Isolation, Identification, and In Vitro Probiotic Characteristics of *Lactobacillus plantarum* from Naturally Fermented Kimchi Juice: A Postprint

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Abstract

A dominant lactic acid bacterium was isolated from naturally fermented pickle juice using MRS solid medium and designated as strain R1. It was identified as Lactobacillus plantarum based on 16S rRNA gene sequence analysis combined with physiological and biochemical characteristics and sugar fermentation tests. Strain R1 exhibited strong acid-producing capability, reducing the pH of MRS liquid medium from 6.14 to 3.59 within 24 h. The fermentation supernatant of strain R1 demonstrated excellent inhibitory effects against Shigella dysenteriae, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella enteritidis, and Escherichia coli. In vitro probiotic assays indicated that strain R1 could tolerate 0.3% bile salts, pH 3.0 acidity, and heat treatment at 60 ℃ for 30 min, and also showed good tolerance to artificial gastric juice and artificial intestinal fluid. Strain R1 was relatively sensitive to cephalosporin and penicillin antibiotics, but insensitive to antibiotics such as norfloxacin, kanamycin, and streptomycin.

Full Text

Isolation, Identification, and In Vitro Probiotic Characteristics of Lactobacillus plantarum from Naturally Fermented Pickle Juice

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Abstract: A dominant lactic acid bacterium was isolated from naturally fermented pickle juice using MRS solid medium and designated as strain R1. Through 16S rRNA gene sequence analysis, combined with physiological and biochemical characterization and sugar fermentation tests, strain R1 was identified as *Lactobacillus plantarum*. The strain exhibited strong acid-producing capability, reducing the pH of MRS broth from 6.14 to 3.59 within 24 hours. The fermentation supernatant of strain R1 demonstrated excellent inhibitory effects against *Shigella dysenteriae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, and *Escherichia coli*. In vitro probiotic assays revealed that strain R1 could tolerate 0.3% bile salts, pH 3.0 acidity, and heat treatment at 60°C for 30 minutes, while also showing good tolerance to artificial gastric and intestinal fluids. The strain was sensitive to cephalosporin and penicillin antibiotics but insensitive to norfloxacin, kanamycin, streptomycin, and other antibiotics.

Keywords: *Lactobacillus plantarum*; antibacterial activity; isolation; identification; probiotic properties

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Lactic acid bacteria are widely distributed probiotics in nature with broad applications in food, medicine, and feed industries [1-2]. *Lactobacillus plantarum*, a member of the *Lactobacillus* genus within the Lactobacillaceae family, is a homofermentative lactic acid bacterium [3]. Numerous studies have demonstrated that *L. plantarum* possesses various probiotic functions, including reducing serum cholesterol concentration [4-7], inhibiting proliferation of intestinal pathogens [8-11], and decreasing nitrite concentration in foods [12-14]. The use of *L. plantarum* and its metabolites as microbial ecological regulators to inhibit common human and animal pathogens has received considerable attention in food safety and animal husbandry [15-17]. Naturally fermented vegetable products serve as excellent sources for isolating and screening *L. plantarum*, and many superior strains have been obtained from such materials [6,12,18].

This study isolated *L. plantarum* with antibacterial activity from naturally fermented pickle juice and determined its taxonomic status. Additionally, since probiotics must withstand adverse gastrointestinal conditions such as gastric acid and bile to exert their beneficial effects in vivo [17,19-20], we evaluated the in vitro probiotic characteristics of the isolated strain to establish a foundation for developing probiotic preparations.

1.1.1 Isolation Material

Naturally fermented pickle juice prepared primarily from cabbage.

1.1.2 Test Pathogens

The pathogenic strains *Escherichia coli* (GIM 1.299), *Shigella dysenteriae* (GIM 1.236), *Staphylococcus aureus* (CGMCC:29213), *Salmonella enteritidis*

(CGMCC:21510), and *Pseudomonas aeruginosa* (GIM 1.443) were preserved in our laboratory.

1.1.3 Culture Media and Reagents

MRS medium and beef extract peptone medium were prepared according to reference [21]. PCR reagents including Taq DNA polymerase, dNTPs, and $MgCl_2$ were purchased from TaKaRa (Japan). Porcine bile salts were from Shanghai Lanji Technology Development Co., Ltd. Pepsin (1:3000) and trypsin (1:250) were from Amresco (USA). Antibiotic filter paper discs were from Hangzhou Microbial Reagent Co., Ltd. All other reagents were analytical grade.

1.2 Instruments and Equipment

UV-2000 UV-Vis spectrophotometer (Unico, Shanghai), HQY-C constant temperature shaking incubator (Jintan Hongke Instrument Factory), PHS-3C pH meter (Shanghai Leici Instrument Factory), T Professional Trio PCR thermal cycler (Biometra, Germany), Gel Doc XR+ gel imaging system (Bio-Rad, USA), and DX50 microscope (Olympus, Japan).

1.3.1 Isolation of Lactic Acid Bacteria

Ten milliliters of naturally fermented pickle juice were aseptically transferred to a flask containing 90 mL sterile water to prepare a 10^{-1} dilution. After mixing, 1 mL was transferred to a tube with 9 mL sterile water to make a 10^{-2} dilution. This process was repeated to obtain 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions. Then, 0.2 mL aliquots of 10^{-4}, 10^{-5}, and 10^{-6} dilutions were spread on MRS solid plates, which were incubated at 37°C for 48 hours. The dominant colony type was selected as strain R1 for further study.

1.3.2 Identification of Strain R1

1.3.2.1 16S rRNA Gene Sequence Analysis

Total DNA of strain R1 was extracted using a bacterial DNA extraction kit, and the 16S rRNA gene was amplified by PCR [22]. Universal bacterial primers were used: 27f (5' -GAGCGGATAACAATTTCACACAGG-3') and 1492r (5' -CGCCAGGGTTTTCCCAGTCACGAC-3'). The 50 µL reaction mixture contained: 1 µL template DNA, 5 µL 10×Taq buffer, 4 µL Mg^{2+} (25 mmol/µL), 4 µL dNTP mixture (20 mmol/µL), 2 µL each primer (25 pmol/µL), 0.5 µL Taq DNA polymerase (5 U/µL), and 31.5 µL sterile double-distilled water. PCR conditions were: 94°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min; final extension at 72°C for 10 min; hold at 10°C for 10 min. PCR products were verified by 0.75% agarose gel electrophoresis and sequenced by GenScript (Nanjing).

Phylogenetic analysis: The 16S rDNA sequence was compared against the NCBI database (http://www.ncbi.nlm.nih.gov) and homologous sequences were down-

loaded from the RDP database (http://rdp.cme.msu.edu/index.jsp). Sequences were aligned using ClustalW in BioEdit, and a phylogenetic tree was constructed using MEGA 6.0 with the neighbor-joining method and 1,000 bootstrap replicates.

1.3.2.2 Physiological and Biochemical Characterization

Physiological and biochemical tests including catalase reaction, nitrate reduction, gelatin liquefaction, V-P test, methyl red test, and sugar fermentation were performed according to the *Manual of Systematic Identification of Common Bacteria* [23].

1.3.3 Growth and Acid Production Curves of Strain R1

Strain R1 was activated in MRS broth for 12 hours, then inoculated at 1% (v/v) into 200 mL MRS broth and incubated at 37°C with shaking at 150 rpm. At regular intervals, 3 mL samples (in triplicate) were collected to measure pH and OD_{600} nm using a pH meter and spectrophotometer, respectively. Growth and acid production curves were plotted with time on the x-axis and pH/OD_{600} nm on the y-axis.

1.3.4 Antibacterial Activity of Strain R1 Fermentation Supernatant

The Oxford cup method was used to assess antibacterial activity. Five pathogenic strains (*S. dysenteriae*, *S. aureus*, *S. enteritidis*, *P. aeruginosa*, and *E. coli*) were cultured to logarithmic phase in liquid beef extract peptone medium. Two hundred microliters of each pathogen suspension were spread on solid beef extract peptone plates. After absorption, three sterile Oxford cups were placed on each plate. Strain R1 was cultured in MRS broth for 24 hours, centrifuged at 6,000 rpm for 10 minutes, and 200 µL of supernatant was added to each cup. Plates were incubated at 37°C for 10 hours, after which inhibition zones were observed and measured. The antibacterial mechanism was preliminarily analyzed by adjusting supernatant pH and adding catalase and protease [24].

1.3.5 In Vitro Probiotic Characteristics of Strain R1

1.3.5.1 Bile Salt Tolerance

Activated strain R1 was inoculated at 1% (v/v) into MRS broth containing 0%, 0.1%, 0.3%, or 0.5% bile salts and incubated at 37°C with shaking at 150 rpm for 26 hours. OD_{600} nm was measured at intervals to plot growth curves and determine bile salt tolerance.

1.3.5.2 Acid Tolerance

MRS broth was adjusted to pH 1.0, 2.0, 3.0, or 5.0 using 1 mol/L HCl. Activated strain R1 was inoculated at 1% (v/v) into each pH-adjusted medium and

incubated at 37°C. OD_{600} nm was measured periodically to plot growth curves under different pH conditions.

1.3.5.3 Heat Tolerance

Activated strain R1 culture was divided into three portions and treated at 40°C, 60°C, or 80°C for 30 minutes. Viable cell counts were determined by dilution plating before and after treatment to calculate survival rates.

1.3.5.4 Tolerance to Artificial Gastric and Intestinal Fluids

Artificial gastric and intestinal fluids were prepared according to reference [19]. Activated strain R1 was inoculated at 1% (v/v) into 100 mL of artificial gastric or intestinal fluid and incubated at 37°C with shaking at 150 rpm. Viable counts were performed at 0, 30, and 180 minutes.

1.3.5.5 Antibiotic Sensitivity

The filter paper disc method was used [25]. Strain R1 was cultured to logarithmic phase in MRS broth, and 200 µL was spread on MRS agar plates. Antibiotic-containing filter paper discs were placed on the plates, which were incubated at 37°C for 24 hours. Inhibition zones were measured (three replicates per antibiotic). Antibiotic contents per disc were: cefaclor 30 µg, cefixime 5 µg, cephradine 20 µg, ampicillin 10 µg, penicillin 10 U, amoxicillin 20 µg, azithromycin 15 µg, kanamycin 30 µg, streptomycin 10 µg, tetracycline 30 µg, furazolidone 300 µg, norfloxacin 10 µg, and bacitracin 0.04 U.

1.4 Data Processing and Analysis

Data were processed and analyzed using Excel 2007 and DPS v6.50 software. Duncan' s new multiple range test was used to determine significant differences between groups ($P < 0.05$).

2.1 Isolation and Identification of Lactic Acid Bacteria

A dominant lactic acid bacterium was isolated from naturally fermented pickle juice using MRS solid medium and designated strain R1. On MRS agar, strain R1 formed circular, milky white, convex, opaque colonies with regular edges. Microscopic observation revealed rod-shaped cells occurring singly or in chains [Figure 1: see original paper]. The strain was Gram-positive, catalase-negative, methyl red-negative, V-P-negative, and gelatin liquefaction-negative. It fermented maltose, mannose, galactose, glucose, lactose, and sucrose to produce acid, with high utilization of glucose and galactose. Strain R1 grew well at NaCl concentrations below 5%, with optimal growth temperature and pH of 37°C and 5.0, respectively.

PCR amplification and sequencing of the 16S rRNA gene using universal primers 1492r and 27f yielded a sequence that was submitted to GenBank (accession number KY874048). BLAST analysis revealed 100% similarity with the 16S

rRNA gene of *Lactobacillus plantarum* MXG-68 (accession number KY750314). Phylogenetic analysis using RDP Sequence Match and MEGA 6.0 showed that strain R1 was most closely related to the type strain *L. plantarum* DK0 22T (accession number AJ640078) [Figure 2: see original paper]. Based on these results and physiological-biochemical characteristics, strain R1 was identified as *Lactobacillus plantarum*.

2.2 Growth and Acid Production Curves of Strain R1

The growth and acid production curves of strain R1 in MRS broth over 0–28 hours are shown in [Figure 3: see original paper]. The strain grew slowly during the first 2 hours, then rapidly entered logarithmic phase. Growth slowed after 12 hours, reaching maximum cell density at 14 hours (OD_{600} nm = 2.144), with only a slight decrease after 24 hours. The pH remained relatively stable for the first 2 hours, then dropped rapidly from 6.14 to 3.95 between 2–12 hours, and gradually decreased to 3.59 by 28 hours. These results indicate that strain R1 has a short lag phase and can achieve maximum biomass and low pH within 14 hours, making it suitable for fermentation processes requiring rapid acidification and high cell yields.

2.3 Inhibition of Common Pathogens by Strain R1

Lactic acid bacteria often inhibit pathogens through lactic acid production or secretion of antibacterial substances, making such strains highly sought after. The antibacterial activity of strain R1 fermentation supernatant was tested against common pathogens. The results showed strong inhibitory effects against all tested pathogens, with the highest activity against *S. dysenteriae* (inhibition zone diameter: 20.50 mm), followed by *S. enteritidis* (15.67 mm), *E. coli* (14.17 mm), *S. aureus* (13.33 mm), and *P. aeruginosa* (12.67 mm). The control MRS broth showed no inhibition. Preliminary mechanism analysis by pH adjustment and addition of catalase and proteases revealed that antibacterial activity was primarily dependent on acidic metabolites such as lactic acid. When supernatant pH was adjusted to 5.0, the inhibition zone against *S. aureus* decreased significantly ($P < 0.05$), and no inhibition was observed at pH 7.0. Addition of pepsin, trypsin, or catalase slightly reduced antibacterial activity .

2.4.1 Bile Salt Tolerance of Strain R1

Growth of strain R1 in MRS broth containing different bile salt concentrations is shown in [Figure 4: see original paper]. At 0.1% bile salts, the lag phase extended to 8 hours, with reduced growth rate compared to the control, reaching stationary phase after 24 hours. At 0.3% bile salts, the lag phase further extended to 12 hours, with exponential growth beginning thereafter and reaching stationary phase at 26 hours. At 0.5% bile salts, growth was completely inhibited even after 30 hours. These results demonstrate that strain R1 can tolerate 0.3% bile salt concentration.

2.4.2 Acid Tolerance of Strain R1

Growth of strain R1 in MRS broth at different pH values is presented in [Figure 5: see original paper]. At pH 5.0, the lag phase was only 2 hours, followed by exponential growth and reaching stationary phase by 8 hours. At pH 3.0, the lag phase extended to 4 hours, with growth slowing after 22 hours and lower final cell density compared to pH 5.0. At pH 1.0 and 2.0, growth was completely inhibited even after 24 hours. These findings indicate that strain R1 grows optimally at pH 5.0 and can tolerate pH 3.0.

2.4.3 Heat Tolerance of Strain R1

Viable cell counts after heat treatment at different temperatures for 30 minutes are shown in . Survival rates were 94.70% and 80.60% after treatment at 40°C and 60°C, respectively, indicating good tolerance. However, survival rate dropped to only 1.95% after 80°C treatment, showing high sensitivity to this temperature. Therefore, temperatures above 60°C should be avoided during production, transportation, and application of strain R1.

2.4.4 Tolerance to Artificial Gastric and Intestinal Fluids

As shown in , strain R1 not only survived but also grew in artificial gastric juice, with viable counts increasing from (4.28$±0.65)×10^{5}$ CFU/mL at 0 minutes to (8.35$±0.46)×10^{5}$ CFU/mL at 180 minutes. Similarly, in artificial intestinal fluid, viable counts increased from (2.58$±0.03)×10^{6}$ CFU/mL to (3.66$±0.10)×10^{6}$ CFU/mL within 3 hours. These results demonstrate excellent tolerance to both artificial gastric and intestinal fluids.

2.4.5 Antibiotic Sensitivity of Strain R1

Strain R1 exhibited varying sensitivity to different antibiotics . It was highly sensitive to antibiotics commonly used for respiratory infections, including amoxicillin, ampicillin, and cephalosporins. Sensitivity to furazolidone (used for gastrointestinal infections) was weak, while no sensitivity was observed to norfloxacin, bacitracin, kanamycin, streptomycin, or tetracycline.

3.1 Identification of Strain R1

Taxonomic identification of microbial isolates is essential for understanding their biology. 16S rRNA gene sequence analysis is currently the most accurate and rapid method for bacterial classification. BLAST analysis of strain R1' s 16S rRNA gene sequence against the NCBI database revealed 100% homology with numerous *L. plantarum* sequences, leading to preliminary identification. Many *L. plantarum* strains have been isolated from naturally fermented vegetables [6,12,18], and strain R1 was similarly obtained from pickle juice as a dominant isolate. Its colonial and cellular morphology, Gram reaction, physiological-

biochemical characteristics, and sugar fermentation patterns all matched the description of *L. plantarum* [23], confirming its identification.

3.2 Antibacterial Activity of Strain R1

Lactobacillus plantarum produces large amounts of organic acids during growth [3,26], and some strains produce specific bacteriocins [2,9], both serving as effective biopreservatives that prevent pathogen colonization through pH reduction or biological antagonism. Strain R1 showed strong inhibition against common pathogens including *S. dysenteriae*, *S. enteritidis*, *E. coli*, *S. aureus*, and *P. aeruginosa*, with activity superior to some previously reported lactic acid bacteria [7,15]. Mechanism analysis through pH adjustment and enzyme addition indicated that inhibition was primarily mediated by acidic metabolites such as lactic acid, consistent with its strong acid-producing ability. Zhang et al. [26] reported that 11 *E. coli*-inhibiting lactic acid bacteria strains all exhibited good acid production. Ma et al. [27] identified multiple antibacterial components including lactic acid, acetic acid, succinic acid, and various fatty acids in the supernatant of a broad-spectrum inhibitory *Lactobacillus pentosus* strain. Further identification of the specific acidic compounds in strain R1' s supernatant is warranted.

3.3 In Vitro Probiotic Characteristics of Strain R1

Animal gastric fluid typically has a pH around 3.0, while the intestinal environment is alkaline (pH ~7.6) and contains bile salts and bile acids. Probiotics must tolerate these harsh conditions to survive and proliferate in the digestive tract [19]. Our results show that strain R1 can tolerate pH 3.0 and 0.3% bile salts. Compared with reported *L. plantarum* strains GF103 [19] and DJ-04 [28], strain R1 not only survived but also maintained growth in artificial gastric and intestinal fluids, suggesting superior potential for in vivo survival. Furthermore, probiotics often encounter heating processes during application or antibiotic exposure when the host is ill, which can inhibit their growth and efficacy. Understanding heat and antibiotic sensitivity is therefore crucial for proper usage. Strain R1 tolerated 60°C and was only sensitive to penicillins and cephalosporins, while showing resistance to norfloxacin, bacitracin, kanamycin, streptomycin, and tetracycline.

Conclusion

A lactic acid bacterium strain R1 was isolated from naturally fermented pickle juice, with 16S rRNA gene sequence showing 100% similarity to *Lactobacillus plantarum* MXG-68 (accession number KY750314). Its fermentation supernatant produced inhibition zones of 20.50, 15.67, 14.17, 13.33, and 12.67 mm against *S. dysenteriae*, *S. enteritidis*, *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively. The strain exhibited excellent acid-producing capability, tolerated pH 3.0 and 60°C heat treatment, and showed good tolerance to artificial gastric

and intestinal fluids. Strain R1 was sensitive to cephalosporin and penicillin antibiotics.

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Note: Figure translations are in progress. See original paper for figures.