



**The Preparation of *Lactobacillus* Probiotic as Enteric Coated Capsules  
for Diarrhea Patients**

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Master of Pharmacy in Pharmaceutical Sciences**

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ชื่อวิทยานิพนธ์	การเตรียมแลคโตบาซิลัสโปรไบโอติกบรรจุแคปซูลเคลือบเอนเทอร์ริก สำหรับผู้ป่วยท้องเสีย
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### บทคัดย่อ

เชื้อโปรไบโอติก คือเชื้อจุลินทรีย์ที่มีชีวิต สามารถก่อประโยชน์ต่อร่างกายของสิ่งมีชีวิตที่เชื้ออาศัยอยู่ โดยมีกระบวนการการปรับสมดุลของจุลินทรีย์ในลำไส้จากการส่งเสริมการเจริญของจุลินทรีย์ที่มีประโยชน์และยับยั้งการเจริญของจุลินทรีย์ก่อโรค ในการคัดเลือกเชื้อเพื่อพัฒนาเป็นโปรไบโอติกจำเป็นต้องมีการทดลองในระดับ *in vitro* และ *in vivo* ซึ่งในการศึกษาค้นคว้าครั้งนี้ได้ทำการจำแนกสปีชีส์ของเชื้อ *Lactobacillus* ที่ได้รับการคัดเลือก ศึกษาความสามารถของเชื้อดังกล่าวในการผลิตสารที่มีฤทธิ์ในการยับยั้งเชื้อก่อโรค ศึกษาความไวต่อยาปฏิชีวนะ ศึกษาการทนกรดและทนด่างในระบบทางเดินอาหาร ศึกษาการเกิดการเกาะกลุ่มกันของเชื้อ *Lactobacillus* และเชื้อก่อโรคทางเดินอาหาร ศึกษาการยับยั้งการเกาะของเชื้อก่อโรคนบนเซลล์ผนังลำไส้ (Caco-2 cell line) และศึกษาความคงทนของเชื้อ *Lactobacillus* ในรูปแบบแคปซูล จากการศึกษาเพื่อจำแนกสปีชีส์ของเชื้อ *Lactobacillus* (T23/3) พบว่าเชื้อดังกล่าวเป็นเชื้อ *Lactobacillus* สปีชีส์ *plantarum* ผู้วิจัยนำเชื้อดังกล่าวมาทดสอบคุณสมบัติในการยับยั้งการเจริญเติบโตของเชื้อก่อโรคทางเดินอาหารซึ่งได้แก่ เชื้อ *S. aureus*, *S. typhimurium*, *S. sonnei*, *E. coli* และ *V. cholerae* พบว่าเชื้อ *L. plantarum* (T23/3) สามารถยับยั้งการเจริญเติบโตของเชื้อก่อโรคทางเดินอาหารทั้ง 5 ชนิด นอกจากนี้ในการศึกษาพบว่าเชื้อ *L. plantarum* (T23/3) คือต่อยา erythromycin (15 µg), doxycycline (30 µg), penicillin (10 units), neomycin (30 µg) และ tetracycline (30 µg) ในขณะที่เดียวกันเชื้อดังกล่าวไวต่อยา ampicillin (30 µg) และ chloramphenicol (30 µg) เมื่อศึกษาสมบัติความเป็นโปรไบโอติกพบว่าเชื้อ *L. plantarum* (T23/3) สามารถมีชีวิตอยู่ได้มากกว่า 4 ชั่วโมงเมื่ออยู่ในสภาวะความเป็นกรด pH 4 และ สภาวะความเป็นด่าง pH 8 นอกจากนี้พบว่าเชื้อดังกล่าวสามารถเกิด auto-aggregation มี

surface hydrophobicity ชนิด high hydrophobicity เกิด co-aggregation กับเชื้อก่อโรคในทางเดินอาหารทั้ง 5 ชนิดได้อย่างสมบูรณ์และสามารถยึดเกาะกับเซลล์ของผนังลำไส้ได้ดีกว่าเชื้อก่อโรคทางเดินอาหาร เมื่อศึกษาความคงตัวของเชื้อ *L. plantarum* (T23/3) ในรูปแบบแคปซูลเมื่อเก็บไว้ที่อุณหภูมิ 4°C พบว่าเมื่อเวลาผ่านไป 12 เดือนเชื่อดังกล่าวสามารถมีชีวิตรอดเป็น 100 เปอร์เซ็นต์เมื่อเทียบกับเดือนแรกที่เก็บเชื้อ จากผลการศึกษาวิจัยครั้งนี้ แสดงให้เห็นว่าเชื้อ *L. plantarum* (T23/3) สามารถนำมาพัฒนาเป็น โพรไบโอติกในรูปแบบแคปซูลเพื่อนำไปใช้ในการรักษาอาการท้องเสียได้

<b>Thesis Title</b>	The preparation of <i>Lactobacillus</i> probiotic as enteric coated capsules for diarrhea patients
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### ABSTRACT

Probiotics are live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance. The selection of probiotics before incorporation in diet requires close scrutiny in the form of *in vitro* as well as *in vivo* tests. The present study was undertaken to investigate *in vitro* characteristics of *Lactobacillus* strain. The characterization included identification of lactobacilli strain, determination of inhibitory substances produced, susceptibility to antibiotics, survival in simulated gastrointestinal fluid, aggregation tests, inhibition of pathogens adhesion to Caco-2 cells and stability of lactobacilli in capsule. The results revealed that *Lactobacillus* (T23/3) was identified as *L. plantarum*. This strain had inhibition activity against gastrointestinal pathogens (*S. aureus*, *S. typhimurium*, *S. sonnei*, *E. coli* and *V. cholerae*). *L. plantarum* (T23/3) was sensitive to ampicillin (30 µg) and chloramphenicol (30 µg), while it was resistant to erythromycin (15 µg), doxycycline (30 µg), neomycin (30 µg), penicillin (10 units) and tetracycline (30 µg). The strain could survive after 4 h of incubation in simulated gastric fluid at pH 4 and simulated intestinal fluid at pH 8. *L. plantarum* (T23/3) was self-aggregate from macroscopic granules and has high surface hydrophobicity property with a maximal ammonium

sulfate concentration of 0.5 mol/l. It also co-aggregated with five strains of gastrointestinal pathogens. *L. plantarum* (T23/3) was able to adhere and inhibit the adhesion of *E. coli* and *V. cholerae* to Caco-2 cells. It survived after freeze drying and showed good stability up to 12 months during storage at 4°C. This study suggests that *L. plantarum* (T23/3) has a potential application in gastrointestinal infection.

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## LIST OF ABBREVIATIONS AND SYMBOLS

°C	=	Degree celcius
16s rRNA	=	16s ribosomal ribonucleic acid
µg	=	Microgram
µl	=	Microliter
A	=	Adenine
AAD	=	Antibiotic-associated diarrhea
BHI	=	Brain heart infusion
C	=	Cytosine
CFU	=	Colony forming unit
Caco-2	=	Human colon carcinoma cell line
DNA	=	Deoxyribonucleic acid
EDTA	=	Ethylene diamine tetraacetic acid
FAD	=	Flavoprotein
FBS	=	Fetal bovine serum
g	=	Gram
G	=	Guanine
GI	=	Gastrointestinal
GRAS	=	Generally recognized as safe
HCl	=	Hydrochloric acid
H <sub>2</sub> O <sub>2</sub>	=	Hydrogen peroxide
kDa	=	Kilo Dalton

## LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

L	=	Liter
LAB	=	Lactic acid bacteria
LPS	=	Lipopolysaccharides
m	=	Meter
M	=	Mole
ml	=	Milliliter
mm	=	Millimeter
mM	=	Millimolar
MEM	=	Eagle's minimal essential medium
MRS	=	Man Rogosa and Sharpe
NADH	=	Nicotinamide adenine dinucleotide phosphate
PBS	=	Phosphate buffer Saline
PCR	=	Polymerase chain reaction
SAT	=	Salt aggregation test
SCFAs	=	Short chain fatty acids
SEM	=	Scanning electron microscope
SD	=	Standard deviation
SDS	=	Sodium dodecyl sulfate
SGF	=	Simulated gastric fluid
SIF	=	Simulated intestinal fluid



## LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

T	=	Thymine
TMB	=	Tetramethylbenzidine
TSS	=	Toxin shock syndrome
u	=	Unit
USP	=	United states pharmacopoeia
UV	=	Ultraviolet
v/v	=	Volume by volume
w/v	=	Weight by volume
WHO	=	World health organization

## CHAPTER 1

### INTRODUCTION

#### 1.1 General Introduction

The human GI is a complex ecosystem with many microorganisms. The representative microorganisms include bacteria. The GI tract, where more than 400 bacteria species are found, half of the wet weight of colonic material is due to bacterial cells. Normally, the stomach contains few bacterial ( $10^3$  cfu/ml of gastric fluid) whereas the bacterial concentration increase throughout the gut resulting in the final concentration in the colon of  $10^{12}$  cfu/ml of faeces. Bacterial colonization of gut begins at birth, a sterile status is maintained until the delivery begins. Bacterial continues throughout life, with notable age-specific changes. Once established, the intestinal flora remains relatively stable throughout life (Harsharnjit, 2003). The majority of indigenous flora are beneficial but some are potentially pathogenic. In healthy individuals, a balance exists between good and bad microorganism populations. Disturbance in the microorganism natural balance in gastrointestinal tract is due to antibiotic, radiation therapy, stress and infection. This enhances the predisposition to an increased risk of gastrointestinal infection diseases, for example acute diarrhea and chronic gut diseases (Maurad and Meriem, 2008; Pagnini *et al.*, 2010).

The probiotics are live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance (Ripamonti *et al.*, 2011). Selection of suitable probiotic candidates is the principle basis for improving the bio-therapeutic action and functional properties of probiotic food and pharmaceutical

products (Pan *et al.*, 2009). Probiotics have become a major topic of lactic acid bacteria (LAB). The representative species include *Lactobacillus* spp. because the lactobacilli are a Generally Recognized As Safe (GRAS) and have been used in food and fermented products for long history (Bayane *et al.*, 2010). The essential characteristics for lactobacilli as probiotics are such as acid and bile salt tolerance in gastrointestinal tract (Del Piano *et al.*, 2006). They also need to survive with sufficient numbers during production and storage (Ljungh and Wadstrom, 2006). Furthermore, antibiotic susceptibilities of potential probiotic strains is necessary although lactobacilli associated infection are rare (Zhou *et al.*, 2005; Matto *et al.*, 2007). These strains inhibited growth of potential pathogens by producing compounds such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), lactic acid, short chain fatty acid and bacteriocin (Reid *et al.*, 2004). Other functions of probiotics include competitive exclusion of pathogens from the cell surface, co-aggregation with certain pathogenic bacteria, adherence to epithelial cells and biofilm formation based on auto-aggregation and surface hydrophobicity (Collado *et al.*, 2008). Previous studies indicated that auto-aggregation of probiotic strains is necessary for adherence to gastrointestinal epithelial cells and co-aggregation leads to formation of a barrier that prevents colonization by pathogens (Zhou *et al.*, 2004; Collado *et al.*, 2008). Adhesion to Caco-2 cell lines has been used frequently because it exhibits, *in vitro*, the characteristics of a mature enterocyte. The cultured cell lines have been reported to mimic the *in vivo* conditions of adhesion and infection of pathogenic bacteria (Pan *et al.*, 2009). The delivery of active probiotic cells in capsulated form has received reasonable attention during the last 10 years, since it can reduce losses of sensitive bacteria induced by detrimental external factors such as oxidative or acid stress during

storage and digestion (Donthidi *et al.*, 2010; Sandoval *et al.*, 2010). Drug delivery systems have been developed for intestine specific drug delivery using pH-sensitive polymers (enteric coating polymers). Enteric-coated systems are most commonly used for intestinal drug delivery and constitute majority of commercially available preparations for intestine targeting (Sinha *et al.*, 2006; McConnell *et al.*, 2008). Improved drug delivery systems are required for drug currently in use to treat localized diseases of the intestine. The advantages of targeting drugs specifically to the diarrhea can reduce incidence of systemic side effects, lower dose of drug, supply of the drug to the biophase only when it is required and maintenance of the drug in its intact form as close as possible to the target site (Sadeghi *et al.*, 2009). It is expected that these strains can be applied as probiotic for the treatment of diarrhea from bacterial pathogens in human. Therefore in present study, the feasibility of lactobacilli isolated from human as a probiotic for diarrhea treatment was investigate and its stability as enteric coated capsule form was followed up.

## **1.2 Diarrhea**

Diarrhea is a common medical condition that is characterized by increased frequency of bowel movements and increased liquidity of stool (Fine and Schiller, 1999). Although acute diarrhea is typically self-limiting, it can be severe and can lead to profound dehydration, which can lead to low blood pressure, and damage to the kidneys, heart, liver, brain and other organs. Acute diarrhea remains a major cause of infant mortality around the world. Over 2 million deaths are attributed to acute diarrhea each year world-wide, most of them in the developing world (King *et al.*, 2003; Thapar and Sanderson, 2004; Thielman and Guerrant, 2004). Children and the

elderly are particularly prone to dehydration secondary to diarrhea. Diarrhea has been defined over time by various scientific groups and health organizations in different ways, such as: “the passage of loose unformed stools” or “three looser-than normal stools in a 24-h period” with emphasis on the consistency of stools rather than the number (WHO, 2008). In epidemiological studies, diarrhea is usually defined as the passage of three or more loose or watery stools in a 24-h period (WHO, 2008; Ruiz-Palacios *et al.*, 2006).

There are many causes of infectious diarrhea, which include viruses, bacteria and parasites (Navaneethan and Giannella, 2008). Norovirus is the most common cause of viral diarrhea in adults (Patel *et al.*, 2009) but rotavirus is the most common cause in children under five years old (Greenberg and Estes, 2009). Adenovirus types 40 and 41 and astroviruses cause a significant number of infections (Mitchell, 2002). The campylobacter is a common cause of bacterial diarrhea, but infections by salmonellae, shigellae and some strains of *Escherichia coli* (*E.coli*) are frequent (Viswanathan *et al.*, 2009). In the elderly, particularly those who have been treated with antibiotics for unrelated infections, a toxin produced by *Clostridium difficile* often causes severe diarrhea (Rupnik *et al.*, 2009). Parasites do not often cause diarrhea except for the protozoan *Giardia*, which can cause chronic infections if these are not diagnosed and treated properly (Kiser *et al.*, 2008) and *Entamoeba histolytica* (Dans and Martinez, 2006; Gonzales *et al.*, 2009). Other infectious agents such as parasites and bacterial toxins also occur (Wilson, 2005). In sanitary living conditions where there is ample food and a supply of clean water, healthy person usually recovers from viral infections in a few days. However, for ill or malnourished

individuals, diarrhea can lead to severe dehydration and become life-threatening (Alam and Ashraf, 2003).

### **1.3 Bacterial pathogens**

Ingested pathogenic bacteria can cause gastroenteritis by several mechanisms. They may attach to the intestinal epithelium and elaborate toxin, or they may directly invade and destroy the mucosal cell. This invasion results in intense inflammation and bloody diarrhea and patients typically have fever and severe abdominal pain.

#### ***Staphylococcus aureus***

*Staphylococcus aureus* is a Gram-positive bacteria, catalase-positive. It is a leading cause of soft tissue infection, as well as toxin shock syndrome (TSS) and scalded skin syndrome. The pathogenic effects of *S.aureus* are mainly associated with the toxins it produces. Most of these toxins are produced in the stationary phase of the bacterial growth curve. *S.aureus* has been implicated as causative agents of disease in man and animal by producing a number of extracellular compounds including enterotoxin which is responsible for the symptoms of food poisoning. Infection can be traced to contaminated meats which have not been fully cooked (Ryan and Ray, 2004).

#### ***Shigella sonnei***

*Shigella sonnei* is a non-motile, nonspore-forming, facultative anaerobic Gram-negative bacterium. Its non-motile characteristic means that this species doesn't have flagella to facilitate its movement like many other human enterobacteria. *S. sonnei* is a rod-shape bacterium and is lactose-fermenting bacterium causing

dysentery (Yang *et al.*, 2005). *S. sonnei* is extremely fragile in experimental settings. Its natural habitat is in a low pH environment such as the human gastrointestinal tract. Its optimal environmental temperature is 37°C. Therefore, human's gastrointestinal tract appears to be the only found natural host of *S. sonnei* that is known so far (Potter, 2006).

### ***Escherichia coli***

*Escherichia coli* is a Gram-negative bacteria, facultative anaerobic. They live in the intestinal tracts of the animals in health and disease. Pathogenic strains of *E. coli* can cause severe cases of diarrhea in all age groups by producing a powerful endotoxin. Treating *E.coli* infections with antibiotics may actually place the patient in severe shock which could possibly lead to death. This is due to the fact that more of the bacterium toxin is released when the cell dies (Madigan and Martinko, 2006)

### ***Vibrio cholerae***

*Vibrio* species is a member of the family Vibrionaceae, Gram-negative, catalase-positive and facultative anaerobic. The most important *Vibrio* worldwide is *V.cholerae* which is responsible for the rapidly dehydrating and the often fatal diarrhea seen in developing countries. Typically, infection occurs after ingestion of contaminated water or seafood. The organisms attach to epithelial cells of the proximal small intestine and produce a protein enterotoxin. The toxin causes an increase in intracellular cyclic adenosine monophosphate, which interferes with the absorption of sodium and causes chloride secretion. The cells then lose water which passively flows out of the cells. Less than 10% of ill persons develop typical cholera with signs of moderate or severe dehydration (Faruque and Nair, 2008).

### ***Salmonella typhimurium***

*Salmonella typhimurium* is a pathogenic Gram-negative bacteria predominately found in the intestinal lumen. Its toxicity is due to an outer membrane consisting largely of lipopolysaccharides (LPS) which protect the bacteria from the environment. *Salmonella* produces enterotoxin and cytotoxin. Enterotoxin is released from bacteria cell wall as an enteric fever. *Salmonella* produced cytotoxin by inhibition of protein synthesis and may be at least responsible for damage to the intestinal mucosa inflammation. The acute inflammatory response causes diarrhea and may lead to ulceration and destruction of the mucosa. The bacteria can disseminate from the intestines to cause systemic disease (Ellermeier and Schlauch, 2007; Murray *et al.*, 2009).

## **1.4 Probiotics**

### **1.4.1 Definition of probiotics**

The name probiotic comes from the Greek 'pro bios' which means 'for life'. Probiotics are defined as the living microorganisms administered in a sufficient number to survive in the intestinal ecosystem. They must have a positive effect on the host (Carlos *et al.*, 2010). Lilly and Stillwell in 1965 defined probiotics as “microorganisms promoting the growth of other microorganisms”. Although numerous definitions have been proposed since then, most have failed to be completely satisfactory because they lack properties such as “stabilization of the gut flora” (Goktepe *et al.*, 2006). Finally, since probiotics have been found to be effective in the treatment of some gastrointestinal diseases (Marteau *et al.*, 2001), they can be considered as therapeutic agents. It is clear that a number of definitions of the term probiotic have been used over the years but the one derived by the Food and



Agriculture Organization of the United Nations/World Health Organization (WHO, 2001) and endorsed by the International Scientific Association for Probiotics and Prebiotics (Reid *et al.*, 2003) best exemplifies the breadth and scope of probiotics as they are known today: live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. This definition retains historical elements of the use of living organisms for health purposes but does not restrict the application of the term only to oral probiotics with intestinal outcomes (Reid *et al.*, 2005). The probiotics in use today have not been selected on the basis of all these criteria, but the most commonly used probiotics are the strains of lactic acid bacteria such as *Lactobacillus*, *Bifidobacterium* and *Streptococcus* the first two are known to resist gastric acid, bile salts and pancreatic enzymes, to adhere to colonic mucosa and readily colonize the intestinal tract (Fioramonti *et al.*, 2003)

*Lactobacillus* spp. are also classified as lactic acid bacteria (LAB). They are Gram-positive facultative anaerobes. They are non-spore forming, rod or coccobacilli (Holzapfel *et al.*, 2001). Lactobacilli can be isolated from a large number of sources, for example from human and animal body (oral cavity, stomach, intestine and vagina), plant and material of plant origin, sewage and fermented products (Bernardeau *et al.*, 2008). Lactobacilli are strictly fermentative and acid-resistant. Growth is enhanced by acidic conditions. The final pH in broth cultures is generally below 4.5. They are considered to have beneficial effects on human and animal health. Lactobacilli are composed of a diverse group of homofermentative and heterofermentative species. The main end product of glucose fermentation is lactic acid. Homofermentative lactobacilli such as *L. delbrueckii*, *L. helveticus* and *L. amylovorus* produce more than 85% lactic acid from glucose. Heterofermentative

lactobacilli such as *L. plantarum*, *L. pentosus*, *L. casei*, *L. sake* and *L. ruminis* produce at least 50% lactic acid together with acetic acid, ethanol and carbon dioxide. Lactobacilli produce short chain fatty acids such as acetic acid, butyric acid, propionic acid etc. some strains also produce hydrogen peroxide and they are mostly cited for production of proteinaceous antimicrobial substances or bacteriocins (Aslim *et al.*, 2005).

#### **1.4.2 Health benefits of probiotics**

Since, a number of health benefits have been contributed to products containing probiotic organisms. While, some of these benefits have been well documented and established, others have shown a promising potential in animal models, with human studies required to substantiate these claims. More importantly, health benefits imparted by probiotic bacteria are very strain specific; therefore, there is no universal strain that would provide all proposed benefits, not even strains of the same species. Moreover, not all the strains of the same species are effective against defined health conditions. The strains *L. rhamnosus* GG and *L. casei* Shirota are certainly the most investigated probiotic cultures with the established human health (Azizpour *et al.*, 2009).

##### **1.4.2.1 Diarrhea caused by certain pathogenic bacteria**

Infectious diarrhea is a major world health problem, responsible for several million deaths each year. While, the majority of deaths occur amongst children in developing countries, it is estimated that up to 30% of the population even in developed countries are affected by food-borne diarrhea each year (Azizpour *et al.*, 2009). Probiotics can potentially provide an important means to reduce these problems. It

should be noted that some of the studies referenced below utilize probiotics administered in a non-food form. The strongest evidence of a beneficial effect of defined strains of probiotics has been established using *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB-12 for prevention (Szajewska *et al.*, 2001) and treatment of acute diarrhea mainly caused by pathogenic bacteria in children (Allen *et al.*, 2010). In addition, many bacterial species cause death and morbidity in humans. There is good *in vitro* evidence that certain probiotic strains can inhibit the growth and adhesion of a range of enteropathogens (Gopal *et al.*, 2001) and animal studies have indicated beneficial effects against pathogens such as *Salmonella* (Ogawa *et al.*, 2001). There is evidence from studies on travellers' diarrhea, where some of the causative pathogens have been presumed to be bacterial in nature that benefits can accrue with probiotic administration (Chelikyoussef *et al.*, 2009; Graul *et al.*, 2009; Lin *et al.*, 2009; Pan *et al.*, 2009).

#### **1.4.2.2 Antibiotic-associated diarrhea**

Antibiotic-associated diarrhea (AAD) results from an imbalance in the colonic microbiota caused by antibiotic therapy. Microbiota alteration changes carbohydrate metabolism with decreased short-chain fatty acid absorption resulting in an osmotic diarrhea. Another consequence of antibiotic therapy leading to diarrhea is overgrowth of potentially pathogenic organisms such as *Clostridium difficile*. The cultural product contains the strain *L. rhamnosus* GG, may reduce the risk of antibiotic associated diarrhea, improve stool consistency during antibiotic therapy (Graul *et al.*, 2009; Parkes *et al.*, 2009). Probiotic treatment can reduce the incidence and severity of AAD as indicated in several meta-analyses (D'Souza *et al.*, 2002; Mcfarland, 2006;

Szajewska *et al.*, 2006). Further documentation of these findings through randomized, double blind, placebo-controlled trials are warranted. Efficacy of probiotic AAD prevention is dependent on the probiotic strain used and on the dosage (Doron *et al.*, 2008). Up to a 50% reduction of AAD occurrence has been found (Sazawal *et al.*, 2006). No side-effects have been reported in any of these studies. Caution should, however, be exercised when administering probiotic supplements to immunocompromised individuals or patients who have a compromised intestinal barrier.

#### **1.4.2.3 Lactose intolerance**

The decline of the intestinal  $\beta$ -galactosidase activity is a biological characteristic of the maturing intestine in the majority of the world's population. With the exception of the inhabitants of northern and central Europe and Caucasians in North America and Australia, over 70% of adults worldwide are lactose malabsorbers (De Vrese *et al.*, 2001). Lactose upon ingestion is hydrolyzed by lactase in the brush border membrane of the mucosa of the small intestine into constitutive monosaccharides, glucose and galactose, which are readily absorbed in the blood stream. However, the activity of intestinal lactase in lactose intolerant individuals is usually less than 10% of childhood levels. This decline, termed hypolactasia, causes insufficient lactose digestion in the small intestine, characterized by an increase in blood glucose concentration or hydrogen concentration in breath upon ingestion of 50 g lactose, conditions designated as lactose maldigestion. Hypolactasia and lactose malabsorption accompanied with clinical symptoms, such as bloating, flatulence, nausea, abdominal pain and diarrhoea, are termed lactose intolerance (Sanders, 2000). Symptoms are caused by undigested lactose

in the large intestine, where lactose is fermented by intestinal microflora and osmotically increases the water flow into the lumen. The severity of the symptoms depends primarily on the size of the lactose load ingested. The development of the intolerance symptoms also depends on the rate of lactose transit to the large intestine, influenced by the osmotic and caloric load and the ability of the colonic microflora to ferment lactose (Soomro *et al.*, 2002). Numerous studies have shown that individuals with hypolactasia could tolerate fermented dairy products better than an equivalent quantity in milk (Hertzler and Clancy, 2003; Montalto *et al.*, 2005). Various explanations have been suggested in order to clarify this phenomenon. At least 3 factors appear to be responsible for a better tolerance of lactose in fermented milk including starter culture, intracellular  $\beta$ -galactosidase expressed in these cultures and most importantly and oro-caecal transit time. The traditional cultures used in dairy fermentations utilize lactose as an energy source during growth, thus at least, partially reducing its content in fermented products. Furthermore, the bacterial lactase may resist luminal effectors avoiding denaturation and can be detected in the duodenum and terminal ileum after consumption of products containing live bacteria. The presence of this enzyme may lead to lactose hydrolysis and improved lactose tolerance. On the other hand, other studies not supporting this theory found no difference in digestion and tolerance to lactose in several fermented dairy products with substantially different lactase activities. It was suggested that increased viscosity of fermented milk, in this case yoghurt, slowed gastric emptying and consequently prolonged transit time through the gastrointestinal tract improving absorption and lactose tolerance (Azizpour *et al.*, 2009).

## **1.5 Properties of probiotics**

### **1.5.1 Antimicrobial properties**

The intestinal microflora is a complex ecosystem. Introducing new organisms into this highly competitive environment is difficult. Thus organisms that can produce a product or products that will inhibit the growth or kill existing organisms in the intestinal milieu have a distinct advantage. The growth media filtrates and sonicates from the bacterial cells of prospective probiotics should be tested for bactericidal and bacteriostatic activity in well-plates against a wide variety of pathogens. The ability of probiotics to establish in the GI tract is enhanced by their ability to eliminate competitors. Inhibitory substances produced by lactobacilli are lactic acid, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), short chain fatty acids (SCFAs) and bacteriocins.

#### **1.5.1.1 Lactic acid**

Lactic acid is one of the inhibitory agents produced by lactobacilli. It is the major end product of their carbohydrate catabolism. Lactic acid can be formed either via the Embden-Meyerhof-Parnas (glycolytic) pathway or by the 6-phosphogluconate pathway (Sheedy *et al.*, 2009). On the basis of their hexose catabolism, lactic acid bacteria can be divided into two groups: homofermentative type (using Embden-Meyerhof-Parnas pathway) and heterofermentative type (using 6-phosphogluconate pathway) (Wisselink *et al.*, 2002). Lactobacilli utilize carbohydrates as a main nutritional source and are found in fermenting animal and plant products. The main end-product of glucose fermentation is lactic acid, resulting in a decrease of pH in medium, by one or more units. Besides lactic acid, they also produce acetic acid and hydrogen peroxide. These metabolites make the environment less favorable for the *in*

*in vitro* growth of potentially pathogenic microorganisms, such as *Staphylococcus*, *Pseudomonas* and *Salmonella*. *L. acidophilus* inhibited the growth of seven isolates of *Helicobacter pylori in vitro*, probably due to the production of lactic acid (Reid *et al.*, 2004).

### **1.5.1.2 Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)**

Lactic acid bacteria produce hydrogen peroxide through electron transport via flavin enzymes. The formation of hydrogen peroxide is catalyzed by a very active cytoplasmic flavoprotein (FAD) nicotinamide adenine dinucleotide phosphate (NADH) oxidase in order to remove excess electrons from NADH (formed during glucose breakdown) but without the production of ATP. This hydrogen peroxide can be subsequently reduced to water by flavoprotein NADH peroxidase. Hydrogen peroxide is also synthesized from intermediately formed pyruvate which is reduced to acetyl phosphate and carbon dioxide by a flavin-containing cytoplasmic pyruvate oxidase. Furthermore, hydrogen peroxide can be synthesized from glycerophosphate and even from lactate. While glycerophosphate is reduced under elaboration of hydrogen peroxide to dihydroxyacetone phosphate by a FAD-containing cytoplasmic glycerophosphate oxidase, lactate can be oxidized to pyruvate by lactate oxidase or a NAD-independent flavin-containing lactate hydrogenase which reduces oxygen to hydrogen peroxide. Accumulated pyruvate is then further split to acetate and carbon dioxide. The dismutation of endogenous short-living superoxide anions (O<sub>2</sub><sup>•-</sup>) formed through partial transfer of electrons from sugar metabolism to the dioxygen molecule can also contribute slightly to the accumulation of hydrogen peroxide by the action of a superoxide dismutase present in most lactic acid bacteria or by manganese ions

present in high concentration in the cytoplasm of bacteria lacking superoxide dismutase or by a manganese-containing non heme (lactobacilli). Lactobacilli usually lack heme and therefore cannot utilize the cytochrome system which reduces oxygen to water for terminal oxidation and are absent for the heme protein catalase to degrade hydrogen peroxide. This compound may be released to an environment. Furthermore, in the presence of hydrogen peroxide, these superoxide anions can result in the formation of inhibitory hydroxyl radicals ( $\text{OH}^\bullet$ ). Hydrogen peroxide toxicity may result from the peroxidation of membrane lipids, which would explain the increased membrane permeability caused by hydrogen peroxide,  $\text{O}_2^\bullet$  and  $\text{OH}^\bullet$ . The resulting bactericidal effect of these oxygen metabolites has been attributed not only for their strong oxidizing effect on the bacteria cell but also to the destruction of basic molecular structures of nucleic acids and cell proteins. Finally, the hydrogen peroxide not only itself inhibits undesirable microorganisms, it may also react with other components to form additional inhibitory compounds (Thomas *et al.*, 2003).

#### **1.5.1.3 Short chain fatty acids (SCFAs)**

Short chain fatty acids (SCFAs) or volatile fatty acids are used as a common name for monocarboxylic acids with length upto 6 or sometimes 8 carbon atoms. The molecular weight of SCFAs is low. They are polar soluble in water are weak acids and the pKa value is around 4.8. They are major end products of anaerobic microbial in the large intestine that use the carbohydrate fermentation, among which acetic acid, butyric acid and propionic acid are dominant. These electron products are formed to maintain redox balance during fermentation (Reid *et al.*, 2004). While butyric acid is almost completely used as a direct energy source by the colonic cells, acetic acid and



propionic acid are absorbed in the colon and seem to contribute significantly (Topping and Clifton, 2001). SCFAs produced by colonic fermentation are also suggested to be of importance from a health perspective. Generally, SCFAs lower the colonic pH. This may assist in controlling the proliferation of undesirable pathogen and protecting against colonic carcinogenesis (Gill and Rowland, 2002). Specifically, butyric acid is considered to contribute significantly to the maintenance of the integrity of the colonic mucosa, by serving as a direct energy source for the enterocytes. Studies have suggested that butyrate may excrete a protective role against ulcerative colitis and suppresses the growth of cancer cells isolated from the large intestine (Gill and Rowland, 2002). Acetate promotes the relaxation of resistance vessels in the colonic vasculature, supporting the maintenance of the blood flow to the liver and the colon (Topping and Clifton, 2001). Propionate seems to enhance colonic muscular contraction contributing to laxation and relief of constipation and to stimulate the proliferation of the colonic epithelium enhancing the absorption capacity in large intestine (Topping and Clifton, 2001). The inhibition ability of SCFAs is more bactericidal than dissociated ones due to their ability to penetrate into pathogen cells (Reid *et al.*, 2004).

#### **1.5.1.4. Bacteriocins**

Bacteriocins are commonly active against a wide range of Gram positive bacteria (Cascales *et al.*, 2007). It is also active against Gram negative bacteria such as *E. coli* and *Salmonella* spp. causing the outer membrane damage (De Vuyst and Leroy, 2007). The lethal actions of bacteriocins are divided into three stages: (1) binding of the bacteriocins to specific cells surface receptor: (2) insertion into or

transport across the susceptible cell's membrane: (3) killing of the cells. Bacteriocins 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 (De Vuyst and Leroy, 2007). The associations of hydrophobic patches of bacteriocins with 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 (Brotz *et al.*, 1998). Since ATP has no transport system in the sensitive cells studied and glutamate is not transported by proton motive force driven system the results induced to cells death. The increase in membrane permeability results in the collapse of either or both the transmembrane potential and the pH gradient (Arici *et al.*, 2004).

### **1.5.2 Acid resistance of probiotics**

The environment of stomach may highly affect the survival of lactobacilli. The lactobacilli are surviving during transit in GI tract and then persist in the gut to provide beneficial effect for the host (Chou and Weimer, 1999). The low pH of the stomach and the antimicrobial action of pepsin are known to provide an effective barrier against entry of bacteria into the GI tract (Holzapfel *et al.*, 1998). The pH of the stomach could be as low as 1.5 or as high as 6.0 or above after food intake, but generally ranges from pH 2.5 to 3.5 (Holzapfel *et al.*, 1998). The nature of food in the stomach affects the transit time through the stomach. Normally, food remains in the

stomach between 2 and 4 h (Holzapfel *et al.*, 1998). However, liquids emptying time from the stomach faster than solids and only take about 20 min to pass through the stomach. Usually, the healthy stomach contains relatively small number of bacteria ( $0-10^3$  cfu/ml gastric content) due to the gastric acidity (Holzapfel *et al.*, 1998). In healthy volunteers, most of the ingested bacteria cannot metabolically active at pH less than 4.0 within 30 min. The survival of lactobacilli in acid environment has been tested *in vitro* after 4 h. It was found that *L. rhamnosus* GG showed no loss of viability in gastric juice with pH 3.0-7.0, but there is rapid loss in viability at pH 1.0 (Begley *et al.*, 2005; Del Piano *et al.*, 2006).

### **1.5.3 Bile resistance of probiotics**

Bile is an aqueous solution made up of bile acids, cholesterol, phospholipids, and the pigment biliverdin, which gives the bile its yellow-green color. About 500-700 ml/day of bile acids are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum, after food intake by an individual. Bile plays an essential role in lipid digestion; it emulsifies and solubilizes lipids and functions as biological detergent. Prior to secretion into the duodenum, bile acids are conjugated either with glycine (glycoconjugated) or taurine (tauroconjugated) (Begley *et al.*, 2006). In the colon conjugated bile undergoes various chemical changes including deconjugation, dehydroxylation, dehydrogenation, and deglucuronidation, almost solely by microbial activity (Begley *et al.*, 2006). Bile salt hydrolase (BSH) is usually an intracellular enzyme that catalyses the hydrolysis of glycine and taurine conjugated bile salts into amino acid residue and free bile salt (bile acid). Hydrolysis of bile salts is mediated by various genera of the intestinal microflora, including *Clostridium*,

*Bacteroides*, *Lactobacillus*, *Bifidobacterium* and *Enterococcus* (Grill *et al.*, 2000; Franz *et al.*, 2001). A number of BSH have been identified and characterized in probiotic bacteria, and the ability of probiotic strains has often been included among the criteria for probiotic strain selection (Begley *et al.*, 2006). Bile tolerance of probiotic bacteria can be investigated by incubating them for 24 hrs in a milk-yeast medium containing different concentrations of bile extracts and monitoring cell viability and pH before and after incubation (Goktepe *et al.*, 2006). This assay was used by several authors to assess the bile resistance of potential or already commercialized probiotic lactobacilli. All these studies reported a growth delay of lactobacilli in the presence of oxgall that was strain- and not species-dependent. It has been hypothesized that deconjugation of bile salts is a detoxification mechanism and BSH enzymes play a role in bile tolerance of probiotic organisms in the GI (Begly *et al.*, 2006).

#### **1.5.4 Antibiotic therapy**

Antibiotic is used for prevention and treatment of infection due to its antimicrobial effects. The oral uptake of the antibiotics may affect the intestinal microflora such as causing an overgrowth or superinfection of microorganisms that are resistant strains, may lead to establishment of some potent pathogens. It has been found that lactobacilli and other intestinal microflora decrease after antibiotics therapy (Paola *et al.*, 2005). Antibiotics treatment had reduced numbers of lactobacilli and bifidobacteria, both of which are thought to be positive gut genera and chloramphenicol given orally can reduce the number of lactobacilli 2-3 log cfu/ml (Paola *et al.*, 2005). In addition, the administration of clindamycin, neomycin or

combination of neomycin and metronidazole can also reduce the number of lactobacilli (Katla *et al.*, 2001). Supplement with lactobacilli during or after antibiotics may help to stabilize microflora and maintain the colonization resistance against pathogens, or inhibit the growth of pathogens. It is important that lactobacilli as a supplement during antibiotics treatment must be antibiotic resistant. Maria *et al.*, (2006) treated one group of patients with tetracycline orally incorporating with tetracycline resistant *L. plantarum* (LP-A22) and treated other group with tetracycline alone, they found that the fecal staphylococci in both groups increased at the beginning of antibiotic therapy but continued to increase in the patients taking antibiotic alone. Antibiotics used for treatment of microbial infections affecting normal microbiota of human are well recognized. Probiotics are commonly used as a complementary with antibiotics to alleviate the possible gastrointestinal tract symptoms caused by drug therapy (Oprica and Nord, 2005). The results from several groups showed that probiotic *Lactobacillus* or *Bifidobacterium* strains can reduce side effects of antibiotics for *Helicobacter pylori* eradication (Armuzzi, *et al.*, 2001, Cremonini *et al.*, 2002, Sheu *et al.*, 2002).

### **1.5.5 Antibiotic resistance**

Treatment with antimicrobial agents may cause pronounced disturbances in a normal microflora (Nord *et al.*, 2006). Antibiotics are important in the treatment and prophylaxis of infections. However, it should be considered that some of these agents have a harmful effect on the human microflora, leading to undesirable effects such as overgrowth and superinfections with commensal microorganisms. Suppression of the normal microflora lowers the colonization resistance of pathogens, often leading to

establishment of potentially pathogenic microorganisms such as *Candida* spp. These pathogens are often resistant to the antibiotics and may cause stomatitis or diarrhea. Furthermore, in immunocompromised patients *Candida* may cause systemic infections. Overgrowth by toxin-producing *Clostridium difficile* can give rise to diarrhea, colitis or pseudomembranous colitis. Antimicrobial agents such as cephalosporins, clindamycin and ampicillin have been associated with disease (Norrby *et al.*, 2005; Nord *et al.*, 2006).

Though *Lactobacillus* spp. are classified as GRAS microorganism, it is important to assess the safety of those microorganisms intended for use as probiotics. Because of the serious concerns about the growing level of resistance to antibiotics in regular use in animal and human medicine, one of the aspects which needs to be analysed is antibiotic resistance. Probiotic strains may carry resistance genes which may be transferred to unrelated pathogenic or potentially pathogenic bacteria in the gastrointestinal tract (Teuber *et al.*, 1999). Many lactic acid bacteria are resistant to some antibiotics. Their resistance attributes are often intrinsic and nontransmissible. However, for example, certain strains of *L. fermentum*, *L. plantarum* and *L. reuteri* carry potentially transmissible plasmid-encoded antibiotic resistance gene. The strains carry antibiotic resistance plasmids are considered unsuitable to use as human or animal probiotics (Saarela *et al.*, 2000). Since probiotic are added in different kinds of food and pharmaceutical products, they represent a potential source for the spread of antibiotic resistance genes. On the other hand, the strains with intrinsically antibiotic-resistant may benefit to human hosts whose normal intestinal flora have become unbalanced or greatly reduced in numbers due to the administration of various antibiotics (Ashraf and Shah, 2011). Phenotypes for bacteriocin production and

immunity and drug resistance have been related to extra-chromosomal DNA in several species of lactobacilli (Hummel *et al.*, 2007). Therefore, the possibility of genetic transfer will be limited due to large size of chromosomal DNA in comparison with plasmid gene.

### **1.5.6 Adherence of probiotics bacteria**

Co-aggregation is a process by which genetically-distinct bacteria adhere to one another via specific molecules. Cumulative evidence suggests that such adhesion influences the development of complex multispecies biofilms (Rickard *et al.*, 2003). Bacterial aggregation between microorganisms of the same strain (auto-aggregation) or between genetically divergent strains (co-aggregation) is of considerable importance in several ecological niches, especially in the human gut, where probiotics are to be active (Collado *et al.*, 2007a). A relationship between auto-aggregation and adhesion has been reported for some bifidobacterial spp. (Del Re *et al.*, 2000; Collado *et al.*, 2007b). A correlation between adhesion and hydrophobicity, as measured by microbial adhesion to hydrocarbons, has also been observed in some lactobacilli (Del Re *et al.*, 2000). Furthermore, it has been suggested (Collado *et al.*, 2007b) that inhibitor or bacteriocin-producing LAB, which co-aggregates with pathogens, may constitute an important host defence mechanism against infection. Co-aggregation with potential gut pathogens could therefore contribute to the probiotic properties ascribed to specific LAB.

It is generally agreed that LAB must adhere to intestinal mucus or epithelial cells in order to persist in the gut. The ability of LAB to adhere to mucosal surfaces prevents their rapid removal by gut contraction and subsequent peristaltic flow of

digestive content, and could also confer a competitive advantage. A large body of research has been conducted to screen probiotic bacteria for their ability to attach to intestinal cells (Goktepe *et al.*, 2006). *In vitro* experimentation shows that some strains of *Lactobacillus* adhere to intestinal tissue cultured cells in a species-dependent way. However, other studies concluded that the capacity to adhere to the surface is undoubtedly insufficient by itself to ensure that the microorganisms can colonize the epithelial habitat. Cultured human intestinal cell line models, which express various specific characteristics of cell phenotypes of intestinal epithelium, have been used to study probiotic adhesion. Probiotics are believed to temporarily colonise the intestine by adhering to intestinal surface therefore the adhesive ability of bacteria to intestinal cells has been considered as one of the selection criteria for probiotic strains. Because it is difficult to investigate bacterial adherence *in vivo*, adhesion has been studied using intestinal cell lines of human origin in culture as *in vitro* models for intestinal epithelium. One of these intestinal cell line is the Caco-2 cell line which was originally isolated from a human colon adenocarcinoma (Elina and Seppo, 1998). The Caco-2 cell line spontaneously differentiates under standard culture condition and the differentiated cells express characteristics of mature enterocyte (Liliana *et al.*, 2008).

Adhesion to mammal epithelial cells is a key process for bacteria to survive and colonize in GI. For pathogenic bacteria, the adhesion to epithelium is a critical step, since it allows the release of enzymes and toxins initiating necrotic processes directly into the target cell, thereby facilitating the invasion (Alicja *et al.*, 2008). The epithelial cells of GI are protected from pathogenic bacteria by a number of mechanisms. One of them is a reduction in pathogenic infections through competition



of microbiota for adhesion sites with microbial pathogens and production of components with antimicrobial activity (Ouwehand and Vesterlund, 2003; Baccigalupi *et al.*, 2005). To cause infection, pathogenic bacteria, after penetrating intestinal mucus, must adhere to enterocyte (Sylvester *et al.*, 1996). The initial step of adhesion in the case of pathogenic species is mediated by bacterial fimbriae which recognize certain receptors on eukaryotic cells. Several studies indicate that LAB could prevent the attachment of pathogens, in this way reducing colonization, and prevent infection (Zarate and Nader-Macias, 2006).

## **1.6 The stability of probiotics bacteria**

### **1.6.1 Viability of probiotics**

From a technological point of view, a probiotic formulation should include selected microorganisms with the ability to survive at high levels during the industrial process and remain viable afterwards with unaltered properties for long periods of storage (shelf-life). Stability after conservation and storage has been extensively studied in microorganisms commonly used as starters in the food industry (Wang *et al.*, 2004).

Freeze-drying is a convenient method for the preservation and long term storage of a wide variety of microorganisms. Special precautions are needed for the preservation of the microorganisms sensitive to desiccation, light, oxygen, osmotic pressure and surfactant. Effective protective agents, for example skim milk, lactose and meso inositol are used to suspend cells to be freeze dried in order to protect them against freezing and drying injuries. Along with the protective agents mentioned,

anaerobic bacteria which are sensitive to aerobic freeze drying can be preserved using activated charcoal (5% w/v) in the suspending media (Pikal, 2007). Because of lower transport and storage costs of the dried product, freeze drying is one of the techniques used to obtain stable cultures in term of viability and functional activity. Bacterial survival during the freeze-drying process and the subsequent storage is affected by factors such as initial concentration, growth and drying medium, rehydration and storage conditions. Intrinsic factors such as genus, species and bacterial cell size also have an incidence on the bacterial behavior during freeze-drying and storage (Carvalho *et al.*, 2004).

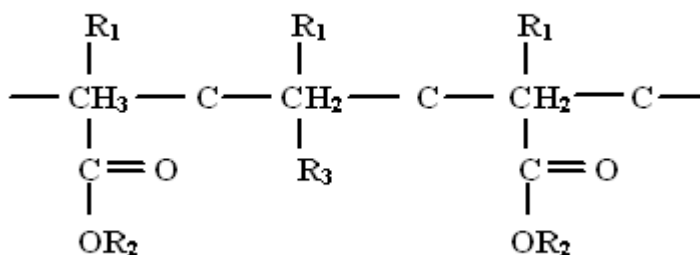
### **1.6.2 Stability probiotics as enteric coated capsules**

Microorganisms introduced orally have to, at least, transiently survive in the stomach and small intestine. Although this appears to be a rather minimal requirement, many bacteria including producing *Lactobacillus* subsp. often do not survive to reach the lower small intestine. The reason for this appears to be low pH of the stomach. In fasting individuals, the pH of the stomach is between 1.0 and 2.0 and most microorganisms, including lactobacilli, can only survive from 30 seconds to several minutes under these conditions. Therefore, in order for a probiotic to be effective, even the selection of strains that can survive in acid at pH 3.0 for sometime would have to be introduced to stability of enteric coated.

Enteric coating is aimed to prevent the formulations from gastric fluid in the stomach and release the drug component in the intestinal region. Based on this approach, enteric coating is suitably applied for drugs which cause gastric irritation or are deteriorated by the gastric fluid or gastric enzyme. With an acid-resistant property,

enteric coating polymers generally possess free carboxylic acid groups on the polymer backbone. They are insoluble in acidic media but become deprotonated and dissolved in basic media at nearly neutral pH values (Krisanin, 2010).

Use of pH-dependent polymers is based on the difference in pH-levels along the GI. The polymers described as pH-dependent in intestinal specific delivery are insoluble at low pH-levels but become increasingly soluble as pH rises (Leopold, 1999). The pH in the GI varies between and within individuals and also between healthy and patients (McConnell, 2008), which could lead to the failure of the system in the treatment. Most commonly used pH-dependent coatings polymers are copolymers methacrylic acid and methyl methacrylate containing carboxyl groups (Eudragit®) as shown in Figure 1.1. Eudragit® L and S are copolymerisates based on methacrylic acid and methyl methacrylate. The ratio of the free carboxyl groups to the esters is about 1:1 for Eudragit® L and 1:2 for Eudragit® S, resulting in enteric coatings with varying dissolution pH values (L = easily soluble, S = sparingly soluble). Preferred solvents are isopropanol, acetone and ethanol, as well as mixtures thereof. Delivery is either in isopropanolic solution with or without added plasticizers or as solvent-free powder under the trade name Eudragit® L 100 or S 100, respectively. The latter product types can be dissolved in organic solvents and mixtures or redispersed in water.



- $\text{R}_1 = \text{CH}_3; \text{H}$   
 $\text{R}_2 = \text{CH}_3, \text{CH}_3\text{CH}_2$   
 $\text{R}_3 = \text{COOH}$  (Eudragit® L and S)  
 $\text{R}_3 = \text{COOCH}_2\text{CH}_2\text{N}(\text{CH}_3)_3\text{Cl}^-$  (Eudragit® RL and RS)

**Figure 1.1** Chemical structures of various formulations of Eudragit®

The application of an enteric coating to a solid dosage form is an established approach to prevent drug release in the stomach and allow release in the small intestine. It is used to preclude the degradation of acid-labile actives in the gastric environment or to protect the stomach from irritant compounds (Agyilirah *et al.*, 1991). The commonly used enteric coatings employ pH-dependent polymers which contain carboxylic acid groups. These remain un-ionized in the low pH environment of the stomach and become ionized in the higher pH conditions of the small intestine, thus initiating dissolution of the coating and allowing drug release (Li-Fang *et al.*, 2009). The *in vitro* dissolution of enteric coated products is usually assessed in compendia pH 6.8-7.4 phosphate buffer. In this medium, drug release is normally rapid (McNamara *et al.*, 2003).

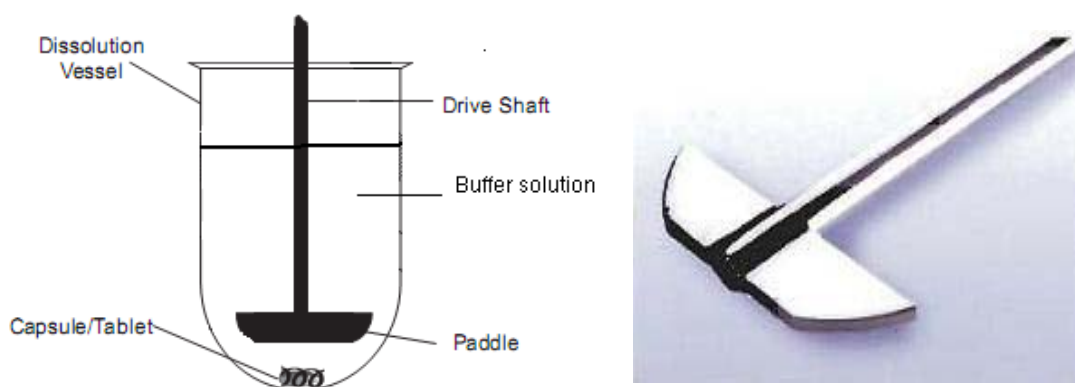
The constituent buffer salts, ionic strength and buffer capacity of the dissolution media have been reported to influence drug release from pH-responsive

polymer coated dosage forms (Chan *et al.*, 2001; Fadda and Basit., 2005; Ibekwa *et al.*, 2006; McConnell *et al.*, 2008; Fadda *et al.*, 2009). The luminal fluids of the small intestine are predominantly buffered by bicarbonate (as well as other ions and luminal constituents such as bile salts, proteins, carbohydrates and food components). Hence, bicarbonate buffers would more closely resemble the environment within the intestine and provide a more physiological medium for the *in vitro* assessment of products designed to release in the small bowel (Li-Fang *et al.*, 2009).

### **1.6.3 *In-vitro* release testing**

In the area of novel drug delivery systems, *in vitro* dissolution testing is important in designing, developing and testing new pharmaceutical formulations, (Siepmann and Peppas, 2001; Crane *et al.*, 2004b). In order to achieve the appropriate concentrations of the desired drug *in vivo*, (i.e. in the target organs and tissues), during the desired period of time, dissolution profiles *in vitro* need to satisfy certain criteria, generally established by the pharmacopoeias (Sun *et al.*, 2003; Crane *et al.*, 2004b). Thus dissolution *in vitro* can be regarded as the first step toward modeling *in vivo* dissolution and absorption. The dissolution rate is measured, in practice, using one of a number of standard dissolution test methods outlined in international pharmacopoeias, such as the European Pharmacopoeia (Eur.Ph, 2006) and United States Pharmacopoeia (USP, 2006). One commonly used dissolution apparatus is the Paddle Dissolution Apparatus (Figure 1.2), known as Apparatus 2, (USP, 2006). However, there are a number of difficulties related to *in vitro* dissolution testing. Very often, the relationship between the formulation and process parameters of a pharmacological compact, and its required *in vitro* dissolution profile, is not entirely

understood, due to the complexity of mass transport at dissolution. Complex mass transport often results in barely trackable effects, like interactions and synergies. For these reasons, experimentation associated with the field of drug design is very costly and time consuming. Thus, modeling drug release can facilitate design of new products, through predictions and selection of key parameters for experimental testing, while helping to gain insight on the phenomena involved in the dissolution process. A specific interest of this thesis is to consider the *in vitro* environment used for dissolution testing, as settings of the dissolution apparatuses are known to affect the process of dissolution.



**Figure 1.2** Schematic representation of the USP (United States Pharmacopoeia) Paddle Apparatus.

### **1.7 Aims and scope of this thesis**

The aim of this study was to identify *Lactobacillus* strain, to characterize *Lactobacillus plantarum* (T23/3) as a probiotic for diarrhea treatment and to investigate its stability as enteric coated capsule. Those properties included anti-gastrointestinal pathogens, susceptibility to antibiotics and simulated gastrointestinal juice and bile tolerance properties, auto-aggregation, surface hydrophobicity, co-aggregation with certain pathogenic bacteria, adhesion of lactobacilli on Caco-2 cells and stability of lactobacilli as enteric coated capsule.

#### **Scope of this thesis;**

1. To identify *Lactobacillus* strain
2. To determine the inhibition on gastrointestinal pathogens
3. To determine the susceptibility to antibiotics
4. To investigate the survival of *Lactobacillus* in simulated gastric fluid and simulated intestinal fluid
5. To investigate the aggregation activity
  - 5.1 auto-aggregation
  - 5.2 surface hydrophobicity
  - 5.3 co-aggregation
6. To investigate the adhesion of *Lactobacillus* on Caco-2 cells
7. To investigate the stability of *Lactobacillus* probiotics as enteric coated capsules

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Bacterial strains and growth condition

The *Lactobacillus plantarum* strain was obtained from Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hatyai, Thailand. The strain was isolated from the feces of healthy elderly volunteers, who had no recent history of gastrointestinal complaint and had not received any antibiotics at least 3 months prior to the sampling. Strain was stored at -80°C in de Man Rogosa Sharpe broth (MRS, Difco, USA), supplemented with 20% (v/v) glycerol until use. For routine analysis strain was subcultured twice in MRS broth for 24 h at 37°C.

For the antimicrobial activity and the inhibition of pathogen adhesion to Caco-2 cells, pathogenic strains of clinical origin like *Escherichia coli*, *Salmonella typhimurium*, *Shigella sonnei*, *Vibrio cholerae* and *Staphylococcus aureus*. These strains were obtained from Department of Pathology, Faculty of Medicine, Songklanakarin Hospital, Southern Thailand. Gastrointestinal pathogens were routinely grown in Brain Heart Infusion broth (BHI, Difco, USA) at 37°C for 18 h.

#### 2.2 Identification of lactobacilli strain by 16s rRNA gene sequencing

*L. plantarum* was cultured in MRS broth (500 ml) and incubated in anaerobic condition at 37°C for 48 h. Cells were harvested at 10,000 g for 5 min at 4°C. The cell pellet was resuspended in 5 ml of solution I [50 mM Tris pH 8, 50 mM EDTA] and



freeze the cell suspension at  $-20^{\circ}\text{C}$  for 30 min. Add 0.5 ml of solution II [250 mM Tris pH 8], 1 ml of lysozyme to the frozen cells, thaw at room temperature, and place on ice for 45 min. Add 1 ml of solution III [0.5% SDS, 50 mM Tris pH 7.5, 0.4 M EDTA], 20  $\mu\text{l}$  of 20 mg/ml proteinase K. Place the mixture in  $50^{\circ}\text{C}$  water bath for 60 min. The cell pellet was suspended in DNA extraction chromosomal DNA.

Extraction of chromosomal DNA was achieved using the protocol of Leenhouts *et al.*, (1990). Extract with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) by vortexing for 1 minutes followed by centrifugation at 8,500 g for 30 min. Supernatant was then transferred to 50 ml tube to which 1/10 volume of 3 M sodium acetate and 2 volumes of absolute alcohol was added, mixed and vortexed. The tube containing above mixture was centrifuged at 9,000 g for 45 min. Discard the supernatant and dissolve the DNA in 0.2 ml ultra-pure water. DNA was further purified by using the DNA purification kit (Amersham Biosciences, USA) and DNA solution was quantified using UV spectrophotometer 260/280 nm.

The primers LacAll-F (5'-TGCCTAATACATGCAAGTC-3') and LacAll-R (5'-CCTTGTTACGACTTCACC-3') were used to amplify nearly the full length of the 16S rRNA gene, corresponding to the conserved 16S rRNA gene regions of *L. plantarum*. PCR amplification was performed with a Perkin-Elmer DNA Thermal Cycler 9600, programmed for 35 cycles, comprising denaturation at  $94^{\circ}\text{C}$  for 45 s, annealing at  $50^{\circ}\text{C}$  for 1 min and elongation at  $72^{\circ}\text{C}$  for 2 min, and followed by a final extension at  $72^{\circ}\text{C}$  for 7 min. An amplicon of about 1500 bp in size was excised from a 1% agarose gel after electrophoresis, purified using the GFX™ Purification Kit (Amersham Biosciences, USA), and cloned into pGEM-T Easy using the procedures recommended by the manufacturer (Promega, USA). The 16S rRNA gene was

sequenced directly using the recombinant plasmids as DNA template, an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA), T7 and SP6 universal primers, and primers designed by a walking strategy (Kaewsrichan *et al.*, 2006). Analysis of alignment and homology for the sequences obtained was performed by BIOEDIT and BLAST programs.

## **2.3 Probiotics property of lactobacilli**

### **2.3.1 Antimicrobial activity**

Inhibitory activity of *L. plantarum* (T23/3) was measured by agar overlay diffusion method (Charteris *et al.*, 1998). Briefly the *L. plantarum* (T23/3) was cultured in MRS broth and incubated anaerobically at 37°C for 24 h. Lactobacilli culture pellets were collected by centrifugation at 15,000 g, for 10 min at 4°C. The cell pellet of this strain was resuspended in phosphate buffered saline (PBS, pH 7.4) to a final concentration of  $1 \times 10^8$  cfu/ml. Ten  $\mu$ l of lactobacilli cell pellet was spotted on BHI agar with 1% of glucose plates and incubated anaerobically at 37°C for 4 days.

For the indicator strains of five gastrointestinal pathogens were i.e., *Escherichia coli*, *Staphylococcus aureus*, *Vibrio cholerae*, *Shigella sonnei* and *Salmonella typhimurium*, which were cultured in BHI broth under aerobic condition at 37°C for 18 h. Pathogens culture pellets were collected by centrifugation at 15,000 g, for 10 min at 4°C. The cell pellet of indicator pathogens was resuspended in PBS to final concentration of  $1 \times 10^8$  cfu/ml. Four ml of inoculum in soft medium was overlaid on the top of the plates of lactobacilli. After incubation at 37°C for 18 h, the plates

were observed for clear zone of inhibition around lactobacilli colonies using antibiotic zone reader. The colonies with antimicrobial activity were collected for further studies.

### **2.3.2 Susceptibility to antibiotics**

Seven antibiotic discs from Oxoid Ltd. (Basingstoke, UK) were used to determine the antibiotic resistance of lactobacilli strains by using agar disc diffusion method (Klare *et al.*, 2007). The antibiotic discs (Oxoid, UK) were ampicillin (30 µg), chloramphenicol (30 µg), doxycycline (30 µg), erythromycin (15 µg), neomycin (30 µg), penicillin (10 unit) and tetracycline (30 µg). The cell pellet of *L. plantarum* (T23/3) was resuspended in PBS to a final concentration of  $1 \times 10^8$  cfu/ml. The adjusted cultures were streaked with sterile cotton swab onto surface of MRS agar plates. The antibiotics discs were placed onto the surface of the plates agar. After incubation under anaerobic condition at 37°C for 24 h, the plates were observed for clear zone of inhibition around lactobacilli colonies with antibiotic zone reader.

### **2.3.3 Survival in simulated gastric fluid and simulated intestinal fluid**

Simulated gastric fluids (SGF) were prepared by suspending pepsin (1:10000, ICN, Sigma, USA) in the PBS to a final concentration of 3 g/l and adjusting at pH 2.0, 3.0, and 4.0 with concentrated hydrochloric acid.

Simulated small intestinal fluids (SIF) were prepared by suspending pancreatin USP (P-1500, Sigma, USA) in the PBS to a final concentration of 1 g/l, with 0.45% bile salts (Oxoid, Sigma, USA), and adjusting the pH to 8.0 with sterile 0.1 mol/l of sodium hydroxide.

The activated culture of *L. plantarum* (T23/3) was centrifuged at 5,000 g, 7 min at 4°C. The cell pellet was washed once with sterile PBS and resuspended in PBS to a final concentration of  $1 \times 10^8$  cfu/ml. One ml of cell pellet suspension of this strain was suspended in either 9 ml SGF or SIF before incubating under anaerobic condition at 37°C. Resistance was assessed in terms of viable colony counts and enumerated on MRS agar plate after incubation at 37°C for 1, 2 and 3 h with SGF, and 1, 2, 3 and 4 h with SIF, respectively.

### **2.3.4 Aggregation activity**

#### **2.3.4.1 Autoaggregation test**

Autoaggregation abilities were measured as described by Andreu *et al.*, (1995) using the autoaggregation percentage. *L. plantarum* (T23/3) culture in de MRS (Diffco, USA) broth under anaerobic condition at 37°C for 24 h. Cells were resuspended in PBS to a final concentration of  $1 \times 10^8$  cfu/ml. A drop was placed on a glass slide and examined microscopically. Autoaggregation was determined as the ability to form aggregates within 2 min. *L. cellobiosus* was isolated from fecal of healthy elderly human and used as a negative control.

#### **2.3.4.2 Surface hydrophobicity**

Surface hydrophobicity of this strain was studied by the salt aggregation test (SAT). The cell pellet of *L. plantarum* (T23/3) was resuspended in 0.02 mol/l of sodium phosphate (pH 6.8, Merck, Germany) to a final concentration of  $10^8$  cfu/ml. Solutions (500 µl) of ammonium sulfate (Merck, Germany) (0.5, 1.5, 2.0 and 4.0 mol/l) were mixed with an equal volume of cell suspension on a glass slide and

examined microscopically. The lowest concentration of ammonium sulfate causing the bacteria to aggregate was defined as the SAT value. Strain was classified into three groups: high surface hydrophobicity (SAT<0.9 mol/l), intermediate hydrophobicity (SAT 0.9-1.5 mol/l) and hydrophilic (SAT >1.5 mol/l) (Andreu *et al.*, 1995).

#### **2.3.4.3 Co-aggregation assay of pathogens with lactobacilli**

The cell pellet of *L. plantarum* (T23/3) and indicator pathogens (*E. coli*, *S. typhimurium*, *S. sonnei*, *V. cholerae* and *S. aureus*) were resuspended in PBS to final concentration of  $1 \times 10^8$  cfu/ml. Aliquots (500  $\mu$ l) of lactobacilli strain were mixed with an equal volume of each indicator pathogens. The co-cultures were shaken in anaerobic condition at 37°C, 100 rpm for 4 h. After incubation, the suspensions were Gram-stained and observed under a microscope. A co-aggregation assay was positive if *L. plantarum* (T23/3) formed aggregates with the other pathogen strains (Reid, 1999).

#### **2.3.5 In vitro adherence assay**

The adherence of lactobacilli strain was examined using enterocyte like Caco-2 cells (ATCC HTB-37, Rockville, USA). Cells were routinely grown in Eagle's minimal essential medium (MEM; Gibco, USA) supplemented with 15% (v/v) fetal bovine serum (FBS; Gibco, USA), antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Gibco, USA). Monolayers were prepared in 24-well tissue culture plates (Corning, USA), seed (1 ml) at a concentration of  $4.5 \times 10^5$  cell/well inside the wells and incubated at 37°C in 5% CO<sub>2</sub> for 15 days with the culture medium changed daily.

For the adherence assay, Caco-2 monolayers were washed two times with PBS 1 ml/ well and pre-incubated with 500  $\mu$ l complete medium without antibiotics for at least 30 min before inoculation with lactobacilli strain. Bacterial  $10^8$  cfu/ ml MEM suspension (500  $\mu$ l) was transferred onto the Caco-2 monolayers. The plate was incubated at 37°C in 5% CO<sub>2</sub> for 1 h. After incubation, the well was washed four times with PBS to release unbound bacteria. The washed monolayer was treated with 1 ml of 0.05% Triton X-100 and incubated at 37°C for 5 min to lyse the cells. The number of viable adhering lactobacilli was determined by plate counting on MRS agar.

To study the effect of lactobacilli treatment on the pathogens interaction with enterocyte like cells, Caco-2 cells were used. Caco-2 cells were seeded in 24-well tissue culture plates and incubated at 37°C in 5% CO<sub>2</sub> for 15 days. Before starting the adhesion assay, the Caco-2 cells were washed twice with PBS 1 ml/ well. Overnight culture of lactobacilli and pathogens (*E. coli* and *V. cholerae*) were obtained and centrifugated at 10,000g for 10 min. Cell pellets were then diluted with MEM without antibiotic to give both of  $10^8$  cfu/ml.

- **In competition assay**, *L. plantarum* (T23/3) 500  $\mu$ l were added simultaneously with *E. coli* 500  $\mu$ l or *V. cholerae* 500  $\mu$ l.
- **In exclusion assay**, *L. plantarum* (T23/3) 500  $\mu$ l were added and incubated at 37 °C in 5% CO<sub>2</sub> for 30 min. Then, *E. coli* 500  $\mu$ l or *V. cholerae* 500  $\mu$ l were added and incubated in the same condition.
- **In displacement assay**, *E. coli* 500  $\mu$ l or *V. cholerae* 500  $\mu$ l were added and incubated at 37 °C in 5% CO<sub>2</sub> for 30 min. Then, *L. plantarum* (T23/3) 500  $\mu$ l were added and incubated in the same condition.

Aliquots (500  $\mu$ l) of lactobacilli strain and equal volume of each indicator pathogens were added Caco-2 monolayers well plate. After incubation at 37°C for 1 h, all wells were washed four times with PBS to release unbound bacteria. The washed monolayer was treated with 1 ml of 0.05% Triton X-100 and incubated at 37°C for 5 min to lyse the cells. For the bacterial adhesion assay Caco-2 cells were seeded on glass cover slips placed for scanning electron microscope (SEM), the number of viable adhering bacteria was determined by plate counting on MRS agar for lactobacilli and BHI agar for gastrointestinal pathogens.

## **2.4 The stability of lactobacilli probiotics as enteric coated capsules**

### **2.4.1 Preparation of *L. plantarum* (T23/3) in freeze dry form**

*L. plantarum* T23/3 of third subculture in MRS broth were incubated at 37°C for 24 h in anaerobic condition. After incubation the inoculates were harvested by centrifugation at 10,000 g for 10 min at 4°C and washed twice with sterile PBS. The cell pellets were suspended with 2% MRS broth 1 L containing 8% lactose (Fluka, Germany). The suspensions obtained with a cell density of about  $10^8$  cfu/ml, sealed with aluminum foil and frozen overnight at -20°C, followed by freeze-drying at -40°C and the final step to 25°C using a freeze-dryer (model FD-300 Airvac Engineering Pty Ltd., Dandenong, Australia). The operating conditions of freeze dry are shown in the Table 2.1. They were then stored in sealed plastic bags in a freezer refrigerator at 4°C.

**Table 2.1** The operating conditions of freeze dryer

Step	Temperature (°C)	Time (min)
1	-40	120
2	-20	300
3	-10	660
4	0	60
5	5	60
6	10	120
7	20	480
E	25	-

#### 2.4.2 Preparation of lactobacilli capsules

The lyophilized powders were consisted of 125 mg containing lactobacilli and lactose was filled into hard gelatin capsule size No.1 using capsule filling machine-model panviv (Prolabo, France). Each batch of the capsules was coated with coating solution (12% Eudragit<sup>®</sup>L-100, 3% Propylene glycol, 1 % Polysorbate 80, 45 % Ethyl alcohol and 39 % Acetone) (Changsan *et al.*, 2010) by spray bottle (Thailand). The spray rate was 3 ml/min with the solution 100 ml per 1,000 capsules and the coating process was done using conventional coating pan. The film was allowed to dry with the help of dryer with an inlet temperature of 35-40°C for 10 min. The capsules were placed in airtight container containing silica-gel desiccants and stored at 4°C under



darkness in refrigerator. Viability was determined at defined time intervals throughout a storage period of 12 months.

### 2.4.3 Viability of lactobacilli

The number of viable lactobacilli before and after freeze-drying and during storage was determined by a plate count method. Lyophilized powders were rehydrated in 1 ml of PBS and these suspensions were serially 2-fold diluted in MRS broth. All dilutions were dropped into MRS agar and colonies were enumerated after incubation of plates under anaerobically at 37°C for 48 h. The colony count (C) was counted. % viability of lyophilized powder was calculated using equation (1). Results were expressed as % viability of lyophilized powder.

$$\% \text{ viability} = \frac{\log [ C_{\text{lyophilization}} \times d ]}{\log [ C_{\text{before lyophilization}} \times d ]} \times 100 \% \quad \text{Equation (1)}$$

When:  $C_{\text{lyophilization}}$  was the colony count of lyophilization powder,  $C_{\text{before lyophilization}}$  was the colony count of viable lactobacilli before lyophilization powder and d was dilution factor.

### 2.4.4 *In-vitro* release studies

Lactobacilli release studies were carried out to assess the ability of capsules to remain intact in the physiological pH conditions of stomach and release the content in intestinal fluid using USP Dissolution Test (Apparatus II, USA) at 50 rpm, 37 ± 0.5°C. The coated formulations (100 capsules per vessel) were tested initially at pH 1.2 for the first 2 h in simulated gastric fluids (SGF) containing 0.1 N hydrochloric

acid 900 ml. Then the simulated intestinal fluids (SIF) containing amount of PBS (pH 7.4) 900 ml which were continued for another 2 h. At time intervals, the numbers of viable lactobacilli in dissolution medium were determined by a plate count method.

## CHAPTER 3

### RESULTS

#### 3.1 Identification of lactobacilli strain

*Lactobacillus* strain was identified to sequencing level using 16s rRNA gene sequences probes because it can be used with a high degree of confidence and they are considered to be one of the fastest way for identification of lactic acid bacteria species in fundamental and applied research. From previous study of Nakpheng (2007), the selected strain (T23/3) was identified to species level using API 50 CHL as *L. plantarum* with 93% confidentiality as shown in the Appendix (Table A.1-A.2). In this study, this strain was confirmed by 16S rRNA sequence system (<http://www.ncbi.nlm.nih.gov/sites/entrez>) under accession number HM051157 (bases 1 to 1,486). 16S rRNA of the isolate was 99% identical with that of other *L. plantarum* strains registered in the GenBank database system as shown in Table 3.1.

#### 3.2 Probiotics property of lactobacilli

##### 3.2.1 Antimicrobial activity

*L. plantarum* (T23/3) was tested for their inhibition activity against gastrointestinal pathogens by overlay method. In this study, the inhibition to five gastrointestinal pathogens i.e., *Escherichia coli*, *Staphylococcus aureus*, *Vibrio cholerae*, *Shigella sonnei* and *Salmonella typhimurium* by *L. plantarum* (T23/3) is shown in Table 3.2. The colony culture of this strain showed antibacterial ability against all indicator pathogen strains.

**Table 3.1** The 16s rRNA gene sequences of the tested *L. plantarum* at the GenBank

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Nucleotide sequence of *L. plantarum* (T23/3) 99% accession No. HM 051157

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ORIGIN

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1 cctgttacg acttcacct aatcatctgt cccaccttag gcggtggtt cctaaaaggt
61 taccaccg actttgggtg ttacaaacte tcatggtgtg acgggcggtg tgtacaaggc
121 ccgggaacgt attcaccgcg gcatgctgat ccgcgattac tagcgattcc gacttcatgt
181 aggcgagtg cagcctacaa tccgaactga gaatggcttt aagagattag ctactctcg
241 cgagttcgca actcgttga ccatccattg tagcacgtgt gtagccagg tcataagggg
301 catgatgatt tgacgtcate cccaccttcc tccggttgt caccggcagt ctcaccagag
361 tgcccaactt aatgctggca actgataata agggttgctc tcgttcgggg acttaacca
421 acatctcacg acacgagctg acgacaacca tgcaccacct gtatccatgt ccccgaaggg
481 aacgtctaat ctcttagatt tgcatagtat gtcaagacct ggtaaggctt ttcgcgtage
541 ttcgaattaa accacatgct ccaccgcttg tgcgggcccc cgtaattcc tttgagttc
601 agccttgctg ccgtactccc caggcggaat gctaatgctg ttagctgcag cactgaaggg
661 cggaaacctt ccaaacctta gcatcctcg tttacggtat ggactaccag ggtatctaat
721 cctgtttgct acccacttct tegagctca gcgtcagtta cagaccagac agccgccttc
781 gccactggtg ttcttcata tatctacgca ttcaccgct acacatggag ttcactgtc
841 ctctctgca ctcaagtctt ccagtttccg atgcacttct tcggttgagc cgaaggcttt
901 cacatcagac ttaaaaaacc gcttgcgctc gctttacgcc caataatcc ggacaacgct
961 tgccacctac gtattaccgc ggctgctggc acgtagttag ccgtggcttt ctggttaaat
1021 accgtcaata cctgaacagt tactctcaga tatgttctt ttaacaaca gagttttacg
1081 agccgaaacc ctcttcaact cagcggcgtg tctccatca gacttctgc cattgtggaa
1141 gattccctac tgctgcctcc cgtaggagtt tgggccgtgt ctacgtcca atgtggccga
1201 ttaccctctc aggtcggcta cgtatcattg ccatggtgag ccgttaccac accatctagc
1261 taatacgccg cgggaccatc caaaagtgat agccgaagcc atcttcaag ctccggaccat
1321 gcggtccaag ttgttatgcg gtattagcat ctgtttccag gtgttatccc ccgcttctgg
1381 gcaggttcc cactgtgtac tcaccagttc gccactcact caaatgtaaa tcatgatgca
1441 agcaccaatc aataccagag ttcgttcgac ttgcatgtat taggca

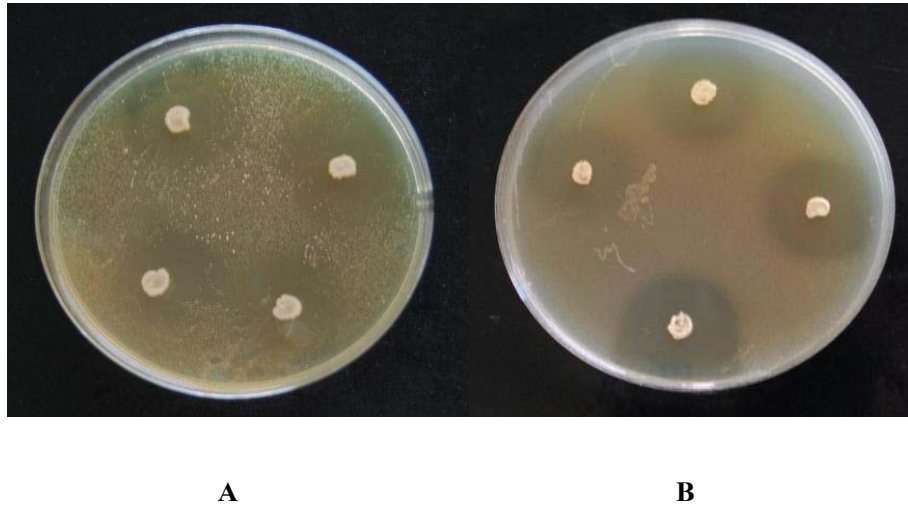
```

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Note: Source: *Lactobacillus plantarum* (T23/3)

Base Count: bases 1 to 1486; 331 a, 435 c, 324 g, 396 t

a= Adenine, c= Cytosine, g= Guanine, t= Thymine



**Figure 3.1** The inhibition zone of *L. plantarum* (T23/3) against gastrointestinal pathogens by overlay method

Note: A. *Staphylococcus aureus*

B. *Escherichia coli*

This result suggests that *L. plantarum* (T23/3) has high inhibitory activity against both Gram positive and Gram negative bacteria as shown in Figure 3.1. The inhibition zone to *E. coli*, *S. typhimurium*, *V. cholerae*, *S. aureus* and *S. sonnei* were between 36-38 mm (Table 3.2). *Lactobacillus* in controlling the proliferation of pathogenic bacteria is in a wide range which suggests the broad spectrum of antibacterial activity.

**Table 3.2** Inhibition zone produced from *L. plantarum* (T23/3) with the activity tested against gastrointestinal pathogens by overlay method

Gastrointestinal pathogens	Inhibition zone (mm)
	(mean $\pm$ sd), n=3
<i>Escherichia coli</i>	38.8 $\pm$ 0.8
<i>Salmonella typhimurium</i>	38.0 $\pm$ 0.7
<i>Shigella sonnei</i>	36.6 $\pm$ 0.8
<i>Staphylococcus aureus</i>	37.8 $\pm$ 0.4
<i>Vibrio cholerae</i>	38.0 $\pm$ 0.7

### 3.2.2 Susceptibility to antibiotics

Susceptibility of *L. plantarum* (T23/3) to antibiotics is shown in Table 3.3. This strain was found to be resistant to erythromycin, doxycycline, neomycin, penicillin G and tetracycline but it was sensitive to ampicillin and chloramphenicol. Since this strains survived during a specific antibiotic treatment (macrolide, tetracycline, penicillin and aminoglycoside), better management is obtained when concurrent therapy is made with probiotics lactobacilli and antibiotics to which they are intrinsically resistant. Susceptibility of *L. plantarum* (T23/3) to antibiotics observes for clear zone of inhibition zone interpretive standard the disc diffusion technique as shown in the Appendix (Table B-1).

**Table 3.3** Antibiotic susceptibility of *L. plantarum* (T23/3)

Antibiotic		Inhibition zone (mm)
Ampicillin	(10 µg)	S (33.5)
Chloramphenicol	(30 µg)	S (29)
Erythromycin	(15 µg)	R (13.5)
Doxycycline	(30 µg)	R (15)
Penicillin G	(10 unit)	R (20)
Neomycin	(30 µg)	R (12)
Tetracycline	(30 µg)	R (10)

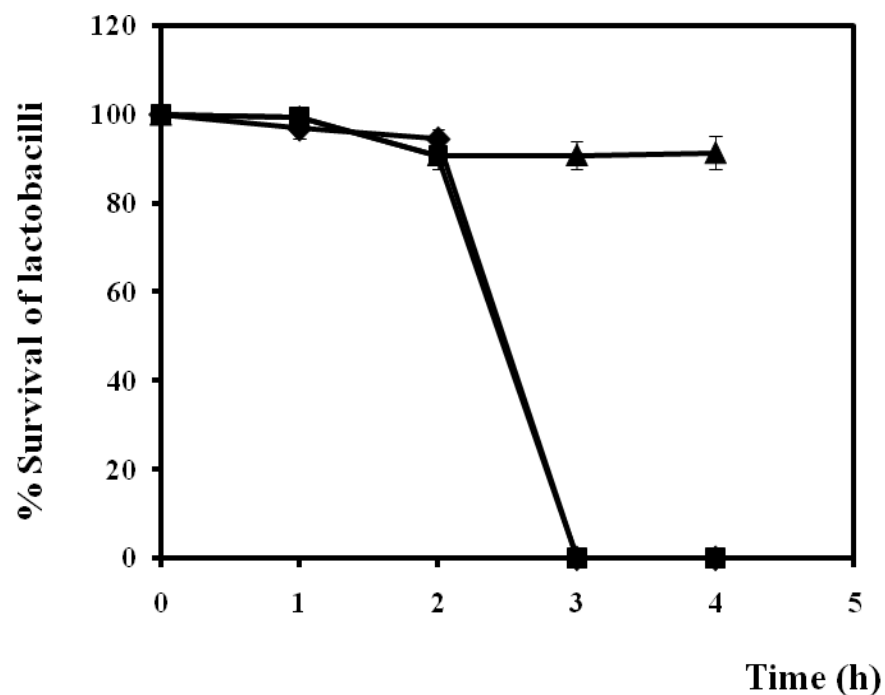
S = Sensitive, R= Resistance

### 3.2.3 Survival in simulated gastrointestinal fluid and simulated intestinal fluid

The viability of *L. plantarum* (T23/3) was not detected after 2 h in SGF at pH 2 and pH 3. At pH 4, its number decreased about 1 log cycle after 2 h as shown in Figure 3.2. The results showed that *L. plantarum* (T23/3) strains were able to retain their viability when exposed to pH value 4.0, but displayed loss of viability at lower pH values. It is not clear whether the decrease of viability conferred by SGF at pH 2 and pH 3 was also due to the pepsin alone, or in synergy with high acidity. The improved viability of microorganism during pH 2.0-4.0 simulated gastric transit with

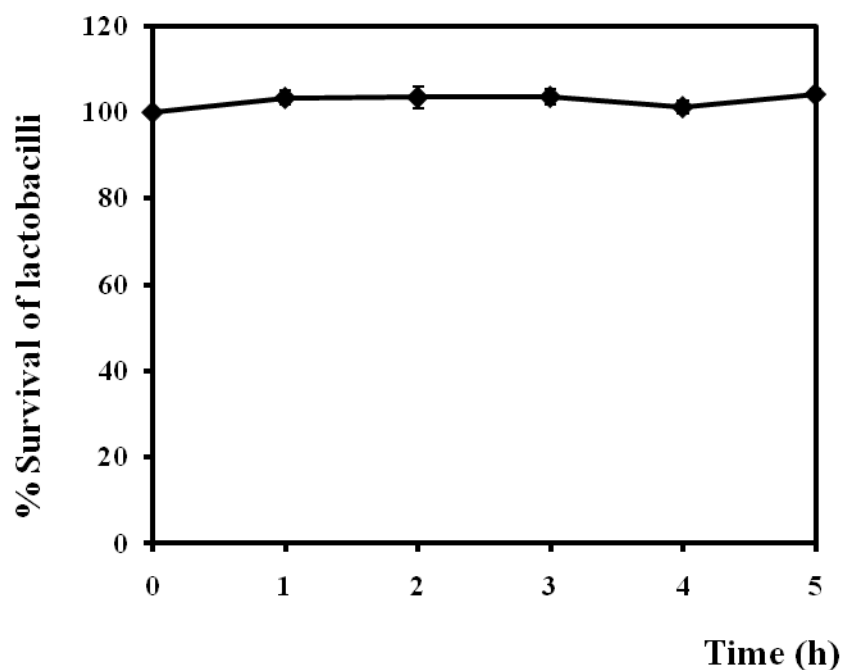
the addition of food indicates that low acid tolerant strains need not be excluded from probiotic application.

In SIF at pH 8.0, the total number of *L. plantarum* (T23/3) was no loss of viability after incubation as shown in Figure 3.3. This strain demonstrated high level of survival in SIF. SIF is essential for probiotic strains to colonize the small intestine. This result indicates that *L. plantarum* (T23/3) can colonize and survive in the small intestine.



**Figure 3.2** Survival of *L. plantarum* (T23/3) in simulated gastric fluid at pH 2 (◆), pH 3 (■), pH 4 (▲) (mean  $\pm$  sd, n = 3).





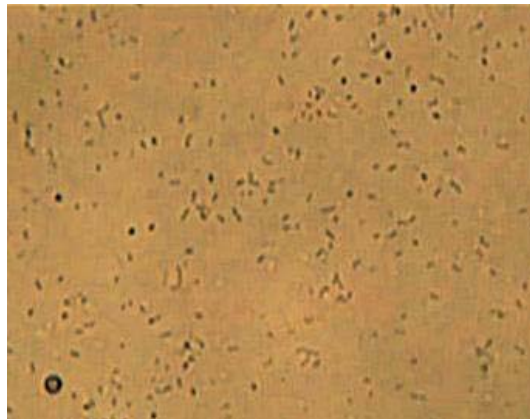
**Figure 3.3** Survival of *L. plantarum* (T23/3) in simulated intestinal fluid at pH 8 (◆) (mean  $\pm$  sd, n = 3).

### 3.2.4 Aggregation activity

#### 3.2.4.1 Autoaggregation and surface hydrophobicity test

As the ability of bacteria to adhere to tissues is an important factor for colonization in different hosts. Our results suggest that a variety of surface structures in these bacteria may be involved in adherence mechanisms in addition to auto-aggregation and hydrophobic properties. Aggregation was not found for *L. cellobiosus* which was isolated from faecal of healthy elderly human and used as a negative control as shown in Figure 3.4. The result of auto-aggregation and surface hydrophobicity assay of *L. plantarum* (T23/3) showed self-aggregation producing

macroscopic granules the phenomenon was clearly observable under the light microscope as shown in Figure 3.5 and this strain showed high surface hydrophobicity by SAT with ammonium sulfate concentration of 0.5 mol/l as shown in Figure 3.6. This result indicates that the *L. plantarum* (T23/3) possess high potential ability to inhibit growth of pathogens by competitive exclusion of pathogens from the cell surface and produce biofilm formation that prevents colonization by pathogens.



**Figure 3.4** *L. cellobiosus* under the light microscope (50X)



**Figure 3.5** *L. plantarum* (T23/3) show self-aggregation under the light microscope (50X)



**Figure 3.6** *L. plantarum* (T23/3) show high surface hydrophobicity by SAT with ammonium sulfate concentration of 0.5 mol/l observable under the light microscope (50X)

#### **3.2.4.2 Co-aggregation**

The result of co-aggregation assay of *L. plantarum* (T23/3) showed co-aggregation and adhesion with five strains of gastrointestinal pathogens *E. coli*, *S. typhimurium*, *S. aureus*, *S. sonnei* and *V. cholerae* observable under the light microscope. Probiotic strains tested showed aggregation abilities with the pathogen strains tested, but the percentage of co-aggregation was demonstrated to be strain specific. Such co-aggregation could be an important factor in maintaining gastrointestinal health because it produces an area around the pathogen where the concentration of antimicrobial substances produced by the lactobacilli is increased.

#### **3.2.5 In vitro adherence assay**

Adhesion of lactobacilli has been claimed to be essential for the exertion of a beneficial probiotic effect in the large intestine. The binding ability of *L. plantarum* (T23/3) was evaluated using the human colon carcinoma cell line (Caco-2) as a cellular model. Recent studies have explained the role of lactobacilli in the prevention and treatment of gastrointestinal disorders. One the important factor is the competitive inhibition of enteropathogen attachment to epithelial cells by lactobacilli. So, In this study, investigated the competitive inhibition of adherence of pathogenic bacteria to Caco-2 cell by adhering *L. plantarum* (T23/3). In this study, after incubation *L. plantarum* (T23/3) showed the strongest adhesion to Caco-2 cells in the highest numbers  $4.5 \times 10^5$  cfu/ml as shown in Figure 3.7 and pathogenic strains (*E. coli* and *V. cholerae*) adhering to Caco-2 cells were shown in Figure 3.8-3.9.

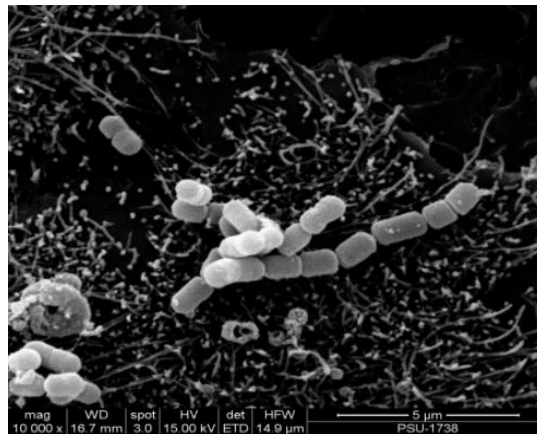
Cell adhesion is multistep process involving contact of the bacterial cell membrane and interacting surface. The competition, exclusion and displacement inhibition of adherence of pathogenic bacteria to Caco-2 cell by adhering *L. plantarum* (T23/3) were studied. The result showed that percent ability to adhesion to Caco-2 cell of *L. plantarum* (T23/3) and pathogenic bacteria (*E. coli* and *V. cholerae*). In competition experiment the percent ability of adhesion to Caco-2 cell of co-culture *L. plantarum* (T23/3) and *E. coli* and co-culture *L. plantarum* (T23/3) and *V. cholerae* were determined. The adhesion percent to Caco-2 cell of *L. plantarum* (T23/3) and *E. coli* in the co-culture were found to be 97.3% and 74.3%, respectively. The adhesion percent to Caco-2 cell of *L. plantarum* (T23/3) and *V. cholerae* in the co-culture were found to be 95.6% and 75.9%, respectively ( $p < 0.01$ ) as shown in Figure 3.10.

In exclusion experiment the percent ability of adhesion to Caco-2 cell of co-culture *L. plantarum* (T23/3) and *E. coli* and co-culture *L. plantarum* (T23/3) and *V. cholerae* were determined. The adhesion percent to Caco-2 cell of *L. plantarum* (T23/3) and *E. coli* in the co-culture were found to be 96.3% and 70.3%, respectively. The adhesion percent to Caco-2 cell of *L. plantarum* (T23/3) and *V. cholerae* in the co-culture were found to be 98.3% and 73.5%, respectively ( $p < 0.01$ ) as shown in Figure 3.11.

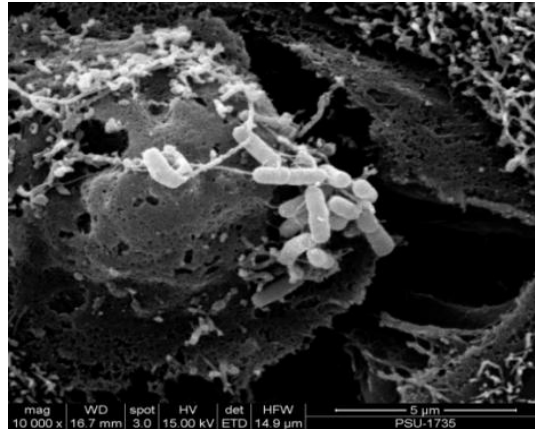
In displacement experiment the percent ability of adhesion to Caco-2 cell of co-culture *L. plantarum* (T23/3) and *E. coli* and co-culture *L. plantarum* (T23/3) and *V. cholerae* were determined. The adhesion percent to Caco-2 cell of *L. plantarum* (T23/3) and *E. coli* in the co-culture were found to be 98.9% and 80.3%, respectively. The adhesion percent to Caco-2 cell of *L. plantarum* (T23/3) and *V. cholerae* in the

co-culture were found to be 97% and 79.5%, respectively ( $p < 0.01$ ) as shown in Figure 3.12.

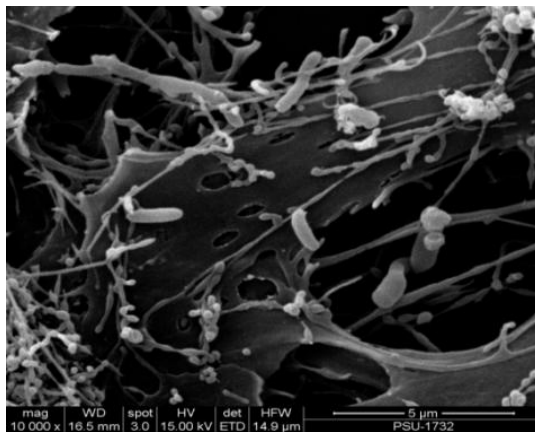
The strain *L. plantarum* (T23/3) shows good adhesion and pathogens inhibition property therefore it was selected for producing a probiotic product.



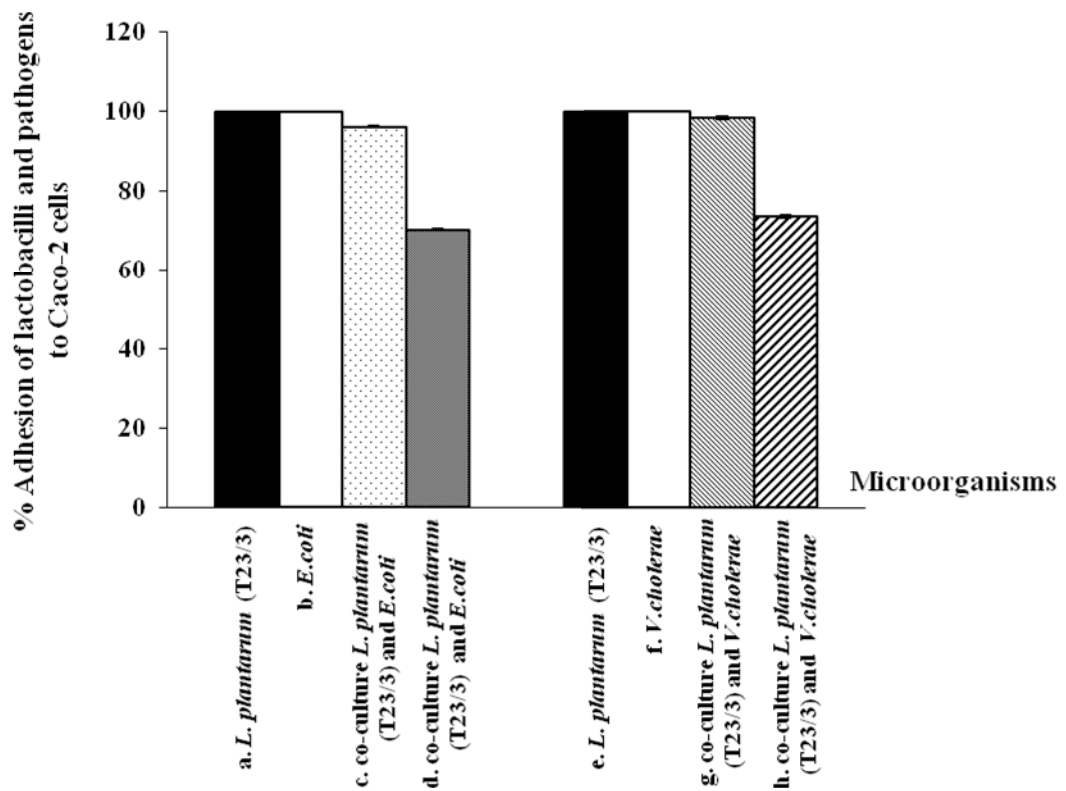
**Figure 3.7** Adhesion of *L. plantarum* (T23/3) to Caco-2 cell observed using SEM.



**Figure 3.8** Adhesion of *E. coli* to Caco-2 cell observed using SEM



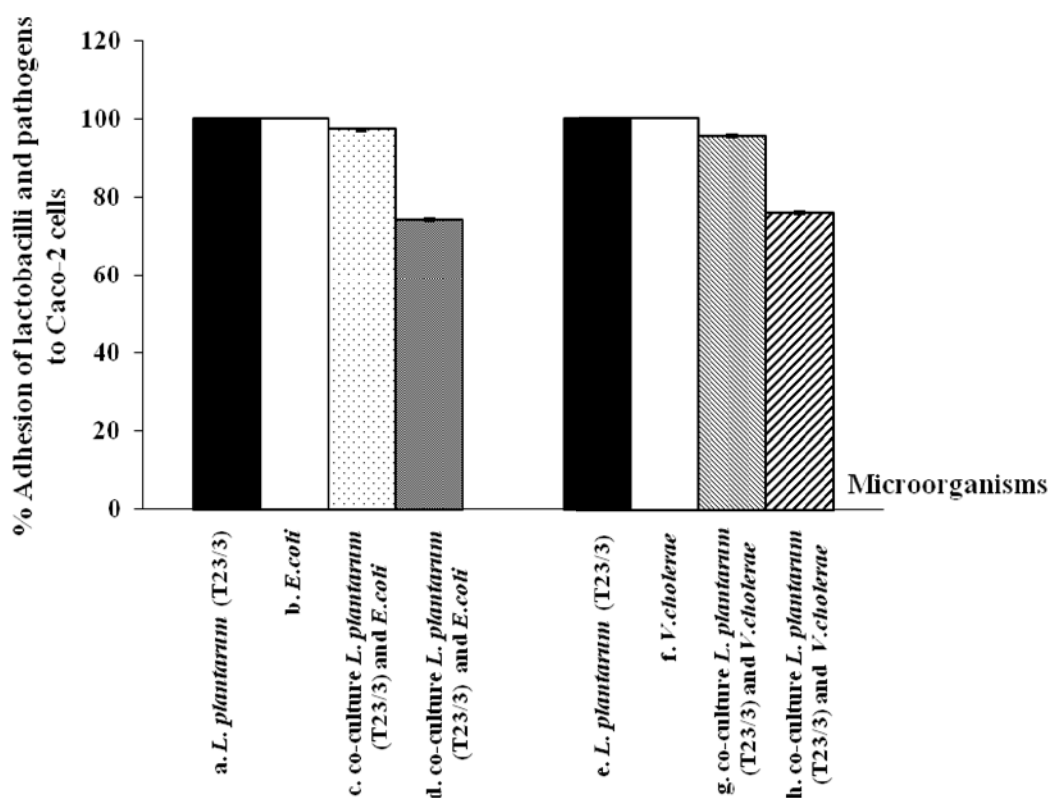
**Figure 3.9** Adhesion of *V. cholerae* to Caco-2 cell observed using SEM



**Figure 3.10** The adhesion ability to Caco-2 cell of *L. plantarum* (T23/3) and pathogens (*E. coli* and *V. cholerae*) in competition experiment (mean  $\pm$  sd, n = 3).

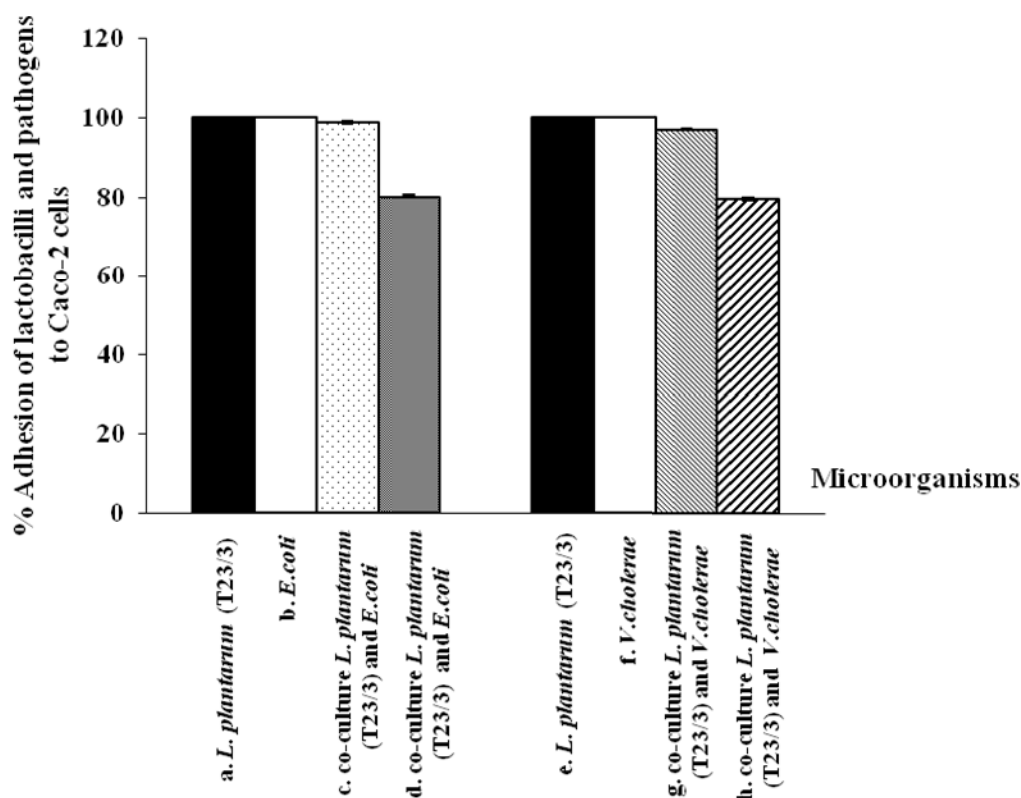
- Note: a. *L. plantarum* (T23/3) (100% *L. plantarum* (T23/3) adhesion)
- b. *E. coli* (100% *E. coli* adhesion)
- c. Co-culture *L. plantarum* (T23/3) and *E. coli* (97.3% *L. plantarum* (T23/3) adhesion)
- d. Co-culture *L. plantarum* (T23/3) and *E. coli* (74.3% *E. coli* adhesion)
- e. *L. plantarum* (T23/3) (100% *L. plantarum* (T23/3) adhesion)
- f. *V. cholerae* (100% *V. cholerae* adhesion)
- g. Co-culture *L. plantarum* (T23/3) and *V. cholerae* (95.6% *L. plantarum* (T23/3) adhesion)
- h. Co-culture *L. plantarum* (T23/3) and *V. cholerae* (75.9% *V. cholerae* adhesion)





**Figure 3.11** The adhesion ability to Caco-2 cell of *L. plantarum* (T23/3) and pathogens (*E. coli* and *V. cholerae*) in exclusion experiment (mean  $\pm$  sd, n = 3).

Note: a. <i>L. plantarum</i> (T23/3)	(100% <i>L. plantarum</i> (T23/3) adhesion)
b. <i>E. coli</i>	(100% <i>E. coli</i> adhesion)
c. Co-culture <i>L. plantarum</i> (T23/3) and <i>E. coli</i>	(96.3% <i>L. plantarum</i> (T23/3) adhesion)
d. Co-culture <i>L. plantarum</i> (T23/3) and <i>E. coli</i>	(70.3% <i>E. coli</i> adhesion)
e. <i>L. plantarum</i> (T23/3)	(100% <i>L. plantarum</i> (T23/3) adhesion)
f. <i>V. cholerae</i>	(100% <i>V. cholerae</i> adhesion)
g. Co-culture <i>L. plantarum</i> (T23/3) and <i>V. cholerae</i>	(98.3% <i>L. plantarum</i> (T23/3) adhesion)
h. Co-culture <i>L. plantarum</i> (T23/3) and <i>V. cholerae</i>	(73.5% <i>V. cholerae</i> adhesion)



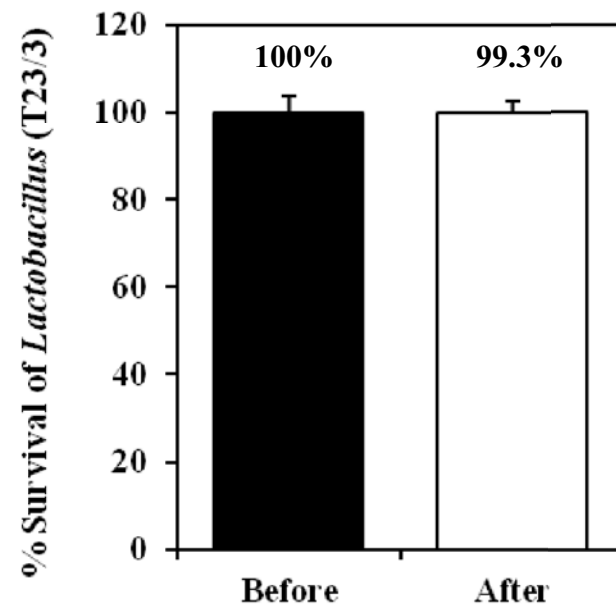
**Figure 3.12** The adhesion ability to Caco-2 cell of *L. plantarum* (T23/3) and pathogens (*E. coli* and *V. cholerae*) in displacement experiment (mean  $\pm$  sd, n = 3).

- Note: a. *L. plantarum* (T23/3) (100% *L. plantarum* (T23/3) adhesion)  
 b. *E. coli* (100% *E. coli* adhesion)  
 c. Co-culture *L. plantarum* (T23/3) and *E. coli* (98.9% *L. plantarum* (T23/3) adhesion)  
 d. Co-culture *L. plantarum* (T23/3) and *E. coli* (80.3% *E. coli* adhesion)  
 e. *L. plantarum* (T23/3) (100% *L. plantarum* (T23/3) adhesion)  
 f. *V. cholerae* (100% *V. cholerae* adhesion)  
 g. Co-culture *L. plantarum* (T23/3) and *V. cholerae* (97% *L. plantarum* (T23/3) adhesion)  
 h. Co-culture *L. plantarum* (T23/3) and *V. cholerae* (79.5% *V. cholerae* adhesion)

### **3.3 The stability of lactobacilli probiotics as enteric coated capsules**

#### **3.3.1 Survival of lactobacilli before and after freeze dry**

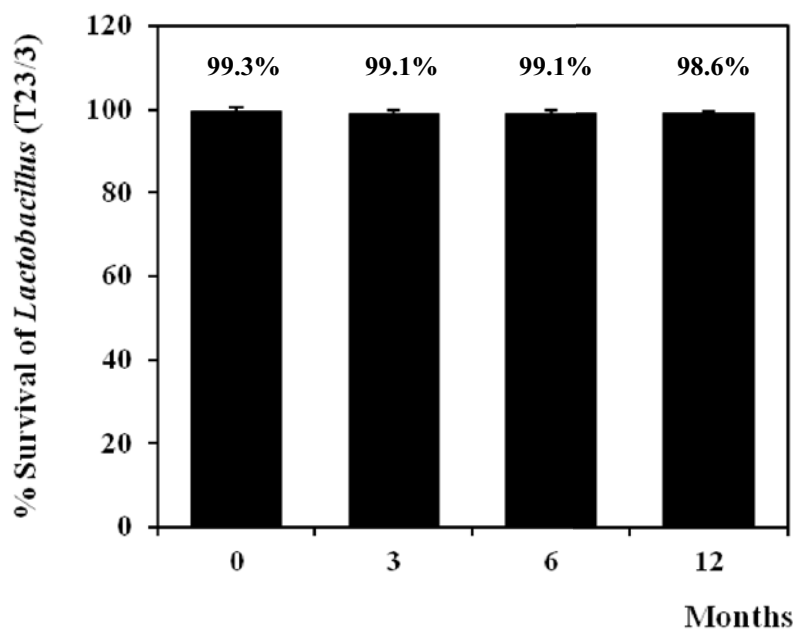
The freeze-drying process is commonly used for the preservation and storage of microorganisms for industrial application. The optimal performance of certain strains should guarantee their potential to survive and stabilize their metabolic activity. Survival rate of the microorganism varied among the strains and the agents used as suspending media. In the present studies, we used lactose as a protective agent and enhanced this survival during storage. In addition, lactose is used in pharmaceutical formulas based on powder to increase their solubility. Thus, for our purpose, lactose would promote the delivery of microorganism growth because they can use this sugar as a carbon source. In this study, *L. plantarum* (T23/3) strain with lactose showed no significant differences ( $p < 0.01$ ) between the survival of this strain before and after lyophilization as shown in Figure 3.13.



**Figure 3.13** Survival of *L. plantarum* (T23/3) before and after freeze drying ( $p < 0.01$ ).

### 3.3.2 Stability of lactobacilli probiotics in hard gelatin capsule

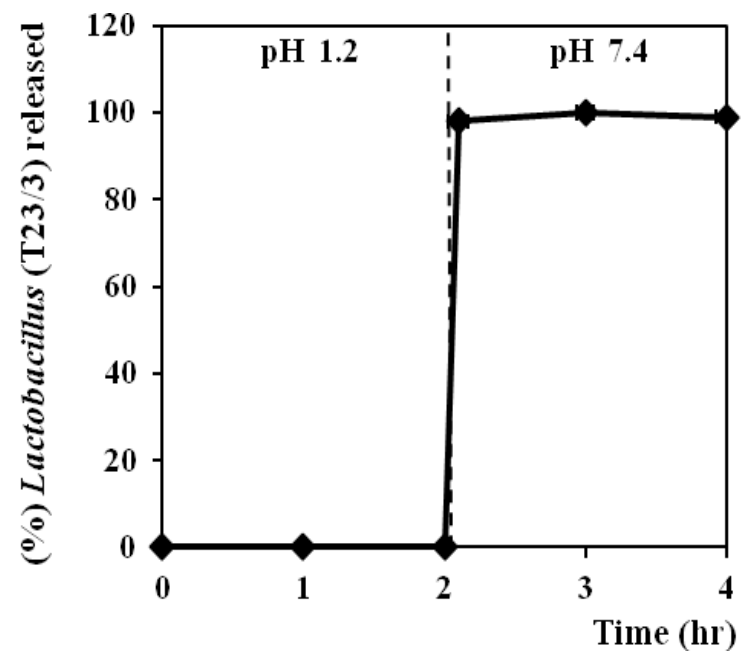
The stability of this strain in capsules containing freeze-dried bacteria with lactose showed no decrease in the viable counts and 100% of the original cells number was recovered after 12 months of storage ( $98.6\% \pm 0.06$ ). The lactose used individually as the drying medium for lactobacilli acted as protective agent and improved the survival of microorganisms during the storage in a significant manner ( $p < 0.01$ ) as shown in Figure 3.14.



**Figure 3.14** Survival of *L. plantarum* (T23/3) stored as freeze-dried powder into Eudragit<sup>®</sup>L-100 coated capsule during 12 months at 4°C ( $p < 0.01$ ) (mean  $\pm$  sd, n = 3).

### 3.3.3 *In-vitro* release studies

In the study, Eudragit<sup>®</sup>L-100 enteric polymer was used to coat the hard gelatin capsules for the targeted delivery of freeze-dried *L.platarum* (T23/3) strain in the small intestine. Eudragit<sup>®</sup>L-100 anionic copolymers of methacrylic acid and acrylates is resistant to the acidic environment present in the stomach but dissolve rapidly in the small intestinal media condition. The *in vitro* release profiles of *L. plantarum* (T23/3) from the freeze dried with enteric-coated capsules are shown in Figure 3.15. At pH 1.2 freeze-dried lactobacilli loaded in the Eudragit<sup>®</sup>L-100 coated capsule did not show any release. In contrast, this capsule dissolved instantly at pH 7.4. The results also demonstrated that Eudragit<sup>®</sup>L-100 can be successfully used from aqueous system to coat capsules for intestine targeted delivery of lactobacilli. The formulation can be adjusted to deliver drug at any other desirable site of the intestinal region of the GI tract on the basis of pH variability.



**Figure 3.15** The release of *L. plantarum* (T23/3) from the freeze-dried in Eudragit<sup>®</sup>L-100 coated capsule to prolonged release using dissolution medium with the pH adjusted to 1.2 and 7.4. (mean  $\pm$  sd, n = 4).

## CHAPTER 4

### DISCUSSION

#### 4.1 Identification lactobacilli strain

The relatively recent introduction of molecular techniques for the detection and quantification of microorganisms has started to create a greater understanding of microbial diversity and its role in nature. Microorganisms can now be grouped according to similarities in their genes, which reflect their evolutionary relationships (Balcazer *et al.*, 2007). The most powerful new approach for the exploration of microbial diversity from complex environmental samples is based on the cloning and sequencing of 16S ribosomal RNA encoding genes (Hugenholtz *et al.*, 1998). An increased knowledge of the structure of these genes, coupled with recent developments in the polymerase chain reaction (PCR), enabled to identify closely related microorganisms by, first, amplifying the 16S rRNA gene directly from isolated colonies using universal primer directed at conserved regions at both ends of the gene, and then sequencing the PCR product (O'Sullivan *et al.*, 2000; Balcazer *et al.*, 2007).

The identification of lactobacilli has been based on colony morphology, Gram-stain reaction, sugar fermentation profiles and enzyme activities. To ensure the identification results, it is needed to be confirmed with other methods such as rRNA sequencing or DNA-DNA hybridization. These methods have been developed to improve the knowledge on generic and supra generic relationship of lactic acid bacteria (Scheifer *et al.*, 1987). In present study, this strain was confirmed to be



*L. plantarum* by genetic methods based on the 16s rRNA gene sequences with 99.0% accuracy. Dickson *et al.*, (2005) reported that 16s rRNA and 32s rRNA sequences probes because it can be used with a high degree of confidence and they considered them to be one of the fastest way for identification of lactic acid bacteria species in fundamental and applied research.

## **4.2 Probiotics property of lactobacilli**

### **4.2.1 Antimicrobial activity**

During the growth of fermentative lactobacilli isolates, pH of the environment gradually decreased due to the conversion of sugars to acid end products. Other microbial inhibitory substances such as lactic acid, short chain fatty acids, hydrogen peroxide and bacteriocin were also produced. In present study, *L. plantarum* (T23/3) inhibited Gram positive bacteria such as *S. aureus* and Gram negative bacteria such as *V. cholera*, *S. sonnei*, *E. coli* and *S. typhimurium in vitro*. The colony culture of *L. plantarum* (T23/3) showed antibacterial ability against all indicator pathogen strains. This result suggests that *L. plantarum* (T23/3) has high inhibitory against both Gram positive and Gram negative bacteria. Generally, the effect of lactobacilli in controlling the proliferation of pathogenic bacteria is by producing a wide range of antibacterial compounds such as organic acids (e.g., lactic acid and acetic acid), hydrogen peroxide, bacteriocins and fatty acid (Lefteris *et al.*, 2006).

Aly and Abo (2006) found that lactobacilli displayed antimicrobial effect toward Gram positive and Gram negative bacteria. Gram positive food borne pathogens such as *S. aureus* and *S. epidermidis* were strongly inhibited by

antimicrobial agent from lactobacilli. Interestingly, the inhibition compound inhibited Gram negative bacteria including food borne pathogens such as *Salmonella*, *S. sonnei* and *E. coli*, respectively.

#### 4.2.2 Sensitivity to antibiotics

Antibiotics resistance has become a serious problem in treatment of infections caused by a variety of microorganisms due to the indiscriminate use of antibiotics in human and veterinary medicine, as well as in animal growth promoters. Detection rate of multi-antibiotic resistant bacteria has increased and resistance is common among strains belonging to the gastrointestinal tract of humans.

One of desirable properties of lactobacilli to be used as probiotic is the resistant to antibiotics. In this study, *L. plantarum* (T23/3) was sensitive to ampicillin and chloramphenicol. However, it was resistant to erythromycin, doxycycline, penicillin G, neomycin and tetracycline. Arici *et al.* (2004) showed that lactobacilli strains of infant feces were sensitive against chloramphenicol, erythromycin, penicillin G and tetracycline. D'Aimmo *et al.*, (2006) found that lactobacilli were susceptible to rifampicin, bacitracin, clindamycin, erythromycin, novobiocin and penicillins and were resistant to nalidixic acid, aztreonam, cycloserine, kanamycin, metronidazole, polymyxin B, and spectinomycin. Previous studies of Katla *et al.*, (2001) indicated that lactobacilli were highly sensitive to ampicillin, cephalothin and erythromycin. If this strain is used as probiotics, they can be taken simultaneously with antibiotics resistant for antibiotic therapy. However, the safety of this strain is essential. The ability to transfer genes for antibiotics resistance must be considered as

an important parameter for the selection of the probiotic strains intended to use in human and animals.

The lactobacilli can effectively protect the natural balance of intestinal microflora during and after therapy by the antibiotics to which they were proved resistant. These strains are also useful in the bio-agents and dairy product manufacture if antibiotics were present in the milk due to antibiotic use in growth promotion and therapy of farm animals. On the other hand, resistant strains may provide resistant genes to other bacteria including pathogens in intestine, especially when antibiotic resistance is plasmid borne. Strains resistance plasmid should not be used either as human or animal probiotics but plasmid-linked antibiotic resistances are not very common among LAB and in most cases antibiotic resistance is not of the transmissible type (Salminen *et al.*, 1998).

The high intrinsic resistance and susceptibility of lactobacillus strains to a range of antibiotics is important. Since this strains survive during a specific antibiotic treatment. In the treatment of gastrointestinal tract infections, better management is obtained when concurrent therapy is made with probiotic lactobacilli and antibiotics to which they are intrinsically resistant. By doing so, intestinal microflora can recover more quickly (Aysun and Candan, 2003).

#### **4.2.3 Survival in simulated gastric fluid**

To reach the intestine, strains must first pass through the stomach, which secretes hydrochloric acid and enzymes. More than two liters of gastric fluid with a pH as low as 1.5 is secreted from cells lining the stomach each day, providing a normally-effective, high-acid barrier against entrance of viable bacteria into the GI.

The effect of gastric pH on bacterial viability and in preventing bacterial colonization of the small intestine is well-studied. Consequently, any probiotic organism that is to survive through the stomach must have a high acid tolerance. In typical acid tolerance tests, the viability of candidate probiotic organisms is determined by exposing them to low pH in a buffer solution or medium for a period of time, during which the number of surviving bacteria remaining is determined (Begley *et al.*, 2005; Del Piano *et al.*, 2006).

This study compared the effect of simulated gastric fluid on the viability of *L. plantarum* (T23/3) selected strain during 4 h simulated gastric transit. There was no loss of viability for *L. plantarum* (T23/3) at pH 4.0, while at pH 2.0 and 3.0 it showed no viability after 2 h. This result is comparable to the finding of Zarate *et al.* (2000), in which lactobacilli strains have shown to survive well at pH 4.0 and these strains lose viability substantially at pH 2.0 and 3.0. The variability of lactobacilli strains to survive at pH 2.0 and 3.0 suggests that the acid tolerance of lactobacilli is strain-specific and pH values of 2.0 and 3.0 could be considered as critical for the selection of potential probiotic. The improved viability of microorganism during pH 2.0 simulated gastric transits with the addition of food indicates that low acid tolerant strains need not be excluded from probiotic application, providing they can be delivered to intestine in high number and preferably as part of buffered food or encapsulated delivery system.

#### **4.2.4 Survival in simulated intestinal fluids**

Small intestinal fluid tolerance is essential for probiotic strains to colonize the small intestine. *L. plantarum* (T23/3) demonstrated high level of small intestinal fluid

tolerance, with no loss of viability after exposure to simulated small intestinal fluid for 5 h at pH 8. From previous study, growth capacity of *L. plantarum* (T23/3) in the presence of bile salt was evaluated. After 4 h, it was able to grow in the PBS containing 0.3% (w/v) oxgall (Nakpheng *et al.*, 2010). Simulated small intestine fluid conditions have little effect on the viability of lactobacilli probiotic. Overall, lactobacilli probiotic showed high capacity of upper gastrointestinal transit tolerance and will provide an alternative source to lactobacilli for future probiotic development.

In probiotic selection, small intestine fluid tolerance is potentially more important than gastric survival. With the development of new delivery systems, evidence clearly demonstrates that acid sensitive strains can be buffered through the stomach. However, to exert a positive effect on the health and well being of a host, probiotics need to colonize and survive in the small intestine (Fernandez *et al.*, 2003; Petros *et al.*, 2006) and it is the condition of this environment that may be an essential selection criteria for future probiotics.

#### **4.2.5 Aggregation activity**

Cell adhesion is a complex process involving contact between the bacterial cell membrane and interacting surfaces. The ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of many bacterial strains used as probiotics. Several researchers have reported investigations on composition, structure and forces of interaction related to bacterial adhesion to intestinal epithelial cells (Del Re *et al.*, 2000) and mucus (Collado *et al.*, 2005). In most cases, aggregation ability is related to cell adherence properties (Del Re *et al.*, 2000). Bacterial aggregation between microorganisms of the same strain (auto-

aggregation) or between genetically different strains (co-aggregation) is of considerable importance in several ecological niches, especially in the human gut, where probiotics are to be active (Jankovic *et al.*, 2003). Therefore, the selected strains not only exhibited for their auto-aggregation and surface hydrophobicity, but also caused co-aggregation and adhesion with pathogenic bacteria (Yan *et al.*, 2010)

The necessary characteristics of *Lactobacillus* strain is to serve as an effective prophylactic agent include prevent of adherence to other intestinal epithelial cells, interference with the adherence of other bacteria, production of bacteriocin and production of H<sub>2</sub>O<sub>2</sub> capable of inhibiting the growth of pathogens (Zhou *et al.*, 2004). *L. plantarum* (T23/3) showed high hydrophobicity, which was similar to that of Liliana *et al.* (2008) work, who concluded that hydrophobicity is an important mechanism in bacterial adherence.

The co-aggregation phenomenon has been observed in bacteria from the human oral cavity and urogenital tract, the mammalian gut and portable water supply systems (Rickard *et al.*, 2003). Furthermore, it has been suggested that inhibitor or bacteriocin producing lactic acid bacteria, which co-aggregate with pathogens, may constitute an important host defense mechanism against infection (Spencer and Chesson, 1994). Co-aggregation with potential gut pathogens could therefore contribute to the probiotic properties ascribed to specific lactic acid bacteria. Different authors had suggested that the cellular aggregation could be positive in promoting the colonization of beneficial microorganisms, as suggested for lactobacilli in the gastrointestinal or vaginal tract (Cesena *et al.*, 2001). Our probiotics strains tested in this study showed co-aggregation abilities with pathogens tested but the percentages depended on each strain (probiotic and pathogen strains).

In order to evaluate cell-cell adherence, a co-aggregation assay was taken and established co-aggregation between selected strains and pathogens. Furthermore, it has been suggested that bacteriocin producing LAB, which co-aggregate with pathogens, may contribute to host defense against infection (Yan *et al.*, 2010).

#### **4.2.6 Adhesion on Caco-2 cells**

The adhesion of probiotic strains to intestinal epithelial cells is considered as a prerequisite feature for attachment and proliferation in the intestinal environment (Kailasapathy and Chin, 2000). Adhesion has also been cited as important for transient colonization (Alander *et al.*, 1999), enhanced healing of the damaged gastric mucosa (Elliott *et al.*, 1998), modulation of the immune system (Perdigon *et al.*, 2002) and antagonism against pathogens (Jin *et al.*, 2000). Caco-2 cell lines have seen extensive use as an *in vitro* model of the human intestinal epithelium for screening probiotic adherence (Gopal *et al.*, 2001), and culture plating has been employed to count the total adhering bacteria to Caco-2 cells (Forestier *et al.*, 2001; Matijasic *et al.*, 2003; Bertazzoni-Minelli *et al.*, 2004; Pennacchia *et al.*, 2006). Some studies have explained the role of lactobacilli in the prevention and treatment of gastrointestinal disorders (Coconier *et al.*, 1998). One important factor is the competitive inhibition of enteropathogen attachment to epithelial cells by lactobacilli. So, we investigated the competitive inhibition of adherence of pathogenic bacteria to Caco-2 cell by adhering *L. plantarum* (T23/3). In this study, after incubation *L. plantarum* (T23/3) showed the strongest adhesion to Caco-2 cells and we investigated the competition, exclusion and displacement inhibition of adherence of pathogenic bacteria to Caco-2 cell by adhering *L. plantarum* (T23/3). This strain was effective as it reduced adhesion of the

*E. coli* and *V. cholerae* to Caco-2 cell. Some studies demonstrated that the ability of some bacterial strains to adhere and colonize the intestinal cell *in vivo* or the cultured intestinal cell *in vitro* is similar. However, it must be considered that Caco-2 cells were only used as a model to study adhesion because of the different morphological, physicochemical and environmental conditions surrounding the epithelial cells in both types of experiences (Pan *et al.*, 2009).

The ability of selected strains of *Lactobacillus* to inhibit the adhesion of pathogenic bacteria is highly specific, and depends on both the probiotic and pathogen strain (Gueimonde *et al.*, 2007). This indicates the need of a case-by-case characterization of the probiotic strains. Except specific antibacterial substances produced by lactic acid bacteria, the inhibition of adhesion could be related to the presence of specific adhesion molecules and receptors for which probiotic and pathogen are competitor. It has to be taken under consideration that observed *in vitro* inhibitory effect of probiotics on pathogen adhesion has to be confirmed *in vivo* (Alicja *et al.*, 2008).

### **4.3 The stability of lactobacilli probiotics as enteric coated capsules**

#### **4.3.1 Viability of probiotics**

The freeze-drying process is commonly used for the preservation and storage of microorganism for industrial application. The optimal performance of certain strains should guarantee their potential to survive and stabilize of their metabolic activity (Otero *et al.*, 2007).



Since the production process could be critical for preserving viable and biological properties of microorganism. A suitable selection of excipients as protecting agents of LAB should be recommendable in the development of probiotic formulations. We assessed in the present study the viability and biological properties of lactobacilli after freeze drying with lactose subsequent storage into gelatin capsules during 12 months. Survival rates of the microorganisms varied among the strains and the agents used as the suspending media. For the microorganisms studied their suspension in lactose for its protective effect and enhanced their survival during storage. Our finding is in accordance with other studies on the viability of different bacterial species commonly used as food starters after freezing, and refrigerated storage (Zayed and Roos, 2004).

Hubalek (2003) has reported that the presence of cryoprotectants is necessary to reduce losses in viability during the freeze-drying process. It is believed that ice formation generates high osmolarity due to high concentration of internal solutes. The removal of water, which affects the properties of many hydrophilic macromolecules in the cells, are the major causes of loss of cell viability during the freeze-drying process (Thammavongs *et al.*, 1996). Hence, cryoprotectants are included with the aim to reduce such adverse effects on the freeze-dried cells. The application of combinations of protective substances led to a better survival than the obtained with excipients individually used (Zayed and Roos., 2004), being lactose the best storage condition for freeze-dried probiotic lactobacilli. Lactose could act as an effective protector due to the presence of hydroxyl groups which provide protection against free radicals and by their water binding capacity that prevents intracellular ice formation (Gagne and Roy, 1993). Lactose is used in pharmaceutical formulas based

on powders to increase their solubility. Thus, for our purpose, lactose would promote the delivery of microorganisms and their growth because they can use this sugar as a carbon source (Otero *et al.*, 2007).

In this study, bacterial survival of product showed good stability up to 12 months after storage in 4°C. Hence, the products should have an acceptable shelf life (Huyghebaert *et al.*, 2005). Despite the importance of viability of these beneficial probiotic, surveys have shown viability of lactobacillus in selection of probiotic (Akalin *et al.*, 2004). Several factors, like acidity of the product, post acidification (acid produced during storage), level of oxygen in the products, sensitivity to antimicrobial substances produced by bacteria, temperature of storage during manufacture and storage, have been found to reduce the viability of probiotics. Thus, maintaining viability of lactobacillus until the products are consumed in order to ensure the delivery of live organisms has been taken as prime importance.

#### **4.3.2 Stability probiotics as enteric coated capsules**

During the early stages of drug development, the limited amount of new chemical entities, sensitive to the gastric fluid or causing gastric irritation, excludes the development of a coated pellet or tablet formulation. Since the coating process of capsules is independent of the capsule content, contrary to coating of pellets and tablets, capsule coating technology can be easily applied (Cole *et al.*, 2002). Enteric coating of capsules are used in the dietary supplement industry for decades (Ogura *et al.*, 1998; Cole *et al.*, 2002). Organic coating of hard gelatin capsules is possible, but it is very sensitive and can lead to shell embrittlement and poor adhesion of the coat to the smooth gelatin surface (Huyghebaert *et al.*, 2004). A pre-coating can reduce

interactions between the gelatin and the enteric polymer but it is time consuming and complicated. Moreover, aqueous coating is preferred over organic coating because of toxicological, environmental and safety-related drawbacks, with hence high manufacturing costs (Cunningham and Fegely, 2001). However, the aqueous coating process of gelatin capsules is very sensitive and requires a very long process time, because of the aqueous solubility of gelatin (Huyghebaert *et al.*, 2004).

The use of Eudragit® for controlled drug delivery has been well known. Depending on the pH, these polymers act as polyelectrolytes which make them suitable for purposes, from gastric or intestinal soluble drug formulations to insoluble but swellable delivery forms, regulated by percentage of charged and non ionized (ether) groups in the structure of these copolymers. Some of them can be considered as polycations (Eudragit® type E, RL, RS, and NE) and the others as polyanions (Eudragit® types L and S) (Moustafine *et al.*, 2005). In this study, the polymer used was Eudragit® L-100 is a 1:1 co-polymer of methacrylic acid and methylmethacrylate. that is insoluble below a pH of 5.0. The enteric reagent was developed as a peroral dosage form with a step-wise release of the coated ingredient within the GI tract as a function of its luminal pH. The gastro resistance of polymethacrylates can be attributed to the exposed ester side chains that are very resistant to hydrolysis, and the pH dependent solubility can be attributed to the step-wise alkaline hydrolysis of the exposed esterified terminal groups (George and Abraham, 2006). However, the Eudragit® polymers are not ionized and solubilized at a specific pH, but rather a range of pH. By varying the type of Eudragit® polymer blend and ratio, the drug release and dissolution kinetics in response to changing pH can be varied accordingly (Lecomte *et al.*, 2005). Eudragit® L-100 was used in this

study for the purpose 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).

#### **4.3.3 *In-vitro* release testing**

When developing dissolution methods for special dosage forms, it is recommended that the compendial apparatus and methods be first used (Siewert *et al.*, 2003). In line with this recommendation, drug release from specific drug delivery systems activated by microflora has been evaluated in USP dissolution apparatus II with slight modification. To better represent the physiological conditions in the GI tract, two media are commonly used: simulated gastric and simulated intestinal fluids. The simulated gastrointestinal fluid usually contains the enzyme that degrades specifically the polysaccharides used in the delivery system (Ugurlu *et al.*, 2007). The duration of testing in each medium was chosen to simulate the transit times in the stomach and small intestine, approximately 2 h and 4 h (Yang, 2008).

The dissolution data obtained from the tested formulations clearly demonstrated that the solubility of the films obtained from the polymers, Eudragit® L-100 and the dissolution profile of the coated capsule in various pH media could be manipulated by changing the ratios of the polymers. The proposed system would allow the formulator a high degree of flexibility to adjust a desired release profile in a targeted pH medium which is not only suitable for intestine targeted delivery of drugs but also applicable to dosage forms containing drugs with variable permeability or absorption windows within the intestinal region of the GI tract (Khan *et al.*, 1999).

In this study, release profiles of capsules demonstrated the pH-dependent release mechanism of the formulations and also confirmed that dissolution occurred due to pore formation which significantly increased at pH 7.4 due to solubilisation of the polymers at this pH. The results also demonstrated that a Eudragit® L-100 was successfully used to coat capsules for intestinal targeted delivery of lactobacilli formulation (Akhgari *et al.*, 2005).

## CHAPTER 5

### CONCLUSION

*Lactobacillus plantarum* (T23/3) in this thesis possessed inhibiting ability against major gastrointestinal pathogens and sensitive to ampicillin and chloramphenicol, while it was resistant to antibiotics commonly prescribed for intestinal infection. *L. plantarum* (T23/3) had its ability to survive in gastric and intestinal environment *in vitro*. This strain forms self-aggregation and has high surface hydrophobicity property which promotes adhesion to intestinal mucosa. It also co-aggregates with several gastrointestinal pathogens and has good adhesion to Caco-2 cells. This strain also interferes with other pathogens adhesion to Caco-2 cells which indicated its competitive binding. These characteristics may enable them to establish themselves in the intestinal tract. *L. plantarum* (T23/3) used in this study retains high viability in appropriate conditions and has good stability up to 12 months during storage in 4°C. According to the obtained results *L. plantarum* (T23/3) was found *in vitro* to possess desirable probiotics properties. This strain is good candidates for further investigation in their protective and therapeutic effects against gastrointestinal infections *in vivo*.

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**APPENDIX**

### Appendix A. Identification of lactobacilli isolates by API 50 CHL

The lactobacilli isolates (T23/3) was identified to species level using API 50 CHL system.

**Table A.1** Carbohydrate fermentation profiles lactobacilli (T23/3) isolates

Sugar	Lactobacilli (T23/3)
Control	-
Glycerol	-
Erythritol	-
D-Arabinose	-
L-Arabinose	-
Ribose	+
D-Xylose	-
L-Xylose	-
Adonitol	-
$\eta$ -Methy-D-xyloside	-
Galactose	+
D-Glucose	+
D-Fructose	+
D-Mannose	+
L-Sorbose	-
Rhamnose	-
Dulcitol	-
Inositol	-
Mannitol	+
Sorbitol	+
$\alpha$ -Methyl-D-Mannoside	+
<i>N</i> -Acetyl-glucosamine	+

**Table A.1** Carbohydrate fermentation profiles lactobacilli isolates (cont.)

<b>Sugar</b>	<b>Lactobacilli (T23/3)</b>
Amydalin	+
Arbutin	+
Esculin	+
Salicin	+
Cellobiose	+
Maltose	+
Lactose	+
Melibiose	-
Sucrose	+
Trehalose	+
Inulin	-
Melezitose	+
D-Raffinose	-
Amidon	-
Glycogen	-
Xylitol	-
$\eta$ -Gentiobiose	+
D-Turanose	-
D-Lyxose	-
D-Tagatose	-
D-Fucose	-
L-Fucose	-
D-Arabitol	-
L-Arabitol	-
Gluconate	+
2-Keto-gluconate	-
5-Keto-gluconate	-

Note: +, positive; -, negative

**Table A.2** Species identification isolates of lactobacilli (T23/3) by the API 50 CHL

Isolates	Species	Probability (%)
T23/3	<i>Lactobacillus plantarum</i>	93

**Appendix B. Susceptibility to antibiotics****Table B.1** Inhibition zone interpretive standard the disc diffusion technique

susceptibility to antibiotic

Antibiotic	Code	Disc Potency	Inhibition zone diameter (mm)		
			Resistant	Intermediate	Sensitive
Ampicillin	AM-10	10 µg	20 or less	21-28	29 or more
Chloramphenical	C-30	30 µg	12 or less	13-17	18 or more
Doxycyclin	D-30	30 µg	15 or less	15-18	19 or more
Erythromycin	E-15	15 µg	14 or less	14-17	18 or more
Neomycin	N-30	30 µg	15 or less	13-16	19 or more
Penicillin	P-10	10 U	20 or less	21-28	29 or more
Tetracyclin	Te-30	30 µg	14 or less	15-18	19 or more

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### List of Publication and Proceeding

Kha, R., Nakpheng, T., Jehtae, K., Kaewnopparat, S. and Srichana, T. Properties of probiotic *Lactobacillus planturum* (T23/3) as potential anti-gastrointestinal pathogens. The 3<sup>rd</sup> BMB international conference from basic to translational researches for better life, The Empress Hotel, Chiang Mai, Thailand. 6-8 April 2011.

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