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Pangenome analysis and "in silico" overview of carbohydrate and vitamin metabolism of *Lactiplantibacillus plantarum* strain TRA56 obtained from lactic-acid fermented beverage known as Shalgam

Ahmet E. Yetiman

Erciyes University, Faculty of Engineering, Food Engineering Department, 38030, Kayseri, Türkiye

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Corresponding Author
Tel.: +90 352 207 6666/ Ext: 32732
E-mail: ahmetyetiman@erciyes.edu.tr

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Abstract

Lactiplantibacillus plantarum is a highly adaptable and versatile species that can be found in a diverse range of niches. It can generate bioactive compounds, including riboflavin, folic acid, and exopolysaccharides, which contribute to the functional qualities of fermented foods. This study aimed to provide a brief evaluation of the overall genetic characteristics, as well as the carbohydrate and vitamin metabolisms of the *Lb. plantarum* TRA56 (The TRA56). Its genome size was 3,242,215 bp with a 44.41% GC content, including 3.030 coding sequences, 62 tRNA genes, 3 rRNA, 4 ncRNA, 1 CRISPR array, 69 pseudogenes, and 2 intact phages. Its genome had 195 singleton genes that differed from those found in other strains analyzed in the pangenome. Moreover, it has been found that TRA56 possesses a facultative heterofermentive carbohydrate metabolism as a result of the existence of 6-phosphofructokinase (*pfk*) and fructose-bisphosphate aldolase (*fbaA*) enzymes. The strain's capacity to synthesize vitamins B2, B5, and B9 has been verified using computational tools. Cholesterol assimilation (46.28±1.6%) and antioxidant activity against DPPH (59.04±0.43%) and $ABTS^{+}$ (77.76±0.33%) were verified via *in vitro* tests. The study of the TRA56's genetic and metabolic characteristics demonstrated its potential as a probiotic food supplement, offering functional advantages to the host.

Introduction

Lactobacilli are a species of bacteria belonging to the lactic acid bacteria group (LAB). They can be found in fermented foods and in the gastrointestinal ecology of both humans and animals (Buron-Moles et al., 2019; Evanovich et al., 2019). Due to their widely proven beneficial effects on health, they are extensively employed in the food biotechnology field. Members of *Lactobacillus* possess the ability to produce diverse metabolic products, including bacteriocins and numerous organic acids. These substances create an unfavorable environment that inhibits the growth of pathogenic and saprophytic microbes. Various kinds of *Lactobacillus* are known to be generally recognized as safe (GRAS) and/or presumably qualified as safe (QPS) because of their putative probiotic characteristics (Seddik et al., 2017). Probiotics, as defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP), are living microorganisms that do not cause disease and provide health advantages to the host when given in sufficient quantities (Hill et al., 2014). Each strain of these microorganisms, identified as probiotics, has distinct and unexpected features that support novel probiotic research. Probiotics' biological activities are strain-specific, and these traits cannot be generalized to other strains of the same species (Lau & Quek, 2024). Thus, when a noteworthy new species is

obtained from any niche, it is necessary to uncover these differences at the genomic level through pangenomic approaches (Wang et al., 2021; Yetiman et al., 2024).

The classification of the genus *Lactobacillus* has been revised based on whole-genome sequence data, leading to the establishment of 25 distinct genera, 23 of which were proposed to be novel (Zheng et al., 2020). Following the implementation of the updated taxonomic classification, *Lactobacillus plantarum* has been reclassified under *Lactiplantibacillus* and renamed *Lactiplantibacillus plantarum* (Qiao et al., 2022; Zheng et al., 2020). *Lb. plantarum* strains are found in a variety of environments, including meat, dairy products, vegetables, wine, silage, gastrointestinal, urogenital, and vaginal tracts, but various studies revealed that sourced strains demonstrated differences at their genetic and physiological levels (Fidanza et al., 2021; Seddik et al., 2017). This ubiquitous characteristic signifies the remarkable adaptability and metabolic pathway variations of *Lb. plantarum* (Fiocco et al., 2010). In addition, *Lb. plantarum* is utilized in the production of fermented vegetables, drinks, kefir, cheese, and fermented meat products (Behera et al., 2020). It enhances the nutritional content, flavor, and preservation of these fermented foods (Russo et al., 2017). Moreover, numerous strains of *Lb. plantarum* are known to have probiotic properties, and they have been exploited for the development of possible live oral vaccines as well as therapeutic and functional foods (Parente et al., 2010). Shalgam is a traditional Turkish beverage made through lactic acid fermentation. It is identifiable by its reddish color, sour-soft taste, and fuzzy appearance (Tanguler et al., 2021). Shalgam's high concentration of lactic acid bacteria in its microbiota has been associated with potential health benefits in combating a variety of health risks and illnesses (Ekinci et al., 2016; Yetiman et al., 2022). Based on several studies, *Lactiplantibacillus plantarum* and *Lactocaseibacillus paracasei* are reported to be prevailing strains in microbial distribution (Agirman et al., 2021; Tanguler & Erten, 2012).

In previous studies, *Lb. plantarum* was isolated from Shalgam, but this does not preclude further research. Even if isolated from the same ecological environment, each new bacterial species may show different characteristics due to evolutionary adaptation and transfer of genetic material between species and strains, depending on their gene numbers and associated metabolic processes (D'Souza et al., 2018; Duar et al., 2017; Xenophontos et al., 2021). On the other hand, a previous pan-genome study revealed significant variations in gene composition and survival strategies amongst 108 genetically clustered *L. plantarum* strains, regardless of habitat (Choi et al., 2018). Therefore, these variations must be understood at the genomic level. Furthermore, to preserve the traditional aspects (microbiota-associated technological properties) of shalgam, it is critical to understand the genomic, physiological, and metabolic characteristics of *Lb. plantarum*, one of major species used in its production (Erginkaya & Turhan, 2016; Kesmen et al., 2012; Tanguler & Erten, 2012). The strain TRA56 has different genomic characteristics from strain DY46 proposed by Yetiman et al. (2022) and other well-known *Lb. plantarum* strains studied, therefore this study aims to explain genomic and certain metabolic properties of *Lb. plantarum* TRA56 by employing pangenomic and other annotative bioinformatic tools.

Materials and Methods

Isolation of bacterial strain and growth conditions

The *Lb. plantarum* TRA56 strain was obtained from a commercially available Turkish fermented shalgam juice (pH: 3.30) produced by Turnib, a local company in Mersin, Türkiye. A 10 mL aliquot of the shalgam sample was diluted with 90 mL of Maximum Recovery Diluent (Merck, GmbH, Darmstadt, Germany) in a Schott container and homogenized for 1 minute using a high-speed vortex (MS-3 Basic, IKA-Werke GmbH, Staufen, Germany). Serial decimal dilutions were made using the same diluent, and 100 µL of each dilution was spread onto MRS agar (Merck). The plates were incubated at 30°C for five days in an anaerobic environment. The TRA56 isolate was chosen from a 10^{-5} dilution and underwent colony purification twice. Subsequently, gram staining and catalase assays were conducted on the pure isolate of the TRA56. Cryopreserved stocks of the TRA56 were cultivated in MRS broth (Merck) containing 25% glycerol and stored at -80°C.

Carbohydrate fermentation assay

The carbohydrate fermentation pattern of the *Lb. plantarum* TRA56 strain were examined via an API 50 CHL kit (BioM´erieux, Marcy l'Etoile, France). The kit contained 49 unique carbohydrate tests, and the analysis was performed as per the instructions provided by the manufacturer.

DNA extraction, identification, Whole-Genome Sequencing, and assembly

DNA extraction was performed by using the Pure Link Genomic DNA Mini Kit (Invitrogen, Thermo-Fisher Scientific, Carlsbad, CA, USA) and identification of the TRA56 using 16S rRNA was performed as explained by Yetiman et al. (2022). The Agilent 5400 Fragment Analyzer was used to determine the gDNA concentration in the TRA56 sample, as well as its integrity and purity. The acquired gDNA was used to prepare a DNA library with the Nextera XT DNA Library Preparation Kit (Illumina, USA) before being transported to the next-generation sequencing platform. The impurities in the library were eliminated following the AMPure XP bead (Beckman Coulter, UK) manufacturer's protocols. The whole genome sequencing was conducted by using the NovaSeq 6000 platform with 2x150 bp pair-end (PE) chemistry. After sequencing, the

whole genome data was retrieved in "fastq.gz" format. Trimmomatic software (v0.36) was used via Kbase for trimming adapter and barcode sequences from genome data (Bolger et al., 2014). Later, trimmed raw data was assembled by using SPAdes v3.13.0 with a CheckM completeness score of 100 as the genome quality parameter. (Prjibelski et al., 2020).

Bioinformatic analyses

Genome annotation was carried out utilizing BV BRC and NCBI-PGAP (prokaryotic genome annotation pipeline; GeneMarkS-2+) for comparison (Olson et al., 2023; Tatusova et al., 2016).

BLAST ring alignment was conducted using BRIG v 0.95 to compare the TRA56 strain with other *Lb. planta rum* strains (Alikhan et al., 2011). Pangenome analysis was fulfilled using the PanACoTA software through panexplorer, with a minimum percentage identity threshold of 80% (Dereeper et al., 2022; Perrin & Rocha, 2021). An alpha value of pangenome was calculated via the *micropan* R package (Snipen & Liland, 2015). Average nucleotide identity (ANI) values were calculated via the Fast ANI tool (Jain et al., 2018). The eggNOG mapper was utilized to predict clusters of orthologous groups (COGs) by analyzing genomic FASTA sequences with the Prodigal Gene Prediction Method (Huerta-Cepas et al., 2019).

Moreover, for easier comparison, the metabolic p athways of the TRA56 were predicted using BlastKOALA and RASTtk to search the KEGG database (Brettin et al., 2015; Kanehisa et al., 2016). A resistome screening was conducted by analyzing the entire genome sequence of the TRA56 strain using the BV-BRC and KEGG databases. The prophage elements in the TRA56 were identified using the PHASTEST (PHAge Search Tool with Enhanced Sequence Translation) webserver (Wishart et al., 2023). The protein-coding sequences retrieved by PHASTEST were subjected to screening against the non-redundant protein (NR) database using protein BLAST. This process aims to identify the genes that have been horizontally transferred. If a gene's homologous protein has a similarity of at least 80% with a bacterium other than *Lb. plantarum*, the gene is classified as being horizontally transmitted. Similar to the phage elements, the resistome was screened for horizontal gene transfer. The secondary metabolite production-responsible gene clusters on the genome of the TRA56 were screened against the antiSMASH (v. 7.1.0) webserver database (Blin et al., 2023). The R Studio environment was utilized for data visualization by employing the *ggplot2*, *pheatmap*, and *upsetR* packages (R-Core-Team, 2021). The whole genome sequence of *Lb. plantarum* TRA56 has been deposited NCBI under accession number of JBBWZX000000000 (accession of contigs ranges between JBBWZX000000000.1-JBBWZX000000000.136).

General probiotic features examination

General probiotic features of *Lb. plantarum* TRA56 have been evaluated by β-hemolysis test on blood agar, cellular auto-aggregation assay, cell-surface hydrophobicity assay, cholesterol assimilation test, and antimicrobial activity assay. The *Lb. plantarum* TRA56's β-hemolytic activity was tested on a Columbia agar plate containing 5% sheep blood. The isolate was streaked on Columbia agar before being incubated at 37°C for 48 hours under anaerobic conditions. The cell surface hydrophobicity and auto-aggregation experiments were performed in accordance with (Krausova et al., 2019). The agar well diffusion technique by using Mueller-Hinton agar (Merck, Germany) was used for examining the bactericidal activity of the cell-free supernatant of Lb. plantarum TRA56 (Mishra & Prasad, 2005). The cell-free supernatant of bacterial cells grown for 48hours was assayed versus *Escherichia coli* O157:H7 (ATCC 43895), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 33019), and *Klebsiella pneumoniae* (ATCC 13883).

Antioxidant activity assays

DDPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) techniques were used to assess the antioxidant activity of the TRA56. Cell-free supernatant (CFS) was extracted from MRS broth by centrifugation at 3500rpm for 15 minutes. The DPPH scavenging activity was assessed utilizing a modified method as outlined by Ozturk et al. (2019). 2 mL freshly-produced 0.2mM DPPH was added to 1 mL four times-diluted CFS to make the reaction mixture. At 25°C, the mixture was incubated in the dark for 30 minutes. The mixture was then centrifuged at 5000 rpm before being measured at 520 nm with five repetitions. The blank control consists of dH_2O and DPPH solution. Scavenging activity % = (1 A_{test}/A_{blank}) x100 formula was utilized to calculate DPPH scavenging activity.

ABTS assay was fulfilled according to Yetiman et al. (2024). ABTS (7 mM) was dissolved in water. After that, the ABTS stock solution was combined with 2.45 mM potassium persulfate (final concentration) to create radical cation ($ABTS^+$), and the mixture was kept in the dark for 16 hours at 25°C before use. The stock solution can be only used for three days. Before implementation, the $ABTS^+$ solution was diluted with phosphate buffer (pH: 7.2) to achieve an absorbance of 0.700±0.03 at 734 nm. Similarly, the CFS was diluted four times in phosphate buffer (pH: 7.2). After that, 40 µL of CFS was added to 4mL of $ABTS^+$ solution, the mixture was left in the dark for 5 minutes, and the absorbance was measured five times. As a blank and control, phosphate buffer (pH: 7.2) was utilized. The ascorbic acid standard curve (0-9 g/mL) was used to predict the percentage of inhibition.

Cholesterol assimilation test

The O-phthaldehyde (OPA) technique, as described by Rudel and Morris (1973), was used to examine the cholesterol consumption by the TRA56 in MRS broth. The TRA56 was cultivated at 37 °C for 24 and 48 hours in MRS broth (0.25% dextrose + 0.3% ox bile containing) supplemented with 100ppm cholesterol (5-cholesten-3-ol (Sigma, Merck GmbH, Darmstadt, Germany), dissolved in 2-propanol). The cultures were centrifuged(3500rpm, 15min, 25°C), and CFS was utilized to measure residual cholesterol. The CFS was then mixed with 2mL of KOH (50% wt/vol) and 3mL of absolute ethanol, vortexed for 1 minute, then heated at 60°C for 15 minutes. After cooling down, the mixture was vortexed for 1 minute with 3mL dH_2O and 5mL hexane. Following that, 2.5 mL of the hexane phase was transferred to another glass tube and kept at 80°C for evaporation. The residue was dissolved in 4 mL of OPA (0.5 mg/mL in glacial acetic acid) and incubated for 10 minutes at 25°C. Later, 2 mL of H_2SO_4 (98%) was gently added and vortexed for 1 minute before the solutions were incubated at 25°C for 10 minutes and absorbance was measured at 550nm (Shimadzu UV-1800 UV/VIS spectrophotometer, Tokyo, Japan). The difference between the control (uninoculated MRS broth) and test samples was used for the calculation of the cholesterol assimilation.

***In vitro* static gastrointestinal simulation**

Lb. plantarum TRA56 has been subjected to gastrointestinal static simulation based on INFOGEST standardized protocol with minor modifications (Brodkorb et al., 2019). Commercially produced sterile peach juice (minimum 10.0 Brix) derived from 50% fruit juice concentrate has been chosen as the food carrier medium for enhancing the survival of TRA56. Initially, a 5mL sample consisting of 2mL of 10^{10} cfu/mL Lb. plantarum TRA56 and 3mL of sterilized peach juice was mixed with 4mL of simulated salivary fluid (SSF), 25µL of $CaCl_2$ (0.3M), and 1,125mL of ddH_2O and the mixture was kept at 37 °C for 2 minutes. After that, 10 mL of bolus (salivary fluid sample mixture) was mixed with 8 mL of simulated gastric fluid (SGF), 5 µL of $CaCl_2$ (0.3 M), 1,985 mL of ddH_2O, and pepsin at a final concentration of 2000 U/mL (Sigma, P7012). The final pH was adjusted to 3.0, and the mixture was incubated for 2 hours at 37 °C. The resultant gastric chyme (20 mL) was thoroughly mixed with 8 mL simulated intestinal fluid (SIF), 64µL of $CaCl_2$ (0.3 M), 5 mL pancreatin (800 U/mL, Sigma, P7545, dissolved in SIF), 3 mL bile salts mixture (Merck, B8756, final concentration 10 mmol/L, dissolved in SIF), and 3,936 mL ddH_2O. The pH was adjusted to 7.0 for the intestinal phase, and the mixture was incubated for 2 hours at 37 °C. The mixture content used during the simulation was homogenized by gentle hand shaking every 15 minutes. The viable cell counts of *Lb. plantarum* in the simulation were assessed hourly from the beginning to the end using serial decimal dilutions and the pour plate technique, with survival quantified as $\log(N/N_0)$, where N signifies the number of viable CFU after each simulated CFU/mL digestion phase, and N_0 indicates the initial viable CFU/mL before digestion.

Results and Discussion

Functional genomic examination

The genome of *Lb. plantarum* TRA56 is a circular chromosome of 3,242,215 bp with a 44.41% GC content, which includes 3.030 coding sequences, 62 tRNA genes, 3 rRNA, 4 ncRNA, 1 CRISPR array region, and 69 pseudogenes (Table S1). At first, the genome of the TRA56 harbors four different putative phage regions (Table S2), which consist of two intact, one questionable, and one incomplete. In the PHASTEST database, putative phage sites are classified and scored as intact (score > 90), incomplete (score 70-90), and questionable (score < 70) based on the amount of matching phage protein (Wishart et al., 2023). The intact phages show high similarity with Oenoco_phiS13_NC_023560 (region 1) and Lactob_Sha1_NC_019489 (region 3), respectively. Lactob_Sha1_NC_019489 was previously reported in the genome of DY46, but the Oenoco_phiS13_NC_023560 was encountered for the first time. Intact phages were found to lack endolysin, which means that they are temperate for *Lb. plantarum* TRA56. Endolysins are phage proteins that quickly degrade the bacterial cell wall, leading to the release of new virus particles (Nazir et al., 2023). On the other hand, all phages identified except Oenoco_phiS13_NC_023560 were found to possess integrase. Phage integrases execute recombination among the attachment sites on phage and bacterial genomes, known as *attL* and *attR*, respectively (Groth et al., 2000). Typically, the existence of the integrase in a bacterial genome serves as a reliable indicator for the presence of pathogenicity islands, phages, and integrative plasmids (Juhas et al., 2009; Yetiman et al., 2022; 2024). The presence of genome gaps and shifts in GC content in Figure 1., verified this finding. In addition, the FastANI calculation results revealed that the TRA56 exhibited a higher level of average nucleotide identity similarity with the genomes of DY46 (Yetiman et al., 2022), ATCC8014 (Zhang et al., 2024), JDM1(Zhang et al., 2012), DOMLa (Wang et al., 2014), and M19 (Fatemizadeh et al., 2023), with percentages of 99.38%, 99.11%, 99.09%, 99.08%, and 99.06%, respectively (Figure 2). The remarkable genomic similarity among strains with varied isolation origins can be attributed to the nomadic lifestyle of *Lb. plantarum* because they can inhabit fruit flies, plants, vertebrate digestive tracts, and dairy products (Duar et al., 2017; Martino et al., 2016).

Subsequently, a pangenome analysis has been conducted using twenty-two different *Lb. plantarum* genomes, including TRA56 (Table S1). The pangenome analysis produced a total of 5826 estimated genes, comprising 1670 strain-specific genes (singletons), 2094 dispensable genes, and 2062 core genes (Figure 3A). The

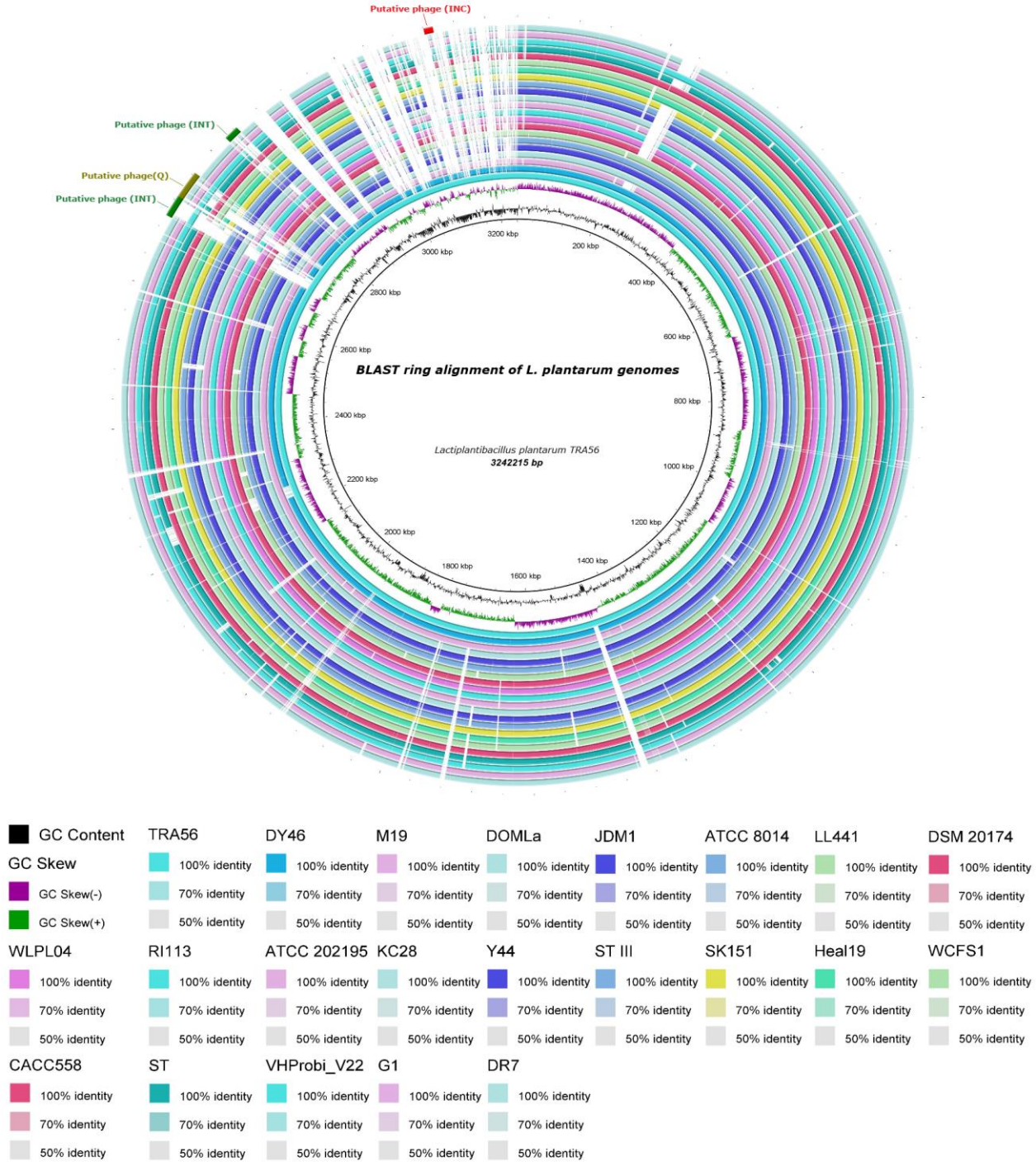

Figure 1. BLAST ring alignment of *Lactiplantibacillus plantarum* genomes. The genomes are sorted from in inner turquoise ring (TRA56) to the outer sky blue ring (DR7). The GC skew (+/−) of the TRA56 genome was displayed in the third inner circle, while the GC content was illustrated in the second inner circle. The genome size was also shown in the first inner circle, preceding the GC content. Prophage regions in the TRA56 genome are represented as different colored arcs [Intacts (INC): green, Questionable (Q): olive, Incomplete (INC): red].

core genome represents 35.4% of the pangenome and is usually linked with primary metabolic processes and housekeeping functions (Figure 4). They can also contain genes that identify the species from others in the genus (Mosquera-Rendon et al., 2016). Conversely, the pangenome curve had a logarithmic pattern and did not approach a plateau. The value of alpha was determined to be 0.699915236874417 via *micropan* R package (Figure 3B). Based on the Heaps law approach proposed by Tettelin et al. (2008), the pangenome is regarded as open due to the presence of an alpha value less than 1 and the observed logarithmic curve trend. Moreover, widely distributed species (*E. coli,* e.g.) possess open pan-genomes, which allow them to continuously acquire genetic material from their surroundings and adapt to severe environments. Thus, functional diversity is enabled (Li et al., 2021). Among the 1670 strain-specific genes, the TRA56 had 195 unique genes (Figure S3), whilst DY46 contained 267 strain-specific genes (Figure S4). Besides, the genomes of LL441 (165), Heal19

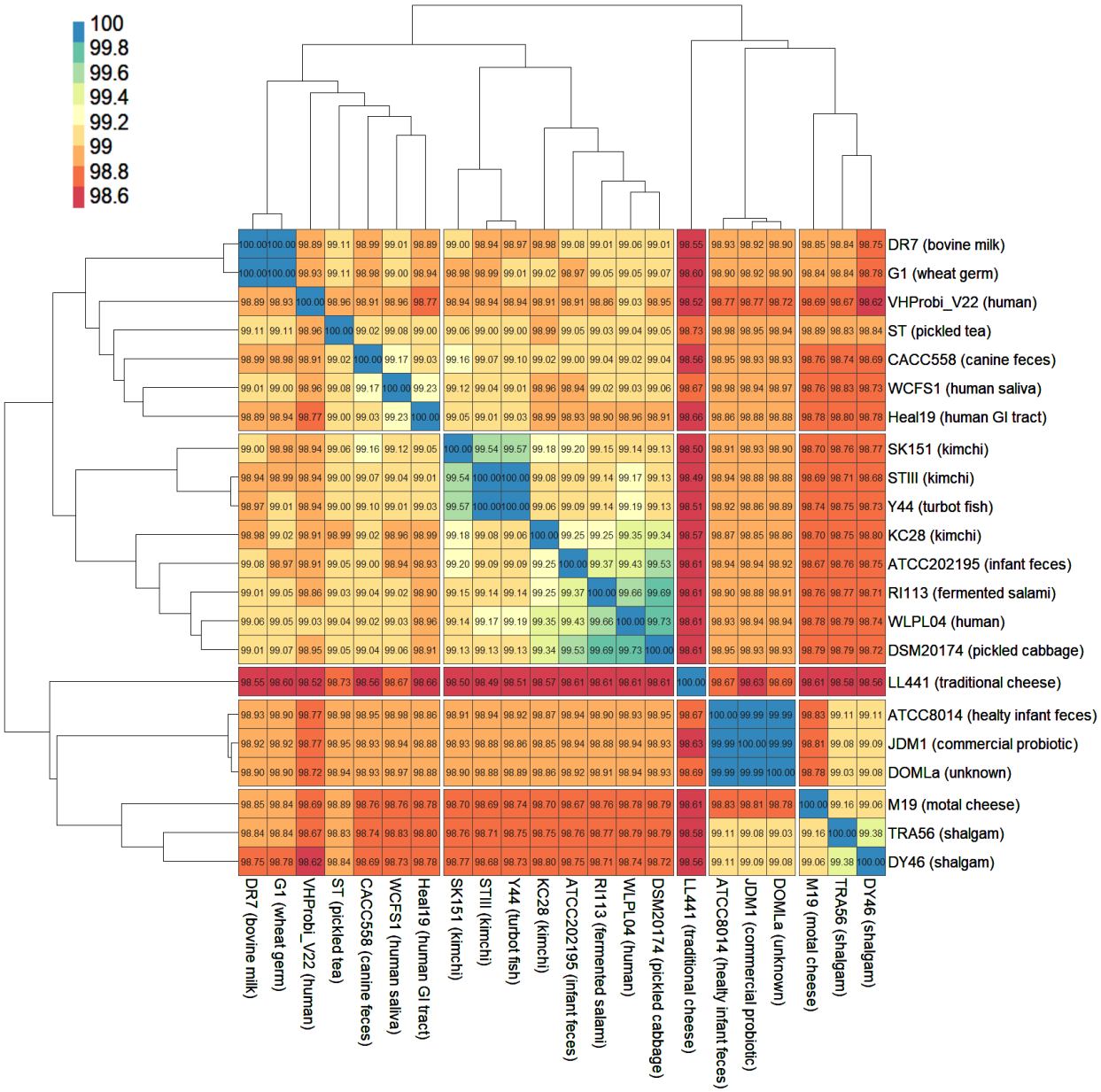

Figure 2. The provided phylogram displays the average nucleotide identity (ANI) between the genome of *Lactiplantibacillus plantarum* TRA56 and other widely recognized *L. plantarum* genomes. The ANI values were computed using the FastANI approach, and the origins of the *Lb. plantarum* genomes were indicated in parentheses next to the genome names.

(124), Vhprobi_v22 (116), WCFS1 (114), and KC28 (110) include over one hundred strain-specific genes. The number of other strain-specific genes, dispensable genes, and core genome COG categories has been summarized in Figure 4. The COG category containing the highest number of genes in the core genome is “E: Amino acid transport and metabolism (264),” whereas the category with the least number of genes is “N: Cell motility (2).” On the other hand, the top eight COG categories with a significant number of genes in the core genome : “R: general function prediction only (227),” “K: transcription (193),” “G: carbohydrate transport and metabolism (190),” “J: translation, ribosomal structure, and biogenesis (145)”, “M: cell wall/membrane/envelope biogenesis(133)”, “S: function unknown (127),” “P: inorganic ion transport and metabolism (117)”, and “L: replication, recombination, and repair (112)”, respectively. The remaining COG categories of the core genome are listed in Figure 4 as a bar graph according to their gene numbers.

The results of the pangenome analysis and the TRA56 genome were subsequently annotated and functional categories were assigned through EggNOG-Mapper and core- and pangenome frequencies of each COG category in the pangenome were summarized in Figure 5. The analysis revealed that "R: General function prediction only (741)" represents the most extensive COG category in the pangenome, while "N: Cell motility (2)" constitutes the category with the fewest genes in the pangenome. In the pangenome pool, it has been determined that other COG categories containing over four hundred genes include "K: Transcription (702)", "E: Amino acid transport and metabolism (689)", "G:

Carbohydrate transport and metabolism (671)", "L: Replication, recombination and repair (562)", and "M: Cell wall/membrane/envelope biogenesis (492)". The remaining COG categories of the pangenome have been sorted based on their frequencies in Figure 5. Besides, Figure 6 summarizes clusters of orthologous groups (COG) of protein-coding sequences connected to individual TRA56 genome.

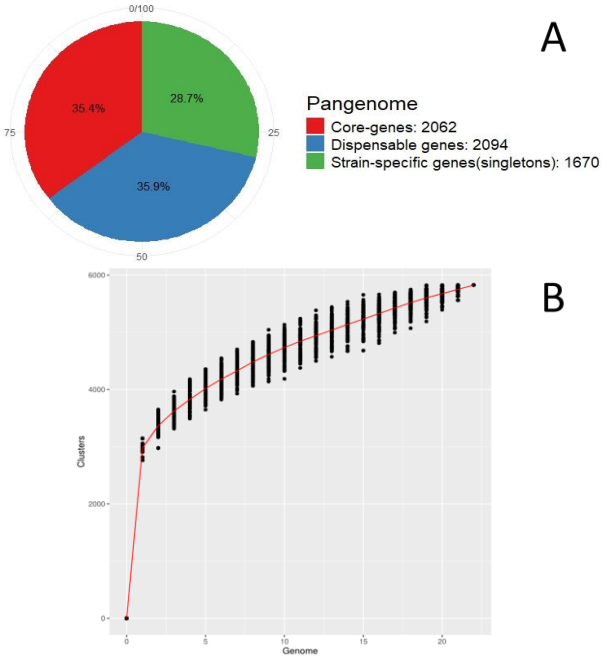

Figure 3. (A) Depictive summary of pangenome of analyzed *L. plantarum* genomes. (B) Rarefaction curve describing cumulative number of gene clusters can be observed in pangenome.

Overview of carbohydrate metabolism

Lactic acid bacteria (LAB) can generate ATP by either a heterofermentative or homofermentative carbohydrate fermentation process, contingent upon the species' preference for particular sugars (Buron-Moles et al., 2019). Understanding the unique metabolic traits of LAB strains is important for accurately predicting their potential uses in industry and the benefits they may have as probiotics (Sharifi-Rad et al., 2020). The API 50 CHL test results proved that the TRA56 strain can metabolize 22 of the 49 examined carbohydrates, as indicated as green in Table 1. *Lactiplantibacillus plantarum* is a versatile and robust species capable of thriving on a diverse array of carbohydrate sources (Seddik et al., 2017). This phenotypic trait is linked to genes that are involved in the metabolism and transportation of carbohydrates. The majority of the transporters involved in carbohydrate metabolism are positioned within the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) (Ganzle & Follador, 2012). As predicted by BlastKOALA, the genome of *Lb. plantarum* TRA56 encodes a wide variety of PTS enzymes, including the general enzyme gene *ptsI* (K08483), the phosphocarrier protein gene *ptsH* (K02784), and 29 genes that generate substrate-specific enzyme II (EII) complexes. Out of the 29 genes, only those that make N-acetyl-D-glucosamine, sucrose, mannitol, cellobiose/diacetylchitobiose, mannose, galactosamine, sorbitol/glucitol, fructose, beta-glucoside, and ascorbate-specific phosphotransferases have all of their parts. Other remaining substrate-specific phosphotransferase genes (N-acetyl-D-galactosamine, glucose, maltose, alpha-glucoside, trehalose, N-acetyl-muramic acid, lactose, sorbose, galactitol, and galactose) do not contain all components of the phosphotransporters. However, their substrate specificity remains unknown. Nevertheless, it is widely recognized that multiple substrates can be transported by one sugar transporter (Kleerebezem et al., 2003). *Lb. plantarum* TRA56 was found to possess both 6-phosphofructokinase (*pfk*) and fructose-bisphosphate aldolase (*fbaA*) enzymes, which play a vital role in the glycolytic pathway. These genes serve as markers that differentiate between homo- and hetero-fermentation (Buron-Moles et al., 2019; Morita et al., 2008). Moreover, there are no genes observed belonging to the *araBAD* operon which is linked with the breakdown of L-arabinose. In addition, the TRA56 possesses an adequate quantity of gene sets necessary for the metabolization of D-ribose, which are linked to the pentose phosphate pathway (Yetiman et al., 2024). These findings validate that the TRA56 exhibits facultative heterofermentative carbohydrate metabolism, which has been previously seen in other *Lb.plantarum* strains (Gao et al., 2020; Liu et al., 2015; Yetiman et al., 2022). On the other hand, the TRA56 genome has five copies of L-lactate dehydrogenase (*ldh*), two copies of D-lactate dehydrogenase (*ldhA*), and one copy of lactate racemase (*larA*). Hence, it can generate and/or transform both isomers of lactate (Desguin et al., 2014). Further, the TRA56 genome carries three copies of the alcohol dehydrogenase (*adh*) gene, which is principally responsible for the production of ethanol (Wang et al., 2022). Apart from the aforementioned genes, the TRA56 possesses several pyruvate depletive enzymes that play a role in the formation of other organic acids and flavor compounds, such as acetate, acetoin, acetaldehyde, and diacetyl (Papagianni, 2012). Beyond that, the CAZYmes have been found in the TRA56 genome are: 115 glycoside hydrolases, 4 enzymes responsible for auxiliary activity, 32 carbohydrate-binding modules, 63 glycosyl transferases, and 4 carbohydrate esterases. Briefly, the evaluation findings indicate that the TRA56 has a favorable ability to break down carbohydrates and possesses an adaptable nature that is specifically suited for the competitive environment of the gastrointestinal system.

Overview of vitamin metabolism

B-group vitamins are essential for human metabolism and their deficiencies are linked to various illnesses and diseases. Furthermore, humans are unable to synthesize them and must obtain them through

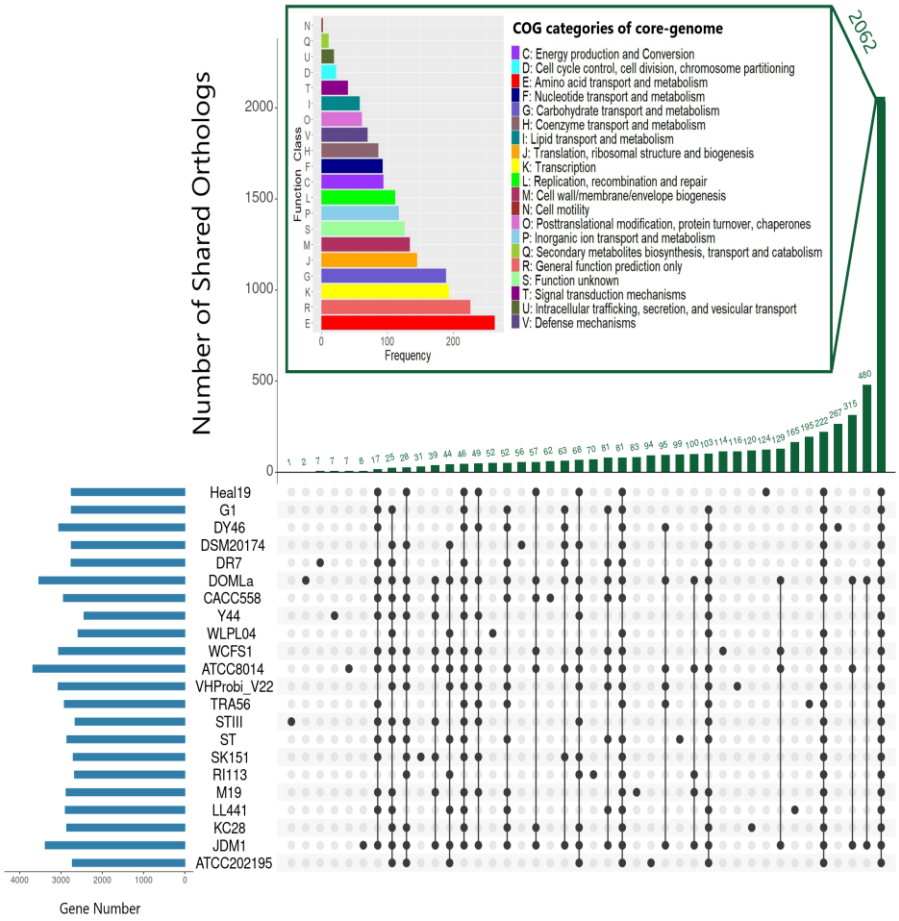

Figure 4. The Upset plot visually represents the number of genes in the core-genome, strain-specific (singletons), and other genes that are considered dispensable. Furthermore, it presents clusters of orthologous groups (COGs) that are linked to the core genome.

external sources. Lactobacilli can generate B-group vitamins, which suggests that they could potentially serve as a substitute for chemically produced vitamins in the fortification of food (Li et al., 2016). In this study, the production capability of *Lb. plantarum* TRA56 for riboflavin (vitamin B2), pantothenate (vitamin B5), and folic acid (vitamin B9) was verified using KEGG and RASTtk pathway annotation. The process of riboflavin biosynthesis begins by utilizing GTP acquired from purine metabolism. It is then facilitated by the *ribA*, *ribD1*, *ribD2*, *PYRP2*, and *RibE* genes until riboflavin is generated. Alternatively, riboflavin can be synthesized by utilizing ribulose-5-phosphate derived from the pentose phosphate pathway with the aid of *ribB*, *ribH*, and *ribE* genes. Afterward, riboflavin is transformed into FMN and FAD through the actions of the *RFK* and *FLAD1* genes, respectively (Arena et al., 2014). The synthesis of pantothenate and CoA starts with the use of 3-methyl-2-oxobutanoate, which is subsequently transformed into (R)-pantothenate by the action of *panE* and *panC* genes. Moreover, (R)-pantothenate can be generated as a byproduct of β-alanine metabolism. Following that, (R)-pantothenate is converted into D-4-phosphopantothenate, which is then turned into Dephospho-CoA with the addition of L-cysteine and the assistance of *coaB*, *PPCDC*, and *PPAT* genes. Finally, Dephospho-CoA is transformed to CoA under the control of the *coaE* gene. CoA is involved in around 100 distinct catabolic and anabolic reactions, which include the breakdown and synthesis of carbohydrates, proteins, lipids, ethanol, bile acids, and xenobiotics (Czumaj et al., 2020). Therefore, pantothenate metabolism becomes more important for both the host, and the microbe. The folate biosynthesis in the TRA56 launches by using GTP as a product of purine metabolism and GTP is converted to folate by chain reactions catalyzed by the enzymes encoded by *folE1*, *folQ*, *folQ3 folB*, *folK*, *folP*, *folC,* and *Dhfr0* genes. Differently, folate can be synthesized from chorismate by reactions of enzymes encoded by the *pabAab* and *pabAc* genes, and the other remaining steps until reaching the folate continue from the *folP* gene. In general, folate stimulates one-carbon metabolism in hosts and microbes, including amino acid homeostasis (methionine, glycine, and serine), biosynthesis of thymidine and purines, redox defense, and epigenetic maintenance (Ducker & Rabinowitz, 2017). Moreover, folate-producing starter cultures have been shown to not only improve folate status but also prevent folate insufficiency in animal models (Laiño et al., 2015). A separate study on *Lb. plantarum* found that fermenting with plant material increased the medium's riboflavin and folate levels (Thompson et al., 2020). Based on these findings, it is possible to hypothesize that

Table 1. Carbohydrate fermentation pattern comparison of *Lactoplantibacillus plantarum* TRA56 to previously described isolates in the literature

Sugar	Strain				Sugar	Strain			
	TRA56	DY46	Y44	ATTC14917		TRA56	DY46	Y44	ATTC14917
Control	-	-	-	-	Esculin ferric citrate	+	+	+	+
Glycerol	-	-	-	-	Salicin	+	+	+	+
Erythritol	-	-	-	-	D-Cellobiose	+	+	+	+
D-Arabinose	-	-	-	-	D-Maltose	+	+	+	+
L-Arabinose	-	-	+	+	D-Lactose	+	+	+	+
D-Ribose	+	+	+	+	D-Melibiose	+	+	+	+
D-Xylose	-	-	-	-	D-Sucrose	+	+	+	+
L-Xylose	-	-	-	-	D-Trehalose	+	+	+	+
Adonitol	-	-	-	-	Inulin	-	-	-	-
Methyl-βD-xylopyranoside	-	-	-	-	D-Melezitose	+	+	+	+
D-Galactose	+	+	+	+	D-Raffinose	-	+	-	+
D-Glucose	+	+	+	+	Amidon (Starch)	-	-	-	-
D-Fructose	+	+	+	+	Glycogen	-	-	-	-
D-Mannose	+	+	+	+	Xylitol	-	-	-	-
D-Sorbose	-	-	-	-	Gentiobiose	+	+	+	+
D-Rhamnose	-	-	-	-	D-Turanose	-	-	+	+
Dulcitol	-	-	-	-	D-Lyxose	-	-	-	-
Inositol	-	-	-	-	D-Tagatose	-	-	-	-
D-Mannitol	+	+	+	+	D-Fucose	-	-	-	-
D-Sorbitol	+	-	+	+	L-Fucose	-	-	-	-
Methyl-αD-mannopyranoside	+	+	+	+	D-Arabitol	-	-	-	-
Methyl-αD-glucopyranoside	-	-	-	-	L-Arabitol	-	-	-	-
N-Acetylglucosamine	+	+	+	+	Gluconate	+	+	+	+
Amygdalin	+	+	+	+	2-Keto-gluconate	-	-	-	-
Arbutin	+	+	+	+	5-Keto-gluconate	-	-	-	-

Lactobacilli such as the TRA56 can boost riboflavin, pantothenate, and folic acid levels in Shalgam, hence contributing to its functional properties.

Safety-related genes assessment

The resistome screening was carried out via the BV-BRC and KEGG databases. The screening results reveal the presence of antibiotic resistance genes related to lincosamides (1), triclosans (1), aminoglycosides (1), peptide antibiotics (4), beta-lactams (9), and vancomycin (7). Initially, the existence and quantity of vancomycin and beta-lactam-linked resistance genes attracted attention. Lactobacilli is widely recognized for its resistance to vancomycin, which arises from a modification of the peptidoglycan terminus, specifically the substitution of D-ala-D-lac for the standard D-ala-D-ala present in vancomycin-sensitive species (Lessard & Walsh, 1999). This alteration leads to decreased vancomycin binding and, therefore, an inability to inhibit cell wall synthesis (Das et al., 2020; Stogios & Savchenko, 2020). This is due to the fact that the *VanX* gene is a precursor of cell wall function and shows high specificity to hydrolyze D-ala-D-ala dipeptides (Lessard & Walsh, 1999; Zhang et al., 2012). Nevertheless, this work has found the presence of vancomycin resistance genes, namely *mraY*, *alr*, *ddl*, *murF*, *vanY*, *murG*, and *vanX*, which have been previously reported (Campedelli et al., 2019; Liu et al., 2015). On the other hand, the presence of beta-lactam resistance associated with *mrcA*, *penP*, *pbp2A*, *abcA*, *oppA*, *oppB*, *oppC*, *oppD*, and *oppF* genes has been confirmed via KEGG. Reports on antimicrobial resistance in Lactobacilli indicate that strains of *L. paracasei*, *L. casei*, *L. brevis*, and *L. plantarum* exhibit high-level resistance to penicillin antibiotics (Florez et al., 2005; Olukoya et al., 1993; Wang et al., 2022). Therefore, it is possible to speculate that penicillin resistance in Lactobacilli might be a growing concern. Furthermore, the *gidB* gene, associated with streptomycin resistance, has been identified. Streptomycin primarily disrupts the synthesis of ribosomal proteins. Streptomycin exerts its action on the small ribosomal subunit, known as 30S, targeting specifically ribosomal proteins S12 and 16S rRNA. *Lb. plantarum* TRA56 synthesizes the enzyme 7-methylguanosine (m-7-G) methyltransferase (*gidB*), which methylates the 16S rRNA from the G527 position in the 530-loop region (Okamoto et al., 2007). Literature suggests that resistance to streptomycin and other aminoglycosides is due to the conserved m-7-G modification in 16S rRNA, mediated by the aforecited gene product (Singh et al., 2020; Okamoto et al., 2007). Subsequently, the bacterial enoyl-ACP reductase enzyme encoding the *fabK* gene, which has a similar function to its other orthologs and confers resistance to the biocide triclosan (Heath & Rock, 2000). The 23S rRNA (guanine(748)-N(1))-methyltransferase (EC

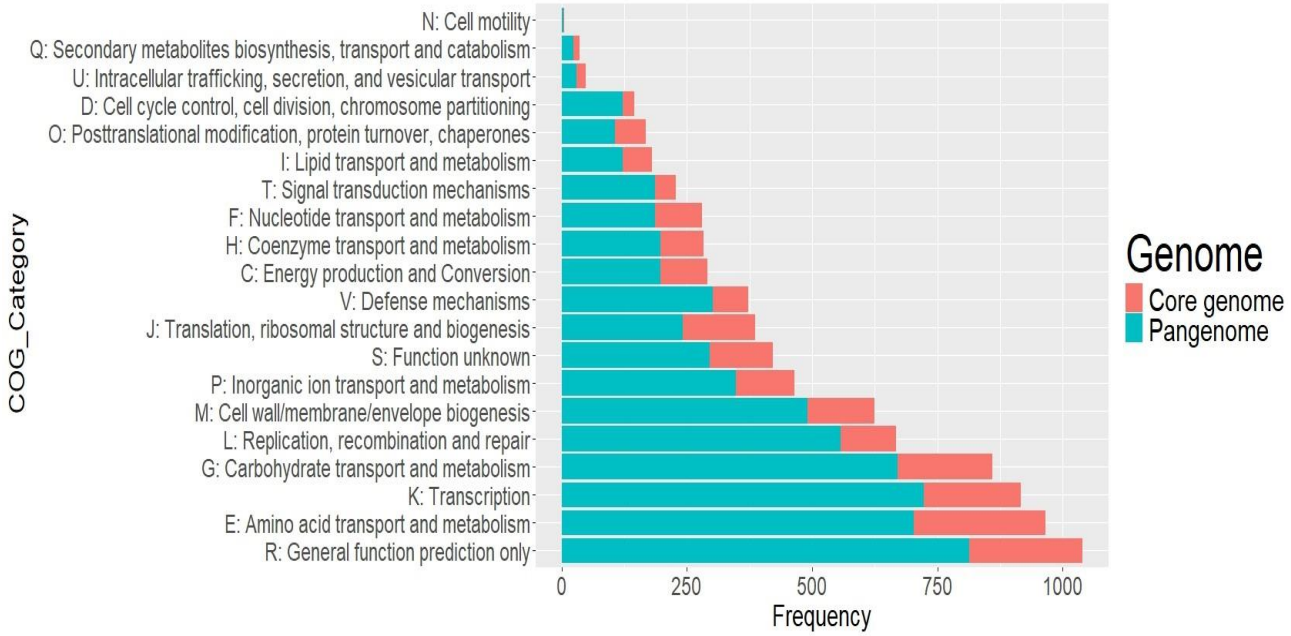

Figure 5. The number of COG categories observed in the core (red) and pangenome (light green) of twenty-two genomes of *Lactiplantibacillus plantarum*.

2.1.1.188) enzyme, encoded by the *RlmA(II)* gene, provides resistance to mycinamicin, tylosin, and lincosamides. The resistance is related to the methylation of nucleotide G748 at the N1-position in the 23s rRNA (Douthwaite et al., 2008). The presence of genes encoding *PgsA*, *MprF*, and *GdpD* proteins, which modify the charge of the cell wall, has been confirmed using BV-BRC. These genes confer resistance to daptomycin and defensin antibiotics (Arias et al., 2011; Friedman et al., 2006; Peleg et al., 2012; Yang et al., 2009). The presence of transmissible antimicrobial resistance genes in probiotics intended for human or animal consumption is problematic due to concerns about the potential spread of these genes in different nutritional environments and the possibility that microbial species may act as reservoirs for these resistance genes (von Wright, 2005). Lactobacilli show intrinsic resistance to a variety of antimicrobial agents, and it is widely recognized that this resistance is not associated with specific safety issues. It is important that antibiotic resistance genes on the bacterial chromosome should not be flanked by integrases or transposases (Yetiman et al., 2024; Zhang et al., 2012). The protein BLAST analysis performed in this study suggests that the target resistome does not contain any horizontally transferred genes (Table S5).

Probiotic features

The probiotic characterization tests were conducted to confirm the presence or absence of traits required for probiotic status. The β-hemolysis assay proved that *Lb. plantarum* TRA56 lacks β-hemolytic ability (Table 2). The cell surface hydrophobicity (CSH) and autoaggregation capacity (AC) were determined as 91.68±0.77% (using xylene) and 95.96±0.14%, respectively. The CSH is important for learning the overall adhesion capacity of probiotics, typically evaluated by measuring bacterial affinity to hydrocarbon solvents such as xylene, n-hexadecane, and hexane (Krausova et al., 2019). Xylene was chosen as the apolar solvent in this study due to its dual characteristics of hydrophobicity and hydrophilicity (Rajoka et al., 2017). Recent studies suggest that bacteria with higher cell surface hydrophobicity exhibit enhanced adhesion to intestinal epithelial cells (de Souza et al., 2019; Goel et al., 2020). *Lb. plantarum* TRA56 demonstrated a superior cell surface hydrophobicity value when compared to *Lb. plantarum* AR326 (11.70%), AR629 (73.93%), AR187 (87.05%), AR171 (89.04%), Dad-13 (78.9%), and Mut-7 (83.5%) strains (Darmastuti et al., 2021; Wang et al., 2018). Additionally, Del Re et al. (2000) revealed that the minimum hydrophobicity value necessary for a probiotic strain is 40%. In this regard, *Lb. plantarum* TRA56 exceeds this requirement. It has been reported that the lipoteichoic acid (LTA) production-responsible genes (*MurJ*, *LtaS*, *LafC*, *LafB*, *LafA*, *dltD*, *dltA*, and *dltB*) are associated with CSH, which is also present in *Lb. plantarum* TRA56 (Yetiman et al., 2024). The autoaggregation capability of probiotic bacteria is important, since it directly correlates with the adherence to epithelial cells and mucosal surfaces, serving as a phenotypic marker for adhesion (Chaffanel et al., 2018). The presence of AC facilitates the formation of cellular aggregates by bacteria, thereby enhancing their persistence in the intestine (Pan et al., 2017). The AC of *Lb. plantarum* TRA56 exceeded that of previously compared strains, including *Lb. plantarum* AR326 (45.53%), AR629 (21.01%), AR187 (41.69%), AR171 (22.96%), Dad-13 (40.9%), and Mut-7 (57.5%). Bacterial cell wall components and related structures, such as mucus-binding proteins, adhesins, surface layer

Table 2. Probiotic characterization test results of *Lb. plantarum* strain TRA56

Characterization Test	**Result**	
β-hemolysis on Blood Agar	Non-hemolytic	
Auto-aggregation (%)	95,96±0,14	
Cell-surface hydrofobicity (%)	91,68±0,77	
Cholesterol assimilation (%)	46,28±1,6	
Antioxidant activity against DPPH (%)	59,04±0,43	
Antioxidant activity against $ABTS^{+}$(%)	77,76±0,33	
Survival behavior of *Lb. plantarum* TRA56, under Static *in vitro* gastrointestinal digestion simulation		
Time*	**Log CFU/mL**	**Survival Rate**
Hour 0. [in simulated gastric fluid (SGF)]	10,79	100%
Hour 1. [in simulated gastric fluid (SGF)]	8,477	78.52%
Hour 2. [in simulated gastric fluid (SGF)]	8,079	74.83%
Hour 3. [in simulated intestinal fluid (SIF)]	9,336	86.48%
Hour 4. [in simulated intestinal fluid (SIF)]	9,372	86.81%
Antimicrobial activity test results		
Test strain	**Zone of inhibiton (mm)**	
Escherichia coli O157:H7 ATCC 43897	16,96±0,61	
Escherichia coli ATCC 25922	15,3±0,4	
Staphylococcus aureus ATCC 25923	16,62±0,44	
Bacillus cereus ATCC 33019	15,46±0,09	
Klebsiella pneumoniae ATCC 11883	18,75±1,11	

*SGF: contains 2000 U/mL pepsin and a working pH is 3.0; SIF: contains 10 mM bile salts, 100 U/mL pancreatin, and a working pH is 7.0. SGF and SIF have been prepared according to the "*INFOGEST static in vitro simulation of gastrointestinal food digestion*" protocol (Brodkorb et al., 2019).

proteins, fibronectin-binding proteins, exopolysaccharides, and lipoteichoic acids, confer advantages to bacteria in terms of colonization and adhesion to host epidermal cells, as well as influencing the CSH and AC values of *Lb. Plantarum* TRA56 (Chaffanel et al., 2018; Haddaji et al., 2015; Pan et al., 2017). Furthermore, the *Lb. plantarum* TRA56 was shown radical scavenging activities of 59.04±0.43% and 77.76±0.33% against DPPH and $ABTS^{+}$ radicals, respectively. DPPH and ABTS are known free radicals that are frequently used to assess the free radical scavenging activity of antioxidants; they are typically analyzed through colorimetric methods. The reduction values of *Lb. plantarum* TRA56 are associated with antioxidant enzymes including *trxA* (thioredoxin), *trxB* (thioredoxin reductase), *tpx* (thiol peroxidase), *dsbA* (thiol-disulfide oxidoreductase), *arsC* (arsenate reductase), *Gor* (glutathione reductase), *nrdH* (glutaredoxin) (Kandasamy et al., 2022; Lu & Holmgren, 2014). Lactobacilli can reduce cholesterol and lipid levels in the bloodstream through various ways. High lipid and cholesterol levels in the bloodstream (hypercholesterolemia) correlate with cardiovascular illnesses, resulting in 2.6 million fatalities and 29.7 million disability-adjusted life years, according to the 2024 Global Patient Safety Report by the WHO (Agolino et al., 2024). Consequently, the fortification of foods with lactic acid bacteria or the direct administration of probiotics has become increasingly important in light of rising cardiovascular health issues (Song et al., 2015). This study has identified comparable cholesterol-lowering effects "*in vitro*." The cholesterol assimilation test findings indicate that the TRA56 utilized 46.28±1.6% of 100 ppm cholesterol throughout a 24-hour period. Previous studies noted cholesterol elimination rates of 23.55%, 54.08%, 25.85%, 22.60%, 24.03%, 36.55%, 49.23%, and 41.97% for *Lb. plantarum* KLDS1.0343, KLDS1.0344, KLDS1.0357, KLDS1.0365, KLDS1.0374, KLDS1.0381, WLPL21, and ZDY04, respectively (Guo et al., 2011; Zhao et al., 2024). The hypocholesterolemic effects are often linked to bile salt hydrolase (*bsh* or *cbh*) enzymes and the production of short-chain fatty acids by bacteria that inhibit enzymes like 3-hydroxy-3-methylglutaryl coenzyme A (HMB-CoA). This enzyme catalyzes the synthesis of mevalonic acid, an essential step in cholesterol biosynthesis in humans and animals (Agolino et al., 2024; Wilson et al., 2013). In the TRA56 genome, two copies of the choloylglycine hydrolase enzyme (EC 3.5.1.24), encoded by the *cbh* gene (RefSeq locus tag: AABC04_04340, AABC04_05680), were observed. For in vitro static digestion simulation, the salivary fluid stage of digestion simulation has been passed. Following the simulated gastric phase, a reduction in cell counts of the TRA56 was observed, which is attributed to the acidic environment and the antimicrobial effects of pepsin (Costa et al., 2017). However, 74.83% of the initial microbial load of the TRA56 survived and this might have been caused by adaptation to a less lethal peach juice acidic environment (pH: 3.85±0.196). Probiotics are frequently advised to be consumed with foods as carriers to safeguard them from the adverse conditions of the gastrointestinal tract (Ranadheera et al., 2010). Therefore, sterile peach juice has been used as a carrier environment for the TRA56. Following the simulated intestinal phase, the cell count was increased by approximately 1.3 log CFU/mL. The presence of choloylglycine hydrolase (*cbh*) enzymes in the TRA56 and prebiotics available in the peach juice carrier environment might be responsible for this trend. After

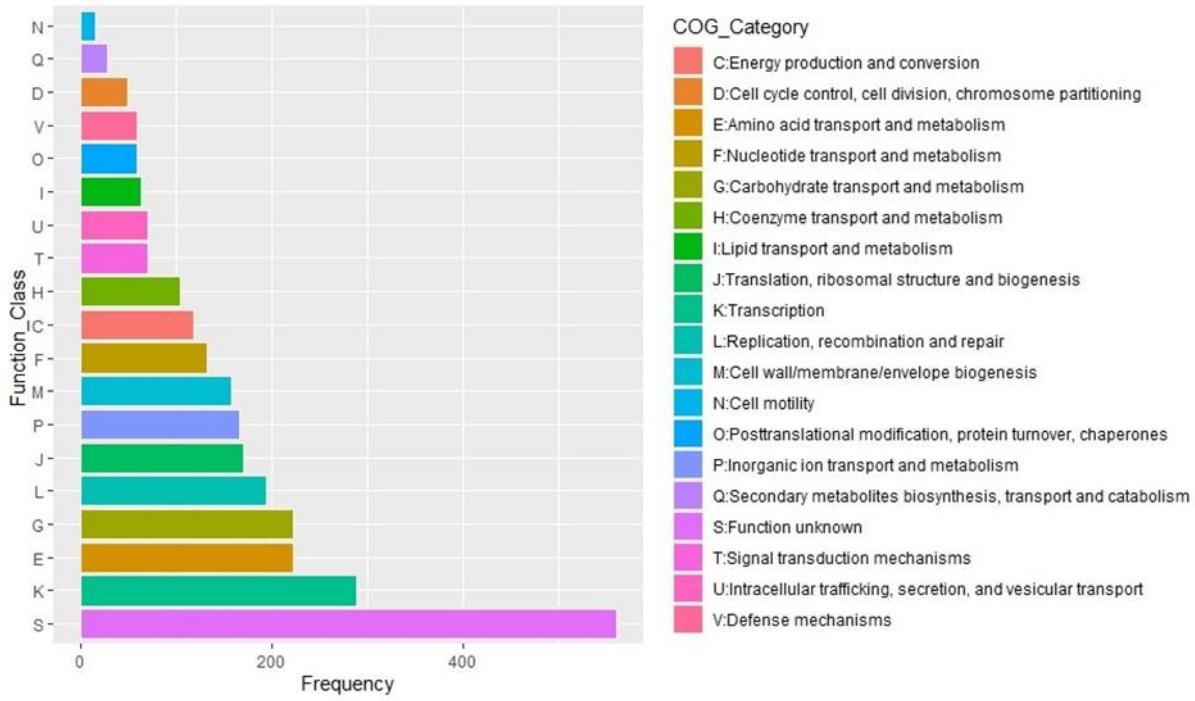

Figure 6. Graphical illustration of the determined numbers of clusters of orthologous groups (COG) of protein-coding sequences in the genome of *Lactiplantibacillus plantarum* TRA56.

digestion, the TRA 56 cell count remained above 9 log CFU/mL. On the other hand, the role of cation-proton antiporters (*NapA*, *NhaC*, *NhaP*), ABC ATPases, F_0F_1-type ATP synthase, and chaperones (*Clp* ATPases) for homeostasis and intracellular pH regulation that have contributed to acid and bile resistance, should not be ignored (Yetiman et al., 2024). Test strains for the antibiogram assay were selected based on their Gram (+/-) category, spore development, and capsule formation status. Assay results indicate that CFS of the TRA56 exhibits a noteworthy zone of inhibition (>15 mm) versus *E. coli* ATCC 25922, *E. coli* O157:H7 ATCC 43897, *K. pneumoniae* ATCC 11883, *S. aureus* ATCC 25923, and *B. cereus* ATCC 33019. Especially for *K. pneumoniae* ATCC 11883 (18,75±1,11 mm), antimicrobial effects of CFS of the TRA56 have come to the forefront. The secondary metabolites produced by TRA56 may be the source of this, as identified through antiSMASH version 7.1.0. The TRA56 genome encodes four distinct gene clusters that are involved in the production of Ripp-like peptides(55% similarity to coagulin) (Le Marrec et al., 2000), cyclic lactone autoinducer peptides for intercellular communication (Wang et al., 2015), Type-3 polyketide synthase (61% similarity to 2,4-diacetylphloroglucinol) (Delany et al., 2000), and terpene synthesis (75% similarity to carotenoid structure compounds) (Lang et al., 1994). The gene clusters will be evaluated extensively in another study on TRA56.

Conclusion

Lactiplantibacillus plantarum is one of the most prevalent bacteria in the Shalgam microbiome. Understanding its functional and metabolic attributes is critical for the preservation of shalgam's traditional traits. This study has used whole genome sequence data to conduct a functional genomic characterization and provided a brief summary of carbohydrate and vitamin metabolism in strain TRA56. The strain possesses four putative phages, all of which exhibit temperate behavior towards it. The analysis of the pangenome revealed that the TRA56 genome had 195 strain-specific genes that are distinct from those found in other compared strains. *Lb. plantarum* can serve as a probiotic or a commercial starter. This study has proven its probiotic capacity both *in silico* and *in vitro*. In this context, TRA56 has a broad spectrum of capacity in metabolizing carbohydrates, which gives it a competitive advantage and makes it the prevailing species in such environments. Additionally, the strain's ability to produce vitamins B2, B5, and B9 has been confirmed through computational tools like as KEGG and RASTtk. Therefore, it may be suggested that Lactobacilli, such as TRA56, have the potential to enhance the amounts of riboflavin, pantothenate, and folic acid in Shalgam, thereby enhancing its functional characteristics. On the other hand, the TRA56 genome has been thoroughly examined for antibiotic resistance against the target resistome, and no sign of transferable antibiotic resistance has been detected in its genome. The findings highlight the importance of evaluating *Lb. plantarum*'s genetic and metabolic traits for the production of shalgam and ensuring consumer safety.

Supplementary Information

https://biotechstudies.org/uploads/BIO-1126_Supp1.docx

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Author Contributions

Ahmet E. Yetiman: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Visualization and Writing and Editing-original draft, Funding Acquisition, Project Administration, Resources.

Conflict of Interest

The author declares that he has no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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