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Isolation and Characterization of *Lactobacillus plantarum* KCC-19 from Crimson Silage

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The present study was to isolate and characterize the lactic acid bacteria (LAB) from crimson silage and investigates their antimicrobial, probiotic and antioxidant potentials. The *Lactobacillus plantarum* KCC-19 was recovered from crimson silage exhibited better antibacterial and antifungal activity and it was deposited in the Korean Collection for type culture (KACC91816P). The secondary metabolites were extracted using organic solvents from spent medium in which KCC-19 was cultivated. The compound was characterized based on the infrared, Mass, ^{13}C nuclear magnetic resonance (NMR), and ^{1}H NMR spectral data. MRS medium supplemented with corn steep liquor and phenylalanine enhanced the production of antimicrobial substances under shake flask cultivation condition. The minimum inhibitory concentration (MIC) of the extracts and isolated compound against food spoilage bacteria and fungi was investigated. The ethyl acetate extract and isolated compound showed significant minimum inhibitory concentration against tested microbes. The HPLC analysis revealed that the amount of free amino acids liberated from fermented skim milk was ranged from 1.81 mg/1000 mL to 120.5 mg/1000 mL. KCC-19 was able to survive in bile salts (0.3%), acidic conditions (pH 3) and non-virulent with the highest percentage of hydrophobicity (100%). On the other hand, the antimicrobial susceptibility pattern was an intrinsic feature of this strain. In addition, KCC-19 shows the significant antioxidant activity. Our results suggested that LAB associated with crimson silage, particularly *L. plantarum* KCC-19, would be valuable sources of antimicrobial, probiotic and antioxidant properties, and hence, KCC-19 was determined to be suitable for application in functional foods.

Key words: *Lactobacillus plantarum*, MIC, Free amino acids, Probiotic properties, Antioxidant activity.

Recently, there has been a growing interest in the use of microorganisms and their metabolites to prevent spoilage and to extend the shelf life of foods. Lactic acid bacteria (LAB) are the organisms of particular interest in bio-preservation. The genus *Lactobacillus* comprises Gram-positive, rod shaped, catalase negative, non-motile, aero-tolerant, acid tolerant, nutritionally fastidious, and non-spore forming microorganisms. Lactobacilli are strictly facultative fermentative anaerobes that are tolerant to very small amounts of oxygen up to stric anaerobic conditions (Singh *et al*. 2009). Lactic acid is the main metabolic acid produced, enabling the members of this genus to

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better adapt to acidic conditions. LAB are known to produce antimicrobial metabolites that inhibit the growth of fungi and other species of bacteria; have a long tradition as starter cultures for different fermented food and feed (Arasu *et al*, 2014). Organic acids, ethanol, hydrogen peroxide, diacetyl bacteriocins and ribosomally synthesized antimicrobial peptides produced by LAB are important metabolites, and have been reported to possess antibacterial and antifungal activities. There are many reports on the production of antibacterial compounds by LAB (Stiles 1996). But the number of reports on antifungal LAB is still low and the majority only describes the inhibitory activities of LAB. Until now very few studies have characterized antifungal compounds or their mechanisms. The evolution and characterization of various LAB strains from fermented food and dairy products has been investigated showing the presence of *Lactobacillus delbrueckii*, *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. fermentum*, *L. curvatus*, *L. plantarum* and *L. helveticus* strains (Bulut *et al.* 2005). Among these LAB strains, *L. plantarum* is widely studied and can produce many antimicrobial substances such as 3-hydroxylated fatty acids, phenyllactic acid, 4- hydroxy-phenyllactic acid, benzoic acid, methylhydantoin, mevalonolactone and several cyclic dipeptides (Niku-Paavola *et al.* 1999; Lavermicocca *et al.* 2000; Magnusson *et al.* 2003; Sjogren *et al.* 2003; Arasu *et al.* 2013). The objective of the present study was to evaluate the antimicrobial potentiality of *Lactobacillus* sp. KCC-19 recovered from crimson silage. The metabolite involved in bio-preservative was identified, and the influence of different nitrogen sources supplement on the metabolite biosynthesis was evaluated. In addition, the probiotic characteristics such as tolerance to low pH, bile salts, antibiotic susceptibility, and free amino acid production capabilities and antioxidant activity were tested *in vitro*.

MATERIALS AND METHODS

Reagents and culture media

Antibiotic discs and culture media were purchased from Himedia, India. API 50CHB and API-ZYM test kits were acquired from Bio-Merieux SA, Marcy I'Etoile, France. Glucose and other chemicals were obtained from Sigma-Aldrich. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and other reagents and MRS medium were purchased from Sigma-Aldrich.

Microorganisms used

Bacteria

Bacillus subtilis (ATCC 7972), *Enterococcus cloacae* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), Staphylococcus (MTCC 3615). Fungi: *Aspergillus clavatus* (KCTC 40071), *A. fumigates* (KCTC 40080), *A. niger* (KCTC 40280), *A. oryzae* (KCTC 44823), *Curvularia lunata* (KCTC 40392), *Fusarium oxysporum* (KCTC 40051), *Gibberella moniliformis* (KCTC 44022), *Humicola grisea* (KCTC 40860), *Penicillium chrysogenum* (KCTC 40399) and *P. roqueforti* (KCTC 41354).

Isolation and identification of antifungal *L. plantarum* KCC-19

Lactobacillus plantarum KCC-19 was isolated from the crimson silage in an experiment following the method described by Arasu *et al.*,(2013). The antifungal inhibition spectrum of KCC-19 was determined by using the agar overlay method and well as the diffusion method, as described by Arasu *et al.*,(2013), on MRS agar plates, using *G.moniliformis* and *A. fumigatus* as an indicator (Arasu *et al.*,2013). KCC-19 was identified based on the biochemical profile and fermentation pattern studies. The biochemical profiles were carried out according to Bergey's manual of systemic bacteriology. The API 50CHL test (BioMerieux, France) was applied for identification by fermentation patterns. The 16S ribosomal DNA gene was amplified from the genomic DNA of the strain KCC-19 by PCR with Taq DNA polymerase, using the following primers: 27 forward primer (5' AGA GTT TGA TCG TGG CTC AG 3') and 1492 reverse primer (3' GGT TAC CTT GTT ACG ACT T 5'). The amplified PCR products were purified by QIAquick® PCR purification Kit (Qiagen Ltd., Crawley, UK). 1500 base pairs were sequenced by Solgent Co. Ltd. (Seoul, Korea). The obtained sequences were subjected to a BLAST search at http://www.ncbinlm-nih.gov/ in the NCBI database. The strain was stored at -80 °C in MRS broth with 20% glycerol. The stock cultures were propagated twice in MRS broth for 18 h before each experiment.

Nucleotide sequence and culture accession numbers

The 16S rRNA sequence of *L. plantaram* KCC-19 was deposited in the NCBI nucleotide sequence database under the accession number KC571201. A *L. plantaram* KCC-19 pure culture was deposited in the Korean Collection for Type Cultures, Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology, Republic of South Korea and was assigned the accession number KACC91758P.

Fungal biomass inhibition effect of *Lactobacillus* sp. KCC-19

Fungal biomass inhibition was examined by inoculating mid-log phase *L. plantaram* KCC-19 KCC-19 into individual 250 ml Erlenmeyer flasks containing 100 mL MRS broth and cultivating them for 48 h at 30°C on an orbital incubator shaker. Cell-free supernatants were collected by centrifugation at 12,000 rpm for 20 min. Aliquots (10 mL) of supernatant with 40 mL of PD broth were placed in 50 mL flasks and inoculated in triplicate with each test fungus. The fungal strains were incubated at 30°C for 5 days. Flasks without fermentation broth were the positive control. The growth performance of all fungal strains was checked separately in PD broth containing 100 mM acetic acid, lactic acid, and succinic acid to ensure that fungal inhibition was not simply due to nutrient exhaustion of the growth medium or acid production. After the incubation, fungal growth was measured by harvesting the cells, which were air-dried on pre-weighed Whatman #1 filter paper. Average fungal biomass were calculated for each test fungus and compared with the fungal biomass of positive controls.

Extraction of antifungal metabolites

Lactobacillus plantarum KCC-19 was cultivated at 30°C for 3 days in a sterile 5 L Erlenmeyer containing 3.0 L MRS broth with 2% glucose. Freshly prepared cells at an OD_{600} of 0.5 were used as the starter culture. At the end of the fermentation cycle, the fermentation medium containing antifungal metabolites was separated by centrifugation at 12,000 rpm for 30 min. The supernatant was extracted with hexane, ethyl acetate and chloroform (3 × 300 mL). The solvent phase was concentrated using a vacuum at 35°C to obtain the crude extract for the antifungal bioassay.

Isolation and identification of antifungal metabolites

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Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of extracts and isolated compound was performed according to the standard reference method (NCCLS 1999).

Influence of different nitrogen sources on antimicrobial metabolite production

To prepare the inoculum for fermentation experiments, one glycerol stock vial was used to inoculate 250 mL flask containing 50 ml MRS medium. The seed activation culture was grown at the 30 °C for 16 h. Two generations of activation cultures were prepared before fermentation for transferring metabolically active cells. This active culture was used as the seed inoclumn. Batch fermentation was performed in 1 L flasks. Then, 10 % (v/v) inoculum was aseptically added to 250 mL of sterilized MRS for fermentation. Agitation was monitored at a low rate (150 rpm) just sufficient to mix the medium. The fermentation temperature was adjusted to 30 °C. Different nitrogen sources such as corn steep liquor (1%), phenylalanine (5 mM) and tryptophan (5 mM) were supplemented in the initial MRS broth in order to study the antimicrobial metabolite production. The fermentation was carried out for 48 h. Samples were withdrawn

periodically to determine the cell growth and concentration of antifungal metabolite - 3-phenyl lactic acid.

Proteolytic activity of KCC-19

The proteolytic activity was measured by growing KCC-19 in 10% skim milk at 30°C for 42 h. The absorbance was read at 650_{nm} with an ELISA reader (Bio-Rad) (Citi *et al.*,1963). The results were expressed as milligrams/milliliter tyrosine by means of reference to a calibration curve.

Quantification of free amino acid produced by KCC-19

KCC-19 was prepared by growing cells in skim milk medium for 24 h at 30°C. About 5 mL of fermented skim milk was mixed with 10 mL 10% (v/v) metaphosphate and centrifuged at 12,000 rpm for 20 min at $4\ ^0$C. After that, 1 mL supernatant was mixed with 1 mL of citric acid buffer (pH 2.2) and quantitatively determined using HPLC

Growth of KCC-19 at low pH

Tolerance to low pH was determined using the plate count method. Briefly, active KCC-19 was grown in MRS broth and was inoculated (1%) in 10 mL of fresh MRS broth adjusted to pH 2.5 with hydrochloric acid (1.0 N) and incubated at 30°C for 3 h. Samples were determined for their initial bacterial population and residual cell population at 0 and 3 h, respectively, by plating suitable dilutions on MRS agar plates. The plates were incubated at 30°C for 48 h, and the number of colonies that grew was counted. The experiment was performed in triplicate.

Bile salts tolerance level of KCC-19

The ability of KCC-19 to grow in the presence of two different bile salts was studied according to the method of Vinderola and Reinheimer, (2003) with slight modification. MRS-thiobroth (MRS supplemented with 0.2% and 0.3% sodium thioglycollate) and MRS-thiobroth supplemented with 0.2% and 0.3% (w/v) Oxgall were freshly prepared and inoculated overnight with 1% suspensions of *Lactobacillus* sp. KCC-19. Samples without Oxgall were used as a control. After 24 h incubation at 30°C, bacterial concentration was checked by a viable count determination on MRS agar by plating suitable dilutions. The experiment was performed in triplicate.

Determination of antibiotic sensitivity and resistance pattern of KCC-19

Antibiotic sensitivity and resistance of KCC-19 was assayed by disc diffusion method (Arasu *et al.* 2013).

Haemolytic activity of KCC-19

Freshly prepared cells at 0.5 $OD_{600\ nm}$ KCC-19 culture were streaked on agar plates containing 5% (w/v) blood, and were incubated for 48 h at 30°C (Maragkoudakis *et al.* 2006).

Evaluation of cell surface hydrophobicity of KCC-19

The cell surface hydrophobicity assay was conducted according to the method described by Lee *et al.* (2011).

Antioxidant activity of KCC-19

The antioxidant effect of KCC-19 was evaluated by DPPH free radical scavenging activity according to the method described by Li *et al.* (2012a).

Analytical methods

The cell concentration was determined by measuring the OD at 600_{nm} in an ELISA reader (Bio-Rad). The quantification of phenyl lactic acid in culture broth was determined by high performance liquid chromatography (Agilent Technologies, USA). The supernatants obtained by centrifuging the culture samples at 10,000 g for 10 min were filtered through the Tuffryn membrane (Acrodisc, Pall Life Sciences) and eluted through a SB-C_{18} column (4.6 mm × 150 mm, 5 µm) at 65°C using methanol/0.05 % trifluoro acetic acid (solvent A) and water/0.05 % trifluoroacetic acid as the mobile phase at 0.5 mL/min. For quantification of amino acids, the chromatographic separation was performed on a Zorbax Eclipse AAA analytical (150 × 4.6 mm i.d., particle size 5 ¼m) column with guard column Zorbax Eclipse AAA 4-Pack (12.5×4.6 mm, i.d.) both purchased from Agilent Technologies. Detection was made at a wavelength of 338_{nm}, and the column oven temperature was set at 40°C. The injection sling was 10 ¼l. The solvent system was delivered at a rate of 2.0 ml/min and consisted of a mixture of (A) sodium dihydrogen phosphate buffer (40 mM) and (B) acetonitrile: methanol:water (45:45:10, v/v/v). The gradient program used as follows: 0-1.9 min, 0% B; 2-21 min, 57% B; 21.1-25 min, 100% B; 25.1-30 min, 0% B. Quantification of the different amino acids was based on peak areas and calculated as equivalents of standard amino acids.

RESULTS

The lactobacillus strain KCC-19 recovered from crimson silage stands for potential inhibitors of bacterial and fungal growth were selected further characterization to use as a probiotic and antioxidants. A total of 155 LAB colonies were isolated from crimson silage and screened for antimicrobial activity against bacterial and spoilage fungus. 28 % of the suspected LAB strains were found to have antifungal effects and 17% of the LAB strains exhibited good antibacterial activity. Among that strain KCC-19 inhibited the growth of food spoilage bacteria such as *B. subtilis*, *E. cloacae*, *S. aureus*, *S. epidermidis* and fungi such as *A. clavatus, A. fumigates, A. niger, A. oryzae*, *C. lunata*, *F. oxysporum*, *G. moniliformis*, *H. grisea*, *P. chrysogenum* and *P. roqueforti*. Strain KCC-19 was Gram-positive, catalase negative, and able to ferment sugars and secrete acid. It exhibited highest antimicrobial activity against all the tested spoilage fungus. Strain KCC-19 was confirmed with the physiological, biochemical characteristics and the information mentioned in the Bergey's manual of systemic bacteriology. The carbohydrate fermentation patterns of the strain were determined by using the API 50 CHL micro identification system. The 16S rRNA gene amplification and sequencing analysis confirmed that the strain KCC-19 shared 100% similarities towards *L. plantarum*. The strain was safely deposited in the culture collection under the accession number KACC91816P.

Shake flask cultivation and extra cellular metabolite production profile of KCC-19

The growth and extra cellular metabolite production profile of the strain KCC-19 and KACC 91096 were evaluated by culturing in MRS medium under aerobic condition. Results revealed that as compared to that of other standard LAB strains, KCC-19 which showed comparatively good growth in the initial stage (Fig. 1). The OD_{600} values recorded for the KCC-19 and KACC 91096 strains after 6 h cultivation was 1.41 and 1.28, respectively. The μmax values achieved with KCC-19 and KACC 91096 were 0.193 and 0.123/h, respectively. The higher growth rate of KCC-19 might be due to activation of the oxidative pathway because oxidative metabolism is responsible for the synthesis of essential cell growth components. The

Table 1. Antibacterial and antifungal activities of the crude extracts and compound obtained from *Lactobacillus plantarum* KCC-19

Microorganism	Minimum Inhibitory Concentration (MIC) (mg/mL)				
	HE	CE	EE	C	S*
Bacteria:	0	0	0	0	0
B. subtilis	-	-	10.00	10.00	2.5
E. cloacae	-	-	10.00	10.00	25
S. aureus	-	-	10.00	>5.00	6.25
S. epidermidis	-	-	5.00	>5.00	50
Fungi:	0	0	0	0	0
A. clavatus	10.00	ND	7.50	2.50	50
A. fumigates	10.00	-	7.50	5.00	50
A. niger	10.00	-	10.00	5.00	25
A. oryzae	-	-	5.00	2.50	25
C. lunata	-	-	10.00	5.00	37.5
F. oxysporum	>10.00	-	5.00	7.50	25
G. moniliformis	10.00	ND	10.00	5.00	75
H. grisea	-	-	-	10.00	100
P. chrysogenum	>10.00	>10.00	5.00	2.50	50
P. roqueforti	>10.00	>10.00	10.00	2.50	25

S (streptomycin), bacterial control reference; S (ketoconazole), fungal control reference; HE, hexane extract; CE, chloroform extract; EA, ethyl acetate extract; C, compound; ND, not determined.

results revealed that after a 24 h cultivation, the pH of the fermented broth declined to 3.7. This clearly indicated that the LAB strains were able to secrete organic acids. KACC 91096 produced comparatively the highest amount of lactic acid after 24 h incubation, whereas, KCC-19 also produced a significant amount of lactic acid, acetic acid and succinic acid.

Fungal biomass inhibition effect of KCC-19

The fermented broth of KCC-19 was evaluated for its antifungal activity after filtration. The spent medium significantly inhibited the growth of fungi, compared with that of MRS controls and an MRS medium with organic acids based on dry weight measurements of fungal biomass (Fig. 2). The results revealed that the supernatant of KCC-19 had better antifungal activity against nine fungal strains. *F. oxysporum*, *A. oryzae*, *A. clavatus* and *A. niger* were the most sensitive among the fungus recorded above 60% of fungal biomass inhibition, whereas, *A. fumigates*, *P. chrysogenum* and *H. grisea* showed the inhibition range from 55.5% to 58.7% respectively, but the other spoilage fungus revealed less activity. The organic acids did not exhibit a significant decrease in the growth of fungal biomass confirm the presence of other metabolites in the spent medium.

Table 2. Quantification of free amino acid contents (mg/1000 mL.) in fermented skim milk by *Lactobacillus plantarum* KCC-19 (*n*=3)

No.[a)]	Amino Acids	Quantity (mg/ 1000 mL)
1	Aspartate	31.08±0.14
2	Glutamate	120.50±0.53
3	Asparagine	27.70±0.60
4	Serine	17.87±0.34
5	Glutamine	9.68±0.59
6	Histidine	4.38±0.13
7	Glycine	23.15±0.77
8	Threonine	11.33±0.577
9	Arginine	45.56±0.51
10	Alanine	9.51±0.47
11	Tyrosine	1.81±0.11
12	Cystine	6.32±0.27
13	Valine	27.03±0.05
14	Norvaline	ND[b)]
15	Methionine	38.13±0.32
16	Tyrptophan	29.50±0.49
17	Phenyl alanine	83.3
18	Isoleucine	30.65±0.43
19	Leucine	73.39±0.52
20	Proline	61.15±0.78
21	Lysine	17.28±0.29

[a)]No., the elution order of free amino acids from HPLC chromatograms. [b)]ND, not detected

Inhibitory properties and identification of the antifungal metabolite

The antimicrobial metabolites were sequentially extracted by using organic solvents such as hexane, chloroform and ethyl acetate. The antibacterial susceptibility pattern of these extracts revealed that the ethyl acetate extract obtained from KCC-19 showed significant activity (Table 1). It exhibited MIC 5 mg/ mL for *S. epidermidis*, whereas, the other extracts showed MIC of 10 mg/ ml respectively. Hexane and chloroform extracts did show activity against the bacterial strains. Hexane and ethyl acetate extracts exhibited a marked antagonistic activity against the fungal strains (ethyl acetate extract showed comparatively better activity than hexane extract). Hexane extract exhibited MIC 10 mg/ mL for *A. clavatus*, *A. fumigatus*, *A. niger* and *G. moniliformis*. *A. oryzae*, *C. lunata*, and *H. grisea* did not show activity. Ethyl acetate extract showed MIC 5 mg/ mL for *A.*

Table 3. Probiotic properties of *Lactobacillus plantarum* KCC-19

pH	Viable count (log CFU/ml) Tolerance level				
	0 min	30 min	60 min	120 min	180 min
Control	8.20+0.07	7.27+0.14	9.90+0.10	10.28+0.25	10.33+0.06
2.5	8.14+0.15	7.96+0.15	7.86+0.06	7.93+0.04	7.8+0.02
3.0	8.30+0.19	8.43+0.05	8.68+0.79	8.91+0.04	9.14+0.04
4.0	8.28+0.06	8.60+0.01	8.76+0.05	8.89+0.01	9.45+0.05

Values are means of triplicate determinations with standard deviations.

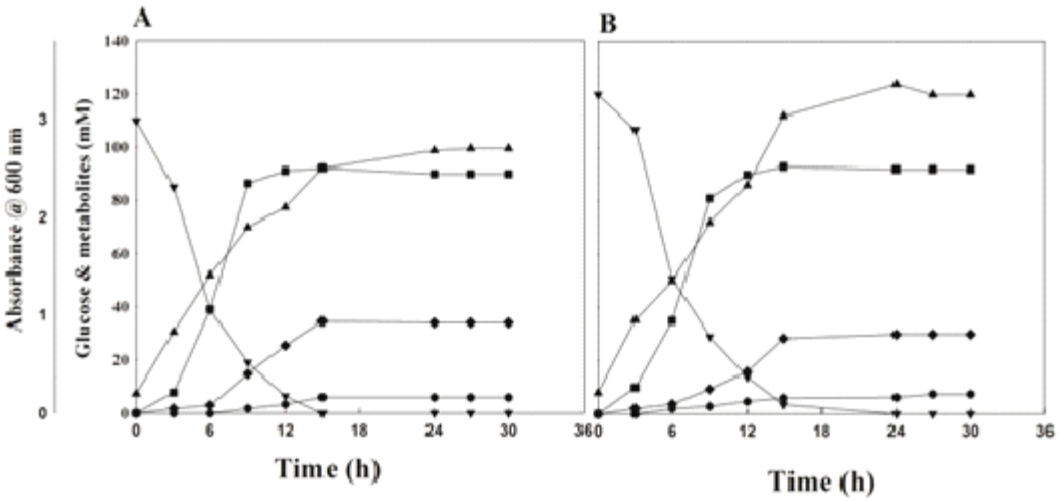
oryzae, F. oxysporum and *P. chrysogenum.* MIC values of ethyl acetate extract showed comparatively better activity than other extracts. The MIC value of 3-phenyl lactic acid recorded as 2.50 mg/ml against *A. clavatus A. oryzae P. chrysogenum P. roqueforti*. These results are compared with the standard broad spectrum antibiotic streptomycin and ketaconazole.

Variation of free amino acids in fermented milk

Proteolytic activity of KCC-19 was determined as 8.3 µg/ mL tyrosine liberation. The amount of individual free amino acids present in

Table 4. Comparative sensitivity pattern of *Lactobacillus plantarum* KCC-19 towards various antibiotics Zones of inhibition were measured after incubating the strains for 48 h at 30°C in MRS agar medium (MRSA).

Antibiotic group	Antimicrobial agent	Disc potency (µg)	Diameter of inhibition zone (mm)*	Status[a]
Aminoglycoside	Amikacin	30	23	S
	Gentamicin	10	26	S
	Kanamycin	30	21	S
	Streptomycin	10	17	S
	Tobramycin	10	18	S
Carboxypenicillin	Carbenicillin	50	24	S
	Ampicillin	50	24	S
	Amoxyclav	10	13	S
	Augmentin	30	27	S
	Dicloxacillin	1	20	S
β-lactamase inhibitor	Imipenem	10	20	S
	Meticillin	5	31	S
	Penicillin	100 U	18	S
	Sulbactam	15	24	S
	Ticarcillin	75	25	S
	Ciprofloxacin	5	22	S
	Gatifloxacin	5	23	S
	Levofloxacin	5	27	S
	Moxifloxacin	5	20	S
Fluroquinolone	Nalidixic acid	30	21	R
	Norfloxacin	10	25	S
	Ofloxacin	5	32	S
	Sparfloxacin	5	28	S
	Cefpodoxime	10	18	S
	Ceftriaxone	30	20	S
	Ceftazidime	30	20	S
Cephalosporin	Cephalothin	30	21	S
	Cefpodoxime	30	27	S
	Cetriaxone	30	18	S
	Cephaloridine	30	20	S
Cephamycin antibiotic	Cefoxitin	30	15	S
Glycopeptide antibiotic	Vancomycin	30	0	R
Polymixin	Colistin	10	24	S
Polyketides	Tetracyclin	100	0	R
Sulphonamide	Co-Trimoxazole	25	27	S
	Sulphafurazole	300	31	S
Lincosamide antibiotic	Clindamycin	2	21	S
	Lincomycin	30	15	S
	Erythromycin	15	0	R
Macrolide antibiotic	Oleandomycin	15	27	S
Nitrofuran antibiotic	Nitrofurantoin	50	34	S



A; *Lactobacillus plantarum* KCC-19, B; *Lactobacillus plantarum* KACC 91096.
1. Symbols: triangle, cell growth (OD at 600 nm); square, residual glucose; square, lactic acid; diamond, acetic acid; circle, succinic acid.

Fig. 1. Growth and metabolite production profiles under aerobic condition

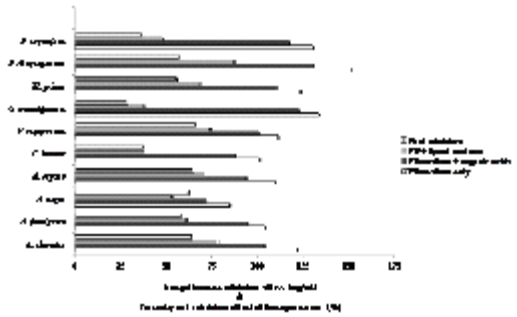
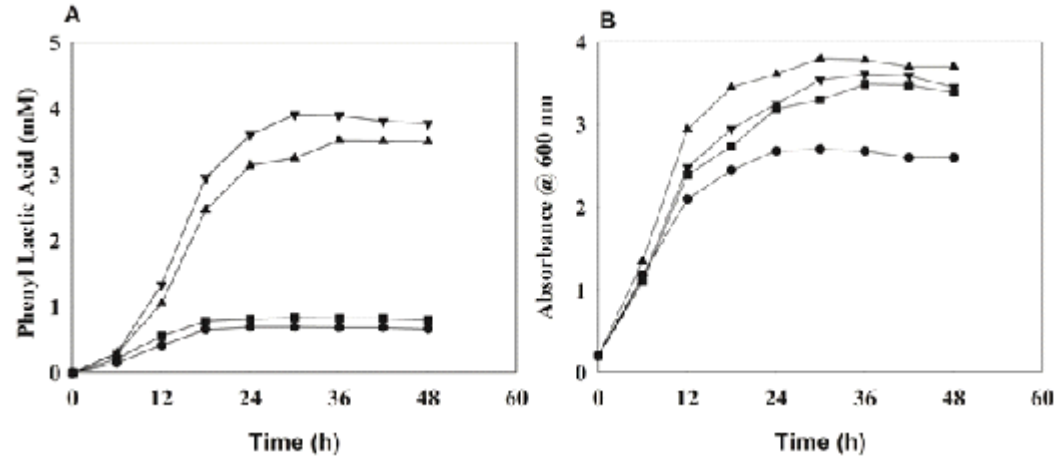


Fig. 2. Fungal biomass inhibition effect of *Lactobacillus plantarum* KCC-19

fermented skim milk was quantified using HPLC. The free amino acids content were ranged from 1.81 mg/1000 mL to 120.5 mg/1000 mL (Table 2). Nine essential amino acids including arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, and valine contributed 48% of the total free amino acids liberated. Among the essential amino acids; phenylalanine documented highest content (83.3 mg/1000 mL) and accounted >26% of the total essential amino acid, whereas histidine documented the lowest



A, phenyl lactic acid production profile; B, growth profile. Symbols: Circle, only MRS; square, MRS with tryptophan; down triangle, MRS with phenylalanine; triangle; MRS with corn steep liquor respectively.

Fig. 3 Growth and phenyl lactic acid production profiles of *Lactobacillus plantarum* KCC-19 using different nitrogen sources under aerobic condition

level (4.38 mg/1000 mL). The major amino acids found in the fermented milk were glutamate (120.5 mg/1000 mL), leucine (73.39 mg/ 1000 mL), arginine (45.56 mg/1000 mL). Norvaline did not detect in the fermented sample.

Probiotic properties of *L. plantaram* KCC-19

The capability of microbes to grow and survive in the gastrointestinal tract is one of the most important characteristics of effective potential probiotic activity. Therefore, the viability of KCC-19 at low pH and high concentrations of oxbile salts were studied. The viabilities of *L. plantarum* KCC-19 at pH 2.5, 3.0 and 4.0 are presented in Table 3. The results indicated that the cell grew well in the control condition and viability was slightly decreased at pH 2.5, whereas, at pH 3.0 and 4.0 the strain exhibited stable growth. The survival ability of the strain in the presence of bile salts (Oxgall (0.2% and 0.3%) and sodium taurocholate (0.2% and 0.3%) is presented in figure 4. The results revealed that the strain was able to grow in the presence of sodium taurocholate and was slightly sensitive to oxgall. The strain showed negative results for haemolysis of agar, and was therefore determined to be in non-virulent in nature and exhibiting high hydrophobicity (100%). All the antibiotics tested in this study inhibited the growth of *L. plantarum* KCC-19 and revealed a similar sensitivity pattern (Table 4). Therefore this strain is considered to be safe for usage as probiotics.

Free radical scavenging activity

The free radical scavenging effect of *L. plantarum* KCC-19 revealed that the activity was dose dependent of DPPH, with the highest radical-scavenging activity (41.22%) at 2.5 mL of cells at 10^9 CFU/mL (Figure 5).

DISCUSSION

In recent years much attention has focused on antimicrobial compound producing LAB isolated from various sources, because metabolites such as bacteriocins and lactic acid derivatives are considered safe as food bio-preservatives and did not cause health hazards to humans (Jeevaratnam *et al.* 2007). The bacterial and fungal strains used in this study have been previously linked to the spoilage of various foods and fruits (Katsumata *et al.* 2002). Also, researchers

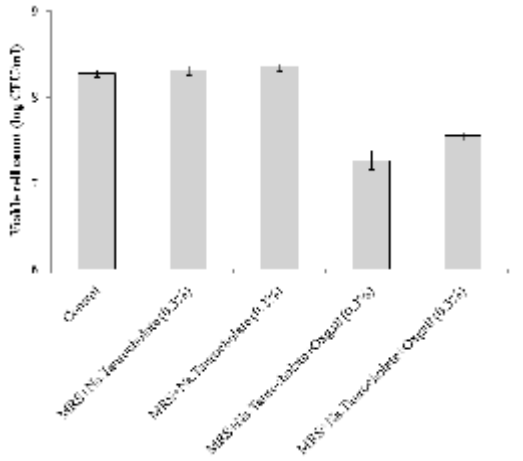


Fig. 4. Cell viability of *Lactobacillus plantarum* KCC-19 in the presence of different concentration of bile salts

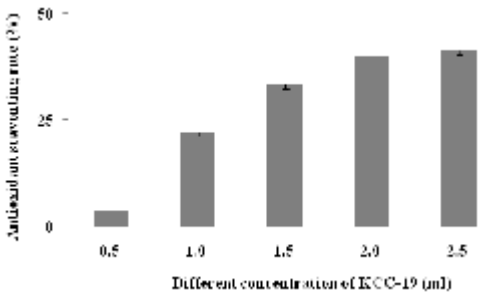


Fig. 5. Antioxidant activities of *Lactobacillus plantarum* KCC-19

reported that *Lactobacillus* strains possessed inhibitory activity against *E. coli*, and it has been confirmed that a symbiotic diet containing probiotics reduces the population of intestinal microorganisms (Liong and Shah 2006). Many reports claimed that the antimicrobial activities depend on microbial community composition as well as environment and growth conditions (Pfeiler and Klaenhammer 2007). In our previous study, most of the *Lactobacillus* strain grows well on MRS agar medium and BCP agar medium (Arasu *et al.* 2013). So MRS and BCP medium were used for isolating *Lactobacillus* strains. During the isolation of antimicrobial metabolite producers, 155

Lactobacillus sp. were screened and we selected *Lactobacillus* sp. KCC-19 that had significant antagonistic activity against the bacteria and spoilage fungus. The strain was able to ferment ribose, galactose, glucose, fructose, mannose, n-acetyl glucosamine, esculin, salicin, cellobiose and gentiobiose, indicating a wide pattern of carbon assimilation. These results were in close agreement with the findings of Abbasiliasi *et al.* (2012). It has been found that the majority of the *Lactobacillus* recovered from silage were resistant to low pH and able to produce antimicrobial metabolites as already reported by Han *et al.* (2012) and Gollop *et al.* (2005). The *Lactobacillus* sp. KCC-19 showed 100% similarity to *Lactobacillus plantaram* based on 16S rRNA gene sequences. The morphological and biochemical characteristics also reflected those of *Lactobacillus* genera.

L. plantarum KCC-19 showed good antifungal activity in solid medium and also in fermented spent broth. The fungal biomass inhibition effect of the spent medium has been confirmed by carrying out by external addition of organic acids because lactic acid can lower the pH and create an unsuitable growth condition for the fungi (Cabo *et al.* 2002; Stiles *et al.* 2002). Organic acids can only penetrate the microbial cell wall and the pKa of lactic acid, acetic acid and 3-phenyl-lactic acid is 3.8, 4.7, and 3.5, respectively (Demuynck, 2004). Li *et al.* (2012b) reported that fumaradimycine 2,6-diphenyl-piperidine an antifungal metabolite isolated from *L.casei* AST18 was active in agar medium and fermentation broth (Li *et al.* 2012b). Our results indicated that the antimicrobial metabolites were extracellular. Purification of the compound was carried out in a silica column, and the compound was chemically characterized by ^{1}H NMR and ^{13}C NMR and identified as 3-Phenyllactic acid. 3-Phenyllactic acid showed moderate activity against tested bacteria and fungi. Previously, 3-phenyllactic acid was well characterized having a broad inhibitory activity against yeast and bacteria (both Gram-positive and Gram-negative) (Dieuleveux and Gueguen 1998). Dalbello *et al.* (2007) reported that lactic acid, phenyllactic acid, and two cyclic dipeptides (cyclo (L-Leu-L-Pro) and cyclo (L-Phe-L-Pro)) were the major components responsible for the antifungal activity of *L. plantarum* FST 1.7. Phenyllactic acid is active against several fungal species (including some mycotoxigenic isolates such as *A. ochraceus*, *P. verrucosum* and *P. citrinum*) and certain contaminating bacteria, namely *Listeria* sp., *S. aureus* and *E. faecalis* (Dalbello *et al.* 2007). The antimicrobial activity of phenyllactic acid would be linked to the lipophility of the undissociated active form. As all organic acids, the rate of dissociation was dependent on the pH. At low pH, the undissociated form can easily pass across the cell membrane and then accumulate within the cytoplasm, thereby causing loss of viability and cell destruction (Torino *et al.* 2001). Gram positive bacteria are more susceptible as they have a more permeable outer peptidoglycan layer (Arasu *et al.* 2013).

Phenyl lactic acid is produced by a wide range of LAB species, such as *Lactobacillus*, *Enterococcus*, *Weissella*, and *Leuconostoc*, but the production varied greatly among strains and species. When grown in MRS medium, most of LAB strain produced less than 1 mM phenyl lactic acid; however, *L. plantarum* and *L. acidophilus* could produce phenyl lactic acid in a range of 1-3.5 mM, respectively (Gerez *et al.* 2010; Rodriguez *et al.* 2012). Shake flask cultivation of KCC-19 using different nitrogen sources revealed that phenylalanine and CSL enhanced the production of phenyl lactic acid. Vermeulen *et al.* (2006) reported that α-ketoglutarate enhanced phenyl lactic acid production in *L. plantarum*.

An important step towards the selection of potential probiotic characters is to investigate the strain behavior under conditions which mimic the gastrointestinal tract (FAO, 2002). The excellent characteristic of KCC-19 to survive in acidic pH and moderate to low bile tolerance similar to that present in the oral and gut conditions was confirmed. In the recent study, bile concentrations ranging from 0.2% to 0.3% were used (Vizoso-Pinto *et al.* 2006), while Mathara *et al.*(2008) established a limit of 0.3% bile to select strains considered to have good resistance (more than 50% growth when compared to the control without bile added to the medium). By following the same criteria, KCC-19 was able to survive under low pH and bile salt concentration, confirming its adaptation ability. Similarly, a strain-dependent tolerance to conditions was also observed in *L. plantarum* strains recovered from Bulgarian cheeses and from Fiore Sardo cheese (Georgieva *et al.* 2008; Pisano

et al. 2008). Antibiotic-resistant strains can be detrimental to the health of humans and animals, because they are capable of transferring antibiotic resistance genes to pathogenic bacteria, which can contaminate raw food products such as meat or milk (Korhonen *et al.* 2010). KCC-19 was susceptible to most of the common antibiotics with high hydrophobicity (100%) and negative results for haemolysis, which is rarely observed in food LAB (Maragkoudakis *et al.* 2006).

An oxidative stress involved in LAB strains are mainly regulated by the antioxidant enzymes, such as SOD, NADH-oxidase and NADH peroxide, and heterologous non-haem catalase. These intracellular enzymes present in microorganisms such as *Streptococcus thermophilus*, *Bifidobacterium longum*, *L.plantarum* and *L. casei* exhibited *in vitro* antioxidant effects after disrupting the bacterial cells (Kullisaar *et al.* 2002). A DPPH free radical scavenging study indicated that the cell surface active compounds of KCC-19 may be involved in antioxidant activity, and that it is directly proportional to cell concentrations confirmed that the antioxidant activity of LAB strains are mainly because of the secretion of extracellular metabolites (Pan and Mei 2010). It is predicted that the cell-surface proteins or polysaccharides of KCC-19 might involved in the antioxidant activity of this strain.

CONCLUSION

In conclusion, this study demonstrated that *L. plantarum* KCC-19 possess good antimicrobial activities against spoilage bacteria such as bacteria, fungi and functional probiotic properties like high tolerance to acidic condition, bile salts and exhibit antioxidant activity. However, this strain has interesting potential for practical application as bio-preservative in the food industry and for agriculture purposes.

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