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ORIGINAL ARTICLE

Potential probiotic properties of lactic acid bacteria isolated from the intestinal mucosa of healthy piglets

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Abstract In the present study, the probiotic properties of 52 lactic acid bacteria strains, isolated from the intestinal mucosa of 60-day-old healthy piglets, were evaluated in vitro in order to acquire probiotics of potential application. Based on acidic and bile salt resistance, 11 lactic acid bacteria strains were selected, among which 1 was identified as *Pediococcus acidilactici*, 3 as *Enterococcus faecium*, 3 as *Lactobacillus rhamnosus*, 2 as *Lactobacillus brevis*, and 2 as *Lactobacillus plantarum* by 16S rRNA gene sequencing. All selected strains were further investigated for transit tolerance in simulated upper gastrointestinal tract, for adhesion capacity to swine intestinal epithelial cells J2 (IPEC-J2), for cell surface characteristics including hydrophobicity, co-aggregation and auto-aggregation, and for antimicrobial activities. Moreover, hemolytic, bile salt hydrolase and biogenic amine-producing abilities were investigated for safety assessment. Two *E. faecium* (WEI-9 and WEI-10) and one *L. plantarum* (WEI-51) exhibited good simulated upper gastrointestinal tract tolerance, and showed high auto-aggregation and co-aggregation with *Escherichia coli* 1570. The strains WEI-9 and WEI-10 demonstrated the highest adherence capacity. The 11 selected strains mentioned above exhibited strong antimicrobial activity against *E. coli* CVCC1570, *Staphylococcus aureus* CVCC1882 and *Salmonella pullorum* AS1.1859. None of the 11 selected strains, except WEI-9 and WEI-33, exhibited bile salt hydrolase, hemolytic or biogenic amine-producing abilities. This work showed that the *E. faecium* WEI-10 and *L. plantarum* WEI-51were found to have the probiotic properties required for use as potential probiotics in animal feed supplements.

Keywords Lactic acid bacteria · Probiotic properties · Tolerance to bile salts · *Enterococcus faecium* · Adhesion ability · Antimicrobial activities

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Introduction

Probiotics are defined as "live microorganisms which, when administrated in adequate amounts, confer a health benefit on the host" (FAO/WHO 2002). The gastrointestinal tract is considered the main sources for isolation of novel promising probiotic microorganisms (Ambadoyiannis et al. 2004). The most studied probiotics belongs to *Lactobacillus* and *Bifidobacterium* genera, and, among them, *Lactobacillus* species are commensal and non-pathogenic inhabitants of human and animal intestine, and have been considered as valuable probiotic microorganisms since they contribute to the inhibition to harmful intestinal bacteria (Walter 2008). A potential probiotic strain is expected to have several desirable properties to exert its beneficial effects. It has been reported that the basic criteria for lactic acid bacteria strains to be used as probiotics include the following: (1) they should be generally recognized as safe (GRAS); (2) they should be acid and bile salt resistant and survive in the gastrointestinal tract; (3) they should be able to adhere to the intestinal epithelium of the hosts; (4) they should demonstrate antimicrobial activity against enteric pathogens; and (5) they should be able to keep their viability during processing and storage (Kumar and Kumar 2015).

Therefore, the aims of the present study were to evaluate, in vitro, the functional and safety characteristics, including resistance to acid and bile, tolerance to simulated upper

gastrointestinal tract, adhesion capacity to IPEC-J2, hydrophobicity, aggregation and co-aggregation, antimicrobial activity, hemolytic, bile salt hydrolase activity and biogenic amine-producing ability, of potential probiotic lactic acid bacteria, isolated from the intestinal mucosa of 60-day-old healthy piglets.

Materials and methods

Bacterial strains and culture conditions

In our previous work, 52 lactic acid bacteria were isolated from the intestinal mucosa of 60-day-old healthy piglets. The method of isolation was as follows: the gastrointestinal digestive tracts and mucosa collected from healthy weaning piglets ($n = 48$, approximate 60-day-old, from farms in the northern part of China) were diluted 10-fold in 0.85% (w/v) NaCl and plated on MRS (de Man-Rogosa Sharpe) agar (Hopebiol, China). Incubation was conducted at 37°C for 48 h. Colonies with different morphological features were picked randomly, purified and stored on MRS agar for further testing. When needed, all 52 lactic acid bacteria were cultured in MRS broth (Hopebiol, China), and incubated statically at 37°C for 24–48 h. Indicator strains for antimicrobial assays were *Escherichia coli* CVCC1570, *Staphylococcus aureus* CVCC1882 and *Salmonella pullorum* AS1.1859, respectively, which were purchased from the China Veterinary Culture Collection Center (CVCC, China). These strains are pathogenic bacteria that may cause gastrointestinal disease of swine or poultry. When needed, these three indicator bacteria were grown in LB broth (Hopebiol, China) at 37°C, 200 rpm for 16–18 h. All the isolated lactic acid bacteria and indicator bacteria were cultured in MRS broth or LB broth respectively, and stored by freezing (−80°C) in MRS or LB broth supplemented with 20% (v/v) sterilized glycerol.

Preparation of simulated gastric juice and small intestinal juice

Simulated gastric juice (SGJ) was prepared as described (Fernandez et al. 2003) from NaCl (125 mmol/L), $NaHCO_3$ (45 mmol/L), KCl (7 mmol/L) and pepsin (3 g/L). The salts were purchased from Sinoreagent (China), and pepsin from Sigma-Aldrich (St. Louis, MO). Aliquots of gastric juice were adjusted to pH value 3.0.

Simulated small intestinal juice (SSIJ) was prepared from 0.1% (w/v) pancreatin (Sigma-Aldrich) and 0.3% (w/v) pig bile salt (Aoboxing Bio-Tech, Beijing, China). Aliquots of small intestinal juice were adjusted to pH value 8.0.

Resistance to low pH

Tolerance to low pH was assessed as described by Anandharaj et al. (2015), with minor modifications. Briefly, resistance to low pH of all isolated strains was examined in MRS broth of pH values 1.0, 2.0 or 3.0 adjusted with hydrochloric acid (1 M). Fresh bacterial cultures from MRS agar slants were inoculated into MRS broth and incubated at 37°C for 14 h. The bacterial cultures were then inoculated into MRS broth of pH values 1.0, 2.0 or 3.0, respectively, at an inoculum size of 1% (v/v), and incubated at 37°C for 11 h. Conventional nonacidified MRS (pH 6.2) was used as a control. Enumeration of the viable cell counts was achieved by surface plating on MRS agar followed by incubation at 37°C for 48 h. The survival rate was calculated as log 10 values of colony-forming units per milliliter (CFU/mL). The experiments were performed in triplicate and mean values were calculated.

Survival rate was calculated as:

$$\%\ \text{Resistance} = \left({}^{\text{Viable cell } \textit{counts in MRS broth at } \text{pH 1.0, 2.0, 3.0}}/_{\text{Viable cell } \textit{counts in MRS broth at } \text{pH 6.2}}\right) \times 100 \quad (1) \qquad (1)$$

Resistance to bile salts

The resistance to bile salts of all strains was assayed following the method of Anandharaj and Sivasankari (2014), with minor modifications. Briefly, tolerance of selected lactic acid bacteria strains to various bile salt concentrations was determined using MRS broth containing bile salts at different concentration (0.1%, 0.3%, 0.5%, w/v). Fresh bacterial cultures from MRS agar slants were inoculated into MRS broth, and incubated at 37°C for 14 h. Bacterial cultures were then inoculated into MRS broth with pig bile salt concentrations of 0.1%, 0.3% or 0.5%, respectively, at an inoculum size of 1% (v/v), and incubated at 37°C for 9 h. MRS broth without bile salts was used as control. Enumeration of the viable cell counts was achieved by surface plating on MRS agar followed by incubation at 37°C for 48 h. The survival rate was calculated as log 10 values of CFU/mL. The experiments were performed in triplicate and mean values were calculated.

Survival rate was calculated as:

$$\%\ \text{Resistance} = \left({}^{\text{Viable cell } \textit{counts in MRS broth with bile salts}}/_{\text{Viable cell } \textit{counts in MRS broth without bile salts}}\right) \times 100 \quad (2)$$

Molecular identification of lactic acid bacteria

On the basis of the resistance to low pH and bile salts characteristics of the 52 lactic acid bacteria described above, 11 strains demonstrating good resistance to low pH and bile salts were subjected to genetic identification using 16S rRNA gene sequencing. Briefly, genomic DNA was extracted by UniversalGen DNA Kit (TianGEN, China). PCR amplification of the 16S rRNA gene was performed using the universal prokaryotic primers SD-Bact-0008-a-S-20 (27 F; 5′ A G A G T T T G A T C C T G G C T C A G 3′) and SD-Bact-1492-a-A-19 (1492R; 5′ TACCTTGTTACGACTT 3′). The polymerase chain reaction (PCR) reaction mix consisted of 1.0 μL template, 2.5 μL 10-fold diluted *Taq* DNA Polymerase Buffer (containing Mg^{2+}), 1 μL deoxynucleoside triphosphate (dNTP, 2.5 mmol/L, TaKaRa), 0.5 μL of each primer (10 μmol/L), 0.125 μL *Taq* DNA polymerase (5 U/μL, TaKaRa) and 25 μL DNase-RNase-free water (ComWin Biotech, China). Each PCR cycling profile consisted of an initial denaturation step (5 min at 95°C), followed by amplification for 30 cycles as follows: denaturation for 1 min at 95°C, annealing for 1 min at 55°C, an extension for 1.5 min at 72°C. PCR was completed with an elongation phase (10 min at 72°C). The amplified PCR products were purified using the QIAquick PCR kit and amplicons were sequenced (forward and reverse sequence) by SinoGenoMax, Beijing, China. The sequence was compared with the nucleotide database of the NCBI GenBank using the BLAST program. A similarity of >99% to the 16S rRNA gene sequence of the known lactic acid bacteria recorded in the database was used as a criterion for the identification.

Resistance to simulated gastric juice

Resistance to simulated gastric juice was determined according to Tulini et al. (2013), with slight modifications. Fresh bacterial cultures from MRS agar slants were inoculated into MRS broth and were incubated at 37°C for 14 h. Fresh cultures were harvested by centrifugation at 7000 *g* for 10 min and re-suspended in 0.85% (w/v) sterile saline solution. Dilutions were made to achieve a final cell density of 10^9 CFU/mL. Thereafter, 0.5 mL each cell suspension and 4.5 mL simulated gastric juice were mixed by vortexing for 10 s and incubated at 37°C for 3 h. The cell suspensions at 0 h were used as a control. To determine viable cell counts, serial dilutions were placed on MRS agar and incubated at 37°C for 48 h before enumeration of CFUs. The survival rate was calculated as log 10 values of CFU/mL. The experiments were performed in triplicate and mean values were calculated.

Survival rate was calculated as:

$$\%\ \text{Resistance} = \left({}^{\text{Viable cell } \textit{counts in simulated gastric juice at } 3\ h}/_{\text{Viable cell } \textit{counts at } 0\ h}\right) \times 100 \quad (3)$$

Resistance to simulated gastrointestinal tract

The resistance to simulated gastrointestinal tract conditions was examined according to the protocol of Fernandez et al. (2003), with slight modifications. Fresh bacterial cultures from the MRS agar slants were inoculated into MRS broth and were incubated at 37°C for 14 h. Fresh cultures were harvested by centrifugation at 7000 *g* for 10 min and re-suspended in 0.85% (w/v) sterile saline solution. Dilutions were made to achieve a final cell density of 10^9 CFU/mL. Thereafter, 0.5 mL each cell suspension and 4.5 mL simulated gastric juice were mixed by vortexing for 10 s and incubated at 37°C for 3 h. Cell suspensions (0.5 mL) treated by simulated gastric juice were taken and mixed with 4.5 mL simulated small intestinal juice. The mixture was again vortexed for 10 s and incubated at 37°C for 6 h. To determine viable cell counts, serial dilutions were placed on MRS agar and incubated at 37°C for 48 h before enumeration of CFUs. The survival rate was calculated as log 10 values of CFU/mL. The experiments were performed in triplicate and mean values were calculated.

Survival rate was calculated as:

$$\%\ \text{Resistance} = \left({}^{\text{Viable cell } \textit{counts in simulated small intestinal juice at } 6\ h}/_{\text{Viable cell } \textit{counts at } 0\ h}\right) \times 100 \quad (4)$$

Adhesion capacity to IPEC-J2

The adhesion capacity to IPEC-J2 of all 11 selected strains was examined as described by Sarem et al. (1996) and Crociani et al. (1995) after slight modification as follows. The IPEC-J2 was donated by Beijing University of Agriculture. The cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; HyClone Laboratories Inc., Logan, UT) supplemented with 10% (v/v) fetal calf serum (Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C and 5% CO_2. For the adhesion assay, cell monolayers were detached from culture dishes by trypsin treatment and then transferred to 6-well cell culture plates. Cells were seeded at a cell density of $2 \times 10^5 - 4 \times 10^5$ cells per well, and grown to confluence. The cell culture medium was changed every day and replaced with fresh supplemented DMEM without antibiotics at least 1 h before the adhesion assay.

Fresh bacterial cultures from MRS agar slants were inoculated into MRS broth and then incubated at 37°C for 14 h. Cell cultures were harvested by centrifugation, washed twice with PBS buffer (pH 7.4), suspended in supplemented DMEM media without antibiotics, and viable cell counts at a concentration of approximate 10^8 CFU/mL were then determined. The adhesion of bacterial strains to IPEC-J2 cell was investigated by adding 1 mL bacterial suspension to wells containing IPEC-J2 monolayer. After incubation at 37°C for 2 h, the IPEC-J2 cell cultures were washed three times with PBS, and then treated with 1 mL 0.1% Triton X-100 for 10 min to lyse the IPEC-J2 cells and release the adhered bacteria. The adhesion ratio was calculated by counting on MRS agar plates. Each adhesion assay was carried out in triplicate. The adhesion ratio (%) was calculated using the formula:

$$\%\ \text{Adhesion ratio} = \left(\text{Viable cell } \textit{counts} \text{ of adhered bacteria} \Big/ \text{Viable cell } \textit{counts} \text{ of suspension before adhesion assay}\right) \times 100 \quad (5)$$

Hydrophobicity assay

The surface hydrophobicity (H%) of the 11 selected strains was performed as described by Solieri et al. (2014) after slight modification as follows. Fresh bacterial cultures from MRS agar slants were inoculated into MRS broth and then incubated at 37°C for 14 h. Cell cultures were harvested by centrifugation, washed twice with PBS solution (pH 7.4), suspended in PBS buffer and adjusted to around 0.5 (A_0) with an approximate concentration of 10^7 CFU/mL by measuring the absorbance at 540 nm as preparation. About 3 mL bacterial suspension and 1 mL toluene were mixed by vortexing for 30 s, and incubated at 37°C for 10 min for temperature equilibration. The mixture was again vortexed briefly and incubated at 37°C for 1 h for phase separation. The aqueous phase was gently taken out for measuring absorbance at 540 nm. The surface hydrophobicity (H%) was calculated using the formula:

$$\text{H}\% = [(A_0 - A_t)/A_0] \times 100 \quad (6)$$

where, A_t and A_0 represented the absorbance of the aqueous phase after mixing and the absorbance of original suspension, respectively.

Auto-aggregation and co-aggregation assay

The auto-aggregation assay was performed according to Solieri et al. (2014) with slight modification as follows. Fresh bacterial cultures from MRS agar slants were inoculated into MRS broth and then incubated at 37°C for 14 h. Cell cultures were harvested by centrifugation, washed twice with PBS solution (pH 7.4), suspended in PBS buffer and adjusted to around 0.5 (A_0) with an approximate concentration of 10^7 CFU/mL by measuring the absorbance at 540 nm as preparation. Cell suspension (4 mL) in PBS buffer was vortexed for 10 s and incubated at room temperature for 5 h. Thereafter, the upper suspension was taken to measure the absorbance at 540 nm. Auto-aggregation (Auto-A%) was expressed as the percentage decrease in absorbance after 5 h relative to that of original suspension as follows:

$$\text{Auto}-\text{A}\% = [(A_0 - A_t)/A_0] \times 100 \quad (7)$$

where A_t and A_0 represent the absorbance of at time 5 h and 0 h, respectively.

Co-aggregation ability (Co-A%) was assayed as reported by Solieri et al. (2014). The pathogen strain used was *E. coli* CVCC1570. The cells suspensions were prepared as above. Equal volumes (2 mL) of suspensions of tested strain and pathogenic strain were mixed together in pairs and vortexed for 10 s. Cell suspensions of each single strain were used as controls. After 5 h at room temperature, absorbance of tested (A_x) strains, pathogenic (A_y) strains and the mixture [$A_{(x+y)}$] were measured at 540 nm. Co-aggregation percentage was calculated using the equation:

$$\text{Co}-\text{A}\% = \left[1 - \frac{A_{(x+y)}}{(A_x + A_y)/2}\right] \times 100 \quad (8)$$

where x and y represented each of the two strains and $(x + y)$ represented the mixture, respectively.

Antimicrobial activity

Antagonism towards various bacterial pathogens was evaluated according to Wu et al. (2014) with slight modification as follows. The tested strain was grown on MRS agar at 37°C for 48 h. Agar containing cultures were then punched using 8 mm hole puncher and spotted onto the surface of LB agar (Hopebiol, China) previously inoculated with 100 μL an overnight culture of *E. coli* CVCC1570 (about 10^8 CFU/mL). Clear zones were measured after overnight incubation at 37°C for 48 h. The 11 strains were further evaluated for their inhibitory activity against the other indicator bacteria, which were *Staphylococcus aureus* CVCC1882 and *Salmonella pullorum* AS1.1859, by the same method. Each strain was tested in triplicate.

Safety characteristics

Antibiotic resistance

The following six antimicrobial agents (Solarbio, China) recommended by the European Food Safety Authority (EFSA 2012a) were used: ampicillin and vancomycin as inhibitors of cell wall synthesis; kanamycin, chloramphenicol, tetracycline and streptomycin as inhibitors of protein synthesis. All antibiotic powders were dissolved in appropriate diluents (CLSI 2012) and filter-sterilized prior to addition to MRS medium. Serial dilutions of antibiotics, ranging from 0.25 μg/mL to 256 μg/mL, were prepared, and vancomycin concentrations of 514 μg/mL and 1028 μg/mL were also tested. MRS broth containing antibiotics at different concentrations was used to prepared each well of a micro-well plate. The methods used were according to CLSI document M100-S22 (CLSI 2012). The inoculum was adjusted to a turbidity equivalent of 0.5 McFarland standard (ca. 10^5 CFU/mL) and was derived from a broth culture that was incubated at 37°C for 18 h. Fresh culture (1 mL) was used to inoculate each well, and incubated at 37°C. Minimal inhibitory concentration (MIC) was determined on MRS broth, since it supported the best growth of all bacteria used in this study. The MIC was defined as the lowest concentration of antibiotic giving a complete inhibition of visible growth in comparison to an antibiotic-free control well. At the same time, the optical density (OD) of cell cultures was evaluated using Microplate Manager Software (Version SkanIt RE for MSS 2.4.2, Thermo) at 600 nm; OD ≤ 0.020 was considered an indicator of transparency.

Evaluation of virulence factors of E. faecium

Strains identified as *E. faecium* and with a MIC value for ampicillin ≤2 mg/L, should be tested for the genetic elements *IS 16*, hyl_{EFM} and *esp*, which are virulence factors and markers that are now considered the most relevant for the assessment of safety (EFSA 2012b). DNA was extracted from bacterial cultures as described previously. The primer sequences are listed in Table 1. The PCR mixture consisted of 1.0 μL template, 2.5 μL diluted 10-fold *Taq* DNA Polymerase Buffer (containing Mg^{2+}, TaKaRa), 1 μL deoxynucleoside triphosphate (dNTP, 2.5 mmol/L, TaKaRa), 0.5 μL of each primer (10 μmol/L), 0.125 μL *Taq* DNA polymerase (5 U/μL, TaKaRa) and 25 μL DNase-RNase-free water (ComWin Biotech, Beijing, China). Each PCR cycling profile consisted of an initial denaturation step (5 min at 95°C), followed by amplification for 35 cycles as follows: denaturation for 1 min at 95°C, annealing for 1 min at 52°C, an extension for 42 s at 72°C. PCR was completed with an elongation phase (10 min at 72°C). PCR samples were analyzed using 0.8% (w/v) agarose gels (Biowest, Barcelona, Spain) in 1 × TBE buffer at 130 V for 50 min ,then visualized with ethidium bromide staining using GelDoc2000 (Bio-Rad, Schiltigheim, France). Banding patterns were analyzed using Quantity One Software (Bio-Rad).

Hemolytic activity

The methods used were according to Lee et al. (2011), with slight modification as follows. Fresh bacterial cultures were streaked in triplicate on a fresh blood agar plate, containing 5% (w/v) sheep blood, and incubated for 48 h at 37°C. Blood agar plates were examined for signs of β-hemolysis (clear zones around colony), α- hemolysis (green-hued zones around colony) and γ- hemolysis (no zones around colony).

Bile salts hydrolysis

The bile salts hydrolysis was assayed as reported by Argyri et al. (2013). Fresh bacterial cultures were streaked on MRS agar containing 0.5% (w/v) taurodeoxycholic acid in triplicate. The hydrolysis effect was indicated by taurodeoxycholic acid precipitated zones in the agar medium below and around a colony, after incubating at 37°C for 48 h.

Biogenic amine-producing ability

The production of biogenic amines (tyramine, histamine and putrescine) was assessed using decarboxylase medium (tryptone, 5 g/L; yeast extract, 5 g/L; beef extract, 5 g/L; sodium chloride, 2.5 g/L; glucose, 0.5 g/L; Tween-80, 1 g/L; $MnSO_4 \cdot H_2O$, 0.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $FeSO_4 \cdot 7H_2O$, 0.04 g/L; thiamine, 0.01 g/L; $K_2HPO_4 \cdot 3H_2O$, 2 g/L; $CaCO_3$, 0.1 g/L; bromcresol purple, 0.06 g/L; pyridoxal-5-phosphate, 0.05 g/L, pH 5.3–5.5) as described by Bover-Cid and Holzapfel (1999). Firstly, fresh bacterial cultures from MRS agar slants were inoculated into MRS broth, incubated at 37°C for 14 h, and then subcultured twice. Thereafter, the cultures were inoculated into MRS broth containing 1% amino acid

Table 1 Primers used for the PCR-based detection of genes responsible for *Enterococcus faecium* virulence

Name	Sequence	Target	Product (bp)	Reference
IS16-F	5′-CATGTTCCACGAACCAGAG-3′	*IS16*	547	Werner et al. (2011)
IS16-R	5′-TCAAAAAGTGGGCTTGGC-3′	*IS16*	547	Werner et al. (2011)
esp14F	5′-CGATAAAGAAGAGAGCGGAG-3′	*esp*	539	Rice et al. (2003)
esp12R	5′-GCAAACTCTACATCCACGTC-3′	*esp*	539	Rice et al. (2003)
hyl_{EFM} forward	5′-GAGTAGAGGAATATCTTAGC-3′	hyl_{EFM}	661	Rice et al. (2003)
hyl_{EFM} reverse	5′-AGGCTCCAATTCTGT-3′	hyl_{EFM}	661	Rice et al. (2003)

precursor (tyrosine, histidine and ornithine, respectively, purchased from Sigma, St. Louis, MO) at an inoculum size of 1% (v/v) and incubated at 37°C for 14 h, and then subcultured five times. Finally, the activated cultures were then used to inoculate to decarboxylation liquid medium at an inoculum size of 1% (v/v), and incubated at 37°C for 4 h, and the color change of the medium was then observed, using MRS broth as a control. Compared to blank MRS broth, medium exhibiting both yellow color and turbidity indicated that the strain was negative for biogenic amine-producing ability, while red or purple medium indicated that the strain was positive for biogenic amine-producing ability.

Multiplex PCR was used to detect genes responsible for biogenic amines production, as follows: DNA was extracted from bacterial cultures as described above. The method described by Coton et al. (2010) was used for simultaneous detection of four biogenic amines genes: histamine (histidine decarboxylase, *hdc*), tyramine (tyrosine decarboxylase, *tyrdc*) and putrescine (via either ornithine decarboxylase, *odc*, or agmatine deiminase, *agdi*) as well as a PCR internal control corresponding to the 16S rRNA coding gene.

Statistical analysis

Results were calculated with mean values, and standard deviations (mean ± SD) were determined from triplicate trials. Statistical significance of the results was evaluated by one-way ANOVA (analysis of variance) and the least significant different (LSD) mean comparison test ($P < 0.05$) using SPSS 17.0 software.

Results and discussion

The intestinal mucosa, the contents and the feces of livestock represent an alternative and readily source of lactic acid bacteria with promising functional properties. It can also be argued that a probiotic strain will function better in an environment similar to that for which it was originally isolated (Saarela et al. 2000). Strompfova and Laukova (2009) revealed 55 potential functional features of isolates from rectal swab samples and feces collected from 40 heads of healthy suckling and weaning piglets. Macias-Rodriguez et al. (2008) isolated 164 lactobacilli from the feces and the associated mucus from the small intestine and cecum of newborn piglets. In the present study, 52 lactic acid bacteria isolated from the intestinal mucosa of 60-day-old healthy piglets were screened for potential probiotic use. Thus, from security considerations, the source of the selected lactobacilli is safe.

It is noteworthy that evaluation of the functional and safety properties of strains in vitro, which including transit tolerance in simulated upper gastrointestinal tract, adhesion capacity, cell surface characteristics, antimicrobial activities, hemolytic, bile salt hydrolase activities, biogenic amine-producing ability, and so on, is considered important for selection of novel probiotic candidates (Fang et al. 2015; Manhar et al. 2016; Tulumoglu et al. 2013).

Resistance to low pH

The 52 lactic acid bacteria isolated here from the intestinal mucosa of 60-day-old healthy piglets were screened for low pH resistance characteristics. The results are reported in Table 2. Among the tested 52 strains, 8 could not survive at pH 1.0 conditions, among which strains WEI-4, WEI-7, WEI-19, WEI-20 and WEI-23 were the most acid-sensitive strains, with the lowest survival rates of 47.79%, 51.28%, 49.34%, 45.04% and 52.97%, respectively, at pH 3.0. Compared to all other strains, WEI-2, WEI-9, WEI-10, WEI-17, WEI-22, WEI-30, WEI-33, WEI-38, WEI-41, WEI-48 and WEI-51 demonstrated better resistance to pH 3.0, with survival rates of 86.63%, 92.61%, 94.92%, 89.49%, 91.94%, 87.29%, 90.69%, 87.10%, 91.05%, 90.75% and 87.23%, respectively.

Potential probiotic strains must tolerate acidic environments and bile secretions in order to successfully pass through the stomach and small intestine. The pH of the gastric juice is around 2.0–3.0, which causes most ingested microorganisms to die (Singh et al. 2012). In this study, when confronted with various pH conditions, most of the strains, except for WEI-2, WEI-9, WEI-10, WEI-17, WEI-22, WEI-30, WEI-33, WEI-38, WEI-41, WEI-48 and WEI-51, showed a decreased survival rate after exposure to pH 3.0 for 11.5 h. Our results are in agreement with those obtained from previous studies,

Table 2 Effect of pH on the viability of tested lactic acid bacteria incubated at various pH ranges (1, 2, 3 and 6.2), expressed as log CFU/mL and survival rate

Strain	Control[a]	pH 1.0		pH 2.0		pH 3.0	
	VC[b]	VC	SR[c](%)	VC	SR(%)	VC	SR(%)
WEI-1	7.89 ± 0.08	1.23 ± 0.46[d]	15.59 ± 4.8	2.46 ± 0.15[d]	31.18 ± 4.27	4.92 ± 0.11[d]	62.36 ± 4.85
WEI-2	8.19 ± 0.15	1.95 ± 0.24[d]	23.81 ± 3.7	3.87 ± 0.26[d]	47.25 ± 3.82	7.26 ± 0.37	86.63 ± 5.92
WEI-3	8.31 ± 0.47	1.46 ± 0.31[d]	17.57 ± 2.6	3.07 ± 0.58[d]	36.94 ± 4.69	6.09 ± 0.34[d]	73.29 ± 8.14
WEI-4	6.55 ± 0.11	-	-	1.03 ± 0.37[d]	15.73 ± 2.73	3.13 ± 0.23[d]	47.79 ± 8.37
WEI-5	7.39 ± 0.23	-	-	1.97 ± 0.58[d]	26.66 ± 4.65	5.01 ± 0.49[d]	67.80 ± 7.41
WEI-6	7.01 ± 0.69	1.02 ± 0.29[d]	14.55 ± 3.9	2.14 ± 0.24[d]	30.53 ± 5.21	4.53 ± 0.16[d]	64.62 ± 4.25
WEI-7	8.23 ± 0.37	1.14 ± 0.42[d]	13.85 ± 2.7	2.23 ± 0.49[d]	27.10 ± 1.92	4.22 ± 0.24[d]	51.28 ± 3.29
WEI-8	7.11 ± 0.29	-	-	1.65 ± 0.28[d]	23.21 ± 4.97	3.89 ± 0.13[d]	54.71 ± 3.81
WEI-9	8.39 ± 0.27	2.41 ± 0.14[d]	28.72 ± 3.4	4.64 ± 0.09[d]	55.30 ± 3.21	7.77 ± 0.32	92.61 ± 5.79
WEI-10	9.26 ± 0.16	2.75 ± 0.09[d]	29.70 ± 4.1	5.46 ± 0.35[d]	58.96 ± 2.93	8.79 ± 0.46	94.92 ± 4.87
WEI-11	8.39 ± 0.39	1.97 ± 0.14[d]	23.48 ± 5.6	3.38 ± 0.18[d]	40.29 ± 3.48	6.37 ± 0.44[d]	75.92 ± 5.74
WEI-12	6.89 ± 0.26	1.01 ± 0.16[d]	14.66 ± 2.4	2.06 ± 0.23[d]	29.90 ± 4.59	4.51 ± 0.12[d]	65.46 ± 6.28
WEI-13	7.56 ± 0.43	1.38 ± 0.25[d]	18.25 ± 2.1	2.98 ± 0.33[d]	39.42 ± 5.17	6.02 ± 0.43[d]	79.63 ± 4.85
WEI-14	7.04 ± 0.29	1.27 ± 0.34[d]	18.04 ± 3.8	2.71 ± 0.14[d]	38.49 ± 2.23	5.23 ± 0.17[d]	74.29 ± 5.13
WEI-15	6.99 ± 0.31	1.12 ± 0.29[d]	16.02 ± 4.2	2.11 ± 0.23[d]	30.19 ± 6.36	4.86 ± 0.13[d]	69.53 ± 6.67
WEI-16	6.72 ± 0.25	-	-	1.67 ± 0.31[d]	24.85 ± 3.14	4.01 ± 0.25[d]	59.67 ± 3.59
WEI-17	8.37 ± 0.24	2.24 ± 0.11[d]	26.76 ± 2.5	4.17 ± 0.22[d]	49.82 ± 5.33	7.49 ± 0.27	89.49 ± 6.28
WEI-18	8.08 ± 0.57	1.41 ± 0.24[d]	17.45 ± 3.2	2.67 ± 0.37[d]	33.04 ± 6.59	5.33 ± 0.27[d]	65.97 ± 7.93
WEI-19	6.79 ± 0.24	-	-	1.25 ± 0.12[d]	18.40 ± 5.38	3.35 ± 0.39[d]	49.34 ± 5.64
WEI-20	6.86 ± 0.32	-	-	1.36 ± 0.18[d]	19.83 ± 4.72	3.09 ± 0.14[d]	45.04 ± 4.83
WEI-21	7.27 ± 0.41	1.01 ± 0.32[d]	13.89 ± 4.4	1.98 ± 0.11[d]	27.24 ± 3.21	4.07 ± 0.13[d]	55.98 ± 4.74
WEI-22	9.31 ± 0.34	2.62 ± 0.22[d]	28.14 ± 3.6	4.96 ± 0.23[d]	53.28 ± 4.11	8.56 ± 0.32	91.94 ± 6.58
WEI-23	6.06 ± 0.69	1.12 ± 0.36[d]	18.48 ± 2.36	1.95 ± 0.36[d]	32.18 ± 4.36	3.21 ± 0.26[d]	52.97 ± 4.99
WEI-24	7.45 ± 0.33	1.53 ± 0.39[d]	20.54 ± 4.69	2.87 ± 0.26[d]	38.52 ± 2.78	5.49 ± 0.36[d]	73.69 ± 5.87
WEI-25	7.32 ± 0.45	1.27 ± 0.45[d]	17.35 ± 2.57	2.54 ± 0.17[d]	34.70 ± 3.45	4.99 ± 0.12[d]	68.17 ± 8.66
WEI-26	7.91 ± 0.39	1.56 ± 0.08[d]	19.72 ± 2.36	2.95 ± 0.08[d]	37.29 ± 3.91	5.68 ± 0.39[d]	71.81 ± 5.94
WEI-27	8.03 ± 0.52	1.29 ± 0.13[d]	16.06 ± 3.15	2.76 ± 0.41[d]	34.37 ± 2.47	5.64 ± 0.29[d]	70.24 ± 6.79
WEI-28	8.34 ± 0.25	1.64 ± 0.24[d]	19.66 ± 2.63	3.28 ± 0.13[d]	39.33 ± 4.52	6.11 ± 0.41[d]	73.26 ± 5.85
WEI-29	7.04 ± 0.16	1.51 ± 0.16[d]	21.45 ± 4.69	2.87 ± 0.25[d]	40.77 ± 3.68	5.27 ± 0.17[d]	74.86 ± 6.24
WEI-30	7.71 ± 0.21	1.97 ± 0.13[d]	25.55 ± 3.15	3.95 ± 0.36[d]	51.23 ± 2.99	6.73 ± 0.23	87.29 ± 5.68
WEI-31	7.56 ± 0.23	1.63 ± 0.36[d]	21.56 ± 3.67	3.26 ± 0.27[d]	43.12 ± 3.84	6.19 ± 0.26[d]	81.88 ± 6.36
WEI-32	6.67 ± 0.44	1.10 ± 0.17[d]	16.49 ± 1.38	2.03 ± 0.32[d]	30.43 ± 2.95	4.13 ± 0.18[d]	61.92 ± 8.59
WEI-33	8.81 ± 0.26	2.27 ± 0.06[d]	25.77 ± 2.49	4.14 ± 0.14[d]	46.99 ± 3.45	7.99 ± 0.45	90.69 ± 6.16
WEI-34	7.92 ± 0.19	1.52 ± 0.15[d]	19.19 ± 4.26	2.99 ± 0.11[d]	37.75 ± 2.85	5.99 ± 0.44[d]	75.63 ± 5.49
WEI-35	7.65 ± 0.43	1.67 ± 0.11[d]	21.83 ± 3.65	3.12 ± 0.25[d]	40.78 ± 5.76	6.04 ± 0.36[d]	78.95 ± 7.38
WEI-36	7.45 ± 0.24	1.31 ± 0.23[d]	17.58 ± 2.72	2.54 ± 0.29[d]	34.09 ± 4.63	5.14 ± 0.15[d]	68.99 ± 4.47
WEI-37	6.63 ± 0.04	-	-	1.32 ± 0.16[d]	19.91 ± 3.21	3.95 ± 0.23[d]	59.58 ± 5.86
WEI-38	8.06 ± 0.22	2.15 ± 0.34[d]	26.67 ± 2.51	3.96 ± 0.27[d]	49.13 ± 2.74	7.02 ± 0.39	87.10 ± 6.27
WEI-39	8.95 ± 0.27	1.73 ± 0.36[d]	19.33 ± 4.16	3.31 ± 0.19[d]	36.98 ± 4.55	6.97 ± 0.27[d]	77.88 ± 6.84
WEI-40	8.02 ± 0.56	1.35 ± 0.48[d]	16.83 ± 3.03	2.37 ± 0.14[d]	29.55 ± 1.96	5.21 ± 0.23[d]	64.96 ± 5.26
WEI-41	9.05 ± 0.22	2.32 ± 0.26[d]	25.64 ± 2.52	4.17 ± 0.39[d]	46.08 ± 2.47	8.24 ± 0.37	91.05 ± 7.95
WEI-42	7.31 ± 0.21	1.53 ± 0.37[d]	20.93 ± 1.74	2.54 ± 0.32[d]	34.75 ± 4.66	5.79 ± 0.07[d]	79.21 ± 6.74
WEI-43	6.85 ± 0.28	1.06 ± 0.21[d]	15.47 ± 2.89	2.06 ± 0.29[d]	30.07 ± 5.02	4.23 ± 0.34[d]	61.75 ± 4.89
WEI-44	6.69 ± 0.57	-	-	1.73 ± 0.06[d]	25.86 ± 3.14	4.08 ± 0.27[d]	60.99 ± 3.72
WEI-45	7.07 ± 0.39	1.25 ± 0.05[d]	17.68 ± 3.76	2.34 ± 0.27[d]	33.10 ± 4.42	4.58 ± 0.19[d]	64.78 ± 4.16
WEI-46	8.58 ± 0.14	1.87 ± 0.12[d]	21.80 ± 2.47	3.79 ± 0.15[d]	44.17 ± 2.36	6.98 ± 0.27[d]	81.35 ± 9.56

Table 2 (continued)

Strain	Control[a]	pH 1.0		pH 2.0		pH 3.0	
	VC[b]	VC	SR[c](%)	VC	SR(%)	VC	SR(%)
WEI-47	8.01 ± 0.19	1.31 ± 0.23[d]	16.35 ± 3.42	2.68 ± 0.24[d]	33.46 ± 4.09	5.49 ± 0.46[d]	68.54 ± 6.84
WEI-48	8.76 ± 0.36	2.42 ± 0.34[d]	27.63 ± 3.32	4.14 ± 0.37[d]	47.26 ± 3.35	7.95 ± 0.44	90.75 ± 7.59
WEI-49	7.74 ± 0.53	1.25 ± 0.16[d]	16.15 ± 4.03	2.39 ± 0.43[d]	30.88 ± 2.57	5.88 ± 0.32[d]	75.97 ± 6.37
WEI-50	7.23 ± 0.26	1.06 ± 0.25[d]	14.66 ± 3.75	2.13 ± 0.15[d]	29.46 ± 3.03	5.06 ± 0.28[d]	69.97 ± 3.76
WEI-51	8.69 ± 0.29	2.28 ± 0.29[d]	26.24 ± 3.63	4.09 ± 0.28[d]	47.07 ± 2.83	7.58 ± 0.25	87.23 ± 6.49
WEI-52	7.67 ± 0.27	1.45 ± 0.19[d]	18.90 ± 2.84	2.79 ± 0.16[d]	36.38 ± 3.69	5.76 ± 0.38[d]	75.10 ± 5.17

[a] Control, cells were grown in pH 6.2 in MRS broth

[b] VC (viable counts in CFU/mL)

[c] SR (survival ratio in %), cell numbers in MRS (pH 1.0-3.0)/cell numbers in control (pH 6.2) × 100

[d] Viable counts after incubating to pH 1.0, 2.0 or 3.0 MRS broth are significantly different from those of the control ($P < 0.05$)

where lactobacilli of animal, human, or fermented food origin, were able to retain their viability when exposed to pH ranged from 1.0 to 3.0 (Tulumoglu et al. 2013; Anandharaj and Sivasankari 2014).

Resistance to bile salts

The 52 strains were further tested for their tolerance to various bile salt concentrations. Table 3 shows viable cell counts (log CFU/mL) and survival percentages of probiotic strains in MRS broth supplemented with 0.1%, 0.3% and 0.5% bile salts. We found that resistance to bile salts varied significantly among the probiotic strains. Among all strains tested, WEI-2 (85.32%), WEI-9 (86.76%), WEI-10 (88.09%), WEI-17 (82.50%), WEI-22 (85.57%), WEI-30(84.34%), WEI-33 (80.98%), WEI-38 (81.26%), WEI-41 (84.14%), WEI-48 (83.59%) and WEI-51 (85.36%) demonstrated higher resistance to 0.3% bile salts after 9 h, but their survival rate decreased dramatically to between 40.29 and 45.42% when exposed to 0.5% bile salts. WEI-3, WEI-25, WEI-32, WEI-34 and WEI-44 were most sensitive to 0.3% bile salts and almost lost their viability in 0.5% bile salts after 9 h. WEI-2, WEI-9, WEI-10, WEI-17, WEI-22, WEI-30, WEI-33, WEI-38, WEI-41, WEI-48 and WEI-51 exhibited greater resistance to bile salts and also survived well under low pH conditions.

Bile tolerance of probiotics has been revealed to be dependent on bile type and on the strain (Liong and Shah 2005); the mean bile concentration is believed to be 0.3% (w/v) (Prasad et al. 1998). In present study, most of the selected strains were found to be resistance to 0.1% (w/v) bile salts, while the viable counts of bacteria reduced with increased concentrations of bile salts. These 11 strains also survived well under low pH conditions, thus we selected these 11 strains to further probiotic evaluation assay, to acquire the most potential probiotics. Many previous studies also reported that a lot of lactobacilli were resistance to 0.3–0.5% bile salts and confirmed that the resistance to low pH and bile salts is a strain-specific trait (Messaoudi et al. 2012; Toscano et al. 2015; Shobharani and Halami 2016), which is in accordance to our study.

Molecular identification of lactic acid bacteria

The 11 strains, all isolated from the intestinal mucosa of 60-day-old healthy piglets, were selected on the basis of the properties described above, and were identified through 16S rRNA gene sequencing. Results demonstrated that the strain WEI-2 (GenBank accession number: KY041755) belonged to *Pediococcus acidilactici* species, the strains WEI-9 (GenBank accession number: KY041756), WEI-10 (GenBank accession number: KY041757) and WEI-48 (GenBank accession number: KY041764) belonged to *E. faecium* species, the strains WEI-17 (GenBank accession number: KY041758), WEI-30 (GenBank accession number: KY041760) and WEI-41 (GenBank accession number: KY041763) belonged to *Lactobacillus rhamnosus* species, the strains WEI-22 (GenBank accession number: KY041759) and WEI-38 (GenBank accession number: KY041762) belonged to *Lactobacillus brevis* species, while the strains WEI-33 (GenBank accession number: KY041761) and WEI-51 (GenBank accession number: KY041765) belonged to *Lactobacillus plantarum*.

Resistance to simulated gastric juice

The same 11 lactic acid bacteria, which exhibited the highest acid and bile resistance, were selected for in vitro resistance to simulated gastric juice (Table 4). The strains WEI-9 and WEI-10 showed the highest resistance to simulated gastric juice, with survival rates above 97%. Followed by WEI-9 and WEI-10, WEI-51 also demonstrated high tolerance to simulated gastric juice after 3 h, and the survival rate reached >90%. Simulated small intestinal juice resistance varied

Table 3 Effect of bile salt concentration (0.1%, 0.3% and 0.5%) on the viability of tested lactic acid bacteria, values expressed as log CFU/mL and survival rate

Strain	Control[a]	0.1% Bile salts		0.3% Bile salts		0.5% Bile salts	
	VC[b]	VC	SR[c](%)	VC	SR(%)	VC	SR(%)
WEI-1	7.32 ± 0.11	5.37 ± 0.36[d]	73.36 ± 3.98	4.29 ± 0.27[d]	58.61 ± 3.76	1.18 ± 0.26[d]	16.12 ± 2.86
WEI-2	8.24 ± 0.28	7.64 ± 0.25	92.72 ± 2.75	7.03 ± 0.38[d]	85.32 ± 4.39	3.32 ± 0.18[d]	40.29 ± 3.79
WEI-3	7.94 ± 0.39	5.74 ± 0.09[d]	72.29 ± 4.27	2.36 ± 0.16[d]	29.72 ± 3.55	-	-
WEI-4	6.02 ± 0.18	4.18 ± 0.24[d]	69.44 ± 1.56	2.51 ± 0.24[d]	41.69 ± 4.91	0.78 ± 0.21[d]	12.96 ± 3.56
WEI-5	7.47 ± 0.34	4.89 ± 0.17[d]	65.46 ± 3.27	3.67 ± 0.05[d]	49.13 ± 5.32	1.26 ± 0.05[d]	16.87 ± 1.89
WEI-6	7.27 ± 0.57	5.85 ± 0.05[d]	80.47 ± 3.94	4.41 ± 0.41[d]	60.66 ± 2.18	1.85 ± 0.36[d]	25.45 ± 3.36
WEI-7	8.01 ± 0.42	6.12 ± 0.31[d]	76.40 ± 2.47	4.65 ± 0.33[d]	58.05 ± 2.67	1.47 ± 0.17[d]	18.35 ± 3.21
WEI-8	7.58 ± 0.23	5.69 ± 0.25[d]	75.07 ± 5.05	3.92 ± 0.26[d]	51.72 ± 3.21	0.78 ± 0.25[d]	10.29 ± 1.92
WEI-9	8.46 ± 0.25	7.89 ± 0.34	93.26 ± 3.73	7.34 ± 0.18[d]	86.76 ± 2.94	3.66 ± 0.14[d]	43.26 ± 2.23
WEI-10	9.07 ± 0.29	8.56 ± 0.45	94.38 ± 2.61	7.99 ± 0.39[d]	88.09 ± 3.67	4.12 ± 0.22[d]	45.42 ± 4.12
WEI-11	8.16 ± 0.42	6.78 ± 0.26[d]	83.09 ± 3.82	5.85 ± 0.41[d]	71.69 ± 2.75	2.72 ± 0.05[d]	33.33 ± 5.15
WEI-12	7.02 ± 0.32	5.37 ± 0.21[d]	76.50 ± 4.92	3.56 ± 0.28[d]	50.71 ± 3.49	1.54 ± 0.14[d]	21.94 ± 2.16
WEI-13	7.13 ± 0.28	5.82 ± 0.14[d]	81.63 ± 5.14	3.07 ± 0.18[d]	43.06 ± 1.48	1.23 ± 0.09[d]	17.25 ± 3.28
WEI-14	6.76 ± 0.27	4.29 ± 0.05[d]	63.46 ± 1.26	2.54 ± 0.05[d]	37.57 ± 1.96	0.52 ± 0.11[d]	7.69 ± 1.54
WEI-15	7.08 ± 0.32	5.21 ± 0.13[d]	73.59 ± 2.93	2.86 ± 0.15[d]	40.40 ± 3.25	1.06 ± 0.26[d]	14.97 ± 3.72
WEI-16	6.89 ± 0.28	4.98 ± 0.27[d]	72.28 ± 2.69	2.63 ± 0.21[d]	38.17 ± 1.69	0.99 ± 0.12[d]	14.37 ± 4.29
WEI-17	8.57 ± 0.29	7.74 ± 0.26	90.32 ± 3.01	7.07 ± 0.32[d]	82.50 ± 2.74	3.56 ± 0.27[d]	41.54 ± 4.83
WEI-18	8.01 ± 0.59	7.03 ± 0.39[d]	87.77 ± 4.83	5.35 ± 0.38[d]	66.79 ± 3.29	2.98 ± 0.39[d]	37.20 ± 1.62
WEI-19	6.73 ± 0.37	5.45 ± 0.26[d]	80.98 ± 4.01	3.17 ± 0.23[d]	47.10 ± 4.92	1.38 ± 0.42[d]	20.51 ± 2.78
WEI-20	6.59 ± 0.51	5.07 ± 0.24[d]	76.93 ± 5.68	2.39 ± 0.15[d]	36.27 ± 5.03	0.62 ± 0.17[d]	9.41 ± 1.01
WEI-21	7.19 ± 0.48	6.16 ± 0.38[d]	85.67 ± 4.02	3.24 ± 0.26[d]	45.06 ± 3.88	1.19 ± 0.37[d]	16.55 ± 1.79
WEI-22	9.22 ± 0.31	8.54 ± 0.33	92.62 ± 3.99	7.89 ± 0.44[d]	85.57 ± 2.95	3.97 ± 0.41[d]	43.06 ± 3.69
WEI-23	6.31 ± 0.54	5.52 ± 0.21[d]	87.48 ± 2.73	2.98 ± 0.36[d]	47.23 ± 3.71	1.32 ± 0.16[d]	20.92 ± 3.32
WEI-24	7.23 ± 0.32	5.89 ± 0.17[d]	81.47 ± 2.49	3.49 ± 0.39[d]	48.27 ± 2.24	1.92 ± 0.08[d]	26.56 ± 4.01
WEI-25	7.56 ± 0.43	5.07 ± 0.08[d]	67.06 ± 4.85	2.01 ± 0.16[d]	26.59 ± 3.17	-	-
WEI-26	7.29 ± 0.26	5.89 ± 0.31[d]	80.80 ± 5.96	2.62 ± 0.27[d]	35.94 ± 2.73	0.68 ± 0.08[d]	9.38 ± 1.72
WEI-27	7.78 ± 0.49	6.36 ± 0.28[d]	81.75 ± 3.27	2.34 ± 0.35[d]	30.08 ± 2.92	0.93 ± 0.15[d]	11.95 ± 2.39
WEI-28	8.28 ± 0.36	7.12 ± 0.44[d]	85.99 ± 4.23	3.57 ± 0.41[d]	43.12 ± 5.75	1.88 ± 0.26[d]	22.71 ± 4.03
WEI-29	7.29 ± 0.22	5.75 ± 0.15[d]	78.88 ± 5.16	2.19 ± 0.44[d]	30.04 ± 3.47	0.31 ± 0.06[d]	4.25 ± 4.89
WEI-30	7.92 ± 0.47	7.51 ± 0.32	94.82 ± 3.46	6.68 ± 0.35[d]	84.34 ± 2.36	3.23 ± 0.27[d]	40.78 ± 4.58
WEI-31	7.36 ± 0.14	6.03 ± 0.09[d]	81.93 ± 2.81	3.13 ± 0.27[d]	42.53 ± 2.01	1.54 ± 0.41[d]	20.92 ± 2.93
WEI-32	6.71 ± 0.49	3.87 ± 0.14[d]	57.68 ± 1.38	1.25 ± 0.09[d]	18.63 ± 3.29	-	-
WEI-33	8.94 ± 0.34	8.08 ± 0.35	90.38 ± 2.92	7.24 ± 0.28[d]	80.98 ± 4.27	3.61 ± 0.31[d]	40.38 ± 2.19
WEI-34	7.58 ± 0.26	5.83 ± 0.25[d]	76.91 ± 3.03	1.94 ± 0.17[d]	25.59 ± 1.48	-	-
WEI-35	7.33 ± 0.41	6.36 ± 0.17[d]	86.77 ± 4.54	3.57 ± 0.15[d]	48.70 ± 3.61	1.56 ± 0.23[d]	21.28 ± 2.54
WEI-36	7.39 ± 0.33	6.24 ± 0.19[d]	84.44 ± 5.25	2.99 ± 0.32[d]	40.46 ± 4.01	1.27 ± 0.42[d]	17.19 ± 2.73
WEI-37	6.51 ± 0.09	4.27 ± 0.21[d]	65.59 ± 1.78	2.16 ± 0.25[d]	33.18 ± 1.16	1.03 ± 0.37[d]	15.82 ± 1.67
WEI-38	8.27 ± 0.26	7.54 ± 0.29	91.17 ± 2.69	6.72 ± 0.16[d]	81.26 ± 2.92	3.45 ± 0.32[d]	41.72 ± 3.41
WEI-39	8.81 ± 0.36	7.41 ± 0.31[d]	84.11 ± 3.12	4.59 ± 0.24[d]	52.10 ± 1.99	2.09 ± 0.48[d]	23.72 ± 2.88
WEI-40	8.47 ± 0.57	6.36 ± 0.47[d]	75.09 ± 3.29	3.97 ± 0.28[d]	46.87 ± 3.28	1.59 ± 0.16[d]	18.77 ± 1.67
WEI-41	8.89 ± 0.31	8.32 ± 0.23	93.59 ± 4.04	7.48 ± 0.17[d]	84.14 ± 4.29	3.83 ± 0.29[d]	43.08 ± 3.46
WEI-42	7.42 ± 0.46	6.45 ± 0.16[d]	86.93 ± 5.27	3.59 ± 0.06[d]	48.38 ± 2.57	1.35 ± 0.17 [d]	18.19 ± 1.61
WEI-43	6.71 ± 0.39	5.68 ± 0.14[d]	84.65 ± 3.89	2.74 ± 0.24[d]	40.83 ± 3.39	0.64 ± 0.26[d]	9.54 ± 1.22
WEI-44	6.48 ± 0.59	3.75 ± 0.06[d]	57.87 ± 2.18	1.75 ± 0.11[d]	27.01 ± 4.25	-	-
WEI-45	7.37 ± 0.41	6.19 ± 0.18[d]	83.99 ± 2.22	3.63 ± 0.13[d]	49.25 ± 1.74	1.44 ± 0.31[d]	19.54 ± 3.92
WEI-46	8.29 ± 0.27	7.31 ± 0.24[d]	88.18 ± 3.64	5.73 ± 0.27[d]	69.12 ± 5.82	2.63 ± 0.07[d]	31.72 ± 5.28

Table 3 (continued)

Strain	Control[a]	0.1% Bile salts		0.3% Bile salts		0.5% Bile salts	
	VC[b]	VC	SR[c](%)	VC	SR(%)	VC	SR(%)
WEI-47	8.17 ± 0.16	6.96 ± 0.13[d]	85.19 ± 4.23	4.59 ± 0.31[d]	56.18 ± 1.38	2.19 ± 0.15[d]	26.81 ± 3.73
WEI-48	8.59 ± 0.29	8.01 ± 0.35	93.24 ± 3.77	7.18 ± 0.38[d]	83.59 ± 3.85	3.52 ± 0.29[d]	40.98 ± 2.54
WEI-49	7.68 ± 0.58	5.39 ± 0.35[d]	70.18 ± 4.63	2.88 ± 0.41[d]	37.50 ± 2.96	0.47 ± 0.16[d]	6.12 ± 1.73
WEI-50	7.19 ± 0.37	6.08 ± 0.29[d]	84.56 ± 5.02	3.76 ± 0.16[d]	52.29 ± 3.73	1.84 ± 0.19[d]	25.59 ± 3.66
WEI-51	8.47 ± 0.36	7.85 ± 0.24	92.68 ± 4.91	7.23 ± 0.13[d]	85.36 ± 2.69	3.64 ± 0.27[d]	42.98 ± 4.57
WEI-52	7.25 ± 0.38	5.99 ± 0.15[d]	82.62 ± 3.25	2.64 ± 0.19[d]	36.41 ± 3.25	1.02 ± 0.21[d]	14.07 ± 2.18

[a] Control, cells were grown in pH 6.2 in MRS broth

[b] VC (viable counts in CFU/mL)

[c] SR (survival rate in %), cell numbers in MRS (0.1%, 0.3% or 0.5% bile salts)/cell numbers in control (without bile salt) × 100

[d] Represents the viable counts after incubating to MRS broth containing 0.1%, 0.3% or 0.5% bile salts are significantly different from those of the control ($P < 0.05$)

significantly among the strains, among which the viability of the strains WEI-9, WEI-10, WEI-48 and WEI-51 was not affected by exposure to small intestinal juice. A good survival rate was found for WEI-9, WEI-10 and WEI-51 strains.

Results of the tolerance to the simulated upper gastrointestinal tract of the 11 selected strains demonstrate that the low value of pH in the gastric compartment has more influence on strain survival than the presence of bile and pancreatic juice. These results are in agreement with previous studies (Solieri et al. 2014; Pitino et al. 2010). Strains WEI-9, WEI-10, WEI-33, WEI-48 and WEI-51 exhibited high in vitro tolerance to simulated gastric juice and small intestinal juice, among which WEI-9, WEI-10 and WEI-33 belong to *E. faecium*, and WEI-48 and WEI-51 belong to *L. plantarum*. Meanwhile, *E. faecium* WEI-9, WEI-10 and WEI-33 showed higher tolerance to simulated gastric juice and small intestinal juice than *L. plantarum* WEI-48 and WEI-51, which indicated that *E. faecium* exhibited higher resistance to simulated gastric juice and small intestinal juice.

Adhesion capacity to IPEC-J2 cells and cell surface characteristics

In the present study, the adhesion to IPEC-J2 cells was determined for 11 tested lactic acid bacteria, and the results are illustrated in Fig. 1. Adhesion capacity to IPEC-J2 cells varied significantly among the 11 lactic acid bacteria, of which the adhesion ratio was from 40% to 95%. The highest adherence capacity (more than 90%) was demonstrated from strains WEI-9 and WEI-10, and had significant difference ($P < 0.05$) compared to that of other strains.

Table 4 Transit tolerance in simulated upper gastrointestinal tract expressed as viable of lactic acid bacteria. Results are expressed as mean ± SD; $n = 3$

Strains	Viable counts (log CFU/mL) and survival rate after simulated gastric transit		Viable counts (log CFU/mL) and survival rate after simulated small intestinal transit again
	0 h	3 h	6 h
WEI-2	8.52 ± 0.20[a]	7.35 ± 0.35[b] (86.27%[d])	6.10 ± 0.11[c] (71.60%)
WEI-9	8.69 ± 0.15 [a]	8.44 ± 0.06 [ab] (97.12%)	8.22 ± 0.26 [b] (94.59%)
WEI-10	8.76 ± 0.18[a]	8.51 ± 0.20[a] (97.15%)	8.41 ± 0.17 [a] (96.00%)
WEI-17	8.07 ± 0.13[a]	5.41 ± 0.28[b] (67.04%)	3.90 ± 0.16[c] (48.33%)
WEI-22	8.37 ± 0.08[a]	6.16 ± 0.14[b] (73.60%)	4.39 ± 0.17[c] (52.45%)
WEI-30	7.97 ± 0.20[a]	4.62 ± 0.35[b] (57.97%)	2.58 ± 0.20[c] (32.37%)
WEI-33	8.73 ± 0.10[a]	7.49 ± 0.17[b] (85.80%)	6.94 ± 0.20[c] (79.50%)
WEI-38	8.14 ± 0.12[a]	3.80 ± 0.18[b] (46.68%)	1.44 ± 0.21[c] (17.69%)
WEI-41	8.21 ± 0.18[a]	2.73 ± 0.18[b] (33.25%)	1.13 ± 0.32[c] (13.76%)
WEI-48	8.48 ± 0.20[a]	6.37 ± 0.18[b] (75.12%)	6.22 ± 0.15[b] (73.35%)
WEI-51	8.24 ± 0.12[a]	7.52 ± 0.11[b] (91.26)	7.57 ± 0.11[b] (91.87%)

[a-c] Significance results of one-way ANOVA analysis of viable counts under different treatment ($P < 0.05$)

[d] Survival rate

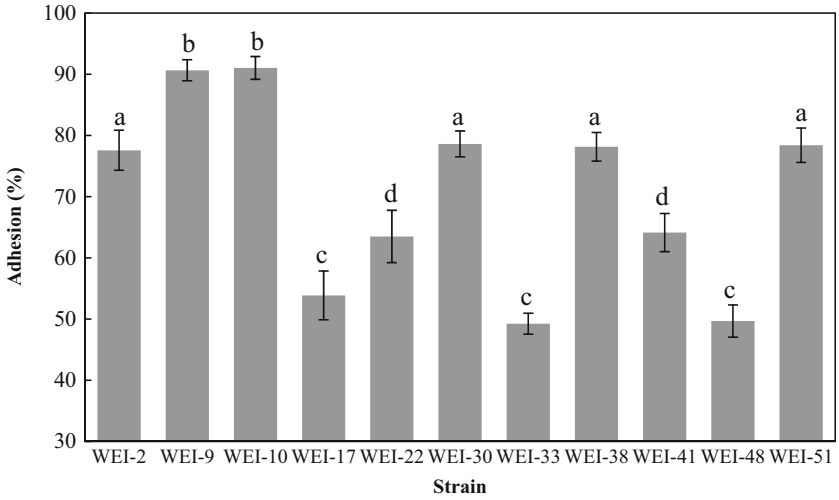

Fig. 1 Adhesion ability to the IPEC-J2 of the selected lactic acid bacteria. Adhesion capacity was calculated as the percentage of adhered lactic acid bacteria in relation to the total number of strains added. *Error bars* Standard deviation from three replications. Values within each column that do not sharing a common letter are significantly different ($P < 0.05$) according to the least significant different (LSD) mean comparison test

The 11 lactic acid bacteria were further evaluated for cell surface hydrophobicity associated to adhesion capacity, and the results are shown in Fig. 2. The cell hydrophobicity of the 11 selected strains ranged from 8% to 92%. Two strains (WEI-9 and WEI-10) exhibited good hydrophobic cell surface, with a percentage higher than 85%, and had significant difference ($P < 0.05$) compared to other strains.

Results of the co-aggregation ability tests of the 11 selected strains with the pathogen *E. coli* CVCC1570 are illustrated in Fig. 2. The highest co-aggregation percentages (above 60%) were displayed by the strain WEI-9 and WEI-10, which exhibits significant difference ($P < 0.05$) compared to other strains.

In this study, the calculated value of auto-aggregation revealed a variable distribution, with percentage ranging from 5% to 70% (Fig. 2). Among the selected strains, WEI-9 and WEI-10 showed the highest auto-aggregation percentages, reaching >55% and with significant difference ($P < 0.05$) compared to other strains. From the results, we observed that WEI-9 and WEI-10 had better cell surface hydrophobicity, co-aggregation and auto-aggregation capacity compared to other strains.

The ability of the strains to adhere to the mucosal surface is another common criterion for probiotic strain selection, since it is related directly to their colonization and persistence in the gastrointestinal tract (Zuo et al. 2016). Our results for bacterial adherence capacity to IPEC-J2 cells revealed adhesion ability of the 11 selected strains, confirming that this property for lactic acid bacteria is strain dependent (Laparra and Sanz 2009). It is interesting to note that the adhesion ability of lactobacilli has been correlated to cell hydrophobicity, which represents a benefit for bacterial maintenance in the gastrointestinal tract (Kos et al. 2003). Many studies have confirmed that auto-aggregation is an important property of probiotic strains for prevention of pathogens. Probiotic strains with epithelial adherence and co-aggregation properties form a barrier

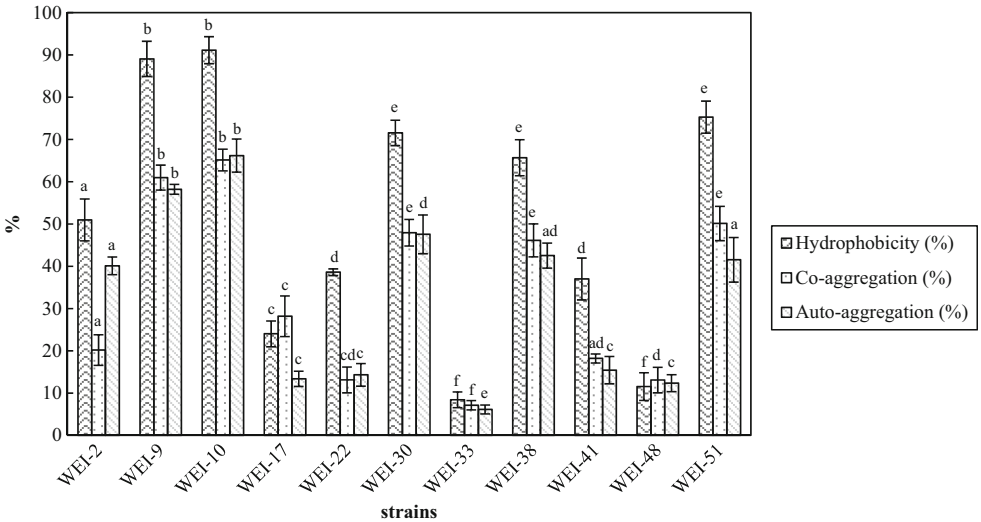

Fig. 2 Hydrophobicity, auto-aggregation and co-aggregation with *Escherichia coli* CVCC1570 of lactic acid bacteria. *Error bars* Standard deviation from three replications. Values within each column that do not sharing a common letter are significantly different ($P < 0.05$) according to the LSD mean comparison test

Table 5 Antimicrobial activity of lactic acid bacteria against common pathogens, hemolytic activity, bile salt hydrolase (BSH) activity and biogenic amine-producing (BAP) ability of the strains

Strains	Indicator strains[a]			Hemolytic activity[e]	BSH activity	BAP ability
	E. coli[b]	*S. pullorum*[c]	*S. aureus*[d]			
WEI-2	++	+	+	γ	-	-
WEI-9	+++	++	++	γ	-	-
WEI-10	++	++	++	γ	-	-
WEI-17	+++	++	++	γ	-	-
WEI-22	++	+	++	γ	-	-
WEI-30	++	+	+	γ	-	-
WEI-33	++	++	+	α	+	-
WEI-38	+++	+	++	γ	-	-
WEI-41	++	+	++	γ	-	-
WEI-48	++	++	++	γ	-	-
WEI-51	+++	++	++	γ	-	-

[a] +: 10 mm < zone < 15 mm; ++: 15 mm < zone < 20 mm; +++: zone > 20 mm

[b] *Escherichia coli* CVCC1570

[c] *Staphylococcus aureus* CVCC1882

[d] *Salmonella pullorum* AS1.1859

[e] α-hemolysis, γ- hemolysis

that inhibits colonization of intestinal pathogens (Anandharaj et al. 2015). This work revealed that strains with high adhesion capacity and hydrophobicity, such as WEI-9, WEI-10 and WEI-51, showed high auto-aggregation and co-aggregation abilities, which is in accordance with previous data (Saarela et al. 2000; Pithva et al. 2014). Nevertheless, conflicting evidence, including that adhesive and aggregative properties as well as hydrophobic characteristics are strain dependent, has been reported previously (Solieri et al. 2014).

Antimicrobial activity

The inhibitory abilities of the lactic acid bacteria against *E. coli* CVCC1570, *Staphylococcus aureus* CVCC1882 and *Salmonella pullorum* AS1.1859, considered in the present study, were shown in Table 5. In particular, all strains indicated forceful antimicrobial activity against *E. coli* CVCC1570 (with inhibition zone higher than 8.0 mm in diameter) and variable activity versus *S. aureus* CVCC1882 and *S. pullorum* AS1.1859 strains (Table 5). All strains inhibited not only the growth of Gram-positive bacteria like *S. aureus*, but also the growth of Gram-negative bacteria like *E. coli* and *S. pullorum*, whereas there was no correlation between the capacity to inhibit Gram-positive indicator strains and the capacity to inhibit Gram-negative indicator strains.

The ability to produce antimicrobial compounds may be one of the key characteristics for competitive exclusion of pathogen survival in the intestine and expression of a probiotic effect for the host (Collado et al. 2005). All 11 selected strains tested showed forceful activity against *E. coli*, in agreement with previous work (Tulumoglu et al. 2013; Strompfova and Laukova 2014). However, as previously reported (Pithva et al. 2014), our results also confirmed the strain-specific nature of the antimicrobial activity, in particular against *S. aureus* CVCC1882 and *S. pullorum* AS1.1859 strains.

Antibiotic resistance

One of the required properties for probiotic strains is their safety for host consumption without harboring acquired and transferable antibiotic resistance. Table 6 shows the MIC values of 11 selected strains to antibiotics. Strains were

Table 6 Minimal inhibitory concentrations (MIC) (in μg/mL) of strains to antibiotics. *A* Ampicillin, *V* vancomycin, *S* streptomycin, *K* kanamycin, *T* tetracycline, *C* chloramphenicol, *R* resistant

Strain	(MIC) (μg/mL)					
	A	V	S	K	T	C
WEI-2	≤2	≥1024	32	128 R	8	4
WEI-9	≤2	≤2	64	512	4	8
WEI-10	≤2	≤2	64	512	4	8
WEI-17	≤2	≥1024	32	128 R	≤0.25	2
WEI-22	≤1	≤2	≤8	32	≤0.25	2
WEI-30	≤2	≥1024	32	128 R	≤0.25	4
WEI-33	≤2	≤2	64	512	4	8
WEI-38	≤1	≤2	≤8	32	≤0.25	2
WEI-41	≤2	≥1024	32	128 R	≤0.25	2
WEI-48	≤2	≥1024	64	32	64 R	4
WEI-51	≤2	≥1024	64	32	16	8

considered resistance when they showed MIC values higher than the MIC breakpoints established by the European Food Safety Authority (EFSA 2012a). None of the strains exhibited resistance to ampicillin, streptomycin, or chloramphenicol. On the contrary, all tested strains except WEI-9, WEI-10 and WEI-33, were resistant to vancomycin (without a specific breakpoint from EFSA), which is not transferable to the other species because it is chromosomally encoded (Morrow et al. 2012). The resistance to vancomycin has been attributed to the presence of D-Ala-D-lactate in their peptidoglycan instead of the normal D-Ala-D-Ala, which is the target of the antibiotic (Monteagudo-Mera et al. 2011). Most of the strains revealed sensitivity to tetracycline, confirming the generally lower resistance of the lactobacilli species towards tetracycline (Maragkoudakis et al. 2006). Regarding the antibiotic kanamycin, four strains were resistant to kanamycin. Resistance to kanamycin has already been reported in several *Lactobacillus* species, and it could be attributable to the absence of cytochrome-mediated electro transport, which mediates drug uptake, and to membrane impermeability (Elkins and Mullis 2004). The low resistance to vancomycin and kanamycin does not represent a safety concern, since the strains exhibited high susceptibility to clinically relevant antibiotics, so could be totally free of transferable antibiotic resistance genes.

Evaluation of virulence factors of *E. faecium*

It is now recognized that *E. faecium* consists of two distinct subpopulations, or clades. One subpopulation [termed community-associated clade B by Palmer et al. (2012) based on whole genome phylogeny] consists predominantly of isolates from the feces of healthy individuals, and is characterized by susceptibility to ampicillin. The other subpopulation (hospital-associated clade A) contains most of the ampicillin-resistant isolates. Comparative genomic hybridization and genome sequencing have revealed the presence of several genes that are enriched in clinical *E. faecium* isolates. One of the genes that is most clearly over-represented in clinical isolates is the insertion sequence *IS16* [hospital associated strain marker, Werner et al. (2011)], which presumably confers a level of genomic flexibility to its host, thereby facilitating the subsequent acquisition of additional elements involved in virulence or antibiotic resistance. Other factors potentially associated with *E. faecium* virulence have also been identified but, among them, ESP [pathogenicity island (PAI) marker, Top et al. 2011; Rice et al. 2003], and the *hyl*-like (Freitas et al. 2010) gene are now considered the most relevant for assessment of safety (EFSA 2012b). The purpose of this assessment was to exclude *E. faecium* strains belonging to the hospital-associated clade from use in animal nutrition because of the hazard they present to a vulnerable subpopulation of consumers. PCR-based detection of genes responsible for antibiotic resistance and virulence was applied to strains belonging to *E. faecium* (WEI-9, WEI-10 and WEI-33). None of the three genetic elements were detected in WEI-9, WEI-10 and WEI-33 (data not shown), indicating that these three *E. faecium* strains belonged to the community-associated clade (B), and can be considered safe.

Hemolytic, bile salts hydrolysis and biogenic amine-producing abilities

Table 5 shows the hemolytic activity, bile salts hydrolysis (BSH) and biogenic amine-producing (BAP) ability of the 11 selected strains. None of the strains examined exhibited β-hemolytic activity when grown in fresh blood agar plates; only the strains WEI-33 exhibited α-hemolysis. The absence of hemolytic activity is considered as a safety prerequisite for selection of a probiotic strain (FAO/WHO 2002). None of the strains, excepted strain WEI-33, exhibited BSH activity. Furthermore, none of the 11 selected strains showed BAP ability.

Up to now, it is not yet completely clear if BSH activity is a desirable trait for promising probiotic strains selection. For one thing, this activity could maximize intestinal survival and persistence of probiotic strains, increasing the overall beneficial effects of the strains (Begley et al. 2006). For another thing, the deconjugation bile salts by BSH enzymes may lead to gastrointestinal malabsorption, and the bile salts deconjugated to free bile acid in the early period may lead a promotion of the occurrence and development of colon cancer, but there is not enough clinical evidence and epidemiological evidence showing that free bile acids are harmful (Vankerckhoven et al. 2008). Our results indicated that all selected strains, except the *E. faecium* WEI-33, were not able to deconjugate bile salts. Moreover, the strains that did not exhibit BSH activity, such as WEI-9, WEI-10 and WEI-51, were able to survive at different bile salts concentrations, confirming that the two activities are not correlated each other, in accordance with previous report data (Moser and Savage 2001).

Biogenic amines have many types, of which histamine and tyramine are the most toxic. Lactic acid bacteria, which produce biogenic amines, belong to *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Leuconostoc*, etc., and lactic acid bacteria applied to additives may also produce tyramine and histamine (Moreno-Arribas et al. 2003). Although lactic acid bacteria producing biogenic amines are distributed in various species, the ability to produce biogenic amines is strain-specific. Phenotype detection of histidine, tyrosine, ornithine decarboxylase activities determined by the qualitative liquid culture method showed that none of the 11 strains had any ability to produce biogenic amines. The detection of biogenic amines producing bacteria by conventional culture techniques is often tedious and unreliable, exhibiting disadvantages such as lack of speed, appearance of false positive/negative results and low

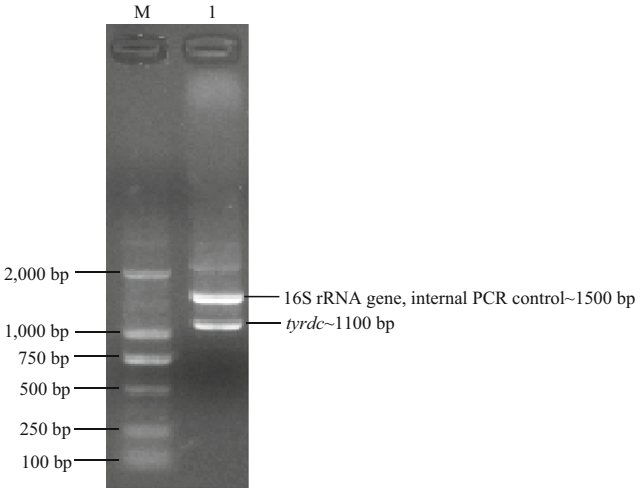

Fig. 3 Simultaneous detection of the *tyrdc*, *odc*, *agdi* and *hdc* genes by multiplex PCR. Lanes: *M* DM 2000 DNA ladder 100 bp to 2 kb linear scale molecular weight marker (ComWin Biotech, China), *1 E. faecium* WEI-9

sensibility (Landeta et al. 2007). As molecular methods are fast, reliable and culture-independent, they represent an interesting alternative for the detection of bacteria producing biogenic amines, a multiplex PCR method was applied to simultaneously detect four genes involved in the production of the main biogenic amines. From the results, the *tyrdc* gene was detected in *E. faecium* WEI-9 (Fig. 3), which was inconsistent with the phenotypic results and further confirmed the accuracy of the molecular methods. The remaining strains were negative for all four target genes.

Conclusions

Following a systematic screening strategy in vitro, the strains *E. faecium* WEI-10 and *L. plantarum* WEI-51 were finally selected based on their promising probiotic properties and absence of unsafe characteristics. These two strains could be taken for further investigation in vivo to elucidate their potential health benefits and for application as novel probiotic strains in animal feed supplements.

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