CHAPTER 1

INTRODUCTION

General Introduction

Recently, an increasing interest has developed in microbiota, that promote a women’s health. In particular, *Lactobacillus* species, they are also classified as lactic acid bacteria (LAB). They are Gram-positive facultative anaerobe, non-spore forming, rod or coccobacilli. Lactobacilli can be isolated from a large number of sources, for example from human and animal body (oral cavity, stomach, intestine and vagina) plant and material of plant origin, sewage and fermented products. *Lactobacillus* spp. presented in the human vagina, have received considerable attention due to their protective and probiotic properties (Andreu, 2004). In vagina, lactobacilli make up a biofilm on the surface of the vaginal mucosa (Reid, 2001) which is considered to protect the vagina against pathogenic microorganisms through different mechanisms such as (1) secretion of organic acids (mainly lactic acid) (2) production of other antimicrobial substances (mainly hydrogen peroxide and bacteriocins), (3) adhere to surface and inhibit the adhesion of pathogens, (4) stimulation of the host’s immune system, (5) competition for nutrients with pathogens (Reid *et al*., 2002). There is a good clinical evidence that the vaginal and urogenital floras play a central role in maintaining both the well-being and the illnesses of women (Hillier *et al*., 1992). When the vaginal lactobacilli are reduced or absent, other microorganisms may grow excessively, thus causing disorders. Indeed, most urogenital infections result from vaginal flora imbalance. Bacterial vaginosis is mainly caused by an association of *Gardnerella vaginalis* with anaerobic bacteria and often mycoplasma. Yeast vaginitis is mainly due to *Candida albicans*. For all these infections excepted yeast vaginitis, the lactobacilli flora is reduced (Buton *et al*., 2003).

*Lactobacillus* spp. to be used in human must be of human origin because the colonization and some benefit effects may be species dependent. They could produce hydrogen peroxide, lactic acid and bacteriocin. The strains with high adhesion ability are preferred because they are more likely to establish in vagina.

The aims of this study were isolation and selection of *Lactobacillus* spp. from vagina of healthy women to use as probiotic against bacterial pathogens in vagina. The selected
strains were evaluated for their antimicrobial activities, detection of their antimicrobial substances, sensitivity to antibiotics, adhesion ability to vagina epithelial cells and formulation and evaluation of vaginal suppository containing promising strain of selected *Lactobacillus* spp.
Review literatures

1. Definition of bacterial vaginosis

Bacterial vaginosis is the common form of vaginal infection among reproductive-age women (Sobel, 1990). The infection has been reported to occur in 15% to 20% of pregnant women. Bacterial vaginosis during pregnant was regarded as a relatively harmless abnormality. Recent work, however, has linked bacterial vaginosis to numerous upper genital tract complications such as preterm labor and delivery, preterm premature rupture of the membranes, chorioamnionitis, and postpartum endometritis (Gravett et al., 1986).

Most 40 years ago Gardner and Dukes (1955) first described a non specific infection of the vagina, with a fishy smell and discharge. They named this infection after the organism that caused 'Haemophilus vaginalis vaginitis'. Because this organism was not only found in women with the typical signs of this infection but also in women who had neither any symptoms nor were sexually active, controversial discussions about the pathological consequences of this disease took place over years. Due to its unstable gram staining the germ was titled 'Corynebacterium vaginale' (Zinneman and Turner, 1963) but in 1980, it was renamed in the honor of Gardner 'G. vaginalis', because its genus belonged neither to Haemophilus nor to Corynebacterium (Greenwood and Pickett, 1980). To date, the controversy continues, because numerous (especially anaerobic) microorganisms are involved in the bacterial vaginosis. Names like un-specific colitis, G. vaginitis, Gardners vaginitis, anaerobic vaginosis show the attempt to define this disease in a better way. Hoyme and Eschenbach (1985) reported that, they will use the name bacterial vaginosis which is defined as follows: a thin, homogeneous, greyish-white discharge, an elevated vaginal pH (more than 4.5), the occurrence of 'clue cells' (Gardner and Dukes, 1955) on microscopic examination of vaginal smears and intensified fishy smell after adding 10% KOH (liberating amines) to vagina fluid specimens.
Microbiology of bacterial vaginosis

Bacterial vaginosis is a clinical syndrome with a complex microbiology characterized by a reduced concentration of normally abundant *Lactobacillus* species and increased concentrations of Gram-negative anaerobic bacteria, particular *G. vaginalis*, *Mobiluncus* species, *Bacteroides* and *Prevotella* species, and *Mycoplasma* species (Hillier, 1993). The relative concentration of these microorganisms varies among women who have this condition. The presence of an altered vaginal flora is believed to exist as a continue range from normal to intermediate of bacterial vaginosis. The bacterial pathogen can cause bacterial vaginosis disease in section.

1.1 Gardnerella vaginalis

*G. vaginalis* is a Gram-negative bacteria catalase-negative. It is an infection of the female tract, often in combination with various anaerobic bacteria. The infection often produces a gray or yellow discharge with a "fishy" odor that increases after washing the genitalia with alkaline soaps. It is assumed that the infection is sexually transmitted. The bacteria are also found in women without a history compatible with a sexually transmitted disease, and often produces no symptoms (Gardner and Dukes, 1955).

1.2 Escherichia coli

*E. coli* is a Gram- negative bacteria, facultatively anaerobic. It is the name of type of bacteria that live in intestine and vagina. Most types of *E. coli* are harmless. However, some types cause a sickness. The worst type of *E. coli* causes bloody diarrhea, and can sometimes cause kidney failure and even death. These problems are most likely to occur in children and in adult weak immune systems (Feng et al., 2002).
1.3 *Candida albicans*

*C. albicans* is fungus that is normally present on the skin and mucus membranes such as the vagina, mouth, or rectum. The fungus also can travel through the bloodstream and effect the throat, intestines, and heart valves. *C. albicans* becomes an infectious agent when there is some change in the body environment that allows it to grow out of control (Redondo-Lopez et al., 1990).

1.4 *Staphylococcus aureus*

*S. aureus* is a Gram-positive coccus, which appears as grape-like clusters when viewed through a microscope and has large, round, golden-yellow colonies, often with \( \beta \)-hemolysis, when grown on blood agar plates. *S. aureus* is catalase positive and thus able to convert hydrogen peroxide \( (H_2O_2) \) to water and oxygen, which makes the catalase test useful to distinguish staphylococci from enterococci and streptococci. *S. aureus* appears to be an increasing problem that clinical laboratories should be aware of. They are as virulent as those producing coagulase and can colonize, cause infections and spread among patients. The latter requires anaerobic conditions for growth, is an infrequent cause of infection (Dhinya and Speck, 1968).

1.5 *Neisseria gonorrhoeae*

*N. gonorrhoeae* is a species of Gram-negative bacteria responsible for the sexually transmitted disease gonorrhoeae. *Neisseria* are highly fastidious cocci. They require special nutrients to survive. These cocci are intracellular and typically appear in pairs (diplococci). Gonorrhoeae symptoms include a purulent (or pus-like) discharge from the genitals which may be foul smelling, a burning sensation during urination and conjunctivitis commonly in neonatal infection, also occasionally in adults. *Neisseria* is usually isolated on a Modified Thayer-Martin culture plate. This plate has antibiotics and nutrients which not only facilitate the growth of *Neisseria* species, but inhibit the growth of Gram-positive organisms and most bacilli. Further
testing to differentiate the species usually includes an oxidase test which will be positive for *Neisseria gonorrhoeae*, and testing with the carbohydrates lactose, sucrose, and glucose. *N. gonorrhoeae* will only oxidize the glucose (Zheng *et al*., 1994).

**1.6 Pseudomonas aeruginosa**

*P. aeruginosa* is a Gram negative bacteria that is commonly found in the environment e.g. soil, water and other moist locations. It is an opportunistic pathogen. The bacterium takes advantage of an individual's weakened immune system to create an infection and this organism also produces tissue-damaging toxins. *P. aeruginosa* causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed (Graham *et al*., 1986).

**1.7 Staphylococcus epidermidis**

*S. epidermidis* is a member of the bacterial genus *Staphylococcus*, consisting of Gram-positive cocci arranged in clusters. It is catalase-positive and coagulase-negative and occurs frequently on the skin of humans and animals and in mucous membranes. The bacterium is responsible for a growing number of infections among hospital patients whose immune systems are weakened. Such infections often start at skin wounds caused by catheters. Little is known about mechanisms of pathogenesis of *S. epidermidis* infections. A characteristic of many pathogenic strains of *S. epidermidis* is the production of a slime resulting in biofilm formation. The slime is predominantly a secreted teichoic acid, normally found in the cell wall of the *Staphylococci*. This ability to form a biofilm on the surface of a prosthetic device is probably a significant determinant of virulence for these bacteria (Hancock and Scott., 2000).
1.8 *Peptostreptococcus* spp.

*Peptostreptococcus* spp. is the only genus among anaerobic Gram-positive coccI encountered in clinical infections. The organisms are part of the normal flora of human mucocutaneous surfaces, including the mouth, intestinal tract, vagina, urethra, and skin. They are isolated with high frequency from all specimen sources. *Peptostreptococcus* infections can occur in all body sites, including the CNS, head, neck, chest, abdomen, pelvis, skin, bone, joint, and soft tissues. Inadequate therapy against these anaerobic bacteria may lead to clinical failures. Because of their fastidiousness, peptostreptococci are difficult to isolate and are often overlooked. Isolating them requires appropriate methods of specimen collection, transportation, and cultivation. Their slow growth and increasing resistance to antimicrobials, in addition to the polymicrobial nature of the infection, complicate treatment (Douglas *et al.*, 2006).

**Diagnosis of bacterial vaginosis**

The standard method for diagnosing bacterial vaginosis uses clinical criteria that were developed by Amsel *et al.* in 1983. A diagnosis of bacterial vaginosis is made when three of the four following clinical signs are present: (1) an elevated vaginal pH level (>4.5), (2) positive amine odor with 10% potassium hydroxide (Whiff test), (3) presence of clue cells (squamous epithelial cells with adherent bacteria) on microscopic examination, and (4) a thin, homogeneous vaginal discharge (Amsel *et al.*, 1983).

**Infection and pregnancy**

Andrews *et al.* (1998) reported that the common infections residing in or acquired through the lower genital tract have been studied to determine if there is a relationship between such lower genital tract infection and spontaneous preterm birth. Infections most studied in this regard include syphilis, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, group B streptococcus, *Trichomonas vaginalis*, genital mycoplasmas, and bacterial vaginosis. In most
In many cases, it is difficult to control for other confounding risk factors that may also be present (Andrews et al., 1998). However, the genital tract condition that has been most consistently associated with spontaneous preterm birth is bacterial vaginosis.

In recent years, increasing attention has been given to the relationship between altered vaginal flora, low birth weight, and preterm birth. Specifically, numerous reports indicate an association between bacterial vaginosis and so-called bacterial vaginosis-associated microorganisms and preterm delivery. Eschenbach et al. (1984) were some of the earliest investigators to examine the association of bacterial vaginosis with preterm labor. They used gas-liquid chromatography (GLC) to diagnose bacterial vaginosis by identifying abnormal patterns of organic acids in vaginal secretions. They reported that bacterial vaginosis was present in 49% of 57 women who delivered before 37 weeks and in 24% of 114 women who delivered at term (P = .001). Subsequently, these investigators performed a larger, prospective trial in 534 women from whom vaginal fluid was obtained for GLC analysis during the second and third trimester (Gravett et al., 1986). Bacterial vaginosis was diagnosed in 102 (19%) of the 534 women, and was associated with an increased risk of preterm labor at less than 37 weeks, preterm premature rupture of the membranes and intraamniotic infection.

Many subsequent studies of bacterial vaginosis, diagnosed by use of clinical criteria or Gram's stain of vaginal secretions, have linked this condition with preterm delivery either as a result of preterm labor or preterm premature rupture of the membranes. In a prospective, observational trial, studied the relationship between bacterial vaginosis in early pregnancy and adverse pregnancy outcomes (Kurki et al., 1992).
Treatment of bacterial vaginosis and impact on preterm delivery

The results of preliminary trials suggest that treatment of BV, in certain high-risk women, decreases the rate of preterm delivery. Morales et al. (1994) performed a randomized trial to determine the effect of metronidazole in patients who had both a history of preterm birth in a preceding pregnancy and bacterial vaginosis. Patients with singleton gestations between 13 and 20 weeks who had a history of preterm labor or premature rupture of membranes were screened for BV. Those with a positive screen were selected randomly to receive metronidazole (250 mg orally three times daily for 7 days) or placebo in a double-blind design. Of 94 eligible patients, 80 were enrolled and completed the study, of whom 44 received metronidazole. Compared with the placebo group, patients who received metronidazole had significantly fewer hospital admissions for preterm labor, 12 (27%) versus 20 (78%); preterm births, 8 (18%) versus 16 (39%); births of infants weighing less than 2,500 g, 6 (14%) versus 12 (33%); and preterm premature rupture of membranes, 2 (5%) versus 12 (33%). They concluded that treatment of bacterial vaginosis with metronidazole was effective in reducing preterm births in patients with a history of a prior preterm delivery.

In a subsequent trial, Hauth et al. (1995) investigated whether treatment with metronidazole and erythromycin during the second trimester would decrease the rate of preterm delivery. A total of 624 women at risk for preterm delivery (prior history of a preterm delivery or weight less than 50 kg before pregnancy) were screened for bacterial vaginosis between 22 and 24 weeks of gestation. Patients were selected randomly (2 to 1) in a double-blind fashion to receive metronidazole and erythromycin or placebo. In this trial, preterm delivery occurred in 29% of women, and bacterial vaginosis was present in 42% at their initial examination. In the overall population, 26% of the women assigned to metronidazole and erythromycin delivered at less than 37 weeks compared with 36% assigned to placebo (P = 0.01). However, the association of metronidazole and erythromycin treatment and reduction in preterm delivery occurred only in women who had positive results for bacterial vaginosis. Treatment with metronidazole and
erythromycin resulted in a reduction in preterm delivery in women with bacterial vaginosis who were at increased risk for preterm delivery either because of a history of prior preterm delivery or because of prepregnancy weight of less than 50 kg.

2. Lactobacilli as good probiotics for prevent bacterial vaginosis

Fuller (1992) defined the probiotic as "live microbial feed supplement with beneficially affects the host animal by improving its vaginal microbial balance. These organisms are non pathogenic, non toxigenic and retain viability during storage (Salmien et al., 1998). Promising probiotic strains include the various bacteria and yeast such as Bifidobacterium, Lactococcus, Enterococcus, Lactobacillus, Streptococcus thermophilus and Saccharomyces.

The dominant presence of Lactobacillus spp. in the urogenital microflora of healthy women and the obliteration of lactobacilli in patients who develop urinary tract infections (Stamey, 1973), bacterial vaginosis and many other genital infections (Hillier et al., 1993) [except candidiasis] has led to a focus on these bacteria. They prefer an acidic environment and help create one by producing lactic and other acids. In general, lactobacilli have not been associated with disease. They have been regarded as nonpathogenic members of the intestinal and urogenital floras (Bibel, 1988). Lactobacilli have long been of interested to the dairy and agriculture industries (Klaenhammer, 1982), although over the past century, studies in relation to human health were sporadic and often inconclusive. There is one obvious question: what properties do these strains possess that make them effective probiotic agents. The answer is not fully known, but some common denominators appear to exist, namely the ability to adhere to and colonize tissues and the capacity to inhibit the pathogenesis of disease causing organisms (Figure 1). Another question can be raised: do we expect an exogenous probiotic strain to colonize the gastrointestinal and urogenital tracts of a given person for a long time and even become part of the normal flora, replacing or coexisting with the endogenous lactobacilli organisms (Korshunov et al., 1985).
Figure 1. By products of lactobacillus metabolism that have an antagonistic effect against urinary and vaginal pathogens; the biosurfactants inhibit adhesion; the acids, bacteriocins, and hydrogen peroxide inhibit growth; and the coaggregation molecules block the spread of the pathogens (Korshunov et al., 1985).

3. Inhibitory substance produced by *Lactobacillus* spp.

3.1 Lactic acid

Lactic acid is one of the inhibitory agents produced by lactobacilli. It is the major end product of their carbohydrate catabolism. Lactic acid can be formed either via the Embden-Meyerhof-Parnas (glycolytic) pathway or by the 6-phosphogluconate pathway (Condon, 1987). On the basis of their hexose catabolism lactic acid bacteria can be divided into two groups: homofermentative types (using Embden-Meyerhof-Parnas pathway) and heterofermentative types (using 6-phosphogluconate pathway) (Orla-Jensen, 1919).

The homofermentative (Figure 2) lactic acid bacteria (lactococci, pediococci, streptococci and homofermentative lactobacilli) metabolize glucose primarily to lactic acid. The homofermentative involves splitting of fructose 1, 6 biphosphate (divided from glucose) into two triose phosphate moieties (glyceraldehydes 3-phosphate and dihydroxyacetone phosphate). These trioses are further converted to pyruvate, which is then reduced to lactate in order to maintain the
electron balance. The splitting reaction is catalyzed by aldolase, a constitutive enzyme in homofermentative lactic acid bacteria. When lactose is used as carbon source and taken up from the medium by an ATP-dependent permease system, it is converted into glucose and galactose by means of a β-galactosidase, or when it is taken up from the medium by the action of the PEP-PTS system, it is hydrolyzed into glucose and galactose 6-phosphate by means of phosphor β-galactosidase. While free galactose is first phosphorylated and further metabolized to glucose 6-phosphate via the Leloir pathway and finally to lactate via the glycolytic pathway, galactose 6-phosphate is utilized through the tagatose 6-phosphate pathway resulting in the production of additional lactic acid (Kandler, 1993).

The heterofermentative pathway (Figure 3) (leuconostocs and heterofermentative lactobacilli) is initiated by the oxidation of glucose 6-phosphate to 6-phosphogluconate, followed by decarboxylation of the hexose moiety. The resulting pentose moiety (xylulose-5 phosphate) is subsequently split by the phosphoketolase enzyme into a triose phosphate (glyceraldehydes-3 phosphate) and acetyl phosphate. Depending on hydrogen acceptors available, the acetyl phosphate is either metabolized to acetic acid with concomitant ATP generation or it is reduced by dehydrogenases to acetaldehyde and ethanol. The triose phosphate is further metabolized via the glycolytic pathway and excreted as lactic acid.

Although homofermentative lactic acid bacteria lack the enzyme phosphoketolase, some homofermentative lactic acid bacteria exhibit a heterofermentative end-product pattern comprising formic acid, acetic acid and ethanol (Cogan et al., 1989). These products are formed due to relatively low intracellular levels of fructose 1, 6-bisphosphate an essential activator of lactate dehydrogenase particularly when they are grown in glucose or lactose-limited non aerated chemostat cultures. These strins are called facultative homofermentative lactic acid bacteria. The optical configuration of lactic acid by lactobacilli can be L (+) or D (-) isomer. The isomer of lactic acid depends on the sterio specificity of NAD⁺ linked lactate dehydrogenase. It has been reported that the L (+) form accounted for 90% of the
total lactic acid (Condon, 1987). The accumulation of lactic acid end products and the concomitant low pH results in a wide inhibitory spectrum including both Gram-positive and Gram-negative bacteria (Adams and Hall, 1988). The specific effect on the microbial cell is also by the accumulation of lactic acid. It’s undissociated form can penetrate the microbial cell and at the higher with essential metabolic functions such as substrate translocation and oxidative phosphorylation, thus reducing the intracellular pH.
Figure 2. Carbohydrate metabolism in homofermentative lactic acid bacteria
Figure 3. Carbohydrate metabolism in heterofermentative lactic acid bacteria
Lactobacilli play an important role in the physiological acidification of vagina (Hill et al., 1985). Lactic acid produced by lactobacilli has long been believed that the basis of a protective role of these organisms against vaginal infection (Hawes et al., 1996). When pH of vagina is lower by such acid from these bacteria, some pathogens such as Neisseria gonorrhoeae are significantly killed (Zheng et al., 1994).

### 3.2 Short chain fatty acids (SCFAs)

Short chain fatty acids (SCFAs) or volatile fatty acids are used as a common name for monocarboxylic acids with length up to 6 or sometimes 8 carbon atoms. The molecular weight of SCFAs is low. Their molecules are polar and soluble in water. They are weak acids and the pKa value is around 4.8. Majority of anaerobic microbial in the large vagina use the carbohydrate fermentation (Wolin and Miller, 1983). SCFAs such as acetate, propionate and butyrate are the major end products of this metabolism (Cumming, 1984). Electron sink products i.e., lactate, ethanol, hydrogen and succinate are also included. These electron sinks products are formed to maintain redox balance during fermentation (Figure 4).

The inhibition ability of SCFAs is more active in low since undissociated forms are more bactericidal than dissociated ones due to their ability to penetrate into bacterial cells. Furthermore, SCFAs are especially antimicrobial under the low oxidation-reduction potential environment (Kashet, 1987).
Figure 4. Carbohydrate metabolism in lactic acid bacteria to acetic acid, butyric acid and propionic acid
3.3 Hydrogen peroxide

Hydrogen peroxide is postulated to have a crucial role in protective against the overgrowth of the pathogens in the vagina, since it can be inhibitory to bacteria, fungi, viruses and mammalian cell. Hydrogen peroxide alone or in combination with halide and peroxides that are present in vaginal secretion has potent toxic properties (Klenbanoff et al., 1991).

In comparison between lactobacilli isolated from normal women and women with bacterial vaginosis, it was found that 96% and 6% of lactobacilli isolated from the former and the latter (Reid et al., 1993), respectively (Eschenbach et al., 1989). It was likely to postulate that hydrogen peroxide producing lactobacilli may prevent bacterial vaginosis through the inhibition of the intravaginal growth of the causative microorganisms.

There are many mechanisms of generation of hydrogen peroxide by lactobacilli during growth (Gotz et al., 1980). Some of these mechanisms are shown below. Lactobacilli produce hydrogen peroxide by using either glycerophosphate oxidase (Reiter et al., 1984) or lactate oxidase (Kandler et al., 1983) to reduce oxygen directly.

\[
\text{Pyruvate oxidase} \\
\text{Pyruvate} + \text{O}_2 + \text{PO}_4^{3+} \rightarrow \text{acetyl phosphate} + \text{CO}_2 + \text{H}_2\text{O}_2
\]

Furthermore, in the presence of hydrogen peroxide, these superoxide anion can result in the formation of inhibitory hydroxyl radical. Hydrogen peroxide toxicity may result from the peroxidation of membrane lipids, which would explain the increased membrane permeability caused by hydrogen peroxidase. The resulting bactericidal effect of these oxygen metabolites has been attributed not only their strong oxidizing effect on the bacteria cell but also to the destruction of basic molecular structures of nucleic acids and cell proteins. Finally, the hydrogen peroxide not only itself inhibits undesirable microorganisms, it may also react with other components to form additional inhibitory compounds (Archibald and Fridovich, 1981).
Gunsalus and Umbreith (1989) were the first to show that *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus* produced hydrogen peroxide. Thereafter, lactic acid bacteria including lactobacilli, lactococci and pediococci were reported to generate hydrogen peroxide. Lactobacilli were found to produce hydrogen peroxide differently among strains even in the same species. Collins and Aramaki (1980) found that strains A and B of *L. acidophilus* produced larger amounts, especially if agitation continuous during growth at 37°C or storage at 4°C. Continuous shaking was required at 4°C for strain C or D to yield high cell concentration and to produce sufficient hydrogen peroxide in order to retard the growth of *Pediococcus fragi*. *L. bulgaricus* and *L. lactis* can produce hydrogen peroxide 2-6 µg/ml at 5°C to inhibit *S. aureus*. *L. plantarum* produces 3-13 µg/ml to prolong the lag period of *Pseudomonas* sp. (Price et al., 1970). Attaie et al. (1987) found that the accumulation of hydrogen peroxide after 2 h of yogurt fermentation was 0.88 µg/ml. This concentration caused a difference in the population of *S. arueus* in yogurts between those with and without catalase. Hydrogen peroxide can be reduced significantly in the presence of catalase. Bucker et al. (1982) and Flower et al. (1977) demonstrated that when *S. aureus* catalase activity was reduced by thermal stress and NaCl, hydrogen peroxide increased during aerobic growth and inability of the cells to destroy this toxic compound resulted in the loss of colony forming ability of this organism.

### 3.4 Bacteriocin

Bacteriocin are proteinaceous, bactericidal, antibiotic-like substance, apparently protein in nature, which are produced by many bacteria (Reeves, 1965). It causes a rapid killing of microbes, often within minutes, and exhibit a broad spectrum of activity, targeting both Gram-positive and - negative bacteria, causing the outer membrane damage (Hancock et al., 2000).
The bacteriocins from lactic acid bacteria have been studied extensively by many workers (Jack et al. 1995). They are divided into four distinct classes (Klaenhammer, 1993):

- **class I**, lantibiotics small (< 5 kDa) membrane active peptides e.g. nisin, lacticin 481, carnocin U149, and lactocin S.

- **class II**, small (<10 kDa), relatively heat stable, non-lanthionine containing peptides and divided into three subclasses, namely IIa, IIb and IIc on the basis of either their distinctive N-terminal sequence, their formation of biocomponent pores or the presence of a functional sulphydryl group e.g., pediocin PA-1, leucocin A.

- **class III**, large (>30 kDa), heat labile proteins that may mimic the physiological activities of bacteriocin e.g., helveticin J, lactacin A and B.

- **class IV**, complex bacteriocins containing lipid or carbohydrate moieties in addition to protein e.g., plantaricin S, leuconocin S, lactocin 27.

Klaenhammer (1993) class II peptide bacteriocins are commonly produced by lactobacilli, while a few class I bacteriocins have been isolated and characterized.

Bacteriocin production is a growth phase dependent process and the other important factors which influence bacteriocin production are: growth medium composition, pH and temperature. The optimum pH for the production of some lactobacilli bacteriocins is usually between 5 and 7 (Muriana et al., 1991).

**Mechanism of action of bacteriocin**

LAB bacteriocins are a heterogeneous group of peptides with different spectra of antimicrobial activity, different genetic determinants and distinct biochemical characteristics (Klaenhammer, 1993). Most bacteriocins are amphiphilic and cationic. A common structural motif may underlay their antimicrobial activity as suggested for other antimicrobial peptides occurring in nature (Ojcius & Young, 1991). Based on bacteriocins amphiphilic characteristics,
there are at least two different mechanisms which may explain their membrane-permeabilization action. Bacteriocins may act by a poration complex in which bacteriocin monomers bind, insert and oligomerize in the cytoplasmic membrane to form a pore with the hydrophilic residues facing inward and the hydrophobic ones facing the hydrophobic regions of phospholipid molecules in the interior of the membrane. Alternatively, bacteriocins may destabilize the integrity of the cytoplasmic membrane in a detergent-like fashion (Figure 5). It is appropriate to say a few words about the binding step. Given the wide spectra of activity displayed by LAB bacteriocins, and that some strains are sensitive to some bacteriocins while insensitive to others, the question of a receptor arises. Some bacteriocins are active both on cells and on lipid bilayers.
Figure 5. Interaction of bacteriocin monomers (ovals) with the cytoplasmic membrane according to the ‘poration complex’ model (A) and the ‘detergent disruption’ model (B) (Ojcius & Young, 1991)
4. Factors affecting on the survival of lactobacilli in vaginal

4.1 pH

The environment of vaginal tract has effect on the survival of lactobacillus. Lactobacilli have been recognized as the predominant microflora of the healthy human vagina to maintain a pH of < 4.5 (Redondo-López et al., 1990). This low pH reduces the risk of colonization by pathogens. Bacterial vaginosis, the most common vaginal pathology worldwide, is characterized by a vaginal pH of > 4.5 and by an overgrowth of anaerobic bacteria (Eschenbach, 1993). An increase in vaginal pH is detrimental to the survival of lactobacilli; therefore, local acidification with lactic acid or lactobacilli is useful for restoration of the vaginal ecosystem (Melis et al., 2000).

Under conditions of good growth for L. acidophilus CRL 1259, the final pH values reached (3.5–4.6) were comparable to those determined in the healthy vagina (Andersch et al., 1986). Boskey et al. (1999) reported that eight vaginal Lactobacillus strains acidified the growth medium to an asymptotic pH of 3.2–4.8. This fact suggests that lactobacilli create an acidic environment that can inhibit the growth of other microorganisms.

Optimal pH and temperature for maximum production of lactic acid were the same as those required for growth. Levels of total lactic acid produced by this microorganism under different culture conditions were higher than those found in vaginal secretions of women (Boskey et al., 2001).

4.2 Antibiotic therapy

Antibiotics were introduced for the treatment of microbial diseases. Since then, the greatest threat to the use of antimicrobial agents for therapy of bacterial infections has been the development of antimicrobial resistance in pathogenic bacteria (Hughes and Datta, 1983).

Antibiotics treatments for nonpregnant women often include intravaginal therapy with an antimicrobial agent. Several topical intravaginal antimicrobial therapies,
including 2% clindamycin single-dose and standard-release vaginal creams and a 0.75% metronidazole vaginal gel, have been approved for use in various patient populations for the treatment of bacterial vaginosis. The therapeutic goal of these treatments is to allow the reestablishment of the normal vaginal environment by decreasing the abnormal flora associated with bacterial vaginosis, while avoiding a negative impact on the growth of the normal *Lactobacillus* species. Clindamycin and metronidazole have both been demonstrated to be effective treatments for bacterial vaginosis, but their effectiveness may be limited by negative effects on the growth of the normal vaginal microflora (Simoes *et al.*, 2001). They have particularly concerned with clindamycin because it has an *in vitro* spectrum of activity that covers lactobacilli (Bayer *et al.*, 1978). However, studies with both clindamycin and metronidazole suggested that high concentrations of these medications, such as those achieved with intravaginal therapy for bacterial vaginosis, may inhibit the growth of *Lactobacillus* species (Aroutcheva *et al.*, 2001). There are relatively few data on the effect of intravaginal clindamycin and metronidazole treatment on *Lactobacillus* species *in vivo*. These data suggest that intravaginal metronidazole has little effect on the growth of lactobacilli, whereas intravaginal clindamycin causes an initial but transient decrease in lactobacilli (Hillier *et al.*, 1993).

**4.3 Antibiotic resistance**

Bacterial resistance to antibiotics is now a major social problem. It is accelerating and increases morbidity, mortality and health-care costs. More prudent use of antibiotics would reduce the selective pressure which favors the development of resistance (Levy, 1992). Recently many investigators have speculated that commensal bacteria may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens (Levy and Salyers, 2002) and are thus very important in our understanding of how antibiotic resistance genes are maintained and spread through bacterial populations (Levy and Miller, 1989). The main
threat associated with these bacteria is that they can transfer resistance genes to pathogenic bacteria.

The greatest threat to the use of antibiotics is the emergence and spread of resistance in pathogenic bacteria that consequently cannot be treated by previously successful regimens. Extensive recent reviews of the application of antibiotics in human and veterinary medicine (WHO, 1997), agriculture (Falkiner, 1998) and aquaculture (Reilly and Kaferstein, 1997) have documented the evolution and enrichment of antibiotic resistant bacteria: the phenomenon is regularly observed upon the introduction of a new antibiotic (Levy, 1997). Development of antibiotic resistance in bacteria is mainly based on two factors, the presence of resistance genes and the selective pressure by the use of antibiotics (Levy, 1992).

### 4.4 Bacterial adhesion

During the first decade of intense research on the adhesion of microorganisms to various substrata a number of points had become clear. The first, there is little doubt that the survival of microorganisms in various niches is dependent on their ability to adhere to surfaces or substrata. The second, the adhesion process involves an interaction between complementary molecules on the respective surfaces of the microbe and substratum. The third, the expression by the organisms of the macromolecules that participate in the adhesion process is under a number of regulatory control mechanisms. Bacteria adhere only to complementary substrata. They adhere by ionic or coulombic interaction, by hydrogen bonding, by the hydrophobic effect (Duncan-Hewitt et al., 1990), and by coordination complexes involving multivalent metal ions.

Adhesion is an essential property and it is the first step of colonization. The adhering of such bacteria to epithelium can act as a defense mechanism of the host. *Lactobacillus* strains possesses high adherence ability is advantage for prevention of adherence and colonization of pathogen. Hallen et al. (1992) reveal in their study that the treatment of 60 patients with bacterial vaginosis with lyophilized *L. acidophilus* which produced hydrogen peroxide was
effective in increasing the colonization of lactobacilli and reducing the number of bacteroides as well as the persistence of bacterial vaginosisis.

Adhesion of lactobacilli depends on the strain, environment and the physical nature of various surface involved (Savage, 1992). The growth phase and culture conditions also affect the extracellular structure of the cells (Cook et al., 1988). Lipoteichoic acid and glycocalyx of the cell wall have been involved in the attachment of lactobacilli (Graham et al., 1986) and it was found that lipoteichoic and surface proteins were affected by growth conditions. A proteinaceous surface layer of L. acidophilus with the strong adherence to the crop and caecum of an adult fowl was responsible for the adhesion (Schneitz et al., 1993). Selection of strains with the capacity of adherence can be base on in vitro study even though it is hard to extrapolate the results to the in vivo situation (Havenaar et al., 1992). It was believed that bacteria showed high adhesion ability to different surfaces, they may have high probability to adhere and colonization in vagina.

5. Suppositories

Long-term antibiotic prophylaxis is the most common method for managing recurrent urinary tract infection (UTI). However, antibiotic use leads to the increased urogenital tract abnormal in patients presence of drug-resistant organism and many patients suffer from yeast vaginitis as a result of the disruption of normal levels of intestinal and vaginal flora. In such case, it has been noted that probiotic, which are define as living microorganisms that can be administered to promote the health of the host (FAO and WHO, 2001) by treating or preventing disease, can be used as an alternative preventative approach. The flora of the urogenital tract is abnormal in patients with recurrent UTI compared with those of healthy women (Schaeffer et al., 1977). This fact leads to the investigation of the role of the flora, particulary lactobacilli, in maintaining urogenital health and reducing the risk of infections. The use of probiotics to restore
the normal vaginal flora and to provide a competitive bacterial barrier is becoming increasingly acceptable (Uehara et al., 2006).

Suppositories are solid dosage form intended for insertion into body orifices where they melt, soften, or dissolve and exert localized or systemic effects. Suppositories are commonly employed rectally, vaginally, and occasionally urethrally. They have various shapes and weights. The shape and size of a suppository must be such that it is capable of being easily inserted into the intended body orifice without causing undue distension, and once inserted, it must be retained for the appropriate period of time.

**Appearance:** When suppositories are cut longitudinally and examined with the naked eye the internal and external surfaces are uniform in appearance.

**Disintegration:** Unless otherwise stated in the monograph, suppositories comply with the disintegration test for suppository.

**Suppository base**

Analogous to the ointment bases, suppository bases play an important role in the release of the medication they hold and therefore in the availability of the drug for absorption for systemic effect or for localized action. Of course, one of the first requisite for a suppository base is that it remains solid at room temperature but softens, melts, or dissolves readily at body temperature so that the drug it contains may be made fully available soon after insertion. Certain bases are more efficient in drug release than others.

Vaginal suppositories are usually globular or oviform and weigh about 5 g each. They are made from water soluble or water miscible bases such as polyethylene glycol or glycerinated gelatin (USP 27, 2007).

**Polyethylene glycol suppository base**

Several combinations of polyethylene glycols having melting temperatures above body temperature have been used as suppository base. Drug release from these bases depends on dissolution rather than on melting, there are significantly fewer problems in
preparation and storage than exist with melting-type vehicles. However, high concentrations of higher molecular weight polyethylene glycols may lengthen dissolution time, resulting in problems with retention. Labels on polyethylene glycols suppositories should contain directions that they be moistened with water before inserting. Although they can be stored without refrigeration, they should be packaged in tightly closed containers (USP 27, 2007).

In the preparation of vaginal suppositories, the most commonly used base consists of combinations of the various molecular weight polyethylene glycols. This base is frequently added surfactants and preservatives, commonly the parabens. Many of the vaginal suppositories and other types of vaginal dosage forms are buffered to an acid pH, usually around pH 4.5 which resembles that of the normal vagina. This acidity discourages pathogenic organisms and at the same time provides a favorable environment for eventual recolonization by the acid producing bacilli normally found in the vagina (Howard et al., 1995).

Lactobacillus is viable and instable during the heating process of suppository. Therefore, in this study hollow-type suppository was formulated in stead of conventional suppository. Hollow-type suppository have some advantages over conventional suppositories such as they can carry either powdered or solution forms of drugs and they eliminate the effect of the heating process on the survival of lactobacilli or drug during the preparation of the suppository (Kaewnopparat et al., 2004). Watanabe et al. (1986) demonstrated that drug was release more rapidly from a hollow-type suppository than from conventional suppository.