BRAZILIAN JOURNAL OF FOOD TECHNOLOGY

ISSN 1981-6723
Online version

ORIGINAL ARTICLE

Tapping the potential of lactic acid bacteria: optimizing gamma-aminobutyric acid production for enhanced health benefits in fermented milk

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Cite as: Wicaksono, S., Nuraida, L., & Faridah, D. N. (2024). Tapping the potential of lactic acid bacteria: optimizing gamma-aminobutyric acid production for enhanced health benefits in fermented milk. *Brazilian Journal of Food Technology*, *27*, e2024015. https://doi.org/10.1590/1981-6723.01524

Abstract

Gamma-aminobutyric acid (GABA) is a non-protein amino acid with numerous health benefits, such as reducing hypertension, treating diabetes, and providing neuroprotection. GABA can be produced by lactic acid bacteria (LAB), but not all LAB produce GABA. Therefore, it is necessary to screen LAB isolates with the potential to be GABA producers. GABA was analyzed qualitatively using thin-layer chromatography (TLC) and quantitatively by Ultraviolet-visible (UV-Vis) spectrophotometer. Ten LAB isolates were evaluated for their ability to produce GABA. Among these, *Pediococcus acidilactici* YKP4 produced the highest concentration of GABA. *P. acidilactici* YKP4 as the highest GABA producer and *Lacticaseibacillus rhamnosus* BD2 as a probiotic candidate were further studied to increase GABA production in *de Man Rogosa Sharpe* (MRS) broth by providing monosodium glutamate (MSG) as the precursor and applying different initial pH and incubation time. The highest production of GABA by *P. acidilactici* YKP4 (3.30±0.03 g/L) was obtained by the addition of 1% MSG while that of *L. rhamnosus* BD2 (2.67±0.07 g/L) by 2% MSG with initial pH of 5.0 and incubation time of 72 h. Application of both LAB as a starter culture for milk fermentation showed that GABA production in milk was lower than in MRS broth. Enrichment of milk with MSG increased GABA from 1.80±0.03 g/L to 1.94±0.02 g/L in *P. acidilactici* YKP4 with 1% MSG, and from 1.15±0.03 g/L to 1.33±0.04 g/L in *L. rhamnosus* BD2 with 2% MSG. Besides, *P. acidilactic*i YKP4 and *L. rhamnosus* BD2 were considered as potential LAB for GABA production and can be applied to ferment milk as a functional food.

Keywords: Fermentation; Functional food; GABA-producing LAB; *L. rhamnosus*; *P. acidilactici*; Starter culture.

Highlights

- *P. acidilactici* YKP4 and *L. rhamnosus* BD2 were LAB as potential GABA producers
- MSG concentration, initial pH, and incubation time affected GABA production in both LAB
- Both LAB can be applied to enrich GABA in fermented milk.

1 Introduction

Gamma-aminobutyric acid (GABA) is a bioactive, non-protein amino acid that acts as an inhibitory neurotransmitter in the central nervous system of mammals and has additional health-related functional properties. GABA has numerous bio-activities, such as providing neuroprotection (Li et al., 2016), reducing hypertension (Kawakami et al., 2018; Suwanmanon & Hsieh, 2014), treating diabetes (Shang et al., 2018; Untereiner et al., 2019), and possessing anticancer properties (Song et al., 2016). It also acts as an antioxidant (Tang et al., 2018; Zhu et al., 2019), anti-inflammatory (Sokovic Bajic et al., 2019), and protects the intestinal wall (Chen et al., 2015). Additionally, it prevents sleep difficulties and depression (Ngo & Vo, 2019). GABA is present in both animals and plants, but its concentration is relatively low, around µg/g in animals and mg/g in plants (Ramos-Ruiz et al., 2018). Microorganisms synthesize GABA through glutamate decarboxylation, a process catalyzed by the enzyme glutamic acid decarboxylase (GAD; EC 4.1.1.15) (Wu & Shah, 2017). Microorganisms are preferable to animals and plants for producing GABA because they grow faster, can produce GABA quicker, and require less space for cultivation (Cui et al., 2020). Besides being more efficient, GABA production by microorganisms is a more promising method than chemical synthesis because it is eco-friendly, requires lower costs, and obtains higher yields (Zhao et al., 2014). Lactic acid bacteria (LAB) are the most widely used for GABA production among microorganisms.

Generally, LAB are widely used as starter cultures for food fermentation due to their specific metabolism activities and they are generally recognized as safe (GRAS) bacteria (Luo et al., 2021). The production of GABA by LAB has made it possible to create fermented foods with high GABA concentrations, resulting in improved functional properties. Several fermented foods, including yoghurt (Shan et al., 2015), black soybean milk (Ko et al., 2013), litchi juice (Wang et al., 2021), fermented mulberry juice (Kanklai et al., 2020), *som-pak* (a Thai fermented vegetable) (Tanamool et al., 2020), and *kung-som* (a Thai fermented shrimp) (Sanchart et al., 2017), have been enriched with GABA through the use of GABA-producing LAB as starter cultures.

Currently, the isolation and characterization of GABA-producing LAB from different fermented foods have been widely investigated and discussed by Yogeswara et al. (2020) and Cui et al. (2020). Nonetheless, screening the most efficient GABA-producing LAB remains imperative due to the varied LAB groups present in fermented foods, which depend on the production methods and raw ingredients used (Seo et al., 2013). Production of GABA by LAB relies on the LAB strain and is affected by the presence of precursor (monosodium glutamate/MSG), pH of the medium, and incubation time (Binh et al., 2014; Lim et al., 2017). The concentration of glutamate in MRS broth, which is generally utilized for LAB cultivation, is minimal (Wu & Shah, 2015). The pH of the medium is crucial factor for GABA production, with the optimal pH range for GABA production being between 4.5 and 5.5 (Yogeswara et al., 2020). Previous research has demonstrated the addition of MSG (Tanamool et al., 2020), initial pH (Li et al., 2010; Lim et al., 2017), and incubation time (Binh et al., 2014) as potential means to enhance GABA production in LAB.

Indonesia has a variety of fermented foods that involve LAB in their production. The LAB used in this study was obtained from fermented foods such as tempeh, fermented mustard, sticky rice tape, and other sources such as kefir granules and breast milk. Two potential probiotics, *Lacticaseibacillus rhamnosus* BD2, and *Lactobacillus kefiri* YK4, were derived from kefir granules (Yusuf et al., 2019) and were found to possess high proteolytic activities (Rubak et al., 2020). LAB isolated from kefir granules produced antioxidant peptides (Yusuf et al., 2021) and angiotensin-converting enzyme (ACE) inhibitors (Rubak et al., 2020). Nevertheless, the ability of these LAB to produce GABA is yet to be determined. Therefore, this study aims to obtain LAB isolates from different foods to determine their potential as GABA producers and identify the factors that contribute to the GABA production in LAB.

2 Material & methods

2.1 LAB isolates and LAB growth medium

The LAB isolates used in this study were obtained from the SEAFAST (Southeast Asian Food and Agricultural Science and Technology) Center, IPB University. The isolates of kefir granules origin were *L.*

kefiri YK4, *L. rhamnosus* BD2, *Pediococcus acidilactici* YKP4, *Lactococcus lactis* subsp. *lactis* BD17, and *Limosilactobacillus fermentum* JK13 (Yusuf et al., 2019), while that from tempeh were *Lactiplantibacillus plantarum* 1W22408 and *L. fermentum* S206 (Touw, 2014), from fermented mustard were *L. plantarum* 4C261 (Kurnia, 2018), and LAB isolate from sticky rice tape, namely *L. fermentum* BK27 (Hasanah et al., 2019; Nurchandra, 2018). In addition to that, an isolate from breast milk, *L. rhamnosus* R23 (Nuraida et al., 2012) was used in this study. These LAB were cultivated in *de Man Rogosa Sharpe* (MRS) broth (Oxoid, UK) and quantified using MRS agar (Oxoid, UK).

2.2 Inoculum preparation

Inoculum preparation refers to Yusuf et al. (2019) with slight modifications. LAB isolates were refreshed in MRS broth and incubated in an incubator (MMM Medcenter Einrichtungen GmbH Incucell, Germany) at 37 °C for 24 hours with an inoculum concentration of 10^7 CFU/mL.

2.3 Screening of GABA-producing LAB

As much as 1% (v/v) of a fresh culture of LAB was inoculated into 100 mL MRS broth (in 100 mL Schott Duran bottles) containing 1% (w/v) MSG (HiMedia, India). Before sterilization, the initial pH was adjusted to 5.0 (Yang et al., 2018) by addition of HCl 1 N (Merck, Germany) gradually. The pH was measured using a pH meter (Horiba Laqua-PH1100, Japan). Incubation was carried out for 48 hours at 37 °C (Rayavarapu et al., 2021). Uninoculated MRS broth was used as a control. GABA was analyzed qualitatively and quantitatively.

Qualitative analysis of GABA was performed by thin layer chromatography (TLC) referring to Qiu et al. (2010) with slight modification. A 1 mL culture was taken and centrifuged (Labtron Refrigerated Centrifuge LRF-B20, UK) at 8000 rpm for 10 min at 4 °C. A total of 0.5 µL of supernatant, GABA standard (1%) (Sigma-Aldrich, USA), and MSG solution (1%) were plated on silica gel 60 F_{254} plates (Merck, Germany). The mobile phase consisted of a mixture of butanol (Merck, Germany), acetic acid (Merck, Germany), and distilled water in a ratio of 5:3:2, respectively. The plate was then sprayed with 1.2% ninhydrin (Merck, Germany) and dried in an oven (Memmert UN55 53L, Germany) at 90 °C for 7 minutes. GABA produced by LAB is indicated by the same Rf value as the GABA standard (Yogeswara et al., 2021). To determine the LAB capable of producing the highest GABA, a quantitative analysis of GABA produced by LAB was then carried out.

Quantitative analysis of GABA was performed based on the method of Li et al. (2009) with slight modifications. The spot separated on the TLC plate representing GABA was scraped off and dissolved in a solution containing 1 mL of 75% (v/v) ethanol (Merck, Germany) and 0.6% (w/v) cupric sulfate (Merck, Germany) in a 38:2 (v/v) ratio at 40 °C. Samples were measured for absorbance with a UV-Vis spectrophotometer (Hitachi U-2800, Japan) at a wavelength of 512 nm using a 1 mL cuvette. GABA concentration was calculated based on the GABA standard curve which had a concentration range of 0.5-4.5 g/L and a coefficient of determination (R^2) of 0.99. LAB with the highest GABA production and probiotic candidate LAB were used in the next stage.

2.4 Effect of precursor (MSG) on GABA production

LAB inoculum of 1% (v/v) was inoculated into 100 mL MRS broth with an initial pH of 5.0 and MSG concentrations of 0, 1, 2, and 3% (w/v) in 100 mL Schott Duran bottles (Tanamool et al., 2020) and incubated at 37 °C for 72 hours (Binh et al., 2014; Sanchart et al., 2017). GABA concentration was analyzed quantitatively.

2.5 Effect of initial pH of the medium and incubation time on GABA production

A fresh LAB culture of 1% (v/v) was inoculated into 100 mL of sterile MRS broth (in 100 mL Schott Duran bottles) with the best MSG concentration obtained in the previous stage. The initial pH value of the medium was set to 4.5; 5.0; and 5.5. Incubation was carried out for 0, 24, 48, and 72 hours at 37 °C. Analyses

were carried out for LAB count, pH, and GABA concentration. The number of LAB was enumerated using the pour plate method in MRS agar, and incubated at 37 °C for 48 hours. Only plates containing colonies from 25-250 were used for calculation of LAB count (Maturin & Peeler, 2001).

2.6 Application in fermented milk

Inoculum preparation refers to Nursini et al. (2022) with slight modifications. The starter culture was prepared by inoculating fresh LAB cultures at 2% (v/v) into reconstituted skim cow's milk (Sunlac, Malaysia) (14% total solids) and incubated for 24 hours at 37 °C. The starter culture was then inoculated at 5% (v/v) into the production medium (50 mL reconstituted skim cow's milk in 100 mL Schott Duran bottles) with and without addition of MSG of 1% for *P. acidilactici* YKP4 and 2% for *L. rhamnosus* BD2 based on the concentration resulted in the highest GABA concentration. Incubation was carried out at 37 °C for 72 hours. Analysis was conducted on GABA concentration, LAB count, and culture pH at the end of incubation. For GABA analysis, 10 mL of fermented milk was centrifuged (6000 rpm for 30 minutes at 4 °C), then the supernatant was filtered using a 0.22 µm pore size membrane filter (Han et al., 2020). The concentration of GABA in the supernatant was analyzed using the method previously described.

2.7 Statistical analysis

The experiment was conducted with two repetitions. Statistical analysis was performed using SPSS 16 software with a one-way Analysis of Variance (ANOVA) test for the first, second, and third stages. If a significant difference was found, the Duncan Multiple Range Test (DMRT) was conducted at a 5% significance level. For the fourth stage, an independent sample t-test was performed at a 5% significance level.

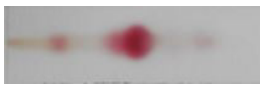
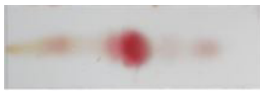
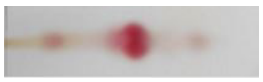
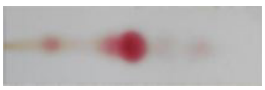
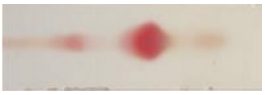
3 Results & discussion

3.1 Screening of GABA-producing LAB

All LAB isolates, derived from various foods, are capable of producing GABA, as demonstrated by the GABA spot with an identical Rf value to the standard GABA Rf value (Table 1). The quantitative GABA analysis was conducted to identify high GABA-producing LAB. The concentration of GABA produced in this study ranged from 1.05 to 2.32 g/L, which is notably higher than that of Park et al. (2014) who used *L. plantarum* K154, producing 0.20 g/L of GABA. In the previous study, 3% MSG concentration was employed, and the LAB was incubated at 37 °C for 18 hours (Park et al., 2014). This variation may arise as GABA production is affected by LAB strains and the conditions for fermentation (Cui et al., 2020).

The results showed that *P. acidilactici* YKP4 produced the highest concentration of GABA among the LAB, reaching a concentration of 2.30 g/L after 48 hours of incubation. Additionally, previous studies have indicated that *P. acidilactici* DS15 and *P. pentosaceus* HN8 produced GABA concentrations of 0.31 g/L and 9.06 g/L, respectively (Anggraini et al., 2019; Ratanaburee et al., 2013). On the other hand, *L. rhamnosus* BD2, *L. kefiri* YK4, and *L. plantarum* 4C261 as probiotic candidates LAB (Kurnia, 2018; Yusuf et al., 2019) produced GABA amounted to 1.33, 1.32, and 1.31 g/L, respectively, that were not significantly different ($p > 0.05$). Previous research has shown that *L. rhamnosus* BD2 has high α-glucosidase inhibitory activity, as well as the ability to produce antioxidant peptides (Yusuf et al., 2021) and reduce cholesterol levels *in vitro* (Yusuf et al., 2019). The production of GABA by *L. rhamnosus* BD2 enhances the physiological function of LAB besides inhibiting pathogenic bacteria causing enteric infections. For the subsequent stage, two LAB isolates were selected: *P. acidilactici* YKP4 as high GABA producer and *L. rhamnosus* BD2, considered a potential probiotic candidate.

Table 1. GABA-producing LAB from various foods after 48 hours of incubation at 37 °C using MRS broth medium and 1% MSG concentration.

No	Lactic Acid Bacteria	Sources	TLC Results	Rf Value	GABA Concentration (g/L)*
1	*Lactiplantibacillus plantarum* 1W22408	Tempeh		0.65	1.17 ± 0.01^{e}
2	*Limosilactobacillus fermentum* S206	Tempeh		0.65	1.17 ± 0.03^{e}
3	*Lactobacillus kefiri* YK4	Kefir granules		0.65	1.31 ± 0.05^{d}
4	*Lacticaseibacillus rhamnosus* BD2	Kefir granules		0.65	1.33 ± 0.06^{cd}
5	*Pediococcus acidilactici* YKP4	Kefir granules		0.65	2.32 ± 0.06^{a}
6	*Lactococcus lactis* subsp. *lactis* BD17	Kefir granules		0.65	1.05 ± 0.01^{f}
7	*Lacticaseibacillus rhamnosus* R23	Breast milk		0.65	1.20 ± 0.04^{e}
8	*Limosilactobacillus fermentum* JK13	Kefir granules		0.65	1.41 ± 0.03^{c}
9	*Lactiplantibacillus plantarum* 4C261	Fermented mustards		0.65	1.31 ± 0.03^{d}
10	*Limosilactobacillus fermentum* BK27	Sticky rice tape		0.65	1.69 ± 0.02^{b}
11	Medium without culture	-		-	-
12	MSG	-		0.54	-
13	GABA Standard	-		0.65	-

*Numbers in the same column followed by a different letter indicate a significant difference at the 5% level, as determined by the Duncan test with two replicates.

3.2 Effect of precursor addition (MSG) on GABA production

Figure 1 demonstrated that supplementing MSG enhanced GABA production in both chosen LAB. The GAD enzyme in LAB decarboxylates glutamate into GABA, hence adding MSG stimulates GABA production. LAB utilizes glutamate as a protective mechanism against acidic compounds that accumulate in the MRS broth medium. Undissociated acidic compounds can efficiently enter the cell membrane. The pH

value inside the cell is higher than that outside the cell, causing the acid to dissociate into protons (H^+) and acid ions. This results in a decrease in pH inside the cell (Cotter & Hill, 2003). To counteract this, LAB utilizes glutamate to form GABA and balance the pH inside the cell. Glutamate present in the MRS broth medium enters the cell through GadC, and then is decarboxylated by GadA/GadB, consuming protons in the process to form GABA. The produced GABA is subsequently discharged by the cell via GadC (Yogeswara et al., 2020). This maintains the pH balance of the cell, resulting in efficient LAB metabolism, allowing LAB to survive in an acidic environment.

The highest GABA yield for *P. acidilactici* YKP4 was 3.25 g/L reached by the addition of 1% MSG concentration. The results of 1% addition of MSG obtained in this step was higher than presented in Table 1 was considered due to application of longer fermentation time in this step. When the concentration of MSG added to the medium exceeded 1%, it decreased the production of GABA. In the case of *L. rhamnosus* BD2, the highest GABA production (2.52 g/L) was reached with addition of a 2% MSG, but decreased with concentrations greater than 2% (Figure 1). This suggests that MSG concentrations above 1% suppress GABA production in *P. acidilactici* YKP4, whereas MSG concentrations above 2% inhibit GABA production in *L. rhamnosus* BD2. As shown in the study by Seo et al. (2013), *L. brevis* 340G produced 7.09 g/L of GABA at a concentration of 3% MSG, which decreases when MSG concentrations are above 3%. High MSG concentrations elevate the osmotic pressure of LAB cells leading to water loss and eventually cellular dehydration. When cells lack water, LAB growth is inhibited and their metabolism is disrupted, including a decrease in GABA production (Stecker et al., 2022; Yang et al., 2008). However, other LAB were able to adapt to high concentrations of MSG. *L. brevis* CRL 1942 was capable of generating a significant GABA yield of ≈26 g/L by utilizing an MSG concentration of 4.6% (Villegas et al., 2016). These findings suggest that specific concentrations of MSG may promote GABA production. In addition to the concentration of MSG, the concentration of GAD enzyme and its catalytic properties may influence the efficiency of GAD enzyme in producing GABA (Mazzoli et al., 2010). The concentration of MSG that increased GABA production by both LAB isolates in this study was lower compared to the findings of other studies. Based on the results, the MSG concentration used in the next stage was 1% for *P. acidilactici* YKP4 and 2% for *L. rhamnosus* BD2.

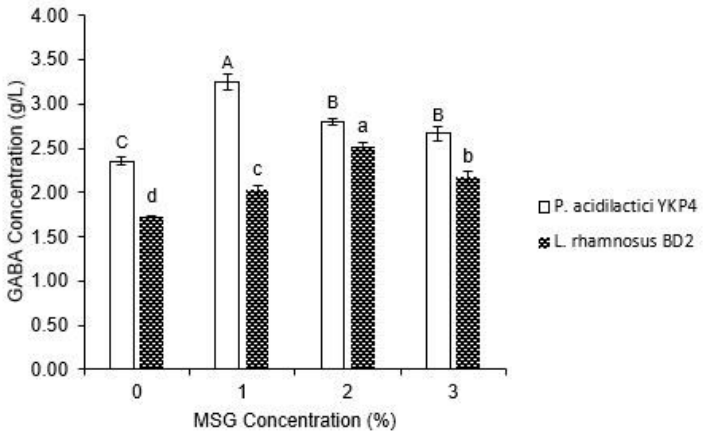


Figure 1. GABA production by *P. acidilactici* YKP4 and *L. rhamnosus* BD2 in MRS broth with various concentrations of MSG after 72 hours of incubation time at 37 °C. Different letters indicate significant differences at the 5% test level using the Duncan test with two replicates.

3.3 Effect of initial pH of the medium and incubation time on GABA production

The study revealed an increase in GABA concentration throughout the 72-hour incubation time. It could be noted that *P. acidilactici* YKP4 exhibited greater GABA production than *L. rhamnosus* BD2 during the incubation time (Figures 2 and 3). The highest GABA concentration with a concentration of 3.30 g/L was

achieved by *P. acidilactici* YKP4 at 72 hours incubation time, initial pH of 5.0, and 1% MSG concentration. In *L. rhamnosus* BD2, the maximum GABA concentration was 2.67 g/L achieved after 72 hours of incubation, with an initial pH of 5.0 and 2% MSG concentration.

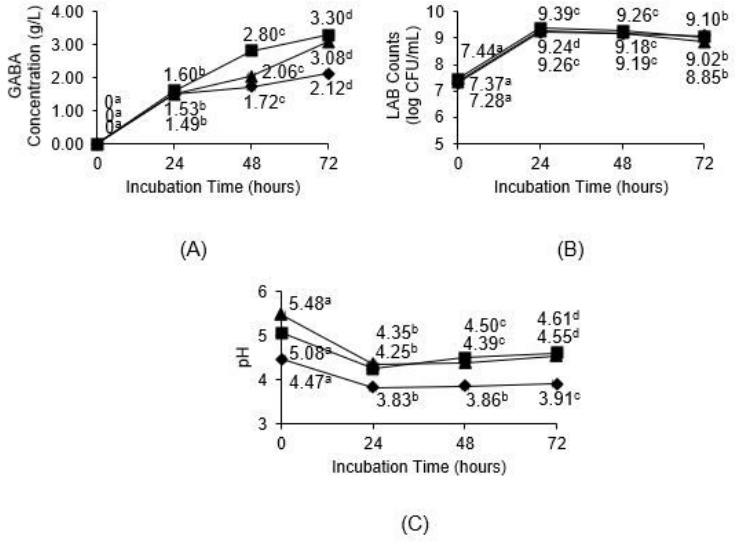


Figure 2. Effect of incubation time and initial pH (◆) 4.5, (■) 5.0, and (▲) 5.5 of MRS broth containing 1% MSG on GABA production (A), total LAB (B), and pH change (C) by *P. acidilactici* YKP4 at 37 °C for 72 hours. Different superscripts indicate significant differences between incubation times at the 5% test level using the Duncan test with two replicates.

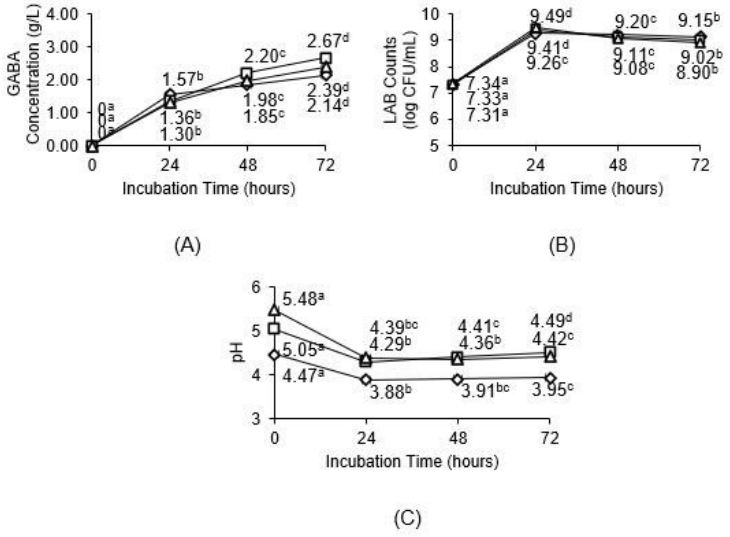


Figure 3. Effect of incubation time and initial pH (◇) 4.5, (□) 5.0, and (△) 5.5 of MRS broth containing 1% MSG on GABA production (A), total LAB (B), and pH change (C) by *L. rhamnosus* BD2 at 37 °C for 72 hours. Different superscripts indicate significant differences between incubation times at the 5% test level using the Duncan test with two replicates.

Based on the results of this study, the growth of *P. acidilactici* YKP4 and *L. rhamnosus* BD2 exhibited a similar pattern at varying pH levels (Figures 2 and 3). Both LAB experienced a steep increase in growth for the initial 24 hours and slightly decreased until the end of the 72-hour incubation. In contrast to the growth GABA production continuously increased after the growth ceased. Lim et al. (2018) found that as LAB transition to the stationary phase, they express GadA, GadB, and GadC genes, which allow them to produce GABA. The initial pH value of the medium did not affect the growth of the LAB (Figures 2B and 3B), however, it was observed that GABA production (Table 2) was affected, with the highest amount achieved when the initial pH

was 5.0 after 72 h incubation that significantly different with other pHs. Although GABA production serves as a mechanism of protection for LAB against acidic conditions in order to maintain their viability, however when the initial pH was too low, the GABA production was lower, especially for *P. acidilactici* YKP4.

Table 2. Effect of pH on the GABA production and viable cells of *P. acidilactici* YKP4 and *L. rhamnosus* BD2 after 72 h incubation in MRS broth with addition of MSG.

Initial pH	*P. acidilactici* YKP4		*L. rhamnosus* BD2	
	GABA Concentration (g/L)*	Viable Cells Count (log CFU/mL)*	GABA Concentration (g/L)*	Viable Cells Count (log CFU/mL)*
4.5	2.12^{c}	9.10^{a}	2.14^{c}	9.15^{a}
5.0	3.30^{a}	9.02^{a}	2.67^{a}	9.02^{ab}
5.5	3.08^{b}	8.85^{b}	2.39^{b}	8.90^{b}

*Numbers in the same column followed by a different letter indicate a significant difference at the 5% level, as determined by the Duncan test with two replicates.

Figures 2 and 3 show that the pH of cultures decreased after 24 hours of incubation. This was due to a sharp increase in LAB numbers, resulting in acid production and subsequent pH decrease. At 48 and 72 hours of incubation, the pH increased slightly, and GABA production continued to increase, while LAB growth ceased. This may occur because the acidic MSG undergoes decarboxylation by the GAD enzyme present in the cytoplasm to produce alkaline GABA, which is then released into the MRS medium via the GABA antiporter. This aligns with prior studies indicating that alkaline GABA is transported into the growth medium via the antiporter, leading to a slight increase in pH in the medium. The increase in pH cannot be attributed to ammonia production, as LAB lacks glutamate dehydrogenase which produces NH_3 (Cotter & Hill, 2003; Seo et al., 2013). Furthermore, pH is one of the most important factors in GABA production, as GAD activity in LAB is very active at pH 4.5-5.5 (Yogeswara et al., 2020). Li et al. (2010) conducted research revealing that the initial pH of fermentation has an effect on GABA production. Nonetheless, the optimal initial pH for GABA production differs for each species or strain. *L. brevis* NCL912 has the ability to produce GABA with maximum yield at an initial pH of 5.0, as reported by Li et al. (2010). In addition, *L. brevis* HYE1 can produce GABA optimally at an initial pH of 4.74, according to Lim et al. (2017), and *L. paracasei* 15C has been found to produce GABA with maximum production at an initial pH of 5.5, as reported by Franciosi et al. (2015).

During this study, the production of GABA was also influenced by the incubation time. Within the 24–72-hour incubation period, GABA production consistently increased despite the inhibition and cessation of LAB growth (reaching the stationary phase). This suggests that LAB growth during the stationary phase does not affect GABA production. When the growth of LAB is deceased, they actively employ glutamate to generate GABA to protect themselves from acidic conditions. The pH value at the end of the incubation time shows the acidification of the MRS medium, achieving 4.6 in *P. acidilactici* YKP4 and 4.5 in *L. rhamnosus* BD2 from the initial pH of 5.0 (Figures 2C and 3C). This pH condition brought the growth of both LAB isolates to a halt. The growth conditions are in compliance with the study carried out by Mataragas et al. (2003) which indicated the inhibition of the growth of *Leuconostoc mesentroides* L124 and *Lactobacillus curvatus* 442 when the pH level is below 5.0.

The research found that the greatest time for GABA production was at 72 hours of incubation. The present results correspond with previous studies (Binh et al., 2014; Sanchart et al., 2017) which also demonstrated that 72 hours was the most effective incubation period for GABA production. Sanchart et al. (2017) discovered that *L. futsaii* CS3 produced the highest concentration of GABA, approximately 25 g/L, in MRS medium after 72 hours of incubation. Another study demonstrated that incubation for more than 72 hours did not result in significant differences in GABA production (Binh et al., 2014). The results of this and previous studies suggest that environmental conditions during incubation greatly influence GABA production and that each LAB have unique capabilities in this respect.

3.4 Application in fermented milk

The addition of MSG to skim milk resulted in increased GABA production by both cultures (Figure 4). Specifically, the addition of 1% MSG into skim milk increased GABA production significantly ($p < 0.05$) by *P. acidilactici* YKP4 from 1.80±0.03 g/L to 1.94±0.02 g/L, meanwhile, the addition of 2% MSG also increased significantly ($p < 0.05$) GABA production by *L. rhamnosus* BD2 from 1.15±0.03 g/L to 1.33±0.04 g/L. Using skim milk supplemented with MSG as a substrate for fermentation, previous research showed an increase in GABA production by different species of LAB. Seo et al. (2013) found that GABA production by *L. brevis* 340G increased from 0.08 g/L to 0.48 g/L after 48 hours of incubation in skim milk with the addition of 1% MSG. Research carried out by Somkuti et al. (2012) also demonstrated an increase in GABA production in skim milk supplemented with 1% MSG by *Streptococcus thermophilus* ST110 from 0.003 g/L to 0.07 g/L. The content of free glutamate present in skim milk is inadequate to produce significant levels of GABA (Seo et al., 2013), thus, the addition of glutamate increases the precursor for GABA production that results in significant increases in GABA production in skim milk. Although in the present research GABA production (1.94 g/L) in skim milk with the addition of MSG was higher than that of reported in the previous studies (Seo et al., 2013; Somkuti et al., 2012), however, the percentage increased in GABA production was lower than those two previous research (8% vs 500% and 2233%, respectively). This may be due to a different composition of skim milk and incubation temperature, besides different cultures between the present research and two previous studies. Increase of MSG concentration from 1% to 1.5% in combination with addition of casein hydrolysate (1 g/L) in skim milk has been demonstrated to increase 93% (from 2.80 g/L to 5.40 g/L) GABA production by *S. thermophilus* (Han et al., 2020). This research confirms that beside LAB strains and fermentation conditions that affect GABA production, enrichment of nutrient may also needed to increase GABA production.

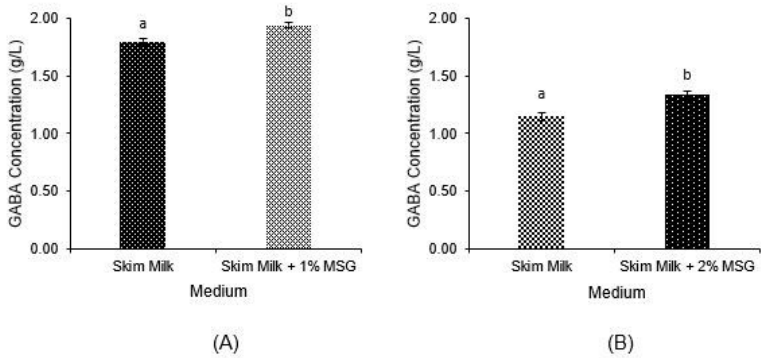


Figure 4. GABA production by *P. acidilactici* YKP4 (A) and *L. rhamnosus* BD2 (B) in skim milk and skim milk + MSG medium after 72 hours incubation time at 37 °C. The different letters indicate significant differences based on a t-test with a 5% significance level with two replicates.

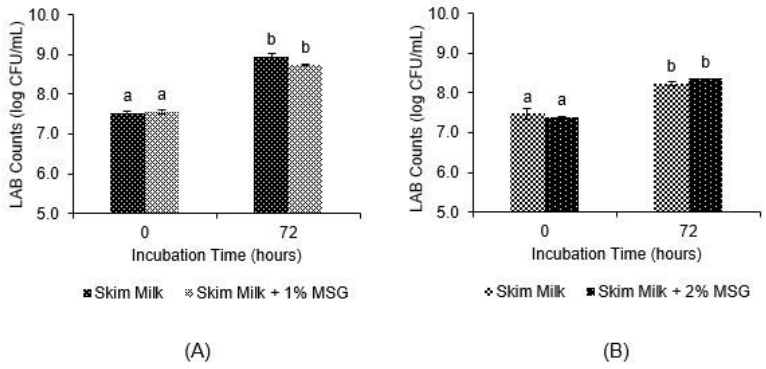


Figure 5. Total LAB of *P. acidilactici* YKP4 (A) and *L. rhamnosus* BD2 (B) in skim milk and skim milk + MSG medium after 72 hours incubation time at 37 °C. The different letters indicate significant differences based on a t-test with a 5% significance level with two replicates.

Furthermore, there was no notable difference in the total number of LAB between the skim milk medium containing MSG and the one without MSG ($p > 0.05$) after the incubation time ended for both *P. acidilactici* YKP4 and *L. rhamnosus* BD2 (Figure 5). These findings suggest that MSG did not impede LAB growth at the end of the incubation period (Figure 5), but did affect GABA production (Figure 4). GABA production by both LAB isolates in skim milk supplemented with MSG was comparatively lower than in MRS medium supplemented with MSG (refer to Table 3). This is believed to be attributed to dissimilarities in the initial pH value employed. Specifically, while the initial pH of the MRS + MSG medium was brought down to pH 5.0 by a gradual addition of HCl, the initial pH of the skim milk + MSG medium could not be regulated due to clotting when acid was included, resulting in an initial measurement of pH 6.5 (Figure 6). The initial pH value significantly affects GABA production, with pH 4.5-5.5 being the most conducive for GABA production (Yogeswara et al., 2020). These findings align with those of Seo et al. (2013), who noted lower GABA production by *L. brevis* 340G in skim milk + MSG medium (0.48 g/L) compared to the optimized MRS medium (7.09 g/L). LAB isolates generate acid during fermentation, resulting in reduced pH levels of milk. The final pH value of skim milk added with MSG, both fermented by *P. acidilactici* YKP4 and *L. rhamnosus* BD2, tended to be higher than without MSG (Figure 6) and this correlated with the production of GABA as a mechanism for reducing acid in the medium.

Table 3. Comparison of GABA levels in MRS + MSG and skim milk + MSG medium after 72 hours of incubation at 37 °C.

LAB Isolates	GABA Concentration (g/L)	
	MRS + MSG	Skim Milk + MSG
P. acidilactici YKP4	3.30	1.94
L. rhamnosus BD2	2.67	1.33

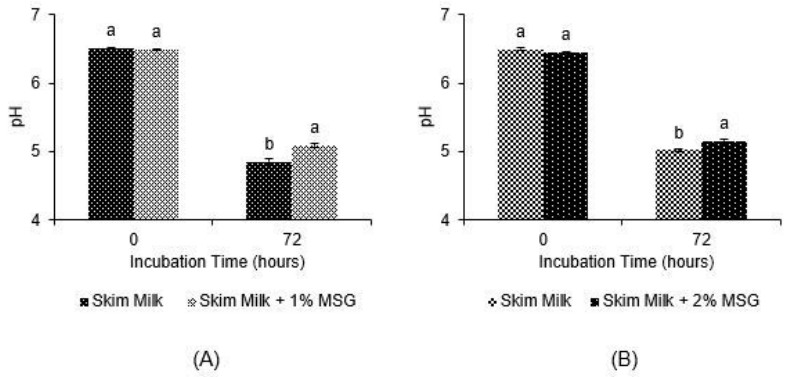


Figure 6. Decrease in pH of skim milk and skim milk + MSG media fermented by *P. acidilactici* YKP4 (A) and *L. rhamnosus* BD2 (B) after 72 hours incubation time at 37 °C. The different letters indicate significant differences based on a t-test with a 5% significance level with two replicates.

4 Conclusions

Lactic acid bacteria are promising GABA producers, however, its capability relies on the strain and fermentation conditions. Indeed, *P. acidilactici* YKP4 was found to be the highest GABA producer among the isolates of kefir grain origin. However, *L. rhamnosus* BD2, a potential probiotic candidate, also showed as a promising GABA producer. The ability to produce GABA will enhance its health beneficial effect. MSG as a precursor plays an important role in increasing GABA production. As can be seen, different LAB responses differently to the precursor. MSG concentration of 1% was sufficient to increase GABA production by *P. acidilactici* YKP4 while *L. rhamnosus* BD2 needed 2%. Initial pH and incubation time were responded similarly by the two LAB with the maximum production at an initial pH of 5.0 and 72 h fermentation.

Considering the growing demand for fermented food as functional food with specific health beneficial effect, enriched fermented milk with GABA is becoming one of the promising product. Thus, *P. acidilactici* YKP4 and *L. rhamnosus* BD2 are the potential to be used as starter culture for GABA-enriched fermented milk. However, further exploration is needed to optimize the MSG concentration and sensory aspects of the fermented milk.

Acknowledgements

The authors are grateful to the staff of the SEAFAST Center Laboratory and the Biopharmaca Laboratory, IPB University, for their valuable assistance and support in executing this research.

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Funding: Directorate General of Higher Education, Research and Technology; Ministry of Education, Culture, Research and Technology under the Master Thesis Research scheme with number 082/E5/PG.02.00.PT/2022 dated May 10th, 2022.

Received: Feb. 07, 2024; Accepted: May 28, 2024

Associate Editor: Felipe Alves de Almeida.