




Gamma-aminobutyric acid (GABA) production in milk fermented by specific wild lactic acid bacteria strains isolated from artisanal Mexican cheeses

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Abstract

Purpose: The purpose of this study was to screen wild GABA-producing lactic acid bacteria (LAB) isolated from artisanal Mexican cheeses and to evaluate the fermentation conditions for the enhancement of the GABA yield in fermented milk.

Methods: A qualitative test was carried out to select the GABA-producing LAB and the GABA was quantified by reversed-phase high-performance liquid chromatography in fermented milk (FM). Two inoculum concentrations (10^7 and 10^9 CFU/mL), two incubation temperatures (30 and 37 °C), three glutamate concentrations (1, 3, and 5 g/L), and three pyridoxal 5'-phosphate (PLP) concentrations (0, 100, and 200 μM) were assessed to establish suitable conditions to enhance the GABA yield in FM.

Results: Results showed that, from a total of 94 LAB strains, fermented milk with two *Lactococcus lactis* strains (L-571 or L-572) presented the highest GABA production. However, 37 °C of incubation and 10^9 CFU/mL and 3 g/L of glutamate significantly led the highest GABA yield in FM with L-571. Further studies are needed to establish the optimum conditions for producing GABA by this strain, and in vivo studies may reveal its potential use as GABA-producing culture.

Conclusion: These results highlight the importance of wild LAB strains in order to generate new alternatives and opportunities in the development of functional foods containing GABA.

Keywords: GABA, Lactic acid bacteria, Artisanal Mexican cheeses, Fermented milk

Introduction

In recent years, interest in the production of GABA has increased because of its multiple beneficial effects. GABA may act as an inhibitory neurotransmitter and also have antianxiety, antidepressant, and antihypertensive properties. Additionally, the consumption of GABA may inhibit the proliferation of cancer cells, be used in the treatment of Alzheimer's and Parkinson's disease, regulate thyroid

hormone levels, and improve the immune system (Diana et al. 2014a). Recent research has focused on natural ways of producing GABA as a bioactive compound for use in the pharmaceuticals and food industries. In particular, fermentation by LAB may produce GABA in its most natural and suitable form (Xu et al. 2017).

LAB produces GABA during fermentation as a defense mechanism to maintain viability under acidic conditions. The glutamate decarboxylase (GAD) of LAB uses pyridoxal 5'-phosphate (PLP) as a cofactor; when activated, it catalyzes the α -decarboxylation of glutamic acid or its salts present in the medium (Shi and Li 2011; Xu et al. 2017). The GABA-producing ability of different LAB strains may vary and depends on different fermentation

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parameters or additives, including pH, temperature, cultivation time, PLP addition, and medium composition (Song and Yu 2017).

Various GABA-producing bacteria have been isolated from tea leaves, fermented fish, kimchi, and yogurt (Komatsuzaki et al. 2005; Jeng et al. 2007; Lu et al. 2008). Further studies have focused on isolating GABA from new sources or on screening new GABA-producing LAB with a high biosynthesis capacity performed by wild strains isolated from nature (Franciosi et al. 2015). Actually, recent investigations reported a high concentration of GABA in “Crema de Chiapas cheese,” a Mexican artisanal cheese, suggesting the presence of LAB with the capability to synthesize GABA (Gonzalez-Gonzalez et al. 2019). In this sense, artisanal Mexican dairy products possess an undiscovered variety of LAB that may be a rich source of strains with considerable GABA production capacity. Therefore, to provide a basis for the potential development of new health-promoting fermented dairy products, the aim of the present study was to screen wild GABA-producing LAB isolated from artisanal Mexican cheeses and to evaluate the fermentation conditions for the enhancement of the GABA yield in fermented milk (FM).

Materials and methods

Strains and growth conditions

A total of 94 LAB strains of different genera (*Lactobacillus* spp., *Lactococcus* spp., and *Streptococcus* spp.) were assessed. All the strain cultures used (stored at -80°C in glycerol 10%) are part of the Dairy Laboratory collection in the Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD, Hermosillo, Sonora, Mexico). These strains were isolated from the processes of three artisanal Mexican cheeses (*Lactobacillus* spp. from Cocido cheese (Heredia 2011), *Lactococcus* spp. from Chihuahua cheese (Gutierrez 2008), and *Streptococcus* spp. from Fresco cheese (Serrano 2012)). The *Lactococcus* strains were reactivated by inoculating 1% (v/v) of each culture in sterile M17 broth (BD Difco; Spark, MD, USA) supplemented at 5% (v/v) with a sterile 10% lactose solution (BD Difco, Spark, MD, USA). The *Lactobacillus* and *Streptococcus* strains were reactivated by inoculating 1% (v/v) with MRS broth (BD Difco; Spark, MD, USA). The strains were then incubated for 24 h at 30°C and 37°C , respectively. This procedure was repeated twice to obtain fresh cultures with a final concentration of 10^7 CFU/mL (Rodríguez-Figueroa et al. 2010).

Qualitative test for the detection of GABA-producing strains

To select strains that potentially produce GABA, a GAD colorimetric assay was carried out. For this purpose, 5

mL of each fresh culture was centrifuged (Sorvall ST 16 centrifuge, Thermo Scientific, Chelmsford, MA, USA) at $4696\times g$ for 20 min at 25°C . The pellet was recovered and resuspended in 5 mL of a 0.9% NaCl solution. Afterwards, the suspension was centrifuged again under the same conditions and the pellet was homogenized with 0.5 mL of a GAD solution (1 g of 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), followed by an incubation at 37°C during 4 h under anaerobic conditions (Cotter et al. 2001). After this time, the colorimetric reaction was visually observed and all the strains that turned the color green to blue were selected as it was shown by the *Lactobacillus brevis* (NBRC 12005) strain used as a positive control (Lacroix et al. 2013).

Production of FM

The selected GABA-producing strains were evaluated for their ability to produce GABA in FM. A starter culture was prepared from each strain by adding fresh culture (3%, v/v) into 5 mL of 10% (p/v) sterile (110°C , 10 min) reconstituted commercial semi-skimmed milk (ALPURA®), which its composition was of 46.9% of total carbohydrates, 29.5% of protein, and 13% of fat. The inoculated milk was incubated for 12 h at 30°C (*Lactococcus* spp.) or 37°C (*Lactobacillus* spp.). After the incubation period, a 3% (v/v) aliquot of this starter culture was taken and added into 10 mL of 10% (w/v) sterile reconstituted semi-skimmed milk and finally incubated for 48 h at 30°C or 37°C . pH was determined at the time of milk inoculation (0 h) and at 48 h of fermentation.

Preparation of aqueous extracts

After the incubation process, the fermentation was stopped by applying heat treatment (75°C , 15 min) followed by a quick cooling in order to inactivate LAB. Samples of 10 mL were collected from each FM and centrifuged (Thermo Scientific, Chelmsford, MA, USA) at $4696\times g$ for 1 h at 4°C , and the supernatants were recovered and storage at -80°C for further analyses (Beltrán-Barrientos et al. 2018b).

Quantification of GABA in FM

The GABA production in aqueous extracts from FM was quantified by reversed-phase high-performance liquid chromatography in a Series 1260 Infinity HPLC system (Agilent Technology, Waldbronn, Germany). Aliquots of 2 mL were centrifuged (Eppendorf, Hauppauge, NY, USA) at $12000\times g$ for 10 min at room temperature to remove solids and bacterial cells and were then ultra-filtered through membranes with a pore size of < 3 kDa (Pall Life Sciences, Port Washington, NY, USA) (Wu and Shah 2016). Afterwards, a derivatization of the

extracts was made in a precolumn following the methodology of the 6-aminoquinolyl-n-hydroxy succinimidyl carbamate commercial kit AccQ-Tag (Waters Corporation, Ciudad de México, México).

Chromatographic separation was performed using an AccQ-Tag C18 column (1.7 μm , 2.1 \times 100 mm) maintained at a constant temperature (37 °C). Mobile phase A consisted of an AccQ-Tag ultra-eluent A, and phase B consisted of AccQ-Tag ultra-eluent B. Finally, phase C consisted of an eluent of Milli-Q water. The gradient separation times were set as follows: (A:B:C), 0 min, (100:0:0), 0.5 min (100:0:0), 18 min (95:5:0), 19 min (91:9:0), 29.5 min (83:17:0), 38 min (0:60:40), and 41 min (100:0:0). Detection was performed by UV absorbance at 254 nm to quantify GABA (Diana et al. 2014b).

Strategies to enhance the GABA production

The LAB strains that showed the highest GABA production were selected to assess the conditions to enhance the production of GABA in FM. The selected LAB strains were subjected to evaluate the conditions of fermentation in milk (enriched with 1 g/L of glutamate): two concentrations of inoculum (10^7 and 10^9 CFU/mL) and two different incubation temperatures (30 or 37 °C). After selecting those fermentation conditions with the highest GABA production, three concentrations of glutamate (1, 3, and 5 g/L) and three concentrations of PLP (0, 100, and 200 μM) were assessed one factor at a time, to establish the suitable conditions to enhance the GABA production for each strain. The milk was enriched with these additives before the fermentation process. It is important to mention that preliminary results (data not shown) revealed that the selected conditions (glutamate, PLP concentrations, temperatures, and inoculum concentrations) did not affect the survival of lactic acid bacteria. The quantification of GABA was performed as described in the previous section.

Statistical analysis

A one-way analysis of variance (ANOVA), through a completely randomized design, was used to analyze data corresponding to pH and GABA concentration in order to select the best strains, glutamate concentration, and PLP concentration. Additionally, a general linear model (GLM) ANOVA was applied to select the fermentation conditions (inoculum concentration and incubation temperature). All these analyses were applied to determine which conditions may exert a greater effect on GABA production in FM. All the experiments were carried out in triplicate. When differences ($p \leq 0.05$) among means were found, the Tukey-Kramer test was applied and all the analyses were performed by using the NCSS 2007 statistical software (Hintze 2006). The figures were made using the program GraphPad Prism version 5.0.

Results and discussion

Qualitative colorimetric assay of GABA-producing strains

The colorimetric assay allowed us to analyze qualitatively the strains that may potentially synthesize GABA in the culture medium or food matrix (Yu et al. 2011). The results indicated that, from the 54 *Lactobacillus* spp., 16 strains (29.6%) and, from 8 *Lactococcus* spp., 3 strains (37.5%) were able to produce GABA; however, *Streptococcus* spp. strains did not show the capacity to synthesize GABA (Table 1). Since this colorimetric assay uses the green bromocresol compound, which is highly sensitive to proton consumption and referring to the color difference, qualitatively indicates which strains display the production of GABA. This behavior of LAB is directly related to the degree of activity of GAD, since this enzyme is responsible to convert glutamate to GABA. Therefore, the 19 positive GABA-producing strains (20.2% from 94 strains assessed) were closely related to the presence and activation of the GAD enzyme (Komatsuzaki et al. 2008).

The production of GABA by fermenting microorganisms is a defense mechanism in response to different types of stress, mainly exposure to low pH, osmotic

Table 1 Lactic acid bacteria used for the selection of GABA-producing strains

	Strains codes					
<i>Lactobacillus</i> spp. ^a	J20	J22	J23	J24	J25	
	J26	J27	J28	J31	J32	
	J34*	J37	J38	MQ22*	SD22	
	SQ31	LR32	LR14	CR24*	SQ33	
	LQ24*	MR34	LM32	S3	SS22*	
	SS11	LS12	SQ24	L20	L8	
	LD12*	QR24*	L6	S37	LR24	
	QR33*	LD24*	LM12	LS24*	SD11*	
	SR31*	QR21*	MQ31*	CD32*	CD33*	
	S33	S26	B26	L49	L21	
	L2 Lb C	L2 Lb D	C2 Lb B	C2 Lb C		
	<i>Streptococcus</i> spp. ^b	L1 St A	L2 St A	L2 St B	L2 St C	Q2 St A
		Q2 St B	Q2 St C	Q2 St D	St26	St 27
St 28		St 32	St 42	St 45	St 50	
St 53		St 54	St 35	St 36	St 38	
St 39		St 40	St 41	St 44	Q7 St A	
Q7 St A		Q7 St B	Q7 St C	Q7 St D	L7 St A	
L7 St D		S7 St D	C7 St B			
<i>Lactococcus</i> spp. ^c	L-572*	L-599	Q3	L-600	L-598*	
	L-571*	E3	C1			

^aGABA-producing strains

^bLAB strains isolated from Cocido cheese

^cLAB strains isolated from Fresco cheese

^dLAB strains isolated from Chihuahua cheese

stress, and nutrient deficiency during fermentation. The GAD system converts the glutamic acid molecule in GABA while consuming an intracellular proton (hydrogen ion) (Feehily and Karatzas 2013). The net effect is to reduce the concentration of protons within the cell, decreasing the acidification of the cytoplasm and diminishing any changes in pH (Le Vo et al. 2012).

Furthermore, *Lactobacillus* is one of the genera most reported as GABA producers and these strains have been isolated from milk, cheese, fermented milk, and vegetable beverages, and the presence of the GAD has been identified (Siragusa et al. 2007; Di Cagno et al. 2010; Nejati et al. 2013; Franciosi et al. 2015). Even though studies have reported *Lactococcus* spp. as GABA producers, which have been mainly isolated from dairy products and vegetables, only a few of them have focused on the GAD activity (Lu et al. 2009; Nejati et al. 2013); however, interestingly, 37% from the *Lactococcus* spp. strains assessed in the present work showed this enzymatic activity by the qualitative test.

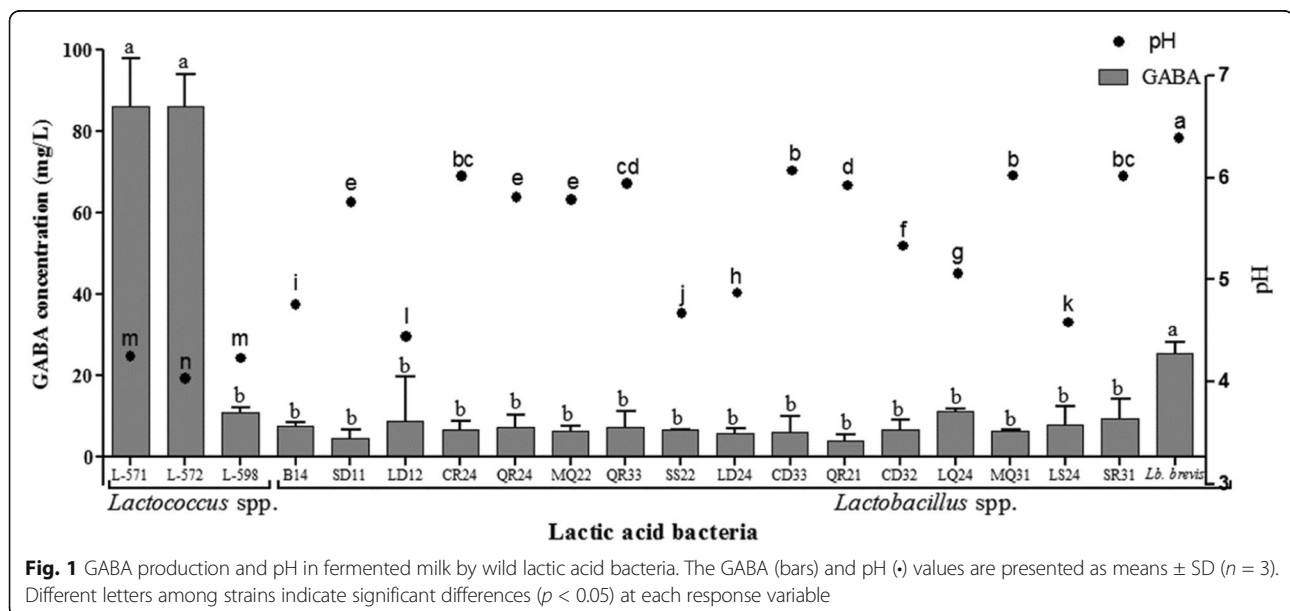
On the other hand, *Streptococcus* spp. strains have been scarcely reported as GABA producers. In a previous study, the optimum conditions for GAD activity by a specific strain of *Streptococcus salivarius* were assessed (Yang et al. 2008). In the present study, *Streptococcus* spp. was found as non-GABA-producing strains. The screening of strains as GABA producers through the use of molecular techniques has been applied to identify the GadA and GadB genes, which encodes the expression of the GAD enzyme in the strains (Sanchart et al. 2017). Nevertheless, it is also reported that the presence of GAD⁺ phenotypes of non-GABA-producing strains related to *Streptococcus* spp. genus may be usually found (Somkuti et al. 2012). Consequently, the 19 selected

strains and the positive control *Lactobacillus brevis* NBRC 12005 were used to ferment milk and to determine the resulting GABA concentrations.

GABA quantification in FM

The concentration of GABA was variable among the fermented milk by each strain (Fig. 1). Overall, after 48 h of fermentation, the FMs with *Lactobacillus* spp. strains produced 4.2–11.2 mg/L of GABA and were not significantly different ($p > 0.05$) among them. These concentrations were lower ($p < 0.05$) than that of *Lactobacillus brevis* NBRC 12005, used as a positive control, which was found to generate 25.7 ± 2.26 mg/L of GABA. This prior strain is known to present high GAD activity; therefore, it is widely used in qualitative and quantitative methods to determine potential GABA-producing strains (Sheng-Yuan et al. 2006).

Although the strains assessed produced GABA in lower concentrations, several studies have reported that *Lactobacillus* spp. are capable of producing different amounts of GABA. Thus, various studies have been carried out in order to evaluate the production of GABA in different substrates, such as culture media, vegetable beverages, meat products, seafood, and mainly dairy products (Di Cagno et al. 2010; Ratanaburee et al. 2013; Shan et al. 2015; Taoka et al. 2019). All these studies report a wide range of GABA production, which naturally depends mainly on the glutamate concentration in the food matrix and the LAB strain used; however, the highest GABA productions have been displayed when the fermentation conditions were modified. For example, yogurt added with *Lactobacillus plantarum* NDC75017 showed 500 mg/kg of GABA production, which was related to the activity of the GAD enzyme. However, it



was shown that biomass of the bacterium during fermentation as well as glutamate concentration, positively influenced the GABA production reaching 3145.6 mg/kg (Shan et al. 2015). In another study, *Lactobacillus brevis* 877G produced 1.95 g/L of GABA in skim milk. The use of a mixture of *Lactobacillus brevis* 877G and *Lactobacillus sakei* 795 (1:1) further enhanced GABA production to 2.32 g/L (Seo et al. 2013). It is important to mention that the co-fermentation of different bacteria may increase GABA production due to the competition between the strains in the medium or stress generated in the environment, which may therefore be important conditions to enhance the GABA production.

On the other hand, the FM with *Lactococcus* L-598 showed a GABA production of 11.0 ± 1.18 mg/L and was not significantly different ($p > 0.05$) from those of *Lactobacillus* spp. assessed in the present work. Furthermore, FM by L-571 and L-572 produced the highest concentration of GABA (86.0 ± 11.9 and 86.2 ± 7.7 mg/L, respectively) and were not significantly different ($p > 0.05$) between them, but were significantly different ($p < 0.05$) from all the FM. Additionally, it is also important to highlight that these FM produced 3.4-fold GABA than the FM by *Lactobacillus brevis*.

Notably, the use of *Lactococcus* spp. strains has become increasingly important in the production and/or fermentation of dairy products for the generation of bioactive compounds (Hagi et al. 2016). Nevertheless, to the best of our knowledge, only a few studies have been focus on the GABA production by *Lactococcus* spp. strains. In this sense, Nomura et al. (1999) previously reported that fermented skim milk with two *Lactococcus lactis* strains ATCC 19435 or ATCC 13675 were able to produce GABA equivalent to 21.69 mg/L and equivalent to 23.30 mg/L, respectively. Moreover, another study reported that *Lactococcus lactis* NCDO 2118, isolated from frozen peas, produced 20.62 mg/L of GABA in a chemically defined medium (Mazzoli et al. 2010). Although GABA production of the mentioned studies was similar to our positive control strain, *Lactobacillus brevis* NBRC 12005 (25.7 mg/L), in the present study, our results reveal that our *Lactococcus* strains presented a high potential to being considered as GABA producers. On the other hand, some studies have been carried out using GABA-producing *Lactococcus* spp. strains in different substrates, such as culture media and vegetable beverages (Lu et al. 2009; Laroute et al. 2016); however, dairy products have been the most assessed (Gardner-Fortier et al. 2013; Nejati et al. 2013). In this sense, *Lactococcus* spp. strains were evaluated to produce GABA under different fermentation conditions, and it was revealed that they presented a high potential as GABA producers if suitable conditions are established, such as the adjustment of the substrate concentration, temperature, and pH (Lu et al. 2009).

The acidifying capacity of *Lactococcus* spp. strains were also determined in our study, and we found diverse capacities, ranging 4–6 of pH (Fig. 1), suggesting a strain-dependent behavior. Overall, the FM with the *Lactococcus* spp. were significantly lower ($p < 0.05$) than those of *Lactobacillus* spp., and specifically strains L-571 and L-571 showed the lowest pH, suggesting that the acidifying capacity may be related with the capacity to produce GABA. In fact, it has been reported that the GAD enzyme optimally acts on the bioconversion of glutamic acid to GABA at a pH of 4.4 (Huang et al. 2016), which may explain the high production of strains L-571 and L-571. Nevertheless, L-598, LD12 and LS24 presented pH values lower than 4.5, presenting significantly lower ($p < 0.05$) concentrations of GABA compared to the FM with L-571 or L-572 strains. In these particular cases, the low GABA production may be associated with other factors, such as their optimum growing conditions (Minervini et al. 2009) or the expression of the GAD enzyme of the strains (Li et al. 2013). In addition, some authors have suggested that in some strains, there may be deficiencies in the transport system of glutamate-GABA towards the inside and outside of the cell (Feehily and Karatzas 2013) or that the assimilated glutamate could be used in other metabolic pathways, which would limit the production of GABA (Fernandez and Zuniga 2006).

In another study, the acidifying potential of *Lactococcus* spp. strains was analyzed in order to evaluate how acidity influences the biosynthesis of GABA in FM enriched with glutamate. The results showed that 70 mg/L of GABA were produced in FM (Gardner-Fortier et al. 2013). Different LAB isolated from Japanese fermented products (sea products) were assessed for their potential to produce GABA at different pH ranges. The highest GABA production was found at a pH between 5 and 6 (8000 mg/kg), decreasing the concentration of GABA at lower pH (< 1000 mg/kg) and the level of production was specific to each strain (Barla et al. 2016).

The current research trend of assessing GABA production in food matrices is focused on the standardization and/or optimization of fermentation processes in order to increase GABA concentrations (Tajabadi et al. 2015). In this sense, our results showed that *L. lactis* were promising; however, since it is reported that 100–120 mg/L of GABA is required to exert beneficial effects (Inoue et al. 2003), strategies to enhance the GABA yield should be applied in an attempt to achieve this target. According to the results of the present study, the *Lactococcus* spp. strains L-571 and L-572 presented the greatest potential for producing GABA; hence, they were selected to evaluate whether the modification of fermentation conditions or the co-fermentation of these strains would increase the GABA production.

Modification of fermentation conditions to increase GABA synthesis in FM

It has been widely reported that GABA production by LAB is affected by culture conditions and the medium composition (Li and Cao 2010). Therefore, in the present study, the fermentation conditions and medium compositions were modified to enhance GABA production. The selected working strains were NRRL B-50571 (L-571) and NRRL B-50572 (L-572).

The results of the fermentation conditions (inoculum concentration and incubation temperature) are depicted in Fig. 2. L-571 showed the highest GABA production with a concentration of inoculum of 10^7 CFU/mL (30 °C) and 10^9 CFU/mL (37 °C) with 915.6 ± 133.2 and 897.7 ± 6.3 mg/L, respectively, and were not significantly ($p > 0.05$) between them. However, FM with an inoculum of 10^9 CFU/mL incubated at 37 °C during 48 h was selected for further analysis. Likewise, the same fermentation conditions were selected for L-572, since they allowed to significantly ($p < 0.05$) generate the highest GABA production (138.2 ± 24.2 mg/L).

Overall, according to the temperature and inoculum concentrations assessed, 37 °C and 10^9 CFU/mL were the conditions that displayed the best GABA production, since they allowed to enhance 10-fold and 1.6-fold of GABA production for L-571 and L-572, respectively, in milk enriched with 1% of glutamate. Therefore, these conditions were selected for each FM. It was previously reported that the optimal temperature for GABA production with LAB ranges 30–50 °C and was concluded that it is strain-dependent (Li and Cao 2010). In fact, the optimum fermentation conditions for some LAB may not match the optimum GABA synthesis conditions (Dhakal et al. 2012), since it is widely reported that

Lactococcus lactis spp. optimally grows at 30–32 °C (Chen et al. 2015); however, 37 °C allowed the highest GABA production.

Therefore, controlling the fermentation process may be used to optimize the GABA production. In this sense, Yang et al. (2008) carried out a controlled experiment in two stages testing different pH and temperature conditions to achieve optimum GABA production. The GAD activity for *Streptococcus salivarius* subsp. *thermophilus* Y2 was evaluated and they reported that after adjusting the optimal temperature at 40 °C and pH at 4.5, the GAD activity allowed an increase in GABA production up to 1.76-fold (Yang et al. 2008).

To the best of our knowledge, there are only a few reports on the effect of different inoculum concentrations in terms of poblational density for optimizing GABA production in FM. However, a study carried out an optimization analysis of GABA production in a chickpea-based beverage using *Lactobacillus plantarum* M-6 and the inoculum (on the basis of 10^7 CFU/mL) was added in different concentrations (3%, 5%, and 7%). They concluded that inoculation at 7% resulted in the highest GABA production, because a higher concentration of inoculum may produce more cellular biomass, possibly allowing greater conversion of glutamate to GABA (Li et al. 2016). Similarly, in the present study, results indicated that the more cellular biomass, the more GABA production was enhanced. Lastly, the selected fermentation conditions for each FM were used for further analyses, in order to assess the effect of different glutamate concentrations on the GABA production. In addition, a co-fermentation of both strains (L-571/L-572, 1:1) was also assessed.

The results of the effect of different glutamate concentrations on the GABA production are shown in Fig. 3. It was observed that by enriching glutamate concentration

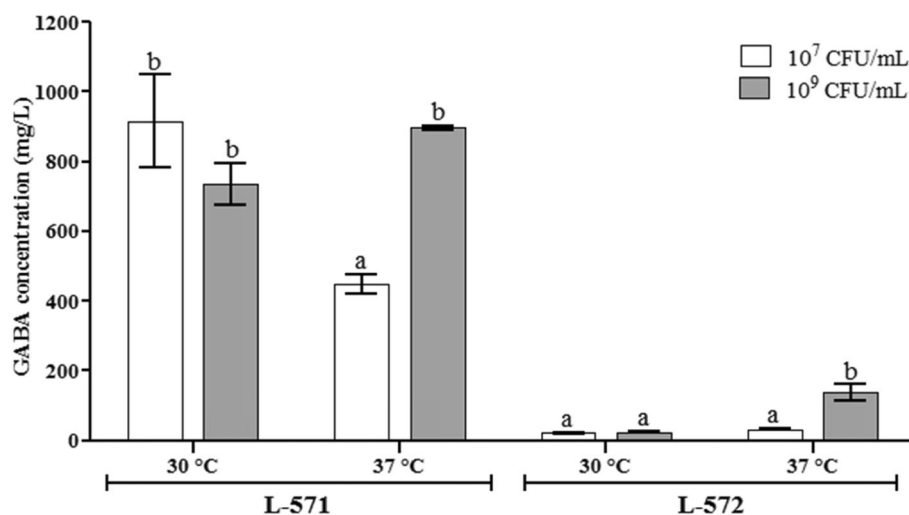


Fig. 2 GABA concentrations in fermented milk using L-571 and L-572 strains at different fermentation conditions. The results show means \pm SD ($n=3$). Different letters, among fermentation condition for each strain, indicate significant differences ($p < 0.05$)

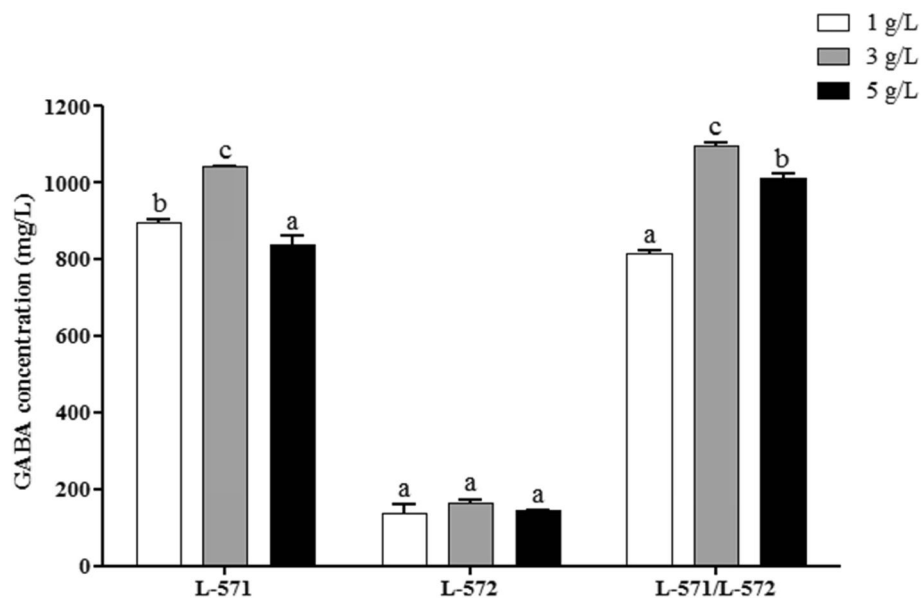


Fig. 3 GABA production in fermented milk using L-571, L-572 and L-571/L-572 strains at different glutamate concentrations. The results show means \pm SD ($n=3$). Different letters, among glutamate concentrations for each FM (L-571, L-572 or L-571/L-572), indicate significant differences ($p < 0.05$)

in milk from 1 to 3 g/L, FM with L-571 and L-571/L-572 significantly ($p < 0.05$) enhanced the GABA production up to 16% and 34.5% more, respectively. Nevertheless, when milk was enriched up to 5 g/L of glutamate, GABA production significantly decreased in both FMs. On the other hand, FM with L-572 did not show significant differences ($p > 0.05$) among the glutamate concentrations.

Several studies have reported that enriching milk with glutamate positively influence GABA production (Dhakal et al. 2012). In this sense, the effects of fermentation

conditions, including glutamate enrichment, were assessed in an attempt to enhance the GABA yield by a specific strain of *Lactococcus lactis*. They concluded that glutamate was the most significant factor influencing GABA yield and the optimal condition was at 15 g/L of enrichment achieving 7.2 g/L of GABA (Lu et al. 2009). These findings clearly reveal that a high GABA yield may be displayed by *Lactococcus* spp.; however, diverse results have been reported by different strains of lactic acid bacteria cultured in a wide variety of matrices and fermentation conditions

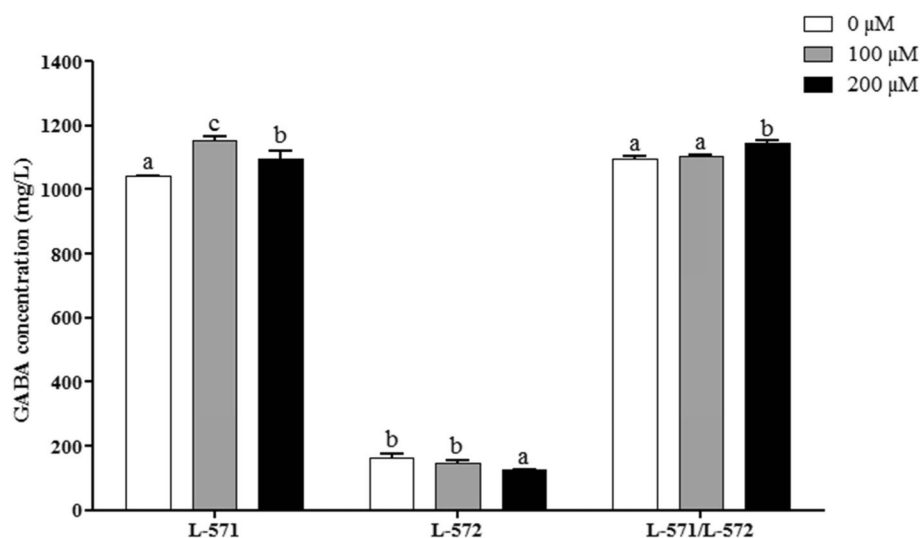


Fig. 4 GABA production of fermented milk with L-571, L-572 and L-571/L-572 strains at different PLP concentrations. The results show means \pm SD ($n=3$). Different letters, among PLP concentrations for each FM (L-571, L-572 or L-571/L-572), indicate significant differences ($p < 0.05$)

(Dhakal et al. 2012), which denotes that this activity is strain-dependent. Specifically, in fermented milk, the GABA concentrations obtained in the present study were superior to those reported by other authors who encountered a GABA production of 258.7 mg/L by *Lactococcus lactis* subsp. *cremoris* and 629 mg/L by *Lactobacillus plantarum* NTU 102 following the addition of 10 g/L of glutamate (Tung et al. 2011).

Since the enrichment of milk with 3 mg/L of glutamate showed the best GABA production in the present study, this condition was selected for further analyses with different PLP concentrations in order to enhance the GABA yield. PLP is an enzymatic cofactor that bacteria use to improve the efficiency or activity of the GAD enzyme, thereby favoring the production of GABA (Li et al. 2016). Although it has been previously reported

that PLP may be present in milk in low concentrations (3 μM) (Schimdt et al. 2017), in the present study, the PLP concentrations of 100 and 200 μM as an additive in milk were assessed. The results, depicted in Fig. 4, showed that the enrichment with 100 μM of PLP significantly ($p < 0.05$) enhanced the GABA yield in FM with L-571, increasing up to 10.7%. However, when milk was enriched with 200 μM of PLP, L-571 significantly ($p < 0.05$) decreased by 4.9% its yield. In contrast, L-571/L-572 significantly ($p < 0.05$) improved its production with 200 μM , achieving an increase of 4.5%. Conversely, although FM with the singular culture of L-572 did not show improvement when 100 μM of PLP was added to the milk, GABA yield was significantly ($p < 0.05$) decreased 23.9% when 200 μM of PLP was added.

These results revealed that to enrich milk with 100 μM of PLP before the fermentation process, it may

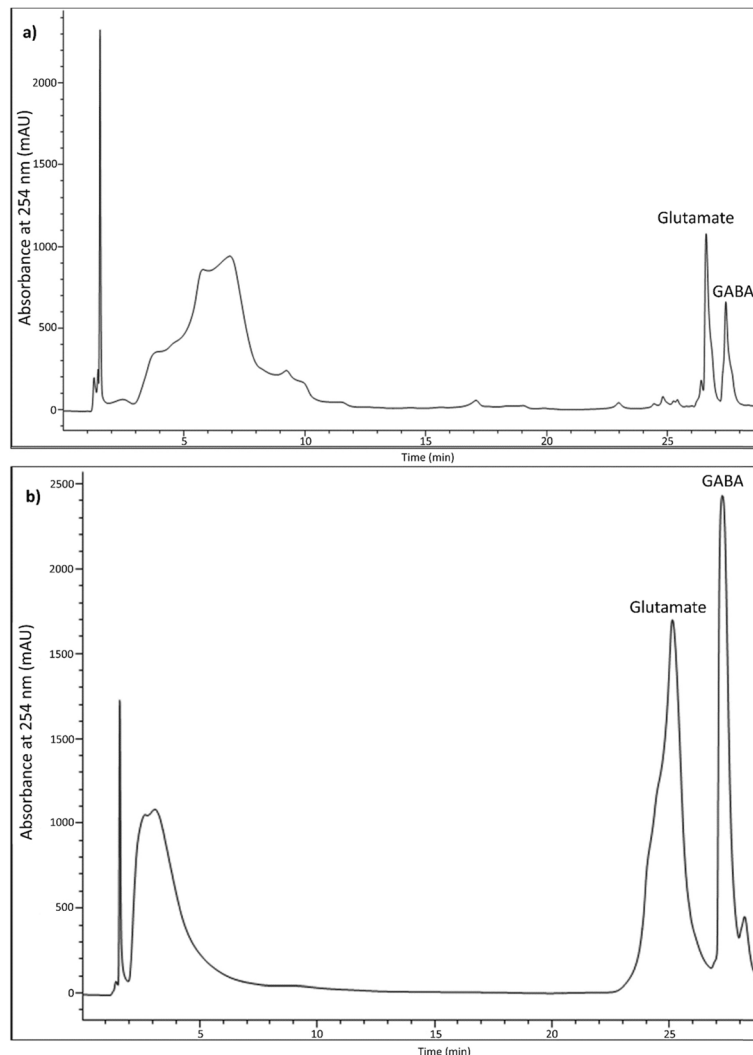


Fig. 5 Chromatographic profile of GABA production in fermented milk with *L. lactis* L-571 under unmodified (30 °C of incubation, 10^7 CFU/mL of inoculum, 0 g/L of glutamate, and 0 μM of PLP) (a) and modified (37 °C of incubation, 10^9 CFU/mL of inoculum, 3 g/L of glutamate, and 100 μM of PLP) (b) fermentation conditions

maintain or even increase the GABA production by L-571, L-572, or L-571/L-572. However, to increase more than 100 μM may lead to improve the GABA yield, as it was shown for L-571/L-572 or be decreased as in FM with L-572. Similar studies with *Lactobacillus brevis* and *Lactobacillus plantarum* were carried out by assessing four different concentrations of PLP, and it was found that the optimum concentration was 200 μM (Gomaa 2015). Therefore, PLP may be considered as an additive in order to enhance the GABA yield.

Notably, in the present study, we managed to increase the GABA present in FM, which is associated with beneficial health effects such as the antihypertensive effect (Inoue et al. 2003; Hayakawa et al. 2004) by more than 20-fold using the strains L-571 and L-571/L-572 compared to the GABA concentrations previously reported (Beltrán-Barrientos et al. 2018a). Nevertheless, it is interesting to note that different GABA production capacities may be exerted by *Lactococcus* spp. as it was shown in the present study by L-571 and L-572. The lower capacity of the last strain may be due to the oversaturation of the glutamate in medium, since it increases the osmotic stress or deficiencies in the transport of GABA or glutamate to outside or inside the cellular cytoplasm of the strain (Wang et al. 2018). Conversely, when two or more strains coexist in the same medium, there is greater competition for nutrients and greater osmotic stress, which may positively influence the fermentation processes for GABA synthesis (Xu et al. 2017). In this case, after selecting the suitable conditions, our study disclosed that the co-fermentation L-571/L-572 (1147.4 ± 6.7 mg/L) did not significantly ($p > 0.05$) enhance the GABA production displayed by the singular culture of L-571 (1153.1 ± 13.5 mg/L). Actually, the FM of the strain L-571 achieved to increase the GABA yield up to 13.4-fold compared when conditions were not adjusted (Fig. 5). Therefore, *Lactococcus lactis* L-571 seems to be the strain predominant in the production of GABA and further studies may determine their optimum yield taken into account these specific considerations.

Conclusions

In this study, we revealed that specific wild GABA-producing strains, isolated from artisanal Mexican cheeses, may exert an enhancement of GABA production in FM by adjusting their fermentation conditions. *Lactococcus lactis* strains L-571 and L-572 showed the highest production without modified fermentation conditions. However, after assessing the fermentation conditions, FM with L-571 showed the highest GABA production and its yield was significantly enhanced at 37 °C of incubation, 10^9 CFU/mL of inoculum, 3 g/L of glutamate, and 100 μM of PLP. Thus, further studies are needed to establish the optimum conditions for producing GABA by this strain and in vivo studies may reveal its potential use as GABA-producing culture for fermented functional foods. Our results allowed us to highlight the importance of wild

LAB strains isolated from natural sources, such as artisanal Mexican cheeses, in order to generate new alternatives and opportunities in the development of foods containing GABA with potentially beneficial effects.

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Authors' contributions

Conceptualization: AFGC. Experimental design: AFGC, BVC. Laboratory analysis: ASE. Supervision: AFGC, MMM, AHM, SGSA, GAGA. Data analysis and interpretation: ASE, RRD, LMBB. Manuscript drafting: ASE, LMBB. Manuscript review & editing: All authors. Manuscript approval: All authors read and approved the final manuscript.

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Ethics approval and consent to participate

N/A

Consent for publication

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Competing interests

The authors declare that they have no competing interest.

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