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Research article

Genomic comparison of gamma-aminobutyric acid-producing *Levilactobacillus brevis* and *Companilactobacillus zhachilii* strains from Thai fermented foods

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Abstract

Importance of the work: The GABA-producing *Companilactobacillus zhachilii* isolated from fermented pork (*'nham'*) demonstrates both efficacy and safety in GABA production.

Objectives: To identify the GABA-producing strains ASK-1 and ANB-1 to compare their genomes with those of *Levilactobacillus brevis* and *Companilactobacillus zhachilii* strains.

Materials and Methods: Two fermented food samples were collected and used for the isolation of lactic acid bacteria. The strains obtained were evaluated for GABA production. The selected strains were identified based on their phenotypic and genotypic characteristics. Genomic comparisons of the GABA-producing strains were performed using genome sequencing, assembly, annotation and comparative analysis. This analysis included the examination of CRISPR regions, antimicrobial genes, functional attributes and genome visualization.

Results: The GABA-producing lactic acid bacteria (ASK-1 and ANB-1) were isolated from traditional Thai fermented foods ('s*ai-krog-prieo*' and '*nham*'). The strains ASK-1 and ANB-1 produced 9.12 ± 0.51 g/L GABA and 12.73 ± 0.57 g/L GABA, respectively. On the basis of phenotypic characteristics and 16S rRNA gene sequencing, strain ASK-1 presented 100% sequence similarity to *Levilactobacillus brevis* ATCC 14687^T, whereas ANB-1 presented 99.93% similarity to *Companilactobacillus zhachilii* HBUAS52074^T. Genome analysis identified ASK-1 as *L. brevis* ATCC 367, with an average nucleotide identity based on a BLAST (ANIb) value of 98.86% and ANB-1 with an ANIb value of 96.92%, to *C. zhachilii* HBUAS52074^T. Additionally, genomic studies revealed GABA-producing genes (*gadA* and *gadB*) via the KofamKOALA-KEGG Orthology database.

Main finding: *Levilactobacillus brevis* ASK-1 and *Companilactobacillus zhachilii* ANB-1 efficiently and safely produced GABA and exhibited probiotic properties, making them promising starter cultures for functional food production in both local food and industrial fermentation processes.

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Introduction

γ-Aminobutyric acid (GABA), or 4-aminobutanoate, is a naturally occurring, nonproteinogenic amino acid synthesized by most living organisms; specifically, it serves as the primary inhibitory neurotransmitter in the mammalian central nervous system (Walls et al., 2015) and provides various health benefits, including anxiety reduction, antidepressant effects (Chih et al., 2013), cholesterol-lowering properties (Xie et al., 2014; Kingkaew, 2023), improved sleep quality, diabetes management, obesity control and hormonal regulation. Additionally, GABA is associated with the development and prevention of neurological disorders, such as schizophrenia, Alzheimer's disease and Parkinson's disease, as well as antihypertensive effects (Özdemir, 2021; Salminen et al., 2021).

GABA-producing lactic acid bacteria (LAB) have gained considerable attention in the food industry because of their Generally Recognized as Safe (GRAS) status and fermentation applications (Icer et al., 2024). LAB metabolites, including GABA, are increasingly marketed as postbiotics (Hebert et al., 2015; Kaur et al., 2023). Following the reclassification of *Lactobacillus* species by Zheng et al. (2020), many LAB species have been revised.

In Thailand, LAB play a vital role in the fermentation of traditional foods. The Thai fermented pork sausage ('*sai-krog-prieo'*) contains species such as *Lactiplantibacillus pentosus, L. plantarum, L. brevis, Limosilactobacillus fermentum, Pediococcus acidilactici, P. pentosaceus, Enterococcus hirae, Weissella confusa, W. cibaria, W. kimchi* and *Companilactobacillus farciminis*. Fermented pork ('*nham'*) includes *L. pentosus, L. plantarum, Latilactobacillus sakei, P. acidilactici, P. pentosaceus, Leuconostoc* spp., *L. fermentum* and *Lactococcus lactis* (Sriphochanart and Skolpap, 2010; Ratanaburee et al., 2013).

Several LAB strains isolated from fermented meats have produced GABA. For example, *Levilactobacillus namurensis, L. plantarum* and *P. pentosaceus* were obtained from *nham* (Krongkeha, 2022) and *L. plantarum* was obtained from Vietnamese fermented meat ('*nem chua'*), according to Duyen et al. (2022). Other GABA-producing strains, such as *L. plantarum, P. acidilactici, W. hellenica, L. pentosus* and *Latilactobacillus sakei*, have been identified in Turkish fermented sausage ('*sucuk'*), according to Kamiloğlu et al. (2022). Additionally, GABA-producing LAB have been reported across diverse sources (Cui et al., 2020; Icer et al., 2024).

Currently, whole-genome approaches offer rapid, cost-effective biological insights, facilitating pan-genomic analysis, reference construction and future cross-species refinements (Rungsirivanich et al., 2025)

This study aimed to identify and compare the genomes of selected GABA-producing LAB strains. Specifically, the *gad* genes of the strains ASK-1, ANB-1 and HBUAS52074^T (NCBI database at https://www.ncbi.nlm.nih.gov/) were analyzed and characterized.

Materials and Methods

Isolation and screening of γ-aminobutyric acid-producing strains

Two samples were collected from fermented foods in Bangkok, Thailand. LAB were isolated using an enrichment approach. Samples (each 5 g) were enriched in 45 mL of Man Rogosa Sharpe (MRS broth; 1% proteose peptone, 1% beef extract, 0.5% yeast extract, 2% dextrose, 0.1% polysorbate 80, 0.2% ammonium citrate, 0.5% sodium acetate, 0.01% $MgSO_4.7H_2O$, 0.005% $MnSO_4.4H_2O$, 0.2% K_2HPO_4, pH 6.5; Difco; USA in 100 mL Duran flasks and incubated at 30°C for 24 hr. The cultures were obtained via the streak plate technique on MRS agar supplemented with 0.3% $CaCO_3$ and incubated at 30°C for 48 hr. Colonies surrounded by a clear zone were picked and streaked on new MRS agar plates for purification. Pure cultures were maintained with 20% glycerol at -80°C or a lyophilization technique for further study (Phuengjayaem et al., 2021).

Active cultures were prepared by transferring the pure culture from glycerol stock into 5 ml MRS broth and incubating at 30°C for 24 hr. Then, each inoculum (10% volume per volume, v/v) was transferred to 10 mL of glucose-yeast extract-peptone (GYP broth; 1% glucose, 1% yeast extract, 0.5% polypeptone, 0.2% sodium acetate, 20 parts per million, ppm, $MgSO_4.7H_2O$, 1 ppm $MnSO_4.4H_2O$, 1 ppm $FeSO_4.7H_2O$ and 1 ppm NaCl, pH 6.8) supplemented with 1% monosodium glutamate (MSG) and cultivated at 30°C for 72 hr. The culture broth was centrifuged at 10,000 revolutions per minute (rpm) for 5 min and 2 µL aliquots of the supernatant were spotted onto thin-layer chromatography (TLC) plates (aluminum sheet silica gel 60 F254; Merck; Germany). Qualitative GABA analysis was based on TLC and followed the method described by Phuengjayaem et al. (2023).

Quantitative analysis of γ-aminobutyric acid using high-performance liquid chromatography

To assess GABA synthesis efficiency, each inoculum (10% v/v) from an 18-hr-old culture was transferred to 10 mL of GYP broth supplemented with (3% w/v) MSG and incubated at 30°C for 72 hr. The standard and samples were centrifuged at 8,000 rpm for 15 min. The supernatant was derivatized and analyzed for GABA concentration using high-performance liquid chromatography (HPLC), following the derivatization method and mobile phase preparation described by Silva et al. (2009).

The HPLC system included a Varian Prostar pump (Model 210; Granite Quarry, NC, USA) and a fluorescence detector (FLD; Model 363; USA) set at an excitation wavelength of 337 nm and an emission wavelength of 454 nm. A C18 analytical column (150 mm × 4.6 mm internal diameter, 3 µm particle size, Hibar-Futigsanle RT; Germany) was used for separation. GABA production experiments were conducted in triplicate. Statistical analyses were performed using the SPSS version 18.0 software (IBM Corporation; USA). Variance analysis was carried out using analysis of variance and significant differences between mean values ($p < 0.05$) were determined based on Duncan's multiple range test.

Identification of strains

Phenotypic characterization

The selected strains were evaluated using Gram staining, cell morphology observation, and analysis of colony appearance. These evaluations were conducted using cells grown on MRS agar plates incubated at 30°C for 24 hr. Biochemical characteristics were assessed by evaluating growth at temperatures of 40–45°C, tolerance to NaCl concentrations of 6%, 8%, 9% and 10% (w/v) and growth at pH values of 3.5, 8.5, 9.0 and 9.6. Additional biochemical tests consisted of catalase activity and carbohydrate fermentation patterns, as described by Phuengjayaem et al. (2021).

Genotypic characterization

DNA extraction and purification were performed according to the protocol of Saito and Miura (1963). The 16S rRNA gene sequences were amplified using PCR via the primers 20F (5′-AGTTTGATCCTGGCTC-3′) and 1530R (5′-AAGGAGGTGATCCAGCC-3′). The amplified PCR products were sequenced using a DNA sequencer (Macrogen; South Korea) with universal primers, consisting of 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′).

Sequence similarity values for strains ASK-1 (1,566 bp) and ANB-1 (1,567 bp) were calculated via the EzBioCloud platform (Yoon et al., 2017) and analyzed using BLAST software from GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment and editing were conducted using BioEdit version 7.2 (https://bioedit.software.informer.com/7.2/). A phylogenetic tree was constructed using the neighbor-joining method in the MEGA version 11 software (Tamura et al., 2021), with bootstrap analysis performed with 1,000 replications to assess the confidence values of individual branches (Felsenstein, 1985). The identified sequences were deposited in the DNA Data Bank of Japan (DDBJ) database.

Genome annotation alignment and comparative analysis

The bacterial strains were cultivated in MRS broth as previously described and the cells were harvested via refrigerated centrifugation at 10,000×*g* using a SIGMA 2K15 centrifuge (Germany). The genomic DNA was extracted following the protocol outlined by Phuengjayaem et al. (2020). The genome sequences of strains ASK-1 and ANB-1 were generated using the Illumina MiSeq platform (Illumina, Inc.; CA, USA) with 2 × 250 bp paired-end reads. The reads were assembled into contigs using the SPAdes version 3.12 software (Bankevich et al., 2012). The draft genome sequences of strains ASK-1 and ANB-1 are publicly available in GenBank under accession numbers JAJAWJ000000000 and JAESIV000000000, respectively.

Sequence similarity values for the strains ASK-1 (genome size: 2,343,673 bp) and ANB-1 (genome size: 2,748,085 bp), as well as their related reference strains, were computed using the EzBioCloud tool (Yoon et al., 2017). The average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values were analyzed using the JSpeciesWS web server tool (Richter et al., 2016). ANI and dDDH values >95% and >70%, respectively, indicate the same species (Kim et al., 2014; Lee et al., 2016).

Genome annotation was performed via the RAST server (Aziz et al., 2008) and the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) at https://www.ncbi.nlm.nih.gov/refseq/annotation_prok/. Comparative genomic analyses were conducted on the SEED Viewer platform (Aziz et al., 2012). Alignments were performed using the CLUSTAL X program available in the NCBI BLAST suite (Thompson et al., 1997).

The pan-genome was constructed using the OrthoVenn3 server (Sun et al., 2023). Genes related to GABA production pathways were predicted through the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.kegg.jp; Kanehisa et al., 2016) and the KofamKOALA database (https://www.genome.jp/tools/kofamkoala/; Aramaki et al., 2020) using default parameters and all identified hits.

The web-based bioinformatics platform of the Center for Genomic Epidemiology (CGE) tools was utilized to predict plasmid, pathogenicity and virulence genes (Cosentino et al., 2013; Carattoli et al., 2014; Joensen et al., 2014; Malberg et al., 2020). Antibiotic resistance genes were identified using the Comprehensive Antibiotic Resistance Database (CARD: https://card.mcmaster.ca/; Alcock et al., 2020). The presence of CRISPR regions in the genomes was determined using the DFAST server (https://dfast.ddbj.nig.ac.jp/; Tanizawa et al., 2018). Biosynthesis-related gene clusters for antimicrobial peptides were analyzed using BAGEL4 (Bacteriocin Genome Mining Tool; http://bagel4.molgenrug.nl/; van Heel et al., 2018).

Results and Discussion

Isolation and screening of γ-aminobutyric acid-producing strains

From the two samples collected in Bangkok, strain ASK-1 was isolated from fermented pork sausage (*sai-krog-prieo*) and strain ANB-1 was isolated from fermented pork (*nham*). Both demonstrated the ability to produce GABA, as confirmed through qualitative thin-layer chromatography (TLC). GABA production was identified by comparing the spots on the TLC plates to those of standard GABA and MSG. Among the tested strains, ANB-1 and ASK-1 produced the most prominent GABA spots on the TLC plates (Fig. 1). These findings were consistent with other studies (Lee and Kim, 2023; Chen et al., 2024b).

The GABA production of the two selected strains was further quantified using HPLC. The GABA standard curves revealed a strong linear relationship (coefficient of determination (R^2) = 0.9811) between peak areas and GABA concentrations in the range 2–64 mg/L. HPLC chromatograms were obtained for both the GABA standard solution and the GABA produced based on the decarboxylation reactions of the tested strains.

In triplicate experiments, strain ANB-1 produced 12.73 ± 0.57 g/L GABA, whereas strain ASK-1 produced 9.12 ± 0.51 g/L GABA. Both strains had significant potential for GABA production and were selected for further study as potential starter cultures for the food industry.

In addition, other species within the genus *Lactobacillus* have been reported to produce high concentrations of GABA. For example, *L. rhamnosus* GG, *Lactococcus lactis* subsp. *lactis* from fermented adzuki bean milk and *P. pentosaceus* NH102 and HN8 from fermented pork (*nham*) have been reported to produce GABA at concentrations of 1.13, 3.68, 8.39 and 9.06 g/L, respectively (Ratanaburee et al., 2013; Song and Yu, 2018). In comparison, *L. brevis* isolated from kimchi and pickled Chinese vegetables produced GABA at concentrations in the range 19.07–61 g/L (Zhang et al., 2012; Wu and Shah, 2015). Additionally, *L. plantarum* NDC75017 from a traditional fermented dairy product produced 3,145.6 mg/kg GABA on the basis of the substrate (Shan et al., 2015).

Identification of strains

Two rod-shaped strains (ASK-1 and ANB-1) were identified as Gram-positive, catalase-negative bacteria. The colonies on MRS agar plates were circular, had a low degree of convergence and presented entire margins. On the basis of their phenotypic and biochemical characteristics, both strains grew optimally at 30°C. Phylogenetic analysis, based on 16S rRNA gene sequence similarity, placed both strains in a monophyletic cluster within the genera *Levilactobacillus* and *Companilactobacillus,* respectively (Fig. 2).

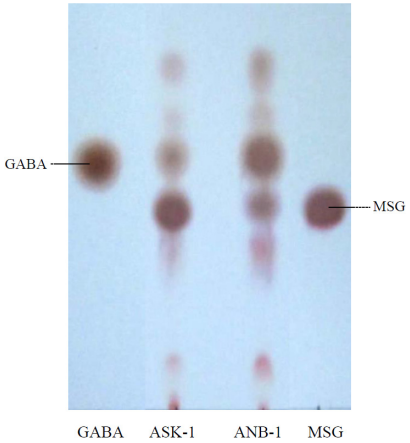

Fig. 1 Chromatogram of γ-aminobutyric acid (GABA) production by representative isolates using thin-layer chromatography, where Lane 1 = GABA standard (10 mg/mL); Lane 2 = strain ASK-1; Lane 3 = strain ANB-1; and Lane 4 = monosodium glutamate (MSG) standard (10 mg/mL).

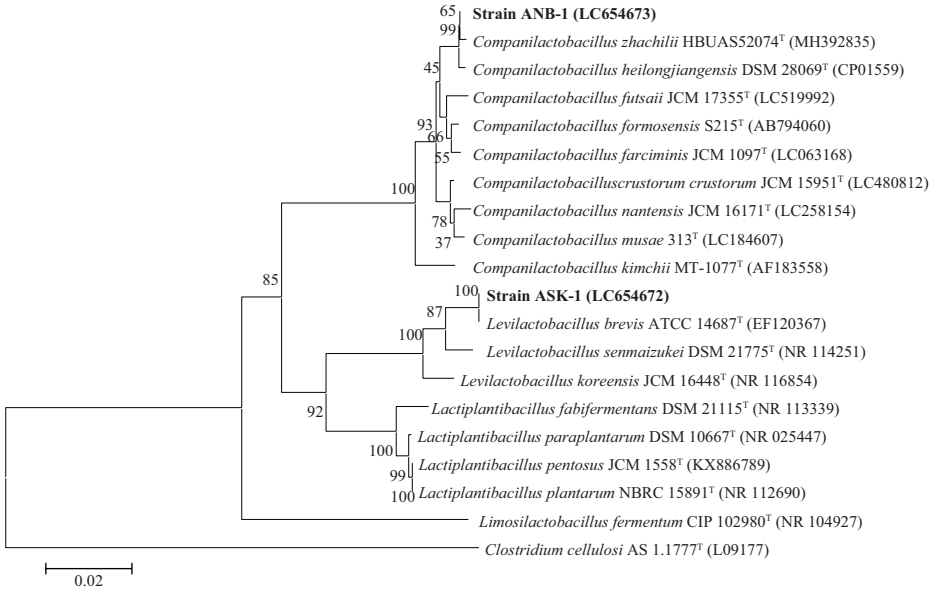

Fig. 2 Neighbor-joining tree based on 16S rRNA gene sequences of strains ASK-1 and ANB-1 (in bold), showing the relationships among *Lactobacillus* and related species, where numbers on branches represent percentage of bootstrap values from 1,000 replicates, *Clostridium cellulosi* AS 1.1777^T was the outgroup and scale bar represents 0.02 substitutions per nucleotide position.

Strain ASK-1 (LC654672) was able to grow at pH 8.5, 45°C and in 6% and 8% NaCl and produced acid from D-maltose and D-xylose. The differential characteristics of strain ASK-1 compared with those of related *Lactobacillus* species are summarized in Table 1. These phenotypic and biochemical characteristics are consistent with those reported elsewhere (Ehrmann et al., 2010; Bui et al., 2011; Chao et al., 2012; Yi et al., 2013). The 16S rRNA gene sequence of strain ASK-1 (1,566 bp) had 100% similarity with that of *Levilactobacillus brevis* ATCC 14687^T (Fig. 2). Therefore, strain ASK-1 was identified as *Levilactobacillus brevis*.

Table 1 Differential characteristics between strains ASK-1 and ANB-1 and related type strains: strain 1, ASK-1 (data from this study); strain 2, *L. brevis* LMG 6906^T; strain 3, ANB-1 (data from this study); strain 4, *C. zhachilii* HBUAS52074^T; strain 5, *L. senmaizukei* NBRC 103853^T; strain 6, *L. koreensis* DCY50^T; strain 7, *C. heilongjiangensis* DSM 28069^T; strain 8, *C. farciminis* KCTC 3681^T; and strain 9, *C. futsaii* JCM 17355^T.

Characteristic	1	2[a]	3	4[b]	5[c]	6[d]	7[e]	8[f]	9[g]
Isolation source	Sai-krog-prieo	Human feces	Nham	Zha-Chili	Japanese pickle	Cabbage kimchi	Pickle	Sausage	Fermented mustard
Growth in 10% NaCl	-	-	+	w	-	+	w	+	w
Acid production from:									
D-Cellobiose	w	-	w	+	+	-	+	+	+
Lactose	+	w	w	+	-	-	+	+	+
D-maltose	+	+	w	+	-	+	+	+	w
D-mannose	w	-	w	+	+	-	ND	+	+
D-melibiose	+	+	-	-	-	+	-	-	-
D-melezitose	-	-	ND	+	-	ND	-	+	-
Sucrose	w	-	-	-	+	-	+	+	+
D-xylose	w	+	-	-	-	+	ND	-	-
DNA G+C content (mol%)	45.9	46	36.3	36.3	46	49	37.5	36.4	36.3

+ = positive; w = weakly positive; - = negative reaction; ND = no data available.

Data from a, Bui et al. (2011), Ehrmann et al. (2010), Chao et al. (2012), Yi et al. (2013); b, Zhang et al. (2019); c, Bui et al. (2011), Yi et al. (2013); d, Bui et al. (2011), Yi et al. (2013); e, Gu et al. (2013), Chen et al. (2017); f, Bui et al. (2011), Heo et al. (2018); g, Chao et al. (2012), Heo et al. (2018).

Strain ANB-1 (LC654673) grew at pH 9, 40°C and 10% NaCl. It produced acid from D-cellobiose, lactose, D-maltose, D-mannose and D-melezitose. The differential characteristics of strain ANB-1 compared with related species are shown in Table 1. These phenotypic and biochemical characteristics were consistent with those reported elsewhere (Zhang et al., 2019). The 16S rRNA gene sequence of strain ANB-1 (1,567 bp) had 99.94% similarity with that of *Companilactobacillus zhachilii* HBUAS52074^T (Fig. 2). Thus, strain ANB-1 was identified as *Companilactobacillus zhachilii*.

Genome annotation and alignment

The draft genomes of strains ASK-1 and ANB-1, which are publicly available in GenBank, were annotated and analyzed. Comparative analysis between strains ASK-1 and *Levilactobacillus brevis* ATCC 367 revealed ANI (ANIb and ANIm) values of 98.86% and 99.23%, respectively, and a dDDH value of 93.5%. These results confirmed the identification of strain ASK-1 as *L. brevis* on the basis of species-level thresholds for dDDH (≥70%), according to Auch et al. (2010) and ANI (≥95%, according to Kim et al. (2014) and Chun et al. (2018). Similarly, strain ANB-1 presented ANI values (ANIb and ANIm) of 96.92% and 97.92%, respectively, and a dDDH value of 78.7% with *Companilactobacillus zhachilii* HBUAS52074^T, confirming its identification as *C. zhachilii*.

Genome assembly statistics are summarized in Table 2, comparing *Levilactobacillus brevis* ASK-1, *L. brevis* ATCC 367, *L. brevis* BDGP6, *Companilactobacillus zhachilii* ANB-1 and *C. zhachilii* HBUAS52074^T (obtained from NCBI). GABA production by the two strains was quantitatively analyzed, with *C. zhachilii* ANB-1 having significantly higher levels of GABA than *L. brevis* ASK-1. Genome analysis confirmed the presence of the *gadA* and *gadB* genes in both strains, which were identified via the KofamKOALA-KEGG Orthology database. KofamKOALA, a web server for assigning KEGG orthologs (KOs) to protein sequences, uses profile hidden Markov models (KOfam) with adaptive score thresholds for accurate and efficient KO assignments (Aramaki et al., 2020; Zhang et al., 2023; Chen et al., 2024a).

Table 2 Genome characteristics of *Levilactobacillus brevis* ASK-1, *L. brevis* ATCC 367, *L. brevis* BDGP6, *Companilactobacillus zhachilii* ANB-1 and *C. zhachilii* HBUAS52074^T (obtained from NCBI). γ-aminobutyric acid predictions, antibiotic resistance genes, plasmid, pathogenicity and virulence genes, production of antimicrobial peptide gene clusters and genome analysis of presence of CRISPR region.

Attribute/Strain	ASK-1	ATCC 367	BDGP6	ANB-1	HBUAS52074^T
Accession no.	JAJAW000000000	GCA_000014465.1	GCA_002762175.1	JAESIV000000000	GCA_003606365.2
Genome size (bp)	2,343,673	2,340,228	2,785,111	2,748,085	2,714,973
Plasmids	2(rep28, 98, 100% identity)	0	0	1(rep28, 98.65% identity)	0
G+C content (mol%)	45.9	46.1	45.6	36.3	36.3
Genome coverage	1000x	no data	70x	225x	100x
N50	117,944	2,291,220	2,785,111	800,126	2,714,973
L50	6	1	1	2	1
Number of contigs (with PEGs)	73	3	1	36	1
No. of subsystems	318	314	300	308	295
No. of coding sequences	2,275	2,286	2,755	2,473	2,496
No. of RNAs	59	80	91	53	68
GAD gene analysis	*gad A, B*	*gad A, B*	*gad A, B*	*gad A, B*	*gad A, B*
Probability of being a human pathogen	0.186	0.189	0.179	0.207	0.182
Input proteome coverage (%)	10.63	19.54	3.57	0.28	0.24
Matched pathogenic families	0	0	0	0	0
Matched non-pathogenic families	242	446	100	7	6
Conclusion	Non-human pathogen	Non-human pathogen	Non-human pathogen	Non-human pathogen	Non-human pathogen
Virulence gene	0	0	0	0	0
Antibiotic resistance gene (ARGs)					
CARD:					
Number of perfect hits	0	0	0	0	0
Number of strict hits	1	2	2	5	5
Number of loose hits	156	157	165	174	171
ResFinder	No resistance	No resistance	No resistance	No resistance	No resistance
Putative bacteriocin genes	0	0	0	Carnobacteriocin-B2	Carnobacteriocin-B2
Number of CRISPRs	5	3	1	2	1

Pan-genome analysis using the OrthoVenn3 server identified orthologous clusters of predicted proteins among *C. zhachilii* ANB-1, *C. zhachilii* HBUAS52074^{T}, *L. brevis* ASK-1, *L. brevis* ATCC 367 and *L. brevis* BDGP6. The distribution of orthologous clusters and a summary of detected proteins are illustrated in Fig. 3.

Analysis using VirulenceFinder, PlasmidFinder, ResFinder, PathogenFinder and CARD revealed that *C. zhachilii* ANB-1, *C. zhachilii* HBUAS52074^{T}, *L. brevis* ASK-1, *L. brevis* ATCC 367 and *L. brevis* BDGP6 strains did not harbor virulence genes but did contain antibiotic resistance genes. PathogenFinder analysis indicated low probabilities of 0.186 for *L. brevis* ASK-1 and 0.207 for *C. zhachilii* ANB-1 for being classified as human pathogens (Table 2). Additionally, genome analysis using the DFAST web-based tool identified CRISPR regions in both strains.

Biogenic amine (BA) production potential was evaluated based on Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of genes associated with BA formation, such as those encoding histidine decarboxylase, ornithine decarboxylase, lysine decarboxylase and tyrosine decarboxylase. The results revealed that neither strain harbored the genes required for BA production, which was consistent with other findings (Phuengjayaem et al., 2023).

BAGEL4 analysis identified putative bacteriocin genes, including a carnobacteriocin-B2 immunity protein (51.2% similarity to Carnocin_CP52) in *Companilactobacillus zhachilii* strains ANB-1 and HBUAS52074^{T}. This finding suggested that ANB-1 may produce carnobacteriocin-B2, which has inhibitory activity against *Listeria monocytogenes*, similar to *Carnobacterium piscicola* (syn. *C. maltaromaticum*), as reported (Pilchová et al., 2016). CRISPR-Cas systems are widely studied for their pivotal role in bacterial adaptive immunity (Devi et al., 2022). Collectively, these findings confirmed that *C. zhachilii* ANB-1 and *Levilactobacillus brevis* ASK-1 were effective γ-aminobutyric acid (GABA) producers with favorable safety profiles, highlighting their strong potential as starter cultures for food fermentation and functional food applications.

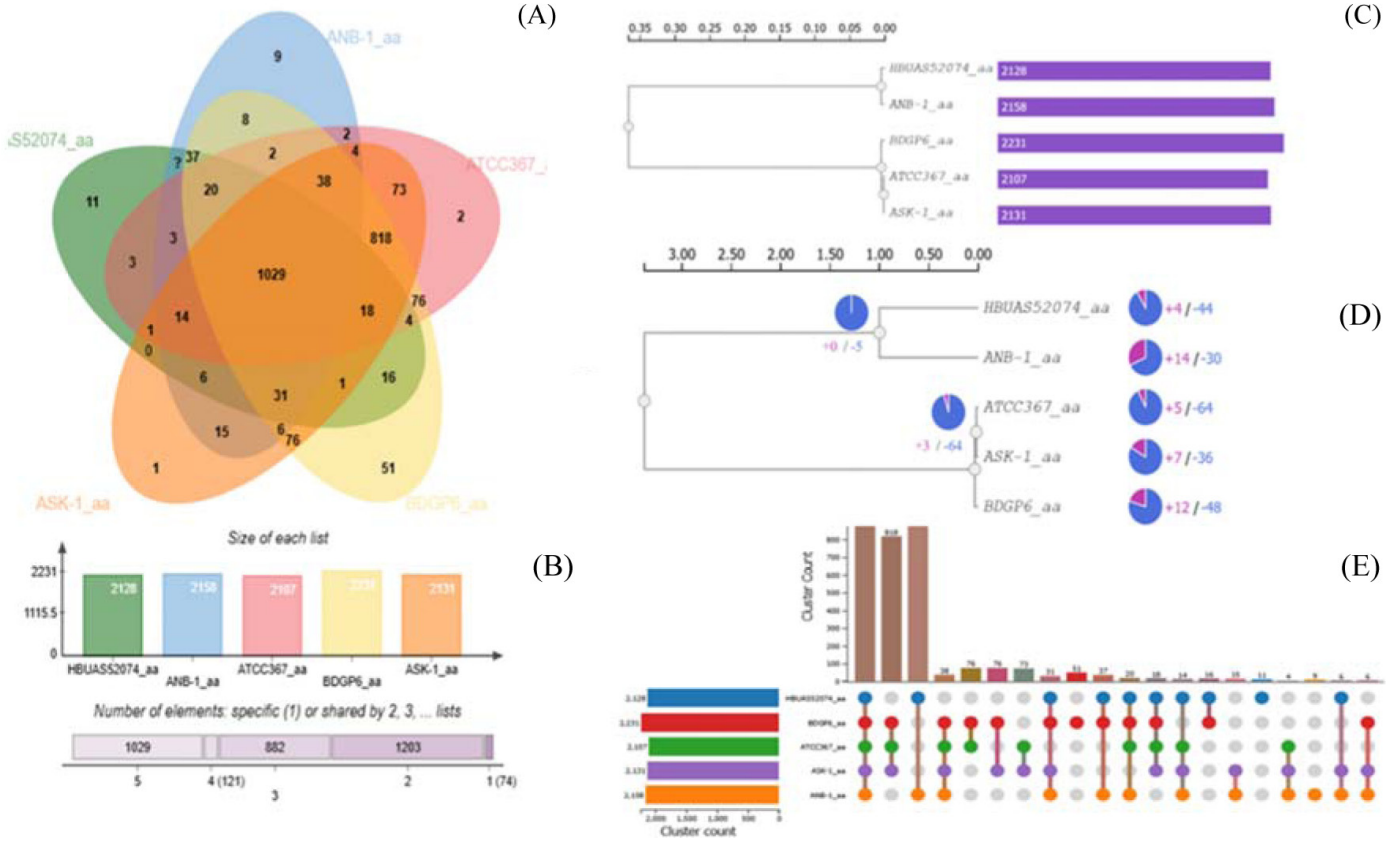

Fig. 3 Summary of pan-genome analysis of orthologs conducted using OrthoVenn3 server: (A) Venn diagram illustrating distribution of orthologous clusters among strains *L. brevis* ASK-1, *L. brevis* ATCC 367, *L. brevis* BDGP6, *C. zhachilii* ANB-1 and *C. zhachilii* HBUAS52074^{T}; (B) summary of proteins detected in each strain; (C) phylogenetic analysis of protein comparisons among each strain and closely related type strains from NCBI; (D) comparison of protein expansion and contraction among each strain and closely related strains of *L. brevis* and *C. zhachilii*; (E) orthologous proteome comparison of each strain and closely related type strains of the genus, based on OrthoVenn3.

Conclusion

Two selected strains of GABA-producing LAB were isolated from Thai fermented foods. Strain ANB-1 had greater GABA production (12.73 ± 0.57 g/L) than strain ASK-1 (9.42 ± 0.28 g/L). Genome analysis revealed that strains ANB-1, HBUAS52074^T and ASK-1 harbored the *gadA* and *gadB* genes responsible for GABA production, as identified using a KofamKOALA-KEGG Orthology Search in the KEGG database. This approach was more effective in confirming GABA production capabilities than using the RAST server. Notably, strain ANB-1, which was isolated from *nham* (a traditional Thai fermented food), was identified as *Companilactobacillus zhachilii*, marking the first report of this species being isolated from *nham*. Strain ASK-1, which was isolated from *sai-krog-prieo*, was identified as *Levilactobacillus brevis*. Both strains had high efficiency in GABA production and favorable safety profiles suitable for food applications. Further studies are recommended to optimize GABA production, evaluate probiotic properties and explore the potential of these strains for functional food applications and industrial food fermentation, both locally and globally.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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