International Food Research Journal 27(3): 407 - 416 (June 2020)
Journal homepage: *http://www.ifrj.upm.edu.my*

Characterisation and *in vitro* antioxidant activity of probiotic *Lactobacillus* isolated from Inner Mongolia "Hurood" cheese

Ishaq, M., Ji, W., Wu, T., Liu, C.-L., Fang, L. and *Min, W.-H

College of Food Science and Engineering, Jilin Agricultural University, Changchun 130118, PR China

Article history

Received: 28 August 2019
Received in revised form: 26 March 2020
Accepted: 8 May 2020

Keywords

Lactobacillus, antioxidant activity, probiotics, Hurood

Abstract

Probiotic bacteria possess tremendous capabilities that confer extensive health benefits by maintaining the gastrointestinal microbiota of the host organism. In the present work, we comprehensively investigated the probiotic characteristics and influence of selected *Lactobacillus* strains as antioxidative agents isolated from Inner Mongolian Cheese. For this purpose, the physiological properties, acid and bile tolerance, microbial adhesion to hydrocarbons, antimicrobial activity, and resistance of bacterial strains to six antibiotics were investigated. *In vitro* antioxidant analysis of selected LAB strains was performed, measuring scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radicals (OH^-). Furthermore, 16S rRNA sequencing was used to identify four presumptive LAB strains. All four strains showed significant survival at pH 2.0 - 3.0 for 4 h, and with the addition of ox gall at 0.1 - 0.5% for 4 h. However, the cell viability of strain LP38 was $\log_{10}$ 5.01 colony forming unit (CFU)/mL at pH 2.0, and $\log_{10}$ 8.97 CFU/mL at pH 3.0, after 4 h of incubation. Strain LP38 showed more stability with 0.1% ox gall ($\log_{10}$ 9.78 CFU/mL) than with 0.3 or 0.5% ox gall (w/v). Strains NS24 and LP38 showed better adherence and hydrophobicity to different solvents including xylene, vinyl acetate, and chloroform. Additionally, the antimicrobial results of strain LP38 against all five groups of pathogenic bacteria showed relatively higher inhibition than that by the other strains. Similarly, *in vitro* scavenging activity revealed that strain LP38 exhibited the highest hydroxyl (66.66% at 10^{10} CFU/mL) and 2,2-diphenyl-1-picrylhydrazyl (45.12%) free radicals. Overall, our findings provide new insights into the identification of *Lactobacillus* associated with potential probiotic properties.

Introduction

Lactic acid bacteria (LAB) are widely and naturally distributed in soil, water, manure, sewage, silage, and plants, and have considerable probiotic properties. Probiotic microorganisms positively affect the health of the host system by promoting microbial balance in the intestine, a phenomenon known as a live microbial supplement (Fuller, 1989; Hoque *et al.,* 2010). Probiotics are associated with multiple applications, especially in fields related to health effects in animals and humans. Probiotic products contain a wide variety of enzymes, vitamins, therapeutic capsules, and various kinds of fermented foods coupled with microorganisms. Human consumable products are mostly produced in fermented milk. Recent studies have shown a defensive response by gut flora accompanying oral consumption of probiotic microorganisms. Previous studies have suggested that probiotics associated with useful effects in gut microbial disorders also raise crucial concerns in clinical trials of these products. Some probiotic products have been shown to be positive therapeutic agents, producing antibiotic sensitivity in bacteria that cause acute diarrhoea (Saad *et al.,* 2013).

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial activity targets enteric undesirables and pathogens (Klaenhammer and Kullen, 1999). The antimicrobial effects of LAB occur through the production of substances such as organic acids, carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances, and bacteriocins (Wouters *et al.,* 2002; Çakır, 2003; Quwehand and Vesterlund, 2004). The probiotic ability of LAB and their associated functional properties are unique and strain-dependent. Therefore, it is important to consider these properties more comprehensively (Salminen and Von Wright, 2004). During the selection of appropriate probiotic strains, several properties such as acid and bile tolerance, antimicrobial sensitivity against serious pathogenic bacteria, survival through the gastrointestinal

*Corresponding author.
Email: minwh2000@jlau.edu.cn

tract, and adherence to intestinal surfaces should be considered (Ouwehand *et al.,* 2001).

Recently, probiotics have been shown to exhibit hypocholesterolaemic activities by minimising cholesterol levels in the plasma, preventing diarrhoea, and boosting the immune system (FAO and WHO, 2002; Díaz-Ropero *et al.,* 2007; Kotani *et al.,* 2010). Several other mechanisms of probiotics have also been reported to exhibit various beneficial effects on the host system. These mechanisms primarily involve reduction of luminal pH, immune revoking, secretion of antimicrobial substances, competition with pathogens for adhesion sites, and toxin inactivation (Salminen et al., 2010). Despite the existing literature, there is no study available on the explicit mechanism of LAB from Inner Mongolian cheese, which exhibits a striking probiotic potential. In the present work, we aimed to identify potential *Lactobacillus* strains isolated from Inner Mongolian cheese and investigate their probiotic properties.

Materials and methods

Experimental material and bacterial strains

Four *Lactobacillus* strains, namely KC96, LP88, NS24, and LP38, were isolated from the traditional cheese of Inner Mongolia in China. These isolates were primarily subjected to microscopic observation followed by Gram-staining and then catalase reactions. The identified Gram-positive isolates associated with negative catalase reaction and non-motile bacterial rods were screened and then subjected to species level identification by using 16S rRNA sequence analysis. In addition, *L. rhamnosus* GG (LGG) was used as a reference strain.

The samples were collected in sterile carriers and stored on ice until delivery to the laboratory. Once delivered to the laboratory, they were taken to the procedure for isolation. Pour plate technique was used to isolate the microorganisms. Samples were used directly and also diluted to 10^{-1}, 10^{-2}, and 10^{-3} using sterile peptone water. One mL of aliquot of the samples and dilutions were plated onto MRS (Man, Rogosa, and Sharpe) agar (pH 6.2 and 5.5), TPY (Trypticase Phytone Yeast) agar (pH 6.5), and MRS-cysteine agar (pH 5.5). The plates were incubated at 37°C for 3 d under anaerobic conditions (in anaerobe jar using Oxoid anaerogen compact). The use of these mediums was aimed to isolate and enumerate *lactobacilli*, *streptococci*, and *enterococii*. After incubation, individual colonies were selected and transferred into sterile broth media. The following step is purifying the selected colonies with streak plate technique. The isolates were examined based on their colony morphology, catalase reaction, and Gram reaction. Gram-positive and catalase negative cocci and bacilli colonies were stored in glycerol stocks, and served as lactic acid bacteria.

Resistance to pH and bile concentration

The acid and bile resistance was determined according to Anderson *et al.* (2010) with slight changes. The *lactobacilli* were cultured overnight and then harvested through centrifugation at 4,000 *g* for 10 min at 4°C. A two-step washing with sterile saline solution (0.85% NaCl (w/v)) was performed followed by resuspension of the pellets in MRS broth (pH 6.6) or MRS adjusted to pH 2 or 3. The resultant mixtures were then incubated at 37°C for 4 h. For bile tolerance assays, *lactobacilli* were supplemented with MRS broth containing three different concentrations of ox gall (Sigma, USA); 0.1, 0.3, and 0.5% (w/v). The viable counts of *lactobacilli* were investigated using the plate count method on MRS agar. Finally, the plates were subjected to further incubation at 37°C for 48 h under anaerobic conditions.

Bacterial adhesion to hydrocarbons

The hydrocarbon assay of bacterial adhesion was investigated using the protocol described by Zoueki *et al.* (2010) with moderate changes. Selected strains were cultivated in the MRS broth at 37°C for 18 h, and harvested with centrifugation at 4,000 *g* for 10 min at 4°C. The pellets were subjected to a double step washing with PBS (pH 7.4), and the cell density for each sample was adjusted up to OD_{600} = 0.4 (A_0). The final cell suspension was mixed with xylene (1 mL), chloroform, and vinyl acetate. The suspension mixture was thoroughly vortexed for 1 min, and allowed to separate into two phases. The determination of the aqueous phase absorbance was observed at 600 nm (A_1). The cell surface hydrophobicity (H%) was measured using Eq. 1:

$$H\% = (A_0 - A_1)/A_0 \times 100 \quad \text{(Eq. 1)}$$

Three categories, including low (0 - 35%), medium (36 - 70%), and high (71 - 100%) were classified based on the bacterial surface's hydrophobic characteristics.

Antimicrobial activity

To investigate the antimicrobial activity of the selected LAB strains, five enteric pathogens namely *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Salmonella typhi* were used as indicator strains. The agar disc diffusion assay was performed following the instructions given by Bokhari *et al.* (2017). Nutrient

agar plates were prepared. Inoculum of 0.2 mL from the broth containing each of the indicator pathogens was poured onto each of the agar plates. The disc was placed on the agar plate, and then 0.5 mL of LAB strain under study was poured onto the disc on agar plates (five strains in each plate), and then the nutrient agar plates were incubated at 37°C for 24 h. At the end of the incubation, the plates were observed for the presence or absence of inhibition zones around the disc.

Determination of antibiotic susceptibility

The sensitivity of the LAB strains to antibiotics namely gentamicin (GM), vancomycin (VA), ampicillin (AM), penicillin (P), levofloxacin (LVX), and tetracycline (TE) was tested by disc diffusion method (Das *et al.,* 2016). Tests were performed in triplicate.

In vitro antioxidant assay of the isolated LAB strains
Preparation of strains and intracellular cell-free extracts

The five selected LAB strains were cultured in MRS broth at 37°C for 18 h, and harvested by centrifugation (6,000 *g*, 10 min, 4°C), followed by two consecutive washing with PBS. The cells were resuspended again in PBS solution. The bacterial load of the cell pellet was adjusted to 10^{10} CFU/mL. Following the protocol of Lin and Yen (1999) with slight modifications, the intracellular cell-free extracts were prepared. Bacterial cells exhibiting 10^{10} CFU/mL were further incubated with lysozyme (1 mg/mL) at 37°C for 30 min and then subjected to ultrasonication (JY92-IIDN, Ningbo Scientz Biotechnology Co. Ltd., China). The disruption by sonication was investigated using five times (1 min) duration within ice bath. The cell debris was removed by centrifugation (8,000 *g*, 10 min, 4°C), and then the resulting supernatant was used as the intracellular cell-free extract of the selected LAB strains.

Hydroxyl ion scavenging capacity of the probiotic bacteria

Hydroxyl ion (OH-) scavenging analysis was performed following the procedures described by Wang *et al.* (2008). OH- was prepared based on Fenton reaction using the mechanism of $FeSO_4$ and H_2O_2. The reaction mixture contained 0.5 mL of $FeSO_4$ (8 mM), 0.5 mL distilled water, and 0.8 ml H_2O_2 (6 mM). Each bacterial treatment (1.0 mL) and 0.2 mL sodium salicylate (20 mM) was mixed and added to the reaction mixture, and then incubated at 37°C for 1 h. The mixture absorbance was observed at 562 nm. The scavenging capacity of the cells was measured using Eq. 2:

$$\text{Scavenging or inhibition rate \%} = [1 - (A_1 - A_2)/A_0] \times 100 \quad \text{(Eq. 2)}$$

where, A_0 = absorbance obtained from the control group, A_1 = absorbance after addition of the sample, and A_2 = absorbance value without sodium salicylate.

Scavenging activity of DPPH free radical

The DPPH free radical-scavenging activity of LAB strains was investigated following the method presented by Kao and Chen (2006) with minor modifications. Briefly, 1.0 mL of LAB strain exhibiting 10^{10} CFU/mL, was subjected to 2.0 mL of ethanolic DPPH radical solution (0.05 mM). The reaction mixture was regularly mixed and then transferred to a dark room at 37°C for 30 min. The de-ionised water and DPPH solution alone were used as controls, while the blanks were prepared by adding only ethanol and the cells. The resulting solutions were then analysed by measuring the absorbance at 517 nm following centrifugation at 8,000 *g* for 10 min. Each experiment was performed in independent biological replicates. The DPPH scavenging capacity was calculated using Eq. 3:

$$\text{Scavenging activity (\%)} = [1 - (A_{\text{sample}} - A_{\text{blank}})/A_{\text{control}}] \times 100 \quad \text{(Eq. 3)}$$

Identification of Lactobacillus strains using PCR

DNA was extracted from the overnight LAB cultures using Takara DNA Kit following the manufacturer instruction. The amplification of the *Lactobacillus* specific gene was performed with LA tag polymerase (Takara Beijing, China) using the primers set as follow: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) as described by Das *et al.* (2016) with slight modifications. A 20 µL PCR mix was prepared to consist of 2 µL of sample (template) DNA, 2 µL of 10× PCR buffer, 2 µL of dNTPs, 1 µL of each primer (forward and reverse), 0.4 µL LA *Taq* polymerase, and 11.6 µL double distilled water. The PCR conditions were: initial step of denaturation at 94°C for 2 min followed by 35 cycles of heating at 94°C (20 s), primer annealing at 53°C (20 s), and extension at 70°C for 1.5 min. The final extension step was carried out at 70°C for 5 min. The presence of specific bands was confirmed on 1% agarose gel electrophoresis using 2 and 15 kb DNA ladder. The resultant PCR products were sent for Sanger sequencing to confirm any base mutation. Upon successful sequencing, the resultant amplicons were further analysed through phylogenetic analysis by investigating sequence homology using BLASTn and Clustal W for multiple sequence alignment.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993), and a phylogenetic tree was created by using MEGA 7 software (Kumar *et al.,* 2016).

Statistical analysis

For all the experiment, tests were performed in triplicate. All data were expressed as means ± standard deviations. Statistical comparisons were made using the statistical software package Statistix 8.1. The significant differences between treatments were tested by analysis of variance (ANOVA) followed by a comparison between treatments performed using least significance difference (LSD) method, with levels of significance $p < 0.05$.

Results

Cell viability in gastric juice

Cell viability was determined for the strains (KC96, LP88, NS24, LP38, and a reference strain (LGG) at different pH's and bile concentrations using the pour plate method (Anderson *et al.,* 2010) in which colony forming units (CFUs) were measured 48 h after incubation at 37°C. The results are shown in Table 1, indicating that most of the LAB isolates were more sensitive to pH 2 but less sensitive to pH 3. All strains showed significant survival when incubated at pH 3, and among them, strain LP38 survived significantly better ($p < 0.05$) than other strains. At pH 2, the cell viability was measured at $\log_{10}$ 5.01 ± 0.01 CFU/mL, while it was $\log_{10}$ 8.97 ± 0.03 CFU/mL at pH 3 after incubation for 4 h. In addition, the viability of strain LP38 in bile medium containing different concentrations of ox gall was also found to be higher than that of most of the selected strains. Strain LP38 showed more stability at 0.1% ox gall ($\log_{10}$ 9.78 ± 0.03 CFU/mL) than at 0.3 and 0.5% ox gall (w/v). From these results, it was concluded that strain LP38 was the most competent in the gastric environment from the strains tested.

Bacterial adhesion

Bacterial adhesion to hydrocarbons was determined following the instructions of Zoueki *et al.* (2010) with minor modifications. LGG was used as a reference strain to characterise the cell surface hydrophobicity of the selected strains under different solvents such as xylene, chloroform, and vinyl acetate. The results shown in Table 2 indicate that the maximum hydrophobicity was of strain LP38 when compared with the other strains. The basic (electron donor) and acidic (electron acceptor) properties of the selected bacterial cell surface were analysed by calculating the bacterial separation from chloroform, a monopolar acidic solvent (electron acceptor), and

Table 1. The comparative survival effects of *Lactobacillus* strains (log10 CFU/mL) against acid and bile tolerance.

Strains	pH 2.0 (4 h)	pH 3.0 (4 h)	0.1% ox gall (4 h)	0.3% ox gall (4 h)	0.5% ox gall (4 h)
KC96	4.66 ± 0.01^{b}	8.34 ± 0.02^{e}	9.34 ± 0.02^{b}	7.72 ± 0.07^{d}	7.36 ± 0.03^{d}
LP88	4.44 ± 0.02^{c}	8.77 ± 0.05^{c}	9.45 ± 0.06^{a}	8.34 ± 0.05^{a}	7.68 ± 0.01^{b}
NS24	4.95 ± 0.02^{a}	8.29 ± 0.06^{a}	9.51 ± 0.02^{a}	8.32 ± 0.06^{a}	7.48 ± 0.01^{c}
LP38	5.01 ± 0.01^{a}	8.97 ± 0.03^{b}	9.78 ± 0.03^{a}	8.56 ± 0.02^{c}	7.97 ± 0.02^{a}
LGG	4.61 ± 0.05^{b}	8.66 ± 0.02^{d}	9.35 ± 0.01^{b}	8.19 ± 0.01^{b}	7.65 ± 0.01^{b}

Data are means ± standard deviations. Means in the same column with the same letters do not significantly differ ($p < 0.05$).

Table 2. Surface hydrophobicity (H%) of *Lactobacillus* strains in different hydrocarbons.

Strains	H% in Xylene	H% in Chloroform	H% in Vinyl acetate
KC96	48.84 ± 1.97^{b}	53.82 ± 2.39^{bc}	48.05 ± 1.86^{a}
LP88	45.55 ± 1.84^{c}	60.01 ± 0.10^{ab}	45.69 ± 1.08^{ab}
NS24	51.15 ± 0.68^{b}	63.17 ± 1.45^{a}	41.74 ± 2.09^{bc}
LP38	58.09 ± 0.58^{a}	65.25 ± 0.54^{a}	49.40 ± 3.13^{a}
LGG	33.25 ± 0.87^{d}	48.08 ± 3.35^{c}	37.09 ± 2.60^{c}

Data are means ± standard deviations. Means in the same column with the same letters do not significantly differ ($p < 0.05$).

vinyl acetate, a basic monopolar solvent (electron donor). Collectively, our findings suggest that strains NS24 and LP38 showed various degrees of hydrophobicity in response to chloroform supplementation

Antimicrobial activity

An agar disk diffusion assay was used to detect antimicrobial properties according to Bokhari *et al.* (2017) of the LAB isolates (Table 3) against five pathogenic bacteria namely *Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Salmonella typhi*. The inhibition zone diameter was determined, which indicated the antimicrobial effect of the selected strains against each pathogen. Strain LP38 showed a high range of inhibition zone diameters against the tested pathogens.

Antibiotic susceptibility

Antibiotic susceptibility was determined according to Das *et al.* (2016). The results in Table 4 show that the tested strain KC96 was resistant to all antibiotics; isolate LP88 was susceptible to (AM) and (P); isolate NS24 was susceptible to (AM), (P), and (TE); isolate LP38 was susceptible to (AM) and (P); and the reference strain LGG was susceptible to (P) and (TE).

.

Hydroxyl radical scavenging capacity

The results of hydroxyl radical scavenging using the intracellular cell-free extracts of the five selected LAB isolates are shown in Figure 1A. All five strains showed considerable hydroxyl radical scavenging activity in a dose-dependent manner with a test concentration of 10^{10} CFU/mL. LP38 demonstrated the maximum hydroxyl radical scavenging capacity, up to 66.66% at 10^{10} CFU/mL. Strains KC96, LP88, NS24, and reference strain LGG also effectively scavenged hydroxyl radicals.

DPPH free radical scavenging assay

The ability of the five strains to scavenge DPPH free radicals was measured at 10^{10} CFU/mL (Figure 1B). Strain LP38 showed the highest radical scavenging activity (45.12%). The other strains showed minimum DPPH radical scavenging activities, including the reference strain LGG.

Identification of Lactobacillus strains using PCR

Four potential probiotics were identified by PCR amplification using 16S rRNA as a reference gene. Investigation of PCR products on 1% agarose gel revealed a band size between 1,400 and 1,600 bp. Based on multiple sequence alignment results, the identified bacteria were designated to the genus *Lactobacillus*. For further molecular identification,

Table 3. Antibacterial activity of *Lactobacillus* strains.

Strains	*Klebsiella pneumonia*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aurous*	*Salmonella typhii*
KC96	8.33 ± 0.34^{c}	7.03 ± 0.13^{c}	8.09 ± 0.21^{c}	4.31 ± 0.13^{b}	6.55 ± 0.24^{c}
LP88	7.08 ± 0.08^{d}	4.62 ± 0.45^{d}	6.97 ± 0.13^{d}	3.75 ± 0.29^{c}	5.45 ± 0.22^{d}
NS24	7.00 ± 0.22^{d}	6.84 ± 0.21^{c}	8.43 ± 0.37^{c}	4.22 ± 0.17^{b}	4.95 ± 0.08^{d}
LP38	11.24 ± 0.20^{a}	10.04 ± 0.28^{a}	10.00 ± 0.11^{a}	4.32 ± 0.11^{b}	9.23 ± 0.20^{a}
LGG	9.48 ± 0.29^{b}	8.39 ± 0.36^{b}	9.12 ± 0.10^{b}	5.10 ± 0.23^{a}	8.49 ± 0.33^{b}

Data are means ± standard deviations. Means in the same column with the same letters do not significantly differ ($p < 0.05$).

Table 4. Growth of LAB strains on MRS agar plate against antibiotics.

Strains	Gentamicin (GM)	Vancomycin (VA)	Ampicillin (AM)	Penicillin (p)	Levofloxacin (LVX)	Tetracycline (TE)
KC96	R	R	R	R	R	R
LP88	R	R	S	S	R	S
NS24	R	R	S	S	R	S
LP38	R	R	S	S	R	R
LGG	R	R	R	S	R	S

Note: R = Resistance; S = Susceptible.

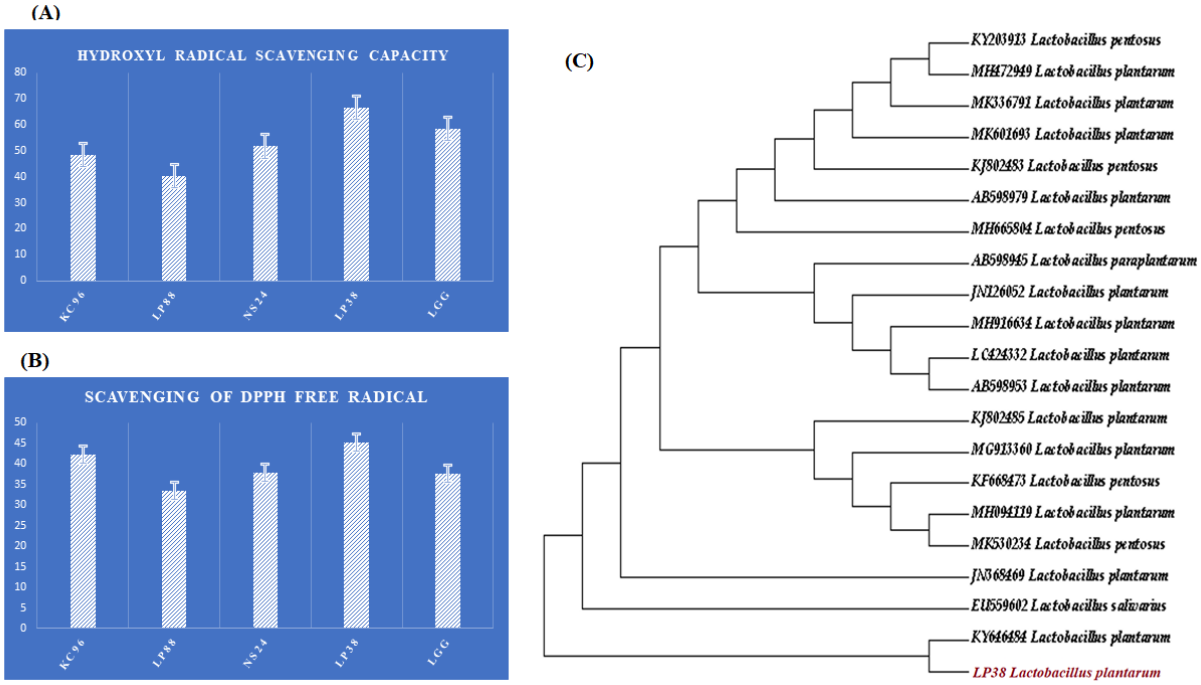

Figure 1. (A) The representation of free radical scavenging capacity of the Hydroxyl ion of tested LAB strains, (B) DPPH free radical scavenging activity of tested LAB strains obtained during our study, and (C) The identification of evolutionary relationship of *L. plantarum* LP38 through molecular phylogenetic analysis using Maximum Likelihood (ML) with 1000 bootstrap method. The reconstruction of the phylogenetic tree consists of similar sequences of *L. plantarum* with those of other *Lactobacillus* species obtained from GenBank (NCBI).

phylogenetic analysis was performed using 16S rRNA sequences. A maximum likelihood phylogenetic tree was created to analyse the evolutionary history of the selected isolates (Figure 1C), and strain LP38 showed high homology with *L. plantarum* KY646484.

Discussion

In the present work, different LAB strains of potential probiotic bacteria were characterised, and a competent *Lactobacillus* strain was identified. To characterise different potential LAB strains, the viability of cells in the gastric environment, cell surface hydrophobicity, antioxidant activity, antimicrobial activity, and antibiotic activity were determined.

Cell viability was determined for LAB strains (KC96, LP88, NS24, LP38, and control LGG) at different pH's and bile concentrations using the pour plate method. In our results, LAB strain LP38 was the most competent in the gastric environment. In a previous study, the bile concentration present in the intestine was shown to affect the viability of LAB (Lin *et al.,* 2007). Although human bile juice composition has been shown to be different from that of 0.3% ox gall solution, most researchers have used ox gall as a substitute for human bile in laboratory testing (Lin *et al.,* 2006). In the present work, we supplemented the selected LAB strains in MRS broth with 0.1, 0.3, and 0.5% ox gall to analyse their bile tolerance. The effect of bile was tested on the growth of the selected isolates after 4 h of incubation (Table 1). Previous studies have shown the disruption of various biomolecules within the cell including DNA, proteins, and fatty acids by hydrochloric acid present in the human stomach (Hassanzadazar *et al.,* 2012). These studies also showed that a low bile concentration and low pH could lead to metabolic inhibition of LAB, thus causing a reduction in their growth and viability. Similarly, encountering gastric acid with pH less than 2 after 4 h incubation may also lead to the inhibition of bacterial viability (Hassanzadazar *et al.,* 2012).

Bacterial adhesion to hydrocarbons was analysed following a protocol described by Zoueki *et al.* (2010) with some modifications. Our results showed that as compared to the other LAB strains, LAB strain LP38 was highly hydrophobic (Table 2). Cell hydrophobicity is a key factor in bacterial adhesion to host tissues. A previous study identified that the sensitivity of bacterial surface hydrophobicity to certain environmental factors such as pH and ionic strength might affect their adherence potential in response to various hydrophobic substrates (Popat *et al.,* 2007; Puckett *et al.,* 2010). In the present work, we efficiently identified LAB strains demonstrating

high levels of cell-surface hydrophobicity (Table 2).

The production of antimicrobial compounds against five pathogens namely *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Salmonella typhi* was determined by agar well diffusion assay. The use of LAB as natural bioprotective agents has been well-documented in several studies (Zuo *et al.,* 2016). The ability to produce various antimicrobial compounds is a property of bacteria through which they can significantly reduce pathogenic microorganisms in the host intestine, thus, producing a probiotic effect in the host system (Argyri *et al.,* 2013). In the present work, antimicrobial activity of the isolated LAB strains was tested against the listed pathogens, and almost all of the tested LAB strains were observed to inhibit their growth (Table 3). As compared to the other tested LAB strains, the LAB strain LP38 exhibited the maximum inhibitory effect against the pathogens. Our results are consistent with those of previous studies, in which LAB bacteria were found to have a highly inhibitory effect on Gram-negative pathogens (Bokhari *et al.,* 2017).

The antibiotic activity of the LAB strains was determined by the method of Das *et al.* (2016). LAB strain KC96 was resistant to all antibiotics; LAB strain LP88 was susceptible to (AM) and (P); LAB strain NS24 was susceptible to (AM), (P), and (TE); LAB strain LP38 was susceptible to (AM) and (P); and the reference strain LGG was susceptible to (P) and (TE). Resistance of our strains to gentamicin and vancomycin is similar to that of LAB isolates reported previously (Argyri *et al.,* 2013), and resistance to vancomycin and levofloxacin is also similar (Argyri *et al.,* 2013).

All the selected strains in the present work demonstrated considerable scavenging of certain free radicals. However, we observed two strains with notably increased antioxidant activities in a dose-dependent manner (Figure 1A). Previous studies have shown that oxidative stress is associated with pathology in cancer, arthritis, cirrhosis, atherosclerosis, and emphysema (Mosley *et al.,* 2006; Alpsoy and Yalvac, 2011). A growing number of studies have shown that the use of *Lactobacillus* and *Bifidobacterium* spp. as natural antioxidants is crucial in promoting human health by minimising the risk of reactive oxygen species accumulation during food ingestion (Mosley *et al.,* 2006; Lin *et al.,* 2009; Lee *et al.,* 2010).

Furthermore, evidence has shown that the antioxidant capacity of cell-free extract is much higher than that of intact cells (Chen *et al.,* 2010). Therefore, we prepared cell-free extracts of four *Lactobacillus* isolates to investigate antioxidant assays, including hydroxyl and DPPH, *in vitro*. Based on our findings, it is clear that the cell-free extracts of LAB strain LP38 (Figure 1A and 1B) demonstrated an increasing tendency toward hydroxyl radicals and DPPH scavenging. The free radical scavenging capacity of our LAB strains could be due to the presence of intracellular antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase. However, some non-enzymatic antioxidant components may also be produced in the cell lysates of LAB, including glutathione and thioredoxin. Moreover, the antioxidant ability of intact LAB cells may be associated with the production of certain compounds on the cell surface, including extracellular polysaccharides produced by *Lactococcus lactis* subsp. *lactis* 12 (Pan and Mei, 2010), *Bifidobacteria*, RH (Xu *et al.,* 2011), and lipoteichoic acid from the cell surface of *Bifidobacteria* (Yi *et al.,* 2009).

Several traditional methods have been used to identify natural probiotics in the gastrointestinal tract of humans such as serotyping, colony morphology, and fermentation patterns. These classical techniques are highly recommended for selection, differentiation, and biochemical assessment. However, with the help of recent advances in molecular detection and typing, discovery and identification of novel bacterial strains are becoming easier and more reliable. Furthermore, molecular detection strategies are strong enough to differentiate members of closely related species. A variety of molecular detection methods have been adopted to classify the strains, including species-specific hybridisation probes (Klaenhammer and Kullen, 1999). Other PCR-based approaches include PCR ribotyping, PCR-RFLP, RAPD, and REP-PCR. Among all these techniques, the most efficient and powerful is molecular tool (Bulut, 2003). Nonetheless, amidst all these techniques, the most efficient and powerful molecular tool is sanger sequencing (Coeuret *et al.,* 2003). Woese (1987) was the first to characterise new strains following 16S rRNA sequencing. Since then, phylogenetic classification of bacteria has developed simply by using targeted oligonucleotide probes for 16S or 23S rRNA. The 16S rRNA gene is approximately 1,540 bp, and it contains different regions exhibiting structural conservation. Owing to the broad range of probe specificity associated with different species, it is important to highlight the role of the 16S rRNA gene to clarify phylogenetic relationships between different groups of microorganisms and classify them more accurately (Charteris *et al.,* 1997; Holzapfel *et al.,* 1998; Çakır, 2003).

Conclusion

Our findings suggest that at least one of the selected *Lactobacillus* strains from Inner Mongolian cheese is a probiotic with considerable antioxidant capacity. The investigated strains exhibited prominent tolerance against acid and bile, and showed remarkable microbial adhesion and antimicrobial properties. The *Lactobacillus* strain LP38 demonstrated the highest antioxidant activity among the strains tested, indicating its potential role as a crucial supplement in food to prevent various diseases. Further research is required on the fermented products of these strains to highlight their health benefits. It is possible that there are additional LAB strains present in Inner Mongolian cheese that still need to be identified and characterised.

Acknowledgement

The present work was financially supported by the Jilin Provincial Science and Technology Department Outstanding Young Talents Fund Project (Grant no. 20190103130JH).

References

Alpsoy, L. and Yalvac, M. E. 2011. Key roles of vitamins A, C, and E in aflatoxin B1-induced oxidative stress. Vitamins and Hormones 86: 287-305.

Anderson, R. C., Cookson, A. L., McNabb, W. C., Kelly, W. J. and Roy, N. C. 2010. *Lactobacillus plantarum* DSM 2648 is a potential probiotic that enhances intestinal barrier function. FEMS Microbiology Letters 309(2): 184-192.

Argyri, A. A., Zoumpopoulou, G., Karatzas, K. A., Tsakalidou, E., Nychas, G. J., Panagou, E. Z. and Tassou, C. C. 2013. Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. Food Microbiology 33(2): 282-291.

Bokhari, D. M., Khan, H., Riaz, M. N., Ullah, A. and Yunus, A. W. 2017. Probiotic characterization of the *Lactobacillus* isolates from the gastrointestinal tract of poultry in Pakistan. Pakistan Journal of Agricultural Sciences 54(4): 881-886.

Bulut, Ç. 2003. Isolation and molecular characterization of lactic acid bacteria from cheese. Turkey: İzmir Institute of Technology, MSc thesis.

Çakır, İ. 2003. Determination of some probiotic properties on *Lactobacilli* and *Bifidobacteria*. Turkey: Ankara University, PhD thesis.

Charteris, W. P., Kelly, P. M., Morelli, L. and Collins, J. K. 1997. Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. International Journal of Food Microbiology 35(1): 1-27.

Chen, L. S., Ma, Y., Chen, L. J., Zhao, C. H., Maubois, J. L., Jiang, T. M., ... and He, S. H. 2010. Antioxidant activity of two yeasts and their attenuation effect on 4-nitroquinoline 1-oxide induced *in vitro* lipid peroxidation. International Journal of Food Science and Technology 45(3): 555-561.

Coeuret, V., Dubernet, S., Bernardeau, M., Gueguen, M. and Vernoux, J. P. 2003. Isolation, characterisation and identification of *lactobacilli* focusing mainly on cheeses and other dairy products. Le Lait 83(4): 269-306.

Das, P., Khowala, S. and Biswas, S. 2016. *In vitro* probiotic characterization of *Lactobacillus casei* isolated from marine samples. LWT 73: 383-390.

Díaz-Ropero, M. P., Martin, R., Sierra, S., Lara-Villoslada, F., Rodriguez, J. M., Xaus, J. and Olivares, M. 2007. Two *Lactobacillus* strains, isolated from breast milk, differently modulate the immune response. Journal of Applied Microbiology 102(2): 337-343.

Food and Agriculture Organization (FAO) and World Health Organization (WHO). 2002. Guidelines for the evaluation of probiotics in food. Report of a Joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. Canada: FAO and WHO.

Fuller, R. 1989. Probiotics in man and animals. The Journal of Applied Bacteriology 66(5): 365-378.

Hassanzadazar, H., Ehsani, A., Mardani, K. and Hesari, J. 2012. Investigation of antibacterial, acid and bile tolerance properties of *lactobacilli* isolated from Koozeh cheese. Veterinary Research Forum 3(3): 181-185.

Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U. and Huis in't Veld, J. H. 1998. Overview of gut flora and probiotics. International Journal of Food Microbiology 41(2): 85-101.

Hoque, M. Z., Akter, F., Hossain, K. M., Rahman, M. S. M., Billah, M. M. and Islam, K. M. D. 2010. Isolation, identification and analysis of probiotic properties of *Lactobacillus* spp. from selective regional yoghurts. World Journal of Dairy and Food Sciences 5(1): 39-46.

Kao, T. H. and Chen, B. H. 2006. Functional components in soybean cake and their effects on antioxidant activity. Journal of Agricultural and Food Chemistry 54(20): 7544-7555.

Klaenhammer, T. R. and Kullen, M. J. 1999.

Selection and design of probiotics. International Journal of Food Microbiology 50(1-2): 45-57.

Kotani, Y., Shinkai, S., Okamatsu, H., Toba, M., Ogawa, K., Yoshida, H., ... and Kohda, N. 2010. Oral intake of *Lactobacillus pentosus* strain b240 accelerates salivary immunoglobulin A secretion in the elderly: a randomized, placebo-controlled, double-blind trial. Immunity and Ageing 7: article ID 11.

Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33(7): 1870-1874.

Lee, B.-J., Kim, J.-S., Kang, Y. M., Lim, J.-H., Kim, Y.-M., Lee, M.-S., ... and Je, J.-Y. 2010. Antioxidant activity and γ-aminobutyric acid (GABA) content in sea tangle fermented by *Lactobacillus brevis* BJ20 isolated from traditional fermented foods. Food Chemistry 122(1): 271-276.

Lin, M. Y. and Yen, C. L. 1999. Antioxidative ability of lactic acid bacteria. Journal of Agricultural and Food Chemistry 47(4): 1460-1466.

Lin, P. W., Myers, L. E., Ray, L., Song, S. C., Nasr, T. R., Berardinelli, A. J., ... and Neish, A. S. 2009. *Lactobacillus rhamnosus* blocks inflammatory signaling *in vivo* via reactive oxygen species generation. Free Radical Biology and Medicine 47(8): 1205-1211.

Lin, W. H., Hwang, C. F., Chen, L. W. and Tsen, H. Y. 2006. Viable counts, characteristic evaluation for commercial lactic acid bacteria products. Food Microbiology 23(1): 74-81.

Lin, W. H., Yu, B., Jang, S. H. and Tsen, H. Y. 2007. Different probiotic properties for *Lactobacillus fermentum* strains isolated from swine and poultry. Anaerobe 13(3-4): 107-113.

Mosley, R. L., Benner, E. J., Kadiu, I., Thomas, M., Boska, M. D., Hasan, K., ... and Gendelman, H. E. 2006. Neuroinflammation, oxidative stress, and the pathogenesis of Parkinson's disease. Clinical Neuroscience Research 6(5): 261-281.

Ouwehand, A. C., Tuomola, E. M., Tölkkö, S. and Salminen, S. 2001. Assessment of adhesion properties of novel probiotic strains to human intestinal mucus. International Journal of Food Microbiology 64(1-2): 119-126.

Pan, D. and Mei, X. 2010. Antioxidant activity of an exopolysaccharide purified from *Lactococcus lactis* subsp. lactis 12. Carbohydrate Polymers 80(3): 908-914.

Popat, K. C., Eltgroth, M., LaTempa, T. J., Grimes, C. A. and Desai, T. A. 2007. Decreased *Staphylococcus epidermis* adhesion and increased osteoblast functionality on antibiotic-loaded titania nanotubes. Biomaterials 28(32): 4880-4888.

Puckett, S. D., Taylor, E., Raimondo, T. and Webster, T. J. 2010. The relationship between the nanostructure of titanium surfaces and bacterial attachment. Biomaterials 31(4): 706-713.

Quwehand, A. and Vesterlund, S. 2004. Antimicrobial components from lactic acid bacteria. In Salminen, S., von Wright, A. and Ouwehand, A. (eds). Lactic Acid Bacteria Microbiological and Functional Aspects. United States: Marcel Dekker Inc.

Saad, N., Delattre, C., Urdaci, M., Schmitter, J. M. and Bressollier, P. 2013. An overview of the last advances in probiotic and prebiotic field. LWT - Food Science and Technology 50(1): 1-16.

Salminen, S., Nybom, S., Meriluoto, J., Collado, M. C., Vesterlund, S. and El-Nezami, H. 2010. Interaction of probiotics and pathogens - benefits to human health? Current Opinion in Biotechnology 21(2): 157-167.

Salminen, S. and von Wright, A. 2004. Lactic acid bacteria: microbiological and functional aspects. United States: CRC Press.

Tamura, K. and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10(3): 512-526.

Wang, H., Gao, X. D., Zhou, G. C., Cai, L. and Yao, W. B. 2008. *In vitro* and *in vivo* antioxidant activity of aqueous extract from *Choerospondias axillaris* fruit. Food Chemistry 106(3): 888-895.

Woese, C. R. 1987. Bacterial evolution. Microbiology Reviews 51(2): 221-271.

Wouters, J. T. M., Ayad, E. H. E., Hugenholtz, J. and Smit, G. 2002. Microbes from raw milk for fermented dairy products. International Dairy Journal 12(2-3): 91-109.

Xu, R., Shen, Q., Ding, X., Gao, W. and Li, P. 2011. Chemical characterization and antioxidant activity of an exopolysaccharide fraction isolated from *Bifidobacterium animalis* RH. European Food Research and Technology 232: 231-240.

Yi, Z. J., Fu, Y. R., Li, M., Gao, K. S. and Zhang, X. G. 2009. Effect of LTA isolated from bifidobacteria on D-galactose-induced aging. Experimental Gerontology 44(2): 760-765.

Zoueki, C. W., Tufenkji, N. and Ghoshal, S. 2010. A modified microbial adhesion to hydrocarbons assay to account for the presence of hydrocarbon droplets. Journal of Colloid and Interface Science 344(2): 492-496.

Zuo, F., Yu, R., Feng, X., Chen, L., Zeng, Z.,

Khaskheli, G. B., … and Chen, S. 2016. Characterization and *in vitro* properties of potential probiotic Bifidobacterium strains isolated from breast-fed infant feces. Annals of Microbiology 66: 1027-1037.