Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

https://doi.org/10.17113/ftb.64.02.26.9241 original scientific paper

Development of Gamma-Aminobutyric Acid (GABA) Rich and Probiotic Fermented Soymilk Using *Lactiplantibacillus plantarum* W12 as a Starter Culture

Running title: Fermented Soymilk with GABA and Probiotics

Nguyen Cao Cuong[1], Bir Bahadur Thapa[2], Ngoc Hieu Nguyen[3], Tran Thanh Quynh Anh[1], Pham Hoang Son Hung[4], Peter Vandamme[5] and Do Thi Bich Thuy[6,7*]

[1]Faculty of Engineering and Food Technology, University of Agriculture and Forestry, Hue University, 102 Phung Hung Street, Thua Thien Hue 530000, Vietnam
[2]Molecular Biotechnology Laboratory, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Nakhon Ratchasima 30000, Thailand
[3]University of Medicine and Pharmacy at Ho Chi Minh City, 217 Hong Bang Street, District 5, Ho Chi Minh City 700000, Vietnam
[4]Faculty of Animal Sciences and Veterinary Medicine, University of Agriculture and Forestry, Hue University, 102 Phung Hung Street, Thua Thien Hue 530000, Vietnam
[5]Laboratory of Microbiology, Department of Biochemistry and Microbiology, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium
[6]Institute of Research and Development, Duy Tan University, 254 Nguyen Van Linh Street, Da Nang 50312, Vietnam
[7]School of Engineering and Technology, Duy Tan University, 254 Nguyen Van Linh Street, Da Nang 50312, Vietnam

Received: 19 June 2025
Accepted: 21 March 2026

Copyright© 2026 Authors retain copyright and grant the FTB journal the right of first publication under CC-BY 4.0 licence that allows others to share the work with an acknowledgement of the work's authorship and initial publication in the journal

*Corresponding author:
Phone: +84914091340
E-mail: dotbichthuy@duytan.edu.vn

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

SUMMARY

Research background. Fermented soymilk has emerged as a potential functional food due to its nutritional and health-promoting properties. Enhancing its functionality through enrichment with gamma-aminobutyric acid (GABA), a neurotransmitter with various health benefits, is an area of active research. This study aimed to develop a novel GABA-enriched fermented soymilk using a newly isolated *Lactiplantibacillus plantarum* strain with dual probiotic and GABA-producing capabilities.

Experimental approach. Five *L. plantarum* strains isolated from Vietnamese soybean whey underwent screening for GABA production and probiotic characteristics. Strain W12, exhibiting superior performance, was selected for optimization. Response surface methodology employing central composite design optimized monosodium glutamate (MSG) and sucrose concentrations for maximal GABA yield. A time-course study was then conducted to monitor bacterial growth, pH changes, organic acid production, and GABA accumulation during fermentation under the optimized conditions.

Results and conclusions. L. plantarum W12 demonstrated exceptional probiotic traits: 97.06 % survival at pH=2.5, 96.54 % survival in bile salts/pancreatin, 97.34 % pepsin tolerance, 96.67 % auto-aggregation, and 85.45 % hydrophobicity in chloroform. Initial GABA production reached (9.07±0.40) mM. Response surface optimization predicted maximum GABA concentration of 34.24 mM at 1.564 mg/mL MSG and 10.93 % sucrose, experimentally validated at (34.46±0.98) mM (15 h, 45 °C). Lactic acid dominated organic acid production ((182.37±7.94) mg/g at 18 h), with viable cell counts exceeding 7.9 log CFU/mL, meeting probiotic thresholds.

Novelty and scientific contribution. This study presents the first comprehensive characterization of *L. plantarum* W12, a strain combining exceptional GABA biosynthesis with robust probiotic properties. The systematic RSM optimization framework and detailed metabolic profiling provide reproducible protocols for developing multifunctional fermented soy beverages with applications in neurological and gastrointestinal health promotion.

Keywords: gamma-aminobutyric acid; fermented soymilk; *Lactiplantibacillus plantarum*; response surface methodology; glutamate decarboxylase; functional food

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

INTRODUCTION

Lactic acid bacteria (LAB) are important microorganisms in industry and have a key role as starter cultures in many fermented food products. They convert carbohydrate compounds to lactic acid as the major end products of fermentation processes. In addition, they enhance fermented food quality by creating flavour and aroma through changing lipids and proteins into esters, alcohols, and organic acids [*1*]. LAB in intestinal tracts produce essential vitamins (e.g., thiamine, niacin, folic acid, pyridoxine, vitamin B12), and beneficial enzymes like lactase, and release free amino acids and short-chain fatty acids [*2*]. These beneficial bacteria also contribute to gut health by inhibiting the growth of pathogenic bacteria through competitive exclusion and reducing the risk of diarrhoea.

Recently, LAB have garnered attention for their ability to produce gamma-aminobutyric acid (GABA), a non-proteinaceous amino acid compound present throughout the human body [*3*], a neurotransmitter crucial for different functions. This GABA-producing capacity makes LAB fermentation a promising avenue for developing functional foods with enhanced health benefits. GABA exerts multiple physiological effects in the human body, including regulation of blood pressure through vasodilation [*4*], reduction of anxiety and stress by modulating neural excitability [*5*], improvement of sleep quality, enhancement of immune function, and potential benefits in managing diabetes through improved insulin secretion and glucose metabolism [*6*]. Recent studies have also demonstrated GABA's role in maintaining intestinal homeostasis, where it attenuates ischemia-reperfusion-induced alterations in intestinal immunity through increased IgA secretion and enhanced antioxidant activity [*7*]. These diverse health-promoting effects make GABA-enriched functional foods particularly attractive for preventive nutrition and wellness applications.

Soybeans, rich in antioxidants like phenolics and isoflavones, are recognized as a potential functional food and offer benefits such as cardiovascular protection, antidiabetic effects, antioxidant properties, anticancer properties, and blood pressure regulation [*8*]. Soymilk, a soybean extract, is a nutritious food containing high-quality protein, unsaturated fatty acids, lecithin, and isoflavones, while being cholesterol-free and lactose-free, making it suitable for individuals with lactose intolerance [*9*]. As a plant-based alternative to cow's milk yogurt, fermented soymilk offers health benefits, including protection against cancer and diabetes, improved memory, and enhanced wound healing [*10*].

Recently, LAB fermentation of soymilk has attracted scientific interest. Studies have shown that fermentation with *Lactiplantibacillus plantarum* Y16 increases the antioxidant capacity of soymilk [*11*]. Research has also focused on the influence of added carbohydrates on the volatile organic

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

compound profile of fermented soymilk, impacting sensory characteristics [*12*]. Furthermore, fermentation with *L. brevis* and *L. plantarum* has been shown to alter soymilk composition, reducing isoflavones while increasing aglycones, ornithine, enzyme activity (pancreatic lipase, α-amylase, α-glucosidase), and antioxidant capacity [*13*]. Studies have also explored the impact of different LAB strains, such as *Lactobacillus fermentum* SMN10-3(A) and *Lactococcus lactis* SMN15-6(B), on GABA content and flavour in fermented soymilk, and revealed that combining strains can enhance the GABA yield [*3*].

Supplementing soymilk with cell-free supernatant (CFS) from *Lactiplantibacillus plantarum* BC112 has been shown to significantly enhance GABA production by *Enterococcus faecium* through quorum sensing modulation and upregulation of glutamate decarboxylase (GAD) gene expression [*14*]. The CFS contains secreted metabolites and signaling molecules that can influence the metabolic activity and gene expression of co-cultured GABA-producing strains, thereby increasing overall GABA yield in fermented products. Optimization of medium composition and culture conditions for maximizing GABA yield in soymilk fermented with *L. plantarum* Lp3, including investigating the effects of strain ratios, monosodium glutamate (MSG) concentration, fermentation time, and temperature, has been conducted using response surface methodology [*15*].

Among LAB, *L. plantarum* is particularly attractive for its diverse health-promoting properties, including probiotic potential, antioxidant, anti-obesity, anticancer, and antidiabetic effects [*16*]. The present study aims to develop a novel synbiotic soymilk that is GABA-enriched and has probiotic properties by using *L. plantarum* W12. This strain was isolated from soybean whey selected for its high GABA production and probiotic potential. We investigated the optimization of MSG and sucrose supplementation to maximize GABA production in the fermented soymilk. Furthermore, we analysed changes in pH, GABA content, organic acids, and LAB viability during fermentation to understand *L. plantarum* W12 metabolism during soymilk fermentation.

MATERIALS AND METHODS

Samples

Samples (*N*=20) were collected from soybean whey obtained from five traditional tofu manufacturers in Hue City, Vietnam (located in Thuan Hoa, Phu Xuan, Vi Da, An Cuu, and Kim Long Wards) during March–April 2023. Tofu was produced using traditional methods involving soaking

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

whole soybeans (local Vina variety) for 8-12 h, grinding with water (1:8 ratio), filtering to obtain soymilk, and coagulating with 20–22 % of whey from the previous batch (pH=4.0-4.5). The soybean whey, a byproduct of tofu coagulation, was collected immediately after pressing and transported on ice to the laboratory within 2 h of production. Samples were assigned unique identification codes (TW-01 to TW-20) and stored at −80 °C until bacterial isolation.

Isolation of lactic acid bacteria (LAB)

Fifteen grams of each soybean whey sample were aseptically ground and homogenized in 135 mL of Ringer's solution (Sigma-Aldrich, Milan, Italy) using a stomacher (Bag Mixer 400; Interscience, Saint Nom, France) at maximum speed for 2 min. Serial decimal dilutions of the homogenate were plated onto MRS agar (Oxoid, Milan, Italy) and incubated anaerobically at 37 °C for 48 h. Fifteen to twenty colonies per sample were randomly selected and purified by repeated streaking on MRS agar. Gram staining and catalase testing were performed on all isolates. Gram-positive and catalase-negative isolates were tentatively identified as LAB and were stored in Microbank™ vials (Pro-Lab Diagnostics, Richmond Hill, ON, Canada) at -80 °C until further analysis.

Pathogenic indicator strains

The pathogenic indicator strains used in this study were obtained from standard culture collections to ensure reproducibility and biosafety. The following strains were used: *Escherichia coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, USA); *Staphylococcus aureus* ATCC 25923; and *Salmonella typhimurium* ATCC 14028. All strains were maintained on Tryptic Soy Agar (TSA, Oxoid) at 4 °C with monthly subculturing, and working cultures were prepared fresh before each assay by overnight incubation in Tryptic Soy Broth (TSB, Oxoid) at 37 °C.

MALDI-TOF MS analysis

Putative LAB isolates were identified through MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with the MBT Compass Explorer software v. 4.1 and the commercial Bruker BDAL (MSP-11897) and the proprietary LM UGENT ID (MSP-6102) databases. Following the manufacturer's protocols, fresh colonies were transferred directly to MALDI

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

target plates using the direct transfer method. One microliter of 70 % formic acid was added, followed by 1 µL of HCCA matrix solution (α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile/2.5 % trifluoroacetic acid). Spectra were acquired in positive linear mode (m/z=2 000–20 000). Score values ≥2.0 indicated highly probable species-level identification, scores 1.7-1.99 indicated reliable genus-level identification, and scores <1.7 were considered unidentified.

Preparation of inoculants

LAB strains were activated by culturing in MRS broth (Sigma-Aldrich) at 37 °C for 24 h. Cells were harvested by centrifugation at 10 000×*g* and 4 °C for 5 min (Eppendorf Centrifuge 5424R, Eppendorf AG, Hamburg, Germany). The pellets were washed and resuspended in peptone water to achieve a final concentration of 8–9 log CFU/mL using the absorbance at 600 nm as a standard of cell density. This suspension served as the inoculum for soymilk fermentation.

Soymilk fermentation

Soymilk (100 mL, 4 % protein content) was used as the base medium for all fermentation experiments. The soymilk was supplemented with MSG and/or carbon sources as described below, inoculated with each of the five selected LAB strains (*L. plantarum* W1, W3, W4, W5, and W12) individually at an initial cell density of 8 log CFU/mL.

Initial GABA screening

For initial screening of GABA-producing capacity, the five selected *L. plantarum* strains (W1, W3, W4, W5, and W12) were cultured in MRS broth supplemented with 1 % (m/V) MSG at 37 °C for 24 h.

MSG concentration optimization

To determine the optimal MSG concentration for GABA production by *L. plantarum* W12 in soymilk, MSG was added at concentrations of 0, 0.5, 1.0, 1.5, and 2.0 % (m/V). Fermentation

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

temperature increased to 45 °C for final optimization to reduce fermentation time to 15 h while maintaining bacterial viability. GABA concentration was measured by HPLC.

Carbon source screening

To evaluate the effect of different carbon sources on GABA production, soymilk supplemented with 1.5 % *(m/V)* MSG was further supplemented with 5 % *(m/V)* of individual carbon sources: lactose, sucrose, glucose, or maltose. A control without additional carbon source was also prepared. Each supplemented soymilk was inoculated with *L. plantarum* W12 and fermented at 43 °C for 15 h. GABA concentration was determined by HPLC.

Sucrose concentration optimization

Based on carbon source screening results, the optimal sucrose concentration was investigated. Soymilk containing 1.5 % (*m/V*) MSG was supplemented with sucrose at concentrations of 0, 5, 10, 15, and 20 % (*m*/*V*). Fermentation conditions and GABA analysis were performed as described above. Fermentation conditions and GABA analysis were performed as described above.

Time-course fermentation study

For final product characterization, *L. plantarum* W12 was inoculated into soymilk containing optimized concentrations of MSG (1.564 mg/mL) and sucrose (10.93 %, *m*/*V*) at an initial cell density of $5\cdot10^6$ CFU/mL. Fermentation was conducted at 43 °C with an initial pH=6.0. Samples were collected at 0, 3, 6, 9, 12, 15, and 18 h for analysis of cell growth, pH, GABA concentration, and organic acid production.

Enumeration of viable cells

Viable cell counts were determined using the pour plate method. For fermented soymilk samples, 20 g of sample were homogenized with 180 mL of sterile diluent containing 0.1 % (*m*/*V*) Bacto Peptone (Difco Laboratories, Detroit, MI, USA) and 0.9% (*m*/*V*) NaCl. For liquid samples (inoculums and fermentation broths), samples were directly diluted in sterile 0.85 % (*m*/*V*) NaCl

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

solution. Serial ten-fold dilutions were prepared, and 1-mL aliquots of appropriate dilutions were plated onto MRS agar in duplicate using the pour plate technique. Plates were incubated anaerobically at 37 °C for 48 h. Viable cell enumeration was performed according to standard methods. Results are expressed as $\log_{10}$ CFU/mL for liquid samples or log CFU/g for fermented soymilk and represent the mean of three independent experiments.

Chemical analysis by HPLC

Sample preparation

Two different sample preparation methods were used depending on the analyte and sample matrix:

For fermented soymilk: This extraction procedure was adapted from Costa *et al.* [*17*]. Briefly, four grams were accurately weighed and mixed with 5 mL of HPLC-grade water (Sigma-Aldrich). This mixture was then further diluted with 25 mL of HPLC-grade acetonitrile (Sigma-Aldrich). The resulting solution was filtered through a 0.45 μm membrane filter to remove any particulate matter. A 500 μL aliquot of the filtrate was then used for HPLC analysis.

For MRS broth cultures: LAB strains were cultivated in MRS broth. The supernatants were collected after centrifugation at 10 000×*g*, 4 °C for 15 min. Proteins were precipitated by adding 3 % (*m/V*) sulfosalicylic acid, followed by a second centrifugation step. The resulting protein-free supernatant was then used for GABA derivatization, as described in section GABA Quantification.

Organic acid analysis

Organic acid analysis was performed using a Shimadzu HPLC system equipped with an Alltech OA-1000 Organic Acid column (6.5×300 mm) and a UV detector set at 210 nm. The mobile phase consisted of 0.01 N H_2SO_4 at a flow rate of 0.4 mL/min, and the column temperature was maintained at 35 °C. Reference organic acids (Sigma-Aldrich) were used for calibration and quantification.

GABA quantification

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

GABA analysis was performed following a method adapted from Thuy *et al.* [*18*]. Briefly, GABA in the prepared samples was derivatized with 4 mM dabsyl chloride (for soymilk samples) or 4 mM dabsyl chloride (4-dimethylaminoazobenzen-4-sulfonyl chloride, for MRS broth samples). Dabsyl-GABA was quantified using a Shimadzu LC-20A HPLC system equipped with an SPD-20A UV-Vis detector (465 nm). Separation occurred on a Supelco C18 column (250 m×4,6 mm i.d., 5 µm particle size) maintained at 55 °C. The mobile phase consisted of an isocratic mixture of 25 mM ammonium acetate (0.1 % acetic acid) and acetonitrile (26:74 *V*/*V*) at a 1 mL/min flow rate. GABA concentrations were determined via a calibration curve (0–10 mM).

Probiotic properties assessment

Following the method by Li *et al.* [*19*], the following assessments were done to analyse probiotic properties:

Low pH tolerance assay

LAB cells were harvested from overnight cultures by centrifugation (10 000×*g*, 4 °C, 15 min) and resuspended in a sterile solution of 0.1 % (w/B) bacto Peptone (Difco) and 0.9 % (*m*/*V*) NaCl. One millilitre cell suspension was then inoculated into MRS broth and adjusted to pH 2.5. The initial absorbance (A_{T0}) was measured at 600 nm. Following incubation at 37 °C for 4 h, the absorbance was measured again (A_{T4}). Survival percentage (H/%) was calculated using the following equation:

$$H=(A_{T4}/A_{T0})\cdot 100 \qquad /1/$$

Bile salts and pancreatin tolerance assay

LAB cells from overnight cultures in MRS broth were harvested by centrifugation (10 000×*g*, 4 °C, 15 min) and resuspended. One millilitre of the cell suspension was inoculated into a sterile saline solution (0.85 % NaCl, *m*/*V*) containing 1 mg/mL pancreatin (Sigma-Aldrich, South Korea) and 0.3 % (*m*/*V*) bile salts (Sigma-Aldrich, South Korea). The solution was incubated at 37 °C. Absorbance measurements were taken initially (A_{T0}) and after inoculating for 6 h (A_{T6}). Tolerance was calculated as survival percentage (H/%), using the following equation:

$$H=(A_{T6}/A_{T0})\cdot 100 \qquad /2/$$

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Pepsin tolerance assay

After 24 h incubation, LAB cultures were centrifuged (400×*g*, 4 °C, 6 min). Simulated gastric fluid was prepared by adding pepsin (Sigma-Aldrich, South Korea) to a 0.85 % NaCl solution to achieve a final concentration of 3 mg/mL and adjusting the pH to 2.5. The cell pellet was resuspended in the simulated gastric fluid, vortexed, and the initial absorbance was measured at 600 nm (A_o). After incubation at 37 °C for 4 h, the absorbance was measured again (A_4). Survival percentage (H/%) was calculated using the following equation:

$$H=(A_4/A_o)\cdot 100 \quad /3/$$

Antibiotic susceptibility assay

Antibiotic susceptibility was determined using the disc diffusion method by Oh *et al.* [*20*] and interpreted according to the guidelines established by the European Food Safety Authority (EFSA) for *Lactiplantibacillus* species [*21*]. Antibiotic discs (vancomycin 30 µg, clindamycin 2 µg, tetracycline 30 µg, ampicillin 10 µg, streptomycin 10 µg, penicillin 10 µg, chloramphenicol 30 µg, erythromycin 15 µg, kanamycin 30 µg, and gentamicin 10 µg) were placed on agar plates (Agar powder Bacteriological Grade GRM026 Himedia, India) inoculated with the LAB strains. After 24 h incubation at 37 °C, the diameters of the inhibition zones were measured.

Aggregation properties

Auto-aggregation: Following the method by Li *et al.* [*19*], overnight LAB cultures were centrifuged (10 000×*g*, 4 °C, 10 min), and pellets were resuspended in 1 mL of phosphate-buffered saline (PBS). Initial absorbance (A_0) was measured at 600 nm. After incubation at 37 °C for 20 h, the absorbance of the upper suspension (A_{20}) was measured. Autoaggregation (%) was calculated using the following equation:

$$\text{Autoaggregation}=[(A_0-A_{20})/A_0]\cdot 100 \quad /4/$$

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Co-aggregation: LAB cells from 24-hour cultures in MRS broth were mixed with equal volumes (1.5 mL) of pathogenic strains (*Staphylococcus aureus, Escherichia coli,* and *Salmonella typhimurium*). Mixtures were incubated at 37 °C for 5 h. Absorbance of the supernatants (A_{mix}) was measured at 600 nm. The absorbance of the individual LAB ($A_{probiotic}$) and pathogen ($A_{pathogen}$) suspensions was measured. Co-aggregation (%) was calculated using the following equation:

$$\text{Co-aggregation}=[1-A_{mix}/(A_{probiotic}+A_{pathogens})/2]\cdot 100 \qquad /5/$$

Hydrophobicity assay

LAB cultures grown in MRS broth at 37 °C for 24 h were centrifuged (400×*g*, 4 °C, 10 min). Pellets were resuspended in 0.1 M KNO_3. The initial absorbance (A_0) was measured at 600 nm. Two millilitres cell suspension were mixed with 1 mL of organic solvent (chloroform, ethyl acetate, or xylene). After 10-min incubation, 2-min vortexing, and 20 min. The absorbance of the aqueous phase (A_1) was measured. Cell surface hydrophobicity (%) was calculated using the equation below according to Oh *et al.* [*20*]:

$$\text{Hydrophobicity}=((A_0-A_1)/A_0)\cdot 100 \qquad /6/$$

Evaluation of antimicrobial activity

The antimicrobial activity was conducted using the “agar spot” method of Hernandez *et al.* [*22*]. Pathogen indicator strains included *S. aureus, S. typhimurium,* and *E. coli*. A suspension of LAB cells was prepared by mixing the overnight LAB biomass in 0.85 % NaCl. This suspension (5 µL) was dropped onto MRS agar surfaces and allowed to dry and grow at 37 °C for 18 h. Cells of pathogenic bacteria were suspended in 1 % agar at approximately 37 °C. These agar suspensions were immediately poured onto the spot-inoculated MRS agar plates, left to solidify, and then incubated at 37 °C for 24–48 h. The diameters of the clear zones of inhibition of pathogenic bacteria around the spots were determined by a vernier calliper. Positive controls consisted of MRS agar plates inoculated with known antimicrobial-producing *L. plantarum* reference strain (ATCC 14917), while negative controls consisted of uninoculated MRS agar spots overlaid with pathogen-containing agar. Inhibition zones were measured only when they exceeded 2 mm beyond the LAB colony margin to ensure specific antimicrobial activity.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Optimization using response surface methodology (RSM)

Central composite design (CCD) of response surface methodology (RSM) was employed to optimize the concentrations of MSG and sucrose for maximizing GABA production. The two independent variables were X_1: MSG concentration (mg/mL) and X_2: sucrose concentration (%, *m/V*). The experimental design consisted of 13 runs, including 4 factorial points, 4 axial points for rotatable design, and 5 centre points. The two independent variables were studied at three different levels (Table 1).

The coded values were transformed into actual values (Table 2) using the following equations:

X_1 (actual, mg/mL)=1.5+0.5·X_1 (coded) /7/

X_2 (actual, % *m/V*)=10+5·X_2 (coded) /8/

The second-order polynomial model was:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{12}X_1X_2 \quad /9/$$

where Y represents the predicted GABA yield (mM); X_1 and X_2 represent the coded values of MSG and sucrose concentrations, respectively; β_0 is the intercept term; β_1 and β_2 are the linear coefficients; β_{11} and β_{22} are the quadratic coefficients; and β_{12} is the interaction coefficient between MSG and sucrose.

Statistical analysis

Response surface methodology (RSM) was employed to analyse the experimental data and fit a second-order polynomial model [*23*]. Design-Expert software v. 12.0.3.0 was used for the experimental design, data analysis, and optimization procedures [*24*]. Model adequacy was evaluated using analysis of variance (ANOVA), considering the model F-value, lack-of-fit test, and coefficient of determination (R^2). Significance of model terms was determined using p-values (α=0.05), with only significant terms included in the reduced models. Non-significant linear terms were retained if corresponding quadratic or interaction terms were significant, following established hierarchical model reduction principles. All experiments were performed in triplicate, and the results are presented as mean±standard deviation. One-way ANOVA and Tukey's HSD post-hoc test were used to compare means, with statistical significance set at $p\leq0.05$.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

RESULTS AND DISCUSSION

Isolation and identification of L. plantarum strains

Assessment of absence of catalase activity and positive Gram-stain yielded 30 presumptive LAB isolates. Five isolates exhibiting GABA-producing capabilities (see below), *i.e.* the isolates W1 (R-49778), W3 (R-49768), W4 (R-49769), W5 (R-49770), and W12 (R-49779), were identified as *Lactiplantibacillus plantarum* by MALDI-TOF MS with high score values.

GABA production capacity

The GABA production capacity of the five selected *L. plantarum* strains was assessed (Fig. 1a). *L. plantarum* W12 exhibited the highest GABA production ((9.07±0.40) mM), significantly higher than the other isolates ($p \le 0.05$). *L. plantarum* W5 also showed substantial GABA production ((7.27±0.39) mM). Isolates W1, W3, and W4 produced lower GABA concentrations ((3.58±0.20), (2.92±0.46) and (5.45±0.60) mM, respectively).

GABA production varied significantly among *L. plantarum* strains, likely due to differences in genetic background, glutamate decarboxylase (GAD) activity, and associated metabolic pathways [*25*]. Among them, *L. plantarum* W12 showed the highest GABA yield and was selected for further development of GABA-enriched fermented soymilk.

The initial GABA production by *L. plantarum* W12 in MRS broth (9.07±0.40 mM at 24 h with 1% MSG) was lower than that reported by Harnentis *et al.* (211.17 mM under fully optimized conditions: pH=5.5, 36 °C, 500 mM glutamate (50× higher substrate than our study), 84 h fermentation time (5.6× longer) with yeast extract and glucose supplementation) [*26*], but fell within the range reported by Ledashcheva *et al.* (7.03–54.21 mM in MRS broth with 1 % MSG, with strain GB111 achieving 54.21 mM) [*27*] and was lower than the optimized value by Thuy *et al.* (25.52 mM under optimized conditions: $5 \cdot 10^6$ CFU/mL, 2 % MSG, pH=7, 35 °C, 48 h in MRS broth) [*18*]. More importantly, under optimized fermentation conditions in soymilk, W12 achieved a maximum GABA yield of (34.46±0.98) mM (3.55 g/L), which was substantially higher than the 1.76 mg/mL (17.07 mM) reported by Xia *et al.* in co-fermented soymilk using *L. fermentum* SMN10-3(A) and *L. lactis* SMN15-6(B) at a 2:1 ratio [*3*]. This demonstrates that *L. plantarum* W12 exhibits superior GABA-producing capacity specifically in soymilk fermentation compared to previously reported strains.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Probiotic potential of L. plantarum strains

The present study investigated the probiotic potential of *L. plantarum* strains W1, W3, W4, W5, and W12 by assessing their gastrointestinal tolerance, autoaggregation and co-aggregation abilities, cell surface hydrophobicity, and antagonistic effects against pathogenic bacteria. As described below, *L. plantarum* W12 exhibited the most promising probiotic characteristics and was subsequently selected for soymilk fermentation studies.

Gastrointestinal tolerance

Survival under simulated gastrointestinal conditions is crucial for probiotic efficacy. All tested strains demonstrated considerable tolerance to low pH, bile salts, pancreatin, and pepsin (Table 3). *L. plantarum* W3 and W12 exhibited the highest survival rates in low pH and bile salt/pancreatin conditions (over 95 and 96 % survival, respectively). While all strains showed good pepsin tolerance (above 84 %), W1 and W12 displayed the highest survival rates, exceeding 97 %. These results suggest that these strains possess robust resistance to the harsh conditions of the gastrointestinal tract. Acid tolerance is a critical probiotic trait, enabling survival through the stomach. At low pH (<4.5), proton influx requires substantial ATP for homeostasis, potentially leading to metabolic disruption and cell death [*28*]. Gastric pH can drop to 1–2 during fasting [*29*]. Thus, a cutoff of pH 3.0 for 3 h was used to simulate gastric conditions. All *L. plantarum* strains assessed survived well at pH<3.0. Bile tolerance is also essential for probiotic efficacy. Bile salts exert antimicrobial effects by disrupting bacterial membranes [*30*].

Aggregation

Autoaggregation, the ability of bacterial cells to clump together, and coaggregation, the ability to adhere to pathogenic bacteria, are important for colonization and competitive exclusion of pathogens in the gut. In Table 3, *L. plantarum* W12 displayed the highest autoaggregation ability (96.67 %), significantly higher than the other strains. While W12 also showed strong coaggregation with *S. aureus* (20.82 %) and *S. typhimurium* (14.39 %), W3 demonstrated the highest coaggregation with *S. typhimurium* (17.74 %). These varying co-aggregation profiles suggest strain-specific interactions with different pathogens.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Probiotic activity is also linked to the ability to aggregate and adhere to host tissues. Autoaggregation and coaggregation help form a barrier against pathogens [*31*]. These traits were evident in the *L. plantarum* strains tested, supporting their probiotic potential [*32*].

Hydrophobicity

Cell surface hydrophobicity influences the adherence of probiotic bacteria to intestinal epithelial cells, contributing to colonization. Table 3 (hydrophobicity) shows that *L. plantarum* W12 exhibited the highest hydrophobicity with chloroform (85.45 %) and ethyl acetate (12.71 %), while W1 showed the highest hydrophobicity with xylene (9.79 %). The observed variations in hydrophobicity across different solvents suggest differences in cell surface composition among the strains. Surface hydrophobicity is correlated with aggregation and adhesion capabilities [*33*]. Hydrophobicity was particularly high in strains W1 and W12, as assessed in chloroform. Bile exposure reduced hydrophobicity, likely affecting adhesion, consistent with the previous report [*34*].

Antagonistic effect

The ability to inhibit pathogenic bacteria is a key probiotic trait. *L. plantarum* W3 and W12 (Table 3, antimicrobial activity) demonstrated the strongest antagonistic activity against all tested pathogens (*E. coli*, *Salmonella*, and *S. aureus*), as evidenced by the largest zones of inhibition. W12 notably exhibited the largest inhibition zones against *S. aureus* (28 mm) and *Salmonella* (14.33 mm), while W3 showed the strongest effect against *E. coli* (8.33 mm). These results highlight the potential of these strains to control the growth of common foodborne pathogens.

Based on the comprehensive evaluation of these probiotic properties, *L. plantarum* W12 emerged as the most promising candidate. Its superior performance in GABA production, combined with its high gastrointestinal tolerance, autoaggregation, coaggregation with specific pathogens, and strong antagonistic activity, justifies its selection for further investigation in soymilk fermentation. This strain holds significant potential for developing functional foods with enhanced probiotic benefits. Antibacterial activity of *L. plantarum* is well-documented [*35*], primarily through organic acid production and pH reduction [*36*]. W12 showed antagonistic effects against *E. coli, S. aureus,* and *S. typhimurium*, likely due to its high GABA output and acidification. All tested strains produced inhibition

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

zones significantly larger than negative controls (no inhibition zone), and positive controls using *L. plantarum* ATCC 14917 showed comparable inhibition patterns, validating the assay methodology.

The results presented here demonstrate that *L. plantarum* W12 successfully integrates two critical functional properties: exceptional GABA biosynthesis capacity and robust probiotic characteristics. The strain's high survival rates under simulated gastrointestinal conditions (>96 % for pH, bile/pancreatin, and pepsin challenges, Table 3), strong auto-aggregation (96.67 %), and broad-spectrum antimicrobial activity against foodborne pathogens (inhibition zones of 28.00 mm against *S. aureus*, 14.33 mm against *Salmonella*, and 3.00 mm against *E. coli*) provide confidence in its probiotic efficacy. Simultaneously, the optimized GABA yield of 34.46 mM represents a 3.8-fold improvement over the initial screening value (9.07 mM) and positions this fermented soymilk among the highest GABA-containing plant-based fermented products reported to date.

The viable cell counts exceeding 7.9 $\log_{10}$ CFU/mL at the end of fermentation ensures adequate probiotic dosage (typically ≥10^7 CFU/mL for claimed health benefits) [*37*], while the high GABA concentration provides therapeutic potential for neurological and cardiovascular benefits. This dual functionality, combined with the favourable sensory properties (mild acidity, clean flavor) and extended shelf life conferred by organic acid preservation, positions this product as a promising candidate for commercialization as a functional, health-promoting beverage. Future research should focus on *in vivo* validation of GABA bioavailability and probiotic colonization efficiency through animal models and clinical trials, as well as shelf-life stability studies and consumer acceptance testing to facilitate commercial development.

Applications of L. plantarum W12 in soymilk fermentation

Factors influencing GABA biosynthesis

The initial cell density ($5\cdot10^6$ CFU/mL), temperature (45 °C), initial pH (7), and fermentation time (15 h) were kept constant to investigate the specific effects of supplemental MSG concentration and carbon source supplementation on GABA biosynthesis.

Effects of additional MSG concentration in soymilk significantly impacted GABA production by *L. plantarum* W12 (Fig. 1b).

GABA production increased with increasing MSG supplementation up to 1.5 % (*m/V*), reaching a maximum of (21.40±0.60) mM. However, further increasing the MSG concentration to 2 % did not

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

result in a further increase in GABA and showed a slight decrease, suggesting a potential inhibitory effect at higher concentrations or substrate saturation. This finding aligns with other studies that have reported an optimal MSG concentration for GABA production in LAB fermentations [*38*]. The observed decrease in GABA production at 2 % MSG could be due to substrate inhibition of glutamate decarboxylase or feedback inhibition mechanisms within the metabolic pathway [*39*]. Therefore, 1.5 % MSG supplementation is the optimal concentration for maximizing GABA production by *L. plantarum* W12 under these fermentation conditions. While high glutamate concentrations can enhance GAD activity [*40*], excessive levels may disrupt metabolism via osmotic stress. Our data suggest GABA production peaked at 15 h, followed by a decline, possibly due to substrate depletion, GABA degradation, or GAD feedback inhibition [*41*].

The results (Fig. 1c) indicate that the type of carbon source (lactose, sucrose, glucose or maltose) significantly affected GABA production. Supplementation with sucrose resulted in the highest GABA production ((29.29±0.84) mM), significantly exceeding all other treatments ($p \leq 0.05$). Glucose supplementation also led to a substantial increase in GABA ((26.42±0.53) mM), while lactose and maltose supplementation resulted in GABA levels comparable to the control (no added carbon source) ((24.33±1.21) and (22.76±0.68) mM, respectively).

The observed differences in GABA production with different carbon sources can be attributed to variations in their metabolic pathways and how efficiently they support the growth and metabolic activity of *L. plantarum* W12. Sucrose, being a disaccharide composed of glucose and fructose, may provide a more sustained release of readily metabolizable sugars, leading to enhanced GABA production [*42*]. The lower GABA levels observed with lactose and maltose could be due to differences in their uptake and utilization by *L. plantarum* W12 or potential catabolite repression effects. The selection of an appropriate carbon source is therefore crucial for optimizing GABA production in soymilk fermentation by *L. plantarum* W12.

In the next step, the optimal sucrose concentration for GABA production was further investigated. Varying concentrations of sucrose (0, 5, 10, 15 and 20 %, *m/V*) were added to the MRS medium containing 1.5 % MSG. The results (Fig. 1d) of *L. plantarum* W12 demonstrate a clear influence of sucrose concentration on GABA production. GABA production increased with increasing sucrose concentration up to 10 %, reaching a maximum of (33.60±1.44) mM. However, further increases in sucrose concentration (15 and 20 %) resulted in a decrease in GABA production

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

((30.79±1.17) and (27.61±0.71) mM, respectively). This suggests that while sucrose enhances GABA production up to a certain point, excessive sucrose concentrations can have a detrimental effect.

The observed decrease at higher concentrations could be attributed to several factors, including osmotic stress, substrate inhibition, and a shift in metabolic flux. High sucrose concentrations can create osmotic stress for the bacteria, inhibiting their growth and metabolic activity [*43*]. While sucrose is beneficial, excessive amounts could potentially inhibit enzymes involved in GABA metabolism or related pathways. High sugar concentrations might redirect metabolic flux away from GABA production towards other metabolic pathways, such as those involved in exopolysaccharide production or other stress responses [*44*]. The results indicate that 10 % sucrose supplementation is optimal for maximizing GABA production by *L. plantarum* W12 under these fermentation conditions. This finding is valuable for developing an efficient and cost-effective fermentation strategy for GABA-enriched soymilk production.

Optimization of screened variables using central composite design

The optimization of MSG and sucrose concentrations for enhanced GABA production by *L. plantarum* W12 in soymilk was conducted using a CCD of RSM. The CCD consisted of 13 experimental runs, including factorial, axial, and centre points (Table 2). The independent variables were MSG concentration (X_1) and sucrose concentration (X_2), with GABA yield (Y, mM) as the response variable. GABA yield varied significantly depending on the concentrations of MSG and sucrose.

P-values less than 0.05 indicate model terms are significant. Values greater than 0.10 indicate the model terms are not significant. In the analysis of variance [*45*], the response surface model optimizing GABA yield by *L. plantarum* W12 in soymilk was optimized using a central composite design (CCD) of response surface methodology (RSM) with MSG (X_1) and sucrose (X_2) concentrations as independent variables (Table 2). The quadratic model for GABA yield (Y, mM) was highly significant ($F=39.65$, $p<0.0001$, $R^2=96.59$ %), with significant terms for MSG ($p=0.0338$), sucrose ($p=0.0284$), their interaction ($p=0.0319$), and quadratic terms for both MSG ($p<0.0001$) and sucrose ($p<0.0001$) (Table 4 and Table 5).

The coded values for MSG (X_1) and sucrose (X_2) were transformed to actual values using Eq. 7 and Eq. 8.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

The following second-order polynomial regression model was fitted to the data:

$$Y=33.958-1.744X_1+1.824X_2-7.881X_1^2-6.187X_2^2-2.505X_1X_2 \quad /10/$$

where Y is the GABA yield (mM), and X_1 and X_2 are the coded values for MSG and sucrose concentrations, respectively.

Response surface methodology (RSM) was employed to visualize and optimize the combined effects of MSG (mg/mL) and sucrose (%, *m/V*) concentrations on GABA yield (mM). The 3D and contour plots (Fig. 2a and Fig. 2b) revealed a non-linear relationship between the independent variables and GABA yield, with a clear optimal region indicated by the curved surface and concentric rings, respectively. Model optimization predicted a maximum GABA yield (Y_{max}) of 34.237 mM at 1.564 mg/mL MSG and 10.93 % sucrose. These optimized conditions were experimentally validated. Optimization results were: Y_{max}=34.237 mM, X_1(MSG)=1.564 mg/mL, and X_2(sucrose)=10.93 %./

Time course study of cell growth, pH, organic acid, and GABA content

A time course study was conducted to monitor the changes in pH, cell growth, organic acid production, and GABA concentration during soymilk fermentation by *L. plantarum* W12 under optimized conditions (initial cell density $5\cdot10^6$ CFU/mL, 45 °C, initial pH=6.0). Key factors influencing microbial GABA biosynthesis include cell density, pH, temperature, fermentation time, and glutamate availability [*18*].

The primary objective of this study was to optimize GABA production by *L. plantarum* W12 in soymilk. The time course of GABA production was monitored throughout the 18-hour fermentation (Fig. 3a). The results demonstrate that GABA production is time-dependent. Initially, GABA levels were negligible, but they increased steadily over time, reaching a maximum of (34.46±0.98) mM at 15 h. A slight decrease in GABA concentration was observed at 18 h ((33.46±0.66) mM), although this difference was not statistically significant ($p>0.05$). The observed increase in GABA production correlates with the growth of *L. plantarum* W12 and the decrease in pH. This suggests that active bacterial metabolism and the acidic environment created by organic acid production are favourable for GABA biosynthesis [*46*].

L. plantarum W12 reached a GABA level of 9.069 mM under optimal conditions and demonstrated strong acid/bile resistance, high hydrophobicity, and antimicrobial activity, supporting its suitability for functional food applications. The observed pH decline (from ~6.0 to 4.6 over 18 h)

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

reflects active metabolism and organic acid production, notably lactic acid, which contributes to preservation and sensory properties [*47*]. At 15–18 h, W12 entered a stationary phase with maximum GABA accumulation. GAD activity, optimal at low pH, may be suppressed as GABA production increases at medium pH. Mutant GAD enzymes with broader pH activity have been proposed to overcome this limitation [*48*]. Recently, Kubota *et al.* revealed that GABA attenuates ischemia-reperfusion-induced alterations in intestinal immunity through increased IgA secretion, alpha-defensin-5 expression, and small intestinal superoxide dismutase activity [*7*]. From there, it shows that *L. plantarum* W12 in our study is a potential probiotic that can be used to produce functional foods to enhance intestinal health.

Cell growth is a critical factor in fermentation as it relates to the production of metabolites, including GABA. The growth of *L. plantarum* W12 in soymilk was monitored over the 18-hour fermentation period (Fig. 3b). The data show a typical growth curve for *L. plantarum* W12 under these conditions. The initial cell density (log CFU/mL) of 6.7 increased rapidly during the first 12 h, reaching 7.8. After 12 h, the growth rate slowed, and the cell density reached 7.9 by the end of the 18 h fermentation. This indicates that *L. plantarum* W12 efficiently utilizes the soymilk medium supplemented with MSG and sucrose for growth. The observed growth pattern suggests that the bacteria entered the stationary phase after 12 h, due to nutrient depletion or the accumulation of inhibitory metabolites [*49*]. It is important to note that the production of GABA is often associated with bacterial growth and metabolic activity [*50*]. Therefore, understanding the growth dynamics of *L. plantarum* W12 is crucial for optimizing GABA production. The correlation between cell growth and GABA concentration will be further investigated in subsequent analyses. This will help determine the optimal fermentation time for maximizing GABA yield while considering the cost and efficiency of the process.

Monitoring pH changes during fermentation is crucial as it reflects the metabolic activity of the bacteria and can influence enzyme activity, including glutamate decarboxylase, which is responsible for GABA production [*51*]. The pH of soymilk decreased significantly throughout the fermentation period (Fig. 3c).

Regarding organic acid profiles, concentrations of lactic acid, acetic acid, pyruvic acid, propionic acid, and formic acid were monitored over the 18-hour fermentation period (Fig. 3d–Fig. 3g). Lactic acid (Fig. 3d) was the dominant organic acid produced, consistent with the metabolic activity of *L. plantarum* W12, a lactic acid bacterium. Its concentration increased steadily throughout

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

fermentation, reaching a maximum of (182.37±7.94) mg/g at 18 h. This progressive increase in lactic acid is directly related to the decrease in pH observed. Acetic acid (Fig. 3e) was produced at much lower concentrations than lactic acid, reaching approximately 1.10 mg/g by the end of fermentation. Pyruvic acid concentration also increased gradually during fermentation, reaching 0.12 mg/g at 18 h. Pyruvic acid (Fig. 3f) is an important intermediate in various metabolic pathways, including lactic acid fermentation [*52*]. Propionic acid (Fig. 3g) levels remained stable throughout fermentation, fluctuating around 0.25 mg/g. Formic acid (Fig. 3h) production showed a different pattern, with an initial increase followed by a decrease. The highest concentration (0.02 mg/g) was observed at 9 h.

Our results showing lactic acid as the predominant byproduct ((182.37±7.94) mg/g) with minor amounts of acetic acid (1.10 mg/g) align with the typical homofermentative metabolism of *L. plantarum*. This metabolic pattern is crucial for both product quality and GABA production efficiency. The high lactic acid: acetic acid ratio (165:1) confirms the homofermentative nature of *L. plantarum* W12, contrasting sharply with heterofermentative LAB such as *L. brevis*, which produce higher acetic acid proportions alongside lactic acid and generate additional metabolic byproducts including ethanol and CO_2 [*53*].

The dominance of lactic acid in our fermented soymilk contributes several advantages: (*i*) desirable sensory properties, including mild acidity (pH=4.6) and a clean flavor profile without the sharp, vinegary notes associated with high acetic acid content; (*ii*) effective preservation through controlled pH reduction, inhibiting the growth of spoilage organisms and foodborne pathogens; (*iii*) favourable metabolic environment for GAD enzyme activity, as the enzyme shows optimal activity at pH=4.5–5.5, which is maintained during mid-to-late fermentation stages; and (*iv*) minimal production of off-flavor compounds, as evidenced by the low levels of other organic acids (pyruvic acid 0.12 mg/g, propionic acid 0.25 mg/g, and formic acid 0.02 mg/g). These low concentrations of minor organic acids indicate efficient metabolic flux toward lactic acid production, minimizing potentially undesirable flavor compounds and ensuring a clean, acceptable taste profile for the final product [*54*].

The temporal dynamics of organic acid production during fermentation (Fig. 3d–Fig. 3h) revealed coordinated metabolic processes: lactic acid accumulation paralleled GABA production and pH decline, suggesting coupling between energy metabolism and glutamate decarboxylation. This relationship is consistent with the GAD-mediated acid resistance mechanism, where GABA synthesis consumes intracellular protons, helping maintain cellular pH homeostasis during lactic acid fermentation [*55*]. The stable low levels of propionic acid and the transient peak of formic acid at 9 h

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

(followed by a decline) indicate active secondary metabolism without accumulation of undesirable metabolites.

CONCLUSIONS

Among five *Lactiplantibacillus plantarum* strains examined, *L. plantarum* W12 showed the most promising performance, possessing superior probiotic traits and achieving the highest GABA production ((34.46±0.98) mM) under optimized fermentation conditions (1.564 mg/mL MSG, 10.93 % sucrose, 15 h at 43 °C). The GABA-enriched fermented soymilk produced using *L. plantarum* W12 represents a functional food product with dual benefits: high levels of bioactive GABA for neurological and cardiovascular health support, combined with viable probiotic bacteria (>7.9 log CFU/mL) for gastrointestinal health promotion. The optimized fermentation process yields a product with favourable sensory characteristics (pH=4.6, mild lactic acid flavor) and extended shelf life due to organic acid preservation. The homofermentative metabolic profile of W12, characterized by high lactic acid production and minimal off-flavor compounds, ensures both product quality and optimal conditions for GABA biosynthesis through maintenance of favorable pH ranges for glutamate decarboxylase activity. Therefore, these results strongly suggest that *L. plantarum* W12 is an excellent candidate for commercial-scale production of GABA-enriched fermented soymilk. This is a multifunctional food containing GABA (at concentrations exceeding those in most reported plant-based fermented products), as well as probiotic mechanisms positions this product competitively in the functional food market.

To fully assess its safety and health benefits, however, further *in vivo* studies—including animal models and clinical trials specifically, future studies should evaluate: (*i*) GABA bioavailability through pharmacokinetic studies in animal models to confirm absorption and blood-brain barrier penetration; (*ii*) probiotic persistence and colonization in human gastrointestinal tract using molecular tracking methods such as strain-specific PCR or whole-genome sequencing; (*iii*) sensory acceptability and shelf-life stability under commercial storage conditions (4 °C, 21 days) including assessment of GABA stability and viable cell counts; and (*iv*) scale-up feasibility and cost-effectiveness analysis for commercial production, including evaluation of substrate costs, fermentation efficiency at industrial scale, and regulatory compliance pathways for health claim substantiation.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

ChatGPT (GPT-4, OpenAI) was used to assist with English grammar and language refinement. No scientific content, data interpretation, or conclusions were generated by the tool. The authors are fully responsible for the manuscript.

FUNDING

This work was not funded by any specific grants.

AUTHORS' CONTRIBUTION

Nguyen Cao Cuong contributed to the study design, data collection, and preparation of the first draft of the manuscript. Bir Bahadur Thapa contributed to data analysis, prepared the graphs, and drafted the manuscript. Ngoc Hieu Nguyen, Tran Thanh Quynh Anh, and Pham Hoang Son Hung contributed to the experiment design, collected the data, and edited the manuscript. Peter Vandamme contributed to the identification of isolates and revised the manuscript. Do Thi Bich Thuy served as the project manager, contributed to the study design, and edited the manuscript. All authors engaged in the final approval of the version to be published.

ORCID ID

N.C. Cuong https://orcid.org/0000-0003-0288-8202

B.B. Thapa https://orcid.org/0000-0002-8700-1502

N.H. Nguyen https://orcid.org/0000-0001-6091-3491

T.T.Q. Anh https://orcid.org/0009-0004-0268-5979

P.H.S. Hung https://orcid.org/0000-0002-6629-1379

P. Vandamme https://orcid.org/0000-0002-5581-7937

D.T.B. Thuy https://orcid.org/0000-0002-4723-5179

REFERENCES

1. Tangyu M, Muller J, Bolten CJ, Wittmann C. Fermentation of plant-based milk alternatives for improved flavour and nutritional value. Appl Microbiol Biot. 2019;103(23):9263-75.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

https://doi.org/10.1007/s00253-019-10175-9

2. Khayatan D, Nouri K, Momtaz S, Roufogalis BD, Alidadi M, Jamialahmadi T*, et al.* Plant-derived fermented products: An interesting concept for human health. Curr Dev Nutr. 2024;8(5):102162.

https://doi.org/10.1016/j.cdnut.2024.102162

3. Xia Y, Zha M, Feng C, Li Y, Chen Y, Shuang Q. Effect of a co-fermentation system with high-GABA-yielding strains on soymilk properties: microbiological, physicochemical, and aromatic characterisations. Food Chem. 2023;423.

https://doi.org/10.1016/j.foodchem.2023.136245

4. Inoue K, Shirai T, Ochiai H, Kasao M, Hayakawa K, Kimura M*, et al.* Blood-pressure-lowering effect of a novel fermented milk containing γ-aminobutyric acid (GABA) in mild hypertensives. Eur J Clin Nutr. 2003;57(3):490-95.

https://doi.org/10.1038/sj.ejcn.1601555

5. Hepsomali P, Groeger JA, Nishihira J, Scholey A. Effects of oral gamma-aminobutyric acid (GABA) administration on stress and sleep in humans: A systematic review. Front Neurosci. 2020;14:559962.

https://doi.org/10.3389/fnins.2020.00923

6. Abdou AM, Higashiguchi S, Horie K, Kim M, Hatta H, Yokogoshi H. Relaxation and immunity enhancement effects of γ-Aminobutyric acid (GABA) administration in humans. BioFactors. 2006;26(3):201-08.

https://doi.org/https://doi.org/10.1002/biof.5520260305

7. Kubota A, Kobayashi M, Sarashina S, Takeno R, Yasuda G, Narumi K*, et al.* Gamma-aminobutyric acid (GABA) attenuates ischemia reperfusion-induced alterations in intestinal immunity. Biol Pharm Bull. 2018;41(12):1874-78.

https://doi.org/10.1248/bpb.b18-00338

8. Kim I-S, Kim C-H, Yang W-S. Physiologically active molecules and functional properties of soybeans in human health—A current perspective. Int J Mol Sci. 2021;22(8):4054.

https://doi.org/10.3390/ijms22084054

9. Lai L-R, Hsieh S-C, Huang H-Y, Chou C-C. Effect of lactic fermentation on the total phenolic, saponin and phytic acid contents as well as anti-colon cancer cell proliferation activity of soymilk. J Biosci Bioeng. 2013;115(5):552-56.

https://doi.org/10.1016/j.jbiosc.2012.11.022

10. Chuang Y-C, Cheng M-C, Lee C-C, Chiou T-Y, Tsai T-Y. Effect of ethanol extract from *Lactobacillus plantarum* TWK10-fermented soymilk on wound healing in streptozotocin-induced diabetic rat. AMB Express. 2019;9(1):163.

https://doi.org/10.1186/s13568-019-0886-2

11. Wang Y, Guo H, Wu A, Ju C, Jiang J, Chen J. Multiple-strain Lactobacillus-fermented soymilk with antioxidant capacity and delicate flavour. Int J Food Sci Tech. 2021;56(11):6052-61.

https://doi.org/10.1111/ijfs.15253

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

12. Yu G, Hua Y, Zhang C, Li X, Kong X, Chen Y. Characterization of the volatile organic compounds and sensory properties of fermented soymilks as affected by carbohydrates and starter cultures. LWT-Food Sci Technol. 2024;202:116264.

https://doi.org/10.1016/j.lwt.2024.116264

13. Hwang CE, Kim SC, Kim DH, Lee HY, Suh HK, Cho KM*, et al.* Enhancement of isoflavone aglycone, amino acid, and CLA contents in fermented soybean yogurts using different strains: Screening of antioxidant and digestive enzyme inhibition properties. Food Chem. 2021;340:128199.

https://doi.org/10.1016/j.foodchem.2020.128199

14. Tan X, Zhang S, Liu J, Xiang W, Zhang Q, Tang J. Effect of *Lactiplantibacillus plantarum* cell-free supernatant on the physiology, quorum sensing, transcription, and enhanced GABA production of Enterococcus faecium. LWT-Food Sci Technol. 2024;198:115986.

https://doi.org/10.1016/j.lwt.2024.115986

15. Wang X, Wang Y, Nan B, Cao Y, Piao C, Li X*, et al.* Optimization of fermentation for gamma-aminobutyric acid (GABA) production by *Lactiplantibacillus plantarum* Lp3 and the development of fermented soymilk. LWT-Food Sci Technol. 2024;195:115841.

https://doi.org/10.1016/j.lwt.2024.115841

16. Arasu MV, Al-Dhabi NA, Ilavenil S, Choi KC, Srigopalram S. In vitro importance of probiotic *Lactobacillus plantarum r*elated to medical field. Saudi J Biol Sci. 2016;23(1, Supplement):S6-S10.

https://doi.org/10.1016/j.sjbs.2015.09.022

17. Costa MPd, Frasao BdS, Lima BRCdC, Rodrigues BL, Junior CAC. Simultaneous analysis of carbohydrates and organic acids by HPLC-DAD-RI for monitoring goat's milk yogurts fermentation. Talanta. 2016;152:162-70.

https://doi.org/10.1016/j.talanta.2016.01.061

18. Thuy DTB, An NT, Jayasena V, Vandamme P. A comprehensive investigation into the production of gamma-aminobutyric acid by *Limosilactobacillus fermentum* NG16, a tuna gut isolate. Acta Aliment Hung. 2022;51(2):302-11.

https://doi.org/10.1556/066.2022.00065

19. Li M, Wang Y, Cui H, Li Y, Sun Y, Qiu H-J. Characterization of lactic acid bacteria isolated from the gastrointestinal tract of a Wild Boar as potential probiotics. Front Vet Sci. 2020;Volume 7 - 2020.

https://doi.org/10.3389/fvets.2020.00049

20. Oh A, Daliri EB-M, Oh DH. Screening for potential probiotic bacteria from Korean fermented soybean paste: In vitro and Caenorhabditis elegans model testing. LWT-Food Sci Technol. 2018;88:132-38.

https://doi.org/10.1016/j.lwt.2017.10.007

21. Hazards EPoB, Allende A, Alvarez-Ordóñez A, Bortolaia V, Bover-Cid S, De Cesare A*, et al.* Update of the list of qualified presumption of safety (QPS) recommended microbiological agents

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

intentionally added to food or feed as notified to EFSA 22: Suitability of taxonomic units notified to EFSA until March 2025. EFSA J. 2025;23(7):e9510.

https://doi.org/10.2903/j.efsa.2025.9510

22. Hernández D, Cardell E, Zárate V. Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of plantaricin TF711, a bacteriocin-like substance produced by *Lactobacillus plantarum* TF711. J Appl Microbiol. 2005;99(1):77-84.

https://doi.org/10.1111/j.1365-2672.2005.02576.x

23. Myers RH, Montgomery DC, Anderson-Cook CM. Response surface methodology: process and product optimization using designed experiments. Fourth ed. Hoboken: John Wiley & Sons; 2016.

24. Stat-Ease Inc. Design-Expert® Software Version 12. Minneapolis, MN, USA: Stat-Ease, Inc.; 2019.

25. Cataldo PG, Urquiza Martinez MP, Villena J, Kitazawa H, Saavedra L, Hebert EM. Comprehensive characterization of gamma-aminobutyric acid (GABA) production by *Levilactobacillus brevis* CRL 2013: insights from physiology, genomics, and proteomics. Front Microbiol. 2024;15:1408624.

https://doi.org/10.3389/fmicb.2024.1408624

26. Harnentis H, Nurmiati N, Marlida Y, Adzitey F, Huda N. gamma-Aminobutyric acid production by selected lactic acid bacteria isolate of an Indonesian indigenous fermented buffalo milk (dadih) origin. Vet World. 2019;12(8):1352-57.

https://doi.org/10.14202/vetworld.2019.1352-1357

27. Ledashcheva T, Dinh Thi Thu T, Nguyen Cong T, Vo Thi Hoai T, Do Thi T, Do Thi Thu H*, et al.* Production of gamma-aminobutyric acid by lactic acid bacteria of marine origin from Vietnam. E3S Web Conf. 2023;407.

https://doi.org/10.1051/e3sconf/202340704010

28. Călinoiu L-F, Vodnar D-C, Precup G. The probiotic bacteria viability under different conditions. B UASVM-Food Sci Te. 2016;73(2):55-60.

https://doi.org/10.15835/buasvmcn-fst:12448

29. Schneider F, Grimm M, Koziolek M, Modeß C, Dokter A, Roustom T*, et al.* Resolving the physiological conditions in bioavailability and bioequivalence studies: Comparison of fasted and fed state. Eur J Pharm Biopharm. 2016;108:214-19.

https://doi.org/10.1016/j.ejpb.2016.09.009

30. Sannasiddappa TH, Lund PA, Clarke SR. In vitro antibacterial activity of unconjugated and conjugated bile salts on Staphylococcus aureus. Frontiers in Microbiology. 2017;8:1581.

https://doi.org/10.3389/fmicb.2017.01581

31. Trunk T, Khalil HS, Leo JC. Bacterial autoaggregation. AIMS Microbiol. 2018;4(1):140.

https://doi.org/10.3934/microbiol.2018.1.140

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

32. Rocchetti MT, Russo P, Capozzi V, Drider D, Spano G, Fiocco D. Bioprospecting antimicrobials from *Lactiplantibacillus plantarum*: Key factors underlying its probiotic action. Int J Mol Sci. 2021;22(21):12076.

https://doi.org/10.3390/ijms222112076

33. Du Y, Li H, Shao J, Wu T, Xu W, Hu X*, et al.* Adhesion and colonization of the probiotic *Lactobacillus plantarum* HC-2 in the intestine of Litopenaeus vannamei are associated with bacterial surface proteins. Frontiers in Microbiology. 2022;13:878874.

https://doi.org/10.3389/fmicb.2022.878874

34. Mantzourani I, Chondrou P, Bontsidis C, Karolidou K, Terpou A, Alexopoulos A*, et al.* Assessment of the probiotic potential of lactic acid bacteria isolated from kefir grains: Evaluation of adhesion and antiproliferative properties in in vitro experimental systems. Ann Microbiol. 2019;69:751-63.

https://doi.org/10.1007/s13213-019-01467-6

35. Aguilar C, Klotz B. Effect of the temperature on the antagonistic activity of lactic acid bacteria against *Escherichia coli* and *Listeria monocytogenes*. J Food Safety. 2010;30(4):996-1015.

https://doi.org/10.1111/j.1745-4565.2010.00257.x

36. Reis J, Paula A, Casarotti S, Penna A. Lactic acid bacteria antimicrobial compounds: characteristics and applications. Food Eng Rev. 2012;4:124-40.

https://doi.org/10.1007/s12393-012-9051-2

37. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B*, et al.* The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11(8):506-14.

https://doi.org/10.1038/nrgastro.2014.66

38. Zhu F, Hu S, Mei L. Production and quality evaluation of a novel gamma-aminobutyric acid-enriched yogurt. Front Nutr. 2024;11:1404743.

https://doi.org/10.3389/fnut.2024.1404743

39. Iorizzo M, Paventi G, Di Martino C. Biosynthesis of Gamma-aminobutyric acid (GABA) by *Lactiplantibacillus plantarum* in fermented food production. Curr Issues Mol Biol. 2023;46(1):200-20.

https://doi.org/10.3390/cimb46010015

40. Bai Q, Chai M, Gu Z, Cao X, Li Y, Liu K. 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. 2009;116(1):152-57.

https://doi.org/10.1016/j.foodchem.2009.02.022

41. Pannerchelvan S, Rios-Solis L, Faizal Wong FW, Zaidan UH, Wasoh H, Mohamed MS*, et al.* Strategies for improvement of gamma-aminobutyric acid (GABA) biosynthesis via lactic acid bacteria (LAB) fermentation. Food Funct. 2023;14(9):3929-48.

https://doi.org/10.1039/d2fo03936b

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

42. Cha X, Ding J, Ba W, You S, Qi W, Su R. High production of gamma-aminobutyric acid by activating the xyl operon of *Lactobacillus brevis*. ACS Omega. 2023;8(8):8101-09.

https://doi.org/10.1021/acsomega.2c08272

43. Liu X, Wang Z, Xiao J, Zhou X, Xu Y. Osmotic stress tolerance and transcriptome analysis of Gluconobacter oxydans to extra-high titers of glucose. Front Microbiol. 2022;13:977024.

https://doi.org/10.3389/fmicb.2022.977024

44. Yao C, Liu Y, Hu X, Wang X. Improve gamma-aminobutyric acid production in *Corynebacterium glutamicum* by optimizing the metabolic flux. Syst Microbiol Biomanufacturing. 2021;2(2):305-16.

https://doi.org/10.1007/s43393-021-00062-8

45. Naydenova G, Popova-Krumova P, Danova S, Yankov D. Lactic acid production from distiller's dried grains dilute acid hydrolysates. Fermentation. 2024;10(11).

https://doi.org/10.3390/fermentation10110581

46. Xue C, Ting W-W, Ng IS. A novel toggle switch under acid-base regulation for tunable gamma-aminobutyric acid (GABA) production in *Escherichia coli*. J Taiwan Inst Chem E. 2024;157:105442.

https://doi.org/10.1016/j.jtice.2024.105442

47. Karpiński TM, Ożarowski M. Plant organic acids as natural inhibitors of foodborne pathogens. Appl Sci-Basel. 2024;14(14).

https://doi.org/10.3390/app14146340

48. Xu N, Wei L, Liu J. Biotechnological advances and perspectives of gamma-aminobutyric acid production. World J Microb Biot. 2017;33:1-11.

https://doi.org/10.1007/s11274-017-2234-5

49. Zhang Q, Chen Y, Shi H. Bacterial strategies in prolonged stationary phase: Tradeoff between cell growth, maintenance, and recycling. Phys Rev Res. 2023;5(1).

https://doi.org/10.1103/PhysRevResearch.5.013119

50. Rehman A, Di Benedetto G, Bird JK, Dabene V, Vadakumchery L, May A*, et al.* Development of a workflow for the selection, identification and optimization of lactic acid bacteria with high gamma-aminobutyric acid production. Sci Rep. 2023;13(1):13663.

https://doi.org/10.1038/s41598-023-40808-z

51. Rault A, Bouix M, Béal C. Fermentation pH influences the physiological-state dynamics of *Lactobacillus bulgaricus* CFL1 during pH-controlled culture. Appl Environ Microbiol. 2009;75(13):4374-81.

https://doi.org/10.1128/AEM.02725-08

52. Luo Q, Ding N, Liu Y, Zhang H, Fang Y, Yin L. Metabolic engineering of microorganisms to produce pyruvate and derived compounds. Molecules. 2023;28(3).

https://doi.org/10.3390/molecules28031418

53. Pessione E. Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. Front Cell Infect Microbiol. 2012;2:86.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

https://doi.org/10.3389/fcimb.2012.00086

54. McSweeney PL, Sousa MJ. Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. Le Lait. 2000;80(3):293-324.

https://doi.org/10.1051/lait:2000127

55. Feehily C, Karatzas K. Role of glutamate metabolism in bacterial responses towards acid and other stresses. J Appl Microbiol. 2013;114(1):11-24.

https://doi.org/10.1111/j.1365-2672.2012.05434.x

Table 1. Ranges of the two independent variables used in RSM

Independent variables		Levels				
		−α	−1	0	1	+α
X_1	MSG (mg/mL)	0.79	1.00	1.50	2.00	2.21
X_2	Sucrose (%, *m/V*)	2.93	5.00	10.00	15.0	17.07

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Table 2. Experimental plan for optimization of GABA yield using RSM

Experiment[1]	Variable (Coded[2])		Variable Net Value		GABA yield (mM)	
	X_1	X_2	X_1	X_2	Actual	Predicted
1	-1	-1	1.00000	5.000	17.82	17.30
2	1	-1	2.00000	5.000	16.33	18.83
3	-1	1	1.00000	15.000	27.28	25.96
4	1	1	2.00000	15.000	15.77	17.46
5	-1.41421	0	0.79289	10.000	19.12	20.66
6	1.41421	0	2.20711	10.000	18.45	15.73
7	0	-1.41421	1.50000	2.9289	20.16	19.00
8	0	1.41421	1.50000	17.0711	24.19	24.16
9	0	0	1.50000	10.0000	34.00	33.96
10	0	0	1.50000	10.0000	32.98	33.96
11	0	0	1.50000	10.0000	33.59	33.96
12	0	0	1.50000	10.0000	34.11	33.96
13	0	0	1.50000	10.0000	35.10	33.96

[1]The experiment was conducted randomly
[2]X_1: monosodium glutamate (MSG mg/mL); X_2: sucrose (%, *m/V*)

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Table 3. Comprehensive probiotic properties of *L. plantarum* strains isolated from soybean whey

Survival / Strain	Gastrointestinal tolerance (Survival/%)			Hydrophobicity/%			Antagonistic zone diameter/mm			Auto-aggregation/%	Coaggregation with pathogenic bacteria/%		
	Low pH	Bile/pancreatin	Pepsin	Xylene	Chloroform	Ethyl acetate	*E. coli*	*S. aureus*	*S. typhimurium*	*L. plantarum*	*E. coli*	*S. aureus*	*S. typhimurium*
L. plantarum W1	(92.05±3.38)^{c}	(89.27±1.95)^{b}	(97.72±1.29)^{a}	(9.79±0.29)^{a}	(68.33±2.78)^{b}	(7.30±1.17)^{b}	(2.67±0.58)^{c}	(15.67±0.57)^{c}	(12.00±1.00)^{b}	(93.72±2.28)^{a}	(6.73±0.64)^{b}	(11.42±1.05)^{c}	(0.11±0.04)^{d}
L. plantarum W3	(98.01±2.35)^{a}	(95.20±3.19)^{a}	(88.06±2.87)^{b}	(0.16±0.03)^{e}	(10.58±1.47)^{d}	(3.70±1.21)^{d}	(8.33±1.16)^{a}	(26.67±1.53)^{a}	(15.00±1.00)^{a}	(87.06±1.94)^{b}	(5.45±0.52)^{c}	(2.74±0.53)^{e}	(17.74±1.56)^{a}
L. plantarum W4	(87.05±2.57)^{d}	(93.13±1.27)^{b}	(84.22±2.27)^{c}	(5.47±0.56)^{b}	(2.62±0.68)^{e}	(5.83±1.05)^{bc}	(2.67±0.58)^{c}	(16.33±1.53)^{c}	(5.33±0.58)^{d}	(83.22±1.81)^{c}	(0.19±0.03)^{d}	(18.31±0.92)^{b}	(0.66±0.21)^{d}
L. plantarum W5	(93.12±1.24)^{bc}	(95.34±1.64)^{a}	(87.94±1.19)^{b}	(3.96±0.22)^{c}	(16.68±0.99)^{c}	(4.378±0.51)^{cd}	(5.00±1.00)^{b}	(20.67±1.53)^{b}	(7.00±1.00)^{c}	(87.27±0.95)^{b}	(4.84±0.60)^{c}	(8.79±1.11)^{d}	(11.34±1.36)^{c}
L. plantarum W12	(97.06±1.98)^{ab}	(96.54±1.02)^{a}	(97.34±1.73)^{a}	(2.87±0.43)^{d}	(85.45±0.55)^{a}	(12.71±0.62)^{a}	(3.00±1.00)^{c}	(28.00±1.00)^{a}	(14.33±0.58)^{a}	(96.67±1.82)^{a}	(8.30±0.63)^{a}	(20.82±1.58)^{a}	(14.39±0.88)^{b}

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Values are presented as mean±standard deviation. Different superscript letters within a column indicate significant differences ($p \leq 0.05$, Duncan's multiple range test)

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Table 4. Test of significance for regression coefficients

Term	Coeff	SE coeff	T-value	P-value	VIF
Constant	33.958	0.838	40.51	0.000	
A	-1.744	0.663	-2.63	0.034	1.00
B	1.824	0.663	2.75	0.028	1.00
A×A	-7.881	0.711	-11.09	0.000	1.02
B×B	-6.187	0.711	-8.71	0.000	1.02
A×B	-2.505	0.937	-2.67	0.032	1.00
	Model summary				
	Standard deviation of the residuals (s)		1.87427	Mean	25.30
	R-squared (R^2)		96.59 %	CV/%	7.41
	Adjusted R-squared (Adj. R^2)		94.15 %		
	Predicted R-squared (Pred. R^2)		77.61 %		

The results were obtained using the Design Expert software v. 12.0.3.0 [*24*]. A=MSG (mg/mL), B=sucrose (%, m/V)

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Table 5. ANOVA for the quadratic model of GABA yield

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	696.47	5	139.29	39.65	< 0.0001	significant
A	24.33	1	24.33	6.93	0.0338	
B	26.62	1	26.62	7.58	0.0284	
AB	25.10	1	25.10	7.14	0.0319	
A^2	432.03	1	432.03	122.99	< 0.0001	
B^2	266.32	1	266.32	75.81	< 0.0001	
Residual	24.59	7	3.51			
Lack of Fit	22.17	3	7.39	12.22	0.0175	significant
Pure Error	2.42	4	0.6048			
Cor Total	721.06	12				

The results were obtained using Design Expert software v. 12.0.3.0 [24]. A=MSG (mg/mL), B=sucrose (%, *m*/*V*)

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

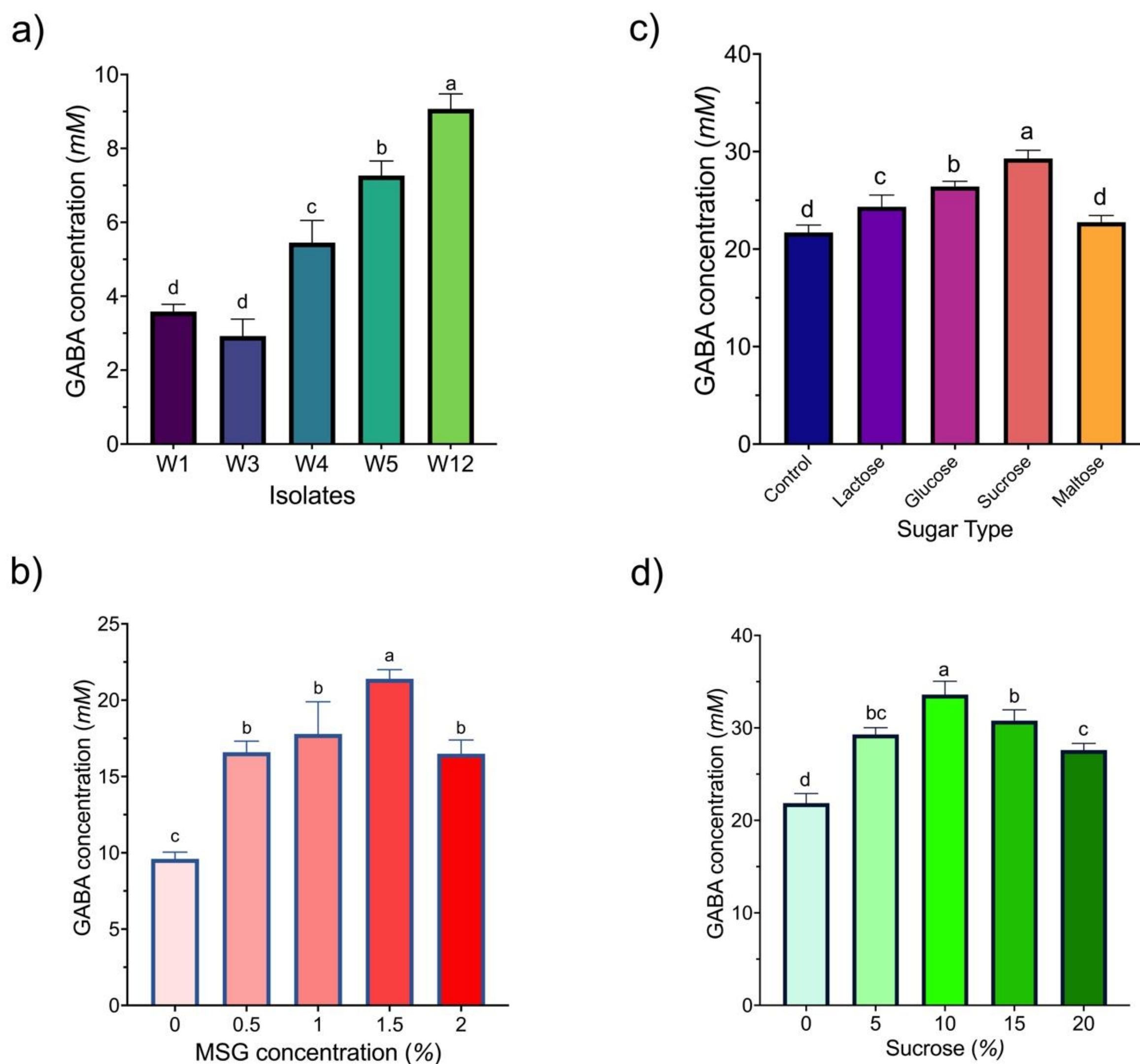

Fig. 1. GABA production optimization by *L. plantarum* strains: a) GABA production capacity of five *L. plantarum* isolates (W1, W3, W4, W5, W12) in MRS broth supplemented with 1 % MSG after 24 h fermentation at 37 °C, b) effect of MSG concentration (0–2 %, *m/V*) on GABA production by *L. plantarum* W12 in soymilk fermented at 43 °C for 15 h, c) effect of carbon source type (5 %, *m/V* lactose, sucrose, glucose, maltose) on GABA production by *L. plantarum W12* in soymilk containing 1.5 % MSG, d) effect of sucrose concentration (0–20 %, *m/V*) on GABA production by *L. plantarum* W12 in soymilk containing 1.5 % MSG. Data are presented as mean±standard deviation (*N*=3). Different letters indicate significant differences ($p \leq 0.05$, Duncan's multiple range test)

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

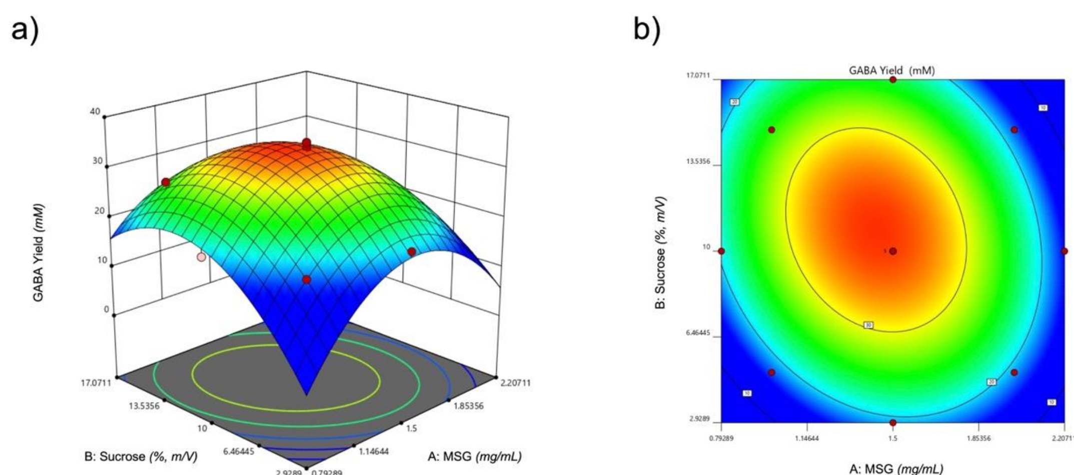

Fig. 2. Response surface methodology optimization of GABA production by *L. plantarum* W12: a) three-dimensional response surface plot showing the combined effects of MSG (mg/mL) and sucrose (%, *m/V*) concentrations on GABA yield (mM). The curved surface indicates a clear optimal region with maximum GABA production at intermediate levels of both variables, b) two-dimensional contour plot displaying the same optimization landscape, where concentric rings represent GABA yield iso-response lines. The optimal conditions (1.564 mg/mL MSG and 10.93 % sucrose) are located at the center of the highest contour region, predicting a maximum GABA yield of 34.24 mM. Panel labels (a, b) are positioned in the left corner outside each graph

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

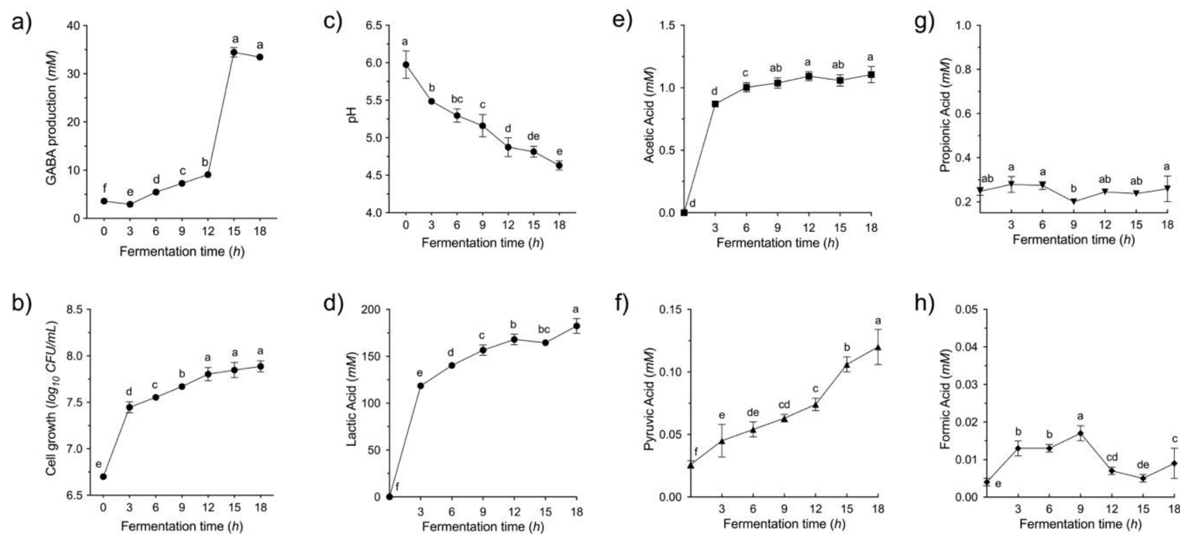

Fig. 3. Time-course analysis of *L. plantarum* W12 soymilk fermentation under optimized conditions (1.564 mg/mL MSG, 10.93 % sucrose, 43 °C, initial pH=6.0, initial cell density $5\cdot10^6$ CFU/mL). Changes in: a) GABA concentration (mM), b) cell growth (log CFU/mL), c) pH, d) lactic acid concentration (mg/g), e) acetic acid concentration (mg/g), f) pyruvic acid concentration (mg/g), g) propionic acid concentration (mg/g), and h) formic acid concentration (mg/g) were monitored over 18 h. Samples were collected at 0, 3, 6, 9, 12, 15, and 18 h. Data are presented as mean±standard deviation (*N*=3). Different letters at each time point indicate significant differences ($p\leq0.05$, Duncan's multiple range test)