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BENEFICIAL EFFECT OF A PROBIOTIC *LACTOBACILLUS FERMENTUM* CFR 2195 IN TRINITROBENZENESULFONATE INDUCED COLITIS IN RAT

Basavanna Girishkumar[1], David Elisha Henry[1], Krishnapura Srinivasan[2],
Siddalingaiya Gurudutt Prapulla[1]*
[1]Department of Fermentation Technology & Bioengineering
[2] Department of Biochemistry & Nutrition
Central Food Technological Research Institute, CSIR, Mysore–570020, India
*E-mail: prapullasg@yahoo.co.in

Abstract
The intestinal anti-inflammatory effects of L. fermentum CFR 2195, isolated from healthy breast fed infant's fecal matter was evaluated. Ulcerative colitis was induced in Wistar rats by intracolonic administration of TNBS. L. fermentum CFR 2195 was administered orally ($5x10^8$ colony-forming units) to rats daily for 3 weeks, starting 2 weeks prior to the induction of ulcerative colitis. Normal control group of rats were fed solely with basal diet. Probiotic control group was fed with basal diet along with probiotic administration. Among the colitis induced rats, the TNBS control group was fed with basal diet whereas, the TNBS-probiotic group was fed with basal diet along with probiotic administration. Colonic damage was evaluated both biochemically, histologically and the luminal contents were used for the microbiological studies. The intestinal anti-inflammatory effects of L. fermentum CFR 2195 were evidenced by a significant reduction in colonic myeloperoxidase activity (P<0·05) and also in histopathological changes. The results regarding the microbiological analysis revealed that when the lactobacilli: pathogen ratio was evaluated, the inflammatory condition did result in a significant decrease (P<0·05) in comparison with normal control group; the administration of probiotic L. fermentum CFR 2195 resulted in the restoration of the normal balance of lactobacilli: pathogen ratio. Treatment with L. fermentum CFR 2195 also significantly counteracted the colonic glutathione depletion induced due to the oxidative stress caused by the inflammatory process. In conclusion, it can be stated that pretreatment with L. fermentum CFR 2195 has shown anti-inflammatory activity in the intestines of TNBS model of rat colitis.

Keywords: experimental colitis, trinitrobenzenesulfonic acid, *L. fermentum* CFR 2195, probiotic

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1. INTRODUCTION

Probiotics, defined as 'live microorganisms which when administered in adequate amounts, confer a health benefit on the host', have become a major research area in recent years. Majority of the representative probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacterium*. Beneficial effects conferred by probiotics include inhibition of pathogenic bacteria, such as *Salmonella, Shigella, Pseudomonas* and *Helicobacter* (Servin, 2004; Sgouras et al., 2004). Numerous health benefits of probiotics such as reduction of lactose intolerance (Adolfsson et al., 2004), management of allergic diseases (Weston, et al., 2005), anticancer effects (Commane, et al., 2005), modulation of immune responses (Hart et al., 2004) and prevention of inflammatory bowel diseases have been reported. There has been an increase in the use of probiotic strains either as fermented food commodities or in lyophilized form, both as supplements and also as pharmaceutical preparations. There is a need for establishing health claims attributed to probiotics, prior to their envisaged applications (Holzapfel and Schillenger, 2002). General aspects of probiotics, including origin, identity, safety and antibiotic resistance are considered to be of major importance. It is generally known that certain probiotics can exert beneficial effects; nevertheless the mechanisms underlying these effects are less known. The mechanisms can vary from one probiotic to another and combinations of events may be involved. Modes of action so far accepted include the production of antimicrobial substances, the competition for nutrients, the

competitive exclusion of pathogen binding and the modulation of the immune system (Parvez et al., 2006). Among them, the best documented is the production of antimicrobial compound(s), while the most challenging seems to be the modulation of immune responses (Peran et al., 2006). 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 an increasing experimental evidence to support a role for luminal bacteria in the initiation and progression of these intestinal conditions (Shanahan, 2000). This could justify the remission achieved in intestinal inflammation, after treatment with antibiotics such as metronidazole or ciprofloxacin (Chung and Peppercorn, 1999). A possible therapeutic approach for IBD is the administration of probiotics to the subjects. It has been reported that administration of a mixture of probiotics prolongs remission in ulcerative colitis (Venturi et al., 1999). Different mechanisms have been proposed with regard to the therapeutic effects exerted by probiotics. Probiotics may either 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 (Tannock ,et al., 2000) or probiotics may modulate the host defences by influencing the intestinal immune system (Schultz, et al., 2003). These microorganisms have also been reported to positively affect the intestinal barrier function (Otte and Podolsky, 2004). Although the results obtained after probiotic treatment in both human IBD and experimental colitis are promising, further studies are required to fully understand the concept of the use of probiotics for the treatment of IBD. Additionally, not all probiotics exhibit similar activities in reducing intestinal inflammation (Shibolet , et al., 2002). 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.

Mucosa of the gastrointestinal tract is endowed with several antioxidant defense systems whose function is to neutralize the injurious effects of continuous formation of reactive oxygen species (ROS). Among these defense networks, endogenous sulfhydryls, mainly reduced glutathione (GSH) have a major role in gastric mucosal cytoprotection against oxidative stress in a variety of models. The reductive metabolism of trinitrobenzene sulfonic acid (TNBS) has been shown to generate ROS (Goldin et al., 1997; Martensson et al., 1990). A decrease in the levels of antioxidants, including that of GSH has been observed in mucosa from patients and experimental animal models of colitis (Holmes et al., 1998). Myeloperoxidase (MPO) is a peroxidase enzyme most abundantly present in neutrophil granulocytes. MPO activity is suggested to be a valuable marker of neutrophil infiltration (Grisham et al., 1990). In the present study, probiotic *Lactobacillus fermentum* CFR 2195 was evaluated for its beneficial effects particularly, the intestinal anti-inflammatory activity in TNBS induced experimental colitis in rats.

2. MATERIAL AND METHODS

All chemicals used in the present study were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. The microbiological media were from HiMedia Labs Pvt. Ltd. (Mumbai, India). Skim milk powder was obtained from the local dairy. Halothane I.P. 85 (2-bromo-2-chloro-1,1,1-Trifluroethane, 0.01% w/w of thymol), used for the study was from Raman and Well Pvt. Ltd., Daman. Edible grade casein was obtained from Nimesh Corporation (Mumbai, India). All other chemicals and solvents used were of analytical grade.

Preparation and administration of probiotic strain

L. fermentum CFR 2195, isolated from the faecal matter of healthy breast fed infants were

maintained as glycerol stocks at 4 ^{o}C. The strain was freshly subcultured in de Man, Rogosa & Sharpe (MRS) broth (37 ^{o}C for 24 h). For probiotic treatment, appropriate concentrations of the strain were prepared by serially diluting the strain in skim milk. For the feeding, bacteria were suspended in skim milk (10^8 colony forming unit/mL), prepared fresh prior to feeding.

Animal treatment

The animal study was carried out by taking all appropriate precautions and by strictly following the guidelines with regard to the use of animals for experimental purpose after due approval from the Institutional Animal Ethics Committee (CFTRI, Mysore, India). Female Wistar rats (180-200 g) were obtained from the animal production facility of this institute, and maintained under standard conditions. The rats were fed with basal diet *ad libitium.* The basal diet consisted (%) of maize starch, 54; casein, 21; refined peanut oil, 10; powdered cane sugar, 10; Bernhardt-Tommarelli modified salt mixture, 4; and National Research Council (NRC) vitamin mixture, 1.

The rats were randomly assigned into four groups (n=6). The first group (Normal Control) was fed with the basal diet; without either probiotic or TNBS treatment. The second group (Probiotic Control) was fed with the basal diet and received the probiotic orally but were not treated with TNBS. The third group (TNBS Control) was fed with basal diet and treated with TNBS did not receive the probiotic. The fourth group (TNBS Probiotic) was fed with basal diet and treated with TNBS received the probiotic orally.

The rats of Probiotic-Control and TNBS-Probiotic groups were administered daily for two weeks with probiotic suspended in skim milk (0.6 mL) orally by intubation (5 x 10^8 colony-forming units suspended in 0·6 ml skimmed milk). At the end of two weeks, the rats in TNBS-Control and TNBS-Probiotic groups were fasted overnight and anesthetized with halothane and were rendered colitic by the intra-colonic administration of TNBS (0.6 mL TNBS + 0.3 mL ethanol) with the aid of a Teflon canula (Morris, et al., 1989). Saline (0.9% NaCl) instead of TNBS was administered intra-colonically to the remaining two groups. Body weight, food intake as well as stool consistency were recorded throughout the period of the experiment. The rats were sacrificed one week after the induction of colitis with an overdose of halothane. Liver, kidney and spleen were quickly excised, weighed and stored at -20 ^{o}C in airtight vials. The colon was excised and assessed for damage. Blood samples were also collected from all rats for analysis of haematological parameters and lipid profile.

Assessment of colonic damage

The colon from the sacrificed rats was removed aseptically and opened longitudinally and the luminal contents were collected for the analysis of vital parameters *viz.* pH and microbiological load. The weight and length of the colon were also recorded. The colon was observed for macroscopically visible damage on a 0-10 scale by three observers who were unaware of the treatment based on the criteria mentioned by (Bell, et al., 1995) which took into account the extent, as well as the severity of the damage to the colon. Representative sections of the colon displaying inflammation and sections adjacent to the gross macroscopic damage were fixed in 10% formal-saline. Appropriate cross sections were selected and embedded in paraffin. Equivalent colonic segments were also obtained from the Normal-Control as well as Control-Probiotic control. Colonic sections of 5µm thickness were taken at different levels and stained with haematoxylin and eosin. The histopathological damage was scored on a 0-27 scale by two pathologists, who were blinded to the experimental groups, according to the criteria described by Camuesco et al., 2005. The colon was subsequently divided into two segments for biochemical analysis: one for Glutathione (GSH) assay and the other for Myeloperoxidase (MPO) activity, and stored at –80^{o}C.

Myeloperoxidase (MPO) activity

The extent of neutrophil infiltration was determined by myeloperoxidase (MPO) assay (Krawisz, et al., 1984). The colon (4 cm) was

homogenized in 1 mL saline. A 200 µL aliquot was centrifuged (15,000 rpm, 10 min) and the pellet resuspended in hexadecyltrimethylammonium bromide (HTAB) buffer. The sample was vortexed to release MPO from the tissue. Samples were again centrifuged (15,000 rpm, 2 min) and 50 µL of the supernatant added, in duplicate, to a 96- well plate. *o*-Diansidine reaction mixture and hydrogen peroxide were added to the test wells and absorbance of the reaction mixture was measured at 450 nm at 1 min intervals for 15 min using a spectrophotometer (Shimadzu Corp., Kyoto, Japan). The results were expressed as MPO units per g of wet tissue; one unit of MPO activity was defined as that degrading 1µmol hydrogen peroxide/min at 25^{o}C.

Glutathione content

Glutathione content was determined using glutathione assay kit (Sigma Chemical Co.) as per the instructions provided. Colonic samples were homogenised in ice cold 5-sulfosalicylic acid (5% w/v), and the supernatants were assayed for total glutathione content (Andersen, 1985) and expressed as nmol/g tissue.

Microbiological studies

Luminal contents were weighed, homogenized and serially diluted in sterile normal saline. Appropriate dilutions of the homogenates were plated on to MRS agar for lactobacilli. Coliforms and Enterobacteria were also enumerated using MacConkey agar by spread plate technique; incubated at 37^{o}C for 24 h. After incubation, the final count was expressed as $\log_{10}$ colony forming units/ gram of the luminal content.

Statistical analysis

Statistical analyses were performed using the SPSS software package V11.5 (SPSS Inc., Chicago, USA). Comparisons between groups were made by Pair-wise ANOVA with a Holmes post-hoc test, with a family-wise significance level of 0.05. All other data were compared by one-way ANOVA with a Tukey's LSD post hoc test with $p < 0.05$ considered significant.

3. RESULTS

Effect of probiotic *L. fermentum* CFR 2195 administration on body and tissue weights in experimental colitic rats

Intracolonic administration of TNBS resulted in an acute intestinal inflammation in the rats characterised by anorexia, loss of weight and diarrhoea, which gradually increased. One week after colitis induction, body weight was reduced by 4·28% in the TNBS-treated rats, whereas in control rats it was increased by 4·55 % ($p < 0·05$). The daily administration of probiotics for two weeks prior to induction of colitis showed no significant differences in the weight gain of rats belonging to TNBS-Probiotic group compared with normal-control group (Fig.1).

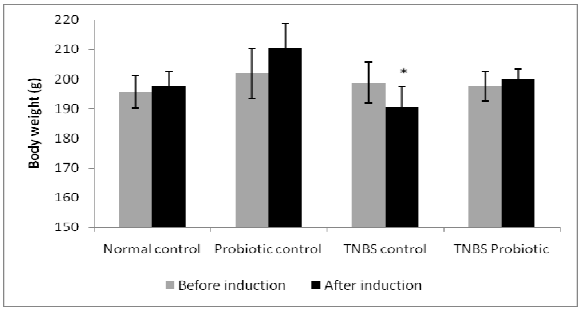


Fig.1 Effect of probiotic treatment on body weights in TNBS-induced colitic rats

Values are mean with their standard errors for six rats per group.

* Significantly different when compared to that of Normal control group ($p < 0·05$).

^ Significantly different when compared to that of TNBS control group ($p < 0·05$).

The effect of probiotic supplementation either in inhibiting the anorexia or reducing the weight loss in the acute phase of inflammation was not very significant, but the weight of the animals was restored at the end of the regimen, as evident by an increase of 1.13% in colitic rats which received the probiotic *L. fermentum.* The anorexia and the inflammatory response caused a significant increase in the weight of the colon, which was not alleviated by treatment of *L. fermentum* CFR 2195 while other organs such as liver, kidney and spleen showed no significant differences (Table 1).

Table 1 Effect of probiotic treatment on tissue weights in TNBS-induced colitic rats

Animal group	Liver	Spleen	Kidney	Colon
Normal Control	6.0 ± 0.33	0.45 ± 0.05	1.45 ± 0.08	1.15 ± 0.04
Probiotic-Control	6.1 ± 0.35	0.51 ± 0.07	1.35 ± 0.06	1.10 ± 0.07
TNBS-Control	6.5 ± 0.32	0.55 ± 0.10	1.40 ± 0.07	1.55 ± 0.08*
TNBS-Probiotic	5.5 ± 0.23	0.55 ± 0.05	1.50 ± 0.08	1.15 ± 0.05**

Values (g/ kg body weight) are mean with their standard errors for six rats per group.
* Significantly different from that of the respective Normal-Control group ($p < 0{\cdot}05$)
**Significantly different from that of the respective TNBS-Control group ($p < 0{\cdot}05$)

Table 2 Effect of probiotic treatment on diarrhoea, damage score, extent of inflammatory lesion along the colon and changes in colon weight in TNBS-induced colitic rats

Animal group	Diarrhea (%)	Damage score (0-10)		Extent of damage (cm)	Colon weight (mg/cm)
		Median	Range		
Normal Control	0	0	0	0	81.6 ± 1.61
Probiotic-Control	0	0	0	0	82.6 ± 1.12
TNBS-Control	80	6.5	5 to 8	3.16 ± 0.13	199.0 ± 2.19*
TNBS-Probiotic	20**	6	3 to 7	1.85 ± 0.08**	137.5 ± 1.18**

Values are mean with their standard errors for six rats per group.
* Significantly different compared to that of the Normal Control group ($p < 0{\cdot}05$).
**Significantly different compared to that of the TNBS-Control group ($p < 0{\cdot}05$).

Table 3 Effect of probiotic treatment on activity of colonic myeloperoxidase and glutathione content in TNBS-induced colitic rats

Animal group	MPO (units/g tissue)	GSH (nmol/g tissue)
Normal Control	18.8 ± 0.46	1235.0 ± 4.61
Probiotic Control	19.5 ± 0.21	1216.7 ± 10.2
TNBS Control	154.1 ± 0.78*	666.7 ± 5.23*
TNBS Probiotic	75.0 ± 0.4**	967.2 ± 7.47**

Values are mean with their standard errors for six rats per group.
* Significantly different from that of the Normal control group ($p < 0{\cdot}05$)
**Significantly different from that of the TNBS control group ($p < 0{\cdot}05$)

Effect of probiotic *L. fermentum* CFR 2195 administration on experimental induction of inflammatory colitis

Pretreatment of animals with *L. fermentum* CFR 2195 resulted in amelioration of diarrhoea, which was evident by a significantly lower incidence (20%) after seven days when compared with TNBS control rats (80%; $p < 0{\cdot}05$) (Table 2). The macroscopic evaluation of the colonic segments one week after induction of colitis revealed the beneficial effect of probiotic. This was substantiated by a

significant reduction in the ratio of colonic weight: length ($p < 0{\cdot}05$) (Table 2), as well as by a significantly lower colonic damage score as compared to that of TNBS control rats. The beneficial effect of probiotic was evidenced by a significant reduction in these inflammatory parameters in TNBS probiotic group as compared to TNBS control group. 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 14·1. The histological analysis of the colonic specimens from TNBS probiotic group revealed a marked recovery of the intestinal architecture compared to TNBS control, with a score of 8.15 ($p < 0{\cdot}05$ vs. TNBS control group). Thus, based on these observations it could be inferred that most of the samples (4 out of 6) showed almost complete restoration of the epithelial cell layer, in contrast to the extensive ulceration observed in TNBS control group. 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 histological studies revealed that *L. fermentum CFR 2195* was efficient in promoting the recovery of colonic tissue.

Effect of probiotic *L. fermentum CFR 2195* administration on colonic biochemical parameters in experimental colitic rats

The lower leukocyte infiltration was assessed biochemically by the reduction in colonic MPO activity, a marker for neutrophil infiltration which was found to be enhanced in the TNBS control group (Table 3). In addition, probiotic treated colitic rat (TNBS-Probiotic group) showed a significant increase in colonic glutathione content (Table 3), which is depleted in colitic rats as a consequence of the colonic oxidative stress caused by the TNBS induced inflammation. The observed results with respect to the GSH content and the MPO activity in the two groups of rats treated with *L. fermentum* CFR 2195 and the TNBS-control colitic rats were statistically significant ($p < 0{\cdot}05$). The biochemical analysis of the colonic specimens confirmed the intestinal anti-inflammatory effect exerted by *L. fermentum* CFR 2195.

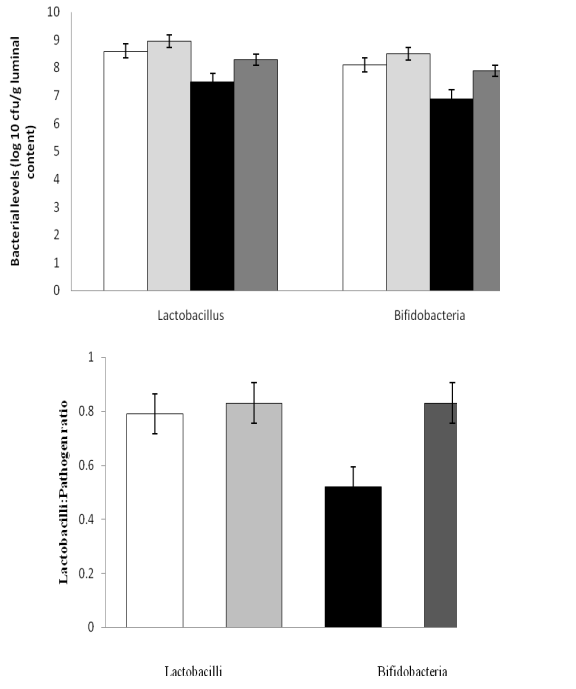
Effect of probiotic *L. fermentum* CFR 2195 administration on colonic bacterial profile in experimental colitic rats

The colitis induced by the administration of TNBS resulted in a significant reduction in fecal LAB count as compared to normal control rats ($p < 0.05$). TNBS probiotic group showed higher counts of LAB species in colonic contents than TNBS control group, however the difference in the bacterial counts of normal control and probiotic control groups were not statistically different (Fig. 2). Significant differences were observed in the population of potential pathogenic bacteria such as enterobacteria or coliforms in the fecal matter of TNBS control and TNBS probiotic groups.

TNBS-induced colitis resulted in a significant reduction in colonic lactobacilli, together with an increase in coliforms and enterobacteria ($p < 0{\cdot}05$) (Fig.2). TNBS probiotic group showed higher counts of lactobacilli in the colonic contents than in TNBS control group; the same were not statistically different from that of non-colitic and probiotic control groups (Fig.2A). The amounts of potential faecal pathogenic bacteria such as enterobacteria or coliforms among both the colitic groups were not statistically different. As expected, when the lactobacilli: pathogen ratio was evaluated, the inflammatory condition did result in a significant decrease in comparison with normal control group; the administration of probiotic *L. fermentum* CFR 2195 resulted in the restoration of the normal balance of lactobacilli: pathogen ratio (Fig.2B).

4. DISCUSSION

The results of the present study are supportive of the concept of use of dietary probiotics as adjuvant (Sartor, 2004) and the efficacy of *L. fermentum* CFR 2195 for use in probiotic therapy against IBD.



Normal control, Probiotic control , TNBS control, TNBS Probiotic.

Fig. 2 Effect of probiotic treatment on (A) bacteria levels (lactobacilli and bifidobacteria) and on (B) lactobacilli : pathogen ratio in TNBS-induced colitic rats

Values are mean with their standard errors represented by vertical bars.

*Significantly different from that of Normal control group ($p < 0{\cdot}05$)

^ Mean value was significantly different from that of TNBS control group ($p < 0{\cdot}05$)

This encouraging observation widens the scope of its application and suggests inclusion of *L. fermentum* CFR 2195 a potent probiotic to the list of probiotics that have already been reported to attenuate the development of colonic injury in experimental and human IBD (Schultz and Sartor , 2000). Oral administration of the probiotic facilitated recovery from TNBS-induced colonic damage. TNBS is a hapten, which when bound with a substance of high molecular tissue proteins, turns into an antigen. It has been shown to elicit immunologic responses and induce generation of colitis (Fidler, 1985). The probiotic *L. fermentum* CFR 2195 ameliorated some of the clinical manifestation of this experimental model such as anorexia or diarrhoea and the macroscopic colonic damage. The probiotic significantly attenuated the incidence of diarrhoea and adhesions, reduced colonic weight: length ratio, damage score and extension. The reduction in the etiology of diarrhea exerted by *L. fermentum* CFR 2195 can be a consequence of improvement of the gut epithelial cell barrier function, thus contributing to its intestinal anti-inflammatory effect, as has been proposed with other probiotics (Gionchetti, et al., 2005). The microscopic evaluation showed that the restoration in the epithelial lining was more evident in the rats administered with *L. fermentum*. This may be interesting since a barrier disruption leads to increased stimulation by luminal antigens. In this regard, mucosal inflammation can be considered as a self-perpetuating process in which the disruption of epithelial layer plays a central role (Heymann et al., 1994).

The probiotic *L. fermentum* CFR 2195 was able to reduce neutrophil infiltration in the inflamed colon as observed microscopically; *L. fermentum* CFR 2195 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. The ability of the administered probiotic to reduce granulocyte infiltration, indicated by the reduction in MPO activity was confirmed histologically, since the level of leukocyte infiltrate in the colonic mucosa was lower in probiotic administered colitic animals than in the corresponding TNBS control group. 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 externalisation of circulating granulocytes contribute markedly to the colonic injury in the current model of IBD. Glutathione is an important constituent of intracellular protective mechanism against various noxious stimuli including oxidative stress and it is the main component of endogenous nonprotein sulfhydryl pool, known to be a major low molecular weight scavenger

of free radicals in cytoplasm (Wu CC , et al., 2004). Depletion of GSH in colonic tissue happens in accordance with tissue damage during the process of colitis. Treatment of TNBS colitic rats with probiotic *L. fermentum* CFR 2195 counteracted the depletion of colonic glutathione levels observed in TNBS control group. The ability of the probiotic under study to attenuate the depletion of GSH levels may play a crucial role in the intestinal anti-inflammatory effect of the probiotic. Because, a situation of intense oxidative stress 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 different experimental models of rat colitis, including the TNBS induced colitis (Galvez, et al., 2003). 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).The probiotic *L. fermentum* CFR 2195 was evaluated here for its ability to modify colonic microflora, which gets altered as a consequence of TNBS-induced inflammation (Peran, et al., 2006). In this regard, the probiotic treatment restored the pathogenic bacteria: lactobacilli ratio in the colon. This effect could definitively contribute to the beneficial effect exerted by probiotic *L. fermentum* CFR 2195 in the TNBS-induced experimental colitis. 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 animal models (Garcia-Lafuente, et al., 1997) and in human subjects (Cummings, et al., 2003).

5. CONCLUSIONS

In conclusion, probiotic *L. fermentum* CFR 2195 has shown anti-inflammatory activity in the intestines of TNBS-induced rat colitis. However, the probiotic showed 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. The colonization of the probiotic *L. fermentum* CFR 2195 in the colonic lumen would result in positive effects in the intestinal conditions, probably derived from their immunomodulatory properties. Further pre-clinical and clinical studies are warranted to convincingly advocate the use of the selected strain of *L. fermentum* CFR 2195 in the probiotic treatment of IBD.

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