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ORIGINAL PAPER

Gamma-aminobutyric acid-producing abilities of lactococcal strains isolated from old-style cheese starters

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Abstract A previous study showed the ability of old-style cheese starters to produce large amounts of γ-aminobutyric acid (GABA). This work reports the identification of GABA-producing strains and the effect of the main cheese matrix parameters (NaCl, glutamic acid, pH, oxygen) having an influence on GABA production. Out of a total of 50 individual bacterial strains contained in two old-style cheese starters, nine were able to produce GABA and were identified as either *Lactococcus lactis* ssp. *lactis* or *L. lactis* ssp. *lactis* biovar *diacetylactis*. Strains ULAAC-A13 and ULAAC-A23 were able to produce up to 500 mg of GABA per 100 mL of fermented milk containing 2% NaCl and 367 mg per 100 mL of glutamate. Moreover, the low residual glutamate concentration indicates the almost total conversion of glutamate into GABA. GABA was also detected in four commercial cheeses at concentrations varying from 10 to 97 mg of GABA per 30 g of cheese and at a concentration of 29 mg of GABA per 30 g of a cheese slurry ripened with ULAAC-A old-style starter. In that cheese slurry, GABA concentrations were above the levels previously proven to be effective for lowering blood pressure in humans. Both individual GABA-producing strains or the old-style starters containing the GABA strains seem to be promising for GABA production in hard or semi-hard cheeses with prevailing conditions for GABA production.

Keywords Gamma-aminobutyric acid · Glutamate · Lactococcus lactis

1 Introduction

Lactic starters used for cheesemaking, mainly lactococcal strains, are responsible for the acidification of cheese milk to the desired pH during manufacturing. In addition,

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starter bacteria play an important role in the maturation and flavor development of cheeses (Farkye et al. 1995). During cheese ripening, casein is degraded into peptides and amino acids by the proteolytic enzymes of the starter bacteria. Free amino acids (FAA) are substrates for a series of catabolic reactions that generate many important flavor compounds. Some amino acids such as glutamate may subsequently undergo decarboxylation to produce carbon dioxide and γ-aminobutyric acid (GABA) by a glutamate decarboxylase (GAD) enzyme, which is activated in acidic and anaerobic conditions (Nomura et al. 1998).

A well-known inhibitory neurotransmitter in the central nervous system, GABA, has long been reported to lower blood pressure by intravenous administration in experimental animals (Lacerda et al. 2003; Stanton 1963; Takahashi et al. 1955) and in human subjects (Elliott and Hobbiger 1959). Therefore, the effect of GABA in food products on human health is of current interest (Tsukatani et al. 2005). A number of fermented foods naturally contain high concentrations of GABA, including fermented tea, tempeh, kimchi, and yogurt (Aoki et al. 2003a, b). Several organisms isolated from these products have been found to produce GABA, namely *Lactococcus lactis* (Nomura et al. 1998, 1999a, b), *Lactobacillus paracasei* (Komatsuzaki et al. 2005), *Lactobacillus brevis* (Ueno et al. 1997), and *Rhizopus* (Aoki et al. 2003a, b). With respect to dairy products, fermented milk containing GABA has been effective for decreasing blood pressure in moderately hypertensive humans (Inoue et al. 2003) and spontaneously hypertensive rats (Aoki et al. 2003a; Hayakawa et al. 2004). Consequently, the screening of GABA-producing lactic acid bacteria and the production of food enriched with GABA are of great interest (Aoki et al. 2003b; Kono and Himeno 2000; Nomura et al. 1998; Park et al. 2005; Tsukatani et al. 2005). However, few data are available regarding the presence of GABA in cheeses, even though lactic acid bacteria are used for cheese manufacturing. The same observation could be drawn regarding the parameters having an effect on GABA production. To our knowledge, only Komatsuzaki et al. (2005) studied the effect of pH and pyridoxal 5-phosphate (PLP), a coenzyne of GAD enzyme, on GABA production by lactic acid bacteria.

In a recent work, Lacroix et al. (2010) studied the characteristics of 13 old-style cheese starters collected in 1968 from various Canadian cheese producers. The starters usually originated from a neighboring cheese factory that had been having success with reliable acid development and good quality cheese. These starters were of interest for two reasons: first, the starters may contain some form of partial resistance to bacteriophages, and second, the starters could contain some uniquely valuable strains providing unique flavor production (the factories were in an area that had produced cheeses famous for more than a century for developing good strong Cheddar flavor). Lacroix et al. (2010) observed that two of the starters (ULAAC-A and ULAAC-H) demonstrated the ability to generate large amounts of GABA, which contributed up to 15% of the total amino acids. Since the ripening conditions of hard cheese seem to be favorable to the production of GABA (presence of glutamate released from casein proteolysis, low pH, and anaerobiosis), the objective of this study was to optimize for GABA production certain conditions that would occur in a cheese matrix.

2 Materials and methods

2.1 Materials

All chemical reagents and amino acids were purchased from Sigma Aldrich (St. Louis, MO, USA). The M17 broth was purchased from Difco (Detroit, MI, USA).

2.2 Bacterial strains

Two starters (ULAAC-A and ULAAC-H) that had previously been collected in 1968 from various Canadian cheese factories and were subsequently identified for their ability to produce GABA were obtained from Agriculture and Agri-Food Canada (Ottawa, ON, Canada). Each starter was maintained separately in sterile 11% (*w*/*v*) reconstituted skim milk with 20% glycerol (*v*/*v*) and kept frozen in cryovials at −80 °C until required. The individual strains composing the starters were previously isolated and kept frozen in sterile 11% (*w*/*v*) reconstituted skim milk with glycerol (20%).

2.3 Glutamate decarboxylase (GAD) assay

A rapid colorimetric test (Cotter et al. 2001) was adapted to lactococci and allowed the detection of GABA-producing strains. Individual lactococcal strains isolated from starters ULAAC-A (20 strains) and ULAAC-H (30 strains) were tested as described by Cotter et al. (2001) and Olier et al. (2004) with some modifications. Volumes (5 mL) of overnight cultures grown in GM17 medium (M17 broth+0.5% [*w*/*v*] glucose) at 30 °C were centrifuged (5,000×*g*, 20 min, 25 °C) and washed once with 5 mL of 0.9% (*w*/*v*) NaCl solution. The cells were centrifuged again and finally resuspended in 0.5 mL of the GAD reagent solution containing 1 g of L-glutamic acid, 0.3 mL of Triton X-100, 90 g of NaCl, and 0.05 g of bromocresol green in 1 L of distilled water adjusted to pH 4. After 4 h at 37 °C under anaerobic conditions, no change in color (yellow) was considered to be a negative result. Development of a green or blue color was considered to indicate low or high GAD activity, respectively. Three independent experiments were carried out for each strain tested (total of 50 strains).

2.4 Acidifying activity

The acidification abilities of the GABA-producing strains were tested in 10 mL of 12% (*w*/*v*) sterile reconstituted skim milk. The final pH of the milk was recorded after 6 h of incubation at 30 °C. The growth curves at 30 and 40 °C in the GM17 medium containing 2% or 4% (*w*/*v*) NaCl were determined for 10 h of fermentation.

2.5 Polymerase chain reaction identification

Bacterial strains with GAD activity were identified by polymerase chain reaction (PCR) analysis. Volumes (10 mL) of overnight cultures grown in GM17 medium at

30 °C were used for the isolation of genomic DNA according to the method described by Leenhouts et al. (1990). Genotypic differentiation of the *Lactococcus lactis* subspecies and biovar was performed according to the method of Beimfohr et al. (1997). Table 1 describes the PCR primers used.

2.6 Overproduction of GABA in fermented milk

Overnight cultures of either strain ULAAC-A13 or ULAAC-A23 (from starter ULAAC-A) grown in 12% (*w*/*v*) reconstituted skim milk at 30 °C were inoculated at 2% (*v*/*v*) into 10 mL of 12% (*w*/*v*) reconstituted skim milk containing PLP (50 μM) and different concentrations of NaCl (0%, 2%, or 4%) and glutamate (0, 147, 367, or 735 mg per 100 mL). These concentrations represent 0, 10, 25, or 50 $mmol.L^{-1}$ of glutamate, respectively. Incubations were done in 20-mL headspace vials (Agilent Technologies, Palo Alto, CA, USA) sealed with 20-mm aluminum crimp caps with PTFE/Si septa (Agilent Technologies) to maintain anaerobic conditions. The vials were incubated for 5 days at 30 °C to accelerate and extend the formation of GABA before the contents were analyzed by gas chromatography–flame ionization detection or gas chromatography–mass spectrometry (GC-MS).

2.7 Quantification of GABA and glutamate

The amounts of GABA produced by the nine strains and the residual concentrations of glutamate were quantified after 5 days at 30 °C. The vials were uncapped, and the milk samples were transferred to 15-mL tubes and centrifuged at 5,000×*g* for 20 min at 25 °C. The supernatants were collected in cryovials and frozen at −20 °C until needed. Derivatization of these supernatant fractions was performed with the EZ:faast kit (Phenomenex, Torrance, CA, USA) following the manufacturer's recommendations. The derivatized samples were then analyzed by GC (HP 5890A Gas Chromatograph, Hewlett Packard, Avondale, PA, USA) with a flame ionization detector port. A 2-μL volume of each 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 $°C.min^{-1}$. For quantification purposes, calibration curves were made for GABA and glutamate (10 $nmol.L^{-1}$ per 100 μL to 2,560 $nmol.L^{-1}$ per 100 μL) with norvaline (20 $nmol.L^{-1}$ per 100 μL) as the internal standard.

Table 1 Primers used for polymerase chain reaction analysis

Primer pairs	Lactococcus lactis ssp. cremoris (bp)	L. lactis ssp. lactis (bp)	L. lactis ssp. lactis biovar diacetylactis (bp)
Lchis1F/Lchis2R	556	0	0
LLhis3F/LLhis4R	0	343	0
Lhis5F/Lhis6R	1,149	934	934

bp base pairs

2.8 Identification of GABA and glutamate

Identification of GABA was carried out with a gas chromatograph and a mass spectrometer (GC-MS 5973 Network Mass Selective Detector, Agilent Technologies) following the GC derivatization and separation procedure described above. The main ions used to distinguish between and identify GABA and glutamate (according to Phenomenex) were 130, 144, and 172 for GABA and 84, 170, and 230 for glutamate.

2.9 Determination of GABA and glutamate in commercial cheeses

Two different lots of nine different commercial cheeses were purchased from a local supermarket and analyzed for their GABA and glutamate concentrations. For comparison purposes, a cheese slurry (Lacroix et al. 2010) was ripened for 15 days at 30 °C by starter ULAAC-A and analyzed by the same method.

Free amino acids in the water-soluble nitrogen fraction of the cheeses (Kuchroo and Fox 1982) were quantified. Briefly, the cheeses were grated, and a 20-g sample was weighed in a plastic Stomacher bag. A 40-mL volume of distilled water was added to obtain a 1:2 ratio, and the mixture was homogenized for 10 min in a Stomacher before being placed in a water bath at 40 °C for 1 h. The cheese homogenates were then transferred to 15-mL tubes and centrifuged at 5,000 × *g* for 20 min at 4 °C. The upper fat layer was removed, and the supernatant was pipetted into cryovials and either used for analyses or kept frozen until needed. Quantification and identification of FAA, including GABA and glutamate, was done as described above. The results were expressed either as a percentage of total FAA or as milligrams of GABA per 30 g of cheese (because 30 g is representative of a normal daily portion of cheese). Using a PHM 84 Research pH Meter (Radiometer, Copenhagen, Denmark), the pH of the cheeses was measured in a mixture of 10 g of cheese and 10 mL of deionized water (Kuchroo and Fox 1982).

2.10 Statistical analysis

Statistical analyses were conducted using the SAS/Stat 8.01 (proc GLM) package (SAS Institute Inc., Cary, NC, USA). For the GABA overproduction assays with strains ULAAC-A13 and ULAAC-A23, multiple comparisons were performed on the least squares means of a two-factor (three NaCl concentrations and four glutamate concentrations) factorial plan design (3 × 4). To stabilize variance, analyses of GABA concentrations were carried out on their $\log_{10}$ transformations. The means of the GABA concentrations measured were examined by analysis of variance conducted with Tukey's test with an α significance level of 0.05. Six independent experiments were repeated for each strain and each treatment.

3 Results and discussion

3.1 Glutamate decarboxylase activity

The physiological role of glutamate decarboxylation is likely related to the regulation of intracellular pH. Indeed, GAD has been found to play a major role in the acid

resistance of bacteria. During lactococci cell growth, the intracellular cell pH decreases owing to the accumulation of organic acids. When the cell is exposed to low pH, the GAD system converts a molecule of glutamate to GABA while consuming an intracellular proton. The net effect is to reduce the proton concentration within the cell, thus alleviating acidification of the cytoplasm (Cotter et al. 2001). Since starters ULAAC-A and ULAAC-H were composed of 20 and 30 individual strains, respectively, identification of the strains with the highest capacity to produce GABA was undertaken. Table 2 shows the GAD activity for each strain, which is classified as not detectable (yellow coloration), low (green coloration), or high (blue coloration). Among the 50 strains, nine exhibited high GAD activity. These strains were strains 13 and 23 from starter ULAAC-A and strains 02, 12, 13, 15, 20, 24, and 27 from starter ULAAC-H.

The old-style starters studied here were traditionally used for the production of raw-milk Cheddar cheeses. Seed cultures were transferred from 1 day to the next in the cheese factory without modern special protection, in much the same way as in the previous century. Depending on how the starters were kept and used by the cheese producers at that time, it is possible that several strains have developed a strong acid tolerance response as reflected by the activity of the GAD enzyme (Budin-Verneuil et al. 2004; O'Sullivan and Condon 1997).

3.2 Genotypic and acidifying characteristics of GABA-producing strains

The nine GABA-producing strains were identified and found to all belong to *L. lactis* (Table 3). Moreover, the seven strains from starter ULAAC-H were identified as biovar *diacetylactis*. Growth curves made in GM17 broth at 40 and 30 °C with 4% NaCl (results not shown) showed that the nine GABA-producing strains were able to grow with 4% salt (at a slow rate) and at 40 °C, which is another phenotypic criterion that allows the subspecies *cremoris* to be distinguished from the subspecies *lactis* (Nomura et al. 1999a). Nomura et al. (2000) found that the *gadB* gene encoding GAD was detected in *L. lactis* ssp. *cremoris* but was only poorly expressed and inactivated by a frameshift mutation, as compared to the activity of *L. lactis* ssp. *lactis* GAD. Therefore, the activity of the GAD enzyme has been reported as a novel criterion for distinguishing *L. lactis* ssp. *lactis* from *L. lactis* ssp. *cremoris* (Nomura et al. 1999a). Moreover, only strains A13 and A23 were able to lower milk pH below 5 after 6 h at 30 °C (Table 3). Because of these characteristics, these two strains are of particular interest for cheese manufacture and were selected for the remainder of the study.

Table 2 Glutamate decarboxylase activity of the individual lactococcal strains of starters ULAAC-A and ULAAC-H

Activity	Starter ULAAC-A	Starter ULAAC-H
Not detectable (n=0)		
Low (n=41)	02, 05, 08, 10, 12, 14, 15, 16, 17, 18, 19, 20, 21, 24, 25, 26, 29, 30	01, 03, 04, 05, 06, 07, 08, 09, 10, 11, 14, 16, 17, 18, 19, 21, 22, 23, 25, 26, 28, 29, 30
High (n=9)	13, 23	02, 12, 13, 15, 20, 24, 27

Table 3 Genotypic and acidifying characteristics of the nine γ-aminobutyric acid-producing strains

Strain	PCR identification	pH after 6 h at 30 °C	pH after 10 h at 30 °C
ULAAC-A13	*L. lactis* ssp. *lactis*	4.7	4.5
ULAAC-A23	*L. lactis* ssp. *lactis*	4.7	4.4
ULAAC-H02	*L. lactis* ssp. *lactis* biovar *diacetylactis*	5.7	4.8
ULAAC-H12	*L. lactis* ssp. *lactis* biovar *diacetylactis*	5.6	4.8
ULAAC-H13	*L. lactis* ssp. *lactis* biovar *diacetylactis*	5.6	4.8
ULAAC-H15	*L. lactis* ssp. *lactis* biovar *diacetylactis*	5.6	4.8
ULAAC-H20	*L. lactis* ssp. *lactis* biovar *diacetylactis*	5.7	4.8
ULAAC-H24	*L. lactis* ssp. *lactis* biovar *diacetylactis*	5.7	4.6
ULAAC-H27	*L. lactis* ssp. *lactis* biovar *diacetylactis*	5.7	4.9

Values are the mean of two repetitions. *PCR* polymerase chain reaction

3.3 Effect of glutamate and NaCl on GABA production

Since foodstuffs containing a naturally high concentration of glutamate are used by lactic acid bacteria to produce GABA (Tsukatani et al. 2005), and because of the relatively high sodium chloride concentration present in the cheese matrix, the effect of glutamate concentration and NaCl on the GABA-producing abilities of strains A13 and A23 was investigated.

The results showed that GABA production by bacterial strains ULAAC-A13 (Fig. 1) and ULAAC-A23 (Fig. 2) increased with glutamate concentration. Theoretically, when 147, 367, and 735 mg per 100 mL of glutamate were added, bioconversion would result in the production of 103, 257, and 515 mg of GABA per 100 mL, respectively. In products without any salt addition, GABA generally reached levels that suggested complete bioconversion of the glutamate that had been added. The only exception occurred with strain ULAAC-A23 at 735 mg per 100 mL, for which bioconversion was only 55% (Fig. 2).

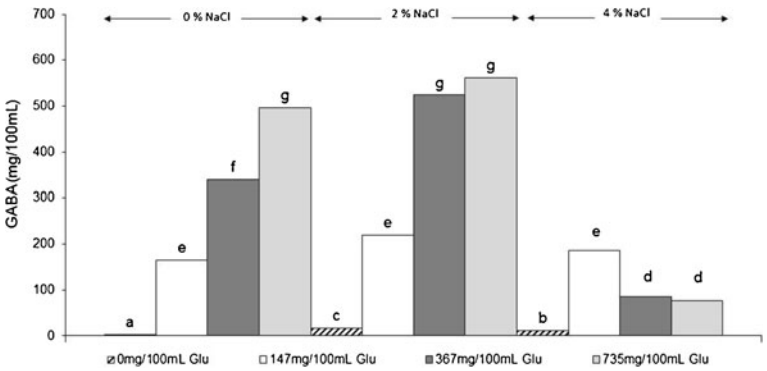

Fig. 1 Overproduction of γ-aminobutyric acid (GABA) by strain ULAAC-A13 after 5 days of incubation at 30 °C in milk containing different concentrations of NaCl and glutamate (Glu). Column means with the same letter are not statistically different ($P>0.05$)

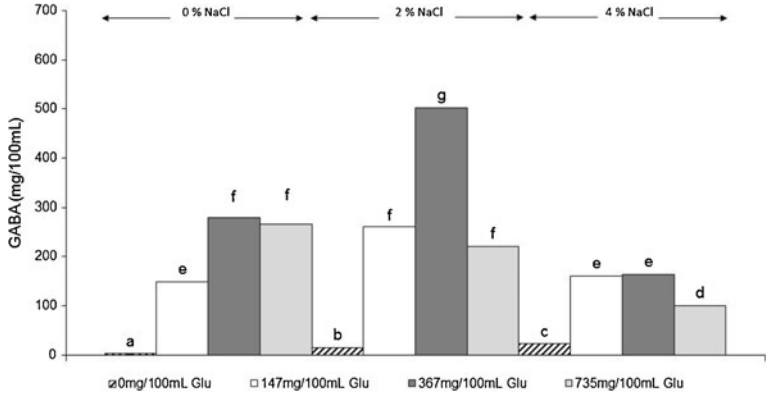

Fig. 2 Overproduction of γ-aminobutyric acid (GABA) by strain ULAAC-A23 after 5 days of incubation at 30 °C in milk containing different concentrations of NaCl and glutamate (Glu). Column means with the same letter are not statistically different ($P > 0.05$)

A different picture emerged at 2% NaCl. In this medium, when 147 or 367 mg per 100 mL of glutamate was added, the GABA levels produced by strain ULAAC-A13 were twofold higher than the 103 and 257 mg of GABA per 100 mL expected from total bioconversion. These results suggest that proteolysis occurred and that a significant quantity of the resulting glutamate was converted to GABA. In this hypothesis, however, important GABA production should also have been noted in fermented milks with no glutamate enrichment. Although there were indeed higher GABA levels in the controls without glutamate when 2% or 4% salt was added (Figs. 1 and 2), the additional quantities produced, 30 mg per 100 mL at most, do not account for all the overproduction noted at 2% salt and 367 mg per 100 mL of glutamate. This observation remains difficult to explain. Some changes in the environment induced by 2% NaCl such as water activity reducing and most probably toxicity of the chlorine ion could have stressed the cells, resulting in the enhancement of the stress response such as the GABA-producing system.

In contrast to 2% salt addition, adding 4% salt significantly reduced the production of GABA, particularly when 367 and 735 mg per 100 mL of glutamate were added (Figs. 1 and 2). This reduction in GABA occurred for both strains. In the presence of 4% NaCl, only the addition of 147 mg per 100 mL (strain A13) and 147 mg per 100 mL or 367 mg per 100 mL (strain A23) of glutamate seemed to enable a high level of conversion to GABA. Since cell growth of both A13 and A23 strains was inhibited by 4% NaCl (results not shown) and GABA detected (Figs. 1 and 2), it seems that GABA production is partially growth dependent.

Overall, the highest GABA yields produced by both strains were obtained with the combination of 367 mg per 100 mL of glutamate and 2% NaCl. In contrast, residual glutamate remained very high in the presence of 4% NaCl or when 735 mg per 100 mL of glutamate was added, regardless of the salt concentration (Figs. 1 and 2).

The positive effect of glutamate and 2% NaCl on GABA levels is presumably related to the expression of the *gadCB* gene, which is highest at the onset of the stationary growth phase in the presence of NaCl and glutamate and at low pH (Sanders et al. 1998; Small and Waterman 1998). It is likely that this NaCl- and glutamate-dependent acid resistance mechanism of *L. lactis* is optimally active under

conditions in which it is needed to maintain viability (Nomura et al. 1999b). However, *L. lactis gadCB* expression in the presence of chloride remains unclear. High NaCl levels, which cause an osmotic stress, could provide cross-protection against severe acid challenges (Sanders et al. 1998). An analogous stress protection mechanism induced by similar conditions in microorganisms such as *L. lactis* and *Shigella flexneri* may point towards a common stressful natural environment. Both organisms can enter the gastrointestinal tract, where they deal with the highly acidic and chloride-rich conditions of the stomach. *L. lactis* is able to pass through the gastrointestinal tract (Klijn et al. 1995), but only in low numbers. The *gadCB* acid resistance system may play a significant role under these conditions. The *gadCB* gene may thus also be important for the survival of lactococcal cells in cheese, as high levels of both NaCl and glutamate are present (Sanders et al. 1998). It is noteworthy that the addition to the medium of 50 $\mu mol.L^{-1}$ of PLP probably had an impact on the production of GABA, accelerating its formation. Indeed, Komatsuzaki et al. (2005) found that GABA production by a strain of *Lactobacillus paracasei* was further improved by the addition of 147 or 735 mg per 100 mL of PLP to the culture medium.

Inoue et al. (2003) and Hayakawa et al. (2004) studied the effect of a fermented milk product containing 10 to 12 mg of GABA per 100 mL of milk and found a significant decrease in both the systolic and diastolic blood pressure of mildly hypertensive humans and spontaneously hypertensive rats after 2 to 4 weeks of daily intake. Other studies reported GABA production of 31 $mg.kg^{-1}$ after 3.5 h of fermentation in goat milk (Minervini et al. 2009) and 2,500 $mg.kg^{-1}$ after 24 h of fermentation in soy milk supplemented with 74 mg per 100 mL of glutamic acid (Park and Oh 2007). In the present study, the combination of 367 mg per 100 mL of glutamate with 2% NaCl made it possible to obtain 500 mg of GABA per 100 mL of fermented milk with both strains A13 and A23, which is about 50 times higher than the amounts found to be effective for decreasing blood pressure (Inoue et al. 2003). Based on these results, it is thus reasonable to estimate that a cheese manufactured with a starter containing one of GABA-producing strains A13 or A23 would contain enough GABA to result in a significant effect on blood pressure. Cheeses are typically salted at approximately 2%, which would appear to be an ideal salt condition for GABA production. In practice, however, salt accumulates in the aqueous fraction of cheese and is therefore found in a concentrated form. For example, adding 2% salt to a Cheddar cheese with 36% moisture actually results in a 5.5% salt-to-moisture level. Data from this study suggest that this salt level is unfavorable for GABA production. Analyses on commercial cheeses were therefore carried out to ascertain their GABA content.

3.4 GABA and glutamate concentrations in commercial cheeses

Cheese appears to be a suitable matrix for the establishment of good conditions for GABA production by lactic acid bacteria. Indeed, the low oxygen level, low pH, and availability of glutamate during ripening favor the activity of the GAD enzyme. In addition to the nine commercial cheeses analyzed, a cheese slurry ripened for 15 days by the old-style starter ULAAC-A containing strains A13 and A23 was subsequently compared with commercial cheeses. In a previous study, Lacroix et al. (2010) showed that 15 days of accelerated ripening in a model cheese was approximately equivalent

to 3 or 4 months of ripening in Cheddar cheese (mild), which was also in agreement with the results obtained by Harper and Kristoffersen (1970). Since the different cheeses analyzed varied greatly in their stage of ripening and extent of proteolysis, the concentration of GABA was expressed as a percentage of total FAA. Gamma-aminobutyric acid was also expressed as milligrams per 30 g of cheese, which corresponds to daily cheese intake.

The occurrence of GABA at concentrations varying from 10 to 97 mg of GABA per 30 g of cheese was detected in four of the nine commercial cheeses (Table 4). A significant (P=0.002) inverse relationship (R^2=0.71) was noted between the GABA and glutamate concentrations. This observation suggests that glutamate is the precursor of GABA in cheese as well. Similarly, Siragusa et al. (2007) detected the presence of GABA in 22 different Italian cheese varieties, in amounts ranging from 0.001 to 12 mg·per 30 g. In the present study, the highest GABA-to-FAA ratio (up to 23%) was detected in Gouda cheese (Table 4). However, when the GABA concentration was expressed as milligrams per 30 g of cheese, Danish Havarti contained the highest level of GABA (97 mg per 30 g). These results are likely due to the more extensive ripening and proteolysis undergone in Havarti cheese, reflected in its high amount of total FAA (104 μmol.mL^{-1}), which allowed the liberation of high amounts of glutamate. Interestingly, Gouda cheese and the cheese slurry contained the highest GABA-to-FAA ratios, and the residual glutamate levels in these cheeses represented only 2.6% (Gouda) and 3.0% (cheese slurry) of total FAA, indicating the almost total conversion of glutamate into GABA. Moreover, because the amounts of total FAA in these cheeses were low (17 and 11 μmol.mL^{-1}), the GABA concentration obtained would probably have been higher if the ripening period had been extended.

Given that a daily oral consumption of 10 to 12 mg of GABA was found to be effective for lowering blood pressure in mildly hypertensive humans (Hayakawa et al. 2004; Inoue et al. 2003), the concentrations of GABA measured in a 30-g portion of cheese (ranging from 10 to 97 mg) could be sufficient to have an effect when cheese is consumed on a daily basis. Cheese contains many compounds, however, including

Table 4 Concentrations of γ-aminobutyric acid (GABA) and glutamate in commercial cheeses and in a cheese slurry ripened with ULAAC-A old-style starter

Cheeses	pH	GABA (mg per 30 g)	GABA/total FAA (%)	Glutamate/total FAA (%)	Total FAA (μmol.mL^{-1})
Blue cheese	5.9	ND	ND	18.3	87.6
Port-Salut	5.0	10.1	4.2	16.4	33.4
St-Paulin	4.8	17.9	14.1	8.1	14.2
Gouda	4.8	38.7	22.7	2.6	17.0
Camembert	6.3	ND	ND	10.2	14.3
Havarti	6.3	97.4	8.6	12.7	104.1
Cheddar, 1 year	4.7	ND	ND	28.1	73.5
Emmental	5.4	ND	ND	20.8	79.1
Havarti, light	5.3	ND	ND	17.3	45.1
ULAAC-A cheese slurry	4.8	29.1	19.5	3.0	11.2

Values are the mean of two repetitions. *FAA* free amino acids, *ND* not detected

bioactive peptides, and their effect would probably be difficult to distinguish from those of GABA if a clinical study were to be undertaken. Although the mechanism underlying the hypotensive action of GABA has not been fully elucidated (Inoue et al. 2003), it has been proven that, unlike bioactive peptides, GABA does not have any activity on angiotensin-converting enzyme.

Overall, using data for the ten cheeses of Table 4 and assigning a value of "0 mg/30" when GABA was not detected (ND), no correlation was found between the FAA levels and the amount of GABA produced (R=0.25; P=0.44). However, when the regression analysis is only carried out with the five cheeses where GABA was indeed produced, then the relationship between GABA and FFA levels becomes statistically significant (R=0.88; P=0.046). Therefore, although proteolysis is a prerequisite for the production of glutamate and its subsequent conversion to GABA, other factors must be expressed as well. Obviously, the presence of GAD-producing cultures is required, but that factor was not ascertained in this study. Nevertheless, it can be hypothesized that the five cheeses where GABA was not detected (Table 4) did not contain GAD-producing cultures, and that when there is indeed GABA produced, there is a relationship with the proteolysis level. In further studies examining the relationship between GABA and cheese proteolysis, the procedures need to include analyses of the presence and content of GAD-producing strains in order to verify this assumption.

Furthermore, it is noteworthy that the higher GABA-to-total FAA ratios were observed in cheeses with a pH below 5.0. This tends to confirm the potential effect of low pH on the percentage of GABA bioconversion.

3.5 Influence of cheese pH and oxygen permeability

Nomura et al. (1999b) detected the activity of the GAD enzyme in *L. lactis* between pH 4.0 and 5.5, with optimal activity at pH 4.7. Above pH 5.5, no GAD enzyme activity was detected. Therefore, a relationship between cheese pH and the GABA concentrations measured was expected to be found in the present work. The cheeses containing GABA as well as the cheese slurry all had a pH below 5, with the exception of Danish Havarti (pH 6.3) (Table 4). Nomura et al. (1998) also observed the occurrence of GABA in blue cheese and Gouda cheese with pH values of 6.8 and 5.7, respectively. These authors hypothesized that GABA production by the starter had already occurred when the pH value began to increase owing to the consumption of lactic acid and the liberation of ammonia and various amines during the growth of fungi in blue cheese. It is thus likely that the GABA concentrations found in Havarti cheese were formed in the early stages of ripening, before the pH increased. However, the absence of GABA in the Camembert and blue cheese analyzed in this study (Table 4) was probably due to the fact that the starters used for their production did not have any active GAD enzyme. The same explanation is applicable for the Cheddar cheese and light Havarti cheese. These data highlight the need to ascertain the presence of GAD-producing strains in order to obtain GABA, as was carried out in the first part of this study.

A number of genetic systems are known to be co-induced by acidity and anaerobiosis, including amino acid degradation and adaptation to acidic or basic conditions (Olson 1993). One of the most important conditions for the induction of GAD activity is the absence of oxygen. Indeed, Small et al. (1994) discovered that a mutant of *Escherichia coli* was acid sensitive when grown aerobically but could develop an

effective acid-inducible tolerance response, owing to its GAD enzyme, when grown anaerobically (Bearson et al. 1997). In the present study, the pressing stage during manufacturing and the low oxygen permeability of the four GABA-containing cheeses were favorable to the establishment of anaerobiosis, with all the cheeses being hard or semi-hard. Moreover, the crust of St-Paulin and Port-Salut and the wax of Gouda protect the center of the cheeses from oxygen.

4 Conclusion

Many fermented foods contain GABA owing to the action of bacterial GAD enzyme under acidic conditions. Some strains of lactic acid bacteria, particularly *L. lactis* ssp. *lactis*, are known to possess an active GAD and a good capacity to produce GABA. In this study, nine GABA-producing lactococcal strains were identified in old-style cheese starters. In addition to their GABA-producing abilities, two of them exhibited high acidifying activity and can thus eventually be used for the manufacture of GABA-containing cheeses. Moreover, hard and semi-hard cheeses seem to be suitable matrices for the establishment of the optimal conditions for GABA production. Further work should be undertaken to verify the ability of the GABA-producing strains to produce GABA under cheese manufacturing conditions.

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References

Aoki H, Furuya Y, Endo Y, Fujimoto K (2003a) Effect of γ-aminobutyric acid-enriched tempeh-like fermented soybean (GABA-tempeh) on the blood pressure of spontaneously hypertensive rats. Biosci Biotechnol Biochem 67:1806–1808

Aoki H, Uda I, Tagami K, Furuya Y, Endo Y, Fujimoto K (2003b) The production of a new *tempeh*-like fermented soybean containing a high level of γ-aminobutyric acid by anaerobic incubation with *Rhizopus*. Biosci Biotechnol Biochem 67:1018–1023

Bearson S, Bearson B, Foster JW (1997) Acid stress response in enterobacteria. FEMS Microbiol Lett 147:173–180

Beimfohr C, Ludwig W, Schleifer KH (1997) Rapid genotypic differentiation of *Lactococcus lactis* subspecies and biovar. Syst Appl Microbiol 20:216–221

Budin-Verneuil A, Maguin E, Auffray Y, Ehrlich SD, Pichereau V (2004) An essential role for arginine catabolism in the acid tolerance of *Lactococcus lactis* MG1363. Lait 84:61–68

Cotter PD, Gahan CGM, Hill C (2001) A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Mol Microbiol 40:465–475

Elliott KAC, Hobbiger F (1959) Gamma aminobutyric acid: circulatory and respiratory effects in different species; re-investigation of the anti-strychnine action in mice. J Physiol 146:70–84

Farkye NY, Madkor SA, Hatkins HG (1995) Proteolytic abilities of some lactic acid bacteria in a model cheese system. Int Dairy J 5:715–725

Harper WJ, Kristoffersen T (1970) Biochemical aspects of flavor development in Cheddar cheese slurries. J Agric Food Chem 18:563–566

Hayakawa K, Kimura M, Kasaha K, Matsumoto K, Sansawa H, Yamori Y (2004) Effect of a γ-aminobutyric acid-enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar–Kyoto rats. British J Nutr 92:411–417

Inoue K, Shirai T, Ochiai H, Kasao M, Hayakawa K, Kimura M, Sansawa H (2003) Blood-pressure-lowering effect of a novel fermented milk containing γ-aminobutyric acid (GABA) in mild hypertensives. Eur J Clin Nutr 57:490–495

Klijn N, Weerkamp AH, de Vos WM (1995) Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. Appl Environ Microbiol 61:2771–2774

Komatsuzaki N, Shima J, Kawamoto S, Momose H, Toshinori K (2005) Production of γ-aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. Food Microbiol 22:497–504

Kono I, Himeno K (2000) Changes in γ-aminobutyric acid content during *beni-koji* making. Biosci Biotechnol Biochem 64:617–619

Kuchroo CN, Fox PF (1982) Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. Milchwissenschaft 37:331–335

Lacerda JEC, Campos RR, Araujo GC, Andreatta-Van Leyen S, Lopes OU, Guertzenstein PG (2003) Cardiovascular responses to microinjections of GABA or anesthetics into the rostral ventrolateral medulla of conscious and anesthetized rats. Braz J Med Biol Res 36:1269–1277

Lacroix N, St-Gelais D, Champagne CP, Fortin J, Vuillemard JC (2010) Characterization of aromatic properties of old-style cheese starters. J Dairy Sci 93:3427–3441

Leenhouts KJ, Kok J, Venema G (1990) Stability of integrated plasmids in the chromosome of *Lactococcus lactis*. Appl Environ Microbiol 56:2726–2735

Minervini F, Bilancia MT, Siragusa S, Gobbetti M, Caponio F (2009) Fermented goats' milk produced with selected multiple starters as a potentially functional food. Food Microbiol 26:559–564

Nomura M, Kimoto H, Someya Y, Furukawa S, Suzuki I (1998) Production of γ-aminobutyric acid by cheese starters during cheese ripening. J Dairy Sci 81:1486–1491

Nomura M, Kimoto H, Someya Y, Suzuki I (1999a) Novel characteristic for distinguishing *Lactococcus lactis* subsp. *lactis* from subsp. *cremoris*. Int J Syst Bacteriol 49:163–166

Nomura M, Kobayashi M, Ohmomo S, Okamoto T (2000) Inactivation of the glutamate decarboxylase gene in *Lactococcus lactis* subsp. *cremoris*. Appl Environ Microbiol 66:2235–2237

Nomura M, Nakajima I, Fujita Y, Kobayashi M, Kimoto H, Suzuki I, Aso H (1999b) *Lactococcus lactis* contains only one glutamate decarboxylase gene. Microbiology 145:1375–1380

O'Sullivan E, Condon S (1997) Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis*. Appl Environ Microbiol 63:4210–4215

Olier M, Rousseaux S, Piveteau P, Lemaître JP, Rousset A, Guzzo J (2004) Screening of glutamate decarboxylase activity and bile salt resistance of human asymptomatic carriage, clinical, food, and environmental isolates of *Listeria monocytogenes*. Int J Food Microbiol 93:87–99

Olson ER (1993) Influence of pH on bacterial gene expression. Mol Microbiol 8:5–14

Park KB, Ji GE, Park MS, Oh SH (2005) Expression of rice glutamate decarboxylase in *Bifidobacterium longum* enhances γ-aminobutyric acid production. Biotechnol Lett 27:1681–1684

Park KB, Oh SH (2007) Production of yogurt with enhanced levels of gamma-aminobutyric acid and valuable nutrients using lactic acid bacteria and germinated soybean extract. Bioresource Technol 98:1675–1679

Sanders JW, Leenhouts K, Burghoorn J, Brands JR, Venema G, Kok J (1998) A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation. Mol Microbiol 27:299–310

Siragusa S, De Angelis M, Di Cagno R, Rizzello CG, Coda R, Gobbetti M (2007) Synthesis of γ-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. Appl Environ Microbiol 73:7283–7290

Small P, Blankenhorn D, Welty D, Zinser E, Slonczewski JL (1994) Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of rpoS and growth pH. J Bacteriol 176:1729–1737

Small PLC, Waterman SR (1998) Acid stress, anaerobiosis and *gadCB*: lessons from *Lactococcus lactis* and *Escherichia coli*. Trends Microbiol 6:214–216

Stanton HC (1963) Mode of action of gamma amino butyric acid on the cardiovascular system. Arch Int Pharmacodyn Ther 143:195–204

Takahashi H, Tiba M, Iino M, Takayasu T (1955) The effect of γ-aminobutyric acid on blood pressure. Jpn J Physiol 5:334–341

Tsukatani T, Higuchi T, Matsumoto K (2005) Enzyme-based microtiter plate assay for γ-aminobutyric acid: application to the screening of γ-aminobutyric acid-producing lactic acid bacteria. Anal Chim Acta 540:293–297

Ueno Y, Hayakawa K, Takahashi S, Oda K (1997) Purification and characterization of glutamate decarboxylase from *Lactobacillus brevis* IFO 12005. Biosci Biotech Biochem 61:1168–1171