CHAPTER 4

DISCUSSION

1. Isolation of vaginal lactobacilli

MRS broth and MRS agar were used as isolation media for vaginal lactobacilli. Although both of them are suitable media for lactobacilli but other bacteria can grow. None lactobacilli grew on MRS agar showed different in morphology and physiology such as cocci shape, catalase negative or spore-forming. Lactobacilli are known as facultative or anaerobe, most of selected isolates of lactobacilli can survive when exposed to oxygen in ambient air. It is indicated that these isolates tolerate to oxygen and it is easier to culture these bacteria than obligate anaerobe ones.

2. Screening for antimicrobial activity of isolated *Lactobacillus* spp.

The finding of this study suggest that 16 isolates, from 80 isolates i.e., *Lactobacillus* sp. SK1, SK2, SK3,....SK16 show inhibition activity against reference strains of *E. coli* ATCC 25923 and *S. aureus* ATCC 25922. These isolates may produce some inhibitory substances i.e., lactic acid, short chain fatty acids, hydrogen peroxide or bacteriocin and release them to the agar medium in over lay method assay. Besides of 16 isolates, they show no inhibition zone or very small inhibition zone. It is indicated that only particular isolates could produce inhibitory substances in high quantity enough to inhibit tested bacteria. Unfortunately, none of them could inhibit *C. albicans* NCPF 3153. The ability of *Lactobacillus* spp. to inhibit growth of other bacteria has been attribution to the secretion of lactic acid, other organic acids including acetic acid, hydrogen peroxide, and an inhibitory protein (Radler and Brohl, 1984). Many investigators believed that lactic acid production is a primary mechanism in maintaining the equilibrium of a healthy vaginal ecosystem (Aroutcheva et al., 2001). When 16 isolates were cultured in MRS broth and cell free supernatants were tested for antibacterial activity, they also
show the same activity as growing in MRS agar. However, the activity of all supernatants active only when their pH at 4.29-4.70 but they lost activity at pH 6.5. The activity of inhibitory substances in supernatant depended on the pH. Skarin et al, (1986) and Dembele et al., (1998) have demonstrated that activity of bacteriocin is influenced by the hydrogen ion concentration in the environment. These investigators demonstrated that at a low pH the *Lactobacillus* sp. bacteriocin was active and decreased dramatically as the hydrogen ion concentration decreased. The activity of hydrogen peroxide also found to be pH dependent. Hydrogen peroxide was stable in acid environment and degraded as the hydrogen ion concentration decreased (Fontaine et al., 1990). Therefore, as the pH of the vaginal decreases, bacteriocin looses its effectiveness, hydrogen peroxide is degraded, and lactobacilli cannot compete against the other bacteria. From the result, increasing of concentration provided increasing of inhibition zone.

3. Sensitivity to antibiotics

Lactic acid bacteria widely used as probiotics or in starter cultures have the potential to serve as a host of antibiotic resistance genes with the risk of transferring the genes in many lactic bacteria and other pathogenic bacteria. Most of studies have been focused on food as vehicles of antibiotic resistance genes. However, there have been very few systemic studies to investigate acquired antibiotic resistance in lactic acid bacteria. Most data exist on opportunistic pathogenic enterococci, while the number of reports on lactococci and lactobacilli is limited. Recently, it is expanding due to increased interest in probiotic lactic acid bacteria and genetic modification of lactic acid bacteria for different purposes. Lactobacilli, pediococci and *Leuconostoc* spp. have been reported to have a high natural resistance to vancomycin, a properties that is useful to separate them form other Gram-positive bacteria (Hamiliton-Miller and Shah, 1998, Simpson et al., 1988). The results from this experiment also showed that *Lactobacillus* sp. SK5, SK6, SK7 and SK 8 were also resistance to vancomycin. Some lactobacilli have a high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin,
metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethoprim/sulphamethoxazole, and vancomycin (Danielsen and Wind, 2003). For a number of lactobacilli, a very high frequency of spontaneous mutation to nitrofurazone (10^{-5}), kanamycin, and streptomycin was found (Curragh and collins, 1992). From these data Mathur and Singh (2005) concluded that intergenous and interspecies differences exist, and consequently identification at species level is required in order to interpret phenotypic susceptibility data. The susceptibility profile of *Lactobacillus* sp. SK5, SK6, SK7 and SK8 to antibiotics are also different. These results are supported by the study of Danielsen and Wind, 2003. The study was undertaken to establish the levels of susceptibility of *Lactobacillus* spp. to various antimicrobial agents and it was shown to be species dependent. The ability to transfer genes for antibiotics resistance must be considered as an important parameter for the selection of the probiotic strains intend to use in human and animals. If *Lactobacillus* sp. SK5, SK6, SK7 and SK8 are intended to use as probiotic they must be proved for their impossible to transfer antibiotic resistant to other bacteria.

4. Hydrogen peroxide production

*Lactobacillus* sp. strains that produce hydrogen peroxide have been isolated from 79% to 96% of women with a healthy vaginal ecosystem (Silva *et al.*, 1987). For select the most suitable strain, hydrogen peroxide production, an intrinsic protective mechanism in the vaginal compartment, was measured for *Lactobacillus* sp. SK5, SK6, SK7 and SK8. The results from this study indicated that *Lactobacillus* sp. SK5 produce higher concentration of hydrogen peroxide than *Lactobacillus* sp. SK6, while *Lactobacillus* sp. SK7 and SK8 did not produce this substance. *Lactobacillus* spp. and hydrogen peroxide that they produce are increasing recognized as essential components of a healthy microflora environment. Hydrogen peroxide is toxic to many microorganisms at concentrations that are typical in the vaginal fluid, and thus provide an intrinsic protective mechanism in the vagina (Eschenbach *et al.*, 1989, Beigi *et al.*, 2005).
Hydrogen peroxide producing lactobacilli predominate in the normal vagina but are seldom found in the vagina of patients with bacterial vaginosis. Hydrogen peroxide is known to inhibit the growth of some bacteria and may be involved in the control of normal flora. *Lactobacillus* sp. SK5 and SK 6 like other lactobacilli, they lack the heme group and do not utilize the cytochrome system for terminal oxidation. They possess flavoproteins, which transform oxygen into hydrogen peroxide. This mechanism, together with the absence of the catalase hemoprotein, generates hydrogen peroxide in amount that exceed the capability of the organism to degrade it. It has been proposed that the production of hydrogen peroxide may explain the success of lactobacilli as vaginal colonizers and that it is an inhibitory mechanism that can inhibit or eliminate other members of the microbiota, particularly those that lack or low level of catalase or peroxidase enzymes (Aroucheva *et al.*, 2001).

### 5. Adhesion of *Lactobacillus* sp. to HeLa cell

The possibility of utilizing lactobacilli in the maintenance of a healthy state in the human female urogenital tract is based on the capacity of these probiotic bacteria to produce a barrier population. One of the main criteria for selecting probiotic strains is their ability to adhere to the vaginal surface. Adhesion of microorganisms to epithelial cells represent and essential step for colonization and persistence in a specific site. Since adhesive properties vary considerably between *Lactobacillus* strains (Reid and Cook, 1987, Chauviere *et al.*, 1992, Mardh and Westrom, 1976, Sobel *et al.*, 1981), Four isolates of *Lactobacillus* sp. SK5, SK6, SK7 and SK8 were evaluated for their adhesion capacity to HeLa cells. These cells have been used as in vitro model of the human vaginal epithelium to screen adhering *Lactobacillus* strains. The results indicated that all strain of *Lactobacillus* spp. were able to adhere to the HeLa cells, exhibiting different degree in their attachment. *Lactobacillus* sp. SK6 and SK7 were less adhesive, than *Lactobacillus* sp. SK5 and SK8.
6. The effect of the presence of bioadhesive polymers on the adherence of *Lactobacillus* spp. to HeLa cells

In order to achieve the best degree of *Lactobacillus* spp. adhesion to the cell surface, the effect of the different bioadhesive polymers on the adhesion of *Lactobacillus* spp. were examined. The compounds were used at the half of the maximum concentration at which that could be solubilized, and were added to medium during the adhesion step. The results showed that the presence of Na-CMC and PVP at the concentration of 100 mg/ml, produce highly enhancement in the adhesion of four strains (*Lactobacillus* sp. SK5, SK6, SK7 and SK8). HPMC-E50 showed little enhancement of adhesion of *Lactobacillus* sp. SK5 and SK7 but it has no effect on the adhesion of *Lactobacillus* sp. SK6 and SK8. These results were different from the experiment of Maggi *et al.*, 2000. They found that in the presence of HPMC-LV and HPMC-HV at the concentration of 1 mg/ml, produced an enhancement in the adhesion of five lactobacilli strains i.e., CD2, FV2, FV6, FV8 and FV9 to HeLa monolayer. In this study, among three biopolymer, PVP is the best enhancement in the adhesion of *Lactobacillus* sp. SK5, SK6, SK7 and SK8. Adhesion will the best when viscosity of PVP is maximum.

7. Activity of dialysis supernatant of *Lactobacillus* sp. SK5

The dialyzed supernatant of *Lactobacillus* sp. SK5 showed inhibition activity against tested bacteria. It is indicated that the high molecular weight molecule (s) that supposed to be bacteriocin remained in dialysis tube and low molecular weight molecules such as organic acids and hydrogen peroxide were eliminated. *Lactobacillus* sp. SK 5 bacteriocin inhibited *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeruginosa*, *E. coli*, *N. gonorrhoeae* and *S. epidermidis*. These findings are similar the results reported by other investigator (McGroatry and Reid, 1988, Nagy *et al.*, 1991 and McGroarty, 1993). They showed that bacteriocin produced by vaginal lactobacilli appears to have a broad spectrum of activity and inhibits a wide rage of Gram-positive and Gram-negative bacteria. *Lactobacillus* sp. SK5 bacteriocin was active at low pH
(about 3.8-4.0) like bacteriocin from *L. delbrueckii* subsp. *bulgaricus* CFR 2028 was active against toxigenic strain of *B. cereus* at pH 3.5-3.8 (Balasubramanyam *et al.*, 1998). The present study demonstrated that inhibitory substances produced by *Lactobacillus* sp. SK 5 exhibited all 3 mechanisms of bacterial antagonism. These 3 factors, organic acids, hydrogen peroxide and bacteriocin, act synergistically to suppress the growth of the tested bacteria in primary screening for antimicrobial activity. *Lactobacillus* spp. SK5 is very promising strain against vaginal bacterial pathogens.

**8. Resistance to feminine hygiene and spermaticide**

From this study, MICs of six feminine hygiene products are 0.391-1.562% and their MBC are 0.391-3.125%. They showed high antimicrobial activity against *Lactobacillus* sp. SK5. These products are used for hygiene purpose of female genital. They should have active against some bacterial as well as *Lactobacillus* sp. These products may not have significant effect to *Lactobacillus* sp. in the inner part of vagina if they are used to clean external part of genital organ. Some other products may be used for douching and they will kill normal flora i.e., *Lactobacillus* spp. and others. Often use of antimicrobial douching may effect on balancing of ecosystem of vagina and leading to vaginal infection.

Spermicides, such as Nonoxynol-9 (N-9) based contraceptive methods, have been known to be toxic to lactobacilli (Watts *et al.*, 1999). Nonoxynol-9 is the active compound in many spermicidal formulas. It is a nonionic detergent, which reduces the superficial tension of the membrane of the human spermatozoon, causing loss motility, diminution of its glycolytic power and alteration in permeability. It also affects the lipidic content of the membrane of human spermatozoon. Nonoxynol-9 is generally used at concentrations of 5% in creams. It is possible that the presence of nonoxynol-9 could affect the ecological balance of the vagina through the inhibition of the protective of lactobacilli, especially those that produce hydrogen peroxide (Richardson *et al.*, 1998). Some studies have shown that lactobacilli present resistance or
sensitive to this substance. In present study, the minimal inhibitory concentration of nonxynol-9 is 6.25 %v/v and minimal bactericidal concentration is 12.5 %v/v. It is indicated that *Lactobacillus* sp. SK5 sensitive to nonoxynol-9. Liliana *et al.* (2006) reported that *L. acidophilus* and *L. fermentum*, among the lactobacilli isolated from vagina, presented the largest number of strains sensitive to nonoxynol-9. In vitro studies showed that most lactobacilli are eradicated by expose to a low dose of nonoxynol-9, whereas uropathogens grow and prosper in high concentrations of this compound (McGroarty *et al.*, 1990).

### 9. Evaluation of physical properties of *Lactobacillus* sp. SK5 vaginal suppository

The viability of *Lactobacillus* sp. SK5 before and after storage in refrigerator at 4 °C for 3 months were evaluated. The viable of this *Lactobacillus* sp. SK5 decreased about 2 log cycles from 1.32x10^8 to 1.10x10^6 cfu/g. In this study, the decrease in *Lactobacillus* sp. SK5 viability may be due to heating process during the preparation of vaginal suppository and the long exposure of oxygen, remained in the cavity of suppository.

The average weight is 2.23±0.063 g. Therefore, the weight variation of vaginal suppository was met the BP of vaginal suppository requirement. According to the BP (2007) the average weight is determined by weighing 20 suppositories. When weighed singly, no suppository deviates from the average weight by more than 5% except that two may deviate by not more than 10%.

The thermogram of mixed PEGs base, which showed a broad endothermic peak between 35.07-44.03 °C, corresponding to the melting range of the base, indicating that the vaginal suppository start melting at the temperature close to the body temperature.

Figure 13 showed the release profile of *Lactobacillus* sp. SK5 from hollow-type suppository. This study showed that the *Lactobacillus* sp. SK5 did not release in the first 25
minutes because only PEGs base is slowly dissolved while *Lactobacillus* sp. SK5 still remain in cavity. The release of *Lactobacillus* sp. SK5 will increase after 25 minutes. This may be due to the water solubility and erosion mechanism of the base, which allows the *Lactobacillus* sp. SK5 to be released. Vinita *et al.*, (2005) reported that the melting temperature of PEG 1000 (melting range 37-40 °C) is slightly higher than body temperature, requiring more time for release of active substance. Hosny *et al.*, (1996) reported that the release of drug from mixed PEGs base may be due to water solubility of the base, which allows the drug to be released by both diffusion and erosion mechanisms.