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Determination of optimal conditions for γ-aminobutyric acid production by *Lactococcus lactis* ssp. *lactis*

Catherine Gardner-Fortier [a,b], Daniel St-Gelais [a,b,*], Claude P. Champagne [a,b], Jean-Christophe Vuillemard [b]

[a] Food Research and Development Centre, Agriculture and Agri-Food Canada, 3600 Casavant Boulevard West, Saint-Hyacinthe, Quebec, Canada J2S 8E3
[b] Institut sur la nutrition et les aliments fonctionnels, Centre de recherche STELA, Université Laval, Quebec City, Quebec, Canada G1V 0A6

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ABSTRACT

The biocompatibility of nine proteinase-negative strains of *Lactococcus lactis* ssp. *lactis* with the ability to produce γ-aminobutyric acid (GABA) and four proteinase-positive (Prt^+) strains of *Lactococcus lactis* ssp. *cremoris* under simulated Cheddar cheese manufacturing conditions resulted in the selection of the Prt^+ strain *L. lactis* ssp. *cremoris* W62 for mixed cultures with the nine GABA strains. Of the nine mixed cultures inoculated in milk enriched with glutamate, two blends produced between 7.0 and 7.5 mg of GABA per 100 mL of milk. The effect of pH, salt-to-moisture (SM) ratio, and glutamate concentration on the production of GABA was evaluated using cheese slurry models. The production of GABA increased with glutamate concentration but was lower for a SM level of 4.5%. Low pH was the key factor for GABA production. Combining the optimal factors (pH 4.8, 3.0 mg glutamate, SM 3.0%) gave 23 mg GABA per 30 g of cheese curd.

1. Introduction

Lactic acid bacteria used in the manufacture of Cheddar cheese are usually strains of *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* selected for their capacity to acidify milk and produce aroma compounds. Among lactococci, some are non-proteolytic proteinase-negative (Prt^-) variants. In milk, the Prt^- variants cannot grow, or stop growing, in the absence of free amino acids and peptides. However, in mixed cultures containing proteinase-positive (Prt^+) strains with the ability to hydrolyse caseins into peptides and amino acids (Thomas & Prichard, 1987), the Prt^- strains are able to grow. Mixed cultures of Prt^+/Prt^- strains are nevertheless used in the cheese industry and thus modulate the acidity and flavour of the curd (Juillard, Furlan, Foucaud, & Richard, 1996; Stadhouders, Toepoel, & Wouters, 1988).

However, some lactic acid bacteria can produce metabolites that have an antagonistic effect against other strains. Therefore, creation of a starter formulation requires prior knowledge of the interactions among the strains to prevent imbalances during their growth in milk and during cheese ripening, which could lead to undesirable changes in cheese flavour and texture (Stadhouders, 1986). Studies on the biocompatibility among strains of mesophilic or thermophilic starters have been reported previously (Juillard, Furlan, Foucaud & Richard, 1996). An automated spectrophotometry technique, based on microplate readers, has commonly been used to study the kinetics of bacterial growth (Begot, Desnier, Daudin, Labadie, & Lebert, 1996; Dalgaard, Ross, Kamperman, Neumeyer, & McMeekin, 1994) as well as to reveal the presence of growth factors or inhibitory agents within a medium (Skyttä, Haikara, & Mattila-Sandholm, 1993). More recently, this technique has been used to study the interactions between mesophilic and thermophilic lactic acid bacteria (Champagne, Gagnon, St-Gelais, & Vuillemard, 2009).

Among the strains of lactic acid bacteria that have been isolated and characterised, some have demonstrated the ability to produce γ-aminobutyric acid (GABA) (Lacroix, St-Gelais, Champagne, Fortin, & Vuillemard, 2010; Nomura, Kimoto, Someya, Furukawa, & Suzuki, 1998; Nomura, Kimoto, Someya, & Suzuki, 1999a). This acid is a neurotransmitter that is well known for its hypotensive effect on the central nervous system and has been the subject of studies in humans (Hayakawa et al., 2004; Stanton, 1963). It has been shown that administering fermented milk containing GABA to hypertensive patients results in a significant decrease in blood pressure (Inoue et al., 2003; Kajimoto et al., 2004). Characterisation of glutamate decarboxylase, the enzyme responsible for converting glutamate into GABA, has pointed to the effect of certain factors

* Corresponding author. Tel.: +1 450 768 3321.
E-mail address: daniel.st-gelais@agr.gc.ca (D. St-Gelais).

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that promote production of GABA. Some of these parameters are low pH (below 5.0), glutamate concentration, absence of oxygen, and presence of NaCl (Cotter & Hill, 2003; Nomura et al., 1998, Nomura et al., 1999b).

Lacroix et al. (2010) studied the characteristics of 13 old-style cheese starters collected in 1968 from various Canadian cheese factories. Approximately 360 lactococci strains were isolated from these undefined old-style starters. Among these strains, nine Prt^- strains of *L. lactis* ssp. *lactis* demonstrated the ability to produce GABA (Lacroix, 2008). To design a GABA-producing mixed-strain starter, the aim of this study was to use automated spectrophotometry to determine the level of biocompatibility of the nine Prt^- strains of *L. lactis* ssp. *lactis* that produce GABA and some commercial Prt^+ strains of *L. lactis* ssp. *cremoris*. Additionally, the impact of certain ripening parameters, such as pH, salt-to-moisture (S/M) ratio, and glutamate concentration on the production of GABA in cheese slurry models was studied.

2. Materials and methods

2.1. Preparation of starters

Four commercial Prt^+ strains of *L. lactis* ssp. *cremoris* (LL074, LL225, and LL390: DSM Food Specialties Inc., Parsippany, NJ, USA; and W62: Wisby, Danlac, Airdrie, AB, Canada) and nine Prt^- strains of *L. lactis* ssp. *lactis* (Université Laval, Agriculture and Agri-Food Canada lactic acid bacteria culture collection) were used in this study. The GABA strains (ULAAC-A13, ULAAC-A23, ULAAC-H02, ULAAC-H12, ULAAC-H13, ULAAC-H15, ULAAC-H20, ULAAC-H24, and ULAAC-H27, referred to by the last three characters of their names) were isolated from old-style cheese starters that had been collected from Canadian cheese factories (Lacroix, 2008) and were selected for their capacity to produce GABA in fermented milk (Lacroix et al., 2010).

Each lactic acid strain was kept at −20 °C in reconstituted skim milk (20% dry matter) to which 5% (w/v) sucrose and 0.35% (w/v) ascorbic acid were added. Working cultures were obtained after two transfers into autoclaved (110 °C, 10 min) 12% (w/v) reconstituted skim milk. For the first transfer for all strains, the inoculum size was 1.0% (v/v), and the incubation was performed at 21 °C for 16 h. For the second transfer for all strains, the inoculum size was 1.5% (v/v), and the incubation was performed at 21 °C for 15 h. For the nine Prt^- *L. lactis* ssp. *lactis* strains, the reconstituted skim milk was supplemented with 0.2% (w/v) yeast extract (BD-Difco Laboratories, Detroit, MI, USA).

After the second subculture, a bacterial count was performed on M17 agar plates (CM0817, Oxoid Ltd., Basingstoke, Hampshire, UK). Samples of the culture were diluted in 0.1% peptone water containing 3 g of glass beads to break the chains of lactococci (St-Gelais, Roy, & Haché, 1992), and the plates were incubated anaerobically at 30 °C for 48 h. This step was repeated three times.

2.2. Biocompatibility test

2.2.1. Preparation of cell-free wheys

The cell-free wheys (CFWs) were prepared as follows. For each strain, test tubes containing 40 mL of commercial microfiltered and pasteurised skim milk (Lactancia PürFiltre, Parmalat, Victoriaville, QC, Canada) adjusted to 32 °C were each inoculated with a working strain to obtain an initial population of 1.0×10^7 cfu mL^{-1}. After inoculation, 0.01% (w/v) of double-strength rennet (CHY-MAX Extra, Chr. Hansen's Laboratory LDT, Mississauga, ON, Canada) was added. The contents were blended by carrying out tube inversions, and the tubes were then placed in a temperature-programmable water bath (VWR Scientific, Bridgeport, NJ, USA). The temperature profile of the incubation, simulating cheddar cheese production, was described by Champagne et al. (2009).

At the end of incubation, the tubes were cooled in an ice-water bath and centrifuged at 4470 × *g* for 30 min at 4 °C (Centra GP8R IEC centrifuge, ThermoFisher Scientific, Nepean, ON, Canada), and the supernatant (whey) was recovered. All supernatants were adjusted to pH 6.5, treated to eliminate phosphate minerals, and sterilised as described by Champagne et al. (2009). The pH-neutralised cell-free supernatant extracts were kept at 4 °C until use.

Control milk samples without lactic cultures were acidified to pH 5.2 with 0.75% (w/v) glucono-δ-lactone (GDL) (Sigma, Steinheim, Germany) and coagulated with rennet.

2.2.2. Automated spectrophotometry assays

Each working strain of *L. cremoris* and *L. lactis* was used to inoculate M17 broth (1% v/v). After 15 h of incubation at 21 °C, the M17 cultures were centrifuged at 4470 × *g* for 30 min at 4 °C (Centra GP8R IEC centrifuge), and the cell pellet was washed twice in a 0.9% (w/v) NaCl solution to eliminate M17 broth nutrients. Finally, the cell pellets were resuspended in a 0.9% NaCl solution to adjust the bacterial population to 1.0×10^8 cfu mL^{-1} for all strains.

Subsequently, 200 μL of each neutralised cell-free supernatant extract (*L. cremoris* and *L. lactis*) was randomly distributed, in duplicate, into the wells of a microplate (Honeycomb, Labsystems, Helsinki, Finland) and inoculated with 20 μL of each cell suspension (*L. cremoris* and *L. lactis*) to obtain a final population of 1.0×10^7 cfu per 220 μL. The microplates were then placed in a Bioscreen C unit (Labsystems) and incubated at 30 °C for 24 h. The Bioscreen system was set to take optical density (OD) readings (600 nm) of each well every 15 min after the plate had been shaken for 10 s at the high level. Three independent assays were carried out. The time–OD curves were typical of bacteria growth curves, with lag, exponential, and stationary phases. Each curve was modelled according to the following sigmoid equation:

$$OD = \frac{a-d}{1+\left(\frac{t}{c}\right)^b} \times d \quad (1)$$

where OD = optical density, a = initial OD, b = slope coefficient, c = symmetric parameter, d = maximum OD (OD_{max}), and, t = time (h). The first and second derivatives of this equation are used to determine, respectively, T_{lag}, which is defined as the time when OD indicates an increase by 0.01 units, and μ_{max}, which is the maximum rate at the inflexion point. An example of modelling curves is presented in Fig. 1. In this figure, the raw data are also presented but only values obtained at every 2 h were used to improve the quality of the presentation. The R^2 values for the 135 curves (9 GABA strains × 5 cell-free whey × 3 replicates) were all above 0.98. This indicated a very good fit between the predicted and experimental data.

2.3. Production of GABA in milk

To evaluate the capacity of lactococci to produce GABA in milk, 10 mL of sterilised 12% (w/v) reconstituted skim milk enriched with 1.47 mg mL^{-1} of glutamic acid (L-glutamic acid, Sigma) were poured into a 20 mL headspace vial (Agilent Technologies, Palo Alto, CA, USA). Each vial was inoculated at a rate of 1% (v/v) with each working lactococcal strain. The vials were sealed with a 20 mm aluminum crimp cap equipped with a PTFE/Si septum (Agilent Technologies) and incubated at 30 °C for 5 d. After incubation, the vials were centrifuged at 5000 × *g* for 20 min at 25 °C (Sorvall RC-5B Refrigerated Superspeed centrifuge, GMI Inc., Ramsay, MN, USA).

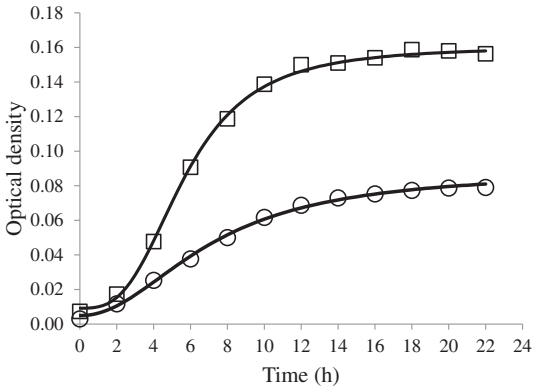

Fig. 1. Optical density curves obtained for the H13 γ-aminobutyric acid strain in the LL225 (○) and W62 (□) cell-free whey. (Symbols represent raw data, whereas lines were obtained with a curve-fitting equation). Although readings were taken every 15 min to carry out a shaking step and prevent cell sedimentation, for reasons of clarity, only the OD data obtained at the 2 h intervals were used to prepare the figure.

Each supernatant was poured into a cryovial and kept at −20 °C until analysis.

2.4. Cheese slurry test

2.4.1. Lactococcal strains

For the cheese slurry test, the W62-A23 and W62-H13 mixed strains were selected for their combined growing ability as determined by the biocompatibility test. Additionally, the A23 and H13 Prt^- strains produced a high concentration of GABA in fermented milk. These strains were prepared as described above.

2.4.2. Production of cheese powder

Cheese powder was obtained from the manufacture of starter-free cheese curd as described by Lacroix et al. (2010) with modifications. About 400 L of whole milk was pasteurised at 72 °C for 20 s before being poured into a cheese vat and brought to 30 °C. A 33% (w/v) calcium chloride solution was added to the milk (20 mL per 100 L of milk). After 30 min, a solution consisting of 1.35 kg of GDL (Aldrich, St. Louis, MO, USA) and 80 mL of double-strength rennet (Maxiren, Gist Grocades, Seclin, France) prepared in 5.33 L of water was added to the milk. The milk was agitated for 1 min after renneting and left to coagulate for 30 min. Cooking of the coagulum began immediately after cutting and consisted of increasing the temperature under gentle agitation from 30 °C to 38 °C over a period of 30 min. Once the final temperature was reached, it was maintained for 30 min, and the whey was then drained (pH around 5.70). The resulting unsalted curd was placed in square moulds covered with cheesecloth and pressed at 300 kPa for 2 h at room temperature. After milling, the curd was frozen at −32 °C before lyophilisation. The lyophilised starter-free cheese was then reduced to a powder with a disc grinder adjusted to the finest grind (GlenMills Inc., Clifton, NJ, USA). The cheese powder (500 g portions) was packed in plastic bags, irradiated at 5 kGy for 2 h to reduce microbial contamination, and kept frozen (−32 °C) until use.

2.4.3. Preparation of cheese slurries

Cheese slurries simulating Cheddar cheese composition were used to determine the effect of two S/M ratios (S/M = 3.0 and 4.5%), two glutamate concentrations (GLU = 1.0 and 3.0 mg g^{-1}), and three pH levels (4.8, 5.1, and 5.4) on the production of GABA. Cheese slurries were prepared as described by Lacroix et al. (2010) with some modifications. The moisture content targeted in all cheese slurries was 38%. To obtain the targeted pH levels (4.8, 5.1, and 5.4) and to maintain these pH values during ripening, 33 mL of citrate-phosphate buffer (0.1 M $C_6H_8O_7$ and 0.2 M Na_2HPO_4) was used, with 3.5, 2, and 1 g of GDL added, respectively (Gomori, 1955). Then, 58.5, 60, and 61 g of cheese powder, respectively, was dispersed in the pH-controlled solutions. To obtain S/M ratios of 3.0 and 4.5%, 1.14 and 1.71 g of NaCl was added to 32.8 and 32.3 g of the GDL buffer solutions, respectively, that had previously been sterilised through a 0.2 μm filter (Analytical filter unit, 150 mL, Thermo Scientific, Rochester, NY, USA). Finally, each preparation was adjusted to the final glutamate concentration (1 and 3 mg g^{-1}, respectively). Thereafter, all cheese slurries were inoculated with 1.3 g of the W62 strain (final concentration of 1.3×10^8 cfu mL^{-1}) and 2.7 g of the A23 or H13 GABA strain (final concentration of 2.7×10^8 cfu mL^{-1}). The final preparations (100 g) were put in sterile plastic bags and homogenised for 3 min at 25 °C (Stomacher, Seward Ltd., London, UK). The homogenised cheese slurries were transferred aseptically into 120-mL sterilised amber glass bottles with polypropylene caps with PTFE liners (EP Scientific amber straight-sided jars, Fisher Scientific, Nepean, ON, Canada). The headspace was flushed with filtered nitrogen. Cheese slurries without starter (starter replaced with sterilised deionised water) were used as the control. All cheese slurries were incubated at 30 °C for 10 d. Samples were aseptically taken after 1 and 10 d.

2.5. GABA and glutamate analyses

The concentration of GABA in milk was determined using milk supernatant samples. In cheese slurries, the concentrations of GABA and glutamate were determined in the water soluble nitrogen extract (WSN). After extraction (Kuchroo & Fox, 1982), the WSN solutions were centrifuged at 5000 × *g* for 30 min at 4 °C (Sorvall RC-5B Refrigerated Superspeed centrifuge, GMI Inc.). The supernatants were filtered with a 0.45 μm filter (PVDF, SF5511-06 Canadian Life Science, Peterborough, ON, Canada). Each filtered supernatant was poured into a cryovial and kept at −20 °C until analysis.

Derivatisation of milk and cheese supernatant samples was carried out with the GC Physiological Amino Acid Analysis EZ:faast kit (Phenomenex, Torrance, CA, USA) following the manufacturer's recommended procedure. For the determination of GABA, derivatised samples were analysed by gas chromatography–mass spectrometry (GC–MS) (GC MS 5973 Network Mass Selective Detector, Agilent Technologies) with the flame ionisation detector port connected to a headspace sampler (G1888 Network Headspace Sampler, Agilent Technologies). Then, 2 μL of the sample was injected into a Varian Factor Four Capillary Column (Variant Inc., Mississauga, ON, Canada) with a split ratio of 1:10 and helium as the carrier gas (2.0 mL min^{-1}). The temperature of the oven program began at 35 °C for 1 min and increased to 200 °C at a rate of 6 °C min^{-1}. After 3 min, the temperature was increased further to 250 °C at a rate of 10 °C min^{-1}. The GABA was identified by comparing its spectral mass to the National Institute of Standards and Technology Mass Spectral Database of 1998.

For the determination of glutamate, derivatised samples were analysed with a gas chromatograph (GC 6890N, Agilent Technologies, Santa Clara, CA, USA) with a flame ionisation detector port. Then, 2 μL of the sample was injected into a Zebron ZB-AAA GC column (Phenomenex) with a split ratio of 1:15 and helium as the carrier gas (1.5 mL min^{-1}). The temperature of the oven program began at 110 °C and increased to 320 °C at a rate of 32 °C min^{-1}.

2.6. Microbiological analysis

The populations of *L. cremoris* and *L. lactis* in cheese slurries were determined on Reddy agar medium (Reddy, Vedamuthu,

Washam, & Reinbold, 1972), on which it is possible to distinguish between *L. cremoris* and *L. lactis*. Cheese slurries (11 g) were diluted in 99 mL of 0.1% peptone water and homogenised using a Stomacher (Model 400, Seward Ltd.). To cause disintegration of long chains of lactococci, 3 g of 4-mm solid-glass beads were used in all subsequent dilution bottles, which were vigorously shaken 40 times before inoculation on Reddy agar medium (St-Gelais et al., 1992). The Reddy agar plates were incubated anaerobically at 30 °C for 48 h. White colonies were identified as GABA *L. lactis*, and yellow colonies were identified as *L. cremoris*.

2.7. Statistical methods

Analysis of variance was performed according to a split-plot design to determine the effect of CFW on the growth of different lactococcal strains. Cell-free whey was the main plot, and starter was the subplot. A factorial design was used to determine the effect of the strain starter composition on the production of GABA in milk. Finally, a split-split-plot design was used to determine the effects of pH, S/M ratio, and glutamate concentration on the production of GABA by lactococcal strains in cheese slurries. The main plot was the S/M ratio, the subplot was the pH and the glutamate concentration, and the sub-subplots were the lactococcal starters. All experiments were replicated three times, and significant differences were tested at $P \leq 0.05$. Statistical analyses were carried out with the general linear models procedure of SAS (SAS Institute, 1999).

3. Results and discussion

3.1. Biocompatibility between the GABA strain and L. lactis ssp. cremoris CFW

Fig. 1 presents the evolution of OD during the growth of the H13 GABA strain in the LL225 and W62 CFW. The curves were strictly ascending but curvilinear with an asymptotic maximum value. The growth patterns were different depending on the type of CFW and the type of GABA strain used (results not shown). These differences were also observed for the other parameters, namely T_{lag} (Fig. 2), μ_{max} (Fig. 3), and OD_{max} (Fig. 4).

For the T_{lag} parameter (Fig. 2), a significant interaction between the types of CFW and the GABA strains was observed ($P \leq 0.05$). The T_{lag} values were significantly lower in the LL390 CFW but higher in the W62 CFW. In the LL390 CFW, however, the A13 and H02 strains showed the same T_{lag} values as those measured in the LL074 and LL225 CFW. The composition of the GDL, LL074, LL225, and LL390 CFW did not significantly modify the T_{lag} values of the GABA strains, with the exception of the H24 and H27 strains. For these strains, the T_{lag} values were significantly higher in the LL074 and W62 CFW. In general, the T_{lag} values were higher in the W62 CFW, probably owing to the depletion of some nutritive elements that the GABA strains need to begin their growth. Since their T_{lag} values were lower in the LL225 and LL390 CFW than in the control GDL medium, the H24 and H27 GABA strains benefited from metabolites released during LL225 and LL390 cell growth.

For the μ_{max} parameter (Fig. 3), a significant interaction between the types of CFW and the GABA strains was also observed ($P \leq 0.05$). The μ_{max} values observed in the W62 and LL074 CFW were statistically similar and higher than those measured in the other CFW. The highest μ_{max} values were obtained for the A13, A23, H24, and H27 strains in the W62 and LL074 CFW. Moreover, the GABA strains reached higher μ_{max} values in the GDL CFW than in the LL225 and LL390 CFW, with the exception of the H24 and H27 strains in the LL390 CFW. Hence, the majority of the GABA strains seemed to grow better in the LL074 and W62 CFW. The μ_{max} parameter is dependent on pH, temperature, concentration of nutrients, and the proteolytic activities of Prt^+ strains (Béal, Juillard, de Roissart, Richard, & Sauraux, 1994). These results indicate that the LL074 and W62 strains, compared with the LL225 and LL390 strains, could have produced or did not use some of the peptides and amino acids in milk that Prt^- GABA strains need to grow (St-Gelais, Roy, Haché, Desjardins, & Gauthier, 1993). However, these results could also be due to the production of inhibitive substances such as diplococcin or lactococcin A (Klaenhammer, 1993) by the LL225 and LL390 strains, which would reduce the cell growth rate of the GABA strains.

For the OD_{max} parameter (Fig. 4), significant effects of the types of CFW and the GABA strains were observed ($P \leq 0.05$). No significant interaction was observed. The highest OD_{max} values were obtained in the W62 CFW. In general, the H02, A23, A13, and H13 GABA strains exhibited the highest OD_{max} values. These results are different from those obtained for the μ_{max} parameter, given that the highest μ_{max} values were reached in the LL074 and W62 CFW. This phenomenon was also observed by Champagne et al. (2009). The nutrients present in the medium, the nutritional needs of the strain, its metabolism, and its adaptive capacity, such as in the presence of inhibitive substances (Champagne et al., 2009; Juillard & Richard, 1989), have a direct impact on the μ_{max} and OD_{max} parameters. Amino acids and free peptides would generate a higher

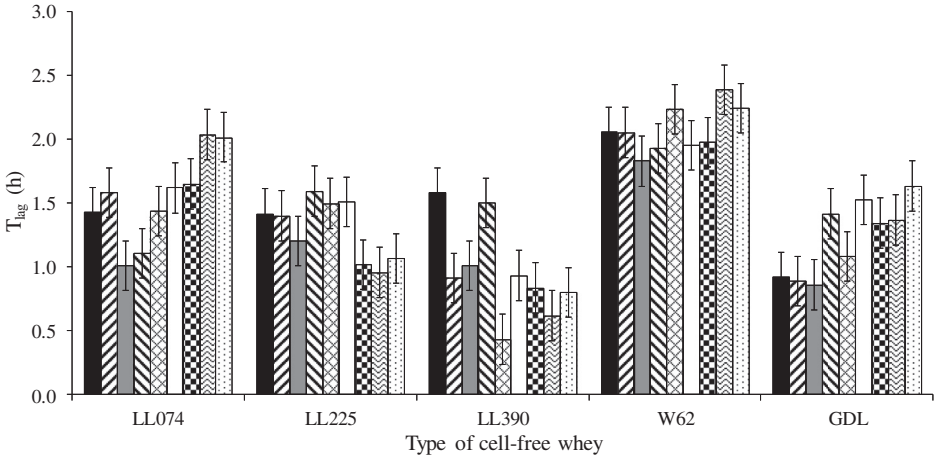

Fig. 2. Values for T_{lag} (time when optical density indicates an increase by 0.01 units) obtained for the γ-aminobutyric acid strains (■, A13; ▨, A23; ▩, H02; ▧, H12; ▨, H13; □, H15; ▨, H20; ▨, H24; ▨, H27) in the LL074, LL225, LL390, W62, and glucono-δ-lactone (GDL) cell-free whey. Error bars represent the standard error of the mean.

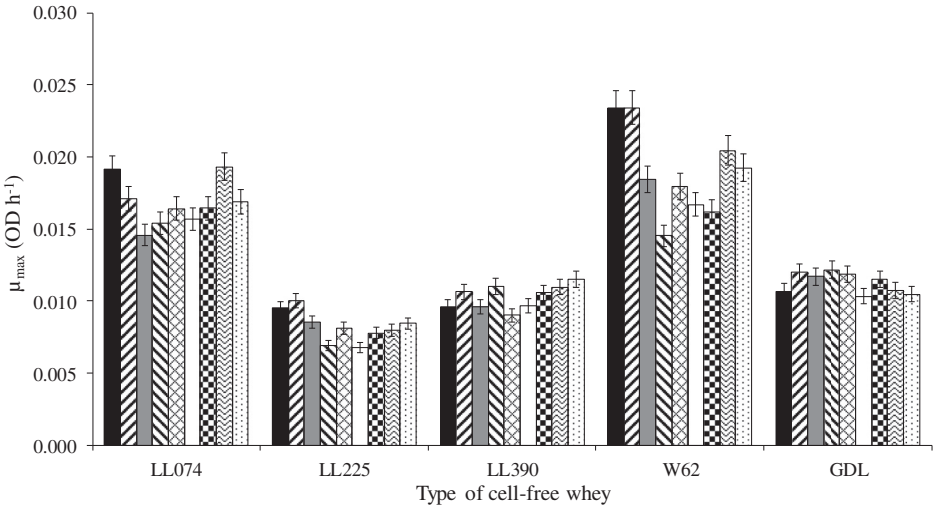

Fig. 3. Values for μ_{max} (maximum rate at the inflexion point) obtained for the γ-aminobutyric acid strains (■, A13; ▨, A23; ■, H02; ▨, H12; ▨, H13; □, H15; ▨, H20; ▨, H24; ▨, H27) in the LL074, LL225, LL390, W62, and glucono-δ-lactone (GDL) cell-free whey. OD, optical density. Error bars represent the standard error of the mean.

μ_{max}, whereas the OD_{max} would be governed by the concentration of nitrogen in milk (Champagne et al., 2009). The composition and the concentration of these nutrients would thus be more favourable in the W62 CFW for all the GABA strains. Among the GABA strains, the A13 and A23 strains showed superior values for the μ_{max} and OD_{max} parameters.

The choice of lactococcal strains used to formulate a starter depends on the synergy among strains. Their biocompatibility allows acidification of milk and limits the imbalance between strains during the production of cheese and during cheese ripening, which increases the quality of cheese. Therefore, considering the results obtained for the μ_{max} and OD_{max} parameters, the W62 strain seems to be the most favourable *L. lactis* ssp. *cremoris* strain for the growth of the GABA-producing strains.

3.2. *Biocompatibility between* L. lactis *ssp.* cremoris *W62 and GABA strain CFW*

The T_{lag}, μ_{max}, and OD_{max} values obtained for the W62 strain in the A13, A23, H02, H12, H13, H15, H20, H24, and H27 CFW are presented in Fig. 5. The T_{lag}, μ_{max}, and OD_{max} values observed in the GABA CFW were not significantly different. In addition, the T_{lag} and μ_{max} values obtained for the W62 strain in the GABA strain CFW were similar to those of the GABA strains in the W62 CFW (Figs. 2–4). However, the OD_{max} values (0.041 OD h^{-1}) observed for the W62 strain in the GABA CFW were very low compared with the OD_{max} values (0.14 OD h^{-1}) obtained with the GABA strains in the W62 CFW (Fig. 4). As previously mentioned, the OD_{max} parameter is influenced mainly by the concentration of nitrogen (Champagne et al., 2009). The Prt^+ W62 strain can produce peptides and amino acids in the W62 CFW that are then available for the GABA strain cell growth, resulting in higher OD_{max} values (Fig. 4). In contrast, the Prt^- GABA strains used in this study did not produce peptides and amino acids (St-Gelais et al., 1993). As a result, the OD_{max} value obtained for the W62 strain in the GABA Prt^- CFW was low (Fig. 5). Stadhouders et al. (1988) showed that mixed cultures of Prt^+ and Prt^- lactococci could be used together to produce good quality cheese. Hence, the W62 Prt^+ strain could be used with a biocompatible GABA Prt^- strain to obtain an active cheese starter.

3.3. *Production of GABA in milk*

The production of GABA by the nine GABA strains in milk enriched with glutamate is presented in Fig. 6. The production of

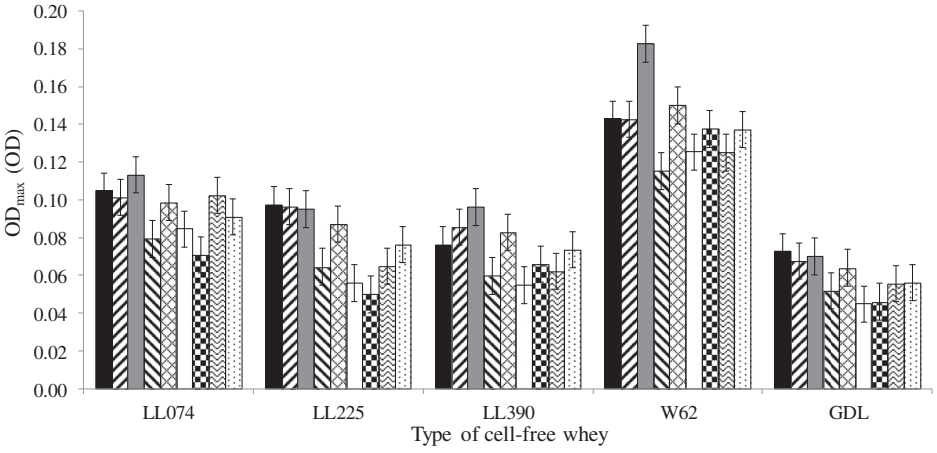

Fig. 4. Values for OD_{max} (maximum optical density) obtained for the γ-aminobutyric acid strains (■, A13; ▨, A23; ■, H02; ▨, H12; ▨, H13; □, H15; ▨, H20; ▨, H24; ▨, H27) in the LL074, LL225, LL390, W62, and glucono-δ-lactone (GDL) cell-free whey. OD, optical density. Error bars represent the standard error of the mean.

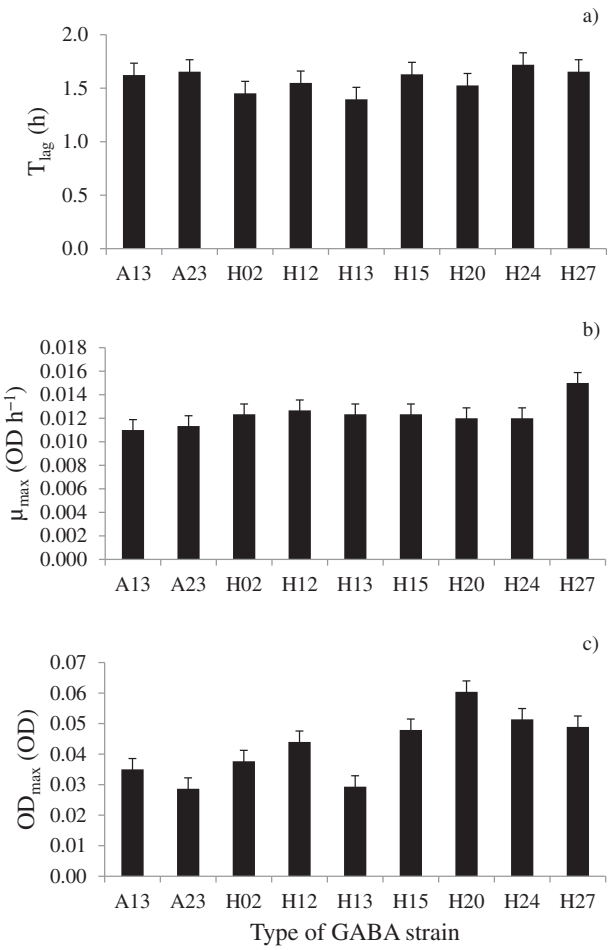

Fig. 5. Values for T_{lag} (time when optical density indicates an increase by 0.01 units; a), μ_{max} (maximum rate at the inflexion point; b), and OD_{max} (maximum optical density; c) of the W62 strain in the γ-aminobutyric acid (GABA) strain cell-free wheys. OD, 0ptical density. Error bars represent the standard error of the mean.

GABA after 5 d of incubation was significantly different ($P \leq 0.05$) depending on the type of strain. The A13, H02, H13, and H20 strains, and the A23 strain in particular, produced the highest GABA concentrations, which were similar to those obtained by Inoue et al. (2003) and Hayakawa et al. (2004).

3.4. Production of GABA in cheese slurries

The initial populations of both the GABA strains (A23 and H13) and the W62 strain in all cheese slurries were, respectively, 8.43 and 8.01 log cfu g^{-1}. After 10 d, the log reduction for both GABA stains in cheese slurries was 8.20 log cfu g^{-1}, while the population of the W62 strain was not detected in all cheese slurries (data not shown). In addition, for both GABA stains the log reduction was statistically similar for both S/M ratios. It is known that the viability of lactic acid bacteria is affected by the S/M ratio in cheese (Mistry & Kasperson, 1998). It is also known, however, that some lactococcal strains are more resistant to salt (osmotic stress) than others (Rallu, Gruss, & Maguin, 1996). In this study, the W62 strain was more sensitive to salt than both GABA strains. Thus, to respond to osmotic and chlorine stress, GABA strains and W62 strain would have different mechanisms of adaptation (Kim, Ren, & Dunn, 1999). The reductions in the populations of the A23, H13, and W62 strains in cheese slurries after 10 d of ripening are similar to those observed with other lactococci in cheese slurries (Farkye, Madkor, & Atkins, 1995). In addition, the evolution of the populations of the A23, H13, and W62 strains in cheese slurries was also representative of true cheddar cheese ripening, considering that 10 d at 30 °C in a cheese slurry model corresponds to a seven-month-old cheese (Farkye et al., 1995).

The effects of S/M ratio, pH, and glutamate concentration in cheese slurries on the production of GABA by the A23 and H13 strains is presented in Fig. 7. A significant quadruple interaction between S/M ratio, pH, glutamate concentration, and type of strain was observed ($P \leq 0.05$). No GABA was detected in cheese slurries inoculated with the W62 strain. This result is similar to the observation made by Nomura et al. (1999a), who concluded that *L. lactis* ssp. *cremoris* could not produce GABA, probably because glutamate dehydrogenase is rare in *L. lactis* (Lapujade, Cocaign-Bousquet, & Loubiere, 1998). The *L. lactis* ssp. *lactis* A23 and H13 strains could produce GABA in cheese slurries, with the amounts being slightly higher with the A23 strain. This result was similar to that obtained in fermented milk (Fig. 6). In cheese slurries, the effect of glutamate on the production of GABA was dependent on

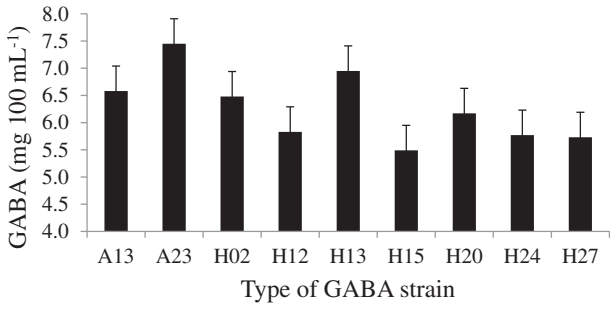

Fig. 6. Concentration of γ-aminobutyric acid (GABA) in milk enriched with 1.47 mg mL^{-1} of glutamate produced by the GABA strains after 5 d at 30 °C. Error bars represent the standard error of the mean.

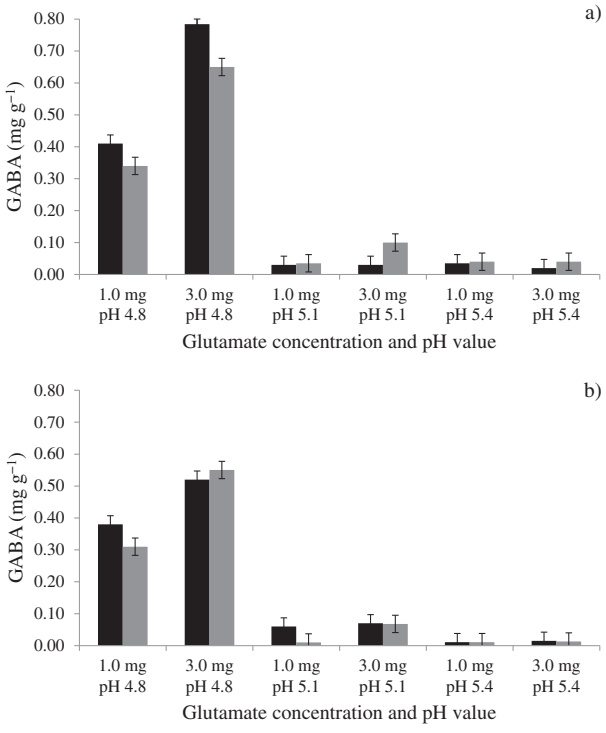

Fig. 7. Concentrations of γ-aminobutyric acid (GABA) after 10 d at 30 °C in cheese slurries made using A23 (■) and H13 (■) at salt-to-moisture ratios of 3.0% (a) and 4.5% (b). Error bars represent the standard error of the mean.

pH, S/M ratio, and strain. At pH 4.8, with 1 mg g^{-1} of glutamate, the S/M ratio did not influence the amounts of GABA produced by the A23 and H13 strains. With 3 mg g^{-1} of glutamate, however, the concentration of GABA was higher at an S/M ratio of 3.0%, particularly in the case of the A23 strain. The production of GABA increased with the concentration of glutamate only at pH 4.8. At pH 5.1 and 5.4, the amount produced was very low. These results are similar to those obtained by Nomura et al. (1998). Thus, the presence of glutamate is necessary for GABA production, and the critical factor is the pH, because the activity of glutamate dehydrogenase in the bacterium is optimal between pH 4.7 and 4.9 (Nomura et al., 1998). In fact, the glutamate dehydrogenase in lactic acid bacteria is activated at a low pH, as H^+ protons are consumed for GABA production and therefore reduce the acidification in the cytoplasm (Cotter & Hill, 2003). However, during the production of GABA, some carbon dioxide is generated (Cotter & Hill, 2003). The presence of this gas in cheese could create the formation of fissures in Cheddar cheese, which result in low-grade Cheddar cheese.

It is known that, during cheddar cheese ripening, casein is hydrolysed and the production of glutamate increases (Lynch, McSweeney, Fox, Cogan, & Drinan, 1997). In this study, glutamate (1 and 3 mg g^{-1}) was added to cheese slurries, and its evolution during ripening was significantly ($P \leq 0.05$) affected by pH. After 10 d of ripening, the concentration of glutamate increased by 25% at pH 5.4 and 5.1 but decreased by 22% at pH 4.8 (results not shown). At pH 5.4 and 5.1, the glutamate dehydrogenase activity in the bacterium was low, and the glutamate produced during ripening probably increased faster than it was used by the A23 and H13 strains to produce GABA. At pH 4.8, an opposite trend was observed. It is noteworthy that GABA was produced in cheese slurries even though an extensive loss of viability occurs with the GABA-producing strains (Fig. 7). It remains to be ascertained, however, if GABA was produced only at the beginning of incubation, when cfu levels were high, or if it was still produced after cell viability loss by the glutamate decarboxylase enzymes released in the cheese matrix.

4. Conclusion

Some Prt^- strains of *L. lactis* ssp. *lactis* produce glutamate dehydrogenase, an enzyme that allows glutamate to be transformed into GABA. To produce cheese, however, Prt^- strains of lactococci must be combined with Prt^+ strains (Stadhouders et al., 1988). Among the nine GABA strains evaluated in this study, two of them (A23 and H13) were selected because they were able to produce the highest concentrations of GABA and were also biocompatible with the W62 Prt^+ strain. Data from this study contribute to the field by confirming the following observations previously reported in the literature: the selection of *L. lactis* strains with glutamate dehydrogenase activity is required for GABA production; GABA production is higher at pH levels under 5.0; and there is a correlation between glutamate concentration and GABA production. This study also contributes novel data to the field: high GABA levels can be reached with mixed starters containing a GABA-producing strain selected for biocompatibility with an acidifying Prt^+ strain; GABA yield is markedly increased at acid pH in the presence of a 3% S/M ratio; and GABA could be produced in a cheese slurry even if an extensive loss of viability of GABA-producing strains occurs.

The results obtained indicate that, to produce a GABA-enriched cheese, some ripening parameters such as pH and S/M ratio should be adjusted. Because the production of GABA increased with the glutamate concentration, the cheese ripening time should be long enough to allow the production of sufficient glutamate (3.0 mg g^{-1} minimum) to obtain high concentrations of GABA. The impact of these selected parameters (pH 4.8 and S/M 3.0%) on the production of GABA during cheese manufacturing and ripening remains to be evaluated. However, because the production of carbon dioxide during the conversion of glutamate to GABA and because the pH and S/M ratio for commercial Cheddar cheese should ideally be kept between 5.2–5.4 and 4.0–5.5%, respectively, Cheddar cheese would not be the best choice to produce a commercial GABA-enriched cheese. GABA starters should be used to produce acid cheese with high moisture content. Finally, the GABA concentrations in cheese that would lead to an antihypertensive effect in humans will need to be determined in future studies.

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