中国传统乳制品中乳杆菌的益生菌潜力评估

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摘 要: 目的 评估从中国传统乳制品中分离出的 9 株乳杆菌的功能及其益生菌潜力。**方法** 从中国传统乳制品中分离纯化得到乳杆菌菌株, 测试其益生菌潜力, 如溶血活性和抗氧化活性等。**结果** 所有分离的乳杆菌均可耐受低 pH 值(pH 2.0、2.5 和 3.0)和高胆盐条件(0.3%、0.5%和 1%)。分离的乳杆菌对 10 种抗生素的敏感性,的测试结果表明, 所有分离株对万古霉素均没有抗性, 此外大多数分离株对青霉素, 氨苄青霉素, 链霉素和四环素具有抗性。所有乳杆菌对 HT-29 细胞具有优异的粘附能力, 其值介于 10.4%~39.0%之间, 并且对 DPPH 自由基和超氧阴离子自由基具有出色的抗氧化活性。**结论** 从中国乳制品中分离出的乳杆菌具有作为各种产品中益生菌的潜力。

关键词: 乳杆菌; 中国传统乳制品; 益生菌潜力

Evaluation of probiotics potential of *Lactobacilli* isolated from Chinese traditional dairy products

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ABSTRACT: Objective To evaluate the functional and probiotic potential of 9 *Lactobacillus* strains isolated from Chinese traditional dairy products. **Methods** The strains were isolated and purified from Chinese traditional dairy products. Probiotic potential of *Lactobacillus* like hemolytic activity, antioxidant activity and so on was tested. **Results** All the isolated *Lactobacillus* strains tolerated low pH values (pH 2.0, 2.5 and 3.0) and high bile salt conditions (0.3%, 0.5% and 1%). The susceptibility of isolated *Lactobacillus* strains was tested to 10 antibiotics; but the results shows that none of isolates were resistant to vancomycin, while on the other hand most of the isolates were resistant to penicillin, ampicillin, streptomycin, and tetracycline. All *Lactobacillus* isolates exhibited excellent adherence ability to HT-29 cell with values ranged from 10.4%–39.0% as well as excellent antioxidant activity against DPPH free radical and superoxide anions free radical, respectively. **Conclusion** The *Lactobacillus* strains isolated from Chinese dairy products have excellent potential for use as probiotics in various products.

KEY WORDS: *Lactobacillus*; Chinese traditional dairy products; probiotic potential

基金项目: 深圳市海外高层次人才创新创业计划孔雀技术创新项目(KQJSCX20180328100801771)、深圳市基础研究面上项目(JCYJ20190808145613154)

Fund: Supported by the Special Fund for Development of Strategic Emerging Industries in Shenzhen (KQJSCX20180328100801771, JCYJ20190808145613154)

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1 Introduction

The Chinese traditional dairy products make an important contribution to Chinese diet due to their inexpensiveness and the enhancement of nutritional quality[1]. The Chinese traditional dairy products have existed for thousands of years and various lactic acid bacterial species have been linked with the Chinese traditional dairy products[2]. As the lactic acid bacteria are considered as the normal inhabitants of the gastrointestinal tract, the recognition of their potential health-promoting effect has led to the promotion of various traditional Chinese dairy products[3]. The research on *Lactobacillus* strain isolated from traditional dairy products shows a long history of safe use[4]. For example, the *Lactobacillus casei* Zhang is a potential probiotic strain isolated from Koumiss with high bile salt resistance, acid resistance, cholesterol reduction, and antimicrobial activity[5]. Furthermore, the isolation, characterization, and their biological potential from Chinese traditional food products has been extensively reviewed elsewhere[2].

The probiotic has become of a crucial topic of lactic acid bacteria over the past 10 years, including the genera *Lactobacillus* and *Bifidobacterium*[6]. Recently, there has been emergent awareness that the consumption of *Lactobacillus* strains isolated from Chinese traditional dairy product has various health promoting effects. To provide the required benefit to the host health, the isolated *Lactobacillus* strain must have ability to pass through the physical and chemical barrier of the gastrointestinal tract[7]. Also, to serve as probiotic, the *Lactobacilli* strain must have ability to survive in sufficient number during production and storage of the desired products[8,9].

Probiotic microbes are the “live microorganisms” when administered in sufficient quantity, which can offer various health benefits to the host including modulation of the immune system, rearrangement of gastrointestinal microflora, and inhibit the growth of harmful microorganisms[10]. Lactic acid bacteria are consider as the most important probiotic bacteria and have compatibility with the human digestive system because of their natural resistance abilities to low pH values and high bile salt conditions[11,12].

The survival and colonization of probiotic strain in gastrointestinal tract are critical to confirm the health benefit to the host[13]. The *Lactobacillus* strain requires certain cell surface properties including cell surface hydrophobicity to colonize the intestine[14]. Furthermore, the presumptive *Lactobacillus* strain also has functional attributes such as antioxidant, cholesterol reduction, and immune system modulation to exert its useful physiologic functions[15]. In this study, the 9 *Lactobacillus* strains were isolated from traditional Chinese dairy products and were screened for desirable probiotics potentials such as acid resistance, bile resistance, phenol resistance, antibiotics resistance, antioxidant activity, cell surface hydrophobicity, adhesion to intestinal epithelial cells, and cholesterol lowering abilities, in order to get a better understanding for the composition of Chinese traditional food products microbiota.

2 Materials and methods

2.1 Materials

2.1.1 Samples

Twenty samples of Chinese traditional dairy products [yak’s milk (4), Koumiss (5), cheese (4), and Kefir grains (7)] were collected from Tibetan autonomous region of Qinghai province, P.R China. Samples were collected in sterile tubes and kept in a mini freezer at -15 ℃ and were processed immediately upon receipt in the laboratory.

2.1.2 Reagent

de Man-Rogosa-Sharpe, DPPH radical and Columbia blood agar (Merck, New York, United State); *L*-cysteine, Bile acid, phenol, cholesterol and Dulbecco’s modified eagle medium (Sigma-Aldrich, Germany); genomic DNA purification kit (TransGen Biotech Co., Ltd., Beijing). All the reagents were of analytical grades.

2.1.3 Instrument

TC 512 Thermocycler (Techne, England); Whatman No.1 filter paper (Sigma Aldrich, China); Uv-Vis spectrophotometer (PerkinElmer, United States); dialysis membrane (Sigma, Beijing).

2.2 Methods

2.2.1 Isolation and identification of *Lactobacillus*

Samples were serially diluted in phosphate buffer saline (PBS, pH 7.2) and small aliquots (50 μL) of each dilution was spread on de MRS, supplemented with 0.05% *L*-cysteine (MRS_c) agar plates. The plates were incubated anaerobically at 37 ℃ for 72 h. The colonies with different morphologies were selected and re-streaked on freshly prepared MRS_c agar plates for several generations in order to isolate the purified individual bacterial colonies. All isolated were tested by further gram staining and catalase test, and the isolates showing gram-positive and catalase negative were presumed to be *Lactobacillus*. The identification of *Lactobacillus* isolates was performed by sequencing the 16S rDNA gene. The total genomic DNA was extracted using the Genomic DNA purification kit, following the manufacturer's instructions. The primers used for amplifying the 16S rDNA sequences are forward 5″-AGAGTTTGATCCTGGCTC AG-3″ and reverse 5″-CCGTCAATTCCTTTGAGTTT-3”. The fragments were amplified in a Techne-TC 512 Thermocycler under the following conditions: 95 ℃ for 1 min, 30 cycles of 95 ℃ for 30 s, 55 ℃ for 30 s, and finally 72 ℃ for 5 min. The amplified fragment was screened on an agarose gel and sequenced by the Guangzhou IGE Biotechnology Ltd, Shenzhen, China. All obtained sequences were tested via the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were deposited into Gene Bank. Sequence alignment was performed via ClustalW2 (http://www.ebi.ac.uk/Tool/mas/clustalw2/) and a phylogenetic tree was constructed via neighbour-joining and maximum-composite likelihood methods using Mega 6.0 software (http://megasoftware.net/).

2.2.2 Probiotic potential of *Lactobacillus*

(1)Survival under low pH and high bile salt conditions

The ability of *Lactobacillus* surviving at pH 2.0, 2.5, 3.0, and 6.5 (control) was evaluated following the method described in[16]. Meanwhile, the bile salt tolerance of *Lactobacillus* surviving for 3 h in acidic conditions were determined using MRS_c broth containing 0.3%, 0.5% and 1% of bile (*w*/*V*) following the method described in [17] with the MRS_c broth without bile salt as the control group. Resistance to acidic condition as well as bile salt conditions was evaluated by plate count on MRS_c agar following the formula (1).

$$\text{Growth rate } (\%)=(N_1/N_0)\times 100 \quad (1)$$

Where, N_1 is the total count of viable bacterial cells in the MRS_c broth after treatment, and N_0 is the total count of viable bacterial cells in the MRS_c broth before treatment.

(2)Phenol tolerance

The phenol tolerance assay was performed by following the method previously reported in [11] with slightly modification. Briefly, 24 h old culture of *Lactobacillus* isolates (1%) were inoculated in to freshly prepared MRS_c broth supplemented with 0.2% and 0.4% phenol, with the MRS_c broth without phenol used as control group. After 24 h incubation at 37 ℃, the phenol resistance was evaluated by measuring the absorbance (*A*) $OD_{630\ nm}$ following the formula (2).

$$\text{Phenol tolerance } (\%)=(A_1/A_0)\times 100 \quad (2)$$

Where, A_1 is the absorbance of cultures after treatment and A_0 is the absorbance of culture before treatment.

(3)Hemolytic activity

Lactobacillus isolates were streaked on the surface of Columbia blood agar plate (Sigma, China) supplemented with 5% sheep blood and the plates were incubated for 72 h at 37 ℃. After incubation, the plates were examined for the hemolytic activity[18].

(4)Exopolysaccharide production

Lactobacillus cultures were grown in a flask containing 100 mL freshly prepared MRS_c broth supplemented with 3% (*w*/*V*) glucose and incubated at 37 °C for 72 h. Bacterial cells were removed via centrifugation (7000 *g* for 10 min) and 2 volume of pre-chilled ethanol was added in to one volume of supernatants for exopolysaccharide (EPS) precipitation. The precipitated EPS was recovered via centrifugation (10000 *g* for 35 min) at 4 °C , dialyzed (6000-8000 Da) for 48 h and then lyophilized. The total amount of sugar was measured following the phenol sulphuric methods using glucose as an standard.

(5)Cell surface hydrophobicity

Cells at stationary phase were harvested by centrifugation (8000 *g* for 5 min), washed twice with PBS (pH 7.2) and finally resuspended in PBS (pH 7.2) to reached an optical density of 0.6±0.02 at 630 nm (A_0). The 1 mL of xylene was mixed with 1 mL of cell suspension, and allows standing at room temperature for 30 min to form a 2-phase system. Aqueous phase was carefully removed and its absorbance at 630 nm (A_1) was measured. The cell surface hydrophobicity (%) was measured by following the formula (3).

$$\text{cell surface hydrophobicity } (\%)=(1-A_1/A_0)\times 100 \quad (3)$$

(6)Antioxidant activity

1)Preparation of intact cell

Overnight *Lactobacillus* cultures were centrifuged (7000 *g* for 15 min) 4 ℃. Cell pellet was washed thrice with PBS (pH 7.2) and finally resuspended in PBS (pH 7.2) at a final concentration of 1×10^6 CFU/mL for antioxidant analysis.

2)Superoxide anion scavenging assay

The superoxide anion scavenging assay was carried out via pyrogallol autoxidation with modification[13]. Briefly, *Lactobacillus* cell suspension (100 μL) in PBS (pH 7.2) at a final concentration of 1×10^6 CFU/mL and 900 μL water was immediately mixed with 2 mL of Tric-HCl buffer (pH 8.1). Sterile distilled water was used as control instead of *Lactobacillus* cell suspension. Then, 50 μL pyrogallol solutions (10 mmol/mL) were added and autoxidation was evaluated by measuring the absorbance of control and sample at 330 nm after 10 min incubation. The superoxide anion scavenging ability of *Lactobacillus* tested was calculated via the formula (4).

$$\text{Scavenging ability } (\%)=(\Delta A_0-\Delta A)\times 100/\Delta A_0 \quad (4)$$

Where, ΔA_0 and ΔA are the autoxidation rates of the pyrogallol before and after the addition of the sample and deionized water, respectively.

3)DPPH radical scavenging activity

3 mL of *Lactobacillus* cell suspension and 3 mL of 0.5 mmol/L ethanolic solution of DPPH radical were mixed and incubated at room temperature for 30 min in dark. After incubation, the reaction mixture was centrifuged (10000 *g* for 20 min) at 4 ℃ and the absorbance of supernatants at 517 nm was measured. The DPPH scavenging ability was calculated by following the formula (5).

$$\text{Scavenging ability } (\%)=(1-OD_{sample}/OD_{control})\times 100 \quad (5)$$

Where, OD_{Sample} and $OD_{Control}$ are absorbance of samples and of distilled water, respectively, mixed with DPPH solution.

(7)Cholesterol lowering ability

Lactobacillus isolates (1%, *V*/*V*) was inoculated into 10 mL of freshly prepared MRS_c broth supplemented with 0.2% sodium thiogylcollate, 0.3% oxgall, and 100 μL/mL of water soluble cholesterol. Each tube was incubated at 37 ℃ for 24 h. The cells were removed by centrifugation (10000 *g* for 35 min) at 4 ℃. The o-phthalaldehyde[19] method for measuring cholesterol was used to determine the amount of cholesterol in the spent broth and uninoculated sterile broth.

(8)Adhesion assay

The HT-29 cells were grown in cell culture bottles using the Dulbecco's modified eagle medium (DMEM) supplemented with 2 mmol/L *L*-glutamine, 10% heat inactivated fetal bovine serum, 100 μg streptomycin/mL, 1% non-essential amino acid and 100 Ul penicillin/mL. HT-29 cells were subsequently seeded into 24 wells culture plates at a concentration of 2.5×10^5 cells per well and allowed to differentiate for 3 d, changed the medium for every day. The cells were incubated at 37 ℃ in 5% CO_2 atmosphere. The overnight cultures of the *Lactobacillus* isolates were centrifuged, washed twice using PBS (0.1 mol/L, pH 7.2) and resuspended in the same buffer to an appropriate dilution (absorbance OD_{630} 0.2, approximately 2×10^8 CFU/mL). After that, the

bacterial cell was added in to each cell well and the plates were incubated at 37 ℃ for 4 h. After incubation, the cells were washed with PBS (0.1 mol/L, pH 7.2) and lysed with 0.1% Triton X-100 solution. The cells lysate were serially diluted and spread on MRS_c agar plates. The plates were incubated at 37 ℃ for 3 d. The percentage of bacterial adhesion was calculated as formula (6).

Adhesion(%)= (adhered bacteria/total added bacteria)×100 (6)

2.2.3 Statistical analysis

Values were given as mean values and standard deviation with triplicate determinations. Significant ANOVA results were followed up with Tukey's Multiple Comparison Test in all assays and if $P<0.05$, the difference was statistically significant.

3 Results and analysis

3.1 Isolation and identification of *Lactobacillus*

After culturing (24 h at 37 ℃ anaerobic conditions), a total of 60 bacterial isolates were isolated from samples of Chinese traditional dairy products. Nine out of an assemblage of 60 bacterial isolates on MRS_c agar plates showed an appearance of *Lactobacillus*. All isolates were Gram's positive, catalase negative, rod shaped, and mesophilic. The isolates were identified based upon the sequence of 16S rRNA gene and the obtained sequences showed similarity with those of known species available in NCBI database. The isolates were identified as *Lactobacillus kefiri* (MSR101 and MSR103), *Lactobacillus hordei* (MSR102), *Lactobacillus brevis* (MSR104), *Lactobacillus oryzae* (MSR105), *Lactobacillus parabuchneri* (MSR106 and MSR107), *Lactobacillus puchneri* (MSR108), and *Lactobacillus fructivorans* (MSR109). A phylogenetic relationship was constructed based on their 16S rRNA sequences by following the neighbor joining methods (Fig.1). The filled circles indicated the strains from NCBI and the empty circles indicated the isolated *Lactobacillus* strains used for tree construction. Our finding in an agreement with various studies showing the presence of *lactobacillus* species in Chinese traditional dairy products[1].

3.2 Survival under low pH and high bile salt conditions

In order to exert their beneficial effects, the orally ingested *Lactobacillus* must remain alive under condition similar to stomach and intestine of the host. In other word, the probiotic strain must survive to the acidic stomach conditions (pH 1-3) and the bile salts secreted in to luminal content in the small intestine[18]. To evaluate the resistance against gastric conditions, all the *Lactobacillus* isolates were screened for their ability to survive at low pH values (pH 2.0, 2.5, and 3.0). All the *Lactobacillus* isolates revealed high survival rate (>45% at pH 2.0, >55% at pH 2.5, and >78% at pH 3.0). Furthermore, the results presented in Fig.2A showed that the all *Lactobacillus* isolates had the ability to grow in both acidic and neutral environment.

From Fig.2B, it could be known that all *Lactobacillus* isolates showed excellent survival rate (>54%) at 0.3% and (>46%) at 0.5% bile salt concentrations. Furthermore, all isolates showed survival rate (>33%) at very high bile salt (1%) conditions. The bile salt concentration used in this study (0.3%) was considered physiological and often chose as critical for evaluation of probiotic strain resistance to bile salt[20]. In general, the results were consistent with the previous reports, which meant that the gut tolerance was not necessarily linked to species, but strain specific.

3.3 Phenol tolerance

During metabolism, aromatic amino acid undergoes deamination process via gut bacteria, leading to the formation of phenol as by product, which have bacteriostatic action. To serve as probiotics, the *Lactobacillus* strains must have survival ability against toxic metabolites (phenol) produced during the digestion process[21]. The *Lactobacillus* isolates showed varying degree of sensitivity toward different phenol concentration (0.2%-0.4%) (Fig.3). At concentration of 0.2%, all the *Lactobacillus* isolates showed highest tolerance, ranging from 92% to 69%. While on the other hand, at 0.4% concentration, all *Lactobacillus* isolates showed varying degree of tolerance, ranging from 57% to 38%. The results were consistent with the previously reports showing the tolerance of *Lactobacillus* strains to various concentration of phenol[11].

3.4 Hemolytic activity and exopolysaccharide production

All *Lactobacillus* isolates were tested for their hemolytic activity (the lysis of blood cells) and the results showed that none of *Lactobacillus* isolates hemolytic activity. All the tested 9 *Lactobacillus* isolates were hemolytic negative and they might therefore be considered safe with regards to hemolytic activity (Table 1). The results were very much in accordance with the previously studies reporting that no *Lactobacillus* culture possessed hemolytic activity[22].

The probiotic strains can secret the extracellular microbial polysaccharide which plays important role in various fermented food[13]. The EPS can also protect the probiotic strains from unfavorable environmental condition. The EPS can serve as protective agent against phagocytosis and antibacterial agents and It also play a vital role in cell surface attachment[23]. Recently, it was reported that the *Lactobacillus* strain having high EPS producing ability could exhibit high bile and acid tolerance[24]. Table 1 showed the potential of EPS production of all *Lactobacillus* isolates. Based on the results, all *Lactobacillus* isolates exhibited the ability to produce the EPS. The results in this study were consistent with the previous study reporting that the probiotic *Lactobacillus* strains producing EPS[21].

3.5 Cell surface hydrophobicity

The cell surface hydrophobicity associates with the adhesion potential of probiotics *Lactobacillus* strain and plays an vital role in colonization of probiotic strain to gastrointestinal tract. A minimum value of 40% hydrophobicity is an essential requirement for a probiotic strain. In present study, all the *Lactobacillus* isolates exhibited excellent hydrophobicity potential with xylene. The *Lactobacillus* isolates (MSR102,

MSR104, and MSR108) showed higher hydrophobicity (84.2%, 90.5%, and 81.3%) respectively compared to Lactobacillus isolates MSR101 (69.8%), MSR103 (66.2%), MSR105 (59.8%), MSR106 (73.5%), and (MSR109 65.1%) respectively. While the *Lactobacillus* isolate MSR107 had the lowest hydrophobicity (43.5%) compared with other isolates. The results are consistent with the previously reported study[25,26].

3.6 Antioxidant activity

To serve as probiotic, the *Lactobacillus* strain needs to tolerate the exogenous and endogenous oxidative stress. The antioxidant potential of *Lactobacillus* strain protects the host microflora from the attack by free radicals when *Lactobacillus* colonize and propagate in the gastrointestinal tract[13]. Furthermore, the antioxidant agents produced by the microflora play a vital role in prevention of various life threatening diseases including diabetes, cardiovascular disease, and ulcer. All the tested *Lactobacillus* isolates exhibited strong antioxidant activity against free radicals as shown in (Fig.4). The scavenging rate of superoxide anion radicals ranged from 44.1% to 17.5% (Fig.4A), while scavenging rate of DPPH radicals ranged from 80.4% to 50.4% (Fig.4B). The results demonstrated that the all *Lactobacillus* isolates expressed excellent antioxidant activity in a strain specific manner.

3.7 Cholesterol lowering ability

The high serum cholesterol level can be considered as an important factor in various pathological conditions such as cardiovascular disease[27,28]. People are looking for safe and effective alternative therapy for serum cholesterol reduction as the use of drugs may cause side effect[13]. The results showed that the cholesterol concentration in culture broth decreased after the inoculation of the tested *Lactobacillus* isolates in the culture medium. Among the tested *Lactobacillus* isolates, the MSR102, MSR103, MSR106, MSR105, and MSR107 had higher cholesterol removal rate(40.2%, 58.1%, 65.8%, 41.5%, and 45.7% respectively). While on the other hand, the MSR101, MSR104, MSR108, and MSR109 had lower cholesterol removal rate. Our results were consistent with the previous results that the *Lactobacilli/Bifidobacteria* spp could reduce human blood cholesterol level[29].

3.8 Adhesion assay

The adhesion ability of *Lactobacillus* provides various benefits to the host such as the modulation of immune system and exclusion of pathogenic microbes. Furthermore, it is also an important criteria for the selection of probiotic strain. The adhesion ability of the tested *Lactobacillus* isolates to HT-29 cells was verified using the plating method and it might vary in a strain dependent manner. The isolates MSR101 and MSR104 had higher adhesion ability (34.4% and 39.0% respectively) to HT-29 cell compared with other isolates MSR102 (26.4%), MSR103 (18.1%), MSR105 (11.6%), MSR106 (22.3%), MSR107 (27.8%), MSR108 (12.6%), and MSR109 (10.4%). Our results were consistent or even better than the previously reported studies[30].

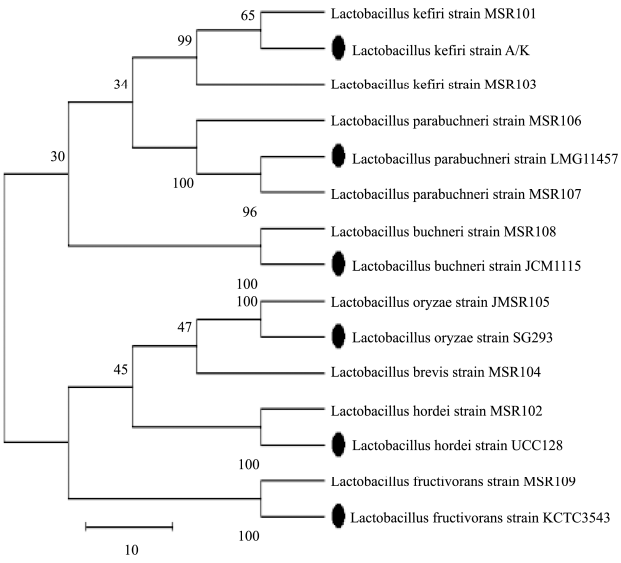
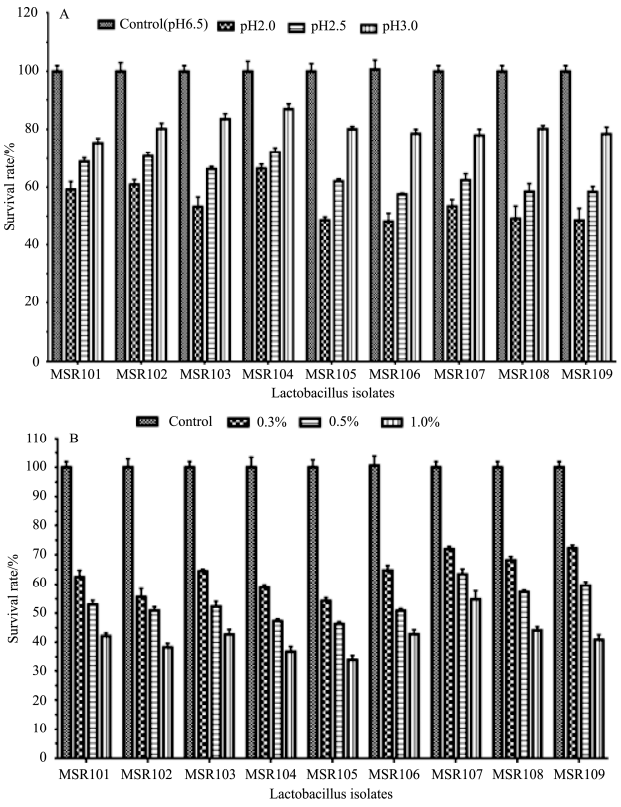


Fig.1　Phylogenetic tree



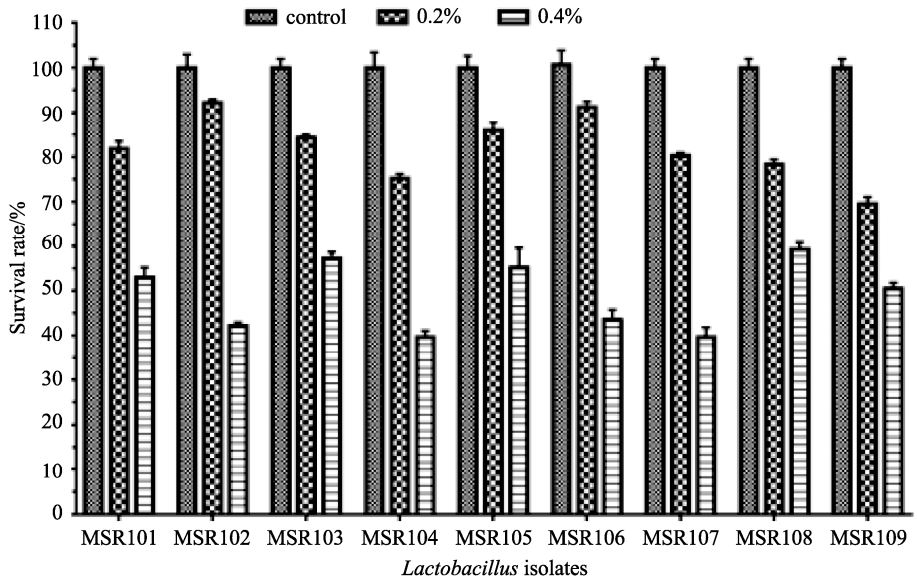
Note: The experiments were performed in triplicate and values were represented as mean±*SD*.

Fig.2　Survival rate under different pH (A) and bile salt (B) conditions

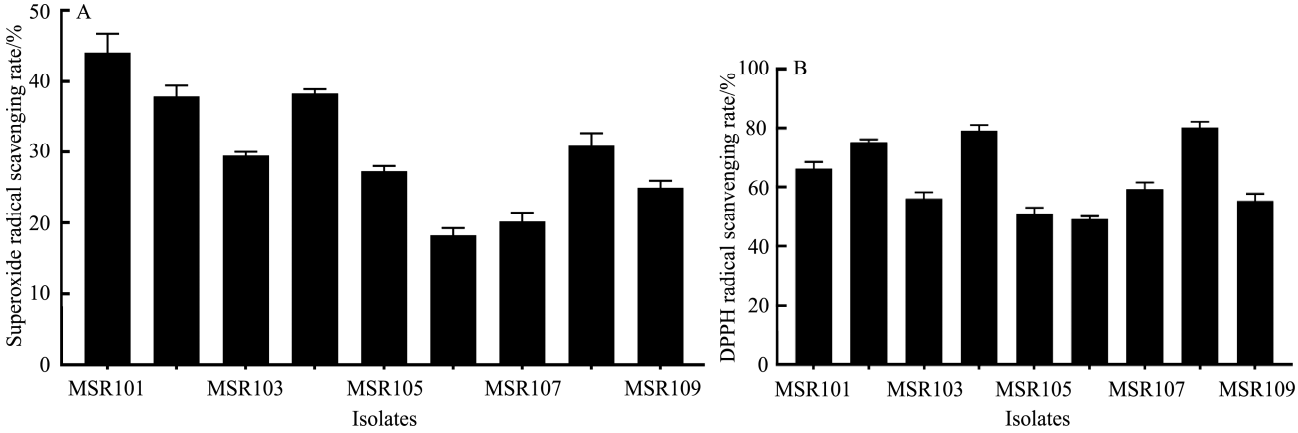
4 Conclusion

In this study, the probiotic potential of 9 *Lactobacillus* strains (MSR101, MSR102, MSR103, MSR104, MSR105, MSR106, MSR107, MSR108, and MSR109) isolated from

Chinese traditional food products were examined in term of survival rate under gut conditions, antibiotic resistance, antioxidant activity, hydrophobicity, hemolytic activity, cholesterol reduction, and adhesion to HT-29 cells. The results showed that isolated *Lactobacillus* strains exhibited excellent mean bile salt tolerance, acid tolerance, antibiotic resistance, cholesterol reduction, and antioxidant activities. Therefore, the results suggested that these isolated *Lactobacillus* strains can be used as functional starter cultures due to their excellent probiotic potential. However, further *in vivo* researches are needed to evaluate the probiotic potential such as antioxidant activity and cholesterol reduction abilities to establish the theoretical basis for the developing of new starter functional culture and antioxidant nutraceutical.



Note: The experiments were performed in triplicate and values were represented as mean±*SD*.
Fig.3 Survival rate under different phenol concentrations.



Note: A: Superoxide anion radicals scavenging rate; B: DPPH radicals scavenging rate.
Fig.4 Antioxidant activity of isolated *Lactobacillus* isolates.

Table 1 Hemolytic activity and EPS production abilities of *Lactobacillus* isolates

Lactobacillus isolates	Origin	Hemolytic activity	EPS production
MSR101	Kefir grain	-	+
MSR102	Kefir grain	-	+
MSR103	Kefir grain	-	+
MSR104	Koumiss	-	+
MSR105	cheese	-	+

Continued Table 1

Lactobacillus isolates	Origin	Hemolytic activity	EPS production
MSR106	cheese	-	+
MSR107	cheese	-	+
MSR108	cheese	-	+
MSR109	yak's milk	-	+

Note: *(-)= negative and (+)= positive.

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