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Probiogenomic Analysis of *Lactiplantibacillus* sp. LM14-2 from Fermented Mussel (*Hoi-dong*), and Evaluation of its Cholesterol-lowering and Immunomodulation Effects

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Abstract

Lactiplantibacillus sp. LM14-2, isolated from Thai-fermented mussel (*Hoi-dong*), showed attractive probiotic properties. This strain was identified as *Lactiplantibacillus plantarum* based on its phenotypic, chemotaxonomic, and genetic characteristics including whole-genome sequencing (WGS). The draft genome sequence was analyzed and annotated for the molecular mechanisms involved in the safety assessment, the adaptation and adhesion of *L. plantarum* LM14-2 to the gastrointestinal tract (GIT), and the beneficial genes involved in bacteria–host interactions. The *L. plantarum* LM14-2 exhibited bile salt hydrolase (BSH) activity, assimilated cholesterol at 86.07 ± 5.03%, stimulated the secretion of interleukin-12, interferon-gamma, and human beta defensin-2, and induced nitric oxide production. In addition, *L. plantarum* LM14-2 showed excellent gastrointestinal tolerance and adhesion ability to Caco-2 cells. Furthermore, the in silico analysis showed that *L. plantarum* LM14-2 was a non-human pathogen and did not contain antibiotic resistance genes or plasmids. *L. plantarum* LM14-2 also contained potential genes associated with various probiotic characteristics and health-promoting effects. Consequently, this study suggested that *L. plantarum* LM14-2 could be considered safe, with potential probiotic properties and health-promoting impacts, which could facilitate its probiotic application.

Keywords Fermented mussel · *Lactiplantibacillus plantarum* · Probiotics · Probiogenomic · Cholesterol-lowering · Immunomodulation

Introduction

Lactic acid bacteria (LAB) have been used as probiotics and defined as a healthy diet as well as a way to provide health benefits such as cholesterol-lowering and immunomodulation effects. A reduction of 1% in serum cholesterol may alleviate the risk of coronary artery disease by 2 to 3% [1]. In addition, LAB immunomodulation is still of interest since it boosts immunity and is used to prevent and treat some immune diseases. Interleukin-12 (IL-12) is a pro-inflammatory cytokine involved in preventing infection and cancer [2]; IFN-γ plays a role in host defense against intracellular pathogens. Moreover, human beta defensin-2 (hBD2), human antimicrobial peptides, serve vital functions in the prevention of infection [3]. Nitric oxide (NO) also plays an important role in the prevention of infection and tumor cell proliferation [4]. There have been several studies of *Lactobacillus* and other LAB-inducing IL-12, IFN-γ, hBD2, and NO production [3, 5–8].

According to FAO/WHO [9], the probiotics must be isolated from safe sources, accurately identified, and characterized at the species level. Conventional methods and whole-genome sequencing (WGS) technology are required to accurately identify *Lactobacillus* species. Furthermore, WGS also plays an attractive and important role in assessing of pathogenicity and virulence, antibiotic-resistance genes, gastrointestinal tolerance genes, and health-promoting genes [10, 11].

In the investigation of LAB in Thai traditional fermented mussel (*Hoi-dong*), a darkish orange semi-solid with a sour (pH 3.1–6.1) and salty taste that was made of *Perna viridis* (*Hoi-ma-laeng-poo*) [12, 13], a strain LM14-2 was isolated and might be an attractive source for evaluating the possibility of discovering new potential probiotic isolates. Furthermore, no investigations have yet been published on the

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probiogenomic, cholesterol-lowering, and immunomodulation activities. Hence, this study aims to evaluate cholesterol-lowering and immunomodulation effects, probiotic properties, and the reliability of the omics approach for probiogenomic and safety assessment of *Lactiplantibacillus* sp. LM14-2.

Materials and Methods

Characterization of Strain

Strain LM14-2 was isolated from *Hoi-dong* (traditionally fermented mussel), which was collected from Chonburi province in Thailand. It was characterized based on the phenotypic and chemotaxonomic characteristics as previously described by E. Kingkaew et al. [14]. The genomic DNA was extracted using the Wizard Genomic DNA Purification kit (Promega Corporation, USA) according to the manufacturer's protocol with some modifications. The modifications included the addition of 25 U/mL mutanolysin (Sigma-Aldrich, USA) in the 1 mg/mL lysozyme resuspending solution, incubation at 37 ℃ overnight for complete cell lysis, and additional centrifugation at 10,000 *g* for 3 min in the protein precipitation step. The DNA pellet was dissolved in DNase/RNase-free water. The concentration and quality of the DNA were measured using a NanoDrop ND-1000 spectrophotometer. At Omics Science and Bioinformatics Center, Chulalongkorn University, library preparation and genome sequencing of the strain LM14-2 were performed by Nextera XT DNA prep kit and Illumina Miseq sequencer. The quality of raw reads was checked using FASTQC software (Galaxy Version 1.1.5). Adaptors and poor-quality reads were removed using Trim Galore (Galaxy Version 0.6.3); filtered reads were used as an input for Unicycler, genome assembly program (Galaxy Version 0.4.8.0). The genomic quality and contamination were evaluated by CheckM [15]. The sequence similarity values between the strain LM14-2 and their related reference strains were computed using the EzBiocloud tool [16]. Then, the average nucleotide identity (ANI) and the digital DNA-DNA hybridization (dDDH) values were analyzed using the JSpeciesWS web server tool [17, 18] and the Genome-to-Genome Distance Calculator (GGDC 2.1) using the BLAST+ method with formular 2 [19]. The ANI of >95% and DDH of >70% are considered to be the same species [20]. A phylogenetic tree based on a whole-genome sequence was constructed by using the TYGS web server (https://tygs.dsmz.de/) [21]. Furthermore, a genomic circular map was constructed using CGView Server [22].

Gene Prediction and Functional Annotation

The draft genome was annotated by using the DFAST sever [23], Rapid Annotation Server Technology (RAST) [24], PATRIC [25], and the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [26]. Plasmid was detected by PlasmidFinder [27]. The PATRIC, CheckM, RAST, DFAST, NCBI, and PlasmidFinder tools were used for the determination of genomic features (Table 1). Antibiotic resistance genes were determined using the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca) [28] and ResFinder web-based tool [29]. The pathogenicity was predicted by PathogenFinder web-based tool [30], and the PHAge Search Tool Enhanced Release (PHASTER) was used to identify and annotate putative prophage sequences [31]. Identification of carbohydrate-active enzymes was performed using the dbCAN meta server (https://bcb.unl.edu/dbCAN2/blast.php) with HMMER: biosequence analysis with profile hidden Markov models (version: 3.3.2), and all data generated in dbCAN were based on the family classification from the CAZy database (http://www.cazy.org/) [32, 33]. The biosynthesis of antimicrobial peptides gene clusters was detected and visualized by BAGEL4 (BActeriocin GEnome mining tool; http://bagel4.molgenrug.nl) [34]. Genes responsible for virulence and undesirable characteristics may be identified using public databases and manually inspected to confirm its identity and function. Precautions are required in the interpretation of the findings since genes involved in survival and adaptation should not be treated as virulence genes for non-pathogenic bacteria. The search uses the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp) [35] for the pathways and genes.

In Vitro Probiotic Assays

Preparation of LAB Cell Suspension

The cell suspension was prepared for studying probiotic characteristics. The strain LM14-2 was cultured twice in MRS broth for 24 h at 30 ℃. Then, the cells were harvested by centrifugation at 14,000 rpm for 10 min at 4 ℃, washed twice with phosphate buffer (0.1 M, pH 7.2, containing 0.85% (w/v) NaCl), and resuspended in phosphate buffer (0.1 M, pH 7) to get a cell suspension with $A_{600} = 1$ and 10^9 CFU/mL.

Bile Salt Hydrolases Activity

The BSH activity was evaluated as described by Shehata et al. [36]. On MRS agar supplemented with 0.037% (w/v) calcium chloride ($CaCl_2$) and 0.5% (w/v) taurodeoxycholic acid sodium salt (TDCA), 20 µl of cell suspension was spotted. Plates were incubated anaerobically for 72 h at 37 ℃. The BSH activity was indicated by the formation of halos around colonies or white opaque colonies. The non-modified MRS served as the control.

Table 1 Genomic features of *Lactiplantibacillus plantarum* LM14-2, *L. plantarum* 299V, and *Lacticaseibacillus rhamnosus* GG (ATCC 53103)

Attribute	LM14-2	299V	GG (ATCC 53103)
Source	Thai-fermented mussel (*Hoi-dong*)	Healthy human intestinal mucosa	Fecal samples of a healthy human
Accession no.	JALPQH000000000[F]	LEAV00000000[F]	FM179322[F]
Genome size (bp)	3,311,812[c]	3,302,055[c]	3,010,111[c]
Plasmids	0[E]	2 (rep28, 98.17% identity; rep38, 99.0% identity)[E]	0[E]
Genome qualities:			
Genome quality	Good[a]	Good[a]	Poor[a]
Completeness (%)	99.35[b]	99.35[b]	99.49[b]
Coarse consistency	97.9[a]	98[a]	99.4[a]
Fine consistency	96.1[a]	96.2[a]	98.3[a]
G+C content (%)	44.3[c]	44.4[c]	46.7[c]
Genome coverage	250×	48×	No data
N50	331,723[c]	173,004[c]	-
L50	4[c]	8[c]	1[c]
No. of contig	40[c]	67[c]	1[c]
No. of subsystem	230[c]	232[c]	231[c]
No. of coding sequences	3,254[c]	3,264[c]	3,009[c]
No. of RNA	67[c]	60[c]	72[c]
No. of CRISPRS	0[D]	0[D]	1[D]

[a]Data obtained from PATRIC
[b]Data obtained from CheckM
[c]Data obtained from RAST web-based tool
[D]Data obtained from DFAST annotation
[E]Data obtained from PlasmidFinder
[F]Data obtained from NCBI

Cholesterol Assimilation

The cholesterol assimilation ability of strain LM14-2 was determined in MRS broth supplemented with cholesterol-polyethylene glycol (PEG) 600 (Sigma, India) at a final concentration of 100 μg/mL; 1% of cell suspension was inoculated into MRS-cholesterol-PEG 600 and incubated anaerobically for 24 h at 37 ℃. The cholesterol in MRS broth was extracted using the method described in [37]. The quantity of residual cholesterol was measured using the modified technique of L. L. Rudel and M. Morris [38]. The following cholesterol concentrations were used to construct a standard absorbance curve with cholesterol concentrations: 0 μg/mL, 3.125 μg/mL, 6.25 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL, and 125 μg/mL in MRS. The cholesterol concentration was compared to the standard curve constructed using the cholesterol stock solution. The assimilate cholesterol ability was presented as the cholesterol assimilated (%) at each incubation as follows:

$$\text{Cholesterol assimilated } (\mu g/ml) = [\text{Cholesterol } (\mu g/ml)]_{0h} - [\text{Cholesterol } (\mu g/ml)]_{24h}$$

$$\% \text{ Cholesterol assimilated} = \left[\frac{\text{Cholesterol assimilated}(\mu g/ml)}{\text{Cholesterol } (\mu g/ml)_{0h}}\right] \times 100$$

Gastrointestinal Transit Tolerance

An in vitro assay that simulated the gastrointestinal fluids and conditions was prepared and conducted following the procedure of M. Minekus et al. [39]. Briefly, the cell suspension was mixed with simulated gastric fluid (SGF) containing pepsin 2,000 U/mL and incubated anaerobically for 3 h at pH 3 and 37 ℃. The samples were collected at 0 h (initial time) and 3 h (gastric-emptying time) of incubation time for viably bacterial enumeration. After the incubated time of the gastric phase, the gastric chyme was transferred, mixed with simulated intestinal fluid (SIF) containing pancreatin (based on trypsin activity at 100 U/mL) and 10 mM of bile, and incubated anaerobically for 5 h at pH 7 and 37 ℃. The samples were collected at 0 h (initial time) and 5 h (small intestinal-emptying time) of incubation time for viably bacterial enumeration. The number of viable LAB was quantified using a serial 10-fold dilution and spot plate technique. The *Lacticaseibacillus rhamnosus* GG was used as control.

The viable bacteria were reported as logarithms of colony-forming units per milliliter (log CFU/mL).

Adhesion Ability

The strain LM14-2 was selected to investigate the adhesion ability, with slight modifications procedure of Q. Han et al. [40]. Caco-2 cells were routinely grown at 37 ℃ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (PS) in a humidified condition of 95% air and 5% CO_2. Caco-2 cells were seeded into 24-well tissue culture plates at a concentration of 5×10^5 cells/mL for the adhesion test. The tissue plates were incubated at 37 ℃ in 5% CO_2 until the Caco-2 cells developed a confluent-differentiated monolayer. Caco-2 cells were washed twice in PBS, and the bacterial cells were collected by centrifugation at 14,000 rpm for 10 min at 4 ℃, and bacterial cells were resuspended in DMEM without antibiotics. The bacterial cell suspension was inoculated into a well and incubated for 90 min at 37 ℃ in a 5% CO_2. After the incubation period, Caco-2 cells were washed three times with PBS to eliminate unbounded bacterial cells. Then, the Caco-2 cells were lysed with 0.05% Triton-X100 solution. The number of adherent bacteria was enumerated by the spot plate technique on the MRS agar and then incubated at 37 ℃ on MRS agar for 48 h. The *L. rhamnosus* GG was used as a control. The adherent ability was calculated according to the following equation:

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

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

The Immunomodulation Effects

The immunomodulation effect of strain LM14-2 was determined by the procedure of Y. Hosaka et al. [41].

Preparation of Sterilized Lactic Acid Bacteria Powder

The strain LM14-2 was cultivated in MRS broth and incubated at 30 ℃ for 24 h, according to the procedure of Y. Hosaka et al. [41]. The cultures were heated at 100 ℃ for 20 min, and the heat-killed cells were collected by centrifugation at 1,000 rpm for 10 min. After that, the heat-killed cells were washed with sterile distilled water and the heat-killed cells were collected by centrifugation at 1,000 rpm for 10 min. Next, the heat-killed cells were lyophilized to prepare powder. The heat-killed cell powders were suspended in PBS to 200 μg/mL to prepare the test sample.

Cell Culture and Cell Differentiation

RAW264.7 cells were cultured in DMEM supplemented with 5% FBS and 0.25% PS at 37 ℃ in 5% CO_2. Professor Shinichi Yokota of Sapporo Medical University School of Medicine provided the Caco-2 cells. Caco-2 cells were grown in DMEM supplemented with 5% FBS and 0.25% PS in a 5% CO_2 at 37 ℃. THP-1 cells were grown in RPMI 1640 media supplemented with 10% FBS and 0.20% PS in a 5% CO_2 incubator at 37 ℃.

Caco-2 cells (1.5×10^5 cells) were cultivated for 72 h in cell culture inserts (Falcon, 24-Well Hanging Inserts 0.4 m). After 72 h, the solution containing 5 mM sodium butyrate was replaced, and the cells were cultured for 96 h to promote differentiation. Differentiated cells were evaluated by transepithelial electrical resistance (TEER) using Millicell-ERS (Merk), and differentiated cells (TEER values >400 Ωxcm^2) were used. THP-1 cells were seeded on a multi-well plate (24 well, Falcon) and incubated for 72 h in media supplementing 100 ng/mL cholecalciferol (vitamin D_3) and 10 nM phorbol12-myristate13-acetate (PMA) to differentiate into macrophage-like cells. Following differentiation, Caco-2 and THP-1 cells were co-cultured in Transwell.

Measurement of NO Production

The nitric oxide (NO) production was measured as described by Y. Yang et al. [42]. RAW264.7 cells were suspended in DMEM medium (5% FBS + 0.2% PS) at a concentration of 3×10^5 cells/mL, seeded in each 24-well multi-well plate, and incubated for 24 h at 37 ℃ in a 5% CO_2. The sample was examined at a final concentration of 20 μg/mL to stimulate the cells. PBS was used as a negative control, while LPS (10 μg/mL) (Fujifilm Wako) was used as a positive control. After 24 h of stimulation, the supernatant was collected and centrifuged at 12,000 rpm for 20 min and determined by Griess reaction as reported by K.-S. Baek et al. [43]; 100 μl of each Griess reagent, medium supernatant sample, and 1.56 to 100 μM of sodium nitrite standard solution were added to 96-well microplates and incubated for 20 min at room temperature. The absorbance at 550 nm was measured in a microplate reader. The amount of nitrite in the medium supernatant was enumerated using a calibration curve generated from the sodium nitrite standard solution.

Intestinal Immunity Model

An in vitro intestinal immune model was simulated by co-culture of cell culture inserts (apical side) and multi-well plates (basal side). The samples were suspended in RPMI 1640 medium that was added to the apical side (final concentration 20 μg/mL), and the cells were stimulated in a 5% CO_2 incubator at 37 ℃ for 2 days. After incubation, the basal side

of the medium was collected and centrifuged at 12,000 rpm for 20 min. For determination of IL-12 and IFN-γ concentration, proteins were precipitated by adding a 25% volume of 100% trichloroacetic acid (TCA) to the culture medium supernatant sample. The precipitated proteins were washed with acetone to eliminate TCA and dissolved in 1X sample buffer for protein enrichment after heat treatment at 100 ℃ for 2 min. SDS-PAGE was used to separate the proteins performed according to U. K. Laemmli [44], and the target proteins were detected by Western blot according to the method reported by H. Towbin et al. [45]. Calibration curves were prepared with IL-12 and IFN-γ standard to calculate the production of IL-12 and IFN-γ. The IL-12 and IFN-γ concentration were corrected by measuring β-actin as an endogenous control. The PVDF membrane was reacted with an IL-12p35 antibody [46] (diluted 500 times) with an anti-rabbit AP IgG (BioRad, diluted 10,000 times) or an anti-rabbit HRP IgG (Biorad, diluted 15,000 times). Samples were treated with a BCIP-NBT solution (Nacalai Tesque) or an ECL Plus Western Blotting Detection System (Cytiva). The immunoblotting bands obtained were analyzed by ImageJ. For hBD2, unenriched medium supernatant was measured by the Dot blot, and the amount of produced hBD2 was corrected from the amount of total protein by CBB staining. The values were evaluated relative to the no stimulation test section with PBS.

Statistical Analysis

All experiments were done in triplicates, and the results were described as the mean ± standard deviation. All data were analyzed by independent *t*-test using SPSS 22.0 software. For the comparison between initial time and gastric/small intestinal-emptying time, paired *t*-test was used for analysis. The significant differences were accepted at *P*-value ≤0.05

Results

Characterization of Strain

Strain LM14-2 was Gram-stain-positive and facultatively anaerobic rods. Growth occurred at 15–45 ℃, pH 3.0–8.0, and in the presence of 1–8% (w/v) NaCl. It did not produce gas from glucose. LM14-2 produced DL-lactic acid from glucose. Negative for catalase, arginine hydrolysis, and nitrate reduction. Strain LM14-2 contained *meso*-DAP in the cell wall peptidoglycan. In API 50 CH tests, acid formation is described in Supplementary Table 1. Based on the full 16S rRNA gene sequence (1,567 bp), strain LM14-2 was closely related to *L. plantarum* ATCC 14917^T, *L. argentoratensis* DSM 16365^T, *L. pentosus* DSM 20314^T, and *L. paraplantarum* DSM 10667^T with 100%, 100%, 99.93%, and 99.73% similarity, respectively.

In phylogenomic tree analysis (Supplementary Fig. 1), strain LM14-2 was formed in the same cluster within several *L. plantarum* strains. Strain LM14-2 was closely related to *L. plantarum* DSM 20174^T or ATCC 14917^T with a digital DNA–DNA hybridization (dDDH) value of 93.7%, which was the highest dDDH value observed with closely related species (Supplementary Table 2). The values of ANIb and ANIm between strain LM14-2 and *L. plantarum* DSM 20174^T or ATCC 14917^T were 98.2% and 99.27%, respectively (Supplementary Table 2). The strain LM14-2 was identified as *L. plantarum*. The genomic features of *L. plantarum* LM14-2 (accession no. JALPQH000000000), *L. plantarum* 299V (accession no. LEAV00000000), and *L. rhamnosus* GG (accession no. FM179322) (Table 1) lead to a circular genome map construction (Fig. 1). The draft genome sequence of strain LM14-2 was 3,311,812 bp, with a genomic DNA G + C content of 44.3%, N_{50} of 331,723, L_{50} of 4, and genome coverage of 250×. CheckM showed 99.35% genome completeness. The Prokaryotic Genome Annotation Pipeline annotation identified 3,115 genes (total), 2,999 are genes (coding), 41 pseudo genes (total), 76 genes (RNA), 70 tRNAs, and 4 ncRNAs; while the Rapid Annotation of microbial genomes using Subsystems Technology (RAST) showed 3,254 (no. of CDSs) and 67 RNA genes. Furthermore, DDBJ Fast Annotation and Submission Tool (DFAST) did not detect CRISPRS in strain LM14-2, and the subsystem is shown in Supplementary Fig. 2.

Safety Assessment

These genomic findings demonstrate the probiotic potential of strain LM14-2. The *L. plantarum* LM14-2 was predicted as a non-human pathogen by PathogenFinder web-based tool (Table 2). From the DFAST annotation database, the virulence-associated genes were detected, such as exopolysaccharides biosynthesis protein (*cps2B* and *cps4B*) and capsular polysaccharide biosynthesis protein (*cpsE*). Although these genes were identified as virulence elements, these genes play a key role in stress defense. In addition, the hemolysin III gene (*hlyIII*) was found in the genome of the strain LM14-2. The hemolysis test using sheep-blood agar demonstrated a hazy zone of hemolysis around the growth of bacteria, similar to the zone surrounding the probiotic *L. rhamnosus* GG, indicating that the two strains had comparable hemolysis activity. From the Comprehensive Antibiotic Resistance Database (CARD), PlasmidFinder and PHASTER web-based tools, the strain LM14-2 did not contain AR genes and plasmid. Three prophage regions were found (see Supplementary information (PHASTER.xlsx) for details).

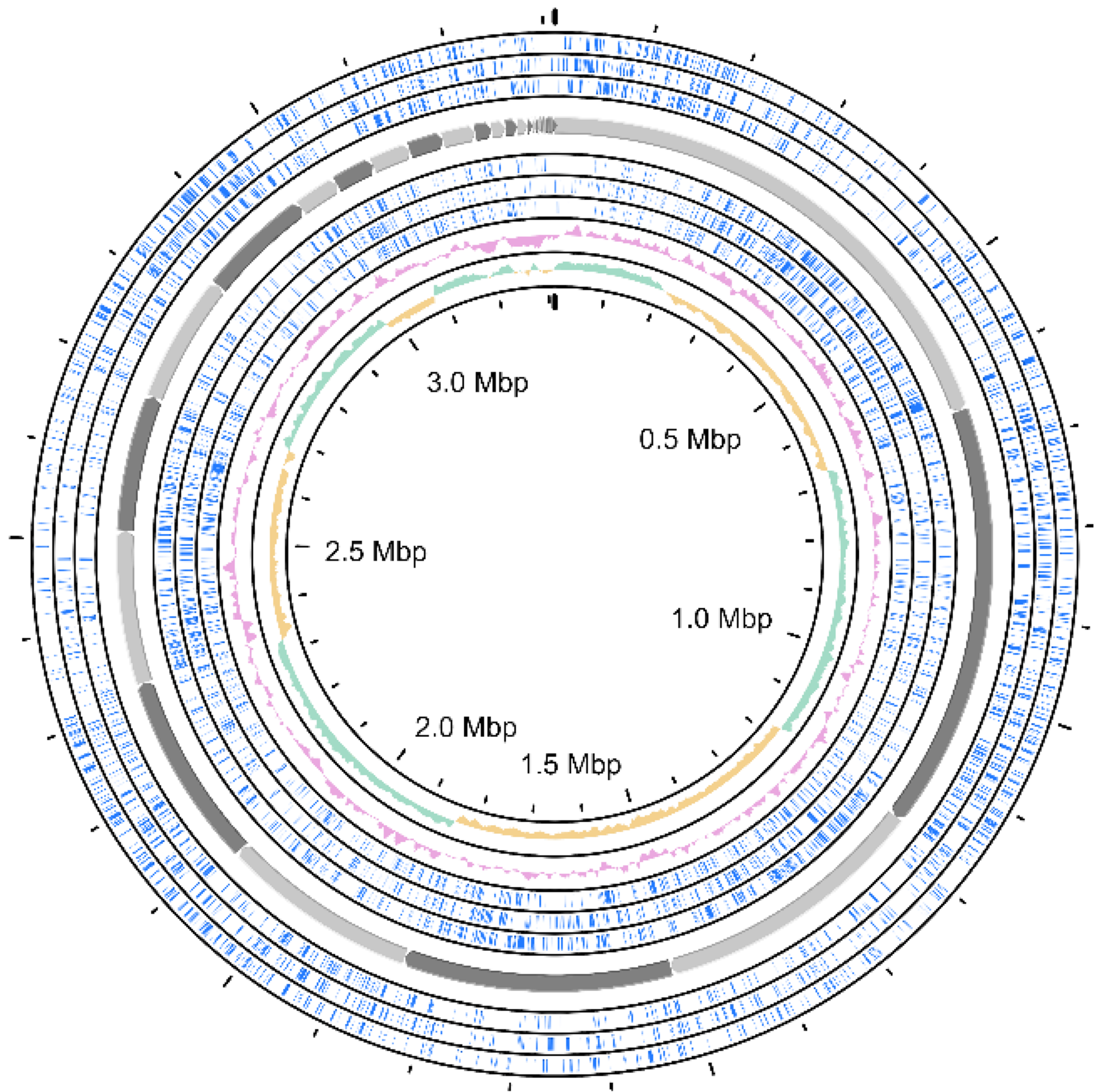

Fig. 1 Circular genomic map of *L. plantarum* LM14-2. The information is indicated as follows: open reading frames (ORFs) (blue), GC skew (+) (green), GC skew (−) (yellow), and GC content (pink)

Carbohydrate-active Enzyme Analysis

The genome of strain LM14-2 comprised 101 carbohydrate-active enzyme genes (Supplementary information, Cazy_LM14-2.xlsx), including 39 glycosyltransferase (GT) genes, 51 glycoside hydrolase (GH) genes, five carbohydrates esterase (CE) genes, three carbohydrate-binding molecules (CBMs), and three auxiliary activities (AA).

Identification of Genes Associated Stress Response, Microbe-Host Interactions, and Bacteriocin Biosynthesis

The genomic data of *L. plantarum* LM14-2 discovered subsystems and genes coding for stress response, adhesion, nutritional synthesis, cholesterol-lowering effect, lactate synthesis, transcriptional regulators, and metabolic

Table 2 Pathogenicity prediction, prophage detection, and antibiotic resistance genes (ARGs) analysis from PathogenFinder of CGE and PHASTER (default program settings applied)

Attribute/strain	*L. plantarum* LM14-2	*L. plantarum* 299v	*L. rhamnosus* GG
Probability of being a human pathogen	0.187	0.185	0.198
Input proteome coverage (%)	0.49	0.48	40.5
Matched pathogenic families	0	0	0
Matched not pathogenic families	15	15	1,147
Conclusion	Non-human pathogen	Non-human pathogen	Non-human pathogen
No. of phage	3	4	5
Antibiotic resistance genes (ARGs)			
CARD			
No. of perfect hits	0	0	0
No. of strict hits	0	0	0
No. of loose hits	192	194	207
ResFinder	No resistance	No resistance	No resistance

rearrangement, all of which contribute to probiotic characteristics, survival in acidic and bile salt conditions, and prevention of pathogen colonization in a gut environment (Table 3). Furthermore, this study discovered that strain LM14-2 possesses six genes that are crucial for the production of the class IIb bacteriocin: plantaricin NC8-α, F, E, J, NC8-β, and K with 98.60 bit-score, 107.07 bit-score, 112.46 bit-score, 112.46 bit-score, 112.85 bit-score, 114.01 bit-score, respectively (Fig. 2); however, this did not contain the other genes for bacteriocin production, including *plnA*, *plnB*, *plnC*, *plnL*, *plnN*, *plnO*, *plnQ*, *plnR*, *plnT*, *plnU*, *plnV*, *plnW*, and *plnX* genes, which are also essential for bacteriocin production. In addition, the no antimicrobial activity of this strain was confirmed by in vitro study. The plantaricin genes of strain LM14-2 and reference strains are shown in Table 4.

Cholesterol-lowering Effects

Bile Salt Hydrolase Activity

L. plantarum LM14-2 exhibited bile salt hydrolase activity by the formation of white opaque colonies and contained the choloylglycine hydrolase (also known as bile salt hydrolase; *bsh*) gene. This study demonstrated that BSH-producing LAB could be found from non-human origin.

Cholesterol Assimilation

In this study, strain LM14-2 showed a great cholesterol assimilation ability at 86.07 ± 5.03% with no statistically significant difference compared to *L. rhamnosus* GG (81.40 ± 4.00%) (Table 5).

The Gastrointestinal Transit Tolerance

The strain LM14-2 was incubated in simulated gastric condition supplemented with pepsin (2,000 U/mL) at pH 3 for 3 h, and the viability was reduced from 9.44 ± 0.03 to 7.93 ± 0.08 log CFU, while the viability of *L. rhamnosus* GG was reduced from 9.38 ± 0.04 to 7.78 ± 0.18 log CFU (Table 5). After that, strain LM14-2 was transited and incubated in an intestinal condition supplemented with pancreatin (based on trypsin activity 100 U/mL) at pH 7 for 5 h. The viability of strain LM14-2 showed a log reduction of 1.79 compared with the log CFU in intestinal condition at 0 h. While *L. rhamnosus* GG showed a log reduction of 1.85 compared with the log CFU in intestinal condition at 0 h.

The Adhesion Ability

L. plantarum LM14-2 and *L. rhamnosus* GG showed adhesion ability at 1.91 ± 0.88% and 1.26 ± 0.22%, respectively (Table 5). The adhesion ability of strain LM14-2 was not statistically significantly different from the adhesion ability of *L. rhamnosus* GG.

Immunomodulation Effects

The immunomodulatory effects of the *L. plantarum* LM14-2 are shown in Fig. 3. The *L. plantarum* LM14-2 stimulated IL-12, IFN-γ, and NO production at 35.49 ± 6.85 ng/mL, 44.89 ± 14.61 ng/mL, and 19.98 ± 0.28 µM, respectively. Furthermore, the hBD2 expression was also enhanced by *L. plantarum* LM14-2 (2.15 ± 0.07 of relative value). These results indicated that the dead cell of *L. plantarum* LM14-2 still had immunomodulatory effects.

Table 3 Potential genes associated with various probiotic characteristics from *L. plantarum* LM14-2 genome

Putative function	Genes	Gene product
Modulation of immune system/acid stress	*clpB*	Potential immunogenic proteins
	lspA	Lipoprotein signal peptidase
	tuf	Elongation factor Tu
Nutritional synthesis and several essential processes	*ccpA*	Catabolite control protein A
Cholesterol-lowering effect/Bile resistance	*bsh*	Choloylglycine hydrolase
Adhesion or interaction with the host	*srtA*	Class A sortase
	dltD	D-alanyl-lipoteichoic acid biosynthesis protein DltD
	dltA	D-alanylation of LTA
	mub	Mucus-binding protein
	glnH1	Glutamine ABC transporter substrate-binding protein
	lspA	Lipoprotein signal peptidase
	tuf	Elongation factor Tu
	mtsA	Manganese ABC transporter substrate-binding protein
	eno2	Enolase 2
	gapB	Type I glyceraldehyde-3-phosphate dehydrogenase
	groS	Co-chaperonin GroES
	groL	Chaperonin GroEL
	glnA	Glutamine synthase
	pgi	Glucose-6-isomerase
Acid stress	*atpC*	ATP synthase subunit epsilon
	atpD	ATP synthase subunit beta
	atpA	ATP synthase subunit alpha
	atpG	ATP synthase subunit gamma
	atpH	ATP synthase subunit delta
	atpF	ATP synthase subunit B
	atpB	ATP synthase subunit A
	atpE	ATP synthase subunit C
	recA	Protein RecA (recombinase A)
	relA	GTP pyrophosphokinase
	groS	Co-chaperonin GroES
	groL	Chaperonin GroEL
	aspS	Aspartate-tRNA ligase
Acid stress/bile resistance	*gpmA1*	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 1
	gpmA2	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2
	dnaK	Chaperone protein DnaK
	dnaJ	Chaperone protein DnaJ
	glmU	Bifunctional UDP-N acetylglucosamine diphosphorylase/glucosamine phosphate
	luxS	S-ribosylhomocysteine lyase
	gadB	Glutamate decarboxylase; GABA transporter
Bile resistance	*nagB*	Glucosamine-6-phosphate deaminase
	pyrG	CTP synthase
	argS	Arginine-tRNA ligase
	rpsC	30S ribosomal protein S3

Table 3 (continued)

Putative function	Genes	Gene product
	rpsE	30S ribosomal protein S5
	rplD	50S ribosomal protein L4
	rplE	50S ribosomal protein L5
	rplF	50S ribosomal protein L6
DNA and protein protection and repair	*dps1*	DNA starvation/stationary phase protection protein
	dps2	DNA starvation/stationary phase protection protein
	msrB	Peptide methionine sulfoxide reductase MsrB
Fatty acid synthesis	*fabD*	Malonyl CoA-acyl-carrier-protein transacylase
	fabH1	3-oxoacyl-[acyl-carrier-protein] synthase III protein 1
	fabH2	3-oxoacyl-[acyl-carrier-protein] synthase III protein 2
	fabF	3-oxoacyl-[acyl-carrier-protein] synthase II
	fabI	Enoyl-[acyl-carrier-protein] reductase [NADH]
	accC1	acetyl-CoA carboxylase biotin carboxylase subunit
	accC2	acetyl-CoA carboxylase biotin carboxylase subunit
Vitamin synthesis:	**Subsystem:** Biotin biosynthesis (Biotin: B_7)	
	Subsystem: Folate biosynthesis (Folate: B_9)	
	Subsystem: NAD and NADP cofactor biosynthesis global (Niacin: B_3)	
	Subsystem: Coenzyme A biosynthesis (Panthothenate: B_5)	
	Subsystem: Pyridoxin biosynthesis (Pyridoxine: B_6)	
	Subsystem: Thiamin biosynthesis (Thiamin: B_1)	
	Subsystem: Riboflavin metabolism (Riboflavin: B_2)	
Lactate synthesis	*mdh*	Malate dehydrogenase
Transcriptional regulator	*ctsR*	Transcriptional regulator CtsR
	hrcA	Heat-inducible transcriptional repressor HrcA
Metabolic rearrangement	*aldB*	Alpha-acetolactate decarboxylase

Discussion

This study highlighted the characterization, probiogenomic, cholesterol-lowering, and immunomodulation effects of *L. plantarum* LM14-2 isolated from Thai fermented mussel (*Hoi-dong*). The strain LM14-2 was identified as *L. plantarum* based on the dDDH and ANI values [47] that were above the species boundary value (ANI > 95–96%) [48]. For these reasons, the characteristics and whole-genome analysis supported strain LM14-2 as representing a member of *Lactiplantibacillus plantarum*.

The results of genomic sequences are necessary for comprehensive safety assessments and proposal of probiotic application of strain. The prediction of pathogenicity found that strain LM14-2 was predicted as a non-human pathogen. Exo- and capsular-polysaccharide genes related to virulence factors were detected. However, exo- and capsular-polysaccharides protein (*cps2B*, *cps4B*, and *cpsE*) and polysaccharides are involved in stress tolerance. Exopolysaccharide help bacteria survive osmotic, desiccation, and oxidative stress conditions [11, 49–51] and play a role in cryoprotection [52]. In addition, exopolysaccharides play a role in cell adhesion [53]. Besides, capsular polysaccharides are involved in discovering the strain-specific properties important for probiotic action, such as stress resistance, adhesion, and the defense mechanism of the host [11, 49]. Capsular polysaccharides have been discovered in bacterial colonization in the digestive tract, and they play a role in modulating the immune system [54]. Notably, virulence factors (i.e., adhesions) were also encoded in the genomes of commensal bacterial [49]. Additionally, the hemolysin III gene (*hlyIII*) was also observed in various commercial probiotics, including an accepted Generally Recognized as Safe (GRAS) probiotic strain *L. plantarum* 299V, *L. rhamnosus* GG, and numerous other *Lactobacillus* strains in the GenBank [55]. Generally, the strains containing the *hlyIII* gene have been proven safe and are commercially available

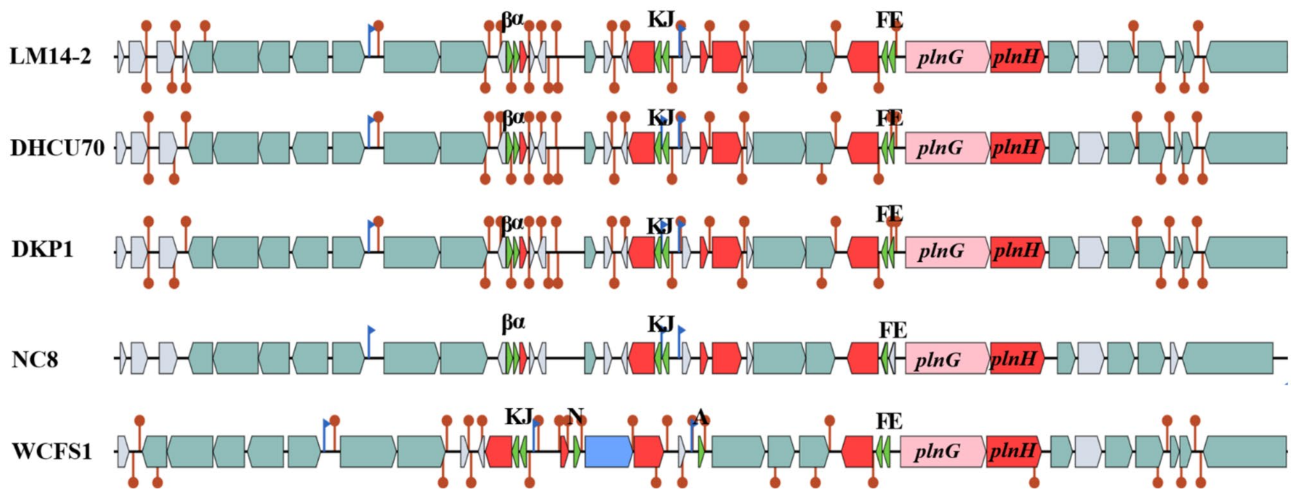

Fig. 2 Genetic organization of *pln* locus of *L. plantarum* LM14-2; *L. plantarum* DHCU70; *L. plantarum* DKP1; *L. plantarum* NC8; and *L. plantarum* WCFS1; Indicators: α – *NC8α*, β – *NC8β*, J – *plnJ*, K – *plnK*, E – *plnE*, F – *plnF*; red color indicated immunity protein. Data generated from the BAGEL4 tool

worldwide. The bacterium containing this gene should not pose a safety risk, provided that no other pathogenesis genes are observable in the genome. There are numerous reports on hemolysin III and its safety in lactobacilli; hence, this toxin protein is not considered of significant concern [56–58]. Nevertheless, these virulence genes could be deemed advantageous to the bacterium without pathogenesis mechanisms. These genes were identified as virulence factors in the virulence factor database as they were also involved in pathogenic bacterial adaptation, survival, or attachment in the hostile/host environment. These genes could be regarded as beneficial to the bacterium since they increase bacterial fitness and may be desirable where live cells are needed. Not only the enhancing role of bacterial endurance but also these genes were observed in the genomes of several commensal bacteria [50, 55]. The concern of AR genes in beneficial bacteria is their transfer possibility to other pathogens, which may cause serious problems. This study focused on mobile elements, plasmids and bacteriophages, because they are the most plausible vehicles for inter-cellular genetic exchange. The strain LM14-2 did not contain AR genes and plasmid; consequently, no *oriT* was found in any of the plasmids, indicating that LM14-2 is incapable of self-transmission through conjugative transfer. No ARGs (antibiotic resistance genes) were located in

Table 4 Comparison of *pln* genes of *L. plantarum* LM14-2, DHCU70, DKP1, NC8, and WCFS1

pln genes	Function	*L. plantarum* strains				
		LM14-2	DHCU70[a]	DKP1[a]	NC8[a]	WCFS1[a]
plnA	Induction pheromone	−	−	−	−	+
plnB	Histidine protein kinase	−	−	−	−	+
plnC	Response regulator	−	−	−	−	+
plnD	Response regulator	+	+	+	+	+
plnEF	Prebacteriocin with GC leader	+	+	+	+	+
plnG	ABC Transporter	+	+	+	+	+
plnH	Accessory protein	+	+	+	+	+
plnJK	Prebacteriocin with GC leader	+	+	+	+	+
plnL	Immunity protein	−	+	+	+	+
plnMN	Prebacteriocin with GC leader	+ (−N)	−	−	−	+
plnNC8α	Prebacteriocin with GC leader	+	+	−	+	−
plnNC8β	Prebacteriocin with GC leader	+	+	+	+	−
plnNC8IF	Induction pheromone	−	+	+	+	−
plnNC8HK	Histidine protein kinase	−	+	+	+	−

[a]Data obtained from the study of Goel A et al. [76]

The (−N) indicated the lack of *plnN*

Table 5 Cholesterol-lowering effects, gastrointestinal tolerance and its adhesion ability of *L. plantarum* LM14-2 and *L. rhamnosus* GG

Cholesterol-lowering effects, gastrointestinal tolerance, and adhesion ability		
	***L. plantarum* LM14-2**	***L. rhamnosus* GG**
Cholesterol-lowering effects:		
Bile salt hydrolase (BSH)	+	+
Cholesterol assimilation (%)	86.07 ± 5.03	81.40 ± 4.00
Gastrointestinal tolerance (Log CFU/mL)		
Simulated gastric phase (pepsin 2,000 U/mL, pH 3)		
0 h	9.44 ± 0.03	9.38 ± 0.04
3 h	7.93 ± 0.08*	7.78 ± 0.18*
Simulated intestinal phase (pancreatin based on trypsin activity at 100 U/mL, pH 7)		
0 h	6.87 ± 0.11	6.68 ± 0.25
5 h	5.08 ± 0.04*,**	4.83 ± 0.13*,**
Adhesion ability (%)	1.91 ± 0.88	1.26 ± 0.22

"+" showed bile salt hydrolase activity

*Significant difference in the comparison between initial time and gastric/small intestinal-emptying time ($P \leq 0.05$)

**Significant difference in the comparison between *L. plantarum* LM14-2 and *L. rhamnosus* GG ($P \leq 0.05$)

the prophage regions. As a result of these findings, it was established that the absence of AR genes in LM14-2 does not present a risk of transfer to other bacteria; therefore, the strain does not provide a safety issue for the functional and transferrable ARG characteristic.

The presence of genes involved in carbohydrate metabolism in strain LM14-2 is essential for its potential adaptability to the environment of the gut environment and its interaction with the human host, hence enhancing its survival, competitiveness, and longevity. The strain LM14-2 comprised several carbohydrate-active enzyme genes. Consequently, strain LM14-2 could use several mono- and polysaccharides as energy sources and produce complex compounds. Oligosaccharides are the source of prebiotics, which are related to human gut health [59]. The presence of GH13 and GH32 are key oligosaccharide-degrading enzymes. Furthermore, GH families play essential roles in synthesizing oligosaccharides that may be preferentially utilized as prebiotics by strain LM14-2 and other probiotic bacteria [60]. In addition, glycosyltransferases catalyze the conversion of sugars, which is necessary for constructing surface structures recognized by host immune systems [61]. Thus, strain LM14-2 containing the numerous GT genes could be a potential probiotic, especially for immune stimulation and pathogen prevention.

For potential genes associated with various probiotic characteristics, sortase class A (*srtA*) is involved for the LPXTG proteins covalently to the cell wall and plays a role in adherence to host surfaces. Mucus-binding proteins contribute to the adherence of bacteria to the intestinal mucosa [62]. Stain LM14-2 harbors gene coding for mucus-binding protein *mub*. Thirty-two genes responsible for acid and bile

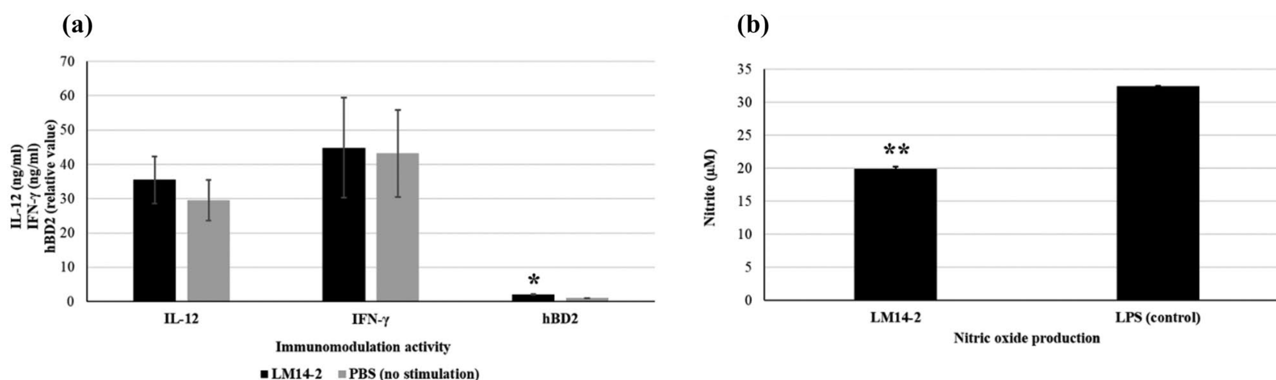

Fig. 3 The immunomodulation effects of *L. plantarum* LM14-2; **a** the level of IL-12 and IFN-γ production and the relative value of hBD2; **b** NO production. Results are expressed as means ± SD. *$P < 0.05$, compared to PBS (no stimulation) within each column; **$P < 0.05$, compared to LPS (positive control)

salt stress were identified (Table 3). The F1F0-ATPase is encoded by the *atp* operon, which comprises the following genes: *atpC*, *atpD*, *atpG*, *atpH*, *atpF*, *atpB*, *atpE*, and *atpA*. The atp genes are vital for the survival or tolerance of acidic environments. The "*atp*" operon is primarily related to the pumping of protons [63]. S-Ribosylhomocysteinase (*luxS*) plays an essential role in the adhesion ability and the autoinducer-2 synthesis that promotes stress resistance [64]. Additionally, moonlighting protein genes or multifunctional protein genes, such as elongation factor Tu and chaperonin GroEL, has been associated with adhesion to epithelial cells and immunomodulation [60]. Furthermore, probiotics perform an essential function in the host gut by synthesizing micronutrients and factors such as amino acids, fatty acids, oligosaccharides, vitamins, and enzymes. The strain LM14-2 comprised subsystems and genes responsible for synthesizing and metabolizing of amino acids and derivatives, carbohydrates, fatty acids, lipids, cofactors, prosthetic groups, and vitamins (Table 3). The seven subsystems are responsible for vitamin biosynthesis, including biotin, folate, niacin, pantothenate, pyridoxine, thiamin, and riboflavin. Overall, the draft genome information of *L. plantarum* LM14-2 will help us properly comprehend the molecular basis for its probiotic effects. The absence of antimicrobial activity was explained by the lack of several genes, including *plnA*, *plnB*, *plnC*, *plnL*, *plnN*, *plnO*, *plnQ*, *plnR*, *plnT*, *plnU*, *plnV*, *plnW*, and *plnX* genes, which are also essential for bacteriocin production, transcriptional regulation, and bacteriocin secretion [65, 66]. The incomplete plantaricin gene cluster, in agreement with in vitro findings, where no bacteriocin-like activity was detected. This finding is consistent with the previous study of Tegopoulos et al. [67]. Thus, the desirable probiotic characteristics of strain LM14-2 were also supported by the in silico findings.

For the cholesterol-lowering effects, the presence of the *bsh* gene and BSH activity indicated the cholesterol-lowering effects (lower total and low-density lipoprotein; LDL cholesterol). In the Guidelines for the Evaluation of Probiotics in Food issued by FAO/WHO, bile salt hydrolase activity is recognized as an additional criterion for selecting probiotics and safety assurance [9, 68]. Conversely, a high level of deconjugated bile may impair lipid digestion, disrupt normal intestinal conditions, induce gallstone formation, and may be further modified to carcinogenic secondary bile salts. After considering all the advantages and risks using the scientific evidence revealed above, this study suggested that the bile salt deconjugation property could be desirable when the strain could not modify the deconjugated bile into harmful secondary bile products. Aside from the choloylglycine hydrolase, no genes associated with the secondary bile salts biosynthesis were discovered in LM14-2. Regarding its inability to produce harmful secondary bile products, this study considers LM14-2 to pose no safety issues from this property. Moreover, the strain LM14-2 showed cholesterol assimilation ability. The BSH and cholesterol assimilation activities are the cholesterol removal mechanisms and desirable characteristics of probiotics [69]. Because probiotics can consume cholesterol for their metabolism, as a result, luminal cholesterol levels available for absorption are reduced.

For the immunomodulatory effects, the advantages of dead/dormant cells of probiotics include a reduced risk of probiotic sepsis and drug resistance, as well as a longer shelf-life because there is no need to preserve the storage system to preserve the viability of the probiotics [70, 71]. The immunomodulation ability of heat-killed LM14-2 cells is consistent with the previous studies [41, 72, 73]. In addition, the defensins activation may be an appealing novel treatment method for strengthening innate immunity. As a result, strain LM14-2 could enhance immunity [74, 75].

Conclusion

This study is the first to investigate the characterization, probiogenomic analysis, cholesterol-lowering, and immunomodulation effects of *L. plantarum* LM14-2 isolated from *Hoi-dong*. The strain LM14-2 exhibited BSH activity by forming an opaque white colony and potentially assimilated cholesterol by more than 75%. It could tolerate gastrointestinal conditions and greatly adhered to Caco-2 cells. Additionally, the *L. plantarum* LM14-2 provided immunostimulatory effects. *L. plantarum* LM14-2 could stimulate the production of IL-12, IFN-γ, and NO and promote the hBD2 secretion.

Furthermore, the in silico analysis of *L. plantarum* LM14-2 emphasized the attractiveness of this strain as a potential probiotic. This strain was considered safe, and it did not contain antibiotic resistance genes, plasmid, and virulence elements. In addition, it is composed of various genes that play roles in acid and bile salt tolerance, adhesion, and other positive benefits. Therefore, the combination of in vitro and in silico analysis suggested that *L. plantarum* LM14-2 is considered a potential probiotic because it exhibited health-promoting effects and probiotic characteristics. Also, the genomic data of this strain supported the desirable features.

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Author Contribution Engkarat Kingkaew: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review and editing visualization. Hiroshi Konno: resources, writing – review and editing, supervision. Yoshihito Hosaka: methodology, validation, formal analysis, data curation, writing – review and editing, visualization. Somboon Tanasupawat: validation, resources, data curation, writing – review and editing, supervision and project administration.

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Declarations

Competing interests The authors declare no competing interests.

Ethics Approval This article does not contain any studies with human participants and/or animals performed by the authors. Formal consent is not required in this research.

Conflict of Interest The authors declare no competing interests.

References

1. Albano C, Morandi S, Silvetti T et al (2018) Lactic acid bacteria with cholesterol-lowering properties for dairy applications: In vitro and in situ activity. J Dairy Sci 101:10807–10818
2. Thamacharoensuk T, Taweechotipatr M, Kajikawa A et al (2017) Induction of cellular immunity interleukin-12, antiproliferative effect, and related probiotic properties of lactic acid bacteria isolated in Thailand. Ann Microbiol 67:511–518
3. Kobatake E, Kabuki T (2019) S-Layer protein of *Lactobacillus helveticus* SBT2171 promotes human β-defensin 2 expression via TLR2–JNK signaling. Front Microbiol 2414
4. Wang B, Wei H, Yuan J et al (2008) Identification of a surface protein *from Lactobacillus reuteri* JCM1081 that adheres to porcine gastric mucin and human enterocyte-like HT-29 cells. Curr Microbiol 57:33–38
5. Moon P-D, Lee JS, Kim H-Y et al (2019) Heat-treated *Lactobacillus plantarum* increases the immune responses through activation of natural killer cells and macrophages on in vivo and in vitro models. J Med Microbiol 68:467–474
6. Nakai H, Hirose Y, Murosaki S, Yoshikai Y (2019) *Lactobacillus plantarum* L-137 upregulates hyaluronic acid production in epidermal cells and fibroblasts in mice. Microbiol Immunol 63:367–378
7. Kmonickova E, Kverka M, Tlaskalová-Hogenová H et al (2012) Stimulation of nitric oxide, cytokine and prostaglandin production by low-molecular weight fractions of probiotic Lactobacillus casei lysate. Neuro Endocrinol Lett 33:166–72
8. Surayot U, Wang J, Seesuriyachan P et al (2014) Exopolysaccharides from lactic acid bacteria: structural analysis, molecular weight effect on immunomodulation. Int J Biol Macromol 68:233–240
9. FAO/WHO (2002) Guidelines for the evaluation of probiotics in food. FAO/WHO Working Group:1-11
10. Khullar G, Det-udom R, Prombutar P, Prakitchaiwattana C (2022) Probiogenomic analysis and safety assessment of *Bacillus* isolates using Omics approach in combination with In-vitro. LWT 159:113216
11. Diale MO, Kayitesi E, Serepa-Dlamini MH (2021) Genome in silico and in vitro analysis of the probiotic properties of a bacterial endophyte, *Bacillus Paranthracis* strain MHSD3. Front Genet 2108
12. Phithakpol B, Varanyanond W, Reungmaneepaitoon S, Wood H (1995) The traditional fermented foods of Thailand ASEAN Food Handling Bureau. Kuala Lumpur. 157
13. Tanasupawat S, Komagata K (1995) Lactic acid bacteria in fermented foods in Thailand. World J Microbiol Biotechnol 11:253–256
14. Kingkaew E, Nuhwa R, Piluk J et al (2020) *Terrilactibacillus tamarindi* sp. nov., isolated from bark of *Tamarindus indica*. Int J Syst Evol Microbiol 70:4145–4150
15. Parks DH, Imelfort M, Skennerton CT et al (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res 25:1043–1055
16. Yoon S-H, Ha S-M, Kwon S et al (2017) Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int J Syst Evol Microbiol 67:1613
17. Richter M, Rosselló-Móra R (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci 106:19126–19131
18. Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J (2016) JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics 32:929–931
19. Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC bioinformatics 14:1–14
20. Kim M, Oh H-S, Park S-C, Chun J (2014) Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. Int J Syst Evol Microbiol 64:346–351
21. Meier-Kolthoff JP, Göker M (2019) TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. Nat Commun 10:1–10
22. Grant JR, Stothard P (2008) The CGView server: a comparative genomics tool for circular genomes. Nucleic Acids Res 36:181–184
23. Tanizawa Y, Fujisawa T, Nakamura Y (2018) DFAST: a flexible prokaryotic genome annotation pipeline for faster genome publication. Bioinformatics 34:1037–1039
24. Aziz RK, Bartels D, Best AA et al (2008) The RAST server: rapid annotations using subsystems technology. BMC genomics 9:1–15
25. Davis JJ, Wattam AR, Aziz RK et al (2020) The PATRIC Bioinformatics Resource Center: expanding data and analysis capabilities. Nucleic Acids Res 48:606–612
26. Tatusova T, DiCuccio M, Badretdin A et al (2016) NCBI prokaryotic genome annotation pipeline. Nucleic acids research 44:6614–6624
27. Carattoli A, Zankari E, Garcìa-Fernandez A et al (2014) PlasmidFinder and pMLST: in silico detection and typing of plasmids. Antimicrob Agents Chemother
28. Alcock BP, Raphenya AR, Lau TTY et al (2020) CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res 48:517–525
29. Bortolaia V, Kaas RS, Ruppe E et al (2020) ResFinder 4.0 for predictions of phenotypes from genotypes. J Antimicrob Chemother 75:3491–3500
30. Cosentino S, Voldby Larsen M, Møller Aarestrup F, Lund O (2013) PathogenFinder-distinguishing friend from foe using bacterial whole genome sequence data. PloS one 8:e77302
31. Arndt D, Grant JR, Marcu A et al (2016) PHASTER: a better, faster version of the PHAST phage search tool. Nucleic Acids Res 44:16–21
32. Cantarel BL, Coutinho PM, Rancurel C et al (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Res 37:233–8
33. Zhang H, Yohe T, Huang L et al (2018) dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. Nucleic Acids Res 46:95–101
34. van Heel AJ, de Jong A, Song C et al (2018) BAGEL4: a user-friendly web server to thoroughly mine RiPPs and bacteriocins. Nucleic Acids Res 46:278–281

35. Kanehisa M, Sato Y, Morishima K (2016) BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. J Mol Biol 428:726–731
36. Shehata MG, El Sohaimy SA, El-Sahn MA, Youssef MM (2016) Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. Ann Agric Sci 61(1):65–75
37. Tomaro-Duchesneau C, Jones ML, Shah D et al (2014) Cholesterol assimilation by *Lactobacillus* probiotic bacteria: an In Vitro investigation. Biomed Res Int Artn 380316. https://doi.org/10.1155/2014/380316
38. Rudel LL, Morris M (1973) Determination of cholesterol using o-phthalaldehyde. J Lipid Re 14:364–366
39. Minekus M, Alminger M, Alvito P et al (2014) A standardised static in vitro digestion method suitable for food–an international consensus. Food & Funct 5:1113–1124
40. Han Q, Kong BH, Chen Q et al (2017) In vitro comparison of probiotic properties of lactic acid bacteria isolated from Harbin dry sausages and selected probiotics. J Funct Foods 32:391–400
41. Hosaka Y, Itoh K, Matsutani S, Kawate S et al (2021) Fermented food Tempeh induces interleukin 12 and enhances macrophage phagocytosis. J Food Biochem 45:e13958
42. Yang Y, Xing R, Liu S et al (2018) Immunostimulatory effects of sulfated chitosans on RAW 264.7 mouse macrophages via the activation of PI3 K/Akt signaling pathway. Int J Biol Macromol 108:1310–1321
43. Baek K-S, Hong YD, Kim Y et al (2015) Anti-inflammatory activity of AP-SF, a ginsenoside-enriched fraction, from Korean ginseng. J Ginseng Res 39:155–161
44. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
45. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci 76:4350–4354
46. Hosaka Y, Matsutani S, Kawate S et al (2021) Expression of difficult-to-express proteins, human IL-12 and IFN-γ. Am J Mol Biol 11:29–37
47. Auch AF, von Jan M, Klenk H-P, Göker M (2010) Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. Stand Genom Sci 2:117–134
48. Lee I, Kim YO, Park S-C, Chun J (2016) OrthoANI: an improved algorithm and software for calculating average nucleotide identity. Int J Syst Evol Microbiol 66:1100–1103
49. Lebeer S, Verhoeven TLA, Francius G et al (2009) Identification of a gene cluster for the biosynthesis of a long, galactose-rich exopolysaccharide in *Lactobacillus rhamnosus* GG and functional analysis of the priming glycosyltransferase. Appl Environ Microbiol 75:3554–3563
50. Ho Sui SJ, Fedynak A, Hsiao WW et al (2009) The association of virulence factors with genomic islands. PloS one 4:e8094
51. Liu Y, Lai Q, Du J et al (2017) Genetic diversity and population structure of the *Bacillus cereus* group bacteria from diverse marine environments. Sci Rep 7(1–1):52
52. Casillo A, Parrilli E, Sannino F et al (2017) Structure-activity relationship of the exopolysaccharide from a psychrophilic bacterium: a strategy for cryoprotection. Carbohydr Polym. 156:364–71
53. Caro-Astorga J, Álvarez-Mena A, Hierrezuelo J et al (2020) Two genomic regions encoding exopolysaccharide production systems have complementary functions in *B. cereus* multicellularity and host interaction. Sci Rep 10:1–5
54. Porter NT, Canales P, Peterson DA et al (2017) A subset of polysaccharide capsules in the human symbiont Bacteroides thetaiotaomicron promote increased competitive fitness in the mouse gut. Cell Host Microbe 22:494–506
55. Chokesajjawatee N, Santiyanont P, Chantarasakha K et al (2020) Safety assessment of a nham starter culture *Lactobacillus plantarum* BCC9546 via whole-genome analysis. Sci Rep 10:1–12
56. Senan S, Prajapati JB, Joshi CG (2015) Feasibility of genome-wide screening for biosafety assessment of probiotics: a case study of *Lactobacillus helveticus* MTCC 5463. Probiotics Antimicrob Proteins 7:249–58
57. Surachat K, Sangket U, Deachamag P et al (2017) In silico analysis of protein toxin and bacteriocins from *Lactobacillus paracasei* SD1 genome and available online databases. PloS one 8:e0183548
58. Tan LL, Tan CH, Ng NK et al (2022) Potential probiotic strains from milk and water kefir grains in Singapore—use for defense against enteric bacterial pathogens. Front Microbiol 13
59. Tarrah A, Pakroo S, Lemos Junior WJF et al (2020) Complete genome sequence and Carbohydrates-Active EnZymes (CAZymes) analysis of *Lactobacillus paracasei* DTA72, a potential probiotic strain with strong capability to use inulin. Curr Microbiol 77:2867–2875
60. Abriouel H, Pérez Montoro B, Casimiro-Soriguer CS et al (2017) Insight into potential probiotic markers predicted in *Lactobacillus pentosus* MP-10 genome sequence. Front Microbiol 8:891
61. Chung W-H, Kang J, Lim MY et al (2018) Complete genome sequence and genomic characterization of *Lactobacillus acidophilus* LA1 (11869BP). Front Pharm 9:83
62. Lehri B, Seddon AM, Karlyshev AV (2015) *Lactobacillus fermentum* 3872 genome sequencing reveals plasmid and chromosomal genes potentially involved in a probiotic activity. FEMS Microbiol Lett 362:fnv068
63. Duary RK, Batish VK, Grover S (2010) Expression of the atpD gene in probiotic *Lactobacillus plantarum* strains under in vitro acidic conditions using RT-qPCR. Res Microbiol 161:399–405
64. Liu L, Wu R, Zhang J, Li P (2018) Overexpression of luxS promotes stress resistance and biofilm formation of *Lactobacillus paraplantarum* L-ZS9 by regulating the expression of multiple genes. Front Microbiol 2628
65. Diep DB, Straume D, Kjos M et al (2009) An overview of the mosaic bacteriocin pln loci from *Lactobacillus plantarum*. Peptides 30:1562–1574
66. Tai HF, Foo HL, Abdul Rahim R et al (2015) Molecular characterisation of new organisation of *pln*EF and plw loci of bacteriocin genes harbour concomitantly in *Lactobacillus plantarum* I-UL4. Microb Cell Fact 14:1–3
67. Tegopoulos K, Stergiou OS, Kiousi DE et al (2021) Genomic and phylogenetic analysis of *Lactiplantibacillus plantarum* L125, and evaluation of its anti-proliferative and cytotoxic activity in cancer cells. Biomedicines 9:1718
68. Miremadi F, Ayyash M, Sherkat F, Stojanovska L (2014) Cholesterol reduction mechanisms and fatty acid composition of cellular membranes of probiotic *Lactobacilli* and *Bifidobacteria*. J Funct Foods 9:295–305
69. Ishimwe N, Daliri EB, Lee BH et al (2015) The perspective on cholesterol-lowering mechanisms of probiotics. Mol Nutr & Food Res 59:94–105
70. Shripada R, Gayatri A-J, Sanjay P (2020) Chapter 5 – paraprobiotics, p 39-49. *In* Faintuch J, Faintuch S (ed), Precision Medicine for Investigators, Practitioners and Providers. https://doi.org/10.1016/B978-0-12-819178-1.00005-8. Academic Press
71. Zendeboodi F, Khorshidian N, Mortazavian AM, da Cruz AG (2020) Probiotic: conceptualization from a new approach. Curr Opin Food Sci 32:103–123
72. Chen CY, Tsen HY, Lin CL et al (2013) Enhancement of the immune response against *Salmonella* infection of mice by heat-killed multispecies combinations of lactic acid bacteria. J Med Microbiol 11:1657–64
73. Reilly N, Poylin V, Menconi M et al (2007) Probiotics potentiate IL-6 production in IL-1β-treated Caco-2 cells through a heat

shock-dependent mechanism. Am J Physiol Regul, Integr Comp Physiol 3:R1169-79
74. Kang C-H, Kim J-S, Kim H et al (2021) Heat-killed lactic acid bacteria inhibit nitric oxide production via inducible nitric oxide synthase and cyclooxygenase-2 in RAW 264.7 Cells. Probiotics Antimicrob Proteins 13:1530–1538
75. Kato I, Tanaka K, Yokokura T (1999) Lactic acid bacterium potently induces the production of interleukin-12 and interferon-γ by mouse splenocytes. Int J Immunopharmacol 21:121–131
76. Goel A, Halami PM, Tamang JP (2020) Genome analysis of *Lactobacillus plantarum* isolated from some Indian fermented foods for bacteriocin production and probiotic marker genes. Front Microbiol 40

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