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Probiotic and genomic evaluation of *Lactiplantibacillus plantarum* T31 isolated from *tempoyak* in mung bean substrate

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Abstract. *Fadhilah QG, Elizabeht A, Falencia M, Santoso I, Maryanto AE, Hemelda NM, Wahid MH. 2025. Probiotic and genomic evaluation of* Lactiplantibacillus plantarum *T31 isolated from* tempoyak *in mung bean substrate. Biodiversitas 26: 4435-4444.* Traditional fermented foods in Indonesia contain diverse beneficial microorganisms, including Lactic Acid Bacteria (LAB), which play an important role. LAB T31 has been isolated from *tempoyak* and evaluated for probiotic characters. In this research, the isolate was evaluated as a potential probiotic in a mung bean substrate to develop a healthy fermented drink. The result showed that LAB T31 grew well on the mung bean substrate. The population increased from 10^7 CFU/mL to 10^9 CFU/mL after 24 hours of incubation. The viability results showed that the LAB T31 population remained 10^8 CFU/mL after 30 days at 10°C. Cell-free filtrate of fermented mung bean substrate showed antibacterial activity against the tested bacteria. The diameter of inhibition increases from 24 hours (15-17 mm) to 48 hours (16-18 mm) of incubation. Whole genome sequences (WGS) of the isolate were performed using DNBseq technology, and the re-identification using JspeciesWS and FastANI showed that the strain belonged to *Lactiplantibacillus plantarum*. Analysis of the antibacterial assay test result showed that the LAB T31 genome encoded an antimicrobial peptide known as plantaricin. The hydrophobicity and auto-aggregation reached 38.86% and 36.92%, respectively, after 24 hours of incubation. Functional annotation reported adhesin genes, and the isolate did not suggest hemolytic activity on blood agar. These results supported WGS analysis using KEGG since the reported genes are not related to hemolytic ability and virulence factors. Moreover, antimicrobial resistance analysis showed that the isolate had a *vanY* gene, with a similarity level of 33.33%. LAB T31 was a potential isolate as a probiotic agent in the mung bean substrate.

Keywords: *Lactiplantibacillus plantarum* T31, mung bean fermentation, probiotic, *tempoyak*, whole genome sequencing

INTRODUCTION

Indonesia has diverse traditional fermented foods originating from various regions. These foods are typically crafted through spontaneous fermentation, a process that involve the activity of indigenous microorganisms. These microorganisms play an important role in shaping texture, flavor, and nutrition. The diversity of traditional fermented foods and their associated microbes represents a valuable resource for further exploration. Among these, lactic acid bacteria (LAB) are of particular interest due to their significant role in fermentation and their well-documented health-promoting properties as a probiotic agent. Murwani et al. (2024) reported that LAB, such as *Tetragenococcus halophilus*, *Levilactobacillus brevis* and *Lactiplantibacillus plantarum*, could be isolated from traditional fermented foods in Kalimantan Island, such as *cincalok*, *tempoyak*, and *mandai.* Another study by Santoso et al. (2024) reported that *L. plantarum* TM2 can be isolated from black glutinous rice *tapai*. LABs of these beneficial microorganisms are often included in the spontaneous fermentation of foods and drinks (Santoso et al. 2024).

Lactic acid bacteria support digestive and immune health (Ayivi et al. 2020). Common probiotic LAB include *Lactobacillus acidophilus*, *L. fermentum*, and *L. reuteri* (Sarita et al. 2024). Indigenous LAB from traditional Indonesian fermented foods offer potential for probiotic use and conservation. Fadhilah et al. (2025, data not shown) successfully isolated *Lactiplantibacillus pentosus* T31 from *tempoyak*, a traditional fermented product made from durian (*Durio zibethinus*). *L. pentosus* T31 isolate showed promising potential as a probiotic agent due to its characteristics, such as the ability to grow at high temperatures (optimal at 37°C), tolerate various bile concentrations (up to 1%), and withstand high NaCl concentrations (up to 6%). The potential indigenous LAB will be developed to produce a healthy fermented drink by using local mung bean.

Mung bean (*Vigna radiata*) is a nutrient-rich legume classified as a superfood (Huang et al. 2024; Sehrawat et al. 2024). It contains high carbohydrates (62.3%), low fat (1.9%) (An et al. 2024), protein, polyphenols, and vitamins (Sun et al. 2025). In Indonesia, production reached 234.7 tons in 2018 but has since fluctuated due to climate and land degradation (Fitriani and Taryono 2021). Mung bean is mainly used in porridge but also as a healthy food product. The seeds have significant potential as a substrate for probiotic beverages (Liang et al. 2022). Research by Sun et al. (2025) reported that mung bean is an ideal material for producing fermented plant-based milk. Fermentation by *L. plantarum* SF28 can increase or maintain stable levels of

polyphenols, thereby enhancing its nutritional value and safety.

Food safety is important in food fermentation processes. Most LAB belongs to the Generally Recognized As Safe (GRAS) category (Kamarinou et al. 2022; Huidrom et al. 2024). Giles-Gómez et al. (2024) reported that products with *L. plantarum*, *L. rhamnosus*, *S. thermophilus*, and *L. acidophilus* are listed in the latest GRAS notice and Food and Drug Administration (FDA) letters. The GRAS status of LAB shows that they are not only safe but also beneficial to health, as they produce bioactive compounds such as antimicrobial agents, organic acids, fatty acids, and related substances (Mathur et al. 2020).

The identity of strains involved in traditional food fermentation is often unclear. However, it is crucial for ensuring safety and functionality. Conventional identification based on phenotypic traits is unreliable due to environmental influence (Clarridge 2004). Likewise, 16S rRNA sequencing struggles to distinguish closely related strains (Church et al. 2020). Whole Genome Sequences (WGS) is a powerful tool, providing detailed genomic insights for accurate identification and safety evaluations for strains (Stevens et al. 2022; Mispelaere et al. 2024). Therefore, this research aimed to evaluate mung bean as a substrate for fermentation, focusing on the growth and viability of the T31 isolate. The strain was previously identified as *L. pentosus* T31 based on its 16S rRNA sequence (Fadhilah et al. 2025, data not shown), but further analysis using WGS is required to confirm its identity and assess its probiotic safety.

MATERIALS AND METHODS

Fermentation and growth of LAB T31 isolate

Mung bean substrate was washed and prepared by soaking seeds in water at a 1:1 (w/v) ratio overnight. Subsequently, the soaked beans were boiled in water at a 1:2 (w/v) ratio, followed by filtration through a cheesecloth. The resulting filtrate was supplemented with 4% (w/v) sucrose and sterilized at 121°C for 15 minutes. The sterile mung bean substrate was inoculated with the LAB T31 isolate. Specifically, 1% (v/v) of a 24-hour-old LAB T31 isolate in de-Man Rogosa Sharpe Broth (MRSB) medium was inoculated into 50 mL substrate. Furthermore, fermentation was carried out at 37°C for 1-3 days. The growth of LAB T31 isolate during fermentation was estimated using Total Plate Count (TPC) with the spread method (inoculum 0.1 mL/plate) on de-Man Rogosa Sharpe Agar (MRSA) medium. The fermentation substrate after 3 days was stored in the refrigerator at 10°C, and the viability of LAB T31 cells was observed up to 30 days with a certain time interval.

Antibacterial activity of the LAB T31 isolate

Antibacterial activity test of the mung bean fermented filtrate of LAB T31 isolate was carried out using the diffusion cylinder method against Gram-positive (*Bacillus cereus* and *Kocuria rhizophila*) and Gram-negative bacteria (*Escherichia coli* and *Salmonella typhi*). The test was carried out on the medium of Nutrient Agar (NA), and bacterial tests were swabbed on the surface. Cell-free filtrates of the LAB T31 isolate were obtained by centrifugation of the fermented mung bean substrate (6,000 rpm, 15 min) and filtered using a filter syringe (0.45 µm). In addition, 250 µL of cell-free filtrate was inserted into the cylinder, and the test was carried out in triplicate across two batches. The NA medium was incubated for 24 hours, and the clear zone formed was measured using a caliper.

Whole genome sequence assembly of the LAB T31 isolate

WGS of the LAB T31 isolate was performed using the BGI/MGI platform. This platform adopted Digital Nanoball Sequencing (DNBseq) technology. The total DNA of LAB T31 isolate was obtained using ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research), following the manufacturer's protocol. The extracted DNA was stored at -20°C for preservation and future analysis. Meanwhile, the completeness and quality of the genome of the LAB T31 isolate were assessed through CheckM (Parks et al. 2015).

Library preparation and sequencing were carried out using DNA sequencing (DNBSEQ) library high-throughput technology developed by MGI. This technology used a unique library preparation method known as DNB (DNA Nanoball) technology, which offered several advantages over traditional sequencing methods in terms of accuracy and cost-efficiency. Meanwhile, sequencing was carried out using DNBSEQ technology based on Combinatorial Probe-Anchor Synthesis (cPAS), and the length of the fragment read was 150 bp (PE150).

Re-identification of LAB T31 isolate

The identity of the strain used as a fermentation agent should be precisely determined. Relatively new methods, such as WGS, have become very important because the genomic information obtained ensures safety in the production of fermented foods (Chokesajjawatee et al. 2020). In this research, the identity of the strain used should be precisely determined. Re-identification of the LAB T31 isolate was performed based on WGS of the isolate using the JspeciesWS web-based bioinformatics tools application (https://jspecies.ribohost.com/jspeciesws/) (Yin et al. 2023). The application is based on Average Nucleotide Identity (ANI) calculated from the nucleotide similarity between two complete genomes. The phylogenetic tree of the LAB T31 isolate was constructed with Automated Multi Locus Species Tree (AutoMLST) (https://automlst.ziemertlab.com/).

Detection of bacteriocin

Analysis of the antibacterial peptide gene related to bacteriocin production was carried out with BAGEL Version 4 (http://bagel4.molgenrug.nl/) (Li et al. 2022). Bagel is a bioinformatics tool used to analyze the genomes of bacteria to obtain genes related to the production of bacteriocin.

Probiotic characteristics of LAB T31 isolate

According to Fadhilah et al. (2025, data not shown), some phenotypic characters of LAB T31 isolate had been observed. Further research was conducted to characterize

LAB T31 isolate related to the safety of food fermentation agents using the analysis of WGS and experimental tests.

Auto-aggregation and hydrophobicity assay and related gene analysis

Auto-aggregation assay was performed by inoculating MRSB medium with approximately 1% (v/v) of a 24-hour-old LAB T31 isolate and incubating for 18 hours at 37°C. Subsequently, the cells were collected by centrifugation at 6,000 rpm for 15 minutes and washed three times with 1X phosphate-buffered saline (PBS). After washing, the cells were resuspended in PBS to achieve an OD600 nm of 0.5-0.6 (A_x). The suspension was incubated at 37°C, with measurements taken at different incubation times (3, 6, and 24 hours) (A_y). For each measurement, the absorbance at OD600 nm of the upper part of the bacterial suspension was recorded. Meanwhile, the auto-aggregation rate was calculated following the method of Li et al. (2020) using the formula:

$$\text{Auto} - \text{aggregation } (\%) = \frac{A_x - A_y}{A_x} \times 100$$

Where:

A_x : Initial absorbance

A_y : Absorbance after incubation (3, 6, and 24 hours)

The hydrophobicity assay was performed according to the protocols described by Darmastuti et al. (2021) and Sadeghi et al. (2022), with modifications. In this context, the microbial adhesion to hydrocarbons (MATH) method was used. An 18-hour-old culture of LAB T31 isolate, grown in MRS Broth (MRSB), was centrifuged at 6,000 rpm for 15 minutes to harvest the cells. The collected cells were washed three times with Phosphate Urea Magnesium (PUM) buffer (composition comprises 2.22% (w/v) $K_2HPO_4 \cdot 2H_2O$, 0.726% (w/v) KH_2PO_4, 0.18% (w/v) urea, and 0.02% (w/v) $MgSO_4 \cdot 7H_2O$). The cells were resuspended in PUM buffer, and the absorbance was adjusted to an initial optical density (OD_{600}) of 0.5-0.6 (A_0). Subsequently, 1 mL of xylene was added to 5 mL of the cell suspension and vortexed. The mixture was incubated at 37°C, and after 1 hour, the absorbance of the aqueous (upper) phase (A_t) was measured. The surface hydrophobicity percentage was calculated with the formula:

$$\text{Hydrophobicity } (\%) = \frac{A_0 - A_t}{A_0} \times 100$$

Where:

A_o : Initial absorbance

A_t : Absorbance after incubation

The analysis of WGS data to identify genes in LAB T31 genome related to cell attachment functions in host tissues was conducted using the evolutionary genealogy of genes known as Non-supervised Orthologous Groups (EggNOG) (http://eggnog-mapper.embl.de/) (Son et al. 2021). This application served as a comprehensive database and computational tool for analyzing and annotating genes and proteins.

Hemolytic activity assay and virulence factors analysis

LAB T31 isolate was streaked on a blood agar medium to evaluate the hemolytic activity. After inoculation, the medium was incubated for 48 hours at 37°C. The formation of a clear zone around the colony indicated a hemolytic reaction (Huligere et al. 2023). The existence of genes related to hemolytic activity and virulence factors was also analyzed through WGS using KEGG (https://www.genome.jp/kegg/) (Oh et al. 2022).

Antimicrobial resistance gene analysis

The possibility of the Antimicrobial Resistance (AMR) gene of LAB T31 isolate was also analyzed through WGS using the Resistance Gene Identifier (RGI) tool provided by the Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/).

RESULTS AND DISCUSSION

Fermentation and growth of LAB T31 isolate

The starter culture was grown in MRSB medium for 24 hours, and TPC results showed a population of 63.80×10^8 CFU/mL. A total of 1% (v/v) inoculum was inoculated into 50 mL of the mung bean fermentation substrate, with an initial pH of the medium was 7.0 upon inoculation. Therefore, the starting population at the beginning of fermentation was approximately 63.80×10^6 or 6×10^7 CFU/mL. This amount is sufficient to serve as an inoculum for yogurt fermentation (Mani-López et al. 2014). Subsequently, the probiotic bacteria were found to grow successfully. Mani-López et al. (2014) reported that the probiotic bacteria population after fermentation often exceeded 10^8 CFU/mL.

LAB T31 grew well in the mung bean substrate, reaching 2.78×10^9 CFU/mL after 24 h of incubation, with the medium pH decreasing to 3.0 (Table 1). Similarly, Matsubara et al. (2024) reported that *L. reuteri* in soy germ-soymilk reached 2.57×10^8 CFU/g after 24 h of fermentation (pH 3.43). Although the LAB T31 population declined slightly during 48-72 h of incubation, it remained at 3.2×10^8 CFU/mL for up to 30 days of refrigerated storage (Figure 1), with the pH stable at 3.0. These results indicate that mung bean as a fermentation substrate can sustain the LAB T31 population. This finding is consistent with Matsubara et al. (2024), who observed a slight decrease in *L. reuteri* to 2×10^8 CFU/g. In both cases, the viable counts met the minimum requirement for probiotic products, as defined by the National Yogurt Association (Talwalkar and Kailasapathy 2004).

Antibacterial activity of LAB T31 isolate

In this research, the LAB T31 isolate was observed to produce antibacterial compounds in fermented mung bean. The activity was assayed during 24-, 48-, and 72-hour fermentation, as presented in Table 2. Antibacterial activity values represent the averages of triplicates from two batches.

Whole genome sequence assembly of LAB T31 isolate

The results of agarose gel electrophoresis indicate that DNA from the LAB T31 isolate was successfully isolated, but slight degradation was observed (Figure 2). Measurement

using the Pinch Fluorometer with BR DNA Assay showed DNA concentration of 99.1 ng/μL. The sequencing data were stored in Fastq format and filtered using Fastp, which provides high-quality information, ensuring the accuracy and reliability of our bioinformatics analysis. De novo assembly was carried out using SPAdes, producing contigs and scaffolds. The quality of the scaffolds was evaluated using QUAST, and the assembly data are summarized in Table 3. Genome annotation using Prokka identified 2,869 coding sequences and 59 RNA molecules, including 55 tRNAs, 1 tmRNA, and 3 rRNAs. Finally, the genome was visualized in a circular format (Figure 3) using the Proksee tool.

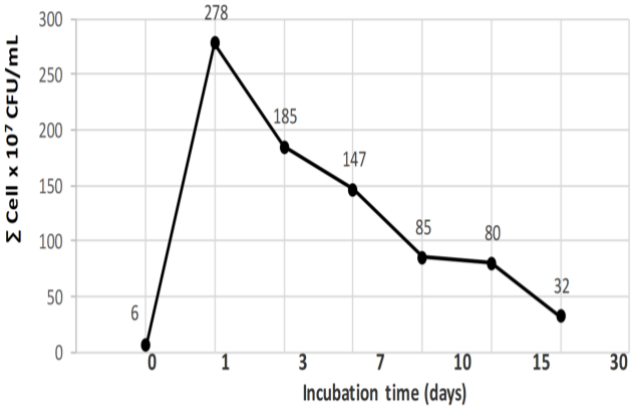

Figure 1. Population of LAB T31 isolate in fermented mung bean substrate

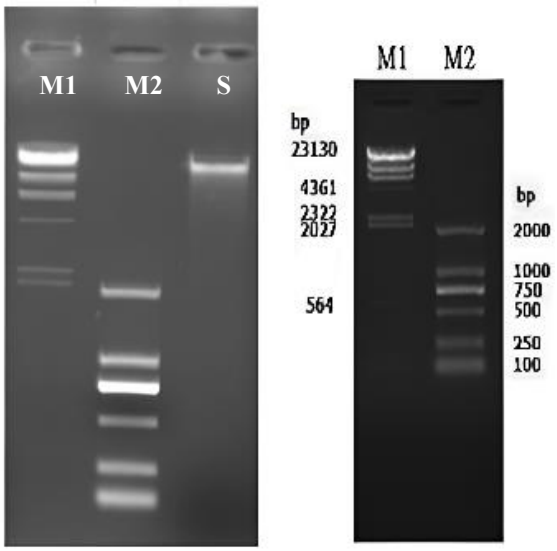

Figure 2. Results of DNA isolation of LAB T31 isolate. M1: λ-Hind III digest (Takara); M2: D2000 (Tiangen); S: Sampel LAB T31 isolate

Table 3. Genome assembly results of LAB T31 isolate

Description	LAB T31 isolate
Contig	231
GC content	44.65
Contig N50	291,990
Contig L50	4
Genome Length (bp)	3,095,505

Table 1. Growth population of LAB T31 isolate in fermented mung bean substrate

	Fermentation (days)						
	T_0	T_1	T_3	T_7	T_{10}	T_{15}	T_{30}
Dilution	10^7	10^7	10^6	10^6	10^6	10^6	10^6
Average/plate	63.80	27.80	184.60	147.20	85.20	79.60	32.20
∑ population CFU/mL	6×10^7	2.78×10^9	1.85×10^9	1.47×10^9	8.5×10^8	8.0×10^8	3.2×10^8

Note: T: Incubation time (days): 0, 1, 3, 7, 10, 15, 30

Table 2. Antibacterial activity assay results of LAB T31 isolate

Incubation (hours)	Batch	Clear zone (mm)			
		Bacillus cereus	*Kocuria rhizophila*	*Escherichia coli*	*Salmonella typhi*
24	1	18.05	14.55	18.45	18.33
	2	13.20	16.33	17.00	17.20
	average	15.63±3.43	15.44±1.26	17.73±1.03	17.77±0.80
48	1	19.16	17.45	16.30	17.34
	2	17.33	17.55	16.90	18.55
	average	18.25±1.29	17.50±0.70	16.60±0.42	17.95±0.86
72	1	17.54	16.20	16.60	14.63
	2	17.44	13.42	14.82	14.52
	average	17.49±0.07	14.81±1.97	15.71±1.26	14.58±0.08

Re-identification of LAB T31 isolate

In this research, the methods used for the re-identification of LAB T31 isolate were JSpeciesWS and FastANI through Average Nucleotide Identity (ANI). JSpeciesWS analysis showed that LAB T31 isolate had the highest Z-score of 0.99957 and 0.99949 when compared to *L. plantarum* DmCS_001 (non-type strain) and strain *L. plantarum* DSM 20174, respectively. These results showed a high similarity between the query (LAB T31 isolate) and the reference genome of *L. plantarum*.

The results of FastANI analysis were consistent with those obtained using JSpeciesWS. FastANI calculations showed that the LAB T31 isolate shared 99.0991% similarity with *L. plantarum* (GCF_0099131655.1), whereas similarity to *L. pentosus* (GCF_003641185.1) was lower at 82.5260%. These results confirm that the T31 isolate is highly similar to *L. plantarum*, highlighting that 16S rRNA sequencing alone is insufficient to distinguish between these closely related species. Huang et al. (2021) reported that *L. plantarum* and *L. pentosus* are closely related, with *L. pentosus* sharing extremely high sequence identity with the full-length 16S rRNA gene of *L. plantarum*. Based on FastANI calculations, the T31 isolate, previously identified as *L. pentosus*, exhibits a high degree of similarity to *L. plantarum* (99.0991%). A phylogenetic tree for the T31 isolate was constructed using AutoMLST and is shown in Figure 4.

Detection of bacteriocin

Analysis using BAGEL4 revealed that *L. plantarum* T31 isolate contained the genes for plantaricins (*pln*), including *plnA*, *plnE*, *plnF*, *plnJ*, *plnK*, and *plnN* (Figure 5). These genes are related to the antimicrobial peptide plantaricin. This finding aligns with Li et al. (2024), who reported variation in plantaricin gene profiles among *L. plantarum* strains, illustrated by GKM3 (*plnA*, *plnE*, *plnF*, *plnJ*), GKD7 (*plnE*, *plnF*), and GKK1 (*plnE*, *plnF*, *plnK*). In addition to the presence of plantaricin, the analysis with BAGEL4 also showed the existence of the lantibiotic gene (*lanT*) of *L. plantarum* T31 isolate.

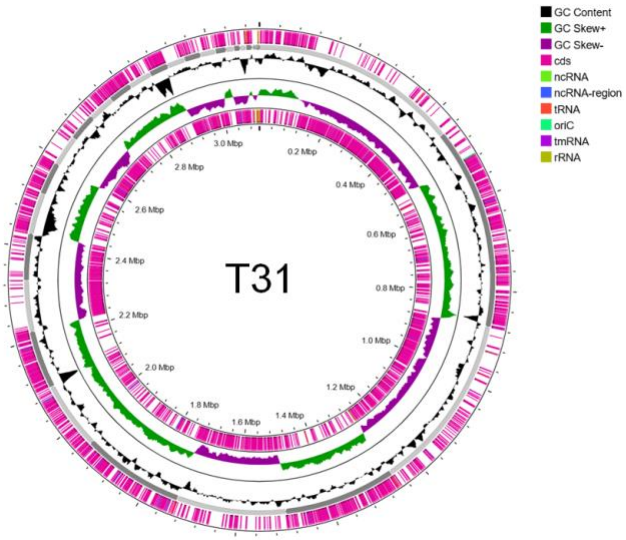

Figure 3. Whole genome sequences of LAB T31 isolate

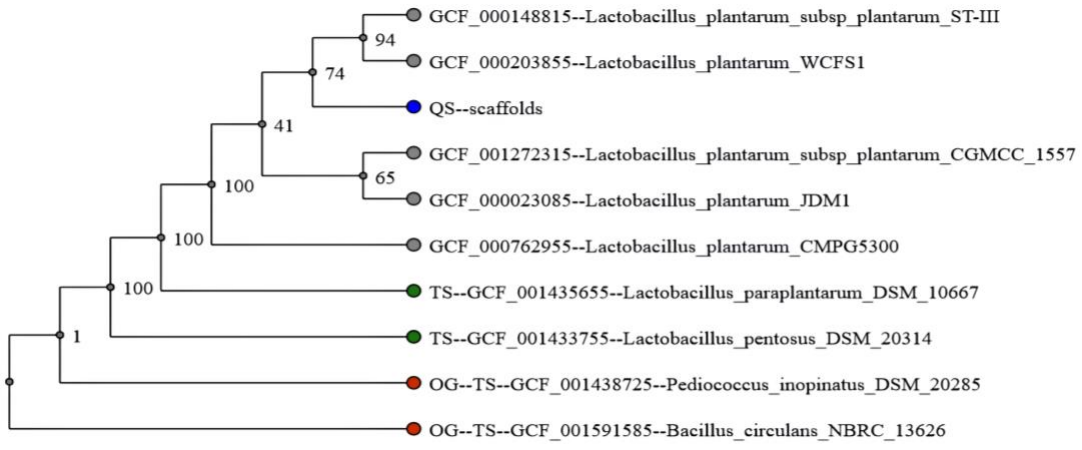

Figure 4. Phylogenetic tree of *Lactiplantibacillus plantarum* LAB T31 isolate

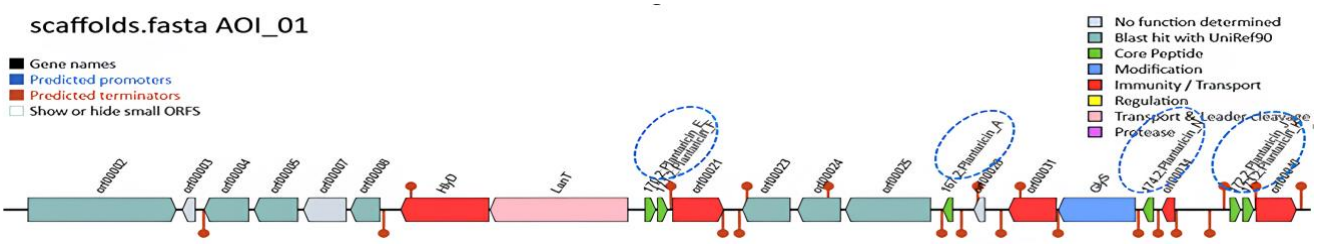

Figure 5. The analysis of the antimicrobial peptide of *Lactiplantibacillus plantarum* T31 isolate

Probiotic characteristics of *Lactiplantibacillus plantarum* T31 isolate

Auto-aggregation and hydrophobicity assay and related gene analysis

Lactiplantibacillus plantarum T31 isolate can perform auto-aggregation and hydrophobicity, as shown in Table 4. After 24 hours of incubation, the average percentages of auto-aggregation and hydrophobicity were 36.92% and 38.86%, respectively. Auto-aggregation ability of *L. plantarum* T31 increased over time and was supported by WGS analysis. The result of EggNOG described the isolate as an adhesin gene, such as mucin binding protein (*MuCBP*), cohesin (M) protein, fibrinogen binding protein, and putative adhesin.

Hemolytic activity and virulence factors analysis

WGS analysis using the KEGG database reported that the *L. plantarum* T31 isolate lacked genes associated with hemolytic activity. Experimental testing on blood agar medium also confirmed the absence of hemolytic activity since no hemolysis was observed (Figure 6). Based on genomic analysis and experimental results, *L. plantarum* T31 isolate did not possess hemolytic potential.

Antimicrobial resistance gene analysis

AMR gene analysis was conducted using the RGI tool in the CARD platform. The analysis identified the presence of the *vanY* gene in the *L. plantarum* T31 isolate, which was part of the glycopeptide resistance gene cluster (Table 5). This gene was associated with resistance to glycopeptide antibiotics, including vancomycin (Chokesajjawatee et al. 2020).

Discussion

The research results showed that *L. plantarum* T31 isolate reported strong growth in mung bean substrates, as indicated by a significant decrease in pH following 24 hours of incubation. The pH of the fermentation medium, which initially was 7.0, then declined to 3.0, reflecting active fermentation. The *L. plantarum* T31 isolate effectively utilized nutrients in mung beans, particularly when supplemented with 4% sucrose (v/v), indicating that mung bean substrates were a viable medium for probiotics. This aligns with An et al. (2024), who reported that mung beans support LAB growth and the production of plant-based probiotic beverages, such as yogurt. *L. plantarum* YI-Y2013 was shown to mitigate the characteristic "beany" odor (Yi et al. 2021), while *L. plantarum* 299v effectively fermented organic chickpeas and green and red lentils, producing legume-derived beverages with potential health benefits (Skrzypczak et al. 2024). During fermentation, pH declined, indicating active bacterial metabolism and lactic acid production, and the bacterial population increased to approximately 10^8 CFU/mL after 72 hours.

The population of *L. plantarum* T31 remained stable at 10^8 CFU/mL after 30 days of refrigerated storage, consistent with Matsubara et al. (2024). Probiotic viability is influenced by factors such as bacterial strain, mixed culture composition, and storage conditions (Mani-López et al. 2014). The National Yogurt Association requires a minimum of 10^8 CFU/mL for yogurts to be labeled as containing "live and active cultures" (Talwalkar and Kailasapathy 2004), while FAO/WHO guidelines define probiotic products as containing at least 10^6 CFU/g per 100-g serving (Skrzypczak et al. 2024). Although viable cell counts may decline during storage due to reduced metabolic activity, the T31 isolate maintained sufficient levels to meet these standards (WHO and FAO 2002).

Figure 6. Hemolytic activity of *Lactiplantibacillus plantarum* T31 isolate on blood agar medium

Table 4. The average percentage of *Lactiplantibacillus plantarum* T31 isolate auto-aggregation and hydrophobicity

	Auto-aggregation (%)			Hydrophobicity (%)
	T_3	T_6	T_{24}	
Batch 1	15.29	20.24	37.37	39.09
Batch 2	12.17	21.87	36.47	38.62
Average	13.73	21.01	36.92	38.86

Note: T: Time (hours). 3, 6, 24: Incubation time (hours)

Table 5. Results of AMR gene analysis with RGI of *Lactiplantibacillus plantarum* T31 isolate

RGI criteria	ARO term	Detection criteria	AMR gene family	Drug class	Resistance mechanism	Identity of matching region (%)	Length of reference sequences (%)
Strict	*vanY* gene in *vanG* cluster	Protein homolog model	*vanY*, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration	33.33	92.11

The antimicrobial test indicated that *L. plantarum* T31 isolate can produce antimicrobial compounds, presumed to be peptides, specifically bacteriocins, synthesized from ribosomes and produced by both Gram-positive and Gram-negative bacteria. However, many bacteriocins are predominantly reported to be produced by Gram-positive bacteria, particularly LAB (Simons et al. 2020). Some bacteriocins are effective against both Gram-positive and Gram-negative bacteria (Syaputri and Iwahashi 2020). Bacteriocins are considered primary metabolites, produced during the growth or log phase (Younas et al. 2022). Since both Gram-positive and Gram-negative bacterial strains showed catalase activity (Fadhilah et al. 2025, data not shown), the antimicrobial effect observed was not attributed to hydrogen peroxide.

Based on TPC of *L. plantarum* T31 isolate, the strain grows well in mung bean substrate, with the growth maintained until the third day of fermentation. Therefore, the data of *L. plantarum* T31 isolate and the resulting antibacterial activity reported a positive correlation. *L. plantarum* synthesizes antibacterial compounds, notably bacteriocins. Furthermore, it can also produce extracellular polymeric substances (EPS), such as plantaricin. These compounds disrupt the integrity of microbial cell membranes, leading to reduced EPS production, inhibition of membrane-associated protein formation, membrane thinning, pore formation, leakage, and rupture of intracellular contents, ultimately resulting in microbial cell death (Lin et al. 2025).

Analysis of bacteriocin detection reported that *L. plantarum* T31 isolate contained genes for plantaricins. The key probiotic characteristic of *L. plantarum* group was the ability to produce bacteriocins. These antimicrobial peptides or protein compounds inhibit the growth of other bacteria, particularly pathogens or undesirable microbes. Bacteriocins enhance the competitiveness of probiotics in the gut microbiota by suppressing the growth of pathogenic bacteria. An example of a bacteriocin produced by *L. plantarum* is plantaricin, which is effective against a wide range of Gram-positive and Gram-negative pathogens (Giles-Gómez et al. 2024).

Plantaricin A (*plnA*) is a signaling peptide initially synthesized as a precursor that induces the expression of *plnEF* and *plnJK* genes. *PlnA* is known to bind to lipopolysaccharides, compromising the integrity of the outer membrane of *E. coli* and enhancing antimicrobial efficacy (Meng et al. 2022). Even though *PlnA* shows strain-specific antagonistic activity, the antimicrobial potency is comparatively lower than *plnEF* and *plnJK* (Meng et al. 2022). Plantaricin E (*plnE*) and F (*plnF*) form a two-peptide bacteriocin system, characterized by amphiphilic α-helical structures. Similarly, *plnJ* and *plnK* also encode two-peptide bacteriocins classified under Class IIb (Syaputri and Iwahashi 2020). Plantaricins EF and JK are biosynthesized as pre-peptides and subjected to cleavage during transport to the cell surface to become active. These gene pairs work synergistically, enhancing antimicrobial activity by disrupting the target cell membrane through pore formation, which leads to ion leakage and cell death (Butorac et al. 2020). In addition, *plnN* encodes an independent bacteriocin-like peptide with a double-glycine leader motif typical of Class II bacteriocins. The mature *PlnN* peptide contains a putative amphiphilic α-helical domain, a structural feature associated with membrane-disrupting antimicrobial activity (Anderssen et al. 1998). *PlnN* exerts direct antimicrobial effects by compromising the integrity of bacterial membranes (Isaac et al. 2024).

L. plantarum T31 isolate has also been reported to contain lantibiotic genes, which are a type of antimicrobial peptide. The compounds have a unique structure because of the presence of uncommon amino acid residues, such as lanthionine and methyllanthionine. Their biosynthesis includes gene clusters that encode precursor peptides, modification enzymes, immunity proteins, and transporters such as *lanT* (Sandiford 2020; McAuliffe et al. 2021). Even though *lanT* is essential for lantibiotic production, its presence is insufficient. Other components, such as *lanM* or *lanBC*, are also required (Singh and Sareen 2014).

L. plantarum T31 isolate showed auto-aggregation and hydrophobicity activity after 18 hours of fermentation. Hydrophobicity and auto-aggregation capabilities are important factors for LAB probiotic cells to bind effectively to host epithelial cells. These characteristics have been proposed as key selection criteria for potential probiotic strains (WHO and FAO 2001; Darmastuti et al. 2021). Generally, auto-aggregation (%) is classified into high (>70%), medium (20-70%), and low (<20%) (Darmastuti et al. 2021). Based on these categories, *L. plantarum* T31 isolate falls into the medium category.

Auto-aggregation appears to be the first step in the adhesion process, allowing bacteria to form a barrier that prevents the adhesion of undesirable bacteria. The bacterial cells grow and form aggregates and colonies. The structure of bacterial aggregates or colonies can assist in the adhesion process of LAB in the gastrointestinal tract. However, adhesion is a complex process of non-specific and specific ligand-receptor interactions. Bacteria that colonize the gastrointestinal tract subsequently perform the function of probiotics. The auto-aggregation characteristic of probiotic bacteria reflects the ability to adhere to host tissues. This is also related to the capacity to inhibit pathogens from attaching to the same site (Zawistowska-Rojek et al. 2022; Wang et al. 2024). The results of gene analysis using EggNOG supported auto-aggregation and hydrophobicity assays. *L. plantarum* T31 isolate contained three genes related to adhesin ability. The cohesin (*M*) protein has an adhesin-like function, while fibronectin-binding proteins (*FbpA*) bind to fibronectin, a glycoprotein in the extracellular matrix of host tissues. These proteins play a crucial role in bacterial adhesion, pathogenesis, and immune evasion.

Hemolytic activity and virulence factor analysis results confirm that the *L. plantarum* T31 isolate has the potential to be a probiotic agent. The absence of hemolytic activity is considered a necessary condition for assessing the safety of potential probiotic isolates (WHO and FAO 2006). Hemolytic activity can lyse the red blood cells of the host. Therefore, the exclusion of isolates presenting γ-hemolysis is necessary (Shivani and Sathiavelu 2024). These results were consistent with previous research, where no hemolytic

activity was observed in LAB isolates of dairy origin (Zhang et al. 2023).

The analysis of AMR-related genes proved that the *L. plantarum* T31 isolate had the potential to be a probiotic agent. Results from CARD analysis reported that the *L. plantarum* T31 isolate harbored only one AMR gene, *vanY*, associated with resistance to vancomycin. CARD is a curated and comprehensive database that contains detailed information on AMR genes identified across various microorganisms. This database includes data on resistance mechanisms, antibiotic classes, gene structures, and the functional relationships between genes. RGI tool, which uses CARD data, predicts potential AMR genes in a given genome by performing sequence balance or k-mer-based analysis. RGI identifies genes and provides insights into the type and mechanism of antibiotic resistance present in the organism through these methods.

Vancomycin exerts its antibacterial effect by binding to peptidoglycan precursors during bacterial cell wall synthesis, inhibiting the formation and weakening of the structure. However, the *vanY* gene enables bacteria to produce D-Ala-D-Ala carboxypeptidase to modify the terminal residues of peptidoglycan precursors and reduce vancomycin binding affinity (Kandasamy et al. 2022). This modification allows bacterial cells to maintain cell wall integrity even under antibiotic pressure. Several *L. plantarum* strains possess intrinsic resistance to vancomycin (Kwon et al. 2021). However, the *vanY* gene does not fully account for this resistance. The presence is closely associated with the capacity to persist in environments containing glycopeptide antibiotics rather than being the sole determinant of resistance. The occurrence of the AMR gene *vanY* in the genome does not automatically imply that bacterial cells are resistant. The phenomenon of resistance to related antibiotics is significantly influenced by the level of gene expression and the type of substrate (Kandasamy et al. 2022; Kingkaew et al. 2023).

In conclusion, *L. plantarum* T31 isolated from *tempoyak* showed potential as a probiotic agent for fermenting mung bean substrate. The strain could grow in the mung bean substrate, and the population remained viable for up to 30 days. Safety and functional evaluation through WGS analysis and in vitro assays showed that *L. plantarum* T31 fulfilled the criteria required to be classified as a probiotic strain. Further studies should evaluate the nutritional content of the fermented mung bean substrate.

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