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Postbiotics produced at laboratory and industrial level as potential functional food ingredients with the capacity to protect mice against *Salmonella* infection

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Running title: Protection of postbiotics against *Salmonella*.

ABSTRACT

Aim: To determine the protective capacity against *Salmonella* infection in mice of the cell-free fraction (postbiotic) of fermented milk, produced at laboratory and industrial level.

Methods and Results: The proteolytic activity (PA) of five commercial cultures and eleven autochthonous *Lactobacillus* strains was evaluated. The DSM-100H culture displayed the highest PA and it was selected for further studies. The capacity of the postbiotics produced by pH-controlled fermentation to stimulate the production of secretory-IgA in faeces and to protect mice against *Salmonella* infection was evaluated. A significant increase of S-IgA in faeces of mice fed 14 days the postbiotic obtained at the laboratory (F36) was detected compared to control animals. A significantly higher survival was observed in mice fed the F36 and the FiSD (industrial product) compared to controls.

Conclusion: The postbiotics obtained showed immunomodulatory and protective capacity against *Salmonella* infection in mice.

Significance and Impact of the Study: The pH-controlled milk fermentation by the proteolytic DSM-100H culture could be a suitable strategy to obtain a food ingredient to be added to a given food matrix, not adequate to host viable cells of probiotics, to confer it enhanced functionality and thus expand the functional food market.

Keywords: functional food ingredient**,** fermented milk**,** postbiotics**,** proteolytic activity, *Salmonella*.

Introduction

The increasing perception about the relationship between diet and health led consumers to be more interested in preventing diseases and improving wellness through the intake of functional foods (Hafeez *et al*. 2014, Lorenzo *et al*. 2018). Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill *et al*. 2014) Fermented milks containing probiotic bacteria lead the market of functional foods. The beneficial effects on health of these products containing live microorganisms are supported by scientific data (Lollo *et al*. 2013; 2015; Baray and Ozcan 2017; Shafi *et al*. 2018). In the last years, many studies have investigated the role of food ingredients such as food-derived peptides on health (Hannu 2006; Hayes *et al*. 2007). Dietary proteins could be a source of physiologically active sequences (Haque *et al*. 2009). Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately positively influence health (Kitts and Weiler 2003). Nowadays, milk proteins are considered one of the most important sources of bioactive peptides (Korhonen 2009; Mohanty *et al*. 2016a, Zanutto-Elgui *et al*. 2019). Beyond peptides, other components produced during milk fermentation (exopolysaccharides, bacteriocins, organic acids), present in the cell-free supernatant or cell-free fraction, can also have healthy effects such as positive immune modulation, antimicrobial activity, among others (Aloglu and Öner 2011; Lorenzo *et al*. 2018). New terms, which do not imply bacterial viability, such as paraprobiotic (non-viable or inactivated probiotics) and postbiotic [metabiotics, biogenics, or simply metabolites/CFS (cell-free supernatants); refers to soluble factors (products or metabolic byproducts) secreted by live bacteria or released after bacterial lysis] have currently emerged (Tsilingiri and Rescigno 2013; Almada *et al*. 2016; Aguilar-Toalá *et al*. 2018). Postbiotic administration increased the levels of gut IgA in the small and large intestine lamina propria of mice that lead to the diminution of the severity of enteric

infections caused by pathogens such as *Salmonella enteritidis* serovar Typhimurium or *Escherichia coli* in mice (Maldonado Galdeano *et al*. 2007; Medici *et al*. 2005). The possible use in food of postbiotics instead of viable microorganisms such as probiotics might have some technological advantages as functionality does not rely on cell viability. The use of this kind of functional ingredients would allow achieving a longer shelf-life, easier storage, handling and transportation and reduced requirements for refrigerated storage (Vinderola 2008). One way to produce postbiotics from milk is through the fermentation with proteolytic starter cultures, where maintenance of pH at values close to neutrality enhances peptide release from milk proteins (Vinderola *et al*. 2007b). This work aimed at comparing the capacity of postbiotics produced by pH-controlled fermentation of milk, manufactured at laboratory and industrial levels, to stimulate the production of secretory-IgA in the gut and to protect mice against *Salmonella* Typhimurium infection.

Materials and Methods

Strains, commercial cultures, culture media and conditions

Five frozen or freeze-dried commercial cultures of thermophilic lactobacilli and eleven autochthonous *Lactobacillus* strains were used. The commercial cultures used were: Delvo-Add 100-H DSF (DSM, Delft, The Netherlands); LH-B02 (Chr. Hansen, Hoersholm, Denmark), Lb-12 (Chr. Hansen, Denmark), Cheestart H 2085 (Biochemical, Buenos Aires, Argentina), Cheestart H 2087 (Biochemical, Buenos Aires, Argentina). The autochthonous strains were: *Lactobacillus helveticus* (Lh 05, Lh 06, Lh 07, and Lh 08), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lb 03, Lb 09, Lb 10, Lb 11, Lb 12) and *Lactobacillus delbrueckii* subsp. *lactis* (Ll 210, Ll 133). Autochthonous lactobacilli strains, isolated from natural whey starters in previous works (Reinheimer *et al*. 1996), belong to the INLAIN collection. Overnight cultures of the strains were obtained in MRS broth (Biokar, Beauvais,

France) or 10% (w/v) reconstituted skim milk (San Regim, Sunchales, Argentina) (43ºC, aerobiosis). When required, cell counts (surface agar plating) were performed on MRS agar (48 h, 43ºC, aerobiosis).

Screening of the proteolytic activity and growth capacity in milk

The proteolytic activity (PA) of the strains and the commercial cultures was determined by the O-Phthaldialdehyde (P1378, Sigma Aldrich, Buenos Aires, Argentina) spectrophotometric assay (OPA Test) (Church *et al*. 1983). Strains were transferred three times in 10% (w/v) skim milk at 43ºC and were inoculated (2% v/v) in the same substrate (10 mL). Commercial cultures were used according to the manufacturer's instructions. Inoculated milk samples were incubated overnight (43°C, aerobiosis) without pH control. After incubation, pH values were measured (pH meter Orion, model SA 720, Beverly, MA, USA) and PA (mean of three independent assays) was expressed as the difference in absorbance (A_{340nm}) between fermented and unfermented samples.

Production of postbiotics at laboratory scale

The strains/commercial cultures with the highest proteolytic activity were used to ferment milk with and without pH control. For pH-controlled milk fermentation, a 2 L stirred tank bioreactor (Sartorius Biostat A Plus) was used. Reconstituted (10 or 20%, w/v) skim milk was inoculated (2% v/v) with an overnight culture of the strain or the corresponding dilution of the commercial starter (according to the manufacturer's instructions). An inoculated sample was removed from the bioreactor for simultaneous incubation without pH control (43ºC) or stirring. The bioreactor was maintained at 43ºC with an agitation rate of 200 rpm and CO_2 sparring (0.2 L min^{-1}). The pH was maintained at a value of 6.0 throughout the fermentation period (20 h) by automatic addition (peristaltic pump) of 2 mol L^{-1} $Ca(OH)_2$. PA

measurements and cell counts (agar plating on MRS, 43ºC, 48 h, aerobiosis) were performed at time 0, 16, 18 and 20 h of culture. The pH-controlled fermented milk was centrifuged (4000 g, 8°C, 30 min) and the supernatant was recovered and stored at -70°C. Milk fermentation was replicated at least twice. The product selected for further analysis was named F36 (pH-controlled milk fermentation using the commercial culture DSM-100H).

Production of postbiotics at industrial scale and spray-drying

According to the previous results obtained in the laboratory, the DSM-100H (Delvo-Add 100-H DSF, DSM) culture was selected for the scaling up. The industrial production of the cell-free supernatant was carried out at the industrial plant of the company Biochemical S.A. (Buenos Aires, Argentina) whereas spray-drying was conducted in the dairy plant of the company SanCor C.U.L. (Sunchales, Santa Fe, Argentina). A 1000 L stainless steel fermenter (La Metalúrgica Industrial - Lampe, Lutz & Cia., Buenos Aires, Argentina) was used. 950 L of 10% (w/v) skim milk were inoculated with the DSM-100H culture according to the manufacturer instructions. 50% (v/v) NH_4OH was used for pH control (pH = 6). No gas sparring was used. Fermentation was carried out at 43ºC for 20 h. The cell-free supernatant was recovered using a continuous centrifuge (Alfa Laval, MB PX 810) (5000 rpm, 5ºC) and transported under refrigeration to SanCor C.U.L. To allow spray-drying, the total solids of the cell-free supernatant were raised from 5.93% (w/v) to 41.00% (w/v) using whole milk concentrate (produced by SanCor C.U.L.). 145 L of the whole milk enriched-cell free supernatant were spray-dried in a Niro Mobile Minor ^{TM} spray drier under the following conditions: inlet air temperature: 160ºC, outlet air temperature: 90ºC, product feeding temperature: 60ºC and product feeding rate: 14 L h^{-1}. The product obtained under these conditions was named FiSD.

Peptide profiles of the postbiotics

The peptide profiles of the cell-free supernatants (postbiotics) of milks fermented with the culture DSM-100H were analyzed by means of reverse phase-high performance liquid chromatography (RP-HPLC).

In order to evaluate the impact of different conditions and scale of fermentation on the peptide profiles, several samples were analyzed. Postbiotics produced at laboratory level derived from the fermentation of: 1) 10% (w/v) skim milk without pH control; 2) 20% (w/v) skim milk without pH control; 3) 10% (w/v) skim milk pH-controlled with $Ca(OH)_2$; 4) 20% (w/v) skim milk pH-controlled with $Ca(OH)_2$; and 5) 10% (w/v) skim milk pH-controlled with NH_4OH. Postbiotics produced at industrial scale were obtained: 1) before spray-drying and 2) spray-dried and reconstituted (FiSD). An unfermented milk supernatant was used as a control. This sample was obtained from reconstituted milk, acidified to pH 3.60 with 85% (v/v) of L-lactic acid syrup (L1250, Sigma Aldrich, Buenos Aires, Argentina) and centrifuged (4000 g, 4°C, 15 min). All the cell-free supernatants were stored at -70°C for further analysis. Spray-dried powder was reconstituted in milk before the analysis.

For HPLC analysis, samples (diluted 1/10 in distilled water) were filtered through 0.45-μm membranes (Millex, Millipore, Sao Paulo, Brazil), and 60 μL portion of each one was injected into the HPLC chromatograph (Series 200 and Flexar, Perkin Elmer, Norwalk, CT). The HPLC equipment consisted of a quaternary pump, an on-line degasser, a column oven and a UV-visible detector (Series 200 and Flexar). Data were collected and processed on a computer with the software Chromera® (Perkin Elmer). Separation was achieved on an Aquapore OD-300 C18 (220 mm × 4.6 mm) analytical column (Perkin Elmer, Norwalk, USA), at 30ºC, and a flow rate of 0.9 mL min^{-1}. The column was equilibrated initially with

100% of solvent A [(0.1% (v/v) trifluoroacetic acid (TFA) in water)]. After 5 min of injection, a gradient was generated by increasing the concentration of solvent B [(0.1% (v/v) TFA in acetonitrile)] from 0 to 60% during 25 min and next maintaining 5 min in this condition; then, the column was returned to starting conditions (100% of solvent A) over 10 min, and these conditions were maintained for 10 min.

In vivo trials

Animals

Eighty-one six-week old male BALB/c mice weighing 20-22 g were obtained from the random inbred colony of the Instituto de Ciencias Veterinarias del Litoral (ICiVet-Litoral, UNL-CONICET), Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe, Argentina). Animals were kept at the INLAIN animal facility for a week before starting the feeding procedures. Each experimental group (depending on the assay) consisted of 7-10-15 mice housed in groups (3, 4 or 5) in plastic cages and kept in a controlled environment (21 ± 1°C and 55 ± 2 % humidity), with a 12 h light/dark cycle.

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 (Cooperación, Buenos Aires, Argentina).

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. 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 considered when using animals.

Secretory IgA (S-IgA) quantification in faeces

The following groups (7 animals/group) were set: a) control group (C): mice that received the unfermented milk supernatant; b) F36 group (F36): mice that received the cell-free supernatant obtained by DSM-100H fermentation in 10% (w/v) skim milk produced in the laboratory and c) F36D group (F36D): mice that received the product F36 diluted 1/10 in tap water. Animals received, by gavage, 300 µl/day/mouse of each product for 35 consecutive days. Faeces samples (approx. 50 mg/mouse) were collected once a week and diluted (1:10) with 500 µL PBS buffer. The samples were processed (UltraturraxT-50® Homogenizer), centrifuged (8000 g, 4ºC, 15 min) and the supernatant was collected to determine the concentration of S-IgA by ELISA.

***Salmonella* Typhimurium survival assays**

A strain of *Salmonella enterica* serovar. Typhimurium was obtained from the Administración Nacional de Laboratorios e Institutos de Salud Dr. Carlos G. Malbrán (Buenos Aires, Argentina). A stock culture of the *Salmonella* strain was inoculated in 5 mL of TS broth (Tryptone Soy, Britania S.A, Buenos Aires, Argentina) for 16 h at 37ºC in aerobiosis.

The capacity of the supernatants (obtained at laboratory and industrial scale) to prevent enteric infection with *Salmonella enterica* was evaluated. In the first assay (evaluation of the product F36), the following groups (10 animals/group) were set: a) control group (C): mice that received the unfermented milk supernatant; b) F36 group (F36): mice that received the cell-free supernatant obtained by DSM-100H fermentation in 10% (w/v) skim milk produced in the laboratory and c) F36D group (F36D): mice that received the product F36 diluted 1/10 in tap water. Animals received, by gavage, 300 µl/day/mouse of

each product for ten consecutive days. After the feeding period, animals were challenged with a single infective dose of 6.30 $\log_{10}$ CFU of *Salmonella*/mouse. Survival to infection was monitored daily for 20 days after challenge. The cumulative mortality during the post-infection period was plotted against time, and the results were expressed as survival (%) to infection [% = ($N_{\text{not-dead mice}}/N_{\text{total mice}}$)*100].

In a second assay (product FiSD), the following groups (15 animals/group) were set: a) control group (C): mice that received reconstituted milk (30% w/v) and b) FiSD group (FiSD): mice that received the cell-free supernatant obtained by DSM-100H fermentation in 10% (w/v) skim milk at industrial level, spray-dried and reconstituted (30% w/v). Animals received, by gavage, 300 µl/day/mouse of each product for ten consecutive days. After the feeding period, animals were challenged with a single infective dose of 6.48 $\log_{10}$ CFU of *Salmonella*/mouse and survival was monitored for 20 days after challenge. The cumulative mortality during the post-infection period was plotted against time, and the results were expressed as survival (%) to infection [% = ($N_{\text{not-dead mice}}/N_{\text{total mice}}$)*100].

Statistical analysis

For the mortality assays, the survival (%) was evaluated with the Log Rank test. The data of the rest of the trials were analysed using one-way ANOVA (SPSS software, SPSS Inc., Chicago, IL, USA). The differences between means were detected by the Duncan's Multiple Range Test (IBM SPSS Statistics, 2013). Data were considered significantly different when $P < 0.05$.

Results

Screening of the proteolytic activity and potential growth in milk

The fermentative capacity of the strains/commercial cultures under study in milk was assessed by measuring the pH and the proteolytic activity (PA) after 20 h of incubation at 43ºC in 10% (w/v) skim milk without pH control (Table 1). Three *L. helveticus* strains (Lh 05, Lh 07 and Lh 08) and *L. bulgaricus* Lb 09 were not able to grow in milk, whereas the commercial cultures DSM-100H and Lb-12 Chr**.** Hansen and the strains *L. helveticus* Lh 06, *L. bulgaricus* Lb 12 and *L. bulgaricus* Lb 03 presented the highest proteolytic activities (absorbance between 0.7 and 1.08), and then they were chosen for further studies. The final pH ranged from 3.86 and 3.35 in all cases. In previous studies in our laboratory (data not published), the culture DSM-100H presented higher immunostimulatory capacity (increase in intestinal secretory IgA) when fed to mice than Lb-12 Chr. Hansen, that is why the former was selected.

Production of postbiotics at laboratory scale (F36)

The fermentation at laboratory level was performed using the most proteolytic strains/commercial culture in reconstituted skim milk (10 and 20% w/v). Cells counts (log_{10} CFU mL^{-1}) of pH-controlled (time = 0, 16 h, 18 h and 20 h) and uncontrolled fermentations (time = 0 and 20 h) are shown in Table 2. Results of the proteolytic activities after 16 h and 18 h of fermentation at pH = 6 are shown in Table 3, and the final PA (20 h) of free and controlled-pH fermentations is shown in Figure 1.

Regarding cell growth, it was observed that the highest cell counts were found after 16 h of fermentation (Table 2). For *L. bulgaricus* Lb 12, a significant loss of viability was evidenced after 20 h of fermentation using 10% or 20% (w/v) skim milk ($P < 0.05$).

Moreover, when fermentations with and without pH control were compared, the counts after 20 h of incubation for *L. bulgaricus* Lb 12 were significantly higher when pH was not controlled using 10% (P = 0.039) or 20% (w/v) (P = 0.001) skim milk, compared to pH-controlled fermentation.

The proteolytic activity of the commercial culture DSM-100H was significantly higher than that obtained for the three autochthonous *Lactobacillus* strains (Table 3 and Figure 1) so it was selected for its use at industrial level and for the *in vivo* trials. After 16 h and 18 h of pH-controlled fermentations with the culture DSM-100H, no significant differences in PA were observed when using 10 or 20% (w/v) skim milk (Table 3). After 20 h of fermentation, the PA of the DSM-100H culture was significantly higher when the fermentation was carried out at pH = 6, than when it was performed without pH control, using 10% and 20% (w/v) skim milk (Figure 1). However, no differences were found between pH-controlled fermentations when using 10% or 20% (w/v) skim milk. On the contrary, a higher milk concentration led to significantly higher PA values for free-pH fermentations (Figure 1).

Production of postbiotics at industrial scale and spray-drying (FiSD)

According to the results obtained at the laboratory, the scaling-up was performed with the DSM-100H culture in 10% (w/v) skim milk. The cell-free supernatant was spray-dried in order to confer stability and easiness of transportation to the product produced at the industry. The global composition of the powder obtained at industrial level after spray drying was: 2.71% (w/v) moisture, 24% (w/v) fat, 29.79% (w/v) protein, 37.4% (w/v) carbohydrates and 6.1% (w/v) ashes.

Peptide profiles of the cell-free supernatants

Cell-free supernatants derived from milk fermentation with the DSM-100H culture were analyzed in order to study the proteolysis of milk proteins and release of peptide fractions to the medium in different conditions and scale of fermentation (Figures 2 and 3). An unfermented milk supernatant was used as a control.

The profiles of unfermented milk supernatant and the cell-free supernatants of milk (10 or 20% w/v) fermented with and without pH control are shown in Figure 2. An increase in the number and height of peaks, above all for those eluted between 14 to 20 min of retention time, was observed in fermented milks due to the proteolytic activity of the culture. This activity was higher when the fermentation was carried out at controlled pH in comparison with samples without pH control. The use of 10 or 20% (w/v) skim milk did not influence on the peptide profiles of pH-controlled fermentations, but in the case of the fermentation without pH control, there was a higher production of peptides at higher milk concentration. All these results are consistent with those of PA, previously described.

In Figure 3, peptides profiles of five samples are compared: unfermented milk supernatant (a), the cell-free supernatants of milk 10% (w/v) fermented at controlled pH with $Ca(OH)_2$ (b) or NH_4OH (c) at laboratory scale, and the cell-free supernatants produced at industrial scale before spray-drying (d), and spray-dried and reconstituted (e). Peptides profiles of all these fermented samples showed a notable increase of peaks with a retention time between 14 to 20 min. Also, some differences in the height and number of peaks were detected between different samples. In this way, the use of NH_4OH to control the pH during fermentation at laboratory scale produced an increase of peaks in the initial part of the chromatogram, and between 20 to 25 min of retention time, in comparison with the employment of $Ca(OH)_2$.

These results suggest an influence of the pH-controlling agent in the proteolytic activity of the strain. The profiles of the postbiotics produced at laboratory or industrial scale (using the same pH-controlling agent) were very similar reflecting a good reproducibility of the fermentation process in the scaling. Regarding the sample produced at industrial scale and spray-dried, some differences were observed. In this profile, a lower height of the characteristics peaks was detected, and additional peaks were found. This fact is probably related to the preparation of the sample for the spray-drying. In effect, the total solids of the cell-free supernatants were increased from 5.93% (w/v) to 41.00% (w/v) with whole milk concentrate, which probably contributed with some peptides to the profiles. However, these changes did not worsen the protective capacity against *Salmonella* infection of this sample, as described below.

Secretory IgA (S-IgA) quantification in faeces

The capacity of the postbiotics obtained at laboratory scale (F36 and F36D), to stimulate the secretion of S-IgA in the gut was determined monitoring this parameter in faeces in mice fed these products (Figure 4). A significant increase in the secretion of S-IgA was detected after 14 days of feeding in mice that received the F36 supernatant, compared to the control group; however, no differences were observed when the supernatant was administered diluted (1/10) (F36D group). Considering the dose-response concept, one may assume that the higher the proteolytic activity, the higher the immunostimulation achieved.

***Salmonella* Typhimurium survival assays**

The capacity of postbiotics produced to protect mice against *Salmonella* infection was assessed. The survival of mice fed the F36 and F36D supernatants during 20 days after infection is shown in Figure 5. Survival of mice that received the cell-free supernatant

obtained by the fermentation of 10% (w/v) skim milk with the commercial culture DSM-100H (F36) was significantly higher (survival > 60%, P = 0.015), compared to the control group. On the contrary, no differences in survival were observed when the 1/10 dilution of the F36 product was used. After 8 days of the challenge, the survival of the control group was only 25%, and all mice died after 15 days of infection. Mice that received the product F36D survived 25% till the end of the assay. The highest levels of S-IgA observed after 14 days of feeding in mice that received the F36 supernatant (Figure 4) could, at least partially, explain the highest protective capacity of the F36 product against *Salmonella* infection.

A second assay was performed to evaluate the protective capacity of the spray-dried cell-free supernatant obtained at industrial scale (Figure 6). A significant (P= 0.017) protective effect was observed in mice treated with this product, compared to the control group. The FiSD group, showed a survival of 85% from day 9 onwards, while the control group had a survival of 80% after 11 days and less than 50% at the end of the assay.

Discussion

As diet is a key factor for health promotion and disease prevention, in the last decades, the food and pharma industries experienced a growing interest in the development of functional foods and supplements that provide health benefits beyond basic nutrition, such as those based on probiotics, prebiotics and the use of dairy hydrolysates containing bioactive peptides (Hernández-Ledesma *et al*. 2014). Fermented dairy products carrying probiotic bacteria are functional food market leaders (Figueroa-González *et al*. 2011), however certain characteristics of probiotics in general (sensitivity to lactic acidity or osmotic pressure, need of low storage temperature) have confined them mainly to dairy products (yoghurt, cheese), limiting the development of other kinds of food products (Burns *et al*. 2010; Peteán *et al*.

2014). Milk proteins are considered one of the most important sources of bioactive peptides (Hafeez *et al*. 2014; Wada and Lönnerdal 2014; Pihlanto 2016). The use of bioactive peptides as a functional component instead of viable microorganisms presents technological advantages such as a longer shelf-life, easier storage, handling, transportation and reduced requirement for refrigerated storage (Vinderola 2008). As a result, several technologies have been developed to produce these bioactive peptides (Hafeez *et al*. 2014). One of the alternatives is through the fermentation of dairy products with proteolytic acid lactic bacteria (LAB) and posterior recovering of the cell-free supernatant. The proteolytic system of LAB during milk fermentation plays a key role in enabling these bacteria to grow in milk, thereby ensuring successful fermentation (Savijoki *et al*. 2006). LAB proteases have an optimum pH of activity between 5.5 and 7.5 (Sadat-Mekmene *et al*. 2011). This fact could explain the increased PA of pH 6-controlled fermentation compared to uncontrolled ones found for the tested strains and commercial cultures. These results are consistent with previous works (Vinderola *et al*. 2007a; Burns *et al*. 2010; Peteán *et al*. 2011). On the other hand, the proteolytic activity values corresponded to the peptide profiles of the supernatants obtained by HPLC. Batista *et al*. (2018) reported proteolytic activities of about 0.7 in probiotic yogurt with the addition of glucose oxidase. Moreover, Cruz *et al*. (2013) proved that yogurts packaged in plastic containers with lower oxygen permeability rates showed a higher proteolysis (0.542–0.777) during the refrigerated storage.

Several studies have demonstrated the role of bioactive peptides in the promotion of the immune function (Vinderola *et al*. 2007a; Hernández-Ledesma *et al*. 2014). Immunomodulatory peptides can enhance immune cell functions, such as antibody synthesis and cytokine regulation, lymphocyte proliferation and natural killer cell activity (Pihlanto 2016). In this sense, different authors have reported that functional foods containing

probiotics and/or bioactive peptides induced the proliferation of IgA-producing cells in the gut (Vinderola 2008; Galdeano *et al*. 2009). The main function of IgA, the most abundant defense in the mucosa-associated immune system (MALT), is to exert the immune exclusion of pathogenic bacteria or viruses (Dongarrà *et al*. 2013).

The postbiotic (F36) produce at the laboratory stimulate the immune system by increasing the secretion of S-IgA in mice. Peteán *et al*. (2014) reported that the administration of the cell-free supernatant of 10 or 20% (w/v) buttermilk, fermented using NaOH or $Ca(OH)_2$ as pH-controlling agents, significantly enhanced the number of IgA producing cells in the lamina propria of the small intestine, compared to control mice. In previous work we demonstrated that buttermilk was a suitable substrate for the fermentation with *L. helveticus* 209, and that the spray-dried cell-free supernatant obtained was able to modulate the gut mucosa *in vivo* (Burns *et al*. 2010). Otani *et al*. (2003) found that faecal and intestinal anti-LPS secretory IgA and total IgA in mice fed a Casein PhosphoPeptide (CPP-III)-added diet were significantly higher than in mice fed the control diet.

When peptides are part of the native protein, they are inactive but the action of enzymes during food processing or gastrointestinal digestion releases and turns them active (Toldrá *et al*. 2018). Literature data report that milk-derived bioactive peptides are effective against several gram-positive and gram-negative bacteria (Mohanty *et al*. 2016b; Zanutto-Elgui *et al*. 2019).

Salmonella infection in mice is considered a heterogeneous and dynamic process dependent on multiple variables that underlie the complex processes that occurred in the host. Even if the dose of *Salmonella* used should induce a mild infection in mice (50 % death in the

control group), this fact is conditioned by several factors involving the pathogen and mice (Zacarías *et al*. 2014). The susceptibility to *Salmonella* infection of control mice was different between the two *in vivo* experiments. It was likely that mouse vendor and batch to batch-dependent variation in gut microbiota composition determines susceptibility to *Salmonella* infection (Ericsson *et al*. 2015).

Both postbiotics produce at laboratory (F36) and industrial level (FiSD) were effective in protecting mice against *Salmonella* infection. These results suggest that the production at different scales and the spray drying process would not interfere with the protective effect. Vinderola (2008) reported that the application of spray-drying to the cell-free fraction of fermented milk allows nutritional and sensorial qualities to be retained, together with an extreme reduction in weight for transportation and an extended shelf-life. A protective effect of the non-bacterial fraction (NBF) of milk fermented with *L. helveticus* R839 against *Salmonella* Typhimurium infection was previously reported by Vinderola *et al*. (2007b). Authors found that mice fed the NBF showed lower levels of liver colonization on day 7 post-challenge, higher luminal contents of specific anti-*Salmonella* S-IgA, and lower numbers of MIP-1α+ cells in the lamina propria.

In the present study, it was demonstrated that the pH-controlled milk fermentation using the proteolytic DSM-100H culture could be a suitable strategy to obtain a cell-free supernatant with immunomodulatory and protective capacity against *Salmonella* infection in mice. These postbiotics may be an attractive alternative as ingredients to produce functional foods especially when the characteristics of the food matrix are not favourable to host viable cells of probiotic microorganisms.

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Conflict of interest

No conflict of interest declared.

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Tables

Table 1. Final pH and proteolytic activity (A_{340nm}) of the different strains/commercial cultures grown in 10% (w/v) skim milk after 20 h of incubation at 43ºC without pH control.

Commercial cultures/Strains	pH*	Proteolytic Activity (A_{340})*
DSM-100H DSF	3.37 ± 0.01	1.03± 0.04
Lb-12 Chr. Hansen	3.58 ± 0.02	1.08± 0.09
LH-B02 Chr. Hansen	3.35 ± 0.03	0.49± 0.01
Biochemical H 2085	3.85 ± 0.37	0.48± 0.09
Biochemical H 2087	3.82 ± 0.27	0.49± 0.07
L. helveticus Lh 05	Slow strain**	
L. helveticus Lh 07	Slow strain**	
L. helveticus Lh 08	Slow strain**	
L. helveticus Lh 06	3.50 ± 0.14	0.70± 0.05
L. bulgaricus Lb 03	3.84 ± 0.11	0.83± 0.04
L. bulgaricus Lb 12	3.44 ± 0.13	0.73± 0.04
L. bulgaricus Lb 09	Slow strain**	
L. bulgaricus Lb 10	3.46 ± 0.02	0.43± 0.06
L. bulgaricus Lb 11	3.36 ± 0.09	0.30± 0.10
L. lactis Ll 210	3.60 ± 0.10	0.47± 0.04
L. lactis Ll 133	3.86 ± 0.06	0.24± 0.09

*Values are the mean of three determinations ± SD. **Not able to grow in milk.

Table 2. Cell counts (log_{10} CFU mL^{-1}) of the selected strains/commercial culture grown in 10% and 20% (w/v) skim milk (SM) at different times with and without pH control.

Commercial culture/strains	Cell counts (log_{10} CFU mL^{-1})									
	10% SM					20% SM				
	pH = 6				Free pH	pH=6				Free pH
	initial	16 h	18 h	20 h	20 h	initial	16 h	18 h	20 h	20 h
DSM-100H	6.28±0.12	8.37±0.43	7.63±0.58	7.60±0.54	7.61±0.88	6.28±0.09	8.22±0.25	7.85±1.43	7.66±0.92	8.74±0.51
L. bulgaricus Lb 03	5.60±0.22	8.32±0.18	7.43±0.55	7.33±0.22	7.48±1.34	5.69±0.36	8.23±0.12	8.56±0.23	8.21±0.35	7.97±0.23
L. bulgaricus Lb 12	6.25±0.78	8.22±0.72	8.18±0.73	7.17±0.61^{*a}	8.86±0.31^{b}	6.81±0.55	8.22±0.27	7.83±0.28	7.26±0.54^{*c}	8.99±0.27^{d}
L. helveticus Lh 06	6.71±0.21	8.84±0.42	8.94±0.52	9.08±0.47	8.76±0.30	5.49±0.15	8.28±0.66	8.29±0.45	8.48±0.36	8.60±0.30

* For each condition (10 or 20% SM), values significantly different compared to that at 16 h ($P < 0.05$). ^{a,b} For 10% SM, values significantly different ($P = 0.039$). ^{c,d} For 20% SM, values significantly different ($P = 0.001$). Values are the mean of three determinations ± SD.

Table 3. Proteolytic activity (A_{340nm}) of the selected strains/commercial culture grown in 10% and 20% (w/v) skim milk (SM) after 16 h and 18 h of fermentation at pH = 6.

Commercial culture/strains	Proteolytic Activity (A_{340})[#]			
	10% SM		20% SM	
	16 h	18 h	16 h	18 h
DSM-100H	0.97 ± 0.37[b]	1.47 ± 0.06[b]	1.78 ± 1.00[b]	1.85 ± 0.80[b]
L. bulgaricus Lb 03	0.27 ± 0.13[a]	0.43 ± 0.04[a]	0.53 ± 0.06[a]	0.57 ± 0.05[a]
L. bulgaricus Lb 12	0.25 ± 0.26[a]	0.29 ± 0.22[a]	0.40 ± 0.20[a]	0.54 ± 0.30[a]
L. helveticus Lh 06	0.24 ± 0.15[a]	0.27 ± 0.16[a]	0.41 ± 0.40[a]	0.42 ± 0.30[a]

[a, b] For each column, values with different superscript letters are significantly different ($P < 0.05$). [#] Values are the mean of three determinations ± SD.

Figure Captions

Figure 1. Proteolytic activities of the commercial culture and the autochthonous strains grown in 10% or 20% (w/v) skim milk (SM) with and without pH control after 20 h of fermentation at 43ºC. Values are the mean of three determinations ± SD. [a,b,c] For each strain, bars with different superscript letters are significantly different ($P < 0.01$).

Figure 2. RP-HPLC profiles of the unfermented milk supernatant (a); the cell-free supernatant of milk (10% w/v) fermented without pH control (b), and with pH controlled with $Ca(OH)_2$ (c); the cell-free supernatant of milk (20% w/v) fermented without pH control (d) and with pH controlled with $Ca(OH)_2$ (e).

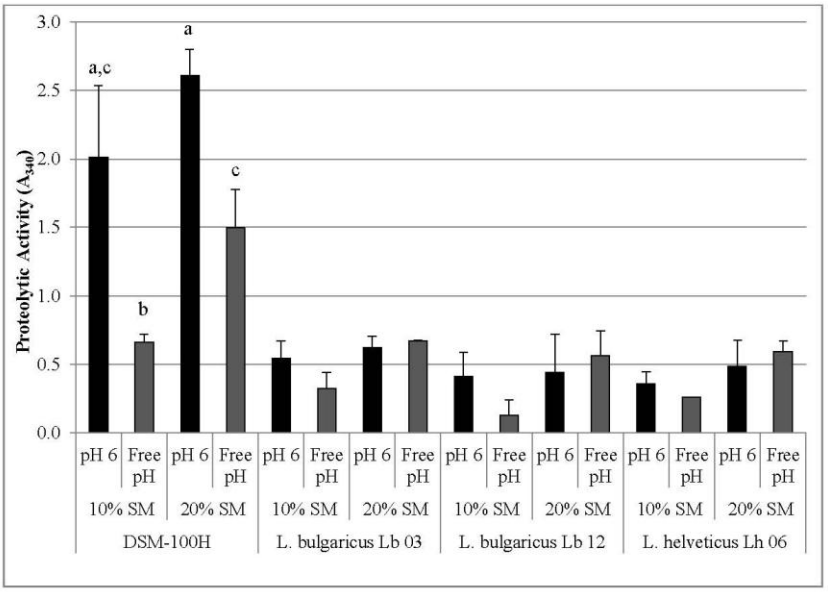
Figure 3. RP-HPLC profiles of the unfermented milk supernatant (a); the cell-free supernatant of milk (10% w/v) fermented at pH 6 controlled with $Ca(OH)_2$ (F36) (b); the cell-free supernatant of milk (10% w/v) fermented at pH 6 controlled with NH_4OH at laboratory scale (c); the cell-free supernatant produced at industrial scale before spray-drying (d); the cell-free supernatant produced at industrial scale spray-dried and reconstituted (FiSD) (e).

Figure 4. Secretory IgA concentration (μg g^{-1} faeces) in mice that received the unfermented milk supernatant (C) (■); the cell-free supernatant obtained by DSM-100H fermentation in 10% (w/v) skim milk (F36) (■); and c) the F36 supernatant diluted 1/10 in tap water (F36D) (■). Values are average ± SD. *Statistically different compared to the control group at the same time ($P < 0.05$).

Figure 5. Survival curve during 20 days post challenge with *S.* Typhimurium of mice that received the unfermented milk supernatant (C) (♦); the cell-free supernatant obtained by DSM-100H fermentation in 10% (w/v) skim milk (F36) (■); and c) the F36 supernatant diluted 1/10 in tap water (F36D) (▲).

Figure 6. Survival curve during 20 days post challenge with *S.* Typhimurium of mice that received reconstituted milk (30% w/v) (C) (♦); the cell-free supernatant obtained by DSM-100H fermentation in 10% (w/v) skim milk at industrial level, spray-dried and reconstituted (FiSD) (■).

Figure 1



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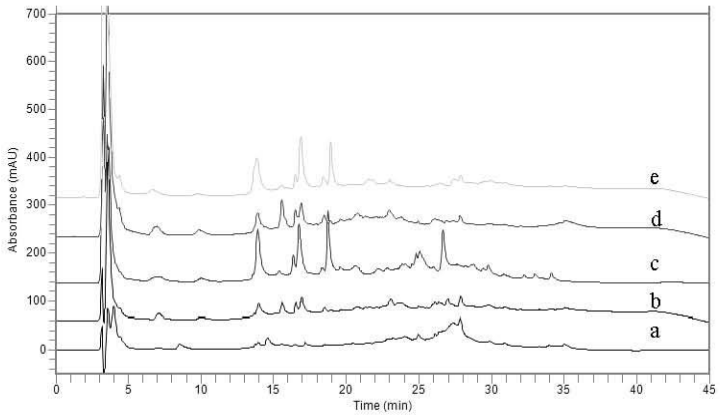

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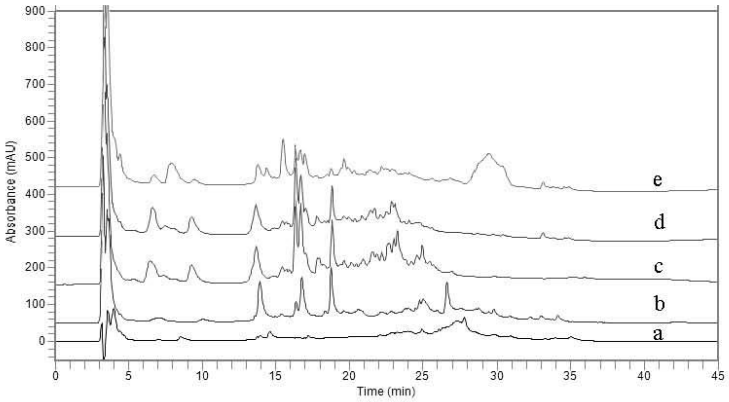

Figure 4.

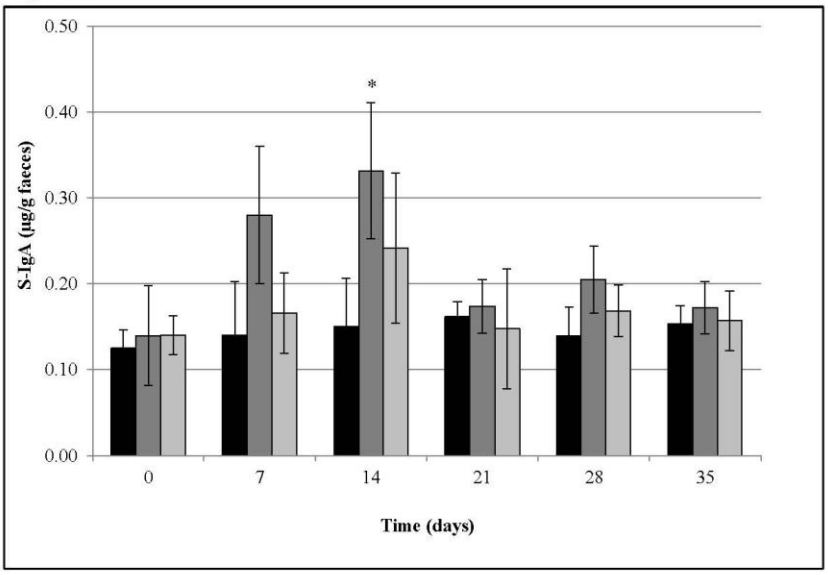

Figure 5.

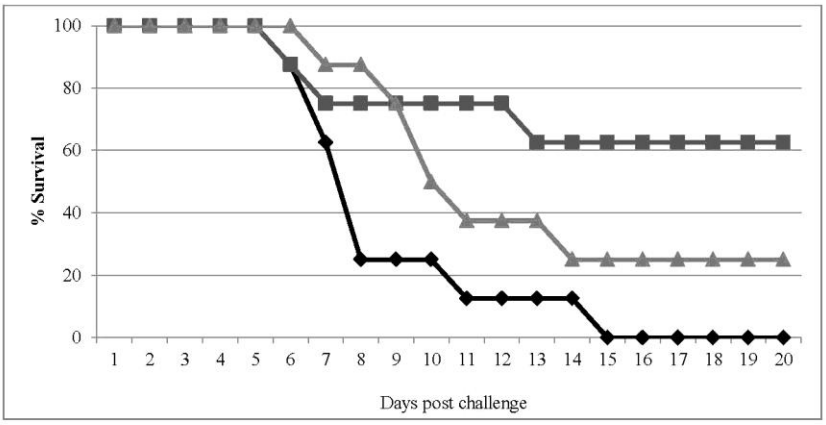

Figure 6.