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ORIGINAL RESEARCH

Influence of technological variables on the functionality of the cell-free fraction of fermented buttermilk

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Buttermilk is a suitable substrate for fermentation with proteolytic strains of Lactobacillus *in order to release peptide fractions able to enhance the gut mucosal immune system. We aimed to determine the influence of the degree of proteolysis of buttermilk proteins on their functionality. Animals received for seven consecutive days the cell-free fraction of 10 or 20% (w/v) buttermilk fermented with* Lactobacillus delbrueckii *subsp.* lactis *210 at pH 6. The pH was controlled either with NaOH or* $Ca(OH)_2$*. No significant differences in the number of IgA-producing cells in the small intestine of mice were found. The functional capacity of the product under study was not affected by the technological variables considered.*

Keywords Functionality, Fermented buttermilk, Cell-free fraction, IgA.

INTRODUCTION

Among functional foods, fermented milks containing probiotic bacteria dominate the global market (Saxelin 2008; Figueroa-González *et al.* 2011). In these products, functionality is given by a combination of their bioactive components such as viable probiotic bacteria and lactic acid starters, peptides derived from milk fermentation and other components such as exopolysaccharides or products derived from lactic acid bacteria metabolism in milk (bacteriocins and organic acids, for example) (Vinderola 2008). Cell viability of probiotic bacteria during production and storage of probiotic foods is a prerequisite in order to achieve their claimed health benefits (Ouwehand and Salminen 1998; Galdeano and Perdigón 2004). Many other foods other than fermented milks or fresh cheeses are not, up to now, suitable vehicles for incorporating probiotic bacteria, as their process of manufacture (high temperature) or storage (room temperature) or their physicochemical characteristics (acidity, water activity, high osmotic pressure) renders them inadequate to carry viable micro-organisms. During milk fermentation, a range of secondary metabolites (bacteriocins, exopolysaccharides) are produced by lactic acid bacteria or released by them from milk proteins, such as bioactive peptides (Vinderola 2008). Many of these components have been associated with health-promoting properties such as mucosal immunomodulation, antihypertensive, antithrombotic, opioid and antimicrobial activities and participation in many nutritional pathways (Silva and Malcata 2005). Buttermilk is a relatively low-cost by-product of the dairy industry obtained during butter manufacturing. In a previous study (Burns *et al.* 2008), we reported the capacity of buttermilk to support the growth of lactobacilli. Although the nutritional and functional value of skim milk components is well understood, buttermilk has only recently gained attention as a potential source of functional ingredients, and the production of buttermilk fractions as value added ingredients would have a great economic impact (Corredig *et al.* 2004). In a previous work (Burns *et al.* 2010), we showed that buttermilk was a suitable substrate for

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fermentation with a proteolytic strain of *Lactobacillus helveticus* and the release of peptide fractions able to be spray-dried and to modulate the gut mucosa *in vivo*. The final goal of these series of works is to obtain, by spray-drying, the bioactive compounds released by an autochthonous strain of *Lactobacillus* in buttermilk, to confer functionality to foods not always able to carry viable probiotic bacteria, such as cereal bars, cookies or too-acidic drinks. Taking into account that many technological variables might influence the functionality of food (Vinderola *et al.* 2011), the aim of this work was to determine the influence of the degree of proteolysis of buttermilk proteins on the functionality of the cell-free fraction of fermented buttermilk.

MATERIALS AND METHODS

Strain, culture media and culture conditions

A strain used in a previous work (Burns *et al.* 2010) was employed in this study. The strain was formerly identified, using sugar fermentation tests, as *Lactobacillus helveticus* 209. For this study, the strain was re-identified using molecular tools (see below). The strain belongs to the culture collection of the INLAIN (Instituto de Lactología Industrial, UNL-CONICET, Santa Fe, Argentina) and had been isolated from natural whey starters (Reinheimer *et al.* 1996). Overnight cultures were obtained in MRS broth (Biokar, Beauvais, France) or in 10% (w/v) reconstituted buttermilk at 43 °C. Buttermilk powder (from fresh neutral buttermilk) was obtained from a local dairy plant. The physicochemical composition of buttermilk (powder) was as follows: lactose: 47 ± 1.3% (w/w), protein: 29 ± 0.8% (w/w), moisture: 3.1 ± 1.1% (w/w), fat: 12 ± 0.4% (w/w), ash: 7.4 ± 0.3% (w/w). When required, cell counts were performed on MRS agar (Biokar) after 48-h incubation at 43 °C in plastic jars under anaerobiosis (GENbox anaer sachets; Biomérieux, Marcy l'Etoile, France).

Identification of the strain

Total DNA of the strain was obtained using the GenElute Bacterial Genomic DNA kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. The identity was analysed by amplifying, sequencing and comparing 1500 bp within its 16S rRNA gene (Edwards *et al.* 1989). PCRs were performed using 1 μL of diluted (1:50) DNA as template, 2.5 U Taq DNA polymerase (GE Healthcare, Little Chalfont, UK), 200 nMdNTPs (GE Healthcare) and 400 nM each primer (Sigma-Genosys, The Woodlands, TX, USA) in a final volume of 50 μL, including a negative control (without template). Amplifications were performed in a GeneAmp PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 2 min at 51 °C and 2 min at 72 °C and a final step of 7 min at 72 °C. The PCR products were separated on 0.8% (w/v) agarose gels in TBE buffer, stained with GelRed (Biotium, Hayward, CA, USA) and visualised under UV light (Sambrook and Russell 2001). The amplicon was purified with MicroSpin Columns (GE Healthcare), and its nucleotide sequence was determined by primer extension at the DNA Sequencing Service of Macrogen (Seoul, Korea). The identity was checked by nucleotide–nucleotide BLAST of the NCBI database (www.ncbi.nlm.nhi.gov/blast).

Preparation of fermented buttermilk and its cell-free fraction

Buttermilk was fermented with the strain under study with and without pH control. Reconstituted buttermilk (10 or 20%, w/v) was inoculated (2%, v/v) with an overnight culture of the strain (in 10%, w/v buttermilk) and incubated without pH control at 43 °C for 22 h (no stirring either). For buttermilk fermented with pH control, a 1.5 L stirred tank bioreactor (LH Fermentation Ltd., Stoke Poges, Bucks, UK) was used. Reconstituted (10% or 20%, w/v) and autoclaved (115 °C, 30 min) buttermilk was inoculated as previously described, the tank being maintained at 43 °C with an agitation rate of 200 rpm and sparging with CO_2 at 0.2 L/min. pH was maintained at 6 throughout the fermentation period (22 h) by automatic addition (peristaltic pump) of 8 M NaOH solution or 8 M $Ca(OH)_2$ suspension (held under constant stirring). Cell counts were performed at time 0, 12, 14, 16, 18, 20 and 22 h of culture on MRS agar plates (43 °C, 48 h, anaerobiosis).

Fermented buttermilk obtained without pH control was centrifuged (4000 × *g*, 4 °C, 15 min). The supernatant (cell-free fraction) was recovered, and a fraction was filtered through a 0.22 μm filter (Millipore, Etobicoke, ON, Canada) and stored at −80 °C. Buttermilk fermented with pH control was acidified to pH 4.60 with 85% DL-lactic acid syrup (Sigma-Aldrich). The acidified buttermilk was centrifuged (4000 × *g*, 4 °C, 15 min), the supernatant was recovered and stored as described above. Buttermilk fermentation, for each different condition, was replicated at least twice.

Proteolytic activity and analysis of fermented buttermilk by reverse-phase high performance liquid chromatography

Proteolytic activity of fermented buttermilk was determined by the o-phthaldialdehyde spectrophotometric assay (OPA Test) (Church *et al.* 1983), and values were expressed as the difference in absorbance at 340 nm between fermented samples and a control of noninoculated buttermilk. Results are the mean of three independent assays. Filter-sterilised samples (0.22-μm filter) of cell-free supernatants obtained as described in the item above were analysed by reverse-phase high performance liquid chromatography (RP-HPLC). A Waters chromatography system (Waters Corporation, Mildford, MA, USA) was used, which was consisted of Waters 1500 Series HPLC pump, Waters 717 plus autosampler, Waters 2487 dual λ

absorbance detector and Waters Breeze System software (Waters Corporation, Mildford, MA, USA). A 100 μL portion of each sample (diluted 1:10 in distilled water) was injected on a Microsorb-MV (250 × 4.6 mm) C18, 300 Å column (Varian Inc., Palo Alto, CA, USA). Two solvents were used for the separation: solvent A, 0.1% (v/v) trifluoroacetic acid (TFA) in water and solvent B, 0.1% (v/v) TFA in acetonitrile. Separations were carried out at 30 °C and a flow rate of 0.9 mL/min under the following conditions: 100% solvent A for 5 min, linear gradient from 0 to 60% of solvent B over 25 min, isocratic step at 60% B for 5 min and finally, a linear gradient from 60 to 0% of solvent B (100% of solvent A) over 10 min. Detection was performed at 214 nm.

In vivo trial

Animals and feeding procedures

Thirty-five 6-week-old female BALB/c mice weighing 19–21 g were obtained from the random bred colony of the Centro de Experimentaciones Biológicas y Bioterio, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe). Animals were kept in the INLAIN animal facility for a week before starting the feeding procedures. Each experimental group consisted of five mice housed together in plastic cages and kept in a controlled environment (21 ± 2 °C and 55 ± 2% humidity), with a 12-h light/dark cycle. Mice were maintained and treated according to the guidelines of the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council (http://grants.nih.gov/grants/olaw/Guide--for-the-care-and-use-of-laboratory-animals.pdf). The animal assay was approved by the Ethical Committee for Animal Experimentation of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe, Argentina). The 3 R's principle was taken into account when using animals.

All mice (grouped into five) received, during seven consecutive days and by gavage, 200 μL/day/mouse of any of the following samples: (i) tap water, (ii) nonfermented buttermilk, (iii) the supernatant of buttermilk fermented without pH control or (iv) the supernatant of pH 6 controlled 10% (w/v) or 20% (w/v) fermented buttermilk, where pH was controlled either with NaOH or with $Ca(OH)_2$. All animals received, simultaneously and *ad libitum*, tap water and a sterile conventional balanced diet containing carbohydrates (460 g/kg) 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.

Assessment of IgA in the small intestine

After the 7-day feeding period, animals were injected intraperitoneally with an anaesthetic mixture containing nine parts of ketamine (100 mg/mL), nine parts of xylazine (20 mg/mL), three parts of acepromazine (10 mg/mL) and 79 parts of sterile saline solution. Animals were sacrificed by cervical dislocation. Liver was removed and homogenised in 5 mL of sterile PBS. One millilitre of liver homogenate was pour plated onto MacConkey agar (safety assay: translocation of enterobacteria to liver). Plates were incubated at 37 °C for 24 h in aerobiosis. The small intestine was recovered for intestinal fluid obtention. Small intestine was flushed with 2 mL of cold PBS buffer containing a cocktail of protease inhibitors. Small intestinal fluid was recovered, centrifuged (10 000 × *g*, 15 min, 4 °C) and stored at −70 °C for secretory IgA quantification by ELISA (Vinderola *et al.* 2007). Flushed intestines were prepared for fixation, histological preparation and paraffin inclusion, according to Vinderola *et al.* (2005). Paraffin sections (4 mm) were stained with haematoxylin–eosin and then examined by light microscopy (double blind observations). 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 alpha-chain-specific anti-mouse IgA fluorescein isothiocyanate (FITC) conjugate (Sigma). Histological slices were deparaffinised and rehydrated in a series of decreasing ethanol concentrations (from absolute alcohol to 70% (v/v) alcohol). Deparaffinised 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 two times with PBS and examined using a fluorescent light (Hg lamp) microscope (Nikon Eclipse E200, Nikon Instruments Inc., Melville, NY, USA). The results were expressed as the number of positive cells (fluorescent cells)/10 fields. Positive (fluorescent) cells were counted with a magnification of 400× (double blind counts). Data were reported as the mean of three counts (each one in a different histological slice) for each animal.

Statistical analysis

The data were analysed by one-way ANOVA using the SPSS software (SPSS Inc., Chicago, IL, USA). The differences between means were detected by the Tukey's multiple range test (version 15.0; SPSS Inc., Chicago, IL, USA). Data were considered significantly different when P is <0.05.

RESULTS AND DISCUSSION

The functional foods industry is experiencing a constant expansion due to the growing demand for new foods and supplements with a proven scientific basis of efficacy on health and consumer interest in functional foods remains strong (Katan and De Roos 2004; Kapsak *et al.* 2011). Attention concerning this category of foods has grown, and new products have appeared in the European market, and interest has turned to define the standards and guidelines for the development and promotion of this kind of foods (Serafini *et al.* 2012). In this context, the production of

novel functional additives for the expansion of the functional food market beyond existing probiotics, prebiotics and other functional ingredients is expected to be welcomed by the food industry. Even though fermented dairy products carrying probiotic bacteria are functional food market leaders (Figueroa-González *et al.* 2011), certain characteristics of specific probiotic strains (high sensitivity to acids or osmotic pressure, need of low storage temperature) have confined them mainly to dairy products (yoghurt, cheese, frozen desserts), limiting the development of other kinds of food products. The health benefits of fermented dairy products containing probiotic bacteria are exerted by a combination of the effects of live micro-organisms (probiotic and lactic acid starter bacteria) and substances produced in milk or derived from milk fermentation, such as bioactive peptides, exopolysaccharides, bacteriocins and organic acids (Clare and Swaigood 2000; Cobb and Kasper 2005; Stanton *et al.* 2005; Beermann and Hartung 2013). In previous works (Burns *et al.* 2008, 2010), it was demonstrated that buttermilk is an adequate substrate for the production of biomass of probiotic lactobacilli and for its fermentation with proteolytic strains of lactic acid starter lactobacilli in order to release bioactive components able to enhance the gut mucosal barrier mediated by IgA. These activities are part of a major project aiming at developing a low-cost technologically simple functional powder carrying the beneficial metabolites left in fermented milk by the proteolytic action of specific strains of lactobacilli and at the same time able to be added to those foods where probiotic bacteria cannot be incorporated for reasons of poor viability. In this work, we aimed at determining the influence of the degree of proteolysis on the functional capacity of the cell-free fraction of pH controlled fermented milk. *Lactobacillus delbrueckii* subsp. *lactis* 210 was grown in buttermilk at different substrate concentrations (10 and 20%, w/v) and at pH 6 constant, controlled by the addition of NaOH or $Ca(OH)_2$. Figure 1 shows the cell counts of this strain grown under the conditions described. The maximum cell concentration was achieved by hour 12 or 14 when pH was controlled with $Ca(OH)_2$ or NaOH, respectively. Maximum biomass concentration was significantly higher ($P < 0.05$) when the pH was controlled with NaOH during fermentation and compared with $Ca(OH)_2$ the difference was approximately 1 log order and 1.3 log orders at the 10 and 20% (v/v) buttermilk concentrations, respectively.

By the end of the fermentation (22 h), cell counts decreased by approximately 1 log order for NaOH controlled fermentation and by approximately 1.8–2.4 log orders when the pH was maintained at 6 with $Ca(OH)_2$. In a previous work, it was observed that pH controlled buttermilk fermentation led to an increase in the salt content (as Na and Ca lactate) of the fermentation medium (Burns *et al.* 2010), which led to an increase of the osmotic pressure. Lactic acid bacteria display acid resistance mechanisms to survive the by-products of their own metabolism (i.e. lactic acid). For example, in *L. delbrueckii* subsp. *bulgaricus*, during the acid adaptation present in the fermentation of milk to obtain yoghurt, the expression of chaperon proteins GroES, GroEL, HrcA, GrpE, DnaK, DnaJ, ClpE, ClpP and ClpL was induced (Serrazanetti *et al.* 2009). These proteins are known to confer enhance protection against adverse conditions (Derzelle *et al.* 2003). It is a commonly observed fact that pre-exposure to one stress may also confer protection against other stresses, a phenomenon termed 'cross-adaptation' (Lou and Yousef 1996; O'Driscoll *et al.* 1996). For example, bifidobacteria grown at pH 5 (lactic acid stress) were more resistant to simulated gastric digestion than cultures grown at pH 6 (Vinderola *et al.* 2012). In this context, we might hypothesise that the lack of exposure of cells to lactic acid stress due to the maintenance of pH at a constant value of 6 failed to induce cross-protection to the osmotic stress exerted by the increasing salt content (Na or Ca lactate) of the medium along fermentation, which led to cell death as observed in Figure 1. Consistent with previous findings (Shelef and Potluri 1995; Aran 2001), calcium lactate was more inhibitory towards cells compared to sodium lactate, both derived from $Ca(OH)_2$ or NaOH addition to the fermentation medium for pH control. Cell inhibition is induced by a combination of mechanisms not all completely understood (Shelef 1994).

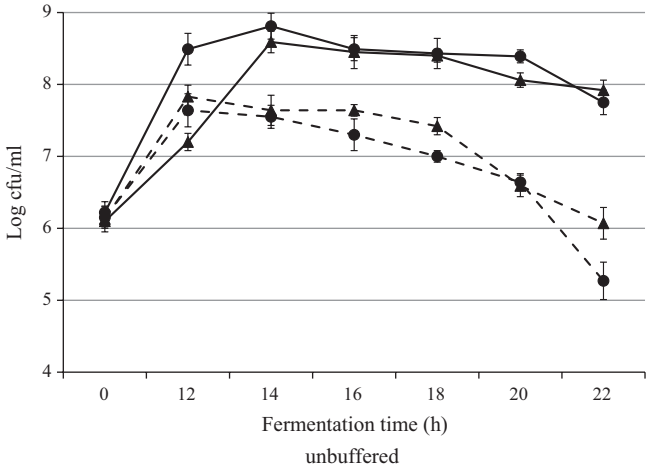

Figure 1 Cell counts of *L. delbrueckii* subsp. *lactis* 210 during the fermentation of 10% (w/v) (▲) or 20% (w/v) (●) buttermilk at pH 6 controlled with NaOH (straight line) or $Ca(OH)_2$ (dashed line).

The proteolytic activity increased during fermentation (data not shown) to reach final values of 0.830 ± 0.024 [10% (w/v) buttermilk, $Ca(OH)_2$], 1.418 ± 0.018 [20% (w/v) buttermilk, $Ca(OH)_2$], 3.516 ± 0.122 [10% (w/v) buttermilk, NaOH] and 3.215 ± 0.048 [20% (w/v) buttermilk, NaOH]. Proteolytic activity in the $Ca(OH)_2$ controlled fermentation was significantly lower than its counterpart in NaOH controlled fermentation. The higher inhibitory capacity of

calcium salts compared to sodium salts on protease activity had been reported (Armenteros *et al.* 2009). Proteolytic activity in the $Ca(OH)_2$ controlled fermentation was significantly higher ($P < 0.05$) when 20% (w/v) buttermilk was used, compared to 10% (w/v). However, no significant differences were observed in proteolytic activity values when NaOH was used as pH control agent, suggesting that the maximal proteolytic capacity of the strain has been achieved in NaOH controlled fermentation independently of the substrate concentration or that an artifact (saturation) of the technique employed to measure proteolytic activity might have occurred.

Cell-free supernatants derived from buttermilk fermentation with and without pH control were analysed by RP-HPLC to study the degree of proteolysis of buttermilk proteins and the peptide fractions released to the culture medium (Figure 2). Fermentation of buttermilk carried out without pH control (Figure 2b) scarcely modified the RP-HPLC profile of unfermented buttermilk (Figure 2a), increasing slightly the peptide fractions that appeared from minute 16 onwards. When fermentation of 20% (w/v) buttermilk was carried out at pH 6 for 22 h using $Ca(OH)_2$ (Figure 2c) or NaOH (Figure 2d) as pH control agents, there was an evident increase in the peptide fractions, which appeared as 5–6 main peaks detected from minutes 16 onwards and in the peaks that appeared at minute 5. The increased peptide content of pH 6 controlled fermentation compared to unbuffered culture is due to an enhanced proteolytic activity of *Lactobacillus* proteases which have an optimum pH of activity between 5.5 and 7.5 (Sadat-Mekmene *et al.* 2011). The peaks were higher in NaOH controlled fermentation compared to $Ca(OH)_2$ controlled fermentation, correlating with the enhanced proteolytic activity when NaOH was used. The fact that doubling substrate concentration did not double proteolytic activity can also be noticed by integrating the area under the profiles, which gives an indirect measure of total peptide concentration. If we assume that the unbuffered fermentation area has an arbitrary value of one, then $Ca(OH)_2$-controlled 10% (w/v) buttermilk fermentation increased the peptide content in 3.16-fold, whereas its counterpart, $Ca(OH)_2$-controlled 20% (w/v) buttermilk fermentation, increased the peptide content in 4.41-fold. Finally, 20% (w/v) buttermilk fermentation controlled with NaOH increased the peptide content in 6.31-fold, compared to unbuffered fermentation of 20% (w/v) buttermilk.

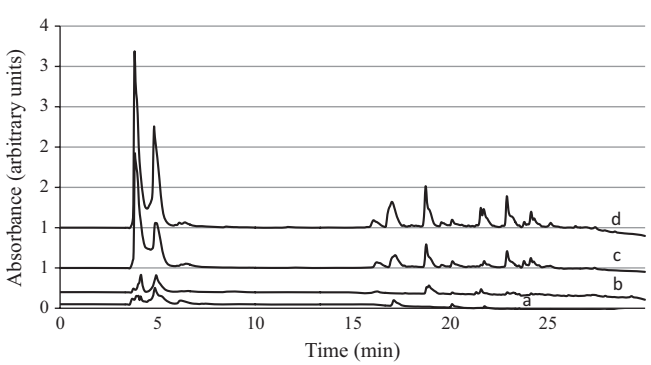

Figure 2 RP-HPLC profile of unfermented buttermilk supernatant (a), cell free fraction of buttermilk fermented without pH control (unbuffered) (b) or buttermilk fermented at pH 6 using $Ca(OH)_2$ (c) or NaOH (d). In all cases 20% (w/v) buttermilk was used and fermentation was conducted for 22 h.

We aimed then to determine whether these different degrees of proteolysis of buttermilk proteins, achieved by different substrate concentration or pH control agents, might influence the capacity of the fermented buttermilk supernatant to enhance the gut mucosal immunity mediated by IgA previously reported (Burns *et al.* 2010). Immunoglobulin A (IgA) is the main immunoglobulin in the gut surfaces where its main function is to exert the immune exclusion of pathogenic bacteria or viruses by intimate cooperation with the innate nonspecific defence mechanisms (Brandtzaeg *et al.* 1987). As a functional trait, the induction of the proliferation of intestinal IgA-producing cells is a desired attribute for functional foods containing probiotic bacteria or bioactive peptides (Vinderola 2008; Galdeano *et al.* 2009). Mice received orally the supernatant of fermented buttermilk for seven consecutive days, and IgA was assessed by two different methodologies in the small intestine: enumeration of the number of IgA-producing cells on histological slices (immunohistochemistry) and quantification of secretory IgA in the small intestine fluid (ELISA test). The administration of nonfermented buttermilk or unbuffered fermented buttermilk induced no changes in the number of IgA-producing cells compared to control mice (Figure 3). The administration of the cell-free supernatant of 10 or 20% (w/v) buttermilk, fermented under the pH control of NaOH or $Ca(OH)_2$, significantly enhanced ($P < 0.05$) the number of IgA-producing cells, compared to control mice. However, no significant differences ($P > 0.05$) were observed among these groups. Taking into account the dose response general concept, one might assume that the higher the proteolytic activity, the higher the immunostimulation achieved. However, the gut mucosal immune system does not always respond in a linear way to challenge with functional foods. For example, in a previous work, we showed that challenging small intestine epithelial cells with 10^7 or 10^8 cfu of *Lactobacillus casei* CRL431 induced the same amount of IL-6 on the eukaryotic cell culture (Vinderola *et al.* 2005). In another study, no difference in production of IL-8 was observed in Caco-2 cells challenged with 10^7 or 10^8 cfu/mL of *Helycobacter pylori* (Myllyluoma *et al.* 2008). Comparing the RP-HPLC profiles of unbuffered fermented 20% (w/v) buttermilk to pH 6 controlled fermentations, it seems that there is a threshold amount of released peptides necessary to enhance the number of IgA-producing cells. At the same time, it seems there is also a limited capacity in the proliferation capacity of IgA-producing cells in the small intestine.

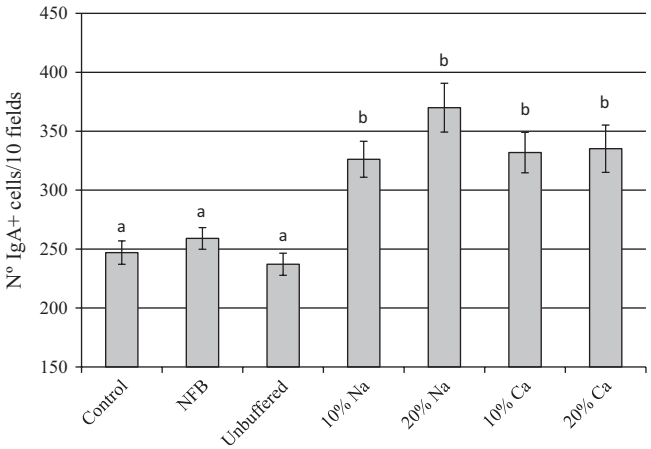

Figure 3 Effect of the oral administration (7 days) of the cell free supernatant of buttermilk fermented (pH 6 constant) with *L. delbrueckii* subsp. *lactis* 210 on the number of IgA + cells in the small intestine lamina propria of mice, compared to control mice (tap water). NFB group: mice that received non-fermented buttermilk. Unbuffered fraction group: mice that received the cell free fraction of unbuffered fermented 20% (w/v) buttermilk. pH-controlled group: 10% (w/v) or 20% (w/v) buttermilk was fermented for 22 h using NaOH or $Ca(OH)_2$ as pH-controlling agent. [a,b]Columns with different superscript are significantly different ($P < 0.05$).

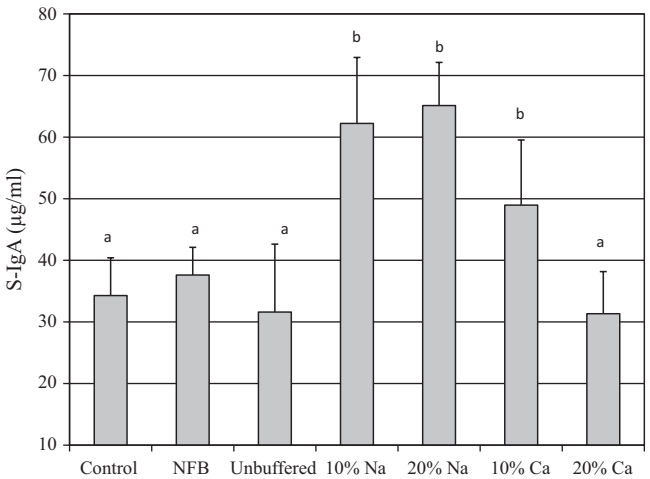

Figure 4 Effect of the oral administration (7 days) of the cell free supernatant of buttermilk fermented (pH 6 constant) with *L. delbrueckii* subsp. *lactis* 210 on the content of secretory IgA in the small intestine fluid, compared to control mice (tap water). NFB group: mice that received non-fermented buttermilk. Unbuffered fraction group: mice that received the cell free fraction of unbuffered fermented 20% (w/v) buttermilk. pH-controlled group: 10% (w/v) or 20% (w/v) buttermilk was fermented for 22 h using NaOH or $Ca(OH)_2$ as pH-controlling agent. [a,b]Columns with different superscript are significantly different ($P < 0.05$).

However, other immune parameters not measured in this study (cytokines, other immune cells) might have been triggered in a more dose dependant way by the degree of proteolysis. The immunoenhancing capacity of the cell-free fraction of fermented buttermilk on IgA was also assessed by quantifying secretory IgA in the small intestine fluid of mice. The administration of nonfermented buttermilk or unbuffered fermented buttermilk supernatant induced no changes in small intestine content of secretory IgA (Figure 4), compared to control mice. The administration of the cell-free supernatant of 10 or 20% (w/v) fermented buttermilk under the pH control of NaOH or 10% (w/v) buttermilk controlled with $Ca(OH)_2$ significantly enhanced ($P < 0.05$) the concentration of secretory IgA in the small intestine. Again, no significant differences ($P > 0.05$) were observed among these groups. It is worth mentioning that the different groups of animals were sacrificed in the same order that they appear in Figures 3 and 4 (from the left to the right), within a time period of ca. 5 h starting at 9 a.m. on the day of sacrifice. Chronobiology is a field of biology that examines periodic (cyclic) phenomena in living organisms and their adaptation to solar- and lunar-related rhythms. Motor and secretory activities, as well as the rhythm of cell proliferation in the gastrointestinal tract, are subject to many circadian rhythms (Konturek *et al.* 2011). Comparing results in Figures 3 and 4 (no differences on the number of IgA-producing cells among groups that received the cell-free fraction of pH controlled fermented buttermilk in Figure 3) and the fact that many immune parameters

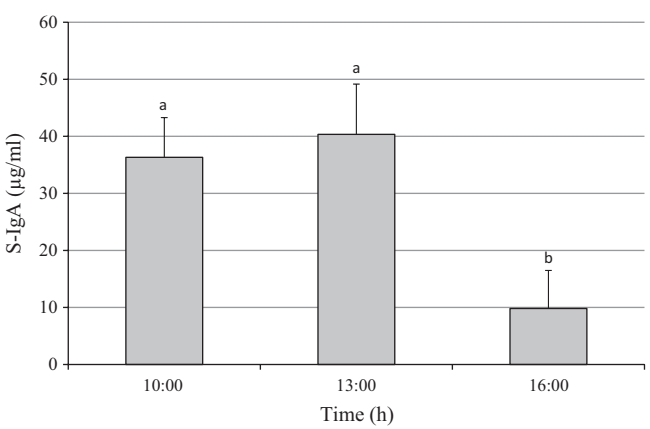

Figure 5 Content of secretory IgA in the small intestine fluid of control mice at different day hours. [a,b]Columns with different superscript are significantly different ($P < 0.05$).

change during the day (Guan *et al.* 2005; Dimitrov *et al.* 2007), we wondered whether the lack of enhanced content of secretory IgA in the group 20% (w/v) buttermilk Ca $(OH)_2$ might have been related to possible interference of circadian cycles in mice. To study this possibility, another animal trial was conducted. Three groups of five control mice (not treated) were sacrificed in the same day at three different times: 09:00, 13:00 and 16:00 h. Small intestine was recovered, and secretory IgA was quantified in the small intestine fluid as described in materials and methods.

Figure 5 shows that the secretory IgA content in the intestinal fluid changes during the day from 09:00 to 16:00 h, which might have negatively influenced the results of the last experimental group [20% (w/v) buttermilk $Ca(OH)_2$] in Figure 4. For future animal trials in our laboratory, we decided to program (shift) feeding periods in order to sacrifice few experimental groups on the same day, within a time period of not more than 1–2 h (shifted feeding periods). The influence of circadian cycles on IgA in the gut must be further studied to determine whether the effects of functional foods might be affected by this phenomenon.

In previous work (Burns *et al.* 2010), it was observed that pH control of the fermentation using NaOH dramatically increased the content of sodium in the final product. The product under study will be used, once optimised, for the development of new functional foods. The increased sodium content when NaOH is used might be regarded as a negative issue taking into account the health problems caused by hypertension related to a high sodium chloride intake (Mitsnefes *et al.* 2006). In this study, replacement of NaOH by $Ca(OH)_2$ for pH control did not negatively impact on the functional capacity of fermented buttermilk cell-free supernatant, at least from the point of view of IgA production in the small intestine. Additionally, the increased content of total solids, from 10% (w/v) to 20% (w/v), did not adversely affect strain growth capacity (due to possible osmotic pressure problems). Functional capacity was not affected. The possibility of conducting fermentation in the presence of a higher content of total solids might be considered as a positive fact since during spray-drying (the technological process adopted for dehydrating the cell-free supernatant), higher amounts of total solids are needed to operate the spray dryer equipment (Burns *et al.* 2010).

CONCLUSIONS

In order to obtain a cell-free fraction of fermented buttermilk with the functional capacity of enhancing mucosal IgA in the small intestine, fermentation with *L. delbrueckii* subsp. *lactis* 210 could be performed using either 10 or 20% (w/v) buttermilk with NaOH or $Ca(OH)_2$ as pH-controlling agents.

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