Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Suitability of buttermilk for fermentation with *Lactobacillus helveticus* and production of a functional peptide-enriched powder by spray-drying

P. Burns[1], F. Molinari[1], A. Beccaria[2], R. Páez[3], C. Meinardi[1], J. Reinheimer[1] and G. Vinderola[1]

1 Instituto de Lactología Industrial (INLAIN, UNL-CONICET), Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santa Fe, Argentina
2 Laboratorio de Fermentaciones, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Paraje El Pozo, Santa Fe, Argentina
3 Estación Experimental Agropecuaria, INTA, Rafaela, Santa Fe, Argentina

Keywords
buttermilk, *Lactobacillus*, peptides, spray-drying.

Correspondence
Gabriel Vinderola, Instituto de Lactología Industrial (INLAIN, UNL-CONICET), Facultad de Ingeniería Química, 1° de Mayo 3250, Santa Fe (3000), Argentina.
E-mail: gvinde@fiq.unl.edu.ar

2009/2209: received 26 December 2009, revised 14 April 2010 and accepted 20 April 2010

doi:10.1111/j.1365-2672.2010.04761.x

Abstract

Aim: To ferment buttermilk, a low-cost by-product of the manufacture of butter, with a proteolytic strain of *Lactobacillus helveticus*, to enhance its value by the production of a functional peptide-enriched powder.
Methods and Results: Buttermilk was fermented with *Lact. helveticus* 209, a strain chosen for its high proteolytic activity. To enhance the release of peptidic fractions, during fermentation pH was kept at 6 by using NaOH, $Ca(CO)_3$ or $Ca(OH)_2$. Cell-free supernatant was recovered by centrifugation, supplemented or not with maltodextrin and spray-dried. The profile of peptidic fractions released was studied by RP-HPLC. The lactose, Na and Ca content was also determined. The powder obtained was administered to BALB/c mice for 5 or 7 consecutive days, resulting in the proliferation of IgA-producing cells in the small intestine mucosa of the animals.
Conclusions: Buttermilk is a suitable substrate for the fermentation with *Lact. helveticus* 209 and the release of peptide fractions able to be spray-dried and to modulate the gut mucosa *in vivo*.
Significance and Impact of the Study: A powder enriched with peptides released from buttermilk proteins, with potential applications as a functional food additive, was obtained by spray-drying. A novel use of buttermilk as substrate for lactic fermentation is reported.

Introduction

The definition of probiotic bacteria adopted by the joint FAO/WHO working group (FAO/WHO 2002) establishes that they are '*live microorganisms which when administered in adequate amounts confer a health benefit on the host*'. Cell viability of probiotic bacteria is a prerequisite for probiotic food to achieve their claimed health benefits (Ouwehand and Salminen 1998; Galdeano and Perdigón 2004). Because the massive introduction of probiotics into the food market, fermented dairy products have been chosen as the main vehicles for the incorporation of viable probiotic bacteria. However, many other foods are not, up to now, a suitable vehicle for incorporating probiotic bacteria, because their process of manufacture (high temperature) or storage (room temperature) or their physicochemical characteristics (high acidity, too low water activity) render them inadequate to carry viable micro-organisms. During milk fermentation, lactic acid bacteria produce a range of secondary metabolites (bacteriocins, exopolysaccharides) and release peptides from milk proteins (Vinderola *et al.* 2008), many of which 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). Previous studies (Vinderola *et al.* 2006a,b, 2007a,b) have pointed out that the administration to mice of only the cell-free fraction of some fermented milks induced beneficial effects that resembled those

described for fermented milks containing viable bacteria. Buttermilk is a 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). Given that spray-drying is routinely used to dry food proteins, among many other applications (Fellows 2000), it could be used as a low cost accessible process to produce dried powders from the cell-free fraction of fermented milks with functional properties (Vinderola 2008). It would be then possible to add the dried cell-free fraction directly to many food matrixes, especially those with low water activity or high acidity where the probiotic viability would be seriously threatened. The aim of this work was to study the suitability of buttermilk for pH-controlled fermentation with *Lactobacillus helveticus* and the release of potentially bioactive peptides for the production of a peptide-enriched powder for further use in functional foods.

Materials and methods

Strains, culture media and incubation conditions

Nine strains of *Lact. helveticus* (ATCC 15807, CNRZ 892, CNRZ 303, CNRZ 241, HLM1, 133, 138, 200 and 209) were screened for their proteolytic activity in milk and buttermilk. Strains belong to the culture collection of the INLAIN and had been previously isolated from natural whey starters (Reinheimer *et al.* 1996) or acquired from international culture collections. Overnight cultures of lactobacilli were obtained in MRS (de Man, Rogosa and Sharpe) broth (Biokar, Beauvais, France) or in 8% (w/v) reconstituted buttermilk at 43°C. Buttermilk powder (from fresh neutral buttermilk) was received from a local dairy plant. The physicochemical composition of buttermilk (powder) was 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) and ash: 7·4 ± 0·3% (w/w). When required, cell counts were performed on MRS agar (Biokar) after 48-h incubation at 43°C under anaerobiosis (GENbox anaer sachets; Biomérieux, Marcy l'Etoile, France) in plastic jars.

Selection of a strain of *Lactobacillus helveticus* with high proteolytic activity in buttermilk

Acidification and proteolytic activity for the nine *Lact. helveticus* strains under study were determined by inoculation (2%, v/v) of the strains (previously transferred three times in 8% buttermilk at 43°C) in sterile (autoclaved at 115°C for 30 min) reconstituted buttermilk at percentages of 4, 8, 12, 16, 20 or 25% and incubation for 24 h at 43°C, without pH control. pH values were measured with a pH meter (model SA 720; Orion, Beverly, MA, USA). Proteolytic activity 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 (A_{340}) between strain cultures and a control of noninoculated buttermilk. Results are the mean of three independent assays.

Preparation of fermented buttermilk and its cell-free fraction

Buttermilk was fermented by *Lact. helveticus* 209 with and without pH control. Reconstituted buttermilk (8%) was inoculated (2%, v/v) with an overnight culture of *Lact. helveticus* 209 and fermented without pH control at 43°C for 24 h. For buttermilk fermented with pH control a 1·5-l stirred tank bioreactor (LH Fermentation, Hayward, CA) was used. Reconstituted (8% or 16%) buttermilk was inoculated as previously described, the tank being maintained at 43°C with an agitation rate of 250 rpm and sparging with CO_2 at 0·2 l min^{-1}. pH was maintained at 6 throughout the fermentation period (24 or 48 h) by automatic addition of 8 mol l^{-1} NaOH or 4 mol l^{-1} $Ca(OH)_2$ with a peristaltic pump. Internal pH control was also assessed by adding 3·4% (w/v) solid $CaCO_3$ (Cicarelli, Santa Fe, Argentina) to the culture medium before autoclaving.

The fermented buttermilk obtained without pH control was centrifuged at 4000 ***g*** and 4°C for 15 min using an IEC Multi RF centrifuge (Thermo Electron Corporation, Waltham, MA, USA). The supernatant (cell-free fraction) was recovered, filtered through a 0·22-μm filter (Millipore, Etobicoke, ON, Canada) and stored at −80°C. Buttermilk fermented with pH control was acidified (or not) to pH 3·60 with 85% DL-lactic acid syrup (Sigma-Aldrich, St. Louis, MO, USA). The acidified (or not) buttermilk was centrifuged, and the supernatant being recovered and stored as described earlier. Total solids and residual lactose were determined for the cell-free supernatants (acidified or not) by the gravimetric and Fehling-Causse-Bonnans (AOAC, 1990) methods, respectively. Buttermilk fermentation, for each different condition, was replicated at least twice.

Spray-drying of cell-free supernatants

Before spray-drying and to increase total solids, cell-free supernatants were either supplemented with food-grade

maltodextrin (Ledesma S.A.A.I., Buenos Aires, Argentina) or, alternatively, water was partially evaporated by using a laboratory-scale rotary evaporator Büchi R-210 (Flawil, Switzerland) (see Table 3 for conditions used). The amount of maltodextrin added (20–40% w/v) was within the values suggested by the manufacturer of the spray-drier for an adequate performance of the drying process. Cell-free supernatants were warmed to 37°C and spray-dried with a laboratory-scale spray-drier (Büchi mini spray dryer model B-290) under the following conditions: inlet air temperature, 119°C, feeding by a two-fluid nozzle, outlet air temperature 64°C and flux of feeding, 6 ml min^{-1}. Powders were then stored in sealed polyethylene bags at 4°C for further HPLC analysis, moisture and sodium content determinations. Spray-drying, for each different condition, was replicated at least twice.

Determination of moisture content

The moisture content of spray-dried powders was determined gravimetrically in duplicate by oven drying 1–3 g of sample at 102·0 ± 2·0°C (IDF Standard 26A. 1993. International Dairy Federation. Brussels).

Determination of sodium and calcium contents

The sodium content in cell-free supernatants was determined in duplicate by flame photometry (*Flame Emission Photometric Method – Standard Methods for Examination of Water and Wastewater*, 21st edn, 2005). Calcium content was determined volumetrically by EDTA titration.

Proteolytic analysis of fermented buttermilk by reverse-phase high performance liquid chromatography (RP-HPLC)

Filter sterilized samples of unfermented buttermilk supernatant and fermented buttermilk supernatant with, and without, pH control (NaOH or $CaCO_3$) for 24 and 48 h were analysed by reverse-phase HPLC. Unfermented buttermilk supernatant was prepared as follows. Reconstituted buttermilk was acidified to pH 3·60 with 85% DL-lactic acid syrup and centrifuged (4000 ***g***, 4°C, 15 min). The supernatant was recovered, filtered and stored as previously described. Spray-dried powders were reconstituted (to the corresponding concentration of total solids determined for each supernatant) in distilled water, filtered and analysed by RP-HPLC as well. A 50-μl portion of each sample (diluted 1 : 10 in distilled water) was loaded on a Chrompack HPLC (250 × 4·6 mm and 300 Å diameter) column (Varian Inc., Palo Alto, CA, USA) in an RP-HPLC system (Isco model 2360; Isco Inc., Lincoln, NE, USA). Two solvents were used for the separation: solvent A, 0·1% (v/v) trifluoracetic acid (TFA) in water, and solvent B, 0·1% (v/v) TFA in acetonitrile, under the following conditions: 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 B to obtain 100% solvent B after 3 min. Proteins and peptides were eluted with a flow rate of 0·9 ml min^{-1}, and their concentrations (in arbitrary units of absorbance) in the eluate were monitored at 214 nm using a V4® absorbance detector (Isco Inc.).

In vivo trial

Animals

Sixty 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 (National Academy Press, 1996).

Feeding procedures

The following groups were set: (i) a control group that received by gavege 200 μl day^{-1} per mouse of tap water for 7 consecutive days; (ii) a group that received (for 2, 5 or 7 consecutive days) 200 μl day^{-1} per mouse of nonfermented buttermilk supernatant obtained by acidification, with 85% (w/v) commercial lactic acid, of an 8% (w/v) buttermilk solution (group named 'NFB'); (iii) a group that received (by gavage for 2, 5 or 7 consecutive days) 200 μl day^{-1} per mouse of supernatant of buttermilk fermented without pH control, obtained by centrifugation (group named 'pH free'); (iv) two groups that received (by gavage for 2, 5 or 7 consecutive days) 200 μl day^{-1} per mouse of pH-controlled supernatant [with $Ca(OH)_2$], either in the liquid form (group named 'pH controlled') or the spray-dried powder reconstituted to its original volume with distilled water (group named 'pH controlled-SD'). All animals received, simultaneously and *ad libitum*, tap water and a sterile conventional balanced diet containing proteins, 230 g kg^{-1}; raw fibre, 60 g kg^{-1}; total minerals,

100 g kg^{-1}; Ca, 13 g kg^{-1}; P, 8 g kg^{-1}; water, 120 g kg^{-1}; and vitamins.

Immunofluorescence test for IgA-producing cells enumeration

After each feeding period, animals were anesthetized and killed by cervical dislocation. The small intestine was removed for histological preparation by paraffin inclusion, according to previous reports (Vinderola *et al.* 2005). The number of IgA-producing (IgA+) cells was determined on histological slices of samples from the ileum near Peyer's patches according to Vinderola *et al.* (2007a). Data were presented as the mean value of three tissue slices for each animal and each feeding period.

Statistical analysis

One way ANOVA of the data was performed with the SPSS software (SPSS Inc., Chicago, IL, USA). The differences between means were detected by the Duncan's Multiple Range Test (SPSS, 1996). Data were considered significantly different when $P < 0{\cdot}05$.

Results

The growth performance of *Lact. helveticus* strains in buttermilk was assessed by measuring pH and proteolytic activity after 24 h of incubation at 43°C in 8% buttermilk without pH control (Table 1). Two strains showed a negligible growth in this substrate, whereas *Lact. helveticus* 209 and CNRZ 303 showed the highest proteolytic activity. As *Lact. helveticus* 209 was a local isolate, it was chosen for further studies. To determine whether a buttermilk concentration increase would enhance the growth of *Lact. helveticus* 209, this strain was cultured for 24 h in buttermilk reconstituted to 4, 8, 12, 16, 20 and 25% (w/v), without pH control, and pH and proteolytic activity were measured (Table 2). According to the parameters measured, growth was not satisfactory in buttermilk at the lowest concentration assessed (4%). No significant differences were observed in cell counts for *Lact. helveticus* 209 when grown in buttermilk at concentrations ranging from 8 to 25%. Significantly, lower pH values were observed when buttermilk was reconstituted to 4 or 8% compared to 25% (w/v), possibly because of the buffering capacity of buttermilk solids. However, proteolytic activity increased with the increase in substrate concentration.

Table 1 Final pH and proteolytic activity of *Lactobacillus helveticus* strains grown in 8% reconstituted buttermilk after 24 h of incubation at 43°C

Lact. helveticus strain	pH*	Proteolytic activity (A_{340})*
ATCC 15807	3·29 ± 0·40	$0{\cdot}18 \pm 0{\cdot}06^{b}$
CNRZ 892	4·81 ± 0·64	$0{\cdot}02 \pm 0{\cdot}01^{a}$
CNRZ 303	3·38 ± 0·02	$0{\cdot}39 \pm 0{\cdot}02^{c}$
CNRZ 241	3·37 ± 0·06	$0{\cdot}19 \pm 0{\cdot}02^{b}$
HLM1	4·99 ± 0·06	0
133	3·86 ± 0·06	$0{\cdot}24 \pm 0{\cdot}09^{b}$
138	3·23 ± 0·21	$0{\cdot}26 \pm 0{\cdot}08^{b}$
200	3·47 ± 0·05	$0{\cdot}19 \pm 0{\cdot}09^{b}$
209	3·60 ± 0·10	$0{\cdot}47 \pm 0{\cdot}04^{c}$

Values with different superscript letters are significantly different ($P < 0{\cdot}05$).

*Values are the mean of three determinations ± standard deviation.

Table 2 Colony counts, pH and proteolytic activity of *Lactobacillus helveticus* 209 grown in reconstituted buttermilk, at different concentrations, after 24 h of incubation at 43°C

Buttermilk concentration (% w/v)	Colony counts (Log cfu mL^{-1})*	pH*	Proteolytic activity (A_{340})*
4	$7{\cdot}0 \pm 0{\cdot}3^{a}$	$3{\cdot}96 \pm 0{\cdot}11^{a}$	$0{\cdot}24 \pm 0{\cdot}05^{a}$
8	$8{\cdot}3 \pm 0{\cdot}2^{b}$	$3{\cdot}63 \pm 0{\cdot}09^{b}$	$0{\cdot}49 \pm 0{\cdot}09^{b}$
12	$8{\cdot}3 \pm 0{\cdot}1^{b}$	$3{\cdot}64 \pm 0{\cdot}06^{b}$	$0{\cdot}80 \pm 0{\cdot}13^{c}$
16	$8{\cdot}2 \pm 0{\cdot}4^{b}$	$3{\cdot}79 \pm 0{\cdot}08^{b,c}$	$1{\cdot}20 \pm 0{\cdot}15^{d}$
20	$8{\cdot}3 \pm 0{\cdot}4^{b}$	$3{\cdot}78 \pm 0{\cdot}10^{b,c}$	$1{\cdot}25 \pm 0{\cdot}11^{d}$
25	$8{\cdot}7 \pm 0{\cdot}3^{b}$	$3{\cdot}86 \pm 0{\cdot}11^{c}$	$1{\cdot}52 \pm 0{\cdot}07^{e}$

Values in columns with different superscript are significantly different ($P < 0{\cdot}05$).

*Values are the mean of three determinations ± standard deviation.

Considering the previous results, different pH-controlled fermentations of buttermilk were performed under the conditions described in Table 3. External pH control was assessed by using NaOH or $Ca(OH)_2$, internal pH control with $CaCO_3$ and mixed pH control with NaOH and $CaCO_3$, where granulated $CaCO_3$ was incorporated to the fermentation medium, and pH was set at 5·2 to reduce the incidence of NaOH in the neutralization process. Maintaining pH at 6, as opposed to a fermentation of 24 h without pH control (Table 2), resulted in an almost fivefold increase of the proteolytic activity (fermentation 1, Table 3). Yet, doubling the fermentation time (48 h instead of 24 h, in fermentation 2) or doubling the buttermilk concentration (16% instead of 8%, in fermentation 3) also resulted in an approximately 10-fold increase in the proteolytic activity, when compared to a culture without pH control. Internal pH control (fermentation 4), achieved by the addition of insoluble $CaCO_3$ to the culture medium, resulted in the same proteolytic activity as that achieved by controlling pH with NaOH exclusively (fermentation 1). However, even when the substrate concentration was doubled (fermentations 5 and 6, in which $CaCO_3$ or $Ca(OH)_2$ were used as neutralizing agents, respectively), the proteolytic

Table 3 Microbiological and physicochemical characteristics of buttermilk fermented with *Lactobacillus helveticus* 209 with pH control

Fermentation number	Buttermilk concentration (% w/v)	Fermentation time (h)	pH control agent	External pH control set at pH	Log cfu ml^{-1}	Proteolytic activity*	Lactose in supernatant (% w/v)	Total solids in supernatant (% w/v)	Sodium (g l^{-1})	Calcium (g l^{-1})	Final total solids†	Powder moisture (% w/v)
1	8	24	8 mol l^{-1} NaOH	6	8·1 ± 0·2^{a}	2·3 ± 0·1^{a}	<0·20	9·7 ± 0·3^{a}	7·8 ± 0·2^{a}	0·6 ± 0·1^{a}	19·8% ± 1·0^{a}	6·1% ± 0·2^{a}
2	8	48	8 mol l^{-1} NaOH	6	8·6 ± 0·1^{b}	5·2 ± 0·3^{b}	<0·20	9·1 ± 0·4^{a}	7·9 ± 0·3^{a}	0·7 ± 0·2^{a}	20·1% ± 1·2^{a}	6·0% ± 0·1^{a}
3	16	24	8 mol l^{-1} NaOH	6	8·2 ± 0·1^{a}	4·9 ± 0·4^{b}	0·41 ± 0·15	14·7 ± 0·5^{b}	12·3 ± 0·1^{b}	0·9 ± 0·1^{a}	41·7% ± 2·1^{b}	6·2% ± 0·2^{a}
4	8	24	4·2% $CaCO_3$ + 8 mol l^{-1} NaOH	5·2	8·3 ± 0·1^{a}	2·3 ± 0·2^{a}	0·32 ± 0·09	12·5 ± 0·3^{c}	1·7 ± 0·1^{c}	11·8 ± 0·4^{b}	42·0% ± 1·8^{b}	5·2% ± 0·1^{b}
5	16	24	4·2% $CaCO_3$	No pH control	7·9 ± 0·2^{a}	2·4 ± 0·3^{a}	0·37 ± 0·18	13·2 ± 0·4^{c}	0·6 ± 0·2^{d}	11·3 ± 0·5^{b}	22·5% ± 0·9^{c}	5·1% ± 0·2^{b}
6	16	24	$Ca(OH)_2$	6	8·1 ± 0·2^{a}	1·8 ± 0·4^{a}	0·36 ± 0·11	12·7 ± 0·2^{c}	0·5 ± 0·1^{d}	10·2 ± 0·3^{b}	22·7% ± 0·8^{c}	4·9% ± 0·1^{b}

Values in columns with different superscript are significantly different ($P < 0·05$).
*Proteolytic activities are expressed as the difference in absorbance at 340 nm between the sample of fermented buttermilk and a control of noninoculated buttermilk.
†Obtained by the addition of food-grade maltodextrin (fermentations 1 to 4) or by evaporation (fermentations 5 and 6).

activity achieved was lower than that achieved using NaOH for the same substrate concentration (fermentation 3). In spite of the high protein content of the media (2·3–4·6 g l^{-1}), no foam control was necessary. The mild conditions in the bioreactor operation (gas flow 0·2 vvm and agitation rate of 250 rev min^{-1}) avoided the problems created by excessive foam. As expected, final sodium and calcium contents in the cell-free supernatants were in accordance with the use of NaOH, $CaCO_3$ or $Ca(OH)_2$ for pH control.

As already mentioned, total solids were increased before spray-drying either by addition of maltodextrin or by partial water removal by evaporation. Besides, it was not possible to spray-dry cell-free supernatants where lactic acid had been added because the product gradually attached to the drying chamber during the process. Then, only neutral centrifuged (not acidified with commercial lactic acid) fermented buttermilk was successfully spray-dried. The addition of maltodextrin from 20% to approximately 42% resulted in a better performance of the drying process, because in fermentations 3 and 4 the dried powder obtained did not partially attached to the glass walls of the drying chamber, as occurred in fermentations 1 and 2. As regards the partial removal of water by evaporation intended to avoid the addition of an extra ingredient (maltodextrin), the white dried powder obtained not only was as easily removed from the spray-drier as the powders obtained from fermentations 3 and 4 but also was easily and rapidly soluble in water (at room temperature) until, at least, 40–45% (w/v).

Cell-free supernatants derived from buttermilk fermentation with *Lact. helveticus* 209 – with and without pH control – and the reconstituted dried-powder were analysed by RP-HPLC to study the degree of proteolysis of buttermilk proteins and the peptide fractions released to the culture medium (Fig. 1). Fermentation of buttermilk carried out without pH control (Fig. 1b) scarcely modified the RP-HPLC profile of buttermilk (Fig. 1a), increasing slightly the peptide fractions that appeared from minute 20 onwards. When fermentation of 8% (w/v) buttermilk was carried out at pH 6 for 24 h (Fig. 1c), there was an evident increase in the peptide fractions, which appeared as four main peaks detected at 11, 18, 22 and 25 min. The height of those peaks enhanced when fermentation was carried out at pH 6 for 48 h (Fig. 1e) or at pH 6 for 24 h with 16% (w/v) buttermilk (data not shown). There was a decrease in the height and a slight increase in the retention time (shift of peaks to the right) of those four peaks after spray-drying the cell-free supernatants (Fig. 1d,f). Finally, Fig. 2 shows the results of the animal trial. When compared to control values, the number of IgA+ cells significantly increased for the feeding periods of 5 and 7 consecutive days, but to different

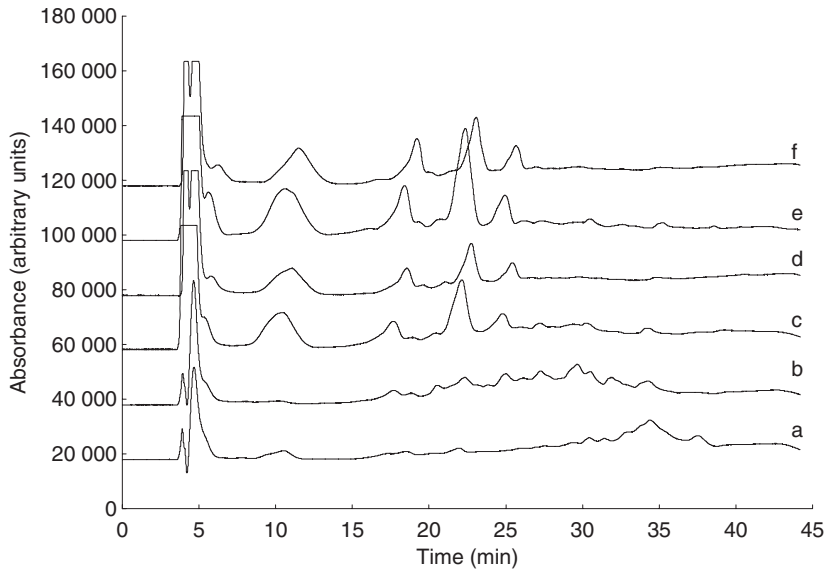

Figure 1 RP-HPLC profile of unfermented buttermilk supernatant (a), cell-free fractions of buttermilk (8% w/v) fermented without pH control (b), buttermilk (8% w/v) fermented at constant pH 6 for 24 h (c) or 48 h (e), or reconstituted spray-dried supernatant of buttermilk (8% w/v) fermented at pH 6 for 24 h (d) or 48 h (f).

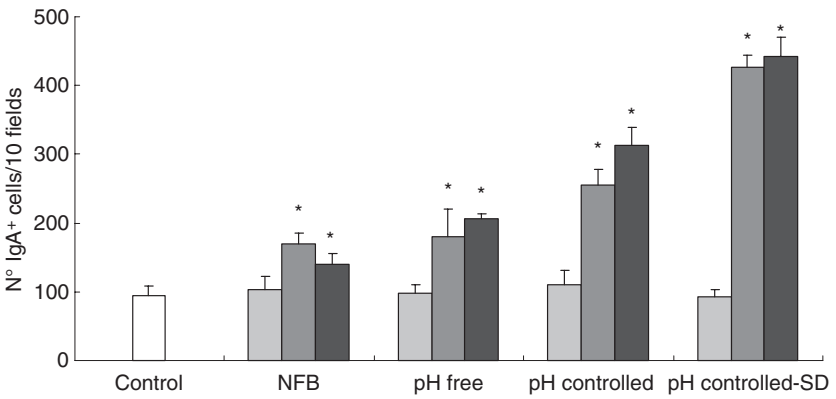

Figure 2 Number of IgA-producing cells in the small intestine lamina propria of control mice (□) and in animals that received nonfermented buttermilk supernatant (NFB), the supernatant of pH-free fermented buttermilk (pH free) or the supernatant of pH-controlled [$Ca(OH)_2$] fermented buttermilk as liquid (pH controlled) or as spray-dried powder (pH controlled – SD), for 2 (▨), 5 (▩) or 7 (■) consecutive days. * Significantly different from the control value ($P < 0{\cdot}05$).

extent. The highest immunostimulation was achieved with the supernatant derived from buttermilk fermentation where pH was controlled at a constant value of 6 with $Ca(OH)_2$ and administered to animals as a reconstituted spray-dried powder for 5 or 7 consecutive days.

Discussion

The industry of functional foods is experiencing a constant expansion because there is plenty of demand for new food and supplements with proven scientific basis of efficacy (Katan and De Roos 2004). In this context, novel additives might be welcome for the expansion of the functional food market beyond existing probiotics, prebiotics and other functional ingredients. Fermented dairy products containing probiotic bacteria lead the market of functional foods. However, certain characteristics of many probiotic bacteria (high sensitivity to acids or osmotic pressure, need of low storage temperature) have conditioned their incorporation almost exclusively to dairy products (yoghurt, cheese, frozen desserts) or certain cereal- or vegetable-based drinks, limiting the development of other kind of food products.

The health benefits of fermented dairy products containing probiotic bacteria are exerted either directly by live micro-organisms (probiotic effect) or indirectly by microbial metabolites, such as bioactive peptides, exopolysaccharides, bacteriocins and organic acids (Clare and Swaigood 2000; Cobb and Kasper 2005), produced during the fermentation process (Stanton *et al.* (2005).

Buttermilk, a by-product of butter manufacture, is a highly nutritious drink (Spreer 1998). Nutritional value and reasonable cost are key motivators for the food industry to use buttermilk-derived products. Buttermilk is used, in some north European countries, to produce a fermented milk drink (Saxelin *et al.* 2003). However, there is yet an interest in new economic utilizations of the large quantities of this by-product produced by the dairy

industry, because of the environmental problem caused by its high organic matter content (Amrane 2001). Some uses of buttermilk for production of functional ingredients were reported. Corredig *et al.* (2004) studied the potential of microfiltration for isolating milk fat globule membrane fractions from buttermilk as food ingredients, whereas Curda *et al.* (2006) used buttermilk to convert its lactose content into prebiotic galacto-oligosaccharides, producing buttermilk with 70 g kg^{-1} of galacto-oligosaccharides, suggested to be used as component of fermented products with probiotic microflora. In a previous work (Burns *et al.* 2008), it was demonstrated that reconstituted buttermilk is an adequate substrate for the production of biomass of probiotic lactobacilli. Given that the chemical composition of buttermilk powder resembles that of skim milk powder, and that it costs three to four times less (Ambrosini S., Milkaut S.A., Franck, Santa Fe, Argentina, personal communication), we determined its suitability for the growth of strains of *Lactobacillus*. The research focused mainly on increasing the content of potentially functional peptides, to further develop a low-cost functional powder carrying some of the beneficial metabolites contained in probiotic fermented milks, but able to be added to those foods were probiotic bacteria cannot be added presently for reasons of poor viability.

Lactobacillus helveticus is considered one of the lactic acid bacteria species with the highest proteolytic activity (Luoma *et al.* 2001). After screening the proteolytic activity of nine strains of this species, we chose that with the highest proteolytic activity in buttermilk, which would be the most adequate for releasing bioactive sequences from buttermilk proteins. *Lactobacillus helveticus* 209 was found to grow in buttermilk up to a concentration of 25% (w/v), which would allow the use of smaller devices when scaling-up the process in the future. This strain was also found to reach a maximum in cell growth but the proteolytic activity was favoured as substrate concentration or fermentation time increased (Table 2), certainly because of the exposure – of a higher amount of proteins or for a longer period of time – of buttermilk proteins to the proteolytic activity of cell-wall anchored proteases in *Lactobacillus* (Savijoki *et al.* 2006). The balance of costs will determine, in the future, whether an increase in substrate concentration and/or fermentation time would be advisable to increase the content of peptidic sequences in the fermentation medium.

In accordance with a previous work (Vinderola *et al.* 2007a), the amount of peptidic fractions released increased when pH was kept at 6 during fermentation, this being the optimum pH for the activity of lactic acid bacteria proteases (Frey *et al.* 1986). Other strategies successfully explored in this study to enhance the content of peptide fractions were the extension of the fermentation for 48 h and the increase in the concentration of buttermilk from 8% to 16% (w/v).

Taking into account the health problems caused by hypertension related to a high sodium chloride intake (Mark and Mitsnefes 2006), we assessed the use of $CaCO_3$ or $Ca(OH)_2$ for internal and external, respectively, pH control to decrease the quantity of sodium salts in the final dried powder. A very significant diminution of the sodium content and an increase in the calcium content in the cell-free supernatant was achieved. However, the presence of Ca was linked to a reduced proteolytic activity. The delayed action of proteolytic activity in the presence of calcium has been reported (Dey *et al.* 2006). In our case, the mechanism of the partial inhibition of proteolytic activity by calcium remains unknown. Additionaly, technical problems might arise at larger scales at the spray-drying step because of the formation of calcium lactate in the fermentation medium if calcium salts are used (Mimouni *et al.* 2007).

Maltodextrin was incorporated to buttermilk cell-free fraction to increase the total solids for an adequate performance of the spray-drying process, according to the instructions of the equipment's manufacturer. The powder obtained presented desired characteristics such as high solubilizing capacity in water and a negligible hygroscopicity, probably because of the low content of remaining lactose after fermentation. However, the moisture content of powders was still above 4%, the level suggested for long-term powder storage life and stability (Gardiner *et al.* 2000). As an alternative to the use of an extra ingredient (maltodextrin) to increase the total solids before spray-drying, we assessed the suitability of water removal by evaporation. The obtained concentration of the cell-free supernatant was also effective for the increase of total solids. The impossibility of drying the acidified cell-free fraction of fermented buttermilk might be because of the presence of added commercial lactic acid because this is a very hygroscopic and thermoplastic acid that makes the drying process very difficult (GEA Niro, GEA Process Engineering A/S personal communication). Then, the spray-drying of the cell-free fraction of fermented buttermilk was performed after the centrifugation of the fermented substrate without acidification to pH 3·6.

The oral administration of the nonbacterial fraction of nonfermented buttermilk or buttermilk fermented with or without pH control modulated the gut mucosa immune response. There was an increase in the number of IgA+ cells in the lamina propria of the small intestine. The effect induced by nonfermented buttermilk supernatant might be attributed to the content of peptides naturally present in buttermilk. The main function of IgA in the gut is to exert the immune exclusion of pathogenic bacteria or viruses by intimate cooperation with the innate

nonspecific defence mechanisms (Brandtzaeg *et al.* 1987). The induction of the proliferation of IgA+ cells at the gut epithelium is a desired attribute for functional foods, like those containing probiotic bacteria (Galdeano *et al.* 2009) or bioactive peptides (Vinderola *et al.* 2008). The highest increase in the number of IgA+ cells was observed in those animals that received the cell-free supernatant of buttermilk fermented with *Lact. helveticus* 209 under pH 6 – controlled, spray-dried and reconstituted. The enhancement of IgA production was even higher than in animals that received the same product before spray-drying. The interaction of peptides with carbohydrates and lipids as well as the influence of the processing conditions (especially heating) on peptide activity and bioavailability should also be investigated to evaluate the chemical safety of new additives (Korhonen and Pihlanto 2003). The RP-HPLC analysis of spray-dried, and reconstituted, powders revealed a shift and a decrease in the height of the main peaks observed, maybe because of a possible reactivity of peptides during the spray-drying process. Additionally, the *in vivo* studies showed a greater capacity of the spray-dried powder to induce the proliferation of IgA+ cells. We hypothesize that the heating process during spray-drying might somewhat, at least partially; induce the formation of peptide aggregates that could have presented thereafter a higher retention time in the HPLC column. The peptide aggregates could have also behaved, *in vivo*, more as particulate rather than as soluble antigens, enhancing their interaction capacity with the immune cells associated with the gut (Perdigón *et al.* 2002). This hypothesis must still be confirmed in future. The use of a dried cell-free fraction of fermented milks opens the possibility to extend some of the functional effects observed for fermented milks to other dairy or nondairy foods, broadening the market of functional foods (Vinderola 2008).

Conclusions

We concluded that buttermilk was a suitable low-cost substrate for the growth of *Lact. helveticus* 209 and the release, by pH-controlled fermentation, of peptide fractions with immunomodulating capacity. The cell-free fraction was easily recovered by centrifugation and handled for spray-drying, added of maltodextrin or concentrated by evaporation, for the production of a peptide-enriched powder with potential for the development of a functional powder for foods. A novel application of buttermilk is reported.

Acknowledgements

This work was partially supported by the following funds: Programación CAI+D 2006 37-203 (Universidad Nacional del Litoral, Santa Fe, Argentina), Proyecto PICT 2004 no. 09 20358 and PICT Jóvenes 2005 32118 from the Agencia Nacional de Promoción Científica y Tecnológica, Proyecto PIP 5321 from CONICET and by the programme Cambio de Escala 'Curso de Acción para la Transferencia Tecnológica – CATT, Programa Cambio de Escala'. Resolución C.S. 299/08.

References

Amrane, A. (2001) Batch cultures of supplemented whey permeate using *Lactobacillus helveticus*: unstructured model for biomass formation, substrate consumption and lactic acid production. *Enzyme Microb Technol* **28**, 827–834.

AOAC (1990) *Official Methods of Analysis*, 15th edn. Arlington, VA: Association of Official Analytical Chemists.

Brandtzaeg, P., Bjerke, K., Kett, K., Kvale, D., Rognum, T., Scott, H., Sollid, L. and Valnes, K. (1987) Production and secretion of immunoglobulins in the gastrointestinal tract. *Ann Allergy* **59**, 21–39.

Burns, P., Molinari, F., Vinderola, G. and Reinheimer, J. (2008) Suitability of whey and buttermilk for the production and frozen storage of cultures of probiotic lactobacilli. *Int J Dairy Technol* **61**, 156–164.

Church, F.C., Swaisgood, H.E., Porter, D.H. and Catignain, G.L. (1983) Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *J Dairy Sci* **66**, 1219–1227.

Clare, D.A. and Swaigood, H.E. (2000) Bioactive milk peptides: a prospectus. *J Dairy Sci* **83**, 1187–1195.

Cobb, B.A. and Kasper, D.L. (2005) Coming age: carbohydrates and immunity. *Eur J Immunol* **35**, 352–356.

Corredig, M., Roesch, R.R. and Dalgleish, D.G. (2004) Production of a novel ingredient from buttermilk. *J Dairy Sci* **86**, 2744–2750.

Curda, L., Rudolfova, J., Stetina, J. and Dryak, B. (2006) Dried buttermilk containing galactooligosaccharides – process layout and its verification. *J Food Eng* **77**, 468–471.

Dey, R., Bhattacharya, J. and Datta, S.C. (2006) Calcium-dependent proteolytic activity of a cysteine protease caldonopain is detected during *Leishmania* infection. *Mol Cell Biochem* **281**, 27–33.

FAO/WHO (2002) Guidelines for the evaluation of probiotics in food. Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report. Available from: ftp://ftp.fao.org/es/esn/food/wgreport2.pdf (accessed 8 May 2010).

Fellows, P.J. (2000) *Dehydration, Food Processing Technology*. Cambridge: Woodhead Publishing Limited.

Frey, J.P., Marth, E.H., Johnson, M.E. and Olson, N.F. (1986) Peptidases and proteases of lactobacilli associated with cheese. *Milchwissenschaft* **41**, 622–627.

Galdeano, C.M. and Perdigón, G. (2004) Role of viability of probiotic strains in their persistence in the gut and in

mucosal immune stimulation. *J Appl Microbiol* **97**, 673–681.

Galdeano, C.M., de Leblanc Ade, M., Carmuega, E., Weill, R. and Perdigón, G. (2009) Mechanisms involved in the immunostimulation by probiotic fermented milk. *J Dairy Res* **76**, 446–454.

Gardiner, G.E., O'Sullivan, E., Kelly, J., Auty, M.A.E., Fitzgerald, G.F., Collins, J.K., Ross, R.P. and Stanton, C. (2000) Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. *Appl Environ Microbiol* **66**, 2605–2612.

Katan, M.B. and De Roos, N.M. (2004) Promises and problems of functional foods. *Crit Rev Food Sci Nutr* **44**, 369–377.

Korhonen, H. and Pihlanto, A. (2003) Food-derived bioactive peptides – opportunities for designing future foods. *Curr Pharmacol Des* **9**, 1297–1308.

Luoma, S., Peltoniemi, K. and Joutsjoki, V. (2001) Expression of six peptidases from *Lactobacillus helveticus* in *Lactococcus lactis*. *Appl Environ Microbiol* **67**, 1232–1238.

Mark, M. and Mitsnefes, M.D. (2006) Hypertension in children and adolescents. *Pediatr Clin North Am* **53**, 493–512.

Mimouni, A., Bouhallab, S., Famelart, M.H., Naegele, D. and Schuck, P. (2007) The formation of calcium lactate crystals is responsible for concentrated acid whey thickening. *J Dairy Sci* **90**, 57–65.

Ouwehand, A.C. and Salminen, S.J. (1998) The health effects of cultured milk products with viable and non-viable bacteria. *Int Dairy J* **8**, 749–758.

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

Reinheimer, J.A., Quiberoni, A., Tailliez, P., Binetti, A.G. and Suárez, V.B. (1996) The lactic acid microflora of natural whey starters used in Argentina for hard cheese production. *Int Dairy J* **6**, 869–879.

Savijoki, K., Ingmer, H. and Varmanen, P. (2006) Proteolytic systems of lactic acid bacteria. *Appl Microbiol Biotechnol* **71**, 394–406.

Saxelin, M., Kopela, R. and Mayra-Makinen, A. (2003) Functional dairy products. In *Dairy Processing. Improving Quality* ed. Smit, G. pp. 229–243 Cambridge: Woodhead Publishing Limited.

Silva, S.V. and Malcata, F.X. (2005) Caseins as source of bioactive peptides. *Int Dairy J* **15**, 1–15.

Spreer, E. (1998) *Milk and Dairy Product Technology*. New York: Marcel Dekker.

SPSS. (1996) *Version 7.5 for Windows, student version*. New York. Release 6 Dec 1996. Chicago, IL: SPSS Inc.

Stanton, C., Ross, R.P., Fitzgerald, G.F. and Van Sinderen, D. (2005) Fermented functional foods based on probiotics and their biogenic metabolites. *Curr Opin Biotechnol* **16**, 198–203.

Vinderola, G. (2008) Dried cell-free fraction of fermented milks: new functional additives for the food industry. *Trends Food Sci Technol* **19**, 40–46.

Vinderola, C.G., Duarte, J., Thangavel, D., Perdigón, G., Farnworth, E. and Matar, C. (2005) Immunomodulating capacity of kefir. *J Dairy Res* **72**, 195–202.

Vinderola, C.G., Perdigón, G., Duarte, J., Farnworth, E. and Matar, C. (2006a) Effects of the oral administration of the products derived from milk fermentation by kefir microflora on the immune stimulation. *J Dairy Res* **73**, 472–479.

Vinderola, C.G., Perdigón, G., Duarte, J., Farnworth, E. and Matar, C. (2006b) Effects of kefir fractions on innate immunity. *Immunobiology* **211**, 149–156.

Vinderola, G., Matar, C., Palacios, J. and Perdigón, G. (2007a) Mucosal immunomodulation by the non-bacterial fraction of milk fermented by *Lactobacillus helveticus* R389. *Int J Food Microbiol* **115**, 180–186.

Vinderola, G., Matar, C. and Perdigón, G. (2007b) Milk fermented by *Lactobacillus helveticus* R389 and its non-bacterial fraction confer enhanced protection against *Salmonella enteritidis* serovar. Typhimurium infection in mice. *Immunobiology* **212**, 107–118.

Vinderola, G., de Moreno de LeBlanc, A., Perdigón, G. and Matar, C. (2008) Biologically active peptides released in fermented milk: role and functions. In *Handbook of Fermented Functional Foods*, 2nd edn, ed. Farnworth, E.R. pp. 209–242 Boca Raton: CRC Press, Taylor and Francis Group.