Anaerobe 32 (2015) 90–97

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Clinical microbiology

In-vitro assessment of the probiotic potential of *Lactobacillus plantarum* KCC-24 isolated from Italian rye-grass (*Lolium multiflorum*) forage

Mayakrishnan Vijayakumar [a, †], Soundharrajan Ilavenil [a, †], Da Hye Kim [b], Mariadhas Valan Arasu [c], Kannappan Priya [d], Ki Choon Choi [a, *]

[a] Grassland and Forage Division, National Institute of Animal Science, Rural Development Administration, Cheonan 330-801, Republic of Korea
[b] The United Graduate School of Agricultural Sciences, Tottori University, 680-8553 Japan
[c] Department of Botany and Microbiology, Addiriyah Chair for Environmental Studies, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia
[d] Department of Biochemistry, PSG College of Arts and Science, Affiliated to Bharathiar University, Coimbatore 614-014, Tamil Nadu, India

ARTICLE INFO

Article history:
Received 14 November 2014
Received in revised form
29 December 2014
Accepted 6 January 2015
Available online 7 January 2015

Keywords:
L. plantarum KCC-24
Antifungal
Probiotics
Auto-aggregation
Antioxidant

ABSTRACT

The aim of the present study was to determine the probiotic potential of the lactic acid bacteria *Lactobacillus plantarum* KCC-24 (*L. plantarum* KCC-24), that was isolated and characterized from Italian ryegrass (*Lolium multiflorum*) forage. The following experiments were performed to assess the probiotic characteristics such as antifungal activity, antibiotic susceptibility, resistance to low pH, stimulated gastric juice and bile salts, proteolytic activity, auto-aggregation, cell surface hydrophobicity, and *in vitro* antioxidant property. The isolated *L. plantarum* KCC-24 exhibited significant antifungal activity against the various fungal strains of *Aspergillus fumigatus* (73.43%), *Penicillium chrysogenum* (59.04%), *Penicillium roqueforti* (56.67%), *Botrytis elliptica* (40.23%), *Fusarium oxysporum* (52.47%) and it was susceptible to numerous antibiotics, survived in low pH, was resistant to stimulated gastric juices and bile salts (0.3% w/v). Moreover, *L. plantarum* KCC-24 exhibited good proteolytic activity. In addition *L. plantarum* KCC-24 showed potent antioxidant and hydrogen peroxide resistant property. In conclusion, the isolated *L. plantarum* KCC-24 exhibited several characteristics to prove it's excellent as a potential probiotic candidate for developing quality food for ruminant animals and human.

1. Introduction

The demand of lactic acid producing bacteria (LAB) in food, pharmaceutical and chemical industries is increasing due to their ability to prevent food spoilage, and their make up in production of biodegradable polymers and green solvents. The industrial production of lactic acid occurs by chemical synthesis or microbial fermentation. Chemically produced lactic acid is always a racemic mixture. Whereas, a biologically produced lactic acid is optically pure and can be obtained using lactic acid producing bacteria. Nowadays, increasing attention to the health benefits of consuming probiotic micro-organisms such as lactic acid producing bacteria has been increased worldwide. Probiotics are defined as live micro-organisms that, when is administrated in sufficient amounts, provides a health benefit to the host. It plays an important role in improving physiological functions, competing with potential pathogenic bacteria for nutrition, killing harmful bacteria, and boosting the immune system [1]. There are many micro-organisms that can be classified as probiotics belonging to the *Lactobacillus* and *Bifidobacterium* genera. Among them, *Lactobacilli* are considered an enteric organism in the industrial processing of fermented dairy, meat, vegetable and cereal products.

Lactobacillus plantarum is a lactic acid producing, gram positive, non-spore forming, fermentative bacteria, that grows anaerobically, and exist in a wide range of environmental places including some agitated foods, and gastrointestinal and urogenital tract of animals. It has many physiological, biochemical, and genetic properties. These bacteria can produce antimicrobial compounds such as peptides, exo-polysaccharides, secondary metabolites, and

* Corresponding author.
E-mail address: choiwh@korea.kr (K.C. Choi).
† They are contributed equally to this work.

http://dx.doi.org/10.1016/j.anaerobe.2015.01.003
1075-9964/

organic acids such as, acetoin reuterin, reutericyclin, hydrogen peroxide, and diacetyl [2]. It plays an important role in the food industry and consumers for the production and consumption of fermented foods and beverage due to their long and safety application [3]. Therefore, whole genome sequencing of probiotics elucidated their complex nutritional requirements [4]. The physiological properties of probiotic micro-organism associated with pH reduction, the production of some digestive enzymes and vitamins, production of antibacterial substances and other unidentified substances, and the reconstruction of normal intestinal micro-flora after it has been damaged by disease [5]. According to the guidelines put forth by the World Health Organization, for a food to attain a probiotic status, micro-organisms have to meet a number of principles related to their safety and biological properties. In terms of safety, the probiotic micro-organism should not be pathogenic, and should not be able to transfer antibiotic resistance genes and sustain genetic stability. As a functional component in food, they should provide acid-base stability, be resistant to digestive enzymes, have antagonist properties, be anti-carcinogenic, stimulate the immune system, produce vitamins and enzymes, and improve the bioavailability of food [6]. Therefore, the aim of the present study was to assess the potential probiotic of *L. plantarum* KCC-24, that was isolated and characterized from Italian rye-grass (*Lolium multiflorum*) forage.

2. Materials and methods

2.1. Sample collection and isolation of lactic acid bacteria

Italian rye-grass (*L. multiflorum*) forage was collected from various places in Cheonan, South Korea and the sample stored in the laboratory for the isolation of the lactic acid producing bacteria *L. plantarum* as a potential probiotic. Isolated *Lactobacillus* strain data were recorded in the laboratory for further reference. For isolation, the sample was mixed with distilled water and centrifuged (Hanil centrifuge MF 300, Korea) at 8000 × g for 15 min to remove heavy particles. The supernatant was serially diluted with distilled water and poured on MRS (de Man, Rosga, and Shape) agar petri dish plates that were incubated at 30 °C for 48 h [7]. After the incubation period, we isolated 10 lactic acid bacterial strains. Among these, a single strain KCC-24 showed a good antifungal and probiotic activity (data not shown). Therefore, we selected this strain for further experiment and it was deposited into the Korean Agricultural collection and type culture (KACC91962P), Korea Research Institute of Bioscience and Biotechnology, Republic of Korea. The purified strain was labeled KCC-24 and stored at −80 °C in MRS broth with 40% glycerol for further use.

2.2. PCR amplification of the 16S rRNA and molecular identification of lactobacillus strain

The 16S ribosomal DNA gene sequencing was done at the Sol-Gent Co (Seoul, South Korea) by the method of Sanger et al. [8]. Genomic DNA was isolated from *L. plantarum* KCC-24 and purified by QIAquick® PCR purification kit (Qiagen Ltd., Crawley, UK), followed by the amplicons were sequenced with Taq DNA polymerase using the 27 forward primer (5′ AGA GTT TGA TCG TGG CTC AG 3′) and the 1492 reverse primer (3′ GCT TAC CTT GTT ACG ACT T 5′). The thermocycler conditions are as follows; initial denaturation of target DNA at 95 °C for 10 min followed by 30 cycle amplification, annealing at 58 °C for 1 min, elongation at 72 °C for 2 min and cooled to 4 °C. The obtained sequence homologies were analyzed by comparing with those sequences was obtained in the BLAST. During the similarities comparison, sequence was required an initial threshold 99% homology when comparing with raw sequence. The obtained 16s rRNA sequence of KCC-24 was deposited into NCBI Genbank and assigned the NCBI accession number is KM396462.

2.3. Preliminary screening of antifungal activity of isolated L. plantarum KCC-24

The Aspergillus *fumigatus* (KACC 40080), *Penicillium chrysogenum* (KACC 40399), *Penicillium roqueforti* (KACC 41354), *Botrytis elliptica* (KACC 43461), and *Fusarium oxysporum* (KACC 40051) were obtained from Korean Agricultural Culture Collection (KACC), South Korea. The antifungal activity of isolated *L. plantarum* KCC-24 was determined by the agar diffusion method of Valan et al. [7] with slight modifications. Briefly, Petri dish plates were prepared with 30 mL of sterilized MRS media followed by overnight culture of isolated *L. plantarum* on the surface of MRS media and incubated at 30 °C for 48 h to allow colonies development. Thereafter, 10 mL of potato dextrose agar (PDA) containing 50 μL of fungal suspension was poured over the MRS media on the same plate. The plates were incubated at 37 °C for 72 h under aerobic condition and clear inhibition zones were recorded. The experiment was repeated three times.

2.4. Antifungal activity of isolated L. plantarum KCC-24 by microdilution method

Antifungal activity of KCC-24 was analyzed by the method of Lavermicocca et al. [9] with slight modification. Breifly190 μL of fermentative metabolites of KCC-24 was dispensed in to 96 well and then 10 μL conidial suspensions were inoculated. Without fermentative metabolites considered as a control. All the experimental plates were incubated at 30 ± 2 °C for 72 h. Fungal growth was measured at 580 nm using microplate reader. Here, growth of fungi in control was considered as a 100% growth. From that we have calculated the percentage of fungal growth inhibition.

2.5. Biochemical analysis of isolated L. plantarum KCC-24 by API 50 CHB system

The overnight culture of isolated *L. plantarum* KCC-24 was used to analyze its biochemical and physiological properties. Phenotypes were analyzed by API 50 CHB system (bioMerieux, Inc, USA), and the strip was prepared and used according to the manufacturer's instructions. The strips were kept in an incubator at 37 °C for 48 h. After the incubation period, the results were noted.

2.6. Antibiotic susceptibility analysis of isolated L. plantarum KCC-24

Antibiotic sensitivity and resistance were analyzed according to the agar diffusion method of Bauer et al. [10]. Twenty five ml of sterilized MRS media was poured into the petri dish plates and allowed to sit for 10 min. Then the overnight culture of *L. plantarum* KCC-24 was swabbed on the MRS media. After the media solidified, the antibiotic disc was placed on the top of MRS media and left for 30 min at room temperature for antibiotic diffusion. The plates were incubated at 37 °C for 48 h, and after the incubation period, the zones of inhibition were observed.

2.7. Evaluation of probiotic characteristics of isolated L. plantarum KCC-24

2.7.1. Tolerance to low pH

Low pH tolerance of *L. plantarum* KCC-24 was evaluated, according to the method described by Tambekar and Bhutada [11].

Briefly, the isolated bacterial culture was inoculated into the sterilized MRS broth of various pH (2, 3, 4, and 5.7) and then the broth was incubated at 37 °C for 48 h. After the incubation, 100 μL of inoculum from each tube was poured onto the MRS medium by the pour plate method. The plates were incubated at 30 °C for 48 h and the colonies were counted.

2.7.2. Tolerance to stimulated gastric juice

The analysis of *L. plantarum* KCC-24 resistance against stimulated gastric juice was evaluated by the method of Charteris et al. [12]. The stimulated gastric juice was prepared with 3 mg/mL of pepsin and 0.5% w/v sodium chloride, water, and the pH were adjusted to 2 and 3. Thirty milliliters of overnight bacterial culture was centrifuged at 6000 × g for 20 min. After centrifugation, the supernatant was removed and the cells were washed twice with 10 mL of 50 mM K_2HPO_4 and were resuspended in 3 mL of K_2HPO_4. Then 9 mL of gastric juice of varying pH was mixed with 1 mL of cell suspension and incubated 37 °C for 3 h. Then 100 μL of cell suspension was poured on the MRS medium at different time intervals and the plates were incubated at 30 °C for 48 h. The number of total viable cells was counted.

2.7.3. Resistance to bile salts

The measurement of growth resistance of isolated *L. plantarum* KCC-24 against two bile salts was studied by the following modified method of Vinderola and Reinheimer [13]. Two kinds of MRS broth were prepared with 0.3% oxgall and 0.3% sodium thioglycollate, and both were inoculated with 1% bacterial culture suspension and incubated at 37 °C for 48 h. After the incubation period, the optical density of the cell culture at 600 nm was compared that of the control (without bile salts).

2.7.4. Screening of proteolytic activity of isolated L. plantarum KCC-24

Proteolytic activity was assayed by the method of Rajagopal and Sandine [14]. Three milliliters of overnight bacterial culture was centrifuged at 4000 × g (4 °C) for 10 min. The collected bacteria were used as a source of protease enzyme in this assay. The agar plates were prepared with 1% agar, 0.5% peptone, 0.3% beef extract, and 0.5% skim milk. Five microliters of bacteria was spotted on the agar media and plates were incubated at 30 °C for 48 h. The clear zone demonstrated the isolated bacteria ability to produce proteases enzyme.

2.7.5. Auto-aggregation assay

Aggregation between the cells membrane and interacting surface were performed according to the method described by Del Re et al. [15] with some modification. The overnight MRS broth bacterial isolate was centrifuged at 8000 × g for 10 min, and the pellet was resuspended with PBS (pH 7) to a concentration of approximately 10^8 CFU mL^{-1}. Four milliliters of cell suspension was mixed by vortexing for 10 s and auto-aggregation was analyzed during the 3 h incubation at room temperature. Each hour, 100 μL of upper cell suspension was mixed with 3.9 mL of PBS and the absorbance at 600 nm was measured. Auto-aggregation was calculated by the following equation:

1-(A_t/A_0) x 10, whereas A_t represents the absorbance at time $_t = 1$, 2 and 3and A_0 = absorbance at $_t = 0$.

2.7.6. Determination of cell surface hydrophobicity

To determine the hydrophobicity of *L. plantarum* KCC-24, a hydrophobicity assay was performed according to the protocol of Rosenberg et al. [16], which measure a microorganism ability to bind to the hydrocarbons from the phosphate buffer solution. A day old of isolated bacterial culture was centrifuged at 5000 × g for 15 min. The cell suspension was collected and washed with PBS (pH 7), the absorbance at measured at 540 nm. One milliliter of cell suspension was mixed with equal volume of hydrocarbons (xylene and chloroform), and the absorbance was measured at 540 nm. After 30 s, the aqueous solution was measured and compared with the initial measurements. The hydrophobicity was calculated using the following formula:

$$\text{Hydrophobicity}\% = (OD_{initial} - OD_{final})/OD_{initial} \times 100$$

2.8. Antioxidant properties of isolated L. plantarum KCC-24

2.8.1. Determination of DPPH free radical scavenging activity

The DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging capacity of isolated *L. plantarum* KCC-24 was measured by the modified method of Chen et al. [17]. Briefly, various 10, 20, 30, 40 and 50 μL concentrations of freshly prepared isolated cells (10^9 CFU/ml) were mixed with 1 mL of (0.05 mM) DPPH solution then vortex and incubated in a dark place at room temperature for 30 min. DPPH solution was used as a control. A combination of methanol and cells were used as blank. The absorbance was measured at 517 nm.

The DPPH scavenging capacity was calculated using the following formula:

$$\text{DPPH scavenging capacity } (\%) = \left[(A_{sample} - A_{blank})/A_{control}\right] \times 100$$

2.8.2. Determination of resistance to hydrogen peroxide

This assay was performed according to the method of Buchmeier et al. [18] with slight modifications. Briefly, 10 mL of overnight growing bacterial culture was centrifuged, and the pellets were collected and mixed with 10 ml 0.9% of NaCl in various conical flask with different concentrations (0.5, 1.0 and 1.5 mM) of H_2O_2. The bacterial cell viability was measured initially and after 30 min of incubation at 37 °C by using the BCP agar plate with respective dilution of the sample. The experiments were repeated three times.

2.9. Statistical analysis

All the numerical data were obtained from three independent experiments and these data analysis was carried out with MS-Excel and SPSS 16 statistical analysis (SPSS Inc., Chicago, IL, USA). The results were presented as mean ± standard of error. The significant difference between the means was analyzed using One-Way ANOVA with a significant level of $P < 0.05$.

3. Results

3.1. Isolation and identification of L. plantarum KCC-24

The aim of the present study was to isolate and, identify probiotic *L. plantarum* from Italian rye-grass forage collected from Cheonan, Republic of Korea. A totally 10 *Lactobacillus* strains were isolated from the Italian ryegrass forage. Among these, single strain showed a potent antifungal and probiotic properties. It named as KCC-24. Therefore we selected the KCC-24 for further characterization. The 16S rRNA from selected strain was sequenced and it was compared with the information in the NCBI database. The BLAST result showed that the isolated lactic acid bacteria KCC-24 as

L. plantarum which displayed a similarity of ≥99% to other *L. plantarum* strains. The 16sRNA sequence was submitted to the NCBI Genebank data base (KM396462).

3.2. Physiological and biochemical characterization of L. plantarum KCC-24

After 48 h grown, isolated *L. plantarum* KCC-24 bacterium was observed by visually and microscopically. The *L. plantarum* KCC-24 was a creamy color, rod shaped, anaerobic, gram positive, and non spore forming bacteria. The bacterial culture was identified by biochemically using API 50CH (BioMerieux, Marcy L'Etoile, France). The isolated bacterial culture KCC-24 had ability to ferment the numerous carbohydrate such as L-Arabinose, D-Ribose, D-Galactose, D-Glucose, D-Fructose, etc., and non fermented the glycerol, erthritol, D-Arabinose, D-xylose, L-xylose, etc., (Table 1) and the *L. plantarum* KCC-24 produced the different types of intra and extra cellular enzymes (Table 2).

Table 1
Biochemical characterization of isolated *L. plantarum* KCC-24 by API 50 CHB system.

Name of carbohydrates	*L. plantarum* KCC-24
Glycerol	−
Erythritol	−
D-Arabinose	−
L-Arabinose	+
D-Ribose	+
D-Xylose	−
L-Xylose	−
D-Adonitol	−
Methyl- β D-xiloside	−
D-Galactose	+
D-Glucose	+
D-Fructose	+
D-Mannose	+
L-Sorbose	−
L-Rhamnose	−
Dulcitol	−
Inositol	−
D-Mannitol	+
D-Sorbitol	−
Methyl- α D-mannoside	+
Methyl- α D-glucoside	−
N-acetyl glucosamine	+
Amygdalin	+
Arbutin	+
Esculin ferric citrate	−
Salicin	+
D-Celiobiose	+
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-ketogluconate	−
Potassium 5-ketogluconate	+

+: Positive response; −: Negative response.

3.3. Antifungal activity of isolated L. plantarum KCC-24

The antifungal activity of the spent media of isolated *L. plantarum* KCC-24 was determined against various fungi. From the study, the pore plate method results revealed that the isolated bacterial culture was able to inhibit the growth of fungal pathogens, which was observed visually. The micro dilution method results also confirmed the antifungal activity against *Aspergillus fumigatus* (73.43 ± 0.96), *P. chrysogenum* (59.04 ± 0.74), *P. roqueforti* (56.67 ± 0.72), *B. elliptica* (40.23 ± 0.43), and *F. oxysporum* (52.47 ± 0.68) (Fig. 1).

3.4. Susceptibility of L. plantarum to antibiotics

An important requirement of probiotic strains is that they should not take genes that confer antibiotic resistance. The antibiotic susceptibility of the isolated KCC-24 strain was determined using various antibiotic agents. The results showed that the isolated KCC-24 strain was susceptibility to chloramphenicol, nitrofurantoin, tetracycline, dicloxacillin, ampicillin, cefalexin, cefuroxime also resistance to kanamycin, sulphafurazole, colistin methane sulphonate, amikacin, gentamicin, cefoxitin, and co-Trimoxazole (Table 3). Further, the isolated *L. plantarum* KCC-24 produced a significant amount of fermentative acids such as lactic acid, acetic acid and succinic acid (Table 4).

3.5. Probiotic properties of isolated L. plantarum KCC-24

We have analyzed the survival ability of KCC-24 in low pH and bile salt environmental conditions because as a probiotic must be survived in the low pH and bile salts conditions in digestive tract. *L. plantarum* KCC-24 was survived in the acidic conditions such as pH 5.7, 4.0, 3.0, and 2.0 after 3 h of incubation (Fig. 2a). No viable cells were found at 1.5 pH. However, the bacterial count did not change within 1 h at pH 5.7 to 2.0. The *L. plantarum* KCC-24 showed significant resistance property against gastric juice stress (Fig. 2b). For bile salts tolerant study, we used oxgall (0.3%) and sodium taurocholate (0.3%). The results showed that the KCC-24 had ability to grow in the presence of bile salts (Fig. 2c).

Table 2
Intra and extra cellular enzyme production by isolated *L. plantarum* KCC-24.

Intra and extra cellular enzymes	*L. plantarum* KCC-24
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.

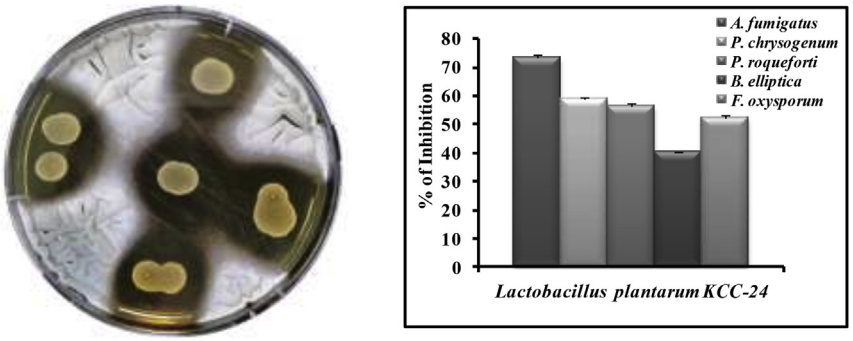

Fig. 1. Inhibition of *A. fumigatus*, *P. chrysogenum*, *P. roqueforti*, *B. elliptica* and *F. oxysporum* growth by isolated *L. plantarum* KCC-24.

3.6. Proteolytic activity of L. plantarum KCC-24

The proteolytic activity of isolated *L. plantarum* KCC-24 was analyzed on an agar plate containing skim milk. The *L. plantarum* showed proteolytic activity by forming a clear zone on the skim milk medium (Fig. 3).

3.7. Sedimentation rate and cell surface hydrophobicity

The aggregation and hydrophobicity properties were used to determine the interaction ability of bacteria with epithelial cells and desirable bacteria. As shown in Fig. 4, the isolated *L. plantarum* KCC-24 revealed strong auto-aggregation property of 50–73 % after 3 h incubation period. Furthermore, we analyzed cell surface hydrophobicity using xylene and chloroform. Based on the results, *L. plantarum* exhibited a significant cell surface hydrophobicity in xylene (41.13%) and chloroform (24.17%).

3.8. Antioxidant activity of L. plantarum KCC-24

Fig. 5a shows the DPPH radical scavenging activity of isolated *L. plantarum* KCC-24 cells free extract. The scavenging activity of cell free extract significantly increased in a dose dependent manner. This result was comparable with ascorbic acid. From the results, we concluded that the DPPH radical scavenging property of *L. plantarum* may be due to the production of secondary metabolites. Fig. 5b shows the impact of H_2O_2 on the viability of isolated bacteria of *L. plantarum* KCC-24 showed moderate resistance against various concentrations of H_2O_2.

4. Discussion

L. plantarum is a gram positive, non pathogenic, lactic acid producing bacteria, and have already exhibited several functions of a probiotic bacteria, such as existing in human gastrointestinal tract and saliva [19]. It is generally found in fermented food products, considered a harmless (GRAS- generally recognized as safe) microorganism for human consumption [20] and also plays an important role in the food products. Because of these properties, several researchers have been paying attention to lactic acid producing bacteria such as *L. plantarum* as a probiotic because of the increasing demand for probiotic products. In addition, *L. plantarum* was shown to possess probiotic properties with a wide range of health benefits, such as reducting cholesterol, pain, intestinal infection, regulating the immune system, and alleviating constipation [21]. At present, only a few of lactic acid producing bacterial strains such as *L. plantarum* 299v and *L. plantarum* Lp01 are commercially available for probiotic applications and these strains have been clinically proven to have probiotic properties in human intestinal tract [22].

The growing concern about human pathogens is a major issue worldwide. Therefore, searching for new antimicrobial compounds to treat pathogens is an important task. Many researchers have established the antifungal efficacy of lactic acid producing bacteria against several strains of fungi s by using the disc diffusion method. We examined the antifungal activity of isolated *L. plantarum* against *A. fumigatus, P. chrysogenum*, *P. roqueforti*, and *Botrytis elliptic* and *F. oxysporum*. KCC-24 exhibited strong fungal growth inhibition against *A. fumigatus* followed by *P. chrysogenum*, *P. roqueforti, and F. oxysporum* and *B. elliptic*. Generally, antimicrobial activity has been attributed to the production of various antimicrobial substance or metabolites such as organic acids, H_2O_2, ethanol, acetoin and bacteriocins etc. Among these, lactic acid, acetic acid, H_2O_2 and bacteriocins are the most powerful antimicrobial agents which are produced by the probiotics [23]. The present study, KCC-24 produced a significant amount of lactic acid, succinic acid and acetic acid. It is an important reason behind this antifungal activity of KCC-24.

One of the important required properties for acting as a good probiotic strains is their safety for human consumption without harboring acquired and transferable antibiotic resistance [24]. The present study, KCC-24 strain exhibited a susceptibility to chloramphenicol, nitrofurantoin, tetracycline, dicloxacillin, ampicillin, cefalexin, cefuroxime also resistance to kanamycin, sulphafurazole, colistin methane sulphonate, amikacin, gentamicin, cefoxitin, and co-Trimoxazole. Generally, lactobacillus strains exhibited resistance to amino glycoside group antibiotic such as gentamicin, kanamycin, neomycin, streptomycin and susceptibility to penicillin, ampicillin, erythromycin, novobiocin Chloramphenicol, rifampin, tetracycline antibiotics. KCC-24 showed similar susceptibility patterns to those of pervious investigations [24,25]. Hydrolysis of milk casein into smaller peptides and free amino acids by the action of the bacterial protease and peptidases are playing an important role chess ripening industries [26]. Present study, *L. plantarum* KCC-24 produced different types of proteolytic and glycolytic enzymes. This could be useful in the fermented food products related industries.

One more important criteria need to be met for a probiotic organism is their resistance to low pH of GI tract [27]. Generally bacteria are very sensitive to the low pH in the stomach [28], but a number of lactic acid producing bacteria can survive and grow in the lower pH because the transport lactic acid and protons to the cell's exterior. Present results showed that the isolated *L. plantarum* KCC-24 had significant tolerant property against low pH. Our study result was supported by Goldin et al. [29] reported that the

Table 3
Antibiotic susceptibility of isolated *L. plantarum* KCC-24.

Name of antibiotics	Disc potency (μg)	Susceptibility by *L. plantarum* KCC-24
Chloramphenicol (C)	50	S
Kanamycin (K)	30	R
Nitrofurantoin (NIT)	50	S
Tetracycline (TE)	100	S
Streptomycin (S)	25	R
Sulphafurazole (SF)	300	R
Colistin methane sulphonate (CL)	100	R
Dicloxacillin (D/C)	1	S
Ampicillin (AMP)	10	S
Amikacin (AK)	30	R
Gentamicin (GEN)	10	R
Cefoxitin (CX)	30	R
Cefalexin (CN)	30	M
Cefuroxime (CXM)	30	S
Co-Trimoxazole (COT)	25	R

<8 mm Moderate = M; > 10 mm Susceptibility = S; R = Resistant.

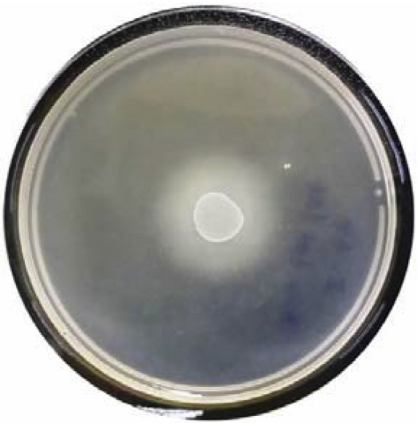

Fig. 3. Proteolytic activity of *L. plantarum* KCC-24 was observed on skim milk.

Table 4
Fermentative acids production of isolated *Lactobacillus plantarum* KCC-24.

Name of fermentative acids	Aerobic condition (mg/mL)	Micro-aerobic condition (mg/mL)
Lactic acid	124.90 ± 0.77	160.71 ± 2.25
Acetic acid	23.87 ± 0.93	25.68 ± 1.25
Succinic acid	3.624 ± 0.15	2.25 ± 0.16

The results are expressed as mean ± SD of three replicates.

digestive systems and these results agree with those of Turchi et al. [30]. Resistance to bile salts is an important property required for LAB to be functionally effective in the small intestine, and plays an important role in the gut's defense mechanism [31]. The *L. plantarum* KCC-24 exhibited a significant bile salts tolerant property. It suggested that the KCC-24 had ability to grow in the intestinal conditions.

The isolated *L. plantarum* KCC-24 exhibited proteolytic activity by releasing protease enzyme, which was confirmed by the for-

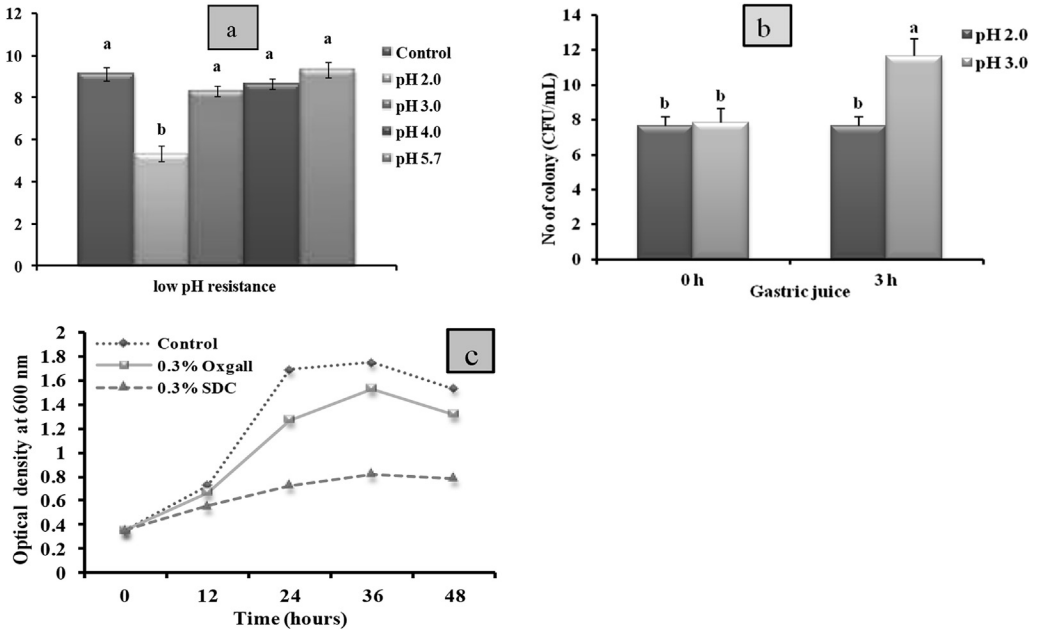

Fig. 2. a Showed the effects of acidity on the viability of *L. plantarum* KCC-24 at pH 2.0, 3.0, 4.0 and 5.7 after 0 min and 3 h. b showed the resistance to the stimulated gastric juice at pH 2 & 3 for 0 min and 3 h interval. c revealed the tolerance to bile salts at various time intervals. The results were expressed as mean of three replicates ± STD. [ab]different letters within treatment represent significant difference ($p < 0.05$).

Lactobacillus GG survived at pH 3 for 4 h incubation. Charteris et al. [12] demonstrated that *Lactobacillus* GG could grow at pH 2.3 for 3 h. The *L. plantarum* KCC-24 had ability to survive in the artificial gastric juices stress conditions. These results suggested the isolated *L. plantarum* had survival ability against harsh condition of mation of a clear zone on the medium. During the fermentation process, milk protein was hydrolyzed by proteolytic enzymes such as proteinases and peptidases, enhancing the amount of free amino groups and peptidase. Amino acids and peptidases levels are low in milk, so the probiotic bacterium depends on the proteolytic system

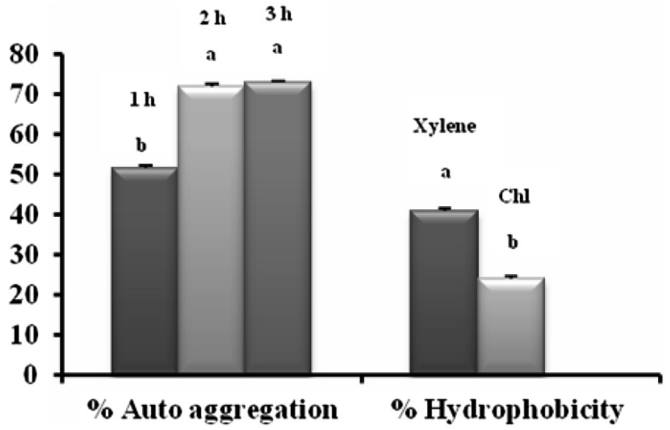

Fig. 4. Ability of *L. plantarum* KCC-24 on auto-aggregation and cell surface hydrophobicity. The results were expressed as mean of three replicates ± STD. [ab]different letters within treatment represent significant difference ($p < 0.05$).

commonly used to detect free radical scavenging activity [35]. The present study, cell free supernatant of KCC-24 showed significant free radical scavenging activity in a concentration dependent manner. It confirmed that the free radical scavenging activity of KCC-24.

In summary, *L. plantarum* KCC-24 showed significant antifungal activity against various pathogens, and displayed all of the properties of a probiotic, including resistance to pH, gastric juice, and bile salts in conditions mimicking the gastrointestinal tract. Furthermore, *L. plantarum* KCC-24 showed strong aggregation activity, which is related to its ability to adhere. Additionally, *L. plantarum* KCC-24 demonstrated the antioxidant activities by DPPH radical scavenging and resistance to hydrogen peroxide. To the best of our knowledge, *L. plantarum* KCC-24 isolated and characterized from Italian rye-grass forage met all the criteria required for consideration as a potential of probiotic. Therefore, *L. plantarum* KCC-24 could be useful for developing the quality food for ruminant animals and human.

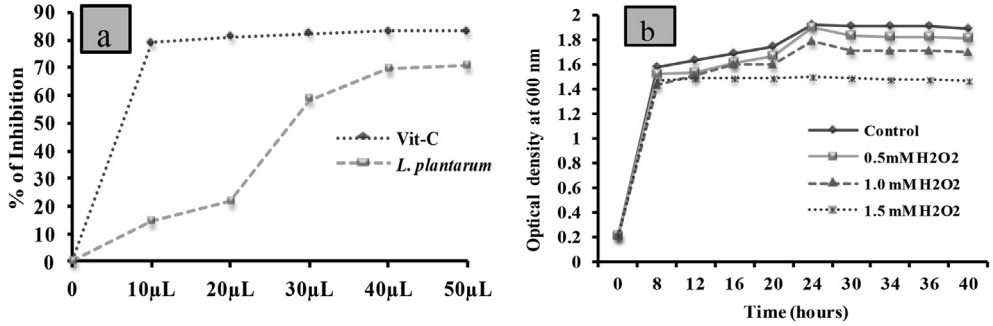

Fig. 5. a DPPH radical scavenging activity of *L. plantarum* KCC-24, b resistant of *L. plantarum* KCC-24 against H_2O_2 treatment. The results were expressed as mean of three replicates ± STD.

in the growth medium. Christensen and Steele [32] reported that impairment in the growth rate in milk is due to the loss of proteolytic enzymatic activity.

Cell adhesion may be defined as process by which interaction between the cell surfaces and membranes of the other microorganism. The ability to adhere to epithelial cells and mucosal surface is another important property in the selection of potential probiotic micro-organisms. It plays an important role in the removal of pathogens [33] and immunomodulation [34]. Classes of bacterial surface structures are involved in the aggregation with bacteria of the same strain. The present study, isolated *L. plantarum* KCC-24 showed potent auto-aggregation and it may play an important role in the preventing pathogenic bacteria from forming biofilms and in eliminating them from the GI tract. Cell surface hydrophobicity is a vital mechanism mainly involved in the auto-aggregation process. In this study, the isolated *L. plantarum* KCC-24 showed significant cell surface hydrophobicity. Generally, *Lactobacillus* bacteria with a high negatively charged cell surface has minimal adherence, whereas a cell surface with a low negative charge will strongly adhere. An increased concentration of salt ions decreases the number of water molecules to interact with the bacterial cell wall.

Free radicals may be defined as unavoidable molecules (end product) produced during normal metabolism. They are highly unstable, reactive, and can oxidize biomolecules, which results in oxidative damage. An antioxidant defense mechanism is important mechanism to scavenge free radicals. The DPPH radical assay is

Acknowledgments

This work was carried out with the support of Cooperative Research Program for Agriculture Science & Technology Development (Project title: Development of establishment and improvement of productivity in silvopastoral system; Project No. "PJ010186032014" Rural Development Administration, Republic of Korea. This study was supported by (2014) Postdoctoral Fellowship Program of (National Institute of Animal Science), Rural Development Administration, Republic of Korea.

References

[1] T.R. Klaenhammer, R. Barrangou, B.L. Buck, M.A. Azcarate peril, E. Altermann, Genomic features of lactic acid bacteria effecting bioprocessing and health, FEMS Microb. Heal 29 (3) (2005) 393–409.
[2] S.D. Tororov, M. Botes, C. Guigas, U. Cchillinger, I. Wiid, M.B. Wachsman, et al., Boza a natural source of probiotic lactic acid bacteria, J. Appl. Microbiol. 104 (2) (2008) 465–477.
[3] E. Caplice, G.F. Fitzgerald, Food fermentation: role of microorganisms in food production and preservation, Int. J. Food Microbiol. 50 (1–2) (1999) 131–149.
[4] V.M. Guchte, S. Penaud, C. Grimaldi, V. Barbe, K. Bryson, P. Nicolas, The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution, Proc. Natl. Acad. Sci. U. S. A. 103 (24) (2006) 9274–9279.
[5] W.H. Holzapfel, U. Schillinger, Introduction to pre and probiotics, Food Res. Int. 35 (2) (2002) 109–116.
[6] A.C. Ouwehand, P.V. Kirjavainen, C. Shor, S. Salminen, Probiotics: mechanisms and established effects, Int. Dairy J. 9 (1) (1999) 43–52.
[7] M. Valan arasu, M.W. Jung, S. Ilavenil, M. Jane, D.H. Kim, K.D. Lee, H.S. Park, T.Y. Hur, G.J. Choi, Y.C. Lim, N.A. Al-Dhabi, K.C. Choi, Isolation and characterization of antifungal compound from *Lactobacillus plantarum* KCC-10 from

forage silage with potential beneficial properties, J. Appl. Microbiol. 115 (5) (2013) 1172–1185.
[8] F. Sanger, S. Nicklen, A. Coulson, DNA sequencing with chain terminating inhibitors, Proc. Natl. Acad. Sci. U. S. A. 74 (12) (1977) 5463–5467.
[9] P. Lavermicocca, F. Valerio, A. Visconti, Antifungal activity of phenyllactic acid against molds isolated from bakery products, Appl. Environ. Microb. 69 (1) (2003) 634–640.
[10] A.W. Bauer, W.M. Kirby, J.C. Sherris, M. Turck, Antibiotic susceptibility testing by a standardized single disc method, Am. J. Clin. Pathol. 45 (4) (1996) 493–496.
[11] D.H. Tambekar, S.A. Bhutada, Studies on antimicrobial activity and characteristics of bacteriocins produced by *Lactobacillus* strains isolated from milk of domestic animals, Int. J. Microbiol. 8 (2) (2010) 1–6.
[12] W.P. Charteris, P.M. Kelly, L. Morelli, J.K. Collins, Development and application of an *in-vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract, J. Appl. Microbiol. 84 (5) (1998) 759–768.
[13] C.G. Vinderola, J.A. Reinheimer, Lactic acid starter and probiotic bacteria: a comparative *in-vitro* study of probiotic characteristics and biological barrier resistance, Food Res. Int. 36 (9–10) (2003) 895–904.
[14] S.N. Rajagopal, W.E. Sandine, Associative growth and proteolysis of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in skim milk, J. Dairy Sci. 73 (4) (1990) 894–899.
[15] B. Del Re, B. Sgorbati, M. Miglioli, D. Palenzona, Adhesion, auto-aggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*, Lett. Appl. Microbiol. 31 (6) (2000) 438–442.
[16] M. Rosenberg, D. Gutnick, E. Rosenberg, Adherence of bacteria to hydrocarbons: a simple method for measuring cell surface hydrophobicity, FEMS Microbiol. Lett. 9 (1) (1980) 29–33.
[17] F.A. Chen, A.B. Wu, P. Shieh, D.H. Kuo, C.Y. Hsieh, Evaluation of antioxidant activity of *Ruellia tuberosa*, Food Chem. 94 (1) (2006) 14–18.
[18] N. Buchmeier, S. Bossie, C.Y. Chen, F.C. Fang, D.G. Guiney, S.J. Libby, SlyA- a transcriptional regulator of *salmonella typhimurium* is required for resistance to oxidative stress and is expressed in the intra cellular environment of macrophages, Infect. Immun. 65 (9) (1997) 3725–3730.
[19] H. Michida, S. Tamalampudi, S.S. Pandiella, C. Webb, H. Fukuda, A. Kondo, Effect of cereal extracts and cereal fiber on viability of *Lactobacillus plantarum* under gastrointestinal tract conditions, Biochem. Eng. 28 (1) (2006) 73–78.
[20] G.B. Brinques, M.A.Z. Ayub, Effect of microencapsulation on survival of *Lactobacillus plantarum* in simulated gastrointestinal tract conditions, refrigeration and yogurt, J. Food Eng. 103 (2) (2011) 123–128.
[21] S. Sirilum, C. Chaiyasust, D. Kantachote, P. Luxananil, Characterization of non human origin probiotic *Lactobacillus plantarum* with cholesterol lowering property, Afr. J. Microbiol. Res. 4 (10) (2010) 994–1000.
[22] G. Giraffa, N. Chanishvili, Y. Widyastuti, Importance of *Lactobacilli* in food and feed biotechnology, Res. Microbiol. 161 (6) (2010) 480–487.
[23] A. Esayas, B. Fekadu, S. Amutha, Isolation and characterizing of inhibitory substance producing lactic acid bacteria from Ergo, Ethiopian traditional fermented milk, Afr. J. Microbiol. Res. 2 (2008) 229–234.
[24] J.S. Zhou, C.J. Pillidge, K. Gopal, H.S. Gill, Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains, Int. J. Food Microbiol. 98 (1) (2005) 211–217.
[25] N. Karasu, O. Simsek, A.H. Con, Technological and probiotic characteristics of *Lactobacillus plantarum* strains isolated from traditionally produced fermentative vegetables, Ann. Microbiol. 60 (2010) 227–234.
[26] J.I. Pitt, A.D. Hocking, Fungi and Food Spoilage, Chapman and Hall, New York, N.Y, 1999.
[27] R. Havenaar, B. Ten Brink, J.H.C. Huisint Veld, Selection of strains for probiotic use, in: Probiotics: Sci. Bas, 1992, pp. 209–224.
[28] P.L. Conway, S.L. Gorbach, B.R. Goldin, Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells, J. Dairy Sci. 70 (1) (1987) 1–12.
[29] B.R. Goldin, S.L. Gorbach, M. Saxelin, S. Barakat, L. Gaulthieri, S. Salminen, Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract, Dig. Dis. Sci. 37 (1) (1992) 121–128.
[30] B. Turchi, S. Mancini, F. Fratini, F. Pedonese, R. Nuvoloni, F. Bertelloni, V.V. Ebani, D. Cerri, Preliminary evaluation of probiotic potential of *Lactobacillus plantarum* strains isolated from Italian food products, World J. Microbiol. Biotechnol. 29 (10) (2013) 1913–1922.
[31] M. Succi, P. Tremonte, A. Reale, E. Sorrentino, L. Grazia, S. Pacifico, R. Coppola, Bile salt and acid tolerance of *Lactobacillus rhamnosus* strain isolated from *Parmigiano reggiano* cheese, FEMS Microbiol. Lett. 244 (1) (2005) 129–137.
[32] J.E. Christensen, J.L. Steele, Impaired growth rates in milk *Lactobacillus helveticus* peptidase mutants can be overcome by use of amino acid supplements, J. Bacteriol. 185 (11) (2003) 3297–3306.
[33] Y.K. Lee, K.Y. Puong, A.C. Ouwehand, S. Salminen, Displacement of bacterial pathogens from mucus and caco-2 cell surface by *Lactobacilli*, J. Med. Microbiol. 52 (Pt 10) (2003) 925–930.
[34] E.J. Schiffrin, F. Rochat, H. Linkamster, J.M. Aeschlimann, A. Donnethughes, Immunomodulation of human blood cells following the ingestion of lactic acid bacteria, J. Dairy Sci. 78 (3) (1995) 491–497.
[35] M. Vijayakumar, V. Selvi, S. Krishnakumari, K. Priya, A. Noorlidah, Elucidation of free radical scavenging potential of *Lagenaria siceraria* (Molina) Sandl fruit extract assayed by different methodologies-*in-vitro*, Asian Pac. J. Trop. Med. 6 (1) (2013) 20–26.