

รายงานสรุปผลการวิจัยฉบับสมบูรณ์

ผลการยึดเกาะและฤทธิ์การยับยั้งของ *Lactobacillus fermentum* SK5

ต่อเชื้อแบคทีเรียก่อโรคในช่องคลอดและการผลิตเชื้อในรูปของยาเหน็บช่องคลอด

**Adhesion and inhibitory effects of *Lactobacillus fermentum* SK5  
against vaginal bacterial pathogens and preparation of  
microbial vaginal suppository**

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โครงการวิจัยนี้ได้รับทุนสนับสนุนจากงบประมาณมหาวิทยาลัยสงขลานครินทร์

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**ชื่อโครงการวิจัย** ผลการยึดเกาะและฤทธิ์การยับยั้งของ *Lactobacillus fermentum* SK5 ต่อเชื้อแบคทีเรียก่อโรคในช่องคลอดและการผลิตเชื้อในรูปของยาเหน็บช่องคลอด  
Adhesion and inhibitory effects of *Lactobacillus fermentum* SK5 against vaginal bacterial pathogens and preparation of microbial vaginal suppository

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## KEY PERFORMANCE INDEX

เป้าหมาย ผลผลิต ตัวชี้วัด และผลการดำเนินงานโครงการวิจัย

แบบวิจัย 16

ลำดับ	ตัวชี้วัด	หน่วยนับ						หมายเหตุ
		ปีที่ 1		ปีที่ 2		ปีที่ 3		
		เป้าหมาย	ผลที่ได้	เป้าหมาย	ผลที่ได้	เป้าหมาย	ผลที่ได้	
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	1.1 จำนวนผลงานตีพิมพ์ (ชิ้น)							
	- ระดับชาติ	1	1					อยู่ใน ขั้นตอน การ เตรียม ต้นฉบับ
	- ระดับนานาชาติ	1	1					อยู่ใน ขั้นตอน review
	1.2 จำนวนผลงานที่นำเสนอ ในที่ประชุม/สัมมนา (ชิ้น)							
	- ระดับชาติ	1	1					
	- ระดับนานาชาติ	1	1					
	1.3 อื่น ๆ (ไปรตระบุ)							
<b>2</b>	<b>ผลผลิตเชิงผลิตภัณฑ์/นวัตกรรม</b>							
	2.1 จำนวนโครงการวิจัยต่อ ยอด (โครงการ)							

ลำดับ	ตัวชี้วัด	หน่วยนับ						หมายเหตุ
		ปีที่ 1		ปีที่ 2		ปีที่ 3		
	2.2 จำนวนโครงการขอทุน ขนาดใหญ่จากแหล่งทุน ภายนอก (โครงการ/เงิน)							เช่น ชื่อ แหล่งทุน ที่เสนอ ขอ
	2.3 จำนวนผลิตภัณฑ์/ นวัตกรรม (ชิ้น/ระบุชื่อ)							
	2.4 เอกสารการยื่นขอ สิทธิบัตร/ อนุสิทธิบัตร (เรื่อง)							
	2.5 เทคโนโลยีที่สามารถนำไป ถ่ายทอดได้  - จำนวนโครงการ/จำนวน ครั้ง  - จำนวนผู้ได้รับประโยชน์ (คน/ชุมชน)							
	2.6 ข้อเสนอเชิงนโยบาย/ ข้อเสนอเพื่อแก้ไขปัญหา (จำนวน ข้อเสนอ)							
	2.7 การนำไปใช้ประโยชน์ อย่างอื่น ที่หน่วยงานที่นำไปใช้ ประโยชน์มีเอกสารรับรอง (เรื่อง)							
	2.8 อื่น ๆ							

## บทคัดย่อ

Lactobacilli ในช่องคลอดมีบทบาทสำคัญต่อสุขภาพของช่องคลอด เช่น ช่วยควบคุม pH ยับยั้งเชื้อก่อโรคในช่องคลอด และช่วยเสริมภูมิคุ้มกัน เป็นต้น *Lactobacillus fermentum* SK5 แยกจากช่องคลอดผู้หญิงสุขภาพดีและไม่เป็นโรคในช่องคลอด มีฤทธิ์ในการยับยั้งเชื้อก่อโรคในช่องคลอด ได้แก่ *Gardnerella vaginalis* และ *Bacteroides vulgatus* DMST 15535 สารสำคัญที่มีฤทธิ์ในการยับยั้งเชื้อเป็นสารโปรตีนในกลุ่มแบคเทอริโอซิน ซึ่งออกฤทธิ์ได้ดีในสภาวะที่เป็นกรด (pH 5) แต่ฤทธิ์จะลดลงเล็กน้อยเมื่อได้รับความร้อนสูง (100-121 °ซ.) หรือถูกย่อยด้วยเอนไซม์ทริปซิน แบคเทอริโอซินจาก *L. fermentum* SK5 ถูกทำให้กึ่งบริสุทธิ์และได้ % yield เท่ากับ 18.27 *L. fermentum* SK5 สามารถอยู่รอดในของเหลวล้างช่องคลอดได้ดี มีคุณสมบัติการเกาะกลุ่มกันเองและมีผิวเซลล์ที่ไม่ชอบน้ำซึ่งจะช่วยให้เชื้อยึดเกาะกับผนังช่องคลอดได้ดีและสร้าง biofilm ปกป้องเยื่อช่องคลอดจากการยึดเกาะของเชื้อก่อโรค นอกจากนี้ *L. fermentum* SK5 สามารถยึดเกาะบนเซลล์ HeLa และลดการยึดเกาะของ *G. vaginalis* บนเซลล์ชนิดเดียวกันนี้ *L. fermentum* SK5 สามารถป้องกันการยึดเกาะและการเจริญของ *G. vaginalis* บนเซลล์เยื่อช่องคลอดได้ดี เมื่อนำ *L. fermentum* SK5 มาเตรียมเป็นยาเหน็บช่องคลอดชนิดกวาง พบว่ามีเชื้ออยู่ 8.36 log cfu/แท่ง มีน้ำหนักเฉลี่ย 2.0785 กรัม/แท่ง มีจุดหลอมเหลวอยู่ในช่วง 35-44 °ซ. ปลดปล่อยตัวเชื้อได้หลังจากเวลา 25 นาที ในระหว่างการเก็บรักษายาเหน็บช่องคลอดไว้ที่อุณหภูมิ 4 °ซ. เป็นเวลา 3 เดือน เชื้อยังคงมีชีวิตอยู่รอดได้สูงและยังคงมีฤทธิ์ในการยับยั้งเชื้อ *G. vaginalis* ได้ดี จากการศึกษาในครั้งนี้พบว่า *L. fermentum* SK5 แสดงคุณสมบัติของการเป็นโปรไบโอติกที่ดี ซึ่งสามารถจะพัฒนาเพื่อใช้ในการป้องกันเชื้อก่อโรคในช่องคลอดได้

## ABSTRACT

Lactobacilli in vagina play important roles for the health of vagina by various activities such as control vaginal pH, inhibit vaginal pathogens and enhance immune response. *Lactobacillus fermentum* SK5 isolated from healthy women's vaginal tract had an antimicrobial activity against vaginal pathogens *Gardnerella vaginalis* and *Bacteroides vulgatus* DMST 15535. The high molecular weight antimicrobial substance was protein and supposed to be bacteriocin. Its activity was optimum at pH 5 but slightly reduced after exposed to heat (100-121°C) or trypsin. The bacteriocin from *L. fermentum* SK5 was partially purified and had 18.27% yield of the initial activity. *L. fermentum* SK5 survived in vaginal fluid and had good autoaggregation characteristic and a high surface hydrophobicity that enhanced its adhesion ability to epithelial cells and for biofilm formation in vagina. This lactobacillus showed coaggregation with *G. vaginalis* to affect its adhesion and colonization. *L. fermentum* SK5 showed high adhesion ability to HeLa cell line and the adhesion of *G. vaginalis* to this cell line was decreased in the presence of these bacteria. These incidences provided evidence of the possible colonization of *L. fermentum* SK5 that would prevent binding and growth of *G. vaginalis* onto vaginal epithelial cells. *L. fermentum* SK5 was prepared as hollow-type vaginal suppository that contained 8.36 log cfu of bacteria with average weight 2.08 g, melting point 35-44 °C, the release time of this *Lactobacillus* from the suppository after 25 min and showed good viability and activity against *G. vaginalis* during storage for 3 months. It is likely that this lactobacillus strain could be a potential probiotic candidate for beneficial use in preventing against vaginal microbial infections.

# CHAPTER 1

## INTRODUCTION

### 1.1 Background and Rationale

Recently, an increasing interest has developed in probiotic bacteria that possess health-promoting effects. In particular, *Lactobacillus* species are Gram-positive facultative anaerobe, non-spore forming and non-flagellated rods. They are a major part of the lactic acid bacteria (LAB) group because most of their members convert lactose and other sugars to lactic acid. Lactobacilli are normal flora in the oral cavity, gastrointestinal (GI) tract and vagina. They play an important role for the health of the GI tract and vagina through different mechanisms including secrete organic acid (lactic acid and short chain fatty acids) (Røssland *et al.*, 2005), produce other antimicrobial substances, i.e. hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Sethi *et al.*, 2006; Mijac *et al.*, 2006), bacteriocin (Rojo-Bezarez *et al.*, 2007; Todorov *et al.*, 2007; Lash *et al.*, 2005; Trinetta *et al.*, 2008), adhere to surface and inhibit the adhesion of pathogens (Maragkoudakis *et al.*, 2006; Tsai *et al.*, 2005; Ramiah *et al.*, 2008; Collado *et al.*, 2007; Gueimonde *et al.*, 2006), stimulate the host's immune system (Kaur *et al.*, 2002) and compete for nutrients with pathogens (McNaught and MacFie, 2001). Furthermore, challenging uropathogenic *Escherichia coli* biofilms with lactobacilli *in vitro* caused significant *E. coli* killing, and *E. coli* biofilms challenged with spent media of *L. rhamnosus* GR-1 caused a marked decrease of cell density and increased cell death (McMillan *et al.*, 2011).

There is the evidence that lactobacilli have been shown to inhibit *in vitro* growth of many pathogens and have been used as probiotics to treat a broad range of GI and/or vaginal disorders (Matu *et al.*, 2010). The GI and urogenital tracts are complex microbial habitats, which for the most part, are infection-free throughout life. The composition, dynamics and structure of the normal flora biofilms appear to play a role in protecting the host from infectious upset. Specifically, lactobacilli and the gut bifidobacteria, have been found to possess properties which enhance the host's ability to compete against pathogens (Reid *et al.*, 1998). The use of oral formulations of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 has been associated with normalization of the vaginal environment within 28 to 60 days after the initiation of treatment (Reid and Burton, 2002; Reid *et al.*, 2003). Moreover, evidence of fecal and vaginal colonization by these strains was observed after 14 days of oral administration (McLean and Rosenstein, 2000). Reid *et al.*, (2001) demonstrated that oral probiotics can resolve urogenital infections. This may have occurred due to the microorganisms themselves ascending to the vagina from the rectal area, or by altering the ability of pathogens to transfer to the vagina. Lee *et al.*, (2007) demonstrated that oral administration of lactobacilli can be as effective as a daily treatment with antibiotics for the treatment of urinary tract infections.



## **1.2 Probiotics**

### **1.2.1 Definition of probiotics**

The Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) (2001) define probiotics as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host”.

### **1.2.2 Health benefits of probiotics**

#### **Prevention of pathogens infection in GI tract**

Protective effects of probiotics against GI infections have been observed. The mechanisms may include the production of acids, H<sub>2</sub>O<sub>2</sub> or antimicrobial substances, competition for nutrients or adhesion receptors and stimulation of the immune system. Marianelli *et al.* (2010) suggested that *L. rhamnosus* GG ATCC 53103 had the antimicrobial effect against *Salmonella enterica* subsp. *enterica* serovar typhimurium 1344. Its antagonistic activity involved both lactic acid and secreted non-lactic acid molecules. *L. acidophilus* strain LAP5 was with antagonistic effect against *Salmonella choleraesuis*. The LAP5 cell culture showed a higher inhibitory effect on the invasion of *S. choleraesuis* to Caco-2 cells than the spent culture supernatant of LAP5 did. Also, the pH, organic acids or the bacteriocin may play the role of antagonistic effect. However, adhesion of LAP5 cells

to Caco-2 cell line may also play roles to reduce the invasion of *S. choleraesuis* (Lin *et al.*, 2008).

### **Prevention of pathogens infection in vagina**

The role of *Lactobacillus* probiotics in the female urogenital tract as a barrier to infection is of considerable interest. These organisms are believed to contribute to the control of vaginal microbiota by competing with other microorganisms for adherence to epithelial cells and by producing antimicrobial compounds. These bactericidal compounds include organic acid, which lowers the vaginal pH, H<sub>2</sub>O<sub>2</sub>, bacteriocin-like substances and possibly biosurfactants (Boris and Barbés, 2000). Xu *et al.* (2008) investigated the indigenous lactobacilli from the vagina of pregnant women and to screen the isolates with antagonistic potential against pathogenic microorganisms. The results were shown that 78 lactobacilli strains could produce hydrogen peroxide, in which 68%, 80%, 80%, and 88% had antagonistic effects against *Candida albicans* CMCC98001, *Staphylococcus aureus* CMCC26003, *Escherichia coli* CMCC44113, and *Pseudomonas aeruginosa* CMCC10110, respectively. The most commonly isolated species from vagina of Chinese pregnant women were *L. acidophilus* and *L. crispatus*. Most of *L. acidophilus* and *L. crispatus* produce a high H<sub>2</sub>O<sub>2</sub> level.

### **Alleviation of lactose intolerance**

Lactose maldigestion is frequent in adults (primary lactose maldigestion) and in subjects with small bowel resection or enteritis (secondary

lactose maldigestion). Symptoms include loose stools, abdominal bloating, pain, flatulence, nausea and borborygmi. Individuals with lactose maldigestion can tolerate lactose present in yoghurt to a much greater degree than the same amount of lactose in raw milk (Marteau *et al.*, 2002). Two different, though not exclusive, mechanisms of action have been put forward to explain this finding. Yoghurt and probiotic LAB contain high levels of lactase, which is released within the intestinal lumen when these bacteria are lysed by bile secretions. Lactase then acts on the ingested lactose, thus relieving maldigestion symptoms. The reduced intestinal transit time of yoghurt might also allow slower digestion of lactose, so reducing the symptomatology.

### **Reduction of the risk associated with colon cancer**

Colon cancer is a multi-factorial and complex neoplasm involving both genetics and environmental factors. There seems to be a strong relationship between colon cancer, diet and intestinal microflora. The rupture of the intestinal microflora equilibrium due to a bad diet seems to be related to an increase in the risk of developing colon cancer. Probiotics may modulate several major intestinal functions potentially associated with the development of colon cancer preventing the growth of deleterious organisms, producing anti-carcinogenic substances and moving the balance of gut bacteria in favour of the ones beneficial for the organism (Iannitti and Palmieri, 2010).

### **Reduction of serum cholesterol level**

Hypercholesterol is a risk factor for cardiovascular disease leading cause of death in many countries. It is important to develop new ways of reducing

serum cholesterol. The reduction of serum cholesterol could be an important health benefit of LAB, as a 1% reduction in serum cholesterol is associated with an estimated reduction of 2 to 3% in the risk of coronary artery disease. Cholesterol-lowering effects may be due in part to the deconjugation of bile salts by strains of bacteria that produce the enzyme bile salt hydrolase (BSH). As deconjugated bile salts are more readily excreted in the feces than conjugated bile salts, bacteria with BSH activity may effectively reduce serum cholesterol by enhancing the excretion of bile salts, with a consequent increase in the synthesis of bile salts from serum cholesterol; or by decreasing the solubility of cholesterol, and thus reducing its uptake from the gut (Nguyen *et al.*, 2007).

### **Modulation of the immune system**

Probiotics can influence the immune system by products like metabolites, cell wall components and DNA. Immune modulatory effects might be even achieved with dead probiotic bacteria or just probiotics-derived components like peptidoglycan fragments or DNA. Probiotic products are recognized by host cells sensitive for these because they are equipped with recognition receptors. The main target cells in that context are therefore gut epithelial and gut-associated immune cells. The interaction of probiotics with host (epithelial) cells by adhesion itself might already trigger a signaling cascade leading to immune modulation. Alternatively, release of soluble factors can trigger signaling cascades in immune cells or in epithelial cells which subsequently affect immune cells (Oelschlaeger, 2010).

### **1.2.3 Selection criteria for probiotics**

#### **Survival in vagina (applicable to probiotics for vaginal use)**

Lactic acid production maintains the vaginal pH at about 4.5 or less, is considered the major mode of action of *Lactobacillus* against vaginal infections (Boskey *et al.*, 1999). Therefore, *Lactobacillus* probiotics used in vaginal tract should survive at this pH.

#### **Adherence to mucus and/or human epithelial cells and cell lines and ability to reduce pathogen adhesion to surfaces**

The ability of probiotics to adhere to epithelial cells is a desirable quality. This is the first step in colonisation and improving their beneficial health effect. A number of probiotics have been shown to strongly adhere to human cell lines including *L. rhamnosus* GG, *L. acidophilus* LA-1 and a variety of *Bifidobacteria* (Gopal *et al.*, 2001). There are also the studies demonstrated the ability of probiotic to inhibit adherence by pathogenic organisms. For example, *L. acidophilus* RY2 showed ability to inhibit Enteroaggregative *Escherichia coli* adhesion to Caco-2 cells (Lin *et al.*, 2009). The competition between lactobacilli and gonococci for adherence to human epithelial cervical cells was investigated. This study showed that gonococci lost when competed with lactobacilli (Vielfort *et al.*, 2008).

#### **Antimicrobial activity against potentially pathogenic bacteria**

*Lactobacillus* probiotics inhibit pathogens infection by producing antimicrobial substances, i.e. lactic acid, short chain fatty acids (acetic acid, propionic

acid and butyric acid), H<sub>2</sub>O<sub>2</sub> and bacteriocin. Røssland *et al.* (2005) showed that co-culture of *Lactobacillus* (five strains) or *Lactococcus* (two strains) with *Bacillus cereus*, organic acids and other potentially antimicrobial metabolites are produced. The strains that produced lactic acid fastest inhibited *B. cereus* best. Juárez Tomás *et al.* (2011) assessed the inhibitory activity of 38 vaginal *Lactobacillus* strains against urogenital pathogens. The lactobacilli assayed inhibited the growth of various urogenital pathogens, except *Candida albicans*, mainly due to the effects of both organic acids and H<sub>2</sub>O<sub>2</sub>. In another research, 21 strains of lactobacilli were isolated from newborn infant feces. *L. rhamnosus* (seven strains), *L. paracasei* ssp. *paracasei* (four strains), *L. fermentum* (four strains), *L. buchneri* (two strains), *L. brevis* (one strain), *L. curvatus* (one strain) and *Lactobacillus* sp. (two strains) isolated from infant faeces were analysed for antimicrobial activities. It was found that some of these isolates have capability of producing broad-spectrum bacteriocin and/or bacteriocin-like substances against some food contaminants and pathogenic bacteria (Arici *et al.*, 2004).

### 1.3 Bacterial vaginosis

Bacterial vaginosis (BV) is the most common cause of abnormal vaginal discharge in women of child-bearing age. It is a syndrome of unknown cause characterized by depletion of the normal *Lactobacillus* population and an overgrowth of vaginal anaerobes, accompanied by loss of the usual vaginal acidity (Hay, 2005).

### 1.3.1 Aetiology and pathogenesis

The aetiology of BV is probably multifactorial, and the condition is not regarded as sexually transmitted infections, though it is sexually associated. One factor is an increase in vaginal pH from the normal 3.5–4.5 to 7.0, which reduces the inhibitory effect of hydrogen peroxide on anaerobic growth. This is associated with loss of lactobacilli and an up to one thousandfold increase in the concentration of several organisms, most commonly *Gardnerella vaginalis*, *Bacteroides (Prevotella)* spp., *Mobiluncus* spp. and *Mycoplasma hominis*. Hormonal changes and inoculation with organisms from a partner might be important.

### 1.3.2 Diagnosis

BV should be suspected in any woman presenting with an offensive, typically fishy-smelling vaginal discharge. Speculum examination shows a thin, homogeneous, white or yellow discharge adherent to the walls of the vagina. *Gardnerella* can be found in low concentrations in more than 50% of women without BV; therefore, culture has a poor specificity and should not be used for routine diagnosis.

#### **Amsel criteria**

Amsel criteria have been the mainstay of diagnosis. A wet-mount examination is used that involves mixing vaginal fluid with a drop of saline and observing it under oil immersion at high power (x 800). Amsel criteria for the

diagnosis of BV conclude vaginal pH > 4.5, release of a fishy smell on addition of alkali (10% potassium hydroxide), characteristic discharge on examination and presence of 'clue cells' on microscopy. At least three of the four criteria must be fulfilled to make a diagnosis of BV.

### **Gram-staining**

Examination of a Gram-stained vaginal smear is a quick and relatively simple means of confirming the diagnosis of BV. Typical lactobacilli are large, Gram-positive rods with blunt ends. *Gardnerella* is usually a Gram-negative coccus. The normal flora includes plentiful lactobacilli (Figure 1), whereas in BV there are large numbers of Gram-negative cocci and small rods (Figure 2).

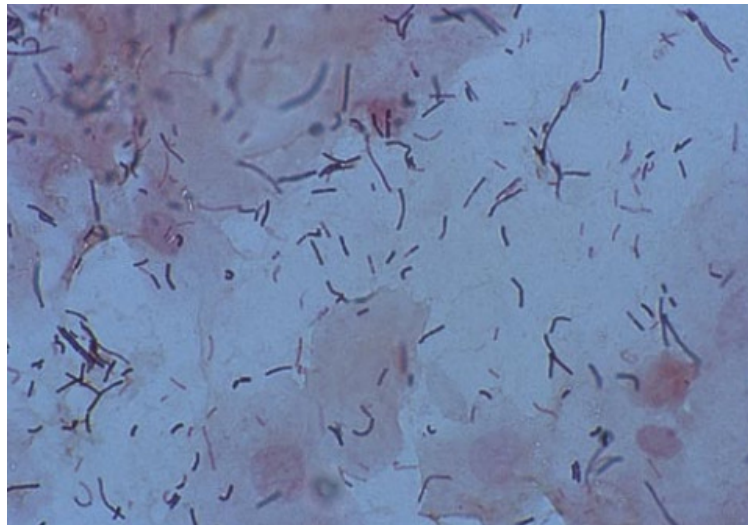


Figure 1. Gram-stained vaginal smear from a woman with normal flora. Epithelial cells and their nuclei can be seen clearly. Gram-positive rods are typical of lactobacilli (Hay, 2005).



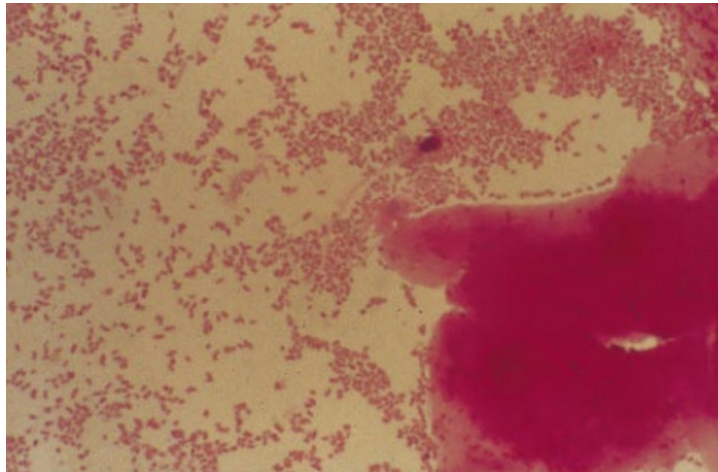


Figure 2. Gram-stained vaginal smear from a woman with BV (Hay, 2005)

### 1.3.3 Management

Antibiotics with good anti-anaerobic activity should be an effective treatment for BV, and metronidazole and clindamycin are obvious choices. Theoretically, an antibiotic that is not active against lactobacilli (e.g. metronidazole) might facilitate more rapid restoration of the vaginal flora than one that is active against these organisms (e.g. clindamycin). However, clindamycin has better activity against *M. hominis*, *Mobiluncus* spp. and *G. vaginalis* than does metronidazole.

### 1.3.4 Lactobacilli as good probiotics for prevent BV

Lactobacilli are the most well-known markers of normal vaginal flora. Their ability to produce an acid pH in the vagina (mainly due to the acidification enzyme hydrogen peroxidase) and bacteriocins that kill off other bacteria makes them prime candidates for the surveillance of vaginal health. There are many different

strains of lactobacilli present in the vagina, the most frequent being *L. jensenii*, *L. gasseri*, *L. iners* and *L. crispatus*, and there is a wide variation in species and relative numbers of species according to the population studied. In general, where lactobacilli predominate, other bacteria and parasites such as *Trichomonas* are not abundant. On the other hand, lactobacillus deficient conditions are associated with the development of numerous infectious conditions such as BV and aerobic vaginitis, and promote the transmission of sexually transmitted diseases including human immunodeficiency virus and elevated risk of preterm labor (PTL) (Donders, 2007).

Figure 3 illustrates how probiotic lactobacilli can potentially interfere with these PTL processes based upon a number of studies which have indirectly examined the mechanisms of action (Reid and Bocking, 2003).

- By adhering to the vaginal epithelium and interfering with pathogen adhesion, invasion/translocation, growth and survival; this includes deposition of collagen-binding proteins that could interfere with matrix metalloproteinase (MMP)-2 and MMP-9 degradation of tissue collagen; it includes displacing *Gardnerella* and preventing cytotoxin release and elevation of insulin-like growth factor binding protein-1 (IGFBP-1) indicative of PTL.
- Through enhancement of anti-inflammatory cytokines via the intestine and vagina that block the pathway to Cox-2 and prostaglandins and increase sIgA to inhibit pathogen colonization.
- Reducing the pH to make the vaginal environment more conducive to lactobacilli growth and better able to prevent BV recurrence. If certain strains of probiotic lactobacilli are given orally, there may be

additional benefits such as degradation of lipids and increase in conjugated linoleic acid as well as modulation of inflammation and reduction in pathogen emergence from the rectum to the vagina.

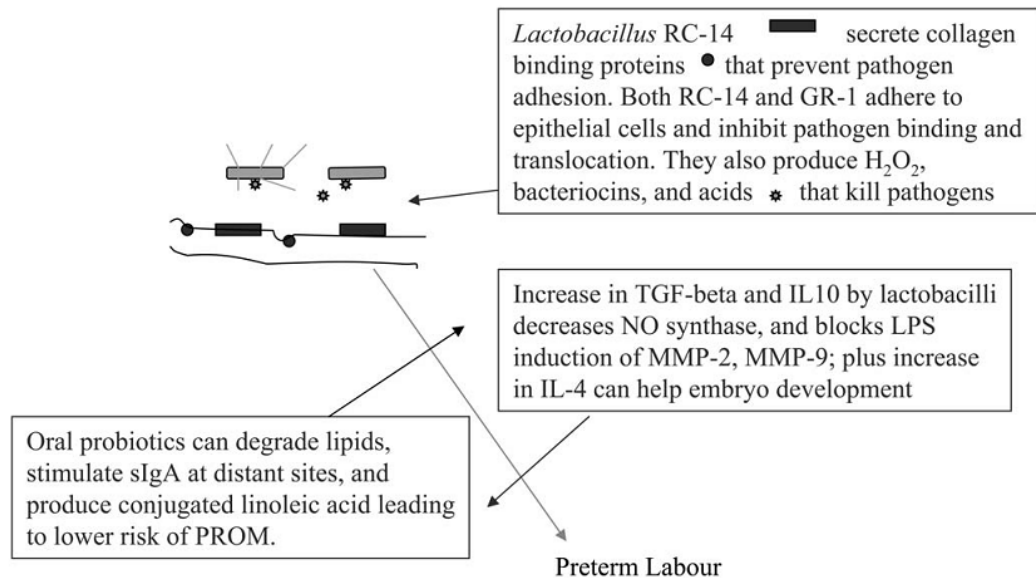


Figure 3. Potential pathways in which lactobacilli could reduce the risk of preterm labor (Reid and Bocking, 2003).

## **1.4 Factors affecting on the survival of Lactobacilli in vaginal tract**

### **Vaginal pH**

Normal microflora predominantly Lactobacilli produce sufficient lactic acid to acidify vaginal secretions to pH 3.5–4.5. This value is maintained by the Lactobacilli which convert glycogen from exfoliated epithelial cells into lactic acid (Valenta, 2005). This low pH reduces the risk of colonization by pathogens. 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).

## **1.5 Aggregation properties**

The ability to autoaggregate, cell-surface hydrophobicity and co-aggregation with pathogen strains are used for preliminary screening in order to identify potentially probiotic bacteria suitable for human or animal use (Collado *et al.*, 2008).

### **1.5.1 Autoaggregation and cell-surface hydrophobicity**

In order to express beneficial effects, probiotic bacteria need to achieve an adequate mass through aggregation. The cellular aggregation could be positive in promoting the colonization of beneficial microorganisms, as suggested for Lactobacilli in the vaginal tract. Consequently, the ability of probiotics to aggregate is

a desirable property. Bacterial aggregation between microorganisms of the same strain (autoaggregation) and hydrophobicity are related to cell adherence properties (Boris *et al.*, 1997; Del Re *et al.*, 2000; Kos *et al.*, 2003). Adhesion is a complex process involving non-specific (hydrophobicity) and specific ligand-receptor mechanisms. Adherence of bacterial cells is usually related to cell surface characteristics.

### **1.5.2 Coaggregation**

The aggregation between genetically different strains (coaggregation) as coaggregation of probiotic strains with pathogens is of considerable importance in therapeutic manipulation of the aberrant vaginal microbiota. Coaggregation abilities depend on each strain (probiotic and pathogen strains) and time of coaggregation (Collado *et al.*, 2008). Many authors have reported that the coaggregation abilities of *Lactobacillus* species might enable it to form a barrier that prevents colonization by pathogenic bacteria (Pelletier *et al.*, 1997; Bao *et al.*, 2010).

### **1.6 Adherence of Lactobacilli to vaginal epithelial cells and interference with pathogens**

The ability to adhere to epithelial cells is an important criterion for probiotics because only strains which can adhere to mucosal cells might be successful with respect to colonization (Merk *et al.*, 2005). To evaluate adhesion ability of probiotic strains on vaginal epithelial cells, HeLa cells have been used as *in vitro*

model. Tropcheva *et al.* (2011) examined the adhesion potential of pre-selected strain *L. plantarum* AC131. The result showed that *L. plantarum* AC131 was classified as strongly adhesive to HeLa cell line.

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 pathogens. Mastromarino *et al.* (2002) showed coaggregation of strains of *L. salivarius* and *L. gasseri* with *G. vaginalis* and *C. albicans* and suggested, that coaggregation is an important factor for a healthy urogenital flora because of the production of a microenvironment around a pathogen with consecutive rise of inhibiting substances produced by lactobacilli (Figure 4).

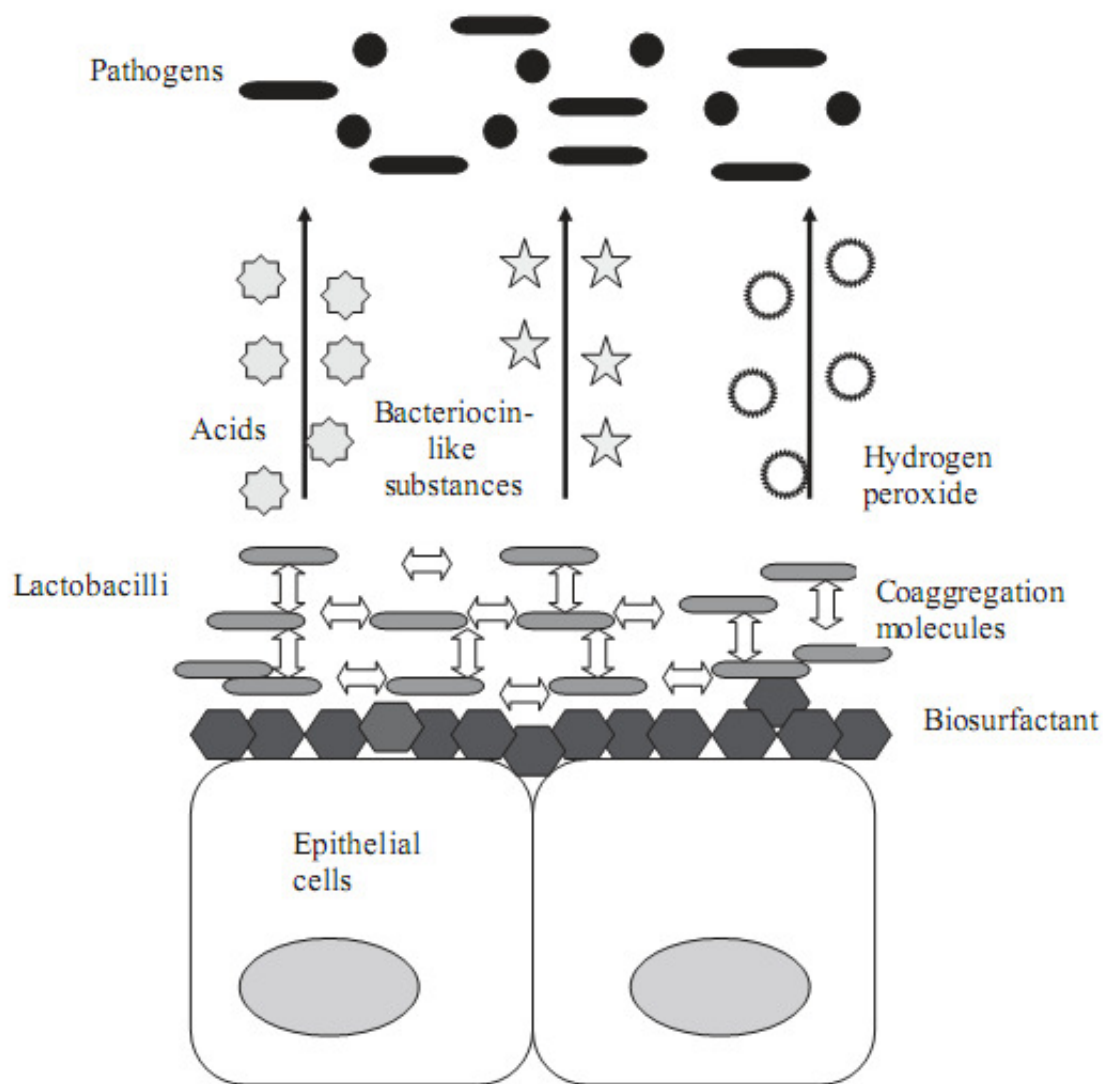


Figure 4. Competition of normal flora and pathogens. Lactobacilli can enhance host defence by occupying vaginal epithelia in large numbers and diversity, by competing for essential nutrients or for epithelial attachment sites, production of antimicrobial compounds such as bacteriocin-like substances, acids,  $H_2O_2$ , and biosurfactants (Mastromarino *et al.*, 2002).

## **1.7 Inhibitory substances produced by Lactobacilli.**

### **1.7.1 Fatty acids**

Organic acids such as lactic, acetic and propionic acids produced as end products which provide an acidic environment unfavourable for the growth of many pathogenic and spoilage microorganisms. Acids are generally thought to exert their antimicrobial effect by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions (Ross *et al.*, 2002). They have a very broad mode of action and inhibit both gram-positive and gram-negative bacteria as well as yeast and moulds (Caplice and Fitzgerald, 1999).

The vaginas of healthy premenopausal women show a pH of 3.5–4.5. Vaginal epithelial cells maintain this acidity by producing fatty acids, which are released into the secretions. Lactobacilli contribute to the vaginal acidity by also producing lactic acid and other fatty acids (Boris and Barbés, 2000). A low vaginal pH seems to be an important factor in controlling the composition of microbiota (Merk *et al.*, 2005). Vaginal fluid was examined for antimicrobial components and the results showed that the vaginal fluid with the highest levels of antimicrobial activity against *Escherichia coli* also correlated with both low pH and high lactic acid content (Valore *et al.*, 2002).



### 1.7.2 Hydrogen peroxide

Most lactobacilli are able to produce  $H_2O_2$ , which has a toxic potential towards other bacteria but to the producing bacteria themselves. Lactobacilli and other lactic-acid-producing bacteria lack heme and thus do not utilize the cytochrome system (which reduces oxygen to water) for terminal oxidation. Lactobacilli utilize flavoproteins, which generally convert oxygen to  $H_2O_2$ . This mechanism, together with the absence of the heme protein catalase, generally results in the formation of  $H_2O_2$  in amounts which are in excess of the capacity of the organism to degrade it. The  $H_2O_2$  formed may inhibit or kill other members of the microbiota, particularly those which lack or have low levels of  $H_2O_2$ -scavenging enzymes, such as catalase peroxidase. The microbicidal activity of  $H_2O_2$  is considerably increased by the enzyme peroxidase in the presence of a halide ion (Eschenbach *et al.* 1989).

### 1.7.3 Bacteriocin

Bacteriocins are ribosomally synthesized, extracellularly released low-molecular-mass peptides or proteins (usually 30-60 amino acids) which have a bactericidal or bacteriostatic effect on other bacteria such as closely related bacteriocin producing species, food spoilage bacteria, and food-borne pathogens (Cheikhoussef *et al.*, 2009).

### **Classification of bacteriocin**

Bacteriocins of LAB are classified into four classes based on primary structure, molecular mass, heat stability, and molecular organization (Deraz *et al.*, 2005).

Class I bacteriocins (lantibiotics) are small (< 5 kDa) and heat-stable peptides containing thioether amino acids (lanthionine and  $\beta$ -methyl lanthionine).

Class II bacteriocins are small, heat-stable, non-lantibiotic peptides (< 10 kDa).

Class III comprises of large (> 30 kDa), heat labile bacteriocins.

Class IV is large, complex bacteriocins containing lipid or carbohydrate groups.

### **Mechanisms for bacteriocin action**

Most bacteriocins are amphiphilic and cationic. Based on bacteriocins amphiphilic characteristics, there are at least two different mechanisms which may explain their membrane-permeabilization action (Figure 5). Bacteriocin molecules contain a region of positively charged amino acids that are thought to interact electrostatically with the negatively charged polar head groups of the phospholipids of cell membrane. It was thought to contribute to the initial binding with the target membrane. The association of hydrophobic patches of bacteriocins and the hydrophobic membrane dissipate the proton motive force (PMF) of the target cell by forming a pore through the cytoplasmic membrane which causes in the rapid efflux of small cytoplasmic compounds, e.g., amino acid, potassium, inorganic phosphate, preaccumulated rubidium and glutamate and flux of essential energy (ATP). Since

ATP has no transport system in the sensitive cells and glutamate is not transported by proton motive force driven system, the result induced to cells death. Alternatively, bacteriocins may destabilize the integrity of the cytoplasmic membrane in a detergent-like fashion (Montville *et al.*, 1995).

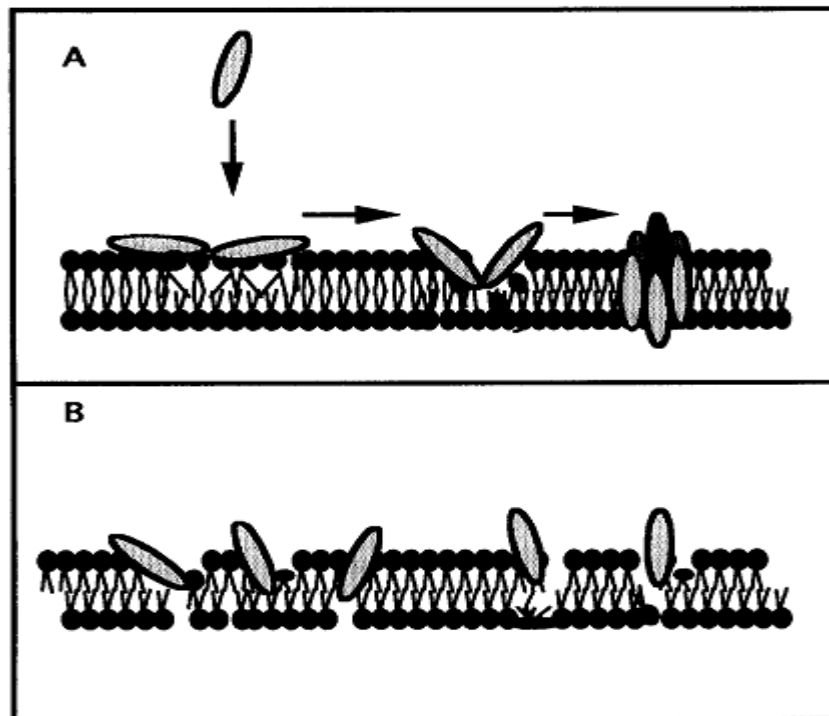


Figure 5. Interaction of bacteriocin monomers (ovals) with the cytoplasmic membrane according to the “poration complex” model (A) and the “detergent disruption” model (B) (Montville *et al.*, 1995)

## 1.8 Vaginal suppository

Traditionally, solutions, suppositories, gels, foams and tablets have been used as vaginal formulations. More recently, vaginal ring has been introduced for hormone replacement and contraceptive therapy. Ideally, the choice of vaginal drug administration depends on the applicability of the intended effect. For a local effect to occur, semi-solid or fast dissolving solid system will be required. For a topical effect, generally, a bioadhesive dosage form or intravaginal ring system would be more preferable (Hussain and Ahsan, 2005).

The local application of products that contain lactobacillus is likely to reduce vaginal infections as confirmed by clinical studies (Hallen *et al.*, 1992; Shalev *et al.*, 1996). Currently the products for vaginal delivery of *Lactobacillus* include dairy products (yogurt, acidophilus milk, etc.) and commercially available powders and tablets containing *Lactobacillus*. These products often have poor patient compliance for several reasons such as irritation, discomfort, and leakage at the application site. In that case, a suppository dosage form containing *Lactobacillus* would be a better option. A vaginal suppository has certain advantages: dose uniformity can be maintained, insertion into the vagina without irritation is possible, and a large volume of dissolution fluid is not required for the release of active substance (Kale *et al.*, 2005). The hollow-type suppository was developed by Watanabe *et al.* (1986) in order to evaluate the effectiveness of the drug when administered rectally. This suppository has a hollow cavity which drugs in the form of powder, liquid, or solid could be placed. The advantage of using the hollow-type suppository for preparation of *Lactobacillus* vaginal suppository is that it can

eliminate the effect of the heating process on the survival of *Lactobacillus* during preparation and interactions between *Lactobacillus* and the suppository materials can be essentially eliminated (Watanabe and Matsumoto, 1984). *Lactobacillus* vaginal hollow-type suppository was shown in Figure 6.

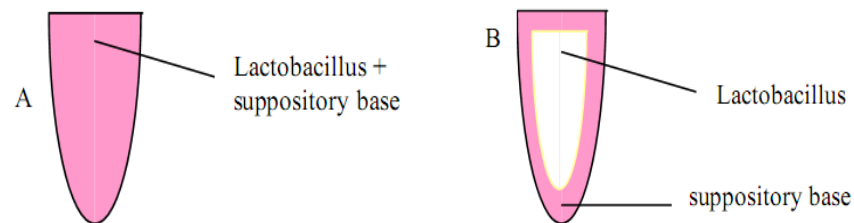


Figure 6. Schematic illustration of conventional suppository (A) and hollow-type suppository (B)

### Suppository base

Collett (1990) described that the properties of an ideal suppository base are:

- Melts at body temperature or dissolves in body fluids
- Non-toxic and non-irritant
- Compatible with any medicament
- Releases any medicament readily
- Easily molded and removed from the mold
- Stable to heating above the melting point
- Resistant to handling
- Stable on storage

### **Polyethylene glycol suppository base**

Polyethylene glycols (PEGs) are polymers of ethylene oxide and water, prepared to various chain lengths, molecular weights and physical states. They are available in a number of molecular weight ranges, the more commonly used being PEG 200, 400, 600, 1000, 1500, 1540, 3350, 4000, 6000 and 8000. The numerical designations refer to the average molecular weights of each of the polymers (Ansel, 1995). Mixture of PEG may be used as bases for suppositories. Their physical properties can be varied by suitable mixtures of high and low polymers. High polymers yield products that disintegrate and release their drug slowly. Softer, less brittle preparations that disperse and liberate their drug more quickly are obtained by mixing high with either medium or medium and low polymers, or by adding plasticizers. Advantages of PEG base include:

- No laxative effect
- Microbial contamination less likely
- Preparation is convenient. The base contracts slightly on cooling and no lubricant is necessary.
- Melting point generally above body temperature. Cool storage is therefore not so critical, they are suitable for hot climates and less likely to melt on handling. The high melting point also means that the base does not melt in the body but dissolves and disperses the medication slowly, providing a sustained effect.
- Produce high-viscosity solutions. This means that after dispersing in the body, leakage is less likely.
- Give products with clean smooth appearance

## 1.9 Objectives

- 1.9.1 To isolate and partial purify of antimicrobial substances (bacteriocin)
- 1.9.2 To characterize probiotic properties of *Lactobacillus fermentum* SK5 including survival through vaginal tracts, aggregation properties, adhesion ability to epithelial cells, inhibition of pathogens adhesion
- 1.9.3 To formulate and evaluate hollow-type vaginal suppository containing *L. fermentum* SK5

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Preparation of bacterial strains

##### 2.1.1 Preparation of *Lactobacillus* spp.

*L. fermentum* SK5 was one among the isolates that were taken by swabbing from the posterior zone of the vaginal fornix of healthy, premenopausal and non-menstruating women who were also negatively diagnosed for vaginosis, vaginitis, hepatitis and HIV. These women were those who came for the normal vaginal check at Songkla Hospital, Thailand. *L. fermentum* SK5 was identified by the API 50 CHL system (BioMerieux, France) and confirmed by 16S rDNA analysis by the National Center for Genetic Engineering and Biotechnology, Thailand, and registered in the GenBank database system <http://www.ncbi.nlm.nih.gov/> under accession number JQ894941. The 16S rDNA gene analysis showed a 98.9% probability of identity to *L. fermentum* strains available in the GenBank database system. *L. fermentum* SK5 was cultured in Mann Rogosa and Sharpe (MRS) broth (Difco, USA) and incubated in anaerobe jar containing gas pak microbiology anaerocult® A (Merck, Germany) at 37 °C for 18 and 48 h used in section 2.4-2.5. *L. rhamnosus* GG ATCC 53103 was cultured in MRS broth and incubated in aerobic condition at 37 °C for 48 h used in section 2.4.5. Bacterial cells were harvested by centrifuging 2,000 × g at 4 °C for 10 min.



### 2.1.2 Preparation of vaginal bacterial pathogens

*Gardnerella vaginalis* was cultured in Brain Heart Infusion (BHI) broth (Difco, USA) at 37 °C for 18 h and 24 h used in section 2.2-2.4. *Bacteroides fragilis* DMST 19152 and *Bacteroides vulgatus* DMST 15535 were cultured in BHI supplemented with 0.5% yeast extract (Merck, Germany) and 15 µg/ml hemin (Sigma-Aldrich, USA) and incubated in anaerobic condition at 37 °C for 48 h used in section 2.2. *Bacteroides ureolyticus* ATCC 33387, *Mobiluncus curtisii* ATCC 35241 and *Prevotella bivia* ATCC 29303 were cultured in Brucella broth (Difco, USA) supplemented with 0.2% vitamin K and 15 µg/ml hemin (Sigma-Aldrich, USA) and incubated in anaerobic condition at 37 °C for 48 h used in section 2.2. Bacterial cells were harvested by centrifuging 2,000 × g at 4 °C for 10 min.

## 2.2 Antimicrobial activity of dialyzed culture fluid produced by *L. fermentum* SK5

*L. fermentum* SK5 was grown in MRS broth and incubated in anaerobic condition at 37 °C for 5 days. After incubation, cell-free supernatant was obtained by centrifuging at 2,000 × g at 4 °C for 30 min. Cell-free supernatant was dialyzed across dialysis tube with 3,500 Da molecular weight cut-off against 20 mM sodium acetate buffer (Ajax Finechem Pty Ltd, Australia), pH 5.0 to remove low molecular weight molecules including H<sub>2</sub>O<sub>2</sub> and organic acids. After dialysis, the supernatant was collected and then lyophilized with lyophilizer. The lyophilized powder (assumed bacteriocin-like substance) was dissolved with distilled water to

make concentration of 1 g/ml. Six mm diameter paper disc was applied with 50 µl of dialyzed culture fluid and placed to dry in air before use.

The disc diffusion test was used for assaying the antibacterial activity of dialyzed culture fluid produced by *L. fermentum* SK5 against vaginal bacterial pathogens. *G. vaginalis* (24 h cultured broth), *B. fragilis* DMST 19152, *B. vulgatus* DMST 15535, *B. ureolyticus* ATCC 33387, *M. curtisii* ATCC 35241 and *P. bivia* ATCC 29303 in section 2.1.2 were adjusted with normal saline to give turbidity equal to 0.5 McFarland standard ( $10^8$  cfu/ml). 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 Muller Hinton Agar (MHA, Difco, USA) (for *G. vaginalis*), BHI supplemented agar (for *B. fragilis* DMST 19152 and *B. vulgatus* DMST 15535) and Brucella supplemented agar (for *B. ureolyticus* ATCC 33387, *M. curtisii* ATCC 35241 and *P. bivia* ATCC 29303) were inoculated by streaking the swab over the surface. Streaking was repeated three 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 h (for *G. vaginalis*) and 48 h in anaerobic condition (for *B. fragilis* DMST 19152, *B. vulgatus* DMST 15535, *B. ureolyticus* ATCC 33387, *M. curtisii* ATCC 35241 and *P. bivia* ATCC 29303). The inhibition zone diameter was measured by antibiotic zone reader (Fisher-Lilly, USA). *G. vaginalis* was used as an indicator strain in following experiments.

## 2.3 Isolation and characterization of bacteriocin from *L. fermentum* SK5

### 2.3.1 Bacteriocin biosynthesis

*L. fermentum* SK5 was cultured in MRS broth and incubated in anaerobic condition at 37 °C. At appropriate intervals, cell-free supernatant was obtained by centrifuging 2,000 × g at 4° C for 30 min and dialyzed across dialysis tube with 3,500 Da molecular weight cut-off against 20 mM sodium acetate buffer, pH 5.0. After dialysis, the supernatant was collected and lyophilized with lyophilizer. The antimicrobial activity of lyophilized powder was examined by microbroth dilution method. The lyophilized powder was dissolved with 20 mM sodium acetate buffer pH 5.0 to make concentration of 1000 µg/ml and made two-fold serial dilution with Mueller Hinton broth (MHB, Difco, USA). *G. vaginalis* (24 h cultured broth) in section 2.1.2 was adjusted with 0.85% sterile normal saline solution to give turbidity equal to 0.5 McFarland standard and the adjusted inoculum was diluted 1:100. Each well of 96 wells microtiter plate contained: 50 µl of dialyzed culture fluid at two-fold serial dilution and 50 µl of *G. vaginalis* (5x10<sup>5</sup> cfu/ml as final concentration). Positive growth control of *G. vaginalis* was included in this microtiter plate. The microtiter plate was incubated in anaerobic condition at 37 °C. After 16-20 h of incubation, the plate was examined and minimal inhibitory concentrations (MICs) were determined. The endpoint MIC is the lowest concentration of sample at which the microorganism tested does not demonstrate visible growth compared with the growth in the control well. The wells showing MIC value was examined the minimum bactericidal concentration (MBC) endpoint by plate counting on MHA. Count colonies from MBC

plates being equal to or less than the determined colony count from the original inoculum plates will be considered as a 99.9% kill or bactericidal result.

### **2.3.2 Characterization of bacteriocin**

To evaluate structural modifications produced by dialyzed culture fluid on *G. vaginalis*, *G. vaginalis* cell pellet in section 2.1.2 (18 h cultured cells) was washed once with phosphate buffered saline (PBS, Sigma-Aldrich, USA), pH 7.4 and the cell pellet was resuspended in 1 ml dialyzed culture fluid. The cells were incubated for 18 h at 37 °C, harvested (2,000 × g, 4 °C, 10 min) and washed three times with PBS, pH 7.4. The cells were smeared onto a coated slide and fixed with 2.5% (w/v) glutaraldehyde (Sigma-Aldrich, USA) in 0.1 mol/l PBS, pH 7.4 at room temperature for 2 h. After two washes with PBS pH 7.4, cells were dehydrated in a graded series of ethanol, starting with 50, followed by 60, 70, 80, 90% (v/v) and finally absolute alcohol. Cells were dried in a critical point drier (CPD 7501, Polaron, United Kingdom) and coated with gold. The specimens were then examined by scanning electron microscopy (SEM, JSM-5800LV, JEOL, Japan).

To study the chemical characterization of dialyzed culture fluid, free amino groups were determined by the ninhydrin test. For the sensitivity to heat, pH and proteolytic enzyme of dialyzed culture fluid produced by *L. fermentum* SK5, trypsin was added in the dialyzed culture fluid at final concentration of 1 mg/ml. The sample was incubated at 37 °C for 3 h and the residual activity was checked by disc diffusion test as described above. The control (dialyzed culture fluid) was used. Sensitivity to heat, the dialyzed culture fluid was treated in water bath at 60, 80, 100

and 121 °C (autoclave), 15 psi for 10 min. After, samples were cooled to 4 °C for 1 h and checked for the residual activity. For sensitivity to pH, the dialyzed culture fluid was adjusted to pH value from 2 to 9 using 50 mM different buffer (glycine-HCl buffer (Merck, Germany), pH 2-3, sodium acetate buffer pH 4-5, phosphate buffer (Merck, Germany), pH 6-7, Tris-HCl buffer (Merck, Germany), pH 8-9). Then samples were checked for the residual activity. The negative controls (buffer solution pH 2, 3, 4, 5, 6, 7, 8 and 9) were used. *G. vaginalis* was used as an indicator strain for examination of antibacterial activity by disc diffusion test as describe in section 2.2.

### **2.3.3 Partial purification of bacteriocin**

*L. fermentum* SK5 was grown in MRS broth and incubated in anaerobic condition at 37 °C for 5 days. After incubation, cell-free supernatant was obtained by centrifuging at 2,000 × g at 4 °C for 30 min. Cell-free supernatant was dialyzed across dialysis tube with 3,500 Da molecular weight cut-off against 20 mM sodium acetate buffer, pH 5.0 to remove low molecular weight molecules including H<sub>2</sub>O<sub>2</sub> and organic acids. After dialysis, the supernatant was collected and then lyophilized with lyophilizer.

The lyophilized powder was dissolved in 20 mM sodium acetate buffer, pH 5.0 and loaded on the top of Sephacryl S-100 column (Amersham Bioscience, UK) which equilibrated with 20 mM sodium acetate buffer, pH 5.0 at a flow rate of 1 ml/min. The elution profile was monitored the absorbance at 280 nm. Fractions containing bacteriocin were pooled and applied to a cation-exchanger SP-Sepharose fast flow (HiTrap, Sweden) equilibrated with 20 mM sodium acetate

buffer, pH 5.0. After washing the unbound protein, the bacteriocin was eluted using a linear gradient of 0-1 M NaCl in the same buffer. The flow rate was maintained at 1 ml/min.

Total protein in each step was determined by Bradford assay. Bacteriocin activity was determined by using microbroth dilution method (as described in section 2.3.1) with *G. vaginalis* and showed as the arbitrary unit (AU). One AU was defined as the maximum dilution factor of purified bacteriocin that inhibited the 50% of *G. vaginalis* growth.

## **2.4 Probiotic properties of *L. fermentum* SK5**

### **2.4.1 Survival in simulated vaginal fluid (SVF)**

SVF was prepared by dissolving 68.04 g of sodium acetate in sterile distilled water 1000 ml and the result solution was adjusted with glacial acetic acid to pH 4.4 based on British Pharmacopoeia (BP 2007). *L. fermentum* SK5 cell pellet in section 2.1.1 (18 h cultured cells) was washed once with sterile PBS, pH 7.4 and resuspended in SVF, pH 4.4. After incubation in anaerobic condition at 37 °C, viable cells were enumerated at 0, 1, 2 and 3 h on MRS agar. Survival percentage was calculated according to the following equation:

$$\text{Survival (\%)} = (N_1/N_0) \times 100\%$$

$N_1$ : The total viable count of *L. fermentum* SK5 after treatment

$N_0$ : The total viable count of *L. fermentum* SK5 before treatment

### 2.4.2 Autoaggregation test

PBS was prepared and adjusted the pH to 6.2 with 1N HCl. The buffer solution was sterilized by autoclaving at 121 °C, 15 psi for 15 min. *L. fermentum* SK5 cell pellet in section 2.1.1 (18 h cultured cells) was washed once with sterile PBS, pH 7.4 and resuspended in PBS, pH 6.2. A drop of cell suspension was placed on a glass microscope slide and autoaggregation was observed under microscope. Autoaggregation test was positive if *L. fermentum* SK5 formed aggregates within 2 min.

### 2.4.3 Surface hydrophobicity

Surface hydrophobicity was studied by the salt aggregation test (SAT). 0.02 mol/L sodium phosphate (Merck, Germany) was prepared and adjusted the pH to 6.8 with 1N HCl. *L. fermentum* SK5 cell pellet in section 2.1.1 (18 h cultured cells) was washed once with sterile PBS, pH 7.4 and resuspended in 0.02 mol/l sodium phosphate, pH 6.8. Solutions of ammonium sulfate (4.0, 2.0, 1.5, 0.5 mol/l) (Merck, Germany) were mixed with an equal volume of bacterial cell suspension on a glass slide. The lowest final concentration of ammonium sulfate causing the bacteria to aggregate was defined as the SAT value. Bacterial strains were classified into three groups:

High surface hydrophobicity (SAT < 0.9 mol/l)

Intermediate hydrophobicity (SAT 0.9-1.5 mol/l)

Hydrophilic (SAT > 1.5 mol/l)

Adherent lactobacilli show high surface hydrophobicity, whereas nonadherent lactobacilli are much more hydrophilic.

#### **2.4.4 Coaggregation assay**

*L. fermentum* SK5 cell pellet in section 2.1.1 (18 h cultured cells) was washed once with sterile PBS, pH 7.4 and resuspended in PBS, pH 7.4. *G. vaginalis* cell pellet in section 2.1.2 (18 h cultured cells) was washed once with sterile PBS, pH 7.4 and resuspended in PBS, pH 7.4. *L. fermentum* SK5 suspension were mixed with an equal volume of the *E. coli* or *G. vaginalis* suspension by shaking at 100 rpm for 4 h and incubated at 37 °C. The mixture was Gram-stained and coaggregation was observed under a microscope. A coaggregation assay was positive if *L. fermentum* SK5 formed aggregates with *G. vaginalis*.

#### **2.4.5 Adhesion of *L. fermentum* SK5 on HeLa cells**

HeLa cells (ATCC CCL-2.2™) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with L-glutamine, 10% fetal calf serum (Gibco, USA) and 100 U/ml of penicillin G and streptomycin sulfate (Gibco, USA) and incubated at 37 °C in 5% CO<sub>2</sub>.

The confluence cells were added in 24 wells tissue culture plate (Corning Inc, USA) at a concentration of approximately  $4.5 \times 10^5$  cells/ml and grown as described above 4-5 days until confluence. Prior adhesion assay, cell monolayer was washed twice with medium without antibiotics.



In adhesion assay, *L. fermentum* SK5 cell pellet in section 2.1.1 (48 h cultured cells) was resuspended in medium without antibiotics and added into the wells of cell monolayer to the total volume of 1 ml medium. The inoculated tissue culture plate was incubated at 37 °C in 5% CO<sub>2</sub> for 1 h. Unbound bacterial cells were discharged by washing three times with PBS. Then, cell culture was lysed by 0.05% Triton X-100 (Sigma-Aldrich, USA) for 5 min to free *L. fermentum* SK5, and adherent *L. fermentum* SK5 was enumerated by plate counting on MRS agar. MRS plates were incubated in anaerobic condition at 37 °C for 48 h. *L. rhamnosus* GG ATCC 53103 cell pellet in section 2.1.1 was used as a probiotic control strain. Adhesion percentage was calculated according to the following equation:

$$\text{Adhesion (\%)} = (N_1/N_0) \times 100\%$$

N<sub>1</sub>: Amount of adherent bacterial cells

N<sub>0</sub>: Amount of added bacterial cells

#### **2.4.6 Adhesion effect of *L. fermentum* SK5 on vaginal bacterial pathogens adhesion on HeLa cells**

HeLa cells were cultured and added in 24 wells tissue culture plate at a concentration of approximately  $4.5 \times 10^5$  cells/ml. *L. fermentum* SK5 cell pellet in section 2.1.1 (48 h cultured cells) and *G. vaginalis* cell pellet in section 2.1.2 (24 h cultured cells) were separately resuspended in medium without antibiotics.

In competitive assay, *L. fermentum* SK5 were added simultaneously with *G. vaginalis* and incubated at 37 °C in 5% CO<sub>2</sub> for 1 h.

In exclusion assay, *L. fermentum* SK5 were added and incubated at 37 °C in 5% CO<sub>2</sub> for 30 min. *G. vaginalis* were then added and incubated in the same condition.

In displacement assay, *G. vaginalis* were added and incubated at 37 °C in 5% CO<sub>2</sub> for 30 min. *L. fermentum* SK5 were then added and incubated in the same condition.

The adherent *G. vaginalis* were enumerated by plate counting on MHA. MHA plates were incubated in aerobic condition at 37 °C for 24 h. *G. vaginalis* suspension without *L. fermentum* SK5 was used as the control. Adhesion percentage was calculated as described in section 2.4.5.

## **2.5 Preparation of *L. fermentum* SK5 hollow-type vaginal suppository**

### **2.5.1 Production of *L. fermentum* SK5 cells**

*L. fermentum* SK5 was prepared in lyophilized form used in formulation. Cryoprotectants were used to protect *L. fermentum* SK5 cells during lyophilization process. To select suitable cryoprotectants, *L. fermentum* SK5 cell pellet in section 2.1.1 (48 h cultured cells) was resuspended in cryoprotectant solution (skim milk, inulin and galactooligosaccharide (GOS)) at the concentration of 2, 4, 6, 8 and 10% (w/v). These suspensions were lyophilized by lyophilizer. Viable cells were enumerated before and after lyophilization.

To evaluate effect of prebiotic used as cryoprotectant on the growth of *L. fermentum* SK5, the bacteria (1%) in section 2.1.1 (48 h cultured broth) was

inoculated into quarter strength MRS broth supplemented with prebiotics (inulin and GOS) at the concentration of 1, 2 and 3% (w/v) and incubated under anaerobic condition at 37 °C for 48 h. Growth was assessed by measuring the optical density at 580 nm at various time intervals.

*L. fermentum* SK5 lyophilized powder was prepared by using cryoprotectant that showed high survival and well improved the growth of this strain.

### **2.5.2 Preparation of *L. fermentum* SK5 hollow-type vaginal suppository**

Vaginal suppository was prepared by fusion method (molding method) using the mixture of PEGs (PEG 400 and PEG 4000 in the ratio of 55:45) as the suppository base. The suppository base was melted in boiling water bath. After melting, the base was cooled down to the temperature approximately 45 °C. The base was poured into a suppository mold equipped with cylindrical tube in the center and allowed to solidify at room temperature. Before the base completely solidified, the cylindrical tube was removed and the hollow cavity of suppository was obtained. The lyophilized powder of *L. fermentum* SK5 was added into the suppository cavity. The open end of the suppository was sealed with melted base.

### **2.5.3 Evaluation of *L. fermentum* SK5 hollow-type vaginal suppository**

#### **Uniformity of weight**

Uniformity of weight determination was performed based on BP 2007. Twenty vaginal suppositories were weighed individually and then the average weight was calculated.

#### **Differential scanning calorimetry (DSC)**

The thermal properties of pulverized PEGs base (the mixed of PEG 400:PEG 4000, ratio 55:45) 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.

#### ***In vitro* release study**

*In vitro* release of *L. fermentum* SK5 from vaginal suppository was studied in 20×150 mm test tube containing 6 ml sodium acetate 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 interval time until 1 h. The average amount of *L. fermentum* SK5 was calculated.

#### **Stability**

Vaginal suppositories were kept at 4° C. The viability and antibacterial activity of *L. fermentum* SK5 were determined every month until 3 months.

## CHAPTER 3

### RESULTS AND DISCUSSIONS

#### 3.1 Antimicrobial activity of dialyzed culture fluid produced by *L. fermentum* SK5

Dialyzed culture fluid from *L. fermentum* SK5 inhibited *G. vaginalis* and *B. vulgatus* DMST 15535 with inhibition zones of  $9.75 \pm 0.71$  and  $21.00 \pm 1.15$  mm, respectively. However, there was no activity against other tested vaginal pathogens i.e., *B. fragilis* DMST 19152, *B. ureolyticus* ATCC 33387, *M. curtisii* ATCC 35241 and *P. bivia* ATCC 29303 (Table 1). The ability to inhibit the growth of pathogenic bacteria is also considered as a desirable feature for probiotic bacteria. Previous studies have demonstrated diverse growth inhibition of different pathogens by many LAB strains Lahteinen *et al.*, 2010; Kirtzalidou *et al.*, 2011; Juarez Tomas *et al.*, 2011). The isolation site of the bacterial strain did not seem to significantly affect the inhibition of the pathogens. In this study, *L. fermentum* SK5 isolated from vagina did not inhibit all tested vaginal pathogens but inhibited only *G. vaginalis* and *B. vulgatus* DMST 15535.

Table 1 Antimicrobial activity of dialyzed culture fluid produced by *L. fermentum* SK5

Bacterial vaginal pathogens	Inhibition zone (mm) $\pm$ SD
<i>Gardnerella vaginalis</i>	9.75 $\pm$ 0.71
<i>Bacteroides fragilis</i> DMST 19152	-
<i>Bacteroides vulgatus</i> DMST 15535	21.00 $\pm$ 1.15
<i>Bacteroides ureolyticus</i> ATCC 33387	-
<i>Mobiluncus curtisii</i> ATCC 35241	-
<i>Prevotella bivia</i> ATCC 29303	-

(-): No inhibition zone

### 3.2 Isolation and characterization of bacteriocin from *L. fermentum* SK5

#### 3.2.1 Bacteriocin biosynthesis

During first four days, bacteriocin from *L. fermentum* SK5 was not detected. However, *L. fermentum* SK5 showed the production of bacteriocin after 5 days of incubation at 37 °C under anaerobic condition. Bacteriocin activity against *G. vaginalis* was shown at the same MIC and MBC values (250  $\mu$ g/ml). These results indicated that suitable production time of bacteriocin produced by *L. fermentum* SK5

was 5 days of incubation. The bacteriocin production depends on probiotic strain. *L. pentosus* 31-1 produced bacteriocin named pentocin 31-1 at 640 arbitrary units (AU)/ml in MRS broth in the early stationary phase (i.e. after 24 h) (Liu *et al.*, 2008). Bacteriocin production of *L. plantarum* Strain LR/14 followed a growth-associated pattern. It started during log phase, reaching maximum during the stationary phase (~22 h) (Tiwari and Srivastava, 2008). Deraz *et al.* (2005) reported that *L. acidophilus* DSM 20079 produced bacteriocin during the transition from the exponential to the stationary phase. Significant bacteriocin activity was detected after 10 h and the activity were highest after 11–13 h.

### 3.2.2 Characterization of bacteriocin

Dialyzed culture fluid from *L. fermentum* SK5 inhibited *G. vaginalis*. SEM images of treated pathogen bacterial cells indicated that *G. vaginalis* cells were lysed and had shrunk (Figure 7). On the treatment of dialyzed culture fluid with ninhydrin, a blue-violet dye was formed to indicate the presence of primary or secondary amines with a high molecular weight. When it was treated with trypsin (1 mg/ml), the diameter of the inhibition zone was slightly reduced (Table 2). Treated by heating dialysed culture fluid at different temperatures from 60 to 121 °C, the diameter of inhibition zone was reduced. However, more than 97% of the activity against *G. vaginalis* was still present after heating up to 121 °C (Table 2). This indicated that some of the inhibitory activity could be associated with a protein-like compound supposed to be bacteriocin. The optimum activity of dialyzed culture fluid

was at pH 5. At the pH value of 2, 7, 8 and 9, no antimicrobial activity was observed (Table 2).

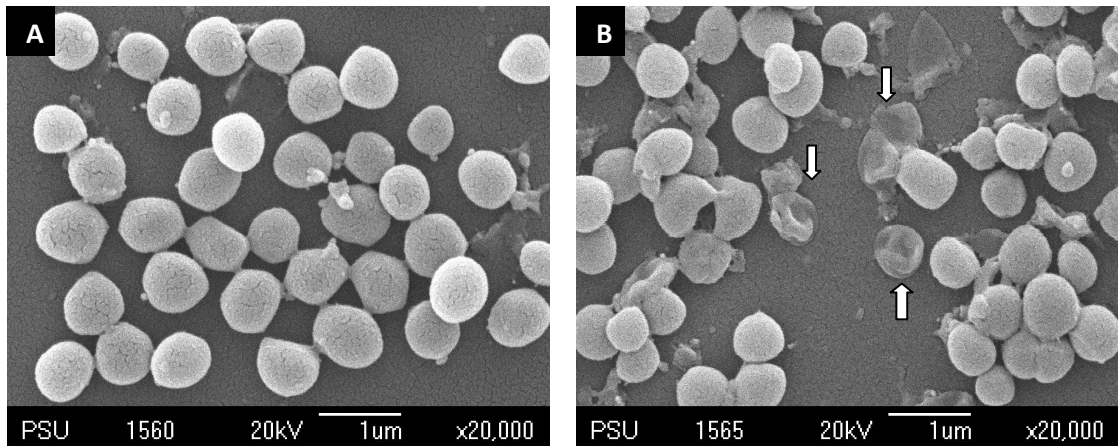


Figure 7. Examination by scanning electron microscopy (SEM) of *G. vaginalis*. (A) *G. vaginalis* treated with phosphate buffer saline, pH 7.4. (B) *G. vaginalis* treated with dialyzed culture fluid from *L. fermentum* SK5 (pointing arrow).



Table 2. Effect of trypsin, heat and pH on the antibacterial activity of a dialyzed culture fluid from *L. fermentum* SK5

Treatment		Inhibition zone (mm) $\pm$ SD
		<i>G. vaginalis</i>
Dialyzed culture fluid (untreated)		9.75 $\pm$ 0.50
Enzyme	Trypsin (1 mg/ml)	8.75 $\pm$ 0.50*
Heat ( $^{\circ}$ C)	60	8.25 $\pm$ 0.50*
	80	8.00 $\pm$ 0*
	100	8.00 $\pm$ 0*
	121	8.00 $\pm$ 0*
pH	2	0 *
	3	8.00 $\pm$ 0*
	4	8.25 $\pm$ 0.50*
	5	9.25 $\pm$ 0.50
	6	9.25 $\pm$ 0.50
	7	0*
	8	0*
	9	0*

\* The mean difference is significant at the 0.05 level when compared with the dialyzed culture fluid.

Some strains of lactobacilli have been introduced as probiotics with beneficial effects on their hosts. The inhibition of infections by the microbial pathogenic *G. vaginalis* by *L. fermentum* SK5 by producing antimicrobial substances leads to some health benefits. SEM examination of *G. vaginalis* cells treated with a dialysed culture fluid showed pore formation. Ninhydrin reacts quantitatively with primary and secondary amines to form a Schiff's base at temperatures of at least 100 °C. Following this, an oxidative deamidation and a hydrolysis occur, which leads to a ninhydrin derivative carrying an amino group. This derivative reacts with an un-derivatized ninhydrin to form a blue-violet dye known as "Ruhemann's purple" (González-González *et al.*, 2011). In this study, substances in dialyzed culture fluid reacted with ninhydrin reagent to form a blue-violet dye. This confirmed that part of the inhibitory substances was likely a high molecular weight protein or lipoprotein. Many lactic acid bacteria are known to produce bacteriocin-like molecules that inhibit Gram-positive and Gram-negative foodborne pathogens (Ponce *et al.*, 2008; Voravuthikunchai *et al.*, 2006). This purported bacteriocin from *L. fermentum* SK5 was active at an acidic pH with an optimum pH 5 but was only slightly sensitive to proteolytic enzymes and heat. Other studies have demonstrated that bacteriocin-like substances produced by *L. acidophilus* YIT 0154 were heat stable with optimum activity at neutral and acidic pH but they were destroyed by proteases (Yamato *et al.*, 2003). *L. plantarum* strain LR/14 produced a bacteriocin that was stable at a high temperature, was active in the pH range of 2.0–6.0 and was destroyed by proteolytic enzymes (Tiwari and Srivastava, 2008).

### 3.2.3 Partial purification of bacteriocin

The inhibitory substance produced from *L. fermentum* SK5 is indicated that the high molecular weight molecule that supposed to be bacteriocin remained after low molecular weight molecules such as organic acids and H<sub>2</sub>O<sub>2</sub> were removed by dialysis. Bacteriocin from *L. fermentum* SK5 was partially purified by a three- step purification protocol (Table 3). At the first step, bacteriocin after dialysis and freeze-dry showed total activity of 1540 AU/ml. When this sample was applied on Sephacryl-S100, the bacteriocin activity was 695 AU/ml. Finally, the active fraction from second step was load into SP-Sepharose column and the activity was shown at 281.35 AU/ml. The recovery was 18.27% of the initial activity.

Table 3. Partial purification of bacteriocin produced by *L. fermentum* SK5

Purification step	Total volume (ml)	Total activity (AU/ml)	Yeild (%)
Solution of <i>L. fermentum</i> SK5 lyophilized powder	5	1540	100
Sephacryl-S100	5	695	45.13
SP-Sepharose	5	281.35	18.27

In this study, *L. fermentum* SK5 produced bacteriocin and it was partially purified using dialysis, Sephacryl-S100 and SP-Sepharose chromatography. There were also many bacteriocins isolated and purified from different probiotic strains. A novel bacteriocin, sakacin LSJ618, produced by the strain *L. sakei* LSJ618. After partial purification by ammonium sulfate precipitation and Sephadex G-25 chromatography, the molecular weight of sakacin LSJ618 was determined to be 5.2 kDa by Tricine-SDS-PAGE (Jiang *et al.*, 2012). Hata *et al.*, 2010 reported a new bacteriocin produced by *L. plantarum* A-1, plantaricin ASM1 (PASM1). The bacteriocin was purified using cation exchange, hydrophobic interaction, and reverse-phase high-performance liquid chromatography. The activity of the purified bacteriocin was obtained as one fraction. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of the fraction showed a mass of 5045.7 Da. *L. fermentum* strain L23 produced a small bacteriocin, designated bacteriocin L23, with an estimated molecular mass of < 7000 Da. The bacteriocin was isolated from cell-free culture supernatant fluid and purified by a process of ammonium sulfate precipitation, gel filtration, thin-layer chromatography, bioautography, and reversed-phase HPLC (Pascual *et al.*, 2008).

### 3.3 Probiotic properties of *L. fermentum* SK5

#### 3.3.1 Survival in simulated vaginal fluid (SVF)

*L. fermentum* SK5 survived in SVF, pH 4.4. There still had more than 99% survival rate at examined condition (Figure 8).

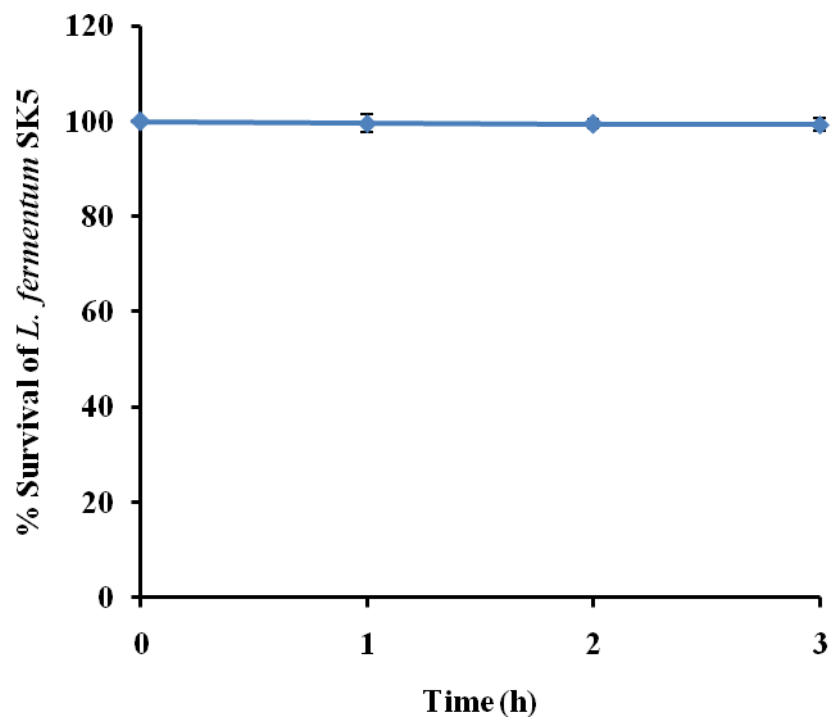


Figure 8. Survival of *L. fermentum* SK5 in simulated vaginal fluid (SVF)

The low vaginal pH appears as the primary mechanism in controlling the composition of microbiota. Because of the finding that the vaginas of reproductive-aged women typically have a pH of approximately 4–4.5, it has commonly been believed that this degree of acidity strictly limited the microbiota to

acidophilic or aciduric species such as *Lactobacillus* spp (Boris and Barbés, 2000). In this study, *L. fermentum* SK5 survived in SVF, pH 4.4. This indicated that *L. fermentum* SK5 can be survive in SVF if they are applied to vagina as a probiotic for prevention or treatment of vaginal infection.

### 3.3.2 Aggregation and surface hydrophobicity

*L. fermentum* SK5 showed autoaggregation by forming tiny granules on a glass microscope slide within 2 min of suspension in PBS, pH 6.2 (Figure 9). In mixed cultures, *L. fermentum* SK5 coaggregated with *G. vaginalis* (Figure 10). It was also classified as having a high surface hydrophobicity with a SAT value  $< 0.9$  mol/l.

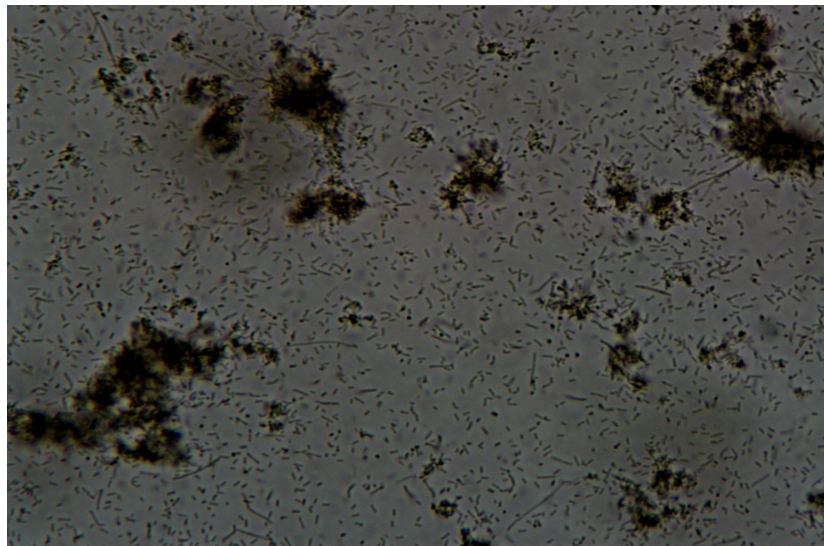


Figure 9. Microscopic observation of *L. fermentum* SK5 forming aggregates

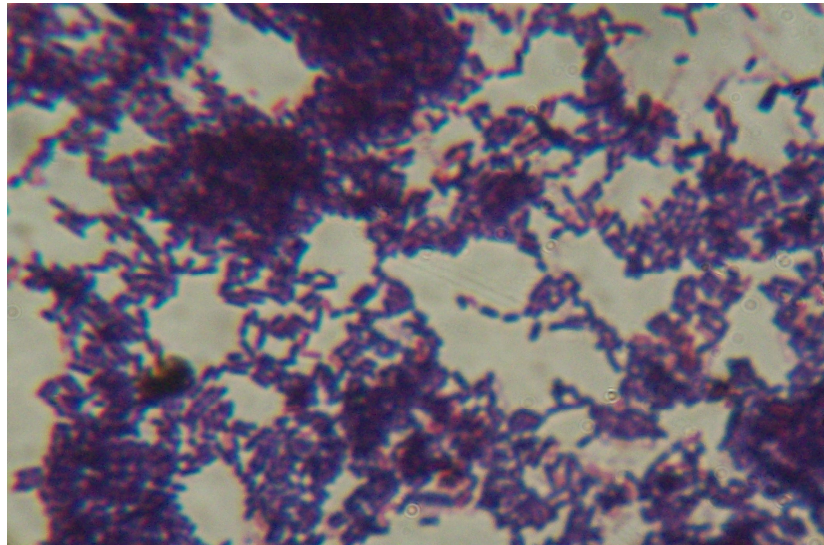


Figure 10. Microscopic observation of coaggregation between *L. fermentum* SK5 and *G. vaginalis*

A previous study had indicated that adherence to epithelial cells and biofilm formation was related to its ability to autoaggregate and its surface hydrophobicity. Autoaggregation may substantially increase the colonization potential of lactobacilli in the environments in which they have a short residence time. Adherent lactobacilli show a high surface hydrophobicity, whereas nonadherent lactobacilli are much more hydrophilic. The formation of a probiotic bacterial film may contribute to the exclusion of pathogens from the mucosa. In addition, coaggregation and adhesion lead to the formation of a barrier that prevents colonization by pathogens (Boris *et al.*, 1998). In this study, *L. fermentum* SK5 showed autoaggregation and a high surface hydrophobicity. Its good adhesion to the tested cell cultures would have to be due to these two characteristics although the molecular mechanisms underlying these autoaggregations by this bacterial strain are

unknown. Autoaggregation of *L. fermentum* SK5 may favor colonization of the gastrointestinal and vaginal epithelium through the formation of a bacterial film and this may also contribute to the exclusion of pathogens from those epithelia. The non-specific hydrophobicity surface interaction between the microorganism and the host cell may be one of the main adhesion mechanisms for *L. fermentum* SK5. Although a high cell surface hydrophobicity may play an initial role in the adhesion of bacteria to the epithelial cells and extracellular matrix protein (Zareba *et al.*, 1997), many studies have demonstrated that this non-specific initial interaction is weak and reversible and precedes the subsequent more stable adhesion process mediated by more specific mechanisms that involve cell-surface proteins and lipoteichoic acids (Granato *et al.*, 1999; Rojas *et al.*, 2002; Roos and Jonsson, 2002). Schillinger *et al.*, (2005) found that *L. acidophilus* BFE 719 with an extremely low hydrophobicity was also able to bind to HT29MTC cells better than *L. rhamnosus* GG that had a higher cell surface hydrophobicity. They concluded that cell hydrophobicity may be helpful for adhesion, but it was obviously not a prerequisite for a strong adhesive capacity. *L. fermentum* SK5 coaggregated with vaginal pathogen. The coaggregation could be an important factor that interferes with the ability of the pathogens to adhere to receptors on the epithelial surface. During coaggregation, lactobacillus could control a microenvironment around the pathogens and increase the concentration of excreted inhibiting substances such as acids, H<sub>2</sub>O<sub>2</sub>, bacteriocin-like substances (Barrons and Tassone, 2008). Mastromarino *et al.*, (2002) evaluated 10 strains of lactobacilli for vaginal probiotic activity and they found various degrees of coaggregation with *Candida albicans* and *G. vaginalis*.



### 3.3.3 Adhesion of *L. fermentum* SK5 on HeLa cells

The ability of *L. fermentum* SK5 to adhere to HeLa cells was compared to the commercial strain, *L. rhamnosus* GG ATCC 53103. *L. fermentum* SK5 adhered to HeLa cells with rates of 93.21%, whereas the adherence of *L. rhamnosus* GG ATCC 53103 to the same cell lines was 86.63%. The ability to adhere to the epithelial cells is one of the main criteria for selecting probiotic strains. This ability is regarded as a prerequisite to exert beneficial effects. The difficulties of assessing the adherence of probiotic strains *in vivo* have led to the development of *in vitro* adherence assays (Blum *et al.*, 1999). In this study, HeLa cells were used. HeLa cells being human cervical carcinoma cells are representative of vaginal epithelium cells. *L. fermentum* SK5 was able to adhere to the tested cells. This indicated that *L. fermentum* SK5 adhered to and colonized vaginal epithelium cells and provided inhibition against pathogens using various mechanisms. *L. rhamnosus* GG ATCC 53103 is known as the commercial probiotic strain and is used in many studies about probiotic properties. *L. fermentum* SK5 had significantly higher adhesive properties to HeLa cells than *L. rhamnosus* GG ATCC 53103. This may be due to the cell adherence specificity. In consideration of adherence to HeLa cells, *L. fermentum* SK5 showed appreciably more adhering ability than *L. rhamnosus* GG ATCC 53103. This occurrence might be due to the source specificity for such adherence. The result was in agreement with the results obtained by Duary *et al.*, (2011). HeLa cells have been used as an *in vitro* adhesion model for other studies. For example, Tropcheva *et al.*, (2011) evaluated the adhesion potential of a pre-selected strain of *L. plantarum* AC131 to HeLa cells. In this case, the strain AC131 was classified as being strongly adhesive to HeLa cells.

### 3.3.4 Adhesion effect of *L. fermentum* SK5 on vaginal bacterial pathogens adhesion on HeLa cells

*L. fermentum* SK5 produced an inhibition of the adhesion of *G. vaginalis* to HeLa cells (Figure 11). The adhesion of *G. vaginalis* to HeLa cells was 76.82% (Figure 11). *L. fermentum* SK5 significantly reduced the adhesion of *G. vaginalis* to HeLa cells by 15, 18 and 11% in the competition, exclusion and displacement assays, respectively.

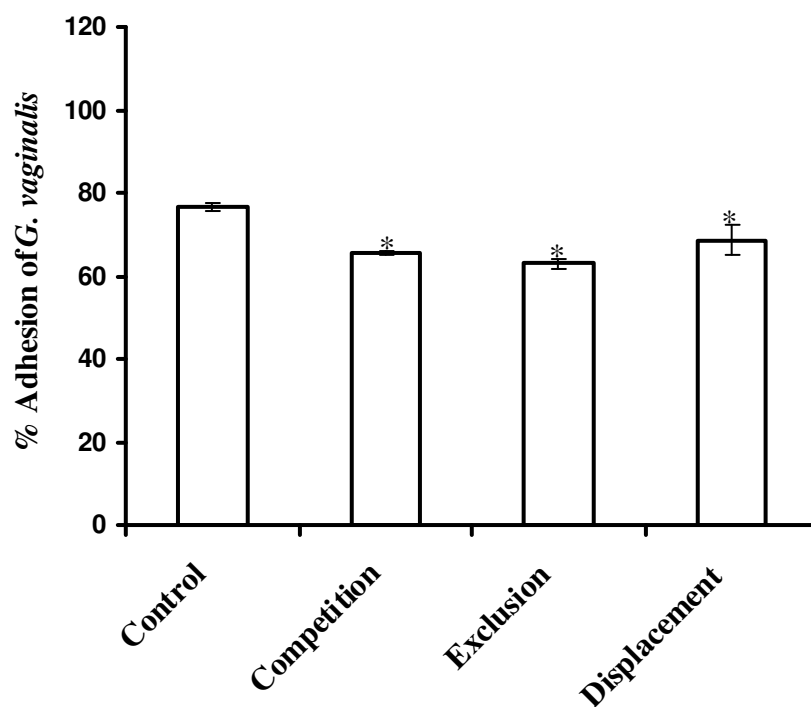


Figure 11. Inhibition of *G. vaginalis* adhesion to HeLa cells by *L. fermentum* SK5.

\* =  $p < 0.05$  (compared with *G. vaginalis* alone)

The mechanisms involved in protection against pathogen adhesion have been proposed to be either non-specific hindrance of receptors for pathogens or competition with pathogens for the binding sites (Matijašić *et al.*, 2006). Such inhibitory activity is specific depending on both the probiotic and pathogenic strains (Collado *et al.*, 2005). Gueimonde *et al.* (2006) reported a very high specificity in inhibiting adhesion of enteropathogens by different lactobacilli. This means that there is a need for a case-by-case assessment in selecting the strains with an ability to inhibit certain pathogens. In the present study, *L. fermentum* SK5 inhibited the adhesion of *G. vaginalis* to HeLa cells. It indicated the specificity of *L. fermentum* SK5 to inhibit the adhesion of *G. vaginalis*. The inhibition of *G. vaginalis* adhesion by *L. fermentum* SK5 was shown by all performed assays including competition, exclusion and displacement assays. The best adhesion inhibition of this pathogen was through exclusion mechanism. This result indicates that the balance of normal probiotic *Lactobacillus* may provide a good protection against the adhesion of pathogens to epithelium cells. The ability to compete, exclude and displace pathogens from mucus by specific probiotic strains has been reported in other studies. For example, *L. rhamnosus* GG and *L. casei* Shirota were able to compete with, exclude and displace pathogenic gastrointestinal (GI) bacteria, but the degree of inhibition of adhesion was bacterial strain-dependent (Lee *et al.*, 2003).

### 3.4 Preparation of *L. fermentum* SK5 hollow-type vaginal suppository

#### 3.4.1 Production of *L. fermentum* SK5 cells

##### Effect of cryoprotectants on survival of *L. fermentum* SK5

The effect of skim milk, inulin and GOS on survival of *L. fermentum* SK5 was evaluated. The results showed that skim milk offered 72.1-90.4% survival rate of *L. fermentum* SK5 and the highest viability was shown at 10% skim milk (Figure 12). The best survival of *L. fermentum* SK5 was 91.1 and 88.6% at 4% inulin and 4% GOS, respectively (Figure 12).

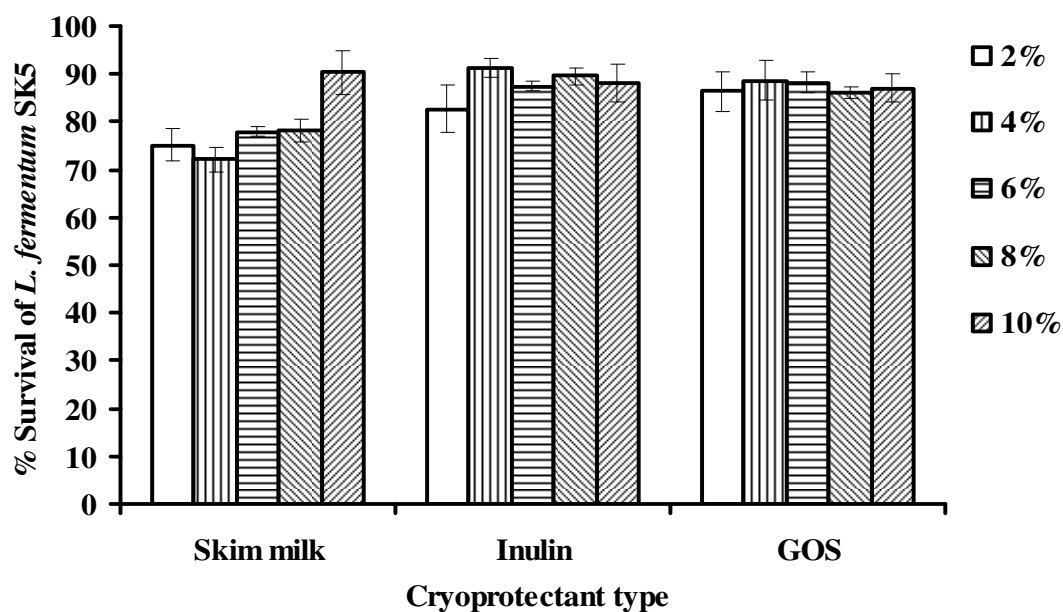


Figure 12. Effect of cryoprotectants on survival of *L. fermentum* SK5

Freeze-drying has for long been considered as a suitable dehydration process for bacteria, with the ultimate goal of achieving a solid and stable final formulation. For most LAB cultures of commercial interest for the dairy industry, skim milk powder is selected as drying medium because it prevents cellular injury by stabilizing the cell membrane constituents, creates a porous structure in the freeze-dried product that makes rehydration easier and contains proteins that provide a protective coating for the cells (Carvalho *et al.*, 2004). Skim milk should use as a cryoprotectant for preparation of *L. fermentum* SK5 powder. However, skim milk may enhance the growth of both Lactobacilli probiotics and pathogens. Indeed, the cryoprotectants should increase only the growth of Lactobacilli. Prebiotics were therefore used as cryoprotectants for probiotic formulation. Inulin and GOS were prebiotic used in this study. Inulin and GOS gave the greater survival rate than skim milk. The best survival of *L. fermentum* SK5 in 4% inulin was not significant comparing in 4% GOS. *L. fermentum* SK5 lyophilized powder may prepare in either 4% inulin or 4% GOS.

#### **Effect of prebioic on the growth of *L. fermentum* SK5**

Figure 13 and 14 showed the effect of prebiotics inulin and GOS on the growth of *L. fermentum* SK5. GOS enhanced the growth of *L. fermentum* SK5 better than inulin.

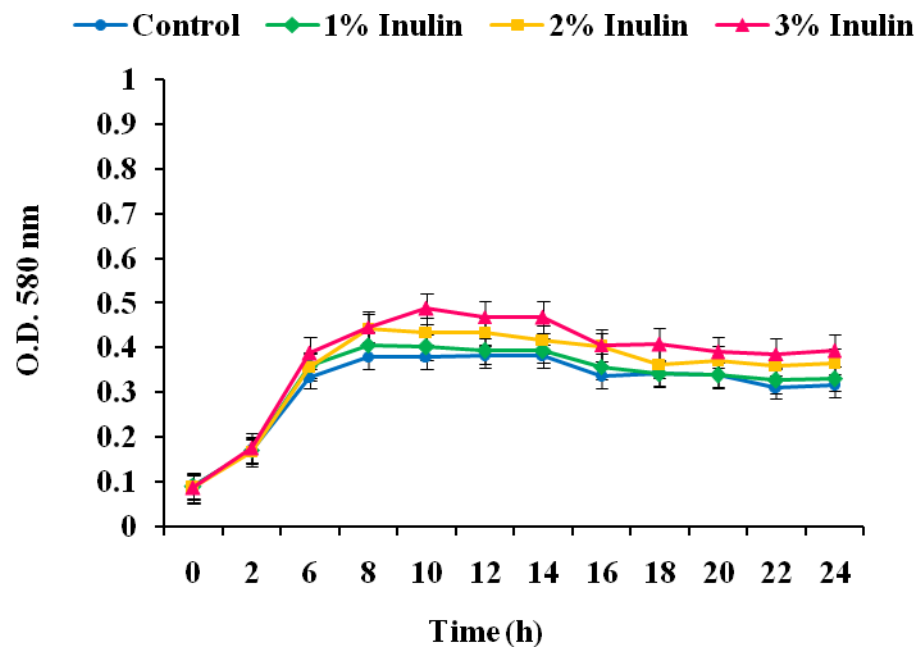


Figure 13. Effect of inulin on the growth of *L. fermentum* SK5

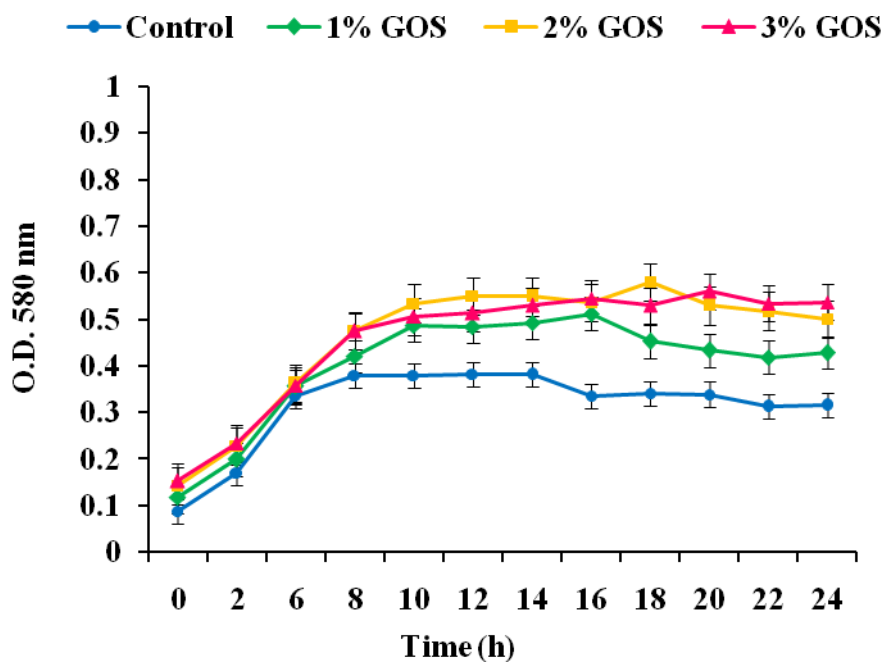


Figure 14. Effect of GOS on the growth of *L. fermentum* SK5

Inulin and GOS have demonstrated to be efficient cryoprotectants of *L. fermentum* SK5 during lyophilization. However, GOS was good prebiotic for *L. fermentum* SK5. In this study, *L. fermentum* SK5 lyophilized powder was prepared in 4% GOS as cryoprotectant and viability of *L. fermentum* SK5 in this powder was 8.36 log cfu/ml. The combined form of a probiotic and a prebiotic are known as “Synbiotics” (Gibson and Roberfroid, 1995). *L. fermentum* SK5 lyophilized powder was self-protected synbiotic because prebiotics act as protective molecules (Tymczyszyn *et al.*, 2011).

#### 3.4.2 Preparation of *L. fermentum* SK5 hollow-type vaginal suppository

*L. fermentum* SK5 hollow-type vaginal suppository had a uniform, smooth but appeared waxy surface (Figure 15).



Figure 15. Appearance of *L. fermentum* SK5 hollow-type vaginal suppository

Polyethylene glycols (PEGs) having average molecular weights of 200, 400 and 600 are clear and colorless liquids. Those having average molecular weights of greater than 1000 are wax-like, white solids with the hardness increasing with an increase in the molecular weight. In this study, *L. fermentum* SK5 hollow-type vaginal

suppository was prepared using mixture of PEG 400 and PEG 4000 as suppository base (Ansel, 1995). PEG 4000 was wax-like solid resulting in the surface of *L. fermentum* SK5 hollow-type vaginal suppository appeared waxy.

### **3.4.3 Evaluation of *L. fermentum* SK5 hollow-type vaginal suppository**

#### **Uniformity of weight**

The weight of vaginal suppositories was in the range of 2.00-2.25 g (Table 4). The weight variation of vaginal suppositories was within the acceptable limit. The weight of each suppository was less than the average weight  $\pm 5\%$  ( $2.0785 \pm 0.10$  g) and there were only two suppositories that the weight was more than the average weight  $\pm 10\%$  ( $2.0785 \pm 0.20$  g).



Table 4. Weight of *L. fermentum* SK5 vaginal suppositories

Sample	Weight (g)
1	2.05
2	2.07
3	2.25
4	2.13
5	2.15
6	2.22
7	2.04
8	2.03
9	2.04
10	2.06
11	2.08
12	2.00
13	2.07
14	2.06
15	2.04
16	2.09
17	2.05
18	2.04
19	2.08
20	2.02
Average	$2.0785 \pm 0.06$

### DSC thermogram of PEGs base

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

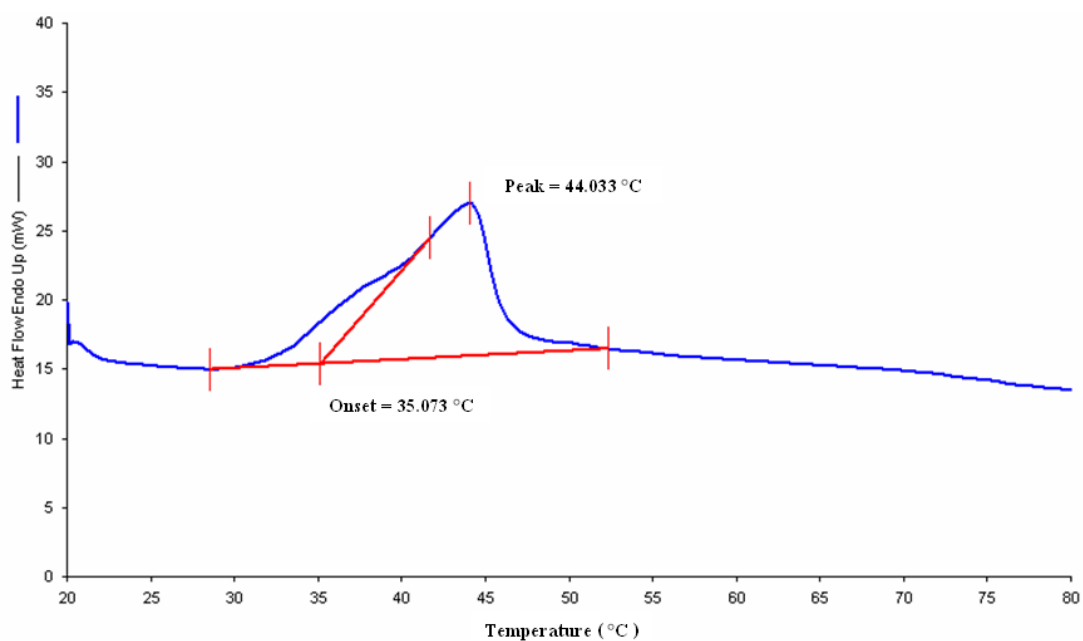


Figure 16. DSC thermogram of suppository base (the mixed of PEG 400:PEG 4000, ratio55:45)

### *In vitro* release study

*In vitro* release study, the release profile of *L. fermentum* SK5 from vaginal suppository is shown in Figure 17. The release of *L. fermentum* SK5 did not detected in the first 25 min. After that, the release of *L. fermentum* SK5 was increased as a function of time.

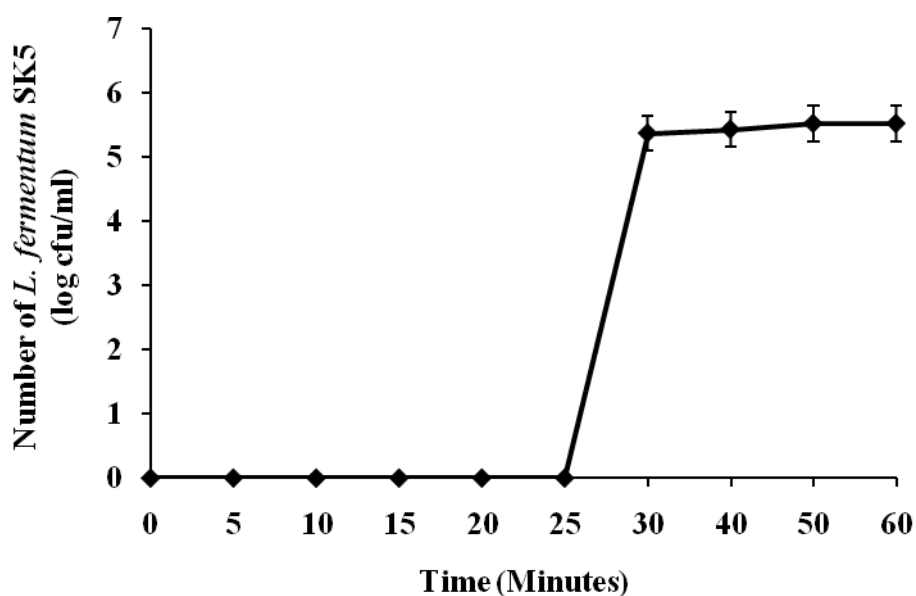


Figure 17. *In vitro* release of *L. fermentum* SK5 from hollow-type vaginal suppositories

The *in vitro* release time test revealed that the time required melting the whole suppository. In this study, vaginal suppository was prepared from PEG mixture (PEG 400 and PEG 400) having melting point considerably higher than that of body temperature. Therefore, vaginal suppository required time for release of *L. fermentum* SK5. The result showed that *L. fermentum* SK5 was initially released after 25 min.

### **Stability**

The viability of *L. fermentum* SK5 from vaginal suppository before and after storage at 4° C for 3 months was evaluated. The *L. fermentum* SK5 vaginal suppository before storage contained 8.36 log cfu/ml. After storage for 3 months, *L. fermentum* SK5 vaginal suppository contained 8.27 log cfu/ml (Figure 18). The viable of *L. fermentum* SK5 decreased a little amount from 8.36 to 8.27 log cfu/ml after storage of 3 months. It may be due to the long exposure of oxygen remained in the cavity of suppository.

*L. fermentum* SK5 from vaginal suppository showed antibacterial activity against *G. vaginalis* with inhibition zone 11.40-12.60 mm during storage of 3 months (Table 5). This activity correlated with its high survival rate.

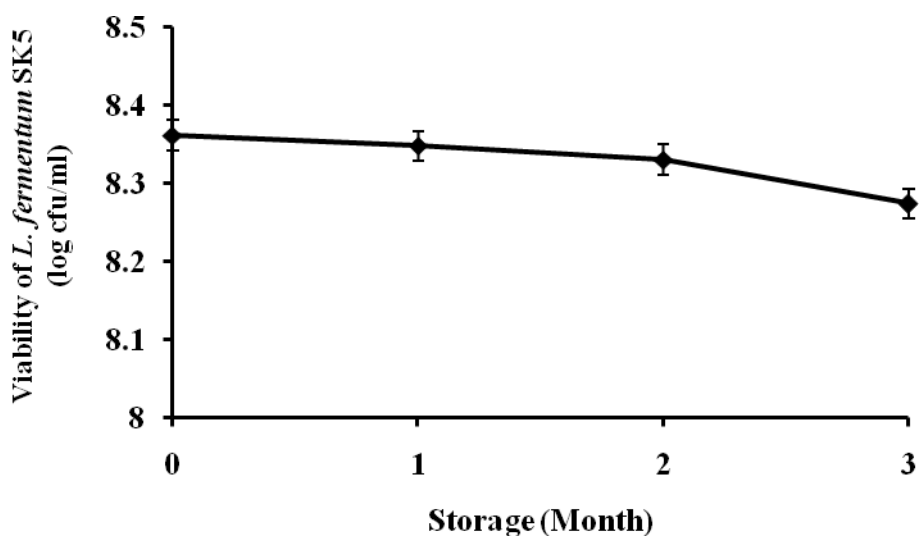


Figure 18. Viability of *L. fermentum* SK5 from hollow-type vaginal suppositories

Table 5. Antibacterial of *L. fermentum* SK5 from vaginal suppositories

Storage (month)	Inhibition zone (mm) $\pm$ SD
	<i>G. vaginalis</i>
0	12.60 $\pm$ 0.40
1	12.00 $\pm$ 1.85
2	11.95 $\pm$ 0.47
3	11.40 $\pm$ 0.28

## CHAPTER 4

### CONCLUSIONS

*L. fermentum* SK5 isolated from healthy women's vaginal tracts showed inhibition activity against vaginal pathogenic *Gardnerella vaginalis* and *Bacteroides vulgatus* DMST 15535. The high molecular weight antimicrobial substance isolated from 5 days cultured *L. fermentum* SK5. This substance active at optimum pH 5 but its activity was slightly reduced after exposure to heat or proteolytic enzyme. When *G. vaginalis* was treated with the inhibitory substance, the cells were lysed and had shrunk with the rough cell surface. The substance was supposed to be a bacteriocin. The bacteriocin from *L. fermentum* SK5 was partially purified using dialysis, Sephacryl-S100 and SP-Sepharose chromatography. The partial purified bacteriocin had 18.27% of the initial activity. *L. fermentum* SK5 showed good probiotic properties including survived in simulated vaginal fluid (SVF), autoaggregated, showed high surface hydrophobicity, coaggregated with *G. vaginalis*, adhered and inhibited *G. vaginalis* adhesion to HeLa cells (vaginal epithelial cells). *L. fermentum* SK5 was prepared in lyophilized form using skim milk, inulin and galactooligosaccharide (GOS) at concentration of 2, 4, 6, 8 and 10% (w/v). After lyophilization, survival of *L. fermentum* SK5 was the highest in 10% skim milk, 4% inulin and 4% GOS. Inulin and GOS improved the survival of *L. fermentum* SK5 better than skim milk. Evaluation of prebiotic inulin and GOS on the growth of *L. fermentum* SK5, *L. fermentum* SK5 was more grown in GOS than inulin. From these results, *L. fermentum* SK5 lyophilized powder was prepared using 4% GOS as cryoprotectant. This powder was formulated 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 8.36 log cfu of *L. fermentum* SK5 with average weight 2.0785 g, melting point 35-44 °C and the release time of this *Lactobacillus* from the suppository after 25 min. During storage for 3 months, *L. fermentum* SK5 showed good viability and activity against *G. vaginalis*.

In this study, *L. fermentum* SK5 exhibited good properties including producing of bacteriocin to inhibit vaginal pathogens, survival in vaginal fluid, autoaggregation, high surface hydrophobicity, coaggregation with pathogens, adhesion and inhibition pathogen adhesion on vaginal epithelium and formulation as hollow-type vaginal suppository. *L. fermentum* SK5 showed the high promising properties of probiotic against vaginal bacterial pathogens.

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## **APPENDIX**

***In vitro* probiotic properties of *Lactobacillus fermentum* SK5 isolated from the healthy woman's vagina against gastrointestinal pathogenic *Escherichia coli* and vaginal pathogenic *Gardnerella vaginalis***

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## ABSTRACT

A lactobacillus strain isolated from a healthy woman's vaginal tract was examined *in vitro* for its probiotic potential. This strain was identified as *Lactobacillus fermentum* SK5 that was able to survive at pH 3-4 and 0.1-0.2% bile, and unaffected by pepsin ( $3 \text{ g l}^{-1}$ ) and pancreatin ( $1 \text{ g l}^{-1}$ ), but was susceptible to all tested antibiotics except metronidazole. *L. fermentum* SK5 had an antimicrobial potential against gastrointestinal pathogenic *Escherichia coli* and vaginal pathogenic *Gardnerella vaginalis*. The effective substance was suspected to be a bacteriocin-like compound with a molecular weight of more than 10 kDa, but hydrogen peroxide was also detected. Further studies revealed that *L. fermentum* SK5 had good autoaggregation characteristic and a high surface hydrophobicity that enhanced its adhesion ability to epithelial cells and for biofilm formation. This lactobacillus showed coaggregation with *E. coli* and *G. vaginalis* to affect their adhesion and colonization. The potential of *L. fermentum* SK5 to adhere to and decrease the adherence of the studied *E. coli* and *G. vaginalis* was demonstrated in HeLa, HT-29 and Caco-2 cells. These incidences provided evidence of the possible colonization of *L. fermentum* SK5 that would prevent binding and growth of *E. coli* and *G. vaginalis* onto intestinal and vaginal epithelial cells. With Regard to such inhibition of potential pathogenic microorganisms by *L. fermentum* SK5 through coaggregation and antimicrobial substances, it is likely that this lactobacillus strain could be a potential probiotic candidate for beneficial use in protecting against gastrointestinal and vaginal microbial infections.



*Keywords:*

*Lactobacillus fermentum* SK5

Probiotic property

Gastrointestinal pathogens

Vaginal pathogens

## 1. Introduction

Among the normal flora of the human gastrointestinal and urogenital tracts, lactobacilli are one of the most interesting organisms for use as probiotics. The Food and Agriculture Organization of the United Nations and World Health Organization defines probiotics as live microorganisms that when administered in adequate amounts, confers beneficial effects on the health of the host [1]. Specific probiotic *Lactobacillus* spp. have been documented for their health effects [2] by various mechanisms. They can inhibit pathogenic infections by the production of antimicrobial compounds such as lactic acid, short chain fatty acids, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), bacteriocin-like substances and biosurfactants [3,4,5,6]. Other mechanisms proposed for their antimicrobial activity are competition for nutrient [7,8], ability to adhere to the surface of the intestinal and vaginal epithelial cells to inhibit the adhesion of pathogens [9] and stimulate the immune system via interactions with immune cells [10,11,12,13]. Furthermore, challenging uropathogenic *E. coli* biofilms with lactobacilli *in vitro* caused significant *E. coli* killing, and *E. coli* biofilms challenged with spent media of *L. rhamnosus* GR-1 caused a marked decrease of cell density and increased cell death [14].

There is much evidence that has shown that some gastrointestinal lactobacilli ascend to the vagina. The use of oral formulations of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 has been associated with normalization of the vaginal environment within 28 to 60 days after the initiation of treatment [8,15]. Moreover, evidence of fecal and vaginal colonization by these strains was observed after 14 days of oral administration [16]. Oral probiotics can resolve urogenital infections [17]. This may have occurred due to the microorganisms themselves ascending to the vagina from the

rectal area, or by altering the ability of pathogens to transfer to the vagina. Oral administration of lactobacilli can be as effective as a daily treatment with antibiotics for the treatment of urinary tract infections [18].

Since probiotic effects on the host are strain specific, it is very likely that better performing strains may be selected using the specific sources of strain origin and a use selection process [19]. Before probiotic strains exert their beneficial effects on the host, they have to be able to survive during passage through the host's gastrointestinal tract. They should have high acid and bile tolerance properties, adherence to host epithelial cells and interference with the adherence of pathogens, self aggregation and production of inhibitory substances capable to inhibit the growth of pathogens [20]. The ability of probiotic strains to adhere to the epithelial surface of the intestine and vagina and subsequent short or long-term colonization has always been used as common criteria for selection of probiotic strains. Enterocyte-like HT-29 and Caco-2 cells have been successfully used for *in vitro* studies on the mechanism of cellular adhesion of probiotics for intestine [21,22], while HeLa cells have been used for probiotic bacteria to bind to the vagina [23].

The aim of this study was to investigate the *in vitro* probiotic properties of *Lactobacillus fermentum* SK5 isolated from a healthy woman's vagina that could be used as an oral and vaginal probiotic. This study focused on its antipathogenic activities, tolerance to simulated gastrointestinal conditions, adhesion ability and ability to inhibit adhesion of gastrointestinal and urogenital pathogens to HeLa, HT-29 and Caco-2 cell lines.

## 2. Materials and Methods

### 2.1. Bacterial strains

*L. fermentum* SK5 was one among the isolates that were taken by swabbing from the posterior zone of the vaginal fornix of healthy, premenopausal and non-menstruating women who were also negatively diagnosed for vaginosis, vaginitis, hepatitis and HIV. These women were those who came for the normal vaginal check at Songkla Hospital, Thailand. *L. fermentum* SK5 was identified by the API 50 CHL system (BioMerieux, France) and confirmed by 16S rDNA analysis by the National Center for Genetic Engineering and Biotechnology, Thailand, and registered in the GenBank database system <http://www.ncbi.nlm.nih.gov/> under accession number JQ894941. The 16S rDNA gene analysis showed a 98.9% probability of identity to *L. fermentum* strains available in the GenBank database system. *L. fermentum* SK5 was kept at -80 °C in Mann Rogosa and Shape (MRS) broth (Difco, USA) containing 25% (v/v) glycerol as a cryoprotectant. Gastropathogenic *Escherichia coli* and vaginal pathogenic *Gardnerella vaginalis* were obtained from Songkla Hospital, Thailand. They were kept at -80 °C in brain heart infusion (BHI) broth (Difco, USA) containing 25% (v/v) glycerol.

### 2.2. Inhibition of *E. coli* and *G. vaginalis*

The disc diffusion test was used for assaying the antibacterial activity of *L. fermentum* SK5 against *E. coli* and *G. vaginalis*. The cultural broth of *L. fermentum* SK5 was obtained from the culture grown in MRS broth at 37 °C for 3 d in an anaerobic condition. Cell-free supernatant was obtained by centrifuging the culture broth at 2,000 × g, 30 min, 4 °C. The supernatant was dialyzed across a membrane with a 10 kDa molecular weight cut-off against 20 mM sodium acetate buffer (Ajax

Finechem Pty Ltd, Australia), pH 5.0 at ten times of supernatant volume. The buffer was changed every 6 h until 24 h and the dialyzed culture fluid was freeze-dried. *E. coli* and *G. vaginalis* were grown in BHI broth for 24 h at 37 °C. Each culture was transferred to 0.85% (w/v) normal saline solution and its turbidity was adjusted to be equivalent to the 0.5 McFarland standard with approximately  $10^8$  cfu ml<sup>-1</sup> of indicator bacteria. Using sterile swabs, this suspension was then inoculated onto the agar surface of Mueller-Hinton agar (Difco, USA). The dialyzed culture fluid 50 µl was gradually applied on the disc and allowed to dry in ambient air before placing on the inoculated plates. All disc-containing plates were incubated at 37 °C for 18 h. The diameter of the inhibition zone was measured using an antibiotic zone reader (Fisher-Lilly, USA).

To evaluate structural modifications produced by dialyzed culture fluid on *E. coli* and *G. vaginalis*, a cell pellet from overnight (18 h) *E. coli* or *G. vaginalis* cultures were washed once with phosphate buffered saline (PBS, Sigma-Aldrich, USA), pH 7.4 and the cell pellet was resuspended in 1 ml dialyzed culture fluid. The cells were incubated for 18 h at 37 °C, harvested ( $2,000 \times g$ , 10 min, 4 °C) and washed three times with PBS, pH 7.4. The cells were smeared onto a coated slide and fixed with 2.5% (w/v) glutaraldehyde (Sigma-Aldrich, USA) in 0.1 mol l<sup>-1</sup> PBS, pH 7.4, for 2 h at room temperature. After two washes with PBS pH 7.4, cells were dehydrated in a graded series of ethanol, starting with 50, followed by 60, 70, 80, 90% (v/v) and finally absolute alcohol. Cells were dried in a critical point drier (CPD 7501, Polaron, United Kingdom) and coated with gold. The specimens were then examined by scanning electron microscopy (SEM, JSM-5800LV, JEOL, Japan).

### 2.3. Characterization of dialyzed culture fluid

To study the chemical characterization of dialyzed culture fluid, free amino groups were determined by the ninhydrin test. For the sensitivity to the proteolytic enzyme, the dialyzed culture fluid was treated with trypsin ( $1 \text{ mg ml}^{-1}$ ) and incubated for 3 h at  $37 \text{ }^\circ\text{C}$ . In other tests, the dialyzed culture fluid was heated in a water bath at 60, 80, 100 and  $121 \text{ }^\circ\text{C}$  (autoclave) for 10 min, followed by cooling at  $4 \text{ }^\circ\text{C}$  for 1 h. To test the effect of pH, the dialyzed culture fluid was adjusted to pH value from 2 to 9 using 50 mM different buffer (glycine-HCl buffer (Merck, Germany), pH 2-3, sodium acetate buffer pH 4-5, phosphate buffer (Merck, Germany), pH 6-7, Tris-HCl buffer (Merck, Germany), pH 8-9). After treatments, the residual activity against *E. coli* and *G. vaginalis* was checked using the disc diffusion test.

### 2.4. Detection of hydrogen peroxide production

*L. fermentum* SK5 was tested for its ability to produce  $\text{H}_2\text{O}_2$  by the qualitative method [24]. Plates of MRS agar contained  $5 \text{ mg ml}^{-1}$  hemin,  $1 \text{ mg ml}^{-1}$  vitamin K,  $0.01 \text{ mg ml}^{-1}$  horseradish peroxidase (Sigma-Aldrich, USA) and  $0.05 \text{ mg ml}^{-1}$  3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich, USA) was spotted with a loop of the culture and incubated anaerobically at  $37 \text{ }^\circ\text{C}$  for 48 h. After incubation, the plates were exposed to air and observed for blue or brown colonies indicated  $\text{H}_2\text{O}_2$ -producing colonies. With regard to the color intensity, the bacteria were classified to be strong (blue), medium (brown), weak (light brown) or negative (white color) for  $\text{H}_2\text{O}_2$  production.

### 2.5. Antibiotic susceptibility assay

The antibiotic susceptibility of *L. fermentum* SK5 was determined by the disc diffusion method [25]. *L. fermentum* SK5 was grown with MRS broth at  $37 \text{ }^\circ\text{C}$  for 48

h in anaerobic condition. The tested antibiotic discs (Oxoid, England) included ampicillin (10 µg), cefoperazone (75 µg), ceftazidime (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), clindamycin (30 µg), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), metronidazole (30 µg), neomycin (30 µg), norfloxacin (10 µg), penicillin G (10 units), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (30 µg). The discs were placed on the inoculated MRS agar plate. Inhibition zones after incubation were measured, and interpreted to be susceptible, moderately susceptible, or resistant.

#### *2.6. Survival under conditions that simulated the human gastrointestinal tract*

The resistance of *L. fermentum* SK5 under low pH conditions and simulation of the human GI tract was tested as previously described method [26]. Briefly, bacterial cells from an 18-h culture were harvested by centrifugation at  $2,000 \times g$  for 5 min at 4 °C, washed once with PBS, pH 7.4, before being resuspended ( $10^8$  cfu ml<sup>-1</sup>) in the following tested solutions. For the test on resistance to low pH conditions, bacterial cells were resuspended in PBS, pH 2, 3 and 4 (adjusted with 1N HCl). For the test on resistance to bile salts, bacterial cells were resuspended in PBS, pH 7.4 supplemented with 0.1, 0.2, 0.3 and 0.4% (w/v) oxgall (Sigma-Aldrich, USA). For the test on resistance to pepsin and pancreatin, bacterial cells were resuspended in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). SGF was prepared by supplementing sterilized PBS pH 2, 3 and 4 (adjusted with 1N HCl) with pepsin (Sigma-Aldrich, USA) to a final concentration of 3 g l<sup>-1</sup>. SIF was prepared by supplementing sterilized PBS, pH 6.8 and 8 (adjusted with 1N NaOH) with pancreatin (Sigma-Aldrich, USA) to a final concentration of 1 g l<sup>-1</sup>. Cells in low pH solutions and SGF were incubated at 37 °C for 0, 1, 2 and 3 h, and those in bile solution and

SIF were incubated for 0, 1, 2, 3 and 4 h. The resistance of *L. fermentum* SK5 in every condition was assessed in terms of viable colony count on MRS agar after the treatment. Survival rates were calculated according to the following equation:

$$\text{Survival rate (\%)} = (N_1/N_0) \times 100\%$$

$N_1$ : The total viable count of bacterial cell after treatment

$N_0$ : The total viable count of bacterial cell before treatment

## 2.7. Aggregation test

### 2.7.1. Autoaggregation test

The autoaggregation ability of *L. fermentum* SK5 was determined [27]. Bacterial cells were grown in MRS broth at 37 °C for 18 h in an anaerobic condition. They were resuspended in PBS, pH 6.2 to a final concentration of  $10^8$  cfu ml<sup>-1</sup>. The microbial suspension was dropped onto a glass slide and autoaggregation was observed with a light microscope. If the cells aggregated within 2 min, autoaggregation was considered positive.

### 2.7.2. Surface hydrophobicity

The surface hydrophobicity of the bacterial cells was determined by the salt aggregation test (SAT) [20]. *L. fermentum* SK5 cells were resuspended in 0.02 mol l<sup>-1</sup> of sodium phosphate (Merck, Germany), pH 6.8 to a final concentration of  $10^9$  cfu ml<sup>-1</sup>. Solutions of ammonium sulfate (Merck, Germany) at 4.0, 2.0, 1.5 and 0.5 mol l<sup>-1</sup> were mixed with an equal volume of cell suspension on a glass slide. The lowest final concentration of ammonium sulfate that caused the bacteria to aggregate was defined as the SAT value. From this value, the tested strain was identified to have a high surface hydrophobic, intermediate hydrophobic and hydrophilic if the SAT values were < 0.9 mol l<sup>-1</sup>, 0.9 - 1.5 mol l<sup>-1</sup> and > 1.5 mol l<sup>-1</sup>, respectively.



### 2.7.3. Coaggregation assay

*L. fermentum* SK5 was tested for its ability to coaggregate with the pathogens [28]. One ml of *L. fermentum* SK5 suspension ( $10^9$  cfu in PBS) was homogeneously mixed with 1 ml of *E. coli* or *G. vaginalis* suspension ( $10^9$  cfu in PBS) and mixed by vortex for 15 s and then incubated in a 24-well tissue culture plates (Corning Inc, USA) for 4 h at 37 °C with gentle agitation. The treated cells were Gram-stained and observed under a light microscope for coaggregation.

## 2.8. Adhesion to HeLa, HT-29 and Caco-2 cells

### 2.8.1. HeLa, HT-29 and Caco-2 cells culture

HeLa (ATCC CCL-2.2™), HT-29 (ATCC HTB-38™) and Caco-2 cells (ATCC HTB 37™) were purchased from the American Type Culture Collection (USA). HeLa and HT-29 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with L-glutamine, 10% (v/v) fetal bovine serum (Gibco, USA), 100 U ml<sup>-1</sup> of penicillin G and 100 µg ml<sup>-1</sup> streptomycin sulfate (Gibco, USA). Caco-2 cells were routinely cultured in Eagle's minimal essential medium (MEM, Gibco, USA) with L-glutamine, 15% (v/v) fetal bovine serum, 100 U ml<sup>-1</sup> of penicillin G and 100 µg ml<sup>-1</sup> streptomycin sulfate. All cultured cells were incubated under 5% (v/v) CO<sub>2</sub> at 37 °C. Cells were seeded at a concentration of  $4.5 \times 10^5$  cells well<sup>-1</sup> in 24-well tissue culture plates. HeLa and HT-29 cells were cultured for 7 d to reach confluence and used in the adhesion assay. Caco-2 cells were maintained for 21 d to allow for good differentiation and used in the adhesion assay [22].

### 2.8.2. Adhesion assay

For the adhesion assay, HeLa, HT-29 and Caco-2 monolayer were washed twice with PBS, pH 7.4 before use. The suspension of *L. fermentum* SK5 ( $10^9$  cfu ml<sup>-1</sup> in DMEM for HeLa and HT-29 cells or MEM for Caco-2 cells) was added into the wells of HeLa, HT-29 and Caco-2 monolayer in a total volume of 1 ml of medium. The inoculated tissue culture plate was incubated under 5% (v/v) CO<sub>2</sub> at 37 °C for 1 h. Unbound bacterial cells were washed twice with PBS, pH 7.4. Cell monolayers were lysed with 0.05% (v/v) Triton X-100 (Sigma-Aldrich, USA) at 37 °C for 5 min. Adherent lactobacilli were enumerated by plate counting on MRS agar. This assay was tested in triplicate. *L. rhamnosus* GG ATCC 53103 was used as the adhesion control. The adhesion percentage was calculated according to the following equation:

$$\text{Adhesion (\%)} = (N_1/N_0) \times 100\%$$

N<sub>1</sub>: Amount of adherent bacterial cells

N<sub>0</sub>: Amount of added bacterial cells

### 2.9. Inhibition of pathogens adhesion to HeLa, HT-29 and Caco-2 cells

The inhibition of the adhesion of potential pathogens by *L. fermentum* SK5 on HeLa, HT-29 and Caco-2 monolayer was performed in 24-well tissue culture plates. *E. coli* and *G. vaginalis* were cultured in BHI broth at 37 °C for 24 h. *L. fermentum* SK5 was cultured in MRS broth in anaerobic condition at 37 °C for 48 h. Bacterial cell pellets were collected and washed once with PBS pH 7.4. *E. coli*, *G. vaginalis* and *L. fermentum* SK5 were adjusted to  $10^9$  cfu ml<sup>-1</sup> with DMEM or MEM, for the adhesion assay.

In the competition assay, *L. fermentum* SK5 was added simultaneously with the pathogens [29]. To examine the exclusion and displacement of the pathogens by

*L. fermentum* SK5 or vice versa, each incubated monolayer cells with the tested strains was washed, added with the second strain and incubated for another 30 min [30]. Adherent *E. coli* and *G. vaginalis* were enumerated by plate counting on McConkey agar (Difco, USA) and BHI agar, respectively. McConkey and BHI plates were incubated in aerobic conditions at 37 °C for 24 h.

HT-29 cells grown on glass cover slips were used for the SEM studies. After the bacterial adhesion assays, cells were processed and observed by SEM (as described in previous section).

#### 2.10. Statistical analysis

All data were expressed as a mean  $\pm$  SD. The Student's *t* test was used for statistical analysis by comparing treatment groups versus the control group. Results were regarded as statistically significant at  $p < 0.05$ .

### 3. Results

#### 3.1. Inhibition of *E. coli* and *G. vaginalis*

Dialyzed culture fluid from *L. fermentum* SK5 inhibited *E. coli* and *G. vaginalis* with inhibition zones of  $13.50 \pm 0.71$  and  $9.75 \pm 0.50$  mm, respectively (Table 1). SEM images of treated pathogen bacterial cells indicated that *E. coli* and *G. vaginalis* cells were lysed and had shrunk (Fig. 1). On treat dialyzed culture fluid with ninhydrin a blue-violet dye was formed to indicate the presence of primary or secondary amines with a high molecular weight. When treated with trypsin ( $1 \text{ mg ml}^{-1}$ ) the diameter of the inhibition zone was slightly reduced (Table 1). Heating at different temperatures from 60 to 121 °C also reduced the diameter of the inhibition zone but more than 59% of the activity against *E. coli* was still present and 97% against *G. vaginalis* after heating up to 121 °C (Table 1). This indicated that some of the inhibitory activity could be associated with a protein-like compound, perhaps a bacteriocin. Adjusting the pH to values of between 2 and 7 indicated an optimum inhibitory activity at pH 5. At a pH value of 8 and 9 no inhibition was observed (Table 1).

#### 3.2. Detection of hydrogen peroxide production

*L. fermentum* SK5 was classified as a strong  $\text{H}_2\text{O}_2$  producer because of the production of an intense blue color.

#### 3.3. Antibiotic susceptibility assay

In consideration of the antibiotic sensitivity of *L. fermentum* SK5, this bacterial strain was sensitive to most tested antibiotics. These included those in the group of the beta-lactam antibiotics (ampicillin, cephalothin and penicillin G), third generation cephalosporin (cefoperazoe and ceftazidime), the aminoglycosides

antibiotics (gentamicin, kanamycin, neomycin and streptomycin) and tetracyclines (doxycycline and tetracycline) as well as other individual drug i.e., chloramphenicol, clindamycin, erythromycin, norfloxacin and vancomycin. It showed only resistance to metronidazole.

#### 3.4. Survival under conditions simulating the human GI tract

The acid tolerance profile of *L. fermentum* SK5 (Fig. 2A) showed that after 1 h at every test pH there was virtually no effect on cell numbers, after 2 h there was virtually no viable cells left at pH 2 but with little change in numbers at pH 3, and after 3 h numbers fell to only 96.57% at pH 3 with again no change at pH 4.

As for resistance to bile salts (oxgall) in PBS (Fig. 2B) no viable *L. fermentum* SK5 cells were detected after exposure to 0.3 and 0.4% bile salts for 2 h. Exposure to 0.2% oxgall over 4 h reduced cell numbers gradually by about 30% and exposure to 0.1% oxgall over 4 h reduced numbers by only 8.5%.

The resistance of *L. fermentum* SK5 to pepsin and pancreatin is shown in Fig. 2C and 2D, respectively. When exposed to pepsin (SGF) there was still more than 99% survival at pH 3 and 4 after 3 h but at pH 2 the survival rate was decreased to only 70.48% whereas in the absence of pepsin at pH 2 all cells died. Exposure to pancreatin (SIF) at either pH 6.8 or 8 had virtually no effect on viable cell numbers.

#### 3.5. Aggregation and surface hydrophobicity

*L. fermentum* SK5 showed autoaggregation by forming tiny granules on a glass microscope slide within 2 min of suspension in PBS (pH 6.2). In mixed cultures, *L. fermentum* SK5 coaggregated with both *E. coli* and *G. vaginalis*. It was also classified as having a high surface hydrophobicity with a SAT value  $< 0.9 \text{ mol l}^{-1}$ .

#### 3.6. Adhesion of *L. fermentum* SK5 to HeLa, HT-29 and Caco-2 cells

The ability of *L. fermentum* SK5 to adhere to HeLa, HT-29 and Caco-2 cells compared to the commercial strain, *L. rhamnosus* GG ATCC 53103, was shown in Fig. 3. *L. fermentum* SK5 adhered to HeLa, HT-29 and Caco-2 cells with rates of 93.21, 92.26 and 93.32%, respectively, whereas the adherence of *L. rhamnosus* GG ATCC 53103 to the same cell lines was 86.63, 79.91 and 98.09%, respectively.

### 3.7. Inhibition of pathogen adhesion to HeLa, HT-29 and Caco-2 cells

*L. fermentum* SK5 produced an inhibition of the adhesion of *E. coli* and *G. vaginalis* to HeLa, HT-29 and Caco-2 cells (Fig. 4). The adhesion of *E. coli* to HeLa, HT-29 and Caco-2 cells was 86.23, 85.28 and 87.14%, respectively (Fig. 5A), whereas the adhesion of *G. vaginalis* to HeLa, HT-29 and Caco-2 cells was 76.82, 90.77 and 79.28%, respectively (Fig. 5B). *L. fermentum* SK5 significantly reduced the adhesion of *E. coli* to HeLa cells by 10, 13 and 9% in the competition, exclusion and displacement assays, respectively. With the HT-29 cells, the adhesion of *E. coli* was significantly reduced by 13, 16 and 9% in those three assays, respectively, and with Caco-2 cells the significant inhibition was 14, 19 and 12%, respectively. The adhesion of *G. vaginalis* to HeLa cells was significantly reduced by 15, 18 and 11% in the competition, exclusion and displacement assays, respectively. The adhesion to HT-29 cells was significantly reduced by 8, 17 and 11% in those three assays, respectively. The significant reduction of *G. vaginalis* adhesion to Caco-2 cells was 12, 15 and 10%, respectively.

#### 4. Discussion

Some strains of lactobacilli have been introduced as probiotics with beneficial effects on their hosts. The inhibition of infections by the microbial pathogens *E. coli* and *G. vaginalis* by *L. fermentum* SK5 by producing antimicrobial substances leads to some health benefits. SEM examination of *E. coli* and *G. vaginalis* cells treated with a dialysed culture fluid showed pore formation. Ninhydrin reacts quantitatively with primary and secondary amines to form a Schiff's base at temperatures of at least 100 °C. Following this, an oxidative deamidation and a hydrolysis occur, which leads to a ninhydrin derivative carrying an amino group. This derivative reacts with an un-derivatized ninhydrin to form a blue-violet dye known as "Ruhemann's purple" [31]. In this study, substances in dialyzed culture fluid reacted with ninhydrin reagent to form a blue-violet dye. This confirmed that one of the inhibitory substances was most likely a high molecular weight protein or lipoprotein maybe a bacteriocin as all low molecular weight molecules such as organic acids and H<sub>2</sub>O<sub>2</sub> had been removed by dialysis. Many lactic acid bacteria are known to produce bacteriocin-like molecules that inhibit Gram-positive and Gram-negative foodborne pathogens [32,33]. This purported bacteriocin from *L. fermentum* SK5 was active at an acidic pH with an optimum pH 5 but was only slightly sensitive to proteolytic enzymes and heat. Other studies have demonstrated that bacteriocin-like substances produced by *L. acidophilus* YIT 0154 were heat stable with optimum activity at neutral and acidic pH but were destroyed by proteases [34]. *L. plantarum* strain LR/14 produced a bacteriocin that was stable at a high temperature, was active in the pH range of 2.0–6.0 and was destroyed by proteolytic enzymes [35].

The vagina of healthy women is a typical balanced ecosystem with a microbial flora that consists of more than 95% lactobacilli. There were strong evidences that lactobacilli vaginal flora can prevent urogenital tract infections including recurrent urinary tract infections and bacterial vaginosis (BV) [14,36,37]. BV is the most common cause of abnormal vaginal discharge in women of child-bearing age. It is a syndrome of unknown cause characterized by depletion of the normal *Lactobacillus* population and an overgrowth of vaginal anaerobes, accompanied by loss of the usual vaginal acidity [38]. H<sub>2</sub>O<sub>2</sub>-producing lactobacilli can protect women against the development of BV [39,40,41]. In this study, *L. fermentum* SK5 was a strong H<sub>2</sub>O<sub>2</sub> producer. The colonies producing H<sub>2</sub>O<sub>2</sub> formed a blue or brown color in the medium because the horseradish peroxidase oxidized the tetramethylbenzidine in the presence of H<sub>2</sub>O<sub>2</sub>. The result indicated that *L. fermentum* SK5 may be suitable to use as a vaginal probiotic to prevent vaginal pathogen infections.

Some lactic acid bacteria have the potential to serve as a host for antibiotic resistance genes leading to the risk of transferring these genes to other lactic acid and pathogenic bacteria [2]. However, some studies have also demonstrated that many of these resistance attributes are intrinsic and nontransmissible [42,43,44,2]. Some lactobacilli such as *L. fermentum*, *L. plantarum*, and *L. reuteri* strains [45,46,47,48] are known to carry potentially transmissible plasmid-encoded antibiotic resistance gene. The transfer of plasmid and conjugative transposons to and from lactic acid bacteria have been reviewed [49]. It has been suggested probably even requested, that any strains harbouring antibiotic resistance plasmids are not suitable for use as human and animal probiotics since they may transmit antibiotic resistance genes to pathogenic or potentially pathogenic bacteria in the gut and vagina [50,51]. *L.*



*fermentum* SK5 was sensitive to all tested antibiotics except for metronidazole so is unlikely to be a host for transferable antibiotic resistance genes. The resistance to metronidazole of *L. fermentum* SK5 will be subjected to further analysis by genetic methods to establish if the antibiotic resistance genes of this strain are present on the chromosome or on plasmids and if resistance can be transferred to other strains, species and even genera. There are other situations when intrinsically antibiotic-resistant probiotic strains may be an advantage to patients. They can be given at the same time as the antibiotic treatment. In treating of a urogenital tract and gastrointestinal tract infection with lactobacilli probiotic together with the antibiotic to which they were intrinsically resistant facilitated a quick recover of the normal microflora [52]. If antibiotic resistance genes of *L. fermentum* SK5 are not present on plasmids, it may be used together with metronidazole for treatment of vaginal infections.

To exert a better health effect, the lactobacilli need to resist the harsh conditions of the stomach and upper intestine [53]. The low pH of the stomach and the antimicrobial action of pepsin are known to provide an effective barrier for the entry of bacteria into the GI tract. The pH of the stomach can be as low as 1.5 or as high as 6.0 or above after food intake, but it generally ranges from pH 2.5 to 3.5 [54]. In the present study, it was observed that *L. fermentum* SK5 did not survive for more than 2 h at pH 2, but could survive appreciably in pH 3 and 4. Pepsin also had no effect on *L. fermentum* SK5. Bile salts are toxic to bacterial cells, since they disorganize the structure of the cell membrane and bile salt tolerance is considered to be one of the properties required for lactic acid bacteria to survive in the small intestine [55]. Tolerance to pancreatin in the small intestine is also needed. *L.*

*fermentum* SK5 survived in 0.1 and 0.2% of bile salts and with pancreatin at pH 6.8 and 8. This means that this bacterial strain is likely to survive in the stomach and intestine juices. These results are similar to a previous study, where *L. casei* Zhang had a high tolerance to simulated gastric, intestine juices and bile salts [56].

A previous study had indicated that adherence to epithelial cells and biofilm formation was related to its ability to autoaggregate and its surface hydrophobicity. Autoaggregation may substantially increase the colonization potential of lactobacilli in the environments in which they have a short residence time. Adherent lactobacilli show a high surface hydrophobicity, whereas nonadherent lactobacilli are much more hydrophilic. The formation of a probiotic bacterial film may contribute to the exclusion of pathogens from the mucosa. In addition, coaggregation and adhesion lead to the formation of a barrier that prevents colonization by pathogens [57]. In this study, *L. fermentum* SK5 showed autoaggregation and a high surface hydrophobicity. Its good adhesion to the tested cell cultures would have to be due to these two characteristics although the molecular mechanisms underlying these autoaggregations by this bacterial strain are unknown. Cell surface hydrophobicity was correlated to autoaggregation activity in 12 homofermentative *Lactobacillus* strains [58]. The autoaggregation of one strain was mediated by the presence of an aggregate promoting factor (APF). *L. plantarum* 4B2 isolated from the human intestine that showed strong autoaggregation [59] contained an APF gene and product. An APF from *L. gasseri* was different from that from *L. acidophilus* and *L. jensenii* [57]. The APF of *L. gasseri* was a protein while the APF in the other two strains was lipoprotein. They also indicated that the aggregation-promoting molecules seemed to form a continuous layer around the cells when observed by negative staining before

and after treatment with protease K or lipase. Autoaggregation of *L. fermentum* SK5 may favor colonization of the gastrointestinal and vaginal epithelium through the formation of a bacterial film and this may also contribute to the exclusion of pathogens from those epithelia. The non-specific hydrophobicity surface interaction between the microorganism and the host cell may be one of the main adhesion mechanisms for *L. fermentum* SK5. Although a high cell surface hydrophobicity may play an initial role in the adhesion of bacteria to the epithelial cells and extracellular matrix protein [60], many studies have demonstrated that this non-specific initial interaction is weak and reversible and precedes the subsequent more stable adhesion process mediated by more specific mechanisms that involve cell-surface proteins and lipoteichoic acids [61,62,63]. *L. acidophilus* BFE 719 with an extremely low hydrophobicity was also able to bind to HT29MTC cells better than *L. rhamnosus* GG that had a higher cell surface hydrophobicity [64]. They concluded that cell hydrophobicity may be helpful for adhesion, but it was obviously not a prerequisite for a strong adhesive capacity. *L. fermentum* SK5 coaggregated with both gastrointestinal and vaginal pathogens. The coaggregation could be an important factor that interferes with the ability of the pathogens to adhere to receptors on the epithelial surface. During coaggregation, lactobacillus could control a microenvironment around the pathogens and increase the concentration of excreted inhibiting substances such as acids, H<sub>2</sub>O<sub>2</sub>, bacteriocin-like substances [65]. Ten strains of lactobacilli for vaginal probiotic activity and they found various degrees of coaggregation with *Candida albicans* and *G. vaginalis* [66].

The ability to adhere to the epithelial cells is one of the main criteria for selecting probiotic strains. This ability is regarded as a prerequisite to exert beneficial

effects. The difficulties of assessing the adherence of probiotic strains *in vivo* have led to the development of *in vitro* adherence assays [67]. In this study, HeLa, HT-29 and Caco-2 cells were used. HeLa cells being human cervical carcinoma cells are representative of vaginal epithelium cells. HT-29 and Caco-2 cells are derived from colon carcinomas and represent the major cell phenotypes found in the human intestinal mucosa, namely enterocytes and goblet cells. Both HT-29 and Caco-2 cells show the typical characteristics of enterocytic differentiation [68]. HT-29 cells that produce a large quantity of mucus are representative for the mucus secreting small intestinal epithelium cells. Caco-2 cells do not secrete mucus, therefore, these cells are representative of the non-mucus secreting small intestine-type enterocytes [69]. *L. fermentum* SK5 was able to adhere to the three tested cells. This indicated that *L. fermentum* SK5 adhered to and colonized intestinal and vaginal epithelium cells and provided inhibition against pathogens using various mechanisms. *L. rhamnosus* GG ATCC 53103 is known as the commercial probiotic strain and is used in many studies about probiotic properties. *L. fermentum* SK5 had significantly higher adhesive properties to HeLa and HT-29 cells than *L. rhamnosus* GG ATCC 53103 but had a lower adherence to Caco-2 cells. This may be due to the cell adherence specificity. In consideration of adherence to HeLa cells, *L. fermentum* SK5 showed appreciably more adhering ability than *L. rhamnosus* GG ATCC 53103. This occurrence might be due to the source specificity for such adherence. The result was in agreement with previous study [70]. HeLa cells have been used as an *in vitro* adhesion model for other studies. For example, the adhesion potential of a pre-selected strain of *L. plantarum* AC131 to HeLa cells was evaluated [71]. In this case, the strain AC131 was classified as being strongly adhesive to HeLa cells. With regard to HT-29 and

Caco-2 cells, they were derived from a colon carcinoma but the adhering ability of the tested *Lactobacillus* strains to these two cultured cells was different which might be due to the different protein and lipid compositions of these two epithelial cells. Besides, it was observed that the tested lactobacilli, *L. fermentum* SK5 and *L. rhamnosus* GG ATCC 53103, showed better adherence on Caco-2 cells (non-mucus secreting) than to HT-29 cells (mucus secreting). This indicated that Caco-2 cells have a higher affinity than HT-29 cells for lactobacilli and the mucus produced from HT-29 cells did not enhance the adhesion ability of either *L. fermentum* SK5 or *L. rhamnosus* GG ATCC 53103. There are studies that showed the adhering ability of *Lactobacillus* probiotics to HT-29 and Caco-2 cells [72,73,74]. *Lactobacillus* from fermented sausages had good adhesion capability to human intestinal Caco-2 cell lines [75].

The mechanisms involved in protection against pathogen adhesion have been proposed to be either non-specific hindrance of receptors for pathogens or competition with pathogens for the binding sites [76]. Such inhibitory activity is specific depending on both the probiotic and pathogenic strains [77]. A very high specificity in inhibiting adhesion of enteropathogens by different lactobacilli was reported [78]. This means that there is a need for a case-by-case assessment in selecting the strains with an ability to inhibit certain pathogens. In the present study, *L. fermentum* SK5 inhibited the adhesion of both *E. coli* and *G. vaginalis* to HeLa, HT-29 and Caco-2 cells. It indicated the specificity of *L. fermentum* SK5 to inhibit the adhesion of *E. coli* and *G. vaginalis*. The inhibition of *E. coli* and *G. vaginalis* adhesion by *L. fermentum* SK5 was shown by all performed assays including competition, exclusion and displacement assays. The best adhesion inhibition of these

two pathogens was through exclusion mechanism. This result indicates that the balance of normal probiotic *Lactobacillus* may provide a good protection against the adhesion of pathogens to epithelium cells. The ability to compete, exclude and displace pathogens from mucus by specific probiotic strains has been reported in other studies. For example, *L. rhamnosus* GG and *L. casei* Shirota were able to compete with, exclude and displace pathogenic gastrointestinal (GI) bacteria, but the degree of inhibition of adhesion was bacterial strain-dependent [79].

In conclusion, the present study performed by *in vitro* tests revealed that *L. fermentum* SK5 possessed desirable probiotic properties. Of most importance were the findings, that *L. fermentum* SK5 inhibited both *E. coli* and *G. vaginalis*, two pathogens found in the GI tract and vagina, respectively. It is likely that *L. fermentum* SK5 could be a good probiotic candidate for use in preventing *E. coli* and *G. vaginalis* infections.

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Table 1. Effect of trypsin, heat and pH on the antibacterial activity of a dialyzed culture fluid from *L. fermentum* SK5.

Fig. 1. Examination by scanning electron microscopy (SEM) of *E. coli* and *G. vaginalis*. *E. coli* (A) and *G. vaginalis* (C) treated with phosphate buffer saline, pH 7.4. *E. coli* (B) and *G. vaginalis* (D) treated with dialyzed culture fluid from *L. fermentum* SK5 (pointing arrow).

Fig. 2. Survival of *L. fermentum* SK5 in simulated human GI tract conditions: the presence of acid (A): pH 2 (◆), pH 3 (●), pH 4 (▲), bile-containing buffer (B): 1% oxgall (◆), 2% oxgall (●), 3% oxgall (▲), 4% oxgall (×), simulated gastric fluid (C): SGF pH 2 (◆), SGF pH 3 (●), SGF pH 4 (▲), simulated intestinal fluid (D): SIF pH 6.8 (◆), SIF pH 8 (●). \* =  $p < 0.05$  (compared with before treatment).

Fig. 3. Adhesion of *L. fermentum* SK5 (■) to HeLa, HT-29 and Caco-2 cells. \* =  $p < 0.05$  (compared to binding of *L. rhamnosus* GG ATCC 53103 (□)).

Fig. 4. Examination by scanning electron microscopy (SEM) of *L. fermentum* SK5 inhibiting pathogen adhesion to HT-29 cells. Adhesion of *E. coli* alone to HT-29 cell monolayer (A). Adherence of *L. fermentum* SK5 inhibiting *E. coli* (pointing arrow) adhesion to HT-29 cell monolayer (B). Adhesion of *G. vaginalis* alone to HT-29 cell monolayer (C). Adherence of *L. fermentum* SK5 inhibiting *G. vaginalis* adhesion to HT-29 cell monolayer (D).

Fig. 5. Inhibition of *E. coli* (A) and *G. vaginalis* (B) adhesion to HeLa, HT-29 and Caco-2 cells by *L. fermentum* SK5: Competition (■), Exclusion (▢), Displacement (⊠). \* =  $p < 0.05$  (compared with *E. coli* alone or *G. vaginalis* alone (□)).

Table 1

Treatment		Inhibition zone (mm) $\pm$ SD	
		<i>E. coli</i>	<i>G. vaginalis</i>
Dialyzed culture fluid (untreated)		13.50 $\pm$ 0.71	9.75 $\pm$ 0.50
Enzyme	Trypsin (1 mg/ml)	10.50 $\pm$ 0.58*	8.75 $\pm$ 0.50*
Heat ( $^{\circ}$ C)	60	10.00 $\pm$ 0*	8.25 $\pm$ 0.50*
	80	9.50 $\pm$ 0.58*	8.00 $\pm$ 0*
	100	9.50 $\pm$ 1.00*	8.00 $\pm$ 0*
	121	9.00 $\pm$ 0*	8.00 $\pm$ 0*
pH	2	12.50 $\pm$ 0.58	0*
	3	12.00 $\pm$ 0.82*	8.00 $\pm$ 0*
	4	10.75 $\pm$ 0.50*	8.25 $\pm$ 0.50*
	5	9.50 $\pm$ 0.58*	9.25 $\pm$ 0.50
	6	8.75 $\pm$ 0.96*	9.25 $\pm$ 0.50
	7	8.75 $\pm$ 0.50*	0*
	8	0*	0*
	9	0*	0*

\* The mean difference is significant at the 0.05 level when compared with the dialyzed culture fluid.

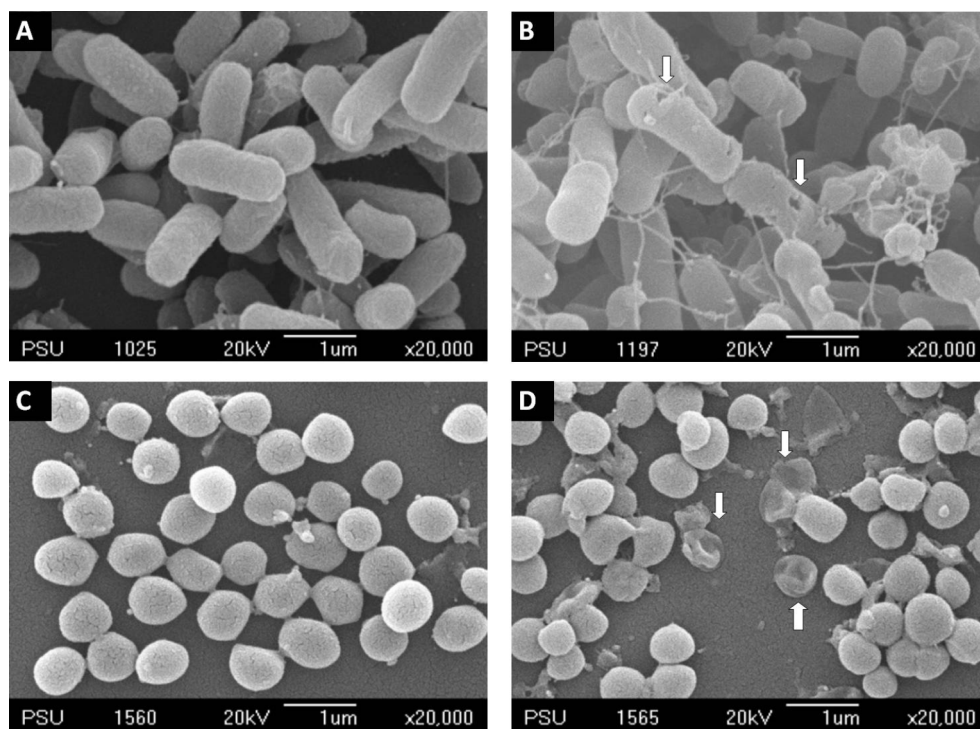


Fig. 1

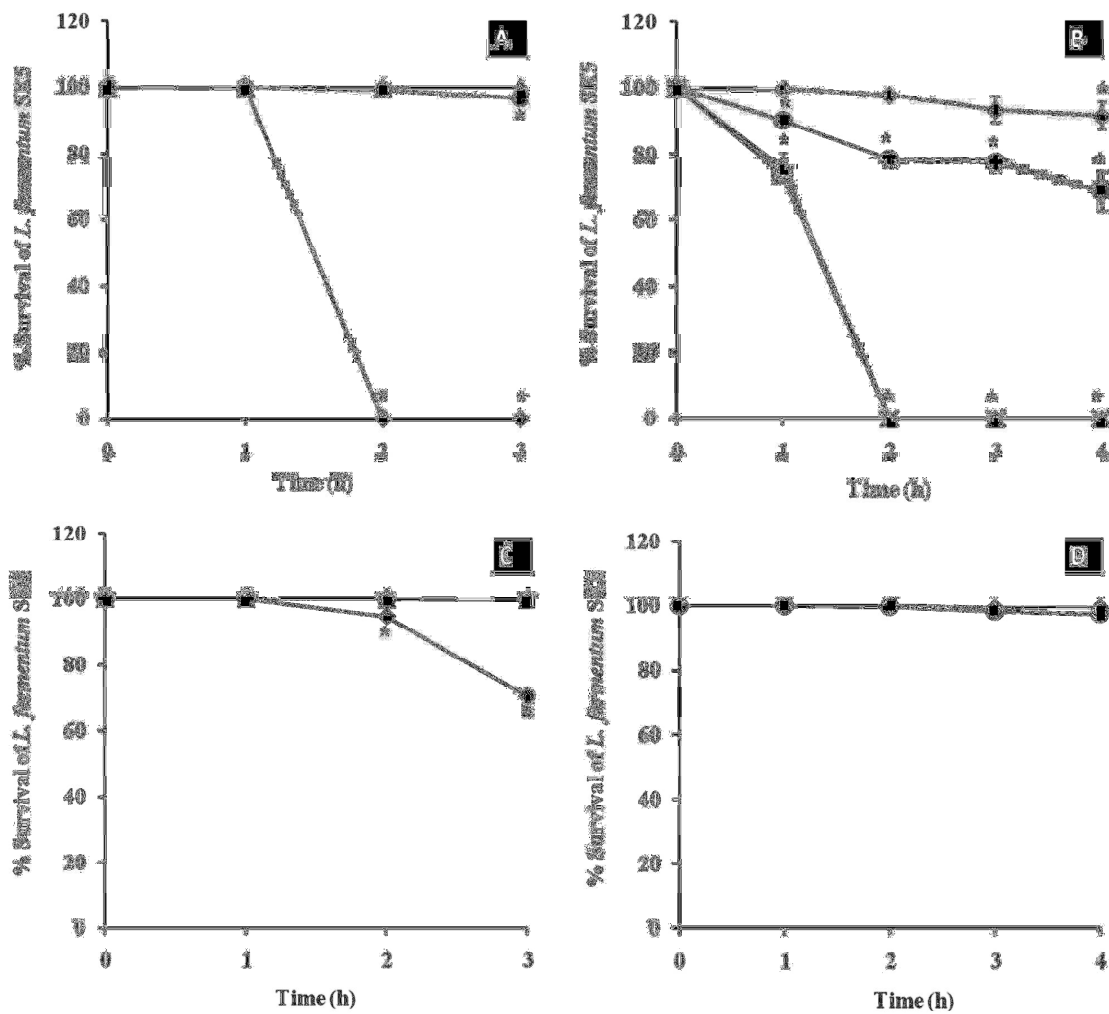


Fig. 2

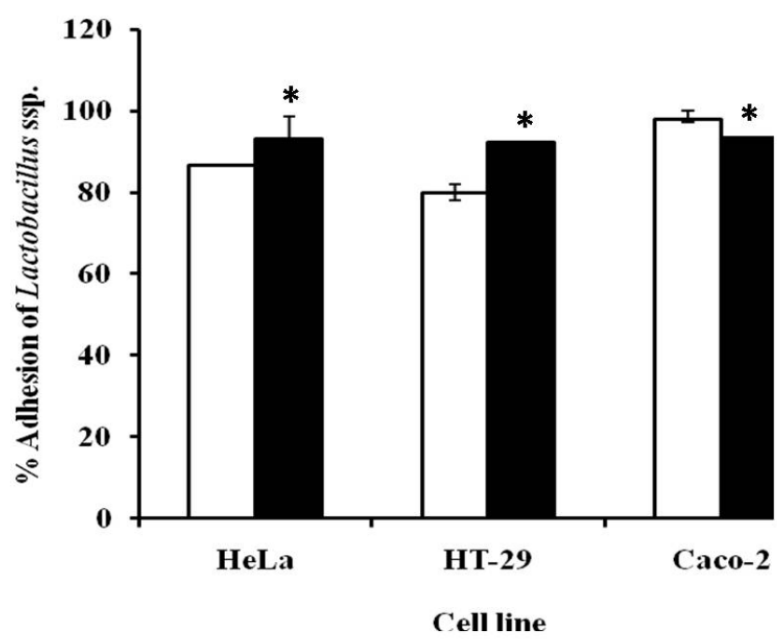


Fig. 3

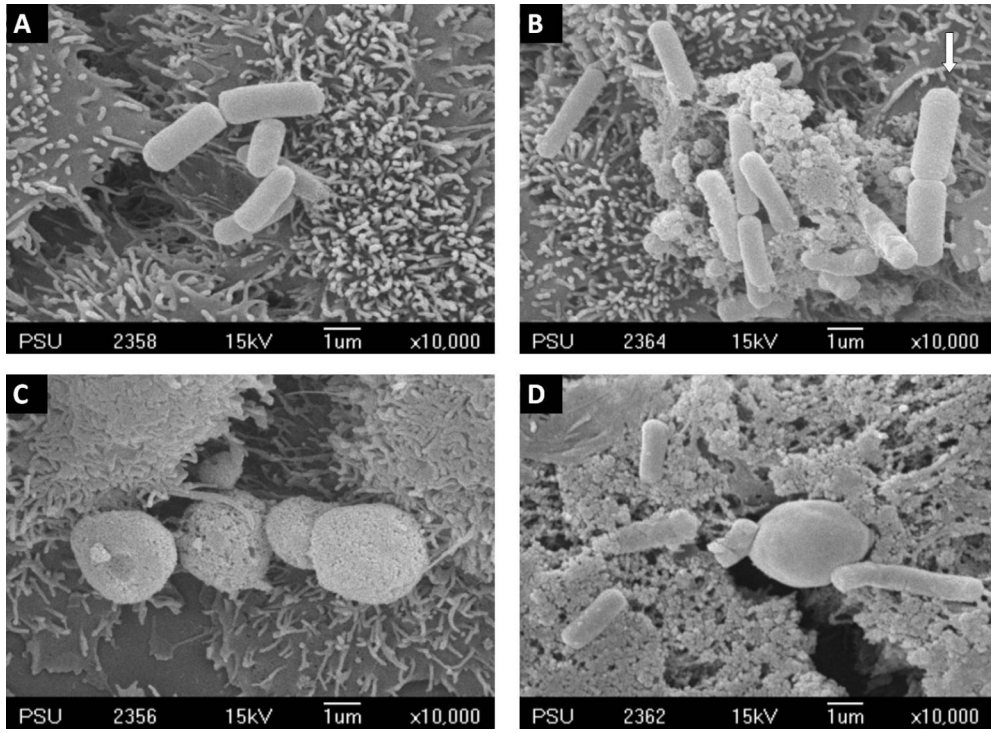


Fig. 4



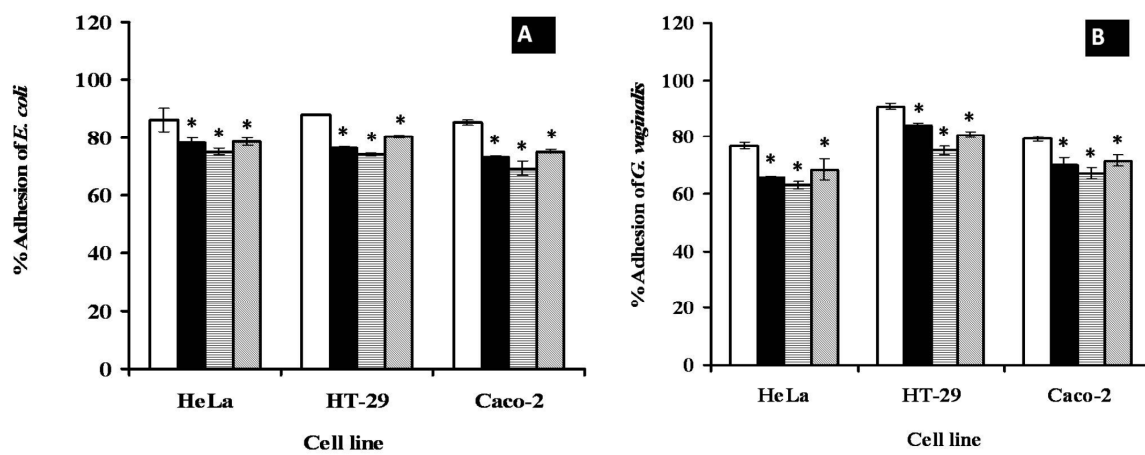


Fig. 5