Preliminary characterization of *Lactobacillus salivarius* K7 for probiotic properties

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Abstract

Lactobacillus salivarius K7 was isolated from chicken intestine. This strain produced bacteriocins against several gram-positive bacteria. Its inhibitory activities and probiotic properties were performed. Strain survival *in vitro* study was demonstrated in pH range of 2, 2.5, 3 and 3.5, concentrations of ox-bile at 0, 3, 6, 9 and 12%, concentrations of bile salts at 0, 0.3, 0.6 and 1%, and also fresh chicken bile in MRS at 3%. Survival of this strain in gastrointestinal tract model at pH 2, 3, 4 and 7 and cell free supernatant (CFS, pH 4) inhibitory effect on indicator strains was determined. Moreover, antibiotic resistant of this strain was examined. The results showed that *Lb. salivarius* K7 survived in bile salt concentration of 0, 0.3, 0.6 and 1.0% at 9.32, 2.57, 1.18 and 0.46 log cfu/ml, respectively. In addition to pH tolerant, *Lb. salivarius* K7 could be able to grow in culture at pH 2.5, 3 and 3.5 with viable cell count of 2.65, 7.11 and 7.23 log cfu/ml, respectively. Survival of *Lb. salivarius* K7 in 3% fresh chicken bile revealed slightly decreased in cell number from 9.29 to 8.03 log cfu/ml after exposed for 24 h, while this culture could tolerate to ox-bile up to 12%. This strain was completely destroyed in the presence of gastric juice at pH 2, however at pH 3 and 4 this strain survived in gastrointestinal tract model for 180 min. Antibiotic resistance properties tests showed that *Lb. salivarius* K7 resisted to Gentamycin, Kanamycin, Naldixic acid, Neomycin, Norfloxacin, Oxolinic acid, Tetracyclin, Oxytatracyclin and Streptomycin. This study indicated that *Lb. salivarius* K7 could be used as probiotic.

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บทคัดย่อ

Lactobacillus salivarius K7 แยกได้จากลำไส้ไก่ สามารถสร้างสารแบคเทอริโอซินไปยับยั้งเชื้อแบคทีเรียแกรมบวกชนิดอื่นๆ หลายชนิด จากคุณสมบัติดังกล่าวจึงได้ทำการศึกษาคุณสมบัติการเป็นโปรไบโอติกของเชื้อดังกล่าว โดยทำการศึกษาในหลอดทดลองถึงการมีชีวิตรอดของเชื้อเมื่ออยู่ในอาหารเลี้ยงเชื้อที่มีค่าความเป็นกรดด่าง 2, 2.5, 3 และ 3.5 ox-bile ความเข้มข้น 0, 3, 6, 9 และ 12 เปอร์เซ็นต์ bile salts ความเข้มข้น 0, 0.3, 0.6 และ 1 เปอร์เซ็นต์ และน้ำดีไก่สดความเข้มข้น 3 เปอร์เซ็นต์ในอาหารเหลว MRS ทดสอบการมีชีวิตรอดของเชื้อในระบบทางเดินอาหารจำลองในน้ำย่อยของกระเพาะที่ค่าความเป็นกรดด่าง 2, 3, 4 และ 7 และความสามารถในการยับยั้งเชื้อแบคทีเรียทดสอบ โดยใช้น้ำเลี้ยงเชื้อส่วนใส (ค่าความเป็นกรดด่าง 4) ผลการทดลองพบว่า *Lb. salivarius* K7 มีชีวิตรอดใน bile salts ความเข้มข้น 0, 0.3, 0.6 และ 1 เปอร์เซ็นต์ โดยมีชีวิตเหลือรอด 9.32, 2.57, 1.18 และ 0.46 log cfu/ml ตามลำดับ สามารถทนและเจริญในอาหารที่มีค่า pH 2.5, 3 และ 3.5 โดยมีจำนวน 2.65, 7.11 และ 7.23 log cfu/ml ตามลำดับ *Lb. salivarius* K7 มีจำนวนลดลงเล็กน้อยเมื่อเลี้ยงในอาหารที่มีน้ำดีไก่สด 3 เปอร์เซ็นต์โดยมีจำนวนลดลงจากเชื้อเริ่มต้น 9.29 เหลือ 8.03 log cfu/ml เมื่อเวลาผ่านไป 24 ชั่วโมง ในขณะที่เชื้อสามารถทนใน ox-bile ความเข้มข้น 12 เปอร์เซ็นต์ เชื้อไม่สามารถมีชีวิตรอดเมื่ออยู่ในกระเพาะจำลองที่มีค่าความเป็นกรดด่าง 2 อย่างไรก็ตามพบว่า เชื้อสามารถมีชีวิตรอดได้เมื่ออยู่ในกระเพาะจำลองที่มีค่าความเป็นกรดด่าง 3 และ 4 เป็นเวลา 180 นาที การทดสอบความต้านทานต่อยาปฏิชีวนะพบว่า *Lb. salivarius* K7 สามารถต้านทานต่อยาปฏิชีวนะ Gentamycin, Kanamycin, Naldixic acid, Neomycin, Norfloxacin, Oxolinic acid, Tetracyclin, Oxytatracyclin และ Streptomycin การศึกษาในครั้งนี้ชี้ให้เห็นว่าเชื้อ *Lb. salivarius* K7 สามารถนำไปใช้เป็นโปรไบโอติกได้

คำสำคัญ: ระบบทางเดินอาหารจำลอง *Lactobacillus salivarius* โปรไบโอติก

Keywords: gastrointestinal tract model, *Lactobacillus salivarius*, probiotic.

Introduction

Lactic acid bacteria are regarded as a major group of probiotic bacteria (Collins et al., 1998). The probiotic concept has been defined as a live micro-organisms that when administered in adequate amounts confer a health benefit on the host (Guarner et al., 2005). Several beneficial functions have been suggested for probiotic bacteria such as inhibition of intestinal pathogenic bacteria by production of organic acids and pH reduction, prevention of pathogens adherence to the intestinal mucosa, production of bacteriocins and immune stimulation (Vassu et al., 2001). The probiotic bacteria properties must be able to colonize in gastrointestinal tract, survive on low pH of the stomach and bile acids in the intestine, and compete against other microorganisms in the gastrointestinal tract (Erkkilä and Petäjä, 2000). *Lb. salivarius* is a species frequently found in the intestinal tract of various mammals (Slover, 2008). It could produce bacteriocins that inhibited pathogens and could play an important role of the gastrointestinal tract ecology (Robredo and Torres, 2000). *Lb. salivarius* offers promising possibilities as a probiotic, because of their ability to inhibit growth of *Salmonella enteritidis* and *E. coli*, their high adhesion to intestinal mucosal, and their resistance to bile salts and acidic conditions (Garriga et al., 1998).

Lb. salivarius K7 was isolated from chicken intestine. It produced bacteriocin Abp 118 beta which showed inhibitory activity against *Lb. sakei* subsp. *sakei* JCM 1157^T, *L. mesenteroides* subsp. *mesenteroides* JCM 6124^T and *B. coagulans* JCM 2257^T (Pilasombut et al., 2006). Therefore, the study of *Lb. salivarius* K7 strain properties as probiotic agent was carried on.

Materials and Methods

Bacterial strain and growth conditions

Lb. salivarius K7 was propagated in MRS broth (de Man Rogosa and Sharp; Merck, Germany) at 37°C for 16 hr under anaerobic condition for optimum growth as previous studied (Pilasombut, 2006). List of other bacterial strains, media and their growth condition was shown in Table 1.

Detection of antibacterial activity

The antibacterial activity was carried out by spot-on-lawn method (Ennahar et al., 1999). Cell-free supernatant (CFS, pH 4) was obtained by centrifugation at 4000 x g for 20 min and then sterilized by filter (0.2 µm, Pall, U.S.A.). Antibacterial activities were test by spotting 10 µl of CFS onto the surface of agar plate which was overlaid with 5 ml of soft agar (0.8-1% w/v) seeded with 10 µl of freshly grown indicator strains (about 10^7 cfu/ml). After overnight incubation at proper temperature as shown in Table 1, inhibition zone was observed.

Determination of acid tolerance

A modified method of Hyronimus et al., (2000) was applied in this study. A suspension of overnight culture of *Lb. salivarius* K7 (2% v/v) was mixed with MRS broth pH 2.0, 2.5, 3.0 and 3.5. After each mixture was incubated at 37°C for 16 hr, viable cell count was determined by plating serial dilution on MRS agar containing 0.5% $CaCO_3$. These plates were then incubated at 37°C in an anaerobic atmosphere for 48 hr. The survival rate was expressed as the percentage of bacterial cell count (log cfu/ml) at initial compared to those at final after 16 hr incubation. Each experiment was performed in triplicate.

Determination of bile salt and ox-bile tolerance

This test was done by the methods of Walker and Gilliland (1993) with some modification. The overnight culture (2% v/v) was inoculated to MRS broth containing 0, 0.3, 0.6 or 1% (w/v) bile salts (Sigma, New Zealand) and 0, 3, 6, 9 or 12% (w/v) ox-bile (Fluka, Switzerland). The mixtures were incubated at 37°C under anaerobic condition for 16 hr. The procedure of cell count determination was similar to those described for the acid tolerance test. The property of bile tolerance was compared as cell survival percentage of the bacterial count in MRS broth with bile to those without bile after 16 hr incubation. Each experiment was performed in triplicate.

Determination of fresh chicken bile tolerance

The method of Gilliland et al., (1984) with some modification was used to determine fresh chicken bile tolerance. 2% (v/v) of the overnight culture with was inoculated to MRS broth containing 3% fresh chicken bile (w/v). Subsequently, the cultures were incubated at 37°C for 0, 0.5, 1, 1.5, 2, 3, 6 and 24 hr. Growths in control treatment (without bile) and tested culture (3% fresh chicken bile) were monitored by measuring absorbance at 600 nm using a spectrophotometer. Investigation of cell survival was determined as described in the acid tolerance test.

Determination of artificial gastric and intestinal fluids tolerance

Simulated gastric digestion was tested essentially as described in Zárate et al., (2000). *Lb. salivarius* K7 was inoculated in 100 ml of MRS broth at 2% (v/v) and incubated at 37°C for 16 hr. After washing in sterile saline solution (0.9% NaCl) and centrifugation, the cell suspension was added to 100 ml of artificial gastric juice with the following composition: NaCl 125 mM, KCl 7 mM, $NaHCO_3$ 45 mM, and pepsin (Fluka, Switzerland) 3 g /l. The final pH was adjusted with HCl solution to pH 2, 3, 4, and 7. The bacterial suspension was agitated at 200 rpm/min to simulate peristalsis. Aliquots were taken for enumeration of viable at 0, 30, 60, 90 and 180 min by spread plate technique with MRS agar containing 0.5% $CaCO_3$. Simulated intestinal fluid was prepared by suspending the cells (after 180 min of gastric digestion) in 0.1% (w/v) pancreatin (Fluka, Switzerland) and 0.15 % (w/v) bile salts (Sigma, New Zealand) in water and adjusted it to pH 8.0 with 1 N NaOH solution. The suspension was incubated as described above and samples for total viable counts were taken at 0, 30, 60, 90 and 180 min using spread plate technique with MRS agar containing 0.5% $CaCO_3$. The experiment was performed in triplicate and mean were calculated.

Antibiotic resistance test

The antibiotic resistance tests used in this study was done according to the agar disc diffusion method by the National Committee for Clinical Laboratory Standards (NCCLS, 1990). The list of antibiotic (Oxoid, England) used was shown in Table 3. *Lb. salivarius* K7 culture was transferred to 5 ml of MRS broth and incubated at 37°C until turbidity of 0.5 MacFarland standard appeared. The swabs of bacterial suspension were streaked in three directions over entire surface of each agar plate, later different antibiotic discs were placed on agar and anaerobic incubated at 37°C for 16 hr. The diameters of inhibition zones were measured after incubation, and compared with those in interpretative standard chart (NCCLS. 1990). The results were reported as resistance (R), intermediate (I) and susceptible (S).

Results and Discussion

Antimicrobial activity

The study revealed that CFS of *Lb. salivarius* K7 could inhibit *B. coagulans* JCM 2257^T, *B. coagulans* TISTR 1447, *L. innocua* ATCC 33090^T, *Br. campestris* NBRC 11547^T, *A. hydrophila* TISTR 1321 and *S. typhimurium* TISTR 292 (Table 1). Interestingly, it was noticed that this strain showed inhibition against both gram positive and gram negative bacteria.

Lactic acid bacteria (LAB) could produce various antibacterial compounds, such as organic acids, hydrogen peroxide, diacetyl and bacteriocins (Holzapfel and Wood, 1995). These antimicrobial substances could inhibit the growth of other microorganisms (Huot et al., 1996). The result was similar to Pilasombut et al., (2005) who reported that cell free supernatant of isolates J9-2, J6-1, D2-6, D3-9, D1-8, D7-3, D2-8, I4-9, I4-8, C4-4 and C3-3 which were isolated from chicken intestine showed inhibitory activity against *S.* Enteritidis DMST 17368.

Table 1. List of indicator strains, their growth condition and antibacterial activity of *Lb. salivarius* K7 against indicator strains

Indicator strains	Media	Temperature (˚C)	Condition	Antibacterial activity K7
Lactic acid bacteria				
Lactobacillus plantarum ATCC 14917^T	MRS	30	anaerobic	-
Lactobacillus sakei subsp. *sakei* JCM 1157^T	MRS	30	anaerobic	-
Lactobacillus sakei TISTR 890	MRS	37	anaerobic	-
Lactococcus cremoris TISTR 1344	MRS	30	anaerobic	-
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 6124^T	MRS	30	anaerobic	-
Leuconostoc mesenteroides TISTR 942	MRS	30	anaerobic	-
Enterococcus feacalis JCM 5803^T	MRS	37	anaerobic	-
Enterococcus feacalis TISTR 888	MRS	37	anaerobic	-
Streptococcus sp. TISTR 1030	MRS	30	aerobic	-
Other gram positive bacteria				
Bacillus coagulans JCM 2257^T	TSB-YE	37	aerobic	+
Bacillus coagulans TISTR 1447	TSB-YE	37	aerobic	+
Listeria innocua ATCC 33090^T	TSB-YE	37	aerobic	+
Brochotrix campestris NBRC 11547^T	TSB-YE	26	aerobic	+
Staphylococcus aureus TISTR 118	TSB-YE	37	aerobic	-
Other gram negative bacteria				
Pseudomonas fluorescens JCM 5963^T	TSB-YE	26	aerobic	-
Pseudomonas fluorescens TISTR 358	TSB-YE	26	aerobic	-
Aeromonas hydrophila TISTR 1321	NB	30	aerobic	+
Salmonella typhimurium TISTR 292	TSB-YE	37	aerobic	+
Escherichia coli TISTR 780	TSB-YE	37	aerobic	-

ATCC = American Type Culture Collection, Rockville. Md
JCM = Japanese culture of Microorganisms, Wako, Japan
NRBC = National Institute of Technology and Evaluation (NITE) Biological Resource Center
TISTR = Thailand Institute of Scientific and Technological Research
K7 = *Lb. salivarius* K7
TSB – YE = Tryptic soy broth + 0.6% Yeast extract (Merck, Germany)
NB = Nutrient broth (Merck, Germany)
MRS = de man Rogosa and Sharpe (Merck, Germany)
\+ = Inhibition zone
\- = Not inhibition

Acid tolerance and bile tolerance

Lb. salivarius K7 was able to withstand in MRS media at pH 2.5, 3 and 3.5 with survival rate at 28.51%, 76.63% and 77.88% respectively (Table 2), whereas, at pH 2 cells could not survive. Moreover, cell also survived in bile salts at 1% and in ox-bile up to 12%. However, increasing of bile salts concentration from 0.3% to 1% resulted in a survival rate decline from 27.58% to 4.94%. On the contrary, increasing of ox-bile concentration from 3% to 12%, the survival rate escalated from 57.80% to 72.19%. Comparisons of *Lb. salivarius* K7 growth rates at 24 hr in the control and in culture with fresh chicken bile at 3% concentration for also revealed that fresh chicken bile had inhibitory effect with initial cells about 7 log cfu/ml and about 8 log cfu/ml in fresh chicken bile, while 9.29 log cfu/ml in control (Figure 1).

Resistance to pH is of great importance in survival and growth of bacteria in the stomach and thus, is a prerequisite property for probiotics. The pH of the gastric juice in chickens can be as low as 0.5-2.0 (Ehrmann et al., 2002). When the stomach is non-fasting, e.g. after meal, the gastric pH is usually raised up to 3.0 or more (Erkkilä and Petäjä, 2000). In this study, the media with pH 2.0 was used to represent the extreme acid condition of human stomach as in the case of fasting period. The media with pH 3.0 used in this study represented the pH of non-fasting gastric condition. After exposition to various pH values for 16 hr, *Lb. salivarius* K7 did not grow at pH 2.0 and exhibited low viability at pH 2.5. This result differed from Ehrmann et al., (2002) who reported that *Lb. salivarius* TMW 1.992 could survive after incubation at pH 1.0 for 1 hr and incubation period of 4 hr at pH 3.0 and pH 2.0. Jin et al., (1998) reported that the time required for feed to pass through the entire alimentary canal of chicken is as short as 2.5 hr. In addition, Erkkilä and Petäjä, (2000) reported that the time required for feed to pass through the stomachin human was 2-4 hr. Thus, these experiments must be confirmed with long-term viability of their presence in the gastrointestinal tract.

The relevant physiological concentration of human bile was range from 0.3% (Dunne et al., 1999) to 0.5% (Zavaglia et al., 1998). *Lb. salivarius* K7 showed a low survival rate at bile salt concentration up to 1%, while at ox-bile and 3% fresh chicken bile exhibited no effect on viable cell of this strain. Ehrmann et al., (2002) reported that in the presence of 7 mM/l taurocholic acid slightly decrease viable cell of *Lb. salivarius* TMW 1.970 and TMW 1.992 was found. The addition of ox gall (2%), on the other hand, inhibited the growth of both strains of *Lb. salivarius.*

Table 2. Survival of *Lb. salivarius* K7 grown in MRS at 37 ˚C for 16 hr under various pH, concentration of bile salts and ox-bile

Treatments	Viable cells (log cfu/ml)	Survival rate (%)
pH value		
control	9.28	100.00
2.0	0.00	0.00
2.5	2.65	28.51
3.0	7.11	76.63
3.5	7.23	77.88
Bile salts concentration		
0%	9.32	100.00
0.3%	2.57	27.58
0.6%	1.18	12.66
1.0%	0.46	4.94
Ox-bile concentration		
0%	9.17	100.00
3%	5.30	57.80
6%	6.30	68.70
9%	6.82	74.37
12%	6.62	72.19

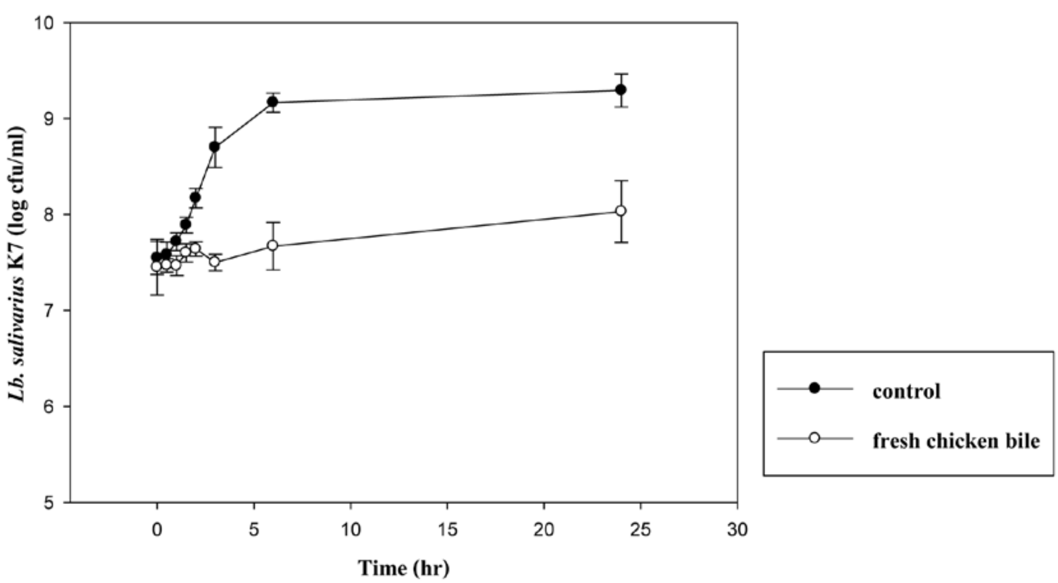

Figure 1. Viability of *Lb. salivarius* K7 in the presence of fresh chicken bile 3%

log cfu K7 control = viable cell count (log cfu/ml) of *Lb. salivarius* K7 in control group

log cfu K7 bile = viable cell count (log cfu/ml) of *Lb. salivarius* K7 in 3% fresh chicken bile

Artificial gastric and intestinal fluids tolerance

The study of *Lb. salivarius* K7 survival in artificial gastric at pH 2, 3, 4, and 7 was demonstrated. The outcome at pH 2 showed no survival after 30 min of exposure. On the other hand, at pH 3, 4 and 7 a viability of *Lb. salivarius* K7 decreased according to prolonged exposure to 180 min (Figure 2). Moreover, the tolerance of this strain against intestinal fluids showed that a viability of *Lb. salivarius* K7 decreased during 180 min incubation (Figure 2).

Survival of microorganisms in the gastrointestinal conditions represents one of the most important criteria for selecting lactic acid bacteria as probiotic. *In vivo*, it is expected that successive digestions exert a stronger effect than either of these alone (Zárate et al., 2000). In this study, both acid and bile stresses were assayed in a sequential way, also simulating the gastrointestinal movement. The result was similar to Gänzle et al., (1999) who reported that *Lb. curvatus* was rapidly killed in the gastric compartment at pH 2.0. Zárate et al., (2000) found that propionibacteria survived at pH 4 in gastric and intestinal digestion, and only *P. acidipropionici* Q4 was sensitive to digestion at pH 3. At pH 2, viable cell counts decreased rapidly during the gastric trail.

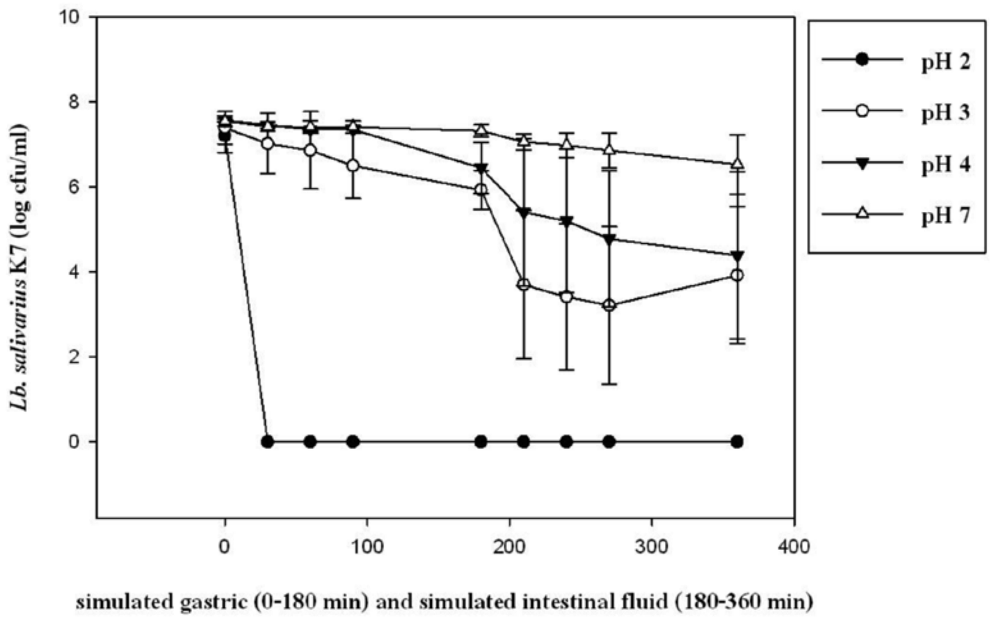

Figure 2. Viability of *Lb. salivarius* K7 in the presence of simulated gastric juice pH 2, 3, 4 and 7 (0-180 min) and simulated intestinal fluid (180-360 min)

Antibiotic resistance

The results in Table 3 revealed that *Lb. salivarius* K7 was resisted to gentamycin, kanamycin, nalidixic acid, neomycin, norfloxacin, oxolinic acid, tetracyclin, oxytatracyclin and streptomycin. However, this strain was susceptible to ampicillins, cephalothin, erythomycin, nitrofurantoin, sulfamethoxazole/trimethoprim, penicillin G and amoxycillin.

Antibiotics used in this study were commonly used in human medicines and in farming practice.

Antibiotic resistance of probiotics was conducted with purpose of examining their ability to survive if they were taken simultaneously with an antibiotic therapy. The result was similar to D'Aimmo, Modesto and Biavati, (2007) that *Lb. acidophilus, Lb. casei, Lb. delbrueckii* subsp. *bulgaricus* were susceptible to ampicillin G, rifampicin and resisted to aztreonam, cycloserin, kanamycin, nalidixic acid, polymycin B and spectinomycin.

Table 3. Antimicrobial susceptibility of *Lb. salivarius* K7

Antibiotic tested	Disk content (μg)	Antimicrobial susceptibility	
		Clear zone diameter (mm)	Acceptable inhibitory
Ampicillins	10	25	S
Chloramphenicol	30	16	I
Cephalothin	30	28	S
Erythomycin	15	26	S
Gentamycin	10	8	R
Kanamycin	30	0	R
Nalidixic acid	30	0	R
Neomycin	30	9	R
Nitrofurantoin	300	26	S
Norfloxacin	10	0	R
Novobiocin	5	15	I
Oxolinic acid	2	0	R
Tetracyclin	30	8	R
Sulfamethoxazole/ Trimethoprim	25/1.5	16	S
Oxytetracyclin	30	8	R
Penicillin G	10	28	S
Amoxycillin	10	26	S
Streptomycin	10	0	R

S = Susceptible: the infection may respond to the treatment at the normal dosage.

I = Intermediate: the result is equivocal and if the bacterium is not fully susceptible to an alternative drug, then the test should be repeated.

R = Resistant: the bacterium is not inhibited by the usually achievable systemic concentrations of the antimicrobial agent and efficacy has not been reliable in clinical studies.

Conclusion

Lb. salivarius K7 was the strain that exhibited acid and bile tolerant as well as strong inhibition of some pathogens and resistance to a wide range of clinical important antibiotics. The result from this study suggested that this bacteriocin producer strain could potentially be applied as probiotic starter for fermented meat products.

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