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Antimicrobial activity, genome analysis, and gene cluster encoding the plantaricin production of *Lactiplantibacillus plantarum* ZBK1-5

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ABSTRACT: This study aimed to explore bacteriocin-producing lactic acid bacteria (LAB), identify the genes influencing bacteriocin synthesis, and assess their probiotic potential via safety evaluations both *in vitro* and *in silico*. The strain ZBK1-5, isolated from pickled ginger, was identified as *Lactiplantibacillus plantarum*. The bacteriocin ZBK1-5 exhibited the highest antimicrobial activity with 6,400 AU/ml at 18 h, predominantly effective against Gram-positive bacteria and stable within pH 2–9. Significantly, the genome analysis confirmed the existence of bacteriocin synthesis genes, specifically plantaricin KJ, EF, A, and N. Simultaneously, the safety evaluations showed its low risk of antibiotic resistance gene transfer, thereby emphasizing the safety profile of the strain ZBK1-5. The genetic components associated with the ability of *L. plantarum* ZBK1-5 to survive and adapt in gastrointestinal conditions provide additional evidence (16 genes related to acid stress and 9 genes associated with bile resistance), supporting its potential as a probiotic candidate. These findings were validated through *in vitro* digestion conditions, where the viability of the cells was observed to be 68.26, 66.62, and 60.91% during transit through the oral, gastrointestinal, and small intestinal phases, respectively. Additionally, the strain ZBK1-5 showed a 90.39% adhesion rate in the Caco-2 cell line. The unique characteristics of plantaricin produced by *L. plantarum* ZBK1-5 exhibit potent antibacterial activity, rapid time to production compared to other known plantaricins, and strain safety for application and upscaling. This research study offers significant scientific insights into *L. plantarum* ZBK1-5, a plantaricin producer, emphasizing its promising potential for future applications.

KEYWORDS: bacteriocins, genomics, lactic acid bacteria, plantaricin, safety evaluation

INTRODUCTION

Bacteriocin-producing lactic acid bacteria (LAB) have gained significant attention due to their potential applications in various fields, including food preservation, probiotics, and biomedical industries [1]. Most of LAB considered Generally Recognized as Safe (GRAS) and widely isolated from fermented foods and healthy animal feces, they have the potential to inhibit pathogens and demonstrate probiotic properties, ensuring safety and benefiting both human health and animals [2]. The utilization of bacteriocin-producing LAB as probiotics presents a two-fold benefit by conferring advantageous impacts on the host organism while concurrently inhibiting the growth of pathogens through the synthesis of bacteriocins [3]. This phenomenon not only promotes the overall well-being of the host organism but also provides an essential role in maintaining the balance within the gastrointestinal tract.

However, before considering the application of bacteriocinogenic LAB as probiotics, a comprehensive safety evaluation is necessary to determine their suitability for human consumption [4]. Whole-genome analysis is currently a practical approach for safety evaluation, allowing a comprehensive assessment of possible risks associated with the presence of specific genes and other genetic components [5]. It provides valuable insights into identifying and characterizing antibiotic resistance genes, potential virulence factors, and other genetic elements that could represent risks to the safety of LAB strains.

This study aimed to investigate bacteriocin-producing LAB derived from Thai fermented food sources, pickled ginger, provide significant scientific knowledge regarding the genetic factors influencing bacteriocin synthesis, and assess their probiotic potential via a comprehensive *in silico* safety evaluation.

MATERIAL AND METHODS

Isolation and screening of bacteriocin-producing lactic acid bacteria

Two samples of pickled ginger (*Khing dong*) were collected from Bangkok, Thailand. Ten grams of sample was suspended and homogenized in 90 ml of De Man

Rogosa Sharpe (MRS) broth and incubated aerobically at 30 °C for 72 h. A 20 µl of culture broth was streaked on MRS agar supplemented with 0.3% (w/v) $CaCO_3$ and incubated under the same conditions. The colonies surrounding the clear zone were chosen for purification based on their distinct morphologies. This procedure was repeated until pure cultures were obtained. For further research, the isolated LAB were stored at −20 °C in 40% (v/v) glycerol and lyophilized with 10% (w/v) skim milk.

Isolated strains were activated twice and incubated at 30 °C for 24 h in MRS broth. The culture supernatant was collected through centrifugation at 10,000 × *g* for 10 min at 4 °C. The cell-free supernatant (CFS) was adjusted to pH 6.5 ± 0.1 with 1 M NaOH. The antimicrobial assay was conducted utilizing the spot-on-lawn technique [6]. *Latilactobacillus sakei* JCM 1157^T was used as an indicator strain.

The antimicrobial activity was determined by calculating the reciprocal of the highest two-fold serial dilution that resulted in a transparent inhibition zone of the indicator strain measuring above 9.0 mm by using the following formula:

$$\text{Antimicrobial activity (AU/ml)} = (2^N)\times 100 \quad (1)$$

where AU is an arbitrary unit, and N is the highest two-fold serial dilution, showing a transparent inhibition zone of the indicator strain.

Characterization and identification of bacteriocin-producing strain

The bacteriocin-producing isolates were characterized after incubating at 30 °C for 24 h. The selected strains were evaluated on MRS agar plates to determine their morphological and biochemical characteristics: catalase activity, aesculin hydrolysis, nitrate reduction, arginine hydrolysis, gas production from glucose, and acid production from fermentation of sugars including L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, D-lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-raffinose, D-ribose, D-sorbitol, D-sucrose, D-trehalose, D-xylose, L-rhamnose, and salicin, as well as growth at 15, 30, and 45 °C in 4, 6, and 8% NaCl and pH 3, 6, and 9. The physiological and biochemical characteristics were conducted using the previously described method by Tanasupawat et al [7]. The hemolysis assay was performed on blood agar plates, followed by an incubation at 30 °C for 48 h.

The amplification of the 16S rRNA gene sequences of the selected strains was performed using the polymerase chain reaction (PCR) technique using the universal primer 27F (5′-GAGTTTGATCATGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Subsequently, DNA was purified using the GenepHlow™ Gel/PCR Kit Quick Protocol. The PCR products were analyzed by a DNA sequencer (Macrogen, Korea). Species identification was accomplished using the EzBiocloud tool [8]. The sequence of isolate ZBK1-5 was deposited in DDBJ (DNA Data Bank of Japan, Mishima, Japan).

Bacterial growth dynamics and antimicrobial peptide production

The antimicrobial activity of bacteriocin was investigated following the method by Woraprayote et al [9] with modifications. One percentage of the overnight selected strain was inoculated into 200 ml of MRS broth and then incubated at 30 °C. Samples were collected and recorded at 3 h intervals for 24 h. The growth was measured using a spectrophotometer at 600 nm, while the changes in pH were monitored using a pH meter. The antimicrobial activity was assessed using the critical dilution spot-on-lawn assay, reporting in arbitrary units per milliliter (AU/ml).

Antimicrobial spectrum

The cell-free supernatant (CFS) of selected strain was further tested by spot-on-lawn assay using 6 Gram-positive bacteria (*Enterococcus faecalis* JCM 5803^T, *Listeria inocua* ATCC 33090^T, *L. monocytogenes* ATCC 19115, *Kocuria rhizophila* MIII, *Pediococcus pentosaceus* JCM 5885, and *Staphylococcus aureus* ATCC 23235) and 5 Gram-negative bacteria (*Escherichia coli* O157:H7, *E. coli* ATCC 25922, *E. coli* ATCC 35401, *E. coli* F18, and *Salmonella* Typhimurium ATCC 13311).

Effect of temperature, pH, and enzyme on the activity of bacteriocin

The effect of pH on the stability of bacteriocin was examined by manipulating the CFS to different pH levels, specifically 2, 3, 5, 7, 9, and 11, using 1 M HCl or 1 M NaOH. The samples were incubated at 37 °C for 5 h. Subsequently, the pH was readjusted to 6.5–7.0 before conducting the antimicrobial test.

The effect of thermal conditions on the stability of bacteriocin was examined by placing the CFS of bacteriocin in incubation at 100 °C for 5, 10, 20, 30, and 40 min. Subsequently, the samples were maintained at room temperature before an antimicrobial assessment was conducted.

The sensitivity of bacteriocin to a proteolytic enzyme, proteinase K, was tested by subjecting the CFS to incubation in the presence of 1.0 mg/ml of the enzyme at 37 °C for 5 h [10]. The sample underwent thermal treatment at 100 °C for 10 min to render the enzyme inactive. The control group used the conditioned media of the bacteriocin without the enzyme. The antimicrobial activities of the control group and the treated samples were assessed through a spot-on-lawn assay, utilizing *L. sakei* JCM 1157^T as the indicator strain.

The zone of inhibition was measured and reported in arbitrary units per milliliter (AU/ml) [9].

Adhesion assay

The Caco-2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum and incubated at 37 °C in 5% CO_2. Then, it was seeded at 1×10^5 cells/well in a 24-well plate and incubated overnight at the abovementioned condition. Subsequently, a volume of 0.1 ml, containing approximately 10^9 CFU/ml, of the strain ZBK1-5 and *Lacticaseibacillus rhamnosus* GG (LGG), serving as control, was inoculated into the Caco-2 cells. Following a 90 min incubation period at 37 °C under 5% CO_2 atmosphere, wells were gently washed 3 times with PBS buffer. This step aimed to eliminate any bacteria not attached to the wells. Caco-2 cells and adhered bacteria were subsequently dislodged using 1.0 ml of 0.1% Triton-x solution, and the attached bacteria were quantified through plating on MRS agar. The viability of the strain ZBK1-5 was investigated following the method of Alp and Kuleaşan [11] with modifications.

Adhesion was evaluated by calculating the percentage of bacteria that adhered relative to the total number of bacteria introduced by using the following formula:

$$\text{Adhesion percentage}(\%) = \frac{N_t}{N_0} \times 100 \qquad (2)$$

where N_t is the log number of adherent LAB cells to the Caco-2 cell, and N_0 is the log total number of LAB cells inoculated.

In vitro bacterial survival under simulated gastrointestinal conditions

The analysis followed the INFOGEST static *in vitro* digestion procedure as outlined by Brodkorb et al [12] with adaptations to simulate oral, gastric, and small intestinal digestion phases at 37 °C with gentle agitation to approximate physiological conditions inside the oral-gastro-intestinal tract. Digestion-like electrolyte solutions were added to each stage of the *in vitro* digestion process.

Aliquots of 5.0 ml of cell suspension were added with 8 ml simulated saliva juice and 2 ml alpha-amylase solution to simulate the condition of the mouth, remaining in this condition for 5 min and then adjusting the pH to 3 before proceeding to the next step. In the stomach stage, 4 mg/ml of porcine stomach pepsin (SRLChem, India) was prepared in simulated gastric juice. A volume of 8 ml of this solution was added into the cell suspension and then incubated for 2 h. Afterward, the sample was immediately analyzed for surviving cell counts during the stomach passage simulation. In the small intestinal phase, a solution that includes 1 mg/ml of porcine pancreatic pancreatin (Sigma, USA) and 8.452 g/l of porcine bile extract (Sigma) was prepared in $NaHCO_3$ 0.1 M, and the pH was adjusted to 7.0. A volume of 16 ml of intestinal juice was added into the mixture and then incubated for 4 h. The samples were taken for surviving cell counts at 0, 120, and 240 min [13].

Genome analysis

The genomic DNA of strain ZBK1-5 was extracted using the Wizard Genomic DNA Purification kit (Promega Corporation, USA). The Nodai Genome Research Centre at Tokyo University of Agriculture, Japan, performed whole-genome sequencing of strain ZBK1-5 utilizing the Illumina MiSeq and Oxford Nanopore platforms. Both short-read and long-read DNA sequencing techniques were hybridized and used to analyze genomic DNA. Short-read and long-read sequences were integrated by utilizing the *de novo* assemblers: SPAdes and Unicycler (Galaxy Version 0.5.0+galaxy1, database version 2022-09-24), to assemble the whole-genome sequence [14]. The evaluation of genomic quality and contamination was conducted using CheckM [15]. The computation of sequence similarity values between the strains and their corresponding reference strains was performed using the EzBiocloud tool. The average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values were analyzed with the JSpeciesWS web server tool (Version 4.1.1, database version 2023-12-21) [16] and the Genome-to-Genome Distance Calculator (GGDC 2.1, database version 2023) with the BLAST+ method using formula 2 [17]. According to Kim et al [18], when the ANI is greater than 95% and the dDDH is greater than 70%, it is generally regarded that the organisms in question belong to the same species. A phylogenetic tree was constructed using the TYGS web server (version v391, database version 2023) [19], utilizing whole-genome sequences. Furthermore, a genomic circular map was generated utilizing the Proksee tools (version 1.1.1, database version 2023) [20].

Determination of antimicrobial peptide genes and probiotic properties of strain ZBK1-5

The whole genome was annotated. The antibacterial peptide genes or clusters were determined by the BActeriocin GEnome mining tool, BAGEL4 (version 4.0, database version 2023) [21]. Additionally, identifying the essential genes related to probiotic properties was predicted from RAST and the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations.

Safety assessment assay

Antimicrobial resistance (AMR) gene identification

The study worked with 4 publicly available databases to identify the genetic determinants responsible for AMR in the genome. These databases include the Comprehensive Antibiotic Resistance Database (CARD) (RGI 6.0.2, CARD 3.2.7, database version 2023) [22].

Additionally, the ResFinder database (version 2.0, database version 2022-06-30) was used [23]. The DFAST server (version 1.6, database version 2022-03-24) [24] and the KEGG database (version 3.0, database version 2023-11-01) were also employed [25], specifically utilizing the BlastKOALA search tool (version 3.0, database version 2023-11-01) [26] to examine the genetic information under the category "Brite ko01504: Antimicrobial resistance genes".

Mobile genetic element identification

The study additionally focused on assessing the potential transferability of AMR genes within the genome using the Center for Genomic Epidemiology web-based tool (https://www.genomicepidemiology.org). The analysis of their occurrence in 2 mobile genetic elements, plasmids and bacteriophages, was conducted using PlasmidFinder (version 2.0.1, database version 2023-01-18). The identification of prophages within the genome was conducted utilizing the PHASTER tool. The study assessed plasmid self-transmission via conjugation, identifying transfer origins using oriTfinder.

Virulence factors and undesirable gene identification

The BlastKOALA search tool, available in the KEGG database [25], was employed to examine the presence of virulence factors and toxin genes in the genome of strain ZBK1-5.

RESULTS AND DISCUSSION

Isolation and screening of bacteriocin-producing lactic acid bacteria

Twelve LAB strains were isolated from pickled ginger in Bangkok, Thailand. They demonstrated antimicrobial activity in the range of 100–400 AU/ml after cultivation at 30 °C for 24 h in MRS broth. Among them, the CFS of strain ZBK1-5 presented the maximum antimicrobial activity (400 AU/ml) against *L. sakei* JCM 1157^{T}. Furthermore, the antimicrobial activity was lost after treatment of the CFS with Proteinase K, indicating the proteinaceous nature of bacteriocin. Consequently, strain ZBK1-5 was selected as the representative strain for subsequent investigations.

Characterization and identification of bacteriocin-producing strain

The strain ZBK1-5 was Gram-stain-positive, rod-shaped. On MRS agar plates, the colonies of strain ZBK1-5 were white with a smooth surface. The growth occurred at 15–45 °C, pH 3.0–6.0, and in the presence of 4–6% (w/v) NaCl, while a weak growth response was observed at pH 9.0, indicating adaptability across a wide pH range. The optimization for growth was observed at 30 °C, pH 6.0. It did not produce gas from glucose and was negative for catalase, aesculin hydrolase, and nitrate reduction, while being positive for arginine hydrolysis and alpha hemolysis on blood agar. The observed carbohydrate fermentation results were intriguing, as strain ZBK1-5 exhibited the ability to produce acid from almost all sugars except for D-raffinose, L-rhamnose, and D-xylose, which indicated its capacity to utilize a wide range of carbohydrates, suggesting it could potentially use various prebiotics. Based on 16S rRNA genes, the isolate ZBK1-5 (1,458 bp) was closely related to both *Lactiplantibacillus plantarum* ATCC 14917^{T} and *L. argentoratensis* DSM 16365^{T} with 100% similarity. The 16S rRNA gene sequence of strain ZBK1-5 was deposited in the DDBJ accession number LC769879 (https://www.ncbi.nlm.nih.gov/nuccore/LC769879).

Bacterial growth dynamics and antimicrobial peptide production

The dynamics of bacteriocin production and pH were investigated in MRS broth at 30 °C (Fig. 1). The bacteriocin was initially synthesized at 12 h (800 AU/ml) and reached its highest activity at 18 h (6,400 AU/ml) while the pH was lowered from 6.87 to 3.80. The results indicated that the bacteriocin was synthesized during the exponential growth phase, similar to the study of Gaspar et al [27]. The reduction in antimicrobial activity following an extended period of incubation may be related to the enzymatic degradation by proteolytic enzymes, alterations in the surrounding environmental conditions [28], adsorption into the producing strain [29], or aggregation [30].

However, the strain of *L. plantarum* encompasses both bacteriocinogenic and non-bacteriocinogenic varieties. In addition, varied characteristics of plantaricins include differing antimicrobial activity profiles and production kinetics. Bacteriocin ZBK1-5 exhibited unique characteristics such as higher antimicrobial effectiveness (6400 AU/ml) and rapid production time (18 h). In comparison, some from other known plantaricins may require extended incubation periods to reach peak activity (48 h) [31]. Plantaricin ZBK1-5 demonstrates a rapid onset of activity, beneficial for applications requiring timely antimicrobial intervention such as food preservation or probiotic development. Moreover, the observed safety profile of strain ZBK1-5 further supports its suitability for practical applications, suggesting its potential for utilization in various industrial settings.

Antimicrobial spectrum

The antimicrobial spectrum showed that bacteria belonging to the LAB group were sensitive to bacteriocin ZBK1-5 since it inhibited *P. pentosaceus* JCM 5885 (200 AU/ml). The results related to the core characteristic of bacteriocin: the ability to inhibit closely related bacterial strains [5]. Moreover, its wide range antibacterial activity explicitly targeted Gram-positive

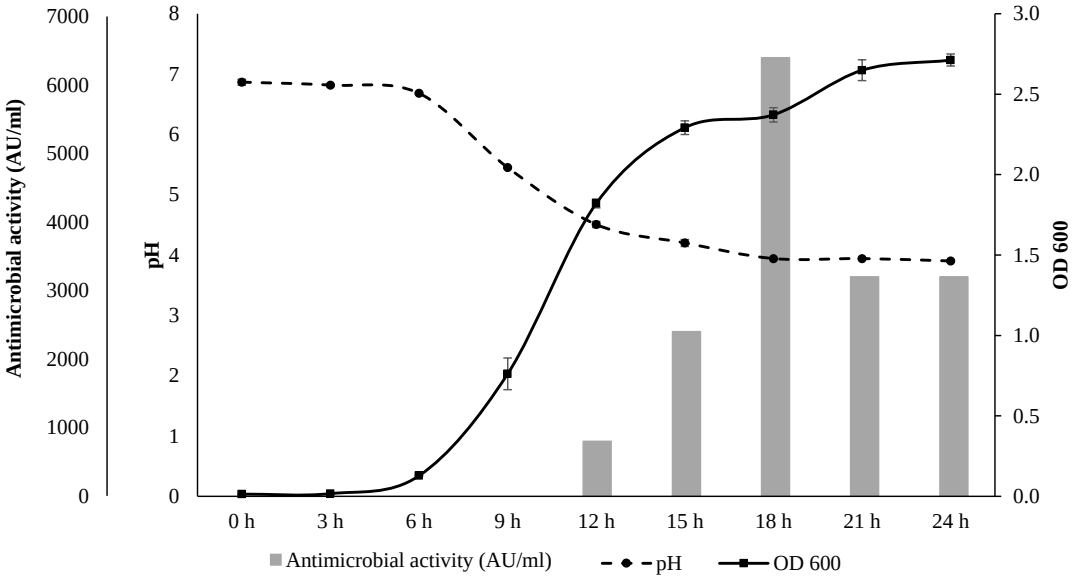

Fig. 1 Bacterial growth dynamics and bacteriocin production of *L. plantarum* ZBK1-5 cultured in MRS broth at 30 °C.

bacteria. Significantly, a multitude of pathogens was effectively eliminated, encompassing *L. innocua* ATCC 33090^{T} (400 AU/ml), *L. monocytogenes* ATCC 19115 (400 AU/ml), *S. aureus* (1600 AU/ml) ATCC 23235, *Enterococcus faecalis* JCM 5803^{T} (100 AU/ml), and *Kocuria rhizophila* MIII (100 AU/ml). Nevertheless, it was observed that Gram-negative bacteria, specifically *E. coli* O157:H7, *E. coli* ATCC 25922, *E. coli* ATCC 35401, *E. coli* F18, and *Salmonella* Typhimurium ATCC 13311, exhibited resistance to the inhibitory properties. These findings emphasized the precise and specific action against related bacterial strains [2], pointing up their potential as natural antimicrobial agents. The increasing consumer preference for chemical-free food products, driven by health-consciousness, is reshaping the market. Notably, bacteriocin production is becoming a crucial criterion in choosing probiotic strains.

Effect of temperature, pH, and enzyme on the activity of bacteriocin

The assessment of bacteriocin activity from CFS demonstrated its susceptibility to proteinase K. The bacteriocin was rendered completely inactive by proteinase K, thereby confirming the proteinaceous nature of this antimicrobial substance. The antimicrobial activity stability was observed under a wide pH range (pH 2–9 at 37 °C) and elevated temperature (100 °C for 0–30 min). Nonetheless, the completed inactivation was observed when the bacteriocin was exposed to pH 11 at 37 °C and maintained at 100 °C for 40 min. Bacteriocin ZBK1-5 was hypothesized to belong to the bacteriocin class II, specifically plantaricin, due to its noticeable attributes, including thermal and pH resistance.

Due to its rich nutritional matrix, fresh meat has a high perishability, making it a prime environment for food-borne pathogens, including *L. monocytogenes* and spoiling bacteria [32]. The intrinsic thermal stability of bacteriocin ZBK1-5 and its efficacy against *L. monocytogenes* may be appropriate for food packaging applications. Also, it could offer a promising solution to meet consumer demands for meat preservation that is high-quality, safe, easy to handle, and less chemically preserved.

Adhesion assay

The strain ZBK1-5 exhibited an adhesion rate of 90.39%. It was hypothesized that strain ZBK1-5 can encode certain cell-surface factors that play a role in adhering to mucus or intestinal epithelial cells (IECs). On the other hand, *L. rhamnosus* GG, a probiotic strain, demonstrated an adhesion rate of 67.63%.

In vitro bacterial survival under simulated gastrointestinal conditions

The viability of probiotic bacteria is essential for their beneficial health effects, as they need to survive food processing and storage and pass through the stomach with increased acidity as well as enzymes and bile salts in the small intestine. The Thailand Food and Drug Administration (Thai FDA) recommends that probiotics should contain at least 6 log CFU/g to be effective. A study monitored the viability of strain ZBK1-5 throughout the digestive process. Initially, 15.91 log CFU/ml was detected, but after passing

through the oral phase, the stomach phase (after 120 min), and the small intestine (after 240 min), the viability decreased to 10.86 log CFU/ml (68.26% viable cells), 10.60 log CFU/ml (66.62% viable cells), and 9.69 log CFU/ml (60.91% viable cells), respectively. The findings revealed a decline in viable cell count following passage through the oral phase, which stabilized upon transit through the stomach and small intestine phases. Moreover, this stability indicates suitability for colonizing the intestine and potentially conferring health benefits upon the host.

Lactiplantibacillus plantarum ZBK1-5 genome analysis

The genome size of strain ZBK1-5 (JAUTDJ000000000) was 3,445,826 bp with 44.11% GC content and 3,371 coding sequences. It comprised 6 contigs with an N50 value of 3,296,517 bp. A single CRISPR repeat with a length of 85 bp was identified, while no plasmids were detected from PlasmidFinder database version 2023-01-18 (Supplementary Table S1).

According to the analysis of the 16S rRNA gene sequence, it was found that the strain ZBK1-5 was closely related to *L. plantarum* ATCC 14917^T and *L. argentoratensis* DSM 16365^T with 100% similarity in both cases. Therefore, a whole-genomic analysis was performed to confirm the species identification of strain ZBK1-5. The phylogenomic tree depicts that the strain ZBK1-5 shared cluster with *L. plantarum* ATCC 14917^T and *L. plantarum* DSM 20174^T (noted that both strains are identical (Type strains) that have been deposited in various culture collections) (Fig. 2). The values of ANIm between strain ZBK1-5 and closely related strain including *L. plantarum* DSM 20174^T, *L. plantarum* subsp. *plantarum* ATCC 14917^T, and *L. argentoratensis* DSM 16365^T were 99.24, 99.23, and 95.66%, respectively. These ANI values are higher than the cut-off value at 95–96%, as proposed by Richter and Rosselló-Móra [16]. The investigation revealed a close genetic relationship between strain ZBK1-5 and *L. plantarum* DSM 20174^T and *L. plantarum* subsp. *plantarum* ATCC 14917^T, with dDDH values of 93.0% and 92.9%, respectively (Supplementary Table S2). The dDDH values exceeded the established threshold of 70%, commonly used for species delineation [18]. Meanwhile, the dDDH value between strain ZBK1-5 and *L. argentoratensis* DSM 16365^T was 62.8%, which falls below the recommended cut-off value. The circular map of *L. plantarum* ZBK1-5 is depicted in Fig. 3.

These results suggest that depending only on the analysis of the 16S rRNA gene was insufficient to precisely identify bacterial species within this particular group, especially in the case of *L. plantarum* and *L. argentoratensis*. Therefore, all genomic data provided evidence that the strain ZBK1-5 was identified as *L. plantarum*.

Determination of antimicrobial peptide genes and probiotic properties of strain ZBK1-5

BAGEL4 identified 2 bacteriocin genetic clusters in the genome of strain ZBK1-5. The result showed the presence of a gene cluster encoding the production of Plantaricin. This cluster includes 18 genes (Table 1): Plantaricin K, J, N, A, F, and E (Structural gene Plantaricin KJ, N, A, and EF) with bit scores 114.005, 112.464, 108.227, 92.434, 107.071 and 112.464, respectively. The genes for immunity (*PlnM* and *PlnI*), transport (*LanT*, *PlnG*, and *PlnS*), modification (*PlnO* and *PlnY*), and regulation (*PlnB*, *PlnC*, and *PlnD*) were all fully functioning and had all the supporting components needed for expression. The group of genes of Plantaricin is shown in Fig. 4a. Plantaricin A is part of the operon regulating gene cluster expression in bacteriocin synthesis, while Plantaricin N functions as a putative prebacteriocin, possessing a GG-leader sequence that promotes the export of two-peptide bacteriocins Plantaricin KJ and EF. The presence of the 5 genetic clusters of plantaricin (*pln*) mentioned previously was considered a unique characteristic of *L. plantarum* [33]. These clusters were typically found in bacteriocin operon (*plnNEFI*) and transportation operon, serving as common preservative components. The smaller conservative parts include a cluster of genes responsible for regulating the expression of Plantaricin, while the remaining 2 or 3 groups of bacteriocin operons were also present.

Furthermore, the BAGEL4 analysis found the second cluster: Plantaricin W (*Plwβ* and *Plwα*) genes and identified the presence of a modification gene (*LanM*) associated with the modification of Plantaricin W. The presence of genes involved in activating extracellular proteins, specifically the protease (*EpiP*) (Fig. 4b). However, further gene group searches showed that the immunity genes and genes associated with bacteriocin expression were not detected. Hence, it was impossible to definitively ascertain whether *L. plantarum* ZBK1-5 synthesizes Plantaricin W (*Plwβ* and *Plwα*) (Fig. 4b).

From the mentioned results, *L. plantarum* ZBK1-5 exhibited a complete genetic similarity of 100% in the genes responsible for the production of Plantaricin KJ, EF, A, and N. Two-peptide bacteriocins plantaricin KJ and EF are the main bacteriocins.

Probiotic strains are essential microorganisms that can adjust to challenging conditions and endure acidic and bile salt conditions. The genomic data of *L. plantarum* ZBK1-5 demonstrated the presence of genes responsible for modulation of the immune system (3 genes), adhesion or interaction with the host (7 genes), acid stress (16 genes), bile resistance (9 genes), fatty acid synthesis (3 genes), lactate synthesis (1 gene), and transcriptional regulator (2 genes). These genetic elements contribute to survival in acidic pH and prevent pathogen colonization in a gut environment (Supplementary Table S3).

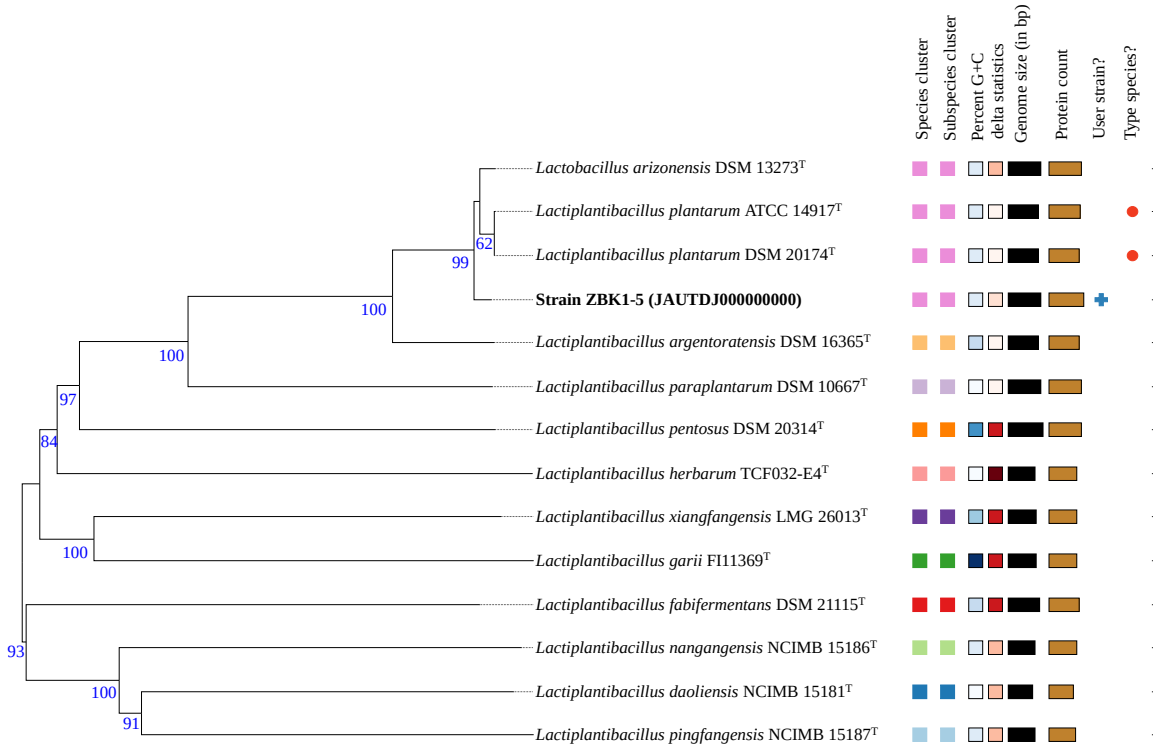

Fig. 2 Phylogenomic tree of strain ZBK1-5 and closely related type strains constructed by the Type (Strain) Genome Server (TYGS).

Table 1 The genes encoding the production of Plantaricin KJ, N, A, and EF in the *L. plantarum* ZBK1-5 genome.

Gene name	Function	Similarity (%)	Gnen length (bp)
Bacteriocin Core Peptide			
173.2;Plantaricin_K (Bit score 114.005)	ComC; Bacteriocin_IIc; 173.2;Plantaricin_K	100	171
172.2;Plantaricin_J (Bit score 112.464)	172.2;Plantaricin_J	100	165
174.2;Plantaricin_N (Bit score 108.227)	Bacteriocin_IIc; 174.2;Plantaricin_N	100	165
167.2;Plantaricin_A (Bit score 92.4337)	Antimicrobial17; Bacteriocin_IIc; 167.2;Plantaricin_A	100	153
171.2;Plantaricin_F (Bit score 107.071)	ggmotif; Lactococcin; Bacteriocin_IIc; 171.2;Plantaricin_F	100	156
170.2;Plantaricin_E (Bit score 112.464)	170.2;Plantaricin_E	100	168
Modification			
GlyS	*PlnO*	100	1,197
orf00063	*PlnY* Homologous to plasmid maintenance system antidote proteins	100	291
Immunity			
orf00021	Putative bacteriocin Immunity protein	32.02	666
orf00027	Immunity protein *PlnM*	100	198
orf00031	Immunity protein *PlnI*	29.86	744
orf00042	Immunity protein *PlnI*	99.22	789
Transport & Leader cleavage			
LanT	Bacteriocin ABC-transporter, ATP-binding, and permease protein *PlnG*	99.85	2,070
HlyD	ABC-transporter *PlnH*	99.56	1,374
orf00055	*PlnS*	94.12	687
Regulon			
orf00036	*PlnB* Bacteriocin production-related histidine kinase	28.39	1,326
orf00037	Response regulator *PlnC*, activator	97.30	666
orf00038	response regulator *PlnD*	96.76	741

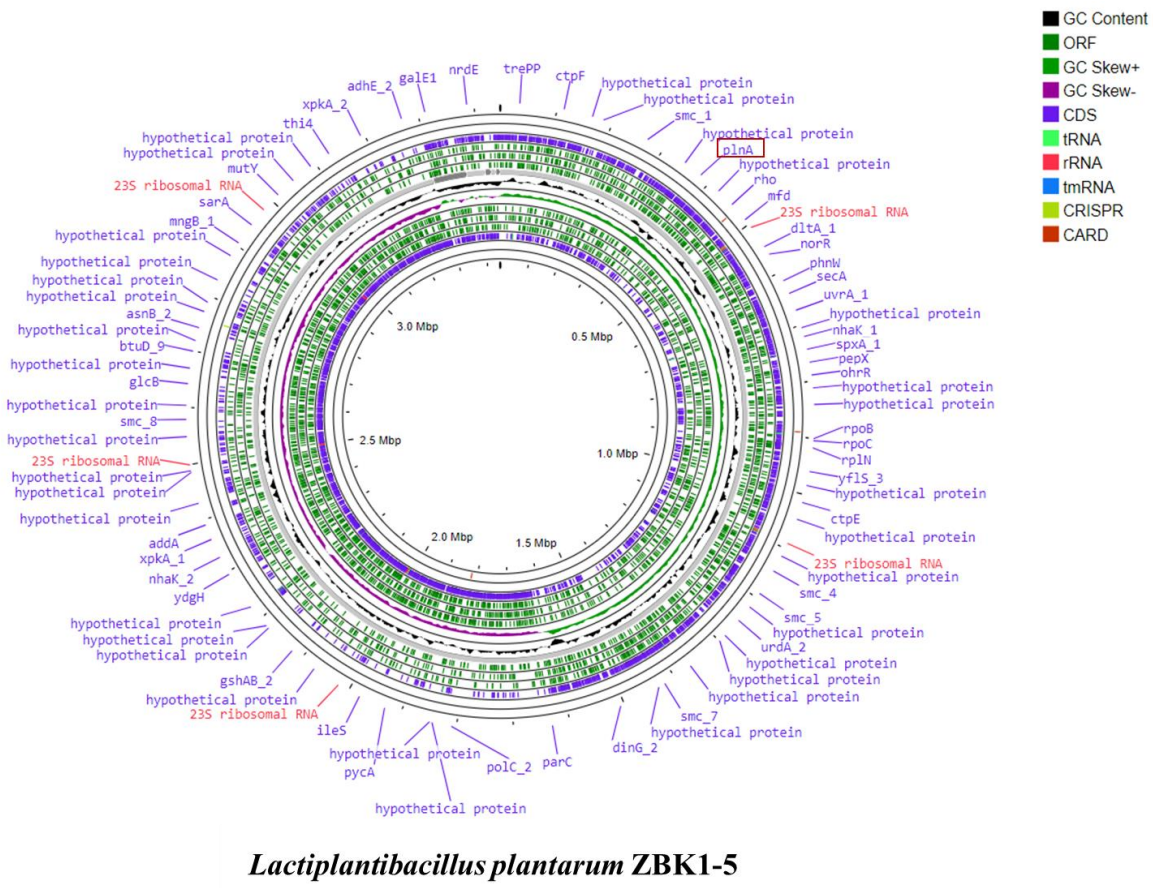

Fig. 3 A circular genomic map of *L. plantarum* ZBK1-5 constructed utilizing the Proksee server. The visualization of gene features on genomes was represented as arrows rotating along the circumference.

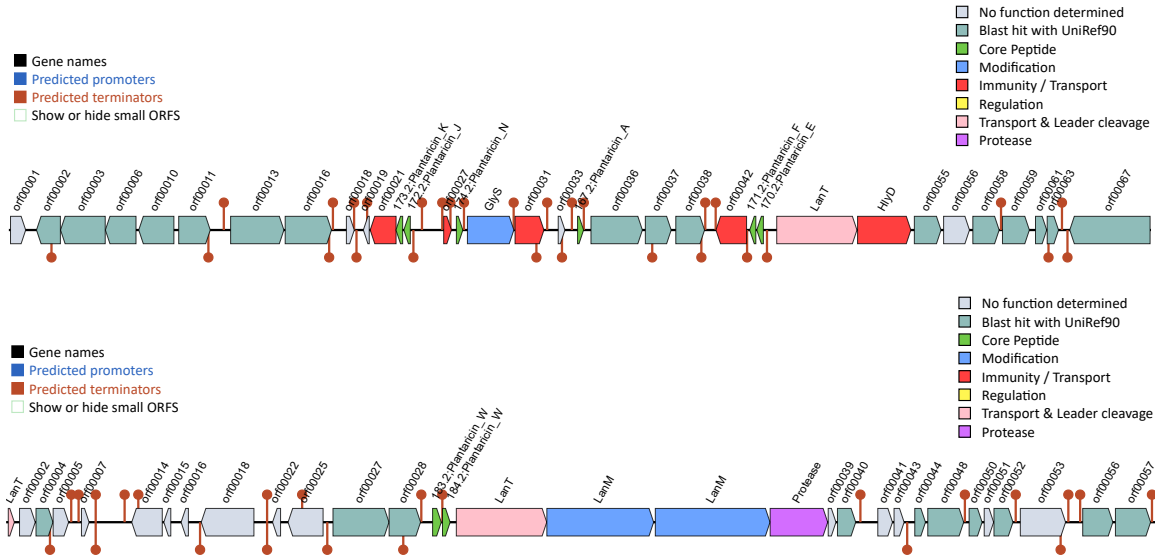

Fig. 4 The gene cluster encoding the production of Plantaricin KJ, N, A, and EF (a) and Plantaricin W (*Plwβ* and *Plwα*) (b) in strain ZBK1-5 using the online BAGEL4 web-based tool.

The presence of adhesins within probiotic cell walls is crucial for adhesion. Additionally, surface proteins dependent on sortase contribute to the adhesion ability and the maintenance of intestinal homeostasis [34]. A comprehensive set of 12 genes associated with acid and bile salt stress were identified. Among these genes, the atp operon encodes the F_1F_0-ATPase, a proton-pumping enzyme essential in maintaining a neutral pH [35]. The enzyme S-Ribosylhomocysteinase (*luxS*) is vital in synthesizing autoinducer-2, facilitating stress resistance and adherence to intestinal epithelial cells [36]. The elongation factor Tu and chaperonin GroEL have been related to the processes of adhesion and immunomodulation [37]. Probiotics are known to have an essential function within the gastrointestinal tract of the host organism, as they are involved in synthesizing various micronutrients and substances such as amino acids, fatty acids, oligosaccharides, vitamins, and enzymes. Gaining insights into the molecular mechanisms underlying the probiotic effects of *L. plantarum* ZBK1-5 will impressively contribute to future application development.

Nevertheless, the intricacy of the host-microbe interactions or the environmental variables in the gut may not be considered by *in silico* analysis, which is dependent on predictions and correlations. In conclusion, even though *in silico* research can offer insightful information about possible probiotic qualities of a substance, it is typically insufficient to determine the microorganism probiotic status. Complete validation of probiotic capabilities usually requires both *in vitro* and *in vivo* investigations.

Safety assessment of *Lactiplantibacillus plantarum* ZBK1-5

To perform thorough safety assessments for probiotic applications, it is essential to obtain genomic sequences for the proposed strain [38]. The genomic findings presented in this study provided evidence for the probiotic capabilities of strain ZBK1-5, showing that it has a group of genes potentially associated with various probiotic characteristics. According to the results shown in Table 2 of the PathogenFinder analysis, it was determined that *L. plantarum* ZBK1-5 was classified as a non-human pathogen, confirming its suitability for probiotic application.

Antimicrobial resistance (AMR) gene identification

The ResFinder database identified no antibiotic-resistant genes. According to the CARD tool, it did not identify any antibiotic-resistant gene within the genome of *L. plantarum* ZBK1-5 with perfect and strict program settings. However, when using a strict criterion (perfect/strict/loose option in CARD), one gene, *vanY*, was identified. A total of 197 genes were identified from CARD as being associated with antibiotic resistance found in the loose criterion. These genes exhibited a range of 19–61% identity and 16.0–30.7% coverage. As a result of the low stringency of the search criteria, most of the retrieved results did not match the actual AMR genes.

Nevertheless, a single gene exhibiting 29.63% similarity to a vancomycin resistance gene (*vanY*) was detected from the strict hits. Notably, *vanY* is commonly associated with microbial metabolisms such as lactic acid production. The Resfinder and CARD databases focus primarily on antimicrobial resistance genes found in pathogenic bacteria, frequently neglecting those found in non-pathogenic bacteria.

Meanwhile, the KEGG database search identified 7 AMR-related genes in the ZBK1-5 (Supplementary Table S4), including the *vanY* gene. The genome contains genes linked to efflux pumps, potentially causing multidrug resistance. Additionally, it harbors vancomycin (*vanX*) and macrolide (*msrA*) resistance genes, possibly conferring resistance to erythromycin and beta-lactam antibiotics. According to Chokesajjawatee et al [5] explained that AMR genes in the genome do not necessarily ensure resistance; therefore, gene expression and substrate specificity significantly affect the resistance phenotype. The fact that all the AMR-related genes discovered in strain ZBK1-5 were also found in other probiotics, including strains 299V, JDM1, ST-III, and WCFS154, suggests that probiotics commonly contain these genes. Therefore, it has been noted in some earlier investigations that LAB strains may still show phenotypic susceptibility to common antibiotics despite AMR genes, highlighting the possibility that the link between phenotype and genotype is not always exact.

Further *in vitro* investigations on antibiotic resistance may be necessary, as various factors, including the level of gene expression and the specificity of the expressed products towards different substrates, can influence the modulation of genes associated with antibiotic resistance.

Mobile genetic elements identification

Antibiotic resistance on mobile genetic elements such as plasmids or phage regions can transfer between bacteria, posing a greater risk than intrinsic resistance. Strain *L. plantarum* ZBK1-5 contained no plasmids (undetected plasmids were in the PlasmidFinder database). This result suggested that antibiotic resistance genes of this strain were incapable of self-transmission through conjugative transfer. Upon conducting an analysis of antibiotic-resistance genes within the genome of the ZBK1-5 strain, it was observed that reported genes were located on the chromosome and not within the phage regions. Therefore, it was determined that strain ZBK1-5 shows a diminished possibility of horizontal gene transfer of antibiotic resistance.

Table 2 Pathogenicity prediction, prophage detection, and antibiotic resistance genes analysis from PathogenFinder of CGE and CGE, PHASTER, CARD, and ResFinder of *L. plantarum* ZBK1-5 and *L. plantarum* 299V.

Attribute/Strain	*L. plantarum* ZBK1-5	*L. plantarum* 299V
Probability of being a human pathogen	0.201	0.185
Input proteome coverage (%)	0.65	0.48
Matched pathogenic family	0	0
Matched not pathogenic family	21	15
Conclusion	Non-human pathogen	Non-human pathogen
No. of phage	6	4
No. of Plasmid	0	2 (rep28, 98.17% identity; rep38, 99.0% identity)
Antibiotic resistance gene (ARG)		
CARD:		
- No. of perfect hit	0	0
- No. of strict hit	1	0
- No. of loose hit	197	194
ResFinder	*ClpL*	No resistance

Virulence factors and undesirable gene identification

This analysis discovered that the hemolysin III gene was the only toxin gene present in the genome of *L. plantarum* ZBK1-5. The identification of the hemolysin gene was verified through a manual investigation, which revealed a 100% sequence identity to the *Lactobacillus* hemolysin III family. This finding further confirms the predicted nature of the protein as a membrane channel-forming protein. It was essential to mention that the gene was also detected in various commercially available probiotics, including the GRAS probiotic strain *L. plantarum* 299V, which is widely used in commercial applications as well as numerous other *Lactobacillus* strains recorded in the GenBank database. The present study involved the utilization of sheep-blood agar for hemolysis experiments. The results revealed the presence of an obscure zone of hemolysis surrounding the bacterial growth, which was similar to the surrounding area of the probiotic strain *L. plantarum* 299V (alpha hemolysis) as observed in the experiments studied by Chokesajjawatee et al [5]. These results suggest a similarity in the hemolysis showed by both strains. Given the wide prevalence of the hemolysin III gene in various species of *Lactobacillus* and the well-known safety and commercial availability of strains containing this gene in numerous countries, it is reasonable to conclude that the presence of this gene in the bacterium does not present a safety concern as long as no other pathogenic genes are detected in the genome.

The search was conducted on the KEGG database; 2 specific genes associated with the production of D-lactic acid were found within the genome of *L. plantarum* ZBK1-5. These genes were identified as lactate racemase (*larA*) and D-lactate dehydrogenase (*ldhA*). D-lactic acid is a crucial component of peptidoglycan. The synthesis of D-lactic acid in the cell walls of various gram-positive bacteria such as *L. plantarum* has been regarded as an inherent property. Moreover, it is noteworthy that D-lactic-producing bacteria such as *Lactobacillus* and *Bifidobacterium* species are indigenous microbial communities commonly present in the human gastrointestinal tract. These bacteria are also frequently encountered in various food sources, including Yogurt and other fermented products, which have been consumed safely for an extended period.

Furthermore, it should be noted that the bacterial species *Lactobacillus* probiotics have been identified as safe and are classified in the qualified presumptive of safety (QPS) system [39]. Hence, it is advisable to exercise general precautions when consuming D-lactic-producing bacteria, particularly for individuals at a heightened risk of developing D-lactic acidosis such as patients diagnosed with short-bowel syndrome or carbohydrate malabsorption. Carbohydrate malabsorption refers to the impaired ability of the body to absorb carbohydrates effectively.

Furthermore, the KEGG database was used to analyze the genome of *L. plantarum* ZBK1-5, revealing the presence of choloylglycine hydrolase. This gene indicates the ability of organisms to deconjugate bile salts, which can be advantageous for the survival of bacteria and reduce cholesterol levels. However, higher levels of deconjugated bile salts can negatively affect digestion, disturb the intestinal environment, and potentially generate carcinogenic secondary bile salts, potentially promoting colorectal cancer development [40]. Notably, the study found no genes related to the biosynthesis of secondary bile salts, other than choloylglycine hydrolase, in *L. plantarum* ZBK1-5. The result suggested that the inability of the strain to produce harmful secondary bile products could enhance its survival, indicating no safety issues due to its inherent characteristics.

Lastly, no genes related to the synthesis of biogenic amines, including cadaverine, putrescine, spermidine, spermine, ornithine, histamine, tyramine, and

tryptamine, were found in the genetic analysis of ZBK1-5. Therefore, it could be concluded that this specific strain cannot generate biogenic amines, thus reducing potential food safety hazards.

CONCLUSION

In this study, strain ZBK1-5, isolated from pickled ginger, produces bacteriocins, including plantaricin KJ, EF, A, and N, with potent antimicrobial activity against Gram-positive bacteria. The *in vitro* result confirmed that bacteriocin ZBK1-5 exhibited inhibitory effects against *L. monocytogenes*, *L. innocua*, and *S. aureus*, significant contributors to food spoilage and poisoning. In addition, the result of genome analysis highlights that the strain ZBK1-5 also exhibited probiotic properties with existing gene-related probiotic properties, and safety evaluations indicated a low risk of antibiotic resistance gene transfer. *In vitro* investigations demonstrated that strain ZBK1-5 exhibited a high adhesion rate and was tolerant to acid and bile, which was attributed to its survival in simulated gastrointestinal conditions. This underscores its considerable promise for probiotic applications. In future research, the spectrum of bacteriocin will be extended for use in medical, agricultural, aquatic, food, and industrial applications.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2024.046.

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Appendix A. Supplementary data

Table S1 Genomic features of *L. plantarum* ZBK1-5 and *L. plantarum* 299V.

Attribute	*L. plantarum* ZBK1-5	*L. plantarum* 299V
Source	Pickled ginger	Healthy human intestinal mucosa
Accession no.	JAUTDJ000000000	LEAV00000000
Genome size (bp)	3,445,826	3,302,055
Plasmids	0	2 (rep28, 98.17% identity; rep38, 99.0% identity)
G+C content (%)	44.11	44.4
Genome coverage	200x	48x
N_{50}	3,296,517	173,004
L_{50}	1	8
No. of contig	6	67
No. of subsystem	232	232
No. of coding sequences	3,371	3,264
No. of RNA	87	60
No. of CRISPRS	1	0

Table S2 Genomic data: 1, strain ZBK1-5; 2, *Lactiplantibacillus plantarum* DSM 20174^T (GCA_014131735.1); 3, *Lactiplantibacillus plantarum* subsp. *plantarum* ATCC 14917^T (GCA_000143745.1); 4, *Lactiplantibacillus argentoratensis* DSM 16365^T (GCA_001435215.1); and 5, *Lactiplantibacillus plantarum* subsp. *argentoratensis* DSM 16365^T (GCA_003641165.1).

Query genome	Reference genome	ANIb	ANIm	% dDDH	Model C.I. (%)	Distance	Prob. DDH ⩾ 70%	G+C difference
1	2	98.80	99.24	93.0	91.1–94.5	0.0088	96.70	0.39
1	3	98.73	99.23	92.9	90.9–94.5	0.0090	96.67	0.37
1	4	94.81	95.66	62.8	59.9–65.6	0.0469	61.20	0.91
1	5	94.83	95.71	63.2	60.3–66.0	0.0463	62.25	0.86

C.I., confidential interval; ANI, average nucleotide identity; dDDH, digital DNA-DNA hybridization; and Prob. DDH, probability DNA-DNA hybridization.

Table S3 Potential genes associated with probiotic characteristics in the *L. plantarum* ZBK1-5 genome.

Putative function	KEGG_ID	Gene Name
Modulation of immune system/Acid stress	K03695	*clpB*; ATP-dependent Clp protease ATP-binding subunit ClpB
	K03101	*lspA*; signal peptidase II [EC:3.4.23.36]
	K02358	*tuf*, TUFM; elongation factor Tu
Adhesion or interaction with the host	K07284	*srtA*; sortase A [EC:3.4.22.70]
	K03740	*dltD*; D-alanyl-lipoteichoic acid biosynthesis protein DltD
	K03367	*dltA*; V/D-alanine-poly(phosphoribitol) ligase subunit 1
	K03101	*lspA*; signal peptidase II [EC:3.4.23.36]
	K02358	*tuf*, TUFM; elongation factor Tu
	K01915	*glnA*, GLUL; glutamine synthetase [EC:6.3.1.2]
	K01810	*GPI*, pgi; glucose-6-phosphate isomerase [EC:5.3.1.9]
Acid stress	K02114	ATPF1E, *atpC*; F-type H+-transporting ATPase subunit epsilon
	K02112	ATPF1B, *atpD*; F-type H+/Na+-transporting ATPase subunit beta [EC:7.1.2.2 7.2.2.1]
	K02111	ATPF1A, *atpA*; F-type H+/Na+-transporting ATPase subunit alpha [EC:7.1.2.2 7.2.2.1]
	K02115	ATPF1G, *atpG*; F-type H+-transporting ATPase subunit gamma
	K02113	ATPF1D, *atpH*; F-type H+-transporting ATPase subunit delta
	K02109	ATPF0B, *atpF*; F-type H+-transporting ATPase subunit b
	K02108	ATPF0A, *atpB*; F-type H+-transporting ATPase subunit a
	K02110	ATPF0C, *atpE*; F-type H+-transporting ATPase subunit c
	K03553	*recA*; recombination protein RecA
	K07816	*relA*; E2.7.6.5; GTP pyrophosphokinase [EC:2.7.6.5]
	K04077	*groL*; groEL, HSPD1; chaperonin GroEL [EC:5.6.1.7]
	K01876	*aspS*; DARS2, aspS; aspartyl-tRNA synthetase [EC:6.1.1.12]
	K04043	*dnaK*, HSPA9; molecular chaperone DnaK
	K03686	*dnaJ*; molecular chaperone DnaJ
	K04042	*glmU*; bifunctional UDP-N-acetylglucosamine pyrophosphorylase/ glucosamine-1-phosphate N-acetyltransferase [EC:2.7.7.23 2.3.1.157]
	K07173	*luxS*; S-ribosylhomocysteine lyase [EC:4.4.1.21]
Bile resistance	K02564	*nagB*, GNPDA; glucosamine-6-phosphate deaminase [EC:3.5.99.6]
	K01937	*pyrG*, CTPS; CTP synthase [EC:6.3.4.2]
	K01887	*argS*; RARS, argS; arginyl-tRNA synthetase [EC:6.1.1.19]
	K02982	RP-S3, *rpsC*; small subunit ribosomal protein S3
	K02988	RP-S5, MRPS5, *rpsE*; small subunit ribosomal protein S5
	K02926	RP-L4, MRPL4, *rplD*; large subunit ribosomal protein L4
	K02931	RP-L5, MRPL5, *rplE*; large subunit ribosomal protein L5
	K02933	RP-L6, MRPL6, *rplF*; large subunit ribosomal protein L6
	K07305	*msrB*; peptide-methionine (R)-S-oxide reductase [EC:1.8.4.12]
Fatty acid synthesis	K00645	*fabD*, MCAT, MCT1; [acyl-carrier-protein] S-malonyltransferase [EC:2.3.1.39]
	K09458	*fabF*, OXSM, CEM1; 3-oxoacyl-[acyl-carrier-protein] synthase II [EC:2.3.1.179]
	K00208	*fabI*; enoyl-[acyl-carrier protein] reductase I [EC:1.3.1.9 1.3.1.10]
Lactate synthesis	K00027	ME2, *sfcA*, *maeA*; malate dehydrogenase (oxaloacetate-decarboxylating) [EC:1.1.1.38]
Transcriptional regulator	K03708	*ctsR*; transcriptional regulator of stress and heat shock response
	K03705	*hrcA*; heat-inducible transcriptional repressor

Table S4 Antimicrobial resistance gene list in the ZBK1-5 genome.

No.	Function	KEGG ID	Gene (length)	Gene name
1	Macrolide resistance	K18231	lpl:lp_0215 (506) (196433..197953)	*msr*, *vmlR*; macrolide transport system ATP-binding/permease protein
2	Phenicol resistance	K19271	lpl:lp_1787 (222) (1620066..1620734)	*catA*; chloramphenicol O-acetyltransferase type A [EC:2.3.1.28]
3	beta-Lactam resistance	K17836	lpl:lp_2341 (376) (2116268..2117398)	*penP*; beta-lactamase class A [EC:3.5.2.6]
4	Vancomycin resistance	K07260	lpl:lp_1010 (257) (931038..931811)	*vanY*; zinc D-Ala-D-Ala carboxypeptidase [EC:3.4.17.14]
5	Vancomycin resistance	K08641	lpl:lp_0769 (185) (702348..702905)	*vanX*; zinc D-Ala-D-Ala dipeptidase [EC:3.4.13.22]
6	Multidrug resistance	K18104	lpl:lp_2497 (587) (2227238..2229001)	*bmrA*; ATP-binding cassette, subfamily B, bacterial AbcA/BmrA [EC:7.6.2.2]
7	Multidrug resistance	K18907	lpl:lp_1407 (483) (1288236..1289687)	*norG*; GntR family transcriptional regulator, regulator for abcA and norABC

The data was obtained from the KEGG database.