

## Bacteriocin-producing lactic acid bacteria as a probiotic potential from Thai indigenous chickens

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**ABSTRACT:** Bacteriocin-producing lactic acid bacteria (LAB) were isolated and screened from the gastrointestinal tract (GIT) of Thai indigenous chickens. The bacteriocinogenic activities and the primary probiotic properties were determined. The bacteriocins produced by 14 strains of selected LAB displayed inhibitory activity against indicator strains after the supernatants were neutralized with NaOH in the following species: *Lactobacillus sakei* subsp. *sakei* JCM1157, *Enterococcus faecalis* VanB, *Bacillus* sp., and *Listeria monocytogenes*. The antagonistic activity of selected LAB was inactivated or decreased after being treated with proteolytic enzymes ( $\alpha$ -chymotrypsin and trypsin). CR5-1 strain exhibited the highest level of activity (5120 AU/ml) in the stationary phase against *L. sakei* subsp. *sakei* JCM1157 in MRS broth at 37°C. The nine isolates of selected LAB were investigated for primary probiotic properties. The survival of the nine isolates was found to decrease approximately by 3 log CFU/ml after passing through the gastrointestinal conditions. All isolates exhibited protein digestion on agar plates but no isolates showed the ability to digest starch and lipid. Most of them showed high susceptibilities to some antibiotics (penicillin G, tetracycline and erythromycin). Thirteen LAB strains producing bacteriocin with strongly inhibitory activity were identified as *Lactobacillus salivarius* and only one strain was identified by 16S rDNA sequence analysis as *Lactobacillus agilis*.

**Keywords:** poultry; gastrointestinal tract; probiotic; antibiotic susceptibility

Lactic acid bacteria (LAB) are common microorganisms in foods and also constitute the natural intestinal microbiota of humans and most animals (Rojo-Bezares et al., 2006). LAB produce a wide range of antimicrobial metabolites which include organic acids, diacetyl, hydrogen peroxide, antibiotics and bacteriocins. Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptides which have a bactericidal or bacteriostatic effect on other (usually closely related) species. They are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanisms vary and may include pore formation of cell wall or cytoplasmic membrane, degradation of cellular DNA, disruption through specific cleavage of 16S rRNA, and inhibition of peptidoglycan syn-

thesis (Todorov et al., 2011). At first, bacteriocins received much attention mainly because of their potential application as food additives in the control of food spoilage and pathogenic food-borne microorganisms (Cleveland et al., 2001; Bromberg et al., 2005). Later, the effects of bacteriocin or bacteriocin-producing probiotics on animal performance have been studied (Stern et al., 2006).

Probiotics have received increasing attention as an alternative to in-feed antibiotics and for the purpose of improving productivity in the poultry industry (Shin et al., 2008). LAB, particularly *Lactobacillus* spp., is one of the probiotic groups which make up a large group of microorganisms in the GIT of all humans and animals. They can be tolerant to acid and bile, adhere to the intestinal epithelium of the hosts; they show an antagonistic

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activity against pathogenic bacteria and keep their viability during processing and storage (Musikasang et al., 2009).

LAB are well known for their production of bacteriocins. They have the potential to be used in the food and feed industry to substitute for chemical preservation (Gao et al., 2010). Bacteria intended for probiotic use should be screened for antibiotic resistance to avoid any potential carriage of undesirable antibiotic resistance into the intestinal environment (Huyghebaert et al., 2011). To avoid a source of antibiotic resistant LAB, Thai indigenous chickens not fed any commercial feed, were used as a source of bacteriocin-producing LAB and probiotic strains.

In general, for the production of Thai indigenous chickens backyard or village production systems by homesteads (extensive system) are preferred since the production costs are very low. The chickens are allowed to scavenge on their own for resources

around the homestead during the day and this is supplemented with concentrated feeds in the evening when they come back to roost and shelter for night (Wattanachant, 2008). In this rearing system, the chickens are not fed any commercial feed and antibiotics. Native chickens are well-suited to small-farm conditions; they have a good resistance to diseases and they tolerate a large variety of locally available feeds (Haitook et al., 2003). Therefore, there is an intestine compost of a large variety of bacteria species in Thai indigenous chickens due to the kind of feeds and rearing positions. For this reason, Thai indigenous chickens were chosen for LAB screening in this study to isolate many species of LAB and to avoid a source of antibiotic-resistant LAB.

The purpose of this study was to isolate and screen bacteriocin-producing LAB from the GIT of Thai indigenous chickens. The bacteriocinogenic activities and the primary probiotic properties were also studied.

Table 1. Microorganisms, strains and growth conditions

Microorganisms	Gram stain	Growth medium	Growth conditions (h/°C)
<i>Lactobacillus sakei</i> subsp. <i>sakei</i> JCM1157 <sup>a</sup>	+	MRS	24/37
<i>Pediococcus pentosaceus</i> DMST 18752 <sup>b</sup>	+	MRS	24/37
<i>Enterococcus faecalis</i> VanB <sup>c</sup>	+	MRS	24/37
<i>Listeria monocytogenes</i> <sup>d</sup>	+	TSB	24/37
<i>Listeria monocytogenes</i> DMST 17303 <sup>b</sup>	+	TSB	24/37
<i>Bacillus</i> sp. <sup>d</sup>	+	TSB	24/30
<i>Bacillus cereus</i> DMST 5040 <sup>b</sup>	+	TSB	24/30
<i>Staphylococcus aureus</i> <sup>d</sup>	+	TSB	24/37
<i>Staphylococcus aureus</i> DMST 8840 <sup>b</sup>	+	TSB	24/37
<i>Salmonella</i> Stanley 42 <sup>c</sup>	–	TSB	24/37
<i>Salmonella</i> Typhimurium DMST 16809 <sup>b</sup>	–	TSB	24/37
<i>Salmonella</i> Enteritidis DMST 15676 <sup>b</sup>	–	TSB	24/37
<i>Escherichia coli</i> <sup>d</sup>	–	TSB	24/37
<i>Escherichia coli</i> DMST 4212 <sup>b</sup>	–	TSB	24/37
<i>Vibrio parahaemolyticus</i> <sup>d</sup>	–	TSB	24/37
<i>Pasteurella multocida</i> DMST 16357 <sup>b</sup>	–	TSB	24/37

<sup>a</sup>obtained from the Japan Collection of Microorganisms, Japan

<sup>b</sup>obtained from Culture Collection for Medical Microorganism, Department of Medical Sciences, Thailand

<sup>c</sup>obtained from The WHO Global Salm-Surv Regional Centre of Excellence, South-East Asia and Western Pacific, Faculty of Veterinary Science, Chulalongkorn University, Thailand

<sup>d</sup>obtained from Songklanagarind Hospital, Prince of Songkla University, Thailand

MRS = de Man, Rogosa and Sharpe, TSB = Tryptic Soy Broth

## MATERIAL AND METHODS

### Bacterial strains and culture conditions

Reference strains of pathogenic and spoilage microorganisms were chosen to test antimicrobial activity of the strains presumed to produce bacteriocin or bacteriocin-like substances (Table 1). All strains were stored at  $-20^{\circ}\text{C}$  in their respective media with added glycerol (25%).

### Isolation of LAB from chicken intestinal tract

The gastrointestinal tracts (crop, small intestine, large intestine and cecum) of six Thai indigenous chickens were used as LAB sources. 25 g of each section of the gut was homogenized in 225 ml phosphate-buffered saline (PBS: 50mM  $\text{KH}_2\text{HPO}_4$ , 50mM  $\text{K}_2\text{HPO}_4 \times 3\text{H}_2\text{O}$ , 0.85% NaCl, pH 7.0) for 5 min using the Stomacher® 400 Circulator (Seward Ltd., Worthing, UK). Appropriate serial dilutions were plated onto MRS agar (HiMedia Laboratories, Pvt. Ltd., India) supplemented with 0.02% bromocresol purple (Ajax Finechem, Australia) and incubated anaerobically at  $37^{\circ}\text{C}$  for 24 h. Colonies which exhibited a clear halo were randomly selected from the highest dilutions of each MRS agar plate. Bacterial colonies were then purified 2–3 times by restreaking on MRS agar. The pure cultures were characterized using Gram stain, cell morphology and catalase reaction tests. Gram-positive and catalase-negative isolates were stored at  $-20^{\circ}\text{C}$  in MRS broth supplemented with 25% (v/v) glycerol. For routine analysis, the strains were subcultured twice in MRS broth at  $37^{\circ}\text{C}$  for 24 h (Musikasang et al., 2009).

### Antimicrobial assay and estimation of bacteriocinogenic activity

**Primary screening.** Antimicrobial activity was determined by the agar-spot test method using *L. sakei* subsp. *sakei* JCM1157 as the indicator strain. Briefly, each isolate of LAB was spotted on MRS agar and incubated under anaerobic conditions at  $37^{\circ}\text{C}$  for 24 h. Then, the plate was overlaid with 10 ml of soft MRS agar (1% agar, Fluka, USA) seeded with 250  $\mu\text{l}$  overnight culture of *L. sakei* subsp. *sakei* JCM1157 strain. This was incubated under the same condi-

tions at  $37^{\circ}\text{C}$  for 24 h. Inhibition was detected as a clear zone around the tested organism.

**Secondary screening.** The selected strains from primary screening were grown in MRS broth at  $37^{\circ}\text{C}$  for 24 h. Cell-free supernatants were collected by centrifugation (8500 rpm, 10 min,  $4^{\circ}\text{C}$ ) of overnight MRS broth cultures. The pH value of the culture supernatants was adjusted to 6.5 with NaOH (6M) to eliminate the effect of organic acids. After adding catalase (300 U/ml) (Fluka, USA), the cell-free supernatants (pH 6.5) were incubated at  $37^{\circ}\text{C}$  for 1 h to eliminate the effect of hydrogen peroxide. Then they were heated at  $90^{\circ}\text{C}$  for 10 min to stop the enzyme reaction. The same supernatants without catalase were used as control and all culture supernatants were filter-sterilized to eliminate the possible presence of viable cells (Gurban Oglu Gulahmadov et al., 2006).

The inhibition activity was examined by means of the diameters of inhibition zones using the agar well diffusion method (Batdorj et al., 2006). Briefly, 50  $\mu\text{l}$  of cell-free supernatants were placed into wells (6.0 mm in diameter) on the appropriate media agar plates (Table 1) seeded with indicator strains (final concentration  $10^6$  CFU/ml). After incubation for 24 h, the diameters of inhibitory zones were measured.

The proteinaceous character of the bacteriocin substance was confirmed by treating neutralized cell free supernatant (NCFS) with  $\alpha$ -chymotrypsin (Fluka, USA) and trypsin (Fluka, Switzerland) at a final concentration of 1 mg/ml at  $37^{\circ}\text{C}$  for 3 h. After stopping the enzyme activity by heat, the agar well diffusion method was used to estimate the bacteriocinogenic activity.

### Production of bacteriocin from selected LAB

An overnight culture of selected bacteriocin-producing LAB was inoculated ( $\text{OD}_{600} = 0.5$ , 1%) into 1 l of MRS broth and cultivated with moderate stirring (50 rev/min) at  $37^{\circ}\text{C}$  for 24 h. Samples were taken every 3 hours for 24 h and changes in optical density (600 nm), pH value, viable count and bacteriocin activity were measured. To quantify the bacteriocin activity, the agar well diffusion method was used. NCFS was serially diluted twofold in sterile deionized water and 50  $\mu\text{l}$  of each dilution was added into the wells. The titre was defined as  $2^n$ , where  $n$  is the reciprocal of the highest dilution that resulted in inhibition of the indicator strain. Thus, the arbitrary unit (AU)

of antibacterial activity per ml was defined as  $(2^n \times 1000 \mu\text{l})/50 \mu\text{l}$  (Cheikhoussef et al., 2009). *L. sakei* subsp. *sakei* JCM1157 was used as a bacteriocin sensitive indicator strain to determine bacteriocin activity levels.

### Resistance to simulated intestinal juice after sequential incubation in simulated gastric juice of isolated LAB

A simulated gastric juice was prepared by suspending 3 mg/ml pepsin (Fluka, Japan) in sterile saline (0.85% NaCl, w/v) and the pH was adjusted to 3.0 with 1.0M HCl. Twenty-four hour 1.0 ml cultures of the strains (Table 2) were subjected to centrifugation in Eppendorf Centrifuge 5415R (Eppendorf AG, Hamburg, Germany) at 8500 rpm for 10 min. They were then washed twice with sterile saline before being re-suspended in simulated gastric juice. Resistance was assessed in terms of the viable colony count and enumerated after incubation at 42°C for 2 h. After 120 min of gastric digestion, cells were harvested and suspended in simulated intestinal fluid which contained 1 mg/ml pancreatin (Sigma-Aldrich Chemie GmbH, Germany) and 0.5% freeze-dried chicken bile at pH 8.0. The suspension was incubated at 42°C for 6 h and the viable count was determined by drop plating (modified from Musikasang et al., 2009).

### Starch, protein and lipid digesting capabilities

Three modified MRS agars were prepared as follows: MRS agar containing 2% skimmed milk (HiMedia Laboratories Pvt. Ltd., India), MRS agar without dextrose containing 1% palm oil (Fluka, USA), and MRS agar without dextrose containing 2% soluble starch (Labchem, Ajax Finechem, Australia). These media were used for detecting the protein, lipid and starch digesting capabilities of selected LAB strains, respectively. The overnight cultures of LAB (10  $\mu\text{l}$ ) were dropped on the modified MRS agar and incubated at 42°C for 24 h. Then the diameters of the halo zone on the agar plate were measured. The digesting capability of the tested strains was classified as positive when the diameters of the clear zone were more than 1 mm. Each assay was performed in triplicate (modified from Musikasang et al., 2009).

### Antibiotic susceptibility

Nine isolates of selected LAB (S6-10, CE5-3, CE5-10, CR5-15, CR5-1, CR6-1, CR6-3, L3-7, and L5-4) (Table 2) were tested for resistance to four antimicrobial agents. These were: penicillin G (Fluka, Austria) ( $\beta$ -lactam group, inhibitors of cell wall synthesis), tetracycline (Fluka, China) (inhibi-

Table 2. Source and some characteristics of selected bacteriocin-producing lactic acid bacteria

Strain	Source	Gram-stain	Shape	Catalase test
CR5-1	crop	+	short rod	–
CR5-15	crop	+	short rod	–
CR6-1	crop	+	short rod	–
CR6-3	crop	+	short rod	–
S4-17	small intestine	+	short rod	–
S4-18	small intestine	+	short rod	–
S6-10	small intestine	+	short rod	–
L3-2	large intestine	+	short rod	–
L3-7	large intestine	+	short rod	–
L5-4	large intestine	+	short rod	–
CE4-4	cecum	+	short rod	–
CE4-5	cecum	+	short rod	–
CE5-3	cecum	+	short rod	–
CE5-10	cecum	+	short rod	–

tors of protein synthesis), chloramphenicol (Sigma-Aldrich Chemie GmbH, Germany) (inhibitors of protein or mRNA synthesis, broad spectrum) and erythromycin (Fluka, Italy) (inhibitors of protein synthesis – Gram positive spectrum). Each of the antibiotic powders was weighed, dissolved, diluted in appropriate diluents and filter sterilized prior to the addition to MRS medium. Serial dilutions of antibiotics ranging from 256 to 0.25 µg/ml for penicillin G, tetracycline, and chloramphenicol and 320 to 2.5 µg/ml for erythromycin were prepared.

Minimal inhibitory concentration (MIC) values for all bacterial isolates were determined by the broth microdilution procedure. The MRS broth containing antibiotics was added to micro-well plate and serial twofold dilutions were prepared. The final concentration of inoculum was adjusted to  $10^5$  CFU/ml. The inoculum was derived from a broth culture which was incubated at 42°C for 24 h and 20 µl of the inoculum was used to inoculate each well. The trays were covered and incubated at 42°C for 24 h. Then minimal bactericidal concentration (MBC) values were measured by streaking on MRS agar and incubation at 42°C for 24 h. The experiments were replicated at least three times to verify the reproducibility of the methodology when using the above-mentioned conditions (D'Aimmo et al., 2007).

### Strain identification

To identify bacteriocin-producing strains, the selected isolates were finally identified along the full length of 16S rDNA sequence analysis. Some isolates were identified by 16S rDNA  $V_3$  regions of bacterial sequence. PCR amplifications were performed in a total reaction volume of 50 µl containing 0.2 µM of each of the primers, 25 µl of EmeralAmp Mastermix (Takara, Japan), Dnase and Rnase free water 14 µl (Takara, Japan), and 1 µl of template DNA. All amplification reactions were performed in a PCR Sprint temperature cycling system (Techne, UK) using the following amplification conditions: the first cycle was preceded by incubation at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 50°C for 30 s and at 72°C for 1.5 min. The reactions were terminated with 5 min of incubation at 72°C, and then chilled to 4°C. 10 µl of the PCR products were subjected to electrophoresis in a 1% agarose gel (ISC Bio Express®, USA) and were subsequently visualized by UV illumination after staining with SYBR Safe (Invitrogen™, USA). The oligonucleotide

primers (AITbiotech Pte Ltd., Singapore) used in this study are as follows: Forward primer 8F: 5' AGA GTT TGA TCC TGG CTC AG 3'; Reverse primer 1492R: 5' GGC TAC CTT GTT ACG ACT T 3'; yielding a product of approximately 1500 base pairs (bp). Forward primer 341F: 5' CCT ACG GGA GGC AGC AG 3' and Reverse primer 518R: 5' ATT ACC GCG GCT GCT GG 3' giving a product of 192 bp. The PCR fragments of the 16S rDNA were purified and then subsequently sequenced (1<sup>st</sup> BASE Pte Ltd., Singapore). Sequences were analysed with the sequencing software Chromaspro from Technelysium that opens chromatogram files produced by the Applied Biosystems equipment. The sequences obtained were sent to the National Centre for Biotechnology Information (NCBI) to be analysed for the nucleotide-nucleotide BLAST database in FASTA format (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (modified from González et al., 2007).

## RESULTS AND DISCUSSION

### Isolation of LAB from chicken intestinal tract

In total, 307 LAB strains were isolated from the GIT of six Thai indigenous chickens. All of them were isolated from four gut parts: 66 isolates were isolated from crop, 72 from small intestine, 71 from large intestine, and 98 isolates were isolated from caecum. They were Gram-positive, catalase negative, mostly short rod-shaped and facultatively anaerobic bacteria. The results were in accordance with previous studies establishing that the major LAB in native chicken or poultry intestinal tract were rod shaped (Lima et al., 2007; Musikasang et al., 2009).

### Antimicrobial activity of bacteriocin-producing LAB

Only 14 isolates (L3-2, L3-7, S4-17, S4-18, CE4-4, CE4-5, CR5-1, CR5-15, L5-4, CE5-3, CE5-10, CR6-1, CR6-3, and S6-10) (Table 2) out of the 307 isolates of selected LAB could inhibit *L. sakei* subsp. *sakei* JCM1157. This was used as an indicator strain for the primary bacteriocin screening step.

For the secondary screening, the neutralized supernatants from 14 strains of selected LAB exhibited inhibitory activity against indicator strains including *L. sakei* subsp. *sakei* JCM1157, *P. pentosaceus* DMST

Table 3. Inhibitory activity of selected lactic acid bacteria strains against bacterial indicator strains after eliminating effect of organic acid and H<sub>2</sub>O<sub>2</sub> by using agar well diffusion method

Indicator strains	Antibacterial activity														
	L3-2	L3-7	S4-17	S4-18	CE4-4	CE4-5	CR5-1	CR5-15	L5-4	CE5-3	CE5-10	CR6-1	CR6-3	S6-10	
<i>Lactobacillus sakei</i> subsp. <i>sakei</i> JCM1157	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
<i>Pediococcus pentosaceus</i> DMST 18752	+	+	+	+	+	+	+	+	+	−	−	+	+	+	
<i>Enterococcus faecalis</i> VanB	+	+	+	+	+	+	+	+	+	−	−	+	+	+	
<i>Listeria monocytogenes</i>	+	+	+	+	+	+	+	+	+	−	+	−	+	+	
<i>Listeria monocytogenes</i> DMST 17303	+	+	+	+	+	+	+	+	+	−	−	+	+	+	
<i>Bacillus</i> sp.	−	−	−	−	−	−	+	+	+	−	−	−	+	+	
<i>Bacillus cereus</i> DMST 5040	−	−	−	−	−	−	+	+	−	−	−	−	−	+	
<i>Staphylococcus aureus</i>	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
<i>Staphylococcus aureus</i> DMST 8840	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
<i>Salmonella</i> Stanley 42	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
<i>Salmonella</i> Typhimurium DMST 16809	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
<i>Salmonella</i> Enteritidis DMST 15676	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
<i>Escherichia coli</i>	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
<i>Escherichia coli</i> DMST 4212	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
<i>Vibrio parahaemolyticus</i>	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
<i>Pasteurella multocida</i> DMST 16357	−	−	−	−	−	−	−	−	−	−	−	−	−	−	

+ = inhibition activity, – = none inhibition activity

18752, *Ent. faecalis* VanB, *Lis. monocytogenes*, *Lis. monocytogenes* DMST 17303, *Bacillus* sp. and *B. cereus* DMST 5040 (Table 3). However, the NCFS of selected LAB could not inhibit *S. aureus*, *S. aureus* DMST 8840, *Sal.* Stanley 42, *Sal.* Typhimurium DMST 16809, *Sal.* Enteritidis DMST 15676, *E. coli*, *E. coli* DMST 4212, *V. parahaemolyticus*, and *P. multocida* DMST 16357.

The proteinaceous character of the bacteriocin substance was confirmed. After treating NCFS with  $\alpha$ -chymotrypsin, the inhibitory activity toward *L. sakei* subsp. *sakei* JCM1157 of 12 selected LAB strains disappeared (Table 4). The destruction of the antimicrobial activity by proteases suggested that the compound in the supernatant could be a peptide or bacteriocin (Bromberg et al., 2005). In addition,

the inhibitory activities of NCFS after being treated with trypsin were decreased 0.5 fold (Table 3).

The LAB isolated from chickens could produce many kinds of bacteriocins such as acidocin A which is secreted by *Lactobacillus acidophilus* TK9201 (Kanatani et al., 1995). Bacteriocin OR7 is produced by *L. salivarius* (NRRL B-30514) (Cole et al., 2006; Stern et al., 2006). *Ent. faecium* EF55 isolated from the gastrointestinal tract of chickens produced enterocins A and P (Strompfová and Lauková, 2007). In addition, bacteriocins produced from LAB strains were also reported to inhibit the growth of pathogenic bacteria in many studies (Ogunbanwo et al., 2004; De Kwaadsteniet et al., 2005; Cole et al., 2006; Pilasombut et al., 2006; Stern et al., 2006; Lima et al., 2007; Gao et al., 2010).

Table 4. Inhibitory activity of selected lactic acid bacteria strains against *Lactobacillus sakei* subsp. *sakei* JCM1157 after eliminating effect of organic acid, H<sub>2</sub>O<sub>2</sub> and effect of protease enzyme on antagonistic activity by using agar well diffusion method

Strains	Supernatants	Inhibitory activity (mm)	Strains	Supernatants	Inhibitory activity (mm)
CR5-1	NC	14.50	S6-10	NC	10.75
	NCT	10.50		NCT	5.25
	NCC	5.50		NCC	–
CR5-15	NC	12.75	L3-2	NC	10.00
	NCT	4.25		NCT	4.00
	NCC	–		NCC	–
L5-4	NC	11.50	L3-7	NC	9.00
	NCT	7.00		NCT	3.00
	NCC	4.25		NCC	–
CE5-3	NC	7.00	S4-17	NC	9.00
	NCT	–		NCT	4.00
	NCC	–		NCC	–
CE5-10	NC	1.50	S4-18	NC	10.50
	NCT	–		NCT	3.50
	NCC	–		NCC	–
CR6-1	NC	3.00	CE4-4	NC	10.50
	NCT	–		NCT	3.00
	NCC	–		NCC	–
CR6-3	NC	12.25	CE4-5	NC	7.50
	NCT	6.00		NCT	3.00
	NCC	–		NCC	–

NC = neutralized supernatant + catalase, NCT = neutralized supernatant + catalase + trypsin, NCC = neutralized supernatant + catalase +  $\alpha$ -chymotrypsin

In our study, the bacteriocins produced from selected LAB showed strongly inhibitory activity toward many Gram-positive bacteria. However, all of the Gram-negative indicators (Table 1) used were not inhibited by any of the bacteriocins produced (Table 3). Bacteriocins produced by LAB have attracted great interest in terms of safety, but most of them only inhibit some Gram-positive pathogenic bacteria. However, some bacteriocins are effective against Gram-negative spoilage and pathogenic bacteria such as sakacin C2 produced by *Lactobacillus sakei* C2 strongly inhibited *E. coli* ATCC 25922. This activity against many Gram-negative bacteria was not frequently seen in bacteriocins from LAB (Gao et al., 2010). In addition, the combination of bacte-

riocin with some natural antimicrobial compounds is able to enhance the inhibition of Gram-negative pathogen such as interactions of nisin, lysozyme, and EDTA had an effect on the growth of *Sal. typhimurium* (Gill and Holley, 2000).

Gram-negative spoilage organisms and pathogens are especially problematic due to their inherent resistance to some antimicrobial agents such as bacteriocins. This inability is due to the protective outer membrane which covers the cytoplasmic membrane and peptidoglycan layer of the cells. This asymmetrical membrane contains glycerophospholipids and lipopolysaccharide (LPS) molecules in which the anchoring of divalent cations is involved. Treatment with metal-chelating

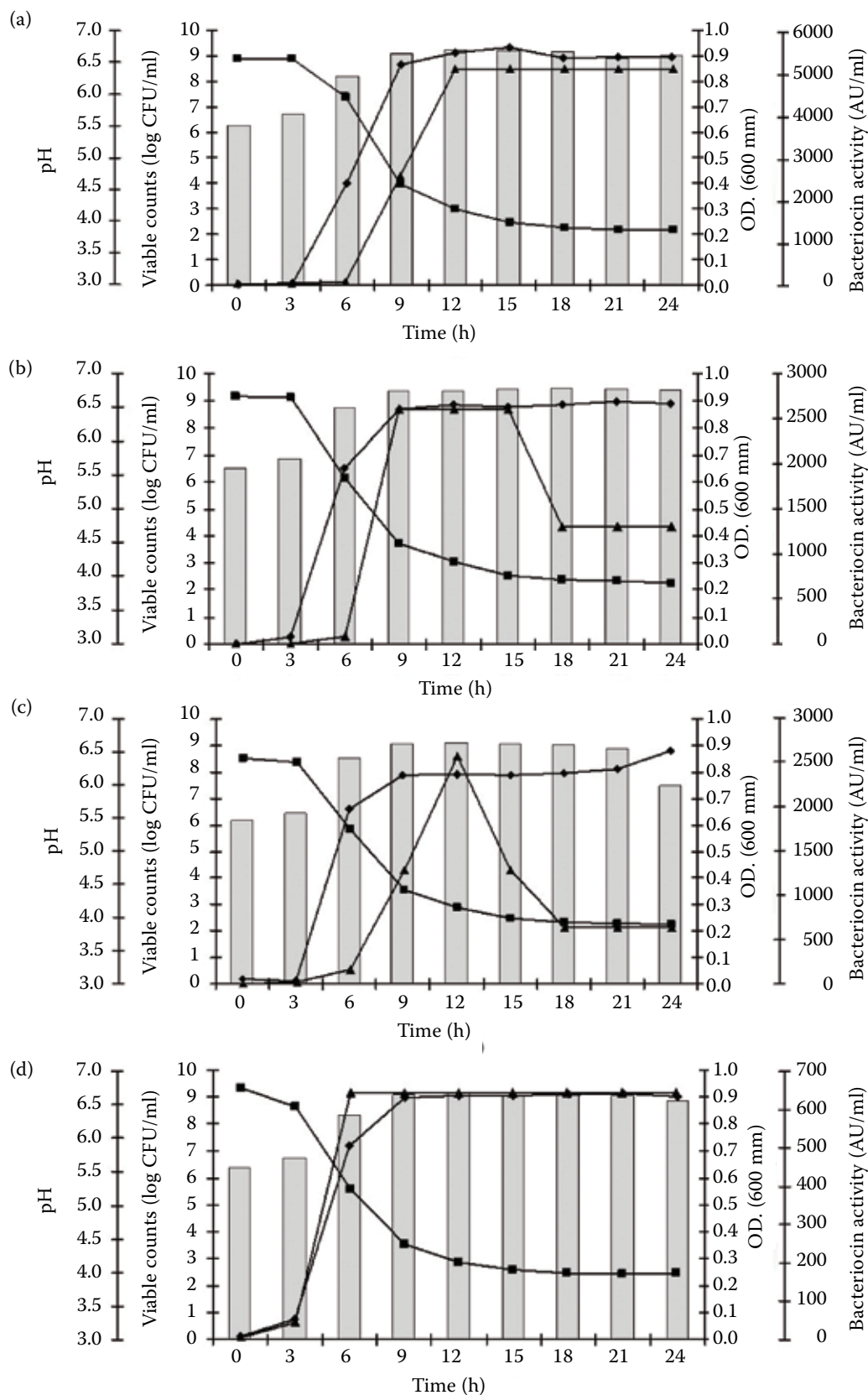


Figure 1. Bacteriocin production during the growth of CR5-1 (a), CE4-4 (b), CR6-3 (c), and CR5-15 (d) strains in MRS broth at 37°C. The viable counts (bar), optical density, absorbance at 600 nm (♦) and pH (■) of the culture were measured at the time intervals as indicated. The antibacterial activity (▲) was also assayed and expressed as AU/ml



agents such as EDTA generally results in removal by chelation of divalent cations with a consequent disruption of the outer membrane. Lactic acid and its salts are also potent outer membrane disintegrating agents as evidenced by their ability to cause LPS release. These permeabilizers increase susceptibility to hydrophobic substances such as bacteriocins by enabling them to penetrate the cell wall (Alakomi et al., 2003; Castellano et al., 2008).

### Production kinetics of bacteriocin from selected LAB

Out of the 14 selected LAB tested, four strains (CR5-1, CE4-4, CR6-3, and CR5-15) showed relatively high levels of antimicrobial activity against *L. sakei* subsp. *sakei* JCM1157 (Figure 1).

Bacteriocin produced from strain CR5-1 gave the highest level of activity against *L. sakei* subsp. *sakei* JCM1157 when cultured in MRS broth at 37°C. This strain started to produce bacteriocin of about 40 AU/ml at 6 h during the middle logarithmic growth phase at a cell count of 8.19 log CFU/ml. Bacteriocin concentration reached the maximum (5120 AU/ml) after 12 h of incubation at a cell count of 9.26 log CFU/ml. This was at the beginning of the stationary phase of growth, the level of inhibition thereafter remained constant (Figure 1a). Both the CE4-4 and CR6-3 strains showed inhibitory activity against *L. sakei* subsp. *sakei* JCM1157 at 2560 AU/ml after 9 and 12 h of incubation, respectively. However, the production started to decrease after 15 and 12 h, respectively, which may be due to proteolytic degradation or changes in the envi-

ronmental conditions (Huang et al., 2009). During bacterial growth, the pH of the medium dropped from pH 6.57 (0 h) to pH 3.85 (24 h) (Figure 1b, c).

### Resistance to simulated intestinal juice after sequential incubation in a simulated gastric juice of isolated LAB

Tolerance to low pH, bile, and pancreatic fluid *in vitro* is expected to predict the survival of a strain in the conditions present in the gut. All of the nine strains (CE5-3, CR6-3, CR5-1, S6-10, CE5-10, CR5-15, L5-4, CR6-1, and L3-7) (Table 2) could survive after the sequential method was studied. The viable LAB cell numbers initially decreased approximately 3 log CFU/ml for most strains (Figure 2). However, the lab cells could survive over  $10^6$  CFU/ml even after 2 h of exposure to the simulated gastric juice (pH 2.5) and their number slightly decreased after 4 h incubation in the simulated intestinal juice. It corresponds with previous studies reporting that LAB isolated from chickens were less stable in the gastric juice conditions (pH 2.6) (Lin et al., 2007; Musikasang et al., 2009). However, most of the isolated LAB were reduced to some extent at pH 3.0 after 2 h of incubation (data not shown). The pH levels of the gastric juice may vary from 2.0 to 3.5 depending on the feeding time, the growing stage or the kind of animal (Yu and Tsen, 1993).

Intestinal origin has been reported to be a relevant criterion for selecting probiotic strains. This is because probiotic strains may be expected to function better in an environment similar to that from which they were originally isolated. However, technologi-

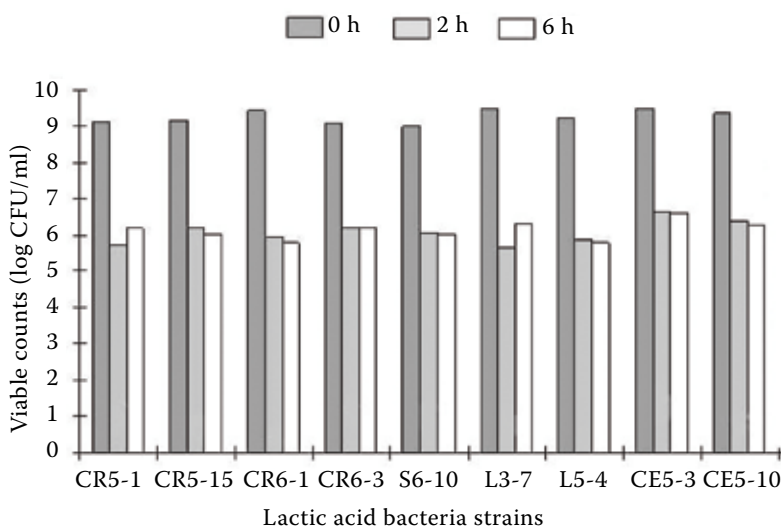


Figure 2. Survival of selected lactic acid bacteria in the presence of 0.5% freeze-dried chicken bile and pancreatin (1 mg/ml) at pH 8.0 (4 h) after sequential incubation in simulated gastric juice (pH 2.5, containing pepsin 3 mg/ml, for 2 h)

cal aspects such as the viability in the product have to be considered in the probiotic selection (Saarela et al., 2000; Ruiz-Moyano et al., 2008).

### Starch, protein, and lipid digestive capabilities

The agar plate assays were used to study the digestive capability of nine isolates (L3-7, CR5-1, CR5-15, L5-4, CE5-3, CE5-10, CR6-1, CR6-3, and S6-10) of the selected bacteriocin-producing LAB. Sterilized skimmed milk, palm oil, and soluble starch were used for detecting protein, lipid and starch digestive capabilities, respectively. All the nine isolates exhibited protein digestion. In particular, the L3-7 strain showed the highest activity observed from the size of the clear halo. However, neither starch nor lipid digestions were detected (data not shown).

The beneficial effects of enzymes in the *Lactobacillus* show the importance of enzymatic activities in bacterial strains of chicken probiotics. Probiotics exhibiting enzymatic activities can improve digestion especially in newly hatched chicks (Taheri et al., 2001). In addition, *Lactobacillus* producing high-active amylase could improve the feed conversion ratio in pigs (Lee et al., 2001). Utilization of some LAB strains in nutrition is due to their production of enzymes such as  $\alpha$ -amylase, phytase, lecitinase, lipase, and/or protease.

### Antibiotic susceptibility

Table 5 shows MIC and MBC values obtained from different antibiotics tested in our selected LAB strains. Penicillin G MIC values detected in all LAB strains were low (MIC < 0.25  $\mu\text{g/ml}$ ) and their MBC values were also low (MIC range from < 0.25 to 4  $\mu\text{g/ml}$ ). Similarly to penicillin G, all LAB strains showed high susceptibility to erythromycin (MIC < 0.25  $\mu\text{g/ml}$ ) and tetracycline (MIC range was from < 0.25 to 4  $\mu\text{g/ml}$ ). L3-7, L5-4, CR5-1, CR5-15, CE5-10, and S6-10 strains showed high MBC values of erythromycin (MBC range from 20 to 320  $\mu\text{g/ml}$ ). The other tested strains (CE5-3, CR6-1, and CR6-3) exhibited a lower range of MBC values (< 0.25 to 5  $\mu\text{g/ml}$ ). Tetracycline MBC values detected in most of our strains were generally very high. However, the CR5-15 and CR6-3 strains showing tetracycline MBC values < 0.25  $\mu\text{g/ml}$  were the exception. All selected LAB strains showed a moderate resistance to chloramphenicol (MBC 8  $\mu\text{g/ml}$ ) and most of them exhibited high MBC values (64–128  $\mu\text{g/ml}$ ).

In this study, MIC values of the selected LAB were mostly lower than 0.25–8  $\mu\text{g/ml}$ , so the bacteria may be considered moderately resistant (Walsh, 2003; D'Aimmo et al., 2007). Therefore, the probability of resistances of antibiotic genes to environmental changes may be low.

Bacteria can develop numerous strategies to counteract the action of antibiotics, although most of them can be included in three types of resist-

Table 5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antibiotics toward selected lactic acid bacteria (in  $\mu\text{m/ml}$ )

Strains	Antibiotic susceptibility							
	penicillin G		tetracycline		chloramphenicol		erythromycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
L3-7	< 0.25	< 0.25	1.0	64	8	64	< 0.25	40
L5-4	< 0.25	< 0.25	4.0	64	8	128	< 0.25	40
CR5-1	< 0.25	< 0.25	4.0	128	8	256	< 0.25	20
CR5-15	< 0.25	1.0	< 0.25	< 0.25	8	> 256	< 0.25	40
CE5-3	< 0.25	< 0.25	1.0	16	8	16	< 0.25	5
CE5-10	< 0.25	4.0	0.5	128	8	256	< 0.25	40
CR6-1	< 0.25	0.5	1.0	64	8	64	< 0.25	< 2.5
CR6-3	< 0.25	< 0.25	< 0.25	< 0.25	4	8	< 0.25	< 2.5
S6-10	< 0.25	< 0.25	1.0	128	8	> 256	< 0.25	320

ance mechanisms. These are: (1) inactivation of the antibiotics by some enzyme(s), (2) active efflux pumps that extrude the molecule outside the cell, and (3) modification of the subcellular target where the antibiotics bind (Walsh, 2003; Rojo-Bezares et al., 2006).

### Strain identification

The 14 bacteriocin-producing LAB strains were all Gram-positive, catalase-negative and facultatively anaerobic short rods. They were identified by a comparison of the full length of 16S rDNA sequence or 16S rDNA V<sub>3</sub> region sequence. Strains CE4-4, CE4-5, CE5-3, CR5-1, CR5-15, L3-2, L3-7, S4-17, and S4-18 were similar to *Lactobacillus salivarius* at 100%. The CE5-10, CR6-3, L5-4, and S6-10 strains were identified as *L. salivarius* with 97, 97, 99 and 99% similarity, respectively. Strain CR6-1 was similar to *L. agilis* at 99%. The rDNA sequences were deposited in DDBJ/ EMBL/GenBank under accession numbers AB612958, AB612959, AB612960, AB612962, AB612963, AB612966, AB612967, AB612969, AB612970, AB612961, AB612965, AB612968, AB612971, and AB612964, respectively.

*Lactobacillus salivarius* is able to produce many kinds of bacteriocins such as Abp 118 alpha and beta from *L. salivarius* UCC118 (Flynn et al., 2002). Salivaricin CRL 1328 was produced by *L. salivarius* CRL 1328 which was isolated from a healthy human vagina (Pingitore et al., 2009). Bacteriocin OR7 was also produced by *L. salivarius* (NRRL B-30514) (Cole et al., 2006; Stern et al., 2006). In addition, *L. salivarius* K7 isolated from the chicken intestinal tract could produce bacteriocin FK22 which was homologous to Abp 118 beta produced by *L. salivarius* UCC118 (Pilasombut et al., 2006).

### CONCLUSION

The results indicate that the 14 LAB strains of 307 isolates showed bacteriocin activity. Thirteen isolates were identified as *L. salivarius*. Only one strain was identified as *L. agilis*. The bacteriocins produced from the selected LAB showed many strongly inhibited Gram-positive bacteria. However, all the Gram-negative indicators used were not inhibited by any of the bacteriocins produced. In this research, it has been demonstrated

that bacteriocin-producing LAB have some good primary probiotic properties. Based on the physiological characteristics of *L. salivarius* and biological properties of bacteriocin, the strain may be used as probiotics for poultry. Future investigation is warranted on *in vivo* probiotic properties on poultry production of *L. salivarius* CR5-1. Additional experiments proving the safety of the strain and its bacteriocin need to be undertaken.

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