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Isolation and characterization of *Lactobacillus salivarius* MTC 1026 as a potential probiotic

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Researchers are becoming more interested in studying probiotics at present due to their benefit to human and animal health. In this study, newly isolated strains from human feces were evaluated for probiotic properties. A total of sixty isolated strains were collected from feces and six out of sixty isolated strains could inhibit the growth of *Salmonella typhi* and *Salmonella typhimurium*. The strain which gave the best inhibitory effect was selected for further characterization as a probiotic strain. The identification of this strain was analyzed on the basis of morphological and biochemical characteristics and 16S rDNA gene sequence. This strain was Gram-positive, rod shaped, and catalase and oxidase-negative, and produced acids from D-glucose, D-fructose, lactose, mannitol, sorbitol, inulin and starch. It could not hydrolyze esculin or red blood cells. Based on its 16S rDNA gene sequence, it was *Lactobacillus salivarius*, and so was called *L. salivarius* MTC 1026 in this study, and was closely related with *L. salivarius* DSPV 344T isolated from the calf gut. It was able to survive in gastric and small intestinal juices at pH 2.0 and 1.0% bile salt for several hours and also could grow at 45°C. Moreover, this strain showed inhibitory activity against a variety of food-borne pathogens. It was highly sensitive to piperacillin, chloramphenicol, ampicillin, erythromycin and ceftazidime. After plasmid curing, this strain was susceptible to gentamicin, amikacin, norfloxacin and ciprofloxacin. *L. salivarius* MTC 1026 could significantly inhibit the adhesion process of *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311 on Caco-2 monolayers in a competition assay and also reduced invasion of both pathogens (4-log cfu/ml) in an exclusion and displacement assay. Therefore, it was clearly demonstrated in this study that *L. salivarius* MTC 1026 has shown promising properties as a candidate for a potential probiotic for applications in humans and animals.

Key Words—adhesion assay; antimicrobial activity; *Lactobacillus salivarius*; probiotic

Introduction

Probiotic bacteria are defined by FAO/WHO as “Live microorganisms which when administered in adequate amount confer a health benefit” (Fuller, 1986; Reid et

al., 2005). Probiotics are characterized by numerous antagonistic effects against the Gram-positive and Gram-negative pathogens. In addition, probiotics are also believed to reduce the risk of disorders from bacteria in the gastrointestinal tract (Dunne, 2001) and prevent diarrhea (Fuller, 1991; Fuller and Gibson, 1997; Goldin, 1998). A number of requirements have been identified for a bacterial strain to be a potential probiotic such as human origin (Dunne et al., 2001; Fuller, 1989), ability to survive through the gastrointestinal tract, competition of adhesion to the epithelium in the gastrointestinal tract and production of antibacterial

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substances such as lactic and acetic acid, hydrogen peroxide and bacteriocin (Bielecka et al., 1998). Required characteristics include resistance to gastric acid and bile salt (Fuller, 1989; Fuller and Gibson, 1997; Phalakornkule and Tanasupawat, 2006–2007).

Nowadays, probiotics are widely used in fermented dairy products. They may also be applied in other fermented products like vegetables and meats. The beneficial properties of probiotics can improve product values (Kalantzopoulos, 1997). The major factor in effective probiotic usage in foods is the long-term survival of the culture in the food product during its shelf-life and during transit through the acid and bile salt conditions in the gastrointestinal tract (Dunne, 2001; Dunne et al., 2001). Many different strains are used as probiotics in food including *Lactobacillus salivarius*, *Lactobacillus casei*, *Bifidobacterium longum*, *Enterococcus* sp. and *Saccharomyces cerevisiae*. The most generally used strains are members of the lactic acid bacteria (Fuller, 1989). For probiotics in humans, safety is the key criterion for applying usage. Thus, probiotics should be of human origin and representative strains which are generally recognized as safe (GRAS) (Goldin, 1998; Salminen et al., 1998). *Lactobacilli* and *Bifidobacteria* are usually considered safe, based on their taxonomic pattern (Ouwehand et al., 2002). Moreover, probiotics are commonly used as viable microbial feed supplements that improve the intestinal microbial balance in the host animal (Fuller, 1989). The report by Fuller shows that probiotics have beneficial effects on cattle, pigs and chickens, including improvement of the health, faster growth rate and increased production of goods such as milk and eggs. *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Enterococcus* and yeast, as probiotic strains, have been as additive animal feeds (Fuller, 1989). Therefore, the objective of this study was to screen the microbial strains from human origin to be used as potential probiotics.

Materials and Methods

Culture condition for microbial strains. The microorganisms used in this study, were supplied by the Microbiology Laboratory, Faculty of Pharmacy, Mahidol University, Thailand. Pathogenic bacterial strains were cultivated in Brain Heart Infusion broth (BHI; Difco) at 37°C for 18–24 h. Isolated strains were cultured in De Man, Rogosa and Sharpe (MRS; Difco).

Isolation of the probiotic strain. Feces sample col-

lected from children at the age of 5 was added into phosphate buffer (pH 7.0) as an initial dilution. The sample solution was prepared in serial dilutions up to 10^{-4} . Then, each of serial dilutions was plated onto MRS agar and incubated at 37°C for 24–48 h under anaerobic conditions. After that, single colonies were randomly selected in MRS broth and incubated under the same conditions. The selected colonies were isolated by repeated streaking on MRS agar. Finally, the overnight cultures of all isolated colonies were stored at –80°C in MRS broth containing 30% glycerol. Before being used, isolated strains were refreshed in MRS broth at 37°C for 24 h under anaerobic conditions and subcultured on MRS agar.

Antimicrobial activity screening assay. The serial dilutions of the sample were prepared and plated onto MRS broth and then on a randomly picked single colony on another plate as the lower layer. Plates were incubated under anaerobic conditions at 37°C for 24 h. Next, the overnight growth of *Salmonella typhi* and *Salmonella typhimurium* was adjusted to an optical density of 0.2 at 590 nm wavelength by a spectrophotometer. Then, aliquots of *Salmonella* spp. as pathogenic strains were mixed with 0.75% BHI agar and poured on the lower layer agar. After being incubated at 37°C for 24 h, the inhibition zones around the colonies were measured.

Phenotype characteristics. Characteristics of the isolated strain were determined by Gram stain reaction. Oxidase activity was examined by using *Pseudomonas aeruginosa* ATCC 9027 as the positive control and *E. coli* ATCC 25922 as the negative control. *Staphylococcus aureus* ATCC 25923 was the positive control and *Enterococcus* sp. from the Microbiology Laboratory, Faculty of Pharmacy, Mahidol University, Thailand was the negative control for the catalase test. Red blood cells and bile-esculin hydrolysis were also determined. The acid production was carried out by carbohydrate fermentation using D-glucose, D-fructose, lactose, mannitol, sorbitol, inulin and starch, which were investigated by adding each sugar in cystine tryptic agar (CTA) to a final concentration of 10 g/L (Martin et al., 2006).

Amplification of 16S rDNA, nucleotide sequencing and sequence analysis. The 16S rDNA gene was amplified by using polymerase chain reaction (PCR) with a thermal cycle. The PCR reaction contained $1 \times$ PCR buffer pH 8.8 (10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris-HCl, 2 mM MgSO_4 , 0.1% Triton X-100), 0.4 μM de-

oxynucleoside triphosphate (dNTP), 0.4 μM of each primer and 1 unit of *Taq* polymerase (Biolab®). Deionized water was used for adjusting the final solution to 20 μl . The universal oligonucleotide primers in this reaction were UFUL primer (5'-GCC TAA CAC ATGCAA GTC GA-3') and URUL primer (5'-CGT ATT ACC GCGGCT GCT GG-3') (Buntin et al., 2008; Phalakornkule et al., 2006–2007). The thermocycle program was as follows: 95°C for 5 min; 30 cycles of 95°C for 5 min, 55°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. The bacterial 16S rDNA was purified by ethanol precipitation. The mixed solution was centrifuged at 11,000 $\times g$, at room temperature, for 15 min. The precipitant was washed with 70% ethanol, and then centrifuged for removal and dried at 95°C for 2–3 min. The dried precipitant was dissolved in 10–15 μl Hi-Di formamide, boiled at 95°C for 2 min, and cooled with ice for 5 min. The thermocycle program was as follows: 95°C for 5 min; 30 cycles of 95°C for 30 s, 50°C for 10 s, and 60°C for 4 min; and a final extension step at 72°C for 4 min. The 16S rDNA gene was sequenced by using a BigDye v.3.1 cycle sequencing kit (Applied Biosystems) with the primer sets used previously (Thompson et al., 1997). The nucleotide sequencing was determined with automated sequencing. Finally, nucleotide sequences of 16S rDNA were aligned and analyzed by using the BLAST program (Buntin et al., 2008).

Determination of antimicrobial activity against various pathogens. Inhibitory activity of the potential probiotic strains was determined by using 13 pathogenic strains as indicator microorganisms (*Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus* sp., *Klebsiella pneumoniae*, *Shigella flexneri*, *Shigella sonnei*, *Salmonella typhi*, *Salmonella typhimurium* ATCC 13311, *Salmonella paratyphi* A, *Salmonella paratyphi* B, *Proteus vulgaris* and *Pseudomonas aeruginosa* ATCC 9027). In brief, all of pathogenic strains were inoculated onto BHI broth at 37°C for 24 h. The overnight culture of pathogenic bacteria was adjusted to an optical density of 0.2 at 590 nm wavelength by a spectrophotometer. The inhibitory activity of pure isolated strains was screened by the agar spot test (Gu et al., 2008; Schillinger and Lucke, 1989). For pure isolated strains, sterile MRS broth was inoculated with 10⁸–10⁹ cfu/ml viable culture of each pure isolate and incubated at 37°C for 24 h. Overnight cultures of tested strains were spotted on 1.5% agar medium and incu-

bated overnight. Next, indicator strains were mixed into 0.75% wormed BHI, poured onto the overnight tested strain plates, and incubated at 37°C for 1–2 days, depending on indicator strains. After incubation, the diameter of the inhibition zone around spots was measured. Inhibition zones of <5, >5, >8 and >10 mm were classified as strains of no (–); mild (+); strong (++) and very strong (+++) inhibition, respectively (Gu et al., 2008).

Determination of bacteriocin activity. The inhibitory activity of probiotic strains used various pathogenic strains as indicator organisms: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Salmonella typhimurium* ATCC 13311, *Pseudomonas aeruginosa* ATCC 9027, *Lactobacillus plantarum* ATCC 14917, *Lactobacillus casei* BEC 36987, *Lactobacillus sakei* ATCC 15521 and *Lactobacillus salivarius* MTC 1026. Inhibitory effect of probiotic strains were detected by agar well diffusion assay. Firstly, the overnight isolated strains were collected from the supernatant by centrifugation at 10,000 $\times g$ for 20 min at 4°C. Next, their supernatants were concentrated up to 5-fold by speed vacuum at 4°C and supplemented with 0.05 $\mu\text{g/ml}$ catalase enzyme in concentrated supernatants. Finally, each of the concentrated supernatants was filtered through 0.2 μm pore size. Initially tested, the overnight culture of indicator strains which were adjusted to OD 0.2 in BHI and MRS broth, was added to soft BHI agar (0.75%) and overlaid on base agar base and punched into this agar. After that, 5 $\times$ concentrated supernatants of isolated strains were dropped into a well. Finally, all plates were incubated at 37°C for 1–2 days, depending on the indicator strains. After incubation, the inhibition zones around the wells were measured and reported as average inhibition diameter in millimeters.

Effect of temperature on bacterial survival. Isolated strains were cultivated in MRS broth at 37°C for 24 h. After that, the overnight culture was adjusted to an optical density of 0.5 at 590 nm wavelength by a spectrophotometer. Aliquots of culture were added to MRS broth and incubated for 24 h at various temperatures: 37°C, 45°C, 55°C and 65°C. After that, an aliquot of each cell culture was taken for determining the number of surviving cells by the plate count method and compared with the starting point.

In vitro survival in gastric juices. An aliquot of each washed cell suspension was added to simulated gastric juices at various pHs (2.0, 3.0, 4.0, 6.0 and 7.0) and

then vortexed vigorously for 10 s before being incubated at 37°C (Huang and Adams, 2004). After 0, 90 and 180 min, an aliquot of the cell culture was taken for determining the number of surviving cells by the plate count method.

In vitro survival in small intestinal juices. An aliquot of each washed cell suspension was added to 0.15%, 3.0% and 1.0% oxagall bile salt juices, pH 8.0 and without oxagall bile salt juice as a control and then vortexed vigorously for 10 s before being incubated at 37°C (Huang and Adams, 2004). At 0, 90 and 180 min, an aliquot of the cell culture was taken for determining the number of surviving cells by the plate count method.

Antibiotic susceptibility test. The antibiotic susceptibility test in this study was determined by the agar diffusion method using 3 groups of antibiotics (Xu et al., 2008). In group A, 5 antibiotics (penicillin, ampicillin, piperacillin, ceftazidime, and bacitracin) were grouped as activity of inhibiting cell wall synthesis. In group B, 6 antibiotics (gentamicin, streptomycin, tetracycline, erythromycin, chloramphenicol, azithromycin, vancomycin, and amikacin) were grouped as property of inhibiting the synthesis of protein. In group C, 3 antibiotics (nalidixic acid, ciprofloxacin, and norfloxacin) were grouped as property of inhibiting the synthesis of nucleic acid. *L. salivarius* MTC 1026 was refreshed in MRS broth at 37°C for 24–48 h under anaerobic conditions. Then, the overnight culture was adjusted to the optical density of 0.2 at 590 nm wavelength. After that, the suspension of the tested strain was spread onto an MRS agar plate and left to dry for 10–15 min. Sterile antibiotic discs were placed onto the surface of plates before being incubated at 37°C for 24–48 h under anaerobic conditions. After incubation, the inhibition zone around each antibiotic disc was measured and the average inhibition diameters were presented in millimeters.

Curing of plasmids. The plasmid curing of *L. salivarius* MTC 1026 was performed by using protoplast formation (Rosander et al., 2008). An overnight culture of the *L. salivarius* MTC 1026 in MRS broth was adjusted to an optical density of 0.1 at 590 nm wavelength by a spectrophotometer and then used as an inoculum in 10 ml fresh MRS culture. The growth of this strain at an optical density of 0.7–0.8 was used in this study after incubation at 37°C. In brief, the cells were harvested by centrifuging at 3,000 × *g* for 10 min, and washed with ultrafiltration water. The cell solution

was re-centrifuged and resuspended in a protoplast buffer at pH 7.0: 0.2 M sodium phosphate, 0.5 M sucrose, and 20 mM MgCl₂. After centrifugation, whole cells were mixed with an equal volume of protoplast buffer containing 10 mg/ml lysozyme (Sigma). Protoplast cells were collected by centrifugation at 3,000 × *g* for 15 min after incubation at 37°C for 1 h. Then, protoplast cells were washed in protoplast buffer and the solution carefully discarded after re-centrifugation. The protoplast cells were resuspended in protoplast buffer and diluted cells with protoplast buffer were plated on MRS with 0.5 M sucrose for regenerating cells. Simultaneously, the number of total cells was determined by colony forming unit (CFU) counting after 24 h incubation at 37°C. Regenerated colonies were picked up on MRS agar and MRS agar supplemented with 10 µg/ml of various antibiotics for identifying the cured bacteria. The curing plasmid was isolated by considering the non-growth bacteria on MRS containing antibiotics. Antibiotic susceptibility was determined after curing.

Caco-2 cell culture. Caco-2 cells were obtained from the American Type Culture Collection (ATCC® HTB-37™) and were cultured in Dulbecco's Modified Eagle's minimal medium (DMEM) (JR Scientific) supplemented with 10% (v/v) heat-inactivated (30 min at 56°C) fetal calf serum (JR Scientific), 1% 100 U/ml penicillin, and 100 µg/ml streptomycin (JR Scientific) at 37°C in a humidified atmosphere of 5% CO₂/95% air. Confluent Caco-2 cells were maintained for 2 weeks prior to the adhesion assay. Caco-2 cells were transferred with 0.25% trypsin (JR Scientific) into a 24-well tissue culture plates at a concentration of 10⁵ seeded cells per well (1 ml) to monolayer for adhesion and adhesion-inhibition assay. The cell culture medium was changed every other day and replaced by fresh non-supplement DMEM for 1 h before the adhesion assay (Matijašić et al., 2006; Todoriki et al., 2001).

Adhesion assay. Caco-2 monolayers were washed twice with sterile phosphate buffer saline (PBS; pH 7.0) and cultured with 0.5 ml of bacterial suspension (10^{9–10} cfu/ml) for 1 h (Matijašić et al., 2006; Todoriki et al., 2001). Non-adhering bacterial strains were removed by washing with PBS (pH 7.0). Caco-2 cells were covered by agitating with a pipette in sterile PBS (pH 7.0). Adherent bacteria were counted by the plating method. *L. plantarum* ATCC 14917 as control and *L. salivarius* MTC 1026 were determined on MRS agar and pathogen strains (*E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311) were determined on BHI agar.

Experiments (Yu et al., 2011) were performed under different conditions: (a) competition assay: 0.5 ml of *Lactobacilli* suspensions (10^{9-10} cfu/ml) and 0.5 ml of *E. coli* ATCC 25922 or *S. typhimurium* ATCC 13311 suspension (10^{9-10} cfu/ml) were simultaneously mixed and co-incubated with a Caco-2 monolayer in each well and incubated at 37°C for 1 h; (b) exclusion assay: 0.5 ml of *Lactobacilli* suspensions (10^{9-10} cfu/ml) was first preincubated with a Caco-2 monolayer in each well for 1 h and then 0.5 ml of *E. coli* ATCC 25922 or *S. typhimurium* ATCC 13311 suspension (10^{9-10} cfu/ml) was added in each well and incubated for 1 h at 37°C; (c) displacement assay: 0.5 ml of *E. coli* ATCC 25922 or *S. typhimurium* ATCC 13311 suspension (10^{9-10} cfu/ml) was applied in each well and incubated for 1 h at 37°C before adding 0.5 ml of *Lactobacilli* suspensions (10^{9-10} cfu/ml) and incubating under the previous conditions. Caco-2 monolayers were washed with sterile PBS (pH 7.0). Caco-2 cells were covered by agitating with a pipette in PBS (pH 7.0). The lysed cells were plated on selective medium to determine the number of surviving cell by colony plate counting. MacConkey agar (Difco) was used for detecting *E. coli* ATCC 25922, Bismuth sulfite agar (Difco), Xylose lysine deoxycolate agar (Difco) and Brilliant-Green phenol red lactose agar (Difco) for detecting *S. typhimurium* ATCC 13311 and MRS agar for detecting *Lactobacilli* strains.

Results

Screening and identification of *L. salivarius* MTC 1026

About 60 strains isolated from feces of children on MRS medium were screened for potential probiotic properties. The results of the antimicrobial activity screening assay showed that 6 out of 60 isolated strains could inhibit *S. typhi* and *S. typhimurium* growth. The isolated MTC 1026 strain was selected to be identified and evaluated for its probiotic properties. The isolated MTC 1026 strain was Gram-positive, rod-shaped and oxidase and catalase negative. It could not hydrolyze bile-esculin or red blood cells. In addition, this strain could produce acid by fermenting D-glucose, D-fructose, lactose, mannitol, sorbitol, inulin and starch as its carbon source (Table 1). Most phenotypic characteristics suggested that this isolated strain could belong to genus *Lactobacillus*. The isolated strain were further identified by PCR amplification with a universal bacterial primer. The sequence of 16S

Table 1. Phenotypic characteristics of *Lactobacillus salivarius* MTC 1026.

Test	Reaction or characteristic
Morphology	Rod
Gram reaction	+
Cell arrangement	Single, chain and group
Catalase	–
Oxidase	–
Fermentation	
D-Glucose	+
D-Fructose	+
Lactose	+
Mannitol	+
Sorbitol	+
Inulin	+
Starch	+
Bile-esculin	–
Red blood cells	–

Note: (+): positive reaction, (–): negative reaction.

rDNA from the tested strain was aligned with the nucleotide BLAST (blastn) program in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). The result showed that the isolated strain could be *L. salivarius* and was closely related with the 16S rDNA sequence of *L. salivarius* DSPV 344T. Therefore, MTC 1026 was called *L. salivarius* MTC 1026 in this study.

Determination of antimicrobial activity of *L. salivarius* MTC 1026

Antimicrobial activity of isolated strains was determined by the agar spot test. Most of the isolated strains presented weak inhibitory effect on the growth of 13 pathogenic strains. However, *L. salivarius* MTC 1026 could strongly and broadly inhibit all pathogens. The best inhibiting effect of this strain was found against *Salmonella paratyphi* A. *L. plantarum* ATCC 14916 was also tested as the probiotic control and the results appeared in a similar pattern (Table 2).

Determination of bacteriocin activity of *L. salivarius* MTC 1026

Bacteriocin activity of selected strains was studied by the agar well diffusion method because one probiotic property is the ability of a bacteriocin producer to inhibit pathogen growth. The result showed that *L. salivarius* MTC 1026 could inhibit 9 pathogenic strains (Table 3). *S. aureus* ATCC 25923 strain was the most

Table 2. Antimicrobial activity of *Lactobacillus* spp. against pathogenic bacteria by agar spot test.

Pathogen strains	Inhibitory zones (ϕ = mm)	
	<i>L. salivarius</i> MTC 1026	<i>L. plantarum</i> ATCC 14917
<i>Bacillus cereus</i> ATCC 11778	(20.33 $\pm$ 0.58)	(25.00 $\pm$ 0.00)
<i>Escherichia coli</i> ATCC 25922	(18.33 $\pm$ 0.58)	(19.33 $\pm$ 0.58)
<i>Staphylococcus aureus</i> ATCC 25923	(14.00 $\pm$ 0.00)	(15.00 $\pm$ 0.00)
<i>Enterococcus</i> sp.	(16.33 $\pm$ 0.58)	(18.67 $\pm$ 0.58)
<i>Klebsiella pneumoniae</i>	(20.33 $\pm$ 0.58)	(20.00 $\pm$ 0.00)
<i>Shigella flexneri</i>	(19.00 $\pm$ 0.00)	(20.00 $\pm$ 0.00)
<i>Shigella sonnei</i>	(15.00 $\pm$ 0.00)	(15.00 $\pm$ 0.00)
<i>Salmonella typhi</i>	(18.00 $\pm$ 0.00)	(18.00 $\pm$ 0.00)
<i>Salmonella typhimurium</i> ATCC 13311	(19.00 $\pm$ 0.00)	(19.33 $\pm$ 1.15)
<i>Salmonella paratyphi</i> A	(22.00 $\pm$ 0.00)	(25.00 $\pm$ 0.00)
<i>Salmonella paratyphi</i> B	(19.00 $\pm$ 0.00)	(19.00 $\pm$ 0.00)
<i>Proteus vulgaris</i>	(20.00 $\pm$ 0.00)	(20.00 $\pm$ 0.00)
<i>Pseudomonas aeruginosa</i> ATCC 9027	(14.33 $\pm$ 0.58)	(16.33 $\pm$ 0.58)
<i>Salmonella enteritidis</i>	(18.00 $\pm$ 0.00)	(18.00 $\pm$ 0.00)
<i>Staphylococcus epidermidis</i>	(17.00 $\pm$ 0.00)	(12.00 $\pm$ 0.00)

*Results are shown as mean $\pm$ SD.

Table 3. Bacteriocin activity of *Lactobacillus* spp. against pathogenic bacteria.

Pathogen strains	Inhibitory zones (ϕ = mm)	
	<i>L. salivarius</i> MTC 1026	<i>L. plantarum</i> ATCC 14917
<i>Staphylococcus aureus</i> ATCC 25923	13.33 $\pm$ 0.58	14.33 $\pm$ 0.58
<i>Escherichia coli</i> ATCC 25922	12.67 $\pm$ 0.58	13.33 $\pm$ 0.58
<i>Lactobacillus salivarius</i> MTC 1026	12.67 $\pm$ 0.58	12.33 $\pm$ 0.58
<i>Lactobacillus plantarum</i> ATCC 14917	12.33 $\pm$ 0.58	12.33 $\pm$ 0.58
<i>Bacillus cereus</i> ATCC 11778	11.67 $\pm$ 0.58	12.67 $\pm$ 0.58
<i>Enterococcus faecalis</i> MTC 1032	11.67 $\pm$ 0.58	11.33 $\pm$ 0.58
<i>Pseudomonas aeruginosa</i> ATCC 9027	10.67 $\pm$ 0.58	13.33 $\pm$ 0.58
<i>Lactobacillus casei</i> BEC 36987	10.67 $\pm$ 0.58	11.67 $\pm$ 0.58
<i>Salmonella typhimurium</i> ATCC 13311	10.33 $\pm$ 0.58	11.67 $\pm$ 0.58
<i>Lactobacillus sakei</i> ATCC 15521	7.67 $\pm$ 0.58	13.33 $\pm$ 0.58

*Results are shown as mean $\pm$ SD.

inhibited by the strain isolated. When compared with the reference strain, the result of *L. salivarius* MTC 1026 showed no difference.

Effect of temperature on the survival of *L. salivarius* MTC 1026

One of the properties that is highly valued for commercial probiotics is the ability to resist high temperatures. To examine the survival ability at a range of temperatures, the survival rate of *L. salivarius* MTC 1026 was determined when cultivated at 25°C, 37°C, 45°C, and 55°C (Fig. 1). The survival rate of the tested strain varied during the incubation time. The result showed

that the optimum temperature for growth of *L. salivarius* MTC 1026 was at 45°C. In addition, *L. plantarum* ATCC 14917 was able to survive at 25°C, 37°C, and 45°C as shown by its 1-log increase. Lastly, both *L. salivarius* MTC 1026 and *L. plantarum* ATCC 14917 had the same pattern in their survival ability at 55°C.

Effect of simulated gastric on *L. salivarius* MTC 1026 viability

The capability of the selected probiotic strain to survive under gastrointestinal tract conditions had to be considered as well. Therefore, this study was performed to determine the effect of various pHs on bac-

Fig. 1. Effect of temperature on the growth of *L. salivarius* MTC 1026 (A) and *L. plantarum* ATCC 14917 (B) after incubation at various temperatures (25°C, 37°C, 45°C, and 55°C).

In the bar graph, the turbidity of cells is shown as follows: A at 25°C, B at 37°C, C at 45°C, and D at 55°C. In the line graph, the numbers of surviving cells (cfu/ml) are presented as follows: ▲ at 25°C, * at 37°C, ◇ at 45°C, and ● at 55°C.

terial survival. It was demonstrated that *L. salivarius* MTC 1026 showed lower survival ability in simulated gastric juices at pH 2 than under other conditions (Fig. 2). Comparing the level of survival cells during 180 min, it could be concluded that this strain was the most sensitive at pH 3. Its cell count was reduced by about 7-log (4.33×10^5 cfu/ml) when compared with the initial cell number of 2.32×10^{12} cfu/ml. At pH 4 and 6, the growth time of this strain was longer than at pH 3. The most favorable pH for all isolates strains was 7, which did not differ from the *L. plantarum* ATCC 14917 control.

Effect of simulated small intestinal transit with different bile salt concentrations on *L. salivarius* MTC 1026 viability

L. salivarius MTC 1026 isolated from human feces was also tested for its ability to grow in various bile salt concentrations. The result showed the reduction of vi-

Fig. 2. Effect of artificial gastric juices on the growth of *L. salivarius* MTC 1026 (A) and *L. plantarum* ATCC 14917 (B) during 180 min of various pH levels at pH 2.0, pH 3.0, pH 4.0, pH 6.0 and pH 7.0.

In the bar graph the turbidity of cells is shown as follows: A at pH 2.0, B at pH 3.0, C at pH 4.0, D at pH 6.0 and E at pH 7.0. In the line graph, the numbers of surviving cells (cfu/ml) are presented as follows: △ at pH 2.0, ◆ at pH 3.0, * at pH 4.0, ● at pH 6.0 and ■ at pH 7.0.

able cells by about 3-log at 0.3% and 1.0% bile salt after 180 min while a slight reduction was detected at 0.15% bile salt (Fig. 3). The reference strain, *L. plantarum* ATCC 14917, exhibited the reduction of survival cells by about 4-log in all tested bile salt concentrations.

Antibiotic susceptibility test

One important point of probiotic properties is the capacity to resist several antibiotics, particularly when used after antibiotic treatment (Fuller, 1991; Kilic et al., 2005; Salminen et al., 1998; Xu et al., 2008). *L. salivarius* MTC 1026 was highly sensitive to piperacillin as displayed by large clear zone diameters of above 30 mm (Table 4), while it showed some sensitivity to chloramphenicol (27 mm), ampicillin and erythromycin in group A antibiotics, which were categorized by

Fig. 3. Effect of small intestinal juice on the growth of *L. salivarius* MTC 1026 (A) and *L. plantarum* ATCC 14917 (B) during 180 min of various bile salt concentrations: 0, 0.15, 0.30 and 1.0% bile salt.

In the bar graph, the turbidity of cells is shown as follows: A 0% bile salt, B 0.15% bile salt, C 0.30% bile salt, and D bile salt as a control. In the line graph, the number of surviving cells (cfu/ml) is presented as follows: $\blacktriangle$ at 0% bile salt, $*$ 0.15% bile salt, $\blacklozenge$ 0.30% bile salt, and $\circ$ 1.0% bile salt.

Table 4. Antibiotic susceptibility of *Lactobacillus salivarius* MTC 1026.

Antimicrobial agents	Inhibition zone of <i>L. salivarius</i> MTC 1026 ($\phi = 6$ mm)	
	Normal cells	Protoplast cells
Piperacillin (PRL 100)	34.00 $\pm$ 0.00	36.33 $\pm$ 0.58
Chloramphenicol (C 30)	27.00 $\pm$ 0.00	30.67 $\pm$ 0.58
Ampicillin (Am 10)	26.00 $\pm$ 0.00	32.33 $\pm$ 0.58
Erythromycin (E 30)	25.00 $\pm$ 0.00	25.00 $\pm$ 0.00
Ceftazidime (CAZ 30)	20.00 $\pm$ 0.00	20.00 $\pm$ 0.00
Azithromycin (AZM 15)	19.00 $\pm$ 0.00	19.00 $\pm$ 0.00
Tetracycline (TE 30)	12.00 $\pm$ 0.00	13.33 $\pm$ 0.58
Bacitracin (B 10)	8.00 $\pm$ 0.00	8.00 $\pm$ 0.00
Penicillin (P 10)	ND	ND
Gentamicin (CN 10)	ND	10.67 $\pm$ 0.58
Streptomycin (S 10)	ND	ND
Amikacin (AK 30)	ND	9.67 $\pm$ 0.58
Vancomycin (VA 30)	ND	ND
Nalidixic acid (NA 30)	ND	ND
Ciprofloxacin (CIP 5)	ND	8.67 $\pm$ 0.58
Norfloxacin (NOR 10)	ND	9.33 $\pm$ 0.58

ND: not detected.

the property of inhibiting the synthesis of cell walls. The results showed that *L. salivarius* MTC 1026 was strongly sensitive to piperacillin, ampicillin and ceftazidime. It was slightly sensitive to bacitracin (8.00 ± 0.00 mm). This strain was sensitive to group B antibiotics, which were classified by the property of inhibiting protein synthesis and included chloramphenicol, erythromycin, azithromycin and tetracycline. On the other hand, *L. salivarius* MTC 1026 was resistant to penicillin, gentamicin, streptomycin, amikacin, vancomycin, nalidixic acid, ciprofloxacin and norfloxacin.

Curing of plasmids

The key point of considering probiotic strains in food application was long-term safety assessment. Antibiotic resistance of bacteria strains was the main point that had to be solved to prevent transporting antibiotic-resistant genes to the host. Therefore, plasmid curing was performed to remove antibiotic-resistance-carrying plasmids. After curing plasmids by protoplast formation, the results showed that *L. salivarius* MTC 1026 was less resistant to gentamicin, amikacin, norfloxacin and ciprofloxacin when compared with untreated bacteria cells (Table 4). Simultaneously, susceptibility to piperacillin, chloramphenicol, ampicillin and tetracycline in this strain was increased after the curing. *L. salivarius* MTC 1026 was strongly sensitive to piperacillin, chloramphenicol, ampicillin, erythromycin, and ceftazidime as revealed by large clear zone diameters of above 20 mm and slightly sensitive to amikacin, norfloxacin, ciprofloxacin and bacitracin by clear zone diameters under 10 mm.

Inhibition of pathogenic adhesion on human epithelial colorectal adenocarcinoma cells (Caco-2)

L. salivarius MTC 1026 was selected to study its effect on pathogen adhesion on Caco-2 cells for probiotic properties. The results indicated that *L. salivarius* MTC 1026 could adhere on Caco-2 cells and the adhesion rate was 9-log cfu/ml when compared with the 10-log cfu/ml initial cells, which did not differ from *L. plantarum* ATCC 14917 as the control (Fig. 4). The adhesion of *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311 to Caco-2 cells significantly decreased in competition, exclusion and displacement assays, while the adhesion rate of *L. salivarius* MTC 1026 showed no significant difference. In addition, this selected strain could significantly prevent the invasion process of *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311 on

Fig. 4A. Competition assay.

In the bar graph, the numbers of surviving bacterial cells are shown as follows: A the cell number of *L. plantarum* ATCC 14917, B the cell number of *E. coli* ATCC 25922 coincubated with *L. plantarum* ATCC 14917, C the cell number of *L. salivarius* MTC 1026, and D the cell number of *E. coli* ATCC 25922 coincubated with *L. salivarius* MTC 1026. In the line graph, the numbers of surviving bacterial cells are presented as follows: ▲ the cell number of *L. plantarum* ATCC 14917, ◇ the cell number of *S. typhimurium* ATCC 13311 coincubated with *L. plantarum* ATCC 14917, × the cell number of *L. salivarius* MTC 1026, and ○ the cell number of *S. typhimurium* ATCC 13311 coincubated with *L. salivarius* MTC 1026.

Fig. 4B. Exclusion assay.

In the bar graph, the numbers of surviving bacterial cells are shown as follows: A the cell number of *L. plantarum* ATCC 14917, B the cell number of *E. coli* ATCC 25922 coincubated with *L. plantarum* ATCC 14917, C the cell number of *L. salivarius* MTC 1026, and D the cell number of *E. coli* ATCC 25922 coincubated with *L. salivarius* MTC 1026. In the line graph, the number of surviving bacterial cells is presented as follows: ▲ the cell number of *L. plantarum* ATCC 14917, ◇ the cell number of *S. typhimurium* ATCC 13311 coincubated with *L. plantarum* ATCC 14917, × the cell number of *L. salivarius* MTC 1026, and ○ the cell number of *S. typhimurium* ATCC 13311 coincubated with *L. salivarius* MTC 1026.

Fig. 4C. Displacement assay.

In the bar graph, the numbers of surviving bacterial cells are shown as follows: A the cell number of *L. plantarum* ATCC 14917, B the cell number of *E. coli* ATCC 25922 coincubated with *L. plantarum* ATCC 14917, C the cell number of *L. salivarius* MTC 1026, and D the cell number of *E. coli* ATCC 25922 coincubated with *L. salivarius* MTC 1026. In the line graph, the numbers of surviving bacterial cells are presented as follows: ▲ the cell number of *L. plantarum* ATCC 14917, ◇ the cell number of *S. typhimurium* ATCC 13311 coincubated with *L. plantarum* ATCC 14917, × the cell number of *L. salivarius* MTC 1026, and ○ the cell number of *S. typhimurium* ATCC 13311 coincubated with *L. salivarius* MTC 1026.

Caco-2. The *E. coli* ATCC 25922 cell number was reduced about 6-log when compared with the initial cell number of 10-log cfu/ml and the *S. typhimurium* ATCC 13311 cell number was reduced about 4–5-log when compared with the initial cell number of 10-log cfu/ml.

Discussion

The selection of a microbial strain to be used as a probiotic is a rather complex process. *Lactobacillus* sp. or LAB of human origin is more attractive than other sources due to its modulation to the environment of the human gastrointestinal tract. In this study, *L. salivarius* MTC 1026 was originally screened from human feces. The presence of this species in infants has been repeatedly observed (Martin et al., 2006). In fact, finding *L. salivarius* in human feces is not surprising but the important point is discovering its probiotic property. *L. salivarius* is found growing naturally in the oral cavities (Busarcevic et al., 2008; Koll et al., 2008), intestines (Ryan et al., 2008), vagina (Juarez et al., 2002; Kilic et al., 2005; Vera et al., 2009), and feces of animals (Nemcova et al., 1997; Robredo and Torres, 2000) such as calves, chickens, pigs (Lahtinen et al., 2010; Yun et al., 2009) and humans (Maldonado et al.,

2009). In addition, it was also found in the breast milk of mothers (Maldonado et al., 2010; Martin et al., 2006). It is considered to be non-pathogenic strain and may be used to produce lactic acid in fermented foods. It is used as a probiotic in order to reduce the levels of pathogenic bacteria in the gastrointestinal tract such as *Helicobacter pylori* (Ryan et al., 2008), which causes gastritis, gastric and duodenal ulcers and gastric cancer (Uemura et al., 2001), and inhibits the growth of *S. typhimurium*, *C. perfringens*, and *E. coli* in chicken feed (Murry et al., 2004). The identification of the isolated *L. salivarius* MTC 1026 was analyzed on the basis of morphological and biochemical characteristics, and 16S rDNA gene sequence. This strain was Gram-positive, rod shaped, catalase and oxidase-negative, grew at 25–45°C and produced acids from D-glucose, D-fructose, lactose, mannitol, sorbitol, inulin, and starch. It could not hydrolyze esculin or red blood cells. Based on its 16S rDNA gene sequence, the isolated strain was closely related to *L. salivarius* DSPV 344T, which is the predominant species of *Lactobacillus* in the intestinal microbial ecosystem of young calves. This species was also detected by Schneider et al. in calves (Schneider et al., 2004) and it could provide beneficial effects as inocula for animal when administered as a

probiotic strain (Lorena et al., 2010). However, the safety of *L. salivarius* DSPV 344T has not been reported.

In this study, *L. salivarius* MTC 1026 could survive during 180 min at pH 2.0 of simulated gastric juice and 1.0% bile salt of simulated small intestinal juice. The data on its survival was similar to those obtained with other feces *Lactobacilli* in the in vitro model (Martin et al., 2005). Many studies have tried to prevent gastrointestinal infections by using probiotics (Buntin et al., 2008; Pascual et al., 1999; Reid et al., 2005; Ryan et al., 2008; Yanagida et al., 2005). Our data showed that *L. salivarius* MTC 1026 had antimicrobial activity against 13 pathogenic strains. The high antimicrobial potential exhibited by *L. salivarius* MTC 1026 was related with other studies (Dunne et al., 1999; Lee et al., 2003).

The *L. salivarius* strain is under consideration as a potential probiotic bacterium (Maldonado et al., 2009; Ocana et al., 1999a; Pascual et al., 1999) and is found in a number of commercial probiotic products (Phalakornkule and Tanasupawat, 2006–2007). The production of hydrogen peroxide by *L. salivarius* CECT 5713 has been reported (Martin et al., 2006) and could inhibit the growth of *S. aureus* through its H₂O₂ production (Ocana et al., 1999b). The highest antimicrobial activity was against *S. paratyphi* A but its low inhibitory effect against *S. aureus* and inhibitory effect may be due to acid, bacteriocin-like substances (Ocana et al., 1999b) or both in combination with other metabolites such as hydrogen peroxide and short chain fatty acids (Ahrne et al., 2005; Meimandipour et al., 2010). *L. salivarius* UCC118 is the most cultivated, not only in in vitro assay but also in vivo assay and clinical trials, by which its probiotic function and, particularly, its anti-inflammatory effects have been demonstrated (Castellazzi et al., 2007; Dunne et al., 1999, 2001; Flynn et al., 2002; McCarthy et al., 2003).

Due to the wide use of probiotics for human foods, there are many conditions about safety assessment for commercial application (Bernardeau et al., 2008; Dunne et al., 2001; Fuller, 1989) including recent guidelines which consider transferable antibiotic resistance in novel commercial strains to be the major hazard (Bernardeau et al., 2008; Vankerckhoven et al., 2008). In fact, antibiotic resistance in *Lactobacillus* sp., is a major concern. *Lactobacillus* sp. in foods should not transfer antibiotic resistance genes from foods to intestinal human microflora. Therefore, we attempted

to remove the antibiotic resistance plasmid from *L. salivarius* MTC 1026. Generally, *Lactobacillus* strains are sensitive to the antibiotics inhibiting cell wall synthesis, such as ampicillin, except cephalosporins (Amor et al., 2007). The tested strain in this study also showed a high susceptibility toward antibiotics that inhibited cell wall synthesis including piperacillin, ampicillin, and ceftazidime. Moreover, this strain was slightly sensitive to gentamicin and amikacin, grouped as inhibitors of protein synthesis. It was also present in *Lactobacilli* strains isolated from two Italian hard cheeses (Belletti et al., 2009). *Lactobacilli* isolated from Parmigiano Reggiano cheese were reported to be resistant to other antibiotics such as gentamicin and penicillin G and showed that resistance toward penicillin G was widespread among probiotics including *L. rhamnosus*, *L. reuteri*, and *L. plantarum* (Temmerman et al., 2003). *L. salivarius* MTC 1026 presented a similar pattern but it could not resist gentamicin after plasmid curing. Normally, *Lactobacillus* strains seem to be intrinsically resistant to ciprofloxacin and nalidixic acid, which were grouped by their property of inhibiting the synthesis of nucleic acid (Hummel et al., 2007) but *L. salivarius* MTC 1026 was susceptible to ciprofloxacin and nalidixic acid after curing.

The effect of *L. salivarius* MTC 1026 on pathogen adhesion to human intestinal cells was studied in vitro. The Caco-2 cell line is an intestinal epithelium which was originally isolated from colon adenocarcinoma of humans (Fogh et al., 1977). Inhibition of the pathogen invasion into epithelial cells is a critical defense to prevent gastrointestinal infection. In this report, *E. coli* ATCC 25922 adhesion was strongly inhibited by *L. salivarius* MTC 1026, more than by *S. typhimurium* ATCC 13311 by about 1-log. Gueimonde et al. (2006) showed that *L. casei* TMC 0409 and *L. acidophilus* TMC 0356 could inhibit significantly the adhesion of *S. typhimurium* ATCC 29631 but had no effect on the adhesion of *E. coli* strain NCTC 8603. Moreover, it has been reported that adhesion of *E. coli* K88 was lowered significantly by six *Lactobacillus* strains including *L. brevis*, D11, D14, R4, C2, and D17 in both competition and exclusion assays. The *S. typhimurium* SL1344 invasion rate on Caco-2 cells was reduced significantly in competition, exclusion and displacement assays (Yu et al., 2011).

Therefore, considering the probiotic properties of isolated strains, the data from this study demonstrate that *L. salivarius* MTC1026 is a satisfactory candidate

for a useful probiotic for application in humans and animals.

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