Probiotic Properties of *Lactobacillus fermentum* SK5 and Pharmaceutical Preparation Design

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Pharmaceutical Sciences
Prince of Songkla University
2013

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ชื่อวิทยานิพนธ์ คุณสมบัติโพรไบโอติกของเชื้อ *Lactobacillus fermentum* SK5 และการออกแบบเภสัชภัณฑ์
ผู้เขียน นางสาวณัฐกาญจน์ แดงมณี
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บทคัดย่อ

Lactobacillus fermentum SK5 เป็นเชื้อที่แยกได้จากช่องคลอดของผู้หญิงสุขภาพดี มีฤทธิ์ยับยั้งเชื้อก่อโรคในทางเดินอาหาร ได้แก่ *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Shigella sonnei* และ *Vibrio cholerae* และเชื้อก่อโรคในช่องคลอด ได้แก่ *Gardnerella vaginalis*, *Bacteroides fragilis* DMST 19152, *Bacteroides vulgatus* DMST 15535, *Bacteroides ureolyticus* ATCC 33387, *Mobiluncus curtisii* ATCC 35241 และ *Prevotella bivia* ATCC 29303 ซึ่งสารที่ยับยั้งเชื้อก่อโรคที่เชื้อนี้ผลิตขึ้น ได้แก่ กรดอินทรีย์ต่างๆ ไฮโดรเจนเปอร์ออกไซด์ และสารโมเลกุลใหญ่ที่มีน้ำหนักโมเลกุลสูงกว่า 10 กิโลดัลตัน *L. fermentum* SK5 สามารถอยู่รอดในสภาวะทางเดินอาหารและช่องคลอดจำลองได้ดี และไวต่อยาปฏิชีวนะทุกตัวที่ทดสอบยกเว้นยาเมโทรนิดาโซล เชื้อนี้มีคุณสมบัติในการรวมกลุ่มกันเองได้ดี (autoaggregation) ผิวของเชื้อมีความไม่ชอบน้ำสูง (high surface hydrophobicity) รวมกลุ่มกับเชื้อ *E. coli* และ *G. vaginalis* ได้ดี (coaggregation) สามารถยึดเกาะกับเซลล์เยื่อบุช่องคลอด (HeLa) และเซลล์ผนังลำไส้ (HT-29 และ Caco-2) ได้ดี และยับยั้งการเกาะของเชื้อ *E. coli* และ *G. vaginalis* บนเซลล์เหล่านี้ได้ จากการศึกษาการเพิ่มการเจริญของเชื้อแลคโตบาซิลลัสและเชื้อก่อโรคพบว่า อินนูลินและกาแลคโตโอลิโกแซคคาไรด์เพิ่มการเจริญของเชื้อแลคโตบาซิลลัสเท่านั้น ในขณะที่สกิมมิลค์เพิ่มการเจริญทั้งเชื้อแลคโตบาซิลลัสและเชื้อก่อโรค กาแลคโตโอลิโกแซคคาไรด์เพิ่มการเจริญของเชื้อแลคโตบาซิลลัสได้มากกว่าอินนูลินอย่างมีนัยสำคัญ ในระหว่างกระบวนการทำแห้งแบบแช่เยือกแข็งพบว่า อินนูลิน 4 เปอร์เซ็นต์น้ำหนักต่อปริมาตร กาแลคโตโอลิโกแซคคาไรด์ 4 เปอร์เซ็นต์น้ำหนักต่อปริมาตร และสกิมมิลค์ 10 เปอร์เซ็นต์น้ำหนักต่อปริมาตร ให้อัตราการอยู่รอดของเชื้อ *L. fermentum* SK5 ดีที่สุด ซึ่งกาแลคโตโอลิโกแซคคาไรด์ 4 เปอร์เซ็นต์น้ำหนักต่อปริมาตร ได้รับการคัดเลือกเพื่อนำมาใช้เป็นสารพรีไบโอติกและ cryoprotectant ในการเตรียมเชื้อ *L. fermentum* SK5 ในรูปผงแห้ง ได้นำผงแห้งของเชื้อ *L. fermentum* SK5 มาเตรียมเป็นแคปซูลเคลือบให้แตกตัวในลำไส้ (enteric coating) (มีเชื้อ 8.39 log cfu ต่อแคปซูล) โดยใช้สารยูดราจิต (Eudragit®

L100) เป็นสารเคลือบ และเตรียมเป็นยาเหน็บช่องคลอดชนิดกลวง (มีเชื้อ 8.39 log cfu ต่อแท่ง) โดยใช้ส่วนผสมของพอลิเอทิลีนกลัยคอลเป็นยาพื้น แคปซูลเคลือบให้แตกตัวในลำไส้และยาเหน็บช่องคลอดชนิดกลวงที่ได้มีรูปลักษณ์ การปลดปล่อยตัวเชื้อแลคโตบาซิลลัส และฤทธิ์ในการยับยั้งเชื้อก่อโรคที่ดี เชื้อแลคโตบาซิลลัสยังคงมีอัตราการอยู่รอดสูงในระหว่างการเก็บรักษาเภสัชภัณฑ์ทั้งสองชนิดที่อุณหภูมิ 4 องศาเซลเซียสเป็นเวลา 6 เดือน จากการศึกษาครั้งนี้พบว่าเชื้อ *L. fermentum* SK5 มีคุณสมบัติการเป็นโพรไบโอติกที่ดีเพื่อนำไปใช้เป็นเภสัชภัณฑ์สำหรับการป้องกันการติดเชื้อก่อโรคในทางเดินอาหารและช่องคลอดได้

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Academic Year 2013

ABSTRACT

Lactobacillus fermentum SK5 was isolated from a vaginal tract of a healthy woman. It had an antimicrobial activity against gastrointestinal pathogens, i.e. *Escherichia coli, Pseudomonas aeruginosa, Salmonella* Typhimurium, *Shigella sonnei* and *Vibrio cholerae* and bacterial vaginosis pathogens, i.e. *Gardnerella vaginalis, Bacteroides fragilis* DMST 19152, *Bacteroides vulgatus* DMST 15535, *Bacteroides ureolyticus* ATCC 33387, *Mobiluncus curtisii* ATCC 35241 and *Prevotella bivia* ATCC 29303. The effective compounds were organic acids, hydrogen peroxide and a high molecular weight substance of more than 10 kDa. *L. fermentum* SK5 was able to survive under conditions simulating the human gastrointestinal tract and vagina. It was susceptible to all tested antibiotics except metronidazole. *L. fermentum* SK5 had a good autoaggregation characteristic, a high surface hydrophobicity and a coaggregation with *E. coli* and *G. vaginalis*. It showed high adhesion ability to HeLa, HT-29 and Caco-2 cells and inhibited the adhesion of *E. coli* and *G. vaginalis* to these cells. Growth promotion studies revealed that inulin and galactooligosaccharide (GOS) increased only the growth of *Lactobacillus* but skim milk increased both the growth of *Lactobacillus* and pathogens. GOS increased

the growth of *Lactobacillus* significantly better than inulin. During the lyophilzation process, 4% (w/v) inulin, 4% (w/v) GOS and 10% (w/v) skim milk gave the best survival rate of *L. fermentum* SK5. Four percent (w/v) GOS was selected as the prebiotic and cryoprotectant for preparation of a *L. fermentum* SK5 lyophilized powder. *L. fermentum* SK5 lyophilized powder was formulated as an enteric coated capsule (8.39 log cfu/capsule) using Eudragit® L100 as coating media and a hollow-type vaginal suppository (8.39 log cfu/suppository) using a mixture of polyethylene glycols (PEGs) as a base. The appearance, bacterial release and antimicrobial activity of enteric coated capsule and suppository were acceptable. The survival rate of *Lactobacillus* in these formulas was stable during storage at 4 °C for 6 months. It is likely that this lactobacillus strain could be a potential probiotic candidate for beneficial use as pharmaceutical products protecting against gastrointestinal and vaginal pathogens infections.

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LIST OF ABBREVIATIONS AND SYMBOLS

ATCC	American Type Culture Collection
˚C	degree Celcius
cfu	colony forming unit
CMCC	Center for Medical Culture Collection
d	day
g	gram
h	hour
i.e.	id est
kDa	kiloDalton
l	liter
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimole/liter
M	mole/liter
N	normality
nm	nanometer
rpm	revolutions per minute
v/v	volume by volume
w/v	weigh by volume
µg	microgram

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

µl	microliter
%	percent

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 mechanism including secrete organic acids (lactic acid and short chain fatty acids) (Røssland *et al.*, 2005; Lee *et al*., 2011), produce other antimicrobial substances, i.e. hydrogen peroxide (H_2O_2) (Ammor *et al*., 2006; Pascual *et al*., 2006; Anukam and Reid, 2007), bacteriocin (Vera Pingitore *et al*., 2009; Agaliya and Jeevaratnam, 2013; Ghanbari *et al*., 2013), adhere to surface and inhibit the adhesion of pathogens (Gueimonde *et al.*, 2006; Collado *et al.*, 2007; Ramiah *et al.*, 2008), stimulate the host's immune system (Zoumpopoulou *et al*., 2008; Kotzamanidis *et al*., 2010, Lee *et al*., 2011; O'Shea *et al*., 2012) 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 pathogens (Jara *et al*., 2011; Khler *et al*., 2012; Zabihollahi *et al*., 2012). 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*., (2001a) 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.

Lactobacilli probiotics play important roles in the production of fermented food products (dairy, vegetable, meat and wine) and pharmaceutical products. Dried preparations have advantages of long-term preservation and convenience in handling, storage, marketing and consumption. The industrial of LAB as starter and/or probiotic cultures relies heavily on the ability to concentrate and

preserve them, so as to guarantee long-term delivery of viable and functional cultures (Carvalho *et al*., 2003a; De Giulio *et al*., 2005; Li *et al*., 2009). Lyophilization is often used for preservation and storage of biological samples; however, it brings about undesirable side-effects, such as denaturation of sensitive proteins and decreased viability of many cell types (Carvalho *et al*., 2003b; Jalali *et al*., 2012). Protective additives have an important role in the conservation of viability. A good protectant should provide cryoprotection of cells during lyophilization, be easily dried, and provide a good matrix to allow stability and ease of rehydration (Costa *et al*., 2000). Various groups of substances have been tested for their protective action, including polyols, polysaccharides, disaccharides, amino acids and protein hydrolysates, proteins, minerals, salts of organic acids and vitamins-complex media (Strasser *et al*., 2009; Li *et al*., 2011; Nag and Das, 2013). Moreover, many prebiotic carbohydrates and plant fibers have been reported to improve bacterial survival during freezing and dehydration processes (Saarela *et al*., 2006; Hongpattarakere *et al*., 2012).

Commercial literature on various probiotic products suggests that they can be taken before meals, during meals or after meals or even without meals. Traditionally, in the food industry, probiotics have been delivered as part of a whole fermented dairy product such as a yogurt, kefir or sweet acidophilus milk. In the nutritional supplement industries, they are often delivered in capsules or powders. There are very little literature discusses the appropriate conditions for assuring the survival of probiotic microbes delivered in a capsule. Tompkins *et al*. (2011) reported that probiotic capsules without enteric-coated, should be taken with a food or beverage with at least 1% w/w fat content to ensure the highest survival of viable

microbes arriving in the small intestine. Enteric-coated delivery systems are able to provide lag phases starting on gastric emptying, with the coating dissolution, and lasting throughout the small intestinal transit time (Gazzaniga *et al*., 2006). Gastric resistance is generally obtained by means of polymers with pH-dependent solubility; the most widely employed are acrylic and metacrylic acid copolymers (e.g. Eudragit® L, S and FS), polyvinyl acetate phthalate (PVAP) and cellulose derivatives (e.g. cellulose acetate phthalate, CAP; hydroxypropyl methyl cellulose acetate succinate, HPMCAS) (McGinity and Felton, 2008). Capsule coated with Kollicoat® IR (polyvinyl alcohol–polyethylene glycol graft copolymer) and/or Explotab® CLV (sodium starch glycolate) could be manufactured, and a promising performance was achieved with appropriate gastric resistance in pH 1.2 medium and break-up in pH 6.8 within 1 h (Zema *et al*., 2013). Nakpheng *et al*. (2012) reported that freeze-dried *Lactobacillus plantarum* T23/3 loaded into the Eudragit® L100 coated capsule did not release the probiotic content in SGF pH 1.2 over 2 h but dissolved, released the *L. plantarum* 23/3 instantly in the SIF at pH 7.4. The results demonstrated that Eudragit® L100 coated capsules were successful for intestinal delivery of lactobacilli.

Different types of formulations for delivery of vaginal probiotics are commercially available, but the main group of vaginal probiotic delivery forms is lyophilized powders compacted into tablets, lyophilized powders filled into capsules and lyophilized powders with a gelatin-based encapsulation. Also tampons impregnated with lactobacilli are commercially available. Vaginal lactobacilli protect the female urogenital tract from pathogen colonization. Therefore, they can contribute to the prevention of genitourinary tract infection. Many studies have been published

describing the relationships between bacterial vaginosis and lactobacilli (Reid *et al*., 2001b; Reid *et al*., 2001c; Reid and Burton, 2002). Studies utilizing *Lactobacillus*-vaginal tablets have been used for treatment of human vaginal infection (Mastromarino *et al*., 2002). *Lactobacillus* vaginal capsules are effective in treating BV and in re-establishing a *Lactobacillus*-predominant flora (Reid *et al*., 2003). However, these commercial products often have many problems such as irritation, discomfort, and leakage at the application site. An optimal formulation for vaginal delivery of probiotic strains should (1) have a long retention time to maximize release of the probiotic strain, (2) have a proper spreading over the vaginal vault to establish colonization of the probiotic strain on different sites of the vaginal tract, (3) not cause harm to the normal vaginal microflora and mucosa, (4) be easy to administer and (5) not cause discomfort to the patient (Santiago *et al*., 2009). Alternative formulation was developed as *Lactobacillus* vaginal suppository that resolves poor patient compliance of traditional vaginal dosage forms. The preventative effect of *Lactobacillus* vaginal suppositories on recurrent urogenital tract infections was exhibited (Reid *et al*., 1992).

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

1.2.2.1 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_2O_2 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).

1.2.2.2 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_2O_2, 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* CMCC 98001, *Staphylococcus aureus* CMCC 26003, *Escherichia coli* CMCC 44113, and *Pseudomonas aeruginosa* CMCC 10110, 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_2O_2 level. There were other studies that report inhibition of vaginal pathogens by lactobacilli (Juárez Tomás *et al*., 2003; Saunders *et al*., 2007).

1.2.2.3 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 and nausea. 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.

1.2.2.4 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).

1.2.2.5 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).

1.2.2.6 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 Properties of probiotics

1.2.3.1 Antimicrobial activity against potentially pathogenic bacteria

Lactobacillus probiotics inhibit pathogens infection by producing antimicrobial substances. 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 stronger inhibited *B. cereus*. Juárez Tomás *et al*. (2011) assessed the inhibitory activity of 38 vaginal *Lactobacillus* strains against urogenital pathogens. The lactobacilli inhibited the growth of various urogenital pathogens, except *Candida albicans*, mainly due to the effects of both organic acids and H_2O_2. 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).

Inhibitory substances produced by Lactobacilli.

Fatty acids

Organic acids such as lactic acid, short chain fatty acids (acetic acid, propionic acid and butyric acid) 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).

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_20_2. This mechanism, together with the absence of the heme protein catalase, generally results in the formation of H_20_2 in amounts which are in excess of the capacity of the organism to degrade it. The

H_20_2 formed may inhibit or kill other members of the microbiota, particularly those which lack or have low levels of H_20_2-scavenging enzymes, such as catalase peroxidase. The microbicidal activity of H_20_2 is considerably increased by the enzyme peroxidase in the presence of a halide ion (Eschenbach *et al*. 1989).

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 (Cheikhyoussef *et al.*, 2009).

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 β-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.

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 1.1). 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).

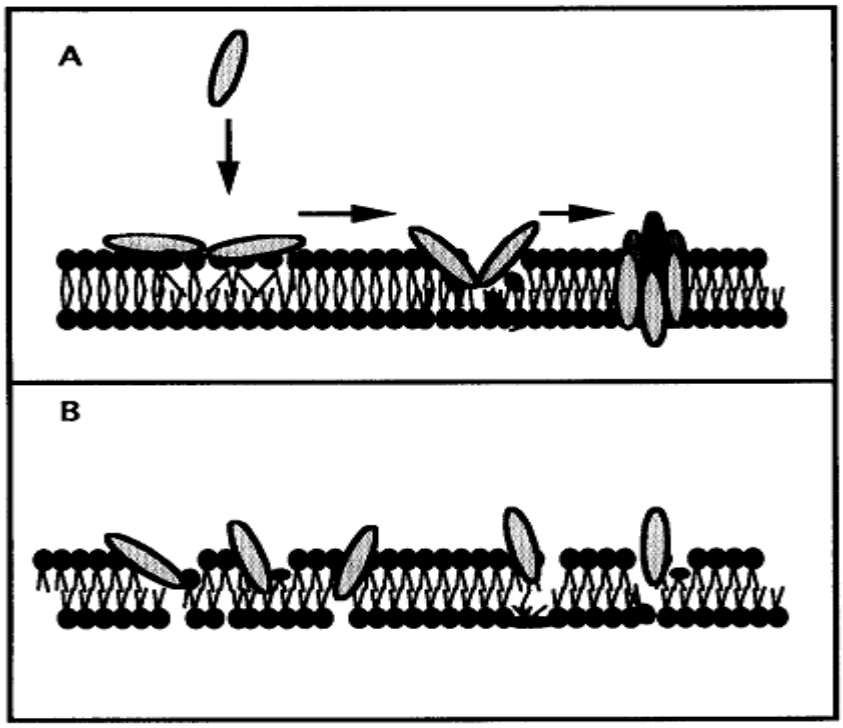

Figure 1.1 Interaction of bacteriocin monomers (ovals) with the cytoplasmic membrane (Montville *et al.*, 1995)

1.2.3.2 Antibiotic resistance

Antibiotics are introduced for the treatment of microbial diseases but the use of antimicrobial agents for therapy of bacterial infections has been the development of antimicrobial resistance in pathogenic bacteria (Mathur and Singh, 2005). Antibiotic resistance genes located on conjugative or mobilizable plasmids and transposons can be found in species living in habitats, e.g. human and LAB (Schwarz and Chaslus-Dancla, 2001; Nordmann and Poirel, 2005; Pruden *et al*., 2006). Although, LAB may act as reservoirs of antibiotic resistance genes can be transferred via the food chain or within the GI tract to pathogenic bacteria (Egervarn, 2009). They are generally considered to be safe for human use according to basic principle for "Generally Recognised as Safe" (GRAS) status (Ji *et al*., 2013). The resistances of LAB are often intrinsic and nontransmissible (Curragh and Collins, 1992; Adams and Marteau, 1995; Charteris *et al*., 1998; Salminen *et al*., 1998). However, some LAB may carry potentially transmissible plasmid-encoded antibiotic resistance genes, as shown for example in certain *L. fermentum, L. plantarum* and *L. reuteri* strains (Ishiwa and Iwata, 1980; Ahn *et al*., 1992; Tannock *et al*., 1994; Fons *et al*., 1997). New species and more specific strains of probiotic bacteria are constantly identified. It cannot be assumed that these novel probiotic organisms share the historical safety of tested or traditional strains. Therefore, new strains should be carefully assessed and tested for safety.

1.2.3.3 Survival in gastrointestinal tract

The environment of stomach may highly affect the survival of Lactobacilli. The survival of bacteria in gastric juice depends on their ability to

tolerate low pH. The pH of excreted HCl in stomach is 0.9. However, the presence of food raises the pH value to the level of pH 3. After the ingestion of food it takes 2-4 h for the stomach to empty (Goldin and Gorbach, 1992).

In the intestine, bile plays an important role in emulsify lipids, which enables intra-luminal lipolysis and absorption of lipids nutrient and have a detergent-like function (Liong and Shah, 2005). Since the cell membranes of microorganism are composed of lipids and fatty acids, the bile salts are critical to them. However, some microorganisms are able to reduce this detergent effect by their ability of hydrolyzed bile salts by BSH and thus to decrease their solubility (Knarreborg *et al.*, 2002).

To reach the intestine, strains must first pass through the stomach, which secretes hydrochloric acid and enzyme. More than two litres of gastric juice is secreted each day, with a pH as low as 1.5 providing a barrier to the entrance into the gut of bacteria (Morelli, 2000). The survival of potential probiotic strains to gastric juice depends on their intrinsic resistance to the hostile environment but also on the host and the ingestion vector. Foods with a high level of fat and the presence of certain proteins in the food may protect the bacteria from stomach acid and thus increase survival to gastric transit (Zarate *et al*., 2000). A mechanism proposed for the beneficial effect of foods and food ingredients is the increase in the pH of gastric contents resulting from the addition of food (Charteris *et al*., 1998). To exert a positive effect on the health of a host, probiotics also need to colonise and survive in the intestine which contains bile salt and enzyme affecting on their viability (Ouwehand, 2002). *L. acidophilus* NIT isolated from infant feces was examined for resistance to pH 2–4. It was observed that *L. acidophilus* NIT had certain resistance

ability to acid. Its high survival was shown at pH 4.0 (Pan *et al.*, 2009). Maragkoudakis *et al.* (2006) examined *in vitro* probiotic potential of twenty-nine *Lactobacillus* strains from dairy origin. Only a few strains were able to survive in the presence of pepsin, while all were unaffected by pancreatin. In addition, the ability to survive the action of bile salts is generally included among the criteria used to select potentially probiotic strains. *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese were tested the resistance to bile salts. These strains evidenced a good survival in presence of 1.0%, 1.5% and 2.0% bile salts (Succi *et al.*, 2005). Although, the bile salt resistant lactobacilli can be selected by testing their survivability in the presence of bile salt and their growth in selected medium containing various levels of bile (Chung *et al.*, 1999). A concentration of 0.15-0.3% of bile salt has been recommended as a suitable concentration for selecting probiotics bacteria for human use (Erkkila and Petaja, 2000).

1.2.3.4 Survival in vagina (applicable to probiotics for vaginal use)

Normal microflora predominantly Lactobacilli produce sufficient lactic acid to acidify vaginal secretions to pH 3.5–4.5 (Boskey *et al.*, 1999). 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). *Lactobacillus* probiotics used in vaginal tract should survive at this vaginal pH.

1.2.3.5 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).

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 GI or 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.

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 GI or 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).

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

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). 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). *In vitro* models involving human intestinal epithelial cell lines, mostly Caco-2, HT29 and mucus-secreting HT29-MTX cells have been used to assess the adhesion properties of potential probiotic strains (Collado *et al*., 2010). Adhesion and colonisation properties of three probiotic strains namely, *L. rhamnosus* DR20, *L. acidophilus* HN017, and *B. lactis* DR10, were determined in vitro using the differentiated human intestinal cell-lines including HT-29, Caco-2, and HT29-MTX, and compared with properties of *L. acidophilus* LA-1 and *L. rhamnosus* GG (two commercial probiotic strains). All three strains showed strong adhesion with the human intestinal cell lines *in vitro*. The adhesion indices of three strains were not significantly different from the values obtained from the two commercial probiotic strains *L. acidophilus* LA-1 and *L. rhamnosus* GG (Gopal *et al*., 2001). 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. The mechanisms of action of probiotic to prevent GI infection are likely to be multifactorial processes as shown in Figure 1.2 (Collado *et al*., 2010). 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 1.3). The adhesion of pathogens was interfered by lactobacilli probiotic via competition, exclusion and displacement mechanisms (Ren *et al*., 2012; Dhanani and Bagchi, 2013; Woo and Ahn, 2013). *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).

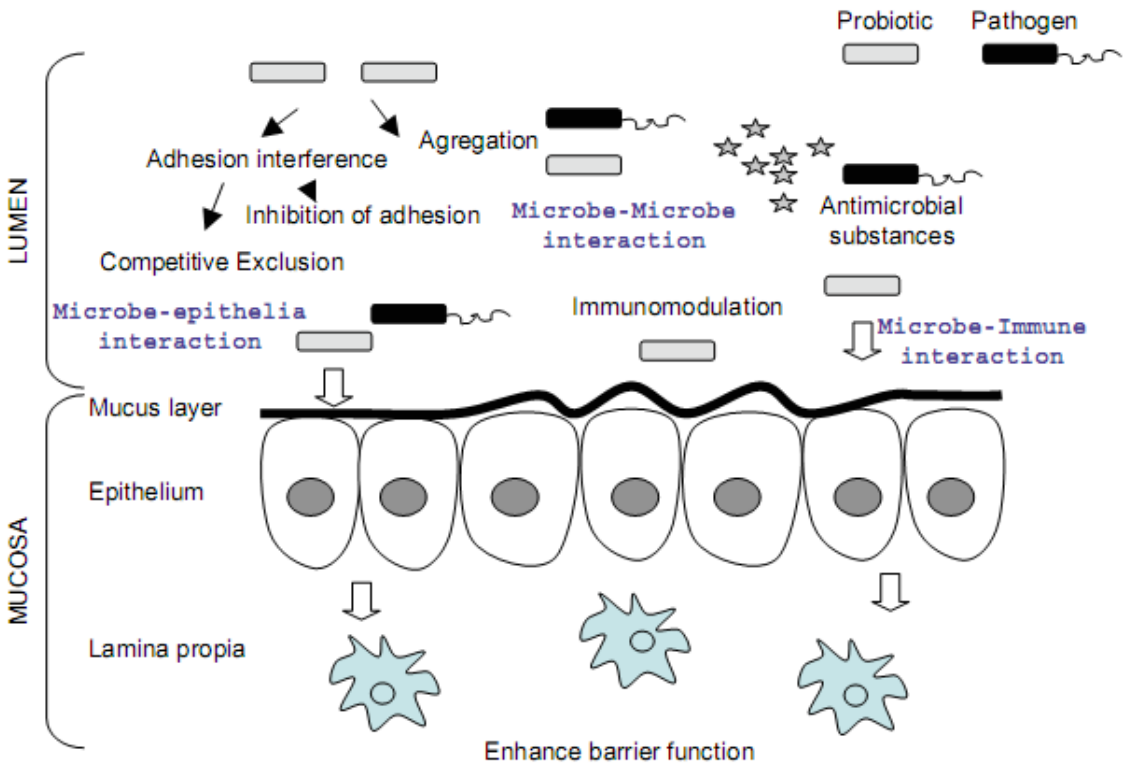

Figure 1.2 Mechanisms of probiotics against pathogen infection (Collado *et al.*, 2010)

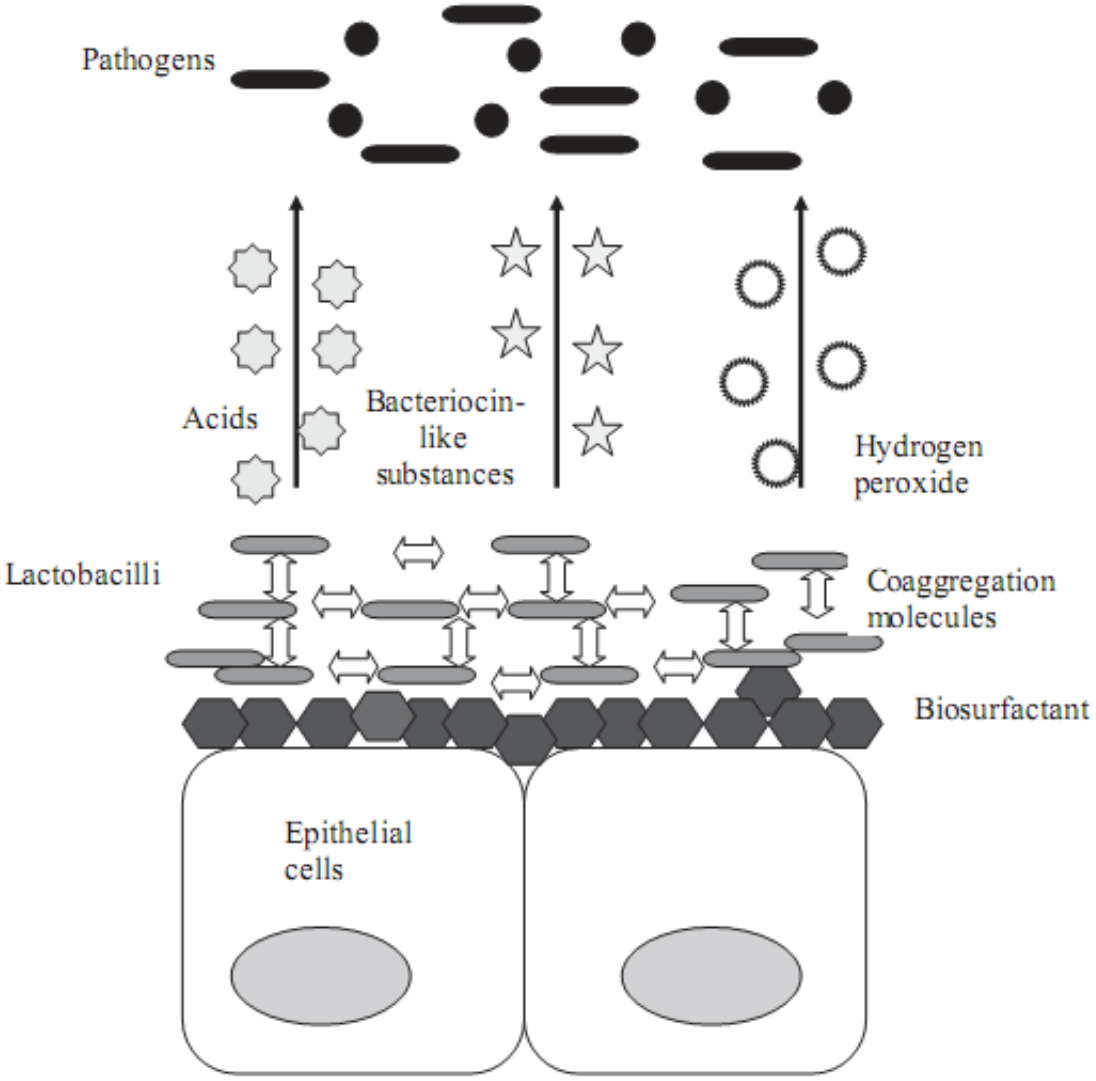

Figure 1.3 Competition of normal flora and pathogens (Mastromarino *et al*., 2002)

1.3 Bacterial gastroenteritis

1.3.1 Pathogenesis

Bacteria cause gastroenteritis by one of three principal processes shown in Table 1.1 (Edgeworth, 2005).

Production of preformed toxins

These usually induce vomiting and abdominal cramps within a few hours.

Secretion of toxins after adhering to intestinal epithelium

Non-invasive bacteria that secrete toxins usually cause a syndrome of watery diarrhea without fever, and stool samples do not contain blood or mucus.

Invasion of the intestinal mucosa causes either dysentry or enteric fever.

Dysentry is an invasive disease of the colon and terminal ileum causing fever, lower abdominal pain, tenesmus and passage of small-volume stools containing blood, mucus and pus.

Enteric fever is characterized by variable episodes of diarrhoea associated with fever and bacteraemia.

Table 1.1 Major bacterial causes of gastroenteritis and the most commonly associated clinical syndromes

Intoxication	Watery diarrhoea	Dysentery	Enteric fever
Staphylococcus aureus	*Vibrio cholerae*	*Shigella* spp.	*Salmonella* Typhi
Bacillus cereus	*Salmonella* spp.	Entero-invasive *Escherichia coli*	*Salmonella* Paratyphi
Clostridium perfringens	Enterotoxigenic *Escherichia coli*	Enterohaemorrhagic *Escherichia coli*	
	Enteropathogenic *Escherichia coli*	*Campylobacter*	
	Clostridium perfringens	*Yersinia*	
	Listeria monocytogenes		
	Bacillus cereus		

1.3.2 Diagnosis

It is important to send a stool sample to the laboratory from all patients with moderate-to-severe diarrhoea, particularly when the symptoms suggest invasive disease. Pathogens in stool samples are identified by culture on agar, which usually takes 2 days; antibiotic sensitivities are available the following day.

1.3.3 Management

Supportive therapy is the mainstay of management of severe gastroenteritis. The aim is to prevent dehydration, renal failure and metabolic derangement. Ciprofloxacin is the antibiotic of choice in adults (Dryden *et al*., 1996).

1.3.4 Lactobacilli as good probiotics for prevent gastrointestinal infections

In general, many mechanisms have been suggested by which Lactobacilli probiotics prevent the detrimental effect of gastrointestinal pathogens including competition for limited nutrients, inhibition of epithelial and mucosal adherence of pathogens, inhibition of epithelial invasion by pathogens, production of antimicrobial substances and/or the stimulation of mucosal immunity (Vasiljevic and Shah, 2008). Lactobacilli probiotic produce a wide range of antibacterial compounds including organic acids (e.g. lactic acid and acetic acid), H_2O_2, bacteriocins. Lactic and acetic acids are the main organic acids produced during the growth of probiotics and their pH lowering effect in the gastrointestinal tract has a bacteriocidal or bacteriostatic effect. Low molecular mass compounds such as lactic acid have been reported to be inhibitory towards Gram-negative pathogenic bacteria (Alakomi *et al*., 2000). Moreover, a heat-stable, low molecular weight antibacterial substance different from LAB was present in the cell-free culture supernatant resulting in the inactivation of a wide range of Gram-negative bacteria and inhibition of the adhesion to and invasion of Caco-2 cells by *Salmonella enteric* ser. Typhimurium (Coconnier *et al*., 2000; Liévin-LeMoal *et al*., 2002). Also, probiotics like many other LAB can produce

various bacteriocins. Recently, Corr *et al*. (2007) showed that *L. salivarius* produced bacteriocin Abp118 against *Listeria monocytogenes*.

1.4 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).

1.4.1 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.

1.4.2 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 1.4), whereas in BV there are large numbers of Gram-negative cocci and small rods (Figure 1.5).

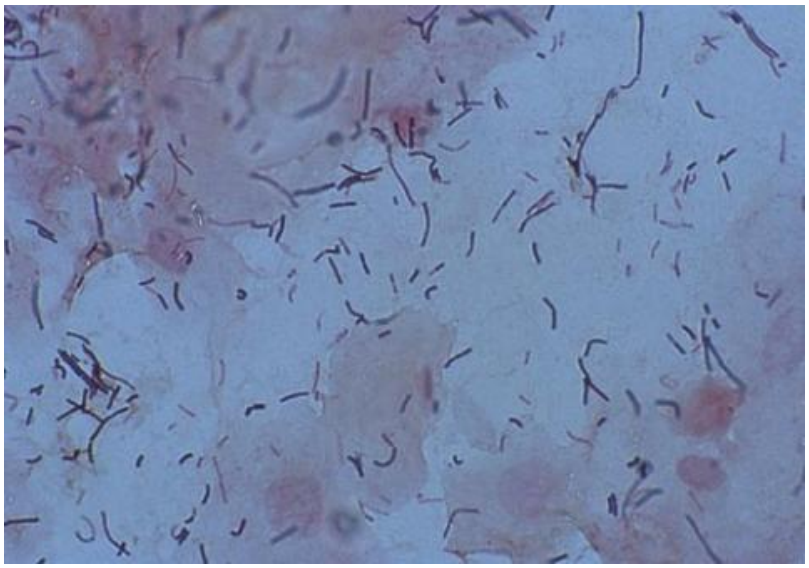

Figure 1.4 Gram-stained vaginal smear from a woman with normal flora (Hay, 2005).

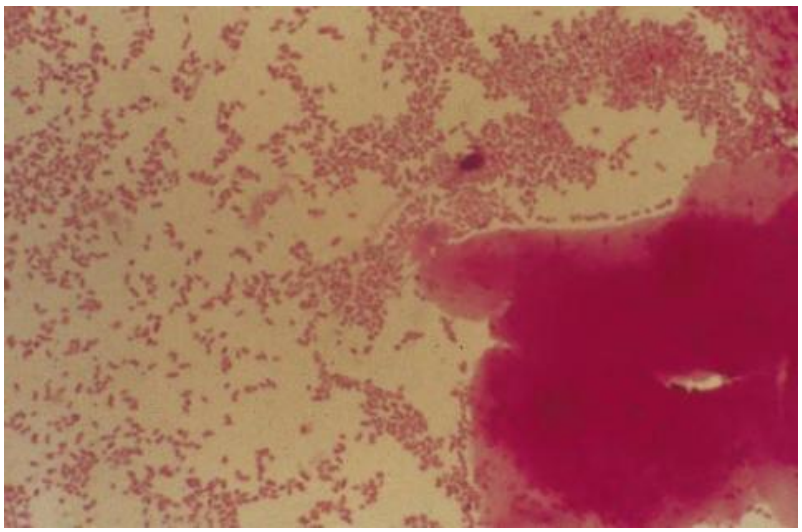

Figure 1.5 Gram-stained vaginal smear from a woman with BV (Hay, 2005)

1.4.3 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.

1.4.4 Lactobacilli as good probiotics for prevent vaginal infections

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 1.6 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.

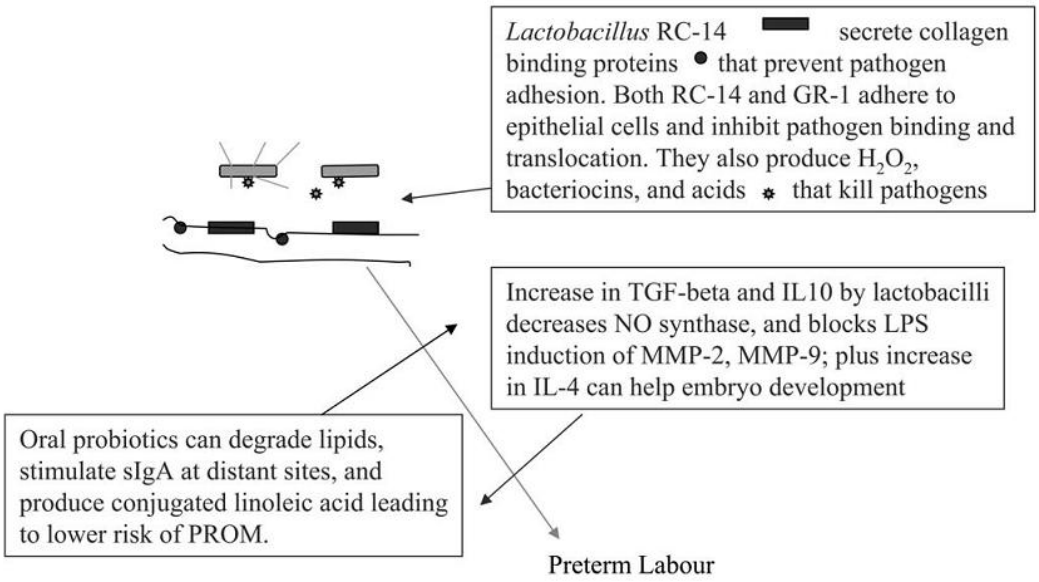

Figure 1.6 Potential pathways in which lactobacilli could reduce the risk of preterm labor (Reid and Bocking, 2003).

1.5 Viability of Lactobacilli probiotics

1.5.1 Low survival rate of probiotic cells after lyophilization

Lyophilization is a process extensively used for preservation and long-term storage of biological samples. However, during this process the cells experience extreme environmental conditions such as low temperature and low water activity that produce structural and physiological injury to the bacterial cells resulting in the loss of viability of many species (Carvalho *et al*., 2002). Damage to biological systems resulting from lyophilization can be attributed primarily to changes in the physical

state of membrane lipids or to changes in the structure of sensitive proteins. Lyophilization therefore decreases the viability of probiotic cells (Higl *et al*., 2007; Zayed and Roos, 2004).

1.5.2 Enhancing the viability of probiotic cells

1.5.2.1 Cryoprotectant

The important role of probiotics as starters in the dairy and pharmaceutical products highlights the requirement of appropriate processes for their preservation. Lyophilization has been the method of choice for the long-term storage of stable cultures in terms of viability and functional activity (Carvalho *et al*., 2003c; Morgan *et al*., 2006; Meng *et al*., 2008). However, during this process the number of viable bacteria is dramatically reduced due to the decrease in water activity, loss of water being responsible for the cell damage. Membranes, nucleic acids and certain enzymes have been identified as the cellular targets for this damage (Carvalho *et al*., 2004a). To avoid these damages, the cryoprotectants have been used. The cryoprotectant is a compound that is added to prevent or reduce cell death during lyophilization and subsequent storage. Several compounds have been examined as protective agents during lyophilization (Abadias *et al*., 2001; Desmond *et al*., 2002; Oldenhof *et al*., 2005).

Skim milk powder is selected as cryoprotectant agent for lactobacilli probiotics because it prevents cellular injury by stabilizing the cell membrane constituents (Castro *et al*., 1996; Selmer-Olsen *et al*., 1999), creates a porous structure in the lyophilized product that makes rehydration easier and contains proteins that

provide a protective coating for the cells (Abadias *et al*., 2001). The cryoprotectant agents are differently permeable to the cells, which in turn affect the mechanism of their protective effect (Carvalho *et al*., 2004b). Three categories may be identified including penetration of both the cell wall and the cytoplasmatic membrane (e.g. dimethyl sulfoxide and glycerol), penetration of the cell wall, but not the membrane (e.g. oligosaccharides, amino acids and low molecular weight polymers) and no penetration of the cell wall, or no direct interaction with the cell wall or membrane (e.g. polymers with high molecular weight, such as proteins and polysaccharides).

1.5.2.2 Prebiotics used as cryoprotectant

Prebitics are defined as non-digestible food ingredients that affect the host beneficially by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health (Gibson and Roberfroid, 1995). In previous studies, various prebiotics were also used as protective agents. The addition of the prebiotics fructooligosaccharides (FOS), galactooligosaccharide (GOS), inulin and polydextrose also enhanced survival of lactobacilli during drying process. Schwab *et al*., (2007) investigated the influence of the addition of sucrose, FOS, inulin and skim milk on the viability and membrane integrity of *Lactobacillus reuteri* TMW1.106 during freezing, freeze-drying and storage. The result demonstrated that sucrose, FOS and skim milk significantly enhanced survival of exponential-phase cells of *L. reuteri* during freeze-drying. The reconstituted skim milk with prebiotic substances, i.e. Raftilose® P95 (inulin) and Polydextrose as a spray drying carrier also resulted in a high level of survival of *Lactobacillus rhamnosus* GG (ATCC 53103) (Ananta *et al*., 2005). Moreover, the

supplementation of probiotic *Lactobacillus delbrueckii* subsp. *bulgaricus* with GOS allows the production of self-protected synbiotic products, GOS exerting both a prebiotic and protecting effect. (Tymczyszyn *et al*., 2011).

1.6 Enteric coated capsule

Delivery of drugs through oral admission to the colon has many important applications in the field of pharmacotherapy. This may be achieved by different approaches including the use of pro-drugs, the time–clock system, pH sensitive coating and biodegradable matrices. Among these approaches, pH sensitive coating has been widely used. This approach involves making drug tablets, pellets or capsules coated with a pH sensitive material. The pH sensitive material is insoluble or almost impermeable in dissolution liquids of low pH but can dissolve in those with pH ranging from 5 to 7 (Chan *et al*., 2001).

Methylacrylic acid–methylmethacrylate copolymers, which are also known as Eudragit, have been used as a pH sensitive coating material to protect drug substances prior to delivery to the human intestines. Eudragit® L100 are anionic copolymer based on methylacrylic acid and methylmethacrylate, dissolve at pH higher than 6. This makes it a suitable coating material for the colonic drug delivery.

GI tract resistant capsules are commonly used for several purposes. Among these is the protection of the active substance from being destroyed by the gastric contents - either enzymes or highly acidic gastric fluids (Pina *et al*., 1996).

To be effective, orally administered probiotics should be efficiently implanted in the intestine and adhesion to the intestinal mucosa is considered one of

the beneficial health effects of probiotics. This requires that the cells survive during the preparation of dosage forms and passage through the acidic environment. Reaching the intestine, these microorganisms should be able to establish themselves, remain viable and perform their beneficial actions. In this context, oral formulations have to protect the bioactive agent from the gastric acidity and to deliver it to the intestinal site (Calinescu and Mateescu, 2008).

1.7 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 1.7.

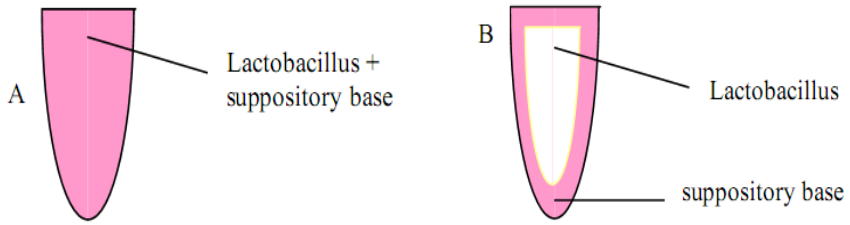

Figure 1.7 Schematic illustration of conventional suppository (A) and hollow-type suppository (B)

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 and 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 use 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 bases include no laxative effect, microbial contamination less likely, preparation us 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 do not melt in the body but dissolve and disperse the medication slowly, providing a sustained effect, produce high-viscosity solutions that means after dispersing in the body, leakage is less likely and give products with clean smooth appearance.

1.8 Objectives

1.8.1 To characterize probiotic properties of *Lactobacillus fermentum* SK5 including antimicrobial activity against gastrointestinal and bacterial vaginosis pathogens, production of hydrogen peroxide and a high molecular weight inhibitory substance, antibiotic susceptibility, survival under conditions that simulated the human gastrointestinal tract and vagina, aggregation properties, adhesion ability to epithelial cells and inhibition of pathogens adhesion.

1.8.2 To examine effect of prebiotics on the growth of *L. fermentum* SK5, gastrointestinal and bacterial vaginosis pathogens.

1.8.3 To select cryoprotectant for preparation of *L. fermentum* SK5 lyophilized powder.

1.8.4 To formulate and evaluate enteric coated capsule and hollow-type vaginal suppository containing *L. fermentum* SK5.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions

2.1.1 *Lactobacillus* strain

Lactobacillus 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 anaerobic jar containing gas pak microbiology anaerocult® A (Merck, Germany) at 37 °C for 48 h.

2.1.2 Gastrointestinal and bacterial vaginosis pathogens

Gastrointestinal pathogens, i.e. *Escherichia coli, Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Shigella sonnei* and *Vibrio cholerae* were

obtained from Songklanagarind hospital, Thailand. These pathogens were cultured in Brain Heart Infusion (BHI) broth (Difco, USA) at 37 °C for 24 h.

Gardnerella vaginalis was obtained from Songklanagarind hospital, Thailand. *Bacteroides fragilis* DMST 19152, *Bacteroides vulgatus* DMST 15535 were purchased from Department of Medical Sciences, Ministry of Public Health, Thailand. *Bacteroides ureolyticus* ATCC 33387, *Mobiluncus curtisii* ATCC 35241 and *Prevotella bivia* ATCC 29303 were purchased from the American Type Culture Collection, USA. All of bacterial vaginosis pathogens were cultured in BHI broth supplemented with 0.5% (w/v) yeast extract (Merck, Germany) and 15 μg/ml hemin (Sigma-Aldrich, USA). The incubation of these strain was in anaerobic jar containing gas pak microbiology anaerocult® A at 37 °C for 48 h.

2.2 Probiotic properties of *L. fermentum* SK5

2.2.1 Antimicrobial activity of *L. fermentum* SK5 against gastrointestinal and bacterial vaginosis pathogens

A spot-on-lawn method was used to determine the antimicrobial activity of *L. fermentum* SK5 against gastrointestinal and bacterial vaginosis pathogens. *L. fermentum* SK5 was spotted (5 μl) on the surface of MRS agar in a 10 cm plates and incubated anaerobically at 37 °C for 48 h. One ml of a 48 h of pathogen cultures (ca. 1×10^8 cfu/ml) was mixed with 100 ml of BHI soft agar (1% (w/v) agar, for gastrointestinal pathogens) and BHI soft agar supplemented with 0.5%

(w/v) yeast extract and 15 μg/ml hemin (for bacterial vaginosis pathogens). Four ml of the mixtures were poured into the plate to cover the lactobacilli spots and incubated in culture condition. After incubation, inhibition zones were measured. Each test was performed in quadruplet.

2.2.2 Detection of hydrogen peroxide production

L. fermentum SK5 was tested for its ability to produce H_2O_2 by the qualitative method. Plates of MRS agar contained 5 mg/ml hemin, 1 mg/ml vitamin K, 0.01 mg/ml horseradish peroxidase (Sigma-Aldrich, USA) and 0.05 mg/ml 3,3´,5,5´-tetramethylbenzidine (Sigma-Aldrich, USA) was spotted with a loop of the culture and incubated anaerobically at 37 °C for 48 h. After incubation, the plates were exposed to air and observed for blue or brown colonies indicated H_2O_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 H_2O_2 production.

2.2.3 Detection of a high molecular weight inhibitory substance produced by *L. fermentum* SK5

To detect a high molecular weight inhibitory substance, MRS cultured broth of *L. fermentum* SK5 (3 days) was centrifuged at 2,000 × g at 4 °C for 30 min. Cell-free supernatant was dialyzed across dialysis tube with 10 kDa 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 organic acids and H_2O_2. The buffer was changed every 6 h until 24 h. The dialyzed supernatant was lyophilized by freezing at -40 °C for 2 h followed by 18 h of primary drying at -40 °C, 8 h of secondary drying at -10 °C and the final step to 25 °C using a freeze-dryer (model FD-300 Airvac Engineering Pty Ltd., Dandenong, Australia) and its antimicrobial activity was examined by disc diffusion test. The lyophilized powder was resuspened with 20 mM sodium acetate buffer pH 5.0 to make concentration of 1 mg/ml and 50 µl of dialyzed culture fluid was gradually applied onto 6 mm diameter paper disc and placed to dry in air before use. Cultured broth of gastrointestinal and bacterial vaginosis pathogens was adjusted with normal saline to give turbidity equal to 0.5 McFarland standard (10^8 cfu/ml). The plates of BHI agar and BHI agar supplemented with 0.5% (w/v) yeast extract and 15 µg/ml hemin were inoculated by streaking the swab of gastrointestinal and bacterial vaginosis pathogens over the surface, respectively. The discs containing dialyzed culture fluid were placed on the agar surface and incubated in cultured condition. The inhibition zone diameter was measured. Norfloxacin (10 µg) and metronidazole (5 µg) antibiotic discs were tested against gastrointestinal and bacterial vaginosis pathogens, respectively.

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 was washed once with phosphate buffered saline (PBS, Sigma-Aldrich, USA), pH 7.4 and the cell pellet was resuspended in 1 ml high molecular weight inhibitory substance. The cells were incubated for 18 h at 37 °C, harvested (2,000 × 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 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).

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 mg/ml) and incubated for 3 h at 37 °C. In other tests, the dialyzed culture fluid was heated in a water bath at 60, 80, 100 and 121 °C (autoclave) for 10 min, followed by cooling at 4 °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.

2.2.4 Antibiotic susceptibility assay

The antibiotic susceptibility of *L. fermentum* SK5 was determined by the disc diffusion method. 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.

2.2.5 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 (Maragkoudakis *et al*., 2006). Bacterial cells from an 18-h culture were harvested by centrifugation at 2,000 × g for 5 min at 4 °C, washed once with PBS, pH 7.4 before being resuspended (10^8 cfu/ml) 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. 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. 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

2.2.6 Survival under conditions that simulated the human vagina

Simulated vaginal fluid (SVF) was prepared by dissolving 68.04 g of sodium acetate (Ajax Finechem Pty Ltd, Australia) in sterile distilled water 1000 ml and the result solution was adjusted with glacial acetic acid to pH 4.4. Bacterial cells from an 18-h *L. fermentum* SK5 culture were harvested by centrifugation at 2,000 × g for 5 min at 4 °C, washed once with PBS, pH 7.4 and resuspended (10^8 cfu/ml) in SVF, pH 4.4. After incubation in anaerobic condition at 37 °C, viable cells were

enumerated at 0, 1, 2 and 3 h. Survival rate was calculated as described in section 2.2.5.

2.2.7 Autoaggregation test

The autoaggregation ability of *L. fermentum* SK5 was determined as previously described (Pascual *et al*., 2008). Bacterial cells were grown in MRS broth at 37 °C for 18 h in an anaerobic condition. Bacterial cells were resuspended in PBS, pH 6.2 to a final concentration of 10^8 cfu/ml. 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.

2.2.8 Surface hydrophobicity

The surface hydrophobicity of the bacterial cells was determined by the salt aggregation test, SAT (Pascual *et al*., 2008). *L. fermentum* SK5 cells were resuspended in 0.02 mol/l of sodium phosphate (Merck, Germany), pH 6.8 to a final concentration of 10^9 cfu/ml. Solutions of ammonium sulfate (Merck, Germany) at 4.0, 2.0, 1.5 and 0.5 mol/l 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 < 0.5 mol/l, 0.5 - 1.5 mol/l and > 1.5 mol/l, respectively.

2.2.9 Coaggregation assay

L. fermentum SK5 was tested for its ability to coaggregate with the pathogens (Pascual *et al*., 2008). 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.

2.2.10 Adhesion of *L. fermentum* SK5 on HeLa, HT-29 and Caco-2 cells

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. 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 of penicillin G and 100 μg/ml 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 of penicillin G and 100 μg/ml streptomycin sulfate. All cultured cells were incubated under 5% (v/v) CO_2 at 37 °C. Cells were seeded at a concentration of 4.5 x 10^5 cells/well in 24-well tissue culture plates. HeLa and HT-29 cells were cultured for 7 days to reach confluence and used in the adhesion assay. Caco-2 cells were

maintained for 21 days to allow for good differentiation and used in the 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 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_2 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_1: Amount of adherent bacterial cells

N_0: Amount of added bacterial cells

2.2.11 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. Bacterial cell pellets of *E. coli*, *G. vaginalis* and *L. fermentum* SK5 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 with DMEM or MEM, for the adhesion assay.

In the competition assay, *L. fermentum* SK5 was added simultaneously with the pathogens (Gagnon *et al*., 2004). 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 (Lee *et al*., 2000). Adherent *E. coli* and *G. vaginalis* were enumerated by plate counting on McConkey agar (Difco, USA) and BHI agar supplemented with 0.5% (w/v) yeast extract and 15 μg/ml hemin, respectively.

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 section 2.2.3.

2.3 Effect of inulin, galactooligosaccharide and skim milk on the growth of *L. fermentum* SK5, gastrointestinal and bacterial vaginosis pathogens

Inulin, galactooligosaccharide (GOS) and skim milk were tested for their effect on the growth of *Lactobacillus* and pathogens. *L. fermentum* SK5 (1% (v/v)) was inoculated into a quarter strength MRS broth supplemented with inulin, GOS or skim milk at the concentration of 1, 2 and 4% (w/v). Gastrointestinal pathogens were added into a quarter strength BHI broth supplemented with inulin, GOS or skim milk at the concentration of 1, 2 and 4% (w/v). Bacterial vaginosis pathogens were added into a quarter strength BHI broth supplemented with 0.5%

(w/v) yeast extract, 15 μg/ml hemin that also contained inulin, GOS or skim milk at the same concentration. The incubation was carried out under cultured condition for 24 h. The bacterial growth was assessed by measuring the optical density at 580 nm at various time intervals.

2.4 Selection of cryoprotectant for preparation of *L. fermentum* SK5 lyophilized powder

To prepare *L. fermentum* SK5 in lyophilized form, cryoprotectants were used to protect bacterial cells during lyophilization process. Inulin, GOS and skim milk were used in this experiment. *L. fermentum* SK5 cultured broth (48 h) was centrifuged at 2,000 × g at 4 °C for 10 min. Bacterial cell pellet was resuspended in inulin, GOS and skim milk at the concentration of 2, 4, 6, 8 and 10% (w/v). These suspensions were lyophilized and viable cells were enumerated before and after lyophilization.

2.5 Formulation and evaluation of *L. fermentum* SK5 enteric coated capsule and hollow-type vaginal suppository

2.5.1 Production of *L. fermentum* SK5 lyophilized cells

To incorporate *L. fermentum* SK5 into enteric coated capsules or hollow-type vaginal suppositories, it should be first prepared as a lyophilized cell powder. The lactobacillus was cultured and the cell pellet was resuspended in

selected cryoprotectant solution that gave the highest survival rate of bacteria and showed good prebiotic property. This cell suspension was then lyophilized.

2.5.2 Preparation of *L. fermentum* SK5 enteric coated capsule

One hundred milligrams of *L. fermentum* SK5 lyophilized powder was filled into the hard gelatin capsule size No.1 using capsule filling machine. The capsule was coated with Eudragit® L100 solution (15% w/v Eudragit® L100, 5% v/v propylene glycol, 3% v/v sorbitan monooleate, 45% v/v ethyl alcohol and 32% v/v acetone) using spray bottle. The spray rate was 3 ml/min with the solution of 50 ml/500 capsules and the coating process was done using conventional coating pan.

2.5.3 Evaluation of *L. fermentum* SK5 enteric coated capsule

Uniformity of weight

Uniformity of weight determination was performed based on British Pharmacopoeia (BP). Twenty enteric coated capsules were weighed individually and then the average weight was calculated.

In vitro release study

In vitro release of *L. fermentum* SK5 from enteric coated capsule was tested by using dissolution apparatus with rotating paddle. One hundred capsules was placed in the vessel containing SGF, pH 1.2 maintained at 37 °C as the dissolution medium for 2 h and the speed used was 50 rpm. After this time, the capsules were

immediately immersed in SIF, pH 7.4 for 2 h. At interval time, the amount of *L. fermentum* SK5 was enumerated on MRS agar.

Inhibitory activity of enteric coated capsules against gastrointestinal pathogens

Co-culture method was used to examine the inhibitory activity of enteric coated capsules against gastrointestinal pathogens. *E. coli* or *V. cholerae* at concentration of 10^4 and 10^6 cfu/ml was added in 20×150 mm test tubes containing 20 ml of BHI broth. The enteric coated capsule was then added in these tubes and incubated at 37 °C. The number of bacterial cells was enumerated at interval time until 24 h.

Stability

Enteric coated capsules were kept in a sterile plastic wear at 4 °C. The texture of capsules and viability of *L. fermentum* SK5 were determined every month until 6 months.

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

The vaginal suppository were 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 (Kaewsrichan *et al*., 2007). The suppository base was melted in boiling water bath. After melting, the base was cooled down to the temperature approximately 45 °C. It 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 (100 mg) was added into the suppository cavity. The open end of the suppository was sealed with melted base.

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

Uniformity of weight

The test for the uniformity of weight determination was performed based on BP. Twenty vaginal suppositories were weighed individually and then the average weight was calculated.

Melting point measurement of suppository base

The thermal properties of pulverized PEGs base (the mixture 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 a nitrogen gas purge.

In vitro release study

In vitro release of *L. fermentum* SK5 from the vaginal suppository was studied in a 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.

Inhibitory activity of vaginal suppository against bacterial vaginosis pathogens

The inhibitory activity of the vaginal suppositories was examined by the co-culture method. Twenty ml of medium in 20×150 mm test tubes containing *G. vaginalis* or *B. vulgatus* DMST 15535 at concentrations of 10^4 and 10^6 cfu/ml were added with suppository and incubated under anaerobic condition at 37 °C. In the first hour of incubation, the mixture was shaken at 50 rpm to enhance the melting of the suppository base. The amount of bacterial cells was enumerated at intervals of time until 48 h.

Stability

Vaginal suppositories were wrapped in aluminium foil and kept in a sterile plastic bag at 4 °C. The appearance of suppository and viability of *L. fermentum* SK5 were determined every month until 6 months.

CHAPTER 3

RESULTS

3.1 Probiotic properties of *Lactobacillus fermentum* SK5

3.1.1 Antimicrobial activity of *L. fermentum* SK5 against gastrointestinal and bacterial vaginosis pathogens

L. fermentum SK5 produced antimicrobial substances to inhibit gastrointestinal pathogens, i.e. *E. coli*, *P. aeruginosa*, *S.* Typhimurium, *S. sonnei*, *V. cholerae* and bacterial vaginosis pathogens, i.e. *G. vaginalis*, *B. fragilis* DMST 19152, *B. vulgatus* DMST 15535, *B. ureolyticus* ATCC 33387, *M. curtisii* ATCC 35241 and *P. bivia* ATCC 29303 (Table 3.1).

3.1.2 Detection of hydrogen peroxide production

L. fermentum SK5 produced an intense blue color. According to the previous criteria, it was classified as a strong H_2O_2 producer.

Table 3.1 Antimicrobial activity of *L. fermentum* SK5 against gastrointestinal and bacterial vaginosis pathogens using spot-on-lawn method

Pathogens	Inhibition zone (mm) ± SD
E. coli	32.75 ± 2.22
P. aeruginosa	30.50 ± 1.00
S. Typhimurium	29.50 ± 1.91
S. sonnei	30.00 ± 0
V. cholerae	30.50 ± 1.91
G. vaginalis	17.05 ± 0.10
B. fragilis DMST 19152	21.80 ± 0.54
B. vulgatus DMST 15535	29.50 ± 0.58
B. ureolyticus ATCC 33387	27.15 ± 0.19
M. curtisii ATCC 35241	35.10 ± 0.12
P. bivia ATCC 29303	32.25 ± 0.50

3.1.3 Detection of a high molecular weight inhibitory substance produced by *L. fermentum* SK5

After dialysis to eliminate low molecular weight inhibitory substances such as lactic acid, short chain fatty acids and H_2O_2, the dialyzed culture fluid from *L.*

fermentum SK5 inhibited *E. coli* and *V. cholerae* with inhibition zones of 13.50 ± 0.71 and 17.00 ± 1.15 mm, respectively (Table 3.2). All of tested gastrointestinal pathogens were also sensitive to norfloxacin. Moreover, the dialyzed culture fluid showed inhibitory activity against *G. vaginalis* and *B. vulgatus* DMST 15535 with inhibition zones of 9.75 ± 0.50 and 21.00 ± 1.15 mm, respectively (Table 3.2). The result of antibiotic sensitivity test showed that *G. vaginalis*, *B. fragilis* DMST 19152 and *B. vulgatus* DMST 15535 were sensitive to metronidazole, but *B. ureolyticus* ATCC 33387, *M. curtisii* ATCC 35241 and *P. bivia* ATCC 29303 were resistant.

SEM images of treated pathogen bacterial cells indicated that *E. coli* and *G. vaginalis* cells were lysed and had shrunk (Figure 3.1). 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 mg/ml) the diameter of the inhibition zone was slightly reduced (Table 3.3). 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 3.3). This indicated that some of the inhibitory activities could be associated with a protein-like compound, perhaps a bacteriocin. This bacteriocin was active at pH values between 2 and 7. At a pH value of 8 and 9, no inhibition was observed (Table 3.3).

Table 3.2 Antimicrobial activity of a high molecular weight inhibitory substance produced by *L. fermentum* SK5 against gastrointestinal and bacterial vaginosis pathogens

Pathogens	Inhibition zone (mm) ± SD		
	High molecular weight substance > 10 kDa (50 μg/disc)	Norfloxacin (10 μg/disc)	Metronidazole (5 μg/disc)
E. coli	13.50 ± 0.71	38.00 ± 0	ND
P. aeruginosa	-	38.00 ± 0	ND
S. Typhimurium	-	23.00 ± 1.15	ND
S. sonnei	-	30.00 ± 0	ND
V. cholerae	17.00 ± 1.15	32.00 ± 0	ND
G. vaginalis	9.75 ± 0.50	ND	47.50 ± 2.89
B. fragilis DMST 19152	-	ND	57.50 ± 2.89
B. vulgatus DMST 15535	21.00 ± 1.15	ND	57.50 ± 2.89
B. ureolyticus ATCC 33387	-	ND	-
M. curtisii ATCC 35241	-	ND	-
P. bivia ATCC 29303	-	ND	-

(-): No inhibition, ND: Not determine

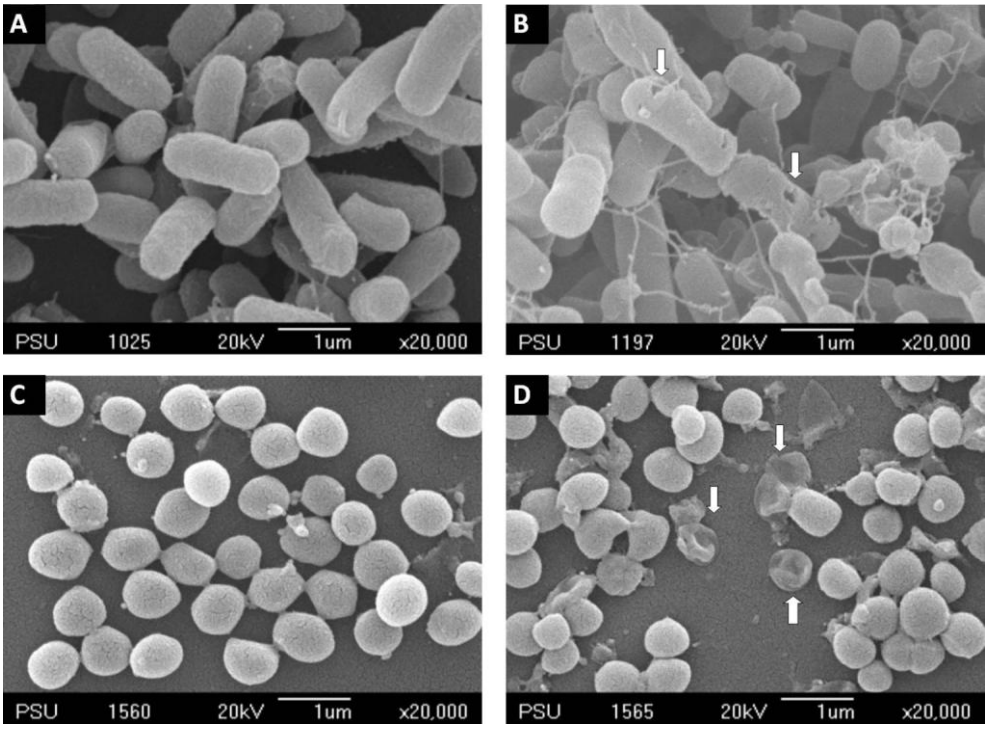

Figure 3.1 Examination by scanning electron microscopy (SEM) of *E. coli* and *G. vaginalis*. *E. coli* (A) and *G. vaginalis* (C) treated with phosphate buffer saline, pH 7.4. *E. coli* (B) and *G. vaginalis* (D) treated with dialyzed culture fluid from *L. fermentum* SK5 (Pointing arrows indicated the lysed bacterial cells)

Table 3.3 Effect of trypsin, heat and pH on the antibacterial activity of a dialyzed culture fluid from *L. fermentum* SK5

Treatment		Inhibition zone (mm) ± SD	
		E. coli	*G. vaginalis*
Dialyzed culture fluid (untreated, pH 6.25)		13.50 ± 0.71	9.75 ± 0.50
Enzyme	Trypsin (1 mg/ml)	10.50 ± 0.58*	8.75 ± 0.50*
Heat (°C)	60	10.00 ± 0*	8.25 ± 0.50*
	80	9.50 ± 0.58*	8.00 ± 0*
	100	9.50 ± 1.00*	8.00 ± 0*
	121	9.00 ± 0*	8.00 ± 0*
pH	2	12.50 ± 0.58	8.25 ± 0.50*
	3	12.00 ± 0.82*	8.25 ± 0.58*
	4	10.75 ± 0.50*	8.25 ± 0.50*
	5	9.50 ± 0.58*	9.25 ± 0.50
	6	8.75 ± 0.96*	9.25 ± 0.50
	7	8.75 ± 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.

3.1.4 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 (cefoperazone 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, norfloxazin and vancomycin. It showed only resistance to metronidazole.

Table 3.4 Antibiotic sensitivity of *L. fermentum* SK5

Antibiotic disc	Inhibition zone (mm) ± SD (n=4)	Antibiotic resistant pattern
Ampicillin	43.10 ± 0.26	S
Bacitracin	35.20 ± 0.54	S
Cefoperazone	35.25 ± 0.87	S
Ceftazidime	32.10 ± 1.73	S
Cephalothin	39.95 ± 0.50	S
Chloramphenicol	35.05 ± 0.55	S
Clindamycin	24.80 ± 0.43	S
Doxycycline	35.45 ± 0.50	S
Erythromycin	40.10 ± 0.26	S
Gentamicin	40.20 ± 1.07	S
Kanamycin	30.45 ± 1.05	S
Metronidazole	-	R
Neomycin	32.90 ± 0.38	S
Norfloxacin	18.05 ± 0.30	S
Penicillin G	39.90 ± 0.26	S
Streptomycin	26.20 ± 0.28	S
Tetracycline	31.15 ± 0.34	S

3.1.5 Survival under conditions simulating the human GI tract

The acid tolerance profile of *L. fermentum* SK5 (Figure 3.2A) showed that after 1 h at every tested 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 (Figure 3.2B) 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 Figure 3.2C and 3.2D, 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.

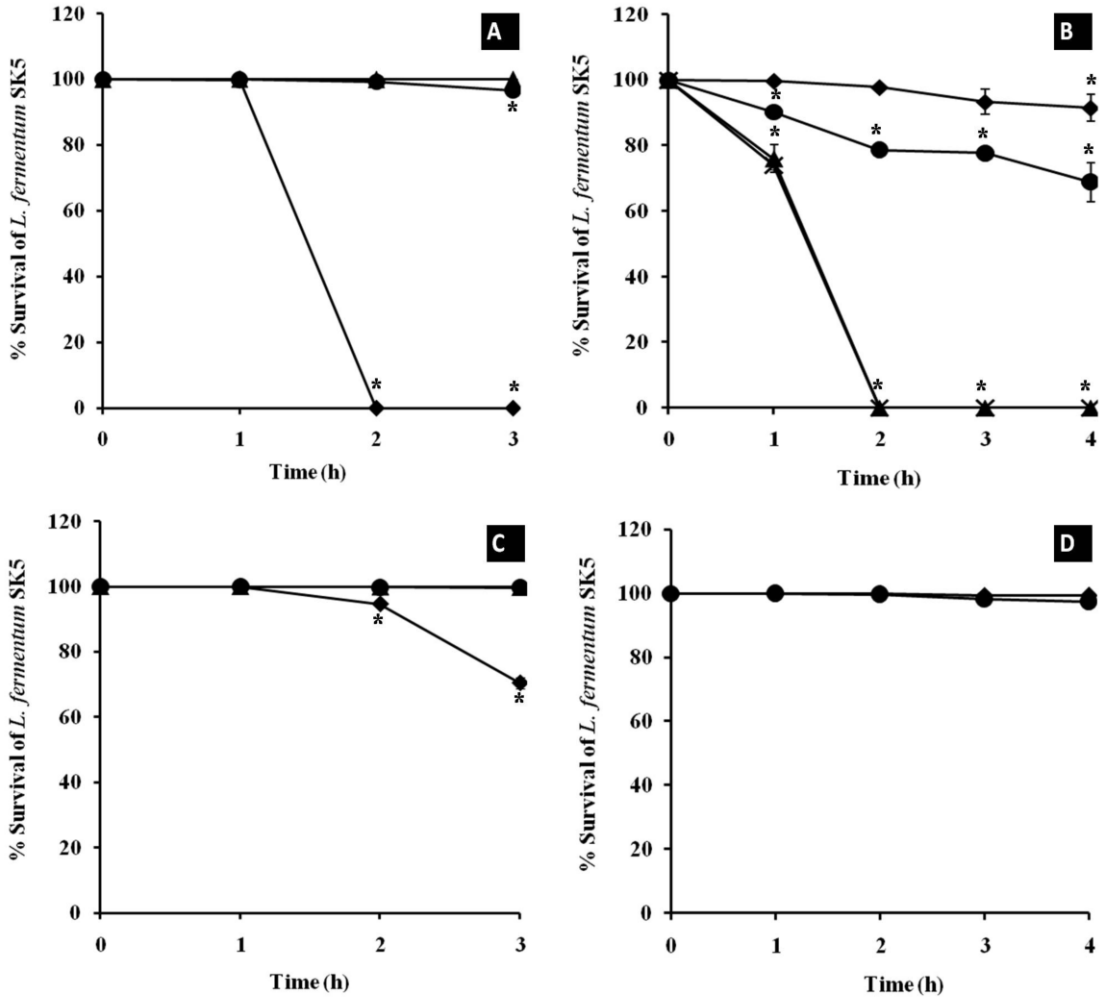

Figure 3.2 Survival of *L. fermentum* SK5 in simulated human GI tract conditions: the presence of acid (A): pH 2 (♦), pH 3 (●), pH 4 (▲), bile-containing buffer (B): 0.1% oxgall (♦), 0.2% oxgall (●), 0.3% oxgall (▲), 0.4% oxgall (×), simulated gastric fluid (C): SGF pH 2 (♦), SGF pH 3 (●), SGF pH 4 (▲), simulated intestinal fluid (D): SIF pH 6.8 (♦), SIF pH 8 (●). * = $p < 0.05$ (compared with before treatment)

3.1.6 Survival under conditions that simulated the human vagina

L. fermentum SK5 survived in SVF, pH 4.4. There still had more than 99% survival rate at examined condition (Figure 3.3).

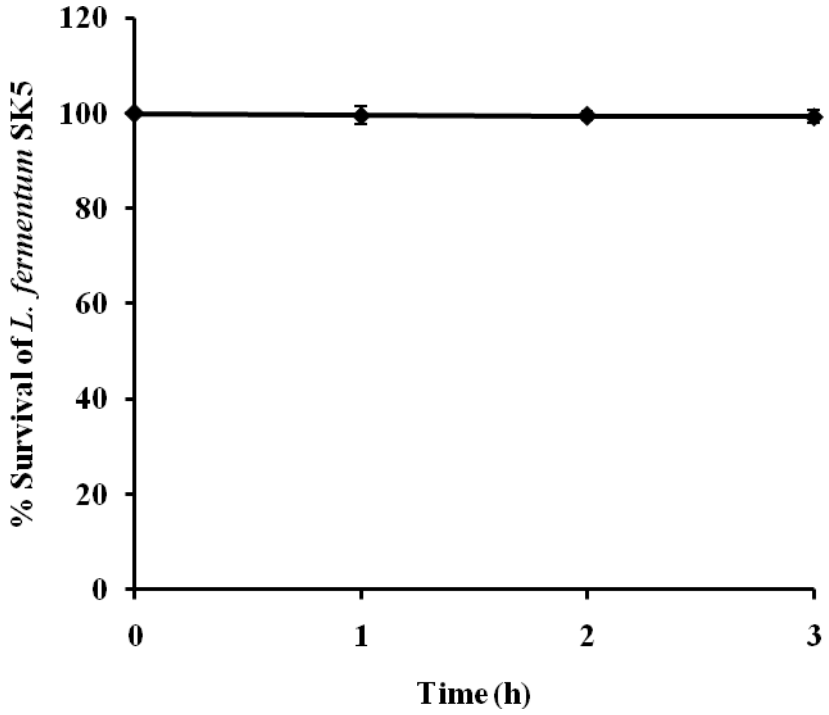

Figure 3.3 Survival of *L. fermentum* SK5 in simulated vaginal fluid (SVF)

3.1.7 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 3.4). It was also classified as having a high surface hydrophobicity with a SAT value < 0.9

mol/l (Figure 3.5). In mixed cultures, *L. fermentum* SK5 coaggregated with both *E. coli* and *G. vaginalis* (Figure 3.6).

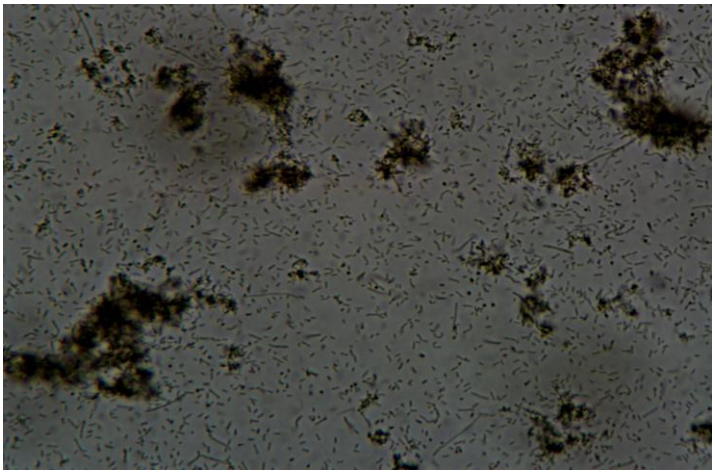

Figure 3.4 Microscopic observation of *L. fermentum* SK5 forming aggregates

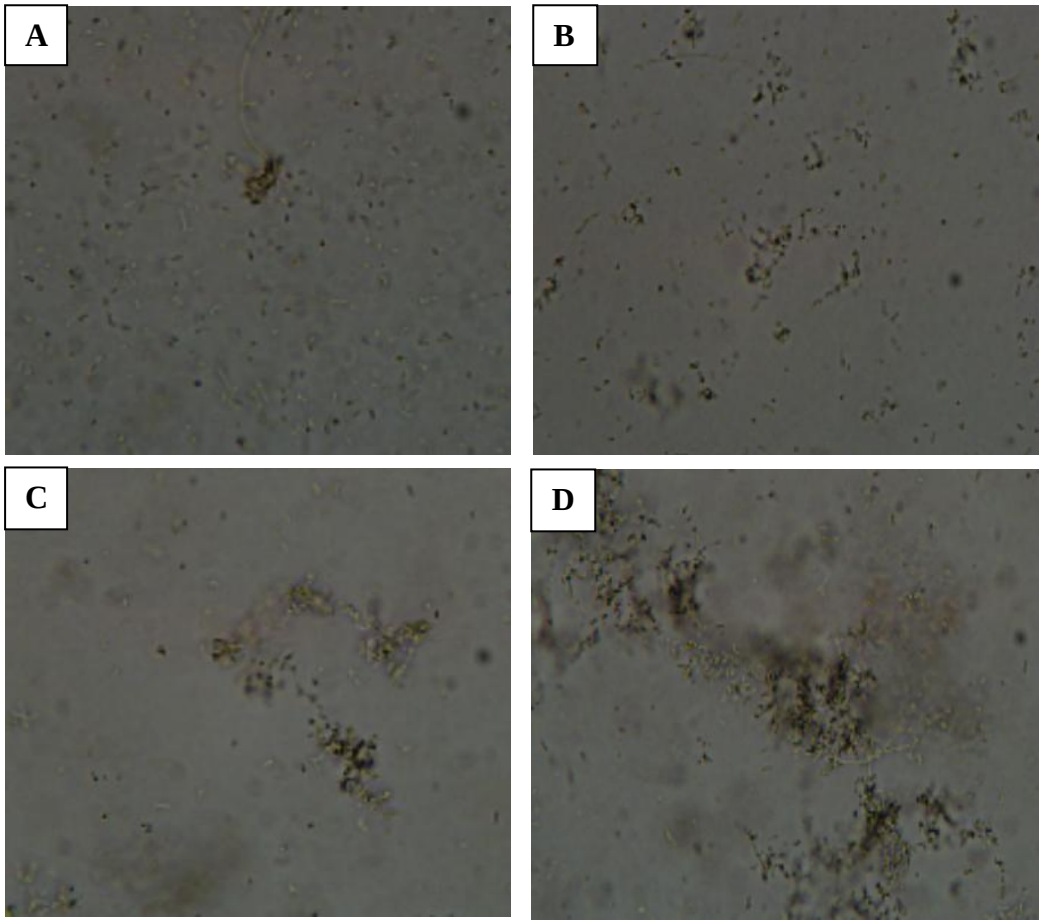

Figure 3.5 *L. fermentum* SK5 formed aggregates at 0.5 (A), 1.5 (B), 2.0 (C) and 4.0 (D) mol/l ammonium sulfate

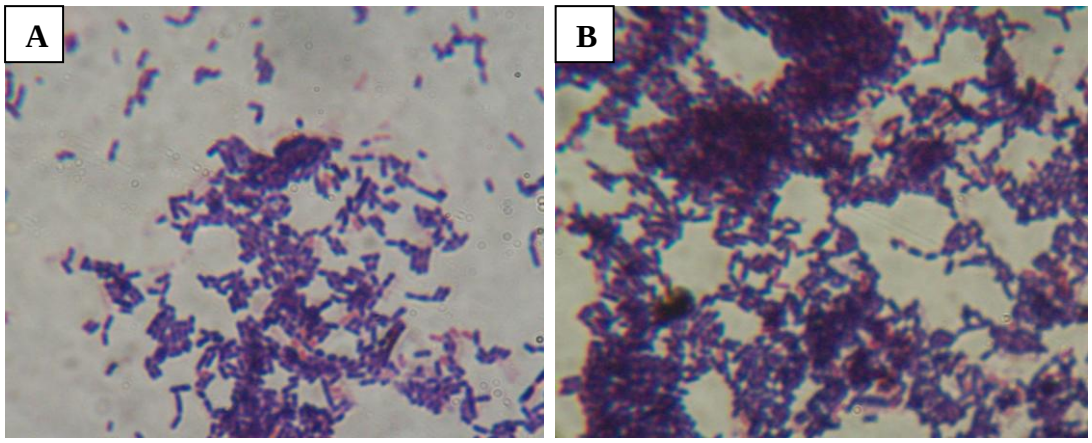

Figure 3.6 Microscopic observation of coaggregation between *L. fermentum* SK5 (violet color) and pathogens (red color), *E. coli* (A), *G. vaginalis* (B)

3.1.8 Adhesion of *L. fermentum* SK5 on 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 Figure 3.7. *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.

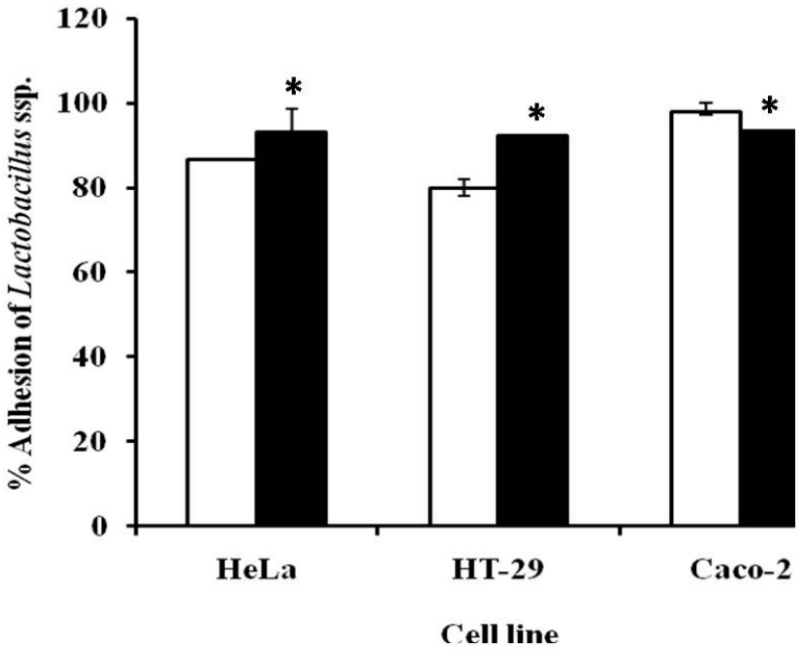

Figure 3.7 Adhesion of *L. fermentum* SK5 (■) to HeLa, HT-29 and Caco-2 cells. * = $p < 0.05$ (compared to binding of *L. rhamnosus* GG ATCC 53103 (□)).

3.1.9 Inhibition of pathogens 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 (Figure 3.8). The adhesion of *E. coli* to HeLa, HT-29 and Caco-2 cells was 86.23, 85.28 and 87.14%, respectively (Figure 3.9A), whereas the adhesion of *G. vaginalis* to HeLa, HT-29 and Caco-2 cells was 76.82, 90.77 and 79.28%, respectively (Figure 3.9B). *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.

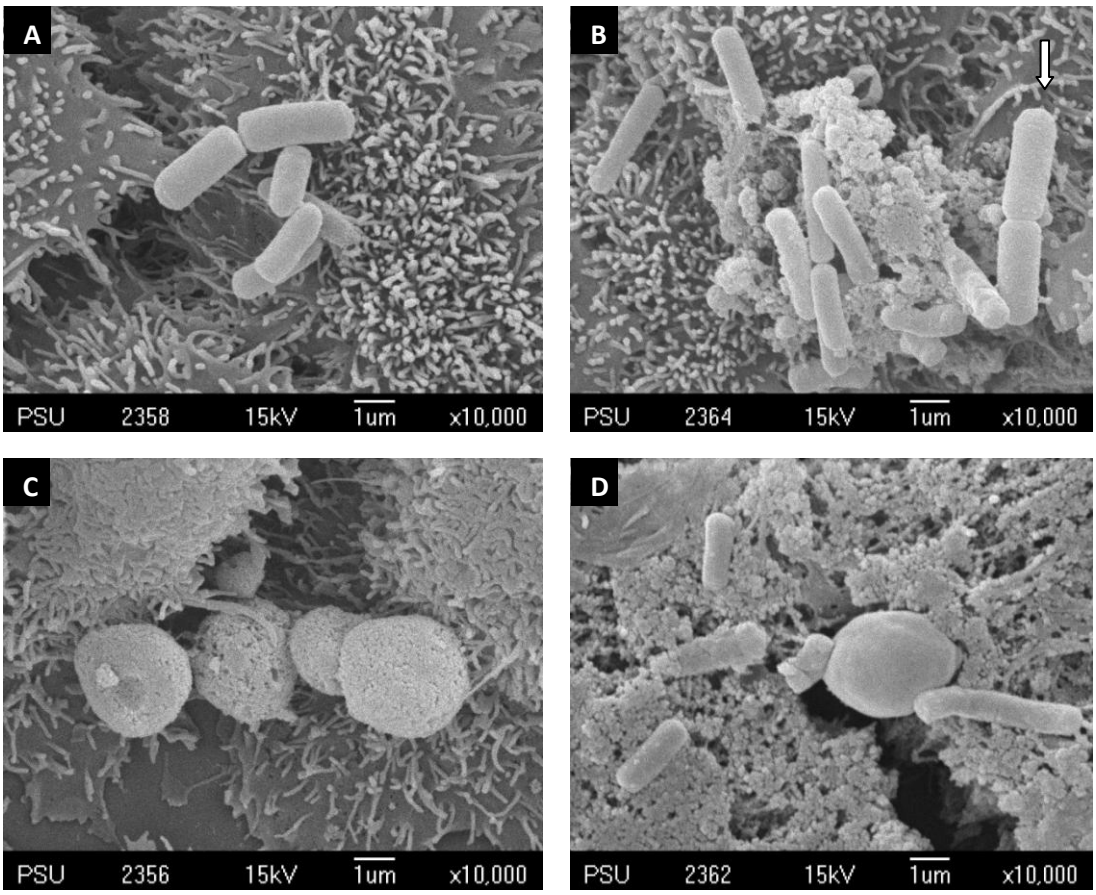

Figure 3.8 Examination by scanning electron microscopy (SEM) of *L. fermentum* SK5 inhibiting pathogen adhesion to HT-29 cells. Adhesion of *E. coli* alone to HT-29 cell monolayer (A). Adherence of *L. fermentum* SK5 inhibiting *E. coli* (pointing arrow) adhesion to HT-29 cell monolayer (B). Adhesion 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).

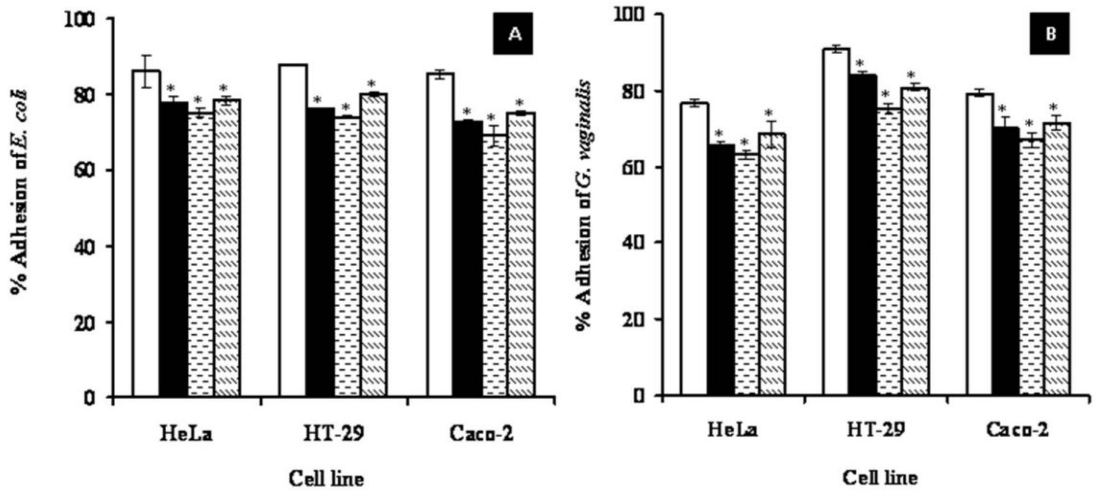

Figure 3.9 Inhibition of *E. coli* (A) and *G. vaginalis* (B) adhesion to HeLa, HT-29 and Caco-2 cells by *L. fermentum* SK5: Competition (■), Exclusion (▨), Displacement (▨). * = $p < 0.05$ (compared with *E. coli* alone or *G. vaginalis* alone (□)).

3.2 Effect of inulin, galactooligosaccharide and skim milk on the growth of *L. fermentum* SK5, gastrointestinal and bacterial vaginosis pathogens

Figure 3.10A, B and C showed the effect of inulin, GOS and skim milk on the growth of *L. fermentum* SK5. Inulin enhanced the growth of *L. fermentum* SK5 less than GOS. Skim milk produced the highest growth promotion of *L. fermentum* SK5. Inulin and GOS did not promote the growth of all tested pathogens, whereas skim milk stimulated the growth of all pathogens. Figure 3.11A, B and C showed the effect of inulin, GOS and skim milk on the growth of *E. coli*. The effect of inulin, and GOS on the growth of other tested pathogens showed the same result as *E. coli* (not

data shown). Figure 3.12-3.21 showed the effect of skim milk on the growth of other tested pathogens.

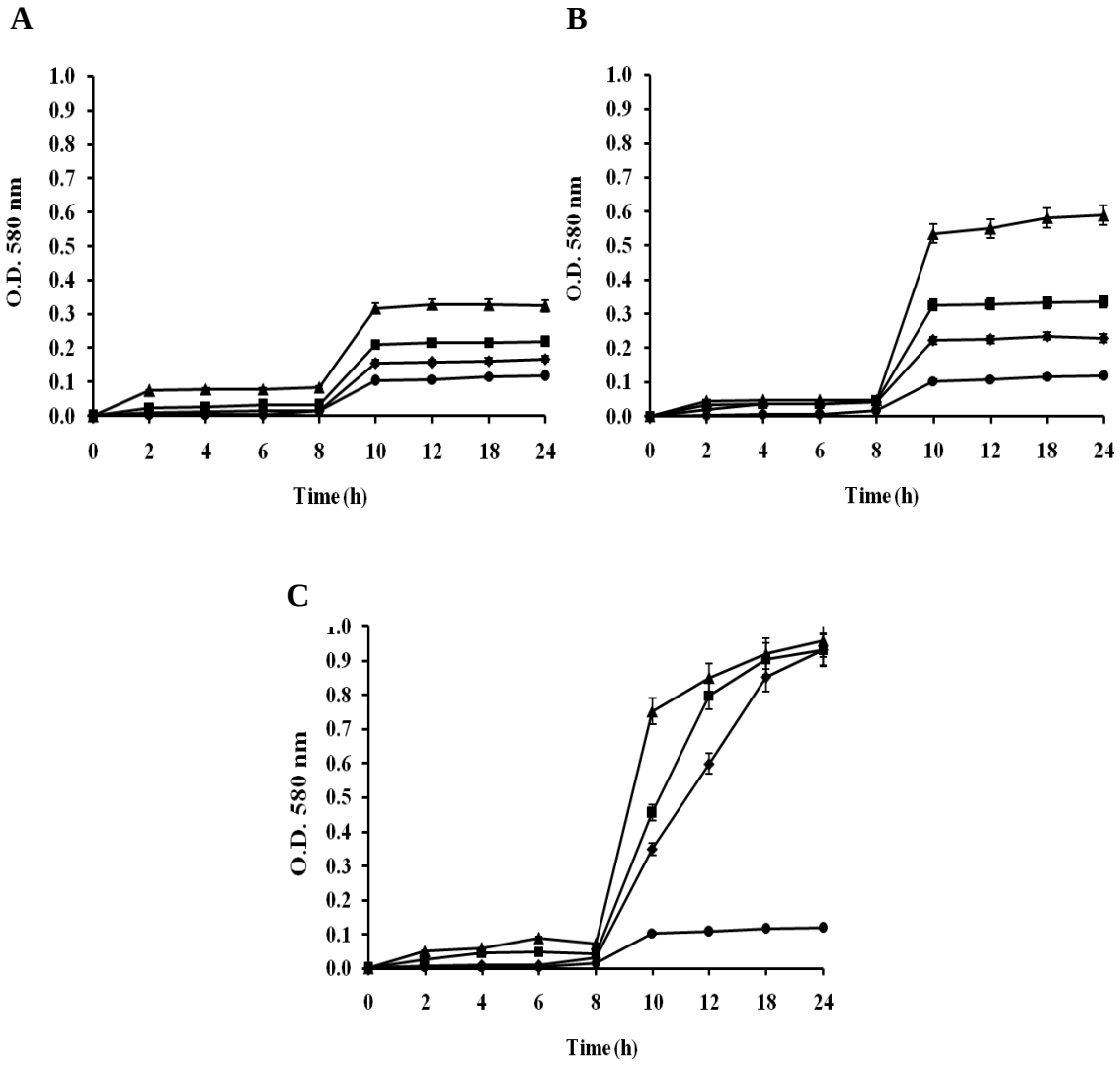

Figure 3.10 Effect of inulin (A), GOS (B) and skim milk (C) supplemented in a quarter strength of MRS broth on the growth of *L. fermentum* SK5: 0% (●), 1% (♦), 2% (■), 4% (▲)

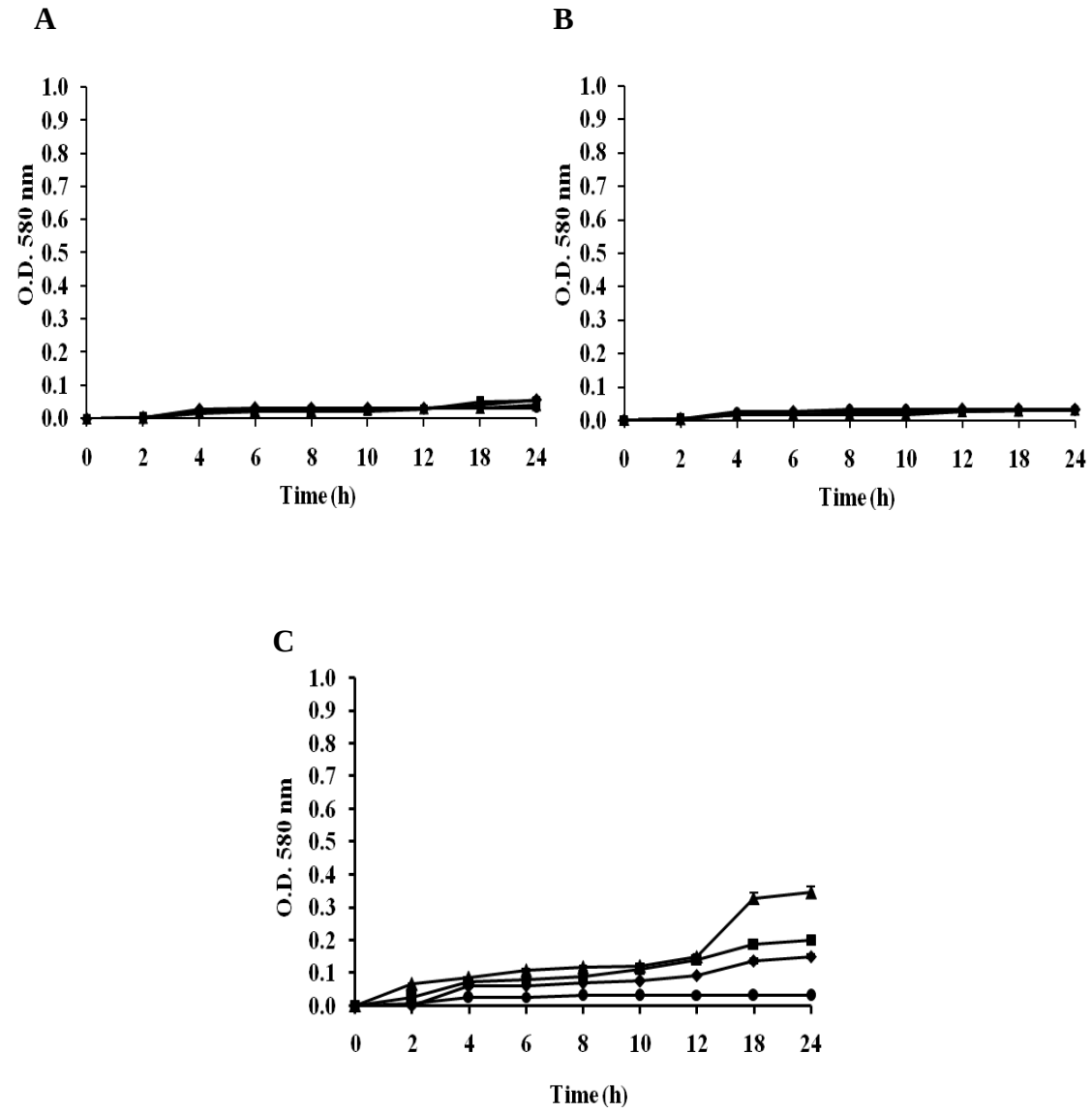

Figure 3.11 Effect of inulin (A), GOS (B) and skim milk (C) supplemented in a quarter strength of BHI broth on the growth of *E. coli*: 0% (●), 1% (♦), 2% (■), 4% (▲)

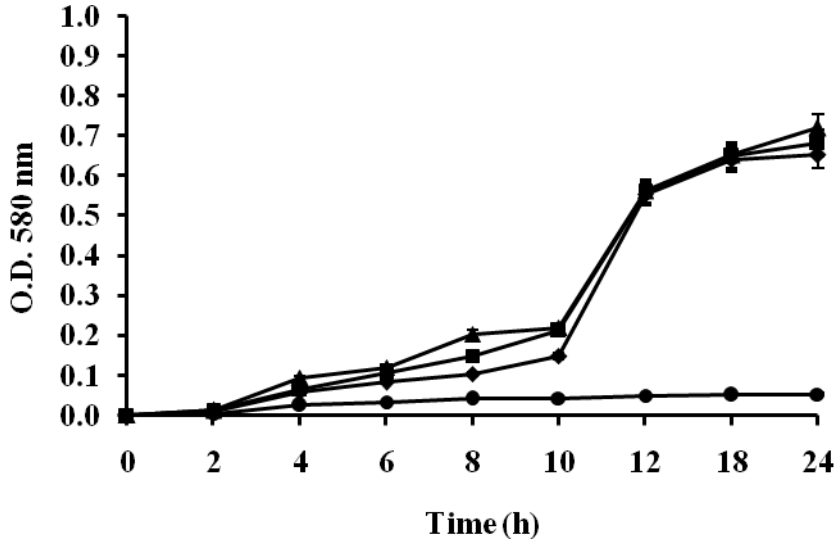

Figure 3.12 Effect of skim milk supplemented in a quarter strength of BHI broth on the growth of *P. aeruginosa*: 0% (•), 1% (♦), 2% (■), 4% (▲)

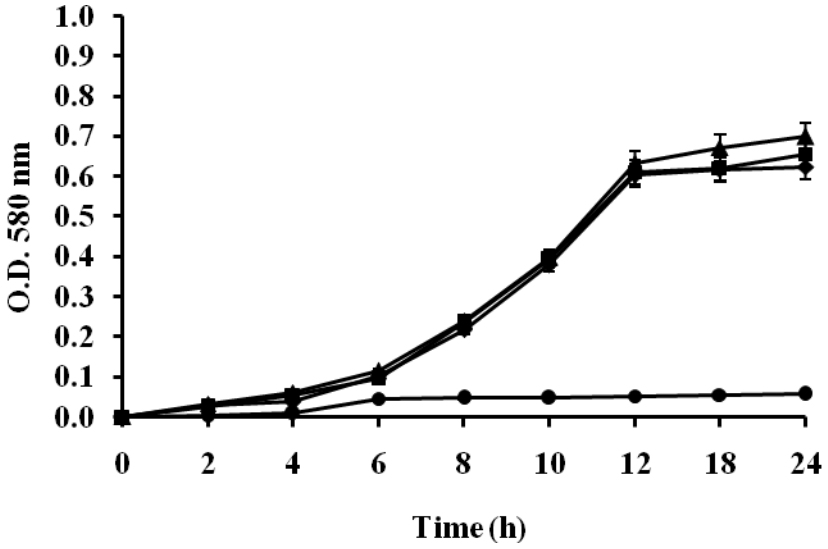

Figure 3.13 Effect of skim milk supplemented in a quarter strength of BHI broth on the growth of *S.* Typhimurium: 0% (•), 1% (♦), 2% (■), 4% (▲)

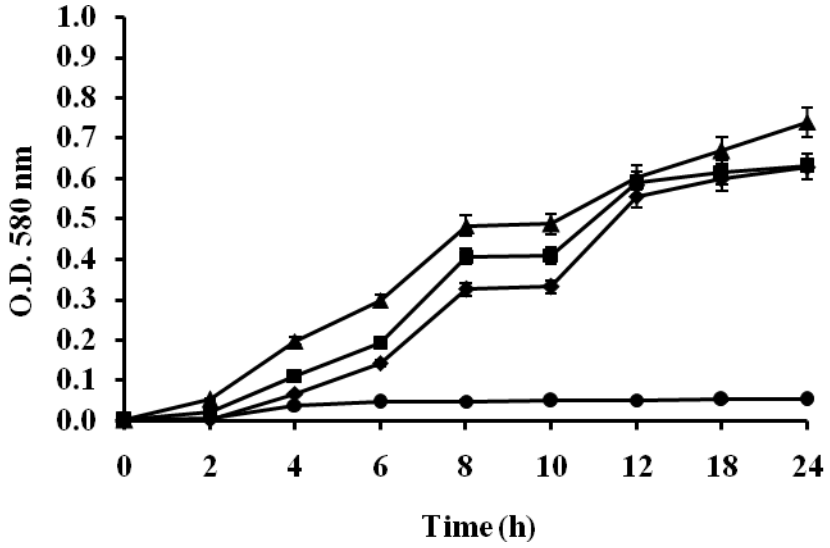

Figure 3.14 Effect of skim milk supplemented in a quarter strength of BHI broth on the growth of *S. sonnei*: 0% (●), 1% (♦), 2% (■), 4% (▲)

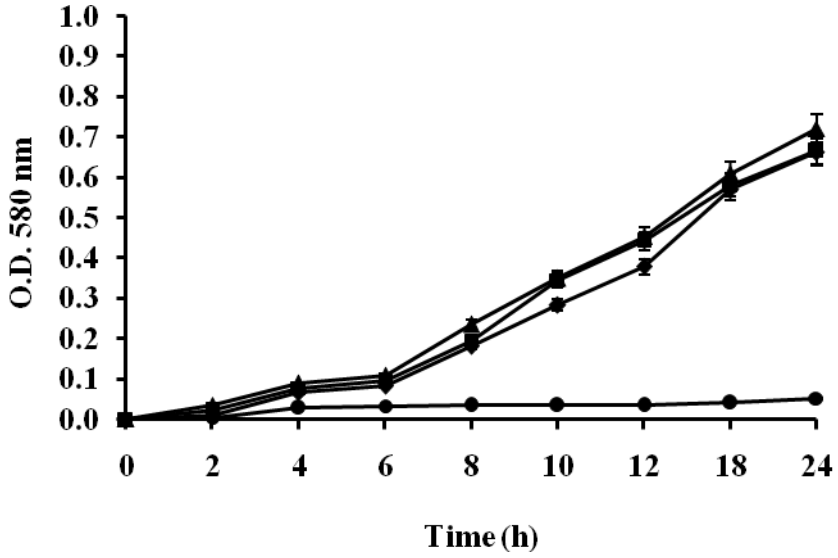

Figure 3.15 Effect of skim milk supplemented in a quarter strength of BHI broth on the growth of *V. cholerae*: 0% (•), 1% (♦), 2% (■), 4% (▲)

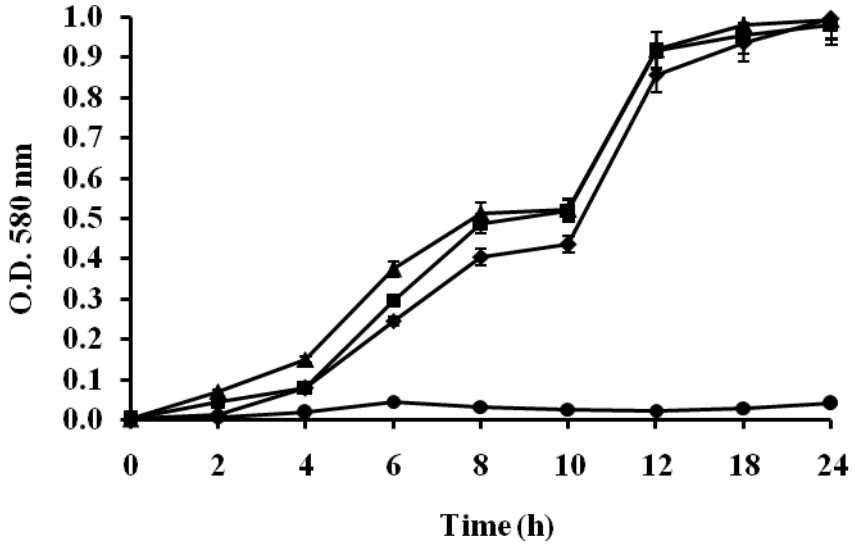

Figure 3.16 Effect of skim milk supplemented in a quarter strength of BHI broth containing 0.5% (w/v) yeast extract, 15 μg/ml hemin on the growth of *G. vaginalis*: 0% (●), 1% (♦), 2% (■), 4% (▲)

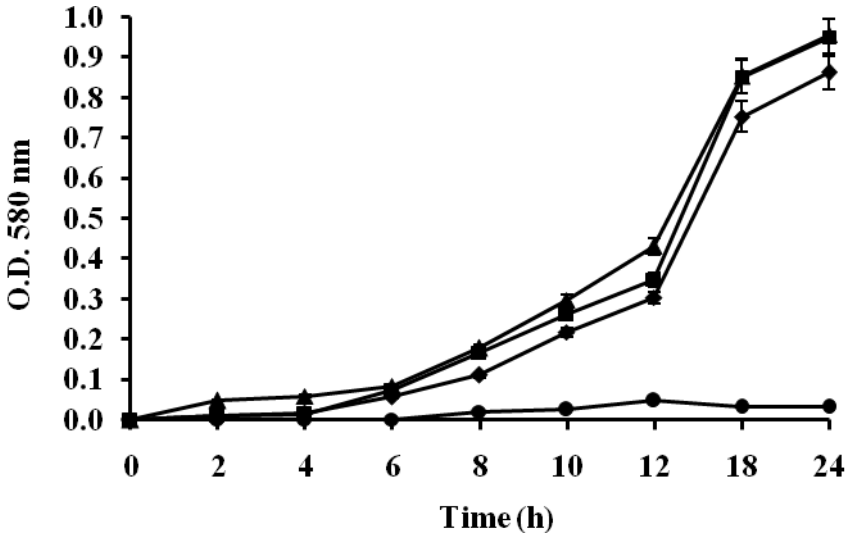

Figure 3.17 Effect of skim milk supplemented in a quarter strength of BHI broth containing 0.5% (w/v) yeast extract, 15 μg/ml hemin on the growth of *B. fragilis* DMST 19152: 0% (●), 1% (♦), 2% (■), 4% (▲)

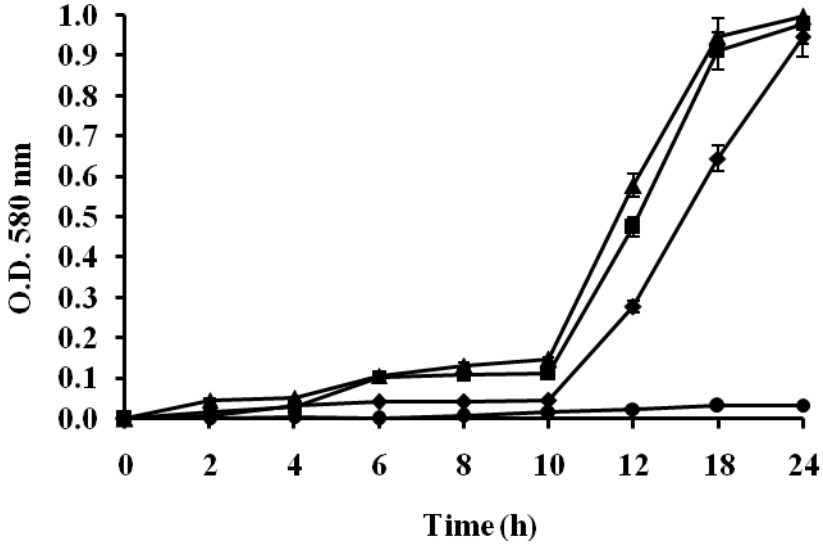

Figure 3.18 Effect of skim milk supplemented in a quarter strength of BHI broth containing 0.5% (w/v) yeast extract, 15 μg/ml hemin on the growth of *B. vulgatus* DMST 15535: 0% (●), 1% (♦), 2% (■), 4% (▲)

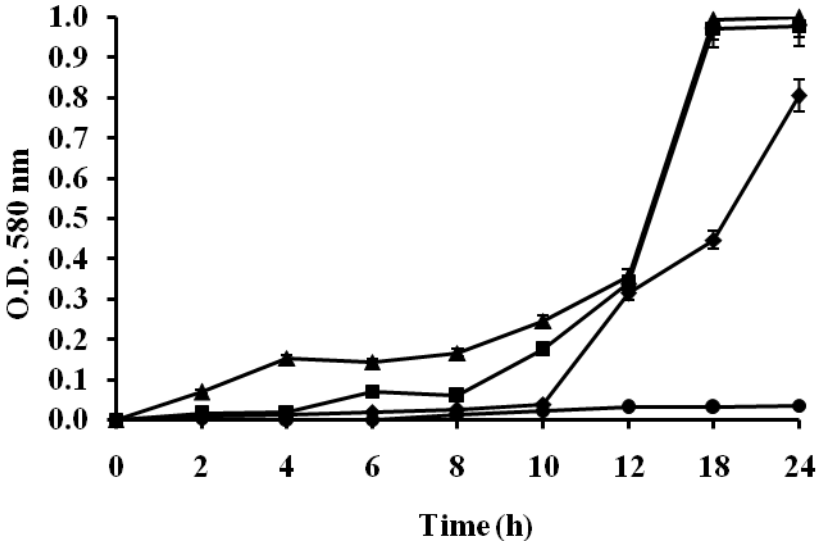

Figure 3.19 Effect of skim milk supplemented in a quarter strength of BHI broth containing 0.5% (w/v) yeast extract, 15 μg/ml hemin on the growth of *B. ureolyticus* ATCC 33387: 0% (•), 1% (♦), 2% (■), 4% (▲)

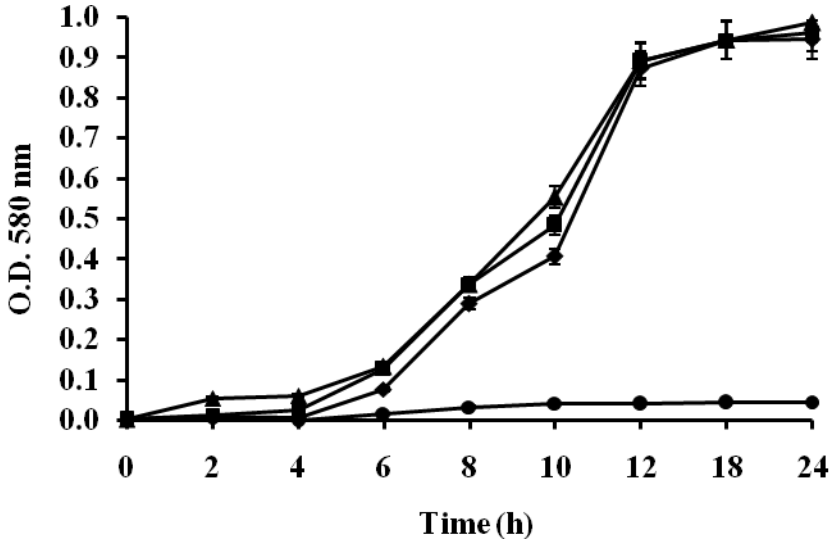

Figure 3.20 Effect of skim milk supplemented in a quarter strength of BHI broth containing 0.5% (w/v) yeast extract, 15 μg/ml hemin on the growth of *M. curtisii* ATCC 35241: 0% (●), 1% (♦), 2% (■), 4% (▲)

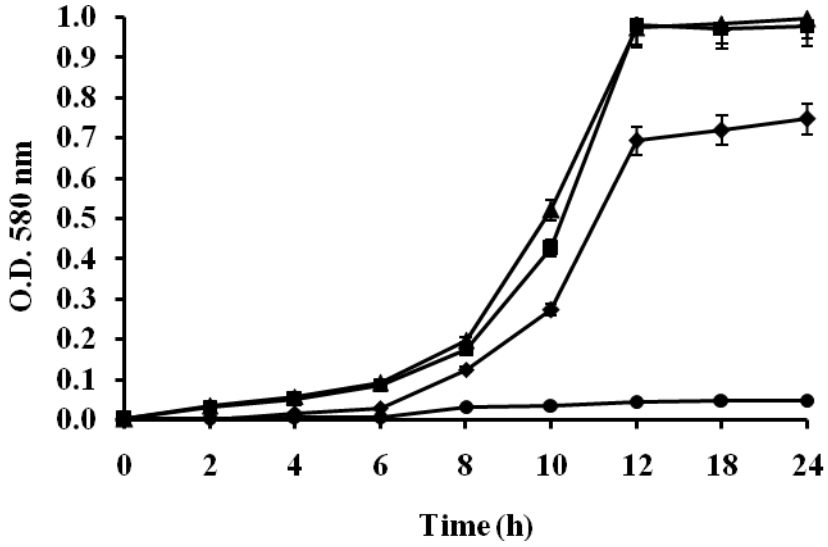

Figure 3.21 Effect of skim milk supplemented in a quarter strength of BHI broth containing 0.5% (w/v) yeast extract, 15 µg/ml hemin on the growth of *P. bivia* ATCC 29303: 0% (●), 1% (♦), 2% (■), 4% (▲)

3.3 Selection of cryoprotectant for preparation of *L. fermentum* SK5 lyophilized powder

The effect of inulin, GOS and skim milk on survival of *L. fermentum* SK5 was evaluated. The results showed that the best survival rate of *L. fermentum* SK5 was 91.1 and 88.6% in 4% (w/v) inulin and 4% (w/v) GOS, respectively. However, its survival rate was not significantly different at all tested concentration of inulin and GOS (Figure 3.22). Skim milk produced a 72.1-90.4% survival rate of *L. fermentum* SK5 and the highest viability was shown in 10% (w/v) skim milk. Its survival rate at 2 and 4% (w/v) skim milk was significantly less than that of 10% (w/v) (Figure 3.22). In addition, the survival rate of *L. fermentum* SK5 at 4% (w/v) inulin and GOS was significantly more than that of 4% (w/v) skim milk.

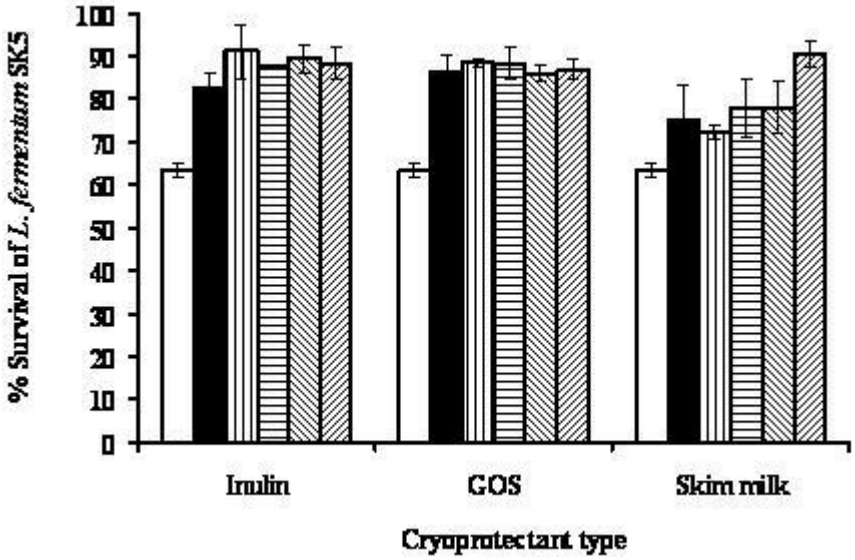

Figure 3.22 Cryoprotectant effect of inulin, GOS and skim milk on survival of *L. fermentum* SK5 during lyophilization process: 0% (□), 2% (■), 4% (▥), 6% (▤), 8% (▧), 10% (▨)

3.4 Formulation and evaluation of *L. fermentum* SK5 enteric coated capsule and hollow-type vaginal suppository

3.4.1 Production of *L. fermentum* SK5 lyophilized cells

L. fermentum SK5 lyophilized powder was prepared in 4% (w/v) GOS as cryoprotectant and viability of *L. fermentum* SK5 was 8.39 log cfu/ml.

3.4.2 Preparation of *L. fermentum* SK5 enteric coated capsule

The appearance of enteric coated capsules was shown in figure 3.23.

Figure 3.23 Appearance of *L. fermentum* SK5 enteric coated capsule

3.4.3 Evaluation of *L. fermentum* SK5 enteric coated capsule

Uniformity of weight

The weight of enteric coated capsules was in the range of 101.9-109.8 mg (Table 3.5).

***In vitro* release study**

In vitro release study, the release profile of *L. fermentum* SK5 from enteric coated capsules shown in Figure 3.24. At firstly 2 h in SGF pH 1.2 the release of *L. fermentum* SK5 was not detected. After that, the release of *L. fermentum* SK5 in SIF pH 7.4 was gradually increased.

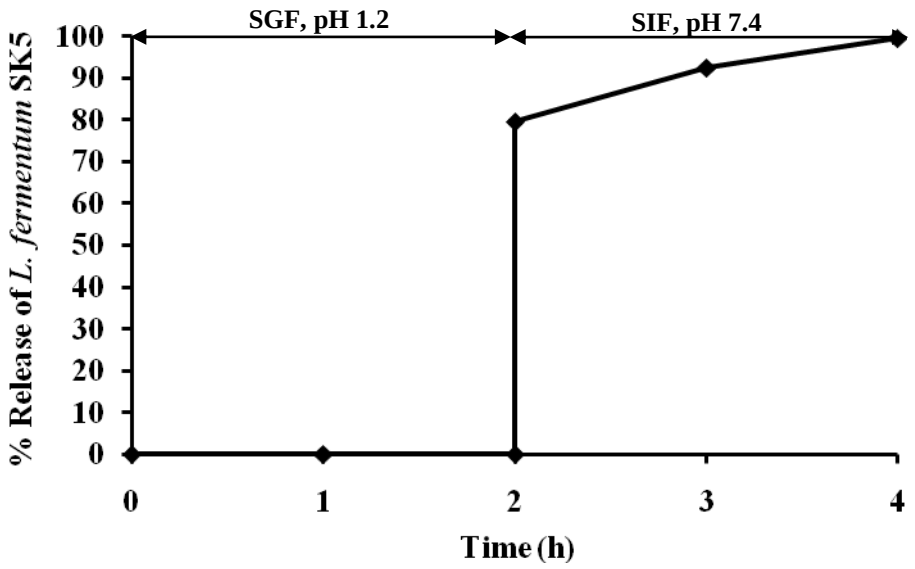

Figure 3.24 *In vitro* release of *L. fermentum* SK5 from enteric coated capsules

Table 3.5 Weight of *L. fermentum* SK5 enteric coated capsules

Capsule No.	Weight (mg)
1	106.3
2	108.1
3	109.2
4	103.6
5	102.1
6	103.7
7	101.9
8	107.2
9	104.0
10	108.3
11	106.1
12	105.6
13	108.4
14	109.8
15	108.0
16	103.7
17	102.7
18	105.0
19	104.5
20	105.9
Average	105.7 ± 2.4

Inhibitory activity of enteric coated capsules against gastrointestinal pathogens

The mixed cultures of *L. fermentum* SK5 released from the capsule and *E. coli* or *V. cholerae* were shown in Figure 3.25 and Figure 3.26, respectively. Both pathogens inoculated at 10^4 or 10^6 cfu/ml, complete inhibition of *E. coli* growth was observed after 18 h (Figure 3.25), whereas 100% inhibition of *V. cholerae* was observed at 20 h (Figure 3.26).

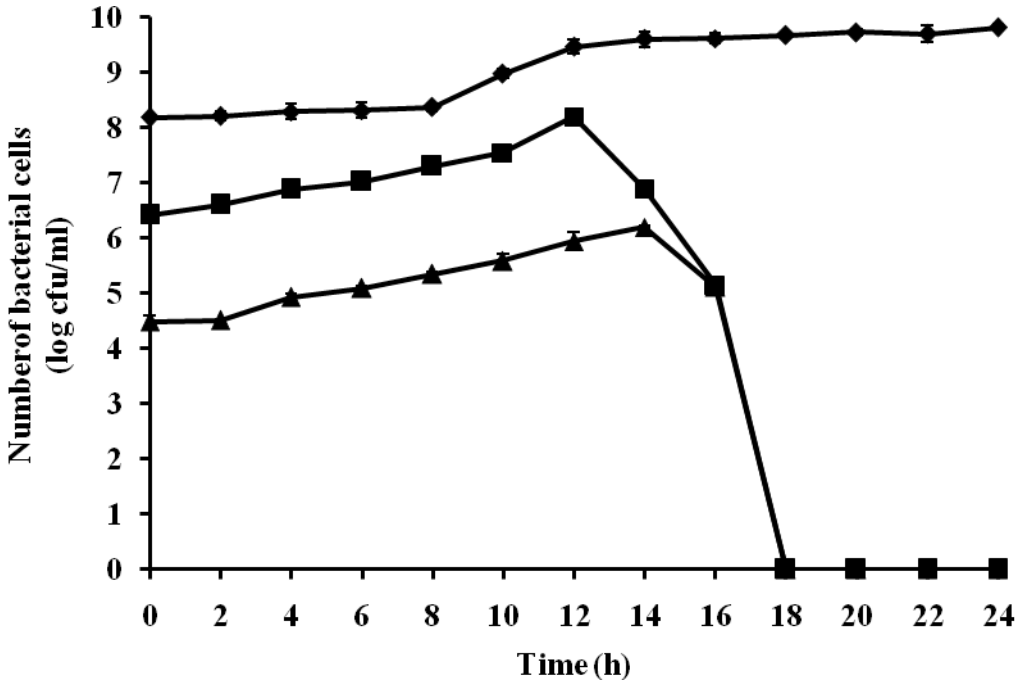

Figure 3.25 Inhibitory effect of *L. fermentum* SK5 released from the enteric coated capsule against *E. coli* co-cultured in BHI broth, *L. fermentum* SK5 (♦), 10^6 cfu/ml *E. coli* (■), 10^4 cfu/ml *E. coli* (▲)

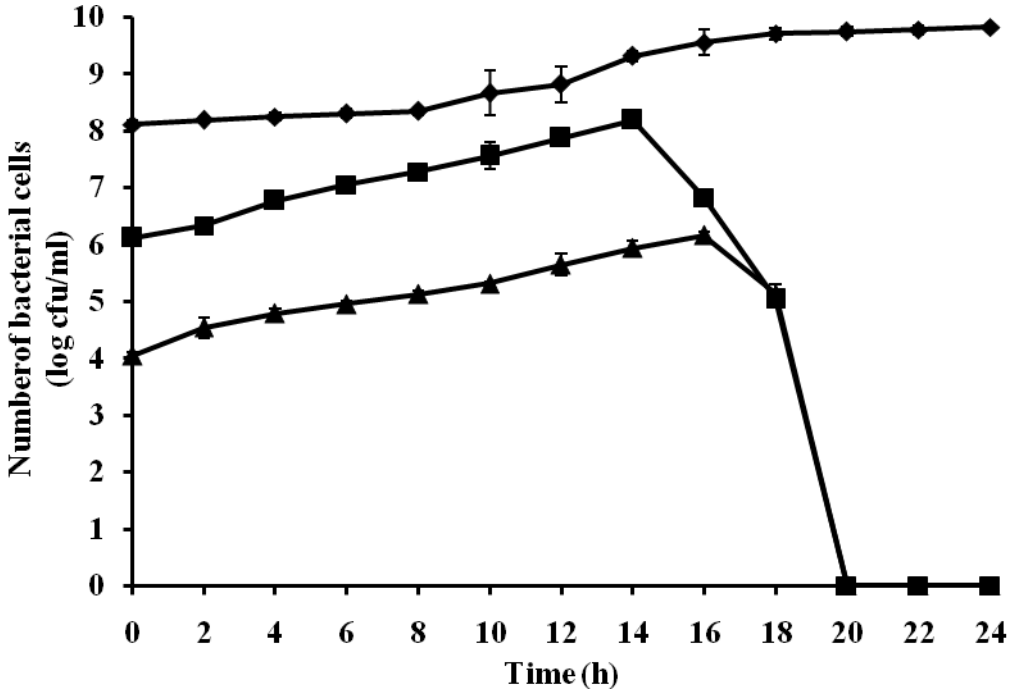

Figure 3.26 Inhibitory effect of *L. fermentum* SK5 released from the enteric coated capsule against *V. cholerae* co-cultured in BHI broth, *L. fermentum* SK5 (♦), 10^6 cfu/ml *V. cholera*e (■), 10^4 cfu/ml *V. cholerae* (▲)

Stability

After storage at 4 °C for 6 months the viability of *L. fermentum* SK5 in enteric coated capsule was 96.38-98.38 % (Figure 3.27).

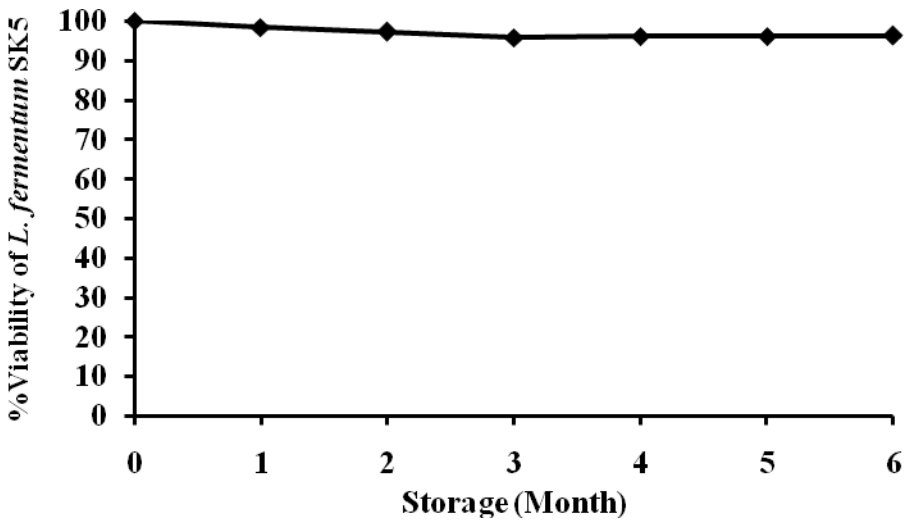

Figure 3.27 Viability of *L. fermentum* SK5 from enteric coated capsule**s**

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

L. fermentum SK5 hollow-type vaginal suppository had a uniform, smooth and waxy surface (Figure 3.28).

Figure 3.28 Appearance of *L. fermentum* SK5 hollow-type vaginal suppository

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

Uniformity of weight

The weight of hollow-type vaginal suppositories was in the range of 2.00-2.25 g (Table 3.6).

Table 3.6 Weight of *L. fermentum* SK5 vaginal suppositories

Suppository No.	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	2.08 ± 0.06

Melting point measurement of suppository base

The DSC thermogram of PEGs showed a broad endothermic peak between 35.07-44.03 °C indicating the melting point of PEGs base (Figure 3.29)

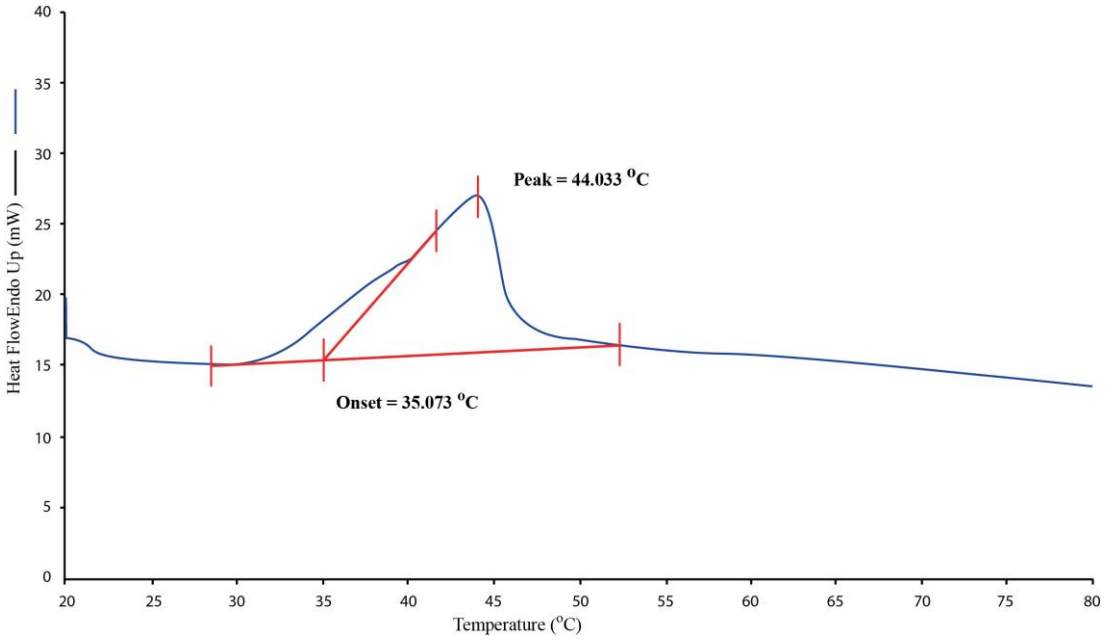

Figure 3.29 DSC thermogram of suppository base (the mixture of PEG 400:PEG 4000, ratio55:45)

***In vitro* release study**

In vitro release study, the release profile of *L. fermentum* SK5 from vaginal suppositories is shown in Figure 3.30. No bacterial release was detected in the first 25 min but over the next 5 min, i.e. 25-30 min virtually all was released.

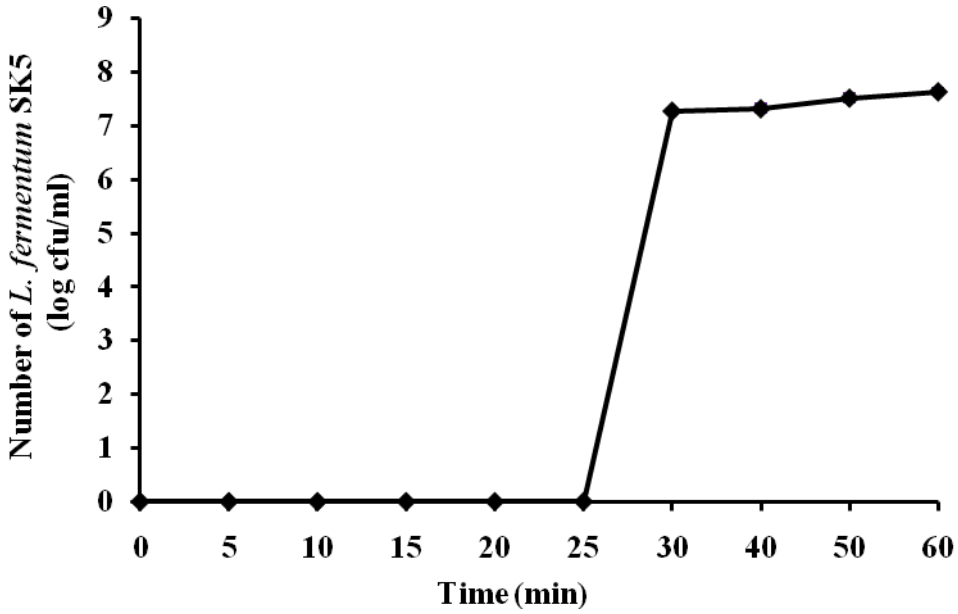

Figure 3.30 *In vitro* release of *L. fermentum* SK5 from hollow-type vaginal suppositories

Inhibitory activity of vaginal suppository against bacterial vaginosis pathogens

The mixed cultures of *L. fermentum* SK5 from suppository and *G. vaginalis* or *B. vulgatus* DMST 15535 were shown in Figure 3.31 and 3.32, respectively. Both pathogens inoculated at 10^4 or 10^6 cfu/ml, were completely eliminated after 48 h whereas the *L. fermentum* SK5 remained relatively constant over the 48 h period at about 10^9 cfu/ml. *G. vaginalis* grew until about 14 h and reached a population of about 10^9 cfu/ml then began to decrease between 14 -20 h and was completely eliminated at 44 h (Figure 3.31). In contrast the *B. vulgatus* DMST 15535 population increased to about 10^7 cfu/ml at about 32 h and was then completely eliminated by 48 h (Figure 3.32).

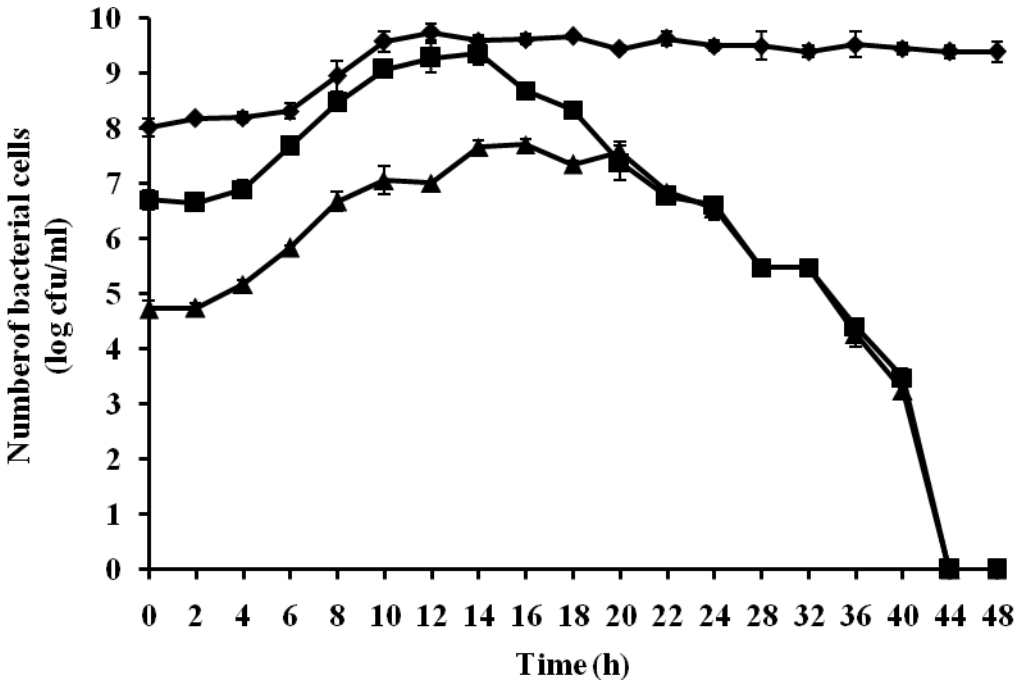

Figure 3.31 Inhibitory effect of *L. fermentum* SK5 released from hollow-type vaginal suppository against *G. vaginalis* co-cultured in BHI broth containing 0.5% (w/v) yeast extract, 15 µg/ml hemin: *L. fermentum* SK5 (♦), 6 log cfu/ml *G. vaginalis* (■), 4 log cfu/ml *G. vaginalis* (▲)

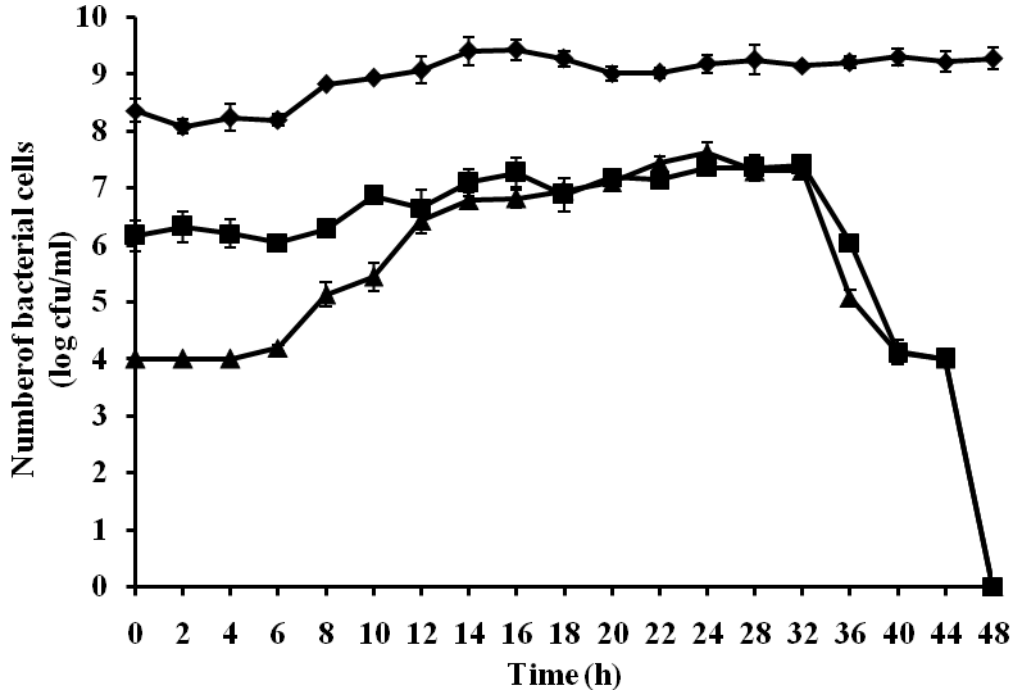

Figure 3.32 Inhibitory effect of *L. fermentum* SK5 released from hollow-type vaginal suppository against *B. vulgatus* DMST 15535 co-cultured in BHI broth containing 0.5% (w/v) yeast extract, 15 µg/ml hemin: *L. fermentum* SK5 (♦), 6 log cfu/ml *B. vulgatus* DMST 15535 (■), 4 log cfu/ml *B. vulgatus* DMST 15535 (▲)

Stability

After storage at 4 °C for 6 months, the viability of *L. fermentum* SK5 in hollow-type vaginal suppository was 96.49-99.81% (Figure 3.33).

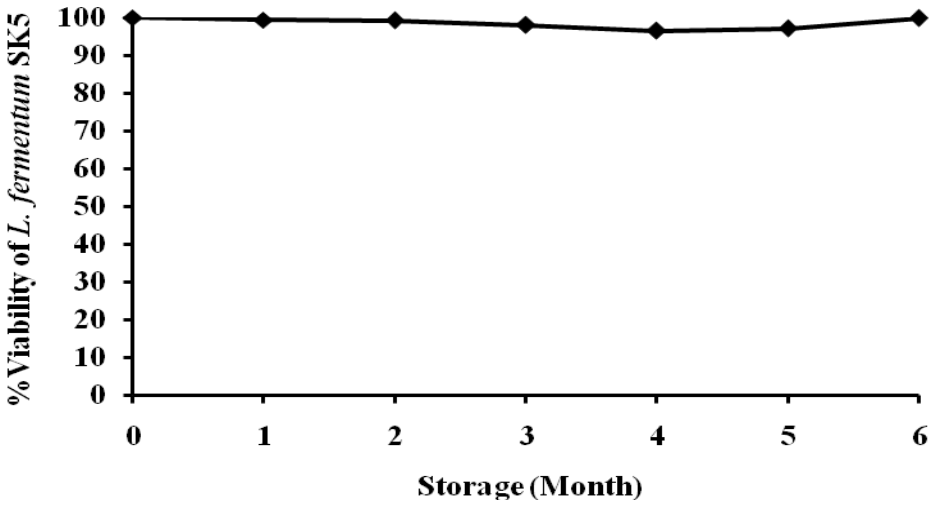

Figure 3.33 Viability of *L. fermentum* SK5 from hollow-type vaginal suppositories

CHAPTER 4

DISCUSSIONS

4.1 Probiotic properties of *Lactobacillus fermentum* SK5

4.1.1 Antimicrobial activity of *L. fermentum* SK5 against gastrointestinal and bacterial vaginosis pathogens

The ability to inhibit the growth of gastrointestinal and vaginal pathogens is considered as one of desirable properties of lactic acid bacteria intended to use as probiotic in gastrointestinal tract and vagina. Previous studies have demonstrated diverse growth inhibition of different gastrointestinal and vaginal pathogens by many lactic acid bacterial strains (Kirtzalidou *et al*., 2011; Juárez Tomás *et al*., 2011; Messaoudi *et al*., 2012; Jena *et al*., 2013). General inhibitory substances produced by these bacteria include lactic acid and short chain fatty acids. Some strains also produce H_2O_2 and/or bacteriocin that enhance the inhibition activity (Lebeer *et al*., 2008; Yang *et al*., 2012; Rushdy and Gomaa, 2013). In this study, *L. fermentum* SK5 inhibited gastrointestinal pathogens, i.e. *E. coli*, *P. aeruginosa*, *S.* Typhimurium, *S. sonnei* and *V. cholerae*. This result indicated that *L. fermentum* SK5 may be used as a probiotic for prevention and treatment of gastrointestinal infections. Moreover, *L. fermentum* SK5 inhibited bacterial vaginosis pathogens including *G. vaginalis, B. fragilis* DMST 19152, *B. vulgatus* DMST 15535, *B. ureolyticus* ATCC 33387, *M. curtisii* ATCC 35241 and *P. bivia* ATCC 29303. According to the high antibacterial activity of *L. fermentum* SK5, it is a suitable probiotic candidate used for prevention or treatment of bacterial vaginosis.

4.1.2 Detection of hydrogen peroxide production

H_2O_2 is one of the active compounds produced by vaginal lactobacillus. There were strong evidences that H_2O_2 producing lactobacilli can protect women against the development of bacterial vaginosis (Xu *et al*., 2008; Muench *et al*., 2009; Lin *et al*., 2011). In this study, H_2O_2 should play an important role to inhibit tested bacterial vaginosis pathogens since *L. fermentum* SK5 was a strong H_2O_2 producer. Moreover, Pridmore *et al*., 2008 reported that human intestinal isolate *L. johnsonni* NCC533 produced H_2O_2 was effective in killing the model pathogen *Salmonella enterica* serovar Typhimurium SL1344 *in vitro*. H_2O_2 was one of antibacterial substances produced by *L. fermentum* SK5 causing inhibition of tested gastrointestinal pathogens. These results indicated that *L. fermentum* SK5 may be suitable for use as a probiotic to prevent vaginal and gastrointestinal pathogen infections.

4.1.3 Detection of a high molecular weight inhibitory substance produced by *L. fermentum* SK5

The results of present study confirmed that one of the inhibitory substances produced by *L. fermentum* SK5 was most likely a high molecular weight inhibitory substance perhaps a bacteriocin-like substance as all low molecular weight molecules such as organic acids and H_2O_2 had been removed by dialysis. This bacteriocin-like substance inhibited specific strain of *E. coli*, *V. cholerae*, *G. vaginalis* and *B. vulgatus* DMST 15535. All tested gastrointestinal pathogens were also

sensitive to norfloxacin. Although metronidazole is the first drug of choice for treatment of bacterial vaginosis, it inhibited only some tested pathogens, i.e. *G. vaginalis*, *B. fragilis* DMST 19152 and *B. vulgatus* DMST 15535. Liu *et al*., (2008) reported that pentocin 31-1, an anti-Listeria bacteriocin showed a wide range of antimicrobial activity against *Listeria* spp., *Staphylococcus* spp., *Bacillus* spp., *Lactobacillus* spp., *Streptococcus* spp., *Pediococcus* spp. and *Escherichia* spp. All *Listeria* strains tested, including *Listeria monocytogenes*, were highly sensitive to the bacteriocin. Previous study demonstrated that bacteriocin-producing probiotic may be a potential candidate for alternative agents to control vaginal tract infections. The result showed that *L. fermentum* isolated from vagina of healthy woman produced bacteriocin HV6b to inhibit *G. vaginalis* (Kaur *et al*., 2013).

The bacteriocin-like substance produced by *L. fermentum* SK5 reacted with pathogenic cell surfaces (*E. coli* and *G. vaginalis*) and showed pore formation similar to the action of bacteriocin isolated from other Lactobacillus species (Ponce *et al*., 2008; Pei *et al*., 2013; Sharafi *et al*., 2013). Moreover, this substance reacted with ninhydrin reagent to form a blue-violet dye. This confirmed that it was most likely a high molecular weight protein. However, this protein-like compound in this substance may be protein that was the composition of medium. This should further approve that the protein was produced by *L. fermenyum* SK5 or obtained from medium. This purported bacteriocin from *L. fermentum* SK5 was active at pH 2-7 but was only slightly sensitive to proteolytic enzymes and heat. Its activity was the highest at pH 2 and decreased as the pH was increased to 7. There was no activity at pH 8 and 9.

Many bacteriocins have been reported to have greater bactericidal activity at low pH (Banerjee *et al*., 2013; Srinivasan *et al*., 2013; Trivedi *et al*., 2013). They can possess either a positive or negative net charge depending upon the environmental pH. As the pH drops below 6, Lys and Arg will have a net positive charge and influence bioactivity by increasing the likelihood to bind to the negative charge in the bacterial cell wall. It has been suggested that the pH-induced alterations of net charge might facilitate translocation of some bacteriocin molecules through the cell wall (Jack *et al*., 1995). It is important to note that the bioactivity of the compound in distilled water (pH 6.25) against *E. coli* produced an inhibition zone of 13.50 mm which was much higher than that of the pH 5 of compound in buffering solution (9.50 mm). The lower activity in this case may be explained by the presence of the extra salts in the buffering system that might compete with the binding and transport sites on the bacterial cell walls. In a similar manner the activity of the compound against *G. vaginalis* disappeared when the pH was 7 or above. Whereas from pH 2-6 the activity was only slightly decreased at the lower pH values although the difference was not significantly different.

4.1.4 Antibiotic susceptibility assay

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. However, some studies have also demonstrated that many of these resistance attributes are intrinsic and nontransmissible (Salminen *et al*.,

1998). Probiotic strains that have intrinsically antibiotic-resistant traits may be an advantage to patients. They can be given at the same time as the antibiotic treatment (Cebeci and Gürakan, 2003). *L. fermentum* SK5 was sensitive to all tested antibiotics except for metronidazole. It may be co-administered with metronidazole for treatment of vaginal infections.

4.1.5 Survival under conditions simulating the human GI tract

To exert a better health effect, the lactobacilli need to resist the harsh conditions of the stomach and upper intestine (Morelli, 2007). 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 (Holzapfel *et al*., 1998). 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 (Succi *et al*., 2005). 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. A concentration of 0.15–0.3% of bile salt has been recommended as a suitable concentration for selecting probiotic bacteria for human use. In this study, no viable cells were detected at 0.3 and 0.4% of bile salt. The

tolerant ability may be different depend on strain specificity. *L. fermentum* SK5 was tolerant to low pH and bile salt and survived in gastric and intestinal fluid. This means that this bacterial strain is likely to survive in the stomach and intestine juices. These results are similar to many previous studies, where *Lactobacillus* had a high tolerance to simulated gastric, intestine juices and bile salts (Turchi *et al*., 2013; Ryu and Chang, 2013; Yu *et al*., 2013).

4.1.6 Survival under conditions that simulated the human vagina

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 could formulate as vaginal products to use in preventing vaginal infections.

4.1.7 Aggregation and surface hydrophobicity

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 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_2O_2, 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*.

4.1.8 Adhesion of *L. fermentum* SK5 on HeLa, HT-29 and Caco-2 cells

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, 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 (Gopal *et al*., 2001). 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 (Lewandowska *et al*., 2005). *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 the results obtained by Duary *et al*., (2011). HeLa cells have been used as an *in vitro* adhesion model for other studies (Atassi *et al*., 2006; Martín *et al*., 2012; Lavilla-Lerma *et al*., 2013). 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. 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 (Jensen *et al*., 2012; Lin *et al*., 2008; Tsai *et al*., 2008). Pennacchia *et al*., (2006) showed that *Lactobacillus* from fermented sausages had good adhesion capability to human intestinal Caco-2 cell lines.

4.1.9 Inhibition of pathogens adhesion to HeLa, HT-29 and Caco-2 cells

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 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 indicated 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 (Candela *et al*., 2008; Stöber *et al*., 2010; Zhang *et al*., 2010). Lee *et al*., (2003) reported that *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.

4.2 Effect of inulin, galactooligosaccharide and skim milk on the growth of *L. fermentum* SK5, gastrointestinal and bacterial vaginosis pathogens

The effects of inulin, GOS and skim milk on the growth of *L. fermentum* SK5, gastrointestinal and bacterial vaginosis pathogens were studied. The result showed that GOS enhanced the growth of *Lactobacillus* more so than inulin but inulin and GOS did not increase the growth of the pathogens. Inulin and GOS are carbohydrate-based well-known food ingredients with prebiotic properties. Previous studies have reported the positive effect of inulin and GOS on the growth of lactobacilli (Martinez *et al*., 2011; Rezaei *et al*., 2012; Hernandez-Hernandez *et al*., 2012). In this case, GOS was a good prebiotic for *L. fermentum* SK5 growth. Skim milk also enhanced the growth of *L. fermentum* SK5 and the pathogens. This

suggested that skim milk should not be used as prebiotic for *Lactobacillus* product used in the gastrointestinal tract and vagina.

4.3 Selection of cryoprotectant for preparation of *L. fermentum* SK5 lyophilized powder

Pharmaceutical probiotic products are usually prepared from dried bacterial cell powder. Lyophilization process is mostly used to prepare the cell powder by using cryoprotectant to protect the cell during lyophilization process and storage (Carvalho *et al*., 2004a). In this study, the effect of inulin, GOS and skim milk on survival of *L. fermentum* SK5 was evaluated. Inulin and GOS showed the best survival of *L. fermentum* SK5 at the same concentration of 4% (w/v). Both of them can be used as cryoprotectant for lyphilization of this *Lactobacillus*. Ten percent (w/v) skim milk increased the same survival rate of *L. fermentum* SK5 as did inulin and GOS. Skim milk has been used as pharmaceutical cryoprotectants and/or excipients in many *Lactobacillus* probiotic formulations such as capsules, vaginal tablets and vaginal suppositories (Mastromarino *et al*., 2002; Zaráte and Nader-Macias, 2006; Kaewsrichan *et al*., 2007). However, in this case skim milk increased not only the growth of *Lactobacillus* but also increased the growth of tested pathogens. For this reason, it should not be used as a cryoprotectant or excipient in probiotic formulation intended to use in the vagina. GOS was therefore the optimum prebiotic and cryoprotectant for *L. fermentum* SK5 lyophilized powder preparation. 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). There were other studies showed that prebiotics used as protective agents for lyophilization process (Capela *et al*., 2006). The viability of freeze-dried *Lactobacillus rhamnosus* IMC 501® and *Lactobacillus paracasei* IMC 502® using different protective agents (i.e. glycerine, mannitol, sorbitol, inulin, dextrin, Crystalean®) was determined and compared with semi skimmed milk (SSM) control. No significant differences were observed between the tested protectants and the control (SSM) during storage at refrigerated conditions (Savini *et al*., 2010).

In this study, prebiotics were used as the cryoprotectants to protect bacterial cells during lyophilization process. According to the results, GOS significantly increased the growth of *L. fermentum* SK5 higher than inulin and both of them did not enhance the growth of tested pathogens. Four percentage (w/v) GOS significantly increased the growth of *L. fermentum* SK5. Skim milk stimulated not only the growth of *Lactobacillu*s but also pathogens. Therefore, GOS was a good prebiotic for enhancement of *L. fermentum* SK5 growth. The survival rate of *L. fermentum* SK5 was not significantly different at all tested concentrations of inulin and GOS used as cryoprotectant. Therefore, inulin or GOS may be used as cryoprotectant at all tested concentrations. Although 10% (w/v) skim milk showed the highest survival rate of *L. fermentum* SK5 it was not suitable to be used as prebiotic. Therefore, 4% (w/v) GOS was the optimum prebiotic and cryoprotectant for preparation of *L. fermentum* SK5 lyophilized powder.

4.4 Formulation and evaluation of *L. fermentum* SK5 enteric coated capsule and hollow-type vaginal suppository

4.4.1 Preparation and evaluation of *L. fermentum* SK5 enteric coated capsule

L. fermentum SK5 enteric coated capsule had good appearance. The uniformity of weigh was accepted under BP criteria. *L. fermentum* SK5 released from enteric coated capsule in SIF, pH 7.4. This result demonstrated that Eudragit® L100 can be used as coated solution for *Lactobacillus* capsule to deliver to intestine. Eudragit® L100 is a 1:1 co-polymer of methacrylic acid and methylmethacrylate that is insoluble below a pH of 5.0. It was used for pore formation, creating channels for alkaline media to penetrate the coating, causing dissolution of the polymer coat, and releasing the capsule contents (George and Abraham, 2006). *L. fermentum* SK5 from the capsule showed antimicrobial activity against gastrointestinal pathogenic *E. coli* and *V. cholerae.* It due to antimicrobial substances produced by *L. fermentum* SK5 such as lactic acid, short chain fatty acids, H_2O_2 and a high molecular weight inhibitory substance. After storage at 4 °C for 6 months, *L. fermentum* SK5 enteric coated capsules were stable in term of viability and antimicrobial activity of this *Lactobacillus.* It was due to GOS was efficient protective agent for long term preservation of *Lactobacillus* (Tymczyszyn *et al*., 2011)

4.4.2 Preparation and evaluation of *L. fermentum* SK5 hollow-type vaginal suppository

Hollow-type vaginal suppositories of *L. fermentum* SK5 showed satisfy appearance with a uniform, smooth and waxy surface. The uniformity of weight was accepted under BP criteria. The thermogram of the mixed PEGs base, showed a broad endothermic peak between 35.07-44.03 °C that corresponded to the melting range of the base, and indicated that the *L. fermentum* SK5 hollow-type vaginal suppository started to melt at the temperature close to the body temperature. The mixture of PEG 400:PEG 4000 in the ratio 55:45 is suitable as a vaginal suppository base in term of its melting point and bacterial cell release. The *L. fermentum* SK5 suppository showed antimicrobial activity against vaginal pathogenic *G. vaginalis* and *B. vulgatus* DMST 15535. It was due to antimicrobial substances produced by *L. fermentum* SK5 such as lactic acid, short chain fatty acids, H_2O_2 and a high molecular weight inhibitory substance. The appearance of the formulation, the viable and the antimicrobial activity of *L. fermentum* SK5 from suppository were stable after storage at least 6 months. The high survival rate was caused by the protective activity of GOS and ability of suppository base to protect the cells from exposure to oxygen.

CHAPTER 5

CONCLUSIONS

Lactobacillus fermentum SK5 isolated from vagina of a healthy woman showed good probiotic properties. This strain inhibited gastrointestinal pathogens, i.e. *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Shigella sonnei* and *Vibrio cholerae* and bacterial vaginosis pathogens, i.e. *Gardnerella vaginalis*, *Bacteroides fragilis* DMST 19152, *Bacteroides vulgatus* DMST 15535, *Bacteroides ureolyticus* ATCC 33387, *Mobiluncus curtisii* ATCC 35241 and *Prevotella bivia* ATCC 29303. The antimicrobial substances produced by *L. fermentum* SK5 including organic acids, hydrogen peroxide and a high molecular weight antimicrobial substance. *E. coli* and *G. vaginalis* cells treated with the high molecular weight antimicrobial substance were lysed and had shrunk with the rough cell surface. This substance also showed positive result of ninhydrin test and it was only slightly sensitive to proteolytic enzymes and heat. It was active at acidic and neutral pH. The substance was supposed to be a bacteriocin-like compound. *L. fermentum* SK5 was sensitive to all of tested antibiotics except metronidazole. It showed high survival under conditions simulating the human GI tract and vagina, a good autoaggregation, a high surface hydrophobicity and a coaggregattion with *E. coli* and *G. vaginalis*. In addition, *L. fermentum* SK5 had capability to adhere to HeLa, HT-29 and Caco-2 cells and inhibited the adhesion of *E. coli* and *G. vaginalis* on these three cells.

Inulin, GOS and skim milk affected on the growth of *L. fermentum* SK5, gastrointestinal and bacterial vaginosis pathogens. GOS improved the growth of

L. fermentum SK5 significantly better than inulin but both GOS and inulin did not enhance the growth of tested pathogens. In case of skim milk, it increased both the growth of *L. fermentum* SK5 and tested pathogens. GOS therefore was a good prebiotic for the promotion of *L. fermentum* SK5 growth. *L. fermentum* SK5 was prepared in lyophilized form using inulin, GOS and skim milk at concentration of 2, 4, 6, 8 and 10% (w/v). After lyophilization, survival of *L. fermentum* SK5 was the highest at 4% (w/v) inulin, 4% (w/v) GOS and 10% (w/v) skim milk. Four percent (w/v) GOS was used as a cryoprotectant for preparation of *L. fermentum* SK5 lyophilized powder and viability of *L. fermentum* SK5 was 8.39 log cfu/ml.

L. fermentum SK5 lyophilized powder was formulated as enteric coated capsule using Eudragit® L100 as coating solution. The enteric coated capsule had the weight in range 101.9-109.8 mg. *L. fermentum* SK5 released from the capsule in SIF pH 7.4 and showed inhibitory activity against gastrointestinal pathogens. After storage at 4 °C for 6 months, the enteric coated capsule showed high survival and good antimicrobial activity of *L. fermentum* SK5. Moreover, *L. fermentum* SK5 lyophilized powder was formulated as hollow-type vaginal suppository using combination of different molecular weight PEGs as a suppository base. The suppository had average weight 2.0785 g, melting point of suppository base 35.07-44.03 °C, the release time of this *Lactobacillus* from the suppository after 25 min and inhibitory activity against bacterial vaginosis pathogens. During storage at 4 °C for 6 months, the suppository had good appearance and *L. fermentum* SK5 from the suppository showed good viability and antimicrobial activity.

L. fermentum SK5 tends to be an effective probiotic candidate for the beneficial use as an enteric coated capsule and a hollow-type vaginal suppository for prevention or treatment of bacterial gastroenteritis and bacterial vaginosis.

BIBLIOGRAPHY

Abadias, M., Benabarre, A., Teixidó, N., Usall, J., Viñas, I. Effect of freeze-drying and protectants on viability of the biocontrol yeast *Candida sake*. International Journal of Food Microbiology. 2001; 65: 173–182.

Adams, M.R., Marteau, P. On the safety of lactic acid bacteria from food. International Journal of Food Microbiology. 1995; 27: 263-264.

Agaliya, P.J., Jeevaratnam, K. Characterisation of the bacteriocins produced by two probiotic *Lactobacillus* isolates from *idli* batter. Annals of Microbiology. 2013; 1-11.

Ahn, C., Thompson, D.C., Duncan, C., Stiles, M.E. Mobilization and location of the genetic determinant of chloramphenicol resistance from *Lactobacillus plantarum* ca TC2R. Plasmid. 1992; 27: 169-176.

Alakomi, H.L., Skytta, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., Helander, I.M. Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. Applied and Environmental Microbiology. 2000; 66: 2001–2005.

Ammor, S., Tauveron, G., Dufour, E., Chevallier, I. Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility: 1--Screening and characterization of the antibacterial compounds. Food Control. 2006; 17(6): 454-461.

Ananta, E., Volkert, M., Knorr, D. Cellular injuries and storage stability of spray-dried *Lactobacillus rhamnosus* GG. International Dairy Journal. 2005; 15: 399–409.

Ansel, H.C. Pharmaceutical dosage form and drug delivery systems. 6th ed. Philadelphia: Williams & Wilkins. 1995; 424-442.

Anukam K.C., Reid G. *Lactobacillus plantarum* and *Lactobacillus fermentum* with probiotic potentials isolated from the vagina of healthy Nigerian women. Research Journal of Microbiology. 2007; 2(1): 81-87.

Arici, M., Bilgina, B., Sagdic, O., Ozdemir, C., Some characteristics of *Lactobacillus* isolates from infant faeces. Food Microbiology. 2004; 21: 19–24.

Atassi, F., Brassart, D., Grob, P., Graf, F., Servin, A.L. *In vitro* antibacterial activity of *Lactobacillus helveticus* strain KS300 against diarrhoeagenic, uropathogenic and vaginosis-associated bacteria. Journal of Applied Microbiology. 2006; 101(3): 647-654.

Banerjee, S.P., Dora, K.C., Chowdhury, S. Detection, partial purification and characterization of bacteriocin produced by *Lactobacillus brevis* FPTLB3 isolated from freshwater fish: bacteriocin from *Lb. brevis* FPTLB3. Journal of Food Science and Technology. 2013; 50(1): 17-25.

Bao, Y., Zhang, Y., Zhang, Y., Liu, Y., Wang, S., Dong, X., Wang, Y., Zhang, H. Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. Food Control. 2010; 21: 695-701.

Barrons, R., Tassone, D. Use of *Lactobacillus* probiotics for bacterial genitourinary infections in women: a review. Clinical Therapeutics. 2008; 30: 453-468.

Blum, S., Reniero, R., Schiffrin, E.J., Crittenden, R., Mattila-Sandholm, T., Ouwehand, A.C., Salminen, S., von Wright, A., Saarela, M., Saxelin, M.,

Collins, K., Morelli, L. Adhesion studies for probiotics: need for validation and refinement. Trends in Food Science & Technology. 1999; 10: 405-410.

Boris, S., Barbés, C. Role played by lactobacilli in controlling the population of vaginal pathogens. Microbes and Infection. 2000; 2: 543-546.

Boris, S., Suárez, J.E., Barbés, C. Characterization of the aggregation promoting factor from *Lactobacillus gasseri*, a vaginal isolate. Journal of Applied Microbiology.1997; 83: 413–420.

Boris, S., Suárez, J.E., Vazquez, F., Barbés, C. Adherence of human lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infection and Immunity. 1998; 66: 1985-1989.

Boskey, E.R., Telsch, K.M., Whaley, K.J., Moench, T.R., Cone, R.A. Acid production by vaginal flora *in vitro* is consistent with the rate and extent of vaginal acidification. Infection and Immunity. 1999; 67: 5170-5175.

British Pharmacopoeia. Uniformity of weight (Ph. Eur. Method 2.9.5) London: The Stationary Office. 2011; V/1: A326.

Calinescu, C., Mateescu, M.A. Carboxymethyl high amylose starch: chitosan self-stabilized matrix for probiotic colon delivery. European Journal of Pharmaceutics and Biopharmaceutics. 2008; 70: 582-589.

Candela, M., Perna, F., Carnevali, P., Vitali, B., Ciati, R., Gionchetti, P., Rizzello, F., Campieri, M., Brigidi, P. Interaction of probiotic *Lactobacillus* and *Bifidobacterium* strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8

production. International Journal of Food Microbiology. 2008; 125(3): 286-292.

Capela, P., Hay, T.K.C., Shah, N.P. Effect of cryoprotectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt. Food Research International. 2006; 39(2): 203-211.

Caplice, E., Fitzgerald, G.F. Food fermentations: role of microorganisms in food production and preservation. International Journal of Food Microbiology. 1999; 50: 131–149.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X., Gibbs, P. Survival of freeze-dried *Lactobacillus plantarum* and *Lactobacillus rhamnosus* during storage in the presence of protectants. Biotechnology Letters. 2002; 24: 1587–1591.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X., Gibbs, P. Protective effect of sorbitol and monosodium glutamate during storage of freeze-dried lactic acid bacteria. Lait. 2003a; 83(3): 203-210.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X., Gibbs, P. Effect of various growth media upon survival during storage of freeze-dried *Enterococcus faecalis* and *Enterococcus durans*. Journal of Applied Microbiology. 2003b; 94(6): 947-952.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X., Gibbs, P. Impedimetric method for estimating the residual activity of freeze-dried *Lactobacillus delbrueckii* ssp. *bulgaricus*. International Dairy Journal. 2003c; 13: 463–468.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X., Gibbs, P. Relevant factors for the preparation of freeze-dried lactic acid bacteria. International Dairy Journal. 2004a; 14: 835-847.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X., Gibbs, P. Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freeze-dried *Lactobacillus delbrueckii* ssp. *bulgaricus*. Biotechnology Progress. 2004b; 20: 248–254.

Castro, H.P., Teixeira, P.M., Kirby, R. Changes in the membrane of *Lactobacillus bulgaricus* during storage following freeze-drying. Biotechnology Letters. 1996; 18: 99–104.

Cebeci, A., Gürakan, C. Properties of potential probiotic *Lactobacillus plantarum* strains. Food Microbiology. 2003; 20: 511-518.

Chan, W.A., Boswell, C.D., Zhang, Z. Comparison of the release profiles of a water soluble drug carried by Eudragit-coated capsules in different *in-vitro* dissolution liquids. Powder Technology. 2001; 119: 26-32.

Charteris, W.P., Kelly, P.M., Morelli, .L, Collins, J.K. Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. Journal of Food Protection. 1998; 61: 1636-1643.

Charteris, W.P., Nelly, P.M., Morelli, L., Collins, J.K. Development of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. Journal of Applied Bacteriology. 1998; 84: 759–768.

Cheikhyoussef, A., Pogori, N., Chen, H., Tian, F., Chen, W., Tang, J., Zhang, H. Antimicrobial activity and partial characterization of bacteriocin-like inhibitory substances (BLIS) produced by *Bifidobacterium infantis* BCRC 14602. Food Control. 2009; 20: 553-559.

Chung, H.S., Kim, Y.B., Chun, S.L., Ji, G.E. Screening and selection of acid and bile resistant bifidobacteria. International Journal of Food Microbiology. 1999; 47: 25-32.

Coconnier, M.H., Lievin, V., Lorrot, M., Servin, A.L. Antagonistic activity of *Lactobacillus acidophilus* LB against intracellular *Salmonella enterica* serovar Typhimurium infecting human enterocyte-like Caco-2/TC-7 cells. Applied and Environmental Microbiology. 2000; 66: 1152–1157.

Collado, M.C., Gueimonde, M., Hernández, M., Sanz, Y., Salminen, S. Adhesion of selected *Bifidobacterium* strains to human intestinal mucus and its role in enteropathogen exclusion. Journal of Food Protection. 2005; 68: 2672-2678.

Collado, M.C., Meriluoto, J., Salminen, S. *In vitro* analysis of probiotic strain combinations to inhibit pathogen adhesion to human intestinal mucus. Food Research International. 2007; 40: 629-636.

Collado, M.C., Meriluoto, J., Salminen, S. Adhesion and aggregation properties of probiotic and pathogen strains. European Food Research and Technology. 2008; 226: 1065–1073.

Collado, M.C., Meriluoto, J., Salminen, S. Probiotics in adhesion of pathogens: mechanisms of action. Bioactive foods in promoting health: probiotics and

prebiotics. Watson, R.R., Preedy, V.R. (eds.). Academic Press, Elsevier Inc., Chennai, India. 2010; 353-370.

Collett, D.M. Suppositories and Pessaries. Collett, D.M., Aulton, M.E. (eds.). Pharmaceutical Practice. Hong Kong, Churchill Livingstone. 1990; 135-144.

Corr, S.C., Li, Y., Riedel, C.U., O'Toole, P.W., Hill, C., Gahan, C.G. Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104: 7617–7621.

Costa, E., Usall, J., Teixidó, N., Garcia, N., Viñas, I. Effect of protective agents rehydration media and initial cell concentration on viability of Pantoea agglomerans strain CPA-2 subjected to freeze-drying. Journal of Applied Microbiology. 2000; 89(5): 793-800.

Curragh, H.J., Collins, M.A. High levels of spontaneous drug resistance in *Lactobacillus*. Journal of Applied Bacteriology. 1992; 73: 31-36.

De Giulio, B., Orlando, P., Barba, G., Coppola, R., De Rosa, M., Sada, A., De Prisco, P.P., Nazzaro, F. Use of alginate and cryo-protective sugars to improve the viability of lactic acid bacteria after freezing and freeze-drying. World Journal of Microbiology and Biotechnology. 2005; 21(5): 739-746.

Del Re, B., Sgorbati, B., Miglioli, M., Palenzona, D. Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Letters in Applied Microbiology. 2000; 31: 438–442.

Deraz, S.F., Karlsson, E.N., Hedstrom, M., Andersson, M.M., Mattiasson, B. Purification and characterisation of acidocin D20079, a bacteriocin produced by *Lactobacillus acidophilus* DSM 20079. Journal of Biotechnology. 2005; 117: 343-354.

Desmond, C., Ross, R.P., O'Callaghan, E., Fitzgerald, G., Stanton, C. Improved survival of *Lactobacillus paracasei* NFBC 338 in spray-dried powders containing gum acacia. Journal of Applied Microbiology. 2002; 93: 1003–1011.

Dhanani, A.S., Bagchi, T. *Lactobacillus plantarum* CS24.2 prevents *Escherichia coli* adhesion to HT-29 cells and also down-regulates enteropathogen-induced tumor necrosis factor-α and interleukin-8 expression. Microbiology and Immunology. 2013; 57(4): 309-315.

Donders, G.G.G. Definition and classification of abnormal vaginal flora. Best Practice & Research Clinical Obstetrics & Gynaecology. 2007; 21: 355-373.

Dryden, M.A., Gabb, R.J.E., Wright, S.K. Empirical treatment of severe acute community acquired gastroenteritis with ciprofloxacin. Clinical Infectious Diseases. 1996; 22: 1019-1025.

Duary, R.K., Rajput, Y.S., Batish, V.K., Grover, S. Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. Indian Journal of Medical Research. 2011; 134: 664-671.

Edgeworth, J.D. Bacterial gastroenteritis. Medicine. 2005; 33: 73-77.

Egervarn, M. Antibiotic resistance in *Lactobacillus reuteri* and *Lactobacillus plantarum*. Doctoral Thesis, Swedish University of Agricultural Sciences Uppsala. 2009.

Erkkila, S., Petaja, E. Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. Meat Science. 2000; 55: 297-300.

Eschenbach, D.A., Davick, P.R., Williams, B.L., Klenanoff, S.J., Young-Smith, K., Critchlow, C.M., Holmes, K.K. Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. Journal of Clinical Microbiology. 1989; 27: 251-256.

FAO/WHO. Evaluation of health and nutritional properties of probiotics in food, including powder milk with live lactic acid bacteria. Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report. 2001; 1-34.

Fons, M., Hege, T., Ladire, M., Raibaud, P., Ducluzeau, R., Maguin, E. Isolation and characterization of a plasmid from *Lactobacillus fermentum* conferring erythromycin resistance. Plasmid. 1997; 37: 199-203.

Gagnon, M., Kheadr, E.E., Le Blay, G., Fliss, I. *In vitro* inhibition of *Escherichia coli* 015:H7 by bifidobacterial strains of human origin. International Journal of Food Microbiology. 2004; 92: 69-78.

Gazzaniga, A., Maroni, A., Sangalli, M.E., Zema, L. Time-controlled oral delivery systems for colon targeting. Expert Opinion on Drug Delivery. 2006; 3: 583-597.

George, M., Abraham, T.E. Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan – A review. Journal of Controlled Release. 2006; 114: 1–14.

Ghanbari, M., Jami, M., Kneifel, W., Domig, K.J. Antimicrobial activity and partial characterization of bacteriocins produced by lactobacilli isolated from Sturgeon fish. Food Control. 2013; 32(2): 379-385.

Gibson, G.R., Roberfroid, M.R. Dietary modulation of the human colonic microbial: Introducing the concept of prebiotics. Journal of Nutrition. 1995; 125: 1401-1412.

Goldin, B., Gorbach, S. Probiotics for humans. Fuller, R. (ed.). Probiotics-the scientific basis. London: Chapman and Hall. 1992; 355-376.

Gopal, P.K., Prasad, J., Smart, J., Gill, H.S. *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. International Journal of Food Microbiology. 2001; 67: 207-216.

Granato, D., Perotti, F., Masserey, I., Rouvet, M., Golliard, M., Servin, A., Brassart, D. Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. Applied and Environmental Microbiology. 1999; 65: 1071-1077.

Gueimonde, M., Jalonen, L., He, F., Hiramatsu, M., Salminen, S. Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli. Food Research International. 2006; 39: 467-471.

Hay, P. Bacterial vaginosis. Medicine. 2005; 33: 58-61.

Hallen, A., Jarstran, C., Pahlson, C. Treatment of bacterial vaginosis with lactobacilli. Sexually Transmitted Diseases. 1992; 19: 146–148.

Hernandez-Hernandez, O., Muthaiyan, A., Moreno, F.J., Montilla, A., Sanz, M.L., Ricke, S.C. Effect of prebiotic carbohydrates on the growth and tolerance of *Lactobacillus*. Food Microbiology. 2012; 30(2): 355-361.

Higl, B., Kurtmann, L., Carlsen, C.U., Ratjen, J., Forst, P., Skibsted, L.H., Kulozik, U., Risbo, J. Impact of water activity, temperature and physical state on the storage stability of *Lactobacillus paracasei* ssp. *paracasei* freeze-dried in a lactose matrix, Biotechnology Progress. 2007; 23: 794–800.

Holzapfel, W.H., Haberer, P., Snel, J., Schillinger, U., Huis in't Veld, J.H.J. Overview of gut flora and probiotics. International Journal of Food Microbiology. 1998; 41: 85-101.

Hongpattarakere, T., Rattanaubon, P., Buntin, N. Improvement of freeze-dried *Lactobacillus plantarum* survival using water extracts and crude fibers from food crops. Food and Bioprocess Technology. 2012: 1-12.

Hussain, A., Ahsan, F. The vagina as a route for systemic drug delivery. Journal of Controlled Release. 2005; 103: 301-313.

Iannitti, T., Palmieri, B. Therapeutical use of probiotic formulations in clinical practice. Clinical Nutrition. 2010; 29: 701-725.

Ishiwa, H., Iwata, S. Drug resistance plasmids in *Lactobacillus fermetum*. Journal of General and Applied Microbiology. 1980; 26: 71-74.

Jack, R.W., Tagg, J.R., Ray, B. Bacteriocins of Gram-positive bacteria. Microbiological Reviews. 1995; 59: 171–200.

Jalali, M., Abedi, D., Varshosaz, J., Najjarzadeh, M., Mirlohi, M., Tavakoli, N. Stability evaluation of freeze-dried *Lactobacillus paracasei* subsp. *tolerance* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in oral capsules. Research in Pharmaceutical Sciences. 2012; 7(1): 31-36.

Jara, S., Sánchez, M., Vera, R., Cofré, J., Castro, E. The inhibitory activity of *Lactobacillus* spp. isolated from breast milk on gastrointestinal pathogenic bacteria of nosocomial origin. Anaerobe. 2011; 17(6): 474-477.

Jena, P.K., Trivedi, D., Chaudhary, H., Sahoo, T.K., Seshadri, S. Bacteriocin PJ4 active against enteric pathogen produced by *Lactobacillus helveticus* PJ4 isolated from gut microflora of wistar rat (*Rattus norvegicus*): partial purification and characterization of bacteriocin. Applied Biochemistry and Biotechnology. 2013; 169: 2088-2100.

Jensen, H., Grimmer, S., Naterstad, K., Axelsson, L. *In vitro* testing of commercial and potential probiotic lactic acid bacteria. International Journal of Food Microbiology. 2012; 153: 216–222.

Ji, Y., Kim, H., Park, H., Lee, J., Lee, H., Shin, H., Kim, B., Franz, C.M.A.P., Holzapfel, W.H. Functionality and safety of lactic bacterial strains from Korean kimchi. Food Control. 2013; 31(2): 467-473.

Juárez Tomás, M.S., Ocaña, V.S., Wiese, B., Nader-Macías, M.E. Growth and lactic acid production by vaginal *Lactobacillus acidophilus* CRL 1259 and inhibition of uropathogenic *Escherichia coli*. Journal of Medical Microbiology. 2003; 52: 1117-1124.

Juárez Tomás, M.S., Saralegui Duhart, C.I., De Gregorio, P.R., Vera Pingitore, E., Nader-Macías, M.E. Urogenital pathogen inhibition and compatibility between vaginal *Lactobacillus* strains to be considered as probiotic candidates. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2011; 159: 399-406.

Kaewsrichan, J., Chandarajoti, K., Kaewnopparat, S., Kaewnopparat, N. Evaluation of Lactobacilli containing suppository formulation for probiotic use. Mahidol University Journal of Pharmaceutical Sciences. 2007; 34: 1-8.

Kale, V.V., Trivedi, R.V., Wate, S.P., Bhusari, K.P. Development and evaluation of a suppository formulation containing *Lactobacillus* and its application in vaginal diseases. Annals New York Academy of Sciences. 2005; 1056: 359-365.

Kaur, B., Balgir, P., Mittu, B., Chauhan, A., Kumar, B. Purification and physicochemical characterization of anti-gardnerella vaginalis bacteriocin hv6b produced by *Lactobacillus fermentum* isolate from human vaginal

ecosystem. American Journal of Biochemistry and Molecular Biology. 2013; 3(1): 91-100.

Khler, G.A., Assefa, S., Reid, G. Probiotic interference of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 with the opportunistic fungal pathogen *Candida albicans*. Infectious Diseases in Obstetrics and Gynecology. 2012; 2012: 1-14.

Kirtzalidou, E., Pramateftaki, P., Kotsou, M., Kyriacou, A. Screening for lactobacilli with probiotic properties in the infant gut microbiota. Anaerobe. 2011; 17: 440-443.

Knarreborg, A., Engberg, R.M., Jensen, S.K., Jensen, B.B. Quantitative determination of bile salt hydrolase activity in bacteria isolated from the small intestine of chickens. Applied and Environmental Microbiology. 2002; 68: 6425-6428.

Kos, B., Suskovic, J., Vukovic, S., Simpraga, M., Frece, J., Matosic, S. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. Journal of Applied Microbiology. 2003; 94: 981–987.

Kotzamanidis, C., Kourelis, A., Litopoulou-Tzanetaki, E., Tzanetakis, N., Yiangou, M. Evaluation of adhesion capacity, cell surface traits and immunomodulatory activity of presumptive probiotic *Lactobacillus* strains. International Journal of Food Microbiology. 2010; 140: 154-163.

Lavilla-Lerma, L., Pérez-Pulido, R., Martínez-Bueno, M., Maqueda, M., Valdivia, E. Characterization of functional, safety, and gut survival related characteristics

of *Lactobacillus* strains isolated from farmhouse goat's milk cheeses. International Journal of Food Microbiology. 2013; 163(2-3): 136-145.

Lebeer, S., Vanderleyden, J., De Keersmaecker, S.C.J. Genes and molecules of Lactobacilli supporting probiotic action. Microbiology and Molecular Biology Reviews. 2008; 72: 728–764.

Lee, H., Yoon, H., Ji, Y., Kim, H., Park, H., Lee, J., Shin, H., Holzapfel, W. Functional properties of *Lactobacillus* strains isolated from kimchi. International Journal of Food Microbiology. 2011; 145(1): 155-161.

Lee, J., Yun, H.S., Cho, K.W., Oh, S., Kim, S.H., Chun, T., Kim, B., Whang, K.Y. Evaluation of probiotic characteristics of newly isolated *Lactobacillus* spp.: immune modulation and longevity. International Journal of Food Microbiology. 2011; 148(2): 80-86.

Lee, S.J., Shim, Y.H., Cho, S.J., Lee, J.W. Probiotics prophylaxis in children with persistent primary vesicoureteral reflux. Pediatric Nephrology. 2007; 22: 1315-1320.

Lee, Y.K., Lim, C.Y., Teng, W.L., Ouwehand, A.C., Tuomola, E.M., Salminen, S. Quantitative approach in the study of adhesion of lactic acid bacteria to intestinal cells and their competition with enterobacteria. Applied and Environmental Microbiology. 2000; 66: 3692-3697.

Lee, Y.K., Puong, K.Y., Ouwehand, A.C., Salminen, S. Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. Journal of Medical Microbiology. 2003; 52: 925-930.

Lewandowska, M., Olejnik, A., Neumann, M., Krêpulec, A., Piotrowska, J., Teresiak, A., GrajekLin, W. Comparative *in vitro* study on the adhesion of probiotic and pathogenic bacteria to different human intestinal cell lines. Biotechnologia. 2005; 2: 215-233.

Li, B., Tian, F., Liu, X., Zhao, J., Zhang, H., Chen, W. Effects of cryoprotectants on viability of *Lactobacillus reuteri* CICC6226. Applied Microbiology and Biotechnology. 2011; 92(3): 609-616.

Li, C., Zhao, J.L., Wang, Y.T., Han, X., Liu, N. Synthesis of cyclopropane fatty acid and its effect on freeze-drying survival of *Lactobacillus bulgaricus* L2 at different growth conditions. World Journal of Microbiology and Biotechnology. 2009; 25(9): 1659-1665.

Liévin-LeMoal, V., Amsellem, R., Servin, A.L., Coconnier, M.H. *Lactobacillus acidophilus* (strain LB) from the resident adult human gastrointestinal microflora exerts activity against brush border damage promoted by a diarrhoeagenic *Escherichia coli* in human enterocyte-like cells. Gut. 2002; 50: 803–811.

Lin, C.-K., Tsai, H.-C., Lin, P.-P., Tsen, H.-Y., Tsai, C.-C. *Lactobacillus acidophilus* LAP5 able to inhibit the *Salmonella choleraesuis* invasion to the human Caco-2 epithelial cell. Anaerobe. 2008; 14: 251-255.

Lin, L., Yang, H.-I., Su, J. Vaginal lactobacillus distributions and functions in pregnant women. African Journal of Microbiology Research. 2011; 5: 1224-1230.

Lin, P.-P., Hsieh, Y.-M., Tsai, C.-C. Antagonistic activity of *Lactobacillus acidophilus* RY2 isolated from healthy infancy feces on the growth and adhesion characteristics of enteroaggregative *Escherichia coli*. Anaerobe. 2009; 15: 122-126.

Liong, M.T., Shah, N.P. Bile salt deconjugation and BSH activity of five bifidobacterial strains and their cholesterol co-precipitating properties. Food Research International. 2005; 38: 135-142.

Liu, G., Lv, Y., Li, P., Zhou, K., Zhang, J. Pentocin 31-1, an anti-*Listeria* bacteriocin produced by *Lactobacillus pentosus* 31-1 isolated from Xuan-Wei Ham, a traditional China fermented meat product. Food Control. 2008; 19: 353-359.

Maragkoudakis, P.A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., Tsakalidou, E. Probiotic potential of *Lactobacillus* strains isolated from dairy products. International Dairy Journal. 2006; 16: 189-199.

Marianelli, C., Cifani, N., Pasquali, P. Evaluation of antimicrobial activity of probiotic bacteria against *Salmonella enterica* subsp. *enterica* serovar typhimurium 1344 in a common medium under different environmental conditions. Research in Microbiology. 2010; 161: 673-680.

Marteau, P., Seksik, P., Jian, R. Probiotics and intestinal health: a clinical perspective. British Journal of Nutrition. 2002; 88: s51-s57.

Martín, R., Sánchez, B., Suárez, J.E., Urdaci, M.C. Characterization of the adherence properties of human Lactobacilli strains to be used as vaginal probiotics. FEMS Microbiology Letters. 2012; 328(2): 166-173.

Martinez, R.C.R., Aynaou, A.-E., Albrecht, S., Schols, H.A., De Martinis, E.C.P., Zoetendal, E.G. Venema, K., Saad, S.M.I., Smidt, H. *In vitro* evaluation of gastrointestinal survival of *Lactobacillus amylovorus* DSM 16698 alone and combined with galactooligosaccharides, milk and/or *Bifidobacterium animalis* subsp. *lactis* Bb-12. International Journal of Food Microbiology. 2011; 149: 152-158.

Mastromarino, P., Brigidi, P., Macchia, S., Maggi, L., Pirovano, F., Trinchieri, V., Conte, U., Matteuzzi, D. Characterization and selection of vaginal *Lactobacillus* strains for the preparation of vaginal tablets. Journal of Applied Microbiology. 2002; 93: 884-893.

Mathur, S., Singh, R. Antibiotic resistance in food lactic acid bacteria-A review. International Journal of Food Microbiology. 2005; 105: 281-295.

Matijašić, B.B., Narat, M., Peternel, M.Z., Rogelj, I. Ability of *Lactobacillus gasseri* K7 to inhibit *Escherichia coli* adhesion *in vitro* on Caco-2 cells and *ex vivo* on pigs' jejunal tissue. International Journal of Food Microbiology. 2006; 107: 92-96.

Matu, M.N., Orinda, G.O., Njagi, E.N.M., Cohen, C.R., Bukusi, E.A. *In vitro* inhibitory activity of human vaginal lactobacilli against pathogenic bacteria associated with bacterial vaginosis in Kenyan women. Anaerobe. 2010; 16: 210-215.

McGinity, J.W., Felton, L.A. (Eds.). Aqueous polymeric coatings for pharmaceutical dosage forms, third ed. Informa Healthcare, New York. 2008.

McLean, N.W., Rosenstein, I.J. Characterization and selection of a *Lactobacillus* species to re-colonise the vagina of women with recurrent bacterial vaginosis. Journal of Medical Microbiology. 2000; 49: 543-552.

McMillan, A., Dell, M., Zellar, M.P., Cribby, S., Martz, S., Hong, E., Fu, F., Abbas, A., Dang, T., Miller, W., Reid, G. Disruption of urogenital biofilms by lactobacilli. Colloids and Surfaces B: Biointerfaces. 2011; 86: 58-64.

McNaught, C.E., MacFie, J. Probiotics in clinical practice: a critical review of the evidence. Nutrition Research. 2001; 21: 343-353.

Melis, G.B., Ibba, M.T., Steri, B., Kotsonis, P., Matta, V., Paoletti, A.M. Role of pH as a regulator of vaginal physiological environment. Minerva Ginecologica. 2000; 52: 111–121.

Meng, X.C., Stanton, C., Fitzgerald, G.F., Daly, C., Ross, R.P. Anhydrobiotics: the challenges of drying probiotic cultures. Food Chemistry. 2008; 106: 1406–1416.

Merk, K., Borelli, C., Korting, H.C. *Lactobacilli* - bacteria-host interactions with special regard to the urogenital tract. International Journal of Medical Microbiology. 2005; 295: 9-18.

Messaoudi, S., Kergourlay, G., Dalgalarrondo, M., Choiset, Y., Ferchichi, M., Prèvost, H., Pilet, M.-F., Chobert, J.-M., Manai, M., Dousset, X. Purification and characterization of a new bacteriocin active against

Campylobacter produced by *Lactobacillus salivarius* SMXD51. Food Microbiology. 2012; 32(1): 129-134.

Montville, T.J., Winkowski, K., Ludescher, R.D. Models and mechanisms for bacteriocin action and application International Dairy Journal. 1995; 5: 797-814.

Morelli, L. *In vitro* selection of probiotic Lactobacilli: a critical appraisal. Current Issues in Intestinal Microbiology. 2000; 1: 59-67.

Morelli, L. *In vitro* assessment of probiotic bacteria: from survival to functionality. International Dairy Journal. 2007; 17: 1278-1283.

Morgan, C.A., Herman, N., White, P.A., Vesey, G. Preservation of micro-organisms by drying: a review. Journal of Microbiological Methods. 2006; 66: 183-193.

Muench, D.F., Kuch, D.J., Wu, H., Begum, A.A., Veit, S.J., Pelletier, M.E., Soler-García, A.A., Jerse, A.E. Hydrogen peroxide-producing lactobacilli inhibit gonococci *in vitro* but not during experimental genital tract infection. The Journal of Infectious Diseases. 2009; 199: 1369-1378.

Nag, A., Das, S. Effect of trehalose and lactose as cryoprotectant during freeze-drying, *in vitro* gastro-intestinal transit and survival of microencapsulated freeze-dried *Lactobacillus casei* 431 cells. International Journal of Dairy Technology. 2013; 66(2): 162-169.

Nakpheng, T., Kha, R., Balekar, N., Kaewnopparat, S., Srichana, T. *Lactobacillus plantarum* T23/3 as a potential probiotic strain isolated from feces. Thai Journal of Pharmaceutical Sciences. 2012; 36(3): 83-93.

Nguyen, T.D.T., Kang, J.H., Lee, M.S. Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. International Journal of Food Microbiology. 2007; 113: 358-361.

Nordmann, P., Poirel, L. Emergence of plasmid-mediated resistance to quinolones in *Enterobacteriaceae*. Journal of Antimicrobial Chemotherapy. 2005; 56: 463-469.

Oelschlaeger, T.A. Mechanisms of probiotic actions – A review. International Journal of Medical Microbiology. 2010; 300: 57-62.

Oldenhof, H., Wolkers, W., Fonseca, F., Passot, S., Marin, M. Effect of sucrose and maltodextrin on the physical properties and survival of air-dried *Lactobacillus bulgaricus*: an in situ Fourier Transform Infrared Spectroscopy study. Biotechnology Progress. 2005; 21: 885–892.

O'Shea, E.F., Cotter, P.D., Stanton, C., Ross, R.P., Hill, C. Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanisms: bacteriocins and conjugated linoleic acid. International Journal of Food Microbiology. 2012; 152(3): 189-205.

Ouwehand, A.C., Salminen, S., Isolauri, E. Probiotics: an overview of beneficial effects. Antonie van Leeuwenhoek. 2002; 82: 279–289.

Pan, X., Chen, F., Wu, T., Tang, H., Zhao, Z. The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT. Food Control. 2009; 20: 598-602.

Pascual, L.M., Daniele, M.B., Pajaro, C., Barberis, L. *Lactobacillus* species isolated from the vagina: identification, hydrogen peroxide production and nonoxynol-9 resistance. Contraception. 2006; 73(1): 78-81.

Pascual, L.M., Daniele, M.B., Ruiz, F., Giordano, W., Pájaro, C., Barberis, L. *Lactobacillus rhamnosus* L60, a potential probiotic isolated from the human vagina. Journal of General and Applied Microbiology. 2008; 54: 141-148.

Pelletier, C., Bouley, C., Cayuela, C., Bouttier, S., Bourlioux, P., Bellon-Fontaine, M.N. Cell surface characteristics of *Lactobacillus casei* subsp. *casei*, *Lactobacillus paracasei* subsp. paracasei, and *Lactobacillus rhamnosus* strains. Applied and Environmental Microbiology. 1997; 63: 1725-1731.

Pennacchia, C., Vaughan, E.E., Villani, F. Potential probiotic *Lactobacillus* strains from fermented sausages: further investigations on their probiotic properties. Meat Science. 2006; 73: 90-101.

Pei, J., Yuan, Y., Yue, T. Characterization of bacteriocin bificin C6165: a novel bacteriocin. Journal of Applied Microbiology. 2013; 34: 168-176.

Pina, M.E., Sousa, A.T., Brojo, A.P. Enteric coating of hard gelatin capsules. Part 1. Application of hydroalcoholic solutions of formaldehyde in preparation of gastro-resistant capsules. International Journal of Pharmaceutics. 1996; 133: 139-148.

Ponce, A.G., Moreira, M.R., del Valle, C.E., Roura, S.I. Preliminary characterization of bacteriocin-like substances from lactic acid bacteria isolated from organic leafy vegetables. LWT - Food Science and Technology. 2008; 41: 432-441.

Pridmore, R.D., Pittet, A.C., Praplan, F., Cavadini ,C. Hydrogen peroxide production by *Lactobacillus johnsonii* NCC 533 and its role in anti-*Salmonella* activity. FEMS Microbiology Letters. 2008; 283(2): 210-215.

Pruden, A., Pie, R., Storteboom, H., Carlson, K.H. Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. Environmental Science and Technology. 2006; 40: 7445-7450.

Ramiah, K., van Reenen, C.A., Dicks, L.M.T. Surface-bound proteins of *Lactobacillus plantarum* 423 that contribute to adhesion of Caco-2 cells and their role in competitive exclusion and displacement of *Clostridium sporogenes* and *Enterococcus faecalis*. Research in Microbiology. 2008; 159: 470-475.

Reid, G., Bocking, A. The potential for probiotics to prevent bacterial vaginosis and preterm labor. American Journal of Obstetrics and Gynecology. 2003; 189: 1202-1208.

Reid, G., Bruce, A.W., Fraser, N., Heinemann, C., Owen, J., Henning, B. Oral probiotics can resolve urogenital infections. FEMS Immunology and Medical Microbiology. 2001a; 30: 49-52.

Reid, G., Beuerman, D., Heinemann, C., Bruce, A.W. Probiotic *Lactobacillus* dose required to restore and maintain a normal vaginal flora. FEMS Immunology and Medical Microbiology. 2001b; 32(1): 37-41.

Reid, G., Howard, J., Gan, B.S. Can bacterial interference prevent infection? Trends in Microbiology. 2001c; 9(9): 424-428.

Reid, G., Bruce, A.W., Taylor, M. Influence of three-day antimicrobial therapy and lactobacillus vaginal suppositories on recurrence or urinary tract infections. Clinical Therapeutics. 1992; 14(1): 11-16.

Reid, G., Burton, J. Use of *Lactobacillus* to prevent infection by pathogenic bacteria. Microbes and Infection. 2002; 4: 319-324.

Reid, G., Charbonneau, D., Erb, J., Kochanowski, B., Beuerman, D., Poehner, R., Bruce, A.W. Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. FEMS Immunology and Medical Microbiology. 2003; 35: 131-134.

Ren, D., Li, C., Qin, Y., Yin, R., Li, X., Tian, M., Du, S., Gou, H., Liu, C., Zhu, N., Sun, D., Li, Y., Jin, N. Inhibition of *Staphylococcus aureus* adherence to Caco-2 cells by lactobacilli and cell surface properties that influence attachment. Anaerobe. 2012; 18(5): 508-515.

Rezaei, R., Khomeiri, M., Aalami, M., Kashaninejad, M. Effect of inulin on the physicochemical properties, flow behavior and probiotic survival of frozen yogurt. Journal of Food Science and Technology. 2012; 1-6.

Rojas, M., Ascencio, F., Conway, P.L. Purification and characterization of a surface protein from *Lactobacillus fermentum* 104R that binds to porcine small intestinal mucus and gastric mucin. Applied and Environmental Microbiology. 2002; 68: 2330-2336.

Roos, S., Jonsson, H. High-molecular mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. Microbiology. 2002; 148: 433-442.

Ross, R.P., Morgan, S., Hill, C. Preservation and fermentation: past, present and future. International Journal of Food Microbiology. 2002; 79: 3-16.

Røssland, E., Langsrud, T., Granum, P.E., Sørhaug, T. Production of antimicrobial metabolites by strains of *Lactobacillus* or *Lactococcus* co-cultured with *Bacillus cereus* in milk. International Journal of Food Microbiology. 2005; 98: 193-200.

Rushdy, A.A., Gomaa, E.Z. Antimicrobial compounds produced by probiotic *Lactobacillus brevis* isolated from dairy products. Annals of Microbiology. 2013; 63(1): 81-90.

Ryu, E.H., Chang, H.C. *In vitro* study of potentially probiotic lactic acid bacteria strains isolated from kimchi. Annals of Microbiology. 2013; 1-9.

Saarela, M., Virkajärvi, I., Nohynek, L., Vaari, A., Mättö, J. Fibres as carriers for *Lactobacillus rhamnosus* during freeze-drying and storage in apple juice and chocolate-coated breakfast cereals. International Journal of Food Microbiology. 2006; 112(2): 171-178.

Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W.M., Fonde'n, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S.E., Sandholm, T.M. Demonstration of safety of probiotics - a review. International Journal of Food Microbiology. 1998; 44: 93-106.

Santiago, G.L.dS., Verstraelen, H., Poelvoorde, N., Corte, S.D., Claeys, G., Trog, M., Backer, E.D., Saerens, B., Vervaet, C., Boeck, F.D., Bortel, L.V., Remon, J.P., Temmerman, M., Vaneechoutte, M., Verhelst, R. A pilot study evaluating the safety of vaginal administration of a multi-particulate pellet formulation. European Journal of Pharmaceutics and Biopharmaceutics. 2009; 73(3): 399-403.

Santivarangkna, C., Kulozik, U., Foerst, P. Alternative drying processes for the industrial preservation of lactic acid starter cultures. Biotechnology Progress. 2007; 23: 302–315.

Saunders, S., Bocking, A., Challis, J., Reid, G. Effect of *Lactobacillus* challenge on *Gardnerella vaginalis* biofilms. Colloids and Surfaces B: Biointerfaces. 2007; 55(2): 138-142.

Savini, M., Cecchini, C., Verdenelli, M.C., Silvi, S., Orpianesi, C., Cresci, A. Pilot-scale production and viability analysis of freeze-dried probiotic bacteria using different protective agents. Nutrients. 2010; 2(3): 330-339.

Schwab, C., Vogel, R., Gänzle, M.G. Influence of oligosaccharides on the viability and membrane properties of *Lactobacillus reuteri* TMW1.106 during freeze-drying. Cryobiology. 2007; 55(2): 108-114.

Schwarz, S., Chaslus-Dancla, E. Use of antimicrobials in veterinary medicine and mechanisms of resistance. Veterinary Research. 2001; 32: 201-225.

Selmer-Olsen, E., Birkeland, S.-E., Sørhaug, T. Effect of protective solutes on leakage from and survival of immobilized *Lactobacillus* subjected to drying, storage and rehydration. Journalof Applied Microbiology. 1999; 87: 429–437.

Schillinger, U., Guigas, C., Holzapfel, W.H. *In vitro* adherence and other properties of lactobacilli used in probiotic yoghurt-like products. International Dairy Journal. 2005; 15: 1289-1297.

Shalev, E. Battino, S., Weiner, E., Colodner, R., Keness, Y. Ingestion of yogurt containing *Lactobacillus acidophilus* compared with pasteurised yogurt as prophylaxis for recurrent candidial vaginitis and bacterial vaginosis. Archives of Family Medicine. 1996; 5: 593-596.

Sharafi, H., Maleki, H., Ahmadian, G., Zahiri, H.S., Sajedinejad, N., Houshmand, B., Vali, H., Noghabi, K.A. Antibacterial activity and probiotic potential of *Lactobacillus plantarum* HKN01: A new insight into the morphological changes of antibacterial compound-treated *Escherichia coli* by electron

microscopy. Journal of Microbiology and Biotechnology. 2013; 23(2): 225-236.

Srinivasan, R., Kumawat, D.K., Kumar, S., Saxena, A.K. Purification and characterization of a bacteriocin from *Lactobacillus rhamnosus* L34. Annals of Microbiology. 2013; 63(1): 387-392.

Stöber, H., Maier, E., Schmidt, H. Protective effects of Lactobacilli, Bifidobacteria and Staphylococci on the infection of cultured HT29 cells with different enterohemorrhagic *Escherichia coli* serotypes are strain-specific. International Journal of Food Microbiology. 2010; 144(1): 133-140.

Strasser, S., Neureiter, M., Geppl, M., Braun, R., Danner, H. Influence of lyophilization, fluidized bed drying, addition of protectants, and storage on the viability of lactic acid bacteria. Journal of Applied Microbiology. 2009; 107(1): 167-177.

Succi, M., Tremonte, P., Reale, A., Sorrentino, E., Grazia, L., Pacifico, S., Coppola, R. Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. FEMS Microbiology Letters. 2005; 244: 129-137.

Tannock, G.W., Luchansky, J.B., Miller, L., Connell, H., Thode- Andersen, S., Mercer, A.A., Klaenhammer, T.R. Molecular characterization of a plasmid-borne (pGT633) erythromycin resistance determinant (ermGT) from *Lactobacillus reuteri* 100- 63. Plasmid. 1994; 31: 60-71.

Trivedi, D., Jena, P.k., Patel, J.k., Seshadri, S. Partial purification and characterization of a bacteriocin DT24 produced by probiotic vaginal *Lactobacillus brevis* DT24 and determination of its anti-uropathogenic *Escherichia coli* potential. Probiotics and Antimicrobial Proteins. 2013; 1-10.

Tropcheva, R., Georgieva, R., Danova, S. Adhesion ability of *Lactobacillus plantarum* AC131. Biotechnology & Biotechnological Equipment. 2011; 25: S121-S124.

Tsai, C.-C., Lin, P.-P., Hsieh, Y.-M. Three *Lactobacillus* strains from healthy infant stool inhibit enterotoxigenic *Escherichia coli* grown *in vitro*. Anaerobe. 2008; 14: 61-67.

Tompkins, T.A., Mainville, I., Arcand, Y. The impact of meals on a probiotic during transit through a model of the human upper gastrointestinal tract. Beneficial Microbes. 2011; 2(4): 295-303.

Turchi, B., Mancini, S., Fratini, F., Pedonese, F., Nuvoloni, R., Bertelloni, F., Ebani, V.V. Cerri, D. Preliminary evaluation of probiotic potential of *Lactobacillus plantarum* strains isolated from Italian food products. World Journal of Microbiology and Biotechnology. 2013; 1-10.

Tymczyszyn, E.E., Gerbino, E., Illanes, A., Gómez-Zavaglia, A. Galacto-oligosaccharides as protective molecules in the preservation of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Cryobiology. 2011; 62: 123-129.

Valenta, C. The use of mucoadhesive polymers in vaginal delivery. Advanced Drug Delivery Reviews. 2005; 57: 1692-1712.

Valore, E.V., Park, C.H., Igreti, S.L., Ganz, T. Antimicrobial components of vaginal fluid. American Journal of Obstetrics and Gynecology. 2002; 187: 561-568.

Vasiljevic, T., Shah, N.P. Probiotics--From Metchnikoff to bioactives. International Dairy Journal. 2008; 18: 714-728.

Vera Pingitore, E., Hébert, E.M., Nader-Macías, M.E., Sesma, F. Characterization of salivaricin CRL 1328, a two-peptide bacteriocin produced by *Lactobacillus salivarius* CRL 1328 isolated from the human vagina. Research in Microbiology. 2009; 160(6): 401-408.

Vielfort, K., Sjolinder, H., Roos, S., Jonsson, H., Aro, H. Adherence of clinically isolated lactobacilli to human cervical cells in competition with *Neisseria gonorrhoeae*. Microbes and Infection. 2008; 10: 1325-1334.

Watanabe, Y., Matsumoto, M. Pharmaceutical evaluation of hollow type suppository. I. Brilliant blue FCF release characteristics of oleagenous hollow type suppository. Journal of the Pharmaceutical Society of Japan. 1984; 104: 479-484.

Watanabe, Y., Matsumoto, Y., Baba, K., Matsumoto, M. Pharmaceutical evaluation of hollow type suppositories. IV. Improvement of bioavailability of propranolol in rabbits after rectal administration. Journal of Pharmacobio-dynamics. 1986; 9: 526-531.

Woo, J., Ahn, J. Probiotic-mediated competition, exclusion and displacement in biofilm formation by food-borne pathogens. Letters in Applied Microbiology. 2013; 56(4): 307-313.

Xu, H.-Y., Tian, W.-H., Wan, C.-X., Jia, L.-J., Wang, L.-Y., Yuan, J., Liu, C.-M., Zeng, M., Wei, H. Antagonistic potential against pathogenic microorganisms and hydrogen peroxide production of indigenous Lactobacilli isolated from vagina of Chinese pregnant women. Biomedical and Environmental Sciences. 2008; 21: 365-371.

Yang, E., Fan, L., Jiang, Y., Doucette, C., Fillmore, S. Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. AMB Express. 2012; 2(1): 1-12.

Yu, Z., Zhang, X., Li, S., Li, C., Li, D., Yang, Z. Evaluation of probiotic properties of *Lactobacillus plantarum* strains isolated from Chinese sauerkraut. World Journal of Microbiology and Biotechnology. 2013; 29(3): 489-498.

Zabihollahi, R., Motevaseli, E., Sadat, S.M., Azizi-Saraji, A.R., Asaadi-Dalaie, S., Modarressi, M.H. Inhibition of HIV and HSV infection by vaginal lactobacilli *in vitro* and *in vivo*. DARU, Journal of Pharmaceutical Sciences. 2012; 20(1).

Zarate, G., Chaia, A.P., Gonzalez, S., Oliver, G. Viability and beta-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. Journal of Food Protection. 2000; 63: 1214–1221.

Zaráte, G., Nader-Macias, M.E. Viability and biological properties of probiotic vaginal lactobacilli after lyophilization and refrigerated storage into gelatin capsules. Process Biochemistry. 2006; 41: 1779-1785.

Zareba, T.W., Pascu, C., Hryniewicz, W., Waldström, T. Binding of extracellular matrix proteins by enterococci. Current Microbiology. 1997; 34: 6-11.

Zayed, G., Roos, Y.H. Influence of trehalose and moisture content on survival of *Lactobacillus salivarius* subjected to freeze-drying and storage. Process Biochemistry. 2004; 39: 1081-1086.

Zema, L., Loreti, G., Melocchi, A., Maroni, A., Palugan, L., Gazzaniga, A. Gastroresistant capsular device prepared by injection molding. International Journal of Pharmaceutics. 2013; 440(2): 264-272.

Zhang, Y.-C., Zhang, L.-W., Tuo, Y.-F., Guo, C.-F., Yi, H.-X., Li, J.-Y., Han, X., Du, M. Inhibition of *Shigella sonnei* adherence to HT-29 cells by lactobacilli from Chinese fermented food and preliminary characterization of S-layer protein involvement. Research in Microbiology. 2010; 161(8): 667-672.

Zoumpopoulou, G., Foligne, B., Christodoulou, K., Grangette, C., Pot, B., Tsakalidou, E. *Lactobacillus fermentum* ACA-DC 179 displays probiotic potential *in vitro* and protects against trinitrobenzene sulfonic acid (TNBS)-induced colitis and *Salmonella* infection in murine models. International Journal of Food Microbiology. 2008; 121(1): 18-26.