CHAPTER IV

MATERIALS AND METHODS

1. Lactobacilli

1.1 Collection of the microorganisms

All isolates of *Lactobacillus* were collected from vagina of goats and foods from the former project of Dr. Siriwoot Sookkhee. The microorganisms were recovered in Mann Rogosa Sharpe broth (MRS broth; Merck™; Merck KGaA, Darmstalt, Germany) at 37°C under 5% CO_2 atmosphere for 48 hrs and were isolated on Rogosa SL agar (Rogosa; Difco™; Bacton Dickinson, Sparks, MD.) at 37°C under 5% CO_2 atmosphere for 36 hrs. Each colony was kept in glycerol medium as an aliquot for storing in the -20°C freezer until use. There were totally of 172 isolates recovered from samples on Rogosa agar at the above condition. Six lactobacilli isolates include, *Lactobacillus plantarum* strain L541, *Lactobacillus pentosus* strain LSS, *Lactobacillus rhamnosus* strain L055, *Lactobacillus paracasei* strain L108, *Lactobacillus acidophilus* strain L1034 and *Lactobacillus casei* strain L330 which kindly offered from Emeritus Prof. Dr. Malyn Chulasiri were also carried out to determine the antimicrobial activity in the present study.

1.2 Primary antimicrobial screening

Staphylococcus aureus ATCC 25923, *Sarcina lutea* ATCC 9341, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633 were used to determine the primary antimicrobial activity (131). Each tested strain was separately cultured in Tryptic Soy broth (TSB; Merck™; Merck KGaA) at 37°C for 24 hrs before adjusting a turbidity of the culture equal to McFarland No. 0.5. The suspension was swabbed evently onto Tryptic Soy agar (TSA; Oxoid®; Basingstoke, Hampshire, England). Sterile stainless cup was placed on each plate. Diameter of the cup is 6.0 mm. Each culture of lactobacillus was cultured in MRS broth at 37°C under 5% CO_2 atmosphere for 36 hrs, and then centrifuged at 4,800 rpm at 4°C for 30 min to sediment the cells. Cell - free supernatant was collected into a new tube and filled into the cylinder cup on the swabbed agar before incubated at 37°C for 24 hrs. Among 172 isolates of lactic acid bacteria, 12 isolates which exhibited the largest inhibition zones were recruited for the further screening test.

1.3 Secondary antimicrobial screening

Twelve isolates of the selected *Lactobacillus* which possessed the potent antimicrobial activity against 4 tested indicator strains were carried out to determine the broad spectrum activity. Three standard strains of *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 and 3 laboratory strains of *Proteus vulgaris* strain u201, *Acinetobacter baumannii* strain u46 and *Streptococcus agalactiae* strain u304 and 38 clinical isolates of *E. coli* were used as the tested indicators in the antimicrobial activity assay. TSB and TSA were used as the culture media for *E. coli*, *E. faecalis*, *P. aeruginosa*, *P. vulgaris* and *A. baumannii*

under the aerobic condition for 24 hrs. Brain - Heart Infusion broth (BHI; Bacto™; Becton Dickinson) and Blood agar (BA) were used as the culture media for *S. agalactiae* under the aerobic condition for 24 hrs. The agar cup diffusion assay (132) was performed to determine the antimicrobial activities toward these bacteria on Mueller - Hinton agar (MHA; Merck™; Merck KGaA) plate, except *S. agalactiae* was cultured on BA plate. The preparation of this assay was similar to the agar diffusion assay. Each cell - free supernatant of lactobacillus was freshly prepared and filled into the cylinder cup which placed on the swabbed agar before incubated at 37°C for 24 hrs. Two isolates which exhibited the largest inhibition zones were selected for the further studies and called as the potent antimicrobial isolates.

1.4 Identification of potent antimicrobial isolates

The potent antimicrobial isolates of lactic acid bacteria were identified according to their biochemical profiles by using the API - 50 CHL test kit (API®50 CHL; BioMérieux, Durham, NC). Each isolate was cultured on MRS agar at 37°C under the 5% CO_2 atmosphere for 36 hrs. The colonies were picked up by using a swab and then prepared a heavy suspension in the MRS broth. The inoculum was adjusted the optical turbidity as equal to McFarland Standard No. 2 by transfering a certain number of drops of bacterial suspension into the MRS broth. Two drops of the suspension were inoculated into the API - 50 CHL medium test kit (API®50 CHL Medium; BioMérieux) and the suspension was filled into the biochemical test kit and covered with mineral oil. The culture was incubated at 37°C under aerobic atmosphere for 48 hrs. The result was recorded at 24 and 48 hrs of incubation times and the biochemical profile obtained from each isolate was identified using the

identification software (API®50 CHB/E databases; BioMérieux). The standard strains of lactobacilli, namely, *Lactobacillus casei* TISTR 330 and *Lactobacillus rhamnosus* TISTR 108 that provided by TISTR Culture Collection Bangkok MIRCEN, Thailand Institute of Scientific and Technological Research (TISTR) were tested in parallel.

1.5 Characterization of cell - free supernatants

1.5.1 Preparation of cell - free supernatants

The amount of 10^3 CFU/ml of each isolate was inoculated into MRS broth and cultured at 37°C under CO_2 atmosphere for 36 hrs. The cell - free supernatant was harvested by centrifugation the fresh culture with 4,800 rpm at 4°C for 30 min before transferring to a new tube.

1.5.2 Hydrogen peroxide (H_2O_2) determination (133)

The concentration of hydrogen peroxide in the cell-free supernatant was determined by using Quantitative Peroxide Assay Kit (PeroXOquant™; Pierce, Rockford, IL). Briefly, 200 µl of working reagent were added to 20 µl of the cell - free supernatants in separated well. The reaction was mixed and placed for 15 - 20 min at room temperature. The absorbance was measured by using spectrophotometer at 595 nm. The concentration was calculated according to the hydrogen peroxide standard curve. In these assays, hydroperoxides convert the Fe^{2+} to Fe^{3+} at acidic pH. With the aqueous - compatible formulation, peroxide first reacts with sorbitol, converting it to a peroxyl radical, which in turn initiates Fe^{2+} oxidation to Fe^{3+}. In the lipid compatible formulation, the peroxide converts the Fe^{2+} to Fe^{3+} directly. In a sulfuric acid solution, the Fe^{3+} complexes with the xylenol orange dye to yield a purple product with maximum absorbance at 560 nm. The molar extinction

coefficient of the xylenol orange - Fe^{3+} complex is 1.5 x 10^4 $M^{-1}cm^{-1}$ in 25 mM H_2SO_4 at room temperature. When using microplates, the best wavelength for measurement is 595 nm (best signal - to - noise). The maximum absorbance of the assay components before addition of peroxide is at 430 nm.

1.5.3 The cut - off concentration of H_2O_2

The standard H_2O_2 (PeroXOquant™; Pierce) was prepared in serial 2 - fold dilutions. The antimicrobial activity of the diluted H_2O_2 was tested against *E. coli* ATCC 25922 by agar - cup diffusion assay. The standard curve between the hydrogen peroxide concentration (x - axis) and antimicrobial activity (y - axis) was done. The minimal concentration of H_2O_2 which demonstrated the antimicrobial activity was called as the H_2O_2 cut - off point. If the concentration of H_2O_2 in the cell - free supernatant was more than the cut - off point, the antimicrobial activity of this supernatant may partly be due to the containing H_2O_2.

1.5.4 Lactic acid determination

The concentration of lactic acid in the cell-free supernatant was measured by using Gutmann's method (134). The reagents were prepared as shown in Table 5. Four reagents were mixed. The absorbance was monitored by using spectrophotometer (Synergy™ HT; BioTek Instruments, Winooski, VT.) at 340 nm (A_{340nm}) and recorded the initial absorbance (Ai) of both the test and blank. Afterward, 50 µg/ml of L - lactic dehydrogenase (Roche; Roche Diagnostics; Basel, Switzerland) were added immediately and mixed thoroughly. The absorbances of reactions were monitored by using spectrophotometer at 340 nm and recorded the final absorbance (Af) of both the test and blank. A difference absorbance was calculated as follows:

Different absorbance = Af - Ai

The standard curve of lactic acid concentration was plotted between the different absorbance of standard and its concentration in µg/ml. Lactic acid concentration of each sample was determined by the standard curve.

The kit measures the concentration of lactate dehydrogenase (LDH) using a direct, plate - based, colorimetric reaction. It provides accurate results even in complex liquid measures. When sample is added to the reaction mix, the LDH in the sample converts the lactate and NAD^+ in the mix to pyruvate and NADH. The production of latter product is directly monitored by measuring the increase in absorbance of the reaction at 340 nm over a 5 minute time interval.

1.5.5 The cut - off concentration of lactic acid

The lactic acid standard (Roche; Roche Diagnostics) was prepared in serial 2 - fold dilutions. The antimicrobial activity of the diluted lactic acid standard was tested against *E. coli* ATCC 25922 by using the agar - cup diffusion assay. The standard curve between the hydrogen peroxide concentration (x - axis) and antimicrobial activity (y - axis) was done. The lactic acid cut - off point was defined as the lowest amount of lactic acid that produced a clear inhibition zone. If the concentration of the cell - free supernatant was more than the cut - off point, the antimicrobial activity of this supernatant may partly be due to the containing lactic acid.

Table 5 The composition of reagent for determination of lactic acid

Reagent	Standard test (µl)	Blank (µl)
Deionized Water	35	135
Reagent A (Buffer)	140	140
Reagent B (Lactate)	100	-
Reagent C (β-NAD)	15	15

1.5.6 Total protein determination (135)

Total protein concentration was determined by Qubit® Fluorometer (Invitrogen; Carlsbad, CA.). Working solution was prepared by combining 1 µl Quant - iT Reagent with 199 µL Quant - iT Buffer for the protein sample. The standard solutions No. 1, No. 2 and No. 3 were prepared by mixing 190 µl working solution with 10 µl of the standard solution from the kit. Two µl of the samples was added with 198 µl of the working solution. Concentration of the standard solutions No. 1, No. 2 and No. 3 were 0, 200 and 400 ng/µl in TE buffer with 2 mM sodium azide, respectively. Reaction was carefully mixed by vortex mixer for 2 - 3 seconds to prevent the bubble formation and then incubated for 15 min at room temperature. The reading given by the Qubit fluorometer was recorded. The concentration of the original sample was also calculated by this equipment.

1.6 Characterization of crude bacteriocins

1.6.1 Preparation of cell - free supernatants

The amount of 10^3 CFU/ml of each lactobacillus isolate was inoculated into MRS broth and cultured at 37°C under CO_2 atmosphere for 36 hrs. The cell - free

supernatant was harvested by centrifugation the fresh culture with 4,800 rpm at 4°C for 30 min before transferring to a new tube.

1.6.2 Extraction of crude bacteriocins using ammonium sulphate precipitation (136)

Forty percent (w/v) of AR grade ammonium sulphate (Merck™; Merck KGaA) were separately added into 100 ml of each freshly prepared cell - free supernatant and MRS broth by stirring on magnetic stirrer at 4°C for 1 hr. The precipitant was harvested by centrifuged with 4,200 rpm at 4°C for 30 min. The supernatant was removed. The protein pellet was resuspended with sterilized Phosphate Buffer Saline (PBS) pH. 7.2 in the double volume of the pellet. The dissolved protein was dialyzed with the sterilized PBS pH 7.2 by using the dialysis membrane under the cut off ≤ 7.5 kDa (Snake Skin® Pleated Dialysis Tubing; Pierce Biotechnology, Rockford, IL) at 4°C for 24 hrs. This dialysis was done on the continuous magnetic stirrer. The dialysis buffer was changed every 6 hrs. The dialysed protein was ultrafiltered through the desalting column (HiTrap™ Desalting; Amersham Biosciences AB, Uppsala, Sweden). The desalting protein was eluted with 1 column volume of the sterilized PBS pH 7.2, and then concentrated using the Vivaspin 6 (Vivascience AG; Hannover, Germany) pass through membrane at the cut off ≤ 10 kDa under the condition of 3,000 rpm at 4°C for 30 min. The concentration of protein was determined by Qubit® fluorometer. It was aliquoted and stored at -20°C until use.

1.6.3 Determination of protein pattern with one - dimensional polyacrylamide gel electrophoresis (137)

Five µg of crude proteins from lactobacilli were separately mixed with 6X Loading Dye Solution (MassRuler™; Fermentas, Burlington, ON, Canada) and heated at 95°C for 20 min in the heat box. The denatured protein was loaded into a well of 4% SDS - polyacrylamide gel as the stacking gel and 12% SDS - polyacrylamide gel as the separating gel. The proteins were performed for electrophoresis in the vertical SDS - PAGE chamber (miniVE; Amersham Bioscience AB) under 80 volts constant current condition for stacking gel and 100 volt for separating gel in running buffer pH 8.3 until the dye reached the bottom of the separating gel. The proteins were visualized by silver stain kit (PlusOne™; GE Healthcare, Biosciences AB) (137). The gel was removed from the glass plates and soaked in fixing solution for 60 min. The fixing solution was removed and the gel was placed in sensitizing solution with gentle shaking for 60 min. The gels was rinsed four times in distilled water for 15 min for each time. It was soaked in silver solution with gentle shaking for 60 min and then washed two times in distilled water for 1 min for each wash. The gel was soaked in developing solution until bands were visible approximately 2 to 10 min. It was transferred to the stopping solution and leaved for 60 min. Finally, the gel was soaked in preservation solution for 60 min. It was dried overnight in wrapping cellophane.

2. ESBL producing uropathogenic Enterobacteriaceae and their β - lactamases

2.1 Collection and identification

All isolates of the uropathogenic Enterobacteriaceae were collected from the Microbiology Section, Central Diagnostic Laboratory, Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiangmai University during June to December 2007. The clinical pathogens were isolated from urine samples. They were identified and tested the antimicrobial susceptibility by microbiologists of the Central Diagnostic Laboratory. The resistant isolates were collected and isolated to a single colony on TSA at 37°C for 24 hrs. The isolated colony was cultured in TSB at optimal condition and kept in 20% glycerol medium as an aliquot for storing in the -20°C freezer until use. The freshly cultures on TSA were carried out to identify.

2.1.1 Gram staining

The morphology of microorganisms was studied by Hucker's modification of gram - staining (138). Sterile water was dropped on the slide. An aliquot of isolated colony was picked and then smeared with a sterile wire needle into the drop. Smear was air - dried and passed through a flame two or three times. The slide was allowed to cool down before staining. The fixed smear was flooded with the crystal violet solution for 10 sec. Then, crystal violet was decanted and the slides were rinsed gently under the running water. The excess water was rinsed off with iodine solution, and then the slide was flooded with fresh iodine solution for 10 sec. After rinsed gently with a flow of tap water, slide was then gently decolorized for 1 - 2 sec. The excess decolorizer was removed with a flow of tap water. The slide was flooded with safranin for 10 sec. The excess counterstain was removed with a gentle flow of tap

water. Slide was drained and air - dried in an upright position. The smears were examined under a microscopy. Gram staining could differentiate the bacteria into two groups, those which retain the crystal violet (gram - positive) and those that take the color of safranin O (gram - negative).

2.1.2 Lactose fermentation test

An aliquot of colony was streaked on MacConkey agar (Oxoid®) and incubated at 37°C for 18 - 20 hrs. Gram - negative lactose - fermenters could produce pink colonies, while gram - negative non lactose - fermenters demonstrated colourless colonies.

2.1.3 TSI reaction test

An aliquot of colony was streaked on slant surface and stabbed into the Triple Sugar Iron (TSI; Merck™; Merck KGaA) agar slant. They were incubated at 37°C for 18 - 20 hrs. Carbohydrate fermentation is detected by the presence of gas and a visible color change (from red to yellow) of the pH indicator, phenol red. The production of hydrogen sulfide is indicated by the presence of a precipitate that blackens the medium in the butt of the tube.

2.1.4 Motility/Lysine decarboxylase/Lysine deaminase/Indole tests

An aliquot of colony was stabbed into the Motility Indole Lysine (MIL; MerckTM; Merck KGaA) semi - solid medium. It was incubated at 37°C for 18 - 20 hrs. Growth of motile organisms extends out from the line of inoculation, while non - motile organisms grow only along the stab line.

The bromcresol purple is used as the pH indicator to detect the decarboxylase activity. When inoculated with an organism that ferments dextrose, acids are produced that lower the pH, causing the indicator in the medium to change from

purple to yellow. The acidic pH also stimulates enzyme activity. Organisms that possess a specific decarboxylase degrade the amino acid provided in the medium, yielding a corresponding amine. Lysine decarboxylation yields cadaverine. The production of these amines elevates the pH and causes the medium in the bottom portion of the tube to return to a purple color. If the organism being tested does not produce decarboxylase, the medium remains yellow (acidic) with a purple or red color on the top.

Lysine deamination produces a color change in the upper portion of this medium. Oxidative deamination of lysine yields a compound that reacts with ferric ammonium citrate, producing a burgundy red color in the top centrimeter of the medium while the bottom portion of the medium remains acidic.

Indole can be detected in the medium by adding Kovacs' reagent to the agar surface. The indole combines with the *p* - dimethylamino - benzaldehyde of Kovacs' reagent and produces a red complex. Indole is produced in this medium by organisms that possess the enzyme tryptophanase. Tryptophanase degrades the typtophan present in the casein peptone, yielding indole (139).

2.1.5 Urease test

An aliquot of colony was streaked on slant surface of the Urea agar (BBL™; Becton Dickinson, Sparks, MD.). It was incubated at 37°C for 18 - 20 hrs. Enzymatic digest of gelatin provides nitrogen, carbon, and amino acids required for organism growth in the Urea agar. Dextrose is an energy source. Sodium chloride maintains the osmotic balance of the medium. Monopotassium phosphate is the buffer. Urea provides a nitrogen source for organisms producing urease. The splitting of urea by urease causes the release of ammonia, increasing pH of the

medium to the alkaline side. This is indicated by a color change of the pH indicator, Phenol Red, from yellow (pH 6.8) to red (pH 8.1). Agar is added as a supplement to solidify the medium (140).

2.1.6 Citrate utilization test

An aliquot of colony was streaked on slant surface of Simmon's Citrate agar (MerckTM; Merck KGaA). It was incubated at 37°C for 18 - 20 hrs. The bacteria can utilize sodium citrate as the sole carbon source and then produce an alkaline reaction in the color change from green to blue by bromthymol blue used as the indicator (141).

2.2 Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed by using the agar disc diffusion method on MHA at 37°C for 18 - 20 hrs following the recommendation of CLSI 2006 guidelines (142). The uropathogenic Enterobacteriaceae were tested their susceptibility to 23 antimicrobial agents (Oxoid®) as shown in Table 6. The isolates which demonstrated the resistance more than 10 antibiotics were selected for the further studies.

2.3 Classification of the uropathogenic ESBL - producing bacteria

2.3.1 Screening and confirmation test by agar - disc diffusion

Antimicrobial susceptibility test was performed by the agar disc diffusion assay on MHA at 37°C under aerobic atmosphere for 18 hrs following the CLSI 2006 guidelines (Table 7). Cefotaxime and ceftazidime discs were carried out to determine

the susceptibility in the ESBL screening test. Ceftazidime and ceftazidime/clavulanic acid discs were carried out to determine the susceptibility in the Confirmatory test.

Table 6 Antimicrobial agents which recommended for *Enterobacteriaceae* following the CLSI 2006 guidelines (142)

Antibiotics	Abbreviations	Contents (μg)	Zone diameters (mm)		
			R	I	S
A: primary test and report					
Ampicillin	AM	10	≤ 13	14 - 16	≥ 17
Cephalothin	CF	30	≤ 14	15 - 17	≥ 18
Gentamicin	GM	10	≤ 12	13 - 14	≥ 15
B: primary test and report selectively					
Amikacin	AK	30	≤ 14	15 - 16	≥ 17
Amoxicillin/ clavulanic acid	AMC	20/10	≤ 13	14-17	≥ 18
Cefamandole	MA	30	≤ 14	15 - 17	≥ 18
Cefepime	FEP	30	≤ 13	15 - 16	≥ 17
Cefoxitin	FOX	30	≤ 14	15 - 17	≥ 18
Cefotaxime	CTX	30	≤ 14	15 - 22	≥ 23
Ciprofloxacin	CIP	5	≤ 15	16 - 20	≥ 21
Imipenem	IPM	10	≤ 13	14 - 15	≥ 16
Piperacillin	PRL	100	≤ 17	18 - 20	≥ 21
Trimethoprim/ sulfamethoxazole	STX	1.25/23.75	≤ 10	11 - 15	≥ 16
C: supplemental report selectively					
Ceftazidime	CAZ	30	≤ 14	15 - 17	≥ 18
Chloramphenicol	C	30	≤ 12	13 - 17	≥ 18
Kanamycin	K	30	≤ 13	14 - 17	≥ 18

Table 6 (continued)

Antibiotics	Abbreviations	Contents (µg)	Zone diameters (mm)		
			R	I	S
Netilmicin	NET	30	≤ 12	13 - 14	≥ 15
Tetracycline	TE	30	≤ 14	15 - 18	≥ 19
Tobramycin	TOB	10	≤ 12	13 - 14	≥ 15
U: supplemental for urine only					
Norfloxaxin	NOR	10	≤ 12	13 - 16	≥ 17
Nitrofurantoin	F/M	300	≤ 14	15 - 16	≥ 17
Trimethoprim	W	5	≤ 10	11 - 15	≥ 16
Carbenicillin	CAR	100	≤ 19	20 - 22	≥ 23

R, resistance; I, intermediate susceptible; S, susceptible

Table 7 Antimicrobial agents which recommended for testing the ESBL - producing bacteria according to the CLSI 2006 guidelines (142)

Tests	Antibiotics	Abbreviations	Contents (µg)	Positive result of zone (mm)
Screening	Ceftazidime	CAZ	30	≤ 22
	Cefotaxime	CTX	30	≤ 27
Confirmation	Ceftazidime/ clavulanic acid	CAZ/CLA	30/10	A difference of ≥5 mm in the zone diameter
	Ceftazidime	CAZ	30	
	Cefotaxime/ clavulanic acid	CTX/CLA	30/10	
	Cefotaxime	CTX	30	

2.3.2 Keyhole phenomenon detection by expanded double disc diffusion synergy test (143)

All strains were additionally subjected to an expanded double disc diffusion synergy test. A distance of 25 mm. between discs was regarded as optimal to observe a keyhole phenomenon. This distance also allowed incorporation of double disc synergy with discs of cefotaxime, ceftazidime, cefpodoxime and cefepime were placed around an amoxicillin/clavulanic acid disc (Figure 8). An expanded double disc diffusion synergy test was performed on MHA at 37°C under aerobic atmosphere for 18 hrs. A keyhole phenomenon was regarded as positive for ESBL production.

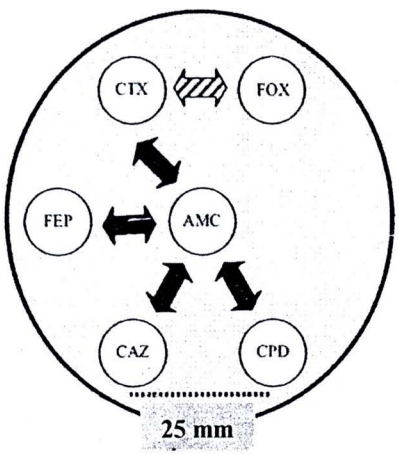

Figure 8 Expanded double disc diffusion synergy test (143). Black arrows indicate the probable keyhole phenomenon due to the synergism of each tested antibiotic toward clavulanate. Striped arrow indicates the probable blunted zone due to the antagonism of cefoxitin (FOX, cefoxitin 30 μg/disc) towards cefotaxime. For the investigative drugs, tests were performed only on those strains giving zones of ≥ 15 mm in disk diffusion assay.

2.3.3 AmpC β - lactamases detection by double disc antagonist test

AmpC β - lactamases production was performed by using the double disc antagonist test (144) with discs of cefotaxime and cefoxitin on MHA at 37°C for 18 hrs. A distance between disce was 25 mm. The presence of a blunted zone of cefotaxime adjacent to cefoxitin was indicated the inducibility of the AmpC enzyme (Figure 9).

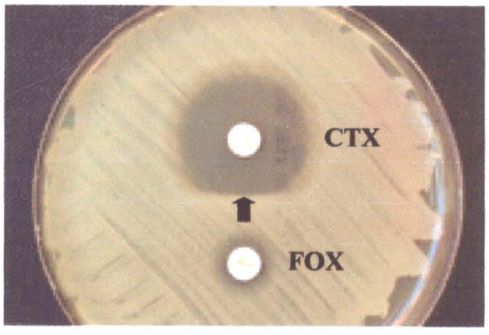

Figure 9 A blunted zone (black arrows) of cefoxitin - cefotaxime antagonist test; CTX, cefotaxime 30 μg/disc; FOX, cefoxitin 30 μg/disc (144)

2.3.4 Definition of resistance types (145)

According to the characteristics of β - lactamase production, the resistance of these tested bacterial strains were grouped as described below and in Table 8.

Group 1: Derepressed AmpC producers

Group 2: Partially derepressed AmpC producers

Group 3: (Partly) Derepressed AmpC producers with ESBL production

Group 4: ESBL production with keyhole phenomenon

Group 5: Inducible AmpC producers

The isolates with ESBL production were selected. The minimum inhibitory concentration (MIC) of ceftazidime was determined by broth microdilution assay following the recommendation of CLSI 2006 guidelines(142). The two - fold serial dilutions of ceftazidime in MHB were prepared. The bacterial suspension was then added to each well to achieve final bacterial cells of 5 x 10^6 CFU/ml. Well containing bacterial culture without antibiotic was also tested as the growth control. Microtiter plate was incubated for 18 hrs at 37°C. Well showed clear medium was indicated as MIC. The highly resistant isolates, 50 mg/ml of ceftazidime as final concentration, were selected for the further studies.

Table 8 Characterization of resistance types (145)

Characterizatics	Group 1	Group 2	Group 3	Group 4	Group 5
ESBL production	-ve	-ve	+ve	+ve	-ve
Resistant to FOX	R	R	R	S	R or S
Resistant to CTX	R	R	R	R or S	S
Double disc antagonism	-ve	+ve	-ve	-ve	+ve
Keyhole phenomenon*				+ve*	

Resistant or intermediate susceptible to CTX, zone diameter ≤22 mm.; Resistant of FOX, zone diameter < 14 mm.; Susceptible to FOX, zone diameter ≥18 mm.;

Keyhole phenomenon in double disc synergy test at least 1 agent of broad - spectrum cephalosporin

2.4 Characterization of β - lactamase production

2.4.1 Analysis of growth curve and generation time (146)

The amounts of 10^6 CFU/ml of the selected isolates were used as the starting inoculums for their growth curve determination in BHI broth for 18 hrs. Their cultures were collected every 6 hrs. The viable count was determined by spreading on MacConkey agar and incubating for 18 hrs at 37°C. The recovered colonies were counted in the colony forming unit and the generation time was also calculated during the period of late - log phase as the following formula,

$$\text{Generation time} = \frac{0.3 \times T}{\text{Log Bf} - \text{Log Bi}}$$

Bf, final number bacteria (CFU/ml); Bi, initial number bacteria (CFU/ml);T, total time

2.4.2 Extraction of β - lactamase contained periplasmic proteins (147)

Bacterial strains were cultured in MHB supplemented with ampicillin at the sub - minimal inhibition concentration (sub - MIC) at 37°C for 16 hrs. The cells were harvested in late - log phase or early stationary phase by centrifuged at 4,000 rpm for 10 min at room temperature. Forty μg of cell pellets were resuspended harvested in 80 μl of PeriPreps Periplasting Buffer (PeriPreps Periplasting kit; Epicentre® Biotechnologies, Madison, WI.) by vortex mixing until the cell suspension was homogeneous. The suspension was incubated for 5 min at room temperature. One hundred and twenty μl of purified water (PeriPreps Periplasting kit; Epicentre® Biotechnologies) was added at 4°C and mixed by inversion and incubated for 10 min on ice. It was centrifuged at 8,000 rpm for 15 min at room temperature. The supernatant containing the periplasmic fraction was transfered to a clean tube.

2.4.3 Determination of the protein concentration

Qubit® Fluorometer was carried out to determine the concentration of the extracted protein as described in 1.5.6.

2.4.4 One - dimensional polyacrylamide gel electrophoresis (136)

Protein pattern of β - lactamase in the SDS - PAGE gel was detected by silver staining as described above.

2.4.5 Determination of β - lactamase activity by spectrophotometric nitrocefin assay (148)

The microdilution method was used to determine the β - lactamase activity. One hundred and twenty μg/ml of nitrocefin (Oxoid®) was two - fold diluted with phosphate buffer pH 7.0 for preparing the working solution. Twenty μl of each sample was added into each well of microdilution plates. The standard β - lactamase, benzylpenicillinase (Sigma, St. Louis, MO.), at 3.125 μg/ml was used as the positive control. Phosphate buffer solution was used as a negative control. The reaction was started by the addition of 20 μl of the samples and controls in each well containing the working solution. The amide bond in the β - lactam ring of nitrocefin was hydrolyzed by β - lactamase samples. The color change from yellow to red was appeared. The change in optical density at a wavelength of 486 nm was measured with a microplate reader (Synergy™ HT; BioTek Instruments). The reaction was incubated for 10 min before reading the optical density. β - lactamase International Unit (IU) of each sample was calculated from its optical density according to the curve of the standard β - lactamase. For the standard curve, a serial 2 - fold dilution of benzylpenicillinase was done in duplicate.

2.4.6 The determination of MIC of the extracted β - lactamase to inhibit antibiotic activity

The broth microdilution technique was performed to determine MICs of *E. coli* ATCC 25922. Two - folds serial dilutions of β - lactamase in 100 µl PBS pH 7.2 were prepared. PBS pH 7.2 was used as the parallel experiment. *E. coli* ATCC 25922 was cultured in TSB at 37°C for 18 hrs before adjusting a turbidity of the culture equal to McFarland standard No 0.5. The suspension was swabbed evenly onto MHA plates. The extracted β - lactamase was serially 2 - fold diluted with PBS, pH 7.2. Twenty µl of each dilution was added on ceftazidime disc and they were left in sterile plate until dry. The dry discs were put onto the swabbed MHA plates and then incubated at 37°C for 24 hrs. The PBS was used as a negative control. The inhibition zone was measured. The differences in the average zones of inhibition were used to evaluate the antimicrobial activities of each sample.

3. The effects of lactobacillus culture and its agents on crude bacteriocin toward the growth of the uropathogenic bacteria and their β - lactamases

3.1 Preparation of bacterial cells and its agents

Preparation of potent lactobacillus cell

Each isolate of the potent lactobacillus was cultured in MRS broth under 5% CO_2 atmosphere at 37°C for 36 hrs. It's turbidity of the culture was adjusted to equal as a McFarland standard No. 2.0 in a new tube with BHI broth.

Preparation of crude bacteriocins

The amount of 10^3 CFU/ml of each isolate was inoculated into MRS broth and cultured at 37°C under CO_2 atmosphere for 36 hrs. The cell - free supernatant was harvested by centrifuging the fresh culture with 4,800 rpm at 4°C for 30 min before transferring to a new tube. The crude bacteriocins were prepared by the ammonium sulphate precipitating, dialyzing, desalting, concentrating and determining the total proteins as described in 1.6.2.

Preparation of tested uropathogenic bacteria

The selected strains were inoculated into TSB and cultured at 37°C for 24 hrs before adjusting a turbidity of the culture equal to a McFarland standard No. 0.5 by diluting with BHI broth.

Preparation of crude β - lactamase

These selected strains were cultured in MHB supplemented with ampicillin at their sub MIC (sterile nadicillin; Nida Pharma Incorporation Co Ltd., Bangkok, Thailand) at 37°C for 16 hrs. The cells were in log phase or early stationary phase. Periplasmic proteins from bacterial culture was preparing by using PeriPreps™ Periplasting Kit as described in 2.4.2. Qubit® fluorometer was used as the instrument for determination the concentration of the extracted protein. Crude β - lactamase samples were adjusted to have equal concentrations of total protein for further studies.

3.2 Determination of the lactobacillus culture's effect toward the growth of the uropathogenic bacteria (149)

The potent Lactobacillus cells equivalent as 1 x 10^7 CFU/ml were prepared and then diluted to a serial 2 - fold dilution in MRS broth. Afterwards, 100 µl of each

dilution was put into microtiter plates. It was incubated for 12 hrs at 37°C under 5% CO_2 atmosphere before adding 1 x 10^5 CFU/ml of the 18 hrs culture of uropathogenic bacteria cell. The growth of tested uropathogen was further analyzed in BHI broth at 37°C under 5% CO_2 atmosphere for 12 hrs. The culture was collected every 6 hrs of the incubation. The viable count was determined by spreading 30 µl of culture on MacConkey agar and then incubating at 37°C for 18 hrs. The recovered colonies were counted in the colony forming unit.

3.3 Determination of effect of the lactobacillus's agent toward the growth of the uropathogenic bacteria (150)

One hundred µl of the crude bacteriocins was put into microtiter plates. The uropathogenic bacteria cells equivalent as 1 x 10^5 CFU/ml were prepared and then diluted to a serial 2 - fold dilution in BHI broth. Afterwards, one hundred µl of each bacteria was put into the cultured microtiter plates. The growth of tested uropathogen was analyzed at 37°C under 5% CO_2 atmosphere for 12 hrs. The culture was collected at 0, 6 and 12 hrs of the incubation. The viable count was determined by spreading 30 µl of culture on MacConkey agar and then incubating at 37°C for 18 hrs. The recovered colonies were counted in the colony forming unit.

3.4 Determination of effects of crude bacteriocin on the β - lactamase of the uropathogenic bacteria (151)

Fifty µl of the diluted crude bacteriocins and 50 µg of crude β - lactamases were added into the well of microtiter plate. The phosphate buffer pH 7.0 was used as

the control. They were incubated at 37°C for 30 min. β - lactamase activity was determined by spectrophotometric nitrocefin assay (148). Five hundred μg/ml of nitrocefin was diluted phosphate buffer pH 7.0 for preparing the working solution. Twenty μl of each incubated sample was added into each well of microdilution plates. The benzylpenicillinase at 3.125 μg/ml was used as the positive control. The phosphate buffer was used as a negative control. The reaction was started by the addition of 20 μl of the prepared working solution in each well containing the samples and controls. The change in optical density at a wavelength of 486 nm was determined with a microplate reader. The reaction was incubated for 10 min in the dark condition before reading the optical density again. Inhibition percentage of β - lactamase activity of each sample was calculated from its optical density after analyzed with the β - lactamase standard curve.