Isolation and Investigation of the Effect of *Lactobacillus* spp. on Vaginal Bacterial Pathogens and Formulation of Vaginal Suppositories

Sukanya Settharaksa

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Thesis Title	Isolation and Investigation of the Effect of *Lactobacillus* spp. On Vaginal Bacterial Pathogens and Formulation of Vaginal Suppositories
Author	Miss Sukanya Settharaksa
Major Program	Pharmaceutical Sciences

Major Advisor

.....

(Asst. Prof. Dr. Sanae Kaewnopparat)

Co-advisor

.....

(Assoc. Prof. Nattha Kaewnopparat)

Examining Committee :

.....Chairperson

(Asst. Prof. Dr. Wimonman Sriroongrueng)

.....Committee

(Asst. Prof. Dr. Sanae Kaewnopparat)

.....Committee

(Assoc. Prof. Nattha Kaewnopparat)

.....Committee

(Asst. Prof. Dr. Nimit Worakul)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Pharmacy Degree in Pharmaceutical Sciences

….....

(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

ชื่อวิทยานิพนธ์	การแยกเชื้อและการศึกษาผลของเชื้อ *Lactobacillus* spp. ต่อแบคทีเรียที่ก่อโรคในช่องคลอดและการเตรียมยาเหน็บช่องคลอด *Lactobacillus* spp.
ผู้เขียน	นางสาวสุกัญญา เศรษฐรักษา
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บทคัดย่อ

Lactobacilli ในช่องคลอดมีบทบาทสำคัญต่อสุขภาพของช่องคลอด เช่น ช่วยควบคุม pH, ยับยั้งเชื้อก่อโรคในช่องคลอด และช่วยเสริมภูมิคุ้มกันเป็นต้น การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อแยก *Lactobacillus* spp. มาใช้เป็น probiotic เพื่อต้านเชื้อก่อโรคในช่องคลอด โดยแยกเชื้อจากช่องคลอดผู้หญิงสุขภาพดีและไม่เป็นโรคในช่องคลอด จำนวน 120 คน ซึ่งสามารถแยกเชื้อ *Lactobacillus* spp. ได้จำนวน 80 isolates และพบว่ามี 16 isolates ที่มีฤทธิ์ในการยับยั้งเชื้อ *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 แต่ไม่สามารถยับยั้งเชื้อ *Candida albicans* NCPF 3153 ได้ จากเชื้อ 16 isolates มีเชื้อ 4 isolates คือ *Lactobacillus* sp. SK5, SK6, SK7 และ SK8 ได้รับคัดเลือกมาศึกษาความไวของเชื้อกับยาปฏิชีวนะ พบว่าเชื้อทุกตัวไวต่อ ampicillin, bacitracin, cefoperazone, ceftazidime, cephalothin, chloramphenicol, erythromycin, penicillin G และ tetracycline และดื้อต่อ kanamycin, ketoconazole, metronidazole, norfloxacin, nystatin, streptomycin, vancomycin เมื่อนำเชื้อทั้ง 4 isolates มาทดสอบความสามารถในการผลิต H_2O_2 และความสามารถในการยึดเกาะกับ HeLa cell พบว่า *Lactobacillus* sp. SK5 สามารถผลิต H_2O_2 และยึดเกาะกับ HeLa cell ได้ดีที่สุด เมื่อดูการส่งเสริมการเกาะของเชื้อด้วยพอลิเมอร์ต่าง ๆ คือ hydroxypropylmethylcellulose (HPMC-E50), sodiumcarboxymethylcellulose (Na-CMC) และ polyvinylpyrrolidone (PVP) พบว่า PVP เพิ่มการเกาะของเชื้อดีที่สุด เมื่อนำ *Lactobacillus* sp. SK5 มาจำแนกหาสปีชีส์ด้วย 16S rDNA พบว่าเป็น *L. fermentum* นอกจากนี้สารโมเลกุลใหญ่ที่ผลิตโดยเชื้อนี้สามารถยับยั้งแบคทีเรียก่อโรคในช่องคลอดคือ *Pseudomonas aeruginosa, S. aureus, Gardnerella vaginalis, E. coli* และ *Neisseria gonorrhoeae* ได้ ซึ่งเชื่อว่าเป็นแบคเทอริโอซิน เมื่อนำ *Lactobacillus* sp. SK5 มาเตรียมเป็นยาเหน็บช่องคลอดชนิดกลวงโดยใช้ส่วนผสมของ PEG เป็นยาพื้น เมื่อประเมินผลยาเหน็บที่ได้พบว่ามีเชื้ออยู่แท่งละ 1.32×10^8 cfu มีน้ำหนักเฉลี่ย 2.32 กรัม มีจุดหลอมเหลวอยู่ในช่วง 35-44 °ซ. สามารถแตกกระจายตัวได้ในเวลา 8.5 นาที และปลดปล่อยตัวเชื้อได้หลังจากเวลา 25 นาที ยาเหน็บช่องคลอดที่เตรียมได้นี้มีคุณสมบัติต่างๆ ดี จากการศึกษาครั้ง

นี้พบว่า *Lactobacillus* sp. SK5 มีคุณสมบัติที่ดีหลายประการซึ่งควรจะนำมาพัฒนาเป็น probiotic เพื่อต้านเชื้อแบคทีเรียก่อโรคในช่องคลอดต่อไป

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ABSTRACT

Lactobacilli in vagina play an important roles for the healthy of vagina by various activities such as control vaginal pH, inhibit vaginal pathogens and enhance immune response. The aim of this study was to isolate *Lactobacillus* spp. to be used as probiotic against vaginal pathogens. From 120 women vaginal samples, eighty *Lactobacillus* spp. were isolated. Sixteen isolates showed inhibition activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 but none of them could inhibit *Candida albicans* NCPF3153**.** Four of sixteen isolates, *Lactobacillus* sp. SK5, SK6, SK7 and SK8, were tested for their sensitivity to antibiotics. They were all sensitive to ampicillin, bacitracin, cefoperazone, ceftazidime, cephalothin, chloramphenicol, erythromcycin, penicillin G, tetracycline and resistance to kanamycin, ketoconazole, metronidazole, norfloxacin, nystatin, streptomycin and vancomycin. Among them, *Lactobacillus* sp., SK5 was the best H_2O_2 producer and showed the highest adhesion ability to HeLa cells. When bioadhesive polymer such as hydroxypropylmethylcellulose (HPMC-E50), sodiumcarboxymethylcellulose (Na-CMC) and polyvinylpyrrolidone (PVP) were tested for bacterial adhesion enhancement, it was found that PVP was the best one. *Lactobacillus* sp. SK5 was later identified as *L. fermentum* by 16S rDNA. Furthermore, high molecular weight substances produced by this bacterium inhibited vaginal pathogenic bacteria i.e., *Pseudomonas aeruginosa, Staphylococcus epidermidis, Gardnerella vaginalis, E. coli* and *Neisseria gonorrhoeae*. This substance (s) is expected to be a bacteriocin. Hollow-type vaginal suppository containing *Lactobacillus* sp. SK5 was prepared using combination of PEGs as the suppository base. Each suppository contained $1.32x10^8$ cfu of bacteria with average weight 2.32 g, melting point 35-44 $^{\circ}$C., disintegration time 8.5 min and bacteria were released from the suppository after 25 minutes. The properties of suppository are

acceptable. *Lactobacillus* sp. SK5 is a promising candidate that could be developed as a probiotic against vaginal bacterial pathogens.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	percent
µg	microgram
µl	microliter
µm	micrometer
cfu	colony forming unit
cm	centimeter
Da	Dalton
g	gram
kDa	kiloDalton
l	liter
mg	milligram
ml	milliliter
mm	millimeter
N	normal
^{o}C	degree Celcius
SD	standard deviation
SEM	scanning electron microscopy
v/v	volume by volume
w/v	weight by volume

CHAPTER 1

INTRODUCTION

General Introduction

Recently, an increasing interest has developed in microbiota, that promote a women' 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 alblicans

C. alblicans 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 blood stream and effect the throat, intestines, and heart valves. *C. alblicans* becomes an infectious agent when there is some charge 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 β-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 *Neiserria 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

cases, conflicting reports are available to support or refute the association of specific lower genital tract infections with preterm birth. 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 a1*. (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).

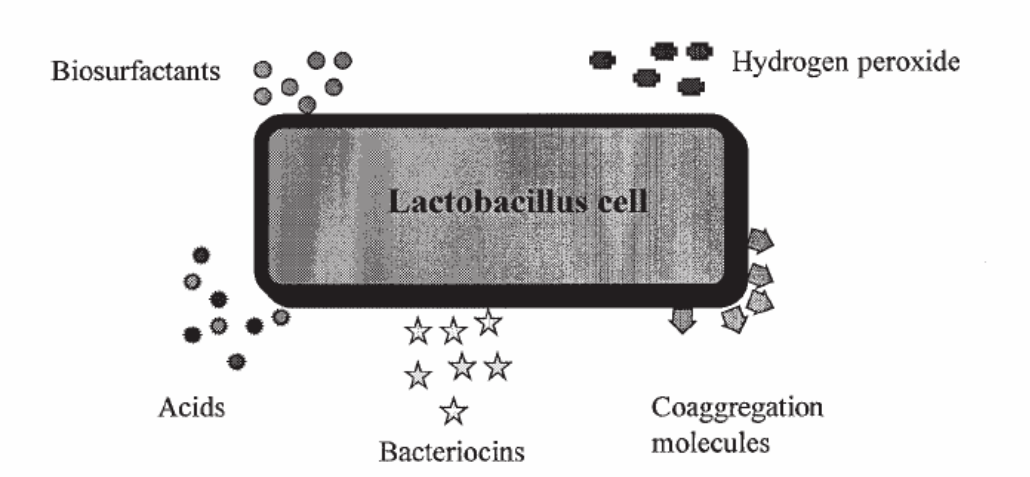


Figure 1. By products of lactobacillus metabolism that have an antagonistic effect against urinary and vaginal pathogens; the biosurfactants inhibit adhesion; the acids, bacteriocins, and hydrogen peroxide inhibit growth; and the coaggregation molecules block the spread of the pathogens (Korshunov *et 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 futher 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 acerated 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.

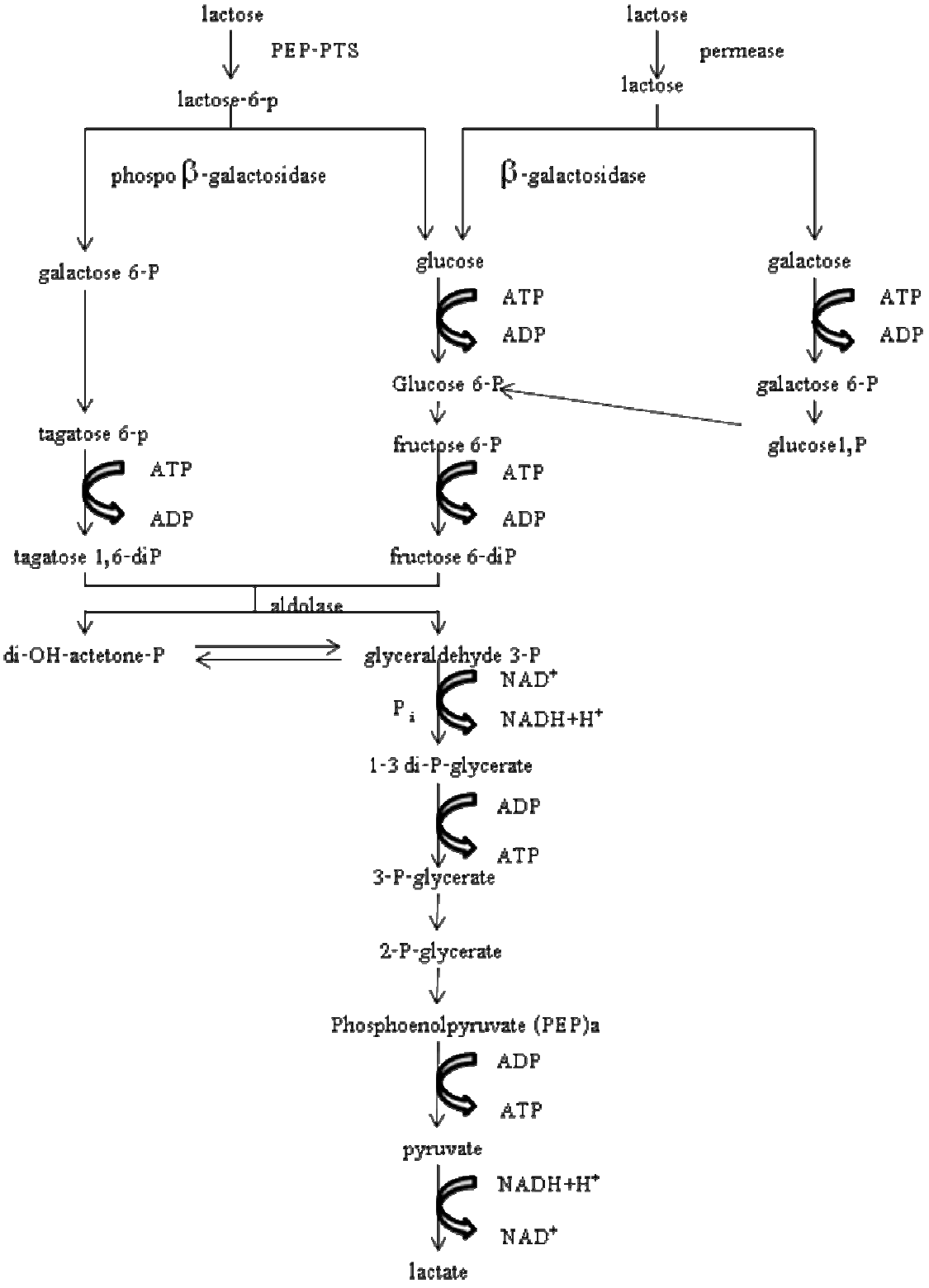


Figure 2. Carbohydrate metabolism in homofermentative lactic acid bacteria

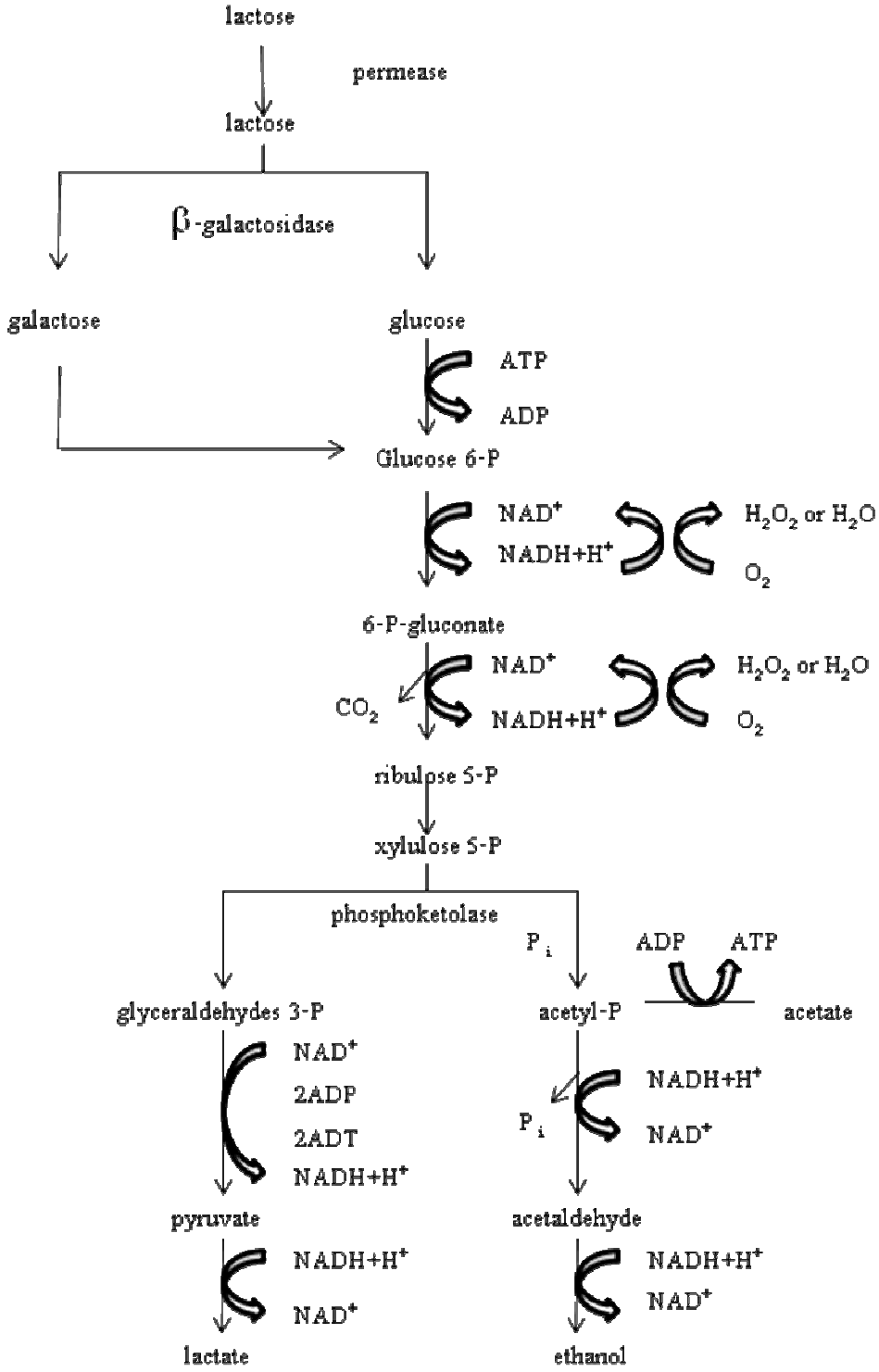


Figure 3. Carbohydrate metabolism in heterofermentative lactic acid bacteria

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 moleculars 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).

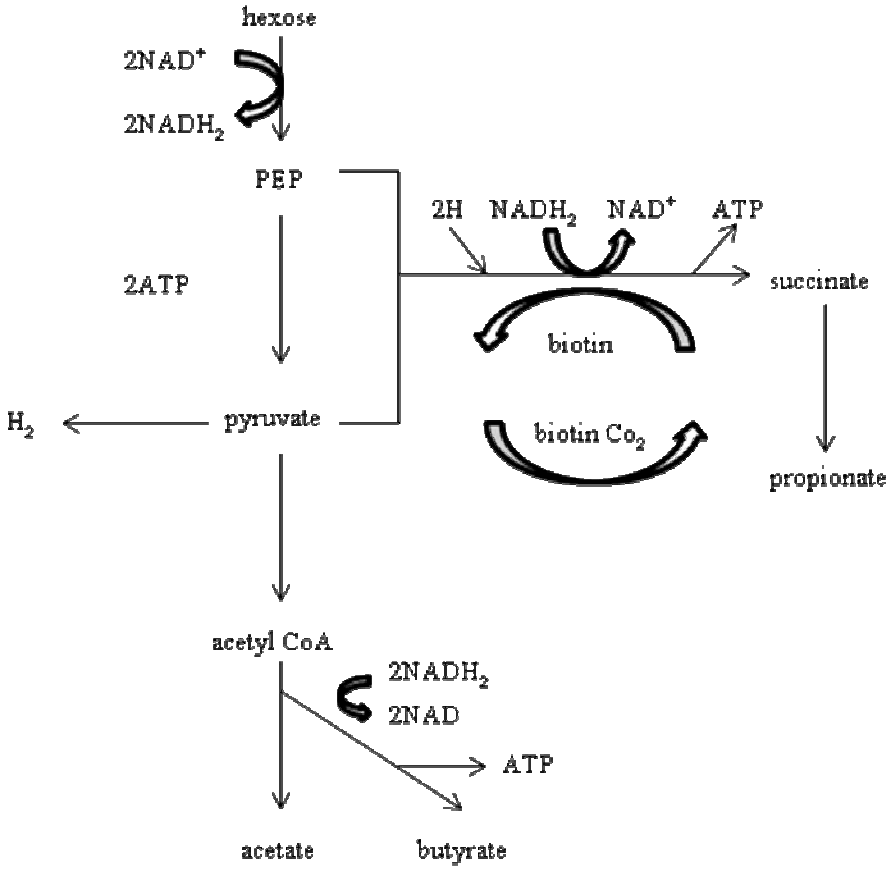


Figure 4. Carbohydrate metabolism in lactic acid bacteria to acetic acid, butyric acid and propionic acid

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.

$$\text{Pyruvate} + O_2 + PO_4^{3+} \xrightarrow{\text{Pyruvate oxidase}} \text{acetyl phosphate} + CO_2 + H_2O_2$$

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. There after, 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 devided 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 sulhydryl 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.

Klaenhanmmer (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.

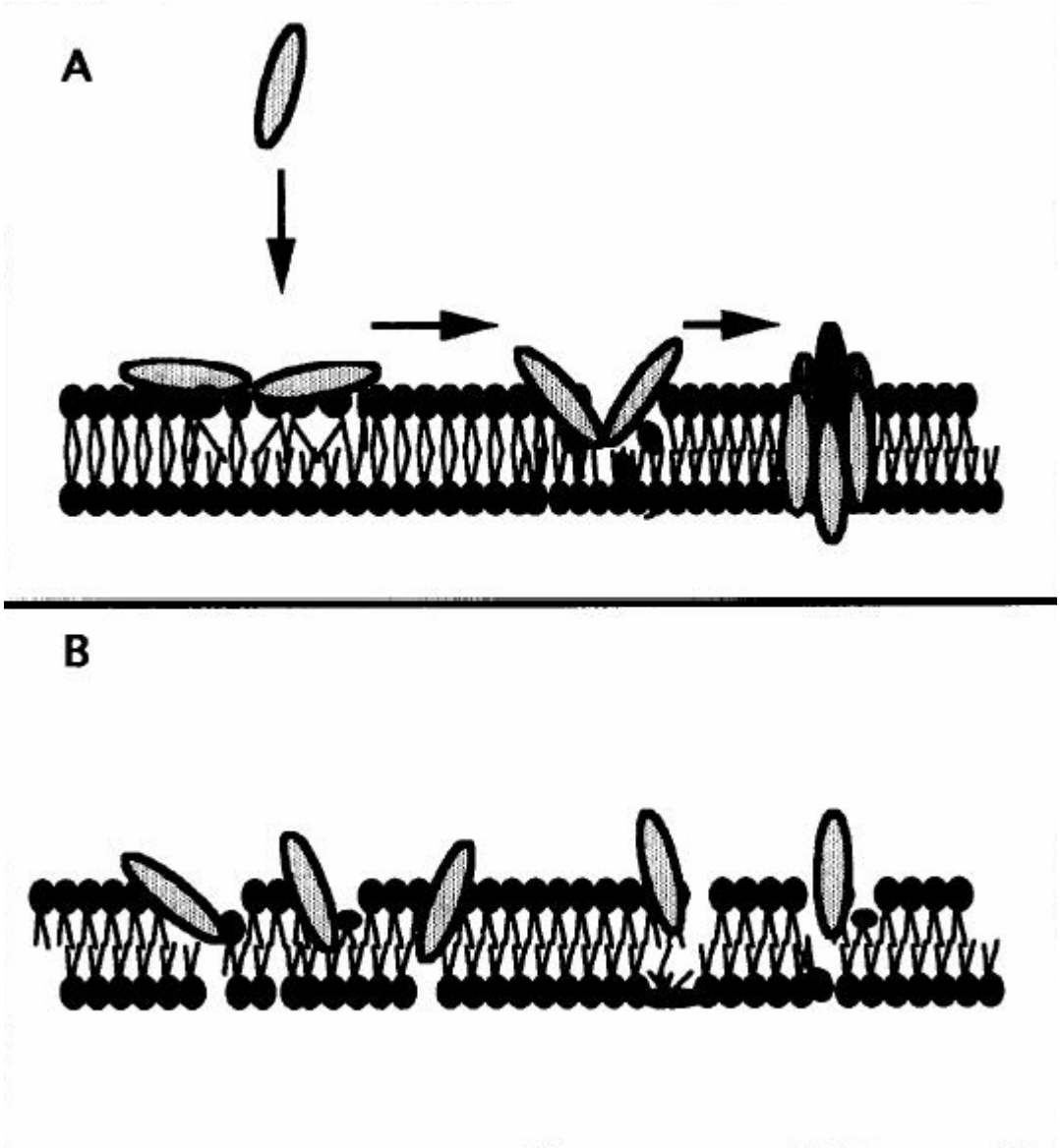


Figure 5. Interaction of bacteriocin monomers (ovals) with the cytoplasmic membrane according to the 'poration complex' model **(A)** and the 'detergent disruption' model (B) (Ojcius & Young, 1991)

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-Lo´ pez *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 particulary 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 vaginosis.

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 approporiate 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.

CHAPTER 2

MATERIALS AND METHODS

1. Lactobacilli isolation and preservation of lactobacilli isolates

Vaginal samples for lactobacilli isolation were obtained from Songkhla Hospital in the South of Thailand. The women involved in this study were aged 18–40 years old. They were premenopausal, nonmenstruating, lack of vaginosis and vaginitis, and negative hepatitis and HIV serologies. The samples were taken with sterile cotton swabs from the posterior zone of the fonix of the vagina. The swabbing process was performed by doctor and nurse. The cotton swabs were immediately transferred into MRSB (Man Rogosa and Sharpe Broth) (Merck) and incubated in anaerobe jar containing gas pak microbiology anaerocult® A (Merck) at 37 °C for 24 hours. The growing bacteria in MRSB were diluted with half strength of MRSB to obtain dilution $10^{-1} – 10^{-6}$. Ten µl of appropriate dilution was spreaded on MRSA (Man Rogosa and Sharpe Agar) (Merck) plate and incubated in anaerobic condition at 37 °C for 48 hours. Five to ten colonies growing on MRSA surface were selected for Gram staining, catalase test and observed for spore formation. The colonies with Gram positive rod, catalase negative and no spore forming were assumed as *Lactobacillus* spp.

Preservation of *Lactobacillus* spp. at -80 °C

The isolates *Lactobacillus* spp. were subcultured by restreaking on MRSA plates and incubated under anaerobic condition at 37 °C for 48 hours. The growth colonies were transferred into MRSB and incubated in the same condition for 24 hours. The sterile glycerol was added to the culture to make 20% v/v final concentration. The cultures were dispersed about 1 ml into sterile cryotubes. They were placed in refrigerator about 2 hours then kept in -80 °C freezer.

Preservation of *Lactobacillus* spp. in lyophilized form

After growing *Lactobacillus* spp. isolates on MRSA plate under anaerobic condition at 37 °C for 48 hours. The colonies were washed from the agar surface with sterile 20%w/v skim milk and transferred 100 µl with micropipette to sterile glass ampule and plugged with sterile cotton about 2.5 cm below the mouth. The ampules were placed in -80 °C freezer for 24 hours and immediately connected to lyophilizer for 6 hours until the samples completely dried. The ampules were sealed by flaming above the cotton plug. The lyophilized cultures were kept in refrigerator and tested for their survival and purity.

2. Antimicrobial screening of isolated *Lactobacillus* spp. against bacteria and yeast by overlay method

Selected *Lactobacillus* spp. were tested for their antimicrobial activity against standard reference strains of Gram negative and Gram positive bacteria i.e., *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 by overlay method as following details. Furthermore, they were also tested for antifungal activity against *Candida albicans* NCPF3153.

2.1 Inoculation of *Lactobacillus* spp. on MRSA plates

The isolates *Lactobacillus* spp. kept at -80 °C were cultured in MRSB in anaerobic condition at 37 °C for 48 hours. The cultures were streaked on MRSA and incubated in anaerobic condition at 37 °C for 48 hours. The isolated colonies from MRSA were picked up and smeared on new MRSA with diameter 6 mm and incubated in anaerobic condition at 37 °C for 48 hours. Each sample was smeared two spots on each of two MRSA plates.

2.2 Preparation of tested reference standard strains

The standard reference strains of bacteria and yeast used in this experiment were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* NCPF 3153. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were cultured on BHIA (Brain Heart Infusion Agar) (Merck) and *C. albicans* NCPF 3153 was cultured on SDA (Sabouraud Dextrose Agar) (Merck). The bacteria and yeast cultures were incubated under aerobic condition at 37 °C for 24 hours. The growth colonies on agar surfaces were harvested by washing with sterile normal saline and adjusted with sterile normal saline to obtain turbidity equal to 0.5 McFarland turbidity standard for bacteria and 2.0 McFarland turbidity standard for yeast.

2.3 Inoculation reference standard strains on *Lactobacillus* spp. colonies

MHA (Mueller-Hinton Agar) (Merck) and SDA media were used for bacteria and yeast, respectively. They were sterilized and cooled down to 50 °C before adding 0.1% of the turbidity adjusted strains. Five millilitres of tested cultures were overlayed on the lactobacilli colonies. The plates were incubated at 37 °C for 18 hours for bacteria and 24 hours for yeast. The inhibition zones around *Lactobacillus* spp. colonies were observed. The colonies with antimicrobial activity were collected for further studies.

3. Antimicrobial activities test of cell-free supernatant from selected *Lactobacillus* spp. by disc diffusion method

3.1 Preparation of *Lactobacillus* spp. cell-free supernatant

The selected isolates of *Lactobacillus* spp. were grown in 10 ml MRSB. They were incubated in anaerobic condition at 37 °C for 48 hours. After inoculation, cell-free supernatant was obtained by centrifuging the culture at 6000 g for 15 minutes. The cell-free

supernatants were measured for their pH and a part of them was adjusted with 1 N NaOH to pH 6.5. The original supernatant and supernatant with pH 6.5 were lyophilized to obtain dry powder. The dry powder was then reconstituted with distilled water to make twenty times (20x) of original supernatant concentration. Twenty microliters of 20x supernatant was added to 6 mm sterile paper disc and tested for its antimicrobial activity.

3.2 Preparation of tested microorganisms

The same microorganisms used in **Section 2** i.e., *E.* coli ATCC 25922, *S. aureus* ATCC 25923 and *C. albicans* NCPF 3153 were used again in this experiment. Bacteria were grown on MHA and yeast was grown on SDA, at 37 °C for 24 hours. Sterile normal saline was used to wash growing colonies from the agar surface and the turbidity of each tested microorganism was adjusted to obtain turbidity comparable to 0.5 and 2.0 McFarland turbidity standard for bacteria and yeast, respectively, using sterile normal saline.

3.3 Inoculation of tested microorganisms on agar surface

A sterile cotton swab was dipped into the inoculum prepared in **Section 3.2** and the excess was removed by rotating the swab several times against inside the wall of the tube above the fluid level. The surface of MHA plates were inoculated by streaking the swab over the surface. Streaking were repeated 3 times and for each time the plate were rotated 60 degrees**.**

3.4 Application of 20x supernatant disc on inoculated agar surfaces

The discs prepared in **Section 3.1** were placed on inoculated agar surface and the plates were incubated at 37 °C for 24 hours. The inhibition zone diameter around the discs were measured by using antibiotic zone reader.

4. Sensitivity of four selectes *Lactobacillus* spp. to antibiotics

From the experiment in **Section 2 and 3** *Lactobacillus* spp. isolates named *Lactobacillus* sp. SK5, SK6, SK7 and SK8 showed high activity against bacteria but not yeast were selected for further investigation of their activity. The *Lactobacillus* sp. SK5, SK6, SK7 and SK8 were grown in MRSB and incubated in anaerobic condition at 37 °C for 48 hours. The cultures were adjusted with normal saline to give turbidity equal to 0.5 McFarland turbidity standard. The adjusted cultures were streaked with sterile cotton swab on MHA surface. Streaking was repeated 3 times and for each time the plate was rotated 60 degrees**.** The antibiotic discs i.e., ampicillin (10 µg), bacitracin (10 µg), cefoperazone (15 µg), ceftazidime (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), clindamycin (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), ketoconazole (30 µg), metronidazole (30 µg), norfloxacin (10 µg), nystatin (2000 IU), penicillin G (10 IU), streptomycin (10 µg), tetracycline (30 µg), and vancomycin (30 µg), were placed on the surface agar and incubated in anaerobic condition at 37 °C for 24 hours. The inhibition zone diameters were measured in milliliter by antibiotic zone reader.

5. Detection of hydrogen peroxide production of four selected *Lactobacillus* spp.

Four selected lactobacilli i.e., *Lactobacillus* sp. SK5, SK6, SK7 and SK8 were tested for their ability to produce hydrogen peroxide. The agar plate of MRSA containing 5 mg/ml hemin, 1 mg/ml vitamin K, 0.01 mg/ml horseradish peroxidase (Sigma), and 0.25 mg/ml tetramethylbenzidine (Sigma) as indicator were prepared. One loop of the culture was spotted on agar surface and incubated anaerobically at 37 °C, 48 hours. After incubation the plates were exposed to air. Hydrogen peroxide producing colonies showed blue or brown color. According to the color intensity, the strains were classified as strong (blue), medium (brown), weak (light

brown) or negative (white color) for hydrogen peroxide production. The colonies producing hydrogen peroxide form blue or brown color in the medium because the horseradish peroxidase oxidized the tetramethylbenzidine in the present of hydrogen peroxide.

6. Adhesion ability of four selected *Lactobacillus* spp. to vaginal epithelial

6.1 Cultivation of HeLa cell

HeLa cell is a human cervical carcinoma cell. It was purchased from ATCC (P.O. Box 1549, Manassas, VA 20108-1549, 703-365-2007 FAX: 703-365-2570, email: sales@atcc.org) HeLa cell was routinely grown in Eagle's minimal essential medium (25 mM glucose, 10 mM sodium pyruvate) (Gibco), supplement with 6% heat-inactivated fetal calf serum (Gibco), and 100 U/ml of penicillin G and streptomycin sulfate. Cells were stored in liquid nitrogen with 10% dimethysulfoxide (DMSO) as cryoprotectant. The frozen cells in cryotube were taken from liquid nitrogen and warmed in 37 $^{\circ}$C water bath for a few seconds until a portion of frozen medium melt. Approximately 0.5 ml of fresh medium was added and transferred into 10 ml of medium. The mixture was centrifuged at 1,000 g at room temperature for 5 minutes. The supernatant was discarded and cells were resuspended in 5 ml of fresh medium. Cell suspension was inoculated into 50 ml tissue culture flask and incubated in 90% air + 10% CO_2. The cells were checked under a inverted microscope every day and the medium was changed every 2-3 days.

6.2 Passage of HeLa cell

When the cells grew to 80% confluence, the culture medium was discarded and 5 ml of phosphate buffer pH 7.4 was added into the flask to wash the cells. The cells were washed two times before adding 3 ml of trypsin EDTA (1:25) to cover the cell surface and incubated at 37 $^{\circ}$C, 90% air + 10% CO_2 for 1 minute. Cells were separated to a single cell and detached the

cells from the plastic surface by tapping the side of the flask with hand. Seven ml of medium was added into the flask and removed the cells suspension into 15 ml centrifuge tube. The cell suspension was centrifuged at 1,000 g at room temperature for 5 minutes. After discarding the supernatant, the cells were resuspended with 2-3 ml fresh medium and the viable cells were counted. A hundred µl of the cell suspension was mixed with equal volume of 0.4%w/v trypan blue and incubated at room temperature for 5 minutes. The mixture was transferred to haemocytometer and counted for the number of viable and dead cells under inverted microscope. The viable cells were noticed with no color while nonviable cells were blue color. The concentration of the cells was calculated and diluted with the medium to the required concentration before dispensing into the new culture flask for continuous cultivation or to the tissue culture plate for adhesion assay.

6.3 Preservation of HeLa cell

One ml of dimethylsulfoxide (DMSO) was added into 9 ml of growth medium without antibiotic. After mixing, the tube was placed to cool down in the refrigerator. The cells with 80% growth confluence in 50 ml culture flask were used and their surfaces were washed twice with 10 ml PBS. Three ml trypsin EDTA was added and incubated at 37 $^{\circ}$C, 90% air + 10% CO_2 for 1 minute. Cells were detached from the plastic surface and 10 ml of fresh medium without DMSO was added into the flask. Cell suspension was transferred to 50 ml sterile centrifuge tube and centrifuged at 1,000 g, at room temperature for 5 minutes. The cell pellet was resuspended with 5.5 ml medium and then, the cool medium with DMSO was added dropwise into the cell suspension while the container was being shaken. One ml of the cell suspension with 5% final concentration of DMSO was dispensed to each cryotube and placed in -80 $^{\circ}$C freezer to be kept over night before storing in liquid nitrogen.

6.4 Preparation of *Lactobacillus* spp. cell for adhesion assay

Before each adhesion assay, four selected lactobacilli i.e., *Lactobacillus* sp. SK5, SK6, SK7 and SK8 were cultured in MRSB and incubated in anaerobic condition at 37 $^{\circ}$C 48 hours. *Lactobacillus* spp. cultures were centrifuged at 3,000 g for 10 minutes and washed two times using PBS pH 7.4. The cells were resuspended in 5 ml MEM medium and adjusted to $3x10^8$ cfu/ml before using for adhesion assay.

6.5 The effect of bioadhesive polymer of *Lactobacillus* sp. SK5, SK6, SK7 and SK8 on HeLa cell

Each well of 6-well tissue culture plate was inoculated with $5x10^5$ HeLa cells and incubated at 37 $^{\circ}$C, 90% air + 10% CO_2. The medium was changed every 2-3 days. After 7 days of cultivation or full confluence, the cells were used for adhesion assay. The cell monolayer surface was washed twice with PBS pH 7.4 before adding 1 ml of *Lactobacillus* sp. SK5, SK6, SK7 and SK8 $3x10^8$ cfu. For studying the effect of bioadhesive, i.e., Hydroxypropylmethylcellulose (HPMC-E50), sodiumcarboxymethylcellulose (Na-CMC) and polyvinylpyrrolidone (PVP) on adhesion of *Lactobacillus* sp. SK5, SK6, SK7 and SK8 to HeLa cells, their solutions were prepared in MEM medium to obtain the concentration of 1 mg/ml, 0.5 mg/ml and 100 mg/ml for HPMC-E50, Na-CMC and PVP, respectively. The *Lactobacillus* sp. SK5, SK6, SK7 and SK8 cells were added into each solution to make the concentration of $3x10^8$ cfu/ml before adding 1 ml of the mixture into each well of HeLa cell culture. Three wells were used for each bacterium. The inoculated tissue culture plate was incubated at 37 $^{\circ}$C, 90% air + 10% CO_2 for 1 hour. The culture medium was discarded and the cell surfaces were washed four times with 3 ml PBS pH 7.4 to remove all non-adhering *Lactobacillus* sp. SK5, SK6, SK7 and SK8 Each well was added with 1 ml of 0.05% triton X-100 water solution and incubated for 10 minutes to lyse the HeLa cells. The mixture containing lysed HeLa cells and *Lactobacillus* sp.

SK5, SK6, SK7 and SK8 were prepared 10-fold dilution with half strength MRSB and 0.1 ml of each dilution was spreaded on MRSA surface and incubated in anaerobic condition at 37 °C for 2 days and the number of viable adhering cells were evaluated.

7. Identification of selected lactobacilli using 16 S rDNA sequencing

7.1 Bacterial culture and genomic DNA purification

From the experiment in **Section 2, 3, 4, 5, and 6** *Lactobacillus* isolates named *Lactobacillus* sp. SK5 that showed promising activity against bacterial and had high adhesion to HeLa cell was selected to identify for its species. *Lactobacillus* sp. SK5 was cultured in 10 ml of MRSB and incubated under anaerobic condition at 37 °C for 48 hours. The culture was centrifuged at 3,000 g for 2 minutes. The pellet was resuspended in 1 ml of 0.01 M Sodium-phosphate buffer in 20% sucrose (pH 7.0) and lysozyme to 2.5 mg/ml, and incubated at 37 °C for overnight. Nine microliters of 1% SDS was added and incubated at 37 °C for 30 minutes. Phenol and chloroform were added to extract lysed cells and DNA was precipitated by ethanol. DNA was spooled out by glass rod and washed once with 80% ethanol before drying.

7.2 PCR reaction

The prokaryotic 16S rDNA gene was amplified by PCR with a thermal cycler. The PCR reaction contained 100 µl of final solution consisting of 10 µl 10x *Tag* buffer, 2 mM of each deoxynucleoside triphosphate (dNTP) 10 µl, 10 pmol of each primer 4 µl, 5 unit of Taq polymerase 0.5 µl, optimal concentration template 4 µl and water 59.5 µl. The oligonucleotide primers used for the bacterial 16S rRNA gene were: 20F; 5' GAG TTT GAT CCT GGC TCA G 3' and 1500R, 5' GTT ACC TTG TTA CGA CTT 3'. The thermocycle program was as follows: 94 °C for 3 minutes, 25 cycles of 94 °C for 1 minute, 50 °C for 1 minutes, and 72 °C for 2 minutes and a final extension step at 72 °C for 3 minutes.

7.3 Purification of PCR product by using QIA quick

Amplification products were detected by electrophoresis on a 0.8% agarose gel, staining with ethidium bromide. Reaction products which displayed bands corresponding to the correctly size product (about 1500 base pair) were purified using QIAquick PCR purification kit (QIAgen, German).

7.4 Sequencing reaction

For sequencing of PCR products, approximately 1500 nucleotides proximal to the 5' end of the rDNA were targeted using primer 20F; 5'GAG TTT GAT CCT GGC TCA G 3' and 520 R; 5' GTA TTA CCG CGG CTG CTG 3'. The thermocycle program was as follows: 96 °C for 30 second, 25 cycles of 96 °C for 10 second, 50 °C for 5 second, and 60 °C for 4 minutes.

7.5 Precipitation of DNA after sequencing reaction

The 10 µl of 16S rDNA gene amplicons were purified by ethanol precipitation 80 µl and incubated at room temperature for 15 minutes. The supernatant was centrifugation at 15,000 rpm for 20 minutes, washed the precipitant with 70% ethanol 250 µl, centrifuged to remove the ethanol and dried the samples in heat box or thermal cycle at 90 °C for 1 minute.

7.6 Preparing of loading samples for capillary electrophoresis

The dried precipitant was dissolved in 20 µl of TSR (Terminator sequencing reagent), and then boiled at 95 °C for 2 minutes and placed on ice immediately ready to load on instrument. Nucleotide sequencing was carried out with an automated DNA sequencer Nucleotide sequences of 16S rDNA were compared with the BLAST program (Altschul *et al*., 1990).

8. Antimicrobial activity of dialysed supernatant of selected isolate *Lactobacillus* sp. SK5

The *Lactobacillus* sp. SK5 was grown in MRSB and incubated in anaerobic condition at 37 °C for five days. After incubation, cell-free supernatant was obtained by centrifuging 6,000 g for 15 minutes. Fifty ml of supernatant was dialysed in dialysis tube with molecular weight 3,500 Da cut-off against 5 liters of sodium acetate buffer pH 4.2 performed on a magnetic stirrer plate at 4 °C for 24 hours. The buffer was changed two times. After dialysis, the supernatant was collected and then lyophilized with lyophilizer. The lyophilized supernatant was dissolved with distilled water to make fifty times (50x) concentration of original supernatant. Six mm diameter paper disc was applied with 50 µl of concentrated supernatant and placed to dry in air before use.

Two bacteria reference standard strains i.e., *E.* coli ATCC 25922 and *S. aureus* ATCC 25923 and 5 clinical isolates of vaginal pathogens i.e., *Pseudomonas aeruginosa, Staphylococcus epidermidis, Gardnerella vaginalis, Escherichia coli* and *Neisseria gonorrhoea* were used to test the activity of dialyzed supernatant of *Lactobacillus* sp. SK5. They were cultured in BHIA at 37 °C for 24 hours. The cultures were harvested by normal saline and adjusted with normal saline to give turbidity equal to 0.5 McFarland turbidity standard. A sterile cotton swab was dipped into the inoculum and the excess was removed by rotating the swab several times against inside wall of the tube above fluid level. The surface of MHA plates was inoculated by streaking the swab over the surface. Streaking was repeated 3 times and for each time the plate was rotated 60 degrees**.** The discs were placed on the agar surface and incubated at 37 °C for 24 hours. The inhibition zone diameter was measured by antibiotic zone reader.

9. Sensitivity of *Lactobacillus* sp. SK5 to feminine hygiene and spermicide

Lactobacillus sp. SK5 was tested for its sensitivity to six brands of feminine hygiene i.e., Lactacid, Dettol, Oriental princess, Mistine, Carefree, and Eucerine, commercial traded in the market and a spermicide nonoxinal-9. Most of feminine hygiene products were used for cleaning up female genital and they may have antimicrobial activity. Unfortunately, their active ingredients were not shown on the products. To study the sensitivity of selected lactobacilli to these materials, the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were investigated. The MIC was performed in 96 wells sterile plastic plate (Corning Incorporated, USA). The well in row 1-12 was filled with 50 µl of MRSB. The tested solution 50 µl was added into well in row 1, after mixing 50 µl of the mixture was transferred to well in row 2 and continue making two-fold serial dilution up to well in row 10 and from well 10 discarded 50 µl of the mixture. The over night cultures of *Lactobacillus* sp. SK5 in MRSB was adjusted with normal saline to obtain turbidity equivalent to 0.5 McFarland turbidity standard. Fifty µl of adjusted culture was added into well 1-11. Well 11 and well 12 were used as positive control and negative control, respectively. Plates were incubated in anaerobic condition at 37 $^{\circ}$C for 24 hours. The lowest concentration that showed no growth was considered as the MIC (Fernanda *et al*., 2007).

MBC of feminine hygiene and spermicide to *Lactobacillu*s sp. SK5

According to the results from MIC test, 100 µl of content in the well without visible growth was inoculated on MHA surface and spreaded across the agar surface with sterile bent glass rods. The plated were incubated overnight at 37 $^{\circ}$C for the MBC test. MBC was determined from the plate with colony count that represent 0.1% of original inoculums (i.e., 99% reduction).

10. Viability of selected *Lactobacillus* sp. SK5 in lyophilized form

Lactobacillus sp. SK5 was grown in MRSB and incubated under anaerobic condition at 37 °C for 48 hours. The growth culture was streaked densely on MRSA and incubated in anaerobic condition at 37 °C for 48 hours. The colonies on the agar surface were washed and dispersed in three different solutions i.e., 8% w/v skim milk, 8% w/v lactose and 8% w/v malt extract. They were freezed and lyophilized using the method described in **section 8**. The number of viable *Lactobacillus* sp. SK5 before and after lyophilization was determined by plate count method.

11. Preparation of *Lactobacillus* sp. SK5 vaginal suppository

Vaginal suppositories were prepared by fusion method (moulding method) using the mixture of PEGs (PEG 400 and PEG 4000 in the ratio of 55:45) as the suppository base. The base was melted in boiling water bath. After melting, the base was cooled down to the temperature approximately 45 °C. Then it was poured into a suppository mold equipped with cylindrical tube in the center and allowed to stand for 30 minutes at room temperature to solidify. Before it completely solidify, the cylindrical tube was removed and the hollow cavity of suppository was obtained. The lyophilized powder of *Lactobacillus* sp. SK5 9.17×10^8 cfu/ml in skim milk was added into the suppository cavity. The open end of the suppository was sealed with melted base. After removing from the mold, the suppository was wrapped with aluminum foil and put in plastic bag before keeping in refrigerator. The suppository was evaluated for weight, melting point, disintegration time and *in vitro Lactobacillus* sp. SK5 release study.

Evaluation of physical properties of suppositories

11.1 Viability of *Lactobacillus* sp. SK5 from vaginal suppository

The *Lactobacillus* sp. SK5 vaginal suppository kept in refrigerator at 4 °C for 3 months was cut with sterile knife and then *Lactobacillus* sp. SK5 powder from cavity of suppository was taken out. Two hundred and eighty nine mg of *Lactobacillus* sp. SK5 powder from fresh prepared and aged vaginal suppository was dissolved with MRS broth and diluted with half strength of MRS broth to obtain dilution $10^{-2} – 10^{-6}$. One hundred µl of appropriate dilution was poured in plate and melted agar was added on the *Lactobacillus* sp. SK5. They were incubated in anaerobic condition at 37 °C for 48 hours.

11.2 Uniformity of weight

Twenty vaginal suppositories were weighed individually. The average weight was calculated.

11.3 Differential scanning calorimetry (DSC)

The thermal properties of pulverized PEGs base (the mixed of PEG 400: PEG 4000, ratio55: 45) were studied on a differential scanning calorimetry (Perkin Elmer DSC, Norwalk Connection, USA). Sample was heated in closed aluminium crimp cells at the rate of 10 °C /min under nitrogen purge.

11.4 Disintegration time

Disintegration time of suppository was tested by using disintegration apparatus for disintegration test. The apparatus consists of a basket-rack assembly, a 1000 ml low-form beaker for the immersion of fluid, a thermostatic arrangement for heating the fluid between

37±2 °C, and device for rising and lowering the basket in the immersion fluid with a constant frequency rate between 29-32 cycles per minute through the distance of not less than 5.3 cm and not more than 5.7 cm. The volume of the fluid in the vessel in such that the highest point of the upward stroke the wire mesh remains at least 2.5 cm below the surface of the fluid and descends to not less than 2.5 cm from the bottom of the vessel on the downward stroke. The basket-rack assembly consists of six open-ended transparent tubes, each 7.75±0.25 cm long and having an inside diameter of 20.7 to 23 cm. Attached to the lower end of each tube is a woven stainless steel wire cloth, which have a plain square weave with 1.8-2.2 mm mesh apertures and with wire diameter of 0.60 to 0.65 mm.

For disintegration test of suppository, place one suppository in each of six tubes of the basket and operate the apparatus, using water maintained at 37±2 °C as the immersion fluid. The suppository in each tube was observed until completely disintegrate.

11.5 *In vitro* release studies

In vitro release of *Lactobacillus* sp. SK5 from vaginal suppository was studied in 20x150 mm test tube containing 6 ml citrate buffer pH 4.4 as the test medium. The tube was placed in 37 °C water bath with gentle shaking (50 rpm). The suppository was placed in the tube and 1 ml of medium was collected at 5, 10, 15, 20, 25, 30, 40, 50 and 60 minutes. The same amount of fresh medium was replaced into the tube in each time of sampling. The samples from each point of time were counted for bacterial content by spread plate method.

CHAPTER 3

RESULTS

1. Lactobacilli isolation

From 120 healthy women, the vaginal samples were diluted and cultured on MRSA plates. Two hundred and ninety four colonies were primary picked up from MRSA surface. Most of them showed small colonies without pigment, white or cream color as shown in Figure 6. All of them were later tested for Gram staining, catalase production and checked for spore forming. Only eighty of them showed lactobacilli properties with Gram positive, catalase negative, non spore forming, and the cell morphology vary from short to long rod under microscope as shown in Figure 7. They were kept in MRSB containing 20%v/v glycerol and stored in -80 $^{\circ}$C for future studies and also stored in lyophilized form for long term preservation.

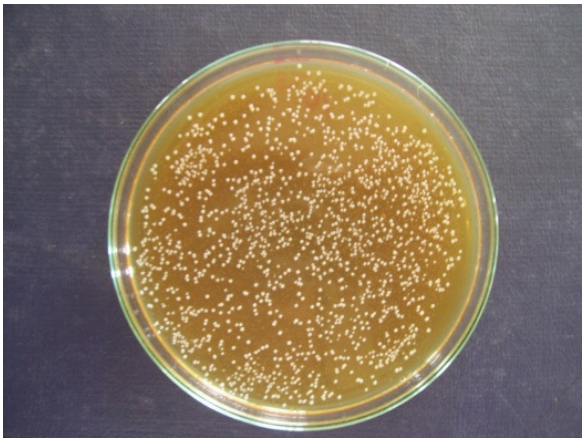


Figure 6. Colonies of bacteria from vaginal samples growing on MRS agar

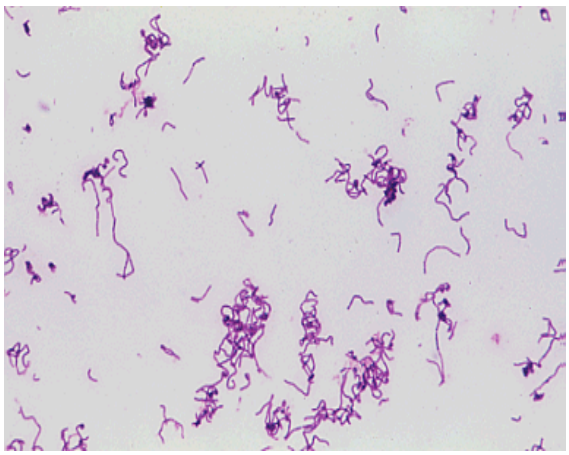


Figure 7. Morphology of isolated *Lactobacillus* spp. from vagina

2. Screening of isolated *Lactobacillus* spp. against bacteria and yeast by overlay method

From 80 isolates, they were screened for their antimicrobial activity against standard reference strains of bacteria and yeast i.e., *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *C. albican*s NCPF 3153 by overlay method. There are 16 isolates showed inhibition to *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 but none of them inhibited *C. albicans* NCPF 3153. The inhibition zone diameters were between 15.9 mm to 30.7 mm. The isolates with antibacterial activity were named *Lactobacillus* sp. SK1, SK2, SK3…and SK16. Their inhibition zones are shown in Table 1. and the inhibition zone of *Lactobacillus* sp. SK5 against *E. coli* ATCC 25922 is shown in Figure 8.

Table 1. Inhibition zone of 16 isolates of *Lactobacillus* spp. against standard reference strains

Isolates	Inhibition zone (mm)		
	E. coli ATCC 25922	*S. aureus* ATCC 25923	*C. albicans* NCPF 3153
Lactobacillus sp. SK1	23.30±2.35	24.40±0.16	-
Lactobacillus sp. SK2	21.0±3.80	28.20±2.71	-
Lactobacillus sp. SK3	26.40±1.02	23.50±0.57	-
Lactobacillus sp. SK4	27.20±2.17	27.80±6.32	-
Lactobacillus sp. SK5	27.40±1.18	30.70±3.14	-
Lactobacillus sp. SK6	27.80±2.48	26.30±3.49	-
Lactobacillus sp. SK7	25.70±3.56	29.50±1.28	-
Lactobacillus sp. SK8	30.50±3.72	23.60±2.59	-
Lactobacillus sp. SK9	15.90±1.36	17.00±2.99	-
Lactobacillus sp. SK10	17.10±1.98	13.80±0.59	-
Lactobacillus sp. SK11	25.70±3.63	22.70±0.89	-
Lactobacillus sp. SK12	17.80±1.73	25.20±2.78	-
Lactobacillus sp. SK13	26.10±1.72	20.0±2.35	-
Lactobacillus sp. SK14	25.40±0.69	27.30±2.44	-
Lactobacillus sp. SK15	22.60±0.70	24.90±1.87	-
Lactobacillus sp. SK16	17.50±1.07	17.40±1.44	-

* Note - = no inhibition zone

E. coli = Escherichia coli

S. aureus = Staphylococcus aureus

C. albicans = Canida albicans

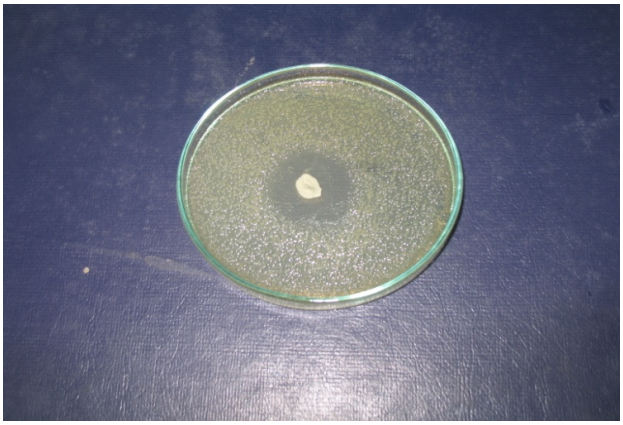


Figure 8. Inhibition zone of *Lactobacillus* sp. SK5 against *Escherichia coli* ATCC 25922

3. Antibacterial activities of cell-free supernatant from selected *Lactobacillus* spp.

After culturing in MRSB, the concentrated (20x) cell-free supernatants of 16 isolates i.e., *Lactobacillus* sp. SK1, SK2, SK3,…and SK16 were tested against standard reference strains of bacteria and yeast by disc diffusion method. From the results in Table 2., all of them inhibited *E. coli* ATCC 29522 and *S. aureus* ATCC 29523 but none of them inhibit *C. albicans* NCPF 3153. The inhibition zones were between 7.30 mm to 11.33 mm. The samples of inhibition zones from *Lactobacillus* sp. SK5 against *E. coli* ATCC 29522 are shown in Figure 9. The pH of all cell free supernatant was between 4.29 to 4.70. It is indicated that all isolates produce organic acids and they reduce the pH of MRSB from pH 6.5 to lower pH. All cell-free supernatants at pH 4.29 to 4.70 have inhibition activity against bacteria but they could not inhibit *C. albicans* NCPF 3153. However, when pH of all supernatants was adjusted to 6.5, they loss their activity to bacteria. Since *Lactobacillus* sp. SK5 showed the largest inhibition zone to *E. coli* ATCC 29522, its supernatant was used in further study.

Table 2. Inhibition zones of concentrated (20X) supernatant of 16 isolated *Lactobacillus* spp. against standard reference strains by disc diffusion method

Isolates	cell-free supernatant pH	Inhibition zone (mm)		
		E. coli ATCC 29522	*S. aureus* ATCC 29523	*C. albicans* NCPF 3153
Lactobacillus sp. SK1	4.70	8.16±0.05	7.37 ±0.01	-
Lactobacillus sp. SK2	4.64	7.37±0.05	7.37±0.05	-
Lactobacillus sp. SK3	4.38	7.46±0.05	7.57±0.05	-
Lactobacillus sp. SK4	4.33	8.37±0.05	8.23±0.05	-
Lactobacillus sp. SK5	4.32	11.33±0.11	9.50±0.26	-
Lactobacillus sp. SK6	4.31	8.60±0.10	10.10±0.05	-
Lactobacillus sp. SK7	4.32	7.83±0.11	9.50±0.26	-
Lactobacillus sp. SK8	4.32	7.40±0.30	11.17±0.15	-
Lactobacillus sp. SK9	4.31	7.97±0.35	8.20±0.28	-
Lactobacillus sp. SK10	4.35	7.53±0.11	7.63±0.30	-
Lactobacillus sp. SK11	4.33	8.50±0.30	8.33±1.36	-
Lactobacillus sp. SK12	4.45	8.77±0.47	9.80±0.42	-
Lactobacillus sp. SK13	4.33	8.20±0.82	7.83±0.49	-
Lactobacillus sp. SK14	4.32	8.00±0.35	7.30±0.10	-
Lactobacillus sp. SK15	4.29	8.27±0.15	7.33±0.05	-
Lactobacillus sp. SK16	4.30	8.20±0.10	7.47±0.05	-

* Note - = no inhibition zone

E. coli = Escherichia coli

S. aureus = Staphylococcus aureus

C. albicans = Canida albicans

Figure 9. Inhibition zone of concentrated (20x) cell-free supernatant of *Lactobacillus* sp. SK5 against *Escherichia coli* ATCC 25922

4. Sensitivity to antibiotics

According to data shown in Table 3., *Lactobacillus* sp. SK5, SK6, SK7 and SK8 were sensitive to ampicillin, bacitracin, cefoperazone, ceftazidime, cephalothin, chloramphenicol, erythromcycin, penicillin G and tetracycline. Their inhibition zone diameters were 23.1-40.0 mm. These four isolates were resistance to kanamycin, ketoconazole, metronidazole, norfloxacin, nystatin, streptomycin and vancomycin. Most of them showed no inhibition zone or small inhibition zone (9.2-14.3 mm) to resistance antibiotics. *Lactobacillus* sp. SK5, SK6 and SK8 were sensitive to clindamycin while *Lactobacillus* sp. SK7 resistance to this antibiotic. For gentamycin, *Lactobacillus* sp. SK5, SK6 and SK 7 were sensitive to this antibiotic but not *Lactobacillus* sp. SK8.

Table 3. Antibiotic sensitivity of *Lactobacillus* sp. SK5, SK6, SK7 and SK8

	Sensitivity (inhibition diameter, mm)			
Antibiotics	*Lactobacillus* sp. SK5	*Lactobacillus* sp. SK6	*Lactobacillus* sp. SK7	*Lactobacillus* sp. SK8
Ampicillin (10µg)	S(40)	S(39.5)	S(39)	S(36)
Bacitracin (10µg)	S(28.1)	S(28.5)	S(33)	S(32.9)
Cefoperazone (75µg)	S(33.2)	S(33)	S(30.5)	S(33.5)
Ceftazidime (30µg)	S(27.8)	S(30.1)	S(29.1)	S(29.7)
Cephalothin (30µg)	S(34)	S(36.5)	S(33.6)	S(35.1)
Chloramphenicol (30µg)	S(33.6)	S(33.6)	S(32)	S(33.3)
Clindamycin (30µg)	S(24.9)	S(23.1)	R(-)	S(31.6)
Erythromycin (15µg)	S(32.2)	S(32.5)	S(30.5)	S(32.5)
Gentamycin (10µg)	R(14.4)	R(12.5)	S(21.4)	R(16.9)
Kanamycin (30µg)	R(10.5)	R(-)	R(12.8)	R(-)
Ketoconazole (30µg)	R(-)	R(-)	R(-)	R(-)
Metronidazole (30µg)	R(-)	R(-)	R(-)	R(-)
Norfloxacin (10µg)	R(-)	R(-)	R(-)	R(-)
Nystatin (2000 IU)	R(-)	R(-)	R(-)	R(-)
Penicillin G (10µg)	S(40)	S(39.5)	S(38)	S(40)
Streptomycin (10µg)	R(-)	R(9.2)	R(11.7)	R(14.3)
Tetracycline (30µg)	S(31.3)	S(30)	S(33.2)	S(32.5)
Vancomycin (30µg)	R(-)	R(-)	R(-)	R(-)

Note : S = sensitive, R = resistance, - = no inhibition zone

5. Detection of hydrogen peroxide production strain

Four isolates i.e., *Lactobacillus* sp. SK5, SK6 SK7 and SK8 were tested for their ability to produce hydrogen peroxide. Results in Table 4. showed that *Lactobacillus* sp. SK5 and SK6 colonies had blue and brown color, respectively while *Lactobacillus* sp. SK7 and SK8 had white color. It is indicated that *Lactobacillus* sp. SK5 and SK6 produced hydrogen peroxide. *Lactobacillus* sp. SK5 showed higher capacity to produce this substance than *Lactobacillus* sp. SK6 while SK7 and SK8 could not produce hydrogen peroxide.

Table 4. The color of colony of *Lactobacillus* spp. growing on agar plate for detection of hydrogen peroxide production

Isolates	Colony color (level of H_2O production)
Lactobacillus sp. SK5	blue (high)
Lactobacillus sp. SK6	brown (medium)
Lactobacillus sp. SK7	white (no)
Lactobacillus sp. SK8	white (no)

6. Adhesion ability of *Lactobacillus* spp. isolates to HeLa cell

The adhesion of *Lactobacillus* sp. SK5, SK6, SK7 and SK8 to HeLa cells, a cell line which originated from a human carcinoma of the cervix, were studied. 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 lactobacilli was examined. The compounds were used at half of the maximal concentration at which these could be solubilized, and were added to culture medium during the adhesion step. The results are shown in Figure 10, 11, 12 and 13. The HeLa cells were grown on cover glass with the surface area of 22x22 mm^2 and $3x10^8$ cfu of *Lactobacillus* sp. SK5, SK6, SK7 and SK8 were added to the cell surface for adhesion test. The adhesion of control were $5.13x10^5$, $2.76x10^5$, $1.9x10^5$ and $3.69x10^5$ for *Lactobacillus* sp. SK5, SK6, SK7 and SK8, respectively. When bacterial cells suspended in medium containing bioadhesive polymer i.e., sodiumcarboxymethylcellulose (Na-CMC), hydroxylpropylmethyl-cellulose (HPMC-E50) and polyvinylpyrrolidone (PVP) were tested for adhesion, it was found that the number of adhesion cells increase. The adhesion bacterial cell numbers were $1.21x10^6$, $6.63x10^5$, $1.64x10^6$ cfu for *Lactobacillus* sp. SK5, $7.87x10^5$, $2.8x10^5$, $1.11x10^6$ for *Lactobacillus* sp. SK6, $1.48x10^5$, $2.46x10^5$, $7.23x10^5$ for *Lactobacillus* sp. SK7 and $3.69x10^5$, $8.50x10^5$, $3.29x10^5$ for *Lactobacillus* sp. SK8 from Na-CMC, HPMC-E50 and PVP, respectively. PVP gave the highest activity of adhesion enhancement, followed by Na-CMC and HPMC-E50.

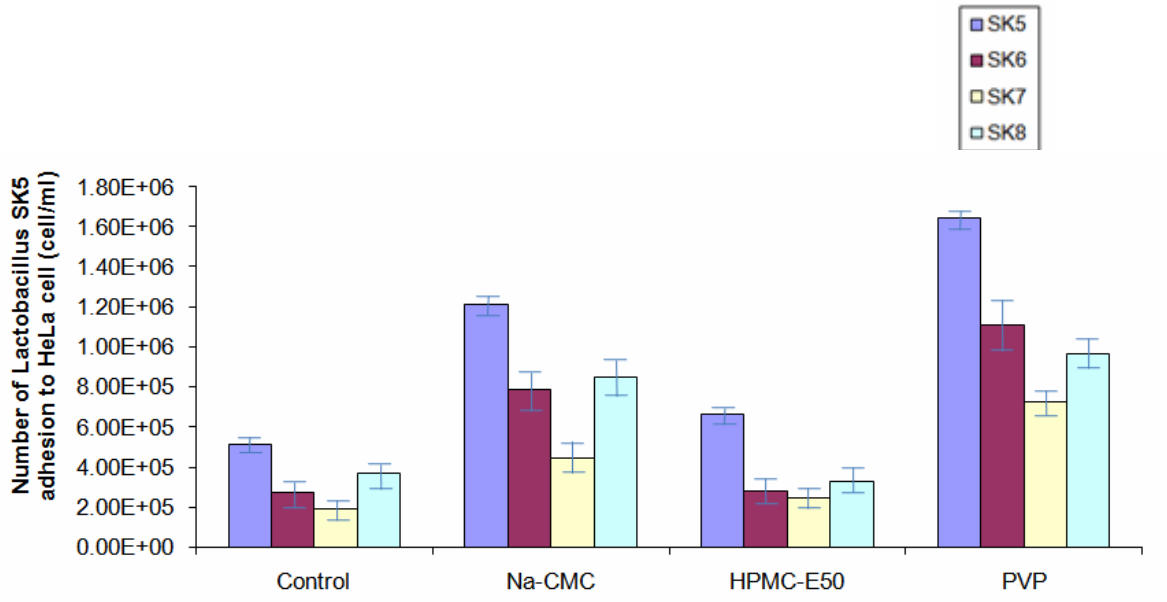


Figure 10. Adhesion of *Lactobacillus* sp. SK5, SK6, SK7 and SK8 with and without bioadhesive to HeLa cell

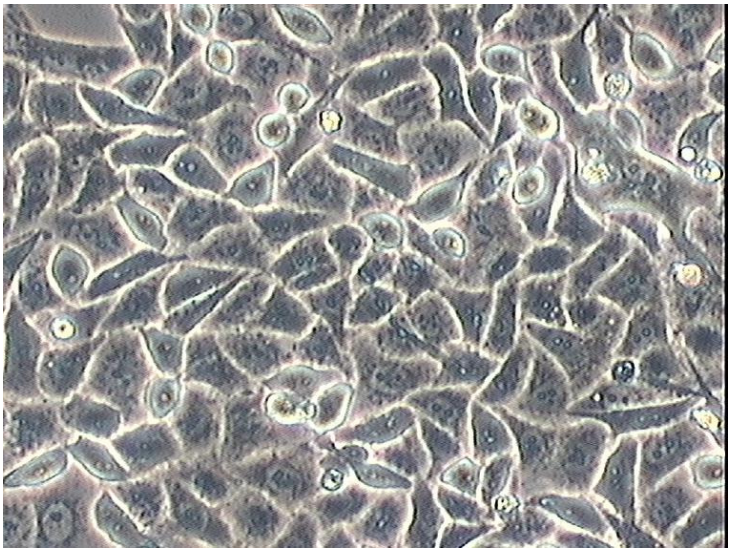


Figure 11. HeLa cell monolayer under inverted microscope

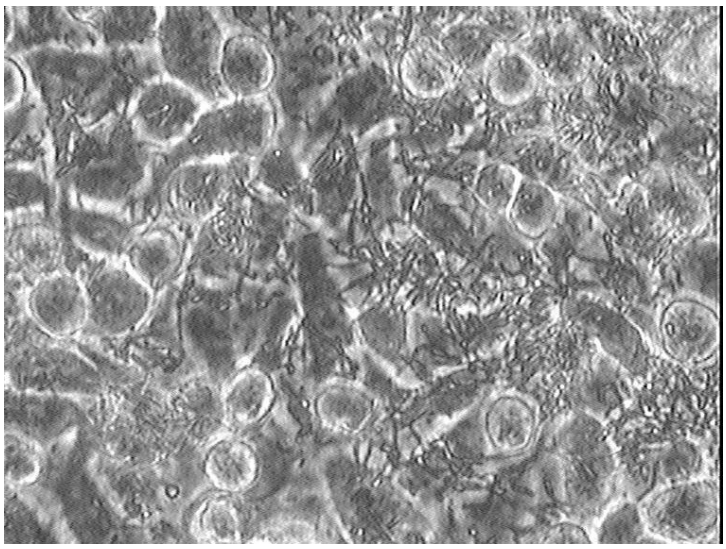
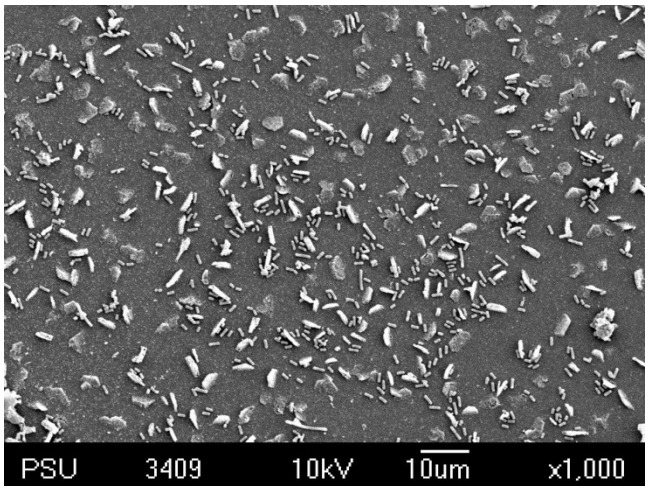
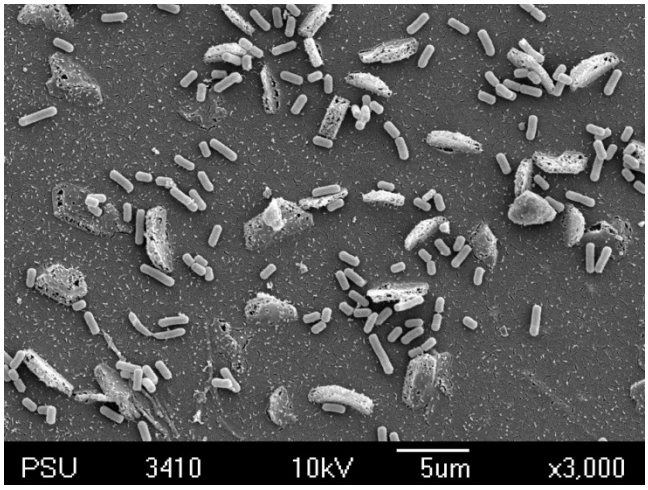


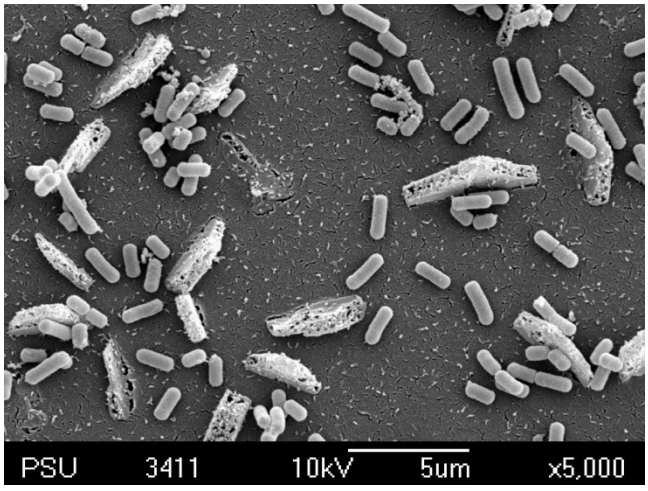
Figure 12. Adhesion of *Lactobacillus* sp. SK5 to HeLa cell monolayer under inverted microscope



(A)



(B)



(C)

Figure 13. Adhesion of *Lactobacillus* sp. SK5 to HeLa cell monolayer under SEM (A) 1,000x, (B) 3,000x, (C) 5,000x

7. 16 S rDNA sequencing of the isolated *Lactobacillus* sp. SK5

Genotypic work was therefore carried out to identify this microorganism. This involved extraction of its DNA and amplification, through PCR, of the gene responsible for 16S rDNA of the base in this gene approximately 1500 were sequenced and showed the *Lactobacillus* sp. SK5 to be a close relative of *L. fermentum,* which has probability 98.9%.

8. Antimicrobial activity of dialysed supernatant

From 16 isolates of lactobacilli with antibacterial activity, *Lactobacillus* sp. SK5 was selected for this study. The dialyzed *Lactobacillus* sp. SK5 supernatant was found to be the yellow bulky powder as shown in Figure 14. The data in Table 5. show that reconstituted cell-free supernatant (50x) of *Lactobacillus* sp. SK5 inhibited *E. coli* ATCC 25922*, S. aureus* ATCC 25923 and clinical isolates pathogens i.e., *P. aeruginosa, S. epidermidis, G. vaginalis, E. coli, N. gonorrhoeae* with the inhibition zone sizes between 8.97±0.63 – 23.58±2.67 mm.

Table 5. Inhibition zone of reconstituted dialysed supernatant (50x) of *Lactobacillus* sp. SK5 against standard reference strains and pathogens

Standard reference strains and pathogens	Inhibition zone (mm)
Escherichia coli ATCC 29522	23.58±2.67
Staphylococcus aureus ATCC 29523	14.02±0.56
Pseudomonas aeruginosa	10.52±0.52
Staphylococcus epidermidis	11.88±0.74
Gardnerella vaginalis	9.89±1.04
Escherichia coli	21.88±1.78
Neisseria gonorrhoeae	8.97±0.63

Figure 14. Lyophilized powder of *Lactobacillus* sp. SK5 supernatant

9. Sensitivity of *Lactobacillus* sp. SK5 to feminine hygiene and spermicide

All of six feminine hygiene products have antimicrobial activity against *Lactobacillus* sp. SK5. Table 6 showed that Mistine, Carefree and Eucerine have the same MIC (0.391%v/v) and MBC (0.391% v/v). Lactacid, Dettol and Oriental princess have MIC 1.562% v/v, 0.781% v/v and 1.562% v/v, respectively and they have the same MBC of 3.125% v/v. Nonoxynol-9 has MIC and MBC 6.25% v/v and 12.5% v/v, respectively.

Table 6. Sensitivity of *Lactobacillus* sp. SK5 to feminine hygiene products and spermicide nonoxynol-9

Feminine hygiene	Concentration (%v/v)	
	MIC	**MBC**
Lactacid	0.781	1.562
Dettol	0.781	3.125
Oriental princess	1.562	3.125
Mistine	0.391	0.391
Carefree	0.391	0.391
Eucerine	0.391	0.391
Nonoxynol-9 (spermicidal)	6.25	12.5

10. Viability of *Lactobacillus* sp SK5 in skim milk, lactose and malt extract

In this experiment 8% w/v skim milk, lactose and malt extract were used as suspending medium for *Lactobacillus* sp. SK5. Before lyophilization each medium contained $1.25x10^{9}$, $1.32x10^{8}$ and $1.02x10^{9}$ cfu/ml in skim milk, lactose and malt extract, respectively. After lyophilization, the viability of *Lactobacillus* sp. SK5 was evaluated. There were $9.17x10^{8}$, $8.38x10^{7}$ and $3.72x10^{7}$ cfu/ml found in skim milk powder, lactose powder and malt extract powder, respectively. The viable cells decreased about 1 log in skim milk but it decreased about 2 logs in lactose and malt extract. It can be concluded that skim milk is the most suitable medium compound for preparation *Lactobacillus* sp. SK5 in lyophilized form.

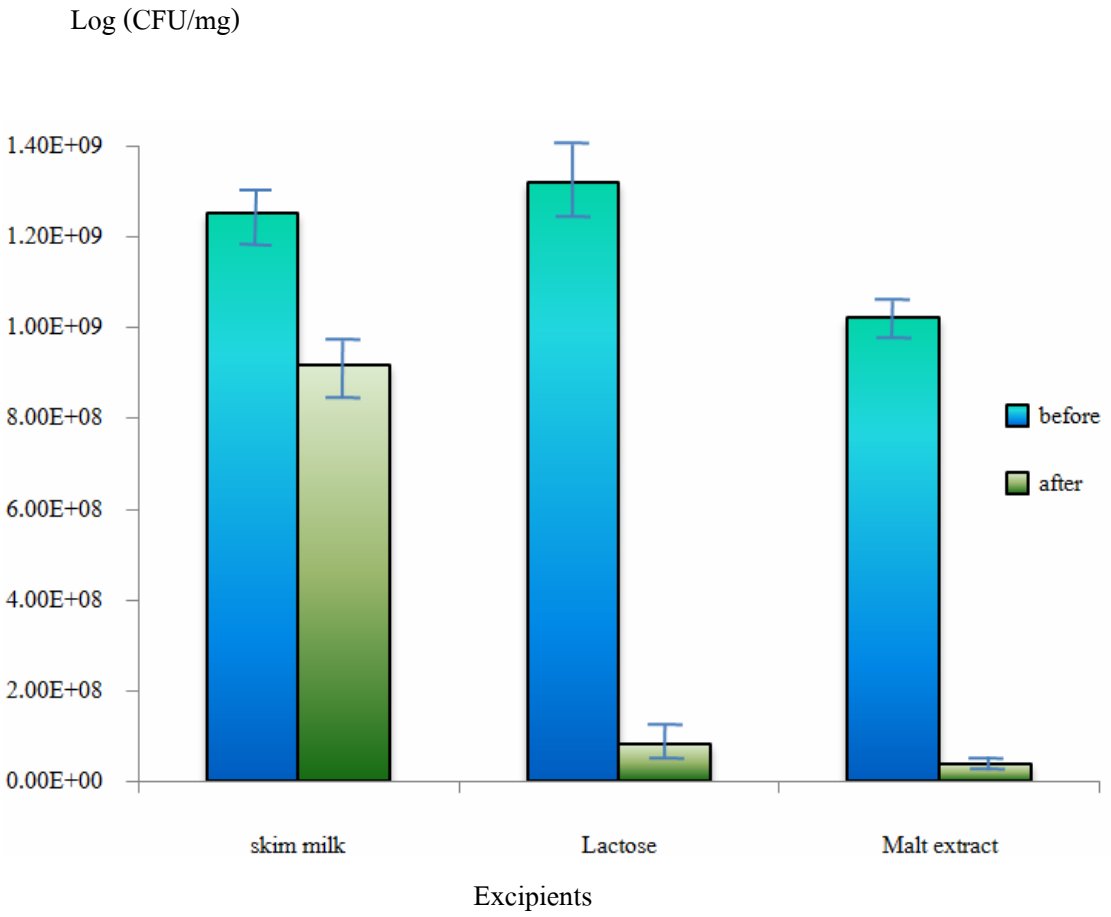


Figure 15. Viability of *Lactobacillus* sp. SK5 before and after freeze-drying with different excipients

11. Preparation of *Lactobacillus* sp. SK5 vaginal suppository

The viability of *Lactobacillus* sp.SK5 from vaginal suppository before and after storage in refrigerator for 3 months was evaluated. The *Lactobacillus* sp. SK5 vaginal suppository before storage contains $1.32x10^{8}$ cfu/suppository. After storage for 3 months, *Lactobacillus* sp. SK5 vaginal suppository contains $1.10x10^{6}$ cfu/ suppository.

The weight of vaginal suppositories were in the range of 2.109-2.400 g and the average weight is 2.23±0.063 g as shown in Table 7.

Table 7. Weight of *Lactobacillus* sp. SK5 vaginal suppositories

Sample	Weight (g)
1	2.172
2	2.206
3	2.252
4	2.232
5	2.224
6	2.210
7	2.140
8	2.109
9	2.147
10	2.251
11	2.278
12	2.204
13	2.221
14	2.314
15	2.251
16	2.278
17	2.400
18	2.216
19	2.240
20	2.256
Average	2.23±0.063

The disintegration time of *Lactobacillus* sp. SK5 vaginal suppositories were between 7.41-10.21 minutes and the average disintegration time shown in Table 8. was 8.51±1.08 minutes.

Table 8. Disintegration time of *Lactobacillus* sp. SK5 vaginal suppositories

Sample	Disintegration time (min)
1	7.41
2	7.5
3	10.21
4	9.29
5	8.57
6	8.08
Average	8.51±1.08

Figure 16. showed the DSC thermogram of PEGs base. It showed a broad endothermic peak between 35.07-44.03 °C indicating the melting point of PEGs base and Figure 17. showed the appearance of vaginal suppository.

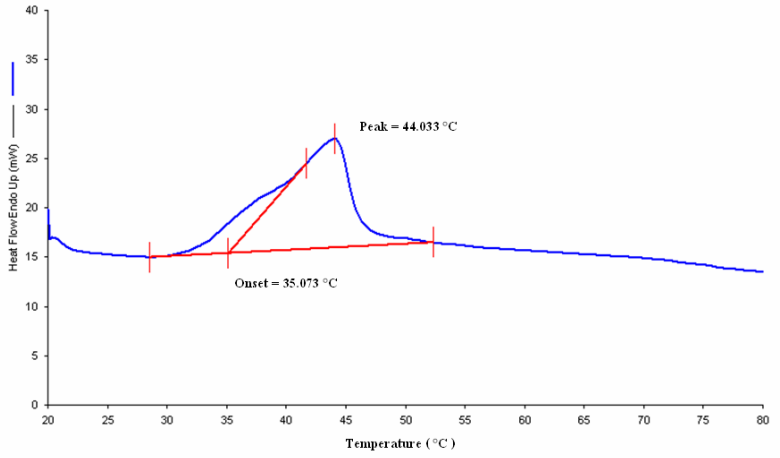


Figure 16. DSC thermogram of suppository base (the mixed of PEG 400: PEG 4000, ratio55: 45)

Figure 17. Appearance of *Lactobacillus* sp. SK5 vaginal suppository

***In vitro* release study**

The release profile of *Lactobacillus* sp. SK5 from vaginal suppository is shown in Figure 18. The release of *Lactobacillus* sp. SK5 did not detected in the first 25 minutes. After that, the release of *Lactobacillus* sp. SK5 was increased as a function of time.

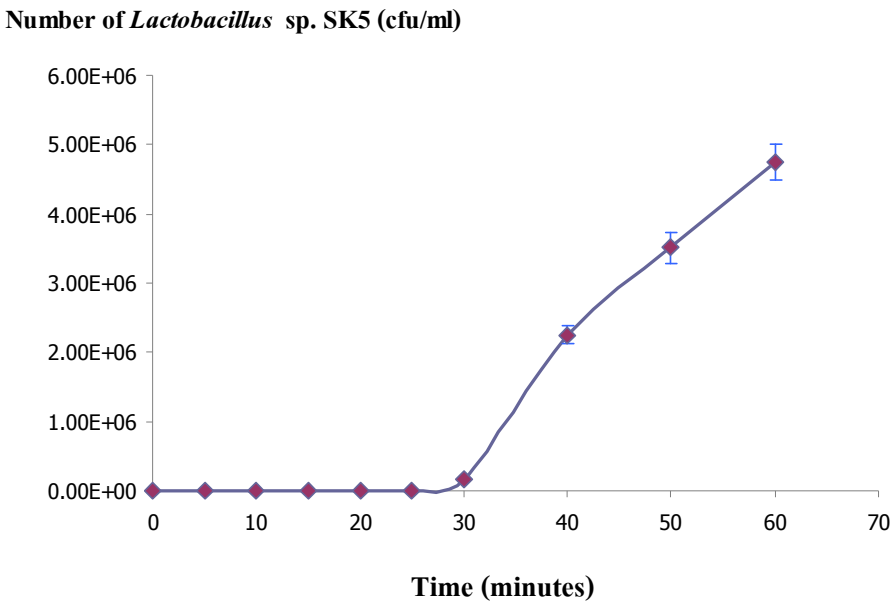


Figure 18. *In vitro* release of *Lactobacillus* sp. SK5 from hollow-type vaginal suppositories

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 bactericin 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 vancomysin. 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 (Daneilsen 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 $^{\circ}$C for 3 months were evaluated. The viable of this *Lactobacillus* sp. SK5 decreased about 2 log cycles from 1.32×10^{8} to 1.10×10^{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 $^{\circ}$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 $^{\circ}$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.

CHAPTER 5

CONCLUSIONS

Two hundred and forty nine *Lactobacillus* spp. were isolated from vagina of healthy women. Sixteen isolates showed inhibition activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 but none of them could inhibit *Candida albicans* NCPF3153. Among them, *Lactobacillus* sp. SK5, SK6, SK7 and SK8 with high inhibition activities were tested for sensitivity to antibiotics. They were all sensitive to ampicillin, bacitracin, cefoperazone, ceftazidime, cephalothin, chloramphenicol, erythromycin, penicillin G and tetracycline. These four isolates were resistance to kanamycin, ketoconazole, metronidazole, norfloxacin, nystatin, streptomycin and vancomycin. They were tested for hydrogen peroxide production ability. *Lactobacillus* sp. SK5 was better hydrogen peroxide producer compared with *Lactobacillus* sp. SK6 while *Lactobacillus* sp. SK7 and SK8 could not produce this substance. To compare adhesion ability with human vaginal epithelial HeLa cells, *Lactobacillus* sp. SK5 showed the highest adhesion activity follow by *Lactobacillus* sp. SK8, SK6 and SK7, respectively. Some bioadhesive polymers i.e., hydroxypropylmethylcellulose (HPMC-E50), sodiumcarboxymethylcellulose (Na-CMC) and polyvinylpyrrolidone (PVP) were used to study for adhesion enhancement of these bacteria to HeLa cells. From this result, PVP could increase adhesion of all bacteria and had better activity than HPMC-E50 and Na-CMC. *Lactobacillus* sp. SK5 with high hydrogen peroxide production and high adhesion ability was identified for species using 16 S rDNA. It was *L. fermentum*. *Lactobacillus* sp. SK 5 was later cultured in MRS broth and the cell free supernatant was dialysed in dialysis tube with molecular weight cut off 3,500. The remain substance showed inhibition activity against vaginal isolates of bacterial pathogens i.e., *Pseudomonas aeruginosa, Staphylococcus epidermidis, Gardnerella vaginalis, E. coli* and *Neisseria gonorrhoeae*. This high molecular weight substance was supposed to be bacteriocin.

Lactobacillus sp. SK5 was lyophilized with 8% w/v solution of skim milk, lactose and malt extract. Survival of the bacterial cells after lyophilzation was highest in skim milk. Lyophilized *Lactobacillus* sp. SK5 in skim milk powder was prepared as hollow-type vaginal suppository using combination of different molecular weight PEGs as a suppository base. The suppository was evaluated for its properties. Each suppository contained $1.32x10^{8}$ cfu of bacteria with average weigh 2.32 g, melting point 35-44 $^{\circ}$C, disintegration time 8.5 minutes and bacteria were released from the suppository after 25 minutes. The properties of suppository are acceptable. From its promising properties such as high inhibition activity against vaginal bacterial pathogens, produce hydrogen peroxide and high molecular weight inhibitory substances, high adhesion ability to vaginal epithelial cells and can be formulated as hollow-type vaginal suppository, *Lactobacillus* sp. SK5 should be intensively studied in other aspects in order to use as probiotic against vaginal bacterial pathogens.

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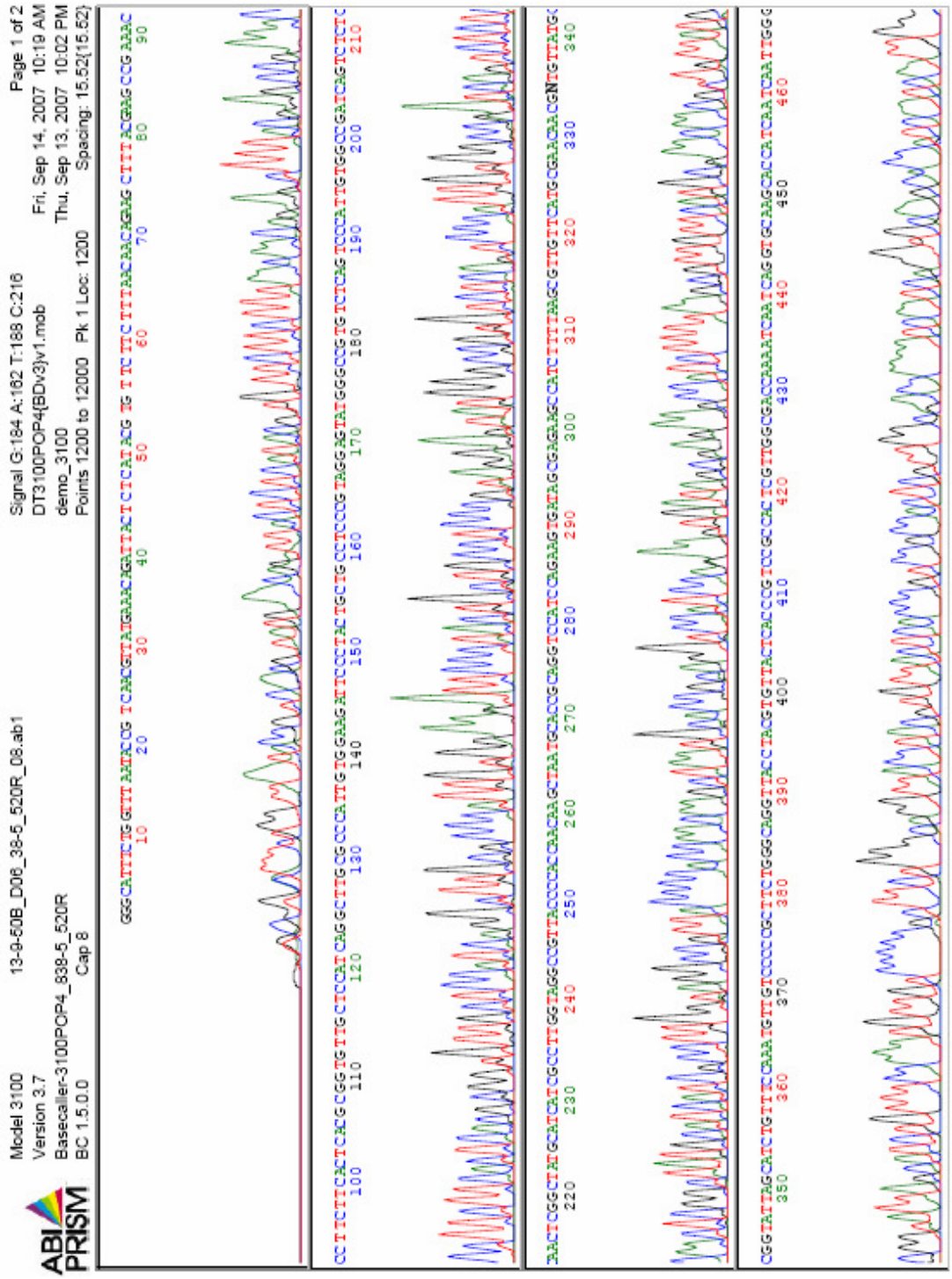
APPENDIX - A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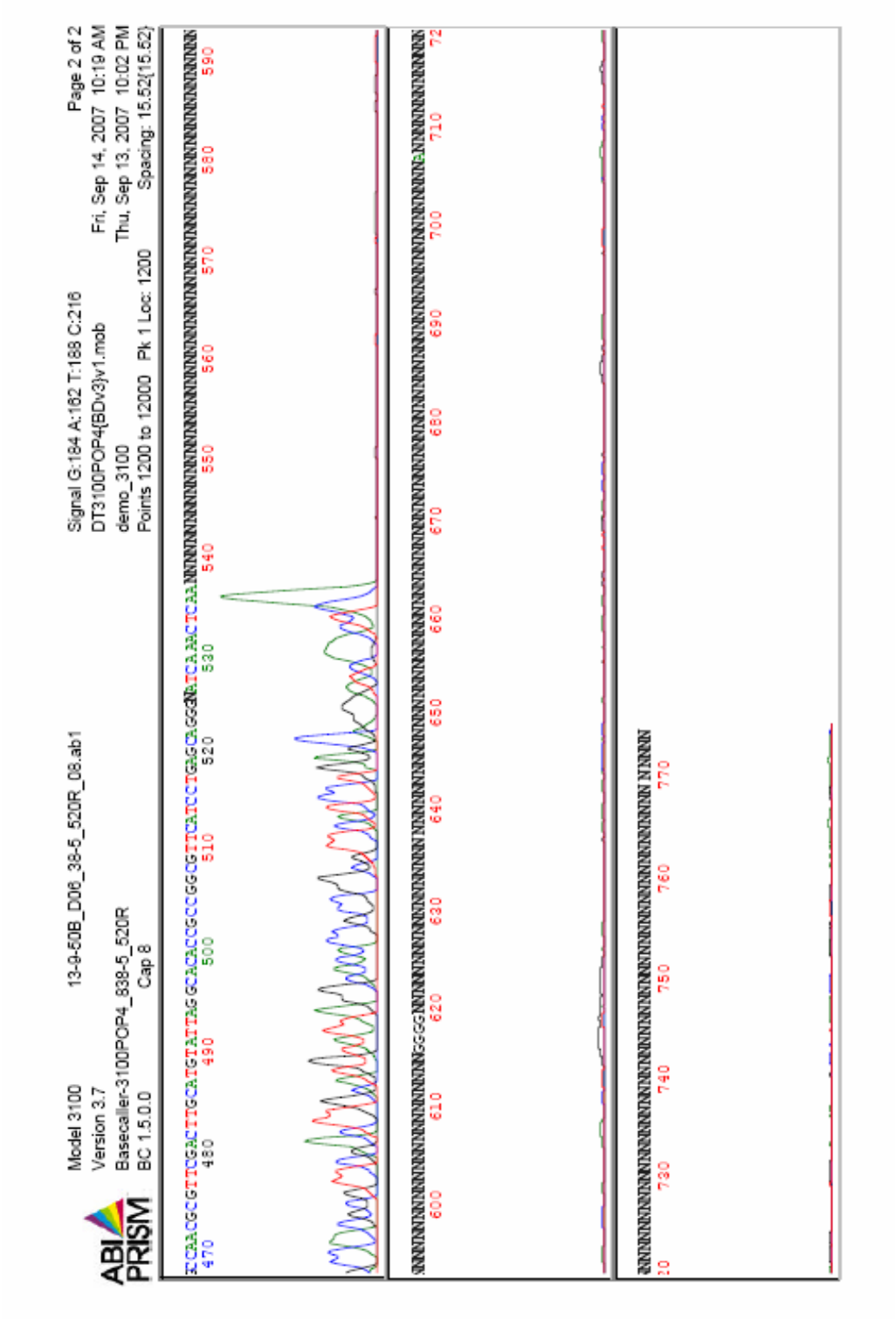
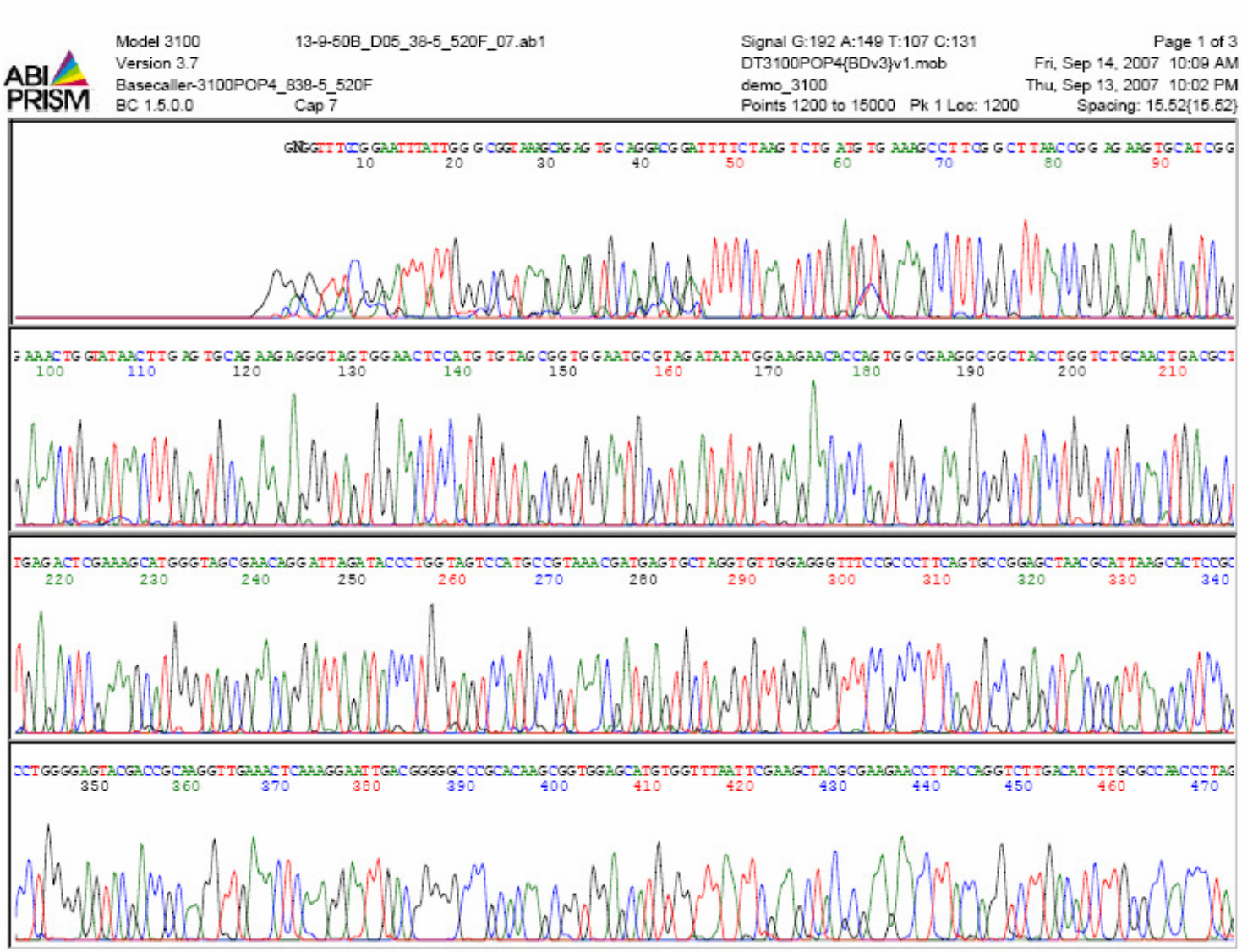


Figure 19. DNA sequence of *Lactobacillus* sp. SK5 in the plasmid 520R (5' GTA TTA CCG CGG CTG CTG 3')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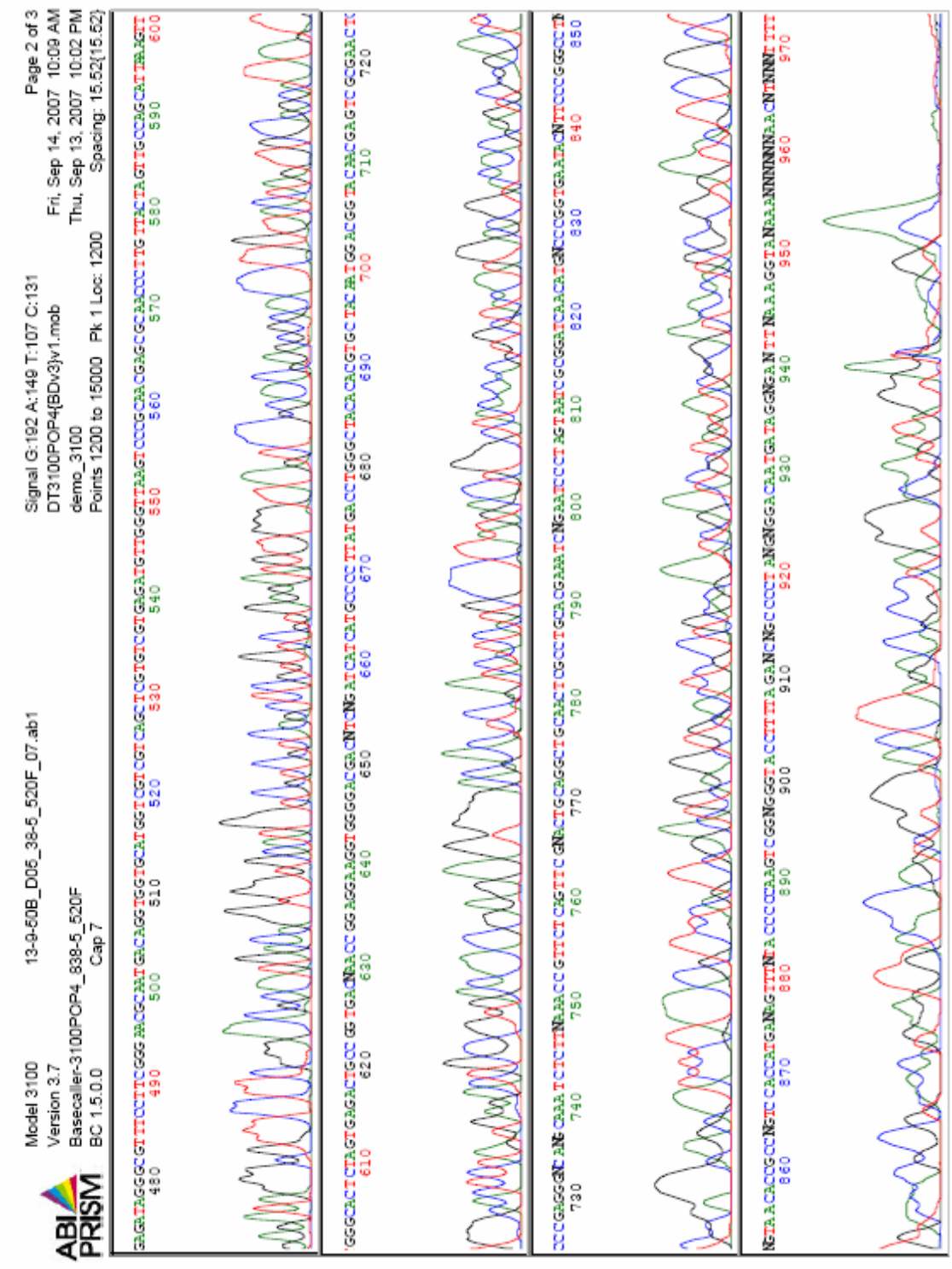
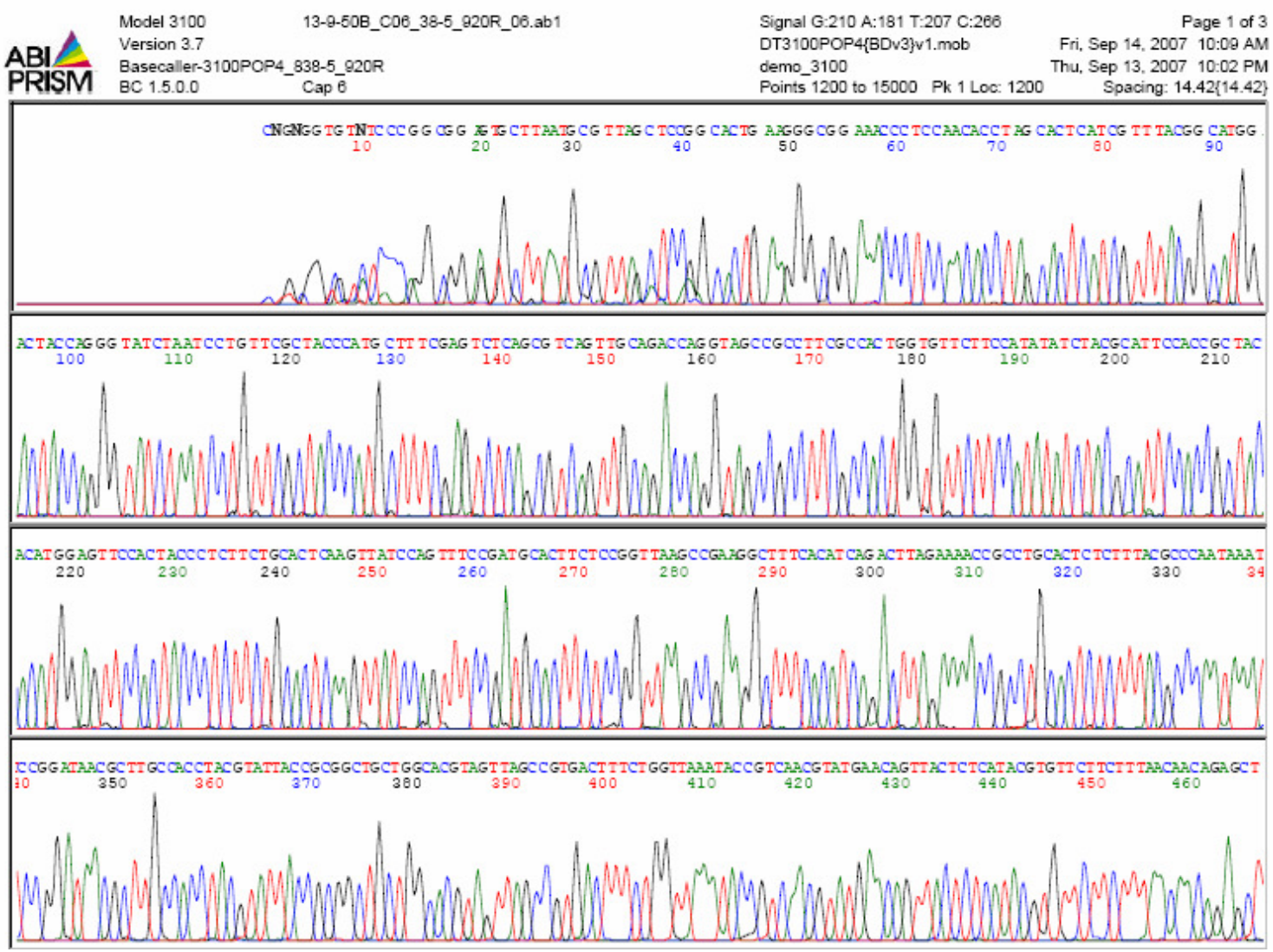


Figure 20. DNA sequence of *Lactobacillus* sp. SK5 in the plasmid 520F (5' CAG CAG CCG CGG TAA TAC 3')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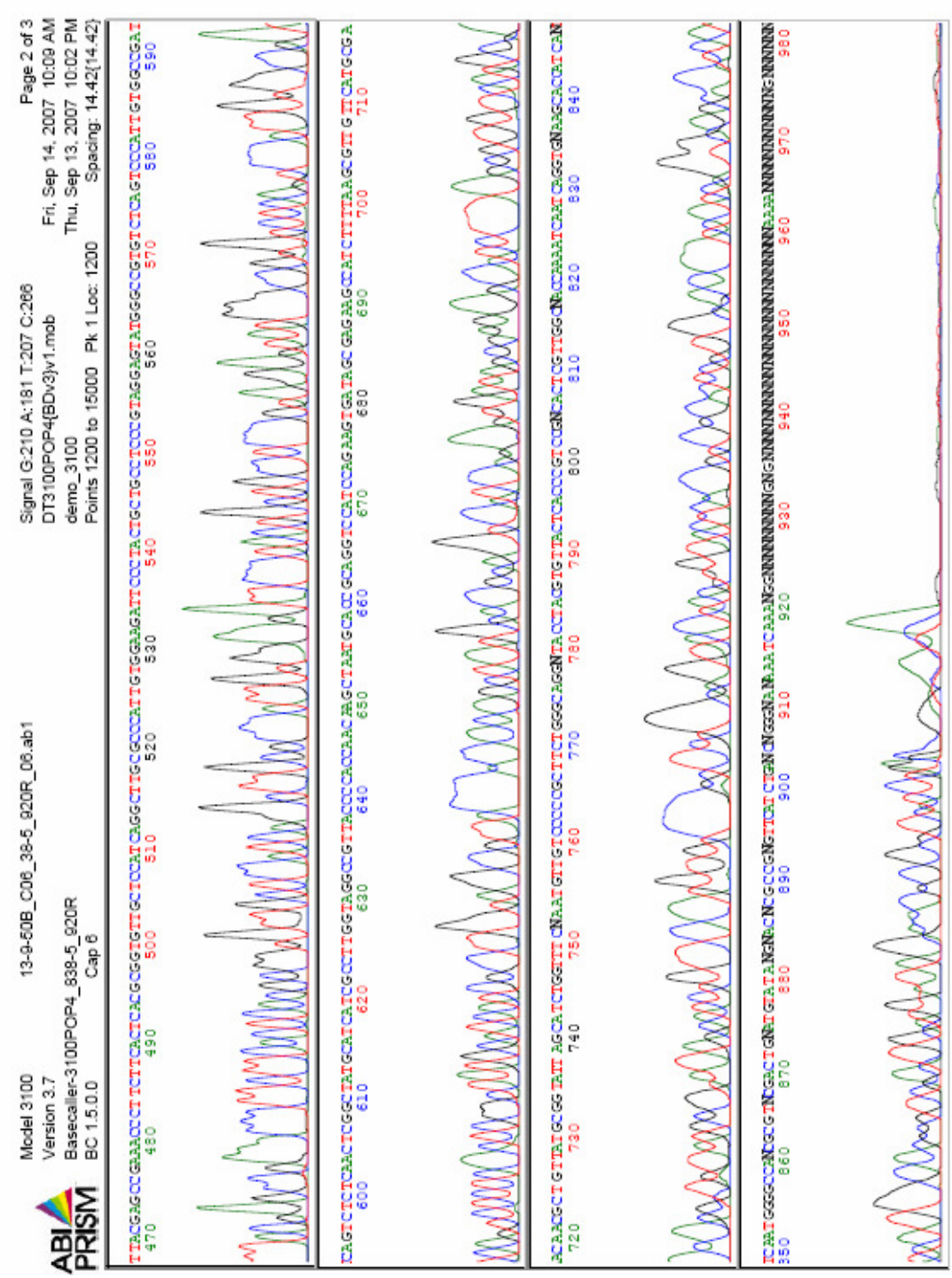
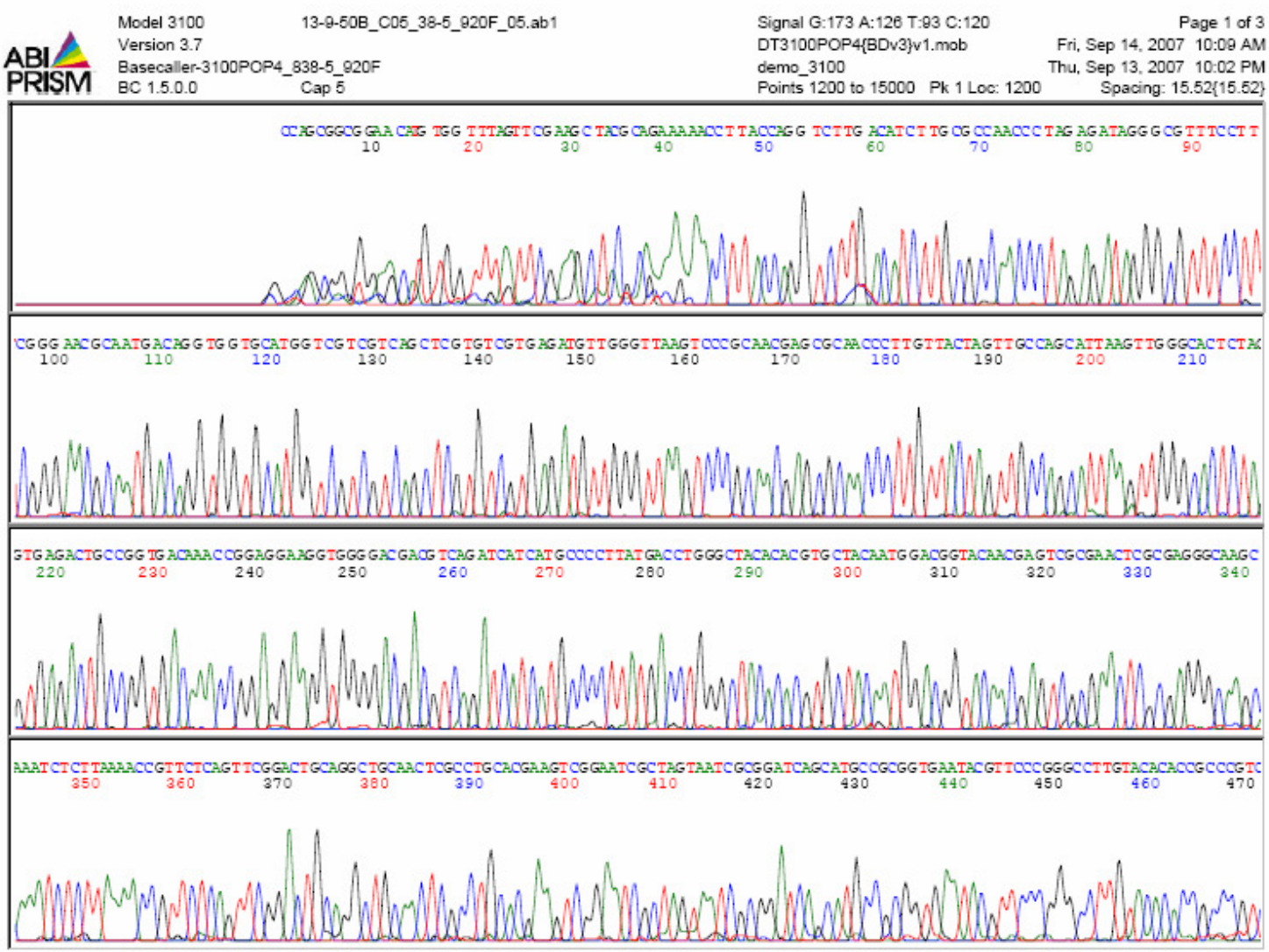


Figure 21. DNA sequence of *Lactobacillus* sp. SK5 in the plasmid 920R (5' CCG TCA ATT CAT TTG AGT TT 3')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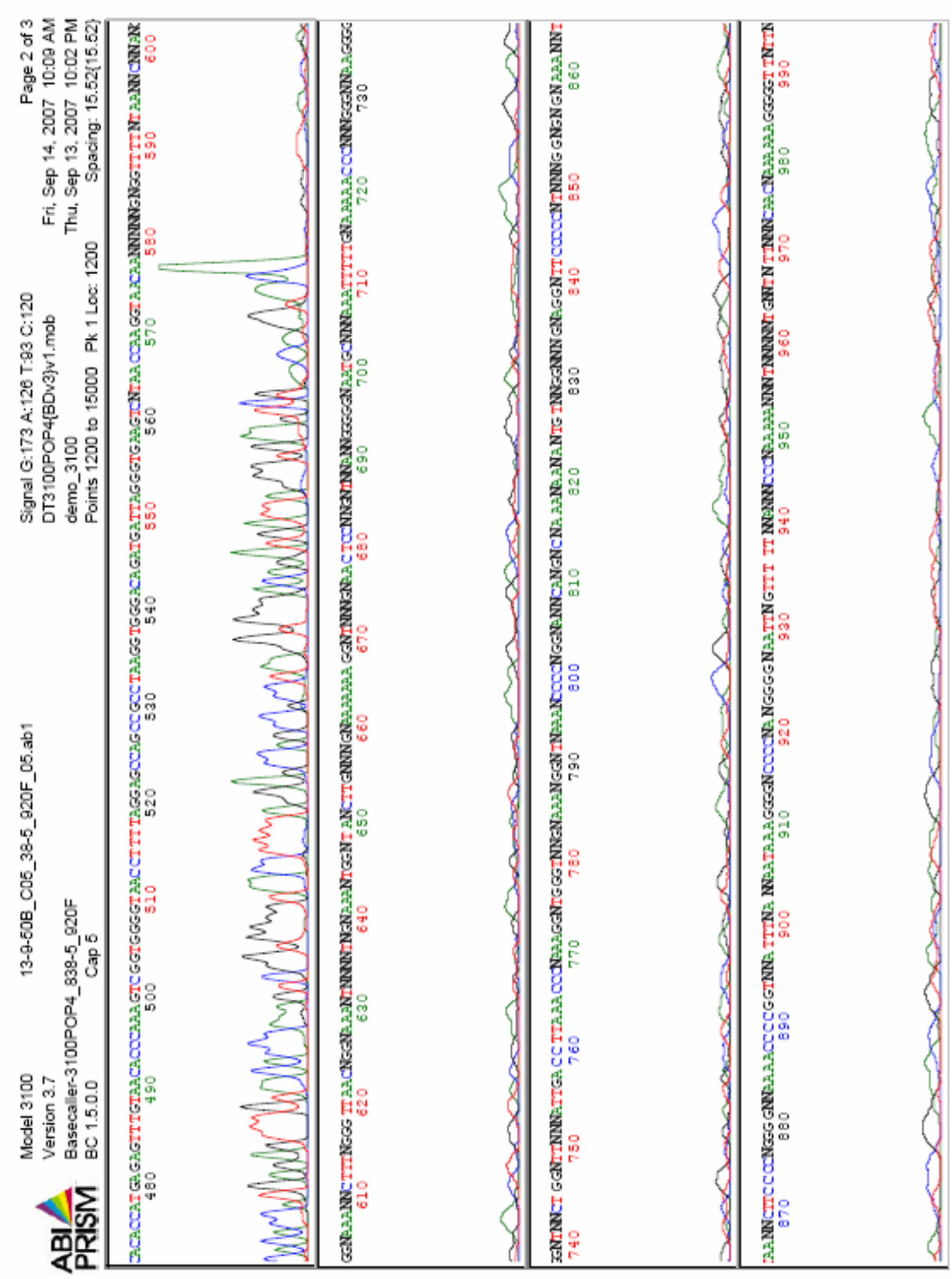


Figure 22. DNA sequence of *Lactobacillus* sp. SK5 in the plasmid 920F (5’ AAA CTA AAA TGA ATT GAC GG 3’)

APPENDIX - B

Media

MRS-broth (Lactobacilli broth act to MAN, ROGOSA SHARP for microbiology)

Composition type (g/litre)

Peptone from casein	10.0	g
Meat extract	8.0	g
Yeast extract	4.0	g
D-glucose	20.0	g
Di-potassium hydrogen phosphate	2.0	g
Tween 80	1.0	g
Di-ammonium hydrogen citrate	2.0	g
Sodium acetate	5.0	g
Magnesium sulfate	0.2	g
Manganese	0.04	g

Preparation

Suspend 52.2 g in 1 litre of demin. water; autoclave (15 min. at 118 ^{o}C).

pH: 5.7±0.2 at 25^{o} C

Sabouraud -2% dextrose broth (SDB)

Composition type (g/litre)

Peptone from meat	5.0	g
Peptone from casein	5.0	g
D (+) glucose	20.0	g

Preparation

Suspend 30 g in 1 litre of demineralized water; autoclave (15 min. at 121 ^{o}C)

Sabouraud -4% dextrose agar (SDA)

Composition type (g/litre)

Peptone	10.0	g
D (+) glucose	40.0	g
Agar-Agar	15.0	g

Preparation

Suspend 65 g in 1 litre of demin. water by heating in a boiling water bath or in a current of stream; autoclave (15 min. at 121 ^{o} C). Do not overheat.

Bacto™ Brain Heart Infusion

Composition type (g/litre)

Calf brains, infusion from 200 g	7.7	g
Beef heart, infusion from 250g	9.8	g
Proteose peptone	10.0	g
Dextrose	2.0	g
Sodium chloride	5.0	g
Disodium phosphate	2.5	g

Preparation

Suspend 37 g of the powder in 1 L of purified water mix thoroughly. Heat with frequent agitation and boil for 1 minutes to completely dissolve the powder. Autoclave (121^{o} C for 15 minutes) Final pH 7.4±0.2

Mueller-Hinton broth

Composition type (g/litre)

Infusion from meat	2.0	g
Casein hydrolysate	17.0	g
Starch	1.5	g

Preparation

Suspend 21 g in 1 litre of demin. water; dispense into tube; autoclave (121^{o} C for 15 minutes) Final pH 7.4±0.2 at 25^{o} C

Meuller Hinton Agar

Composition type (g/litre)

Beef extract powder	2.0	g
Acid digest of casein	17.5	g
Starch	1.5	g
Agar	17.0	g

Preparation

Suspend 38 g of the powder in 1 L of purified water. Mix thoroughly Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121 ^{o}C for 15 minutes.

Adjust and/or supplemented as required to meet performance criteria Final pH 7.3±0.1

Agar-Agar

Granulated, purified and free from inhibitors for microbiology

Preparation

For solid culture media which can be inoculated on the surface, approx. 12-15 g/l; which pH values below 6.0 up to 20g/l; for semi-solid culture media, approx 3-8 g/l; for liquid culture media of low viscosity, approx. 0.5-1 g/l.

Skim milk

Adjusted and/or supplemented as required to meet performance criteria. Final pH 6.3±2

Preparation

Suspend 100 g of the powder in 1 L of purified water warm, if necessary. Autoclave at 121 ^{o}C for 15 minutes.

Microbiology Anaerocult A

Anaerocult A contains components which chemically bind oxygen quickly and completely, creating and oxygen-free (anaerobic) milieu and a CO_2 atmosphere.

Composition

Kieselguhr

Iron powder

Citron acid

Sodium carbonate

Sodium carbonate

The chemical mixture inside the sachet contains free crystalline silica. In case of damage to the sachet do not inhale dust. Repeated inhalation can cause severe harm to health. Contact with the eyes may cause irritations.

Direction

Anaerocult A is put into the anaerobic jar

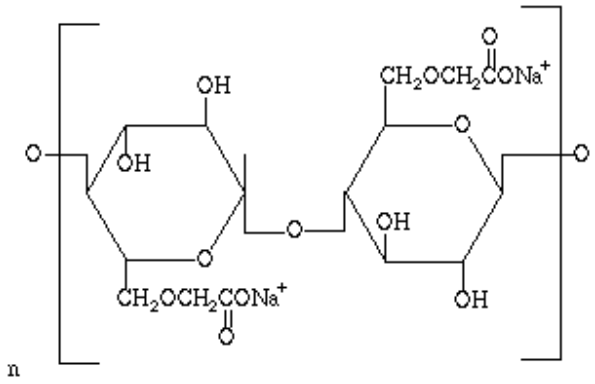
Polyethylene Glycol 400 (PEG 400)

PEG 400 is a low molecular weight. It is a clear, colorless, viscous liquid. Due in part to its low toxicity, PEG 400 is widely used in a variety of pharmaceutical formulations.

Polyethylene Glycol 4000 (PEG 4000)

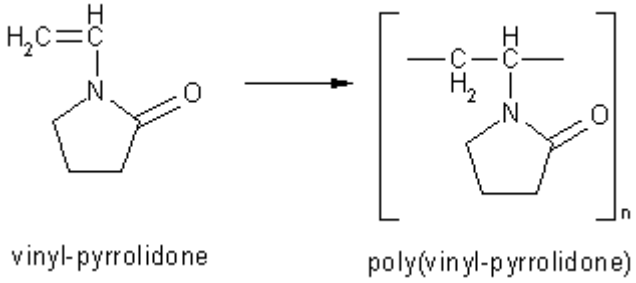
PEG - 4000 is a high molecular weight polymer of ethylene oxide and is a blend of polymers with different degrees of polymerization. Like all other PEGs, PEG-4000 is readily soluble in water. So water can be the most economical solvent for this, apart from other organic solvents. PEG-4000 acts as binder & dry lubricant due to its laminar structure and therefore can be used in the manufacture of pills and tablets for certain pharmaceutical preparations.

Sodium carboxymethyl cellulose (Na-CMC)



Sodium carboxy methyl cellulose is an anionic water soluble polymer derived from cellulose. It is odorless, tasteless and nontoxic. It has the following properties making it useful in a wide variety of applications.

Polyvinyl pyrrolidone (PVP)



PVP is soluble in water and other polar solvents. In water it has the useful property of Newtonian viscosity. When dry it is a light flaky powder, which readily absorbs up to 18% of its weight in atmospheric water. In solution, it has excellent wetting properties and readily forms films. This makes it good as a coating or an additive to coatings.

Minimum Essential Medium Eagle (MEM) of HeLa cells

Incomplete media

Ingredient	1 L	Storage
MEM	1 pack	2-8°C
Sodium bicarbonate	22 g	RT
1 N NaOH or 1 N HCl	Adjusted to pH 7.0	RT
Sterile water	Adjusted to1 L	

Sterile by filtration (0.22 μm)

Store at 2-8°C

Phosphate buffer saline

NaCl	80 g
$Na_2HPO_4 \cdot 12H_2O$ (Na_2HPO_4 11.5 g , NaH_2PO_4 9.7 g, Add water 1,000 ml)	29 g
KCl	2 g
KH_2PO_4 (K_2HPO_4 2.55 g)	2 g
Autoclave 121°C, 30 min	

Ultra water pure 900 ml

PBS (concentrated) 100 ml (PBS were diluted 10 fold) and then filtrate solution

Trypsin-EDTA solution

0.25 trypsin	0.5	g
0.02% EDTA·2Na	0.04	g
PBS	200	ml

Citrate buffer, pH 4.4, 0.1 mol/L

Put 56 mL of citric acid solution and 44 ml of sodium citrate solution in a beaker and allow the solution to equilibrate at 37 ^{o}C in water bath. Check the pH with a pH meter and adjust to pH 4.40 (±0.05) by adding citric acid solution or sodium citrate solution. Transfer the solution quantitatively to a 200 ml volumetric flask, dilute to the calibration mark with distilled water, and store at 4 ^{o}C.