LWT - Food Science and Technology 81 (2017) 111–117

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Technological challenges in the production of a probiotic pasta filata soft cheese

Facundo Cuffia [a], Guillermo George [a], Pablo Renzulli [b], Jorge Reinheimer [a], Carlos Meinardi [a], Patricia Burns [a, *]

[a] Instituto de Lactología Industrial (INLAIN, UNL-CONICET), Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santiago del Estero 2829, Santa Fe 3000, Argentina
[b] Mastellone Hnos. S.A., General Rodríguez (B1748), Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 9 January 2017
Received in revised form
15 March 2017
Accepted 22 March 2017
Available online 24 March 2017

Keywords:
Technology
Cheese
Pasta filata
Probiotic

ABSTRACT

The aim of this study was to adjust technological parameters: acidification of the curd (pH 5.25) and time (2, 5, 10 and 20 min) and stretching temperature (58, 62.5 and 68 °C) in order to make a pasta filata cheese carrying a probiotic bacterium at levels higher than 10^7 CFU/g. A control and a probiotic cheese were produced. *Lactobacillus rhamnosus* GG was used and its resistance to simulated gastrointestinal digestion (SGD) was evaluated. Gross composition and pH, microbiological analysis, proteolysis, physicochemical and sensory characteristics, volatile compounds, organic acids and sugar profiles were also determined. The probiotic remained above 3×10^7 CFU/g during its shelf life and exhibited high resistance to SGD (matrix protection of about 60%). The addition of the probiotic increased secondary proteolysis (about 30% for SN fraction in trichloracetic and phosphotungstic acids) and the production of diacetyl, acetoin, lactic and acetic acids. Sensory characteristics (smell, astringency, acid taste and residual flavor) were also modified. The development of a probiotic Fior di Latte cheese that might contribute to disease prevention and generate improvements in sensory characteristics compared to traditional products would allow expanding the market of functional foods.

1. Introduction

Functional foods are those which contain some health promoting components which go beyond the traditional nutrients (Granato, Branco, Nazzaro, Cruz, & Faria, 2010). One way to achieve this is by adding probiotics. A probiotic food is a processed product which contains viable probiotic microorganisms in a suitable matrix and in sufficient concentration (Castro, Tornadijo, Fresno, & Sandoval, 2014). 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'. *Lactobacillus rhamnosus* GG (ATCC 53103, LGG®) is one of the most investigated probiotic strains in the world. It has been widely studied in humans and laboratory animals for a variety of uses. Moreover, it is a well attested clinical bacterial strain widely used as probiotic culture in dairy foods. The ability of *L. rhamnosus* GG to survive and colonize in the gastrointestinal tract (GIT) has been shown for both adults and children (Jia, Chen, Chen, & Ding, 2015).

Nowadays there is a great interest of food industries for the development of dairy products containing probiotic bacteria, which may additionally provide essential nutrients such as calcium and proteins (Angiolillo, Conte, Faccia, Zambrini, & Del Nobile, 2014).

Cheeses have a number of advantages over yogurt and fermented milks as a delivery system for viable probiotic microorganisms, because they generally have higher pH and buffering capacity, a solid and consistent matrix and relatively higher fat content. These attributes protect probiotic bacteria during storage and passage through the GIT (Burns et al., 2008). Even though some cheese varieties have been studied as vehicles for probiotic microorganisms (Albenzio et al., 2013; Burns et al., 2015; Dantas et al., 2016; Felicio et al., 2016; Ong, Henriksson, & Shah, 2007), there are very few published studies (and to our knowledge, no product on the market) in which the incorporation of probiotic microorganisms to fresh pasta filata cheese (Fior di Latte type) was evaluated. Besides, previously reported data suggest the addition of microencapsulated (Angiolillo et al., 2014; Ortakci, Broadbent, McManus,

* Corresponding author.
E-mail address: pburns@fbcb.unl.edu.ar (P. Burns).

http://dx.doi.org/10.1016/j.lwt.2017.03.039
0023-6438/

& McMahon, 2012) or heat-adapted probiotics (Minervini et al., 2012), strategies that could present some difficulties for their industrial application. The novelty of this work lies in the adaptation of the production process, together with the selection of a suitable bacterium, allowing the manufacture of a probiotic cheese by means of a simple technology which could be easily transferred to the industrial sector.

Fior di Latte (a high-moisture cow milk Mozzarella cheese) can be manufactured through chemical acidification of the curd or by using commercial (*S. thermophilus*) or natural whey starter cultures. It is a white rindless-cheese, with a characteristic shine on the surface and sweet taste (with a slight taste of lactic acid). It presents high humidity (45–55%) and the fat content is about 18–20% (Ghitti, Bianchi, & Rottigni, 1996). It is a cheese that is usually consumed within a few days of being prepared. Nevertheless, several challenges are related to the addition of probiotics to Fior di Latte cheese, the most important being the survival of these bacteria during cheese making (high temperature of water and time of stretching) and storage. Another challenge found when adding a probiotic into a food is to maintain the sensory attributes of the product (Minervini et al., 2012).

The aim of this work was to modify the traditional cheese making technology in order to make a pasta filata soft cheese carrying the probiotic *L. rhamnosus* GG at levels higher than 10^7 CFU/g during its shelf life.

2. Materials and methods

2.1. Strains and growth conditions

Four commercial probiotic bacteria (*Lactobacillus paracasei* A13, *Lactobacillus rhamnosus* GG, *Lactobacillus paracasei* C, Bifidobacterium animalis subsp. lactis D) and the autochthonous Bifidobacterium animalis subsp. lactis INL1 (isolated from human breast milk, Zacarías, Binetti, Laco, Reinheimer, & Vinderola, 2011) belonging to the INLAIN collection were used. Lactobacillus and Bifidobacterium were propagated for 24 h at 37 °C, in the Man, Rogosa and Sharpe broth (MRS; Biokar, Beauvais, France) or MRS with 0.1% (w/v) L-cysteine hydrochloride (Biopack, Argentina) (MRSc), aerobiosis or anaerobiosis (Mitsubishi™ AnaeroPack-Anaero), respectively. Real names of *Lactobacillus paracasei* C and Bifidobacterium animalis subsp. lactis D are not given because of confidentiality reasons.

2.2. Heat resistance

During cheese making the curd is dipped in hot water so, in order to select the most resistant bacterium, their heat tolerance was evaluated in 20% (w/v) skim milk (Difco, Becton, Dickinson and Company, Sparks, MD, USA) acidified with lactic acid (pH 5.25 ± 0.05) at 60 °C for 10 min (to simulate conditions during stretching). An overnight culture of each bacterium (20 h) was centrifuged (6000 g, 10 min, 8 °C), washed twice with PBS buffer (pH 7.20) and resuspended in skim milk at a final concentration of 1×10^7 CFU/mL. Immediately after heat treatment, cells were chilled on ice. Viable cell counts were performed before and after 10 min exposure to 60 °C in MRS or MRSc agar (48 h, 37 °C, aerobiosis or anaerobiosis). Experiments were carried out in duplicate.

2.3. Cheese making and setting of technological parameters

Each cheese making day, 120 L of raw milk at 4 °C (provided by MILKAUT S.A., Franck, Santa Fe, Argentina) were pasteurized at 65 °C for 20 min and cooled to 39 °C (coagulation temperature). Two types of pasta filata cheese were manufactured: (1) Control cheese (CC: without a probiotic bacterium) and (2) Probiotic cheese (PC: with the selected (more heat-tolerant) probiotic bacterium, *L. rhamnosus* GG). The freeze-dried starter culture Streptococcus thermophilus ST-M6 (Chr. Hansen Inc., Denmark) was added at a concentration of 2×10^6 CFU/mL of milk. The probiotic was cultivated in 1 L MRS broth (20 h, 37 °C, aerobiosis). After growth, cells were harvested by centrifugation (6000 g, 10 min, 8 °C), washed twice with PBS buffer (pH 7.20), resuspended in 100 mL of pasteurized milk and added at a final concentration of 5×10^7 CFU/mL. After 10 min, chymosin [(Chy-Max, Inc. Chr Hansen, Denmark. 183 International Milk-Clotting Units (IMCU/mL))] was added. The amount of rennet (0.3 mL/L of milk) was enough to obtain the proper firmness for cutting the curd in 20–25 min. At this time, the curd was cut in the adequate grain size (~20 mm). After 15 min, the mixture was stirred gently for 15 min to achieve proper moisture and left standing for about 70 min until a pH of 5.6. After that, the whey was removed, the curd was placed on table for approximately 30 min until a pH of 5.20 (±0.02) and cut in slices of c.a. 1 cm thick. Stretching of the curd was performed over 10 min in water at 76.0 °C ± 0.5 °C, 81.0 ± 0.5 °C and 86.0 ± 0.5 °C (core temperature of the curd of 58.0 ± 0.5 °C, 62.5 ± 0.5 °C and 68.0 ± 0.5 °C, respectively). The stretched curd cheeses were formed automatically by a mechanical extruder and immediately placed in brine 3.5% (w/v) at 4 °C for 40 min. Finally, cheeses were vacuum-packed and stored in a chamber at 4 °C for 15 days. Cheese making was carried out in triplicate.

2.4. Microbiological analysis

Viable cell count of total lactic acid bacteria (LAB) in milk and curd was performed in plate count agar (PCA, Britania, Buenos Aires, Argentina) + 10% (w/v) skim milk powder (Difco) at 37 °C for 48 h, whereas *L. rhamnosus* GG was enumerated in MRS agar (37 °C, 48 h, aerobiosis). It has been checked that the starter culture would not grow in MRS agar. Microbiological analysis of cheeses was performed after 1, 7 and 15 days of ripening. At the end of the shelf life the level of coliforms and moulds and yeasts was also determined. Coliforms were counted in lactose violet red bile agar (VRBA, Biokar, Beauvais, France) at 37 °C, 24 h, aerobiosis. Moulds and yeasts were enumerated in chloramphenicol glucose agar (Biokar, Beauvais, France) at 25 °C, 7 days, aerobiosis. Microbiological analysis was performed in triplicate.

2.5. Gross composition and pH measurement

Cheese samples were grated and analyzed for fat matter by Gerber van Gulik method (ISO, 2008), moisture by oven-drying at 102 °C (ISO, 2004) and total protein by Kjeldahl method (ISO, 2011) using a Digestion System 6 (1007 Digester, Tekator, Switzerland) and BÜCHI Distillation Unit B-324 (Sweden). The pH of cheese slurry, prepared by blending a mix 1:1 of grated cheese in H_2O according to the American Public Health Association (APHA) (Bradley et al., 1993) was measured with a pH meter (Orion Research Incorporated, United States) after calibrating with fresh pH 4.0 and 7.0 standard buffers. Cheese composition was analyzed at day 1 and 15. Analyses were carried out in triplicate.

2.6. Proteolysis assessment and volatile profiles

2.6.1. Analysis of soluble nitrogen

Cheese samples were treated to obtain crude citrate extract and soluble fractions at pH 4.6, in trichloracetic acid TCA 12% (v/v) and phosphotungstic acid PTA 2.5% (v/v), according to Hynes et al. (2003). The crude cheese extract was obtained by adding 20 mL of sodium citrate 0.5 M–10 g of cheese and grounding to homogeneity using a pestle. Deionized water was added to ~90 mL, and

the pH was adjusted to 4.6. After centrifugation (3000 g, 15 min), the soluble fraction volume was adjusted to 100 mL. The TCA 12% and PTA 2.5% soluble fractions were obtained from 4.6 soluble fraction according to Gripon, Desmazeaud, Le Bars, and Bergère (1975). The nitrogen content was determined in triplicate by the macro-Kjeldahl method (ISO, 2011).

2.6.2. Analysis of volatile compounds

Prior to the analysis, the samples were cut in cubes and finely grated. Volatile compounds were analyzed by gas chromatography (GC) used solid-phase micro-extraction (SPME) as extraction technique. Cheese samples (5 g) were transferred to GC vials at low temperature (4 °C). The vials were thermostatized at 40 ± 1 °C for 10 min, and then a Carboxen®/Polydimethylsiloxane (CAR/PDMS) 75 μm (Supelco Inc. Bellefonte, PA, USA) fiber was exposed into the headspace for 30 min. The gas chromatography system (Agilent J&W, Agilent Technologies, USA) consisted of HP Innowax column (60 m × 0.25 mm × 0.25 μm), FID detector set at 290 °C. The oven temperature program was as follows: 45 °C (5 min), increase at 8 °C/min to 150 °C (3 min) and finally, an increase at 10 °C/min to 250 °C (5 min). Hydrogen was used as carrier gas at a flow rate of 2.0 mL/min. Volatile compounds were identified by their retention time of standard compounds (Sigma–Aldrich, Italy) or bibliographical data (Wolf, Urgeghe, Addis, Zalazar, & Piredda, 2008; Ziino, Condurso, Romeo, Guiffrida, & Verzera, 2005). Analyses were carried out in triplicate.

2.6.3. Analysis of organic acids and sugars

Organic acids, lactose and galactose were analyzed by high-performance liquid chromatography (HPLC). The HPLC system consisted of a quaternary pump, an on-line degasser, a manual injector, an UV/VIS detector set 210 nm all Series 200 (Perkin Elmer, Norwalk, CT, USA) and a refractive index detector Serie Flexar (Perkin Elmer, Norwalk, CT, USA). The analyses were performed isocratically at 0.6 mL/min and 65 °C with a mobile phase of 0.01 mol/L H_2SO_4 at a flow rate of 0.6 mL/min on an Aminex HPX-87H column (300 × 7.8 mm) from Bio-Rad equipped with a cation H^+ microguard cartridge. The cheese sample (5 g) was blended with distilled water (15 mL) using mortar and pestle and incubated at 40 °C for 1 h. The suspension was centrifuged (3000 g, 30 min) and filtered through fast flow filter paper. The filtered solution was adjusted to a final volume of 25 mL. Samples were diluted in bidistilled water, filtered through 0.45 μm membranes (Millex, Millipore, São Paulo, Brazil), and injected into HPLC (Bergamini, Wolf, Perotti, & Zalazar, 2010).

2.7. Sensory analysis

At the end of ripening (15 d), a descriptive sensory evaluation was made in individual cabins, arranged in a room equipped for this purpose. Both cheeses were presented simultaneously, identified with random numbers. Three replicates were performed. The analysis was made by 8 trained evaluators (4 women and 4 men) using unstructured scales anchored at the extremes with ten lengths of balance. The parameters evaluated were: aroma, color, appearance of mass, elasticity, mouthfeel, cream flavor, salty taste, bitter taste, acid taste and residual flavor and residual taste.

2.8. Resistance of L. rhamnosus GG to simulated gastrointestinal digestion

The resistance of *L. rhamnosus* GG to SGD, as pure culture or in probiotic cheese (after 15 d of ripening) was performed according to Vinderola et al. (2011) with some modifications. Twenty grams of PC or 200 μL of an overnight culture (20 h) of *L. rhamnosus* GG were mixed with 20 or 40 mL of sodium citrate sterile solution and homogenized in a stomacher. The amounts were adjusted to have the same initial concentration of the strain (~10^6 CFU/mL). Gastric digestion (GD) was performed in 'saliva-gastric' solution + 0.3% (w/v) porcine pepsin (Sigma) pH 3.0 (37 °C, 90 min). Duodenal digestion (DD) was performed in 1% (v/v) bovine bile (Sigma) buffer pH 8 (37 °C, 10 min). Intestinal digestion (ID) was carried out in 0.3% (v/v) bovine bile + 0.1% (w/v) porcine pancreatin (Sigma) buffer pH 8 (37 °C, 90 min). Cell counts were made in MRS agar after GD, DD and ID. Plates were incubated at 37 °C, 48 h. Analyses were performed in duplicate.

2.9. Statistical analysis

The results were processed by analysis of variance (ANOVA) using Statgraphics Plus v3.0 (Statistical Graphics Corp.). When significant differences were present ($p < 0.05$), Duncan's test was applied to detect homogeneous groups of means, using the same software.

3. Results and discussions

3.1. Heat resistance

During pasta filata cheese making, one of the most important challenges to be overcome by probiotics is the high temperature of water during stretching. The selection of a heat-resistant bacterium could be a good strategy to avoid a large loss of viability. Cell death was strain-dependent. Both bifidobacteria strains were the most sensitive with a viability loss of more than 3 $\log_{10}$ CFU/mL. *L. rhamnosus* GG was the most heat resistant bacterium (cell death < 1 $\log_{10}$ CFU/mL) so it was selected as adjunct culture for incorporation during the manufacture of Fior di Latte cheese (Table 1).

3.2. Cheese making and setting of technological parameters

In order to set the technological parameters during cheese making, stretching of the curd was made at 58 °C, 62.5 and 68 °C for 2, 5, 10 and 20 min. Results of viability loss of *L. rhamnosus* GG due to the combined treatments (after 1 day of storage) are shown in Table 2. After 20 min of stretching cheeses lost their typical texture, the mass was hard and dry. Nevertheless, good results were obtained after 5 and 10 min. At 68 °C and after 2 min of stretching a viability loss of 2 $\log_{10}$ CFU/g was observed for *L. rhamnosus* GG. After 10 min, its final concentration was 4.8 $\log_{10}$ CFU/g (viability loss of 2.7 $\log_{10}$ CFU/g).

At 58 °C a viability loss of the probiotic of only 0.26 $\log_{10}$ CFU/g was observed after 10 min of stretching but since the range of pH in which the curd can be properly stretched decreases with temperature, a temperature of 62.5 ± 0.5 °C (and 10 min) was chosen.

Table 1
Heat resistance of probiotic bacteria resuspended in 20% (w/v) skim milk (pH 5.25 ± 0.05) after 10 min at 60 °C. Values are expressed as cell death (N_0-N_f $\log_{10}$ CFU/mL) ± SD (n = 2).

Probiotic bacteria	Cell death (Δ $\log_{10}$ CFU/mL) ± SD
L. paracasei A13	2.81 ± 0.08
L. paracasei C	2.92 ± 0.28
L. rhamnosus GG	0.95 ± 0.05
B. lactis D	3.46 ± 1.40
B. lactis INL1	3.70 ± 0.08

N_0 and N_f: cell count before and after heat treatment ($\log_{10}$ CFU/mL), respectively.

Table 2
Viability loss of *L. rhamnosus* GG (log_{10} CFU/g ± SD) in cheese after 1 day of storage at 4 °C (n = 3).

Stretching temperature (°C)	Viability loss (log_{10} CFU/g)			
	Stretching time (min)			
	2	5	10	20
58.5 ± 0.5	0.21 ± 0.01	0.23 ± 0.06	0.26 ± 0.09	–
62.5 ± 0.5	0.37 ± 0.04	0.39 ± 0.10	0.44 ± 0.12	3.83 ± 0.05
68.0 ± 0.5	2.00 ± 0.02	2.55 ± 0.08	2.70 ± 0.07	–

3.3. Microbiological analysis

The level of total LAB in pasteurized milk, after the addition of the starter culture, was 6.34 ± 0.04 log_{10} CFU/mL and in the curd of 9.54 ± 0.04 log_{10} CFU/g. The level of *L. rhamnosus* GG was 7.59 ± 0.01 log_{10} CFU/mL and 8.57 ± 0.03 log_{10} CFU/g in milk and curd, respectively. Microbiological analysis of CC and PC after 2 and 10 min of stretching and 1, 7 and 15 days of ripening are shown in Table 3. After 10 min of stretching (day 1) there was a cell load reduction of total LAB of 0.16 ± 0.11 log_{10} CFU/g in both CC and PC and levels remain almost unchanged till the end of the shelf life (15 d). For L. rhamnosus GG the cell death was 0.44 ± 0.12 log_{10} CFU/g. After 15 days of ripening (and 10 min of stretching) the level of *L. rhamnosus* GG remains at 7.55 ± 0.09 log_{10} CFU/g exceeding the level of probiotic bacteria required for a probiotic food. This means that with a daily consumption of 100 g of this Fior di Latte cheese a person would assume a quantity of probiotic bacteria equal to 10^9 CFU/100 g. After 15 days of vacuum-storage at 4 °C, no viability loss of the probiotic bacterium was observed at stretching time = 2 min and a viability loss of 0.21 log_{10} CFU/g was observed at stretching time = 10 min. The viability of *L. rhamnosus* GG was not affected by the presence of *S. thermophilus* ST-M6. The level of coliforms was <1 log_{10} CFU/g in all cheeses and that of moulds and yeasts vary from <1 log_{10} CFU/g to 1.30 ± 0.43 log_{10} CFU/g, indicating an acceptable cell load of these spoilage microorganisms (Table 3).

These results indicate that the setting of the technological variables was satisfactory in order to maintain the viability of *L. rhamnosus* GG in levels higher than 7.5 log_{10} CFU/g during cheese making and until the end of the shelf life of the Fior di Latte cheese (above the level expected for a probiotic food in which the probiotic bacterium must be present, at least, at 6–7 log_{10} CFU/g, Angiolillo et al., 2014; Gomes da Cruz, Buriti, Batista de Souza, Fonseca Faria, & Saad, 2009).

As mentioned before, there are very few works where the addition of probiotic bacteria to fresh pasta filata cheeses is evaluated. Angiolillo et al. (2014) have formulated a symbiotic Fior di Latte cheese with an edible sodium alginate coating as carrier of probiotic (*L. rhamnosus*) and prebiotic fructo-oligosaccharide substances (FOS). Minervini et al. (2012) reported the production of probiotic Fior di Latte cheeses with two heat-adapted probiotic lactobacilli (L. delbrueckii subsp. bulgaricus SP5 and L. paracasei BGP1). Both strains showed high survival (8.0 log_{10} CFU/g of cheese) during 14 days of storage at 4 °C. Ortakci et al. (2012) studied the addition of an erythromycin-resistant strain of probiotic *Lactobacillus paracasei* ssp. paracasei LBC-1 (LBC-1e) to part-skim Mozzarella cheese in alginate microencapsulated or free form at a level of 10^8 and 10^7 CFU/g, respectively. Hot stretching caused slight log reductions of 0.4 and 0.2 in the numbers of free and encapsulated Lb. paracasei LBC-1e, respectively.

3.4. Gross composition and pH measurement

Values of fat, protein, moisture and pH at the beginning and at the end of ripening are shown in Table 4. No differences ($p > 0.05$) in any of the parameters evaluated for both CC and PC cheeses, either at the beginning or at the end of the ripening process, were found and the values agree with others reported by different authors (Minervini et al., 2012; Ortakci et al., 2012).

3.5. Proteolysis assessment and volatile profiles

3.5.1. Soluble nitrogen

Levels of SN-4.6 (soluble nitrogen pH 4.6) showed no significant differences between cheeses. The values of the SN-TCA (soluble nitrogen in trichloracetic acid) and SN-PTA (SN in phosphotungstic acid) fractions were significantly higher in cheeses containing *L. rhamnosus* GG at the end of the ripening period, being the percentage ratio of SN-TCA/SN-4.6 greater than 40% and 30% in PC and CC, respectively. On the other hand, the percentage NS-PTA/NS-4.6 ratio was similar for both cheeses (about 15%) (Table 5). L. rhamnosus GG had no significant impact on primary proteolysis, which was evidenced by the values of SN-pH 4.6, but increased secondary proteolysis.

Certain bacteria such as *Lactobacillus helveticus*, *L. rhamnosus* GG and Lactobacillus delbrueckii subsp. bulgaricus have the capacity to generate peptides during dairy fermentation driven by their proteolytic system (Rodríguez-Serrano et al., 2014). Minervini et al. (2012) reported that after 14 d of storage, Fior di Latte cheeses made with the addition of probiotic strains were characterized by the highest levels of free aminoacids (FAA), including ornithine. FAA are the main factor responsible for the background flavor of cheeses and contribute, as precursors, to the formation of volatile compounds that influence taste and aroma.

Table 3
Microbiological analysis (log_{10} CFU/g ± SD) of cheeses during the refrigerated storage at 4 °C (n = 3).

Cheese	Stretching time (min)	Cell count (log_{10} CFU/g)							
		T = 1 d		T = 7 d		T = 15 d			
		Total LAB	*L. rhamnosus* GG	Total LAB	*L. rhamnosus* GG	Total LAB	*L. rhamnosus* GG	Coliforms	Moulds and Yeasts
Control	2	9.47 ± 0.11	–	9.31 ± 0.05	–	9.35 ± 0.04		<1	<1
	10	9.23 ± 0.08	–	9.11 ± 0.16	–	9.14 ± 0.09		<1	<1
Probiotic	2	9.45 ± 0.03	8.20 ± 0.04	9.50 ± 0.04	8.25 ± 0.07	9.50 ± 0.02	8.35 ± 0.17	<1	1.30 ± 0.43
	10	9.38 ± 0.02	7.76 ± 0.17	9.26 ± 0.09	7.64 ± 0.03	9.37 ± 0.01	7.55 ± 0.09	<1	<1

Table 4
Gross composition (g/g of cheese) and pH in control (CC) and probiotic (PC) pasta filata soft cheeses at the beginning and at the end of ripening (15 d). Values are mean ± SD (n = 3).

Cheese	Moisture		Fat		Protein		pH	
	1 d	15 d	1 d	15 d	1 d	15 d	1 d	15 d
CC	52.6 ± 0.8	52.6 ± 0.8	20.8 ± 0.3	20.9 ± 0.6	24.1 ± 0.5	23.4 ± 0.3	5.5 ± 0.1	5.3 ± 0.1
PC	52.7 ± 0.2	53.1 ± 0.8	20.0 ± 0.4	20.9 ± 0.3	23.4 ± 0.6	22.9 ± 0.2	5.3 ± 0.1	5.3 ± 0.1

Table 5
Values of soluble nitrogen (SN) at pH 4.6 in trichloroacetic acid (TCA) and in phosphotungstic acid (PTA) expressed as percentage of total nitrogen (TN) and the percentage ratio of these fractions in control and probiotic cheeses at the beginning (T_0) and at the end of ripening (15 d) (T_f). Values are means ± SD (n = 3).

	Time	Control	Probiotic
SN-4.6/TN	T_0	2.54 ± 0.21^a	2.27 ± 0.07^a
	T_f	3.71 ± 0.40^a	3.27 ± 0.26^a
SN-TCA/TN	T_0	1.08 ± 0.07^a	1.08 ± 0.02^a
	T_f	1.25 ± 0.05^a	1.42 ± 0.05^b
SN-PTA/TN	T_0	0.40 ± 0.01^a	0.40 ± 0.02^a
	T_f	0.44 ± 0.03^a	0.52 ± 0.03^b
SN-TCA/SN-4.6 (%)	T_0	38.96	49.20
	T_f	33.66	43.43
SN-PTA/SN-4.6 (%)	T_0	15.60	17.40
	T_f	11.80	16.00

Values with different superscript letters in the same row are significantly different (p < 0.05).

3.5.2. Volatile compounds

At the end of the ripening period the presence of 17 compounds was revealed: six acids (acetic, butyric, hexanoic, octanoic, decanoic and dodecanoic); five ketones (2-propanone, diacetyl, acetoin, 2-hexanone and 2-heptanone); four alcohols (ethanol, 1-propanol, 2-pentanol and 1-hexanol) and two esters (ethyl acetate and ethyl butanoate). Overall, only slight differences were found in the profiles of volatile compounds considering the proportions of the different chemical groups (Fig. 1).

In both cheeses, ketones were the largest group, reaching proportions of 55 and 69% for CC and PC, respectively. Acetoin was the most representative compound of this group. Acids group, reached ~25% of the volatile fraction, wherein the butyric, hexanoic and octanoic acids were the most representative.

Alcohols reached proportions of 12% and 5% while the esters reached values of 7% and 2% for CC and PC, respectively. In these groups, the production of ethanol and ethyl acetate stood out.

Statistical comparison of the areas values of each compound between both cheeses showed that in the presence of *L. rhamnosus* GG the production of diacetyl, acetoin and acetic acid increased significantly (p < 0.05) (Fig. 2). Diacetyl and acetoin are important compounds in the aroma of many cheeses, the first typically occur only in small amount, whereas acetoin production is much higher (McSweeney, 2004). Despite its importance as aroma compound, fully reactions that result in the formation of diacetyl remain unclear.

3.5.3. Organic acids and sugars

Citric, orotic, succinic, lactic and formic acids were quantified. Lactic acid was predominant and its concentration was significantly higher (p < 0.05) in PC. The addition of *L. rhamnosus* GG had a significant influence on the levels of citric and succinic acids, since it was able to consume citric and produce succinic acid (Fig. 3A and B).

Concentrations of formic and orotic acids were similar for both cheeses (data not shown). Lactic acid contributes to pH decreased, which directly affects the degree of mineralization of casein and, therefore, impacts on cheese texture. Moreover, it indirectly influences the flavor, by modifying the activity of enzymes and their influence on the retention of coagulant in the curd (McSweeney, 2011; Slattery, O'Callaghan, Fitzgerald, Beresford, & Ross, 2010).

L. rhamnosus GG was able to consume citric acid. Weinrichter, Sollberg, Ginzinger, Jaros, and Rohm (2004) demonstrated that the utilization of *L. rhamnosus* 58/2 in the production of a Swiss type cheese resulted in the total consumption of citrate after 4 weeks. The concentration of citrate in milk is relatively low (1750 mg/L) and most (94%) is lost in the whey during cheese making. However, the remaining citrate is retained in the curd and it is vital for the development of flavor. Some mesophilic LAB are

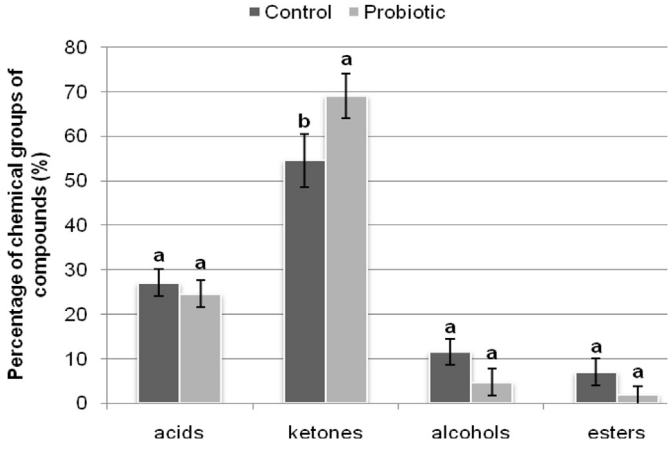

Fig. 1. Percentage of chemical groups of compounds identified in cheeses made with and without the incorporation of *L. rhamnosus* GG at the end of its ripening period (15 d). Values with different superscript letters in the same bars to a same compound are significantly different (p < 0.05).

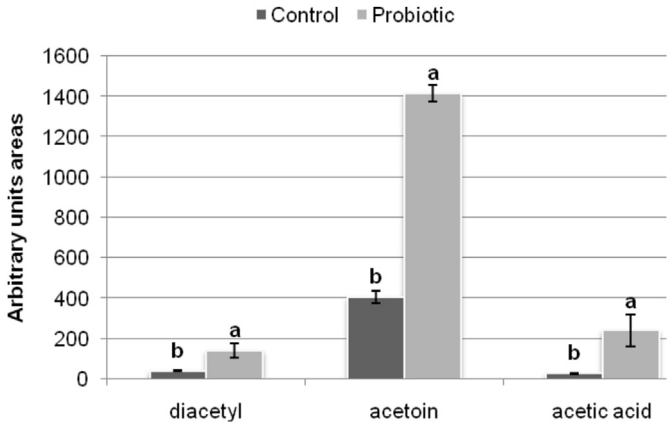

Fig. 2. Arbitrary unit's areas of diacetyl, acetoin and acetic acid in cheeses made with and without the incorporation *L. rhamnosus* GG at the end of its period of maturation (15 d). Values with different superscript letters in the same bars to a same compound are significantly different (p < 0.05).

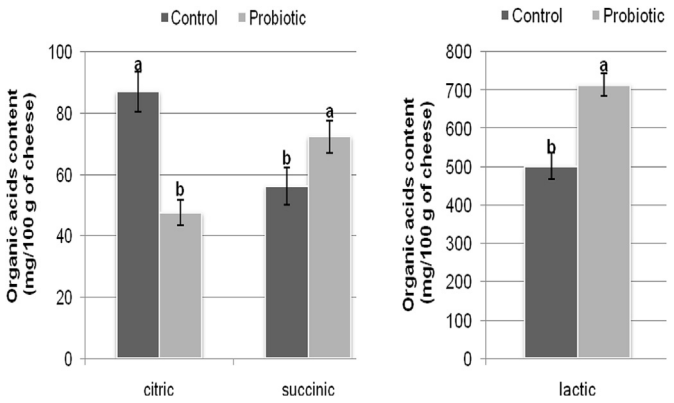

Fig. 3. A. Concentration of citric and succinic acids (mg/100 g of cheese) in control and probiotic cheeses at the end of the ripening period (15 d). Values with different superscript letters in the same bars to a same compound are significantly different ($p < 0.05$).
B. Concentration of lactic acid (mg/100 g of cheese) in control and probiotic cheeses at the end of the ripening period (15 d). Values with different superscript letters in the same bars to a same compound are significantly different ($p < 0.05$).

able to metabolize citrate, which leads to the formation of a series of compounds having aromatics properties such as acetoin, diacetyl, 2,3-butanediol and acetic acid; and their impact is significant in cheese quality (Fox, Guinee, Cogan, & McSweeney, 2000).

At the end of the maturing process, no glucose or lactose was detected in any of the cheeses and the concentration of galactose was about 40 mg/100 g of cheese. Lactose metabolism to lactic acid or lactate is a key step in the production of cheese and is the principal technological role of the primary ferment. High concentrations of galactose found in cheeses indicate that the *S. thermophilus* strain used as starter, was not able to metabolize this sugar (St-Gelais, Lessard, Champagne, & Vuillemard, 2009).

3.5.4. Sensory characteristics

Fig. 4 represents the scores for sensory descriptors evaluated in cheeses after 15 d of ripening. Differences in cohesiveness, odor, astringency, acid taste, and residual flavor were found. The presence of *L. rhamnosus* GG significantly increased ($p < 0.05$) values for odor, astringency, acid taste and residual flavor while cohesiveness was significantly higher in control cheeses.

Sensory analysis is fundamental to determine the quality of the product which impacts directly on consumers (Carpenter, Lyon, &

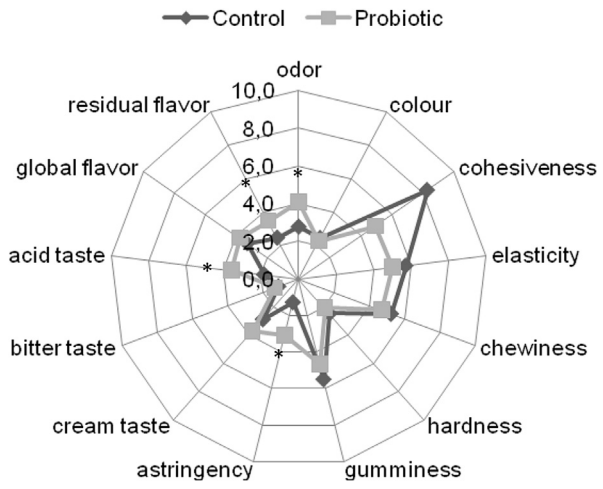

Fig. 4. Scores of sensory descriptors in cheeses at the end of the ripening period (15 d). * Values statistically different ($p < 0.05$).

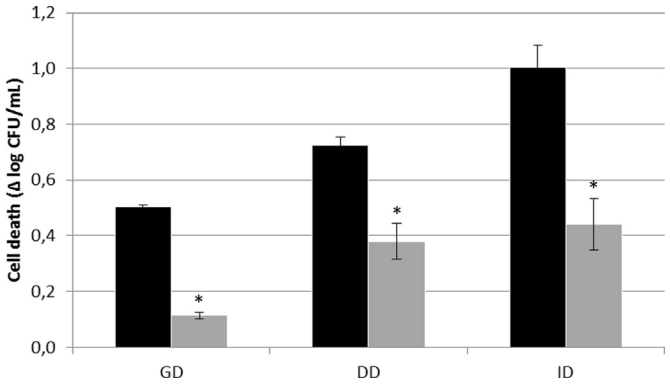

Fig. 5. Resistance of *L. rhamnosus* GG to SGD. Cell death (N_0-N_f $\log_{10}$ CFU/mL) ± SEM of *L. rhamnosus* GG as pure culture (■) and added to the probiotic cheese (■) after gastric (GD), duodenal (DD) and intestinal (ID) digestion. * Values statistically different ($p < 0.05$).

Hadsell, 2000, pp. 1–11). . A food added with probiotic cultures should not display impaired sensory characteristics or reduction in consumer acceptance in comparison to the original product (Cruz et al., 2010). In particular, the highest values found in the acidic taste are closely linked with the increase in the production of lactic and acetic acids observed by the incorporation of the probiotic strain.

3.6. Resistance of L. rhamnosus GG to simulated gastrointestinal digestion

Probiotic microorganisms must survive in food products above a standard level until the time of consumption and arrive in an adequate amount to the large intestine where they exert their beneficial effects (Ortakci et al., 2012). Fig. 5 shows the cell load reduction following the SGD of *L. rhamnosus* GG as pure culture or added to the PC (after 15 days of ripening). There was significantly less cell load reduction when the probiotic was incorporated into the cheese matrix after gastric, duodenal and intestinal digestion indicating that this matrix creates a buffer against the high acidic environment in the GIT generating a more favorable environment (Castro et al., 2014; Gomes da Cruz et al., 2009).

4. Conclusions

This work demonstrates that the setting of the technological variables (acidification of the curd and time and temperature of stretching) was satisfactory in order to maintain the viability of *L. rhamnosus* GG during cheese making and storage at 4 °C. Hot stretching during cheese manufacture caused a reduction of 0.44 $\log_{10}$ CFU/g. The probiotic strain remained above 7.55 $\log_{10}$ CFU/g during its shelf life and exhibited high resistance to SGD. The addition of the probiotic increased secondary proteolysis and the production of diacetyl, acetoin, lactic and acetic acids. Sensory characteristics such as smell, astringency, acid taste and residual flavor were also modified.

Acknowledgments

This work was supported by the project: “Desarrollo de una tecnología para la elaboración de un queso de pasta hilada con adición de bacterias probióticas como actividad rentable y generadora de alto valor agregado”. 44-143208. Secretaría de Políticas Universitarias (SPU) del Ministerio de Educación de la Nación Argentina. Facundo Cuffia is postdoctoral fellow of CONICET (Argentina).

References

Albenzio, M., Santillo, A., Caroprese, M., Ruggieri, D., Napolitano, F., & Sevi, A. (2013). Physicochemical properties of scamorza ewe milk cheese manufactured with different probiotic cultures. *Journal Dairy of Science, 96*, 2781–2791.

Angiolillo, L., Conte, A., Faccia, M., Zambrini, A., & Del Nobile, M. (2014). A new method to produce symbiotic fiordilatte cheese. *Innovative Food Science and Emerging Technologies, 22*, 180–187.

Bergamini, C., Wolf, I., Perotti, M., & Zalazar, C. (2010). Characterisation of biochemical changes during ripening in Argentinean sheep cheeses. *Small Ruminant Research, 94*, 79–89.

Bradley, R., Arnold, E., Barbano, D., Semerad, R., Smith, D., & Vines, B. (1993). Chemical and physical methods. In R. Marshall (Ed.), *Standard methods for the examination of dairy products* (pp. 433–532). Washington, DC: American Public Health Association (APHA).

Burns, P., Patrignani, F., Serrazanetti, D., Vinderola, G. C., Reinheimer, J. A., Lanciotti, R., et al. (2008). Probiotic crescenza cheese containing *Lactobacillus casei* and *Lactobacillus acidophilus* manufactured with high-pressure homogenized milk. *Journal of Dairy Science, 91*, 500–512.

Burns, P. G., Patrignani, F., Tabanelli, G., Vinderola, G. C., Siroli, L., Reinheimer, et al. (2015). Potential of high-pressure homogenisation on probiotic Caciotta cheese quality and functionality. *Journal of Functional Foods, 13*, 126–136.

Carpenter, R. P., Lyon, D. H., & Hadsell, T. A. (2000). *Guidelines for sensory analysis in food products development and quality control*. What is sensory analysis used for? (2nd ed.). Gaithersburg, Maryland: Aspen Publisher

Castro, J. M., Tornadijo, M. E., Fresno, J. M., & Sandoval, H. (2014). Review article. biocheese: A food probiotic carrier. *BioMed Research International*, 1–11. Article ID 723056.

Cruz, A. G., Cadena, R. S., Walter, E. H. M., Mortazavian, A., Granato, D., Faria, J. A. F., et al. (2010). Sensory analysis: Relevance for prebiotic, probiotic, and symbiotic product development. *Comprehensive Reviews in Food Science and Food Safety, 9*, 358–373.

Dantas, A. B., Jesus, V. F., Silva, R., Almada, C. N., Esmerino, E. A., Cappato, L. P., et al. (2016). Manufacture of probiotic minas frescal cheese with *Lactobacillus casei* zhang. *Journal of Dairy Science, 99*, 18–30.

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. http://www.fao.org/es/ESN/food/foodandfood_probio_en.stmS.

Felicio, T. L., Esmerino, E. A., Vidal, V. A. S., Cappato, L. P., Garcia, R. K. A., Cavalcanti, R. N., et al. (2016). Physico-chemical changes during storage and sensory acceptance of low sodium probiotic minas cheese added with arginine. *Food Chemistry, 196*, 628–637.

Fox, P., Guinee, T., Cogan, T., & McSweeney, P. (2000). Biochemistry of cheese ripening. In P. F. Fox (Ed.), *Fundamentals of cheese science* (pp. 236–281). Maryland: Aspen publishers.

Ghitti, G. C., Bianchi, B., & Rottigni, C. (1996). *Il formaggio Mozzarella*. Milano, Italia: Editori Centro Sperimentale del Latte. Codice SBN MIL0308233. (Total pages 61).

Gomes da Cruz, A., Buriti, A. F. C., Batista de Souza, C. H., Fonseca Faria, J. A., & Saad, S. M. I. (2009). Probiotic cheese: Health benefits, technological and stability aspects. *Trends in Food Science & Technology, 20*, 344–354.

Granato, D., Branco, G. F., Nazzaro, F., Cruz, A. G., & Faria, J. A. F. (2010). Functional foods and nondairy probiotic food development: Trends, concepts, and products. *Comprehensive Reviews in Food Science and Food Safety, 9*, 292–302.

Gripon, J., Desmazeaud, M., Le Bars, D., & Bergère, J. (1975). Etude du rôle des microorganismes et des enzymes au cours de la maturation des fromages. II. – influence de la présure commerciale. *Le Lait, 55*, 502–516.

Hynes, E., Bach, C., Lamberet, G., Ogierb, J., Son, O., & Delacroix-Buchet, A. (2003). Contribution of starter lactococci and adjunct lactobacilli to proteolysis, volatile profiles and sensory characteristics of washed-curd cheese. *Le Lait, 83*, 31–43.

ISO. (2004). *Cheese and processed cheese - determination of the total solids content* (Reference method). ISO 5534-IDF 4. Geneva, Switzerland: International Organization for Standardization.

ISO. (2008). *Cheese - determination of fat content - van Gulik method*. ISO 3433-IDF 222. Geneva, Switzerland: International Organization for Standardization.

ISO. (2011). *Milk and milk products - determination of nitrogen content - part 1: Kjeldahl principle and crude protein calculation*. ISO 8968-IDF 20. Geneva, Switzerland: International Organization for Standardization.

Jia, R., Chen, H., Chen, H., & Ding, W. (2015). Effects of fermentation with *Lactobacillus rhamnosus* GG on product quality and fatty acids of goat milk yogurt. *Journal of Dairy Science, 99*, 221–227.

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology, 57*, 127–144.

McSweeney, P. (2011). Biochemistry of cheese ripening. In H. Rogisnski, J. Fuquay, & P. Fox (Eds.), *Vol. 1. Encyclopedia of dairy sciences* (pp. 667–675). United Kingdom: Academic Press.

Minervini, F., Siragusa, S., Faccia, M., Dal Bello, F., Gobbetti, M., & De Angelis, M. (2012). Manufacture of Fior di Latte cheese by incorporation of probiotic lactobacilli. *Journal of Dairy Science, 95*, 508–520.

Ong, L., Henriksson, A., & Shah, N. P. (2007). Proteolytic pattern and organic acid profiles of probiotic cheddar cheese as influenced by probiotic strains of *Lactobacillus acidophilus*, Lb. paracasei, Lb. casei or Bifidobacterium sp. *International Dairy Journal, 17*, 67–78.

Ortakci, F., Broadbent, J. R., McManus, W. R., & McMahon, D. J. (2012). Survival of microencapsulated probiotic *Lactobacillus paracasei* LBC-1e during manufacture of Mozzarella cheese and simulated gastric digestion. *Journal of Dairy Science, 95*, 6274–6281.

Rodríguez-Serrano, G., González-Olivares, L., López-Cuellar, Z., Añorve-Morga, J., Franco-Fernández, M., Castañeda-Ovando, A., et al. (2014). Viability and proteolytic capacity of *Lactobacillus bulgaricus* 2772 and *Lactobacillus rhamnosus* GG during cheese ripening. *Journal of Biosciences and Medicines, 2*, 7–12.

Slattery, L., O'Callaghan, J., Fitzgerald, G., Beresford, T., & Ross, R. (2010). Invited review: *Lactobacillus helveticus* - a thermophilic dairy starter related to gut bacteria. *Journal of Dairy Science, 93*, 4435–4454.

St-Gelais, D., Lessard, J., Champagne, C. P., & Vuillemard, J. C. (2009). Production of fresh cheddar cheese curds with controlled postacidification and enhanced flavor. *Journal of Dairy Science, 92*, 1856–1863.

Vinderola, G., Céspedes, M., Mateolli, D., Cárdenas, P., Lescano, M., Aimaretti, N., et al. (2011). Changes in gastric resistance of *Lactobacillus casei* in flavoured commercial fermented milks during refrigerated storage. *International Journal of Dairy Technology, 64*(2), 269–275.

Weinrichter, B., Sollberg, H., Ginzinger, W., Jaros, D., & Rohm, H. (2004). Adjunct starter properties affect characteristic features of Swiss-type cheeses. *Molecular Nutrition and Food Research, 48*, 73–79.

Wolf, I. V., Urgeghe, P., Addis, M., Zalazar, C., & Piredda, G. (2008). Aplicación de la microextracción en fase sólida al estudio de la fracción volátil de quesos de origen italiano. *Revista Argentina de Lactología, 25*, 55–83.

Zacarías, M. F., Binetti, A., Laco, M., Reinheimer, J., & Vinderola, G. (2011). Preliminary technological and probiotic characterization of bifidobacteria isolated from breast milk for use in dairy products. *International Dairy Journal, 21*, 548–555.

Ziino, M., Condurso, C., Romeo, V., Guiffrida, D., & Verzera, A. (2005). Characterization of "Provola dei Nebrodi", a typical Sicilian cheese, by volatiles analysis using SPME-GC/MS. *International Dairy Journal, 15*, 585–593.