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Identification and in vitro assessment of potential probiotic characteristics and antibacterial effects of *Lactobacillus plantarum* subsp. *plantarum* SKI19, a bacteriocinogenic strain isolated from Thai fermented pork sausage

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Abstract A total of 2257 lactic acid bacteria were preliminarily screened for antagonistic activity against *Lactobacillus sakei* subsp. *sakei* JCM 1157. Strain SKI19 was selected and identified at the subspecies level as *Lactobacillus plantarum* subsp. *plantarum* SKI19, using *16S* rRNA gene sequence analysis combined with *recA* and *dnaK* genes' amplification. Antibacterial activity of SKI19 was completely lost after treatment of neutralized cell free culture supernatant with proteolytic enzymes, suggesting that SKI19 produced a bacteriocin-like substance that inhibited not only closely related species, but was also effective against *Listeria monocytogenes* DMST 17303. Viewed under scanning electron microscope, cell membranes of the indicator strain appeared to collapse after exposure to the bacteriocin-like substance. In vitro tests concerning probiotic properties, SKI19 survived under simulated gastrointestinal tract conditions, and adhesion of its cell surface to xylene and chloroform was 90.14 and 89.85%, respectively. Complete inhibition by SKI19 against pathogenic bacteria (*Escherichia coli* DMST 4212, *L. monocytogenes* DMST 17303, and *Staphylococcus aureus* DMST 8840) was observed in co-cultivation under anaerobic conditions. A safety assessment showed that SKI19 was susceptible to several antibiotics and had no haemolytic activity. PCR amplification of virulence factors with the specific primers for *ace*, *asa1*, *cylL$_S$*, *efaA$_{fs}$*, *hyl*, and *gelE* genes were negative for SKI19. Also, SKI19 did not harbor any *hdc*, *tdc*, *odc* or *ldc* genes involved in biogenic amine production. The results reveal that SKI19 has probiotic potential and antibacterial activity, and is safe for further application in certain food products.

Keywords Bacteriocin · *Lactobacillus plantarum* subsp. *plantarum* · Probiotic · Inhibition · Sausage

Introduction

Lactic acid bacteria (LAB), used in fermenting food, have the "generally regarded as safe" (GRAS) status, and are found in natural environments (Belicova et al. 2013). Physiological characteristics principally associated with them are substrate utilization, metabolic capabilities, and probiotic properties. Besides producing lactic acid during fermentation, LAB also release antimicrobial substances including bacteriocins. These have gained much attention as safe biopreservatives, since they are degraded by protease in the gastrointestinal tract (GI). These antimicrobial compounds are ribosomally synthesized and extracellularly released low molecular mass peptides or peptide complexes (usually 30–60 amino acids) having bactericidal or biostatic activity. Some bacteriocins appear to inhibit different indicator bacteria, including food poisoning microorganisms, foodborne pathogenic bacteria, and closely related species (Swetwiwathana and Visessanguan 2015). Furthermore, LAB have been qualified as potential probiotics, which is of interest because of health and safety concerns that require development of food products that promote healthy well-being beyond basic nutrition. Probiotics are defined as "Live microorganisms which when

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administered in adequate amount confer a health benefit on the host" (FAO/WHO 2002). In order to be qualified as a candidate probiotic, a strain should be screened both for its essential functional properties (resistance to gastric acidity and bile salt, antagonistic effect against pathogenic bacteria, and adhesion to gut tissues), and for its safety properties (antibiotic resistance, biogenic amine production, virulence factors, and haemolytic activity) (Belicova et al. 2013). Use as probiotics in fermented dairy or sausage products has been documented for *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Bifidobacterium longum* (Vuyst et al. 2008).

Sai krok e-san mu is a traditional Thai fermented pork sausage, basically made of pork, cooked rice, garlic, salt, and sugar. Various microorganisms, predominantly LAB, are involved in the fermentation. Visessanguan et al. (2015) found *L. plantarum*, *Lactobacillus pentosus*, *Lactobacillus sakei*, and *P. pentosaceus*, as predominant in meat fermentation. However, meat products fermented by naturally occurring LAB can potentially be contaminated by pathogenic bacteria such as *Salmonella* spp., *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, and *Listeria monocytogenes* (Paukatong and Kunawasen 2001). Chemical preservatives inhibit harmful bacteria, but long-term exposure to artificial preservatives may adversely impact the consumer's health. This suggests that bacteriocin-producing strains could be used as natural preservatives that inhibit the growth of and the spoilage by pathogenic microorganisms, and the strains' probiotic properties could allow their use as starter cultures.

The objectives of the present study were to screen LAB isolated from sai krok e-san mu. A chosen strain was further identified and preliminarily characterized for antibacterial compounds and for inhibitory spectrum. Its probiotic potential (survivability in the gut environment, bacterial adhesion to hydrocarbons, and co-cultivation) was also evaluated. Haemolysis, susceptibility to antibiotics, presence of virulence factors, and biogenic amines were also examined, to determine its safety for probiotic use.

Materials and methods

Bacterial strains and growth media

The genetically closely related species used as indicator strains, namely *L. sakei* subsp. *sakei* JCM 1157, *L. plantarum* TISTR 875, and *P. pentosaceus* TISTR 413, were purchased from the Japan Collection of Microorganisms (JCM) and the Thailand Institute of Scientific and Technological Research (TISTR), respectively. *Enterococcus faecium* CKF12 and *E. lactis* CKF16 were from our own

cultured stocks, whereas *E. faecalis* CM6CR07 and *E. faecalis* CF1GI14 were kindly provided by Dr. Noraphat Hwanhlem (Hwanhlem et al. 2017). These indicator strains were cultured in de Man, Rogosa and Sharpe (MRS) medium under aerobic conditions at 37 °C. The food spoilage and foodborne pathogenic bacteria used in this research were based on the Thai Community Product Standards. All of them, including Gram-positive and Gram-negative bacteria, were purchased from the Department of Medical Science, Ministry of Public Health, Thailand (DMST). *E. coli* DMST 4212 was cultured in Luria–Bertani (LB) medium. *B. cereus* DMST 5040 and *S. aureus* DMST 8840 were cultured in nutrient broth (NB). *Clostridium perfringens* DMST 16637 was cultured in Thioglycolate broth (TB) under anaerobic conditions (HiMedia, India). *L. monocytogenes* DMST 17303 and *Salmonella* were cultured in trypticase soy broth (TSB). The strains were cultured for 18–24 h at 37 °C and subcultured at least twice prior to the experiments. All strains used in this study were maintained as stock cultures at − 80 °C in 30% glycerol.

Isolation and screening of bacteriocin-producing LAB

Ten freshly prepared sai krok e-san mu samples were bought from local markets in Songkhla province. Samples (25 g) of sai krok e-san mu were added to 225 ml of normal saline solution (0.85% NaCl). Appropriate decimal dilutions (10^5–10^6 CFU/ml) were prepared in normal saline solution and poured onto molten MRS agar (45 °C) containing 1% $CaCO_3$. The plates were incubated at 37 °C for 2–3 days under microaerophilic conditions. The LAB strains that showed clear zones were picked for re-streaking three times to obtain single colonies. Antagonistic activity detection was performed by the agar spot assay. Briefly, 5 µl of overnight cultures in MRS broth were spotted onto bacteriocin screening medium and incubated at 37 °C for 24 h under anaerobic conditions. LAB colonies were overlaid with 10 ml of semi-soft MRS (0.75% agar) containing *L. sakei* subsp. *sakei* JCM 1157 (10^6 CFU/ml). Those LAB colonies that showed clear zones of growth inhibition were then examined by microscopy. Isolates that exhibited Gram-positive, catalase-negative, and non-motile cells were selected for further investigation. Production of antibacterial substances by the selected LAB strains was determined by the drop plate method. Overnight culture of LAB was centrifuged at $10,000 \times g$ for 10 min at 4 °C and cell-free culture supernatant was filtered through a 0.22 µm filter. Inhibition was observed by spotting 10 µl of the supernatant onto semi-soft agar seeded with *L. sakei* subsp. *sakei* JCM 1157, and then incubating anaerobically overnight at 37 °C. That LAB strain

showing the maximal inhibition zone of the indicator strain was selected for further identification and characterization.

Identification of selected LAB strain SKI19

The bacterial cells were extracted using the TIANamp Genomic DNA kit (TIANGEN, China), following the manufacturer's instructions. The primers and the PCR conditions used for the amplification of *16S* rRNA, *recA*, and *dnaK* genes are summarized in Table 1. The PCR products were analyzed by electrophoresis in 1% agarose gel and were directly visualized under ultraviolet light. The amplified PCR products were purified by using a PCR purification kit (Vivantis, Malaysia), following the manufacturer's instructions prior to sequencing (1st BASE, Malaysia). The obtained sequences were edited by BioEdit

Sequence Alignment Editor V 7.2.1 program, and were compared to sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST). Phylogenetic trees were constructed using the neighbor-joining method in the Molecular evolutionary genetics analysis (MEGA 7.0) software.

Characterization of antibacterial compounds and inhibitory spectrum

SKI19 was cultured in MRS broth (100 ml) and incubated at 37 °C for 18–24 h. Then, the entire culture broth was centrifuged at $10,000 \times g$ for 10 min at 4 °C. The supernatant obtained was used in the experimental trials as follows: (1) the cell free supernatant (CFS) was used directly; (2) the CFS was neutralized to pH 6.5 using 6 N NaOH to

Table 1 Primers and PCR conditions used for the identification and screening of virulence and biogenic amine genes

Target gene	Primer (5′–3′)	AT (°C)	Size (bp)	References
Genetic markers				
16S rRNA	27F: AGAGTTTGATCCTGGCTCAG	50	1500	Gong et al. (2010)
	1492R: ACGGCTACCTTGTTACGACTT			
recA	planF: CCG TTTATGCGGAACACCTA	56	322	Torriani et al. (2001)
	pREV: CGGGATTACCA AAC ATCAC			
dnaK	Lpdnak-500F3: CCGTTCTTRTCRATRTCRAA	58	1100	Huang et al. (2010)
	Lpdnak-1710R5: GAAAYY CAAGTYGGHGAAGT			
Virulence genes				
ace	ACE-F: GAATTGAGCAAAAGTTCAATCG	56	1008	Martín-Platero et al. (2009)
	ACE-R: GTCTGTCTTTTCACTTGTTTC			
asa1	ASA11: GCACGCTATTACGAACTATGA	56	375	Vankerckhoven et al. (2008)
	ASA12: TAAGAAAGAACATCACCACGA			
cylL$_S$	CylL$_S$1: GAAGCACAGTGCTAAATAAGG	55	580	Semedo et al. (2003)
	CylL$_S$2: GTATAAGAGGGCTAGTTTCAC			
efaA$_{fs}$	TE5: GACAGACCCTCACGAATA	54	705	Eaton and Gasson.(2001)
	TE6: AGTTCATCATGCTGTAGTA			
gelE	GEL11: TATGACAATGCTTTTTGGGAT	45	213	Vankerckhoven et al. (2008)
	GEL12: AGATGCACCCGAAATAATATA			
hyl	HYLn1: ACAGAAGAGCTGCAGGAAATG	56	576	Vankerckhoven et al. (2008)
	HYLn2:GACTGACGTCCAAGTTTCCAA			
Biogenic amine genes				
hdc	HIS1-F: GGNATNGTNWSNTAYGAYMGNGCNGA	46	372	de las Rivas et al. (2006)
	HIS1-R: ATNGCDATNGCNSWCCANACNCCRTA			
tdc	TDC-F: TGGYTNGTNCCNCARACNAARCAYTA	55	825	de las Rivas et al. (2006)
	TDC-R: ACRTARTCNACCATRTTRAARTCNGG			
odc	PUT1-F:GTNTTYAAYGCNGAYAARCANTAYTTYGT	54	1446	de las Rivas et al. (2006)
	PUT1-R: ATNGARTTNAGTTCRCAYTTYTCNGG			
ldc	CAD2-R: CAYRTNCCNGGNCAYAA	52	1185	de las Rivas et al. (2006)
	CAD2-F: GGDATNCCNGGNGGRTA			

AT annealing temperature, *bp* base pair

exclude the antimicrobial effect of organic acids (NCFS); (3) the NCFS was treated with catalase (200 unit/ml) to eliminate the inhibitory effect of hydrogen peroxide (H_2O_2) and incubated at 37 °C for 1 h (NCFSC); and (4) proteolytic enzymes were added to NCFSC, including α-chymotrypsin, trypsin, proteinase K, or pronase E (1 mg/ml), and incubated at 37 °C for 3 h (NCFSC-proteolytic enzymes). All the samples were filter-sterilized (0.22 μm) and heated for 5 min at 90 °C before testing. Antibacterial activity spectrum was determined using an agar well diffusion assay. Soft agar media (1% agar, 45 °C) were first seeded with all the indicator strains (10^6 CFU/ml) and were poured into sterile petri dishes. Aliquots (50 μl) of each treatment were dispensed into each well and incubated at 37 °C for 24 h. Fifty microliters of MRS broth was applied as the control treatment, and the result reported is the diameter of inhibition zone, measured using a Vernier caliper.

Scanning electron microscopy (SEM)

Bacterial cells for SEM were prepared as described by Khan and Kang (2016) with minor modifications. Briefly, *L. monocytogenes* DMST 17303, as the test indicator, was cultured in TSB broth at 37 °C for 7 h to approximately 10^6 CFU/ml. Thereafter, the bacterial cells were treated with and without NCFS at equal proportions for 2 h. Then, the cells were centrifuged at $5000 \times g$ for 15 min at 4 °C and washed three times using 0.1 M phosphate buffer saline (PBS, pH 7.2). A thin smear was prepared on a glass slide and fixed in 2.5% glutaraldehyde for 1 h. Then the fixed samples were washed twice with 0.1 M PBS (pH 7.2) followed by washing twice with sterile distilled water for 15 min each time. In the next step, dehydration was performed using an ethanol series from 50 to 100%, with further drying using CO_2 at 4 °C. The dried cells were sputter coated with gold and observed under SEM (Quanta 400, Czech Republic).

Tolerance to simulated human gastrointestinal tract (GI)

Survival of SKI19 under simulated GI tract conditions was assessed as described by Yu et al. (2013), with slight modifications. Freshly prepared culture was centrifuged at $5000 \times g$ for 5 min at 4 °C, and washed twice with 0.1 M PBS (pH 7.0). To simulate the hydrolysis of bacterium in the human oral cavity, the obtained cell pellets were resuspended in a sterile electrolyte solution (SES) containing 100 μg/ml lysozyme, and incubated at 37 °C for 10 min. To test for gastric juice tolerance, the bacterial suspension was centrifuged and washed twice with 0.1 M PBS (pH 7.0). Then, it was re-suspended in 0.1 M PBS (pH

2.5) containing 0.3% pepsin and incubated at 37 °C in a water bath for 4 h. Subsequently, the tolerance to the simulated small intestine was assessed. The bacterial suspension was centrifuged and washed twice with 0.1 M PBS (pH 7.0), before re-suspension in 0.1 M PBS (pH 8.0) containing 0.1% porcine pancreatin (Sigma, Germany) and 0.3% oxgall bile salt (Merck, Germany), and it was then incubated at 37 °C for 4 h. For cell counting, the sample was diluted and spread on MRS agar plates containing 0.01% bromocresol purple (Ajax Finechem, Australia). Survival counts for gastric tolerance and for intestinal tolerance were determined at the initial time (0 h) and at 1, 2, 3 and 4 h of exposure. The values are expressed as log CFU/ml.

Bacterial adhesion to hydrocarbons (BATH)

Bacterial adhesion to hydrocarbons was determined as described by Pieniz et al. (2015). Briefly, SKI19 was harvested by centrifugation at $5000 \times g$ for 5 min at 4 °C. The cells were washed twice and suspended in 10 mM PBS (pH 7.2). Initial OD_{600} nm (A_0) was adjusted to 0.5 for optimizing the bacterial cells to 10^7 CFU/ml. Then, an equal volume of either xylene or chloroform was added to the bacterial cells and vortexed well for 5 min. The two phases were separated after incubation at room temperature for 1 h. The aqueous phase was carefully removed and measured at OD_{600} nm. The percentage of BATH was calculated according to the formula below:

$$\mathrm{BATH}(\%) = [(A_0 - A)/A_0] \times 100$$

A_0 is the absorbance before mixing with xylene or chloroform, A is the absorbance after mixing with xylene or chloroform.

Competitiveness of SKI19 in co-cultivation with pathogenic bacteria

Co-cultivation of SKI19 with *E. coli* DMST 4212, *S. aureus* DMST 8840, and *L. monocytogenes* DMST 17303, was assessed as described by Hongpattarakere et al. (2012). The co-cultivations were evaluated in a minimal medium supplemented with 1% glucose (pH 6.5). For each experiment, the strains were inoculated in equal proportions of 10^5 CFU/ml for the co-cultures and for the monocultures that were used as controls. Each sample was incubated at 37 °C for 48 h under anaerobic conditions. After 0, 6, 12, 18, 24, 36, and 48 h of incubation, samples were collected to analyze for pH, SKI19, and remaining pathogenic bacteria. Ten-fold serial dilutions were spread-plated onto selective agars for enumeration of each individual strain. Growth of SKI19 was enumerated on MRS agar, while the enumerations of the pathogenic bacteria (*E. coli* DMST

4212, *S. aureus* DMST 8840, and *L. monocytogenes* DMST 17303) were determined on violet red bile agar, Bird-Parker agar, and PALCAM agar (HiMedia, India), respectively. The results are expressed as logCFU/ml.

Haemolytic activity

Fresh bacterial culture was streaked on TSA agar containing 5% human blood (Songklanakarin hospital, Thailand), and incubated at 37 °C for 48 h. The haemolytic reaction was labeled by observation of: clear zones around the colonies (β-haemolysis); green-hued zones around colonies (α-haemolysis); or no clear zone around the colonies (γ-haemolysis). The γ–haemolysis was considered as a negative case with missing haemolytic activity (Pieniz et al. 2015).

Susceptibility to antibiotics

Antibiotic susceptibility of SKI19 was determined according to the technical guidelines of the European Food Safety Authority (EFSA 2012), using the broth microdilution method. Briefly, SKI19 colonies were suspended in 10 ml sterile saline solution to a turbidity of 1.0 on McFarland scale, and diluted 500-fold in LSM medium (Iso-sensitestbroth:MRS, 9:1). Fifty-microliter aliquots of the diluted bacterial suspension were added to each well (containing the various antibiotic test concentrations in each 50 µl volume of LMS broth per well). The antibiotics tested in the concentration range 0.125–256 µg/ml were ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, penicillin G, polymycin B, rifampicin, and tetracycline. SKI19 inoculated in MRS broth was used as a positive control, and bacteria-free case was used as a negative control. After 24 h incubation at 37 °C, the minimum inhibitory concentration (MIC) was defined as the lowest antibiotic concentration that inhibited visual bacterial growth. The growth inhibition of SKI19 was measured by OD_{600} nm using a microplate reader (Biotex, USA). The breakpoints (cutoff values) were used to distinguish susceptible and resistant strains. The strain with higher MIC than the respective cutoff value was considered resistant to an antibiotic.

Detection of virulence and biogenic amine genes

The presence of virulence factors was checked by PCR. The virulence genes detected were *ace* (encodes the adhesion collagen protein), *asa1* (encodes the aggregation substance), *efaA_{fs}* (encodes the cell wall adhesion), *hyl* (encodes the hyaluronidase), *cylL_S* (encodes the cytolysin structural subunit), and *gelE* (encodes the protease GelE with gelatinase activity). The presence of biogenic amine genes encoding the decarboxylase enzymes involved in biogenic amine production was also investigated. The genes analyzed were *hdc*, encoding for histidine decarboxylase; *tdc*, encoding for tyrosine decardoxylase; *odc*, encoding for ornithine decarboxylase; and *ldc*, encoding for lysine decarboxylase. The amplified products were resolved by electrophoresis through a 1% agarose gel. The primer sequences and the PCR conditions are given in Table 1.

Statistical analysis

Statistical analysis was performed using SPSS statistical software (version 15.0; SPSS, Inc., Chicago, IL). One-way analysis of variance (ANOVA) was considered statistically significant at $P < 0.05$. All data are presented as mean ± standard deviation.

Results and discussion

Isolation and screening of potential bacteriocin-producing LAB

A total of 2257 LAB strains were directly isolated from 10 sai krok e-san mu samples. However, in the primary screening by the agar spot assay only 46 colonies produced inhibition zones against *L. sakei* subsp. *sakei* JCM 1157, with the radius ranging from 9 to 17 mm. Further investigation by the drop plate method revealed 16 strains of LAB with the radius of inhibition zone larger than 7 mm (data not shown). Of these isolates, SKI19 exhibited the largest 12 mm inhibition zone. Therefore, the strain SKI19 was selected for further experiments.

Identification of selected LAB strain SKI19

SKI19 is a rod-shaped, catalase negative, non-spore-forming, and Gram-positive bacterium (data not shown). Based on the *16S* rRNA gene sequence, the strain was identified as *L. plantarum*, due to 99% sequence similarity in the GenBank database (the GenBank accession number is KY762263). However, the *16S* rRNA gene sequence may not be sufficient to distinguish among the species *L. plantarum*, *L. pentosus*, and *L. paraplantarum*, since these species are closely related and share highly homologous phenotypes (Torriani et al. 2001) (Fig. 1a). The *recA* gene could be used to distinguish among them, and it had sequence similarity to *L. plantarum* subsp. *plantarum* (the GenBank accession number is KY762265) (Fig. 1b). The phylogenetic tree based on the *dnaK* gene obviously confirmed the distinction of SKI19 from *L. plantarum* subsp. *argentoratensis*, and indicated 99% homology to *L.*
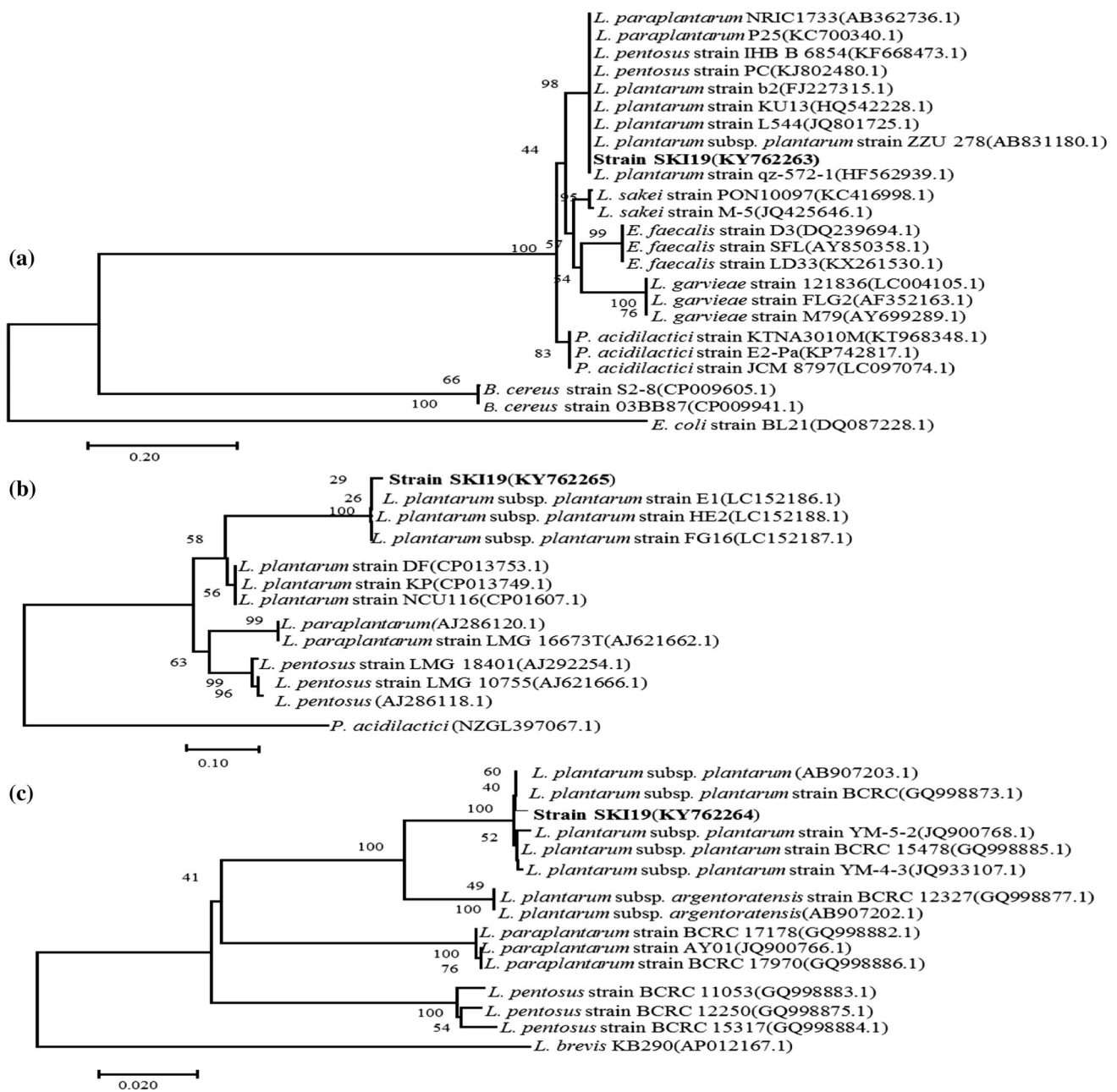
(a)

L. paraplantarum NRIC1733(AB362736.1)
L. paraplantarum P25(KC700340.1)
L. pentosus strain IHB B 6854(KF668473.1)
L. pentosus strain PC(KJ802480.1)
L. plantarum strain b2(FJ227315.1)
L. plantarum strain KU13(HQ542228.1)
L. plantarum strain L544(JQ801725.1)
L. plantarum subsp. plantarum strain ZZU 278(AB831180.1)
Strain SKI19(KY762263)
L. plantarum strain qz-572-1(HF562939.1)
L. sakei strain PON10097(KC416998.1)
L. sakei strain M-5(JQ425646.1)
E. faecalis strain D3(DQ239694.1)
E. faecalis strain SFL(AY850358.1)
E. faecalis strain LD33(KX261530.1)
L. garvieae strain 121836(LC004105.1)
L. garvieae strain FLG2(AF352163.1)
L. garvieae strain M79(AY699289.1)
P. acidilactici strain KTNA3010M(KT968348.1)
P. acidilactici strain E2-Pa(KP742817.1)
P. acidilactici strain JCM 8797(LC097074.1)
B. cereus strain S2-8(CP009605.1)
B. cereus strain 03BB87(CP009941.1)
E. coli strain BL21(DQ087228.1)

0.20

(b)

Strain SKI19(KY762265)
L. plantarum subsp. plantarum strain E1(LC152186.1)
L. plantarum subsp. plantarum strain HE2(LC152188.1)
L. plantarum subsp. plantarum strain FG16(LC152187.1)
L. plantarum strain DF(CP013753.1)
L. plantarum strain KP(CP013749.1)
L. plantarum strain NCU116(CP01607.1)
L. paraplantarum(AJ286120.1)
L. paraplantarum strain LMG 16673T(AJ621662.1)
L. pentosus strain LMG 18401(AJ292254.1)
L. pentosus strain LMG 10755(AJ621666.1)
L. pentosus (AJ286118.1)
P. acidilactici(NZGL397067.1)

0.10

(c)

L. plantarum subsp. plantarum (AB907203.1)
L. plantarum subsp. plantarum strain BCRC(GQ998873.1)
Strain SKI19(KY762264)
L. plantarum subsp. plantarum strain YM-5-2(JQ900768.1)
L. plantarum subsp. plantarum strain BCRC 15478(GQ998885.1)
L. plantarum subsp. plantarum strain YM-4-3(JQ933107.1)
L. plantarum subsp. argentoratensis strain BCRC 12327(GQ998877.1)
L. plantarum subsp. argentoratensis(AB907202.1)
L. paraplantarum strain BCRC 17178(GQ998882.1)
L. paraplantarum strain AY01(JQ900766.1)
L. paraplantarum strain BCRC 17970(GQ998886.1)
L. pentosus strain BCRC 11053(GQ998883.1)
L. pentosus strain BCRC 12250(GQ998875.1)
L. pentosus strain BCRC 15317(GQ998884.1)
L. brevis KB290(AP012167.1)

0.020

Fig. 1 Phylogenetic trees were constructed by the neighbor-joining method, kimura's two-parameter model. SKI19 had 99% nucleotide homology with *L. plantarum* strains and *L. plantarum* subsp. *plantarum* strains. *E. coli* strain BL21, *P. acidilactici*, and *L. brevis* KB290 were chosen as an out group for *16S* rRNA, *recA*, and *dnaK* genes, respectively

plantarum subsp. *plantarum* (the GenBank accession number is KY762264) (Fig. 1c). Thus, our data verified that the sequences of *recA* and *dnaK* genes provided resolution at a high discrimination level, particularly the *dnaK* gene. This suggests the *dnaK* gene sequence could be applied as a phylogenetic marker for accurate and rapid classification of *L. plantarum* strains at the subspecies level. This result is in agreement with Huang et al. (2010),

where the *dnaK* gene sequence clearly distinguished between the phenotypically closely related species *L. plantarum* subsp. *plantarum*, *L. plantarum* subsp. *argentoratensis*, *L. paraplantarum*, *L. pentosus*, and *L. fabifermentans*. Therefore, based on *16S* rRNA gene sequence analysis combined with *recA* and *dnaK* genes' amplification, SKI19 was potentially identified as *L. plantarum* subsp. *plantarum* SKI19.

Characterization of antibacterial compounds and inhibitory spectrum

The inhibitory spectrum of antibacterial compounds produced by SKI19 was assayed by the agar well diffusion method summarized in Table 2. The CFS in direct use exhibited inhibitory activity against the closely related bacteria *Enterococcus* spp. and *L. sakei* subsp. *sakei* JCM 1157. Also, it inhibited food spoilage and foodborne pathogenic bacteria, including Gram-positive bacteria (*B. cereus* DMST 5040, *C. perfringens* DMST 1663, *L. monocytogenes* DMST 17303, and *S. aureus* DMST 8840) and Gram-negative bacteria (*E. coli* DMST 4212, *S.* Typhimurium DMST 15674, and *S.* Enteritidis DMST 15676). The inhibition is probably due to the production of antibacterial compounds, like lactic and acetic acids, diacetyl, hydrogen peroxide (H_2O_2), bacteriocin, or their combination. After excluding the effects of organic acids (NCFS) and H_2O_2 (NCFSC), SKI19 showed unchanged activity in inhibiting the closely related bacteria. This indicates that SKI19 excreted a bacteriocin-like inhibitory substance (BLIS) (Aslim et al. 2005). Moreover, the BLIS was also effective against the foodborne bacterium *L. monocytogenes* DMST 17303, since the class IIa bacteriocins include bacteriocin-like *Listeria*-active peptides with a conserved N-terminal sequence Tyr–Gly–Asn–Gly–Val,

and two cysteines forming a disulfide bridge in the N-terminal half of the peptide (Cleveland et al. 2001). Thus, our results indicate that the anti-*Listeria* organism in the inhibition region is predominantly this BLIS. In addition, the BLIS did not inhibit the tested probiotic strains such as *L. plantarum* TISTR 875 and *P. pentosaceus* TISTR 413, suggesting an interesting technological application in fermented foods for control of *Listeria* without affecting probiotic cultures (Barbosa et al. 2014). However, no inhibition by BLIS was observed for Gram-negative bacteria, due to their supplementary layers that make the outer membrane act as a permeability barrier hindering bacteriocin action (Gong et al. 2010). A similar observation has been reported regarding plantaricin NC8 (Maldonado et al. 2003) produced by *L. plantarum* NC8, which appeared to inhibit only Gram-positive bacteria. In contrast, plantaricin MG (Gong et al. 2010) was active against both Gram-positive and Gram-negative bacteria. This difference may depend on the bacteriocin-producing strain (Powell et al. 2007). Complete inactivation of the BLIS by treatments with proteolytic enzymes, including α-chymotrypsin, trypsin, proteinase K, and pronase E, provides preliminary affirmation that SKI19 is a bactericocin-producing strain.

Table 2 The inhibitory spectrum of antibacterial compounds produced by SKI19

Indicator strain	Diameter of inhibition zone (mm)*			
	CFS	NCFS	NCFSC	NCFSC-proteolytic enzymes
Closely related species				
Enterococcus faecalis CF1GI14	18 ± 0.20[Aa]**	16 ± 0.12[Aa]	14.5 ± 0.15[Ab]	0.00 ± 0.00
Enterococcus faecalis CM6CR07	17 ± 0.10[Aa]	15 ± 0.03[Ab]	13.5 ± 0.11[Bb]	0.00 ± 0.00
Enterococcus faecium CKF12	16.5 ± 0.05[Aa]	13.5 ± 0.05[Bb]	12 ± 0.13[Bb]	0.00 ± 0.00
Enterococcus lactis CKF16	18.5 ± 0.21[Aa]	17 ± 0.10[Aa]	17 ± 0.10[Aa]	0.00 ± 0.00
Lactobacillus plantarum TISTR 875	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lactobacillus sakei subsp. *sakei* JCM 1157	19 ± 0.12[Aa]	18 ± 0.15[Aa]	18 ± 0.15[Aa]	0.00 ± 0.00
Pediococcus pentosaceus TISTR 413	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Food spoilage and foodborne pathogenic bacteria				
Bacillus cereus DMST 5040	11 ± 0.15[Ab]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Clostridium perfringens DMST 16637	9 ± 0.15[Ac]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Escherichia coli DMST 4212	10 ± 0.12[Ab]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Listeria monocytogenes DMST 17303	20 ± 0.10[Aa]	18 ± 0.05[Aa]	18 ± 0.05[Aa]	0.00 ± 0.00
Salmonella Enteritidis DMST 15676	11 ± 0.12[Ab]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Salmonella Typhimurium DMST 15674	10.5 ± 0.12[Ab]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Staphylococcus aureus DMST 8840	12.5 ± 0.14[Ab]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

*Values are given as mean ± standard deviation (SD) from triplicate experiments

**Significant differences in row are indicated by different capital letters and significant differences in column are presented by different small letters ($P < 0.05$)

Observation of changes in target bacterial cell morphology viewed by SEM

The SEM study allowed observation of the irregular cell morphology of anti-*Listeria* bacteriocin SKI19 treated *L. monocytogenes* DMST 17303, with untreated *L. monocytogenes* DMST 17303 serving as a control. The cell surfaces of the control were smooth and intact, with regular appearance (Fig. 2a), while membrane of the indictor strain appeared shrunken, corrugated, and changed in shape, after treatment at MIC (100 µl) of anti-*Listeria* bacteriocin SKI19 for 2 h, indicating that the cell wall probably was destroyed. Pores appeared in the cells, which led to cytoplasm leakage resulting in cell death (white arrows) (Fig. 2b). This provides evidence that the bactericidal activity exerted by anti-*Listeria* bacteriocin SKI19 was associated with damage to the cell membranes, as it destroyed the integrity of the membrane and caused subsequent cell lysis. This matches prior observations of other bacteriocins (Khan and Kang 2016).

Survival in simulated human gastrointestinal tract

Tolerance of SKI19 to the gastrointestinal tract was assessed by simulating exposure to gastric acid and passage through the small intestine by incubation at 37 °C for 4 h. In the presence of simulated gastric juice at pH 2.5, it was observed that SKI19 initially showed no obvious difference ($P < 0.05$) in cell count to the control. After incubation for 2, 3, and 4 h, the cell viability had declined to 8.13, 7.12 and 6.84 log CFU/ml, respectively. In the presence of simulated small intestinal juice at pH 8.0, the viable cell counts were approximately 3 log units below the control (6.60 log CFU/ml), and remained stable over the observation time span. Gobbetti et al. (2010) theorized that a potentially probiotic microorganism with benefits to the consumer's health must reach the intestine with a large number of viable cells, between 10^5 and 10^6 CFU/ml. The pH in the stomach before a meal is typically between 2.5 and 3.5, and after a meal about 4.5 (Huang and Adams 2004). Our results were satisfactory in that SKI19 survived
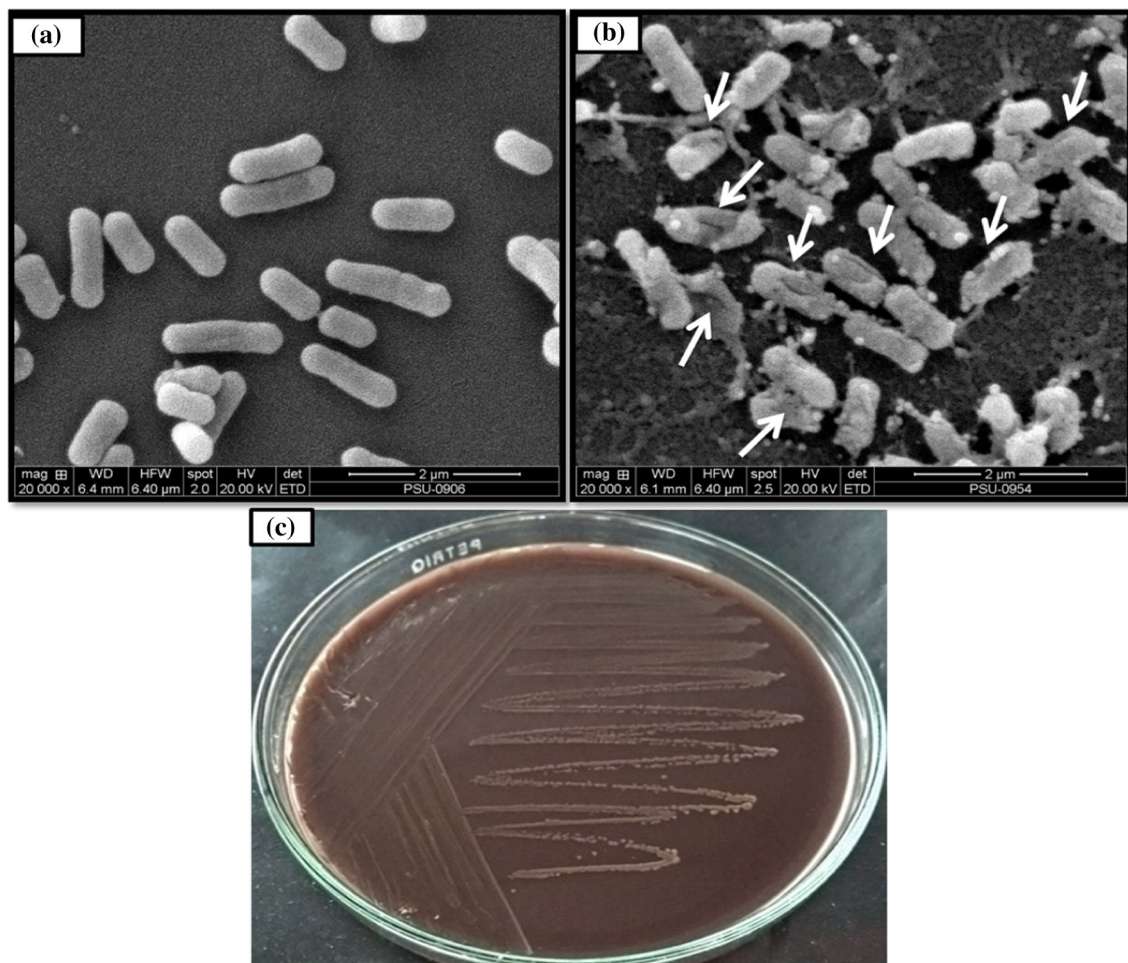

Fig. 2 SEM of *L. monocytogenes* DMST 17303 with untreated (control) and treated anti-*Listeria* bacteriocin SKI19 (**a**, **b**). The absence of haemolytic activity in SKI19 (**c**)

both simulated gastric and intestinal juices, and therefore might be appropriate for future development as a probiotic.

Hydrophobicity

The hydrophobicity of SKI19 was evaluated using two solvents. Xylene was used as an apolar (bacterial adhesion), while chloroform was considered a monopolar acid, an electron acceptor to the bacteria. SKI19 showed high adhesion to xylene at 90.14% whereas the cell wall adhered to acidic chloroform at 89.85%. The hydrophobic components of bacteria are generally present in their outer membrane and act hydrophobically due to net negative surface charge. These hydrophobic interactions are essential in the adhesion of bacteria to epithelial cells. The hydrophobicity of the cell surface could be affected by the growth medium, the age of the bacteria, the bacterial surface structures, and the composition changes of proteins from environmental stresses (Kaushik et al. 2009). Our results reveal that SKI19 has potential to adhere to and colonize on gut epithelial cells in the human intestine.

Inhibitory activity of SKI19 in co-cultivation with pathogenic bacteria

Probiotic strains inhibit pathogens by consuming nutrients that the pathogens need. This inhibition depends on the rate of nutrient absorption, the inherent metabolic velocity, the growth rate, and the excretion of specific inhibitors (Balcázar et al. 2006). SKI19 showed strong inhibitory effects against *E. coli* DMST 4212 and *S. aureus* DMST 8840, as the pathogen cell counts fell rapidly by approximately 3 log units at 24 h, and further to below detection limit after 36 h of incubation, and the pH level dropped to approximately 3.6 in these co-culture studies (Fig. 3a, b). The inhibition of those pathogens likely coincided with the decreased pH level. This observation correlates with the characterization of antibacterial compounds, as in Table 2, where organic acids are the major agents contributing to the antibacterial effect. Kongnum and Hongpattarakere (2012) reported that *L. plantarum* MR03.12 completely inactivated *Vibrio harveyi* to no detection around 18–24 h of co-culture. Also, *L. plantarum* or *L. brevis* totally inactivated *Salmonella* spp. in mixed cultures after 24 h or 48 h, respectively (Szala et al. 2012), indicating that organic acids produced by LAB are the main factors inhibiting the growth of Gram-negative bacteria, as observed also in our study. Moreover, SKI19 exhibited highly significant inhibitory effects against *L. monocytogenes* DMST 17303. The cell concentration sharply decreased by about 3 log units at 18 h to complete inhibition after 24 h of incubation in the co-culture (Fig. 3c). This reveals that the inhibition of SKI19 towards *L. monocytogenes* DMST 17303 is
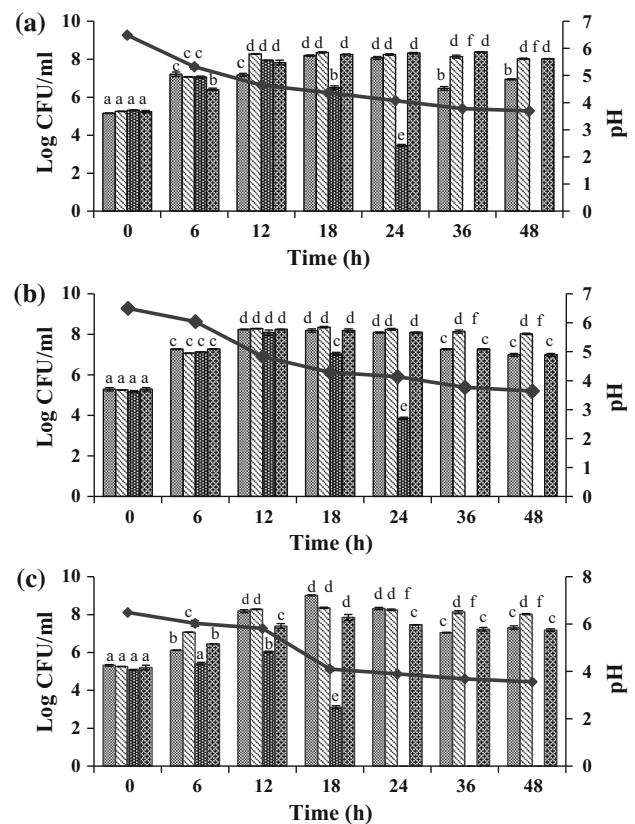

Fig. 3 Growth of *E. coli* DMST 4212 (**a**), *S. aureus* DMST 8840 (**b**), and *L. monocytogenes* DMST 17303 (**c**) in co-culture system. ▨ Pathogen alone; ▨ SKI19 alone; ▨ Pathogen in co-culture; ▨ SKI19 in co-culture; ◆ pH in co-cultivation. The results (Mean ± S.D) of three experiments with lowercase letters (**a–f**) indicate a significant difference (*P* <0.05)

probably caused by a combination of antibacterial compounds, as mentioned in Table 2. SKI19 growth in the co-culture remained steady until the end of fermentation, while SKI19 and pathogen cell concentrations in mono-culture remained at constant levels (10^6–10^8 CFU/ml) throughout the experiment. Our results thus clearly highlight that SKI19 has a very high competitive ability against foodborne pathogenic bacteria, which is pertinent to its potential application in food products.

Haemolytic activity

The absence of haemolytic activity is required for safety of probiotic strains (FAO/WHO 2002). In the present study, SKI19 showed no haemolysis of blood cells (γ-hemolysis, Fig. 2c). Our result is consistent with the study of Oh and Jung (2015), in which *L. plantarum* and *Pediococcus* strains exhibited no haemolytic activity.
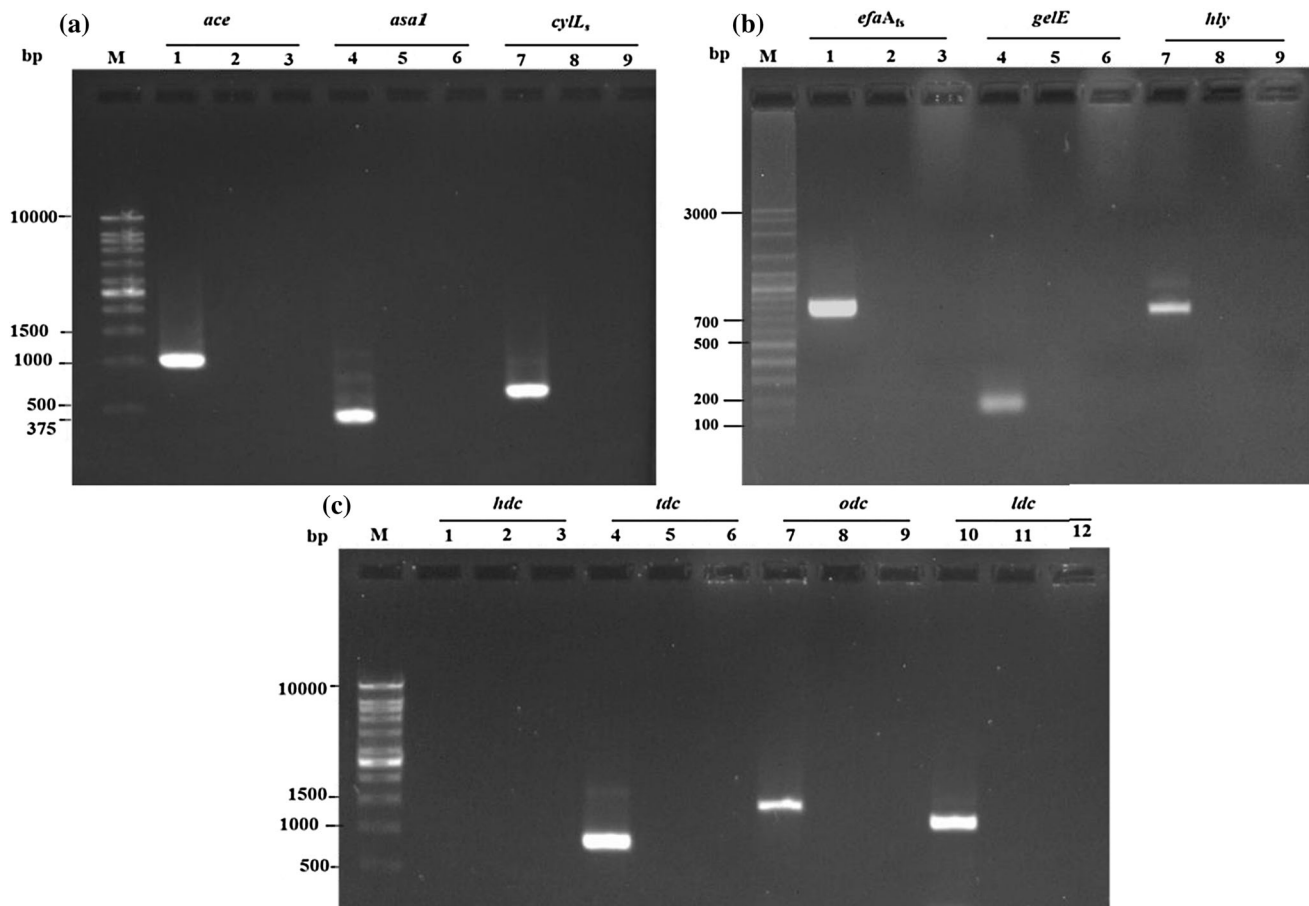
Fig. 4 Amplification of virulence genes (**a**, **b**) and biogenic amine genes (**c**) in SKI19 by PCR. Lanes 1, 4, 7, 10: products of positive controls; Lanes 2, 5, 8, 11: products of negative controls; Lanes 3, 6, 9, 12: the absence of target genes in SKI19. Lane M: 100 bp and 1 Kb DNA markers

Susceptibility to antibiotics

Antibiotic resistance is assessed relative to the MIC breakpoints as given by EFSA (2012). SKI19 was susceptible to ampicillin and penicillin that inhibit cell wall synthesis. However, resistance to polymixin B was observed in this study. Susceptibility to protein synthesis inhibitors was observed for chloramphenicol, clindamycin, erythromycin, gentamicin, and tetracycline, but not for kanamycin. Also, SKI19 was sensitive to rifampicin that inhibits nucleic acid synthesis. In a similar study by Yu et al. (2013), antibiotic resistance to cell wall (polymyxin B) and protein synthesis inhibitors (kanamycin) was observed in *L. plantarum* strains. Most lactobacilli carry natural or intrinsic resistance to certain antibiotics. The various mechanisms involved in intrinsic resistance to aminoglycosides lead to absence of cytochrome-mediated electron transport, thus preventing uptake of antibiotics. However, the probiotics with intrinsic antibiotic resistance, like resistance to glycopeptides and aminoglycosides that are antibiotics regularly administered to humans, could be beneficial to maintain the gastrointestinal tract balance and

avoid antibiotic-induced diarrheas (Fraqueza 2015). It seems reasonable to conclude that consumption of SKI19 does not present a health risk to humans due to its antibiotic resistance.

Detecting the presence of virulence and biogenic amine genes

Although LAB are food-grade organisms with a long history of safe use, potential virulence factors or capacity to produce toxic compounds (such as biogenic amines) must be considered and assessed. To guarantee the safe use of SKI19 as either probiotic or starter culture, the absence of virulence and biogenic amine genes must be proven. SKI19 did not harbor any of the virulence genes (*ace*, *asa1*, *cylL$_S$*, *efaA$_{fs}$*, *hyl*, and *gelE*), whereas the expected PCR products were observed for *E. faecalis* CM6CR07 and *E. faecalis* CF1GI14 used as positive controls (Fig. 4a, b). This agrees with the results of Landeta et al. (2013) who detected none of these virulence genes in *L. plantarum* strains. The presence of virulence factors causes various risks of infections. The *ace* gene affects adhesion to collagen and is

expressed conditionally after growth in serum. The $efaA_{fs}$ gene is expressed for cell wall adhesions in serum (H-Kittikun et al. 2015). The *asa1* gene is located on pheromone-responsive plasmids and contributes to the severity of endocarditis in animal models. Cytolysin coding gene ($cylL_S$) is a bacterial toxin expressed by haemolysis and contributes to the severity of enterococcal disease in humans. The *gelE* gene has been shown to exacerbate endocarditis in an animal model. The *hyl* gene is associated with invasive disease (Vankerckhoven et al. 2008).

The presence of biogenic amines in foods is considered potentially unsafe to human health. SKI19 was PCR-negative for the *hdc*, *tdc*, *odc*, and *ldc* genes tested, whereas the expected PCR products were observed for *E. faecium* CE5-1 (*odc* and *tdc* genes) and *S. aureus* DMST 8840 (*ldc* gene) used as positive controls (Fig. 4c). An analysis without the positive control showed absence of *hdc* gene. This result agrees with what was reported by Landeta et al. (2013), namely the absence of biogenic amine genes in *L. plantarum* strains. However, some *L. plantarum* strains (CK06, LM11, and ZS11) were found to decarboxylate tyrosine into tyramine. Histamine and tyramine cause toxic effects by their vasoactive and psychoactive properties and are the most common biogenic amines present in fermented foods and starter cultures, in which the presence, activity, and specificity of decarboxylases are strain-specific (Belicova et al. 2013). Putrescine and cadaverine cause toxic effects due to their relevant biogenic amines by histamine and tyramine (Perin et al. 2014). Our results prove that SKI19 is safe for consumption.

Conclusion

Lactobacillus plantarum subsp. *plantarum* SKI19 was isolated from sai krok e-san mu, a traditional Thai fermented pork sausage, and was found to have BLIS acting against closely related bacteria as well as the foodborne pathogenic bacterium *L. monocytogenes* DMST 17303. An evaluation of potential probiotic properties and safety characteristics demonstrated that SKI19 is a candidate probiotic for application in certain food products. However, further studies are necessary to characterize its BLIS, with a view to the biopreservation of meat products and related foods.

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