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Lactobacillus delbrueckii subsp. *bulgaricus* CRL 454 cleaves allergenic peptides of β-lactoglobulin

Micaela Pescuma [a], Elvira M. Hébert [a], Thomas Haertlé [b], Jean-Marc Chobert [b], Fernanda Mozzi [a,*], Graciela Font de Valdez [a,c,*]

[a] Centro de Referencia para Lactobacilos (CERELA)-CONICET, Chacabuco 145, 4000 San Miguel de Tucumán, Argentina
[b] UR 1268, Biopolymères Interactions Assemblages, INRA, Fonctions et Interactions des Protéines, Rue de la Géraudière, B.P. 71627, 44316 Nantes Cedex 3, France
[c] Cátedra de Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina

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ABSTRACT

Whey, a cheese by-product used as a food additive, is produced worldwide at 40.7 million tons per year. β-Lactoglobulin (BLG), the main whey protein, is poorly digested and is highly allergenic. We aimed to study the contribution of *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 454 to BLG digestion and to analyse its ability to degrade the main allergenic sequences of this protein. Pre-hydrolysis of BLG by *L. delbrueckii* subsp. *bulgaricus* CRL 454 increases digestion of BLG assayed by an *in vitro* simulated gastrointestinal system. Moreover, peptides from hydrolysis of the allergenic sequences V41-K60, Y102-R124, C121-L140 and L149-I162 were found when BLG was hydrolysed by this strain. Interestingly, peptides possessing antioxidant, ACE inhibitory, antimicrobial and immuno-modulating properties were found in BLG degraded by both the *Lactobacillus* strain and digestive enzymes. To conclude, pre-hydrolysis of BLG by *L. delbrueckii* subsp. *bulgaricus* CRL 454 has a positive effect on BLG digestion and could diminish allergenic reactions.

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1. Introduction

Whey is the main by-product of the food industry, the total worldwide production of whey is 40.7 million tons per year (Prazeres, Carvalho, & Rivas, 2012). Whey proteins are used for supplementing several foods due to their low cost and high nutritional value. Whey is a common ingredient of breads, crackers, meat, beverages, sauces, soups and dairy products because of its emulsification capacity, high solubility, gelling, whipping/foaming, viscosity and water binding properties (Johnson, 2000).

Despite the interesting applications of whey, the main whey protein β-lactoglobulin (BLG) is hardly degraded by digestive enzymes because of its rigid structure (Maier, Okun, Pittner, & Lindner, 2006). Moreover, this protein is one of the main milk allergens, which harbours the three very well-known epitopes V41-K60, Y102-R124, C121-L140 and L149-Y162 (Cerecedo et al., 2008; Prioult, Pecquet, & Fliss, 2004; Sélo et al., 1999). Allergy is a growing concern in developed countries where its incidence is high. Although allergy prevention implies mainly removing the allergenic food from the patient's diet, this strategy is not possible in infants who cannot be breastfed. Instead, infants receive a highly hydrolysed milk formula or an amino acid formula free of lactose, since this sugar can be contaminated with milk proteins (Niggemann, Nilsson, & Friedrichs, 2008). Lactose is known to promote colonisation of the gut with beneficial biota mainly composed of lactic acid bacteria (LAB) and bifidobacteria. While amino acid formulas are less nutritive, highly hydrolysed formula have been shown to not be entirely effective in preventing allergy symptoms (Puerta, Diez-Masa, & De Frutos, 2006).

LAB are widely used in the fermented food industry due to their beneficial, technological and health-promoting properties, such as improving the sensorial characteristics of fermented products, prolonging their shelf-life as well as imparting special features to food including probiotic capacity or the release of compounds with health-promoting effects (Das & Goyal, 2012). LAB have an efficient proteolytic system composed of a cell envelope proteinase (which initiates protein degradation), a transport system and several intracellular peptidases. In this respect, certain LAB are able to degrade milk proteins (Do Carmo et al., 2011; Hebert et al., 2008). Moreover, Jędrychowski and Wróblewska (2005) have shown that milk fermentation by LAB could reduce 99% of some

* Corresponding authors. Address: Centro de Referencia para Lactobacilos (CERELA)-CONICET, Chacabuco 145, 4000 San Miguel de Tucumán, Argentina.
E-mail addresses: fmozzi@cerela.org.ar (F. Mozzi), gfont@cerela.org.ar (G. Font de Valdez).

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whey protein allergenicity; however, immunoreactivity of BLG was only slightly reduced. On the other hand, Prioult, Pecquet, and Fliss (2005) reported that hydrolysis of BLG trypsin-digests by a *Bifidobacteria* strain could reduce allergenicity of this protein.

The aim of this study was to evaluate the role of the yoghurt strain *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 454 on BLG digestion and its ability to degrade the main allergenic sequences of this protein.

2. Materials and methods

2.1. Microorganism

The strain *L. delbrueckii* subsp. *bulgaricus* CRL 454 used in this work was 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 skimmed milk containing 0.5% (w/v) yeast extract, 1.0% (w/v) glucose and 10% (v/v) glycerol for a maximum of one year, and at −80 °C for longer periods. All strains of the collection were stored freeze dried.

2.2. Hydrolysis of BLG

2.2.1. Non-proliferating cells

L. delbrueckii subsp. *bulgaricus* CRL 454 was inoculated in a chemically defined medium (CDM) containing 5 mM $CaCl_2$ (Hebert, Raya, & De Giori, 2000) at an initial optical density at 560 nm (OD_{560}) of 0.07 (Spectronic 2000, Bausch & Lomb, Rochester, NY, USA). The CDM was used to avoid inhibition of LAB proteinase activity by small peptides usually present in complex culture media such as MRS. Cells grown in 300 ml CDM were harvested by centrifugation (10,000*g*, 4 °C, for 10 min) at the exponential growth phase (OD_{560} = 0.65), washed twice (the same volume of the initial culture was used) with 0.85% (w/v) saline solution supplemented with 10 mM $CaCl_2$, and suspended to a final OD_{560} of 10 in 100 mM sodium phosphate (pH 7.0) with 5 mM $CaCl_2$.

Cell suspensions were kept at 37 °C for 30 min for amino acid starvation; non-proliferating cells of *L. delbrueckii* subsp. *bulgaricus* CRL 454 were incubated with bovine BLG (3 mg/ml) (ICN Biomedicals, Lot R17384, Eschwege, Germany, isolated from bovine milk) in a cell/protein ratio of 2/1 (v/v). BLG was previously dissolved in 100 mM sodium phosphate (pH 7.0) supplemented with 5 mM $CaCl_2$ and heated at 80 °C for 30 min to simulate industrial heat treatment of whey to avoid microbial contamination of whey-based drink formulations (Fialho et al., 1999; Pescuma, Hebert, Mozzi, & Font de Valdez, 2008). The cell-protein mixture was incubated for 18 h at 37 °C; samples were withdrawn at 2, 4, 8 and 18 h and centrifuged (10,000*g*, 10 min, 4 °C). Proteins present in the supernatant were analysed by Tricine SDS–PAGE, RP-HPLC and LC–MS/MS as previously described (Pescuma, Hebert, Mozzi, & Font de Valdez, 2007; Pescuma et al., 2009).

2.2.2. In vitro simulated gastrointestinal digestion

Hydrolysis of BLG by digestive enzymes was performed in two successive steps. First, BLG or BLG previously hydrolysed by *L. delbrueckii* subsp. *bulgaricus* CRL 454 (BLGh) were incubated with pepsin to mimic gastric digestion and finally, BLG or BLGh were hydrolysed by a mixture of trypsin and chymotrypsin (T/C), this time to mimic pancreatic digestion in the intestine.

2.2.2.1. Peptic digestion. Seventy microlitres of pepsin (2080 U/mg protein, Sigma Chemical CO, MO, USA) at a 1 mg/ml concentration dissolved in 0.2 N HCl was added to 3 ml of BLG or BLGh (1 mg/ml) and incubated at 37 °C during 2 h with agitation (30 rpm). For simulating *in vivo* gastric digestion, the pH of the dispersion was progressively reduced from 7.0 to 2.0 within 2 h by manually adding 80 μl of 1 N HCl every 30 min. Hydrolysis was stopped by raising the pH to 8.0 with 2.0 N NaOH. Samples were withdrawn after 1 and 2 h incubation periods and stored at −20 °C until further analysis by Tricine SDS–PAGE.

2.2.2.2. Pancreatic digestion. Intestinal digestion was carried out by adding to peptic digests, 43 μl of a trypsin (12400 U/mg protein, Sigma) and chymotrypsin (40–50 U/mg protein, ICN, Biomedicals Inc., Illkirch, France) mixture (1/2.3, w/w), at a weight concentration of 2.5–1 mg/ml of BLG or BLGh. Flasks were incubated in a water bath at 37 °C with agitation (30 rpm). Samples were freeze-dried and preserved until further protein analysis by reverse phase high performance liquid chromatography (RP-HPLC) and liquid chromatography coupled to mass spectrometry (LC–MS/MS).

2.3. Reverse phase-high performance liquid chromatography (RP-HPLC) analyses of BLG hydrolysates

RP-HPLC was performed using an Alliance 2695 system (Waters, Millford, MA, USA). A Symmetry C_{18} column (3.9 × 150 mm, 300 Å, 5 μm, Waters, Milford, MA) was equilibrated with an aqueous trifluoroacetic acid (TFA) (0.055%) solution at a flow rate of 0.15 ml/min. For peptide analysis a flow rate of 0.15 ml/min, solvent A (0.055% TFA) and solvent B (80% acetonitrile (v/v) in ultra-pure water, and 0.045% TFA, (v/v)) were used. Elution was performed by applying 0% B from 0 to 5 min, then a linear gradient from 5 to 35 min and 0–100% B from 37 to 40 min.

2.4. Identification of the peptides released from BLG hydrolysates by LC–MS/MS mass spectrometry

Prior to injection, some samples were reduced. For reduction, samples obtained as described above were lyophilized, dissolved in a reduction buffer (50 mM Tris, 10 mM dithiothreitol, 6 M guanidinium chloride, pH 8.5), flushed with nitrogen and incubated at 45 °C for 1 h.

Alkylation of free SH-groups was performed by adding 1 M iodoacetamide to a final concentration of 50 mM. After incubation for 30 min at room temperature in the dark, the reaction was stopped by adding TFA to a final concentration of 0.1% (v/v). Samples were desalted by solid phase extraction (SPE) on a sep-Pak C_{18} cartridge (Waters). After rinsing the cartridge with a TFA (0.11% v/v) solution, 5 ml of sample was loaded on the cartridge and peptides were eluted using a water, acetonitrile, TFA (20/80/0.09, v/v/v) solution. Acetonitrile was evaporated by using a speed vac concentrator.

HPLC was performed on a Waters HPLC system (Waters 616 pump controlled by a Waters 600 controller) coupled to a Finnigan LCQ ion trap spectrophotometer (Finnigan MAT, San Jose, CA). The same column and elution conditions for RP-HPLC were used for the MS/MS analysis. Spectra were acquired in automated MS/MS mode. The scan rate for MS mode was set between 400 and 2000 Da. Peptide identification was performed using the Mascot software (version 2.5, Matrix Science) on the MS/MS ion search mode with the following parameters: enzyme: none; peptide mass tolerance: 1 Da; fixed modification: carbamidomethyl (C); variable modification: oxidation (M).

3. Results

3.1. BLG hydrolysis by LAB

Non-proliferating cells of *L. delbrueckii* subsp. *bulgaricus* CRL 454 were able to degrade BLG (57%) after the first 2 h of incubation;

proteolysis continued up to 70% during the incubation period (18 h) (Fig. 1a). Two main bands with MW between 11.6 and 3.0 kDa were released as determined by Tricine–SDS PAGE and densitometric analysis of electrophoretic bands (Fig. 1a). Samples were analysed by LC–MS/MS in two different manners; by injecting *L. delbrueckii* subsp. *bulgaricus* CRL 454 BLG degraded samples without modifications (to avoid losing small size peptides) and by reversed phase after reduction and carboxymethylation to identify peptides linked by disulphide bridges. Sequence coverage after mass spectrometry analysis was almost complete except for the peptides L1-V3 and V92-L93. Twenty-five peptides originating from BLG degradation were identified by means of LC–MS/MS (Table 1). Forty-four percent of the peptides obtained had glutamine or glutamic acid on their amino-terminus and five of them had alanine on their carboxyl-terminus. Seven peptides were identified as part of the sequence V41-K60 and five of L149-I162, indicating that these protein sequences were more susceptible to hydrolysis than the remaining parts of the protein. In contrast, the fraction K101-S150 was the most resistant to the *L. delbrueckii* subsp. *bulgaricus* CRL 454 proteinase. Nevertheless, this bacterium was able to cleave BLG throughout the protein sequence. The MW of the released peptides, as assessed by LC–MS/MS, were between 561.26 and 3313.7 kDa. Interestingly, *L. delbrueckii* subsp. *bulgaricus* CRL 454 could hydrolyse the main allergenic epitopes of BLG V41-K60, Y102-R124, C121-L140 and L149-I162 (Table 1, Fig. 1b). Although the sequence C121-L140 was truncated in the peptide A111-A132, it could still be observed encrypted in peptide Q120-A142.

3.2. Simulated in vitro digestion

Pepsin could hydrolyse 55.5% of BLG after 2 h of incubation, releasing peptides with MW lower than 9.4 kDa. This high degradation percentage was probably due to the heat treatment applied to BLG before incubation with pepsin. Degradation of BLG by pepsin was higher when it previously hydrolysed by non-proliferating cells of *L. delbrueckii* subsp. *bulgaricus* CRL 454 (BLGh) reaching 83.5% degradation (Fig. 2).

Further hydrolysis of BLG by pancreatic enzymes was carried out; results showed that the protein was completely degraded after 6 h incubation, independently of being previously degraded by the *Lactobacillus* strain (Fig. 3a). However, when BLG was previously cleaved by *L. delbrueckii* subsp. *bulgaricus* CRL 454 (BLGh), hydrophobic peaks were less intense. BLGh peaks appearing between 25 and 32 min retention time were less intense than those of BLG, while only slight differences between hydrophilic peaks of both samples were observed.

Twenty-nine peptides were identified by LC–MS/MS in the BLG hydrolysates obtained with digestive enzymes; most of these corresponded to the amino-terminus of the protein, for which the sequence coverage after LC–MS/MS was complete (between amino acids L1-E89). It is noteworthy that peptides LLFCMENSAEPEQSLV and ALKALPMHIRL, present in the BLG primary structure, were not detected after hydrolysis (Table 2, Fig. 3b). When BLGh was hydrolysed by pancreatic enzymes, 47 peptides comprising almost the entire protein sequence were identified (Table 3, Fig. 3b). The larger amount of peptides corresponded to the BLG sequence

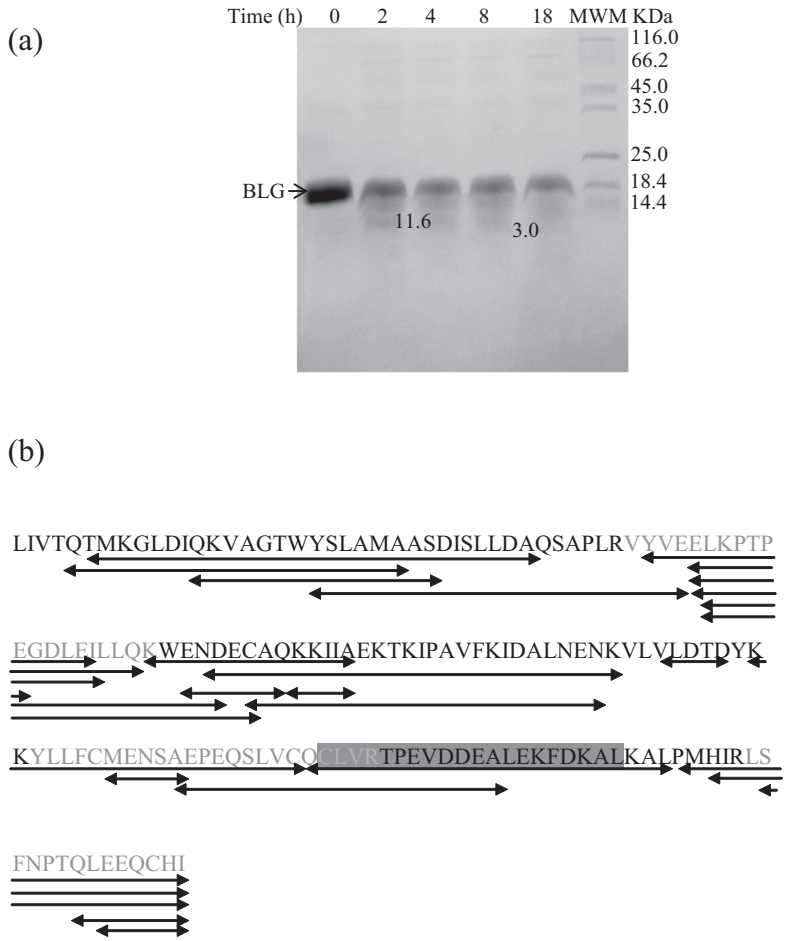

Fig. 1. (a) BLG degradation by *L. delbrueckii* subsp. *bulgaricus* CRL 454 determined by SDS–PAGE. (b) Location of identified peptides (arrows) in the primary structure of BLG released by *L. delbrueckii* subsp. *bulgaricus* CRL 454 proteinase. Allergenic sequences V41-K60, Y102-R124 and L149-I162 appear in grey letters. Epitope C121-L140 is highlighted in dark grey.

Table 1
Peptides identified by LC–MS/MS in BLGh (BLG hydrolysates by *L. delbrueckii* subsp. *bulgaricus* CRL 454).

Observed	Mr Expect	Mr Calc	Delta	Score	Peptide	Peptide identity	Allergenic sequence*
562.00	561.00	561.26	−0.3	17	L.VLDTD.Y	94–98	
567.84	566.83	566.20	0.63	13	C.MENSA.E (M)	107–111	C (102–124)
629.00	627.99	628.43	−0.4	19	A.QKKII.A	67–72	
644.91	643.90	644.22	−0.3	13	L.EEQCHI	157–162	C (149–162)
679.40	678.39	679.21	−0.3	12	W.ENDECA.Q	61–67	
813.76	812.76	812.43	0.33	31	E.ELKPTPE.G	45–51	C (41–60)
1056.25	1055.2	1055.50	−0.2	13	T.QLEEQCHI	155–162	C (149–162)
727.23	1452.4	1452.80	−0.3	44	E.ELKPTPEGDLEIL.L	45–57	C (41–60)
528.82	1583.4	1582.80	0.65	16	I.QKVAGTWYSLAMAAS.D	13–27	
801.8	1601.6	1601.70	−0.1	43	L.SFNPTQLEEQCHI	150–162	C (149–162)
539.82	1616.4	1617.80	−1.4	15	V.YVEELKPTPEGDLE.I	42–55	C (41–60)
554.52	1660.5	1660.80	−0.3	14	Q.KWENDECAQKKII.A	60–72	
848.08	1694.2	1693.90	0.24	53	E.ELKPTPEGDLEILLQ.K	45–59	C (41–60)
1061.63	2121.3	2121.00	0.21	42	M.HIRLSFNPTQLEEQCHI	146–162	C (149–162)
727.23	2178.7	2179.20	−0.5	18	E.LKPTPEGDLEILLQKWENG.E	46–64	C (41–60)
765.70	2294.1	2292.10	1.96	11	L.PMHIRLSFNPTQLEEQCHI	144–162	C (149–162)
1165.37	2328.7	2327.20	1.55	12	T.QTMKGLDIQKVAGTWYSLAMA.A (M)	5–25	
801.80	2402.4	2402.10	0.31	14	S.AEPEQSLACQCLVRTPEVDDEA.L	111–132	C (121–140)
820.75	2459.2	2461.10	−1.9	14	Y.KKYLLFCMENSAEPEQSLVC.Q (M)	100–119	C (102–124)
848.08	2541.2	2540.30	0.98	47	E.ELKPTPEGDLEILLQKWENGEC.A	45–66	C (41–60)
892.53	2674.6	2674.40	0.21	13	C.QCLVRTPEVDDEALEKFDKALKA.L	120–142	C (102–124) N(121–140)
895.40	2683.2	2682.40	0.81	16	W.YSLAMAASDISLLDAQSAPLRVYVE.E	20–44	C (41–60)
910.79	2729.3	2727.50	1.81	18	E.CAQKKIIAEKTKIPAVFKIDALNE.N	66–89	
1048.11	3141.3	3141.70	−0.4	12	E.NGECAQKKIIAEKTKIPAVFKIDALNEN.K	63–90	
1105.90	3314.7	3313.70	1.02	12	V.TQTMKGLDIQKVAGTWYSLAMAASDISLLDA.Q (M)	4–34	

* C indicates that the allergenic epitope has been cleaved and N not cleaved.

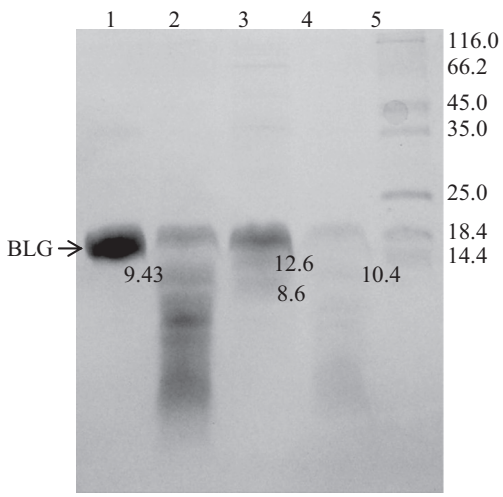

Fig. 2. SDS–PAGE showing BLG hydrolysis. Lanes: (1) BLG; (2) BLG hydrolysed by pepsin; (3) BLG hydrolysed by *L. delbrueckii* subsp. *bulgaricus* CRL 454; (4) BLG hydrolysed by *L. delbrueckii* subsp. *bulgaricus* CRL 454 and pepsin; (5) molecular weight marker.

between the amino acids K41 and Y99, while smaller amounts of peptides were found for the carboxy- and amino-terminal parts of the protein. Twelve peptides (highlighted in grey in Tables 2 and 3) detected after intestinal degradation of BLG and BLGh were found in both samples, suggesting that these peptides were released by the digestive enzymes. Moreover, some of the peptides found were almost identical in both samples (BLG and BLGh) since only one amino acid less was found in the peptides D33-R40 and I84-N90 coming from BLGh (Fig. 3b). On the other hand, 2 peptides previously identified in the BLG hydrolysates by *L. delbrueckii* subsp. *bulgaricus* CRL 454 were also found in BLGh subjected to digestive enzymes (highlighted in dark grey in Tables 1 and 3). Moreover, some peptides, which most likely came from the digestion of peptides released by *L. delbrueckii* subsp. *bulgaricus* CRL 454, were identified as these peptides, and were only found in the BLGh samples after digestion (Table 3). In this respect, peptides K8-K14, G9-K14, V15-Y20 and V15-W19 could be released from Q5-A25 or T4-A34, while peptides K71-K75, K70-A86, T76-A80 and I78-F82 could be part of C66-E89 or N63-N90. Interestingly, peptide Q115-Q120 could be part of A110-A132 and peptide C106-L117 could have been released from K100-C119. Peptides A110-A132 and C106-L117, released from BLG by the studied LAB strain, are part of the allergenic sequence Y102-R124. Moreover, peptides V123-F136, T125-K135 and T125-F136 may have been degraded from Q120-A142, while peptides L149-T154 and N152-I162 could be part of the larger peptides P144-I162 or H146-I162, which contain part of the allergenic epitope L149-I162. In this respect, the allergenic epitope Q121-L140 was also cleaved during *in vitro* digestion of both BLG and BLGh, releasing several peptides with different sequences except for T125-K135, which was found in both samples.

4. Discussion

Protein susceptibility to hydrolysis determines its biological value, meaning the amount of protein that can be used by the body to synthesise its own proteins. Moreover, the degree of hydrolysis of an allergenic protein and the sequences of the peptides released will determine their ability to cross the intestinal barrier and their reactivity (Peyron, Mouécoucou, Frémont, Sanchez, & Gontard, 2006).

Here we demonstrated that the yoghurt strain *L. delbrueckii* subsp. *bulgaricus* CRL 454 was able to degrade the allergenic whey protein BLG and to release mainly hydrophilic peptides. Also, other lactobacilli were capable of releasing mainly hydrophilic peptides as a consequence of milk protein (casein and BLG) hydrolysis (Espeche Turbay, De Giori, & Hebert, 2009; Hebert et al., 2008; Pescuma, Hebert, Mozzi, & Font de Valdez, 2010; Pescuma et al., 2007, 2008). However, only a few reports showed that LAB were able to degrade BLG in its main epitopes and/or to lower the protein immunoreactivity (Kleber, Weirich, & Hinrichs, 2005; Pescuma et al., 2009; Pescuma et al., 2011). In contrast to what has been found for other strains (Pescuma et al., 2009; Pescuma

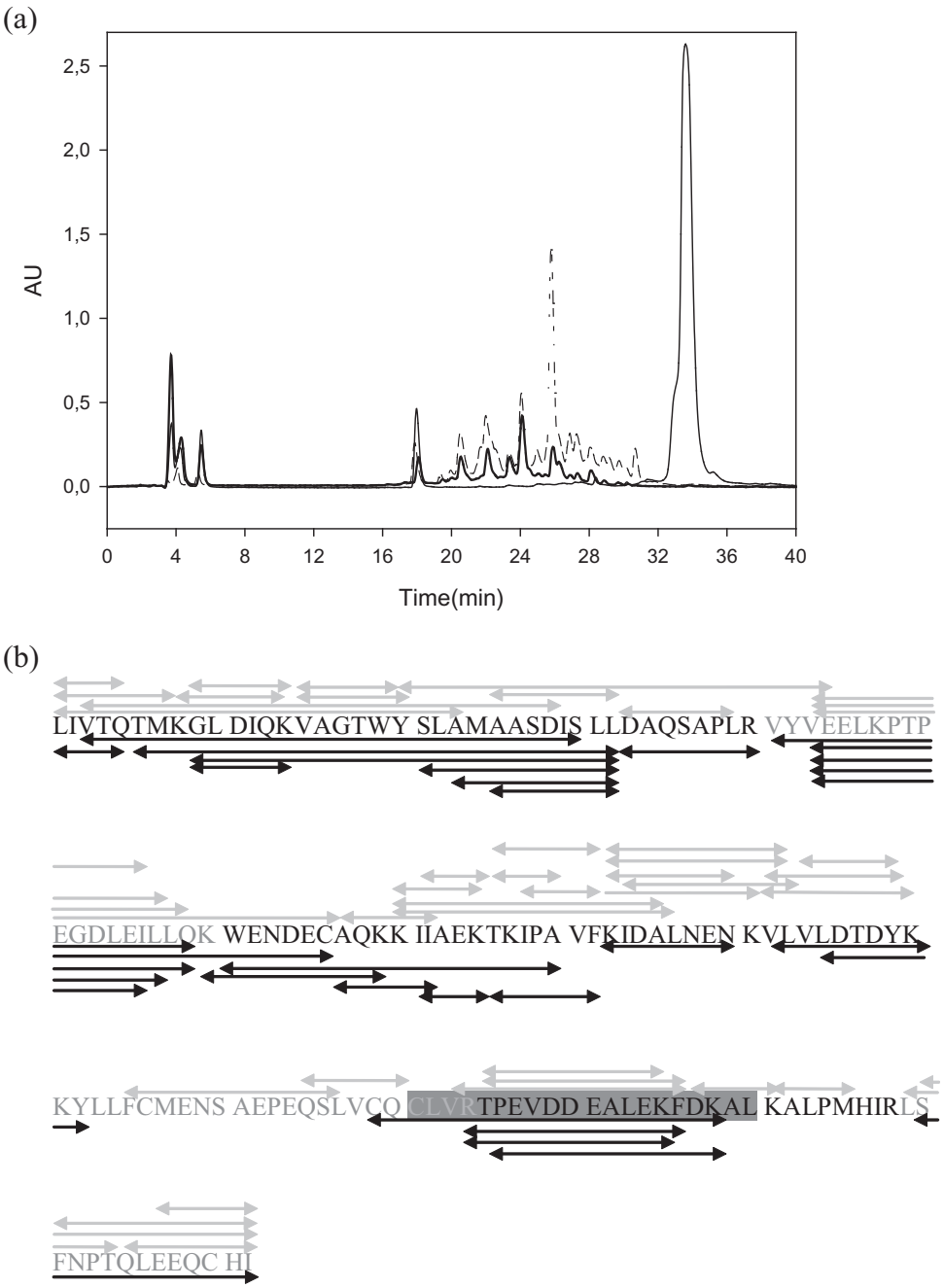

Fig. 3. (a) BLG hydrolysed by simulated *in vitro* digestion (dashed line); BLGh hydrolysed by simulated *in vitro* digestion (solid bold line), BLG control sample (solid line). (b) Location of identified peptides in the primary structure of BLG released by simulated *in vitro* digestion (black arrows), and by *L. delbrueckii* subsp. *bulgaricus* CRL 454 proteinase and simulated *in vitro* digestion (grey arrows). Allergenic sequences V41-K60, Y102-R124 and L149-I162 appear in grey letters. Epitope C121-L140 is highlighted in dark grey.

et al., 2011), the sum of the identified peptides from BLG degradation by *L. delbrueckii* subsp. *bulgaricus* CRL 454 covers almost the entire sequence of this protein. These results provide full information about the sites where the cell-envelope proteinase of *L. delbrueckii* subsp. *bulgaricus* CRL 454 can hydrolyse BLG. On the contrary, the strain *L. delbrueckii* subsp. *bulgaricus* CRL 656 degraded mostly the BLG amino terminal part (Pescuma et al., 2011) while *Lactobacillus acidophilus* CRL 636 cleaved the carboxy-terminal part of the molecule (Pescuma et al., 2009). These findings confirm that microbial hydrolysis of BLG is strain-dependent and that the strain used here would be more suitable for improving the biological value of this protein.

Previous results (Pescuma et al., 2013) showed that *L. delbrueckii* subsp. *bulgaricus* CRL 454 and *L. acidophilus* CRL 636 harboured a mixed PI-PIII type proteinase, while proteinase of *L. delbrueckii* subsp. *bulgaricus* CRL 656 belonged to PI type. Although strains CRL 454 and CRL 636 had the same type of proteinase, their ability to degrade different (milk and vegetable) proteins as well as the peptides released were different. Moreover, *L. acidophilus* CRL 636 had a low capacity to degrade the S-Ala substrate while the *L. delbrueckii* subsp. *bulgaricus* strains displayed high affinity towards it. On the whole, *L. delbrueckii* subsp. *bulgaricus* CRL 454 would be more suitable for improving the biological value of this protein.

Table 2
Peptides identified by LC–MS/MS in BLG hydrolysates obtained by *in vitro* simulated gastrointestinal digestion.

Observed	Mr (Expect)	Mr (Calc)	Delta	Score	Peptide	Peptide Identity	Allergenic sequence*
573.14	572.13	572.35	-0.22	20	LIVTQ.T	1-5	
573.21	572.20	572.35	-0.15	18	K.IIAEK.T	71-75	
673.43	672.42	672.38	0.04	22	K.GLDIQK.V	9-14	
701.11	700.10	699.46	0.63	14	C.AQKKII.A	67-73	
775.26	774.26	774.46	-0.21	23	K.TKIPAVF.K	76-82	
789.19	788.19	788.43	-0.24	25	M.AASDISLL.D	25-32	
857.33	856.33	856.44	-0.11	53	L.DAQSAPLR.V	33-40	
991.25	990.24	990.51	-0.27	36	L.AMAASDISLL.D	23-32	
522.87	1043.73	1043.56	0.17	60	F.KIDALNEN.V	83-90	
522.83	1043.65	1044.51	-0.86	15	V.LDTDYKKY.L	95-102	
1066.69	1065.69	1064.58	1.11	31	K.VLVLDTDYK.K	92-100	
1191.29	1190.28	1190.62	-0.34	21	Y.SLAMAASDISLL.D	21-32	
623.37	1244.20	1244.58	0.15	71	R.TPEVDDEALEK.F	125-135	C (121-140)
624.37	1306.73	1307.55	-0.81	26	Q.KWENDECAQK.K	60-69	
1392.51	1391.50	1391.65	-0.15	50	R.TPEVDDEALEKF.D	125-136	C (121-140)
750.97	1499.92	1499.75	0.17	54	L.VRTPEVDDEALEK.F	123-135	C (121-140)
803.08	1604.14	1602.70	1.44	52	L.SFNPTQLEEQCHI	150-162	C (149-162)
818.52	1635.02	1634.77	0.24	78	R.TPEVDDEALEKFDK.A	125-138	C (121-140)
841.55	1681.09	1680.88	0.21	67	Y.VEELKPTPEGDLEIL.L	43-57	C (41-60)
897.98	1793.94	1793.97	-0.02	84	Y.VEELKPTPEGDLEILL.Q	43-58	C (41-60)
962.13	1922.25	1922.02	0.23	44	Y.VEELKPTPEGDLEILLQ.K	43-59	C (41-60)
972.55	1943.8	1943.01	0.07	25	R.VYVEELKPTPEGDLEILLQ.K	41-59	C (41-60)
818.52	2452.53	2453.11	-0.57	47	V.CQCLVRTPEVDDEALEKFDK.A	119-138	C (102-124) C(121-140)
841.56	2521.64	2522.31	-0.66	16	K.GLDIQKVAGTWYSLAMAASDISLL.D	9-32	C (41-60)
962.13	2883.38	2884.37	-0.99	34	Y.VEELKPTPEGDLEILLQKWENDEC.A	43-66	C (41-60)
972.55	2914.62	2914.48	0.14	16	Q.TMKGLDIQKVAGTWYSLAMAASDISLL.D	5-32	
974.00	2914.62	2914.48	0.14	16	I.VTQTMKGLDIQKVAGTWYSLAMAASDIS.L	3-30	
1065.36	2128.71	2128.12	0.59	15	W.ENGECAQKKIIAEKTKIPA.V	62-80	
841.56	2521.64	2522.31	-0.66	47	A.CQCLVRTPEVDDEALEKFDK.A	119-138	C (102-124) C (121-140)

Peptides identified in both BLG and BLGh samples after *in vitro* simulated digestion appear highlighted in light grey.
* C indicates that the allergenic epitope has been hydrolysed.

As reported by other authors (Picariello et al., 2013; Théolier, Hammami, Labelle, Fliss, & Jean, 2013) and confirmed here as well, BLG was partially degraded by pepsin and greatly susceptible to intestinal degradation (trypsin–chymotrypsin). A highly positive effect on the BLG hydrolysis degree was observed when it was previously incubated with *L. delbrueckii* subsp. *bulgaricus* CRL 454. Several peptides obtained were identical in both BLG and BLGh after *in vitro* digestion; which could be due to two separate break-down pathways as a consequence of incomplete hydrolysis of BLG by *L. delbrueckii* subsp. *bulgaricus* CRL 454 in the BLGh samples. However, not all peptides identified from BLG were found in BLGh hydrolysates when both were degraded by gastrointestinal enzymes; the reason could be the low concentration of some peptides in BLGh samples.

Several health-promoting benefits have been attributed to whey-derived peptides, including antihypertensive, antioxidant, antimicrobial and immuno-modulating effects. These bioactive peptides are encrypted in the protein primary structures and are only active when they are released in the media by proteolytic enzymes or chemical treatments (Fitzgerald & Murray, 2006). In this respect, the antioxidant peptide V92-K100 (Conway, Gauthier, & Pouliot, 2013) was found in both BLG and BLGh samples after being hydrolysed by pepsin, trypsin and chymotrypsin. Interestingly, an additional bioactive peptide (V15-Q20) previously reported to have ACE inhibitory, antimicrobial and immuno-modulating activities (Hernandez-Ledesma, Ramos, & Gomez-Ruiz, 2011; Pellegrini, Dettling, Thomas, & Hunziker, 2001) was found only in BLGh samples after gastric/pancreatic digestion.

Concerning the allergenic epitopes of BLG, the main allergenic sequences V41-K60, Y102-R124 and C121-L140 were truncated in both BLG and BLGh samples when degraded by digestive enzymes. Four of the peptides obtained from the V41-K60 epitope were the same in both samples. However, the peptide 41–59 comprising almost the entire epitope was found only in the BLG hydrolysate and not in BLGh. One peptide (C119-K138) from the Y102-R124 epitope was found in BLG submitted to digestive enzymes, while three peptides containing part of this sequence were detected when BLGh was used. Finally, one peptide coming from the epitope L149-I162 was found in the BLG hydrolysate (S150-I162), while five peptides bearing part of this epitope were detected in BLGh. The results shown here demonstrate that the allergenic peptide Y102-R124 was more resistant to the hydrolysis conditions used in this study.

Different peptide profiles were obtained between BLG and BLGh hydrolysates after trypsin/chymotrypsin digestion, with less intense peaks eluting between 24 and 32 min, probably indicating the presence of smaller size peptides. This result suggests that the pre-treatment of BLG with rationally selected LAB strains could increase protein digestibility, releasing smaller size peptides. In agreement with these results, Ehn, Allmere, Telemo, Bengtsson,

Table 3
Peptides identified by LC–MS/MS in BLGh hydrolysates obtained by *in vitro* simulated gastrointestinal digestion.

Observed	Mr Exp	Mr Calc	Delta	Score	Peptide	Peptide Identity	Allergenic sequence (*)
533.07	523.07	523.30	-0.2	34	K.VAGTW.Y	15-19	
573.25	572.24	572.40	-0.11	21	K.IIAEK.T	71-75	
645.04	644.03	644.20	-0.19	13	L.EEQCH.I	157-161	C (149-162)
696.54	695.53	695.30	0.2	13	K.VAGTWY.S	15-20	
700.97	699.96	699.50	0.5	13	C.AQKKII.A	67-72	
725.07	724.06	724.30	-0.27	26	L.VLDTDY.K	94-99	
775.24	774.23	774.50	-0.23	19	K.TKIPAVF.K	76-82	
789.14	788.14	788.40	-0.24	27	M.AASDISLL.D	25-32	
628.95	1255.90	1256.00	0.16	50	F.KIDALNENKVL.V	83-93	
824.94	1647.90	1647.00	1.05	82	L.VRTPEVDDEALEKF.D	123-136	C (121-140)
841.50	1681.00	1681.00	0.11	61	Y.VEELKPTPEGDLEIL.L	43-57	C (41-60)
898.02	1794.00	1794.00	0.06	75	Y.VEELKPTPEGDLEILL.Q	43-58	C (41-60)
628.95	1883.80	1884.00	-0.32	31	K.KIIAEKTKIPAVFKIDA.L	70-86	
962.17	1922.30	1922.00	0.31	61	Y.VEELKPTPEGDLEILLQ.K	43-59	C (41-60)
870.25	2607.70	2606.00	1.31	15	LIVTQTMKGLDIQKVAGTWYSLA.M	1-23	
962.18	2883.50	2884.00	-0.93	20	T.WYSLAMAASDISLLDAQSAPLRVYVE.E (M)	19-44	
972.52	2914.50	2913.00	1.08	15	I.VTQTMKGLDIQKVAGTWYSLAMAASDI.S (M)	3-29	
702,11	701,1	700,3	0.76	12	L.DAQSAPL.R	33-39	
529,09	528,08	528,3	-0.25	23	K.TKIPA.V	76-80	
559,21	558,21	558,3	-0.11	22	L.KALPM.H	141-145	
573,12	572,11	572,4	-0.25	13	LIVTQ.T	1-5	
574,23	573,22	573,4	-0.13	17	F.DKALK.A	137-141	C(121-140)
673,24	672,23	672,4	-0.15	23	K.GLDIQK.V	9-14	
678,30	677,3	676,3	0.98	19	E.QSLVCQ.C	115-120	
696,19	695,18	695,3	-0.15	13	K.VAGTWY.S	17-21	
701,35	700,34	700,5	-0.11	26	K.KIIAEK.T	70-75	
726,17	725,16	724,3	0.83	35	L.VLDTDYK.K	94-100	
801.31	800.30	800.50	-0.17	37	M.KGLDIQK.V	8-14	
805.16	804.15	804.40	-0.29	29	LIVTQTM.K	1-7	
522.83	1043.60	1044.00	0.08	57	F.KIDALNENK.V	82-90	
623.45	1244.90	1245.00	0.31	55	R.TPEVDDEALEKF.D	125-136	C(121-140)
628.95	1255.90	1256.00	0.16	50	F.KIDALNENKVL.V	82-92	
628.95	1883.80	1884.00	-0.32	31	K.KIIAEKTKIPAVFKIDA.L	70-86	
546.16	545.15	545.30	-0.17	27	K.IPAVF.K	78-82	
678.13	677.12	677.30	-0.22	24	R.LSFNPT.Q	149-154	C (149-162)
784.20	788.20	787.40	0.82	28	K.IDALNEN.K	84-90	
805.16	804.15	804.40	-0.29	29	LIVTQTM.K	1-7	
916.24	915.23	915.50	-0.23	30	K.IDALNENK.V	84-91	
916.27	915.26	915.50	-0.21	26	F.KIDALNEN.K	83-90	
937.22	936.21	936.50	-0.27	26	K.VLVLDTDY.K	92-99	
1056.31	1055.30	1056.00	-0.17	16	T.QLEEQCHI	155-162	C (149-162)
1128.33	1127.30	1128.00	-0.3	23	K.IDALNENVL.V	84-93	
1245.30	1244.30	1245.00	-0.29	39	R.TPEVDDEALEK.F	125-135	C(121-140)
1368.41	1367.40	1368.00	-0.21	47	F.NPTQLEEQCHI	152-162	C (149-162)
1394.50	1393.50	1394.00	-0.05	45	F.CMENSAEPEQSL.V	106-117	C (102-124)
802.10	1602.20	1602.00	0.48	60	L.SFNPTQLEQCHI	150-162	C (149-162)
962.51	2884.50	2883.00	1.11	65	Y.VEELKPTPEGDLEILLQKWENDEC.A	43-66	C (41-60)

Peptides identified in both BLGh and BLGh after *in vitro* simulated digestion samples appear highlighted in dark grey. Peptides detected after *in vitro* simulated digestion of BLG and of BLGh samples appear highlighted in light grey.
(*) C indicates that the allergenic epitope has been hydrolyzed.

and Ekstrand (2005) have shown that whey protein hydrolysis by trypsin generated mainly hydrophobic peptides, while hydrolysis with the strains *Lactobacillus helveticus* 174 and 192 released mostly hydrophilic peptides.

The results obtained in this work have shown that pre-hydrolysis of BLG by *L. delbrueckii* subsp. *bulgaricus* CRL 454 had a positive effect on this protein's digestion, and could also diminish allergic reactions by generating peptides from the three main epitopes with the consequent decrease of the BLG immuno-reactivity. Moreover, bioactive peptides with antioxidant and ACE inhibitory activities may be released by the action of this *Lactobacillus* strain. Interestingly, the entire sequence of BLG could be found in the obtained hydrolysates making possible to identify all the cleavage sites of this protein by using the *L. delbrueckii* subsp. *bulgaricus* CRL

454 cell-associated proteinase. In this study a commercial bovine BLG was used in order to analyse its degradation without interference with other whey proteins, which makes analysis much easier (RP-HPLC, LC–MS/MS). However, in previous studies we have shown that this strain was able to degrade BLG present in whey protein concentrates and in whey (Pescuma et al., 2007; Pescuma et al., 2008). As this LAB species is commonly used in the fermented dairy industry, *L. delbrueckii* subsp. *bulgaricus* CRL 454 is an excellent candidate to be included in the formulation of novel hypoallergenic dairy foods with specific health-promoting properties. Further *in vivo* trials are needed to go forward with these observations.

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