Evaluation of improved γ -aminobutyric acid production in yogurt using *Lactobacillus plantarum* NDC75017

Y. Shan,*† C. X. Man,*‡ X. Han,§ L. Li,† Y. Guo,† Y. Deng,† T. Li,† L. W. Zhang,§ and Y. J. Jiang*†‡¹

*National Research Center of Dairy Engineering and Technology, Northeast Agricultural University, Harbin 150086, China

†Key Laboratory of Dairy Science, Ministry of Education, Department of Food Science, Northeast Agricultural University, Harbin 150030, China

‡Synergetic Innovation Center of Food Safety and Nutrition, Harbin 150030, China

§College of Food Science and Engineering, Harbin Institute of Technology, Harbin 150090, China

ABSTRACT

Most γ -aminobutyric acid (GABA)-producing microorganisms are lactic acid bacteria (LAB), but the yield of GABA is limited in most of these GABA-producing strains. In this study, the production of GABA was carried out by using *Lactobacillus plantarum* NDC75017, a strain screened from traditional fermented dairy products in China. Concentrations of substrate (L-monosodium glutamate, L-MSG) and coenzyme (pyridoxal-5-phosphate, PLP) of glutamate decarboxylase (GAD) and culture temperature were investigated to evaluate their effects on GABA yield of *Lb. plantarum* NDC75017. The results indicated that GABA production was related to GAD activity and biomass of *Lb. plantarum* NDC75017. Response surface methodology was used to optimize conditions of GABA production. The optimal factors for GABA production were L-MSG at 80 mM, PLP at 18 μ M, and a culture temperature of 36°C. Under these conditions, production of GABA was maximized at 314.56 mg/100 g. Addition of *Lb. plantarum* NDC75017 to a commercial starter culture led to higher GABA production in fermented yogurt. Flavor and texture of the prepared yogurt and the control yogurt did not differ significantly. Thus, *Lb. plantarum* NDC75017 has good potential for manufacture of GABA-enriched fermented milk products.

Key words: γ -aminobutyric acid (GABA), *Lactobacillus plantarum* NDC75017, glutamate decarboxylase (GAD), yogurt

INTRODUCTION

γ -Aminobutyric acid (GABA) is a 4-carbon, non-protein AA from microorganisms in plants and animals, and it acts as the major inhibitory neurotransmitter of the central nervous system (Wong et al., 2003). γ -Aminobutyric acid also exerts positive effects in the

treatment of stress (Vaiva et al., 2004) and hypertension (Tsai et al., 2006), and in the prevention of diabetes (Hagiwara et al., 2004). In addition, it has diuretic and tranquilizer effects (Komatsuzaki et al., 2005). Consequently, GABA has been classified as a bioactive component in food and pharmaceutical products (Sawai et al., 2001). To realize the beneficial functions of GABA, many attempts have been made to synthesize GABA chemically or biologically (Inoue et al., 2003; Choi et al., 2006). Biosynthetic methods of GABA may be more promising than chemical synthetic methods, because the former have a simple reaction procedure, high catalytic efficiency, mild reaction conditions, and environmental compatibility (Huang et al., 2007).

Many microorganisms can produce GABA, including bacteria, fungi, and yeasts. Among the GABA-producing bacteria, the lactic acid bacteria (LAB) are commonly used in many fermented foods. In recent decades, several LAB species have been isolated from traditional fermented foods and screened for their capacity to synthesize GABA, including *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Lactobacillus buchneri*, *Lactobacillus helveticus*, and *Lactobacillus brevis* (Yokoyama et al., 2002; Cho et al., 2007; Siragusa et al., 2007; Lu et al., 2009; Sun et al., 2009). The GABA-producing ability of LAB varies among different species and strains (Park and Oh, 2006; Komatsuzaki et al., 2008; Kim et al., 2009; Coda et al., 2010). Yields of GABA are affected by several factors, including activity of glutamate decarboxylase (GAD; EC 4.1.1.15), related to LAB and fermentation conditions (Huang et al., 2007; Ko et al., 2013).

Different fermentation factors affect production of GABA by LAB. These factors include pH, temperature, culture time, nutritional additives in media, and various fermentation ingredients. Li et al. (2010a) found that glucose, soy peptone, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, and Tween-80 were key factors affecting GABA production. When concentrations of glucose, soy peptone, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, and Tween-80 were 55.25 g/L, 30.25 g/L, 0.0061 g/L, and 1.38 mL/L, respectively, the maximum yield (345.83 mmol/L) of GABA by *Lb. brevis* NCL912

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¹Corresponding author: yujun_jiang@163.com

was reached, which was 130% higher than the yield of GABA under basal conditions. Battaglioli et al. (2003) and Di Cagno et al. (2010) reported that GAD catalyzes the conversion of L-glutamate (or its salts) into GABA through a single step of α -decarboxylation. For GAD reaction activity in biotransformation, Yang et al. (2008) reported that at optimal culture conditions of 40°C and pH 4.5, GABA production by *Streptococcus salivarius* ssp. *thermophilus* Y₂ was increased up to 1.76-fold, reaching to $7,984.75 \pm 293.33$ mg/L. For that reason, adjusting the fermentation conditions to maximize GAD activity is essential to maximizing GABA production (Dhakal et al., 2012).

In this study, we optimized 3 factors: L-monosodium glutamate (L-MSG) concentration, pyridoxal-5-phosphate (PLP) concentration, and culture temperature to enhance GABA production using *Lb. plantarum* NDC75017. The objective was to develop a GABA-enriched yogurt using *Lb. plantarum* NDC75017 as a starter culture.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

A GABA-producing LAB strain of *Lb. plantarum* NDC75017 was isolated from a traditional fermented dairy product from Inner Mongolia, China (Zhou, 2010). Skim milk was reconstituted to 12% (wt/vol; Nestle Company, Heilongjiang, China) and sterilized at 115°C for 15 min. *Lactobacillus plantarum* NDC75017 was inoculated (2%, vol/vol) into sterilized reconstituted skim milk and cultured at 30°C for 48 h. One milliliter of the cultured sample was then added to 9.0 mL of 0.85% sterilized physiological saline, and appropriate dilutions were made. Then, 0.1 mL of each dilution was spread on de Man, Rogosa, and Sharpe plate count agar using a micropipette (in triplicate) and inoculated for 48 h at 37°C. Visible colonies were then counted on plates containing 30 to 300 colonies, and biomass was expressed as log colony-forming units per milliliter (Park and Oh, 2007). A commercial starter culture YC-X100 (Chr. Hansen Inc., Beijing, China), which consisted of *Streptococcus thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*, was used as a control.

Factors Affecting GABA Synthesis

Effect of L-MSG on GABA Production. Different concentrations of L-MSG were added to 12% (wt/vol) reconstituted skim milk that contained 20 μ M PLP. The final concentrations of L-MSG were 0, 25, 50, 100, 150, and 200 mM, respectively. All reconstituted skim milk samples were sterilized at 115°C for 15 min.

About 2% (vol/vol) of *Lb. plantarum* NDC75017 was inoculated into the skim milk. The initial cell density of *Lb. plantarum* NDC75017 in skim milk was 10^8 cfu/mL and it was fermented at 30°C for 48 h. After 48 h, GAD activity, biomass of *Lb. plantarum* NDC75017, and GABA yield were measured.

Effect of PLP on GABA Synthesis. Different concentrations of PLP were added to 12% (wt/vol) reconstituted skim milk that contained 50 mM L-MSG. The final concentrations of PLP were varied from 0 to 100 μ M. Thereafter, GAD activity, biomass of *Lb. plantarum* NDC75017, and GABA yield in fermented culture were measured.

Effect of Culture Temperature on GABA Synthesis. To determine the effect of culture temperature, the *Lb. plantarum* NDC75017 was inoculated into reconstituted skim milk that contained 50 mM L-MSG and 20 μ M PLP. These samples were cultured for 48 h at temperatures ranging from 25°C to 45°C. The, GAD activity, biomass, and GABA yield were measured.

Determination of GAD Activity

Cells of *Lb. plantarum* NDC75017 were collected by centrifugation ($8,000 \times g$ for 20 min at 4°C), washed with 0.9% NaCl 3 times, suspended in 10 times the pellet weight of 0.9% NaCl, and stirred with a magnetic stirrer for 10 min. One milliliter of suspension was incubated with 2 mL of L-MSG (100 mM) and 2 mL of acetate buffer (100 mM, pH 5.8) for 30 min. The reaction temperature was 30°C. To terminate the reaction, 8 mL of absolute ethanol was added at -20°C . The suspension was centrifuged ($8,000 \times g$ for 20 min at 4°C). The GABA content in the enzyme mixture was measured using ultra-performance liquid chromatography (UPLC, Acquity UPLC H-Class, Waters Corporation, Milford, MA). One unit of enzyme activity of GAD was defined as the amount of enzyme that produced 1 μ mol of GABA in 1 min (Siragusa et al., 2007). The assay for GAD activity was done in triplicate.

GABA Assay

Accumulation of GABA in yogurt was assessed as follows. First, casein was separated from yogurt by centrifugation twice (each at $10,000 \times g$ for 15 min at 4°C). Then, 1 M potassium ferrocyanide and 0.25 M zinc acetate were used to precipitate the other proteins. Potassium ferrocyanide (2.5 mL) was added to the supernatant sample, and the sample was incubated at the room temperature for 3 min. Then, 2.5 mL of zinc acetate was added to the sample. The mixture was incubated at room temperature for 5 min and centrifuged twice (each at $10,000 \times g$ for 15 min at 4°C), and

Table 1. Independent variables (factors A, B, and C) and their coded and actual values used in response surface methodology optimization

Code level	Independent variable		
	A: Temperature (°C)	B: Pyridoxal-5-phosphate (μM)	C: L-Sodium glutamate (mM)
−1	28	10	50
0	35	20	75
1	42	30	100

the supernatant was then collected. Analysis by UPLC was performed to determine the content of GABA in suspension (Rea et al., 2005). The sample extraction, derivation of GABA standard solution, and UPLC analysis method were according to Kim et al. (2009).

Response Surface Methodology

A Box-Behnken design of response surface methodology (**RSM**) was used to optimize the 3 most significant variables (L-MSG concentration, PLP concentration, and culture temperature), which were further screened by Plackett-Burman design to determine the enhancement of GABA yield. The 3 independent factors were investigated at 3 different coded levels (−1, 0, and +1; see Table 1) under the experimental design shown in Table 2. In total, 17 experiments were performed and analyzed using RSM and the statistical software package (Design Expert 8.05b, Stat-Ease Inc., Minneapolis, MN). The design matrix plus the corresponding experimental data are shown in Table 2. The experimental results were analyzed by quadratic stepwise regression to fit the second-order equation [Eq. 1]:

$$Y = \beta_0 + \sum_{i=1}^3 B_i x_i + \sum_{i=1}^3 B_{ii} x_i^2 + \sum_{i=1}^2 \sum_{i < j}^3 B_{ij} X_i X_j, \quad [1]$$

where Y denotes response observed for treatment combination $X = (x_1, x_2, \dots, x_p)$ for p factors, β_0 represents the model intercept, and the parameters of B_i , B_{ii} , and B_{ij} represents the regression coefficients of variables for linear, quadratic, and interaction regression terms, in that order. Analysis of variance was conducted to determine the significance of model and regression coefficients. The quality of the polynomial equation was judged by the coefficient of determination R^2 , and the statistical significance was checked using the F -test. The significance of regression coefficients was tested using a t -test. The response surface and contour plots of the model-predicted responses were used to assess the relationships between the significant variables (Lu

et al., 2008; Gao et al., 2009; Li et al., 2010b; Tung et al., 2011).

Fermented Milk Preparation

A single-strain starter culture of *Lb. plantarum* NDC75017, the commercial starter-culture YC-X100, and the combination of these were separately used to make yogurts. The 3 types of starter cultures were inoculated separately in sterilized reconstituted skim milk and incubated at 36°C. Inocula of 10^8 cfu/mL from each starter culture were used. When the pH reached 4.5, the samples were rapidly cooled to 4 to 6°C in an ice-bath and stored at 4°C for 12 h. The pH of each sample was determined using a pH meter (Corning 120 pH meter, Corning Inc., Corning, NY). Then, acetaldehyde and diacetyl concentrations were determined by gas chromatography (Agilent, Santa Clara, CA) as described by Pourahmad et al. (2011). Water-holding capacity (**WHC**) was determined by the procedure described by Keogh and O’Kennedy (1998). Before measuring yogurt consistency, previously incubated samples at 4°C were adapted to 25°C for 30 min according to Xanthopoulos et al. (2012), using a Brookfield RVDV-II+ Viscometer (Brookfield Engineering Laboratories Inc., Middleboro, MA).

Sensory Evaluation

The method used was according to Han et al. (2014) with minor modifications. Briefly, 50 trained panelists were asked to evaluate the appearance, aroma, texture, flavor, and taste of the fermented milk using 9-point scores (from 1 to 9). The ratings were presented on a 9-point hedonic scale ranging from 9 (“like extremely”) to 1 (“dislike extremely”).

Statistical Analysis

Data of triplicates were expressed as the means $\pm$ standard deviation (SD). The statistical significance of the differences between the 2 means was evaluated

Table 2. Box-Behnken design matrix and experimental and predicted values of optimizing fermented conditions of γ -aminobutyric acid (GABA) production by *Lactobacillus plantarum* NDC75017

Trial	Factor ¹			GABA yield (mg/100 g)	
	A	B	C	Experimental	Predicted
1	42	20	50	209.08	203.93
2	35	20	75	189.67	195.87
3	42	30	75	137.93	131.73
4	35	30	100	231.29	236.45
5	35	20	75	110.78	115.83
6	28	20	100	160.66	154.36
7	42	20	100	160.64	166.95
8	35	20	75	230.13	225.08
9	28	30	75	160.01	160.11
10	35	20	75	185.84	186.99
11	28	20	50	264.86	263.71
12	42	10	75	205.32	205.22
13	35	10	100	312.15	306.41
14	35	20	75	310.57	306.41
15	35	10	50	301.23	306.41
16	35	30	50	309.34	306.41
17	28	10	75	298.74	306.41

¹Factor A = temperature ($^{\circ}\text{C}$); factor B = pyridoxal-5-phosphate concentration (μM); factor C = L-monosodium glutamate concentration (mM).

using *t*-test. Values of $P < 0.05$ were considered significant. Optimal culture conditions for GABA yield were determined using RSM, and calculations were performed using RSM software (Design Expert 8.05, Stat-Ease Inc.).

RESULTS

Effect of L-MSG Concentration on GABA Yield, GAD Activity, and Biomass of *Lb. plantarum* NDC75017

The effects of L-MSG addition to skim milk medium on GABA yield, GAD activity, and biomass of *Lb. plantarum* NDC75017 are presented in Figure 1. Yield of GABA, GAD activity, and biomass of *Lb. plantarum* NDC75017 increased as the concentration of L-MSG reached 75 mM . When L-MSG increased above 75 mM , the yield of GABA, GAD activity, and the viable count of *Lb. plantarum* NDC75017 all declined. Hence, the concentration of L-MSG had a significant effect on GABA yield, GAD activity, and biomass of *Lb. plantarum* NDC75017 ($P < 0.05$). When the concentration of L-MSG was 75 mM , the 3 factors were maximal.

Effect of PLP Concentration on GABA Yield, GAD Activity, and Biomass of *Lb. plantarum* NDC75017

The effects of PLP concentration on GABA yield, GAD activity, and the biomass of *Lb. plantarum* NDC75017 were investigated. The results showed that GAD activity increased with the increment of PLP from 0 to 20 μM . When the concentration of PLP was

about 20 μM , maximum GAD activity was achieved. Meanwhile, the yield of GABA was reached the highest level of $165.6 \pm 0.3 \mu\text{g}/100 \text{ g}$ (Figure 2). We detected no significant change in the viable counts of *Lb. plantarum* NDC75017 ($P > 0.05$).

Effect of Culture Temperature on GABA Yield, GAD Activity, and Biomass of *Lb. plantarum* NDC75017

The effects of culture temperature on the yield of GABA, *Lb. plantarum* NDC75017 growth, and GAD

Figure 1. Change in glutamate decarboxylase (GAD; Δ) activity, biomass of *Lactobacillus plantarum* NDC75017 ($\log \text{cfu}/\text{mL}$; $\diamond$), and γ -aminobutyric acid (GABA; $\blacksquare$) concentration produced in skim milk medium containing 20 μM pyridoxal-5-phosphate (PLP) at 30°C induced by different concentrations of L-sodium glutamate (L-MSG). Data expressed as mean $\pm$ SD from 3 independent experiments.

Figure 2. Change in glutamate decarboxylase (GAD) activity (Δ), biomass of *Lactobacillus plantarum* NDC75017 (log cfu/mL; ◇), and γ-aminobutyric acid (GABA; ■) concentration produced in skim milk medium containing 100 mM L-sodium glutamate at 30°C induced by different concentrations of pyridoxal-5-phosphate (PLP). Data expressed as mean ± SD from 3 independent experiments.

Figure 3. Change in glutamate decarboxylase (GAD) activity (Δ), biomass of *Lactobacillus plantarum* NDC75017 (log cfu/mL; ◇), and γ-aminobutyric acid (GABA; ■) concentration produced in skim milk medium containing 100 mM L-sodium glutamate (L-MSG) and 20 μM PLP at 25, 30, 35, 40, and 45°C, respectively. Data expressed as mean ± SD from 3 independent experiments.

activity are shown in Figure 3. The optimal temperature for GAD activity was 40°C, but the yield of GABA and the growth of *Lb. plantarum* NDC75017 were not maximal at this temperature. The highest GABA yield and the highest count of *L. plantarum* NDC75017 were obtained at 35°C and 30°C, respectively.

RSM Analysis of GABA Yield

Using the designed experimental data, the polynomial model for GABA yield (Y) was regressed by considering only the significant terms, as represented below [Eq. 2]:

$$Y = -2305.12 + 102.49A + 6.70B + 17.40C + 0.40AB + 0.03AC - 0.08BC - 1.55A^2 - 0.40B^2 - 0.10C^2, \quad [2]$$

where Y is the predicted GABA yield, A is the culture temperature, B is the PLP concentration, and C is the L-MSG concentration. The values of R^2 and adjusted R^2 (being measures of fitness of the regressed model) were 0.9804 and 0.9553, respectively. These values indicate that the accuracy and general ability of the polynomial model were good. In Table 3, the P -values of the model indicate that the obtained experimental data fitted well with the model ($P < 0.0001$). The coefficient of variation indicates the degree of precision with which the comparison between treatments was made. Generally, a model can be considered reasonably reproducible if its coefficient of variation is not greater than 10% (Zhao et al., 2014). In present study, the coefficient of variation

(3.43%) demonstrated that the performed experiment was highly reliable.

The response surface curves were plotted to explain the interaction of the variables and to determine the optimum level of each variable for maximum responses. As shown in Figure 4a–c, the response surface plots illustrate the pair-wise interaction of the 3 variables. These surface plots demonstrate interactions between each pair of variables. The interactions between culture temperature (A) and PLP concentration (B), as well as between PLP concentration (B) and L-MSG concentration (C) were all significant, with P -value of 0.0002 and 0.0008, respectively. Surprisingly, the interaction between culture temperature (A) and L-MSG concentration (C) was not significant ($P = 0.2397$), indicating that the interaction of L-MSG concentration and culture temperature did not play a crucial role in GABA yield.

By applying these statistical methods for optimization of GABA production, the optimal combinations of culture substrate, coenzyme, and culture temperature for maximum GABA yield were 80 mM L-MSG, 18 μM PLP, and culture temperature of 36°C. The experimental GABA yield was the same as the predicted value, and thus the model proved adequate. The maximum value of GABA yield predicted from this model was 312.43 mg/100 g. To verify the predicted results, the experiment was performed under the optimized conditions, and the experimental value of GABA yield was 314.56 mg/100 g, indicating that experimental and predicted values of GABA yield were in good agreement. Therefore, the strain used seems to have high potential use for industrial applications.

Table 3. Statistical ANOVA for regression model of response surface methodology for optimizing fermented conditions of γ -aminobutyric acid (GABA) accumulation by *Lactobacillus plantarum* NDC75017¹

Source	Sum of squares	df	Mean square	F-value	P > F
Model	70,952.99	9	7,883.67	135.36	0.0001
A	4,671.49	1	4,671.58	80.21	0.0001
B	499.91	1	499.91	8.58	0.0220
C	7,421.27	1	7,421.27	127.43	0.0001
AB	3,179.27	1	3,179.268	54.59	0.0002
AC	96.14	1	96.14	1.65	0.2397
BC	1,822.01	1	1,822.01	31.28	0.0008
A ²	24,598.71	1	24,598.71	422.37	0.0001
B ²	6,073.36	1	6,073.36	104.28	0.0001
C ²	17,473.01	1	17,473.01	300.02	0.0001
Residual	407.68	7	58.24		
Cor. total	71,360.67	16			

¹A = temperature (°C); B = pyridoxal-5-phosphate concentration (μ M); C = L-monosodium glutamate concentration (mM); Cor. total = corrected total; R² = 0.9943, adjusted R² = 0.9869, predicted R² = 0.9378.

Production and Characterization of the Fermented Skim Milk

The *Lb. plantarum* NDC75017 starter culture and the mixed-starter culture (composed of a commercial starter culture YC-X100 and *Lb. plantarum* NDC75017) were inoculated in skim milk, which contained both L-MSG (80 mM) and PLP (18 μ M). Yogurt fermented by the commercial starter culture YC-X100 was used as a control sample. The samples were incubated at 36°C until the pH decreased to 4.5 ± 0.1 . Fermentation was stopped immediately in the ice-bath and maintained at 4°C for further analyses. The GABA yield and fermented milk parameters are shown in Table 4. The production of GABA by mixed-starter culture was 231.23 ± 2.32 mg/100 g, significantly lower than that in yogurt fermented only by *Lb. plantarum* NDC75017 as a starter culture (289.23 ± 1.47 mg/100 g; $P < 0.05$).

The scores for attributes flavor, WHC, and cohesiveness were significantly lower when using *Lb. plantarum* NDC75017 alone to ferment the yogurt compared with the yogurt fermented by the other 2 starter cultures ($P < 0.05$). The yogurt fermented by the mixed-starter culture was not significantly different from the control sample in WHC and cohesiveness. However, the concentrations of acetaldehyde (13.34 ± 0.72 mg/L) and diacetyl (12.45 ± 0.54 mg/L) were higher in yogurt fermented by the mixed-starter culture than in the control sample ($P < 0.05$). As shown in Table 4, the yogurt fermented by a mixed-starter culture with higher GABA production had a similar texture to the control sample ($P > 0.05$).

Sensory Evaluation

The scores for sensory characteristics of the fermented milk samples are presented in Table 5. We detected

no significant differences ($P > 0.05$) in appearance, odor, acidity, thickness, or fluidity among mixed-starter culture yogurt and the control yogurts. The overall acceptance scores were not significantly different ($P > 0.05$) between the yogurt fermented by mixed-starter culture and the control sample. However, we did detect a significant difference ($P < 0.05$) in taste between the yogurt fermented by mixed-starter culture and the control sample. Thus, adding *Lb. plantarum* NDC75017 into commercial starter culture for milk fermentation had no significant effect ($P > 0.05$) on sensory evaluation in general.

DISCUSSION

The ability to produce GABA varies widely among strains of LAB and is affected by the fermentation conditions (Di Cagno et al., 2010). The GABA-producing strain *Lb. plantarum* NDC75017 was screened from traditional dairy products of Inner Mongolia, China. Our results confirmed that the concentrations of L-MSG and PLP plus culture temperature play important roles in enhancing GABA yield.

L-Monosodium glutamate, as a substrate of GAD, is catalyzed to GABA by decarboxylation (Jeng et al., 2007; Tamura et al., 2010), and GAD is the key enzyme in the conversion of L-MSG to GABA (Hiraga et al., 2008). From Figure 1, it can be seen that L-MSG concentration had a significant effect on GAD activity and yield of GABA. Interestingly, GABA concentration showed a gradual increase with the addition of L-MSG from 0 to 75 mM. With the addition of L-MSG (75 mM) to skim milk, the yield of GABA reached 287 mg/100 g, which was significantly higher than in fermented milk without L-MSG ($P < 0.05$). According Seo et al. (2013), the GABA production of *Lb. brevis* 340G is enhanced by increasing the MSG concentration

Figure 4. Three-dimensional response surface plots and 2-dimensional contour plots for γ -aminobutyric acid (GABA) yield by *Lactobacillus plantarum* NDC75017 showing variable interactions of (a) pyridoxal-5-phosphate (PLP) concentration (μ M) and fermentation temperature ($^{\circ}$ C); (b) L-sodium glutamate (L-MSG) concentration (mM) and fermentation temperature ($^{\circ}$ C); (c) L-MSG concentration (mM) and PLP (μ M) concentration. GABA yield was expressed in mg/100 g. Color version available online.

up to 3% in de Man, Rogosa, and Sharpe medium. However, when the concentration of MSG exceeds 3%, it has a negative effect on GABA production. This is a clear indication that MSG in a particular concentration range can increase the GABA yield of LAB.

The irreversible decarboxylation of glutamate to GABA is catalyzed by GAD. Promoting GAD activity

is helpful in stimulating GABA accumulation (Shelp et al., 1999). In Figure 1, we show that GAD reached its highest activity when 75 mM of L-MSG was added to skim milk. This result was the same as those of Bai et al. (2009) and Guo et al. (2012).

High cell density could increase the yield of GABA through synthesis of GAD. Figure 1 showed that when

Table 4. Characteristics of yogurt manufactured with commercial starter culture YC-X100, *Lactobacillus plantarum* NDC75017, or a mixed-starter culture (YC-X100 + NDC75017)

Analysis	Fermented milk type		
	YC-X100 (control)	YC-X100 + NDC75017	NDC75017
Yogurt curd time (h)	5.75 $\pm$ 0.23 ^a	5.75 $\pm$ 0.33 ^a	9.00 $\pm$ 0.55 ^b
GABA ¹ production (mg/100 g)	ND ²	231.23 $\pm$ 2.32 ^a	289.23 $\pm$ 1.47 ^b
Diacetyl (mg/L)	10.56 $\pm$ 0.33 ^b	12.45 $\pm$ 0.54 ^c	3.45 $\pm$ 0.24 ^a
Acetaldehyde (mg/L)	12.23 $\pm$ 0.55 ^b	13.34 $\pm$ 0.72 ^c	3.02 $\pm$ 0.45 ^a
Cohesiveness (mPa)	4,193 $\pm$ 19 ^b	4,392 $\pm$ 13 ^b	2,032 $\pm$ 14 ^a
Water-holding capacity (%)	81.0 $\pm$ 1.4 ^b	79.3 $\pm$ 2.7 ^b	62.35 $\pm$ 1.5 ^a

^{a-c}Values with different letters within the same row differ significantly ($P < 0.05$).

¹ γ -Aminobutyric acid.

²Not detected.

the concentration of L-MSG did not exceed 75 mM, the biomass of *Lb. plantarum* NDC75017 was increased with increasing L-MSG concentration ($P < 0.05$). The biomass of *Lb. plantarum* NDC75017 reached its maximum level of 9.89 ± 0.24 log cfu/mL when adding 75 mM L-MSG to skim milk. However, when the concentration of L-MSG exceeded 75 mM, growth of *Lb. plantarum* NDC75017 was inhibited ($P < 0.05$). This finding was similar to the results of Seo et al. (2013), in which cell growth of *Lb. brevis* 340G was increased by adding MSG from 0 to 2% ($P < 0.05$). Moreover, when concentration of MSG exceeded 5%, cell growth was inhibited ($P < 0.05$). Cell growth was highest when adding 2 to 5% MSG ($P > 0.05$). Monosodium glutamate appears to be a minor nutritional element for growth of *Lb. plantarum* NDC75017. Interestingly, LAB may be able to utilize the GAD system to maintain neutral cytoplasmic pH, especially when the external pH decreases. During this stage, the decarboxylation of glutamate within the LAB cell consumes an intracellular proton. Neutral cytoplasmic pH is more suitable to bacterial growth than an acidic environment (Li et al., 2010b). Therefore, certain concentrations of

MSG may benefit the growth of several LAB species. However, excess L-MSG increases the osmotic pressure of the cells, disturbs bacteria metabolism, and inhibits cell growth (Yang et al., 2008).

Glutamate decarboxylase, which forms GABA from glutamic acid, is activated by the coenzyme PLP and catalyzes the irreversible α -decarboxylation of glutamate to produce GABA (Kook et al., 2010). Therefore, we investigated the relationship between PLP concentration and GABA production. Yang et al. (2008) reported that PLP played a useful role in increasing GABA production. Komatsuzaki et al. (2005) reported that adding 10 μ M PLP into MRS medium or to milk being fermented by *Lb. paracasei* could enhance GABA yield. A similar relationship between PLP concentration and GABA production was found in our study.

Nevertheless, PLP appears to have a different effect on GABA production for different LAB. Li et al. (2010b) reported that adding PLP had no effect on cell growth or production of GABA when *Lb. brevis* NCL912 was used in the fermentation process. As shown in Figure 2, PLP affected GABA yield and GAD activity but had no significant effect on the biomass

Table 5. Sensory characteristics of fermented yogurts (means $\pm$ SD for $n = 3$) manufactured with commercial starter culture YC-X100, *Lactobacillus plantarum* NDC75017, or a mixed-starter culture (YC-X100 + NDC75017)

Item	Fermented milk type		
	YC-X100	YC-X100 + NDC75017	NDC75017
Appearance	6.23 $\pm$ 0.46 ^b	6.29 $\pm$ 0.28 ^b	5.02 $\pm$ 0.31 ^a
Odor	6.67 $\pm$ 0.36 ^b	6.45 $\pm$ 0.39 ^b	5.65 $\pm$ 0.14 ^a
Acidity	6.34 $\pm$ 0.08 ^b	6.25 $\pm$ 0.45 ^b	5.43 $\pm$ 0.24 ^a
Thickness	7.43 $\pm$ 0.55 ^b	7.36 $\pm$ 0.12 ^b	6.87 $\pm$ 0.40 ^a
Fluidness	7.09 $\pm$ 0.25 ^b	7.16 $\pm$ 0.27 ^b	6.23 $\pm$ 0.38 ^a
Tasty	7.34 $\pm$ 0.43 ^c	6.89 $\pm$ 0.48 ^b	6.34 $\pm$ 0.47 ^a
Overall acceptance ¹	6.85 $\pm$ 0.33 ^b	6.73 $\pm$ 0.30 ^b	5.92 $\pm$ 0.24 ^a

^{a-c}Values with different letters within the same row differ significantly ($P < 0.05$).

¹Where 1 = most undesirable, 5 = neither desirable nor undesirable, 9 = most desirable.

of *Lb. plantarum* NDC75017 ($P > 0.05$), although the biomass was maximal when PLP concentration was 20 μM . This finding indicated that PLP could not be used as a nutritional factor for the growth of *Lb. plantarum* NDC75017. Meanwhile, this result verified that GAD activity was correlated with GABA production. In our experiment, we demonstrated that GAD activity of *Lb. plantarum* NDC75017 was PLP enzyme-dependent, in agreement with Tong et al. (2002). The addition of PLP to the culture medium could increase the GAD activity of *Lb. paracasei* NFRI7415 (Komatsuzaki et al., 2005). However, additional PLP had little effect on the GAD activity of *Lb. brevis* CGMCC1306, which might be because the GAD of *Lb. brevis* CGMCC1306 is bound firmly to the coenzyme PLP and exists mostly as holo-GAD (Huang et al., 2007; Fan et al., 2012).

Temperature control is necessary for bioconversion (Zhang et al., 2012). An elevated temperature could increase the reaction rate and influence the final yield of GABA. In our research, the yield of GABA was 175.6 mg/100 g when *Lb. plantarum* NDC75017 was incubated at 35°C. Di Cagno et al. (2010) indicated that the highest yield of GABA (497 mg/L) was achieved at an incubation temperature of 30°C for 72 h. Lu et al. (2008) and Komatsuzaki et al. (2005) also demonstrated that the optimum production temperatures of *Lc. lactis* and *Lb. paracasei* NFRI 7415 were 34°C and 37°C, and the corresponding GABA yields were 0.27 and 31.14 mg/mL, respectively. Thus, different LAB have different optimal GABA-producing temperatures. Li and Cao (2010) indicate that most of the optimal temperatures for GAD activity of LAB are within the range of 30 to 50°C. It is known that the higher temperatures cause enzyme inactivation and cell aging. In this study, the optimum GAD activity of *Lb. plantarum* NDC75017 was at 40°C (Figure 3). The optimum temperatures of *Lb. brevis* CGMCC 1306 and *Lb. brevis* IFO 12005 in terms of GAD activity were 37°C and 30°C, respectively (Ueno et al., 1997; Huang et al., 2007).

In this study, the optimum growth temperature of *Lb. plantarum* NDC75017 was 30°C, the same as that of *Lb. brevis* TCCC13007 (Zhang et al., 2012). However, the optimal temperature in terms of GAD activity was 40°C, and GABA synthesis was related to GAD activity. Meanwhile, a high cell density could increase GAD production and a high yield of GABA may be obtained (Li and Cao, 2010). Therefore, the viable count is very necessary for the bioconversion.

Response surface methodology has been successfully used in many recent studies on the optimization of GABA yield (Lu et al., 2009; Li et al., 2010a). In our assessment, the optimal conditions for GABA yield were L-MSG at 80 mM, PLP at 18 μM , and culture temperature of 36°C. The maximum GABA yield obtained

was 314.56 mg/100 g, which was 1.92-fold higher than the result under non-optimized conditions (L-MSG at 50 mM, PLP at 20 μM , and culture temperature at 30°C). Tung et al. (2011) used a Box-Behnken design combined with RSM to optimize the culture medium of *Lb. plantarum* NTU102. Their results indicated that *Lb. plantarum* NTU102 produced the highest level of GABA (629 mg/L) at 37°C in rehydrated skim milk supplemented with 1% MSG.

The literature indicates many investigations with studies on GABA-producing lactobacilli strains. Among them, many species have been used to enhance GABA production of milk-based products. As shown in Table 6, *Lb. plantarum* C48 and *Lb. helveticus* ND01 isolated from cheese and koumiss, respectively, are some of the higher GABA-producing strains. In those experiments, these strains were inoculated in skim milk and their respective GABA yields were 4.31 mg/100 mL and 16.51 mg/100 mL (Sun et al., 2009; Servili et al., 2011). Various LAB species have different GABA production abilities and different fermentation requirements (Kook and Cho, 2013). *Lactobacillus plantarum* NDC75017 was isolated from traditional fermented dairy products and screened for its capacity to synthesize GABA. Therefore, it may be more suitable for GABA production compared with strains isolated from nondairy sources (Seo et al., 2013). Pyridoxal-5-phosphate is a necessary coenzyme of GAD but its concentration has not been optimized in some studies (Di Cagno et al., 2010; Tung et al., 2011). In our study, the 3 key factors—L-MSG, PLP, and culture temperature—were optimized by RSM. The optimal conditions of *Lb. plantarum* NDC75017 obtained in this paper could be used in further development for functional foods.

The characteristics and sensory evaluations of milk samples fermented by either the commercial starter culture YC-X100 (control) or the mixed-starter culture (*Lb. plantarum* NDC75017 combined with YC-X100) were compared in this study. Milk fermented by the mixed-starter culture did not differ from the control sample ($P > 0.05$), particularly in WHC and viscosity. However, we did detect a significant difference ($P < 0.05$) in taste between the mixed-starter culture yogurts and the control yogurts. When 80 mM of L-MSG was added to the sterilized skim milk, a salty note was perceived in the flavor, in agreement with results of Ko et al. (2013). The transformation percentage of L-MSG was 80.81% (relative SD = 1.46%), which is similar to results found by Rizzello et al. (2010). Overall, the addition of L-MSG at 80 mM had no significant effect on overall acceptance of the yogurts.

As for the concentrations of diacetyl and acetaldehyde in yogurts fermented by the mixed-starter culture, both were higher than those in the control sample ($P <$

Table 6. Production of γ -aminobutyric acid (GABA) by different *Lactobacillus* strains in different milk base mediums

Microorganism	Isolation source	Major components of culture medium ¹	GABA production	Reference
<i>Lactobacillus plantarum</i> NDC75017	Fermented dairy production	12% skim milk, 80 mM L-MSG, 18 μ M PLP	314.56 mg/100 g	This work
<i>Lactobacillus plantarum</i> C48 and <i>Lactobacillus paracasei</i> 15N	Cheese	Functional milk beverage	6.70 mg/100 mL	Servili et al., 2011
<i>Lactobacillus helveticus</i> ND01	Koumiss	Skim milk	16.51 mg/100 mL	Sun et al., 2009
<i>Lactobacillus plantarum</i> PU11	Cheese	Milk, 5 g/L yeast extract, 20 mM glutamic acid	7.74 mg/100 g	Nejati et al., 2013
<i>Lactobacillus casei</i> Zhang	Koumiss	Cheddar cheese	67.74 mg/100 g	Wang et al., 2010
<i>Lactobacillus plantarum</i> NTU102	Homemade Korean-style cabbage pickle	12% skim milk, 1% MSG	62.90 mg/100 mL	Tung et al., 2011
<i>Lactobacillus plantarum</i> 1288	Not provided	Goat milk	1.28 mg/100 g	Minervini et al., 2009
<i>Lactobacillus crispatus</i> RMK567	Whole milk	12% skim milk, 0.1% MSG	74.56 mg/100 mL	Kook and Cho, 2013
<i>Lactobacillus</i> spp. OPY-1	Yogurt	20% brown rice powder, 18% whole milk, 2% skim milk, 5% MSG	83.0 mg/100 mL	Kook and Cho, 2013
<i>Lactobacillus brevis</i> 340G	Kimchi	Skim milk, 1% MSG	47.84 mg/100 mL	Seo et al., 2013

¹MSG = monosodium glutamate; PLP = pyridoxal-5-phosphate.

0.05). The GABA yield of yogurt fermented by mixed-starter culture reached 231.23 mg/100 g. Consequently, the addition of *Lb. plantarum* NDC75017 to the commercial starter culture would enhance yield of GABA with no significant effect on yogurt flavor.

Recently, some studies have reported that products containing GABA caused a decrease in blood pressure in hypertensive humans and spontaneously hypertensive rats (Inoue et al., 2003; Liu et al., 2011). Daily intake of fermented milk (10 mg of GABA) for 12 wk decreased blood pressure by 17.4 mmHg in hypertensive patients (Inoue et al., 2003). In the current study, the GABA yield of yogurt fermented by *Lb. plantarum* NDC75017 and commercial starter culture reached 231.23 mg/100 g. The amount of GABA in yogurt synthesized by *Lb. plantarum* NDC75017 was higher than the minimum effective daily dose resulting in a positive effect in the study of Inoue et al. (2003). Thus, *Lb. plantarum* NDC75017 could be used in co-fermentation with other LAB starter strains to produce functional dairy foods containing elevated levels of GABA.

CONCLUSIONS

The GABA yield of *Lb. plantarum* NDC75017 was investigated by adding different concentrations of L-MSG and PLP and culturing at different temperatures. The results revealed that high GAD activity and high biomass could enhance GABA production of *Lb. plantarum* NDC75017. Response surface methodology was used to optimize GABA production conditions. When the L-MSG concentration was fixed at 80 mM and PLP 18 μ M, and under a culture temperature of 36°C, GABA yield of *Lb. plantarum* NDC75017 was maximal, at 314.56 mg/100 g. Under these optimal conditions, yogurt made using *Lb. plantarum* NDC75017 combined with commercial starter culture contained higher concentrations of GABA than control yogurt. A significant difference in taste was noted but no significant effect on the overall acceptance of the products compared with the control samples. Therefore, *Lb. plantarum* NDC75017 has potential to enrich dairy products with GABA.

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REFERENCES

- Bai, Q. Y., M. Q. Chai, Z. X. Gu, X. H. Cao, Y. Li, and K. L. Liu. 2009. Effects of components in culture medium on glutamate decarboxylase activity and γ -aminobutyric acid accumulation in foxtail millet (*Setaria italica* L.) during germination. *Food Chem.* 116:152–157.
- Battaglioli, G., H. Liu, and D. L. Martin. 2003. Kinetic differences between the isoforms of glutamate decarboxylase: Implications for the regulation of GABA synthesis. *J. Neurochem.* 86:879–887.
- Cho, Y. R., J. Y. Chang, and H. C. Chang. 2007. Production of gamma-aminobutyric acid (GABA) by *Lactobacillus buchneri* isolated from kimchi and its neuroprotective effect on neuronal cells. *J. Microbiol. Biotechnol.* 17:104–109.
- Choi, S. I., J. W. Lee, S. M. Park, M. Y. Lee, J. I. Ge, M. S. Park, and T. R. Heo. 2006. Improvement of γ -aminobutyric acid (GABA) production using cell entrapment of *Lactobacillus brevis* GABA 057. *J. Microbiol. Biotechnol.* 16:562–568.
- Coda, R., C. G. Rizzello, and M. Gobbetti. 2010. Use of sourdough fermentation and pseudo-cereals and leguminous flours for the making of a functional bread enriched of γ -aminobutyric acid (GABA). *Int. J. Food Microbiol.* 137:236–245.
- Dhakal, R., V. K. Bajpai, and K. H. Baek. 2012. Production of GABA (γ -aminobutyric acid) by microorganisms: A review. *Braz. J. Microbiol.* 43:1230–1241.
- Di Cagno, R., F. Mazzacane, C. G. Rizzello, M. De Angelis, G. Giuliani, M. Meloni, B. De Servi, and M. Gobbetti. 2010. Synthesis of γ -aminobutyric acid (GABA) by *Lactobacillus plantarum* DSM19463: Functional grape must beverage and dermatological applications. *Appl. Microbiol. Biotechnol.* 86:731–741.
- Fan, E. Y., J. Huang, S. Hu, L. H. Mei, and K. Yu. 2012. Cloning, sequencing and expression of a glutamate decarboxylase gene from the GABA-producing strain *Lactobacillus brevis* CGMCC1306. *Ann. Microbiol.* 62:689–698.
- Gao, H., M. Liu, J. Liu, H. Q. Dai, X. L. Zhou, X. Y. Liu, Y. Zhuo, W. Q. Zhanga, and L. X. Zhang. 2009. Medium optimization for the production of avermectin B1a by *Streptomyces avermitilis* 14-12A using response surface methodology. *Bioresour. Technol.* 100:4012–4016.
- Guo, Y., R. Yang, H. Chen, Y. Song, and Z. Gu. 2012. Accumulation of γ -aminobutyric acid in germinated soybean (*Glycine max* L.) in relation to glutamate decarboxylase and diamine oxidase activity induced by additives under hypoxia. *Eur. Food Res. Technol.* 234:679–687.
- Hagiwara, H., T. Seki, and T. Ariga. 2004. The effect of pre-germinated brown rice intake on blood glucose and PAI-1 levels in streptozotocin-induced diabetic rats. *Biosci. Biotechnol. Biochem.* 68:444–447.
- Han, X., L. Zhang, P. Yu, H. Yi, and Y. C. Zhang. 2014. Potential of LAB starter culture isolated from Chinese traditional fermented foods for yogurt production. *Int. Dairy J.* 34:247–251.
- Hiraga, K., Y. Ueno, and K. Oda. 2008. Glutamate decarboxylase from *Lactobacillus brevis*: Activation by ammonium sulfate. *Biosci. Biotechnol. Biochem.* 72:1299–1306.
- Huang, J., L. Mei, H. Wu, and D. Lin. 2007. Biosynthesis of γ -aminobutyric acid (GABA) using immobilized whole cells of *Lactobacillus brevis*. *World J. Microbiol. Biotechnol.* 23:865–871.
- Inoue, K., T. Shirai, H. Ochiai, M. Kasao, K. Hayakawa, M. Kimura, and H. Sansawa. 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.
- Jeng, K. C., C. S. Chen, Y. P. Fang, R. C. Hou, and Y. S. Chen. 2007. Effect of microbial fermentation on content of statin, GABA, and polyphenols in Pu-Erh tea. *J. Agric. Food Chem.* 55:8787–8792.
- Keogh, M. K., and B. T. O'Kennedy. 1998. Rheology of stirred yogurt as affected by added milk fat, protein and hydrocolloids. *J. Food Sci.* 63:108–112.
- Kim, J. Y., M. Y. Lee, G. E. Ji, Y. S. Lee, and K. T. Hwang. 2009. Production of γ -aminobutyric acid in black raspberry juice during fermentation by *Lactobacillus brevis* GABA100. *Int. J. Food Microbiol.* 130:12–16.
- Ko, C. Y., H. T. V. Lin, and G. J. Tsai. 2013. Gamma-aminobutyric acid production in black soybean milk by *Lactobacillus brevis* FPA 3709 and the antidepressant effect of the fermented product on a forced swimming rat model. *Process Biochem.* 48:559–568.
- Komatsuzaki, N., T. Nakamura, T. Kimura, and J. Shima. 2008. Characterization of glutamate decarboxylase from a high gamma-aminobutyric acid (GABA)-producer, *Lactobacillus paracasei*. *Biosci. Biotechnol. Biochem.* 72:278–285.
- Komatsuzaki, N., J. Shima, S. Kawamoto, H. Momose, and T. Kimura. 2005. Production of γ -aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. *Food Microbiol.* 22:497–504.
- Kook, M. C., and S. C. Cho. 2013. Production of GABA (gamma amino butyric acid) by lactic acid bacteria. *Korean J. Food Sci. Anim. Resour.* 33:377–389.
- Kook, M. C., M. J. Seo, C. I. Cheigh, S. J. Lee, Y. R. Pyun, and H. Park. 2010. Enhancement of γ -aminobutyric acid production by *Lactobacillus sakei* B2-16 expressing glutamate decarboxylase from *Lactobacillus plantarum* ATCC 14917. *J. Korean Soc. Appl. Biol.* 53:816–820.
- Li, H., and Y. S. Cao. 2010. Lactic acid bacterial cell factories for gamma-aminobutyric acid. *Amino Acids* 39:1107–1116.
- Li, H., T. Qiu, D. Gao, and Y. Cao. 2010a. Medium optimization for yield of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912. *Amino Acids* 38:1439–1445.
- Li, H., T. Qiu, G. Huang, and Y. Cao. 2010b. Production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation. *Microb. Cell Fact.* 9:85–89.
- Liu, C. F., Y. T. Tung, C. L. Wu, B. H. Lee, W. H. Hsu, and T. M. Pan. 2011. Antihypertensive effects of *Lactobacillus*-fermented milk orally administered to spontaneously hypertensive rats. *J. Agric. Food Chem.* 59:4537–4543.
- Lu, X., Z. Chen, Z. Gu, and Y. Han. 2008. Isolation of γ -aminobutyric acid-producing bacteria and optimization of fermentative medium. *Biochem. Eng. J.* 41:48–52.
- Lu, X., C. Xie, and Z. Gu. 2009. Optimisation of fermentative parameters for GABA enrichment by *Lactococcus lactis*. *Czech J. Food Sci.* 27:433–442.
- Minervini, F., M. T. Bilancia, S. Siragusa, M. Gobbetti, and F. Caponio. 2009. Fermented goats' milk produced with selected multiple starters as a potentially functional food. *Food Microbiol.* 26:559–564.
- Nejati, F., C. G. Rizzello, R. Di Cagno, M. Sheikh-Zeinoddin, A. Diviccaro, F. Minervini, and M. Gobbetti. 2013. Manufacture of a functional fermented milk enriched of angiotensin-I converting enzyme (ACE)-inhibitory peptides and γ -amino butyric acid (GABA). *Lebenson. Wiss. Technol.* 51:183–189.
- Park, K. B., and S. H. Oh. 2006. Isolation and characterization of *Lactobacillus buchneri* strains with high gamma-aminobutyric acid producing capacity from naturally aged cheese. *Food Sci. Biotechnol.* 15:86–90.
- Park, K. B., and S. H. Oh. 2007. Production of yogurt with enhanced levels of gamma-aminobutyric acid and valuable nutrients using lactic acid bacteria and germinated soybean extract. *Bioresour. Technol.* 98:1675–1679.
- Pourahmad, R., A. Moghimi, S. Dadkhah, and M. M. Assadi. 2011. Evaluation of flavor and aroma compounds amounts in kefir from soymilk. *World Appl. Sci. J.* 15:673–676.
- Rea, K., T. I. Cremers, and B. H. Westerink. 2005. HPLC conditions are critical for the detection of GABA by Microdialysis. *J. Neurochem.* 94:672–679.

- Rizzello, C. G., L. Nionelli, R. Coda, M. D. Angelis, and M. Gobbetti. 2010. Effect of sourdough fermentation on stabilization, and chemical and nutritional characteristics of wheat germ. *Food Chem.* 119:1079–1089.
- Sawai, Y., Y. Yamaguchi, D. Miyana, and H. Yoshitomi. 2001. Cycling treatment of anaerobic and aerobic incubation increases the content of γ -aminobutyric acid in tea shoots. *Amino Acids* 20:331–334.
- Seo, M. J., J. Y. Lee, Y. D. Nam, S. Y. Lee, S. L. Park, S. H. Yi, M. H. Lee, S. W. Roh, H. J. Choi, and S. I. Lim. 2013. Production of γ -aminobutyric acid by *Lactobacillus brevis* 340G isolated from kimchi and its application to skim milk. *Food Engineering Progress* 4:418–423.
- Servili, M., C. G. Rizzello, A. Taticchi, S. Esposto, S. Urbani, F. Mazzacane, I. Di Maio, R. Selvaggini, M. Gobbetti, and R. Di Cagno. 2011. Functional milk beverage fortified with phenolic compounds extracted from olive vegetation water, and fermented with functional lactic acid bacteria. *Int. J. Food Microbiol.* 147:45–52.
- Shelp, B. J., A. W. Bown, and M. D. McLean. 1999. Metabolism and functions of gamma-aminobutyric acid. *Trends Plant Sci.* 4:446–452.
- Siragusa, S., M. De Angelis, R. Di Cagno, C. G. Rizzello, R. Coda, and M. Gobbetti. 2007. Synthesis of γ -aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. *Appl. Environ. Microbiol.* 73:7283–7290.
- Sun, T. S., S. P. Zhao, H. K. Wang, C. K. Cai, Y. F. Chen, and H. P. Zhang. 2009. ACE-inhibitory activity and gamma-aminobutyric acid content of fermented skim milk medium by *Lactobacillus helveticus* isolated from Xinjiang koumiss in China. *Eur. Food Res. Technol.* 228:607–612.
- Tamura, T., M. Noda, M. Ozaki, M. Maruyama, Y. Matoba, T. Kumagai, and M. Sugiyama. 2010. Establishment of an efficient fermentation system of gamma aminobutyric acid by a lactic acid bacterium, *Enterococcus avium* G-15, isolated from carrot leaves. *Biol. Pharm. Bull.* 33:1673–1679.
- Tong, J. C., I. R. Mackay, J. Chin, R. H. Law, K. Fayad, and M. J. Rowley. 2002. Enzymatic characterization of a recombinant isoform hybrid of glutamic acid decarboxylase (rGAD67/65) expressed in yeast. *J. Biotechnol.* 97:183–190.
- Tsai, J. S., Y. S. Lin, B. S. Pan, and T. J. Chen. 2006. Antihypertensive peptides and γ -aminobutyric acid from prozyme 6 facilitated lactic acid bacteria fermentation of soymilk. *Process Biochem.* 41:1282–1288.
- Tung, Y. T., B. H. Lee, C. F. Liu, and T. M. Pan. 2011. Optimization of culture condition for ACEI and GABA production by lactic acid bacteria. *J. Food Sci.* 76:M585–591.
- Ueno, Y., K. Hayakawa, S. Takahashi, and K. Oda. 1997. Purification and characterization of glutamate decarboxylase from *Lactobacillus brevis* IFO12005. *Biosci. Biotechnol. Biochem.* 61:1168–1171.
- Vaiva, G., P. Thomas, F. Ducrocq, M. Fontaine, V. Boss, P. Devos, C. Rasle, O. Cottencin, A. Brunet, P. Laffargue, and M. Goude-mand. 2004. Low post trauma GABA plasma levels as a predictive factor in the development of acute posttraumatic stress disorder. *Biol. Psychiatry* 55:250–254.
- Wang, H. K., C. Dong, Y. F. Chen, L. M. Cui, and H. P. Zhang. 2010. A new probiotic Cheddar cheese with high ACE-inhibitory activity and γ -aminobutyric acid content produced with koumiss-derived *Lactobacillus casei* Zhang. *Food Technol. Biotechnol.* 48:62–70.
- Wong, C. G., T. Bottiglieri, and O. Carter Snead III. 2003. GABA, γ -hydroxybutyric acid, and neurological disease. *Ann. Neurol.* 54(Suppl. 6):S3–12.
- Xanthopoulos, V., C. G. Ipsilandis, and N. Tzanetakis. 2012. Use of a selected multi-strain potential probiotic culture for the manufacture of set-type yogurt from caprine milk. *Small Rumin. Res.* 106:145–153.
- Yang, S. Y., F. X. Lü, Z. X. Lu, X. M. Bie, Y. Jiao, L. J. Sun, and B. Yu. 2008. Production of γ -aminobutyric acid by *Streptococcus salivarius* ssp. *thermophilus* Y₂ under submerged fermentation. *Amino Acids* 34:473–478.
- Yokoyama, S., J. Hiramatsu, and K. Hayakawa. 2002. Production of γ -aminobutyric acid from alcohol distillery lees by *Lactobacillus brevis* IFO-12005. *J. Biosci. Bioeng.* 93:95–97.
- Zhang, Y., L. Song, Q. Gao, S. M. Yu, L. Li, and N. F. Gao. 2012. The two-step biotransformation of monosodium glutamate to GABA by *Lactobacillus brevis* growing and resting cells. *Appl. Microbiol. Biotechnol.* 94:1619–1627.
- Zhao, H. Z., J. W. Liu, F. X. Lv, R. Ye, X. M. Bie, C. Zhang, and Z. X. Lu. 2014. Enzymatic synthesis of lard-based ascorbyl esters in a packed-bed reactor: Optimization by response surface methodology and evaluation of antioxidant properties. *Lebenson. Wiss. Technol.* 57:393–399.
- Zhou, Y. Q. 2010. The screening and identification of γ -aminobutyric acid-producing lactic acid bacteria from traditional dairy products in Inner Mongolia. MS Thesis. Northeast Agriculture University, Harbin, China.