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Study for Probiotic Properties of *Lactobacillus salivarius* KL-D4 Isolated from Duck Intestine

Rumjuankiat, K.[1], Ngamyeesoon, N.[2], Swetwiwathana, A.[3] and Pilasombut, K.[4*]

[1]Faculty of Biotechnology, College of Agriculture Innovation Biotechnology and Food, Rangsit University, LakHok, MueangPathumThani District, PathumThani 12000, Thailand; [2]Division of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, 10520, Thailand.; [3]Division of Fermentation Technology, Faculty of Agro-Industry, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand; [4]Department of Animal Production Technology and Fisheries, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, 10520, Thailand.

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Abstract *Lactobacillus salivarius* KL-D4 isolated from duck intestine, could produce bacteriocin against several pathogenic and spoilage bacteria. The study aimed to evaluate the probiotic potential of *L.salivarius* KL-D4 as preliminary study for probiotic properties. The survival of *L.salivarius* KL-D4 was performed in 1-5%NaCl concentration, pH range of 2-10 and bile salt concentrations at 0.3%, 0.6% and 0.9%. The survival rate of this strain was determined at pH 2, 3, 4 and 7 in simulated gastrointestinal tract. Furthermore, the susceptibility to antibiotic was also investigated. It was found that this strain could tolerate to NaCl (1-5%), pH 3-10 and bile salt (0.3-0.9%). Additionally, it was able to survive in simulated gastric juice and showed the survival rate approximately 42%, 85% and 98% at pH 3, 4 and 7, respectively. The tolerance of *L. salivarius* KL-D4 in intestinal fluids at pH 3, 4 and 7 with a viability count about log 2.0, 4.8 and 6.8 CFU/ml, respectively. Moreover, this strain was also sensitive to ampicillins, chloramphenicol, cephalothin, erythomycin, nitrofurantoin, sulfamethoxazole/trimethoprim, penicillin G and amoxicillin. Therefore, *L. salivarius* KL-D4 can be probiotic alternative in the future.

Keywords: *Lactobacillus salivarius*, Probiotic, Duck Intestine

Introduction

Lactic acid bacteria (LAB) are defined as a cluster of lactic acid producing, non spore forming, low G+C content and Gram-positive bacteria (Gómez *et al*., 2016). They can produce antibacterial substance and are considered as generally recognized as safe (GRAS), resulting in a great

[*]**Coressponding author:** Pilasombut, K.; **Email:** komkhae.pi@kmitl.ac.th.

biotechnological potential in the food industry (Alvarez-Sieiro*et al.,* 2016). LAB is widely used as starter culture including cheeses, yoghurts, fermented milk (Hoque*et al.,* 2010), Chinese fermented cabbage (Zhao *et al.,* 2016), kimchi (Jung *et al.,* 2012), fermented oyster mushrooms (Liu *et al.*, 2016) and Thai traditional fermented sausage such as Nham (Kingcha*et al.,* 2012, Swetwiwathana and Visessanguan, 2015), Plaa-som (Saithong*et al.,* 2010) and Som-fug (Kongkiattikajorn, 2015). Furthermore, LAB can be used as biopreservative for creamy filling (Therdtatha*et al.,* 2016) or used as sanitizer (Rumjuankiat*et al.,* 2017). In addition, LAB are commonly studied as probiotic for the past few decades because they are desirable microflora of the gastrointestinal tract (Angmo *et al.*, 2016).

Probiotics are living microorganisms that provide health benefits to host (FAO/WHO, 2014). Generally, probiotics should possess some properties such as contributing colonization resistance, supporting the intestinal barrier including tight junction expression or secretion of mucus, instructing the intestinal microbiota organization and activity (Gómez *et al.*, 2016). Thus, the functional requirements of probiotics is necessary to investigate such as tolerance to acid, resistance to gastric acidity and bile salts or production of antimicrobial compound that comprised lactic acid, acetic acid, hydrogen peroxide or bacteriocins(Shehata *et al.*, 2016).

The genus *Lactobacillus* belongs to the large group of LAB and widely used in food both as starters in fermented food and probiotics (Arena *et al.*, 2016). Among lactobacilli, *Lactobacillus salivarius* have been extensively used for probiotic. It is a promising probiotic usually isolated fromhuman feces (Martín *et al.*, 2006, Tinrat *et al.*, 2011), human milk (Langa*et al.,* 2012), mammalian digestive tract (Neville and O'Toole, 2010), or avian gastrointestinal tracts (Kergourlay *et al.*, 2012, Nouri *et al.*, 2010). Many of *Lactobacillus salivarius* are producers of unmodified bacteriocins of sub-classes IIa, IIb and IId(O'Shea *et al.*, 2011). Moreover, there are a lot of mechanisms of *Lactobacillus salivarius*enhance intestinal health (Messaoudi *et al.*, 2013).

In previous study, Therdtatha*et al.* (2016) have been report about *Lactobacillus salivarius* KL-D4, which was a new strain isolated from duck intestine, could produce bacteriocin called salivaricin KLD. Its partial purified bacteriocin was tested on the artificial contamination of creamy filling and showed the inhibition against *Bacillus cereus*, *Enterococcus faecalis*, *Pseudomonas stutzeri*, *Staphylococcus* sp. and *Stenotrophomonas*sp. It seems that, salivaricin KLD might be a possible biopreservative for food industry. Therefore, the present study aimed on evaluation of potential bacteriocin-

producing strains, *Lactobacillus salivarius* KL-D4, to investigate various probiotic properties.

Materials and methods

Bacterial strains and growth conditions

Lactobacillus salivarius KL-D4 was cultivated in MRS medium (de Man, Rogosa and Sharpe, Merck, Germany) at 30 °C for 16 h without agitation. Indicator strains used for the antibacterial assay were propagated according to the growth conditions shown in Table 1.

Table 1. List of indicator strains and their growth conditions

Indicator strains	Medium	Temperature (°C)
Lactic acid bacteria		
*Lactobacillussakei*subsp. *sakei*JCM 1157^T	MRS	30
Pediococcusdextrinicus JCM 5887^T	MRS	30
Lactobacillus plantarum ATCC 14917	MRS	30
*Enterococcus faecium*JCM 5804^T	MRS	30
Other Gram-positive bacteria		
Listeria innocua ATCC 33090^T	TSB-YE	37
Bacillus coagulans JCM 2257 ^{T}	TSB-YE*	37
Bacillus subtilis subsp. *subtilis*JCM 1465^T	TSB-YE*	37
Gram-negative bacteria		
*Salmonella enterica*serovarEnteritidis DMST 17368	TSB-YE*	37
*Salmonella*Typhimurium TISTR 292	TSB-YE*	37
Pseudomonas fluorescens TISTR 358	TSB-YE*	26
Pseudomonas fluorescens JCM 5963^T	TSB-YE*	26
Escherichia coli O157:H7	NB*	37

ATCC, American Type Culture Collection, Rockville, Md, USA; JCM, Japan Collection of Microoganisms, Wako, Japan; TISTR, *Thailand Institute of Scientific and Technological Research*; MRS, De Man, Rogosa and Sharpe broth (Merck, Germany); TSB-YE, Tryptic Soy Broth (Merck, Germany) containing 0.6% Yeast extract (Merck, Germany); NB, Nutrient broth (Merck, Germany)

* Growth under agitation at 200 rpm.

Antibacterial activity assay

Bacterial inhibition

The pathogenic and spoilage bacteria inhibition by *L. salivarius* KL-D4 was determined using agar well diffusion assay (Ranganath and Sharmila, 2016). The pathogenic and spoilage bacteria including *Salmonella*

*enterica*serovarEnteritidis DMST 17368, *Salmonella*Typhimurium TISTR 292 and *Escherichia coli* O157:H7 were used as indicators (Table 1). Each indicator strain was lawn MRS agar plate using sterile cotton swab and incubated for 15 min. The culture of *L. salivarius* KL-D4 was centrifuged at 12,000 rpm for 10 min. Subsequently, 50 µl of the cell free supernatant (CFS) was transferred into agar well which was made using sterile cork borer and incubated overnight. The diameter of the inhibition zone was measured.

Antimicrobial inhibition

The antimicrobial substances of *L. salivarius* KL-D4 was determined for its inhibition activities against 9 indicators which were listed in Table 1. MRS agar plate was overlaid with 7 ml of MRS soft agar (1%agar, w/v) mixed with 30 ml of each indicator (approximately 10^6 CFU/ml). After setting, 10 µl of the CFS was spotted onto the surface and incubated overnight at the optimum temperature. The inhibition zone was determined using the spot-on-lawn method according to the modified method of Ennahar *et al*. (2001).

Investigation of L. salivarius KL-D4 tolerance

pH tolerance

This method was modified according to the method of Hoque*et al*. (2010). Two percent of fresh *L. salivarius* KL-D4 was transferred to 10 ml of MRS broth adjusted to different pH at 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 with 0.5 mol l^{-1}HCl or 0.5 mol l^{-1}NaOH, and incubated at 37°C for 18 hr. After incubation, the growth was investigated by observing the turbidity. Double positive sign (++), positive sign (+) and negative sign (-) was indicated for maximum growth, normal growth and no growth, respectively.

NaCl tolerance

The NaCl tolerance was investigated by preparing MRS broth containing various concentrations (0%, 1%, 2%, 3%, 4%, 5%, 6% and 7%) of NaCl. Two percent of fresh *L. salivarius* KL-D4 was inoculated in each test tube and incubated at 37°C for 18 hr. MRS broth without NaCl was used as a control. All samples were incubated at 37°C for 18 hr. The turbidity of the maximum growth were determined as double positive sign (++), normal growth as a positive sign (+) while no growth as a negative sign (-) according to the modified method of Hoque *et al*. (2010).

Bile tolerance

The culture was grown in MRS broth at 30ºC for 18 hr then inoculated into 10 ml of MRS broth contained bile salts (Sigma-Aldrich, St. Louis, MO, USA) at various concentration (0%, 0.3%, 0.6% and 0.9%). All samples were incubated at 30ºC for 18 hr. Subsequently, 100 µl of each sample was spread onto MRS agar and incubated at 30ºC for 18 hr under anaerobic condition. Tolerance to bile salts were assayed by growing culture in MRS plate in duplicate. The method used for testing was similar to the modified method of Bakari *et al*. (2011).

In vitro simulated gastric juice and bile salt solution

The capability of *L. salivarius* KL-D4 to tolerate the simulated gastric juice and bile salt solution was determined as described by Zárate*et al*. (2000). Two percent of *L. salivarius* KL-D4 was inoculated in 100 ml of MRS broth and incubated at 30°C for 16 hr. The culture was centrifuged at 4,000 rpm, 4°C for 10 min and washed in sterile saline buffer (NaCl, 0.9% w/v). Then, dissolved the cell in 100 ml of artificial gastric juice (125mM NaCl, 7mM KCl, 45 mM $NaHCO_3$, 3% pepsin adjusted the final pH to 2, 3, and 4 with HCl). The cell survival was considered in simulated gastric juice by standard plate count. To simulate peristalsis and the normal temperature of human body, the suspension was incubated at 37°C with agitation (200 rpm.) for 30, 60, 90 and 180 min. An aliquot of the suspension was enumerated for survival cell. Subsequently, 50 ml of the suspension was centrifuged at 4,000 rpm, 4°C for 10 min, discarded the supernatant and suspended the pellets in 50 ml of intestinal fluid including 0.1% pancreatin USP (Sigma-Aldrich, St. Louis, MO, USA) and 0.15% bile salts (Sigma-Aldrich, St. Louis, MO, USA) adjusted pH to 8.0 with 5 N NaOH. The suspensions were incubated for 0, 30, 60, and 180 min. The cell survival was calculated equation below:

$$\%\text{bacterial survival} = N_{survival} x\ 100/N_{initial}$$

Where $N_{survival}$ is the number of bacterial survival at 0, 30, 60, 90 and 180 min (log cfu/ml) and $N_{initial}$ is the number of initial cell (log cfu/ml).

Safety assessment

The antibiotic resistance was investigated using agar overlay disc diffusion test according to the modified method of Tulumoglu*et al.* 2013. *L. salivarius* KL-D4was propagated in 10 mL MRS broth at 30 °C overnight.

After incubation, the culture was adjusted to 0.5 McFarland and swabbed onto MRS agar plate. Subsequently, antibiotic discs (Oxoid, England) such as Ampicillins (10 µg), Chloramphenicol (30 µg), Cephalothin (30 µg), Erythomycin (15 µg), Gentamycin(10 µg), Kanamycin (30 µg), Naldixic acid (30 µg), Neomycin (30 µg), Nitrofurantoin (300 µg), Norfloxacin (10 µg), Novabincin (5 µg), Oxolinicacid (2 µg), Tetracyclin (30 µg), Sulfamethoxazole/Trimethoprim (25 µg/1.5 µg)andOxytetracyclin (30 µg) were placed on the swabbed plates with sterile conditions. After 24 h incubation at 30 °C, the diameter of inhibition zone was measured for its susceptibility. The interpretation were designated by the CLSI (Ferraro, 2001).

Results

Inhibition spectra of CFS produced by L. salivarius KL-D4

The antimicrobial substance of *L. salivarius* KL-D4 were determined against three pathogenic bacteria, five food spoilage and related four LABas shown in Table 2.The CFS of*L. salivarius* KL-D4 against both potential Gram-positive and Gram-negative bacteria. Especially, *Salmonella enterica*serovarEnteritidis DMST 17368, *Salmonella*Typhimurium TISTR 292 and *Escherichia coli* O157:H7, which were Gram-negative bacteria pathogens, were shown with inhibition zone approximately 15, 19 and 16 mm, respectively. The most sensitive strains to KL-D4 was*L. sakei* subsp. *sakei* JCM 1157^T,whereas*P. dextrinicus* JCM 5887^T was the most resistant.

Table 2. Antimicrobial spectrum of *L. salivarius* KL-D4 against pathogenic bacteria, food spoilage and related LAB

Indicator strains	Inhibition zone (mm)
*Lactobacillussakei*subsp. *sakei*JCM 1157^T	25
Pediococcusdextrinicus JCM 5887^T	6
Lactobacillus plantarum ATCC 14917	13
*Enterococcus faecium*JCM 5804^T	12
Listeria innocua ATCC 33090^T	22
Bacillus coagulans JCM 2257 ^{T}	12
Bacillus subtilis subsp. *subtilis*JCM 1465^T	20
*Salmonella enterica*serovarEnteritidis DMST 17368	15
*Salmonella*Typhimurium TISTR 292	19
Pseudomonas fluorescens TISTR 358	16
Pseudomonas fluorescens JCM 5963^T	17
Escherichia coli O157:H7	16

pH, NaCl and bile tolerance

The effect of various pH, NaCl and bile salt concentrations on the viability of *L. salivarius* KL-D4 was shown in Table 3. This strain could survive in wide range conditions of pH (3-10), NaCl (1%-5%) and bile salt (0.3%-0.9%). Moreover, *L. salivarius* KL-D4 well growth at pH 6-8, 1%-2% of NaCl and 0.3% bile salt concentrations compared with control sample.

Table 3. The viability of *L. salivarius* KL-D4 under various pH, NaCl and bile salt concentrations.

Treatments	Growth level
pH value	
2.0	-
3.0	+
4.0	+
5.0	+
6.0	++
7.0	++
8.0	++
9.0	+
10.0	+
NaCl concentration	
0%	++
1%	++
2%	++
3%	+
4%	+
5%	+
6%	-
7%	-
Bile salt concentration	
0%	++
0.3%	++
0.6%	+
0.9%	+

Legend: ++, maximum growth; +, normal growth; -, no growth

Survival of bacteria in simulated gastric digestion

The survival of *L. salivarius* KL-D4 in artificial gastric and intestinal fluid was evaluated. This strain could not survive in simulated gastric at pH 2 after inoculation, on the contrary it could survive for 180 min at pH 3, 4 and 7, with a viability count about log 3.7, 6.7 and 7.8 CFU/ml, respectively, as shown

in Figure 1A. Furthermore, it showed a survival rate approximately 42%, 85% and 98% at pH 3, 4 and 7, respectively. In addition, *L. salivarius* KL-D4 tolerated to intestinal fluids at pH 3, 4 and 7 with a viability count about log 2.0, 4.8 and 6.8 CFU/ml, respectively. After 180 min, the viability count of *L. salivarius* KL-D4 in intestinal fluid decreased from a various viability count in gastric digestion to log 2, 4.8 and 6.8 CFU/ml and the survival rate indicated about 25%, 60.8% and 84.9% at pH 3, 4 and 7, respectively (Figure 1B).

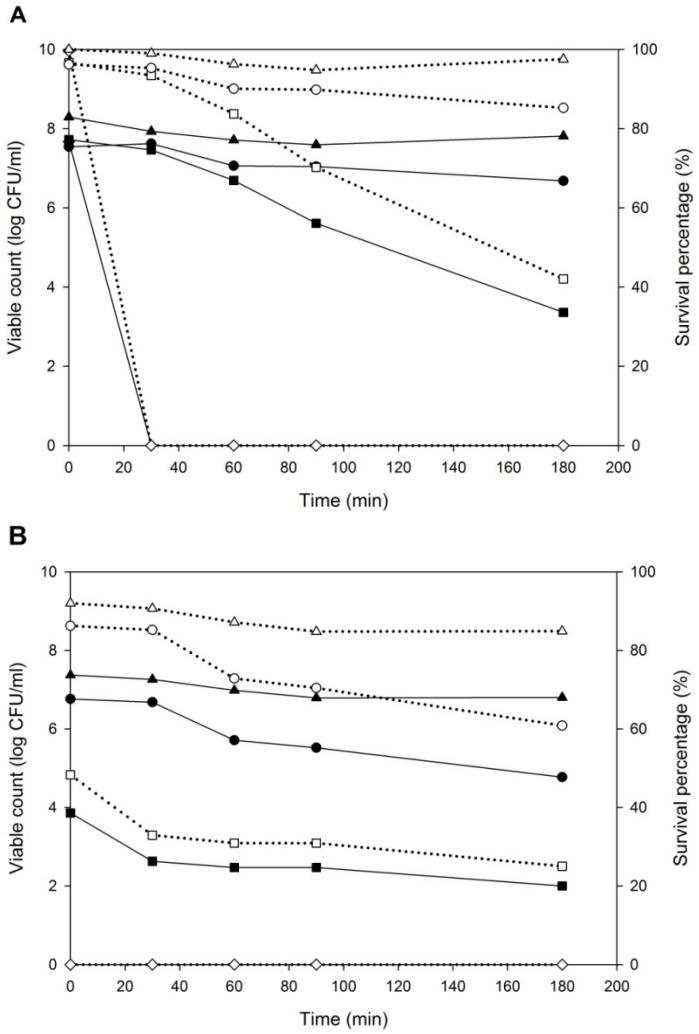

Figure 1. The viable count (log cfu/ml) and survival percentage (%) of *L. salivarius* KL-D4 determined by (A) simulated gastric and (B) intestinal fluid at 0, 30, 60, 90 and 180 min. The viable counts was evaluated at pH 2 (—◆—), pH 3 (—■—), pH 4 (—●—) and pH 7 (—▲—). The survival percentage was investigated at pH 2 (···◇···), pH 3 (···□···), pH 4 (···○···) and pH 7 (···△···).

Antibiotic resistance

Antibiotic susceptibility of *L. salivarius*KL-D4 was investigated according to the anti-microbial drug sensitivity standard of CLSI criteria. There were 18 antibiotics for the sensitivity test as shown in Table 4. *L. salivarius*KL-D4 was generally susceptable to 8 antibiotics including ampicillins, chloramphenicol, cephalothin, erythomycin, nitrofurantoin, sulfamethoxazole/trimethoprim, penicillin G and amoxycillin. On the other hand, *L. salivarius*KL-D4 could resist to tetracyclin, especially gentamycin, kanamycin, naldixic acid, neomycin, norfloxacin, oxolinic acid, oxytetracyclin and streptomycin were extremely resisted by this strain.

Table 4, Antimicrobial susceptibility of *L. salivarius* KL-D4

Antimicrobial agents	Disk content (µg)	Zone diameter (mm)	Acceptable inhibitory
Ampicillins	10	30	S
Chloramphenicol	30	29	S
Cephalothin	30	30	S
Erythomycin	15	25	S
Gentamycin	10	0	R
Kanamycin	30	0	R
Naldixic acid	30	0	R
Neomycin	30	0	R
Nitrofurantoin	300	19	S
Norfloxacin	10	0	R
Novabincin	5	15	I
Oxolinic acid	2	0	R
Tetracyclin	30	9	R
Sulfamethoxazole/Trimethoprim	25/1.5	20	S
Oxytetracyclin	30	0	R
Penicillin G	10	30	S
Amoxycillin	10	30	S
Streptomycin	10	0	R

Discussion

Although many researchers have been reported about the probiotic properties and safety using lactobacilli, it should not ignore the assessment of appropriate *in vitro* assays (Borriello *et al.*, 2003, Messaoudi *et al.*, 2013, Sanders *et al.*, 2010), especially *Lactobacillus sarivarius*. Several *L.sarivarius* strains comprised *L.sarivarius*(Martín *et al.*, 2006), *L.sarivarius*W24(Koninkx *et al.,* 2010), *L. salivarius* C3 (Kirtzalidou *et al.*, 2011) and *L.*

salivarius REN(Wang *et al.*, 2017) were considered as potential probiotics. Not only *L.sarivarius* was proved as probiotics, but some strain of *L.sarivarius* also can produce bacteriocin such as *L. salivarius* K7 (Narakaew *et al.*, 2010, Pilasombut *et al.*, 2006), *L. salivarius* subsp. *salivarius* UCC118 (Flynn *et al.*, 2007), *L. salivarius* DPC6005 (Walsh *et al.*, 2008) or *L. salivarius* SMXD51 (Saint-Cyr *et al.*, 2017). In previous study, *L. salivarius* KL-D4 was confirmed that it could produce bacteriocin and consequently showed the activity of microbial inhibition (Therdtatha *et al.*, 2016). However, bacteriocin of *L. salivarius* KL-D4, which was neutralized, could not inhibit *E. coli* O157:H7 while its CFS without pH adjustment showed the inhibition of this pathogenic strain in this experiment. Therefore, our results demonstrated that *L. salivarius* KL-D4 may have good probiotic because it against both Gram positive and Gram negative bacteria. Moreover, *L. salivarius* KL-D4 could inhibit all tested pathogenic bacteria, especially *Escherichia coli*, *Salmonella*Typhimurium or *Staphylococcus aureus*. Thesepathogenicbacteriawere occasionally found in gastrointestinal tract and might cause gastroenteritis (Klayraung *et al.*, 2008).

NaCl is an inhibitory substance because it could affect physiology, enzymatic, water activity and metabolism of the bacterial cell when it was cultivated in a high salt concentration (Ibourahema *et al.*, 2008, Liu *et al.*, 1998, Menconi *et al.*, 2014). LAB strains with high osmotolerance would be a requirement strain (Ibourahema *et al.*, 2008) because when LAB produced lactic acid, alkali would be pumped into the broth to prevent an excessive reduction in pH. Therefore, the free acid would be converted to its salt form and then increasing the osmotic pressure on the bacterial cells (Menconi *et al.*, 2014). In our results, *L. salivarius* KL-D4 was able to tolerate NaCl concentration approximately 1-5% and supposed that could be a high osmotolerance strain, thereby resulting in the strain would be desirable as a commercial strain.

pH is a crucial factors that affect bacterial growth. However, some LAB strains can grow in both acidic and alkaline (Hoque *et al.*, 2010). In our experiment, the effect of pH ranging from 2 to 10 on the survival strain of *L. salivarius* KL-D4 was studied. This strain could survive in pH 3-5 and the growth could dramatically rise from pH 6 to pH 8. Subsequently, the growth decreased when it was cultured in pH 9-10. However, the potential probiotics should resist to acid at least pH 3.0 (Fernández*et al.,* 2003, Sahadeva*et al.,* 2011) because bacterial strain have to tolerate for gastric stress and could survive for longer period in food which have a high acid content without larger reduction in humans (Shehata*et al.,* 2016).

Not only the ability of survival cell in low pH condition, but the capability to tolerate the high concentration of bile salt of the upper gastrointestinal tract

is considered as good indicators also(Kizerwetter-Świda and Binek, 2016). Normally, the maximum concentration of bile salt in intestinal tract of healthy men is around 0.3% (Hoque *et al.*, 2010) while the concentration of bile salt in the small intestine is approximately 0.2%-2.0% (Klayraung *et al.*, 2008). In this experiment, the concentrations of 0.3%, 0.6% and 0.9% bile salts were used. Our strain, *L. salivarius* KL-D4, was able to tolerate up to 0.9% of bile salts concentration.

However, the ability of bacterial survival when they pass through the stomach is the first requirement for probiotics (Fernández *et al.*, 2003).The viability and survival rates at pH 2 of*L. salivarius* KL-D4in gastric is generally low (less than 1%) and could not survive in intestinal fluid. The viability and survival rate of *L. salivarius* KL-D4 was gradually decreased from 7.72 to 3.36 log CFU/ml and 93.37% to 42.05% at pH 3 for 180 min, respectively. The viable cell count in intestinal fluid was about 2 log CFU/ml and showed calculated survival rate about 25% at 180 min. These results indicated that there were the viability of cell in simulated gastrointestinal condition. Although *L. salivarius* KL-D4 demonstrated that it could not tolerate to pH 2 in gastric and intestinal fluid, it might be survive when consumed in food or encapsulated using different biopolymeric substances (Ashraf and Smith, 2016, Chávarri *et al.*, 2010).

In our experiment, *L. salivarius* KL-D4 was susceptible to ampicillins, chloramphenicol, cephalothin, erythomycin, nitrofurantoin, sulfamethoxazole/trimethoprim, penicillin G and amoxicillin. Another criterion to be a good source of probiotics is antibiotic susceptibility. This is a crucial point because bacteria, which used as probiotics, may serve as host of antibiotic resistance gene, resulting in transferred to pathogenic bacteria (Klayraung *et al.*, 2008).

In conclusion, *L. salivarius* KL-D4 exhibits broad spectrum inhibition and tolerate to 1-5%NaCl, pH 3-10, 0.3%-0.9% bile salt concentration and also survive in simulated gastric digestion. In addition, this strain sensitive to ampicillins, chloramphenicol, cephalothin, erythomycin, nitrofurantoin, sulfamethoxazole/trimethoprim, penicillin G and amoxycillin. Therefore, *L. salivarius* KL-D4 might be a potential probiotic strains in the future.

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