacillus reuteri	989*	206	1351	135	42·0*†	7·0
L. fermentum	882*†‡	114	1490†	112	53·1*†	11·0

* Mean value was significantly different from that of the non-colitic group ($P<0·05$).
† Mean value was significantly different from that of the TNBS control group ($P<0·05$).
‡ Mean value was significantly different from that of the *L. reuteri* group ($P<0·05$).
§ One unit of MPO activity was defined as that degrading 1 μmol H_2O_2/min at 25°C.

TNBS colitis also resulted in a significant reduction in colonic lactobacilli and bifidobacteria counts ($P<0·05$; Fig. 1), together with an increase in coliforms and enterobacteria ($P<0·05$; data not shown) in comparison with normal rats. Probiotic-treated colitic rats showed higher counts of lactobacilli and bifidobacteria species in the colonic contents than in control colitic rats, without showing statistical differences with the non-colitic control group (Fig. 1 (A)). No statistical differences were observed in the amount of faecal potential pathogenic bacteria such as enterobacteria or coliforms among the three colitic groups (data not shown). As expected, when the lactobacilli:pathogen ratio was evaluated, the inflammatory process did result in a significant decrease in comparison with normal rats; the administration of *L. fermentum* or *L. reuteri* resulted in the normalisation of this ratio (Fig. 1 (B)).

Discussion

The results obtained in the present study are supportive of the helpfulness of the dietary incorporation of probiotics in IBD therapy (Sartor, 2004). Furthermore, they confirm the intestinal anti-inflammatory activity previously shown by this strain of *L. fermentum* (CECT5716) (Peran *et al.* 2006) as well as by other strains of *L. reuteri* (Mao *et al.* 1996; Madsen *et al.* 1999; Holma *et al.* 2001; Moller *et al.* 2005), although the present study is the first that describes the efficacy of *L. reuteri* ATCC55730 in the TNBS model of rat colitis.

Both probiotics ameliorated some of the clinical manifestations of this colitis experimental model such as anorexia or diarrhoea and the macroscopic colonic damage; however, *L. fermentum* treatment seemed to be more effective. In fact, this probiotic significantly attenuated the incidence of diarrhoea and adhesions, increased thymus weight and reduced the colonic weight:length ratio as well as the damage score and extension. On the contrary, *L. reuteri* treatment did not show significant modifications on most of these parameters; only the colonic weight:length ratio was significantly reduced in comparison with untreated colitic rats.

The reduction in the diarrhoeic process exerted by *L. fermentum* can be a consequence of an improvement of the gut epithelial cell barrier function, thus contributing to its intestinal anti-inflammatory effect, as has been proposed to occur with other probiotics (Gionchetti *et al.* 2005). In fact,

Table 4. Effects of probiotic treatment on faecal pH and moisture, and on colonic short-chain fatty acid production in trinitrobenzenesulfonic acid (TNBS) experimental colitis in rats

(Mean values and standard deviations for ten rats per group)

	Faecal pH		Faecal moisture (%)§		Total SCFA (mg/l)		Acetate (mg/l)		Propionate (mg/l)		Butyrate (mg/l)	
Group	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Non-colitic	7·05	0·06	68·9	3·5	10 388	3655	6922	2917	2335	708	1130	267
TNBS control	7·26	0·04	72·2	1·4	7662	1290	4978	847	1858	322	825	177
Lactobacillus reuteri	7·32	0·06	78·5*	1·1	2821*†	75	1822*†	44	556*†	40	299*†	20
L. fermentum	7·31	0·03	76·1	1·1	9659‡	2298	6830‡	1888	2896‡	908	1028‡	279

* Mean value was significantly different from that of the non-colitic group ($P<0·05$).
† Mean value was significantly different from that of the TNBS control group ($P<0·05$).
‡ Mean value was significantly different from that of the *L. reuteri* group ($P<0·05$).
§ Faecal moisture was expressed as the proportion in water content expressed in %.

microscopic evaluation showed that the restoration in the epithelial lining was more evident in the rats administered *L. fermentum* (80 % of the samples showed complete restoration) than in those that received *L. reuteri* (40 %). This may be interesting since a barrier disruption leads to increased stimulation by luminal antigens. In this regard, mucosal inflammation can be considered a self-perpetuating process in which the disruption of the epithelial layer plays a central role (Heyman *et al.* 1994).

L. fermentum and *L. reuteri* were able to reduce neutrophil infiltration in the inflamed colon, as was observed in the microscopic analysis, although only *L. fermentum* treatment significantly decreased colonic MPO activity. The inhibition of neutrophil infiltration can account for their intestinal anti-inflammatory effect, given the important role attributed to these cells in the inflammatory process.

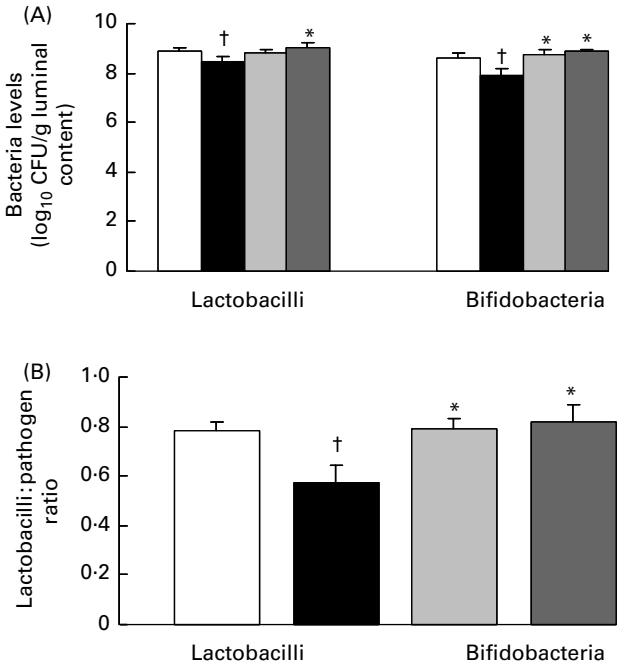

Fig. 1. Effects of probiotic treatment (5×10^8 colony-forming units (CFU)/rat·per d) on (A) bacteria levels (lactobacilli and bifidobacteria) and on (B) lactobacilli:pathogen ratio in trinitrobenzenesulfonic acid (TNBS) experimental colitis in rats. (□), Non-colitic group; (■),TNBS control group; (▒), *Lactobacillus reuteri*-treated group; (▒), *L. fermentum*-treated group. Values are means, with their standard errors represented by vertical bars. *Mean value was significantly different from that of the TNBS control group ($P<0·05$). †Mean value was significantly different from that of the non-colitic group ($P<0·01$).

L. fermentum treatment of TNBS colitic rats counteracted the depletion of colonic glutathione levels that took place in control colitic animals. This activity may play a crucial role in the intestinal anti-inflammatory effect of the probiotic because a situation of intense oxidative insult is an important mechanism for tissue damage during chronic intestinal inflammation and thus a common feature in human IBD (Grisham, 1994) as well as in the different experimental models of rat colitis, including the TNBS (Galvez *et al.* 2003) and the dextran sodium sulfate (Camuesco *et al.* 2004) models. The effect exerted by this probiotic could be due to its ability to release glutathione and the antioxidant dipeptide γ-Glu-Cys (Peran *et al.* 2006).

When other pro-inflammatory mediators were evaluated, *L. fermentum* and *L. reuteri* were able to significantly reduce colonic TNFα production. This may be relevant since this cytokine plays a key role in intestinal inflammation, and different drugs capable of interfering with the activity of this mediator are being developed for IBD therapy (Rutgeerts *et al.* 2004). Previous *in vitro* studies have also shown the ability of different probiotic, including *L. casei*, *L. bulgaricus*, *L. fermentum* or *L. salivarius* ssp. *salivarius*, to down regulate TNFα production (Borruel *et al.* 2002; Peran *et al.* 2005, 2006).

A common feature of both probiotics assayed is their ability to modify colonic microflora, which was altered as a consequence of the TNBS-induced inflammatory process (Peran *et al.* 2006). In this regard, the probiotic treatment restored the pathogenic bacteria:lactobacilli ratio. This effect could definitively contribute to the beneficial effect exerted by these probiotics in the TNBS model of experimental colitis. In fact, it has been previously described that the increase in *Lactobacillus* sp. levels reduces the concentration of adherent and translocated bacteria and attenuates the colitis in IL-10 gene-deficient mice (Madsen *et al.* 1999). This could prevent the pathogenic effect of other species that may contribute to the generation of an exacerbated immune response in intestinal inflammation, as proposed both in experimental models (Garcia-Lafuente *et al.* 1997) and in human subjects (Cummings *et al.* 2003).

However, the colonic SCFA content profiles shown by the two probiotics were different. Thus, *L. fermentum* was able to significantly counteract the decrease in colonic SCFA production observed in TNBS colitic rats, whereas *L. reuteri* treatment reduced even more the SCFA production despite its effect on colonic microbiota. The effect of *L. fermentum* on butyrate production is very interesting since it has been proposed that the inflammatory process results in an alteration of the intestinal epithelial cell function, including colonic SCFA utilisation, mainly butyrate, which is considered the most important SCFA for colonocyte metabolism (Mortensen & Clausen, 1996; Rodriguez-Cabezas *et al.* 2002).

In conclusion, *L. fermentum* and *L. reuteri* have shown intestinal anti-inflammatory activity in the TNBS model of rat colitis. However, each probiotic shows its own anti-inflammatory profile, confirming that not all probiotics present the same efficacy as anti-inflammatory agents, and do not share the same mechanisms of action. Of note, *L. fermentum* can be considered more effective than *L. reuteri*, a probiotic with reputed efficacy in promoting beneficial effects on human health (Valeur *et al.* 2004). Both probiotics can be found in breast milk, and although the doses administered to rats in the present study are higher than those probably incorporated in the infant by breast milk, the present results suggest that the colonisation of these probiotics in the colonic lumen would result in beneficial preventative effects in these intestinal conditions, probably derived from their immunomodulatory properties. Human clinical studies will be required in order to confirm these results.

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