

Research Article

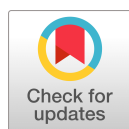
In Vitro Assessment of Probiotic Properties for Lactic Acid Bacteria Isolated from Korean Traditional Fermented Food, *Kimchi*

Eui-Sang Cho¹, Jeonghye Chun¹, Jeongin Park², Mibang Kim¹,
Chi Young Hwang¹, Deok Jun Yoon¹, Inonge Noni Siziya³, Dong-Ho Seo^{3,*}
and Myung-Ji Seo^{1,2,*}

¹Department of Bioengineering and Nano-Bioengineering, Graduate School of Incheon National University, Incheon 22012, Korea

²Division of Bioengineering, Incheon National University, Incheon 22012, Korea

³Department of Food Science and Technology, College of Agriculture and Life Sciences, Jeonbuk National University, Jeonju 54896, Korea



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*Corresponding author :

Dong-Ho Seo
Department of Food Science and
Technology, College of
Agriculture and Life Sciences,
Jeonbuk National University,
Jeonju 54896, Korea.
Tel: +82-63-270-2571,
E-mail: dhseo@jnu.ac.kr

Myung-Ji Seo
Division of Bioengineering,
Incheon National University,
Incheon 22012, Korea.
Tel: +82-32-835-8267,
E-mail: mjseo@inu.ac.kr

ORCID

Eui-Sang Cho
<https://orcid.org/0000-0001-6033-2240>
Jeonghye Chun
<https://orcid.org/0000-0002-1451-2192>
Jeongin Park
<https://orcid.org/0000-0002-5281-6066>
Mibang Kim
<https://orcid.org/0000-0003-0317-6821>
Chi Young Hwang
<https://orcid.org/0000-0001-9132-0293>
Deok Jun Yoon
<https://orcid.org/0000-0001-9770-0713>
Inonge Noni Siziya
<https://orcid.org/0000-0002-0014-2557>
Dong-Ho Seo
<https://orcid.org/0000-0003-0423-5686>
Myung-Ji Seo
<https://orcid.org/0000-0003-4100-1504>

Abstract

In this study, thirty-eight lactic acid bacteria (LAB) strains were isolated from *Kimchi* (Korean traditional fermented food) and screened for probiotic characterization in a series of *in vitro* tests, including resistance to low pH, tolerance to bile salts, hydrophobicity, antibiotic resistance and antioxidant activity. Firstly, twenty-two strains displayed survival in acid and bile passage conditions. Secondly, the strains KCCP 11349 and KCCP 11356 were observed to have high cell surface hydrophobicity with 63.9 and 98.8 %, respectively. Functionality of these potential probiotic isolates was supported by their antioxidant activity. Altogether, the strain KCCP 11349 showed characteristics similar to or even better than reference strain *Lactobacillus rhamnosus* GG. Finally, 16S rRNA gene sequencing was conducted to identify potential probiotic strains and KCCP 11349 was identified as *Lactobacillus plantarum* subsp. *plantarum*.

Keywords

Kimchi, lactic acid bacteria, probiotic

Introduction

Nowadays, there is considerable interest in functional foods with regards to their beneficial effects on human health. Accordingly, the preference for foods containing probiotics is constantly increasing, mainly due to the numerous publications made that have linked them to the promotion of positive effects on health (Aureli *et al.*, 2011).

Kimchi is a Korean traditional fermented food classified as one of the healthiest foods as it is rich in vitamins, and has a variety of beneficial effects such as aiding in digestion, and cancer prevention. Several researchers have previously isolated and studied various probiotics from kimchi, particularly Lactic acid bacteria (LAB) (Choi *et al.*, 2002; Hwang

et al., 2000; Kim *et al.*, 2002). With regards to LAB and other ingredients used in kimchi, the nutritional, functional and organoleptic profiles change as fermentation progresses and when consumed, kimchi helps to restore the probiotic microbiota in the gut.

Probiotics are officially defined as: “live microorganisms which, when administered in adequate amounts, exert a beneficial effect on the health of the consumer” (FAO/WHO, 2002). Probiotics can modulate host immune responses to promote a healthy gastrointestinal tract. LAB are the most prominent microorganisms applied as probiotics (Rivera-Espinoza and Gallardo-Navarro, 2010) and they have been studied as sources of antibacterial, anticancer (Shin *et al.*, 1998), anti-diabetic (Yadav *et al.*, 2007), anti-obesity (Arora *et al.*, 2013; Kang *et al.*, 2013) and anti-oxidant (Das and Goyal, 2015; Spyropoulos *et al.*, 2011) components. The antioxidant activity of some LAB used as food components and probiotics may have a substantial impact on human health (Lin *et al.*, 2000, Oxman *et al.*, 2000).

To assess such possibilities, the aim of this study was to isolate potential probiotic strains from kimchi which show probiotic characterization and evaluate them in a series of *in vitro* tests, including low pH resistance, tolerance to bile salts, hydrophobicity, resistance to antibiotics and anti-oxidant activity.

Materials and Methods

Isolation and identification of bacteria from kimchi

Homemade kimchi was collected and serial dilutions were made in saline. The saline mixtures were spread on de Man, Rogosa and Sharpe (MRS) agar followed by overnight incubation at 30°C. To obtain a pure colony, a single representative colony was transferred to a new MRS agar plate at least three times. The pure isolates were preserved at -80°C in 25% (w/v) glycerol suspension for long-term storage.

Acid and bile salt tolerance

To determine the tolerance level of isolated LAB in low pH conditions and bile salt conditions we used the method

described by Pieniz *et al.* (2004) with slight modifications. An overnight culture of potential probiotics was centrifuged at 12,000 ×g for 5 min at 4°C. The cell pellet washed with 0.85% NaCl (w/v) solution and suspended in 0.85% NaCl (w/v) solution adjusted to pH 2.0, prior to being incubated at 37°C for 2 h. After incubation viable cell count was determined on MRS agar plate. For bile salt tolerance, a similar protocol was followed. Cell pellet was dissolved in 0.85% NaCl (w/v) solution supplemented with 1.5% and 2.0% bile salt (BD) and further incubated at 37°C for 4 h. After incubation the cell viable count was determined on MRS agar plate. The strains with acid and bile salt tolerance were primarily selected and the following experiments were conducted on the selected strains.

Bacterial adhesion to hydrocarbons (BATH)

BATH test was performed according to an established protocol with simple modifications (Collado *et al.*, 2007). Bacterial cells were washed with 0.85% NaCl (w/v) solution and re-suspended in the same solution. Initial OD₆₀₀ adjusted to 0.2 to 0.3. One milliliter of toluene was added to bacterial cells and vortexed well for about 5 min, prior to being incubated for 1 h at room temperature. When two distinct phases were observed, the aqueous phase was removed and the reading was noted. BATH% was calculated as follows:

$$\text{BATH\%} = (A - A_0) / A \times 100$$

where A and A₀ represent absorbance readings after and before mixing with toluene.

Antibiotic susceptibility test

The antibiotic susceptibilities of strains isolated from kimchi were tested using paper disc diffusion methodology (Bauer *et al.*, 1996). Overnight culture of isolates was spread onto the MRS agar plate and discs containing specific antibiotic concentrations were placed onto them. The plates were incubated overnight at 37°C for 24 h and the inhibition zones were identified. Antibiotic concentrations used in this experiment were as follows: Ampicillin (100 µg/mL), ciprofloxacin (10 µg/mL), erythromycin (25 µg/mL), gentamicin (30 µg/mL), lincomycin (15 µg/mL),



novobiocin (10 µg/mL), streptomycin (50 µg/mL) and tetracyclin (30 µg/mL).

Antioxidant properties of LAB isolates in MRS broth

The isolates were inoculated into 20 mL of MRS broth (n=3) and incubated overnight at 37°C. After centrifugation (15,000 ×g for 10 min at 4°C), the scavenging activity of the isolates was determined using the supernatant. 2,2-diphenyl-1-picrylhydrazyl (DPPH) was added to the supernatant and the antioxidant activity was quantified using a colorimetric microplate assay described by Kim *et al.* (2019). Prepared DPPH solution (0.2 mM in ethanol; Sigma Aldrich, USA) was mixed with bacterial supernatant. The reaction mixture was incubated in the dark at room temperature for 30 min. The DPPH radical scavenging activity was then detected by measuring the absorbance at 517 nm using a microplate reader. The scavenging ability was calculated as follows:

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

Identification of selected LAB

Identification of isolates was performed at the 16S rRNA gene sequencing facility of Macrogen Co., Ltd. (Seoul, Korea). The obtained DNA sequence was analyzed by using BLAST program provided by GenBank (<https://www.ncbi.nlm.nih.gov/>) and EzBioCloud server (<http://www.ezbiocloud.net/>) (Yoon *et al.*, 2017) for phylogenetic analysis. Phylogenetic analysis was conducted by MEGA 7 (Kumar *et al.*, 2016) after a gap deletion and multiple alignments of data via the CLUSTAL W program (Thompson *et al.*, 1994). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1980), and neighbor-joining (NJ) algorithm was used to construct the phylogenetic trees.

Results and Discussion

Isolation and screening of probiotic strains with acid and bile salt resistant

Thirty-eight isolates of LAB isolated from kimchi were tested for their abilities to resist high acidity at the low pH

of 2.0 and respective bile salt concentrations of 1% and 2% (w/v). All isolates were observed to have survival rates above 75% with tolerance to 1% and 2% bile salt (w/v), and of the isolates that were screened for tolerance to pH 2.0, twenty-two had an adequate survival rate of between 20 and 60% (Table 1). The bile and pH tolerance of microbiota within the gastric juice are considered among the main factors affecting the survival of probiotic bacteria upon passage through the stomach to the intestine. Interestingly, all of the strains showed high survival rate in bile salts at 1% and 2%, particularly the strain KCCP 11349, which also presented adequate viability in pH 2 and was comparable to the reference strain. Collectively, twenty-two strains of LAB recorded probiotic potential and were selected for further study.

Bacterial adhesion to hydrocarbon (Hydrophobicity)

The selected strains on the basis of their survival under *in vitro* GIT conditions, were evaluated for their hydrophobicity toward toluene that may reflect the colonization potential of the organism in the intestinal lumen. The degree of hydrophobicity was high for KCCP 11356 with the highest hydrophobicity value of 98.8% (Table 2). This value was significantly higher than *Lactobacillus rhamnosus* GG (82.8%). The hydrophobicity of the outer surface of microorganisms has been implicated in the attachment of bacteria to host tissue. This property may provide an important competitive advantage in maintaining bacteria in the human gastrointestinal tract (Singh *et al.*, 2012). Several tested strains exhibited weak hydrophobicity values for toluene, while hydrophobicity values of KCCP 11349 and KCCP 11356 presented strong hydrophobicity (63.9±0.6 and 98.8±0.3%, respectively). The variation in hydrophobicity has been reported in other probiotic strains and has been elucidated by the fact that attachment depends upon the origin of the strains as well as their surface properties (Abushelaibi *et al.*, 2017).

Antibiotic susceptibility test

The selected strains were resistant to erythromycin but only four strains were resistant to novobiocin (Table 3).



Table 1. Assessment of viability of the isolated strains after exposure to low pH and bile salts. In all tests, the probiotic *L. rhamnosus* GG KCTC 5033 served as a reference strain

Number	Strains	Initial counts	After 2 h (pH 2.0)		After 4 h (Bile salts 1%, w/v)		After 4 h (Bile salts 2%, w/v)	
			Log (cfu/mL)	Survival (%)	Log (cfu/mL)	Survival (%)	Log (cfu/mL)	Survival (%)
1	LGG	7.5	4.5	60.0	6.8	90.8	6.7	89.1
2	JK226	9.4	5.6	59.5	9.2	97.7	9.0	96.2
3	JK756	9.0	5.5	60.9	7.6	85.1	7.7	85.5
4	KCCP11031	9.0	3.1	34.6	8.3	92.2	8.3	92.0
5	KCCP11156	10.6	4.6	43.4	9.2	86.3	9.4	88.8
6	KCCP11178	9.3	2.0	20.9	9.1	97.0	9.0	96.3
7	KCCP11179	9.4	0.0	0.0	9.0	95.6	9.1	97.2
8	KCCP11180	9.5	0.0	0.0	9.2	97.4	9.3	98.6
9	KCCP11181	9.4	0.0	0.0	9.2	98.7	9.2	98.7
10	KCCP11183	9.3	2.5	26.6	8.8	94.6	9.0	96.1
11	KCCP11184	9.4	2.2	23.5	8.9	94.8	9.0	95.5
12	KCCP11186	9.3	2.4	26.2	8.8	94.8	8.7	93.1
13	KCCP11209	9.6	0.0	0.0	8.9	93.3	7.9	82.3
14	KCCP11213	9.3	1.7	17.9	8.9	96.0	9.0	96.8
15	KCCP11218	9.3	1.1	12.1	9.0	96.9	9.1	98.2
16	KCCP11219	9.3	0.0	0.0	9.1	98.4	9.1	98.5
17	KCCP11222	9.4	1.1	12.0	9.1	97.4	8.8	94.2
18	KCCP11223	8.4	2.3	27.9	7.7	91.8	7.8	93.1
19	KCCP11298	9.1	0.0	0.0	8.7	94.8	8.6	94.4
20	KCCP11303	9.3	3.0	32.1	8.5	91.4	8.6	91.7
21	KCCP11308	9.2	0.5	5.7	8.9	96.7	8.9	96.8
22	KCCP11309	9.2	5.1	55.6	8.8	95.3	8.9	96.5
23	KCCP11316	9.1	0.0	0.0	7.9	87.0	7.9	86.9
24	KCCP11317	9.4	2.8	29.6	8.7	92.9	8.6	92.4
25	KCCP11318	9.1	3.1	34.5	8.1	89.9	8.2	90.5
26	KCCP11319	9.3	4.6	50.0	8.9	96.4	9.0	96.6
27	KCCP11324	8.2	1.8	22.1	7.9	95.4	8.0	97.3
28	KCCP11349	9.3	5.8	62.4	8.9	95.6	8.7	93.3
29	KCCP11354	9.0	3.9	43.4	8.8	97.7	8.8	97.9
30	KCCP11355	9.1	0.0	0.0	8.9	98.2	8.7	95.9
31	KCCP11356	9.0	3.9	43.4	8.8	97.7	8.8	97.9
32	KCCP11370	8.7	1.0	11.5	8.2	94.0	7.8	89.4
33	KCCP11376	10.2	0.5	5.1	8.0	78.6	7.8	75.9
34	KCCP11446	9.3	4.3	46.0	8.8	94.5	9.0	96.2
35	66	9.0	3.6	40.3	8.7	97.1	8.6	96.2
36	70	9.3	4.8	51.8	8.7	93.7	8.7	93.9
37	75	8.5	0.0	0.0	7.8	92.3	7.7	91.3
38	78	8.2	2.2	27.1	6.8	82.7	6.8	82.4
39	115	9.2	0.0	0.0	7.7	83.6	7.6	82.9

**Table 2.** Percent hydrophobicity of selected strains to toluene

Number	Strains	Hydrophobicity (%)
1	LGG	82.8±0.3
2	JK226	6.5±3.0
3	JK756	0.1±0.1
4	KCCP11031	3.1±1.3
5	KCCP11178	1.6±1.0
6	KCCP11183	4.7±1.4
7	KCCP11184	1.3±0.6
8	KCCP11186	0.4±0.2
9	KCCP11213	0.7±0.7
10	KCCP11223	0.4±0.2
11	KCCP11226	96.8±0.8
12	KCCP11303	2.3±1.9
13	KCCP11309	1.8±1.4
14	KCCP11317	2.6±1.4
15	KCCP11318	0.2±0.1
16	KCCP11319	3.5±2.2
17	KCCP11324	2.5±1.2
18	KCCP11349	63.9±0.6
19	KCCP11354	7.0±1.0
20	KCCP11356	98.8±0.3
21	KCCP11446	6.1±0.2
22	66	18.7±0.3
23	70	2.4±1.0
24	78	17.1±3.3

While the resistances to ampicillin and gentamicin was similar to that of the LAB isolates reported previously (Argyri *et al.*, 2013), the other antibiotics showed different patterns for each strain.

Antioxidant properties of LAB isolates in MRS broth

The DPPH-scavenging activity of the strains is shown in Table 4. With the exception of JK226, the potential probiotic strains had a higher antioxidant potential when compared to the reference strain (*L. rhamnosus* GG). The maximum DPPH radical inhibition activity was observed the strain KCCP 11303 (86.2±1.4). From the above results we could conclude that of the antioxidant potential of probiotic strains depends on the type of strain. These

Table 3. Antibiotic susceptibility profiles of potential probiotic strain

Number	Strains	Antibiotics resistant							
		Amp	Ery	Gen	Cip	Lin	Nov	Tet	Strep
1	LGG	S ^a	R ^b	S	S	S	S	S	S
2	JK226	S	R	R	R	R	S	R	R
3	JK756	S	R	R	R	R	S	R	S
4	KCCP11031	S	R	R	R	S	S	R	S
5	KCCP11178	R	R	R	R	S	R	S	R
6	KCCP11183	R	R	R	R	S	S	S	R
7	KCCP11184	R	R	R	R	S	S	S	R
8	KCCP11186	S	R	R	R	S	R	R	R
9	KCCP11213	R	R	R	R	S	S	S	S
10	KCCP11223	S	R	R	R	S	R	R	R
11	KCCP11303	R	R	R	R	S	S	S	S
12	KCCP11309	R	R	S	R	S	S	S	S
13	KCCP11317	R	R	R	R	S	S	S	R
14	KCCP11318	S	R	R	R	S	R	S	R
15	KCCP11319	R	R	R	R	S	S	S	S
16	KCCP11324	S	R	R	R	S	R	R	R
17	KCCP11349	S	R	R	R	R	S	R	R
18	KCCP11354	S	R	R	R	R	S	S	S
19	KCCP11356	S	R	R	R	S	S	R	S
20	KCCP11446	S	R	R	R	S	S	S	S
21	66	S	R	S	S	S	S	R	S
22	70	S	R	S	S	S	S	S	S
23	78	S	R	S	S	S	S	S	S

^aSensitive, ^bResistant.

results imply that the metabolic products of LAB might be contributing to the higher antioxidant potential.

Identification of selected LAB by 16S RNA gene sequencing

Twenty-two potential probiotics were identified by 16S rRNA gene sequence. The isolates underwent molecular phylogeny analysis and phylogenetic tree construction to identify the LAB to a species level based on the 16S rDNA sequences from evolutionary distances by neighbor-joining method. The phylogenetic tree of the 22 isolates with reference strain is shown in Fig. 1. Almost all the isolates belonged to the genera *Pediococcus* and *Lactobacillus*,

Table 4. Antioxidant activity of potential probiotic strain

Number	Strains	DPPH radical scavenging activity
1	LGG	53.7±2.6
2	JK226	51.6±4.1
3	JK756	83.4±0.9
4	KCCP11031	78.5±7.4
5	KCCP11178	65.9±7.1
6	KCCP11183	69.6±12.5
7	KCCP11184	75.9±4.4
8	KCCP11186	76.3±2.9
9	KCCP11213	75.6±6.4
10	KCCP11223	80.2±2.8
11	KCCP11303	86.2±1.4
12	KCCP11309	82.3±1.9
13	KCCP11317	75.5±3.4
14	KCCP11318	83.8±6.6
15	KCCP11319	83.9±4.2
16	KCCP11324	82.1±3.1
17	KCCP11349	57.4±4.7
18	KCCP11354	63.1±4.0
19	KCCP11356	57.4±5.4
20	KCCP11446	58.4±2.0
21	66	61.5±6.7
22	70	80.3±5.6
23	78	77.4±0.9

while some belonged to *Leuconostoc* and *Weissella* (Fig. 1).

Conclusion

Thirty eight of LAB strains isolated from kimchi were evaluated for their potential probiotic properties. Twenty-two strains were selected with respect to their bile salts and low pH tolerance, and analyzed for their antibiotic susceptibility, and antioxidant activity. Our results indicated that one of the strains, identified by phylogenetic analysis as *Lactobacillus plantarum* subsp. *plantarum* KCCP 11349, possessed desirable probiotic properties, which were close to, and in some cases better than, the commercial probiotic *L. rhamnosus* GG. However, according to the Guidelines for the Evaluation of Probiotics in Food by FAO/WHO (FAO/WHO, 2002), it is necessary to conduct additional *in vitro* and *in vivo* experiments to further establish its probiotic character. Also, further studies are required to explore the health benefit of this strain in fermented foods made with this isolate.

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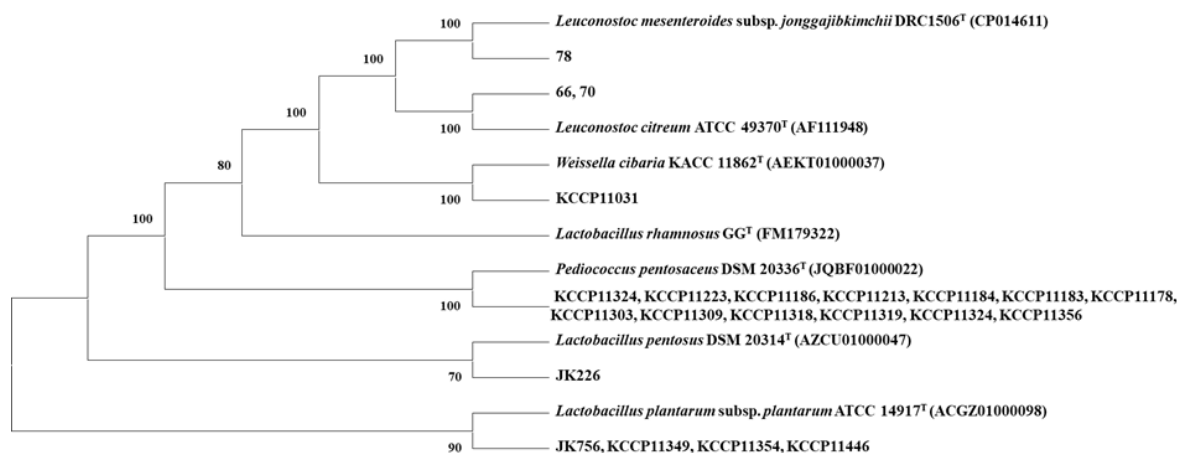


Fig. 1. Neighbor-joining phylogenetic tree, based on the 16S rRNA gene sequences, showing the position and relationship of strains isolated from kimchi with probiotic potential. Numbers at nodes indicate bootstrap values (>70%) calculated based on the NJ algorithms for the branch point based on 1000 replications. Bar, 0.01 substitutions per nucleotide position.



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Conflict of Interest

The authors declare that there is no conflict of interest.

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