Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Comparative study of the protective capacity against *Salmonella* infection between probiotic and nonprobiotic lactobacilli

N.A. Castillo[1], A. de Moreno de LeBlanc[1], C.M. Galdeano[1,2] and G. Perdigón[1,2]

1 Centro de Referencia para Lactobacilos, CERELA-CONICET, Tucumán, Argentina
2 Cátedra de Inmunología, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina

Keywords
enterobacteria, immunology, lactic acid bacteria, probiotics, *Salmonella*.

Correspondence
Gabriela Perdigón, CERELA-CONICET, Chacabuco 145, San Miguel de Tucumán, Tucumán T4000ILC, Argentina.
E-mail: perdigon@cerela.org.ar

2013/1593: received 6 September 2012, revised 24 October 2012 and accepted 10 November 2012

doi:10.1111/jam.12074

Abstract

Aims: To investigate the immunoprotective ability of three *Lactobacilli* strains against *Salmonella enterica* serovar Typhimurium in a mouse model. To identify the probiotic properties involved in the protection against infection caused by this pathogen.

Methods and Results: The immunomodulatory effect of three different lactobacilli strains: *Lactobacillus* (*Lact.*) *casei* CRL 431 (probiotic bacterium), *Lact. delbrueckii* subsp. *bulgaricus* CRL 423 (*Lact. bulgaricus*) and *Lact. acidophilus* CRL 730 was compared using a mouse model of *Salmonella* infection. *Lactobacillus casei* continuous administration improved animal survival, diminished pathogen spreading outside the intestine, attenuated the intestinal inflammation, modulated cytokine profile previous and postinfection and increased the expression and secretion of IgA in the gut. Additionally, the administration of this lactobacilli increased peritoneal, Peyer's patches and spleen macrophages' phagocytic activity in healthy mice and monocyte chemotactic protein (MCP-1) released by intestinal epithelial cells in an *in vitro* assay. Although *Lact. acidophilus* increased the number of IgA-secreting cells previous and postinfection, and *Lact. bulgaricus* increased MCP-1 released by intestinal epithelial cells and the phagocytic activity of macrophages, these effects alone were not enough to confer protection against *Salmonella* Typhimurium infection in mouse.

Conclusions: Probiotic strain *Lact. casei* CRL 431 was the one that induced protection against *Salmonella*, by increasing the intestinal barrier function and by decreasing the local inflammatory response.

Significance and Impact of the Study: *Salmonella* spp. constitutes an important agent of foodborne diseases in the world. Not all lactobacilli, even with some immunostimulating properties at gut level, can protect against *Salmonella* infection. *Lactobacillus casei* CRL 431, a probiotic bacterium, could be useful as an oral mucosal adjuvant of the immune system to improve gut health, especially in the prevention or amelioration of *Salmonella* infections. We demonstrated that there is not a unique mechanism by which this protective effect was exerted.

Introduction

Numerous studies propose the use of probiotics to improve gut health (Heselmans *et al.* 2005) in the treatment of inflammatory bowel diseases (Chaves *et al.* 2011) and in the prevention of antibiotic-induced diarrhoea (Song *et al.* 2011). One of their foremost allegations is their ability to protect against several infectious agents, especially against enteropathogens (Cremonini *et al.* 2002; Maragkoudakis *et al.* 2010). The protective effect of

certain probiotic strains against specific pathogens is undeniable; however, the scientific basis of the ways through which probiotics confer protection must be well established. A great number of effects have been proposed in such protective effect. Some of these are the stabilization of the gut mucosal barrier (Yan *et al.* 2007), the stimulation of goblet cells for mucus secretion (Dogi and Perdigón 2006), the competition for nutrients, the secretion of antimicrobial substances (bacteriocins), and the modulation of the mucosal and systemic immune responses (Lebeer *et al.* 2008).

Salmonella spp. is one of the principal causative agents of poisoning and foodborne disease in the world. Enteric infections caused by this genus are one of the major causes of morbidity and mortality in infants in developing countries. *Salmonella* spp. can cause a wide variety of diseases, going from mild gastroenteritis to typhoid fever. The nature and severity of the infection developed depend on many factors, including the serovar involved, the virulence of the strain, the infective dose, the species, the age and immune status of the host.

Lactobacillus (*Lact.*) *casei* CRL 431 is a probiotic strain that was evaluated in clinical studies performed in humans documenting its effects in various conditions (Gaon *et al.* 2003; De Vrese *et al.* 2005; Vlieger *et al.* 2009; Rizzardini *et al.* 2011). Previous works performed by our research group demonstrated, using animal models, the ability of *Lactobacillus* (*Lact.*) *casei* CRL 431 to modulate the mucosal immune system (Perdigón *et al.* 2002a; Maldonado Galdeano and Perdigon 2004; Vinderola *et al.* 2005) and its protective capacity against *Salmonella enterica* serovar Typhimurium (*Salm.* Typhimurium) infection in murine models (Gauffin Cano and Perdigón 2003; De Moreno De Leblanc *et al.* 2010; Castillo *et al.* 2011). In these studies, BALB/c mice were employed due to *Salm.* Typhimurium develops in them the same pathogenesis and symptoms that typhoidal and nontyphoidal *Salmonellae* in humans, making it a valuable model for the study of this disease (Santos *et al.* 2001). Other potentially probiotic strains studied by our laboratory were *Lact. delbrueckii* subsp. *bulgaricus* CRL 423 and *Lact. acidophilus* CRL 730. These bacterial strains showed immunomodulatory properties in different animal models (Perdigón *et al.* 1992, 2002b; Gauffin Cano *et al.* 2002), and it was reported that these bacteria combined with other strains presented variable protective ability against *Salmonella* infection in different *in vitro* and *in vivo* assays (Valdez *et al.* 2001). Based on these previous reports, we hypothesized that even when a probiotic product or a probiotic strain may present immunomodulatory properties, this does not guarantee a protective effect against a particular pathogen, and that there is a set of strain-specific effects, which would be vital for a probiotic strain to confer protection against certain enteropathogen. Considering these facts, the aim of this study was to investigate the ability of each *Lactobacillus* strain to stimulate the intestinal immune system and to protect against *Salmonella* Typhimurium infection in a mouse model. A comparative study was carried out to determine what effects on the mucosal immune system would be desired in a probiotic strain to achieve protection against this particular pathogen.

Materials and methods

Bacterial strains, media and growth conditions

Lactobacilli strains were obtained from the CERELA Culture Collection (Centro de Referencia para Lactobacilos, San Miguel de Tucumán, Argentina). Overnight cultures of *Lact. acidophilus* CRL 730 or *Lact. delbrueckii* subsp. *bulgaricus* CRL 423 were grown at 37°C in sterile LAPTg broth (tryptone, 10 g l^{-1}; yeast extract, 10 g l^{-1}; peptone, 15 g l^{-1}; glucose 10 g l^{-1}; Tween 80, 1 ml l^{-1}) and for *Lact. casei* CRL 431 overnight cultures were grown in sterile Mann–Rogosa–Sharp (MRS; Britania, Buenos Aires, Argentina) broth. After incubation, cells were harvested by centrifugation at 5000 ***g*** for 10 min, washed three times with fresh phosphate-buffered saline (PBS) and resuspended in sterile 10% (v/v) nonfat milk. Each *Lactobacillus* strain was adjusted to 1×10^8 CFU ml^{-1}.

Salmonella Typhimurium strain was obtained from the Bacteriology Department of the Hospital del Niño Jesús (San Miguel de Tucumán, Argentina). An aliquot (200 μl) from an overnight culture was placed in 5 ml of sterile brain heart infusion (BHI) broth (Britania) and grown at 37°C. After incubation, *Salmonella* was adjusted to 1×10^8 CFU ml^{-1} in sterile PBS.

Animals and experimental design

To evaluate the protective ability of these three lactobacilli strains against *Salm.* Typhimurium, 5–6-week-old BALB/c male mice weighing 22–31 g were obtained from the closed random bred colony, maintained at CERELA (Centro de Referencia para Lactobacilos, San Miguel de Tucumán, Argentina). Three to four mice were housed together per cage, in metal cages kept in a controlled atmosphere (22 ± 2°C; 55 ± 2% relative humidity) with a 12-h light/dark cycle. Mice were fed *ad libitum* with a conventional balanced diet.

The experimental protocol contained three experimental groups, one for each tested bacterium (*Lact. acidophilus* CRL 730, *Lact. delbrueckii* subsp. *bulgaricus* CRL 423 or *Lact. casei* CRL 431). Each lactobacillus strain was

administered in the drinking water at a final concentration of 1×10^8 CFU ml^{-1}. This count was periodically controlled at the beginning and after 24 h of dilution in water to avoid modifications of more than one logarithmic unit. Basal samples (three mice per group) were collected after 7 days of lactobacilli administration. The rest of the mice were challenged with 100 μl of 1×10^8 CFU ml^{-1} of *Salm.* Typhimurium orally, using a gavage′s syringe. After challenge, each group was divided into two test groups: one continued receiving the *Lactobacillus* strain (continuous administration) and the other not (preventive administration). In addition, two control groups without special feeding were performed: one of them was challenged with *Salmonella* (infected control, IC) and the other was not challenged with the pathogen (normal control, NC). Mice were weighed throughout the experiment until the day 10 postinfection; animals were sacrificed 7 and 10 days postchallenge to obtain the samples (three mice per group and assay). Small and large intestine, liver and spleen were removed, and small intestine fluids were collected to perform the different assays. To evaluate the mortality rate, the same protocols detailed above were carried out for each lactic acid bacteria comparing with the infection control (two test groups and one control group for each assay) using 10 mice per group, and the number of deaths was registered. The protocol design was preapproved by the Animal Protection Committee of CERELA. All experiments were performed following institutional guidelines for the care and use of animals and complied with the current laws of Argentina.

Colonization assays

The large intestine, spleen and liver were aseptically removed, weighed and placed into sterile tubes containing 5 ml of peptone water (0·1%). The samples were immediately homogenized under sterile conditions using a micro homogenizer (MSE, England). Serial dilutions were made and spread onto the surface of MacConkey agar (Britania) for the liver and spleen samples, and Salmonella-Shigella agar (Britania) for the large intestine samples. The plates were then incubated aerobically at 37°C for 18 h. Results were expressed as Log CFU per gram of organ.

Histological evaluations of the small intestines

The small intestines were removed, washed with 0·85% NaCl, cut in pieces and prepared for histological studies, following the technique described by Sainte-Marie (1961) (Sainte-Marie 1961). Serial paraffin sections (4 μm) were stained with haematoxylin–eosin for light microscopy examination. The extent of intestinal damage and inflammation was assessed using the following histopathological grading system:

Grade 0: Histological findings identical to normal mice.

Grade 1: Mild mucosal and/or submucosal inflammatory infiltrate (admixture of neutrophils) and oedema. Muscularis mucosae intact.

Grade 2: Grade 1 changes involving 50% of the specimen.

Grade 3: Prominent inflammatory infiltrate and oedema (neutrophils usually predominating) frequently with deeper areas of ulceration extending through the muscularis mucosae into the submucosa. Rare inflammatory cells invading the muscularis mucosae but without muscle necrosis.

Grade 4: Grade 3 changes involving 50% of the specimen.

Grade 5: Extensive ulceration with coagulative necrosis bordered inferiorly by numerous neutrophils and lesser numbers of mononuclear cells. Necrosis extends deeply into the muscularis propria.

This was a blind analysis performed by a pathologist.

Immunofluorescence assay for IgA-secreting cells in small intestine tissues

The number of IgA-secreting cells was determined on histological slices using a direct immunofluorescence assay. After deparaffinization using xylene and rehydration in a decreasing gradient of ethanol, tissue sections (4 μm) were incubated with a 1 : 100 dilution of α-chain monospecific antibody conjugated with fluorescein isothiocyanate (FITC; Sigma, St Louis, MO, USA) for 30 min and observed with a fluorescent light microscope. Fluorescent cells were counted in 30 fields at 1000 × magnification, and results were expressed as the number of fluorescent cells in 10 fields of view. The number of fluorescent cells was counted for two different investigators (by blind counts) covering different portions of each sample.

Determination of total and specific secretory IgA in intestinal fluids

Intestinal fluids were collected from the small intestines with 1 ml of 0·85% NaCl and immediately centrifuged at 5000 ***g*** during 15 min at 4°C. The supernatant was recovered and ELISA was used to measure the concentration of total secretory IgA (S-IgA) according to the technique described by Leblanc *et al.* (2004). The optical density was read at 450 nm using a VERSA Max Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

For the specific anti-*Salmonella* S-IgA antibodies determinations, plates were coated with 50 μl of a suspension of concentrated and heat-inactivated *Salm.* Typhimurium solution (10^{10} CFU ml^{-1}) and incubated overnight at 4°C. Nonspecific protein-binding sites were blocked with PBS containing 0·5% nonfat milk. The test and control samples from the intestinal fluid were diluted in 0·5% nonfat milk in PBS and then incubated at room temperature for 2 h. After washing with PBS containing 0·05% Tween 20, the plates were incubated 1 h with peroxidase-conjugated anti-IgA-specific antibodies. Plates were again washed and the tetramethylbenzidine (TMB) reagent was added. The reaction was stopped with H_2SO_4 (2 N). The absorbance was read at 450 nm.

Determination of cytokine-producing cells in the lamina propria of the small intestine

Tissue sections (4 μm) of small intestine from each mouse were obtained as described above and used to analyse cytokine-producing cells by an indirect immunofluorescence assay. The sections were incubated with a blocking solution of bovine serum albumin (BSA)/PBS, washed with PBS and incubated with normal goat serum (Sigma) to prevent nonspecific staining. Rabbit anti-mouse TNFα, IFNγ, IL-10 and IL-6 (Peprotech, Inc., Rocky Hill, NJ, USA) polyclonal antibodies (diluted in saponin/PBS) were applied to the tissue sections for 105 min at room temperature (25°C). The sections were then treated 1 h with diluted goat anti-rabbit antibody conjugated with FITC (Jackson Immuno Research Labs, Inc., West Grove, PA, USA). The number of fluorescent cells was counted by two individual blind counts per sample in 30 fields of view, and the results were expressed as the number of fluorescent cells in 10 fields of view as seen with 1000 × magnification using a fluorescent light microscope.

Determination of TNFα, IFNγ, IL-10 and IL-6 in the small intestine fluid

Intestinal fluid supernatants were recovered as explained previously for S-IgA. For cytokine determination, TNFα, IFNγ, IL-10 and IL-6 BD OptEIA mouse cytokine ELISA sets (BD Bioscience, San Diego, CA, USA) were used according to the manufacturer instructions. Results were expressed as concentration of each cytokine in the intestinal fluid (pg ml^{-1}).

Isolation of macrophages from peritoneum, Peyer's patches and spleen. Determination of phagocytic activity

Peritoneal macrophages were obtained according to Valdez *et al.* (2001). Macrophages were extracted from peritoneal cavity with 5 ml of sterile PBS pH 7·4 containing 100 μg ml^{-1} of gentamicin (Gm). For the isolation of macrophages from Peyer's patches, the protocol described by Galdeano and Perdigón (2006) was used. The small intestine of each mouse was removed, washed and the Peyer's patches were excised in Hank's buffered salt solution (HBSS; Sigma-Aldrich) containing heat-inactivated foetal bovine serum (FBS). The epithelium cells were separated with an HBSS/FBS solution containing EDTA. The sediments were incubated with dispase/DNAse solution and the mononuclear cells were recovered. These cells were collected from the supernatant and washed with RPMI 1640 medium (Sigma). For spleen macrophages isolation, each spleen was recovered and aseptically disrupted in 5 ml of HBSS solution containing FBS. The cells were harvested by centrifugation at 800–1000 ***g*** for 15 min at 4°C. The resulting pellets were gently mixed with 2 ml of sterile red blood cell lysing buffer (Sigma) for 2 min. The haemolysis was stopped with PBS. The samples were again centrifuged and resuspended in RPMI-1640 medium (Sigma) containing FBS.

The adherent cells (macrophages and DC) were separated from the other mononuclear cells using their adherence property to glass slides. Phagocytosis assays were performed using *Saccharomyces* (*S.*) *boulardii* suspension (Hansen CBS 5926 from Floratil, MERCK Quimica, Argentina) at a concentration of 10^7 cell ml^{-1}. Phagocytosis was performed by *ex vivo* assay using equal volumes of opsonized *S. boulardii* mixed with 10^6 cells ml^{-1} of macrophages. The mixture was incubated for 30 min, at 37°C. Phagocytosis was expressed as the percentage of phagocyting macrophages in 200 cells count using an optical microscope.

Isolation and culture of intestinal epithelial cells. *In vitro* assay

Intestinal epithelial cells (IECs) were isolated from NC mice. Animals were sacrificed, and a midline incision was made to remove intestines from the duodenum to the ileum. Intestines were washed with cold PBS + gentamicin (Gm) 100 μg ml^{-1} to remove the content. The Peyer's patches and the mesentery were discarded, and the rest of the intestine was opened along the length of the antimesenteric borders to expose the mucosal surface. The samples were washed five times with ice-cold HBSS + Gm 100 μg ml^{-1} (4°C). Each sample was cut into small segments and placed in ice-cold HBSS + FBS 8% + Gm 100 μg ml^{-1}. The samples were transferred to a tube with HBSS + FBS 8% + Gm 100 μg ml^{-1} + DL-Dithiothreitol 1 mmol/l^{-1} (DTT; Biochemika, Fluka-Sigma-Aldrich) + EDTA 10 mmol/l^{-1} and incubated 15 min at 4°C. Supernatants were discarded, and the

pellets were incubated in Erlenmeyers with 15 ml of HBSS + FBS 8% + Genta 100 μg ml^{-1} + EDTA 30 mmol/l^{-1}, using a magnetic bar to shake the samples during 15 min. Supernatants were separated and left to settle for 2 min; this allowed the sedimentation of nondigested large fragments. The supernatants containing IECs were collected and centrifuged at 300 ***g*** during 5 min. The pellets were washed twice and resuspended with Dulbecco's Modified Eagle Medium (DMEM High Glucose 1X; Gibco-Life Technologies Corporation, Grand Island, NY, USA). Trypan blue (0·4%) exclusion was used to assess cell viability.

Determination of IL-6 and MCP-1 levels in a culture supernatant of IECs challenged with the different lactobacilli or with *Salmonella*. *In vitro* assay

IECs were adjusted at a concentration of 1×10^6 cells ml^{-1} in DMEM + FBS 10% and were placed in 6-well tissue culture plates (2 ml per well). Cells were stabilized 1 h (37°C, 5% CO_2). Each well was inoculated with a cellular suspension of *Salm.* Typhimurium, *Lact. casei* CRL 431, *Lact. delbrueckii* subsp. *bulgaricus* CRL 423 or *Lact. acidophilus* CRL 730 (IEC: bacteria ratio was 1 : 10). LPS from *Salm.* Typhimurium at a final concentration of 5 μg ml^{-1} (lyophilized powder; Sigma) was used as a positive control. Challenged cells were incubated 6 or 18 h (for *Salmonella* or lactobacilli, respectively) at 37°C and 5% CO_2. Culture supernatants were recovered to determine the cytokine IL-6 and the chemokine MCP-1 using commercially available BD OptEIATM mouse cytokine ELISA sets (BD Biosciences), according to manufacturer's instructions. Results were expressed as concentration of the cytokine or the chemokine in the culture supernatant (pg ml^{-1}).

Statistical analysis

The experimental protocol was performed three times, and all the results (from the three trials) were analysed together ($N = 9$). Statistical analyses were performed using MINITAB 14 software (Minitab Inc., State College, PA, USA). Unless for the mortality rate, a factorial experimental design (replicates – dietary regimen – time point) was used for the rest of the experiments. Comparisons were accomplished by an ANOVA general linear model followed by a Tukey's post hoc test, and $P < 0{\cdot}05$ was considered significant.

Results

Determination of body weight, mortality rate and pathogen counts in different organs. Histological study of small intestine

None of the lactobacilli tested induced significant variations in animal body weights, compared to the infection control group (IC) (Table 1). Seven days postchallenge, the groups that received a preventive administration of *Lact. acidophilus* or *Lact. casei* and the mice fed continuously with *Lact. bulgaricus* or *Lact. casei* showed a significant decrease in mortality percentage, compared to the

Table 1 Changes in body weight and mortality percentage in mice receiving the different lactobacilli and challenged with the pathogen

	Body weight (g)			Mortality percentage (%)	
Group	Basal	7-d PCh	10-d PCh	7-d PCh	10-d PCh
Infection control	26·59 ± 1·08^a	25·58 ± 2·11^a	26·50 ± 2·72^a	27	73
Lactobacillus acidophilus					
Preventive	22·22 ± 1·61^b	24·57 ± 2·89^a	22·46 ± 2·19^a	10*	70
Continuous	22·21 ± 0·97^b	25·66 ± 3·66^a	24·61 ± 1·77^a	20	80
Lact. bulgaricus					
Preventive	28·51 ± 0·89^a	27·88 ± 2·73^a	24·96 ± 0·10^a	30	90
Continuous	30·31 ± 1·66^c	30·78 ± 3·95^a	26·29 ± 3·59^a	10*	60
Lact. casei					
Preventive	26·39 ± 0·37^a	23·29 ± 2·71^a	23·03 ± 1·50^a	7*	50
Continuous	27·87 ± 0·82^a	23·53 ± 2·51^a	28·16 ± 4·90^a	0*	7*

Results are expressed as means ± SD of the body weight of infection control mice and the test groups at three time points: the day of the infection (basal) and 7 and 10 days postchallenge (7 and 10-day PCh). Data of body weight correspond to $N = 9$ animals per group from three separate experiments. Means for each value in a column without a common letter differ significantly ($P < 0{\cdot}05$). Mortality percentages were determined in separate experiments for each lactobacillus comparing with the infected control (IC) group (10 mice per group). For each test group, the mortality percentage was related to 10 mice (100%), and for IC group, the values represent the mean of three trials (10 mice per trial).

*Means a decrease in the mortality percentage of 50% or less than the value observed for the IC group in each column.

IC group, while 10 days postinfection, only the group fed continuously with *Lact. casei* maintained this significantly lower rate of mortality compared to the IC group (Table 1). Pathogen translocation to liver and spleen and its counts in large intestine did not decrease in the groups that received a preventive administration of lactobacilli compared to the IC group (Table 2). With regard to continuous administration, the decrease on mortality in the group given *Lact. bulgaricus* was not accompanied by less counts of *Salmonella* in large intestine, liver or spleen, 7 days postchallenge. Only the group fed continuously with *Lact. casei* showed a significant diminution of pathogen counts in spleen (7 and 10 days postchallenge), liver and large intestine (10 days postchallenge) compared to the IC group (Table 2).

Histological studies by haematoxylin–eosin staining on small intestine slides revealed a significant decrease in the damage score with moderate inflammation and less cellular infiltration in both groups that received *Lact. casei* (2·3 ± 0·6 for preventive and 1·7 ± 0·3 for continuous administration), and in the group fed continuously with *Lact. bulgaricus* (2·7 ± 0·6), compared to the IC group (3·8 ± 0·3), 7 days postchallenge (Fig. 1 and Table 3). It was also observed an increased cellularity in the intestines of the groups that received a preventive feeding with *Lact. acidophilus* or *Lact. casei*, and in the groups fed continuously *Lact. bulgaricus* or *Lact. casei*, without significant changes in the architecture of the villi (Fig. 1f,i–k, respectively). In contrast, small intestine of mice from the rest of the infected groups (continuous feeding with *Lact. acidophilus* and preventive administration of *Lact. bulgaricus*) showed the same inflammatory profile that the mice from IC group (Table 3), with high polymorphonuclear and red blood cell infiltration (Fig. 1E–G–H, respectively), shortening and widening of the villi.

Determination of IgA-secreting cells in the lamina propria and IgA release to the lumen of the small intestine

Lactobacillus acidophilus CRL 730 and *Lact. casei* CRL 431 but not *Lact. bulgaricus* CRL 423 administration increased the number of IgA-secreting cells in the lamina propria of the small intestine of healthy mice (basal sample) compared to NC group; only *Lact. casei* was able to enhance the secretion of total S-IgA at this time point (Fig. 2a,b). Seven days postchallenge, the continuous administration with *Lact. acidophilus* or *Lact. casei* maintained increased the IgA+ cell numbers compared to the NC group, and the values were significantly higher than those observed for the IC (Fig. 2a). Ten days postchallenge, no significant differences were observed between the different test groups and IC.

Concerning total S-IgA levels, no significant differences were observed between infected groups and healthy mice 7 days postchallenge, while reached values significantly higher than the NC group in all the infected groups (treated and control) 10 days postinfection (Fig. 2b).

Specific anti-*Salm.* Typhimurium S-IgA increased significantly in both groups of mice fed with *Lact. casei* (preventive and continuous administration) compared to the IC group, 7 and 10 days postchallenge. Specific S-IgA levels in the intestinal fluids of the mice from groups that received *Lact. acidophilus* or *Lact. bulgaricus* were maintained similar to the IC group (Fig. 2c).

Table 2 *Salmonella* Typhimurium counts in spleen, liver and large intestine of mice that received different lactobacilli

		Preventive administration			Continuous administration		
Organ	IC	*Lactobacillus acidophilus*	*Lact. bulgaricus*	*Lact. casei*	*Lact. acidophilus*	*Lact. bulgaricus*	*Lact. casei*
Spleen							
7-day PCh	7·4 ± 0·7	7·1 ± 1·3	7·9 ± 0·5	8·6 ± 0·6	6·6 ± 0·7	7·0 ± 1·9	3·3 ± 0·4*
10-day PCh	7·0 ± 1·0	8·2 ± 0·3	9·4 ± 0·0	8·6 ± 0·6	7·3 ± 1·0	6·8 ± 0·0	3·5 ± 0·3*
Liver							
7-day PCh	6·5 ± 0·9	5·2 ± 2·9	7·6 ± 0·3	8·6 ± 0·2*	5·0 ± 2·8	6·8 ± 2·1	6·2 ± 0·3
10-day PCh	7·4 ± 1·7	8·6 ± 0·1	8·7 ± 0·1	7·4 ± 2·3	6·9 ± 2·0	7·3 ± 0·1	1·6 ± 0·0*
LI							
7-day PCh	5·5 ± 0·9	5·5 ± 1·6	6·5 ± 0·0*	6·8 ± 2·5	3·9 ± 1·8	6·9 ± 0·4	3·8 ± 1·3
10-day PCh	6·7 ± 0·7	8·4 ± 0·2	9·2 ± 0·1	8·1 ± 2·0	6·9 ± 0·1	6·3 ± 0·8	1·1 ± 0·3*

IC, infection control group; LI, large intestine.

Results show the colony counts of *Salmonella* Typhimurium in different organs, 7 and 10 days postchallenge (7- and 10-day PCh). They are expressed as means ($\log_{10}$ CFU g^{-1} of organ) ± SD of results of $N = 9$ animals from three separate experiments.

*For each organ, means values significantly different from those of the IC group ($P < 0·05$).

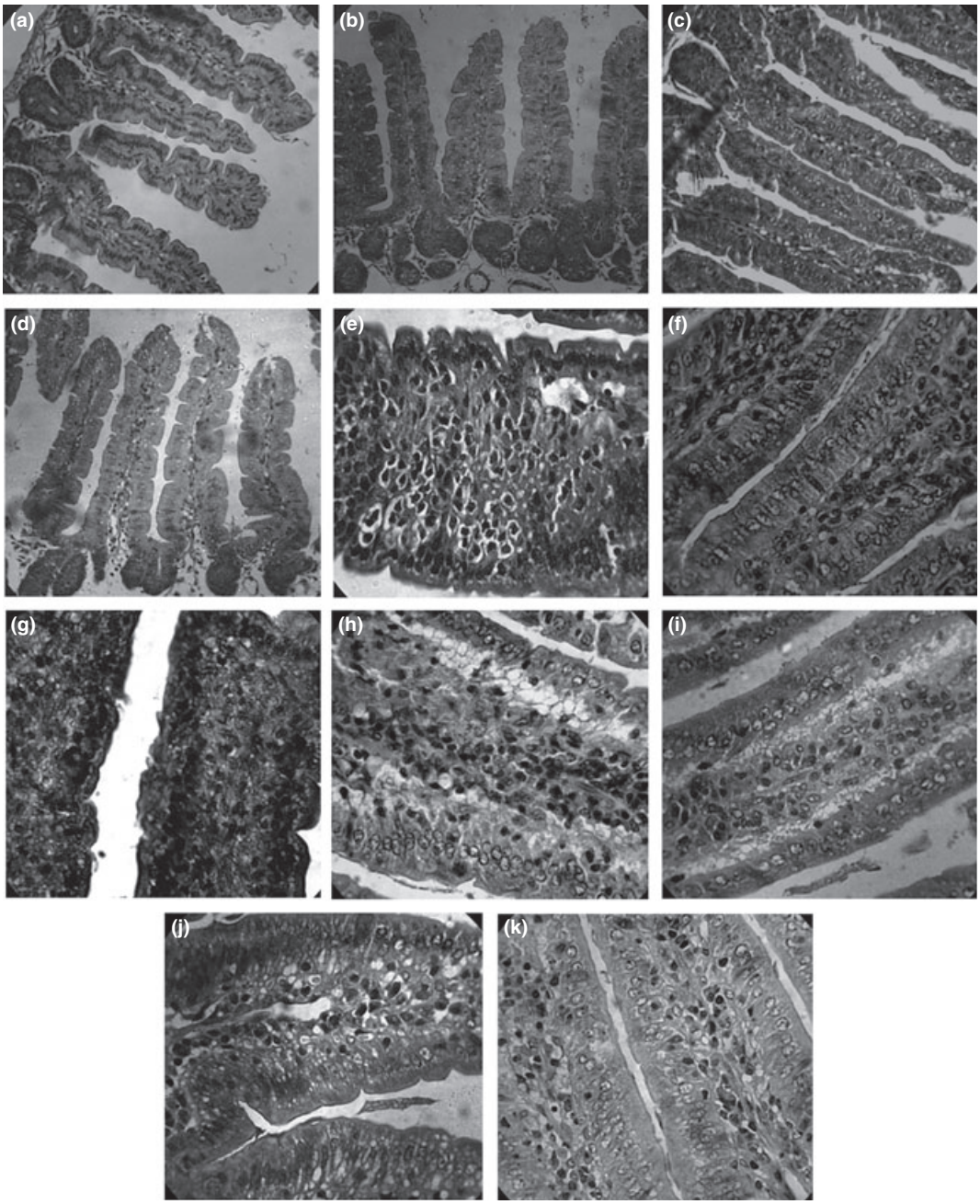

Figure 1 Histological study of the small intestine. The samples were obtained 7 days postchallenge. The microphotographs are representative slices from small intestine of mice, stained with haematoxylin–eosin. Samples were obtained before infection for normal control group (a) or mice that received *Lactobacillus acidophilus* (b), *Lact. bulgaricus* (c) or *Lact. casei* (d) during 10 days. The rest of the figures correspond to mice sacrificed 7 days postinfection. (e) IC group, where severe mucosal damages are observed with loss of villi and influx of red blood and polymorphonuclear cells in the mucosa and submucosa. (f, i, j and k) Mice that received preventive *Lact. acidophilus*, continuously *Lact. bulgaricus* and preventive and continuously *Lact. casei*, respectively, where the tissue maintained the typical structure of the small intestine and there is infiltration of mononuclear cells, and lower infiltration of polymorphonuclear cells in specific areas. (g and h) Mice feed continuously with *Lact. acidophilus* and/or preventively with *Lact. bulgaricus*, respectively, which showed similar histology changes than the infection control group. (a–d) Were taken at 400×, and (e–k) were taken at 1000× of magnification for a best appreciation of the changes.

Table 3 Histological damage score of small intestine sections from mice receiving the different lactobacilli and challenge with *Salmonella*

Group	Sample		
	Basal	7 days PCh	10 days PCh
Infection Control	$0{\cdot}3 \pm 0{\cdot}2^{a}$	$3{\cdot}8 \pm 0{\cdot}3^{a}$	$4{\cdot}0 \pm 0{\cdot}6^{a}$
Lactobacillus acidophilus			
Preventive	$0{\cdot}6 \pm 0{\cdot}2^{a,b}$	$2{\cdot}7 \pm 0{\cdot}6^{b,c}$	$3{\cdot}5 \pm 0{\cdot}5^{a,b}$
Continuous		$3{\cdot}3 \pm 0{\cdot}6^{a,b,c}$	$3{\cdot}8 \pm 0{\cdot}4^{a}$
Lact. bulgaricus			
Preventive	$1{\cdot}0 \pm 0{\cdot}4^{b}$	$3{\cdot}5 \pm 0{\cdot}5^{a,b}$	$3{\cdot}3 \pm 0{\cdot}7^{a,b,c}$
Continuous		$2{\cdot}7 \pm 0{\cdot}6^{b,c}$	$2{\cdot}6 \pm 0{\cdot}7^{b,c,d}$
Lact. casei			
Preventive	$0{\cdot}5 \pm 0{\cdot}3^{a,b}$	$2{\cdot}3 \pm 0{\cdot}6^{c,d}$	$2{\cdot}0 \pm 0{\cdot}6^{c,d}$
Continuous		$1{\cdot}7 \pm 0{\cdot}3^{d}$	$1{\cdot}6 \pm 0{\cdot}5^{d}$

Results are expressed as the means ± SD of the damage score. For basal sample (before the infection), preventive and continuous administrations are not separated and one value is showed for each lactic acid bacteria. The preventive group corresponds to mice that received the bacterial supplementation during 7 days before the infection; the continuous group correspond to mice given bacterial supplementation before (during 7 days) and after infection. Means for each value in a column without a common letter differ significantly ($P < 0{\cdot}05$). Data correspond to the means ± SD of results of $N = 9$ animals from three separate experiments. PCh, postchallenge with *Salmonella*.

Cytokine profile in the lamina propria of the small intestine induced by the different lactobacilli administered previous and postchallenge with *Salmonella*

After 7 days of feeding (basal samples), there was a significant increase of IFNγ-producing cells in the lamina propria of the small intestine of healthy mice that received *Lact. bulgaricus* or *Lact. casei* compared to the NC (Fig. 3a), whereas the number of TNFα, IL-6 and IL-10-producing cells was similar in the three experimental groups and in the NC (Fig. 3–b–d).

For 7 days postinfection, the number of IFNγ-producing cells was significantly increased in mice that received continuous administration of *Lact. casei* compared to the IC, while the rest of the groups showed no differences (Fig. 3a). The number of TNFα-producing cells in both groups given *Lact. casei* remained similar to the NC and was significantly lower than the IC. In the rest of the groups (fed with *Lact. bulgaricus* or *Lact. acidophilus*), the number of TNFα-producing cells was similar to the IC group (Fig. 3b). The number of IL-6-producing cells did not modify in the mice fed with *Lact. acidophilus*, *Lact. bulgaricus* or *Lact. casei* compared to the IC. Nevertheless, the number of cells positive for this cytokine was significantly increased in the groups that received a preventive feeding with *Lact. bulgaricus* or in both groups given *Lact. casei* compared to the NC (Fig. 3c). As regard to the number of IL-10-producing cells, only the groups fed with *Lact. casei* (preventive and continuous administration) and the group fed continuously with *Lact. bulgaricus* showed significant increases compared to the IC (Fig. 3d).

Cytokine concentration in the small intestine fluid

The results obtained previous to the infection after 7 days of lactobacilli administration showed that all the groups maintained similar concentrations of IFNγ in the small intestine fluid, *Lact. acidophilus* administration induced increased release of TNFα, *Lact. bulgaricus* increased the release of TNFα and decreased IL-10 levels, and *Lact. casei* increased the levels of IL-6, with regard to the NC group (Fig. 4–a–d).

Seven days postchallenge, IFNγ levels decreased significantly in the IC group and in the groups given *Lact. bulgaricus* and *Lact. acidophilus*, compared to the NC. Instead, IFNγ levels in both groups given *Lact. casei* remained similar to the NC, and these levels were significantly higher than the observed for the IC group (Fig. 4a).

Seven days postchallenge, only the groups fed with *Lact. bulgaricus* (preventive and continuous administration) showed levels of TNFα significantly higher than both controls [IC and NC, (Fig. 4b)]. For IL-6 levels, 7 days postinfection, it was observed significant increases in all the infected groups (treated and control) compared to the NC, being the concentration in the IC group significantly higher than in the groups that received lactobacilli (Fig. 4c).

Seven days postchallenge, IL-10 levels remained similar to the NC in the mice that received *Lact. casei* previous to *Salmonella* challenge (preventive administration), while the group fed continuously with this *Lactobacillus* showed lower values than NC (Fig. 4d). Nevertheless, both groups given *Lact. casei* showed values of IL-10 significantly higher than the IC group. The mice fed with *Lact. bulgaricus* or *Lact. acidophilus* showed similar levels of IL-10 than the IC, 7 days postchallenge, and these levels were significantly lower than those observed in healthy mice (basal data).

Effect of lactobacilli on macrophage phagocytic activity

The phagocytic activity using *S. boulardii* as antigen was analysed in basal samples after 7 days of feeding with *Lact. acidophilus*, *Lact. bulgaricus* and *Lact. casei*. The administration of *Lact. bulgaricus* or *Lact. casei* to healthy mice during 7 days increased significantly ($P < 0{\cdot}05$) the phagocytic activity of macrophages isolated from peritoneal, Peyer's patches and spleen, compared to NC mice,

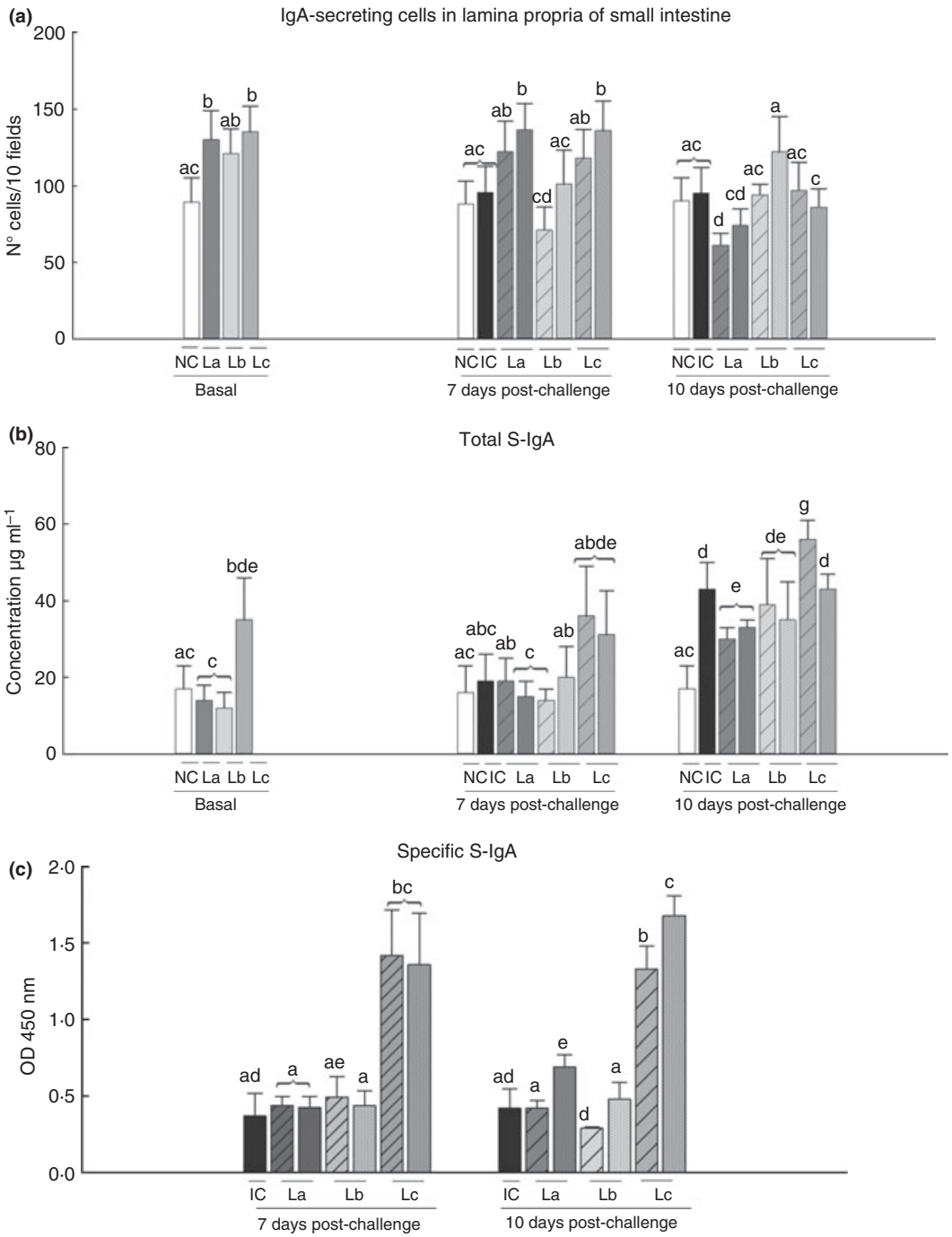

Figure 2 Effect of the oral administration of lactobacilli and *Salmonella* challenge on S-IgA in the small intestine. IgA-secreting cells in the lamina propria of the small intestine (a) were evaluated by immunofluorescence in histological sections. Results are expressed as number of IgA-positive cells per 10 fields (1000×). Total S-IgA (b) and specific anti-*Salm.* Typhimurium S-IgA (b) levels were determined in small intestine content by ELISA. For total IgA, the results are expressed as concentration (μg m^{-1}), related to the standard curve. For specific S-IgA, the results are expressed as OD (450 nm). The samples were obtained before the infection (basal) and 7 and 10 days postinfection. For the groups that received lactobacilli, diagonal bars represent preventive administration (before the infection), and dotted bars represent continuous feeding (before and after infection). Data correspond to the means ± SD of $N = 9$ animals from three separate experiments. For each figure, means for each value without a common letter differ significantly ($P < 0{\cdot}05$). C, normal control; IC, infection control; La, *Lactobacillus acidophilus*; Lb, *Lact. bulgaricus*; Lc, *Lact. casei*.

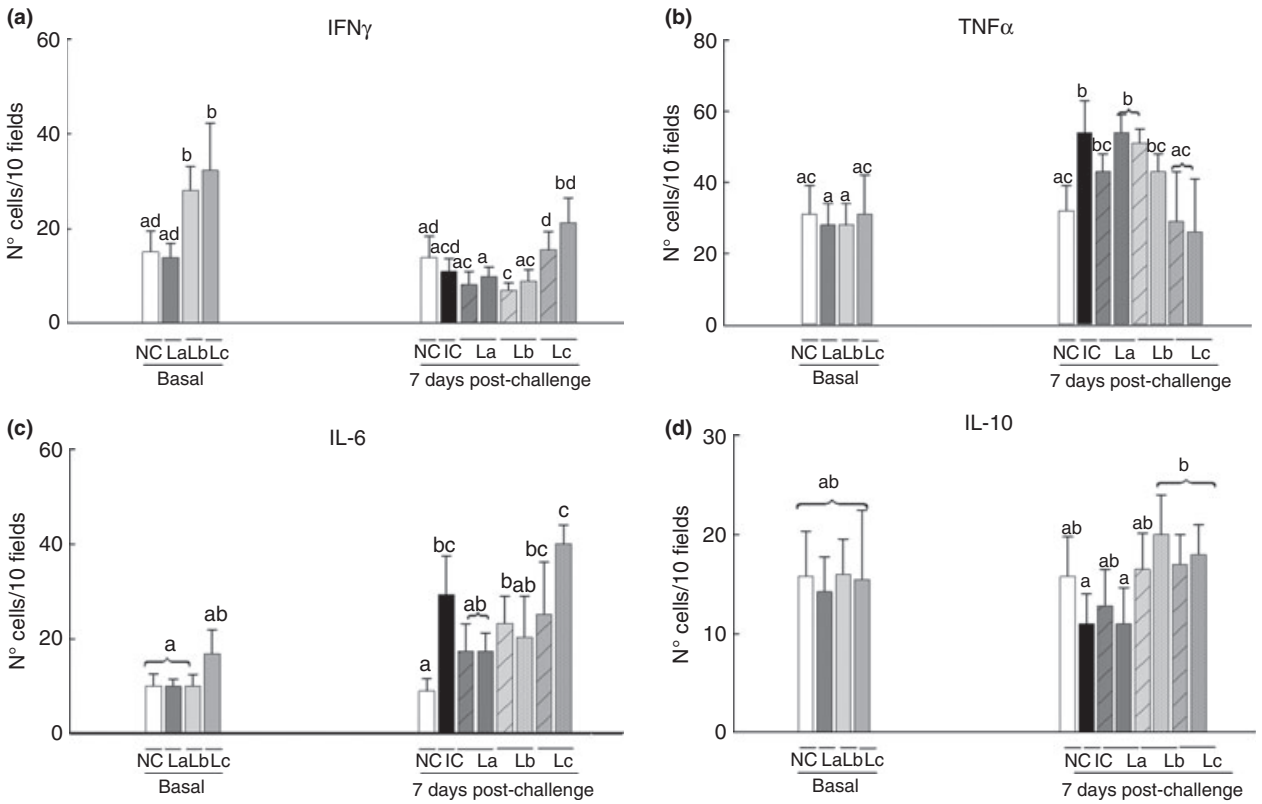

Figure 3 Determination of the number of cytokine-secreting cells in the lamina propria of small intestine obtained from mice treated with different lactobacilli and/or challenged with *Salmonella,* by indirect immunofluorescence. Samples were taken before the infection (basal) and 7 days postchallenge. For the groups that received lactobacilli, diagonal bars represent preventive administration (before the infection), and dotted bars represent continuous feeding (before and after infection). The results are expressed as the means of the total number of positive cells counted in 10 fields at 1000 × magnification. Data correspond to the means ± SD of $N = 9$ animals from three separate experiments. For each cytokine, IFNγ (a), TNFα (b), IL-6 (c) and IL-10 (d), mean values without a common letter differ significantly ($P < 0{\cdot}05$). NC, normal control; IC, infection control; La, *Lactobacillus acidophilus*; Lb, *Lact. bulgaricus*; Lc, *Lact. casei*.

whereas mice fed *Lact. acidophilus* showed no significant increase ($P < 0{\cdot}05$) of phagocytosis compared to NC mice (Table 3).

Determination of IL-6 and MCP-1 levels in culture supernatant of IECs. *In vitro* assay

None of the three strains of lactobacilli assayed induced significant increases in IL-6 secretion by the IECs compared to the NC, while *Salm.* Typhimurium induced an increased release of IL-6 compared to NC or to cells incubated with the different lactobacilli. Nevertheless, IECs significantly increased secretion of MCP-1 after incubation with *Salmonella*, *Lact. casei* or *Lact. bulgaricus*, compared to NC or cells incubated with *Lact. acidophilus* (Fig. 5b). There were no significant differences between the levels of MCP-1 secreted by IECs incubated with *Salmonella*, *Lact. casei* and *Lact. bulgaricus*.

Discussion

It is well documented that oral administration of certain probiotic strains can be effective in the prevention or amelioration of some infectious diseases, mainly at the gastrointestinal level (Song *et al.* 2011; Tsai *et al.* 2011). To contribute the knowledge in this area, we conducted a comparative study of three lactobacilli strains, one probiotic bacterium and two potentiality probiotic strains, selected by the capacity to stimulate the intestinal immunity, to establish which probiotic properties are involved in the protection against *Salm.* Typhimurium infection in a mouse model. Continuous bacterial administration was selected instead of postinfection administration because a previous work using a probiotic fermented milk showed that continuous feeding induced better protection than the one performed after infection (De Moreno De Leblanc *et al.* 2010). The preventive effect of bacterial

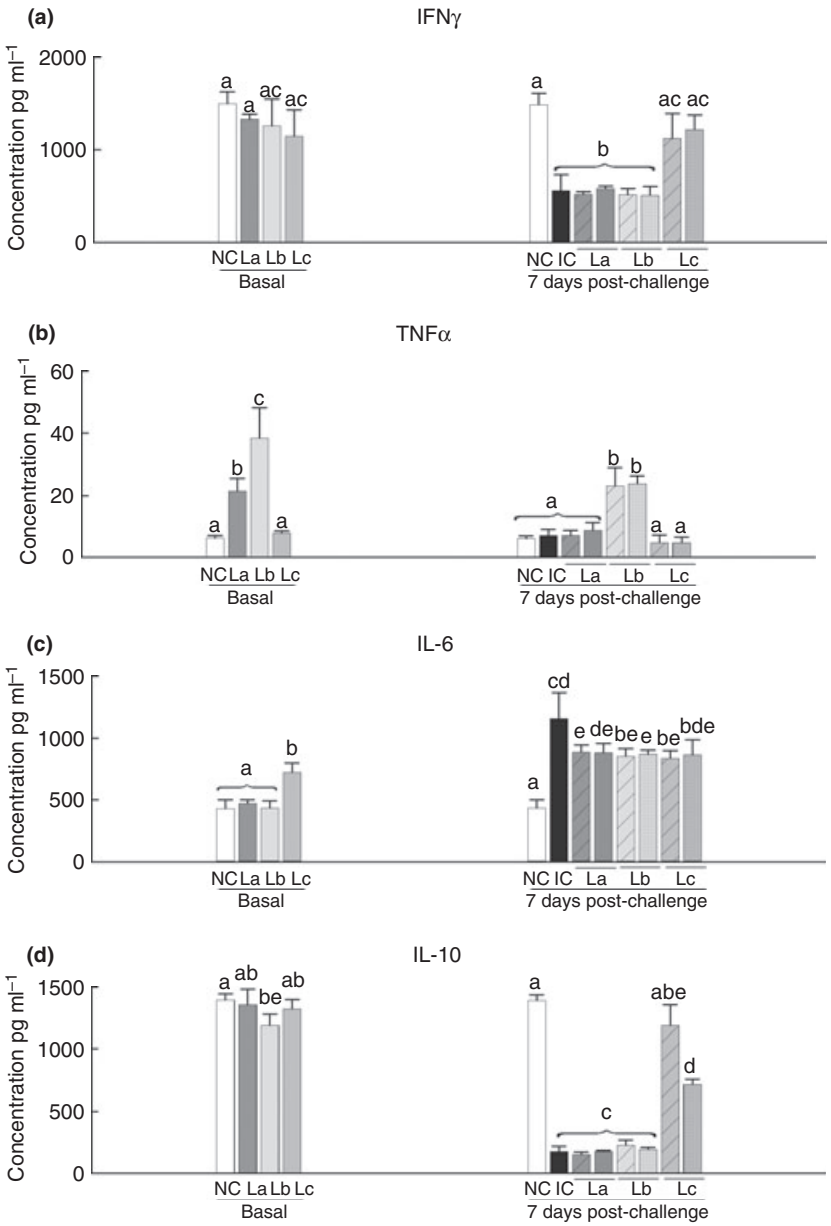

Figure 4 Determination of cytokine concentrations in small intestine fluids obtained from mice treated with the different lactobacilli and/or challenged with *Salmonella* by ELISA test. Samples were taken before the infection (basal) and 7 days postchallenge. For the groups that received lactobacilli, diagonal bars represent preventive administration (before the infection), and dotted bars represent continuous feeding (before and after infection). Data correspond to the means ± SD of $N = 9$ animals from three separate experiments. For each cytokine, IFNγ (a), TNFα (b), IL-6 (c) and IL-10 (d), mean values without a common letter differ significantly ($P < 0{\cdot}05$). NC, normal control; IC, infection control; La, *Lactobacillus acidophilus*; Lb, *Lact. bulgaricus*; Lc, *Lact. casei*.

administration (before *Salmonella* challenge) was also analysed. It was demonstrated that only the continuous administration (previous and postchallenge) of the probiotic strain *Lact. casei* CRL 431 protected against *Salmonella* infection. This protection was related to a lower intestinal inflammatory response, with significant decreases in mortality rates, 7 and 10 days postinfection. Other authors found similar results with another probiotic strain able to attenuate inflammation against *Salmonella* infection in an experimental mouse model (Silva *et al.* 2004). The group fed continuously (previous and postinfection) with *Lact. bulgaricus* CRL 423 also showed a significant decrease of the mortality percentage 7 days postchallenge, which agreed with the lower levels of tissue

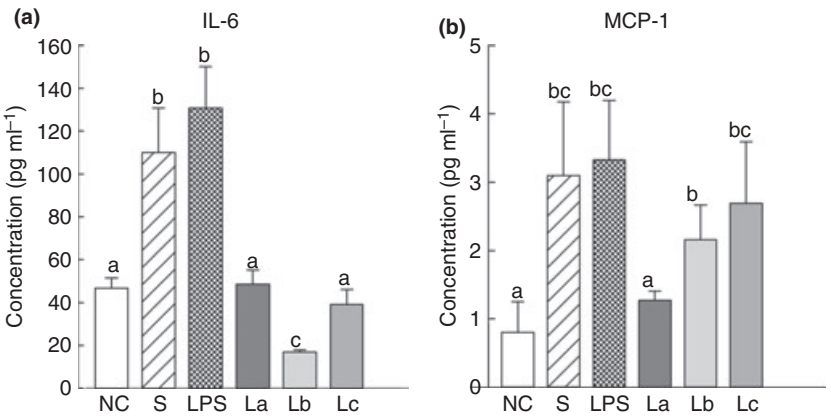

Figure 5 Determination of IL-6 (a) and MCP-1 (b) concentration in culture supernatants of IECs by ELISA. *In vitro* assay. The IECs were isolated from mice of the normal control (NC) group and were incubated (*in vitro*) without stimuli (NC); with *Salmonella* (S) or with the different lactobacilli [*Lactobacillus acidophilus* (La), *Lact. bulgaricus* (Lb) or *Lact. casei* (Lc)]. LPS was used as a positive control. Each bar corresponds to the means ± SD of $N = 9$ data, from three separate experiments. Mean values without a common letter differ significantly ($P < 0{\cdot}05$).

inflammation observed at this time point. Nevertheless, for this group, mortality raised to levels similar to the IC group 10 days postchallenge. Therefore, even though *Lact. bulgaricus* CRL 423 has reported immunomodulatory properties at the intestinal level (Maldonado Galdeano and Perdigon 2004), in this model of infection the administration of this strain was considered ineffective. This result agrees with other reports indicating that the lactobacilli with immunomodulatory properties do not always present *in vivo* protective effects against a particular pathogen (Olah *et al.* 2007).

The analysis of different immunological parameters was performed to know differences in the response induced by the administration of each *lactobacillus* and their relationships with the protection or not against *Salmonella*. Recent studies showed the importance of total S-IgA in the protection of mucosal surfaces by immune exclusion (Mantis and Forbes 2010). Specific S-IgA also plays an important role in the protection against certain pathogens, and its principal role is to protect against re-infection (Allam *et al.* 2011). It was observed that oral administration of *Lact. acidophilus* CRL 730 or *Lact. casei* CRL 431 increased significantly the number of IgA-secreting cells in the lamina propria of the small intestine in healthy mice compared to the control (basal data), but only *Lact. casei* CRL 431 was able to increase the secretion of total S-IgA into the intestinal lumen of the mice. This observation shows that the increase in the number of IgA-secreting cells is not enough to assure high levels of S-IgA and agrees with results obtained by other authors (Macpherson *et al.* 2005; Lara-Villoslada *et al.* 2007). Postchallenge, the most important observation was the significant increase of specific anti-*Salmonella* S-IgA concentration, induced only in the groups given *Lact. casei* CRL 431 compared to the rest of the infected groups. Similar results were found in mice fed with two lactobacilli strains and challenged with *Salm*. Enteritidis (Jain *et al.* 2008). These results demonstrated the importance of the increases of both total S-IgA concentration prior to *Salmonella* challenge and of specific anti-*Salmonella* S-IgA postchallenge to have the best protection. These results agree with the lower spread of the pathogen to the deep tissues and the less inflammatory response found in the animals given *Lact. casei* CRL 431.

According to previous studies where the administration of the three lactobacilli induced different cytokine profiles at the intestinal level of healthy mice (Perdigón *et al.* 2002b), this same analysis was performed in our infection model. Basal sample and 7 days postchallenge were the sample selected for this analysis, and IL-6, IFNγ, TNFα and IL-10 were assessed in all the groups to enquire the role of each cytokine in the effect observed against *Salmonella* and in the subsequent course of disease. TNFα and IFNγ are pro-inflammatory cytokines, which play an important role in immune surveillance but under pathological processes, their increased levels may compromise the epithelial barrier function (Turner 2006). IL-10 is a regulatory cytokine that shows opposite effects to TNFα (Kwon *et al.* 2010) and is required to increase S-IgA secretion (Schultz and Coffman 1991). The increased levels of TNFα in the intestinal fluids of the groups given *Lact. bulgaricus* or *Lact. acidophilus* and the increased number of IFNγ-secreting cells in the mice given *Lact. casei* or *Lact. bulgaricus*, without changes in IL-10 levels before infection, suggest that lactobacilli administration

induces a 'physiological inflammation state' in healthy mice that allows quick and adapted response to an infectious stress (Sansonetti and Medzhitov 2009). IL-6 was also analysed due to its dual role: at low concentrations, it induces S-IgA production, but when it is deregulated, it induces a strong pro-inflammatory response (Naugler and Karin 2008). In our model, it was found that before the infection, only the group fed with *Lact. casei* CRL 431 increased the secretion of IL-6 compared to the NC mice, and this enhancement was accompanied by increased levels of total S-IgA.

After infection, the more relevant variations in cytokine profiles were observed. The significant increases in the number of TNFα-secreting cells and/or in the release of this cytokine to the intestinal fluid were accompanied by significant decreases of IL-10 levels in the IC group and in the groups fed previous to the infection with *Lact. bulgaricus* or previous and postinfection with *Lact. acidophilus*, indicating an important inflammatory process developed in these groups, which agrees with the histological changes observed. In contrast, the mice fed continuously with *Lact. bulgaricus* presented a higher number of IL-10-secreting cells in comparison with the groups previously mentioned, and in this group, milder histological changes were observed. Finally, in mice fed with *Lact. casei* CRL 431 (previous and continuous administration), TNFα production was significantly lower, and IL-10 levels were significantly higher than the IC group. This cytokine profile appears to be involved in the protection observed against *Salm.* Typhimurium. As regard to IFNγ, all the groups challenged with *Salmonella* showed a significant decrease of this cytokine, except the group fed continuously with *Lact. casei*, which maintained an increased number of IFNγ-producing cells in the lamina propria and increased secretion to the intestinal lumen. Previous reports demonstrate that IFNγ is a cytokine involved in a series of innate and acquired immune defence mechanisms of the host, including the increased expression of the secretory component necessary for IgA secretion; it stimulates macrophages and restrains the intracellular replication of pathogens by induction of microbicidal activity dependent on NADPH oxidase and iNOS-mediated activity, thus limiting bacterial dissemination (Vazquez-Torres *et al.* 2000; Rosenberger and Finlay 2002). In agreement with these previous reports, the IFNγ increase in the group fed continuously with *Lact. casei* was accompanied by increases in S-IgA secretion and decreased pathogen dissemination to deeper tissues. With regard to IL-6, all the infected groups showed a significant increase in the release of this cytokine into the small intestine fluid, compared to the basal data, being the highest levels obtained from IC group. These results, accompanied by increased release of IL-10 in mice given *Lact. casei* CRL 431, show that the probiotic administration was able to maintain an increased production of certain cytokines in the intestine, which could be used by the host when would be required but was modulated to avoid an inflammatory process.

The effect of lactobacilli administration on macrophage activation, through the assessment of their phagocytic activity, was evaluated (Table 4). The results showed that *Lact. bulgaricus* CRL 423 and *Lact. casei* CRL 431 stimulated macrophages obtained from Peyer's patches, peritoneum and spleen, by inducing an increase in their phagocytic activity against *Saccharomyces* spp. These results agree with previous reports, where the oral administration of specific *Lactobacillus* strains enhanced phagocytic activity of different leucocytes (Schiffrin *et al.* 1995). Although *Lact. bulgaricus* CRL 423 was able to stimulate these cells of the innate immunity, the lack of protection observed in the mice that received this lactobacillus suggests that the macrophage activation by itself is not enough to confer protection against *Salmonella* infection. The macrophage activation is only a step to afford a protective effect against this pathogen.

Finally, the stimulation of the IEC was analysed considering that these cells are in constant contact with micro-organisms that inhabit or come into the intestinal lumen. They act as important 'sentinels' of the mucosal immune system against infections and possibly expand the local inflammatory response through secretion of cytokines and chemokines, while limiting the inflammatory response to the normal microbiota (Kagnoff and Eckmann 1997; Parlesak *et al.* 2004). Probiotic bacterial suspensions administered orally may

Table 4 Influence of lactobacilli administration on phagocytic activity of macrophages

Group	Peritoneal macrophages	Peyer's patches macrophages	Spleen macrophages
NC	19 ± 4	18 ± 4	16 ± 1
Lactobacillus acidophilus	19 ± 3	22 ± 6	18 ± 3
Lact. bulgaricus	29 ± 3*	40 ± 6*	29 ± 6*
Lact. casei	33 ± 1*	37 ± 1*	23 ± 3*

NC, normal control group.

Basal samples (after 7 days of bacterial administration) were used to isolate macrophages from peritoneum, Peyer's patches and spleen of mice. The activity of these cells was determined by phagocytosis of *Saccharomyces boulardii*. The mean values correspond to the percentage of phagocyting macrophages in 200 counted cells. Data correspond to the means ± SD of $N = 9$ animals obtained from three separate experiments.

*Mean values were significantly different from those of the NC group ($P < 0{\cdot}05$).

interact with the intestinal mucosa, resulting in activation of IECs to induce cytokine release (Vinderola *et al.* 2005).

In our work, it was observed that *Salm.* Typhimurium was the only bacterium able to induce a significant increase of IL-6 levels by the IECs compared to control cells, or to the cells incubated with different lactobacilli. The lack of response observed in the IECs after incubation with the lactobacilli strains could be related to an adaptive tolerance developed in the small intestine against these bacteria, to prevent an inflammatory reaction and show that these cells are not responsible for the IL-6 increases observed in the previous analysis. Similar results were reported in previous studies with *Lact. casei*, *Lact. helveticus* and the enteropathogen *Escherichia coli*, where only the stimulus with *E. coli* was able to induce a significant increase of IL-6 levels produced by a culture of IECs (Vinderola *et al.* 2005). Other studies also found no increase in the secretion of IL-6 after contact of several lactobacillus strains with Caco-2 cells co-cultured with leucocytes (Morita *et al.* 2002).

The monocyte chemotactic protein MCP-1 is a chemokine dependent of MyD88 pathway, responsible for the recruitment of monocytes, dendritic cells and memory T lymphocytes (Rydstrom and Wick 2009). In the present study, it was observed that IECs significantly increased MCP-1 secretion after *Lact. casei* or *Lact. bulgaricus* co-incubation and after incubation with *Salmonella*, compared to control cells. The increases of MCP-1 after *Salmonella in vitro* incubation agreed with an *in vivo* study where oral infection with *Salmonella* recruited phagocytes in the Peyer's patches and mesenteric lymph nodes, and this recruitment correlated with increased MCP-1 levels (Rydstrom and Wick 2009) and shows also the importance of macrophage recruitment for the protective effect of the lactobacillus administration.

Conclusion

The present work showed the comparative effect of the three lactobacilli tested (*Lact. acidophilus* CRL 730, *Lact. bulgaricus* CRL 423 and *Lact. casei* CRL 431), in the protection against *Salmonella* infection. Only the probiotic strain *Lact. casei* CRL 431 was able to protect against this pathogen, by increasing the intestinal barrier function and by decreasing the local inflammatory response. This strain could be useful as an oral mucosal adjuvant to protect against pathogens as *Salmonella*.

Although *Lact. bulgaricus* CRL 423 showed immunostimulating properties, through the activation of macrophages and increased levels of certain cytokines, these parameters were not enough to control the pathogen-induced infection.

The results obtained indicate that in the defence mediated by lactobacilli against *Salmonella*, the protective capacity depends on a set of related effects. Among them, the increases of total S-IgA and specific S-IgA are essential. We believe that this fact could be considered as a first biological marker for selection of probiotic strains, for the prevention or amelioration of *Salmonella* infections. Another relevant parameter would be the induction of a cytokine profile with high levels of IFNγ, regulated increases of IL-6 and not increased, but sustained levels of IL-10 and TNFα. Intestinal epithelial cells also play a significant role in the protective activity of a probiotic against *Salmonella* through MCP-1 release, which increases macrophage infiltration, instead of PMN cells, in lamina propria of the small intestine.

The results obtained also reinforce the theory that probiotic efficacy against a given pathogen is strain specific and depends on the biology of the infection induced by the pathogen. Further studies performed in IECs and other immune cells such as macrophages and dendritic cells are currently underway to find new explanations about the effect of each *Lactobacillus* strain in the protection against *Salmonella*.

Acknowledgements

This work was financially supported by Consejo de Investigación de la Universidad Nacional de Tucumán (CIUNT 26/D442), and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 0652), Argentina. None of the authors have a conflict of interest for the publication of the manuscript.

References

Allam, U.S., Krishna, M.G., Lahiri, A., Joy, O. and Chakravortty, D. (2011) Salmonella enterica serovar Typhimurium lacking hfq gene confers protective immunity against murine typhoid. *PLoS One* **6**, e16667.

Castillo, N.A., Perdigon, G. and De Moreno De Leblanc, A. (2011) Oral administration of a probiotic Lactobacillus modulates cytokine production and TLR expression improving the immune response against *Salmonella enterica* serovar Typhimurium infection in mice. *BMC Microbiol* **11**, 177.

Chaves, S., Perdigon, G. and De Moreno De Leblanc, A. (2011) Yoghurt consumption regulates the immune cells implicated in acute intestinal inflammation and prevents the recurrence of the inflammatory process in a mouse model. *J Food Prot* **74**, 801–811.

Cremonini, F., Di Caro, S., Covino, M., Armuzzi, A., Gabrielli, M., Santarelli, L., Nista, E.C., Cammarota, G. *et al.* (2002)

Effect of different probiotic preparations on anti-helicobacter pylori therapy-related side effects: a parallel group, triple blind, placebo-controlled study. *Am J Gastroenterol* **97**, 2744–2749.

De Moreno De Leblanc, A., Castillo, N.A. and Perdigon, G. (2010) Anti-infective mechanisms induced by a probiotic Lactobacillus strain against *Salmonella enterica* serovar Typhimurium infection. *Int J Food Microbiol* **138**, 223–231.

De Vrese, M., Rautenberg, P., Laue, C., Koopmans, M., Herremans, T. and Schrezenmeir, J. (2005) Probiotic bacteria stimulate virus-specific neutralizing antibodies following a booster polio vaccination. *Eur J Nutr* **44**, 406–413.

Dogi, C.A. and Perdigón, G. (2006) Importance of the host specificity in the selection of probiotic bacteria. *J Dairy Res* **73**, 357–366.

Galdeano, C.M. and Perdigón, G. (2006) The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. *Clin Vaccine Immunol* **13**, 219–226.

Gaon, D., Garcia, H., Winter, L., Rodriguez, N., Quintas, R., Gonzalez, S.N. and Oliver, G. (2003) Effect of Lactobacillus strains and *Saccharomyces boulardii* on persistent diarrhea in children. *Medicina (B Aires)* **63**, 293–298.

Gauffin Cano, P. and Perdigón, G. (2003) Probiotics induce resistance to enteropathogens in a re-nourished mouse model. *J Dairy Res* **70**, 433–440.

Gauffin Cano, P., Aguero, G. and Perdigón, G. (2002) Immunological effects of yogurt addition to a re-nutrition diet in a malnutrition experimental model. *J Dairy Res* **69**, 303–316.

Heselmans, M., Reid, G., Akkermans, L.M., Savelkoul, H., Timmerman, H. and Rombouts, F.M. (2005) Gut flora in health and disease: potential role of probiotics. *Curr Issues Intest Microbiol* **6**, 1–7.

Jain, S., Yadav, H., Sinha, P.R., Naito, Y. and Marotta, F. (2008) Dahi containing probiotic *Lactobacillus acidophilus* and *Lactobacillus casei* has a protective effect against *Salmonella enteritidis* infection in mice. *Int J Immunopathol Pharmacol* **21**, 1021–1029.

Kagnoff, M.F. and Eckmann, L. (1997) Epithelial cells as sensors for microbial infection. *J Clin Invest* **100**, 6–10.

Kwon, H.K., Lee, C.G., So, J.S., Chae, C.S., Hwang, J.S., Sahoo, A., Nam, J.H., Rhee, J.H. *et al.* (2010) Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. *Proc Natl Acad Sci U S A* **107**, 2159–2164.

Lara-Villoslada, F., Sierra, S., Boza, J., Xaus, J. and Olivares, M. (2007) [Beneficial effects of consumption of a dairy product containing two probiotic strains, *Lactobacillus coryniformis* CECT5711 and *Lactobacillus gasseri* CECT5714 in healthy children]. *Nutr Hosp* **22**, 496–502.

Lebeer, S., Vanderleyden, J. and De Keersmaecker, S.C. (2008) Genes and molecules of lactobacilli supporting probiotic action. *Microbiol Mol Biol Rev* **72**, 728–764. Table of Contents.

Leblanc, J., Fliss, I. and Matar, C. (2004) Induction of a humoral immune response following an *Escherichia coli* O157:H7 infection with an immunomodulatory peptidic fraction derived from *Lactobacillus helveticus*-fermented milk. *Clin Diagn Lab Immunol* **11**, 1171–1181.

Macpherson, A.J., Geuking, M.B. and Mccoy, K.D. (2005) Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria. *Immunology* **115**, 153–162.

Maldonado Galdeano, C. and Perdigon, G. (2004) Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation. *J Appl Microbiol* **97**, 673–681.

Mantis, N.J. and Forbes, S.J. (2010) Secretory IgA: arresting microbial pathogens at epithelial borders. *Immunol Invest* **39**, 383–406.

Maragkoudakis, P.A., Chingwaru, W., Gradisnik, L., Tsakalidou, E. and Cencic, A. (2010) Lactic acid bacteria efficiently protect human and animal intestinal epithelial and immune cells from enteric virus infection. *Int J Food Microbiol* **141**(Suppl. 1), S91–S97.

Morita, H., He, F., Fuse, T., Ouwehand, A.C., Hashimoto, H., Hosoda, M., Mizumachi, K. and Kurisaki, J. (2002) Adhesion of lactic acid bacteria to caco-2 cells and their effect on cytokine secretion. *Microbiol Immunol* **46**, 293–297.

Naugler, W.E. and Karin, M. (2008) The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. *Trends Mol Med* **14**, 109–119.

Olah, A., Belagyi, T., Poto, L., Romics, L. Jr and Bengmark, S. (2007) Synbiotic control of inflammation and infection in severe acute pancreatitis: a prospective, randomized, double blind study. *Hepatogastroenterology* **54**, 590–594.

Parlesak, A., Haller, D., Brinz, S., Baeuerlein, A. and Bode, C. (2004) Modulation of cytokine release by differentiated CACO-2 cells in a compartmentalized coculture model with mononuclear leucocytes and nonpathogenic bacteria. *Scand J Immunol* **60**, 477–485.

Perdigón, G., Alvarez, S. and Medici, M. (1992) Systemic and local augmentation of the immune response in mice by feeding with milk fermented with Lactobacillus acidophilus and/or Lactobacillus casei. In *Foods, Nutrition, and Immunity: Effects of Dairy and Fermented Milk Products: 2nd Bio-Inova/EIBET Workshop* eds Paubert-Braquet, M., Dupont, G. and Paoletti, R. pp. 66–76. Paris: Karger.

Perdigón, G., De Moreno De Leblanc, A., Valdez, J. and Rachid, M. (2002a) Role of yoghurt in the prevention of colon cancer. *Eur J Clin Nutr* **56**(Suppl. 3), S65–S68.

Perdigón, G., Maldonado Galdeano, C., Valdez, J.C. and Medici, M. (2002b) Interaction of lactic acid bacteria with the gut immune system. *Eur J Clin Nutr* **56**(Suppl. 4), S21–S26.

Rizzardini, G., Eskesen, D., Calder, P.C., Capetti, A., Jespersen, L. and Clerici, M. (2011) Evaluation of the immune

benefits of two probiotic strains *Bifidobacterium animalis* ssp. *lactis*, BB-12(R) and *Lactobacillus paracasei* ssp. *paracasei*, *L. casei* 431(R) in an influenza vaccination model: a randomised, double-blind, placebo-controlled study. *Br J Nutr* **107**, 876–884.

Rosenberger, C.M. and Finlay, B.B. (2002) Macrophages inhibit Salmonella typhimurium replication through MEK/ERK kinase and phagocyte NADPH oxidase activities. *J Biol Chem* **277**, 18753–18762.

Rydstrom, A. and Wick, M.J. (2009) Monocyte and neutrophil recruitment during oral Salmonella infection is driven by MyD88-derived chemokines. *Eur J Immunol* **39**, 3019–3030.

Sainte-Marie, G. (1961) A paraffin embedding technique for studies employing immunofluorescence. *J Histochem Cytochem* **25**, 0–256.

Sansonetti, P.J. and Medzhitov, R. (2009) Learning tolerance while fighting ignorance. *Cell* **138**, 416–420.

Santos, R.L., Zhang, S., Tsolis, R.M., Kingsley, R.A., Adams, L. G. and Baumler, A.J. (2001) Animal models of Salmonella infections: enteritis versus typhoid fever. *Microbes Infect* **3**, 1335–1344.

Schiffrin, E.J., Rochat, F., Link-Amster, H., Aeschlimann, J.M. and Donnet-Hughes, A. (1995) Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. *J Dairy Sci* **78**, 491–497.

Schultz, C.L. and Coffman, R.L. (1991) Control of isotype switching by T cells and cytokines. *Curr Opin Immunol* **3**, 350–354.

Silva, A.M., Barbosa, F.H., Duarte, R., Vieira, L.Q., Arantes, R. M. and Nicoli, J.R. (2004) Effect of *Bifidobacterium longum* ingestion on experimental salmonellosis in mice. *J Appl Microbiol* **97**, 29–37.

Song, H.J., Kim, J.Y., Jung, S.A., Kim, S.E., Park, H.S., Jeong, Y., Hong, S.P., Cheon, J.H. *et al.* (2011) Effect of probiotic *Lactobacillus* (Lacidofil(R) cap) for the prevention of antibiotic-associated diarrhea: a prospective, randomized, double-blind, multicenter study. *J Korean Med Sci* **25**, 1784–1791.

Tsai, C.C., Liang, H.W., Yu, B., Hsieh, C.C., Hwang, C.F., Chen, M.H. and Tsen, H.Y. (2011) The relative efficacy of different strain combinations of lactic acid bacteria in the reduction of populations of *Salmonella enterica* Typhimurium in the livers and spleens of mice. *FEMS Immunol Med Microbiol* **63**, 44–53.

Turner, J.R. (2006) Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application. *Am J Pathol* **169**, 1901–1909.

Valdez, J., Rachid, M., Gobbato, N. and Perdigón, G. (2001) Lactic acid bacteria induce apoptosis inhibition in *Salmonella typhimurium* infected macrophages. *Food Agric Immunol* **13**, 189–197.

Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H. and Fang, F.C. (2000) Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J Exp Med* **192**, 227–236.

Vinderola, G., Matar, C. and Perdigón, G. (2005) Role of intestinal epithelial cells in immune effects mediated by gram-positive probiotic bacteria: involvement of toll-like receptors. *Clin Diagn Lab Immunol* **12**, 1075–1084.

Vlieger, A.M., Robroch, A., Van Buuren, S., Kiers, J., Rijkers, G., Benninga, M.A. and Te Biesebeke, R. (2009) Tolerance and safety of *Lactobacillus paracasei* ssp. *paracasei* in combination with *Bifidobacterium animalis* ssp. *lactis* in a prebiotic-containing infant formula: a randomised controlled trial. *Br J Nutr* **102**, 869–875.

Yan, F., Cao, H., Cover, T.L., Whitehead, R., Washington, M. K. and Polk, D.B. (2007) Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. *Gastroenterology* **132**, 562–575.