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ORIGINAL CONTRIBUTION

The immunomodulatory properties of viable *Lactobacillus salivarius* ssp. *salivarius* CECT5713 are not restricted to the large intestine

Belén Arribas · Natividad Garrido-Mesa · Laura Perán · Desirée Camuesco · Mònica Comalada · Elvira Bailón · Mónica Olivares · Jordi Xaus · Laurens Kruidenier · Ian R. Sanderson · Antonio Zarzuelo · Maria Elena Rodríguez-Cabezas · Julio Gálvez

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Abstract
Purpose The aim of this study was to better characterise the biological effects of *Lactobacillus salivarius* ssp. *salivarius* CECT5713, a probiotic with immunomodulatory properties.
Methods Live or dead probiotic was assayed in the TNBS model of rat colitis to determine whether viability was a requisite to exert the beneficial effects. In vitro studies were also performed in Caco-2 cells to evaluate its effects on epithelial cell recovery and IL-8 production. Finally, the probiotic was assayed in the LPS model of septic shock in mice to establish its effects when there is an altered systemic immune response.
Results The viability of the probiotic was required for its anti-inflammatory activity. The probiotic inhibited IL-8 production in stimulated Caco-2 cells and facilitated the recovery of damaged intestinal epithelium. In LPS-treated mice, the probiotic inhibited the production of TNFα in plasma and lungs and increased the hepatic glutathione content. These effects were associated with an improvement in the altered production of the T-cell cytokines in splenocytes, by reducing IL-2 and IL-5 and by increasing IL-10. Finally, it reduced the increased plasma IgG production in LPS-treated mice.
Conclusion The anti-inflammatory effects of viable *L. salivarius* ssp. *salivarius* CECT5713 are not restricted to the gastrointestinal tract.

Keywords *L. salivarius* ssp. *salivarius* CECT5713 · Mice LPS septic shock · Cytokines · Immunoglobulin · Intestinal anti-inflammatory activity · TNBS rat colitis · Caco-2 cells

Introduction

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [1]. Different mechanisms have been proposed to be involved in these beneficial effects [1]. Firstly, probiotics are able to colonise the human gastrointestinal tract and modify the composition of the normal intestinal microflora and prevent the growth or epithelial binding and invasion of enteric pathogenic bacteria, through its ability to decrease luminal pH, to promote the secretion of bactericidal proteins and/or to stimulate mucin production. Secondly, probiotics exert immunoregulatory activities, by inducing protective cytokines (IL-10 and TGF-β) or by suppressing proinflammatory cytokines, like TNFα, in the intestinal mucosa. And thirdly, these microorganisms improve the intestinal barrier function by decreasing mucosal permeability. Most of the

E. Rodríguez-Cabezas and J. Gálvez contributed equally to the supervision of the study.

B. Arribas · N. Garrido-Mesa · L. Perán · E. Bailón · J. Xaus · A. Zarzuelo · M. E. Rodríguez-Cabezas · J. Gálvez (✉)
Centro de Investigaciones Biomédicas en Red, Enfermedades Hepáticas y Digestivas (CIBER-EHD), Department of Pharmacology, Center for Biomedical Research, University of Granada, Avenida del Conocimiento s/n, 18100 Armilla, Granada, Spain
e-mail: jgalvez@ugr.es

D. Camuesco · L. Kruidenier · I. R. Sanderson
Institute of Cell and Molecular Science Queen Mary College, University of London, London, UK

M. Comalada
Institute of Research in Biomedicine (IRB), Macrophage Biology Group, Barcelona, Spain

M. Olivares
Department of Immunology and Animal Sciences, Biosearch Life, Granada, Spain

beneficial effects reported for probiotics are related to intestinal conditions [2], and among them, special attention has been lately considered to their use in the management of inflammatory bowel disease (IBD) [3]. However, the characterisation of probiotics that also exert systemic immunomodulatory effects may be of great interest, since the prevalence of extraintestinal diseases in IBD patients is around 6% (excluding arthritis), presenting many of them an immunological basis, like primary sclerosing cholangitis, ankylosing spondylitis, iritis/uveitis, pyoderma gangrenosum and erythema nodosum [4].

Lactobacillus salivarius ssp. *salivarius* is a probiotic with immunomodulatory properties. Different strains of this probiotic have been reported to exert intestinal anti-inflammatory effects in experimental models of colitis [5, 6], an effect associated with downregulation of proinflammatory cytokines, including IFNγ and TNFα, which play a key role in IBD. The beneficial effects of *L. salivarius* ssp. *salivarius* may be also ascribed to its ability to produce antimicrobial substances, such as bacteriocins [7, 8], thus limiting the deleterious effect of potential pathogens. However, other mechanisms can be also involved in its intestinal anti-inflammatory effects. The aim of this study is to better characterise the biological effects of *L. salivarius* ssp. *salivarius* CECT5713. First, based on different studies reporting that the viability of probiotics is not essential to exert its anti-inflammatory effect [9], we have tested whether the administration of viable *L. salivarius* ssp. *salivarius* is necessary to obtain a beneficial effect in the TNBS model of rat colitis. Second, we have evaluated the activity of this probiotic on the intestinal epithelial cell activity, since a defect in the epithelial integrity has been considered as an initial and crucial step in the intestinal inflammatory response [10]; moreover, epithelial cells actively participate in the immune response taking place in the intestinal inflammatory process, given their ability to act as antigen-presenting cells and/or to promote the release of proinflammatory cytokines like IL-8 [11]. Finally, we have also investigated the effects of this probiotic in the lipopolysaccharide (LPS)-induced septic shock in mice, in order to establish whether, in addition to its intestinal anti-inflammatory activity, it shows beneficial effects when an alteration in the systemic immune response occurs.

Materials and methods

Reagents and animals

All chemicals, including LPS from *Escherichia coli* serotype 055:B5, were obtained from Sigma Chemical (Madrid, Spain), unless otherwise stated. *Lactobacillus salivarius* ssp. *salivarius* CECT5713 was provided by Biosearch Life S.A. (Granada, Spain), and it was normally grown in MRS media at 37 C in 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. Enterotoxigenic *Escherichia coli* was purchased from the ATCC collection, grown in tryptone soya broth overnight, and the concentration of the suspension was measured by turbidimetry.

Female HsdHan Wistar rats (200–210 g) or male BALB/c AnNHsd mice (20–22 g) obtained from the Laboratory Animal Service of the University of Granada were housed in makrolon cages, maintained in air-conditioned animal quarters, which were monitored according to the recommendations from 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. The monitoring of 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).

Intestinal anti-inflammatory effect of live or dead *Lactobacillus salivarius* ssp. *salivarius* in the TNBS model of rat colitis

The rats were randomly assigned to four groups ($n = 10$); two of them (non-colitic and control groups) received orally PBS solution (1 mL) and the other two (treated groups) received the probiotic orally at the concentration of $5{\cdot}10^8$ colony forming units (CFU) suspended in 1 mL of PBS solution, live or dead, the latter after heating the microorganisms at 95°C for 30 min. The suspension was administered 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 [12]. Briefly, they were anaesthetized with halothane 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. 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), and the weight/length ratio determined. The colon was scored for macroscopically visible damage on a 0–10 scale by two observers unaware of the treatment, according to the criteria previously reported [12], which takes into account the extent as well as the severity of colonic damage. The colon was subsequently divided into different segments for biochemical determinations. Three fragments were frozen at −80 °C for myeloperoxidase (MPO) activity, TNFα production and inducible nitric oxide synthase (iNOS) expression, and another sample was weighed and frozen in 1 mL of 50 g/L trichloroacetic acid for total glutathione content determinations.

Effects of *Lactobacillus salivarius* ssp *salivarius* on epithelial cell recovery and IL-8 production in Caco-2 cells

The human colonic epithelial cell line Caco-2 (American Type Culture Collection, Manassas, VA, USA) was used as a model of intestinal epithelium. The cells were grown until confluence in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS) (Boëhringer Mannheim, Barcelona, Spain), 2 mmol/L L-glutamine, 50.000U/L penicillin/streptomycin and 2.5 mg/L amphotericin B, in a humidified 5% CO_2 atmosphere at 37 °C; 24 h before performing the experiments, they were cultured in the absence of FBS, and before adding the bacteria, the medium with antibiotics was removed, and the cells were washed twice with warm sterile PBS. Then, fresh antibiotic-free medium was added. Two different experiments were performed with these cells: evaluation of epithelial regeneration in an in vitro model of wound healing and inhibition of IL-8 release after cell activation with two different stimuli: IL-1β and enterotoxigenic *Escherichia coli*. For the evaluation of the epithelial regeneration, we used the traditional wound-healing scrape assay. Briefly, cells were cultured in 6-well plates until confluence, when cell monolayers were disrupted by scrapping them with a pipette tip. The width of the wound was measured with the help of a scale in the ocular of the microscope at time 0 as well as 24 h after. Once the wound had been induced, the cells were incubated in the presence of the probiotic (10^8 CFU/mL) for 1 h, this was removed by washing, and the wound was left to recover for the remaining 23 h; some of the cells were not incubated with the probiotic, but we followed the same protocol. The percentage of recovery was established for each well. In another set of experiments, we studied the production of IL-8 in Caco-2 cells after their stimulation with IL-1β (1 ng/mL) or with a suspension of enterotoxigenic *Escherichia coli* at the final concentration of 10^8 CFU/mL. Bacteria was removed after 1 h by washing twice with culture medium without FBS, and the cells were then incubated in the presence of the probiotic (10^8 CFU/mL) for 1 h, the probiotic being removed and the cells washed twice with the culture medium without FBS. Twenty four hours after, the supernatants were collected, centrifuged and frozen until determination of IL-8 content, which was performed by ELISA (Biosource, Nivelles, Belgium), according to the instructions provided by the manufacturer.

Evaluation of the effects of *Lactobacillus salivarius* ssp. *salivarius* in the LPS-induced model of septic shock in mice

The mice were randomly assigned to three groups ($n = 10$); two of them (healthy and control groups) received tap water and the other (treated group) received the probiotic suspended daily in the drinking water at the final concentration of 10^8 CFU/mL. Orally gavage was avoided in mice to prevent any stressful situation; however, the total daily dose of probiotic administered to mice was quite similar to that used for rats ($5{\cdot}10^8$ CFU), since water intake for each mouse was approximately 5 mL per day. Food and water intake was recorded daily for all groups. Two weeks after starting the experiment, endotoxic shock was induced in treated and control mice with an intraperitoneal injection of LPS (20 mg/kg) to a final volume of 200 µL; healthy mice received sterile saline solution. Previous assays have revealed that this dose of LPS did not induce the death of any mouse in the following 24 h. Then, mice were anaesthetized with halothane, blood samples were taken from the retro-orbital venous plexus and then killed immediately. The following tissues were quickly removed and weighed: spleen, lungs and liver. Colon specimens, after weighing and measuring their length, were frozen at −80 °C for the evaluation of inducible NO synthase (iNOS) expression. Liver samples were frozen in 1 mL of 50 g/L trichloroacetic acid for total glutathione content determination. One of the lungs was immediately processed for the measurement of TNFα levels.

Biochemical 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 [13]; 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/min at 25 °C. Total glutathione content was quantified in liver with the recycling assay described by Anderson [14], and the results were expressed as nmol/g wet tissue. Lung or colonic samples for TNFα 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 9,000 $\times g$ for 30 s at 4 °C; the supernatants were frozen at −80 °C until cytokine assay. TNFα was quantified by ELISA assay (R&D Systems, Abingdon, UK for rat samples, and Biosource, Nivelles, Belgium, for mouse samples), and the results were expressed as pg/g wet tissue. Colonic specimens from mice were also used for protein extraction to evaluate iNOS expression by Western blotting, which was performed as described elsewhere [15]. Blood samples were centrifuged (600 $\times g$ for 10 min), and plasma TNFα and IgG levels were quantified by ELISA assay (Biosource, Nivelles, Belgium).

Spleen-derived cell culture

In order to obtain primary lymphocyte cultures, mice spleens were immediately disaggregated in DMEM plus 1% penicillin/streptomycin/amphotericin after collection, centrifuged (600 $\times g$, 5 min) and erythrocytes were lysed with a lysis buffer (NH_4Cl 1.7 mol/L, $KHCO_3$ 0.12 mol/L, ethylenediamine tetraacetic acid 9 mmol/L) for 30 min at 4 °C. Resting cells were counted using a hemocytometer and cultured to perform stimulation assays in the culture medium (DMEM+10% FBS). Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Spleen-derived lymphocytes were cultured in 6-well plates ($1x10^7$ cells/well) in 3 mL of media and stimulated with concanavalin A (Con A) (5 μg/mL) or LPS (50 μg/mL) to activate T or B cells, correspondingly, and supernatants were collected after 48 h or 72 h, respectively, and frozen until ELISA analysis. Cytokine production (IL-2, IL-5 and IL-10) by T cells and IgG secretion by B cells were measured with commercial murine ELISA kits (R&D Systems, Abingdon, UK for cytokines; Bethyl, Montgomery, TX, USA for IgG), following the manufacturers' protocols. In addition, RNA was isolated from spleen-derived T cells using TRIzol® Reagent (Gibco-BRL, USA) and subsequently converted to cDNA using First-Strand cDNA Synthesis Kit (Amersham, Biosciences). The cDNA was amplified with appropriate primers, and the polymerase chain reaction was performed as described previously [16]. The numbers of amplification cycles were as follows: 30 for TNFα, 28 for IL-1β and 35 for IL-10, and 25 for β-actin.

Statistical analysis

The results are expressed as mean ± s.e. mean. Differences among means were tested for statistical significance using one-way analysis of variance (ANOVA) and *post hoc* least significance tests. Macroscopic score data were expressed as median (range) and analysed by the Mann–Whitney test. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Maryland), with statistical significance set at $P < 0.05$.

Results

Viable *Lactobacillus salivarius* ssp. *salivarius* CECT5713 is required for its intestinal anti-inflammatory activity in the TNBS model of rat colitis

The administration of the probiotic *Lactobacillus salivarius* ssp. *salivarius* CECT5713, live or dead, for 2 weeks before colitis induction did not result in any symptom of diarrhoea or affect weight evolution (data not shown). However, once the colitis was induced, only the rats treated with viable probiotic showed an overall lower impact of TNBS-induced colonic damage compared to the TNBS control group. The anti-inflammatory effect was evidenced macroscopically by a significantly lower colonic damage score than that of control rats ($P < 0.05$), with a significant reduction of the extent of colonic necrosis and/or inflammation (Table 1). However, this anti-inflammatory effect was not associated with a significant reduction of the colonic weight/length ratio between both colitic groups, an index of colonic oedema that is increased significantly as a consequence of the inflammatory process (Table 1). The treatment of colitic rats with the dead probiotic was not able to significantly improve any of the macroscopic parameters evaluated in comparison with untreated control rats, even showing statistical differences with the group of colitic rats treated with viable *L. salivarius* ssp. *salivarius* CECT5713 (Table 1). Biochemically, the preventative beneficial effects showed by the live probiotic were evidenced by the reduction of colonic MPO activity, a marker

Table 1 Effects of viable or dead *Lactobacillus salivarius* ssp. *salivarius* CECT5713 ($5{\cdot}10^8$ CFU/rat·day) treatment on macroscopic damage score, extent of the inflammatory lesion along the colon and in colon weight in TNBS experimental colitis in rats

Group ($n = 10$)	Damage score(0–10)	Extent of damage(cm)	Colon weight (mg/cm)
Non-colitic	0	0	65.4 ± 1.2
TNBS control	7 (6–8)	3.7 ± 0.2	217.2 ± 17.0
TNBS live probiotic	5 (3–8)*,#	2.5 ± 0.5*,#	149.6 ± 13.8*,#
TNBS dead probiotic	7.5 (6–8)	4.4 ± 0.3	189.7 ± 19.3

Damage score for each rat was assigned according to the criteria described in [12] and data are expressed as median (range). Extent of damage and colon weight data are expressed as mean ± SE. *$P<0.05$ versus TNBS control; #$P<0.05$ versus TNBS colitic rats treated with dead probiotic. All TNBS colitic groups differ significantly from non-colitic group ($P < 0.01$, not shown)

Table 2 Effects of viable or dead *Lactobacillus salivarius* ssp. *salivarius* CECT5713 ($5{\cdot}10^8$ CFU/rat·day) treatment on colonic myeloperoxidase (MPO) activity, glutathione (GSH) levels and TNFα production in TNBS experimental colitis in rats

Group ($n = 10$)	MPO activity (U/g tissue)	GSH (nmol/g tissue)	TNFα (pg/g tissue)
Non-colitic	51.3 ± 4.9	1,913 ± 186	740 ± 61
TNBS control	515.5 ± 48.9	1,195 ± 71	1,424 ± 100
TNBS live probiotic	319.0 ± 53.8*	1,509 ± 105*,#	981 ± 143*
TNBS dead probiotic	468.9 ± 73.6	1,091 ± 28	1,117 ± 113

Data are expressed as mean ± SE. *$P < 0.05$ versus TNBS control and TNBS colitic rats treated with dead probiotic. All colitic groups differ significantly from non-colitic group ($P < 0.01$, not shown)

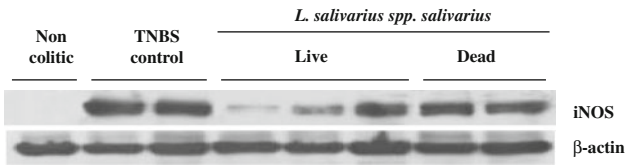

Fig. 1 Effect of *Lactobacillus salivarius* ssp. *salivarius* CECT5713 administration ($5{\cdot}10^8$ CFU/rat·day), live or dead, on colonic iNOS expression (Western blot) in TNBS colitic rats

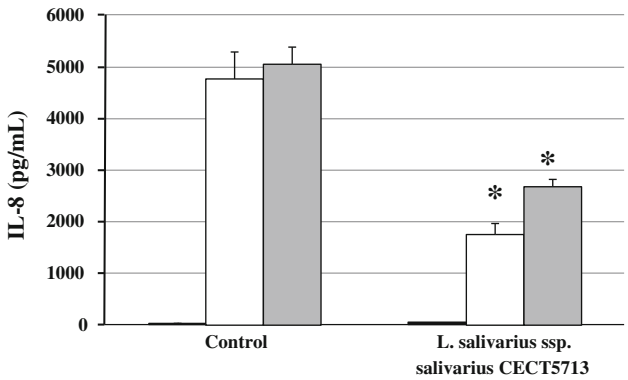

Fig. 2 Effects of *Lactobacillus salivarius* ssp. *salivarius* CECT5713 (10^8 CFU/mL) on IL-8 production in Caco-2 cells in basal conditions (*black bars*), or stimulated with IL-1β (1 ng/mL) (*white bars*) or with a suspension of enterotoxigenic *Escherichia coli* (10^8 CFU/mL) (*grey bars*). The concentrations (means ± SE) of the cytokine were analysed by ELISA (data from four independent experiments). *$P < 0.05$ versus control cells without probiotic treatment

of neutrophil infiltration that was enhanced in the TNBS control group (Table 2), suggesting a lower leucocyte infiltration into the inflamed tissue. No significant modification in this enzyme activity was evidenced in the colitic rats treated with the dead probiotic, although no differences were obtained when compared with the group of colitic rats receiving the live probiotic. In comparison with non-colitic animals, the colonic inflammation induced by TNBS was characterised by a decreased content in GSH and by increased levels of TNFα (Table 2). The treatment with viable *L. salivarius* ssp. *salivarius* CECT5713 resulted in a significant increase in the colonic GSH content as well as by a reduction of colonic TNFα, whereas the administration of dead probiotic did not result in a significant improvement in any of the biochemical parameters studied (Table 2). Furthermore, when colonic iNOS expression was evaluated, only viable probiotic was able to reduce the increased expression of this enzyme in colitic rats (Fig. 1).

Lactobacillus salivarius ssp. *salivarius* CECT5713 inhibits IL-8 production in stimulated Caco-2 cells and facilitates the recovery in an in vitro model of damaged intestinal epithelium

The incubation of confluent Caco-2 cells with the probiotic *Lactobacillus salivarius* ssp. *salivarius* CECT5713 (10^8 CFU/mL) for 1 h did not result in a significant modification of the release of IL-8 in comparison with its basal production (45.2 ± 3.0 pg/mL versus 47.8 ± 2.3 pg/mL in the probiotic-treated cells). The stimulation of Caco-2 cells with IL-1β or with enterotoxigenic *E.coli* for one hour resulted in an increased production of IL-8 ($P < 0.01$ versus untreated cells). The incubation of the Caco-2 cells with the probiotic significantly inhibited the increased production of this cytokine induced by both stimuli (Fig. 2). When a wound was induced in the Caco-2 cell monolayer, the percentage of recovery of the monolayer after 24 h was 15.1 ± 5.1%. The pre-treatment with *L. salivarius* ssp. *salivarius* CECT5713 (10^8 CFU/mL) for 1 h significantly facilitated the healing in comparison with untreated cells, showing a percentage of regeneration of 34.9 ± 10.2% ($P < 0.05$ versus untreated cells).

Preventative effects of *L. salivarius* ssp. *salivarius* CECT5713 in the LPS-induced model of septic shock in mice

The probiotic administration to mice for 2 weeks did not affect body weight gain (data not shown), and no sign of toxic effects was observed, accordingly to that observed in rats. After 2 weeks of probiotic treatment, mice received an i.p. injection of a sub-lethal dose of 20 mg/kg of LPS. Within 8 h, the mice from the control group showed evident symptoms of endotoxic shock, including decreased motor activity, ruffled fur and ocular exudates. At that time, those mice pre-treated with the probiotic showed a better general appearance and motor activity, although signs of illness were evident when compared with the healthy ones. All mice survived 24 h after LPS injection, when they were killed after exsanguination. The macroscopic tissue modifications observed as a consequence of

the septic shock included significant increases in colon weight/length ratio as well as in spleen and lungs weights when compared with healthy mice ($P < 0.05$; Table 3), whereas no significant modification was observed in the weights of liver and kidneys (Table 3). The probiotic treatment only resulted in a significant reduction in colon weight/length ratio, without showing statistical differences with the healthy group (Table 3).

The beneficial effects exerted by the probiotic in this experimental model of septic shock were evidenced biochemically. Thus, plasma and lung TNFα levels were significantly increased 24 h after LPS administration in comparison with normal mice, while the group of mice receiving *L. salivarius* ssp. *salivarius* CECT5713 for 2 weeks prior to LPS injection resulted in a significant reduction in TNFα production when compared with untreated control mice (Fig. 3). The endotoxic shock induced by LPS is associated with a systemic free radical overproduction, which clearly contributes to tissue damage. One of the manifestations of this situation of oxidative stress is the depletion of the antioxidant peptide glutathione. In fact, 24 h after LPS administration, a significant decrease in the hepatic glutathione levels were observed in septic mice in comparison with the healthy group ($P < 0.01$ versus healthy group; Fig. 3). The beneficial preventative effect exerted by the probiotic resulted in a significant increase in the hepatic content of glutathione when compared with untreated control septic mice ($P < 0.05$ versus control group; Fig. 3). Similarly, the probiotic administration prevented the increased expression of colonic iNOS, observed in control septic mice when compared with the healthy ones (Fig. 3).

Table 3 Effects of *Lactobacillus salivarius* ssp. *salivarius* CECT5713 ($5{\cdot}10^8$ CFU/mice·day) treatment on tissue weights in LPS-induced septic shock in mice

Group ($n = 10$)	Liver (mg/g mice)	Kidney (mg/g mice)	Colon (mg/cm)	Spleen (mg/g mice)	Lungs (mg/g mice)
Healthy	49.9 ± 1.7	19.2± 0.5	23.9 ± 0.9	4.3 ± 0.2	5.8 ± 0.3
Control	51.3 ± 2.1	20.2± 1.4	30.9 ± 0.8$^{\#}$	4.8 ± 0.2$^{\#}$	7.8 ± 0.3$^{\#}$
Probiotic	50.9 ± 1.2	18.7± 0.8	25.6 ± 1.3*	4.5 ± 0.1	7.7 ± 0.2$^{\#}$

Data are expressed as mean ± SE. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus healthy group

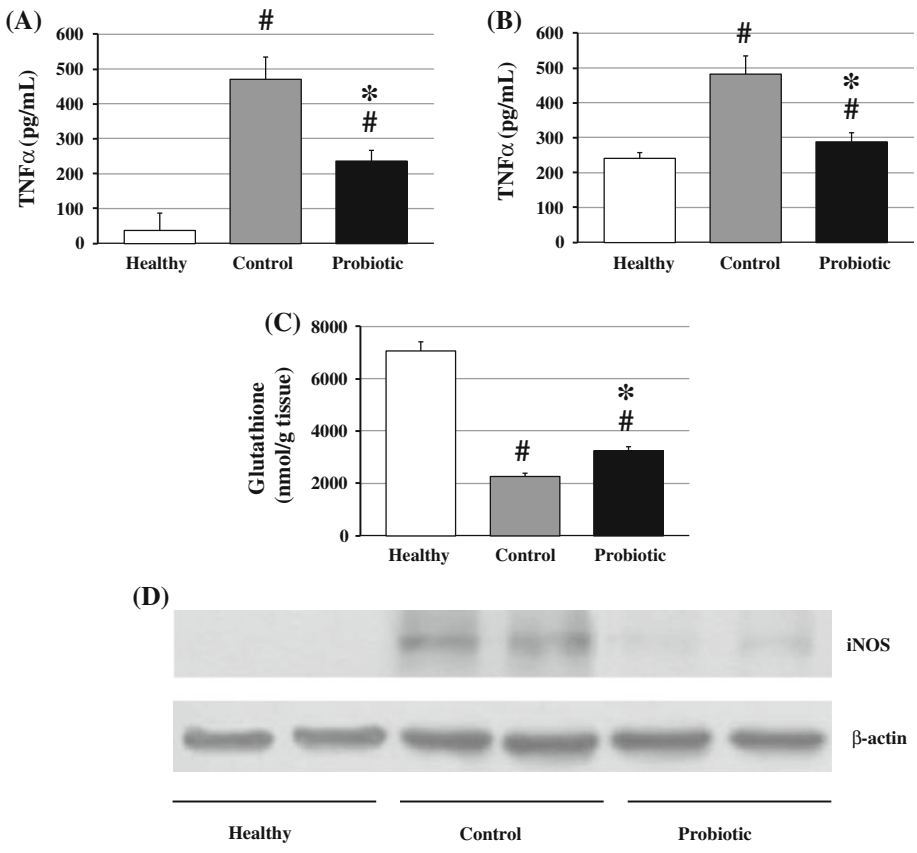

Fig. 3 Effect of *Lactobacillus salivarius* ssp. *salivarius* CECT5713 administration ($5{\cdot}10^8$ CFU/mice·day) on TNFα production in LPS-induced septic shock in mice in plasma **a** and lungs **b**; on hepatic glutathione content **c**; and on colonic iNOS expression (Western blot) **d**. TNFα production and glutatione content values are expressed as means ± SE ($n = 10$). $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus healthy group

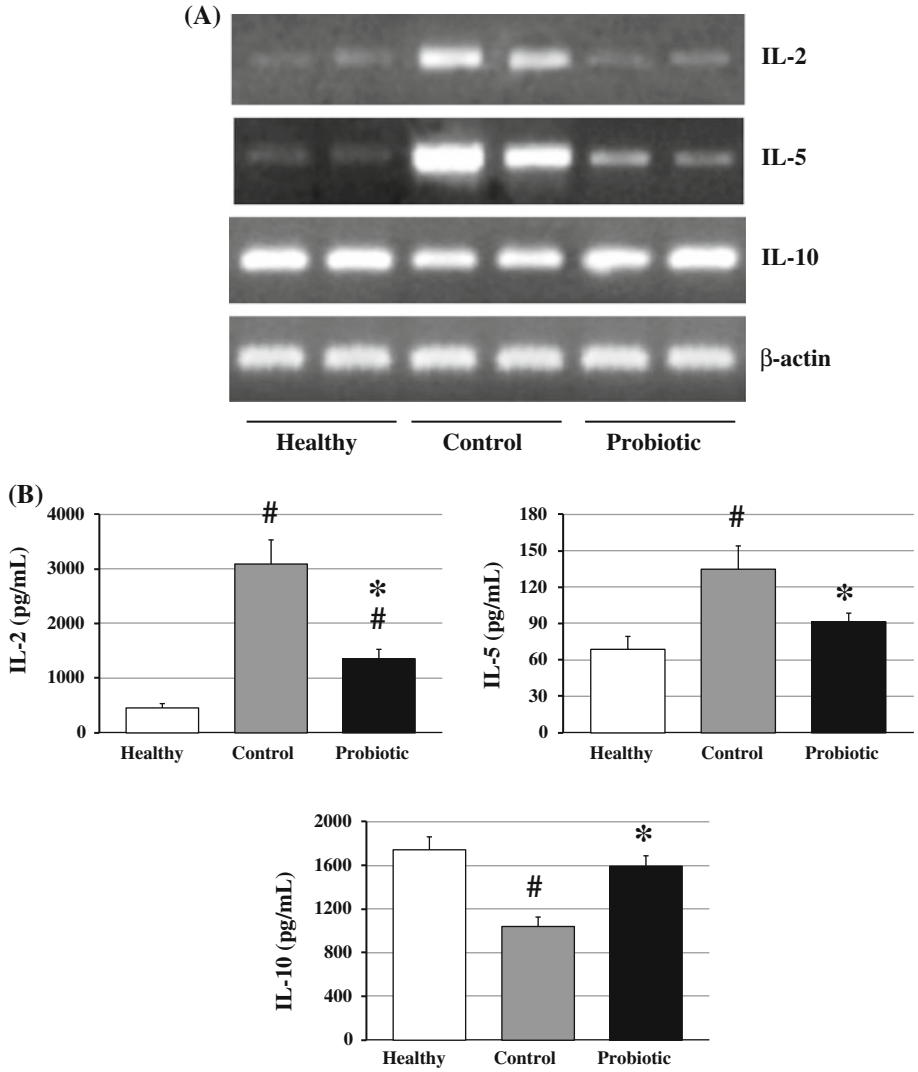

Fig. 4 Effects of *Lactobacillus salivarius* ssp. *salivarius* CECT5713 administration ($5{\cdot}10^8$ CFU/mice·day) on the expression (RT–PCR) **a** and on the secretion **b** of the cytokines IL-2, IL-5 and IL-10 in Concanavalin (Con A)-activated splenocytes obtained from mice after LPS-induced septic shock. Splenocytes were incubated with Con A 5 (μg/mL) for 48 h and the concentrations (means ± SE) of the cytokines in the supernatant were analysed by ELISA ($n = 10$). $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus healthy group

To further evaluate the effects of the probiotic treatment on altered immunologic response after LPS administration, the expression (by RT–PCR) and the production (by ELISA) of different Th1 and Th2 cytokines (IL-2 and IL-5, respectively) as well as that of the anti-inflammatory cytokine IL-10 were analysed in Con A-stimulated splenocytes. The results revealed that cytokine expression was altered in Con A-stimulated splenocytes from septic mice (Fig. 4), which was confirmed when their production was analysed in the cell supernatants (Fig. 4); whereas, an increased expression of IL-2 and IL-5 was observed in control mice in comparison with healthy ones, a decreased expression of the anti-inflammatory cytokine IL-10 was detected. Finally, plasma IgG production was also increased as a result of the LPS-induced septic shock (12.9 ± 0.7 nmol/mL versus 18.8 ± 0.7; $P < 0.05$) and the pre-treatment with *L. salivarius* ssp. *salivarius* was able to completely prevent it (11.7 ± 1.3; $P < 0.05$ versus untreated LPS control group).

Discussion

In the last decade, there have been reported many studies showing the results obtained after probiotic treatment in both human IBD and experimental colitis, which seem to be promising. However, it is obvious that all probiotics are not equally beneficial, each may have individual mechanisms of action and host characteristics may determine which probiotic species and even strain may be optimal. In fact, several studies have shown that not all probiotics exert constant intestinal anti-inflammatory effects in experimental models of intestinal inflammation [17]. For this reason, it is very interesting to characterise the biological

effects of a given probiotic in order to achieve the more complete profile of the bacteria as possible. Among probiotics, two different strains of *L. salivarius* ssp. *salivarius*, UCC118 and CECT5713, have been proven to exert intestinal anti-inflammatory activity in the experimental models of colitis [5, 6], and these studies revealed that the probiotic shows immunomodulatory properties that can account to the reported beneficial effects. However, other mechanisms reported to play a key role in other probiotics need to be explored for these bacteria.

The concept of probiotic includes the term 'live microorganisms' in its definition. Although some studies have proposed that the viability of probiotics is not essential to exert its anti-inflammatory effect, their effects may be due to their immunostimulatory DNA [9]. However, this is not a constant feature in all probiotics since different in vitro studies have reported that viable probiotic is required to exert anti-inflammatory effect [18, 19]. In fact, when the intestinal anti-inflammatory effects of live and heat-inactivated *L. salivarius* ssp. *salivarius* CECT5713 were compared in the TNBS model of rat colitis, we observed that viable probiotic administration is essential for displaying the beneficial effects in this model of rat colitis. Thus, all the histological and biochemical properties (MPO activity, glutathione content, TNFα levels and iNOS expression) were clearly improved only when live probiotic was administered to colitic rats, showing a similar profile to that previously reported for this probiotic [6].

We have also analysed the effects that *L. salivarius* ssp. *salivarius* CECT5713 can induce on an in vitro model of intestinal epithelial regeneration. The results revealed that these lactobacilli are able to facilitate the recovery of a wound induced in a Caco-2 cell monolayer. This seems to be of importance in the beneficial effects ascribed to this probiotic, since an initial defect in epithelial integrity has been proposed to play a key role in the development and/or maintaining of the exacerbated immune response that occurs in IBD [20]. It has been proposed that the increased permeability allows the entry of bacteria, which secondly activate the intestine-resident immune cells, including macrophages, lymphocytes and epithelial cells, with the subsequent production and release of cytokines, like IL-1β, TNFα and IL-6 among others, which, in turn, affects the epithelial cells further reducing epithelial barrier function [21]. Thus, the ability of this *L. salivarius* ssp. *salivarius* CECT5713 to decrease cytokine production can account for its beneficial effect on mucosal integrity, as it has been demonstrated previously by reducing the release of the cytokines, TNF-α and IL-12, in LPS-stimulated macrophages [6, 22]. Other strain of this probiotic (UCC118) has been shown to be able to reduce IL-8 secretion in HT-29 epithelial cells after incubation with *Salmonella typhimurium* [23]. In the present study, it has been shown that *L. salivarius* ssp. *salivarius* CECT5713 was able to significantly reduce the induced IL-8 secretion in other intestinal epithelial cell line, Caco-2 cells, and with two different stimuli, IL-1β and enterotoxigenic *E. coli*.

Most of the studies performed with probiotics have been focused on their beneficial effects on the gastrointestinal tract. However, it is more and more evident that although these microorganisms may exert their beneficial effects due to the well-known ability to modulate intestinal microbiota and affect the intestine immune response, these effects are not restricted to the gastrointestinal tract. In fact, different probiotics have been reported to ameliorate the inflammation associated with rheumatoid arthritis [24, 25], or atopic disease [26, 27] or septic shock [28, 29]. Similarly, Sheil et al. [30] reported that systemic administration of *L. salivarius* UCC118 had an anti-inflammatory effect on colitis in IL-10 KO mice, demonstrating that the oral route is not mandatory for its effect, although the effect was of comparable, but not superior, magnitude to that previously reported using the oral route of administration. In the same study, the authors demonstrated that subcutaneous administration of this probiotic had a preventative effect in ameliorating the severity of collagen-induced arthritis in mice. However, it would be interesting to know whether oral administration of viable *L. salivarius* ssp. *salivarius* CECT5713 shows its beneficial effect in a situation of systemic altered immune, which would be an interesting effect given the high prevalence of extraintestinal diseases that occur in IBD patients [4].

For this purpose, we assayed this probiotic in an experimental model of sepsis induced in mice by i.p. administration of LPS, and the results revealed that the oral administration of the probiotic resulted in a significant preventative effect. TNFα has a critical role in the development of LPS-mediated shock in mice, and the excess of this cytokine promotes the major alterations observed in this situation, including vasodilatation, impaired coagulation and fibrinolysis [31]. The administration of the probiotic *L. salivarius* ssp. *salivarius* CECT5713 clearly improved the altered immune response and decreased the production of inflammatory mediators associated to endotoxic shock induced by LPS. Thus, the probiotic was able to downregulate the increased levels of TNFα both in plasma and lungs. The ability of this probiotic to reduce TNFα production in inflammatory conditions has been also demonstrated in the present study when assayed in the TNBS model of rat colitis. Similarly, to that previously reported for this probiotic in different experimental models of rodent colitis [5, 6], the beneficial effects showed by this strain of *L. salivarius* ssp. *salivarius* were associated with an improvement in the altered immune response induced by LPS. The onset of sepsis is characterised by

hyperactivation of the inflammatory cascade, in which T-cell activation and proliferation plays a key role. The activation of T cells results in the secretion of cytokines with inflammatory properties, including IL-2 (from type 1 helper T cell), IL-5 (from type 2 helper T cell) or cytokines with anti-inflammatory properties, like IL-10. Consequently, the altered immune response induced in mice after LPS i.p. administration was evidenced by an increased production of IL-2 in Con A-stimulated splenocytes, similarly to that previously described in this experimental model of septic shock [32]. The probiotic treatment reduced the production of this cytokine, suggesting that splenic T cells are part of the mechanism by which it exerts the beneficial effect, in accordance with previous observations reported for different probiotics in the experimental TNBS model of rat colitis [33], which represents an experimental model of T cell-mediated intestinal inflammation [10]. Correspondingly, the expression of the Th2 cytokine IL-5 was upregulated in Con A-stimulated splenocytes from control septic mice, whereas probiotic treatment downregulated its expression. When the production of IL-10 was evaluated in mice splenocytes stimulated with Con A, a decrease was observed in control mice when compared to the healthy group, thus reflecting the failure of this anti-inflammatory cytokine to control systemic inflammatory situation following the upregulated release of pro-inflammatory cytokines. The pre-treatment of septic mice with this probiotic resulted in a significant increased production of IL-10, thus contributing to the downregulation of pro-inflammatory cytokines, including TNF-α, observed after probiotic treatment to septic mice. In addition to T-cell activation, B cells, through the production of immunoglobulins, have been also involved in septic shock [34]. Thus, the LPS- induced septic shock in mice is associated with a significant increase in IgG antibodies, reflecting the presence of a second defence line that attempts to eliminate harmful antigens. The pre-treatment with *L. salivarius* ssp. *salivarius* resulted in a decreased production of plasmatic, thus confirming the immunomodulatory effects observed with this probiotic.

In conclusion, viable *L. salivarius* ssp. *salivarius* CECT5713 facilitates intestinal epithelial regeneration and possesses immunomodulatory properties that protect against TNBS-induced colitis in rats and LPS-induced organ damage in mice. Therefore, the anti-inflammatory effects of this probiotic are not restricted to the gastrointestinal tract.

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