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ORIGINAL ARTICLE

Probiotic activity of *Enterococcus faecalis* CECT7121: effects on mucosal immunity and intestinal epithelial cells

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Abstract

Aims: To analyse the effect of *Enterococcus faecalis* CECT7121 on intestinal epithelial cells (IECs) and its effects on the mucosal immune response.
Methods and Results: *Enterococcus faecalis* CECT7121 showed a high adhesion capacity to completely and heterogeneously differentiated human intestinal epithelial cell line (Caco-2 cells). In addition, the contact of this bacterium with Caco-2 cells did not induce inflammatory chemokines (IL-8 and CCL-20). The presence of IgA^+ and $IL\text{-}6^+$ cells in the small intestine, as well as the production of inflammatory cytokines (TNFα, IL-6 and IL-12) in the gut, was determined after intragastric inoculation of *Ent. faecalis* CECT7121 in BALB/c mice. The administration of *Ent. faecalis* CECT7121 increased the number of IgA^+ cells in the intestinal lamina propria without modifying the percentage of $IL\text{-}6^+$ cells. No differences were observed in the cytokines measured in the intestinal extracts between probiotic-treated and control mice.
Conclusions: *Enterococcus faecalis* CECT7121 stimulates local mucosal immunity and adheres to IECs without inducing inflammatory signals.
Significance and Impact of the Study: Our results indicate that, apart from its already reported systemic immune activity, *Ent. faecalis* CECT7121 has a modulatory effect at a local level.

Introduction

Many studies have been performed to assess the usefulness of probiotics in different physiological or pathological conditions. Probiotics have been employed to control infections and intestinal inflammatory diseases, to avoid and treat allergy processes, to prevent several types of cancers and to stimulate the immune system (Chiang and Pan 2012; Hardy *et al.* 2013; Sarowska *et al.* 2013). Probiotics are live micro-organisms that, upon their administration, are able to influence the composition and activity of the intestinal microbiota, to improve the nonspecific intestinal barrier and to reinforce or modulate the mucosal and systemic immune responses (Maldonado Galdeano *et al.* 2007).

The mechanisms by which probiotic micro-organisms exert these beneficial effects are varied and, generally, they are strain-dependent. Each probiotic species, and within each species, each strain, may have different activities. Probiotics may be able to either inhibit or kill pathogenic bacteria by producing short-chain fatty acids, hydrogen peroxide, nitric oxide or bacteriocins (Sparo *et al.* 2006; Nishitani *et al.* 2009). They can also compete for adhesion sites in the gastrointestinal tract (Oelschlaeger 2010).

The mucosal defence against bacteria is essential to maintain the host's homoeostasis. In the gut, the main colonized mucosa, the protection against enteropathogens partially depends on the commensal flora and probiotic strains have a fundamental role in such protection (Adams 2010). Probiotic micro-organisms have direct effects on the immune system. The immunological properties of probiotic micro-organisms have been studied and numerous works show that various strains of lactic acid bacteria are

able to enhance both systemic and mucosal immunity (Shida *et al.* 2011; Chiang and Pan 2012). Furthermore, it has been reported that while some probiotic strains stimulate the innate immune response, others act on the adaptive arm (Maldonado Galdeano and Perdigón 2006).

The mucosal-associated lymphoid tissue (MALT), and more specifically the gastrointestinal tract, is continuously exposed to different stimuli from the external environment (Giorgetti *et al.* 2015). These stimuli can either be dietary antigens as well as commensal or pathogenic bacteria (Turner 2009). Therefore, the intestinal mucosa is a crucial site for the induction and regulation of the immune response (Gross *et al.* 2015).

The interactions between the MALT and the external environment occur mainly at the epithelial lining of the mucosa. Intestinal epithelial cells (IECs) are the first defence line against pathogenic bacteria and it is known that they play a crucial role in the cross-talk between commensal flora and probiotic micro-organisms (Thomas and Versalovic 2010). Probiotics may exert their effects on IECs by several mechanisms: by enhancing their capacity as a physical barrier, by increasing the mucin production, by inducing the production of antimicrobial substances, or by modulating signalling pathways (Paolillo *et al.* 2009; Lebeer *et al.* 2010; Thomas and Versalovic 2010).

In previous works, we have demonstrated that *Enterococcus faecalis* CECT7121, a nonpathogenic environmental bacterium, implants and remains in the intestinal mucosa of BALB/c mice for at least 18 days (Castro *et al.* 2007). We have also demonstrated the adjuvant effect of *Ent. faecalis* CECT7121 and its broad pro-Th1 immunomodulatory activity, conferring beneficial effects on different biological models: *Salmonella* infection, specific immune response induced by vaccination, challenge with lymphoma LBC cells and ovalbumin allergic response (Castro *et al.* 2007, 2008, 2010, 2012). However, the effect of this bacterium at the local level remains to be studied. Thus, in this work we analysed the effect of *Ent. faecalis* CECT7121 on IECs as well as on the mucosal immune response. *In vitro* studies were carried out employing Caco-2 cells (a human intestinal epithelial cell line) to study the adhesion of *Ent. faecalis* CECT7121 and its effect on the expression of inflammatory molecules. Additionally, the percentage of IgA^+ and IL-6^+ cells in murine intestine and cytokine levels in gut extracts, after the intragastric administration of *Ent. faecalis* CECT7121, were measured.

Materials and methods

Epithelial cell line cultures

Human colonic epithelial cell line (Caco-2) and Caco-2 cells stably transfected with a luciferase reporter construction under the control of the CCL20 promoter (Caco-2 ccl20:luc) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 2 mmol l^{-1} L-glutamine (MP Biomedicals Inc., Solon, OH, USA), 100 U ml^{-1} penicillin (Gibco), 100 μg ml^{-1} streptomycin (Gibco), 25 mmol l^{-1} HEPES (Gibco), 1% nonessential amino acids (Gibco) and 10% fetal calf serum (Natocor, Argentina). Epithelial cell lines were seeded at $1{\cdot}0 \times 10^5$ cells per well in 96- or 24-well plates and cultured in a 5% CO_2 atmosphere at 37°C. For viability assays, undifferentiated Caco-2 cells were used. For adherence assays, Caco-2 cells were used at 7 or 14 days after reaching confluence, whereas for the assessment of IL-8 and CCL20 secretion, cells were used at 14 days post confluence. Caco-2 ccl20:luc were used when they reached confluence.

Enterococcus faecalis CECT7121 suspensions

Enterococcus faecalis CECT7121 was grown in Triptone Soy Broth (Biokar Diagnostics, France) at 35°C for 18 h. After incubation, the culture was harvested by centrifugation at 5800 ***g*** for 15 min (4°C) and washed three times with sterile PBS. *Enterococcus faecalis* CECT7121 suspensions were prepared using standard growth curves which were constructed by plotting the OD_{620} values *vs* agar plate counts of freshly prepared, serially diluted cultures. To obtain heat-killed *Ent. faecalis* CECT7121, the bacterial suspension was heated at 80°C for 1 h. *Enterococcus faecalis* CECT7121 soluble lysate was obtained according to Molina *et al.* (2015) and was employed in specific antibody determination. Biochemical tests were routinely performed for identification, and contamination/inactivation was controlled employing sheep blood and chromogenic agar plates (Laboratorios Britania, Bs.As., Argentina).

Viability assays

Twenty four hours post seeding, cultivated Caco-2 cells (0·1 ml per well) were coincubated with the same volume of *Ent. faecalis* CECT7121 in a wide multiplicities of infection (MOI) of 0·1–10 000 bacteria per cell. Untreated cells were used as controls. After 24 h at 37°C and 5% CO_2, supernatants were removed, fresh medium and 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg ml^{-1} in PBS; Sigma) were added, followed by incubation for 4 h at 37°C in a humid, 5% CO_2 atmosphere. After this period, the supernatants were removed again, and the insoluble formazan crystals were dissolved in 100 μl of DMSO. The absorbance was read in an ELISA plate reader (Multiskan EX; Thermo Scientific, FL, USA) at 570 nm.

Adhesion assays

An *Ent. faecalis* CECT7121 suspension was washed three times with PBS and then suspended in free-antibiotic DMEM. Before adding the bacterial strain, Caco-2 cells (7 or 14 days in confluence) were washed three times with nonsupplemented medium and maintained overnight in free antibiotic DMEM.

Caco-2 cells were coincubated with *Ent. faecalis* CECT7121 at MOIs of 20, 200, 500 and 1000 bacteria per cell. After 2 h at 37°C and 5% CO_2, cells were washed with nonsupplemented DMEM to eliminate nonadherent bacteria and lysed with 1 ml per well of 0·1% Triton X100 (Sigma). Serial dilutions of the lysates were plated in tryptone soy agar to enumerate CFU. For quantification of intracellular bacteria, cells coincubated with *Ent. faecalis* CECT7121 were washed and incubated for another 2 h in the presence of penicillin (100 U ml^{-1}; Gibco) and streptomycin (100 μg ml^{-1}; Gibco). After that, cells were lysed and CFU quantified.

Bacterial adhesion was calculated as the percentage of adherent bacteria in relation to the total number of bacteria added. Experiments were performed in duplicates and repeated three times.

Stimulation assay with *Enterococcus faecalis* CECT7121

After 14 days postconfluence, Caco-2 cells were put in contact with different concentrations of *Ent. faecalis* CECT7121 (MOIs: 1, 10, 100 and 500) or *Escherichia coli* ATCC 25922 (*E. coli*, MOI 100) for 3 h (37°C, 5% CO_2). Next, Caco-2 cells were washed out with nonsupplemented DMEM and maintained in culture for another 21 h. A preincubation assay was also performed: Caco-2 cells were preincubated with different MOIs of *Ent. faecalis* CECT7121 for 2 h and then, *E. coli* stimulus (MOI 100) was added at each well. After 2 h of additional incubation, cells were washed, fresh DMEM was added and the incubation was continued for another 20 h. When incubations were concluded, supernatants were removed and stored at –80°C. Experiments were performed in duplicates and repeated three times.

For the analysis of ccl20 promoter induction, confluent Caco-2 ccl20:luc were cocultured for 1 h with a suspension of *Ent. faecalis* CECT7121 (OD_{620} = 1·00 and 0·10). OD_{620} = 1·00 value corresponds to approx. 1×10^9 CFU ml^{-1}. Then, cells were stimulated for 6 h with flagellin from *Salmonella enterica* serovar Enteritidis (1 μg ml^{-1}). After incubation, samples were homogenized and luciferase activity was measured in a Labsystems Luminoskan TL Plus luminometer (Promega, Madison, WI) as described Romanin *et al.* (2010). Luminescence was expressed as a percentage of the mean of stimulated control. Experiments were performed in duplicates and repeated twice.

Animals

Female BALB/c mice were obtained from the Animal Facility at the *Facultad de Ciencias Veterinarias* (Universidad de Buenos Aires, Argentina) and kept in the Animal Facility of our Institute (conventional facility). The animals employed in this study were 6- to 8- weeks old and weighed approx. 20–25 g. All animal experiments were approved by the *Instituto de Estudios de la Inmunidad Humoral 'Prof. Dr. Ricardo A. Margni'* (IDEHU, CONICET-UBA). Mice were housed (n = 5 mice/cage) under specific conditions according to the 'Guide for the Care and Use of Laboratory Animals' (2011), with a 12-h light/dark cycle at controlled air temperature (20–22°C). Animals were allowed to have food and water *ad libitum*. Mice were sacrificed by cervical dislocation. Every effort was made to minimize animal suffering.

Enterococcus faecalis CECT7121 intragastric administration

Bacterial suspensions were prepared in sterile PBS at approx. 3×10^8 CFU ml^{-1} to be used in *in vivo* studies. BALB/c mice (n = 5 per group distributed at random) were intragastrically inoculated with 0·2 ml per mouse with either sterile PBS or live *Ent. faecalis* CECT7121 during three consecutive days, as previously described (Castro *et al.* 2007).

Extraction of gut samples: histological and Immunohistochemical studies

After 48 h from the last inoculation of *Ent. faecalis* CECT7121, mice were sacrificed and the small and large intestine of each animal were removed. Small intestines were used for histological and immunohistochemical studies while large intestines were employed to obtain colon extracts for determination of cytokine levels. After removing, small and large intestines were washed several times with PBS to remove faecal remnants.

Small intestines were cut open longitudinally at the mesenteric attachment and subsequently the so called 'Swiss rolls' were made (Herías *et al.* 2005). After fixation for 2 days in 96% ethanol, samples were processed for paraffin embedding. Five micrometer thick sections were cut and stained with H&E. For immunohistochemical studies, standard protocols were employed with minor modifications (Hoffman 2002). Briefly, after deparaffination with an immersion in xylene and rehydration in a graded ethanol series, endogenous peroxidase activity was eliminated by incubation with 3% v/v H_2O_2 for 30 min. Fc receptors were blocked by the addition of a human immunoglobulin solution previously prepared in our laboratory. Detection of IgA^+ and IL-6^+ cells was carried out

by incubating overnight at 4°C with a horseradish peroxidase (HRP)-conjugated goat IgG anti-mouse IgA serum (Bethyl, TX, USA) and a biotinylated anti-mouse IL-6 monoclonal antibody (BD Biosciences, CA, USA) plus streptavidin-HRP (BD, CA, USA) respectively. Slides were washed three times with PBS, incubated for 10–15 min at room temperature with diaminobenzidine (1 mg ml^{-1}; Sigma) in TRIS buffer (1 mol l^{-1}, pH: 7·20) plus H_2O_2 in the dark, and then, counterstained with haematoxylin. Positive cells were analysed by the point-counting method described by Ichihara *et al.* (1993). Briefly, photographs of each slide were taken. Each photograph was then placed on a grid made up of 52 horizontal lines and 38 vertical lines for a total of 1976 intersections. The number of intersections with total cells (TC) and with IgA^+ cells or IL-6^+ cells (IgAC, IL-6C) was counted and the percentages of IgA^+ and IL-6^+ cells were calculated as IgAC/TC × 100, or IL-6C/TC × 100, respectively. At least, 10 photographs of each animal (control or *Ent. faecalis* CECT7121) were counted and the results expressed as mean of percentages.

Intestinal extracts: levels of *Enterococcus faecalis* CECT71721 specific antibodies

Small and large intestines were cut in small pieces and placed in contact with an extraction solution (4·68 g NaCl; 74·4 mg EDTA; 3·4 mg phenylmethanesulfonyl fluoride (Sigma); 1 g serum bovine albumin (Sigma); 100 μl Tween 20 (Biopack, Argentina); and protease inhibitor cocktail (Roche, Germany) in 200 ml deionized water). After three freeze-thawing cycles, mixtures were incubated in agitation at 4°C for 18 h. Intestinal extracts were obtained after centrifugation at 12 000 ***g*** and supernatants were stored at −80°C until analysed.

Specific IgA antibody levels were measured by indirect ELISA in pure individual intestinal extract samples. Briefly, *Ent. faecalis* CECT7121 soluble lysate was employed as coating antigen at 10 μg protein ml^{-1} in phosphate-buffered saline (PBS). Antibodies were detected using an HRP-conjugated anti-mouse IgA serum (Bethyl Laboratories Inc). All determinations were done in duplicates. Absorbance values were obtained after spectrophotometric reading at dual 450–570 nm wavelengths in an ELISA plate reader. Control values were those obtained from intestinal extract of untreated mice.

Determination of cytokine levels by ELISA

Commercial kits were employed to determine levels of murine IL-6, IL-10, IL-12p40 and TNFα (BD Biosciences), as well as human IL-8 (BD) and CCL20 (R&D Systems, Minneapolis, MN, USA). Cytokine concentrations were tested individually and mean values were expressed in pg ml^{-1} derived from standard curves with the corresponding recombinant cytokine standards. Results are presented as concentration means ± SEM.

Statistical analysis

To analyse cytokine levels, a linear regression of ELISA absorbance values of standards was performed. Differences between data were analysed employing One-way ANOVA. Comparisons between groups were done with Student–Newman–Keuls' multiple comparison test, and those against a control group were performed with Dunnett's test. When two data groups were compared, the Student's *t* test was carried out. All analyses were performed using GraphPad Prism 5.00 for Windows (GraphPad Software, CA, USA). Values were considered significantly different at $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, or $^{***}P < 0{\cdot}001$. Tendency was defined in this work as a clear proximity to the statistical significance.

Results

Effect of *Enterococcus faecalis* CECT71721 on the viability of intestinal epithelial cells

To study if *Ent. faecalis* CECT7121 affects the viability of human IECs, the tetrazolium dye assay was carried out. The MTT assay is a colorimetric test for assessing cell metabolic activity in which viable cells with an active metabolism are able to convert MTT into the purple-coloured product, formazan. In contrast, when cells die they lose the ability to convert MTT into formazan. For the Caco-2 cell line, the levels of formazan in cells coincubated with heat-killed *Ent. faecalis* CECT7121 were comparable to those of untreated cells (Fig. 1) demonstrating that *Ent. faecalis* CECT7121 suspensions did not have an effect on the viability of Caco-2 cells.

Adherence of *Enterococcus faecalis* CECT7121 to human epithelial cells

The human intestinal Caco-2 cell line has been broadly employed to study the adhesion of probiotics to epithelial cells. In order to analyse the ability of *Ent. faecalis* CECT7121 to adhere to human epithelial cells, completely (more of 14 days in culture) or heterogeneously (7 days in culture) differentiated Caco-2 cells were incubated with different MOIs of the bacterium.

Enterococcus faecalis CECT7121 showed a strong adherence capacity to the epithelial cells in both, completely and heterogeneously differentiated cells (Fig. 2a,b). In the heterogeneously differentiated cells, the adherence of

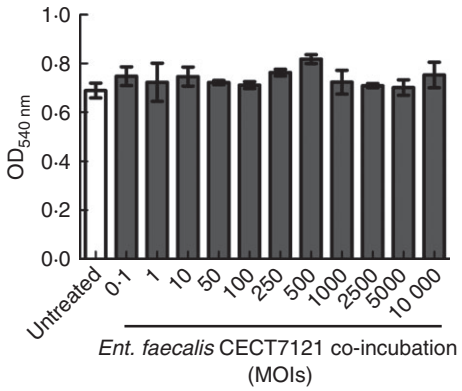

Figure 1 Effect of *Enterococcus faecalis* CECT71721 on the viability of intestinal epithelial cells. Twenty-four hours post seeding, cultivated Caco-2 cells were coincubated with *Ent. faecalis* CECT7121 in a wide multiplicities of infection (MOI: 0·1 to 10 000 bacteria per cell). Non-stimulated cells (untreated) were used as controls. After 24 h at 37°C and 5% CO_2, supernatants were removed, and the metabolic activity was measured by tetrazolium dye assay. Bars indicate mean of OD values ± SEM. Experiments were performed with duplicate determinations, and repeated three times.

Ent. faecalis CECT7121 was dose-dependent although this tendency was not statistically significant (Fig. 2b).

Then, the results of adherence obtained with completely or heterogeneously differentiated Caco-2 cells were compared. No differences were found in the adhesion of the same MOI of *Ent. faecalis* CECT7121 between the cells with a different differentiation grade. However, at MOIs 200 a clear tendency towards a higher adherence was observed in the heterogeneously differentiated cells (Fig. 2c, $P = 0{\cdot}0772$).

After treatment with antibiotics, no live *Ent. faecalis* CECT7121 were detected in Caco-2 cells (data not shown). This result indicates that *Ent. faecalis* CECT7121 does not internalize in the epithelial cells.

ccl20 promoter induction, IL-8 and CCL20 secretion by Caco-2 cells after exposure to *Enterococcus faecalis* CECT7121

IECs secrete a variety of cytokines which control the extent and length of the immune response, particularly during contact with enteropathogens. To investigate the ability of *Ent. faecalis* CECT7121 to modulate inflammatory reaction in human intestinal cell lines we analysed ccl20 promoter induction as well as IL-8 and CCL20 secretion by Caco-2 cells after exposure to this strain. Interleukin-8 and CCL20 are released by epithelial cells under inflammatory stimuli. IL-8 is a neutrophil chemoattractant, whereas CCL20 selectively attracts effector and memory T cells, immature dendritic cells and naïve B cells.

The ccl20-promoter in Caco-2 cells is known to be highly inducible upon stimulation with pro-inflammatory molecules such as TLR agonists. Flagellin, a potent TLR-5 inductor, induced the increase of the basal luciferase activity (Fig. 3, $P < 0{\cdot}001$). Preincubation of Caco-2 ccl20:luc reporter cells with viable *Ent. faecalis* CECT7121 produced a strong inhibition of luciferase activity induced by flagellin ($P < 0{\cdot}001$). The inhibitory effect was only observed when the viable bacterium was preincubated, since the addition of heat-killed *Ent. faecalis* CECT7121 did not cause any change (data not shown).

In our study, nonstimulated Caco-2 cells secreted CCL-20 and IL-8 proteins at a detectable level. When CCL20 was measured in the culture supernatants, coincubation with *Ent. faecalis* CECT7121 produced a mild decrease in the CCL20 levels compared to the levels measured in the supernatants of the cells incubated in medium alone, although it did not reach statistical significance (Fig. 4a). Preincubation of Caco-2 cells with *Ent. faecalis* CECT7121 induced CCL20 levels, that numerically, were lower than the levels induced by *E. coli*

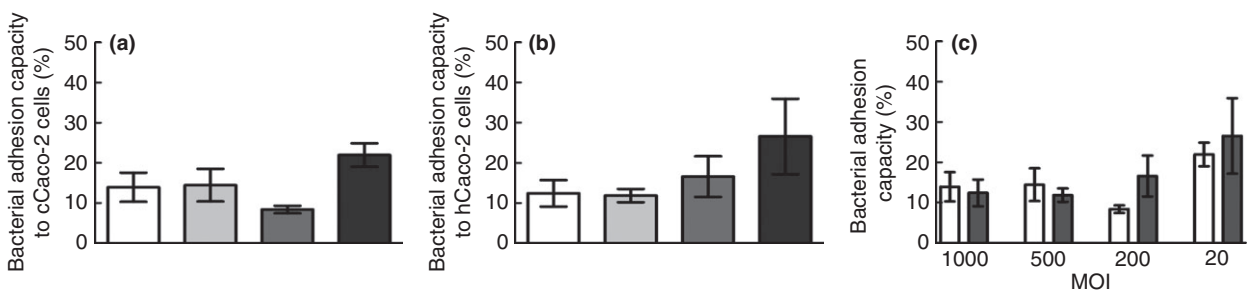

Figure 2 Adhesion of *Enterococcus faecalis* CECT7121 to Caco-2 cells. Completely (a) and heterogeneously (b) differentiated Caco-2 cells (cCaco-2 and hCaco-2, respectively) were coincubated for 2 h with *Ent. faecalis* CECT7121 at multiplicities of infection (MOI) of 1000, 500, 200 and 20 bacteria per cell. Adhesion capacity was calculated as the percentage of adhering bacteria in relation to the total number of bacteria added. Experiments were performed in duplicate determinations and repeated three times. In the panel c, a comparison of adhesion percentage of *Ent. faecalis* CECT7121 to cells with different grade of differentiation was performed. Results are expressed as mean ± SEM. (a, b) (□) MOI 1000; (▢) MOI 500; (▩) MOI 200 and (■) MOI 20. (c) (□) cCaco-2 cells (14 days post-confluence) and (■) hCaco-2 cells (7 days post-confluence).

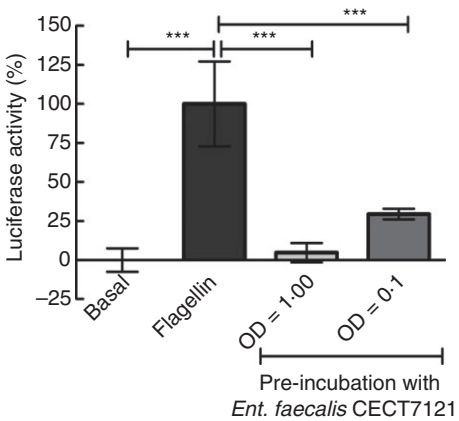

Figure 3 Effects of *Enterococcus faecalis* CECT7121 on ccl20 promoter induction. Confluent Caco-2 ccl20:luc were cocultured for 1 h with a suspension (OD_{620} = 1·00 and 0·10) of *Ent. faecalis* CECT7121 (OD = 1·00 corresponds to approx. 1×10^9 CFU ml^{-1}). Then, cells were stimulated with flagellin from *Salmonella enterica* serovar Enteritidis (1 μg ml^{-1}) for 6 h. After incubation, samples were homogenized and luciferase activity was measured in a luminometer. Luminescence was expressed as percentage of the mean of stimulated control. Bars indicate mean ± SEM. Experiments were performed with duplicate determinations, and repeated twice. ***$P < 0{\cdot}001$, One-way ANOVA followed by Dunnett's post-test (flagellin as control column).

stimulus but, this reduction did not achieve statistical significance (Fig. 4b).

We observed that the coincubation of viable *Ent. faecalis* CECT7121 with differentiated Caco-2 cells for 3 h did not induce the secretion of IL-8 above baseline levels. Only an increase in the release of IL-8 was observed when MOI 100 was employed (Fig. 5, $P < 0{\cdot}05$). *Escherichia coli* (MOI 100) employed as control induced a high IL-8 secretion ($P < 0{\cdot}01$).

Effects of *Enterococcus faecalis* CECT7121 on mucosal immune system

Since the gut mucosa plays an essential role as a physical barrier preventing the entry of pathogens and toxic substances to systemic tissues, any potential damage to the integrity of the mucosa caused by the administration of probiotic micro-organisms must be ruled out. BALB/c mice were intragastrically inoculated with *Ent. faecalis* CECT7121 and after 48 h they were sacrificed. Paraffin-embedded sections of small intestine were then prepared to assess the number of IgA^+ and IL-6^+ cells by immunohistochemistry.

The inoculation of *Ent. faecalis* CECT7121 did not modify the morphology of the intestinal mucosa as compared to nontreated mice. Intestinal villi were intact, well-conserved and no inflammatory infiltrate was observed (Fig. 6a,b).

To investigate whether *Ent. faecalis* CECT7121 is able to stimulate the mucosal immune system, the presence of IgA^+ and IL-6^+ cells in the small intestine were analysed. *Enterococcus faecalis* CECT7121-treated and control mice showed specific staining for IgA in the lamina propria of villi (Fig. 6c,d). The percentage of IgA^+ cells in the lamina propria was higher in bacterium-treated mice than in control ones (Fig. 6e, $P < 0{\cdot}001$). Regarding the mucosal expression of IL-6, the amount of IL-6^+ cells was similar between *Ent. faecalis* CECT7121-treated mice and control animals (Fig. 6f).

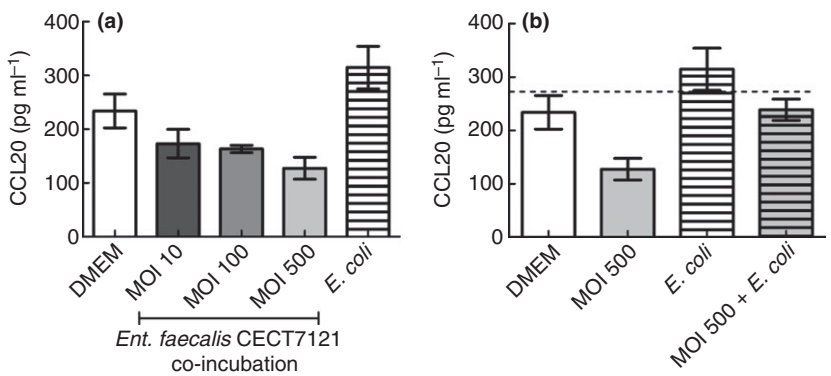

Figure 4 Effects of *Enterococcus faecalis* CECT7121 on CCL20 secretion. (a) Caco-2 cells (14 days in culture post confluence) were put in contact with different concentrations of *Ent. faecalis* CECT7121 (MOIs 10, 100 and 500) for 3 h (37°C, 5% CO_2). After that, Caco-2 cells were washed with non supplemented Dulbecco's Modified Eagle's Medium (DMEM) and maintained in culture for another 21 h. CCL20 levels were measured by ELISA in supernatants. (b) Caco-2 cells were preincubated with *Ent. faecalis* CECT7121 (MOI 500) for 2 h and then, *Escherichia coli* stimulus was added. After 2 h of additional incubation, cells were washed, fresh DMEM was added and the incubation was continued for other 20 h. CCL20 levels were measured by ELISA in supernatants. Dotted line indicates mean + SEM of cells incubated only with culture medium (DMEM).

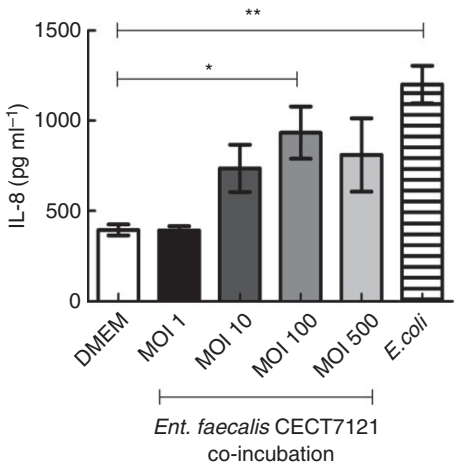

Figure 5 Effects of *Enterococcus faecalis* CECT7121 on IL-8 secretion. Caco-2 cells (14 days in culture post confluence) were put in contact with different concentrations of *Ent. faecalis* CECT7121 (MOIs 1, 10, 100 and 500) for 3 h (37°C, 5% CO_2). After that, Caco-2 cells were washed with nonsupplemented Dulbecco's Modified Eagle's Medium (DMEM) and maintained in culture for other 21 h. IL-8 levels were measured by ELISA in supernatants. Experiments were performed with duplicate determinations, and repeated three times. Bars indicate mean ± SEM. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$, One-way ANOVA followed by Dunnett's post-test (DMEM as control column).

To determine whether a specific immune response was elicited after *Ent. faecalis* CECT7121 inoculation, antibodies to *Ent. faecalis* CECT7121 were measured in the intestinal extracts. No differences were observed in the OD_{450} values between control and *Ent. faecalis* CECT7121 treated mice (Control: 0·206 ± 0·080 *vs* *Ent. faecalis* CECT7121: 0·165 ± 0·032).

When levels of inflammatory cytokines (IL-6, TNFα and IL-12) were measured in the intestinal extracts (small and large intestine), no significant differences were found between samples of probiotic-treated and control animals (Table 1).

Discussion

Enterococcus faecalis CECT7121 is a probiotic strain that, after intragastric administration, it implants and persists in the gastrointestinal tract of BALB/c mice (Castro *et al.* 2007). *Enterococcus faecalis* CECT7121 produces a bacteriocin with a broad inhibitory spectrum on Gram-positive bacteria (but it did not display any inhibitory activity on lactic acid bacteria) and some strains of Gram-negative bacteria (Sparo *et al.* 2008). Many strains of the genus *Enterococcus* are emerging pathogens while others may be considered safe (Foulquie′ Moreno *et al.* 2006), thus, the absence of virulence factors is an essential condition for the utilization of *Enterococcus* strains as probiotics. *Ent. faecalis* CECT7121 does not have β-lactamase activity and shows susceptibility to ampicillin, tetracycline, chloramphenicol, vancomycin and teicoplamin. Moreover, resistance to high levels of the aminoglycoside antibiotics, gentamicin and streptomycin, could also be ruled out (Sparo *et al.* 2008). Previously, we have demonstrated that *Ent. faecalis* CECT7121 does not express virulence factors, does not produce biogenic amines as undesirable compounds and is devoid of a capsule. In addition, the innocuousness of *Ent. faecalis* CECT7121 was confirmed previously by an *in vivo* pathogenicity assay in BALB/c mice. We demonstrated that the LD50 obtained for *Ent. faecalis* CECT7121 was comparable to the LD50 obtained with other environmental strains of *Ent. faecalis* (Sparo *et al.* 2008). In the last years, we have demonstrated that *Ent. faecalis* CECT7121 promote beneficial effects on different experimental models (Castro *et al.* 2007, 2008, 2010, 2012). In this work, we study the effect of this bacterium at the local level.

Probiotics benefit the host by interacting with a variety of cell types. The interaction of probiotics with epithelial cells might trigger a signalling cascade leading to immune modulation (Lin *et al.* 2009). To exert their effects in the intestine, probiotics should be able to survive the passage through the gastrointestinal tract. Moreover, one of the most important requisites of probiotic bacteria is that they must be able to adhere to the mucosal surface of the IECs (Nueno-Palop and Narbad 2011). Caco-2 and HT-29 cell lines, both derived from a human colon adenocarcinoma, are commonly used models representing features of the human intestinal epithelium although they show some differences (Liévin-Le Moal and Servin 2013). Caco-2 cells can differentiate spontaneously *in vitro* under standard culture conditions thereby exhibiting enterocyte-like structural and functional characteristic (Lenaerts *et al.* 2007). In contrast, HT-29 cannot differentiate spontaneously *in vitro* under standard conditions, representing undifferentiated colonic epithelial cells, but several mucus-secreting HT-29 cell clones have been established (Lenaerts *et al.* 2007; Liévin-Le Moal and Servin 2013). These human epithelial cell lines have been widely used to assess the adhesive properties of probiotic strains (Candela *et al.* 2008; Mathias *et al.* 2010; Sun *et al.* 2012). In a recent study, other authors have demonstrated that the adhesion capacity of probiotics to epithelial cell lines varies from species to species (Jensen *et al.* 2012). These authors have demonstrated that several strains of *Lactobacillus* and *Pediococcus* can attach to the epithelial cells and observed a variation in adhesion percentages that ranged from 1 to 25% depending on the bacterium employed. In the present work, we observed that *Ent. faecalis* CECT7121 shows *in vitro* a high binding

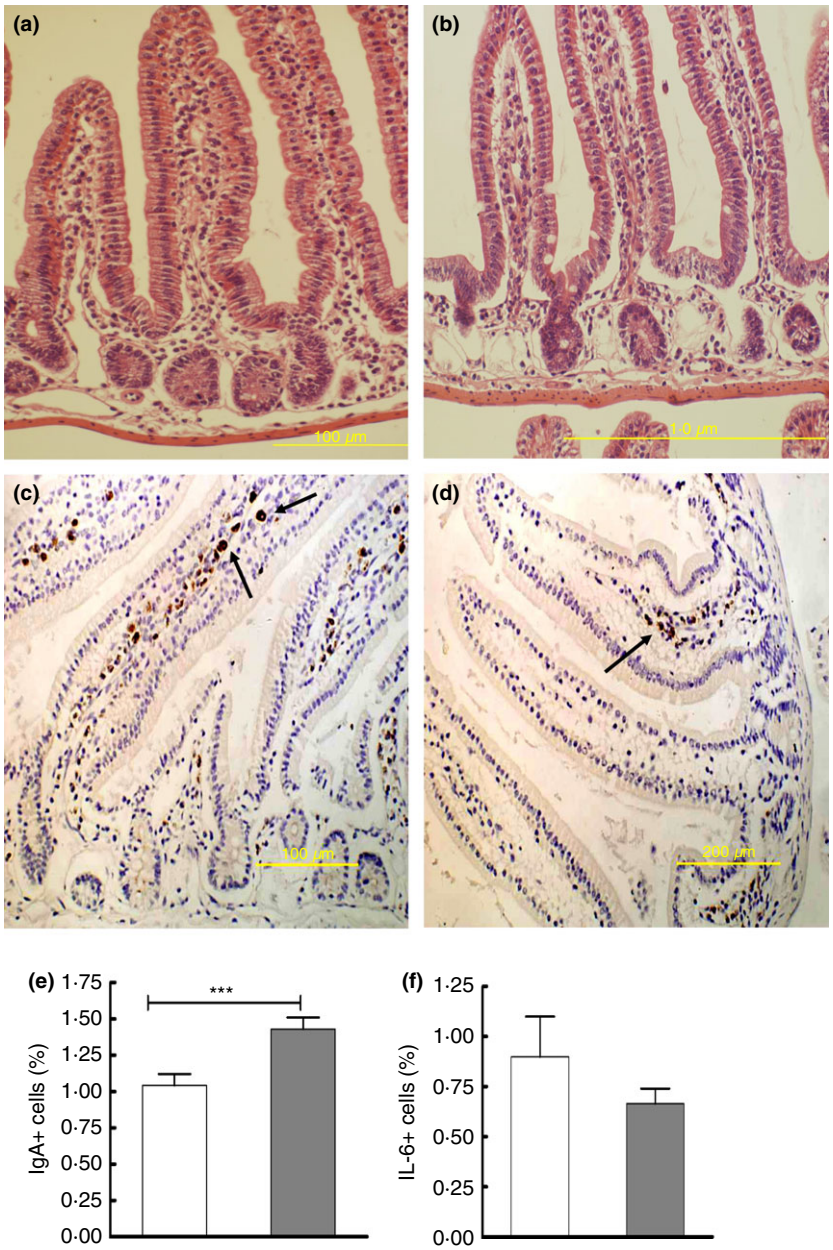

Figure 6 Effects of *Enterococcus faecalis* CECT7121 on murine small intestine. BALB/c mice (n = 5 per group) were intragastrically inoculated with 0·2 ml of either sterile PBS (Control mice) or live *Ent. faecalis* CECT7121 (*Ent. faecalis* CECT71721-treated mice) during three consecutive days. After 48 h from the last inoculation, mice were sacrificed and the small intestine of each animal was removed. Histological slides from *Ent. faecalis* CECT7121-treated (a) or Control (b) mice were stained with H&E. Immunohistochemical staining for IgA on slides from *Ent. faecalis* CECT7121-treated (c) or Control (d) was performed. Arrows show positive staining to IgA. Percent of IgA^+ cells and $IL\text{-}6^+$ cells in the lamina propria of the small intestine of mice that received PBS or *Ent. faecalis* CECT7121 are shown in panel e and f respectively. Bars indicate mean ± SEM. ***$P < 0{\cdot}001$, Student´s t test. (□) Control mice and (■) *Ent. faecalis* CECT7121-treated mice.

capacity to Caco-2 human cells, with average adherence percentages ranging from 9 to 27%, values similar to those reported by Jensen *et al.* (2012). It is known that results of *in vitro* adhesion assays are difficult to extrapolate to the situation in human gastrointestinal tract where the host's defence system, the peristaltic flow and the mucosal shedding, among other physiological phenomena, may affect the bacterial adhesion process. Although the high capacity of *Ent. faecalis* CECT7121 to implant and persist after its *in vivo* inoculation in mice (Castro *et al.* 2007) and its adhesion to Caco-2 cells described in this work could make us to speculate that this probiotic strain could show persistence after its administration into humans, this aspect deserves further investigation. Mucin-secreting HT-29 cells, providing an environment closer to human gut, could be a valuable tool to deeper investigate *in vitro* the adhesion properties of *Ent. faecalis* CECT7121.

Epithelial Caco-2 cells present different characteristics and properties according to their grade of differentiation and, although they are a colonic tumour-derived cell line, it differentiates postconfluence into a polarized monolayer with properties of foetal ilium epithelial cells (Engle *et al.* 1998). When Caco-2 cells are not differentiated they

Table 1 Effects of *Enterococcus faecalis* CECT7121 on murine small and large intestine

	Small intestine			Large intestine		
	TNFα	IL-6	IL-12	TNFα	IL-6	IL-12
Control	101·4 ± 6·5	66·7 ± 5·8	165·5 ± 21·3	125·2 ± 15·5	1658 ± 638	429·9 ± 34·1
Ent. faecalis CECT7121	119·7 ± 17·0	70·5 ± 5·5	161·6 ± 9·7	103·5 ± 20·9	1356 ± 306	389·0 ± 42·9

BALB/c mice (n = 5–10 per group) were intragastrically inoculated with 0·2 ml per mouse of either sterile PBS (Control) or live *Ent. faecalis* CECT7121 (*Ent. faecalis* CECT71721) during three consecutive days. After 48 h from the last inoculation, mice were sacrificed and the small and large intestines of each animal were removed. Levels of TNFα, IL-6, and IL-12 were measured by ELISA in gut extracts and expressed as pg ml^{-1} mean ± SEM of animals belonging to the same experimental group.

show a colonic-type cell but, after 6 days in culture, these cells start to develop an enterocyte morphology characterized by the formation of a brush border containing numerous microvilli, the presence of occluding junctions at the apical surface and a tall and more compact cell shape (Engle *et al.* 1998; Ganan *et al.* 2010). A fully differentiated monolayer is obtained after 14 days of culture, exhibiting the characteristics of mature enterocytes with a dome-like appearance. Taking these properties into consideration, we also decided to explore the adhesion capacity of *Ent. faecalis* CECT7121 to Caco-2 cells at different differentiation stages. Therefore, adhesion assays were performed on cells differentiated after 7 or 14 days in culture in order to represent the colonic and enterocyte features respectively. No differences were observed in the adhesion capacity of *Ent. faecalis* CECT7121 in cells with different differentiation grades indicating that the probiotic strain is able to adhere to both, colonic cells and enterocytes. These observations are in accordance with our previous results obtained in experiments of *in vivo* adhesion to murine large intestine, demonstrating the capacity of this strain to adhere to both, small and large intestine (Castro *et al.* 2007).

By the internalization assay, we demonstrated that *Ent. faecalis* CECT7121 adheres to epithelial cells but this strain does not show cellular invasion to the human epithelial cell. The lack of invasive capacity was not surprising since in an *in vivo* murine model, *Ent. faecalis* CECT7121 was not found in other organs (blood, spleen, kidneys and liver) after implantation in the murine intestine, thus indicating that it is incapable to invade and translocate (Castro *et al.* 2007). In addition, we have demonstrated that this bacterium strain does not induce cytotoxicity in IECs even when high MOIs were employed. A traditional explanation for the differential immune recognition of pathogenic *vs* commensal bacteria is that only pathogenic bacteria have evolved mechanisms to invade the epithelial cell barrier and survive within host tissues, whereas commensal bacteria lack such virulence or pathogenicity genes (Westendorf *et al.* 2010). Up to this point our results indicate that *Ent. faecalis* CECT7121 is able to adhere to human IECs. This bacterial adhesion does not cause bacterial internalization nor affect the cell viability.

IECs play a crucial role in the maintenance of mucosal homoeostasis, and actively sample commensal bacteria, pathogens and other antigens. These cells express different Toll-like receptors, are able to secrete cytokines and chemokines, and they have developed a variety of strategies to prevent bacterial invasion. The interaction between probiotics with enterocytes is important for the controlled production of cytokines secreted by epithelial cells. It has been shown that like the microbiota, some probiotics induce expression of inflammatory molecules, whereas others stimulate the production of anti-inflammatory mediators (Delcenserie *et al.* 2008; Rescigno 2011). However, it is generally considered that probiotic microbes can help to preserve the intestinal homeostasis by down-modulating the immune response.

In order to study whether *Ent. faecalis* CECT7121 is able to modulate the inflammation at the epithelial level, we studied the effect of this strain on the secretion of IL-8, a neutrophil chemoattractant, and the induction of the promoter of CCL20 in Caco-2 cells. Additionally, CCL20 secretion also was determined. Human intestinal cell lines have been shown to constitutively produce IL-8 (Wang *et al.* 2008). We found that *Ent. faecalis* CECT7121 did not induce a strong release of this chemokine. In fact, the levels of IL-8 measured in culture supernatants were different from the baseline levels only when the bacteria: Caco-2 cell ratio was 100. This result is in line with the lack of inflammatory infiltrate in the murine intestine after *Ent. faecalis* CECT7121 inoculation.

The constitutive expression of CCL20 has clearly been demonstrated in a variety of normal human mucosa-associated tissues, especially in the mucosa epithelial cells (Schutyser *et al.* 2003). CCL20 is responsible for the chemoattraction of immature dendritic cells, effector/memory T cells and B cells. Furthermore, the presence of inflammatory mediators causes the up-regulation of CCL20 mRNA and protein expression both *in vitro* and *in vivo* (Schutyser *et al.* 2003). For example, the stimulus

with flagellin, a TLR-5 agonist, is known to induce a high increase of CCL20 expression by Caco-2 cells. In this work, the preincubation with *Ent. faecalis* CECT7121 inhibited the flagellin-induced activation of the ccl20-luc reporter in Caco-2 cells. Other authors have also observed that probiotics are able to down-regulate the intestinal epithelial innate immune response. Using the same reporter system as that employed in this work, Romanin *et al.* (2010) have found that a broad variety of yeasts from kefir were able to inhibit the expression of CCL20. Likewise, *Lactobacillus crispatus* strains are able to down-regulate the transcription levels of CCL20 gene induced by the stimulation of HT-29 epithelial cells with *Salmonella braenderup* (Sun *et al.* 2012). Additionally, we also measured the levels of CCL20 protein secreted by Caco-2 cells. Although it did not reach statistical significance, low levels of CCL20 were found in the culture supernatants when epithelial cells were coincubated with *Ent. faecalis* CECT7121 compared with untreated cells. As previously mentioned, this probiotic strain did not have cytotoxic effect on IECs. With this observation we could confirm that the results obtained working with Caco-2 cells were not due to the reduction of cell viability but a direct modulatory effect mediated by *Ent. faecalis* CECT7121.

The maintenance of the gut integrity is particularly important for the homeostasis of the immune system, as a great diversity of dietary antigens and microbes interact with the luminal surface of the mucosal barrier. Just as their resistance to acid and bile, ability to colonize the gastrointestinal tract, and their clinical efficacy, the activity of probiotics on the intestinal mucosa is bacteria dependent (Madsen 2006). It has been reported that while *Lactobacillus paracasei* and *Lactobacillus rhamnosus* do not modify the mucosal tissue, the contact with *Lactobacillus plantarum* results in clear tissue deterioration (Tsilingiri *et al.* 2012). In a previous work, we have demonstrated that the administration of *Ent. faecalis* CECT7121 during three consecutive days allows its implantation and its persistence in the murine intestine (Castro *et al.* 2007). In this work, the effect of *Ent. faecalis* CECT7121 on the morphology of the intestinal villi was studied. The histological evaluation confirmed that the transient colonization of *Ent. faecalis* CECT7121 on the murine gut does not alter its integrity. The architecture of intestinal villi of probiotic-treated mice was not affected by the bacterium, as compared to non-treated animals. In addition, no inflammatory infiltrate was found. The overall healthy condition of probiotic-treated mice would allow us to assume that the intragastric inoculation of this bacterium does not perturb the anti-inflammatory condition of normal intestinal mucosa.

Many commensal and probiotic micro-organisms are apparently well able to stimulate the production of IgA by B cells (Macpherson and Uhr 2004; Dogi *et al.* 2008; Sanz and De Palma 2009; Maldonado Galdeano *et al.* 2011). It is known that IgA antibodies play a crucial role in the gut due to their protective effects against pathogens by inhibiting bacterial adherence, neutralizing viruses and blocking toxins. The development of the classical T cell-dependent antibody response takes at least 5–7 days to develop. On the other hand, it is also known that specialized B cell subsets can rapidly produce IgM as well as IgG or IgA in a CD4+ T cell and CD40L-independent manner (Cerruti 2008). It has been reported that, the intestinal T cell-independent IgA production in mice may involve B1 cells and that this production preferentially targets commensal bacteria. A probable site where B cells undergo this T cell-independent IgA class switching is the intestinal lamina propria (Cerruti 2008; Sutherland and Fagarasan 2012). *Enterococcus faecalis* CECT7121 administration induced an increase in the number of IgA^+ cells in the mice gut. The enhanced intestinal IgA production provides a defence for the mucosal surface and it is one of the beneficial effects associated with probiotic consumption. In our study, we observed this stimulatory effect as soon as 2 days after the last inoculation of *Ent. faecalis* CECT7121. Probably, this rapid expansion in the IgA^+ clones could be the consequence of the T cell-independent IgA class switch. While an increase in total IgA+ cells in the murine lamina propria after the inoculation of *Ent. faecalis* CECT7121 was observed, we did not find an increase in specific IgA levels in the intestinal fluids. IgA without any known antigen specificity can be produced in normal colonized mice in the absence of antigenic stimulation (Bemark *et al.* 2012). These nonspecific SIgA are also called natural IgA. In fact, the lack of specific IgA against probiotic bacterium was also observed by other authors. Maldonado Galdeano and Perdigón (2006) did not detect secretory IgA specific for *Lactobacillus casei* in the intestinal fluid and they suggest that the probiotic administration would not induce antigenic presentation with production of specific antibodies. In this line, recently we have demonstrated that cells of mesenteric lymph nodes and spleen from *Ent. faecalis* CECT7121-treated mice did not proliferate when this strain was employed as stimulus (Molina *et al.* 2015). The lack of specific proliferation in both lymphoid organs could be in line with the capacity of this probiotic bacterium to behave as a commensal micro-organism without inducing uncontrolled inflammatory responses.

Working with a novel polarized culture system of human healthy and Intestinal Bowel Disease intestinal mucosa Tsilingiri *et al.* (2012) have tested the action of three different strains of *Lactobacillus*. In the normal intestinal mucosa, these authors have observed that the cytokine secretion profile of probiotic-stimulated samples

was similar to that of negative controls. Similarly, in our study the cytokine levels measured were found to be similar between intestinal extracts obtained from *Ent. faecalis* CECT7121-treated mice and control mice. IL-6 is produced in a NF-κB-dependent manner by innate immune cells within the lamina propria in response to intestinal injury (Grivennikov *et al.* 2009). We demonstrated that the administration of *Ent. faecalis* CECT7121 did not increase the percentage of IL-6^+ cells in the lamina propria of treated mice. This result shows the noninflammatory effect of *Ent. faecalis* CECT7121 on the intestinal mucosa.

Ibnou-Zekri *et al.* (2003) have suggested that the influence of probiotics on humoral immunity may be partially determined by the colonizing properties of the strain. They demonstrated that although *Lactobacillus johnsonii* and *Lact. paracasei* displayed similar adhesion capacities to Caco-2 cells, *Lact. johnsonii* was a better colonizer in the intestines of gnobiotic mice, and a more efficient inducer of intestinal IgA than *Lact. paracasei*. In our study, *Ent. faecalis* CECT7121 showed a strong adherence capacity both *in vivo* (intestine of BALB/c mice) and *in vitro* (Caco-2 cells) and in addition, this probiotic strain was able to increase the proportion of IgA^+ cells in the small intestine.

In this work, we have studied different aspects of the interaction of *Ent. faecalis* CECT7121 with IECs and the mucosal immune system. We have demonstrated that this probiotic strain is able to stimulate the mucosal immune system increasing the number of IgA^+ cells in the lamina propria without inducing an epithelial inflammatory response. Our results indicate that *Ent. faecalis* CECT7121 has a modulatory effect at the local level, which adds to its already reported systemic immunomodulatory activities. Our previous work with this bacterial strain combined with the results presented here, allow us to speculate that *Ent. faecalis* CECT7121 may be employed as an immunomodulatory therapy for the treatment of inflammatory conditions such as, food allergies and inflammatory bowel diseases.

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Conflict of Interest

The authors report no conflict of interest in the preparation of this manuscript.

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