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Milk fermented by *Lactobacillus helveticus* R389 and its non-bacterial fraction confer enhanced protection against *Salmonella enteritidis* serovar Typhimurium infection in mice

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Abstract

Bacterial infections in the gastrointestinal tract represent a major global health problem, even in the presence of normally effective mucosal immune mechanisms. Milk fermented by *Lactobacillus helveticus* R389 (FM) or its non-bacterial fraction obtained by milk fermentation at controlled pH 6 (NBF) are able to activate the small intestine mucosal immune response according to previous studies. In this work we aimed at comparing their protection capacity against an infection by *Salmonella enteritidis* serovar Typhimurium and at studying the mechanisms involved. In a completely randomized design, BALB/c mice received FM or NBF for 2, 5 or 7 consecutive days, followed by a single oral challenge with *S.* Typhimurium (10^7 cells/mouse). The increase in the number of IgA+ cells in the lamina propria of the small intestine, after the feeding periods, was accompanied by an increase in the luminal content of total S-IgA. However, no antibodies were produced against the NBF. In mice given the FM or the NBF for 7 consecutive days, lower levels of liver colonization on day 7 post-challenge with *S.* Typhimurium, higher luminal contents of specific anti-*Salmonella* S-IgA, higher percentages of survival to infection and lower numbers of MIP-1α+ cells in the lamina propria were observed. In this work we observed that in both the FM or the NBF there are active principles that confer enhanced protection against *S.* Typhimurium infection. However, the mechanisms underlying mucosal immunomodulation and protection are different. In those mechanisms, the mucosal immune response would seem to be more involved than the competitive or exclusion mechanisms between *L. helveticus* R389 and *S. enteritidis* serovar Typhimurium.

Keywords: Fermented milks; Lactic acid bacteria; Mucosal immune system; Non-bacterial fraction; *Salmonella enteritidis* serovar Typhimurium

Introduction

Bacterial infections in the gastrointestinal tract represent a major global health problem, even in the presence of normally effective mucosal immune mechanisms, and are

Abbreviations: FM, milk fermented by *L. helveticus* R389; NBF, non-bacterial fraction of milk fermented by *L. helveticus* R389 (at controlled pH 6)

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important targets for vaccine development. Enteric tract infections are among the leading causes of illness and death worldwide, especially in infants and preschool children in developing countries (Holmgren and Svennerholm, 2004). *Salmonella* serotypes are associated with three different human disease syndromes: bacteremia, typhoid fever and enterocolitis. Mouse septicemia after the oral administration of *S.* Typhimurium is generally considered a reasonable reflection of typhoid fever in *S.* Typhi infected patients (Zhang et al., 2003).

The original definition of probiotics given by Fuller (1989) states that probiotics are live microbial feed additions that beneficially affect the host by improving its intestinal microbial balance. The definition gave by FAO/WHO (2002) established that probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. However, these definitions were broadened by Lee et al. (1999), who stated that probiotics are preparations of viable cells or foods containing viable bacterial cultures or components of bacterial cells that exert beneficial effects on the health of the host. Many probiotics have been reported to be useful in the treatment of disturbed intestinal microflora and diarrheal diseases (Asahara et al., 2004). It has been suggested that the possible actions of probiotics against enteric pathogens involve a pH reduction in the gut, direct antagonism by production of antimicrobial compounds, competition for adhesion sites and available nutrients and stimulation of the immune system (Collins and Gibson, 1999). Shu et al. (2000) and Gill et al. (2001) reported that selected probiotic strains orally administered to mice conferred enhanced resistance against *Salmonella enteritidis* serovar Typhimurium infection. In our laboratory, it was demonstrated that the oral administration of milk fermented by *Lactobacillus casei* and *Lactobacillus acidophilus* (Perdigón et al., 1990), yoghurt (Perdigón et al., 1991) or its microflora (Valdez et al., 2001) and the strain *L. casei* CRL 431 itself (Perdigón et al., 1993, 1995) were effective in the prevention of *S.* Typhimurium infection in conventional and in malnourished/re-nourished mice (Gauffin-Cano and Perdigón, 2003). Not only fermented milks or lactic acid bacteria were reported to activate immune cells and to confer enhanced protection against enteropathogens. Ng and Griffiths (2002) reported that the release of cytokines by macrophages could be enhanced by cell-free fractions of fermented milks. LeBlanc et al. (2004) showed that the peptide fraction derived from milk fermented by *L. helveticus* R389 was effective in improving the humoral response against *E. coli* O157:H7. In a previous work (Vinderola et al., 2006a), we observed that the microflora derived from the fermented milk kefir as well as its non-bacterial fraction induced different cytokine patterns in peritoneal macrophages and in adherent cells from Peyer's patches. Milk fermented by *L. helveticus* R389 has the capacity to activate the immune system associated to the gut as well as at distal sites (Matar et al., 2001; LeBlanc et al., 2002), including the mammary glands (de Moreno de LeBlanc et al., 2005). In previous works (Matar et al., 2001; Vinderola et al., 2006b), we observed the capacity of the milk fermented by *L. helveticus* R389 and its non-bacterial fraction to activate the small intestine mucosal immune response. These previous studies led us to analyze the effects, against a *Salmonella* infection, of milk fermented by *L. helveticus* R389 obtained as previously reported (Matar et al., 2001) and of the non-bacterial fraction of a milk fermented by the same strain in a fermentation where pH was kept at a value of 6 in order to increase the release of peptides, as previously reported (Vinderola et al., 2006b). In this work we aimed at assessing whether or not the major productions of metabolites induced in special conditions of fermentation are able to confer enhanced protection against a *S.* Typhimurium infection and at studying some of the mechanisms involved in the protection against infection to determine the contribution of the potentially bioactive metabolites contained in the non-bacterial fraction of a fermented milk.

Materials and methods

Animals and bacterial strains

Six-to-eight week-old BALB/c mice weighing from 20 to 25 g were obtained from the random bred colony kept by our department at CERELA.

The strain *L. helveticus* R389, isolated from Swiss cheese, was used for this study. Overnight cultures (37 °C, aerobic conditions) were obtained in 12% skimmed milk (Difco, Becton Dickinson and Company, Sparks, MD, USA) or in MRS broth (Britania, Buenos Aires, Argentina).

A human isolate of *S. enteritidis* serovar Typhimurium was kindly provided by the Departamento de Bacteriología of the Instituto de Microbiología (Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán). The strain was maintained at −80 °C in Brain Heart Infusion (BHI) broth (Britania, Buenos Aires, Argentina) with 12% glycerol. Overnight cultures (37 °C, aerobiosis) were obtained in BHI broth.

Samples for the feeding trials

The samples were: (1) milk fermented by *L. helveticus* R389 (from now on referred to as FM), obtained by the inoculation (2%) of a fresh (overnight 18 h) culture of this strain into 12% skimmed milk followed by overnight incubation (18 h, 37 °C, aerobiosis); this fermented milk was prepared daily for the feeding trials; (2) the

non-bacterial fraction from milk fermented by *L. helveticus* R389 (from now on referred to as NBF). To obtain the NBF, 12% skimmed milk (1 l) was inoculated (2%) with an overnight (18 h) culture of *L. helveticus* R389 and fermented using a Bioflo Model 70 13 70 biofermentor (New Brunswick Scientific) at 37 °C with an agitation rate of 100 rpm and CO_2 spurging (10 lb/in^2; 0.2 l/min). The pH was buffered to 6.00 by the automatic addition of 8 M NaOH as required during the fermentation. In order to obtain the non-bacterial fraction of the fermented milk, the pH was dropped to 3.7 with 85% DL-lactic acid syrup (Sigma-Aldrich, St. Louis, MO, USA) and the fermented milk was then centrifuged (3500*g*, 4 °C 15 min, IEC B-22 M centrifuge) and the supernatant (non-bacterial fraction) was kept frozen (−80 °C) until use. An enumeration of *L. helveticus* was performed on MRS agar (37 °C aerobics, 72 h incubation) to determine the presence of viable cells in samples of FM or NBF (prior to conservation at −80 °C). The protein content of the non-bacterial fraction was determined by the Bradford technique (Quick Start Bradford Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

Feeding procedures and challenge with *S.* Typhimurium

A completely randomized design was chosen for this study. Groups of mice (5 animals/group) received the FM, in replacement of drinking water, for 2, 5 or 7 consecutive days. Other groups of mice received the NBF by gavage (intragastrical intubation) (100 μg of total protein/day/mouse) for 2, 5 or 7 consecutive days and drinking water ad libitum. The control group received 12% non-fat milk (NFM). Mice from control group or from FM group also received 200 μl of drinking water daily by gavage to account for the possible stress imposed by the gavage technique. All animals received, simultaneously and ad libitum, a sterile conventional balanced diet (proteins 230 g/kg, raw fiber 60 g/kg, total minerals 100 g/kg, Ca 13 g/kg, P 8 g/kg, moisture and vitamins 120 g/kg). Animals were fed in a way that treated mice (2, 5 or 7 days of FM or NBF administration) and control animals were sacrificed on the same day. Each experimental group consisted of 5 mice housed in metallic cages kept in a controlled atmosphere (temperature 22±2 °C; humidity 55±2%) with a 12 h light/dark cycle.

Colonization assay and morbidity after *Salmonella enteritidis* serovar Typhimurium infection

Groups of animals (5 animals/group) that received the FM or the NBF for 2, 5 or 7 consecutive days and control mice were orally challenged with a single dose of a fresh culture (5 h incubation, 37 °C, aerobiosis) of *Salmonella enteritidis* serovar Typhimurium (10^7 cells/mouse). For the colonization assay, the liver was removed on days 4 and 7 post-challenge. Then it was homogenized in 0.1% peptone water and serial dilutions (10^{-1}–10^{-4}) were plated in MacConkey agar. Plates were incubated at 37 °C for 48 h. Results from five animals for each day post-challenge were expressed as the number (log) of CFU/liver, since the liver is an organ normally devoid of bacteria. For the morbidity studies, groups of mice (15 mice/group) received the FM or the NBF for 2, 5 or 7 consecutive days in the conditions described above. A control group (15 mice) received 12% skimmed milk. Treated and control groups were challenged in the same way with *S.* Typhimurium. Results were expressed as the percentage of animals that survived the infection.

Determination by Double Antibody Sandwich-Enzyme-Linked Immuno Sorbant Assay (DAS-ELISA) of the luminal content of total secretory IgA (S-IgA)

After each feeding period (2, 5 or 7 consecutive days) with the FM or with the NBF, treated and control animals were anesthetized and sacrificed by cervical dislocation. The small intestine from each mouse was recovered (by truncation at the stomach/duodenum junction and the ileum/ascending colon junction) and its content was flushed with 5 ml PBS. Particulate material was removed by centrifugation (10,000*g* for 10 min at 4 °C). The supernatant fluid was stored (−80 °C) until use. Total IgA antibodies were detected by standard Double Antibody Sandwich Enzyme-Linked Immuno Sorbant Assay (DAS-ELISA). Briefly, affinity purified monoclonal goat anti-IgA (α-chain specific, Sigma) was added (50 μl/well) at 1.25 μg/well in 50 mM carbonate–bicarbonate buffer pH 9.6 to 96-well, flat-bottomed Nunc-Immuno Plate™ Maxi Sorp™ plates (Nalge Nunc International, USA) and incubated at 37 °C for 1 h. The plates were then washed three times with PBS containing 0.05% Tween-20 (PBS-T) and blocked for 1 h at 25 °C with 0.5% skimmed milk in PBS. Plates were washed (5 times, PBS-T) and incubated for 2 h at 37 °C with 50 μl of different dilutions of standard kappa IgA (Sigma) (for the construction of the calibration curve) or 50 μl of serially diluted samples (in PBS 0.5% skimmed milk) of the intestinal fluid. Plates were washed (7 times, PBS-T) and incubated in the presence of 50 μl/well of anti-mouse IgA (α chain specific) peroxidase conjugate (Sigma) at 1.25 μg/well for 1 h at 37 °C. Plates were washed (7 times, PBS-T) and 100 μl of the substrate (4 g *o*-phenylenediamine dihydrochloride/l in 0.05 M phosphate-citrate buffer, pH 5) were added to each well.

The reaction was stopped 20 min later by the addition of 100 µl of 2 N H_2SO_4/well. The absorbance (493 nm) was read in an ELISA microplate reader (Versa max, Molecular Devices).

Determination of secretory-IgA (S-IgA) specific against *S.* Typhimurium by ELISA in the intestinal fluid of infected animals

The ELISA test was performed in the fluid of the small intestine of control and treated animals on days 4 and 7 after the infection with *S.* Typhimurium to determine the presence of specific S-IgA against the enteropathogen. A fresh culture of *S.* Typhimurium was washed twice with PBS, resuspended to a concentration of 5×10^9 CFU/ml in PBS solution and heat-inactivated (80 °C, 30 min for 3 consecutive days). ELISA plates were coated with 50 µl/well of the suspension of heat-killed *S.* Typhimurium to a concentration of 5×10^8 CFU/ml in 0.1 M Na_2CO_3 buffer, pH 9.6. Plates were incubated overnight at 5 °C and then washed twice with PBS-T (200 µl/well) and blocked (50 µl/well) with 0.5% skimmed milk in PBS (1 h, room temperature). Plates were washed 3 times with PBS-T and different dilutions (1/10, 1/100 or 1/1000) of the intestinal fluid were added to each well in triplicate. Plates were incubated for 1 h at 37 °C and washed 5 times with PBS-T. Fifty microliter of an anti-mouse IgA (α-chain specific, Sigma) conjugated with peroxidase were added to each well (1.25 µg/well). Plates were incubated for 1 h at 37 °C and then washed seven times with PBS-T. A hundred microliter of the substrate (4 g *o*-phenylene-diamine dihydrochloride/l in 0.05 M phosphate-citrate buffer, pH 5) were added to each well. The reaction was stopped after 20 min with 100 µl of 2 N H_2SO_4. The absorbance (493 nm) was read in an ELISA microplate reader (Versa max, Molecular Devices). Results were expressed as ng of anti-*S.* Typhimurium IgA/ml using a standard curve.

Immunofluorescence test for B population (IgA + cells) identification

After each feeding period (2, 5 or 7 consecutive days) and at days 4 and 7 post-infection with *S.* Typhimurium, treated and control animals were anesthetized and sacrificed by cervical dislocation. The small intestine was removed for histological preparation following Sainte-Marie's technique (Sainte-Marie, 1962) for paraffin inclusion. The number of IgA producing (IgA +) cells was determined on histological slices of samples from the ileum region near Peyer's patches of mice that received the FM or the NBF at the end of each feeding period or on days 4 and 7 post-challenge with *S.* Typhimurium. The immunofluorescence test was performed using α-chain specific anti-mouse IgA FITC conjugate (Sigma). Histological slices were deparaffinized and rehydrated in a graded ethanol series. Deparaffinized histological samples were incubated with a dilution (1/100) of the antibody in PBS solution and incubated in the dark for 30 min at 37 °C. Then, samples were washed two times with PBS solution and examined using a fluorescent light microscope. The results were expressed as the number of IgA + cells (positive: fluorescent cell) per 10 fields (magnification 1000 ×). Data represent the mean of three histological slices for each animal (5 animals per group) for each feeding period.

Immunofluorescence test for MIP-1α + cells identification

The number of MIP-1α + cells (Macrophage Inflammatory Protein-1α) was studied by an indirect immunofluorescence method on histological slices of the small intestine of mice that received the FM or the NBF at the end of each feeding period and on days 4 and 7 post-challenge with *S.* Typhimurium. Histological slices were deparaffinized and rehydrated in a graded series of ethanol, and then incubated for 30 min in a 1% blocking solution of BSA (Jackson Immuno Research, West Grove, PA, USA) at room temperature. Histological slices were then incubated for 60 min at 37 °C with rabbit anti-mouse MIP-1α (Peprotech Inc., Rocky Hill, NJ, USA) polyclonal antibodies diluted 1:100 in PBS containing 0.1% saponin (Sigma). The incubation was followed by two washes with PBS-Saponin solution and, finally, sections were incubated for 45 min at 37 °C with a dilution (1:100) of goat anti-rabbit antibody conjugated with FITC (Jackson Immuno Research) in PBS-Saponin. The incubation was followed by two washes with PBS-Saponin solution. The results were expressed as the number of MIP-1α-producing cells (positive: fluorescent cell) per 10 fields (magnification 1000 ×). Data represent the mean of three histological slices for each animal for each feeding period.

Electron microscopy studies

Bacterial attachment to small intestine epithelial cells (SIEC) was determined qualitatively by electron microscopy examination as described by Zárate et al. (2002). Briefly, SIEC suspensions (10^6 organoids/ml) were obtained as described below in this section. Single or mixed cultures of *L. helveticus* R389 or *S.* Typhimurium were added to a final concentration of 10^{10} CFU/ml. Bacterial-SIEC suspensions were allowed to stand at room temperature for 30 min. The mixtures were then centrifuged (1000*g*, 4 °C 10 min) and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After

fixation, the sample was washed three times with 0.1 M cacodylate buffer at 10 min-intervals and postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 2 h. Bacterial-SIEC suspensions were then washed three more times with the same buffer. Dehydration was carried out with ascending concentrations of acetone (35%, 50%, 75%, 95% and 100%). The cells were finally dried in a critical point drier (HCP-2, Hitachi, Tokyo, Japan) for 20 min and coated with gold using a scanning electron microcopy coating unit (E5100). The samples were examined with a scanning electron microscope (JEOL JSM 35CF, JEOL Co. Ltd., Akishima, Japan).

Primary culture of mouse small intestine epithelial cells (SIEC) for the challenge with *L. helveticus* R389 and *S.* Typhimurium and IL-6 determination

Preparation of primary cultures of SIEC was performed as described previously (Vinderola et al., 2005). SIEC suspensions were transferred to 96-well cell culture plates (200 µl/well) and single or mixed cultures of *L. helveticus* R389 or *S. typhimurium* were added to a final concentration of 10^6 or 10^7 CFU/ml. LPS (Sigma-Aldrich) was used as a positive control at a final concentration of 1 µg/ml. Plates were incubated for 8 h (37 °C, 5% CO_2). An in vitro toxicology assay kit, MTT based (Sigma-Aldrich) and trypan blue (0.4%) exclusion were used to assess cell viability. Supernatants were recovered for IL-6 determination using the mouse IL-6 ELISA Set (BD OptEIA, BD Biosciences Pharmingen, San Diego, CA, USA).

Statistical analysis

A completely randomized design was used in this study. Data were analyzed using the one-way ANOVA procedure of SPSS software. The differences between means were detected by Duncan's Multiple Range Test. Data were considered significantly different when $P<0.05$.

Results

The microbiological screening of FM and NBF showed that the former contained 4.5×10^8 CFU/ml of viable *L. helveticus* R389 whereas the latter contained less than 1×10^3 CFU/ml of *L. helveticus* R389. We considered the NBF as a supernatant practically devoid of bacteria due to the low number of viable microorganisms in it. From an immunological point of view, the number of non-commensal bacteria in a food able to influence the gut associated lymphoid tissue should be higher than 1×10^6 CFU/ml (Gauffin-Cano and Perdigón, 2003). The effects of the oral administration of FM or NBF to conventional mice for 2, 5 or 7 consecutive days on the number of IgA+ cells in the lamina propria of the small intestine are shown in Fig. 1. There was a significant increase in this parameter for all feeding periods assayed, being more noticeable after 7 days of the oral administration of the NBF.

The luminal content of total secretory IgA (S-IgA) in mice that received FM or NBF before challenge with *S.* Typhimurium is shown in Fig. 2. The content of S-IgA in the lumen reached a maximum in animals that received FM for 5 days or NBF for 2 days. Then, these values significantly diminished or returned to control values by day 7.

After the feeding periods with FM or the NBF, treated and control mice were orally challenged with a single infective dose of *S.* Typhimurium. On days 4 and 7 after challenge, livers were removed to perform the

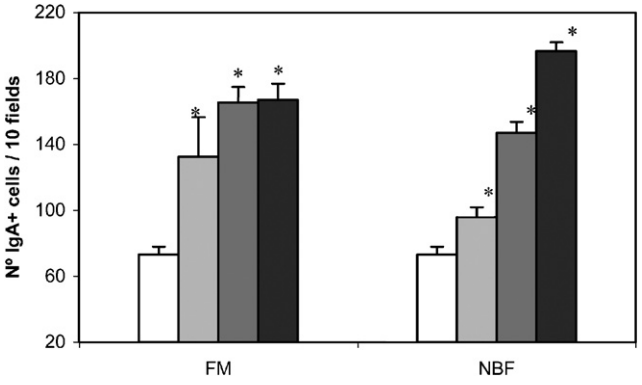

Fig. 1. Number of IgA+ cells in the small intestine lamina propria of mice that received FM or NBF for 2 (light gray colums), 5 (dark gray columns) or 7 (black columns) consecutive days. *Significantly different from the corresponding control (white columns) group ($P<0.05$).

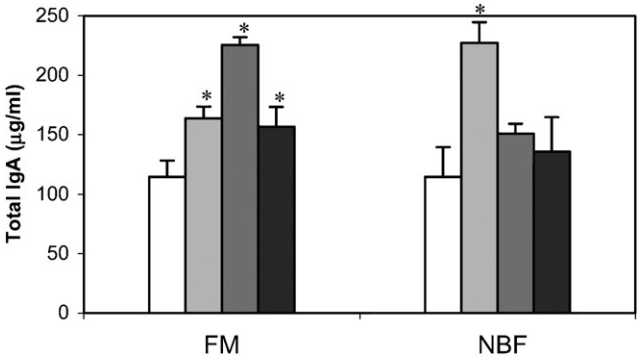

Fig. 2. Effect of the oral administration of FM or NBF for 2 (light gray columns), 5 (dark gray columns) or 7 (black columns) consecutive days on the content of total secretory IgA in the intestinal fluid of mice, compared to control animals (white columns). *Significantly different from the control ($P<0.05$).

colonization assay. Fig. 3 shows the number of viable bacteria in the liver of infected control and treated mice. On day 7 after *S.* Typhimurium infection, there was a significant decrease in the colonization levels in the liver of animals that received FM for 5 or 7 days or NBF for 2, 5 or 7 consecutive days compared to the untreated challenged controls. Simultaneously, there was a significant increase in the luminal content of specific anti-*Salmonella* S-IgA on day 7 after infection in mice that received FM for 2 or 7 consecutive days or NBF for 2, 5 or 7 consecutive days compared to the untreated challenged controls (Fig. 4). Fig. 5 shows the morbidity of control and treated animals after infection with *S.* Typhimurium. In addition to lower colonization levels in liver than other groups, the mice that received FM or NBF for 7 days showed the highest percentage of survival to the infection. On the whole, animals treated with NBF showed a higher percentage of survival than the ones that received FM.

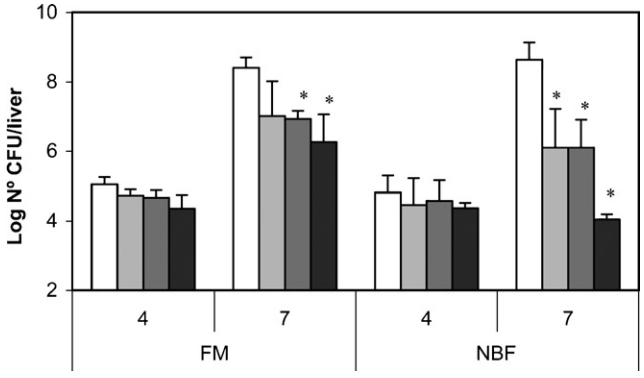

Fig. 3. Colonization assay. Number of CFU of *S.* Typhimurium/liver on days 4 and 7 post-infection in mice that received FM or NBF for 2 (light gray columns), 5 (dark gray columns) or 7 (black columns) consecutive days before infection. *Significantly different from the corresponding control (white columns) group ($P<0.05$).

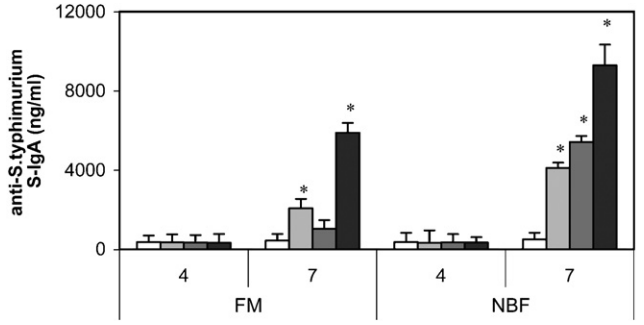

Fig. 4. Specific secretory IgA anti-*S.* Typhimurium in the intestinal fluid on days 4 and 7 post-infection in mice that received FM or NBF for 2 (light gray columns), 5 (dark gray columns) or 7 (black columns) consecutive days before infection. *Significantly different from the corresponding control (white columns) group ($P<0.05$).

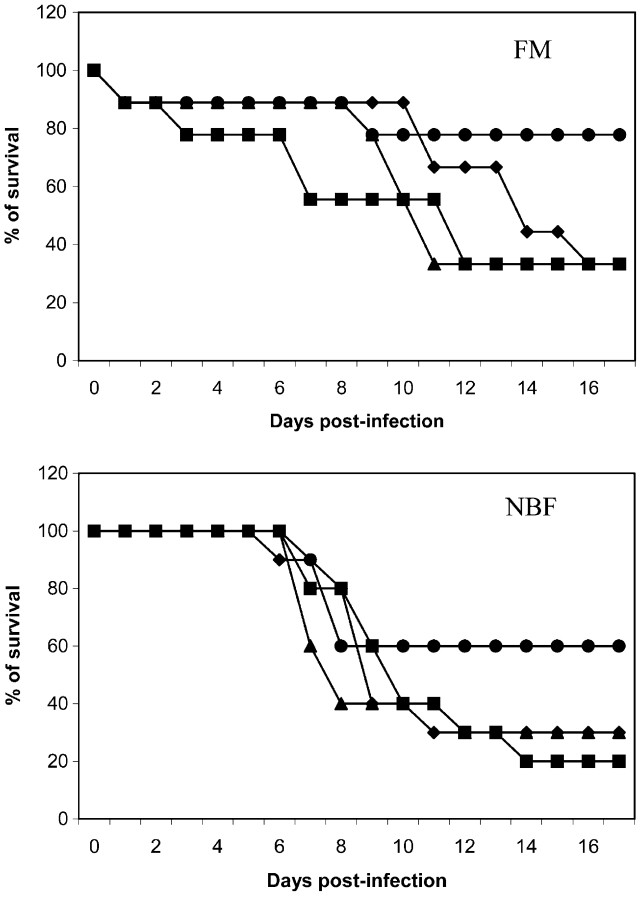

Fig. 5. Survival of mice that received FM (top panel) or NBF (bottom panel) for 2 (▲), 5 (♦) or 7 (●) consecutive days before infection with *S.* Typhimurium compared to the control group (■). 15 mice were used in each group.

Fig. 6 shows the number of IgA+ cells in the lamina propria of mice after the challenge with *S.* Typhimurium. The number of IgA+ cells, which had been already enhanced by the oral administration of FM and the NBF (Fig. 1), continued to increase during the course of infection in untreated challenged control animals, reaching a maximum of approximately 140 to 170 IgA+ cells/10 fields. In animals that received FM for 2 and 7 days or NBF for 2, 5 or 7 days, the number of IgA+ cells was significantly higher than in the corresponding control mice on day 7 after infection.

The number of MIP-1α+ cells in the small intestine lamina propria was not modified by the oral administration of FM or NBF, compared to control mice (Fig. 7). However, on days 4 and 7 after the infection with *S.* Typhimurium, there was a significant increase in this parameter in control mice compared to pre-infection values. In animals that received FM for 2, 5 or 7 consecutive days or NBF for 7 days, there was a significant decrease in the number of MIP-1α+ cells on day 4 after infection, compared to the untreated challenged mice. In all these cases, the number of

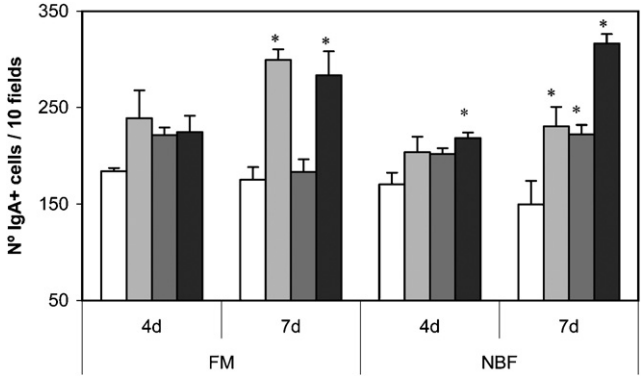

Fig. 6. Number of IgA+ cells in the small intestine lamina propria on days 4 and 7 post-infection with *S*. Typhimurium in mice that received FM or NBF for 2 (light gray columns), 5 (dark gray columns) or 7 (black columns) consecutive days. *Significantly different from the corresponding control (white columns) group ($P<0.05$).

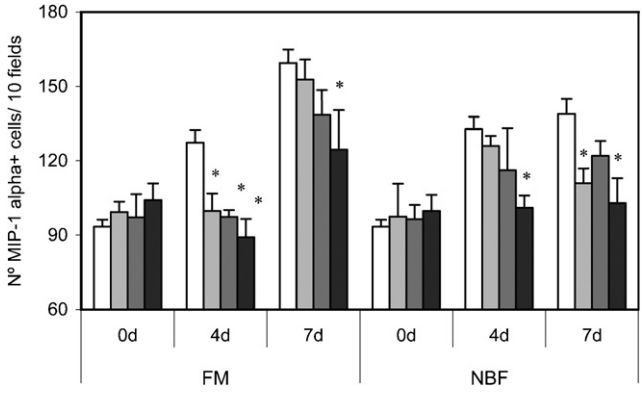

Fig. 7. Number of MIP-1α+ cells in the small intestine lamina propria on days 4 and 7 post-infection with *S*. Typhimurium in mice received FM or NBF for 2 (light gray columns), 5 (dark gray columns) or 7 (black columns) consecutive days. *Significantly different from the corresponding control (white columns) group ($P<0.05$).

MIP-1α+ cells was similar to control values prior to infection. On day 7 post-challenge, a significant decrease in the number of MIP-1α+ cells was observed in animals that received FM for 7 days or NBF for 2 or 7 consecutive days.

The interaction of *L. helveticus* R389 and *S*. Typhimurium with small intestine epithelial cells was studied by electron microscopy. Fig. 8A shows enterocytes isolated from the small intestine of conventional mice. Pure cultures of *L. helveticus* R389 or *S*. Typhimurium added to these isolates were able to adhere to their surface, as shown in Fig. 8B and C, respectively. When *L. helveticus* R389 and *S*. Typhimurium were added simultaneously to freshly isolated small intestine epithelial cells, both microorganisms could be seen attached to the surface of the enterocytes (Fig. 8D). Even when small intestine epithelial cells were coated with *L. helveticus* R389 for 30 min prior to the addition of *S*. Typhimurium (Fig. 8E), the enteropathogen could still be observed, attached to the enterocytes next to *L. helveticus* R389 cells.

When freshly isolated small intestinal epithelial cells were cultured in the presence of pure cultures of *L. helveticus* R389 or *S*. Typhimurium, there was a significant increase in the production of IL-6 by enterocytes in the presence of both microorganisms as compared to the unchallenged control (Fig. 9). When *L. helveticus* R389 was added at a final concentration of 10^7 or 10^6 CFU/ml there were no differences in IL-6 production. However, for *S*. Typhimurium, IL-6 secretion was more strongly stimulated at a concentration of 10^7 CFU/ml than at a concentration of 10^6 CFU/ml.

Discussion

Enteric infections of bacterial etiology are a worldwide medical problem. They are one of the major causes of infant mortality in developing countries and constitute a permanent risk for travelers. The widespread use of antibiotics in public health to control gastrointestinal infections implies the emergence of drug-resistant strains and the potential for development of chronic toxicity. The modification of the intestinal milieu by the oral administration of lactic acid bacteria or their fermented milks may thus constitute an alternative for the obtainment of prophylactic effects against intestinal infections (Perdigón et al., 2004). Brovko et al. (2003) showed that fermented milks are able to ameliorate the clinical symptoms of *Salmonella* infected mice, whereas their cell-free fractions are able to enhance cytokine release in macrophage (Ng and Griffiths, 2002). In this context, we first studied the capacity of milk fermented by *L. helveticus* R389 – obtained by overnight (18 h) fermentation of milk – and its non-bacterial fraction – obtained by the fermentation of milk at controlled pH 6 – in the activation of the mucosal immunity and in the prevention of an infection by *S*. Typhimurium.

The significant increase in the number of IgA+ cells (Fig. 1) in the lamina propria of the small intestine after the oral administration of milk fermented by *L. helveticus* R389 (FM) or its non-bacterial fraction (NBF) for all feeding periods assessed was accompanied by a significant increase in the luminal content of total S-IgA, especially for FM. In a previous work (Vinderola et al., 2006b), we showed that this enhanced "natural" or "polyvalent" S-IgA was not directed against the non-bacterial fraction of the fermented milk. Galdeano and Perdigón (2006) also demonstrated that no specific antibodies were raised against the probiotic strain

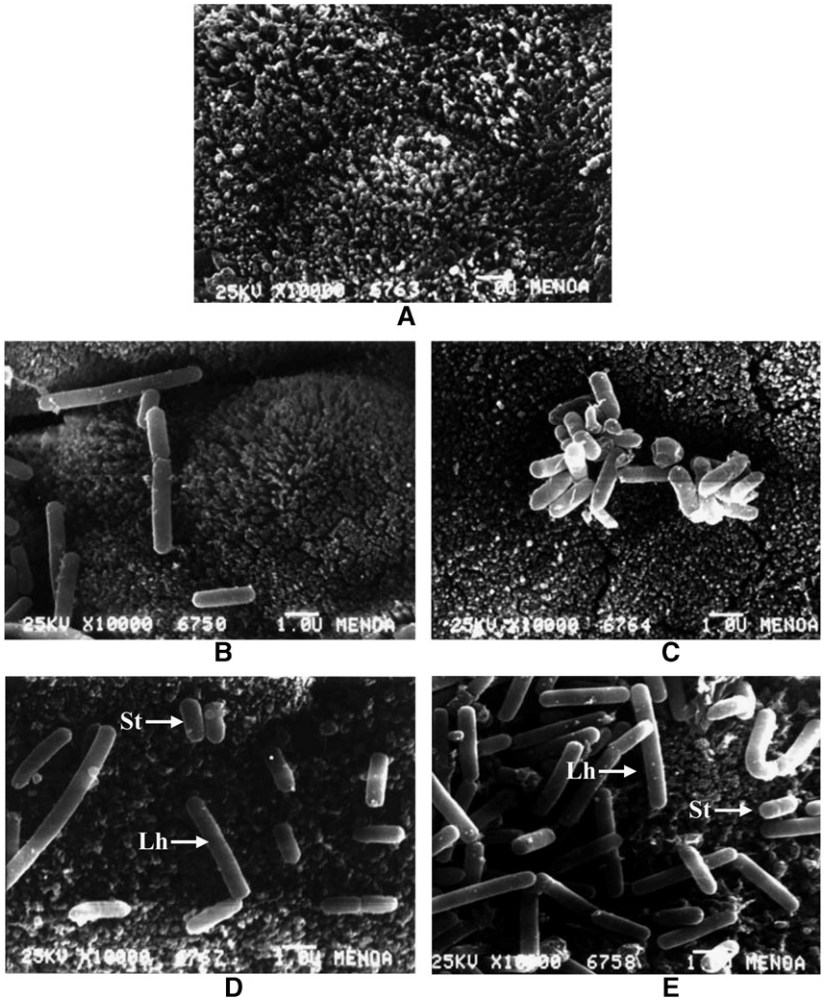

Fig. 8. Scanning electron micrograph of (A) control small intestine epithelial cells before the adhesion assay, pure cultures of *L. helveticus* R389 (B) and (C) *S.* Typhimurium attached to the surface of SIEC, a mixture (D) of *L. helveticus* R389 (Lh) and *S.* Typhimurium (St) and (E) *S.* Typhimurium (St) on small intestine epithelial cells previously coated with *L. helveticus* R389 (Lh) for 30 min at room temperature. Magnification $\times 10000$.

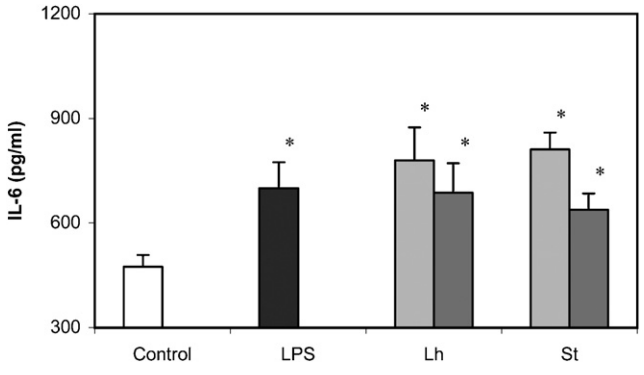

Fig. 9. IL-6 production by small intestine epithelial cells challenged with LPS (1 μg/ml), *L. helveticus* R389 (Lh) or *S.* Typhimurium (St) at concentrations of 10^7 (light gray columns) or 10^6 (dark gray columns) CFU/ml. *Significantly different from unchallenged control ($P<0.05$).

L. casei CRL 431 when orally administered to mice. Natural IgA antibodies provide an early innate immune response against bacterial and virus invaders (Macpherson et al., 2000). In this work, we observed that the enhanced production of luminal S-IgA (Fig. 2) was downregulated and by the end of the 7 day feeding period it returned almost to control values, while the number of IgA+ cells was still significantly higher than that of the controls. The biological relevance of this phenomenon might be that, in the absence of pathogenic threat, there is no need for the organism to maintain a significantly high rate of S-IgA secretion to keep intestinal homeostasis. Nevertheless, it seems probable that there is still an improved state of immunological surveillance exerted by the significantly higher number of IgA+ cells in the lamina propria compared to control values.

Several studies have investigated the use of immunomodulating probiotics for the control of microbial enteropathogens by utilizing rodent infection/challenge models. A large volume of research in this area has focused on the use of *Salmonella enteritidis* serovar Typhimurium as a model pathogen in mice, the first studies being the ones carried out by Perdigón's team in the early 1990s (Cross, 2002). In our laboratory we demonstrated that the oral administration of milk fermented by *Lactobacillus casei* and *Lactobacillus acidophilus* (Perdigón et al., 1990), yoghurt (Perdigón et al., 1991) or yoghurt microflora (Valdez et al., 2001) and *Lactobacillus casei* CRL 431 itself (Perdigón et al., 1993, 1995) was effective in the prevention of a *S.* Typhimurium infection in both conventional and malnourished/re-nourished mice (Gauffin-Cano and Perdigón, 2003). Several specific and non-specific mechanisms were reported to be involved such as the production of anti-*Salmonella* antibodies, the increase in the non-specific local immunity mediated by pro-inflammatory cytokines, the increased phagocytic activity of macrophages and the inhibition of apoptosis in infected macrophages (Perdigón et al., 2004).

Certain differences were observed in our study in the protective capacity against *Salmonella* infection exerted by FM or NBF. In mice given the latter for 7 consecutive days, lower levels of liver colonization on day 7 post-challenge (Fig. 3), higher luminal contents of specific anti-*Salmonella* S-IgA (Fig. 4) and a higher percentage of survival to infection were observed. Even though both FM and NBF provided enhanced protection against *S.* Typhimurium infection, and even when some characteristic of this preventive capacity can be shared between FM and NBF, it seems that the mechanisms underlying mucosal immunomodulation and protection are different. However, all these differences remain to be determined. The regulatory capacity of peptides derived from milk proteins has been largely reported (Meisel and Bockelmann, 1999). In a previous work (Vinderola et al., 2006a), we observed that the non-bacterial fraction of the fermented milk kefir was more effective in inducing IL-10 secretion by cells from the innate immune system than its bacterial counterpart. We hypothesize that after fermentation at controlled pH 6, there was an increase in the content of, mainly, bioactive peptides but also in other bioactive molecules (lactulose, galactoligosaccarides) in the NBF (Vinderola et al., 2006b). This fact might be the reason for the better protection that the NBF provided against the infection.

In agreement with these previous studies, we also observed the enhanced production of anti-*Salmonella* specific antibodies in animals that received FM or NBF (Fig. 4). This effect was more evident with NBF, maybe due to the metabolites produced at pH 6. The relative significance of luminal IgA antibodies in the protection against invasive enteric bacterial infections is less well defined as yet. There appears to be a potential complementary or alternative role for both cell-mediated immune mechanisms and antibodies of other isotypes. For protection against mucosal invasive infections such as those caused by *Salmonella*, cell-mediated immune responses seem to play the major role in clearing an already established infection, whereas mucosal antibodies would be more important for preventing infection. The improved state of immunological surveillance achieved in the small intestine lamina propria by the enhanced numbers of IgA+ cells continued to increase during infection, providing the necessary S-IgA for *S.* Typhimurium elimination from the host.

A boggling array of chemokines (LIX, MIG, IP-10, MIP-2, MIP-1α, MIP-1β, MCP-1, etc.) produced to different degrees by a wide variety of cell types in response to injury, endogenous mediators and exogenous stimulants has been described and detected at local inflammatory sites (Qiu et al., 2001), a characteristic of this array being its strong chemotactic capacity for the recruitment of polymorphonuclear neutrophils (PMNs) into the local inflammatory site (Hidemura et al., 2003). In untreated/challenged control animals, there was an increase in the number of MIP-1α+ cells after the infection (Fig. 7). However, it seems that the oral administration of FM or the NBF for 7 days acted in an anti-inflammatory manner, since it prevented the increase in the number of MIP-1α+ cells. Since PMNs represent the first line of host defense against microbial infection, the oral administration of FM or NBF prevented a possible over-recruitment of PMNs into the local inflammatory site, to protect tissues against extra damage due to inflammation.

An alternative contributory mechanism for the protection against *Salmonella* infection might have been carried out by dietary calcium, present in both FM and NBF. In the intestine, dietary calcium exerts cytoprotective effects on epithelial cells. Dietary calcium decreases the cytotoxicity of intestinal contents and intestinal epitheliolysis by precipitating cytotoxic surfactants such as bile acids and fatty acids. Decreased luminal cytotoxicity might not only strengthen the barrier function of the gut mucosa but also reinforce the protective endogenous microflora (Bovee-Houdenhoven et al., 1997). It was demonstrated that dietary calcium improves colonization resistance and reduces the severity of gut-derived systemic infections by enteropathogens such as *Salmonella* (Bovee-Houdenhoven et al., 1996) and *E. coli* (Bovee-Houdenhoven et al., 2003).

The first critical step for the bacterial pathogen in establishing an infection at a mucosal surface is the colonization of the mucosal epithelium (Holmgren and Svennerholm, 2004). One factor that has been generally

used as a selection criterion for probiotic formulation is the ability of strains to adhere to the host's gut epithelia. However, it should be pointed out that it may not be an essential attribute for achieving the probiotic effect (Bibiloni et al., 1999). When we studied the interaction of *L. helveticus* R389 and *S.* Typhimurium with small intestine epithelial cell isolates using an electron microscope, we observed that both strains were able to establish intimate contact with the enterocyte surface with no apparent competition for adhesion sites when added simultaneously to enterocyte isolates or no apparent exclusion of *S.* Typhimurium by *L. helveticus* R389 (Fig. 8) when enterocytes were previously coated with the probiotic strain, since in all cases both strains were observed attached to enterocytes close to one another. It was suggested that although adhesion to epithelial surfaces is considered an important prerequisite for probiotic activity, high adhesiveness may not guarantee protective properties in a selected strain (Bibiloni et al., 1999). In addition to the classic adhesin–receptor interactions between a pathogen and enterocytes, mucosal colonization may be facilitated by the interaction of mucosal cells and specific molecules on pathogens called pathogen-associated molecular patterns (PAMPs) (Holmgren and Svennerholm, 2004). The immune system detects microorganisms by discriminating between self and non-self organisms. This is achieved by the Toll-like receptors (TLRs), which provide considerable specificity for microbial pathogens and discrimination between pathogens and the host while providing an immediate response system in the setting of danger (Abreu and Arditi, 2004). TLR4 recognizes lipopolysaccharide and Gram(-) bacteria, while TLR2 recognizes a variety of microbial components such as peptidoglycan and lipoteichoic acids from Gram(+) bacteria (Neeser et al., 2000). In a previous work (Vinderola et al., 2005), we observed that *L. helveticus* R389 adhered to TLR4, which might afford a better protection against enteric infections by competition with pathogens for adhesion sites. In line with this observation, Neeser et al. (2000) reported that *L. johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. However, photographic evidence allowed us to see that, even when *L. helveticus* R389 shares some adhesion sites with pathogens and even when it was used to coat enterocyte isolates, adhesion sites might still be accessible for *S.* Typhimurium to infect the host. Similar IL-6 levels were induced by *L. helveticus* R389 and *S.* Typhimurium when added to IEC cultures (Fig. 9); however, the gut response to the presence of the probiotic *L. helveticus* R389 was far different from the response obtained with the pathogen *S.* Typhimurium. It is known that IL-6 can behave as a proinflammatory cytokine (Takeda et al., 2003) but at the same time it is involved in the terminal differentiation of B-cells to plasmocytes (Goodrich and McGee, 1998). In a previous work (Vinderola et al., 2005), we demonstrated that IL-6 can be equally induced by pathogenic and probiotic bacteria. However, other proinflammmatory cytokines such as IL-1α are induced in the presence of pathogenic bacteria that trigger the inflammatory response against an enteropathogen (Vinderola et al., 2005).

In this work we observed that in both the milk fermented by *L. helveticus* R389 and the fermented milk fraction devoid of bacteria there are active principles involving not only the probiotic bacteria but also the biological metabolites produced during the fermentation of milk that confer enhanced protection against *S.* Typhimurium infection. We only found differences between FM and NBF in the production of specific S-IgA and in the number of MIP-1α producing cells. In the present work we also demonstrated that in the mechanisms underlying the enhanced protection, the mucosal immune response would seem to be more involved than the competitive or exclusion mechanisms between *L. helveticus* R389 and *S.* Typhimurium.

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References

Abreu, M.T., Arditi, M., 2004. Innate immunity and toll-like receptors: clinical implication of basic science research. J. Pediatr. 144, 421–429.

Asahara, T., Shimizu, K., Nomoto, K., Hamabata, T., Ozawa, A., Takeda, T., 2004. Probiotic bifidobacteria protect mice from lethal infection with shiga toxin-producing *Escherichia coli* O157:H7. Infect. Immun. 72, 2240–2247.

Bibiloni, R., Pérez, P.F., de Antoni, G.L., 1999. Will a high adhering capacity in a probiotic strain guarantee exclusion of pathogens from intestinal epithelia? Anaerobe 5, 519–524.

Bovee-Houdenhoven, I.M., Termont, D., Dekker, R., Van Der Meer, R., 1996. Calcium in milk and fermentation by yoghurt bacteria increase the resistance of rats to *Salmonella* infection. Gut 38, 59–65.

Bovee-Houdenhoven, I.M., Termont, D.S., Weerkamp, A.H., Faassen-Peters, M.A., Van Der Meer, R., 1997. Dietary calcium inhibits the intestinal colonization and translocation of *Salmonella* in rats. Gastroenterology 113, 550–557.

Bovee-Houdenhoven, I.M., Lettink-Wissink, M.L., Van Doesburg, W., Witteman, B.J., Van Der Meer, R., 2003. Diarrhea caused by enterotoxigenic *Escherichia coli* infection of humans is inhibited by dietary calcium. Gastroenterology 125, 469–476.

Brovko, L.Y., Vandenende, C., Chu, B., Ng, K.Y., Brooks, A., Griffiths, M.W., 2003. In vivo assessment of effect of fermented milk diet on course of infection in mice with bioluminescent *Salmonella*. J. Food Prot. 66, 2160–2163.

Collins, M.D., Gibson, G.R., 1999. Probiotics, prebiotics, and synbiotics, approaches for modulating the microbial ecology of the gut. Am. J. Clin. Nut. 69, 1052S–1057S.

Cross, M.L., 2002. Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. FEMS Immunol. Med. Microbiol. 34, 245–253.

de Moreno de LeBlanc, A., Matar, C., Theriault, C., Perdigón, G., 2005. Effects of milk fermented by *Lactobacillus helveticus* R389 on immmune cells associated to mammary glands in normal and a breast cancer model. Immunobiology 210, 349–358.

FAO/WHO, 2002. Joint FAO/WHO Working group report on drafting guidelines for the evaluation of probiotics in food, London, Ontario, Canada. April 30 and May 1, 2002.

Fuller, R., 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66, 365–378.

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

Gauffin-Cano, P., Perdigón, G., 2003. Probiotics induce resistance to enteropathogens in a re-nourished mouse model. J. Dairy Res. 70, 440–443.

Gill, H.S., Shu, Q., Lin, H., Rutherfurd, K.J., Cross, M.L., 2001. Protection against translocating *Salmonella typhimurium* infection in mice by feeding the immuno-enhancing probiotic *Lactobacillus rhamnosus* strain HN001. Med. Microbiol. Immunol. 190, 97–104.

Goodrich, M.E., McGee, D.W., 1998. Regulation of mucosal B cell immunoglobulin secretion by intestinal epithelial cell-derived cytokines. Cytokine 10, 948–955.

Hidemura, A., Saito, H., Fukatsu, K., Matsuda, T., Kitayama, J., Ikeda, S., Kang, W., Nagawa, H., 2003. Oral administration of *Bifidobacterium longum* culture condensate in a diet-restricted murine peritonitis model enhances polymorphonuclear neutrophil recruitment into the local inflammatory site. Nutrition 19, 270–274.

Holmgren, J., Svennerholm, A.M., 2004. Mucosal immunity to bacteria. In: Mestecky, J., Lamm, M.E., Strober, W., Bienenstock, J., McGhee, J.R., Mayer, L. (Eds.), Mucosal Immunology. Academic Press, New York, pp. 783–797.

LeBlanc, J.G., Matar, C., Valdéz, J.C., LeBlanc, J., Perdigón, G., 2002. Immunomodulating effects of peptidic fractions issued from milk fermented with *Lactobacillus helveticus*. J. Dairy Sci. 85, 2733–2742.

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

Lee, Y.K., Nomoto, K., Salminen, S., Gorbach, S.L., 1999. Handbook of Probiotics. Wiley, New York, pp. 1–22.

Macpherson, A., Gatto, D., Sainsbury, E., Harriman, G., Hengartner, H., Zinkernagel, R., 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science 288, 2222–2226.

Matar, C., Valdez, J.C., Medina, M., Rachid, M., Perdigón, G., 2001. Immunomodulating effect of milks fermented by *Lactobacillus helveticus* and its non-proteolytic variant. J. Dairy Res. 68, 601–609.

Meisel, H., Bockelmann, W., 1999. Bioactive peptides encrypted in milk proteins, proteolytic activation and thropho-functional properties. Antoine Van Leeuwenhoek 76, 207–215.

Neeser, J.R., Granato, D., Rouvet, M., Servin, A., Teneberg, S., Karlsson, K.A., 2000. *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. Glycobiology 10, 1193–1199.

Ng, K.Y., Griffiths, M.W., 2002. Enhancement of macrophage cytokine release by cell-free fractions of fermented milk. Milchwissenschaft 57, 66–70.

Perdigón, G., Nader de Macías, M.E., Alvarez, S., Oliver, G., Pesce de Ruiz Holgado, A.A., 1990. Prevention of gastrointestinal infection using immunobiological methods with milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus*. J. Dairy Res. 57, 255–264.

Perdigón, G., Alvarez, S., Nader de Macias, M.E., Savoy de Giori, G., Medici, M., Nuñez de Kairuz, M., 1991. Behaviour of natural and heated yogurt in the immune system and preventive capacity on enteric infections. Milchwissenschaft 46, 411–416.

Perdigón, G., Alvarez, S., Medici, M., Pesce de Ruiz Holgado, A.A., 1993. Influence of the use of *Lactobacillus casei* as an oral adjuvant on the levels of secretory immunoglobulin a during an infection with *Salmonella typhimurium*. Food Agric. Immunol. 5, 27–37.

Perdigón, G., Alvarez, S., Gobbato, N., de Budeguer, M.V., Pesce de Ruiz Holgado, A.A., 1995. Comparative effect of the adjuvant capacity of *Lactobacillus casei* and lipopolysaccharide on the intestinal secretory antibody response and resistance to *Salmonella* infection in mice. Food Agric. Immunol. 7, 283–294.

Perdigón, G., Maldonado-Galdeano, C., de Moreno de LeBlanc, A., Vinderola, C.G., Medici, M., Bibas Bonet, M.E., 2004. Immunomodulation of mucosal immune response by probiotics. Curr. Trends Immunol. 6, 69–85.

Qiu, B., Frait, K.A., Reich, F., Komuniecki, E., Chensue, S.W., 2001. Chemokine expression dynamics in mycobacterial (type-1) and schistosomal (type-2) antigen-elicited pulmonary franuloma formation. Am. J. Pathol. 158, 1503–1505.

Sainte-Marie, G.A., 1962. A paraffin embedding technique for studies employing immunofluorescence. J. Histochem. Cytochem. 10, 250–256.

Shu, Q., Lin, H., Rutherfurd, K.J., Fenwick, S.G., Prasad, J., Gospal, P.K., Gill, H.S., 2000. Dietary *Bifidobacterium lactis* (HN019) enhances resistance to oral *Salmonella typhimurium* infection in mice. Microbiol. Immunol. 44, 213–222.

Takeda, K., Kaisho, T., Akira, S., 2003. Toll-like receptors. Annu. Rev. Immunol. 21, 335–376.

Valdez, J.C., Rachid, M., Gobbato, N., Perdigón, G., 2001. Lactic acid bacteria induce apoptosis inhibition in

Salmonella typhimurium infected macrophages. Food Agric. Immunol. 13, 189–197.

Vinderola, C.G., Matar, C., Perdigón, G., 2005. Role of intestinal epithelial cells in the immune effects mediated by Gram (+) probiotic bacteria. Involvement of Toll-like receptors. Clin. Diag. Lab. Immunol. 12, 1075–1084.

Vinderola, C.G., Perdigón, G., Duarte, J., Farnworth, E., Matar, C., 2006a. Effects of kefir fractions on innate imunity. Immunobiology 211, 149–156.

Vinderola, G., Matar, C., Perdigón, G., 2006b. Mucosal immunomodulation by the non-bacterial fraction of milk fermented by *L. helveticus* R389. Int. J. Food Microbiol., in press.

Zárate, G., Morata de Ambrosini, V., Pérez Chaia, A., González, S., 2002. Some factors affecting the adherence of probiotic *Propionibacterium acidopropionici* CRL 1198 to intestinal epithelial cells. Can. J. Microbiol. 48, 449–457.

Zhang, S., Kingsley, R.A., Santos, R.L., Andrews-Polymenis, H., Raffatellu, M., Figuereido, J., Nunes, J., Tsolis, R.M., Adams, L.G., Baumler, A.J., 2003. Molecular pathogenesis of *Salmonella enterica* serotype Typhimurium-induced diarrhea. Infect. Immun. 71, 1–12.