

Original Research Article

Screening and Probiotic Characterization of a Cholesterol-Lowering *Lactacaseibacillus rhamnosus* Strain Isolated from Traditional Fermented Pickle Brine

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Abstract: Hypercholesterolemia is a major risk factor for cardiovascular diseases, and probiotic intervention offers a promising alternative to pharmacotherapy. This study aimed to isolate lactic acid bacteria (LAB) with high cholesterol-lowering capacity from traditional fermented pickle brine and comprehensively evaluate their probiotic properties. LAB were isolated using MRS agar, and their in vitro cholesterol removal ability was determined by the o-phthalaldehyde colorimetric method. The selected strain was identified by morphological observation, Gram staining, and 16S rRNA gene sequencing. Probiotic characteristics, including antimicrobial activity, tolerance to simulated gastrointestinal fluids, acid and bile salt resistance, auto-aggregation, hydrophobicity, and DPPH radical scavenging activity, were systematically assessed. Twelve presumptive LAB strains were obtained, among which strain LH3 exhibited the highest cholesterol removal rate (66.71%). It was identified as *Lactacaseibacillus rhamnosus* based on phenotypic and phylogenetic analyses. LH3 showed strong inhibitory activity against *Staphylococcus aureus* (inhibition zone 25.29 mm) and moderate activity against *Escherichia coli* (17.85 mm). Survival rates in simulated gastric and intestinal juices were 90.23% and 98.79%, respectively. At pH 3.0, survival was 69.43%; in 0.15% and 0.3% bile salt, survival rates were 73.15% and 42.96%, respectively. Auto-aggregation was 20.67%, hydrophobicity 50.09%, and DPPH scavenging activity 68.89%. *L. rhamnosus* LH3 possesses excellent in vitro cholesterol-lowering ability and favorable probiotic traits, making it a potential candidate for developing functional probiotic products targeting hypercholesterolemia.

Keyword: *Lactacaseibacillus rhamnosus*, Cholesterol Reduction, Probiotic Properties, Screening, Pickle Brine.

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1. INTRODUCTION

Cholesterol is an essential lipid for human physiology, serving as a key component of cell membranes and a precursor for bile acids, steroid hormones, and vitamin D (Chunfeng, 2011). However, modern dietary patterns high in fat and calories have led to elevated blood cholesterol levels worldwide. Epidemiological data indicate that each 1 mmol/L increase in total serum cholesterol is associated with an approximately 35% higher risk of cardiovascular events (Jingxue, 2010). Hypercholesterolemia is a major independent risk factor for atherosclerosis, coronary heart disease, and stroke, contributing to about 30% of all deaths in China, with a trend toward younger age of onset (Yadidi, 2017).

Current cholesterol-lowering strategies include dietary modification, physical exercise, and pharmacotherapy. While lifestyle interventions are safe, they require long-term adherence and show poor patient compliance. Statins and other lipid-lowering drugs are effective but may cause adverse effects such as hepatotoxicity and myopathy, and their high cost limits prophylactic use (Shanshan, Zhining, Weifeng, & Fenggeng, 2019). Hence, there is growing interest in safe, sustainable, and cost-effective alternatives, among which probiotics have emerged as a promising option.

Probiotics are live microorganisms that confer health benefits when administered in adequate amounts (Danxia, 2020). Numerous studies have demonstrated that certain *Lactobacillus* and *Bifidobacterium* strains

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can lower cholesterol via multiple mechanisms: assimilating cholesterol in the medium or gut; deconjugating bile salts by bile salt hydrolase (BSH), leading to co-precipitation of free bile salts with cholesterol and reduced micellar solubility; producing short-chain fatty acids (e.g., propionate) from fermentable carbohydrates, which inhibits hepatic cholesterol synthesis; and modulating gene expression involved in cholesterol metabolism (Changqing & Lina, 2007; Lim, Kim, & Lee, 2004; Remagni *et al.*, 2013). These findings provide a theoretical basis for using probiotics to manage hypercholesterolemia.

Several cholesterol-lowering probiotic strains have been isolated from fermented dairy products, kimchi, and the human intestine. For instance, *Lactocaseibacillus casei* S1 from fermented foods showed 68.74% cholesterol removal (Ying *et al.*, 2018); *Lactiplantibacillus plantarum* LAB4 from pickle brine achieved 42.72% (Mingyong, Tao, & Qianqian, 2014); and *Lactobacillus sakei* ADM14 from kimchi exhibited anti-adipogenic effects (Wanqiang, Linlin, Jianxin, Hao, & Wei, 2019). However, most studies remain at the in vitro or animal model stage, and commercially available strains specifically designed for cholesterol reduction are still limited. Moreover, probiotic traits such as acid and bile tolerance vary greatly among strains, highlighting the need to explore novel indigenous strains with robust functional properties (Muhua, Jie, Rui, & Xiaoguang, 2021).

Traditional Sichuan pickle brine is a rich source of diverse lactic acid bacteria, making it an ideal natural reservoir for probiotic screening (Yun, 2015). In this study, we isolated LAB from traditional pickle brine and screened for high in vitro cholesterol-lowering activity. The selected strain was systematically identified, and its probiotic potential was comprehensively evaluated by simulating gastrointestinal conditions, acid and bile salt resistance, antimicrobial activity, auto-aggregation, surface hydrophobicity, and antioxidant capacity. The aim was to provide a candidate strain and experimental basis for developing new functional probiotic formulations.

2. MATERIALS AND METHODS

2.1. Samples and Reagents

Pickle brine was collected from traditional fermentation processes in Yibin, Sichuan, China. Cholesterol ($\geq 99\%$) was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). o-Phthalaldehyde, pepsin (1:3000), trypsin (1:250), and bile salts were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China), Solarbio Science & Technology Co., Ltd. (Beijing, China), and Chengdu Kelong Chemical Reagent Factory, respectively. All other reagents were of analytical grade.

MRS medium (containing 10 g peptone, 10 g beef extract, 5 g yeast extract, 20 g glucose, 2 g K_2HPO_4 ,

5 g sodium acetate, 2 g ammonium citrate dibasic, 0.58 g $MgSO_4 \cdot 7H_2O$, 1 mL Tween-80, 20 g agar, and 1 L distilled water, sterilized at 115°C for 20 min) was used for isolation and cultivation. MRS-cholesterol medium was prepared by adding sterile-filtered cholesterol (0.1 mg/mL final concentration, dissolved in absolute ethanol) into cooled MRS broth.

2.2. Isolation and Purification of LAB

The pickle brine was allowed to settle for 1 h, and 100 μ L of the supernatant was mixed with 900 μ L sterile PBS, serially diluted (10^{-3} to 10^{-5}), and 50 μ L of each dilution was spread onto MRS agar plates. Plates were incubated anaerobically at 37°C for 48 h. Single colonies with distinct morphologies were picked and streak-purified 3–4 times on MRS agar until uniform colonies were obtained. Purified strains were cultured in MRS broth at 37°C for 24 h, then mixed with equal volumes of 50% glycerol and stored at -80°C .

2.3. In Vitro Cholesterol Removal Assay

Each strain was activated for two passages, then inoculated (1%, v/v) into MRS-cholesterol broth (initial cholesterol 0.1 mg/mL) and incubated statically at 37°C for 24 h. Cultures were centrifuged at 5,000 rpm for 10 min, and 50 μ L of supernatant was used to determine residual cholesterol by the o-phthalaldehyde method. Briefly, the supernatant was mixed with 0.2 mL of o-phthalaldehyde reagent (1 mg/mL in ethanol) and 4.0 mL of mixed acid (glacial acetic acid: concentrated sulfuric acid = 1:1). After 15 min at room temperature, absorbance at 550 nm was measured. A standard curve was constructed using cholesterol standards (0–0.1 mg/mL), yielding the regression equation $y = 1.6523x + 0.0028$ ($R^2 = 0.9992$). Cholesterol removal rate (%) = $[(\text{initial} - \text{residual})/\text{initial}] \times 100$. All tests were performed in triplicate.

2.4. Strain Identification

Morphological Characterization:

Colony morphology (color, size, edge, surface) on MRS agar was observed. Gram staining was performed on cells from the logarithmic phase, and morphology was examined under an oil-immersion microscope (1000 $\times$).

Molecular Identification:

Genomic DNA was extracted using a bacterial DNA kit. The 16S rRNA gene was amplified with universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR reactions (25 μ L) contained 12.5 μ L 2 $\times$ Taq Master Mix, 0.5 μ L each primer, 2 μ L DNA template, and ddH₂O to volume. Amplification conditions: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 30 s, 55°C for 35 s, 72°C for 90 s; and final extension at 72°C for 10 min. Products were checked by 1% agarose gel electrophoresis and sequenced by Sangon Biotech (Shanghai, China). Sequences were compared using NCBI BLAST, and a

phylogenetic tree was constructed with MEGA 7.0 using the Neighbor-Joining method.

2.5. Probiotic Property Evaluation

Antimicrobial Activity:

The Oxford cup double-layer agar method (Zhenyu, Haiying, Mengtao, Xiaokai, & Gang, 2020) was used. Indicator strains (*E. coli* ATCC 25922 and *S. aureus* ATCC 25923) were inoculated (1%) into 1.7% water agar plates. After solidification, sterile Oxford cups were placed, and semi-solid MRS medium containing indicators was poured. After solidification, cups were removed, and 100 µL of filter-sterilized cell-free supernatant of LH3 (centrifuged and 0.22 µm filtered) was added to each well. Plates were kept at 4°C for 30 min for diffusion, then incubated at 37°C for 48 h, and inhibition zone diameters were measured.

Tolerance to Simulated Gastrointestinal Fluids:

Simulated gastric fluid (SGF, pH 3.0) contained 0.35 g pepsin and 0.2 g NaCl per 100 mL; simulated intestinal fluid (SIF, pH 6.8) contained 0.68 g KH₂PO₄ and 0.1 g trypsin per 100 mL. Both were filter-sterilized. Cells were washed twice with PBS and resuspended in sterile saline. One milliliter of cell suspension was added to 9 mL of SGF or SIF and incubated at 37°C with shaking for 3 h. Viable counts were determined by serial dilution and plating on MRS agar. Survival rate (%) = (viable count after treatment / initial viable count) × 100.

Acid Tolerance:

Cells were inoculated (1%) into MRS broth adjusted to pH 3.0 (with 1 mol/L HCl) and natural pH (~6.4) as control, incubated at 37°C for 24 h, and OD₆₀₀ was measured. Survival rate (%) = [(OD_{3.0} - blank) / (OD_{natural} - blank)] × 100.

Bile Salt Tolerance:

MRS broth was supplemented with 0.15% or 0.3% bile salts, inoculated with 1% cell suspension, incubated at 37°C for 24 h, and OD₆₀₀ was recorded. Survival rate (%) = (OD_{experimental} / OD_{control}) × 100.

Auto-Aggregation:

Cells were washed twice with PBS, resuspended in saline, and adjusted to OD₆₀₀ ≈ 0.5 (A₀).

Four milliliters of suspension was incubated statically at 37°C for 2 h. The upper layer was carefully taken and OD₆₀₀ measured (A_t). Auto-aggregation (%) = [(A₀ - A_t) / A₀] × 100.

Hydrophobicity:

Cell suspension (OD₆₀₀ ≈ 0.5, A₀) was mixed with xylene (3:1, v/v), vortexed for 2 min, and allowed to stand for 20 min. The aqueous phase was collected and OD₆₀₀ measured (A). Hydrophobicity (%) = [(A₀ - A) / A₀] × 100.

DPPH Radical Scavenging Activity:

Two milliliters of cell suspension (~10⁸ CFU/mL) was mixed with 2 mL of 0.2 mmol/L DPPH-ethanol solution (prepared in dark). After 30 min incubation at room temperature in the dark, the mixture was centrifuged at 5,000 rpm for 10 min, and absorbance at 517 nm (A₁) was measured. Controls used ethanol instead of DPPH (A₀) and PBS instead of sample (A₂). Scavenging rate (%) = [1 - (A₁ - A₀) / A₂] × 100 (Weibin, Jing, Zhiqiang, Lenzhong, & Xin, 2024).

2.6. Statistical Analysis

All data are expressed as mean ± standard deviation (SD). One-way ANOVA was performed using SPSS 27.0, and differences were considered significant at P < 0.05.

3. RESULTS

3.1. Isolation and Screening of Cholesterol-Lowering LAB

Twelve presumptive LAB strains (LH1–LH12) with distinct colony morphologies were isolated from pickle brine on MRS agar. The cholesterol standard curve showed excellent linearity ($y = 1.6523x + 0.0028$, $R^2 = 0.9992$). After 24 h cultivation in MRS-cholesterol broth, cholesterol removal rates varied significantly among strains (Table 1). Strain LH3 exhibited the highest removal rate of 66.71% ± 1.08%, significantly ($P < 0.05$) greater than the others. Strains LH1 (57.14%), LH5 (53.89%), and LH6 (53.14%) also exceeded 50% removal. The remaining strains showed lower capacities, with rates below 34% or even below 22%. Based on its superior and stable performance, LH3 was selected for further characterization.

Table 1: Cholesterol Removal Rate of Bacterial Strains in Vitro

Strain	m	n	Cholesterol removal rate
LH1	0.700	0.300	57.14%±0.85b
LH2	0.700	0.487	30.43%±1.10g
LH3	0.700	0.233	66.71%±1.08a
LH4	0.700	0.481	31.29%±0.73f
LH5	0.700	0.323	53.89%±0.92c
LH6	0.700	0.328	53.14%±0.72d
LH7	0.700	0.602	14.00%±1.33k
LH8	0.700	0.579	17.29%±1.14i
LH9	0.700	0.596	14.89%±1.39j

Strain	m	n	Cholesterol removal rate
LH10	0.700	0.552	21.14%±1.02h
LH11	0.700	0.487	30.43%±1.26g
LH12	0.700	0.481	33.86%±0.88e

Note: m represents the cholesterol content in the culture medium before cultivation; n represents the cholesterol content in the culture medium after cultivation. The cholesterol removal rate values are expressed as mean ± standard deviation. Different lowercase letters indicate significant differences between the same column ($p < 0.05$).

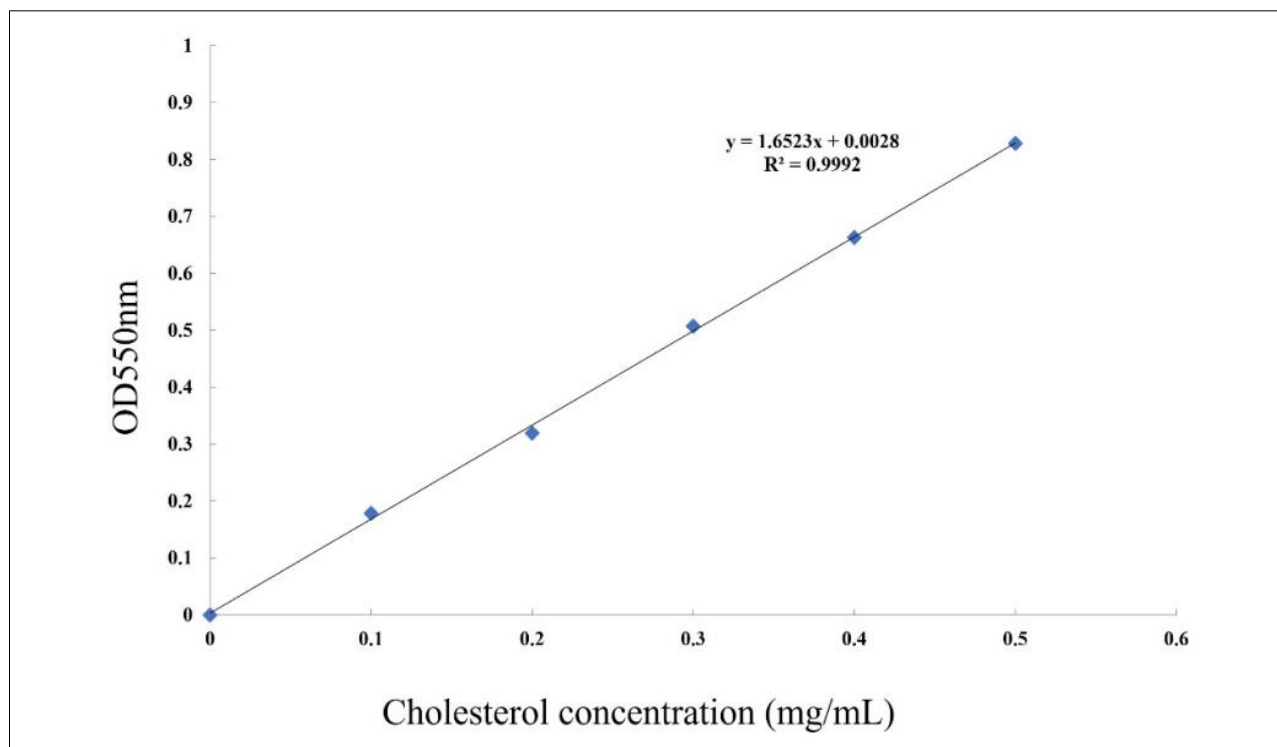


Figure 1: Cholesterol Standard Curve

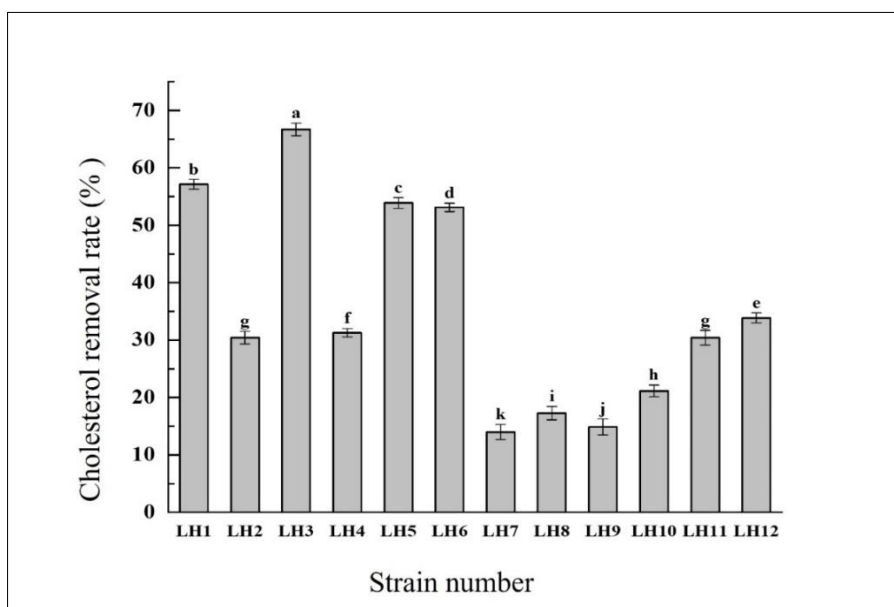


Figure 2: Cholesterol removal rate of the strain in vitro

3.2. Identification of Strain LH3

Colonies of LH3 on MRS agar were creamy white, round, convex, smooth, moist, opaque, with entire margins and about 1–2 mm in diameter. Gram staining

revealed short rods, occurring singly, in pairs, or in short chains, purple-stained, indicating Gram-positive bacilli. These features matched the genus *Lactobacillus*.

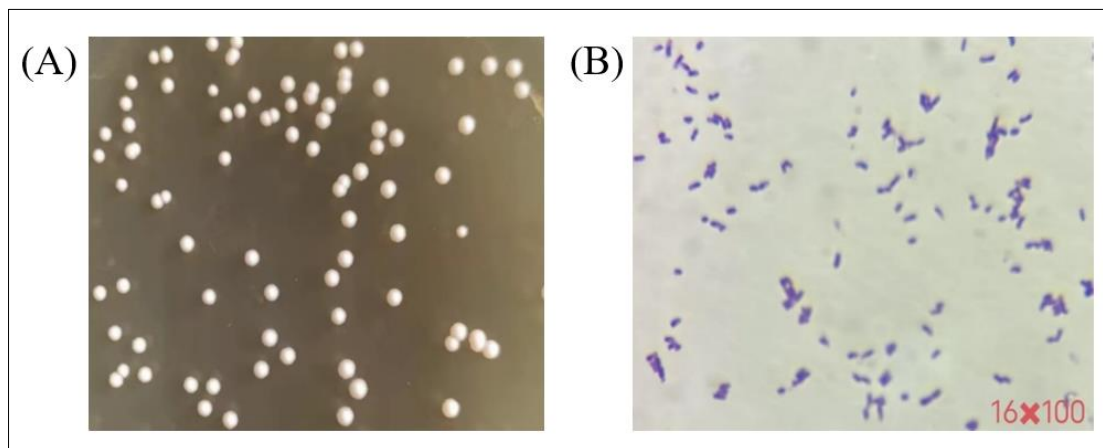


Figure 3: Morphological characteristics of strain LH3: (a) Morphological features of the colony; (b) Gram staining microscopic examination

The 16S rRNA gene sequence (approx. 1,500 bp) showed 100% similarity to *Lacticaseibacillus rhamnosus* (accession NR_113332.1) in BLAST. Phylogenetic analysis (Neighbor-Joining tree) placed

LH3 within the clade of *L. rhamnosus* with high bootstrap support (100%). Therefore, strain LH3 was conclusively identified as *Lacticaseibacillus rhamnosus*.

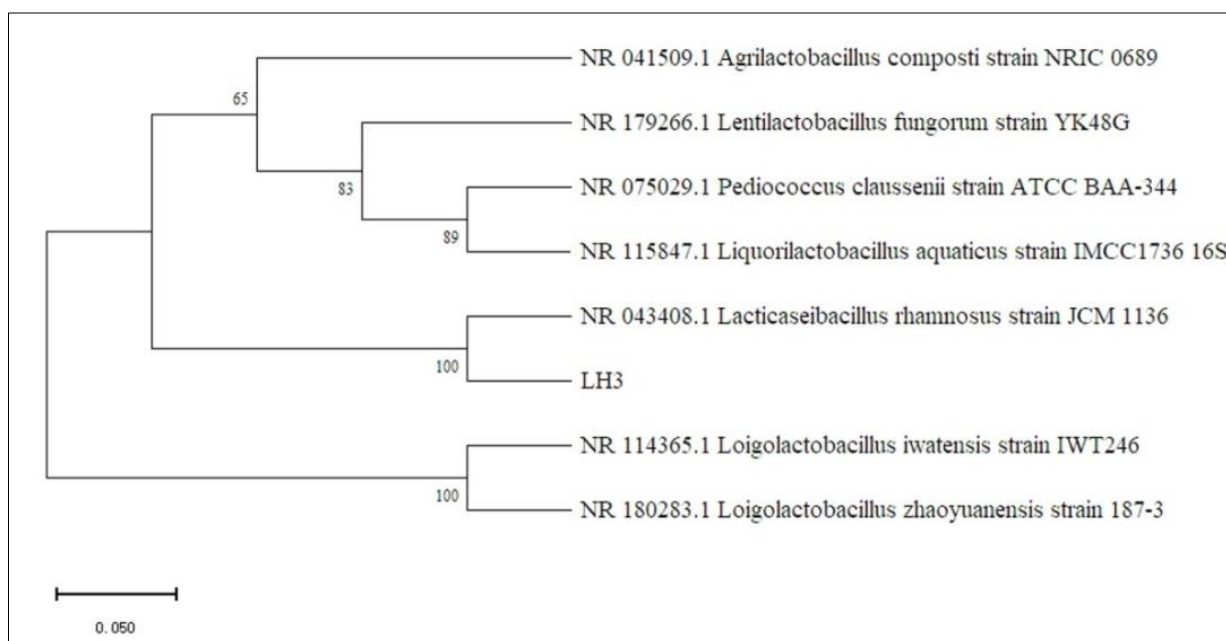


Figure 4: Phylogenetic tree of the LH3 system

3.3. Probiotic Properties of LH3

3.3.1. Antimicrobial Activity

The cell-free supernatant of LH3 exhibited clear inhibition zones against both indicators (Table 2).

Against *S. aureus*, the zone diameter was 25.29 mm (highly sensitive), and against *E. coli*, it was 17.85 mm (moderately sensitive).

Table 2: Antibacterial Activity of LH3 Strain

Strain	Bactericidal zone diameter (mm)	
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
LH3	17.85±0.07	25.29±0.08

3.3.2. Tolerance to Simulated Gastrointestinal Fluids

After 3 h exposure, LH3 survival in SGF (pH 3.0 with pepsin) was 90.23% ± 1.25%, and in SIF (pH

6.8 with trypsin) was 98.79% ± 0.89% (Figure 5), indicating strong resistance to digestive enzymes.

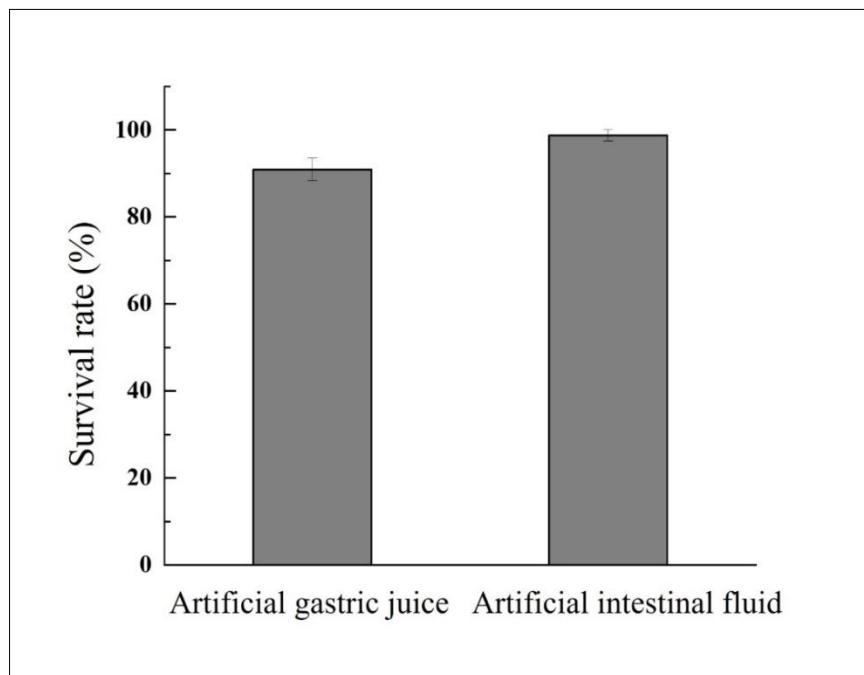


Figure 5: Survival Rate of Artificially Simulated Gastrointestinal Bacterial Strains

3.3.3. Acid and Bile Salt Tolerance

At pH 3.0 for 24 h, survival was $69.43\% \pm 1.52\%$ (Figure 6). In 0.15% bile salts, survival was $73.15\% \pm$

1.34% , while at 0.3% it decreased to $42.96\% \pm 1.67\%$, showing a clear concentration-dependent decline ($P < 0.05$).

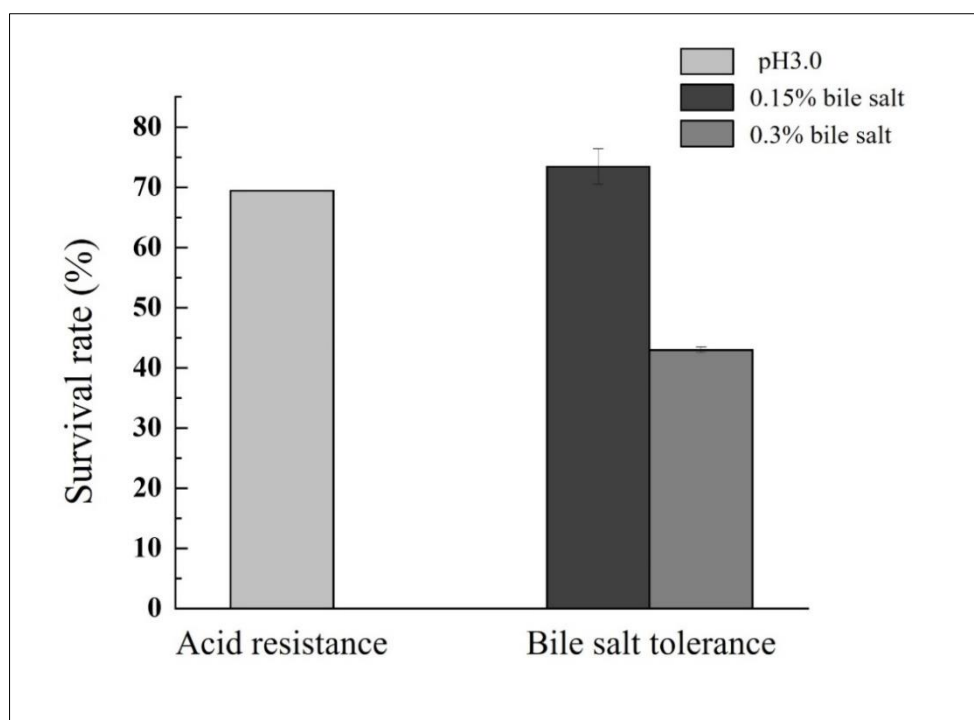


Figure 6: Acid tolerance and bile salt tolerance of *Lactobacillus rhamnosus* LH3

3.3.4. Auto-Aggregation, Hydrophobicity, and DPPH Scavenging

After 2 h static incubation, auto-aggregation was $20.67\% \pm 0.98\%$. Surface hydrophobicity toward

xylene was $50.09\% \pm 1.22\%$, indicating a highly hydrophobic surface. DPPH radical scavenging activity reached $68.89\% \pm 1.09\%$ (Figure 3).

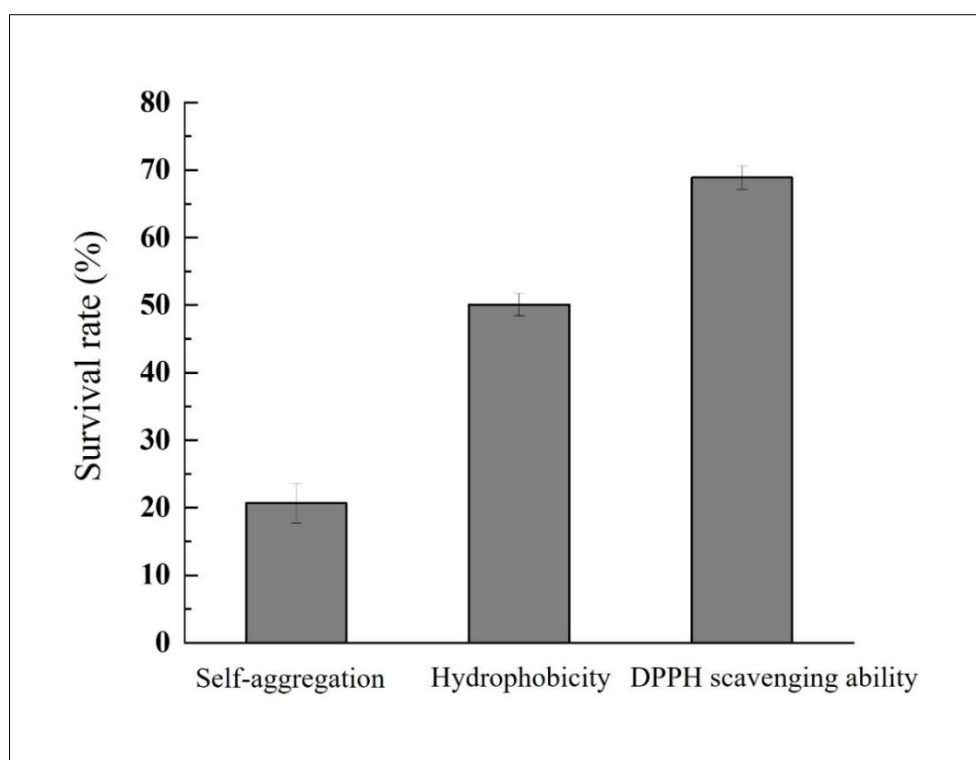


Figure 7: Evaluation of the probiotic performance of *Lactobacillus rhamnosus* LH3

4. DISCUSSION

In this study, we successfully isolated a potent cholesterol-lowering LAB strain, *L. rhamnosus* LH3, from traditional pickle brine. Its cholesterol removal rate (66.71%) is comparable to or higher than many previously reported strains, such as *L. casei* S1 (68.74%)(Jiahao *et al.*, 2023), *L. plantarum* LAB4 (42.72%)(Ziyu *et al.*, 2022), and other isolates from fermented vegetables(Yu *et al.*, 2023; Ziyuan *et al.*, 2020). This high activity may be attributed to efficient cholesterol assimilation, BSH activity, or coprecipitation with deconjugated bile salts, though the exact mechanisms require further investigation.

The probiotic properties of LH3 are remarkably well-balanced. Its strong survival in SGF (90%) and SIF (99%) outperforms many reported strains, e.g., *L. rhamnosus* strains often show 70–85% survival under similar conditions(Qiu *et al.*, 2023; Yiting, Hongxing, Yuanhong, Junhua, & Shun, 2018). The acid tolerance at pH 3.0 (69%) and bile tolerance at 0.3% (43%) are also satisfactory, as the physiological bile salt concentration in the human small intestine ranges from 0.03% to 0.3%. These traits ensure that LH3 can reach the intestine in viable counts sufficient to exert benefits.

The antimicrobial activity against *S. aureus* (inhibition zone >25 mm) suggests that LH3 produces potent bacteriocins or organic acids that effectively inhibit Gram-positive pathogens, which is consistent with the known antimicrobial spectrum of many lactobacilli(Guoqiang, Ziwen, & Kaimei, 2007). The moderate effect against *E. coli* may be limited by the

outer membrane barrier, but still indicates a broad-spectrum potential.

Auto-aggregation and hydrophobicity are widely used proxies for intestinal adhesion. LH3's hydrophobicity (50.09%) falls into the high-hydrophobicity category (>50% according to Kos *et al.*, [criteria]), predicting good adhesion to epithelial cells. Although its auto-aggregation (20.67%) is modest at 2 h, it is likely to increase with longer incubation, as observed in other studies(Ziyu *et al.*, 2022). This adhesive potential, combined with its acid tolerance, supports its ability to colonize the gut transiently.

The DPPH scavenging rate (68.89%) indicates notable antioxidant activity, which may help reduce oxidative stress associated with hyperlipidemia. Many probiotics possess antioxidant enzymes (SOD, catalase) or produce exopolysaccharides with radical-scavenging properties(Yujun, Dong, Lanfeng, & Ruixia, 2013). LH3's dual functionality – cholesterol reduction and antioxidant capacity – makes it a promising candidate for combating metabolic syndrome.

Compared to commercial probiotic strains, LH3 is a wild-type isolate from a traditional fermented food, which often exhibits better environmental adaptability and metabolic diversity. This strain could be developed into a functional food ingredient or dietary supplement. However, further studies are needed to elucidate the molecular mechanism of cholesterol lowering (e.g., gene expression profiling, BSH activity assays), evaluate its safety (e.g., antibiotic resistance transfer, hemolytic

activity), and validate its efficacy in animal models or human clinical trials.

5. CONCLUSIONS

Lactacaseibacillus rhamnosus LH3, isolated from traditional Sichuan pickle brine, demonstrated excellent in vitro cholesterol removal (66.71%) and comprehensive probiotic properties, including high tolerance to simulated gastrointestinal conditions, acid and bile salts, antimicrobial activity against pathogens, favorable adhesive potential (hydrophobicity 50.09%, auto-aggregation 20.67%), and strong antioxidant capacity (DPPH scavenging 68.89%). These findings indicate that LH3 is a valuable candidate strain for developing functional probiotic products aimed at managing hypercholesterolemia and associated oxidative stress.

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Credit Authorship Contribution Statement

Jiacheng Li: Methodology, Investigation, Data curation, Formal analysis, Writing—original draft. Lijuan Yang: Conceptualization, Project administration, Supervision, Funding acquisition, Writing—original draft, Writing—review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability: Data will be made available on request.

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