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Identification and Characterisation of Potential Probiotic Lactic Acid Bacteria Extracted from Pig Faeces

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Abstract

Given that probiotics always have host-homologous and strain-specific effects on the hosts, lactic acid bacteria extracted and identified from porcine specimens can be potentially developed as probiotics for pig production. We aimed to identify lactic acid bacteria that are potentially probiotic, have good capacity of inhibiting pathogenic bacteria in intestine and are promising to be used as substitutes for antibiotics in pig production. Potential probiotic strains were extracted from 15 fecal specimens collected from 15 apparently healthy pigs, and were identified via 16S rDNA sequencing. The antimicrobial activity, tolerance to acid and bile salts, Caco-2 cell adhesiveness and susceptibility to antibiotics of the isolates were evaluated in vitro, and oral toxicity of the isolates were evaluated in mice. One *Lactiplantibacillus plantarum* (BJR2), two *Lacticaseibacillus casei* (HJD and TH2), one *Lacticaseibacillus rhamnosus* (MRS1), and two *Enterococcus faecium* (S-3 and S-4-H) were extracted from healthy pigs and underwent 16S rDNA sequencing identification. *L. plantarum* BJR2 and *L. casei* HJD exhibited broad-spectrum and higher antimicrobial activity against indicator enteric pathogens, including *Salmonella choleraesuis* CVCC 2139, *Escherichia coli* (O147:K89) CVCC 199, *Escherichia coli* (O141:K99) CVCC 223 and *Escherichia coli* (O139) CVCC 1496, among 6 tested strains. In addition, both *L. plantarum* BJR2 and *L. casei* HJD exhibited good tolerance to low pH (pH 2.5 and pH 3.5) and 0.30% bile salts, had relatively strong Caco-2 adhesiveness and carried no transferable resistant genes against antibiotics encoded by plasmid. In safety trials, these two isolates had no α or β-hemolysis activity, and were proved safe through oral toxicity tests in mice. It is concluded that *L. plantarum* BJR2 and *L. casei* HJD are potential probiotic candidate strains and their probiotic effects need to be further studied in pigs.

Keywords: Lactic Acid Bacteria, Probiotic, Antimicrobial Activity, Pigs

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INTRODUCTION

In swine industry, antibiotics are effective in improving feed conversion rate and decreasing disease-associated mortality.[1,2] However, the immoderate and indiscriminate utilization of antibiotic growth promoters in the breeding industry increases the number of pathogenic strains resistant to antibiotics in animals and humans.[3,4] And for this reason, the European Union and China have prohibited antibiotic growth promoter usage in the breeding industry starting from January 2006 and January 2020, respectively. To make the swine industry sustainable, it is imperative to developing antibiotic substitutes for animal production. So far, Some potential antibiotic alternatives, such as probiotics,[5,6] organic acids,[7] plant extracts and antimicrobial peptides,[8,9] have been applied as feed additives for livestock production and proven beneficial to animal health. Among these additives, probiotics were considered to be the promising alternatives to antibiotic growth promoters because they are safe and can beneficially affect the health status of hosts through keeping microbial balance in their intestine.[10,11] Probiotics refer to living microorganisms that can benefit the health of hosts when applied at a sufficient amount.[12] Recent investigations have indicated the benefits of probiotics including their effects on the inhibition of growth of pathogenic microorganisms,[13,14] on the improvement of feed conversion rate and meat quality,[15] and on the enhancement of immune response.[16] Several mechanisms have been suggested to be the explanation for probiotics' effects, such as producing organic acid,[17] releasing antimicrobial substances,[18] competitively excluding pathogenic bacteria,[19] producing digestive enzymes, nutrients and growth factors, and stimulating immune response.[20, 21]

Lactic acid bacteria (LAB) have originally been defined as Gram-positive, microaerophilic microorganisms primarily converting hexose sugars into lactic acid. They are a group of diverse bacteria belonging to genera *Lactobacillus*, *Enterococcus*, *Streptococcus* and some other microbes. LAB have been widely used as probiotics in swine production.[22] It was proved that the probiotic strain *Lactobacillus johnsonii* L531 could enhance the intestinal health of piglings within the crucial weaning period because of its capacity to control *Salmonella* infection and maintain metabolic homeostasis.[23] Dowarah et al.[24] identified *Pediococcus acidilactici* FT28 as probiotics that could improve the physicochemical characteristics and carcass quality of pork while maintaining normal levels of blood metabolites.

Although many studies have been reported on probiotics, only a few kinds of microorganisms have been applied as probiotics in swine production, actually more probiotic strains are available for development. In addition, probiotics, depending on the strain, can exhibit varied beneficial effects and characteristics. Therefore, we first isolated LAB from pig faeces and evaluated in vitro the potential of these strains as probiotics for replacement of antibiotics in swine production. The antibacterial strains were further investigated for their probiotic characteristics, including tolerance to acid and bile salts, Caco-2 cell adhesiveness, antibiotic susceptibility, and safety.

MATERIALS AND METHODS

Bacterial Strains and Caco-2 Cells

We obtained *Salmonella choleraesuis* CVCC (China Veterinary Culture Collection Center) 2139, *Staphylococcus aureus* CVCC 546, *Escherichia coli* (O147:K89) CVCC 199, *Escherichia coli* (O141:K99) CVCC 223 and *Escherichia coli* (O139) CVCC 1496 from CVCC (Beijing, China), *Escherichia coli* (O157:H7) CICC (Center of Industrial Culture Collection) 21530 from CICC (BeiJing, China), and *Escherichia coli* ATCC 25922 from China Center for Type Culture Collection (CCTCC) (WuHan, China). These strains were aerobically cultured in Luria-Bertani (LB) broth for 24 h at 37°C. Human Caco-2 cells (a colon epithelial cancer cell line) were purchased from Shanghai BoGu Biotech Co., Ltd.

The cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) plus 10% (v/v) fetal bovine serum and 100 U/mL streptomycin-penicillin mixture in a 5% CO_2 incubator.

Isolation of LAB

Fifteen fecal specimens were obtained from 15 apparently healthy pigs fed commercial

feed without antibiotic growth promoters. Each of the samples (5 g) was mixed with 45 mL sterile 0.9% (w/v) NaCl and shaken at 150 rpm for 30 min. The mixture was filtered with cotton gauze and then the filtrate was serially 10-fold diluted from 10^{-1} to 10^{-6}. One hundred microliters of serial dilutions including 10^{-4}, 10^{-5} and 10^{-6} were separately plated on de Man, Rogosa and Sharpe (MRS) agar plates, which were anaerobically or aerobically incubated for 48 h at 37°C. Different colonies were purified and stored in MRS broth added with 15% (v/v) glycerol at -70°C for downstream testing.

Identification of LAB

The isolates were preliminarily characterized via Gram stain affinity and catalase assays. For purification of genomic DNA, the Bacteria Genomic DNA Kit (ComWin Biotech Co., Ltd., Beijing, China) was utilized. The 16S rDNA sequence was amplified from extracts of isolates by the universal primers 16sRp1 (5'-AGAGTTTGATCATGGCTCAG-3') and 16sRp2 (5'-GTGTGACGGGCG GTGTGTAC-3'). Each 25-µL PCR system contained 12.5 µL 2× PCR Master Mix (ComWin Biotech Co., Ltd., Beijing, China), 0.5 µL upstream and downstream primers (10 µM), 2 µL template DNA, and 9.5 µL ddH_2O. The thermocycling program consisted of an initial step of 5 min at 94°C; 35 rounds of 30 s at 94°C, 30 s at 57°C, and 2 min at 72°C; and a final step of 72°C for 10 min. The products (~1,380 bp) of PCR were purified using a DNA purification Kit (TIANGEN Biotech, Beijing, China) and sequenced by Sangon Biotech Co., Ltd., after which the results were submitted to NCBI GenBank.

Antimicrobial Activity

The isolates' antimicrobial activity against *Salmonella choleraesuis* CVCC 2139, *Staphylococcus aureus* CVCC 546, *Escherichia coli* (O147:K89) CVCC 199, *Escherichia coli* (O141:K99) CVCC 223, *Escherichia coli* (O139) CVCC 1496 and *Escherichia coli* (O157:H7) CICC 21530 was analyzed via agar well diffusion assay according to a protocol of Argyri et al.[25] Briefly, nutrient agar was melted, mixed with an overnight grown indicator culture, and filled into petri dishes with a diameter of 90 mm. When the agar solidified, four wells (with a diameter of 6 mm) were made for each dish. The isolates were cultured overnight and centrifuged for 10 min at 7000 rpm to collect cell-free supernatants (CFS), which were filtered with 0.22-µm membrane filters. Afterwards, each well on the agar plates was filled with 200 µL of the CFS. After incubation for 24 h at 37°C, we determined the isolates' antimicrobial activity by measuring the area of clear zone surrounding each well on the plates. Each sample was tested three times independently.

Tolerance to Acidic pH

Tolerance of isolates to acidic pH was appraised using a protocol of Delgado et al.,[26] with some modifications. Briefly, overnight grown isolates were washed by centrifugation and incubated for 90 min in sterile 0.9% (w/v) NaCl with pH adjusted to 2.5, 3.5 and 4.5 using 0.1 mol/L HCl. One hundred microliters cellular suspensions treated with different pH were cultured with 10 mL MRS broth and then inoculums were incubated for 16 hr at 37°C. MRS broth with a pH value of 6.2 ± 0.2 was set as the control in this experiment. After incubation, tolerance of isolates was assessed by reading 600-nm optical density (OD) values.

Bile Salts Tolerance

The protocol proposed by Bao et al.[27] was utilized to assess bile salts tolerance. Briefly, one hundred microliters of overnight grown isolates were cultured in 10 mL MRS broth containing various concentrations (0.03, 0.3, or 0.5%, w/v) of porcine bile salts, and then the inoculums were incubated for 16 h at 37°C. The medium lacking bile salts served as the control for this experiment. After incubation, tolerance of isolates were assessed by reading 600-nm optical density (OD) values.

Caco-2 Cell Adhesiveness

Cell adhesiveness was tested following a method of Abhisingha et al.[14] Briefly, overnight grown isolates were centrifuged at 7000 rpm for 5min, and the pellets were washed three times with sterile phosphate-buffered saline (PBS) and resuspended with DMEM. The number of bacterial cells in the initial suspension was decided by plate count. Then 500 µL of the suspensions were applied onto monolayer Caco-2 cells cultured

in 24-well plates, which were subsequently incubated for 90 min at 37°C. After that, the suspensions were withdrew and the cells were rinsed thrice by sterile PBS to eliminate dissociative bacterial cells. A volume of 500 µL 0.1% (v/v) Triton X-100 was supplemented to each well to remove the adherent bacteria, and the number of cell-bound bacteria was decided by plate count. Each isolate was assessed thrice independently.

Antibiotic Susceptibility Assay

The susceptibility of isolates to antibiotics was determined via the Kirby-Bauer method.[28] The isolates and quality control strain (*Escherichia coli* ATCC 25922) were subjected to treatment with beta-lactams (ampicillin, 10 µg/disc; ceftriaxone, 30 µg/disc), macrolides (azithromycin, 15 µg/disc), lincosamides (clindamycin, 2 µg/disc), aminosugars (streptomycin, 10 µg/disc; gentamycin, 10 µg/disc), tetracyclines (tetracycline, 30 µg/disc), glycopeptides (vancomycin, 30 µg/disc), and quinolones (ciprofloxacin, 5 µg/disc; nalidixic acid, 5 µg/disc). Overnight grown bacterial cultures of isolates and quality control strain were spread onto MRS and Mueller-Hinton agar plates respectively, and a confluent layer was created with sterile swabs. After inoculation, the disks with antibiotics were placed on the surface of agar plates, which were subjected to anaerobic or aerobic incubation for 24 h at 37°C, followed by measuring inhibition zone diameter. Three replicates were carried out for each isolate. In accordance with the guideline of the Clinical and Laboratory Standards Institute (CLSI), the test strains were classified, based on their antibiotic susceptibility, as sensitive (S), intermediate (I), or resistant (R).

Plasmid DNA Isolation

Plasmid DNA extraction from isolates was performed by utilizing a TIANprep Mini Plamid Kit (TIANGEN Biotech, Beijing, China), following the provider's protocol. The extracted plasmid DNA was analyzed by electrophoresis (1%).

Hemolysis Assay

Overnight grown isolates were streaked on MRS agar plates containing 5% (w/v) sheep blood and incubated for 24 h at 37°C. Afterwards, hemolysis surrounding the colonies on the plates

Table 1. Antimicrobial activity of the Lactic acid bacteria isolates against indicator microbes (mm)

Isolates	Zone of inhibition(mm)					
	Salmonella choleraesuis CVCC 2139	*Staphylococcus aureus* CVCC 546	*Escherichia coli* (O147:K89) CVCC 199	*Escherichia coli* (O141:K99) CVCC 223	*Escherichia coli* (O139) CVCC 1496	*Escherichia coli* (O157:H7) CICC 21530
Lactiplantibacillus plantarum BJR2	13.61±0.11^{b}	—	14.93±0.32^{a}	15.08±0.46^{a}	16.26±0.57^{a}	16.40±0.27^{b}
Lacticaseibacillus casei HJD	17.83±0.26^{a}	—	15.65±0.45^{a}	12.63±0.53^{b}	13.84±0.37^{b}	17.18±0.45^{b}
Lacticaseibacillus casei TH2	11.46±0.42^{c}	19.32±0.63^{a}	11.28±0.24^{c}	10.88±0.18^{c}	—	11.88±0.21^{c}
Lacticaseibacillus rhamnosus MRS1	14.22±0.51^{b}	—	14.50±0.46^{b}	14.38±0.65^{a}	12.71±0.34^{c}	20.62±0.75^{a}
Enterococcus Faecium S-3	10.77±0.15^{c}	18.62±0.31^{a}	—	10.11±0.17^{c}	10.58±0.38^{d}	10.03±0.21^{d}
Enterococcus Faecium S-4-H	—	10.44±0.26^{b}	10.98±0.33^{c}	12.64±0.42^{b}	10.96±0.26^{d}	11.84±0.43^{c}

Differences were detected with one-way ANOVA; —: NO inhibition; a,b,c,dValues with different superscripts in the same column differ significantly($p<0.05$)

Table 2. Tolerance of the Lactic acid bacteria isolates to acidic conditions represented in mean optical density after 16h

Isolates	pH			
	MRS broth	pH2.5	pH3.5	pH4.5
Lactiplantibacillus plantarum BJR2	0.235±0.031^{c}	0.144±0.012^{a}	0.163±0.010^{a}	0.235±0.011^{b}
Lacticaseibacillus casei HJD	0.364±0.024^{a}	0.122±0.007^{a}	0.140±0.018^{a}	0.356±0.013^{a}
Lacticaseibacillus casei TH2	0.343±0.026^{a}	0.073±0.014^{b}	0.130±0.025^{a}	0.309±0.039^{a}
Lacticaseibacillus rhamnosus MRS1	0.313±0.044^{a}	0.128±0.024^{a}	0.155±0.028^{a}	0.310±0.051^{a}
Enterococcus Faecium S-3	0.304±0.016^{b}	0.007±0.002^{c}	0.014±0.005^{b}	0.014±0.006^{c}
Enterococcus Faecium S-4-H	0.259±0.019^{c}	0.010±0.005^{c}	0.019±0.008^{b}	0.021±0.001^{c}

Differences were detected with one-way ANOVA
a,b,c: Values with different superscripts in the same column differ significantly($p<0.05$)

Table 3. Growth of the Lactic acid bacteria isolates in MRS broth supplemented with different concentrations of bile salts represented in mean optical density after 16h

Isolates	Bile salts concentration			
	MRS broth	0.03%	0.30%	0.50%
Lactiplantibacillus plantarum BJR2	0.348±0.024^{b}	0.299±0.010^{a}	0.181±0.012^{a}	0.086±0.004^{a}
Lacticaseibacillus casei HJD	0.418±0.032^{a}	0.309±0.015^{a}	0.157±0.024^{a}	0.068±0.002^{b}
Lacticaseibacillus casei TH2	0.372±0.017^{a}	0.245±0.020^{a}	0.188±0.017^{a}	0.102±0.021^{a}
Lacticaseibacillus rhamnosus MRS1	0.404±0.063^{a}	0.313±0.052^{a}	0.201±0.020^{a}	0.104±0.019^{a}
Enterococcus Faecium S-3	0.197±0.033^{c}	0.052±0.006^{c}	0.020±0.005^{c}	0.009±0.002^{d}
Enterococcus Faecium S-4-H	0.371±0.048^{a}	0.092±0.010^{b}	0.082±0.003^{b}	0.049±0.004^{c}

Differences were detected with one-way ANOVA
a,b,c,d: Values with different superscripts in the same column differ significantly($p<0.05$)

was evaluated, with *Staphylococcus Aureus* CVCC 546 being the positive control. Three types of hemolysis are classified, for which a clear zone, a green zone, or no zone respectively indicates β-, α-, or γ-hemolysis.

In Vivo Safety Assay

Four-week-old Kunming mice were obtained from Guangxi Medical University and housed in plastic cages at room temperature. After 5 days for environmental adaptation, all mice were randomly distributed in 7 groups including 6 experimental groups and one negative control group (6 mice per group with half male and half female). To prepare inoculums, overnight grown isolates were washed three times by centrifugation and resuspended into 1×10^{9} CFU/mL suspensions by sterile PBS. Each mouse was treated with the candidate strain at a dose of 10 mL/kg by gavage, while the negative control group was given PBS. The experiment lasted 14 days. The mice were weighed on days 0 and 14, and their appetite, appearance, mental state, behavior and mortality were monitored everyday.

Statistical Analysis

We employed SPSS (release 16.0 standard version; SPAA, Inc., Chicago) for data analysis. The data were expressed as mean ± standard deviation (SD), and one-way analysis of variance (ANOVA) and Tukey range test were sequentially performed for comparison of multiple groups of data. $P<0.05$ was considered statistically significant.

RESULTS

LAB Extraction and Identification

Six isolates (BJR2, HJD, TH2, MRS1, S-3, S-4-H) were picked from MRS agar plates; all of them were rod or coccus in shape, Gram-positive,

Table 4. Adhesion rate of the Lactic acid bacteria isolates to Caco-2 cells

Isolates	Adhesion rate (%)
Lactiplantibacillus plantarum BJR2	44.90±2.73[a]
Lacticaseibacillus casei HJD	36.93±3.24[b]
Lacticaseibacillus casei TH2	27.50±1.89[c]
Lacticaseibacillus rhamnosus MRS1	16.00±0.96[d]
Enterococcus Faecium S-3	11.09±0.55[e]
Enterococcus Faecium S-4-H	47.95±1.57[a]

Differences were detected with one-way ANOVA
a,b,c,d,e Values with different superscripts in the same column differ; significantly ($p<0.05$)

and catalase-negative. 16S rDNA sequencing analysis showed that BJR2 was *Lactiplantibacillus plantarun*, HJD and TH2 were *Lacticaseibacillus casei*, MRS1 was *Lacticaseibacillus rhamnosus*, S-3 and S-4-H were *Enterococcus faecium*. Their 16S rDNA sequences were uploaded into GenBank and assigned accession numbers of MZ558169–MZ558174.

The LAB Strains' Antimicrobial Activity

The antimicrobial activity of the isolated LAB against indicator pathogens is displayed in Table 1. The growth of *E. coli* (O141:K99) CVCC 223 and *E. coli* (O157:H7) CICC 21530 was suppressed by all the LAB strains, while *S. aureus* CVCC 546 growth was only suppressed by *L. casei* TH2. *E. faecium* S-3 and *E. faecium* S-4-H. *L. plantarum* BJR2 exhibited the greatest growth-inhibiting effect against *E. coli* (O147:K89) CVCC 199 (14.93 mm), *E. coli* (O141:K99) CVCC 223 (15.08 mm) and *E. coli* (O139) CVCC 1496 (16.26 mm) among the six candidate strains.

Low pH and Bile Salts Tolerance

L. plantarum BJR2, *L. casei* HJD and *L. rhamnosus* MRS1 showed higher tolerance at pH 2.5 and pH 3.5 as compared to the other isolates, whereas *E. Faecium* S-3 and *E. Faecium* S-4-H were highly sensitive to acidic environment (Table 2). The selected isolates' ability to tolerate bile salts is presented in Table 3. The results showed that *L. plantarum* BJR2, *L. casei* HJD, *L. casei* TH2 and *L. rhamnosus* MRS1 tolerated 0.30% bile salts well. In contrast, *E. Faecium* S-3 and *E. Faecium* S-4-H showed low tolerance to bile salts.

Caco-2 Cell Adhesion Ability of the Test Strains

As revealed by Table 4, The test strains showed diverse adhesion abilities. *E. faecium* S-4-H and *L. plantarum* BJR2 had higher adhesion rates (47.95% and 44.90%, respectively) than the other isolates.

Antibiotic Susceptibility Assay

The test strains' susceptibility to various antibiotics is shown in Table 5. *E. faecium* S-4-H, *L. casei* HJD and *L. rhamnosus* MRS1 exhibited higher sensitivity to antibiotics compared with other isolates. All test strains were resistant to streptomycin and nalidixic acid, and *L. plantarum* BJR2 was resistant to all antibiotics used in this study.

Plasmid DNA Isolation of Test Strains

The result of electrophoretic analysis showed that no plasmids were detected in all isolates (data not shown).

Hemolysis Assay

The result showed that all the test strains exhibited no α or β-hemolysis activity when cultured on agar plates containing sheep blood.

***In Vivo* Safety Assay**

During the animal testing, the mice in experimental groups showed no adverse effects in respect to general health conditions compared with the negative control group. As shown in Table 6, the group administered with *L. casei* HJD had significantly higher weight gain ($P < 0.05$) compared with the negative control group at day 14.

DISCUSSION

Intensive pig farming has increased the risk of intestinal disease. Infection by bacteria may represent a key pathogenic mechanism of piglets diarrhea. Neonatal and weaning piglets are susceptible to potentially pathogenic bacteria including *Escherichia coli* and *Salmonella* spp.[29] LAB can release many antimicrobials, such as lactic acid, bacteriocins and hydrogen peroxide, that can significantly inhibit pathogenic bacteria.[30,31] Therefore, we herein aimed to screen and identify potential probiotic LAB strains extracted from

Table 5. The antibiotic susceptibility of the Lactic acid bacteria isolates

Isolates	AMP	CEF	AZI	CLI	STR	GEN	TET	VAN	CIP	NAL
Escherichia coli ATCC 25922	S	S	R	R	S	S	S	R	S	S
Lactiplantibacillus plantarum BJR2	R	R	R	R	R	R	R	R	R	R
Lacticaseibacillus casei HJD	R	I	I	S	R	R	S	R	R	R
Lacticaseibacillus casei TH2	R	R	I	R	R	R	R	R	R	R
Lacticaseibacillus rhamnosus MRS1	S	R	I	S	R	R	S	R	R	R
Enterococcus Faecium S-3	R	R	R	I	R	R	R	R	I	R
Enterococcus Faecium S-4-H	S	R	R	R	R	I	S	I	I	R

AMP ampicillin, CEF ceftriaxone, AZI azithromycin, CLI clindamycin, STR streptomycin, GEN gentamycin, TET tetracycline, VAN vancomycin, CIP ciprofloxacin, NAL nalidixic acid; R resistant, I intermediate, S sensitive

Table 6. Body weight evaluations of experimental mice inoculated by oral gavage with the Lactic acid bacteria isolates

Groups	Body weight (g)		Death No.
	Day 0	Day 14	
PBS	20.87±0.63	31.04±0.60	0
Lactiplantibacillus plantarum BJR2	21.08±1.62	31.36±1.17	0
Lacticaseibacillus casei HJD	20.47±0.29	34.99±1.06*	0
Lacticaseibacillus casei TH2	20.6±0.68	31.81±2.29	0
Lacticaseibacillus rhamnosus MRS1	20.99±1.31	32.18±1.38	0
Enterococcus Faecium S-3	20.68±0.43	31.02±0.59	0
Enterococcus Faecium S-4-H	20.37±0.26	32.33±0.96	0

Significant difference compare with PBS,*P<0.05

fecal samples of apparently healthy pigs, which can exert antimicrobial activity against common enteric pathogenic bacteria. Selected LAB strains exhibited antimicrobial activity against different indicator pathogens. Each of them showed inhibitory activity against five tested indicator strains, displaying a broad antimicrobial spectrum. *L. plantarum* BJR2 displayed higher inhibitory activity against *E. coli* (O147:K89) CVCC 199 (14.93 mm), *E. coli* (O141:K99) CVCC 223 (15.08 mm) and *E. coli* (O139) CVCC 1496 (16.26 mm) than the other test strains. This is consistent with the findings of Piyadeatsoontorn et al.,[32] who observed that *L. plantarum* strains L21 and L80 had strong inhibition to *E. coli* using agar spot assays. Similarly, according to Betancur et al.,[33] *L. Plantarum* CAM6 exhibited the greatest inhibitory effect against *E. coli* strain NBRC 102203 (19.7 mm). *L. casei* HJD exerted a significant growth inhibitory effect against *S. choleraesuis* CVCC 2139 (17.83 mm) and *E. coli* (O147:K89) CVCC 199 (15.65 mm), was stronger than the other isolates. Previous studies revealed that orally-administered recombinant *L. casei* could shape the intestinal probiotics and significantly reduce the incidence of diarrhea in newborn piglets.[34] Yin et al.[35] reported that *L. casei*-fermented feeds could reduce diarrhea severity of pigs challenged by *Salmonella*. *L. plantarum* BJR2 and *L. casei* HJD showed good antimicrobial properties of common intestinal pathogenic bacteria, and had the potential to prevent and treat bacterial infections.

Acid and bile tolerance is one of the primary criteria used for selection of a potential probiotic strain because this property always reflects the survivability of a strain inside the host.[36] In the present research, *L. plantarum* BJR2, *L. casei* HJD and *L. rhamnosus* MRS1 tolerated to low pH (pH=2.5 and 3.5) and 0.30% bile salts well, suggesting that they have the potential ability of surviving the passage through the stomach and into the intestinal tract. This finding is consistent with that of Jia et al.,[6] who found that *L. johnsonii* pZL5c and *L. animalis* pZL8a exhibited good

tolerance to acidic pH (pH=3.0) and a 0.30% porcine bile salt solution in PBS. Betancur et al.[33] reported that all three *L. Plantarum* strains tolerated pH 3.0 and a bile salt concentration as high as 0.15% well, which is similar to our results. Intestinal epithelial cell adhesiveness represents a crucial property of probiotics because it predicts the residence duration of probiotics in host intestine, thereby reflects the probiotics' pathogen clearance and pro-immunologic effects.[37] Among the currently available animal and human intestinal cell lines, including Caco-2, T84, HT-29, IEC-6 and IEC-18,[38] Caco-2 is widely applied in analyses of adhesiveness.[39] In this study, *E. faecium* S-4-H and *L. plantarum* BJR2 had strong adhesion ability to Caco-2 cells, exhibiting adhesion rates of 47.95% and 44.90%, which were higher than those of the other test strains, and *L. casei* HJD showed the second highest adhesion rate (36.93%). These rates were significantly higher than those reported in Jia et al.'s research,[6] in which *L. Johnsonii* pZL8b and *L. animalis* pZL8a respectively displayed relatively lower adhesion rates of 11.52% and 10.25%. One possible explanation is that different bacterial strains being studied in our work.

Antibiotic susceptibility is a vital criterion to select bacteria as probiotics, and antibiotic resistance is a focused issue in world.[40] The resistance of LAB to antibiotics can be either intrinsic resistance, which is not transmissible, or acquired resistance, which usually arises due to bacterial DNA mutations that are potentially transmissible via mobile genetic materials (such as transposons and plasmids).[41] Therefore, it is important to test the presence of antibiotic resistance genes in plasmids or chromosomes.[42] As per our data, all lactobacillus species isolates including *L. plantarum* BJR2, *L. casei* HJD, *L. casei* TH2 and *L. rhamnosus* MRS1 were resistant to streptomycin, vancomycin and ciprofloxacin. Our findings are consistent with those of Sharma et al.,[43] who observed that lactobacillus species exhibited intrinsic resistance to ciprofloxacin, streptomycin, sulphamethoxazole-trimethoprim, and vancomycin. Pei et al.[44] also found that all LAB isolates showed resistance to ciprofloxacin and streptomycin. All tested strains displayed resistance to nalidixic acid, resembling the findings of Jia et al.,[6] who determined that all the isolates were categorized as nalidixic acid resistance. None of our tested strains involved any transferable antibiotic resistance genes carried by plasmid. Therefore, their antibiotic resistance could be innate and would not spread through horizontal gene transfer. The mechanism underlying intrinsic antibiotic resistance of LAB species remains largely unclear and warrants further investigation.[45]

Hemolysis activity could cause anemia, bacteremia and edema, and is a threat to host.[46] So bacterial strains must be evaluated for safety before they can be commercially used as probiotics. We found that all isolates displayed no α or β-hemolysis activity, agreeing with the findings of Zoumpopoulou et al.,[47] who reported that no lactobacillus was found to be hemolytic.

Potential probiotics must be evaluated for safety through oral toxicity tests in animals. In this study, safety tests for all selected strains in mice were performed. The results showed that all mice maintained good physical condition and had no adverse effects. Furthermore, administration of *L. casei* HJD significantly enhanced the weight gain of the mice relative to the control group. These results indicated that all tested strains are potentially safe for consumption, and *L. casei* HJD has the potential to improve performance including feed intake and conversion and weight gain in animals. Further researches are needed in pigs.

CONCLUSION

Both in vitro and in vivo probiotic characterization studies and safety assay in this research suggest that *L. plantarum* BJR2 and *L. casei* HJD are good probiotic candidate strains, which exhibit broad-spectrum and high antimicrobial activity against indicator enteric pathogens. In future studies, their beneficial effects on anti-infection, growth performance, immune and others, and possible negative effects in pigs should be assessed.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

LM, SQ and PZ carried out safety evaluation in mice. WZ, AB, JL and FC performed assessment in vitro. HD, WZ and JW designed the experiments. SQ analyzed the data and wrote the manuscript. JW revised the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by Animal Ethics Committee of the Guangxi Veterinary Research Institute. In this study, all experimental animals used were dealt according to the Animal Ethic Procedures and Guidelines of the People's Republic of China (SYXK2010-0001).

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