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ORIGINAL ARTICLE

Screening for potential probiotic from spontaneously fermented non-dairy foods based on in vitro probiotic and safety properties

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Abstract

The aim of this study was to screen potential probiotic lactic acid bacteria from Chinese spontaneously fermented non-dairy foods by evaluating their probiotic and safety properties. All lactic acid bacteria (LAB) strains were identified by 16S rRNA gene sequencing. The in vitro probiotic tests included survival under low pH and bile salts, cell surface hydrophobicity, auto-aggregation, co-aggregation, antibacterial activity, and adherence ability to cells. The safety properties were evaluated based on hemolytic activity and antibiotic resistance profile. The salt tolerance, growth in litmus milk, and acidification ability were examined on selected potential probiotic LAB strains to investigate their potential use in food fermentation. A total of 122 strains were isolated and identified at the species level by 16S rRNA gene sequencing and included 62 *Lactobacillus plantarum*, 40 *Weissella cibaria*, 12 *Lactobacillus brevis*, 6 *Weissella confusa*, and 2 *Lactobacillus sakei* strains. One *W. cibaria* and nine *L. plantarum* isolates were selected based on their tolerance to low pH and bile salts. The hydrophobicity, auto-aggregation, co-aggregation, and antagonistic activities of these isolates varied greatly. All of the 10 selected strains showed multiple antibiotic resistance phenotypes and no hemolytic activity. The highest adhesion capacity to SW480 cells was observed with *L. plantarum* SK1. The isolates *L. plantarum* SK1, CB9, and CB10 were the most similar strains to *Lactobacillus rhamnosus* GG and selected for their high salt tolerance and acidifying activity. The results revealed strain-specific probiotic properties were and potential probiotics that can be used in the food industry.

Keywords Lactic acid bacteria · Probiotic · Non-dairy food · Probiotic properties

Introduction

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host" (FAO/WHO 2006). The most commonly used probiotics belong to *Bifidobacterium* and lactic acid bacteria (LAB), including various species of *Lactobacillus*, *Enterococcus*, *Lactococcus*, and *Bacillus* (Guarner and Schaafsma 1998; Dicks and Botes 2010). LAB have been classified as "generally recognized as safe" (GRAS) due to their general occurrence in fermented food products and also being an essential part of the human commensal microflora (Argyri et al. 2013). Although many studies have focused on the isolation and evaluation probiotic LAB, the screening of novel probiotic candidates is still desirable since the probiotic features and benefits to human health are strain-specific (Jampaphaeng et al. 2017).

Fermentation has been used for centuries in food production as an effective method to improve the flavor, quality, and preservation properties of food. With the help of diverse microorganisms that exist during the fermentation procedure, the texture, aroma, nutritive compounds, and organoleptic attributes of food change significantly (Kumar et al. 2015). LAB are associated with the fermentation of all many different types of food, i.e., wine, dairy, vegetable, and meat products (Dave and Shah 1997; Moreno-Arribas et al. 2000; Klingberg et al. 2005; Lee et al. 2015). Therefore, isolating microorganisms from fermented food, especially spontaneously fermented food, is an essential way to obtain novel potential probiotic LAB isolates with specific features for industrial products. Up to now, many studies have reported probiotics isolated from human feces, kimchi, and dairy fermented products, such as yogurt, cheese, and other fermented milk beverages (Dave and Shah 1997; Succi et al. 2005; Lee et al. 2012,

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2015). However, to increase the biodiversity of commercial probiotics, there is a need to screen non-dairy probiotics from other spontaneously fermented foods and evaluate their potential to be used in the food industry (Kumar et al. 2015).

Although yet to be proven conclusively, it is believed that probiotics may provide diverse health-promoting effects to human, such as prevention and treatment of pathogenic infections, cholesterol-lowing effects, antioxidant activity, allergy prevention, antiinflammation, and anti-tumor activity (Saarela et al. 2000). To bring out these health benefits, a potential probiotic must be tolerant of low pH and bile salts, must attach to epithelial cells, and must colonize the human intestine (Piano et al. 2006). Hence, acid and bile resistance assays have been used as preliminary screening methods to identify potential probiotics from large numbers of bacterial strains (Guo et al. 2009). Antagonistic activity towards intestinal pathogens is also an important functional requirement of probiotics. Moreover, cell surface hydrophobicity indicates the nonspecific physical-chemical interactions between two surfaces and the physical and chemical characteristics of the bacterial cell surface. Together with auto-aggregation activity, these two probiotic features are related to the ability of a strain to adhere to epithelial cells (Ramos et al. 2013). On the other hand, co-aggregation with pathogens may prevent colonization of the latter in the gut (Del Re et al. 2000). Furthermore, in vitro models have been established in many studies using mammalian epithelial cells to investigate the adhesion of probiotics to the gastrointestinal tract (Kos et al. 2003; Klingberg et al. 2005; Han et al. 2017).

The objective of this study was to evaluate the probiotic properties of LAB isolated from spontaneously fermented non-dairy foods collected in Yangling, China. All selected strains were identified at a species level by 16S rRNA gene sequencing. Probiotic properties were evaluated according to survival in a low pH environment, tolerance to bile salts, surface properties (hydrophobicity, auto-aggregation, and co-aggregation with pathogens), antibiotic resistance, hemolytic activity, antagonistic activity towards pathogenic bacteria, and adhesion ability to the human colon adenocarcinoma SW480 cell line. Salt tolerance, growth in litmus milk, and acidification ability were also evaluated among selected probiotic LAB strains to investigate their potential use in the food industry.

Materials and methods

Sample collection and isolation of potential LAB

Twenty-four spontaneously fermented food samples were collected from four local markets in Yangling, China, including pickle, sauerkraut, sausage, smoked pork, and cured beef. All samples were collected in sterile bags, transported at 4 °C, and examined within 24 h of reaching the laboratory. For isolation of potential LAB, samples were diluted by 10-fold serial dilution with sterile 0.85% saline and mixed thoroughly. Thereafter, 1 mL of 10^{-4}–10^{-6} dilutions was mixed with de Man, Rogosa, and Sharpe (MRS) agar supplemented with 1% $CaCO_3$. After anaerobic incubation at 37 °C for 48 h, colonies presenting various macroscopic and microscopic characteristics with calcium-dissolving rings were collected and sub-cultured on MRS agar for purification. Purified isolates were stored in MRS broth medium (containing 20% glycerol) at − 80 °C. All isolates were sub-cultured twice before use.

Identification of LAB strains

All purified isolates were subjected to Gram staining and catalase testing. Strains that were Gram-positive and catalase-negative were recognized as potential LAB. Further identification was performed by 16S rRNA sequencing. Total genomic DNA was extracted from different isolates using the cetyltrimethylammonium bromide (CTAB) method described by Zhou et al. (2012). In brief, PCR amplification was performed in a total volume of 25 μL, containing 10 mmol/L 10 × PCR buffer, 25 mmol/L $MgCl_2$, 2.5 mmol/L each deoxyribonucleotide triphosphate (dNTP), 25 μmol/L each primer (27f 5′-AGAGTTTGATCCTGGCTCAG-3′, and 1495r 5′-CTAC GGCTACCTTGTTACGA-3′; Yu et al. 2011), 1.25 U Taq polymerase, and 100 ng DNA. The temperature profile was as follows: an initial hold at 94 °C for 2 min; 35 cycles of 94 °C for 1 min, annealing at 56 °C for 1 min, and 72 °C for 2 min; a final extension of 72 °C for 10 min; and finally held at 4 °C. Thereafter, the PCR products were purified and sequenced by Sangon Biotech (Shanghai, China). Assembled sequences were compared with National Center for Biotechnology Information (NCBI) database for species-level identification by the Basic Local Alignment Search Tool (BLAST).

Tolerance to low pH and bile salts

All isolates were cultured in MRS broth for 24 h at 37 °C and resuspended in sterile saline. Tolerance to low pH was determined in MRS broth with pH 2.0 in which pH 7.0 was used as a control. As described by Nishida et al. (2008), the optical density (OD) value at 600 nm was measured before (A_0) and after 24 h (A_{24}) of anaerobic incubation at 37 °C. The acid tolerance of LAB strains was estimated by the survival rate (%) as follows:

$$\text{Survival rate } (\%) = A_{24}/A_0 \times 100\%.$$

Strains that showed a high tolerance to pH 2.0 were selected for the bile tolerance assay as described by Guo et al. (2009) with modifications. Bile tolerance was evaluated based

on the time required to increase the OD_{600} by 0.3 units in MRS broth with and without 0.3% (*w*/*v*) bile salts (Sigma Chemical Co., St. Louis, MO). The difference in time (h) to obtain 0.3 units between the measurements of MRS broth with and without bile was considered as the adaptation time (AT) of the LAB to adapt to bile. All the acid and bile tolerance experiments were performed in triplicate.

Cell surface hydrophobicity

The cell surface hydrophobicity of the LAB was determined by measuring microbial adhesion to hydrocarbons (MATH) as previously described (Kotzamanidis et al. 2010; Ramos et al. 2013). In brief, bacteria cultivated in MRS broth at 37 °C for 24 h were washed twice with sterile saline and resuspended in 3 mL of 0.1 M KNO_3 containing approximately 10^8 CFU/mL of bacteria, and the absorbance was measured at 600 nm (A_0). One milliliter of xylene and chloroform was then added to each cell suspension to form a two-phase system. After a pre-incubation at room temperature for 10 min, the two-phase system was mixed by vortexing for 2 min. The water and hydrocarbon phases were then separated and incubated for 20 min at room temperature. The water phase was carefully removed and its absorbance at 600 nm was measured (A_1). All assays were performed in triplicate. The percentage of cell surface hydrophobicity ($H\%$) was calculated using the following formula:

$$H\% = (1 - A_1/A_0) \times 100\%.$$

Auto-aggregation and co-aggregation

The auto-aggregation assay was performed as described by Del Re et al. (2000) with modifications. LAB cultivated in MRS broth at 37 °C for 24 h were washed twice and resuspended in PBS buffer (10^9 cfu/mL). One milliliter of cell suspension was vortexed for 10 s and incubated at 37 °C for 5 h. Thereafter, an aliquot (100 μL) of the upper suspension was carefully obtained and mixed with 400 μL PBS buffer to measure the absorbance at 600 nm. The percentage of auto-aggregation (AutoA%) was expressed as the percent decrease in absorbance after 5 h (A_5) relative to that of the original suspension (A_0) as follows:

$$\text{AutoA}\ (\%) = (1 - A_5/A_0) \times 100\%$$

The co-aggregation assay was performed as described by Kos et al. (2003). The co-aggregation ability of LAB was analyzed with four pathogenic bacteria strains: *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* ATCC 13076, *Escherichia coli* ATCC 25922, and *Shigella dysenteriae* ATCC 13313. The preparation of cell suspensions was the same as that for auto-aggregation. Equal volumes (1 mL) of cell suspensions of LAB and pathogenic strains were mixed together in pairs and vortexed for 10 s. Cell suspensions of each single strain were set up as controls. After 5 h of incubation at 37 °C, absorbance of the LAB (A_x) and pathogenic strains (A_y) in the control tubes and in the mixture (A_{x+y}) were measured at 600 nm. The assays were performed in triplicate. The co-aggregation percentage (CoA%) was calculated as follows:

$$\text{CoA}\ (\%) = \left[\left(A_x + A_y\right)/2 - A_{x+y}\right]/\left(A_x + A_y/2\right) \times 100\%$$

Antibacterial activity against pathogen bacteria

All selected LAB strains were tested for antibacterial activity against *S. aureus* ATCC 25923, *S. enterica* ATCC 13076, *E. coli* ATCC 25922, and *S. dysenteriae* ATCC 13313. The antibacterial assay was performed with fresh 24 h-culture supernatant from LAB cultivated in MRS broth. The cell-free culture supernatant (CFCS) of LAB strains was collected by centrifugation (1000×g, 5 min, 4 °C), adjusted to pH 6.5 with NaOH, sterilized with 0.22-μm filters, and subjected to antibacterial activity using the well diffusion assay according to Ramos et al. (2013) with modifications. Overnight cultures of four pathogenic bacteria strains in brain-heart infusion (BHI) broth were evenly incorporated into BHI agar. Wells (5 mm diameter) were drilled into the agar after solidification, and 50 μL CFCS from the LAB was transferred into respective wells. The plates were incubated at 37 °C for 24 h, and the antibacterial activity was recorded as the growth-free inhibition zones (diameter in mm) around the well. The antibiotic kanamycin (30 mg/mL) was used as a positive control, while MRS broth adjusted to pH 6.5 was the negative control. The assay was performed in triplicate.

Adherence to SW480 cells

The adhesion capacity of selected LAB strains was investigated using the human colon adenocarcinoma cell line SW480. Cells were propagated in modified Eagle's medium (MEM) supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2.

The adhesion assay was performed as previously described (Klingberg et al. 2005) with modifications. Overnight cultures of the selected LAB strains were harvested by centrifugation (10,000×*g*, 5 min, 4 °C) and resuspended in PBS solution at a concentration of approximately 10^8 CFU/mL. A monolayer of SW480 cells was seeded at 2×10^5 cells/mL and dispensed into a 24-well culture plate. Then, 0.5 mL bacterial suspension was added to the SW480 cells previously washed with Dulbecco's

phosphate-buffered saline (DPBS). The commercial probiotic *Lactobacillus rhamnosus* GG (ATCC 53103) was used as a reference strain. After 1 h of incubation at 37 °C with 5% CO_2, the cells were gently washed three times with PBS solution to remove unbound bacteria. SW480 cells were then lysed using 0.05% trypsin-EDTA solution, and the bacterial counts of viable adherent LAB on the MRS agar plate were determined. The adhesion capacity was described as the number of adhered bacteria (CFU/mL) relative to the total number of bacteria initially added. Each adhesion assay was conducted three times with duplicate determinations.

Hemolytic activity

Fresh LAB cultures were streaked on Columbia agar plates containing 5% (*w*/*v*) sheep blood and incubated for 48 h at 37 °C. The hemolytic activity of the LAB strains was determined according to the signs of α-hemolysis (green zones around colonies), β-hemolysis (clear zones around colonies), or γ-hemolysis (no zones around colonies) on Columbia blood agar plates (Pieniz et al. 2014).

Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC, μg/mL) of eight antibiotics was determined using the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) criteria (2009). Each experiment was performed on MRS agar plate containing different antibiotics, including ampicillin, kanamycin, gentamycin, tetracycline, erythromycin, roxithromycin, lincomycin, and chloramphenicol. Antibiotic concentrations ranged from 0.125 to 512 μg/mL by 2-fold dilutions, and experiments at each concentration were performed in triplicate. All LAB strains were inoculated on MRS agar containing antibiotics at 37 °C for 24 h.

Potential use in fermentation

The salt tolerance, growth in litmus milk, and acidification ability were examined on selected probiotic LAB strains to evaluate their potential use in food fermentation. All tests were performed in triplicate. The salt tolerance of LAB was determined in MRS broth with 0%, 2%, 4%, and 6.5% NaCl (Morandi and Brasca 2012). The OD value at 600 nm was measured before and after 24 h incubation at 37 °C. The growth of LAB in litmus milk was also investigated at 37 °C for 24 h. The color change of the litmus milk was recorded during the fermentation. For acidification ability, LAB strains were inoculated in reconstituted sterile skim milk powder (10% *w*/*v*) at 1%. A pH meter was used to determine the pH value changes in the milk during 24 h of incubation at 37 °C. According to Morandi and Brasca (2012), the acidification rate was calculated as ΔpH:

$$\Delta pH = pH_{\text{zero time}} - pH_{\text{at time}}$$

Values of ΔpH after 6 h (ΔpH6) and 24 h (ΔpH24) were used to compare strain-acidifying activity.

Statistical analysis

Data analysis was conducted using IBM SPSS Statistics for Mac, version 24.0 (IBM Corp., 2016, Armonk, N.Y., USA), and data are presented as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's multiple comparison were used to test the significant differences between means of the treatments at a significance level of $P < 0.05$.

Results and discussion

Isolation and identification of LAB

A total of 182 isolates were collected from 24 spontaneously fermented non-dairy food samples in Yangling, China, including 6 pickles, 6 sauerkrauts, 4 sausages, 3 smoked pork, and 5 cured beef samples. By Gram staining and catalase testing, 122 isolates were confirmed as Gram-positive and catalase-negative. Thereafter, these 122 isolates were identified and categorized into five LAB species by 16S rRNA gene sequencing as follows: 62 *Lactobacillus plantarum*, 40 *Weissella cibaria*, 12 *Lactobacillus brevis*, 6 *Weissella confusa*, and 2 *Lactobacillus sakei* strains. These species have been reported in different types of fermented products (Ammor et al. 2005; Lee et al. 2012; Ramos et al. 2013; Saelim et al. 2017). In our study, *L. plantarum* strains were identified from all of the collected samples, confirming that *L. plantarum* was one of the most common LAB species in fermented food. This result is in agreement with observations reported by Saelim et al. (2017). Remarkably, all of the *L. brevis* strains were isolated from pickles, whereas the *W. confusa* and *L. sakei* strains were only found in meat origin samples.

Tolerance to low pH and bile salts

The tolerance of low pH conditions and bile salts are two essential properties for probiotic LAB to survive in the stomach and the upper part of the intestinal tract (Ramasamy et al. 2012). Thus, acid and bile resistance assays have been used as preliminary screening methods to identify potential probiotics from large numbers of bacterial strains (Argyri et al. 2013; Ding et al. 2017). In our study, the survival rate of 122 LAB

strains was determined at pH 2.0 based on OD value changes. An increase in OD value after incubation indicated that the strain was resistant to acidic conditions with a high survival rate. Thirty-six of 122 strains had survival rates higher than 100% and were selected for further experiments: 24 *L. plantarum*, 8 *W. cibaria*, 2 *W. confusa*, and 2 *L. brevis*. Remarkably, *L. plantarum* PIC10 showed the highest survival rate of 124.38% at pH 2 followed by *L. plantarum* PIC33 and SK5 (Table 1). The results indicated acid tolerance varies greatly among strains. Gilliland et al. (1984) suggested that 0.3% bile salts is considered to be a critical concentration for screening for bile-resistant probiotics. In our study, all of the tested 36 LAB strains were able to grow under 0.3% bile salts pressure, although the adaptation times varied among strains. Isolates *L. plantarum* PIC42 and CB10 and *W. cibaria* CB12 showed the shortest adaptation time of less than one hour, whereas one *W. confusa* and two *L. brevis* isolates from cured beef showed the longest adaptation time of approximately 4 h. Ten LAB strains with short adaptation times and high resistance to bile salts were selected for further investigations (data shown in Table 1). Table 2 presents the species and origin of selected LAB strains. The passage of probiotics through the human gastrointestinal tract is a stressful journey since the low pH and bile salts pressure may affect probiotic cell viability (Corcoran et al. 2008). Studies evaluating the tolerance of probiotics to low pH and bile salts have shown that the related resistant mechanism is strain and species dependent (Angmo et al. 2016; Abushelaibi et al. 2017). In line with our results, Ramos et al. (2013) found that the *L. fermentum*, *L. plantarum*, and *L. brevis* isolates from Brazilian food products exhibited high tolerance to a pH 2 environment. Moreover, the bile salt tolerance of the selected LAB in this study was higher than those reported by Han et al. (2017). As reported by Hyacinta et al. (2015), the bile tolerance of LAB may related to the activity of bile salts hydrolases, which can help to reduce cholesterol level and benefit the human host.

Cell surface properties

Cell surface properties including hydrophobicity and auto-aggregation are regarded as indicative parameters for probiotic cell adhesion to epithelial cells in the human intestine (Kumar et al. 2017). In addition, co-aggregation with pathogenic bacteria represents a barrier to reducing invasion by intestinal pathogens and preventing infection in human hosts (Campana et al. 2017). In our study, all of the selected LAB strains were examined for their surface properties, including cell surface hydrophobicity, auto-aggregation, and co-aggregation (Table 3). Regarding cell surface hydrophobicity, *L. plantarum* CB10 from cured beef showed the highest hydrophobic activity (hydrophobicity value of 40.34%) compared with the rest of the isolates, which showed a moderate hydrophobicity with values ranging from 3.62 to 13.14%. Furthermore, three strains isolated from cured beef (*L. plantarum* CB9 and CB10 and *W. cibaria* CB12) showed the highest auto-aggregation ability of more than 90%, whereas *L. plantarum* PIC19 exhibited the lowest auto-aggregation value of 39.10%. The co-aggregation abilities of LAB strains were analyzed with four pathogenic bacteria: *S. aureus*, *S. enterica*, *E. coli*, and *S. dysenteriae*. The results demonstrated a broad range of variation in co-aggregative phenotypes. The co-aggregative interactions between LAB and *E. coli* were ranged from 2.12 to 20.32%. *L. plantarum* SK1 and SK2 were the most effective strains at co-aggregating with *S. aureus*, whereas *L. plantarum* PIC33 was the most prone strain to co-aggregate with *S. enterica* (18.39%). In general,

Table 1 Tolerance to low pH and bile salt

Isolates	Survival rate (%) at pH 2	Bile tolerance (Time required to increase A_{600} nm with 0.3 units)		
		MRS (h)	MRS + 0.3% bile (h)	AT (h)
SK1	108.12 ± 0.15cd	4.07 ± 0.04	5.30 ± 0.17	1.23 ± 0.14c
SK5	118.59 ± 1.47b	4.80 ± 0.20	6.58 ± 0.08	1.78 ± 0.19b
PIC10	124.38 ± 0.33a	5.20 ± 0.03	7.95 ± 0.26	2.75 ± 0.25a
PIC19	101.39 ± 2.12e	3.96 ± 0.15	5.24 ± 0.09	1.28 ± 0.11c
PIC20	100.36 ± 3.25e	4.45 ± 0.11	5.59 ± 0.05	1.14 ± 0.06cd
PIC33	119.22 ± 1.16a	3.83 ± 0.11	6.25 ± 0.08	2.42 ± 0.09a
PIC42	111.45 ± 2.10c	3.48 ± 0.05	4.23 ± 0.13	0.75 ± 0.09e
CB9	105.85 ± 1.10cd	4.14 ± 0.14	5.16 ± 0.18	1.02 ± 0.12cd
CB10	115.59 ± 0.22bc	4.16 ± 0.15	5.11 ± 0.22	0.95 ± 0.12d
CB12	103.24 ± 1.97de	4.02 ± 0.07	4.85 ± 0.12	0.83 ± 0.08de
Reference LGG	118.40 ± 0.43bc	5.35 ± 0.17	6.50 ± 0.12	1.15 ± 0.07cd

Presented values are means of triplicate determinations; ± indicates standard deviations from the mean. Mean values (± standard deviation) within the same column followed by different lowercase letters differ significantly ($p < 0.05$)

Table 2 Selected LAB strains used for in vitro study of probiotic properties

Identity	Species	Origin
SK1	*Lactobacillus plantarum*	Sauerkraut
SK5	*Lactobacillus plantarum*	Sauerkraut
PIC10	*Lactobacillus plantarum*	Pickle
PIC19	*Lactobacillus plantarum*	Pickle
PIC20	*Lactobacillus plantarum*	Pickle
PIC33	*Lactobacillus plantarum*	Pickle
PIC42	*Lactobacillus plantarum*	Pickle
CB9	*Lactobacillus plantarum*	Cured beef
CB10	*Lactobacillus plantarum*	Cured beef
CB12	*Weissella cibaria*	Cured beef
LGG (ATCC 53103)	*Lactobacillus rhamnosus*	Reference strain

W. cibaria CB12 displayed a low co-aggregation ability to all the four pathogenic bacteria (less than 10%). Although *L. plantarum* CB 10 exhibited the greatest hydrophobicity and auto-aggregation activities, its co-aggregation ability was not as good as that of other *L. plantarum* strains isolated from sauerkraut. According to our results, there was no correlation between hydrophobicity, auto-aggregation, and co-aggregation ability. Similar results were reported by previous studies (Kotzamanidis et al. 2010; Ramos et al. 2013). It has been shown that cellular aggregative activities could promote the colonization of beneficial microorganisms in the vaginal and gastrointestinal tracts of human hosts (Collado et al. 2005; Atassi and Servin 2010). However, the mechanisms of auto-aggregation and co-aggregation remain unclear. Previous studies indicated that probiotic aggregative abilities are strain-specific (Kos et al. 2003; Ramos et al. 2013; Jampaphaeng et al. 2017). There are several factors that may influence the aggregative ability of probiotic LAB, including cell surface charge, cell surface components, the size of the bacterial cell, and environmental conditions (Han et al. 2017). In addition, Goh and Klaenhammer (2010) described genes encoding aggregation-promoting factors in *Lactobacillus acidophilus*.

Antibacterial activity against pathogenic bacteria

The antibacterial activity of probiotic LAB is essential for protecting the host from pathogenic infection. Moreover, the antibacterial activity of LAB is related to their antioxidant activity according to previous studies (González et al. 2007; Ou et al. 2009). In our study, the antagonistic activity of selected LAB against pathogenic bacteria is presented in Table 4. The isolate *L. plantarum* PIC33 showed strong antagonistic activity towards *S. aureus*, *S. enterica*, and *S. dysenteriae*, whereas the highest activity towards *E. coli* was obtained by another *L. plantarum* isolate, SK5. Five *L. plantarum* isolates and *W. cibaria* CB12 showed low inhibitory activity towards *S. enterica* with an inhibition zone of 1–2 mm. The isolate *L. plantarum* PIC19 presented no antagonistic activity towards all four pathogenic bacteria tested. In concordance with our results, Olatunde et al. (2018) found that all the selected strains of LAB significantly inhibited the growth of *Salmonella typhimurium*, *E. coli*, and *S. aureus* used for their study. Although the tested LAB strains showed various antagonistic activities against pathogenic bacteria, the nature of the inhibitory substances remains unknown. Campana et al. (2017) suggested that the production of antibacterial compounds, such as bacteriocins, organic acids, and short chain fatty acids, is one of the mechanisms by which probiotics inhibit the growth of pathogens. Hence, the

Table 3 Surface properties of 10 LAB strains

Strains	Hydrophobicity (%)	Auto-A (%)	Co-A (%)			
			S. aureus	*S. enterica*	*E. coli*	*S. dysenteriae*
SK1	3.98 ± 0.28ef	89.73 ± 0.89d	39.95 ± 0.57b	15.27 ± 0.35ab	12.67 ± 0.71b	16.40 ± 0.45a
SK5	3.70 ± 0.56f	72.20 ± 0.71e	38.07 ± 0.84bc	9.95 ± 0.45c	20.32 ± 0.64a	7.48 ± 0.42b
PIC10	4.41 ± 0.55e	68.96 ± 0.67f	29.86 ± 0.68c	17.19 ± 0.26ab	10.12 ± 0.71c	5.85 ± 0.44c
PIC19	7.11 ± 0.73d	39.10 ± 0.66i	11.64 ± 0.60de	9.19 ± 0.34cd	2.12 ± 0.16g	5.69 ± 0.39c
PIC20	3.88 ± 0.27ef	63.93 ± 0.72gh	11.94 ± 0.63de	3.47 ± 0.56d	4.23 ± 0.27e	3.28 ± 0.47de
PIC33	3.62 ± 0.48f	61.03 ± 0.28h	77.75 ± 0.74a	18.39 ± 0.48a	2.45 ± 0.37fg	3.59 ± 0.25de
PIC42	4.52 ± 0.35de	64.05 ± 0.54g	13.89 ± 0.38d	5.72 ± 0.40d	5.13 ± 0.35d	4.47 ± 0.30d
CB9	13.14 ± 0.29c	92.54 ± 0.84b	33.20 ± 0.59bc	12.53 ± 0.54b	3.06 ± 0.21f	2.60 ± 0.32e
CB10	40.34 ± 1.24b	93.82 ± 0.53ab	18.18 ± 0.66d	16.92 ± 0.48ab	3.33 ± 0.29ef	3.18 ± 0.37de
CB12	7.75 ± 0.40d	91.20 ± 0.73c	9.14 ± 0.34e	9.23 ± 0.54cd	2.85 ± 0.40fg	2.97 ± 0.26e
Reference LGG	45.20 ± 0.53a	95.20 ± 0.44a	25.45 ± 0.49c	9.33 ± 0.72cd	10.24 ± 0.82c	12.31 ± 0.58ab

Presented values are means of triplicate determinations; ± indicates standard deviations from the mean. Mean values (± standard deviation) within the same column followed by different lowercase letters differ significantly ($p < 0.05$)

Table 4 Antibacterial activities of selected LAB strains against 4 pathogen bacteria

LAB strains	*S. aureus*	*S. enterica*	*E. coli*	*S. dysenteriae*
SK1	+	+	–	–
SK5	++	–	++	+
PIC10	+	+	–	+
PIC19	–	–	–	–
PIC20	–	–	–	+
PIC33	+++	+	–	++
PIC42	–	+	–	+
CB9	+	+	–	–
CB10	–	–	+	–
CB12	–	+	+	–

− no inhibition zone, + 1.0 to 2.0 mm, ++ 2.1 to 3.0 mm, +++ ≥ 3.0 mm

antibacterial properties of probiotic LAB can be beneficial for both food preservation and prevention of pathogenic infection in human hosts (Acurcio et al. 2017).

Hemolytic activity

Hemolytic activity is regarded as a safety aspect for the selection of probiotic strains (FAO/WHO 2006). In this study, the hemolytic activities of 10 selected LAB strains were evaluated on blood agar plates, and no hemolytic effects were observed (γ-hemolysis). Our results were in agreement with those reported by Oh and Dong (2015) regarding five *Lactobacillus* species isolated from traditionally fermented millet alcoholic beverages in Korea.

Antibiotic susceptibility profiles of LAB strains

Probiotics are considered to be generally safe and beneficial to the host, but they also may cause bacteria-host interactions and other unwanted effects. The main concern regarding the safety of probiotics is their antibiotic resistances since these strains may transfer antibiotic resistance genes to pathogenic bacteria, which may represent a serious risk for the treatment of infections (Doucet-Populaire et al. 1992; Jacobsen et al. 2007; van Reenen and Dicks 2011). For the safe use of novel isolated probiotic LAB strains, it is necessary to characterize their antibiotic resistance profiles in advance. In our study, the antibiotic resistance profiles of 10 selected LAB strains are presented in Table 5. According to the breakpoints listed in the table, the strains were identified as either sensitive (S, MIC ≤ breakpoint) or resistant (R, MIC > breakpoint). Since the studies of antibiotic resistance of LAB strains were limited, there was a blank of some antibiotic breakpoints in the EFSA handbook. Thus, we suggested some breakpoints in this study and they might change as more strains are tested by other researchers. All 10 strains were susceptible to ampicillin (MIC values range from 0.25 to 2 μg/mL), which was in agreement with similar results observed by other authors (Danielsen and Wind 2003; Pérez Pulido et al. 2005; Nawaz et al. 2011; Palachum et al. 2018). The main mechanism of aminoglycosides is to inhibit protein synthesis or mRNA synthesis in microorganisms. The bacterial cell walls of LAB serve as a natural barrier for aminoglycosides and may be further fortified via acquired mutations. In addition, efflux pumps work to expel aminoglycosides from bacterial cells, and modifications also may cause further resistance to aminoglycosides (Garneau-Tsodikova and Labby 2016). However, a high resistance level to aminoglycosides (kanamycin and gentamycin) was observed in our study. All of the *L. plantarum* isolates were found to be highly resistant to gentamycin, and six of these strains were also resistant to erythromycin with MIC values greater than 1 μg/mL. These results were in concordance with those reported by Zhou et al. (2012) and Adimpong et al. (2012). However, Nawaz et al. (2011) found that *Lactobacillus* isolates in their study were sensitive to kanamycin, which contradicts our results. In addition, high resistance to chloramphenicol was also observed in our study. This is in good concordance with the results of Pérez Pulido et al. (2005) and Nawaz et al. (2011). A high percentage of LAB strains (60%) was sensitive to tetracycline. The resistance level against roxithromycin and lincomycin varied among isolates. Remarkably, two *L. plantarum* isolates CB9 and CB10 were sensitive to six of the eight antibiotics tested in our study.

Adherence to SW480 cells

One of the most important probiotic properties is the ability to adhere human intestine, since to be termed as a probiotic, a selected strain must reach the intestine alive and colonize the colon in abundant numbers (Guo et al. 2016). Adhesion ability to the human colon adenocarcinoma cell line SW480 was evaluated for the 10 selected LAB, and the results are shown in Fig. 1. All tested strains showed a low percentage of adhesion ability (less than 15%). However, the isolate *L. plantarum* SK1 showed the highest adhesion percentage of 14.1% among tested strains ($P < 0.05$), followed by *L. plantarum* CB9 (11.8%) and CB10 (10.3%). However, another *L. plantarum* PIC isolated from pickles showed the lowest percentage of adhesion to SW480 cells (0.7%). Other *L. plantarum* and *W. cibaria* isolates presented moderated adhesion abilities (1.73–7.2%), Generally, the adhesion capacity to SW480 cells was strain-specific and varied within species in our study. Adhesion of LAB to the human colon is a complex process that involves contact between the bacterial cell membrane and interacting surfaces (Han et al. 2017). According to Re et al. (2000), the auto-aggregating abilities of LAB are correlated with their adhesion capacities to epithelial cells, which is a prerequisite for colonization and persistence in the

Table 5 Antibiotic resistance profiles of selected LAB strains and their breakpoints (μg/mL)

Strains and breakpoints	AMP	TET	CHL	ERY	ROX	LIN	GEN	KAN
SK1	S	S	R	S	S	R	R	R
SK5	S	S	R	R	S	S	R	R
PIC10	S	R	R	R	R	S	R	R
PIC19	S	S	R	R	S	R	R	R
PIC20	S	R	R	R	R	R	R	R
PIC33	S	S	R	R	R	S	R	R
PIC42	S	R	R	R	R	R	R	R
CB9	S	S	S	S	S	S	R	R
CB10	S	S	S	S	S	S	R	R
CB12	S	R	R	R	R	R	S	R
Breakpoint for *L. plantarum*	2[a]	32[a]	8[a]	1[a]	2[b]	64[b]	16[a]	64[a]
Breakpoint for *W. cibaria*	2[a]	8[a]	4[a]	1[a]	2[b]	64[b]	16[a]	16[a]

AMP ampicillin, *TET* tetracycline, *CHL* chloramphenicol, *ERY* erythromycin, *ROX* roxithromycin, *LIN* lincomycin, *GEN* gentamycin, *KAN* kanamycin

[a] Breakpoints defined by European Food Safety Authority (EFSA 2012)

[b] Breakpoints suggested by this paper. These breakpoints are suggestions that might change as more strains are tested

gastrointestinal tract. Kos et al. (2003) observed a relationship between auto-aggregation and the adhesion capacity of *Lactobacillus acidophilus* M92, which was mediated by proteinaceous components on the cell surface. Han et al. (2017) found the strains with higher rates of adhesion to Caco-2 cells, such as *L. brevis* R4, *L. acidophilus*, and *L. sake*, also had higher auto-aggregation abilities. However, in our study, high auto-aggregation capacity did not always guarantee a correlation with high adhesion ability to SW480 cells. For example, *L. plantarum* CB9 and CB12 both showed an auto-aggregation (%) of greater than 90%, whereas the adhesion capacity (%) of CB9 was four times of that of CB12. We also found that hydrophobicity values did not correlate with adhesion capacity, which was in agreement with the findings of Zago et al. (2011) and García-Cayuela et al. (2014). However, in a study by Han et al. (2017), the results revealed that in most cases, the LAB with the greater hydrophobicity also had the higher adhesion ability.

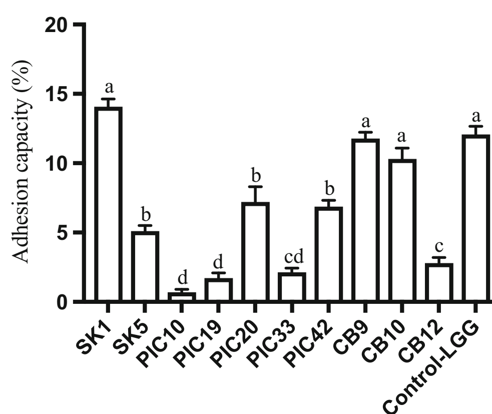

Fig. 1 The adhesion capacity of 10 strains to SW480 cells

Principal component analysis of probiotic properties

The 10 selected LAB isolates were evaluated according to their tolerance to low pH, bile salts and stimulated human GI tract, surface properties, antibacterial activities, antibiotic resistances, and adhesion capacity to SW480 cells. All results obtained from these assays were subject to principal components analysis (PCA). Based on the Cattell scree plot and a variance explained criterion of 60%, six principal components were obtained. The first (PC1) and the second (PC2) principal components explained 30.9% and 21.6% of the total variance, respectively. PC1 represents hydrophobicity, auto-aggregation, adhesion capacity, and part of the antibiotic resistance since higher contributions come from these factors. PC2 is mainly related to bile tolerance and co-aggregation. Figure 2 shows the scatter plot presenting the distribution of the 10 selected LAB and the reference probiotic LGG in the PC1-PC2 plane. Particularly, three *L. plantarum* strains, namely SK1, CB9, and CB10, formed a cluster with the reference probiotic LGG. These strains were characterized by high values of hydrophobicity, auto-aggregation, co-aggregation and adhesion, and low values of bile adaptation time. *L. plantarum* SK1, CB9, and CB10 were identified as the most promising potential probiotic based on PCA analysis. Similarly, other strains of *L. plantarum* have been proven to be able to survive gastric transit and colonize the intestinal tract of humans and other mammals as potential probiotics (Mathara et al. 2008; Ding et al. 2017).

Potential use in fermentation

During food fermentation, probiotic LAB are exposed to a high salt and/or an acidic environment. Hence, the potential

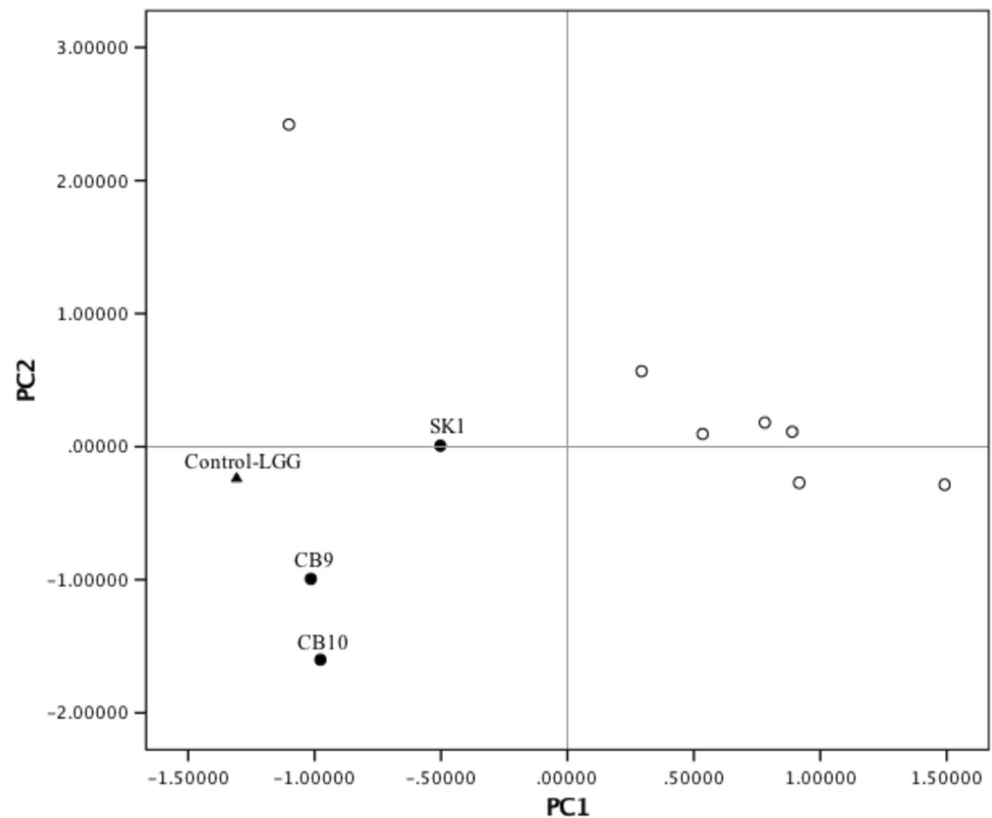

Fig. 2 Principal component analysis (PCA) of probiotic proprieties as tolerance to low pH and bile salt, surface properties, antimicrobial activities, antibiotic resistances, and adhesion capacity to SW480 cells for the selected 10 LAB isolates

probiotic LAB isolates SK1, CB9, and CB10 were further evaluated for their tolerance to these conditions. All three strains showed high salt tolerance, growing in the presence of 6.5% NaCl. Compared with the findings of Morandi and Brasca (2012), our *L. plantarum* strains from non-dairy food were more tolerant to salt pressure than the *Streptococcus thermophilus* isolated from cheese. During the fermentation of litmus milk, the color of the indicator changed from purple to white within 14 h with coagulation in the milk, indicating that our promising probiotics were suitable for milk fermentation. For acidification ability, isolate SK1 presented the highest value of ΔpH after 6 h incubation ($\Delta pH_6 = 1.52 \pm 0.13$), followed by CB10 ($\Delta pH_6 = 1.05 \pm 0.24$) and CB9 ($\Delta pH_6 = 0.89 \pm 0.18$). While after 24 h incubation, the highest acidification rate ΔpH_{24} was found in CB10 (2.78 ± 0.24), slightly higher than SK1 (2.66 ± 0.14) and CB9 (2.65 ± 0.27).

Conclusions

In conclusion, potential probiotic LAB isolates were identified and characterized from different Chinese spontaneously fermented non-dairy foods. The probiotic characteristics of the LAB isolates from cured beef have not been studied before. It was clearly observed that LAB strains belonging to the same species may present different in vitro probiotic characteristics and develop different mechanisms. The results of this work provided a preliminary selection of potential probiotic isolates *L. plantarum* SK1, CB9, and CB10, which could be used as probiotic due to their in vitro probiotic and safety properties. In addition, these strains were confirmed to have high salt tolerance and acidification ability, indicating that they can to be used as starters for producing functional foods. Other in vitro and in vivo tests were required to investigate their specific functional characteristics such as antioxidant activity, antiinflammation activity, and cholesterol-lowing activity.

Authors' contributions M.T. Fan, J. Wang, and X.Y. Wei designed the study. J. Wang, K. Yang, and M.M. Liu performed experiments and collected test data. J. Wang and J. Zhang conducted data analysis. M.T. Fan, J. Wang, and X.Y. Wei drafted the manuscript. All authors revised the manuscript.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals.

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

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