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Antibacterial and Antibiofilm Activity of Lactic Acid Bacteria Isolated from Traditional Artisanal Milk Cheese from Northeast China Against Enteropathogenic Bacteria

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Abstract
The present study aims to investigate the probiotic properties of novel strains of lactic acid bacteria isolated from traditional artisanal milk cheese from Northeast China and to explore their antibacterial activity against enteropathogenic bacteria. Of the 321 isolates, 86 exhibited survival in low pH, resistance to pancreatin, and tolerance to bile salts; of these, 12 inhibited the growth of more than seven enteropathogenic bacteria and exhibited antibiofilm activities against *Staphylococcus aureus* CMCC26003 and/or *Escherichia coli* CVCC230. Based on 16S ribosomal RNA sequence analysis, the 12 isolates were assigned to *Lactobacillus plantarum* (7), *Lactobacillus helveticus* (3), *Pediococcus acidilactici* (1), and *Enterococcus faecium* (1) species. In addition, 5 of the 12 strains were susceptible to most of the tested antibiotics. Furthermore, four strains with sensitivity to antibiotics showed significantly high levels of hydrophobicity similar to or better than the reference strain *Lactobacillus rhamnosus* GG. Moreover, three strains were confirmed safe through non-hemolytic activities and bacterial translocation. Overall, the selected *Lact. plantarum* 27053 and 27172 and *Lact. helveticus* 27058 strains can be considered potential probiotic strains and candidates for further application in functional food and prevention or treatment of gastrointestinal diseases.

Keywords Lactic acid bacteria · Probiotics · Cheese · Antibacterial · Antibiofilm · Enteropathogens

Introduction

Lactic acid bacteria (LAB), especially the species of genus *Lactobacillus*, have recently received attention because of their "generally recognized as safe" status and their potential health-promoting effects as probiotics. The WHO defines probiotics as "live microorganisms that, when consumed in sufficient amounts, confer a health benefit to the host" [1]. Probiotics must survive stressful conditions of the gastrointestinal tract by tolerating acid, bile, and gastric enzymes and must adhere to intestinal epithelial cells to colonize the gut. Moreover, probiotics should have antimicrobial effects against pathogenic microorganisms and desirable antibiotic susceptibility patterns [2].

Many gastrointestinal diseases, such as diarrhea, irritable bowel syndrome, and chronic inflammatory bowel disease, are caused by intestinal microflora imbalance [3], which is an important factor in bacterial translocation and infection. The current treatment of intestinal microbiota imbalance is using antibiotics; however, misuse or overuse of antibiotics contributes to resistance, which is one of the major public health problems worldwide. Another concern is the decreasing efficacy of antibiotics in treating human and animal infections because of the biofilm formation of pathogenic bacteria. Bacterial cells in biofilms are highly protected, less subjected to mutation, represent low metabolic activity, and become resistant to antibiotics [4].

Probiotics of LAB with beneficial properties are useful for food fermentation starters. They improve digestion and assimilation of nutrients [5, 6], modulate the immune system [7], remove toxic substances, and inhibit the growth or invasion of parasites and pathogenic bacteria to prevent gastrointestinal infections [8]. Recently, probiotics have become widely

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recognized as a method to regulate the intestinal bacterial environment and probably offer a natural alternative to antibiotic supplementation. Probiotics are effective for preventing and treating infectious colitis/infectious diarrhea, thereby making probiotics a useful public health intervention [9]. However, the research on LAB-inhibited multiple enteropathogenic bacteria was seldom been analyzed, and little attention has attempted to the effects; moreover, analysis on the effects of LAB on enteropathogenic bacteria biofilm has not been attempted.

The objectives of this study were to isolate LAB from artisanal milk cheese in Northeast China and to investigate their probiotic potencies, such as tolerance to stressful gastrointestinal conditions, antibiotic resistance, antimicrobial and antibiofilm activities, and safety. This study describes the initial step in the selection of local potential probiotic strains of *Lactobacillus* isolated from artisanal milk cheese in Northeast China.

Material and Methods

Isolates, Cultures, and Growth Conditions

LAB were isolated from 11 traditional artisanal milk cheeses collected in Northeast China. Briefly, 1 g of each cheese sample was homogenized in 9 mL sterile saline water. Serially diluted samples were immediately plated on MRS agar (de Man–Rogosa–Sharpe, Hope Bio-Technology Co., Ltd., Qingdao, China), which is selective for *Lactobacillus* spp. Then, the plates were incubated at 37 °C for 48 h under anaerobic conditions [10]. Representative colonies of all morphologies were selected randomly and purified on the same media by subculturing. Catalase-negative, gram-positive, and rod-shaped bacilli were considered presumptive LAB. Three to four colonies of each culture were selected and stored.

Acid, Bile Salts, and Trypsin Tolerance Assays

Stock cultures of LAB isolates were propagated twice in MRS medium at 37 °C for 24 h before the next assay. LAB were inoculated in MRS broths and cultured overnight to evaluate the resistance of LAB isolates to gastric lumen conditions. Thereafter, LAB were adjusted to pH 2.0 with HCl and incubated at 37 °C for 3 h. Cultures inoculated in non-acidified MRS (pH 6.8) served as controls [11]. Similarly, the MRS medium containing 0.4 and 0.8% (*w*/*v*) bile salt (Huankai Microbial Sci. & Tech. Co., Ltd., Guangdong, China) was inoculated with active cultures of LAB at 37 °C for 8 h. The control cultures were grown without bile salt. For the trypsin tolerance test, the MRS medium containing 0.1% (*w*/*v*) trypsin (Thermo Fisher Scientific Inc., USA) was inoculated with active cultures of LAB. Strains grown without trypsin were used as control. Acid, bile salt, and trypsin tolerance values were estimated by comparing the viable LAB isolate counts on MRS agar plates for surviving cells after incubation [12]. The experiments were conducted thrice.

Antimicrobial Activity

The Oxford cup method was performed to study the antimicrobial activity of LAB as described previously by Chen et al. [13] with slight modifications. Cultures of LAB grown anaerobically at 37 °C for 24 h in MRS medium were centrifuged to obtain cell-free culture supernatant (CFCS).

Pathogenic microorganisms, namely, *Staphylococcus aureus* CMCC26003, *Listeria monocytogenes* ATCC19111, *Salmonella typhimurium* CVCC541, *Enterococcus faecalis* ATCC29212, *Pseudomonas aeruginosa* ATCC9027, *Shigella flexneri* ATCC12022, *Yersinia enterocolitica* ATCC9610, *Escherichia coli* CVCC230, and *Clostridium perfringens* CVCC-81, were incubated in Luria–Bertani (LB) broth at 37 °C for 16 h under aerobic or anaerobic conditions.

Then, microbial density was adjusted to 10^6 to 10^7 CFU/mL, and 200 μL aliquots of the organisms were spread on the surface of the LB agar. Then, 200 μL of the LAB supernatant was loaded into an Oxford cup. Plates were incubated at 37 °C for 48 h, and the diameter of the inhibition zones around the cup (including that of the Oxford cup, 7.8 mm) was measured. Then, the LAB isolates were estimated for production of antimicrobial substances, such as organic acids, bacteriocin, and hydrogen peroxide, using the method by Touré et al. [14] with modifications. For the organic acid assay, the CFCS was adjusted to pH 7.0 using $NaHCO_3$ (5% *w*/*v*). The catalase (Sigma-Aldrich Co. LLC., USA) was used to evaluate the ability of LAB to produce hydrogen peroxide. Finally, the CFCS of the LAB was treated with proteinase K (Sigma-Aldrich Co. LLC., USA), and trypsin was used for bacteriocin assay. Ampicillin and sterile MRS broth were used as positive and negative controls, respectively. The experiment was carried out in triplicate.

Antibiofilm Activity

Antibiofilm activity was assessed using a previously published method with some modifications [15]. These enteropathogenic bacteria were cultured overnight with fresh sterile tryptone soya broth (TSB) supplemented with 0.5% (*w*/*v*) glucose. Then, 100 μL of cultures of each bacterium was transferred to 96-well microtiter plates (Guangzhou Jet Bio-Filtration Co., Ltd., Guangzhou, China). LAB bacterial supernatants (100 μL) adjusted to pH 7.0 were added to each well. After incubation for 24 h at 37 °C, the medium was discarded and planktonic cells were removed from each well by gently washing twice with sterile phosphate-buffered saline (PBS).

Thereafter, the biofilms were fixed with 200 μL methanol for 10 min, stained with 200 μL 0.1% crystal violet for 10 min, and rinsed thrice with water gently. Crystal violet attached to the biofilm samples was dissolved with 200 μL 33% acetic acid. The absorbance at 590 nm was measured using a microplate reader as the value of biofilm formation [16]. Experiments were repeated thrice. Enteropathogenic bacteria grown on culture media were used as positive control, and only the TSB medium with 0.5% (*w*/*v*) glucose was used as the negative control. The results were expressed in biomass formation inhibition percentage calculated according to approaches in previous studies [17].

LAB Identification Through 16S rRNA Gene Sequencing

Identification of the selected LAB isolates was confirmed by 16S rRNA sequence analysis. The total genomic DNA of the isolates was extracted using the TIANamp Bacteria DNA Kit (TIANGEN Biotech, Co., Ltd., Beijing, China), following the manufacturer's recommendations. The PCR primer sequences were as follows: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [18].

This polymerase chain reaction (PCR) was performed at a total reaction volume of 25 μL containing 1 μL each of forward and reverse primers, 3 μL 0.2 mM dNTPs, 2.5 μL 10× PCR buffer and 2.5 U r Taq DNA polymeras (TAKARA Biotechnology, Co., Ltd., Dalian, China), 5 μL genomic DNA (approximately 200 ng), and 13 μL water. The PCR program was conducted using an initial denaturation step at 95 °C for 5 min, followed by 25 cycles at 94 °C for 30-s denaturation, annealing at 49 °C for 45 s, plus 2 min of elongation at 72 °C, and finally a 10-min extension step at 72 °C. PCR-amplified products were separated by 0.8% (*w*/*v*) agarose gel electrophoresis with ethidium bromide and visualized under UV light. The PCR products were purified with the TIANgel Midi Purification Kit (TIANGEN Biotech Co., Ltd., Beijing, China) and were sent to the sequencing company (Comate Bioscience Co., Ltd., Changchun, China). The sequences were submitted to GenBank database and compared with other sequences.

Antibiotic Susceptibility

Antibiotic susceptibility patterns of the selected LAB isolates with improved antimicrobial activity were determined using the disk diffusion method according to a previous study [19]. A total of 15 antibiotic agents were used. Antibiotic-containing disks (KONT Biology and Technology., Ltd., Wenzhou, China) placed onto the MRS agar were previously seeded with approximately 200 μL 10^7 CFU/mL) of LAB isolates. After incubation under anaerobic conditions at 37 °C for 48 h, the inhibition zone diameters (mm) were measured. The results were noted according to the Clinical and Laboratory Standards Institute (CLSI 2013). The reference strains of *Staph. aureus* ATCC25923 and *E. coli* ATCC25922 were used as controls. Results were expressed as susceptible, intermediate, or resistant.

Cell Surface Hydrophobicity

The tested bacteria were cultured at 37 °C for 18 h in MRS broth. Cells were harvested and washed twice with PBS at pH 7.0. The cells were resuspended with the same solution. A final concentration of 10^9 CFU/mL Was used. Cell surface hydrophobicity was tested using Solieri et al. [20] method, with slight modifications. The cell suspension in PBS and xylene was added at a ratio of 1:1 and vortexed at 37 °C for 10 min for temperature equilibration. After vortexing, the mixture was again vortexed briefly and left at 37 °C for 5 h to separate the layer. The aqueous phase was gently collected and measured at 600-nm absorbance. Surface hydrophobicity (*H*%) was calculated using Solieri et al. [20] method.

Safety Assessment

Hemolysis Test

To test hemolytic activity, four isolates were streaked on 5% sheep blood agar (Biocell BioTech., Co., Zhengzhou, China) and incubated at 37 °C for 24 h, as described by Argyri et al. [21]. The presence of α-hemolysis or β-hemolysis was confirmed by the formation of greenish or clear zones around the colonies, respectively [22]. *Staph. aureus* CMCC26003 was used as positive control, and *Lactobacillus rhamnosus* GG was used as negative control.

Safety Evaluation

For safety evaluation, BALB/c mice weighing 17–21 g (6 weeks of age) (Beijing Vital River Laboratory Animal Technology Co., Ltd., China) were acclimatized for 2 weeks before testing in an animal housing with ad libitum access to food and water. The experimental protocol (20119) was accepted by the International Recommendations for Animal Welfare and the Ethical Committee for Animal Sciences of the Heilongjiang province.

The selected four strains were tested for safety evaluation in mice following the protocol by Gotteland et al. [23] with slight modifications. Each experimental and control group consisted of five mice. The experimental groups underwent oral gavage with the selected four strains, whereas the positive control group was treated with *Lact. rhamnosus* GG. The vehicle (10% skim milk powder) was used as in the negative control group. A total of 200 μL bacterial suspension in 10% skim milk at ~ 10^{11} CFU/mL concentration was administered

intragastrically once daily for 8 days. Tissue homogenates of mesenteric lymph nodes, liver, and spleen were spread on MRS and LB agar plates (with 8% fetal bovine serum). The plates were incubated at 37 °C for 48 h anaerobically in MRS plates or aerobically in LB plates with 8% fetal bovine serum. Results were expressed as incidence of translocation (number of mice where colonies were detected/total number of mice).

Results

Acid, Bile Salt, and Trypsin Resistance

Biochemical and phenotypical tests identified 321 strains as LAB. As such, 94 strains could survive under acidic condition and were resistant to trypsin. Among the 94 strains, 86 strains survived the bile resistance test.

Antimicrobial Activity

Based on acid, trypsin, and bile salt tolerance, 86 strains were selected and qualified for further antimicrobial activity testing. The results showed that 37 strains could inhibit most of the enteropathogenic bacteria, and 12 of 37 LAB isolates had improved inhibitory effects. As showed in Table 1, the 12 strains exhibited antimicrobial activity against at least seven enteropathogenic bacteria, or even nine enteropathogenic bacteria. The results may indicate that LAB can produce antibacterial active substances. Moreover, the results demonstrated that LAB isolates possessed broad-spectrum antibacterial activity against both gram-positive and gram-negative enteropathogenic bacteria and can inhibit aerobic or anaerobic bacteria. When the CFCS of the LAB strains was adjusted to pH 7.0, the inhibition zones of all strains decreased (Table 1), and the antimicrobial activity of strains 27094, 27181, 27199, and 27208 completely disappeared, thereby indicating that the inhibitory effects of these LAB strains were due to their organic acid productions. After pH adjustment, the CFCS of the other strains was treated with catalase. The inhibition zones of isolates 27071, 27167, 27175, and 27179 were significantly reduced, and strains 27053, 27058, 27170, and 27172 showed that the diameters of the inhibition zones had no obvious differences. This finding indicated that hydrogen peroxide production may be involved in the antimicrobial activity mechanism of strains 27071, 27167, 27175, and 27179. Then, after neutralized acid (pH 7.0) and catalase treatments, the CFCS of strains 27053, 27058, 27170, and 27172 were treated with proteinase k and trypsin. The inhibition zones decreased significantly and completely disappeared. This result showed that CFCS of strains 27053, 27058, 27170, and 27172 can be degraded by protease but not catalase and that the antibacterial protein secreted by these strains may be bacteriocins.

Table 1 Antimicrobial activity of the LAB strains against the enteropathogenic bacteria

Strain	Indicator strains																					
	Staph. aureus		*L. monocytogenes*		*Sdm. typhimurium*		*Ent. faecalis*		*Ps. aeruginosa*		*Sh. flexneri*		*Y. enterocolitica*		*Cl. perfringens*		*E. coli*	
	S[a]	pH7[b]	S	pH 7	S	pH 7	S	pH 7	S	pH 7	S	pH 7	S	pH 7	S	pH 7	S	pH 7
27053	+	+	+	+	+	+	+	−	+	−	+	−	‡	−	+	−	+	−
27058	+	−	+	+	+	−	+	−	+	−	‡	−	‡	+	+	+	+	−
27071	+	−	+	−	+	+	+	−	+	−	‡	−	‡	−	+	−	+	−
27094	+	+	+	−	−	−	+	−	+	+	+	+	+	+	+	−	−	−
27167	+	−	+	+	+	−	+	−	+	−	‡	+	‡	+	+	−	+	+
27170	+	+	+	+	+	+	+	−	+	+	‡	+	+	+	+	+	+	+
27172	+	−	+	+	+	+	‡	−	+	+	+	+	‡	+	+	+	+	+
27175	+	−	+	+	+	−	+	−	+	−	+	−	‡	−	−	−	+	−
27179	+	−	+	+	+	−	+	−	+	+	+	−	‡	−	−	−	+	−
27181	+	+	+	−	+	−	+	+	+	+	+	−	‡	−	−	−	+	−
27199	+	−	+	−	+	−	+	−	+	−	+	−	‡	−	−	−	+	−
27208	−	−	+	−	+	+	+	−	+	−	+	−	‡	−	−	−	+	−

Interpretation of zone inhibition diameter: −, less than 10 mm; +, 10–16 mm; and ++, 17–22 mm

[a] Supernatants

[b] The pH of cell-free culture supernatants (CFCS) was neutralized to pH 7 with $NaHCO_3$ (5% *w/v*)

Antibiofilm Activity

We examined biofilm formation ability of all nine enteropathogenic bacteria; however, only *E. coli* CVCC230 and *Staph. aureus* CMCC26003 exhibited improved ability to form biofilm. Therefore, we used these two strains as in vitro biofilm patterns to investigate the antibiofilm activities of the 12 strains with improved antimicrobial activity. As shown in Fig. 1, almost all isolated strains were able to reduce the biofilm formation of *E. coli* CVCC230 or *S. aureus* CMCC26003, except by isolates 27175 and 27170, respectively. In the case of *E. coli*, the obtained results revealed that the inhibitory rates of the five isolates (27053, 27058, 27071, 27094, 27179) were more than 50%. Isolate 27058 significantly reduced biofilm formation. LAB isolate 27175 was not found to have biofilm inhibiting activity against *E. coli*. For *Staph. aureus*, the three isolates (27175, 27179, 27181) had inhibitory rate higher than 50%. Isolate 27179 was found to markedly inhibit biofilm growth. However, isolate 27170 showed no effect against *Staph. aureus*. From Fig. 1, we can also conclude that the LAB had abilities against gram-positive and gram-negative bacterium biofilm formation, but the abilities were strain dependent. The biofilm of *E. coli* and *Staph. aureus* was inhibited by strains 27053, 27058, 27071, 27094, and 27179. However, different isolates have obviously different inhibiting ability, for instance, isolate 27175 showed a higher activity against *Staph. aureus* biofilm, but 27175 did not affect the biofilm formation of *E. coli*. Remarkably, isolate 27179 markedly inhibited biofilm growth of *E. coli* and *Staph. aureus*, which indicated that 27179 is a good candidate to control enteropathogenic bacteria biofilm formation.

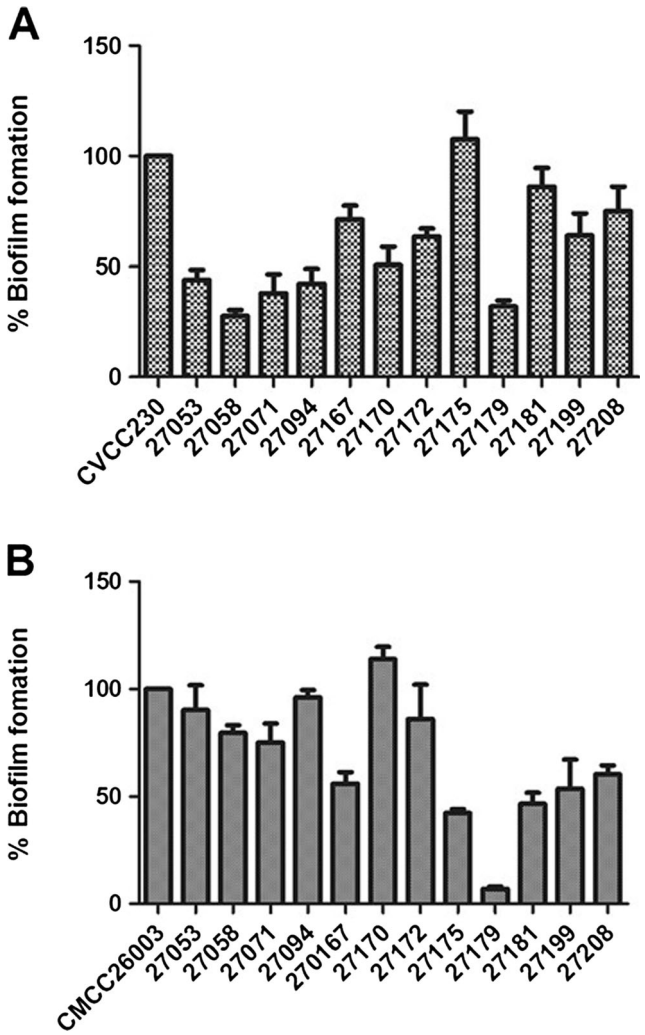

Fig. 1 The effects of pH-neutralized treatment CFCS on *E. coli* CVCC230 (**a**) or *Staph. aureus* CMCC26003 (**b**) biofilm formation. Biofilm formation (OD590) of *E. coli* CVCC230 or *Staph. aureus* CMCC26003 in 96-well plates was quantified in the presence of pH-neutralized treatment CFCS after 24 h. Arbitrary units obtained from *E. coli* CVCC230 or *Staph. aureus* CMCC26003 were set to 100% biofilms, and those made by the other strains were calculated. Error bars represent the standard errors of the mean calculated using data from at least three independent experiments

Identification by 16S rRNA Sequencing

The 12 potential probiotic isolates were identified through 16S rRNA gene sequence analysis. The universal primers 27F and 1492R were used to obtain PCR amplification of the 16S rRNA-encoding genes. The 16S rRNA genes of the 12 isolates were analyzed and compared using NCBI BLAST program. The 12 isolates were identified as *Lactobacillus plantarum* (7 isolates), *Lactobacillus helveticus* (3 isolates), *Pediococcus acidilactici* (1 isolate), and *Enterococcus faecium* (1 isolate). The GenBank accession numbers of the selected 12 isolates are reported in Table 2.

Antibiotic Susceptibility

The antibiotic susceptibilities of the selected 12 isolates with good antibiofilm activities were investigated to evaluate their potential probiotic characteristics. Table 3 shows the antibiotic susceptibility patterns of the strains. All tested strains were sensitive to imipenem and linezolid or moderately sensitive to ampicillin (except *Ent. faecium* 27199), chloramphenicol (except *Lact. plantarum* 27071), and azithromycin (except *Lact. plantarum* 27071, 27094, 27199, *Lact. helveticus* 27181, and *Ent. faecium* 27179). However, all the isolates were resistant to streptomycin, gentamicin, vancomycin, and ciprofloxacin.

Hence, seven strains were discarded because of their antibiotic susceptibility, and isolates 27053, 27058, 27167, 27170, and 27172 were selected for further analysis.

Cell Surface Hydrophobicity

The hydrophobic natures of the cell surface of the selected strains that were sensitive to antibiotics were measured

Table 2 Camparative analysis of 16S rRNA sequences of the isolates using highly matched and closely related species available in NCBI database

Strain	Accession no.	Highly matched bacteria/accession no.	Identity (%)	Database
27053	KX810087	*Lactobacillus plantarum* strain ML5-1/EU807752.1	98	NCBI
27058	KX815871	*Lactobacillus helveticus* strain MJM60419-3/KM485567.1	98	NCBI
27071	KX815872	*Lact. plantarum* strain NGRI0101/LC177235.1	99	NCBI
27094	KX815881	*Lact. plantarum* strain NGRI0101/LC177235.1	99	NCBI
27167	KX815873	*Pediococcus acidilactici* strain KP10/JN592051.1	98	NCBI
27170	KX815874	*Lact. helveticus* strain MJM60419-3/KM485567.1	99	NCBI
27172	KX815875	*Lact. plantarum* strain H2/HQ286594.1	98	NCBI
27175	KX815876	*Lact. plantarum* strain s 21/KY363567	100	NCBI
27179	KX815877	*Enterococcus faecium* strain IN 3531/KC715828.1	98	NCBI
27181	KX815878	*Lact. helveticus* strain GM2/KX430837.1	100	NCBI
27199	KX815879	*Lact. plantarum* strain gp55/KM495881.1	98	NCBI
27208	KX815880	*Lact. plantarum* strain KF/KT025848.1	99	NCBI

photometrically using bacterial adhesion to hydrocarbon assay to assess their cell adherence. In general, isolates 27053, 27058, 27170, and 27172 exhibited high hydrophobicity (i.e., 62, 78, 59, and 65%, respectively) that are similar to or even better than the reference strain *Lact. rhamnosus* GG, and only isolate 27167 possessed 44% hydrophobicity. The results revealed that most isolates had high percentages of cell surface hydrophobicity.

Safety Assessment

Hemolysis Test

In this study, none of the examined strains exhibited α-hemolytic and β-hemolytic activities when grown in 5% sheep blood agar. The absence of hemolytic activity is always considered a safety prerequisite for selecting potential probiotic strains [2].

Table 3 Results of antibiotic susceptibility tests of LAB isolates from artisanal milk cheese in China

Species/strain	Antibiotic														
	AM	CTX	IPM	AZT	GEN	S	TET	AZI	DOX	E	VAN	CIP	CM	C	LZD
Lact. plantarum															
27053	I	R	S	S	R	R	S	S	S	S	R	R	S	S	S
27071	I	R	S	R	R	R	R	R	R	I	R	R	R	R	S
27094	S	R	S	R	R	R	I	R	I	I	R	R	I	S	S
27172	I	R	S	S	R	R	S	S	S	I	R	R	I	I	S
27175	I	R	S	R	R	R	R	I	S	I	R	R	I	S	S
27199	R	R	S	R	R	R	R	R	S	I	R	R	I	S	S
27208	I	R	S	R	R	R	R	S	I	R	R	R	R	I	S
Lact. helveticus															
27058	S	R	S	S	R	R	S	S	S	I	R	R	I	S	S
27170	I	R	S	S	R	R	S	S	S	I	R	R	R	I	S
27181	I	S	S	R	R	R	R	R	R	R	R	R	I	I	S
Ped. acidilactici															
27167	I	R	S	S	R	R	S	I	S	I	R	R	I	S	S
Ent. faecium															
27179	I	I	S	R	R	R	R	R	R	R	R	R	R	I	S

AM ampicillin (10 μg), *CTX* cefotaxime (30 μg), *IPM* imipenem (10 μg), *AZT* aztreonam (30 μg), *GEN* gentamicin (10 μg), *S* streptomycin (10 μg), *TET* tetracycline (30 μg), *AZI* azithromycin (15 μg), *DOX* doxycycline (30 μg), *E* erythromycin (15 μg), *VAN* vancomycin (30 μg), *CIP* ciprofloxacin (5 μg), *CM* clindamycin (2 μg), *C* chloramphenico (30 μg), *LZD* linezolid (30 μg), *R* resistant, *I* intermediate, *S* susceptible

Safety Evaluation in Mice

Isolates 27053, 27058, 27170, and 27172, which have good antibiofilm, antibiotic ability, antimicrobial activity, and hydrophobicity, were used to evaluate safety. The condition of the animals was not affected by the treatments. As shown in Table 4, the prevalence of bacteria in the lymph node, spleen, and liver of the animals treated with the strains that were selected in the current study were similar to or lower than those observed with the commercial probiotics (*Lact. rhamnosus* GG), except for the group treated with the isolate *Lact. helveticus* 27170, which showed a significantly high rate of detection in the MRS medium.

Discussion

Among traditionally fermented dairy products, cheese represents an alternative and readily available source of LAB with promising functional properties and could be beneficial for health. Several studies have suggested that resistance to gastric acidity, trypsin, and bile salts is fundamental for selecting novel probiotic candidates. The aim of this study was to screen probiotic LAB from cheese with high antimicrobial and antibiofilm properties against enteropathogenic bacteria, including *Staph. aureus* CMCC26003, *L. monocytogenes* ATCC19111, *Salm. typhimurium* CVCC541, *Ent. faecalis* ATCC29212, *Ps. aeruginosa* ATCC9027, *Sh. flexneri* ATCC12022, *Y. enterocolitica* ATCC9610, *Cl. perfringens* CVCC-81, and *E. coli* CVCC230. Those bacteria are the most regular pathogens in the intestine and can easily cause enteric infections.

In this study, out of the 321 tested strains, most of the strains exhibited survival in pH 2.0, 94 strains showed good tolerance to pancreatic enzymes, and 86 were unaffected by exposure to bile salts. These results expressed significant resistance under simulated gastrointestinal tract conditions, thereby corroborating with the results of Maragkoudakis et al. [24]. Based on gastrointestinal tract condition tolerance assays, 86 strains with high performance were selected for antimicrobial activity assays, and 12 of the isolates obtained had growth-inhibiting properties against more than seven of the nine indicator bacteria. Inhibition of enteropathogenic bacteria has mostly been attributed to the production of common antimicrobial substances, such as organic acids, hydrogen peroxide, or bacteriocins, by the LAB strains. Previous studies have reported that a competitive exclusion mechanism in vivo is involved in antimicrobial activities, in which probiotic strains compete with pathogens for attachment sites and nutrients, as well as prevent enteropathogenic bacteria colonization [25]. In this study, the antimicrobial activities of the 12 isolated LAB strains were due to their organic acid, hydrogen peroxide production, or bacteriocin production.

Previous studies have shown that the antimicrobial activity of the LAB strains is generally correlated with their species. In the present study, the 12 selected LAB strains belong to *Lact. plantarum*, *Lact. helveticus*, *Ped. acidilactici*, and *Ent. faecium*. These strains are the main groups present in the cheese during ripening. The LAB observed in the present study correlate with the results of other studies [26, 27]. Moreover, the antimicrobial activity of the LAB isolated from cheese was observed in other studies. Georgieva1 et al. [28] showed a broad-spectrum antagonistic effect of *Lact. plantarum* isolated from artisanal Bulgarian white-brined cheese on the growth of *Staph. aureus*, *Ps. aeruginosa*, and *E. coli*. Moreover, LAB strains isolated from sauerkraut [29] and traditional cheese [30] showed antimicrobial activity against *Sh. flexneri*, *Ps. aeruginosa*, *Staph. aureus*, and *L. monocytogen* [31] [32]. Furthermore, *Lactobacillus* isolates inhibit the growths of *Salm. typhimurium*, *E. coli*, and *Cl. perfringens* [33]. Among the 12 selected LAB strains, all isolates inducing the strongest growth inhibition in *Y. enterocolitica*, similar findings of LAB against *Y. enterocolitica* were also reported by Angmo et al. [34]. These observations are partially similar to our results, as our LAB isolates showed statistically significant growth inhibition of indicator bacteria. Then, the 12 strains with good ability to inhibit more than seven enteropathogenic bacteria were selected to evaluate the antibiofilm activity. In the past, studies

Table 4 Prevalence of microorganisms (grown on LB agar with 8% fetal bovine serum) or *Lactobacillus* sp. (grown on MRS agar) in samples of mesenteric lymph nodes, spleen, and liver from the animals treated with the selected LAB strains

	Mesenteric lymph nodes		Spleen		Liver	
	Serum LB[a] (%)	MRS (%)	Serum LB (%)	MRS (%)	Serum LB (%)	MRS (%)
Vehicle	5/5 (100)	1/5 (20)	4/5 (80)	1/5 (20)	2/5 (40)	0/5 (0)
Lact. rhamnosus GG	5/5 (100)	1/5 (20)	3/5 (60)	0/5 (0)	2/5 (40)	0/5 (0)
27053	3/5 (60)	1/5 (20)	3/5 (60)	0/5 (0)	2/5 (40)	1/5 (20)
27058	4/5 (80)	1/5 (20)	2/5 (40)	0/5 (0)	2/5 (40)	0/5 (0)
27170	5/5 (100)	2/5 (40)	4/5 (80)	3/5 (60)	3/5 (60)	2/5 (40)
27172	5/5 (100)	1/5 (20)	3/5 (60)	1/5 (20)	2/5 (40)	0/5 (0)

[a] Serum LB (lysogeny broth) containing 8% heat-inactivated fetal bovine serum

have documented the antibiofilm activity of LAB against oral and vaginal pathogens [35, 36]. Several previous studies reported that *Lactobacillus casei* has good antibiofilm formation properties against enteroaggregative *E. coli* (EAEC) strains and *Lactobacillus fermentum* TCUESC01 and significantly reduces the biofilm formation of *Staph. aureus* under subinhibitory conditions [37, 38].

Based on the data obtained in this study, almost all of the 12 isolates are good candidates for controlling enteropathogenic bacteria biofilm formation. Antibiofilm activities of LAB may be due to its inhibitory substances, such as biosurfactants, H_2O_2, and bacteriocin. However, detailed molecular mechanisms of biofilm inhibitory effect for pathogenic bacteria have not been completely understood and merits further studies.

To strengthen therapeutic or prophylactic applications, the antibiotic susceptibilities of the selected 12 isolates were investigated. The isolates of *Lact. plantarum* 27053 and 27172, *Lact. helveticus* 27058 and 27170, and *Ped. acidilactici* 27167 showed sensitivity to most antibiotics tested, except for gentamicin, streptomycin, vancomycin, ciprofloxacin, and cefotaxime. Several studies have reported that resistance of the LAB strains to antimicrobial agents depends on species and strains [39]. Previous studies have reported that LAB showed a high level of resistance to streptomycin and may have a naturally reduced susceptibility to aminoglycosides, probably due to low cell membrane permeability [16]. According to Nelson et al. [40], *Lactobacillus* and *Pediococcus* are intrinsically resistant to glycopeptides. The resistance to vancomycin has been attributed as chromosomally encoded and not inducible or transferable. Moreover, other studies have provided evidence for the resistance to ciprofloxacin among human fecal *Lactobacillus* isolates, which is a common feature among LAB [41]. Resistance to cefotaxime was reported by Vay et al. [42] and Danielsen et al. [43] for the homofermentative *Lactobacilli* group. Researchers have pointed out that the natural bacterial resistance to antibiotics is not a major risk to animal or human welfare [43]; however, the possibility of the ability to deliver drug resistance needs to be evaluated in future studies.

Cell adhesion is another important aspect of probiotics. Highly hydrophobic bacterial cells have strong interactions with mucosal cells or adhere strongly to epithelial cells or mucus [38]. *Lact. rhamnosus* GG is usually used as a positive control to bind to epithelial cells. Hydrophobicity values observed for *Lact. plantarum* 27053 and 27172 and *Lact. helveticus* 27058 and 27170 ranged from 44–78%, which was similar to or higher than the hydrophobicity value of 59% obtained for *Lact. rhamnosus* GG in the present study.

According to their high levels of antibacterial and antibiofilm activities, antibiotic sensitivity, and adhesion, four strains were finally selected in this study to evaluate safety aspects. None of the examined strains exhibited α-hemolytic or β-hemolytic activities, thereby confirming the safety properties of the probiotic strains [44]. Moreover, bacterial translocation was evaluated in mice. Our results indicated that bacterial translocation between the experimental and control groups receiving the selected probiotic strains or the groups fed with *Lact. rhamnosus* GG had no significant differences. However, *Lact. helveticus* 27170 should be discarded because of a high prevalence of liver bacterial translocation observed in the animals fed with this strain, thereby suggesting that the probability of side effects with the strain could be higher than with other strains.

Conclusions

Among the 321 strains of LAB isolated from traditional artisanal milk cheese samples from Northeast China, 86 were selected for their resistance to gastric conditions and bile salts. They were evaluated according to their antimicrobial, antibiofilm, antibiotic sensitivity, and adhesive properties, and the four most efficient were evaluated for their safety in mice. Three autochthonous strains of *Lact. plantarum* 27053 and 27172 and *Lact. helveticus* 27058 were finally selected for their probiotic properties and safety, thereby allowing their eventual use in future studies. These results contribute to the increase in the diversity of probiotic strains for developing nutraceuticals and functional foods. These results suggested that our *Lactobacillus* strains with probiotic potentials may be useful for preventing or treating of diarrhea, but further in vitro and in vivo studies on these probiotic strains should be conducted.

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Compliance with Ethical Standards The experimental protocol (20119) was accepted by the international recommendations for animal welfare and the Ethical Committee for Animal Sciences of Heilongjiang province.

Conflict of Interest The authors declare that they have no conflict of interest.

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