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Mucosal immunomodulation by the non-bacterial fraction of milk fermented by *Lactobacillus helveticus* R389

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Abstract

The health promoting effects ascribed to probiotic bacteria and fermented dairy products arise not only from bacteria themselves but also from metabolites derived from milk fermentation. Exopolysaccharides produced during milk fermentation and peptides derived from major milk proteins, when released during fermentation, are potential modulators of various regulatory processes in the body. The aim of this work was to increase the knowledge of the previously observed immunomodulating capacity of milk fermented by *Lactobacillus helveticus* R389 by the study of the mucosal immunomodulation exerted by the non-bacterial fraction of the milk fermented at a constant pH6 (NBFpH6). The effects on IL-6 production by small intestine epithelial cells, the profile of IgA+ and cytokine+ cells (IL-2, IL-6, IL-10, TNF-α and IFN-γ) induced in the gut lamina propria, and the levels of total and specific secretory IgA in the lumen of BALB/c mice that received NBFpH6 for 2, 5 or 7 days were examined. There was an increase in the number of IgA+, IL-10+, IL-2+ and IL-6+ cells after all feeding periods. Total S-IgA in the small intestine lumen increased in mice that received NBFpH6 for 2 days. However, no specific antibodies against NBFpH6 were detected. Feeding of NBFpH6 for 7 days significantly ($P<0.05$) enhanced IL-6 secretion by small intestine epithelial cells. NBFpH6 induced a non-specific mucosal response that was down-regulated for protective immunity, enhancing IL-6 production by epithelial cells and IgA production in the small intestine. These events improve the immunological defenses at the intestinal level, increasing host protection against pathologies. Because mucosal immune responses induced by certain dietary antigens play a large part in the prevention of gastrointestinal diseases, the oral administration of a mucosal adjuvant such as NBFpH6 may positively affect the milieu of the intestinal lumen. The opportunity exists then to manipulate the constituents of the lumen of the intestine through dietary means, thereby enhancing the health condition of the host.

Keywords: Fermented milks; Mucosal immune response; *Lactobacillus*; Non-bacterial fraction; Peptides

1. Introduction

The nutritional condition of an individual has a major impact on the immune system. In both healthy and nutritionally deficient persons, dietary supplements have been found to enhance the response of the immune system resulting, in some cases, in fewer days of infectious illnesses (Walrand et al., 2003). The interactions between nutrients and immunity should therefore be analyzed from biological and clinical points of view (de Pablo et al., 2002). The gastrointestinal tract is the only part of the body that normally contacts nutrients before they are absorbed. Although nutritional changes ultimately impinge on most organs, it is the epithelium of the gastrointestinal tract that first encounters any variation in nutrient intake (Sanderson, 1998).

Probiotics are defined as 'live microorganisms which when consumed in adequate numbers confer a health benefit on the host beyond basic nutrition' (Guarner and Schaafsma, 1998). Fermented milks and their related dairy lactic acid bacteria (LAB) have demonstrated health benefits (Perdigón et al., 2001; Lopez-Varela et al., 2002; Gibson et al., 2003; Mercenier et al.,

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2003; Reid et al., 2003; Saikali et al., 2004) and therefore are considered to have probiotic activity. Their beneficial effects can be exerted mainly through two mechanisms: direct effects of live microbial cells (probiotics) or indirect effects via metabolites of these cells (biogenics). Biogenics are defined as food components derived from microbial activity that provide health benefits without involving the intestinal microflora (Takano, 2002). The most important biogenics in fermented milks are peptides, which are not present prior to fermentation. The probiotic effects ascribed to LAB and fermented dairy products arise not only from whole microorganisms and cell wall components but also from metabolites such as peptides and exopolysaccharides produced during fermentation. Peptides derived from major milk proteins during fermentation are potential modulators of various regulatory processes in the body (Meisel and Bockelmann, 1999). Ganjam et al. (1997) reported that cell free yogurt fractions exert antiproliferative effects on cultured mammalian intestinal cells. Matar et al. (2001) demonstrated that immunoenhancing and antimutagenic compounds released during milk fermentation by the proteolitic organism *Lactobacillus helveticus* R389 were not present in milk fermented by a non-proteolytic mutant. LeBlanc et al. (2002) demonstrated that the peptidic fractions released from milk proteins by the same strain were able to decrease the size of a fibrosarcoma in mice. Additionally, peptides released from milk proteins by the same *L. helveticus* strain induced a protective humoral immune response after an *Escherichia coli* O157:H7 infection in mice (LeBlanc et al., 2004).

To interact with the gut-associated immune cells, dietary antigens must first interact with the cells lining the intestine. Ménard et al. (2004) demonstrated that some LAB strains release metabolites that exert an anti-inflammatory effect and influence the activities of intestinal epithelial cells. In a previous work (Vinderola et al., 2006b), we observed that the non-bacterial fraction of kefir, a fermented milk (Farnworth and Mainville, 2003), induced an enhanced *ex vivo* production of the cytokine IL-6 by isolated small intestine epithelial cells. Several factors in milk can affect the expression of genes in the intestinal epithelium, particularly genes that are associated with enterocyte differentiation (Sanderson, 1998). We reported that intestinal epithelial cells from conventional mice produce IL-6 in response to the challenge *in vitro* with certain LAB strains that had demonstrated *in vivo* immunomodulating capacity (Vinderola et al., 2005).

The aim of this work was to increase the knowledge of the immunomodulating capacity of milk fermented by *L. helveticus* R389 through the study of the mucosal immunomodulation exerted by the non-bacterial fraction of this fermented milk.

2. Materials and methods

2.1. Animals and bacterial strains

Six-to-eight week-old male BALB/c mice weighing from 20 to 25 g were obtained from the random bred colony kept by our department at CERELA. All animal protocols were approved by the Animal Protection Committee of CERELA. All experiments comply with the current laws of Argentina.

L. helveticus R389 (Matar et al., 1996, 2001; LeBlanc et al., 2002), isolated from Swiss cheese, was used. Cultures were grown overnight in 12% skim milk (Difco, Becton Dickson and Company, Sparks, MD, USA) at 37 °C, under aerobic conditions.

2.2. Preparation of fermented milks and their non-bacterial fractions

Two types of fermented milk were produced with *L. helveticus* R389. For one type, the milk was fermented without pH control by inoculating 12% skim milk with 2% (v/v) of a culture of *L. helveticus* R389 containing 2×10^6 CFU/ml, and incubating the preparation aerobically at 37 °C for 18 h. The final pH was 3.7. For the second type, the milk was maintained at pH6 throughout the fermentation. Skim milk inoculated as before was fermented in a Bioflo Model 70 13 70 biofermentor (New Brunswick Scientific Edison, NJ, USA) at 37 °C with an agitation rate of 100 rpm and sparging with CO_2 at 0.2 l/min. The pH was maintained at 6.00 by automatic addition of 8 M NaOH.

The non-bacterial fraction (NBF) of the fermented milk obtained by fermentation without pH control was centrifuged at 3500 ×*g* and 4 °C for 15 min using a IEC B-22M centrifuge (IEC, Nedham Heights, MA, USA) and the supernatant was recovered and stored at −80 °C until use. The non-bacterial fraction of the milk fermented with pH control (NBFpH6) was treated with 85% DL-lactic acid syrup (Sigma-Aldrich, St. Louis, MO, USA) to reduce the pH to 3.7. The acidified milk was centrifuged and the supernatant was recovered and stored as was the NBF. The protein contents of the non-bacterial fractions were determined using the Quick Start Bradford Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Reverse-phase high performance liquid chromatography (RP-HPLC) of milks

Samples of unfermented milk and milk acidified to pH 3.7 with 85% lactic acid were centrifuged at 6000 ×*g* for 20 min at 4 °C to obtain their supernatants. Then, the supernatants from unfermented milk and from milk acidified to pH 3.7 and the NBF and the NBFpH6 were filtered using 0.45 and 0.22 μm Millex-GP syringe filter (Millipore, Etobicoke, ON, Canada) and maintained at 4 °C until injection. A 50 μl portion of each sample was loaded on a Spherisorb ODS-2 5 μm column (Phenomenex, Torrance, CA, USA) in a RP-HPLC System (ISCO, Lincoln, NE, USA). The solvents used for the separation were, solvent A, 0.1% trifluoracetic acid (TFA) in water, and solvent B, 0.1% TFA in acetonitrile. The solvent gradient conditions were 100% solvent A for 5 min then progressive administration of solvent B to obtain a mixture of 40% solvent A and 60% solvent B after 25 min, maintenance of this mixture for 5 min and, finally, the mixture was progressively replaced by solvent A to obtain 100% solvent A after 3 min. Proteins and peptides were eluted with a flow rate of 0.9 ml/min and their

concentrations in the eluate were monitored at 214 nm using a V^4 absorbance detector (ISCO).

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of milks and non-bacterial fractions

Electrophoresis was performed as described by Laemmli (1973) on vertical slab gels that measured 16×12×0.75 cm, using a MiniPROTEAN 3 system (Bio-Rad Laboratories). The polyacrylamide gel was composed of a stacking gel of 5% acrylamide in 0.5 M Tris–HCl buffer, pH 6.8 and a resolving gel of 16% acrylamide in 1.5 M Tris–HCl buffer, pH 8.8. Fermented milks and unfermented and acidified milks were each diluted in an equal volume of buffer to obtain a final concentration of 0.5 M Tris–HCl buffer, pH 6.8 with 2% SDS, 0.5% 2-mercaptoethanol, 25% glycerol and 0.5% bromophenol blue. A 20 μl portion of each sample was placed in a separate well of each gel. Electrophoresis was performed at a constant voltage of 150 V until the tracking dye migrated to the bottom of the gel. The gels were stained in a solution of methanol:water: acetic acid 50:50:20, with 0.1% Coomasie blue R-250 (Bio-Rad, Laboratories). Gels were decolorized under continuous shaking in a solution of methanol:water:acetic acid, 25:65:10. The molecular mass marker (Wide Molecular Weight Range Sigma-Marker; Sigma) consisted of myosin (205 kDa), galactosidase (116 kDa), phosphorylase b (97 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa).

2.5. Feeding procedures

Each experimental group consisted of 5 mice housed in metallic cages kept in a controlled environment at a temperature of 22±2 °C with humidity at 55±2%, and with a 12 h light/dark cycle. Mice received NBF or NBFpH6 by gavage, with each mouse receiving 100 μg of total protein per day for 2, 5 or 7 consecutive days. One control group received 200 μl of 12% skim milk instead and the other received 200 μl of phosphate buffered saline, pH 7.4 (PBS; Sigma-Aldrich). All animals received, simultaneously and *ad libitum*, a sterile conventional balanced diet containing proteins, 230 g/kg; raw fibre, 60 g/kg; total minerals, 100 g/kg; Ca, 13 g/kg; P, 8 g/kg; water, 120 g/kg; and vitamins.

2.6. Immunofluorescence test for B (IgA^+ cells) population identification

After each feeding period, treated and control groups were anesthetized and sacrificed by cervical dislocation. The small intestine of each animal was removed for histological preparation by paraffin inclusion (Sainte-Marie, 1962). The number of IgA producing (IgA+) cells was determined on histological slices of samples from the ileum near Peyer's patches. The immunofluorescence test was performed using α-chain specific anti-mouse IgA fluorescein isothiocyanate (FITC) conjugate (Sigma-Aldrich). Histological slices were deparaffinized and rehydrated in a series of ethanol concentrations. Deparaffinized histological samples were treated with a dilution (1/100) of the antibody in PBS and incubated in the dark for 30 min at 37 °C. Then, samples were washed 2 times with PBS and examined using a fluorescent light microscope. The results were expressed as the number of fluorescent, IgA+ cells per 10 fields with magnification of 1000×. Data are presented as the mean value for three tissue slices for each animal for each feeding period.

2.7. Cytokine assay

IL-2+, IL-6+, IL-10+, TNF-α+ and IFN-γ+ were studied by an indirect immunofluorescence method applied to histological slices of the small intestines of mice fed a milk or PBS. Histological slices were deparaffinized and rehydrated in a series of ethanol concentrations, and then were incubated for 30 min in a 1% blocking solution of Bovine Serum Albumin (BSA; Jackson Immuno Research, West Grove, PA, USA) and 30 min in a blocking solution of goat serum (Sigma-Aldrich) diluted 1:100 in PBS, at room temperature. Histological slices were then incubated for 60 min at 37 °C with rabbit anti-mouse IL-2, IL-6, IL-10, TNF-α or IFN-γ polyclonal antibodies (Peprotech, Inc., Rocky Hill, NJ, USA) diluted 1:100 in PBS containing 0.1% Saponin (Sigma). The incubation was followed by two washes with PBS–Saponin solution. Finally, sections were incubated for 45 min at 37 °C with a 1:100 dilution of goat anti-rabbit antibody conjugated with FITC (Jackson Immuno Research) in PBS–Saponin. The incubation was followed by two washes with PBS–Saponin. The results were expressed as the number of fluorescent X-producing cells per 10 fields with magnification of 1000×. Data are presented as the mean values of three histological slices for each animal for each feeding period.

2.8. Primary culture of small intestine epithelial cells from mice fed with NBFpH6

Preparation of primary cultures of enterocytes was performed as described previously (Vinderola et al., 2005). After each feeding period, treated and PBS control animals were anesthetized and sacrificed by cervical dislocation. The last 1/3 portion of the small intestine of PBS control mice and of animals that received the NBFpH6 was removed and placed in Hank's balanced salt solution (HBSS; Sigma-Aldrich) containing 2% glucose (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich) and 0.1 mg/ml streptomycin (Sigma-Aldrich) on ice. Intestines were flushed 6 times with 10 ml of the same buffer, cut into 2- to 3-mm fragments and collected in HBSS. Then, the small intestines were digested in 20 ml of HBSS containing 300 U/ml collagenase (Sigma-Aldrich C-7657) and 0.1 mg/ml dispase (Gibco, Grand Island, NY, USA) at 25 °C, with agitation at 150 rpm for 45 min. Digestion was stopped by the addition of 20 ml of Dulbecco's Modified Eagle Medium (DMEM) without phenol red (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (ATCC, Manassas, VA, USA), epidermal growth factor at 10 ng/ml (US Biological, Swapscott, Mass., USA), Insulin–Transferrin–Selenium-A at

2.50 μg/ml, 0.55 μg/ml and 1.68 pg/ml, respectively, from a 100 x-ready-to-use solution (Gibco), penicillin at 100 U/ml and streptomycin at 0.1 mg/ml. The largest fragments were allowed to settle at the bottom of the flask for 2 min. The supernatant was then transferred to centrifuge tubes and centrifuged for 3 min at 100 *g*. The pellet was washed twice with the culture medium and finally resuspended in the same medium at a concentration of $4–6 \times 10^5$ single cells or clusters of intestinal epithelial cell (IEC)/ml. A 200 μl portion of each organoid suspension was then transferred to a well in each of once 96-well cell culture plates. The plates were incubated for 8 h at 37 °C under an atmosphere containing 5% CO_2. An *in vitro* toxicology assay kit (Sigma Aldrich) based on 3(4, 5 dimethylthiazol-2 YL)-2, 5 diphenyltetrazolium bromide (MTT) and trypan blue (0.4%) exclusion were used to confirm 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).

2.9. Determination by Double antibody sandwich-Enzyme-Linked Immuno Sorbant Assay (DAS-ELISA) of total secretory IgA (S-IgA) and S-IgA specific against proteins and/or peptides of the NBFpH6 in the guts of mice that received NBFpH6

The small intestine from each mouse was recovered by truncation at the stomach/duodenum junction and the ileum/ascending colon junction and its contents was flushed out with 5 ml of PBS. Particulate material was removed by centrifugation at 10,000 ×*g* for 10 min at 4 °C. The supernatant fluid was stored at −80 °C. Total IgA antibodies were detected by standard DAS-ELISA. Briefly, 50 μl of a solution of affinity purified monoclonal goat α-chain specific anti-IgA (Sigma-Aldrich) containing 25 μg antibody/ml in 50 mM carbonate–bicarbonate buffer, pH 9.6, was added to each well in each 96-well, flat-bottomed Nunc-Immuno Plate™ Maxi Sorp™ plate (Nalge Nunc International, Copenahue, Denmark) and the plates were incubated at 37 °C for 1 h. For the determination of specific S-IgA against proteins and/or peptides of the NBFpH6, plates were coated for 1 h at 37 °C with 200 μl/well total protein of 180 μg/ml (Bradford assay) of the NBFpH6 in 50 mM carbonate–bicarbonate buffer, pH 9.6. Plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and then incubated for 1 h at 25 °C with 0.5% skim milk in PBS. They were washed three times with PBS-T, then coated with 50 μl of serial dilutions of standard kappa IgA (Sigma-Aldrich) to obtain the standard curve, or 50 μl of samples of the intestinal fluids serially diluted in PBS plus 0.5% skim milk and incubated at 37 °C for 2 h. The plates were washed five times with PBS-T then the wells were filled with anti-mouse IgA (α chain specific) peroxidase conjugate (Sigma) at 1.25 μg/well (50 μl/well) and the plates were incubated at 37 °C for 1 h. Subsequently, the plates were washed seven times with PBS 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, and the plates were incubated at 37 °C. After 20 min, the reaction was stopped by the addition of 100 μl of 2 N H_2SO_4 to each well. Absorbance at

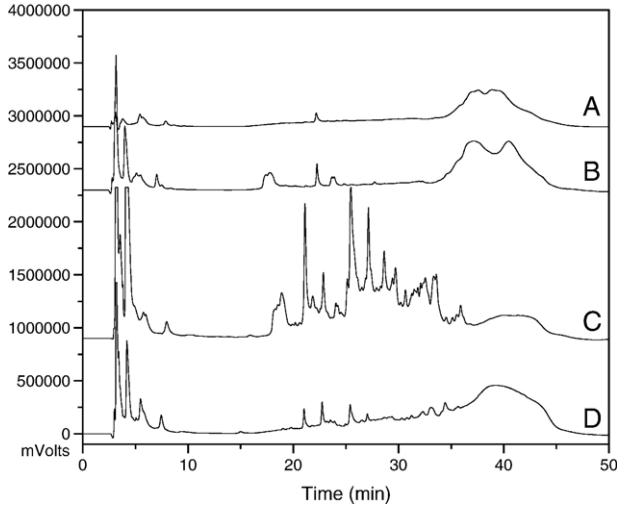

Fig. 1. RP-HPLC profile of unfermented milk (A), milk acidified with HCl (B) and milk fermented by *L. helveticus* R389 with (D) and without (C) pH control.

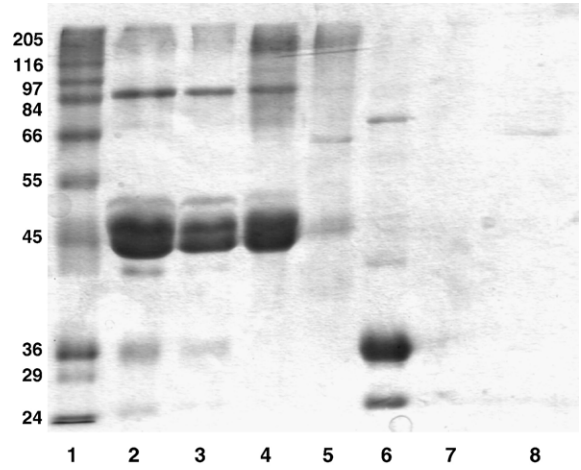

Fig. 2. The SDS-PAGE of 12% milk (2), 12% milk acidified to pH 3.7 with 85% lactic acid (3), milk fermented by *L. helveticus* R389 (no pH control: (4) or pH6-controlled: (5)), supernatant from milk acidified to pH 3.7 (6), non-bacterial fraction of milk fermented by *L. helveticus* R389 (no pH control: (7) or pH6-controlled: (8)). Molecular mass marker (1) in kDa.

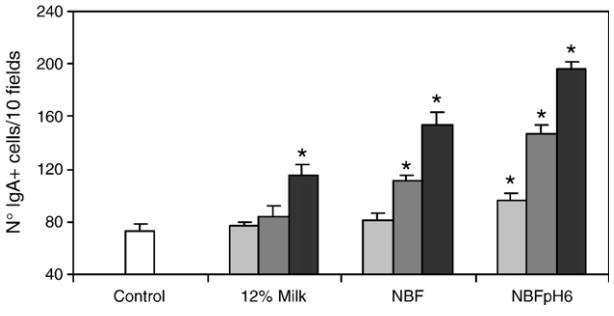

Fig. 3. Effect of the oral administration of the non-bacterial fraction of milk fermented by *L. helveticus* R389 with (NBFpH6) or without (NBF) pH control, for 2 (▒), 5 (▒) or 7 (■) consecutive days, on the number of IgA+ cells in the small intestine lamina propria, compared to PBS control mice (□) or animals that received 12% milk. * Significantly different from the PBS control group ($P<0.05$).

493 nm was read in an ELISA microplate reader (Versa max; Molecular Devices, Sunnyvale, CA, USA).

2.10. Statistical analysis

Data were analyzed using the one-way ANOVA procedure of SPSS software (SPSS Inc., Chicago, IL, USA). The differences between means were detected by Duncan's Multiple Range Test (SPSS, 1996). Data were considered significantly different when P was <0.05.

3. Results

3.1. Characterization of the non-bacterial fractions

The HPLC elution patterns of milk confirmed that *L. helveticus* R389 hydrolyzed milk proteins with the release of peptides and low molecular weight proteins not present before fermentation (Fig. 1A). The proteolysis was greater when the pH was kept at 6.0 (Fig. 1C) than when the pH was not controlled (Fig. 1D). Acidification of milk also resulted in some release of peptides or low mass proteins. The SDS-PAGE analysis of fermented milks and their non-bacterial fractions (Fig. 2) showed that pH6-controlled fermentation of milk by *L. helveticus* R389 produced a greater break down of milk proteins than milk fermentation without pH control or acidification, as indicated by the disappearance of protein bands obtained with non-fermented milk.

3.2. Effects of the non-bacterial fractions on the mucosal immune response

The effects of the oral administration of the non-bacterial fraction of milks fermented by *L. helveticus* R389 on the number of IgA+ cells in the lamina propria of the small intestine are shown in Fig. 3. There was a significant increase ($P<0.05$) in the number of IgA+ cells on day 7 of 12% milk administration and on days 5 and 7 of the oral administration of the NBF. With NBFpH6, there was a significant increase in the number of IgA+ cells after all feeding periods. The number of IgA+ cells at day 7 of NBFpH6 administration was significantly higher ($P<0.05$) than the numbers observed after feeding NBF or 12% milk for the same period.

The content of total S-IgA in the small intestine lumen of mice that received NBFpH6 for 2 days was 232±18 μg/ml. In animals that received NBFpH6 for 5 or 7 days the levels of S-IgA did not differ from PBS control values of 110±12 μg/ml. The content of S-IgA specific against peptides and/or proteins of NBFpH6 at all feeding periods did not differ from the value of 8960±625 ng/ml for PBS control animals.

The cytokine assay showed that at all the times, the numbers of IL-10+, IL-2+ and IL-6+ cells were significantly greater ($P<0.05$) in the intestine of animals that received NBFpH6 than in those that received PBS (Fig. 4).

IL-6 secretion by small intestine epithelial cells from animals that received NBFpH6 or PBS for 7 days were 4040±380 pg/ml or 1702±128 pg/ml, respectively. The difference in the amounts was significant ($P<0.005$).

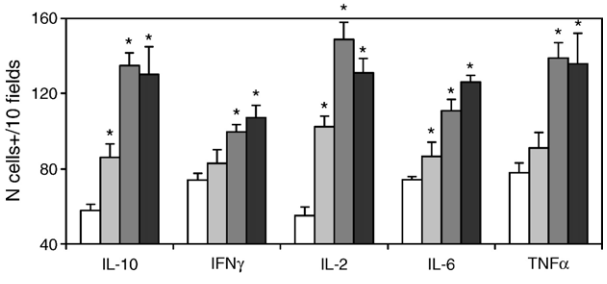

Fig. 4. Effect of the oral administration of the non-bacterial fraction of milk fermented by *L. helveticus* R389 (pH6-controlled) for 2 (▒), 5 (▓) or 7 (■) consecutive days on the number of cytokine+ cells in the small intestine lamina propria compared to the corresponding PBS control (□). * Significantly different from the corresponding control ($P<0.05$).

4. Discussion

L. helveticus is an industrially important thermophilic starter for the fermentation of foods. It is mostly employed for cheese manufacture (Fortina et al., 1998). This species, known for its high proteolytic activities (Luoma et al., 2001), releases oligopeptides from milk proteins (Foucaud and Juillard, 2000). These oligopeptides can be hydrolyzed to bioactive peptides by gastrointestinal enzymes. In our study, the HPLC elution patterns as well as the SDS-PAGE study of milks fermented by *L. helveticus* R389 demonstrated the high proteolytic activity of this strain, which was enhanced when the pH was kept at 6.0 during fermentation. This is due to the optimum pH for activity of lactic acid bacteria proteases being near pH 6.0 (Frey et al., 1986).

The possibility that the gut epithelium may respond to luminal factors by signalling their presence to the mucosal immune system has been largely ignored. It is now recognized that enterocytes secrete cytokines, growth factors and their binding proteins, and may directly present antigens to T cells. Epithelial signalling enables luminal factors to alter immune responses while the integrity of the epithelial barrier is maintained (Sanderson, 1998). Nutrition influences the maintenance of intestinal epithelium integrity and functioning as well as the gut mucosa immune response. For example, total parenteral nutrition leads to loss of the intestinal epithelial barrier function, atrophy of the gut-associated lymphoid tissue and significant decreases of intestinal IgA levels (Keith Hanna et al., 2000; Yang et al., 2002). In this work, the oral administration of NBFpH6 to mice was able to up-regulate IL-6 secretion, which is necessary for B cell terminal differentiation for IgA secreting cells in the gut lamina propria (Goodrich and McGee, 1999).

The oral administration of the non-bacterial fractions of milks fermented by *L. helveticus* R389 modulated the gut mucosa immune response. There was an increase in the number of IgA+ cells in the lamina propria of the small intestine. That increase, which was greater in mice that received NBFpH6 than in the ones that received NBF or non-fermented 12% milk, was probably due to the greater content of bioactive peptides in NBFpH6. Feeding of 12% milk also induced an increase in the number of IgA+ cells

by day 7, possibly due to the presence of endogenous preformed immunoregulatory peptides in bovine milk (Gill et al., 2000) or other factors not controlled in this study. We observed that the greater the amount of peptides formed in milk, the greater the effect on the number of IgA+ cells in the small intestine lamina propria. Since the greatest effects on the number of IgA+ cells were observed when mice received NBFpH6, we decided to further characterize its effects on the gut mucosa. Consequently, NBFpH6 was used in the subsequent studies.

B cell immunoglobulin switching and differentiation to plasmocytes secreting IgA occurs in an environment rich in IL-4, IL-5 and TGF-beta (Blum et al., 1999), while IL-6 promotes terminal differentiation of B cells into plasma cells (Goodrich and McGee, 1999). In this study, the increase in the number of IgA+ cells was accompanied by an increase in the number of IL-10+ and IL-6+ cells. It is known that macrophages and dendritic cells of the lamina propria, as well as other cell types, are producers of IL-6, IL-10, IL-12, IFN-γ and TNF-α (Husband et al., 1999). Additionally, mast cells are a potential source of early-response cytokines, such as TNF-α and IL-4, which are decisive in initiating the immune and inflammatory responses (Féger et al., 2002). Therefore, these cell populations from the innate immune system could have been the source of the cytokines determined in the lamina propria of the small intestine. The simultaneous induction of some pro-inflammatory and regulatory cytokines could be beneficial for the maintenance of a chronic and immunological balanced intestinal inflammation response, which has been termed physiological inflammation (Cebra et al., 2005).

It has been reported that IgA B cells in the lamina propria could arise from B cell populations present in the peritoneal cavity that are different from those in the Peyer's patches (Strober et al., 2005). Lamina propria IgA cells, termed B-1 B cells, were later shown to undergo isotype switching in the absence of T cells. Consequently, B-1 B cells are said to be the origin of "natural" IgA antibodies that provide an early and innate immune response against bacterial and viral invaders (Strober et al., 2005). The main function of secretory IgA in the gut is to exert the immune exclusion of pathogenic bacteria or viruses by intimate cooperation with the innate, non-specific defense mechanisms (Brandtzaeg et al., 1987). In our study, the most significant IL-6 response by small intestine epithelial cells as well as the greatest response in the number of IgA+ cells was achieved after 7 days of oral administration of NBFpH6. On day 2 we observed a peak in S-IgA production, which then returned to control values. We hypothesized that there was a down-regulation of S-IgA production on days 5 and 7, perhaps mediated by the regulatory IL-10 (Lindsay and Hodgson, 2001), which showed an enhanced response. Down-regulation of S-IgA would maintain intestinal homeostasis in the absence of a pathogenic threat to healthy mice and so avoid an unnecessary overactivation of the local immune response. However, the steady increase in the numbers of IgA+ cells in the lamina propria from days 2 to 7 would still ensure an enhanced state of immunological surveillance mediated by an enhanced population of IgA+ cells. Lamina propria B-1 cells are very sensitive to T-cell-independent stimulation, which results in polyclonal stimulation (Bos et al., 2005). When determining whether the S-IgA observed on day 2 showed specificity against the peptides and/or proteins present in the NBFpH6, we observed that there were no differences from control values. This would mean that the enhanced S-IgA was polyvalent or "natural", as termed by Macpherson et al. (2000).

In recent years, our concept of IgA B cell differentiation has changed in that we can no longer consider the IgA response as related exclusively to adaptative immunity, in which the IgA B cell is strictly dependent on a T cell both for its differentiation into an IgA B cell (class-switch differentiation) and for its terminal differentiation into an antibody-producing plasma cell (Strober et al., 2005). The studies of Macpherson et al. (2000, 2001) and Fagarasan et al. (2001) provide strong support for the existence of an alternative (innate) IgA B cell differentiation program based on B-1 B cells and occurring in the lamina propria. Three observations lead us to believe that NBFpH6 acted mainly at the innate immune response level. The absence of a clearly polarized cytokine response, i. e. one not dominantly Th1 or Th2, suggests that the enhanced cytokine response was due to an effect on innate immune cells such as macrophages, dendritic cells or mast cells. The possible local proliferation of IgA+ cells in the lamina propria in response to T-independent stimulation of B-1 cells would involve cells that were not primed at inductive sites, such as Peyer's patches, with repopulation of the lamina propria through the IgA cycle. The absence of specific S-IgA directed against the fraction administered indicates that there was no antigenic presentation. Additionally we previously observed that non-bacterial fractions from the fermented milk kefir were able to stimulate the cytokine response in isolated peritoneal macrophages and in adherent cells from Peyer's patches (Vinderola et al., 2006a). However, we cannot ignore the possible participation of adaptive immunity that might have been induced, or at least an effect on T cells proliferation, since an increase in the number of IL-2+ cells was also observed.

The induction of IgA+ B cells could indicate an enhanced immunosurveillance to prevent intestinal infections or other intestinal pathologies.

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