Journal of Dairy Research (2012) **79** 201–208. doi:10.1017/S0022029912000040

Diversity in growth and protein degradation by dairy relevant lactic acid bacteria species in reconstituted whey

Micaela Pescuma[1], Elvira M. Hébert[1], Elena Bru[1], Graciela Font de Valdez[1,2] and Fernanda Mozzi[1]*

[1] Centro de Referencia para Lactobacilos (CERELA)-CONICET, Chacabuco 145, 4000 San Miguel de Tucumán, Argentina
[2] Cátedra de Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina

Received 12 July 2011; accepted for publication 19 December 2011

The high nutritional value of whey makes it an interesting substrate for the development of fermented foods. The aim of this work was to evaluate the growth and proteolytic activity of sixty-four strains of lactic acid bacteria in whey to further formulate a starter culture for the development of fermented whey-based beverages. Fermentations were performed at 37 °C for 24 h in 10 and 16% (w/v) reconstituted whey powder. Cultivable populations, pH, and proteolytic activity (*o*-phthaldialdehyde test) were determined at 6 and 24 h incubation. Hydrolysis of whey proteins was analysed by Tricine SDS-PAGE. A principal component analysis (PCA) was applied to evaluate the behaviour of strains. Forty-six percent of the strains grew between 1 and 2 Δlog CFU/ml while 19% grew less than 0·9 Δlog CFU/ml in both reconstituted whey solutions. Regarding the proteolytic activity, most of the lactobacilli released amino acids and small peptides during the first 6 h incubation while streptococci consumed the amino acids initially present in whey to sustain growth. Whey proteins were degraded by the studied strains although to different extents. Special attention was paid to the main allergenic whey protein, β-lactoglobulin, which was degraded the most by *Lactobacillus acidophilus* CRL 636 and *Lb. delbrueckii* subsp. *bulgaricus* CRL 656. The strain variability observed and the PCA applied in this study allowed selecting appropriate strains able to improve the nutritional characteristics (through amino group release and protein degradation) and storage (decrease in pH) of whey.

Keywords: Whey, lactic acid bacteria, proteolysis, β-lactoglobulin, whey fermentation.

Whey is a by-product of the cheese industry, which is generally disposed as waste especially in developing countries. Nowadays still 47% of the worldwide produced whey is discarded in rivers, lakes or loaded into the ground causing environmental contamination problems (Athanasiadis et al. 2004). Whey is a source of vitamins, minerals and high quality proteins compared with caseins, egg and soy proteins (Dralić et al. 2005; Smithers, 2008). Currently, some of the produced whey is processed by various methods such as concentration, fractionation (ultrafiltration, microfiltration) and drying resulting in several valuable products such as lactose and whey protein concentrates.

The composition of whey varies according to the milk used, the methods of curd coagulation and the cheese produced; values are usually in the following range (g/l): 45–50 lactose, 6–8 soluble proteins, and 4–5 lipids (Gonzalez Siso, 1996; Gernignon et al. 2010). The functional properties of whey proteins encompass physicochemical features such as solubility, whipping and foaming, emulsification and gelation that make it useful in food products (Ji & Hauque, 2003).

The major constituents of bovine whey proteins are β-lactoglobulin (β-lg) and α-lactalbumin (α-la), which constitute 55–60 and 15–20% of total proteins, respectively; serum albumin and immunoglobulins, lactoferrin, lactoperoxidase and various growth factors are present in lower concentrations. β-Lg is found in the whey fraction of cow's milk but is absent in human, rodents and lagomorphs milk (Iametti et al. 2002). This protein is not degraded by pepsin and may cause allergenicity problems especially in children less than three years of age. Denaturation and proteolysis represent powerful tools for enhancing protein digestibility. Whey protein hydrolysates are physiologically better than intact proteins because their intestinal absorption appears to be more effective (Peñas et al. 2006).

Lactic acid bacteria (LAB) are commonly used as starter cultures in the fermented food industry mainly due to their acidifying capacity but also to their metabolic activity on

*For correspondence; e-mail: fmozzi@cerela.org.ar

proteins, sugars and lipids, thus contributing to food digestibility and preservation as well as to the texture and sensory characteristics of the end product (Khalid & Marth, 1990; Wood, 1997). Although LAB are usually considered to be weakly proteolytic, they do cause significant degree of proteolysis in multiple fermented dairy products (Pritchard & Coolbear, 1993; Liu et al. 2010). These bacteria possess a variety of proteolytic enzymes that enable them to utilize milk proteins as nitrogen source and to ensure their growth during fermentation (Matar et al. 1996). Several proteolytic enzymes have been identified for *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*; also, the whole proteolytic system of *Lactococcus lactis* and several lactobacilli species has been well characterized (Liu et al. 2010; Do Carmo et al. 2011).

The functional food market had a combined value of $16 billion worldwide in 2005 and nearly 43% of this market was entirely made up of fermented dairy products. Nowadays, the fermented beverage market represents a large and growing industry (Hozer & Kirmaci, 2009). Whey-based lactic beverages constitute an emerging segment of nonconventional dairy products (Gallardo-Escamilla et al. 2007). Thus, the production of a functional beverage produced upon whey fermentation by LAB could be a good alternative for whey utilization to improve the whey nutritional and sensory characteristics in a natural and non-expensive way.

In this study sixty-four LAB strains belonging to species commonly used in the elaboration of fermented milks were evaluated for their technological characteristics as well as their capability to degrade whey proteins to select appropriate candidate strains for formulating whey-based drink starter cultures.

Materials and Methods

Microorganisms

The strains belonging to the species *Lb. acidophilus* (13); *Lb. delbrueckii* subsp. *bulgaricus* (15), *Lb. casei* (2), *Lb. paracasei* subsp. *paracasei* (13), *Lb. rhamnosus* (4) and *Str. thermophilus* (17) used in this work were obtained from the Culture Collection of Centro de Referencia para Lactobacilos (CERELA), San Miguel de Tucumán, Argentina. Cultures were stored at −20 °C in 10% (w/v) sterile reconstituted skim milk containing 0·5% (w/v) yeast extract, 1·0% (w/v) glucose, and 10% (v/v) glycerol. The assayed strains were chosen as they belong to LAB species commonly used in the production of fermented milks.

Preparation of reconstituted whey powder

The composition (g/100 g product) of the whey powder (WP) used in this study (kindly provided by DANONE S.A., Buenos Aires, Argentina) was the following: fat, 2·0; protein, 10·0; moisture, 3·0; lactose, 76·5; and sodium salts, 0·40–0·45. Whey (WP) was reconstituted to two different concentrations: 10% (w/v, RW10) and 16% (w/v, RW16) with distilled water, the former one was used by Dralić et al. (2005) to grow LAB while 16% (w/v) was chosen considering the low protein concentration of WP. The pH of the RW was adjusted to 6·7 with 2 M NaOH and the obtained solutions were pasteurized at 80 °C for 30 min (Fiahlo et al. 1999) and stored at 4 °C until used (no longer than one week).

Fermentation conditions

Strains were activated in MRS (Britania, Buenos Aires, Argentina) and transferred twice in RW10 or RW16, respectively, prior to experimental use; 16-h old cultures were used as inoculums at 2% (v/v). Fermentations were performed statically in sealed bottles containing 200 ml RW10 or RW16 solution and incubated at 37 °C for 24 h. Samples were aseptically withdrawn at 0, 6 and at 24 h incubation. Cell viability was determined by plating appropriate dilutions of the cultures in MRS agar (MRS plus 1·5%, w/v agar). Plates were incubated at 37 °C for 48 h and colony-forming units CFU/ml were determined and expressed as log CFU/ml. Decrease in pH was followed with a digital pH meter (Altronix TPX 1, New York, USA). Results of pH and CFU/ml were expressed as the difference between values obtained at the beginning (initial counts were in the order of 10^6 CFU/ml) and the end of fermentation (6 or 24 h). The strains were cultured in duplicate and analyses were done in triplicate.

Proteolytic activity

Proteolytic activity by LAB was measured at 6 and 24 h incubation by the *o*-phthaldialdehyde (OPA) test (Church et al. 1983) measuring the increase in optical density at 340 nm relative to the control using a VERSAmax™ Tunable Microplate reader (Sunnyvale, CA, USA). The OPA solution contained: 2·5 ml 20% (w/v) SDS, 25 ml 100 mM sodium tetraborate, 40 mg OPA (previously dissolved in 1 ml methanol), 100 µl 2-mercaptoethanol and distilled water up to a 50 ml final volume. Fermented samples were incubated with 0·75 M trichloroacetic acid (ratio sample to TCA = 1:3) at 4 °C for 30 min and centrifuged (5000 ***g***, 10 min). A 10 µl supernatant aliquot of this mixture was added to 0·2 ml OPA reagent and incubated at room temperature for 5 min until reading at an optical density of 340 nm in the microplate spectrophotometer. Proteolytic activity was arbitrarily expressed as mM leucine using a standard curve of L-leucine (BDH Chemicals Ltd Poole, England). Proteolytic activity was expressed as the difference between the free-amino group values (using Leu as standard) at the beginning and the end of fermentation (0 and 6 or 24 h).

https://doi.org/10.1017/S0022029912000040 Published online by Cambridge University Press

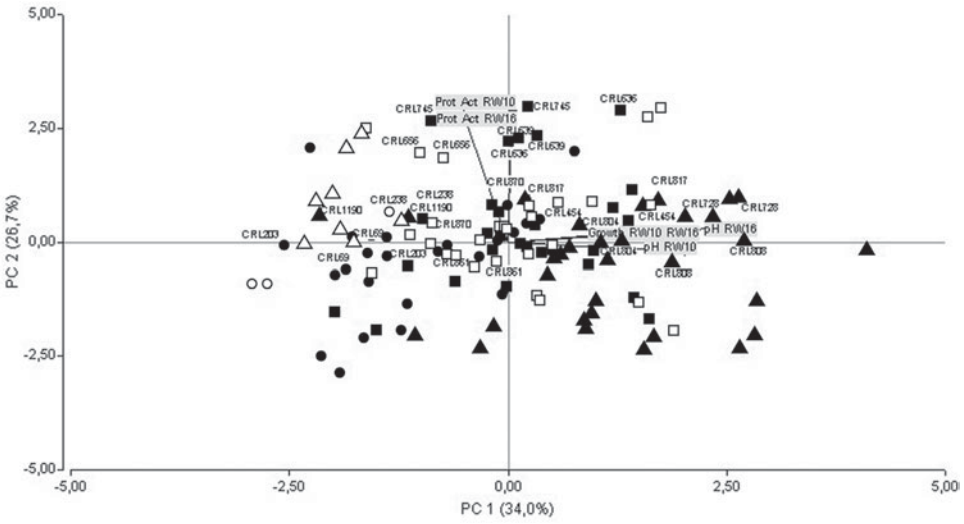

Figure 1. Principal component analysis combining the variables: Growth expressed as ΔCFU/ml (Growth RW10 and Growth RW16), pH (pH RW10 and pH RW16) and proteolytic activity expressed as ΔmM Leu (Prot Act RW10 and Prot Act RW16) of sixty-four LAB strains of *Lb. casei* (○), *Lb. paracasei* (●), *Lb. rhamnosus* (△), *Str. thermophilus* (▲), *Lb. delbrueckii* subsp. *bulgaricus* (□) and *Lb. acidophilus* (■) in RW10 and RW16, incubated at 37 °C during 6 h. The strains were cultured in duplicate and analyses were done in triplicate. Circles represent duplicates of cultures grown in RW10 and RW16 incubated at 37 °C during 6 h.

Whey protein degradation

Whey protein degradation, determined in 24 h-fermentation samples, was measured by Tricine SDS-PAGE (Schagger & Von Jagow, 1987) and performed as follows: fermented whey samples (8 µl) were suspended in 5 µl Laemmli buffer and heated at 100 °C for 5 min. The corresponding blanks (non-fermented, heated RW10 and RW16), and the molecular weight marker (Protein marker, Broad Range, 2–212 kDa, New England Biolabs Inc., Ipswich, MA, USA) were loaded, separately. Tricine SDS-PAGE were carried out on 17% polyacrylamide gels on vertical slab electrophoresis cells (BIO RAD Mini PROTEAN® 3 System, Hercules, CA, USA) for 4 h at 60 V. Coomassie brilliant blue R250 was used to visualize the bands. The degradation of whey protein fractions was evaluated by densitometric analysis of gels by using the QuantiScan software (BIOSOFT 1.5, USA). The percentage of protein hydrolysis was calculated with the values obtained by integrating the peak areas displayed by the software when measuring the band intensities. The molecular weight of the bands was calculated by comparison of the mobility of the bands of the molecular weight marker in the gel using the same software.

Statistical analyses

The obtained data was analysed using principal component analysis (PCA) with INFOSTAT/professional version 2007p. The evaluated variables were ΔpH, growth (Δlog CFU/ml), proteolytic activity (ΔmM Leu) and percentage of protein degradation (variables corresponded to degradation of each protein fraction in whey) in RW10 and RW16. Comparisons between values obtained for each strain were accomplished by Analysis of Variance (ANOVA) general linear model followed by Tukey's *post-hoc* test and $P<0{\cdot}05$ was considered significant.

Results and Discussion

Whey preparation and fermentation

The heat treatment (80 °C for 30 min) applied to RW was sufficient to pasteurize the whey medium as no further cell growth was detected in the RW when plating in MRS agar. To evaluate whether the strains grew better in whey powder reconstituted at 10 (RW10) or 16 (RW16)% (w/v) both concentrations were used.

Whey fermentation by LAB to produce new fermented beverages could be an interesting alternative to solve whey discard. In this work, growth and proteolytic activity of diverse LAB species, usually present in yogurt and fermented milk starters, were analysed for their development in whey.

All the assayed LAB strains were able to grow in both RW10 and RW16, although to different extents. The relative distribution of the assayed strains according to their cell growth, pH values, and proteolytic activity in RW10 and RW16 after incubation at 37 °C for 6 h is shown in Fig. 1. The first principal component (PC 1) displayed most of the strain discrimination (34%) resulting in a horizontal dispersion of the data mainly due to the pH and growth variables, independently of the RW concentration used.

According to the obtained results, growth values (Δlog CFU/ml) were arbitrarily grouped into three categories: values in the range of 0·1–0·9, were considered low; 1–2, moderate, and 2–3, high. Forty-six percent of the total strains

https://doi.org/10.1017/S0022029912000040 Published online by Cambridge University Press

Table 1. Growth and proteolytic activity of selected LAB stains in whey incubated at 37 °C during 6 h

		Proteolysis (ΔmM Leu)		Acidity (ΔpH)		Growth (Δ log CFU/ml)	
LAB (CRL)		RW10	RW16	RW10	RW16	RW10	RW16
Lb. casei†	238	0·09 ± nd^{a}	0·45 ± 0·03^{c}	0·22 ± 0·03^{b}	0·22 ± 0·03^{b}	1·40 ± 0·14^{a}	1·81 ± 0·28^{b}
Lb. paracasei†	69	0·16 ± 0·09^{b}	0·09 ± 0·01^{a}	0·20 ± 0·02^{b}	0·14 ± 0·02^{a}	1·73 ± 0·22^{b}	1·63 ± 0·42^{b}
	203	0·15 ± 0·01^{b}	0·11 ± 0·05^{b}	0·12 ± 0·03^{a}	0·12 ± 0·03^{a}	1·19 ± 0·36^{a}	0·95 ± 0·69^{a}
Lb. acidophilus	636	0·58 ± 0·11^{b}	0·46 ± 0·04^{a}	0·39 ± 0·18^{a}	0·58 ± 0·05^{a}	2·35 ± 0·91^{b}	2·12 ± 0·49^{b}
	639	0·41 ± 0·04^{a}	0·59 ± 0·01^{b}	0·71 ± 0·074^{b}	0·88 ± 0·18^{b}	1·81 ± 0·15^{a}	2·22 ± 0·57^{b}
	745	0·61 ± 0·04^{b}	0·53 ± 0·20^{b}	0·61 ± 0·13^{b}	0·46 ± 0·13^{a}	1·58 ± 0·45^{a}	1·49 ± 0·45^{a}
Str. thermophilus	728	0·19 ± 0·04^{b}	0·16 ± 0·02^{b}	0·74 ± 0·06^{b}	1·16 ± nd^{c}	1·83 ± 0·08^{a}	2·72 ± 0·09^{c}
	804	0·14 ± 0·04^{b}	0·16 ± 0·02^{b}	0·49 ± 0·09^{a}	0·47 ± 0·03^{a}	2·11 ± 0·41^{b}	2·66 ± 0·19^{c}
	808	0·09 ± 0·02^{a}	0·05 ± 0·01^{a}	0·76 ± 0·15^{b}	0·94 ± 0·09^{b}	1·83 ± 0·28^{a}	2·36 ± 0·02^{b}
	817	0·19 ± 0·1^{b}	0·30 ± 0·05^{c}	0·92 ± 0·01^{c}	0·76 ± 0·06^{b}	1·53 ± 0·28^{a}	1·80 ± 0·4^{a}
Lb. bulgaricus	454	0·12 ± 0·03^{b}	0·39 ± 0·01^{c}	0·59 ± 0·25^{c}	0·45 ± 0·25^{b}	2·33 ± 0·36^{c}	2·52 ± 0·07^{c}
	656	0·46 ± 0·13^{c}	0·24 ± 0·02^{b}	0·36 ± 0·1^{a}	0·47 ± nd^{b}	2·00 ± 0·21^{b}	1·68 ± 0·32^{a}
	861	0·07 ± 0·06^{a}	0·14 ± 0·07^{a}	0·41 ± nd^{b}	0·51 ± 0·06^{c}	1·54 ± 0·35^{a}	2·16 ± 0·16^{b}
	870	0·15 ± 0·13^{b}	0·36 ± nd^{c}	0·31 ± 0·06^{a}	0·28 ± 0·01^{a}	1·31 ± 0·86^{a}	2·39 ± 0·22^{c}

Table shows means and SD of replicate cultures. nd = SD < 0·01. Comparisons were done between strains of the same species (letters) († strains belonging to *Lb. casei* and *Lb. paracasei* were considered in the same group and compared between them) in the same RW concentrations. Values with different superscript were considered significantly different after ANOVA analyses and Tukey's *post-hoc* test

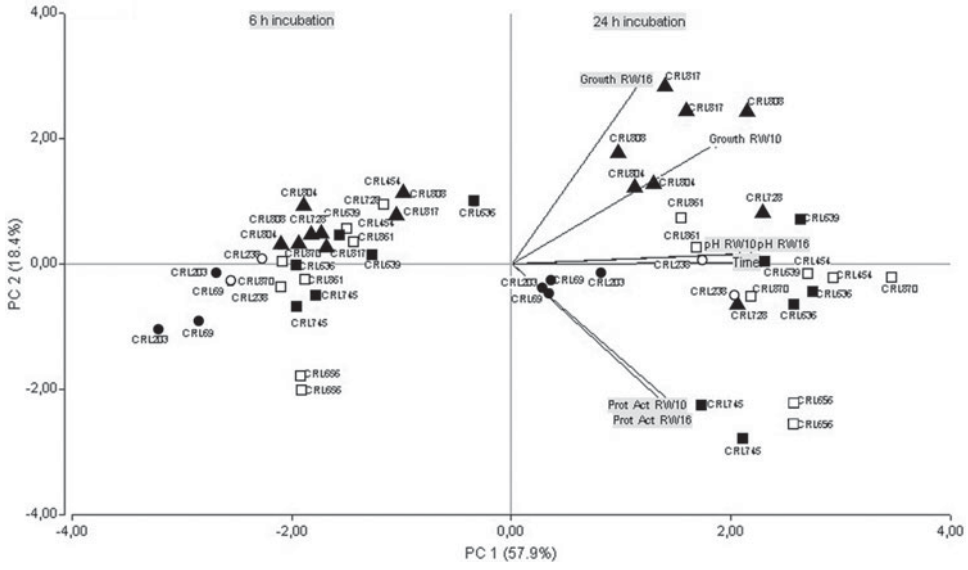

Figure 2. Principal component analysis combining the variables: Growth expressed as ΔCFU/ml (Growth RW10 and Growth RW16), pH (pH RW10 and pH RW16) and proteolytic activity expressed as Δ mM Leu (Prot Act RW10 and Prot Act RW16), and time in h (Time) of 14 strains of *Lb. casei* (○), *Lb. paracasei* (●), *Str. thermophilus* (▲) *Lb. delbrueckii* subsp. *bulgaricus* (□), and *Lb. Acidophilus* (■) in RW10 and RW16 incubated at 37 °C during 6 and 24 h. The strains were cultured in duplicate and analyses were done in triplicate. Circles represent duplicates of cultures grown in RW10 and RW16 incubated at 37 °C during 6 h and 24 h.

showed moderate growth, 35% grew strongly while only 19% grew less than 0·9 Δlog CFU/ml after 6 h of incubation at 37 °C in either RW assayed.

The proteolytic activity of the studied strains was evaluated since the release of amino acids and small peptides increases the nutritional values of whey as it is known that amino acids and small peptides are better absorbed in the intestine than whole proteins (Peñas et al. 2006). The proteolytic activity varied among the assayed LAB strains. High, moderate, and low proteolytic activities were arbitrarily defined as: negative (due to amino acid consumption by the microorganisms) and zero values, were considered low; values between 0·05 and 0·11 ΔmM Leu, moderate; and between 0·12 and 0·65; high. In general, the strains belonging to the species *Lb. casei, Lb. paracasei* and *Lb. rhamnosus* showed moderate proteolytic activity (65% of

https://doi.org/10.1017/S0022029912000040 Published online by Cambridge University Press

Table 2. Behaviour of selected LAB strains in whey incubated at 37 °C during 24 h

		Proteolysis (ΔmM Leu)		Acidity (ΔpH)		Growth (Δlog CFU/ml)	
LAB (CRL)		RW10	RW16	RW10	RW16	RW10	RW16
Lb. casei†	238	$1{\cdot}67 \pm 0{\cdot}07^{b,1}$	$2{\cdot}03 \pm 0{\cdot}08^{b}$	$1{\cdot}22 \pm 0{\cdot}32^{1}$	$0{\cdot}88 \pm 0{\cdot}24^{1}$	$2{\cdot}87 \pm 0{\cdot}25^{2}$	$3{\cdot}55 \pm 0{\cdot}82^{4}$
Lb. paracasei†	69	$0{\cdot}83 \pm 0{\cdot}08^{a}$	$0{\cdot}76 \pm 0{\cdot}16^{a}$	$1{\cdot}40 \pm 0{\cdot}18^{1}$	$0{\cdot}97 \pm 0{\cdot}09^{1}$	$1{\cdot}95 \pm 0{\cdot}11^{1}$	$2{\cdot}00 \pm 0{\cdot}08^{1}$
	203	$0{\cdot}64 \pm 0{\cdot}02^{a}$	$0{\cdot}86 \pm 0{\cdot}06^{a}$	$1{\cdot}40 \pm 0{\cdot}02^{1}$	$1{\cdot}24 \pm 0{\cdot}07^{1}$	$2{\cdot}25 \pm 0{\cdot}48^{1}$	$1{\cdot}80 \pm 0{\cdot}23^{1}$
Lb. acidophilus	636	$0{\cdot}86 \pm 0{\cdot}05^{a}$	$0{\cdot}86 \pm 0{\cdot}04^{a}$	$1{\cdot}73 \pm 0{\cdot}28^{a,2}$	$1{\cdot}98 \pm 0{\cdot}05^{b,2}$	$3{\cdot}53 \pm 0{\cdot}38^{c,5}$	$3{\cdot}02 \pm 0{\cdot}16^{b,3}$
	639	$1{\cdot}44 \pm 0{\cdot}36^{b}$	$2{\cdot}50 \pm 0{\cdot}05^{b}$	$2{\cdot}40 \pm 0{\cdot}07^{b,4}$	$2{\cdot}33 \pm 0{\cdot}09^{c,3}$	$2{\cdot}90 \pm 0{\cdot}37^{b,2}$	$1{\cdot}95 \pm 0{\cdot}36^{a,1}$
	745	$1{\cdot}90 \pm 0{\cdot}09^{b}$	$3{\cdot}37 \pm 0{\cdot}28^{c}$	$1{\cdot}20 \pm 0{\cdot}60^{a,1}$	$1{\cdot}30 \pm 0{\cdot}05^{a,2}$	$2{\cdot}12 \pm 0{\cdot}09^{a,1}$	$1{\cdot}95 \pm 0{\cdot}39^{a,1}$
Str. thermophilus	728	$0{\cdot}15 \pm 0{\cdot}10$	$0{\cdot}21 \pm 0{\cdot}18$	$2{\cdot}04 \pm 0{\cdot}06^{3}$	$2{\cdot}44 \pm 0{\cdot}01^{4}$	$2{\cdot}82 \pm 0{\cdot}30^{2}$	$3{\cdot}72 \pm 1{\cdot}22^{4}$
	804	$0{\cdot}16 \pm 0{\cdot}04$	$0{\cdot}18 \pm 0{\cdot}02$	$2{\cdot}11 \pm 0{\cdot}25^{3}$	$1{\cdot}81 \pm 0{\cdot}01^{2}$	$2{\cdot}47 \pm 0{\cdot}07^{3}$	$3{\cdot}44 \pm 0{\cdot}07^{4}$
	808	$0{\cdot}12 \pm 0{\cdot}12$	$0{\cdot}22 \pm 0{\cdot}01$	$2{\cdot}04 \pm 0{\cdot}23^{3}$	$2{\cdot}07 \pm 0{\cdot}4^{3}$	$3{\cdot}16 \pm 0{\cdot}83^{4}$	$2{\cdot}32 \pm 0{\cdot}13^{2}$
	817	$0{\cdot}18 \pm 0{\cdot}02$	$0{\cdot}30 \pm 0{\cdot}10$	$2{\cdot}15 \pm 0{\cdot}24^{3}$	$2{\cdot}51 \pm \text{nd}^{4}$	$3{\cdot}48 \pm 0{\cdot}17^{5}$	$2{\cdot}51 \pm 0{\cdot}45^{2}$
Lb. bulgaricus	454	$1{\cdot}37 \pm 0{\cdot}19^{c}$	$1{\cdot}90 \pm 0{\cdot}17^{c}$	$2{\cdot}20 \pm 0{\cdot}15^{3}$	$2{\cdot}42 \pm 0{\cdot}02^{4}$	$2{\cdot}82 \pm 0{\cdot}17^{2}$	$3{\cdot}06 \pm 0{\cdot}16^{3}$
	656	$2{\cdot}61 \pm 0{\cdot}43^{d}$	$1{\cdot}90 \pm 0{\cdot}90^{c}$	$2{\cdot}12 \pm 0{,}01^{3}$	$2{\cdot}26 \pm 0{\cdot}14^{3}$	$1{\cdot}32 \pm 0{\cdot}52^{1}$	$2{\cdot}14 \pm 0{\cdot}34^{1}$
	861	$0{\cdot}81 \pm \text{nd}^{b}$	$0{\cdot}87 \pm 0{\cdot}01^{b}$	$2{\cdot}11 \pm 0{\cdot}16^{3}$	$2{\cdot}66 \pm 0{\cdot}57^{4}$	$2{\cdot}57 \pm 0{\cdot}06^{3}$	$2{\cdot}13 \pm 0{\cdot}60^{1}$
	870	$0{\cdot}40 \pm 0{\cdot}05^{a}$	$0{\cdot}41 \pm \text{nd}^{a}$	$2{\cdot}18 \pm 0{\cdot}81^{3}$	$2{\cdot}06 \pm 0{\cdot}08^{3}$	$3{\cdot}14 \pm 0{\cdot}23^{4}$	$2{\cdot}43 \pm 0{\cdot}52^{2}$

Table shows means and SD of replicate cultures. nd = SD < 0·01. Comparisons were done between strains of the same species (letters) († strains belonging to *Lb. casei* and *Lb. paracasei* were considered in the same group and compared between them) or among all the assayed strains (numbers) in the same RW concentrations for the cases cited in the text. Values with different superscript were considered significantly different after ANOVA analyses and Tukey's *post-hoc* test

the strains in RW10 and 82% in RW16) and low acidifying capacity with maximum ΔpH values of 0·5, and were grouped on the left and bottom side of Fig. 1. These species together with *Lb. zeae* are phylogenetically very close and are considered to belong to the *Lb. casei* group; the taxonomical classification of these microorganisms was not clear until 2005 (Svec et al. 2005).

Instead, the strains of *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. acidophilus* were placed throughout Fig. 1 showing high and moderate proteolytic activities and acidifying capacities.

The majority (10 out of 17 strains) of the streptococci showed high acidifying capacity. On the contrary, these strains consumed amino acids and small peptides initially present in whey, resulting in a negative balance of these metabolites (between −0·01 and −0·35 ΔmM Leu) after 6 h incubation in both RW media indicating that in general, the *Str. thermophilus* strains had low proteolytic activity and some of them consumed the amino acids and small peptides initially present in whey. In this respect, Letort et al. (2002) reported that a cell wall-associated proteinase positive *Str. thermophilus* strain consumed amino acids during its growth in milk. Desmazeaud & de Roissart (1994) stated that *Str. thermophilus* strains have complex amino acid requirements and their growth is influenced by the availability and active transport/exchange of free amino acids from the growth medium.

Despite the fact that the strains were originally grouped according to their species, variations of strains within the same species were observed. This finding allowed selecting strains exhibiting the best behaviour belonging to each species or group (as the case of the *Lb. casei, Lb. rhamnosus* and *Lb. paracasei* strains). Thus, 14 strains were selected considering their proteolytic activity, acidifying capacity and/or growth values (Table 1) as follows. From the group comprising *Lb. casei, Lb. rhamnosus* and *Lb. paracasei* the strain *Lb. casei* CRL 238 was included for its high proteolytic activity in RW16 and moderate growth while *Lb. paracasei* CRL 69 and CRL 203 were chosen for their moderate to high growth and proteolytic activity in both RW10 and RW16. The strains *Lb. acidophilus* CRL 636, CRL 639 and CRL 745 showed high OPA values (Table 1) and moderate to high growth in RW10 and RW16. Regarding *Str. thermophilus*, the strain CRL 804 was selected for its high growth and proteolytic activity in whey. Also, the strains CRL 728, CRL 808 and CRL 817 were chosen for their moderate to high growth in whey and their capacity to reduce pH. *Lb. delbrueckii* subsp. *bulgaricus* CRL 454 and CRL 656 were selected for their high growth in both RW10 and RW16, while the strains CRL 870 and CRL 861 were chosen because of their high growth in RW16. Also, the strains CRL 454, CRL 656 and showed high proteolytic activity while CRL 861 showed moderate activity.

The behaviour of the selected fourteen strains was further analysed using a longer incubation period of 24 h. The PCA distribution of these strains for their growth in RW10 and RW16 at 6 and 24 h is shown in Fig. 2; additional information of the pat-hoc statistical analysis of the results is shown in Table 2. The results obtained after 6 h incubation (on the left side of Fig. 2) displayed lower values compared with those from 24 h (right side of Fig. 2) indicating that the strains continued to grow, to digest whey proteins and to acidify the whey media beyond 6 h incubation. After this period, the *Lb. paracasei* strains displayed the lowest growth and acidifying capacity remaining close to the centre of Fig. 2. *Lb. casei* CRL 238 showed better growth and higher proteolytic activity than the *Lb. paracasei* strains after 24 h incubation (Fig. 2, Table 2).

https://doi.org/10.1017/S0022029912000040 Published online by Cambridge University Press

Table 3. Hydrolysis of whey proteins by selected LAB incubated at 37 °C during 24 h

		Hydrolysis (%)											
		RW10					RW16						
		Protein Fraction (kDa)					Protein Fraction (kDa)						
LAB	CRL	80	69	50	25	18	14	80	69	50	25	18	14
Lb. casei†	238	3·9 ± 0·9^{2}	9·8 ± 2·0^{3}	1·4 ± 0·2^{a,1}	13·2 ± 0·7^{4}	6·1 ± 2·8^{b,2}	1·4 ± 0·9^{a,1}	7·3 ± 5·7^{2}	8·1 ± 0·3^{2}	7·0 ± 1·2^{a,2}	11·5 ± 0·2^{3}	5·0 ± 1·3^{c,2}	2·1 ± 0·9^{a,1}
Lb. paracasei†	69	29·0 ± 1·6^{2}	23·4 ± 1·4^{2}	38·9 ± 0·1^{c,3}	0·45 ± 0·2^{1}	6·5 ± 2·4^{b,1}	2·9 ± 0·8^{a,1}	36·8 ± 1·2^{2}	49·9 ± 11·9^{3}	43·2 ± 1·9^{c,3}	0·4 ± 0·1^{1}	3·7 ± 0·5^{b,1}	8·6 ± 3·9^{b,1}
	203	10·9 ± 6·9^{2}	12·1 ± 7·4^{2}	0·2 ± 0·1^{a,1}	20·1 ± 4·1^{3}	1·7 ± 0·3^{a,1}	6·0 ± 1·1^{a,1}	11·6 ± 5·9^{2}	12·8 ± 8·4^{2}	4·2 ± 9·4^{a,1}	29·8 ± 9·5^{3}	1·9 ± 0·2^{a,1}	5·0 ± 2·5^{a,1}
Lb. acidophilus	636	7·7 ± 3·1	21·6 ± 5·5	36·2 ± 8·4^{c}	3·7 ± 1·6	16·1 ± 3·4^{c}	23·2 ± 5·5^{c}	10·8 ± 1·2	31·2 ± 8·0	29·4 ± 1·1^{c}	2·8 ± 0·3	17·7 ± 1·0^{d}	23·5 ± 5·0^{d}
	639	35·0 ± 1·7	32·7 ± 5·8	24·0 ± 3·1^{b}	20·6 ± 0·8	1·8 ± 0·0^{a}	5·4 ± 3·4^{a}	67·2 ± 6·9	61·5 ± 9·7	15·4 ± 0·6^{b}	46·4 ± 6·5	0·7 ± 0·1^{a}	2·8 ± 0·7^{a}
	745	5·0 ± 3·1	38·7 ± 12·4	26·1 ± 3·3^{b}	25·8 ± 3·3	6·2 ± 2·4^{b}	15·2 ± 0·7^{b}	11·4 ± 1·2	31·4 ± 3·3	25·5 ± 4·1^{b}	15·9 ± 2·9	7·2 ± 1·0^{c}	20·5 ± 4·4^{d}
Str. thermophilus	728	40·0 ± 0·2	56·8 ± 4·9	42·4 ± 9·3^{c}	22·2 ± 5·8	7·9 ± 0·3^{b}	22·3 ± 9·8^{c}	27·5 ± 1·6	27·8 ± 6·5	34·1 ± 1·7^{c}	13·7 ± 3·0	6·2 ± 1·2^{c}	20·6 ± 1·0^{d}
	804	23·6 ± 5·7	44·5 ± 11·5	39·7 ± 7·4^{c}	17·2 ± 8·7	8·4 ± 0·7^{b}	12·7 ± 1·4^{b}	16·2 ± 0·7	29·5 ± 0·2	24·1 ± 3·4^{b}	4·9 ± 2·9	3·2 ± 2·8^{b}	13·5 ± 0·1^{c}
	808	46·7 ± 1·5	54·2 ± 2·0	66·6 ± 8·5^{d}	3·6 ± 2·7	4·4 ± 2·4^{b}	8·4 ± 4·1^{b}	48·7 ± 11·9	50·3 ± 7·2	57·6 ± 4·5^{d}	0·4 ± 0·1	7·6 ± 2·4^{b}	13·2 ± 1·1^{c}
	817	6·6 ± 3·3	15·7 ± 5·9	18·2 ± 0·2^{b}	8·2 ± 1·4	1·1 ± 0·9^{a}	0·9 ± 0·3^{a}	6·6 ± 3·3	14·6 ± 2·6	18·2 ± 0·2^{b}	3·2 ± 4·5	4·9 ± 1·5^{b}	2·1 ± 0·2^{a}
Lb. bulgaricus	454	15·7 ± 4·5	37·1 ± 4·7	38·1 ± 10·0^{c}	14·1 ± 5·5	3·2 ± 0·7^{b}	11·4 ± 8·4^{b}	15·6 ± 4·6	20·6 ± 12·7	19·3 ± 4·5^{b}	14·3 ± 0·7	8·5 ± 2·9^{c}	22·8 ± 1·2^{d}
	656	12·8 ± 10·0	31·0 ± 0·7	20·3 ± 3·4^{b}	8·5 ± 3·5	12·2 ± 0·5^{c}	13·2 ± 0·7^{b}	9·5 ± 1·6	33·1 ± 2·9	21·3 ± 1·5^{b}	0·8 ± 0·1	13·9 ± 1·3^{d}	7·6 ± 0·9^{a}
	861	5·6 ± 1·9	25·4 ± 2·6	19·5 ± 0·8^{b}	7·3 ± 4·5	3·7 ± 0·9^{a}	11·4 ± 1·1^{b}	5·9 ± 1·4	24·3 ± 4·2	18·4 ± 2·3^{b}	3·2 ± 1·2	1·2 ± 0·6^{a}	9·6 ± 3·8^{b}
	870	21·6 ± 0·9	17·6 ± 1·0	20·4 ± 1·6^{b}	7·7 ± 4·5	1·6 ± 0·5^{a}	14·8 ± 3·0^{b}	24·5 ± 0·3	22·6 ± 1·9	20·8 ± 0·1^{b}	6·5 ± 4·1	0·4 ± 0·1^{a}	10·4 ± 2·8^{b}

Table shows means and SD of replicate cultures. Comparisons were done between strains for the fractions compared in the text (letters) († strains belonging to *Lb. casei* and *Lb. paracasei* were considered in the same group and compared between them) or between fractions within the same strain (numbers) always for the same RW concentrations. Values with different superscript were considered significantly different after ANOVA analyses and Tukey's *post-hoc* test

Within the *Lb. acidophilus* species, the most proteolytic strain was CRL 745 (right and bottom of Fig. 2), which also showed the lowest acidifying capacity in RW16. Conversely, *Lb. acidophilus* CRL 636 showed the lowest OPA values despite its greatest growth compared with the other strains of the same species (Table 2). Concerning the *Lb. delbrueckii* subsp. *bulgaricus* species, the strains CRL 656 and CRL 454 were the most proteolytic (Table 2).

In general, the *Str. thermophilus* strains showed similar OPA values at 6 and 24 h incubation (Tables 1 & 2), indicating that this species probably consumed the amino acids and small peptides present in whey for sustaining their growth.

Hydrolysis of whey proteins

Peptide availability due to hydrolysis of whey proteins by the selected (14) LAB strains was assessed because of the high nutritional quality of these proteins.

Proteolysis was analysed in RW10 and RW16 in 24 h incubation-samples as no degradation was observed before this period (data not shown). Tricine SDS-PAGE and hydrolysis percentages were determined by using the QuantiScan software. The obtained results are shown in Table 3 and dispersion of the data after statistical analysis is presented in Fig. 3. SDS-PAGE of whey protein hydrolysates obtained with the most representative strains namely *Str. thermophilus* CRL 804 and CRL 808, *Lb. casei* CRL 238 and *Lb. acidophilus* CRL 636 is shown in Fig. 4. On the whole, the majority of the assayed strains digested the protein fractions of 69 and 50 kDa the most; *Str. thermophilus* CRL 808 showed the maximum degradation percentage for the 50 kDa fraction (66·7% in RW10 and 47·8% in RW16, respectively) and was placed on the lower right side of Fig. 3. In contrast, *Lb. casei* CRL 203 and *Lb. paracasei* CRL 238 poorly hydrolysed this fraction and preferentially hydrolysed the 25 kDa fraction (left side of Fig. 3). These results were opposite to those found for *Lb. paracasei* CRL 69, which hydrolysed more the high molecular weight fractions of 80, 69 and 50 kDa (lower right side of Fig. 3). Other authors showed variation on the ability of different strains to hydrolyse the main milk fractions. Ohmiya & Sato (1969) studied the ability of *Lb. helveticus, Lactococcus lactis* and *Lb. delbrueckii* subsp. *bulgaricus* strains to degrade milk proteins and found different casein and whey protein degradation degrees for strains of each species. In addition, Khalid & Marth (1990) reported degradation of different protein fractions between strains of *Lb. plantarum* and *Lb. casei*.

Concerning α-la (14 kg/mol fraction), the second most abundant whey protein, *Lb. acidophilus* CRL 636 and *Str. thermophilus* CRL 728 showed the maximum hydrolysis percentages (up to 23%) in both RW media compared with all the studied strains and were placed on the upper right side of Fig. 3.

Special attention was taken into account for the break-down of β-lg (18 kDa protein) since this protein is recognized

https://doi.org/10.1017/S0022029912000040 Published online by Cambridge University Press

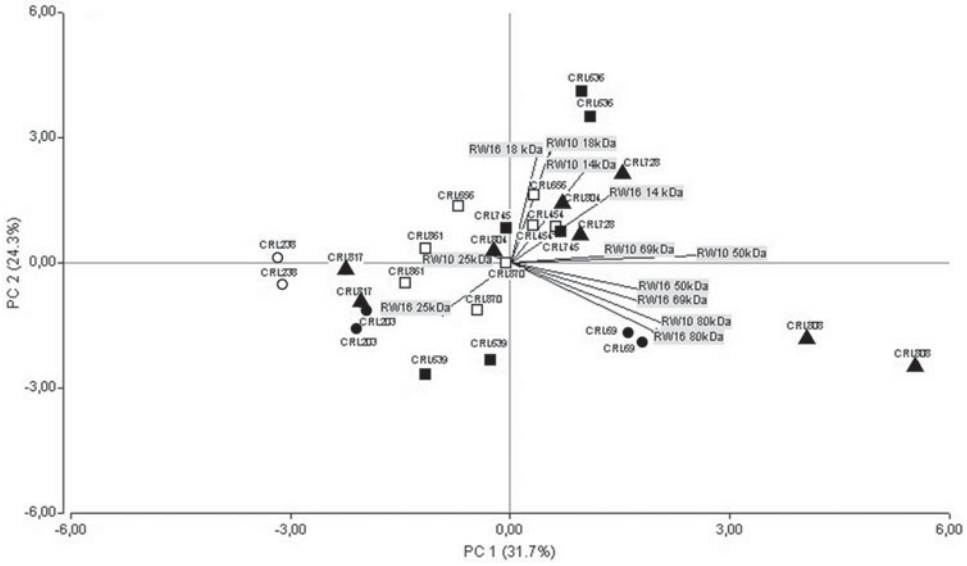

Figure 3. a) Principal component analysis of the data obtained after hydrolysis of whey proteins of: 14 kDa (14 kDa RW10 and 14 kDa RW16), 18 kDa (18 kDa RW10 and 18 kDa RW16), 25 kDa (25 kDa RW10 and 25 kDa RW16), 50 kDa (50 kDa RW10 and 50 kDa RW16), 69 kDa (69 kDa RW10 and 69 kDa RW16) and 80 kDa (80 kDa RW10 and 80 kDa RW16) by the 14 selected LAB strains of *Lb. casei* (○), *Lb. paracasei* (●), *Str. thermophilus* (▲), *Lb. delbrueckii* subsp. *bulgaricus* (□) and *Lb. acidophilus* (■) in RW10 and RW16 incubated at 37 °C, during 24 h. The strains were cultured in duplicate and analyses were done in triplicate. Circles represent duplicate cultures in RW10 and RW16 incubated at 37 °C during 24 h.

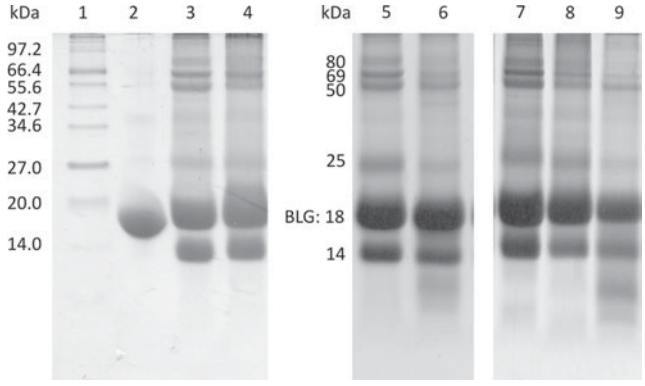

Figure 4. Tricine SDS-PAGE of whey proteins. Lanes, 1 MWM, Broad range molecular weight marker (New England Biolabs); 2 β-lg, β-lactoglobulin (ICN); 3, 5 and 7 non-fermented whey (control); 4, 6, 8 and 9 whey protein hydrolysis by *Str. thermophilus* CRL 808, *Lb. casei* CRL 238, *Str . thermophilus* CRL 804, and *Lb. acidophilus* CRL 636, respectively, in RW10 after 24 h-incubation.

as the major allergen of milk and it is known that hydrolysis of this protein may reduce its allergenicity (Ehn et al. 2005; Bu et al. 2010). On the other hand, whey proteins containing β-lg are added to bovine milk to resemble human milk when preparing infant formula; thus, whey protein hydrolysates obtained by LAB could be an interesting substrate in the formulation of hypoallergenic drinks (Pescuma et al. 2011).

The strains which showed the highest hydrolysis percentages of β-lg were *Lb. acidophilus* CRL 636 (16–18%) and *Lb. delbrueckii* subsp. *bulgaricus* CRL 656 (12–14%) (Table 3) and were placed on the upper side of Fig. 3. Significant differences ($P<0{\cdot}05$) in whey protein degradation between strains of the same species were found. Despite the fact that hydrolysis of β-lg by the studied LAB strains was not higher than 18%, some reports showed that allergenicity of this protein could be reduced after being poorly hydrolysed by LAB during fermentation (Bu et al. 2010; Pescuma et al. 2011).

The extensive work done here with a large number of LAB strains and species together with the PCA applied allowed selecting strains of different species exhibiting good growth and proteolytic activity in whey. *Lb. acidophilus* CRL 636 was selected for its good capacity to hydrolyse β-lg and growth in whey; *Lb. delbrueckii* subsp. *bulgaricus* CRL 656 displayed high whey acidification and proteolytic activity, and the most interesting feature the highest hydrolysis of β-lg; and finally *Str. thermophilus* CRL 728 was able to hydrolyse β-lg and rapidly acidify the whey medium. Reconstitution of whey up to 16% (w/v) (RW16) was selected for further investigations since the selected strains *Lb. delbrueckii* subsp. *bulgaricus* CRL 656 and *Str. thermophilus* CRL 728 grew significantly better ($P<0{\cdot}05$) at this RW concentration.

This work showed an extensive study on growth and proteolytic activity of LAB strains during whey fermentation. The results and the statistical analysis applied allowed the discrimination of different properties within and among species and the selection of appropriate strains to further formulate functional whey-based beverages or products.

https://doi.org/10.1017/S0022029912000040 Published online by Cambridge University Press

The authors acknowledge the financial support of CONICET, ANPCyT and CIUNT from Argentina. We thank to Ing. Ricardo Weill from DANONE S.A., Argentina, for generously providing the whey powder used in this study. M. Pescuma is recipient of a post-doctoral fellowship from CONICET, Argentina.

References

Athanasiadis I, Parakevopoulou A, Blenkas G & Kiosseoglou V 2004 Development of a novel whey beverage by fermentation with kefir granules. Effect of various treatments. *Biotechnology Progress* **20** 1091–1095

Bu G, Luo Y, Zhang Y & Chen F 2010 Effects of fermentation by lactic acid bacteria on the antigenicity of bovine whey proteins. *Journal of Science and Food Agriculture* **90** 2015–2020

Church F, Swaisgood H, Porter D & Catignani G 1983 Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *Journal of Dairy Science* **66** 1219–1227

Desmazeaud M & de Roissart H 1994 Métabolisme général des bactéries lactiques. In *Bactéries Lactiques*, pp. 169–207 (Eds. H de Roissart & FM Luquet). Uriage, France: Lorica

Do Carmo A, da Silva D, De Oliveira M, Borges A, De Carvalho A & De Moraes C 2011 Genes involved in protein metabolism of the probiotic lactic acid bacterium *Lactobacillus delbrueckii* UFV H2b20. *Beneficial Microbes* **2** 209–20

Dralić I, Tratnik L & Bozanić R 2005 Growth and survival of probiotic bacteria in reconstituted whey. *Lait* **85** 1–9

Ehn B, Allmere T, Telemo E, Bengtsson U & Ekstrand B 2005 Modification of IgE binding to β-lactoglobulin by fermentation and proteolysis of cow's milk. *Journal of Agriculture and Food Chemistry* **53** 3743–3748

Fiahlo A, Martins L, Donval M, Leitão J, Ridout M, Jay A, Morris V & Sá-Correia I 1999 Structures and properties of gellan polymers produced by *Sphingomonas paucimobilis* ATTC 31461 from lactose compared with those produced from glucose and from cheese whey. *Applied and Environmental Microbiology* **65** 2485–2491

Gallardo-Escamilla F, Kelly A & Delahunty C 2007 Mouthfeel and flavour of fermented whey with added hydrocolloids. *International Dairy Journal* **17** 308–315

Gernignon G, Schuck P & Jeantet R 2010 Processing of Mozzarella cheese wheys and stretchwaters: a preliminary review. *Dairy Science and Technology* **90** 27–46

Gonzalez Siso M 1996 The biotechnological utilization of cheese whey: a review. *Bioresourse Technology* **57** 1–11

Hozer B & Kirmaci H 2009 Functional milks and dairy beverages. *International Journal of Dairy Technology* **63** 1–15

Iametti S, Rasmussen P, Frøkiær H, Ferranti P, Addeo F & Bonomi F 2002 Proteolysis of bovine β-lactoglobulin during thermal treatment in subdenaturing conditions highlights some structural features of the temperature-modified protein and yields fragments with low immunoreactivity. *European Journal of Biochemistry* **269** 1362–1372

Ji T & Hauque Z 2003 Cheddar whey processing and source: I. Effect on composition and functional properties of whey protein concentrates. *International Journal of Food Technology* **38** 453–461

Khalid N & Marth E 1990 Proteolytic activity by strains of *Lactobacillus plantarum* and *Lactobacillus casei*. *Journal of Dairy Science* **73** 3068–3076

Letort C, Nardi M, Garault P, Monnet V & Juillard V 2002 Casein utilization by *Streptococcus thermophilus* results in a diauxic growth in milk. *Applied and Environmental Microbiology* **68** 3162–3165

Liu M, Bayjanov J, Renckens B, Nauta A & Siezen R 2010 The proteolytic system of lactic acid bacteria revisited: a genomic comparison. *BMC Genomics* **11** 1–15

Matar C, Amiot J, Savoie L & Goulet J 1996 The effect of milk fermentation by *Lactobacillus helveticus* on the release of peptides during in vitro digestion. *Journal of Dairy Science* **79** 971–979

Ohmiya K & Sato Y 1969 Studies on the proteolytic action of dairy lactic acid bacteria. VII. Action of intracellular proteases of *Lactobacillus bulgaricus, Lactobacillus helveticus* or *Streptococcus lactis* on casein. *Agricultural and Biological Chemistry* **33** 669–675

Peñas E, Préstamo G, Baeza M, Martínez-Molero M & Gomez R 2006 Effect of combined high pressure and enzymatic treatments on the hydrolysis and immunoreactivity of dairy whey proteins. *International Dairy Journal* **16** 831–839

Pescuma M, Hébert EM, Rabesona H, Drouet M, Choiset Y, Haertlé T, Mozzi F, Font de Valdez G & Chobert J-M 2011 Proteolytic action of *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 656 reduces antigenic response to bovine β-lactoglobulin. *Food Chemistry* **127** 487–492

Pritchard G & Coolbear T 1993 The physiology and biochemistry of the proteolytic system in lactic acid bacteria. *FEMS Microbiology Reviews* **12** 179–206

Schagger H & Von Jagow G 1987 Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry* **166** 368–379

Smithers G 2008 Whey and whey proteins—from 'gutter-to-gold.' *International Dairy Journal* **18** 695–704

Svec P, Drab V & Sedlacek I 2005 Ribotyping of *Lactobacillus casei* group strains isolated from dairy products. *Folia Microbiologica* **50** 223–228

Wood B 1997 *Microbiology of Fermented Foods*. London, UK: Blackie Academic & Professional