GROWTH PROPERTIES AND BIOACTIVITIES OF LACTOBACILLI IN SOYMILK SUPPLEMENTED WITH B-VITAMINS AND UPON PHYSICAL TREATMENTS

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UNIVERSITI SAINS MALAYSIA
2013

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by

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Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy

May 2013

ACKNOWLEDGMENT

I would like to express my deep and sincere gratitude to my principal supervisor, Assoc. Prof. Dr. Liong Min Tze, for her persistent support and invaluable guidance throughout my studies. Her constructive knowledge and extensive discussions around my work have been of great value for me.

I would also like to thank my co-supervisor, Mdm Wan Nadiah Wan Abdullah and Professor Abdul Karim Alias for all the detailed and helpful comments throughout this work.

I wish to thank the laboratory staff, Mr. Kamaruddin, Mr. Asmaizan and Mr. Maroof among others, for their technical help and also their patience with my persistent and instrumental demands. On top of that, I am extremely grateful to my fellow colleagues and friends for their on-going support; Siok Koon Yeo, Huey Shi Lye, Wai Yee Fung, Chiu Yin Kuan, Ting Jin Lim, Lay Gaik Ooi, Sue Siang Teh, Hui Ting Chan and Sze Yin Sim, who have provided me with advice and encouragements.

I am grateful to acknowledge the financial assistance provided by Universiti Sains Malaysia via the USM RU grant (1001/ PTEKIND/ 811020), USM IPS Research Fund Grant (1002/CIPS/ATTR 3100) and the USM fellowship.

Finally, the most special thanks to my family members for their understanding and endless support that has been my source of strength all the way through the completion of this research.

EWE JOO ANN

MAY 2013

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LIST OF ABBREVIATIONS

ACE = Angiotensin-I Converting Enzyme
ADP = Adenosine diphosphate
ANOVA = Analysis of Variance
ANS = 8-anilino-1-napthalenesulphonic acid

BSH = Bile salt hydrolase
BT = Bioprocess Technology

Ca-EDTA = Calcium-ethylenediamine tetraacetic acid
CEP = Cell envelope protein
CHO = Chinese hamster ovary
CoA = Co-enzyme A
CPD = Cyclobutyl pyrimidine dimers

DBP = Diastolic blood pressure
DNA = Deoxyribonucleic acid
DPH = 1,6-dephenyl-1,3,5-hexatriene
DV = Daily value

FAD = Flavin adenine dinucleotide
FAn = Fluorescence anisotropy
FAO = Food and Agriculture Organization
FMN = Flavin mononucleotide
FTDC = Food Technology Division Culture
FTD = Food Technology Division

GSH = Glutathione-S-transferase
GTP = Guanosine triphosphate

HDL = High density lipoprotein
Hip-His-Leu = Hippuryl-L-Histidyl-L-Leucine Hydrate
HMDS = Hexamethyldisilazane
HPLC = High Performance Liquid Chromatography

IBD = Inflammatory bowel diseases

LAB = Lactic acid bacteria
LDL = Low density lipoprotein

MDA = Malondialdehyde
MRS = de Mann, Rogosa, Sharpe
MRSA = Methicillin-resistant *Staphylococcus aureus*

NAD/NADH = Nicotinamide adenine dinucleotide
NADP/NADPH= Nicotinamide adenine dinucleotide phosphate
ND = Not Detected

OD = Optical density
OPA = o-phtaldialdehyde

pABA = *para*-aminobenzoic acid
PBS = Phosphate-buffered saline
PI = Propidium iodide
PRP = penicillin-resistant *Pneumococcus*

RAS = Rennin-angiotensin system
RDI = Recommended Daily Intake
RFO = Raffinose family oligosaccharides
ROS = Reactive oxygen species

SBP = Systolic blood pressure
SDS = Sodium dodecyl sulfate
SEM = Scanning electron microscopy
sIgA = Secretory immunoglobulin A

TCA = Tricarboxylic acid
TMA-DPH = 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene

USDA = United States Department of Agriculture
UV = Ultraviolet

WHO = World Health Organization

VRE = Vancomycin-resistant *Enterococcus faecalis*

SIFAT PERTUMBUHAN DAN BIOAKTIVITI LAKTOBACILLI DALAM SUSU SOYA YANG DITAMBAH VITAMIN B DAN SELEPAS PENGOLAHAN FIZIKAL

ABSTRAK

Ciri-ciri pertumbuhan, potensi bioaktif dan bio-penukaran isoflavon dalam susu soya diperkaya dengan vitamin B bagi *Lactobacillus acidophilus* BT 1088, *L. fermentum* BT 8219, *L. acidophilus* FTDC 8633 dan *L. gasseri* FTDC 8131 dinilaikan. Kesannya ke atas sifat pertumbuhan didapati bergantung pada strain bakteria serta jenis vitamin B yang ditambahkan. Pertumbuhan sel meningkat kepada hitungan viabel melebihi 7 $\log_{10}$ CFU/mL. Aktiviti spesifik α-galaktosidase meningkat dengan penambahan vitamin B lalu meningkatkan hidrolisis oligosakarida soya, diiringi peningkatan penghasilan laktat dan asetat setelah fermentasi. Aktiviti proteolitis *Lactobacillus* meningkat, menyumbang kepada penghasilan susu soya diperkaya vitamin B terfermen dengan aktiviti perencatan ACE yang lebih tinggi. Aktiviti spesifik β-glukosidase juga meningkat, menyebabkan peningkatan kepekatan aglikon dalam susu soya yang diperkaya.

Penambahan biotin yang telah meningkatkan kandungan aglikon yang tinggi dalam susu soya selepas fermentasi dipilih sebagai suplemen untuk kajian selanjutnya yang melibatkan rawatan fizikal yang bertujuan meningkatkan pertumbuhan serta aktiviti bio-penukaran isoflavon. Pengolahan fizikal, yakni ultrasonikasi, elektroporasi dan radiasi ultra lembayung, ke atas sel-sel bakteria meningkatkan ketelapan membran sel secara signifikan dengan peningkatan keamatan dan jangka masa pengolahan. Pengolahan fizikal telah mengaruh kerosakan oksidatif yang berkait dengan peroksidaan lipid dan perubahan kebendaliran membran. Peningkatan ketelapan membran telah menggalakkan pertumbuhan sel-sel yang terolah sehingga menjangkau 8 $\log_{10}$ CFU/mL, dikaitkan dengan peningkatan aktiviti spesifik β-glukosidase, menyumbang kepada aktiviti bio-penukaran isoflavon serta pengumpulan aglikon isoflavon yang lebih tinggi.

Potensi kesan pengolahan fizikal ini menjejaskan pertumbuhan, bio-penukaran isoflavon dan sifat probiotik tiga sub-kultur sel-sel yang berikutan dikaji. Susu soya diperkaya dengan biotin yang terfermentasi oleh *L. acidophilus* FTDC 8633 yang terolah ultrasonikasi pada 60W selama 2 minit, *L. fermentum* BT 8219 yang terolah elektroporasi pada 7.5 kV/cm selama 3.5 ms, *L. fermentum* BT 8219 yang terolah radiasi ultra lembayung pada 60 J/m^2 masing-masing menunjukkan pengumpulan aglikon isoflavon yang paling tinggi telah dipilih. Pertumbuhan, aktiviti β-glukosidase intrasel dan ekstrasel serta bio-penukaran isoflavon bagi *Lactobacillus* tidak diwarisi oleh sel-sel yang diolah secara fizikal bagi sub-kultur pertama, kedua dan ketiga. Pengolahan fizikal telah menurunkan keupayaan toleransi terhadap asid (pH 2) dan garam hempedu; menurunkan aktiviti perencatan terhadap patogen-patogen tertentu; serta merendahkan kebolehan lekatan, dibandingkan dengan set kawalan; akan tetapi sifat probiotik ini meningkat dalam sel-sel sub-kultur berikutnya.

Kajian ini menunjukkan bahawa penambahan vitamin B dan pengolahan fizikal ke atas *Lactobacillus* dapat meningkatkan penghasilan isoflavon aglikon dalam susu soya selepas fermentasi untuk tujuan pengeluaran makanan berfungsi khusus.

GROWTH PROPERTIES AND BIOACTIVITIES OF LACTOBACILLI IN SOYMILK SUPPLEMENTED WITH B-VITAMINS AND UPON PHYSICAL TREATMENTS

ABSTRACT

The growth characteristics, bioactive potential and bioconversion of isoflavones in soymilk supplemented with B-vitamins of *Lactobacillus acidophilus* BT 1088, *L. fermentum* BT 8219, *L. acidophilus* FTDC 8633 and *L. gasseri* FTDC 8131 were evaluated. The effects on the growth properties were strain- and B-vitamin- dependent. The growth was increased to a viable count exceeding 7 $\log_{10}$ CFU/mL. Supplementation of B-vitamins increased the α-galactosidase specific activities, thus enhanced the hydrolysis of soy oligosaccharides and substantially higher production of lactic and acetic acids after fermentation. Higher proteolytic activities also led to production of lactobacilli-fermented B-vitamin-supplemented soymilk with higher ACE-inhibitory activities. The β-glucosidase specific activities that enhanced the accumulation of isoflavone aglycones upon supplementation were also increased.

Biotin supplementation that resulted in enhanced productions of aglycones in soymilk upon fermentation was selected for subsequent study involving physical treatments, aimed to further enhance the growth of lactobacilli and their isoflavones bioconversion activities. The physical treatments namely ultrasonication, electroporation and UV radiation were used to treat cells at various intensities and durations. The treatments induced oxidative damage on cellular membrane associated with the occurrence of lipid peroxidation and alteration of membrane fluidity, in tandem with increasing treatment intensities and durations. The increased permeability promoted growth of treated cells to beyond 8 $\log_{10}$ CFU/mL after fermentation, associated with enhanced β-glucosidase specific activities. This led to enhanced bioconversion of isoflavones and higher accumulation of isoflavones aglycones.

Potential ramifications of the treatments in affecting the growth, bioconversion of isoflavones and probiotic properties in subsequent three subcultures of cells were evaluated using biotin-soymilk fermented by ultrasonicated *L. acidophilus* FTDC 8633 at 60 W for 2 min, electroporated *L. fermentum* BT 8219 at 7.5 kV/cm for 3.5 ms and UVB treated *L. fermentum* BT 8219 for 60 J/m^2 as these showed utmost accumulation of isoflavone aglycones after fermentation. The growths, intracellular and extracellular β-glucosidase activities and hence aglycones production of the physically treated cells were not passed on by treated cells to the first, second and third subcultures where comparable effects were detected. Physical treatments reducing tolerability towards acid (pH 2) and bile; lowering inhibitory activities against selected pathogens and reducing adhesion ability compared to the control; but these probiotic properties were improved in the subsequent subcultures of the treated cells.

This study shows that supplementation of B-vitamins and physical treatments on lactobacilli could enhance the production of isoflavone aglycones in soymilk after fermentation for the development of functional food.

CHAPTER 1

INTRODUCTION

1.1 Background

Lactobacillus belongs to one of the genera with promising probiotic properties that commonly inhabit the healthy gut. Probiotics have been defined by the FAO/WHO as “live microorganisms which when administered in adequate amounts confer a health benefit to the host” (FAO/WHO, 2001). Conventionally, probiotics have been associated with improvement of intestinal health such as prevention and reduction of diarrhoea, improvement of intestinal microbial balance, protection against intestinal pathogens and alleviation of lactose intolerance symptoms. Over the years, the potential applications of probiotics have been expanded beyond gastrointestinal health to include benefits involving reduction of serum cholesterol, immune system stimulation, anti-hypertensive properties, anti-mutagenic properties and anti-carcinogenic effects (Liong, 2007). Considering the beneficial effects of probiotics, members of the genera *Lactobacillus* are the most commercially used lactic acid bacteria for the production of functional food marketed worldwide.

Soy has been claimed to exhibit health benefits to consumers and numerous soy-based foods have been evaluated as possible probiotic vehicles in various studies (Ng *et al.*, 2008; Liong *et al.*, 2009). Soymilk, the water extract of soybean is a rich source of high quality protein and amino acid. It is also free of lactose which warranted its acceptability by lactose-intolerant consumers, vegetarians and milkallergy individuals (Wang *et al.*, 2002). In addition to these superior nutritional characteristics, soymilk is also considered to have a potential role in the prevention of chronic diseases such as atherosclerosis, cancer, osteoporosis and menopausal disorders (Liu *et al.*, 2002). It has been claimed that soymilk can support the growth and biochemical activities of various lactic acid bacteria (Garro *et al.*, 1998) and bifidobacteria (Chou & Hou, 2000).

B-vitamins are a group of trace organic substances, which are essential for microorganism growth and metabolism. They are needed as enzyme cofactors involved in reactions essential for cellular functions and must be provided in the growth medium if a bacterium is unable to synthesize any of them. Studies have demonstrated that appropriate levels of B-vitamins supplementation have improved the lactic acid production (Xu *et al.*, 2008) and bacteria growth (Nancib *et al.*, 2005; Xu *et al.*, 2008). Apart from microbial growth enhancement, such a supplementation into soymilk could also fulfil the Recommended Daily Intake (RDI) of B-vitamins by a human (FAO/WHO, 2004). A healthy adult is recommended to take 1.5 mg/day of thiamine (vitamin B1), 1.7 mg/day of riboflavin (vitamin B2), 20 mg/day of niacin (vitamin B3), 10 mg/day of pantothenic acid (vitamin B5), 2 mg/day of pyridoxine (vitamin B6), 300 μg/day of biotin (vitamin B7), 400 μg/day of folic acid (vitamin B9) and 6 μg/day of cobalamin (vitamin B12). However, processing of soymilk, such as heat treatment, may result in losses of nutritional compounds especially vitamins (Lešková *et al.*, 2006). One serving (250 mL) of soymilk provides only 10% DV (daily value) of thiamine and riboflavin, 6% DV of niacin, 9% DV of pyridoxine and pantothenic acid, 11% DV of folate, and 0% DV of biotin and cobalamin (USDA, 2009), values that are lower than the RDI. Thus, fortification of B-vitamins in soymilk could aid in achieving the RDI, and is crucial for those with nutrients malabsorption where a higher than average nutritional supplementations is needed.

The consumption of soymilk has been restricted due to the absence of α-galactosidase in the human gut to breakdown the non-digestible α-galactosyl oligosaccharides such as raffinose and stachyose (Tsangalis & Shah, 2004). These intact oligosaccharides pass undigested into the lower intestine where they are metabolized by intestinal microflora, which produces carbon dioxide, hydrogen and methane, leading to flatulence and abdominal pain (Donkor *et al.*, 2007). Studies have proven that fermentation of soymilk with lactic acid cultures possessing α-galactosidase activity such as *L. acidophilus* results in reduced level of raffinose and stachyose (Hsieh & Chou, 2006; Donkor *et al.*, 2007).

Fermented soy products have been claimed as an excellent source of bioactive peptides (Yang *et al.*, 2004). The enrichment of fermented medium with bioactive peptides is attributed to the proteolytic activity of microorganisms. *L. acidophilus* has been reported to exhibit proteolytic activity leading to release amino acids and shorter peptide chains from soy protein to meet their requirement for growth (Liong *et al.*, 2009). Strains of lactobacilli have been found to produce proteinases that could hydrolyse long oligopeptides, liberating angiotensin-I-converting-enzyme (ACE) inhibitory peptides (Gobbetti *et al.*, 2000) with antihypertensive properties (Nakamura *et al.*, 1995). Hence, fermentation of soymilk by lactobacilli could enhance the liberation of bioactive peptides especially with ACE inhibition activity.

The health benefits of soy-based food are attributable to the various functional ingredients in soy, especially isoflavones. Isoflavones are flavonoids occurring abundantly in soybean (Setchell & Cole, 2003) which comprises 80% to 90% of biologically inactive isoflavone glucosides (Wei *et al.*, 2007). The isoflavone glucosides are converted to isoflavone aglycones during soybean processing and upon the addition of β-glucosidase (Toda *et al.*, 2001). It has been reported that lactic acid bacteria such as lactobacilli posses β-glucosidase and thus able to biotransform isoflavone glucosides to aglycones during fermentation of soymilk (Chien *et al.*, 2006; Otieno & Shah, 2007; Raimondi *et al.*, 2009). Izumi *et al.* (2000) stated that soy isoflavone aglycones were absorbed faster and in higher amounts than their glucosides in human. Furthermore, Setchell *et al.* (2002) also reported that isoflavone glucosides were hardly absorbed intact across the intestinal epithelium, and intestinal β-glucosidases are needed for hydrolysis in order to improve the bioavailability of these glucosides. Therefore, the incorporation of β-glucosidase possessing lactobacilli could enhance the bioconversion of isoflavone glucosides to aglycones in soymilk and hence exert health benefits to human upon consumption.

Taking into consideration the health beneficial effects of biologically active isoflavone aglycones, enhanced accumulation of aglycones in fermented soymilk is desirable. However, diffusion of isoflavone glucosides/ enzyme β-glucosidase across the cellular membrane has become the rate-limiting step to achieve effective transformation of isoflavone glucosides to aglycones. The cellular membrane played an important role in controlling the movement of substances in and out of a cell in order to maintain viability and metabolic functions of cells (Chen, 2007). Thus, enhanced membrane permeability is essential to ease the movement of substrates/enzymes across the cytoplasmic membrane to attain increased bioconversion of isoflavones. Physical treatments such as ultrasonication, electroporation and UV radiation have been shown to enhance membrane permeabilization. Each physical treatment has its own characteristic with respect to cell viability, transfer efficiency, general applicability and technical requirements (Stephens & Peppercock, 2001). Exposure of cellular membrane to physical treatment with appropriate intensity could exert permeabilization that is reversible (Kotnik & Miklavčič, 2006). The permeabilization of cell membrane has been shown to induce the release of intracellular metabolites which in turn contributed to higher productivities especially during whole-cell bioprocesses (Nguyen *et al.*, 2009; Tryfona & Bustard, 2008; Hung & Liao, 1996). To our knowledge, the cell permeability issues have been demonstrated well in molecular engineering as well as recombinant DNA technology (Chen, 2007). However, no effort has been made on the cellular membrane of lactobacilli to investigate their isoflavone bioconversion properties by using physical treatments. Furthermore, the prolonged effect of such treatments on subsequent few subcultures of treated cells and their isoflavones bioconversion ability in soymilk remains unknown.

The exposure of cells to physical treatments could alter the surface constituents such as proteins and lipids and the organizations of these constituents within the cell wall and cellular membrane. This has led to alteration in membrane surface following changes in physico-chemical properties of bacteria cells (Schär-Zammaretti & Ubbink, 2003). As a consequence of such modification, the probiotic properties of lactobacilli that are closely related to the structure of the bacterial surface may have been affected, despite it being a promising member of probiotics. To date, no study has been done on the evaluation of probiotic properties on physically treated lactobacilli and the sustainability of the treatment in affecting the probiotic properties for subsequent few subcultures.

1.2 Aims and Objectives of Research

The aim of this study was to evaluate the effects of B-vitamins on the viability, growth characteristics and bioconversion of isoflavones by lactobacilli in soymilk, and the effects of physical treatments on bioconversion of isoflavones in the B-vitamin-supplemented lactobacilli-fermented soymilk. The sustainability of the treatments on the treated cells in affecting the growth and bioconversion of isoflavones for few subcultures were also examined. Thus, the specific objectives were:

1. To examine the effects of B-vitamins on bioactive potentials of lactobacilli-fermented soymilk.

2. To evaluate the effects of physical treatments (ultrasonication, electroporation and UV radiation) on membrane properties, growth and isoflavones bioconversion abilities of lactobacilli in B-vitamin-supplemented soymilk.

3. To investigate the potential inheritance of the effects of physical treatments on lactobacilli based on their growth and bioconversion of isoflavones for the subsequent three subcultures.

4. To examine the effects of physical treatment on probiotic properties of lactobacilli and sustainability of the treatments for the subsequent three subcultures.

CHAPTER 2

LITERATURE REVIEW

2.1 Probiotics

With the increasing accumulation of scientific evidence on the properties, functionality and benefits of probiotics for the enhancement of human health, probiotic bacteria has gained an important role in the context of human nutrition. Probiotics are viable microorganisms that confer health benefits to the host once consumed in adequate amounts, primarily to promote the proliferation of beneficial gastrointestinal indigenous microflora. The important factors that must be possessed by probiotics when being used in food are the capability to survive passage through the digestive tract, acid and bile tolerance and the ability to proliferate in the gut (Stanton *et al.*, 2001). Therefore, probiotics that are predominantly selected for use in the commercial food industries are bacteria that inhabit the human intestinal tract (Crittenden *et al.*, 2005). Strains of lactobacilli such as *Lactobacillus acidophilus, L. casei* and *L. gasseri* are the intestinal microflora that commonly associated with probiotic properties.

Parts of this section have been published:

1. Lye, H. S., Kuan, C. Y., Ewe, J. A., Fung, W. Y. & Liong, M. T. (2009). The improvement of hypertension by probiotics: effects on cholesterol, diabetes, rennin and phytoestrogens. *International Journal of Molecular Sciences*, 10, 3755-3775.
2. Liong, M. T., Yeo, S. K., Kuan, C. Y., Fung, W. Y. & Ewe, J. A. (2009). Antibiotic resistance and probiotics: roles, mechanisms and evidence. In: F. Colombus, (ed). Antibiotic Resistance Hauppauge, New York: Nova Science Publishers, Pp. 5-38.
3. Liong, M. T., Lim, T. J., Yeo, S. K., Ewe, J. A. & Lye, H. S. (2010). Probiotics and Enteric Cancers. In: J. F. J. G. Koninkx, R. Marinsek-Logar, & J. J. Malago, (eds). Probiotics and Enteric Infections, Berlin: Springer-Verlag, Pp. 399-426.

2.1.1 *Lactobacillus*

Lactobacillus is a gram-positive, non-spore-forming and non-flagellated rod-shaped bacterium, varying from long and slender to short coccobacilli, occasionally forming short chains. The optimum growth temperatures of lactobacilli occur within 35−40 ºC. Of all the species of lactobacilli that have been recognized, the most commonly suggested strains for dietary use are *Lactobacillus acidophilus*. Other representative species of *Lactobacillus* are *L. johnsonii, L. bulgaricus, L. gasseri, L. casei, L. rhamnosus* and *L. plantarum* (Ishibashi & Yamazaki, 2001). *Lactobacillus* is either aerotolerant or anaerobic, strictly fermentative and have complex growth requirements. They require carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, minerals and B-vitamin complex (Gomes & Malcata, 1999). Lactobacilli can metabolize glucose moiety chiefly to lactic acid via the homofermentative pathway or to equimolar amounts of lactic acid, CO_2, ethanol, acetate, formate or succinate via the heterofermentative pathway.

2.1.2 General Health Benefits of Probiotics

Lactobacilli are among the predominant members of the intestinal beneficial microbiota which are commonly associated with probiotics. Probiotics such as lactobacilli and bifidobacteria constitute approximately one-third of the bacterial population in the intestinal tract at their optimum level. Therefore, probiotic microorganisms have been regarded as a marker of the stability of the human intestinal microflora, for prevention of many diseases and for maintaining good health (Reyed, 2007).

In general, several mechanisms have been suggested by which probiotics protect the host against intestinal infections. One of the proposed mechanisms is competitive colonization. In order for antibiotic-resistant bacteria to cause infection, these bacteria must colonize the site of the infection. Colonization subsequently progresses to infection when there is a break in the skin or mucous membrane which enables the opportunistic pathogen to evade the host defense. Therefore, the ideal approach to treat antibiotic-resistant infections is by competitive colonization of the infected site with susceptible strains. Probiotics provide a new line of potential therapy because they could effectively decolonize the antibiotic-resistant strains without increasing the risk of future resistance. By encouraging the re-growth and repopulation of probiotic strains, the resistance level could be controlled by out-competing the potentially pathogenic antibiotic-resistant strains.

Colonization with probiotics could possibly counteract the colonization of antibiotic-resistant strains, given the ability of probiotics to bind to the enteric epithelium and inhibit the adhesion of pathogenic strains. Competitive colonization is possible due to the relatively higher adhesion ability of probiotics compared to the antibiotic-resistant strains at the target site. Vesterlund *et al.* (2006) demonstrated that *L. rhamnosus* GG and *Lactococcus lactis* subsp. *lactis* were able to reduce the binding of *Staphylococcus aureus* to human colonic mucosa by 39–44% due to their higher adhesion ability compared to the pathogenic strains. Therefore, probiotics are often chosen for their ability to adhere to epithelial cells. Adherent probiotics could reduce the viability of pathogenic bacteria.

Manley *et al.* (2007) has proposed that competition for nutrients by probiotics could be a potential mechanism to clear intestinal infections. Under normal physiology, the gastrointestinal tract is such a rich source of nutrients that it may seem unfeasible that such mechanism could influence the composition of the gut microflora. Nevertheless, it only requires the absence of one limiting essential nutrient to trigger this mechanism. Under limited nutrient conditions, probiotics could compete more efficiently for the essential nutrient than the antibiotic-resistant strains. This mechanism could have been partly responsible for the effectiveness of probiotics to eliminate or reduce antibiotic-resistant strains in the gut. *L. rhamnosus* GG has been suggested to compete with vancomycin-resistant *Enterococcus faecalis* (VRE) for consumption of essential nutrients (e.g., monosaccharide), leading to slower VRE growth (Manley *et al.*, 2007).

In addition to competitive colonization, probiotics could exert antimicrobial activity against various antibiotic-resistant strains. This antagonistic action is due to the production of antimicrobial substances such as bacteriocin and hydrogen peroxide. The use of bacteriocins is often preferred against the administration of antibiotics, as they are perceived to be more natural due to their long history of safe use in foods. Lacticin, the two-peptide (LtnA1 and LtnA2) lantibiotic produced by *Lactococcus lactis* subsp. *lactis* was reported to act against various Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE) and penicillin-resistant *Pneumococcus* (PRP) (Galvin *et al.*, 1999). The possible mode of action for lacticin towards Gram-positive pathogens involves a lipid II binding step by the LtnA1 peptide, followed by insertion of LtnA2 peptide into the membrane. This leads to the formation of pores and ultimately cell death (Morgan *et al.*, 2005). Therefore, bacteriocin and other antimicrobial peptides produced by probiotics could be promising therapeutic agents to treat antibiotic-resistant infections.

In fact, bacteriocin-producing probiotics could also be used to prevent the growth of antibiotic-resistant strains. The production of these antimicrobial factors in the gut would have an antagonistic effect on the resistant strains, which would confer a competitive advantage to the bacteriocin-producing bacteria in the microenvironment. The competitive exclusion of other microorganisms from this niche would affect the overall composition of the microbiota (Millette *et al.*, 2008). Therefore, it is expected that bacteriocin-producing probiotics could reduce the colonization by antibiotic-resistant strains.

The consumption of probiotics is capable of stimulating the immune system due to the ability of probiotics to enhance both cytokine and secretory immunoglobulin A (sIgA) production. Cytokines play a significant role in stimulating the immune response to pathogens by activating immune cells once a pathogen is encountered. The chief function of sIgA is the prevention of the binding of foreign bacteria to epithelial cells and the penetration of harmful microorganisms (Erickson & Hubbard, 2000). Thus, probiotics could protect the gastrointestinal tract from the invasion of pathogens and opportunistic bacteria, refers to subsequently reducing the risk of infection. In such cases, the use of antibiotics to treat illnesses would be reduced thereby limiting the development of antibiotic resistance. Gorbach (1996) demonstrated that *Lactobacillus* GG fed to adults was effective in treating gastrointestinal illnesses without the need for antibiotics. The preventative potential of probiotics in patients suffering from infectious diarrhea and upper respiratory tract infections has led to the suggestion that they could be used as an alternative to antibiotic treatment, thus lowering the occurrence of antibiotic resistance.

Additionally, probiotics has also been associated with the treatment and prevention of antibiotic-associated diarrhea. The administration of exogenous probiotics has been found to regulate intestinal micobiota and stabilize antibiotic-induced dysbiosis caused by antimicrobial action of antibiotics. Inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn’s disease are associated with infections and alterations of the intestinal biota. Probiotics have been proven to be effective in various clinical trials to treat IBD and strains such as *L. rhamnosus* GG has been used (Zhang *et al.*, 2005).

Several *in-vivo* evidences have indicated that probiotics have been associated with the lowering of serum cholesterol. The administration of probiotic fermented milk (10^9 bacteria per ml) to hypercholesterolemic human subjects has shown a decrease of 50% in serum cholesterol level (Shah, 2007). It has been suggested that probiotics could exert hypocholesterolemic effect via several possible mechanisms such as assimilation by growing cells or binding to the cell surface or incorporation into the cellular membrane (Liong & Shah, 2005a, 2005b). Apart from this, probiotics could deconjugate bile salt by bile salt hydrolase (BSH). The resulting free bile salts have limited re-absorption in the gut due to poor solubilisation and thus are more prone to be excreted in the faeces. This increases the demand for synthesizing new bile salts to replace those lost in faeces (cholesterol is the precursor to bile acids), resulting in a reduction of serum cholesterol.

The ability of probiotics to reduce blood pressure has been elucidated through fermentation of food products in order to release bioactive peptides, such as the angiotensin-I converting enzyme (ACE) inhibitory peptides that play a crucial role in rennin-angiotensin system (RAS). Several evidences from *in vitro* and *in vivo* studies have demonstrated the effects of probiotics on hypertension. A study was performed by Donkor *et al.* (2007) on the proteolytic activity of several dairy lactic acid bacteria cultures and probiotics as determinants of growth and *in vitro* ACE inhibitory activity in milk fermented with these single cultures. The authors reported that both *Bifidobacterium longum* and *Lactobacillus acidophilus* strains showed ACE inhibitory activity during growth. This was also supported by Ong & Shah (2008) who examined the release of ACE inhibitory peptides in Cheddar cheeses made with starter lactococci and probiotics. The authors observed that cheeses made with the addition of *L. casei* and *L. acidophilus* had higher ACE inhibitory activity after 24 weeks at 4 ºC and 8 ºC than those without any probiotic adjunct, probably due to increased proteolysis. Moreover, ACE-inhibitory peptides have also been found in yogurt, cheese and milk fermented with *L. casei* sp. *rhamnosus*, *L. acidophilus* and bifidobacteria strains (Rhyänen *et al.*, 2001). In one of the largest studies involving 94 hypertensive subjects in a double-blind, placebo-controlled, randomized trial, Jauhiainen *et al.* (1996) found that the consumption of 150 mL milk fermented by *L. helveticus* twice a day for 10 weeks could decrease systolic blood pressure (SBP) and diastolic blood pressure (DBP) by 4.1 mm Hg and 1.8 mm Hg, respectively.

Probiotics are postulated to exert anti-carcinogenic effects as well, mainly via the reduction of pro-carcinogenic bacterial enzymes such as β-glucuronidase, nitroreductase and urease, and consequently decrease the risk of tumor development (Gomes & Malcata, 1999). It has been suggested that probiotics could suppress the growth of pathogenic microorganisms which are involved in the production of tumor promoters and pro-carcinogens via lowering pH in the colon (Liong, 2008). Additionally, studies also suggested that tumor suppressing ability of probiotics is attributed to binding properties by fractions of the cell wall skeleton of probiotics on mutagens and the binding of heterocyclic amines by intestinal probiotics (Zhang & Ohta, 1991; Orrhage *et al.*, 1994). Probiotics' anticarcinogenic effect has also been reportedly accredited to their ability to affect intestinal detoxification, transit and immune status (Cabana *et al.,* 2007). In addition, Singh *et al.* (1997) found that the antitumor activities in colon and the reduction in the volume of tumor were attributed to a reduced expression of *ras*-p21 oncoprotein upon consumption of *B. longum*. Probiotics have also been advocated for the prevention and treatment of a diverse range of disorders, from acute gastroenteritis to intestinal neoplasia (Boyle *et al.* 2006). By possessing systemic anti-neoplastic activity, probiotics play a crucial role in the prevention of colorectal cancer (Figure 2.1). Probiotics such as *Lactobacillus, Streptococcus,* and *Lactococcus* and in fermented milk products containing *Bifidobacterium* have shown various degrees of anti-mutagenic activities in the *Salmonella typhimurium* mutagenic assay (Renner and Münzner 1991; Hosoda *et al.* 1992; Abdelali *et al.* 1995).

2.1.3 Production of Vitamins

Probiotics such as *Lactobacillus* sp. are microorganism that colonize the human gastrointestinal tract and are responsible in producing vitamins, short-chain fatty acids and other nutrients for their hosts (Hooper *et al.*, 2002). Vitamins are a group of trace organic substances, which are essential for microorganisms growth and metabolism. The B-vitamins are water-soluble vitamins, comprising of thiamine, riboflavin, niacin, pyridoxine, pantothenate, biotin, folic acid and vitamin B_{12} (cyanocobalamin). They have been reported to synthesize folic acid, niacin, thiamine, riboflavin, pyridoxine, cyanocobalamin and vitamin K, which are slowly absorbed by the body (Gomes & Malcata, 1999). However, the synthesizing ability and the concentrations of the vitamins accumulated are strain dependent. Certain strains of bifidobacteria have shown the ability to synthesize biotin (Noda *et al.*, 1994) but not riboflavin (Biavati & Mattarelli, 2006). B-vitamins could be synthesized *in situ* via fermentation using lactic acid bacteria such as *L. lactis* and *L. bulgaricus* (Hugenholtz & Kleerebezem, 1999; Kleerebezem & Hugenholtz, 2003). Tamine *et al.* (1995) had demonstrated that cultured dairy products contained higher contents of folic acid, niacin, biotin, pantothenic acid, vitamin B_6 and vitamin B_{12} compared to unfermented counterparts.

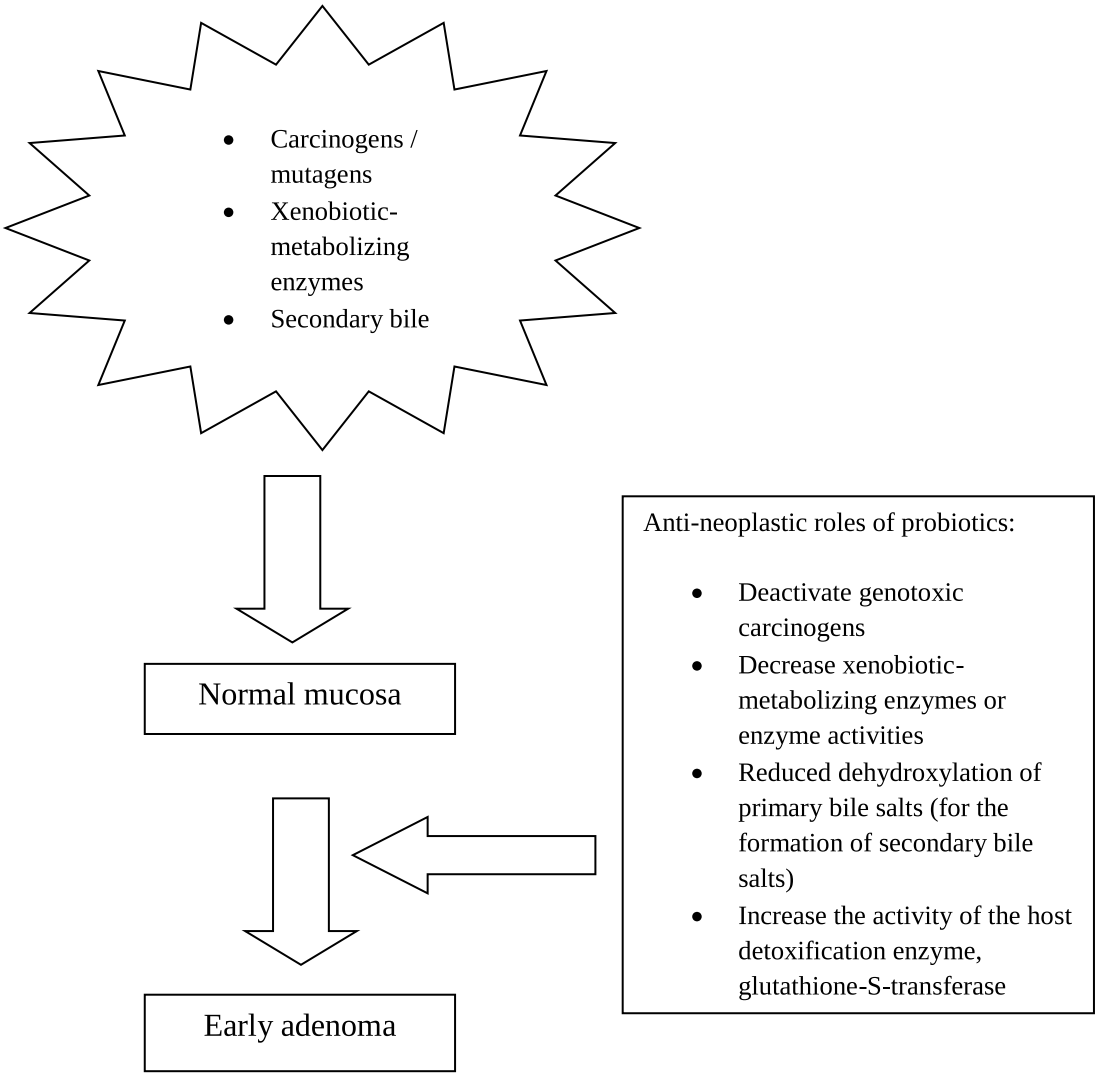

Figure 2.1: The anti-neoplastic activities of probiotics in inhibiting the occurrence of colon carcinogenesis.

B-vitamins have been illustrated to play a crucial role in supporting the growth and metabolism of the probiotics by involving in various cellular functions. Thiamine is essential in the metabolism of carbohydrate. Intracellular thiamine is phosphorylated to form thiamine pyrophosphate that plays a role as a carrier of activated aldehyde groups in decarboxylation and transketolation reactions involved in acetyl-CoA formation, the citric acid cycle, and the branched chain amino acid biosynthesis (Schellenberger, 1998). Riboflavin has been found to be the direct precursor of cofactors for the formation of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Both FMN and FAD are the essential components of redox cofactors of a number of dehydrogenases and oxidases in cellular metabolism of bacteria.

Niacinamide in the form of nicotinamide adenine dinucleotides or its phosphorylated form (NAD, NADH, NADP and NADPH) functions as a cofactor of numerous redox reactions in cellular metabolism in bacteria. They are involved in several classes of NAD-dependent enzymes such as DNA ligase, protein deacetylase and a variety of ADP-ribosyltransferases (Ziegler, 2000). NAD has been reported to be synthesized *de vono* from aspartic acid in bacteria cell (Rodionov *et al.*, 2008). Calcium pantothenate acts as a component of coenzyme A (CoA) in biochemical reactions in which acyl groups are transferred or metabolized. The acyl carrier protein is also a pantothenate-containing cofactor which serves as a specific and soluble cofactor for fatty acid biosynthesis. The enzymes that mediate the synthesis of pantothenate are universal in pantothenate-synthesizing organisms (White, 2003).

Biotin is an essential cofactor in carboxylation, decarboxylation and transcarboxylation reactions in prokaryotes (Hebbeln *et al.*, 2007). During the biosynthesis of fatty acids, biotin serves as a prosthetic group in acetyl CoA carboxylase in catalyzing the synthesis of malonyl coenzyme A (Mandelstam & Mcquillen, 1976). In addition, it also acts as a coenzyme of pyruvate carboxylase that could promote glucose metabolism (Xu *et al.*, 2008). Folate is a vitamin that serves as a cofactor in 1-carbon transfer for DNA synthesis, pantothenate, glycine, serine and methionine (Lin & Young, 2000). It is required in metabolic pathways such as methyl group biogenesis and synthesis of nucleotide, vitamins, and some amino acids. Most bacteria have been reported to synthesis folate *de novo,* which involves a multi-enzyme pathway made up of GTP, *para*-aminobenzoic acid (pABA) and L-glutamate moieties (Crécy-Lagard *et al.,* 2007).

2.2 Soy

Soy (*Glycine max*) is a legume indigenous to East Asia, though the main cultivation areas are now North America (FAO, 2007). Soybean is high in protein and oligosaccharides such as raffinose and stachyose, which is known to promote indigenous microorganisms in the intestinal tract, though they may also cause unpleasant flatulence (Wang *et al.*, 2003). However, the virtues of soybean have been declared; evidenced by the long history of consumption of more than 1000 years. The relatively low cost of soybean protein compared with animal proteins and the simple way it can be fashioned into palatable high protein food such as tofu and soymilk contributes to its role as a an alternative protein source (Ang *et al.*, 1985). It is also low in fat and is an excellent dietary fiber and a variety of micronutrients and phytochemicals (Messina, 1999a).

2.2.1 Soy Foods as Carriers for Probiotics

Soy products have been used to emulate the “probiotic delivery vehicle” role of dairy products. This feature is attributable to the high content of indigestible oligosaccharides present naturally in soy to serve as carbon source for the fermentation of probiotics. After fermentation, the flatulence causing oligosaccharides can be reduced to minimum level upon ingestion. Apart from the high α-galactosyl glucosides, the variable amount of soy protein provides pool of peptides and amino acids essential for growth of probiotics (Shihata & Shah, 2000).

Soymilk, the aqueous extract of whole soybean, is one of the probiotic carriers that have grabbed attention for intensities investigation. Several studies have been performed using soymilk to grow promising probiotics such as *Lactobacillus* and *Bifidobacterium* (Chien *et al.*, 2006; Wei *et al.*, 2007; Otieno & Shah, 2007). These probiotics were able to achieve the cell counts to the suggested level of 10^7 CFU/mL upon fermentation in order to qualify as a probiotic food for humans (Ishibashi & Shimamura, 1993). During the growth of probiotics, the ability of the organisms to utilize the nutrients in the soymilk could lead to an increase in the concentrations of metabolites. Therefore, changes in the concentrations of crude protein, sugars, B-vitamins, organic acids in soymilk were detected (Hou *et al.*, 2000). Donkor *et al.* (2007) also reported that fermented soymilk could be converted into a rich functional product containing probiotics and bioactive compounds such as angiotensin-converting enzymes (ACE) inhibitors.

Probiotics have also been shown to grow well in tofu-based medium as reported by Ng *et al.* (2008). Tofufa is a soft form of tofu that is made via coagulation of soymilk. It is also known as tofu-pudding and generally consumed as an Asian dessert and served with sugar syrup. The growth of probiotics in tofufa has exceeded 10^6 CFU/g and was maintained over storage of 9 days. The presence of probiotics in tofufa has contributed to an increased in the concentration of organic acids which exhibited a preservative effect. In addition, the probiotic strains studied also showed proteolytic activity leading to the production of bioactive peptides with ACE-inhibitory activity.

Soy cream cheese has been developed lately and has been shown as a potential carrier for probiotic with potential antihypertensive property. Soy cream cheese was prepared from tofu and blended with various types of ingredients such as palm oil, carrageenan, pectin, maltodextrin and salt. It could sustain the viability of probiotic strain and was maintained beyond suggested therapeutic level of probiotics of 10^6 CFU/g over storage for 20 days. Due to the metabolic activity of probiotics, the organic acids produced during growth decreased the pH of soy cream cheese which subsequently inhibited the growth of total aerobes and anaerobes (Liong *et al.*, 2009).

Soy waste has been shown as a suitable growth medium base for *L. acidophilus* (Fung *et al.*, 2008b). Soy whey and okara are soy wastes generated from the production of tofu and soy milk. The authors optimized the growth of a probiotic in soy whey medium supplemented with nitrogen sources using response surface methodology. Optimal growth of the probiotics in soy waste medium was found to be 6.15 (OD_{600}) in the presence of nitrogen sources such as meat extract at 7.25 % (w/v), vegetable extract at 4.7 % (w/v) and peptone at 6.85 % (w/v). Utilization of oligosaccharides and reducing sugars and accumulation of acids in the soy waste medium corresponded with the high growth response of probiotics.

Soy yogurt has also been shown to be a suitable medium for delivery of probiotics. Soy yogurt was prepared by addition of yogurt culture such as *L. delbruekii* spp. *bulgaricus* and *Streptococcus thermophilus* into soymilk followed by fermentation at 42 º C to suitable termination pH before being cooled to 4 º C. The selected probiotic cultures inoculated into soy yogurt showed applicable viability and their viability were maintained during prolonged storage. The use of yogurt culture in combination with probiotics has resulted in substantial proteolytic activity and subsequently higher concentration of released bioactive ACE inhibitors, which could confer health benefits to the host (Donkor *et al.*, 2005).

2.2.2 Health Benefits of Soy

Soy rich diets have been associated with lower incidence of atherosclerosis, cancer, hypertension, and osteoporosis as well as the alleviation of menopausal symptoms (Peñalvo *et al.*, 2004). Soy is also an excellent source of phytoestrogens, including the intensely researched isoflavones, daidzein and genistein (Gardner *et al.*, 2001). These isoflavones are involved in a variety of physiological modulation including regulation of cell growth, bone density and plasma lipids (Martin *et al.*, 2001). Additionally, work has also been carried out to further proven the beneficial effects to post-menopausal women, hypertensive, hypercholesterolemic, cardiovascular disease and cancer patients at preventive and curative stages (Xiao, 2008).

Apart from providing an alternative protein source, soy and soy products confer a wide range of other benefits. The hypocholesterolemic effect of soy has been achieved through the cholesterol-lowering properties of soy protein. This property is attributed to the amino acid composition of soy protein and some nonprotein components, such as saponins, isoflavones, and phytic acid that may affect serum cholesterol (Dewell *et al.*, 2006). It has been reported that increased consumption of soy protein has been associated with lower total serum cholesterol, LDL cholesterol and triglyceride concentrations (Anderson *et al.*, 1995). Furthermore, a recent study showed that consumption of soymilk diet resulted in a modest decrease in LDL-cholesterol concentrations and higher HDL-cholesterol (Matthan *et al.*, 2007). Soy protein containing isoflavones has been shown to significantly reduce serum total cholesterol, LDL cholesterol, and triacylglycerol and significantly increase HDL cholesterol (Oboh, 2006; Zhan & Ho, 2005). In addition, soy protein containing enriched or depleted isoflavones has been shown to significantly improve lipid profiles, while the reductions in LDL cholesterol were more significant in hypercholesterolemic than in normocholesterolemic subjects (Taku *et al.*, 2007).

Soy foods have also been claimed to alleviate menopausal symptoms (Duncan *et al.*, 1999). Intake of soy with isoflavones supplementation has been shown to alleviate menopausal symptom such as hot flashes (Han *et al.*, 2002). In addition, soy isoflavones have also been hypothesized to exert estrogenic effects when in a low-estrogen environment, such as in postmenopausal women (Messina, 1999b). The bioactive health beneficial compounds of isoflavones in soy merely credited to its aglycone forms. Several hormone-dependent disorders such as osteoporosis, cardiovascular disease and cancer could be prevented through the consumption of biologically active aglycone forms of isoflavones (Tsangalis *et al.*, 2005). Due to its unique pharmacological profile, soy could also act as a potent estrogen replacement therapy for postmenopausal women (Martin *et al.*, 2001, Han *et al.*, 2002).

Epidemiological studies showed that people with high soy isoflavonoids intake have lower rates of several types of cancers including breast, prostate and colon cancer (American Institute for Cancer Research, 2000). The high intake of isoflavone-rich soy by Asian population is hypothesized to contribute towards their relatively lower incidence of clinical cancers than the Western counterparts (Duncan *et al.*, 1999). The protective effect of soy isoflavones on breast cancer development is suggested to be a hormonal effect involved in lowering circulating levels of unconjugated sex hormones. Isoflavones also could inhibit enzyme activity that would lead to the development of prostate cancer (Cornwell *et al.*, 2004). Apart from soy isoflavones, soy protein isolate have been shown to suppress carcinogen activation contributing to reduce mammary and colon cancer incidences (Badger *et al.*, 2005).

The incidence and risk of osteoporosis is highly associated with postmenopausal women. Estrogen plays an important role in maintaining bone density by regulating the formation and reabsorption of calcium in bone (Nilsson & Gustafsson, 2002; Cornwell *et al.*, 2004). Soy isoflavones have been suggested to be effective in maintaining bone mineral density in postmenopausal women (Morabito *et al.*, 2002). Genistein was found to have modest bone-conserving properties when administered in low doses to ovariectomized, lactating rats (Anderson *et al.*, 1995). A double blind, placebo controlled study of postmenopausal women showed significant increase in bone mineral density at the femoral neck after 12 months of daily administration of 54 mg genistein (Morabito *et al.*, 2002).

2.3 Probiotics in Soymilk

Soymilk has been considered as a good medium for growing probiotics due to the presence of monosaccharide and oligosaccharides which are fermented by most of the probiotic strains. However, the unfavorable beany flavor, flatulence factors and the high contents of indigestible oligosaccharides limit the consumption of soybeans as raw food materials (Garro *et al.*, 2004). Fermentation of soymilk with probiotics not only offers a means of preserving the soymilk but also confers health effects upon ingestion.

2.3.1 Growth of Probiotics in Soymilk

Dairy products have been used as the conventional carriers for probiotics before they are delivered to the human gastrointestinal tract (Klaenhammer *et al.*, 2007). In order to achieve desired therapeutic effects, the level of probiotic bacteria in the probiotic-fermented product must be available in sufficient numbers. As suggested by Schuler-Malyoth *et al.* (1968), at least 10^6 CFU probiotic organisms per milliliter of cultured milk must be present when consumed. By having superior nutritional characteristic such as high quality protein, varying sources of carbohydrates and a small quantity of saturated fatty acids (Scalabrini *et al.*, 1998), soymilk has the potential to support the growth and biochemical activities of various lactic acid bacteria (Garro *et al.*, 1998) and bifidobacteria (Chou & Hou, 2000).

Probiotics have been reported to produce various glycosyl enzymes simultaneously that enable the microorganisms to hydrolyze various glycosidic bonds (Amaretti *et al.*, 2006). The suitability of soymilk as a probiotic carrier is attributed to the ability of probiotics possessing various glycosyl hydrolases which enable them to utilize glycosyl sugars such as raffinose and stachyose for growth.

Liong *et al.* (2009) reported that the ability of probiotics to proliferate in soy-based cream cheese was attributed to their α-galactosidase activity.

Upon hydrolysis by probiotics, the oligosaccharides in soymilk are broken down into simple sugars. The glucose moiety could be metabolized via homofermentative or heterofermentative pathway by lactobacilli while bifidobacteria breakdown glucose via the fructose-6-phosphate shunt. In addition to glucose, lactobacilli and bifidobacteria are also capable of utilizing fructose as a carbon source (Gomes & Malcata, 1999). Caescu *et al.* (2004) demonstrated that intracellular fructose was metabolized via the fructose-6-phosphate phosphoketolase pathway.

A series of metabolic activities occur during the fermentation of probiotics in the soymilk, such as the production of organic acids and a decline in pH. Being homofermentative, both *L. acidophilus* and *L. gasseri* ferment glucose predominantly to lactic acid via the Embden-Meyerhof-Parnas pathway, accompanied by the production of minor amounts of acetic acid while heterofermentative *L. casei* and *L. fermentum* metabolize glucose via pentose phosphate pathway to produce equimolar amount of lactic acid/ethanol and CO_2 as the end-products. On the other hand, the metabolism of carbohydrates by *Bifidobacterium* strains follow the fructose-6-phosphate shunt, yielding 2 moles of lactic acid and 3 moles of acetic acid per mole of glucose in pure glucose medium as carbon source.

2.3.1(a) Improvement of Viability

In order to provide a more favorable growth condition for probiotics, several methods have been applied during the fermentation of soymilk. Owing to glucose being the main carbon source for the metabolism of probiotics, Chou & Hou (2000)