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Antioxidant Activity, γ-Aminobutyric Acid, and Genome Analysis of *Lactiplantibacillus plantarum* FL13-2 from Fermented Rice Flour

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Abstract

Ten rod-shaped isolates of lactic acid bacteria (LAB) from the traditional fermented rice flour, *khao-khab* were identified as belonging to the genus *Lactobacillus* based on phenotypic characteristics. Group I isolates (FL12-1, FL18-1, FL19-1S, FL23-1, FL24-1, FL25-1, and FL26-1) were closely related to *Limosilactobacillus fermentum*, exhibiting 16S rRNA gene sequence similarity of 99.4%–100%. Group II included FL13-2 and FL22-2 (Group IIA) and FL17B (Group IIB), closely related to *Lactiplantibacillus plantarum* (99.9%–100%) and *Lactiplantibacillus pentosus* (100%), respectively. All isolates demonstrated strong antioxidant potential, with 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity—measured by the standard DPPH assay—ranging from 78.49±1.01% to 91.18±3.95%, surpassing that of ascorbic acid. Strain FL13-2, identified as *L. plantarum* through genomic analysis (Average Nucleotide Identity (ANI) 98.5%, digital DNA-DNA hybridization (dDDH) 92.5%), produced 2.43 g/L of γ-aminobutyric acid (GABA). It harbored key biosynthesis and transport genes (*gadB, gadC, gatABCD, pdxK*). Phylogenetic analysis confirmed high sequence similarity of these genes with other LAB strains, supporting their functional roles. The gad system was implicated in acid resistance and GABA production under low pH conditions. Genome annotation revealed bacteriocin genes (Enterocin X β, Plantaricin E, and F) and indicated low pathogenicity and absence of virulence factors. Antimicrobial resistance genes (*vanH, vanT, vanY*) were detected; however, these are intrinsic to LAB and associated with essential cell wall biosynthesis rather than acquired resistance. Additionally, the presence of the qacJ efflux pump and hlyIII

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gene—both commonly found in probiotic strains—further supports the safety profile of FL13-2. These findings highlight *L. plantarum* FL13-2 as a promising multifunctional probiotic candidate with potent antioxidative, GABA-producing, and antimicrobial properties.

Introduction

Gamma-aminobutyric acid (GABA) is a non-protein amino acid that functions as the primary inhibitory neurotransmitter in the vertebrate central nervous system. It helps reduce neuronal excitability, promotes relaxation and sleep, and regulates blood pressure. Although traditionally considered brain-derived, recent research has demonstrated that certain bacteria—particularly gut microbes—also synthesize GABA. In lactic acid bacteria (LAB), GABA is produced via glutamate decarboxylase (GAD)–mediated decarboxylation of glutamate (Dhakal et al., 2012). Through the gut–brain axis, microbially derived GABA may influence mood, sleep quality, and mental health (Liwinski et al., 2023).

GABA occurs naturally in foods such as germinated brown rice, sprouted cereals, spinach, tomatoes, soybeans, and broccoli (Komatsuzaki et al., 2007; Oh et al., 2003). Fermentation further enhances GABA levels; numerous LAB are documented producers, including *Lactococcus lactis, Bifidobacterium adolescentis* (Rehman et al., 2023), *Levilactobacillus brevis* (Kim et al., 2021), *Lactobacillus zymae* GU240 (Lee et al., 2018), and *Lactobacillus plantarum* K255 (Park et al., 2013). *Leuconostoc mesenteroides* strains K1501 and K1627 from kimchi efficiently generate GABA under MSG-supplemented conditions and tolerate simulated gastrointestinal stress, supporting their potential as GABA-producing probiotics (Ahn & Park, 2023). Beyond LAB, several *Bacillus* species (e.g., *B. subtilis, Priestia megaterium, B. cereus, B. coagulans*) also produce GABA, particularly under alkaline fermentation, offering additional routes for functional food development (Wan-Mohtar et al., 2020; Asun et al., 2022).

Thailand hosts a variety of GABA-rich fermented foods—fermented fish (pla-som), fermented shrimp (kung-som), fermented sausage, plant-based ferments, fermented fruit juices, and sweetened glutinous rice (khao-mak)—where microbial activity enhances GABA content (Tanamool et al., 2019; Sanchart et al., 2017; Kanklai et al., 2021; Pakdeeto et al., 2022). Khao-khab is a traditional Thai rice product from Uttaradit Province, consumed directly or used as a wrapper for vegetables and noodles (mee phan). It represents a culturally significant yet underexplored niche for sourcing functional LAB. Additionally, certain LAB strains exhibit strong antioxidant activity, mitigating oxidative stress damage and serving as potential starter cultures to enhance antioxidant properties in fermented foods (Yang et al., 2021a; Łepecka et al., 2023).

Despite growing interest in GABA-producing microorganisms, LAB from Thai khao-khab remain poorly characterized, particularly in terms of their combined antioxidant capacity, GABA production, and genomic attributes. To address this gap, we isolated and identified LAB from khao-khab, assessed their antioxidant activity using the DPPH assay, quantified GABA production, and performed whole-genome analysis of a high-performing *Lactiplantibacillus plantarum* strain. By integrating phenotypic and genomic data, this study provides a scientific foundation for selecting multifunctional probiotic candidates from traditional Thai fermented foods, supporting their potential application in functional foods and nutraceuticals.

Materials and methods

1. Isolation of LAB

Ten fermented rice flour samples were collected from the Laplae District, Uttaradit Province, for the isolation of LAB. For microbial isolation, 10 g of each sample were homogenized with 90 mL of sterile 0.85% NaCl solution to form a uniform suspension. Ten-fold serial dilutions were prepared using the same NaCl solution. To evaluate LAB presence, 1 mL aliquots from each dilution were pipetted into Petri dishes containing 20 mL of melted De Man, Rogosa, and Sharpe (MRS) agar (Difco, USA), supplemented with 0.3 g/100 mL $CaCO_3$. The plates were incubated at 30°C for 3 days. Colonies that exhibited clear zones were considered presumptive LAB and selected for further purification.

2. Identification of isolates

Phenotypic characterization, cell morphology including form, size, arrangement, and colony appearance, was examined from cultures grown on MRS agar after 3 days of incubation. Gas production from D-glucose, arginine hydrolysis, and nitrate reduction were evaluated using established protocols (Tanasupawat et al., 1992; Tanasupawat et al., 1998). All tests were conducted following 72-h incubation at 30°C. Growth at 45°C, under varying initial pH values (4.0, 4.5, 8.0, and 8.5),

and in different NaCl concentrations (4%, 6%, 8%, and 10% w/v) was assessed using MRS broth. Acid production from 0.5% (w/v) of various carbohydrates was determined as described by Tanasupawat et al. (1998).

3. 16S rRNA gene sequencing analysis

Genomic DNA was extracted from LAB isolates cultured in a half-strength MRS (MRSH) broth, using the method of Saito and Miura (1963). The 16S rRNA gene was amplified via polymerase chain reaction (PCR) using primers 20F and 1500R, following the protocol of Lane (1991). PCR conditions included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation (94°C for 1 min), annealing (50°C for 1 min), and extension (72°C for 2 min), with a final extension at 72°C for 3 min. Amplified products were sequenced by Macrogen®, Republic of Korea, using primers 20F (5′-GATTTTGATCCTGGCTCAG-3′) and 1500R (5′-GTTACCTTGTTACGACTT-3′) with Thermo Sequence Dye Terminator cycle sequencing (Amersham Bioscience, Uppsala, Sweden) according to the manufacturer's instructions. The resulting sequences (1307–1352 bp) were aligned with homologous sequences retrieved from BLASTn searches of the GenBank/EMBL/DDBJ databases using CLUSTAL_X (Thompson et al., 1997). Phylogenetic trees were constructed using the Neighbor-Joining method based on gad and gat gene sequences in MEGA version 11 (Tamura et al., 2021), with bootstrap analysis performed using 1,000 replicates (Felsenstein, 1985).

4. DPPH free radical scavenging activity

The antioxidant activity of LAB strains was assessed using the DPPH assay following Kim et al. (2020) with minor modifications. Cell-free supernatants (CFS) were mixed with a 0.05 mM DPPH solution in a 1:2 (v/v) ratio and incubated in the dark at room temperature for 30 min. A control was prepared by mixing ethanol with the DPPH solution. Absorbance was measured at 517 nm using a spectrophotometer (UV-1800, Shimadzu, Japan) and all samples were tested in triplicate. L-ascorbic acid (100 µg/mL) served as a positive control. Antioxidant activity was calculated using the following formula:

$$\text{Scavenging effect (\%)} = [(A_c - A_s)/A_c] \times 100$$

where A_c is the absorbance of the control and A_s is the absorbance of the test sample at 517 nm.

5. Screening of GABA thin layer chromatography (TLC)

LAB isolates were first cultured in MRS broth at 30°C for 24 h, then transferred to GYP broth supplemented with 1% monosodium glutamate (MSG) and incubated at 30°C for 72 h. Supernatants were obtained by centrifuging the culture broth at 10,000 rpm for 5 min. For qualitative GABA detection, supernatants were spotted onto thin-layer chromatography (TLC) plates (aluminum sheet silica gel 60 F254, Merck KGaA, Germany), following the method of Cho et al. (2011).

6. Quantitative analysis of GABA by spectrometry

GABA content was determined using the method of Zhang et al. (2014), with slight modifications. Cultures grown in GYP medium supplemented with 1% MSG were incubated at 30°C for 72 h and then centrifuged at 6,000×*g* (Avanti JXN-26, Beckman Coulter, USA). A 500 µL aliquot of the supernatant was collected and mixed with 200 µL of 0.2 M borate buffer (pH 9.0), 1 mL of 6% phenol, and 800 µL of 4% (w/v) sodium hypochlorite. The mixture was boiled for 10 min, then cooled in an ice bath for 20 min, and gently mixed until a blue color developed. Subsequently, 2 mL of 60% (v/v) ethanol was added, and absorbance was measured 645 nm using a spectrophotometer (U-1900, Hitachi, Japan). GABA concentration was calculated using a standard curve generated from GABA solutions at concentrations of 0, 4, 8, 16, 32, 64, 128, and 256 mg/L. The linear regression equation used was $y = 0.0015x - 0.0009$ ($R^2 = 0.999$).

7. Genome annotation and alignment

As previously described, bacterial strains were cultivated in MRS broth, and cells were harvested using a refrigerated centrifuge. Genomic DNA was extracted using the method of Phuengjayaem et al. (2020). Whole-genome sequencing of strain FL13-2 was performed using 2×250 bp paired-end reads on the Illumina MiSeq platform. Reads were assembled into contigs using SPAdes version 3.12 (Bankevich et al., 2012). The draft genome of strain FL13-2 has been deposited in GenBank under accession number JBOEFN000000000. Genome annotation was initially performed using the RAST server, with further refinement via the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Aziz et al., 2008). Comparative genomic analysis was conducted using the SEED Viewer (Aziz et al., 2012). Average Nucleotide Identity (ANI) values between FL13-2 and related type strains were calculated using the JSpeciesWS online tool. Genes involved in GABA biosynthesis, including *gadA, gadB* and *gadC*, were identified in the genome (Richter et al., 2016).

Results and discussion

1. Identification of isolates

Seven isolates (Group I) were identified as heterofermentative, while 3 isolates (Group II) were homofermentative. All isolates were Gram-positive, non-spore-forming, rod-shaped bacteria classified within the genus *Lactobacillus*. Following the taxonomic reclassification of *Lactobacillus* species by Zheng et al. (2020), many species have been reassigned to newly defined genera. Accordingly, Group I isolates were identified as *Limosilactobacillus fermentum,* whereas the Group II isolates were closely related to *Lactiplantibacillus plantarum* (subgroup IIA) and *Lactiplantibacillus pentosus* (subgroup IIB) (Table 1).

Table 1 Group, isolate number, DPPH radical scavenging capability (%), 16S rRNA gene similarity (%) and identification of isolates

Group	Isolate no.	DPPH Radical Scavenging Capability (%)	Accession no.	Similarity (%)	Identification
I	FL12-1	78.49±1.01*,**	AB985491	100	*L. fermentum*
	FL18-1	84.76±2.02*,**	AB980946	99.9	*L. fermentum*
	FL19-1S	82.18±2.72*,**	AB980948	99.9	*L. fermentum*
	FL23-1	82.50±3.29*,**	LC874650	99.4	*L. fermentum*
	FL24-1	88.04±2.01*,**	LC874651	99.8	*L. fermentum*
	FL25-1	91.18±3.95*,**	LC874652	99.8	*L. fermentum*
	FL26-1	83.30±5.27*,**	LC874653	99.4	*L. fermentum*
IIA	FL13-2	88.36±1.72*,**	LC874649	100	*L. plantarum*
	FL22-2	89.75±3.02*,**	AB980951	99.9	*L. plantarum*
IIB	FL17B	81.08±0.23*,**	AB980952	100	*L. pentosus*
	MRS broth	55.89±0.09			
	L-ascorbic acid (100 µg/mL)	38.51±1.47			

Remark: Data are represented as mean ± standard deviation (SD).
*Significant difference in the comparison between selected strains and sterile MRS broth (p<0.05).
**Significant difference in the comparison between selected strains and L-ascorbic acid (p<0.05).

L. fermentum, L. plantarum, and *L. pentosus* are commonly found in various Thai fermented foods, including sauerkraut, pickled bean sprouts, pickled spring onions, pickled mustard greens, fermented durian, fermented tea leaves, sweetened rice, fermented sausages, fermented fish, and soy sauce mash (Tanasupawat & Komagata, 1995; Tanasupawat et al., 1998; Tanasupawat et al., 2002). *L. plantarum* is also frequently isolated from dairy products and their environments, as well as from sauerkraut, pickled vegetables, sourdough, and the human oral cavity, intestinal tract, and stools (Hammes & Vogel, 1995).

Differentiating among members of the *L. plantarum* group—namely *L. plantarum*, *L. paraplantarum,* and *L. pentosus*—remains challenging. Corsetti et al. (2001) reported that both phenotypic tools (e.g., carbohydrate fermentation profiles) and genetic methods (e.g., 16S rRNA gene sequencing) were insufficient for clear-species level discrimination. However, Tanasupawat et al. (1992) suggested that acid production from glycerol, D-melezitose, and D-xylose could help distinguish *L. plantarum* from L. pentosus, despite their overall genetic similarity.

Based on the carbohydrate fermentation results summarized in Table 2, the Group II isolates in this study exhibited negative acid production from glycerol and D-xylose, consistent with characteristics of *L. plantarum*. However, DNA-DNA hybridization clearly differentiated these isolates from both *L. plantarum* and *L. pentosus.* Therefore, to accurately distinguish *L. paraplantarum* from *L. plantarum* and *L. pentosus,* further investigation using DNA-DNA hybridization, average nucleotide identity (ANI), and additional gene sequence analyses is warranted.

Table 2 Phenotypic characteristics of LAB isolates

Characteristics	Group I (7)	Group IIA (2)	Group IIB (1)
Growth at pH4.0	+	+	+
pH 4.5	+	+	+
pH 8.0	+ (-1)	+	+
pH 8.5	+ (-1)	+	-
Growth in 4% NaCl	+	+	+
6% NaCl	+	+	+
8% NaCl	-	+	+
Growth at 45°C	+	-	-
Arginine hydrolysis	+ (-3)	-	w
Gas from glucose	+	-	-
Acid from:			
D-Amygdalin	-	+	+
L-Arabinose	- (+2)	+	+
D-Cellobiose	-	+	+
D-Galactose	+ (-3)	+	+
Gluconate	w	+	+
Glycerol	-	w (-1)	-
Lactose	+ (-1)	+	+
D-Maltose	+	+	+
D-Mannitol	-	+	+
D-Mannose	- (+3)	+	+
D-Melibiose	+	+	+
D-Melezitose	-	+	-
α-Methyl-D-glucoside	- (+1)	-	-
Raffinose	- (+3)	+	+
D-Ribose	+ (-1)	+	+
Salicin	-	+	+
D-Sorbitol	-	+	+
Sucrose	+	+	+
D-Trehalose	+ (-2)	+	+
D-Xylose	-	-	-

Remark: +, positive; w, weak positive; -, negative reaction. Numbers in parentheses indicate the number of isolates showing the reaction.

2. In *Vitro* antioxidant ability of isolates

Oxidative stress arises when reactive oxygen species (ROS) exceed the body's antioxidant defenses, leading to cellular damage. External antioxidants can help restore redox balance, and various assays employing stable, non-biological radicals are commonly used to assess the free radical scavenging activity of probiotics (Lin & Yen, 1999). Lactic acid bacteria (LAB) are generally recognized as safe microorganisms that produce bioactive compounds, including organic acids and bacteriocins, with minimal side effects (Hamed, 2021). Upon adhering to the intestinal mucosa, both LAB cells and their metabolites contribute to ROS reduction, thereby maintaining redox homeostasis. Consequently, LAB are considered natural antioxidants with nutritional value, safety, and probiotic benefits (Tang et al., 2018).

This study evaluated the antioxidant activity of various LAB isolates using the DPPH radical scavenging assay—a widely accepted, rapid, and reproducible method suitable for high-throughput spectrophotometric screening. The assay detects hydrogen- and electron-donating mechanisms relevant to LAB-derived metabolites. While antioxidant capacity is multifaceted, future work will incorporate additional assays such as 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC).

Seven isolates in group I identified as *L. fermentum* exhibited DPPH scavenging activity ranging from 78.49±1.01% to 91.1891.18±3.95%. Isolates from group IIA (*L. plantarum*) and group IIB (*L. pentosus*) showed activities of 88.36±1.72% to 89.75±3.02%, and 81.08±0.23%, respectively. Among all tested strains, FL25-1 demonstrated the highest activity (91.18%), surpassing the ascorbic acid control (100 µg/mL, 38.51%) as shown in Table 1.

Probiotics are known to produce bioactive compounds with antioxidant properties, which may protect against oxidative stress through strain-specific molecular mechanisms (Kim et al., 2020). Increasing attention has been directed toward cell-free supernatants (CFS), which contain metabolites secreted by viable bacteria (Yang et al., 2021b). CFS are commonly used as in vitro models to evaluate functional effects (De Marco et al., 2018), although probiotics may confer broader health benefits through multiple mechanisms (Chan et al., 2021). Antioxidant components in LAB include exopolysaccharides, bioactive peptides, antioxidant enzymes, and manganese ions. Moreover, gut microflora can generate bioactive dietary antioxidants through enzymatic bioconversion (Davis & Milner, 2009).

3. Quantitative analysis of GABA production using spectrophotometry

This study demonstrated that *Lactiplantibacillus plantarum* strains FL13-2 and FL22-2 could produce GABA (Fig. 1) at concentrations of 2.43 and 2.64 g/L, respectively. These findings align with previous reports on *L. plantarum* K16, which exhibited strong GABA-producing potential and the ability to utilize agri-food by-products as valuable fermentation substrates (Guerrero et al., 2022). Such capabilities highlight promising applications in the development of functional foods or dietary supplements enriched with GABA.

Additionally, Jitpakdee et al. (2021, 2022) reported that *L. plantarum* SPS109 significantly reduced cholesterol levels and enhanced the production of beneficial compounds, including GABA and short-chain fatty acids. Furthermore, the use of starter cultures such as *Levilactobacillus brevis* TISTR 860 and *Lactiplantibacillus plantarum* TISTR 951 in rice slurry fermentation improved product quality and increased GABA content (Chittrakhani et al., 2022).

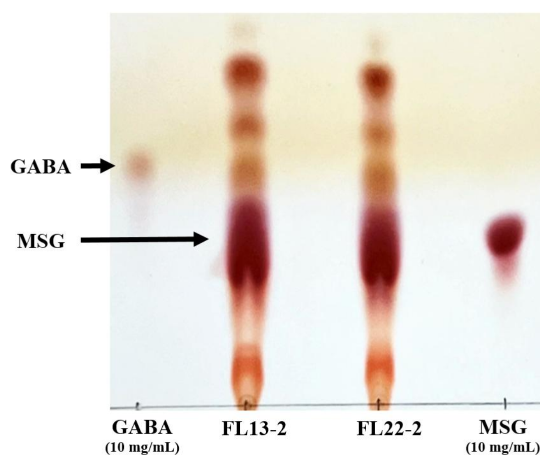


Fig. 1 Thin-layer chromatography (TLC) analysis of GABA production by *Lactiplantibacillus plantarum* strains FL13-2 and FL22-2. Lane 1: GABA standard (10 mg/mL); Lane 2, FL13-2; Lane 3, FL22-2; and Lane 4, monosodium glutamate (MSG) standard (10 mg/mL)

4. Genome analysis of isolate FL13-2

The draft genome of strain FL13-2 comprised 3,514,926 base pairs, with 3,596 coding sequences and a G+C content of 44.1 mol%. The strain exhibited 98.5% Average Nucleotide Identity (ANI) and 92.5% digital DNA–DNA hybridization (dDDH) with *Lactiplantibacillus*

plantarum DSM 20174T. Based on established species delineation thresholds (ANIb≥95% and dDDH≥70%) (Richter et al., 2016), strain FL13-2 was clearly identified as belonging to the same species. A phylogenomic tree reconstructed using the Type (Strain) Genome Server is shown in Fig. 2, and the genomic features of strain FL13-2 and related strains are summarized in Table 3.

Annotation of GABA-related genes revealed the presence of *gadB* (466 amino acids), as a GABA-synthesizing gene; *gatA* (585 amino acids), *gatB* (375 amino acids), *gatC* (312 amino acids), and *gatD* (138 amino acids) as GABA transporter genes; and *pdxK* (232 amino acids) as a gene involved in GABA cofactor biosynthesis. Safety assessment indicated a low pathogenicity score of 0.199, comparable to well-characterized non-pathogenic strains such as DSM 20174 and GPB 7-4, and similar to commercial probiotics, including 299V and LGG (Kingkaew et al., 2023a), supporting its suitability for probiotic applications.

Genomic analysis identified several genes (Fig. 3) typically associated with antimicrobial resistance—*vanH, vanT,* and *vanY*—which are intrinsic to LAB and function in peptidoglycan biosynthesis. Specifically, *vanY* encodes a D-Ala-D-Ala carboxypeptidase that remodels peptidoglycan precursors (Kingkaew et al., 2023b); *vanH* encodes a dehydrogenase converting pyruvate to D-lactate, providing the substrate for D-Ala-D-Lac depsipeptide synthesis (Stogios & Savchenko, 2020); and *vanT* encodes a serine/alanine racemase producing D-alanine, a key component of peptidoglycan (Hols et al., 1997). The *qacJ* gene, encoding a small multidrug resistance (SMR) efflux pump conferring resistance to quaternary ammonium compounds, is also commonly found in *Lactobacillus* species and is not considered a safety concern.

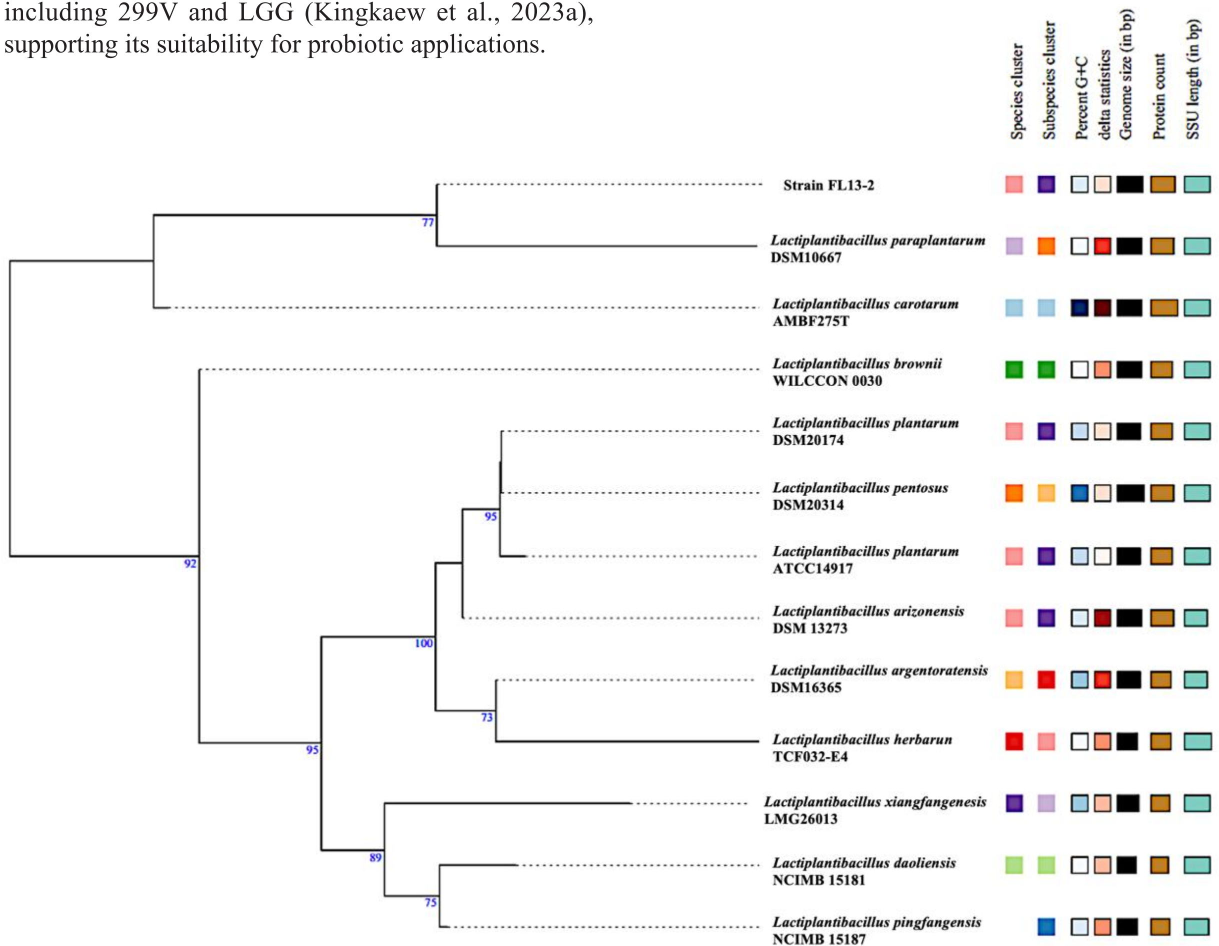


Fig. 2 Phylogenomic tree reconstructed using the Type (Strain) Genome Server based on draft genome sequences of strain FL13-2 and *Lactiplantibacillus* species. Numbers above the branches represent pseudo-bootstrap support values from 100 replications

Table 3 Genomic features of *L. plantarum* FL13-2, *L. plantarum* DSM 20174^{T} and *L. brevis* GPB7-4

Traits / Strains	FL13-2	DSM 20174^{T}	*L. brevis* GPB7-4
Accession number	JBOEFN000000000	ACGZ00000000	JAESIZ000000000
Genome size (bp)	3,514,926	3,242,936	2,320,751
G+C content (mol%)	44.1	44.5	45.9
Genome coverage	345x	Not reported	Not reported
N50	91,661	1	226,033
L50	10	Not reported	4
Number of contigs (with PEGs)	205	1	48
Number of subsystems	235	225	213
Number of coding sequences	3,596	3,145	2,309
Number of RNAs	68	84	57
GABA synthesis genes	*gadB*	*gadB*	*gadB*
GABA transporter genes	*gatA* *gatB_1* *gatB_2* *gatC_1* *gatC_2* *gatD*	*gatA* *gatB* *gatC*	*gatA* *gatB* *gatC* *gatD*
GABA cofactor gene	PDXK		PDXK
Probability of being a human pathogen	0.199	0.19	0.186
Input proteome coverage (%)	0.7	0.43	12.04
Matched pathogenic families	0	0	0
Non-matched pathogenic families	23	13	270
Conclusion	Non-human pathogen	Non-human pathogen	Non-human pathogen
CARD (via Proksee)	*vanY* *vanT* *vanH*	*vanY*	*vanT*
Perfect hits to ARGs	0	0	0
Strict hits to ARGs	2 *vanY* *qacJ*	1 *vanY*	1 *vanT*
Loose hits to ARGs	0	0	0
ResFinder result	No resistance	No resistance	No resistance
Putative bacteriocin genes	Enterocin_X_chain_beta Plantaricin_F Plantaricin_E	Plantaricin_J	Not found

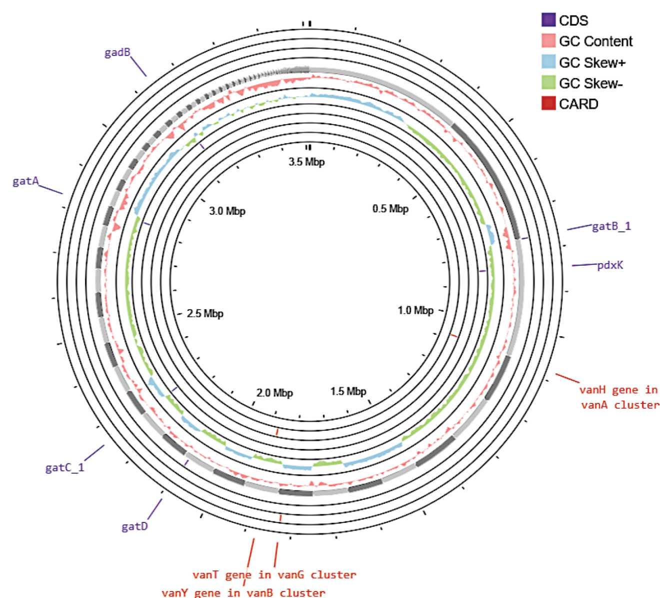
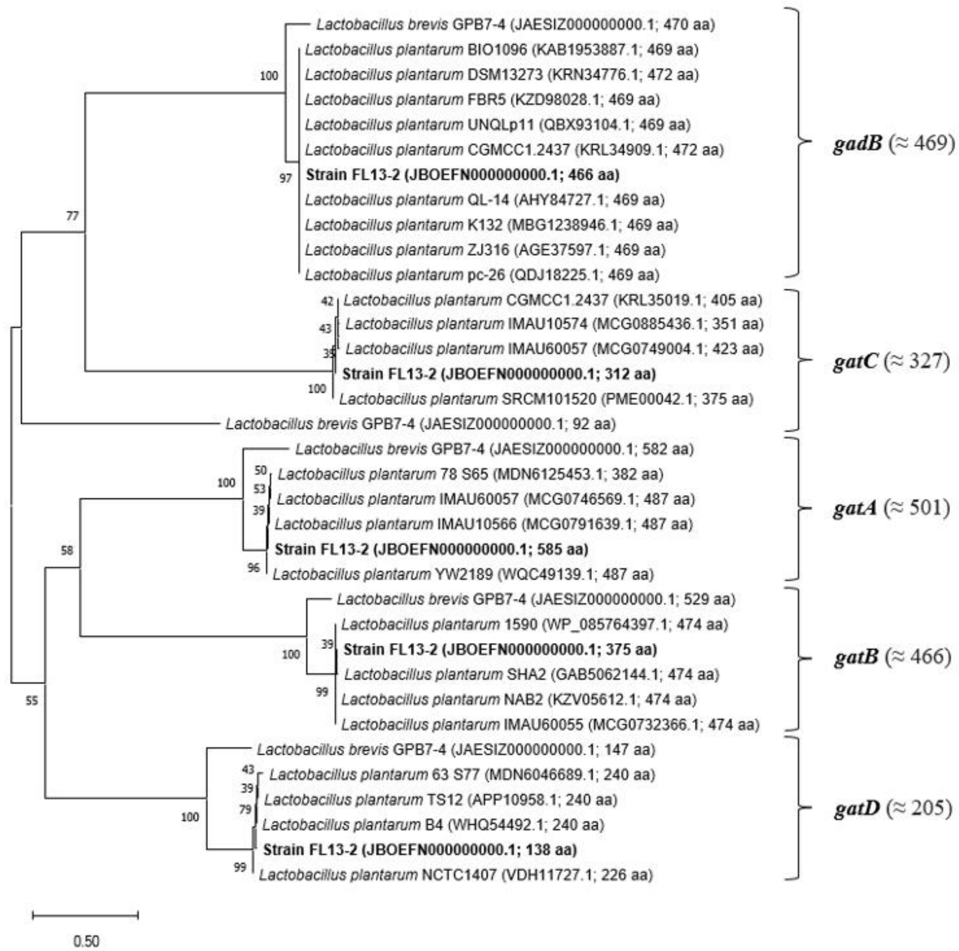


Fig. 3 Circular genomic map of strain FL13-2 highlighting GABA-related genes (*gadB, gatA, gatB, gatC, gatD, pdxK*) and antimicrobial resistance genes (*vanH, vanT, vanY*). Genomic features are color-coded as follows: open reading frames (ORFs) (blue); GC skew (+) (green); GC skew (-) (yellow); and GC content (pink)

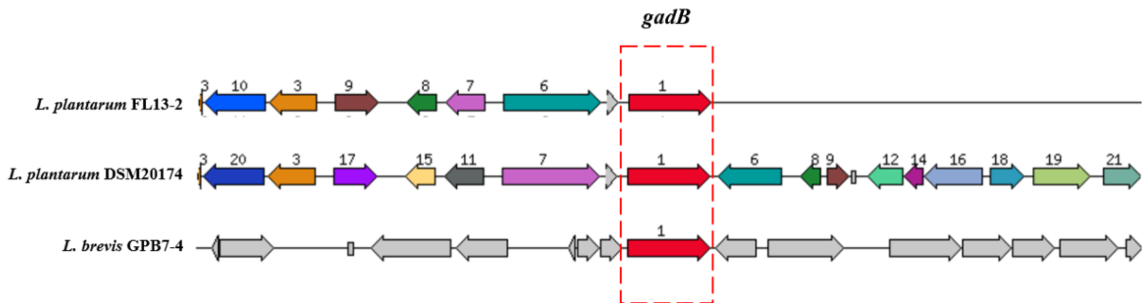
The hemolysin III (*hlyIII*) gene was also detected in FL13-2; however, this gene is widely present in commercial probiotic strains—including *L. plantarum* WCFS1, JDM1, and 299V, as well as *L. rhamnosus* GG—and has been shown to be non-lytic in LAB, with no association to pathogenicity. Its presence is considered intrinsic and not indicative of a safety risk (Kingkaew et al., 2025). Collectively, the identified genes reflect species-specific metabolic and structural functions rather than acquired resistance or virulence traits. In silico screening using VirulenceFinder confirmed the absence of major virulence determinants, including genes encoding enterococcal surface protein (*esp*), collagen adhesion (*ace*), serine protease (*sprE*), gelatinase (*gelE*), and cytolysin (*cylL*).

Several bacteriocin-encoding genes were also identified, including Enterocin X chain β, Plantaricin F, and Plantaricin E. These genes are known to encode antimicrobial peptides that may inhibit the growth of competing or potentially harmful microorganisms. Their presence suggests that the strain possesses natural antimicrobial capabilities, supporting its potential as a probiotic with competitive exclusion properties. However, further studies are required to evaluate antimicrobial activity.

Phylogenetic analysis of GABA-related genes using a neighbor-joining tree with 1,000 bootstrap replicates showed that *gadB* shared amino acid sequence similarities with other LAB strains ranging from 81.5% to 100%. Transporter genes *gatA, gatB, gatC* and *gatD* exhibited sequence similarities of 66.39%–100%, 75.17%–99.78%, 99.09%–100% (within the same species), and 54.18%–99.11%, respectively (Fig. 4A). Moreover, *gadB* was located within a conserved GABA biosynthetic gene cluster in *L. plantarum* FL13-2 and related strains (Fig. 4B).



(a)



(b)

Fig. 4 (a) Neighbor-joining phylogenetic tree based on amino acid sequences of GABA-related genes (*gadB, gatA, gatB, gatC, gatD*) of *L. plantarum* FL13-2 and related strains. Bootstrap support values (1,000 replicates) are shown at branch nodes. GenBank accession numbers and gene lengths are provided in parentheses for each strain. (b) Comparative analysis of GABA-synthesizing gene (*gadB*) in *L. plantarum* FL3-2 and related strains

The *gad* genes encode components of the glutamate decarboxylase (GAD) system, which plays a critical role in maintaining metabolic activity under acidic conditions. When intracellular pH decreases, glutamate (Glu) becomes predominantly non-charged due to protonation of its γ-carboxyl group, facilitating decarboxylation—a proton-consuming reaction that produces GABA and increases cytoplasmic pH. Similarly, low extracellular pH can reduce intracellular pH via activation of the Glu/GABA antiporter system. Under these conditions, protonated glutamate is converted into GABA, which is then exported, helping alleviate acid stress. *GadC* specifically mediates the exchange of intracellular GABA with extracellular glutamate, enabling continuous proton-consuming activity and pH homeostasis.

In addition to synthesis, *gad* genes are involved in GABA transport. Several transporters have been identified, including four GABA transporters (GAT_1, GAT_2, GAT_3, and BGT_1) and 5 glutamate transporters ($EAAT_1$–$EAAT_5$). These transporters regulate extracellular GABA and excitatory amino acid concentrations and supply amino acids essential for cellular metabolism (Phuengjayaem et al., 2023).

Conclusion

This study investigated lactic acid bacteria (LAB) isolated from the traditional fermented rice product khao-khab, focusing on their taxonomy, antioxidant potential, γ-aminobutyric acid (GABA) production, and genomic characteristics. Ten isolates were identified and classified into heterofermentative *L. fermentum* (group I) and homofermentative *L. plantarum* and *L. pentosus* (group II). Although carbohydrate fermentation profiles suggested similarity to *L. plantarum,* DNA–DNA hybridization revealed genetic distinctions, underscoring the need for advanced molecular approaches to differentiate closely related taxa within the *L. plantarum* group.

Functionally, all isolates exhibited strong *in vitro* antioxidant activity (78.49±1.01% to 91.18±3.95% DPPH scavenging), with strain FL25-1 exceeding the activity of ascorbic acid, confirming the antioxidant potential of these LAB strains. Strains FL13-2 and FL22-2 produced high GABA levels (2.43–2.64 g/L), reinforcing their value for functional food development. Whole-genome sequencing of FL13-2 confirmed its identity as *L. plantarum* and revealed a complete, conserved GABA biosynthetic and transport gene cluster, along with bacteriocin-encoding genes and intrinsic antimicrobial resistance genes associated with essential cell wall synthesis rather than pathogenicity.

The integration of traditional fermented food microbiology with genomic analysis in this study highlights multifunctional probiotic candidates that combine antioxidant activity, high GABA production, and antimicrobial potential. These findings provide a strong scientific foundation for the development of *L. plantarum* FL13-2 and related isolates as safe, multifunctional probiotics in functional foods and nutraceuticals. However, further *in vivo* and clinical studies are warranted to confirm their safety, health benefits, and technological performance in real food systems.

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