



Full Length Article

In vitro* Evaluation of Probiotic, Antimicrobial, and Antioxidant Properties of a Novel *Lactobacillus plantarum* Strain Isolated from *Medicago sativa

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Abstract

In fermented food production, lactic acid bacteria (LAB) are the most commonly used organism, as they have been extensively used as probiotics since the last decade. In order to identify a new starter culture, we isolated and characterized LAB from alfalfa (*Medicago sativa*) forage, then evaluated their potential probiotic properties. Among ten isolates, one, namely KCC-34, showed significant cell growth and antifungal activity, and was thus selected for further characterization. The biochemical, physiological, and molecular (16S rRNA sequencing) properties of the selected isolate confirmed that the isolate is *Lactobacillus plantarum* KCC-34. The isolated strain showed a more significant susceptibility against various commonly-used antibiotics. *L. plantarum* KCC-34 exhibited greater antibacterial activity against *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, and had strong antifungal activity against *Aspergillus clavatus*, *Penicillium chrysogenum*, and *Aspergillus flavus*. In addition, the *L. plantarum* KCC-34 strain also had the advantages of cell surface hydrophobicity and aggregation properties. Further, the *L. plantarum* KCC-34 strain proved its ability to survive in the stomach and in the small intestine. Besides, the new *L. plantarum* KCC-34 strain showed good DPPH free radical scavenging activity. Taken together, these features suggest that the novel *L. plantarum* KCC-34 can be potentially useful as a starter culture in fermented food production. © 2018 Friends Science Publishers

Keywords: Antifungal; DPPH; Gastrointestinal tract; *Lactobacillus plantarum*; Probiotics

Introduction

It is believed that the administration of sufficient amounts of viable and non-pathogenic microorganisms can provide health benefits to a host through improving the host's intestinal microbial balance via enhanced nutrient absorption. The human gastrointestinal tract (GIT) has a complex and **potent microbes** that undergoes important changes in bacterial content according to the age, health, and lifestyle of the host (Gill *et al.*, 2001; Isolauri *et al.*, 2004; Ley *et al.*, 2006; Tiihonen *et al.*, 2010). It plays a significant function in inhibiting pathogens and harmful food substances as well as enhancing the immune system while providing beneficial effects to the host (Jankovic *et al.*, 2010). These beneficial effects of the microbes are

associated with the fact that microbial balance in the GIT can lower the risk of gastrointestinal diseases. It has been reported that probiotics can improve lactose digestion, nutrient bioavailability, and prevent allergies in vulnerable individuals (Isolauri *et al.*, 2004). Probiotics are also documented to have anti-carcinogenic, anti-mutagenic, anti-hypertensive, hypocholesterolemic, immunomodulatory, and anti-osteoporosis properties (Chiang and Pan, 2012). In addition, probiotics can prevent relapse in patients with inflammatory bowel diseases and irritable bowel syndrome, and can also reduce the risks of colon, liver, and breast cancers (Prado *et al.*, 2008).

This is referring to the GIT: The advantages of health-promoting probiotic foods for humans have come to be accepted worldwide. According to FAO/WHO, health-

promoting probiotic foods are defined as foods that contains viable and active microorganisms and that have positive health effects on the host when ingested in sufficient amounts (at least 10^6 - 10^7 cfu/g). The majority of probiotic microorganisms are lactic acid-generating bacteria. Of these, *Lactobacillus*, particularly *Lactobacillus plantarum*, is one of the most fundamental anaerobic genera that can improve the health of humans. The genomes of *Lactobacillus* species have been sequenced. A variety of *Lactobacillus* species are being used in several types of fermented foodstuffs. Moreover, metabolic pathway regulation and the functions of numerous genes have been reported. Most researchers believe that the best way to deliver viable probiotics is through fermented dairy products, mainly *via.*, fermented milk and yogurt (De Vuyst *et al.*, 2008; Ruiz-Moyano *et al.*, 2011).

Nowadays, researchers are paying more attention to novel and non-dairy probiotic fermented products that may comprise a useful working base for the advancement of functional foods for humans. Although several well-characterized probiotic *Lactobacillus* microorganisms are commercially available all over the world, the screening of novel candidates for probiotics is still of massive interest for industries manufacturing fermented foodstuffs. The hereditarily changed microorganism has aided the development of many foodstuffs with few limitations. Based on this, isolation of the organism from natural resources should be considered to be the most favorable approach to developing new starter cultures for the production of fermented foods. Therefore, the objectives of this study were to: 1) isolate novel anaerobic *Lactobacillus plantarum* from alfalfa, and 2) identify and evaluate the probiotic characteristics and safety of the novel strain of *Lactobacillus plantarum* for use as a starter for probiotic cultures by using well-known *in-vitro* assays according to the FAO/WHO guidelines.

Materials and Methods

Isolation and Identification of Lactic Acid Bacteria

Medicago sativa (Alfalfa) forages were collected from RDA agriculture farm located in Cheonan, Republic of Korea. The collected samples were kept in a chilled room until processing. About 1 g of forage was suspended in 9 mL of normal saline (0.9%). Then, 100 μ L of diluted sample was added onto the Petri plates consisting of MRS agar (de Man-Rogosa-Shape) medium (HIMEDIA). After that, culture plates were incubated at 37°C for 48 h with 5% CO₂. Colonies which were different from each other were pick up and inoculated on other agar plates. A putative *Lactobacillus* strain was selected and maintained in MRS medium containing 40% glycerol as stock culture and stored at -80°C until further experimentation. For the confirmation of morphology characteristics, the isolated putative *Lactobacillus* strain was subjected to scanning electron microscopy (SEM).

Antibacterial Activity

The antibacterial activity of the novel *Lactobacillus* isolate was measured against various bacteria following the method outlined by Naghmouchi *et al.* (2006) with few modifications. Briefly, a 24 h new broth culture (*L. plantarum*) was centrifuged at 9000 g for 10 min at 4°C. Then, 50 μ L of conidial suspension was added to each well of MRS agar medium. After that, the plates were left at room temperature for 30 min under sterile conditions followed by incubation at 37°C with 5% CO₂ for two days. At the end of the experimentation period, the antibacterial potential was noted by examining the clear zone of inhibition around the well containing culture.

Antifungal Activity

The antifungal activity of the novel *Lactobacillus* strain against various fungal strains was measured using the protocol published by Vijayakumar *et al.* (2015) with few changes. In brief, 25 mL of sterilized MRS agar media was poured into Petri dishes. Then, 50 μ L of new conidial suspension from the 24 h new culture was added to the surface of the MRS media and plates were left for 24 h in incubator so as to allow them to develop colonies. Next, 10 mL of PD agar and 50 mL of the fungal culture were transferred onto the MRS agar medium on the same petri plates. The Petri dishes were then incubated for 78 h in an incubator at 37°C with 5% CO₂. At the end of the experiment, the antagonist activity was measured by the observing clear zone of inhibition.

Molecular Characterization of Isolated *Lactobacillus* Strain

Genomic DNA of the novel *Lactobacillus* strain was extracted and purified using a QIAquick® PCR purification kit (Qiagen Ltd., Crawley, UK). For 16S ribosomal DNA gene sequencing analysis, the total DNA was amplified using forward and reverse primers according to the method published by Sanger *et al.* (1977). The following PCR program conditions were used: 95°C for 10 min, 30 cycles at 95°C for 40, 58°C for 1 min, and 72°C for 2 min. The obtained partial 16S rRNA sequences were compared to those of known *Lactobacillus* strains deposited at GenBank using the BLAST search program. The partial 16S rRNA sequence obtained from the novel *Lactobacillus* strain of this study was deposited in Genbank (accession number-KP091750).

Biochemical and Physiological Characteristics Analysis

The biochemical properties of the new strain were determined following the method outlined by Kozaki *et al.* (1992). The newly-isolated lactic acid and acetic acid produced in the MRS broth culture was measured with a UV spectrometer using the Mega-Zyme assay kit.

Carbohydrate fermentation was determined using API 50 CHB (BIOMERIEUX, Inc, USA) assay. The enzyme production by the new isolates was determined using API-ZYM (BIOMERIEUX, Inc, USA) assay kit.

Antimicrobial Susceptibility

The antimicrobial susceptibility was determined using the standardized single disc agar diffusion method outlined by Bauer *et al.* (1966). For sensitivity analysis, 25 mL of freshly prepared MRS agar media was poured into plate. This was followed by 24 fresh conidial suspensions being swabbed onto the MRS agar media. After that, the antibiotic disc was fixed above the MRS agar media. The plates were then left at 20-25°C for 30 min under sterile conditions; they were incubated at 37 °C with 5% CO₂ for 48 h. After 48 h of incubation, antibacterial activity was measured by observing the zone of inhibition.

Organic Acids Quantification

The isolated *Lactobacillus* strain was grown at 37°C with 5% CO₂ for 48 h. After 48 h of incubation, broth culture was centrifuged at 9000 g for 10 min at 4°C. Conidial suspensions were filtrated using filter paper, and the filtrate was used for the quantification of organic acid. Lactic acid and acetic acid were quantified using the HPLC spectra system (HP110, Agilent USA). The filtrate was stabilized with 5% meta-phosphoric acid then stored at -70°C. The organic acids including lactic acid (HPLC) and acetic acid (Gas chromatography; GC-450, Varian Co., USA), were analyzed (Kristensen *et al.*, 2007).

Evaluation of Probiotic Characteristics of Isolated *Lactobacillus* Strain

Tolerance of the isolate at simulated gastric juice: In order to determine the tolerance of the novel *Lactobacillus* strain isolates against simulated gastric juice, the method published by Charteris *et al.* (1998) was used. Briefly, 20 µL of 24 h new conidial suspensions was centrifuged at 6000 g for 20 min, then the conidial suspensions pellet was washed twice with 50 mM K₂HPO₄. This was followed by 1 mL of freshly prepared bacterial culture being added to the simulated gastric juice (pepsin 3 mg/mL; sodium chloride 0.5% w/v; H₂O) at different pHs (pH 2 and 3) and incubated at 37°C for 48 h with 5% CO₂. Followed by different time periods, 100 µL of this culture was added onto MRS agar medium. The plates were then incubated at 37°C for 48 h. The amount of feasible cells was counted in order to evaluate the strength of the novel *Lactobacillus* strain isolates against simulated gastric juice.

Tolerance of the Isolate to Bile Salts

In order to determine the tolerance of the isolated *Lactobacillus* strain against bile salts such as oxgall and

sodium thioglycollate, the protocol published by Vinderola and Reinheimer (2003) was used with few modifications. Briefly, 1% of 24 h fresh culture was inoculated into the mixture of oxgall (0.3%) and sodium thioglycollate 0.3% containing MRS broth, then incubated at 37°C for 48 h with 5% CO₂. After two days, the culture tolerance was measured at a wavelength of 600 nm. Similar conditions without the presence of bile salts served as a control.

Auto-aggregation

Auto-aggregation assay was carried out using the protocol outlined by Del Re *et al.* (2000) with few modifications. Briefly, the isolate was cultured on sterilized MRS broth at 37°C with 5% CO₂ for 48 h. The fresh culture was then centrifuged at 8000 g for 10 min. The upper layer was removed, and then the pellet was cleaned three times with phosphate saline (PBS) buffer solution (pH 7.0). Cells were then resuspended in freshly-prepared and sterilized PBS in order to get a live cell number at the concentration of 10⁸ CFU/mL. Four milliliters of the cell culture was vortexed for 10 and allowed to stand at 25°C for different time intervals (0, 1, 2 and 3 h). Its growth was determined by measuring OD at a wavelength of 600 nm.

Hydrophobicity

In order to determine the hydrophobicity (adhesion ability to hydrocarbons) of the isolated bacteria, the method published by Rosenberg *et al.* (1980) was used. Briefly, 24 h fresh conidial suspension was centrifuged at 5000 g for 15 min. The collected pellet was then washed with PBS and resuspended in the same solution. One milliliter of hydrocarbons (xylene or chloroform) was added to equal volumes of cell suspension and mixed by vortexing for 2 min, followed by incubation at 37°C for 10 min. The OD of the aqueous phase was measured at a wavelength of 600 nm.

Antioxidant Property (DPPH free radical scavenging activity)

The antioxidative property of the isolated *Lactobacillus* strain was determined using the method outlined by Vijayakumar *et al.* (2013). Briefly, the fresh conidial suspension prepared at different concentrations (10, 20, 30, 40, and 50 µg/mL) was added to 1 mL of DPPH (0.05 mM) solution, mixed by vortexing, and incubated at room temperature for 30 min in a dark place. After incubation, the optical density reflecting antioxidant activity was measured at a wavelength of 517 nm.

Statistical Analysis

All of the experiments were conducted in triplicate. Data were statistically analysed, and comparison was performed using the statistical package of social science (SPSS-16.0, SPSS, Inc., Chicago, IL, USA). The results were represented as mean ± standard error of the mean.

The significant difference between the mean was compared by LSD, and the value was considered to be significant at $p < 0.05$.

Results

Isolation and Molecular Identification of Lactic Acid Bacteria

A total of 10 *Lactobacillus* strains were isolated from alfalfa forage, which was collected from RDA agriculture farm located in Cheonan, Republic of Korea. The biochemical and physiological characteristics of the isolated strains revealed them to be gram-positive, catalase-negative, coccus, non-spore forming, and rod-shaped bacteria. Of these isolates, KCC-34 isolates showed greater antifungal and antibacterial activities than the other strains, suggesting probiotic potential. This novel strain of KCC-34 isolates was then further examined using SEM analysis (Fig. 1). Numerous phylogenetically-related *Lactobacillus* species exhibit similar biochemical characteristics, making the biochemical analysis unfeasible for reliable identification. Therefore, the isolated strain was further confirmed by 16S rRNA sequencing, which confirmed that the KCC-34 strain was a strain of *Lactobacillus* sp, and it was named as *Lactobacillus plantarum* KCC-34 (*L. plantarum* KCC-34). Its similarity was established using BLAST 16S rRNA gene sequence threshold. A phylogenetic tree (provided in the supplementary data) was also constructed to so as determine the genetic relationship between the isolated novel strain and other similar types of the LAB from the NCBI database. The sequence of the unique strain of LAB was submitted to NCBI Genbank with the accession number of KP091750.

Antibacterial and Antifungal Activities

The antibacterial potential of the novel *L. plantarum* KCC-34 strain against various foodborne pathogenic bacteria was determined. Our results showed that the isolated *L. plantarum* KCC-34 strain possessed potent antibacterial activity against *Escherichia coli* (zone of inhibition: 0.9 mm), *Enterococcus faecalis* (zone of inhibition: 0.6 mm), *Staphylococcus aureus* (zone of inhibition: 1.0 mm), and *Pseudomonas aeruginosa* (zone of inhibition: 0.8 mm) (Fig. 2). This bacterial growth might have been inhibited by antibacterial substances such as organic acids (lactic and acetic acid), hydrogen peroxide, and bacteriocins produced by the new LAB strain. The antagonist activity of the recently-isolated *L. plantarum* KCC-34 strain against numerous fungal species was determined through the agar diffusion method (Fig. 3). These results revealed that *L. plantarum* KCC-34 conidial suspension inhibited the growth of fungal pathogens of *Aspergillus clavatus* ($76.9 \pm 0.69\%$), *Penicillium chrysogenum* ($79.71 \pm 1.25\%$), and *Aspergillus flavus* ($77.10 \pm 1.58\%$).



Fig. 1: Scanning electron microscopy photograph of *L. plantarum* KCC-34 morphology after 48 h of growth at 37°C

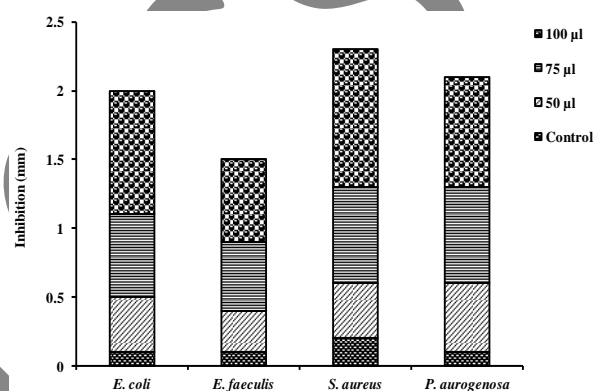


Fig. 2: Antibacterial activity of *L. plantarum* KCC-34 against *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*

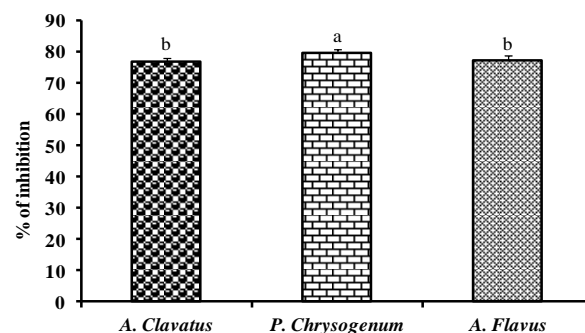


Fig. 3: Antifungal activity of *L. plantarum* KCC-34 strain against *Aspergillus clavatus*, *Penicillium chrysogenum* and *Aspergillus flavus*

Physiological, Biochemical and Antibiotic Susceptibility Analysis

In this study, the 48 h fresh culture of *L. plantarum* KCC-34 strain showed a great ability to utilize several carbohydrates for its growth, including L-arabinose, D-ribose, D-Adonitol, L-rhamnose and inositol, and it did not utilize glycerol,

erthritol, D-arabinose, D-xylose, or L-xylose for its growth (Table 1). In addition, the newly-isolated *L. plantarum* KCC-34 strain produced numerous intracellular and extracellular enzymes (Table 2). The results of our study confirmed that *L. plantarum* KCC-34 strain had greater sensitivities to chloramphenicol, nitrofurantoin, tetracycline, streptomycin, colistin methanesulphonate, dicloxacillin, amikacin, gentamicin, cefoxitin, cefalexin, cefuroxime, and co-trimoxazole, and that it was resistant to kanamycin, sulphafurazole, and ampicillin (Table 3). Overall, the new *L. plantarum* KCC-34 strain showed different susceptibilities and resistances to various antibiotics.

Organic Acids Quantification

HPLC analysis for the novel isolated *L. plantarum* KCC-34 strain was performed in order to identify and quantify the organic acids of lactic acid and acetic acid during the experimental period. In the present study, the novel strain *L. plantarum* KCC-34 produced up to 305.47 ± 0.59 mg/L of lactic acid and 60.82 ± 0.26 mg/L of acetic acid after 24 h of culturing.

Tolerance to Acidic pH and Bile Salts

According to the guidelines of FAO/WHO, for a probiotic to be considered for possible application in foods, it is essential and required for it to have the capacity to live in the stomach and the small intestine. When the novel isolate of *L. plantarum* KCC-34 was exposed to pHs 2 and 3, it showed a significant ability to survive (57-81%) under acidic conditions after 3 h incubation (Fig. 4). Fig. 5 shows the tolerance of the isolated *L. plantarum* KCC-34 strain quite well against bile salts of oxygall (50.28, 60.23%) and sodium deoxycholate (46.69%, 53.99%).

Auto-aggregation and Hydrophobicity

Cell surface properties evaluated by auto-aggregation and hydrophobicity are the essential parameters for probiotic cell adhesion to intestinal epithelial cells. Our results revealed that the novel strain *L. plantarum* KCC-34 displayed the highest score (53-69%) for auto-aggregation (Fig. 6) after 3 h incubation. Fig. 7 shows that the newly isolated *L. plantarum* KCC-34 exhibited significant hydrophobicity against xylene (41.13%) and chloroform (57.28%).

Antioxidant Property (DPPH radical scavenging activity)

The antioxidant activity of the novel strain of *L. plantarum* KCC-34 was determined using DPPH free radical scavenging assay. For this purpose, 24 h of new culture of conidial suspension was used at different concentrations (10, 20, 30, 40 and 50 μ g/mL).

Table 1: Biochemical and physiological characteristics of KCC-34

Name of carbohydrates	KCC-34
Glycerol	+
Erythritol	-
D-Arabinose	-
L-Arabinose	+
D-Ribose	+
D-Xylose	+
L-Xylose	+
D-Adonitol	+
Methyl- β -D-Xylopyranoside	+
D-Galactose	-
D-Glucose	+
D-Fructose	+
D-Mannose	-
L-Sorbose	-
L-Rhamnose	+
Dulcitol	+
Inositol	+
D-Mannitol	-
D-Sorbitol	-
Methyl- α -D-Mannopyranoside	-
Methyl- α -D-Glucopyranoside	+
N-Acetylglucosamine	+
Amygdalin	-
Arbutin	+
Esculin ferric citrate	+
Salicin	+
D-Cellobiose	+
D-Maltose	+
D-Lactose	+
D-Melibiose	+
D-Saccharose	-
D-Trehalose	-
Inulin	+
D-Melezitose	+
D-Raffinose	+
Amidon	+
Glycogen	+
Xylitol	-
Gentiobiose	-
D-Turanose	+
D-Lyxose	+
D-Tagatose	+
D-Fucose	-
L-Fucose	+
D-Arabitol	-
L-Arabitol	+
Potassium gluconate	-
Potassium 2-keto gluconate	-
potassium 5-keto gluconate	+

+: Positive response; -: Negative response

Our results showed that the novel *L. plantarum* KCC-34 strain displayed increased DPPH free radical scavenging activity with increasing concentrations (Fig. 8). In addition, the isolated novel strain showed resistance to various concentrations of H₂O₂ (Fig. 9).

Discussion

According to the guidelines of the food and agriculture organization (FAO) and world health organization (WHO),

Table 2: Extracellular enzymes produced by KCC-34

Extracellular enzymes	KCC-34
Alkaline phosphatase	+++
Esterase (C ₄)	++
Esterase lipase (C ₈)	+++
Lipase (C ₁₄)	+++
Leucine arylamidase	+
Valine arylamidase	+
Cystine arylamidase	+++
Trypsin	+++
α-Chymotrypsin	+++
Acid phosphatase	+++
Naphthol-AS-biphosphohydrolase	+
α-Galactosidase	+
β-Galactosidase	++
β-Glucuronidase	+++
α-Glucosidase	++
β-Glucosidase	++
N-Acetyl-β-glucosaminidase	+++
α-Mannosidase	+
α-Fucosidase	++

+: Weak production: ++; Moderate production: +++; Strong production

Table 3: Antibiotic sensitivities of KCC-34

Name of antibiotics	Conc. (µg)	KCC-34
Chloramphenicol (C)	50	S
Kanamycin (K)	30	R
Nitrofurantoin (NIT)	50	S
Tetracycline (TE)	100	S
Streptomycin (S)	25	S
Sulphafurazole (SF)	300	R
Colistin methane sulphonate (CL)	100	S
Dicloxacillin (D/C)	1	S
Ampicillin (AMP)	10	R
Amikacin (AK)	30	S
Gentamicin (GEN)	10	S
Cefoxitin (CX)	30	S
Cefuroxime (CXM)	30	S
Co-Trimoxazole (COT)	25	S

>10 mm: Susceptibility= S: R=Resistant

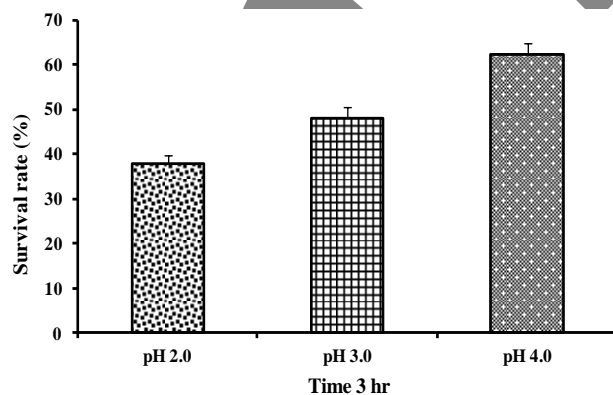


Fig. 4: pH-resistant property of *L. plantarum* KCC-34 against the simulated gastric juice conditions at pHs 2, 3, and 4 after 3 h incubation

16S rRNA sequencing is a suitable and accessible technology for the identification of potential probiotic strains. The novel lactic acid bacterium isolated from alfalfa

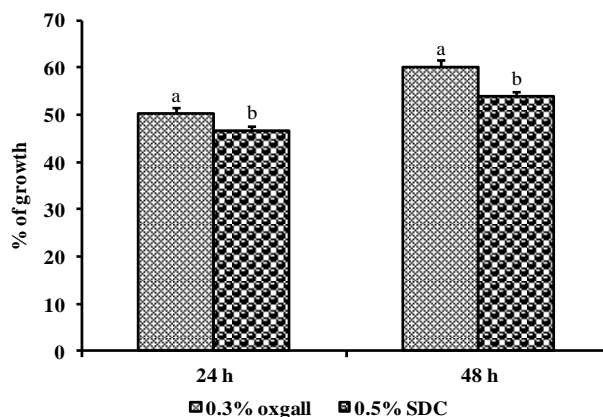


Fig. 5: Bile salts resistance property of *L. plantarum* KCC-34 in the presence of 0.3% oxgall and 0.5 % sodium deoxycholate (SDC) at different time intervals

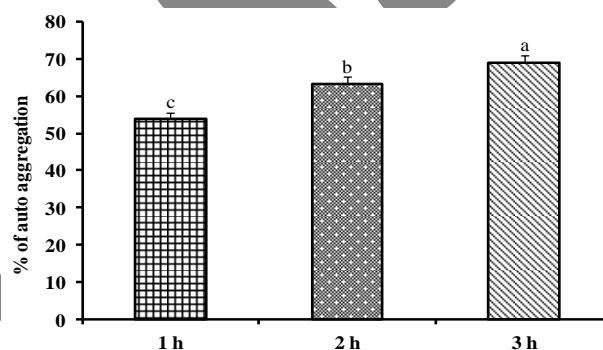


Fig. 6: Ability of *L. plantarum* KCC-34 on auto-aggregation. Results were expressed as mean ± standard error of the mean

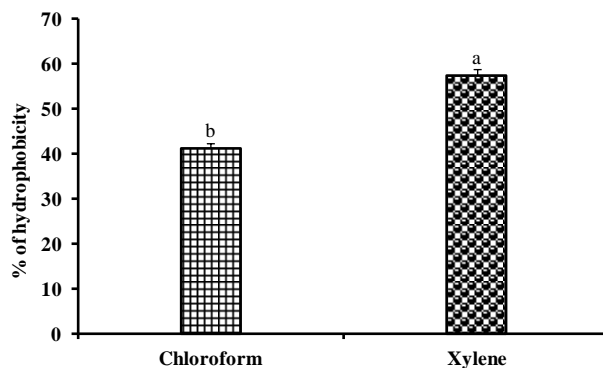


Fig. 7: Cell surface hydrophobicity of *L. plantarum* KCC-34

was identified by SEM and 16S rRNA sequencing using an NCBI tool. It was then compared to sequences deposited in GenBank. The novel strain KCC-34 was confirmed to be a strain of *L. plantarum*. For taxonomical study, the isolated strain shared 99-100% sequencing similarities with other known isolates of *L. plantarum*. It is known that 16S rRNA

sequencing is one of the most robust and accurate technologies for the phylogenetic clustering of potential probiotic microbes (Deng *et al.*, 2008). Although molecular identification has become a reliable method for strain taxonomy, the API phenotypic technique has historically been used as a tool for species identification, as it is less time-consuming and requires less expensive equipment. Therefore, the capability of the isolated potential probiotic bacteria to ferment various carbohydrates contained in an API 50 CHL kit was determined. Among the results of the study, *L. plantarum* KCC-34 was not able to ferment a few of the carbohydrates during the discoloration process. This discoloration process might be caused by insufficient production of desired enzymes by the isolated strain to decompose sugar in the basal medium. Results of identification using the API system and the evaluation of phenotypic rate confirmed that the separated strain belonged to lactic acid bacteria, namely *L. plantarum* (KCC-34). Beyond that, the isolated potential probiotic strain produced a variety of intracellular and extracellular enzymes.

Human pathogens are increasingly becoming major concerns worldwide. Therefore, screening antifungal substances to treat human pathogens is a crucial mission. Several researchers have isolated antifungal competence of lactic acid-producing bacteria against various fungal strains using the agar diffusion method but these are all bacteria that are not commercialized. Therefore, in this study, we also determined the antifungal activity of *L. plantarum* KCC-34 isolate using the agar diffusion method. Among the results of the study, *L. plantarum* KCC-34 possessed great inhibitory activity against *P. chrysogenum*, as well as moderate activity against *A. clavatus* and *A. flavus*. Additionally, the isolated probiotic strain exhibited inhibitory activities against pathogenic bacteria such as *E. coli*, *E. faecalis*, *S. aureus*, and *P. aeruginosa* in the well diffusion tests. The antimicrobial activity of probiotic bacteria has been found to stem from the production of different antimicrobial compounds such as organic acids, bacteriocins, and metabolites. Among them, lactic acid, acetic acid, and bacteriocins have been shown to account for most of the antimicrobial compounds generated by probiotic bacteria (Argyri *et al.*, 2013). Probiotic bacteria capable of producing antimicrobial metabolites are good candidates for food preservation and the growth prevention of foodborne pathogens (Monteagudo-Mera *et al.*, 2012).

Probiotics play an essential role in the microbiota of the human gastrointestinal tract. Therefore, probiotics for people should preferably include bacteria of human origin containing a series of attractive characteristics without harmful traits, such as those considered in the current study. It has already been documented that *L. plantarum* possesses several functions in the GI tract and in saliva. Hence, it is necessary to evaluate the *in vitro* probiotic potential and safety for the isolated probiotic strain of *L. plantarum* KCC-34 before applying in it in an expensive *in vivo* model.

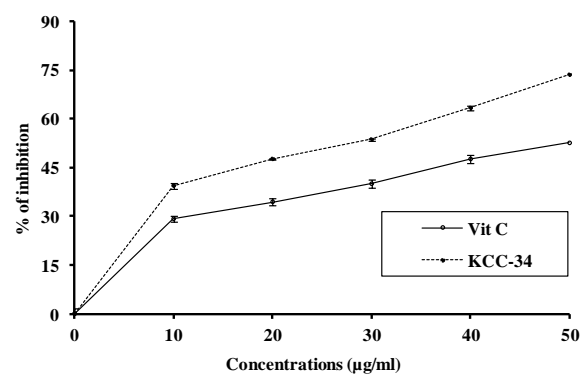


Fig. 8: DPPH radical scavenging activity of *L. plantarum* KCC-34 compared to positive control (vitamin C)

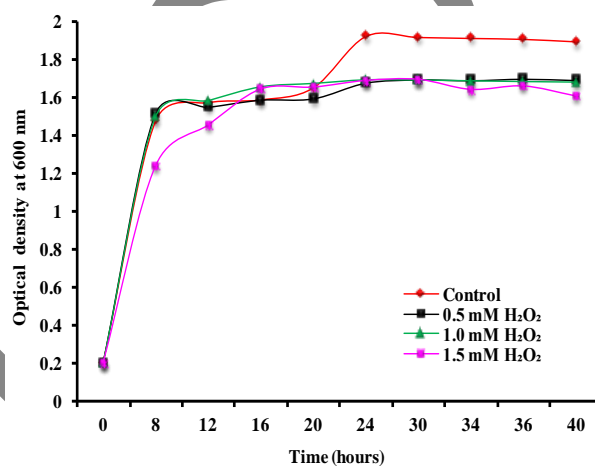


Fig. 9: Resistance of *L. plantarum* KCC-34 against H₂O₂ treatment. The results were expressed as means of three independent replicates

The survival of *L. plantarum* KCC-34 under conditions stimulating gastric juice of GIT, tolerance to acidic pH, bile salts of GIT, auto-aggregation, hydrophobicity, and ability to scavenge free radicals (antioxidative property) are the most important criteria for the selection of probiotics. Tolerance to acidic pH is one of the most vital factors for consideration as a probiotic candidate (Toole and Cooney, 2008). In general, bacteria are very susceptible to the stomach environment. Lactic acid bacteria can survive in an acidic pH environment due to their ability to produce lactic acid. They also have a unique bilayer membrane structure to maintain environmental and cytoplasmic pH as a homeostatic mechanism. However, there is currently no standard protocol for selecting gastric and bile salt tolerance potential of probiotic bacteria (Chung *et al.*, 1999; Afify *et al.*, 2012). Therefore, we used pH 2 and 3 to determine the acidic acid tolerance of the novel probiotic strain in the *in vitro* model. Our results showed that the novel strain *L. plantarum* KCC-34 exhibited great acidic pH tolerance.

Our research results were in agreement with those of Awasti *et al.* (2016) showing that probiotic bacteria of *Lactobacillus* GC had great tolerance to acidic conditions (pH 2 and 3) after 3 and 4 h incubation. The stress adaptation mechanisms produced by acidic surroundings can result in bile salt tolerance (Burns *et al.*, 2010). The survival rate of the novel probiotic strain under high bile salt conditions was determined in this study. Our results showed that the novel strain *L. plantarum* KCC-34 showed good tolerance against oxgall and SDC. Tolerance to bile salts is one of the most important characteristics of the LAB for survival. Their metabolites and growth in GIT have beneficial effects on the host (Charteris *et al.*, 1998; Argyri *et al.*, 2013).

Cell adhesion is a process in which molecules of the cell surface interact with each other. It is one of the essential criteria used to choose probiotic candidates. The adhesion process of probiotic strain plays a significant role in the removal of pathogens and immunomodulation. In this study, *L. plantarum* KCC-34 showed the highest score for auto-aggregation and hydrophobicity compared to control. These two characteristics (auto-aggregation and hydrophobicity) may play substantial roles in cell adhesion. The results of this study were in agreement with those of Clewell *et al.* (2002) showing that the production and aggregation of the substance of LAB can have contact with another cell by binding to the enterococcal binding substance. The *Lactobacillus* strain has low adherence property with a highly negatively-charged surface. However, the cell surface can strongly adhere to a slightly negatively-charged surface. The antioxidant defense mechanism plays an important role in scavenging free radicals by producing substances that inhibit oxidation. DPPH radical scavenging assays are usually used in food biochemistry to determine the scavenging properties of specific microbes, substances, or extracts. Free radicals are unstable, reactive, oxidized biomolecules. They can bring oxidative damage to cells. DPPH is a stable free radical that has a property wherein it can accept unpaired molecules in order to pair and become stable. DPPH has a broad absorption band with a maximum at 517 nm (Lo Scalzo, 2008). The results of the current study showed that *L. plantarum* KCC-34 possessed good DPPH free radical scavenging property, which is in agreement with the results of Vijayakumar *et al.* (2015) showing that *L. plantarum* KCC-24 exhibited increased DPPH free radical scavenging activity with increasing concentration. Our present study confirmed that *L. plantarum* KCC-34 possessed antioxidant activity by scavenging free radicals with hydrogen donating ability.

Conclusion

In summary, we isolated and identified efficient antifungal lactic acid bacteria, *Lactobacillus plantarum* KCC-34, from *Medicago sativa* forage sample, and they were evaluated in terms of probiotic potential. The newly-isolated *L.*

plantarum KCC-34 was further confirmed by SEM and 16S rRNA sequencing. The isolated probiotic bacterial strain *L. plantarum* KCC-34 exhibited greater antifungal activity against *A. clavatus*, *P. chrysogenum*, and *A. flavus*, and also had significant antibacterial activity against *E. coli*, *E. faecalis*, *S. aureus*, and *P. aeruginosa*; this may be due to the production of lactic and acidic acid. This strain displayed good tolerance against simulated gastric juice conditions mimicking the gastrointestinal tract. In addition, the novel probiotic strain exhibited high aggregation activity and hydrophobicity. The *L. plantarum* KCC-34 also showed a higher antioxidant capacity by scavenging free radicals. Therefore, the results of our current study suggest that the *L. plantarum* KCC-34 strain can be a suitable candidature for the development of fermented food production.

Acknowledgements

We thank the Cooperative Research Program for Agricultural Science and Technology Development, Rural Development Administration, Republic of Korea. This study was also supported by Postdoctoral Fellowship Program of National Institute of Animal Science funded by Rural Development Administration, Republic of Korea.

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(Received 06 March 2018; Accepted 19 July 2018)