CHAPTER VI

DISCUSSION

Lactobacilli are the predominant bacteria of a human healthy vagina. Their presences and numbers are influenced by estrogen production, which undergoes age - and menstrual cycle - dependent changes (152). A healthy vaginal ecosystem is predominated by certain species of *Lactobacillus* which exert a significant influence on the microbial ecology of vagina (153 - 154). However, lactobacilli are still used in the fermented food industry as the probiotics for human and animal nutrition (155). Human lactobacilli have long been considered to constitute the probiotics for urogenital tract infection (156, 157). Because of the increase of antibiotics resistance by uropathogens, the inhibitory activity of uropathogens by lactobacilli is considered as the alternative way to treatment (158). There were many studies which reported about the mechanism of lactobacillus to eradicate the pathogens such as the attachment of uropathogens to uroepithelial cells (159), the production of organic acid (160), the secretion of H_2O_2 and the production of bacteriocins (161). Ruiz *et al.* (162) showed that *L. fermentum* strain L23 and *L. rhamnosus* strain L60 produced 2 bacteriocins which displayed a wide inhibitory spectrum including both gram - negative and gram - positive urogenital pathogenic strains and two species of *Candida*. Especially, the tested *E. coli* strains were resistant to ≥ 1 antibiotic.

Likely to the present study, some lactobacilli which collected from goat's vagina and some foods were recovered. A lot of them exhibited the inhibitory activity

against some uropathogenic bacteria. It may be noted that goat's vagina and some food is the source of lactobacilli which acted as probiotic.

Because of the broad spectrum of antimicrobial activity, the activity of supernatant should be active against the basic standard indicators, *S. aureus* ATCC 25923, *S. lutea* ATCC 9341, *E. coli* ATCC 25922 and *B. subtilis* ATCC 6633. They are very susceptible to the antibacterial agent (132). Agar - cup diffusion method which modified by Gordon *et al.* (131) was performed as a gold standard method for determining the antimicrobial activity of the supernatant of lactobacilli. The broad spectrum lactobacilli were recruited as the potent isolates. Among the potent isolates, 2 lactobacilli were identified as *L. plantarum* strain L541 and *L. pentosus* strain LSS after determined with API - 50 CHL kit. *L. plantarum* is a widespread member of the genus *Lactobacillus*, commonly found in many fermented food products as well as anaerobic plant matter. There was particular controversy over the position of some pentose - degrading strains formerly designated *L. pentosus* by Fred *et al.* (163). These species are genotypically closely related and show highly similar phenotypes (164). The genetic heterogeneity of the *L. plantarum* group had been demonstrated by Dellaglio *et al.* (165) on the basis of DNA - DNA hybridization data. Three groups were identified which were later classified as *L. plantarum* (166), *L. pentosus* (167), and *L. paraplantarum* (168).

The present results suggest that *L. plantarum*1 strain L541 and *L. pentosus* strain LSS produced a few amounts of H_2O_2 and lactic acid, but these concentrations were not enough to inhibit those uropathogens. So, it was possible that bacteriocins of these isolates may play a role as the major antimicrobial substance. The high concentration of crude proteins harvested from the cut off membrane $\geq$ 10 kDa could

be observed by the Quant - iT Protein Assay Kit. As well as other studies, *L. plantarum* and *L. pentosus* were also reported as the potent bacteriocin producers. The bacteriocins of *L. plantarum* includes plantaricin B, plantaricin BN, plantaricin A, plantaricin C, plantaricin S and T, plantaricin F, plantaricin C19 and SA6 and other unnamed bacteriocins as reviewed by Olasupo (169). Todorov and Dicks (170) showed that the cell - free supernatant containing bacteriocin ST194BZ, produced by *L. plantarum* ST194BZ, inhibits the growth of *L. casei*, *L. sakei*, *L. delbrueckii* subsp. *bulgaricus*, *E. faecalis*, *E. coli*, *E.cloacae* and *P.aeruginosa*. Strain ST194BZ produces two bacteriocins, ST194BZ of 3.3 kDa and 14.0 kDa, based on tricine - SDS - PAGE. Todorov (171) found that bacteriocin AMA-K which produced by *L. plantarum* AMA-K inhibited the growth of *Enterococcus* spp., *E. coli*, *K. pneumoniae* and *Listeria* spp. Bacteriocin AMA-K shared high homology to pediocin PA-1. *L. plantarum* LPCO10 provided the broadest spectrum of activity (172). This inhibitory compound from this strain was active against some gram - positive bacteria, including clostridia and propionibacteria as well as natural competitors of *L. plantarum* in olive fermentation brines. This substance could be designated as plantaricin S. Plantaricin S was also sensitive to glycolytic and lipolytic enzymes, suggesting that it was a glycolipoprotein. The size of the smallest active form is between 3 and 10 kDa. On the basis of its biological activity, its sensitivity to various enzymes, and its molecular weight (lower than that of plantaricin S) as assessed in SDS - PAGE, plantaricin T appeared different from plantaricin S. Okkers *et al.* (173) found that *L. pentosus* TV35b, isolated from the posterior fornix secretions of the vagina of a prenatal patient, produced a bacteriocin-like peptide (pentocin TV35b), which is inhibitory to *Clostridium sporogenes*, *Clostridium*

tyrobutyricum, *L. curvatus*, *L. fermentum*, *L. sake*, *Listeria innocua*, *Propionibacterium acidipropionici*, *Propionibacterium* sp. and *Candida albicans*. The molecular size of pentocin TV35b was estimated to be between 2.35 and 3.4 kDa, according to tricine - SDS PAGE. Todorov and Dicks in 2007 (174) showed that bacteriocin ST712BZ (14.0 kDa in size), produced by *L.pentosus* ST712BZ, inhibits the growth of *L. casei*, *E. coli*, *P. aeruginosa*, *E. faecalis*, *K. pneumoniae* and *L. curvatus*.

One hundred and forty nine of the uropathogenic enterobacteria were collected from urine specimens over a period of 6 months at Microbiology Section, Central Laboratory Unit, Maharaj Nakorn Chiang Mai hospital in 2007. All of them are the multidrug - resistant Enterobacteriaceae (MDRE). One hundred one isolates of the MDRE which resistant more than 10 antibiotics were identified. We finally got 3 species of MDRE as *E. coli* (74.26%), *K. pneumoniae* (20.79%) and *E. cloacae* (4.95%). As same as the statistic of the hospital in 2007 and 2008 (175 - 176), the organisms frequently isolated from urine were *E. coli* and other gram - negative enterobacteria, especially *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*, *E. cloacae* and *P. mirabilis*. Moreover, ESBL - producing enterobacteria were important. In this statistic report, more than 50% of *E. coli*, *K. pneumoniae* and *E. cloacae* were ESBL - producer. It could be concluded that high prevalence of MDRE in a role of common uropathogenic is found at Maharaj Nakorn Chiang Mai hospital as well as the several studied. MDRE are an important cause of nosocomial infections (177). MDRE producing ESBLs have emerged as an important cause of bloodstream infection in hospitalized patients and UTIs in the community (178). Colonization or infection with MDRE, which are resistant to third - generation cephalosporins and often other

antibiotic classes as well, may result from acquisition of the organism from another patient or through development of resistance in the patient's own previously susceptible strain. Clonal outbreaks of such MDRE have been well described over the past decade and have resulted in substantial morbidity and mortality (179 - 180).

In the analysis of growth curve, the generation time of lactobacilli and uropathogenic bacteria were determined. PeriPreps™ Periplasting Kit recommended that the time appropriated to extract the β - lactamase containing in the periplasm recommend growing bacterial cells to late log phase only, as older cell cultures in stationary phase commonly demonstrate some resistance to lysozyme treatment (147). The cells divide at a constant rate depending upon the composition of the growth medium and the conditions of incubation. Exponential growth cannot be continued forever in a batch culture. Population growth is limited by one of three factors; exhaustion of available nutrients, accumulation of inhibitory metabolites or end products, and the exhaustion of biological space (181). For checking for the release of periplasmic proteins, they were analyzed an aliquot of both the periplasmic and spheroplastic fractions by SDS - PAGE. The presence of unique proteins or the enrichment of specific proteins in each fraction indicates successful fractionation. For example, if the strain of *E. coli* contains a high - copy number plasmid with the ampicillin resistance marker, then the presence of β - lactamase (31.5 kDa) mainly in the periplasmic fraction indicates successful fractionation. Other *E. coli* proteins found in the periplasmic space include alkaline phosphatase (50 kDa) and elongation factor Tu (43 kDa) (147).

β - lactamase activity was also determined by nitrocefin assay. Nitrocefin is a chromogenic cephalosporin substrate and sensitive to hydrolysis by all known

lactamases produced by bacteria. The reaction is based on the production of a colored compound after the substrate, nitrocefin, was exposed to the extracted β - lactamase (182). In present study, the results show that the β - lactamase activities of the induced condition were higher than the non induced condition. All of the tested pathogens, the highest activities of β - lactamases which extracted from CAZ induced conditions were shown. It was suggested that CAZ may be the good inducer for β - lactamases. Unlikely to the previous study (183) showed that most new cephalosporins, like cefotaxime, ceftriaxone and ceftazidime are the poor inducers but ampicillin and cefotetan are the good inducers. In the present result, the activities of AM and CAZ inducers conditions were not significant different in the case of *E. coli* ATCC 25922.

In the present study, these results showed that the 2 potent *Lactobacillus* culture, as *L. plantarum*1 strain L541 and *L. pentosus* strain LSS, and their bacteriocins could completely inhibit the growths of pathogens after 6 hr of exposure. According to the obtained results, *L. plantarum* strain L541 and *L. pentosus* strain LSS were found to be the strongest candidate isolates for vaginal probiotics. Reid *et al.* (184) suggested that the predominance of inhibitor - producing lactobacilli on the urogenital epithelium and the ability of these organisms to interact closely with uropathogens would constitute an important host defense mechanism against infection.

On the other hand, the crude bacteriocins of *L. plantarum*1 strain L541 and *L. pentosus* strain LSS could not affected the β - lactamase activity. The slightly reduction of the β - lactamase activity after mixed with these ESBL containing extracted periplasmic proteins were demonstrated. So, it is possible that the little

inhibition activity might be caused from the other type of β - lactamase. Recently, Simm *et al.* (185) reported that bulgecin A which were antimicrobial glycopeptides produced by *Pseudomonas acidophila*, could competitively inhibited the metallo - β - lactamase (BceII) from *Bacillus cereus*.