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Optimization of batch culture conditions for GABA production by *Lactobacillus brevis* CRL 1942, isolated from quinoa sourdough

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ABSTRACT

γ-aminobutyric acid (GABA) is a four-carbon amino acid, that acts as a major inhibitory neurotransmitter in the central nervous system. Among nineteen lactic acid bacteria strains, isolated from Andean amaranth (A) and Real Hornillos quinoa (Q_r) sourdoughs, *Lactobacillus brevis* CRL 1942 was the most efficient microorganism for the conversion of 53 mM monosodium glutamate (MSG) to GABA, reaching 50 mM after 96 h cultivation. GABA production was enhanced by optimizing culture conditions, such as incubation temperature, time and MSG concentration. A gradual increase of GABA yield was observed at MSG concentrations rising from 0 to 270 mM. In addition, a higher GABA content was observed at 30 °C. GABA production occurred in a time-dependent manner, and greatest amount (~255 mM) was yielded after 48 h in cells grown in MRS with 270 mM MSG at 30 °C, with a conversion rate of ~90%. Cell growth was not affected by MSG addition, implying that the difference in GABA levels could not be attributed to differences in cell numbers. However, addition of glutamate increased viability, indicating a correlation between survival and GABA production. Novel information about LAB with GABA-producing ability is an important breakthrough for the development of health-promoting functional foods.

1. Introduction

γ-aminobutyric acid (GABA) is a ubiquitous non-protein amino acid that is widely distributed among microorganisms, plants and animals. It acts as a major inhibitory neurotransmitter in the central nervous system (Krnjevic, 1974), and has several physiological functions such as neurotransmission, induction of hypotensive, diuretic and tranquilizer effects (Jakobs, Jaeken, & Gibson, 1993; Komatsuzaki, Shima, Kawamoto, Momose, & Kimura, 2005; Pouliot-Mathieu et al., 2013; Somkuti, Renye, & Steinberg, 2012). Furthermore, several studies demonstrated that GABA presented antioxidant, hypolipidemic, and anti-inflammatory properties (Di Cagno et al., 2010; Jeng, Chen, Fang, Hou, & Chen, 2007). Recently, GABA has been used as a major building block for the synthesis of 2-pyrrolidone and biodegradable polyamide nylon 4, opening its application area in the industrial biotechnology (Park et al., 2013).

Due to the potential use of GABA in the food and pharmaceutical fields as a nutritional supplement, the consumption of this amino acid has been actively pursued. The amount of GABA available in vegetables and fruits is quite low and the direct addition of GABA to food is considered unnatural (Kim, Lee, Ji, Lee, & Hwang, 2009; Li & Cao, 2010; Li, Qiu, Huang, & Cao, 2010). Therefore, it is necessary to develop a natural fermentation process to produce and increase GABA levels in food. GABA production by various microorganisms has been reported, including bacteria (Dhakal, Bajpai, & Baek, 2012; Hudec et al., 2015), fungi (Kono & Himeno, 2000) and yeasts (Hao & Schmit, 1993). Recent works have focused their studies on the GABA production ability of lactic acid bacteria (LAB) due to their GRAS (generally recognized as safe) status and their potential use as starter cultures for the manufacture of fermented foods with functional properties (Komatsuzaki et al., 2005; Ratanaburee, Kantachote, Charernjiratrakul, & Sukhoom, 2013; Siragusa et al., 2007). GABA-producing LAB include strains of *Lactococcus* (*Lc.*) *lactis*, *Lactobacillus* (*Lb.*) *brevis*, *Lb. buchneri*, *Lb. helveticus*, *Lb. paracasei*, *Lb. plantarum* and *Streptococcus thermophilus* (Di Cagno et al., 2010; Li & Cao, 2010; Li, Gao, Cao, & Xu, 2008; Lin et al., 2009; Yokoyama, Hiramatsu, & Hayakawa, 2002). *Lb. brevis* strains are the most frequently isolated species from traditional fermented products with the highest GABA productivity (Park & Oh, 2007; Seo et al., 2013). Thus, GABA produced by LAB in appropriate foods can make full use of the health-promoting

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properties of GABA and LAB themselves.

GABA is the result of the conversion of glutamate by glutamate decarboxylase (GAD; EC 4.1.1.15). GAD is a pyridoxal 5′-phosphate (PLP)-dependent intracellular enzyme that catalyzes the decarboxylation of L-glutamate, which is imported into cells by the glutamate GABA antiporter, to produce GABA (Capitani et al., 2003; Sanders et al., 1998).

Domesticated by pre-Hispanic cultures, quinoa (*Chenopodium quinoa*) and amaranth (*Amaranthus caudatus*) crops have been used for feeding of the Andean population since at least 3000 years. These ancestrally grown grains have recently attracted consumer's attention due to their high protein content, nutritional value and gluten-free characteristics. In order to contribute to the development of novel fermented food products with enhanced functional properties, this study aimed to screen GABA-producing LAB, isolated from Andean amaranth (A) and Real Hornillos quinoa (Q_r) sourdoughs. A strain with high capacity to produce GABA, *Lactobacillus brevis* CRL 1942, was selected for further studies. Since fermentation parameters, such as temperature, time and monosodium glutamate (MSG) concentration, play an important role in GABA production, culture conditions were optimized in order to enhance GABA accumulation.

2. Materials and methods

2.1. Microorganisms, media, and growth conditions

The microorganisms used in this study belong to the culture collection of the Centro de Referencia para Lactobacilos (CERELA-CONICET) and were isolated from Andean amaranth (A) and Real Hornillos quinoa (Q_r) sourdoughs (Table 1). MRS broth (Biokar Diagnostics) was used for maintenance of *Lactobacillus* strains and GABA production. In order to investigate GABA production, LAB strains were grown (without shaking) in capped test tubes with 50–270 mM of monosodium glutamate (MSG) at 30 °C.

2.2. Screening of GABA-producing LAB

To analyze GABA production by LAB, strains were grown in MRS medium containing 53 mM of MSG for 96 h at 30 °C. Culture broth was centrifuged at 5000 × *g* for 15 min and GABA production was evaluated from the supernatant fractions using thin layer chromatography (TLC), with a cellulose F aluminum plate (Merck Co.). Briefly, 2 μl of supernatant was spotted onto TLC plates. TLC was conducted using a *n*-butanol:acetic acid:distilled water (5:3:2) solvent mixture, plates were subsequently immerged into 0.4% (w v^{-1}) ninhydrin solution and, then heated to visualize the spots.

Table 1
Lactic acid bacteria strains tested in this study for GABA production and their isolation sources.

Microorganism	Strain code	Source (sourdoughs)
Lactobacillus rhamnosus	CRL 1891	Andean amaranth
Lactococcus lactis	CRL 1895	Real Hornillos quinoa
Enterococcus mundtii	CRL1896	Real Hornillos quinoa
Lactobacillus plantarum	CRL 1898	Real Hornillos quinoa
Enterococcus casseliflavus	CRL 1899	Real Hornillos quinoa
Enterococcus mundtii	CRL 1900	Real Hornillos quinoa
Pediococcus pentosaceus	CRL 1902	Andean amaranth
Enterococcus casseliflavus	CRL 1903	Andean amaranth
Lactobacillus plantarum	CRL 1905	Real Hornillos quinoa
Lactobacillus plantarum	CRL 1906	Real Hornillos quinoa
Lactobacillus brevis	CRL 1942	Real Hornillos quinoa
Lactobacillus brevis	CRL 1951	Real Hornillos quinoa
Enterococcus mundtii	CRL 1953	Andean amaranth
Lactobacillus plantarum	CRL 1956	Andean amaranth
Lactobacillus plantarum	CRL 1957	Andean amaranth
Lactobacillus brevis	CRL 1959	Real Hornillos quinoa
Lactobacillus brevis	CRL 1960	Real Hornillos quinoa
Lactobacillus brevis	CRL 1961	Real Hornillos quinoa
Lactobacillus brevis	CRL 1962	Real Hornillos quinoa

2.3. GABA measurements

Levels of GABA were quantified by HPLC and the GABase method (Tsukatani, Higuchi, & Matsumoto, 2005). The supernatant obtained as described above was filtered through a 0.45 μm membrane filter (Millipore) and the GABA content was analyzed by using a HPLC system equipped with a Hypersil ODS C18 reverse-phase column (Gemini, 110, 150 × 4.6 mm, 5 μm particle). The mobile phases used were A (40 mM NaH_2PO_4, pH 6.4) and B (acetonitrile:methanol:H_2O; 45:45:10, vol:vol:vol). Each sample was derivatized with *o*-phthaldealdehyde 3-mercaptopropionic acid (OPA-3MPA, Schwarz, Roberts, & Pasquali, 2005). OPA-3MPA-derivatized samples were separated at a column temperature of 40 °C and a flow rate of 1.0 ml min^{-1} according to the gradient method (Schwarz et al., 2005). GABA derivatized with OPA-3MPA was detected by a fluorescence detector with excitation at 340 nm, emission at 450 nm. GABA content was calculated from the integrated peak area based on comparison with standard curves constructed using a commercial GABA standard (Sigma–Aldrich Co., St. Louis, MO, USA).

In the GABase method, 0.08 M Tris–HCl buffer (pH 8.9), 5 mM α-ketoglutarate, 3.3 mM 2-mercaptoethanol, 1.2 mM $NADP^+$, and 0.03 U of GABase was added to each well of a 96-well microtiter plate. The mixture was warmed at 25 °C, and then the standard or sample (culture supernatants) solution was added. The formation of NADPH was measured at 340 nm every 1 min for 10 min at 25 °C in a Biotek Synergy HT microplate. The concentration of GABA in samples was calculated from the calibration curve of the standard solutions (0.1, 0.25, 0.5 and 1 mM GABA).

2.4. Optimization of parameters for GABA production

Optimal culture conditions to enhance GABA production were determined by measuring the extracellular GABA content in *Lb. brevis* CRL 1942 with various glutamate concentrations (25, 50, 160, 270 and 400 mM) and cultivation temperatures (22, 25, 30 and 37 °C) in MRS medium. At 45 °C, GABA could not be detected since the strain was not able to grow.

2.5. Bacterial growth, pH and viability

Lb. brevis CRL 1942 was cultivated in MRS broth with or without 270 mM MSG at 30 °C for 144 h. Bacterial growth was determined by measuring culture turbidity at 550 nm at different time intervals. Culture pH was measured at the same intervals. For viable count, samples collected at different times, were diluted to 10^{-7}-10^{-9} and inoculated onto MRS agar plates, which were incubated at 30 °C for 48 h. Viable colonies were counted and expressed as log CFU (colony forming unit) ml^{-1}.

2.6. Statistical analyses

Statistical analyses were performed with the software package Minitab 14 (Minitab Inc.) using ANOVA general linear models followed by Tukey's post hoc test, and $P < 0.05$ was considered significant. Unless otherwise indicated, all values were the means of three independent trials ± standard deviation. No significant differences were observed between individual replicates.

3. Results and discussion

3.1. Screening of GABA-producing LAB

Screening of different microorganisms that have the ability to produce GABA is important for the food industry since it provides natural GABA, a bioactive agent capable of modulating the health functionality and offering the consumer new and attractive food products (Hiraga, Ueno, & Oda, 2008; Komatsuzaki, Nakamura, Kimura, & Shima, 2008; Siragusa et al., 2007). Screening of GABA-producing LAB demonstrated that, among the bacteria analyzed, only 3 strains (*Lb. brevis* CRL 1942, *Lb. plantarum* CRL 1956 and *Lb. plantarum* CRL 1957) clearly showed GABA production (Fig. 1). The extracellular GABA content in these strains was quantified by HPLC, demonstrating that *Lb. brevis* CRL 1942 was the most efficient microorganism for the conversion of MSG to GABA, reaching 50 mM after 96 h cultivation (Fig. 2). This microorganism was selected for further studies.

3.2. Optimization of culture conditions for GABA production

3.2.1. Effect of MSG concentration

GABA production can be improved by adjusting the culture conditions, such as temperature and glutamate concentration, which are considered as the most common fermentation factors that affect GABA synthesis. Taken this into account, GABA production was optimized by measuring the extracellular GABA content in *Lb. brevis* CRL 1942 culture at different glutamate concentrations and cultivation temperatures in MRS medium. Glutamate is transformed in GABA by glutamate decarboxylase in several LAB (Li & Cao, 2010). Therefore, it is necessary the incorporation of sodium glutamate to the medium or foodstuff at a high concentration to produce GABA. Here, a gradual increase of GABA yield was observed when MSG addition increased from 0 to 270 mM (Fig. 3A). However, higher concentrations resulted in a decrease of the amino acid production, maybe due to an increase in the osmotic pressure of the cells, disturbing the bacteria metabolism and generating the decrease of GABA yield (Komatsuzaki et al., 2008). Other authors showed a linear increase of GAD activity with increasing substrate-glutamate concentration in yeasts (Tong et al., 2002), probably caused by increases of the substrate in the vicinity of the cytosolic GAD, which suggests that GAD activity and GABA content were regulated by glutamate addition (Komatsuzaki et al., 2005; Shelp, Bown, & McLean, 1999).

3.2.2. Influence of the temperature on GABA production

The effect of temperature on the GABA yield was also investigated. A higher GABA production was observed at 30 °C than that at 22, 25 and 37 °C, reaching values of ~255 mM within a 96-h culture

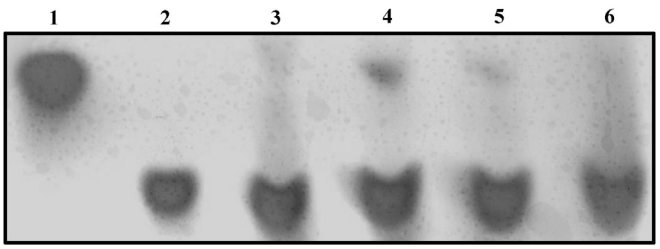

Fig. 1. Thin layer chromatography analysis of GABA synthesis. 1- GABA standard; 2- MSG standard; 3- MRS supplemented with 53 mM MSG (control); 4- Supernatant of *Lb. brevis* CRL 1942, 5- Supernatant of *Lb. plantarum* CRL 1956; 6- Supernatant of *Lb. plantarum* CRL 1957. All strains were grown in MRS medium containing 53 mM MSG at 30 °C for 96 h. Data are representative of three independent experiments.

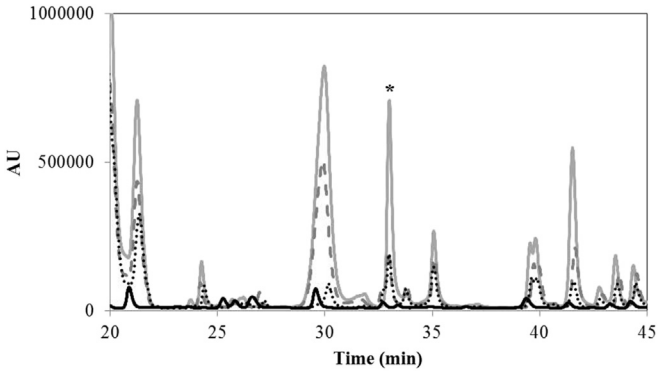

Fig. 2. HPLC chromatogram of GABA production. HPLC profiles from non-inoculated MRS medium (solid black line), *Lb. brevis* CRL 1942 (solid grey line), *Lb. plantarum* CRL 1956 (dashed line) and *Lb. plantarum* CRL 1957 (dotted line) supernatants. Asterisk indicates GABA (RT = ~33 min). HPLC system equipped with a Hypersil ODS C18 reverse-phase column (Gemini, 110 Å, 150 × 4.6 mm, 5 μm particle). Solvent A: 40 mM NaH_2PO_4, pH 6.4. Solvent B: acetonitrile:metanol:H_2O (45:45:10 v/v/v), with a constant flow-rate of 1 ml min^{-1}, excitation at 340 nm and detection at 450 nm. Data are representative of three independent experiments.

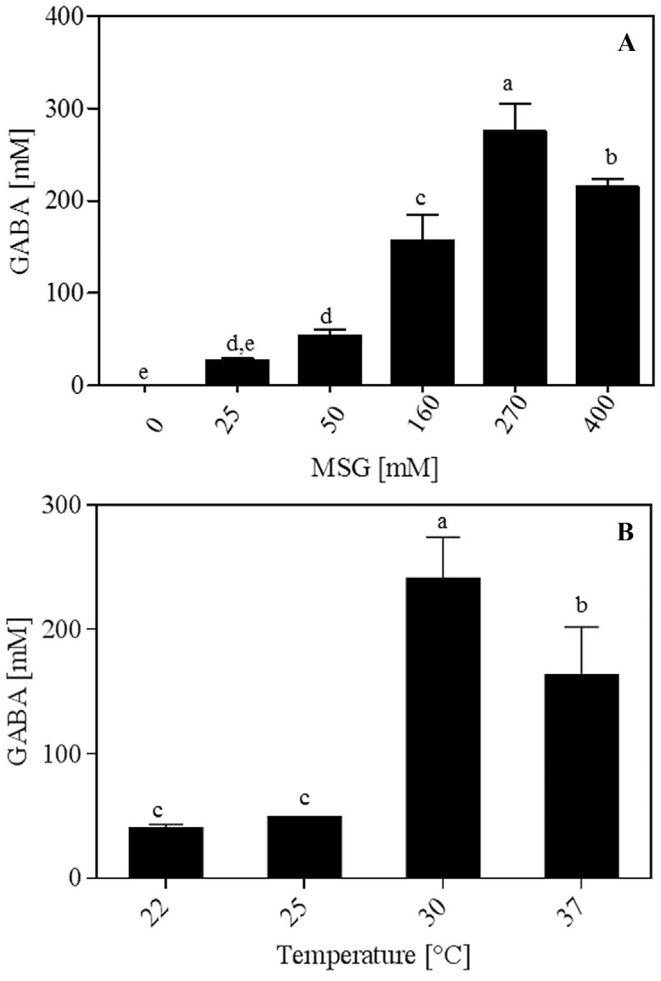

Fig. 3. Effect of MSG concentration and temperature on GABA production by *Lb. brevis* CRL 1942. (A) Cells were grown in MRS medium for 96 h at 30 °C; (B) Cells were grown in MRS medium for 96 h with 270 mM MSG. GABA was quantified by the GABAse method (Sigma) from the cultures supernatants. Each point represents the average ± standard deviation of GABA production from three independent experiments. Means for each time point without a common letter differ significantly ($P < 0.05$).

period with 270 mM MSG (Fig. 3B). This temperature was in agreement with the optimum temperature of purified GAD in *Lb. brevis* (Huang, Mei, Wu, & Lin, 2007; Kim et al., 2009; Park, Jeong, & Kim, 2014). At 45 °C, GABA content could not be detected since cell growth was not observed. Taken together, these results suggest that GABA increase depends on glutamate addition to the culture medium, being 270 mM of MSG and 30 °C the optimal conditions for GABA production by *Lb. brevis* CRL 1942.

3.2.3. Influence of fermentation time on GABA production

Since fermentation time is another key factor to consider for GABA production, a time course analysis following cell growth (OD_{560nm}), cell viability, pH and GABA synthesis of *Lb. brevis* CRL 1942 in MRS medium containing 270 mM MSG at 30 °C was performed (Fig. 4). The stationary growth phase was reached after 24 h of fermentation, with a cell number of 6×10^9 CFU ml^{-1} (Fig. 4A and B). The pH dropped to about 5 within 24 h, and then started to increase (Fig. 4C), concomitant with the appearance of GABA in the culture medium (Fig. 4D). This increase in the pH values would be related to the removal of hydrogen ions by the decarboxylation process and the exchange of extracellular glutamate for a more alkaline substrate, such as GABA, which contributes to local alkalization of the extracellular environment (De Biase & Pennacchietti, 2012). In addition, GABA production occurred in a time-dependent manner, and yielded the greatest amount of GABA (~255 mM) after 48 h, showing a conversion rate of ~90% from the supplied MSG (Fig. 4D). No further increased of GABA was found until 144 h. This may be due since, when cells entered into the exponential phase, the pH begin to decrease, activating the GAD enzyme, which has optimal catalytic activity at low pH (Hiraga et al., 2008; Huang et al., 2007; Lin, 2013). Subsequent, GABA production increases the pH medium, diminishing GAD activity. Some authors have overcame this problem by employing a GAD mutant that has catalytic activity in expanded pH range up to pH 7, enhancing production of GABA by modulating cell growth and enzymatic activity (Choi et al., 2015; Shin et al., 2014). It should be noted that cell growth was not affected in cultures without MSG, when compared to the GABA-producing condition, implying that the difference in GABA levels could not be attributed to differences in cell numbers (Fig. 4A and D). However, addition of glutamate increased viable cells of *Lb. brevis* CRL 1942 by ~1 log, indicating a correlation between survival and GABA production, probably due to the alkalization of the medium by the generated metabolite.

4. Conclusions

In summary, *Lb. brevis* CRL 1942, isolated from quinoa sourdough, was able to produced high levels of GABA in the presence of 270 mM MSG at 30 °C for 48 h, with a conversion efficiency of ~90%. Also, it was demonstrated that initial glutamate concentration and temperature had a significant effect on GABA production, two factors that markedly affected the decarboxylase activity. To our knowledge, the obtained GABA levels are one of the highest among lactobacilli grown in batch culture. Our results provide a scientific basis to industrialized, in an eco-friendly way, production of GABA-enriched sourdoughs. Novel information about LAB with the ability to synthesize high levels of GABA is an important breakthrough for the development of health-promoting functional foods. Furthermore, it has been suggested that due to the beneficial health effects of GABA, the ability of lactobacilli strains, natural inhabitants of the

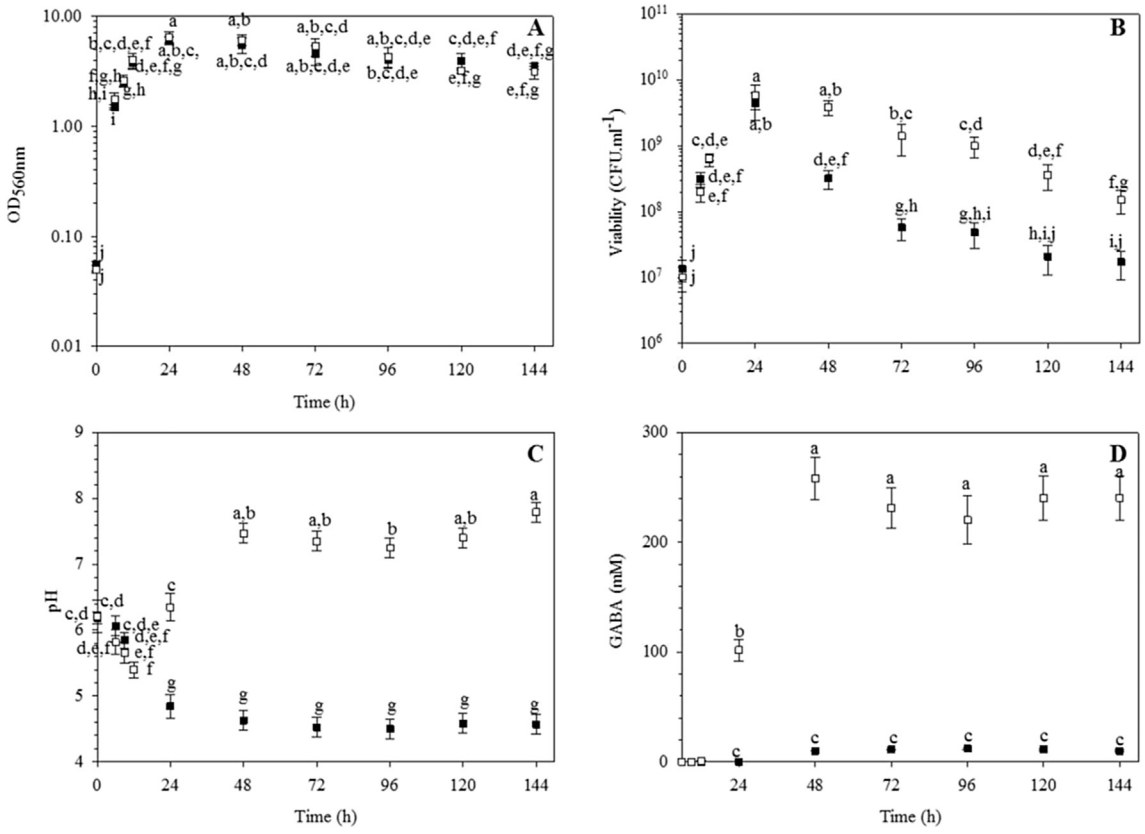

Fig. 4. Time profile of cell growth (A), viability (B), pH (C) and GABA production (D) by *Lb. brevis* CRL 1942. The strain was grown in MRS medium containing 270 mM MSG (□) and without MSG (■), at 30 °C for 144 h. GABA was quantified by the GABase method (Sigma) from the cultures supernatants. Each point represents the average ± standard deviation of GABA production from three independent experiments. Means for each time point without a common letter differ significantly ($P < 0.05$).

intestine, to convert dietary glutamate to GABA, may be considered as a novel probiotic trait.

Acknowledgments

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