Food & Function

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c4fo00938j

The viability of *Lactobacillus fermentum* CECT5716 is not essential to exert intestinal anti-inflammatory properties

Alba Rodríguez-Nogales,[a] Francesca Algieri,[a] Teresa Vezza,[a] Natividad Garrido-Mesa,[a] Mónica Olivares,[b] Mònica Comalada,[c] Carlo Riccardi,[d] M[a] Pilar Utrilla,[a] M[a] Elena Rodríguez-Cabezas†[a] and Julio Galvez*†[a]

Probiotics have been used as alternative therapies in intestinal inflammatory disorders. Many studies have shown that different bacterial probiotic strains possess immuno-modulatory and anti-inflammatory properties. However, there is an increasing interest in the use of non-viable bacteria to reduce the risk of microbial translocation and infection. The aim of this study was to evaluate whether the viability of *L. fermentum* CECT5716 is essential to exert its intestinal anti-inflammatory effect. We compared the preventative effects of viable and non-viable probiotic in the TNBS model of rat colitis. *In vitro* studies were also performed in Caco-2 and RAW 264.7 cells to evaluate the probiotic effects on IL-8, IL-1β and nitrite production, and p44/42 and p38 MAP kinase protein expressions. *In vitro* results revealed a decrease in the stimulated production of pro-inflammatory mediators regardless of the viability of the probiotic. Likewise, both forms of the probiotic administered to colitic rats produced a significant reduction of IL-1β and TNF-α levels and colonic iNOS expression. In conclusion, both live and dead *L. fermentum* CECT5716 have been demonstrated to attenuate the inflammatory process and diminish the production of some of the inflammatory mediators. In fact, the viability of this probiotic did not affect its immuno-modulatory and anti-inflammatory properties.

Received 16th October 2014,
Accepted 7th February 2015

DOI: 10.1039/c4fo00938j

www.rsc.org/foodfunction

Introduction

The human gut is a huge complex ecosystem where microbiota plays many critical roles in maintaining homeostasis. When the intestine microbiota is drastically altered, a process termed dysbiosis takes place, and this has been associated with various intestine conditions like inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS).[1] Therefore, the modification of the gut microbiota following the administration of probiotics has emerged as a promising strategy to alleviate these diseases.[2,3] According to the guidelines of FAO/WHO, probiotics are defined as 'live microorganisms that, when ingested in adequate amounts, exert a health benefit on the host'.[4,5] This definition specifies that probiotic microorganisms must be 'alive', and this specification is supported by an extensive number of studies suggesting that to provide health benefits, probiotic microorganisms must be viable.[6] Nevertheless, other studies have reported that non-viable forms of some probiotics can also exert beneficial effects on the host, most probably due to the capacity of human cells to recognize specific bacterial components or products, including immunostimulatory DNA, cell wall and membrane components like peptidoglycan or lipoteichoic acid, as well as intra- and extracellular polysaccharide products, thus promoting immune responses, which are commonly mediated by the mucosa-associated lymphoid tissue (MALT).[7] Another important issue about probiotics is related to the knowledge of the mechanisms underlying their beneficial effects, which at present is also limited. It is generally accepted that these mechanisms are multifactorial and strain specific, which can be associated with the production of antimicrobial substances, the reduction of luminal pH, the competition for nutrients, the competitive exclusion of pathogen binding, the enhancement of the barrier function and the modulation of the immune system.[8,9]

Different strains of *Lactobacillus fermentum* have been reported to have beneficial properties, particularly for gastrointestinal health. Thus, either *L. fermentum* BR11[10] or *L. fermentum* CECT5716[11] or *L. fermentum* ACA-DC 179[12] have been shown to exert intestinal anti-inflammatory effects in different experimental models of colitis in mice and rats. When consid-

[a]*CIBER-EHD, Department of Pharmacology, ibs.GRANADA, Center for Biomedical Research (CIBM), University of Granada, Granada, Spain. E-mail: jgalvez@ugr.es; Fax: +34-958-248964; Tel: +34-958-241793*
[b]*Research Department, Biosearch S.A., Granada, Spain*
[c]*Institute of Research in Biomedicine (IRB), Barcelona, Spain*
[d]*Department of Clinical and Experimental Medicine, University of Perugia, Italy*
† Both authors contributed equally to the supervision of the study.

ering *L. fermentum* CECT5716, several mechanisms have been proposed to be involved, including its ability to produce antimicrobial substances, such as bacteriocins,[13] thus limiting the deleterious effect of potential pathogens. Also, this strain is able to produce antioxidant compounds, such as glutathione, and short-chain fatty acids, which facilitate the growth of other lactobacilli species.[11] Furthermore, several studies have reported on its immuno-modulatory properties.[14]

The aim of this study was to evaluate whether the viability of *L. fermentum* CECT5716 is essential to exert its intestinal anti-inflammatory effect by comparing the preventative effects of the probiotic under both viable and non-viable conditions in the trinitrobenzenesulfonic acid (TNBS) model of rat colitis, a well-established model of intestinal inflammation with some resemblance to human IBD.[15] In addition, *in vitro* experiments in a human intestinal epithelial cell line were performed to better characterize the biological effects of this probiotic. The results revealed that the viability of *L. fermentum* CECT5716 is not a requisite to show beneficial effects in this experimental model of rat colitis, in which their immuno-modulatory properties on cell mitogen activated protein kinase (MPAK) signalling pathways in epithelial cells may play a role.

Materials and methods

Reagents

All chemicals were obtained from Sigma-Aldrich Quimica (Madrid, Spain), unless otherwise stated. Glutathione reductase was provided by Boehringer Mannheim GmbH (Ingelheim, Germany).

Preparation of the probiotic

Lactobacillus fermentum CECT5716, a human breast milk derived strain,[13] was provided by Biosearch, S.A. (Granada, Spain), and was normally grown in MRS media at 37 °C under anaerobic conditions using the Anaerogen system (Oxoid, Basingstoke, UK). For probiotic treatment, bacteria were prepared daily after their suspension in sterile phosphate-buffered saline (PBS) solution. Dead bacteria were obtained after heating the microorganisms at 95 °C for 30 minutes in a thermoblock MBT250 (ETG Entwicklungs- und Technologie Gesellschaft mbH Ilmenau, Ilmenau, Germany). In order to determine the viability, inactivated probiotic was grown in MRS media at 10^8 UFC ml^{-1} and the bacterial count was carried out at 24, 48 and 72 hours. The viability of inactivated probiotic did not exceed 0.0001%.

In vitro assays

The human colonic epithelial colorectal adenocarcinoma cell line Caco-2 and the mouse macrophage RAW 264.7 cells (obtained from Cell Culture Unit of the University of Granada, Spain) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mmol l^{-1}), penicillin (100 units ml^{-1}) and streptomycin (1 mg ml^{-1}), and maintained at 37 °C in a humidified, 5% CO_2 environment. For experiments, confluent cells in cell culture flasks were trypsinized and seeded on 24-well plates to 70–80% of confluence, and then they were pre-treated for 3 h either with live or dead bacteria suspended in DMEM at 10^8 colony forming units (CFU) ml^{-1}. Cells were stimulated with different stimuli, lipopolysaccharide (LPS) (100 ng ml^{-1}) (RAW 264.7) and interleukin (IL)-1β (1 ng ml^{-1}) (Caco-2) for 24 h to evaluate IL-8, IL-1β and nitrite levels, and for 30 min to evaluate p44/42 and p38 MAP kinase protein expression. Cytokine production was quantified by the ELISA assay (R&D Systems, Abingdon, UK), whereas nitrite determination was performed by the Griess assay as described elsewhere.[16] The p44/42 MAP kinase protein expression in Caco-2 cells was evaluated by immunoblotting[17] (BD, Franklin Lakes, NJ, USA).

In vivo studies

Experimental design. Female Wistar rats (180–200 g) were obtained from Janvier (St Berthevin Cedex, France), housed in makrolon cages and maintained in air-conditioned animal quarters, which were monitored according to the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA), with a 12 h light–dark cycle, and fed standard rodent chow (Panlab A04, Panlab, Barcelona, Spain) and water *ad libitum* throughout the experiment. Monitoring the rats revealed no infection with common murine pathogens during the period of the experiment. This study was carried out in accordance with the Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes of the European Union (86/609/EEC) and approved by the Animal Research and Ethic Committee of the University of Granada (Spain).

The rats were randomly assigned to four groups (n = 10); two of them (non-colitic and control groups) received orally a PBS solution (1 ml) and the other two (treated groups) received the probiotic orally, live or dead, at the concentration of 5 × 10^8 CFU suspended in 1 ml of PBS solution, by means of an oesophageal catheter, daily for 3 weeks. Two weeks after starting the experiment, the rats were fasted overnight, and those from the control and the treated groups were rendered colitic as previously described.[18] Briefly, they were anaesthetized with isofluorane and given 10 mg of TNBS dissolved in 0.25 ml of 50% ethanol (v/v) by means of a Teflon flexible cannula inserted 8 cm through the anus. Rats from the non-colitic group were administered intracolonically 0.25 ml of PBS instead of TNBS. The body weight, water and food intake, as well as stool consistency, were recorded daily throughout the experiment. All rats were killed with an overdose of halothane 1 week after the induction of colitis, and the colon was obtained for the assessment of colonic damage.

Assessment of colonic damage. After the rats were sacrificed, the colon was removed aseptically and placed on an ice-cold plate, the colonic segment was cleaned of fat and mesentery, blotted on filter paper; each specimen was weighed, its length was measured under a constant load (2 g), and the weight/length ratio was determined. The colon was scored for macroscopically visible damage on a 0–10 scale by two

Table 1 Criteria for the assessment of macroscopic colonic damage

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

observers unaware of the treatment, according to the criteria previously reported,[18] which takes into account the extent as well as the severity of colonic damage (Table 1). The colon was subsequently divided into different segments for biochemical determinations, which were frozen at −80 °C for myeloperoxidase (MPO) activity, tumour necrosis factor (TNF)-α, interferon (IFN)-γ and IL-1β production, and inducible nitric oxide synthase (iNOS) expression, except one sample that was weighed and frozen in 1 ml of 50 g l^{-1} trichloroacetic acid for total glutathione content determinations. All biochemical measurements were completed within 1 week from the time of sample collection and were performed in duplicate. MPO activity was measured as previously described;[19] the results were expressed as MPO units per gram of wet tissue and one unit of MPO activity was defined as that degrading 1 μmol hydrogen peroxide per minute at 25 °C. Total glutathione content was quantified in liver with the recycling assay described by Anderson,[20] and the results were expressed as nmol g^{-1} wet tissue. The colonic samples for TNF-α, IFN-γ and IL-1β determination 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 supernatants were frozen at −80 °C until a cytokine assay. TNF-α, IFN-γ and IL-1β were quantified by the ELISA assay (R&D Systems, Abingdon, UK), and the results were expressed as ng g^{-1} wet tissue, respectively. iNOS expression was analyzed by Western blotting as previously described,[21] and control of protein loading and transfer was conducted by detection of the β-actin levels (BD, Franklin Lakes, NJ, USA).

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 a *post hoc* least significance tests. Non-parametric data (score) are expressed as the median (range) and were analyzed using the Mann–Whitney *U*-test. Differences between proportions were analyzed with the chi-square test. All statistical analyses were carried out with the GraphPad Prism version 5.0 (La Jolla, CA, USA), with statistical significance set at $P < 0.05$.

Results

Lactobacillus fermentum CECT5716, live or dead, inhibits the stimulated IL-8 production, p44/42 and p38 MAP kinase protein expression in Caco-2 cells

The incubation of confluent Caco-2 cells with *L. fermentum* CECT5716 (10^8 CFU mL^{-1}), live or dead, for 3 h significantly increased the IL-8 production in comparison with basal conditions, without showing statistical significance when live or dead bacteria are considered (Fig. 1). The incorporation of IL-1β in the cell culture for 24 hours resulted in the stimulated release of IL-8, which was approximately 10-fold higher than that obtained after the incubation of the cells with the probiotics under basal conditions (Fig. 1). When Caco-2 cells were

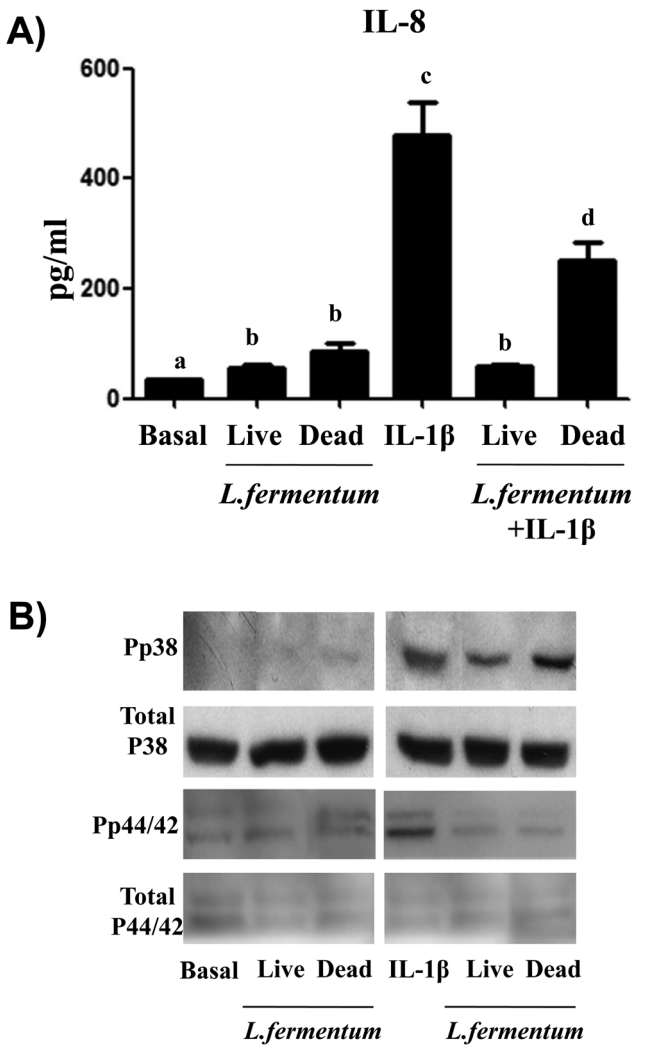

Fig. 1 (A) Effects of *Lactobacillus fermentum* CECT5716, live or dead, on IL-8 production in Caco-2 cells, under basal conditions and stimulated with IL-1β (1 ng ml^{-1}). Data are expressed as means ± SEM. Bars with a different letter differ statistically ($P < 0.05$). (B) Effects of *Lactobacillus fermentum* CECT5716, live or dead, on p44/42 and p38 MAP kinase protein expression in Caco-2 cells under basal conditions and stimulated with IL-1β (1 ng ml^{-1}). The experiments were performed three times, with each individual treatment being run in triplicate.

previously exposed to the probiotic, live or dead, a significant inhibition of the IL-1β-stimulated production of IL-8 was observed; however, in this case, pre-treatment of the cells with live probiotic showed a higher reduction in this cytokine production than pre-treatment with dead bacteria (Fig. 1).

The stimulatory effect of IL-1β on Caco-2 cells was associated with an increased phosphorylation of MAP kinases, both p42/44 ERK and p38 (Fig. 1). The pre-treatment of these cells with live or dead probiotic did not significantly modify the expression of these MAP kinases under basal conditions, but it showed inhibitory effects when they were stimulated with IL-1β, showing a reduced phosphorylation of the MAP kinase p42/44 ERK and p38 when compared with stimulated cells without probiotic (Fig. 1).

Lactobacillus fermentum CECT5716, live or dead, inhibits nitric oxide and IL-1β production in stimulated RAW 264.7 cells

Similarly to what occurred with Caco-2 cells, incubation of confluent RAW 264.7 cells with *Lactobacillus fermentum* CECT5716, live or dead, for 3 h resulted in a significant increase of the release of IL-1β and nitric oxide when compared with those cells without probiotic, showing a significantly higher increase in nitrite production when dead probiotic was administered in comparison with the values obtained using live bacteria (Fig. 2). LPS incorporation into the culture media of these macrophages for 24 hours resulted in an increased production of IL-1β and nitric oxide in comparison with the levels obtained under basal conditions, these being significantly reduced when the cells were previously incubated with the probiotic, either live or dead; however, a reduction in nitrite production was achieved to a lesser extent when dead probiotic was used (Fig. 2).

Intestinal anti-inflammatory effects of *Lactobacillus fermentum* CECT5716, live or dead, in the TNBS model of rat colitis

The administration of the probiotic *Lactobacillus fermentum* CECT5716, live or dead, for 2 weeks before colitis induction did not result in any symptom of diarrhoea or affect the weight evolution in comparison with untreated rats (data not shown). However, the administration of the probiotic to colitic rats resulted in an overall lower impact of the TNBS-induced damage compared to the untreated colitic control group when evaluated one week after the colonic insult. Thus, the intestinal anti-inflammatory effect was evidenced macroscopically by a significant reduction in the colonic damage score in comparison with that of control rats ($P < 0.05$) (Fig. 3), since a significant decrease of the area of colonic necrosis and/or inflammation was observed in both colitic groups treated with the probiotic. However, this anti-inflammatory effect was not associated with significant differences in the colonic weight/length ratio among colitic groups, which was increased significantly as a consequence of the inflammatory process (Fig. 3). The latter contrasts with a previous study reported for this strain of *L. fermentum* in the same model of rat colitis, in which a significant reduction in the colonic weight/length ratio was obtained after probiotic treatment.[11] This could be explained as due to the different experimental conditions at the two time points. There are small quantitative differences in the inflammatory response and in the probiotic effect that may be responsible for the lack of a significant effect after probiotic administration to colitic rats on this ratio in the present study.

Biochemically, the preventative beneficial effects shown by the probiotic, either live or dead, were evidenced by the

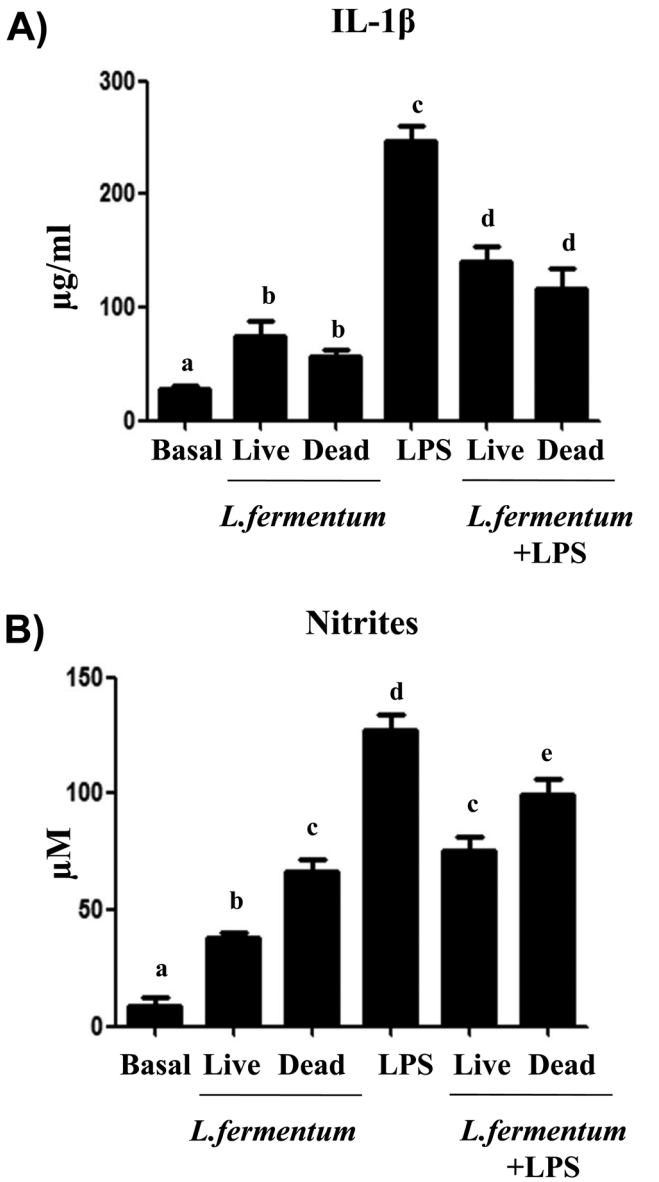

Fig. 2 Effects of *Lactobacillus fermentum* CECT5716, live or dead, on (A) IL-8 production in RAW 264.7 cells, under basal conditions and stimulated with LPS (100 ng ml^{-1}); and (B) nitrite accumulation in RAW 264.7 cells, under basal conditions and stimulated with LPS (100 ng ml^{-1}). Data are expressed as means ± SEM. Bars with a different letter differ statistically ($P < 0.05$). The experiments were performed three times, with each individual treatment being run in triplicate.

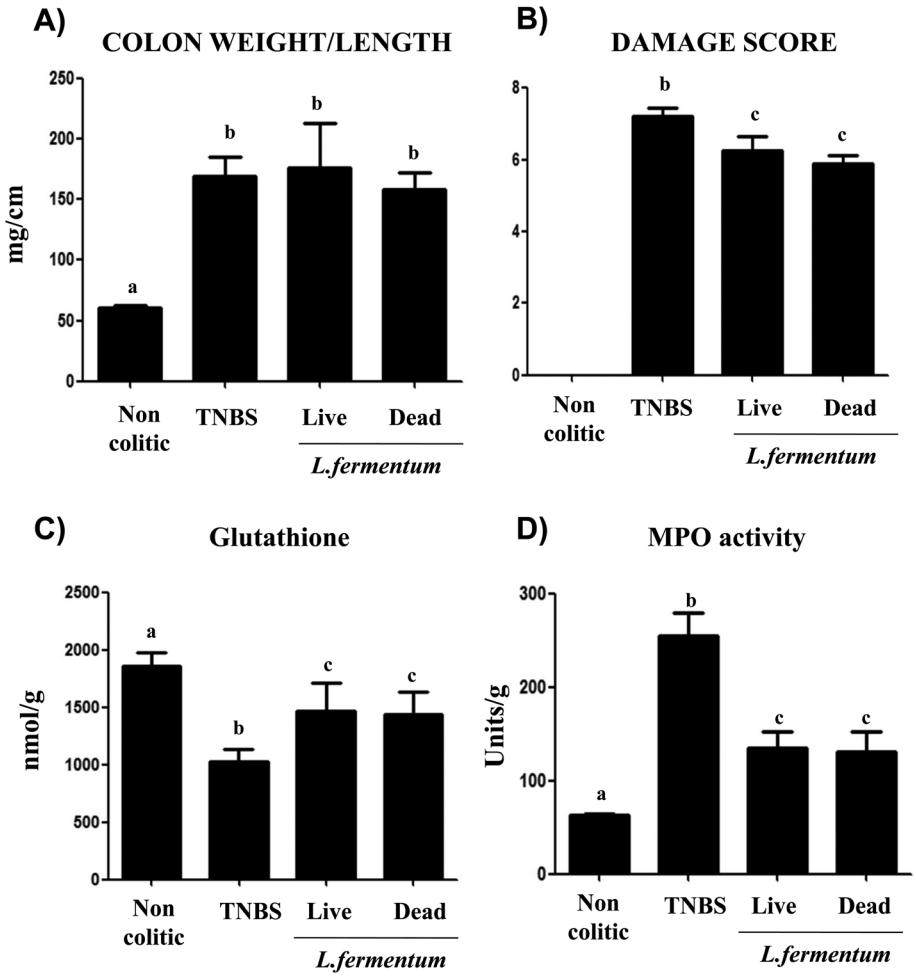

Fig. 3 Effects of *Lactobacillus fermentum* CECT5716, live or dead, on colonic (A) weight/length ratio, (B) macroscopic score, (C) glutathione content and (D) myeloperoxidase (MPO) activity in TNBS rat colitis one week after damage induction. Data (n = 10 per group) are expressed as means ± SEM; groups with a different letter differ statistically ($P < 0.05$).

reduction of the increased colonic MPO activity observed in the colitic control group (Fig. 3), this enzyme activity being a marker of neutrophil infiltration.[22] In addition, colonic inflammation was characterized by a decreased content in glutathione content, most probably as a consequence of the colonic oxidative stress induced by the inflammatory process, as previously reported in this model of experimental colitis[23] (Fig. 3). The treatment with *L. fermentum* CECT5716, live or dead, resulted in a significant increase in the colonic glutathione content, although no significant differences were observed between both treated groups (Fig. 3).

Finally, the colonic inflammatory process induced by TNBS was also associated with increased levels of the pro-inflammatory cytokines TNFα and IL-1β (Fig. 4), as well as by a greater colonic iNOS expression (Fig. 4) in comparison with non-colitic animals. The administration of the probiotic to colitic rats resulted in a significant reduction of both cytokine levels, and a lower colonic iNOS expression when compared to TNBS control animals, without showing differences between viable and dead probiotic (Fig. 4).

Discussion

In the last decade, several studies have supported the potential use of probiotics in human IBD, mainly in pouchitis and ulcerative colitis,[24] thus confirming the preclinical studies performed in experimental models of colitis.[25] Different mechanisms have been involved in these effects, and some of them could be attributed to the interaction of probiotics with other microorganisms, either members of the microbiota or potential pathogens, which result in the restoration of the dysbiosis that characterizes these intestinal conditions.[26] It is evident that this type of interaction is typically dependent on the viability of probiotics. Also, the presence of viable probiotics in the intestinal lumen is required to promote the production of short chain fatty acids (SCFA), which has also been proposed to contribute to the intestinal anti-inflammatory effect.[27] In addition, other mechanisms are related with the cross-talk between probiotics and host cells, clearly contributing to the well-known immuno-modulatory properties ascribed to these beneficial bacteria. However, in contrast to the direct effects

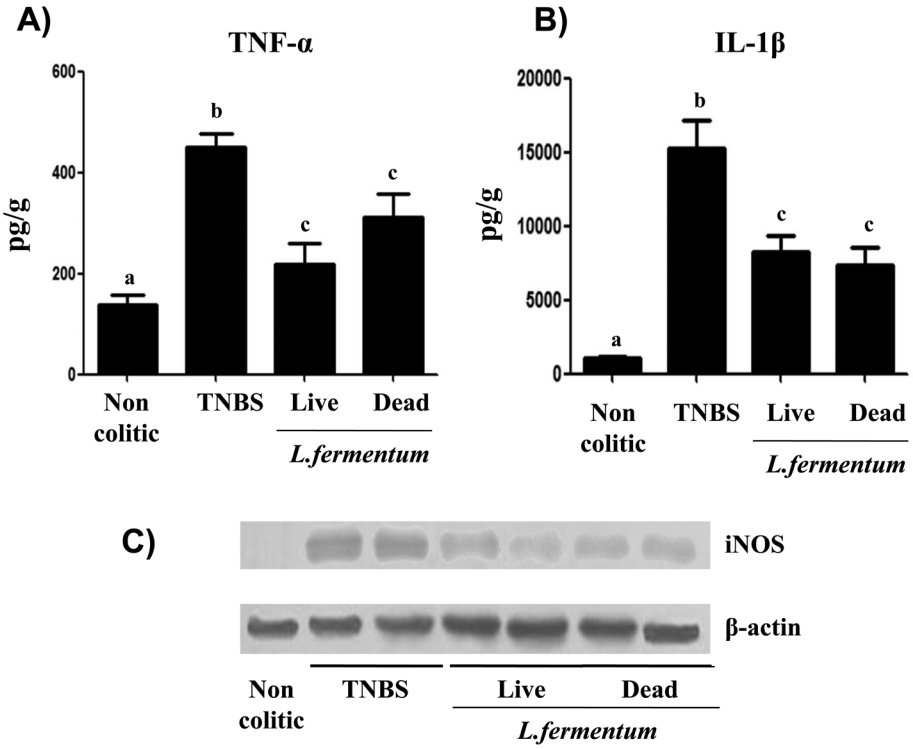

Fig. 4 Effects of *Lactobacillus fermentum* CECT5716, live or dead, on colonic (A) TNFα production, (B) IL-1β production and (C) iNOS expression in TNBS rat colitis one week after damage induction. Data (n = 10 per group) are expressed as means ± SEM; groups with a different letter differ statistically ($P < 0.05$).

exerted by the probiotics on the microbiota composition or SCFA production, their interaction with the host cells is not exclusively dependent on the bacterial viability, due to the capacity of immune cells to recognize specific bacterial components or products, thus promoting the corresponding immunological response.[7] Supporting this, different surface proteins extracted from the *Propionibacterium freudenreichii* ITG P20 surface have been recently characterized and could account for its immuno-modulatory properties, being responsible for the induction of the regulatory cytokines IL-10 and IL-6.[28] In fact, the *in vitro* assays performed in the present study confirm this possibility, since in both intestinal epithelial cells and macrophages, the probiotic viability does not appear to be essential to affect cell activity, both under basal conditions and when the cells are stimulated with IL-1β or LPS, respectively. Furthermore, the cellular mechanisms involved in these effects seem to be similar, because when the expression of the MAP kinases p42/44 ERK and p38 were evaluated in unstimulated or stimulated epithelial cells, both live and dead probiotic showed the same pattern of activities. It is well known that cell-wall components from Gram-negative bacteria such as lipopolysaccharides as well as host-derived cytokines such as IL-1β and TNFα, increase IL-8 secretion from intestinal epithelial cells through the activation of mitogen activated protein kinase (MAPK).[29,30] In consequence, the ability of the probiotic to modulate MAPK activity can justify its effects on IL-8 production in intestinal epithelial cells.

These results confirmed that this strain of *L. fermentum* CECT5716 exhibits one of the important features of potential probiotic candidates, that is, the capacity to modulate the immune response of the host, which clearly contributes to its intestinal anti-inflammatory effect, as stated in the *in vivo* experiments performed in the present study. However, it is interesting to note that these beneficial effects are not exclusively dependent on the probiotic viability, since both live and dead probiotic ameliorated the colonic inflammation induced by instilling TNBS in rats. Previous studies have also revealed that the beneficial effects of probiotics on experimental colitis may be achieved by nonviable bacteria.[31,32] The capacity shown by dead probiotic to display intestinal anti-inflammatory properties would imply the participation of different bacterial components in these effects. Thus, it has been demonstrated that cell walls from Gram-positive bacteria, including *Enterococcus faecium* SF68 and *Lactobacillus casei* Shirota, can exert immuno-modulatory properties by affecting macrophage and B-cell activity.[33,34] Furthermore, it has been also reported that genomic DNA isolated from the probiotic preparation VSL#3 ameliorated the severity of colitis in DSS-, TNBS-induced and spontaneous colitis in IL-10 KO mice, these effects being mediated by TLR-signalling (mainly TLR-9).[31] However, it is important to note that, in contrast, other studies have determined the viability requirements for the probiotic to exert intestinal anti-inflammatory properties in experimental colitis, such as *Lactobacillus salivarius* ssp. *Salivarius* CECT5713.[35]

Classically, the pathogenesis of IBD was mainly attributed to an exacerbated adaptive immune response against antigens present in the luminal environment of the intestine. Most recently, a novel hypothesis has been proposed that this inflammatory disease of the gut may result from a primary defect in intestinal innate immunity, which in turn could cause an imbalance between immune responses and tolerance to the gut microbiota that leads to chronic intestinal inflammation.[36] Considering this, the immune-modulatory properties of the probiotic *L. fermentum* CECT5716, as evidenced both *in vitro* and *in vivo* in the present study, seem to play a key role. Thus, under normal conditions, *i.e.* when the intestinal mucosa is not subjected to any offending agent, the probiotic can promote the strengthening of the immunological barrier by stimulating and maintaining the state of alert of the innate and adaptive immune system. In fact, the *in vitro* assays showed an increased production of the innate cytokines IL-8 and IL-1β, as well as of NO, when either intestinal epithelial cells or macrophages were incubated with probiotic. A similar overproduction of cytokines has been reported for peripheral blood mononuclear cells exposed to well-established probiotic strains of lactobacilli, streptococci, Leuconostoc spp., and *Bifidobacterium breve.*[37,38]

However, in an inflammatory environment, the probiotic is able to decrease the exacerbated immune response, as confirmed both *in vitro* and *in vivo*. *In vitro*, the probiotic inhibited the stimulated production of IL-8 (intestinal epithelial cells), IL-1β and NO (macrophages). *In vivo*, probiotic treatment to colitic rats resulted in a reduced colonic production of the pro-inflammatory cytokines TNFα and IL-1β, as well as a down-regulation of colonic iNOS expression, when compared with the untreated corresponding controls. IL-8 is a chemokine that stimulates migration of neutrophils from intravascular to interstitial sites and can directly activate neutrophils and regulate the expression of neutrophil adhesion molecules.[39] In consequence, the ability of this probiotic to decrease IL-8 production can contribute to inhibit leukocyte infiltration in the inflamed tissue in colitic rats, as evidenced by the lower colonic MPO activity values observed in treated colitic rats in comparison with the untreated colitic group. An increase in neutrophils is a key feature in the pathogenesis of colitis in humans and animals,[40] which once activated by different stimuli, including IFN-γ, promote the release of reactive oxygen species products, such as hydrogen peroxide and hypochlorous acid, leading to a situation of oxidative stress and causing local tissue damage,[41] and corroborated in the present study by a depletion of colonic glutathione content, which was partially prevented after probiotic treatment. In fact, reducing or limiting the influx of these pro-inflammatory cells has previously been demonstrated to attenuate inflammation,[42] and this could be one of the mechanisms involved in the beneficial effect shown by this strain of *L. fermentum* CECT5716. Similarly, different studies have also reported the ability of other probiotics, such as *Enterococcus faecalis*, to attenuate the inflammatory responses and thus prevent inflammatory diseases, such as necrotizing enterocolitis in infants. They are able to inhibit MAPK signaling pathway and, as a result, modulate the expression and production of IL-8.[43] Furthermore, in the DSS-colitis model, similarly to what occurs in patients with IBD, p38 levels are increased in the colonic tissue,[44] and when treated with the p38 inhibitor, mucosal IL-1β and TNF-α levels were reduced,[45] consistent with what has been found for *L. fermentum* CECT5716 treatment in the present study.

In conclusion, *L. fermentum* CECT5716 has been demonstrated to attenuate the inflammatory process with amelioration of the production of some of the mediators involved in the inflammatory response, and this study has revealed that the viability of the probiotic was not required for its anti-inflammatory activity.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitivity (SAF2011-29648) and the Junta de Andalucia (AGR-6826 and CTS 164) with funds from the European Union; F. Algieri is a predoctoral fellow of Junta de Andalucia, and N. Garrido-Mesa is a postdoctoral fellow of Ramón Areces Foundation; M.E. Rodríguez-Cabezas is a postdoctoral fellow of CIBEREHD. The CIBEREHD is funded by the Instituto de Salud Carlos III.

References

1 E. M. Carlisle and M. J. Morowitz, The intestinal microbiome and necrotizing enterocolitis, *Curr. Opin. Pediatr.*, 2013, **25**, 382–387.
2 A. Grishin, S. Papillon, B. Bell, J. Wang and H. R. Ford, The role of the intestinal microbiota in the pathogenesis of necrotizing enterocolitis, *Semin. Pediatr. Surg.*, 2013, **22**, 69–75.
3 K. Whelan and E. M. Quigley, Probiotics in the management of irritable bowel syndrome and inflammatory bowel disease, *Curr. Opin. Gastroenterol.*, 2013, **29**, 184–189.
4 Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria: Report of a Joint FAO WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, American Córdoba Park Hotel, Córdoba, Argentina, 1–4 October 2001, 2001.
5 M. Boirivant and W. Strober, The mechanism of action of probiotics, *Curr. Opin. Gastroenterol.*, 2007, **23**, 679–692.
6 M. Gobbetti, R. D. Cagno and M. De Angelis, Functional microorganisms for functional food quality, *Crit. Rev. Food Sci. Nutr.*, 2010, **50**, 716–727.
7 C. A. Adams, The probiotic paradox: live and dead cells are biological response modifiers, *Nutr. Res. Rev.*, 2010, **23**, 37–46.

8 S. Parvez, K. A. Malik, S. Ah Kang and H. Y. Kim, Probiotics and their fermented food products are beneficial for health, *J. Appl. Microbiol.*, 2006, **100**, 1171–1185.
9 S. E. Power, P. W. O'Toole, C. Stanton, R. P. Ross and G. F. Fitzgerald, Intestinal microbiota, diet and health, *Br. J. Nutr.*, 2014, **111**, 387–402.
10 M. S. Geier, R. N. Butler, P. M. Giffard and G. S. Howarth, Lactobacillus fermentum BR11, a potential new probiotic, alleviates symptoms of colitis induced by dextran sulfate sodium (DSS) in rats, *Int. J. Food Microbiol.*, 2007, **114**, 267–274.
11 L. Peran, D. Camuesco, M. Comalada, A. Nieto, A. Concha, J. L. Adrio, M. Olivares, J. Xaus, A. Zarzuelo and J. Galvez, Lactobacillus fermentum, a probiotic capable to release glutathione, prevents colonic inflammation in the TNBS model of rat colitis, *Int. J. Colorectal Dis.*, 2006, **21**, 737–746.
12 G. Zoumpopoulou, B. Foligne, K. Christodoulou, C. Grangette, B. Pot and E. Tsakalidou, Lactobacillus fermentum ACA-DC 179 displays probiotic potential in vitro and protects against trinitrobenzene sulfonic acid (TNBS)-induced colitis and Salmonella infection in murine models, *Int. J. Food Microbiol.*, 2008, **121**, 18–26.
13 M. Olivares, M. P. Diaz-Ropero, R. Martin, J. M. Rodriguez and J. Xaus, Antimicrobial potential of four Lactobacillus strains isolated from breast milk, *J. Appl. Microbiol.*, 2006, **101**, 72–79.
14 F. J. Pérez-Cano, H. Dong and P. Yaqoob, In vitro immunomodulatory activity of Lactobacillus fermentum CECT5716 and Lactobacillus salivarius CECT5713: two probiotic strains isolated from human breast milk, *Immunobiology*, 2010, 215, 996–1004.
15 C. O. Elson, R. B. Sartor, G. S. Tennyson and R. H. Riddell, Experimental models of inflammatory bowel disease, *Gastroenterology*, 1995, **109**, 1344–1367.
16 D. L. Granger, R. R. Taintor, K. S. Boockvar and J. B. Hibbs Jr., Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction, *Methods Enzymol.*, 1996, **268**, 142–151.
17 T. Hisamatsu and R. W. King, The nature of floral signals in Arabidopsis. II. Roles for FLOWERING LOCUS T (FT) and gibberellin, *J. Exp. Bot.*, 2008, **59**, 3821–3829.
18 D. Camuesco, L. Peran, M. Comalada, A. Nieto, L. C. Di Stasi, M. E. Rodriguez-Cabezas, A. Concha, A. Zarzuelo and J. Galvez, Preventative effects of lactulose in the trinitrobenzenesulphonic acid model of rat colitis, *Inflammatory Bowel Dis.*, 2005, **11**, 265–271.
19 J. E. Krawisz, P. Sharon and W. F. Stenson, Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models, *Gastroenterology*, 1984, **87**, 1344–1350.
20 M. E. Anderson, F. Powrie, R. N. Puri and A. Meister, Glutathione monoethyl ester: preparation, uptake by tissues, and conversion to glutathione, *Arch. Biochem. Biophys.*, 1985, **239**, 538–548.
21 T. Hisamatsu, H. Ogata and T. Hibi, Innate immunity in inflammatory bowel disease: state of the art, *Curr. Opin. Gastroenterol.*, 2008, **24**, 448–454.
22 J. E. Krawisz, P. Sharon and W. F. Stenson, Quantitative Assay for Acute Intestinal Inflammation Based on Myeloperoxidase Activity - Assessment of Inflammation in Rat and Hamster Models, *Gastroenterology*, 1984, **87**, 1344–1350.
23 L. Peran, D. Camuesco, M. Comalada, A. Nieto, A. Concha, J. L. Adrio, M. Olivares, J. Xaus, A. Zarzuelo and J. Galvez, Lactobacillus fermentum, a probiotic capable to release glutathione, prevents colonic inflammation in the TNBS model of rat colitis, *Int. J. Colorectal Dis.*, 2006, **21**, 737–746.
24 R. Fedorak and D. Demeria, Probiotic Bacteria in the Prevention and the Treatment of Inflammatory Bowel Disease, *Gastroenterology Clinics of North America*, 2012, **41**, 821–842.
25 J. Gálvez, M. Comalada and J. Xaus, Prebiotics and Probiotics in Experimental Models of Rodent Colitis: Lessons in Treatment or Prevention of Inflammatory Bowel Diseases, *Bioactive Foods in Promoting Health*, ed. R. R. Watson and V. R. Preedy, Academic Press, Boston, 2010, ch. 35, pp. 589–610.
26 J. Gerritsen, H. Smidt, G. T. Rijkers and W. M. de Vos, Intestinal microbiota in human health and disease: the impact of probiotics, *Genes Nutr.*, 2011, **6**, 209–240.
27 L. Peran, S. Sierra, M. Comalada, F. Lara-Villoslada, E. Bailon, A. Nieto, A. Concha, M. Olivares, A. Zarzuelo, J. Xaus and J. Galvez, A comparative study of the preventative effects exerted by two probiotics, Lactobacillus reuteri and Lactobacillus fermentum, in the trinitrobenzenesulfonic acid model of rat colitis, *Br. J. Nutr.*, 2007, **97**, 96–103.
28 C. Le Marechal, V. Peton, C. Ple, C. Vroland, J. Jardin, V. Briard-Bion, G. Durant, V. Chuat, V. Loux, B. Foligne, S. M. Deutsch, H. Falentin and G. Jan, Surface proteins of Propionibacterium freudenreichii are involved in its anti-inflammatory properties, *J. Proteomics*, 2015, **113**, 447–461.
29 J. M. Otte, E. Cario and D. K. Podolsky, Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells, *Gastroenterology*, 2004, **126**, 1054–1070.
30 H. B. Jijon, A. Buret, C. L. Hirota, M. D. Hollenberg and P. L. Beck, The EGF receptor and HER2 participate in TNF-alpha-dependent MAPK activation and IL-8 secretion in intestinal epithelial cells, *Mediators Inflammation*, 2012, **2012**, 207398.
31 D. Rachmilewitz, K. Katakura, F. Karmeli, T. Hayashi, C. Reinus, B. Rudensky, S. Akira, K. Takeda, J. Lee, K. Takabayashi and E. Raz, Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis, *Gastroenterology*, 2004, **126**, 520–528.
32 L. X. Sang, B. Chang, C. Dai, N. Gao, W. X. Liu and M. Jiang, Heat-killed VSL#3 Ameliorates Dextran Sulfate

Sodium (DSS)-Induced Acute Experimental Colitis in Rats, *Int. J. Mol. Sci.*, 2014, **15**, 15–28.

33 K. A. Baken, J. Ezendam, E. R. Gremmer, A. de Klerk, J. L. Pennings, B. Matthee, A. A. Peijnenburg and H. van Loveren, Evaluation of immunomodulation by Lactobacillus casei Shirota: immune function, autoimmunity and gene expression, *Int. J. Food Microbiol.*, 2006, **112**, 8–18.

34 J. Benyacoub, P. F. Perez, F. Rochat, K. Y. Saudan, G. Reuteler, N. Antille, M. Humen, G. L. De Antoni, C. Cavadini, S. Blum and E. J. Schiffrin, Enterococcus faecium SF68 enhances the immune response to Giardia intestinalis in mice, *J. Nutr.*, 2005, **135**, 1171–1176.

35 B. Arribas, N. Garrido-Mesa, L. Peran, D. Camuesco, M. Comalada, E. Bailon, M. Olivares, J. Xaus, L. Kruidenier, I. R. Sanderson, A. Zarzuelo, M. E. Rodriguez-Cabezas and J. Galvez, The immunomodulatory properties of viable Lactobacillus salivarius ssp. salivarius CECT5713 are not restricted to the large intestine, *Eur. J. Nutr.*, 2012, **51**, 365–374.

36 G. Bamias, D. Corridoni, T. T. Pizarro and F. Cominelli, New insights into the dichotomous role of innate cytokines in gut homeostasis and inflammation, *Cytokine*, 2012, **59**, 451–459.

37 S. B. Gaudana, A. S. Dhanani and T. Bagchi, Probiotic attributes of Lactobacillus strains isolated from food and of human origin, *Br. J. Nutr.*, 2010, **103**, 1620–1628.

38 R. A. Kekkonen, E. Kajasto, M. Miettinen, V. Veckman, R. Korpela and I. Julkunen, Probiotic Leuconostoc mesenteroides ssp. cremoris and Streptococcus thermophilus induce IL-12 and IFN-gamma production, *World J. Gastroenterol.*, 2008, **14**, 1192–1203.

39 A. R. Huber, S. L. Kunkel, R. F. Todd 3rd and S. J. Weiss, Regulation of transendothelial neutrophil migration by endogenous interleukin-8, *Science*, 1991, **254**, 99–102.

40 E. J. Williams, S. Haque, C. Banks, P. Johnson, P. Sarsfield and N. Sheron, Distribution of the interleukin-8 receptors, CXCR1 and CXCR2, in inflamed gut tissue, *J. Pathol.*, 2000, **192**, 533–539.

41 M. J. Steinbeck, J. A. Roth and M. L. Kaeberle, Activation of bovine neutrophils by recombinant interferon-gamma, *Cell. Immunol.*, 1986, **98**, 137–144.

42 S. M. Farooq, R. Stillie, M. Svensson, C. Svanborg, R. M. Strieter and A. W. Stadnyk, Therapeutic effect of blocking CXCR2 on neutrophil recruitment and dextran sodium sulfate-induced colitis, *J. Pharmacol. Exp. Ther.*, 2009, **329**, 123–129.

43 S. Wang, M. L. Hibberd, S. Pettersson and Y. K. Lee, *Enterococcus faecalis* from Healthy Infants Modulates Inflammation through MAPK Signaling Pathways, *PLoS One*, 2014, **9**, e97523.

44 E. Ihara, P. L. Beck, M. Chappellaz, J. Wong, S. A. Medlicott and J. A. MacDonald, Mitogen-activated protein kinase pathways contribute to hypercontractility and increased Ca2+ sensitization in murine experimental colitis, *Mol. Pharmacol.*, 2009, **75**, 1031–1041.

45 E. Hollenbach, M. Neumann, M. Vieth, A. Roessner, P. Malfertheiner and M. Naumann, Inhibition of p38 MAP kinase- and RICK/NF-kappaB-signaling suppresses inflammatory bowel disease, *FASEB J.*, 2004, **18**, 1550–1552.