

## Research Article

# Chive-Fortified Fermentation Enhances Gamma-Aminobutyric Acid Production by *Levilactobacillus brevis* PL9014

<sup>1</sup>J.J. Paek, <sup>2</sup>J. Kim, <sup>2</sup>S. Bae, <sup>3</sup>K.S. Paek, and <sup>1</sup>Y. Lee

<sup>1</sup>PLB&B, Topyung-dong, Guri-shi 11960, Republic of Korea

<sup>2</sup>Department of Chemistry, Seoul Women's University, Seoul 01797, Republic of Korea

<sup>3</sup>PLBio, Chowonjungangro, Seoul 08765, Republic of Korea

**Running title:** Chive-enhanced GABA production with *L. brevis*

This study presents a novel approach for significantly enhancing gamma-aminobutyric acid (GABA) production by supplementing glutamate-containing media with chive (*Allium tuberosum*) during fermentation with *Levilactobacillus brevis* PL9014. *L. brevis* PL9014 demonstrated superior GABA conversion efficiency within two days. In the presence of 1% chive (w/v), the strain produced 13.22 g/L GABA with 5% glutamate (w/v) and some residual substrate, and 10.65 g/L (w/v) GABA with 2% (w/v) glutamate with no detectable residual glutamate. The fermentation was performed at 37 °C without aeration. The resulting product contained high GABA concentration alongside bioactive components from fermented chives, making it suitable for functional foods, dietary supplements, animal feed additives, and cosmetic formulations. This chive-fortified process is cost-effective, environmentally compatible, and readily scalable, providing a practical platform for industrial GABA production without additional post-fermentation glutamate removal. The findings offer a novel approach for optimizing microbial GABA production using plant-based substrates.

**Keywords:** chive, GABA, glutamate, *Levilactobacillus brevis*, psychobiotic.

**Corresponding Author:** Yeonhee Lee, PLB&B, Guri-shi, Korea, E-mail: [yhlee@swu.ac.kr](mailto:yhlee@swu.ac.kr); Tel: +82-031-556-7731; Fax: +82-031-556-7731

## INTRODUCTION

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter and a key postbiotic mediator involved in the gut-brain axis. It plays essential roles in regulating sleep-wake cycles, circadian rhythms, neural synchronization, and stress responses, and low GABA levels are associated with heightened anxiety and impaired sleep quality (Braga, 2024; Gottesmann, 2002; Lewis and Hashimoto, 2007; Nuss, 2015; Wong et al., 2003). Beyond its neurological roles, GABA offers various physiological benefits. It has been shown to reduce blood pressure, an effect recognized by the US FDA (GRAS Notice No. 595). GABA may also improve muscle metabolism and prevent age-related sarcopenia (Jin et al., 2023; Lyssikatos et al., 2023; Song et al., 2024), and exert beneficial dermatological effects such as wrinkle reduction, enhancement of skin elasticity, and photoprotection (Molagoda et al., Uehara et al., 2017; Zhao et al., 2023). Owing to these wide-ranging physiological functions, GABA-enriched materials are increasingly applied in functional foods, dietary supplements,

cosmetics, and other health-related industries.

Although GABA is naturally present in vegetables, fruits, rice, beans, green tea, malt, and similar foods, its concentration is generally too low for practical extraction or formulation. As a result, research has focused on developing efficient chemical and biological synthesis methods for large-scale GABA production (Cui et al., 2020). Recognized as generally safe (GRAS), lactic acid bacteria (LAB)-based biotechnological methods are more efficient than chemical synthesis for GABA production, offering simpler reaction conditions, streamlined procedures, and greater environmental compatibility. GABA produced by LAB demonstrates higher biological activity than that found in natural foods and can be generated in high concentrations at a low cost (Lim et al., 2017).

Previous studies have explored various GABA-producing LAB strains and optimized culture media compositions. However, conventional methods often require high glutamate supplementation,

which results in a large amount of residual glutamate in the final fermentation broth. Given the aversion among consumers to glutamate or monosodium glutamate (MSG), minimizing glutamate content in GABA-containing products is essential.

This study aimed to develop a high-yield, low-residual-glutamate GABA production platform using *Levilactobacillus brevis* PL9014 supplemented with chive powder. Specifically, we evaluated (i) the effect of glutamate concentration on GABA conversion, (ii) the GABA-enhancing potential of various plant materials, (iii) the ability of chive supplementation to increase GABA production while minimizing glutamate residue, and (iv) the feasibility of using food-grade modified MRS medium (mMRS) for industrial applicability. This work introduces a plant-fortified microbial fermentation strategy that enables efficient and scalable GABA production and provides insight into the synergistic effects of chive-enhanced substrates on LAB metabolism.

## Materials and Methods

### Bacterial Strains and Culture Conditions

LABs were cultured in MRS medium (de Man, Rogosa and Sharpe, Difco, BD, USA) containing 1% glucose and 1% glutamate. *L. brevis* PL9014 (KCTC 15410BP) was selected as the primary GABA producer and compared with *L. brevis* KCTC 3498, *Lactococcus lactis* PL186 (KCTC 15383BP). A food-grade modified MRS (mMRS) was prepared using food-use ingredients to evaluate industrial applicability. All cultures were incubated at 37°C for 24–72 h under static conditions. Experiments were conducted in triplicate.

### 16S rRNA Sequencing and Phylogenetic Analysis

The 16S rRNA gene of *L. brevis* PL9014 was amplified by PCR using universal bacterial primers (27F, 5'-AGA GTT TGA TCM TCC CTC AG-3'; 1088R, 5'-GCT CGT TGC GGG ACT TAA CC-3'). PCR amplification was performed using a GeneAmp 9700 thermocycler (Applied Biosystems, USA). The resulting sequence was compared with reference sequences using NCBI BLAST (<https://www.ncbi.nlm.nih.gov>). A phylogenetic tree was constructed to determine the taxonomic position of PL9014 relative to other *Lactobacillus* species.

### Effects of pH and Temperature on Growth and GABA Production

To assess the influence of environmental conditions, PL9014 was cultivated at various initial pH values (4.0–8.0, adjusted using 1 N HCl or NaOH) and temperatures (25–45°C). Growth was monitored visually and by optical density where appropriate. GABA production was evaluated using thin-layer chromatography (TLC) as described below.

### Effect of Glutamate Concentration on GABA Production

To determine the optimal substrate concentration, MSG was added to MRS medium at 0.1–5% (w/v). After 48 h of fermentation at 37°C, culture supernatants were collected by centrifugation

(10,000 × g, 10min). GABA and residual glutamate levels were assessed using TLC and high-performance liquid chromatography (HPLC). All experiments were performed in triplicate.

### Screening of Plants for Enhancement of GABA Production

Various edible plants—including green tea, green onion, barley sprouts, chives, quinoa, banana, green apple, and onion—were checked for their ability to enhance GABA production. Dried plant powders (1% w/v) were added to MRS medium containing 1% glucose (w/v) and 1% MSG (w/v). After 48 h incubation at 37°C, GABA production was analyzed by TLC.

### Comparison of MRS and Food-grade Modified MRS (mMRS) Supplemented with Chive

A comparative analysis was conducted between standard MRS medium and food-grade modified MRS (mMRS) medium, each supplemented with 1% chive powder (w/v), to evaluate their effects on GABA production. The concentration of GABA in the culture supernatant was compared using TLC.

### Thin-Layer Chromatography (TLC) for GABA and Glutamate

TLC was conducted using silica gel 60 F254 plates (Merck, Germany). Samples (0.5 µL) were applied, and chromatographic separation was performed employing a mobile phase composed of *n*-butanol: acetic acid: and water (5:3:2, v/v/v) containing 0.4% (w/v) ninhydrin. To visualize amino acids, plates were dried and heated at 110°C for 5 min. Relative GABA intensity was compared across samples.

### High-Performance Liquid Chromatography (HPLC) Analysis

Quantitative analysis of GABA and glutamate was performed using an HPLC system with a UV detector (Shimadzu, Japan), utilizing a protocol with minor modifications from a previously described method (Pencheva et al., 2022). Each sample (100 µl) was subjected to pre-column derivatization with 1 ml of 0.05% (w/v) dansyl chloride in acetone at 55°C for 60 minutes. Chromatographic separation was achieved using a Shim-pack GISS-HPC18 C18 column, employing a gradient elution system comprising solvent A (methanol) and solvent B (sodium acetate: methanol, 9:1, pH 8.0). Flow rate was 0.1 ml/min, injection volume 10 µL, and column temperature 30°C. Derivatized compounds were detected at 343 nm. Calibration curves were prepared using analytical standards, and GABA concentration was calculated based on the peak area and dilution factors.

## Results

### Identification of *Levilactobacillus brevis* PL9014

The 16S rRNA gene sequence of strain PL9014 showed high identity to *Levilactobacillus brevis* species, confirming its taxonomic

placement. Phylogenetic analysis further positioned PL9014 within the *L. brevis* clade (Fig. 1). The strain has been deposited in the KCTC (Korea collection of Type culture, Korea) as KCTC 15410BP.

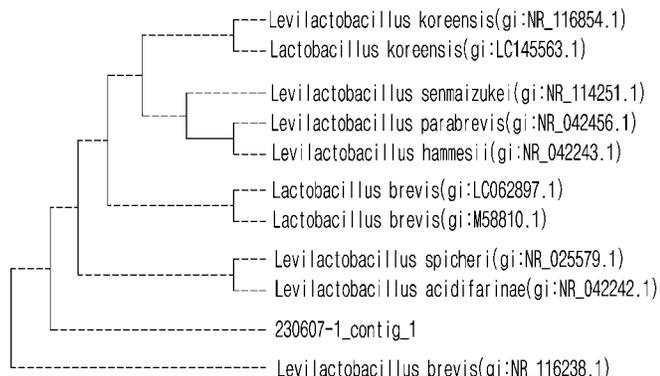


FIGURE 1 Phylogenetic analysis of *Levilactobacillus brevis* PL9014

### Time Course of GABA Production by *L. brevis* PL9014

GABA production by *L. brevis* PL9014 was monitored for 15 days. TLC analysis showed that PL9014 rapidly converted glutamate to GABA, reaching maximal intensity after 48 h (Fig. 2). GABA levels did not increase substantially beyond 48 h, indicating that the strain efficiently completes conversion within two days.

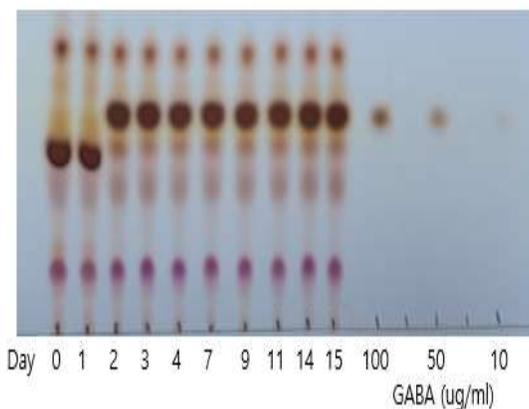
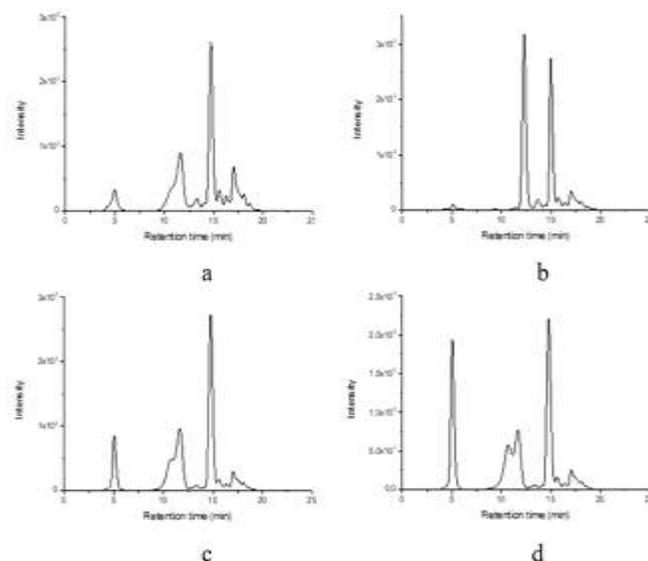


FIGURE 2. Time course for GABA production of *L. brevis* PL9014

### Effect of Glutamate Concentration on GABA Production

*L. brevis* PL9014 efficiently converted glutamate to GABA across a broad substrate range (0.1%-5% w/v). 3% glutamate being the most efficient in terms of conversion rate and the least residual glutamate (Fig. 3). HPLC quantification further confirmed the dose-dependent pattern (Fig. 4, Table 1): 1% glutamate, 4.69 g/L GABA with no detectable glutamate; 2% glutamate, 10.65 g/L

GABA with no detectable glutamate; 5% glutamate, 13.22 g/L GABA with minimal residual substrate; 10% glutamate, 10.79 g/L



GABA. Conversion rate from glutamate to GABA decreased at high concentration of glutamate. For comparison, *L. brevis* KCTC 3498 (Type strain) produced only 0.67-0.8 g/L across the same glutamate range, demonstrating significantly higher productivity of *L. brevis* PL9014.

FIGURE 3. GABA production by *L. brevis* PL9014 at various conc. of glutamate

FIGURE 4. GABA production by *L. brevis* PL9014 in the presence of various concentration of glutamate assayed with HPLC (a, 1% glutamate; b, 2% glutamate; c, 5% glutamate, d, 10% glutamate)

TABLE 1. GABA and glutamate produced at various conc. glutamate by *L. brevis* PL9014

MSG		Retention time(min)	Average peak area	Calculated conc.(ppm)	Dilution factor
1%	Glutamate	4.97814.727	1,659,104	0.0	6
	GABA		7,599,147	4693.3	
2%	Glutamate	5.15415.019	330,819	0.010659.0	16
	GABA		6,511,839		
5%	Glutamate	5.02514.776	2,525,844	0.013222.8	16
	GABA		8,016,790		
10%	Glutamate	5.03514.791	5,110,928	0.010792.8	16
	GABA		6,581,802		

### Effect of pH on Bacterial Growth and GABA Production

*L. brevis* PL9014 exhibited optimal growth at pH 5-7 (Fig. 5a). TLC results showed the highest GABA production within the same pH range (Fig. 5b). GABA production declined sharply below pH 5.

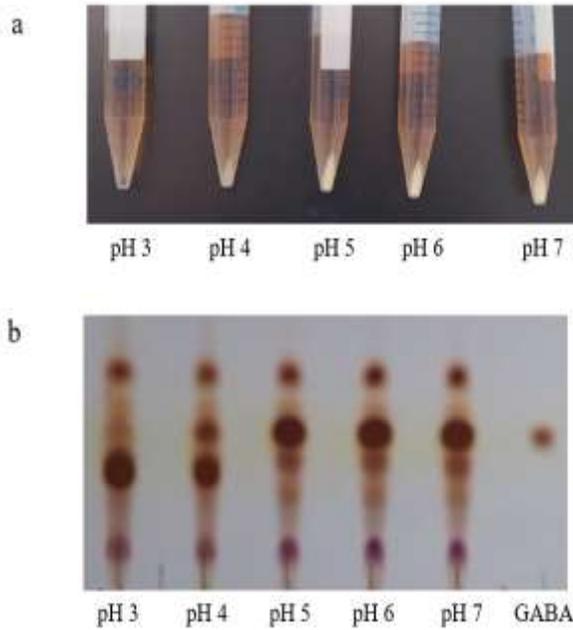


FIGURE 5. Effect of pH on growth and GABA production by *Levilactobacillus brevis* PL9014. (a, growth at various pHs; b, GABA production at various pHs)

### Effect of Temperature on Bacterial Growth and GABA Production

*L. brevis* PL9014 showed optimal growth at temperatures between 30°C and 40°C (Fig. 6a) and TLC analysis revealed that GABA production was maximum at this temperature range (Fig. 6b).

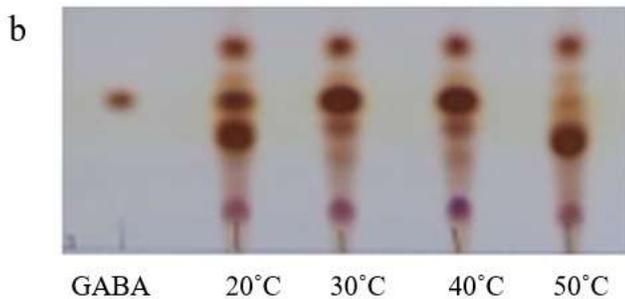


FIGURE 6. Effect of temperature on growth and GABA production by *Lactobacillus brevis* PL9014. (a, growth at various temperatures; b, GABA production at various temperatures).

### Screening Plant for GABA Enhancement

Among more than twenty dried edible plant powders evaluated (1% w/v), chive showed the strongest enhancement of GABA production in two days. Green onion and barely sprouts produced moderate increases, whereas other plant substrates showed little or no effect (Fig. 7).

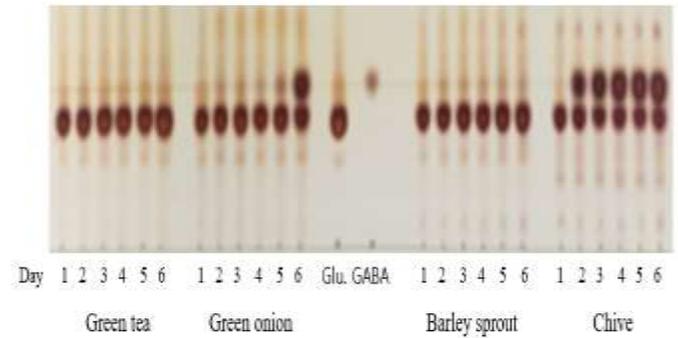


FIGURE 7. Screening Plant for GABA Enhancement

### Chive Supplementation in mMRS for GABA production by *L. brevis* PL9014

Chive supplementation increased GABA production by *L. brevis* PL9014 in mMRS supporting food-grade industrial applicability (Fig. 8). Chive supplementation also reduced residual glutamate at lower substrate concentrations, supporting its role as an effective metabolic enhancer.

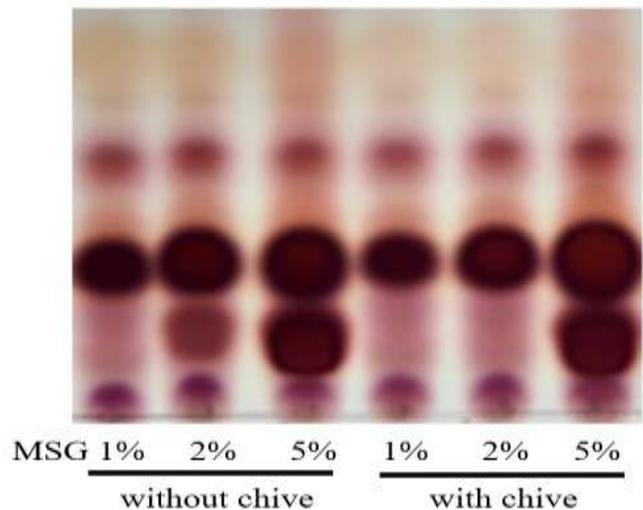


FIGURE 8. Chive Supplementation in mMRS for GABA production by *L. brevis* PL9014

### Discussion

GABA is naturally present in vegetables, grains, and teas, but its concentrations are generally insufficient for functional applications, highlighting the need for efficient production strategies. LABs provide an effective biological approach, enabling large-scale, high-purity GABA synthesis and offer advantages over chemical methods in terms of cost and environmental impact (Dhakal et al., 2012; Icer et al., 2024). From a library of more than 400 LAB strains maintained in our company, *L. brevis* PL9014 exhibited the highest GABA production capacity even without specific medium optimization.

Among the plant materials screened, chive demonstrated the strongest GABA-enhancing effect. Chive contains sulfur compounds, vitamins, minerals, quercetin, and beta-carotene that may support bacterial growth or modulate the glutamate

decarboxylase (GAD) system (Chen et al., 2022; Rana et al., 2021; Tang et al., 2021; Tramejoda et al., 2025). The marked difference between chive and other botanicals indicates a unique metabolic synergy between chive-derived compounds and *L. brevis* PL9014.

The efficiency of PL9014 was further highlighted by its high GABA conversion across a broad range of glutamate concentrations. With 2% glutamate (w/v) and 1% chive (w/v), the strain generated 10.65 g/L GABA with no detectable residual glutamate, representing an optimal balance of substrate economy and product purity. Even at 5% glutamate (w/v), the strain produced 13.22 g/L (w/v) GABA, indicating strong tolerance to substrate loading. In contrast, the reference strain *L. brevis* KCTC 3498 produced less than 1 g/L (w/v) under identical conditions, underscoring the superior metabolic capacity of PL9014.

Environmental conditions also influenced biosynthesis, with optimal GABA production occurring at pH 5-7 and a temperature of 30-40°C. These ranges are consistent with previously reported optimal conditions for LAB-derived GABA production (Dhakal et al., 2012; Icer et al., 2024), supporting the robustness of PL9014 for industrial use. Importantly, comparable results in food-grade modified MRS (mMRS) demonstrate the feasibility of replacing laboratory-grade ingredients with food-grade components without compromising GABA yields. This finding is particularly valuable for the development of scalable, regulatory-compliant fermentation processes for functional materials.

Chive-fortified fermentation offers additional advantages beyond enhanced GABA production. Fermented chive products provide dual functionality, combining high GABA levels with bioactive chive constituents linked to antioxidant, anti-inflammatory, hepatoprotective, and immunomodulatory activities (Arreola et al., 2015; Dai et al., 2023; Michalak et al., 2021; Oh et al., 2021; Shevelev et al., 2023; Sutejo and Efendi, 2015). Such combined functionality expands potential applications into diverse sectors, including functional foods, dietary supplements, animal nutrition, and cosmetic formulations targeting neurological, metabolic, and skin-health benefits. Fermentation with microbes is reported to add nutritional value to plants (Aulesa and Gongora, 2024; Fukasawa et al., 2020; Sapsuha et al., 2024).

Taken together, our findings establish a novel plant-fortified microbial fermentation approach that enables efficient, cost-effective, and environmentally compatible production of GABA with minimal residual glutamate. The use of chive as a metabolic enhancer represents a new strategy for optimizing the performance of GABA-producing strains. Further studies should investigate the mechanistic basis of chive-mediated enhancement, evaluate scale-up conditions such as bioreactor optimization, and assess synergistic biological activities of GABA-enriched and chive-derived components in vitro and in vivo models.

## Conclusion

This study introduces a novel approach that supplements fermentation media with chive (*Allium tuberosum*), markedly enhancing GABA production by *Lactobacillus brevis* PL9014 while minimizing residual glutamate. PL9014 efficiently converted glutamate to GABA across a broad substrate range, achieving 10.65-13.22 g/L (w/v) GABA within two days, particularly when chive was included as a co-substrate.

Comparable enhancement observed in food-grade mMRS further supports the feasibility of this strategy for industrial-scale applications.

Chive-supplemented fermentation provides dual benefits: high GABA yields and the incorporation of chive-derived bioactive compounds, enabling the development of multifunctional materials for the food, supplement, feed, and cosmetic industries. This work establishes a practical and scalable plant-assisted fermentation platform and highlights chive as an effective metabolic enhancer for GABA-producing LAB.

Future studies should investigate the basis of chive-mediated enhancement, optimize bioreactor-scale conditions, and evaluate synergistic biological activities of GABA-chive co-fermented products in vitro and in vivo. Also, modulation of gut microbiota and other beneficial activities with GABA producing probiotic and chives will be researched further, as other previous works did (Paek et al., 2022; Ryšávká et al., 2022).

## Conflict of Interest

The authors have no financial conflicts of interest to declare.

## Funding

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