LWT - Food Science and Technology 51 (2013) 183–189

Contents lists available at SciVerse ScienceDirect

LWT - Food Science and Technology

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

Manufacture of a functional fermented milk enriched of Angiotensin-I Converting Enzyme (ACE)-inhibitory peptides and γ-amino butyric acid (GABA)

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ARTICLE INFO

Article history:
Received 22 March 2012
Received in revised form
31 July 2012
Accepted 18 September 2012

Keywords:
Lactic acid bacteria
Fermented milk
Gamma-aminobutyric acid
Bioactive peptide
Antihypertensive activity

ABSTRACT

This study aimed at developing and characterizing a fermented milk with a potentially anti-hypertensive effect due to the concurrent presence of Angiotensin-I Converting Enzyme (ACE)-inhibitory peptides and γ-amino butyric acid (GABA). Preliminarily, lactic acid bacteria strains were screened based on the capacity of releasing ACE-inhibitory peptides and of synthesizing GABA. The most potent RP-FPLC fraction from the milk fermented with *Lactococcus lactis* DIBCA2 had an ACE-inhibitory activity, expressed in terms of IC_{50}, of 5 ± 2 μg/mL, a value which compares well to those reported for the majority of ACE-inhibitory peptides identified in fermented milks. This fraction contained a mixture of six peptides, five of which shared motifs in common with ACE-inhibitory peptides previously reported. *Lactobacillus plantarum* PU11 was selected as the highest producer of GABA, ca. 77.4 mg/kg after 120 h of milk fermentation. Both the strains were selected and used as starters for consecutive milk fermentation. After fermentation, the milk contained both the lactic acid bacteria at the cell density of ca. 8.0 log CFU/mL, had a pH of ca. 4.45, and showed ACE-inhibitory activity (IC_{50} = 0.70 ± 0.07 mg/mL) and a concentration of GABA (ca. 144.5 mg/kg), which were compatible with the dosage for mild antihypertensive effect.

1. Introduction

The term functional foods was first introduced in Japan in the mid-1980s and refers to processed foods containing ingredients that aid specific body functions, in addition to being nutritious (Hasler, 1998). One way for producing functional foods is fermentation using selected microorganisms. The claimed health benefits of fermented functional foods may be exerted through ingestion of either live microorganisms (probiotics) or biogenic compounds (also called biogenics) produced by microorganisms during fermentation (Gobbetti, Di Cagno, & De Angelis, 2010). Biogenics are defined as food components derived from microbial activity, which provide health benefits without directly involving intestinal microbiota (Takano, 2002).

Some of the most investigated groups of biogenics are the bioactive peptides that are released from food proteins as the result of microbial proteolysis during fermentation. Upon oral administration, bioactive peptides may affect different target systems in the body, also depending on their amino acid sequence. The most attractive sub-group of bioactive peptides are Angiotensin-I Converting Enzyme (ACE)-inhibitory peptides. Several *in vivo* studies have demonstrated the antihypertensive effect of ACE-inhibitory peptides contained in dairy products (for a review see Usinger, Ibsen, & Jensen, 2009).

γ-Aminobutyric acid (GABA) is another biogenic compound that deserved a marked interest during the last decade. GABA is a ubiquitous non-protein amino acid involved in neurotransmission. It may induce hypotension, have diuretic and tranquilizer effects (Wong, Bottiglieri, & Snead, 2003) and show anti-tumorigenic activity (Thaker et al., 2005). Hypotensive effect of GABA is based on a mechanism of action which is different from that of ACE-inhibitory peptides: it inhibits noradrenalin release from peripheral sympathetic nerve terminals; this, in turn, inhibits

Abbreviations: Angiotensin-I Converting Enzyme, ACE; γ-amino butyric acid, GABA; Reversed Phase Fast Protein Liquid Chromatography, RP-FPLC; trifluoroacetic acid, TFA; *o*-phthaldialdehyde, OPA; N-[3-(2-furyl)acryloyl]L-phenylalanyl-glycyl-glycine, FAPGG; nano-Liquid Chromatography-Electrospray Ionisation-Mass Spectra/Mass Spectra, nano-LC-ESI-MS/MS; casein, CN.

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http://dx.doi.org/10.1016/j.lwt.2012.09.017

perivascular nerve stimulation and mediates the hypotensive effect (Hayakawa, Kimura, & Kamata, 2002). Oral administration of GABA-enriched fermented milk beverages decreased the blood pressure in rats (Chen, Tsai, & San Pan, 2007; Liu et al., 2011) or in mildly and moderately hypertensive humans (Inoue et al., 2003). Fermentation of milk by selected lactic acid bacteria was exploited for enriching dairy products with GABA (Chen et al., 2007; Liu et al., 2011).

Since the blood pressure lowering effect was demonstrated *in vivo*, some studies were recently focused on the enrichment of dairy fermented beverages with both ACE-inhibitory peptides and GABA (Liu et al., 2011; Minervini, Bilancia, Siragusa, Gobbetti, & Caponio, 2009; Sun et al., 2009). Fermented beverages enriched of both biogenics potentially have a higher anti-hypertensive effect than their counterpart containing only one of the two biogenics. Fermented goat's milk produced with selected multiple starter had an *in vitro* ACE-inhibitory activity of ca. 73%, but showed a too low concentration of GABA, compared to the dosage (1.36 mg/kg of body weight/day), for anti-hypertensive effect (Minervini et al., 2009). In the other studies the presumptive ACE-inhibitory peptides were not identified and the microbiological, rheology and sensory properties of the fermented milks were not characterized (Liu et al., 2011; Sun et al., 2009).

This study aimed at developing and characterizing a fermented milk with a potentially anti-hypertensive effect due to the concurrent presence of ACE-inhibitory peptides and GABA.

2. Material and methods

2.1. Materials

1-(3-mercapto-(2S)-methyl-1-oxopropyl-)-L-proline (captopril), acetonitrile, Angiotensin-I Converting Enzyme (ACE, EC 3.4.15.1) from rabbit lung, N-[3-(2-furyl)acryloyl]L-phenylalanyl-glycyl-glycine (FAPGG), *o*-phthaldialdehyde (OPA), and trifluoroacetic acid (TFA) were from Sigma–Aldrich Co. LLC (St. Louis, MO). MRS, M17, yeast extract and skim milk powder were from Oxoid Limited (Basingstoke, Hampshire, UK). Buffers and ninhydrin for determination of GABA concentration were from Biochrom Ltd. (Cambridge, UK). The following strains used in this study had been previously isolated from cheeses or fermented milks and belong to the Culture Collection of the Department of Biologia e Chimica Agro-Forestale ed Ambientale of Bari University, Italy: *Enterococcus durans* SDCA4, *Lactobacillus acidophilus* 2949, *Lactobacillus casei* ($FC_1$13 and 2749), *Lactobacillus curvatus* (2770 and 2771), *Lactobacillus delbrueckii* ssp. *bulgaricus* (DIBCA3 and $B_{15}Z$), *Lactobacillus helveticus* (PR4 and $B_{26}W$), *Lactobacillus paracasei* PF6, *Lactobacillus plantarum* ($FC_2$10, FN9, PU11, and 1TP), *Lactobacillus rhamnosus* ($FC_3$6, $FC_3$3, and 510), *Lactobacillus sakei* SAL1, *Lactococcus lactis* (DIBCA2 and DIBCA13), *Pediococcus pentosaceus* C_6F_5, and *Streptococcus thermophilus* CR12. Fresh pasteurised cow milk was purchased from Granarolo S.p.A. (Bologna, Italy).

2.2. Selection of strains based on ACE-inhibitory activity and synthesis of GABA

Strains were routinely propagated in MRS or M17 (only *S. thermophilus* CR12, *E. durans* SDCA4 and *Lc. lactis* strains) broth at 30 or 37 °C for 24 h. Twenty-four hours old cultures of lactic acid bacteria were used to inoculate (10 mL/L) reconstituted (100 g/L) skim milk, containing yeast extract (2 g/L). After overnight incubation, pre-cultures in skim milk were used to inoculate fresh pasteurised milk. Before being inoculated, milk was concentrated at 90 °C for ca. 10 min. After concentration, the milk had the following chemical composition: protein 35.3 g/L, fat 39.5 g/L and lactose 52.6 g/L. The pH was ca. 6.75. The milk was added with yeast extract (5 g/L), rapidly cooled to 30 °C, inoculated (10 mL/L), and fermented at 30 °C or 37 °C for 48 h. After fermentation, the pH of each fermented milk was adjusted to 4.6 (if the pH of the fermented milk was above 4.6). The pH 4.6-soluble fractions of the fermented milks were recovered by centrifugation (10,000 *g*, 4 °C, 10 min), filtered through a 0.45 μm pore size filter, and assayed for ACE-inhibitory activity as afterwards described. Fermentation for the synthesis of GABA was carried out under the same conditions. In details, fresh pasteurised and concentrated milk was added with yeast extract (5 g/L) and glutamic acid (20 mmol/L), inoculated (10 mL/L) with an overnight culture, and fermented at 30 °C or 37 °C for 120 h. The kinetics of growth and acidification of the fermented milks were determined according to Servili et al. (2011).

2.3. Manufacture of the functional fermented milk

Fresh pasteurised and concentrated milk was rapidly cooled to 30 °C, added with yeast extract (5 g/L) and glutamic acid (20 mmol/L), and inoculated (10 mL/L, corresponding to an initial cell density of ca. 7 log CFU/mL) with an overnight skim milk pre-culture of *L. plantarum* PU11. After 8 h of incubation at 30 °C, and the pH had reached a value of ca. 6.0, *Lc. lactis* DIBCA2 was also inoculated (10 mL/L, corresponding to an initial cell density of ca. 7 log CFU/mL), and the fermentation was prolonged until 48 h. After fermentation, milk was cooled down to 4–6 °C and stored at 4 °C for 40 days (Fig. 1).

2.4. Purification of peptides from the pH 4.6-soluble fraction of fermented milk

Aliquots of the pH 4.6-soluble fraction of fermented milk were added with TFA (final concentration of 0.5 mL/L), and centrifuged at 12,000 *g* for 10 min. The supernatant was fractionated through RP-

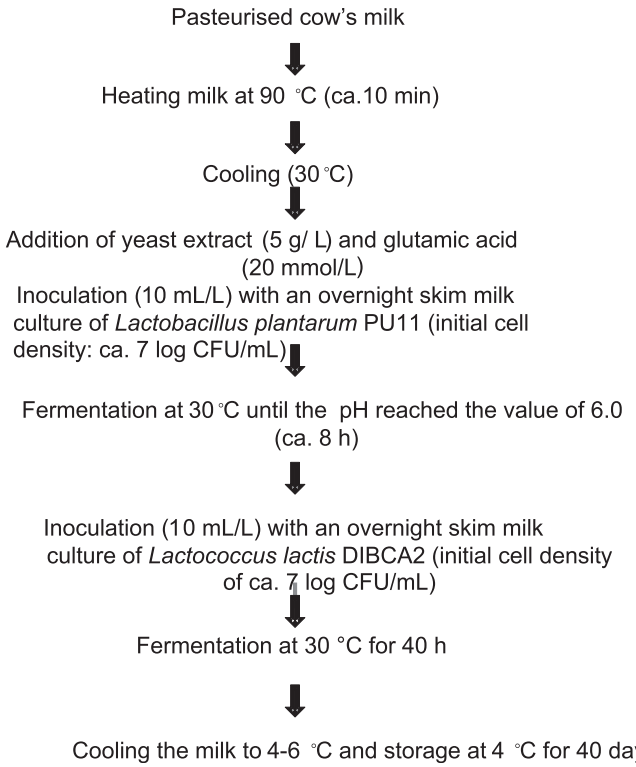

Fig. 1. Protocol for the manufacture of the functional fermented milk started with *Lactobacillus plantarum* PU11 and *Lactococcus lactis* DIBCA2.

FPLC, using the Resource RPC 3 mL column and the ÄKTA FPLC equipment with a UV detector operating at 214 nm (GE Healthcare Bio-Sciences, Uppsala, Sweden). The column was loaded using a 2 mL injection loop. Elution was at a flow rate of 1 mL/min, with a gradient (5–100 mL/100 mL) of acetonitrile in TFA (0.5 mL/L). The concentration of acetonitrile was increased linearly from 5 to 46 mL/100 mL between 16 and 62 min, and from 46 to 100 mL/100 mL between 62 and 72 min. Acetonitrile and water were removed from the collected 2-mL fractions using the vacuum centrifuge (SPD121P SpeedVac Concentrator, Thermo Fisher Scientific Inc., Asheville, NC) and the freeze-dryer (VaCo 10-II, Zirbus Technology GmbH, Bad Grund, Germany), respectively. Freeze dried fractions were dissolved in 600 μL of bi-distilled water and assayed for ACE-inhibitory activity.

The peptide concentration of the fractions was determined by the OPA method elsewhere described (Church, Swaisgood, Porter, & Catignani, 1983).

2.5. Determination of the ACE-inhibitory activity

ACE-inhibitory activity was determined by the method reported first by Holmquist, Bunning, and Riordan (1979), with some modifications. In details, 10 μL of ACE in 10 mmol/L phosphate buffer, containing 0.5 mol/L NaCl, pH 7 (enzyme concentration of 0.05 U/mL), were added to 50 μL of pH 4.6-soluble fraction, RP-FPLC fraction or bi-distilled water (blank) in a micro-cuvette, and incubated at 37 °C for 5 min. Fifty microlitres of FAPGG (0.5 mmol/L in 50 mmol/L Tris–HCl buffer, containing 0.4 mol/L NaCl, pH 7.5) were then added. After incubation (37 °C, 5 min), the absorbance (A_{t0}) of the reaction mixture was read at a wavelength of 340 nm against bi-distilled water. The absorbance (A_{t40}) was read again after incubation of 40 min. The percentage of ACE-inhibitory activity was calculated as follows:

$$\text{ACE inhibitory activity}(\%) = \left(1 - \Delta A_{\text{sample}}/\Delta A_{\text{blank}}\right) \times 100$$

where ΔA_{sample} is $A_{t0} - A_{t40}$ for sample and ΔA_{blank} is $A_{t0} - A_{t40}$ for blank.

ACE inhibitor captopril was used as the reference ACE-inhibitory substance, at the range of concentration of 1–25 nmol/L.

Kinetic constants (K_i) for the inhibition of ACE activity by RP-FPLC fractions were calculated from Dixon plots (Dixon, 1953) and used to determine the IC_{50}, that is the concentration of the ACE-inhibitor needed to inhibit 50% of ACE activity.

To simulate the gastro-intestinal digestion, aliquots (10 μL) of the most active peptide fractions, at inhibitory concentrations, were subjected to sequential hydrolysis by digestive enzymes according to the method described by Pasini, Simonato, Giannattasio, Peruffo, and Curioni (2001). After digestion, samples were freeze-dried, dissolved in 300 μL water and analyzed for ACE inhibitory activity.

2.6. Identification of ACE-inhibitory peptides

Before mass spectrometry analyses, the active fractions were further purified through RP-FPLC. The equipment, the column and the eluent used were the same described in the subsection 2.4. Elution was at a flow rate of 1 mL/min, with a gradient (5–100 mL/100 mL) of acetonitrile in TFA (0.5 mL/L). The concentration of acetonitrile was increased linearly from 5 to 24 mL/100 mL between 6 and 17 min, and from 24 to 30 mL/100 mL between 14 and 44 min. Identification of peptides was carried out by nano-LC-ESI-MS/MS, using a Finningan LCQ Deca XP Max ion trap mass spectrometer (Thermo Electron Co., San Jose, CA) through the nano-ESI interface. According to manufacturer's instrument settings for nano-LC-ESI-MS/MS analyses, MS spectra were automatically taken by Xcalibur software (Thermo Electron Co.), in positive ion mode. MS/MS spectra were processed using the software BioWorks 3.2 (Thermo Electron Co.) generating peaklists suitable for database searches. Peptides were identified using MS/MS ion search of Mascot search engine (Matrix Science Ltd., London, UK) and NCBInr protein database (National Centre for Biotechnology Information, USA). For identification of peptides the following parameters were considered: enzyme: "none"; instrument type: "ESI-trap"; peptide mass tolerance: ±0.1%; and fragment mass tolerance: ±0.5 Da.

2.7. Determination of GABA

The concentration of GABA was determined by a Biochrom 30 series Amino Acid Analyser (Biochrom Ltd.) with a sodium-cation-exchange column (20 by 0.46 cm inner diameter). Sample preparation, analysis and quantification were carried out as elsewhere described (De Angelis et al., 2008).

2.8. Viscosity and sensory analyses

Apparent viscosity of the fermented milks, including a commercial yoghurt (Yogurt intero Mila, Milkon Alto Adige Soc. Agr. Coop., Italy), was determined at 20 ± 1 °C, using a sine wave vibro-viscometer A&D SV-10 (A&D Company, Limited, Tokyo, Japan), at constant frequency of 30 Hz and amplitude of less than 1 mm. The sensory analysis was carried out by a trained panel, familiar with basic sensory evaluation techniques, consisting of 10 members. The following attributes were considered: appearance, aroma, texture, flavour, after taste and overall acceptability. Panellists expressed judgements attributing a score (1 = dislike very much; 2 = dislike; 3 = acceptable; 4 = like; 5 = like very much) for a given attribute as described by Lawless and Heymann (1999).

2.9. Statistical analyses

Three batches of each fermented milk (obtained by three separate fermentation trials) were analyzed in duplicate. Experimental data were subjected to analysis of variance (ANOVA) and pair-comparison of treatment means was achieved using Tukey's procedure at $p < 0.05$, using the software Statistica 7.0 for Windows.

3. Results

3.1. ACE-inhibitory activity

Seven of the lactic acid bacteria strains used did not show appreciable ACE-inhibitory activity during milk fermentation. Overall, fermented milks showed values of IC_{50} ranging between 0.22 ± 0.03 and 1.75 ± 0.03 mg/mL (Table 1). The lowest ($p < 0.05$) values of IC_{50} were found for the pH 4.6-soluble fractions of the milk fermented by *Lc. lactis* DIBCA2 (0.22 ± 0.03 mg/mL) and *L. casei* $FC_1$13 (0.25 ± 0.05 mg/mL), which were selected for further characterization.

The kinetics of growth and acidification during fermentation of the two beverages are shown in Fig. 2A and B. *Lc. lactis* DIBCA2 showed a shorter ($p < 0.05$) latency phase than *L. casei* $FC_1$13 (1.32 ± 0.06 h vs. 1.96 ± 0.08 h), and reached a higher cell density (ca. 9.0 log CFU/mL). Almost 48 h were needed for *L. casei* $FC_1$13 to reach ca. 8.0 log CFU/mL. The maximum growth rate of *Lc. lactis* DIBCA2 and *L. casei* $FC_1$13 was 0.21 ± 0.05 and 0.05 ± 0.02 log CFU/mL h, respectively. Accordingly, *Lc. lactis* DIBCA2 acidified the milk more rapidly than *L. casei* $FC_1$13 (latency phase: 1.01 ± 0.11 h vs.

Table 1
Angiotensin-I Converting Enzyme (ACE)-inhibitory activity (expressed as IC_{50}[a]) of the pH 4.6-soluble fractions from pasteurised and concentrated milk fermented (48 h at 30 or 37 °C) by several lactic acid bacteria strains.

Strain	IC_{50} (mg/mL)[b]
Enterococcus durans SDCA4	0.45 ± 0.04i
Lactobacillus acidophilus 2949	0.73 ± 0.02h
Lactobacillus casei $FC_1$13	0.25 ± 0.05l
Lactobacillus delbrueckii ssp. *bulgaricus* DIBCA3	1.45 ± 0.07c
Lactobacillus delbrueckii ssp. *bulgaricus* $B_{15}Z$	0.78 ± 0.02gh
Lactobacillus helveticus $B_{26}W$	1.46 ± 0.09c
Lactobacillus helveticus PR4	1.02 ± 0.04e
Lactobacillus plantarum $FC_2$10	0.91 ± 0.05f
Lactobacillus plantarum FN9	1.05 ± 0.01de
Lactobacillus rhamnosus $FC_3$3	1.75 ± 0.03a
Lactobacillus rhamnosus $FC_3$6	1.58 ± 0.10b
Lactobacillus rhamnosus 510	0.70 ± 0.03h
Lactobacillus sakei SAL1	1.12 ± 0.07d
Lactococcus lactis DIBCA2	0.22 ± 0.03l
Pediococcus pentosaceus C_6F_5	0.78 ± 0.06gh
Streptococcus thermophilus CR12	0.82 ± 0.04g

[a] IC_{50} is the concentration of peptides needed to inhibit 50% of ACE activity.
[b] Values (mean ± SD, $n = 6$) followed by at least one common letter (a–l) were not significantly different ($p > 0.05$).

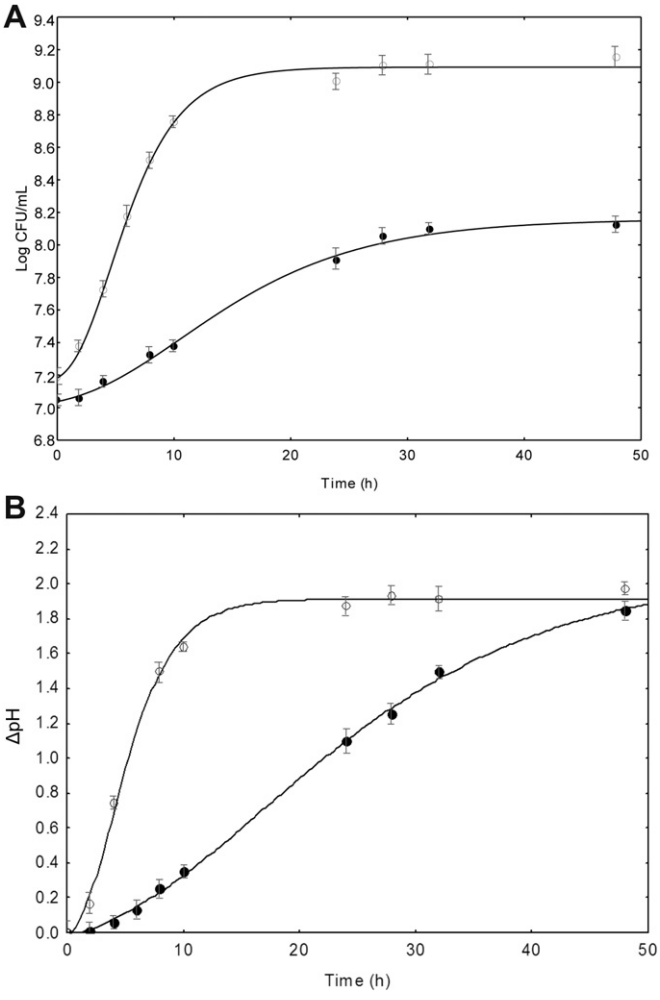

Fig. 2. Kinetics of growth (log CFU/mL) (A) and acidification (ΔpH) (B) of *Lactococcus lactis* DIBCA2 (○) and *Lactobacillus casei* $FC_1$13 (●) during fermentation of pasteurised and concentrated milk at 30 °C for 48 h. Data are the mean of three independent fermentation trials analyzed in duplicate.

2.86 ± 0.09 h; maximum acidification rate: 0.25 ± 0.03 pH/h vs. 0.05 ± 0.01 pH/h). Based on these results, DIBCA2 was chosen as starter for the manufacture of the functional fermented milk and only the fermented milk started with *Lc. lactis* DIBCA2 was further characterized to identify ACE-inhibitory peptides.

Peptides contained in the pH 4.6-soluble fraction were partially purified by RP-FPLC. Table 2 shows the ACE-inhibitory activity, the peptide concentration, and the value of IC_{50} of the most active peptide fractions. IC_{50} ranged from 5 ± 2 to 77 ± 2 μg/mL. Fractions 22 and 24, which eluted at the concentration of acetonitrile between 29.6 and 35.3%, showed the lowest values of IC_{50} (5 ± 2 and 8 ± 3 μg/mL, respectively), which did not significantly ($p > 0.05$) vary after simulated gastro-intestinal digestion (data not shown).

Peptides contained in fraction 22, which had the lowest value of IC_{50} were subjected to a further RP-FPLC step (Fig. 1S), before mass spectrometry analysis. Identification results were subjected to the manual evaluation, as described by Chen, Kwon, Kim, and Zhao (2005), and the validated peptide sequences explained all the major peaks in the MS/MS spectrum. Six peptides, having 4–16 amino acid residues, were identified and their NCBI accession numbers are reported in Table 3. Four peptides derived from β-casein (CN): the neutral LQSW (observed molecular mass of ca. 531 Da) and MFPPQSVLSLSQS (ca. 1408 Da), having the hydrophobic ratio of 50 and 38%, respectively; and LLYQEPVLGP (ca. 1117 Da) and PEQSLVYP (ca. 920 Da), having the total net charge of −1 and the hydrophobic ratio of 40 and 25%, respectively. KPAAVRSPAQILQWQV (ca. 1780 Da) derived from κ-CN, and had a total net charge of +2, and the hydrophobic ratio of 50%. IHAQQK (ca. 723 Da) derived from α_{S1}-CN, and had a total net charge of +2, and the hydrophobic ratio of 33%.

3.2. Synthesis of γ-amino butyric acid (GABA)

Glutamic acid was added to milk because, being the precursor of GABA, it caused an increase of ca. 20% of the concentration of GABA (data not shown). Most of the lactic acid bacteria strains used did not show the capacity to synthesize appreciable levels of GABA. Eight strains synthesized GABA at concentrations ranging from 10.3 ± 0.7 to 77.4 ± 0.7 mg/kg (Table 4). *L. plantarum* PU11 was the best GABA-synthesizing strain. The synthesis of GABA of the previously selected *Lc. lactis* DIBCA2 was 58.7 ± 0.6 mg/kg. The kinetics of growth and acidification of PU11 are shown in Fig. 3. Cell density at 48 h was 8.36 ± 0.12 log CFU/mL, the lag phase lasted 1.05 ± 0.13 h, and the maximum growth rate was of

Table 2
Angiotensin-I Converting Enzyme (ACE)-inhibitory (%) activity, peptide concentration, and IC_{50}[a] values of the most active peptide fractions obtained through RP-FPLC purification of the pH 4.6-soluble fraction of the pasteurised and concentrated milk fermented (48 h at 30 °C) by *Lactococcus lactis* DIBCA2.

Fractions	% ACE-inhibition[b]	Peptide concentration (mg/mL)[b]	IC_{50} (μg/mL)[b]
2	100 ± 0a	10.8 ± 1.5a	77 ± 2a
3	100 ± 0a	9.6 ± 0.9b	64 ± 2b
4	100 ± 0a	4.3 ± 1.0e	43 ± 5c
5	97 ± 2a	2.4 ± 0.8f	48 ± 3c
17	100 ± 0a	8.6 ± 1.8c	21 ± 4d
18	100 ± 0a	5.0 ± 0.9d	12 ± 3e
22	100 ± 0a	1.7 ± 0.6g	5 ± 2fg
24	100 ± 0a	0.8 ± 0.4h	8 ± 3efg
Captopril[c]	–	–	0.003 ± 0.0h

[a] IC_{50} is the concentration of peptide fraction needed to inhibit 50% of ACE activity.
[b] Values (mean ± SD, $n = 6$) in the same column followed by at least one common letter (a–h) were not significantly different ($p > 0.05$).
[c] Captopril was used as positive control.

Table 3
Sequences of peptides contained in fraction 22 of the pH 4.6-soluble fraction of pasteurised and concentrated milk fermented (48 h at 30 °C) by *Lactococcus lactis* DIBCA2.

Sequence[a]	Score	Charge	Observed mass	Calculated mass	Delta	Source protein NCBI accession number
LQSW	26	1	531.265	532.254	−0.989	gi|119388700|qb|ABL74247.1, β-casein 97–100
MFPPQSVLSLSQS	26	3	1408.833	1408.652	0.186	gi|148767917|qb|ABR10906.1, β-casein 114–127
PEQSLVYP	21	2	920.628	920.043	0.585	gi|119388700|ABL74247.1, β-casein 11–18
LLYQEPVLGP	18	2	1117.08	1116.337	0.743	gi|119388700|qb|ABL74247.1, β-casein 148–157
KPAAVRSPAQILQWQV	22	3	1780.483	1780.114	0.369	gi|229416|721588A, para κ-casein 63–78
IHAQQK	21	2	723.749	723.827	−0.078	gi|159793231|ABW98960.1, α_{S1}-casein 3–8

[a] Single-letter amino acid code is used.

0.15 ± 0.03 log CFU/mL h. The difference between the initial and final values of pH was of 2.25 ± 0.15, the lag phase lasted 2.14 ± 0.10 h, and the maximum acidification rate was of 0.12 ± 0.04 ΔpH/h. The concentration of GABA after 48 h of fermentation was not significantly ($p > 0.05$) different from that found after 120 h. Based on the above results, *L. plantarum* PU11 was selected as starter for the manufacture of the functional fermented milk.

3.3. Manufacture and characterization of the functional fermented milk

To enrich the milk with ACE-inhibitory peptides and GABA, *Lc. lactis* DIBCA2 and *L. plantarum* PU11 were used as mixed starters for fermentation. Based on the differences between the maximum growth rates and, especially, on the kinetic of acidifications, the inoculum of the pasteurised and concentrated milk with the two starters was differed (8 h) during time (Fig. 1). Overall, yeast extract (5 g/L) was added to milk to accelerate the growth and acidification of *L. plantarum* PU11. *L. plantarum* PU11 showed a kinetic of growth and related parameters not significantly ($p > 0.05$) different from those found when the strain was used alone (Fig. 4A). When *Lc. lactis* DIBCA2 was used as starter in combination with *L. plantarum* PU11, it showed a significant ($p < 0.05$) decrease of the maximum growth rate (0.10 log ± 0.04 CFU/mL h vs. 0.21 ± 0.05 log CFU/mL h) and a lower ($p < 0.05$) value of cell density (ca. 8.1 log CFU/mL vs. 9.0 log CFU/mL), compared to the cultivation alone. Regarding the kinetic of acidification (Fig. 4B), the difference between the initial and the final values of pH was of 2.30 ± 0.09, the lag phase lasted 2.30 ± 0.10 h, and the maximum acidification rate was of 0.13 ± 0.03 ΔpH/h. After fermentation, the value of pH of the functional fermented milk was ca. 4.45.

The concentration of GABA was ca. 144.5 mg/kg. The pH 4.6-soluble fraction obtained from the functional fermented milk was assayed for ACE-inhibitory activity, showing the value of IC_{50} of 0.70 ± 0.07 mg/mL. After RP-FPLC purification, fraction 22 again showed the highest value of ACE-inhibitory activity, having a value of IC_{50} of 18 ± 1 μg/mL. Nano-LC-ESI-MS/MS analysis of the purified fraction 22 revealed the presence of the same peptides identified in the milk fermented with *Lc. lactis* DIBCA2.

The functional fermented milk had an apparent viscosity value of 120 ± 9 mPa s, which was not significantly ($p > 0.05$) different from that of the commercial yoghurt (110 ± 12 mPa s). Sensory analysis of the functional fermented milk attributed the following scores (scale from 1 to 5): 2.4 ± 0.1, 2.6 ± 0.1, 2.8 ± 0.2, 2.4 ± 0.2 and 2.4 ± 0.1 for appearance, aroma, texture, flavour and after taste, respectively. The panellists observed that the coagulum lacked homogeneity and that a salty note was perceivable in flavour. A score of 2.4 ± 0.2 was given to the overall acceptability.

Table 4
Concentration of γ-amino butyric acid (GABA) of pasteurised and concentrated milk, added with yeast extract (5 g/L) and glutamic acid (20 mmol/L), and fermented (120 h at 30 °C or 37 °C) by several lactic acid bacteria strains.

Strain	GABA (mg/kg)[a]
Lactobacillus casei 2749	24.7 ± 1.0g
Lactobacillus curvatus 2770	31.0 ± 0.6f
Lactobacillus curvatus 2771	51.7 ± 0.6d
Lactobacillus helveticus $B_{26}W$	16.9 ± 1.3h
Lactobacillus helveticus PR4	10.3 ± 0.7i
Lactobacillus paracasei PF6	75.5 ± 0.6b
Lactobacillus plantarum 1 TP	11.1 ± 0.5i
Lactobacillus plantarum $FC_2$10	43.5 ± 0.9e
Lactobacillus plantarum PU11	77.4 ± 0.7a
Lactobacillus rhamnosus $FC_3$6	16.3 ± 1.1g
Lactococcus lactis DIBCA2	58.7 ± 0.6c
Lactococcus lactis DIBCA13	10.1 ± 0.9i

[a] Values (mean ± SD, $n = 6$) followed by at least one common letter (a–i) were not significantly different ($p > 0.05$).

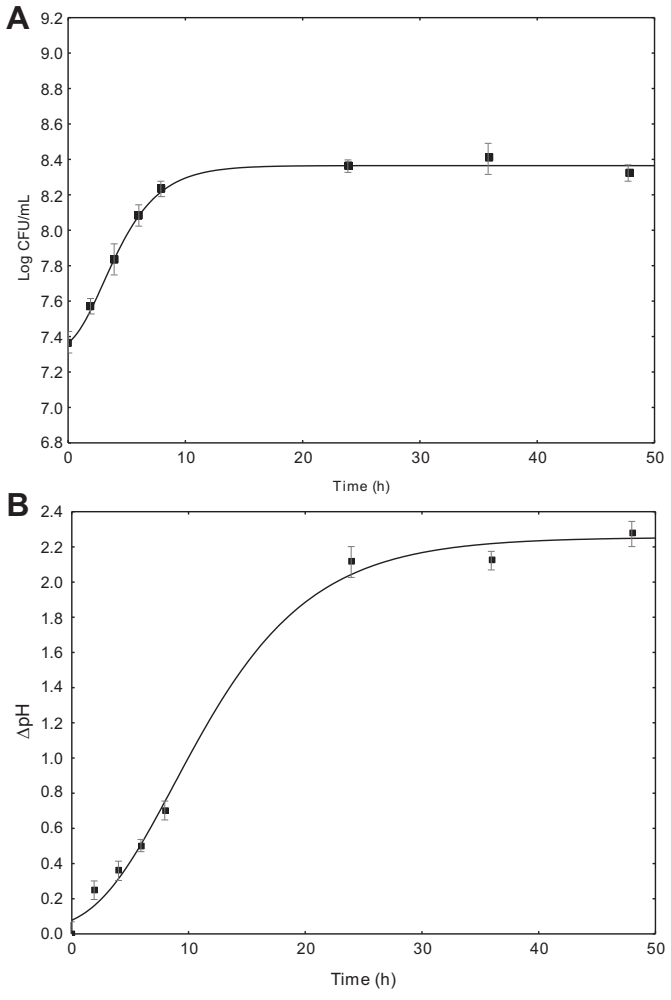

Fig. 3. Kinetics of growth (log CFU/mL) (A) and acidification (ΔpH) (B) of *Lactobacillus plantarum* PU11 during fermentation of pasteurised and concentrated milk at 30 °C for 48 h. Data are the mean of three independent fermentation trials analyzed in duplicate.

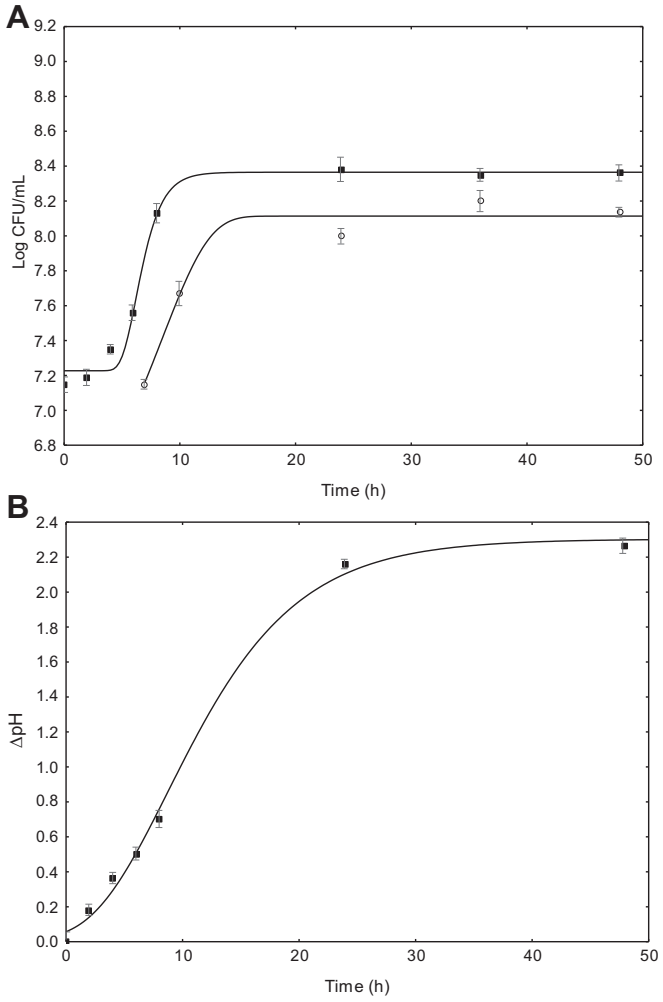

Fig. 4. Kinetics of growth (log CFU/mL) (A) and acidification (ΔpH) (B) of *Lactobacillus plantarum* PU11 (■) and *Lactococcus lactis* DIBCA2 (○) during fermentation of pasteurised and concentrated milk for 48 h at 30 °C. The protocol for the manufacture of the functional fermented milk was according to Fig. 1. Data are the mean of three independent fermentation trials analyzed in duplicate.

The functional fermented milk was stored under refrigerated conditions (ca. 4 °C) for 40 days. After storage, the viability of both the starters maintained almost constant (ca. 8.0 log CFU/mL), the phenomenon of post-acidification was very limited, the ACE-inhibitory activity remained almost constant and the concentration of GABA slightly increased to ca. 165.7 mg/kg. The viscosity and sensory features were confirmed (data not shown).

4. Discussion

Hypertension is considered as one of the major risk factors to develop cardiovascular diseases (arteriosclerosis, stroke and myocardial infraction) and the end-stage of renal disease. Together with the correct lifestyle and diet, the consumption of fermented milks, enriched with targeting functional compounds (e.g., ACE-inhibitory peptides and GABA), could be an important help to prevent hypertension (Usinger et al., 2009).

In the present study, the most common species of dairy lactic acid bacteria were screened for the capacity of generating ACE-inhibitory peptides and synthesizing GABA during milk fermentation. The highest ACE-inhibitory activity was found in the milk fermented with *Lc. lactis* DIBCA2, showing value of IC_{50} comparable to those commonly found in similar dairy products (Pihlanto, Virtanen, & Korhonen, 2010). This species was previously used for enriching fermented milk beverages with ACE-inhibitory peptides (Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000; Nielsen, Martinussen, Flambard, Sørensen, & Otte, 2009). Under the experimental conditions of this study, also *L. casei* $FC_1$13 displayed a considerable ACE-inhibitory activity. The same results were found by other authors (Pihlanto et al., 2010), and *L. casei* 279 or *L. casei* LAFTI L26 were successfully used as adjunct starters for the manufacture of a Cheddar cheese with increased ACE-inhibitory activity (Ong, Henriksson, & Shah, 2007). A mixture of six casein(CN)-derived peptides was identified in the most active fraction from the pH 4.6-soluble fraction of the milk fermented with *Lc. lactis* DIBCA2. Except for the peptide IHAQQK (α_{S1}-CN 3–8), all of them partially share sequences previously related to ACE-inhibition. PEQSLVYP has the four residues at the C-terminus in common with three other ACE-inhibitory peptides (Minervini et al., 2003; Otte, Shalaby, Zakora, Pripp, & El-Shabrawy, 2007; Smacchi & Gobbetti, 1998). LLYQEPVLGP is a part of a larger antihypertensive sequence (β-CN 206–224) liberated by *L. helveticus* CP790 proteinase (Yamamoto, Akino, & Takano, 1994). Besides, the C-terminus (YQEPVLGP) corresponds to the ACE-inhibitory peptides identified both from the *L. helveticus* NCC 2765 CN hydrolysate (Robert, Razaname, Mutter, & Juillerat, 2004) and the milk fermented by *L. rhamnosus* GG (Rokka, Syvaoja, Tuominen, & Korhonen, 1997). PEQSLVYP and LLYQEPVLGP have proline and another hydrophobic residue (Y or L) at the C terminus. Although, the structure–activity relationship of ACE-inhibitory peptides has not yet been clearly established, hydrophobic residues at the three C-terminal positions were often found for ACE competitive inhibitors (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980). The other peptide LQSW (β-CN 97–100) has the three C-terminus residues in common with the longer ACE-inhibitory peptide (β-CN 141–156) liberated through pepsin hydrolysis (Contreras, Carrón, Montero, Ramos, & Recio, 2009). MFPPQSVLSLSQS (β-CN 171–183) contains the sequence (β-CN 172–178) of another ACE-inhibitory peptide previously described (Contreras et al., 2009). The peptide KPAAVRSPAQILQWQV (κ-CN 63–78) has two hydrophobic residues (W and V) at the C terminus and shows, from residue 63 to 68, identity with a longer ACE-inhibitory peptide derived from the same protein upon pepsin hydrolysis (Contreras et al., 2009).

The capacity of synthesizing GABA varied between strains and was related to the acidification of milk. Overall, GABA facilitates cell survival by maintaining the homeostasis of the intracellular pH, through consumption of H^+ ions during decarboxylation of glutamic acid (Small & Waterman, 1998). *L. plantarum* PU11 was the best GABA-synthesizing strain. In a previous study, strains of *L. plantarum* and *L. paracasei* resulted as the most important GABA-synthesizing isolates from 22 Italian cheeses (Siragusa et al., 2007). Fermentation (144 h at 37 °C) of skim milk, containing monosodium glutamate (ca. 60 mmol/L), with a selected strain of *L. plantarum* (NTU 102) resulted in the synthesis of 970 mg/L of GABA (Liu et al., 2011).

Although not always demonstrated (Liu et al., 2011), it may be argued that the antihypertensive activity of a beverage containing both ACE-inhibitory peptides and GABA would be higher than that containing just one of the two biogenics. Since a unique strain that combines both the activities has to be regarded as an exception (Tung, Lee, Liu, & Pan, 2011), a protocol that used both *Lc. lactis* DIBCA2 and *L. plantarum* PU11 was set up to enrich the milk with ACE-inhibitory peptides and GABA. Compared to fermented milks started with single strain, the combined use of both the selected starters showed: (i) the slightly lower cell density of *Lc. lactis* DIBCA2, which was probably due to delayed inoculum (8 h); (ii) the increase (ca. three-fold) of the IC_{50} of ACE-inhibitory activity, which

always corresponded to physiological values (Pihlanto et al., 2010); (iii) and the increase (ca. two-fold) of the concentration of GABA. Notwithstanding the peptides identified in the most active fraction of the milk fermented with *Lc. lactis* DIBCA2 and *L. plantarum* PU11 corresponded to those found in the milk fermented with DIBCA2 only, the lower ACE-inhibitory activity was probably due to some extent hydrolysis, through the peptidases of *L. plantarum* PU11, of peptides other than those identified and contributing to the overall activity of the fermented milk. Compared to the milk fermented with *L. plantarum* PU11 alone, the higher level of GABA was attributed to: (i) stimulation of GABA synthesis through the proteolytic activity of *Lc. lactis* DIBCA2 (Chen et al., 2007; Sun et al., 2009); and (ii) to the complementary synthesis of GABA by *Lc. lactis* DIBCA2. One daily intake (ca. 125 g) of the milk fermented with *Lc. lactis* DIBCA2 and *L. plantarum* PU11 would contain ca. 18 mg of GABA. The daily administration (total of 12 weeks) of 100 g of milk fermented with *L. casei* strain Shirota and *Lc. lactis* YIT 2027, which contained 10–12 mg of GABA, decreased the blood pressure of hypertensive individuals (Inoue et al., 2003).

Although the apparent viscosity was comparable to that of the commercial yoghurt, the functional fermented milk was barely acceptable to panelists. This could be attributed to the length of the fermentation, which was much higher than that used for making yoghurt. The addition of 20 mmol/L glutamic acid caused the salty note of the fermented milk. Nevertheless, the use of flavouring agents and/or sweeteners is a very common practice to enhance the sensory features of functional fermented milks (Allgeyer, Miller, & Lee, 2010).

In conclusion, this study reported the enrichment of milk with both ACE-inhibitory peptides and GABA through fermentation with *Lc. lactis* DIBCA2 and *L. plantarum* PU11. The resulting functional fermented milk would have potential application for the management of mild hypertension.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi:10.1016/j.lwt.2012.09.017.

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