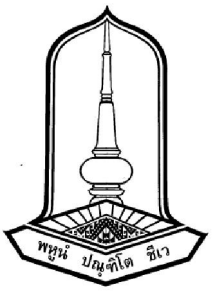

HEALTH-PROMOTING PROPERTIES OF PROBIOTIC *LACTOBACILLUS PENTOSUS* STRAINS USING AS STARTER CULTURE FOR PROBIOTIC SOYA BEVERAGE PRODUCTION

ARUNRUSSAMEE SANGSILA

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology at Mahasarakham University

March 2015

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The examining committee has unanimously approved this dissertation, submitted by Miss Arunrussamee Sangsila, as a partial fulfillment of the requirements for the Doctor of Philosophy in Biotechnology at Mahasarakham University.

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Miss Arunrussamee Sangsila

ชื่อเรื่อง คุณสมบัติการส่งเสริมสุขภาพของโพรไบโอติคสายพันธุ์ *Lactobacillus pentosus* เพื่อใช้เป็นหัวเชื้อเริ่มต้นในการผลิตเครื่องดื่มถั่วเหลืองโพรไบโอติค
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บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อ (1) ศึกษาคุณสมบัติการส่งเสริมสุขภาพของโพรไบโอติคแลคโต-บาซิลัสเพนโทซัส (*Lb. pentosus*) สายพันธุ์ต่าง ๆ (2) ผลิตเครื่องดื่มถั่วเหลืองโพรไบโอติค และศึกษาคุณสมบัติทางเคมี ประเมินการยอมรับทางประสาทสัมผัส และอายุการเก็บรักษาของผลิตภัณฑ์ เชื้อโพรไบโอติคแลคโตบาซิลัสเพนโทซัส จำนวน 8 สายพันธุ์ ที่ใช้ในงานวิจัยนี้ ได้แก่ DM068 JM0812 JM085 UM054 UM055 VM095 VM096 และYM122 ศึกษารูปแบบการเจริญของเชื้อ *Lb. pentosus* ในอาหารเลี้ยงเชื้อ De Man Rogosa Sharpe (MRS) ซึ่งมีน้ำตาลชนิดต่างๆ ได้แก่ แลคโตส (lactose) ราฟิโนส (raffinose) และน้ำตาลฟรุคโตโอลิโกแซคคาร์ไรด์ (fructooligosaccharide) เปรียบเทียบกับน้ำตาลกลูโคส ผลการทดลองพบว่าเชื้อแลคโตบาซิลัสเพนโทซัสจำนวน 5 จาก 8 สายพันธุ์ ได้แก่ UM054 UM055 VM095 VM096 และYM122 สามารถเจริญได้ดีในอาหารเลี้ยงเชื้อที่เติมน้ำตาลโอลิโกแซคคาร์ไรด์ อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อเปรียบเทียบกับกลูโคส โดยค่าการเจริญสูงสุดพบในชั่วโมงที่ 12-15 มีค่า 2.72-2.91 ค่าพีเอช 3.60-3.70 ที่ 24 ชั่วโมง การใช้น้ำตาล (sugar utilization) และการผลิตกรดไขมันสายสั้น (SCFAs production) จะขึ้นอยู่กับชนิดของน้ำตาลที่เติมลงในอาหารเลี้ยงเชื้อและสายพันธุ์ที่ทดสอบ ($p < 0.05$) เชื้อสามารถใช้น้ำตาลกลูโคส (94.38%) ได้ดีกว่าน้ำตาลราฟฟิโนส (68.78%) น้ำตาลแลคโตส (68.34%) และน้ำตาลฟลุคโตโอลิโกแซคคาร์ไรด์ (22.65%) ตามลำดับ เชื้อแบคทีเรียทั้ง 5 สายพันธุ์แสดงความสามารถในการผลิตกรดอินทรีย์และกรดไขมันสายสั้น โดยกรดแลคติค (16.64-18.13 มิลลิกรัมต่อมิลลิลิตร) กรดอะซิติค (89.02-98.49 ไมโครโมลต่อมิลลิลิตร) กรดโพรพิโอนิก (38.31-64.70 ไมโครโมลต่อมิลลิลิตร) และกรดบิวทีริก (37.82-46.08 ไมโครโมลต่อมิลลิลิตร) การทดสอบคุณสมบัติด้านการส่งเสริมสุขภาพ ได้แก่ ค่ากิจกรรมเอนไซม์ BSH ของเชื้อทดสอบโดยวัดจากขนาดของวงตะกอน (precipitation zone) พบว่ามีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) โดยมีค่าอยู่ระหว่าง 8.83-10.17 มิลลิเมตร สายพันธุ์ VM096 VM095 YM122 มีค่ากิจกรรมเอนไซม์ BSH สูงสุด ตามลำดับ ความสามารถจับคอเลสเตอรอลสูงสุด ได้แก่ VM096 (44.71%) UM055 (41.63%) VM095 (39.34%) YM122 (35.68%) ตามลำดับ เชื้อทดสอบทั้งหมดมีความสามารถในการจับไมโครทอกซินซีราลีโนน แตกต่างอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ขึ้นกับระดับความเข้มข้นของซีราลีโนนที่ใช้ทดสอบในสารละลายบัฟเฟอร์ ที่ระดับความเข้มข้นทดสอบสูงสุด (75.70 ไมโครกรัมต่อมิลลิลิตร) ทุกสายพันธุ์มีความสามารถจับซีราลีโนนสูงถึง 60.15-83.17% เชื้อทดสอบมีค่าความสามารถยึดจับเยื่อบุ ระหว่าง 6.24 %-8.20 % ซึ่ง VM096 มีค่าสูงสุด จากผลการทดลองดังกล่าว จึงเลือกสายพันธุ์ VM095 VM096 และ YM122 ซึ่งมีการเจริญที่ดีในน้ำตาลโอลิโกแซค

คาร์ไรด์และผลิตกรดไขมันสายสั้นได้ดี มีกิจกรรมของเอนไซม์ BSH ซึ่งส่งเสริมความสามารถในการจับคอเลสเตอรอล มีสามารถจับเยื่อบุสูงซึ่งเป็นคุณสมบัติที่ช่วยส่งเสริมสุขภาพ อีกทั้งยังมีความสามารถในการจับสารไมโครทอกซินซีลารีโนน มาใช้เพื่อเป็นหัวเชื้อสำหรับการผลิตเครื่องดื่มถั่วเหลืองโพรไบโอติค โดยใช้สายพันธุ์เดี่ยวๆ ในการเตรียมนมถั่วเหลืองหมัก คุณสมบัติของนมถั่วเหลืองหมักที่ได้ พบว่ามีจำนวนเซลล์เพิ่มขึ้นสูงสุดมากกว่า 9 log CFU ต่อมิลลิลิตร ค่าพีเอช (5.31-5.37) และค่าไตเตรทกรด (0.27-0.29%) สายพันธุ์ VM096 จะสามารถใช้วัตถุดิบน้ำนมถั่วเหลืองได้ดีกว่า VM095 และ YM122 ขณะที่ VM095 มีความสามารถผลิตแลคติคและกรดไขมันสายสั้นได้สูงกว่า VM096 และ YM122 อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) เตรียมเครื่องดื่มถั่วเหลืองโพรไบโอติคจากนมถั่วเหลืองหมักแล้วเติมน้ำผึ้งแท้อัตราส่วน 10% โดยปริมาตร ประเมินการยอมรับทางประสาทสัมผัสด้วยวิธี 9-point hedonic scale พบว่าเครื่องดื่มถั่วเหลืองที่หมักด้วยแลคโตบาซิลัสเพนโทซัสสายพันธุ์ VM095 มีคุณสมบัติด้านต่างๆ ดีที่สุด มีจำนวนเซลล์เหลือรอดชีวิตสูงสุดถึง 10 log CFU ต่อมิลลิลิตร ค่าพีเอช 3.49 จากการศึกษานี้แสดงให้เห็นว่าเชื้อแลคโตบาซิลัสเพนโทซัสสายพันธุ์ VM095 มีศักยภาพที่จะนำมาใช้เป็นหัวเชื้อเริ่มต้น เพื่อผลิตผลิตภัณฑ์โพรไบโอติค เนื่องจากเชื้อมีสามารถเจริญได้ดีในเครื่องดื่มถั่วเหลือง มีปริมาณเชื้อเหลือรอดมากกว่า 6 log CFU ต่อมิลลิลิตร นานถึง 4 สัปดาห์ มีคุณสมบัติส่งเสริมสุขภาพและเป็นที่ยอมรับในด้านคุณลักษณะของผลิตภัณฑ์จากผู้ทดสอบชิม ดังนั้นผลิตภัณฑ์เครื่องดื่มถั่วเหลืองโพรไบโอติคจึงเป็นผลิตภัณฑ์ทางเลือกที่เหมาะสำหรับผู้บริโภคกลุ่มมังสวิรัติ และกลุ่มที่มีปัญหาสุขภาพ เช่น ผู้หญิงวัยหมดประจำเดือน ผู้สูงวัย และกลุ่มที่ไม่สามารถดื่มนมโคได้

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TITLE Health-Promoting Properties of Probiotic *Lactobacillus pentosus* Strains Using as Starter Culture for Probiotic Soya Beverage Production

AUTHOR Miss Arunrussamee Sangsila

DEGREE The Doctor of Philosophy degree in Biotechnology

ADVISORS Assist. Prof. Dr. Pariyaporn Itsaranuwat

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UNIVERSITY Mahasarakham University **YEAR** 2015

ABSTRACT

The aims of this study were to:(1) to investigate health-promoting properties of the probiotic *Lb. pentosus* strains, (2) to produce a probiotic soya beverage and study its properties, evaluate its sensory properties, and product's shelf life. The 8 probiotic *Lb. pentosus* strains namely DM068, JM0812, JM085, UM054, UM055, VM095, VM096, and YM122 were used in experiment. The growth profiles of *Lb. pentosus* in MRS medium containing different sugars such as lactose, raffinose, FOS, and glucose (as a control) was observed. The results found that 5 of 8 tested strains (UM054, UM055, VM095, VM096 and YM122) had ability to growth in oligosaccharide sugars (lactose, raffinose and FOS) better than glucose ($p < 0.05$). The maximum growth at 12-15 h in oligosaccharide sugars reached 2.72-2.91, pH values at 24 h were achived 3.60-3.70. Sugar utilisation and SCFAs production of *Lb. pentosus* were dependent on the type of sugar present as well as the strain ($p < 0.05$). The consumption of glucose greater than raffinose, lactose, FOS by 94.83 %, 68.78%, 68.34 %, and 22.65 %, respectively. Those 5 strains were expressed the capability to produce organic acid and SCFAs by lactic acid (16.64-18.13 mg/mL), acetic acid (89.02-98.49 µmol/mL), propionic acid (38.31-64.70 µmol/mL), butyric acid (37.82-46.08 µmol/mL). The health-promoting properties were determined. BSH activities as demonstrated by precipitation zones ($p < 0.05$) with diameters between 8.83-10.17 mm. The greatest precipitation zone was found in VM096 followed by VM095, YM122, respectively. The highest percentage of cholesterol reduced was VM096 (44.71%) follow by UM055 (41.63%), VM095 (39.34%), YM122 (35.68%), respectively. For *In-vitro* ZEA binding abilities, all strains

were showed significant ($p < 0.05$) at various concentration levels of ZEA in buffer solution. At the highest concentration level of ZEA (75.70 μg/mL), tested strains could bind ZEA reached 60.15-83.17%. Cell surface hydrophobicity of all strains was ranged 6.24%-8.20%, VM096 showed the highest value. From the results above, best 3 strains that VM095, VM096, and YM122 were selected for a starter culture due to their had a good growth profile and SCFAs production on oligosaccharide sugar, exhibited the cholesterol binding via BSH activity in culture media, high adhesion ability with health beneficial properties, and also ZEA binding abilities. The fermented soya milk inoculated with individual cultures of VM095, VM096, and YM122 strains were carried out. The maximum increase in cells number in samples is greater than 9 log CFU/mL, pH values of 5.31-5.37, the acidity of 0.27-0.29%. The VM096 strain exploited these substrates more efficiently than YM122 and VM095. In case of lactic acid and SCFAs production, the sample inoculated with VM095 strain had the highest values ($p < 0.05$). The acceptant for soya beverage was considered by using the 9-point hedonic scale. The sample inoculated with VM095 strain had the best properties of sensory score and the viable cells reached the maximum of 10 log CFU/mL, pH value of 3.49. This study showed that *Lb. pentosus* VM095 had the potential to be used as a starter culture for probiotic products due to its ability to grow in soya beverage during storage of 4 weeks to maintain high amount of viable cells to meet a recommended standard of having at least 6 log CFU/mL of viable cells in the probiotic products. Therefore, probiotic soya beverage is an alternative product for vegetarians and health-conscious consumers such as lactose-intolerant, ageing and menopauses women**.**

Keywords: probiotics, health-promoting properties, fermented soya milk, lactic acid bacteria, cholesterol

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LIST OF ABBREVIATION

ANOVA	Analysis of Variance
BSH	Bile Salt Hydrolase
CRD	Completely randomized design
CFU	Colony Forming Units
DMRT	Duncan's Multiple Range Test
DP	Degree of Polymerization
EMP	Embden-Meyerhof-Parnas
FOS	Fructo-oligosaccharides
GRAS	Generally Recognized as Safe
GIT	Gastrointestinal tract
h	hour
HCl	hydrochloric acid
HDL	high-density lipoprotein
HPLC	High Performance Liquid Chromatography
L	Litre
Lb.	*Lactobacillus*
LDL	low-density lipoprotein
LAB	Lactic Acid Bacteria
mL	Milliliter
mg	Milligram
min	minute(s)
MRS medium	De Man, Rogosa and Sharpe medium
NSPs	non-starch polysaccharides
PBS	Sodium phosphate buffer
OD	Optical density
RS	resistant starches
SCFA	Short chain fatty acid
SD	Standard Deviation
s	second(s)
TDCA	Taurodeoxycholic acid
UV	ultraviolet

v/v	volume by volume
w/v	weight by volume
ZEA	Zealarenone
µL	microlitre(s)

CHAPTER 1

INTRODUCTION

1.1 Background

In the present, health-promoting food or functional food has become a popular trend. Functional foods may be produced by adding bioactive compounds into the foods or using beneficial microorganisms in the food processing. In addition to the value added to the product, functional foods would be another choice of healthy diet for consumers. It would reduce consumers' risk of disease or boost consumers' chances of optimal health. Dietary fiber and probiotic food are the examples of functional foods in the market. Probiotic food is food that contains beneficial live microorganisms. These live microorganisms will help improve host's intestinal microbial balance (Gibson and Roberfroid, 1995), enhance nutrition, promote better health and reduce risk of diseases (Lannitti and Palmieri, 2010; Vasiljevic and Shah, 2008; Shah, 2007).

Probiotic is beneficial viable microbes that help promote health, balance microorganism in the digestive system and cure diarrhea. Probiotic also has therapeutic properties such as stimulating immune system, lowering blood cholesterol level, and decreasing the risk of colon cancer (Perdigeon et al., 1998; Kurmann and Rasic, 1991; Fooks et al., 1999; Kailasapathy and Chin, 2000; Kaur et al., 2002; Itsaranuwat et al., 2003; Marona and Pedrigon, 2004). A product that is considered to be probiotic food should contain the probiotic microbes at a concentration of more than 6 log CFU/mL at the time of consumption. To support good health, probiotic bacteria should survive during transit through the stomach until the end of intestine. For this reason, probiotic organism should be resistant to the environments in the digestive system such as acid in the stomach and bile salt. Beside survival in the intestinal tract, probiotic bacteria should have other health promoting characteristics such as producing useful enzymes, activating immune system, decreasing harmful enzyme production, reducing cholesterol level and inhibiting pathogenic bacteria. Probiotic bacteria that are mainly used in industries are lactic acid bacteria (LAB), for instance, *Lactobacillus*, *Bifidobacterium* and *Enterococcus* (Holzapfel and Schillinger, 2002; Vinderola and Reinheimer, 2003). LAB is used in fermented food industry, for example, fermented milk products,

fermented meat products and pickled vegetables. Probiotics are available in the market in some other forms such as capsules, tablets as well. *Lactobacillus* and *Bifidobacterium* are the example of LAB that is used in fermented milk products. LAB in the products helps to detoxify of some pathogenic bacteria in the large intestine and improve lactose digestion. In addition, LAB can prevent diarrheal disease; boost your immune system and lower blood cholesterol level. Several researchers indicated that using LAB as a starter culture in fermented milk production can enhance the nutritional value and therapeutic properties of products (Lannitti and Palmieri, 2010; Parvez et al, 2006). In healthy food industry, it tends to increasingly use probiotics to be the starter cultures in food and beverage productions.

Soya milk is a cheap source of protein compared to animal protein. It is low in saturated fatty acid no cholesterol and high in essential fatty acids such as linoleic and linolenic acid. Furthermore, soya milk contains important substances such as isoflavones and oligosaccharides. Isoflavones are natural flavonoids and act as estrogen which is an essential female hormone. It helps to prevent and cure heal cancer, coronary heart disease and osteoporosis. Many researchers studied the beneficial use of isoflavone to help treat menopause system in women (Dajanta et al., 2009; Phommalth et al., 2008). Oligosaccharides can enhance the growth of probiotic bacteria. Healthy products from soy bean have been developed and interested for a long time because they are not only good for health, but also can be alternative foods for lactose-intolerant person. Cereals and their products for examples, rice, wheat, corn, and soybeans should be kept and stored well. Otherwise, they may be contaminated by mycotoxin from mould, such as Zealarenone (ZEA) which is produced by *Fusarium* sp. (Richardson et al., 1985; Fandohan et al., 2003; Adejumo et al., 2007; Broggi et al., 2007; Zinedine et al., 2007; Ezekiel et al., 2008) ZEA causes brucellosis in animals, decreasing in milk production of mammals and this mycotoxin will be excreted into milk (Witte, 2003). ZEA can lower animals' growth rate and acts like Estrogen, a female hormone. It triggers hyper estrogenic syndromes and affects the hormone system in humans as well (Pfohl-Leszkowicz et al., 1995; Smith et al., 1997; Geraldo et al., 2006; Seeling et al., 2006; Zinedine et al., 2007). According to the reports of El-Nezami et al. (2002); Shetty and Jespersen (2006) and Zinedine et al. (2007) it is found that LAB were able to remove mycotoxin by binding process. Because of the reasons above, it is very important to select probiotic strains that have health-promoting properties and binding

ability to be used as a starter culture in probiotic soy drink production. The developments of health-promoting products from soybean are very beneficial for agro-industry in Thailand because these are not only value added to products, but also can increase choices or be alternative foods for vegetarian and lactose-intolerant consumers.

This research aimed to study health-promoting properties of probiotic bacteria in order to be used as a starter culture for a probiotic soya beverage production. The basic probiotic characteristics of the bacteria in this study were already assessed for the persistence in the gut environment, antagonism against pathogenic bacteria (Kansandee, 2010) and safety in food (Generally Recognized as Safe; GRAS).

1.2 Research objectives

1.2.1 To investigate health-promoting properties of the probiotic bacteria strains, *Lactobacillus pentosus.*

1.2.2 To produce a probiotic soya beverage and study its chemical properties, physical properties, microbial properties, evaluate its sensory properties, and product's shelf life.

1.3 Research hypothesis

1.3.1 *Lb. pentosus* has health-promoting properties such as the reduction of cholesterol level, the binding of mycotoxin. Thus, it is suitable as a starter culture in the production of soya beverage.

1.3.2 The soya beverage product that is produced by probiotic bacteria *Lb. pentosus*, has all good characteristics which are chemical, physical, and microbial properties as well as customers' acceptance.

1.4 Significance of the research

Be able to use potential probiotic bacteria *Lb. pentosus* as a starter culture in the production of the probiotic product with is a health-promoting benefits to be accepted by consumers. This product will be suitable for vegetarians and health-conscious consumers such as lactose-intolerant, ageing and menopauses women**.**

1.5 Scope of study

The scope of this research as the following:

1.5.1 Eight strains of probiotic bacteria were used in the research; *Lb. pentosus* DM068, JM0812, JM085, UM054, UM055, VM095, VM096, and YM122. These bacteria were received from the laboratory of Asst. Prof. Dr. Pariyaporn Itsaranuwat, Department of Biotechnology, Mahasarakham University. Basic probiotic properties were assessed in these bacteria (Kansandee, 2010).

1.5.2 The study of characteristics tested of probiotic bacteria in this work.

1.5.2.1 Study of health-promoting properties of probiotic bacteria;

1) Capacity of carbohydrates utilization by determine of growth pattern, pH changes, sugar contents, and short chain fatty acid (SCFAs) production

2) Bile Salt Hydrolase activity.

3) *In-vitro* cholesterol binding activity.

4) *In vitro* adhesion capacity.

1.5.2.2 *In-vitro* ZEA binding activity.

1.5.3 Study on the use of probiotic *Lb. pentosus* strains with health-promoting properties as a starter culture for probiotic soya beverage production.

1.6 Experimental plan

Completely randomized design (CRD) and analysis of variance were conducted by using Duncan's Multiple Range Test (DMRT) for comparison of the means. All experiments were carried out in triplicates. The statistics used in data analysis are as the following:

1.6.1 Descriptive Statistics

Descriptive statistics used to describe the basic features of a collection of data in the study are:

$\bar{x}$ Mean

% Percentage

SD Standard Deviation

1.6.2 Inferential Statistics

Pair comparison according to DMRT, significant test at P-value $\leq .05$

1.6.3 The research was conducted from May 2013 until March 2014

1.6.4 The research was conducted at the laboratory of:

1) Department of Biotechnology, Faculty of Technology, Mahasarakham University

2) Central Laboratory of Mahasarakham University, and

3) Chemical Engineering Laboratory, Department of Bioprocess & Microbial System, UMR CNRS/INPT/UPS 5503, University of Toulouse – ENSAT, France

1.7 Definitions

1.7.1 Probiotic is a live microorganism or group of microorganisms that can survive in the intestinal tract with beneficial properties to human and animal hosts. Probiotic bacteria can help improve host's intestinal microbial balance and reduce the amount of pathogenic bacteria. A product that is considered to be probiotic food should contain the probiotic bacteria more than 6 log CFU/mL at the time of consumption.

1.7.2 Lactic acid bacteria (LAB) are the bacteria belonging to the family *Lactobacilli Bifidobacteria* that can produce lactic acid. LAB are Gram-positive, non-spore forming cocci or rods. The shape of cell can change depending on the environment. They mostly are microaerophile. Some types are anaerobes because they obtain energy only from the metabolism of sugars in the absence of oxygen. LAB are tolerant to acidic conditions.

1.7.3 The fermented milk is a dairy product that has been fermented with non-pathogenic, non-toxic bacteria which mostly are LAB. The bacteria produce lactic acid during the fermented process by converting lactose sugar into lactic acid. This process will lead to a drop in pH of the product and cause the denaturing of milk proteins to form curds. The product will have a sour taste. The fermented milk is suitable for the person who cannot drink the regular milk because cannot digest lactose sugar (lactose intolerance). The samples of fermented dairy product that use LAB are yoghurt, drinking yoghurt, and cheese.

1.7.4 Starter culture is a microbiological culture, such as fungi, bacteria, or yeast, which has been isolated and purified. It can be one single culture or mixed cultures to perform or start the fermentation process.

1.7.5 Health-promoting properties are the properties of microorganism in the host's gastrointestinal tract that can produce useful substances which are health beneficial to the host, for instance, to help improve the microbial balance in the intestine, reduce blood cholesterol level, and reducing of mycotoxin.

CHAPTER 2

LITERATURE REVIEW

Nowadays, consumers are aware of the link among lifestyle, diet and good health which explains the emerging demand for products that are able to enhance health beyond providing basic nutrition. The list of health benefits accredited to functional food continues to increase and the probiotics are one of the fastest growing categories within food for which scientific researchers have demonstrated therapeutic evidence (Soccol et al., 2010). The nation that food could serve as medicine was first conceived thousands of years ago by the Greek philosopher and father of medicine, Hippocrates, who once wrote: "Let food be your medicine and medicine be your food" (Chow, 2002). Functional foods are defined as: foods that contain some health-promoting components beyond traditional nutrients. In general, the term refers to a food that has been modified in some way to become functional. One way in which foods can be modified to become functional is by the addition of probiotics. Health-benefits derive by the consumption of foods containing probiotic bacteria are well documented and probiotic products are available worldwide.

2.1 Probiotics

2.1.1 Definition of probiotics

The term probiotic is a relatively new word meaning "for life" was originally proposed in 1965 by Lilley and Stillwell (Schmid et al., 2006). It is derived from the Greek language and it is currently used to name bacteria associated with beneficial effects for humans and animals (Fooks et al., 1999). The original observation of the positive role played by some selected bacteria is attributed to Eli Metchnikoff, who suggested, "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff, 1908). Lilly and Stillwell (1965) describe the word probiotic as "substance secreted by one microorganism which stimulates the growth of another." While, Sperti (1971 cited in Itsaranuwat, 2003) was described the term of

probiotic to “tissue extracts that stimulate microbial growth.” Parker (1974) was the first to use the term probiotic in the sense that is used today. He gave the definition of probiotics as “organisms and substances which contribute to intestinal microbial balance”, the definition also including antibiotics. However, Fuller (1989) defined probiotics as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.” This definition stressed the requirement of viability for probiotics and introduced the aspect of a beneficial effect on the host (Itsaranuwat, 2003). The definition of probiotics is continuously argued between microbiologists. According to Salminen (1996 cited in Itsaranuwat, 2003), a probiotic is “a live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host”, whereas in the same year, Schaafsma (1996 cited in Itsaranuwat, 2003) suggested the oral probiotics are “living microorganisms which upon ingestion in certain numbers, exert health effects beyond inherent basic nutrition.” At present, the most generally used definition is that of Fuller (Salminen et al., 1998). However, Diplock et al. (1999 cited in Lee, 2009) puts it as “ probiotic food is functional if they have been satisfactorily demonstrated to beneficially affect one or more target functions in the body beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction in the risk of diseases.” Whereas, Naidu et al. in (1999 cited in Lee, 2009) said “A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract.” next, Tannock in 2000 (Lee, 2009) observed that long-term consumption of probiotics was not associated with any drastic change in the intestinal microbiodata composition, and thus proposed an alternative definition: Microbial cells which transit the GI tract and which, in doing so, benefit the health consumer.”

However, recently, Schrezemeir and de Vrese (2001) defined the term of probiotic as “a product containing viable, defined micro-organisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a complement of the host and by exert beneficial health effects in this host.” In addition, probiotics are defined as live microorganisms when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001). The consumption of probiotic products is

growing very fast all over the world and probiotics are generally considered generally regarded as safe (GRAS). Since the word probiotics refers to microorganisms that are able to confer health benefits on humans and that have been industrially prepared for nutritional and pharmaceutical use.

Probiotics have come to Thailand as dairy products such as yogurt, drinking yogurt, and powder milk since 2001 (Lee et al. 2012). To provide better information relating to its health claim to ensure standards and protect consumers, Thai FDA, Ministry of Public Health Thailand started regulating probiotic usage for food product since 2008 (FDA 403/2551). To assess the properties of probiotics, it is suggested that the following guidelines are needed. For food application, probiotic microorganisms should be able to survive passage through the digestive tract and to proliferate in the gut.

2.1.2 The selection criteria for potential probiotics

A general agreement among scientist has been reached, at least in general terms, on the properties that a strain must have in order to be further tested for human probiotic use (Ouwehund et al., 1999):

(1) Human origin, if intended for human use

(2) Safe for food and clinical use

(3) Survive during gastric transit

(4) Acid and bile stability

(5) Adhesion behavior to gut epithelial tissue

(6) Clinically validated and document health effects

(7) (Good technology properties

The strains generally present into the intestinal flora of the host will be targeted by researchers, who assume that these bacteria have a better chance of out-competing normalflora bacteria and of establishing at a numerically significant level in their new host (Morelli, 2000). Probiotics was consumed orally, in case acid and bile resistance are preferable traits since it is desirable for a probiotic to survive gastrointestinal transit. The adhesion to mucosal surfaces by probiotic organisms is an important ability for the colonization of the human gastrointestinal tract, prevents their elimination by peristalsis and provides a competitive advantage over pathogens (Kos et al., 2003). The adhesion process can be divided into two steps: 1) reversible adhesion

due to long-range forces and 2) subsequent interaction mediating a direct contact between microorganisms and supports surfaces such as the hydrophobic interaction of microorganism and support (Wang and Han, 2007).

2.1.3 The selection of a health-promoting probiotics

The probiotic strains must possess the ability to overcome the extremely low pH and the detergent effect of bile salts, and arrive at the site of action in a viable physiological state. They should be capable of co-aggregation, resistant to gastro intestinal fluid and adhere to the intestinal mucosa. However, besides the various essential characteristics, the organisms should exhibit health benefits with functional properties. The organisms have developed various functional characteristics. Clinically proven, various health effects have been reported for lactobacilli, such as cholesterol reduction, diarrhea prevention, enhancement of lactose intolerance symptoms, anticancer effects, synthesis and enhancing the bioavailability of nutrients and immune-modulatory effects, all of which are considered functional aspects of probiotic criteria. In order to exert their beneficial effect, probiotics must survive in the gastrointestinal (GI) tract, persist in the host, and provide safety for the consumer (De-Vries et al., 2006).

A number of benefits derived from probiotic-containg have been reported (Schaafsma et al., 1998; Fooks et al., 1999) and include the following:

(1) Enhanced lactose digestion

(2) Prevention/treatment of acute rotavirus and antibiotic-induced diarrheas'

(3) Improvement of the balance between microbial populations in the gut

(4) Enhancing the bioavailability of nutrients

(5) Suppression of cancers

(6) Reduction of serum cholesterol/ prevention the risk of coronary heart disease

(7) Detoxificant/binding mycotoxin

The beneficial effects of probiotics likely result from several complexes, interacting mechanisms that will differ for different strains and sites of action. These mechanisms may include competition for binding sites to the intestinal wall, competition for essential nutrients, production of antimicrobial substances, stimulation of *mucin* production, stabilization of the intestinal barrier, improvement of gut transit,

metabolism of nutrients to volatile fatty acids, and immune modulation (immune stimulation and immune regulation). Some of these mechanisms have been demonstrated only through laboratory experiments or animal models and are not substantiated in humans (Fooks et al., 1999).

2.1.4 Mechanisms of probiotic actions

The effects of probiotics maybe classified in three modes of action (Oelschaeger, 2010).

(1) Probiotics might be able to modulate the host's defenses including the innate as well as the acquired immune system. This mode of action is most likely important for the prevention and therapy of infectious diseases but also for the treatment of (chronic) inflammation of the digestive tract or parts thereof. In addition, this probiotic action could be important for the eradication of neoplastic host cells.

(2) Probiotics can also have a direct effect on other microorganisms, commensal and/or pathogenic ones. This principle is in many cases of importance for the prevention and therapy of infections and restoration of the microbial equilibrium in the gut.

(3) Finally, probiotic effects may be based on actions affecting microbial products like toxins, host products e.g. bile salts and food ingredients. Such actions may result in inactivation of toxins and detoxification of host and food components in the gut.

All three modes of probiotic action are in all likelihood involved in infection defense, prevention of cancer and in stabilizing or reconstituting the physiological balance between the intestinal microbiota and its host. However, it has to be stressed that there seems not to be one probiotic exhibiting all three principles, at least not to that extent that it could be a remedy for prevention or therapy of all mentioned kinds of disease. It depends on the metabolic properties, the kind of surface molecules expressed and components to be secreted which probiotic actions a certain probiotic strain might show.

2.1.5 Beneficial health effects of probiotics

The probiotic foods should be safe and must contain the appropriate probiotic organisms in sufficient numbers at the time of consumption. Therefore, the probiotic strains selected should be suitable for large-scale industrial production with the ability

to survive and retain their functionality during production and storage as frozen or dried cultures. It must survive during the food processing operations, and also in the food products into which they are finally formulated (Figure 2.1).

Probiotics provide a number of health benefits mainly through maintenance of normal intestinal microflora, protection against gastrointestinal pathogens, enhancement of the immune system (Gilliland, 1990), reduction of serum cholesterol level and blood pressure (Rasic, 2003), anti-carcinogenic activity (Rasic, 2003), improved utilisation of nutrients and improved nutritional value of food as illustrated in Figure 2.2. Therapeutic applications of probiotics include prevention of infantile diarrhea, urinogenital diseases, osteoporosis, food allergy and atopic diseases; reduction of antibody-induced diarrhea; alleviation of constipation and hypercholesterolemia; control of inflammatory bowel diseases; and protection against colon and bladder cancer (Tripathi and Giri, 2014).

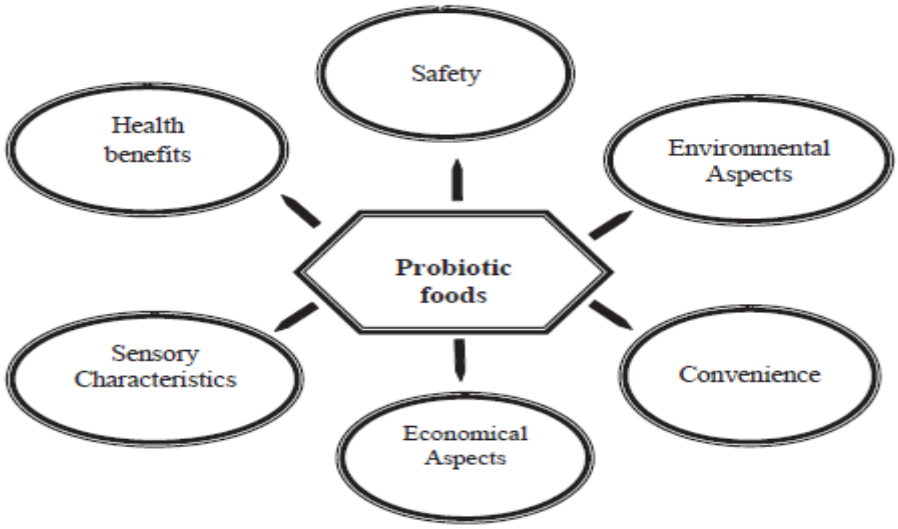

Figure 2.1 Qualitative aspects of probiotic food products

Source: Tripathi and Giri (2014)

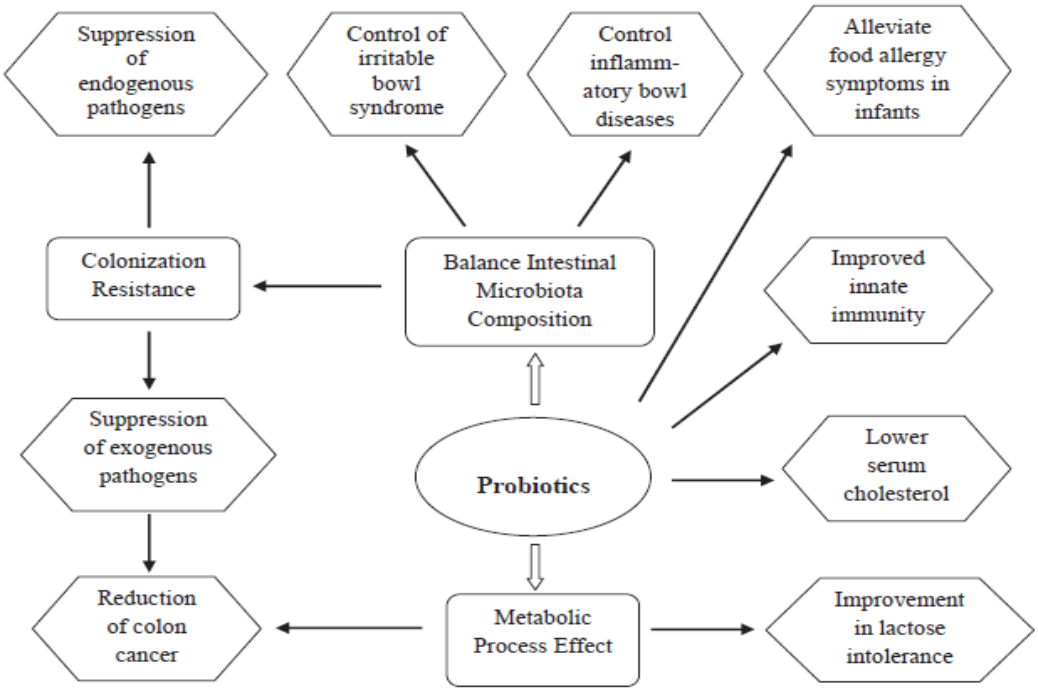

Figure 2.2 Probiotics consumption and health benefits

Source: Parves et al. (2006)

There are several evidences supporting potential clinical applications of probiotics in the prevention and treatment of gastrointestinal, urinogenital tracts and respiratory diseases (Gardiner et al., 2002). Mann and Spoerry (1974) discovered that blood serum cholesterol levels reduced significantly by drinking yogurt fermented with wild strains of *Lactobacillus sp*. Harrison et al. (1975) reported that serum cholesterol decreased by consuming infant formula added with cells of *Lb. acidophilus*. Similarly, Gilliland (1990); Gill and Guarner (2004) showed control of serum cholesterol levels in adult human experiments. It is hypothesized that these benefits may result from the growth and action of the probiotics during the manufacturing of cultured foods, while some may result from the growth and action of certain species of probiotics in the intestinal tract (Rasic, 2003 cited in Tripathi and Giri, 2014).

2.2 Lactic acid bacteria (LAB)

LAB is regarded as a major group of probiotic bacteria (Metchnikoff, 1908; Schrezenmeir and de Verse, 2001) and it constitutes an integral part of the healthy gastrointestinal (GI) microecology and is involved in the host metabolism. Fermentation has been specified as a mechanism of probiotics (Gibson and Fuller, 2000; Metchnikoff, 1908). LAB along with other gut microbiota ferment various substrates like lactose, biogenic amines and allergenic compounds into SCFA and other organic acids and gases (Gibson and Fuller, 2000; Jay, 2000). In addition, it can synthesizes enzymes, vitamins, antioxidants and bacteriocins. With these properties, intestinal LAB constitutes an important mechanism for the metabolism and detoxification of foreign substances entering the body (Salminen, 1990).

2.2.1 Metabolism of LAB fermentation

Two main hexose fermentation pathways are used to classify LAB genera. Under conditions of excess glucose and limited oxygen, homolactic LAB catabolism one mole of glucose in the Embden-Meyerhof-Parnas (EMP) pathway to yield two moles of pyruvate. Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. This process yields two moles ATP per glucose consumed. Representative Homolactic LAB genera include *Lactococcus, Enterococcus, Streptococcus, Pediococcus,* and group I lactobacilli. Heterofermentative LAB use the pentose phosphate pathway, alternatively referred to as the pentose phosphoketolase pathway. One mole of glucose-6-phosphate is initially dehydrogenated to 6-phosphogluconate and subsequently decarboxylated to yield one mole of CO_2. The resulting pentose-5-phosphate is cleaved into one mole glyceraldehyde phosphate (GAP) and one mole acetyl phosphate. GAP is further metabolized to lactate as in homofermentation, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. In theory, end-products (including ATP) are produced in equimolar quantities from the catabolism of one mole of glucose. Obligate heterofermentative LAB include *Leuconostoc, Oenococcus, Weissella*, and group III lactobacilli.

2.2.2 Carbohydate fermentation patterns

2.2.2.1 Homo-and Heterolactic fermentation

Because LAB do not possess a functional respiratory system, they have to obtain their energy by substrate-level phosphorylation. With hexoses there are two basic fermentation pathways homofermentative pathway based on glycolysis or EMP and producing virtually only lactic acid and heterofermentative or heterolactic fermentation (also known as pentose phosphoketolase pathway, hexose monophosphate shunt, or 6-phosphogluconate pathway) producing in addition to lactic acid, significant amounts of CO_2 and ethanol or acetate. Theoretically, homolactic fermentation produces 2 moles of ATP per mole of glucose consumed. In heterolactic fermentation the corresponding yield is only 1 mole of ATP if acetyl phosphate formed as an intermediate is reduced to ethanol. However, if acetyl phosphate is converted to acetic acid in the presence of alternative electron acceptors, an extra ATP is formed (Wright and Axelsson, 2012).

Hexoses other than glucose (mannose, galactose, fructose) enter the major pathways above after different isomerization and phosphorylation steps as either glucose-6-phosphate. For galactose there are two different pathways, depending on whether it enters the cell as galactose-6-phosphate or as free galactose imported by a specific permeate. List of lactobacilli by their different fermentation patterns as were shown in Table 2.1 (Hammes and Vogel, 1995).

The fermentation type is an important taxonomic criterion. The division of lactobacilli in three patterns:

(1) Group I: Obligate homofermentative lactobacilli

(2) Group II lactobacilli are facultatively heterofermentative

(3) Group III lactobacilli are obligate heterofermentative

2.2.2.2 Fermentation of disaccharide

As a case of lactose in milk, lactose is cleaved to glucose and galactose by ß-galactosidase, and both these monosaccharide can subsequently enter the major fermentation pathways. In the case of lactose-specific PEP: PTS system another enzyme, phosphor- ß-D-galactosidase, is needed to split lactose phosphate to glucose and galactose-6-6phosphate. Glucose is then processed by the glycolytic pathway, while galactose-6-phosphate enters the tagarose-6-phosphate pathway (Wright and Axelsson, 2012).

Table 2.1 List of lactobacilli separated by their different fermentation patterns

Fermentation type	Species
obligately homofermentative	*Lb. acidophilus*, *Lb. amylophilus, Lb.amylovorus, Lb. crispatus, Lb. debrueckii* subp. *bulgaricus, Lb. debrueckii* subp. *delbrueckii, Lb. debrueckii* subp. *lactis, Lb. gallinarum, Lb. gasseri, Lb. helverticus, Lb. jensenii, Lb. johnsonii, Lb. kefiranofaciens, Lb. aviaries* subsp. *araffinosus*, *Lb. aviarius* subsp *aviarius, Lb. farciminis,Lb. salivarius* subsp. *salicinus*, *Lb. salivarius* subsp. *salivarius*, *Lb. mali, Lb. ruminis, Lb. sharpeae*
facultatively heterofermentative	*Lb. acetotolerans, Lb. hamster, Lb. alimentarius, Lb. bifermentans, Lb. casei, Lb. coryniformis* subsp. *coryniformis, Lb. coryniformis* subsp. *torquens, Lb.curvatus, Lb. graminis, Lb. homohiochii, Lb. intestinalis, Lb. murinus, Lb. paracasei* subsp. *paracasei, Lb. paracasei* subsp. *tolerans, Lb. rhamnosus, Lb. sake, Lb. agilis, Lb. pentosus, Lb. plantarum*
obligately heterofermentative	*Lb. brevis, Lb. buchneri, Lb. collinoider, Lb. fermentum, Lb. fructivorans, Lb. hilgardii, Lb. kefir, Lb. malefermentans, Lb. oris, Lb. parabuchneri, Lb. reiteri, Lb. pontis, Lb. vaginalis, Lb. suebicus, Lb. vaccinostercus, Lb. sanfrancisco, Lb. connfusus, Lb. confuses, Lb. fructosus, Lb. halotolerans, Lb. viridescens, Lb. kandleri, Lb. kandleri, Lb. minor*

Source: modified from Hammes and Vogel (1995)

2.2.3 Microbial growth

Bacteria grow or multiply in numbers when exposed to a favorable environment such as food. Growth important to isolate an unknown bacteria strain involved in food bioprocessing and studies its physiological, biochemical in order to design methods to controls its growth in food.

2.2.3.1 Growth curve (Roy, 2003)

The growth rate and growth characteristics of bacteria population under a given condition can be graphically represented by counting cell numbers, enumerating CFUs, or measuring OD in a spectrophotometer at a given wavelength (above 300 nm, usually at 600 nm) of a cell suspension. If the CFU values are enumerated at different times of growth and a growth curve is plotted using log10 CFU vs time (log10 CFU is used because of high cell numbers), a plot similar to the one illustrated in Figure 2.3. The plot has several features that represent the conditions of the cells at different times.

Initially, the population does not change (lag phase). During this time, the cells assimilate nutrients and increase in size. Although the population remains unchanged because of change in size, both cell mass and OD show some increase. Following this, the cell number starts increasing, first slowly and then very rapidly. The cells in the population differ initially in metabolic rate and some multiply, and then almost all cells multiply. This is the exponential phase (also called logarithmic phase). Growth rate at the exponential phase follow first-order reaction kinetics and can be use to determine generation time.

Following this, the growth rate slows down and finally the population enters the stationary phase. At this stage, because of nutrient shortage and accumulation of waste products, a few cells die and a few cells multiply, keeping the living population stable. However, if one counts the cells under the microscope or measures cell mass, both may show an increase, as dead cells may remain intact. After the stationary phase, the population enters the death phase, in which the rate of cells death is higher than the rate of cell multiplication. Depending on the strain and conditions of the environment, after a long period of time some cells may still remain viable. This information is important to determine some microorganism's criteria in food, especially controlling spoilage and pathogenic microorganisms in food.

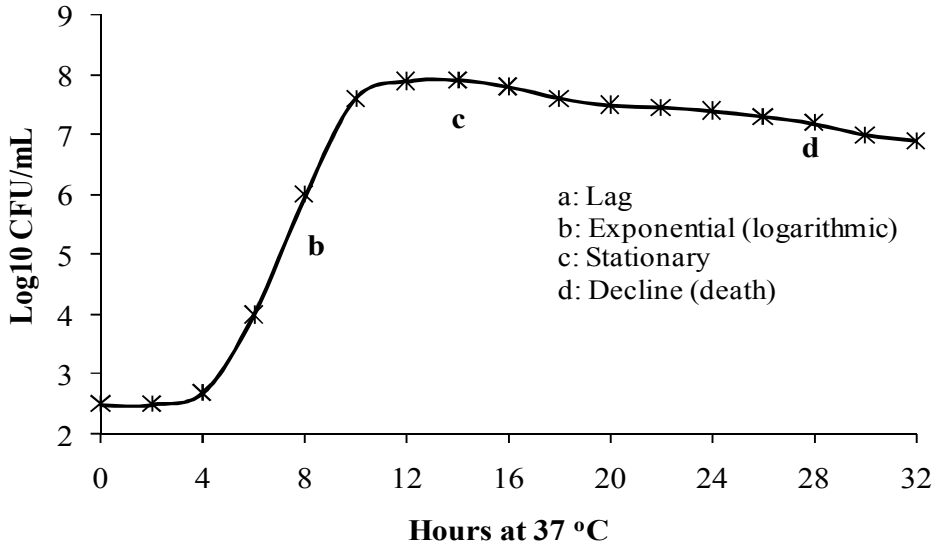

Figure 2.3 Bacterial growth curve showing changes in cell numbers of *Pediococcus acidilactici* H during 32 h incubation at 37°C in a broth.
Source: Ray (2003)

2.2.3.2 Carbohydrates and Growth

Major carbohydrates present in different foods, either naturally or added as ingredients, can be grouped on the basis of chemical nature as follows:

(1) Monosaccharides

Hexoses: glucose, fructose, mannose, galactose

Pentose: xylose, arabinose, ribose, riburose, xylulose

(2) Disaccharides

Lactose (galactose + glucose)

Sucrose (fructose + glucose)

Maltose (glucose + glucose)

(3) Oligosaccharides

Raffinose (glucose + fructose + galactose)

Starchyose (glucose + fructose + galactose + galactose)

(4) Polysaccharides

Starch (glucose units)

Glycogen (glucose units)

Cellulose (fructose units)

Hemicellulose (xylose, galactose, mannose units)

Dextrans (α-1,6 glucose polymer)

Pectins

Gums and mucilages

Lactose is found only in milk and thus can be present in foods made from or with milk and milk products. Pentose, most oligosaccharides, and polysaccharides are naturally present in foods of plant original.

All microorganisms normally found in food metabolize glucose, but their ability to utilize other carbohydrates differs considerably. This is because of the inability of some microorganisms to transport the specific monosaccharides and disaccharides inside the cells and inability to hydrolyze polysaccharides outside the cells.

Food carbohydrates are metabolized by microorganisms principally to supply energy through several metabolic pathways. Some of metabolic products can be used to synthesize cellular components of microorganisms (e.g. to produce amino acids by amination of some keto acids). Microorganisms also produce metabolic by products associated with food spoilage (CO_2 to cause gas defect) or food bioprocessing (lactic acid in fermented foods). Some are also metabolized to produce organic acids, such as lactic, acetic, propionic, and butyric acids, which have an antagonistic effect on the growth and survival of many bacteria (Roy, 2003).

2.2.4 Bacterial structure

The bacterial cell wall provides structural integrity to the cell, but differs from that of all other organisms due to the presence of peptidoglycan (poly-N acetylglucosamine and N-acetylmuramic acid), which is located immediately outside of the cytoplasmic membrane. Peptidoglycan is responsible for the rigidity of the bacterial cell wall, and determines the cell shape. It is also relatively porous and considered as an impermeability barrier to small substrates. The cell walls of all bacteria are not identical. In fact, the cell wall composition is one of the most important factors in the analysis and differentiation of bacterial species. Accordingly, two general types of bacteria exist, of which Gram-positive bacteria (Figure 2.4-2.5) are comprised of a thick peptidoglycan layer connected by amino acid bridges. Imbedded in the Gram-positive cell wall are polyalcohols, known as teichoic acids, some of which are lipid linked to

form lipoteichoic acids. Due to lipoteichoic acids are covalently linked to lipids within the cytoplasmic membrane, they are responsible for linking peptidoglycan to the cytoplasmic membrane. The cross-linked peptidoglycan molecules form a network, which covers the cell like a grid. Teichoic acids give the Gram-positive cell wall an overall negative charge, due to the presence of phosphodiester bonds between the teichoic acid monomers. In general, 90% of the Gram-positive cell wall is comprised of peptidoglycan.

On the contrary, the cell wall of Gram-negative bacteria (Figure 2.4-2.5) is much thinner, and composed of only 10–20% peptidoglycan. In addition, the cell wall contains an additional outer membrane composed of phospholipids and lipopolysaccharides. The highly charged nature of lipopolysaccharides confers an overall negative charge on the Gram-negative cell wall. Sherbert (1978) showed that the anionic functional groups present in the peptidoglycan, teichoic acids and teichuronic acids of Gram-positive bacteria, and the peptidoglycan, phospholipids, and lipopolysaccharides of Gram-negative bacteria were the components primarily responsible for the anionic character and binding capability of the cell wall (Vijayaraghavan and Yun, 2008).

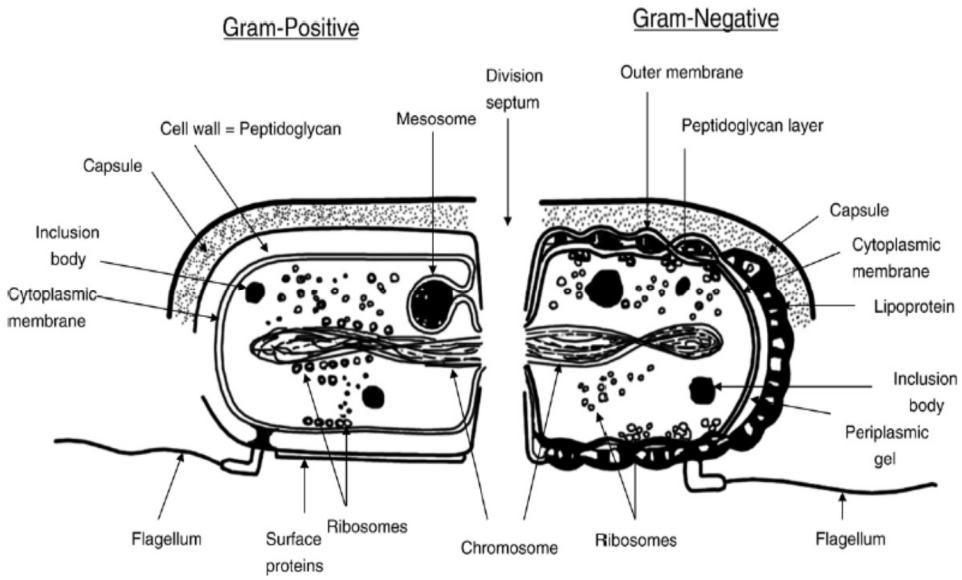

Figure 2.4 Structure of Gram-positive and negative bacteria

Source: Vijayaraghavan and Yun (2008)

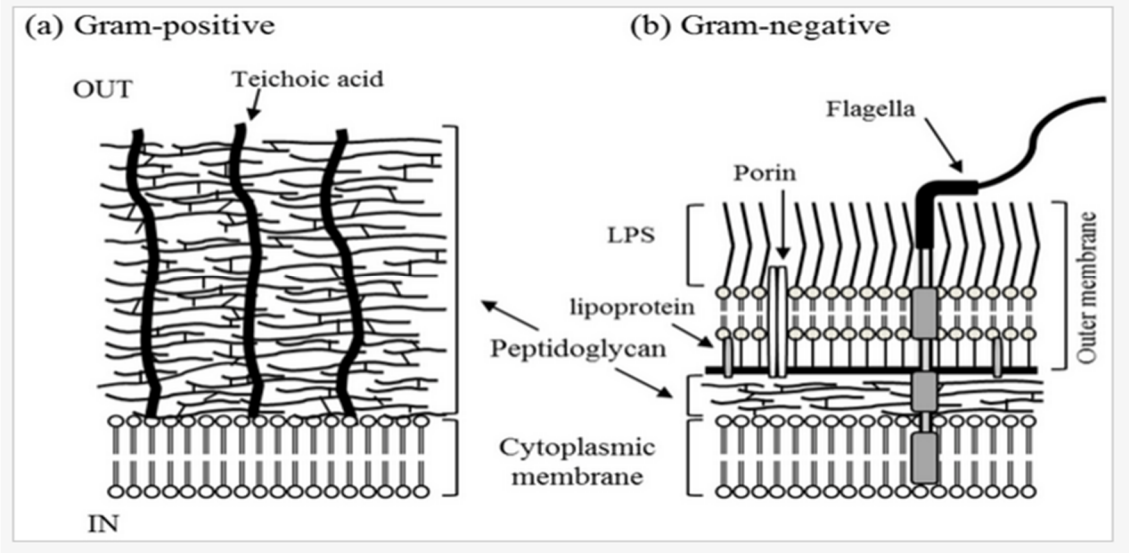

Figure 2.5 Cell wall characteristic of Gram-positive via Gram-negative bacteria.
(a) Gram- positive bacteria have a thick wall composed of peptidogycans; and (b) Gram-negative bacteria have an outer membrane and a thin wall composed of peptidoglycans.
Source: Nakano et al. (2013)

2.2.5 *Lactobacillus* sp.

Lactobacillus is a Gram-positive facultative bacterium. They are a major part of LAB group having the ability to convert lactose and other monosaccharides to lactic acid. Intestinal lactic acid bacteria for humans are closely associated with the host's health because they act as an important biodefense in preventing colonization and subsequent proliferation of pathogenic bacteria in the intestine. Some species of *Lactobacillus* and *Bifidobacterium* that have been claimed as probiotics included *Lb. acidophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. casei*, *Lb. fermentum*, *L.b plantarum*, *Lb. reuteri*, *B. infantis*, *B. breve*, *B. animalis*, *B. adolescentis* and *B. longum*. The gastrointestinal tract of a healthy human is a harsh environment and poses a significant threat to probiotic strains. In addition, low surface tension and immune response also affect the survival of probiotic strains (Gilliland, 1979).

Lactobacillus have several scientifically established and/or clinically proved health effects, such as reduction and prevention of diarrhoea, improvement of the intestinal microbial balance by antimicrobial activity, alleviation of lactose intolerance symptoms, prevention of food allergy, enhancement of immune potency, and

antitumourigenic activities(Liong, 2006). Since milk fermented with lactobacilli was first demonstrated to exhibit hypocholesterolemic effects in humans, various studies have shown that some lactobacilli exhibit cholesterol-reducing ability in human (Liong and Shah, 2005a).

2.2.6 *Lactobacillus pentosus*

Lactobacillus pentosus (*Lb. pentosus*) has since been identified as playing a traditional role in the preparation of many common foods. Also, including raw and soured milks (Kim et al., 2006), fermented cereals and vegetables (Tamminen et al., 2004), fermented meats and fish (Tanasupawat et al., 1998), and fermented beverages such as sake, tea and Scotch malt whiskey (Tanasupawat and Komagata. 1994). *Lb. pentosus* is consumed as live viable colonies in uncooked foods (e.g. soured milks and cheeses) and as dead nonviable cells in fermented foods having a later cooked stage (e.g. sourdough breads); the intentional use and natural presence of *Lb. pentosus* in foods has been international in scope, encompassing countries throughout Europe, Africa and Asia. Similar to other *Lb. pentosus* strains, strain b240 was originally isolated from Mieng, a non-salted fermented tea traditional to northern Thailand (Tanasupawat et al., 2007). Initially identified as a strain of *Lb. plantarum* (a phenotypically similar species), the bacterium was later reclassified as *Lb. pentosus* by genetic analysis using recA (Szabo et al., 2011)

Lb. pentosus is facultatively heterofermentative, as well as the so-called Group II lactobacilli. Gram-positive bacteria, rod shape with rounded ends 1-1.2 × 12-5.0 micron. The characteristics of *Lb. pentosus* are shown in table 2.2.

Table 2.2 Key characteristics of *Lb. pentosus* and *Lb. plantarum* strain

Characteristics	*Lb. pentosus*	*Lb. plantarum*
peptidoglycan type	DAP	DAP
G+C content (mol (%))	46-47.2	44-46
lactic acid isomer (s)	racemic-DL	racemic-DL
growth 15/45 (^{o}C)	+/-	+/-
carbohydrates fermented	amygdalin, L-arabinose, arbutin, galactose, cellobiose, D-fructose, β-gentiobiose, glucanate, D-glucose, glycerol, *N*-acetyl glucosamine, lactose, D-mannose, mannitol, maltose, melibiose, raffinose, ribose, salicin, sorbitol, sucrose, trehalose and D-xylose	amygdalin, L-arabinose, cellobiose, esculin, mannitol, melezitose, melibiose, raffinose, ribose, sorbitol, sucrose,

Symsbols: +, 90% or more of strains are positive; -, 90% or more of strains are negative, DAP, diaminopimelic acid. Parenthesized isomer indicate < 15% of total lactic acid. Source: modified from Zanoni *et al*. (1987); Hammes and Vogel (1995)

2.2.7 Benefits of LAB in food fermentation

Food fermentation is one of the oldest food processing and preservation methods in Asia. Fermentation adds safety, nutritional value and different flavors to what could have otherwise been a bland diet. Foods that are fermented have been subjected to the actions of microorganisms or enzymes, so that desirable biochemical changes have occurred. Moreover, fermentation is a relatively cost-effective, low energy preservation process, which is essential in ensuring the shelf-life and microbiological safety of the product (Liu et al, 2011).

Although the primary purpose was to achieve food safety, fermentation plays at least five roles:

(1) Enrichment of the diet through development of a diversity of flavors, aromas, and textures in food substrates;

(2) Preservation of food through lactic acid, alcoholic, acetic acid, and alkaline fermentations;

(3) Biological enrichment of food substrates with proteins, essential amino acids, essential fatty acids and vitamins;

(4) Detoxification during food fermentation processing; and

(5) Decrease in cooking times and fuel requirements (Steinkraus, 1996).

Fermented dairy products represent about 20% of the total economic value of fermented foods produced world-wide and the market share of such products continues to grow dairy industry is a prime consumer of various LAB strains such as *Lactobacillus, Lactococcus,* and *Leuconostoc*. For this reason, LAB used to be called "milk-souring organisms" (Liu et al, 2011).

2.3 Short chain fatty acids (SCFAs)

Short chain fatty acids (SCFAs) are synthesized by the gastrointestinal microflora. The end products from carbohydrates as substrate, are mainly SCFAs; acetic, propionic and butyric acid together with gases; CO_2, H_2, and methane. SCFAs are carboxylic acids with 1 to 5 carbon atoms that include different other functional groups, such as hydroxyl or dicarboxyl. SCFAs with different carbon chain lengths (acetate (C2), propionate (C3), butyrate (C4), valerate (C5)) are produced in varying amounts depending on the diet and the composition of the intestinal microbiota.Most microorganisms in the colon prefer to ferment carbohydrates and switch to protein fermentation when fermentable carbohydrates are depleted. While carbohydrate fermentation generally leads to health-promoting SCFAs production, protein fermentation yields branched-chain fatty acids and potentially toxic metabolites (e.g. ammonia, amines, phenols, indoles and thiols). In humans, SCFAs such as acetate, propionate and butyrate are produced as the major end products of anaerobic fermentation in large intestine resulting in significant health benefits such as anti-cancerous effect. Also SCFAs are known to fulfill 60–70% of the energy requirement of the colonocytes (Sreenivas and Lele, 2013). SCFAs arise from bacterial fermentation of

carbohydrates, proteins, peptides and glycoprotein precursors. Approximately 80-90% of SCFAs, which are produced from the breakdown of dietary food, are absorbed in colon while the rest are excreted in faces (Huda-Faujan, 2010). SCFAs are rapidly absorbed and have shown to have distinct bioactivity depending on their chain length. With regard to maintenance of colonic health and barrier function. In addition, their important role as fuel for intestinal epithelial cells, butyrate has drawn most attention, as this fatty acid is the major energy source for the colonocytes. However, butyric, acetic, and propionic acids have mainly been emphasized. In particular, butyric acid was addressed to be more beneficial for promoting colonic health and more effective for stimulating the proliferation of intestinal mucosal cells than acetic and propionic acids. Butyric acid is also the main energy substrate for the colonocytes and it has been suggested to play an important role in the prevention and treatment of distal UC, Crohn's disease (CD), and cancer (Huda-Faujan, 2010). Furthermore, butyrate has been shown to have anti-inflammatory properties and to have anti-carcinogenic effects. SCFAs content in faces could be used as a biomarker for the physiological processes in the organisms as well as for the effect of nutritional interventions.

According to Scheppach (1994) about effects of SCFAs on colonic morphology and function (facts and hypotheses) in figure 2.6. Their production during bacterial carbohydrate (starch, fibre) fermentation is well established. SCFAs are the preferred energy substrates of colonocytes, especially in the distal large bowel. Probably linked to this property, they affect a range of mucosal events (absorptive processes, blood flow, mucus release, cellular differentiation and proliferation). These effects of SCFAs are possibly clinically important (adaptation to postoperative conditions, prevention of colitis).

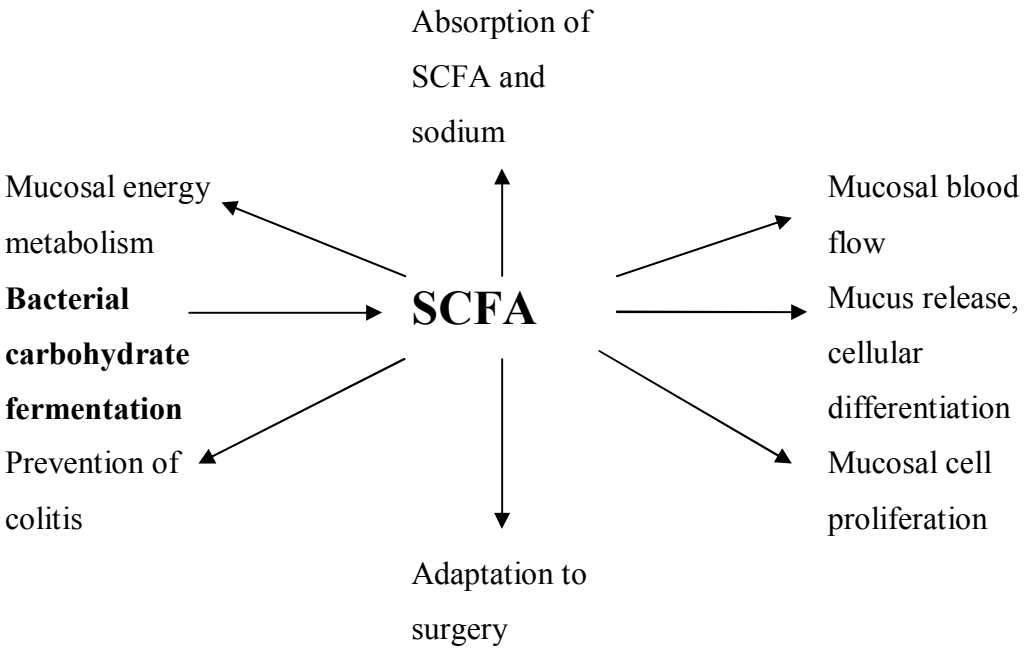

Figure 2.6 Effects of short chain fatty acids (SCFAs) on colonic morphology and function (facts and hypotheses).
Souce: Scheppach (1994)

2.3.1 Implication of substrate

The bacterial species present in the colon use different fermentation pathways, leading to differences in the SCFAs pattern generated. Indigestible carbohydrates that reach the colon are mainly non-starch polysaccharides (NSPs), resistant starches (RS) and certain oligosaccharides. In particular, NSPs are important energy substrates for large intestinal microbial fermentation, and the amount as well as the chemical and structural composition of the carbohydrate is important factors for the microbial activity in the gastrointestinal tract. Most mono- and disaccharides are rapidly absorbed in the upper intestinal tract and provide a readily available source of energy. An exception is lactose, a P-linked disaccharide, which is poorly absorbed by most of the adult population in the world due to low levels of lactase in the small intestine.

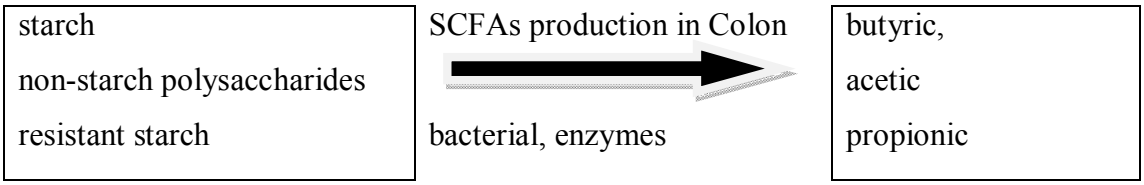

Figure 2.7 The carbohydrates as substrate fermented in the distalileum and colon
Source: Floch (2010)

When lactose is malabsorbed in the small intestine, it reached the colon for fermentation. Resistant peptides may also enter the colon and amino acid fermentation yields branched SCFAs. Starch and NSPs are fermented in the distalileum and colon. Fiber, NSPs (soluble fiber from fruits and vegetables), and prebiotics are fermented primarily by bacterial enzymes as shown in figure 2.7. Humans have essentially no enzymes that permit their production, whereas bacteria extensively ferment and produce them in the molecular ratio of approximately 60:20:20 for acetic, butyric, and propionic. The amount produced varies with different organisms and the availability of different substrates (Floch, 2010).

2.3.2 Nutritional and Health benefits of SCFAs

The SCFAs absorbed from the colon can be utilized as an energy source by the host but they contribute only to a small part (5-10%) of total energy. The colonic mucosa obtains its energy by oxidizing mainly SCFAs in the order of butyric > propionic > acetic acid. The SCFAs that escape metabolism in the colon enter the hepatic portal blood as illustrate in figure 2.8. The liver utilizes acetic acid where it is transferred into Acetyl-CoA, which can act as a precursor for lipogenesis, but also stimulates gluconeogenesis. Acetic acid in low level of concentrations can also be detected in venous blood in peripheral tissues. Propionic acid is mainly metabolized in the liver and has been proposed that may lower plasma cholesterol concentrations by inhibiting hepatic cholesterogenesis. Butyric acid is the main energy for the colonocytes. The colon epithelial cells use butyric acid before they use glucose as a nutrient (Henningsson, 2001).

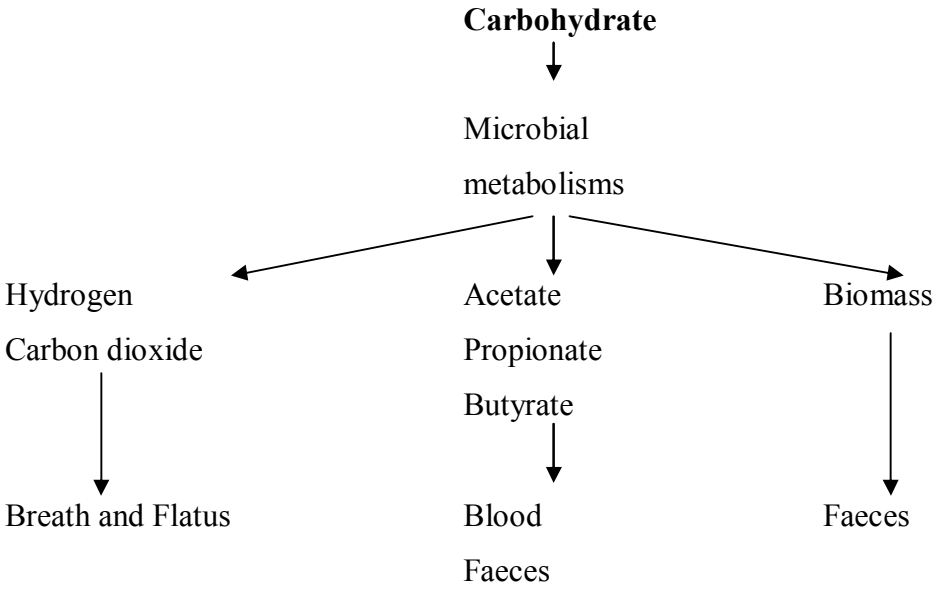

Figure 2.8 Carbohydrate fermentation in the human colon
Source: Henningsson et al. (2001)

Acetic acid is the building block for cholesterol. The functions of propionic acid are not well understood. In humans, the synthesis of cholesterol from acetic acid decreased when propionate was infused rectal. However, they seem to affect the rate of cholesterol metabolism. It is suggested that substrates that can decrease the acetic acid and propionic acid ratio may actually improve lipid risk factors. Furthermore, as much as 5% to 10% of absorbed energy can come from SCFA metabolism. Butyric acid is considered beneficial for gut health, because of it serves the main energy substrate for the colonocytes and metabolized by the cells in preference to glucose or glutamine, accounting for 70% of the total energy demand of the colonic mucosa. It has been reported to be important in the prevention and treatment of diseases of the colonic mucosa, such as distal ulcerative colitis and cancer (Henningsson, 2001). Furthermore, the energy derived from the SCFAs can be beneficial to patients with a short bowel when carbohydrates are metabolized by the microflora to produce SCFAs and then absorbed. In addition, increased SCFAs levels may also increase the solubility of certain minerals, such as calcium, and enhance the absorption and expression of calcium-binding proteins. The entire subject of microorganisms producing substances such as

SCFAs that are beneficial to the host requires further experimentation to see the therapeutic and beneficial effects (Scheppach et al.1995).

2.4 Cholesterol reducing activity

Probiotics have been considered to have potential health-promoting benefits as biotherapeutic agents (Begley et al., 2006). One of the health-promoting benefits of probiotics is their ability to reduce cholesterol (Silirun et al., 2010). Probiotic strains, especially LAB have a major role to play in the cholesterol lowering mechanism. As the cholesterol level keeps increasing in the serum, it leads to cardiac diseases. From many reports, several actions to reduce cholesterol associated with *Lactobacillus* spp. have been described as cholesterol assimilation by the bacteria, cholesterol binding to the bacterial cell wall, and BSH deconjugating of bile salt (Pereira and Gibson, 2002; Kim et al., 2008).

2.4.1 Mechanisms of cholesterol reducing effects

Several mechanisms have been suggested for cholesterol reducing activity of probiotics include deconjugating bile acids through bile salt hydrolase catalysis (Homayouni et al, 2012). Cholesterol is the precursor for the systhesis of new bile acids, the use of cholesterol to synthesize new bile would lead to a decreased concentration of cholesterol in blood. Bacteria tend to take up and assimilate cholesterol for stabilization of their cell membrane and binding cholesterol to cell walls of probiotics in intestine. Conversion of cholesterol into coprostanol (Lye et al., 2010) and SCFA such as propionate produced by probiotic bacteria may also inhibit hepatic cholesterol synthesis and/or redistribution of cholesterol from plasma to the liver (Pereira and Gibson, 2002b) hypocholesterolemic effect via altering the pathways of cholesteryl esters and lipoprotein transporters as described in Figure 2.9 (Ooi and Liong, 2010).

The mechanisms of probiotic activity to reduce cholesterol that have been proposed to three ways (assimilating, binding or by degradation).

(1) assimilate the cholesterol for their own metabolism

(2) cholesterol adherence to the bacterial cell wall or its incorporation into cells and could be bound to the cholesterol molecule,

(3) (probiotics are capable of degrading cholesterol to its catabolic products (Mahrous, 2011).

In addition, the physiological action of the end-products of SCFAs by fermentation, destabilization and co-precipitation of the cholesterol micelles, bile salt hydrolase activity of the lactobacilli, cholesterol oxidize activity, and finally production of some functional peptides.

Bile is a water-soluble end product of cholesterol in the liver, is stored and concentrated in the gallbladder, and released into the duodenum upon ingestion of foods. It consists of cholesterol, phospholipids, conjugated bile acids, bile pigments and electrolytes. Once deconjugating, bile acids are less soluble and absorbed by the intestines, leading to their elimination in the feces. Cholesterol is used to synthesize new bile acids in a homeostatic response, resulting in lowering of serum cholesterol as described in Figure 2.10 (Beglay et al., 2006). Bile salt hydrolase (BSH) is the enzyme responsible for bile salt deconjugation in the enterohepatic circulation. It has been detected in probiotics indigenous to the gastrointestinal tract and able to hydrolyze conjugated glycodeoxycholic acid and taurodeoxycholic acid, leading to the deconjugation of glyco- and tauro-bile acids.

The hypocholesterolemic effect of the probiotics has also been attributed to their ability to bind cholesterol in the small intestines. Usman (1999) reported that strains of *Lb. gasseri* could remove cholesterol from laboratory media via binding onto cellular surfaces. The ability to bind cholesterol appeared to be growth and strain specific. Kimoto *et al.* (2002) evaluated the cholesterol removal by probiotics cells during different growth conditions. Living and growing cells were compared with nongrowing (live but suspended in phosphate buffer) and dead cells by heat-killed. The authors found that although growing cells removed more cholesterol than dead cells, the heat-killed cells could still remove cholesterol from media, indicating that some cholesterol was bound to the cellular surface (Ooi and Liong, 2010).

Reduction of cholesterol, in the added cholesterol media is considered as an indication for the selection of probiotic strains with cholesterol assimilation property

(Gilliland and Walker, 1990; Lin and Chen, 2000). Some researchers have carried out the cholesterol reducing in the MRS media before and after the complete growth of *Lactobacillus* strains as a typical approach (Gilliland and Walker, 1990; Lin and Chen, 2000; Liong and Shah, 2005a). The researchers have been used a colorimetric method base on cholesterol-O-phthalaldehyde reaction, constitute the principles of Rudel and Morris (1973) for determined the cholesterol removal (Mirlohi et al., 2012). Since cholesterol reducing is a health-promoting characteristic, the idea of selection of microbial strains with cholesterol reducing effect has been developed as a tool to introduce new probiotic microorganisms (Madani, 2013).

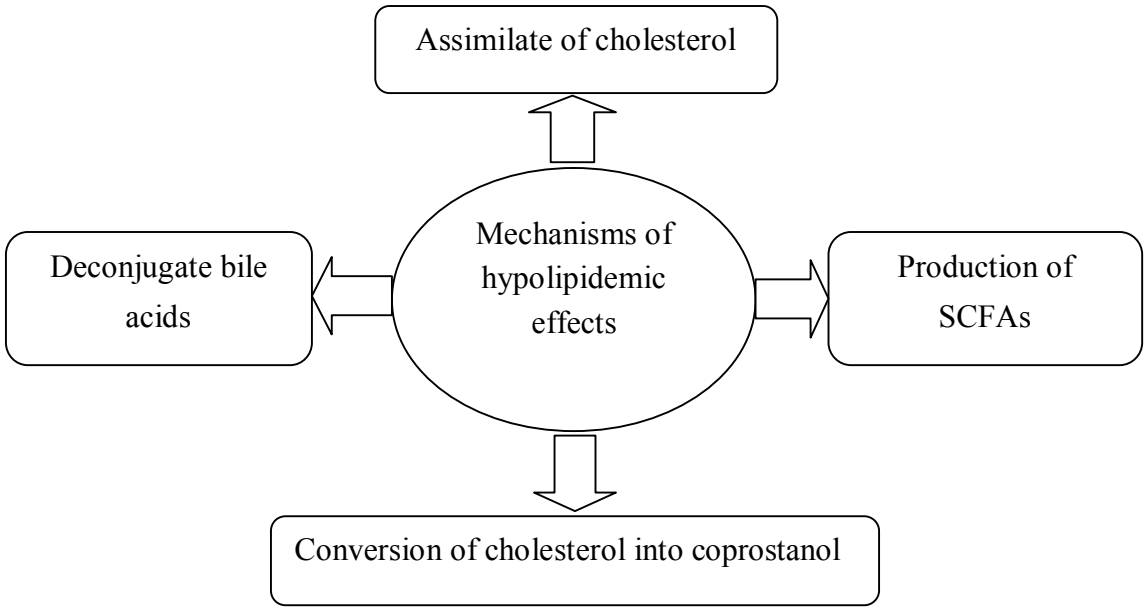

Figure 2.9 Mechanisms of hypocholesterolemic effect
Source: Mahrous (2011)

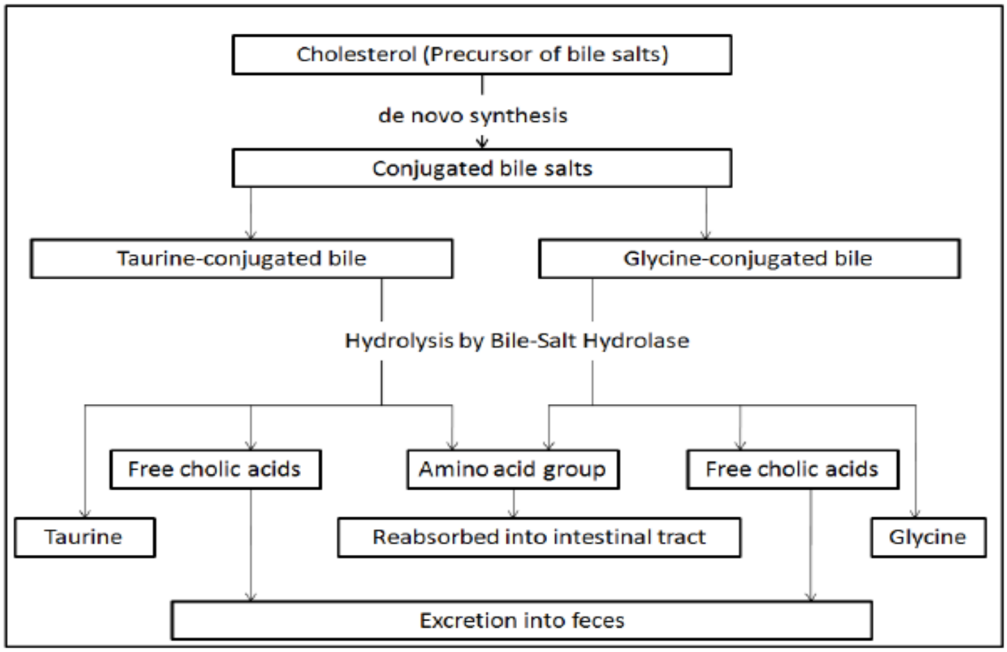

Figure 2.10 Cholesterol as the precursor for the synthesis of new bile acids and the role of bile salt hydrolase for hypocholesterolemic
Source: Ooi and Liong (2010)

2.5 Mycotoxins

Mycotoxins are the secondary metabolites produced from toxigenic fungi recognized as major food and feed contaminants. They are a source of grave concern in food contamination, resulting in mycotoxicosis in humans and animals. Typically, toxin production is influenced by moisture, time, temperature, and food or feed substrates. Contamination can occur throughout the food chain from the field, during harvesting, processing, storage, transportation, and consumption (Ezekiel et al, 2008; Anukul, 2013). The mycotoxins may be cause carcinogenic, mutagenic, teratogenic, estrogenic, neurotoxic, and immunotoxic for animals or humans (Joannis-Cassan et al., 2011). Many of the developed countries have regulations for mycotoxins in food grains and its products. However, the risk of mycotoxin exposures continues in the developing countries due to lack of food security, poverty and malnutrition (Shetty and Jespersen, 2006).

2.5.1 Zearalenone

Zearalenone (ZEA) is an estrogenic mycotoxin that can be produced by several field fungi including *Fusarium* graminearum (Gibberella zeae), *F.* culmorum, *F.* cerealis, *F.* equiseti, *F.* semitectum and *F. crookwellense*. Fungi of the genus *Fusarium* infect cereals pre-harvest in the field during blooming, but growth and toxin production may also occur post-harvest under poor storage conditions. The toxin is common in maize, but because the spores of *Fusarium* are ubiquitous, cereal crops such as barley, oats, wheat (Oliveira et al. 2014; Avantaggiato et al., 2003), rice, sorghum and soybeans are also susceptible to contamination with ZEA, both in the temperate and warmer climate zones (Golinski et al., 2010). Many countries have gathered data on occurrence of ZEA in (mainly grain-based) foods (EFSA, 2004 and Anukul et al , 2013). Depending on climatic, harvest and storage conditions, the concentrations of ZEA found in cereals and cereal products range from less than 1 to over 300 mg/kg, but rarely exceed 10 mg/kg (IARC, 1993).

ZEA not only leads to economic loss by contaminating food and feed, but it causes serious health problems in livestock and humans as well. ZEA causes alterations in the reproductive tract of laboratory animals (mice, rats, guinea pigs) and farm animals. Decreased fertility, increased number of resorptions, reduced litter size, changed weight of adrenal, thyroid and pituitary glands and change in serum levels of progesterone and oestradiol have been observed, but no teratogenic effects were found. Occurrence in mixed feeds associated with hyperoestrogenism has been reported in farm animals, particularly in pigs (Kuiper-Goodman et al. 1987). It can cause estrogenic effects, inducing reproductive toxicological effects in domestic animals and may act as a key factor in certain pregnancy disorders in humans (Zinedine et al., 2007; Lu et al. 2011).

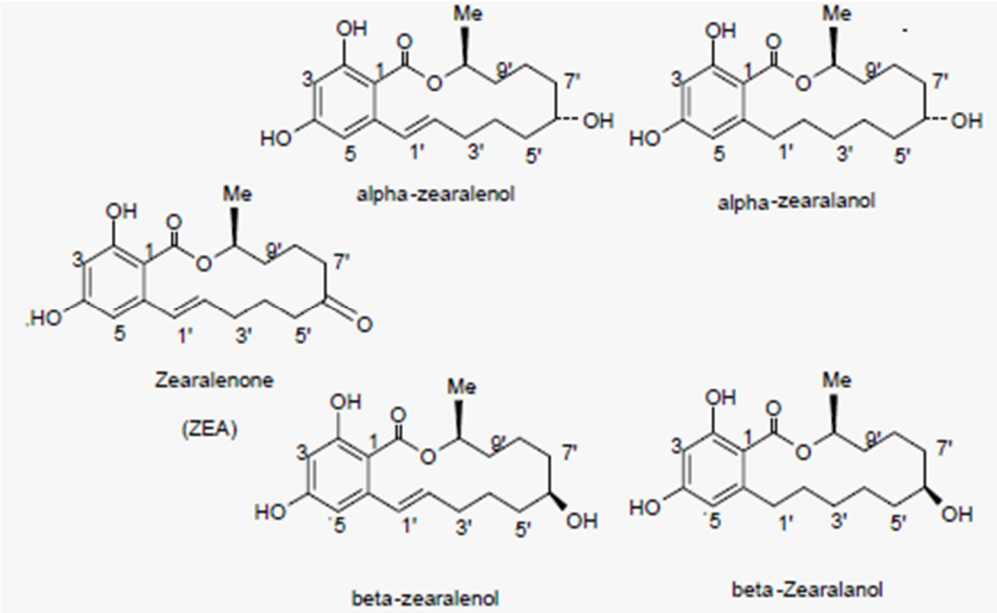

Figure 2.11. Chemical structure of ZEA and its major metabolites
Source: EFSA (2004)

ZEA (formerly denoted F2-toxin) is a resorcyclic acid lactone chemically described as 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcyclic acid lactone ($C_{18}H_{22}O_5$, MW: 318.36) (Figure 2.11). The structure of ZEA allows its binding to mammalian oestrogen receptors (EFSA (2004). Estrogenic syndrome is the major toxic effect of ZEA as its structure resembles that of 17-beta-estradiol, an estrogen hormone. Hyperestrogen, vulvovaginitis, and estrogenic responses from ZEA are observed in estrogenic target cells. An amount of 1 mg/kg in feed can cause estrus in swine. maximum tolerable. ZEA was evaluated by the IARC base on Cancer. Based on inadequate evidence in humans and limited evidence in experimental animals, zearalenone was placed, together with other *Fusarium* toxins, in Group 3 (not classifiable as to their carcinogenicity to humans) (IARC, 1993). The provisional maximum daily intake established by JECFA was 0.5 µg/kg body weight (EFSA, 2004).

2.5.2 ZEA Contaminated in foods product and feeds

From Biomin's mycotoxin survey report 2011, ZEA contamination of was found highest in Asia (53%) of positive samples compared in Europe (35%), South America (28%), Oceania (26%), North America (14%), and Africa (8%), Middle East

(0%), respectively. Never the less, in Asia, the data showed highest by North Asia (63%), Southeast Asia (37%), and South Asia (4%). Average amount of ZEA contamination in Asia was 129 mg/kg, which was still within the range of the maximum limits regulated in Asian countries (Anukul et al, 2013). The regulation limits for ZEA established in various Asian countries are relatively diverse and different from those set by EU regulations. The maximum limit of ZEA in food and feed commodities in Thailand was 30-1000 ppb (all foods), Japan 1000 ppb (compound feeds) whereas, South Korea 200 ppb (grains and processed), 50 ppb (confectionaries), 20 ppb (baby foods). However, the occurrence of this toxin in processed food was largely lower than that found in the EU and in each country's regulation limits except in Indonesia, which showed a slightly higher level (Anukul, 2013).

Zinedine et al. (2007) reported that several studied found ZEA contaminate in cereals and food products in Asia. The contamination of cereals (barley and wheat) with ZEA was found in Japan (Yoshizawa and Jin, 1995; Yoshizawa, 1997) and barley, barley-based foods, corn and corn-based foods (Park et al., 2002) and rice (Park et al., 2005) were found in Korea. In addition, cereals including maize, wheat and rice were reported to contain ZEA in India, (Phillips et al., 1996; Janardhana et al., 1999). Co-contamination of maize with ZEA, NIV, Fumonisins and aflatoxins is an emerging issue in Philippines and Thailand (Yamashita et al., 1995). The contamination of maize-based food and poultry feeds with ZEA was reported in Indonesia (Nuryono et al., 2005). Throughout the globe, ZEA has been detected in a number of cereal crops such as maize, barley, oats, wheat, rice, sorghum, and rye (Zinedine et al., 2007). Depending on climatic and storage conditions, the contents of ZEA vary within the range of 0.001-8.04 mg/kg (wheat), 0.016-0.095 mg/kg (oat), and 0.004-15 mg/kg (barley) (Placinta et al., 1999).

Soybean is an important legume plant cultivated in many parts of the world for its oil and proteins, which extensively used in the manufacture of human foods and animal feedstuffs. Soybean often attacked by fungal infections and cause of mycotoxin contamination during cultivation, post-harvest or process. There is an increasing world consumer demand for high quality and innocuous food and drink products with the lowest possible level of contaminants such as mycotoxins. As a result, the food industry

in the developed world demands raw ingredients of the best quality and that conform to statutory limits where these have been set for mycotoxins (Barros et al, 2011). Several approaches have been developed for decontamination of mycotoxins in foods. Though many approaches are available for mycotoxin decontamination, most of them are not widely available due to high cost or practical difficulties involved in detoxification process (Reddy et al., 2010). However, the methods also affected the taste and the quality of food and the toxin was not in an acceptable level (Yaowapa, 2013). In the present, LAB was investigated and further developed to use as a biosorption for reduce toxins contamination in food industry.

2.5.3 ZEA Binding by LAB

LAB is widely used in foods fermented products and is part of the intestinal microflora. Several investigations indicated that LAB has beneficial health effects in humans (Ouwehand et al., 2002). LAB as biopreservative organisms have been the focus of numerous studies. Generally, LAB is accepted as safe for use in food by the Food and Agricultural Organization of the United States (FAO) and by the European Food Safety Authority (EFSA) who have granted many species with Generally Regarded as Safe (GRAS) and Qualified Presumption of Safety (QPS) status, respectively (Franz et al., 2010). LAB is known to deliver desired technological properties and bioprotection in several different food matrices, concurrently enhancing organoleptic and textural qualities of the final product (Oliveira et al. 2014). One of the effects identified is the protection against toxins contained in foods such as heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, reactive oxygen, and mycotoxins (Fuchs et al., 2008). An increasing interest has been generated by the possibility of using microorganisms to reduce mycotoxins. The ability of a mixed culture of bacteria to degrade completely ZEA from culture media was also reported by Megharaj et al. (1997). Also with other fungal toxins such as ZEA, trichothecenes and fumonisins, binding effects have been observed in chemical analytical investigations (Shetty and Jespersen, 2006). It has been shown earlier by El-Nezami et al. (2002) demonstrated that LAB detoxify aflatoxin B1 (AFB1), which is the most potent known human carcinogen and contributes to the high prevalence of liver cancer in regions such as Central Africa and China. Recently, Mokoena et al. (2005) reported that LAB

fermentation could significantly reduce the concentration of ZEA in maize by 68–75% in fourth days of fermentation

2.5.4 Mechanism of mycotoxin binding by LAB

Cell wall peptidoglycan and polysaccharide are the two most important elements responsible for the binding of mutagens to LAB (Hirayama and Rafter, 2006). Both of these components are expected to be affected by heating and acids. Heating may cause protein denaturation or the formation of Maillard reaction products between polysaccharides and peptides or proteins, whereas under acidic conditions, the glycosidic linkages in polysaccharides break down releasing monomers that may be further fragmented into aldehydes. Acids may also break the amide linkages in peptides and proteins, producing peptides and the component amino acids. The peptidoglycan structure of the cell wall is usually quite thick in LAB but its thickness may be reduced and/or its pore size may be increased via heat and acid treatments. (El-Nezami et al., 2002).

Several LAB have been found to be able to bind mycotoxins *in vitro*/*in vivo* with efficiency depending on the bacterial strain. Mathematical model suggests the attachment of toxins molecules to the surface of the organism and takes two processes into consideration: binding (adsorption) and release (desorption) of toxin to and from the binding site on the surface of the microorganisms (Bueno et al., 2006). The cell walls of some LAB have been reported to be able to bind some mutagenic compounds such as amino acid pyrolysates and heterocyclic amino acids produced during cooking (Dalie et al., 2010). On the basis of the chemical moieties and interactions involved in ZEA and α-zearalenol binding by LAB, it is likely that carbohydrates and proteins were the bacterial cell components involved in the process (El-Nezami et al., 2002).

2.6 Soybean

Soybeans have become an increasingly important agricultural and worldwide annual production in the world. It is widely believed that the soybean originated in China, probably in the north and central regions, 4000-5000 years ago. From China, soybean cultivation spread into Japan, Korea, and throughout Southeast Asia (Liu, 1997). The consumption of soybean in Asian countries has increasing worldwide every

year mainly due to their acclaimed health benefits. In these, soybeans and soy-products are found several phytochemicals and they appear to be the active compounds causing many beneficial health effects. Soybean milk is a widely ingested beverage in East and Southeast Asian countries. In China, annual consumption is reported to be 15-20 kg per person in all forms. While in Thailand, soybean production is not sufficient to meet human and animal needs. In 2010, about 1.8 million tons of soybeans were imported. However, soybean is a widely cultivated crop, most of it is used as the raw material for feedstuff, food protein and widely used in industrial. Several years of rigorous scientific and clinical research has established that most of the components of soybean have beneficial health effects as characterized by its preventive potential for the so-called life-style-related diseases (Dixit et al., 2011).

2.6.1 Soybean seed characteristics

The soybean belongs to the family *Leguminosae*, subfamily *Papilionoideae*, and the genus *Glycine,* L. The cultivated form, named *Glycine max* (L.) Merrill, grows annually. Most mature seeds are made of three basic parts: the seed coat, the embryo, and one or more food storage structures. The embryo contains two pieces of cotyledons that function of food reserve structure (Figure 2.12). The seed coat protects the embryo from fungi and bacteria infection before and after planting. Beside cotyledons, the embryo has three other parts: radical, hypocotyls, and epicotyls. The radical and hypocotyls, together know as hypocotyls radical or germ, are located under the seed coat. During germination, the radical becomes the primary root, whereas the hypocotyl lifts the cotyledons above the soil surface. The epicotyl is the main stem and growing point (Liu, 1997).

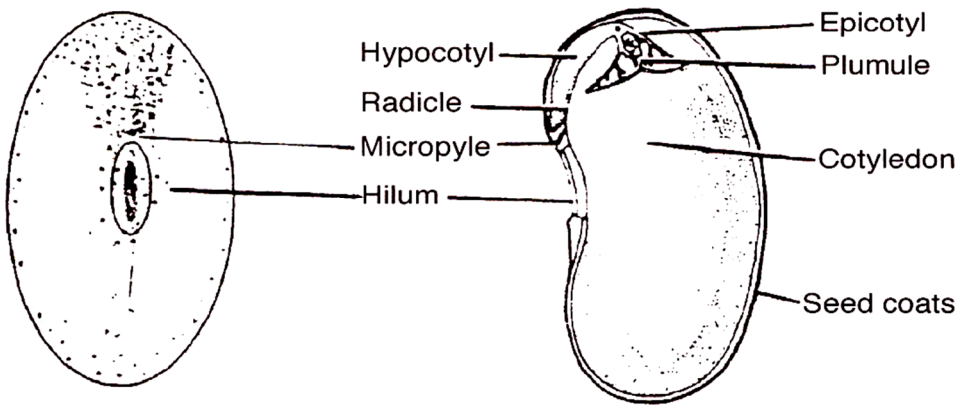

Figure 2.12 Structure of a soybean seed
Source: Liu (1997)

2.6.2 Soybean products

Soybean has been extensively used as important source of dietary protein and oil throughout the world. There are distinct differences in how the East ant the West use soybeans. In the Far East, traditionally, soybeans are made into various foods for human consumption, including tofu, soymilk, soy sprouts, miso, natto, and tempeh, whereas in the West, most soybean are crushed into oil and defatted meal. Although soybean oil is almost all human consumption, soy meal is mainly used as animal feed. Only a small portion is processed into soy protein ingredients including soy flour, concentrated, isolates, and textured soy proteins. These ingredients have functional and nutritional applications in various types of bakery, dairy, and meat products, infant formulas, and the so-called new generation soy foods. Figure 2.13 shows a general outline of soybean food utilisation base on oil beans and food beans (Liu, 1997).

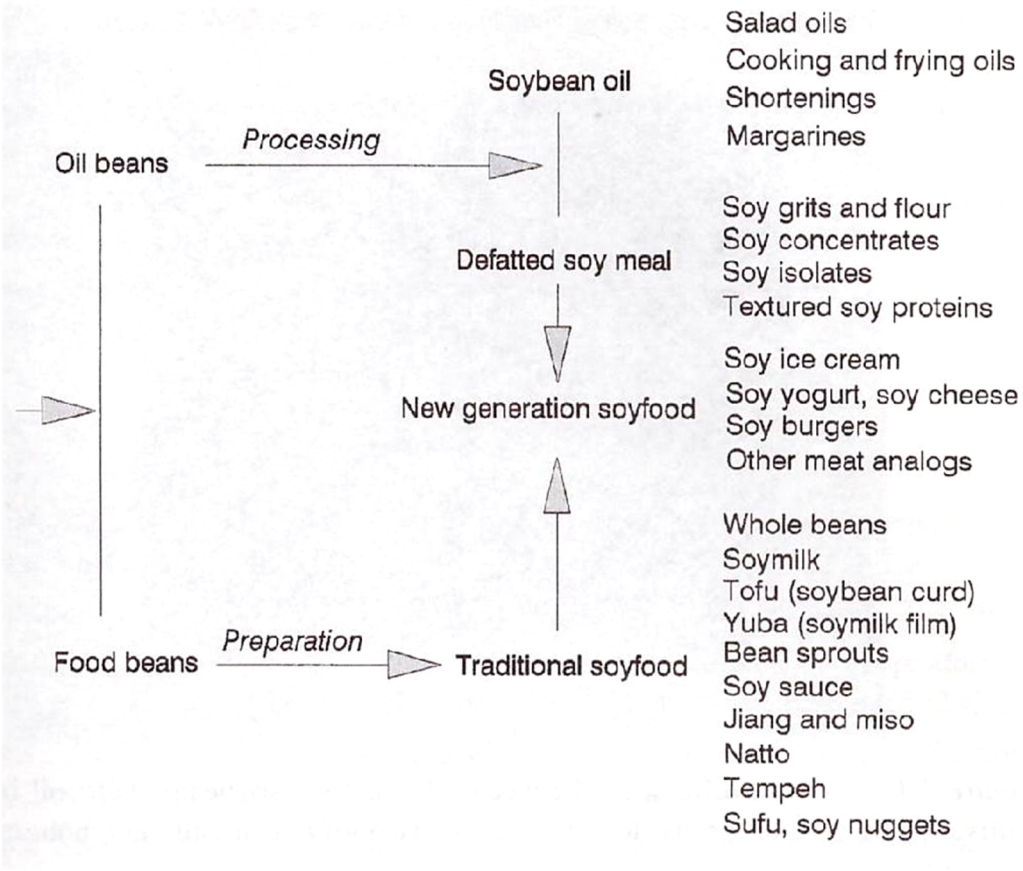

Figure 2.13 A general outline of soybean food use based on classification of oil and food beans

Source: Liu (1997)

2.6.3 Soybean nutritional properties

The soybean is one of the most economical and valuable agricultural commodities because of its unique chemical composition. Proteins and lipids, some vitamins and minerals, are major nutritionally important components of soybeans; carbohydrates are major constituents quantitatively. On average, dry soybean contains roughly 40% protein, 20% oil, 35% soluble (sucrose, raffinose, stachyose, etc.) and insoluble (dietary fiber) carbohydrate and 5% minerals and several other components including vitamins (Liu, 1997; Jooyandeh, 2011). Furthermore, soybeans contain many minor substances, some of which, such as phytase, oligosaccharide, and isoflavones.

2.6.3.1 Proteins

Soybean is good sources of protein, can be good substitutes for animal protein because it offers a complete protein profile. The protein content of soybean has

rich amino acids with a good balance, which is comparable of animal proteins sources like milk and beef. Soybean contains all the essential amino acids (except methionine), which must be supplied in the diet because they cannot be synthesized by the human body. The high sufficient lysine content of soy protein makes it a good complement to cereal proteins, which are low in lysine (Jooyandeh, 2011). Soybean contains 35–40% protein on a dry-weight basis, of which, 90% consists of β-conglycinin (7*S* globulin) and glycinin (11*S* globulin) (Liu, 1997). The amino acid profile and structure of these two main proteins concern much with the physicochemical functions, including emulsification, foaming, gelation and fat binding abilities. These proteins contain all amino acids essential to human nutrition, which makes soy products almost equivalent to animal sources in protein quality but with less saturated fat and no cholesterol. Moreover, soybean is not only high quality protein, but it is now thought to play preventive and therapeutic roles for several diseases (Dixit et al., 2011).

2.6.3.2 Carbohydrates

The carbohydrates of soybeans, containing little starch and hexose, are largely polysaccharides with some oligosaccharides. Carbohydrates make up approximately 35% of the soybeans. Approximately 50% of soy carbohydrates are nonstructural in nature and include: low molecular weight sugars, oligosaccharides and small amounts of starch. The other half comprises polysaccharides that include considerable amounts of pectic polysaccharides. The small amounts of free galactose, glucose, fructose and sucrose make up the low molecular weight sugars. Galacto-oligosaccharides (raffinose, stachyose and verbascose) comprise approximately 5% of the soybeans dry matter, while starch represents less than 1%. Stachyose is a tetraose with a galactose-galactose-glucose-fructose structure, while raffinose is a triose with a structure of galactose-glucose-fructose.

Soybean oligosaccharides; SBO are a group of soluble low molecular weight oligosaccharides in soybean seeds, which include sucrose, stachyose and raffinose. Soybean oligosaccharides are defined as non-digestible oligosaccharides or non-digestible sugars except sucrose since human gastrointestinal tract does not possess α- galactosidase enzyme essential for hydrolysis of the α-1, 6 galactosyl linkages.

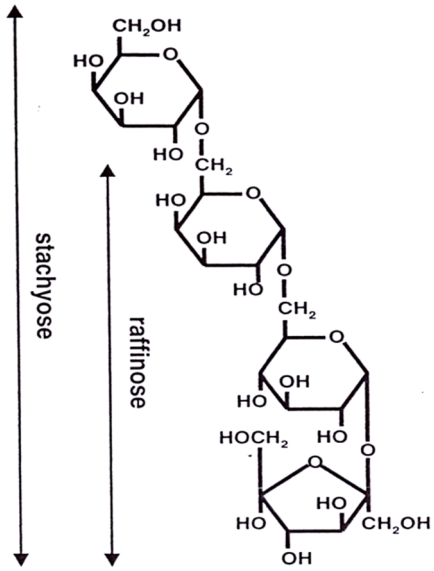

Figure 2.14 Soybean oligosaccharides are extracted directly from soybean whey. The trisaccharide raffinose and the tratasaccharide stachyose are the major oligosaccharide structures
Source: Cristendall (2006).

Therefore, oligosaccharides are supposedly involved in flatulence. The presence of these oligosaccharides impedes the full utilisation of the soybean products. With the progress of oligosaccharides researches, it was found that soybean oligosaccharides are not the direct causes of flatulence. Modern safety tests have proven that oligosaccharides are safe for human consumption. Synthesis SBO: consist mainly of the trisaccharide raffinose and the tratasaccharide stachyose. They are extracted directly from soybean whey rather than being commercially synthesized using enzymatic processes shown in Figure 2.14. Both raffinose and stachyose are resistant to digestion and are readily fermented by bifidobacteria *in vitro* (Cristendall, 2006).

2.6.3.3 Lipids and micronutrient profiles

Soybean oil provides calories, low in saturated fat, rich in the essential fatty acids and is an excellent source of vitamin A and E but contributes insignificant amounts of vitamins D and K. In other word, besides to providing omega-6 fatty acids, soybeans are among the few plant foods that provide omega-3 fat á-linolenic acids (Jooyandeh H. 2011). Linoleic acid in soybean oil is an essential fatty acid belonging to

the omega -6 family of polyunsaturated, which exerts important nutritional and physiological functions. Even the α-linolenic acid is also an essential fatty acid belonging to omega-3 fatty acid family, and plays an important role in the regulation of a number of metabolic pathways (Dixit et al., 2011).

2.6.3.4 Vitamins and minerals

The water-soluble vitamins of soybean mainly include thiamine, riboflavin, niacin, pantothenic acid and folic acid. The main oil soluble vitamins include A (retinol) and E (tocopherol). The Vitamin D and K content is negligible. Vitamin A exists as the pro-vitamin â carotene. The tocopherol content of soybean varies with variety, tocopherol that is excellent natural antioxidants. The major forms of minerals in soybean are sulphates, phosphates and carbonates. Potassium is found in the soybean in the highest concentration, followed by phosphorus, magnesium, sulphur, calcium, chloride and sodium in that order.

2.6.3.5 Isoflavones

Isoflavones is a sub-group of heterocyclic plant phenolic category called flavonoids. The soybean is most abundant source of isoflavones in the nature. Soybean contains three types of isoflavone aglycone; daidzein, genistein and glycitein; each of them present in three glycosidic forms in addition to their aglycone form. Daidzein, genistein and their glycosides contribute to >90% of total isoflavone; whereas glycetein and its glycoside are present as minor component (<10%), only. Isoflavones are structurally similar to mammalian estradiol and can bind to both α and β isoforms of estrogen receptor (ER), thus called phytoestrogens. However, the isoflavones are not essential nutrients that are required to support life, still they exert many beneficial health effects, therefore, are of immense help for maintaining healthy life.

2.6.4 Soybean and health benefits

The health effects of soy components have been extensively studied through human clinical trials, experimental animal studies, and *in vitro* cell culture studies. That is, consuming soy foods as part of the normal diet would be a wise nutritional practice because soybean provides several nutrients and helps prevent cancer, osteoporosis and cardiovascular diseases. Explanations of how soy protein lowers blood cholesterol include:

(1) decrease in cholesterol absorption and increase in bile acid excretion

(2) increase in liver low density lipoprotein (LDL) receptors and faster clearance of LDL from the blood

(3) decrease in hepatic cholesterol synthesis

(4) increase in blood thyroxin and thyroid stimulating levels.

Thus soy helps to prevent osteoporosis by two ways-by daidzein affecting bone resorption and formation directly and by genistein acting as a weak estrogen. Soybeans are also free of milk sugar, that is, lactose. Therefore soy foods provide a wonderful selection of alternatives to dairy foods for people who are lactose intolerant. Moreover, soybean sugars are so effective in promoting the growth of probiotic which play a very important role in promoting health of the colon when these oligosaccharides are replacing common table sugar.

2.7 Honey

Honey was an important food as the available natural sweetener for human along time ago. In present the annual world honey production is about 1.2 million tons, which is less than 1% of the total sugar production. Honey consumption is higher in developed countries, where the home production does not always cover the market demand. Different surveys on nutritional and health aspects of honey have been compiled (Bogdanov et al. 2008).

Key points of honey:

(1) About 95% of the honey dry matter is composed of carbohydrates, mainly fructose and glucose. 5-10 % of the total carbohydrates are oligosaccharides, in total about 25 different di- and trisaccharides.

(2) Besides, honey contains small amounts of proteins, enzymes, amino acids, minerals, trace elements, vitamins, aroma compounds and polyphenols.

(3) Honey has been shown to possess antimicrobial, antiviral, antiparasitory, antiinflammatory, antioxidant, antimutagenic and antitumor effects.

(4) Due to its high carbohydrate content and functional properties honey is an excellent source of energy for athletes.

(5) Most of the health-promoting properties of honey are only achieved by application of rather high doses of honey such as 50 to 80 g per intake.

Application of honey

The application of honey as a food additive is based on its manifold properties. The antibacterial effect of honey counteracts microbial spoilage of food. The antioxidant effect of honey prevents oxidation of food during storage. Other physical and sensory properties make honey a good candidate for an additive to a wide variety of food in term of arising good sensory and rheological properties (Păucean et al. (2011). As a prebiotic, honey contains carbohydrates called oligosaccharides, which may improve gastrointestinal health by stimulating the growth of good bacteria in the colon. Honey has been shown to enhance growth, activity of *Bifidobacteria* in fermented dairy food. (Amiri, 2010)

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Microorganisms

Eight probiotic LAB strains were used in this study; namely *Lb. pentosus* (DM068, JM0812, JM085, UM054, UM055, VM095, VM096, and YM122) (Kansandee, 2010) were obtained from the laboratory of Asst. Prof. Dr. Pariyaporn Itsaranuwat, Department of Biotechnology, Mahasarakham University, Thailand. The strains were maintained in MRS broth (Criterion, USA) with 20% glycerol at -20 °C. They were anaerobically activated twice in MRS broth using 1% (v/v) inoculums for 24 h, at 37 °C, 5% CO_2 incubator prior to use for the experiments.

3.1.2 Materials and media

3.1.2.1 De Man Rogosa Sharpe (MRS) (Citerion, USA)

3.1.2 2 Modified-MRS medium

3.1.2 3 Agar (Merck Darmstadt, Germany)

3.1.2 4 Soybean seed (Rai Thip brand)

3.1.2.5 100% Honey syrup (Suan Jit-radda brand)

3.1.3 Chemicals

3.1.3.1 Chemical reagents

(1) Oxgall (Sigma-Aldrich, USA)

(2) Absolute ethanol (Merck, Germany)

(3) 95 % Ethyl alcohol (Solvent solution Grade A; Commercial grade)

(4) n-Hexadecane; Cetane (Sigma-Aldrich, USA)

(5) Taurodeoxycholic acid; TDCA (Sigma-Aldrich, USA)

(6) *o*-Phthalaldehyde reagent (Sigma-Aldrich, USA)

(7) Sodium chloride (Univar, Ajak Finechem, NSW, Australia)

(8) 0.05 M Sodium acetate buffer (pH 5.0)

(9) 0.85 % NaCl

(10) Acetonitrile HPLC grade (Lab-Scan, Ireland)

(11) Methanol HPLC grade (Lab-Scan, Ireland)

(12) Reverse Osmosis Water for HPLC

3.1.3.2 Standard reagents

(1) Water soluble cholesterol (polyoxyethanyl cholesteryl sebaccate; PCS) (Sigma-Aldrich, USA)

(2) Zeralenone (Sigma-Aldrich, USA)

(3) Propionic acid (Merck, Germany)

(4) Butyric acid (Merck, Germany)

(5) Iso- butyric acid (Merck, Germany)

(6) n-Valeric acid (Merck, Germany)

(7) Lactic acid (Merck, Germany)

(8) Acetic acid (Merck, Germany)

(9) Fructooligosaccharide (FOS) (Sigma-Aldrich, USA)

(10) Maltotetraose (Sigma-Aldrich, USA)

(11) Raffinose (Sigma-Aldrich, USA)

(12) Stachyose (Sigma-Aldrich, USA)

(13) D (-) Maltose (Merck, Germany)

(14) Treharose (Merck, Germany)

(15) Lactose (Sigma-Aldrich, USA)

(16) D (+) glucose (Merck, Germany)

(17) Myo-innositol (Merck, Germany)

(18) Mannose (Merck, Germany)

(19) D (+) galactose (Univar, Ajax Finechem, NSW, Australia)

(20) D (-) fructose (Sigma-Aldrich, USA)

(21) D (+) xylose (Sigma-Aldrich, USA)

(22) Mannitol (Merck, Germany)

(23) Sorbitol (Merck, Germany)

(24) L (-) Rhamnose (Merck, Germany)

(25) L (+) arabinose (Merck, Germany)

(26) D (-) arabinose (Sigma-Aldrich, USA)

3.1.4 Apparatus and Instruments

3.1.4.1 Centrifuge (Beckman Coulter™, Allegra™ X-22R, Germany)

3.1.4.2 Water bath (Memmert, Germany)

3.1.4.3 Membrane filter (Whatman, England)

3.1.4.4 Microscopy (Olympus, BX60, USA)

3.1.4.5 Densimat (bioMérieux, Marcy l'Etoile, France)

3.1.4.6 Micropipette (Biohit, France)

3.1.4.7 Microwave (Turbora, TRX-2021, Thailand)

3.1.4.8 Refrigerator (Hitachi, Thailand)

3.1.4.9 Freezer (Sanyo, Thailand)

3.1.4.10 Hot air oven (Memmert, Germany)

3.1.4.11 Biosafety Cabinet Class II (Telstar Industrial, Biostar plus4, Spain)

3.1.4.12 CO_2 incubator (Contherm, MITRE 4000 Series, Australia)

3.1.4.13 Vortex mixer (Vortex-Genie2, Scientific Industries, USA)

Industries,

G-560E)

3.1.4.14 Autoclave (Hirayama, HICLAVETM, AllegraTM X-22R Cen)

3.1.4.15 UV-Vis spectrophotometer microplate reader (Shimadsu, UV-160A)

3.1.4.16 UV-Vis spectrophotometer microplate reader (SPECTROstar Nano)

3.1.4.17 pH meter (Mettler Toledo, SevenMulti, Switzerland)

3.1.4.18 Ultra sonicator (Cavitator Metter Lectronics crop.,ME11)

3.1.4.19 Balance 2 digits (Mettler Toledo, PB1502-5, Switzerland)

3.1.4.20 Refrigerator microcentrifuge (Eppendorf Centrifuge 5415R, UK)

3.1.4.21 Refrigerator microcentrifuge (Refrigerated Microfuge Sigma 1-15k, UK)

3.1.4.22 Hot plate (Harmony, LMS Laboratory & Medical supplies, VA, USA)

3.1.4.23 Thermostatically controlled shaker (Bioblock Scientific Ping-Pong 74582)

3.1.4.24 HPLC-PDA system (Shimadzu, Japan)

3.1.4.25 HPLC-RID system (Shimadzu, Japan)

3.1.4.26 HPLC system (ICS, France)

3.1.4.27 Aminex HPX-87H column (Bio-Rad, Hercules, CA)

3.1.4.28 C-18 spherisorb column (Prontosil 120-3-C18, 25 cm× 0.4 cm)

3.1.4.29 Inertsil® ODS-3 C18 analytical column (4.6 x 250 mm inside diameter, 5µm)

3.1.4.30 Steriled petri dishes (HYCON, ligand)

3.1.4.31 STERIN 96 well plate steriled

3.1.4.32 Microcentrifuge tube 1.5 mL

3.1.4.33 Sample vial for HPLC

3.1.4.34 Test tube

3.1.4.35 Glass ware

3.1.4.36 Syringe holder

3.1.4.37 Nylon membrane filters, 0.22 (Whatman, Japan)

3.2 Methods

This study was divided into two parts; the first part was studied on health-promoting properties of probiotic *Lb. pentosus* strains and the other one was to examine the use of the probiotic *Lb. pentosus* strains which had good health-promoting properties as a starter culture for probiotic soya beverage production.

Part I Study on health-promoting properties of probiotic *Lb. pentosus* strains.

3.2.1 Growth profile, pH change, sugar utilisation and SCFAs production of probiotic *Lb. pentosus* strains.

3.2.1.1 Culture preparation

Eight strains of *Lb. pentosus* (DM068, JM0812, JM085, UM054, UM055, VM095, VM096, and YM122) were cultured in MRS medium (Citerion, USA) at 37 °C for 24 h, in 5% CO_2 incubator (Contherm, MITRE 4000 Series, Australia). All strains were weekly sub-cultured in MRS broth using 1 % inoculums followed by incubation at 37 °C for 24 h. They were stored at 4 °C before used. The stock cultures were monthly sub-cultured in MRS agar slant. Each strain was sub-cultured twice consequently in steriled MRS broth using 1 % inoculums for 24 h incubation at 37 °C prior to use.

3.2.1.2 FOS, raffinose, lactose (Sigma-Aldrich, USA) were used to replace glucose as a carbon-source in modified-MRS media, namely FOS-MRS medium, Raffinose-MRS medium, and Lactose-MRS medium respectively- (see Appendix A for details). FOS, raffinose, and lactose were added at the same concentration of glucose in modified-MRS media (2 % w/v used in basically formula), mixed together in 1000 mL distilled water, and gently boiled to dissolve the medium completely. One thousand mL of each medium was distributed into Duran bottle (size 250 mL) and sterilized by autoclaving at 121 ^{o}C for 15 min. Thereafter, it was cooled down to be ready for the inoculation with *Lb. pentosus* strains. MRS medium with 2 % (w/v) glucose was used as a control. Triplicate samples of each treatment were prepared.

3.2.1.3 Propagation of *Lb. pentosus* strains and preparation of inoculums Eight strains of *Lb. pentosus* grown in MRS slant agar about 18-24 h were prepared as cell suspensions in steriled normal saline solution (0.85% NaCl). Cell concentrations were adjusted to obtain the final concentration at 3×10^{8} CFU/mL by adjusting turbidity at 1.0 McFarland Units using Densimat (bioMérieux, Marcy l'Etoile, France- (see Appendix C for details) designed to measure the bacteria density and gives values in McFarland Units, proportional to the average value of bacterial concentrations (Itsaranuwat, 2003) (Table 3.1). After that, 2 mL of the cell suspension was transferred to 200 mL the modified-MRS media which prepared as mentioned above. The initial cell concentration in modified-MRS media was approximately 10^{6} CFU/mL. The samples were incubated at 37 °C for 24 h in 5% CO_2 incubator. All experiments were replicated three times.

Table 3.1 Correspondences between McFarland scale/Bacteria concentration/Optical density

Standard McFarland scale	Bacterial concentration X 10^8 CFU/mL	Optical density At 550 nm
0.5	1.5	0.125
1	3	0.25
2	6	0.5
3	9	0.75
4	12	1.00
5	15	1.25
6	18	1.50
7	21	1.75

Source: Itsaranuwat (2003)

3.2.1.4 Growth profile and acid production

The growth and changes in pH were monitored as indicators of growth pattern. The growth profiles of eight strains were measured as optical density (OD) at 600 nm every hour over incubation period by using UV-Vis spectrophotometer microplate reader (SPECTROstar Nano) (see Appendix C) with 96 well plate steriled microplate (STERIN96) at 37 °C of 24 h. Aliquots from each batch were taken at 0, 3, 6, 9, 12, 15, 18, 21, and 24 h to monitor pH value by using a pH meter (model 8417, Hanna Instruments, Singapore). The machine was calibrated before use with buffer solutions of pH 7.0 and 4.0. All experiments were replicated three times.

3.2.1.5 Sugar utilisation

3.2.1.5.1 Samples preparation

To determine sugar contents, 1 mL of each sample was taken at 0, 6, 12, and 24 h of incubation period into 1.5 mL microcentrifuge tube. Samples were centrifuged at 10,000 rpm for 10 min by using the refrigerated microcentrifuge (Refrigerated Microfuge Sigma 1-15k, UK). Supernatant was then filtered though a 0.22 µm pore size nylon membrane HPLC filter (Whatman, Japan) before injecting to HPLC. The experiment was repeated twice.

3.2.1.5.2 Determination of sugars

Sugar contents were determined by modified method described previously by Eyéghé-Bickong et al. (2012) and Liu et al. (2012). Briefly, HPLC analysis was performed on a Shimadzu Prominence System (Shimadzu, Japan) (see Appendix C) equipped with LC-20AD series pumping system and SIL-20A series auto injector system equipped with refractive index detector (RID); RID-10A Series (A waters 410 Milfold, MA) , which was used to simultaneously separate and analyze sugars at wavelength of 210 nm. Aminex HPX-87H (Bio-Rad, Hercules, CA) ion exchange column (300 mm × 7.78 mm) maintained at 85 ^{o}C and protected with a Bio-Rad micro-guard cartridge (30 mm × 4.6 mm). Deionized H_2O with 5 mM H_2SO_4 was used as mobile phases and maintained at a flow rate of 0.5 mL/min. The injection volumn was 20 µL and run time was 55 min.

3.2.1.5.3 Preparation of calibration standard curve

Standard sugar was obtained from Sigma Chemical Co (Sigma-Aldrich, USA). The quantification of sugar in samples was carried out using external calibration standard curve method. The calibration standard curves of 18 sugars were prepared at five concentrations ranging from 0.1 to 10.0 mg/mL (see Appendix D for details). The reference samples were injected repeatedly three times. The calibration curves were constructed by plotting the relative peak area versus sugar concentrations. The concentration of sugar in samples were derived from external standard calibration based on peak are and was expressed as mg/mL.

3.2.1.6 SCFAs production of probiotic *Lb. pentosus* strains

3.2.1.6.1 Samples preparation

The samples for SCFAs determination prepared as described in Section 3.2.1.5. The experiment was repeated in duplicate.

3.2.1.6.2 Determination of SCFAs

SCFAs determination was applied according to the previous method by Itsaranuwat (2008). The SCFAs analyses were carried out by using HPLC. Briefly, 20 µL of samples were injected directly into HPLC RID system (Shimadzu, Japan) LC-20AC Series pumping system; SIL-10AD Series auto injector system with Shimadzu SPD-M20A diode array detector. SCFAs in samples were separated using a Inertsil® ODS-3 C18 analytical column (4.6 x 250 mm inside diameter, 5µm) at 38 °C. The

target compounds were detected using a UV-diode array detector set at wavelength of 210 nm. The mobile phase consisted of 10 mM $NaHPO_4$ buffer (pH 2.5), filtered through 0.22 µm nylon membrane and degassed by sonication for 10 min before use. Flow rate was 1.0 mL/min and injection volumn 20 µL. The SCFA compounds in the samples were identified by comparing their relative retention times and were concentrations were calculated by using peak areas of an external standard.

3.2.1.6.3 Preparation of calibration standard curve

Standard SCFAs such as acetic, propionic, butyric, isobutyric, and *N*-valeric acid were obtained from Merck, Germany. The calibration standard curves were prepared with concentration levels ranging from 0.1 to 1.0 mmol/mL, except lactic acid was used with concentrations ranging from 0.1 to 10.0 mg/mL. SCFAs concentration was derived from external standard calibration and was expressed as mmol/mL.

3.2.3 Screening of strains for Bile Salt Hydrolase (BSH) activity

3.2.3.1 Preparation of strains

Stationary phase growth of *Lb. pentosus* 8 strains were investigated for BSH activity. The strains grew in MRS slant agar for 24 h and cell suspension was prepared in steriled normal saline solution (0.85 % NaCl), the cell concentration was adjusted to have turbidity of 3.0 McFarland Units.

3.2.3.2 BSH activity of *Lb. pentosus* strains

The BSH activity assay of the cultures was evaluated by modified method of Du Toit et al. (1998) and Mahrous (2011). Agar plate assay was developed to detect the BSH activity in lactobacilli. Briefly, MRS agar plates were prepared with 0.5 % (w/v) sodium salt of taurodeoxycholic acid (TDCA; Sigma-Aldrich, USA) as a bile acid and 0.37 g/L of $CaCl_2$ ((Merck, Germany). After sterilization, the plates were poured into steriled plastic petri dishes. Once solidified, the plates were inverted and placed in the anaerobic condition for at least 72 h before use. Steriled filter discs (diameter 0.7 cm) were placed on MRS agar plates and were spotted with a 10-µL of the cell suspension of each tested strain which prepared as mentioned above. All plates were incubated anaerobically at 37 °C for 72 h in 5 % CO_2 incubator. After which, the precipitation zone was observed as an indicator for the BSH activity of the tested strain and diameters of the precipitation zones surrounding filter discs were measured. The

MRS agar plates without supplementation were used as negative controls. Each strain was performed in triplicates.

3.2.4 *In vitro* cholesterol binding activity.

In vitro cholesterol binding activity was performed according to the previous method by Mahrous (2011) and Mirlohi et al. (2012) with slight modifications. Eight strains of *Lb. pentosus* were investigated for cholesterol binding activity. Standard cholesterol (Water soluble cholesterol; polyoxyethanyl-cholesteryl sebacate (PCS), Sigma-Aldrich, USA) was prepared at the concentration of 10 mg/mL in steriled water and filtered with 0.22 µm sterilized membrane. For each culture to be tested, 70 µL of cholesterol solution was added to 10 mL of freshly prepared MRS broth (final cholesterol concentration of 70 µg/mL) supplemented with 0.3 % oxgall (w/v) (Sigma-Aldrich, USA) as a source of bile salt. To the MRS broth, 1 % (v/v) of freshly grown culture of each strain was inoculated and incubated anaerobically at 37 °C for 24 h. An un-inoculated MRS broth at the same condition was used as a negative control. After 24 h of growth, the cells were removed by refrigerator microcentrifuge (Refrigerated Microfuge Sigma 1-15k, UK) at 10,000 g for 10 min at 4 °C. Spent broth was then filtated by using 0.45 mm membrane sterilized. The cholesterol levels were determined in the supernatant using the method of Rudel and Morris (1973) and Liong and Shah (2005). Briefly, 1 mL of the supernatant with 1 mL of 33 % (w/v) KOH and 2 mL of absolute ethanol were placed in a capped test tube, vortexed for 1 min and incubated for 15 min at 37 °C. After incubation, the mixture was removed and cooled under tap water, and then 3 mL of hexane and 3 mL of distilled water were added and mixed for 15 min. One milliliter of the hexane layer was transferred into a dry clean test tube and evaporated under nitrogen gas. The dried material was dissolved in 2 mL of freshly o-phthaladehyde reagent (0.5 mg/mL of glacial acetic acid) (Sigma-Aldrich, USA). After that, 0.5 mL of 12 N H_2SO_4 was added and the mixture was mixed for 1 min. After 10 min, absorbance was measured at 550 nm with a UV-Vis Spectrophotometer microplate reader (Shimadsu, UV-160A). The experiment was repeated in three different runs. The activity of cholesterol binding was calculated as a percentage by the treatment compared with the control from the following equation:

$$\% \text{ cholesterol binding} = [1-(OD_A / OD_B)] \times 100$$

When; OD_A = OD of sample was inoculated the cells

OD_B = OD of un-inoculated MRS broth

3.2.5 ZEA binding ability of *Lb. pentosus* strains in buffer solution

3.2.5.1 Chemical and Reagents

All the organic solvents used in this study were HPLC grade (Fisher scientific, France), MRS media (Criterion, USA), and Standard ZEA was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2.5.2 Preparation of the bacteria cells for toxin binding

Eight probiotic strains were cultivated by steak plate technique in MRS agar media and incubated at 37 °C for 24 h in 5 % CO_2 incubator. Cells were suspended in 0.85% normal saline solution and transferred to microcentrifuge tube (1.5 mL safe-lock). The cells were harvested by centrifugation at 10,000 rpm, 4 °C for 10 min (Eppendorf Centrifuge 5415R, UK). The cell pellets were washed twice with steriled water and suspended in 2 mL of 10% skim milk and the cells suspension was transferred into sterilized serum vials, freeze-dried (Heto power dry PL3000) and stored at -20°C for the next experiment.

3.2.5.3 Preparation of standard solutions ZEA

Standard stock solution of ZEA: stock solution was prepared at 0.1 mg/mL in methanol. The concentration of the ZEA stock solution was determined by measuring the UV absorbance at 236 nm and calculated by using the molar extinction coefficient of 29700 mol^{-1} cm^{-1} (IARC, 1993).

Standard working solutions of ZEA: initial standard solution (0.1 mg/mL) was diluted by 0.05 M sodium acetate buffer (pH 5.0) into 1, 5, 20, 50 and 75 µg/mL and kept at -20 °C for using in the experiment.

0.05 M sodium acetate buffer solution (pH 5.0) was prepared by dissolving 6.5 g of sodium acetate ($C_2H_3NaO_2$-$3H_2O$) in 450 mL of ultrapure water, the pH was adjusted to 5 by adding acetic acid, and then the final volumn was adjusted to 500 mL.

3.2.5.4 Binding step

The experiments were prepared according to the protocol of Joannis-Cassan et al. (2011). All assays were performed in centrifuge tube (1.5-mL, safe-lock). Each tube contained 5 mg of freeze-dried LAB cells and 990 µL of 0.05 M sodium acetate buffer (pH 5.0) was added into the tube. After that, the samples were vortexed for 20 s and rotated at 175 rpm on a thermostatically controlled shaker (Ping-Pong 74582, Fisher Bioblock Scientific, IIIkirch, France) (see Appendix C) at 37 °C for 5 min. Then, 10 µL of ZEA working standard solution (1, 5, 20, 50 and 75 µg/mL) was added to each tube. The final incubation volumn was 1 mL. The suspensions were mixed and incubated on a thermostatically controlled shaker at 37 °C for 15 min at 175 rpm. Subsequently, the suspensions were terminated by centrifugation (Refrigerated Microfuge SIGMA 1-15k, UK) at 9,200 × *g*, at 20 °C for 10 min (Hadjeba-Medjdoub et al, 2009). A control treatment without addition of working standard ZEA solution was included. All experiments were carried out in duplicates.

3.2.5.5 HPLC analysis to determine ZEA binding by *Lb. pentosus* strains

HPLC method was performed according to the protocol of Dall'Asta et al. (2004). HPLC-system (ICS, Bruges, France) consisted with a 20 µL injector loop, a high pressure pump (L-6200A) and an auto sampler (AS-2000A). A C-18 spherisorb column (Prontosil 120-3-C18, 25 cm × 0.4 cm), and a fluorescence detector (Shimadzu Fluorescence Detector RF-10AXK, Japan) was run in a temperature controlled room (25 °C). The mobile phases were gradient condition as described by Faucet-Marquis et al. (2006). Briefly, using the following gradient: solvent A; MeOH/Acetonitile (ACN)/6.5 mM ammonium formate (200/200/600) adjusted to pH 3.5 with formic acid. Solvent B; MeOH/ACN/6.5 mM ammonium formate (350/350/300) adjusted to pH 3.5 with formic acid. Program: T_0 100 % A; T_{25} 30 % A; T_{30} 30 % A; T_{45} 0 % A; T_{55} 0 % A; T_{58} 100 % A at a flow rate of 0.5 mL/min. The spectrofluorimetric detection was 275 nm for ZEA excitation and 450 nm for emission. The calibration curve was obtained by analyzing the peak area of ZEA standard solutions of concentrations in the range between 0.1 to 10 µg/mL. All experiments were carried out in duplicate, and the values are given as mean ± standard deviation.

3.2.6 Adhesion capacity

To study the adhesion capacity of *Lb. pentosus* strains to human intestine cells, *In vitro* cell surface hydrophobicity was tested in all bacteria by using bacterial adherence to hydrocarbon assay modified from the methods of Schillinger et al. (2005) and Klayraung et al. (2008). Briefly, 8 strains of *Lb. pentosus* were grown in MRS broth at 37 °C under anaerobic conditions (5 % CO_2 incubator) about 18-24 h. Cells were harvested by centrifugation at 9,000 rpm, 4 ^{o}C for 10 min, washed twice and resuspended in 0.85 % NaCl. Cell concentrations were adjusted to reach the turbidity at 4.0 McFarland Units (turbidity 1.0 at; OD550) as estimated by Densimat (bioMérieux, Marcy l'Etoile, France). The cell suspension was measured for OD at 600 nm (A_0) with UV-Vis spectrophotometer microplate reader (SPECTROstar Nano). A portion of 0.6 mL of n-hexadecane; Cetane (Sigma-Aldrich, USA) was added to 3 mL of bacterial suspension into a clean test tube with screw cap. The mixture was blended using a vortex mixer at 120 rounds per sec for 10 min. The aqueous and organic phases were allowed to separate at room temperature for 30 min. The aqueous phase in lower layer was carefully removed and OD of the aqueous phase (A_{30}) was measured. Hydrophobicity was calculated from three replicates as OD of the initial cells suspension decreasing due to cells partitioning into a hydrocarbon layer. Therefore, high percentage of cell surface hydrophobicity (% H) shows the strain has adhesion ability and the % H was calculated as follow;

$$\% H = [1-(A_{30} / A_0)] \times 100$$

When; A_0 = OD of cells suspension at 0 min

A_{30} = OD of the aqueous phase at 30 min

Part II Use of probiotic *Lb. pentosus* as starter culture for probiotic soya beverage production.

The selection of three *Lb. pentosus* VM095, VM096, and YM122 strains with health-promoting properties; such as utilisation of sugar as carbon-source in modified MRS media, SCFAs production, cell surface hydrophobicity, cholesterol

reduction and ZEA binding (from Part I) were prepared as starter culture for soybean milk fermentation in this study.

3.2.7 Soya milk fermentation

3.2.7.1 Preparation of soya milk

The soya milk preparation was modified from Carrão-Panizzi et al. (1999) and Sirilun (2012). Dehull soybean seeds (Rai Tip brand) purchased from Big C supermarket, Mahasarakham, Thailand, were washed twice and soaked in distilled water with a weight ratio of 1: 6 w/v for seeds and water, at room temperature overnight. After soaking, water was discarded and the soybean seeds were placed directly into boiling water containing 0.25% $NaHCO_3$, for 3 min. The water was discarded again and the soybean seeds were washed with tap water until final at pH of 6.8-7.2 was reached. The soybean seeds were ground in electric juice blender for 3 min in hot distilled water at 80 ℃ using a weight ratio of 1:5 w/v for seeds and water. The blended solution of soybeans was filtered through double-layered cheese cloth and was then passed through the sieve 100 mesh. A filtrated solution was recognized as soya milk. The soya milk was boiled for 3 min and the volumn 50 mL of soya milk was then filled into Duran bottles size 150 mL (for inoculums preparation) and 200 mL filled into Duran bottles size 500 mL (for soya milk fermentation), and sterilized by autoclaving at 121ºC for 2 min (Itsaranuwat, 2003). The bottles were stored at 4 ºC before use.

3.2.7.2 Preparation of inoculum and fermentation

The strain cultures *Lb. pentosus* VM095, VM096, and YM122 were grown on MRS agar plate about 18-24 h. After that, one loop of cells of each strain was picked-off a plate and added to each bottle of 50 mL soybean milk prepared as mentioned above. The inoculated milks were incubated at 37ºC, 5% CO_2 incubator for 18 h. After first time inoculation, each culture was sub-cultured (5% v/v) into freshly 50 mL of soya milk twice. The activated *Lb. pentosus* strains after two successive transfers were used for the production of cultures for soya milk fermentation. The cultures were prepared by inoculating 5% (v/v) in 200 mL soybean milk and then incubated at 37ºC, 5% CO_2 incubator for 24 h. All fermentations were performed in triplicates. A control consisted of un-inoculated soya milk.

3.2.7.3 Determination of pH value and titratable acidity

Samples at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h of

fermentation period were taken and determined for the pH value and titratable acidity. Changes in pH were recorded using the pH meter (Mettler Toledo, SevenMulti, Switzerland). The acid titration was determined according to the method adapted from Villaluenga and Gómez (2007). Briefly, 1 mL of the soybean milk was transferred in 10 mL of distilled water and titrated with 0.1 M NaOH using phenolphthalein as an indicator. The results were calculated as percentage of equivalent lactic acid (%LA) of fermented soya milk. All data were performed in triplicates and the values were averaged.

$$\%LA = (V_1(mL) \times 0.1(M) \times 90\ (g/mol)\ /V_2\ (mL))/100$$

When; V_1 = used volumn of 0.1 N NaOH for titration

V_2 = volumn of sample

Where the molecular weight of lactic acid is given as 90 g/ mol.

3.2.7.4 Enumeration of *Lb. pentosus* strains

Samples from each batch were taken at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h to monitor cell growth. Enumerations of bacteria were determined by the pour plate technique with appropriate dilutions on selective MRS agar. The plates were anaerobically incubated at 37 °C for 48 h, after which all visible colonies were counted. Results were expressed as the number of log10 CFU/mL of sample. The experiment was done in triplicates.

3.2.7.5 Determination of sugar contents

The fermented soya milk from each batch was taken at 0, 6, 12, and 24 h fermentation period to determine the sugar contents. one mL of each sample in 1.5 mL microcentrifuge tube was centrifuged at 10,000 rpm for 10 min by using the refrigerator microcentrifuge and then filtered though a 0.22 μm pore size nylon membrane HPLC filter (Whatman, Japan). The experiment was repeated twice. The sugar determination was performed as described previously in the section 3.2.1.5.

3.2.7.6 Determination of SCFAs

The samples were prepared according to the procedure in the section 3.2.7.5. The SCFAs determination was carried out described previously in the section 3.2.1.6.

3.2.8 Preparation of soya beverage

Soya beverage were prepared from fermented soya milk as mentioned above in the section 3.2.6.1 and 3.2.7.2 by probiotic strains *Lb. pentosus* (VM095, VM096, YM122). Each batch, 300 mL of soya milk in Duran bottle (500 mL) was inoculated with a single strain 5 % (v/v) and incubated at the same condition as in the section 3.2.7.2. After incubation, each batch was transferred to steriled electric juice blender and 10% honey syrup (100% pure, Suan Jit-radda brand) was added. This was purchased at 7-eleven super store. The soya beverage was blended in electric juice blender for 5 min and was transferred into the sterilized Duran bottle (500 mL), kept at 4 ^{o}C in the refrigerator until sensory test. The sensory test results were compared between 3 batches of the soya beverage (SB095H, SB096H, and SB122H) with 3 batches of the fermented soya milk without honey syrup (SF095, SF096, and SF122) as controls. In all batches, 100 mL of culture in Duran bottle (250 mL) were studied for survival of probiotic strains during storage at 4 ^{o}C for 4 weeks.

3.2.8.1 Sensory evaluation

The sensory properties of probiotic soya beverage already prepared from the section 3.2.8 were evaluated by forty untrained panelists recruited from students of Department of Biotechnology, Mahasarakham University. The samples initially stored at 4 ^{o}C (refrigerator temperature) were removed from refrigeration approximately 1 h prior to evaluation and shaken well to distribute any sediments. Approximately 10 mL of each sample was served into 20 mL plastic cups and were coded with three-digit random numbers from a Random Table (Ott, 1988). Water was available for panelists to rinse their pallet between samples. The test comprising six sensory attributes namely appearance, color, odor, taste, mouth feel, and overall acceptance was given to each panelist. The final sensory evaluation was done by nine-point Hedonic scale to specify their preferences on the samples tested. The scale of satisfaction were as follows: like extremely = 9, like very much = 8, like moderately = 7, like slightly = 6, neither like nor dislike = 5, dislike slightly = 4, dislike moderately = 3, dislike very much = 2, dislike extremely = 1 (Lawless and Heymann, 1999 cited in Itsaranuwat, 2003)-(see Appendix B). The scores were analyzed statistically using Duncan's test for multi comparison of the means.

3.2.8.2 Survival of probiotic strains during storage periods

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Growth profile, pH changes, sugar utilisation and SCFA production by probiotic *Lb. pentosus* strains

A variety of probiotic bacterial strains have ability to use different kinds of various sugars for growth. Evidence suggests that some prebiotic compounds are capable of promoting the growth of probiotics in the colon, since they can pass the upper intestinal region without being hydrolysed (Kneifel et al., 2000). This study investigated on the growth of probiotic *Lb. pentosus* in modified-MRS media, supplemented various sugars for compared with positive control glucose as carbon source and FOS with validated prebiotic activity. Moreover, sugar utilisation and SCFAs production were investgated.

4.1.1 Growth profiles and pH changes

he growth profiles and pH changes of cultured by *Lb. pentosus* namely DM068, JM0812, JM085, UM054, UM055, VM095, VM096, and YM122 were observed during the incubation period in modified-MRS media. The cultures were incubated under anaerobic conditions at 37 ºC and 24 h for all samples. After incubation, the growth intensities of the strains were examined based on optical density (OD) measurements using a spectrophotometer microplate reader (SPECTROstar Nano) at 600 nm (see Appendix C). The results of these analyses are illustrated in Figure 4.1-4.5 as the means ± SD of the triplicate OD and pH measurements for all strains.

The OD of glucose-MRS medium cultured by 8 *Lb. pentosus* strains was presented in Figure 4.1A. The growth patterns of all strains were not significant different ($p > 0.05$) throughout 24 h. The results showed that all strains were quite similar growth curves. The slopes of OD versus incubation time for all of test strains appeared to be similar. The lag phase shortly lasted for 3 h followed by the logarithmic phase of growth from 3-9 h, and the maximum growth was achieved between 9 and 16 h by *Lb. pentosus* UM054, DM068, and UM055 strains at OD 2.62 ± 0.05, 2.59 ± 0.05, and 2.59 ± 0.04 respectively. It appeared that *Lb. pentosus* strains entered the stationary

phase after 16 h until the end of incubation (24 h). Finally, the OD at 24 h found in range between 2.48 ± 0.03 to 2.53 ± 0.00.

In addition, pH values were shown in Figure 4.1B. Similar to the case of the growth curves, the pH profiles of all strains were not significant different ($p > 0.05$). Changes of pH are related to baterial growth behavior, the pH decline at the fastest rate from 6.46 ± 0.00 to 4.45 ± 0.03 from 3 to 12 h and gradually declined to 3.76 ± 0.01 at the end of incubation. This similarity indicated that glucose has no effect on growth behavior of the tested strains. The bacteria can utilise glucose easily as it is monosaccharide. The decrease in pH over time results from the breakdown of glucose to form lactic acid.

Slizewska and Libudzisz (2001 cited in Goderska et al., 2008) demonstrated that glucose is the best source of carbon for *Lb. acidophilus* strain and Goderska K. et al (2008) proved that among the examined carbon sources, *Lb. acidophilus* strain utilized glucose and saccharose best.

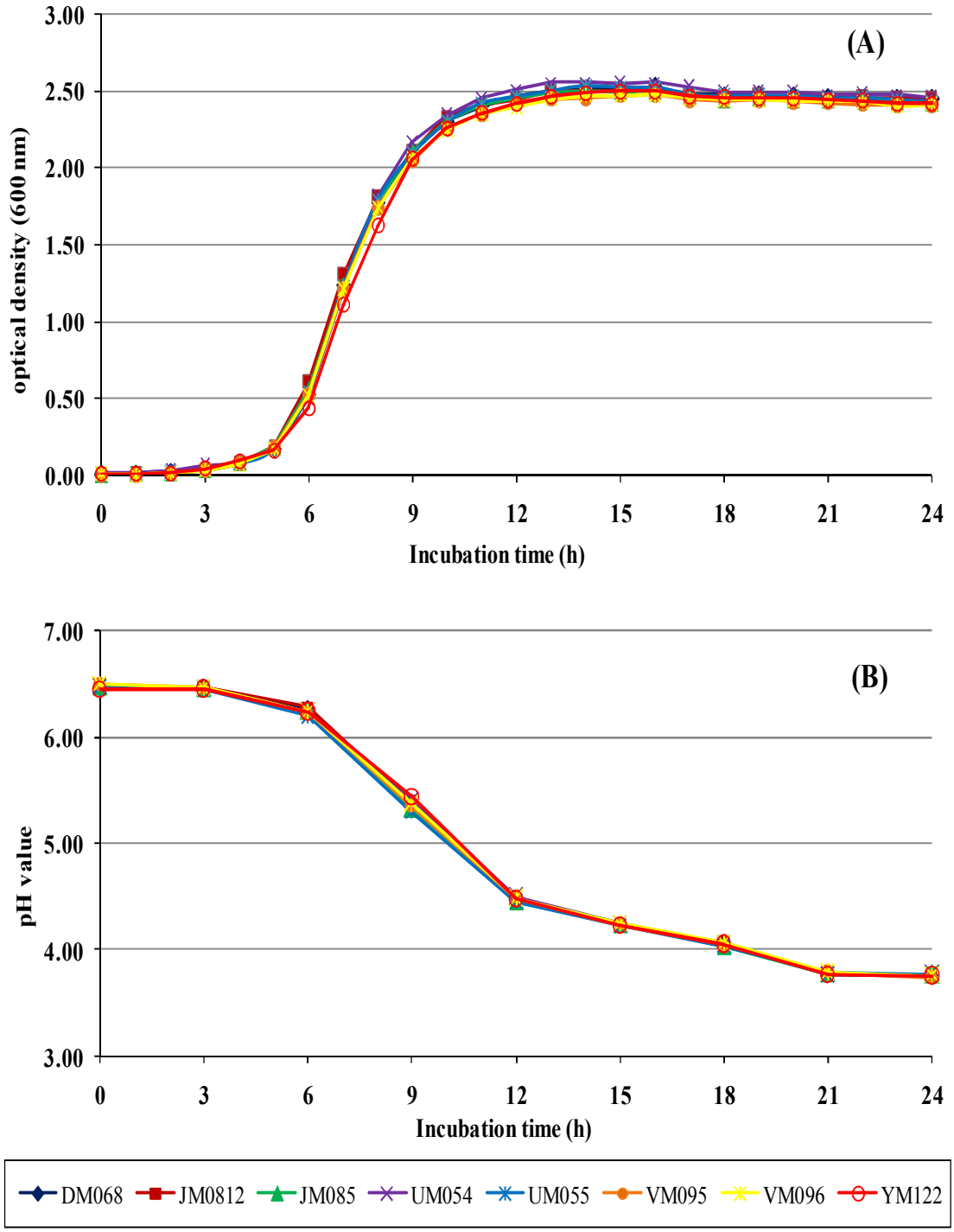

Figure 4.1 Growth profiles (A) and the pH changes (B) in glucose-MRS medium by 8 *Lb. pentosus* strains (◆DM068, ■JM0812, ▲JM085,×UM054,∗UM055, ●VM095, ∗VM096, ○YM122). The results showed mean measurements from triplicate experiments (n = 3). Incubation at 37 ^{o}C for 24 h was performed.

The OD of lactose-MRS media cultured by 8 *Lb. pentosus* strains was showed in Figure 4.2A. We observed that 6 of 8 strains (JM0812, UM054, UM055, VM095, VM096 and YM122) were quite similar growth curves, increased growing significant different ($p < 0.05$). Growth curves of 6 strains above tended to be higher than *Lb. pentosus* DM068 and JM085 strains from 7 - 24 h incubation. It appeared that the lag phase shortly lasted for 3 h followed by logarithmic phase showed a sharp increase between 3-9 h by 6 strains, whereas *Lb. pentosus* DM068 and JM085 strains showed a slightly went up by extend the logarithmic phase and delaying its entrance into the stationary phase. The maximum growth was achieved at 13 h by *Lb. pentosus* VM096, YM122, and JM0182 strains at OD 2.83 ± 0.08, 2.83 ± 0.04, and 2.80 ± 0.07 respectively. It appeared that *Lb. pentosus* strains entered the stationary phase after 16 h until the end of incubation (24 h). Finally, the OD at 24 h was found in range between 1.86 ± 0.16 to 2.57 ± 0.02.

In a similar way, the decrease of pH values (Figure 4.2B) was related to growth curve. The changes pH was significant different ($p < 0.05$) amomg strains from 6 to 24 h incubation. At 24 h incubation, the maximium pH values was achieved at between 3.61±0.01 to 3.63±0.01 by *Lb. pentosus* VM095, VM096, YM122, UM055, and UM054 respectively whereas a slightly changes pH was found at 4.93±0.01 by *Lb. pentosus* DM068 and JM085.

The results indicated that lactose-MRS medium had the differential effect on the growth behavior of the strains by achieved the maximium OD higher compared to glucose-MRS medium. Lactose is a disaccharide or a milk oligosaccharide, a type of nature sugar found in milk and hydrolysed by LAB into galactose and glucose (Venema, 2012) and helps to growth promoting of probiotic bacteria. The decrease in pH over time results from the increasing breakdown of lactose to accumulate lactic acid (Olson and Aryana, 2012). In addition, the experiment results showed that 6 of 8 strains can growth well and has the lower pH values by utilize lactose.

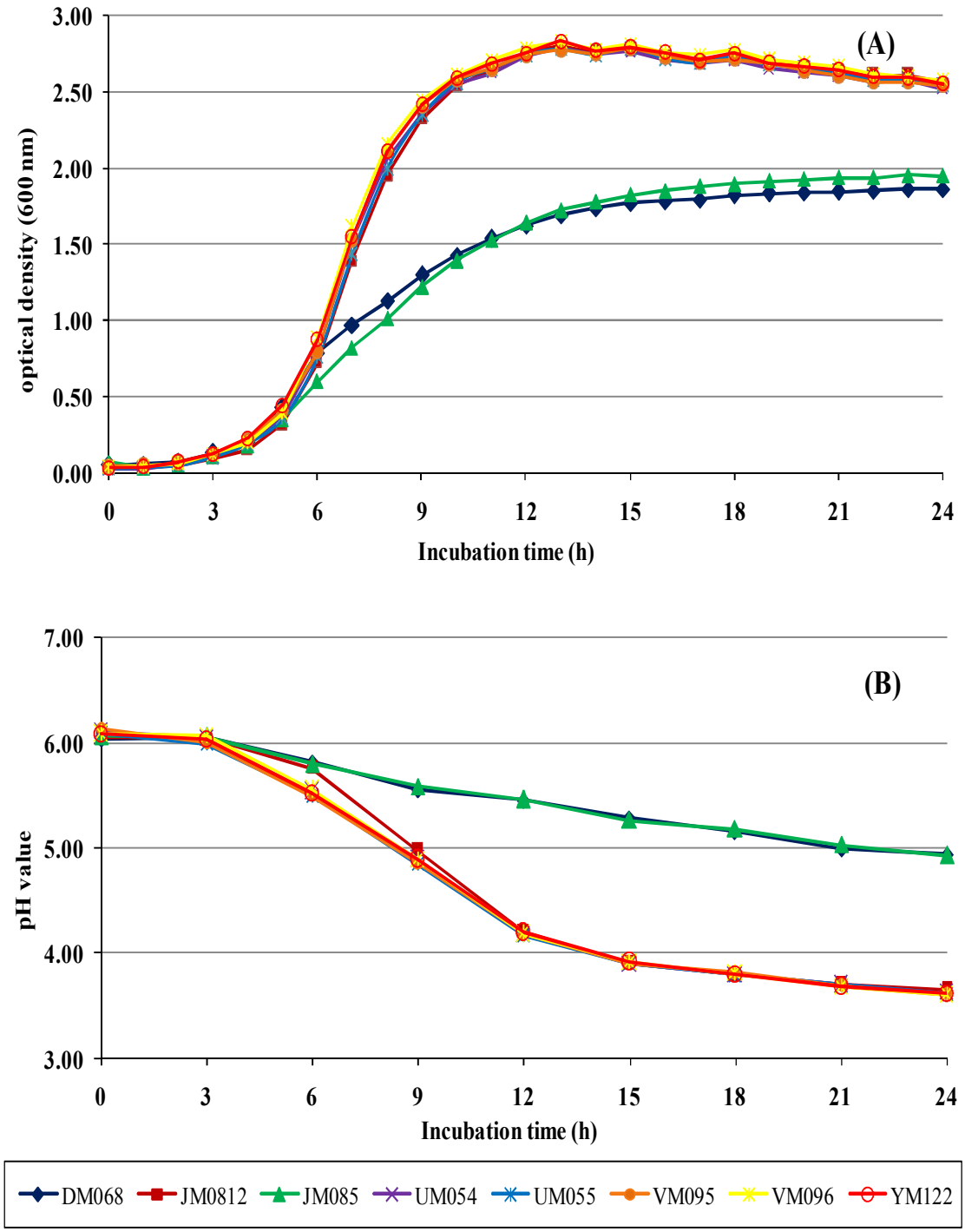

Figure 4.2 Growth profiles (A) and the pH changed (B) in lactose-MRS medium by 8 *Lb. pentosus* strains (◆DM068, ■JM0812, ▲JM085, ×UM054,✳UM055, ●VM095, ✳VM096, ○YM122). The results showed mean measurements from triplicate experiments (n = 3). Incubation at 37 ^{o}C for 24 h was performed.

As shown in Figure 4.3A, eight LAB *Lb. pentosus* strains were compared for their growth in raffinose-MRS medium. The OD was significant different ($p < 0.05$) among strains during 24 h incubation. It was found that 5 strains (YM122, VM096, UM055, UM054 and VM095) showed higher growth rate in raffinose-MRS medium than 3 strains (DM068, JM0812 and JM085) whereas those of 3 strains grown quite well in glucose-MRS medium. It seen that the lag phase shortly lasted for 3 h followed by logarithmic phase showed a sharp increase between 3 h to9 h by 5 strains, then entered the stationary phase after 15 h until 24 h incubation. The maximum growth was achieved between 9 h and 13 h by *Lb. pentosus* YM122, VM096, and UM055 strains at OD 2.93 ± 0.04, 2.93 ± 0.05, and 2.86 ± 0.11 respectively followed by VM095 and UM054. It appeared that *Lb. pentosus* strains entered the stationary phase after 16 h until the end of incubation (24 h). Finally, the OD at 24 h was found in range between 1.99 ± 0.03 to 2.58 ± 0.01. After 13 h, the curve showed decrease in maximum OD levels, indicating that raffinose may be more rapidly exhausted.

Similar the growth pattern, the pH values were significant different ($p < 0.05$) among the tested strains during 24 h incubation (Figure 4.3B). The pH rapidly declined at the 3 to 12 h from 6.20 ± 0.01 to 4.25 ± 0.01 due to LAB substrate consumption for growing and producing of lactic acid at the same time with high growth rate. At the end of incubation 24 h, pH value arranged by those 5 strains as above was approximately 3.70.

Above results demonstrated that raffinose-MRS medium had effect to promote on the growth behavior of some strains in this study by achieved the maximium OD higher compared to glucose-MRS medium. The YM122, VM096, UM055, VM095 and UM054 can growth in raffinose-MRS medium better than DM068, JM0812 and JM085 strains. Raffinose is a oligosaccharide in soybean, a trisaccharide composed of galactose, glucose, and fructose. However, it is the one of main oligosaccharide in soybean that is not digestable by human body but can be hydrolyzed by the enzyme alpha-galactosidase from LAB (Wang et al, 2003).

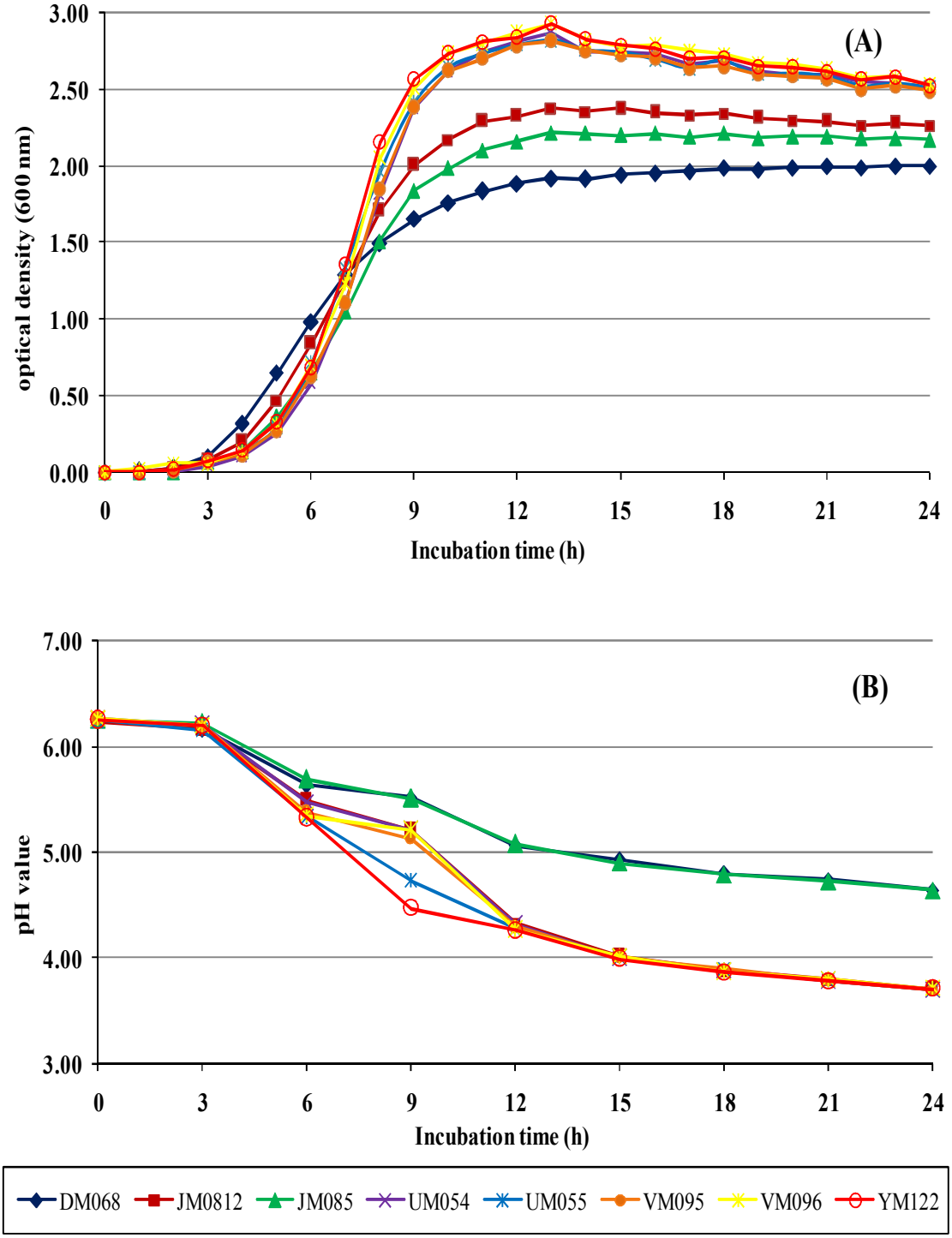

Figure 4.3 Growth profiles (A) and the pH changed (B) in raffinose-MRS medium by *Lb. pentosus* 8 strains (◆DM068, ■JM0812, ▲JM085, ×UM054,✳UM055, ●VM095, ✳VM096, ○YM122). The results showed mean measurements from triplicate experiments (n = 3). Incubation at 37 ^{o}C for 24 h was performed.

As shown in Figure 4.4A-B, the data of growth profiles and changes pH of *Lb. pentosus* 8 strains were observed in FOS-MRS medium. It was found that 6 of 8 strains used in this study including *Lb. pentosus* JM0182, UM054, UM055, VM095, VM096 and YM122 grew very well whereas the strains DM068 and JM085 were slightly growing in FOS-MRS medium. Six strains above increased growing significant different ($p < 0.05$) compared with DM068 and JM085 after 6 h of incubation period. The lag phase is shortly lasted for 3 h and the logarithmic phase showed a sharp increase between 3 to 9 h incubation. The exponential phase contained after 9 h. The maximum cells density was achieved at 13 h by *Lb. pentosus* UM054, JM0812, and UM055 strains at OD 2.95 ± 0.03, 2.94 ± 0.02, and 2.93 ± 0.02 respectively followed by VM096 and VM095. At the end 24 h, the OD was found in range between 1.98 to 2.61.

Similar the growth pattern, the pH values were significant different ($p<0.05$) among the test strains at 6 h until end of incubation at 24 h. The pH rapidly declined at the 3 to 12 h from 6.22 ± 0.05 to 4.20 ± 0.02 by 6 strains as above. At the end of incubation 24 h, the pH declined to 3.66 ± 0.01. FOS in MRS media has the effect on the growth behavior of the some test strains in this study. The JM0812, UM054, VM096, UM055, VM095, YM122 can grow in FOS-MRS medium better than DM068 and JM085 strains.

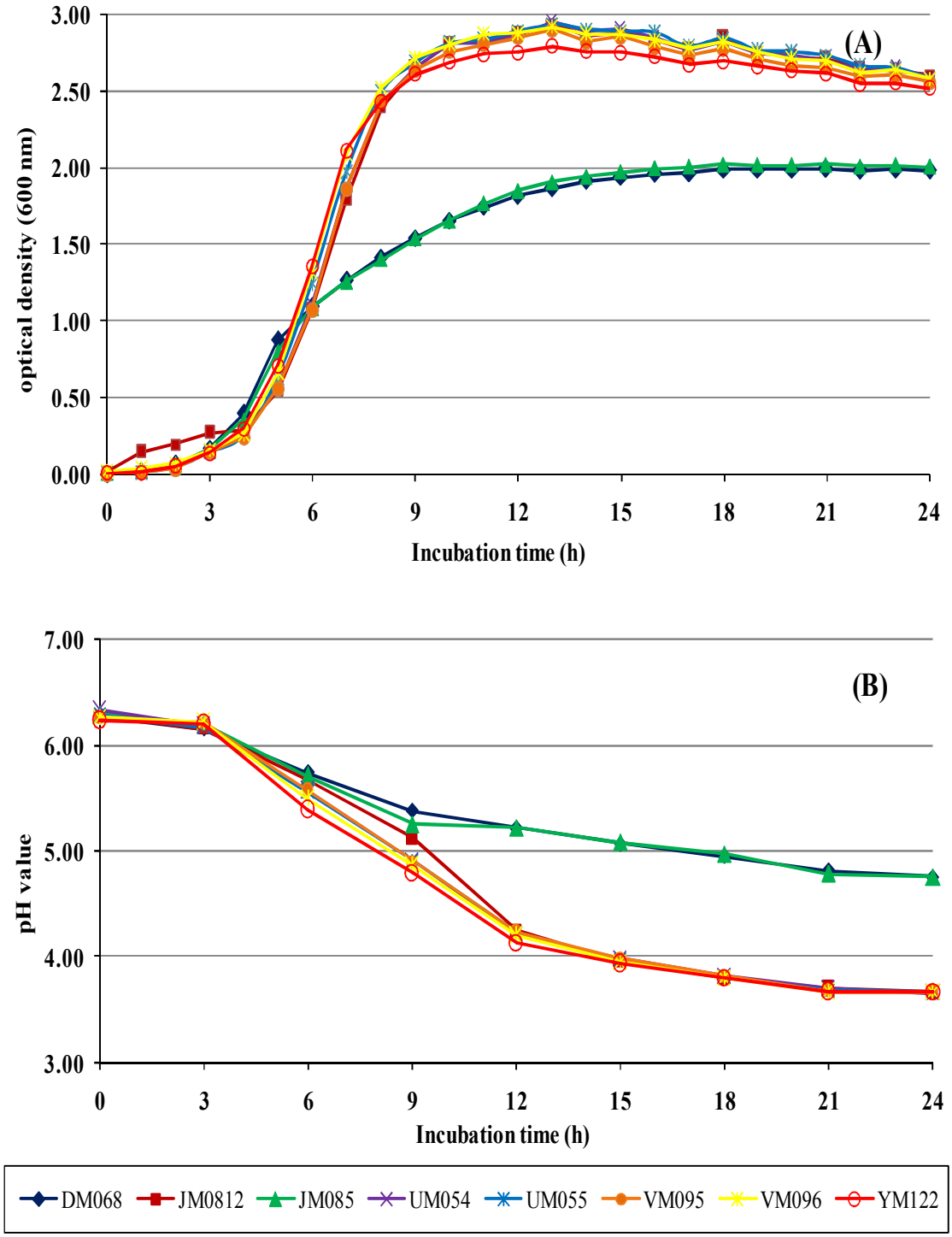

Figure 4.4 Growth profiles (A) and the pH changed (B) in FOS-MRS medium by *Lb. pentosus* 8 strains (◆DM068, ■JM0812, ▲JM085, ×UM054,∗UM055, ●VM095, ✳VM096, ○YM122). The results showed mean measurements from triplicate experiments (n = 3). Incubation at 37 ^{o}C for 24 h was performed.

The growth curves obtained for 8 *Lb. pentosus* strain in different sugars; lactose, raffinose were summaried in Figure 4.5. The experimental medium was compared with glucose as a control in the MRS media and FOS with validated prebiotic activity and no negative control-MRS broth without any carbon source in this study. The results found that the maximum growth differed between sugars tested as a direct carbon source.

The growth rates of *Lb. pentosus* DM068 and JM085 were achieved maximum OD in glucose higher than raffinose, FOS, and lactose significant different ($p < 0.05$). However, the pH values of glucose-MRS declined faster than those of sugars. Glucose was found to be the best carbon source for DM068 and JM085 strains. The maximum growth found at 24 h incubation. Glucose, raffinose, FOS, and lactose reached OD levels of 2.52, 2.00, 1.98, and 1.86, respectivly by DM068 (significant different) whereas, pH values was achieved 3.76, 4.64, 4.76, and 4.93 respectively. Similary, in case of the JM085 strain, the maximum growth found at 15 h, reached OD levels of 2.56, 2.20, 1.97, and 1.82, respectively for gluclose, raffinose, FOS, and lactose-MRS medium. The pH values were 4.24, 4.90, 5.08, and 5.26 respectively. The ending OD and pH values ranges between 1.95-2.49 and 4.93- 3.77.

In better case of JM0812 culture, FOS and lactose were found to be the best carbon source than glucose, whereas raffinose promoted growth lower than glucose by this strain. The maximum growth at 15 h for FOS, lactose, glucose and raffinose-MRS reached OD levels of 2.88, 2.77, 2.57, and 2.37 (significant different) whereas, pH value was achieved 3.95-4.25. On the other hand, the UM054, UM055, VM095, VM096 and YM122 strains had ability to growth in lactose, raffinose and FOS-MRS medium better than glucose. The growth patterns of all 5 strains were quite similarly. Lactose, raffinose and FOS enhanced the growth intensities of 5 strains whereas DM068 and JM085 were not growing well. The maximum growth at 12-15 h found in oligosaccharide sugars, reached approximately 2.72-2.91 whereas OD in glucose reached 2.54-2.62 (significant different). In addition, pH values at 24 h were achived 3.60-3.70.

However, Sánzhes-Zapata et al. (2013) was reported that a very limited growth was observed in the negative control medium (MRS without any carbon source). Glucose was found to be the best substrate to *Lb. acidophilus* grows, followed by FOS and Tiger nut milk (TNLC). Su et al. (2007) reported that glucose was better than FOS in supporting the growth of *Lb. acidophilus*L10. Main sugars in TNLC include sucrose,

fructose, and glucose (in similar amounts) and small amounts of raffinose and glycerol (Sánzhes-Zapata et al., 2013). On the other hand, Crittenden et al. (2001) demonstrated that *Bifidobacterium* grew better with FOS, galactooligosaccharides and xylooligosaccharides than on monosaccharides (as glucose). However, our results showed lactose, raffinose, and FOS-MRS medium enhanced the growth intensities most of tested strains than glucose during the exponential phase of growth. As a result, the effectiveness of a prebiotic depends, therefore on its ability to be selectively fermented by and to support growth of specific targeted organisms (Huebner et al., 2007).

Sugars such as oligosaccharide can be used as a carbon source to promote the growth of *Lb. pentosus* strains. Most of tested strains can be growing in oligosaccharide, they exhibit a faster growth than when grown in monosaccharide (as glucose). These results are also supported by the lower pH and by the faster organic acid production, confirming the relevant property of oligosaccharide as a carbon source for probiotic bacteria growth. These results indicate that soya milk can have a potential to be used for probiotic soya beverages as a fermentable product, but more studies are required to establish the doses, stability and its compatibility with other probiotic microorganisms.

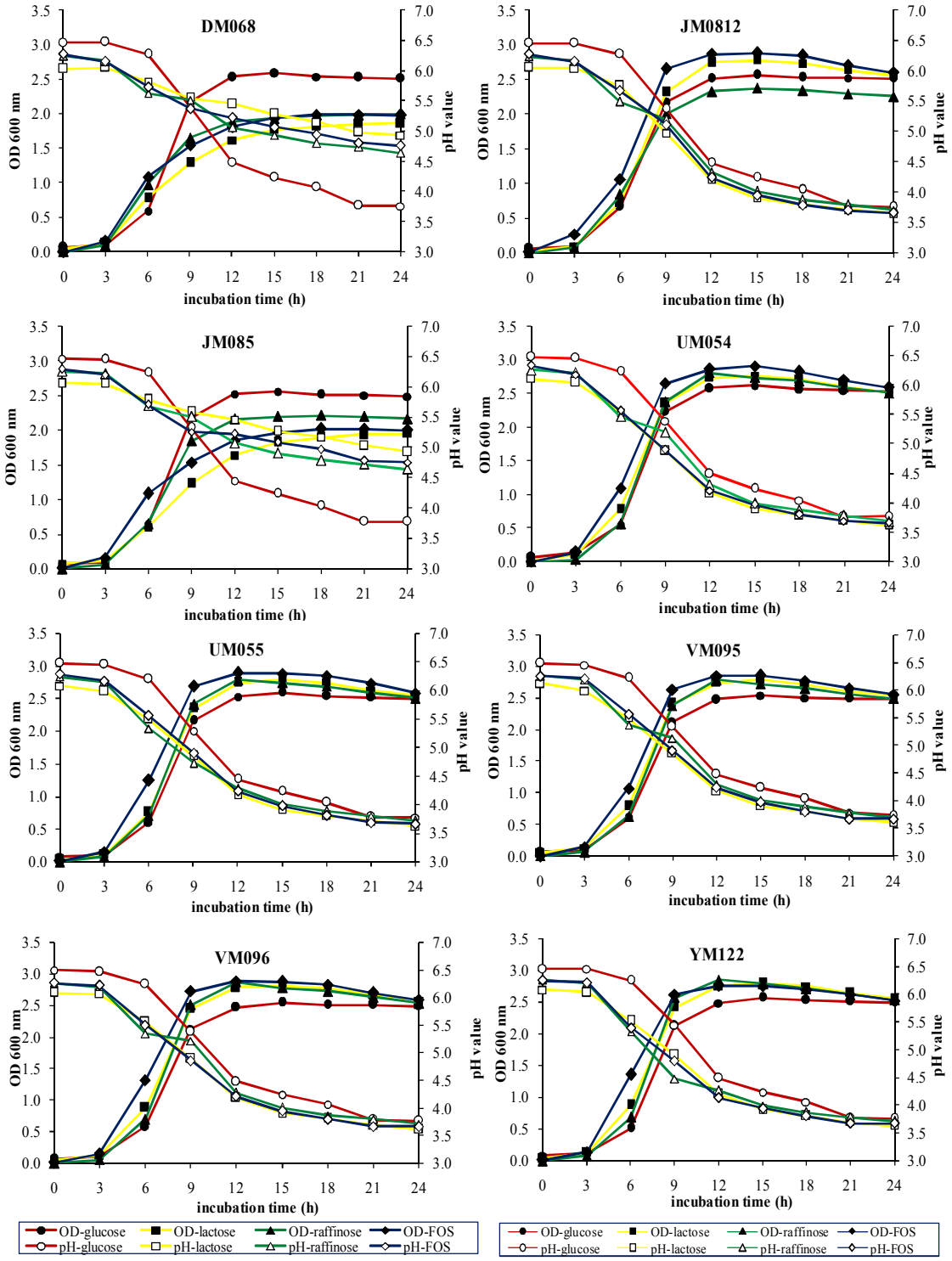

Figure 4.5 Growth behaviors of 8 *Lb. pentosus* strains in MRS with various sugars, lactose (OD= ■; pH=□), raffinose (OD=▲; pH=△), and FOS (OD=◆; pH=◇) as a carbon source and gluclose-MRS medium (OD=●; pH=○) as control. Results were shown as mean measurements from triplicate experiments (n = 3).

4.1.2 Sugar utilisation and SCFAs production by probiotic *Lb. pentosus* strains

4.1.2.1 Glucose utilisation

The capacities of glucose utilisation, lactic acid production and SCFAs production by 8 *Lb. pentosus* strains, namely DM068, JM0812, JM085, UM054, UM055, VM095, VM096, and YM122 in MRS media containing 2% glucose are summarized in Table 4.1. Glucose decreased over 24 h whereas lactic acid increased. The capacity of glucose utilisation for all test strains have quite similar profile with the greater consumption more than 90% (decrease from 17.61 ± 1.66 to 0.91 ± 0.01 mg/mL) of initial concentration of glucose (17.61-16.69 mg/mL in MRS medium). In case of lactic acid production, all strains could produce high amount of lactic acid after first 6 h incubation. The concentration of lactic acid was highest in *Lb. pentosus* DM068 (17.54 ± 0.16 mg/mL) followed by VM095, YM122, and UM054 17.19 ± 0.13, 17.05 ± 0.08 and 17.00 ± 0.05 mg/mL, respectively in MRS media at 24 h incubation.

The ways of probiotic properties are still a hightlight for probiotic selection. Some probiotics influence the change in the profile of fatty acids. SCFAs are the main end-products, which produce by probiotic fermentation from carbohydrate. The major SCFAs are acetic, propionic, butyric and valaric acid. The principal substrates include a wide variety of dietary residues, the main ones being prebiotic sugars.

SCFAs production (acetic, propionic, butyric, iso-butyric, and n-valeric acid) were significant different ($p < 0.05$) among strains at 24 h incubation. Acetic acid produced was ranged between 79.09 ± 1.64 to 90.08 ± 0.44 µmol/mL with YM122 strain showed highest acetic acid produced. Never the less, the production of other SCFAs in the end of incubation with high amount of propionic acid was produced by VM096 (38.31 ± 0.23 µmol/mL), butyric acid by YM122 (37.82 ± 0.12 µmol/mL), iso-butyric by DM068 and JM085 (16.33 ± 0.07 and 16.32 ± 0.02 µmol/mL, respectively), and n-valeric acid by UM054 (181.77 ± 0.00 µmol/mL).

Table 4.1 The capacity of glucose utilisation, lactic acid, and SCFAs production by probiotic *Lb. pentosus* 8 strains in glucose-MRS medium 24 h incubation period.

Incubation Time (h)	*Lb. pentosus* Strains	The capacity of glucose utilisation, lactic acid, and SCFAs production by probiotic *Lb. pentosus* 8 strains						
		Sugar contents (mg/mL)	Lactic acid (mg/mL)	SCFAs (µmol/mL)				
		D(+)Glucose	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Iso-butyric acid	n-valeric acid
0	DM068	17.18±0.05ns	0.17±0.05ns	0.08±0.01b	99.75±10.97b	5.60±0.11d	nd	1500.28±74.04a
	JM0812	17.31±0.38ns	0.14±0.00ns	0.08±0.00b	73.90±5.11cd	2.61±0.01f	nd	543.41±20.51d
	JM085	17.61±1.66ns	0.14±0.01ns	78.20±13.10b	96.22±0.37b	9.93±0.56b	nd	880.95±32.23c
	UM054	17.32±0.26ns	0.23±0.11ns	104.20±9.13a	152.30±15.17a	nd	nd	1041.90±138.54bc
	UM055	17.34±0.19ns	0.14±0.00ns	82.82±1.02b	90.90±10.37bc	10.55±0.02a	nd	1115.59±31.22b
	VM095	17.26±0.19ns	0.14±0.00ns	78.64±4.61b	50.40±5.67f	4.88±0.20e	nd	988.08±37.86bc
	VM096	16.91±0.19ns	0.14±0.00ns	78.47±0.21b	60.33±2.78df	6.05±0.45cd	nd	583.01±77.45d
	YM122	16.69±0.29ns	0.20±0.00ns	81.78±0.73b	65.81±4.64df	6.60±0.09c	nd	1036.66±0.53bc
6	DM068	17.04±0.98ns	0.50±0.10d	97.90±8.42ns	82.36±6.49a	9.72±0.71ns	nd	943.29±52.63bc
	JM0812	16.98±1.04ns	0.92±0.06a	100.86±6.12ns	76.30±1.02a	9.33±0.00ns	nd	830.65±78.40c
	JM085	17.61±0.48ns	0.61±0.04bcd	100.71±0.25ns	82.45±2.83a	8.59±0.05ns	nd	1036.89±49.65ab
	UM054	17.11±0.12ns	0.67±0.09bc	100.55±1.52ns	78.32±1.04a	8.97±0.11ns	nd	1104.11±89.68a
	UM055	16.93±0.12ns	0.74±0.01b	95.97±1.74ns	77.41±2.50a	9.69±0.30ns	nd	1013.32±43.67ab
	VM095	17.06±0.00ns	0.70±0.00bc	100.46±0.43ns	78.80±1.43a	10.19±0.09ns	nd	984.85±4.41ab
	VM096	17.16±0.15ns	0.57±0.04cd	98.75±1.80ns	76.46±2.26a	10.26±0.06ns	nd	984.61±8.25ab
	YM122	17.14±0.14ns	0.57±0.07cd	95.48±0.45ns	56.25±4.45b	8.16±2.73ns	nd	569.62±24.28d
12	DM068	9.36±0.01ab	8.51±0.12bc	102.13±3.42ab	31.92±2.35e	15.12±0.00b	nd	122.97±30.01d
	JM0812	9.39±0.08ab	8.18±0.00c	103.08±1.25a	50.13±3.24c	14.61±0.00b	nd	413.77±51.74c
	JM085	9.18±0.02ab	8.78±0.28ab	96.02±1.78ab	42.72±1.53cd	11.64±2.91b	nd	163.69±20.46d
	UM054	9.53±0.24a	8.38±0.21bc	99.37±6.19ab	43.69±5.32cd	27.84±3.25a	nd	482.08±0.91bc
	UM055	9.09±0.39b	8.96±0.08a	101.13±4.18ab	39.61±0.78d	4.09±0.45c	nd	503.86±67.40b
	VM095	9.45±0.01ab	8.50±0.27bc	103.13±3.59a	37.80±0.67de	7.13±0.67c	nd	687.63±18.11a
	VM096	9.24±0.03ab	8.51±0.16bc	94.66±1.68b	62.95±4.31b	7.69±0.62c	nd	623.94±23.04a
	YM122	9.53±0.09a	8.57±0.04abc	96.03±0.47ab	96.25±3.32a	13.91±0.45b	nd	nd

Table 4.1 Continued

Incubation Time (h)	*Lb. pentosus* Strains	The capacity of glucose utilisation, lactic acid, and SCFAs production by probiotic *Lb. pentosus* 8 strains						
		Sugar contents (mg/mL)	Lactic acid (mg/mL)	SCFAs (µmol/mL)				
		D(+)Glucose	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Iso-butyric acid	n-valeric acid
24	DM068	0.94±0.01a	17.54±0.16a	79.82±2.23c	25.18±5.14c	5.95±0.00dc	16.33±0.07a	110.02±7.98c
	JM0812	0.92±0.00b	16.92±0.08c	83.82±1.99bc	34.43±2.29ab	5.63±0.12dc	15.82±0.02b	122.13±8.71b
	JM085	0.91±0.01b	16.97±0.04c	80.71±1.52bc	34.86±1.04ab	nd	16.32±0.02a	124.64±0.97b
	UM054	0.93±0.00b	17.00±0.05bc	84.81±3.69b	33.15±1.97ab	nd	15.42±0.03c	181.77±0.00a
	UM055	0.91±0.00b	16.90±0.03c	79.09±1.64c	32.69±0.77ab	nd	15.87±0.02b	128.98±0.00b
	VM095	0.92±0.01b	17.19±0.13b	81.88±0.54bc	36.30±4.04ab	8.70±0.15b	14.80±0.08d	nd
	VM096	0.91±0.01b	16.99±0.04bc	82.36±0.91bc	38.31±0.23a	7.98±0.62b	13.92±0.03e	34.96±0.00d
	YM122	0.92±0.01b	17.05±0.08bc	90.08±0.44a	29.72±4.89bc	37.82±0.12a	nd	43.75±0.77d

Note: the mean values ± SD in the same column with the same time of each strain follow with different small letters were significant different ($p < 0.05$), ns= non significant, nd= non detectable

4.1.2.2 Lactose utilisation

The capacity of lactose utilisation by *Lb. pentosus* strains DM068, JM0812, JM085, UM054, UM055, VM095, VM096, and YM122 in MRS medium contains 2% lactose are presented in Table 4.2. The utilisation of lactose in MRS medium was significant different ($p < 0.05$) among strains. Never the less, lactose decreased over time, the small amount of D (+) glucose (range 0.75 ± 0.02 to 1.26 ± 0.04 mg/mL) and D (+) galactose (range 0.07 ± 0.01 to 0.70 ± 0.05 mg/mL) were found in MRS medium due to breakdown of molecule lactose formed to glucose-galactose by bacteria. In the same time, lactic acid and SCFAs was produced from lactose consumption by *Lb. pentosus* strains. During 24 h incubation, the concentration of lactose decreased from 34.50 ± 0.00 to 11.20 ± 0.06 mg/mL and from 36.54 ± 1.59 to 11.57 ± 0.49 mg/mL with highest capacity of lactose utilisation by YM122 and VM096, respectively. However, DM068 and JM085 strains had lower capacity of lactose utilisation.

The lactic acid and SCFAs production by 8 *Lb. pentosus* strains in MRS media contains 2% lactose are also summarized in Table 4.2 In case of lactic acid production, 6 of 8 strains could produced high amount of lactic acid after 6 h incubation except DM068 and JM085 strains. There were significant different ($p < 0.05$) highest lactic acid produced between range 17.46 ± 0.84 to 18.13±0.13 mg/mL at 24 h incubation. Acetic acid decreased during time, the concentration of acetic acid in JM085 and DM068 were high amount 99.33 ± 5.16 and 92.18 ± 5.81 µmol/mL, respectively 24 h. For other SCFAs production, the concentration of propionic, butyric, iso-butyric, and n-valeric acid were significant different ($p < 0.05$) among strains at 24 h incubation. The high concentration of propionic acid that 69.47 ± 1.52, 66.24 ± 5.27 and 64.70 ± 3.38 µmol/mL by JM085, DM068 and VM095, respectively. The high amount of butyric acid was produced by JM085 (16.35 ± 0.49 µmol/mL), iso-butyric produced by only 3 strains that JM085, DM068 and JM0812 (11.70 ± 1.00 to 24.18 ± 1.63 µmol/mL), and n-valeric acid by JM085, JM0812, and VM095 (299.08 ± 7.19 to 338.53 ± 27.84 µmol/mL).

Table 4.2 The capacity of lactose utilisation, lactic acid, and SCFASs production by probiotic *Lb. pentosus* 8 strains in Lactose-MRS medium 24 h incubation period

Incubation Time (h)	*Lb. pentosus* Strains	The capacity of lactose utilisation, lactic acid, and SCFAs production by probiotic *Lb. pentosus* 8 strains								
		Sugar contents (mg/mL)			Lactic acid (mg/mL)	SCFAs (µmol/mL)				
		lactose	D(+)glucose	galactose	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Iso-butyric acid	n-valeric acid
0	DM068	25.67±1.75b	0.59±0.01d	0.58±0.03c	0.32±0.10b	99.20±4.11bc	70.85±0.88c	42.12±8.42bc	nd	794.89±75.46bc
	JM0812	28.32±2.57b	0.74±0.12bcd	0.94±0.10b	0.65±0.29ab	115.37±3.40a	76.81±5.84bc	11.38±0.44ef	nd	709.26±26.39c
	JM085	28.90±0.52b	0.85±0.06abc	0.99±0.05b	0.42±0.04ab	100.37±1.92b	85.06±3.27b	16.15±7.82de	nd	711.84±50.90c
	UM054	26.85±0.15b	0.72±0.08bcd	0.90±0.04b	0.68±0.21a	92.43±0.21c	99.70±0.05a	nd	nd	1117.29±7.22a
	UM055	27.75±1.64b	0.65±0.21cd	0.91±0.09b	0.44±0.04ab	84.09±4.97d	89.33±9.98ab	70.64±11.86a	nd	853.52±15.19b
	VM095	28.95±2.11b	0.82±0.03abcd	0.98±0.06b	0.45±0.02ab	74.22±0.17e	70.94±2.93c	47.92±0.90b	nd	1085.59±77.79a
	VM096	36.54±1.59a	0.97±0.01a	1.20±0.07a	0.51±0.06ab	69.40±2.62ef	48.04±0.23d	28.36±1.35cd	nd	711.81±75.10c
	YM122	34.50±0.33a	0.89±0.00ab	1.18±0.02a	0.52±0.01ab	64.17±3.09f	46.99±8.15d	36.93±1.77bc	nd	536.80±13.94d
6	DM068	25.05±1.11ab	1.18±0.05c	0.42±0.07b	3.81±0.25a	69.39±3.16a	54.54±12.32b	12.83±0.31d	nd	68.83±10.59e
	JM0812	26.58±1.32ab	1.17±0.06c	0.54±0.00ab	1.10±0.04b	66.93±1.80ab	68.38±1.49a	32.86±2.66a	nd	187.14±3.97d
	JM085	28.47±1.78a	1.21±0.04abc	0.59±0.04a	1.21±0.02b	32.93±1.88c	9.86±1.44d	7.82±1.04e	nd	40.93±0.00f
	UM054	24.49±0.77ab	1.18±0.04bc	0.43±0.02b	4.14±0.04a	32.97±2.37c	41.82±1.22c	9.78±0.12de	5.80±1.07	nd
	UM055	25.32±0.42ab	1.35±0.09ab	0.50±0.07ab	3.73±0.24a	60.77±0.63b	54.80±1.75b	17.70±2.11c	11.05±2.81	63.86±9.02ef
	VM095	25.53±0.85ab	1.25±0.05abc	0.46±0.03ab	4.20±0.37a	63.56±4.06ab	60.99±2.99ab	24.77±0.37b	24.42±1.30	478.98±22.37b
	VM096	25.66±1.71ab	1.36±0.12a	0.51±0.11ab	3.93±0.31a	62.30±3.43ab	59.92±3.15ab	20.33±2.42bc	15.73±1.07	319.79±18.50c
	YM122	25.74±1.48ab	1.24±0.04abc	0.48±0.00ab	3.91±0.49a	63.50±5.58ab	62.25±4.17ab	22.46±3.24b	6.45±0.17	539.00±0.00a
12	DM068	26.82±2.91a	1.24±0.08a	0.74±0.09a	1.98±0.03b	60.14±4.82a	68.20±9.18a	43.95±0.68a	nd	345.97±62.02a
	JM0812	19.10±0.83b	0.97±0.04b	0.18±0.03b	10.76±0.46a	52.92±3.49ab	68.58±4.35a	7.87±0.09d	nd	nd
	JM085	26.96±1.47a	1.29±0.07a	0.74±0.02a	2.08±0.18b	59.49±1.38a	68.09±0.12a	17.73±2.50b	18.13±0.00	202.37±7.93bc
	UM054	18.21±0.91b	0.91±0.02b	0.13±0.01b	11.09±0.11a	48.09±4.31b	46.73±2.79b	9.46±1.75cd	nd	nd
	UM055	18.78±0.91b	0.98±0.09b	0.18±0.08b	11.29±0.12a	51.67±2.38b	70.73±5.04a	11.38±1.11cd	nd	nd
	VM095	19.29±0.42b	0.95±0.01b	0.16±0.03b	10.85±0.70a	51.35±2.36b	68.01±5.58a	11.13±1.22cd	nd	157.36±0.00c
	VM096	19.74±1.21b	1.01±0.01b	0.20±0.03b	10.46±0.51a	53.21±0.69ab	68.82±1.67a	8.29±0.14d	7.79±0.25	229.08±14.69b
	YM122	19.43±2.01b	0.99±0.12b	0.17±0.06b	10.81±0.13a	53.98±3.17ab	71.67±6.38a	13.33±3.83c	7.62±0.26	332.82±28.19a

Table 4.2 Continued

Incubation Time (h)	*Lb. pentosus* Strains	The capacity of lactose utilisation, lactic acid, and SCFAs production by probiotic *Lb. pentosus* 8 strains								
		Sugar contents (mg/mL)			Lactic acid (mg/mL)	SCFAs (µmol/mL)				
		lactose	D(+)glucose	galactose	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Iso-butyric acid	n-valeric acid
24	DM068	25.45±0.91a	1.26±0.04a	0.70±0.05a	3.61±0.11c	92.18±5.81a	64.70±3.38a	8.29±0.04i	20.55±0.78b	114.18±22.28d
	JM0812	13.25±0.71c	0.75±0.03d	0.06±0.00c	17.51±0.82a	54.11±2.73b	40.02±12.54bc	7.34±0.13j	11.70±0.00c	309.71±26.80ab
	JM085	22.55±0.54b	1.10±0.03b	0.24±0.03b	7.64±0.66b	99.33±5.16a	69.47±1.52a	16.35±0.49a	24.18±1.63a	338.53±27.84a
	UM054	10.86±0.08d	0.84±0.01cd	0.12±0.00c	17.08±0.13a	50.26±0.51b	21.06±0.92d	11.47±0.57e	nd	238.84±7.71c
	UM055	11.74±0.61d	0.84±0.10cd	0.13±0.10bc	17.90±0.53a	54.56±0.67b	38.34±9.19bc	15.13±0.38b	nd	282.25±6.18bc
	VM095	12.29±0.77cd	0.78±0.00cd	0.07±0.00c	18.13±0.13a	55.72±0.84b	66.24±5.27a	10.46±0.00f	nd	299.08±7.19ab
	VM096	11.57±0.49d	0.90±0.09c	0.17±0.06bc	17.75±1.04a	53.67±5.34b	45.78±6.26b	13.33±0.04c	nd	273.88±23.78bc
	YM122	11.20±0.06d	0.75±0.02d	0.07±0.01c	17.46±0.84a	54.23±0.84b	26.28±0.18cd	12.56±0.34d	nd	282.44±0.85bc

Note: the meanvalues±SD in the same column, in the same time of each strain with different small letters were significant different ($p<0.05$) ns= non significant, nd= non detectable

4.1.2.3 Raffinose utilisation

The raffinose utilisation by *Lb. pentosus* strains DM068, JM0812, JM085, UM054, UM055, VM095, VM096, and YM122 in MRS medium contains 2% raffinose are presented in Table 4.3. The utilisation of raffinose in MRS medium was significant different ($p < 0.05$) among strains at the incubation period. The concentration of raffinose decreased during incubation. At the same time, the concentration of monosaccharide galactose and glucose increased in the culture media. Due to molecule raffinose is trisaccharide formed to glucose + galactose + fructose then raffinose could be breakdown by *Lb. pentosus* strains. However, a small amount of glucose (range 1.79 ± 0.07 to 2.68 ± 0.00 mg/mL) was found only at the initial time incubation maybe molecule raffinose breakdown to form monosaccharide from the preparation culture media process whereas fructose did not detect in this experiment. The amount of raffinose decreased significant ($p < 0.05$) among strains. In the same time, lactic acid and SCFAs was produced from raffinose consumption by bacteria strains. During 24 h incubation, the concentration of raffinose decreased with highest capacity of raffinose utilisation by UM055 and YM122 from 28.96 ± 2.52 to 10.49 ± 0.07 mg/mL and from 28.36 ± 3.58 to 10.30 ± 0.00 mg/mL, respectively whereas the amount of galactose changed from 5.40 ± 0.57 to 2.62 ± 0.00 mg/mL. However, DM068 and JM085 strains had lower capacity of lactose utilisation.

Table 4.3 also present the lactic acid and SCFAs production by *Lb. pentosus* 8 strains in MRS medium contains 2% raffinose. In case of lactic acid production, 6 of 8 strains could produce high amount of lactic acid in the first 6 h incubation except DM068 and JM085 strains. There were increased significant different ($p < 0.05$) highest produced amount of lactic acid between range 17.48 ± 0.840 to 17.93 ± 0.23 mg/mL at 24 h incubation. Also, acetic acid increased during time incubation, but at the end 24 h the concentration of acetic acid dropped from 12 h, it found high amount of acetic acid were 98.49 ± 8.37, 93.31 ± 0.18 and 89.02 ± 9.43 µmol/mL by VM096, UM055, and VM095, respectively at 24 h incubation.

For other SCFAs production, the concentration of propionic, butyric, iso-butyric, and n-valeric acid were significant ($p < 0.05$) among strains at 24 h incubation. The high concentration of propionic acid was 29.49 ± 2.79 µmol/mL by JM0812. The high amount of butyric acid was produced by UM054 (19.98 ± 0.54 µmol/mL), iso-

butyric by JM085 (15.55 ± 2.33µmol/mL), and n-valeric acid by JM0812 (624.22 ± 3.74 µmol/mL).

Table 4.3 The capacity of raffinose utilisation, lactic acid, and SCFAs production by probiotic *Lb. pentosus* 8 strains in Raffinose-MRS medium 24 h incubation period.

Incubation Time (h)	*Lb. pentosus* Strains	The capacity of raffinose utilisation, lactic acid, and SCFAs production by probiotic *Lb. pentosus* 8 strains									
		Sugar contents (mg/mL)				Lactic acid (mg/mL)	SCFAs (μmol/mL)				
		raffinose	D(+) glucose	galactose	D(-) fructose	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Iso-butyric acid	n-valeric acid
0	DM068	23.81±0.63bc	1.79±0.07c	3.57±0.82bc	nd	0.43±0.21ab	93.22±2.97b	268.99±44.74a	32.80±3.28b	nd	414.12±46.93c
	JM0812	21.40±0.67cd	nd	5.40±0.57a	nd	0.46±0.09ab	nd	nd	nd	nd	nd
	JM085	26.46±1.36ab	2.68±0.00a	4.97±0.76ab	nd	0.29±0.06b	96.49±2.49b	280.14±14.52a	20.86±0.05c	nd	491.42±8.75b
	UM054	18.02±0.86d	1.79±0.00c	5.08±0.81ab	nd	0.77±0.06a	104.97±0.60a	nd	22.11±2.92c	nd	504.25±42.89b
	UM055	28.96±2.52a	2.18±0.18b	4.25±0.63bc	nd	0.62±0.37ab	108.61±0.19a	nd	39.20±1.57a	nd	627.16±6.22a
	VM095	17.80±1.63d	1.92±0.00c	3.15±0.38c	nd	0.25±0.02b	104.75±0.06a	nd	29.81±0.93b	nd	599.43±50.46a
	VM096	18.53±0.04d	nd	5.18±0.12a	nd	0.83±0.09a	6.17±0.24c	nd	0.43±0.00d	nd	nd
	YM122	28.36±3.58a	2.28±0.13b	4.42±0.38abc	nd	0.44±0.03ab	95.92±7.52b	13.87±0.00b	20.14±1.83c	nd	20.70±0.95d
6	DM068	24.83±4.63ab	nd	4.74±0.87ns	nd	2.56±0.24e	80.28±7.77a	nd	7.37±0.25bc	7.20±0.01c	237.80±34.52a
	JM0812	21.45±1.58b	nd	5.11±0.38ns	nd	6.44±0.11c	85.57±2.33a	nd	6.19±0.23c	11.38±0.85a	166.95±1.76b
	JM085	27.88±0.41a	nd	5.35±0.04ns	nd	2.90±0.06d	84.44±1.18a	nd	8.63±0.27ab	7.60±0.24c	244.58±1.39a
	UM054	20.62±1.15b	nd	4.93±0.30ns	nd	6.68±0.08abc	81.45±1.82a	nd	4.08±0.06d	4.74±0.10d	128.85±12.18c
	UM055	21.46±1.14b	nd	5.12±0.30ns	nd	6.99±0.21a	82.10±1.49a	nd	4.38±0.04d	4.87±0.18d	146.10±16.08bc
	VM095	21.15±0.30b	nd	5.02±0.14ns	nd	6.81±0.13ab	81.51±1.27a	nd	7.15±1.96bc	7.08±0.29c	122.43±12.39c
	VM096	20.93±0.01b	nd	4.83±0.00ns	nd	6.61±0.04bc	4.90±0.25b	nd	nd	nd	nd
	YM122	20.54±0.42b	nd	5.35±0.13ns	nd	6.97±0.06a	80.97±0.12a	nd	10.16±0.23a	10.52±0.26b	110.43±3.67c
12	DM068	22.43±2.35a	nd	5.22±0.49b	nd	3.55±0.25b	76.05±6.11ab	nd	13.26±2.06a	9.39±1.00a	457.32±47.71a
	JM0812	17.22±0.05b	nd	4.61±0.12c	nd	10.78±0.83a	71.05±0.09b	26.91±6.56a	7.47±1.36b	7.16±0.27bc	224.94±3.68c
	JM085	23.98±0.16a	nd	5.73±0.05a	nd	4.06±0.07b	81.43±3.10a	nd	5.41±1.73b	6.67±1.44c	346.14±3.78b
	UM054	17.65±0.35b	nd	4.74±0.22c	nd	10.44±0.77a	71.30±0.07b	10.19±0.00c	13.99±1.81a	9.58±0.18a	221.96±0.57c
	UM055	16.40±0.20b	nd	4.52±0.01c	nd	11.41±0.47a	72.26±1.95b	nd	12.41±0.00a	9.79±0.16a	198.83±2.00c
	VM095	16.77±0.03b	nd	4.43±0.01c	nd	11.13±0.25a	71.16±2.37b	28.48±0.00a	14.46±0.72a	9.12±0.02ab	192.12±0.68c
	VM096	16.33±0.23b	nd	4.45±0.05c	nd	11.00±0.56a	4.40±0.17c	1.04±0.27d	nd	nd	nd
	YM122	16.73±0.10b	nd	4.43±0.01c	nd	10.95±0.71a	72.09±1.94b	19.61±0.00b	14.74±0.53a	8.75±1.74abc	208.46±12.24c

Table 4.3 Continued

Incubation Time (h)	*Lb. pentosus* Strains	The capacity of raffinose utilisation, lactic acid, and SCFAs production by probiotic *Lb. pentosus* 8 strains									
		Sugar contents (mg/mL)				Lactic acid (mg/mL)	SCFAs (µmol/mL)				
		raffinose	D(+) glucose	galactose	D(-) fructose	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Iso-butyric acid	n-valeric acid
24	DM068	22.29±1.38a	nd	5.83±0.35a	nd	5.35±0.24b	72.50±5.56d	nd	3.06±0.50e	9.22±0.51bc	21.13±2.60f
	JM0812	10.47±0.25b	nd	2.72±0.17b	nd	17.72±0.05a	86.56±0.83abc	22.43±3.43c	15.62±0.47b	10.00±0.20b	624.22±3.74a
	JM085	23.52±1.01a	nd	6.15±0.27a	nd	5.47±0.09b	78.77±4.52cd	29.49±2.79a	13.32±0.50c	15.55±2.33a	nd
	UM054	10.44±0.03b	nd	2.71±0.12b	nd	17.55±0.66a	79.15±2.31cd	26.69±0.97b	19.98±0.54a	7.49±0.60cd	563.41±5.16b
	UM055	10.49±0.07b	nd	2.66±0.02b	nd	17.82±0.29a	93.31±0.18ab	27.59±0.38b	14.23±0.39c	4.88±1.11e	473.33±27.26c
	VM095	10.37±0.20b	nd	2.81±0.08b	nd	17.55±0.31a	89.02±9.43abc	21.76±0.68c	9.68±0.43d	8.33±0.48bc	313.94±9.77d
	VM096	10.58±0.08b	nd	2.78±0.10b	nd	17.48±0.40a	98.49±8.37a	20.38±0.91c	8.58±0.21d	5.13±0.13e	nd
	YM122	10.30±0.00b	nd	2.62±0.00b	nd	17.93±0.23a	82.43±1.95bcd	19.26±0.38c	8.79±0.89d	5.64±0.09de	278.12±2.18e

Note: the mean values ± SD in the same column, in the same time of each strain with different small letters were significant different ($p<0.05$) ns= non significant, nd= non detectable

4.1.2.4 FOS utilisation

The capacity of FOS utilisation lactic acid and SCFAs production by *Lb. pentosus* 8 strains in MRS medium contains 2% FOS are summarized in Table 4.4. At the initial, sugar contents in culture medium was analysis by HPLC. It result found FOS in range between (5.78 ± 0.07 to 6.49 ± 0.05mg/mL), treharose (4.96 ± 1.92 to 6.14 ± 0.30 mg/mL), D (+) glucose (11.67 ± 2.38 to 12.95 ± 0.30 mg/mL), and D (-) fructose (13.13 ± 0.09 to 14.76 ± 0.11 mg/mL). Meanwhile, molecule of FOS was breakdown to monosaccharide glucose, and fructose from the preparation by autoclaving. However, disaccharide treharose came from molecule of glucose attach glucose in the culture media. The utilisation of FOS was significant different ($p < 0.05$) among strains at 24 h incubation. Never the less, FOS slightly decreased over time, treharose, D (+) glucose and fructose were decreased also. At 24 h incubation, the UM055, and UM054 were highest utilized FOS from 6.49 ± 0.05 to 5.02 ± 0.23 mg/mL and from 6.38 ± 0.04 to 5.24 ± 0.19 mg/mL. In addition, the amount of treharose, D (+) glucose and fructose were significant different ($p < 0.05$) among strains at 24 h incubation. Six of eight strains had high capacity of sugar utilisation except DM068 and JM085.

In the same time, whereas sugar decreased lactic acid was increasing by time due to bacteria consumption of sugar and produced organic acid. During 24 h incubation, 6 of 8 strains could produce high amount of lactic acid after first 6 h incubation except DM068 and JM085 strains. There were significant different ($p < 0.05$) highest produced amount of lactic acid between range 16.64 ± 1.10 to 17.54 ± 0.33 mg/mL at 24 h incubation. Acetic acid increased during time incubation, the concentration of propionic, butyric, and n-valeric acid were significant different ($p < 0.05$) among strains at 24 h incubation but iso-butyric did not detect. The high concentration of propionic acid was 60.28 ± 6.12µmol/mL and 56.06 ± 6.39 µmol/mL by UM054 and JM0812, respectively. However, high amount of butyric acid was produced by UM055 and YM122 (46.08 ± 0.25 µmol/mL and 42.85 ± 0.00 µmol/mL), and n-valeric acid was produced by YM122 (614.41 ± 71.85µmol/mL).

Table 4.4 The capacity of FOS utilisation, lactic acid, and SCFAs production by probiotic *Lb. pentosus* 8 strains in FOS-MRS medium 24 h incubation period.

Incubation Time (h)	*Lb. pentosus* Strains	The capacity of FOS utilisation, lactic acid, and SCFAs production by probiotic *Lb. pentosus* 8 strains									
		Sugar contents (mg/mL)				Lactic acid (mg/mL)	SCFAs (µmol/mL)				
		FOS	treharose	D(+) glucose	D(-) fructose	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Iso-butyric acid	n-valeric acid
0	DM068	6.28±0.32ns	6.14±0.30ns	12.93±0.46ns	14.67±0.75ns	0.39±0.03b	45.10±0.58a	40.83±2.09c	19.80±1.05b	nd	362.72±5.21ab
	JM0812	5.81±0.76ns	5.63±0.33ns	12.36±1.11ns	13.89±1.24ns	0.60±0.00b	45.77±2.02a	64.22±0.45a	56.06±6.39a	nd	341.80±5.39ab
	JM085	5.79±0.93ns	4.96±1.92ns	11.67±2.38ns	14.03±1.39ns	0.47±0.15b	48.32±3.95a	50.07±5.32bc	21.14±0.62b	nd	391.18±0.00a
	UM054	6.38±0.04ns	6.00±0.11ns	12.95±0.30ns	14.70±0.29ns	0.62±0.05b	48.86±2.16a	60.94±5.39a	60.28±6.12a	nd	nd
	UM055	6.49±0.05ns	5.96±0.03ns	13.00±0.05ns	14.76±0.11ns	0.66±0.12b	48.08±0.42a	66.54±5.52a	19.28±0.06b	nd	nd
	VM095	5.99±0.30ns	5.61±0.21ns	12.30±0.34ns	13.86±0.48ns	0.67±0.04b	25.84±0.41b	65.42±2.56a	10.57±0.02c	nd	316.03±6.64b
	VM096	5.78±0.07ns	5.34±0.01ns	11.71±0.15ns	13.13±0.09ns	0.60±0.06b	27.06±0.90b	57.21±4.51ab	20.58±0.00b	nd	241.42±58.73c
	YM122	5.85±0.48ns	5.15±0.11ns	11.88±1.07ns	13.19±0.09ns	1.31±0.35a	28.40±1.78b	59.79±5.51ab	22.12±2.79b	nd	239.75±10.13c
6	DM068	5.63±0.21ns	5.08±0.01a	9.87±0.03ns	12.94±0.20ns	2.18±0.06b	116.46±2.38a	32.77±4.02b	19.80±1.05b	nd	253.28±38.37b
	JM0812	5.62±0.21ns	1.90±0.00cd	9.23±0.42ns	12.02±0.55ns	6.66±0.47a	80.47±0.11b	49.43±1.08a	56.06±6.39a	nd	nd
	JM085	5.58±0.34ns	5.00±0.06a	9.79±0.19ns	12.88±0.05ns	2.27±0.22b	115.90±3.46a	37.54±3.43b	21.14±0.62b	nd	495.09±95.61a
	UM054	5.55±0.01ns	1.83±0.04cd	9.18±0.30ns	12.00±0.19ns	6.93±0.21a	115.33±5.82a	51.48±4.39a	60.28±6.12a	nd	nd
	UM055	5.68±0.30ns	1.78±0.13d	9.41±0.73ns	12.16±1.26ns	7.18±1.15a	117.90±15.50a	50.21±6.59a	19.28±0.06b	nd	nd
	VM095	5.56±0.04ns	1.99±0.12bc	9.33±0.36ns	11.90±0.15ns	6.63±0.42a	114.75±6.43a	53.87±2.40a	10.57±0.02c	nd	nd
	VM096	5.47±0.14ns	2.14±0.05b	9.12±0.32ns	11.83±0.43ns	6.51±0.30a	116.61±3.69a	52.45±3.71a	20.58±0.00b	nd	nd
	YM122	5.45±0.30ns	1.73±0.03d	9.27±0.03ns	12.12±0.08ns	7.12±0.11a	117.54±1.56a	54.23±1.32a	22.12±2.79b	nd	nd
12	DM068	5.81±0.10ns	4.28±0.03a	10.31±0.05a	13.45±0.32a	3.53±0.13b	118.54±2.07a	37.77±1.39bc	19.80±1.05b	nd	568.02±41.57a
	JM0812	5.68±0.32ns	1.47±0.07c	8.03±0.91ab	10.65±1.23c	12.17±0.42a	88.33±7.71b	38.02±1.25bc	56.06±6.39a	nd	nd
	JM085	5.36±1.06ns	4.06±0.84ab	9.69±1.74ab	12.57±2.51b	3.29±0.57b	112.76±20.46a	43.70±9.70ab	21.14±0.62b	nd	333.99±59.23b
	UM054	5.74±0.12ns	1.52±0.09c	8.21±0.45ab	10.77±0.96c	11.49±0.33a	86.33±5.78b	47.67±7.97ab	60.28±6.12a	nd	202.16±0.00c
	UM055	5.75±0.17ns	1.54±0.01c	8.16±0.03ab	10.64±0.13c	11.78±0.32a	85.14±4.29b	28.00±4.15c	19.28±0.06b	nd	201.52±0.00c
	VM095	5.37±0.13ns	3.44±0.01b	9.42±1.58ab	11.10±2.16bc	11.25±1.56a	114.75±6.43a	53.87±2.40a	10.57±0.02c	nd	nd
	VM096	5.93±0.09ns	1.55±0.04c	7.78±0.22b	10.69±0.38c	12.31±0.49a	116.61±3.69a	52.45±3.71a	20.58±0.00b	nd	nd
	YM122	5.60±0.36ns	1.54±0.08c	8.04±0.42ab	10.33±0.49c	11.71±0.41a	117.54±1.56a	54.23±1.32a	22.12±2.79b	nd	nd

Table 4.4 Continued

Incubation Time (h)	*Lb. pentosus* Strains	The capacity of FOS utilisation, lactic acid, and SCFAs production by probiotic *Lb. pentosus* 8 strains									
		Sugar contents (mg/mL)				Lactic acid (mg/mL)	SCFAs (µmol/mL)				
		FOS	treharose	D(+) glucose	D(-) fructose	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Iso-butyric acid	n-valeric acid
24	DM068	5.76±0.07ab	3.02±0.07a	10.39±0.30a	13.22±0.16a	4.95±0.18b	110.23±3.02b	19.80±1.05b	0.43±0.00f	nd	354.28±27.74c
	JM0812	5.10±0.12c	1.42±0.01b	6.09±0.11b	7.95±0.01b	17.32±0.37a	79.60±2.66c	56.06±6.39a	3.91±0.00e	nd	78.89±0.00d
	JM085	5.87±0.06a	3.02±0.01a	10.55±0.27a	13.80±0.09a	5.12±0.28b	113.93±5.65ab	21.14±0.62b	0.43±0.00	nd	394.51±6.55c
	UM054	5.24±0.19bc	1.46±0.05b	5.96±0.21b	8.00±0.29b	17.34±0.76a	80.59±3.30c	60.28±6.12a	nd	nd	nd
	UM055	5.02±0.23c	1.51±0.03b	5.91±0.18b	8.01±0.08b	17.54±0.33a	120.58±6.42a	19.28±0.06b	46.08±0.25a	nd	588.85±36.31a
	VM095	5.20±0.19bc	1.47±0.08b	5.92±0.30b	8.06±0.14b	17.54±0.41a	70.71±1.98c	10.57±0.02c	40.35±0.00c	nd	133.68±19.12d
	VM096	4.94±0.47c	1.41±0.12b	5.65±0.56b	7.69±0.70b	16.64±1.10a	121.05±3.58a	20.58±0.00b	25.46±1.95d	nd	475.53±37.15b
	YM122	5.14±0.22c	1.49±0.13b	5.88±0.30b	7.93±0.55b	17.23±1.05a	118.16±4.34ab	22.12±2.79b	42.85±0.00b	nd	614.41±71.85a

Note: the mean values ± SD in the same column, with the same parameter in the same period of each strain with different small letters were significant different ($p<0.05$) ns= non significant, nd= non detectable

Sugar utilisation from different carbohydrates (glucose, lactose, raffinose, and FOS) as a carbon source in MRS media and inoculated with probiotic *Lb. pentosus* 8 strains in 24 h incubation are summarized in Table 4.5. The sugar utilisation was significant different ($p < 0.05$) by time incubation for each strain in the culture medium. The 8 strains had a high capacity to use glucose in culture media more than 90 % (rang 94.49 - 94.83 %). The consumption of glucose in medium greater happened at 12 h due to the growth of *Lb. pentosus* maintained in the exponential phase and reached maximium OD.

Never the less, the capacity lactose utilisation in culture medium was lower than glucose in range 1.22 - 68.34 %. The 6 strains had a capacity of lactose utilisation more than 50 % that were VM096, YM122, UM054, UM055, VM095 and JM0182 by 68.34, 67.54, 59.55, 57.69, 57.55 and 53.21 %, respectively.

The capacity of raffinose utilisation in culture medium was lower than glucose in rang between 6.38 - 68.78%. It can be seen only 3 of 8 strains had a capacity of raffinose utilisation more than 50% were UM055, YM122, and JM0182 by 63.78, 63.68, and 51.07%, respectively.

In addition, the capacity of FOS utilisation in culture meduim was lower than glucose in highest amount 22.65 % by UM055 strain.

Table 4.5 Sugar utilisation from different carbohydrates (glucose, lactose, raffinose, and FOS) as a carbon source in modified-MRS media and inoculated with probiotic *Lb. pentosus* 8 strains in 24 h incubation.

Incubation time (h)	Sugar utilisation by *Lb. pentosus* strains							
	Glucose contents (mg mL^{-1})							
	DM068	JM0812	JM085	UM054	UM055	VM095	VM096	YM122
0	17.18±0.05A	17.31±0.38A	17.61±1.66A	17.32±0.26A	17.34±0.19A	17.26±0.19A	16.91±0.19A	16.69±0.29A
6	17.04±0.98A	16.98±1.04A	17.61±0.48A	17.11±0.12A	16.93±0.12A	17.06±0.00A	17.16±0.15A	17.14±0.14A
12	9.36±0.01B	9.39±0.08B	9.18±0.02B	9.53±0.24B	9.09±0.39B	9.45±0.01B	9.24±0.03B	9.53±0.09B
24	0.94±0.01C	0.92±0.00C	0.91±0.01C	0.93±0.00C	0.91±0.00C	0.92±0.01C	0.91±0.01C	0.92±0.01C
Substrate conversion (%)	94.53	94.69	94.83	94.63	94.75	94.67	94.68	94.49
Incubation time (h)	Lactose contents (mg mL^{-1})							
	DM068	JM0812	JM085	UM054	UM055	VM095	VM096	YM122
0	25.67±1.75A	28.32±2.57A	28.90±0.52A	26.85±0.15A	27.75±1.64A	28.95±2.11A	36.54±1.59A	34.50±0.33A
6	25.05±1.11A	26.58±1.32A	28.47±1.78A	24.49±0.77B	25.32±0.42A	25.53±0.85B	25.66±1.71B	25.74±1.48B
12	24.82±2.91A	19.10±0.83B	26.96±1.47A	18.21±0.91C	18.78±0.91B	19.29±0.42C	19.74±1.21C	19.43±2.01C
24	24.45±0.91A	13.25±0.71C	22.55±0.54B	10.86±0.08D	11.74±0.61C	12.29±0.77D	11.57±0.49D	11.20±0.06D
Substrate conversion (%)	1.22	53.21	21.97	59.55	57.69	57.55	68.34	67.54
Incubation time (h)	Raffinose contents (mg mL^{-1})							
	DM068	JM0812	JM085	UM054	UM055	VM095	VM096	YM122
0	23.81±5.02A	21.40±3.49A	26.46±1.36A	18.02±0.86B	28.96±2.52A	17.80±1.63B	18.53±0.04B	28.36±3.58A
6	24.83±4.63A	21.45±1.58A	27.88±0.41A	20.62±1.15A	21.46±1.14B	21.15±0.30A	20.93±0.01A	20.54±0.42B
12	22.43±2.35A	17.22±0.05A	23.98±0.16B	17.65±0.35B	16.40±0.20C	16.77±0.03B	16.33±0.23C	16.73±0.10C
24	22.29±1.38A	10.47±0.25B	23.52±1.01B	10.44±0.03C	10.49±0.07D	10.37±0.20C	10.58±0.08D	10.30±0.00D
Substrate conversion (%)	6.38	51.07	11.11	42.06	63.78	41.74	42.90	63.68
Incubation time (h)	FOS contents (mg mL^{-1})							
	DM068	JM0812	JM085	UM054	UM055	VM095	VM096	YM122
0	6.28±0.32A	5.81±0.76A	5.79±0.93A	6.38±0.04A	6.49±0.05A	5.99±0.30A	5.78±0.07A	5.85±0.48A
6	5.63±0.21B	5.62±0.21A	5.58±0.34A	5.55±0.01BC	5.68±0.30B	5.56±0.04AB	5.47±0.14AB	5.45±0.30A
12	5.81±0.10AB	5.68±0.32A	5.36±1.06A	5.74±0.12B	5.75±0.17B	5.37±0.13B	5.93±0.09A	5.60±0.36A
24	5.76±0.07AB	5.10±0.12A	5.87±0.06A	5.24±0.19C	5.02±0.23C	5.20±0.19B	4.94±0.47B	5.14±0.22A
Substrate conversion (%)	8.28	12.22	-1.38	17.87	22.65	13.19	14.53	12.13

Note: The mean value ± SD with the same parameter for each strain, with different capital letters in the same row were significant different ($p<0.05$).

4.2 Screening for BSH activity

Cholesterol reducing is a health-promoting characteristic, the idea of selection of probiotic strains. However, cholesterol reducing activity of lactobacilli strain happened by several macchanisms. One of mechanisms through BSH activity has been associated reduction of cholesterol (Corzo and Gilliland, 1999; Liong and Shah, 2005a; Begley et al., 2006). In human, bile acids are synthesized from cholesterol and conjugated to either glycine or taurine in the liver then pass into the intestine, whereas amino acid maybe hydrolyzed from these conjugated bile acid by bacterial enzymes known as conjugated BSH, which expressed by gastrointestinal bacteria of several genera. When BSH-producing lactobacilli were streaked out on MRS plates containing 0.5% TDCA, the taurine-conjugated bile acid was deconjugated, producing deoxycholic acid (Mahrous, 2011).

In this study, 8 strains of the probiotic *Lb. pentosus* were screened for BSH activity by plate assay technique. The cell suspensions of overnight cultures were spotted 10 µL on sterile filter disces (diameter 0.7 cm) and are placed on MRS agar with 0.5% (w/v) TDCA and incubated anaerobically at 37 ^{o}C for 72 h. The deconjugation activity of *Lb. pentosus* strains was presented in Figure 4.6 B-I (disc No. 1-3). The amounts of deoxycholic acid precipitated around disc and diffused into the surrounding medium. Howevre, a positive control disc on MRS agar without 0.5% (w/v) TDCA did not found a precipitated zone around disc. However, the disc (No. 4) without spotted cell suspension also did not found a precipitated zone. All strains exhibited BSH activities as demonstrated by precipitation zones ($p < 0.05$) with diameters between 8.83 ± 0.75 mm to 10.17 ± 0.41 mm (Table 4.6). The greatest precipitation zone was found in *Lb. pentosus* VM 096 followed by VM095, YM122, JM085, and UM055 (10.17 ± 0.41, 9.92 ± 0.58, 9.83 ± 0.41, 9.83 ± 0.41, and 9.67 ± 0.52 mm, respectively) and the strain DM068 had the lowest precipitation of 8.83 ± 0.75 mm. The BSH-positive lactobacilli can be grouped in 3 classes based on the diameters of the precipitation zones. The precipitation zone up to 10 mm was demonstrated by low BSH activity; if the precipitation zone up 11 to 15 mm was demonstrated by medium; the precipitation zone is greater than 16 mm was demonstrated by high BSH activity (Mathara *et al.*, 2008). From the results, this study demonstrated that the 8 *Lb. pentosus* strains showed low BSH activity due to the precipitation zone surrounding the disces up to 10 mm.

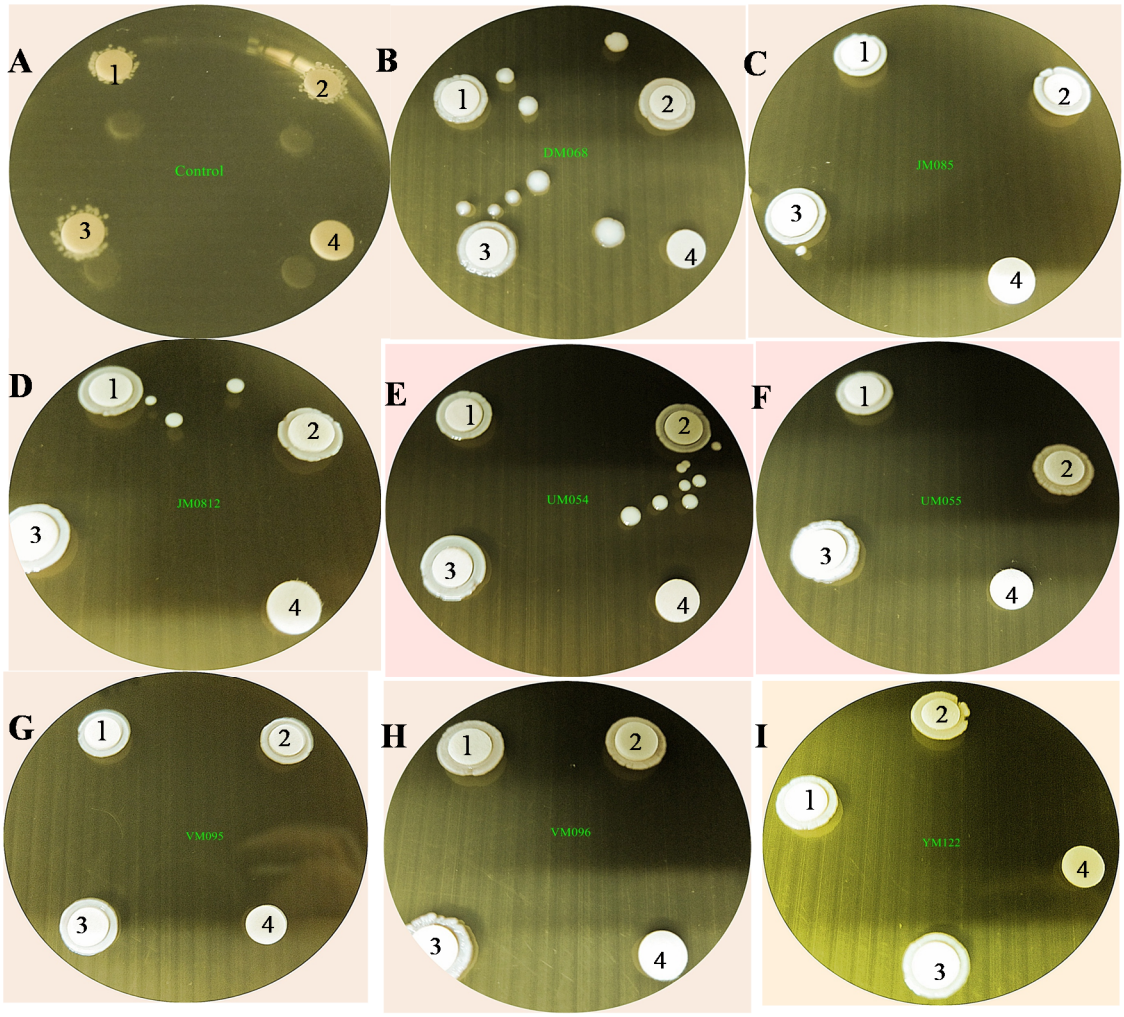

Figure 4.6 Characteristic of BSH activity by probiotic 8 *Lb. pentosus* strains on tested medium. The letters A was a control (MRS medium without 0.5% (w/v) TDCA as positive control), B-I were BSH activity by DM068, JM0812, JM085, UM054, UM055, VM095, VM096, YM122, respectively on MRS medium with 0.5% (w/v) TDCA. The sterile filter disces spotted with 10 µL cell suspensions of each strain (No. 1-3), and without spotted cell suspensions (No. 4) as a negative control.

Table 4.6 BSH activity of probiotic *Lb. pentosus* strains

Lb. pentosus strains	Diameter of precipitation zone (mm.)
DM068	8.83±0.75c
JM0812	9.33±0.52bc
JM085	9.83±0.41ab
UM054	9.33±0.51bc
UM055	9.67±0.52ab
VM095	9.92±0.58ab
VM096	10.17±0.41a
YM122	9.83±0.41ab

Notes: values are the mean ± SD of three independent experiments performed in duplicates. Different letter followed mean values in the same column indicate significant different ($p<0.05$) between the treatments ($n=6$).

These results supported other published work that tested the activity of BSH for some probiotic lactobacilli and all tested strains gave a BSH-positive. Mahrous (2011) reported that *Lb. acidophilus* P106 had greater precipitation followed by *Lb. acidophilus* P110, *Lb. plantarum* P164, and *Lb. pentosus* P191, respectively. However, Sieladie et al. (2011) found that 15 isolates of *Lb. plantarum* from raw cow milk displayed BSH activity and 4 isolates exhibited BSH activity by demonstrated precipitation zone diameter 12 to15 mm and 11 expressing precipitation zone diameter greater than 15 mm. However, Pereira et al. (2003) reported that only 5 of 14 strains of lactobacilli had shown positive BSH activity with precipitation zones different in size. The *Lb. fermentum* KC5b and *Lb. plantarum* NDV^R strain displayed the largest zones. In addition, Silirun et al. (2010) found that 4 of 16 *lactobacillus sp.* (TGCM 15, TGCM 33, SC 359 and LCC 150) displayed BSH activity by providing the precipitation zone around colonies on plate assay. The TGCM 15 and TGCM 33 strain were identified as *Lb. plantarum*. The results suggest that the BSH ability supported the mechanism for the *in vitro* lowering of cholesterol of the cells (Parvez et al., 2006; Kim et al., 2008).

Bile salt deconjugation is an important characteristic as it could play a role in maintaining the equilibrium of the gut microflora in reducing serum cholesterol and in

the production of a detergent shock protein that enables *lactobacillus sp.* to survive exposure to bile (Corzo and Gilliland, 1999). The high BSH activity of *lactobacillus* might have some role in the reduction of the serum cholesterol level. Bile excretion is a major route of eliminating cholesterol from the body, as well as one of the important pathways of cholesterol metabolism (Agaliya and Jeevaratnam, 2012). Liong and Shah (2005a) explained that BSH secreted from lactobacilli were able to catalyze the hydrolysis of glycine-conjuagted bile or taurine-conjuagted bile into amino acid residues and free bile salts (free cholic acids). Free bile salts are less soluble than conjugated bile salts (glycine-conjuagted bile or taurine-conjuagted bile), providing lower absorption in the intestinal lumen and excretion into feces, whereas amino acid group was reabsorbed into intestinal tract. Thus, free bile salts (deconjugation bile acids) can reduce serum cholesterol level by increasing the formations of new bile acids instead of those free bile acids from the enterohepatic circulation and cholesterol act as a precursos of bile salts (Ooi and Liong, 2010; Sirilun et al., 2010).

4.3 *In vitro* cholesterol binding activity

The 8 *Lb. pentosus* strains were measured for their ability to reduce cholesterol *in-vitro* in the presence of bile salts as illustrated in Figure 4.7. The amount of cholesterol reduced ranged 20.31 to 31.30 μg/mL (29.01 ± 1.38 to 44.71 ± 1.33 % reduction). All strains showed a significant ($p < 0.05$) reduction in cholesterol concentration in culture broth. The highest percentage (44.71 ± 1.33%) of cholesterol removed was recorded in strain VM096. The ability to reduce cholesterol of the strains was in the order of UM055, VM095, YM122, and UM054 (41.63 ± 1.12%, 39.34 ± 1.48%, 35.68 ± 1.75%, and 35.19 ± 1.00 %, respectively). The JM085 strain had the lowest ability to reduce cholesterol by 29.01 ± 1.38% reduction.

These results supported the other publish work that observed the ability to reduce cholesterol *in-vitro*. Hyeong et al, (2004) found that *lactobacillus* strains could remove 31.5 to 58.5% cholesterol in the MRS medium with 0.3% oxgall. However, Ramasamy et al (2009) also reported that 12 *lactobacillus* strains were varying able to remove 26.74 to 85.41% cholesterol among the strains. Sirilun et al (2010) demonstrated that the 4 *Lb. plantarum* isolated from food origins were considered as the effective probiotics with cholesterol-lowering property capable of reducing 25.41 to

81.46% from the MRS medium with 0.3% oxgall after 24 h incubation. Several work indicated that *lactobacillus* species were able to reduce cholesterol *in-vitro* via several mechanisms such as an uptake or assimilation of cholesterol by bacteria strains, cholesterol adherence to the bacteria cells wall or its incorporation into bacterial cells, including bile salt deconjugation (Gilliland et al., 1985; Liong and Shah, 2005; Silirun, 2010; Madani et al, 2013). In addition, it can be suggested that the BSH ability of microorganism supported the mechanism for the *in vitro* cholesterol binding of the cells (Parvez et al., 2006; Kim et al., 2008). This corresponded to the report that deconjugated bile salts can co-precipitate in acidic environment at pH lower than 5.5 (Klaver and Van der Meer, 1993; Mathara et al., 2008).

Hypercholesterol is a risk factor for cardiovascular diseases, which is a leading cause of death for human. A 1% reduction in serum cholesterol is estimated to result in 2-3% reduction in the risk of coronary disease. It is suggested that intestinal lactobacilli may reduce serum cholesterol level through bacterial assimilation of cholesterol in the intestine and deconjugation of bile salts. SCFAs produced by lactobacilli may also inhibit hepatic cholesterol systhesis and distribution of cholesterol in the plasma and liver (Collado, 2009)

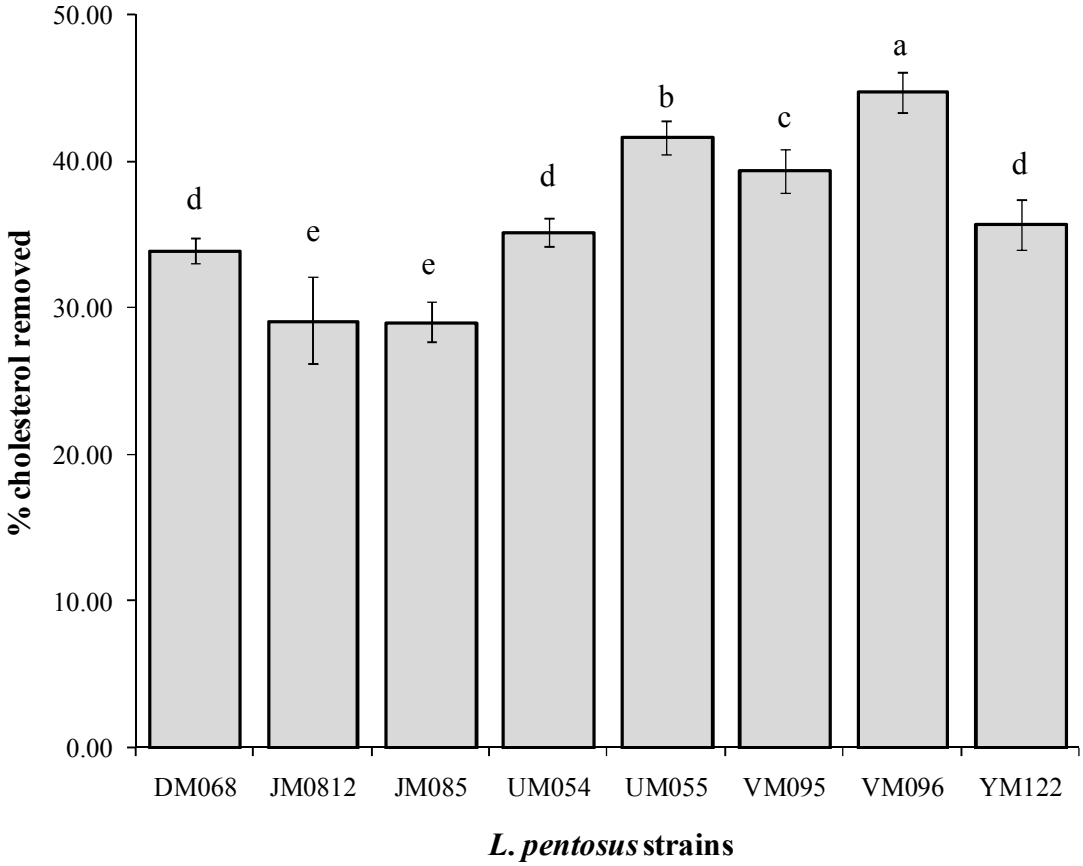

Figure 4.7 Percentage of cholesterol removed by 8 probiotic *Lb. pentosus* strains after 24 h incubation. The error bars indicated the standard deviation (SD) and different superscript letters showed significant different means ($p < 0.05$), n=3.

4.4 ZEA binding ability by *Lb. pentosus* strains in phosphate buffer

ZEA is mycotoxin, which is considered to be causes in economic loss and serious health problems humans and animals as well by contaminated in various cereals crop such as soybean, rice, corn, wheat and grain crops. In Thailand, the report about ZEA contamination in food products had little information. The method to reduce ZEA contamination have been considerable attention but some safe and efficient methods are not practical and too expensive. Several researches found that LAB were able to remove mycotoxin by binding process (Jespersen, 2006) and Zinedine et al, 2007). Therefore, our study was determined of the efficiency of ZEA binding by probiotic 8 *Lb. pentosus* strains which is a first report in Thailand.

The binding ability to ZEA of 8 strains of probiotic *Lb. pentosus* was investigated. Five-milligram dry weight of each strain was tested with various concentration levels of ZEA toxin in sodium acetate buffer solution pH 5.0. The ZEA amount recovered from supernatant indicated the binding ability of the tested strains. The low percentage of toxin remaining indicated high efficiency in adsorption of ZEA into bacterial cells. The binding ability to ZEA of test strains was shown in Figure 4.8 and Table 4.7.

As illustrated in the Figure 4.8, from the initial concentration level of ZEA at 1.10 µg/mL, the result showed 8 strains could bind ZEA more than 40%. All 8 strains were able to bind ZEA between 0.40 ± 0.04 to 0.55 ± 0.01 µg/mL (about 36.18 ± 3.48 % to 49.90 ± 0.74%). The strain *Lb. pentosus* JM085 had the greatest binding ability whereas YM122 had the lowest of binding ability. However, the concentration level of ZEA at 5.51 µg/mL, the ability of test strains to bind ZEA were between 1.35 ± 0.06 to 2.05 ± 0.03 µg/mL (about 24.46 ± 1.10 % to 37.19 ± 0.62%). At the higher initial concentration level of ZEA (23.08 µg/mL) the ability of ZEA was in the range of 7.70 ± 0.11 to10.97 ± 2.37 µg/mL (33.37 ± 0.47 to 47.50 ± 10.27 %). The strains with > 40% of ZEA binding were JM085, UM054 and DM068 (47.50%, 43.18%, and 42.78%, respectively) as present in Table 4.7.

The results of binding tests at the concentration ZEA 51.79 µg/mL found 3 strains were highest ability to bind ZEA reach to 70% that DM068, VM096 and UM055 could bind 75.17%, 70.38% and 70.00%, respectively. However, at the highest concentration level of ZEA (75.70 µg/mL), all strains of *Lb. pentosus* could bind ZEA in between 44.93 ± 16.92 to 62.12 ± 0.61 µg/mL (60.15 ± 22.56% to 83.17 ± 0.83%). We found that 3 strains had capability to bind ZEA higher than 80%. The strain that could greatest detoxify ZEA was UM0812 (83.17%) followed by JM054 (82.02%) and UM055 (81.69%). The results found that the binding abilities of the tested strains were significant different ($p < 0.05$) at various concentration levels of ZEA in buffer solution as shown in Table 4.7. At the highest concentration level of ZEA (74.70 µg/mL), 3 strains of *Lb. pentosus* (JM0812, UM054, and UM055) absorbed more than 80% of ZEA. Never the less, the concentration at 51.79 µg/mL of ZEA, the strains DM068, VM096, and UM055 had the binding capability higher than 70% up. Never the less, testing at the lower level of ZEA concentration (1.10-23.08 µg/mL), the best strain of *Lb. pentosus* could eliminate not more than 50% of ZEA. Regarding to % ZEA binding,

it can be seen that an opposite result of the lowest ZEA concentration level (1.10 μg/mL) was observed comparing to the highest levels (23.08-74.70 μg/mL). Probably the concentration at this point was too low for binding capacity and easy to get error; as a consequence, it was out of the standard curve range.

These results indicated that the binding efficiency of *Lb. pentosus* strains test depended greatly on the initial concentration of toxin in buffer solution. This finding is similar to a report of Fuchs et al (2008) which studied the binding of patulin and ochratoxin from liquid medium by LAB that the eliminations of mycotoxins from liquid medium were increased when the concentrations of the mycotoxins are higher. El-Nezami et al. (2002) stated that *Lb. rhamnosus GG* and *Lb. rhamnosus* LC-705 had ability to bind ZEA and its derivative (α-zearalenone) in liquid medium 38% and 46%, respectively. In contrast, Joannis-cassan et al (2011), who reported that mycotoxin binding by yeasts or yeast cell walls (levels 5 mg/mL), for ZEA, a decrease in the adsorption (%) was noted with the increasing initial concentration whereas AFB1 and OTA were differed with the type and initial concentration of mycotoxin.

The mechanism of mycotoxin binding by LAB has not been clearly described. LAB is gram-positive bacteria. Their thick cell wall consists of many layers of peptidoglycan protein and other components such as teichoic acid (TA), lipoteichoic acid (LTA) and polysaccharide (Delcour et al., 1999). A hypothesis stated that mycotoxin-binding positions were occurred at bacterial cell walls (Zinedine et al., 2007) and found that mycotoxin could be attached by teichoic acid and polysaccharide more than peptidoglycan (Shetty and Jespersen, 2006). These results concluded that binding ability of ZEA by the probiotic bacteria *Lb. pentosus* strains depend on the initial concentration levels of ZEA standard in the buffer solution test.

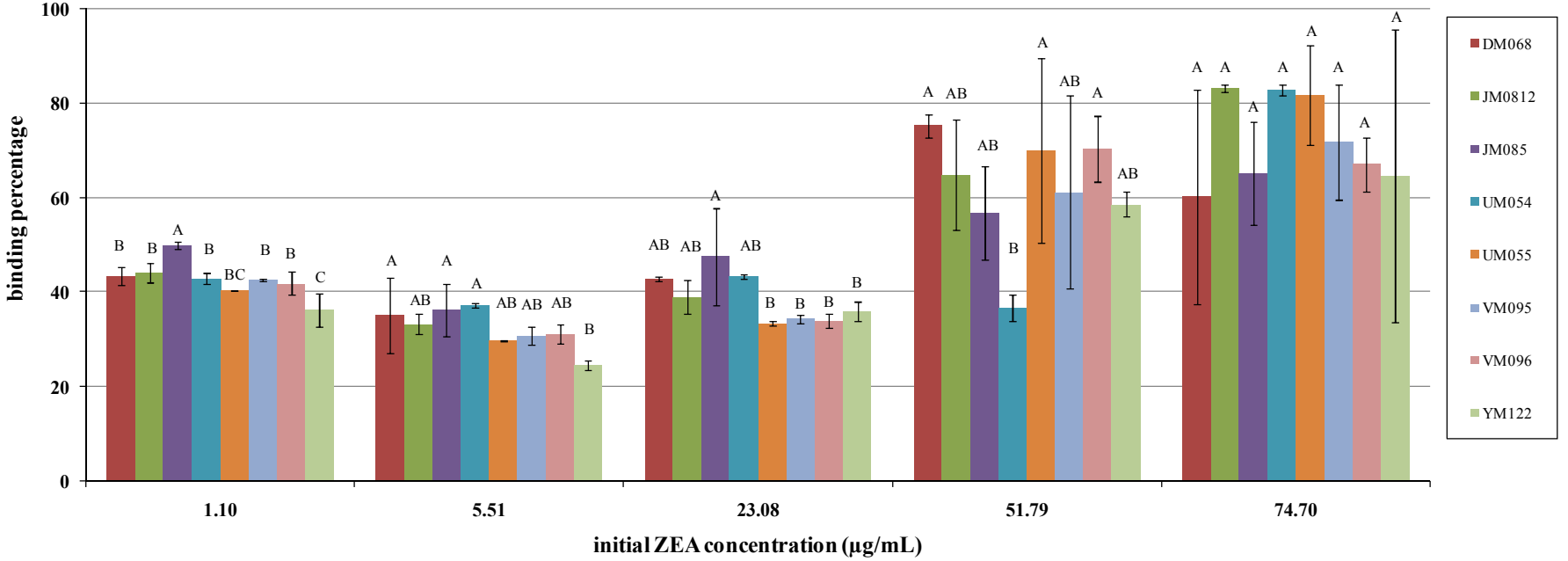

Figure 4.8 The binding ability (%) of ZEA by *Lb. pentosus* strains at 5 levels of ZEA concentration in 0.05 M sodium acetate buffer (pH 5.0). The error bars indicated the standard deviation (SD), n=2

Table 4.7 The ZEA binding ability of *Lb. pentosus* strains in buffer solution pH 5.0, ZEA Remaining concentration (C, remaining; µg/ml) and amout of ZEA adsorbtion (C, adsorbtion; µg/ml), and percentage of ZEA binding (%) at each initial toxin concentration.

Initial concentration of ZEA std (µg/ml)	Bio-adsorption assayed of *Lb. pentosus* strains							
	C, remaining (µg/ml)							
	DM068	JM0812	JM085	UM054	UM055	VM095	VM096	YM122
1.10	0.62±0.02Bb	0.62±0.02Bb	0.55±0.01Cc	0.63±0.01Bd	0.66±0.00ABc	0.63±0.03Bc	0.64±0.03Bc	0.70±0.04Ac
5.51	3.58±0.44Bb	3.68±0.31ABb	3.52±0.31Bc	3.46±0.03Bc	3.87±0.01ABc	3.82±0.11ABc	3.80±0.11ABc	4.16±0.06Ac
23.08	13.21±0.14ABab	14.09±2.37Aa	12.12±2.37Bbc	13.12±0.12ABb	15.38±0.11Aa	15.17±0.34Aabc	15.25±0.34Ab	14.78±0.48Ac
51.79	12.86±1.27Bab	18.23±5.14ABa	22.38±5.14ABab	32.79±1.46Aa	15.55±10.13Ba	20.14±3.61ABab	15.34±3.61Bb	21.46±1.37ABb
74.70	29.76±16.92Aa	12.57±8.11Aa	26.09±8.11Aa	12.87±0.80Ab	13.68±7.90Ab	21.15±4.21Aa	24.64±4.21Aa	26.52±23.12Aa
Initial concentration of ZEA std (µg/ml)	C, adsorption (µg/ml)							
	DM068	JM0812	JM085	UM054	UM055	VM095	VM096	YM122
1.10	0.48±0.02Bb	0.49±0.02Bd	0.55±0.01Ac	0.47±0.01Bd	0.44±0.00Cc	0.47±0.00Bc	0.46±0.03Bd	0.40±0.04Cc
5.51	1.93±0.44Ab	1.83±0.12ABb	1.99±0.31Ac	2.05±0.03Ad	1.64±0.01ABc	1.69±0.10ABc	1.71±0.11ABcd	1.35±0.06Bc
23.08	9.88±0.14ABb	8.99±0.81ABc	10.97±2.37Ac	9.97±0.12ABc	7.70±0.11Bc	7.92±0.20Bc	7.83±0.34Bc	8.30±0.48Bbc
51.79	38.93±1.27Aa	33.56±6.08ABb	29.41±5.14ABb	19.00±1.46Bb	36.24±10.13Ab	31.65±10.62ABb	36.45±3.61Ab	30.33±1.37Aab
74.70	44.93±16.92Aa	62.12±0.61Aa	48.61±8.11Aa	61.83±0.80Aa	61.02±7.90Aa	53.54±9.16Aa	50.06±4.21Aa	48.17±23.12Aa
Initial concentration of ZEA std (µg/ml)	ZEA Binding (%)							
	DM068	JM0812	JM085	UM054	UM055	VM095	VM096	YM122
1.10	43.37±1.95Bb	44.06±2.12Bc	49.90±0.74Aab	42.86±1.13Bb	40.27±0.01BCb	42.55±0.22Bbc	41.86±2.32Bb	36.18±3.48Cab
5.51	35.07±8.05Ab	33.22±2.21ABc	36.15±5.66Ab	37.19±0.62Ac	29.74±0.19ABb	30.68±1.86ABc	31.07±2.01ABb	24.46±1.10Bb
23.08	42.78±0.59ABb	38.97±3.50ABc	47.50±10.27Aab	43.18±0.53ABb	33.37±0.47Bb	34.29±0.86Bbc	33.92±1.47Bb	35.97±2.09Bab
51.79	75.17±2.44Aa	64.81±11.74ABb	56.78±9.93ABab	36.68±2.83Bc	69.98±19.56Aa	61.11±20.50ABab	70.38±6.97Aa	58.57±2.64ABab
74.70	60.15±22.65Aab	83.17±0.82Aa	65.08±10.85Aa	82.78±1.07Aa	81.69±10.58Aa	71.68±12.27Aa	67.02±5.63Aa	64.49±30.96Aa

Note: Values are means ± SD, n=2. Data with different capital letters in the same row and different small letters in the same column were significant different ($p<0.05$).

4.5 Adhesion ability

Probiotics are believed to temporarily colonise the intestine by adherence to intestinal surfaces. The adhesion ability can give information about the possibility of probiotics to colonise and modulate the host immune system. Several mechanisms were reported about the adhesion of microorganisms to intestinal epithelial cells. Cell hydrophobicity is one of factors that may contribute to adhesion of bacterial cells to host tissues (Savage,1992; Ram et al., 2003). This property is an advantage and importance for bacterial maintenance in the human gastrointestinal tract. Therefore, the adhesion ability of bacteria to intestinal cells has been considered as one of the selection criteria for probiotic strains (Salminen et al., 1996).

Eight strains of probiotic *Lb. pentosus* were compared for the *in vitro* cell surface hydrophobicity by determining bacterial adhesion to n-hexadecane. The assay method was modified from Rosenberg et al (1980). As a result of this study, cell surface hydrophobicity values were between 6.24% to 8.20% among bacteria tested as shown in Figure 4.9.

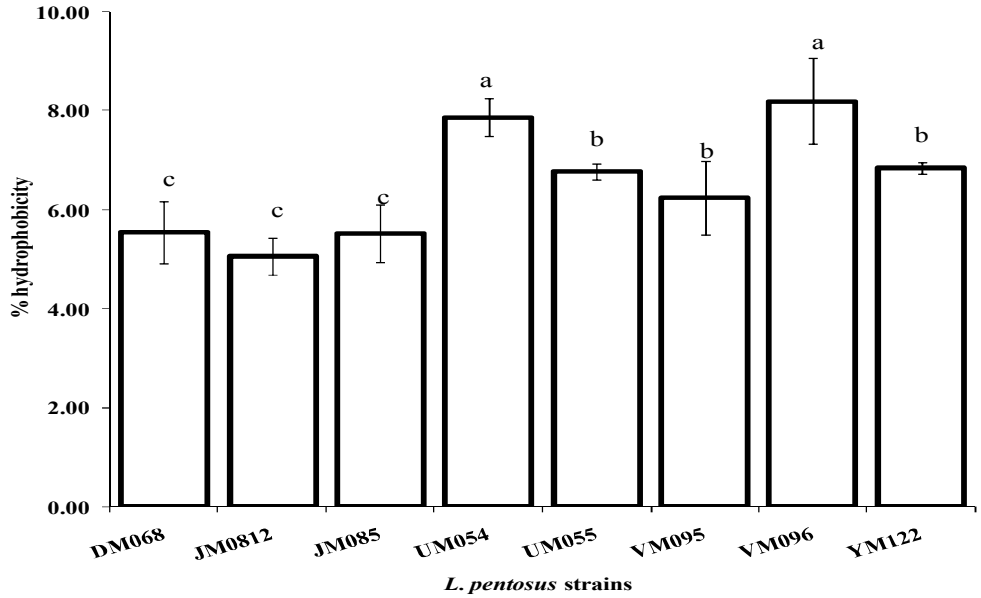

Figure 4.9 Cell surface hydrophobicity of 8 *Lb. pentosus* strains. The values are Mean ± SD of 3independent experiments performed in duplicates. The error bars indicate the standard deviation (SD) and different superscript letters are significant different ($p< 0.05$), n=6.

The results showed that the high value surface hydrophobicity of 8.20% and 7.87% were found for the strains VM096 and UM054, followed by the strains UM055, YM122, and VM095 were 6.85%, 6.77%, and 6.24%, respectively whereas strains DM068, JM0812, and JM085 were shown to have low value for hydrophobicity.

The partitioning of cells between water and hexadecane results from hydrophobic interactions between microorganisms and hydrocarbon. The percentages of adhesion to hexadecane of the strains indicate their surface hydrophobicity (Ly et al., 2010). Probiotic strains with high surface hydrophobicity might exist due to bacterial surface composition and structure. However, Schillinger et al. (2005) found that were between 2% to 94%. Strains of *Lb. acidophilus* tended to exhibit higher hydrophobicity values compared to *Lb. casei* strains and *Lb. rhamnosus* GG. The strains with a high hydrophobicity generally adhered to HT29 MTX cells at a high level but and strain with extremely low hydrophobicity of 2% was also able to adhere HT29 MTX cells at 40%. Hydrophbicity may be helpful for adhesion, but it is obviously not a prerequisite for a strong adherence capacity. Agaliya and Jeevaratnam (2012) concluded that adhesion is a complex process involving non-specific (hydrophobicity) and specific ligand-receptor mechanisms. The determination of microbial adhesion to hexadecane as a way to estimate the ability of a strain to adhere to epithelial cells is a valid qualitative phenomenological approach (Kiely and Olson, 2000). Adherence of bacterial cells is usually related to cell surface characteristics. Cell surface hydrophobicity is a non-specific interaction between microbial cells and host. The initial interaction may be weak, often reversible and precedes subsequent adhesion processes mediated by more specific mechanisms involving cell surface proteins and lipoteichoic acids (Rojas et al., 2002; Ross and Jonsson, 2002). The high hydrophobicity value of microorganism are usually associated with the presence of fibrillar structures on the cell surface and specific cell wall proteins, also cell surface hydrophobicity was related to cell age (the exponential growth phase) (Wang and Han, 2007).

Part II The use of probiotic *Lb. pentosus* as starter culture for probiotic soya beverage production.

The results from the previous section showed that the potential 3 strains of *Lb. pentosus* to be used as starter culture for soya beverage production were *Lb. pentosus* VM095, VM096, and YM122. The soya milk fermentation with a single

culture of each strain was determined at 2 h intervals over 24 h of fermentation. The pH change, the total acid production, bacterial enumeration, sugar and SCFA production by 3 *Lb. pentosus* strains in soya milk were observed.

4.6 Fermented soya milk characteristic

4.6.1 Enumeration of *Lb. pentosus* strains

Table 4.8 Enumeration of *Lb. pentosus* strains in fermented soya milk at 2 h intervals over 24 h of fermentation.

Incubation time (h)	Enumeration of *Lb. pentosus* (logCFU/ mL)		
	VM095	VM096	YM122
0	7.29±0.18b	7.43±0.14ab	7.70±0.17a
2	7.94±0.04ns	7.95±0.06ns	8.02±0.02ns
4	8.10±0.01b	8.13±0.05b	8.26±0.03a
6	9.34±0.06a	9.11±0.07a	9.18±0.11b
8	9.43±0.09a	9.25±0.06ab	9.42±0.10b
10	9.47±0.14ns	9.45±0.13ns	9.46±0.18ns
12	9.88±0.07ns	9.75±0.17ns	9.74±0.12ns
14	9.74±0.12a	9.50±0.04b	9.38±0.26b
16	9.93±0.11ns	9.98±0.06ns	9.97±0.05ns
18	10.15±0.06b	9.41±0.10c	10.32±0.01a
20	9.37±0.14ns	9.41±0.10ns	9.36±0.10ns
22	9.20±0.15ns	9.35±0.12ns	9.36±0.10ns
24	9.26±0.57ns	9.27±0.33ns	9.71±0.11ns

Note: Values are means±SD, n= 3. Different letters indicate significant different ($p<0.05$) between the treatments in the same raw, ns= non significant

The changes of cell numbers of *Lb. pentosus* VM095, VM096, and YM122 during fermentation in soya milk are presented in Table 4.8. The cell numbers of *Lb. pentosus* in the soya milk fermented samples were significantly ($p<0.05$) increased

among strains during fermentation. At 6 h, the cell numbers for VM095, VM096, and YM122 reached to 9.34±0.06, 9.11±0.079, and 9.18±0.11 logCFU/mL, respectively. Maximum counts of cell numbers occurred at 18 h of fermentation for VM095 and YM122 (10.15±0.06 and 10.32±0.01 logCFU/mL) except for VM096 (9.41±0.10 logCFU/mL). The pH values declined from pH 6.35 initially to 5.31 at the 24 h fermentation. It is well known that lactobacilli have complex growth requirements. They require low oxygen tension, fermentable carbohydrates, proteins and their breakdown products, a number of B-complex vitamins, nucleic acid derivatives, unsaturated free fatty acids, and minerals such as magnesium, manganese and iron for their growth. Soya milk contains almost all that requirements, except iron as compared by MRS medium, which can be the reason for poor growth in soymilk than growth medium. In addition, soya milk is scarce in amino acids containing sulphur and this might be reason for their poor growth (Božanić et al., 2008). Wang et al, (2002 cited in Sumarna, 2008) reported that *Lb. delbrueckii* ssp. *bulgaricus* grew poorly in soya milk because they were not able to ferment sucrose and other soy carbohydrates.

In our finding, the tested strains in this study produced low amounts of lactic acid in soya milk and slightly grow up with cell numbers (7 upto 9 logCFU/mL) from the innitial of fermentation until 24 h. However, some LAB grew well in soya milk and produced less organic acids. The low levels of acid in soya milk presumably encouraged cell growth (Liu, 1997). On the other hand, Mital et al (1974) found that *Lb. acidophilus*, *Lb. cellobiosis*, *Lb. pantarum*, which utilised sucrose, could grew well and produced large amount of acid in soya milk. Soya milk was reported previously as a appropriate growth medium for some LAB such as *Lb. plantarum pentosus* SMN, 01*, Lb. plantarum* SMN, 25*, and Lb. plantarum pentosus* FNCC, 235 (Kamaly, 1997; Liu, 1997; and Sumarna, 2008).

4.6.2 Determination of pH value and titratable acidity

This study was carried out to determine the pH value and titratable acidity of soya milk fermented by 3 probiotic bacteria. The titratable acidity was calculated as percentage of lactic acid (w/v) of soya milk fermented. During fermentation of soya milk, the titratable acidity (%TA) increased from 0.12 ± 0.05 to 0.29 ± 0.03 depending on the strain used, meanwhile pH value decreased from 6.32 ± 0.02 to 5.34 ± 0.03 as displayed in Figure 4.10.

The pH values from VM095, VM096, and YM122 strains were not significantly ($p > 0.05$) among strains tested during 24 h fermentation. The initial pH values were 6.32 ± 0.02, 6.33 ± 0.02 and 6.32 ± 0.02, respectively. The pH values of those were decresed rapidly from 4 to10 h fermentation period from pH 6.20 to 5.30. It was observed that the texture of soya milk became slightly curding. At the end, the pH values in fermented soya milk by VM095, VM096, and YM122 strains were 5.31 ± 0.00, 5.37 ± 0.00, and 5.34 ± 0.01, respectively.

However, %TA in soya milk with VM095, VM096, and YM122 was not significantly ($p > 0.05$) among strains.The acidity was 0.12 ± 0.05% at the initial hour and increased rapidly within the 12 h to reach between 0.23 ± 0.04 to 0.26 ± 0.03%. At the same time of a slight increse of pH at 14 h, acidity also slightly droped, and then the development of acidity was increased slowly between 0.27 ± 0.00 to 0.29 ± 0.03% at 24 h fermentation. The highest acidity was observed in case of VM095 (0.29 ± 0.03%) at the end of 24 h fermentation. The pH values of soya milk fermented remained quite stable due to the buffering capacity of the soy protein (Itsaranuwat, 2003). The drop of pH and increasing of acidity was confimed by the growth with maximum changeable of cell number as displayed in Table 4.8.

In general, pH of soya milk dropped from 6.0 to 5.0 or below. Our results are similar to some profiles produced by LAB such as *Lb. plantarum* SMN, 25 and *Lb plantarum pentosus* FNCC,235 which took about 24 h of fermentation to reach pH 5.2 whereas lactic acid was found to be 1.2 mg/mL (Sumarna, 2008). In contrast, Bordignon et al (2004) reported that *Lb. casei* subsp. *casei* JCM 1134, *Lb. casei* subsp. *rhamnosus* IFO3425, and *Lb. delbrueckii* subsp. *bulgaricus* IFO13953 could grow well with lower pH between 4.0-4.74. Lowering pH of the culture and production of lactic acid are essential for soya milk fermented quality (Bordignon et al, 2004) because lactic acid is one of most important compounds in formation of flavor of fermentated products such as soya milk.

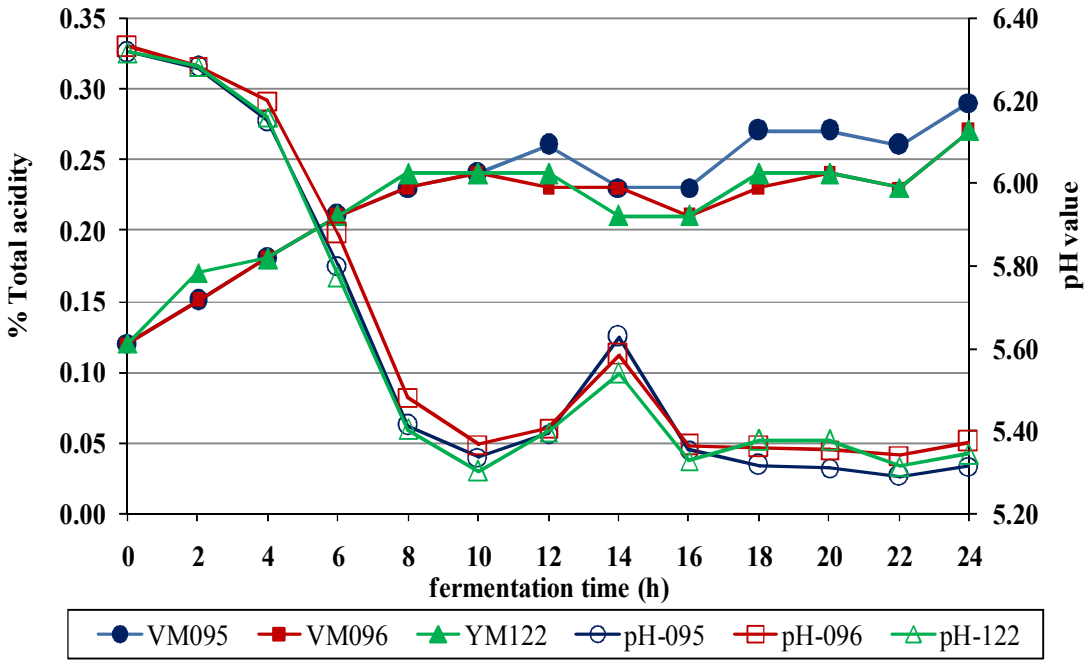

Figure 4.10 The pH change and acid production of *Lb. pentosus* strains VM095 (●=TA; ○= pH), VM096 (■= TA; □= pH), and YM122 (▲= TA; △= pH) in fermented soya milk

4.6.3 Determination of sugar contents in fermented soya milk

Soybean oligosaccharides have prebiotic effects and many reports have shown that their consumption confers to several health benefits, such as lowering of blood cholesterol, increased absorption of minerals, and prevention of some types of cancer (Roberfroid, 2007). However, one factor for the low consumer acceptability was the presence of high levels of non-digestible oligosaccharides. Stachyose and raffinose are the principal oligosaccharides in soya milk. They are believed to cause flatulence in human after eating soybean products. These sugar can be hydrolyzed by β-galactosidase. Several researches have reported that LAB can produce of galactosidase (Wang et al, 2003). *Lactobacilli* are also extensively used as probiotics. Soya milk has been examined as a substrate for the *Lactobacillus* species such as *Lb. casei*, *Lb. fermenti*, *Lb. fermentum*, *Lb. acidophilus* (Garro et al, 1999, 2004; Chumchuere and Robinson, 1999; Wang et al, 2002, 2003; Farnworth et al, 2007).

Changes of stachyose and raffinose in soya milk fermented by *Lb. pentosus* VM095, VM096, and YM122 are shown in Table 4.9. Regardless of *Lb. pentosus* strains used, levels of stachyose and raffinose slightly decreased as the fermentation

time increased, suggesting that all bacterial utilized these sugars. The strains VM096, YM122 and VM095 consumed stachyose significant different ($P < 0.05$) at the end of fermentation period by 47.88% (0.57 ± 0.05 to 0.30 ± 0.01 mg/mL), 37.74% (0.57 ± 0.03 to 0.35 ± 0.02 mg/mL) and 27.10 % (0.52 ± 0.02 to 0.38 ± 0.01 mg/mL) respectively. However, raffinose was reduced 44.50% (0.20 ± 0.02 to 0.11 ± 0.00 mg/mL), 40.56% (0.19 ± 0.05 to 0.11 ± 0.01 mg/mL) and 0% (0.12 ± 0.00 to 0.12 ± 0.00 mg/mL) respectivly. After 24 h, soya milk fermented with VM096 strain contained stachyose 0.30 ± 0.01 mg/mL. The levels were higher than those in soya milk fermented with YM122 and VM095. This showed that VM096 exploited these substrates more efficiently than YM122 and VM095.

Similar observations with soy oligosaccharide have been reported earlier, Chumchuere and Robinson (1999) observed the reduction of stachyose and raffinose levels in the fermented soya milk and they indicated that the utilisation of these sugars varied with the species of LAB. Wang et al (2003) reported that stachyose and raffinose in soya milk was utilized approximately 31.1–50.7% and 9.2–33.1% respectively by the single culture of LAB during a 24h fermentation period.

Bordignon *et al*. (2004) showed that raffinose, was substantially metabolized by LAB strains. The organisms in general metabolized stachyose by over 66% after 24 h with *Lb. plantarum SMN, 25,* and *Lb. plantarum pentosus SMN, 01* showing the highest hydrolysis of 78% and 72.5%, respectively. Moreover, Mital et al. (1974) demonstrated that fermentation of soya milk with lactic cultures possessing galactosidase activity reduced raffinose and stachyose contents.

HPLC analyses in Table 4.9 showed the sugar contents in soya milk fermented by *Lb. pentosus* VM095, VM096, and YM122. D (+) glucose, D (+) galactose, D (-) fructose and treharose contents were maintained from initial fermentation period until the end. These results demonstrated that *Lb. pentosus* strain could utilise stachyose and raffinose then breakdown into form monosaccharide and used various sugars to support their growth in soya milk fermention. On the other hand, the reduction in the content of stachyose, raffinose and an increase in the content of monosaccharide such as glucose, galactose was noted in Table 4.9. In fermented soya milk, the concentration of starchyose and raffinose decreased over time, the concentrations of D (+) glucose, and D (+) galactose in all the fermented milks increased. The D (-) fructose and treharose concentration in soya milk remained low during the 24 h fermentation. The sugar

utilisation results indicated that the 3 strains of *Lb. pentosus* have ability to use raffinose and monosaccharide such as glucose including another mono-sugar such as D (+) galactose, D(-) fructose, and treharose. This may be attributed to the hydrolysis of stachyose, raffinose during fermentation. Therefore, it is not surprising that a higher content of glucose plus galactose and fructose in soya milk cultured with LAB were observed (Chumchuere and Robinson, 1999; Wang et al, 2003; and Yang and Zang, 2009).

Table 4.9 Sugar contents in fermented soya milk by *Lb. pentosus* (VM095, VM096, YM122) strains for 24 h.

Incubation Time (h)	*Lb. pentosus* strains	Type of sugar components (mg/mL) in Soymilk fermentation								
		FOS	Maltotetraose	Starchyose	Raffinose	D(-)Maltose	Treharose	Lactose	Sucrose	D(+)Glucose
0	VM095	nd	nd	0.52±0.02ns	0.12±0.00ns	nd	0.04±0.01b	nd	nd	1.88±0.14ns
	VM096	nd	nd	0.57±0.05ns	0.20±0.02ns	nd	0.06±0.00a	nd	nd	2.02±0.17ns
	YM122	nd	nd	0.57±0.03ns	0.19±0.05ns	nd	0.05±0.01ab	nd	nd	2.20±0.29ns
6	VM095	nd	nd	0.48±0.02ns	0.12±0.01ns	nd	0.07±0.00	nd	nd	1.80±0.10ns
	VM096	nd	nd	0.49±0.01ns	0.19±0.05ns	nd	0.04±0.00	nd	nd	1.70±0.29ns
	YM122	nd	nd	0.51±0.04ns	0.14±0.02ns	nd	0.06±0.00	nd	nd	1.87±0.20ns
12	VM095	nd	nd	0.48±0.00ns	0.14±0.008ns	nd	0.04±0.00b	nd	nd	2.01±0.02b
	VM096	nd	nd	0.50±0.01ns	0.13±0.00ns	nd	0.04±0.00b	nd	nd	2.18±0.02a
	YM122	nd	nd	0.42±0.12ns	0.13±0.01ns	nd	0.06±0.00a	nd	nd	2.19±0.00a
24	VM095	nd	nd	0.38±0.01a	0.12±0.00ns	nd	0.06±0.01ns	nd	nd	2.34±0.11ns
	VM096	nd	nd	0.30±0.01b	0.11±0.01ns	nd	0.05±0.00ns	nd	nd	2.16±0.04ns
	YM122	nd	nd	0.35±0.02a	0.11±0.00ns	nd	0.06±0.00ns	nd	nd	2.23±0.09ns

Note: the meanvalues±SD in the same column, in the same time of each strain with different small letters were significant different ($p<0.05$), ns= non significant, nd= non detectable

Table 4.9 Continued

Incubation Time (h)	*Lb. pentosus* strains	Type of sugar components (mg/mL) in Soy milk fermentation									
		Myo-innositol	Mannose	D(+) Galactose	Xylose	D(-) Fructose	Mannitol	Sorbitol	L(-) Rhamnose	L(+) Arabinose	D(-) Arabinose
0	VM095	nd	nd	0.21±0.01ns	nd	0.04±0.01ns	nd	nd	nd	nd	nd
	VM096	nd	nd	0.18±0.04ns	nd	0.04±0.01ns	nd	nd	nd	nd	nd
	YM122	nd	nd	0.20±0.03ns	nd	0.04±0.00ns	nd	nd	nd	nd	nd
6	VM095	nd	nd	0.19±0.00ns	nd	0.03±0.00b	nd	nd	nd	nd	nd
	VM096	nd	nd	0.19±0.04ns	nd	0.06±0.03a	nd	nd	nd	nd	nd
	YM122	nd	nd	0.17±0.04ns	nd	0.07±0.03a	nd	nd	nd	nd	nd
12	VM095	nd	nd	0.20±0.00c	nd	0.04±0.00	nd	nd	nd	nd	nd
	VM096	nd	nd	0.23±0.00b	nd	nd	nd	nd	nd	nd	nd
	YM122	nd	nd	0.23±0.00a	nd	nd	nd	nd	nd	nd	nd
24	VM095	nd	nd	0.22±0.01ns	nd	0.05±0.00ns	nd	nd	nd	nd	nd
	VM096	nd	nd	0.23±0.01ns	nd	nd	nd	nd	nd	nd	nd
	YM122	nd	nd	0.22±0.00ns	nd	0.05±0.00ns	nd	nd	nd	nd	nd

Note: the meanvalues±SD in the same column, in the same time of each strain with different small letters were significant different ($p<0.05$), ns= non significant, nd= non detectable

4.6.4 SCFAs production in soya milk during fermentation

The amount of SCFAs was measured by HPLC using a LUNA C-18 column (4.6 x 250 mm id., 5µm) at 38 °C with UV detector and 10 mM $NaHPO_4$ buffer (pH 2.5) was used as the mobile phase at a flow rate of 1.0 mL/min. The lactic acid and SCFAs production in soya milk fermentation by *Lb. pentosus* VM095, VM096, and YM122 are presented in Table 4.10

Lactic acid levels in soya milk fermented by VM095 was higher significant different ($p < 0.05$) compared with YM122 and VM096 at 12 h. At the end, lactic acid arranged 0.59 ± 0.01 mg/mL. These results agree with the acidity values and pH changes (Figure 4.10) in fermented soya milk. In addition, the highest enumeration of *Lb. pentosus* strains in soya milk found at 12 h.

In case of SCFAs, acetic acid and propionic acid were decreased significant different ($p < 0.05$) over time fermentation 24 h, acetic acid decreased from to 9.59 ± 0.61 to 2.88 ± 0.07 µmol/mL (for YM122) and propionic acid decreased from 5.77 ± 0.68 to 3.38 ± 0.64 µmol/mL (for VM096). The concentration of the butyric acid and N-valeric acid were increased by time fermentation and iso-butyric acid was not detected the samples cultured by VM096 at the same time. At the end of fermentation, the concentration of acetic acid propionic acid, butyric acid, iso-butyric acid and n-valeric acid by VM095 had higher significant ($p < 0.05$) than VM096 and YM122 about 3.43 ± 0.08, 4.41 ± 0.39, 2.89 ± 0.25, 2.41 ± 0.05, and 28.80 ± 3.90 µmol/mL respectively. The results found that the concentration of starchyose and raffinose decrease over time. As sugar in soya milk was decreasing, the concentrations of lactic acid and some of SCFAs such as butyric, iso butyric, n-valeric acids in the fermented soya milks were increased. In our study iso-butyrate acid was identified only in some samples of soya milk fermented. Their concentrations depend on the cultured of strain used.

SCFAs are carboxylic acids with 1- 6 carbon atoms such as acetic, propionic and butyric acids are mainly formed during microbial fermentation of carbohydrate (Huda-Faujan et al, 2010). It may have specific roles, including beneficial health implications. Butyric acid was addressed to be more beneficial for promoting colonic health and more effective for stimulating the proliferation of intestinal mucosal cells than acetic and propionic acid (Henningsson et al. (2002). It also is the main energy substrate for the colonocytes and play an important role in the prevention of distal UC (Cummings, 1997), Crohn's disease, and cancer (Scheppach et al, 1995; Floch and

Hong-Curtiss, 2002) also, induce of tumor cell lines (Barnard and Warwick, 1993). In addition, SCFAs may have health-promoting effects, both locally in the colon and systemically, e.g. on glucose and cholesterol metabolism (Huda-Faujan et al, 2010).

Table 4.10 SCFAs production in fermented soya milk by *Lb. pentosus* (VM095, VM096, YM122) strains for 24 h.

Incubation Time (h)	*Lb. pentosus* strains	Type of SCFAs components (µmol/mL) in fermented soymilk					
		Lactic acid (mg/mL)	Acetic acid	Propionic acid	N-valeric acid	Butyric acid	Iso-butyric acid
0	VM095	0.42±0.01ns	7.71±0.31b	4.68±0.09ns	8.62±1.39c	0.64±0.00b	1.30±0.10b
	VM096	0.42±0.00ns	9.18±0.02a	5.77±0.68ns	22.02±2.41a	0.77±0.10b	nd
	YM122	0.45±0.01ns	9.59±0.61a	5.02±0.22ns	14.71±0.51b	1.41±0.17a	1.66±0.00a
6	VM095	0.65±0.01a	4.03±0.61b	2.76±0.05c	6.41±1.10b	0.43±0.00	2.13±0.00
	VM096	0.50±0.03b	5.18±0.40ab	4.38±0.07b	56.05±2.79a	0.43±0.00	0.00±0.00
	YM122	0.66±0.01a	5.97±0.01a	4.90±0.14a	61.09±4.69a	1.12±0.00	0.45±0.00
12	VM095	0.78±0.01a	3.54±0.33c	5.21±1.18ns	8.27±0.63c	1.42±0.09ns	0.98±0.10b
	VM096	0.70±0.01b	7.82±0.35a	4.97±0.10ns	14.26±2.30b	1.72±0.47ns	nd
	YM122	0.72±0.00b	4.69±0.21b	4.68±0.24ns	19.50±0.00a	1.64±0.30ns	1.51±0.06a
24	VM095	0.59±0.00ns	3.43±0.08ab	4.41±0.39ns	28.80±3.90a	2.89±0.25a	2.41±0.05a
	VM096	0.59±0.01ns	4.16±0.40a	3.83±0.64ns	24.62±3.21ab	2.43±0.21ab	nd
	YM122	0.59±0.01ns	2.88±0.07c	3.37±0.24ns	19.10±0.10b	2.06±0.17b	1.58±0.36b

Note: the meanvalues±SD in the same column, in the same time of each strain with different small letters were significant different ($p<0.05$)
ns= non significant, nd= non detectable

4.7 Soya beverage properties

Consumption of soybean as a food product is not very popular due to its off-flavor. The other reason is that some people feel uncomfortable upon consumption due to the presence of nondigestible oligosaccharides. To introduce soy-based products to people who are not familiar with its specific flavor and taste, it is required to develop a good flavoured soy-based food. Lactic acid fermentation would be a promising way to enhance soybean flavour because some LABs are effective to reduce the off-flavor of soybean. In addition, supplementation of soybean with flavouring or sweeteners is the other promising way to improve the products properties.

Some researchers have attempted to improve the properties of fermented milk products by addition of natural flavouring or sweeteners such as honey syrup, strawberry, corn syrup are some of the flavors that are quite acceptable. Its functional properties, honey has been gaining interest as a substitute flavouring and sweetener in foods such as yoghurt (Păucean et al., 2011; Roumyan et al., 1996; Chick, 2001). Honey is a rich source of carbohydrates (fructose, glucose, maltose, sucrose etc.). Its low pH value, due to a variety of organic acids, makes honey compatible with much food (Varga, 2006). The effect of honey addition on the 4 basic tastes (sweet, sour, bitter and salty taste). Its properties decreased the sourness of solutions and improved consumer acceptability of sour products, honey can be incorporated into fermented dairy products. Honey flavour is an important quality for its application in food industry and also a selection criterion for the consumer's choice (Bogdanov, 2008).

4.7.1 Sensory evaluation

The results summarised the sensory evaluation for appearance, color, odor, taste, mouth feel, and overall acceptance of fermented soya milk by 3 probiotic *Lb. pentosus* strains VM095, VM096, and YM122. The fermented soya milk without supplementation with 10% (w/v) honey syrup (SF095, SF096, and SF122) and fermented soya milk supplementation with 10% (w/v) honey syrup (SB095H, SB096H, and SB122H) are presented in Table 4.11. The perceived sample quality was represented by the mean score base on a 9-point hedonic scale range from dislike extremely (1) to like extremely (9) and the higher scores show the more desirable sample for panelists. Overall, the results showed that the score on odor, taste, mouth feel, and overall acceptance of SB095H, SB096H, and SB122H were significant ($p <$

0.05) higher in comparison with SF095, SF096, and SF122. The panelists could not detect any different in appearance and color among all samples. However, supplementation with honey syrup was also found to be as good as without honey syrup with the mean scores higher than 6 (like slightly). The mean scores of sensory evaluation for all attributes ranged between 3.10 ± 1.63 and 7.23 ± 1.31 representing the low-range to almost high range of the scale.

The supplementation of 10% (w/v) honey syrup in soya milk fermented showed a significant improvement of odor, taste, mouth feel compared with the samples without honey syrup. Overall acceptance were scored 4.5 (dislike slightly) for the samples without honey syrup whereas samples supplemented with 10% (w/v) honey syrup had significantly ($p < 0.05$) higher scores ranged between 6.93 ± 1.40 to 7.23 ± 1.31 which indicated 'like moderately'. In term of taste, the samples supplemented with 10% (w/v) honey syrup had significantly ($p < 0.05$) higher score ranged between 6.87 ± 1.62 to 7.05 ± 1.53 which indicated 'like moderately' in comparison with the samples without honey syrup which were scored 3.10 to 3.50 (dislike moderately). Odor was scored between 5.20 to 5.60 (neither like nor dislike) for samples without honey syrup while supplementation with 10% (w/v) honey syrup were significantly ($p < 0.05$) higher score ranged between 6.53 ± 1.83 (like slightly) to 7.00 ± 1.71 **(**like moderately). Mouth feel were scored at 5.40 to 5.50 (neither like nor dislike) for samples without honey syrup whereas supplementation with 10% (w/v) honey syrup were significantly ($p < 0.05$) higher scored between 6.83 ± 1.50 to 6.93 ± 1.49 (like moderately). Comparing the overall sensory results, samples supplementation with 10% (w/v) honey syrup showed a more promising soya beverage properties and sample SB095H was highest scored on appearance, color, odor, taste, mouth feel, and overall acceptance by 6.96 ± 1.52, 7.08 ± 1.52, 7.00 ± 1.71, 7.05 ± 1.53, 6.93 ± 1.49, and 7.23 ± 1.31, respectively.

The results obtained in this study indicated that the penelists reacted positively to the properties of soya milk added with honey syrup. The soya milk fermented had the taste and odor properties with the lowest mean score between of 3.10 to 3.53 and 5.20 to 5.60 respectively. The results suggested that soya milk fermented had the sharp/sour taste, flavor-off, which made it unfovourable for panelists. In general, fermentated milk products are characterized by lack of flavor because they possess an alcohol dehydrogenase, which converts acetaldehyde to ethanol (Itsaranuwat, 2003).

These results agreed with previous reports of Amiri (2010), studied on the symbiotic acidophilus milk prepared using starter culture (*Lb. acidophilus, B. bifidum* and *Lb. casei*) and prebiotic additives (oat, inulin, honey) singly or in combination. The results showed, sensory score significant increased with the colour, flavour, texture and overall acceptance of samples when added with inulin (10% w/v) or honey (7% w/v). Similar result was obtained by Riazi and Ziar (2012). They found that honey had a good effect on sensory properties of fermented milk with *bifidobacteria*. The points allocated for colour, body-texture and taste showed that increased in honey content brought about an improvement in the texture, flavour and aroma of the products ($p < 0.05$). On the other hand, Păucean et al. (2011) reported honey addition. They investigated that at the beginning of storage, taste and flavor intensity of kefir-type product has increased significantly ($p < 0.01$) with the honey's level addition. Panelists founded that kefir-type product with 1% (w/v) honey was weak in taste and flavour but the 4% (w/v) honey level was founded too sweet. The flavour intensity of the sample with 2.5% (w/v) added honey was considered optimium. The odour, the colour and the appearance values had no significant ($p > 0.01$) affected by honey addition.

Table 4.11 Comparative sensory evaluation of fermented soy milk (SF) with *Lb. pentosus* strains (VM095, VM096, and YM122), and soya beverage (SB) supplementation with 10% (w/v) honhey syrup (H).

Sample	Appearance^{ns}	color ^{ns}	odor	taste	Mouth-feel	Overall acceptance
SF095	6.58±1.65	7.10±1.45	5.60±1.86b	3.53±1.74b	5.40±1.88b	4.55±1.71b
SF096	6.75±1.64	7.08±1.42	5.20±1.74b	3.10±1.63b	5.48±1.77b	4.58±1.55b
SF122	6.63±1.56	7.00±1.60	5.38±1.73b	3.20±1.77b	5.50±1.77b	4.53±1.43b
SB095H	6.96±1.52	7.08±1.52	7.00±1.71a	7.05±1.53a	6.93±1.49a	7.23±1.31a
SB096H	6.50±1.78	6.53±1.77	6.60±1.69a	6.97±1.56a	6.90±1.43a	6.93±1.40a
SB122H	6.60±1.86	6.53±1.92	6.53±1.83a	6.87±1.62a	6.83±1.50a	6.93±1.61a

Note: Values are the mean ± SD within the same column followed by the different letter indicate significant differences (p<0.05) between the treatments, n = 40. 9= like extremely and 1= dislike extremely

- Soya milk fermented by *Lb. pentosus* VM096, VM096, and YM122 strain without 10% (w/v) honey syrup (coded; SF095, SF096, and SF122, respectively).

- Soya milk fermented by *Lb. pentosus* VM096, VM096, and YM122 strain supplementation with 10% (w/v) honey syrup were soya beverage (coded; SB095H, SB096H, and SB122H, respectively).

4.7.2 Survival of probiotic strains and pH change during storage periods

The survival of probiotic *Lb. pentosus* strains (VM095, VM096, and YM122) and changes of pH in soya beverage (SB095H; SB096H, and SB122H), fermented soya milk (SF095, SF096, and SF122) was investigated during storage 7 day intervals (0, 7, 14, 21, and 28) over 28 days in the refrigerator at 4 ^{o}C. Total colony counts using pour plate techinique and selective media (MRS agar) was used to determine change in viable counts. The changes in viable counts of VM095, VM096, and YM122 strains and pH changes during storage was showed in Table 4.12. The viable cells of *Lb. pentosus* VM095, VM096, and YM122 strain in soya beverage and fermented soya milk were significant ($p < 0.05$) increased. The highest survivals of cells were found in the 7-14 days and survival in soy beverage was higher than fermented soya milk. The result found that the viable cells of all strains were reached 10 logCFU/mL. The initial cell counts were 10.72-10.94 logCFU/mL. Cells was increased between 12.56-13.05 logCFU/mL in 7 days storage. In 21 days, an increasing in viable cells were found in all samples due to their have ability to utilise honey sugar (fructose, glucose, maltose, sucrose etc.) and soybean sugar especially galacto-oligosaccharide, whereas the latter microorganism lacks this ability (Itsaranuwat, 2003). Moreover, the various oligosaccharides found in honey may be responsible for enhanced the growth of some bacterial strains (*Lactobacillus, Streptococcus, Bifidobacterium)* (Bogdanov, 2008). The addition of honey to fermented soya milk was significant ($p < 0.05$) on the growth and survival of *Lb. pentosus* strains at 14 days storage. The final, 28 days storage found that the viable counts of all samples decreased as same as the cells at innitial due to the long time storage, as the storage time increased, the viable counts of bacterial decreased considerably (Sodini et al, 2002).

The pH values of soya beverage was lower than fermented soya milk as presented in Table 4.12. In the presence of honey, pH of SB095H, SB096H, and SB122H were significant different ($p < 0.05$) during storage at 4°C for 28 days. The pH values of SB095H, SB096H, and SB122H decreased from 4.77 ± 0.03 to 3.49 ± 0.02, 4.79 ± 0.02 to 3.64 ± 0.04, and 4.80 ± 0.03 to 3.64 ± 0.04, respectively. It was lower than fermented soya milk without honey due to variety of organic acids in honey (Varga, 2005).

The results supported by Bogdanov (2008), pH of milk products added with 1% and 2.5% honey were decreased by 0.22 and 0.19 units respectively. In any of the

studied levels 1% to 4%, the honey addition increased the viable counts of lactococci at all sampling intervals and bacteria were present at sufficiently high level by 7-8 logCFU/mL during the product shelf-life.

Table 4.12 Survival of *Lb. pentosus* strains in fermented soya milk and soya beverage added 10%(w/v) honey syrup.

Parameter	**Storage time (days)**	Survival of *Lb. pentosus* strains*					
		SF095	SF096	SF122	SB095H	SB096H	SB122H
Viable cell counts (log10 CFU/ mL)	0	10.80±0.06d	10.72±0.07e	10.94±0.04e	10.75±0.05e	10.81±0.09d	10.91±0.08c
	7	12.97±0.02a	12.92±0.08a	12.91±0.02a	12.56±0.07b	12.89±0.04b	13.05±0.04a
	14	12.70±0.08b	12.80±0.07b	12.68±0.07b	13.19±0.13a	13.27±0.03a	13.04±0.10a
	21	12.11±0.14c	12.07±0.03c	12.02±0.04c	12.23±0.04c	12.16±0.11c	12.19±0.06b
	28	10.89±0.08d	11.22±0.06d	11.39±0.03d	11.14±0.12d	10.60±0.07e	10.56±0.06d
pH values	0	5.28±0.03a	5.32±0.04a	5.32±0.02a	4.77±0.03a	4.79±0.02a	4.80±0.03a
	7	4.91±0.02b	4.90±0.01b	4.90±0.01b	3.75±0.03b	3.89±0.04b	3.87±0.02b
	14	4.90±0.01b	4.88±0.02b	4.90±0.01b	3.69±0.01c	3.84±0.02c	3.84±0.04b
	21	4.89±0.01b	4.88±0.02b	4.89±0.02b	3.59±0.01d	3.78±0.01d	3.75±0.03c
	28	4.82±0.02c	4.81±0.01c	4.85±0.02c	3.49±0.02e	3.64±0.04e	3.64±0.04d

Note: Values were Means ± SD from 3 replication. Different letters that followed numbers within the same column in the same parameters indicated significant different (p<0.05) between the treatments. Storage in a refrigerator at 4 ^{o}C for 28 days. *SF095, SF096, and SF122 = cultured with VM096, VM096, and YM122 strain without 10% (w/v) honey syrup. *SB095H, SB096H, and SB122H = cultured with VM096, VM096, and YM122 strain supplementation with 10% (w/v) honey syrup.

About 100 mL of each fermented soya milk (SF) and probiotic soya beverage (SB) were kept in 250 mL of steriled Duran bottles. All six batches were held at 4 ^{o}C in refrigerator for a period 4 weeks (28 days). During the storage time, samples were taken out at 7-day intervals (days 0, 7, 14, 21, and 28) to examine the survival of the probiotic strains and determine pH value. Total colony counts were performed using the pour plate technique and selective media (MRS agar) to determine pH changes and viable counts. Briefly, 1 mL of each sample was taken from each SF and SB. Serial tenfold dilution were prepared in a solution of 0.85 % NaCl (w/v) and suitable dilutions were placed on MRS agar which were subsequently plated (in duplicate) and incubated for 48 h at 37 °C, 5 % CO_2 incubator.

3.3 Statistical analysis

Collected data from the experiments were statistically analyzed by Analysis of Variance using a randomized completely designs with three replications. Experimental results are given as means plus the standard deviation of three parallel measurements. Analysis of variance were conducted to identify differences among means and Duncan's Multiple Range test (DMRT) was employed. All of the statistical analysis was conducted by the SPSS statistical. The level of significant difference was defined at $p<0.05$.

CHAPTER 5

CONCLUSION

The aims of this study were to: (1) Investigate health-promoting properties of the probiotic *Lb. pentosus* strains (2) produce a probiotic soya beverage and study its properties, evaluate its sensory properties, and product's shelf life. To determine the growth profile and pH changes in MRS medium contain different sugar followed by determination of sugar utilisation and SCFAs production by HPLC technique. Then, health-promoting properties were determined such as screening BSH activity and *in vitro* cholesterol binding activity, ZEA binding ability, and the *in vitro* adhesion ability by cell surface hydrophobicity. Then, the tested strains were selected to be inoculants for soya milk fermentation and produce a probiotic soya beverage. The fermented soya milk properties was investigated by enumeration of tested strains, acidity and pH values, sugar utilisation and SCFAs production. Study on soya beverage properties, sensory evaluation test and survival of probiotic during storage 28 days were applied. Finally the following results were obtained;

(1) The growth, sugar utilisation and SCFAs production of *Lb. pentosus* in MRS media were dependent on type of sugar present as well as the strain of *Lb. pentosus.* Five of eight tested strains (UM054, UM055, VM095, VM096 and YM122) had ability to growth in oligosaccharide (lactose, raffinose and FOS-MRS) better than monosaccharide (glucose). The maximum growth at 12-15 h found in oligosaccharide sugars, reached 2.72-2.91, whereas OD in glucose 2.54-2.62. The pH values at 24 h were achived 3.60-3.70. On the other hand, all strains had a higher capacity utilisation of monosaccharide than oligosaccharide (significant different). The consumption of glucose (94.83 %) greater than raffinose (68.78%), lactose (68.34 %), and FOS (22.65 %). However, this study found those 5 strains were expressed the capability to produce organic acid and SCFAs were lactic acid (16.64-18.13 mg/mL), acetic acid (89.02-98.49 µmol/mL), propionic acid (38.31-64.70 µmol/mL), butyric acid (37.82-46.08 µmol/mL), Therefore, hightlight of probiotic selection focusing on probiotic properties. Oligosaccharide is prebiotic sugars and it can be used as a carbon source to promote the growth of probiotic *Lb. pentosus* strains and the main end-products from carbohydrate fermentation by probiotic are SCFAs which can contribute to health benefits.

(2) For BSH activity and *in vitro* cholesterol binding activity, 8 *Lb. pentosus* strains exhibited BSH activities as demonstrated by precipitation zones ($p < 0.05$) with diameters between 8.83 mm to 10.17 mm. The greatest precipitation zone was found in VM 096 followed by VM095, YM122, respectively. The amount of cholesterol reduced ranged 20.31 to 31.30 μg/mL (29.01 to 44.71 % reduction). All strains showed a significant ($p < 0.05$) reduction in cholesterol concentration in culture broth. The highest percentage of cholesterol reduced was VM096 follow by UM055, VM095, YM122, respectively. In this study, the VM096, VM095, and YM122 strains have shown moderate to good for the cholesterol reducing activity relate by BSH enzyme activity from culture media. Those of 3 strains exhibited to decrease cholesterol from media. *lactobacillus* species were able to reduce cholesterol *in-vitro* via several mechanisms such as an uptake or assimilation of cholesterol, cholesterol adherence to cells wall or its incorporation into cells, also bile salt deconjugation (Liong and Shah, 2005; Silirun, 2010). Reduction of cholesterol, in the added cholesterol media is considered as an indication for the selection of probiotic strains with cholesterol assimilation property (Gilliland and Walker, 1990; Lin and Chen, 2000).

(3) *In-vitro* testing of probiotic 8 *Lb. pentosus* strains for ZEA binding and adhesion ability by cell surface hydrophobicity confirmed their health-promoting properties. The binding abilities of 8 tested strains showed significant ($p<0.05$) at various concentration levels of ZEA in buffer solution. At the highest concentration level of ZEA (75.70 μg/mL), all strains could bind ZEA in between 44.93 to 62.12 μg/mL (60.15 % to 83.17%), 3 strains that could greatest detoxify ZEA higher than 80% was JM0812 followed by UM054 and UM055. Never the less, testing at the lower level of ZEA concentration (1.10-23.08 μg/mL), the best strain could eliminate < 50% ZEA. This results supported by Joannis-cassan et al (2011), the level of adsorbent 5.0 μg/mL was sufficient to bind at least 20% of toxins. However, *Lb. rhamnosus GG* and *Lb. rhamnosus* LC-705 had ability to bind ZEA in liquid medium 38% and 46%, respectively (El-Nezami et al., 2002). ZEA is 1 of 5 mycotoxins which are considered to be important in human health and causing economic losses by contaminated in various cereals crop such as soybean, rice, corn, wheat and grain crops (Yazar and Omurtag, 2008; Zinedine et al. 2007) also indirect source of exposure for humans and significant hazard to food chain. The contamination of ZEA showed highest in north Asia (63%), Southeast Asia was second (37%), and South Asia (4%) of positive samples. However, the average amount of ZEA contaminate in Asia was 129 mg/mL, which was within range of the maximum limits

regulated in Asian countries (Anukul et al, 2013). ZEA contamination in Thailand had few informations than other mycotoxins such as AFB1 and OTA. Although, the method to reduce ZEA contamination have been considerable attention but several safe and efficient methods are not practical and too expensive. Therefore, these study of binding ZEA efficiency by probiotic *Lb. pentosus* strains in our study is a first report in Thailand.
For cell surface hydrophobicity test, VM096 shown the highest value follow by UM055 and UM054 while, DM068, JM0812, and JM085 were indicated of low value. The cell surface hydrophobicity values were range 6.24% to 8.20% and had significant ($p<0.05$) among the tested strains. Meanwhile, probiotic which had the high adhesion ability, it possible to colonize and modulate the host immune system. *Lactobacillus* strain has beneficial health effects in humans by the protection against toxins contained in foods (Ouwehand et al., 2002; Saxelin et al., 2005; Fuchs et al., 2008). Cell hydrophobicity could indicate importance for bacterial cells adhesion and maintenance in the human gastrointestinal tract.

Based on the growth profile, sugar utilisation and health-promoting properties of the probiotic *Lb. pentosus* strains from this study, 3 strains were selected to be inoculants for soya milk fermentation. The VM095, VM096, and YM122 were selected to be a starter culture due to the strains were efficiency to use of soy oligosaccharide with exhibited a good growth profile and SCFAs production including the efficient probiotics for health benefit promoting. It could be provide the health benefit promoting on the consumer or provide the potential starter culture of fermented foods or beverages. All these reason will lead to select those strains to be the starter for produce the soya beverages.

(4) Three samples of fermented soya milk which single culture inoculated by VM095, VM096, and YM122 were determined. %TA, pH value had no significant while, maximum increased of cells number greater than 9 log CFU/mL and showed significant ($p<0.05$). The pH values in fermented soya milk arranged 5.31- 5.37 and the acidity between 0.27-0.293%. The VM096 strain exploited these substrates more efficiently than YM122 and VM095. As stachyose and raffinose were decreasing whereas, the concentrations of D (+) glucose, and D(+) galactose in all the fermenting milks increased. In case of lactic acid and SCFAs production, VM095 was significant ($p<0.05$) higher level than YM122 and VM096 From this results confirmed that the fermented soya milk with 3 of *Lb. pentosus* strains could provide the potential health-promoting properties effect on consumer. Thus, all these will produce the soya beverage with improved it properties for consumer acceptance.

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BIOGRAPHY

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Mahasarakham University Scholarships under the program Research for Graduates Research in 2013

(5) The soya beverage products were considered using the 9-point hedonic scale by the 40 tasters. The supplementation 10% (w/v) of honey syrup showed significant ($p<0.05$) higher acceptance score (odor, taste, mouth feel and overall) and viable cells than the samples without honey syrup. The soya beverage inoculated with VM095 strain had the best properties of sensory score and the viable cells reached the maximum to 10 log CFU/mL, pH value 3.49±0.02 store 4 ^{o}C for 28 days storage. This study confirmed of *Lb. pentosus* had ability to growth in soya beverage during storage to maintain high amount of viable cells relate to the suitable dosage used of probiotic microbes recommended at least 6 log CFU/mL of viable cells. Moreover, the starter culture properties and honey syrup with health promoting are usefulness. Therefore, these soya beverage products are very sufficient and suitable for health conscious consumers, vegetarians, and lactose-intolerant consumers.

APPENDICES

APPENDIX A

Culture Media and Chemicals

1. MRS Broth

Ingredients	g/L
Pepton	10.0
Meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1.0 ml
K_2HPO_4	2.0
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.2±0.4. Medium was dispensed into Duran bottles and sterilized by autoclaving at 121°C for 15 min.

2. MRS Agar

Ingredients	g/l
Pepton	10.0
Meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1.0 ml
K_2HPO_4	2.0
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Agar	15.0
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.2±0.4. Medium was sterilized by autoclaving at 121°C for 15 min.

3. Modified-MRS media for testing the sugar utilization

Ingredients	g/l
Pepton	10.0
Meat extract	10.0
Yeast Extract	5.0
Sugar	20.0
Tween 80	1.0 ml
K_2HPO_4	2.0
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Deionized water	1000 ml

** For sugar utilization, in this study used Lactose, Raffinose, and FOS compared with glucose as a control

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 min.

APPENDIX B

Questionnaires for Sensory Evaluation

9-Point Hedonic Scale

Name……Date……Time……...

Product Name **Probiotic soya beverage**

Please rinse your mouth with water before starting observe and taste samples. Observe, tasting and write down the liking scores.

Liking scores

9 = Like extremely
8 = Like very much
7 = Like moderlately
6 = Like slightly
5 = Neither like nor dislike
4 = Dislike slightly
3 = Dislike moderately
2 = Dislike very much
1 = Dislike extemely

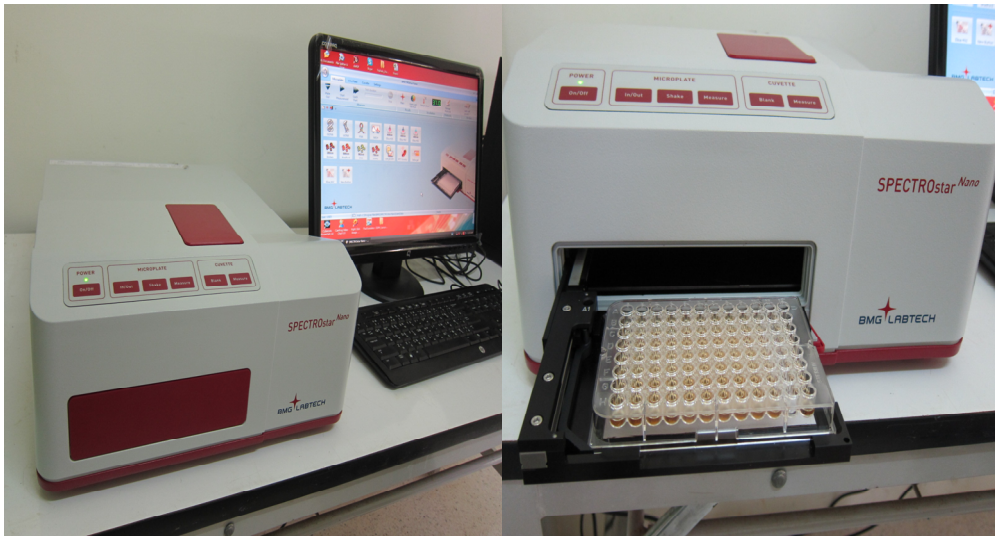
Sample code	Attributes					
	Appearance	color	odor	taste	Mouth-feel	Overall acceptance

Suggestion……
……
……

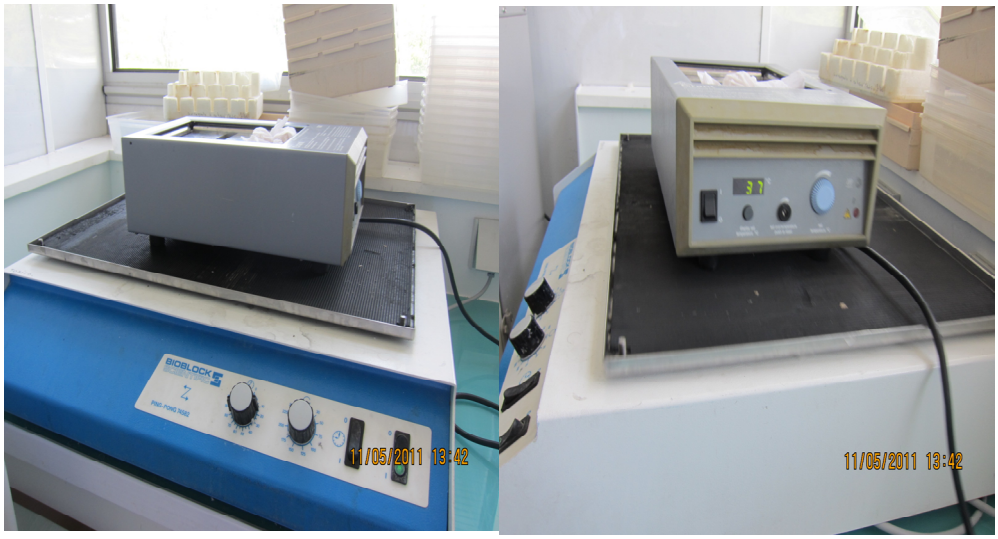
Thank you for your participation

APPENDIX C

Instrument and soya beverage products



UV-Vis spectrophotometer microplate reader (SPECTROstar Nano)



Thermostatically controlled shaker (Bioblock Scientific Ping-Pong 74582)

Densimat (bioMérieux, Marcy l'Etoile, France)

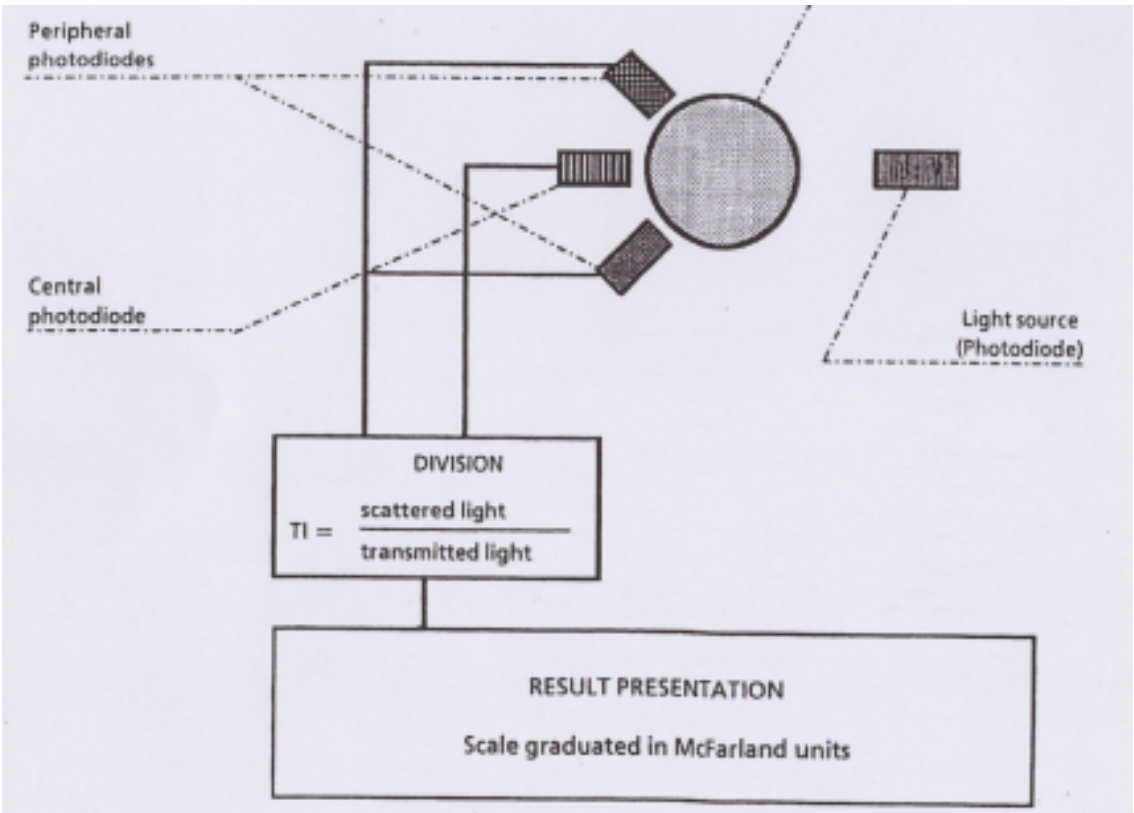


Fig. 1 - Reading Principle of ***DENSIMAT***

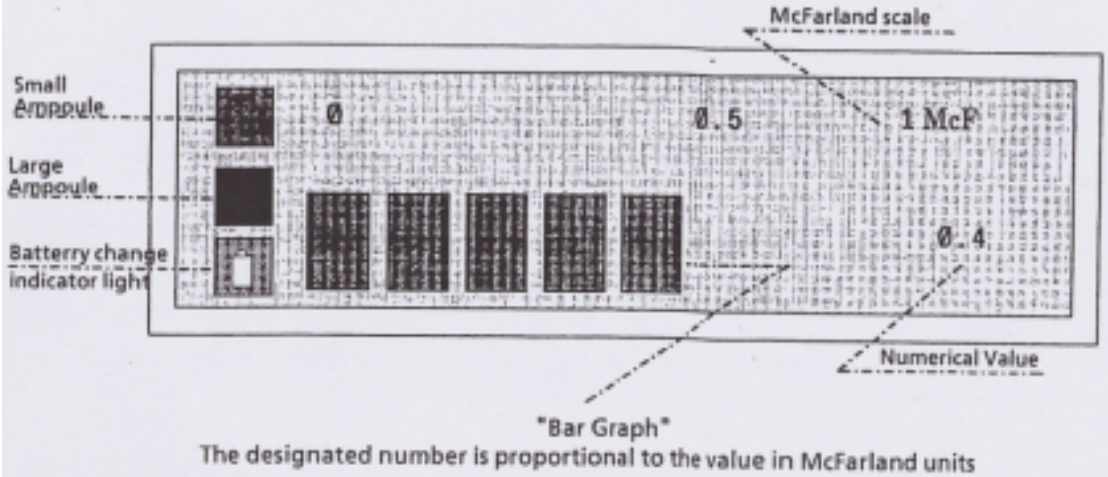


Fig. 2 - ***DENSIMAT*** Display Panel

DENSIMAT enables precise determination of bacteria density by two measurements.

An incident beam of light is passed through the ampoule and two subsequent measurements are obtained:

- Scattered light S (two photodiodes).
- Transmitted light T (one photodiode).

The ratio S/T is directly proportional to the density of the bacteria suspension.

The results are displayed on a mobile graduated scale on a liquid crystal display. The number of squares displayed is proportional to the McFarland value. The corresponding numerical value is shown on the right hand side of the display.

Correspondence McFarland scale/Bacterial concentration/Optical density:

Standard McFarland Scale	Bacterial concentration (1) $\times 10^8$ / mL	Theoretical Optical Density (2) at 550 nm
0.5	1.5	0.125
1	3	0.25
2	6	0.50
3	9	0.75
4	12	1.00
5	15	1.25
6	18	1.50
7	21	1.75

(1) The bacterial concentration varies according to the size of the microorganisms. The figures which are reported represent a mean, valid for the bacteria. For the yeasts, due to their bigger size, these figures have to be divided by 30.

(2) The values correspond to the optical densities of bacterial suspension. The Barium Sulphate (BaSO4) solutions do not present the same optical density since the size and the form of the particles are different from those of bacteria. The light is also diffracted differently.

Innoculum preparation

Fermented Soya Milk

Probiotic Soya Beverage

APPENDIX D

Linear equation and Standard Curves

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1. Linear equation of standard carbohydrates

Order	Carbohydrates	Retention time (min)	Linear equation	R^2
1	FOS	7.543	Y=192,413x-195,907	1.000
2	Maltotetraose	7.990	Y=31,815x+4,205	0.999
3	Starchyose	8.238	Y=255,096x+6,308	1.000
4	Raffinose	9.120	Y=207,604x-12,767	1.000
5	D(-)Maltose	9.272	Y=349,107x+22,798	1.000
6	Treharose	9.285	Y=316,795x+8,251	1.000
7	Lactose	9.465	Y=329,458x-20,649	1.000
8	Sucrose	trace	-	-
9	D(+)Glucose	11.248	Y=345,487x-30,292	1.000
10	Myo-innositol	11.746	Y=337,576x+19,339	1.000
11	Mannose	11.905	Y=335,045x-22,677	1.000
12	D(+) Galactose	11.931	Y=312,622x+6,497	1.000
13	Xylose	12.042	Y=326,602x+3,026	1.000
14	D(-)Fructose	12.104	Y=341,073x-9,360	1.000
15	Mannitol	12.510	Y=315,119x-1,438	1.000
16	Sorbitol	12.688	Y=274,728x+1,715	1.000
17	L(-)Rhamnose	12.742	Y=277,311x-1,713	1.000
18	L(+)Arabinose	13.031	Y=298,992x+3,359	1.000
19	D(-)Arabinose	13.032	Y=290,168x-12,189	1.000

2. Linear equation of standard Lactic acid and SCFAs

Order	SCFA	Retention time (min)	Linear equation	R^2
1	Acetic acid	3.378	y = 4E+07x - 16049	0.900
2	Lactic acid	3.612	y = 6E+07x - 73861	0.999
3	Propionic acid	8.608	y = 5E+07x - 13881	0.900
4	n- Valeric acid	15.610	y = 3E+06x + 23708	0.900
5	Bytaric acid	21.940	y = 6E+07x - 25967	0.900
6	Iso- bytaric acid	22.906	y = 8E+07x - 35802	0.900

3. Standard curve for the responses (peak areas) to Zearalenone doses (µg).