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Screening and Characterization of Bacteriocin Producing Lactic Acid Bacteria Isolated from Chicken Intestine

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ABSTRACT

Five hundred strains of lactic acid bacteria (LAB) were isolated from chicken intestine and screened for their abilities to produce bacteriocins. One strain, K4, produced a bacteriocin with activity against *Lactobacillus sakei* subsp. *sakei* JCM 1157^T, *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124^T, *Bacillus coagulans* JCM 2257^T, *Enterococcus feacalis* JCM 5803^T, *Listeria innocua* ATCC 33090^T and *Brochotrix campestris* NBRC 11547^T. The antibacterial activity was inactivated by proteolytic enzymes, indicating that it has proteinaceous structure of a bacteriocin. Its properties were heat-tolerance at 121°C for 15 min and stable at wide pH range of 3-10. The mode of action of this bacteriocin was clearly bactericidal activity against *Lb. sakei* subsp. *sakei* JCM 1157^T and *B. coagulans* JCM 2257^T whereas it showed bacteriostatic action against *Br. campestris* NBRC 11547^T. In addition, K4 strain was able to grow and produce bacteriocin in MRS containing 4% NaCl and at wide pH range of 3-9. Based on morphological, biochemical characteristics and 16S rDNA nucleotide sequence analysis, K4 strain was identified as *Lactobacillus salivarius.*

Key words: lactic acid bacteria, chicken intestine, bacteriocin

INTRODUCTION

Lactic acid bacteria (LAB) are known to be “generally recognized as safe” (Marteau and Rambaud, 1993) and widely used in food industry for fermentation of dairy, meat and vegetable products (Noonpakdee *et al.*, 2003). In addition, they are able to inhibit the growth of other microorganisms (Huot *et al*., 1996) because they produce various antimicrobial compounds, such as organic acids, hydrogen peroxide and bacteriocins (O’ Sullivan *et al*., 2002). Among them, bacteriocins have attracted great interest as they are safe and effective natural inhibitors against

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pathogenic and food spoilage bacteria in various foods (Delves-Broughton *et al*., 1996) that could replace chemical preservatives (Marrec *et al*., 2000). In addition, LAB were also found to have probiotic potential. They are commonly used as viable microbial feed supplements that affect the host animal by improving its intestinal microflora balance (Hyronimus *et al*., 2000). In chicken, some pathogenic microorganisms were reported to colonize the intestinal tract (Garriga *et al*., 1998). In an effort to control these pathogens, several LAB were isolated from chicken intestine for their probiotic use (Nitisinprasert *et al*., 2000). Therefore, the main objective of this study was to screen and characterize the bacteriocin-producing LAB isolated from chicken intestine.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Strains of bacteriocin producing-LAB grown in de Man-Rogosa-Sharp (MRS) broth (Oxiod, Basingstock, UK) were incubated at 30° C under anaerobic condition for 14 h. The indicator strains used in this study and their growth conditions are shown in Table 1. For long term preservation, all strains were stored at -80°C in 15 % glycerol. Before use, all strains were

Table 1 Antibacterial activity of K4 strain in AU/ml against indicator strains.

Bacteria	Media	Temperature	Antagonistic activity
Lactic acid bacteria			
Lactococcus lactis subsp. *lactis* ATCC 19435^T	MRS	30°C	-
Enterococcus faecium TUA 1344L	MRS	30°C	-
Lactobacillus plantarum ATCC 14917^T	MRS	30°C	-
Pediococcus pentosaceous JCM 5885	MRS	30°C	-
Lactobacillus sakei subsp. *sakei* JCM 1157^T	MRS	30°C	3200
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 6124^T	MRS		100
Enterococcus feacalis JCM 5803^T	MRS	37°C	200
Other Gram-positive bacteria			
Bacillus subtilis JCM 1465^T	TSBYE	30°C	-
Bacillus circulans JCM 2504^T	TSBYE	30°C	-
Bacillus coagulans JCM 2257^T	TSBYE	37°C	1600
Bacillus cereus JCM 2152^T	TSBYE	30°C	-
Staphylococcus epidermis JCM 2414^T	TSBYE	37°C	-
Listeria innocua ATCC 33090^T	TSBYE	37°C	800
Micrococcus luteus IFO 12708	TSBYE	30°C	-
Brochotrix campestris NBRC 11547 ^{T}	TSBYE	26°C	800
Gram-negative bacteria			
Pseudomonas fluorescens JCM 5963^T	TSBYE	26°C	-
Escherichia coli JM109	TSBYE	37°C	-

ATCC = American Type Culture Collection, Rockville, Md
JCM = Japanese culture of Microorganisms, Wako, Japan
JM = Commercial strain from Toyobo, Osaka, Japan
TUA = Tokyu University of Agriculture, Tokyo Japan
IFO = Institute for Fermentation, Osaka, Japan
NBRC = National Institute of Technology and Evaluation (NITE) Biological Resource Center

subcultured overnight in proper media and condition (Table 1). *E. coli* JM109 containing pGEM-T vector (Promega, Medison, USA) was grown in Luria Bertani broth supplemented with 100 μg/ml of ampicillin (Sambrook *et al*., 2001).

Isolation of bacteriocin-producing LAB

LAB strains were isolated from 20 chicken intestines at King Mongkut's Institute of Technology Ladkrabang's farm, Thailand. Ten grams of intestinal content was homogenized in 90 ml diluent (0.85% NaCl) and subsequently ten fold serially dilution was carried out. The dilutions were then spread-plate on MRS agar containing 0.5% $CaCO_3$ in duplicate. The plates were incubated under anaerobic condition at 37°C for 48 h. Only clear zone producing-colonies were randomly selected due to dissolution of $CaCO_3$ by organic acid production. The selected colonies were purified by transferring them from the master plate to a new MRS agar plate for two passages. These colonies were maintained in a MRS broth under anaerobic conditions at 37°C overnight, and then stored at -80°C in 15% glycerol for bacteriocin screening.

Screening of LAB strains for antibacterial activities

The primary screening of five-hundred isolates for bacteriocin producing was carried out by spot-on-lawn method (Ennahar *et al*., 1999). The culture of one isolate, K4 strain was centrifuged at 5369 × g for 10 min. The cell free supernatant (CFS) was neutralized by adjusting to pH 6 by 0.5 M NaOH and then sterilized by filter (0.2 μm, polysulfone, Kanto chemical, Japan). Antibacterial activity was determined by spotting 10 μl of neutralize CFS onto the surface agar plate which was overlaid with 5 ml of soft agar (1% w/v) seeded with 10 μl of freshly-grown indicator strain. After incubating overnight at a proper temperature as shown in Table 1, inhibition zone was observed. The antibacterial activity was expressed in arbitrary unit (AU). The supernatant was two-fold serially diluted in 96-well microtiter plate (Greiner Bio-One, Germany). An arbitrary unit was defined as the reciprocal of the highest dilution producing a clear zone of growth inhibition of the indicator strain. AU was calculated as (1000/10) D where D was the dilution factor (Parente *et al.*, 1995).

Effect of enzyme, pH and heat on the antimicrobial of CFS

On antimicrobial activity, CFS was treated with proteolytic enzymes at the final concentration of 1 mg/ml as follows: trypsin (Sigma, St. Louis, Mo, U.S.A), alpha-chymotrypsin (Sigma), ficin (Sigma), protease type XIII (Sigma), pepsin (Sigma), papain (Merck, Darmstadt, Germany), proteinase K (Merck) and actinase E (Kaken Pharmaceutical, Japan). All samples were adjusted to pH 7 except that treated with pepsin, which was adjusted to pH 3 and then was sterilized by filter (0.2 μm, acrylic copolymer, Kanto Chemical, Japan). The filtrates were incubated at 37°C for 3 h. Residual enzyme activity was finally stopped by boiling for 5 min.

To determine the effect of pH on bacteriocin activity, the supernatant was adjusted to various pH levels of 3-10 and incubated at 30° C for 2 h. All samples were adjusted to pH 6 and sterilized by filtering through 0.22 μm filter. Heat stability was determined by heating the neutralized CFS at 100 °C for 5, 30 min and at 121°C for 15 min. The remaining of antibacterial activity was examined by spot-on-lawn method (Ennahar *et al*., 1999).

Effect of NaCl and pH on growth and production of bacteriocin

NaCl was added to MRS broth at concentrations of 1, 2, 3 and 4%. Similarly, the effect of pH on the production of bacteriocin was determined by adjusting MRS broth to pH 3, 4, 5, 6, 7, 8, 9 and 10. All treatments were then inoculated with 1%(v/v) of K4 strain culture and incubated at 30°C for 16 h. The ability of growth

under all these conditions was evaluated spectrophotometrically by measuring the turbidity at 600 nm. Similarly, pH was measured to determine the ability of growth. Antibacterial activity was evaluated by spot-on-lawn method against *Lb. sakei* subsp. *sakei* JCM 1157^T and *B. coagulans* JCM 2257^T.

Determination of mode of action

Mode of action of bacteriocin produced by K4 strain was carried out by using partially purified bacteriocin which was prepared by Amberlite XAD-16 (Sigma, St. Louis, Mo, U.S.A) to adsorb proteins from CFS as described by Cintas *et al*. (2000). One hundred milliliter of MRS broth was inoculated with 1% (v/v) of indicator strain culture, and then incubated at 30°C for 3 h. Subsequently, partial purified bacteriocin at a final concentration of 100 AU/ml was added to indicator strains culture, *Lb. sakei* subsp. *sakei* JCM 1157^{T}and *Br. campestris* NBRC 11547^T and 1000 AU/ml for *B. coagulans* JCM 2257^T since it was found that 100 AU of partial purified bacteriocin has no inhibitory activity against *B. coagulans* JCM 2257^T. Therefore, concentration of 1000 AU/ml was needed to be applied for mode of action study. The samples were taken at 2 h interval for 8 h. The number of CFU and the optimal density (600 nm) were determined.

Morphological and biochemical characteristics

Cell morphology, Gram stain, catalase activity and gas production of K4 strain were determined according to the method of Forbes *et al*. (1998). Additionally, carbohydrates fermentation pattern was carried out by using API 50 CH kit (BioMerieux, France). The growth of K4 strain was tested at pH 4.5 and 9.6 and at different temperatures of 5, 15, 30, 37, 42, 45 and 50°C. Salt tolerance was observed in MRS containing NaCl concentrations at 6.5 and 18%. Cell turbidity appearance was determined as cell growth.

PCR amplification of 16S rRNA gene of K4 strain

PCR amplification of 16S rDNA was carried out to confirm the identification result. Genomic DNA of K4 or K7 strains were isolated by using Genomic DNA Mag extractor Genome kit (Toyobo, Osaka, Japan). Polymerase Chain Reaction (PCR) amplification was conducted with pair of bacterial universal primer 8f 5'-AGA GTT TGA TCA TGG CTC AG-3' and 1510r 5'-GTG AAG CTT ACG GCT ACC TTG TTA CGA CTT-3' (Martínez-Murcia *et al*., 1995; Christine *et al*., 2002). PCR mixtures composed of 10 X reaction buffer, 25mM $MgCl_2$, 2.5 mM deoxynucleoside triphosphate, 1 U of *Taq* polymerase (Promega, U.S.A), 20 pM of each primer and genomic DNA about 50-200 ng were used as a template for amplification. The PCR conditions were set at 94° C for 5 min, 30 cycles including denaturation at 94°C (30s), annealing at 52°C (30 s), and extension at 72°C (45s) and the final step at 72°C for 7 min.

Screening of *E. coli* JM109 positive clone was determined by colony PCR. Each clone was suspended in 20 µl of 1 X reaction buffer, 25 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphate, 1 U of *Taq* polymerase (Promega, U.S.A) 20 pM of each primer (M13 universal; 5'-CAG GAA ACA GCT ATG ACC -3' and M13 reversal; 5'- AAC AGC TAT CAG CAT G -3'). PCR condition was as follows: denaturation at 94° C for 5 min, 30 cycles of PCR including denaturation at 94°C for 30s, annealing at 52°C for 20 s, and extension at 72°C for 1 min and the final extension at 72°C for 7 min.

Cloning and sequencing of 16S rRNA gene

The PCR product was purified using QIAquick PCR purification kit (Qiagen, U.S.A) and cloned into pGem-T vector system (Promega, U.S.A). The ligation product was transformed into *E. coli* JM109. Positive clone was screened by colony PCR using the same method previously described. The DNA sequences of positive clones

were determined by using the ABI PRISM 3730 XL sequencer with bigdye terminator version 3.1 (Marcogen Ltd., Korea). To eliminate an error of the sequence, the experiment was repeated to sequence different clones and sequenced both strands. A database search from GenBank was done using BLAST program.

RESULTS

Isolation and screening of bacteriocin-producing LAB

Five hundred colonies of LAB were isolated from chicken intestinal content. CFS were adjusted to pH 6 and used for evaluating the antagonistic activity against Gram-positive and Gram-negative bacteria (Table1). K4 strain was found to be bacteriocin-producing LAB. The results showed that CFS of K4 strain exhibited inhibitory activity against *Lb. sakei* subsp. *sakei* JCM 1157^T, *Leu. mesenteroides* subsp. *mesenteroides* JCM 6124^T, *B. coagulans* JCM 2257^T, *E. feacalis* JCM 5803^T, *L. innocua* ATCC 33090^T and *Br. campestris* NBRC 11547 ^{T}. The highest activity of 6400 AU/ml was found on *Lb. sakei* subsp. *sakei* JCM 1157^T.

Effect of enzyme, pH and heat on antibacterial activity of CFS

The CFS antimicrobial activity of K4 strain was completely destroyed by various proteolytic enzymes as showed in Table 2. The antibacterial sustance was found to be stable at wide pH range of 3-10 and heat stable as the antibacterial activity was retained under heat treatment at 100°C up to 30 min. Moreover, it was heat-tolerant at 121°C for 15 min but antibacterial activity was drastically dropped to half of the previous conditions to 800 AU/ml (Table 2).

Effect of NaCl and pH on growth and production of bacteriocin

K4 strain could grow and produce bacteriocin in wide pH range 3-10 and up to 4 % NaCl in MRS broth. However, the highest growth and bacteriocin production was observed at pH 5.5-7 and in 1-2% NaCl when *Lb. sakei* subsp. *sakei* JCM 1157^T was used at target strain while *B. coagulans* JCM 2257^T was at pH 5-7. It was found that the growth of K4 strain and antibacterial activity became diminished when it was cultivated in MRS containing more than 3% NaCl and having pH of 3 to 4 (Table 3).

Table 2 Effect of enzyme, pH and heat on the antibacterial activity produced by K4 strain against *Lb. sakei* subsp. *sakei* JCM 1157^T .

Treatment	Activity unit (AU/ml)
Enzyme stability	
Control pH 3	1600
Control pH 5.5	1600
Control pH 7	1600
Trypsin	-
Alpha-chymotrypsin	-
Papain	-
Ficin	-
Actinase E	-
Proteinase k	-
Pepsin	-
Protease XIII	-
pH stability	
pH 3	3200
pH 4	3200
pH 5	3200
pH 5.5	3200
pH 6	3200
pH 7	3200
pH 8	3200
pH 9	3200
pH 10	3200
Heat stability	
Control	1600
100°C at 5 min	1600
100°C at 30 min	1600
121°C at 15 min	800

Determination of mode of action

The mode of action of the bacteriocin produced by K4 strain against *Lb. sakei* subsp. *sakei* JCM 1157^T, *B. coagulans* JCM 2257^T and *Br. campestris* NBRC 11547^T are shown in Figure 1. A final concentration of 100 AU/ml of partially purified bacteriocin was added into *Lb. sakei* subsp. *sakei* JCM 1157^{T}and *Br. campestris* NBRC 11547^T. In case of *B. coagulans* JCM 2257^T, 1000 AU/ml was used since 100 AU/ml of bacteriocin did not inhibit its growth in liquid culture in the previous test. The bacteriocin was found to have bactericidal activity against *Lb. sakei* subsp. *sakei* JCM 1157^T and *B. coagulans* JCM 2257^T. The optical density (OD 600 nm; data not shown) and cell number determined by plate count method (10^7 CFU/ml) completely decreased after the culture was exposed to bacteriocin for 6 h when *Lb. sakei* subsp. *sakei* JCM 1157^T was used as indicator strain while it that of *B. coagulans* JCM 2257^{T}was inhibited within 2 h, followed by a regrowth at 6 h and gradually higher in 8 h (Figure 1). However, mode of action of partial purified bacteriocin has exhibited bacteriostatic activity against *Br. campestris* NBRC 11547 ^{T} in which initial inoculation of 10^6 CFU/ml and OD 600 nm approached the steady state after 4 h whereas the control strains increased their number at the same period.

Identification of bacteriocin-producing strains

K4 strain was short rod in shape, Gram-positive, catalase negative and did not produce gas from glucose. It could grow at temperature ranging from 30°C to 45 °C and at pH 4.5 and 9.6 but did not grow in 6.5% NaCl. Based on Bergey's manual (Kandler and Weiss, 1986), K4 strain was classified as genus *Lactobacillus* (Table 4). Its carbohydrate fermentation patterns indicated that K4 strain was able to ferment galactose, D-glucose, D-fructose, D-mannose, Mannitol, Sorbitol, N-acetyl-glucosamine, arbutine, esculine, salicine, maltose, lactose, melibiose, saccharose, trehalose, D-raffinose and D-arabitol. Comparison with API database indicated that K4 strain was 99% similar to *Lb. salivarius* with 99%. To confirm its identity, 16S rRNA gene sequence was investigated K4 with universal bacterial primers 8f and 1510r by PCR amplification. About 1500 bp of amplicon from K4 strain was 99% similar to that of *Lb. salivarius* (accession no. AF 420311). As a result, K4 strain was conclusively identified as *Lb. salivarius* K4.

Table 3 Effect of NaCl and pH on bacteriocin production of K4 strain by using *Lb. sakei* subsp. *sakei* JCM 1157^T and *B. coagulans* JCM 2257^T as an indicator strain.

Treatment	OD600nm	pH of culture	*Lb. sakei* AU/ml	*B. coagulans* AU/ml
pH 3	0.27	3.1	400	0
pH 4	1.45	4	800	100
pH 5	2.68	4.5	3200	800
pH 6	3.80	4.5	6400	800
pH 7	4.87	4.5	6400	800
pH 8	4.65	4.5	3200	400
pH 9	4.41	4.8	3200	400
pH 10	4.32	4.9	3200	400
1%NaCl	4.38	4.3	1600	400
2%NaCl	2.96	4.5	1600	400
3%NaCl	2.20	4.5	800	400
4%NaCl	0.66	5.3	400	200

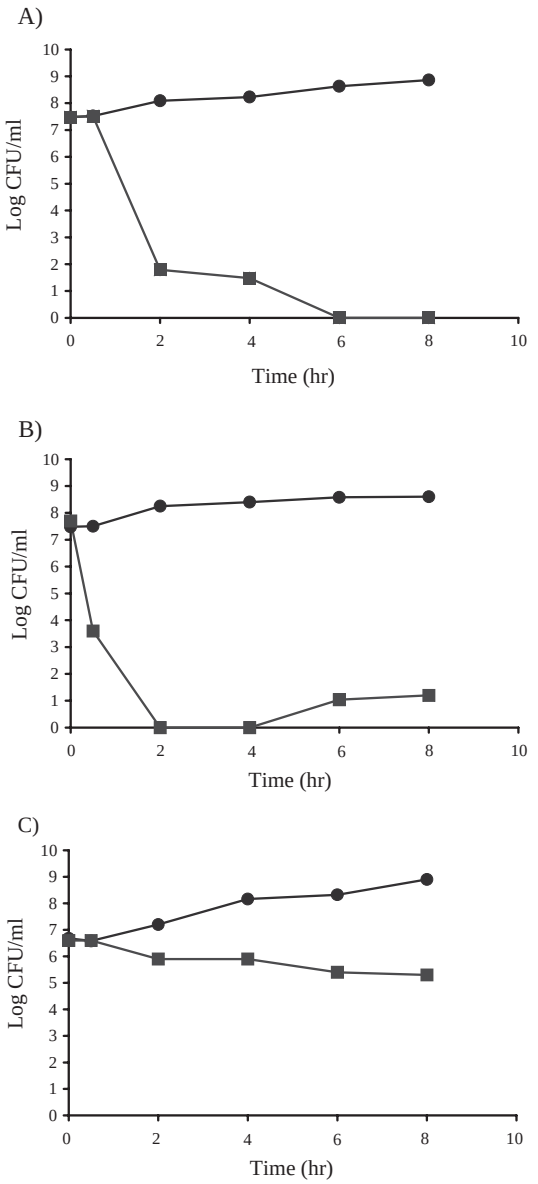

Figure 1 Mode of action of partially purified bacteriocin produced by K4 strain against A) *Lb. sakei* subsp. *sakei* JCM 1157^T B) *B. coagulans* JCM 2257^T C) *Br. campestris* NBRC 11547 ^{T} ■ Indicator strain culture with crude bacteriocin ● Indicator strain culture without crude bacteriocin.

Table 4 Characteristics of K4 strain.

Test	Results
Gram's strain	Positive
Morphology	Short rod
Catalase	Negative
Gas from glucose	-
Growth at 5 °C-15 °C	-
30°C	+
45°C	+
50°C	-
1 %-4 % NaCl	+
6.5% NaCl	-
18% NaCl	-
pH 4.5	+
pH 9.6	+

DISCUSSION

Lactic acid bacteria (LAB) were isolated from chicken intestinal content and screened for their ability to produce bacteriocin. K4 strain was found to produce bacteriocin and was identified as *Lb. salivarius.* This strain showed antibacterial spectrum against only Gram-positive bacteria. *Lb. salivarius* UCC118, on the other hand, exhibited wide spectrum of antibacterial activities. Its CFS displayed antibacterial activity against several Gram-positive bacteria such as *B. subtilis*, *B. cereus, B. thuringiensis, E. faecalis, E. feacium, L. monocytogenes*, *Staphylococcus aureus* and only one strain of Gram-negative, *Pseudomonas fluorescens* (Dunne *et al.*, 1999). Moreover, bacteriocin, Salivaricin B, produced by *Lb. salivarius* M7 was active against several closely related LAB strains, including *Lb. fermentum, Lb. plantarum, Lb. acidophilus* and also other more distantly related Gram-positive bacteria such as *L. monocytogenes L2, Streptococcus feacalis, Staphylococcus epidermis* and *Staphylococcus aureus* (Cataloluk and Gurakan, 2003). The inhibitory activity of K4 strain was inactivated by proteolytic enzymes, indicating that it has

proteinaceous structure of bacteriocin (Klaenhammer, 1988; Vaughan *et al*., 2001). Bacteriocin produced by K4 strain was stable at wide pH range of 3-10 and was heat tolerant. The stability was similar to other bacteriocin-producing *Lb. salivarius* (Cataloluk and Gurakan, 2003). Heat stability is another major feature of low molecular-weight bacteriocins. These properties would be useful for food industrial processing under pasteurization condition. It was observed that most supernatants of bacteriocin-producing strains are resistant to autoclaving conditions and heat treatment (100-121°C). However, some bacteriocins produced by *Lactobacillus* strains (helveticin J) were inactivated by 10-15 min treatment of 60-100°C (Oscáriz and Pisabarro, 2001).

Mode of action of the bacteriocin produced by *Lb. salivarius* K4 was clearly bactericidal against *Lb. sakei* subsp. *sakei* JCM 1157^T and *B. coagulans* JCM 2257^T whereas it exhibited bacteriostatic action against *Br. campestris* NBRC 11547^T. Cataloluk and Gurakan (2003) reported that killing sensitive cells by Salivaricin B could be done in a few minutes for all LAB strains and *L. monocytogenes*. The threshold concentration against LAB strains and *L. monocytogenes* was 50 AU/ml, while over 5000 AU/ml and 300 AU/ml were needed for *Staphylococcus aureus* and *Streptococcus feacalis*, respectively.

It has been reported that *Lb. salivarius* was found as normal flora in chicken intestine (Sarra *et al*., 1992). Several studies have been conducted to evaluate potential of some strains of *Lb. salivarius* for use as probiotic. *Lb. salivarius* A23 was isolated from chicken intestine and showed the ability to colonize the chicken intestinal tract. It was also found to adhere in chicken crop that influenced the indigenous microflora with a reduction of coliforms, indicating its probiotic property (Zacconi *et al*., 1999). Other authors reported that *Salmonella enteritidis* could be completely eliminate from birds after 21 days when *Lb. salivarius* CTC2197 was assessed as probiotic in poultry (Pascual *et al*., 1999). Therefore, these findings suggest the probiotic potential of this K4 strain, and hence deserve further investigation.

CONCLUSION

One out of 500 LAB strains, K4, possessed bacteriocin which showed bactericidal activity against indicator bacterial strains. This bacteriocin was proteinaceous structure with heat tolerant (100-121°C) and its activity was maintained at wide range of pH 3-10. The morphology, biochemical characteristics and 16S rDNA nucleotide sequence of K4 strain was identified and conclusively indicated it as *Lactobacillus salivarius*.

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